SYNTHESIS OF MODIFIED NUCLEOSIDES FOR THE INCORPORATION INTO tRNAs

THÈSE N^{O} 3511 (2006)

PRÉSENTÉE LE 5 MAI 2006

À LA FACULTÉ SCIENCES DE BASE

Laboratoire de chimie des acides nucléiques

SECTION DE CHIMIE ET GÉNIE CHIMIQUE

ÉCOLE POLYTECHNIQUE FÉDÉRALE DE LAUSANNE

POUR L'OBTENTION DU GRADE DE DOCTEUR ÈS SCIENCES

PAR

Sébastien PORCHER

Ingénieur ENSC, Mulhouse, France
DEA de chimie,
et de nationalité française

acceptée sur proposition du jury:

Prof. C. Comninellis, président du jury
Dr S. Pitsch, directeur de thèse
Prof. S. Arseniyadis, rapporteur
Prof. R. Giegé, rapporteur
Prof. K. Johnsson, rapporteur

ÉCOLE POLYTECHNIQUE
FÉDÉRALE DE LAUSANNE

Lausanne, EPFL
2006

The transfer RNA? The biggest leaving group! Pr. Stefan Pitsch

A toutes ces absences…

REMERCIMENTS

La vie est souvent assimilée à une pièce de théâtre. Dans cet art, c'est lors du dernier acte que les acteurs apprennent la véritable teneur des sentiments de leur public. De même pour votre thèse, c'est lors de votre défense que la qualité et la finalité de votre travail prennent tout leur sens. Alors à cet égard, c'est aux membres du jury qu'iront mes premiers remerciements : le Professeur Dr. Simeon Arseniyadis, le Professeur Dr. Richard Giegé, le Professeur Dr. Kai Johnsson, son président le Professeur Dr. Christos Comninellis et naturellement à l'initiateur de ce doctorat, le professeur Dr. Stefan Pitsch. De même, je remercie les institutions du fond national suisse et l'EPFL pour leur soutien.

Toujours en analogie avec le théâtre, après la fin de la représentation, le rideau se baisse sur un travail qui, malgré les apparences, n'aura été que le prolongement de celui de ses prédécesseurs et la somme de toutes les aides apportées par chacune des personnes qui auront bien voulu être de l'aventure. Alors, par ordre d'apparition, mes remerciements vont au Dr. Laure Bouchez qui, à l'instar du petit poucet, aura semé les petits cailloux pour me conduire jusqu'à Lausanne. Puis à Mme Marianne Dang qui a été la première à accueillir mes pas dans les méandres de l'EPFL et, malgré cela, a continué à les accompagner. En toute sincérité.

Je remercie Mme Christine Kupper qui me tint au courant durant trois jours des arrivées toujours imminentes du groupe de recherche en pleine migration depuis Zürich et aura contribué à l'évolution de cette équipe.

Le début de ce doctorat, qui aura finalement duré quatre années, ne commença réellement qu'à l'arrivée de ses premiers doctorants : Alfred Stutz, Damian Ackermann et un jeune thésard aux gestes italiens et à l'accent allemand : Philipp Wenter. Tout au long de sa thèse, Philipp a démontré un dévouement et une capacité de travail incroyable sans jamais faillir. Je ne l'ai vu renoncer qu'une seule fois : m'apprendre l'italien malgré plusieurs tentatives. Désolé d'avoir été cette exception et je pense qu'elle restera la seule de ta carrière. A notre amitié.

Après cette arrivée et ces jours d'installation, le travail commença ponctué d'une multitude de manques de matériels et d'une succession d'ennuis toujours comblés par l'équipe du magasin : Jacques Gremaud, Giovanni Petrucci et Gladys Pache. Je les remercie pour ces quatre années de gentillesse et bonne humeur si salvatrices. Je rends grâce à la patience de Martial Rey malgré l'originalité des problèmes de RMN que je lui présentais.

Enfin, trois mois après la formation du groupe, on vit l'arrivée du savoyard Cyrille Denarié dont la clarté d'âme trancha immédiatement avec la noirceur de sa tenue quotidienne. Ces quelques mots vont à ton amitié si sincère.

En outre, l'arrivée de Cyrille permit d'augmenter une proportion de francophones qui ne cessa jamais de grandir. Ainsi suivit le digne représentant jurassien Daniel Berdat, dont l'amitié continua au-delà de son bref séjour au sein du groupe de recherche. En ton hommage et à nos excès de raclette suisse.

Après lui, deux vaudois arrivèrent montrant des accents contrastés à la mesure de la diversité suisse: à Frédéric Meylan et Luc Reymond. A votre accompagnement pour avoir rendu au laboratoire, tous les accents de ce si magnifique pays qui n'hésita pas à financer mon doctorat.

Sur la fin de la thèse, peut être afin d'équilibrer cette francophonie dominante, le groupe vécu l'arrivée d'un post-doc indien anglophone au nom de Meyyappan. Malgré un nom empreint de philosophie bouddhiste « père de la vérité » ne manqua pas d'apporter à Cyrille, le vocabulaire peu aimable de ce pays. A l'image de cet apport marginal, la thèse a été pour moi un creuset propice aux échanges culturels et l'occasion de discussions enrichissantes. Ces mêmes conversations ont souvent permis des concrétisations de projets tel que celui achevé avec Christian Gossens ou des déblocages de problèmes de synthèse dont il serait difficile aujourd'hui, d'attribuer la paternité. Alors, que toutes les personnes absentes de ce texte et qui sauront se reconnaître, soient remerciées à la mesure de mes sentiments pour leur apport à ma réussite d'aujourd'hui.

RESUME

Plus de 100 nucléosides modifiés présents dans les ARN naturels ont été identifiés à ce jour. Ces modifications sont impliquées dans la structuration de l'ARN, le maintien du cadre de lecture durant la synthèse des protéines et l'interaction correcte de celui-ci avec les complexes enzymatiques. Pour une étude systématique de leur fonction, il apparaît nécessaire de pouvoir incorporer ces modifications à une position donnée de séquences d'ARN en ayant recours à la synthèse chimique.

Nous présentons ici des protocoles optimisés de préparation d'une large variété de ribonucléosides phosphoramidites 2'-*O*-TOM protégés contenant certaines des nucléobases modifiées les plus fréquemment rencontrées, à savoir m^2G, $m^2{}_2G$, m^1A, m^5U, D, m^5C, ψ, m^1I, i^6A, $m^6{}_2A$, m^6A, m^1G, t^6A, I et imG. De plus, les phosphoramidites des nucléosides non-naturels isoG, isoC et 4-desmethyl-5-methylwyosine ont été préparés (Chapitres I, II et III). Pour l'introduction de nucléobases labiles, des conditions de déprotection en combinaison avec l'emploi du nouveau N^2-methoxyacetyl protégé guanosine phosphoramidite, ont été développées (Chapitre V). Pour la première fois, le nucléoside particulièrement sensible wyosine a été incorporé dans un 18mer d'ARN et au sein de la boucle de l'anticodon d'une version tronquée d'un ARN de transfert. Dans ce but, des stratégies de ligation enzymatique, basées sur l'emploi de T4 RNA ligase et de DNA ligase, ont été évaluées et optimisées (Chapitres III et V).

Les propriétés décodantes des anticodons modifiés ont été revues et de nouveaux modèles d'interaction basés sur la formation de liaisons hydrogènes secondaires ont été proposés (Chapitre IV).

Pour la préparation efficace d'analogues d'ARNt estérifiés avec des acides amines non naturels, la synthèse de séquences modifiées d'ARN contenant une 2'-deoxy-2'-thioadenosine 3'-terminale a été développée. En analogie avec la ligation chimique d'oligopeptide, une aminoacylation spécifique spontanée avec un acide aminé faiblement activé sous la forme d'un thioester, a lieu efficacement dans des solutions aqueuses tamponnées ainsi que sous une multitude de conditions. Ce concept pourrait à terme, être employé pour l'aminoacylation directe d'ARNt similairement modifiés (Chapitre VI).

ABSTRACT

More than 100 modified nucleosides have been found in naturally occurring RNA sequences. These modifications are involved in the correct folding, in the maintenance of the reading frame during translation and in the proper interaction with enzymes and protein complexes. For an exhaustive study of their function it would be necessary to incorporate by chemical synthesis such modifications at selected positions of RNA sequences.

Here we present optimized protocols for the preparation of a large variety of 2'-*O*-TOM protected ribonucleoside phosphoramidite building blocks containing the most frequently encountered modified nucleobases m^2G, $m^2{}_2G$, m^1A, m^5U, D, m^5C, ψ, m^1I, i^6A, $m^6{}_2A$, m^6A, m^1G, t^6A, I and imG. In addition, the non-natural ribonucleoside phosphoramidites containing isoG, isoC and 4-desmethyl-5-methylwyosine have been prepared (Chapters I, II and III). For the introduction of base-labile modifications into RNA sequences, modified deprotection conditions in combination with the new N^2-methoxyacetyl protected guanosine phosphoramidite have been developed (Chapter V). For the first time, the sensitive modified nucleoside wyosine was incorporated into a 18mer RNA sequence and into the anticodon loop of a truncated tRNA. For this purpose, enzymatic ligation strategies, based on T4 RNA and T4 DNA ligase, were evaluated and optimized (Chapters III and V).

The decoding properties of modified anticodons have been reviewed and new models, based on the formation of secondary hydrogen bonds have been proposed (Chapter IV).

For the efficient preparation of tRNA analogues esterified with unnatural amino acids, a synthesis of modified RNA sequences containing a 3'-terminal 2'-deoxy-2'-thioadenosine was developed. In analogy to the "native chemical ligation" of oligopeptides, its spontaneous and site-specific aminoacylation with weakly activated amino acid thioesters occurred efficiently in buffered aqueous solutions and under a wide range of conditions. This concept could be employed for a straightforward aminoacylation of analogously modified tRNAs (Chapter VI).

INTRODUCTION

INTRODUCTION

1. Structure and function of nucleic acids

For a long time, cells were viewed as closed systems containing a multitude of free components and steady progresses in analytical methods allowed their progressive identification. The complex and fascinating architecture of cells and their capacity to communicate with their environment has only emerged recently and much more aspects and details about their functioning will eventually be uncovered.

A rough analysis reveals that cells are composed of H_2O (70%), a multitude of small, monomeric compounds such as sugars, nucleotides, amino acids, fatty acids and salts (6%) and, importantly, oligomeric macromolecules (25%, M_W usually several kDa), belonging to the categories of proteins and nucleic acids. For the first time, nucleic acids were identified and described by *Miescher* in 1869 (Doonan 2004), but only in the 1950s their constitution and their basic mode of function was described properly. Since then, the associated field of Molecular Biology is growing steadily, producing new insights and tools for all areas of biological research.

Nucleic acids are oligomers consisting of 5'→3' phosphodiester-linked nucleotide units that are composed of a 2'-deoxy-D-ribose or D-ribose in their furanose forms (in DNA and RNA, respectively) and a heteroaromatic nucleobase *(Figure 1.)*. The nucleobases found in DNA include the purines adenine (A) and guanine (G) and the pyrimidines cytosine (C) and thymine (T), while RNA contains the pyrimidine uracile (U) in place of T. The oligonucleotide chain is composed of a polar, negatively charged sugar-phosphate backbone and an array of hydrophobic nucleobases. This amphiphilic nature, together with the hydrogen bonding and stacking potential of nucleosides, ultimately drives the assembly and maintenance of secondary and tertiary structures within nucleic acids.

The first, global description of the three-dimensional, supramolecular structure and the basic function of nucleic acids has been achieved in 1953 when *Watson* and *Crick* have proposed their model of the DNA double helix (Watson and Crick 1953) (its confirmation by high-resolution single-crystal X-ray analysis was obtained only in 1982 (Dickerson et al. 1982)).

For the first time, structural considerations permitted to deduce the mechanisms of cellular replication and conservation of genetic information. At the same time, the previously observed constant ratios of C/G and A/T (Chargaff et al. 1951) was finally understood, since the association of two DNA single strands is based on the formation of distinctive hydrogen bonds. Thereby, the distribution of acceptor and donor hydrogen bonds allows formation of selective and defined base pairs where C can only be associated with G and A only with T *(Figure 2.)*. The same base pairing rules are found in RNA, which is constituted of almost the same elements undergoing G·C and A·U associations.

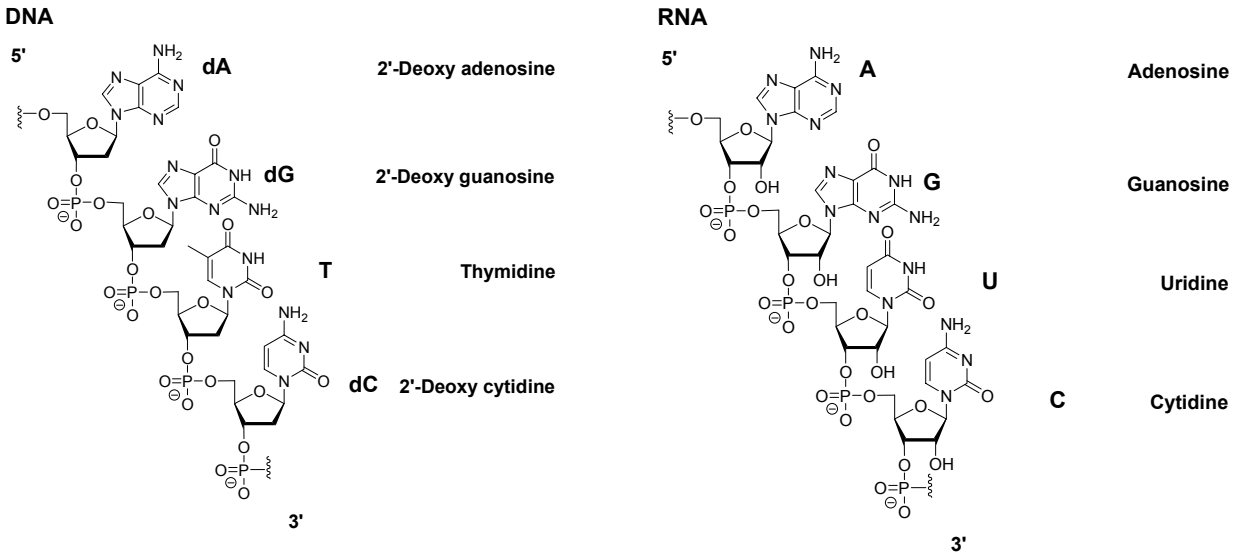


Figure 1: Constitution of DNA (*left*) and of RNA (*right*), with the name of the monoric nucleotide units

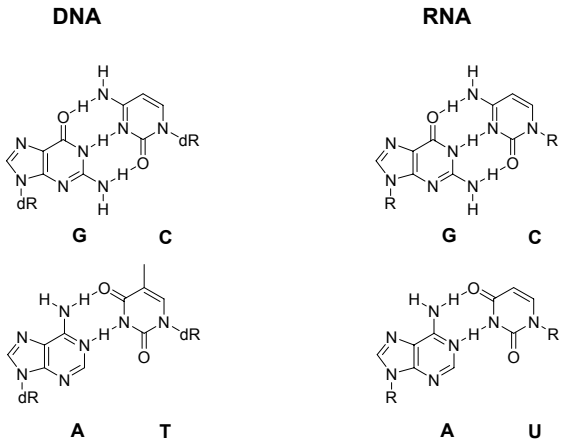


Figure 2: Structure of so-called *Watson-Crick* base pairs in DNA (*left*) and RNA (*right*).

These common features reflect the close relationships and interactions exhibited by these two classes of nucleic acids within the cell. Whereas the genetic information is stocked by DNA, the RNA is responsible for its transport to the site of protein manufacturing. The reasons for this attribution of roles are based on the structural difference existing between these two entities, which is the presence or absence of a HO-group at the 2'-position of the sugar moiety. Absent in DNA and present in RNA, different preferred conformations are adopted by the two closely related classes of nucleic acids. In DNA, the C2'-endo conformation is preferred, whereas RNA preferentially adopts a C3'-endo conformation *(Figure 3.)*, resulting in the (preferred) formation of the B-form double helix structure in DNA and A-form helix structures in RNA *(Figure 4.)*.

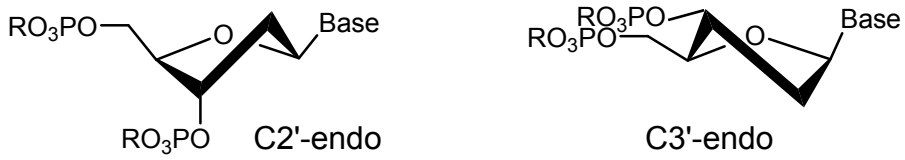


Figure 3: C2' and C3'-endo conformations demonstrated with DNA nucleotides. The inclination of the nucleobase and the progression of the backbone is affected by the conformation of the sugar moiety.

The B-form helix consists in a right handed helical structure with a hydrophobic interior of *Watson-Crick* base pairs stacked nearly perpendicular to the central helix at 3.4 Å intervals, achieving a complete rotation after 10 base pairs (Dickerson et al. 1982) (Wing et al. 1980). The B form of the DNA helix creates two distinct helical grooves, the minor groove and the major groove, providing selective surfaces for the binding of ligands such as proteins or small molecules. Depending on the relative humidity and salt concentration, DNA can also adopt the A-form helix which is characterized by a complete turn after eleven base pairs and a reduced rise per base pair of 2.3 Å (Dickerson et al. 1982) (Frederick et al. 1989).

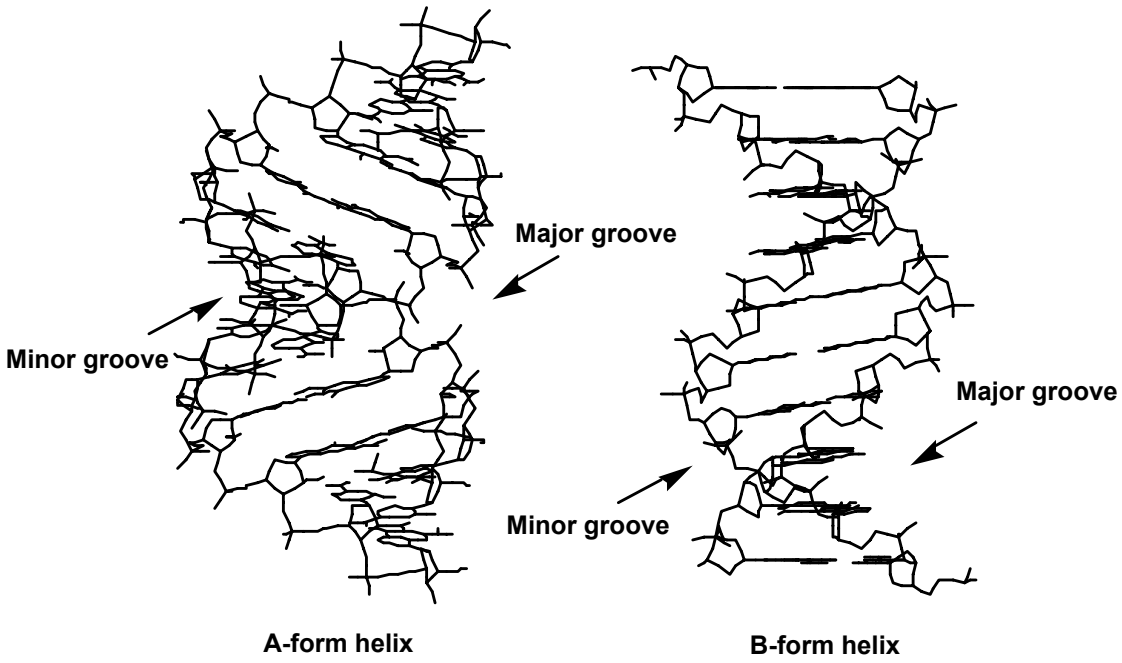


Figure 4: Structure of A form and B form helixes.

The A-type helical orientation is preferred by RNA and results in a deep and narrow major groove and a very shallow and wide minor groove. Another consequence of the presence of a 2'-OH groups in RNA concerns the structure stability of the backbone. RNA is much less stable than DNA specifically under basic conditions which promote strand cleavage by transesterifaction and formation of monomeric nucleotide 2',3'-cyclic phosphodiesters from oligomeric nucleotide 5',3'-linear phosphodiesters *(Figure 5.)*. A multitude of enzymes (RNases) exploit the same mechanism and are devoted to their degradation (Knapinska et al. 2005) which results in a relative short life-cycle of RNA in the cell.

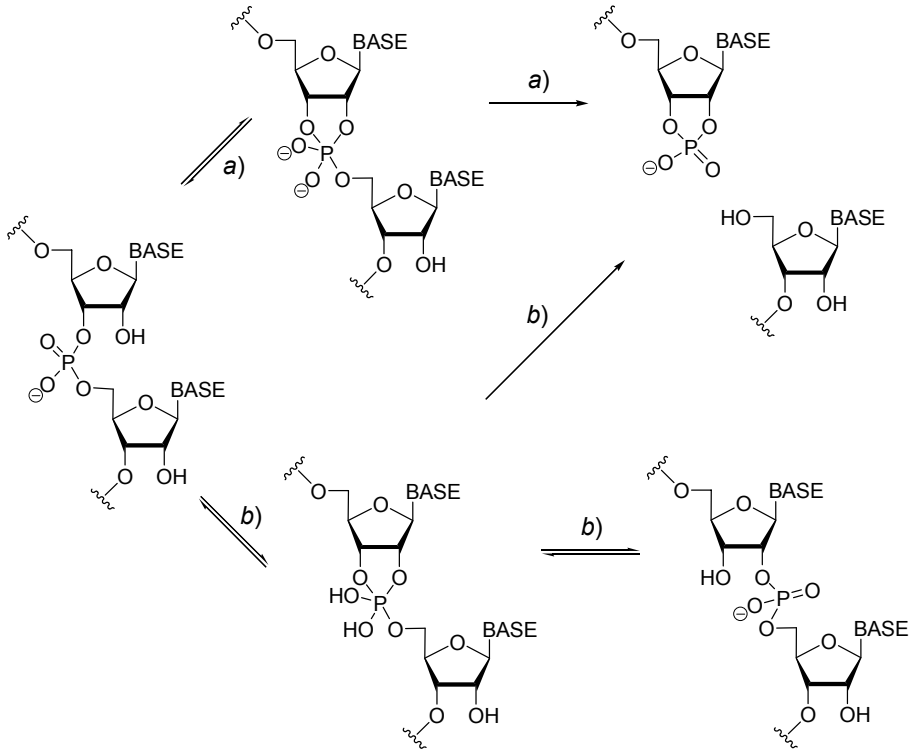


Figure 5: Influence of the 2'-OH group on the stability the phosphodiester bond in RNA. *a*) pH > 12. *b*) pH < 2.

These structural and chemical features confer distinctive biological properties to these two types of nucleic acids. Whereas the role of DNA is well confined to the storage of genetic information, RNA displays a multitude of functions. Most of these functions have been identified only recently by isolation, characterization and study of the wide-ranging family of "non-coding" or "functional" RNAs (ncRNAs, fRNAs). They appear to interfere with the RNA processing, editing, targeting and regulation (Eddy 2001). Among them, small nuclear RNAs (snRNAs) play a central role during the assembly of the mRNA-splicing complexes by associating with proteins to form ribonucleoprotein (RNP) complexes (Nagai et al. 2001). Small nucleolar RNAs (snoRNAs) interact sequence-specifically with rRNA to guide processing and modification by the corresponding RNA-editing enzymes (Lau et al. 2001). Micro-RNAs (miRNAs) have shown to act as posttranscriptional downregulaters of protein expression by inducing the cleavage of specific mRNAs (Elbashir et al. 2001). Very importantly, it has been discovered that the same intrinsic mechanism of gene-silencing can be triggered by artificial short interfering RNAs (siRNAs) (Goldstrohm et al. 2001). The

concepts of gene-expression have been enriched by the discovery of the phenomenon of "alternative splicing" which leads to the generation of alternative proteins from one gene; this process is regulated by consensus sequences (e.g. polypyrimidine tracks) located in intron regions (Winkler and Breaker 2003). Among mRNAs, so-called "riboswitch" motives have been found which regulate or control gene expression on the level of translation or transcription by changing their structure upon binding of metabolites, or 3'-end processing (Nagel and Pleij 2002).

Before these recent developments, the study of RNA was devoted to the three most abundant forms, which are directly involved in the biosynthesis of proteins: the ribosomal RNA (rRNA), the messenger RNA (mRNA) and transfer RNA (tRNA), occurring in all forms of life.

2. The messenger RNA (mRNA)

The mRNAs represent the link between the genotype (DNA) and the phenotype (proteins). It is the carrier of the genetic information (DNA) to the ribosome where this information is expressed as proteins sequences. In prokaryotes, RNA copies (transcripts) of specific DNA sequences (genes) are exclusively constituted of the four canonical nucleotides. In eukaryotes, however, a multitude of modification processes take place. The eukaryotic pre-mRNA obtained from the direct transcription of DNA within the nucleus, is subjected to 5'-end capping, splicing, 3'-end cleavage and polyadenylation (Gu and Lima 2005). Importantly, pre-mRNAs can be spliced differently, depending on the context of the cell, finally resulting in different proteins from the same gene. This process has been termed "alternative splicing" and is responsible for the seemingly paradox situation that the number of proteins greatly exceed the number of genes. The exact comprehension of this phenomenon is extremely challenging and represents a highly active field of research (Moore 2005) (Oberstrass et al. 2005).

However, the mRNAs mainly consist of a linear sequence of the four canonical ribonucleotides, which can be assembled in any possible order. The resulting nucleotide-code

is converted into a sequence of amino acids (proteins) by translation within the ribosome. Thereby, 64 different combinations (= 4^3) of nucleotide triplets are required for encoding the 20 proteinogenic amino acids *(Figure 6.)*. These nucleotide triplets, named codons, have defined functions within the genetic code. Three of them are devoted to stop-signals (UAG, UAA and UGA) for termination of translation, one to a start-signal (methionine) for initiation of translation, and the remaining 60 codons are all encoding for amino acids. There is at least one specific codon for each of the 20 amino acids; some of them are encoded by several codons with different level of degeneracy (4-fold degenerate, e.g. leucine; 2-fold degenerate e.g. histidine and glutamine; only one codon, e.g. tyrosine). The resulting code contained in the mRNA is accurately translated into proteins, with only one in ten thousand amino acids incorporated incorrectly (Thompson and Karim 1982) (Loftfield and Vanderjagt 1972).

	2nd Letter									
	U		C		A		G			
1st Letter	UUU	Phe	UCU	Ser	UAU	Tyr	UGU	Cys	U	3rd Letter
	UUC	Phe	UCC	Ser	UAC	Tyr	UGC	Cys	C	
U	UUA	Leu	UCA	Ser	UAA	Stop	UGA	Stop	A	
	UUG	Leu	UCG	Ser	UAG	Stop	UGG	Trp	G	
	CUU	Leu	CCU	Pro	CAU	His	CGU	Arg	U	
	CUC	Leu	CCC	Pro	CAC	His	CGC	Arg	C	
C	CUA	Leu	CCA	Pro	CAA	Gln	CGA	Arg	A	
	CUG	Leu	CCG	Pro	CAG	Gln	CGG	Arg	G	
	AUU	Ile	ACU	Thr	AAU	Asn	AGU	Ser	U	
	AUC	Ile	ACC	Thr	AAC	Asn	AGC	Ser	C	
A	AUA	Ile	ACA	Thr	AAA	Lys	AGA	Arg	A	
	AUG	Met	ACG	Thr	AAG	Lys	AGG	Arg	G	
	GUU	Val	GCU	Ala	GAU	Asp	GGU	Gly	U	
	GUC	Val	GCC	Ala	GAC	Asp	GGC	Gly	C	
G	GUA	Val	GCA	Ala	GAA	Glu	GGA	Gly	A	
	GUG	Val	GCG	Ala	GAG	Glu	GGG	Gly	G	

Figure 6: The genetic code with the "triplet" codons and their corresponding amino-acid (three letter code).

3. The ribosomal RNA (rRNA)

Ribosomes are huge RNA/protein complexes of almost 2.5MDa size (Dahlberg 1989) (Noller 2005) (Clemons et al. 1999) (Cate et al. 1999) *(Figure 7.)*.

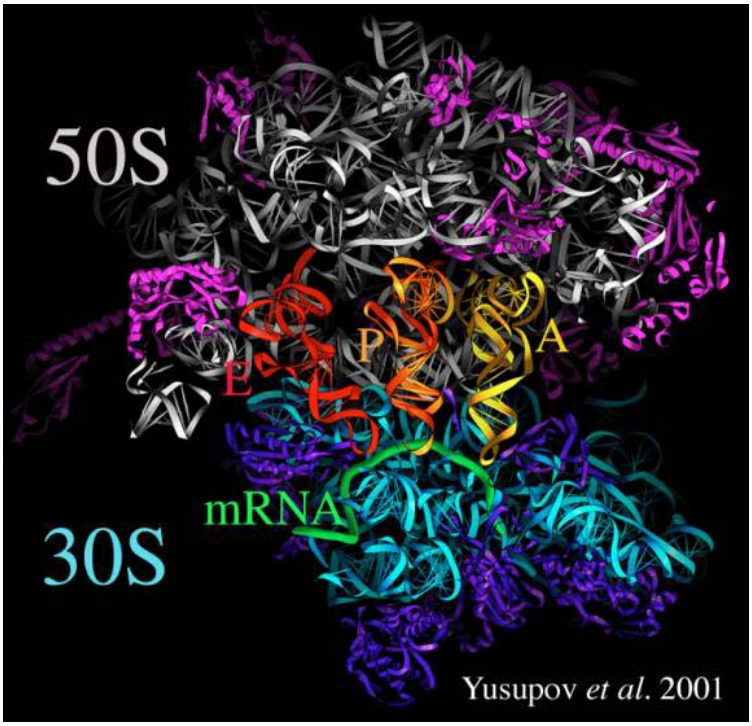


Figure 7: Three dimensional structure of the ribosome with three tRNAs in A, P and E sites taken from (Yusupov et al. 2001))

They consist of two subunits, (the large and the small subunit), containing several rather small proteins, some RNA-sequences of different length (ribosomal RNA, rRNA), depending on the organism *(Figure 8.)*.

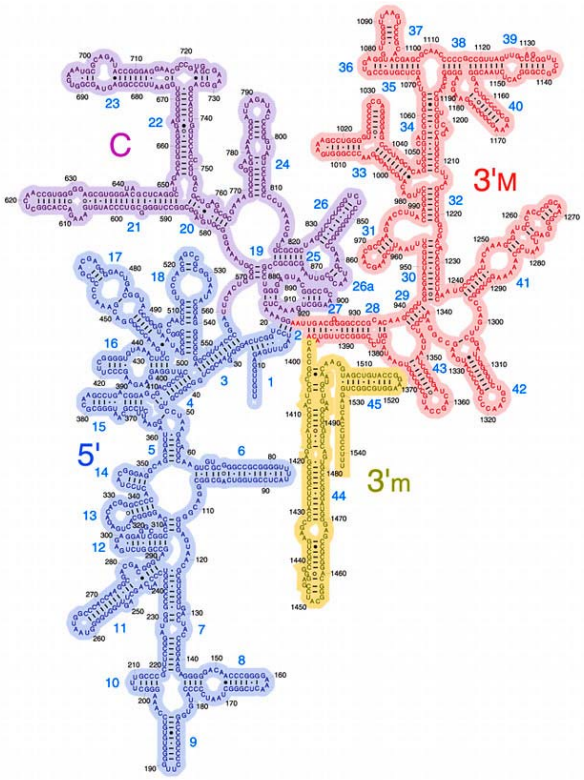


Figure 8: Two-dimensional structure of the ribosomal RNA (rRNA) [1]).

The ribosomes are responsible for bringing together the mRNAs and the aminoacylated tRNAs and for catalyzing the formation of proteins from the amino acids attached to the tRNAs. With the help of several proteins (initiation factors) and a specifically aminoacylated tRNA (with an anticodon complementary to the start-codon of an mRNA and a methionine or *N*-formylmethionine as amino acid), the two subunits are assembled on the 5'-end of the mRNA and translation can start. The elongation step is carried out by first binding an aminoacylated tRNA with an anticodon complementary to the codon following the start-codon *(Figure 9.)*. Next, the amino-group of the amino acid of the aminoacylated tRNA is attacking the carbonyl-group of the methionine (or *N*-formylmethionine) bound to the first tRNA, thereby forming the first peptide-bond of the protein to be synthesized and releasing the methionine from its tRNA. This free (non-aminoacylated) tRNA is now released, the other tRNA, carrying now a dipeptide is translocated (together with the mRNA), and another

[1]) http://www.learner.org/channel/courses/biology/archive/images/1705.html

elongation step takes place in a completely analogous manner. Thereby the peptide is formed by stepwise assembly of each amino acid. The non-aminoacylated tRNA is charged again with the correct amino acid by the corresponding aminoacyl-tRNA synthetase (aaRS) and thereby, continues to participate to the protein synthesis. The peptide continues to grow until one of the three stop-codons is reached *(Figure 10.)*. Since no complementary tRNA is present, the translation stops and eventually a release-factor (a protein with the shape of a tRNA) binds to the ribosome and the stop-codon of the mRNA, and cleaves the ester bond between the full-length protein and the last tRNA by hydrolysis. The resulting complex dissociates into the two ribosomal subunits, the mRNA and the protein.

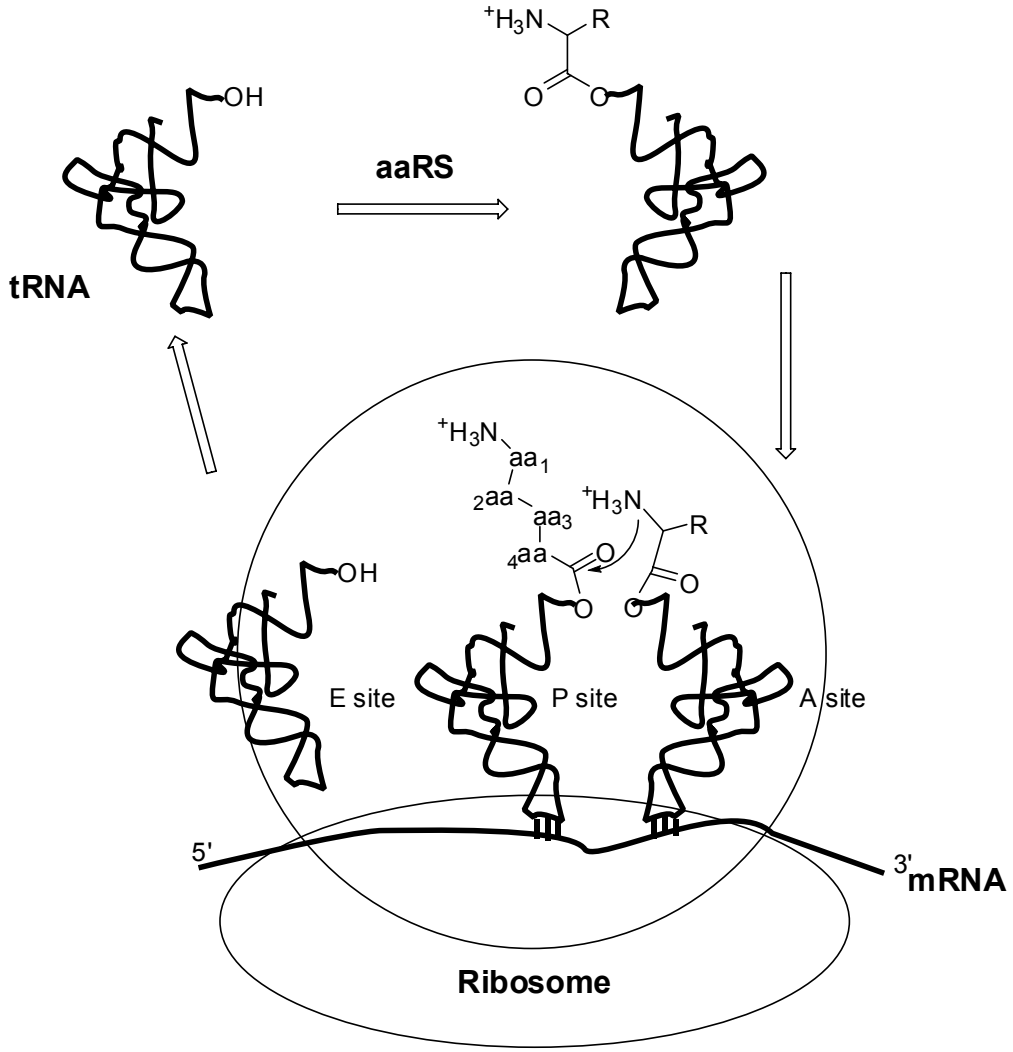


Figure 9: The process of protein synthesis.

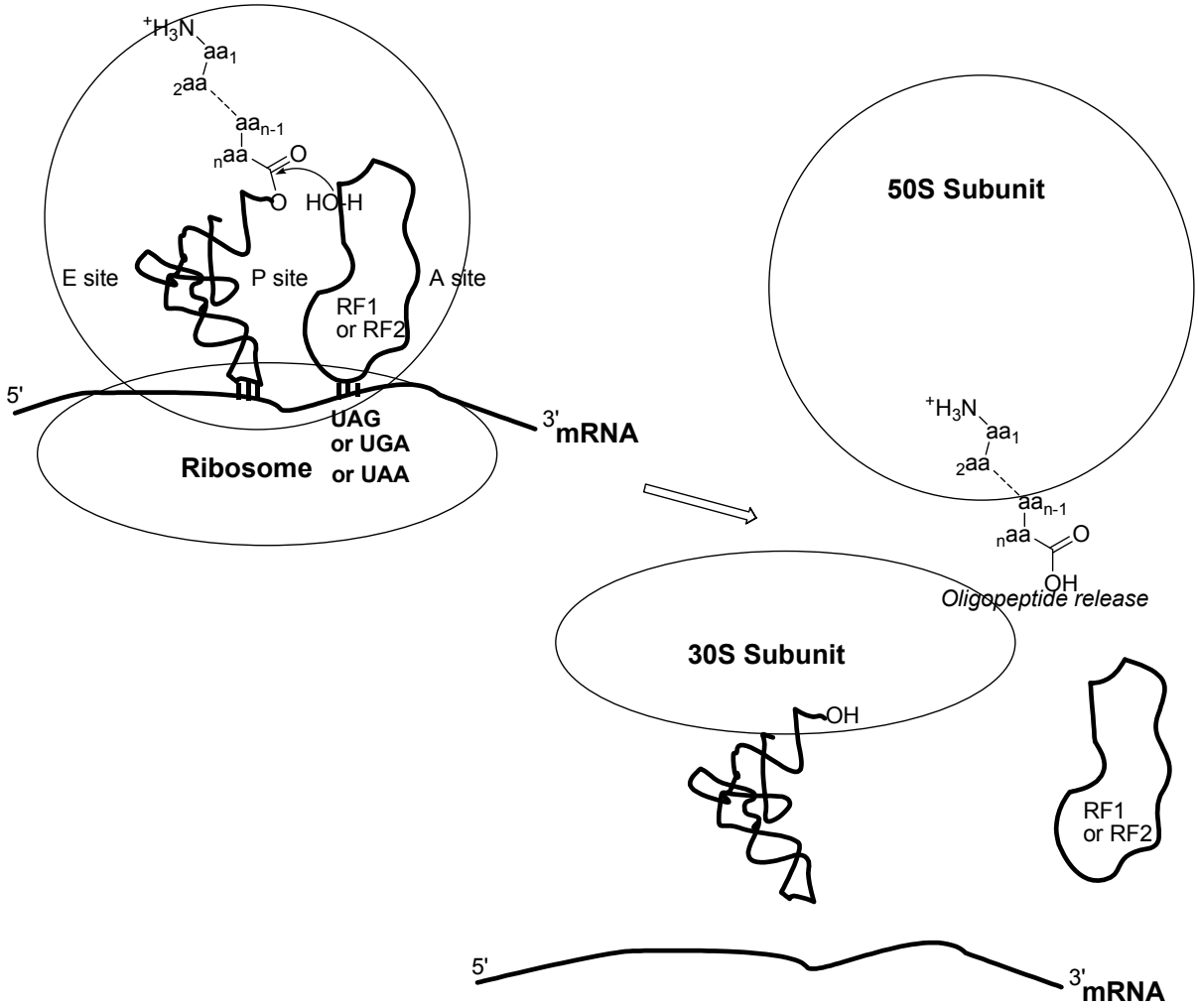


Figure 10: The termination of the protein synthesis.

Interestingly, during the study of the ribosome, it has been demonstrated that the ribosomal RNA supported the catalytic activity of the peptide bond formation (Orgel 2004). This observation has given a significant argument for partisans of the RNA world theory, but it has also given new interest for RNA.

4. The transfer RNA (tRNA)

Transfer RNAs are relatively short RNA sequences which consist of 56 to 95 nucleotides. The cytoplasmic class I tRNAs have a typical length of 76 nucleotides which can be extended up to 86 by the presence of a long variable arm specific of class II tRNAs. Beside these most encountered and conventional lengths, exceptions are found that always correspond to tRNAs displaying special functionalities. Therefore, the $tRNA^{Sec}$ of 95

nucleotides length is designed for the incorporation of selenocysteine, the 21st amino acid, by possessing the unique capacity to read the UGA STOP codon (Commans and Bock 1999). Other tRNAs have a singular structure in relation to their origin tRNAs (Giegé and Frugier 2003) (Florentz et al. 2003), as observed for mitochondrial species that can miss the D arm (for instance in human mitochondrial $tRNA^{Ser}$ (de Bruijn and Klug 1983), the T arm or both. Despite their different lengths, sequences and modifications, they have geometrically very similar secondary cloverleaf and three-dimensional, L-shaped, structures (Kim et al. 1973) (Kim et al. 1974) (Robertus et al. 1974) (Stout et al. 1974) *(Figure 11.)*, even the bizarre mitochondrial tRNAs (de Bruijn and Klug 1983). The presence of conserved (U8, A14, A21, U33, G53, T54, Ψ55, C56, A58, C61, C74, C75 and A76) and semiconserved (Y11, R15, R24, Y32, R37, Y48, R57, and Y60) nucleotides in tRNA *(Figure 12.)*[2]) accounts for its evolutionary conserved L-shaped architecture. Accumulation of sequence and structure information of many tRNAs[3]) has shown that all exhibit the same distance of 100 Å between the ends of the two "L"-arms.

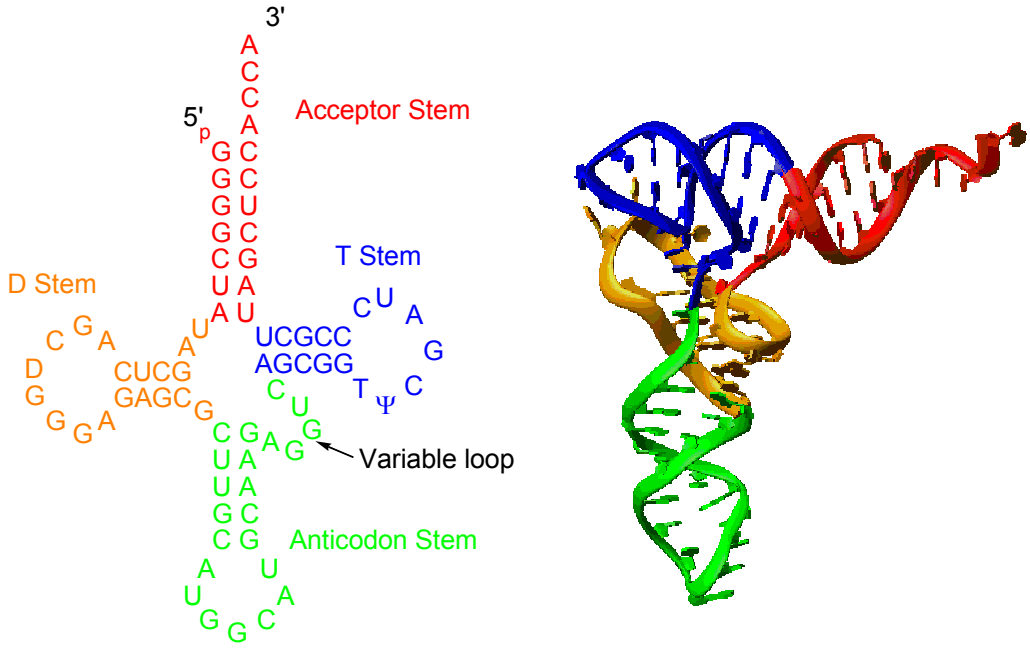


Figure 11: Typical and common cloverleaf secondary structure of tRNAs (*left*) and typical L-shape tertiary structure of tRNAs (*right*) [4]).

[2]) (http://medlib.med.utah.edu/RNAmods/)

[3]) (http://www.uni-bayreuth.de/departments/biochemie/trna/)

[4]) http://www.stanford.edu/~esorin/trna_cartoon.gif

The end of the so-called anticodon branch (comprising the anticodon and D stems) contains a three-nucleotide sequence, named the anticodon, which is complementary to the three-nucleotide codon-sequences of the mRNAs. At the end of the other branch, a specific amino acid is attached enzymatically to the 2'/3'-OH groups of the 3'-terminal adenosine of the highly conserved CCA sequence. Consequently, the tRNAs are at the same time carrying amino acids and decoding the codon-information of the mRNAs, thereby providing the link between information (mRNA-sequence) and sequence-specific assembly of proteins from amino acids. The highly accurate loading of a given tRNA with the corresponding amino acid is carried out by enzymes, named aminoacyl-tRNA synthetases (aaRS) by a two step process. Initially the amino acid and a molecule of ATP are bound to the synthetase where they are covalently linked by ester bond, forming a pyrophosphate and an enzyme bound aminoacyl-adenylate complex. In the second step, the amino acid is then transferred to the 3'-end of the tRNA.

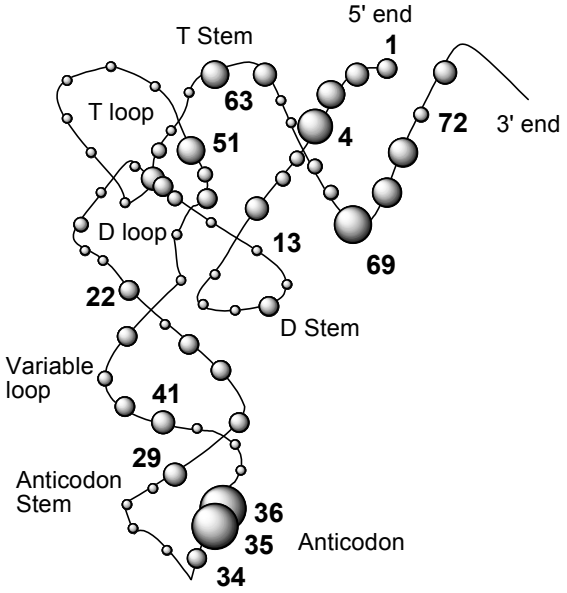
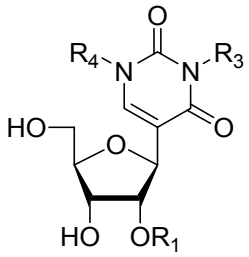


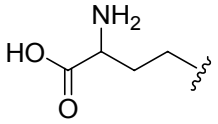
Figure 12: Identity elements in tRNA for recognition by aminoacyl-tRNA synthases; the size of spheres is proportional to the likelihood of putative identity position for aminoacylation (McClain and Nicholas 1987). For an updated view on identity see (Giegé et al. 1998) (Beuning and Musier-Forsyth 1999) (Giegé and Frugier 2003).

The connection of an amino acid to a wrong tRNA would result in false amino acid incorporations into proteins (Chapeville et al. 1962). Accurate tRNA aminoacylation is under the control of identity rules that allow a given tRNA to be recognized by its cognate aminoacyl-tRNA synthetase. For each amino acid specificity, a small number of positive nucleoside determinants, constituting the so-called 'identity sets', confers the aminoacylation identity to the tRNA. Some of these determinants can be considered as 'strong' since their mutation strongly reduces the aminoacylation capacity of the mutant tRNA, others are 'moderate' or 'weak'. Additionally, other nucleosides that may display modifications, can act as negative signals, i.e. antideterminants, by preventing tRNAs to be recognized by noncognate synthetases. As a typical example, aspartate identity for tRNA aspartylation in yeast in given by a set of 6 positive determinants, namely anticodon G34U35C36, C38, G73 and base-pair G10-U25, with anticodon and G73 the strongest determinants and the methyl group of m^1G37 being an antideterminant preventing $tRNA^{Asp}$ to be arginylated by yeast arginyl-tRNA synthetase (reviewed in Giegé and Frugier, 2002). Notice, that modified nucleosides, beside an antideterminant role in some tRNAs, can have a diversity of structural and functional roles in tRNA, such as triggering the correct folding of some tRNAs (Helm et al. 1998) or reducing frame-shift events (Liu et al. 1997) (Wang et al. 2001) (Kowal et al. 2001).

5. The modified nucleosides of tRNAs

In 1951, a modified nucleoside differing from the four canonical nucleosides was isolated for the first time (Cohn and Volkin 1951). Later characterized as pseudouridine, a 5-ribosyl isomer of uridine (Davis et Allen 1957), this modification is widely distributed in most types of RNAs (1 to 2% of total RNA), with the noticeable exception of mRNA (although its absence remains to be definitively proven (Grosjean. 2005)) *(Figure 13.)*. Other modified nucleosides are present in much smaller quantities, but they represent a large diversity of original structures and are mainly found in tRNAs (66% of the known modifications) (Grosjean and Benne 1998) *(Figure 14-18.)*.

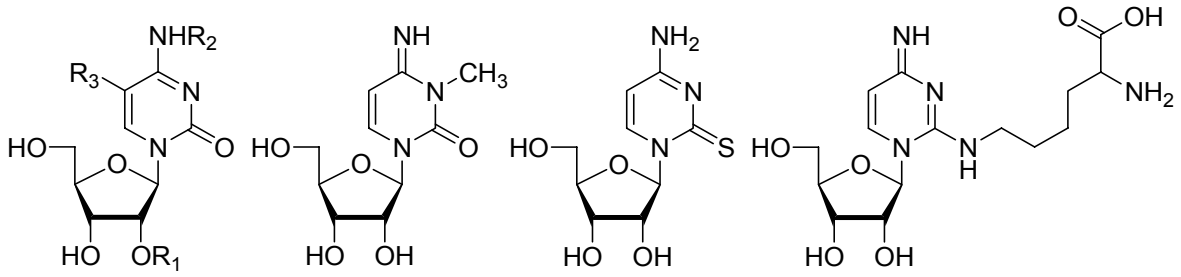




3-amino-3-carboxypropyl (acp)

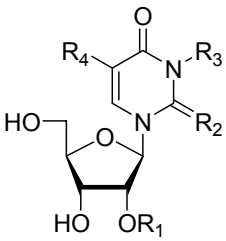
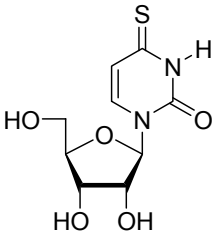
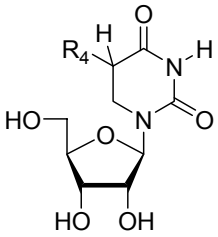
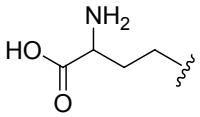
#	Symbol	Name	R1	R3	R4
1	ψ	Pseudouridine	H	H	H
2	ψm	2'-*O*-methylpseudouridine	CH_3	H	H
3	$m^1ψ$	1-methylpseudouridine	H	H	CH_3
4	$m^3ψ$	3-methylpseudouridine	H	CH_3	H
5	$m^1acp^3ψ$	1-methyl-3-(3-amino-3-carboxypropyl)pseudouridine	H	acp	CH_3

Figure 13: The listing of modified nucleosides found in rRNA, mRNA and tRNA: pseudouridine derivatives (name, symbol and structure).



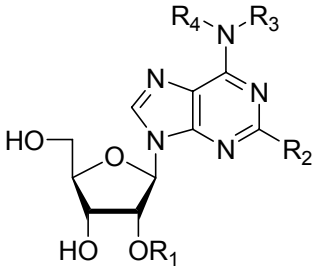
#	Symbol	Name	R1	R3	R4
1	Cm	2'-*O*-methylcytidine	CH_3	H	H
2	m^4C	N^4-methylcytidine	H	CH_3	H
3	m^4Cm	N^4,2'-*O*-dimethylcytidine	CH_3	CH_3	H
4	ac^4C	N^4-acetylcytidine	H	Ac	H
5	ac^4Cm	N^4-acetyl-2'-*O*-methylcytidine	CH_3	Ac	H
6	m^5C	N^5-methylcytidine	H	H	CH_3
7	m^5Cm	N^5,2'-*O*-dimethylcytidine	CH_3	H	CH_3
8	hm^5C	5-hydroxymethylcytidine	H	H	CH_2OH
9	f^5C	5-formylcytidine	H	H	CHO
10	f^5Cm	2'-*O*-methyl-5-formylcytidine	CH_3	H	CHO
11	m^3C	3-methylcytidine	-	-	-
12	s^2C	2-thiocytidine	-	-	-
13	k^2C	Lysidine	-	-	-

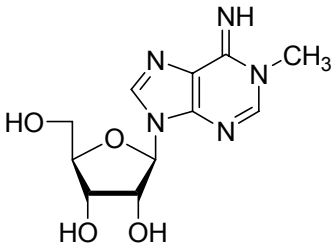
Figure 14: The listing of modified nucleosides found in rRNA, mRNA and tRNA: cytidine derivatives (name, symbol and structure).

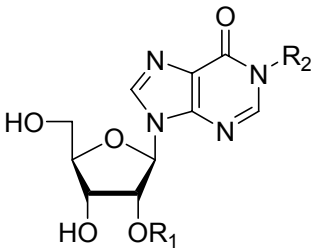





#	Symbol	Name	R1	R2	R3	R4
1	Um	2'-*O*-methyluridine	CH_3	O	H	H
2	s^2U	2-thiouridine	H	S	H	H
3	s^2Um	2-thio-2'-*O*-methyluridine	CH_3	S	H	H
4	m^3U	3-methyluridine	H	O	CH_3	H
5	m^3Um	3,2'-*O*-dimethyluridine	CH_3	O	CH_3	H
6	acp^3U	3-(3-amino-3-carboxypropyl)uridine	H	O	acp	H
7	s^4U	4-thiouridine	-	-	-	-
8	m^5U	ribosylthymine	H	O	H	CH_3
9	m^5Um	5,2'-*O*-dimethyluridine	CH_3	O	H	CH_3
10	m^5s^2U	5-methyl-2-thiouridine	H	S	H	CH_3
11	ho^5U	5-hydroxyuridine	H	O	H	OH
12	mo^5U	5-methoxyuridine	H	O	H	OCH_3
13	cmo^5U	uridine 5-oxyacetic acid	H	O	H	OCH_2COOH
14	$mcmo^5U$	uridine 5-oxyacetic acid methyl ester	H	O	H	OCH_2COOCH_3
15	cm^5U	5-carboxymethyluridine	H	O	H	CH_2COOH
16	mcm^5U	5-methoxycarbonyl methyluridine	H	O	H	CH_2COOCH_3
17	mcm^5Um	5-methoxycarbonylmethyl-2'-*O*-methyl uridine	CH_3	O	H	CH_2COOCH_3
18	mcm^5s2U	5-methoxycarbonylmethyl-2-thiouridine	H	S	H	CH_2COOCH_3
19	ncm^5U	5-carbamoylmethyluridine	H	O	H	CH_2CONH2
20	ncm^5Um	5-carbamoylmethyl-2'-*O*-methyl uridine	CH_3	O	H	CH_2CONH2
21	chm^5U	5-(carboxyhydroxymethyl) uridine	H	O	H	CH(OH)COOH
22	mchm5U	5-(carboxyhydroxymethyl) uridine methyl ester	H	O	H	$CH(OH)COOCH_3$
23	nm^5s2U	5-aminomethyl-2-thiouridine	H	S	H	CH_2NH_2
24	mnm^5U	5-methylaminomethyluridine	H	O	H	CH_2NHCH_3
25	mnm^5s^2U	5-methylaminomethyl-2-thiouridine	H	S	H	CH_2NHCH_3
26	mnm^5se^2U	5-methylaminomethyl-2-selenouridine	H	Se	H	CH_2NHCH_3
27	$cmnm^5U$	5-carboxymethylamino-methyluridine	H	O	H	CH_2NHCH_2COOH
28	$cmnm^5Um$	5-carboxymethylaminomethyl-2'-*O*-methyluridine	CH_3	O	H	CH_2NHCH_2COOH
29	$cmnm^5s^2U$	5-carboxymethylaminomethyl-2-thiouridine	H	S	H	CH_2NHCH_2COOH
30	D	dihydrouridine	H	O	H	H
31	m^5D	dihydrothymine	H	O	H	CH_3

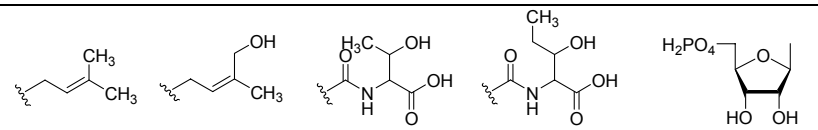
Figure 15: The listing of modified nucleosides found in rRNA, mRNA and tRNA: uridine derivatives (name, symbol and structure).





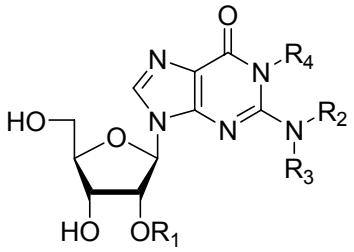


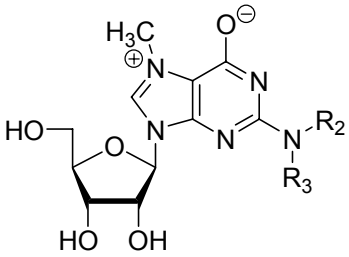
#	Symbol	Name	R1	R2	R3	R4
Adenosine derivatives						
1	Am	2'-*O*-methyladenosine	CH_3	H	H	H
2	m^2A	2-methyladenosine	H	CH_3	H	H
3	m^6A	N^6-methyladenosine	H	H	H	CH_3
4	$m^6{}_2A$	N^6,N^6-dimethyladenosine	H	H	CH_3	CH_3
5	m^6Am	N^6,2'-*O*-dimethyladenosine	CH_3	H	H	CH_3
6	$m^6{}_2Am$	N^6,N^6,2'-*O*-trimethyl adenosine	CH_3	H	CH_3	CH_3
7	ms^2m^6A	2-methylthio-N^6-methyl adenosine	H	CH_3S-	H	CH_3
8	i^6A	N^6-isopentenyladenosine	H	H	H	Dimethylallyl
9	ms^2i^6A	2-methylthio-N^6-isopentenyl adenosine	H	CH_3S-	H	Dimethylallyl
10	io^6A	N^6-(*cis*-hydroxyisopentenyl) adenosine	H	H	H	*cis*-hydroxy-methyl-allyl
11	ms^2io^6A	2-methylthio-N^6-(*cis*-hydroxyisopentenyl) adenosine	H	CH_3S-	H	*cis*-hydroxy-methyl-allyl
12	g^6A	N^6-glycinylcarbamoyl adenosine	H	H	H	-CONH-
13	t^6A	N^6-threonylcarbamoyl adenosine	H	H	H	threonyl carbamoyl
14	m6t6A	N^6-methyl-N^6-threonyl carbamoyladenosine	H	H	CH_3	threonyl carbamoyl
15	ms^2t^6A	2-methylthio-N^6-threonyl carbamoyladenosine	H	CH_3S-	H	threonyl carbamoyl
16	hn^6A	N^6-hydroxynorvalyl carbamoyladenosine	H	H	H	hydroxynorvalyl carbamoyl
17	ms^2hn^6A	2-methylthio-N^6-hydroxynorvalyl carbamoyladenosine	H	CH_3S-	H	hydroxynorvalyl carbamoyl
18	Ar(p)	2'-*O*-ribosyladenosine (phosphate)	2'-O-ribosyl	H	H	H
19	m^1A	1-methyladenosine	-	-	-	-
Inosine derivatives						
20	I	inosine	H	H	-	-
21	Im	2'-*O*-methylinosine	CH_3	H	-	-
22	m^1I	1-methylinosine	H	CH_3	-	-
23	m^1Im	1,2'-*O*-dimethylinosine	CH_3	CH_3	-	-



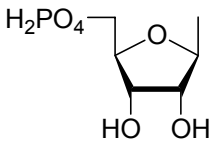
Dimethylallyl (isopentenyl) | cis-hydroxymethyl -methylallyl (cis-hydroxyisopentenyl) | threonylcarbamoyl | hydroxynorvalylcarbamoyl | 2'-O-ribosyl

Figure 16: The listing of modified nucleosides found in rRNA, mRNA and tRNA: adenosine derivatives (name, symbol and structure).



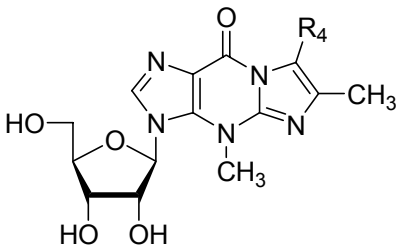


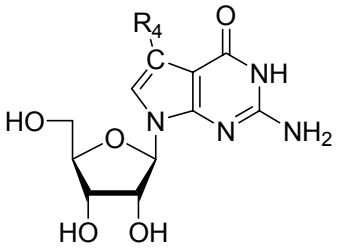
#	Symbol	Name	R1	R2	R3	R4
Guanosine derivatives						
1	Gm	2'-*O*-methylguanosine	CH_3	H	H	H
2	m^1G	1-methylguanosine	H	H	H	CH_3
3	m^2G	N^2-methylguanosine	H	CH_3	H	H
4	$m^2{}_2G$	N^2,N^2-dimethyl guanosine	H	CH_3	CH_3	H
5	m^2Gm	N^2,2'-*O*-dimethylguanosine	CH_3	CH_3	H	H
6	$m^2{}_2Gm$	N^2,N^2,2'-*O*-trimethyl guanosine	CH_3	CH_3	CH_3	H
7	Gr(p)	2'-*O*-ribosylguanosine (phosphate)	2'-O-ribosyl	H	H	H
m^7 Guanosine derivatives						
8	m^7G	7-methylguanosine	-	H	H	-
9	$m^2{}_,{}^7G$	N^2,7-dimethyl guanosine	-	CH_3	H	-
10	$m^2{}_2{}_,{}^7G$	N^2,N^2,7-trimethyl guanosine	-	CH_3	CH_3	-



2'-O-ribosyl

Figure 17: The listing of modified nucleosides found in rRNA, mRNA and tRNA: guanosine derivatives (name, symbol and structure).





#	Symbol	Name	R1	R2	R3	R4
Wyosine derivatives						
1	imG	wyosine	-	-	-	
2	mimG	methylwyosine	-	-	-	CH_3
3	OHyW*	undermodified hydroxywybutosine	-	-	-	S1
4	yW	wybutosine	-	-	-	S2
5	OHyW	hydroxywybutosine	-	-	-	β-hydroxy-S2
6	O_2yW*	peroxywybutosine	-	-	-	β-peroxy-S2
7-deazaguanosine derivatives						
7	Q	queuosine	-	-	-	S3
8	oQ	epoxyqueuosine	-	-	-	epoxy-S3
9	galQ	galactosyl-queuosine	-	-	-	gal-S3
10	manQ	mannosyl-queuosine	-	-	-	man-S3
11	$preQ_0$	7-cyano-7-deazaguanosine	-	-	-	-CN
12	gQ	Archaeosine	-	-	-	$-C(NH_2)=NH$
13	$preQ_1$	7-aminomethyl-7-deazaguanosine	-	-	-	CH_2NH_2

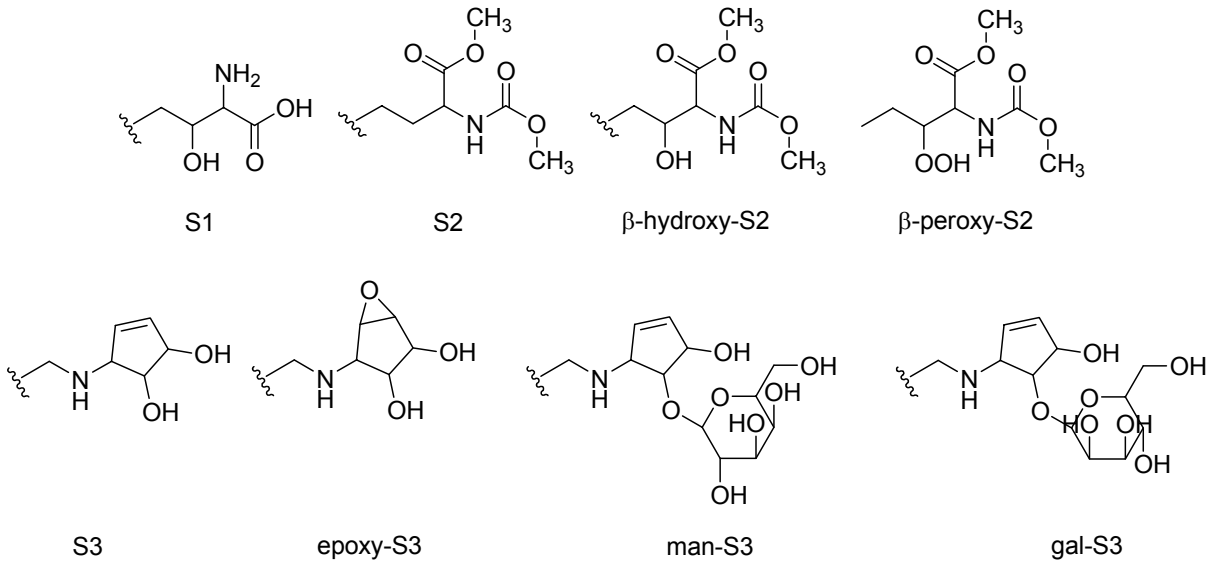


Figure 18: The listing of modified nucleosides found in rRNA, mRNA and tRNA: wyosine derivatives (name, symbol and structure).

Among the known structures, the methylated forms of the canonical nucleotides are structurally the most simple ones *(Figure 14-17.)*. The methylation can occur at the base or at the 2'-*O*-position of the sugar moiety. The latter type of modification is very common and is found in almost all kinds of RNAs (mRNA, snRNA, rRNA and tRNA) and sometimes even in combination with base-methylation (e.g. m^6Am, m^3Um). The nucleobase methylation occurs primarily by replacement of a H-N hydrogen or, to a lesser extent, at nitrogen (m^7G and m^1A), leading to a high sensitivity towards basic media (Hendler et al. 1970) (Helm et al. 1999) (Chapter I). The same chemical instability is also observed for the dihydrouridine nucleobase which has lost its aromatic character by having a saturated C5-C6 bond *(Figure 15.)* (Chapter I and V). More generally, the chemical reactivity is unique for each modified nucleoside, obliging to reconsider synthetic approaches and protecting group for every new compound.

In contrast to their rather simple three-dimensional structure, these modifications render the tRNAs complex chemical structures. Within the sequence of tRNAs different types of modifications are found at different positions, strongly indicating a specific purpose. Guanosine, although often simply methylated (m^2G, $m^2{}_2G$, m^1G) *(Figure 17.)*, can be integrated into an extended imidazole ring, which creates an intriguing tricyclic structure, the wyosine *(Figure 18.)* (Chapter III). Three of the structurally simplest versions of this nucleoside are unique to archaea (imG-14, imG2, mimG) and one is in common with eukarya (imG) (Zhou et al. 2004) (Kalhor et al. 2005). The most elaborated versions of these derivatives have an extended linker at the C^7 position and are exclusively found in eukarya (yW, o_2yW, OHyW, OHyW*). Despite this phyllogenetic distribution, all of these tricyclic compounds are exclusively localized at position 37 of the anticodon loop of phenylalanine tRNAs. Similarly, some of the adenosine derivatives (e.g. i^6A, t^6A) are localized also at position 37 of tRNAs where they appear to have a strong influence on the decoding properties of the tRNA (Agris 2004) (Chapter IV).

Within the anticodon loop, other modifications appeared to affect the decoding capacities of the tRNAs, especially those present at position 34. This position offers a wide variety of

modified nucleosides such as inosine. This nucleoside has constituted the first evidence of the "wobble hypothesis" formulated by Crick in 1966 (Crick 1966). According to this concept, some nucleosides should be able to form base pairs with multiple nucleosides by deviating from the conventional *Watson-Crick* H-bond arrangement (Doonan 2004). With the increasing variety of modified nucleosides identified at this position, such as the modified pyrimidines mnm5U, mcm^{5}Um and mo^{5}U, this model has been continuously extended, but the relationship between tRNA structure and codon readout capacities remains nevertheless poorly understood (Agris 2004). In Chapter IV, the modifications present at the now so-called wobble position (position 34) are reviewed and interaction models, which link the structure to the decoding properties, are proposed.

More generally, modified nucleosides are known to ensure the efficiency of recognition of the tRNA by modification enzymes (including RNase P and aminoacyl-tRNA synthetases), protein factors (initiation, elongation, and termination factors) and the ribosome (Agris 2004). Furthermore, the tRNAs seem to play a fundamental role in other biological pathways such as tetrapyrrole biosynthesis (Schubert et al. 2002), to serve as regulator in aromatic amino acid transport (Buck et al. 1981) or as initiation primers of HIV-1 (Rigourd et al. 2003). However, this diversity of functions remains minor in comparison to its contribution as adaptor molecule and amino acid carrier in the protein synthesis.

6. tRNA based incorporation of unnatural amino acids into proteins.

There is a considerable interest in tRNAs which are loaded with unnatural amino acids because it has been shown that such amino acids can be incorporated site-specifically into proteins, e.g. leading to cross-linking reactions with components of membranes or to be used for fluorescence measurements (Wang, Schultz 2002), leading to enzymes with different catalytic or structural properties than the wild-type enzyme (Wang, Schultz 2002) or to caged proteins which regain their function after photoisomerization (Muranaka et al. 2002). In general, this methodology allows the site-specific introduction of almost every feasible amino acid into proteins and is an extremely important tool for the study of protein-interactions, for

functional and structural assays and for the development of designer-enzymes with artificial properties (Wang and Schultz 2005). The ribosome-mediated incorporation of unnatural amino acids into proteins is usually carried out using a combination of genetically engineered DNA (which is transcribed enzymatically into the corresponding mRNA) and an engineered suppressor-tRNA (Wang and Schultz 2005). The latter carries the desired amino acid and has an anticodon-site which is complementary to a natural stop codon introduced site-specifically into the DNA. In this way, the codon usually leading to a stop of the translation process serves as an active codon, allowing incorporation of the unnatural amino acid into the growing peptide chain (Chapter VI) *(Figure 19.)*.

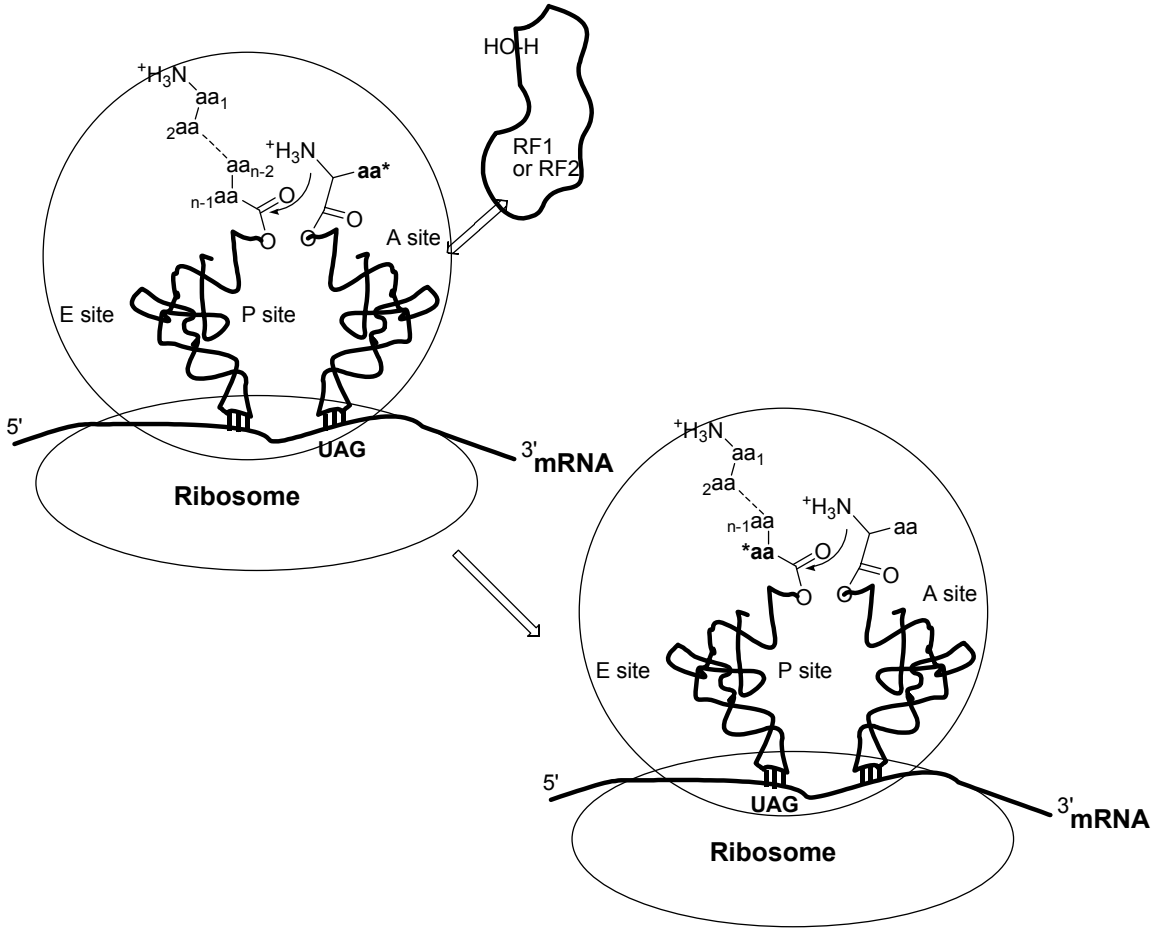
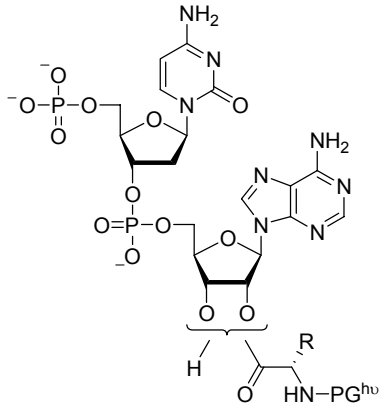


Figure 19. Description of the suppressor tRNA methodology, which exploits the STOP codon. An artificial tRNA has been modified for having the ability to translate a STOP codon as a reading codon.

This approach takes advantage of the fact that the nature of the attached amino acid has no effect on the anticodon-codon recognition with the mRNA (Chapeville et al. 1962). The first

designed tRNA suppressors were elaborated by ligation of a truncated tRNA lacking pCpA end (Heckler et al. 1984) (Heckler et al. 1986) (Roesser et al. 1986) (Heckler et al. 1988) with a suitably protected chemically prepared aminoacylated dimer *(Figure 20.)*.

Structure of the dimer



$PG^{h\upsilon}$ = photocleavable protecting group

Figure 20. Structure of the chemically prepared aminoacylated dimer

A different approach is designed as *in-vivo* process and involves the development of artificial aminoacyl-tRNA-synthetases which recognize the artificial tRNA and connect it to an artificial amino acid (Kiga et al. 2002). This method is in principle very elegant but restricts the choice of the amino acid and has to be adapted to every new combination of tRNA and amino acid. The latest approaches involve ribozymes which catalyze the transfer of the amino acid of short aminoacylated RNA-fragments to a tRNA (Lee et al. 2000), or short peptide nucleic acid (PNA) sequences, which are complementary to the 3'-end of the tRNA, and carry an activated amino acid (connected via a thioester bond and a linker with the PNA), which, after duplex formation, is then transferred to the tRNA (Ninomiya et al. 2004).

During the last years, two concepts to overcome the limitations of the initial suppressor-tRNA approach (based entirely on stop-codons) were developed. The first includes the simultaneous incorporation of unnatural (orthogonal) complementary nucleotides, such as isoC/isoG (Rich 1962) and a thienyl-substituted 2-aminopurine/pyridine-2-one (Fujiwara et al. 2001) (Hirao et al. 2002) into the tRNA and the mRNA respectively (Chapter II). Thereby

new (and orthogonal) codons were created and successfully exploited in the above-mentioned context. The second concept is based on tRNA/mRNA pairs containing four or five complementary RNA-nucleotides as anticodon- and codon-analogues, respectively (Hohsaka et al. 2002) (Hohsaka and Sisido 2002).

The first concept is experimentally more difficult to realize than the second, and consequently only two examples are known so far. In both approaches, the modified mRNA was obtained by transcription of a modified DNA (the modifications were introduced by ligation with a chemically synthesized DNA-fragment), whereas the corresponding tRNA was prepared by ligation of a chemically synthesized RNA-fragment with an enzymatically produced fragment. This approach is very labor-intensive (multi-step preparation of the aminoacylated tRNA) and restricted to modifications, which are transcribed efficiently. The second concept, involving four- or five-nucleotide-codons/anticodons is in principle very simple to realize by standard molecular-biology methods and was already used for the simultaneous introduction of two unnatural amino acids into the same protein (Hohsaka et al. 1999). However, a certain limitation is the requirement to eliminate the corresponding coding triplets from the mRNA.

7. Outlook

The preparation of aminoacylated t-RNA analogues by conventional methods is labor-intensive and can hardly be carried out repetitively or even routinely; they are "of considerable technical difficulty" (Hohsaka and Sisido 2002). Furthermore, protein expression is quite inefficient and only very small quantities of labeled proteins are usually obtained with this approach (typically 5 - 15 percent relative to formation of the parent protein). Although the preparation of artificially aminoacylated tRNAs has been simplified by developping new protecting groups for the synthesis of the dimer (Robertson et al. 1991) (Adams et al. 1989) (Patchornik et al. 1970) (Ellman et al. 1992) (Stutz 2003), it is still the limiting step in this useful strategy. Therefore, new approaches for the aminoacylation of tRNAs should be considered. One such new strategy, which is an adaptation of the "native chemical ligation" of oligopeptides (Dawson and Kent 1997), is presented in Chapter VI.

Since the chemical manipulation of natural tRNA remains a difficult task, most of the work carried out with tRNAs was done with tRNA transcripts which, in contrast to the natural tRNAs, contain only the unmodified nucleosides A, C, G and U. However, in some cases, tRNA transcripts appeared to not bind or poorly to the ribosome (Ashraf et al. 1999) (Ashraf et al. 2000) (Yarian et al. 2000) (Yarian et al. 2002) (vonAhsen et al. 1997) due to the lack of modified nucleosides which normally contribute to the proper folding of a tRNA (Helm et al. 1999) (Chapter I). Furthermore, by using suppressor tRNAs, competition of the tRNAs with the termination-factors (which recognize the stop-codon and subsequently cleave the oligopeptide) takes place, restricting the incorporation efficiency. Therefore, all modifications, which lead to a stronger codon/anticodon pairing, could result in a more efficient introduction of the modified amino acid. In this context particularly relevant are the modified purine-bases in position 37 of the t-RNA-sequences adjacent to the anticodon-site. It has been recognized that these modified nucleobases could be important for a strong codon/anticodon interaction (Agris 2004). They are expected to provide additional pairing-energy by stacking to the last, exposed nucleoside of the codon (Chapter IV). Furthermore, in order to obtain completely orthogonal anticodons, and to avoid competition with the temination factors completely, unnatural codon/anticodon pairs, achievable by synthesizing the corresponding tRNA and mRNA analogues, could be introduced (Chapter II).

The biological introduction of modified nucleosides is under the control of editing enzymes but their application is quite limited since they have not been all identified or purified (Ferré-D'Amaré 2003). Furthermore, the process of the tRNA editing is often highly sequence-dependent, restricting the position choice for their introduction to their natural substrates. Consequently, the flexible preparation of partially modified tRNA derivatives can be achieved only by chemical synthesis (Chapter V). A couple of years ago we created methodologies for the chemical preparation of biologically active aminoacylated tRNAs (Pitsch 2001). Based on the gained experience and on the development of efficient synthetic tools (Stutz 2003) (Wu and Pitsch 1998) (Pitsch et al. 1999) (Pitsch 2001) (Pitsch et al. 2005), we planned to extend

these methodologies to the chemical incorporation of naturally occurring and unnatural modified nucleotides into tRNAs (Chapter V and VI).

CHAPTER I "Synthesis of modified nucleoside phosphoramidite building blocks"

I.1. Introduction

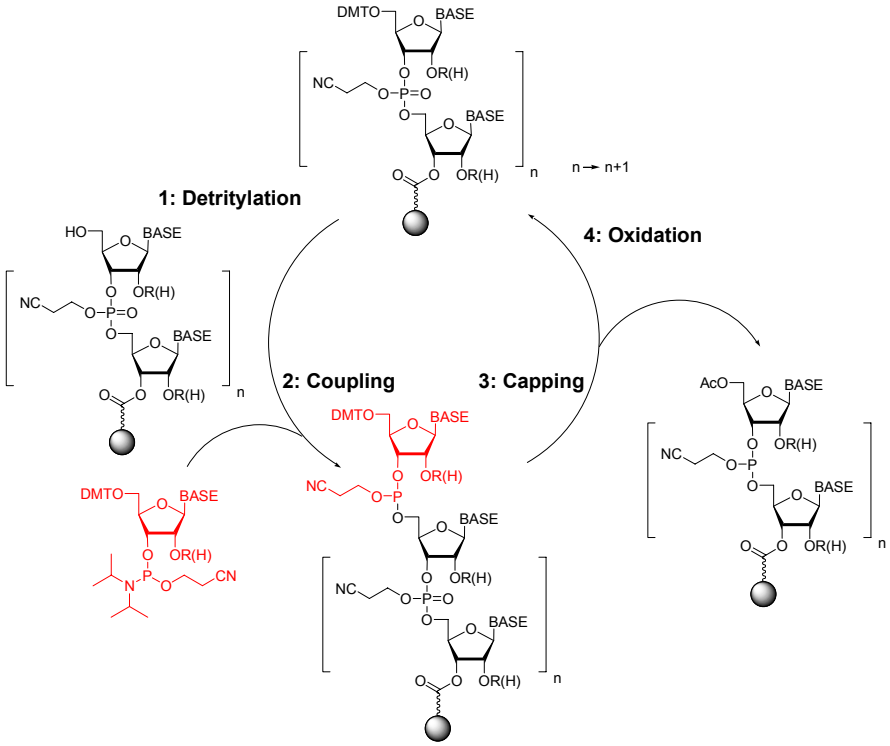
I.1.1. Chemical synthesis of oligonucleotides

This technology, first developed for the preparation of DNA oligonucleotides and then adapted to the preparation of RNA oligonucleotides, is similar to other solid-phase methods, such as peptide synthesis. A controlled pore-glass solid support (CPG), functionalized with a "long chain alkylamine", is bonded with the first (3'-terminal) nucleoside. The solid-phase synthesis is accomplished by stepwise coupling of single building blocks, which are suitably protected and functionalized. The product is then cleaved from the solid support, deprotected and purified. In *Scheme I.1.* such a single reaction cycle is illustrated; the repetition of this cycle leads to the desired oligonucleotide.

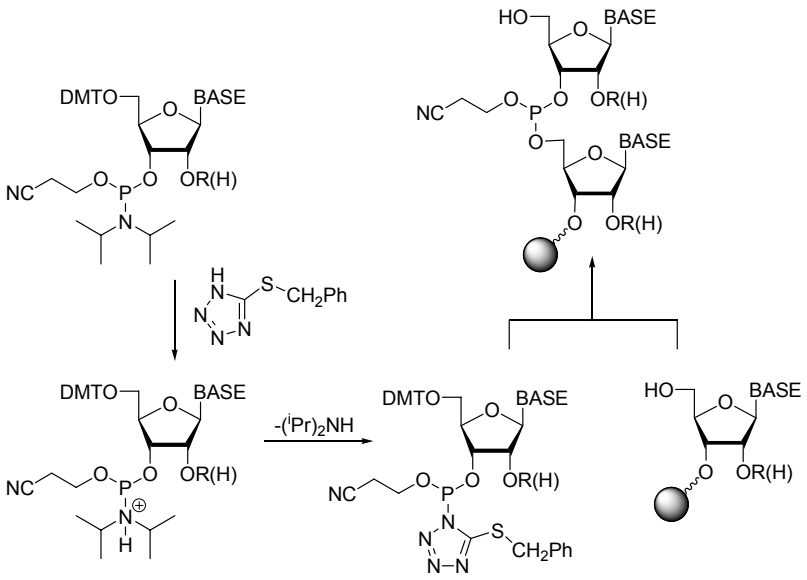
The phosphoramidite building blocks are 5'-*O*-protected with the 4,4'-dimethoxytrityl (= DMT) protecting group, which is removed under acidic conditions (*Scheme I.2.*). This reaction, which constitutes the first step of the cycle, liberates the 5'-OH group. After washing, a mixture of an appropriate phosphoramidite and 1*H*-benzylthio tetrazole is added, which in situ react with each other under the formation of a phosphorotetrazolide. This reactive intermediate is then allowed to react with the previously liberated 5'-OH group of the growing chain. Although quite efficient, usually 90 to 99% of yield, this coupling step leaves a few unreacted 5'-OH groups, which later could lead to the formation of (n-1) sequences that are not easy to remove from the full-length product. In order to prevent an accumulation of these undesired sequences, the unreacted nucleosides are blocked or "capped" by acetylation with acetic anhydride in the presence of *N*-methylimidazole and 2,6-lutidine. The phosphate triester obtained after coupling is prevented from degradation by oxidation with a mixture of iodine, lutidine and water.

After the synthesis, the different protecting groups (cyanoethyl groups, nucleobase protecting groups) and the solid support are cleaved simultaneously by incubation with a 10M solution of methylamine in H_2O/EtOH 1:1 for 6h at room temperature. Filtration of the remaining solid support offers a solubilized mixture of crude sequences containing the shorter (n-1) sequences

and, as main product, the desired sequence. After evaporation, the residue is, for DNA sequence, directly purified by HPLC and, for RNA sequences, treated under specific conditions for removing the 2'-*O*-protecting group.



Scheme I.1. A single cycle of automated oligonucleotide synthesis (OR = suitable protecting group).



Scheme I.2. The activation and coupling step of solid-phase synthesis. (OR = suitable protecting group).

As previously described, the presence of a hydroxyl group at 2'-*O*-position confers special properties to the RNA and especially to its degradation susceptibility towards basic media. Consequently, this position requires a protecting group stable towards basic reagents but also towards acidic media (detritylation cycle during automated synthesis). These requirements incited to employ fluoride-labile protecting groups relying on silyl ethers such as ^{t}BDMS. This protecting group continues to be widely employed but appears less efficient for the preparation of long RNA-sequences (Muller et al. 2004). This severe limitation has been overcome a couple of years ago by introducing the new fluoride labile TOM (={[triisopropylsilyl]oxy}methyl) protecting group (Wu and Pitsch 1998) (Pitsch et al. 1999) (*Figure I.1.*).

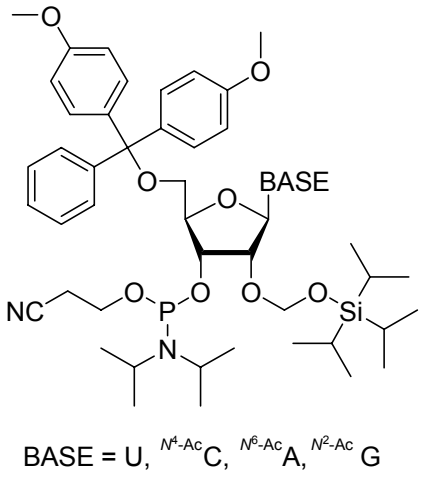


Figure I.1. Structure of 5'-*O*-DMT, 2'-*O*-TOM protected phosphoramidite building blocks.

In contrast to ^{t}BDMS group, the 2'-*O*-TOM group possesses a sterically non-demanding formacetal function preventing any migration or removal during synthesis (Muller et al. 2004) and appears particularly efficient for preparation of long RNA-sequences under DNA coupling conditions (Wu and Pitsch 1998) (Pitsch et al. 1999) (Pitsch et al. 2001). Moreover, its high chemical stability towards a multitude of reagents and reaction conditions *(Figure I.2.)* offers the unique possibility to carry out base transformations in its presence allowing

design of novel and efficient syntheses of 2'-*O*-TOM protected ribonucleoside phosphoramidites of modified nucleobases (Berry et al. 2004) (Hoebartner et al. 2005) (Wu and Pitsch 1998) (Wenter and Pitsch 2003).

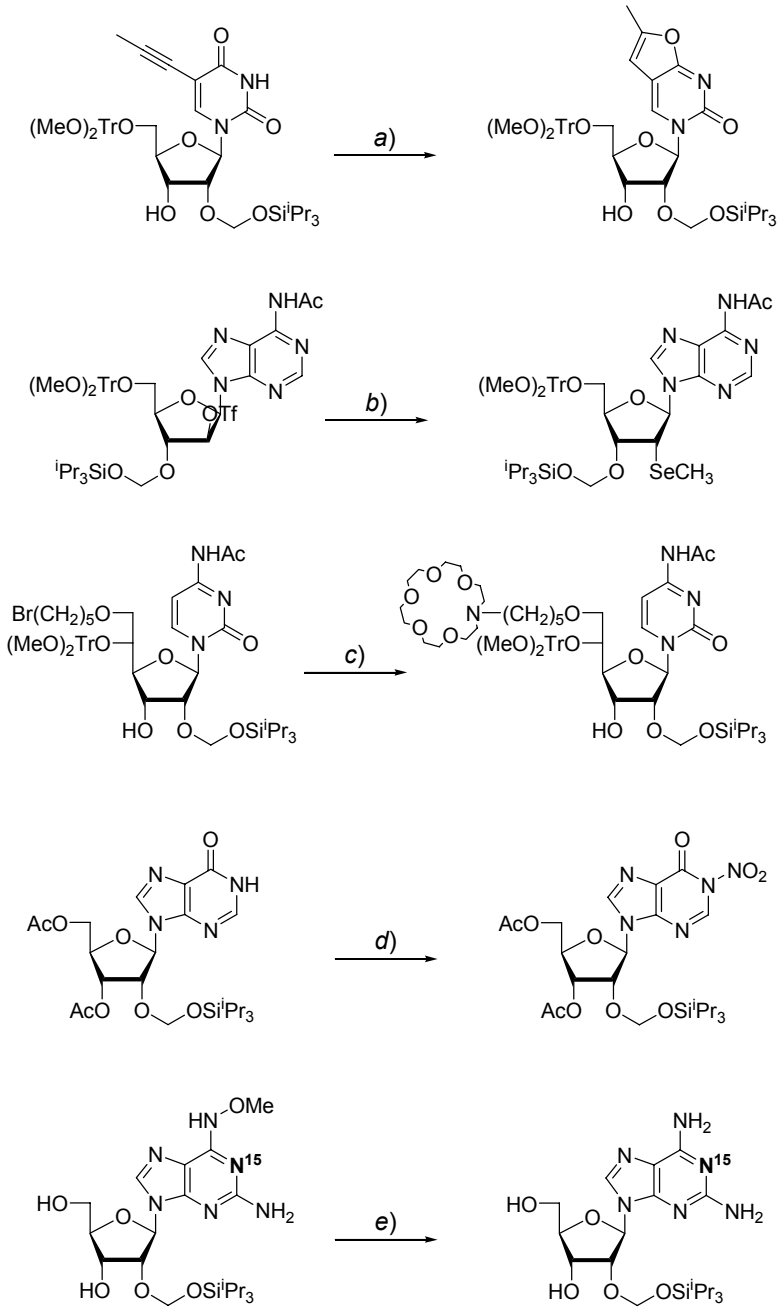


Figure I.2. A few examples of sugar- and base-transformations with 2'-*O*-TOM protected ribonucleosides. *a*) 1. Et_3N, CuI, MeOH, reflux (Berry et al. 2004). *b*) 1. $NaBH_4$, $CH_3SeSeCH_3$, THF, 20° (Hoebartner et al. 2005). *c*) 1. 1-aza-18-crown, Bu_4NI, $(^iPr)_2NEt$, EtOH, 75° (Wu and Pitsch 1998). *d*) 1. $NaNO_2$, AcOH, H_2O, 20° (Wenter and Pitsch 2003). *e*) 1. Ac_2O, pyridine, 100°; 2. NaOH, THF, MeOH, H_2O, 4°; 3. Raney-Ni, THF, MeOH, H_2O, 80° (Wenter and Pitsch 2003).

I.1.3. Aims of the project

During their biosynthesis, tRNAs undergo different levels of processing which mainly consists in the introduction of modified nucleosides (up to 25%) by tRNA-modifying enzymes. This biosynthetic step is so important that *E.coli* devotes about 1% of its genom in coding such enzymes (Björk 1995). Their absence within tRNAs has widespread consequences including incorrect folding (Helm et al. 1998), frameshifting events (Liu et al. 1997) (Wang et al. 2001) (Kowal et al. 2001) and mis-aminoacylation due to reduction of proper interactions with the aminoacyl-tRNA-synthetases (Connolly et al. 2004). For an exhaustive study of their function, it would be necessary to incorporate one or several modifications (natural or not) at selected positions of tRNAs (Nobles et al. 2002). Apart from a pioneering synthesis of the $tRNA^{Ala}$ from yeast, containing m^5U, Ψ and D reported in 1992 (Gasparutto et al. 1992) and the enzymatic incorporation of a single m^1A into a tRNA transcript (Helm et al. 1999), no other attempts to prepare such compounds have been reported. Moreover, these syntheses contain only few modifications in comparison to most of the tRNAs found in Nature which contain modified nucleosides in an extent of up to 25% (Grosjean and Benne 1998). The preparation of such extensively modified full-length tRNA sequences, containing several modified nucleotides, still represents a major challenge. Some of the 80 different known modifications (Björk 1995) (Sprinzl et al. 1998) have been incorporated into short (< 20 mer) model RNA sequences (D (Dalluge et al. 1996), m^1G, m^2_2G, m^2G, m^1I , m^3U, m^4C, m^6A, m^6_2A (Höbartner et al. 2003), m^1A (Mikhailov et al. 2002), I (Green et al. 1991), ψ (Gasparutto et al. 1992) (Chui et al. 2002a), $m^3ψ$ (Chui et al. 2002b), s^4U (Kumar and Davis 1997), mcm^5U (Bajii and Davis 2000), mcm^5s^2U (Bajii and Davis 2000), s^2U (Agris et al. 1995), mnm^5U (Agris et al. 1995), i^6A (Kierzek and Kierzek 2003), ms^2i^6A (Kierzek and Kierzek 2003), mnm^5s^2U (Sundaram et al. 2000) and t^6A (Sundaram et al. 2000) (Boudou et al. 2000)), and it was established that they exhibit unique chemical properties requiring an adaptation of protecting groups and deprotection conditions for their introduction. Most of these modifications were introduced as the corresponding 2'-*O*-^{t}BDMS protected phosphoramidites. In order to profit from the advantages of the 2'-*O*-TOM

based RNA chemistry we prepared the 5'-*O*-DMT, 2'-*O*-TOM protected phosphoramidite building blocks of the most encountered modified nucleosides of the tRNA *(Figure I.18.)*. In the following, the nucleosides are presented in two main categories, by distinguishing the modifications found at the anticodon loop from other tRNA modifications. The chemical syntheses of the nucleosides are also presented in the context of their biosynthesis because often the strategy for preparing them is the same.

I.2. Modified non-anticodon purines

I.2.1. Introduction

The purines of the stem contribute to the structuration of the tRNA (Söll and RajBhandary 1995) (Grosjean and Benne 1998) by favoring or preventing some base pairing. Theoretically investigated for natural or artificial methylated nucleosides (Micura et al. 2001), these properties have been recently highlighted with the m^1A nucleoside which appeared essential for the proper and functional folding of human mitochondrial $tRNA^{Lys}$ (Helm et al. 1999).

I.2.2. Methylated guanosines m^2G, $m^2{}_2G$

I.2.2.1. Biosynthesis

Among the methylated purines, we find $m^2{}_2G$ which is present at position 26 in some eukaryotic tRNAs. The isolation of the related modifying enzyme from yeast has permitted to investigate the editing mechanism and to identify a stepwise process where the two methylations take place within a unique protein (Ellis 1986). This observation has also been confirmed by purification of a G26 methyltransferase from *T. pyriformis* which was found to have both monomethylating and dimethylating activity (Reinhart 1986). Like with other methylating enzymes, the cofactor employed as electrophilic methyl group donor is adenosylmethionine (AdoMet, SAM) (*Figure I.3.*). This observation suggests that the process takes place upon deprotonation of the nitrogen and subsequent nucleophilic attack, but the mechanistic details remain unknown. Nevertheless, the G11-C25 and C10-G24 base pairs have been identified as the main recognition elements (Edqvist 1992, 1994). Furthermore, it

has been suggested that other structural features are required to ensure the favourable orientation of G26 to be recognized by the methyltransferase. The genes related to the methylation of guanosine have been well identified and should soon allow new insights into this process (Armengaud et al. 2004).

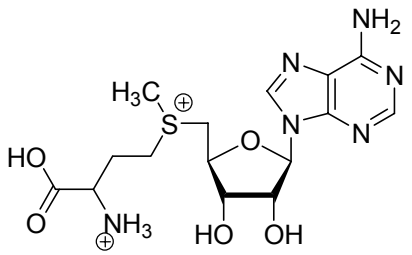


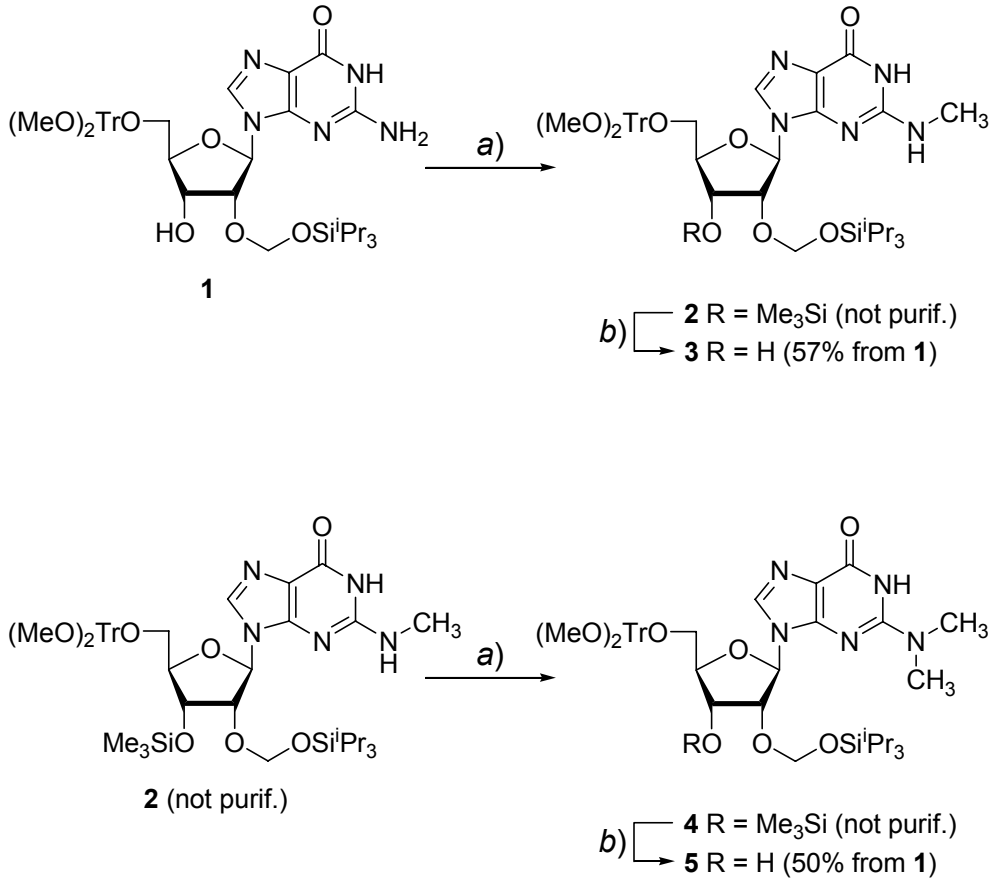
Figure I.3. Structure of S-Adenosylmethionine (AdoMet, SAM).

I.2.2.2. Synthesis

The m^2G and $m^2{}_2G$ nucleosides have been already incorporated into RNA sequences as its 2'-*O*-TOM/*O*-C(6) nitrophenylethyl protected phosphoramidite building block. They have been prepared by nucleophilic aromatic substitution reaction of a protected 2-fluoroinosine derivative with the appropriate amine, $MeNH_2$ for m^2G and Me_2NH for $m^2{}_2G$, respectively (Höbartner et al. 2003). In contrast, we introduced the methyl group into the protected guanosine derivative **1**, by adopting a one-pot method developed by *Sekine* and *Satoh*. First, the nucleoside **1** in Py was treated with Me_3SiCl (→ silylation of *O*-C(6) and *O*-C(3')), and then with 1,3-benzodithiozolium tetrafluoroborate (Sekine and Satoh 1991). The resulting N^2-benzodithiol-2-yl derivative was filtered on silica gel and treated with $(Me_3Si)_3SiH$ (Ballestri et al. 1991) (Ryu et al. 1997) (Apeloig and Nakash 1994) (Chatgilialoglu 1992) /2,2'-azobisisobutyronitrile (AIBN) in refluxing benzene (→ **2**, not isolated)[1]). Finally, the remaining Me_3Si-group of **2** was removed with NH_3 in MeOH and the nucleoside **3** was obtained in a yield of 57% (based on **1**, *(Scheme I.3.)*). The protected N^2,N^2-

[1]) This reduction was originally carried out with Bu_3SnH/AIBN according to (Sekine and Satoh 1991); however, it was difficult to separate the tin-containing byproducts from **26** and **27**.

dimethylguanosine nucleoside **5** was prepared from the crude N^2-monomethylated intermediate **2** by repeating the sequential treatment with 1,3-benzodithiozolium tetrafluoroborate (Sekine and Satoh 1991) and $(Me_3Si)_3SiH$/AIBN (→ **4**, not isolated). After cleavage of the Me_3Si-group with NH_3 in MeOH, the fully protected nucleoside **5** was obtained in a yield of 50% (from **1**, *(Scheme I.3.)*).



Scheme I.3. *a*) 1. Me_3SiCl, pyridine, 20°; then 1,3-benzodithiolylium tetrafluoroborate; 2. $(Me_3Si)_3SiH$, benzene, reflux. *b*) NH_3, MeOH, THF, 20°. **1** was prepared according to (Stutz et al. 2000).

I.2.3. Methylated adenosine m^1A

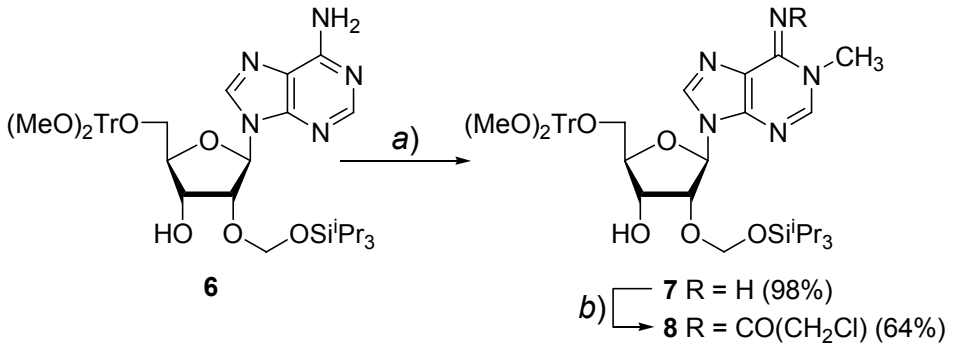
I.2.3.1. Biosynthesis

This modified nucleoside is present in about 25% of all eukaryotic tRNAs, mostly at position 58 and, in a lesser extent, at position 14 in the D-loop and at position 9 of some mitochondrial

tRNAs (Helm et al. 1999). Among the different m^1A methyltransferases, the one related to m^1A58 has been well characterized and it has been established that the TΨC arm constituted the main determinant of the recognition (Grosjean et al. 1996b). Although no experiment has been carried out in order to reveal mechanistic details, the process should not differ from those of other methyltransferases where a deprotonation activates the attacking nitrogen.

I.2.3.2. Synthesis

The corresponding 2'-*O*-^{t}BDMS protected phosphoramidite has been recently prepared by stepwise introduction (1. chloroacetyl, 2. $(MeO)_2Tr$, 3. ^{t}BDMS) of all protecting groups into 1-methyladenosine (Mikhailov et al. 2002). In contrast, we first prepared the N(1)-methylated, 2'-*O*-TOM protected adenosine derivative **7** by treatment of the easily accessible nucleoside **6** (Stutz et al. 2000) with MeI in DMF (98% yield). The chloroacetyl protecting group was then introduced with $(ClCH_2CO)_2O$ in Py and the fully protected 1-methyladenosine nucleoside **8** was obtained in a yield of 64% after cleavage of the concomitantly formed 3'-*O*-chloroacetyl ester with NH_3 in MeOH according to (Mikhailov et al. 2002) *(Scheme I.4.)*.



Scheme I.4. *a*) MeI, DMF, 20°. *b*) 1. $(ClCH_2CO)_2O$, $(CH_2Cl)_2$, pyridine, –15°; 2. NH_3, MeOH, –15°. **6** was prepared according to (Stutz et al. 2000).

I.3. Modified non-anticodon pyrimidines

I.3.1. Introduction

Analogously to the purines of the stem, modified pyrimidines contribute to the structuration of the tRNA or serve as markers for editing enzymes (Söll and RajBhandary 1995) (Grosjean

and Benne 1998). In contrast to all other modified nucleosides, the dihydrouridine (D) does not display a flat aromatic π-system, but a saturated C(5)-C(6) bond. Thereby, it prevents stacking interactions as revealed by NMR investigation of a short synthetic D-containing oligomer (Dalluge et al. 1996) [2]).

I.3.2. Ribothymidine m^5U (= rT)

I.3.2.1. Biosynthesis

5-Methyluridine (ribothymidine, rT) is one of the most conserved modified nucleosides found in tRNAs and is usually located at position 54 in the TψC loop (Söll and RajBhandary 1995) (Grosjean and Benne 1998). This modification is introduced by methylation of uridine with the (m^5U54)methyltransferase (EC 2.1.1.35). Since the sequence of the enzyme is similar to the enzyme thymidylate synthase (EC 2.1.1.45), which generates (d)TMP from (d)UMP, the mechanism of modification has been simply extrapolated (Santi and Hardy 1987). Thereby, a cysteine residue attacks the C6, generating an enolate and a nucleophilic C5, which subsequently allows reaction with an electrophilic methyl group.

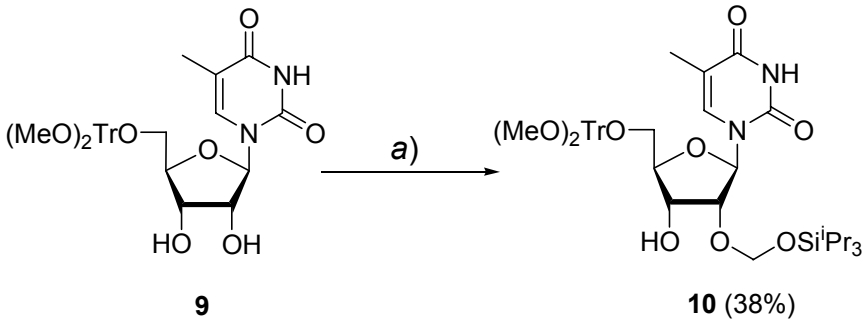
Interestingly, the nature of the methylating reagent depends on the organism. It is often AdoMet but can also be 5,10-methylenetetrahydrofolate (Delk et al. 1980). Furthermore, although the identification elements involved in the process depend on the organism, a same dependence is observed for the nature of identity elements involved in the editing process. Whereas in yeast some specific nucleobases have been identified, in *E.coli* no such sequence specificity has been observed and the interaction depends on the structure of the T arm (Becker et al. 1997).

I.3.2.2. Synthesis

We prepared the 2'-*O*-TOM protected 5-methyluridine **13** from the parent nucleoside in two steps, by first introducing the dimethoxytrityl-group with $(MeO)_2$Tr-Cl in Py

[2]) Revealed by a NMR investigation of a short synthetic D-containing oligomer, which was prepared from the 2'-*O*-^{t}BDMS protected phosphoramidite (Flockerzi et al. 1981).

according to (Gasparutto et al. 1992), followed by alkylation of the product **9** with TOM-Cl under standard conditions (Pitsch et al. 2001) (→ **10**, 38% yield, *(Scheme I.5.)*).



Scheme I.5. *a*) Bu_2SnCl_2, $^{i}Pr_2NEt$, $(CH_2Cl)_2$, 20°; then TOM-Cl, 20°. **9** was prepared according to (Gasparutto et al. 1992).

I.3.3. Dihydrouridine D

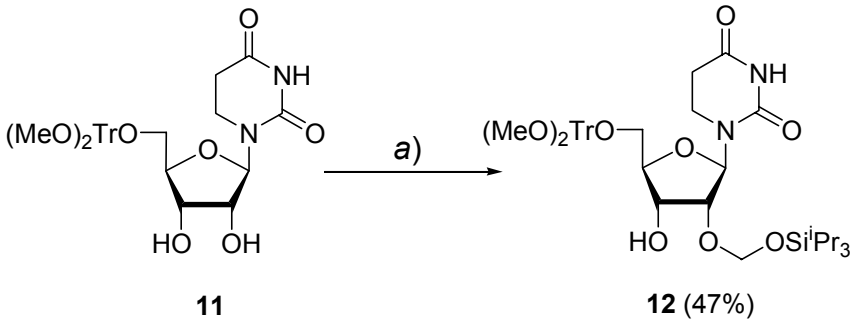
I.3.3.1. Biosynthesis

Widely distributed among eubacteria and eukaryotes, and less among archeaebacteria, the dihydrouridine is found at different positions within tRNAs, such as 16, 17, 20, 20a (rarely 47) (Dalluge et al. 1996). Known for many years and so often encountered that it gave the name to the so-called D-loop, the exact role and the biosynthesis of this nucleoside remain obscure. After its incorporation as nucleoside monophosphate by *E.coli* RNA polymerase (Söll and RajBhandary 1995), an eventual formation during transcription has been suggested, but it appeared soon more reasonable to imagine a "simple enzymatic reduction of the 5,6-double bond" (Grosjean and Benne 1998). This hypothesis has been recently confirmed by isolation of the four specific yeast dihydrouridine synthases, but no further investigations have been carried out yet for elucidation of mechanistic aspects (Xing et al. 2004).

I.3.3.2. Synthesis

We prepared the protected nucleoside **11** in analogy to the reported approach (Flockerzi et al. 1981), consisting in hydrogenation of uridine with Rh/H_2, followed by introduction of the

$(MeO)_2Tr$-group. The 2'-*O*-TOM group was introduced under standard conditions (Pitsch et al. 2001) (→ **12**, 47% yield) *(Scheme I.6.)*[3]).



Scheme I.6. *a*) Bu_2SnCl_2, $^iPr_2NEt$, $(CH_2Cl)_2$, 20°; then TOM-Cl, 80°. **11** was prepared according to (Flockerzi et al. 1981).

I.3.4. 5-Methylcytidine m5C

I.3.4.1. Biosynthesis

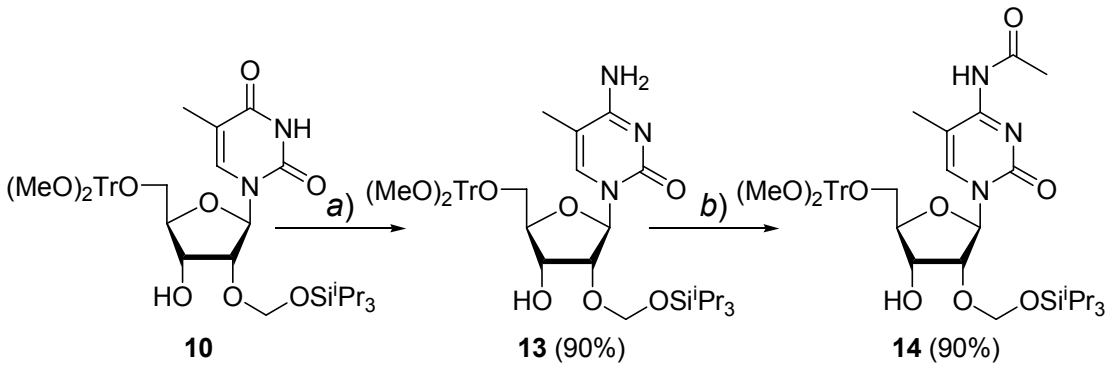
In analogy to the biosynthesis of m^5U, the formation of m^5C occurs by methylation of cytidine. A m^5C40 methyltransferase from human *Hela* cells has been purified and characterized (Keith et al. 1980). The identification of its sequence specificity in yeast suggests that this modification takes place at the level of the precursor tRNA (intron containing tRNA) (Jiang et al. 1997).

I.3.4.2. Synthesis

We prepared the corresponding 2'-*O*-TOM protected nucleoside **13** by adapting one of our recently reported, optimized base-transformation methods (Wenter and Pitsch 2003). The 5-methyluridine nucleoside **10** (see *Scheme 1.5.*) was first acetylated at the 3'-OH-group with Ac_2O in Py, then treated with $(ClC_6H_4O)P(O)Cl_2$, 1*H*-1,2,4-triazole and $^iPr_2NEt$ in MeCN (→ formation of the 4-triazolide derivative), and finally with NH_3 in dioxane/H_2O; after

[3]) Hydrogenation of 2'-*O*-TOM protected uridine provided the corresponding dihydrouridine derivative in excellent yield. This reaction, however, could not be carried out in the presence of a 5'-*O*-$(MeO)_2Tr$-group.

extraction and deacetylation with NaOMe in MeOH, the cytidine nucleoside **13** was isolated in a yield of 90%. It was finally transformed into its N(4)-acetylated derivative **14** by selective *N*-acetylation with Ac_2O in DMF and isolated in a yield of 90% *(Scheme I.7.)*.



Scheme I.7. *a*) 1. Ac_2O, DMAP, pyridine, 20°; 2. 1*H*-1,2,4-triazole, 4-chlorophenyl phosphorodichloridate, $^{i}Pr_2NEt$, MeCN, 20°; 3. NH_3, dioxane, MeCN, H_2O, 20°; 4. NaOH, THF, MeOH, H_2O, 4°. b) Ac_2O, DMF, 20°.

I.3.5. Pseudouridine Ψ

I.3.5.1. Biosynthesis

For a long time, the study of modified ribonucleosides was limited to their most represented forms due to the great quantities of tRNAs required for their isolation. Consequently, the first modified nucleoside to be identified has been the most abundant pseudouridine, which represents almost 2% of the total RNA (Davis and Allen 1957). Present in all variety of RNAs (rRNA, snRNA, snoRNA, tRNA), pseudouridine (ψ), is highly conserved at position 55 in most of the tRNAs in the so-called TψC loop and is also found at other positions where it appears to influence the three-dimensional structure, cell viability or aminoacylation (Arnez et al. 1994) (Davis and Poulter 1991) (Harrigton et al. 1993). This 5-ribosyl isomer of uridine is unique by having a C-C, rather than the usual N-C glycosyl bond that links base and sugar (Charette and Gray 2000). This feature confers different hydrogen bonding capacities to it and influences the conformational preferences. Whereas the free nucleotide shows no real preferential conformation (Davis 1998) (Neumann et al. 1980) (Davis et al. 1998a), NMR and X-ray experiments have demonstrated that it exhibits an extraordinary conformational rigidity

in RNA sequences due to the coordination of a water molecule between N1-H and the 5'-phosphate group (Arnez et al. 1994) (Auffinger and Westhof 1998). This feature improves base stacking interactions and contributes significantly to the structuration of RNAs (Neumann et al. 1980) (Davis et al. 1998a) (Durant and Davis 1999) (Davis 1999). Among the modified nucleosides of the tRNAs, pseudouridine possesses the unique feature to be also present in the middle of the anticodon (position 35) where it is supposed to reinforce the base pairing strength (Johnson and Albeson 1983).

In the tRNAs, the biosynthesis of this modified nucleoside by pseudouridine synthase occurs by recognition of the target-uridine. The isomerization of uridine to pseudouridine requires no ATP or coenzyme and seems to be controlled by only one aspartic acid residue in the active site. However, the mechanistic details of this intriguing base transformation are still heavily debated (Mueller 2002) (Spedaliere et al. 2004).

I.3.5.2. Synthesis

Pseudouridine is still exclusively obtained by isolation from natural sources resulting in a prohibitive price of approximately 2000 CHF per gram. Consequently, some attention was given to its chemical preparation in a multigram scale, but the stereoselective formation of C-nucleosides is still a difficult task. A good process, involving the coupling of an iodinated pyrimidine with a suitably protected ribonolactone was presented by *Grohar* and *Chow* (Grohar and Chow 1999). Their approach gives a 1:1 mixture of α / β anomers in 51% yield.

We have now investigated alternative synthetic approaches to this nucleoside. Specific attention was given to the stereoselective formation of the N-glycosidic bond. Our synthetic plan involved a stereoselective reduction of the acyclic ketone, obtained by coupling the lithiated, protected base with a suitably protected ribonolactone derivative. The cyclisation could then be carried out under Mitsunobu conditions *(Figure I.1.)*.

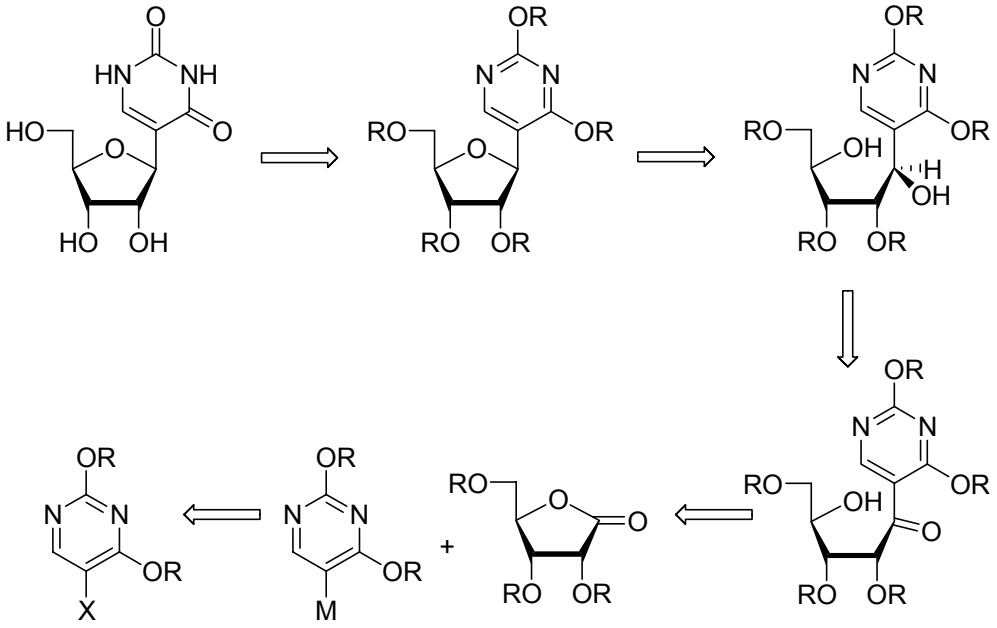
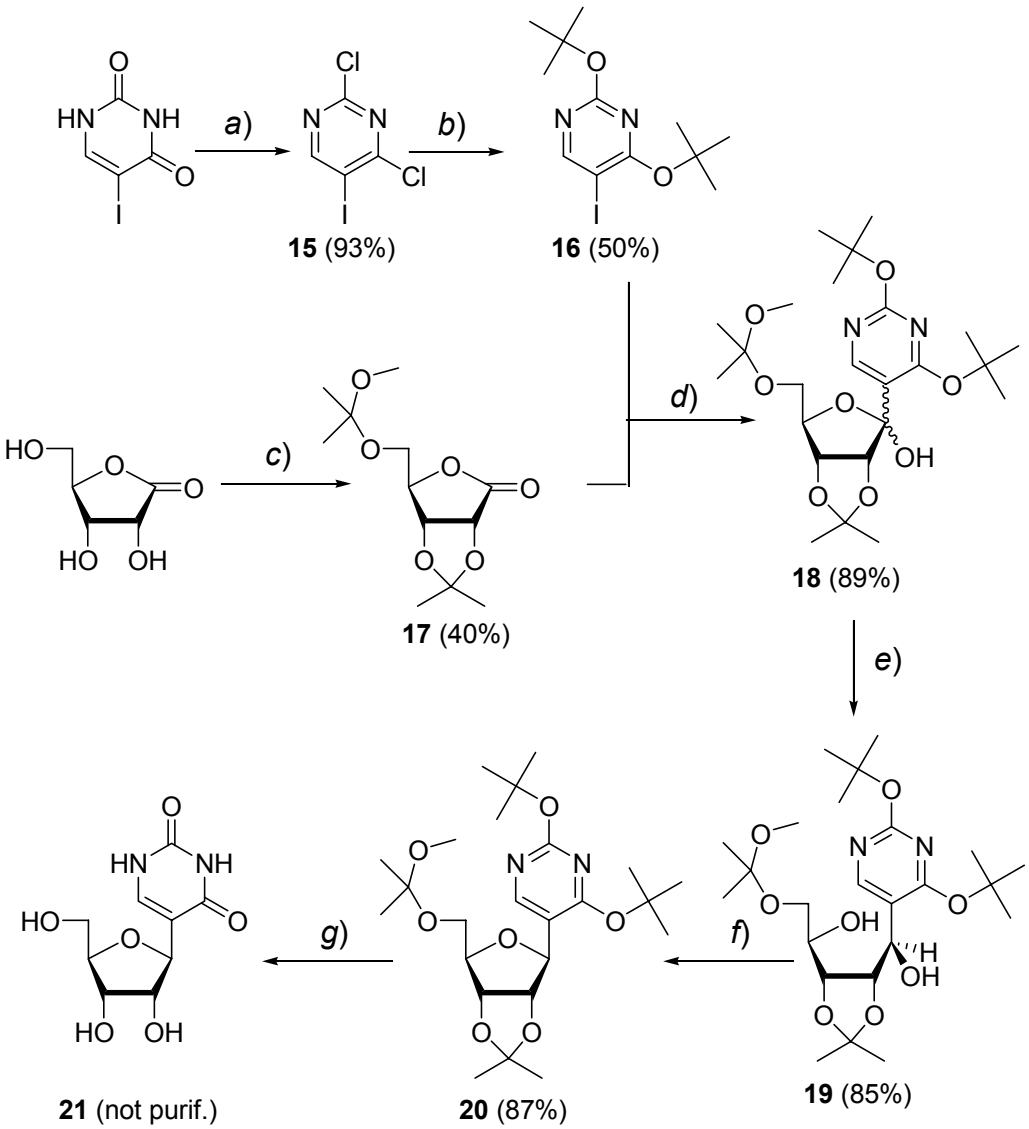


Figure I.1. Retrosynthethic analysis for preparation of the pseudouridine nucleoside.

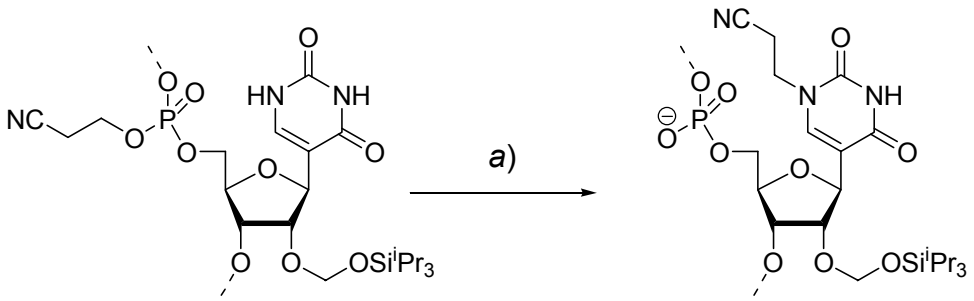
While we were investigating this pathway, a similar synthesis was published (Hanessian and Machaalani 2003). Importantly, there it was described that tert-butoxy protecting groups have to be employed for the introduction of the base, since the usually employed methoxy protecting groups could not be removed without anomerization of the product. All the reactions were incompletely described and usually, in our hands much lower yields were obtained than reported. Therefore all steps required for the preparation of the pseudouridine nucleoside had to be optimized *(Scheme I.8.)*. The 2,4-dichloro-5-iodopyrimidine **15** (93% yield) was prepared from 5-iodouridine and $POCl_3$ according to (Pichat et al. 1971), but purified by chromatography. No exact description for the preparation of the di-tert-butyl derivative **16** was available, but it could be prepared from **15** by chloride substitution with sodium tert-butanolate in tert-BuOH (50% yield). The ketal-protected ribonolactone derivative **17** was prepared from ribonolactone/2,2-dimethoxypropane/TsOH according to (Brown 1968), but only 40% yield were obtained (98% yield reported in (Hanessian and R. Machaalani 2003)). In analogy to the preparation of the 2,4-dimethoxy analogue (Grohar and Chow 1999), the protected base **16** was lithiated with tert-BuLi in THF and then added to the sugar-derivative **17** (89% yield). Under our optimized conditions, this hemiketal-intermediate

18 was then reduced to the corresponding alcohol **19** with *RedAl* (sodium bis(methoxyethoxy)aluminium hydride) in CH_2Cl_2 at –78° (85% yield). This reagent provided a fully stereoselective reduction of **18** and was more convenient to handle than the reported L-Selectride/$ZnCl_2$ complex (Hanessian and Machaalani 2003). Under Mitsunobu conditions, smooth cyclization of **19** to the C-nucleoside **20** occurred (87% yield). Finally, the free pseudouridine was obtained by cleavage of all protecting groups with AcOH/H_2O. This reaction was quantitative and the product **21** was not purified, but directly subjected to the next reaction (*Scheme I.9*).



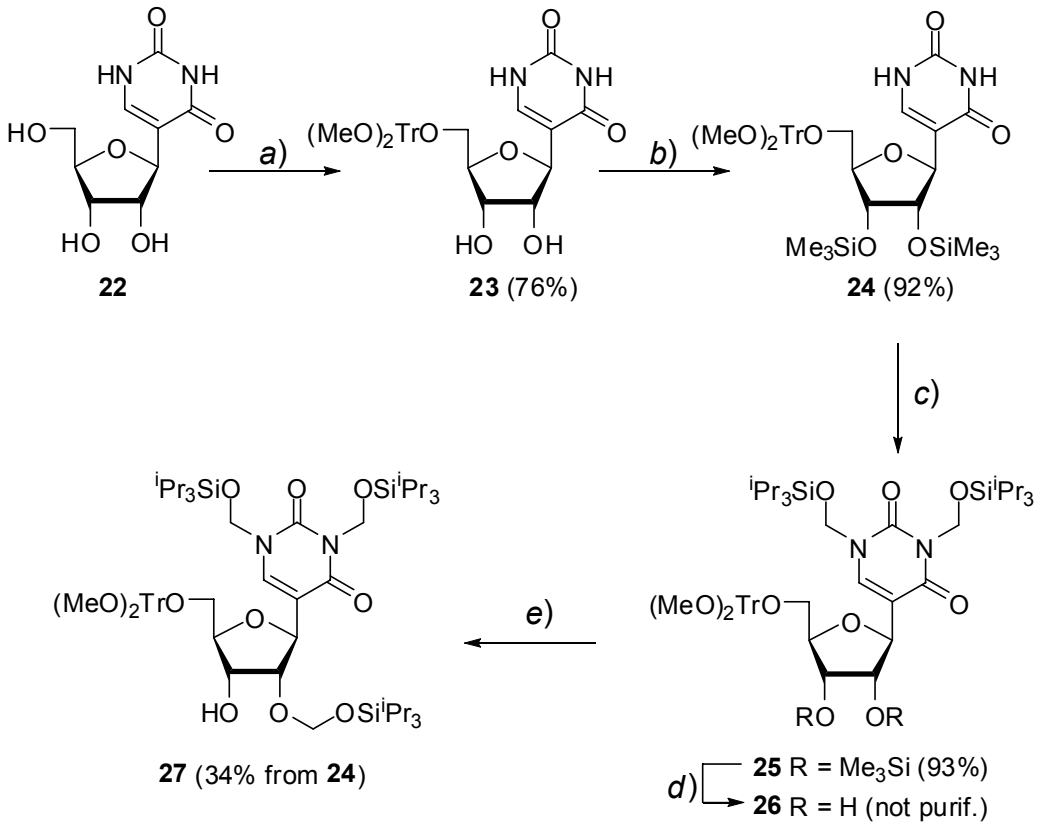
Scheme I.8. *a*) $Cl_3P{=}O$, Et_2NPh, reflux. *b*) ^{t}BuOK, ^{t}BuOH, 20°. *c*) 2,2-dimethoxypropane, TsOH, Na_2SO_4, 20°. *d*) **2**, ^{t}BuLi, THF, -78°; then **3**, THF, -78°. *e*) RedAl, CH_2Cl_2, -78°; then **4**, -78° - 4°. *f*) PPh_3, diisopropylazodicarboxylate, THF, 4°. g) AcOH, H_2O, 50°.

In the literature, contradictory statements about base protecting groups required for introduction of this nucleoside into RNA sequences have been reported. The phosphoramidite with an unprotected base has been employed for the preparation of the fully modified *E.coli* ^{Ala}tRNA (Gasparutto et al. 1992), but other authors underlined the necessity to protect the nucleobase (Bergmann and Pfleiderer 1994) (Pieles et al. 1994). This conclusion seems to be justified upon the recent use of acrylonitrile as derivatization reagent for detection of minor nucleosides, such as pseudouridine by MS analysis (Mengel-Jørgensen and Kirpekar 2002). Acrylonitrile, also generated during phosphate deprotection, results in irreversible N(1) alkylation of pseudouridine *(Scheme I.9.)*. Therefore, we decided to protect the N(1) and N(3) by a suitable protecting group.



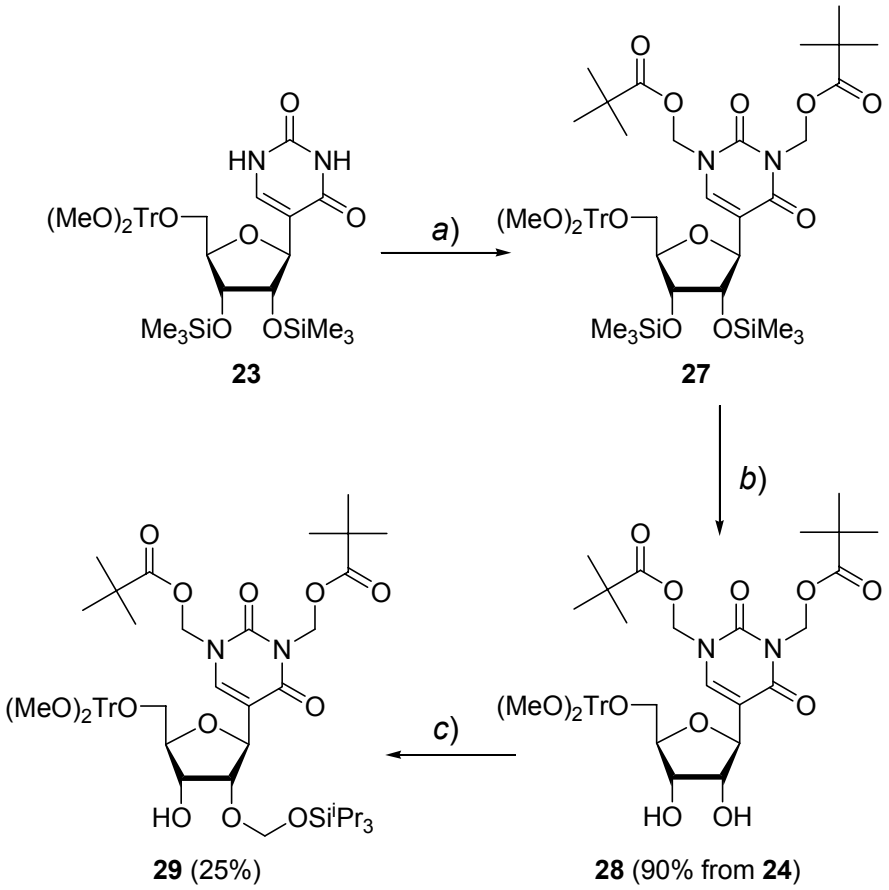
Scheme I.9. *a*) $MeNH_2$, EtOH, H_2O, 20°.

In a first approach, two fluoride-labile TOM groups were introduced *(Scheme I.10.)*. The free pseudouridine nucleoside **21** was first 5'-*O*-dimethoxytritylated with $(MeO)_2TrCl$ in Py (→ **22**, 76% yield) and then 2',3'-di-*O*-trimethylsilylated with Me_3SiCl in Py (→ **23**, 92% yield). Alkylation of 23 with TOM-Cl/K_2CO_3 in DMF provided the N(1),N(3) di-*O*-TOM derivative **24** in 93% yield. The Me_3Si-groups of **24** were then removed with NH_3 in MeOH (→ **25**) and without purification of this intermediate, the 2'-*O*-TOM group was introduced under standard conditions (→ **26**, 34% yield from **24**). Unfortunately, the corresponding phosphoramidite could not be efficiently introduced into RNA sequences. Most probably, the two additional TOM-groups at the nucleobase moiety create too much steric hindrance.



Scheme I.10. *a*) $(MeO)_2TrCl$, pyridine, 20°. *b*) Me_3SiCl, pyridine, 4°. *c*) K_2CO_3, DMF, 20°; then TOM-Cl, 20°. *d*) NH_3, MeOH, 20°. *e*) Bu_2SnCl_2, $^iPr_2NEt$, $(CH_2Cl)_2$, 20°; then TOM-Cl, 65°.

Consequently, we turned to the related POM (pivaloyloxymethyl) protecting group, which has been successfully employed for the preparation of pseudouridine-containing RNA sequences from the corresponding 2'-*O*-Fpmp (=2'-*O*-[1-(2-fluorophenyl)-4-methoxypiperidin-4-yl]) protected phosphoramidites (Pieles et al. 1994) *(Scheme I.11.)*. Incubation of the intermediate **23** with K_2CO_3 and POM-Cl in DMF offered the fully protected derivative **27**. Subsequent removal of the Me_3Si-groups with 1M of TBAF in THF for 70 sec, according to (Pieles et al. 1994) gave the diol **28** in 90% yield (from **23**). Alkylation of **28** with POM-Cl under standard conditions (Pitsch et al. 2001) gave the pseudouridine phosphoramidite precursor **29** in 25% yield.



Scheme I.11. *a*) K_2CO_3, DMF, 20°; then pom-Cl, 20°; *b*) Bu_4NF, THF, 20°. c) Bu_2SnCl_2, $^iPr_2NEt$, $(CH_2Cl)_2$, 20°; then TOM-Cl, 65°.

I.4. Modified purines of the anticodon

I.4.1. Introduction

The position 37 of the anticodon loop is always occupied by an unmodified or modified purine such as m^1I, m^1G, i^6A, t^6A, m^6A, imG. Some of the modifications have been demonstrated to influence the codon-anticodon recognition, probably by a stronger shielding from water and their extended structure (Söll and RajBhandary 1995) (Grosjean and Benne 1998). This ability is also influenced by the hydrophobic character of the nucleobase which is increased by the presence of alkyl substituents, such as methyl groups. Furthermore, for the guanosine-derived nucleoside m^1G, the methyl group has been shown to prevent any base pairing which could result in quadruplet reading, as observed with an unmodified guanosine at this position (Björk et al. 1989). The hypomodification of the nucleobase at position 37 has

often severe consequences on the tRNA reading fidelity. This point will further be discussed in more details in Chapter IV.

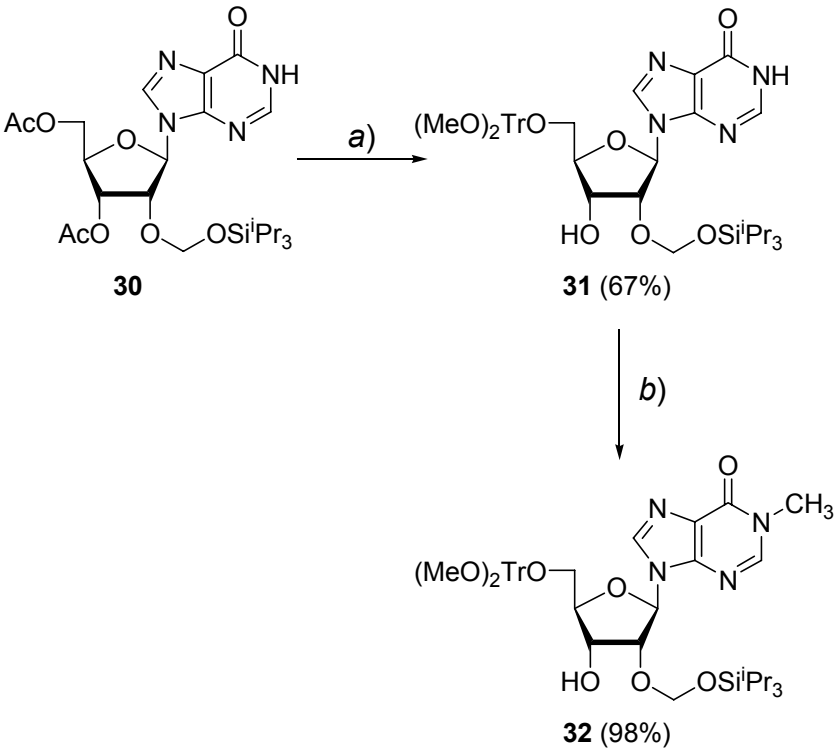
I.4.2. 1-Methylinosine m^1I

I.4.2.1. Biosynthesis

The m^1I nucleoside is exclusively found at position 37 of eukaryotic ^{Ala}tRNA and at position 57 of several archaebacterial tRNAs (Sprinzl et al. 1998). The observed difference of position is also correlated with the mechanism of formation by two distinctive biosynthetic pathways. Whereas in eukaryotes the methylation follows the deamination step, in archaebacteria, the methylation apparently precedes the deamination (Grosjean 1995a and 1996a).

I.4.2.2. Synthesis

The 2'-*O*-TOM protected phosphoramidite of this nucleoside, has already been prepared by introduction of the TOM group into the corresponding nucleoside (Höbartner et al. 2003). As an alternative to this procedure, we efficiently methylated the 2'-*O*-TOM and 5'-*O*-$(MeO)_2$Tr protected inosine derivative **31** with MeI/K_2CO_3 in DMF (→ **32**, 98%) *(Scheme I.12.)*. The building block **31** was prepared in 67% yield from the 2'-*O*-TOM protected analogue **30**, previously synthesized as a common intermediate for the synthesis of ^{15}N-labeled adenosine and guanosine phosphoramidites by nucleobase transformation reactions (Wenter and Pitsch 2003).



Scheme I.12. *a*) 1. NH_3, MeOH, 20°; 2. $(MeO)_2TrCl$, pyridine, 20°. *b*) 1. K_2CO_3, DMF, 20°; then MeI, –15°. **30** was prepared according to (Wenter and Pitsch 2003).

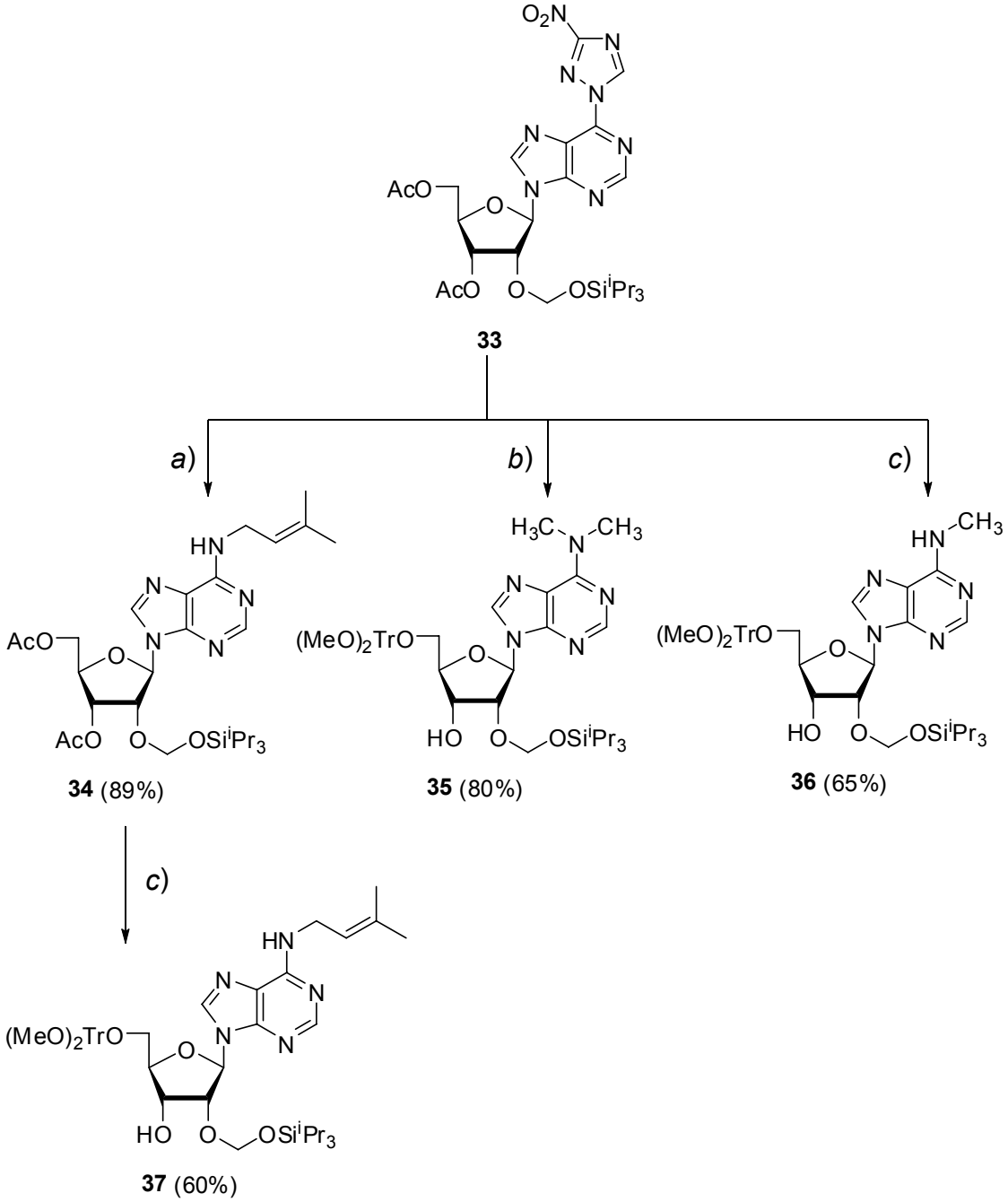
I.4.3. N^6-Isopentenyladenosine i^6A

I.4.3.1. Biosynthesis

This modified nucleoside, present at position 37 of tRNAs, interestingly acts also as a growth factor (cytokinin) in plants (Skoog and Armstrong 1970). This double role is an unique feature amongst the nucleosides present in tRNAs. The role of the isopentenyl moiety in the decoding properties has been extensively investigated and is discussed in the Chapter IV. The i^6A nucleoside has been recently incorporated enzymatically into tRNAs for studying anticodon stem structuration (Cabello-Villegas et al. 2002), and chemically into short RNA-sequences for thermodynamical investigations (Kierzek and Kierzek 2001) (Kierzek and Kierzek 2003). By these studies, it has been demonstrated to contribute significantly to the stabilization of adjacent base pairs and to the maintenance of an open loop structure.

I.4.3.2. Synthesis

The reported methodology involved the preparation of a phosphoramidite building block with a reactive, "convertible" purine derivative, which was converted into the N^6-isopentenyl adenosine upon treatment of the immobilized RNA sequence with isopentenylamine *(Scheme I.13.)*.



Scheme I.13. *a*) 1. Isopent-2-enylamine·HCl, Et_3N, pyridine, 20°. *b*) 1. Me_2NH, EtOH, 20°; 2. $(MeO)_2TrCl$, pyridine, 20°. *c*) 1. $MeNH_2$, EtOH, 20°; 2. $(MeO)_2TrCl$, pyridine, 20°. **33** was prepared according to (Wenter and Pitsch 2003).

We recently reported an optimized method for the preparation of the 2'-*O*-TOM protected, 6-nitrotriazole-substituted adenosine-derivative **33** (Wenter and Pitsch 2003), which served as convenient precursor for the N^6-alkylated adenosine derivatives **35**, **36** and **37**. By treatment of **33** with isopentenylamine in Py/Et_3N, the corresponding N^6-isopentenyl derivative **34** was obtained in a yield of 89%. Deacetylation of this intermediate with NH_3 in MeOH, followed by evaporation and dimethoxytritylation with $(MeO)_2$Tr-Cl in Py gave the fully protected N^6-isopentenyladenosine **37** in 60% yield.

I.4.4. Methylated adenosines m^6A, $m^6{}_2A$

I.4.4.1. Biosynthesis

Whereas m^6A is found exclusively in bacterial tRNAs, $m^6{}_2A$ occurs at the 5'-terminal cap sequence of some mRNAs and in the antibiotic puromycine. The biosynthesis of these two adenosine derivatives should very likely follow the general methylation process with AdoMet as observed for m^1G and m^1A (Grosjean and Benne 1998).

I.4.4.2. Synthesis

In analogy to the preparation of **37**, we treated the 2'-*O*-TOM protected purine-derivative **33** with Me_2NH in EtOH, followed by introduction of the $(MeO)_2$Tr-group under standard conditions and obtained **35** in a yield of 80% *(Scheme I.13.)*.

Likewise to i^6A and $m^6{}_2A$, the 2'-*O*- TOM and 5'-*O*-$(MeO)_2$Tr protected N^6-methyladenosine **36** was obtained again from the intermediate **33**, upon treatment with $MeNH_2$ in EtOH (65 % yield, *Scheme I.13.*).

I.4.5. 1-Methylguanosine m^1G

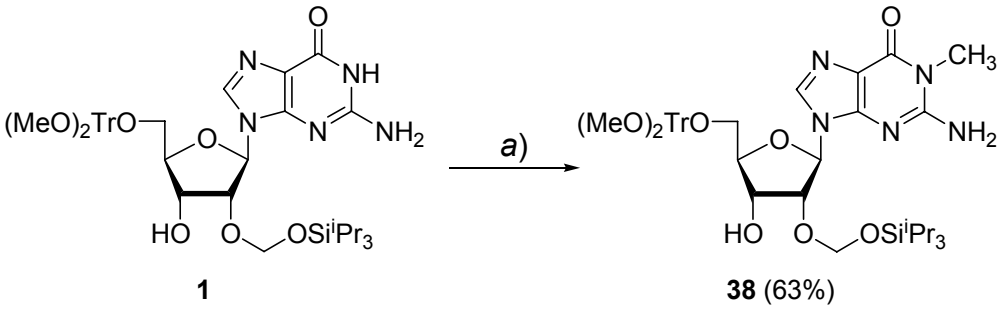
I.4.5.1. Biosynthesis

The m^1G modification is present exclusively at position 37 of tRNAs and is interestingly also the precursor of the wybutosine nucleoside (Droogmans and Grosjean 1987). During its biosynthesis, the parent guanosine is methylated by AdoMet after

deprotonation of the H-N(1) position (Byström et al. 1983) (Hjalmarsson et al. 1983). The main structural requirements for recognition of the corresponding tRNA by the enzyme tRNA(m^1G37)methylransferase is the overall-structure and the target nucleotide G37; the nucleoside at position 38 contributes not to the recognition.

I.4.5.2. Synthesis

A multi-step synthesis of the 2'-*O*-TOM and *N*-acetyl protected 1-methylguanosine phosphoramidite has been reported (Höbartner et al. 2003) but our previous use of unprotected guanosine phosphoramidite (Stutz et al. 2000) suggested that the *N*-acetyl protecting goup was not necessary. Consequently, we prepared the corresponding nucleoside **38** directly from the readily available intermediate **1** (Stutz et al. 2000) by treatment with MeI/K_2CO_3 in DMF at –15° (63% yield, *Scheme I.14*)[4]).



Scheme I.14. *a*) K_2CO_3, DMF, 20°; then MeI, –15°. **1** was prepared according to (Stutz et al. 2000).

I.4.6. *N*-[(9-β-D-ribofuranosyl-9H-purin-6-yl)carbamoyl]threonine t^6A

I.4.6.1. Biosynthesis

N-[(9β-D-ribofuranosyl-9H-purin-6-yl)carbamoyl)]threonine or so called t^6A, is one of the most extensively modified nucleosides. Present in all phyllogenetic domains of life (Archaea, Bacteria and Eukarya (Söll et al. 1995) (Sprinzl et al. 1998)), its abundance has

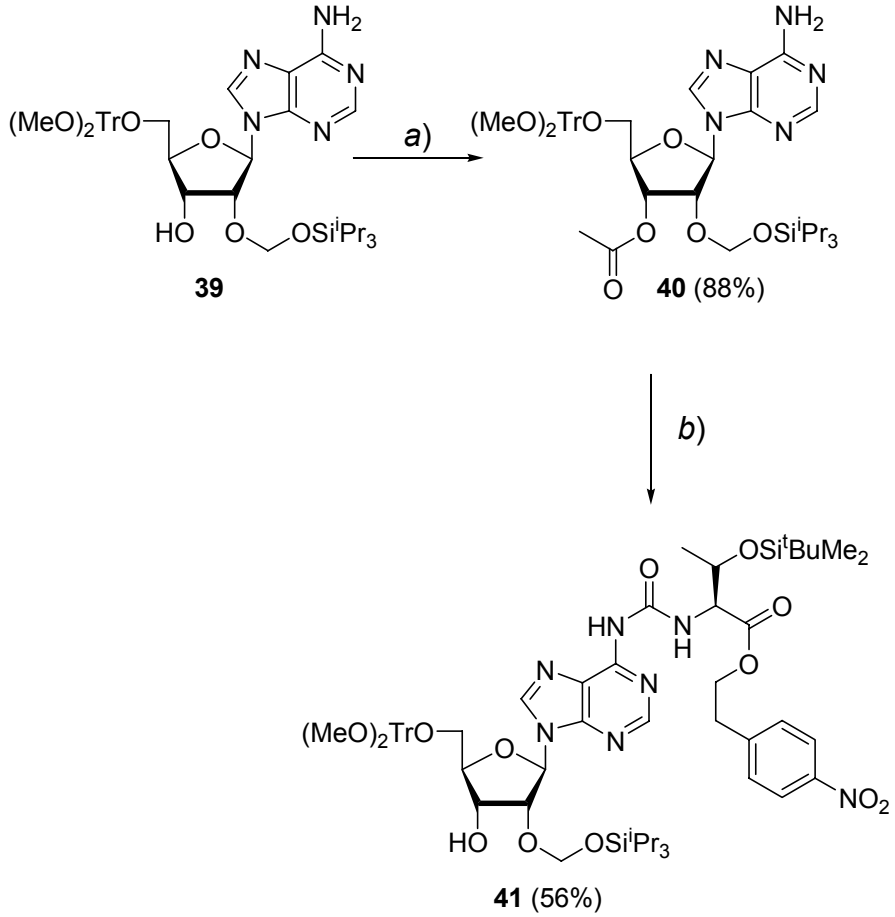
[4]) This modified nucleoside was already incorporated by employing a corresponding N(2)-acetylated buidling block. We found, however that this protecting group is not required, since the N(2)-position of guanosine derivatives is inert under standard coupling conditions (see also (Stutz et al. 2000) for the incorporation of the parent N(2)-unprotected guanosine phosphoramidite into RNA sequences).

aroused several biological investigations (Curran 1998) which permitted to describe its biosynthesis (Chheda et al. 1972) (Powers and Peterkofsky 1972) and to identify the sources of its two starting materials. The threonyl group originates from free L-threonine (Chheda et al. 1972) (Powers et al. 1972) and the carbamoyl moiety originates from HCO_3^- . Furthermore, ATP and Mg^{2+} are required for its incorporation (Elkins et al. 1974). A mechanism involving all these components has been proposed, in which the initiating step would be the formation of an ADP-enzyme-carbamoylphosphate complex. The adenosine 37 induces cleavage of this complex resulting in loss of inorganic phosphate and formation of an N^6-carbamoyladenosine which is again phosphorylated by ATP. Finally, L-threonine is introduced into this active intermediate (Garcia et al. 1998). The enzyme responsible for the incorporation of the amino acid tolerates other structurally similar amino acids and correspondingly modified adenosines containing glycine (g^6A) and hydroxynorvalyl (hn^6A) have been detected (Elkins and Keller 1974).

I.4.6.2. Synthesis

The corresponding 2'-*O*-^{t}BDMS protected phosphoramidite has been prepared already twice (with different protecting groups for the threonine moiety), by first adding the protected amino acid derivative to an activated N^6-carbamoyl- (Sundaram et al. 2000) or a N^6-isocyanato- (Boudou et al. 2000) adenosine, respectively, followed by stepwise introduction of the 5'-*O*-$(MeO)_2$Tr and the 2'-*O*-^{t}BDMS protecting group. In 1999, we have introduced a method for the preparation of *N*-carbamoylated nucleosides, which was applied for the synthesis of ribonucleosides containing photolabile (Stutz and Pitsch 1999) and fluoride labile (Stutz et al. 2000) nucleobase protecting groups, respectively. Meanwhile, we have further optimized this method, which now allows the straightforward synthesis of the nucleoside **41** from the adenosine derivative **39**. Acetylation of the 3'-*O*-position of **39** with Ac_2O in Py gave the derivative **40** in a yield of 88%. This intermediate was treated first with 1,1'-carbonyldi(1,2,4-triazole) and Et_3N in 1,2-dichloroethane, followed by addition of *O*-[(*tert*-butyl)dimethylsilyl]-*l*-threonine 2-(4-nitrophenyl)ethyl ester (prepared according to (Boudou

et al. 2000)). After extraction, the remaining 3'-*O*-acetyl group was cleaved with NH_3 in MeOH and the fully protected t^6A-derivative **41** was isolated in a yield of 56% (based on **40**, *Scheme I.15.*).



Scheme I.15. *a*) 1. Ac_2O, DMAP, pyridine, 20°. *b*) 1,1'-Carbonylbis[1*H*-(1,2,4 triazole)], Et_3N, $(CH_2Cl)_2$, 70°; 2. O^3-[(*tert*-butyl)dimethylsilyl]-*l*-threonine-2-(4-nitrophenyl)ethyl ester, 70°; 3. NH_3, MeOH, 20°; 4. $(MeO)_2TrCl$, pyridine, 20°. **39** was prepared according to (Stutz et al. 2000).

I.4.7. Inosine I

I.4.7.1. Biosynthesis

Inosine was one of the first identified modified nucleosides occurring in tRNAs. Present at position 34 in some tRNAs of bacteria and eukarya, it is totally absent from archaea. Its biosynthesis proceeds through deamination of the related adenosine (Droogmans and Grosjean 1991) by tRNA (I34) deaminase, which is highly sequence dependent. However, other elements, responsible for the three-dimensional folding of the tRNA, are involved in the enzymatic recognition during this editing process (Haumont et al. 1984).

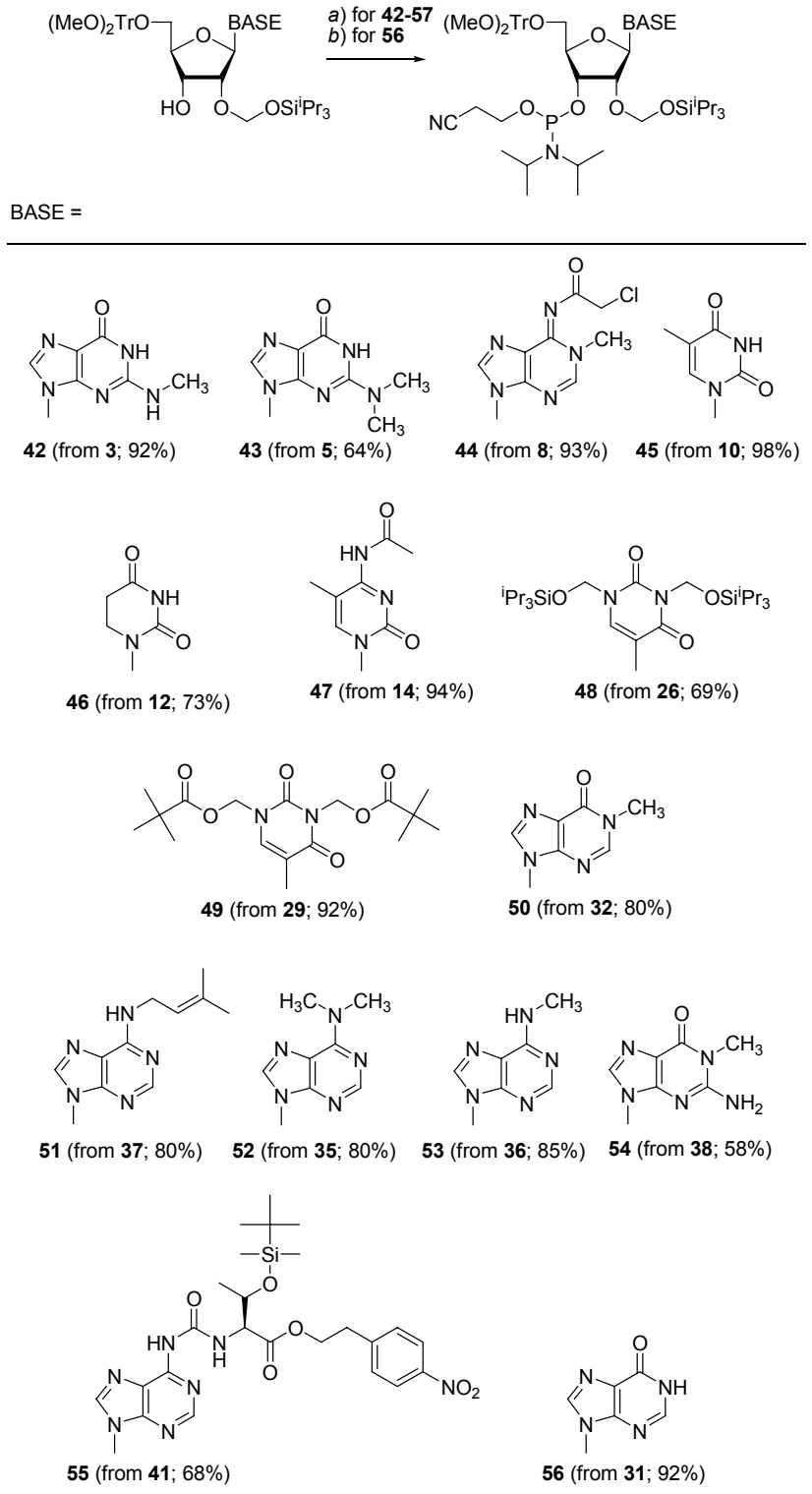
I.4.7.2. Synthesis

The inosine was one of the earliest modified nucleoside to be incorporated into RNA sequences through its 2'-*O*-^{t}BDMS protected phosphoramidite (Green et al. 1991). The synthesis is described in *Scheme I.12.* as precursor for the preparation of m^1I.

I.5. Preparation of phosphoramidites

The protected nucleosides **3, 5, 8, 10, 12, 14, 26, 29, 32, 37, 35, 36, 38, 41** and **31** were finally converted with $^{i}Pr_2NEt$ / 2-cyanoethyl diisopropylphosphoramidochloridite in CH_2Cl_2 into the corresponding phosphoramidites **42** - **57**. However, due to a significant side-reaction at *O*-C(6), the protected inosine phosphoramidite **56** was prepared by a different method, by treating the nucleoside **31** with 2-cyanoethyl tetraisopropylphosphoramidite and 5-(benzylthio)-1*H*-tetrazole (Wu and Pitsch 1998) (Pitsch et al. 1999) (Pitsch et al. 2001) in MeCN (conditions adapted from (Ji et al. 1990)). After chromatography on silica gel, these building blocks were isolated in yields between 50 and 98% *(Scheme I.16.)*[5]).

[5]) The lower yields were obtained from small-scale reactions. High yields of phosphoramidites (>85 %) can usually be obtained only from reactions carried out in a scale > 0.5 mmol. Since for small-scale reactions more equivalents of silica gel have to be employed, the decomposition of these reactive compounds occurs more efficiently.



Scheme I.16. *a*) 2-Cyanoethyl diisopropylphosphoramidochloridite, $^{i}Pr_2NEt$, CH_2Cl_2, 25°. b) 2-Cyanoethyl tetraisopropylphosphoramidite, 5-benzyl-1*H*-tetrazole, MeCN, 20°.

I.6. Conclusion

For an exhaustive study of the role of modified nucleosides within tRNA, it is necessary to be able to incorporate one or several modifications at selected positions (Nobles et al. 2002). This can be achieved only by the preparation of synthetic oligonucleotides using phosphoramidite technology. For this aim, we prepared the 5'-*O*-DMT, 2'-*O*-TOM phosphoramidite building blocks of the most encountered modified nucleosides of tRNAs (5-methyluridine (m^5U), dihydrouridine (D), inosine (I), 1-methylinosine (m^1I), N^6-methyladenosine (m^6A), N^6,N^6-dimethyladenosine ($m^6{}_2A$), N^6-isopentenyladenosine (i^6A), 1-methyladenosine (m^1A), N^6-(1-threonylcarbamoyl)adenosine (t^6A), 1-methylguanosine (m^1G), N^2-methylguanosine (m^2G) and N^2,N^2-dimethylguanosine ($m^2{}_2G$), 5-methylcytidine (m^5C)). Their syntheses have been achieved by the development of original synthetic pathways, exploiting the chemical stability of the 2'-*O*-TOM protecting group. Although these synthetic approaches do not significantly reduce the number of synthetic steps for individual nucleosides, they offer a rapid access to a large set of modified nucleosides prepared from the four commercially available, canonical 2'-*O*-TOM building blocks. In many cases, the synthetic approach is quite similar to the biosynthetic pathway and the modification is carried out after introduction of the 2'-*O*-TOM group. This strategy facilitates considerably the preparation of these compounds, since the introduction of the 2'-*O*-TOM group often results in low yields or appears incompatible with some modified nucleosides.

These phosphoramidite building blocks, available for the investigation of the role of modified nucleosides of the tRNA, could also serve for the preparation of more efficient suppressors tRNAs. Indeed, these compounds which carry an unnatural amino acids, are prepared by biologists as tRNA transcripts which exhibit a lower biological affinity. However, the main limitation of this methodology, which relies on the reading of STOP codon triplet by the ribosome machinery, is the resulting competition with the release factor (See *Introduction*) (Wang and Schultz 2005). In order to reduce this competition, several approaches have been envisaged such as the design of artificial base pairs. Among these base-

pairs, the firstly designed was the isoC-isoG system for which we describe a synthesis (Chapter II).

CHAPTER II "Artificial base pairs: Isocytidine-Isoguanosine"

II.1. Introduction

In the context of attempts to expand the genetic code, new solutions have been proposed for increasing the efficiency of the suppressor tRNA methodology for the introduction of unnatural amino acids into proteins (Wang and Schultz 2005). Thereby, some groups have proposed to "rewrite" the language of protein translation and to create new, orthogonal codon/anticodon pairs to overcome the intrinsic problems associated with the use of stop-anticodons, competing with release factors. These methods implied "quadruplet codons" by preparation of tRNA analogues with a larger anticodon loop containing four decoding bases (Hirao et al. 2002) or codon/anticodon pairs containing unnatural bases. The latter concept was early envisaged (Rich 1962), since it would create new codons that could be used in a very general way, even for the introduction of several unnatural amino acids into proteins. The first example of this strategy was demonstrated with the isoC-isoG base pair, which displays a new arrangement of H-bonds, but the same geometry as the C·G base pair *(Figure II.1.)* (Bain et al. 1992). This system worked effectively during the translation process, but nevertheless, it has never found any broad application and has been recently replaced by the other orthogonal pairing systems such as pyridine-2-one (y) / 2-amino-6-(2-thienyl)purine (s) in the CUs codon / yAG anticodon or pyridine-2-one (y) / 2-amino-6-(*N*,*N*-dimethylamino)purine (x) in the CUx codon / yAG anticodon *(Figure II.2.)* (Fujiwara et al. 2001) (Hirao et al. 2002).

The principal reason to abandon this system was the necessity to chemically modify both mRNAs and tRNAs, which at that time caused severe technical problems. The enzymatic incorporation of these modifications by the corresponding nucleoside triphosphates was difficult and not really satisfactory (Seela et al. 1997). However, considerable progress in the synthesis and ligation of relatively long RNA sequences has been achieved recently, in our group and elsewhere (Pitsch et al. 2005). It could therefore be possible to prepare isoG-isoC based orthogonal translation systems and employ them for the preparation of modified proteins. As an important step towards such projects, involving the preparation of

mRNA/tRNA pairs containing unnatural, isoC-isoG modified codons/anticodons, we have evaluated the synthesis of the corresponding 2'-*O*-TOM protected phosphoramidite building blocks.

Several syntheses of these nucleosides have been reported already, but confusing and contradictory statements in the literature about the stability of the nucleobase protecting groups prompted us to reevaluate their synthesis completely (for a discussion of this subject see (Jurczyk et al. 1998)).

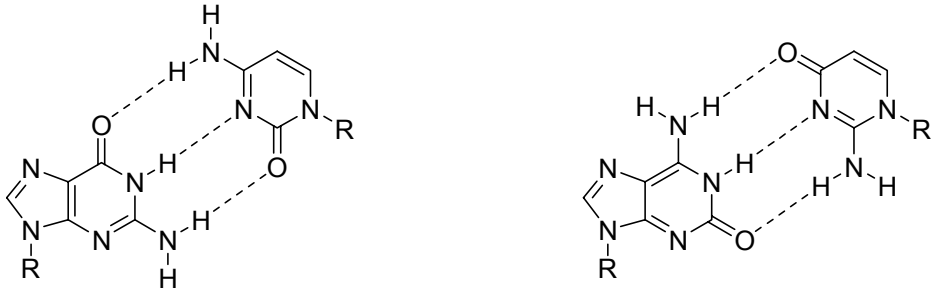


Figure II.1. Iso-cytidine/Isoguanosine base pair in comparison to the conventional Cytidine/Guanosine base pair

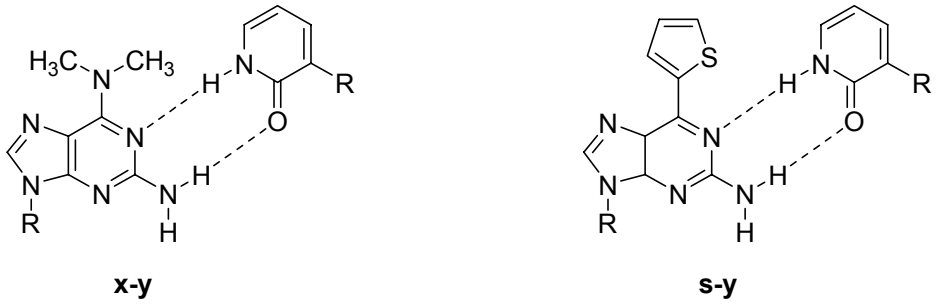


Figure II.2. Unconventional xy and sy base pairs

II.2. Synthesis

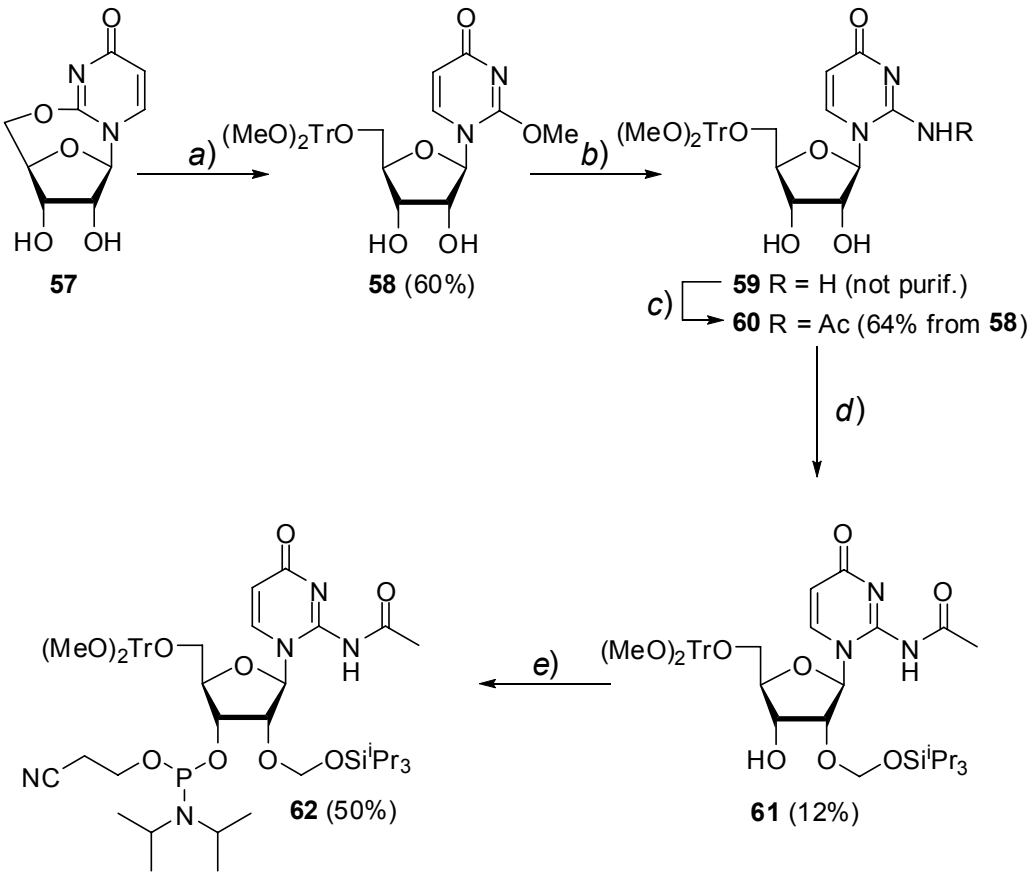
II.2.1. Isocytidine

In the RNA series, both the 2'-*O*-^{t}BDMS, N^2-(dialkylamino)methylidene (= N^2-dialkylformamidine) protected isocytidine (Roberts et al. 1997) (Chen et al. 2001) and 5-methylisocytidine phosphoramidite (Strobel et al. 1994) (Strobel et al. 1996), respectively,

have been prepared and incorporated. The 5-methylisocytidine was systematically used in place of isocytidine in both DNA and RNA series in order to avoid depyrimidination during oligonucleotide deprotection, which was only observed in the DNA series (Jurczyk et al. 1998). Despite these conclusions concerning the 2'-deoxyisocytidine, we prepared the related RNA building block isocytidine in its N^2-acetyl protected form. This choice has been later approved by the publication of chemical preparation of an isocytidine containing RNA sequence (Chen et al. 2001).

In our approach, uridine was first transformed into its cyclic derivative **57** under *Mitsunobu* conditions as reported (Vyle et al. 1998), and then ring-opened under optimized conditions, with MeOH in the presence of Et_3N, resulting in the formation of O^2-methyluridine, which was directly transformed into its $(MeO)_2Tr$-derivative **58** under standard conditions (60% yield from **57**). Substitution of the MeO-group of **58** with liquid NH_3 at 65° gave the isocytidine derivative **59**. After evaporation, the N^2-acetyl protecting group was introduced by first forming the 2',3'-di-*O*-di(trimethylsilyl) derivative with Me_3SiCl in Py, *N*-acetylation with AcCl, extractive work-up and treatment of the intermediate with Bu_4NF in THF ($\rightarrow$ **60**, 64% yield, based on **58**)[1]). Introduction of the 2'-*O*-TOM group into **60** was quite difficult, and even by employing optimized conditions ($^tBu_2SnCl_2$ at 25°, instead of Bu_2SnCl_2 at 70° (Pitsch et al. 2001)) the 2'-*O*-TOM protected isocytidine building block **61** could be obtained only in a low yield of 12% *(Scheme II.1.)*. The protected nucleoside **61** was finally converted with $^iPr_2NEt$ / 2-cyanoethyl diisopropylphosphoramidochloridite in CH_2Cl_2 into the corresponding phosphoramidite **62** in a yield of 50%.

[1]) The traditional, less efficient synthesis involves first preparation of isocytidine (treatment of **57** with NH_3), followed by sequential protection of positions N(2), O-C(5') and O-C(2') (Jurczyk et al. 1998).

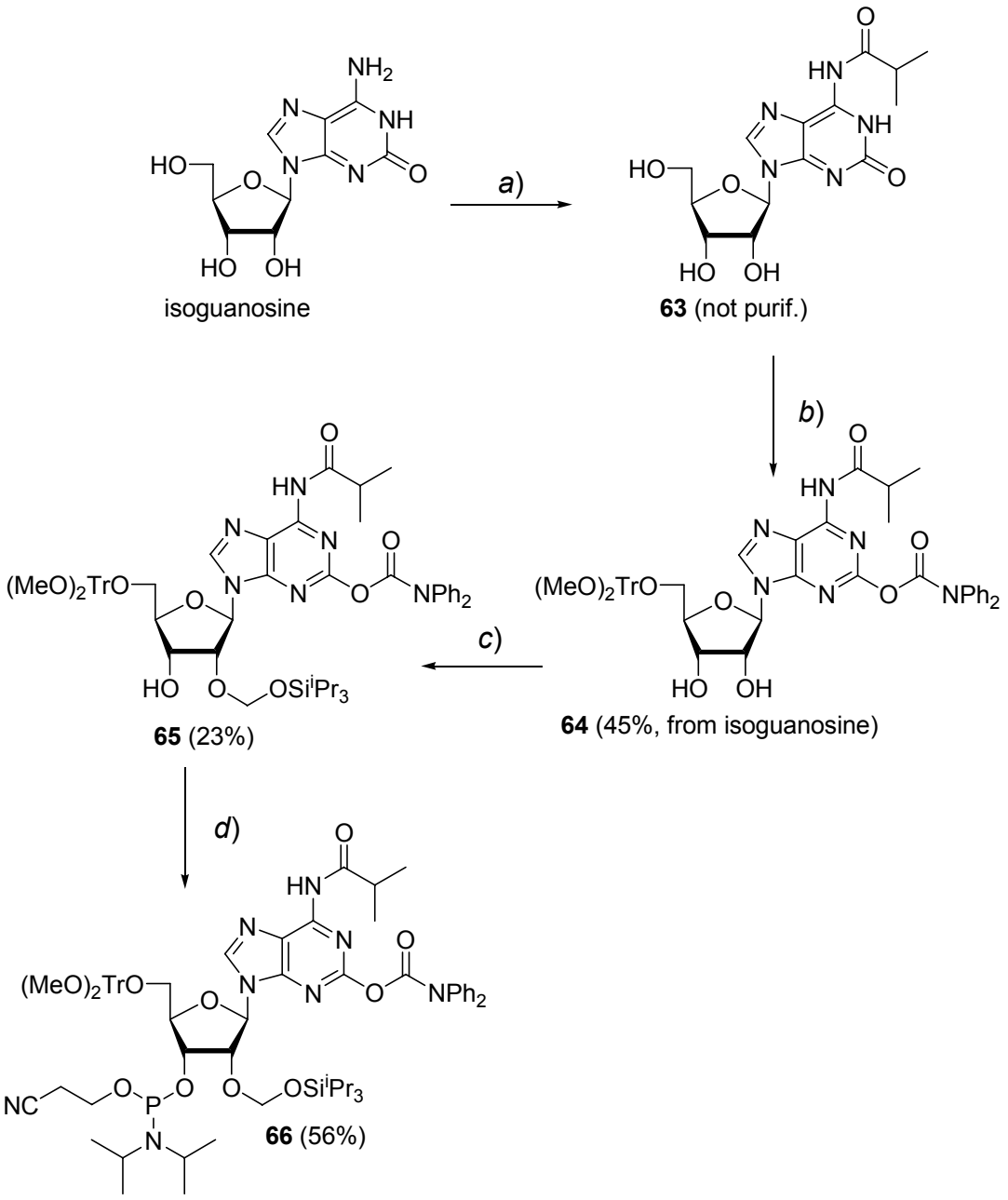


Scheme II.1. *a*) 1. Et_3N, MeOH, 65°; 2. $(MeO)_2TrCl$, pyridine, 20°. *b*) NH_3, 65°. *c*) 1. Me_3SiCl, pyridine, 4°; then AcCl, DMAP (= *N*,*N*-dimethylpyridin-4-amine), pyridine, 20°; 2. Bu_4NF, THF, 20°. *d*) $^tBu_2SnCl_2$, $^iPr_2NEt$, $(CH_2Cl)_2$, 20°; then TOM-Cl, 20°. *e*) 2-Cyanoethyl diisopropylphosphoramidochloridite, $^iPr_2NEt$, CH_2Cl_2, 20°. **57** was prepared according to (Vyle 1998).

II.2.2. Isoguanosine

Several syntheses of 2'-deoxyisoguanosine and isoguanosine phosphoramidites have been published (Chen et al. 2001) (Jurczyk et al. 1998) (Seela et al. 1994). Early, it has been demonstrated that the unprotected isoguanosine was incompatible with the phosphoramidite chemistry (Switzer et al. 1993). Consequently, several protecting groups have been investigated showing that the protection of the O^2-position rendered the N^6-position unreactive (Seela et al. 1997). The result of these studies and the difficulties encountered for introduction of acyl-type groups at N^6-position has lead of the employment of dialkyl-formamidine-type protecting groups in addition to *N*,*N*-diphenylcarbamoyl for *O*-C(2) (Jurczyk et al. 1998). Among the acyl-groups already investigated, the isobutyryl appeared the most suitable, although two different values for the half-life time of deprotection in (25% aq.

ammonia and EtOH 3:1 at 55°) have been reported: 40 min. (Ng et al. 1994), and 8 min. (Seela et al. 1997).



Scheme II.2. *a*) 1. Me_3SiCl, pyridine, 4°; then DMAP, isobutyryl chloride, 20°; 2. AcOH, MeOH, 20°. *b*) $Ph_2N(C{=}O)Cl$, $^{i}Pr_2NEt$, pyridine, 20°; then $(MeO)_2TrCl$, 20°. *c*) Bu_2SnCl_2, $^{i}Pr_2NEt$, $(CH_2Cl)_2$, 20°; then TOM-Cl, 75°. *d*) 2-Cyanoethyl diisopropylphosphoramidochloridite, $^{i}Pr_2NEt$, CH_2Cl_2, 20°.

Despite these reports, we were able to efficiently prepare N^6-(isobutyryl)isoguanosine **63** by treating carefully dried isoguanosine (24 h at 50° and 0.01 mbar, followed by 4Å molecular sieves in Py for 2 h at 25°) first with 10 equiv. Me_3SiCl and 1.2 equiv. isobutyryl chloride, followed by extraction at 4° and subsequent hydrolysis of the Me_3Si-ethers with AcOH in

MeOH[2]) *(Scheme II.2.)*. The crude product **63**, obtained by evaporation was sufficiently pure (^{1}H-NMR: > 90%) to be used directly for the next one-pot reaction sequence. First, the *O*-C(2) position was protected with *N*,*N*-diphenylcarbamoyl chloride in Py, and then the *O*-C(5') position with $(MeO)_2TrCl$ ($\rightarrow$ **64**, 45% yield from isoguanosine). The 2'-*O*-TOM protected derivative **65** was then prepared under standard conditions (Pitsch et al. 2001) (23% yield, *(Scheme II.2.)*).

Furthermore, the removal of the nucleobase protecting groups has been investigated on the 5'-*O*-DMT, O^2-(*N*,*N*-diphenylcarbamoyl)-N^6-isobutyrylisoguanosine. Their full cleavage has been achieved within 15 minutes under standard conditions (12M $MeNH_2$ in H_2O/8M $MeNH_2$ in EtOH 1:1 at 20°). The phosphoramidite building block **66** was prepared as described above from **65**, and isolated in a yield of 56%.

II.3. Conclusion

In attempts to increase the efficiency of the suppressor tRNA methodology, some groups have proposed to "rewrite" the language of protein translation and to create new, orthogonal codon/anticodon pairs containing unnatural bases. For this aim, we prepared the isoC and isoG phosphoramidite building blocks and resolved some of contradictory reports about the stability and the choice of protecting groups.

[2]) This reaction sequence was carried out in analogy to the preparation of N^6-acetyladenosine (Pitsch et al. 2001).

CHAPTER III "Increasing the codon-anticodon strength: the wyosine"

III.1. Introduction.

The poor efficiency of tRNA suppressor elaborated from tRNA transcripts has been mainly attributed to absence of modified nucleosides (Ashraf et al. 1999) (Ashraf et al. 2000) (Yarian et al. 2000) (Yarian et al. 2002) (vonAhsen et al. 1997). Beside their role for the proper functional folding of the tRNA, these modifications are essential for a correct frame-reading and therefore for the accuracy of the protein biosynthesis (Agris 2004). This function is mainly ensured by the fine-tuning of the codon-anticodon pairing provided by the nucleosides at the wobble-position (position 34) and 3'-end adjacent to the anticodon (position 37). This latter position is occupied by a purine derivative which correlates with the base at position 36 (Chapter IV) (Sprinzl et al. 1998). Therefore, all tRNAs with an uridine at position 36 contain a modified adenosine-derivative such as t^6A, ms^2t^6A, m^6t^6A, hn^6A, ms^2hn^6A or g^6A at position 37 and all tRNAs with an adenosine at position 36 contain at position 37 a i^6A, ms^2i^6A, ms^2io^6A or a wyosine-derivative. All three STOP codons (UAG/UAA/UGA Amber/Ochre/Opal) have a uridine at the first position and the introduction of such modifications could be useful for the preparation of a more efficient synthetic suppressor tRNAs. Among the different hydrophobic derivatives present at 3'-end adjacent to adenosine, wyosine-related compounds are those offering the most extended surface area due to their tricyclic structure. All structurally related to guanosine, the different derivatives are exclusive for the phenylalanine tRNAs (UUU and UUC codons) and specific for one type of organism according to their phylogeny (Zhou et al. 2004). One is present in both eukarya and archaea domains (imG), three are unique to archaea (imG-14, imG2, mimG) and the four remaining exhibit an amino acid moiety at position C(7) (yW, o_2yW, OHyW, OHyW*) and are related to eukarya *(Figure III.1.)*. Although most of these nucleosides have been already synthesized for structural confirmation, only 4-desmethylwyosine (Ziomek et al. 2002)[1]) has been prepared as phosphoramidite building block. However, wyosine itself is labile towards

[1]) No synthesis of the corresponding phoshoramidite building block is reported.

acid ($t_{1/2}$ = 95 s at pH 1 and 25°) (Itaya and Harada 1984) and therefore is not fully compatible with the automated assembly of RNA sequences[2]).

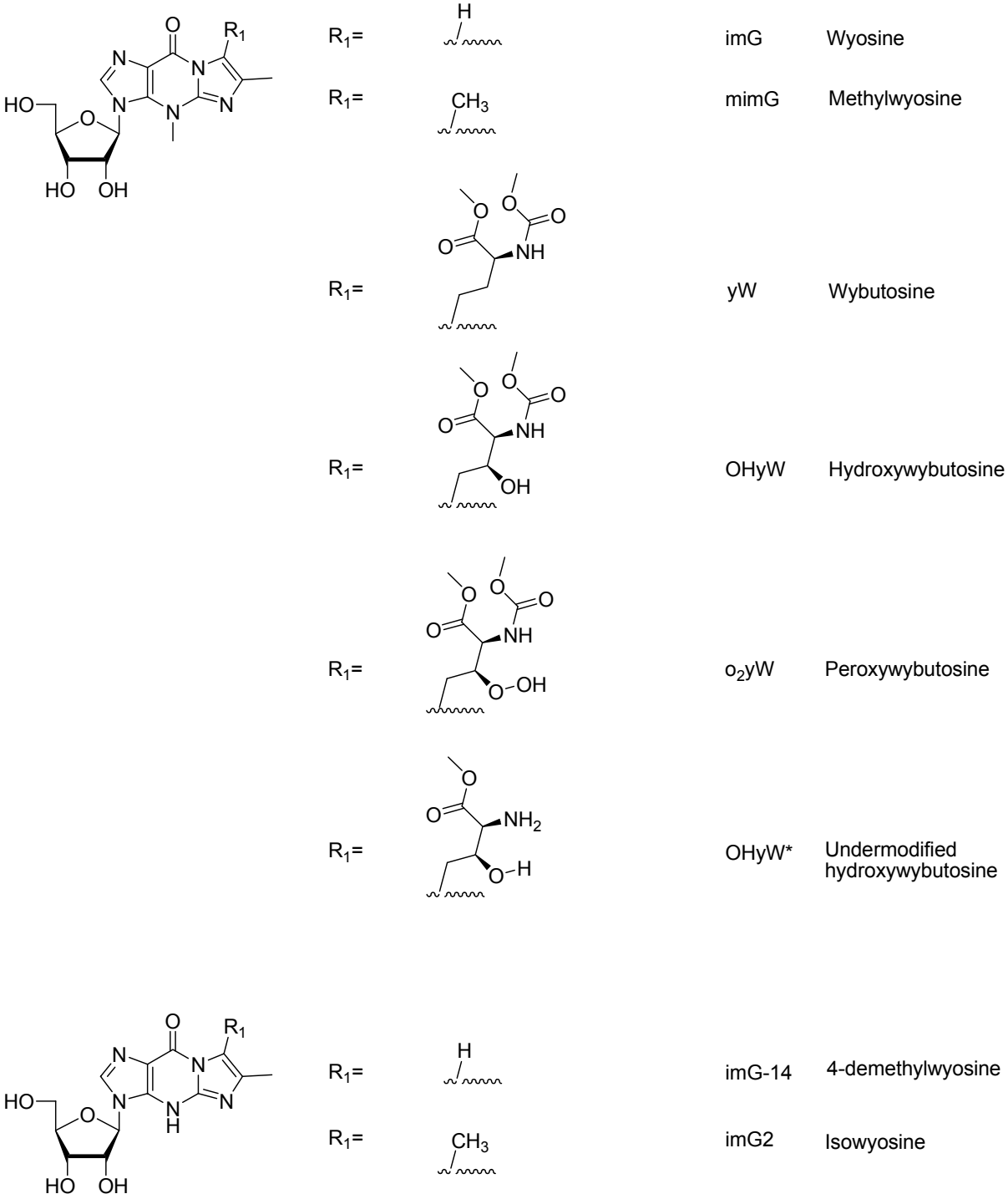


Figure III.1. Structure of naturally occurring wyosine derivatives.

2) Within each cycle of the assembly a detritylation reaction under acidic conditions (e.g. 3% $CHCl_2COOH$ in $(CH_2Cl)_2$ (Pitsch et al. 2001)) is carried out.

We investigated the synthetic pathways for wyosine analogues, which could also be used for the synthesis of two other recently isolated natural wyosine derivatives lacking the N(4)-methyl group, 4-desmethylwyosine (imG-14, unsubstituted at C(7)) and isowyosine (imG2, carrying a Me-group at C(7)). In this context, we also prepared of 4-desmethyl-5-methylwyosine, an isomer of the parent wyosine, which is known to be stable under acidic conditions ($t_{1/2}$ = 690 h at pH 1 and 25°) (Itaya and Harada 1984) *(Figure III.2.)*. The corresponding phosphoramidite building block was incorporated into a model RNA sequence from *Moloney murine leukaemia virus* (Chapter III.3.2.). This RNA sequence was able of self-association ("kissing interaction") through two C·G base pairs stabilized by two adjacent adenines A9 (Kim and Tinoco, Jr. 2000) and in our opinion, this complex is structurally related to the codon-anticodon interaction formed between mRNAs and tRNAs. The 4-desmethyl-5-methyl-wyosine phosphoramidite was successfully incorporated in place of adenosines A9 and the thermodynamic properties of the resulting RNA-sequences were characterized by NMR-spectroscopy. Unfortunately, no "kissing" formation was observed and hence we planned to incorporate the naturally occurring wyosine at the same position. The corresponding phosphoramidite was prepared (Chapter III.2.2.) and again incorporated. Since it can only be incorporated as last nucleotide at the 5'-end, an original enzymatic ligation approach for the incorporation of wyosines into RNA sequences was developed (Chapter III.3.2.3.). Again, the NMR analysis of the wyosine-containing RNA sequence revealed no "kissing" interaction. In order to investigate the origin of the stability of this interaction, a variety of other purine nucleotides were introduced at the same position (Chapter III.3.3.).

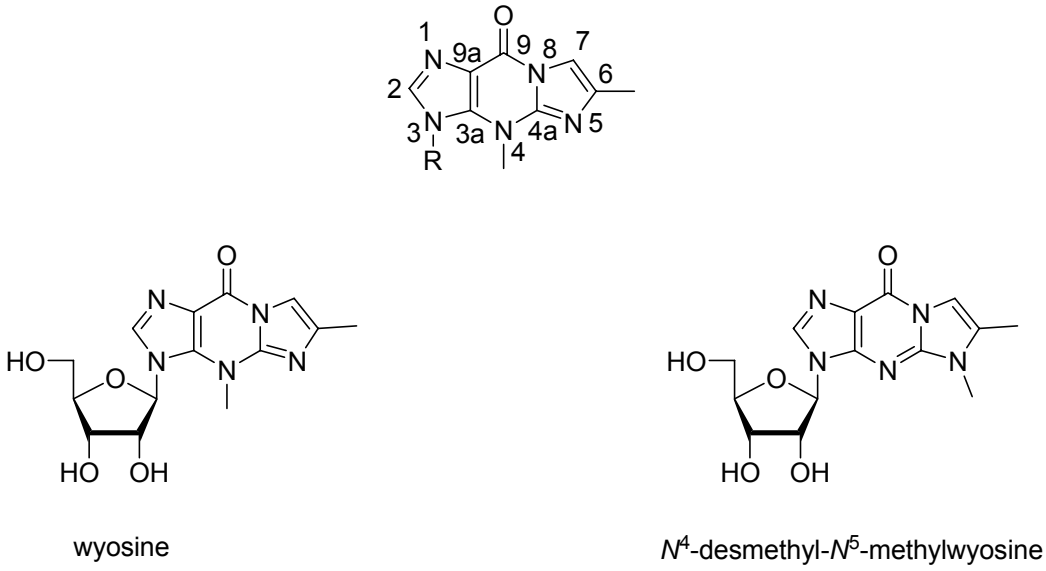
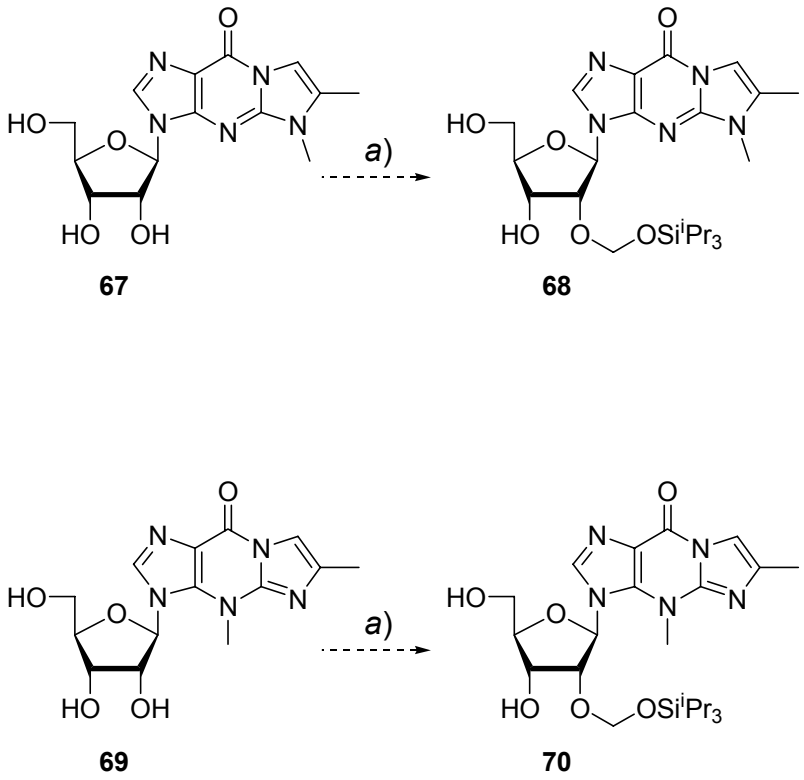


Figure III.2. Structure of wyosine and of its analogue.

III.2. Synthesis of phosphoramidite building blocks

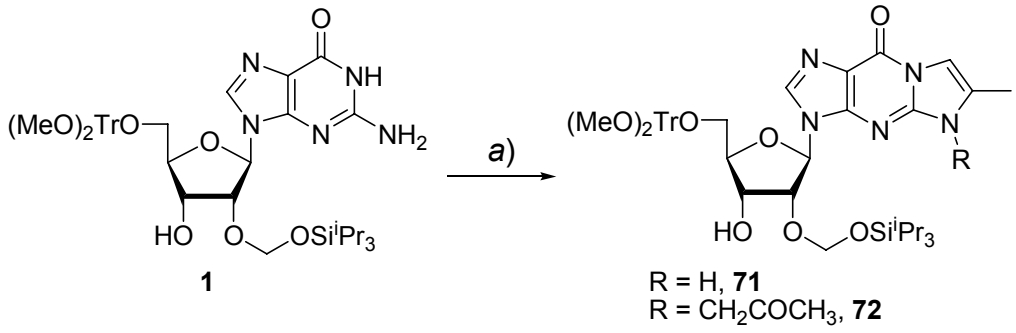
III.2.1. Synthesis of the 4-desmethyl-5-methylwyosine phosphoramidite

Our first attempts to introduce the 2'-*O*-TOM group under established conditions (Pitsch et al. 2001) into the unprotected nucleotides wyosine (Golankiewicz and Folkman 1983) (Bazin et al. 1987) and 4-desmethyl-5-methylwyosine (Golankiewicz and Folkman 1983) (Boryski and Ueda 1985) resulted in depurination and the formation of several other unidentified products *(Scheme III.1.)*. Whereas addition of Bu_2SnCl_2 to wyosine **67** resulted in complete cleavage of the glycosidic bond, the reaction was compatible with the wyosine analogue but gave the desired product among a mixture of unidentified compounds.



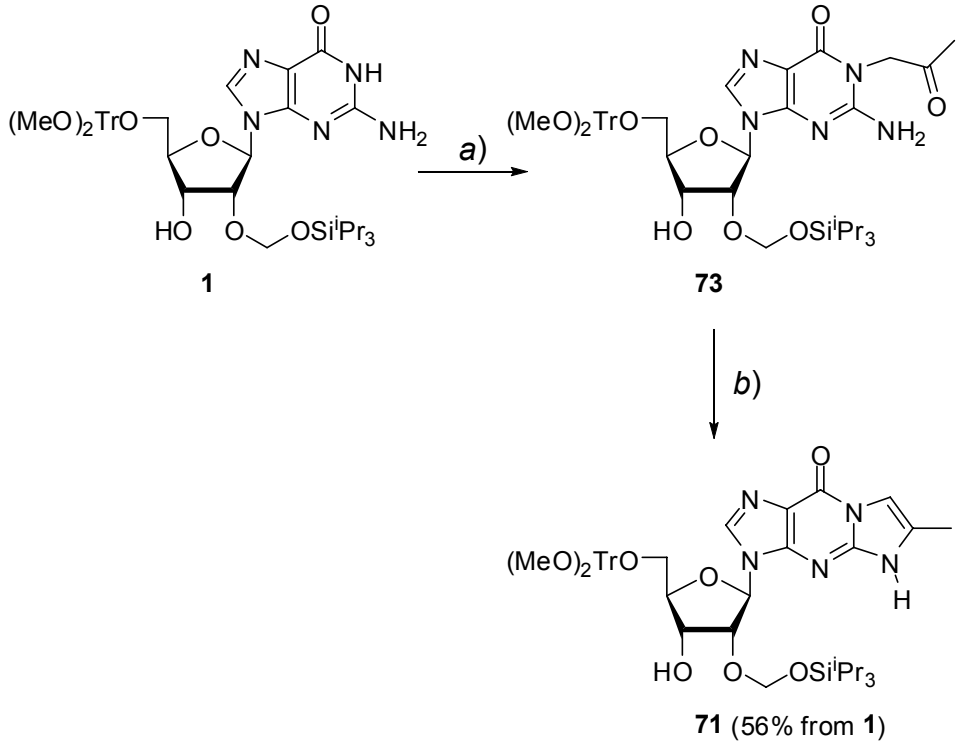
Scheme III.1. *a*) Bu_2SnCl_2, $^{i}Pr_2NEt$, $(CH_2Cl)_2$, 20°; then TOM-Cl, 20°. **67** was prepared according to (Glemarec et al. 1988), **69** according to (Bazin et al. 1987).

These results may be interpreted in terms of the preferred conformation of these nucleosides, since it is now established that the introduction of TOM group introduction requires a syn-conformation of the base. And indeed, whereas the natural wyosine exhibits exclusively an anti-conformation, the analogue preferred the syn-conformation (Sierzputowska-Gracz 1991). Therefore, we decided to prepare the 5'-*O*-DMT, 2'-*O*-TOM wyosine precursor **71** from the 5'-*O*-DMT, 2'-*O*-TOM protected guanosine **1** (Stutz et al. 2000) *(Scheme III.2.)*.



Scheme III.2. *a*) K_2CO_3, KI, DMF, 20°; then bromoacetone, –15° to 20°. **1** prepared according to (Stutz et al. 2000).

Under the conditions reported for the formation of 4-desmethylwyosine from guanosine (NaH/bromoacetone, DMF, –15° (Kasai et al. 1976)), partial decomposition of **1** was observed. This degradation of the product was probably a result of the difficult handling of NaH in terms of molar equivalents (estimated concentration in oily dispersion ca. 50%). By employing milder conditions (K_2CO_3 instead of NaH and addition of KI) this problem was avoided, but now an efficient N(4)-alkylation of the product **71** was observed *(Scheme III.3.)*[3]). Finally, **71** was prepared in two steps: N(1)-alkylation of **1** with K_2CO_3/bromoacetone/KI in DMF at –15°, followed by work-up and extraction gave crude **72**, which then was transformed into the protected 4-desmethylwyosine **73** (56% yield) by dehydration and cyclisation with 4Å molecular sieves in CH_2Cl_2 at 20°.

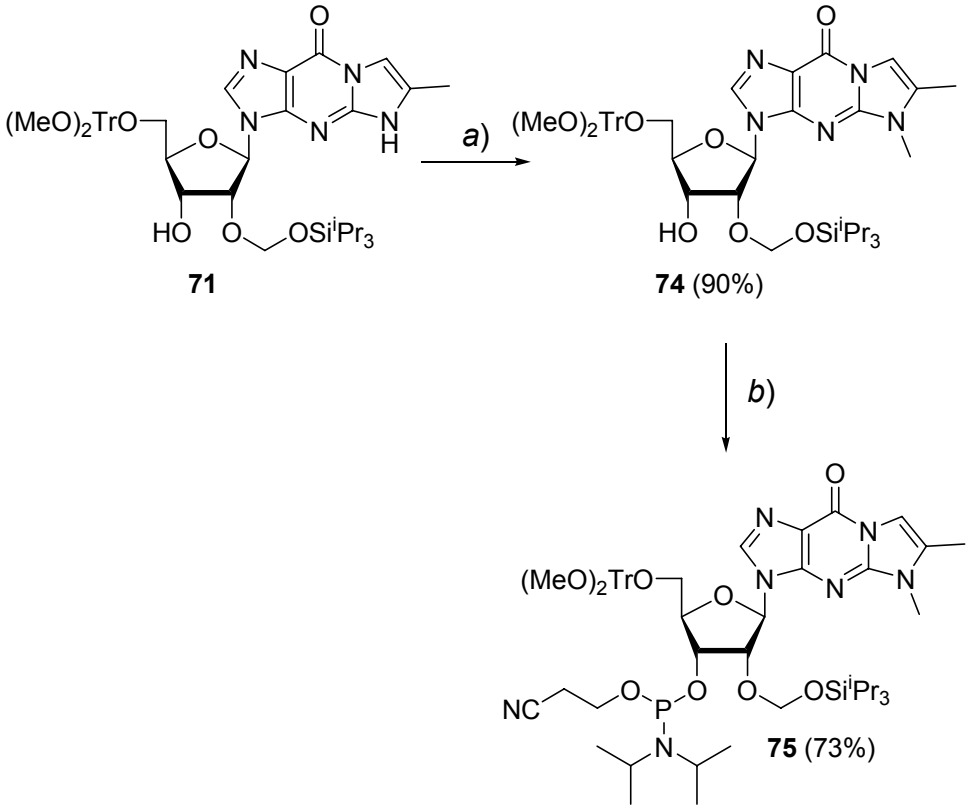


Scheme III.3. *a*) K_2CO_3, KI, DMF, 20°; then bromoacetone, –15°. *b*) molecular sieve, CH_2Cl_2, 20°. **1** prepared according to (Stutz et al. 2000).

All these optimizations permitted to reduce the side product formation to 4% even when the reaction was carried out in a 500 mg scale. N(5)-methylation of **71** (*Scheme III.4.*) with K_2CO_3/MeI in DMF at –20° according to (Boryski and Ueda 1985) gave **74** (90% yield),

[3]) H-N(4) in desmethylwyosine has a pK_a value of 3.24.

which was then transformed into the corresponding 4-desmethyl-5-methylwyosine phosphoramidite building block **75** (73% yield).



Scheme III.4. *a*) K_2CO_3, DMF, 20°; then MeI, –15°. *b*) 2-Cyanoethyl diisopropylphosphoramidochloridite, $^{i}Pr_2NEt$, CH_2Cl_2, 20°.

III.2.2. Synthesis of the wyosine phosphoramidite

According the planned strategy for the incorporation of a wyosine at the interior of a RNA sequence, a 5'-OH-wyosine-ending sequence must be obtained after deprotection, subsequently 5'-O-phosphorylated with a phosphokinase, such as T4 PNK and then ligated to the 3'-OH position of another sequence *(Figure III.3.)*.

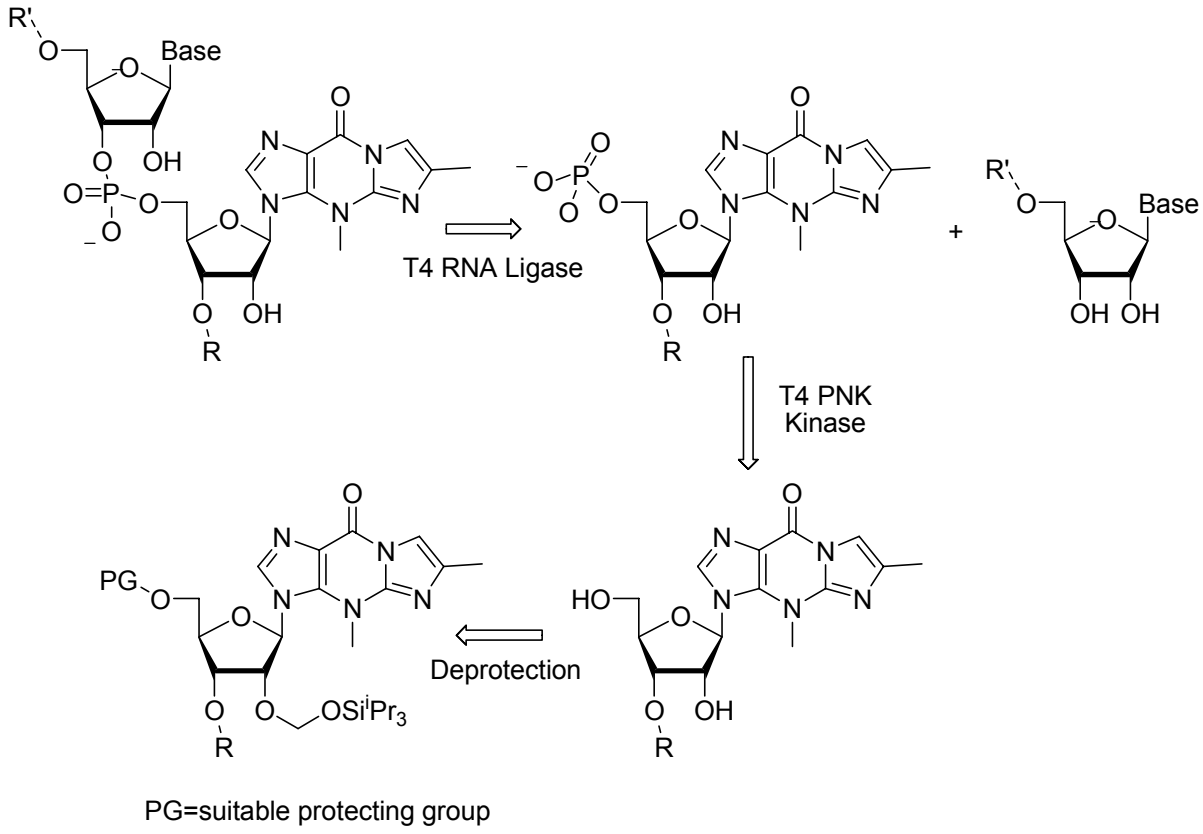
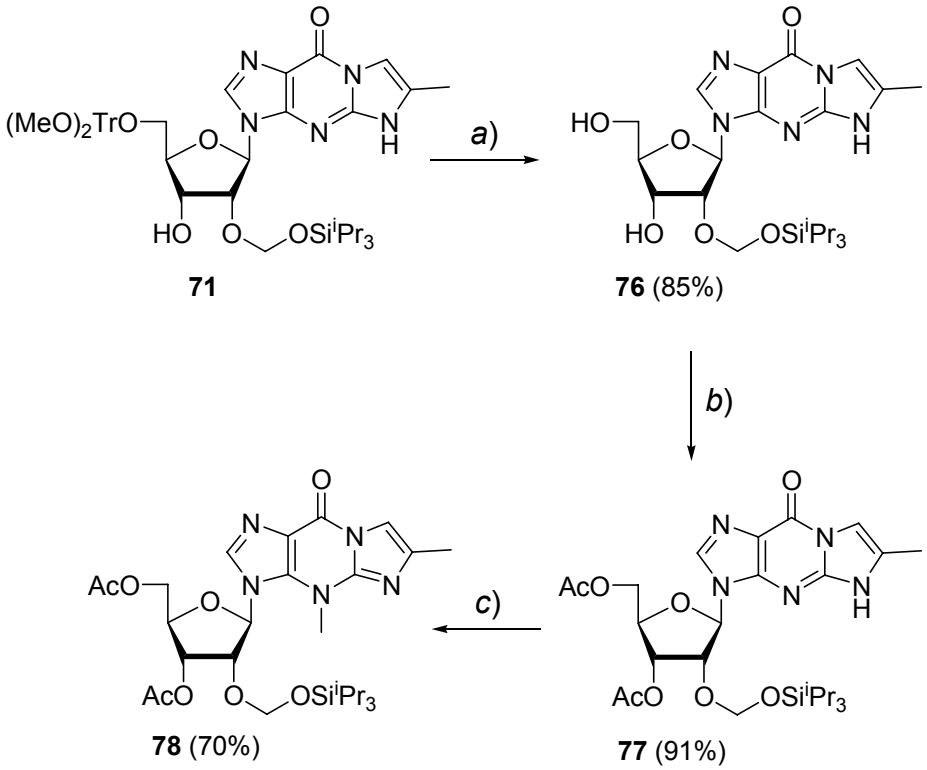


Figure III.3. Strategy for the incorporation of wyosine into RNA oligonucleotides.

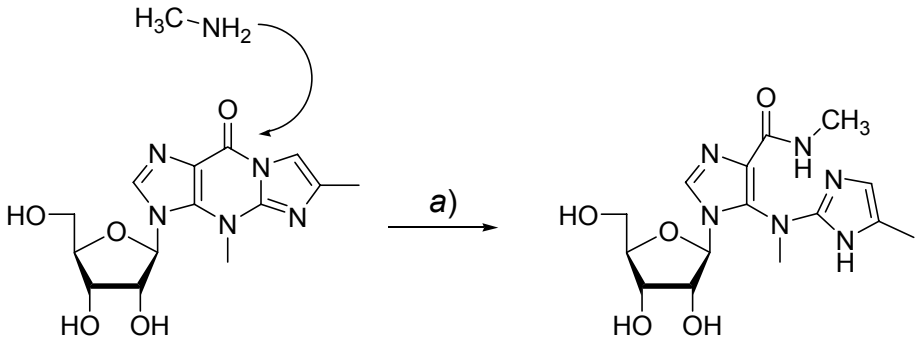
In order to obtain a wyosine with a free 5'-OH group (after deprotection) several protecting groups could be envisaged, including base-labile (e.g. FMOC and various esters) and fluoride-labile (silyl ethers) groups. It was planned to prepare the wyosine phosphoramidite from the intermediate **71**, already obtained in the context of the synthesis of the wyosine analogue. Prior to N(4)-methylation, all HO-groups had to be protected, e.g. by acetylation.

Removal of the $(MeO)_2Tr$ group of **71** with $CHCl_2COOH$ in $(CH_2Cl)_2$ gave **76** in 85% yield. Treatment of this intermediate with Ac_2O in pyridine, followed by selective N(4)-deacetylation under optimized conditions (Golankiewicz and Folkman 1983), with $MeOH/H_2O$/pyridine 1:1:1, resulted in the formation of the 3',5'-di-*O*-acetylated derivative **77** (91% yield). Efficient formation of the protected wyosine **78** (70% yield) was achieved by N(5)-methylation with CH_2I_2/Et_2Zn in Et_2O at 4°, according to (Bazin et al. 1987) *(Scheme III.5.)*.

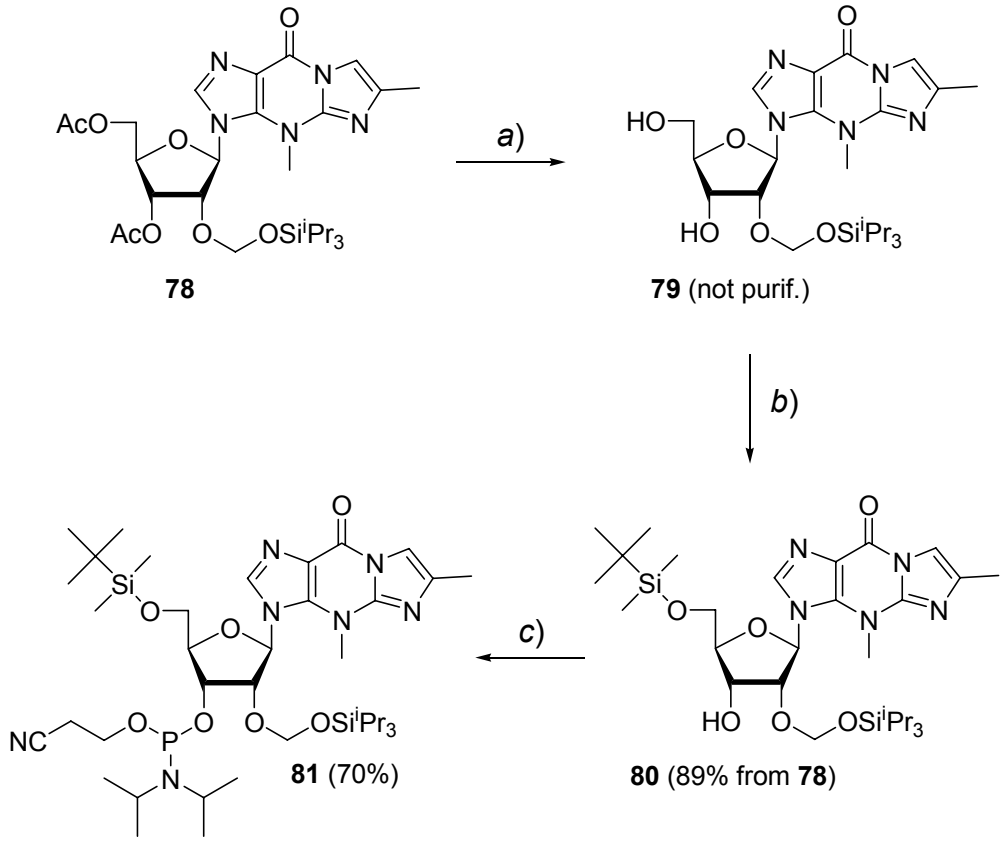


Scheme III.5. *a*) 1. Dichloroacetic acid, CH_2Cl_2, 20°. *b*) 1. Ac_2O, DMAP, pyridine, 20°; 2. pyridine, MeOH, H_2O, 20°. *c*) CH_2I_2, Et_2Zn, Et_2O, 4°.

The removal of acetyl protecting groups appeared to be a critical step since the tricyclic moiety is peculiarly sensitive towards acids and also towards nulceophilic bases. Under strongly basic conditions, the wyosine undergoes a ring opening of the base moiety (Itaya and Harada 1984) as confirmed by MS analysis after incubation with the conventionally used methylamine *(Scheme III.6.)*. Nevertheless, a methanolic solution of ammonia gave a clean cleavage of the acetyl groups without any formation of ring-opened products. The final protection of the 5'-*O*-position with FMOC was unsuccessful. However, the fluoride-labile *tert*-butyldimethylsilyl protecting group could be incorporated under standard conditions, with ^{t}BDMS-Cl/imidazole (→ **80**, 89% yield, from **78**). The phosphoramidite building block **81** was finally obtained in a yield of 70% *(Scheme III.7.)*.



Scheme III.6. *a*) 1. $MeNH_2$, EtOH, H_2O, 20°



78

79 (not purif.)

81 (70%)

80 (89% from **78**)

Scheme III.7. *a*) 1. NH_3, MeOH, 20°. *b*) ^{t}BDMS-Cl, imidazole, DMF, CH_2Cl_2, 4°. *c*) 2-Cyanoethyl diisopropylphosphoramidochloridite, $^i Pr_2NEt$, CH_2Cl_2, 20°.

III.3. Incorporation of wyosine phosphoramidites into oligonucleotides

III.3.1. Investigation of conditions for incorporation

For avoiding the problem of incompatibility of wyosine with the acidic detritylation step of the automated synthesis of oligonucleotides, we designed the compatible wyosine analogue 4-desmethyl-5-methylwyosine. However, its incorporation required other

adaptations, especially for the oxidation step. The usual conditions involve iodine which is incompatible with wyosine and 4-desmethyl-5-methylwyosine, since it results in iodination of position 7 (Glemarec, 1988) or even in degradation of the tricyclic moiety under formation of N^2-methylguanosine (Boryski and Ueda, 1985). So far, only few alternative oxidation reagents for automated synthesis have been investigated, and a 10% solution of *tert*-butylhydroperoxide in acetonitrile (Hayakawa et al. 1986) has been the most widely employed alternative. It is also the most suitable for the incorporation of sensitive nucleosides such as thiol substituted derivatives (Sundaram et al. 2000).

In order to investigate the compatibility of wyosine and of 4-desmethyl-5-wyosine with this oxidative agent, we incubated both nucleosides **74** and **80** at room temperature and monitored the eventual degradation by MS and TLC analysis. Even after 6 h at room temperature less than 5% of degradation has been observed.

Additionally, deprotection of oligonucleotides under standard conditions requires the use of $MeNH_2$ which is known to be incompatible with wyosine and 4-desmethyl-5-methyl-wyosine, as described above. Most of the alternatives are based on aqueous or alcoholic solutions of NH_3, conditions which, as an example, are known to be compatible with the fragile dihydrouridine nucleoside (Chaix et al. 1989) (deprotection with 25% aq. NH_3/EtOH 1:1 at 55° overnight). Such conditions were also compatible with the wyosine and 4-desmethyl-5-methyl-wyosine nucleoside, as experimentally verified.

After establishing conditions for the incorporation and deprotection of wyosine and 4-desmethyl-5-methyl-wyosine, a short model RNA sequence, where a π-stacking interaction could play an important role, was selected as first synthetic target.

III.3.2. Incorporation into a model sequence

III.3.2.1. Introduction

During a study on a retroviral RNA of *Moloney murine leukaemia virus*, *C.-H. Kim* and *I. Tinoco* have observed that a highly conserved GACG tetraloop **S1** formed a stable

homodimeric “kissing complex” through the formation of only two C·G base pairs (Kim and Tinoco Jr. 2000) *(Figure III.4.)*.

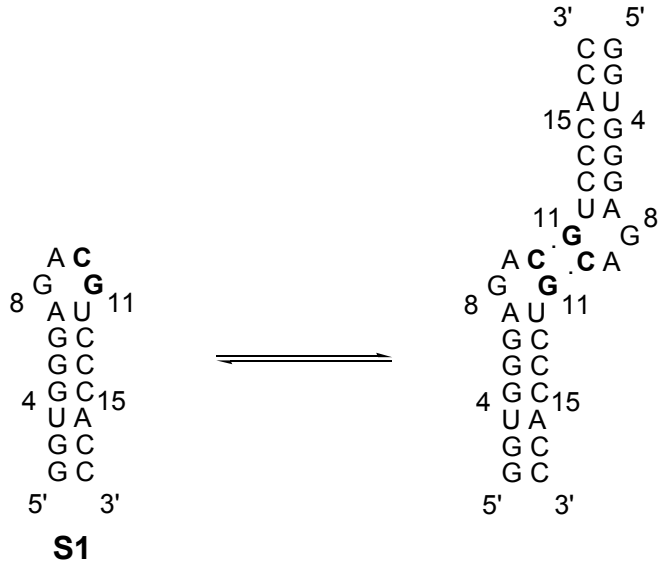


Figure III.4. The “kissing complex”.

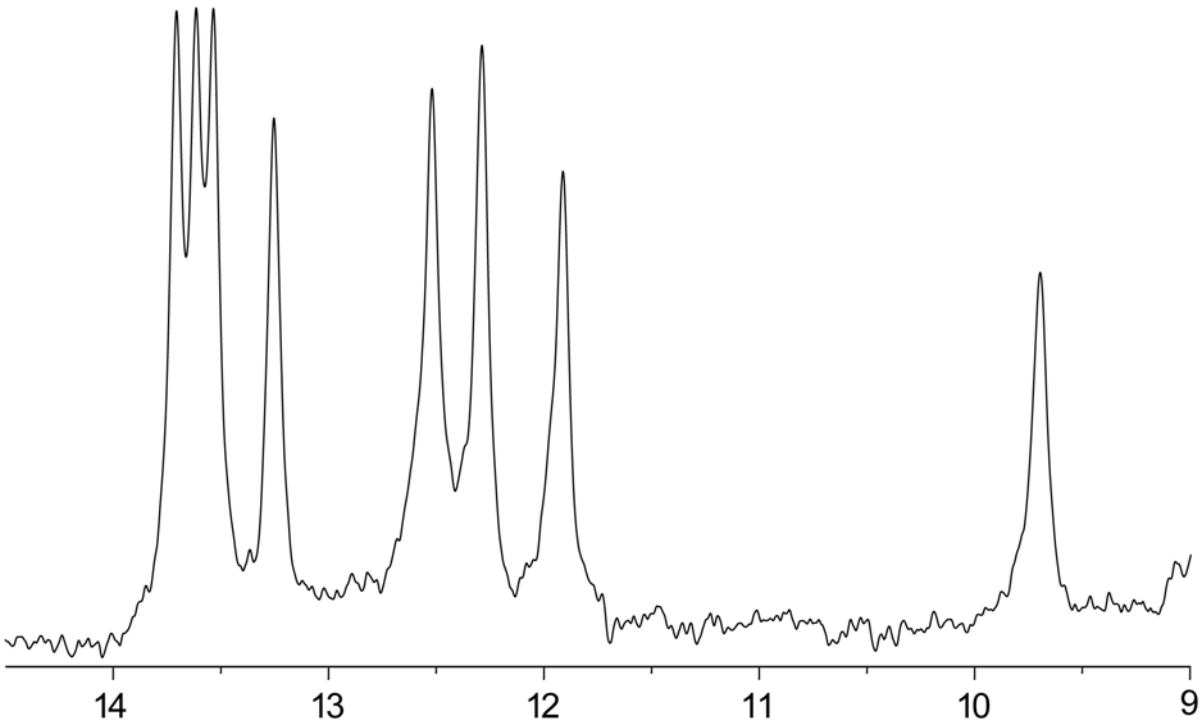


Figure III.5. NMR analysis of the imino protons of the naturally occurring sequence **S1** (scale in ppm).

This complex is quite stable (Tm = 48°), but its stability is poorly understood (Kim and Tinoco Jr. 2000). However, the adenosines 3'-adjacent to the G·C base pair are essential for complex formation. This complex was, in our opinion, structurally related to the codon-anticodon interaction where the adjacent hypermodified purines pay also a great contribution to the stability. Thus, we planned to substitute the A9 of the 18mer RNA sequence by our wyosine analogue 4-desmethyl-5-methylwyosine, which offers a wider π-stacking by its tricyclic structure *(Figure III.6.)*. Later, we have also prepared the corresponding wyosine-containing RNA sequence (Chapter III.3.2.3.).

III.3.2.2. Incorporation of 4-desmethyl-5-methylwyosine and NMR analysis

The preparation of the 4-desmethyl-5-methylwyosine containing RNA sequence **S2** has been carried out with tBuOOH as oxidation reagent (1.1M in MeCN), with a longer oxidation time than described (Kumar and Davis 1995). The deprotection and cleavage of the sequence from the solid support has been achieved by an overnight incubation with 25% aq. NH_3/EtOH 1:1 at 55° (Chaix et al. 1989) and the 2'-*O*-TOM protecting groups were finally removed under standard conditions. According to HPLC analysis and ESI-MS characterization of the main product, the 4-desmethyl-5-methylwyosine was successfully incorporated *(Figure III.6.* and *Figure III.7.)*. For the subsequent NMR analysis, the purified oligonucleotide **S2** was finally converted into the sodium salt form. Imino proton NMR spectroscopy was then carried out at a concentration of 100 μM in potassium arsenate buffer (25mM, pH 7.0) in H_2O/D_2O 9:1. According to these experiments, no kissing complex interaction could be observed at 25° or 10° *(Figure III.8.)*.

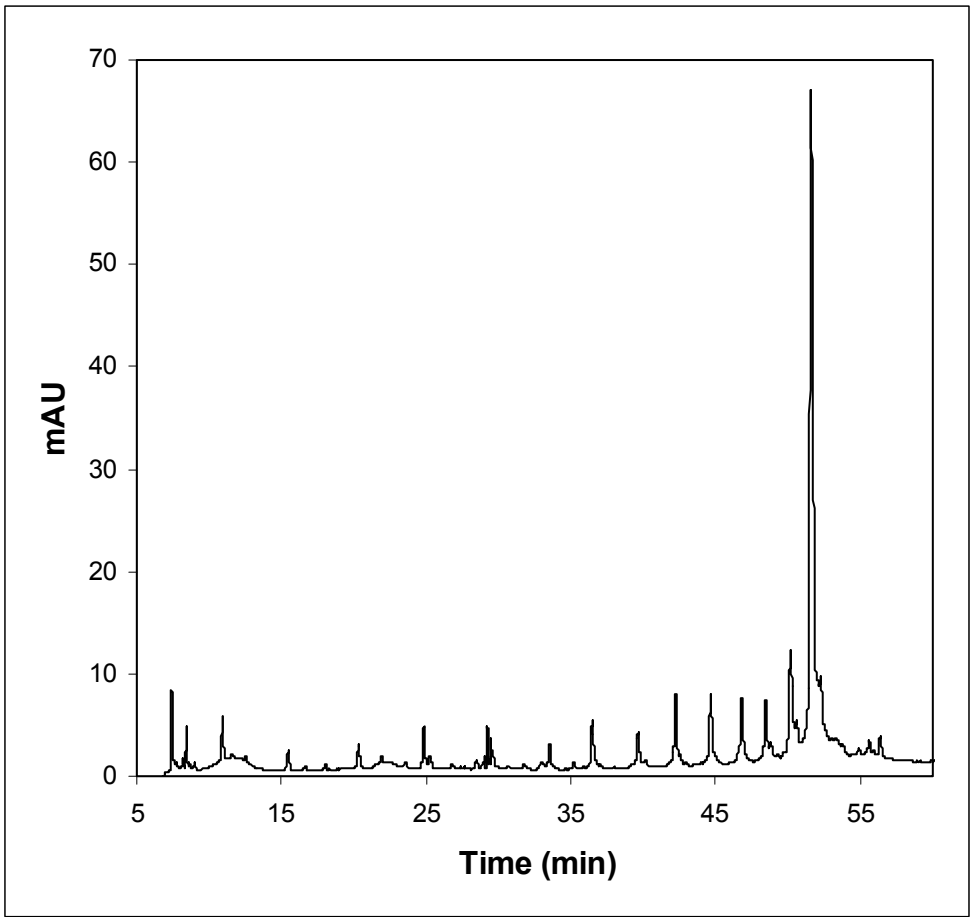


Figure III.6. HPLC trace of the crude wyosine-analogue containing sequence **S2**.

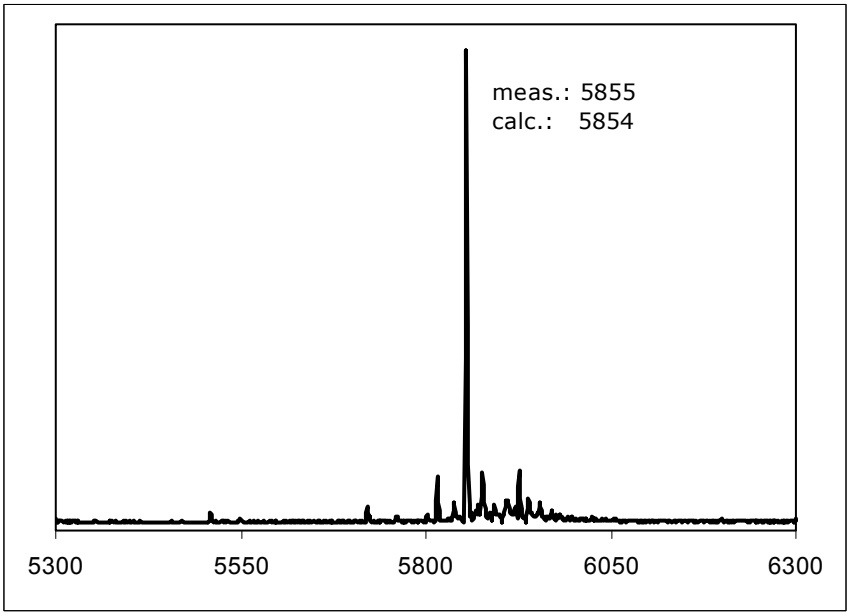


Figure III.7. ESI-MS analysis of the purified wyosine-analogue containing sequence **S2**.

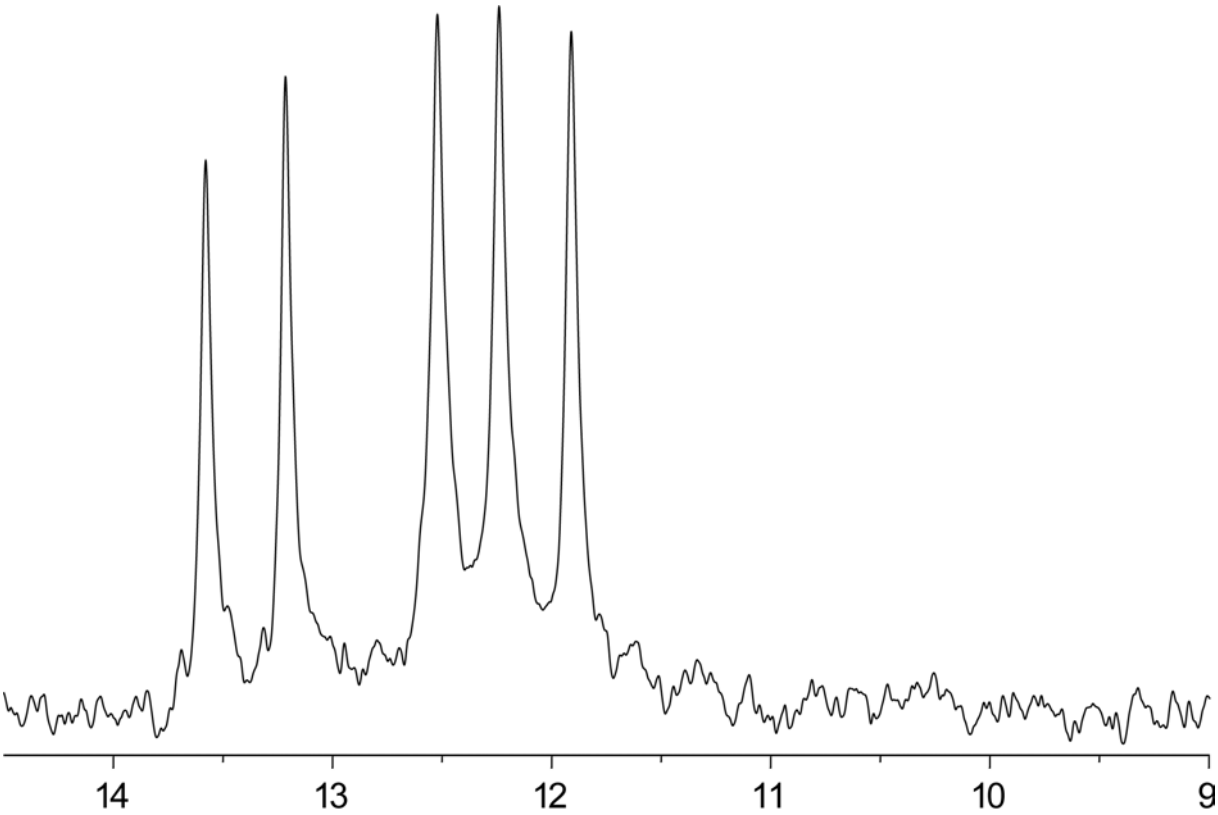


Figure III.8. NMR analysis of the imino protons of the wyosine-analogue containing sequence **S2** (scale in ppm).

III.3.2.3. Incorporation of wyosine and NMR analysis

After these first results with the 4-desmethyl-5-methylwyosine-containing RNA sequence **S2**, which revealed no kissing interaction, we planned to prepare and investigate the corresponding wyosine-containing RNA sequence **S3**. Consequently, we investigated strategies for incorporation of wyosine by a combination of chemical and enzymatic methods. According to our initial concept, the incorporation of wyosine into RNA sequences would include an enzymatic phosphorylation of the free 5'-HO group followed by the ligation with another sequence. For preparation of the sequence **S3**, a ligation of a 8mer RNA sequence **S4** with a 10mer RNA sequence **S6**, containing a 5'-terminal wyosine would have to be carried out *(Figure III.9.)*.

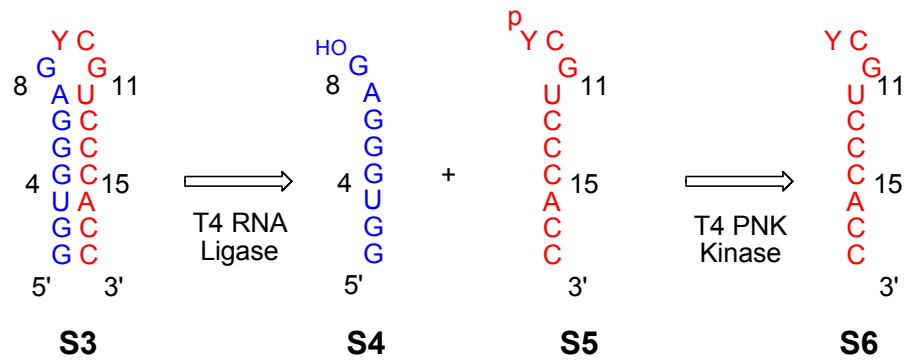


Figure III.9. Synthetic approach for the preparation of the wyosine-containing sequence S3.

Unexpectedly, the wyosine-containing sequence **S6** underwent a partial decomposition under the conditions which have been successfully employed for the deprotection of the 4-desmethyl-5-methyl-wyosine-containing sequence **S2**. Therefore, the aqueous solution of ammonia has been substituted for a dry 12M methanolic solution of ammonia which has been recently employed for incorporation of the sensitive 1-methyladenosine nucleoside (Mikhailov et al. 2002). Before further investigations, we checked if under these conditions the cleavage of the oligonucleotide from solid support did not constitute a rate-limiting step. The experiments were carried out with an uridine immobilized on CPG and revealed a complete cleavage within 8h. However, methanolic ammonia was unable to completely remove the acetyl protecting groups on guanosines. Therefore, guanosines were introduced via the unprotected guanosine phosphoramidite, which had already been successfully employed in our group (Stutz et al. 2000). In addition, ammonia is not nucleophilic enough for constituting a good acceptor of acrylonitrile which is formed during deprotection of the phosphodiesters and can alkylate the nucleobases. Indeed, acrylonitrile has been recently used as derivatisation reagent for MS analysis of pseudouridine and other minor components of tRNAs (Mengel-Jørgensen and Kirpekar 2002). However, it is known that the cyanoethyl protecting groups can be easily removed by washing the solid support with a 10% solution of diethylamine in acetonitrile. But again, in our hands this protocol was not compatible with dihydrouridine and wyosine nucleosides, and has therefore been successfully substituted by diisopropylamine. After this washing-step, the sequence was deprotected and cleaved from

the solid support by action of 12M methanolic solution of ammonia for 14h at 20°. After recovering of the supernatant and evaporation of the solvent, the residue was incubated with a 1M solution of Bu_4NF in THF for 14h. Successive quenching with 1ml of TrisHCl (pH 7.4), partial evaporation of the solvent and desalting on size-exclusion cartidges, the crude sequence **S6** was purified by AE HPLC. The pure 10mer **S6** was quantitatively 5'-*O*-phosphorylated by T4 PNK phosphokinase and isolated by HPLC, giving pure oligonucleotide **S5**.

The ligation of the fragments **S4** and the phosphorylated wyosine-containing **S5** was carried out with T4 RNA ligase according the instructions of the supplier and addition of 1mM ATP. After 24h of incubation at 37°, two RNA sequences were still were still present and only very small amounts of a product with a higher retention time was formed. After isolation and MS analysis, this product has been identified as the cyclized dimer of the 10mer sequence **S3A** *(Figure III.9.)*.

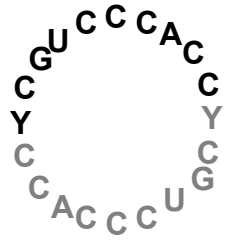


Figure III.9. Supposed structure of the product formed by ligation of fragments **S4** + **S5** by T4 RNA ligase.

In our opinion, this product results from autocomplexation of the guanosine-rich 8mer RNA sequence **S4**, which prevents the required duplex formation with the 10mer sequence **S5**. Nevertheless, this first attempt confirmed the possibility of wyosine incorporation into a sequence and importantly, the high tolerance of the T4 RNA ligase towards the nature of the nucleobase. Consequently, we turned to a template assisted enzymatic ligation for which *F. Meylan* (Meylan 2006) in our group has carried out an intensive optimization.

For this reaction, a 16mer DNA template **S7** was designed and prepared, which is fully complementary to the 8mer RNA sequence **S4** and which can form seven base pairs with the 10mer RNA sequence **S5**; since the tricyclic wyosine can not form any *Watson-Crick*

interactions, an apurinic site derived from 1,3-propandiol (Seela and Kaiser 1987) was introduced at the opposing site of the template *(Figure III.10.)*.

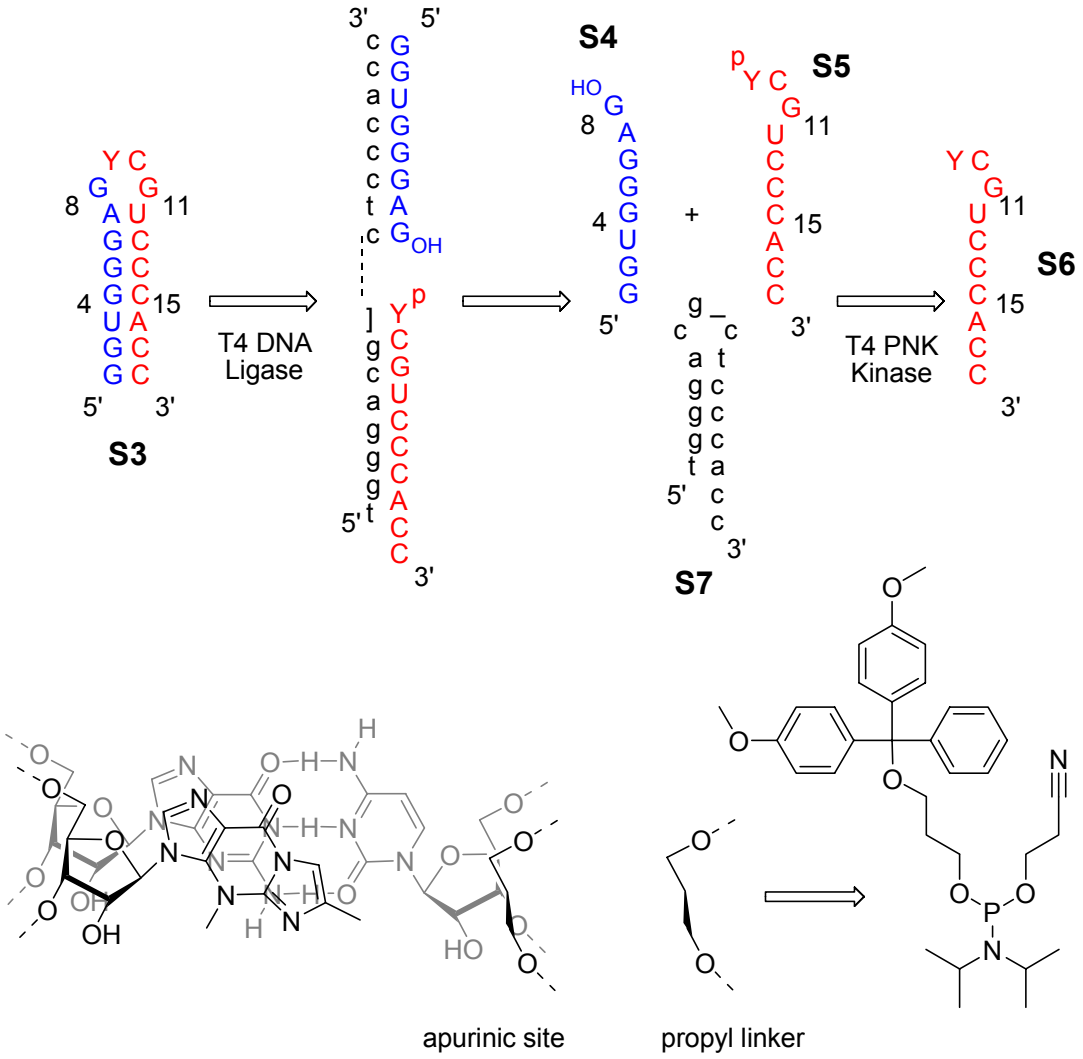


Figure III.10. Synthetic approach for the preparation of the wyosine containing sequence (Y9) through template assisted ligation (T4 DNA ligase). The propyl phosphoramidite building block was prepared according to (Seela and Kaiser 1987).

In a one-pot reaction, the wyosine-containing 10 mer **S6** was phosphorylated with the enzyme T4 PNK and ATP in the same buffer as employed for T4 DNA ligase. After incubation for 90 min at 37°, the enzyme was denaturated by thermal treatment (30 min at 60°) and then the other 8mer substrate sequence **S4** and the template **S7** were added. After a slow cooling to 37°, the enzyme T4 DNA ligase was added and the progress of the ligation reaction was

monitored by HPLC. It seemed to be nearly complete after 4 hours, but longer incubation revealed disappearance of the product **S3** and the formation of higher oligomers. This was probably the result of an incomplete denaturation of the enzyme T4 PNK and the subsequent phosphorylation of the product sequence. As a consequence, the 10 mer sequence **S5** was isolated by HPLC after the enzymatic phosphorylation reaction, thereby removing the enzyme completely.

Incubation of the two substrate sequences **S4** and **S5** (20 μM each) and the template **S7** (30 μM) with T4 DNA ligase (8 Weiss units) in an aqueous buffer (40mM TrisHCl, 2mM $MgCl_2$, 10mM DTT, 0.5mM ATP) at 37° resulted in clean and efficient (90% conversion) formation of the product sequence **S3** after 26 h *(Figure III.11.* and *Figure III.12.)*.

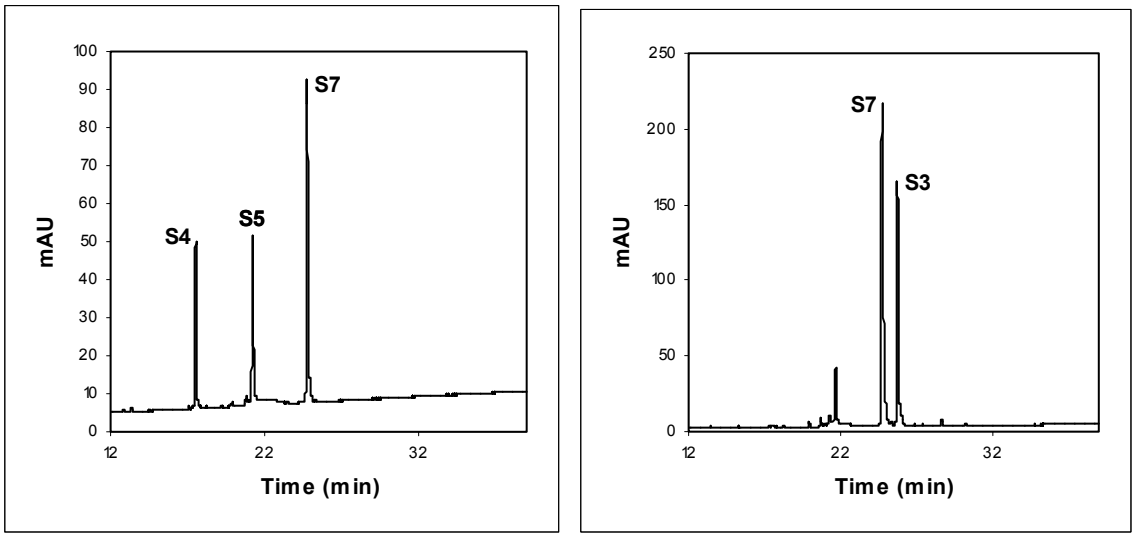


Figure III.11. HPLC traces of the reaction mixture for the preparation of wyosine containing sequence, ligation t=0 (*left*) and t=24h (*right*).

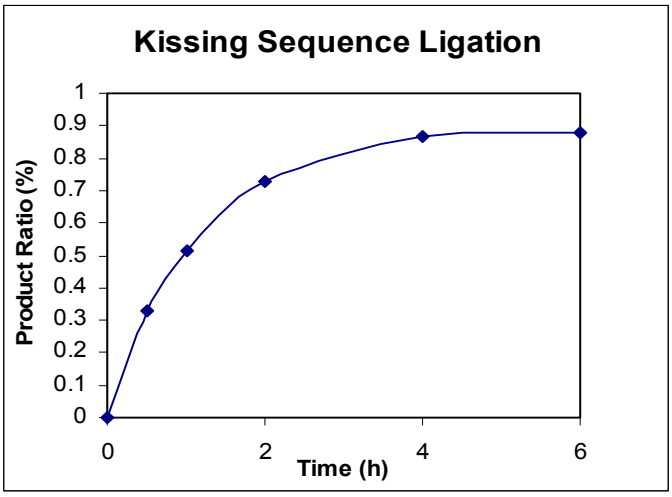


Figure III.12. Time evolution of product formation through template assisted ligation (DNA ligase).

According to these conditions, a large-scale ligation with 96 µmol **S4** and **S5** was carried out and 2 mg (18% yield) of the RNA sequence **S3** were isolated in pure form according to HPLC and ESI-MS analyses *(Figure III.13.)*.

The efficiency of this ligation was surprising, since the activity of T4 DNA ligase is normally affected by base mismatches (Goffin et al. 1987). We concluded that active complex formation between the enzyme and the two substrate strands **S4**, **S5** and the template strand **S7** occurred due to the unique stacking properties of the extended π-system wyosine, thereby mimicking the geometry of a normal *Watson-Crick* base pair.

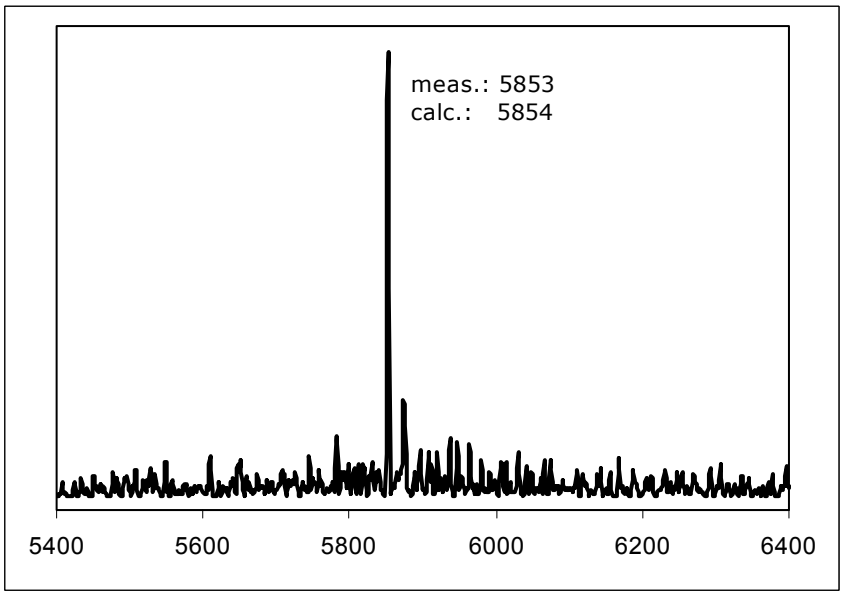


Figure III.13. ESI-MS analysis of the isolated product formed during template assisted ligation with T4 DNA ligase.

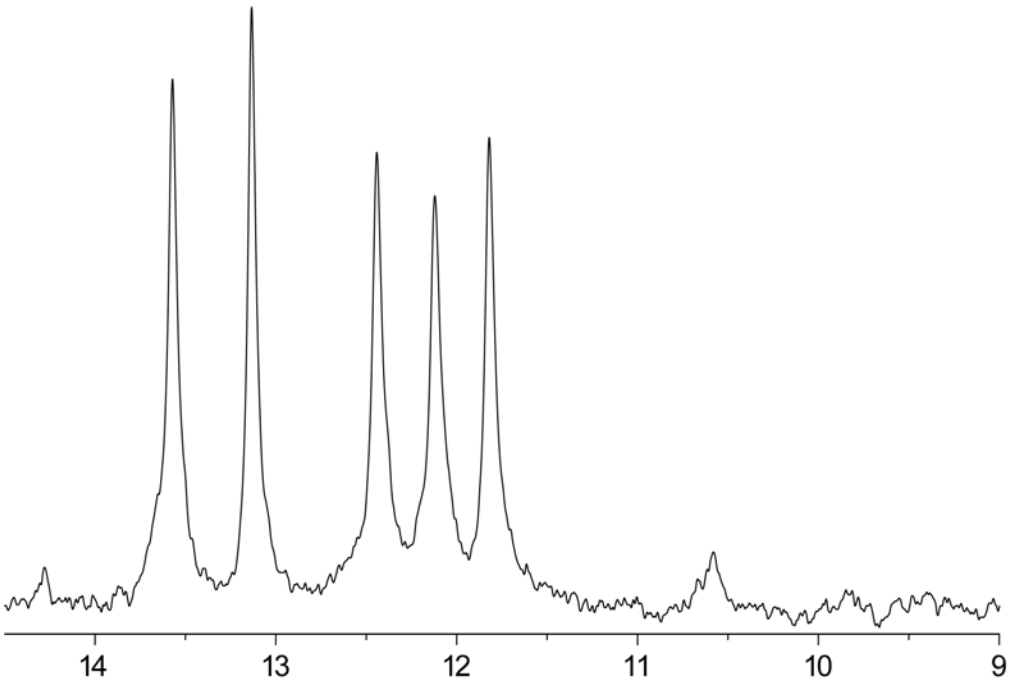


Figure III.14. NMR analysis of the imino protons of the wyosine containing sequence **S3** (scale in ppm).

The 18mer wyosine-containing RNA sequence **S3** was again analyzed by imino proton NMR spectroscopy, but also with this analogue no "kissing" interaction could be detected *(Figure III.14.)*. In order to rationalize this result, some computational investigations were carried out in collaboration with *C. Gossens* (group *U. Röthlisberger*, EPFL). A modelisation of a hypothetic “kissing” complex, in which the adenosines A9 have been substituted for wyosine, revealed an extremely unfavourable steric interaction, preventing the interaction of both hairpins *(Figure III.15.)*.

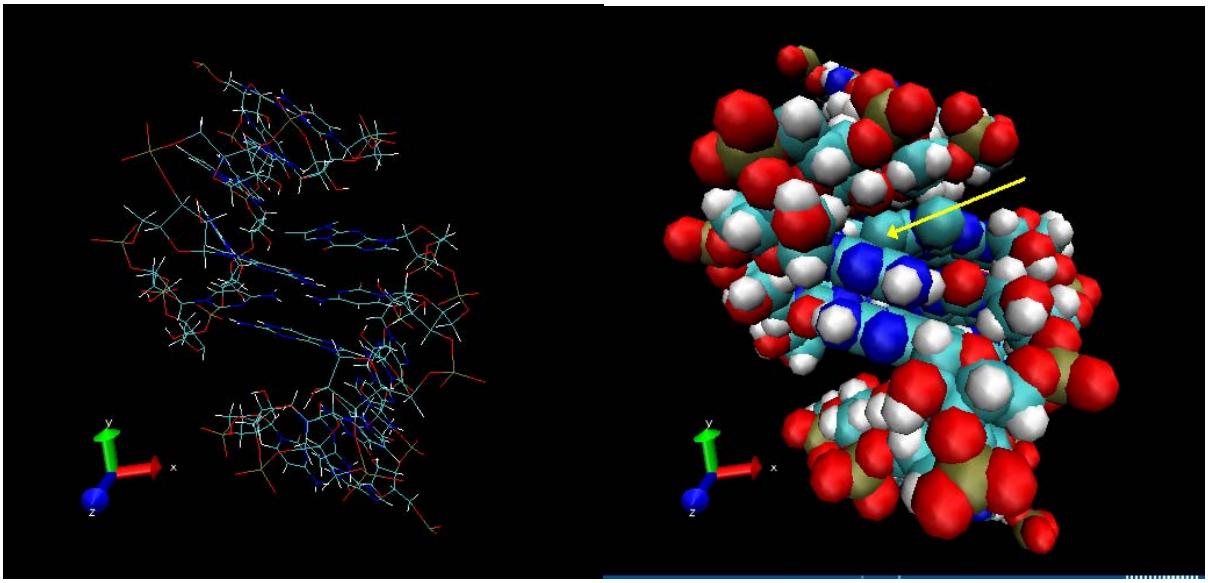


Figure III.15. Modelisation of kissing interaction where naturally occurring adenosines (A9) have been substituted for the wyosine-analogue containing sequence (yW9). Line representation (*left*) and Van der Waals volume representation (*right*). The hydrogen atoms of the wyosine moiety are not represented for clarity. The yellow arrow points out the interpenetration of atomic radii.

III.3.3. Further investigations with the "kissing" complex

In order to eventually better understand the origin of the unexpectedly strong "kissing" interaction, we prepared a series of other analogues by introducing a variety of other modified nucleosides *(Figure III.16.)*.

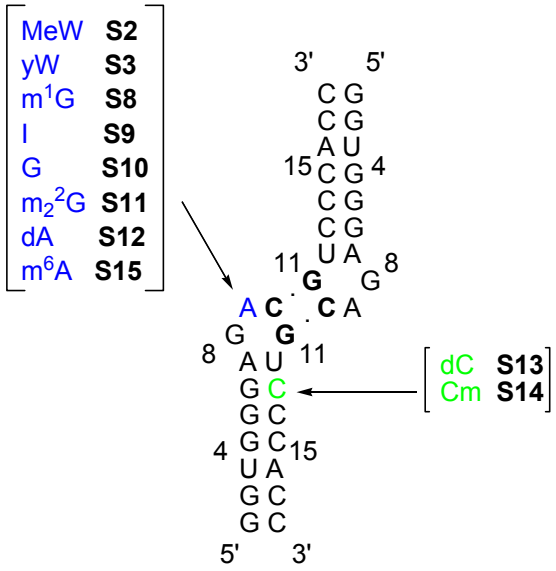


Figure III.16. List of prepared analogues of the “kissing" complex.

First, we limited our study to the adenosine A9 by introduction of naturally occurring purines present in tRNAs for which we have already prepared the phosphoramidite building blocks (Chapter I): 1-methylguanosine **S8**, inosine **S9**, methylguanosine **S10**, and N^2,N^2-methylguanosine **S11**. We have also prepared the **S12** from the 2'-deoxyadenosine. According to imino proton NMR spectra, all these analogues are not undergoing formation of the complex. We then wondered whether the amino function of A9 was involved in interactions with other nucleotides. For this study, other modifications in the immediate environment of this amino group were introduced after consulting the X-ray structure of the complex (pdb file 1F5U). However, neither introduction of 2'-*O*-Me-deoxycytidine at position C13 **S14**, replacement of C13 by 2'-deoxycytidine **S13** or methylation at the amino group of A9 **S15** prevented the formation of the complex *(Table III.1.)*.

5'-GGUGGAGXCGUYCCACC-3'

X	Y	Name	m/z calc.	m/z found	Interaction
A	C	**S1**	_	_	Yes
MeW	C	**S2**	5853.6	5854.1	No
m^1G	C	**S8**	5815.6	5816.0	No
I	C	**S9**	5786.6	5787.0	No
G	C	**S10**	5801.6	5802.0	No
$m^2{}_2G$	C	**S11**	5829.6	5830.0	No
dA	C	**S12**	5769.6	5770.6	Yes
A	dC	**S13**	5769.6	5770.0	Yes
A	mC	**S14**	5799.6	5800.0	Yes
m^6A	C	**S15**	5799.6	5800.0	Yes

Table III.1. List of "kissing" sequence analogues, ESI-MS characterization and identification of "kissing" interactions according to NMR analyses.

III.3.4. Hypothesis

These results inspired us to give a new interpretation of the "kissing" interaction in thermodynamical terms and to rationalize the reported $\Delta G°_{37}$ value of -6.5 kcal mol^{-1} for formation of the complex (Kim and Tinoco Jr. 2000). The authors have commented that this value is nearly equivalent to the energy resulting from the 5'-CGCG-3'/3'-GCGC-5' base

pairing according to the nearest neighbour parameters (Turner et al. 1988). As first hypothesis, we estimated that the A9 contributed to the stabilization of the complex only through its highly favourable π-stacking interaction (Guckian et al. 2000) and reduced the observed value of -6.5 kcal.mol^{-1} by the twice the energetical contribution of a 5'-A-dangling motif adjacent to a C·G base pair (-0.5 kcal.mol^{-1}).

$$\Delta G^{\circ}_{37\ \text{Full complex}} = 2*\Delta G^{\circ}_{37\ \text{stacking A on CG}} + \Delta G^{\circ}_{37\ \text{CG/GC base pairs in the complex}}$$

The new energy of the C·G base pairs involved in the complex are:

$$\Delta G^{\circ}_{37\ \text{CG/GC base pairs in the complex}} = \Delta G^{\circ}_{37\ \text{Full complex}} - 2*\Delta G^{\circ}_{37\ \text{stacking A on CG}} =$$

$$\mathbf{- 6.5 - (-1.0) = - 5.5\ kcal.mol^{-1}}$$

This value is 1.5 kcal mol^{-1} higher than the value describing a double 5'-CG-3'/3'-GC-5' base pair which is - 4 kcal mol^{-1} (Turner et al. 1988).

For explaining the increase of the C·G base pair stability, we included the contribution of the G8 residue which, from NMR refinement carried out by *Kim* and *Tinoco, Jr.* (Kim and Tinoco Jr. 2000), is placed in direct interaction with the N(7) of G11 *(Figure III.14.)*. However, we suspected that this hydrogen bond is not only responsible for the rigidity of the tetraloop but also for the strong base pairs.

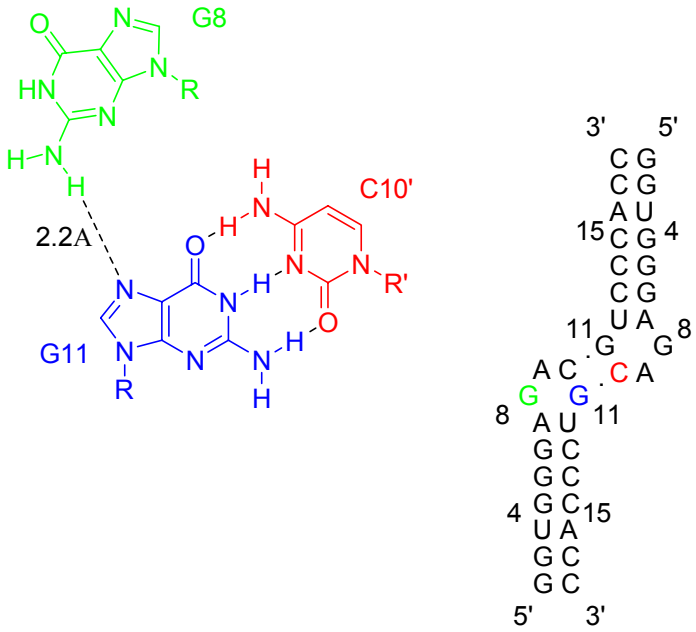


Figure III.17. Relevant interactions involved in the stabilization of the "kissing" complex.

Indeed, the binding of a hydrogen to the lone pair of nitrogen N(7) of guanosine should reduce the electronic density at this position. This effect has been already investigated by computational methods (Burda et al. 1997). During these studies, it has been observed that upon metal-ion binding at N(7), the strength of the G·C base pair could be considerably increased. Consequently, the H-bond between of the N(2)-amino function of the G8 to the N(7) of G11 could be an important contributing factor to the "unexpected stability" of the complex. As definitive proof, we confirmed the special contribution of the amino function of G8 by replacing it with inosine (sequence **S16**). Inosine exhibits a simple hydrogen at position 2 which prevents the formation of the hydrogen bond *(Figure III.18.)* and consequently, prevents the formation of the complex as observed in our NMR study of this sequence.

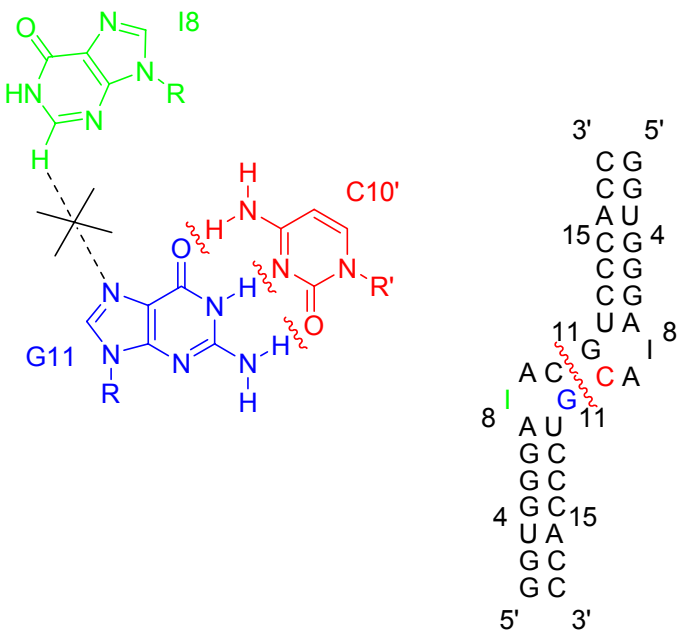


Figure III.18. The introduction of an inosine I(8) **S16** in place of guanosine G(8) **S1** renders the formation of the hydrogen bond between NH_2 of G(8) and the N(7) of G11 impossible, and prevents the "kissing" complex formation.

III.3.5. Evaluation of stacking stabilization by wyosine

The template-mediated ligation of the wyosine-containing RNA sequence **S5** and the RNA sequence **S4** worked surprisingly well, given the unusual arrangement of a wyosine

facing an apurinic residue at the ligation site (Chapter III.3.2.3.). We concluded that active complex formation between the enzyme and the two substrate strands **S4**, **S5** and the template strand **S7** is peculiarly efficient due to the unique stacking properties of the extended π-system wyosine, thereby mimicking the geometry of a normal *Watson-Crick* base pair (*Figure III.10.*). In order to test this hypothesis, we prepared the four analogous 10mer substrate strands **S17** – **S20**, containing the canonical nucleosides at the 5'-position, and submitted them to the same ligation conditions. The course of these reactions was monitored by HPLC *(Figure III.19.)*.

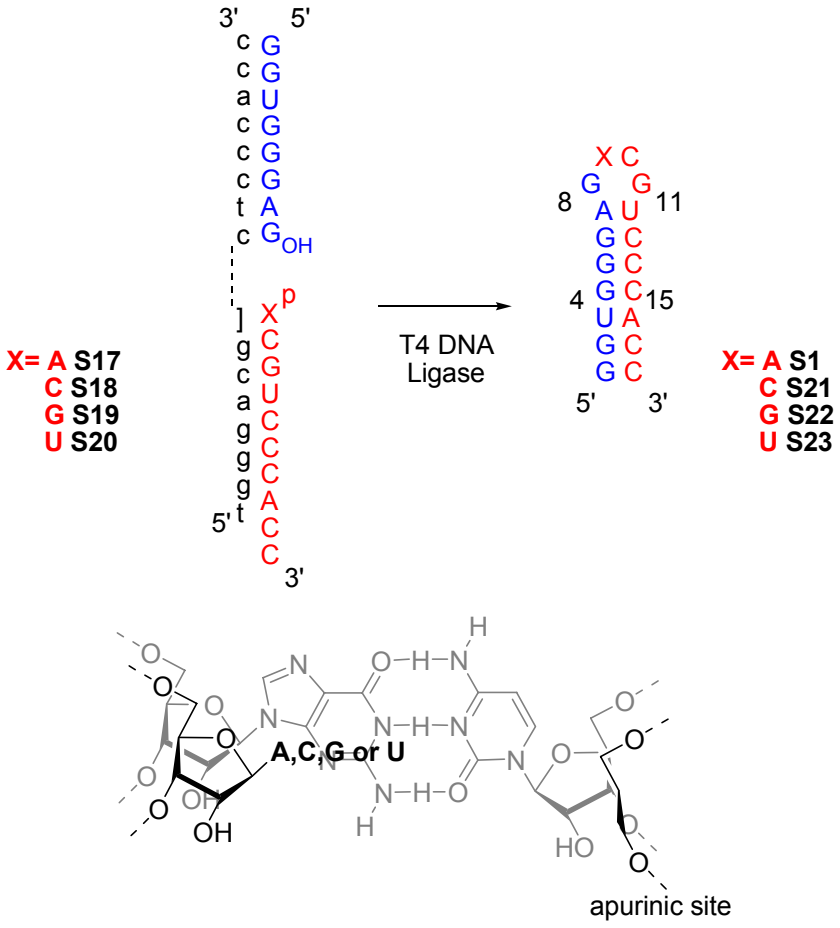


Figure III.19. Sequences prepared for evaluation of the influence of stacking on the ligation reaction.

The ligation yields obtained after 24h reaction time are shown in *(Table III.2.)*. The efficiency of these reactions varied to a great extent, but only with a terminal cytidine no ligation and accumulation of the corresponding 5'AMP-intermediate (Liu et al. 2004)

occurred. In contrast, the other nucleotides seem to show a more predictable behaviour. The lligation yields correlated very well with the Δ*G* values of stacking energies for 3'-dangling nucleotides stacking on a G.C base pair according to the nearest neighbour model (Turner et al. 1988) *(Figure III.20., Table III.2.)*. In other words, the ligation yields reflect the capacity of the unpaired nucleotide to stack with the last base pair of the acceptor sequence, thereby placing it in favourable orientation for ligation.

Ligation Yield (24h)	- ΔG° calc	Stacking motif
0.37	1.1	G**A**/C
0.02	0.4	G**C**/C
0.48	1.3	G**G**/C
0.21	0.6	G**U**/C

Table III.2. Ligation ratio and estimated stacking contribution from increment parameterss of the nearest neighbour model (Turner et al. 1988).

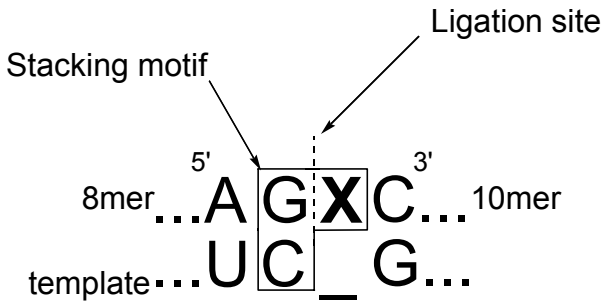


Figure III.20. Configuration at the ligation site.

By plotting the ligation yield after 24 hours against the stacking energy of the corresponding dangling motif (value of the nearest-neighbours model) an almost linear correlation was obtained *(Figure III.18.)*. By linear extrapolation, we then estimated the stacking energy for wyosine to $\Delta G_{stacking}$ = - 2 kcal mol^{-1} (*Figure III.21*).

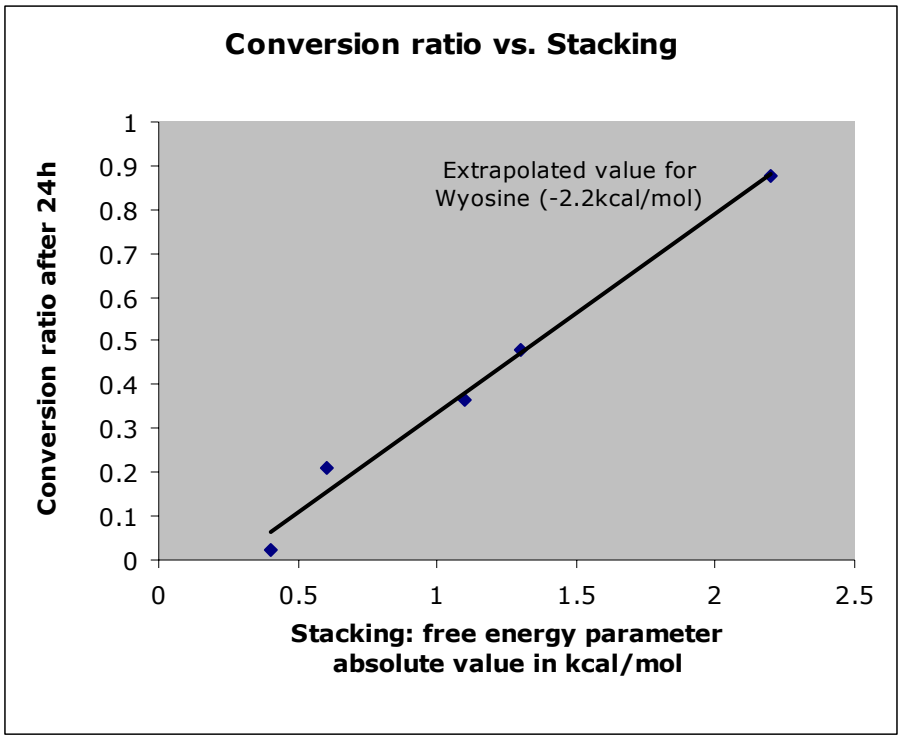


Figure III.21. Plot of ligation yield after 24h against stacking energies according to the nearest neighbour model. Extrapolation for the wyosine nucleoside gave a stacking energy of approximatively -2 kcal mol^{-1}.

III.3.6. Conclusion

Our experimental and theoretical investigations have shown that the "kissing" complex is not a suitable model for the tRNA-mRNA interaction, since it is governed by interactions (such as the cross base pairing of (G8-G11) which do not occur in the anticodon loop. However, the results underline the importance of secondary hydrogen bonds to the formation and stability of RNA interactions, which could be very important in the decoding process. Therefore, we examined in more details the role of wybutosine, a wyosine derivative with an amino acid side-chain capable of undergoing such additional hydrogen bond interactions.

III.4. The Wybutosine

III.4.1. General considerations

Wybutosine and its derivatives belong to the most elaborated modified nucleosides present in tRNAs. The best characterized representative is wybutosine, which is included in a highly modified anticodon loop containing also the four other modified nucleosides Cm32,

Gm34, Y37 and Ψ39. This arrangement ensures the accurate decoding of the phenylalanine UUU and UUC codons. These codons are peculiar among the other codons due to their weak base pairing. Hypomodification of the corresponding tRNAs and also of the "complementary" ^{Asn}tRNAs with AAU and AAC anticodons leads to extensive frameshift (Farabaugh 2000). As an example, the rabbit reticulocyte ^{Phe}tRNA, which is a sequence isoform of the rabbit liver ^{Phe}tRNA, displaying a m^{1}G37 in place of wybutosine, exhibits a favoured -1 frameshift (Carlson et al. 2001). The structural reasons of these decoding properties could be partially explained by a recent NMR study of the synthetic ^{Phe}ASL structure, containing m^{1}G in place of wybutosine (Stuart et al. 2003). It has been shown that the Cm32-U33-Gm34 sequence, exclusively found in eukaryotic ^{Phe}tRNAs, is very rigid and orients the Gm34 base in an unfavourable conformation for base pairing. The resulting inaccessibility of the Gm34 for base pairing is probably maintained upon codon binding, since the A35 and A36 residues are relatively mobile in the hypomodified ^{Phe}ASL (Stuart et al. 2003).

According to our previous considerations, we suggest that the action of wybutosine is not limited to its wider π-stacking with the adjacent base pair A36·UIII (Pongs and Reinwald 1973), since the A35 and A36 are well-structured in the fully modified ^{Phe}tRNA, in contrast to the poorly structured ^{Phe}ASL containing m^{1}G37. Nevertheless, according to *ab initio* studies on the electrostatic potentials of the nucleotides of a Phe-anticodon, wybutosine has electronic properties which affect the stability of the anticodon triplet complex. It has been estimated that the wybutosine provided a strong energetic stabilization to the A35·UII base pair, of up to 50% of the intrinsic base pair energy. In contrast, guanine base placed in position 37 does not significantly affect the energetic of the adjacent base-pairs (Werneck et al 1998). Therefore, by reinforcement of the A·U base pairs, especially of the second one (A35·UII), wybutosine strengthen the phenylalanine anticodon/codon interactions and probably induces a reorientation of the Gm34 for optimal base pairing. Furthermore, the 2'-*O*-methylation of G34 seems favors a C-3'-endo conformation (Venkateswarlu et al. 1999) which allows base pairing with U in addition to C (Ashraf et al. 2000). The same methylation could also prevent formation of a G·G base pair by favouring the anti-conformation (de Leeuw et al. 1980) over

the syn conformation (Söll and RajBhandary 1995). Indeed, ^{Tyr}tRNAs with an unmethylated guanosine in position 34 (Q deficient) are known to partially read through the UAG stop codon (see Chapter IV), which could become deleterious in codon split boxes such as Phe/Leu. Although Gm34 exists in few other tRNAs, it is never associated with the Cm32 modification except in the eukaryotic ^{Phe}tRNA. Consequently, the wybutosine nucleoside is important for the structure of the anticodon loop, especially the Cm32-U33-Gm34-A35-A36 sequence, essentially due to its peculiar electronic properties. Although studies using selectively modified PhetRNA ASL have resulted in a better understanding of the role of the modifications, the contribution of the wybutosine has never been investigated since it could not be synthetically incorporated.

According the previously presented considerations, wybutosine seems to act essentially through its tricyclic structure which affects the electronic potential of the global anticodon loop. This hypothesis seems to be validated by the recent isolation of some archaeal minimalist derivatives of wyosine lacking the 4-methyl group *(Figure III.1.)* and thermodynamic studies carried out with 4-desmethylwyosine (Ziomek et al. 2002). However, wybutosine displays an additional amino acid moiety at position C(7) for which it is difficult to attribute a specific role by from the X-ray structure of a correspondingly modified PhetRNA. However, the wybutosine derivatives, containing such side-chains, are exclusively present in eukaryotes and totally absent in prokaryotes. Consequently, these extended modifications could correlate with structural requirements of eukaryotic ribosomes, for maintaining the accuracy of translation. Indeed, the decoding process involves fundamental interactions of the tRNAs with the 16S rRNA, which serve as reliable phyllogenetic criteria (Woese 1990) and eukaryotic tRNAs undergoe more editing processes than those of bacteria and archaea. Moreover, a different response to a common frameshifting RNA signal has been reported within phylogenetically different organisms. Whereas eukaryotes were sensitive to certain tRNA modifications, prokaryotes did not display any frameshifts (Seung and Kang 2003). Previous computational investigations have led to the hypothesis that the amino acid side-chain could significantly reinforce the adjacent A36·UI base pair by providing

supplementary hydrogen bonding (de Oliviera Neto et al. 1998) (see also Chapter IV) *(Figure III.22.)*.

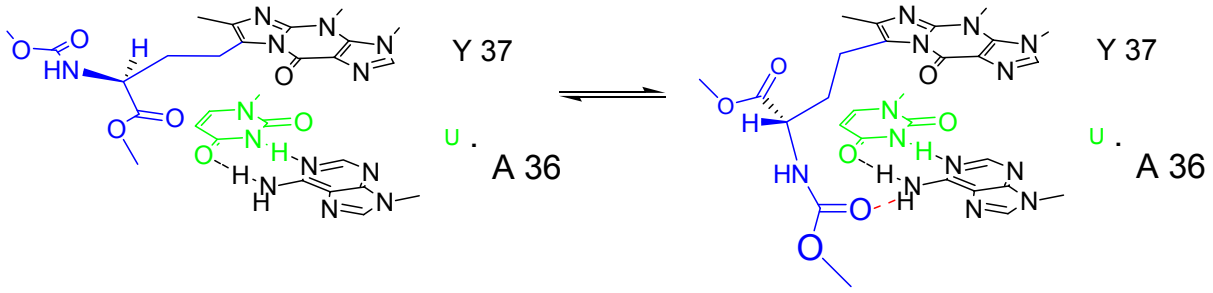


Figure III.22. Wybutosine in interaction with the adjacent A·U base pair according to the proposed model of (de Oliviera Neto et al. 1998) and distinction between the interacting part and non-interacting part of the linker.

III.4.2. Synthesis of wybutosine analogues

III.4.2.1. Introduction

In order to test the hypothesis that secondary hydrogen bonds are important for stabilizing the codon/anticodon interaction and in order to understand at which step of the decoding process they are operative (tRNA selection, translocation, reading frame, proofreading…), it is essential to prepare analogues of wybutosine and to incorporate them into tRNAs.

These nucleosides contain methyl ester side-chains, which are completely incompatible with the automated synthesis of RNA sequences. We therefore planned the synthesis of analogues where the ester groups were replaced by N-methyl amides, expected to be stable under our assembly and deprotection conditions *(Figure III.23.)*.

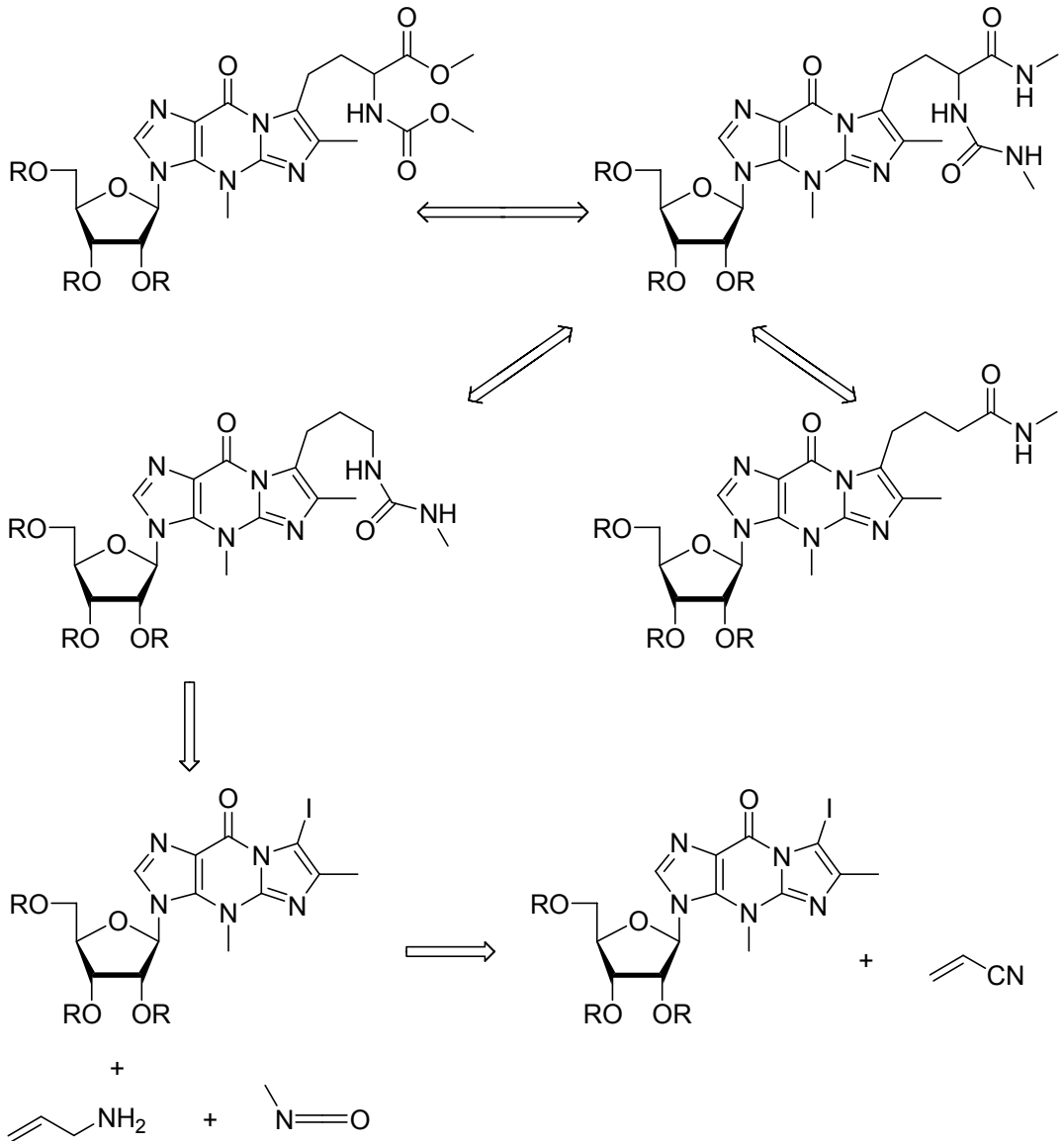
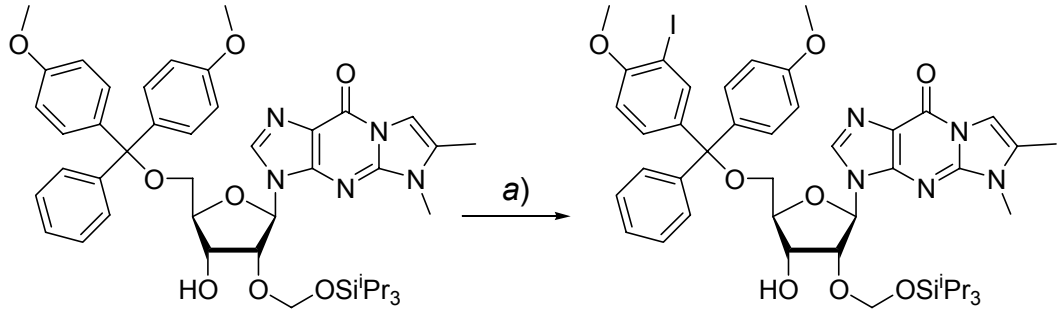


Figure III.23. Synthetic approach for the preparation of wybutosine analogues.

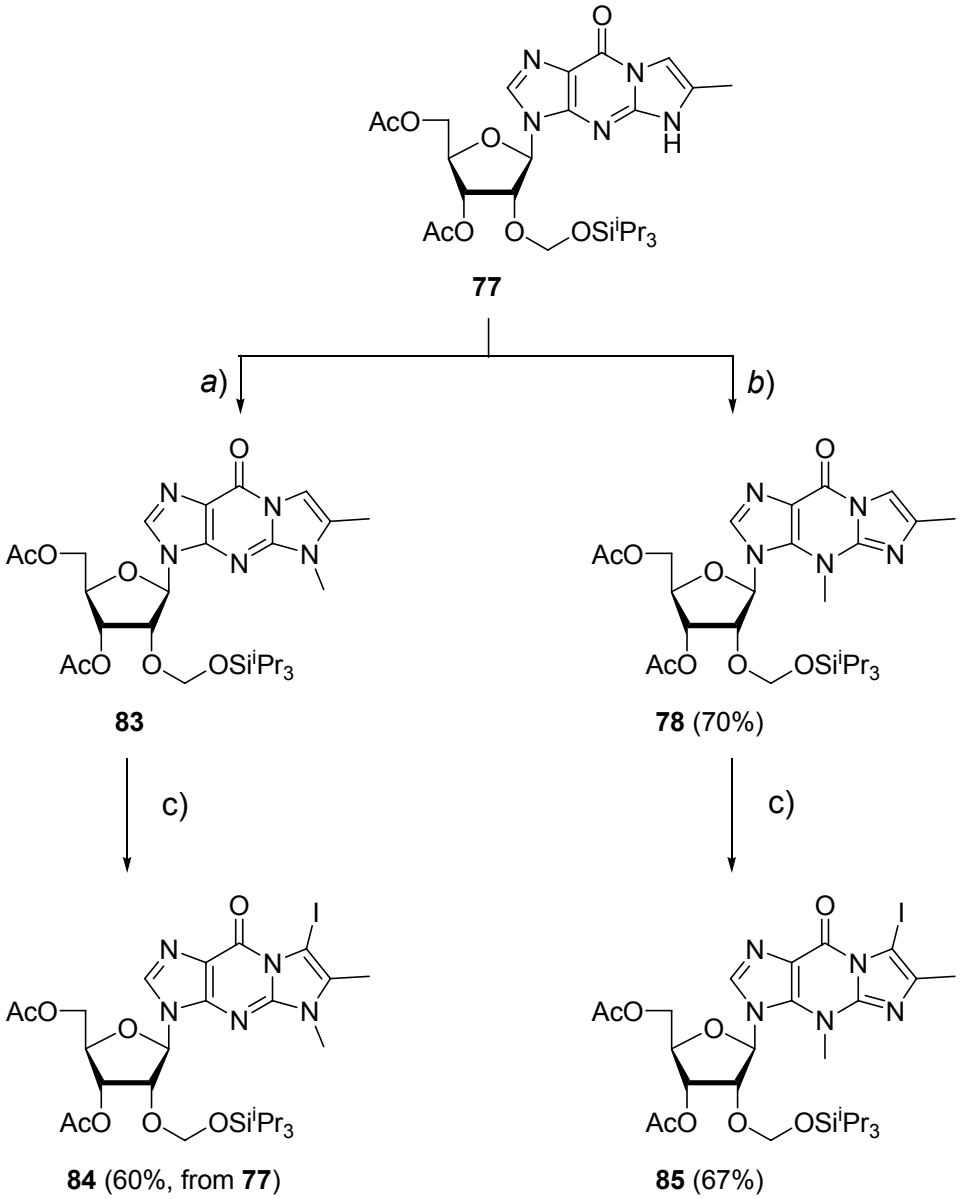
III.4.2.2. Synthesis

In order to investigate the special properties of the wybutosine nucleoside, several attempts for its chemical synhesis have been reported. An efficient alkylation of the C(7) iodinated derivative proceeds under Pd catalysis (Itaya et al. 1988). Treatment of the tritylated wyosine analogue **74** with N-iodoosuccinimide resulted in formation of products exclusively iodinated at the dimethoxytrityl group, as confirmed by the isolation of the non-iodinated nucleoside after acidic cleavage of the DMT-group *(Scheme III.8.)*.



74 **82**

Scheme III.8. *a*) N-Iodosuccinimide, $NaHCO_3$, H_2O, CH_2Cl_2, 20°.



77

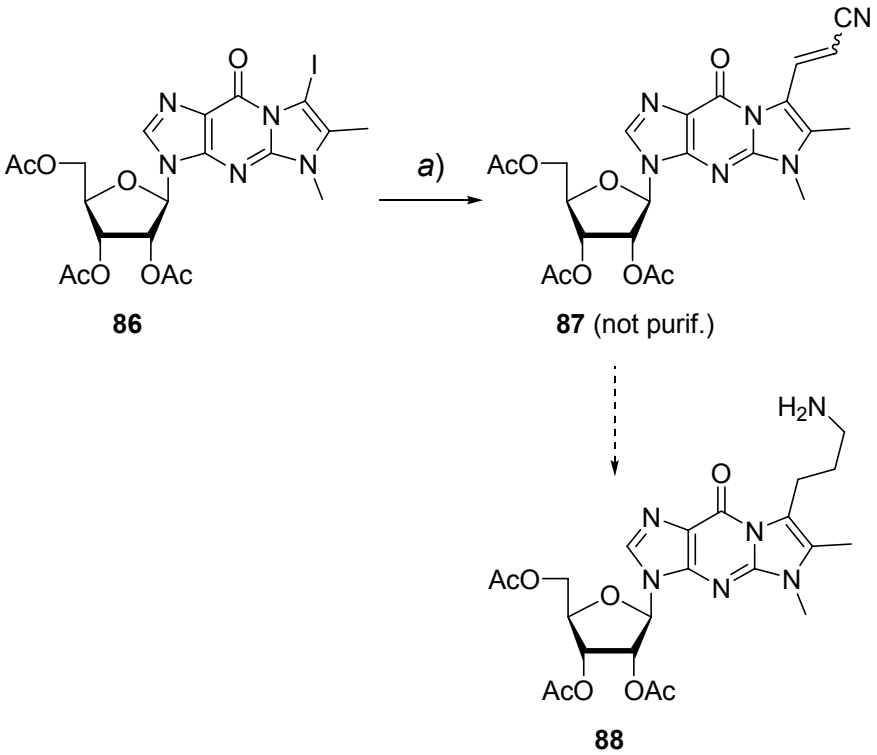
83 **78** (70%)

84 (60%, from **77**) **85** (67%)

Scheme III.9. *a*) K_2CO_3, DMF, 20°; then MeI, 20°; *b*) CH_2I_2, Et_2Zn, Et_2O, 4°; *c*) N-Iodosuccinimide, $NaHCO_3$, H_2O, CH_2Cl_2, 20°.

Therefore, we reconsidered our synthetic strategy and introduced the DMT-group only after iodination *(Scheme III.9.)*. The 5',3'-di-*O*-diacetyl derivatives of wyosine (**78**) and 5-desmethyl-4-methylwyosine (**83**), were effiently N(7)-iodinated with N-iodosuccinimide, which gave a much cleaner reaction than the reported iodine (Boryski and Ueda 1985) and were obtained in yields of 67% and 68%, respectively.

In preliminary studies, the 2',3',5'-Tri-O-acetyl-5-desmethyl-7-iodo-4-methylwyosine **86** (prepared according to (Glemarec et al. 1988)) was alkylated with acrylonitrile in the presence of $Pd(OAc)_2$ according to (Itaya et al. 1988). The resulting 3/2 mixture of *Z/E* derivatives **87** has then been submitted to hydrogenation, but all attempts to reduce it to the saturated amine (H_2/Pd on C or H_2/Pt on C, Raney Ni) failed *(Scheme III.10.)*. We did not carry our further investigations, since the other methods reported for such transformations (e.g. HCl / EtOH under reflux (March 1992a)) are not compatible with the sensitive wyosine.



Scheme III.10. *a*) Bu_4NCl, $NaHCO_3$, $Pd(OAc)_2$, acrylonitrile, DMF, 20°. **86** was prepared according to (Glemarec et al. 1988).

III.4.2.2. Synthesis of wybutosine: new approach and new linkers

In order to overpass these difficulties, we decided to introduce the linkers in a form which was as close as possible to the definitive structure and requiring mild conditions for their introduction. According to these criteria, two new precursors for linker structures have been chosen, N-methylbut-3-enamide and N-allyl-N'-methylurea *(Figure III.22.)*.

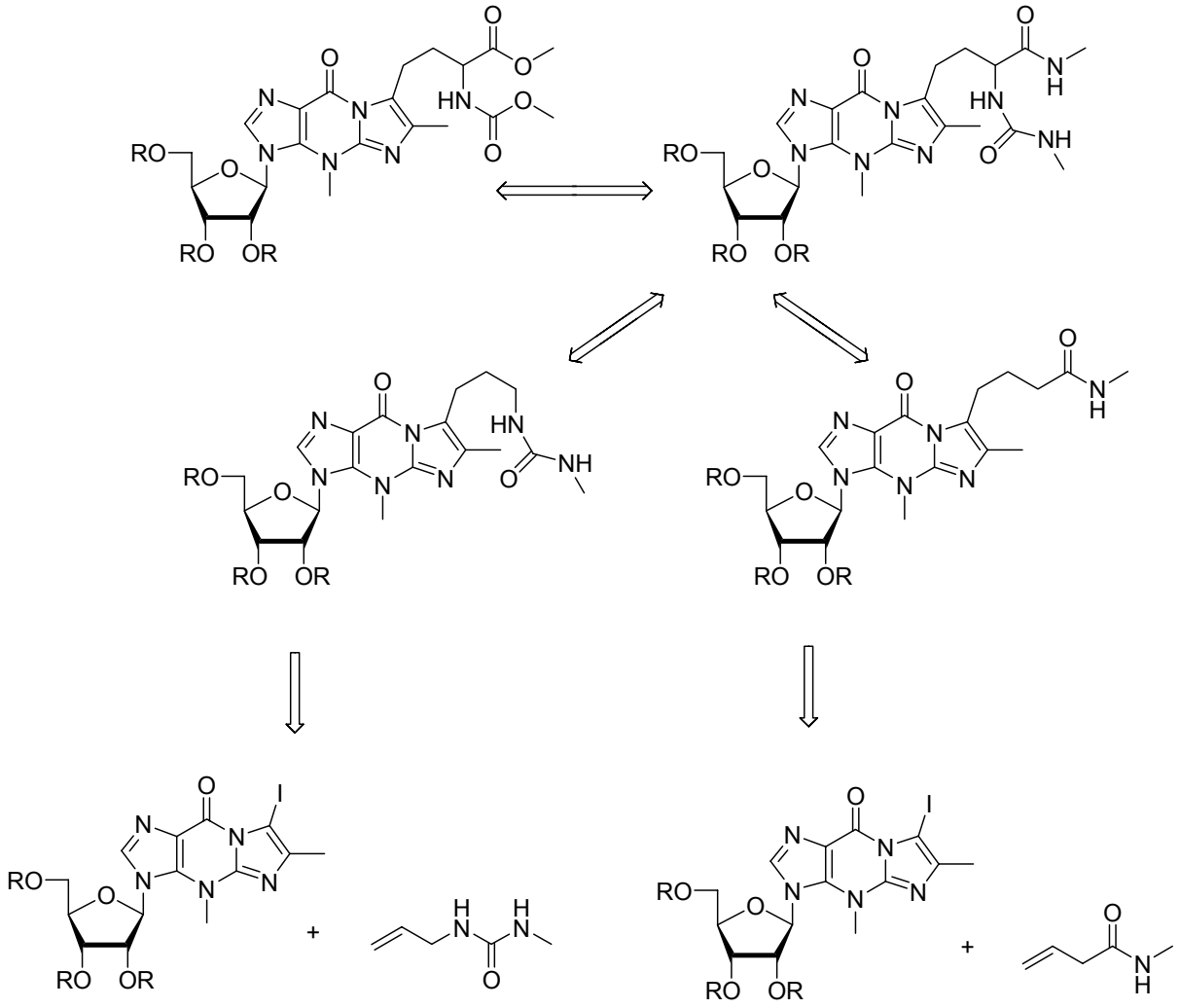
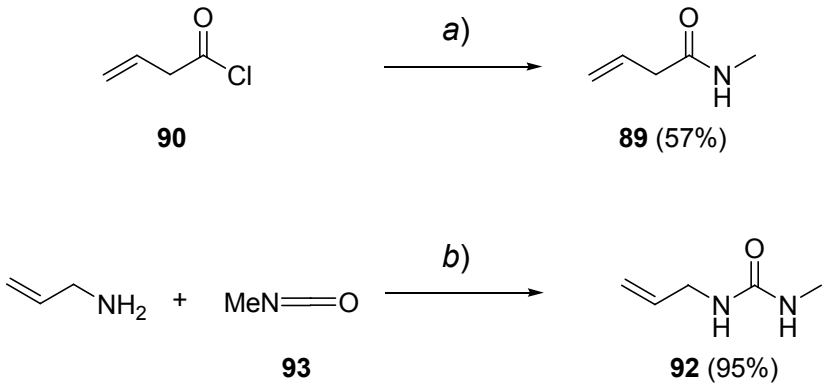


Figure III.24. New strategy for the preparation of wybutosine analogues.

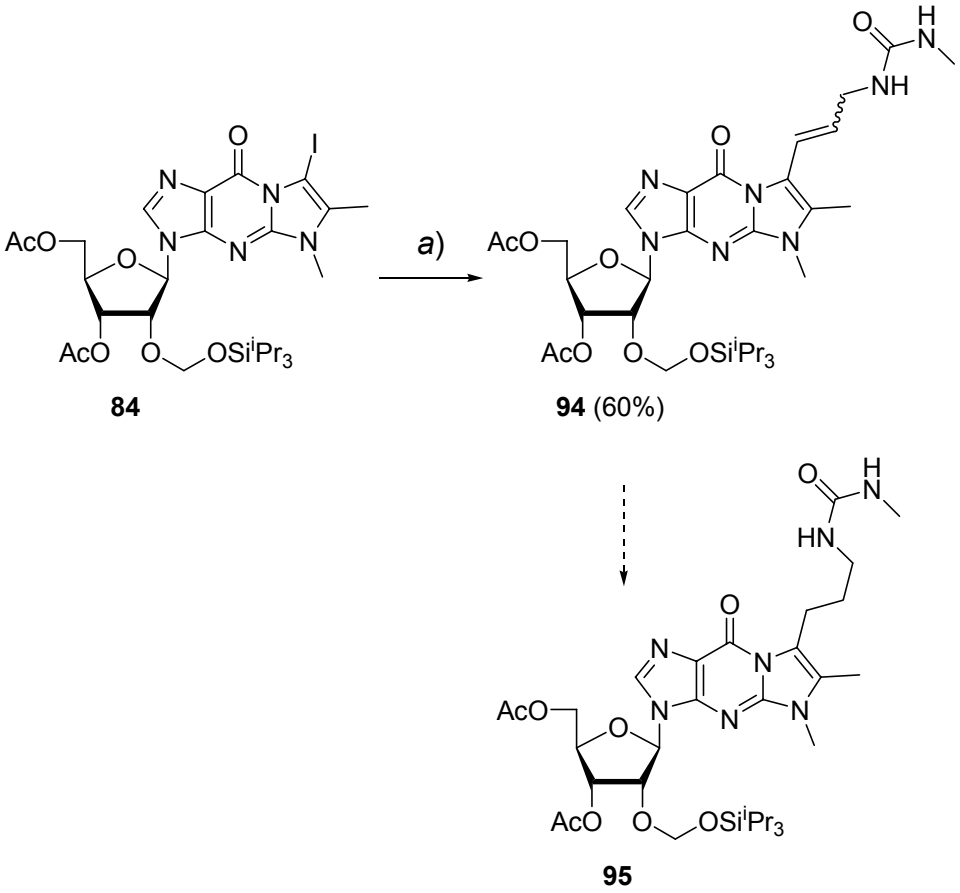
The required side-chain precursor N-methylbut-3-enamide **89** was prepared from the vinylacetic chloride (Ugi and Beck 1961) and $MeNH_2$ in THF (57% yield *(Scheme III.11.)*. The second side-chain analogue consisted in an urea motif and for the preparation of the precursor *N*-allyl-*N*'-methylurea **92** we developed an access based on the reaction of

allylamine with a freshly prepared methylisocyanate solution (95% yield) (Tsoi et al. 1983) *(Scheme III.11.)*.



Scheme III.11. *a*) $MeNH_2$, THF, 4°. *b*) Methylisocyanate, toluene, 20°. **90** was prepared according to (Ugi and Beck 1961).

We then first investigated reaction conditions for the introduction of the *N*-allyl-*N*'-methylurea **92** into the wyosine analogue **84**. The experimental procedure from literature (1.1eq of **92** and heating at 60° (Itaya et al. 1988)), led to a partial decomposition and concomitant formation of the deiodinated derivative **83** *(Scheme III.12.)*. The reaction could be significantly improved by carrying it out at room temperature and employing at least 2.5eq of the alkene reagent **92**. Removal of the Pd by filtration on Celite or drying over $MgSO_4$ resulted in adsorption of the product. However, by avoiding filtration and drying over Na_2SO_4, the N(7)-alkylated wyosine analogue **94** was obtained in a good yield of 60% as a mixture of cis/trans isomers.

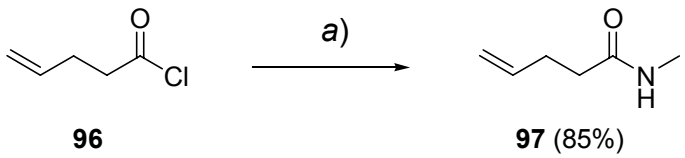


Scheme III.12. *a*) Bu_4NCl, $NaHCO_3$, $Pd(OAc)_2$, **92**, DMF, 20°.

Again, it was not possible to reduce the double bond of **94** with H_2/Pd or H_2/Pt. Therefore, this urea-type linker structure was abandoned and an amide-type linker was chosen as next synthetic target. This side-chain was expected to interact in a similar manner than the side-chain of the natural wybutosine *(Figure III.22.)*.

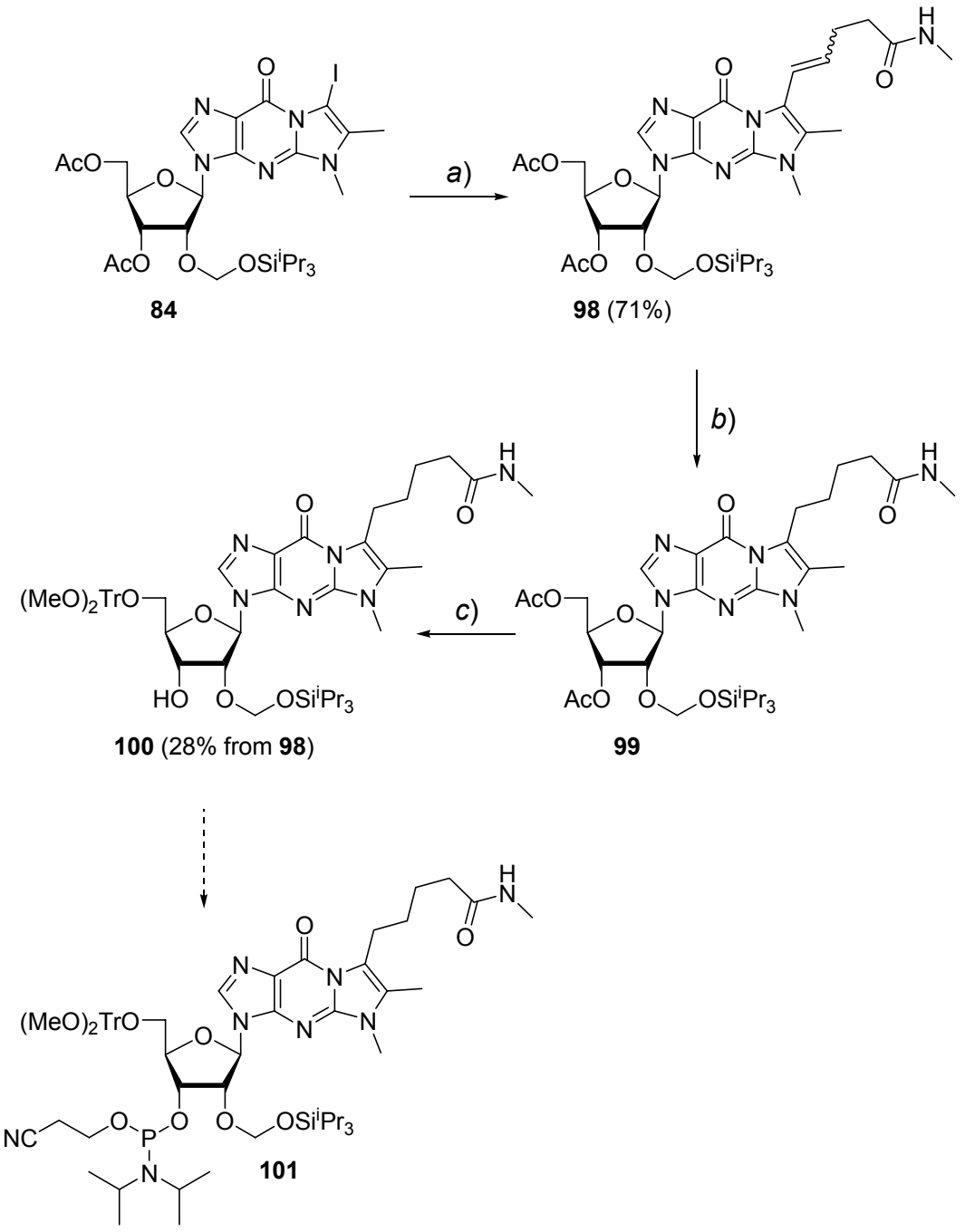
The reagent *N*-methylpent-4-enamide **97** for introduction of the new side-chain was prepared from commercially available 4-pentenoyl chloride **96** and $MeNH_2$ in THF (→ **97**, 85% yield) *(Scheme III.13.)*.

.



Scheme III.13. *a*) $MeNH_2$, THF, 4°.

By employing the previously optimized cross-coupling conditions, with $Pd(OAc)_2$, $NaHCO_3$ and Bu_4NCl, **97** was introduced at C(7) of **84** (→ **98**, 71% yield) *(Scheme III.14.)*. The reduction of the double bond occurred smoothly with H_2/Pd and, without purification of the intermediates, the reduced product was deacetylated with NH_3 in MeOH (→ **99**) and then 5'-*O*-dimethoxytritylated with DMT-Cl in Py. The C(7)-substituted wyosine analogue 100 was thus obtained in a yield of 28% (based on **98**). Unfortunately, due to the small amount of product available and due to time-restrictions, the final synthesis of the corresponding phosphoramidite building block could not be investigated. This transformation could be carried out or by the conventional procedure with 2-cyanoethyl diisopropylphosphoramidochloridite, $^{i}Pr_2NEt$ or with 2-cyanoethyl tetraisopropylphosphoramidite, 5-benzyl-1*H*-tetrazole 20°.



Scheme III.14. *a*) **97**, Bu_4NCl, $NaHCO_3$, $Pd(OAc)_2$, DMF, 20°. *b*) 1. Pd.C, H_2, EtOH, 20°; *c*) NH_3, MeOH, 20°; 3. $(MeO)_2TrCl$, pyridine, 20°.

III.5. Conclusion

The accurate codon (mRNA) recognition by the anticodon of the tRNA is mainly ensured by the fine-tuning of the pairing provided by the nucleosides at the wobble-position (position 34) and 3'-end adjacent to the anticodon (position 37) (Agris 2004), often occupied by hydrophobic derivatives such as wyosine. With the aim to investigate the nature of this interaction, we have prepared and successfully incorporated the wyosine nucleoside into a model RNA sequence from *Moloney murine leukaemia virus*. The synthesis of this wyosine-containing RNA sequence was achieved through ligation (T4 DNA ligase) of two RNA fragments in the presence of a DNA template displaying an apurinic site. Additionally, we have also synthesized several analogues of this oligonucleotide, which permitted to highlight the interactions important for the formation of the "kissing" complex.

CHAPTER IV "Properties of modified nucleosides of the anticodon loop"

IV.1. Introduction

The exact decoding capacities of tRNAs are ensured by a variety of modified nucleosides within the anticodon loop. Despite the isolation and characterization of several tRNA mutants, which are defective in some modifications, an exact correlation between structure of the nucleoside and decoding capacity has not yet been achieved (Agris 2004) (Takai and Yokoyama 2003). Therefore, we decided to review the variety of these modified nucleosides and tried to understand how substituents of the nucleobase could influence the decoding abilities, as revealed by studying modification-defective mutants (Agris 2004). The proposed models emphasize potential hydrogen bonding in the close environment of the canonical codon/anticodon *Watson-Crick* base pairs. In this context, the theoretical study of the "kissing" complex has highlighted the importance of the secondary weak contacts in the stability of nucleobases interactions.

IV.2. *N*-[(9β-D-ribofuranosyl-9H-purin-6-yl)carbamoyl)]threonine (t^6A)

IV.2.1. Introduction

Among the complex nucleosides of the anticodon loop, the most frequent is the t^6A modification (Sprinzl et al. 1998). Its high conservation in all phyllogenetic domains of life (Archaea, Bacteria and Eukarya) (Söll and RajBhandary 1995) aroused several biological experiments (Curran 1998) (Grosjean et al. 1998), which permitted to describe its biosynthesis (Chheda et al. 1972) (Powers and Peterkofsky 1972) and identify the sources of the various components involved in its modification. Adjacent to the 3'-end of the anticodon (position 37) in tRNAs reading codons starting with A (Söll and RajBhandary 1995), it prevents U36 to wobble (Duke et al. 1968), demonstrating its important contribution for proper codon-anticodon recognition (Newmark and Cantor 1968). In 1979, *Nishimura* suggested that this modification was necessary for stabilization of the weak A·U base pair, but without more details (Nishimura 1979). Few investigations about the role of this modification have been carried out despite the recent X-ray analysis of a $tRNA^{Lys}_{UUU}$ anticodon stem-loop

(containing the t^6A and mnm^5s^2U modifications) bound to the 30S ribosomal subunit in the presence of mRNA (Murphy IV et al. 2004).

In the context of this work, we have indepedently developed a model for the t6A interactions in analogy to the model proposed for wybutosine (de Oliviera Neto et al. 1998). Wybutosine is a tricyclic nucleoside that contains an extended "amino acid-linker" (see Chapter III) which could provide an additional H-bond through the carbamoyl side-chain oxygen with one of the hydrogens of the A36 amino group *(Figure IV.1.)*. In our opinion, a similar interaction could occur with the carboxylic oxygen of the side-chain of t^6A.

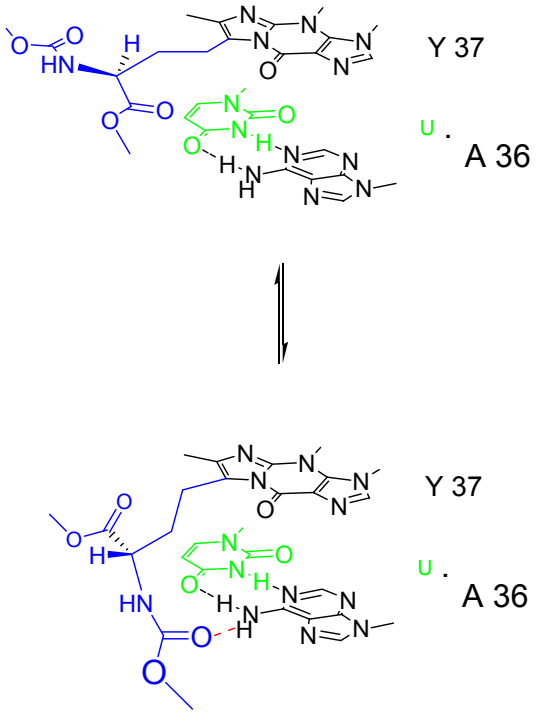


Figure IV.1. Wybutosine in interaction with the adjacent A·U base pair according the proposed model of (de Oliviera Neto et al. 1998).

IV.2.2. Computational investigations

We probed the feasibility of this hypothesis by combining conformational and energetical calculations carried out by *C. Gossens* within the laboratory of computational biochemistry (head: *U Röthlisberger*, EPFL).

The definition of our data set has been elaborated from a NMR study of a $tRNA^{Lys}$ (pdb file 1FL8) (Sundaram et al. 2000). This NMR structure furnished the coordinates of the t^6A modification and also permitted to reconstruct the tRNA-mRNA interaction from an internal A·U base-pair. Our calculations were carried out in vacuum since the nucleobase 3'-end adjacent to the anticodon loop (A site) is expected to be in close proximity to the next tRNA (P site) so excluding most of the water. Moreover, this localisation within the ribosome has been reported to be essentially hydrophobic (Robertson and Wintermeyer 1981). In a first approach, we restrained the global geometry by rotation of the amino acid moiety around the NH(6)-C(10) bound allowing formation of a hydrogen bound between NH(11)-N(1) as observed in X ray data (Parthasarathy 1977) *(Figure IV.2.)*. By rotation around the NH(11)-C(12) bond, the carboxylate, deprotonated under physiologic conditions, appeared to reach the $NH_2(6)$ of the nucleobase adenosine (I) of the mRNA. This hydrogen bond, formed between the atom oxygen COO^- and the proton of the $H_2N(6)$ function is, after energy minimization about 1.82 Å long, which respects the known geometrical constraints (Steiner. 2002). Furthermore, during minimization of energy, the hydroxyl function of the aminoacid moiety appeared to unexpectedly interact with the second oxygen of the carboxylic acid, conferring rigidity to the global structure. However, this interaction, also possible for the hn6A derivative, is probably not essential for functionality of the modified nucleoside, since certain organisms display a g^6A which does not present such hydroxyl groups (Schweizer et al. 1970) *(Figure IV.3.)*.

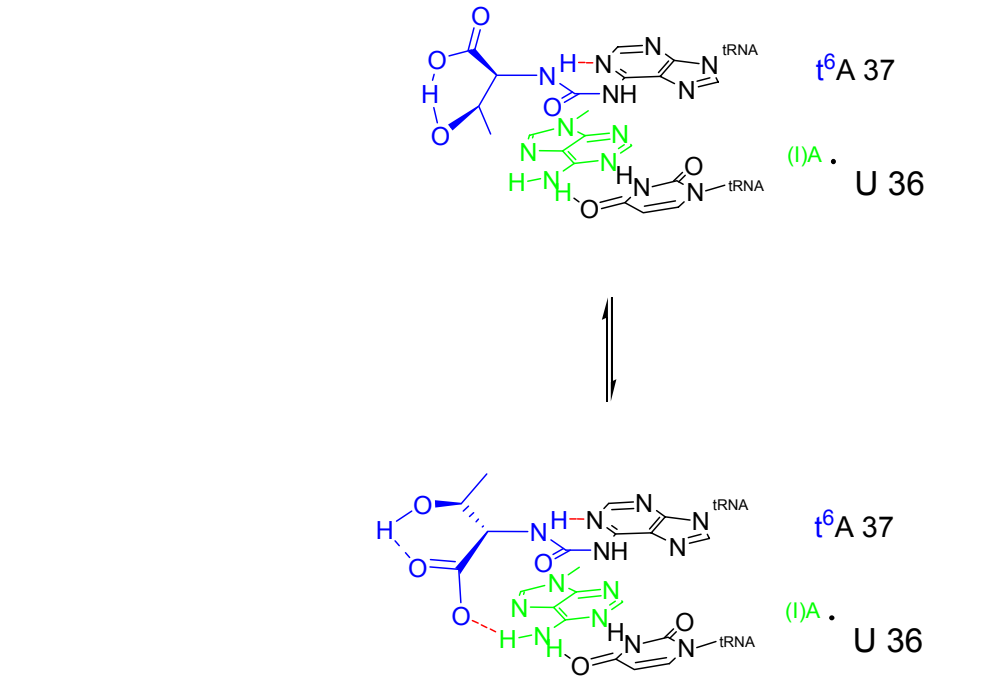


Figure IV.2. Proposed model of interaction of t^6A with the adjacent A·U base pair.

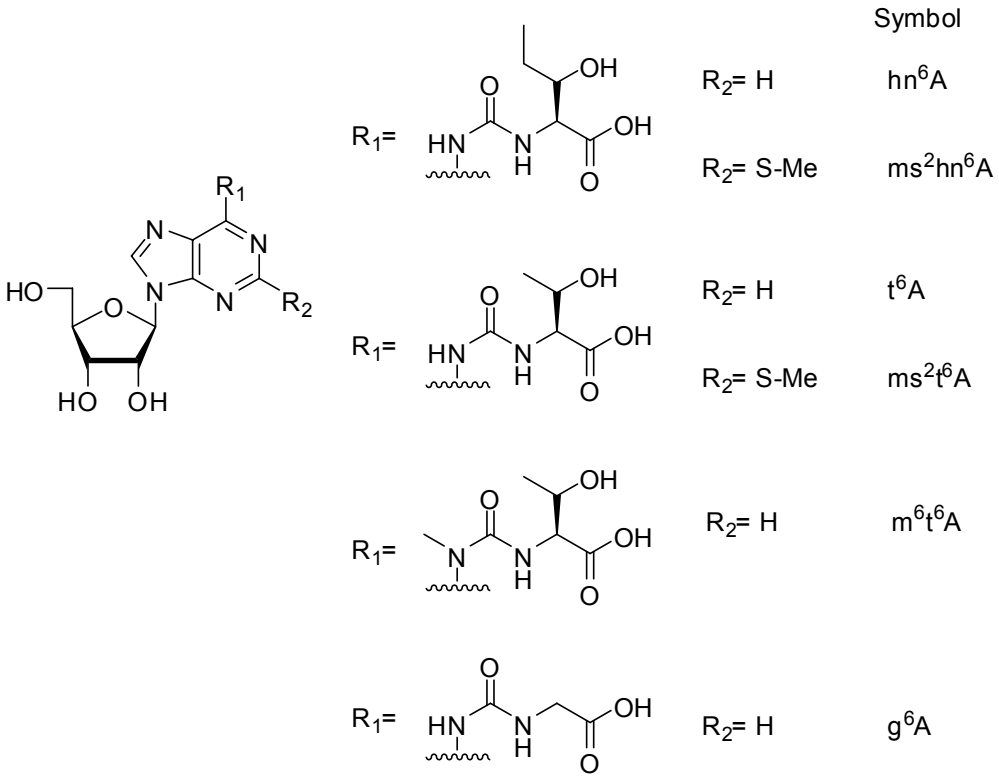


Figure IV.3. Structure of carbamoyl type adenosine derivatives.

We calculated the energy of the **AI**·**U**36 base pair and estimated its new energy in presence of this supplementary hydrogen bond. For this aim, we calculated (see experimental section) the energy of both (**t^{6}A**37/**U**36·**AI**) and (**A**37/**U**36·**AI**) complexes *(Figure IV.4.)*. Therefore, while the (**A**37/**U**36·**AI**) complex exhibits an energy of $E_{A/UA}$ = 14.49 kcal/mol, the t^{6}A containing complex presents an energy of $E_{t6A/UA}$ = 22.98 kcal/mol giving a stabilization energy of 8.49 kcal/mol. ($E_{Stab} = E_{t6A/UA} - E_{A/UA}$). This value, including the contribution of the stacking interaction and of the hypothetical additional hydrogen bond, allows the adjacent A·U base pair energy to reach that of a G·C base pair (22.98 against 24.05 kcal/mol for a G·C base pair).

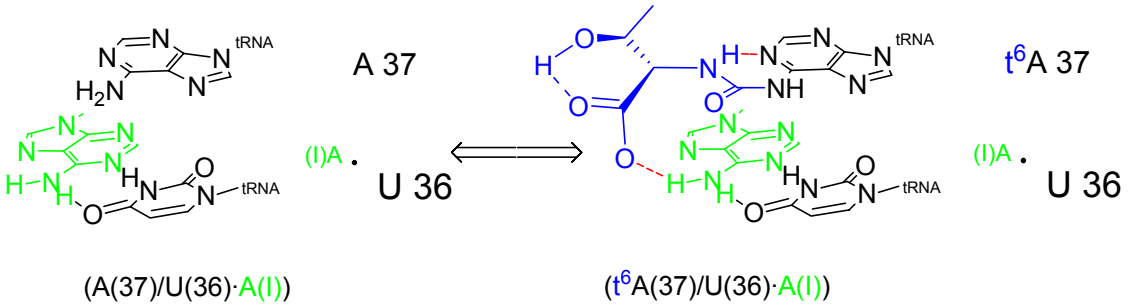


Figure IV.4. Model of complexes used for computational study $E_{A/UA}$ (*left*), compared to $E_{t6A/UA}$ (*right*)

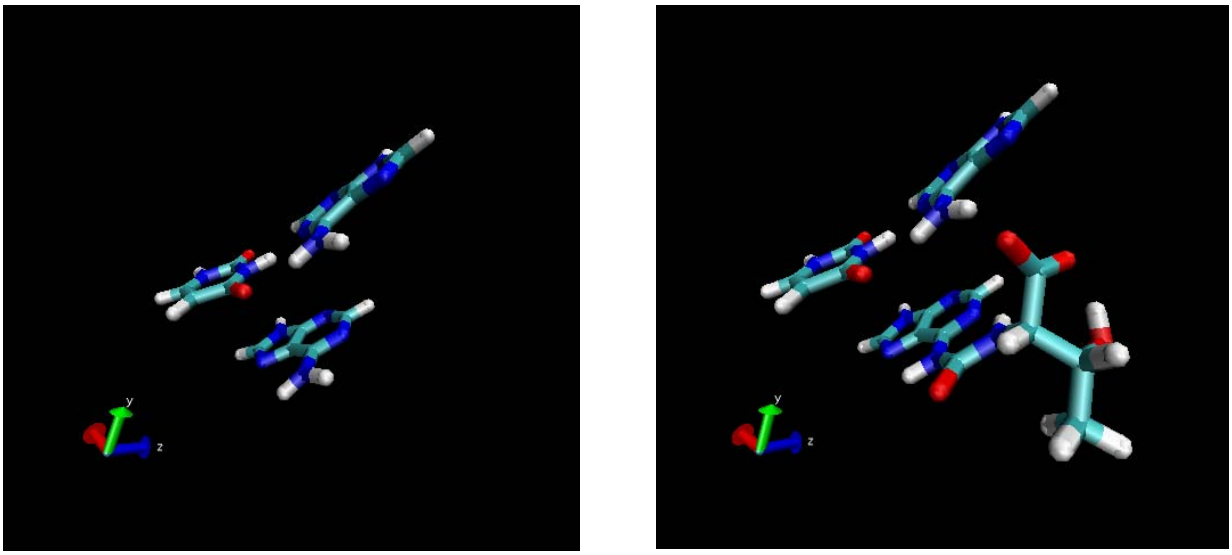


Figure IV.5. Set-up for the estimation of the energy of complex $E_{A/UA}$ (*left*), compared to $E_{t6A/UA}$ (*right*)

IV.2.3. Conclusion

Beyond the increase of the codon-anticodon pairing strength, the additional H-bond interaction provided by t^6A prevents the possible wobbling of the uridine and therefore ensures the reading-frame fidelity. Moreover, if we suppose that the additional interaction stabilizes the adjacent A·U to the same base pair energy as a G·C base pair and if this is a general trend provided by the modifications 3'-adjacent to anticodon, all first letters of the genetic code could be energetically equivalent. Others nucleosides have been found to influence the energetic of the U·A base pair, and among them i^6A derivatives are the most abundant.

IV.3. ms^2i^6A derivatives

IV.3.1. Introduction

As noticed above, the weakness of the A·U base pair could be compensated by the presence of modified nucleosides at position 37 of the anticodon loop. Among the variety of structures, the isoprenylated versions are the most represented (Sprinzl et al. 1998). Within this category, different derivatives have been identified and linked to a linear biosynthesis beginning with i^6A and ending with the ms^2io^6A derivative *(Figure IV.6.)*. According this pathway, the first step consists in prenylation of A37, utilizing Δ^2-isopentenyl pyrophosphate (IPP or dimethylallyl diphosphate) through a carbocationic intermediate (Poulter and Rilling 1976) (Poulter et al., 1976) (Poulter and Satterwhite, 1977). The isolation of the modifying enzyme dimethylallyl (Δ^2-isopentenyl) diphosphate tRNA transferase (MiaA) has revealed the prerequisite of the A36A37A38 sequence and anticodon helical structure flexibility for alkylation (Motorin et al. 1997b). The next step, consisting in methylthiolation of i6A37 to ms2i6A37, involves the MiaB and MiaC enzymatic activities (Björk 1995b) (Björk 1995c) and hydroxylation is dependent of the MiaE gene only present in *S.typhimurium*. Among the mutants defective in one of these genes, those lacking the isopentenyl group (MiaA) have the most severe biological consequences, including decrease of polypeptide chain elongation rates

(Diaz et al. 1987) (Ericson et al. 1991) (Hagervall et al. 1990) decrease of translation efficiency (Hagervall et al. 1990) or increase of codon context sensitivity. Furthermore, the tRNA suppressor harbouring this modification are less efficient (Ericson et al. 1991) (Hagervall et al. 1990) (Björnsson and Isaksson 1993) (Bouadloun et al. 1986) (Connolly and Winkler 1989) (Petrullo et al. 1983) and the number of translation misreading error rates at third or first positions in codons are decreased or increased, respectively.

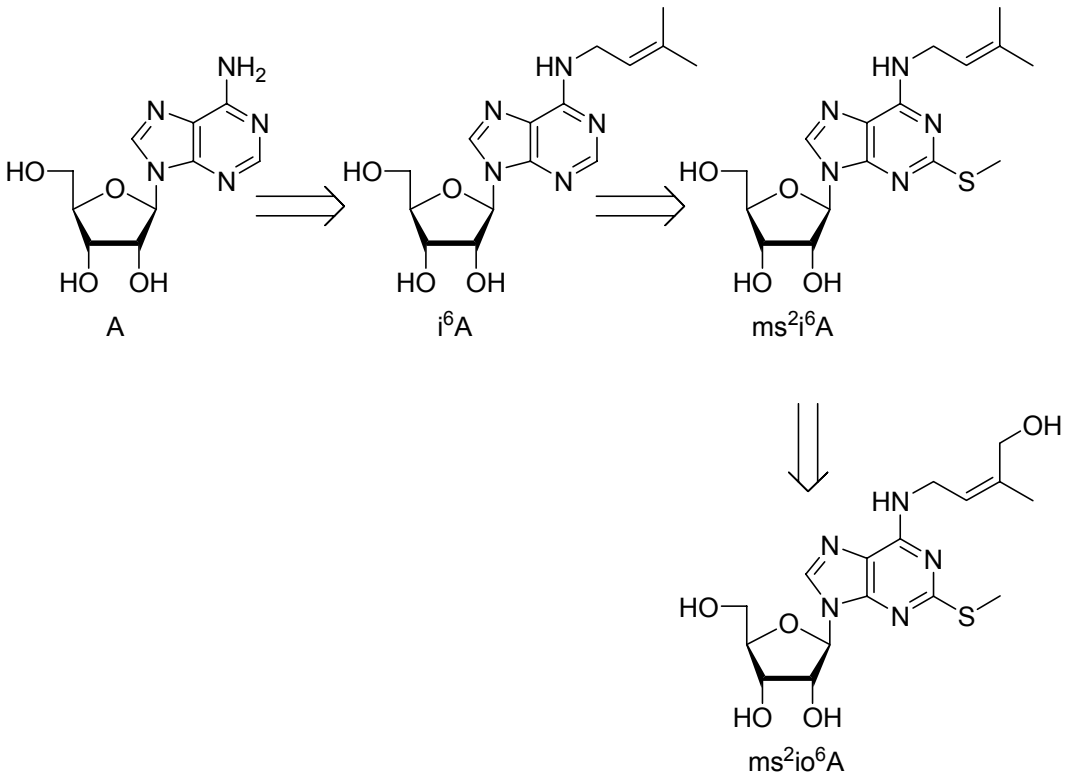


Figure IV.6. Structure of the i^6A derivatives in the context of their biosynthetic pathway.

IV.3.2. State of art

The reasons of these translational consequences have been well investigated concluding that the prenyl group acted mainly through an extended stacking interaction of the purinic ring. This stabilization is also increased by the intramolecular hydrogen bond

N(1)···H-C(11) which has been observed in all conformers of the related free nucleobases (Sonawane, et al. 2002) *(Figure IV.7.)*.

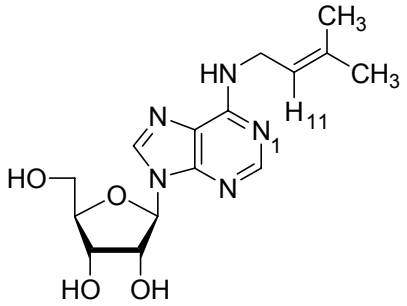


Figure IV.7. Preferred conformation of i^6A derivatives revealing an intramolecular N(1)···H-C(11) hydrogen bond.

The importance of this stabilization has been evaluated through preparation of synthetic oligonucleotides. Whereas 5'-ACAUGU-3' exhibits a Tm of 26°, the modified counterpart appears to be significantly stabilized with a Tm of 38.1° (5'-ACAUGUi^6A-3') (Kierzek and Kierzek 2001). However, the reported stabilization improvement, of around 1.4 kcal/mol, is probably higher, since in this model sequence the i^6A nucleoside stacked on a U·A base pair instead of a (naturally occurring) A·U base pair.

Furthermore, it has been also suggested that the alkyl group could be involved in the structuration of the anticodon loop since the consequence resulting from its absence is highly dependent of the tRNA and the structure of the anticodon loop. Whereas lack of the ms^2i^6A modification inhibits the ability to suppress codons (Pedersen and Curran 1991), the activity of E.coli Phe-tRNA is not affected during the initial selection step but induces a strong proofreading (Diaz and Ehernberg 1991). Furthermore, this tRNA is more likely to frameshift at UUU-Y sites (Schwartz and Curran 1997). Despite the recent NMR study of the related modified ASL, the reasons of this behaviour remain poorly understood. Since this synthetic ASL contains the single i^6A modification instead of Ψ32, Ψ39 and ms^2i^6A37 (Cabello-Villegas et al. 2002), it remains difficult to draw conclusions from this study about the role of the thiomethyl group. Nevertheless, according to investigations carried out by Houssier and Grosjean on tRNAs duplexes, the thiomethyl moiety increases the stability of the complex probably by improvement of stacking interaction (Houssier and Grosjean 1985).

Furthermore, this group appeared to have a stronger effect at the C context (UUU-C) (Esberg and Björk 1995). This sensibility supposes that ms^2 influences the rigidity of the wobble base G34. This hypothesis seems to be well correlated with the signal broadening observed for the G34 base in the i^6A modified ASL, which contrasts with the well-stacked bases of the fully modified anticodon loop containing ms^2i^6A (obtained from X-ray data). Moreover, in the context of a NMR study of the ASL of human tRNA Lys3, it has been proposed that ms^2 is likely needed to compensate the more dynamic U-turn of the ASL displaying t^6A instead of ms^2t^6A (Durant et al. 2005). Furthermore, it has been also proposed that ms^2 could relax or open the loop and expose the *Watson-Crick* faces of the anticodon nucleotides, thereby reducing the thermodynamic barrier to helix formation. This hypothesis could be confirmed by the recent study on synthetic oligonucleotides containing different types of alkylated adenosines, such as ms^2i^6A (Kierzek and Kierzek 2003). The experiments carried out with the model hairpin of tRNATrp from E.coli confirmed that introduction of the thiomethyl group increases the stability of RNA hairpins relative to hairpins containing only N(6)-alkyladenosine. However, this stabilization seems to be strongly influenced by magnesium ions as also noticed in the recent study on a i^6A modified ASL of Phe-tRNA from E.coli (Cabello-Villegas et al. 2002). Despite these reported observations, the role played by ions in the stabilisation of tRNA remains largely unknown.

Beside the demonstrated influences of the prenyl and thiomethyl groups on the decoding properties of the tRNA, the hydroxylation seems to have no translational effect, rendering its utility questionable. However, some observations suggest that this group could serve as "switch signal" from anaerobiosis to aerobiosis. Indeed, it has been observed in *S.typhimurium* that miaE defective mutants were unable to grow aerobically on succinate, fumarate, or malate (Persson et al. 1998). However, the exact biological processes remain largely unknown but could involve the i^6A derivatives as free base or as nucleosides.

IV.3.3. Role of related free base: the cytokinins

The so-called cytokinins are free bases and are known to act as growth factors in plants. Moreover, they can also exert a role in several biological processes such as DNA replication (Mok et al. 2000). Whereas the cytokinins are mainly produced from AMP in plants, in bacteria, the tRNA turnover seems to constitute an important contribution to their production (Prinsen et al. 1997). Consequently, the related cytokinins (ms^2i^6A, ms^2io^6A) issued from the tRNA degradation, could exert a significant effect on the cellular organites such as the yeast alcohol dehydrogenase *(Figure IV.6.)*. This enzyme has indeed been observed to be influenced by io^6A, which inhibits the enzymatic oxidation of ethanol into acetic acid (Zikmanis and Kruce 1990).

IV.3.4. Conclusion

It remains difficult to draw up an accurate scheme of interactions, since the action of these compounds remains poorly understood and information is still "highly fragmented" (Mok et al. 2000). The present conclusions suggest that the modified nucleosides, usually acting for proper codon-anticodon recognition and correct folding of the tRNA, could be also involved in metabolic regulation pathway. This supposed second activity of nucleosides has been also observed for the queuosine which is probably the most intriguing compound among the modified nucleosides.

IV.4. Queuosine

IV.4.1. Introduction

Among all known modifications of the tRNA, the queuosine nucleoside and its related base, queuine, constitute the most intriguing one. First identified in 1968 from *E.coli* $tRNA^{Tyr}$ (Goodman et al. 1968), it has been later identified as specific of the aspartate, asparagine, histidine and tyrosine tRNAs (Nishimura 1983), before its structure was determined in an-eleven year investigation by X ray crystallography (Yokoyama et al. 1979). Consisting of a 7-deazaguanosine residue that is attached to a dihydroxycylopentenediol through a

methylaminomethyl linker, it has been conventionally named 7-(3,4-*trans*-4,5-*cis*-dihydroxy-1-cyclopenten-3-ylaminomethyl)-7-deazaguanosine.

The position 2 of the cylopentenediol can sometimes be further modified by a mannose or a galactose residue, such as in $tRNA^{Asp}$ and $tRNA^{Tyr}$, respectively *(Figure IV.8.)*. Although present in mitochondrial, chloroplastic and cytosolic tRNAs of a variety of organisms (bacteria, animals, plants), queuosine is noticeable absent in yeast (Kasai et al. 1975) (Katze et al. 1982) (Slany and Kersten 1994).

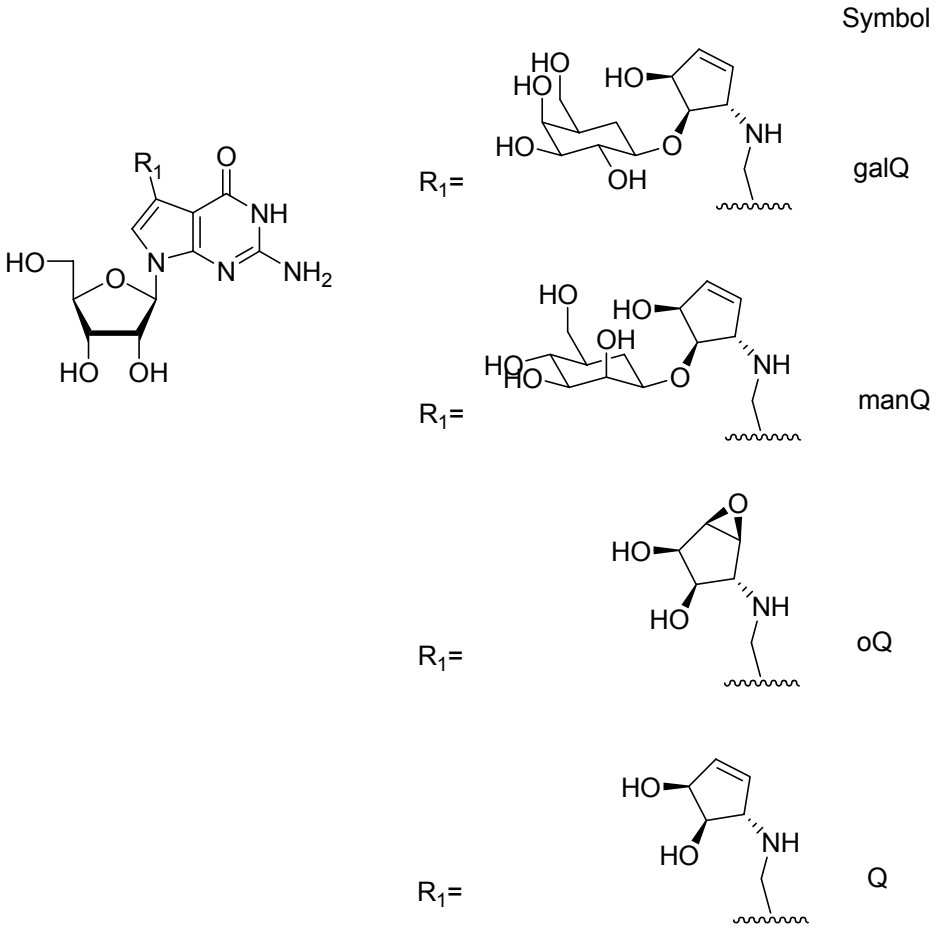


Figure IV.8. Structure of queuosine derivatives.

Despite its wide distribution, the process of queuosine incorporation differs according to the phyllogenetic membership. In contrast to the eukaryotes organisms which require supplementation of the free base from their diet, prokaryotes synthesize queuine *de novo*. As first substrate, the guanosine triphosphate (GTP) is converted into a 7-aminomethyl-7-deazaguanine ($preQ_1$) or a 7-cyano-7-deazaguanine ($preQ_0$) involving the GTP cyclohydrolase

which also acts during dihydroneopterin triphosphate biosynthesis *(Figure IV.9.)*. The $preQ_1$ is enzymatically incorporated into tRNA by replacing the present guanine base in position 34 and submitted to further modifications by introduction of an epoxy-dihydroxycylopentyl residue (Slany and Kersten 1994). This epoxy-Q (or o-Q) derivative, hypothesized to originate from methionine (Katze et al. 1977) (Slany et al. 1993), is converted into the definitive queuosine by an uncharacterized enzyme requiring co-enzyme B_{12} (Phillipson et al. 1987). None of the biosynthetic intermediates are salvaged by bacteria after the tRNA turnover and queuine is lost to the surrounding environment. This metabolic compound serves as substrate for the dependent eukaryotic modification system which has been recently reviewed (Morris and Eliott 2001). From a highly specific queuine transport system and an efficient salvage mechanism, the queuine-tRNA ribosyltransferase (tgt) (TGT, E.C. 2.4.2.29) substitutes the G34 base for queuine within the four queuosine modified tRNAs *(Figure IV.10.)*.

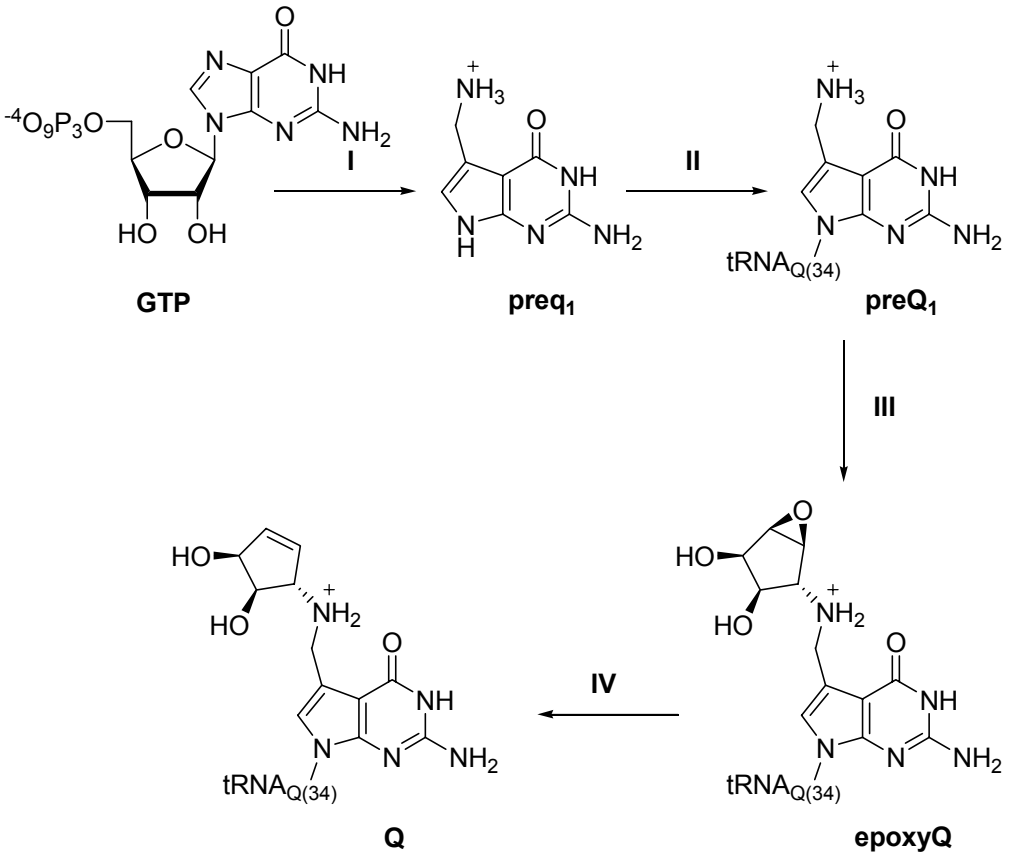


Figure IV.9. Biosynthesis of queuosine in prokaryotic organisms (Morris and Eliott 2001).

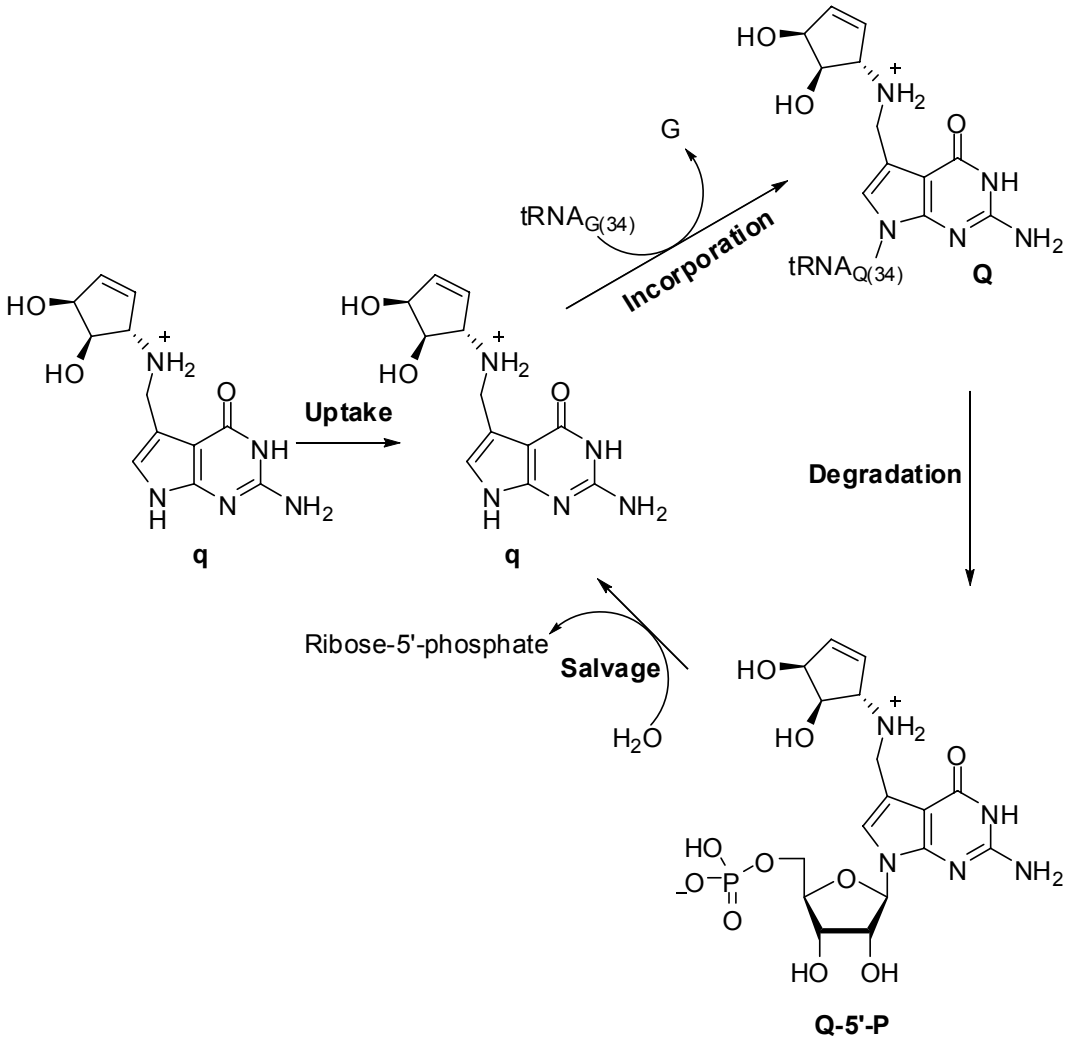


Figure IV.10. Incorporation of queuosine in tRNA in eukaryotic organisms (Morris and Eliott 2001).

IV.4.2. Features of the modifying enzyme: queuine-tRNA ribosyltransferase

As described above, the prokaryotic biosynthesis of queuine requires GTP which is also the substrate for the pteridine production in the cell (Werner-Felmayer et al. 2002). The main consequence of this "biosynthetic" relation is retrieved in their structures, which are quite similar *(Figure IV.11.)*. Therefore, a possible metabolic interference between these two substrates towards the tgt enzyme has been investigated and confirmed for several pteridine derivatives *(Table IV.1.)*.

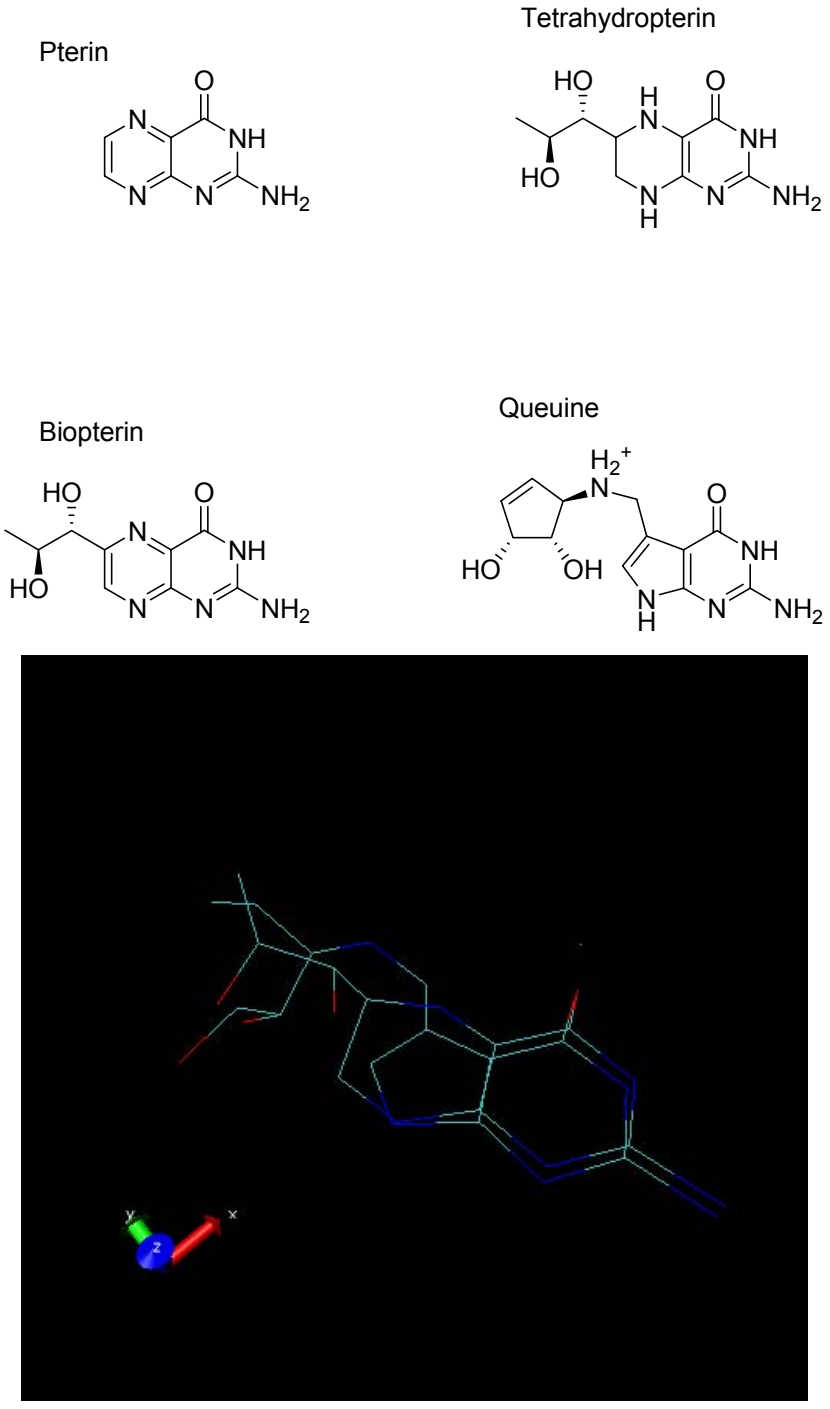


Figure IV.11. Superposition of queuine and pteridine structures.

tgt inhibition	
Compounds	Ki
pterin	0.05µM
biopterin	2µM

Table IV.1. Tgt inhibition by pteridine derivatives. The affinity for the tgt for its substrate (queuine) is almost 0.1µM (Kersten and Kersten 1990).

Although the assays have been carried out in vitro, we can observe an affinity of tgt for pteridines of the same order of magnitude than for its natural substrate queuine. Consequently, the pteridine level disorders observed in rapidly dividing cells (during differentiation or malignant) (Werner-Felmayer et al. 2002), should be responsible for the accumulation of hypomodified tRNAs. Similarly, the action of some substances such as phorbol esters induces the same hypomodification of tRNAs (Seidi et al. 1986). However, this effect is probably linked also to the pteridin metabolism since the phorbol esters induce a transient accumulation of neopterin and biopterin which are known to be efficient tgt inhibitors (Kersten and Kersten et al. 1990).

IV.4.3. Glycosylated derivatives of queuosine and tRNA turnover

The cyclopentendiol ring of queuosine can bear further modifications at position 2 by addition of mannose and galactose residues in the $tRNA^{Asp}$ and $tRNA^{Tyr}$ respectively (Kasai et al. 1976) (Okada et al. 1977) (Haumont et al. 1987). Exclusive to mammalian cells, it has been suggested that these modifications could prevent the rapid tRNA turnover from the efficient salvage mechanism which recycle queuine. Since forgotten, this hypothesis seems to be confirmed by the recent measurement of turnover rates of these both tRNAs. Eight week-old germ free mice fed with queuine free diets exhibit a decrease of 15% of queuosine modification level (Farkas 1980) (Reyniers et al. 1981) in $tRNA^{Asn}$ and $tRNA^{His}$ after 4 weeks. In contrast, the $tRNA^{Asp}$ and $tRNA^{Tyr}$ modification levels were maintained at 100%. As a consequence, the presence of sugar moiety could be understood as “glycosyl tag” for reduction of tRNA turnover rate which reduces accessibility of the Qtrase. Moreover, the nature of the sugar could correspond to the hydration shell mimicry. The mannose and galactose are found in two different tRNAs which differ in their anticodon loop composition but are highly conserved among the tRNA sequences. The mannosyl-queuine is related to the anticodon sequence manQUC and galactosyl-queuine to galQΨA, respectively (Sprinzl et al. 1998).

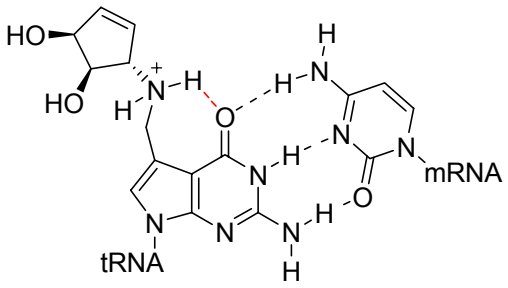
IV.4.4. Role of Queuosine in the decoding properties

Apart from the relatively good knowledge of the biosynthetic pathways for introduction of queuine into tRNAs, most of the biological effects induced by this nucleoside remain poorly understood (Moris and Elliott 2001) except for the consequences of its absence on decoding properties of the tRNAs. Therefore, the $tRNA^{Tyr}$ defective in queuine and displaying a guanosine in place (G34) appeared able to efficiently interact with the UAG STOP codon (Pelham 1978). This process defined as "readthrough", involves a tRNA which misreads a stop codon as sense codon, allowing the synthesis of an extended polypeptide which carries novel activities (Atkins et al. 1990). After this first observation for the tobacco mosaic virus (TMV) (Pelham 1978), it has been suggested that the queuine tRNA deficiency could serve for expression of alternative genes in cells. However, despite several investigations (Cassan and Rousset, 2001) (Beier and Grimm 2001), no similar example in healthy organism has been reported so far and this possible regulatory pathway looks unlikely since most genes essential for E. coli do not end in TAG (Benzer and Champe 1962) (Garen and Siddiqi 1962). Apparently, the editing of the tRNA with queuosine prevents suppression of stop codons by strict recognition of pyrimidine-ending codons. Furthermore, whereas the queuine-deficient tRNAs displaying a guanosine at the wobble position appear to decode preferentially the C over U ending codons, the fully modified (+Q) counterpart shows no bias for either pyrimidine ending codons. This in vivo experiment has been also correlated by the trend observed by *Grosjean* and coworkers (1978) during their dynamical study of complementary-tRNAs complexes *(Table IV.2.)*. These investigations have shown that the pairing with queuosine results always in an improvement of the base pair strength. Q·C base pairs appear almost as stable as the G·C base pair and the Q·U base pair seems to be nearly three times more stable than the G·U counterpart.

Half life of tRNA complexes			
Base pairs			
QC	QU	GC	GU
620ms	200ms	840ms	87ms

Table IV.2. Half-life of tRNA complexes: comparison of Q·C and Q·U base pair to G·C and G·U, respectively.

These properties have been subjected to computational investigations in the context of the ribosome with a queuosine modified tRNA (Morris, et al. 1999) and later on the free nucleotide itself (Sonavane et al. 2002). The energy minimizations carried out in these both studies underlined the presence of an additional hydrogen bond between O(6) of the deaza guanine moiety and the amino function (expected to be protonated at physiological pH) *(Figure IV.12.)*.

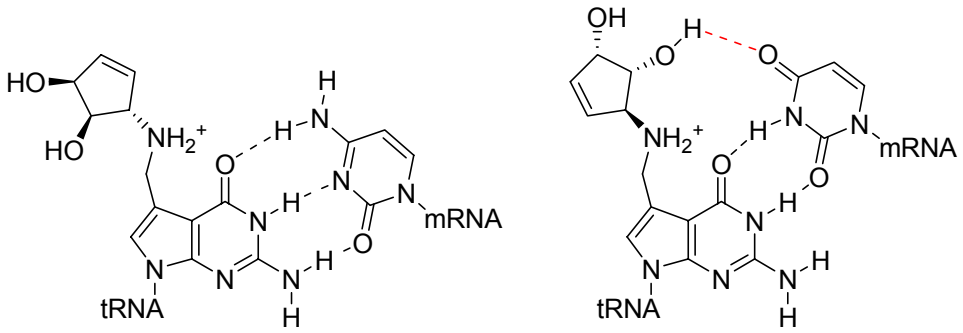


Q·C

Figure IV.12. Model of interaction of the Q·C base pair according the model of (Morris, et al. 1999).

Despite the description of this interaction, the observed decoding properties of queuosine have been attributed to a rigidification of the backbone (Morris, et al. 1999). Nevertheless, the weakening of the Q·C base pair in comparison to G·C could be described in terms of an anticooperative effect of the hydrogen bond (Steiner 2002), or by the presence of C(7) deazaguanosine base in place of guanosine which is known to reduce base pair strength

(Seela and Driller 1989) *(Table IV.3.)*. According to these two criteria we should expect a similar defavourable effect on the Q·U base pair which is surprisingly not observed since this base pair is largely more stable than its G·U counterpart. This observation suggests that other molecular interactions should occur within the close environment of the queuosine. In order to determine the nature of these interactions, we reconstructed a queuosine bound to a uridine in a geometry which should be close to that observed for the G·U base pair *(Figure IV.13.)*. For this purpose, we combined the X-ray data obtained in the context of a $tRNA^{Asp}$ attached to its related aminoacyl-tRNA synthetase (pdb 1C0A) (Eiler et al. 1999), with the data of a well-defined GU mismatch (pdb 1QES) (McDowell et al. 1997). In this new construct, the major groove appeared to be accessible to the HO-group of position 3. At the difference of position 3, the position 2 of the cyclopentenyl moiety remains free to any glycosylation. The hydroxyl, by reaching the O(4) of the uridine of the messenger RNA, should have a stabilizing effect on the base pair since it increases the H-N(3) acidity of the uridine. The new favourable display of π-electrons and the presence of an additional hydrogen bond can be expected to increase notably the strength of the Q·U base pair in comparison to G·U.



Q·C **Q·U**

Figure IV.13. Proposed model of interaction of the Q·U base pair.

Evaluation of base pair strength		
Tm of 8mer dc7G	Tm of 8mer dG	
37°C	46°C	ΔTm = 37/46=0.80
Half life of tRNA complexes		
GC ending	QC ending	
840ms	620ms	Δτ = 620/840=0.74

Table IV.3. Correlation of destabilization induced by C(7) deazaguanine base in place of guanine with the reported values of the Q·C base pair stability.

Unfortunately, this hypothesis has not been submitted to computational investigation since the calculations required would have taken too long.

IV.4.5. Conclusion

The proposed model of interaction of the queuosine requires further investigations that could be accomplished by the design of analogues and their incorporation into RNA sequences through automated synthesis. This challenge will be probably soon possible since several advances have been accomplished in the total synthesis of queuine (Oxenford et al. 2004) (Barnett and Grubb 2000). In contrast to this compound, for which the synthesis has not been yet accomplished, some of the modified nucleosides present at the wobble position have been already prepared and sometimes incorporated. However, their decoding properties remain poorly understood. Often derivatives of uridine, these compounds exhibit peculiar decoding properties which seem to be predictable on the basis of structural considerations. In this context, we reviewed the large variety of modified pyrimidines present at the wobble position and propose models of interaction.

IV.5. Pyrimidines at the wobble position 34

IV.5.1. Introduction

Ribosomal protein synthesis is an extremely accurate process, with only one in ten thousands amino acids incorporated incorrectly (Thompson and Karim 1982). The fidelity of the translation is mainly influenced by the modifications of the tRNA, especially those present in the anticodon loop *(Figure IV.14.)*. Whereas the interaction of the first and second nucleosides of the codon (I and II of mRNA) involve the "rigid" *Watson-Crick* base pairing with tRNA (positions 35 and 36), the last position (position 34) display a variety of modified nucleosides which permits to multiple bases recognition. This concept of "wobbling" was developed by *F. Crick* (Crick 1966) for inosine and uridine nucleosides. Since then, the identification of a large number of modified nucleosides and isolation of their related tRNA modification defective mutants have permitted to redefine "*Crick*'s rules". Whereas the decoding properties of such mutants are well documented (Agris 2004), the correlation between structure of the nucleoside and decoding capacity remains poorly understood (Takai and Yokoyama 2003), especially for the unusual pyrimidines.

To address this issue a series of structural models are proposed for most of the known modified pyrimidines (mnm^5s^2U, mcm^5U, $mchm^5U$, cmo^5U, f^5C, k^2C, $mchm^5U$) placing a particular role on the previously neglected hydrogen bonds which form the base pair.

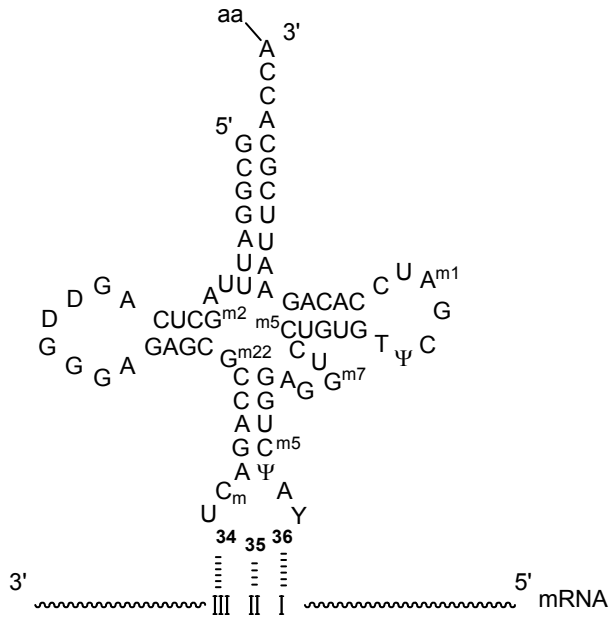


Figure IV.14. Example of tRNA (Yeast Phe tRNA) displaying several modifications (e.g. m^1A, D, m^5C, m^2G…) and carrying an amino acid (aa) at its 3'-end. The bases, 34, 35 and 36 (anticodon loop) interact with the codon triplet III, II and I, respectively.

IV.5.2. Description of decoding properties

IV.5.2.1. Uridines

IV.5.2.1.1. Unmodified uridine

The unmodified uridine has been proposed to exhibit extended "wobble" decoding capacity for the purines bases A and G, by adopting an original geometry beyond the classical *Watson-Crick* base pairing. This model developed by *F. Crick* (Crick 1966), based on an equal repartition between C2'-endo and C3'-endo conformations of the ribose ring (Yokohama et al. 1985) and excluded the U·U and U·C base pair. Although this hypothesis has been confirmed by experiments carried out with *E.coli* cell-free translation assays (Takai et al. 1999), U ending tRNAs anticodons of *Mycoplasma* organism and mitochondria organelles (Björk 1998) seem to have the ability to read all four bases of a family box. For explaining this result a "two out of three" mechanism has been proposed, where only the two first nucleosides are involved in the codon reading (Hagervall 1998). Beside this model, a possible U·U base pair has been also invoked. The first includes a water bridge and requires a N-glycosidic bond propeller twist unfavourable for π-stacking (Lim 1994) *(Figure IV.15)*.

The second model involves the less favorable C2'-endo conformation in a "short wobble" conformation, but maintains the stacking interaction (Yokohama and Nishimura 1995) *(Figure IV.15)*.

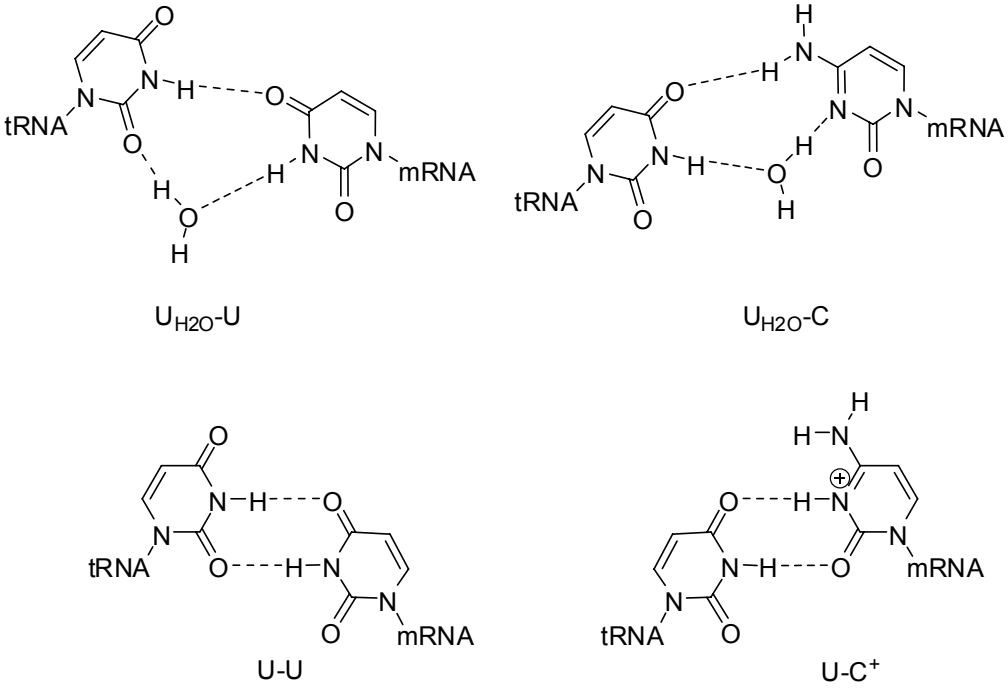
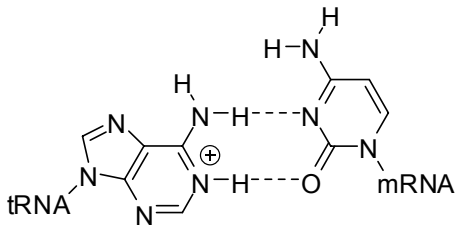


Figure IV.15. Models of U-U and U-C base pairs; *Top*: Requiring a water bridge and a N-glycosidic bond propeller twist unfavourable for π-stacking according (Lim 1994), *Bottom*: U34 should adopt a short C2'-endo conformation for base pairing with U or a protonation of C for base pairing with C according (Yokohama and Nishimura 1995).

Although both models are viable, it has been suggested that the latter one is incompatible with the U·C base pair since it would require a protonated form of C. However, a protonated form of base pair, C·$^+$A, has been already reported in some RNA duplexes (Pan et al. 1998) even crystallized at neutral pH *(Figure IV.16)*. The observed C·$^+$A base pair could explain the observed A-ending codon reading by ^{Ile}tRNA (Grosjean and Björk 2004) in yeast mitochondria. Consequently, in this context, the protonation of N(3) of C (adenosine N(1) pKa=3.52 and cytidine N(3) pKa=4.17) (Saenger 1988) should also be possible but requires further investigations.

The observed extended capacity to read all four bases by U34 containing tRNAs seem to be possible in the solely context of the mitochondrial ribosome. Indeed, this ability observed for mitochondrial tRNAs is totally absent within the cytoplasmic ribosome (Takai et al. 1999). However, in cytoplasmic tRNAs, this weakness is compensated by existence of a wide diversity of modified pyrimidines at position 34 *(Figure IV.17)*[1]). Whereas unmodified uridine exhibits no bias for adopting either C2'-endo or C3'-endo conformations, the substituent of the modified nucleobase confers unique decoding properties by displacing this equilibrium. Despite the wide diversity of original structures, two main categories can be distinguished, differing in the nature of substituent at position 5: the methylene bridged (xm^5U) uridines (often O(2) thiolated: xm^5s^2U) which recognize the purine ending-codons and the oxygen bridged (xo^5U) uridines, which recognize U, A and G ending codons (Mizuno and Sundalingam 1978) *(Table IV.4.)*.



A^+-C

Figure IV.16. Structure of A^+·C base pair where adenosine is protonated (Yokohama and Nishimura 1995).

[1]) Chemical structures and abbreviation of modified nucleosides listed in the introduction can also be found in (http://medstat.med.utah.edu/RNAmods).

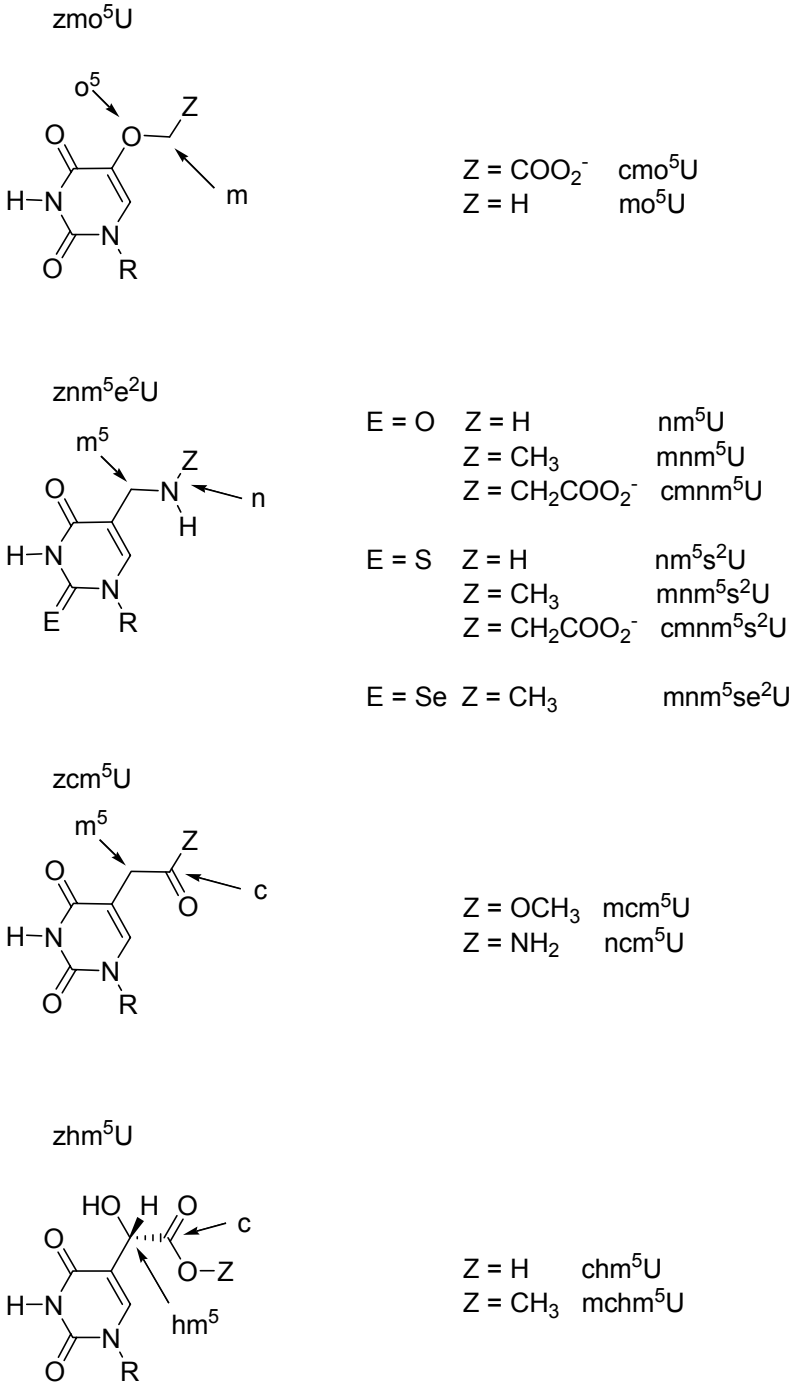


Figure IV.17. Diversity of C(5) and E(2) substituted uridines encountered at wobble position (34) of tRNAs.

IV.5.2.1.2. xm^{5}U modified uridines

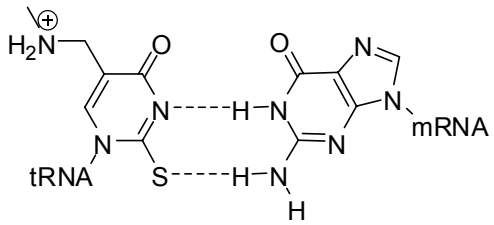
IV.5.2.1.2.1. xnm^{5}U derivatives

The phyllogenetic distinction of the eukaryotic and prokaryotic kingdoms is also evident in the types of xm^{5}U modification seen at tRNA position 34. Those displaying an aminated derivative (xnm^{5}U) are exclusive to prokaryotes and those displaying a carbonyl function (xcm^{5}U) are specific to the eukaryotes (Rozenski et al. 1999). This structural distinction is also apparent in the decoding specificities. Indeed, it was observed that a yeast tRNA Glu, exhibiting a mcm^{5}s^{2}U modification at position 34, was exclusively devoted to the GAA codon (Sekiya et al. 1969) and mcm^{5}U34 was also unable to recognize the G-ending codon (Percudani 2001). The G-ending glutamate codon was found to be recognized by tRNAs with C(34) (Marck and Grosjean 2002), confirming that the eukaryotic xcm^{5}s^{2}U is exclusively devoted to A-ending codons.

On the other hand, the prokaryotic xnm^{5}U modification seem to have decoding capacities, which are extended to both A and G purines, as confirmed by several experiments. Indeed, a synthetic anticodon stem loop containing the mnm^{5}s^{2}U modification has been observed to enhance the A-site binding to the AAA and AAG codons (Yarian et al. 2002). Additionally, well-characterized *E.coli* mutants have enabled measurements of the effects of individual modifications on rates of translation. In mnm^{5}s^{2}U, the s^2 modification appeared to enhance the GAA codon reading with a small effect on GAG, whereas mnm^5 reduces GAA decoding and increases GAG codon reading (Sundaram et al. 2000).

To explain these apparently contradictory experimental data, a model has been proposed where the pKa values have been estimated (Kazuyuki and Shigeyuki 2003), concluding that a partial ionization of the uracil moiety under physiological conditions allows a base pairing with GIII *(Figure IV.18)*. A more pronounced effect for the 2-thio derivative was also predicted by exhibiting a negative charge on sulphur atom conferring an excellent H-bond acceptor capacity. However, with respect to the recent X ray structure of a tRNA$^{Lys}_{UUU}$ stem loop bound to a 30S ribosomal subunit A site, the proposed model seems to be incompatible with the observed base pairing geometry *(Figure IV.19)* (Murphy IV et al.

2004). In this context, no definitive mechanistic explanation of the action of the mnm^5s^2U nucleoside on decoding properties has been proposed due to the absence of consistent electron density (Murphy IV et al. 2004). Nevertheless, the observed local disorder could reflect the fluctuation of two H-bonds sharing the NH_2^+ moiety between the well-characterized NH_2^+ / 2'-OH U(33) bond (Hillen et al. 1978) and the less obvious NH_2^+···O(4) mnm^5s^2U(34) bond *(Figure IV.19)*. This last intramolecular H-bond between the amino subtituent and the O(4) carbonyl group of the uracil base was proposed as a tautomeric form of the negatively charged mnm^5se^2U moiety (Ching 1986). The introduction of "additional proton" in the close proximity to the O(4) should therefore enhance the N(3)-H proton acidity and consequently the H-bond donor character. Different definitions of hydrogen bond between two species A-H···B exist and, among them, this complex can be also described in terms of acidity of A-H and basicity of B (Steiner 2002). An increase of A-H acidity can change a H-bond from weak to strong. The strength of the base pairing with GIII is reinforced by the introduction of the O, S and Se elements at the Y(2) position through two main effects: a local increase of the N(3)-H acidity and the enhancement of the Y(2) atomic radius which favours efficient interaction with the three H-bonds.



mnm^5s^2U-G

Figure IV.18. Model of mnm^5s^2U·G base pair as proposed by *Takai* and *Yokohama* (2003). The electronic withdrawal effect of the amine favours the deprotonation of the uridine moiety allowing a base pairing geometry close to the *Watson-Crick* C·G base pair. The deprotonation is particularly pronounced for the 2-thiolated derivative.

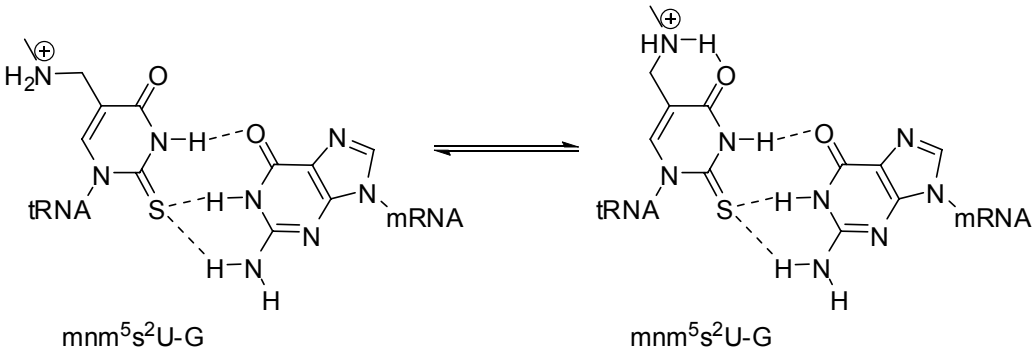


Figure IV.19. Proposed alternative model of mnm^{5}s^{2}U·G base pair deduced from X ray analysis (Murphy IV et al. 2004) and with new hydrogen-bond distribution. At physiological pH the protonoted amine is in close vicinity to the O(4) allowing hydrogen-bond formation which enhances the H-N(3) acidity and stabilizes the mnm^{5}s^{2}U·G base pair.

Although the introduction of the intramolecular H-bond $NH_2^+\cdots O(4)$ favours the base pairing with GIII, it appears to partially weaken the conventional *Watson-Crick* base pair with AIII (Krüger et al. 1998). This effect is mainly due to the anticooperative effect produced by introduction of a second H-bond at the O(4) position (Steiner 2002) which is already involved in the base pair with the amino function of A(III) ($NH_2^+\cdots O(4)\cdot H_2N(6)$) *(Figure IV.20)*.

The cmnm^{5}s^{2}U nucleoside, another widely represented modification, has been isolated during the determination of the biosynthetic pathway to mnm^{5}s^{2}U (Hagervall et al. 1987) (Leipuviene et al. 2004). The relative suppression efficiencies of tRNAs containing each of the intermediates have been evaluated, revealing the following order: mnm^{5}s^{2}U (wild type) > cmnm^{5}s^{2}U > nm5s^{2}U > s^{2}U (Hagervall et al. 1984). The presence of the additional H-bond acceptor COO^- creates a bifurcated H-bond pattern which decreases the $NH_2^+\cdots O(4)$ H-bond strength, reduces the N(3)-H acidity and consequently the suppression efficiency. However, the biosynthetic pathway suggests that cmnm^{5}U does not act as the real effecter of the codon recognition but reflects the composition of the growth medium. Indeed, the formation of mnm^{5}s^{2}U from cmnm^{5}s^{2}U, involving a same polypeptide with two distinct enzyme activities, is severely inhibited by magnesium (Hagervall et al. 1987) and the observed cmnm^{5}s^{2}U modification could be considered only as a biosynthetic intermediate having a reduced impact in the decoding properties.

In contrast to the mnm^{5}y^{2}U derivatives, the xcm^{5}U derivatives do not display the described H-bond and hence they are unable to recognize GIII and consequently they only recognize AIII codons.

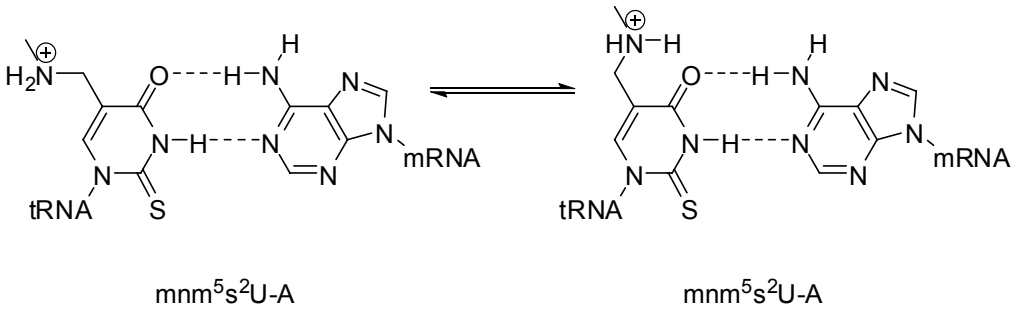


Figure IV.20. Model of mnm^{5}s^{2}U·A base pair. At physiological pH the protonoted amine is in close vicinity to the O(4) allowing hydrogen-bond formation which enhances the H-N(3) acidity and destabilizes the mnm^{5}s^{2}U·A base pair through anticooperative effect (Steiner 2002).

IV.5.2.1.2.2. xhm^{5}U derivatives

Two rare nucleosides amongst the modified uridines could be included in the proposed model, despite their unique structure: the 5-[*S*-methoxycarbonyl(hydroxyl)methyl]uridine mchm^{5}U and 5-[*S*-carboxy(hydroxyl)methyl]uridine chm^{5}U. Isolated from posterior silk glands of Bombyx mori (silkworm) (Kawakami et al. 1979), chm^{5}U seems to be the result of instability of the methylester which suffers partial hydrolysis during the isolation process, and mchm^{5}U is most likely the original component in the intact tRNA molecule (Kawakami et al. 1988). Synthesized for full identification (Nawrot and Malkiewicz 1989), these methylene bridged nucleosides display an hydroxyl group which could interact with the O(4) of the uridine moiety in same manner as described for mnm^{5}s^{2}U *(Figure IV.21)*. The resulting increase in H-N(3) acidity contributes to an efficient reading of GIII codons, in agreement with experimental results (Kawakami et al. 1980). This idea is supported by the isolation of only two glycine tRNAs, one harbouring the presented modification and the other with a

guanosine, without occurrence of a third C34 tRNA (Kawakami et al. 1988) to compensate for a weak GIII ending codon reading. Therefore, in interaction with adenosine, the mchm^{5}U modification should adopt the same geometry as the one described for the mnm^{5}s^{2}U·A base pair *(Figure IV.22)*.

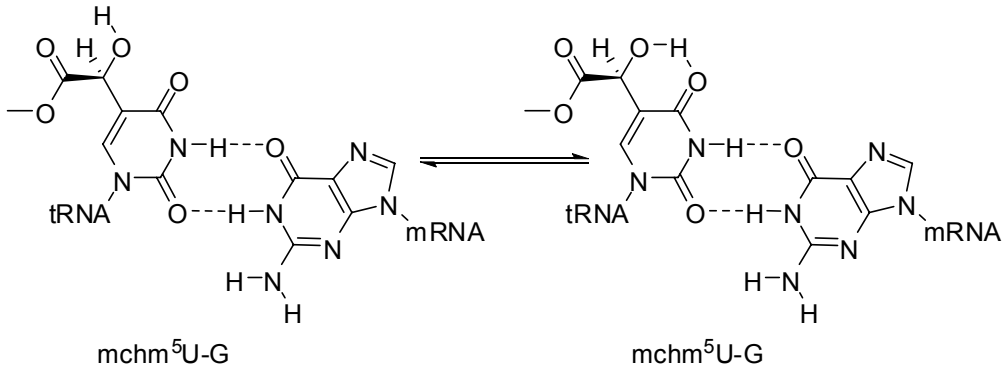


Figure IV.21. The mchm^{5}U·G could adopt the same conformational geometry where, the hydroxyl group of the C(5) alkyl chain behaves in analogy as the protonated amine encountered in the case of mnm^{5}s^{2}U·G base pair.

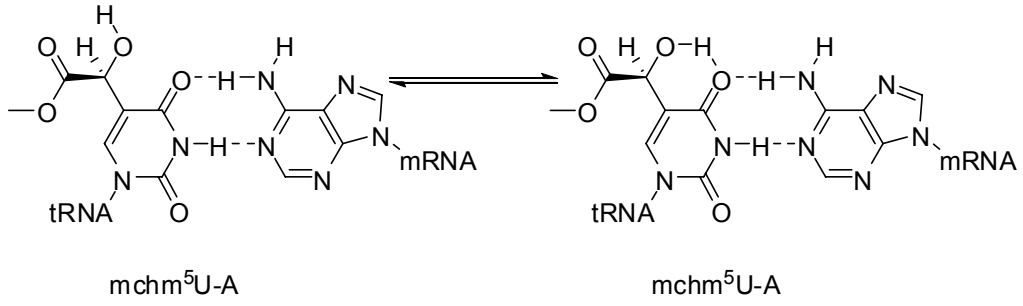


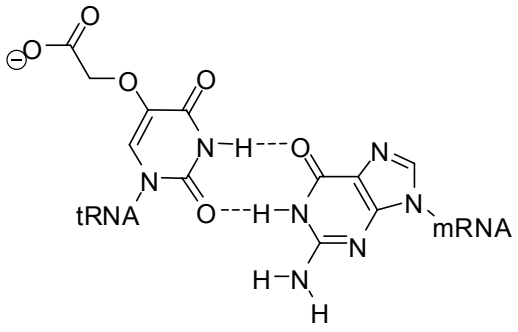
Figure IV.22. The mchm^{5}U·A could adopt the same conformational geometry where the hydroxyl group of the C(5) alkyl chain behaves analogously to the protonated amine encountered in the mnm^{5}s^{2}U·A base pair.

Moreover, despite synthesis of the corresponding 2-thiolated derivative (Nawrot and Malkiewicz 1989), no mchm^5(s^2)U has been identified so far, contributing to the idea that the additional intramolecular H-bond O(4)···Y (Y= NH_2^+, HO) is the main contributor to the observed decoding properties of the mchm^{5}U and mnm^{5}s^{2}U modifications .

IV.5.2.1.3. xo^{5}U modified uridines

In contrast to the methylene bridged uridines, the xo^{5}U34 derivatives exhibit extended decoding properties. Favoring the base pairing with A, U and G, the diversity of oxygen substituted uridines have been recently well reorganized by linking their structure to the biosynthetic pathway (Näsvall et al. 2004). The first hydroxylated ho^{5}U nucleoside is further modified by side chain extensions: starting with mo^{5}U, followed by cmo^{5}U and finishing with mcmo^{5}U (U → ho^{5}U → mo^{5}U → cmo^{5}U → mcmo^{5}U).

From a measure of the pKa value of pmo^{5}U (Shibaev et al. 1975) and a prediction for the other derivatives based on the Hammett equation, (Kazuyuki and Shigeyuki 2003), the estimated pKa value was not sufficient to induce a significant ionization of the base moiety under physiological conditions, predicting a base pairing restricted to purines (A and G). The extended capacity to base pair with U is due to a bias in equilibrium of sugar conformation induced by the (xmo^{5}U x = H, COO^{2-}, COOMe) side chain. Whereas the unmodified uridine displays no bias for either C2'-endo or C3'-endo conformations, the mo^{5}U and cmo^{5}U modifications show a noticeable preference for the C2'-endo conformation (Yokohama et al. 1985) as measured from thermodynamical investigations for the enthalpy differences between C2'- and C3'- endo conformations: 0.1 (pU), -0.28 (pho^{5}U), -0.72 (pmo^{5}U), -0.67 (pcmo^{5}U); (values in kcal.mol^{-1}) (Yokohama et al. 1985). This feature explains the G(III) decoding ability displayed by the mo^{5}U, cmo^{5}U, and consequently mcmo^{5}U, derivatives *(Figure IV.23)*.



cmo^{5}U-G

Figure IV.23. Structure of the cmo^{5}U-G base pairing where the favoured C2'-endo conformation of cmo^{5}U facilitates the base pairing with GIII according to (Yokohama et al. 1985).

Furthermore, an unexpected extension of the decoding capacity of ^{Pro}tRNA for the four nucleobases A, C, G and U has been recently observed in *S.typhimurium*. This anticodon contains cmo^{5}U at the wobble position and, in addition to the reading of A, G, U ending codon reading, it is able to read CIII codons (Näsvall et al. 2004). However, this ability has no real biological significance since the C ending codon is already efficiently read by a ^{Pro}tRNA displaying a G34 at wobble position of its anticodon. This capacity, not observed for the structurally close mo^{5}U modification (Takai et al. 1999), is consequently due to the presence of the carboxylic moiety.

However, the mechanistic details of the C(III) decoding remain difficult to draw-up. As described above, we could expect the formation of a stable cmo^{5}U-C$^+$ base pair where the carboxylic acid function could neutralize the created positive charge of the C$^+$·U base pair directly or indirectly, by water molecule mediation *(Figure IV.24)*.

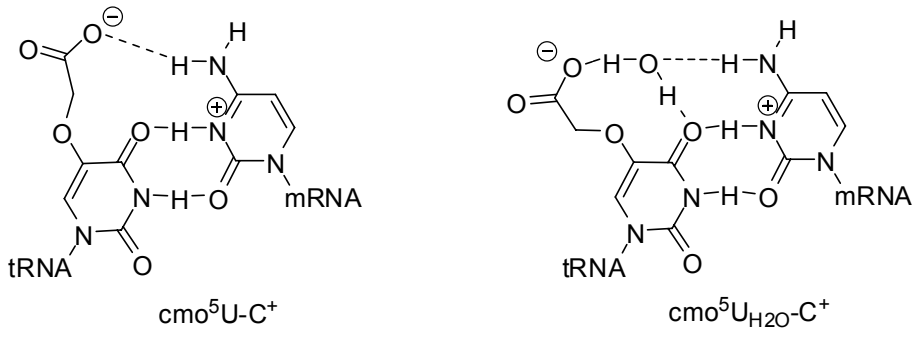


Figure IV.24. Proposed model for the cmo^{5}U-C base pairing where the carboxylic acid function could neutralize the created positive charge of the C$^+$·U base pair directly (*left*) or inderirectly, by water molecule mediation (*right*).

IV.5.3. Cytidines

IV.5.3.1. Lysidine k^2C

The second standard pyrimidine, cytidine, is also modified, but less often than uridine. Among the derivatives, the intriguing lysidine has recently been revisited (Soma et al. 2003) (Grosjean and Björk 2004) several years after its isolation from *E.coli* ^{Ile}tRNA (Muramatsu et al. 1988). Present in *B.subtilis* (Matsugi et al. 1996) and *Mycoplasma capricolum* (Andachi et al. 1989), its total synthesis accompanied its first initial characterization (Muramatsu et al. 1988). Abbreviated to k^2C, this modified nucleoside has a lysine moiety instead of the usual keto group of C which introduces a delocalized positive charge and allows classical base-pairing with adenosine. In contrast to the wobbling mechanism which expands the decoding property of the tRNAs (e.g. inosine, modified uridines), lysidine is unique in its restricted decoding capacity in the crucial split box coding for Methionine and Isoleucine (Grosjean and Björk 2004). In parallel with the structural identification of k^2C, several tautomers or tautomeric forms and base pairing geometries were proposed, but have not been investigated further. A model of twisted geometry has also been suggested but the lysine moiety was not included in the model (Lim 1994). Among the presented models, the planar geometries are likely to be the most energetically favourable because they maintain π-stacking interaction. According to this criterion, two configurations of the H-bonds are possible: one in a conformation close to that observed for the G·U base pair and another in a more conventional A·U *Watson-Crick* base pair *(Figure IV.25)*.

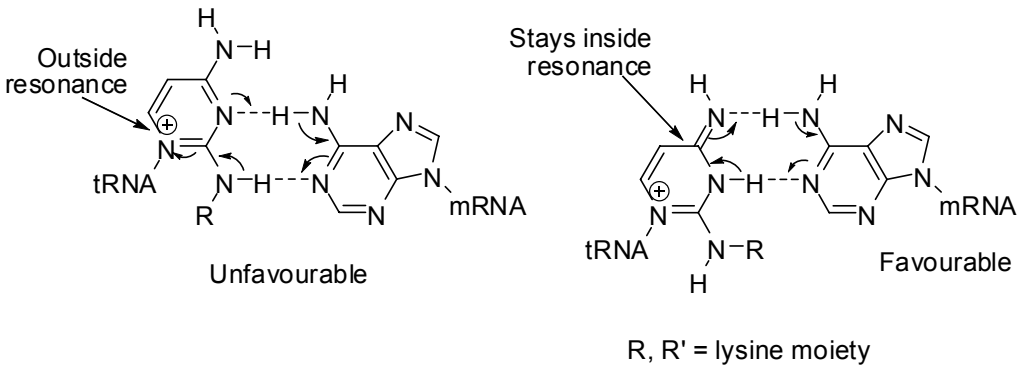


Figure IV.25. The planar geometries in a conformation close to those observed for a G·U base pair (*left*) and another one in a more conventional A·U *Watson-Crick* base pair (*right*) which is preferred for electronic and steric considerations.

Whereas the first one seems to be prohibited due to steric considerations, the second one does not suffer the same restrictions. Moreover, the proposed tautomeric forms (Muramatsu et al. 1988) suggest that the N(3) nitrogen is protonated (6/10 of all tautomeric forms) and, therefore, the context of a A·U base pair, the $H_2N(4)$ function should be in the imino form. Although the presented base pair offers an optimal resonance system, it represents just one of several possible configurations. This feature probably induces some decoding ambiguities within the ribosome when the tRNA is interacting with the codon triplet of the mRNA in the crucial split box Ile/Met (AUN). As well as the ^{Ile}tRNA, the elongator ^{Met}tRNA also contains a modified base N^4-acetylcytidine (ac^4C), weakens the G·C base pair by around 50% so that its strength is about the same as A·U. However, its strength is probably still stronger than the base pair made by k^2C. In order to counter the weakened base pair resulting from the "tautomeric ambiguity", the bulky lysine side-chain might serve to increase the global H-bond strength through a possible interaction of the ammonium function with the nitrogen N(3) of adenosine *(Figure IV.26)*. In view of the resulting possible tautomeric forms, the introduction of a H-bond donor at the position of N(3) should contribute to a compensatory stabilization of the k^2C·A base pair although this remains to be quantified.

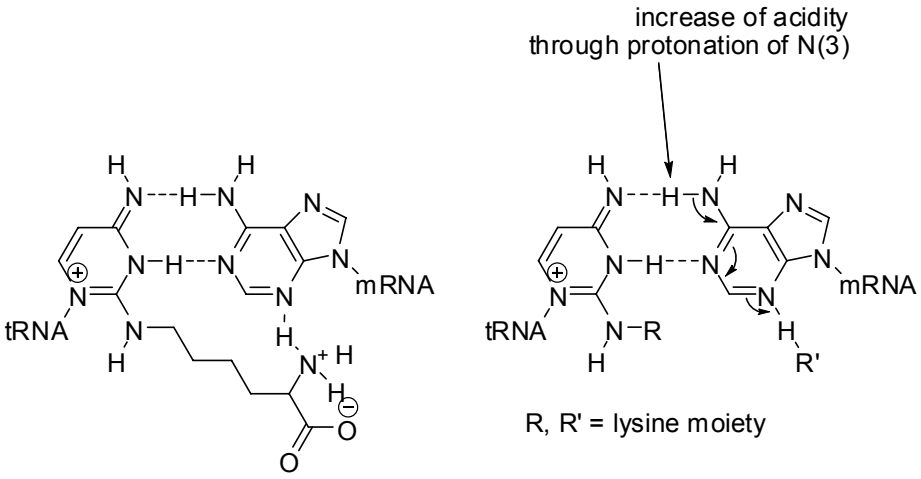
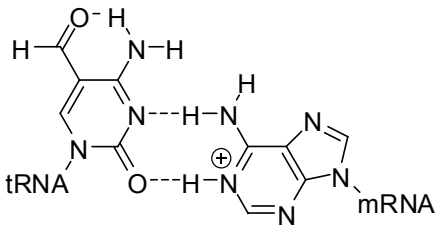


Figure IV.26. Although the presented base pair offers an optimal resonance system, it represents one configuration among several possibilities leading to a probable decoding ambiguity within ribosome when the tRNA is interacting with the codon triplet of the mRNA. The lysine moiety could serve to increase the global hydrogen bond strength through a possible interaction of the ammonium function with the nitrogen N(3) of adenosine. In view of the resulting tautomers, introduction of a hydrogen bond donor at this position could be compensated by significant stabilization of the $k^2C{\cdot}A$ base pair.

IV.5.3.2. 5-Formylcytidine f^5C

The fine-tuning of the decoding properties developed by bacteria is also present in the most puzzling organelle of the cell, the mitochondrion. The mitochondrial protein synthesis has been extensively studied and many diseases have been associated with dysfunction in their gene expression due to dramatic mutagenesis or disorder in tRNA import (Rossignol et al. 2003). Besides having their own genetic code, their original tRNAs structures exhibit some peculiar modifications such as the 5-formylcytidine (abbreviated f^5C) which is present in mitochondrial ^{Met}tRNA of several eukaryotes and decodes AUG and AUA codons. Therefore, in contrast to ac^4C which prevents AUA codon reading, f^5C facilitates this particular decoding but without changing the geometry of the $A^+{\cdot}C$ base pair. The structural reasons of these abilities could be understood in regard to the case of the $A^+{\cdot}C$ base pair. From X ray analysis (Pan et al. 1998) and some NMR studies (Boulard et al. 1995), the $A^+{\cdot}C$ mismatch appeared to interact through two H-bonds *(Figure IV.27)*: the "standard" N(6)-H···N(3) bond and $N^+(1)$-H···O(2) are formed by protonation of A (Sowers et al. 1986), even though the experiments were done at neutral pH (Pan et al. 1998). Further investigations underlined the

special contribution of the adjacent adenosine to the stabilization of the A·C* mismatch (Allawi et al. 1998); this situation corresponds to the case of the ^{Met}tRNAs anticodon (C*AU where C* is a modified cytidine). In order to control the occurrence of the A^{+}·C* mismatch, Nature has developed modifications such as N^4-acetylcytidine which prevents this deleterious mispairing (Stern and Schulman 1978), or other modifications that enable A^{+}·C* base pair formation, such as f^5C (Karino et al. 2001). After structural elucidation of the latter (Moriya et al. 1994), and determination of its conformational properties, a preference of these two modifications for the C-3' endo form (Kawai et al. 1994) has been recognized. The decoding properties are unlikely to be due structural rigidity alone, but also reflect the H-bond acceptor character of the N(3) lone pair. The ^{15}N NMR of the labeled nucleoside gave a direct estimation of this parameter by chemical shift measurements. The values of the ^{15}N(3) chemical shifts of ac^4C and f^5dC have been determined and compared with those of the cytidine and 2'-deoxycytidine. Whereas ac^4C is N(3) deshielded, f^5C shows a reinforcement of the electronic density at this position, indicating greater H-bond acceptor character for f^5C and less for ac^4C (LaFrancois et al. 1998) (Renaud et al. 1988). Therefore, the modification of the same nucleobase with different chemical substituents, such as acetyl and formyl, modulates the decoding properties of the tRNA from non-cognate to cognate.



f^5C-A^{+}

Figure IV.27. The geometry adopted for base pairing between f^5C and A is probably the same as encountered for A^{+}-C, where the N(1) protonation of adenosine is particularly stabilized by the reinforcement of the H-bond acceptor character through the increase of local N(3) electronic density due to the presence of the C(5) formyl alkyl group.

IV.6. Conclusion

The first concept of "wobbling" developed by F. *Crick* (Crick 1966) for inosine and uridine nucleosides has been several times redefined after the isolation of tRNA modification defective mutants. Nevertheless, the correlation between structure of the nucleosides of the wobble position and the decoding capacities of the corresponding tRNA remains poorly understood (Takai and Yokoyama 2003), especially for the unusual pyrimidines (mnm^5s^2U, mcm^5U, $mchm^5U$, cmo^5U, f^5C, k^2C, $mchm^5U$). Our proposed structural models could give new insights in the comprehension of decoding process and arouse computational investigations that we could unfortunately not perform at this time. Nevertheless, we summarized these decoding properties in *(Table IV.4.)*.

First nucleoside of anticodon	Base pairing ability
C, Cm	G and A
k^2C	A
ac^4C	G
f^5C	G and A
U	U, A, G (and C in mitochondria)
xnm^5U	A, G
$mchm^5U$	A, G
xcm^5U	A
xo^5U	U, A, G
cmo^5U	U, A, G and C

Table IV.4. Review of decoding properties

CHAPTER V "Synthesis of a truncated E.coli ^{Ala}tRNA analogue containing dihydrouridine and wyosine"

V.1. Introduction

Although tRNAs obtained by transcription contain no modifications, all structural investigations using thermal melting (Sampson and Uhlenbeck 1988) (Perret et al. 1990) (Derrick and Horowitz 1993), chemical and enzymatic probing in solution (Perret et al. 1990) (Derrick and Horowitz 1993), NMR spectroscopy (Hall et al. 1989) (Chu and Horowitz 1991) (Yue et al. 1994) or X-ray crystallography (Arnez and Steitz 1994) have reported a cloverleaf structure in the presence of magnesium ions. However, this structural tolerance has been recently questioned by the isolation of a tRNA transcript which requires at least one modification, m^1A9, for adopting the canonical cloverleaf structure (Helm et al. 1998) (Helm et al. 1999). Furthermore, in some cases, absence of modified nucleotides led to loss of biological activity by lack of important identity elements for the aminoacyl-tRNA-synthestases (Muramatsu et al. 1988) (Putz et al. 1994) (Ohtsuki et al. 1996). These observations led us to conclude that incorporation of modified nucleosides could not be circumvented for production of optimal suppressor tRNAs.

So far, the preparation of hypermodified tRNAs has been only explored to a very small extent, mainly because of the difficulty to incorporate several modified nucleosides at the same time. Some artificial, but modified suppressor tRNAs have been synthesized through modification of the anticodon loop of natural tRNAs (Wang and Schultz 2005) and two chemical syntheses of hypermodified ^{Ala}tRNAs have been reported. A first rarely known, synthesis of the *yeast* ^{Ala}tRNA achieved by Wang and coworkers in 1984 (Wang 1984), and a second synthesis of the *E.coli* ^{Ala}tRNA *(Figure V.1.)* accomplished in one piece 8 years later (Gasparutto et al. 1992). Since then, no more attempts have been reported. The characterization of the product was carried out by gel-electrophoresis and by a translation assay, which revealed some biological activity. By considering the chemistry employed in this pioneering study, we can conclude that the product was formed in very small yield and that a full purification was impossible. Nowadays, better synthetic methods, leading to much more

full-length sequence and less side-product formation, and better analysis tools, such as LC-ESI MS are available. Nevertheless, the isolation of pure, synthetically prepared 76mer RNA sequences is still impossible, since even the best HPLC columns and conditions are not powerful enough to remove minor side-products, which accumulate in longer sequences. Futhermore, the fragility of most of the modifications requires an adaptation of the standard coupling and deprotection conditions, leading to additional problems. However, shorter RNA sequences, up to 50mers, can be efficiently prepared and purified by HPLC and then combined by general enzymatic ligation methods. The quality of the products can finally be analyzed by mass spectrometry. A successful example of this strategy has been presented for the preparation of the short wyosine-containing RNA sequence **S3** (Chapter III). We then investigated the synthesis of a modified tRNA, derived from *E.coli* ^{Ala}tRNA, containing a dihydrouridine at position 17, a wyosine at position 37 and a CUA anticodon, complementary to the amber STOP codon UAG *(Figure V.2.)*.

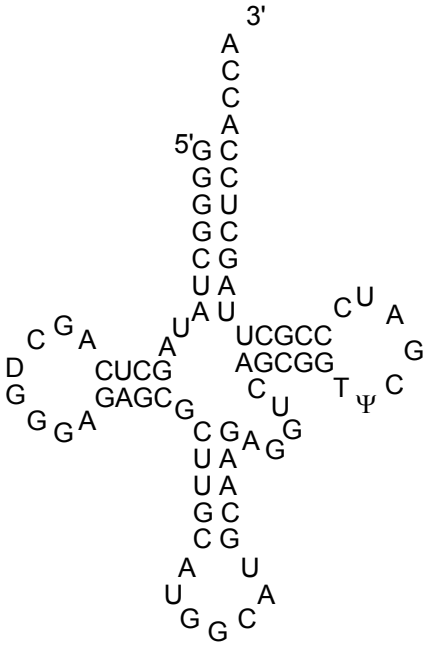


Figure V.1. Structure of the first tRNA fully chemically prepared (^{Ala}tRNA of E.coli) (**S24**) (Gasparutto et al. 1992).

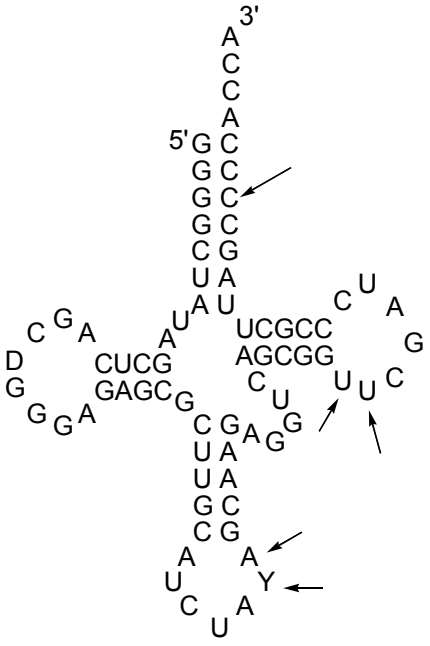


Figure V.2. Structure of the tRNA analogue **S25** planned to be prepared for evaluating different synthetic methodologies (sequence derived from E.coli ^{Ala}tRNA). The arrows show the deviations from the natural structure (Y for wyosine).

V.2. Preparation of the artificial truncated tRNA

V.2.1. Retrosynthesis

The retrosynthetic approach led to three distinctive RNA fragments consisting of 8, 32 and 36 nucleotides *(Figure V.3.)*. The T4-DNA ligase mediated ligation of the unmodified 68mer sequence **S26** with the aminoacylated 8mer sequence **S27** was optimized by *C. Denarie* in our group (Denarie 2006) and should also efficiently work with modified analogues. Therefore, we investigated here the ligation of the two 32mer (**S29**) and 36mer (**S28**) fragments by blunt-end ligation with T4-RNA ligase according to (Ohtsuka et al. 1981) or with T4-DNA ligase in the presence of a suitable template according to Chapter III.

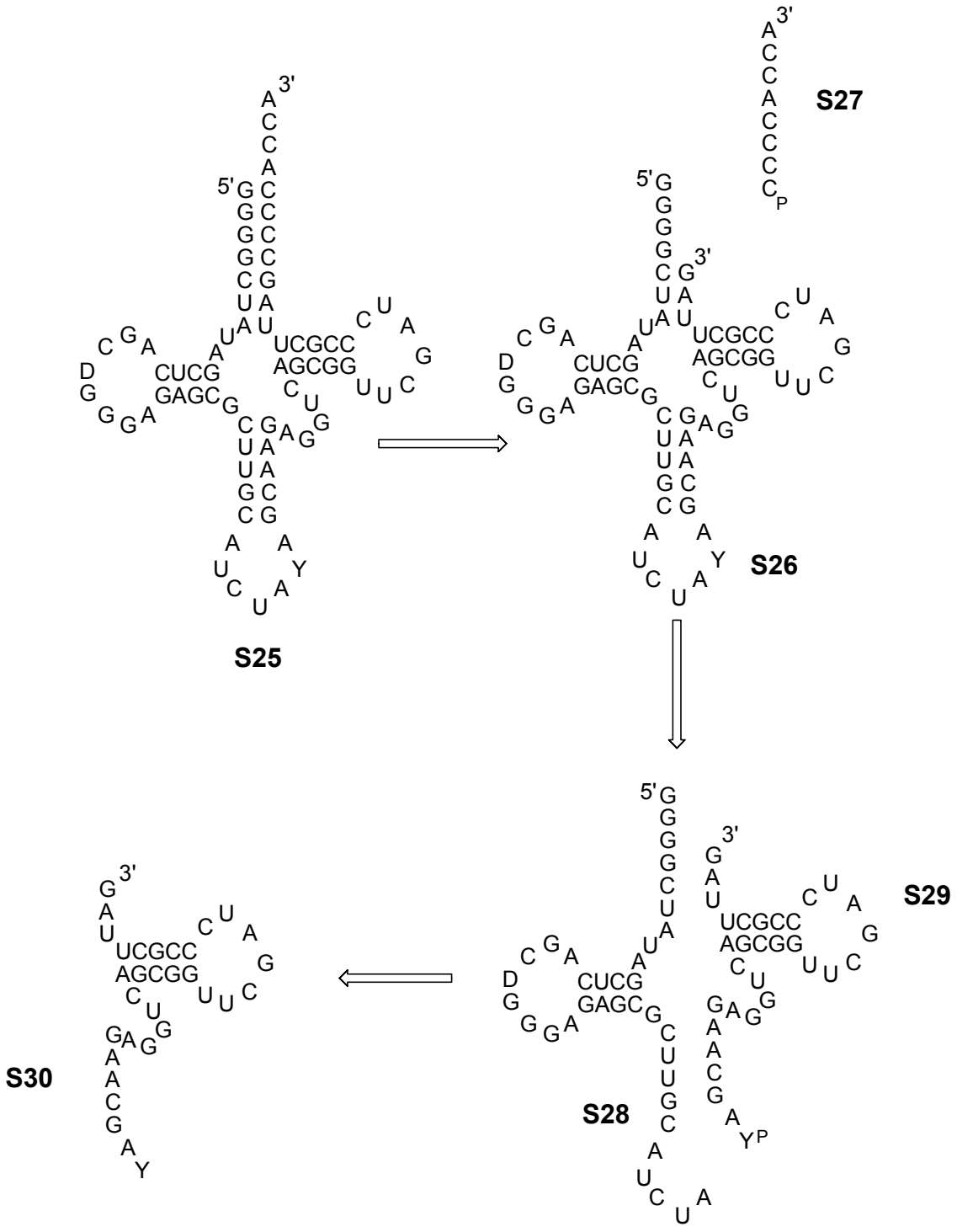
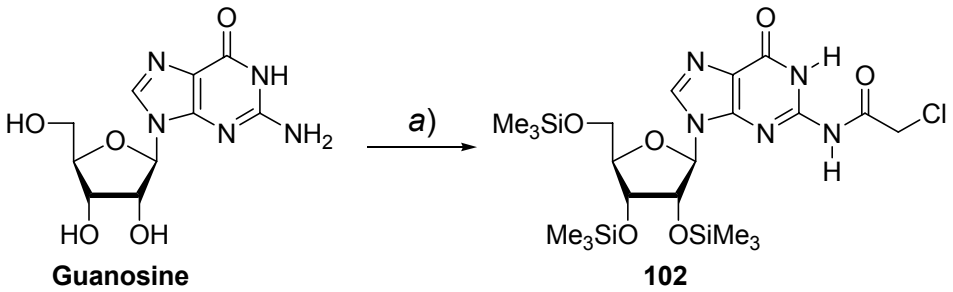


Figure V.3. Retrosynthetic approach for the preparation of the tRNA analogue.

V.2.2. Synthesis of the N^2-methoxyacetyl protected guanosine phosphoramidite

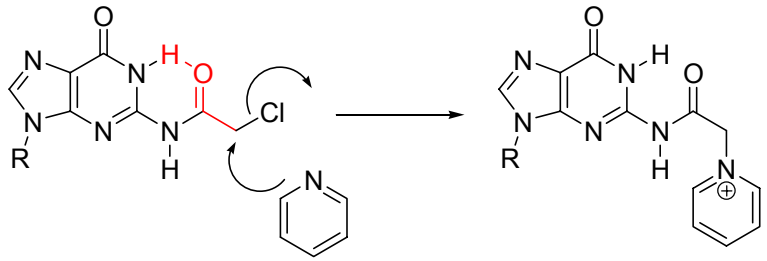
The introduction of dihydrouridine in the 36mer sequence **S28** and wyosine in the 32mer sequence **S30** required mild deprotection conditions and therefore, in analogy to the preparation of the wyosine-containing RNA sequence **S6** (Chapter III), we first employed the

NH_2-C(2) unprotected guanosine phosphoramidite **108** for carrying out the deprotection with NH_3 in MeOH. However, this building block was not compatible with the assembly of the G-tetrade present at the 5'-end of the dihydrouridine containing 36mer sequence **S28**. A more systematic study revealed that the number of consecutively incorporated NH_2-C(2) unprotected guanosines with this building block could not exceed two. In order to overcome this severe limitation, we then investigated new guanosine protecting groups, which can be cleaved under mild conditions, such as NH_3 in methanol. A variety of such protecting groups have been reported (Iyer 2000). Among those, acyl-type protecting groups are preferred, since they show no side-reactions during assembly. We first attempted to introduce the chloroacetyl protecting group into guanosine, since it has been successfully employed for incorporation of 1-methyladenosine (Mikhailov et al. 2002) (Chapter I) *(Scheme V.1.)*. By carrying out the reaction according to the transient silylation strategy (Fan et al. 2004) only a white precipitate was formed, which later was identified by MS analysis as the product of chloride substitution by pyridine *(Scheme V.2.)*.



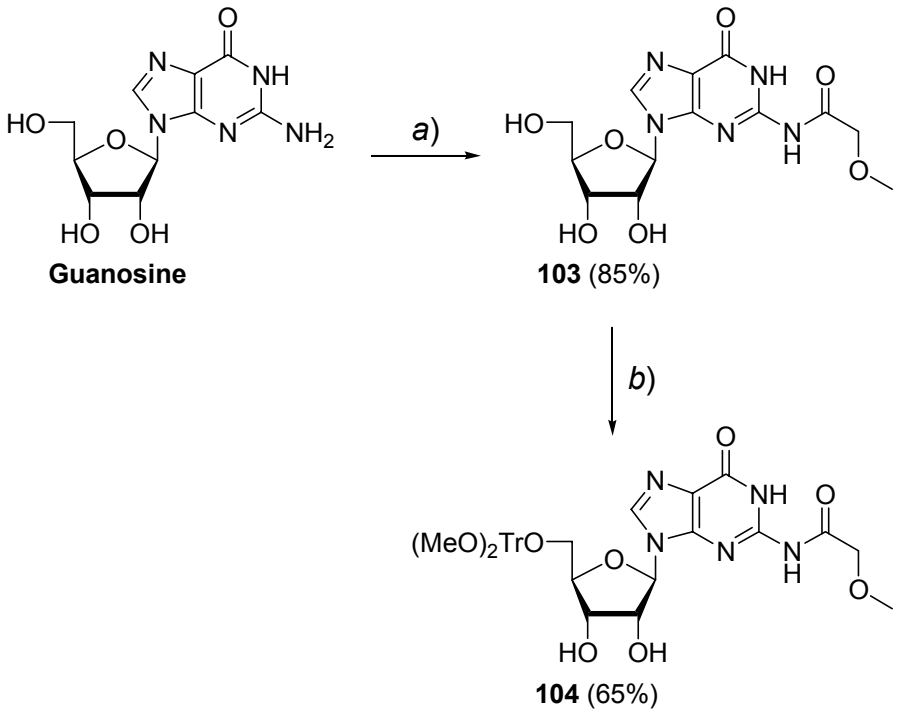
Scheme V.1. Attempted preparation of N^2-chloroacetylguanosine. *a*) Me_3SiCl, pyridine, 4°; then AcCl, DMAP (= *N*,*N*-dimethylpyridin-4-amine), pyridine, 20°.

This difference of reactivity between **102** and the corresponding 1-methyladenosine derivative **8** (Chapter I) could be explained by the formation of an intramolecular hydrogen bond enhancing the local nucleofuge character of the chlorine (*Scheme V.2*).



Scheme V.2. Side product formation during preparation of N^2-chloroacetyl guanosine: supposed structure and mechanism.

In a second attempt, we tried the methoxyacetyl protecting group (MAC) which is known to be labile under mild conditions (Schulhof et al. 1987). Furthermore, it had been already successfully employed for the preparation of p-DNA in our group (Ackermann and Pitsch 2002). The 5'-*O*-DMT, N^2-MAC protected guanosine derivative **104** was prepared according to standard conditions *(Scheme V.3.)*, but unfortunately, it was not possible to introduce the 2'-*O*-TOM group under a variety of conditions. The same behaviour was known from the corresponding N^2-*tert*-butylphenoxyacetyl (PAC)-protected guanosine. According to NMR analyses, both (MAC and PAC) derivatives adopt an anti-conformation, whereas the related N^2-acetyl protected guanosine, which is easily transformed into its 2'-*O*-TOM derivative, adopts a syn-conformation. The same dependance of reactivity on conformation was observed for the wyosine and 4-desmethyl-5-methyl wyosine nucleosides (Chapter III).

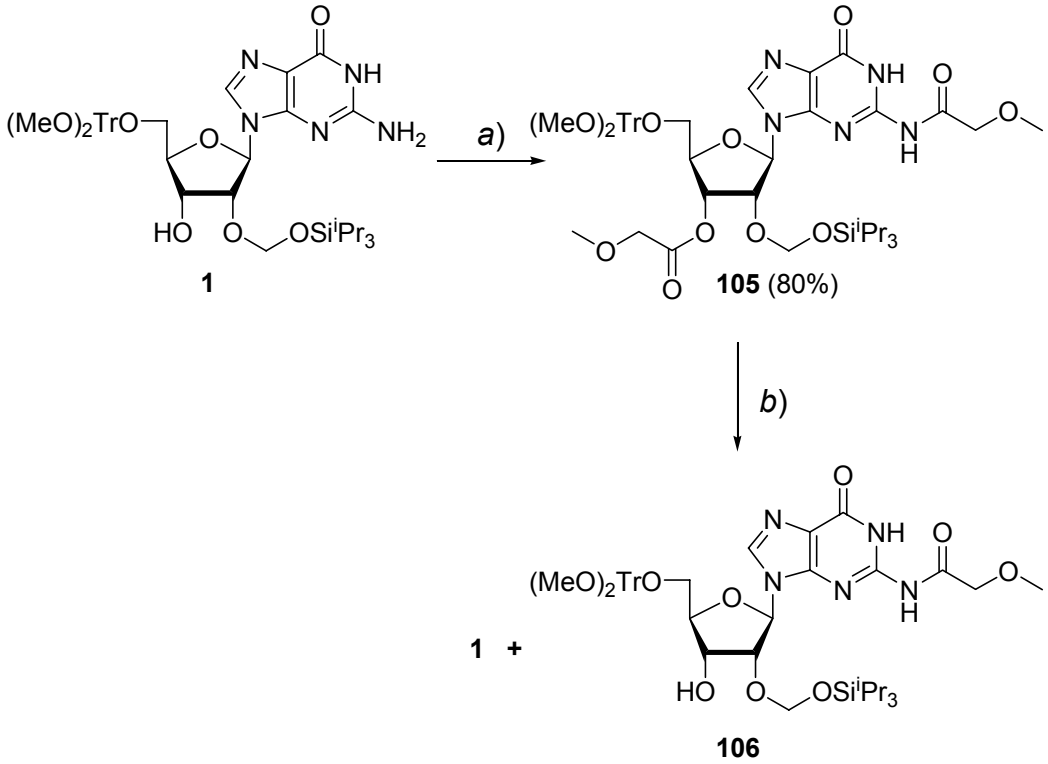


Scheme V.3. Preparation of N^2-methoxyacetyl guanosine. *a*) 1. Me_3SiCl, pyridine, 4°; then methoxyacetyl chloride, DMAP, pyridine, 4°; 2. AcOH, MeOH, 20°; 3. $(MeO)_2TrCl$, pyridine, 20°.

In order to confirm the cleavage properties of the methoxyacetyl-group under mild deprotection conditions, we incubated the derivative **104** in a 12M methanolic solution of NH_3 and found a half-life of 36 min ($k = 0.019\ min^{-1}$), which is acceptable for the preparation of oligonucleotides. It was therefore decided to keep this protecting group and to develop a synthesis of the corresponding phosphoramidite building block. Since the introduction of the 2'-*O*-TOM group was not possible after the introduction of the N^2-MAC group, the reverse order of reactions was investigated.

Acylation of the 5'-*O*-DMT, 2'-*O*-TOM protected guanosine **1** with MAC-Cl in Py gave a complex mixture of products and this approach was abandoned. It is well-known that the acylation of the N^2-position of guanosines is facilited by a transient silylation of the O-C(6) position and in the next attempts, this strategy was succesfully employed. The remaining problem was the choice of protecting group for the 3'-*O*-position of the sugar moiety. Even under the mildest conditions (NH_3 in MeOH, –15°), a 3'-*O*-MAC group could not be

selectively removed in the presence of a N^2-MAC group and it was not possible to introduce a trifluoroacetyl group at the 3'-*O*-position *(Scheme V.4.)*.



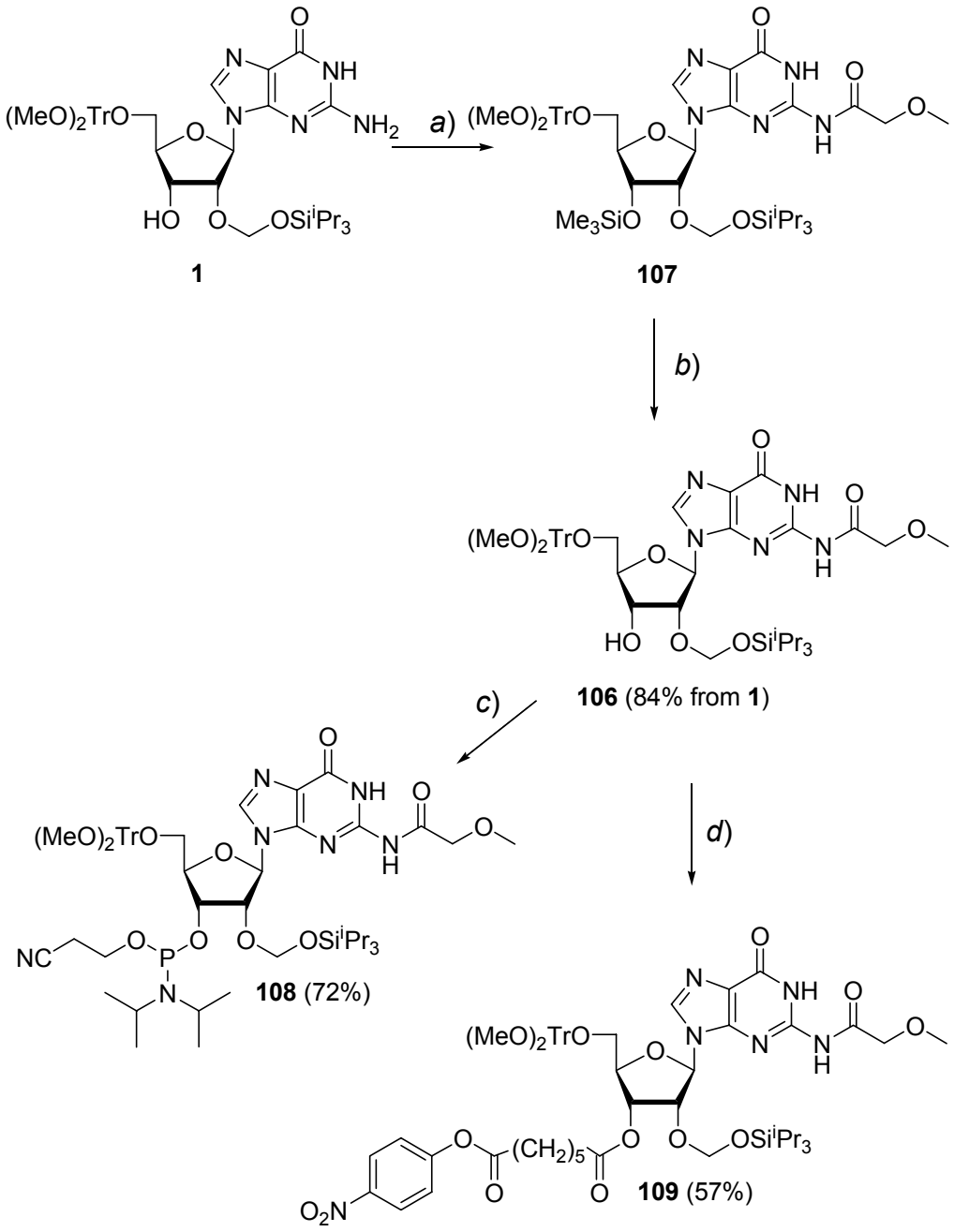
Scheme V.4. Approach for the preparation of 5'-*O*-DMT, 2'-*O*-TOM, N^2-methoxyacetyl guanosine. *a*) 1. Methoxyacetyl chloride, DMAP, pyridine, 4°; then, Me_3SiCl, pyridine, 4°; then methoxyacetyl chloride, pyridine, 4°. *b*) NH_3, MeOH, -15°. **1** was prepared according to (Stutz et al. 2000).

Another possibility was a 3'-*O*-trimethylsilyl protecting group, which was first not considered for compatibility arguments, since the conditions for its removal seemed not to be compatible with the other protecting groups present (5'-*O*-DMT: labile towards acid, 2'-*O*-TOM: labile towards fluoride, N^2-MAC: labile towards base). However, the 3'-*O*-$SiMe_3$, N^2-MAC protected guanosine **107** *(Scheme V.5)* could be efficiently prepared in a one-pot procedure, by first silylating **1** with Me_3Si-Cl in Py, followed by selective *N*-acylation of the O-C(3') and O-C(6)-silylated intermediate with MAC-Cl. A variety of conditions were then investigated to selectively cleave the remaining 3'-*O*-$SiMe_3$ group *(Table V.1.)*.

The recently reported "neutral" conditions with NH_4Cl (Shaabani et al. 2002) gave no silyl cleavage at 25° but full detritylation at higher temperature (80°). We investigated conditions using acidic media but relying on mild acids such as citric acid or acetic acid. We obtained finally selective cleavage of the 3'-*O*-silyl-group with acetic acid (under various conditions: acetone: H_2O 1:1 AcOH pH 5 or AcOH:MeCN:H_2O 1:50:50 at 25°) without affecting the dimethoxytrityl group. By combining these methods, the N^2-MAC protected guanosine **106** could be prepared in 84% yield from **1** and was subsequently transformed into the corresponding phosphoramidite building block **108** *(Scheme V.5.)* Meanwhile, this new building block has been employed at several occasions and was, as an example, used for the successful preparation of a RNA sequence containing an uridine modified with a photocleavable group (Wenter et al. 2006).

Conditions	**Temp./Time**	**Observations** (TLC)
AcOH:MeOH 1:9	r.t./ 30 min	5 % of detritylation
20 % citric acid in MeOH	r.t./ 30 min	90 % of detritylation
Acetone:H_2O 1:1 AcOH pH 5	r.t./ 30 min	Starting Material
	r.t./ 48 h	Product and 1% detritylaion
0.6M NH_4Cl in MeCN:H_2O 7:3	r.t./ 5 h	Starting material
	rx / 30min	100 % of detritylation
AcOH:MeCN:H_2O 1:50:50	r.t./ 5 h	Product and 1% detritylaion

Table V.1. Conditions investigated for selective removal of the 3'-*O*-TMS group of **107**.



Scheme V.5. Preparation of 5'-*O*-DMT, 2'-*O*-TOM, N^2-methoxyacetyl guanosine phosphoramidite and of its activated ester related building block. *a*) 1. Me_3SiCl, pyridine, 4°; then methoxyacetyl chloride, DMAP (= *N*,*N*-dimethylpyridin-4-amine), pyridine, 4°. *b*) AcOH, MeCN, H_2O, 20°. *c*) 2-Cyanoethyl diisopropylphosphoramidochloridite, $^iPr_2NEt$, CH_2Cl_2, 20°. *d*) Bis(4-nitrophenyl) heptanedioate, DMAP, pyridine, 20°.

Furthermore, the activated ester building block **109** was prepared (57% yield) and immobilized on CPG *(Scheme V.5.)*. Importantly, the reported synthetic strategy could be extended to the preparation of any N^2-acyl protected guanosine derivative.

V.2.3. Synthesis of the dihydrouridine-containing 36mer RNA sequence S28

The 36mer RNA sequence **S28**, which contains a G-tetrade motive was successfully assembled from the N^2-MAC-guanosine phosphoramidite **108**, but MS analysis revealed a remaining MAC-group even when deprotected under our usual, relatively harsh conditions (methylamine in ethanol and water). Therefore, additional investigations were carried out on a 21mer model sequence, representing a truncated version of the 36mer RNA sequence **S31-S33**. Under standard conditions with NH_3 in MeOH, 13% and 28% of the mono-acylated product remained. Since no such problems were reported for the preparation of the wyosine-containing 32mer sequence **S30**, this phenomenon was attributed to the G-tetrade motif. Desperately, we then investigated combinations of NH_2-C(2) unprotected and N^2-MAC protected guanosine phosphoramidites. Since a successive incorporation of two unprotected building blocks is not possible, only a few combinations remained: $G^{MAC}G^{NH2}G^{MAC}G^{NH2}$-5'p (**S31**), $G^{NH2}G^{MAC}G^{NH2}G^{MAC}$-5'p (**S32**) and $G^{NH2}G^{MAC}G^{MAC}G^{MAC}$-5'p (**S33**). RNA sequences with all these combinations were assembled, deprotected with NH_3 in MeOH and then analyzed by HPLC *(Figure V.4. and Figure V.5.)* and MS. The last combination, with one NH_2-C(2) unprotected guanosine, followed by three N^2-MAC protected guanosines gave the best results in terms of efficient assembly and deprotection *(Figure V.6.)*.

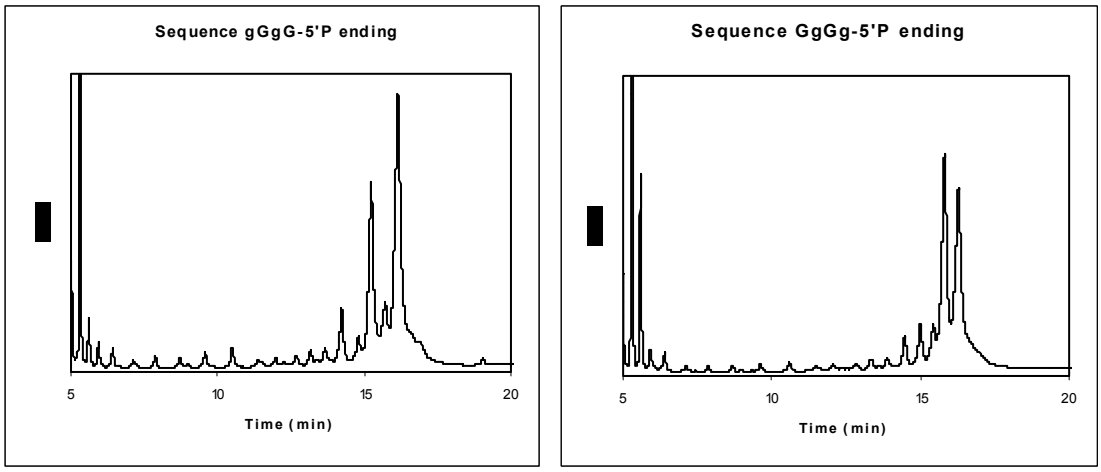


Figure V.5. HPLC traces of the crude products obtained with different G-tetrad motifs ($G^{NH2}G^{MAC}G^{NH2}G^{MAC}$-5'p "**gGgG-5'P**" (**S31**) (*left*), $G^{MAC}G^{NH2}G^{MAC}G^{NH2}$-5'p "**GgGg-5'P**" (**S32**) (*right*)).

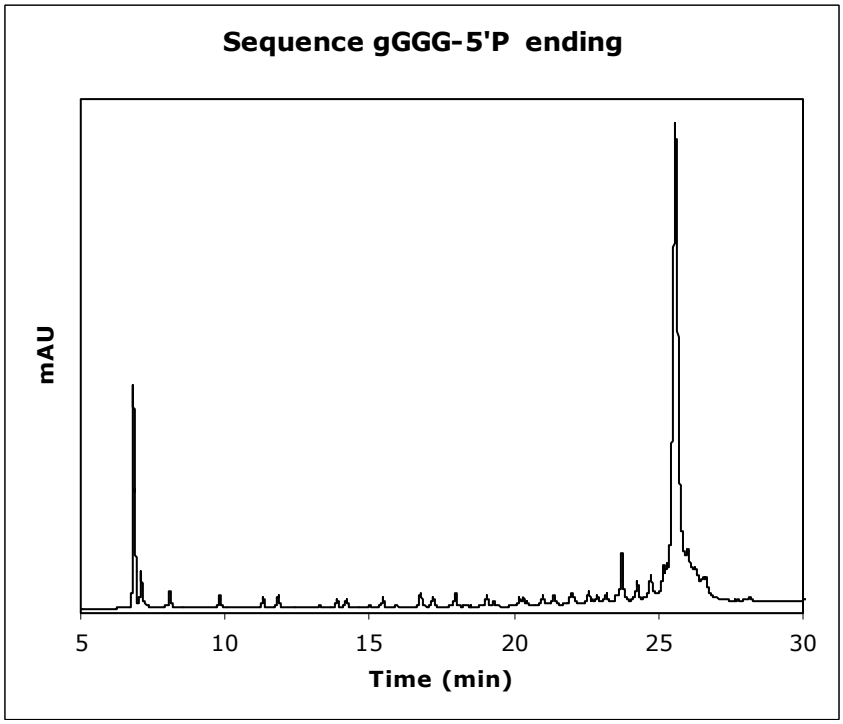


Figure V.6. HPLC trace of the crude product obtained with the optimal combination of guanosine building block $G^{NH2}G^{MAC}G^{MAC}G^{MAC}$-5'p "**gGGG-5'P**" (**S33**).

The optimized conditions developed above were successfully applied to the preparation of the 36mer sequence **S28**, by employing a combination of N^2-MAC protected guanosine and NH_2-C(2) unprotected guanosine phosphoramidites. This latter building block

was introduced at the beginning of the G-tetrad by performing a double coupling cycle. The immobilized sequence was then washed with a 10% solution of diisopropylamine in acetonitrile, deprotected successively with NH_3 in MeOH and 1M TBAF in THF, and then analyzed by HPLC *(Figure V.7.)*. The main product has been isolated and analyzed by ESI-MS *(Figure V.8.)*.

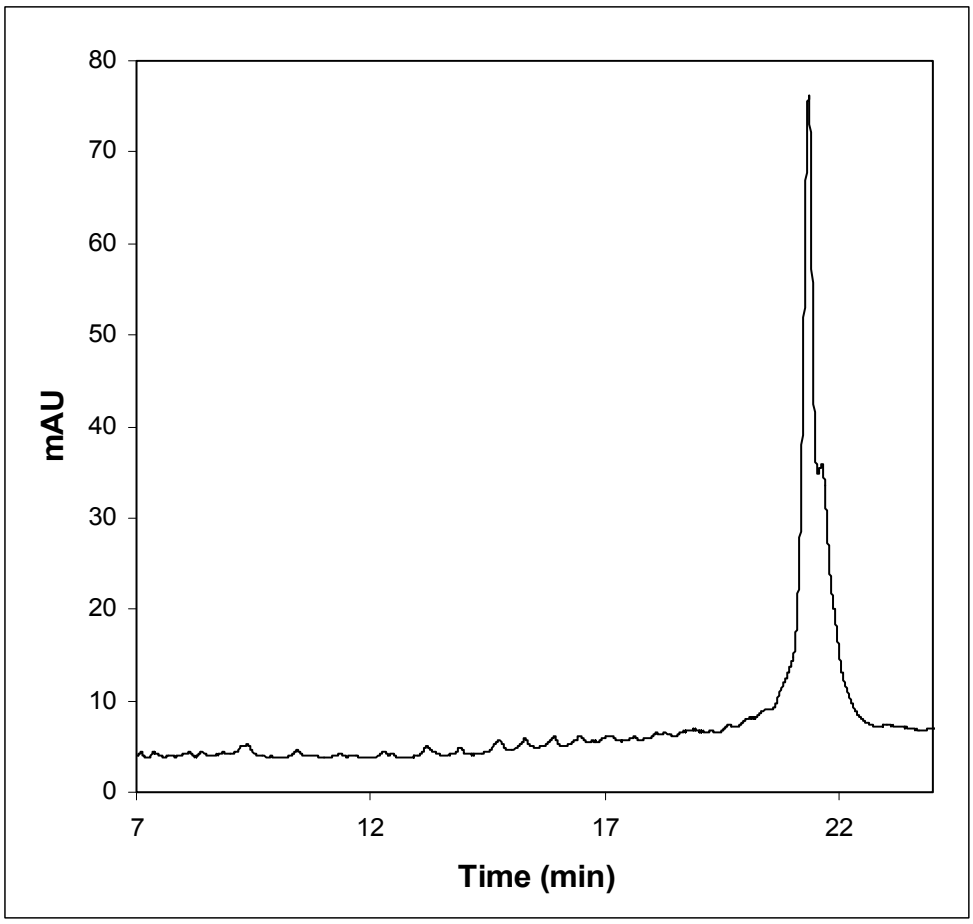


Figure V.7. HPLC trace of the crude product RNA sequence **S28** prepared according the optimized conditions.

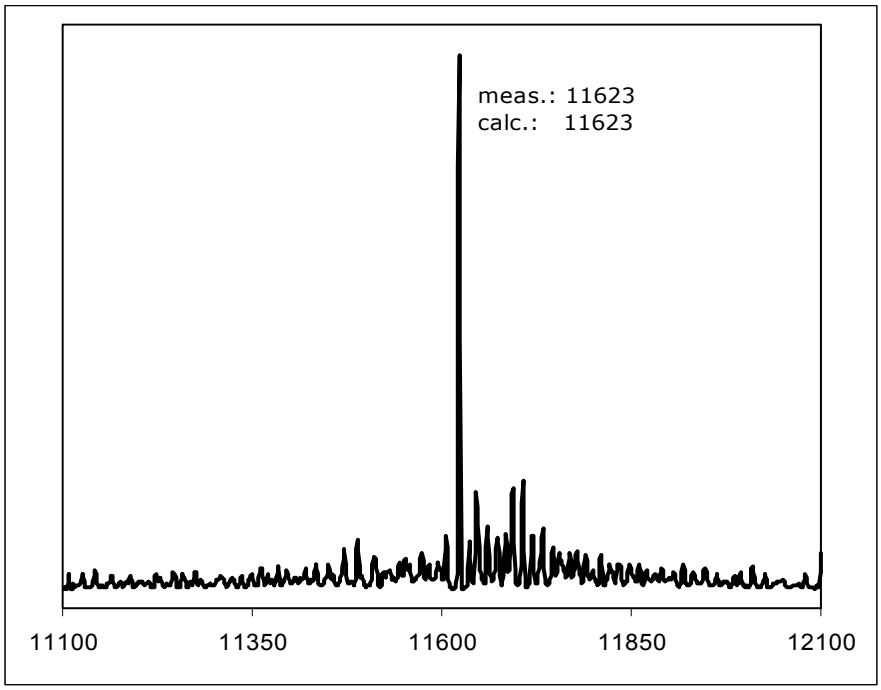


Figure V.8. ESI-MS analysis of the purified sequence **S28**.

V.2.4. Synthesis of the wyosine-containing 32mer RNA sequence S30

The wyosine-containing 32mer RNA sequence **S30** was successfully assembled (HPLC trace and MS analysis, *Figure V.9.* and *V.10.*, respectively) from N^2-MAC-guanosine phosphoramidite **108** according to the methodology developed for the preparation of the wyosine-containing 10mer RNA sequence (Chapter III).

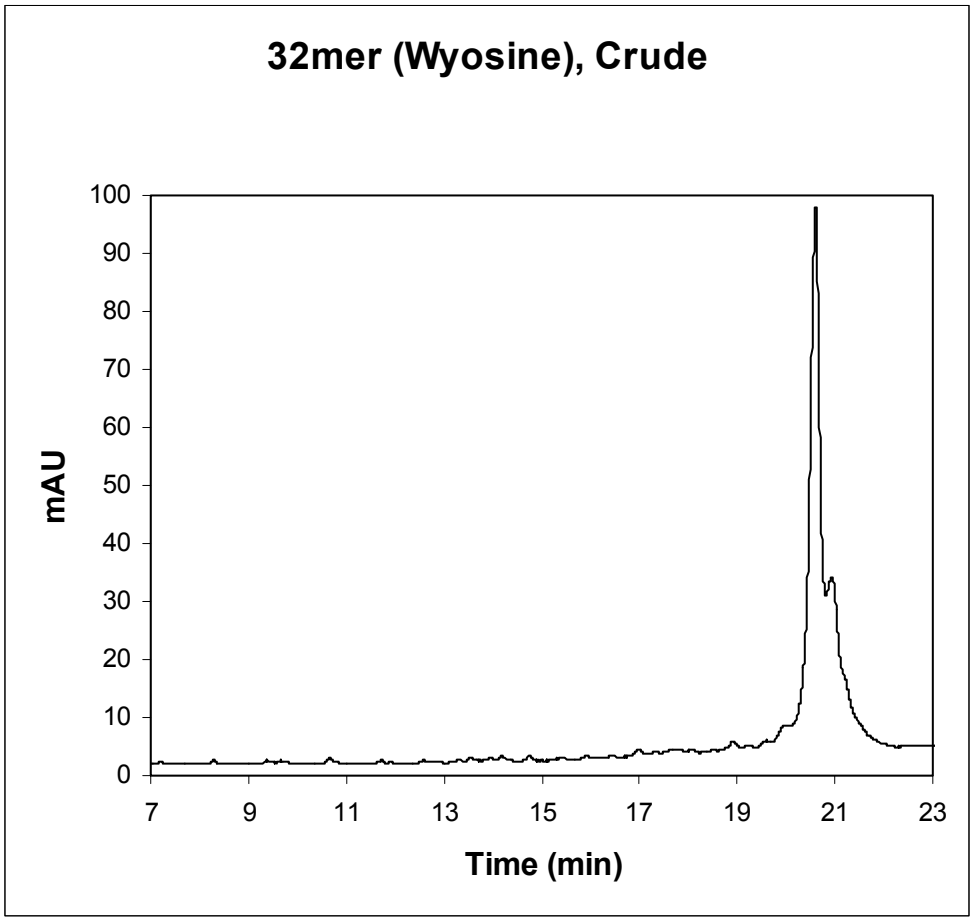


Figure V.9. HPLC trace of the crude product RNA sequence **S30** prepared according the optimized conditions.

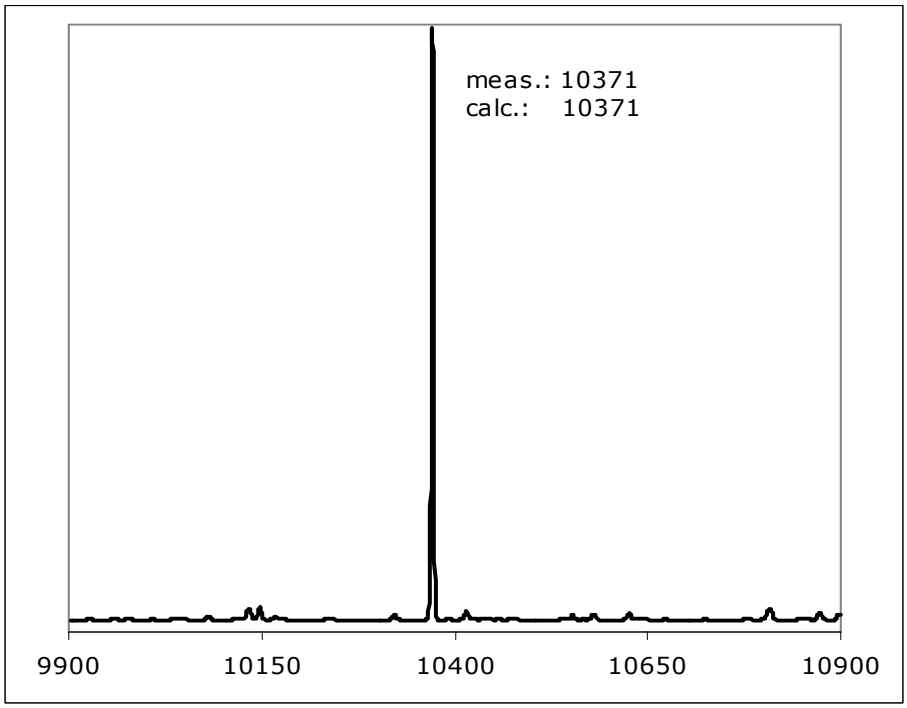


Figure V.10. ESI-MS analysis of the purified sequence **S30**.

V.2.5. Ligation of fragments S28 and S29

The ligation of the two 32mer and 36mer fragments **S29** and **S28** could be carried out or by blunt-end ligation with T4 RNA ligase or by template-assisted ligation with T4 DNA ligase. The first, T4 RNA ligase based approach has been already explored for the preparation of tRNA analogues (Ohtsuka et al. 1981).

The 5'-*O*-phosphorylated, wyosine-containing sequence **S29** was obtained by incubating the purified sequence **30** with ATP and T4 PNK, followed by HPLC purification to remove the enzyme. A 1:1 mixture of the two fragments **S29** and **S28** (final concentration = 20 μM) was heated to 95° and cooled slowly to 4°. Then, the reaction buffer, ATP and the enzyme were added. The ligation reaction was monitored by HPLC *(Figure V.11 and V.12).* Already after a reaction time of 15min at 4°, the 68mer product sequence **S26** was formed efficiently and isolated by HPLC (80% yield).

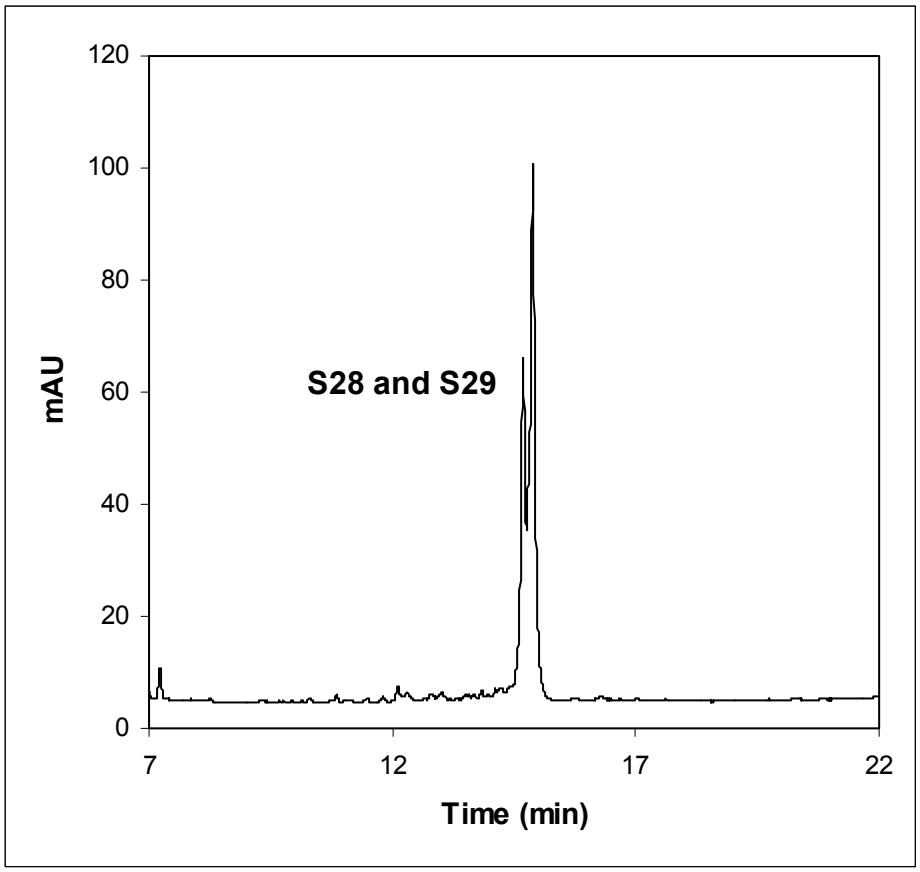


Figure V.11. HPLC trace of the starting materials **S28** + **S29** (before addition of T4 RNA ligase).

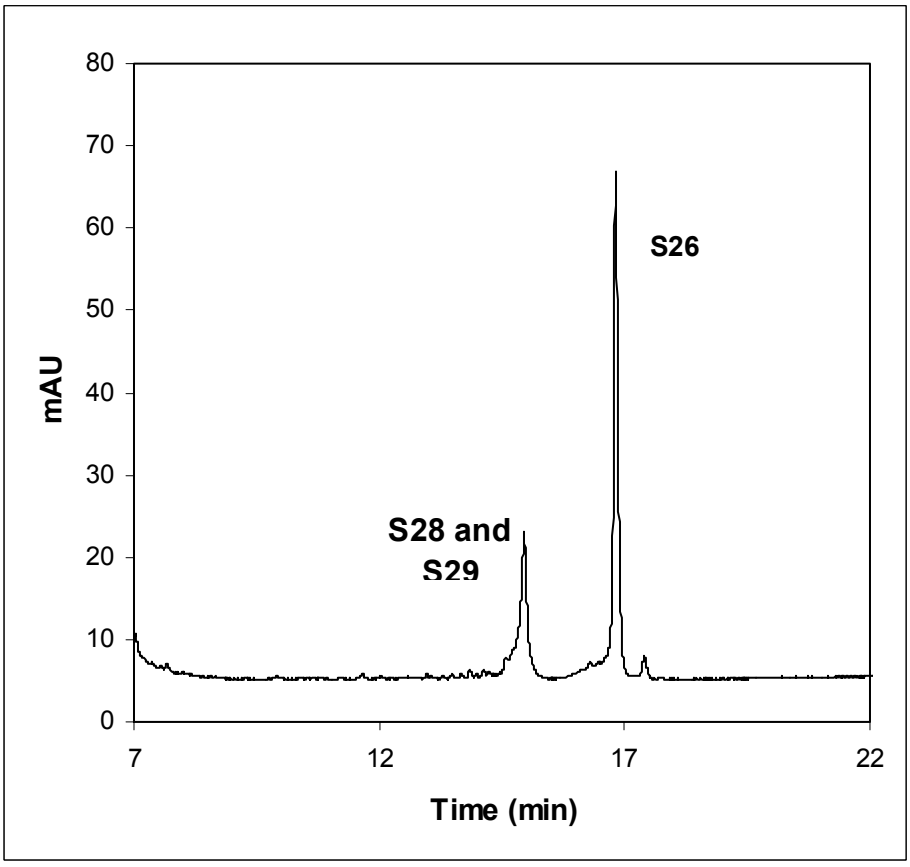


Figure V.12. HPLC trace of the reaction mixture 15min after addition of T4 RNA ligase to **S28** and **S29**.

MS analysis of the isolated product showed a small peak corresponding to the mass of the product sequence **S26** and a main peak corresponding to (**S26** – wyosine base *(Figure V.17.)*). It is well-known that wyosine depurinates easily (Zhao et al. 2003), and therefore this fragment was most probably formed during ionization. The same process was not detected for the much shorter, wyosine-containing sequences **S3**, **S5** and **S6**, because their ionization is much simpler and can be carried out under milder conditions. A loss of the wyosine-base during the enzymatic ligation could be excluded, since under our HPLC-conditions (85°) at least a partial fragmentation at the apurinic site would have occurred. Later (see below), this 68mer RNA sequence **S26** was also prepared by T4 DNA ligase and the same product was obtained. Therefore, the (unlikely) depurinination by T4 RNA ligase can be completely excluded and detection of the apurinic product can be attributed to a MS artifact.

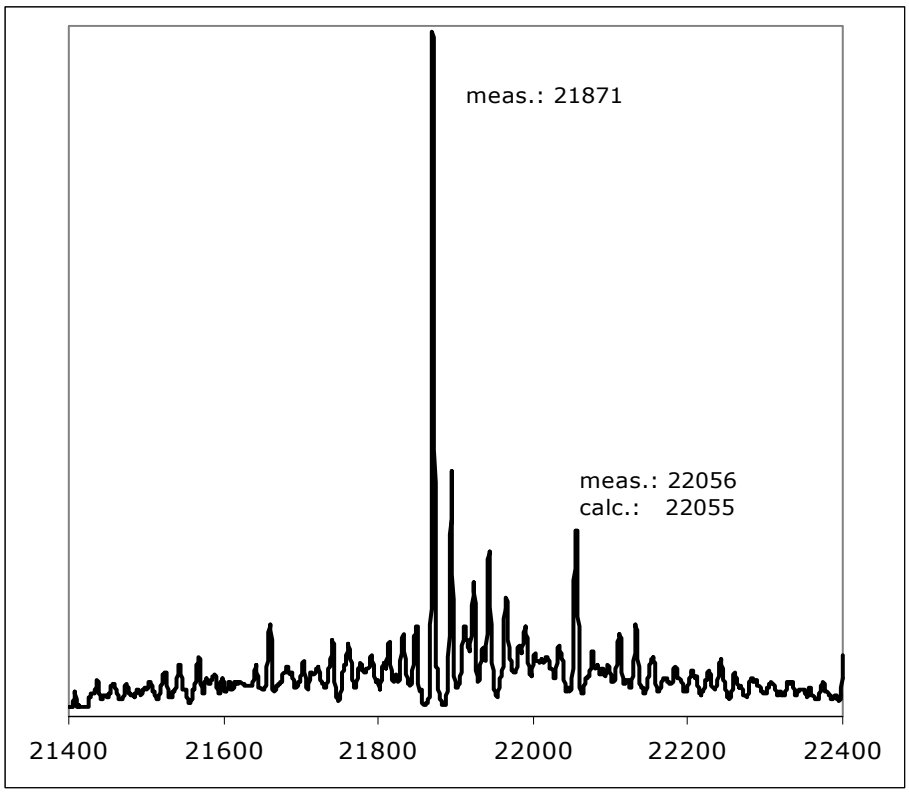


Figure V.17. ESI-MS analysis of the isolated product formed by ligation of **S28** and **S29** by T4 RNA ligase. The main peak (m/z: 21871) corresponds to loss of the wyosine nucleobase, which occurred during MS analysis.

The template-mediated ligation of **S28** and **S29** with T4 DNA ligase was carried out under the same conditions as developed before for the formation of the 18mer RNA sequence **S3** (Chapter III). The template was designed with the help of *F. Meylan* in our group (Meylan 2006) who has shown that 2'-OMe-RNA sequences are often superior templates for such ligation reactions. This design was also used for the 18mer template **S34**, together with an abasic 1,3-propanediol moiety opposite the wyosine *(Figure V.13.)*.

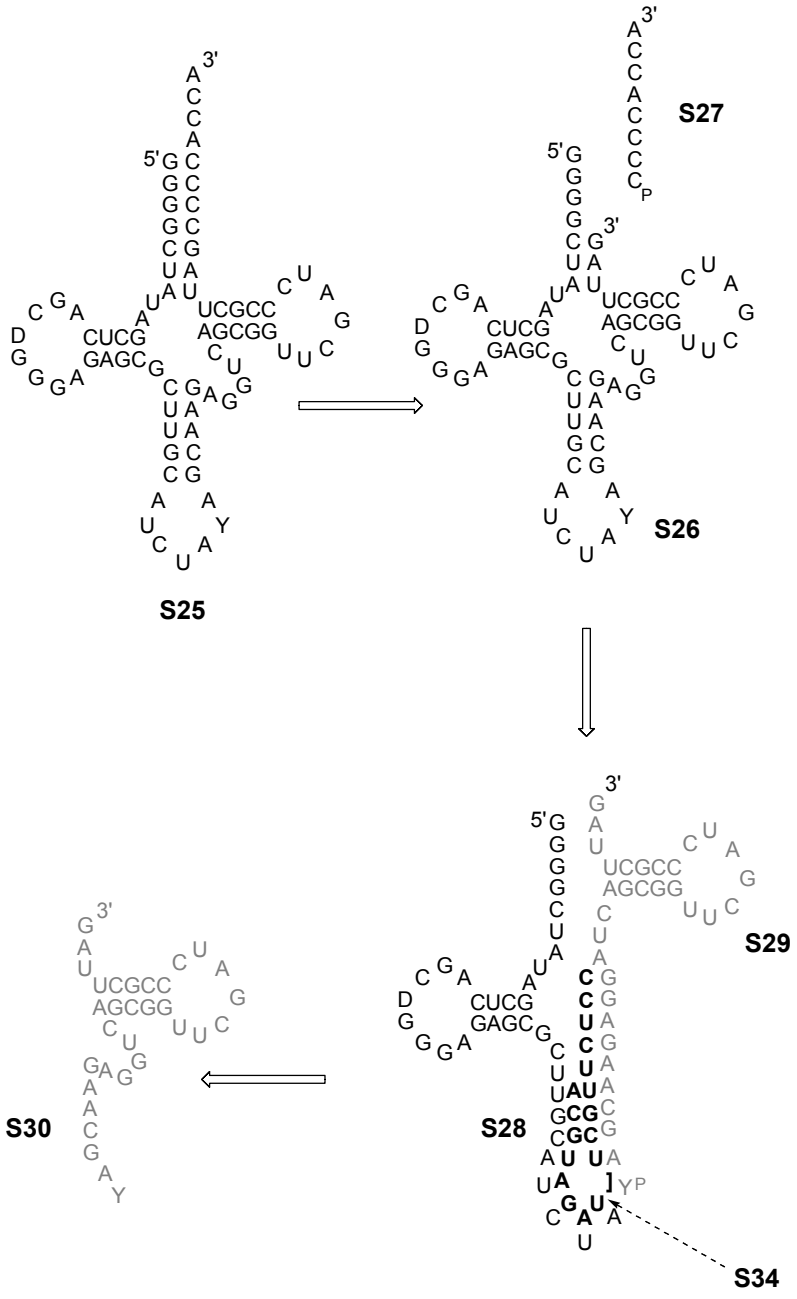


Figure V.13. Design of the 2'-OMe-RNA template used in the template assisted preparation of **S26** with T4 DNA ligase;] stands for a 1,3-propanediol moiety.

The two starting materials **S28** and **S29** were mixed with the template (final concentration 20 + 20 + 30 μM), heated to 95° and slowly cooled to 37°. Then the ATP and the enzyme were added and the course of the reaction was monitored by HPLC *(Figure V.14.* and *Figure V.15.*). Product formation **S26** reached a plateau after 60h at 37°C with around 80% conversion *(Figure V.16.)*. The product of this ligation reaction was coinjection-identical to the product obtained with T4 RNA ligase.

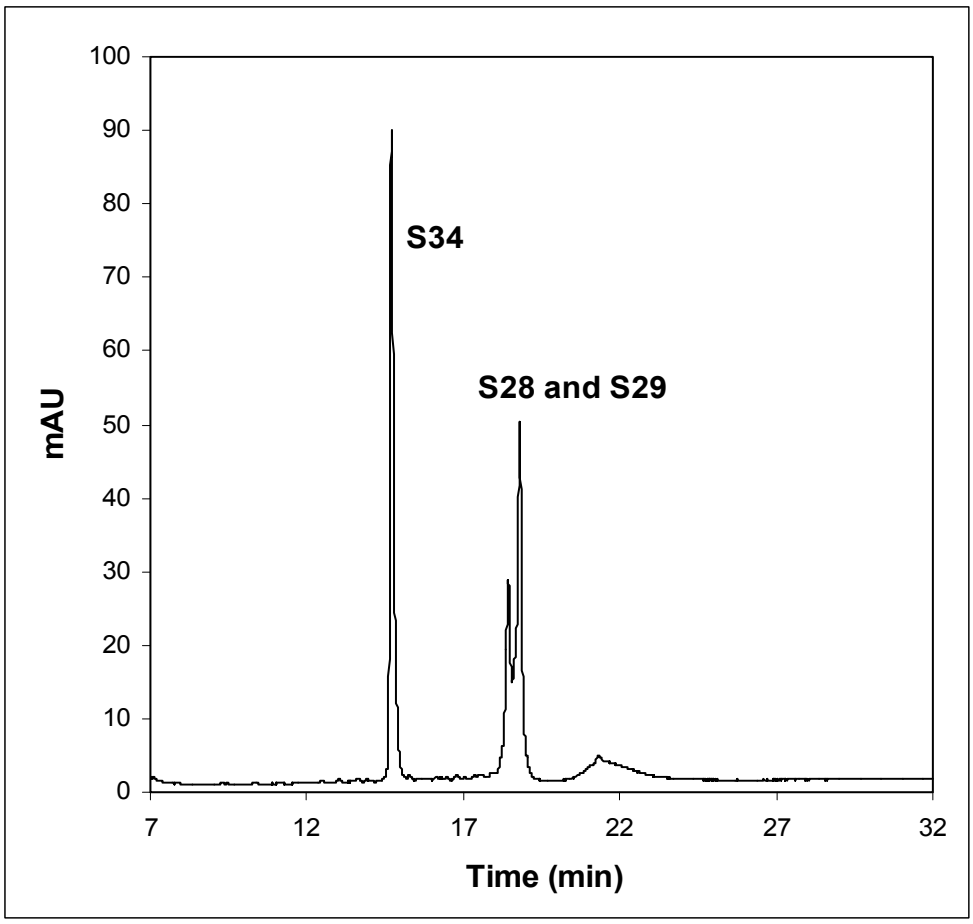


Figure V.14. HPLC trace of the starting materials **S28** + **S29** and the template **S34** subjected to template-mediated ligation with T4 DNA ligase (immediately after addition of enzyme).

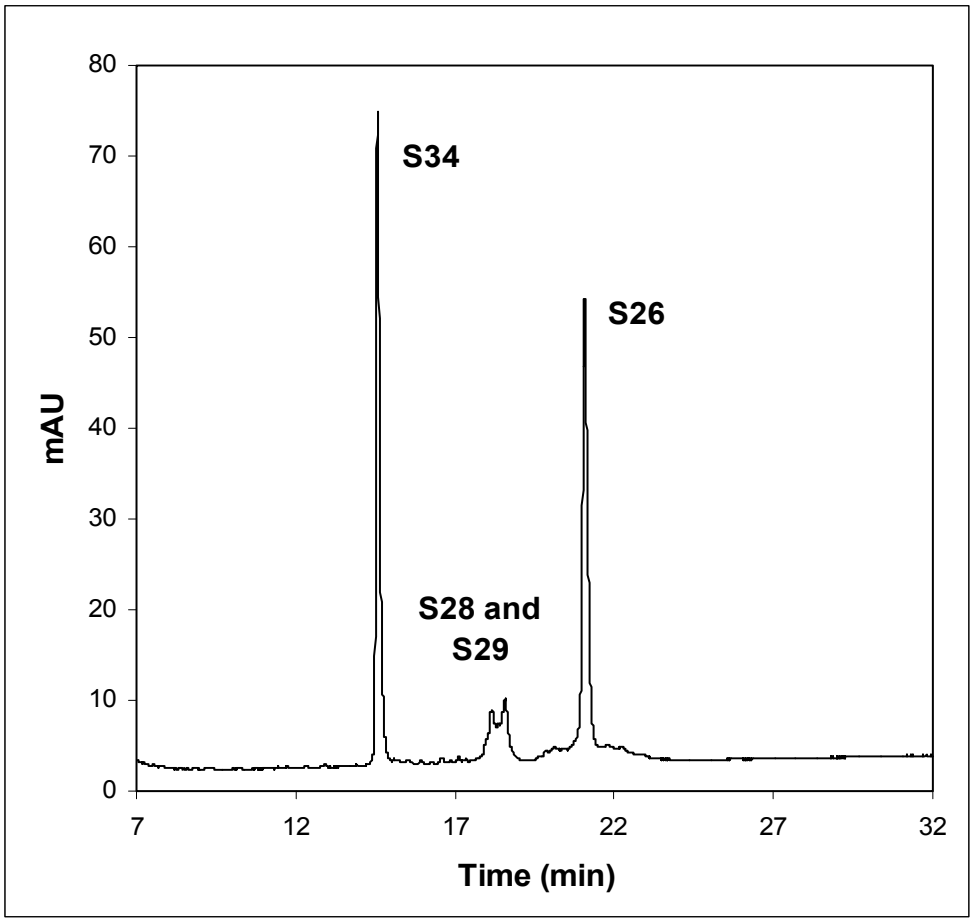


Figure V.15. HPLC trace of the reaction mixture obtained 57 hours after addition of T4 DNA ligase to the mixture of **S28**, **S29** and the template **S34**.

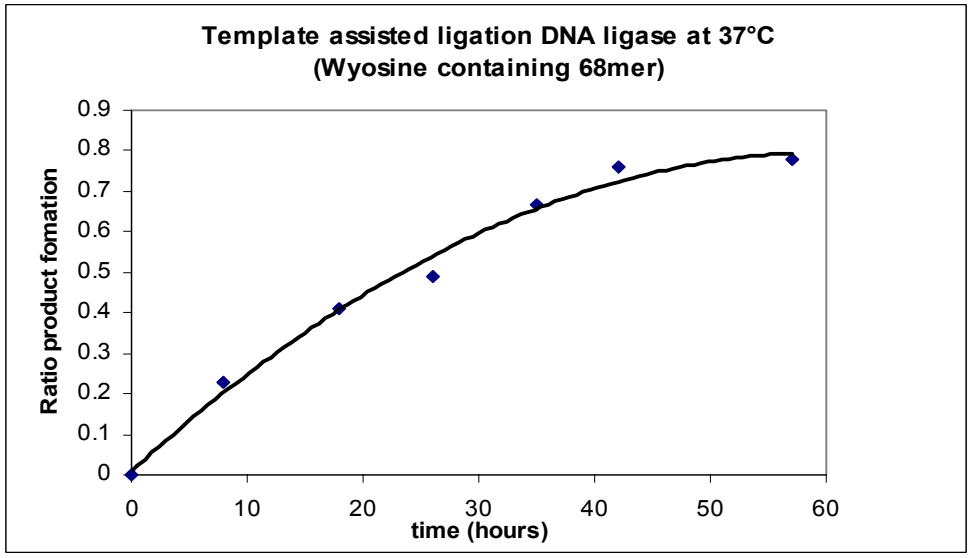


Figure V.16. Time-course of the template-mediated ligation of **S28** and **S29** with the template **S34** and T4 DNA ligase.

V.3. Conclusion

Our methods for the synthesis of modified ribonucleoside phosphoramidites, their incorporation into RNA sequences and their ligation were successfully employed for the synthesis of a truncated tRNA analogue containing two of the most fragile nucleotides (dihydrouridine and wyosine). Specifically, a mild deprotection protocol based on the N^2-MAC protected guanosine phosphoramidite and and efficient ligation with T4 RNA and DNA ligase was developed. With these methods, probably almost any modified, synthetic tRNA could be prepared, since most of the modified nucleosides are chemically more stable than dihydrouridine or wyosine, and therefore easier to introduce. Such tRNA analogues, containing one or several modified nucleotides could be employed for structural and functional studies.

CHAPTER VI "Preparation of a thiol-containing RNA sequence and aminoacylation studies"

VI.1. Introduction

There are several techniques for the site-directed incorporation of nonnatural amino acids into proteins. A variety of strategies for the preparation of the required aminoacylated tRNAs have been developed.

A very elegant approach is designed as *in-vivo* process and involves the development of artificial aminoacyl-tRNA-synthetases which recognize the artificial tRNA and connect it to an artificial amino acid (Wang and Schultz 2005) (Hohsaka and Sisido 2002). The first results were obtained with a pair of an amber suppressor tRNATyr and TyrRS from *Methanococcus jamnaschii* orthogonal to any aaRS/tRNA pair of *E. coli* (Wang et al. 2000) (Wang et al. 2001) and has been extended to a variety of non-natural amino-acids such as the aromatic analogues 2-naphtylalanine (Wang et al. 2002), p-azidophenylalanine (Chin et al. 2002a), p-benzoylphenylalanine (Chin et al. 2002b), to spin-labeled, fluorescent, biotin-containing, electrophilic, allylsubstituted, metal ligands, photocaged and glycosylated (Wang, Schultz 2002) aminoacids have been successfully introduced. In this context it has also been recognized that a mutated *E. coli* TyrRS recognizes m-iodotyrosine more efficiently than tyrosine (Kiga et al. 2002) and transfecting agents for importing aminoacylated suppressor tRNAs in mammalian cells have been developed (Kohrer et al. 2001) (Kowal et al. 2001). This method is in principle very elegant but restricts the choice of the amino acid and has to be adapted to almost every new combination of tRNA and amino acid.

The latest approaches involve ribozymes which catalyze the transfer of the amino acid of short aminoacylated RNA-fragments to a tRNA (Heckler et al. 1983), or short peptide nucleic acid (PNA) sequences, which are complementary to the 3'-end of the tRNA, and carry an activated amino acid (connected via a thioester bond and a linker with the PNA), which, after duplex formation, is then transferred to the tRNA (Ninomiya et al. 2004).

The classical and probably still widest used method, however, consists in an T4 RNA ligase mediated ligation of a truncated tRNA, lacking the 3'-terminal, highly conserved pCpA

residues and a partially protected 3'-*O*-aminoacylated pCpA dimer (Heckler et al. 1984) *(Figure VI.1.)*. Several groups contributed to the development of this methodology, especially by improvement of the aminoacylated dimer synthesis. *Chamberlin* (Bain et al. 1991) and *Schultz* (Robertson et al. 1989) showed that the second cytidine could be replaced by a deoxycytidine (dC) and *Hecht* (Lodder et al. 1998) introduced the iodine removable *N*-allyloxycarbonyl protected amino acid. However, as noticed by *Sisido* and *Hohsaka* (Sisido and Hohsaka 1999) (Hohsaka and Sisido 2002), the synthetic requirements for the preparation of these compounds are too difficult for biochemists.

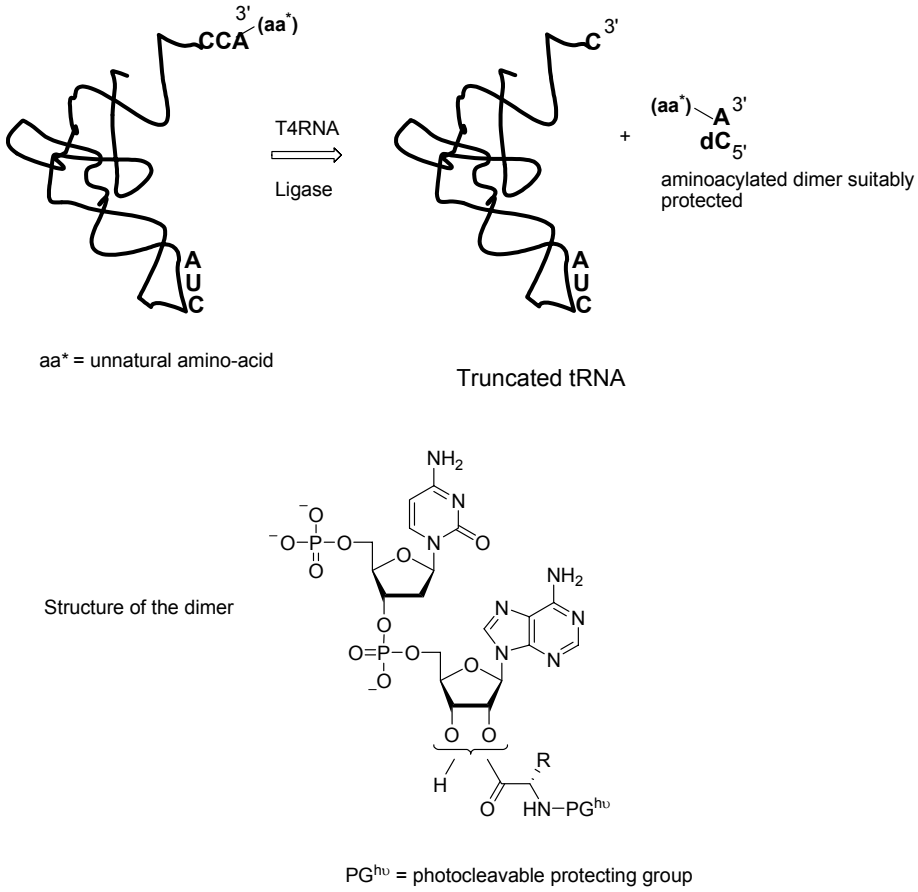


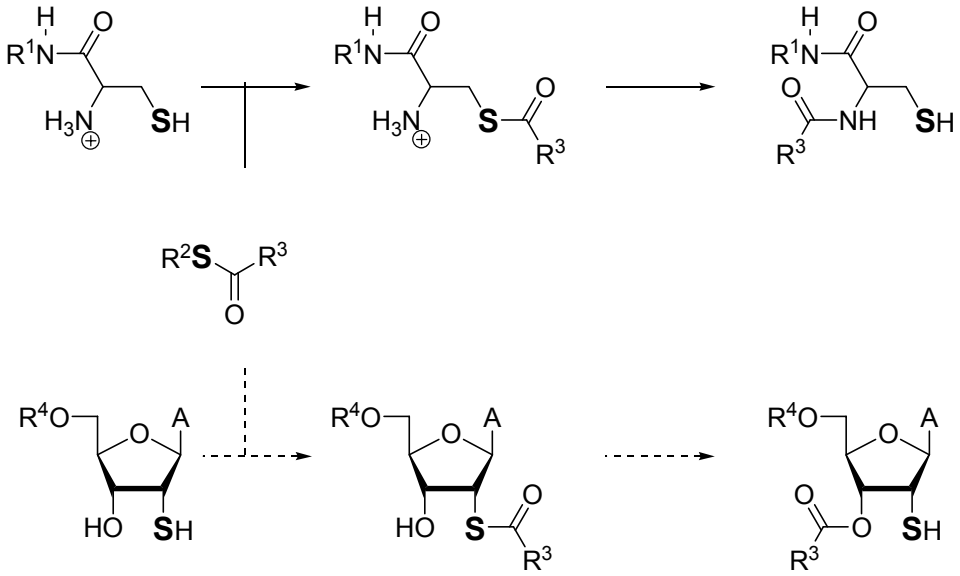
Figure VI.1. Aminoacylated tRNA and the classical synthetic approach

VI.2. A new concept for the preparation of aminoacylated tRNAs

VI.2.1.Concept

In order to facilitate the access to these important and valuable aminoacylated tRNA, we try to prepare and identify biologically active tRNA analogues that are spontaneously aminoacylated by weakly activated amino acids, ideally under the conditions of *in-vitro* translation reactions.

The here presented first concept is related to the so-called "native chemical ligation" where a new amide bond is formed by a fast intermolecular trans-thioesterification reaction between two peptides carrying a *N*-terminal cysteine and a *C*-terminal thioester (Dawson et al. 1994). The analogous process with a 2'-deoxy-2'-thioadenosine derivative and an amino acid thioester would result in the formation of a 3'-*O*-aminoacylated RNA derivative *(Scheme VI.1.)*.



Scheme VI.1. Formation of a amide bond between a cysteine and an amino acid thioester according to the "native chemical ligation" (Dawson et al. 1994) (*top*), as compared to the here presented, analogous formation of an ester bond between a 2'-deoxy-2'-thioadenosine and an amino acid thioester (*bottom*).

In the scope of our retrosynthetic scheme for the preparation of tRNAs (Chapter V), the here reported, exploratory studies were carried out with a 8mer RNA sequence **S27** identical to the 3'-terminal sequence of to $tRNA^{Ala}$ from *E. coli (Figure VI.2.)*.

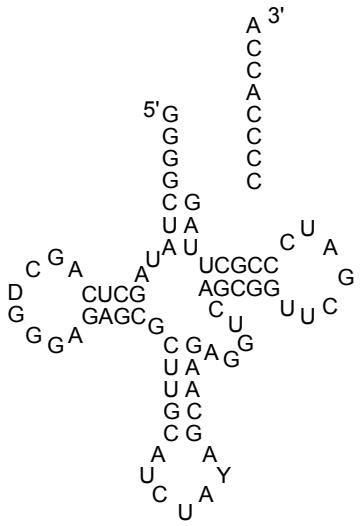


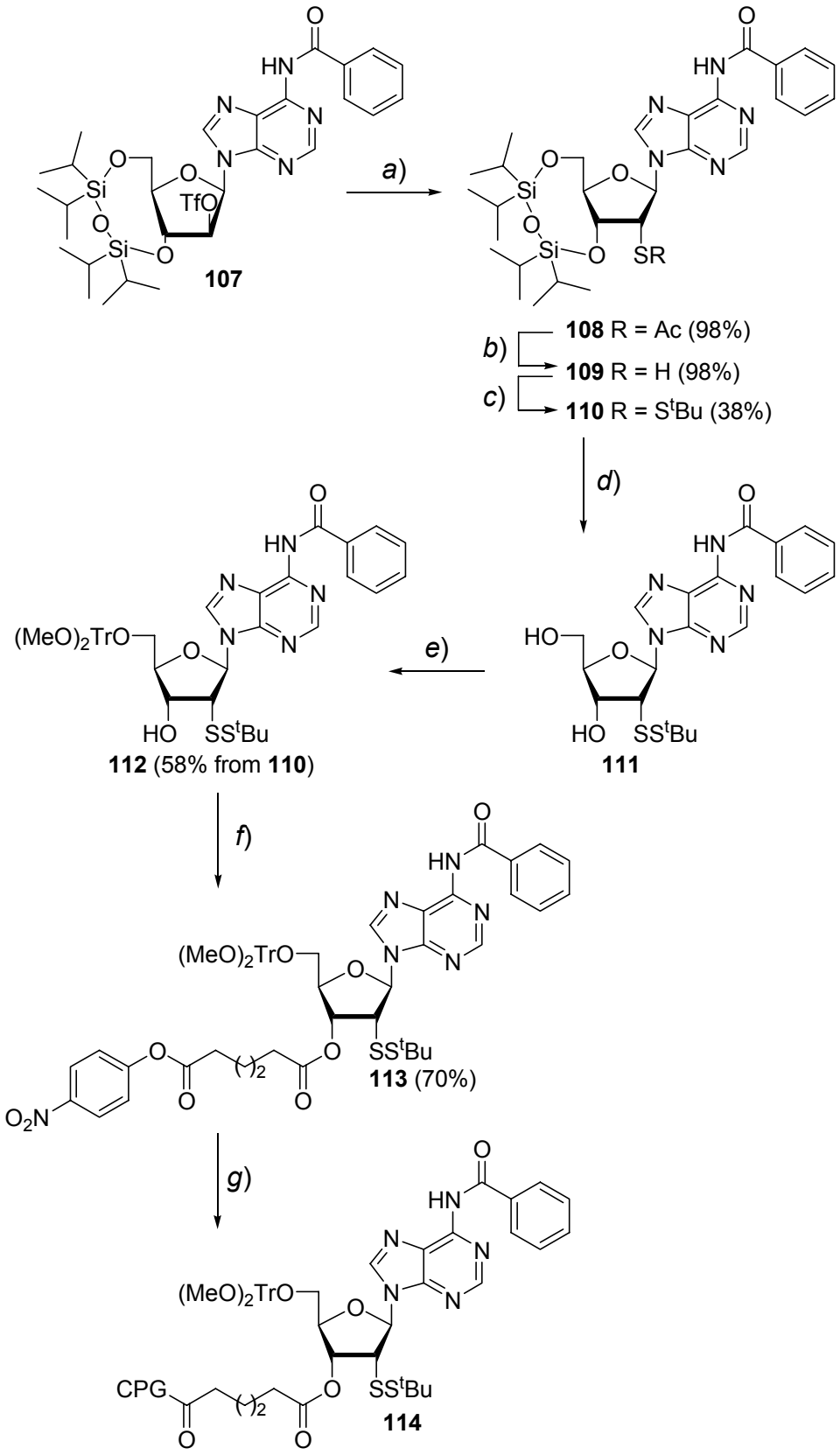
Figure VI.2. 8mer RNA sequence **S27** identical to the 3'-terminal sequence of the *E. coli* $tRNA^{Ala}$.

VI.2.2. First investigations

VI.2.2.1. Synthesis of *tert*-butyl disulfide protected sequence

Introduction of a 3'-terminal 2'-deoxy-2'-thioadenosine required the preparation of a suitably protected and immobilized building blocks, which were prepared from the known, protected arabinonucleoside **107** (Marriott et al. 1991) *(Scheme VI.2.)*. Here, the different methods for preparation of such building blocks are presented.

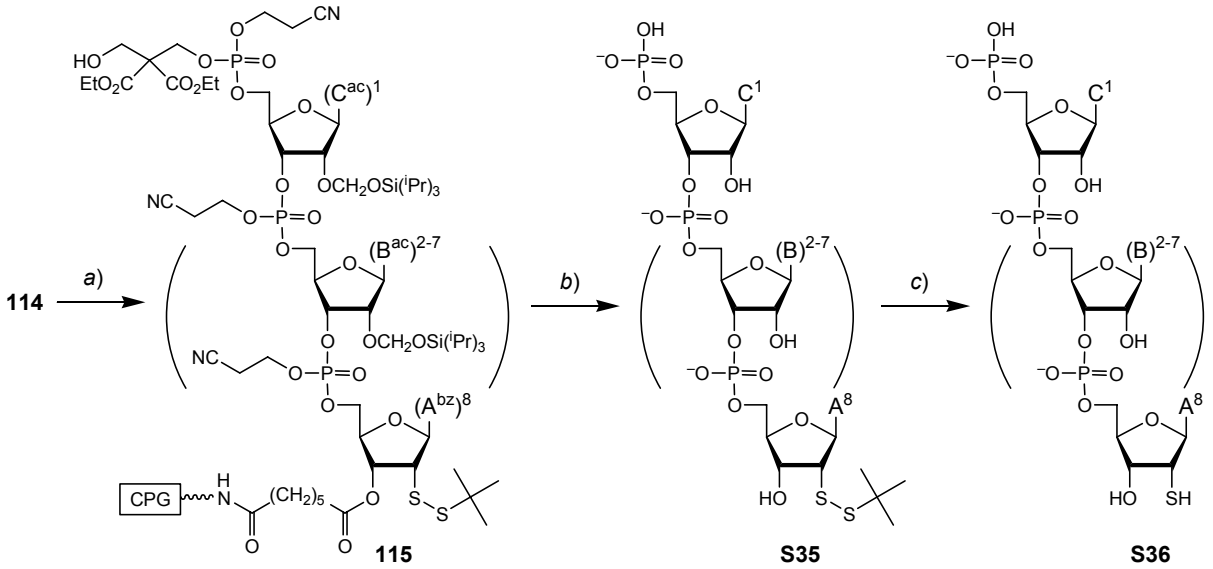
In the first approach, **107** was treated with potassium thioacetate in DMSO. After workup, the crude thioacetate derivative was selectively deacylated to the 2'-thioadenosine **108**. We then encountered some difficulties for the preparation of the *tert*-butyl disulfide derivative **110**, despite a lot of reported methods. The incubation with t-butylsulfide/O_2 in presence of a base (Wallace and Schriesheim 1962), reaction of *tert*-butylsulfide under *Mitsunobu* conditions (Mukaiyama and Takahashi 1968), and activation by methoxycarbonylsulphenyl chloride reagent (Rietman et al. 1994) was unsuccessful, although these methods are routinely used in peptide syntheses. The only successful method, which involved the *in-situ* formation of a transient tert-butylsulfenyl chloride derivative (Derbesy and Harpp 1994), gave the product **110** in a moderate yield of 38%.



Scheme VI.2. *a*) 1. KSAc, DMSO, 20°. *b*) 1. NaOH, THF, MeOH, H_2O, 4°. *c*) 1. SO_2Cl_2, ^tBuSH, pyridine, Et_2O, -78°; then, **3**, THF, pyridine, -78°. *d*) 1. HF·pyridine, pyridine, 20°. *e*) 1. $(MeO)_2TrCl$, pyridine, 20°. *f*) 1. Bis(4-nitrophenyl) heptanedioate, DMAP, pyridine, 20°. *g*) 1. Long-chain-alkylamino CPG, $^iPr_2NEt$, DMF, 20°; 2. Ac_2O, pyridine, 20°. **107** was prepared according to (Mariott et al. 1991).

After cleavage of the silyl-protecting group with HF/Py, extraction and 5'-*O*-dimethoxytritylation with $(MeO)_2$Tr-Cl in Py, the protected 2'-deoxy-2'-thio-adenosine **112** was obtained in 58 % yield. The corresponding activated ester **113** was prepared with bis(4-nitrophenyl)heptandionate in Py (70% yield). Finally, **113** was immobilized on aminoalkyl-functionalized controlled pore glass (CPG) with $^{i}Pr_2NEt$ in DMF, resulting in the solid support **114** with a loading of 39µmol/g.

From **114**, 2'-*O*-TOM protected ribonucleoside phosphoramidites and a commercially available phosphate building block, the 8mer RNA sequence $^{-2}O_3PO$-r(CCCCACCA$^{2'SStBu}$) (**S35**), carrying a monophosphate group at the 5'-end and the disulfide protected 2'-deoxy-2'-thioadenosine at the 3'-end, was assembled by automated synthesis under standard conditions (Pitsch et al. 2001) *(Scheme VI.3.)*. The removal of the nucleobase protecting groups and cleavage from the solid support was achieved under our standard conditions ($MeNH_2$ EtOH/H_2O 1:1, 35°, 3 h and 1M NBu_4F in THF, 14 h). The crude product was purified by anion-exchange (AE) HPLC and desalted. We obtained the pure product **S35** in a yield of 26% (based on solid support **114**).



A^{bz} = N^6-benzoyladenine; C^{ac} = N^4-acetylcytosine; CPG = "alkylamino-functionalized controlled pore glass"

Scheme VI.3. *a*) Assembly of **115** on a oligonucleotide synthesizer with 2'-*O*-tom protected ribonucleosides and 3-[(4,4'-dimethoxytrityl)oxy]-2,2-(ethoxycarbonyl)propyl 2-cyanoethyldiisopropylphosphoramidite according to (S. Pitsch et al. 2001). *b*) 1. 12M $MeNH_2$ in H_2O/ 8M in EtOH, 20°; 3. $Bu_4NF{\cdot}3H_2O$, THF; 4. HPLC purification. *c*) TCEP (= tris(2-carboxyethyl)phosphine) 10mM, pH 7.4 TrisHCl.

VI.2.2.2. Deprotection and aminoacylation studies.

The free thiol-substituted RNA sequence **S36** was formed *in situ* by reductive cleavage of the remaining disulfide protecting group of **S35** (c = 0.5 mM)[1)] with tris(2-carboxyethyl)phosphine (TCEP, c = 10 mM, at pH 7.4). According to HPLC, this reaction was not complete even after 2h of incubation *(Figure VI.3.* and *Figure VI.4.)*. Nevertheless, the crude reaction mixture was treated with 12 equiv. of the activated amino acid thioester thiophenyl phenylalaninate (H-Phe(SPh), **116**[2]) *(Scheme VI.4.)* (added as concentrated solution in DMF) at 25°, and the composition was analyzed by AE-HPLC after 40 min, revealing occurrence of spontaneous aminoacylation *(Figure VI.5.)*.

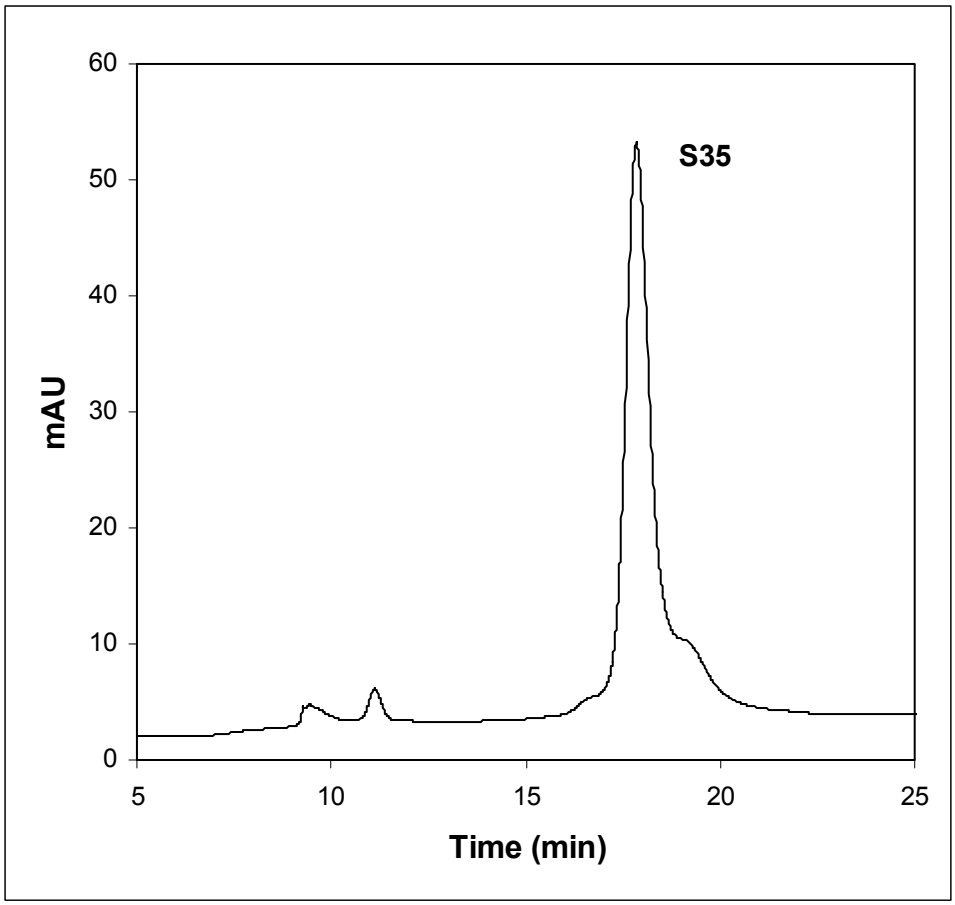


Figure VI.3. Representative AE HPLC trace (detection at 260 nm) of a disulfide-protected starting RNA sequence **S35**. (Time of disulfide deprotection t = 0).

[1]) Such a low concentration was chosen to simulate the aminoacylation of analogously modified tRNAs, which are usually available and required only in very small quantities

[2]) Prepared from commercially available *N*-(*tert*-butoxycarbonyl)-L-phenylalanine 4-nitrophenyl ester according to (Ryan and Chung 1981).

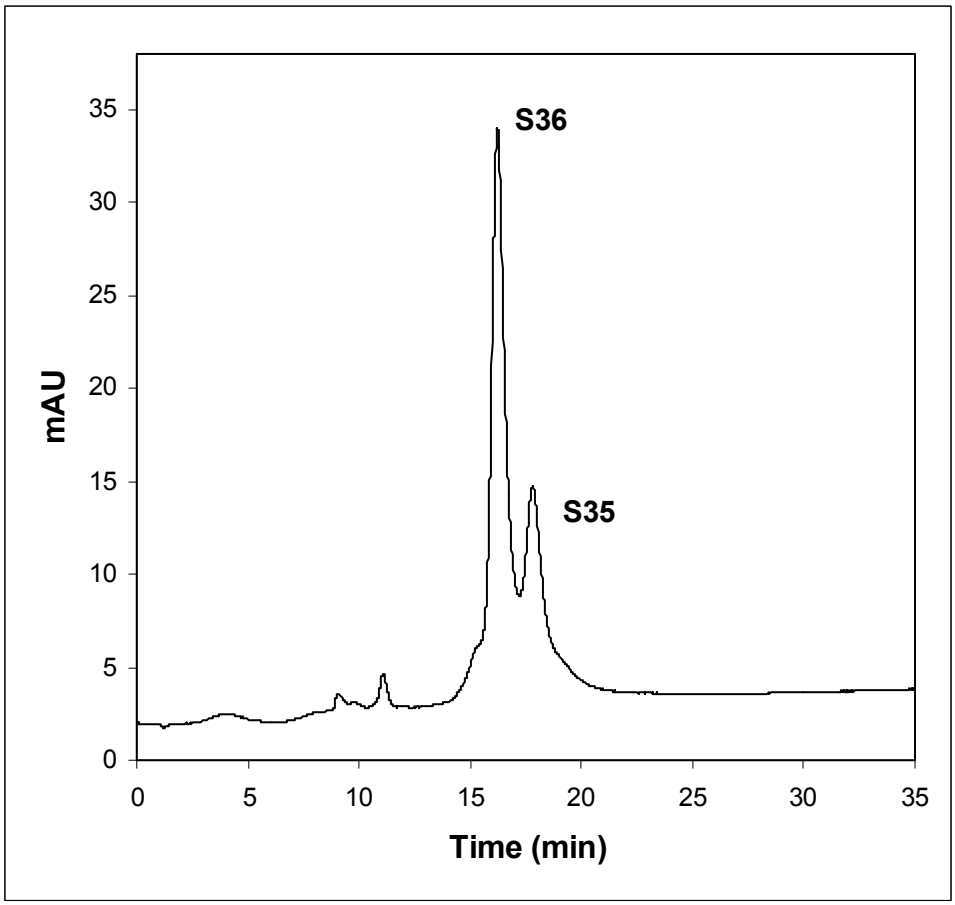
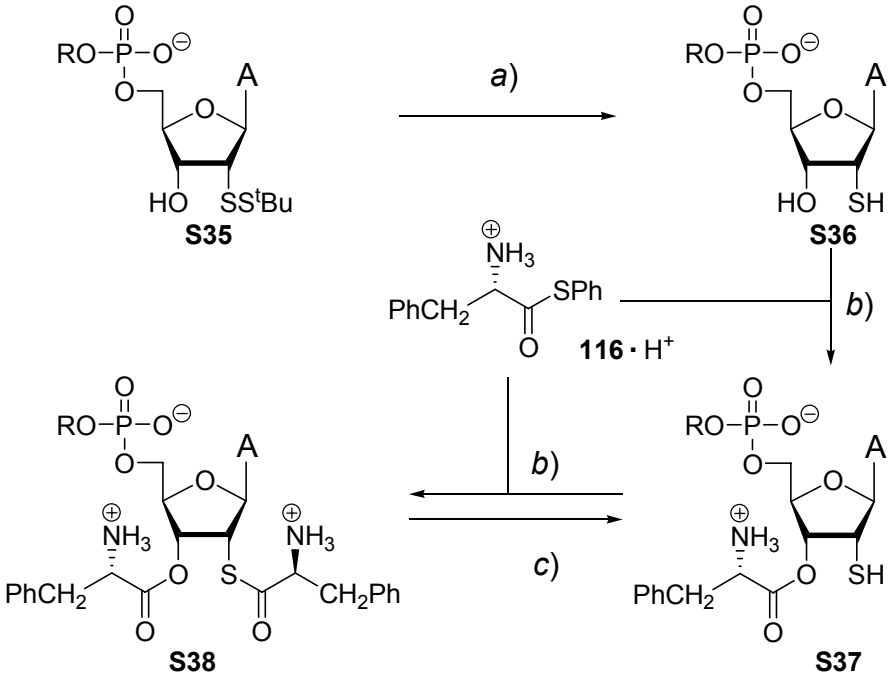


Figure VI.4. Representative AE HPLC trace (detection at 260 nm) of formation of the tert-butyl disulfide-deprotected **S36** from **S35** after 1h.



Scheme VI.4. *a*) TCEP in different aq. buffers, see *Table VI.1. b*) Addition of thiophenyl phenylalaninate (H-Phe(SPh), **116**) in DMF to buffered aq. solutions, see *Table VI.1. c*) NaN_3 in aq. buffer (pH 5.0), see *Figure VI.9.*

Unfortunately, at that time, no ESI-MS analysis was available and the nature of the products (mono- **S37** or di-acylated **S38**) could not be determined. Nevertheless, we were convinced that aminoacylation occurred, since the parent RNA sequence $^{-2}O_3PO$-r(CCCCACCA) was completely inert under these conditions. This observation indicated that the thiol group is involved in and required for the product formation and that the activated amino acid is not interfering with the integrity of the RNA sequence itself.

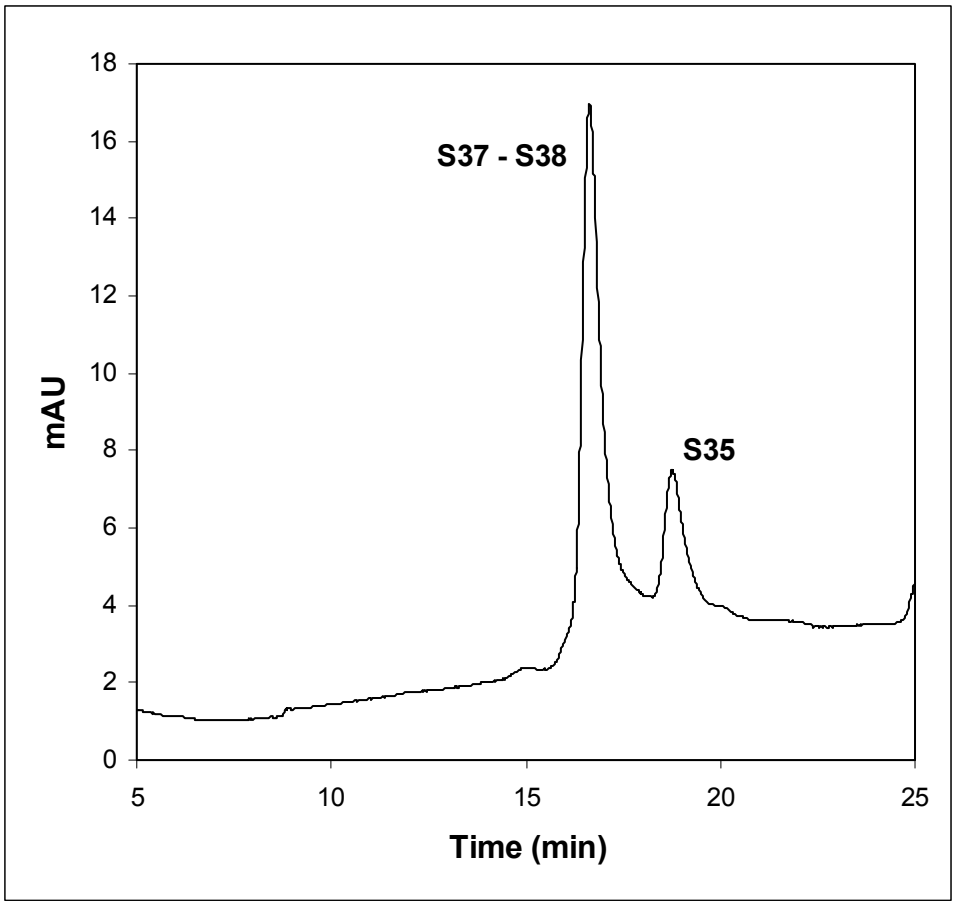
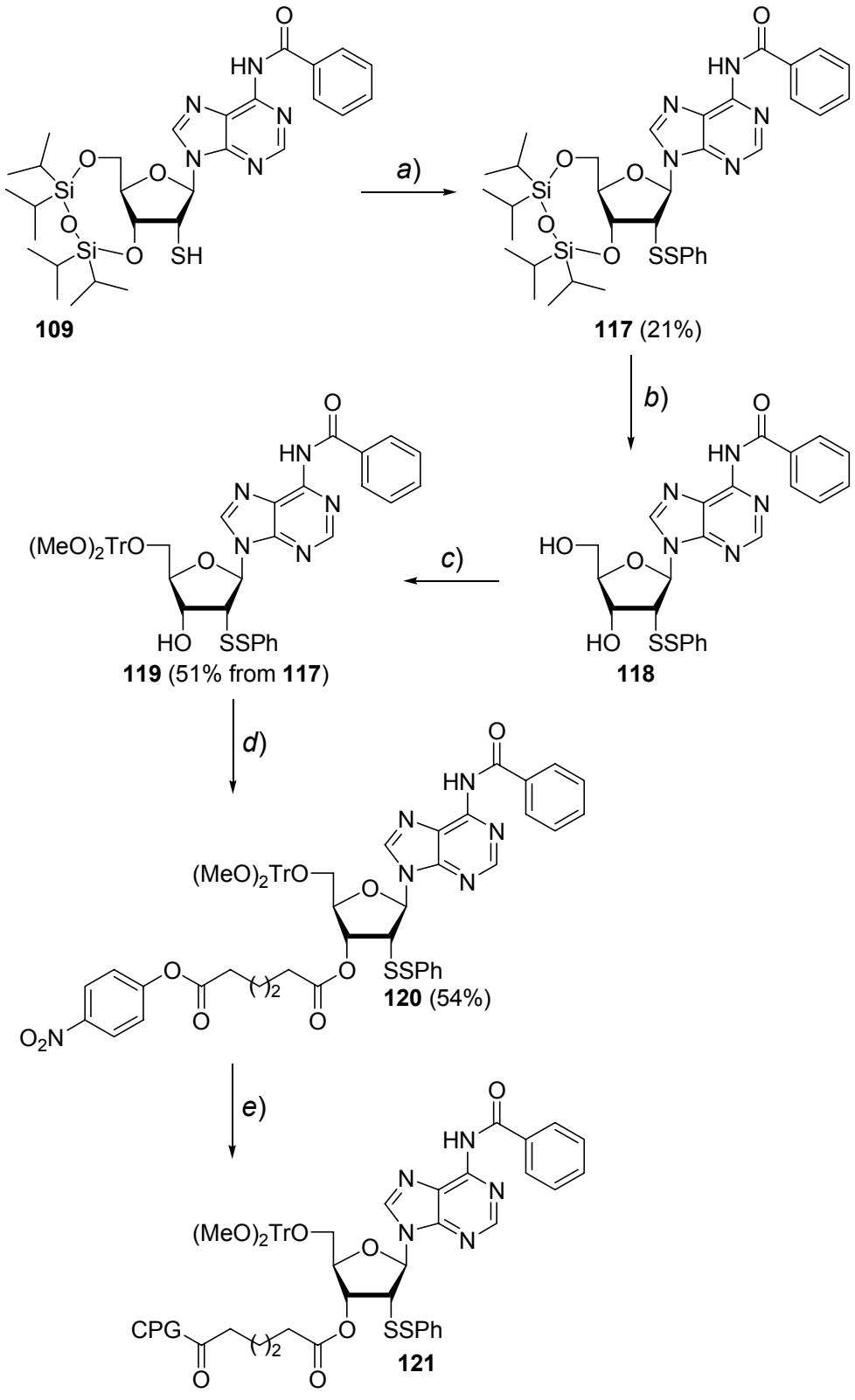


Figure VI.5. AE HPLC trace (detection at 260 nm) of a crude reaction mixture obtained immediately (ca. 1 min) after addition of 12 equiv. H-Phe(SPh) (**116**) to **S36** (obtained from **S35**, see above)**.**

VI.2.3. Improvements of deprotection

VI.2.3.1. Synthesis of phenyl disulfide protected sequence

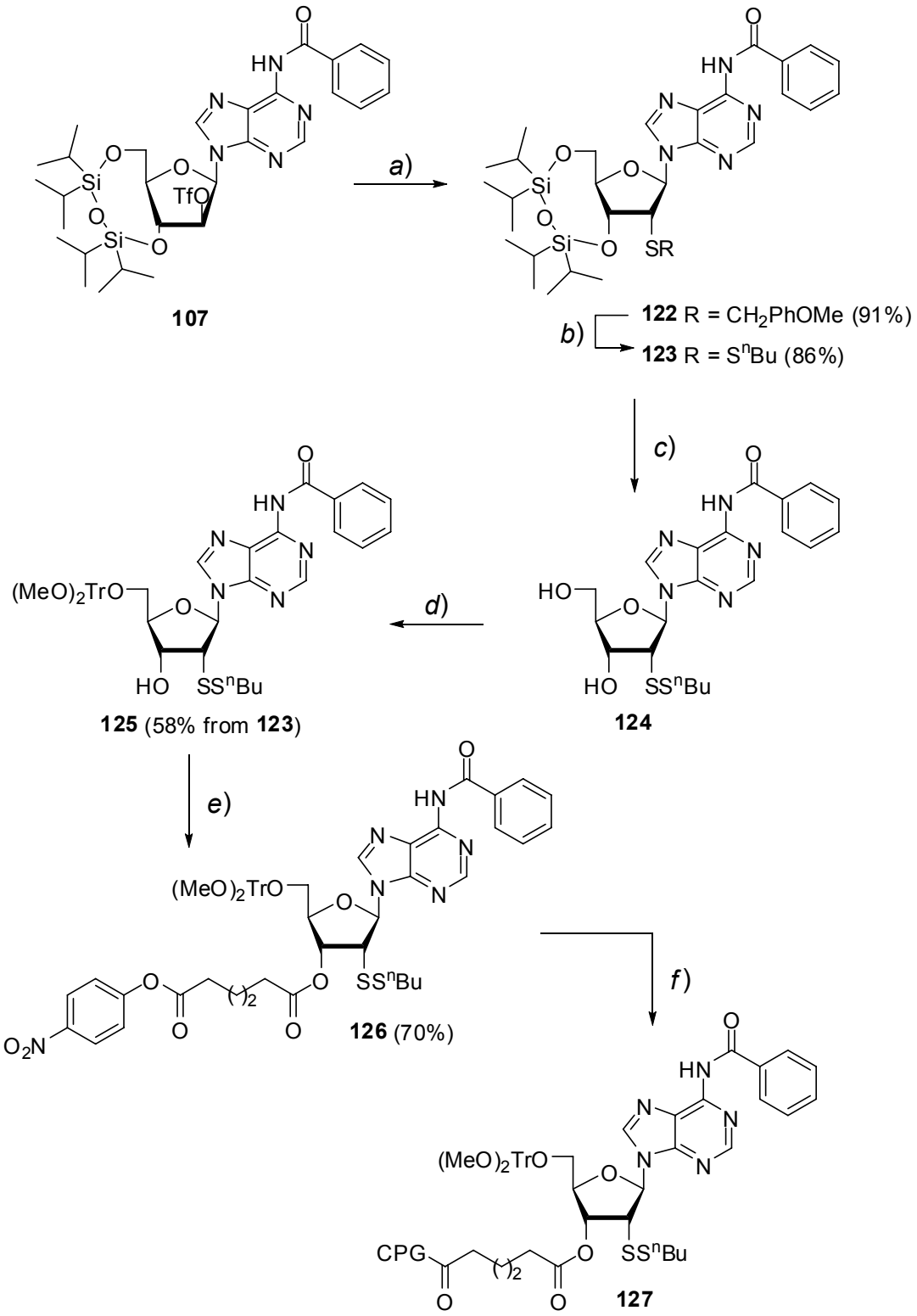
These first results obtained with the tert-butyl disulfide derivative **114** were quite promising, but obviously this protecting group was too stable and could never be completely cleaved under our conditions. Therefore, we investigated other protecting groups for the sulfide moiety, which were easier to cleave. First, the phenyl disulfide derivative **118** was prepared *(Scheme VI.5.)*, for which the kinetics of cleavage in presence of a phosphine had already been investigated (L. E. Overman et al. 1974). From the corresponding solid support **121**, we carried out an automated synthesis of a 8mer oligonucleotide, but this attempt failed, since the phenyldisulfide protecting group was not stable during assembly and/or deprotection of the sequence. Consequently, we investigated the linear butyl disulfide, which was expected to more labile than the tert-butyl disulfide, due to its reduced steric hindrance, and more stable than the phenyl disulfide.



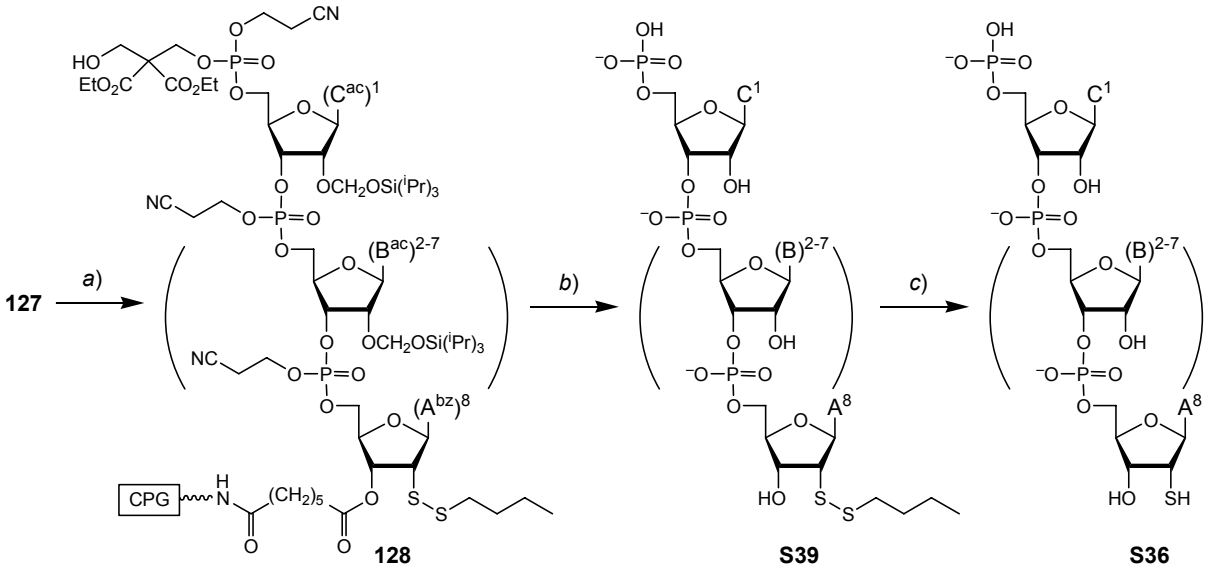
Scheme VI.5. *a*) 1. N-chlorosuccinimide, PhSH, pyridine, Et_2O, -78°; then, **3**, THF, pyridine, -78°. *b*) 1. HF·pyridine, pyridine, 20°. *c*) 1. $(MeO)_2TrCl$, pyridine, 20°. *d*) 1. Bis(4-nitrophenyl) heptanedioate, DMAP, pyridine, 20°. *e*) 1. Long-chain-alkylamino CPG, $^iPr_2NEt$, DMF, 20°; 2. Ac_2O, pyridine, 20°.

VI.2.3.2. Synthesis of butyl disulfide protected sequence

For the preparation of the immobilized n-butyl disulfide protected adenosine derivative **127**, a new synthetic approach was investigated, since the n-butylsulfenylchloride can be much more easily obtained than tert-butylsulfenylchloride (Thea and Cevasco 1988). The 2'-*O*-trifluoromethylsulfonyl derivative **107** was treated with 4-methoxybenzylthiol/NaH in DMSO, according to a reported method (Eleuteri et al. 1996) offering the 4-methoxybenzyl thioether **122** (91 % yield). The efficient formation of the n-butyl disulfide derivative **123** (86% yield) was achieved by incubation with n-butylsulfenylchloride (prepared according to (Thea and Cevasco 1988)) in CH_2Cl_2/AcOH according to (Eleuteri et al. 1996). The rest of the synthesis was carried out in similar manner as described above. After cleavage of the silyl-protecting group with HF/Py, extraction and 5'-*O*-dimethoxytritylation with $(MeO)_2$Tr-Cl in Py, the protected 2'-deoxy-2'-thio-adenosine **125** was obtained (60 % yield). The corresponding activated ester **126** was prepared with bis(4-nitrophenyl)heptandionate in Py (68% yield) *(Scheme VI.6.)*. Finally, **126** was immobilized on aminoalkyl-functionalized controlled pore glass (CPG) with $^{i}Pr_2NEt$ in DMF, resulting in the solid support **127** with a loading of 30µmol/g *(Scheme VI.7.)*. We then prepared the n-butyl disulfide protected RNA sequence **S39** and found that complete removal of the protecting group could indeed be achieved within 30 min *(Table VI.1.)*.



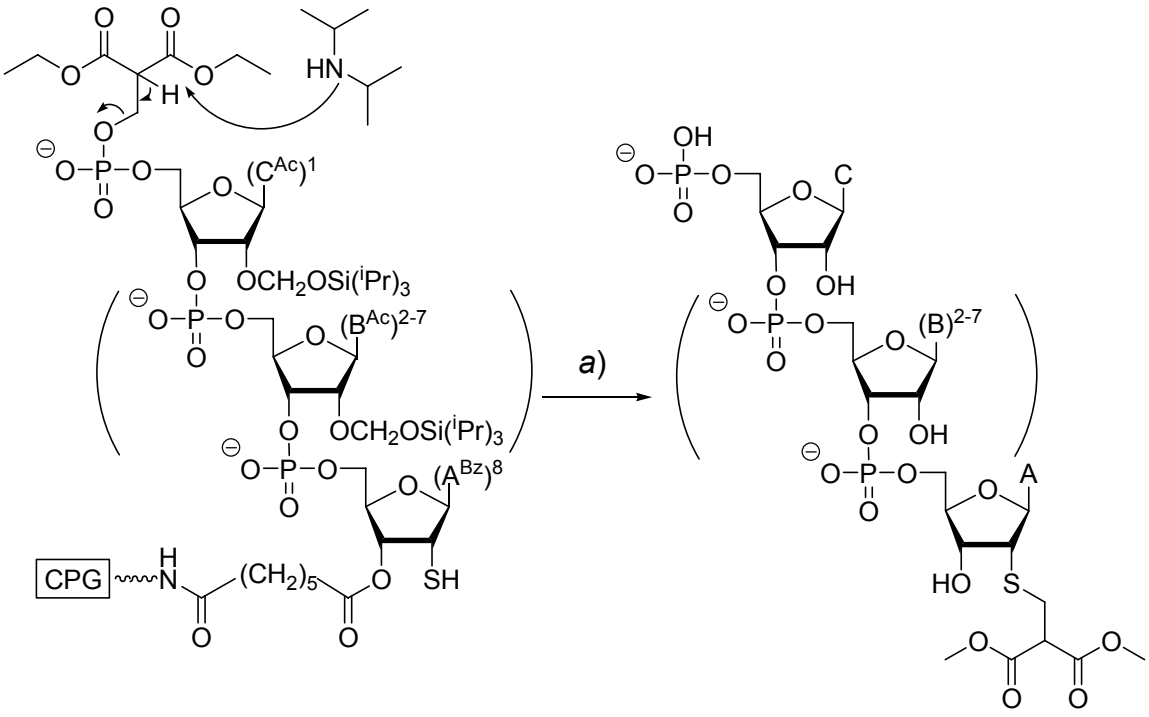
Scheme VI.6. *a*) 1. 4-methoxybenzenemethanethiol, NaH, DMSO, 10°. *b*) 1. BuSCl, AcOH, CH_2Cl_2, 4° – 20°. *c*) 1. HF·pyridine, pyridine, 20°. *d*) 1. $(MeO)_2TrCl$, pyridine, 20°. *e*) 1. Bis(4-nitrophenyl) heptanedioate, DMAP, pyridine, 20°. *f*) 1. Long-chain-alkylamino CPG, $^iPr_2NEt$, DMF, 20°; 2. Ac_2O, pyridine, 20°.



Scheme VI.8. *a*) Assembly on a oligonucleotide synthesizer with 2'-O-tom protected ribonucleosides and 3-[(4,4'-dimethoxytrityl)oxy]-2,2-(ethoxycarbonyl)propyl 2-cyanoethyldiisopropylphosphoramidite according to (S. Pitsch et al. 2001) but employing 20 mM I_2 in THF/pyridine/H_2O 7:2:1 as oxidizing agent. *b*) 1. $(^iPr)_2NH$/MeCN 1:9, 20 min; 2. 12M NH_3, MeOH, 20°; 3. $Bu_4NF{\cdot}3H_2O$, AcOH, THF; 4. HPLC purification. *c*) TCEP (= tris(2-carboxyethyl)phosphine) in different aq. buffers, see *Table VI.1*.

Nevertheless, the initially employed conditions for the deprotection of the 8mer from the solid support were not optimal and therefore we adapted the deprotection protocol. First, the cyanoethyl protecting groups were removed by washing the crude, immobilized sequence **128** with $(^iPr)_2NH$/MeCN 1:9 for 20 min at a flow-rate of 2.5 ml/min, then removal of the nucleobase protecting groups and cleavage from the solid support was achieved with 12M NH_3 in MeOH during 6h at 25°, and finally, the 2'-*O*-TOM protecting groups were removed by 1M $Bu_4NF{\cdot}3H_2O$/0.5M AcOH in THF during 3h at 25°. Even under these optimized conditions (required for a complete deprotection), by-product formation (ca. 30%) was observed. It has been identified as **S39A** (from MS analysis), result of the reaction of the

partially cleaved disulfide with the release product of the protected 5'-phosphate moiety *(Scheme VI.8.)*.



Scheme VI.8. Formation of the side-product **S39A** as deduced from ESI-MS analysis. *a*) 1. $(^{i}Pr)_2NH$/MeCN 1:9, 20 min; 2. 12M NH_3, MeOH, 20°; 3. $Bu_4NF{\cdot}3H_2O$, AcOH, THF; 4. HPLC purification.

After a double HPLC purification (1. anion-exchange (AE); 2. reversed phase (RP)) and desalting, the pure product **S39** was obtained in a yield of 25% (based on solid support **127**). The LC-ESI MS spectrum of this product showed only one signal at *m*/*z* = 2611 amu (calc. for **S39**: 2611 amu). The HPLC and MS traces of purified **S39** are shown in *(Figure VI.6a.)* and *(Figure VI.6e.)* respectively.

VI.2.4. Aminoacylation studies [3])

The free thiol-substituted RNA sequence **S36** was formed *in situ* by reductive cleavage of the remaining disulfide protecting group of **S39** (c = 0.08 mM)[4]) with tris(2-carboxyethyl)phosphine (TCEP, c = 1 – 10 mM, depending on pH) in various aqueous buffers

[3]) Carried out in collaboration with M. Meyappan (Postdoc 2003 - 2005).

[4]) Such a low concentration was chosen to simulate the aminoacylation of analogously modified tRNAs, which are usually available and required only in very small quantities

(pH 7.4 – 3.7) at 25° (for conditions see *Table VI.1.*). This reaction was complete within 30 min, and **S36** was obtained in a purity > 95% according to HPLC and MS analyses (*Figure VI.6b.* and *Figure VI.6f.*, respectively).

In a first set of experiments, the pH dependency of the aminoacylation reaction was investigated (see *Table VI.1.* and *Scheme VI.9.*). Crude **S36** in buffered aqueous solutions (pH 7.4 - 3.7, obtained from **S39** and TCEP as described above) was treated with 12 equiv. of **116** (added as concentrated solution in DMF) at 25°, and the composition of the reaction mixtures was analyzed by RP-HPLC after 10 and 50 min (for an example, obtained at pH 7.4, see *Figure VI.3c.* (10 min) and *Figure VI.6d.* (50 min), respectively). Under all conditions, a fast formation of two slower migrating (less polar) products was observed, which was in agreement with the formation of the monoacylated product **S37** and the diacylated product **S38**, respectively. The HPLC analysis was carried out at pH 3.5, where both products **S37** and **S38** were hydrolytically stable[5]). Additional experiments at 37° and with only 3 or 6 equivalents of **116** were carried out at pH 7.4 and 5.0 *(Table VI.1.)*. The two aminoacylated products **S37** and **S38** were isolated by HPLC and their structure was confirmed by ESI-MS (*Figure VI.6g.* and *VI.6h.*)[6]).

[5])All RP-HPLC analyses were carried out at pH 3.5 with a 0.1M Et_3N-H_3PO_4 buffer. At pH values between 4.0 and 5.5, partial hydrolysis of the diacylated product **S38**, and at pH values between 5.5 and 7.5 partial hydrolysis of both acylated products **S37** and **S38** was observed.

[6]) Our analytical methods (HPLC, MS and hydrolysis studies, see below) do not show whether **S37** is the 3'-*O*- or the 2'-*S*-monoacylated product; however, since esters are thermodynamically much more stable than analogous thioesters, and since it is well-known that the migration of acyl-groups between the 2'-*O* and 3'-*O*-positions of ribonucleosides is an extremely fast reaction (Chladek and Sprinzl 1985), we concluded that **S37** is the 3'-*O*-monoacylated derivative.

Table VI.1. Liberation and aminoacylation of the 2'-deoxy-2'-thioadenosine modified RNA sequence **S36**: Exploratory studies under different conditions[a)]

Conditions[b)]			t = 10 min[c)]			t = 50 min[c)]		
pH	**116** (equiv.)	T [°C]	**S36**	**S37** [%]	**S38**	**S36**	**S37** [%]	**S38**
7.4	12	25	1	18	73	5	37	46
6.5	12	25	3	19	71	3	24	64
5.5	12	25	7	19	70	<1	10	86
5.0	12	25	10	18	70	<1	10	87
4.7	12	25	12	18	68	<1	10	87
3.7	12	25	43	20	36	18	26	55
7.4	6	25	2	24	64	6	78	10
7.4	12	37	1	17	72	5	41	42
5.0	6	25	27	29	43	1	33	65
5.0	3	25	54	20	23	15	35	47
5.0	12	37	2	27	67	1	32	64
5.0	6	37	8	34	53	1	35	60
5.0	3	37	30	29	34	4	41	50

[a)] Detailed procedure in *Exper. Part.* [b)] Incubation of **S39** (c = 0.085 mM, 40µl) with TCEP (c = 1.0 mM at pH 7.4, 6.5; 2.5 mM at pH 5.5, 5.0, 4.7; 10 mM at pH 3.7) in aq. buffers (50 mM each; pH 7.4: Tris-HCl, pH 6.5: H_3PO_4-NaOH, pH 5.5, 5.0, 4.7: AcOH-NaOH, pH 3.7: HCOOH-NaOH) at 25° for 30 min, followed by addition of **116** (indicated equivalents relative to **S39**) in DMF (4 µl). [c)] t = incubation time; quantification by RP-HPLC at 260 nm; t_R (**S36**) = 14 min, t_R (**S37**) = 17 min, t_R (**S38**) = 18 min; values relative to sum of all detected peaks. For an example of such HPLC traces from a reaction carried out pH 7.4 and 25°, see *Fig. VI.6c.* and *VI.6d*.

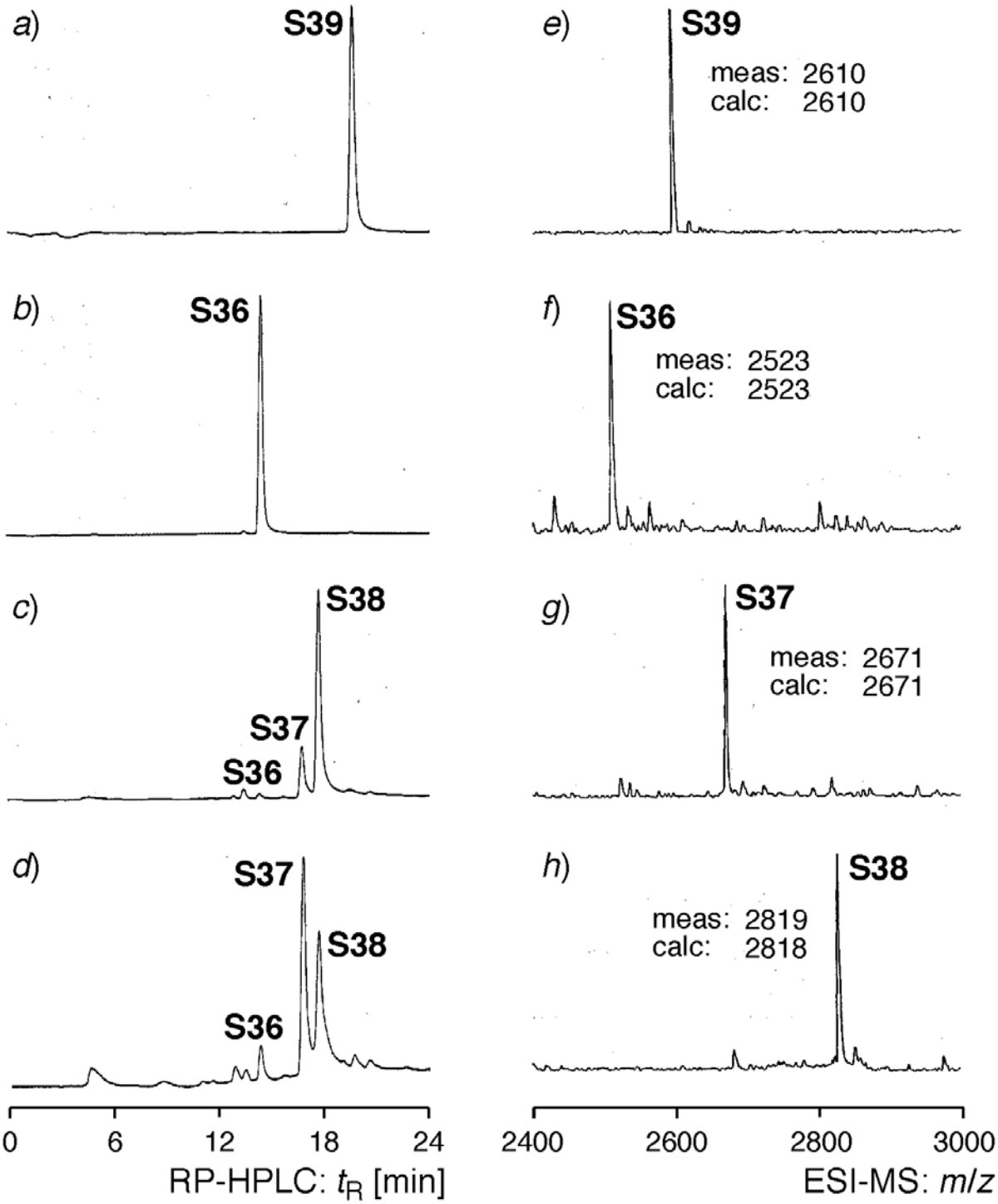


Figure VI.6. *Left*: RP HPLC traces (detection at 260 nm) of a representative aminoacylation reaction carried out at pH 7.4 and 25°: *a*) the disulfide-protected starting RNA sequence **S39**, *b*) 30 min after treatment with TCEP, *c*) 10 min and *d*) 50 min after addition of 12 equiv. H-Phe(SPh) (**116**). *Right*: Deconvoluted ESI-MS spectra (neg. mode) of RNA sequences **S36** – **S39**, for procedures see *Exper. Part*.

At pH-values between 4.7 and 5.5, and at 25°, clean and almost quantitative formation of the monoacylated product **S37** together with the diacylated product **S38** was observed after 50 min. At higher pH-values of 6.5 and 7.4, some unidentified by-products were formed after

50 min. (ca. 10%), but the reactions were complete already after 10 min at 25° (*(Figure VI.6c.)* and *(Figure VI.6d.)*). With only 3 equiv. of **116**, an almost quantitative aminoacylation could be observed after 50 min at pH 5.0 and 37°.

We then focused on two different reaction conditions: the first one typical for *in-vitro* translations, and the second one for the purpose of preparing and isolating aminoacylated RNA sequences. In *(Figure VI.7a.)*, the time course of a reaction carried out with **S36** (c = 0.08 mM) and 12 equiv. of **116** at pH 7.4 and 37° is shown. The composition of the reaction mixture was analyzed by RP-HPLC and the relative amount of products **S37** and **S38** was plotted against reaction time *(Figure VI.7a.)*. After 2 min already, almost quantitative formation of the mono- and diacylated RNA sequences **S37** and **S38** was observed *(Figure VI.7b.)*. Only after about 50 min, they slowly were converted back to the starting material **S36** by hydrolytic cleavage. The plateau between 2 and 50 min indicates the transient nature of the aminoacylated species **S37** and **S38**, which are constantly hydrolyzed and re-acylated, until the activated amino acid is consumed. The rate constants for the cleavage of the ester bond of **S37** and the thioester bond of **S38** at 37° and pH 7.4 (0.1M Tris-HCl) were determined by kinetic studies and are given in *(Figure VI.7b.)*.

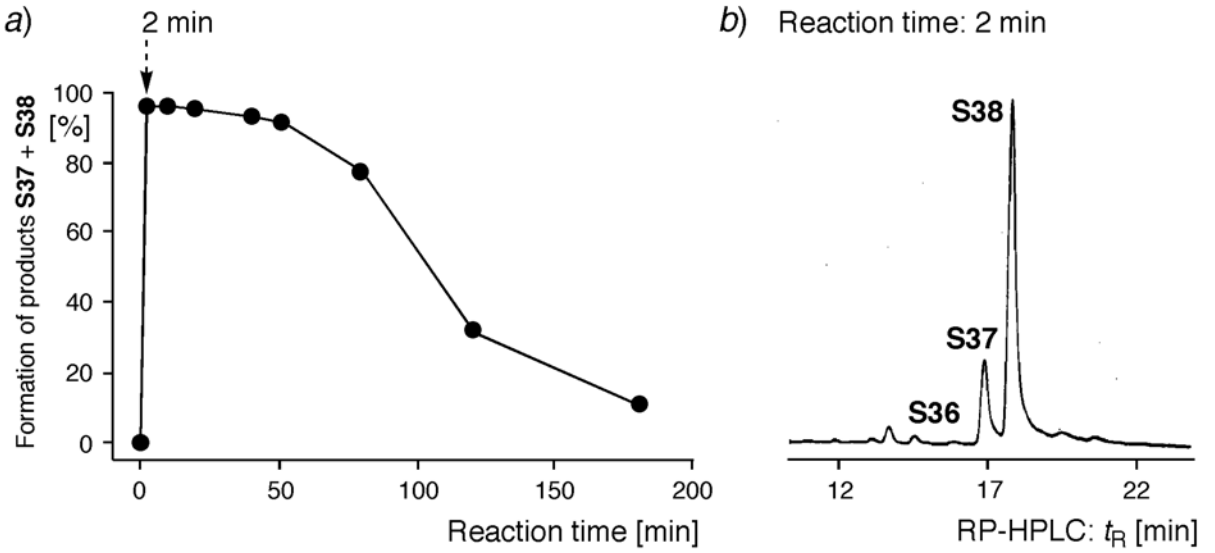


Figure VI.7. Aminoacylation reaction carried out at pH 7.4 and 37° with **S36** and **12** equiv. H-Phe(SPh) (**116**); *a*) Plot of the amount of aminoacylated products (**S37** + **S38**) relative to the sum of all assigned RNA sequences (**S36** + **S37** + **S38**); the data were extracted from HPLC traces. *b*) RP HPLC trace (detection at 260 nm) of the product mixture obtained after 2 min reaction time.

Under the same conditions, but at 25°, the rate constant for the hydrolysis of **S37** to **S36** was $k = 0.02$ min^{-1}, which is about twice the value of an adenosine nucleotide esterified with L-Phe (Stutz et al. 2000)[7]).

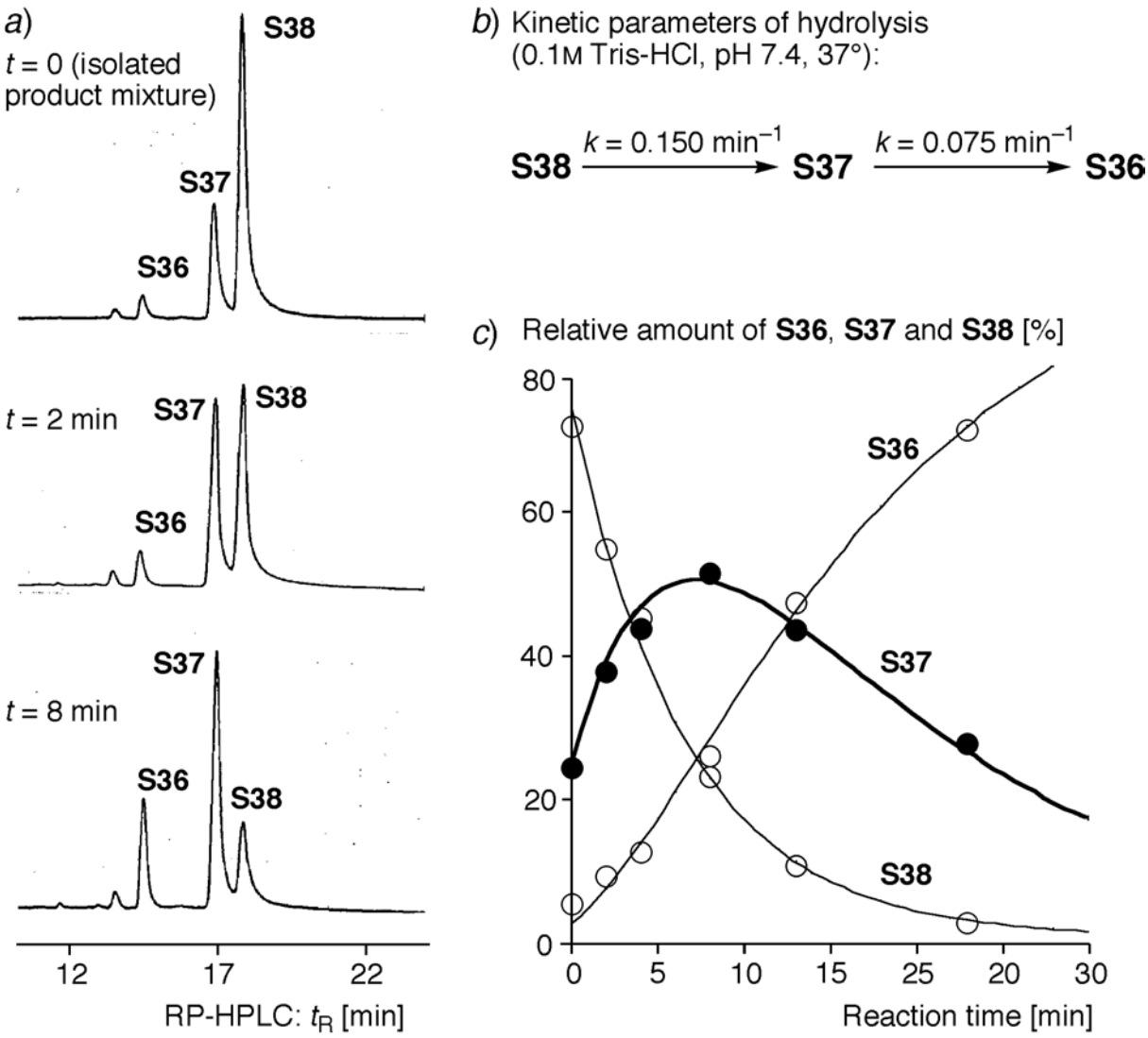


Figure VI.8. *a*) RP-HPLC traces (detection at 260 nm) obtained by incubating an isolated aminoacylation product mixture in 0.1M aq. Tris-HCl, pH 7.4, at 37°; *b*) Independently determined kinetic parameters of hydrolysis for the diacylated product **S38** and the monocylated product **S37**, respectively; *c*) Measured (points) and calculated (lines) composition, respectively, of a hydrolysis reaction mixture obtained according to *a*) at pH 7.4 and 37°: for details see *Exper. Part*.

[7]) Under these conditions, the hydrolysis rate of the activated amino acid **116** is $k = 0.007$ min^{-1} and 0.07 min^{-1} at 25° and 37°, respectively.

In order to simulate the conditions of an *in-vitro* translation reaction, we incubated a desalted mixture of reaction products from an acylation reaction[8]) at pH 7.4 (aqueous 0.1M Tris-HCl buffer) and 37°. Aliquots were removed at different time intervals and analyzed by RP-HPLC (for examples see *Figure VI.8a.*). In *Figure VI.8c.*, the time-dependent composition of the reaction mixture is shown together with the curves calculated from the individually determined hydrolysis rate constants (shown in *Figure VI.8b.*). The hydrolysis of the diacylated **S38** to the monoacylated **S37** occurs twice as fast as the hydrolysis of the latter to **S36**; this results in a predominant occurrence of the relevant monoacylated RNA sequence **S37** after a short incubation at 37° and pH 7.4 *(Figure VI.8c.)*.

An efficient acylation reaction between **S36** and **116** was observed at pH 5.0 *(Table VI.1.)*, where aminoacylated RNAs are considerably stable (Stutz et al. 2000); therefore, we decided to optimize the preparation of **S37** at this pH-value. In order to minimize the formation of side products, we carried out the deprotection and aminoacylation reactions simultaneously. In *Figure VI.9.* the HPLC trace of such a reaction product is shown, which was obtained by incubation of **S39** (0.08 mM) with TCEP (30 equiv.) and **116** (5 equiv.) at pH 5.0 and 37° for 30 min. This HPLC trace reveals the clean and almost quantitative formation of the aminoacylated products **S37** and **S38**. For the selective cleavage of the thioester bond (reaction **S38**→**S37**), a variety of different conditions (pH values, nucleophiles) were investigated.

[8]) The isolated product mixture was obtained from an acylation reaction of **S36** with **116**, carried out at pH 5.0 and at 37°; after 30 min, the reaction mixture was diluted with H_2O and desalted on a size-exclusion cartridge by elution with H_2O. The oligonucleotide containing eluate was stabilized with 1% AcOH and concentrated to a smaller volume by lyophilization.

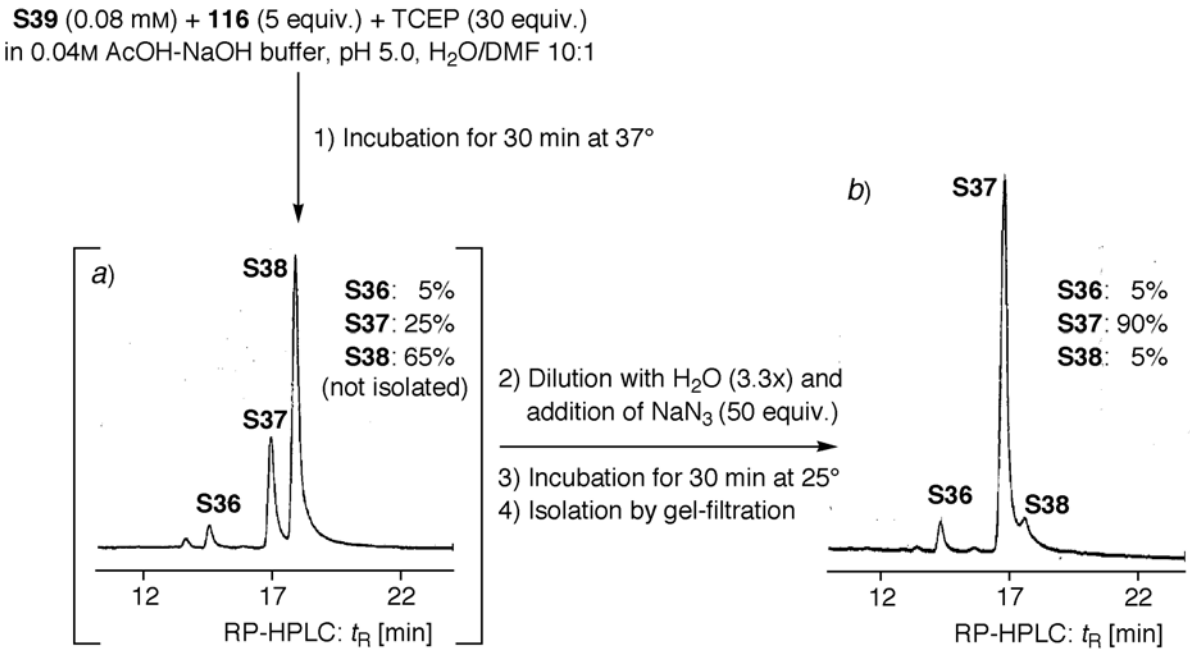


Figure VI.9. Conditions for the preparation and isolation of the monoacylated RNA sequence **S37** and RP HPLC traces (detection at 260 nm) of *a*) the intermediate and *b*) the final product mixture.

The best result was obtained by treating the reaction mixture after 30 min with NaN_3, keeping it for 30 min at 25° and isolating the products by desalting on a size-exclusion cartridge, which removes all non-oligonucleotide components. This protocol furnished a product mixture containing 90% of the desired monoacylated RNA sequence **S37**, together with 5% of **S36** and 5% of **S38**, respectively *(Figure VI.9b.)*. Lyophilization of this aqueous eluate is possible without cleavage of the ester bond, if 1% AcOH is added[9]).

VI.3. Discussion

Mechanistically, it can be assumed that after formation of the 2'-*S*-thioester derivative a fast and almost irreversible migration of the acyl-group to the 3'-*O* position occurs (see *Footnote 7*); subsequently, the liberated 2'-SH group can then undergo another acylation reaction, resulting in formation of the *O*,*S*-diacylated product.

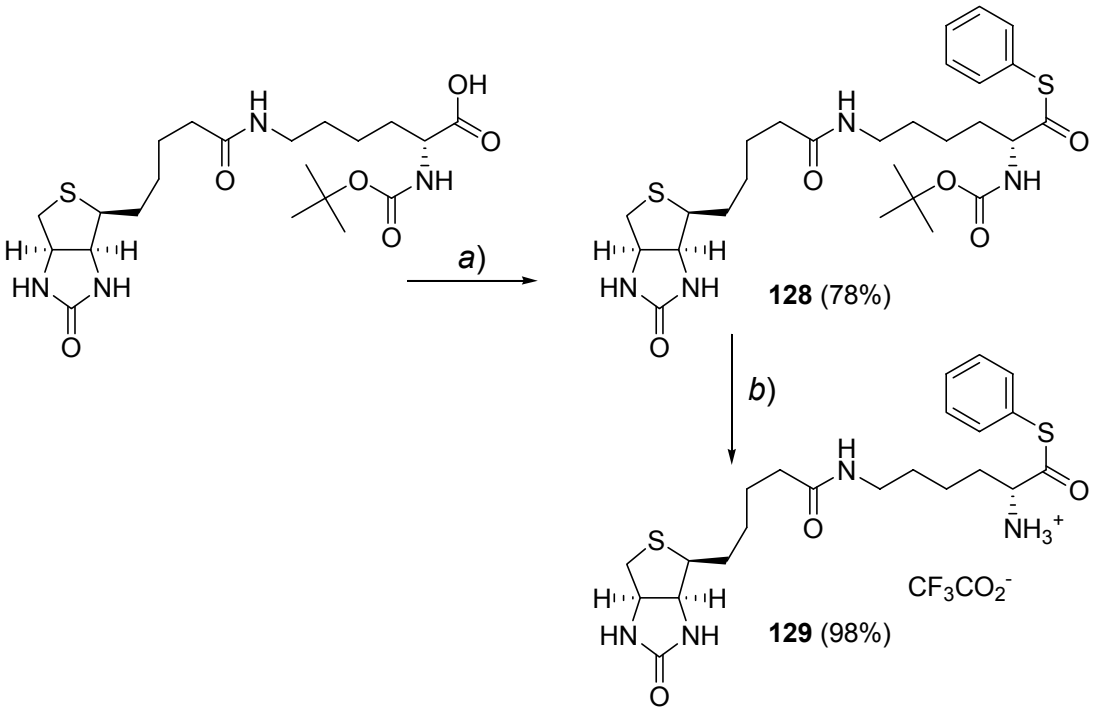
[9]) It was possible to isolate the product **S37** in pure form by RP HPLC (see *Exper. Part*); however, such a purification is not feasible or desirable with analogously prepared aminoacylated tRNA sequences (76mers, available in rather small quantities), for which this method was developed.

At pH 7.4 and 37°, the biologically relevant *O*-monoacylated product is formed as major product *in-situ* by hydrolysis of the *O*,*S*-diacylated precursor. The *O*-monoacylated product can be prepared efficiently by acylation of the modified RNA sequence at pH 5 and 37°, followed by selective cleavage of the concommitantly formed *O*,*S*-diacylated product with NaN_3; its isolation is conveniently carried out by desalting on size-exclusion cartridges, stabilization of the eluate with 1% AcOH, and lyophilization.

VI.4. Conclusion

Among the different processes required for the preparation of aminoacylated suppressor tRNA the aminoacylation step remains probably the most difficult to carry out. Since the first reports of this method, several improvements of this step have been accomplished. Whereas some researchers resorted on biological tools by creating a mutated tRNA/aminoacyl-tRNA synthetase pair, most of them focussed on chemical strategies. In this context, we developed yet another approach, which extends the intriguing concept of "native chemical ligation" of oligopeptides to the straightforward acylation of RNA sequences containing 2'-deoxy-2'-thionucleotides. The here presented method could be applied to translation systems in which a thiol-modified tRNA acts as a catalyst. Our investigations have demonstrated that the method was totally compatible with physiological conditions (pH 7.4 and 37°) and tolerates also larger amino acids, such as biocytin (preparation of the amino acid described in *Scheme VI.10.*). The corresponding 76mer, aminoacylated tRNA was prepared by *C. Denarie* (Denarie 2006) in our group, who first prepared the n-butyl disulfide protected tRNA sequence by enzymatic ligation from **S39** and a 68mer truncated tRNA sequence and then carried out the spontanous aminoacylation with the activated amino acid derivative **129** according to the here developed methods.

Whether such modified aminoacylated tRNAs are tolerated by biological systems, however, remains to be proven and such tests are currently under way (Denarie 2006).



Scheme VI.10. *a*) $^{i}Pr_2NEt$, BOP [= (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate], PhSH, DMF, 20°. *b*) TFA, CH_2Cl_2, 20°.

CONCLUSION

Within this work, a multitude of 2'-*O*-TOM protected phosphoramidite building blocks containing modified nucleobases were prepared by original, highly convergent synthetic approaches. Since the 2'-*O*-TOM group is stable towards a multitude of reagents and reaction conditions, most of the modified building blocks were prepared from the already protected canonical nucleotides by optimized base transformation reactions.

Among these modifications, some are not found in Nature, such as isoC, isoG and the wyosine-analogue 5-desmethyl-4-methylwyosine. These could be useful for the creation of artificial translation systems with orthogonal codons and for investigations involving π-stacking. The here prepared building blocks of the naturally occurring derivatives m^5U, D, I, m^1I, m^6A, $m^6{}_2A$, i^6A, m^1A, t^6A, m^1G, m^2G, $m^2{}_2G$ and m^5C should be incorporated into tRNAs in order to investigate their exact structural and functional role during translation. In this context, a truncated 68mer tRNA was prepared which contained the fragile nucleobase dihydrouridine (D) and, for the first time, also the acid-sensitive wyosine (imG). The latter nucleotide was also incorporated into a RNA sequence, which is forming a particularly stable "kissing" interaction. These studies permitted to partially rationalize the structural features responsible and required for complex formation. Specifically, the importance of secondary interactions for nucleic acid complex formation was impressively demonstrated. Inspired by these results, some alternative models for the mRNA/tRNA decoding process, involving modified nucleotides, such as t^6A were proposed.

In addition, an optimized synthesis of a modified 8mer RNA sequence containing a 3'-terminal 2'-deoxy-2'-thioadenosine was developed. It could be shown that such RNA analogues are spontaneously and site-specifically aminoacylated with a weakly activated amino acid thioester. This concept could be employed for a straightforward aminoacylation of analogously modified tRNAs and therefore considerably facilitate the ribosome-mediated incorporation of unnatural amino acids into proteins.

In conclusion, a variety of efficient synthetic strategies and tools for the preparation of modified RNAs have been developed, which can be used for physical, structural and biological investigations of this highly diverse and fascinating class of biomolecules.

EXPERIMENTAL PART

GENERAL

Reagents and solvents (highest purity) from various suppliers, used without further purification; [(triisopropylsilyl)oxy]methyl chloride (= TOM-Cl) was prepared according to (Pitsch et al. 2001). The 2'-*O*-TOM-protected ribonucleoside and the corresponding phosphoramidites were prepared according to (Pitsch et al. 2001). Py = pyridine, r.t. = room temperature (ca. 20°), DMAP = *N*,*N*-dimethylpyridin-4-amine, TMS-Cl = Me_3Si-Cl, MAC-Cl = MeOAc-Cl, AIBN = 2,2'-azobisisobutyronitrile, MS 4Å = 4Å molecular sieves (activated overnight at 180° and 0.01 mbar), RedAl = Sodium bis(2-methoxyethoxy)aluminium hydride.

Workup implies distribution of the reaction mixture between CH_2Cl_2 and satd. aq. $NaHCO_3$ soln., drying of the organic layer ($MgSO_4$), and evaporation under reduced pressure.

Thin layer chromatography (TLC): precoated silica gel plates from *Merck*, stained by dipping into a soln. of anisaldehyde (10 ml), H_2SO_4 (10 ml), and AcOH (2 ml) in EtOH (180 ml) and subsequent heating with a heat-gun.

Column chromatography (CC): silica gel *60* (230–400 mesh) from *Fluka*.

NMR-spectroscopy: *Bruker* 400 MHz (^{1}H: 400 MHz, ^{13}C: 100 MHz, ^{31}P: 162 MHz). Chemical shift δ in ppm, relative to external standards (^{1}H- and ^{13}C: Me_4Si, ^{31}P: 85% aq. H_3PO_4); coupling constants *J* in Hz; multiplicities (^{13}C) according to DEPT-spectra.

ESI-MS (positive mode): SSQ 710 (*Finnegan*), measurements in MeCN/H_2O/AcOH 50:50:1.

EI-MS: The GC analysis was performed with a WCOT Fused silica column (30m) coupled to a Varian Saturn 2200 mass spectrometer (*Varian*) for the GC-MS analysis (CP3800).

MALDI-MS (pos.mode): Axima CFR Plus (*Kratos*/*Shimadzu*), matrix: 2,4,6-trihydroxyacetophenone, $(NH_4)_2$-citrate.

LC-ESI-MS (neg. mode): Q-Tof-Ultima (*Micromass*/*Waters*) coupled to *Cap*-LC (*Waters*), injection: 2 µl aq. sample (*c* (RNA) = 2.5 µM, c (EDTA) = 1 mM); chromatography on *Xterra*

RP-C18 column (*Waters*, 5 μm, 0.32 x 50 mm; flow 8 μl/min, eluent *A*: 25 mM aq. $Me_2NBu{\cdot}H_2CO_3$ (pH 8.4); eluent *B*: MeCN, elution at 60°, sheath-flow 25 μl/min (MeCN)); gradient *A* → *A*/*B* 1:1 (15 min.); deconvolution by *MaxEnt1*-software.

Standard desalting

The purified sequences were desalted by treating with 1M aq. $Et_3N{\cdot}AcOH$ (pH 7, 5 ml) and applied to *Sepak*-cartridges (*Waters*): after elution of the salts with 0.1M aq. $Et_3N{\cdot}AcOH$ (pH 7, 10 ml), followed by H_2O (20 ml), the Et_3NH^+-form of the sequences were eluted with MeCN/H_2O 1:1 (5 ml) and the solvent evaporated to dryness.

Transformation into sodium salts

The Et_3NH^+-form of RNA sequences were twice dissolved in H_2O (2x 1.5 ml), treated with $NaHCO_3$ (10 equiv. per phosphate group) and evaporated to dryness. The residue was dissolved in H_2O (2 ml) and desalted on 2 *NAP*-columns according to the manufacturer's instructions: aq. soln of RNA sequence.

NMR Experiments

NMR experiments were carried out on a *Bruker* AV 600 MHz spectrometer equipped with a 5 mm TX1-HCN cryogenic probe with z-gradients. The RNA samples (amounts determined spectrophotometrically) were dissolved in 0.3 ml potassium arsenate buffer (25 mM, pH 7.0) in H_2O/D_2O 9:1. Restricted volume Shigemi tubes were used for all experiments. 1D 1H spectra were recorded by combining a water flip-back pulse and 3-9-19 WATERGATE for suppression of the water signal.

CHAPTER I

5'-O-*(4,4'-Dimethoxytrityl)*-N^2*-methyl-2'*-O-*{[triisopropylsilyl)oxy]methyl}guanosine* (**3**).

A soln. of **1** (501 mg, 0.66 mmol, prepared according to (Stutz et al. 2000)) in Py (8 ml) was treated with tms-Cl (200 mg, 2.0 mmol), stirred for 40 min at r.t., treated with 1,3-benzodithiolylium tetrafluoroborate (275 mg, 1.0 mmol) and stirred for 6 h at r.t. Workup and filtration (SiO_2 (11 g), hexane/AcOEt 3:7 → AcOEt) gave a mixture of 3'-O-tms and 3'-OH derivatives as a yellow foam (576 mg, ca. 88%; TLC (CH_2Cl_2/MeOH 9:1): R_f 0.48 and 0.45), which was dissolved in benzene (6 ml), treated with AIBN (49 mg, 0.3 mmol), tris(trimethylsilyl)silane (718 mg, 3.0 mmol) and heated to reflux for 8 h. After evaporation,

the residue was treated dissolved in MeOH (0.3 ml), treated with a satd. soln. of NH_3 in MeOH (2.7 ml) and stirred for 4 h at r.t.. Workup and CC (SiO_2 (10 g), hexane/AcOEt 2:3 → AcOEt then AcOEt/MeOH 95:5 → 9:1) gave **3** (304 mg, 57% from **1**). Pink foam. TLC (CH_2Cl_2/MeOH 9:1): R_f 0.39. ^{1}H-NMR (400 MHz, $CDCl_3$): 0.88–1.08 (*m*, $^iPr_3Si$); 2.78 (br. *s*, MeHN–C(2)); 3.09 (*d*, *J* = 3.1, OH–C(3')); 3.44 (*m*, $H_2C(5')$); 3.77 (*s*, 2 MeO); 4.24 (*m*, H–C(4')); 4.55 (*m*, H–C(3')); 4.82 (*m*, H–C(2')); 5.04 (*d*, *J* = 3.9, OCH_2O); 5.20 (*d*, *J* = 3.9, OCH_2O); 6.06 (*d*, *J* = 4.7, H–C(1')); 6.81 (*d*, *J* = 8.6, 4 arom. H); 7.30–7.46 (*m*, 9 arom. H); 7.69 (*s*, H–C(8)); 12.06 (br. *s*, NH). ^{13}C-NMR (100 MHz, $CDCl_3$): 12.3 (*d*, Me_2CH); 18.2 (*q*, Me_2CH); 28.2 (*q*, *Me*HN–C(2)); 55.6 (*q*, MeO); 63.8 (*t*, C(5')); 71.2 (*d*, C(2')); 82.4 (*d*, C(3')); 84.1 (*d*, C(4')); 86.9 (*d*, C(1')); 87.1 (*s*, arom. C); 91.3 (*t*, OCH_2O); 113.6 (*d*, arom. C); 127.3 (*s*, C(5)); 128.3, 128.6, 128.8, 130.5 (4*d*, arom. C), 136.1, 136.2 (2*s*, arom. C); 136.3 (*d*, C(8)); 145.0 (*s*, arom. C); 152.3 (*s* , C(4)); 153.9 (*s*, C(2)); 158.9 (*s*, C(6)); 159.9 (*s*, arom. C). ESI-MS: 787.74 (100, $[M + H]^+$).

5'-O-(4,4'-Dimethoxytrityl)-N^2,N^2*-dimethyl-2'-O-{[(triisopropylsilyl)oxy]methyl}guanosine* (**5**).

A soln. of **3** (260 mg, 0.3 mmol, obtained from **2** as described above) in Py (2 ml) was treated with tms-Cl (46 mg, 0.8 mmol), stirred for 40 min at r.t, treated with 1,3-benzodithiolylium tetrafluoroborate (137 mg, 0.5 mmol) and stirred for 6 h at r.t. Workup and filtration (SiO_2 (11 g), hexane/AcOEt 3:7 → AcOEt) gave a mixture of 3'-O-tms and 3'-OH derivatives as a yellow foam (288 mg, ca. 94%; TLC (CH_2Cl_2/MeOH 9:1): R_f 0.54 and 0.50), which was dissolved in benzene (3 ml), treated with AIBN (25 mg, 0.15 mmol) and tris(trimethylsilyl)silane (359 mg, 1.5 mmol) and heated to reflux for 8 h. After evaporation, the reaction mixture was dissolved in MeOH (0.3 ml), treated with a satd. soln. of NH_3 in MeOH (2.7 ml) and stirred for 4 h at r.t.. Workup and CC (SiO_2 (5 g), hexane/AcOEt 2:3 → AcOEt then AcOEt/MeOH 95:5 → 9:1) gave **5** (144 mg, 50% from **1**). Pink foam. TLC (CH_2Cl_2/MeOH 9:1): R_f 0.45. ^{1}H-NMR (400 MHz, $CDCl_3$): 0.90–1.28 (*m*, $^iPr_3Si$); 3.03 (*d*, *J* = 3.9, OH–C(3')); 3.16 (*s*, Me_2N); 3.41 (*d*, *J* = 3.9, $H_2C(5')$); 3.79 (*s*, 2 MeO); 4.24 (br. *d*, *J* = 4.7, H–C(4')); 4.52 (br. *d*, *J* = 4.7, H–C(3')); 4.78 (*t*, *J* = 5.5, H–C(2')); 4.98 (*d*, *J* = 4.7, OCH_2O); 5.16 (*d*, *J* = 4.7, OCH_2O); 6.03 (*d*, *J* = 5.4, H–C(1')); 6.81 (*d*, *J* = 9.4, 4 arom. H); 7.19–7.45 (*m*, 9 arom. H); 7.66 (*s*, H–C(8)); 10.64 (br. *s*, NH). ^{13}C-NMR (100 MHz, $CDCl_3$): 12.3 (*d*, Me_2CH); 18.6 (*q*, Me_2CH); 38.5 (*q*, Me_2N); 55.6 (*q*, MeO); 64.1 (*t*, C(5')); 71.4 (*d*, C(2')); 82.3 (*d*, C(3')); 84.1 (*d*, C(4')); 86.7 (*d*, C(1')); 86.9 (*s*, arom. C); 91.2 (*t*, OCH_2O); 113.6 (*d*, arom. C); 127.3 (*s*, C(5)); 128.3, 128.6, 128.7, 130.5 (4*d*, arom. C), 136.1, 136.2 (2*s*,

arom. C); 136.6 (*d*, C(8)); 144.9 (*s*, arom. C); 151.9 (*s*, C(4)); 153.2 (*s*, C(2)); 158.9 (*s*, arom. C); 159.2 (*s*, C(6)). ESI-MS: 800.33 (100, $[M + H]^+$).

5'-O-*(4,4'-Dimethoxytrityl)*-N*1*-*methyl-2'*-O-*{[triisopropylsilyl)oxy]methyl}adenosine* (**7**).
A soln. of **6** (1.00 g, 1.3 mmol) in DMF (15 ml) was treated with MeI (0.08 ml, 1.3 mmol) and stirred for 5 days at r.t. Workup, evaporation and crystallization (acetone) gave **7** (1.02 g, 98%). White solid. M.p. 160° dec. TLC (CH_2Cl_2/MeOH 95:5): R_f 0.05. ^{1}H-NMR (400 MHz, $CDCl_3$): 0.89–1.15 (*m*, $^iPr_3Si$); 1.27 (br. *s*, HN–C(6)); 3.03 (br. *s*, OH–C(3')); 3.38 (*dd*, *J* = 3.7, 11.0, H–C(5')); 3.45 (*dd*, *J* = 3.7, 10.2, H'–C(5')); 3.66 (*s*, CH_3–N(1)); 3.80 (*s*, 2 MeO); 4.29 (*d*, *J* = 2.9, H–C(4')); 4.53 (*m*, H–C(3')); 4.81 (*t*, *J* = 5.9, H–C(2')); 4.96, 5.14 (2*d*, *J* = 5.0, OCH_2O); 6.03 (*d*, *J* = 6.6, H–C(1')); 6.82 (*d*, *J* = 8.8, 4 arom. H); 7.20–7.46 (*m*, 9 arom. H); 7.60 (*s*, H–C(2)); 7.85 (*s*, H–C(8)). ^{13}C-NMR (100 MHz, $CDCl_3$): 11.8 (*d*, Me_2CH); 17.8 (*q*, Me_2CH); 35.9 (*q*, CH_3–N(1)); 55.3 (*q*, MeO); 63.6 (*t*, C(5')); 71.0 (*d*, C(2')); 82.4 (*d*, C(3')); 84.3 (*d*, C(4')); 86.6 (*d*, C(1')); 86.8 (*s*, arom. C); 90.8 (*t*, OCH_2O); 113.2 (*d*, arom. C); 121.8 (*s*, C(5)); 126.9, 127.9, 128.0, 128.2, 130.1 (5*d*, arom. C); 135.63, 135.69 (2*s*, arom. C); 139.3 (*d*, C(8)); 144.6 (*s*, arom. C); 145.6 (*s*, C(4)); 147.3 (*s*, C(2)); 149.1 (*s*, C(6)); 158.6 (*s*, arom. C). ESI-MS: 770.36 (100, $[M + H]^+$).

N*6*-*Chloroacetyl-5'*-O-*(4,4'-dimethoxytrityl)*-N*1*-*methyl-2'*-O- *{[triisopropylsilyl)oxy]methyl} adenosine* (**8**).
A soln. of **7** (1.00 g, 1.3 mmol) in Py/$ClCH_2CH_2Cl$ 1:9 (55 ml) was treated with chloroacetic anhydride (890 mg, 5.2 mmol) and stirred for 1 h at –15°. After workup and evaporation, the residue was dissolved in MeOH (2 ml) and treated with a satd. soln. of NH_3 (7 ml) for 4 h at –15°. Workup and CC (SiO_2 (20 g), hexane/AcOEt 9:1 → AcOEt) gave **8** (0.70 g, 64%). Light yellow foam. TLC (CH_2Cl_2/MeOH 19:1): R_f 0.74. ^{1}H-NMR (400 MHz, $CDCl_3$): 0.99–1.08 (*m*, $^iPr_3Si$); 3.03 (*d*, *J* = 2.9, OH–C(3')); 3.35 (*dd*, *J* = 4.4, 10.3, H–C(5')); 3.46 (*dd*, *J* = 4.4, 10.3, H'–C(5')); 3.64 (s, CH_3–N(1)); 3.81 (*s*, 2 MeO); 4.29 (*m*, H–C(4')); 4.44 (*s*, $ClCH_2$); 4.52 (*m*, H–C(3')); 4.76 (*t*, *J* = 5.1, H–C(2')); 4.93, 5.14 (2*d*, *J* = 5.1, OCH_2O); 6.08 (*d*, *J* = 6.6, H–C(1')); 6.82 (*d*, *J* = 8.8, 4 arom. H); 7.20–7.34 (*m*, 9 arom. H); 7.81 (*s*, H–C(2)); 7.94 (*s*, H–C(8)). ^{13}C-NMR (100 MHz, $CDCl_3$): 11.8 (*d*, Me_2CH); 17.8 (*q*, Me_2CH); 36.8 (*q*, CH_3-N(1)); 46.0 (*t*, CH_2Cl); 55.3 (*q*, MeO); 63.4 (*t*, C(5')); 71.0 (*d*, C(2')); 82.7 (*d*, C(3')); 84.4 (*d*, C(4')); 86.5 (*d*, C(1')); 86.7 (*s*, arom. C); 91.0 (*t*, OCH_2O); 113.2 (*d*, arom. C); 122.4 (*s*, C(5)); 127.6, 127.9, 128.1, 128.3, 130.1 (5*d*, arom. C), 135.63, 135.69 (2*s*, arom. C); 139.3 (*d*, C(8)); 144.6 (*s*, arom. C); 145.6 (*s*, C(4)); 147.3 (*s*, C(6)); 149.1 (*d*, C(2)); 158.6 (*s*, arom. C); 178.09 (s, CO). ESI-MS: 846.74 (70, $[M + H]^+$) 848.74 (30, $[M + H]^+$).

5'-O-*(4,4'-Dimethoxytrityl)-5-methyl-2'*-O-*{[(triisopropylsilyl)oxy]methyl}uridine* (**10**).

A soln. of **9** (1.00 g, 1.8 mmol obtained according to (Gasparutto et al. 1992)) and $^{i}Pr_2NEt$ (0.78 ml, 4.6 mmol) in $ClCH_2CH_2Cl$ (35 ml) was treated with Bu_2SnCl_2 (560 mg, 2.0 mmol), stirred for 30 min at r.t., treated with TOM-Cl (450 mg, 2.0 mmol) and stirred for 12 h at r.t. Workup and CC (SiO_2 (30 g), hexane/AcOEt 9:1 → 1:4) gave **10** (500 mg, 38%). Light yellow foam. TLC (hexane/AcOEt 1:1): R_f 0.47. ^{1}H-NMR (400 MHz, $CDCl_3$): 1.04–1.16 (*m*, $^{i}Pr_3Si$); 1.38 (*s*, CH_3–C(5)); 3.13 (br. *s*, OH–C(3')); 3.40 (*dd*, *J* = 1.5, 11.4, H–C(5')); 3.53 (*dd*, *J* = 1.9, 11.5, H'–C(5')); 3.81 (*s*, 2 MeO); 4.19 (br. *s*, H–C(4')); 4.38 (*t*, *J* = 5.1, H–C(2')); 4.48 (br. *s*, H–C(3')); 5.03 (*d*, *J* = 4.5, OCH_2O); 5.24 (*d*, *J* = 4.5, OCH_2O); 6.14 (*d*, *J* = 8.3, H-C(1')); 6.84–6.86 (*m*, 4 arom. H); 7.26–7.43 (*m*, 9 arom. H); 7.66 (br. *s*, H–C(6)); 8.47 (br. *s*, H–N(3)). ^{13}C-NMR (100 MHz, $CDCl_3$): 12.1 (*d*, Me_2CH); 12.3 (*q*, CH_3–C(5)); 18.2 (*q*, Me_2CH); 55.7 (*q*, MeO); 63.5 (*t*, C(5')); 70.8 (*d*, C(2')); 82.9 (*d*, C(3')); 84.3 (*d*, C(4')); 86.2 (*s*, arom. C); 87.4 (*d*, C(1')); 91.2 (*t*, OCH_2O); 111.7 (*s*, C(5)); 113.6 (*d*, arom. C); 127.6, 128.2, 128.5, 130.6 (4*d*, arom. C); 135.6, 135.8 (2*s*, arom. C); 144.7 (*s*, C(6)); 150.7 (*s*, C(2)); 159.2 (*s*, arom. C); 163.9 (*s*, C(4)). ESI-MS: 373.29 (100, $[M + H]^{2+}$).

5,6-*Dihydro-5'*-O-*(4,4'-dimethoxytrityl)-2'*-O-*{[(triisopropylsilyl)oxy]methyl}uridine* (**12**).

A soln. of **11** (7.20 g, 13.0 mmol, obtained according to (Flockerzi et al. 1981)) and $^{i}Pr_2NEt$ (7.9 ml, 46.0 mmol) in $ClCH_2CH_2Cl$ (50 ml) was treated with Bu_2SnCl_2 (4.39 g, 14.4 mmol), stirred for 30 min at r.t., treated with TOM-Cl (0.45 g, 2.0 mmol), and stirred for 25 min at 80°. Workup and CC (SiO_2 (100 g), hexane/AcOEt 9:1 → 1:1) gave **12** (4.37 g, 47%) as light yellow foam. TLC (hexane/AcOEt 1:1): R_f 0.61. ^{1}H NMR (400 MHz, $CDCl_3$): 1.09-1.28 (*m*, $^{i}Pr_3Si$); 2.39–2.57 (*m*, $H_2C(5)$); 3.04 (*d*, *J* = 3.1, OH–C(3')); 3.31–3.44 (*m*, $H_2C(6)$, $H_2C(5')$); 3.66–3.72 (*m*, H–C(4')); 3.81 (*s*, 2 MeO); 4.26 (*t*, *J* = 5.5, H–C(2')); 4.39–4.40 (*m*, H–C(3')); 5.01 (*d*, *J* = 4.7, OCH_2O); 5.22 (*d*, *J* = 4.7, OCH_2O); 6.03 (*d*, *J* = 6.2, H–C(1')); 6.80–6.82 (*d*, *J* = 9.4, 4 arom. H); 7.22–7.44 (*m*, 9 arom. H). ^{13}C NMR (100MHz, $CDCl_3$): 12.3 (*d*, Me_2CH); 18.2 (*q*, Me_2CH); 31.5 (*t*, C(5)); 36.8 (*t*, C(6)); 55.7 (*q*, MeO); 63.9 (*t*, C(5')); 71.4 (*d*, C(3')); 79.8 (*d*, C(2')); 83.2 (*d*, C(4')); 86.9 (*s*, arom. C); 87.1 (*d*, C(1')); 90.9 (*t*, OCH_2O); 113.6 (*d*, arom. C); 127.4, 128.3, 128.6, 128.7, 130.5 (5*d*, arom. C); 135.9, 136.0 (2*s*, arom. C); 144.9 (*s*, arom. C); 152.5 159.0 (2*s*, C(2), C(4)); 169.9 (*s*, arom. C). ESI-MS: 757.38 (100, $[M + H]^{+}$).

5′-O-*(4,4′-Dimethoxytrityl)-5-methyl-2′*-O-*{[(triisopropylsilyl)oxy]methyl}cytidine* (**13**).

A soln. of **10** (0.65 g, 0.9 mmol) in Py (3.5 ml) was treated with DMAP (22 mg, 0.17 mmol), Ac_2O (0.17 ml, 1.77 mmol) and stirred for 3 h at r.t. Workup and evaporation gave a yellow solid foam (0.63 g), which was carefully dried (14 h at 60°, 0.05 mbar) and dissolved in MeCN (3 ml). Meanwhile, under Ar and at 4°, 4-chlorophenyl phosphorodichloridate (0.65 g, 2.65 mmol) was added dropwise to a suspension of finely powdered 1*H*-1,2,4-triazole (1.07 g, 15.4 mmol, dried by sublimation) in dry MeCN (6 ml). After 15 min at 4°, $^{i}Pr_2NEt$ (2.3 ml, 13.3 mmol) was added and after 40 min at r.t., the reaction mixture was again cooled to 4° and treated with the MeCN-soln. obtained before (3 ml, containing 0.65 g of the intermediate nucleoside). After 6 h at r.t., the soln. was diluted with dioxane (9 ml), treated with satd. aq. NH_3 (13 ml) and stirred for another 3 h at r.t. Extraction with CH_2Cl_2/10% aq. citric acid and satd. aq. $NaHCO_3$ gave a yellow solid foam (0.62 g) which was dissolved in THF/MeOH 5:4 (33 ml), cooled to 4° and treated with 2N NaOH (3.7 ml). After 30 min at 4°, the soln. was treated with AcOH (0.43 ml) and concentrated to 30 ml. Workup and CC (SiO_2 (8 g), CH_2Cl_2 → CH_2Cl_2/MeOH 19:1) gave **13** (0.60 g, 90%). Colorless foam. TLC (CH_2Cl_2/MeOH 15:185): R_f 0.50. ^{1}H-NMR (400 MHz, DMSO): 1.00–1.14 (*m*, $^{i}Pr_3Si$); 1.49 (*s*, CH_3–C(5)); 3.26 (br. *s*, H_2–C(5′)); 3.74 (*s*, 2 MeO); 3.96 (*dd*, *J* = 5.6, 3.2, H–C(4′)); 4.22 (*q*, *J* = 5.2, H–C(3′)); 4.27 (*t*, *J* = 5.4, H–C(2′)); 4.96 (*d*, *J* = 5.2, OCH_2O); 4.99 (*d*, *J* = 5.1, OCH_2O); 5.05 (*d*, *J* = 5.0, HO–C(3′)); 5.99 (*d*, *J* = 5.8, H–C(1′)); 6.89 (*d*, *J* = 8.8, 4 arom. H); 7.22–7.42 (*m*, 9 arom. H, H–C(6)). ^{13}C-NMR (100 MHz, DMSO): 11.9 (*d*, Me_2CH); 13.3 (*q*, *Me*-C(5)); 18.1 (*q*, *Me*$_2$CH); 55.5 (*q*, MeO); 63.9 (*t*, C(5′)); 67.1 (*d*, C(2′)); 78.2 (*d*, C(3′)); 83.4 (*d*, C(4′)); 86.4 (*s*, arom. C); 87.4 (*d*, C(1′)); 88.8 (*t*, OCH_2O); 102.2 (*s*, C(5)); 113.7 (*d*, arom. C); 127.3, 128.2, 128.4 130.2 (4*d*, arom. C); 135.8, 135.9, 138.4 (3*s*, arom. C); 145.1 (*d*, C(6)); 155.6 (*s*, C(2)); 158.6 (*s*, arom. C); 165.8 (*s*, C(4)). ESI-MS: 1491.80 (100, $[2M + 1]^+$).

N^4-*Acetyl-5′*-O-*(4,4′-dimethoxytrityl)-5-methyl-2′*-O-*{[(triisopropylsilyl)oxy]methyl}cytidine* (**14**).

A soln. of **13** (0.6 g, 0.80 mmol) in DMF (3.3 ml) was treated with Ac_2O (113 mg, 1.1 mmol) for 8 h at r.t. Workup and CC (SiO_2 (8 g), hexane/EtOAc 4:1 → 3:7) gave **14** (0.55 g, 90%). Colorless solid foam. TLC (hexane/AcOEt 1:9): R_f 0.52. ^{1}H-NMR (400 MHz, DMSO): 0.87–1.08 (*m*, $^{i}Pr_3Si$); 1.13 (*s*, CH_3–C(5)); 2.25 (*s*, *Me*CO); 3.26 (br. *s*, H_2–C(5′)); 3.74 (*s*, 2 MeO); 4.05 (br. *d*, *J* ≈ 4.6, H–C(4′)); 4.25 (*q*, *J* = 4.5, H–C(3′)); 4.33 (*t*, *J* = 4.7, H–C(2′)); 4.99 (*d*, *J* = 5.2, OCH_2O); 5.04 (*d*, *J* = 5.1, OCH_2O); 5.15 (*d*, *J* = 5.0, OH–C(3′)); 5.99 (*d*, *J* = 4.4, H–C(1′)); 6.89 (*d*, *J* = 8.8, 4 arom. H); 7.23–7.41 (*m*, 9 arom. H); 7.82 (br. *s*, H–C(6)); 9.82 (br.

s, NH–C(4)). ^{13}C-NMR (100 MHz, DMSO): 11.9 (*d*, Me_2CH); 13.8 (*q*, *Me*-C(5)); 18.1 (*q*, Me_2CH); 25.3 (*q*, *Me*CO); 55.5 (*q*, MeO); 63.6 (*t*, C(5')); 69.2 (*d*, C(2')); 78.7 (*d*, C(3')); 83.9 (*d*, C(4')); 86.4 (*d*, C(1')); 88.9 (*s*, arom. C); 90.4 (*t*, OCH_2O); 113.7 (*d*, arom. C); 127.3 (*s*, C(5)); 128.2, 128,4, 130.2, 135.7, 135.9 (5*d*, arom. C); 142.5, 145.0 (2*s*, arom. C); 152.5 (*d*, C(6)); 155.5 (*s*, C(2)); 158.6 (*s*, arom. C); 162.9 (*s*, C(4)); 171.2 (*s*, Me*C*O). ESI-MS: 788.34 (100, $[M + H]^+$).

5-Bromo-2,4-dichloropyrimidine (**15**).

To a mixture of freshly distilled phosphorus oxychloride (3.75 ml, 40.2 mmol) and diethylaniline (0.9 ml, 5.4 mmol) was added 5-bromouracil (0.75 g, 3.9 mmol) followed by reflux (oil bath 130°) for 1 h 40. The resulted dark red soln. was cooled to 25°, poured on a mixture of ice (100ml) and Et_2O (100 ml) and stirred for 30 min. After extraction and evaporation of the solvent, the residue was submitted to CC (SiO_2 (20 g), hexane/CH_2Cl_2 1:1 → 0:1) afforded **15** (0.7 g, 93%). Light yellow oil. TLC (CH_2Cl_2): R_f 0.68. ^{1}H-NMR (400 MHz, $CDCl_3$): 8.70 (*s*, H–C(6)). ^{13}C-NMR (100 MHz, $CDCl_3$): 118.8 (*s*, Br–C(5)); 158.8 (*s*, C(2)); 161.4 (*s*, C(4)); 161.5 (*s*, H–C(6)).

5-bromo-2,4-di-tert-butoxypyrimidine (**16**).

A suspension of potassium tert-butoxyde (2.5 g, 22.2 mmol) in *t*-BuOH (50 ml) was treated dropwise with **15** (1.0 g, 4.4 mmol) and the red suspension was stirred at 25° for 14 h. The reaction mixture was poured on water (100 ml) Et_2O (100 ml) extracted twice with Et_2O (2 x 100 ml). The combined organic layers were washed with brine and dried over Na_2SO_4. The careful evaporation of the solvent furnished a light yellow oil which has been submitted to CC (SiO_2 (40 g), hexane/CH_2Cl_2 9:1 → 1:1). We obtained **16** (0.66 g, 50%). Colourless oil. TLC (CH_2Cl_2): R_f 0.71. ^{1}H-NMR (400 MHz, $CDCl_3$): 1.61 (*s*, Me_3C); 1.67 (*s*, Me_3C); 8.26 (*s*, H–C(6)). ^{13}C-NMR (100 MHz, $CDCl_3$): 28.7 (*q*, Me_3C); 81.2 (*s*, Me_3C); 83.7 (*s*, Me_3C); 100.0 (*s*, Br–C(5)); 159.4 (*d*, H–C(6)); 163.4 (*s*, C(2)); 166.1 (*s*, C(4)). EI-MS: 303-305 (100, $[M]^+$).

2,3-O-*Isopropylidene-5*-O-*(l-methoxy-l-methyl-ethyl)*-D-*ribono-l,4-1actone* (**17**) .

A suspension of *D-ribono-l,4-1actone* (1.50 g, 10.1 mmol) in dry 2,2-dimethoxypropane (40 ml) and Na_2SO_4 (0.75 g) was treated with p-TsOH (30 mg, 0.17 mmol) at 25° for 12 h. After neutralization by addition of K_2CO_3 (48 mg, 0.34 mmol), the reaction mixture was filtered off and the filtrate evaporated to dryness. The residue submitted to CC (SiO_2 (40 g), hexane/CH_2Cl_2 9:1 → 1:1) and recrystallized from hexane offering **17** (1.06 g, 40%) as

colourless needles. TLC (hexane/AcOEt 3:2): R_f 0.74. ^{1}H-NMR (400 MHz, $CDCl_3$): 1.28, 1.30, 1.37, 1.45 (4*s*, 4 Me); 3.16 (*s*, MeO); 3.51 *(dd, J* = 1.6, 10.8, H–C(5)); 3.74 *(dd, J* = 2.3, 10.8, H'–C(5)); 4.66 (*dt*, *J* = 1.6, 2.3, H–C(4)); 4.69 (*d*, *J* = 5.5, H–C(3)); 4.72 (*d*, *J* = 5.5, H–C(2)). ^{13}C-NMR (100 MHz, $CDCl_3$): 26.8, 25.6, 24.4, 23.9 (4*q*, 4 Me); 48.8 (*q*, MeO); 60.2 (*t*, C(5)); 75.70 (*d*, C(2)); 78.5 (*d*, C(3)); 80.9 (*d*, C(4)); 100.5 (*s*, *C*Me_2OC(5)); 113.09 (*s*, *C*Me_2OC(2)C(3)); 174.34 (*s*, C(1)).

l-C-*[2,4-bis(1,1-dimethylethoxy)-5-pyrimidinyl]-5*-O-*(1-methoxy-1-methylethyl)-2,3-O-(1-methylethylidene)-α*-D-*ribofuranose* (**18**).

A soln. of tert-Butyl Lithium (0.4 ml, ca.0.66 mmol) in (8 ml) of dry THF was cooled to -78° and stirred for 30 min giving an intense yellow soln.. Thus, a soln. of **16** (0.101 g, 0.33 mmol) in THF (2 ml) was added over 1 h 30 and stirred at -78° for 1 h after the end of addition. The reaction mixture was treated with a soln. of **17** (0.83 g, 0.31 mmol) in THF (2 ml) added over 1 h 30, and stirred at -78° for 2 h. Workup (H_2O/Et_2O) and CC (SiO_2 (10 g), hexane/AcOEt 4:1 → 1:4) afforded **18** (140 mg, 89%) as a mixture of α/β 1:8. TLC (hexane/AcOEt 1:1): R_f 0.50. ESI-MS: 485.37 (100, $[M + H]^+$).

l-C-*[2,4-bis(1,1-dimethylethoxy)-5-pyrimidinyl]-5*-O-*(1-methoxy-1-methylethyl)-2,3-O-(1-methylethylidene)*-D-*ribitol* (**19**).

Under Argon, a soln. of RedAl® (2 ml, ca. 7 mmol) in CH_2Cl_2 (60 ml) was cooled to -78°, treated dropwise with a soln. of **18** (0.4 g, 0.82 mmol) in CH_2Cl_2 (10 ml) and stirred at -78° for 8 h. Thus, the reaction mixture was allowed to come to 4° 30min, poured on a mixture of Et_2O (100 ml) and a 10% soln. of potassium sodium tartrate (100 ml) and well stirred until to obtain well defined layers. The aqueous layer was extracted with Et_2O (3 x 100 ml), the combined organic layers were washed with brine (150 ml) and dried over Na_2SO_4. CC (SiO_2 (40 g), hexane/AcOEt 9:1 → 2:3) afforded **19** (1.5 g, 85%). Colourless foam. TLC (hexane/AcOEt 1:1): R_f 0.35. ^{1}H-NMR (400 MHz, $CDCl_3$): 1.34 (*s*, Me); 1.39 (*s*, Me_2C); 1.56 (*s*, Me); 1.60 (*s*, Me_3C); 1.62 (*s*, Me_3C); 3.08 (*d*, *J* = 4.7, HO–C(4')); 3.24 (*s*, MeO); 3.26 *(d, J* = 6.7, HO–C(2')); 3.54 *(dd, J* = 6.8, 9.8, H–C(5')); 3.70 (*dd*, *J* = 2.8, 9.8, H'–C(5')); 4.16 (*dd*, *J* = 6.5, 8.2, H–C(3')); 4.24–4.31 (*m*, H–C(4')); 4.33 (*dd*, *J* = 3.9, 6.3, H–C(2')); 5.23 *(d, J* = 6.6, H–C(1')); 8.28 (*s,* H–C(6)). ^{13}C-NMR (100 MHz, $CDCl_3$): 24.8, 24.9, 25.4, 27.3, 28.8, 29.0 (6*q*, 10 Me), 49.1 (*q*, MeO); 63.0 (*t*, C(5')); 65.6 (*d*, C(4')); 69.3 (*d*, C(3')); 77.2 (*d*, C(2')); 78.5 (*d*, C(1')); 80.3 (*s*, *C*Me_3); 82.1 (*s*, *C*Me_3); 100.8 (*s*, *C*Me_2OC(5')); 108.9 (*s*,

CMe_2OC(2')C(3')); 116.0 (*s*, C(5)); 157.2 (*d*, H–C(6)); 163.7 (*s*, C(2)); 166.9 (*s*, C(4)). ESI-MS: 487.37 (100, $[M + H]^+$).

1,4-Anhydro-l-C-*[2,4-bis(1,1-dimethylethoxy)-5-pyrimidinyl]-5*-O-*(1-methoxy-1-methylethyl)-2,3-O-(1-methylethylidene)*-D-*ribitol* (**20**).

A cold soln. of **19** (1.5 g, 3.06 mmol) in THF (75 ml) was treated with triphenylphosphine (1.2 g, 4.59 mmol) and stirred at 25° for 10 min. Thus, the reaction mixture was cooled to 0° and treated dropwise with DIAD (0.93 g, 4.59 mmol) followed by stirring at 0° for 8 h. Evaporation of the solvent and CC (SiO_2 (40 g), hexane/AcOEt 95:5 → 1:1) afforded **20** (1.3 g, 87%) as Colourless foam. TLC (hexane/AcOEt 1:1): R_f 0.72. ^{1}H-NMR (400 MHz, $CDCl_3$): 1.36 (*s*, Me); 1.38 (*s*, Me_2C); 1.60 (*s*, Me); 1.61 (*s*, Me_3C); 1.65 (*s*, Me_3C); 3.23 (*s*, MeO); 3.57 *(dd, J* = 5.9, 10.5, H–C(5')); 3.69 (*dd*, *J* = 3.3, 10.5, H'–C(5')); 4.15 (*m*, H–C(4')); 4.58 (br. *t*, *J* ≈ 5.3, H–C(3')); 4.64 (*dd*, *J* = 4.1, 6.5, H–C(2')); 4.95 *(d, J* = 3.4, H–C(1')); 8.25 (*s,* H–C(6)). ^{13}C-NMR (100 MHz, $CDCl_3$): 22.0, 22.1, 22.3, 24.4, 24.8, 26.1, 28.1, 28.5, 29.0, 32.7 (10 *q*, 10 Me), 49.0 (*q*, MeO); 61.8 (*t*, C(5')); 72.6 (*d*, C(4')); 80.6 (*d*, C(3')); 81.3 (*d*, C(2')); 82.2 (*d*, C(1')); 83.6 (*s*, *C*Me_3); 86.1 (*s*, *C*Me_3); 100.6 (*s*, *C*Me_2OC(5')); 114.1 (*s*, *C*Me_2OC(2')C(3')); 114.6 (*s*, C(5)); 157.3 (*d*, H–C(6)); 164.3 (*s*, C(2)); 167.7 (*s*, C(4)). ESI-MS: 481.37 (100, $[M + H]^+$).

5'-O-*(4,4'-Dimethoxytrityl)pseudouridine* (**22**).

A soln. of **19** (200 mg, 0.41 mmol) in AcOH:H_2O 7:3 (5 ml) was heated at 65° for 5 h. After evaporation of the solvent and coevaporation with water (2 x 5 ml) and toluene (2 x 5ml), the residue was dissolved in Py (3 ml), treated with $(MeO)_2$TrCl (152 mg, 0.45 mmol) and stirred at r.t. for 12 h. Workup and CC (SiO_2 (10 g), hexane/AcOEt 4:1 → 0:1 then AcOEt/MeOH 9:1 → 1:1) offered **22** (170 mg, 76%). Light yellow solid foam. TLC (CH_2Cl_2/MeOH 9:1): R_f 0.42. ^{1}H-NMR (400 MHz, Acetone): 3.24–3.38 (*m*, H_2C(5')); 3.80 (*s*, 2 MeO); 3.90 (br. *s*, HO–C(2')); 4.06 (*q*, *J* = 4.7, H–C(4')); 4.14 (*t*, *J* = 4.7, H–C(3')); 4.20 (br. *q*, *J* ≈ 4.2, H–C(2')); 4.72 (*d*, *J* = 4.4, H–C(1')); 4.82 (br. *s*, HO–C(3')); 6.90 (*d*, *J* = 8.7, 4 arom. H); 7.21–7.56 (*m*, 9 arom. H); 7.72 (*s*, H–C(6)); 10.00, 10.22 (2 br. *s*, 2 NH). ^{13}C-NMR (100 MHz, Acetone): 55.0 (*q*, 2 MeO); 64.2 (*t*, C(5')); 72.1 (*d*, C(3')); 75.7 (*d*, C(2')); 80.0 (*d*, C(4')); 83.1 (*d*, C(1')); 86.3 (*s*, arom. C); 112.5 (*s*, C(5)); 113.1, 113.4 (2*d*, arom. C); 127.0, 128.1, 128.2, 128.5, 130.4, (5*d*, arom. C); 136.5, 136.6 (2*s*, arom. C); 138.6 (*d*, C(6)); 145.7 (*s*, arom. C); 151.1 (*s*, C(2)); 159.1 (*s*, arom. C); 164.5 (*s*, C(4)). ESI-MS: 569.37 (100, $[M + Na]^+$).

5'-O-*(4,4'-Dimethoxytrityl)-2',3'*-O-*bis(trimethylsilyl)pseudouridine* (**23**).

A cold (4°) soln. of **22** (170 mg, 0.41 mmol) in Py (6ml) was treated with TMSCl (0.16 ml, 1.24 mmol) and stirred for 3 h at r.t. Workup gave **23** (196 mg, 92%). Yellow foam. TLC (hexane/AcOEt 9:1): R_f 0.69. ^{1}H-NMR (400 MHz, $CDCl_3$): 0.03, 0.09 (2*s*, 2 Me_3Si); 3.26 (*d*, *J* = 10.2, H–C(5')); 3.66 (*d*, *J* = 10.2, H'–C(5')); 3.97, 3.98 (2*s*, 2 MeO); 4.12 (br. *s*, H–C(4'), H–C(3'), H–C(2')); 4.82 (*s*, H–C(1')); 6.85 (*m*, 4 arom. H); 7.22–7.45 (*m*, 9 arom. H); 7.73 (*s*, H–C(6)); 8.76, 8.25 (2 br. *s*, 2 NH). ^{13}C-NMR (100 MHz, $CDCl_3$): 0.6, 0.8 (2*q*, Me_3Si); 55.7 (*q*, 2 MeO); 62.1 (*t*, C(5')); 71.2 (*d*, C(3')); 76.3 (*d*, C(2')); 80.4 (*d*, C(4')); 80.9 (*d*, C(1')); 86.8 (*s*, arom. C); 113.4 (*s*, C(5)); 113.6, 113.8 (2*d*, arom. C); 127.6, 128.3, 128.7, 129.0, 130.7, 130.8, (6*d*, arom. C); 136.1, 136.4 (2*s*, arom. C); 138.7 (*d*, C(6)); 144.9 (*s*, arom. C); 151.5 (*s*, C(2)); 159.1 (*s*, arom. C); 162.7 (*s*, C(4)). ESI-MS: 689.28 (100, $[M - H]^-$).

5'-O-*(4,4'-Dimethoxytrityl)*-N^1,N^3-*bis{[(triisopropylsilyl)oxy]methyl}-2',3'*-O-*bis(trimethyl silyl)pseudouridine* (**24**).

A soln. of **23** (208 mg, 0.30 mmol) in DMF (4 ml) was treated with K_2CO_3 (198 mg, 1.5 mmol), stirred for 10 min at r.t., treated with TOM-Cl (669 mg, 3.0 mmol) and stirred for 3 h at r.t. Workup gave **24** (303 mg, 93%) without further purification. TLC (hexane/AcOEt 7:3): R_f 0.82. ^{1}H-NMR (400 MHz, $CDCl_3$): 0.02, 0.18 (2*s*, 2 Me_3Si); 0.90–0.96 (*m*, $^iPr_3Si$); 1.04–1.14 (*m*, $^iPr_3Si$); 3.24 (*dd*, *J* = 4.6, 10.2, H–C(5')); 3.45 (*dd*, *J* = 3.4, 10.3, H'–C(5')); 3.81 (*s*, 2 MeO); 3.95 (*dd*, *J* = 4.2, 7.8, H–C(4')); 4.10–4.25 (*m*, H–C(3'), H–C(2')); 4.64 (*d*, *J* = 8.8, $OCH_2N(3)$); 4.82 (*s*, H–C(1')); 5.18 (*d*, *J* = 8.8, $OCH_2N(3)$); 5.59 (*d*, *J* = 8.8, $OCH_2N(1)$); 5.68 (*d*, *J* = 7.9, $OCH_2N(1)$); 6.82 (*m*, 4 arom. H); 7.25–7.52 (*m*, 9 arom. H); 7.58 (*s*, H–C(6)). ^{13}C-NMR (100 MHz, $CDCl_3$): 0.6, 0.8 (2*q*, Me_3Si); 12.2, 12.5 (2*d*, Me_2CH); 18.2, 18.3 (*q*, Me_2CH); 55.6 (*q*, 2 MeO); 63.4 (*t*, C(5')); 65.2 (*t*, $OCH_2N(3)$); 72.1 (*t*, $OCH_2N(1)$); 72.2 (*d*, C(3')); 75.9 (*d*, C(2')); 80.9 (*d*, C(4')); 81.7 (*d*, C(1')); 86.4 (*s*, arom. C); 113.1 (*s*, C(5)); 113.4 (*d*, arom. C); 127.3, 128.2, 128.9, 130.6, 130.7 (5*d*, arom. C); 136.3, 136.4 (2*s*, arom. C); 140.1 (*d*, C(6)); 145.0 (*s*, arom. C); 150.9 (*s*, C(2)); 158.9 (*s*, arom. C); 161.5 (*s*, C(4)). MALDI-MS: 1086.65 (100, $[M + Na]^+$) **.**

5'-O-*(4,4'-Dimethoxytrityl)*-N^1,N^3-*bis{[(triisopropylsilyl)oxy]methyl}-2'*-O-*{[(triisopropyl silyl)oxy]methyl}pseudouridine* (**26**).

A soln. of **24** (303 mg, 0.28 mmol) in MeOH (2 ml) was treated with a satd. soln. of NH_3 in MeOH (6 ml) and stirred for 3 h at r.t. After evaporation, the residue was dissolved in $ClCH_2CH_2Cl$ (7 ml), treated with $^iPr_2NEt$ (0.15 ml, 1.12 mmol) and Bu_2SnCl_2 (95 mg, 0.30

mmol), and stirred at r.t. After 30 min, the reaction mixture was treated with TOM-Cl (75 mg, 0.34 mmol), and stirred for 2 h at 65°. Workup and CC (SiO_2 (10 g), hexane/AcOEt 19:1 → 1:1) offered **26** (108 mg, 34%). Light yellow foam. TLC (hexane/AcOEt 4:1): R_f 0.55. ^{1}H-NMR (400 MHz, $CDCl_3$): 0.81–1.00 (*m*, $^iPr_3Si$); 1.04–1.23 (*m*, $^iPr_3Si$); 3.34–3.46 (*m*, $H_2C(5')$); 3.78 (*s*, 2 MeO); 3.99–4.06 (*m*, H–C(4')); 4.18 (*m*, H–C(3'), H–C(2')); 4.55 (*d*, *J* = 8.9, $OCH_2N(3)$); 4.98 (*s*, H–C(1')); 5.10 (*d*, *J* = 4.7, OCH_2O); 5.24–5.30 (*m*, $OCH_2N(1)$, $OCH_2N(3)$, OCH_2O); 5.61 (*s*, $OCH_2N(1)$); 6.82 (*d*, *J* = 8.8, 4 arom. H); 7.19 (*t*, *J* = 7.2, 1 arom. H); 7.30 (*t*, *J* = 7.2, 2 arom. H); 7.36 (*d*, *J* = 8.5, 4 arom. H); 7.48 (*d*, *J* = 7.5, 2 arom. H); 7.60 (*s*, H–C(6)). ^{13}C-NMR (100 MHz, $CDCl_3$): 12.2, 12.3, 12.4, 12.5, 12.7, 13.0 (6*d*, Me_2CH); 17.8, 18.0, 18.1, 18.2, 18.3 (5*q*, Me_2CH); 55.5 (*q*, 2 MeO); 63.3 (*t*, C(5')); 65.3 (*t*, $OCH_2N(3)$); 70.4 (*t*, $OCH_2N(1)$); 72.4 (*d*, C(3')); 79.5 (*d*, C(2')); 82.1 (*d*, C(4')); 84.7 (*d*, C(1')); 86.5 (*s*, arom. C); 91.1 (*t*, OCH_2O); 113.0 (*s*, C(5)); 113.5 (*d*, arom. C); 127.2, 128.7, 128.8, 129.0, 130.5 (5*d*, arom. C); 136.3, 136.6 (2*s*, arom. C); 139.8 (*d*, C(6)); 145.3 (*s*, arom. C); 150.8 (*s*, C(2)); 158.9 (*s*, arom. C); 161.8 (*s*, C(4)). MALDI-MS: 1128.7 (100, $[M + Na]^+$).

5'-O-*(4,4'-Dimethoxytrityl)*-N^1,N^3-*bis{[(pivaloyl)oxy]methyl}pseudouridine* (**28**).

A soln. of **23** (138 mg, 0.20 mmol) in DMF (2 ml) was treated with K_2CO_3 (138 mg, 1.0 mmol), stirred for 10 min at r.t., treated with pom-Cl (90 mg, 0.6 mmol) and stirred for 3 h at r.t. After workup and evaporation to dryness, the residue was dissolved in THF (0.5 ml) and treated with Bu_4NF (1M) in THF (20 ml) at r.t. for 70 sec. The reaction was quenched by addition of Py/H_2O/MeOH 3:1:1 (1.5 ml). Workup and evaporation of solvent offered **28** (139 mg, 90%). Light yellow foam. TLC (CH_2Cl_2/MeOH 199:1): R_f 0.24. ^{1}H-NMR (400 MHz, $CDCl_3$): 1.15 (*s*, Me_3C); 1.22 (*s*, Me_3C); 3.11 (*s*, HO); 3.29 (*dd*, *J* = 3.5, 10.2, H–C(5')); 3.39 (*dd*, *J* = 3.7, 10.3, H'–C(5')); 3.81 (*s*, 2 MeO); 4.16 (*t*, *J* = 6.1, H–C(4')); 4.20–4.29 (*m*, H–C(3'), H–C(2')); 4.69 (*s*, HO); 4.80 (*d*, *J* = 6.0, H–C(1')); 5.33 (*d*, *J* = 10.3, $OCH_2N(3)$); 5.60 (*d*, *J* = 10.3, $OCH_2N(3)$); 6.00 (*d*, *J* = 9.4, $OCH_2N(1)$); 6.03 (*d*, *J* = 9.4, $OCH_2N(1)$); 6.84 (*d*, *J* = 7.8, 4 arom. H); 7.20–7.46 (*m*, 9 arom. H); 7.70 (*s*, H–C(6)). ^{13}C-NMR (100 MHz, $CDCl_3$): 27.2, 27.4 (2*q*, Me_3CCO); 39.3, 43.9 (2*s*, Me_3CCO); 55.7 (*q*, 2 MeO); 63.9 (*t*, C(5')); 65.1 (*t*, $OCH_2N(3)$); 71.7 (*t*, $OCH_2N(1)$); 73.5 (*d*, C(3')); 76.3 (*d*, C(2')); 78.9 (*d*, C(4')); 84.8 (*d*, C(1')); 86.7 (*s*, arom. C); 113.6 (*d*, arom. C); 113.8 (*s*, C(5)); 127.3, 128.3, 128.5, 130.5 (4*d*, arom. C); 136.1, 136.7 (2*s*, arom. C); 140.5 (*d*, C(6)); 145.2 (*s*, arom. C); 150.2 (*s*, C(2)); 159.0 (*s*, arom. C); 163.5 (*s*, C(4)); 177.7, 178.2 (2*s*, Me_3CCO). ESI-MS: 797.69 (100, $[M + Na]^+$)

5'-O-*(4,4'-Dimethoxytrityl)*-N^1,N^3-*bis{[(pivaloyl)oxy]methyl}-2'*-O-*{[(triisopropylsilyl)oxy]methyl}pseudouridine* (**29**).

A soln. of **28** (290 mg, 0.37 mmol) in $ClCH_2CH_2Cl$ (15 ml) was treated with $^iPr_2NEt$ (0.2 ml, 1.5 mmol) and Bu_2SnCl_2 (126 mg, 0.41 mmol), and stirred at r.t. After 30 min, the reaction mixture was treated with TOM-Cl (100 mg, 0.44 mmol), and stirred for 1.5 h at 65°. Workup and CC (SiO_2 (10 g), hexane/AcOEt 9:1 → 1:1) offered **29** (90 mg, 25%). Light yellow foam. TLC (hexane/AcOEt 1:1): R_f 0.72. ^{1}H-NMR (400 MHz, $CDCl_3$): 0.90–1.12 (*m*, $^iPr_3Si$); 1.14 (*s*, Me_3C); 1.20 (*s*, Me_3C); 3.37 (*d*, *J* = 7.7, HO); 3.44 (*dd*, *J* = 3.9, 14.5, H–C(5')); 3.50 (*dd*, *J* = 2.0, 10.7, H'–C(5')); 3.81 (*s*, 2 MeO); 3.98–4.07 (*m*, H–C(4')); 4.17–4.29 (*m*, H–C(3'), H–C(2')); 4.66 (*d*, *J* = 10.2, $OCH_2N(3)$); 4.97 (*s*, H–C(1')); 5.09 (*d*, *J* = 4.7, OCH_2O); 5.26 (*d*, *J* = 4.7, OCH_2O); 5.42 (*d*, *J* = 10.3, $OCH_2N(3)$); 5.97 (*m*, $OCH_2N(1)$); 6.84 (*d*, *J* = 8.6, 4 arom. H); 7.18–7.40 (*m*, 9 arom. H); 7.73 (*s*, H–C(6)). ^{13}C-NMR (100 MHz, $CDCl_3$): 12.3 (*d*, Me_2CH); 18.2 (*q*, Me_2CH); 27.2, 27.3 (2*q*, Me_3CCO); 39.2, 39.3 (2*s*, Me_3CCO); 55.6 (*q*, 2 MeO); 63.1 (*t*, C(5')); 65.1 (*t*, $OCH_2N(3)$); 70.2 (*t*, $OCH_2N(1)$); 71.7 (*d*, C(3')); 76.8 (*d*, C(2')); 79.0 (*d*, C(4')); 82.2 (*d*, C(1')); 86.7 (*s*, arom. C); 91.0 (*t*, OCH_2O); 113.0 (*s*, C(5)); 113.6 (*d*, arom. C); 127.3, 128.3, 128.8, 129.6, 130.7 (5*d*, arom. C); 136.2, 136.4 (2*s*, arom. C); 141.6 (*d*, C(6)); 145.3 (*s*, arom. C); 150.5 (*s*, C(2)); 159.0 (*s*, arom. C); 161.2 (*s*, C(4)); 177.7, 177.8 (2*s*, Me_3CCO). MALDI-MS: 983.8 (100, [*M* + Na]$^+$)

5'-O-*(4,4'-Dimethoxytrityl)-2'*-O-*{[(triisopropylsilyl)oxy]methyl}inosine* (**31**).

A soln. of **30** (1.0 g, 1.9 mmol, prepared according to (Wenter and Pitsch 2003)) in MeOH (2 ml) was treated with a satd. soln. of NH_3 in MeOH (6 ml) and stirred for 3 h at r.t. After evaporation, the residue was dissolved in Py (5.5 ml), treated with $(MeO)_2TrCl$ (0.78 g, 2.3 mmol) and stirred for 2 h at r.t. Workup and CC (SiO_2 (25 g), hexane/AcOEt 1:1 then CH_2Cl_2/MeOH 99:1 → 9:1) gave **31** (939 mg, 67%). Yellow foam. TLC (CH_2Cl_2/MeOH 1:9): R_f 0.50. ^{1}H-NMR (400 MHz, $CDCl_3$) 0.90–1.10 (*m*, $^iPr_3Si$); 3.10 (*d*, *J* = 3.1, OH–C(3')); 3.42 (*dd*, *J* = 3.9, 10.2, H–C(5')); 3.47 (*dd*, *J* = 3.9, 10.2, H'–C(5')); 3.80 (*s*, 2 MeO); 4.32 (*m*, H–C(4')); 4.55 (*m*, H–C(3')); 4.84 (*t*, *J* = 4.7, H–C(2')); 4.98 (*d*, *J* = 4.7, OCH_2O); 5.17 (*d*, *J* = 4.7, OCH_2O); 6.17 (*d*, *J* = 5.4, H–C(1')); 6.82 (*d*, *J* = 8.8, 4 arom. H); 7.30–7.46 (*m*, 9 arom. H); 7.81 (*s*, H–C(8)); 7.94 (*s*, H–C(2)); 12.98 (br. *s*, NH). ^{13}C-NMR (400MHz, $CDCl_3$): 12.2 (*d*, Me_2CH); 18.2 (*q*, Me_2CH); 55.6 (*q*, MeO); 63.9 (*t*, C(5')); 71.4 (*d*, C(2')); 82.9 (*d*, C(3')); 84.8 (*d*, C(4')); 87.1 (*d*, C(1')); 87.4 (*s*, arom. C); 91.3 (*t*, OCH_2O); 113.6 (*d*, arom. C); 125.7 (*s*, C(5)); 127.4, 128.3, 128.6, 129.5, 130.5 (5*d*, arom. C), 136.0, 136.1 (2*s*, arom. C); 139.5

(*d*, C(8)); 144.9 (*s*, arom. C); 145.2 (*s*, C(4)); 149.3 (*s*, C(2)); 158.9 (*s*, C(6)); 159.6 (*s*, arom. C). ESI-MS: 757.34 (100, $[M + H]^+$).

5'-O-*(4,4'-Dimethoxytrityl)-1-methyl-2'*-O-*{[(triisopropylsilyl)oxy]methyl}inosine* (**32**).
A soln. of **31** (76 mg, 0.1 mmol) in DMF was treated with K_2CO_3 (15 mg, 0.1 mmol), stirred for 1.5 h at –15°, treated with MeI (31 mg, 0.2 mmol) and stirred for 2 h at r.t. Workup, evaporation and CC (SiO_2 (2 g), hexane/AcOEt 3:2 → AcOEt) gave **32** (77 mg, 98%). White solid. TLC (CH_2Cl_2/MeOH 19:1): R_f 0.50. ^{1}H-NMR (400 MHz, $CDCl_3$): 1.00–1.12 (*m*, $^iPr_3Si$); 2.99 (*d*, *J* = 4.7, OH–C(3')); 3.39 (*dd*, *J* = 4.2, 10.4 , H–C(5')); 3.43 (*dd*, *J* = 3.4, 10.4, H'–C(5')); 3.61 (*s*, CH_3–N(1)); 3.78 (*s*, 2 MeO); 4.29 (*q*, *J* = 4.1, H–C(4')); 4.53 (*q*, *J* = 4.2, H–C(3')); 4.82 (*t*, *J* = 5.0, H–C(2')); 4.94, 5.13 (2*d*, *J* = 4.8, OCH_2O); 6.11 (*d*, *J* = 4.9, H–C(1')); 6.81 (*m*, 4 arom. H); 7.18–7.35, 7.41–7.47 (*m*, 9 arom. H); 7.83 (s, H–C(8)); 7.93 (s, H–C(2)). ^{13}C-NMR (100 MHz, $CDCl_3$): 12.2 (*d*, Me_2CH); 18.1 (*q*, Me_2CH); 34.5 (*q*, CH_3–N(1)); 55.6 (*q*, MeO); 64.0 (*t*, C(5')); 71.5 (*d*, C(3')); 82.8 (*d*, C(2')); 84.8 (*d*, C(4')); 86.8 (*s*, arom. C); 87.0 (*d*, C(1')); 91.3 (*t*, OCH_2O); 113.6 (*d*, arom. C); 125.5 (*s*, C(5)); 127.29, 128.23, 128.56, 130.48, 130.51 (5*d*, arom. C); 136.0, 136.1 (2s, arom. C); 139.2 (*d*, C(8)); 145.0 (*s*, arom. C); 147.5 (*s*, C(4)); 148.1 (*s*, C(2)); 157.4 (*s*, C(6)); 159.0 (*s*, arom. C). ESI-MS: 771.40 (100, $[M + H]^+$).

3',5'-Di-O-*acetyl*-N^6-*isopentenyl-2'*-O-*{[(triisopropylsilyl)oxy]methyl}adenosine* (**34**).
A soln. of 3,3-dimethylallylamine·HCl (264 mg, 2.2 mmol) in Py (5 ml) was treated with Et_3N (0.6 ml, 4.3 mmol) and **33** (200 mg, 0.3 mmol, prepared according to (Wenter and Pitsch 2003)), and stirred for 1 h at r.t. Workup and CC (SiO_2 (5 g), hexane/AcOEt 9:1 → 3:7) gave **34** (200 mg, 89%) as yellow foam. TLC (hexane/AcOEt 1:1): R_f 0.50. ^{1}H-NMR (100MHz, $CDCl_3$): 0.89-1.05 (*m*, $^iPr_3Si$); 1.76 (*s*, CH_3); 1.78 (*s*, CH_3); 2.13 (*s*, MeCO); 2.18 (*s*, MeCO); 4.23 (br. *s*, H_2C–NH); 4.37–4.50 (*m*, H–C(3'), H–C(4'), $H_2C(5')$); 4.86 (*d*, *J* = 4.7, OCH_2O); 4.92 (*d*, *J* = 4.7, OCH_2O); 5.22 (*t*, *J* = 6.3, H–C(11)); 5.64 (br. *s*, H–C(2')); 6.13 (*d*, *J* = 5.7, H–C(1')); 7.88 (*s*, H–C(8)); 8.47 (br. *s*, H–C(2)). ^{13}C-NMR (100MHz, $CDCl_3$): 12.1 (*q*, *Me*C=); 12.2 (*d*, Me_2CH); 18.0 (*q*, Me_2CH); 18.4 (*q*, *Me'*C=); 21.2 (*q*, 2 *Me*CO); 26.1 (*t*, CH_2NH); 63.9 (*t*, C(5')); 71.9 (*d*, C(3')); 76.7 (*d*, C(2')); 80.9 (*d*, C(4')); 87.8 (*d*, C(1')); 89.9 (*t*, OCH_2O); 107.6 (*s*, *C*H=C); 120.6 (*s*, C(5)); 137.4 (*s*, Me_2C=); 138.9 (*d*, C(8)); 152.9 (*s*, C(4)); 153.8 (*d*, C(2)); 155.1 (*s*, C(6)); 170.5 (*s*, COMe); 170.8 (*s*, COMe). ESI-MS: 606.80 (100, $[M + H]^+$).

5'-O-*(4,4'-Dimethoxytrityl)*-N^6-*isopentenyl-2'*-O-*{[triisopropylsilyl)oxy]methyl}adenosine* (**37**). A soln. of **34** (100 mg, 0.16 mmol, prepared according to (Wenter and Pitsch 2003)) was treated with a satd. soln. of NH_3 in MeOH (3 ml) for 6 h at r.t. After evaporation, the residue was dissolved in Py (0.7 ml), treated with $(MeO)_2TrCl$ (67 mg, 0.19 mmol) and stirred for 4 h at r.t. Workup and CC (SiO_2 (2 g), hexane/AcOEt 8:2 → 3:7) gave **37** (81 mg, 60%). Yellow foam. TLC (hexane/AcOEt 1:1): R_f 0.53. ^{1}H-NMR (400 MHz, $CDCl_3$): 0.90–1.06 (*m*, $^iPr_3Si$); 1.73 (*s*, CH_3); 1.78 (*s*, CH_3); 2.84 (*s*, HO–C(3')); 3.39 (*dd*, *J* = 4.7, 10.2, H–C(5')); 3.47 (*dd*, *J* = 3.9, 10.2, H'–C(5')); 3.79 (*s*, 2 MeO); 4.01 (*d*, *J* = 4.7, H–C(4')); 4.22–4.24 (*m*, H_2C–NH); 4.69 (*d*, *J* = 4.7, H–C(3')); 5.10 (*s*, OCH_2O); 5.22 (*t*, *J* = 4.7, H–C(11)); 5.42–5.44 (*m*, H–C(2')); 6.17 (*d*, *J* = 4.7, H–C(1')); 6.83–6.87 (*m*, 4 arom. H); 7.22–7.49 (*m*, 9 arom. H); 8.13 (*s*, H–C(8)); 8.19 (br. *s*, H–C(2)); 12.98 (br. *s*, NH). ^{13}C-NMR (100MHz, $CDCl_3$): 12.1 (*d*, Me_2CH); 17.6 (*q*, *Me*$_2$CH); 28.8 (*q*, *Me*C=); 29.9 (*q*, *Me*'C=); 38.4 (*t*, CH_2NH); 54.9 (*q*, MeO); 64.0 (*t*, C(5')); 70.9 (*d*, C(3')); 79.7 (*d*, C(2')); 84.5 (*d*, C(4')); 86.5 (*d*, C(1')); 87.5 (*s*, arom. C); 90.2 (*t*, OCH_2O); 109.4 (*s*, *C*H=C); 113.4 (*d*, arom. C); 122.2 (*s*, C(5)); 127.0, 128.0, 128.5, 130.4, 130.5 (5*d*, arom. C), 134.6, 136.4 (2*s*, arom. C, Me_2C=); 139.8 (*d*, C(8)); 145.2 (*d* ,C(4)); 145.6 (*s*, arom. C); 153.1 (*d*, C(2)); 155.3 (*s*, C(6)); 159.1 (*s*, arom. C). ESI-MS: 824.37 (100, $[M + H]^+$).

5'-O-*(4,4'-Dimethoxytrityl)*-N^6,N^6-*dimethyl*-2'-O-*{[(triisopropylsilyl)oxy]methyl}adenosine* (**35**). A soln. of **33** (0.15 g, 0.2 mmol, prepared according to (Wenter and Pitsch 2003)) was treated with a 33% soln. of Me_2NH in EtOH (3 ml) for 3 h at r.t. After evaporation, the residue was dissolved in Py (2 ml), treated with $(MeO)_2TrCl$ (89 mg, 0.3 mmol) and stirred for 3 h at r.t. Workup and CC (SiO_2 (3 g), hexane/AcOEt 3:2 → AcOEt) gave **35** (148 mg, 80%). Yellow foam. TLC (hexane:AcOEt 1:1): R_f 0.70. ^{1}H-NMR (400 MHz, $CDCl_3$): 1.00–1.11 (*m*, $^iPr_3Si$); 3.05 (*d*, *J* = 4.1, OH–C(3')); 3.36 (*dd*, *J* = 4.3, 10.5, H–C(5')); 3.50 (*dd*, *J* = 3.0, 10.5 , H'–C(5'); 3.45–3.58 (br. *s*, $(CH_3)_2$–N(6)); 3.78, 3.79 (2*s*, 2 MeO); 4.26 (*q*, *J* = 3.7, H–C(4')); 4.48 (*q*, *J* = 4.4, H–C(3')); 4.87 (*t*, *J* = 5.0, H–C(2')); 4.99 (*d*, *J* = 4.7, OCH_2O); 5.15 (*d*, *J* = 4.7, OCH_2O); 6.17 (*d*, *J* = 5.2, H–C(1')); 6.78–6.82 (*m*, 4 arom. H); 7.18–7.46 (*m*, 9 arom. H); 7.93 (*s*, H–C(8)); 8.27 (*s*, H–C(2)). ^{13}C-NMR (100 MHz, $CDCl_3$): 11.8 (*d*, Me_2CH); 17.7 (*q*, *Me*$_2$CH); 38.4 (*q*, 2 CH_3–N(6)); 55.1 (*q*, MeO); 63.4 (*t*, C(5')); 70.7 (*d*, C(3')); 81.8 (*d*, C(2')); 83.8 (*d*, C(4')); 86.4 (*d*, C(1')); 86.8 (*s*, arom. C); 90.7 (*t*, OCH_2O); 113.1 (*d*, arom. C); 120.6 (*s*, C(5)); 126.7, 127.7, 128.1, 130.0, 135.8 (5*d*, arom. C); 136.8 (*d*, C(8)); 144.6 (*d*, C(4)); 144.6, 150.3 (2*s*, arom. C); 152.4 (*d*, C(2)); 154.9 (*s*, C(6)); 158.4 (*s*, arom. C). ESI-MS: 785.41 (100, $[M + H]^+$).

5'-O-*(4,4'-Dimethoxytrityl)*-N^6-*methyl-2'*-O-*{[(triisopropylsilyl)oxy]methyl}adenosine* (**36**). A soln. of **33** (150 mg, 0.2 mmol, prepared according to (Wenter and Pitsch 2003)) was treated with a 33% soln. of $MeNH_2$ in EtOH (3 ml) for 3 h at r.t. After evaporation, the residue was dissolved in Py (2 ml), treated with $(MeO)_2TrCl$ (89 mg, 0.3 mmol) and stirred for 3 h at r.t. Workup and CC (SiO_2 (3 g), hexane/AcOEt 3:2 → AcOEt) gave **36** (120 mg, 65%). Yellow foam. TLC (hexane/AcOEt 1:9): R_f 0.60. ^{1}H-NMR (400 MHz, $CDCl_3$): 0.96–1.05 (*m*, $^iPr_3Si$); 3.06 (*d*, *J* = 3.9, OH–C(3')); 3.19 (*d*, *J* = 4.5, CH_3–N(6)); 3.38 (*dd*, *J* = 4.2, 10.2 , H–C(5')); 3.50 (*dd*, *J* = 3.6, 10.2 , H'–C(5')); 3.78 (*s*, 2 MeO); 4.26 (*q*, *J* = 4.0, H–C(4')); 4.52 (*q*, *J* = 4.2, H–C(3')); 4.93 (*t*, *J* = 5.0, H–C(2')); 4.98 (*d*, *J* = 4.8, OCH_2O); 5.14 (*d*, *J* = 4.8, OCH_2O); 5.75 (br. *s*, NH); 6.14 (*d*, *J* = 5.4, H–C(1')); 6.78 (*m*, 4 arom. H); 7.22–7.44 (*m*, 9 arom. H); 7.93 (*s*, H–C(8)); 8.33 (*s*, H–C(2)). ^{13}C-NMR (100 MHz, $CDCl_3$): 11.8 (*d*, Me_2CH); 17.7 (*q*, Me_2CH); 27.7 (*q*, CH_3–N(6)); 55.1 (*q*, MeO); 63.4 (*t*, C(5')); 70.8 (*d*, C(3')); 81.8 (*d*, C(2')); 84.1 (*d*, C(4')); 86.5 (*d*, C(1')); 87.0 (*s*, arom. C); 90.7 (*t*, OCH_2O); 113.4 (*d*, arom. C); 120.4 (*s*, C(5)); 126.8, 127.8, 128.2, 130.0, 135.8 (5*d*, arom. C); 138.6 (*d*, C(8)); 144.6 (*d*, C(4)); 153.3 (*d*, C(2)); 155.5 (*s*, C(6)); 158.5 (*s*, arom. C). ESI-MS: 769.91 (100, $[M + H]^+$).

5'-O-*(4,4'-Dimethoxytrityl)-1-methyl-2'*-O-*{[triisopropylsilyl)oxy]methyl}guanosine* (**38**). A soln. of **1** (400 mg, 0.5 mmol, prepared according to (Stutz et al. 2000)) in DMF (4 ml) was treated with K_2CO_3 (79 mg, 0.6 mmol), stirred for 2 h at r.t., treated with MeI (162 mg, 1.1 mmol) and stirred for 14 h at –15°. Workup and CC (SiO_2 (10 g), CH_2Cl_2/MeOH 99:1 → 9:1) gave **38** (0.26 g, 63%). Colorless foam. TLC (CH_2Cl_2/MeOH 9:1): R_f 0.72. ^{1}H-NMR (400 MHz, $CDCl_3$): 0.97–1.12 (*m*, $^iPr_3Si$); 1.87 (br. *s*, NH_2); 3.08 (br. *s*, OH–C(3')); 3.34 (*dd*, *J* = 3.2, 10.2, H–C(5')); 3.52 (br. *s*, H'–C(5'), CH_3–N(1)); 3.79 (*s*, 2 MeO); 4.27 (br. *s*, H–C(4')); 4.58 (br. *s*, H–C(3')); 4.92 (br. *s*, H–C(2')); 4.96 (*d*, *J* = 4.4, OCH_2O); 5.14 (*d*, *J* = 5.1, OCH_2O); 5.94 (*d*, *J* = 6.6, H–C(1')); 6.80–6.82 (*m*, 4 arom. H); 7.20–7.36 (*m*, 9 arom. H); 7.65 (*s*, H–C(8)). ^{13}C-NMR (100 MHz, $CDCl_3$): 11.9 (*d*, Me_2CH); 17.8 (*q*, Me_2CH); 28.1 (*q*, CH_3–N(1)); 55.3 (*q*, MeO); 63.6 (*t*, C(5')); 71.2 (*d*, C(2')); 81.6 (*d*, C(3')); 83.9 (*d*, C(4')); 85.9 (*s*, arom. C); 86.5 (*d*, C(1')); 90.9 (*t*, OCH_2O); 113.2 (*d*, arom. C); 118.2 (*s*, C(5)); 126.9, 127.9, 128.2, 130.1 (4*d*, arom. C), 135.7 (*s*, arom. C); 136.6 (*d*, C(8)); 144.6 (*s*, arom. C); 148.8, 153.1 (2*s*, C(2), C(4)); 158.6 (*s*, C(6)). ESI-MS: 786.82 (100, $[M + H]^+$).

3'-O-*Acetyl-5'*-O-*(4,4'-dimethoxytrityl)-2'*-O-*{[(triisopropylsilyl)oxy]methyl}adenosine* (**40**). A soln. of **39** (1.0 g, 1.16 mmol, prepared according to (Pitsch et al. 1999)) in Py (9.3 ml) was treated with DMAP (14 mg, 0.16 mmol) and Ac_2O (143 mg, 1.4 mmol). Workup (1. 10%

citric acid, 2. $NaHCO_3$ soln.) after 30 min at r.t. and CC (SiO_2 (25 g), hexane/AcOEt 2:3 → 1:9 (+ 3% Et_3N)) gave **40** (0.88 g, 88%). Colorless foam. TLC (hexane/AcOEt 1:9): R_f 0.57. ^{1}H-NMR (100MHz, $CDCl_3$): 0.88-1.05 (*m*, $^iPr_3Si$); 2.13 (*s*, MeCO); 3.43 (*dd*, *J* = 6.5, 10.4, H–C(5')); 3.52 (*dd*, *J* = 5.6, 10.4, H'–C(5')); 3.78 (*s*, 2 MeO); 4.34 (*m*, H–C(4')); 4.87 (*s*, OCH_2O); 5.19 (*dd*, *J* = 5.3, 6.9, H–C(2')); 5.51 (*dd*, *J* = 2.5, 5.3, H–C(3')); 5.80 (br. *s*, NH_2); 6.16 (*d*, *J* = 6.9, H–C(1')); 6.78–6.83 (*m*, 4 arom. H); 7.20–7.45 (*m*, 9 arom. H); 7.97 (*s*, H–C(2)); 8.25 (*s*, H–C(8)). ^{13}C-NMR (100 MHz, $CDCl_3$): 11.7 (*d*, Me_2CH); 17.6 (*q*, Me_2CH); 20.9 (*q*, *Me*CO); 55.3 (*q*, MeO); 63.4 (*t*, C(5')); 72.1 (*d*, C(3')); 77.0 (*d*, C(2')); 82.5 (*d*, C(4')); 86.2 (*d*, C(1')); 86.8 (*s*, arom. C); 89.6 (*t*, OCH_2O); 113.2 (*d*, arom. C); 120.1 (*s*, C(5)); 127.0, 127.9, 128.2, 130.1 (4*d*, arom. C), 135.6 (*s*, arom. C); 139.2 (*d*, C(8)); 144.4 (*s*, arom. C); 150.2 (*s*, C(4)); 153.2 (*d*, C(2)); 155.4 (*s*, C(6)); 158.6 (*s*, arom. C); 170.1 (s, CO). ESI-MS: 868.35 (100, $[M + Na]^+$).

N^6-*{{{(1S,2R)-2-{[(tert-butyl)dimethylsilyl]oxy}-1-{[2-(4-nitrophenyl)ethoxy]carbonyl} propyl}amino}carbonyl}-5'*-O-*(4,4'-dimethoxytrityl)-2'*-O-*{[triisopropylsilyl)oxy]methyl} adenosine* (**41**).

A soln. of **40** (400 mg, 0.5 mmol) in $ClCH_2CH_2Cl$ (2 ml) was treated with Et_3N (250 mg, 2.5 mmol) and 1,1'-carbonyl-di(1,2,4-triazole) (120 mg, 0.7 mmol), stirred for 10 min at 70°, treated with of *O*-[(*tert*-butyl)dimethylsilyl]-*l*-threonine-2-(4-nitrophenyl)ethyl ester (280 mg, 0.7 mmol, prepared according to (Boudou et al. 2000)) and stirred for 15 min at 70°. After workup and evaporation, the residue was treated with a satd. soln. of NH_3 in MeOH (5 ml) for 3 h at r.t. Workup and CC (SiO_2 (7 g), hexane/AcOEt 3:2 → AcOEt) gave **41** (326 mg, 56%). Colorless foam. TLC (CH_2Cl_2/MeOH 9:1): R_f 0.25. ^{1}H-NMR (400 MHz, $CDCl_3$): -0.05, -0.03 (2*s*, 2 MeSi); 0.91 (s, ^{t}BuSi); 0.95–1.06 (*m*, $^iPr_3Si$); 1.09 (*d*, *J* = 4.2, Me(γ)); 3.06 (*t*, *J* = 5.7, CH_2CH_2O); 3.15 (*d*, *J* = 3.7, OH–C(3')); 3.14–3.55 (m, $H_2C(5')$); 3.80 (*s*, 2 MeO); 4.30–4.60 (*m*, H–C(4'), H–C(3'), CH(α), CH(β), CH_2CH_2O); 4.97 (*t*, *J* = 5.1, H–C(2')); 5.04 (*d*, *J* = 4.7, OCH_2O); 5.19 (*d*, *J* = 4.7, OCH_2O); 6.24 (*d*, *J* = 6.2, H–C(1')); 6.81 (*d*, *J* = 8.7, 4 arom. H); 7.22–7.44 (*m*, 9 arom. H); 7.22–7.46 (*m*, 13 arom. H); 7.98 (*d*, *J* = 8.8, 2 arom. H); 8.19 (*s*, H–C(8)); 8.42 (*s*, H–C(2)); 9.99 (d, *J* = 8.8, NH–C(β)); 10.02 (br. *s*, NH–C(6)). ^{13}C-NMR (100 MHz, $CDCl_3$): -5.4, -4.3 (*q*, MeSi); 11.8 (*d*, Me_2CH); 17.8 (*q*, Me_2CH); 21.10 (Me(□)); 25.5, (*q*, Me_3CSi); 34.8 (*t*, CH_2CH_2O); 55.2 (*q*, MeO); 59.6 (*t*, CH_2CH_2O); 63.4 (*t*, C(5')); 64.6 (*d*, C(β)); 68.6 (*d*, C(β)); 70.8 (*d*, C(3')); 76.6 (*d*, C(2')); 82.4 (*d*, C(4')); 84.4 (*d*, C(1')); 87.4 (*s*, arom. C); 90.9 (*t*, OCH_2O); 113.2 (*d*, arom. C); 120.9 (*s*, C(5)); 123.6, 126.9, 127.8, 128.2, 129.7 (5*d*, arom. C); 130.1 (*s*, arom. C); 135.7 (*s*, arom. C); 141.7 (*d*, C(8)); 144.6 (*d*,

C(2)); 145.5, (*s*, arom. C); 150.0, (*s*, C(4)); 150.2 (*s*, C(6)); 154.2 (*s*, NH*C*ONH); 158.6 (*s*, arom. C); 170.9 (*s*, *C*OOCH$_2$). ESI-MS: 1164.37 (100, [*M* + H]$^+$).

5'-O-*(4,4'-Dimethoxytrityl)*-N^2-*methyl-2'*-O-*{[(triisopropylsilyl)oxy]methyl}guanosine 3'-(2-CyanoethylDiisopropylphosphoramidite)* (**42**).

A soln. of **3** (75 mg, 0.09 mmol) in CH_2Cl_2 (1 ml) was treated consecutively with $^iPr_2NEt$ (0.04 ml, 0.23 mmol) and cyanoethyl diisopropylphosphoramidochloridite (27 mg, 0.11 mmol). After stirring for 14 h at r.t. the mixture was subjected to CC (SiO_2 (2 g), CH_2Cl_2/MeOH 1:0 → 94:6 (+ 3% Et_3N)): **42** (87 mg, 92%, 1:1 mixture of diastereoisomers). Light pink foam. TLC (CH_2Cl_2/MeOH 9:1): R_f 0.37. ^{1}H-NMR (400 MHz, $CDCl_3$): 0.83–1.02 (*m*, $^iPr_3Si$); 1.06 (*d*, *J* = 6.0, *Me*$_2$CH)$_2$N); 1.17–1.24 (*m*, (*Me*$_2$CH)$_2$N); 1.38 (*t*, *J* = 6.6, *Me*$_2$CH)$_2$N); 2.35 (*t*, *J* = 6.6, 1 H, CH_2CN); 2.61–2.74 (*m*, 1 H, CH_2CN); 3.15 (2*d*, *J* = 7.2, CH_3NH-C(2)); 3.26–3.70 (*m*, 4.5 H, (MeC*H*)$_2$N, H–C(5'), $POCH_2$); 3.777, 3.786 (2*s*, 2 MeO); 3.82–3.98 (*m*, 1.5 H, $POCH_2$); 4.32, 4.36 (2br. *d*, *J* = 4.1, 1 H, H–C(4')); 4.58–4.65 (*m*, 1 H, H–C(3')); 4.92–5.06 (*m*, 3H, H–C(2'), OCH_2O); 6.07 (*d*, *J* = 5.8, 0.5 H, H–C(1')); 6.10 (*d*, *J* = 5.9, 0.5 H, H–C(1')); 6.79–6.84 (*m*, 4 arom. H); 7.16–7.50 (*m*, 9 arom. H); 7.67, 7.68 (2*s*, H–C(8)); 11.9 (br. *s*, H–N(1)). ^{31}P-NMR (162 MHz, $CDCl_3$): 150.4, 150.9. MALDI-MS: 986.29 (100, [*M* + H]$^+$).

5'-O-*(4,4'-Dimethoxytrityl)*-N^2,N^2-*dimethyl-2'*-O-*{[(triisopropylsilyl)oxy]methyl}guanosine 3'-(2-Cyanoethyl Diisopropylphosphoramidite)* (**43**).

As described for **42**, with **5** (140 mg, 0.16 mmol), CH_2Cl_2 (0.7 ml), $^iPr_2NEt$ (0.07 ml, 0.4 mmol) and cyanoethyl diisopropylphosphoramidochloridite (58 mg, 0.24 mmol). CC (SiO_2 (4 g), CH_2Cl_2 → CH_2Cl_2/MeOH 95:5 (+ 3% Et_3N)): **43** (104 mg, 64%, 1:1 mixture of diastereoisomers). Light yellow foam. TLC (CH_2Cl_2/MeOH 9:1): R_f 0.47. ^{1}H-NMR (400 MHz, $CDCl_3$): 0.95–0.97 (1*d*, *J* = 6.6, $^iPr_3Si$); 1.04–1.06 (1*d*, *J* = 6.6, (*Me*$_2$CH)$_2$N); 1.17–1.23 (*m*, (*Me*$_2$CH)$_2$N); 1.27–1.34 (*t*, *J* = 6.6, *Me*$_2$CH)$_2$N); 2.34 (*t*, *J* = 6.6, 1 H, CH_2CN); 2.60–2.72 (*m*, 0.5 H, CH_2CN); 2.34 (*dt*, *J* ≈ 1, 4.4, 0.5 H, CH_2CN); 3.13, 3.14 (2*s*, Me_2N-C(2)); 3.31–3.70 (*m*, 5 H, (MeC*H*)$_2$N, H–C(5'), $POCH_2$, $POCH_2$); 3.786, 3.795 (2*s*, 2 MeO); 3.80–3.96 (*m*, 1.5 H, $POCH_2$); 4.11-4.27 (*m*, 1 H, $POCH_2$); 4.32 (br. *d*, *J* ≈ 3.7, 0.5 H, H–C(4')); 4.36 (br. *d*, *J* ≈ 3.6, 0.5 H, H–C(4')); 4.53–4.60 (*m*, 1 H, H–C(3')); 4.90–5.06 (*m*, 3.0 H, H–C(2'), OCH_2O); 6.03 (*d*, *J* = 6.6, 0.5 H, H–C(1')); 6.07 (*d*, *J* = 5.8, 0.5 H, H–C(1')); 6.78–6.83 (*m*, 4 arom. H); 7.23–7.43 (*m*, 9 arom. H); 7.67 (br. *s*, H–C(8)); 10.9 (br. *s*, H–N(1)). ^{31}P-NMR (162 MHz, $CDCl_3$): 150.4, 150.9. ESI-MS: 1000.36 (100, [*M* + H]$^+$).

N^6-*Chloroacetyl-5'*-O-*(4,4'-dimethoxytrityl)*-N^1-*methyl-2'*-O-*{[(triisopropylsilyl)oxy]methyl} adenosine 3'-(2-Cyanoethyl Diisopropylphosphoramidite)* (**44**).

As described for **42**, with **8** (400 mg, 0.48 mmol), CH_2Cl_2 (4 ml), $^iPr_2NEt$ (0.21 ml, 1.23 mmol) and cyanoethyl diisopropylphosphoramidochloridite (134 mg, 0.56 mmol). CC (SiO_2 (10 g), hexane/AcOEt 4:1 → 1:4 (+ 3% Et_3N)): **44** (462 mg, 93%, 1:1 mixture of diastereoisomers)). Colorless foam. TLC (hexane/AcOEt 1:9): R_f 0.75. ^{1}H-NMR (400 MHz, $CDCl_3$): 0.95–1.02 (*m*, $^iPr_3Si$); 1.08, 1.19 (2*d*, *J* = 7.3, $(Me_2CH)_2N$); 2.37 (*t*, *J* = 6.6, 1 H, CH_2CN); 2.66 (*t*, *J* = 5.8, 1 H, CH_2CN); 3.34 (*dt*, *J* = 4.3, 10.5, 1 H, $POCH_2$); 3.57–3.94 (*m*, 1 H of $POCH_2$, $(MeCH)_2N$, H–C(5')); 3.81 (*s*, MeO); 4.35 (*d*, *J* = 3.6, 0.5 H, H–C(4')); 4.41 (*d*, *J* = 3.6, 0.5 H, H–C(4')); 4.44 (*s*, $ClCH_2$); 4.62 (*dt*, *J* = 4.9, 10.9, H–C(3')); 4.91–5.01 (*m*, H–C(2'), OCH_2O); 6.04, 6.07 (2*d*, *J* = 5.9, H–C(1')); 6.79–6.84 (*m*, 4 arom. H); 7.22–7.38 (*m*, 12 arom. H); 7.74 (*s*, H–C(2)); 7.91, 7.93 (2*s*, H–C(8)). ^{31}P-NMR (162 MHz, $CDCl_3$) 150.3, 150.9. MALDI-MS: 1046.74 (70, $[M + H]^+$) 1048.74 (30, $[M + H]^+$).

5'-O-*(4,4'-Dimethoxytrityl)*-5-*methyl-2'*-O-*{[(triisopropylsilyl)oxy]methyl}uridine 3'-(2-Cyano ethyl) Diisopropylphosphoramidite)* (**45**).

As described for **42**, with **10** (400 mg, 0.13 mmol), CH_2Cl_2 (4 ml), $^iPr_2NEt$ (0.23 ml, 1.35 mmol) and cyanoethyl diisopropylphosphoramidochloridite (139 mg, 0.59 mmol). CC (SiO_2 (12 g), hexane/AcOEt 9:1 → 1:4 (+ 3% Et_3N)): **45** (500 mg, 98%, 1:1 mixture of diastereomers). Colorless foam. TLC (hexane/AcOEt 1:1): R_f 0.71. ^{1}H-NMR (400 MHz, $CDCl_3$): 1.00–1.12 (*m*, $^iPr_3Si$); 1.16–1.32 (*m*, $(Me_2CH)_2N$); 1.46, 1.49 (2*s*, 2 Me–C(5)); 2.36 (*t*, *J* = 6.9, 1 H, CH_2CN); 2.64–2.66 (*dt*, *J* = 3.1, 6.4, CH_2CN); 3.32 (*dt*, *J* = 2.5, 7.1, H–C(5')); 3.46–3.70 (*m*, 4 H $(Me_2CH)_2N$, H'–C(5'), $POCH_2$); 3.80, 3.81 (2*s*, 2 MeO); 3.82–3.97 (*m*, 1 H, $POCH_2$); 4.20 (br. *d*, *J* ≈ 1.5, 0.5 H, H–C(4')); 4.30 (br. *d*, *J* ≈ 2.2, 0.5 H, H–C(4')); 4.45–4.65 (*m*, H–C(2'), H–C(3')); 4.96–5.06 (*m*, OCH_2O); 6.18 (*d*, *J* = 6.6, 0.5 H, H–C(1')); 6.20 (*d*, *J* = 6.5, 0.5 H, H–C(1')); 6.83–6.87 (*m*, 4 arom. H); 7.25–7.38 (*m*, 9 arom. H); 7.41 (br. *s*, H-C(6)); 7.43 (br. *d*, H–N(3)). ^{31}P-NMR (162 MHz, $CDCl_3$): 150.2, 150.9. MALDI-MS: 947.31 (100, $[M + H]^+$).

5,6-*Dihydro-5'*-O-*(4,4'-dimethoxytrityl)-2'*-O-*{[(triisopropylsilyl)oxy]methyl}uridine 3'-(2-Cyanoethyl Diisopropylphosphoramidite)* (**46**).

As described for **42**, with **12** (400 mg, 0.54 mmol), CH_2Cl_2 (5 ml), $^iPr_2NEt$ (0.24 ml, 1.35 mmol) and cyanoethyl diisopropylphosphoramidochloridite (160 mg, 0.65 mmol). CC (SiO_2 (10 g), hexane/AcOEt 4:1 → 1:9 (+ 3% Et_3N)): **46** (367 mg, 73%, 1:1 mixture of diastereomers). Colorless foam. TLC (hexane/AcOEt 7:3): R_f 0.73. ^{1}H-NMR (400 MHz,

$CDCl_3$): 1.03–1.10 (*m*, $^iPr_3Si$); 1.15–1.31 (*m*, $(Me_2CH)_2N$); 2.37 (*t*, 1H, *J* = 6.6, 1 H, CH_2CN); 2.50–2.62 (*m*, 2H, $H_2C(5)$); 2.66 (*dt*, 1H, *J* = 1.8, 6.7, CH_2CN); 3.22–3.27 (*m*, 1H, H–C(6)); 3.37–3.52 (*m*, 2H, H'–C(6), H–C(5')); 3.52–3.77 (*m*, 4 H $(Me_2CH)_2N$, H'–C(5), $POCH_2$); 3.81, 3.82 (2*s*, 2 MeO); 3.82–3.97 (*m*, 1 H, $POCH_2$); 4.12 (br. *d*, *J* ≈ 2.2, 0.5 H, H–C(4')); 4.19 (br. *d*, *J* ≈ 2.7, 0.5 H, H–C(4')); 4.39–4.46 (*m*, H–C(2'), H–C(3')); 4.97 (*d*, *J* = 5.0, 0.5 H, OCH_2O); 5.05 (*q*, *J* = 4.9, 1.5H, OCH_2O); 6.05 (*d*, *J* = 5.9, 0.5 H, H–C(1')); 6.07 (*d*, *J* = 5.7, 0.5 H, H–C(1')); 6.82–6.86 (*m*, 4 arom. H); 7.23–7.44 (*m*, 9 arom. H). ^{31}P-NMR (162 MHz, $CDCl_3$): 150.1, 150.4. MALDI-MS: 935.36 (100, $[M + H]^+$).

N^4*-Acetyl-5'*-O-*(4,4'-dimethoxytrityl)-5-methyl-2'*-O-*{[(triisopropylsilyl)oxy]methyl}cytidine 3'-(2-CyanoethylDiisopropylphosphoramidite)* (**47**).

As described for **42**, with **14** (1.20 g, 1.56 mmol), CH_2Cl_2 (6.3 ml), $^iPr_2NEt$ (0.60 ml, 3.90 mmol) and cyanoethyl diisopropylphosphoramidochloridite (444 mg, 1.88 mmol). CC (SiO_2 (30 g), hexane/AcOEt 1:1 → AcOEt (+ 2% Et_3N)): **51** (1.42 g, 94%, 1:1 mixture of diastereoisomers). Colorless foam. TLC (hexane/AcOEt 3:7): R_f 0.75. ^{1}H-NMR (400 MHz, $CDCl_3$): 0.97–1.08 (*m*, $^iPr_3Si$); 1.08–1.13 (*m*, $(Me_2CH)_2N$); 2.25 (br. *s*, MeCO); 2.51 (br. *s*, Me–C(5)); 2.54 (*t*, *J* = 5.9, 1 H, CH_2CN); 2.75 (br. *q*, *J* = 5.9, 1 H, CH_2CN); 3.42–3.24 (*m*, 1 H of $POCH_2$, $(Me_2CH)_2N$, H–C(5')); 3.66–3.82 (*m*, 1 H, $POCH_2$); 3.73 (*s*, 2 MeO); 7.85 (2br. *s*, H–C(4')); 4.32–4.48 (*m*, H–C(2'), H-C(3')); 4.89–5.04 (*m*, OCH_2O); 6.00 (*d*, *J* = 4.4, H–C(1')); 6.87–6.89 (*m*, 4 arom. H); 7.22–7.43 (*m*, 9 arom. H); 7.85 (*d*, *J* = 8.0, H–C(6)). ^{31}P-NMR (162 MHz, $CDCl_3$): 149.0, 149.5. ESI-MS: 988.30 (100, $[M + H]^+$).

5'-O-*(4,4'-Dimethoxytrityl)*-N^1,N^3*-bis{[(triisopropylsilyl)oxy]methyl}-2'*-O-*{[(triisopropyl silyl)oxy]methyl}pseudouridine 3'-(2-Cyanoethyl Diisopropylphosphoramidite* (**48**).

As described for **42**, with **46** (108 mg, 0.1 mmol), CH_2Cl_2 (1 ml), $^iPr_2NEt$ (0.05 ml, 0.27 mmol) and cyanoethyl diisopropylphosphoramidochloridite (37 mg, 0.15 mmol). CC (SiO_2 (5 g), hexane/AcOEt 99:1 → 4:1 (+ 3% Et_3N)): **48** (88 mg, 69%; 1:1 mixture of diastereoisomers). Colorless foam. TLC (hexane/AcOEt 4:1): R_f 0.65. ^{1}H-NMR (400 MHz, $CDCl_3$): 0.84–1.02 (m, $^iPr_3Si$); 1.04–1.30 (m, $^iPr_3Si$, $(Me_2CH)_2N$, CH_2CN); 2.55–2.65 (m, 0.5 H, CH_2CN); 3.35–3.47 (m, 3 H, $H_2C(5')$, $(Me_2CH)_2N$, $POCH_2$); 3.80 (*s*, 2 MeO); 4.00–4.06 (*m*, H–C(4')); 4.14–4.21 (*m*, H–C(3'), H–C(2')); 4.58 (*d*, *J* = 8.9, $OCH_2N(3)$); 4.99 (*s*, H–C(1')); 5.10 (*d*, *J* = 4.7, OCH_2O); 5.24–5.32 (*m*, $OCH_2N(3)$, $OCH_2N(1)$, OCH_2O); 5.61 (*s*, $OCH_2N(1)$); 6.84 (*d*, *J* = 8.8, 4 arom. H); 7.19–7.32 (*m*, 7 arom. H); 7.34–7.40 (*m*, 7 arom.

H); 7.49 (*d*, *J* = 7.7, 2 arom. H); 7.60 (*s*, H–C(6)). ^{31}P-NMR (162 MHz, $CDCl_3$): 150.4, 150.7. MALDI-MS: 1328.9 (100, $[M + Na]^+$).

5'-O-*(4,4'-Dimethoxytrityl)*-N^1,N^3-*bis{[(pivaloyl)oxy]methyl}-2'*-O-*{[(triisopropylsilyl)oxy]methyl}pseudouridine 3'-(2-Cyanoethyl Diisopropylphosphoramidite* (**49**).

As described for **42**, with **29** (90 mg, 0.1 mmol), CH_2Cl_2 (1 ml), $^iPr_2NEt$ (0.05 ml, 0.27 mmol) and cyanoethyl diisopropylphosphoramidochloridite (34 mg, 0.14 mmol). CC (SiO_2 (5 g), hexane/AcOEt 199:1 → 3:2 (+ 3% Et_3N)): **49** (100 mg, 92%; 1:1 mixture of diastereoisomers). Colorless foam. TLC (hexane/AcOEt 7:3): R_f 0.50. ^{1}H-NMR (400 MHz, $CDCl_3$): 0.87–1.02 (*m*, $^iPr_3Si$); 1.04–1.22 (*m*, $^iPr_3Si$, Me_3C, $(Me_2CH)_2N$, CH_2CN); 2.36–2.42 (m, 0.5 H, CH_2CN); 2.62 (*t*, *J* = 6.0, CH_2CN); 3.35–3.47 (m, 5 H, $H_2C(5')$, $(Me_2CH)_2N$, $POCH_2$); 3.80 (s, 2 MeO); 3.98–4.05 (*m*, 0.5 H, $POCH_2$); 4.18–4.26 (*m*, 1.5 H, H–C(4'), $POCH_2$); 4.36–4.47 (*m*, H–C(3')); 4.49–4.70 (*m*, H–C(2'),); 4.95–5.05 (*m*, H–C(1')); 5.06 (*d*, *J* = 4.7, OCH_2O); 5.09 (*d*, *J* = 4.2, $OCH_2N(3)$); 5.14 (*d*, *J* = 4.2, $OCH_2N(3)$); 5.26 (*d*, *J* = 4.7, OCH_2O); 5.42 (*d*, *J* = 10.0, $OCH_2N(3)$); 5.95 (*d*, *J* = 9.3, $OCH_2N(1)$); 6.85 (*d*, *J* = 8.5, 4 arom. H); 7.16–7.33 (*m*, 7 arom. H); 7.34–7.38 (*m*, 7 arom. H); 7.45–7.50 (*m*, 2 arom. H); 7.73 (*s*, H–C(6)). ^{31}P-NMR (162 MHz, $CDCl_3$): 150.3, 150.9. MALDI-MS: 1184.4 (100, $[M + Na]^+$)

5'-O-*(4,4'-Dimethoxytrityl)-1-methyl-2'*-O-*{[(triisopropylsilyl)oxy]methyl}inosine 3'-(2-CyanoethylDiisopropylphosphoramidite)* (**50**).

As described for **42**, with **32** (77 mg, 0.1 mmol), CH_2Cl_2 (2 ml), $^iPr_2NEt$ (0.04 ml, 0.25 mmol) and cyanoethyl diisopropylphosphoramidochloridite (30 mg, 0.12 mmol). CC (SiO_2 (2 g), hexane/AcOEt 4:1 → 3:2 (+ 3% Et_3N)): **50** (78 mg, 80%, 1:1 mixture of diastereomers). Colorless foam. TLC (hexane/AcOEt 8:2): R_f 0.50. TLC (hexane/AcOEt 1:1): R_f 0.50. ^{1}H-NMR (400 MHz, $CDCl_3$): 0.86–1.05 (m, $^iPr_3Si$); 1.16–1.19 (*m*, $(Me_2CH)_2N$); 2.39 (*t*, *J* = 6.4, 1 H, CH_2CN); 2.67 (*t*, *J* = 6.4, 1 H, CH_2CN); 3.32–3.40 (*m*, 1H, H–C(5')); 3.41–3.51 (*m*, 1H, H'–C(5')); 3.51–3.70 (*m*, 3 H, $(Me_2CH)_2N$, $POCH_2$); 3.60, 3.61 (2*s*, 3H, CH_3–N(1)); 3.79, 3.80 (2*s*, 2 MeO); 3.84–3.97 (*m*, 1 H, $POCH_2$); 4.35, 4.41 (2*q*, *J* = 3.1, H–C(4')); 4.57–4.65 (*m*, H–C(3')); 4.91–4.98 (*m*, 2H, OCH_2O); 5.00–5.04 (*m*, 1H, H–C(2')); 6.05 (*d*, *J* = 6.1, 0.5 H, H–C(1')); 6.09 (*d*, *J* = 6.1, 0.5 H, H–C(1')); 6.75–6.83 (*m*, 4 arom. H); 7.18–7.44 (*m*, 9 arom. H); 7.78 (s, 1H, H–C(8); 7.92, 7.93 (2*s*, 1H, H–C(2)). ^{31}P-NMR (162 MHz, $CDCl_3$): 150.06, 150.73. ESI-MS: 971.83 (100, $[M + H]^+$).

5'-O-*(4,4'-Dimethoxytrityl)*-N^6-*isopentenyl-2'*-O-*{[(triisopropylsilyl)oxy]methyl}adenosine 3'-(2-Cyanoethyl Diisopropylphosphoramidite* (**51**).

As described for **42**, with **37** (90 mg, 0.12 mmol), CH_2Cl_2 (1.5 ml), $^iPr_2NEt$ (0.05 ml, 0.27 mmol) and cyanoethyl diisopropylphosphoramidochloridite (21 mg, 0.18 mmol). CC (SiO_2 (3 g), hexane/AcOEt 9:1 → 2:3 (+ 3% Et_3N)): **51** (90 mg, 80%, 1:1 mixture of diastereoisomers). Light yellow foam. TLC (hexane/AcOEt 1:1): R_f 0.70. ^{1}H-NMR (400 MHz, $CDCl_3$): 0.86–0.98 (*m*, $^iPr_3Si$); 1.11, 1.14, (2*d*, *J* = 6.6, $(Me_2CH)_2N$); 1.22 (*t*, *J* = 5.5, $(Me_2CH)_2N$); 1.76 (s, Me); 1.78 (s, Me); 2.39 (*t*, *J* = 6.5, 1 H, CH_2CN); 2.67 (*dt*, *J* = 2.3, 6.7, 1 H, CH_2CN); 3.32–3.72 (*m*, 1 H of $POCH_2$, $(MeCH)_2N$, H–C(5')); 3.79 (*s*, 2 MeO); 3.84–3.99 (*m*, 1 H, $POCH_2$); 4.22 (br. *s*, H_2C–NH); 4.34 (*d*, *J* = 3.7, H–C(4')); 4.40 (*t*, *J* = 4.0, H–C(4')); 4.67–4.74 (*m*, H–C(3')); 4.94, 4.96 (2*d*, *J* = 5.0, OCH_2O); 4.97–5.04 (*m*, OCH_2O); 5.16–5.23 (*m*, H–C(2')); 5.40 (*t*, *J* = 4.7, H–C(11)); 6.13, 6.16 (2*d*, *J* = 5.7, H–C(1')); 6.77–6.82 (*m*, 4 arom. H); 7.20–7.43 (*m*, 12 arom. H); 7.92, 7.94 (2*s*, H–C(8)); 8.28, 8.30 (2*s*, H–C(2)). ^{31}P-NMR (162 MHz, $CDCl_3$): 150.1, 150.8. MALDI-MS: 1024.36 (100, $[M + H]^+$).

5'-O-*(4,4'-Dimethoxytrityl)*-N^6,N^6-*dimethyl-2'*-O-*{[(triisopropylsilyl)oxy]methyl}adenosine 3'-(2-CyanoethylDiisopropylphosphoramidite)* (**52**).

As described for **42**, with **35** (148 mg, 0.19 mmol), CH_2Cl_2 (3 ml), $^iPr_2NEt$ (0.08 ml, 0.47 mmol) and cyanoethyl diisopropylphosphoramidochloridite (47 mg, 0.23 mmol). CC (SiO_2 (3 g), hexane/AcOEt 9:1 → 1:1 (+ 3% Et_3N)): **52** (148 mg, 80%, 1:1 mixture of diastereomers). Colorless foam. TLC (hexane/AcOEt 2:1): R_f 0.61. ^{1}H-NMR (400 MHz, $CDCl_3$): 0.88–1.10 (*m*, $^iPr_3Si$); 1.12–1.20 (*m*, $(Me_2CH)_2N$); 2.37 (*t*, *J* = 6.5, 1 H, CH_2CN); 2.65 (*t*, *J* = 6.4, 1 H, CH_2CN); 3.45–3.68 (*m*, 11H, $(CH_3)_2N(6)$, $H_2C(5')$, $(Me_2CH)_2N$, $POCH_2$); 3.77, 3.78 (2*s*, 2 MeO); 3.81–3.98 (*m*, 1 H, $POCH_2$); 4.32, 4..38 (2*q*, *J* = 3.1, H–C(4')); 4.67–4.75 (*m*, H–C(3')); 4.92–5.01 (*m*, 2H, OCH_2O); 5.14–5.17 (*m*, 1H, H–C(2')); 6.14 (*d*, *J* = 5.5, 0.5 H, H–C(1')); 6.16 (*d*, *J* = 5.2, 0.5 H, H–C(1')); 6.76–6.80 (*m*, 4 arom. H); 7.20–7.40 (*m*, 9 arom. H); 7.88, 7.90 (2*s*, 1H, H–C(8); 8.22, 8.24 (2*s*, 1H, H–C(2)). ^{31}P-NMR (162 MHz, $CDCl_3$): 149.86, 150.54. ESI-MS: 546.29 (100, $[M + H]^+$).

5'-O-*(4,4'-Dimethoxytrityl)*-N^6-*methyl-2'*-O-*{[(triisopropylsilyl)oxy]methyl}adenosine 3'-(2-Cyanoethyl diisopropylphosphoramidite)* (**53**).

As described for **42**, with **36** (120 mg, 0.16 mmol), CH_2Cl_2 (3 ml), $^iPr_2NEt$ (0.07 ml, 0.39 mmol) and cyanoethyl diisopropylphosphoramidochloridite (39 mg, 0.19 mmol). CC (SiO_2 (3 g), hexane/AcOEt 4:1 → 2:3 (+ 3% Et_3N)): **53** (128 mg, 85%, 1:1 mixture of diastereomers). Colorless foam. TLC (hexane/AcOEt 1:1): R_f 0.50. ^{1}H-NMR (400 MHz, $CDCl_3$): 0.88–1.10

(m, $^{i}Pr_3Si$); 1.16–1.26 (*m*, $(Me_2CH)_2N$); 2.37 (*t*, *J* = 6.5, 1 H, CH_2CN); 2.65 (*t*, *J* = 6.4, 1 H, CH_2CN); 3.20 (br. *s*, 3H, CH_3–N(6)); 3.30–3.33 (*m*, 1H, H–C(5')); 3.49–3.70 (*m*, 4 H, H'–C(5'), $(Me_2CH)_2N$, $POCH_2$); 3.77, 3.78 (2*s*, 2 MeO); 3.86–3.97 (*m*, 1 H, $POCH_2$); 4.32, 4..37 (2*q*, *J* = 3.0, H–C(4')); 4.67–4.75 (*m*, H–C(3')); 4.91–5.01 (*m*, 2H, OCH_2O); 5.16–5.21 (*m*, 1H, H–C(2')); 6.11 (*d*, *J* = 5.5, 0.5 H, H–C(1')); 6.13 (*d*, *J* = 5.2, 0.5 H, H–C(1')); 6.75–6.79 (*m*, 4 arom. H); 7.17–7.41 (*m*, 9 arom. H); 7.90, 7.92 (2*s*, 1H, H–C(8); 8.28, 8.30 (2*s*, 1H, H–C(2)). ^{31}P-NMR (162 MHz, $CDCl_3$): 149.89, 150.58 ppm; ESI-MS: 546.29 (100, $[M + H]^+$).

5'-O-*(4,4'-Dimethoxytrityl)-1-methyl-2'*-O-*{[(triisopropylsilyl)oxy]methyl} guanosine 3'-(2-Cyanoethyl Diisopropylphosphoramidite)* (**54**).

As described for **42**, with **38** (84 mg, 0.11 mmol), CH_2Cl_2 (0.5 ml), $^{i}Pr_2NEt$ (0.03 ml, 0.27 mmol) and cyanoethyl diisopropylphosphoramidochloridite (22 mg, 0.19 mmol). CC (SiO_2 (1.5 g), hexane/AcOEt 2:3 → AcOEt (+ 3% Et_3N)): **54** (61 mg, 58%, 1:1 mixture of diastereoisomers). Light yellow foam. TLC (hexane/AcOEt 1:9): R_f 0.34. ^{1}H-NMR (400 MHz, $CDCl_3$): 0.90–1.00 (*m*, $^{i}Pr_3Si$); 1.06–1.31 (4*d*, *J* = 6.5, $(Me_2CH)_2N$); 2.35 (*t*, *J* = 6.4, 1 H, CH_2CN); 2.69 (*t*, *J* = 6.7, 1 H, CH_2CN); 2.77 (*dd*, *J* = 1.6, 6.3, 0.5 H, H–C(5')); 3.27–3.38 (*m*, 0.5 H, H–C(5')); 3.46, 3.47 (2*s*, 2 Me-N(1)); 3.49–3.72 (*m*, 4.0 H, $(MeCH)_2N$, H–C(5'), $POCH_2$); 3.79 (*s*, 2 MeO); 3.86–4.25 (*m*, $POCH_2$); 4.30 (br. *q*, *J* ≈ 2, 0.5 H, H–C(4')); 4.38 (br. *t*, *J* ≈ 3, 0.5 H, H–C(4')); 4.58–4.68 (*m*, 1 H, H–C(3')); 4.73, 4.80 (2*s*, 1 H, OCH_2O); 4.88–5.15 (*m*, 1.5 H, H–C(2'), OCH_2O); 5.02–5.15 (*m*, 0.5 H, H–C(2')); 5.89 (*d*, *J* = 6.5, 0.5 H, H–C(1')); 5.96 (*d*, *J* = 7.0, 0.5 H, H–C(1')); 6.80–6.82 (*m*, 4 arom. H); 7.21–7.48 (*m*, 9 arom. H, H–C(8)); 7.64, 7.66 (2br. *s*, HN–C(2)). ^{31}P-NMR (162 MHz, $CDCl_3$): 151.3, 151.6. MALDI-MS: 986.31 (100, $[M + H]^+$).

N^6-*{{{(1S,2R)-2-{[(tert-butyl)dimethylsilyl]oxy}-1-{[2-(4-nitrophenyl)ethoxy]carbonyl}propyl}amino}carbonyl}-5'*-O-*(4,4'-dimethoxytrityl)-2'*-O-*{[(triisopropylsilyl)oxy]methyl} adenosine 3'-(2-Cyanoethyl Diisopropylphosphoramidite)* (**55**).

As described for **42**, with **41** (100 mg, 0.08 mmol), CH_2Cl_2 (1 ml), $^{i}Pr_2NEt$ (0.05 ml, 0.21 mmol) and cyanoethyl diisopropylphosphoramidochloridite (24 mg, 0.10 mmol). CC (SiO_2 (3 g), hexane/AcOEt 6:4 → AcOEt (+ 3% Et_3N)): **55** (80 mg, 68%, 1:1 mixture of diastereoisomers). Colorless foam. TLC (hexane/AcOEt 1:9): R_f 0.62. ^{1}H-NMR (400 MHz, $CDCl_3$): -0.03, 0.09 (2*s*, *Me*Si); 0.97–1.08 (*m*, $^{i}Pr_3Si$, ^{t}BuSi); 1.09 (*d*, *J* = 6.6, Me(γ)); 1.10–1.30 (*m*, $(Me_2CH)_2N$); 2.40 (*t*, *J* = 5.9, 1 H, CH_2CN); 2.67 (*t*, *J* = 6.6, 1 H, CH_2CN); 3.07 (t, *J* = 6.6, CH_2CH_2O); 3.35–3.39 (*m*, 1 H of $POCH_2$); 3.51–3.66 (*m*, $(Me_2CH)_2N$, H–C(5'));

3.80–4.00 (*m*, 1 H, $POCH_2$); 3.79 (*s*, 2 MeO); 4.32–4.44 (*m*, H–C(4'), CH_2CH_2O); 4.54 (*d*, *J* = 6.6, CH(β)); 4.58 (*d*, *J* = 8.8, CH(α)); 4.63–4.73 (*m*, H–C(3')); 4.96, 4.97, 5.03 (3*d*, *J* = 4.4, OCH_2O); 5.08–5.13 (*m*, H–C(2')); 6.24 (*d*, *J* = 5.1, H–C(1')); 6.26 (*d*, *J* = 5.8, H–C(1')); 6.79–6.84 (*m*, 4 arom. H); 7.24–7.54 (*m*, 15 arom. H); 8.06 (*d*, *J* = 8.1, 2 arom. H); 8.16, 8.19 (2*s*, H–C(8)); 8.43, 8.44 (2*s*, H–C(2)); 10.04 (*t*, *J* = 9.5, HN–C(6)). ^{31}P-NMR (162 MHz, $CDCl_3$): 150.8, 150.2. ESI-MS: 1364.30 (100, $[M + H]^+$).

5'-O-*(4,4'-Dimethoxytrityl)-2'*-O-*{[(triisopropylsilyl)oxy]methyl}inosine 3'-(2-Cyanoethyl Diisopropylphosphoramidite)* (**56**).

A suspension of **31** (1.5 g, 2 mmol) and 5-benzyl-1*H*-tetrazole (0.43 g, 2.2 mmol, prepared according to (Pitsch et al. 2001)) and MS 4Å, in MeCN (10 ml) was treated with 2-cyanoethyl tetraisopropylphosphoramidite (1.8 g, 6 mmol). The reaction mixture was diluted CH_2Cl_2 (100 ml) and poured into a well-stirred mixture of CH_2Cl_2 (200 ml) and satd. aq. $NaHCO_3$ soln. (250 ml). The organic phase was dried (Na_2SO_4) and evaporated. CC (SiO_2 (40 g), hexane/AcOEt 1:1 → AcOEt (+ 3% Et_3N)) gave **56** (1.75 g, 92%, 1:1 mixture of diastereomers). Colorless foam. TLC (hexane/AcOEt 1:9): R_f 0.48. ^{1}H-NMR (400 MHz, $CDCl_3$): 0.85–1.00 (*m*, $^iPr_3Si$); 1.18–1.31 (*m*, $(Me_2CH)_2N$); 2.40 (*t*, *J* = 6.5, 1 H, CH_2CN); 2.62–2.71 (*dt*, *J* = 1.5, 7.6, CH_2CN); 3.35–3.41 (*m*, $POCH_2$); 3.46–3.74 (*m*, 4 H $(Me_2CH)_2N$, H–C(5'), $POCH_2$); 3.79, 3.80 (2*s*, 2 MeO); 3.85–4.00 (*m*, 1 H, $POCH_2$); 4.38 (br. *d*, *J* = 3.4, 0.5 H, H–C(4')); 4.43 (br. *d*, *J* = 3.6, 0.5 H, H–C(4')); 4.58–4.64 (*m*, H–C(2'), H–C(3')); 4.92–5.09 (*m*, OCH_2O); 6.13 (*d*, *J* = 6.0, 0.5 H, H–C(1')); 6.16 (*d*, *J* = 6.0, 0.5 H, H–C(1')); 6.79–6.84 (*m*, 4 arom. H); 7.19–7.47 (*m*, 9 arom. H); 7.94, 7.95 (2*s*, H-C(8)); 8.01, 8.04 (2*s*, H-C(2));. ^{31}P-NMR (162 MHz, $CDCl_3$): 151.3, 152.1. MALDI-MS: 957.36 (100, $[M + H]^+$).

CHAPTER II

5'-O-*(4,4'-Dimethoxytrityl)*-O^2*-methyluridine* (**58**).

A soln. of **57** (4.67 g, 20.6 mmol, prepared according to (Vyle et al. 1998)) in MeOH (100 ml) was treated with Et_3N (10 ml, 58.4 mmol) and stirred overnight at 70° in an autoclave. Evaporation of the solvent gave a white solid which was dissolved in Py (75 ml), treated with $(MeO)_2TrCl$ (6.93 g, 20.3 mmol) and stirred for 12 h at r.t. Workup and CC (SiO_2 (140 g), CH_2Cl_2/MeOH 19:1 → 17:3) gave **58** (6.15 g, 60%). Yellow foam. TLC (CH_2Cl_2/MeOH 19:1): R_f 0.15. ^{1}H-NMR (400 MHz, $CDCl_3$): 3.44 (*m*, $H_2C(5')$); 3.49 (*s*, OH); 3.78 (*s*, 2 MeO); 3.96 (*s*, MeO–C(2)); 4.19 (*d*, *J* = 3.9, H–C(4')); 4.41 (*t*, *J* = 4.8, H–C(2')); 4.48 (*t*, *J* =

5.1, H–C(3')); 5.59 (*d*, *J* = 7.7, H–C(5)); 5.95 (*d*, *J* = 5.5, H–C(1')); 6.85 (*d*, *J* = 8.8, 4 arom. H); 7.21–7.41 (*m*, 9 arom. H); 7.77 (*d*, *J* = 7.7, H–C(6)). ^{13}C-NMR (100 MHz, $CDCl_3$): 55.3 (*q*, MeO); 55.9 (*q*, MeO–C(2)); 63.0 (*t*, C(5')); 71.1 (*d*, C(3')); 74.7 (*d*, C(2')); 83.9 (*d*, C(4')); 87.2 (*s*, arom. C); 89.7 (*d*, C(1')); 113.4 (*d*, arom. C); 127.1, 128.1, 128.2, 130.1, 130.2 (5*d*, arom. C); 135.1, 135.3 (2*s*, arom. C); 138.4 (*d*, C(6)); 144.3 (*s*, arom. C); 156.3 (*s*, C(2)); 158.7 (*s*, arom. C); 164.1 (*s*, C(4)); 171.6 (*s*, arom. C). ESI-MS: 561.27 (100, $[M + H]^+$).

5'-O-*(4,4'-Dimethoxytrityl)isocytidine* (**59**).

NH_3 (50 ml) was condensed into an autoclave containing **58** (2.50 g, 4.4 mmol). After sealing, the reaction mixture was stirred for 12 h at 65°. Evaporation gave crude **59** (2.07 g) as yellow oil. An analytical sample was obtained by prep. TLC (CH_2Cl_2/MeOH 9:1). TLC (CH_2Cl_2/MeOH 19:1): R_f 0.18. ^{1}H-NMR (400 MHz, DMSO): 3.20–3.26 (*m*, $H_2C(5')$); 3.73 (*s*, 2 MeO); 4.00 (*d*, *J* = 3.0, H–C(4')); 4.07–4.11 (*m*, H–C(3'), H–C(2')); 5.27 (*d*, *J* = 5.1, OH–C(3')); 5.35 (*d*, *J* = 7.4, H–C(5)); 5.52 (*d*, *J* = 5.1, OH–C(2')); 5.60 (*d*, *J* = 4.4, H–C(1')); 6.89 (*d*, *J* = 8.8, 4 arom. H); 7.22–7.37 (*m*, 9 arom. H); 7.53 (*d*, *J* = 7.3, H–C(6)). ^{13}C-NMR (100 MHz, DMSO): 55.6 (*q*, MeO); 63.4 (*t*, C(5')); 69.9 (*d*, C(2')); 74.2 (*d*, C(3')); 83.7 (*d*, C(4')); 86.4 (*s*, arom. C); 90.7 (*d*, C(1')); 107.4 (*d*, C(5)); 113.7 (*d*, arom. C); 127.3, 128.1, 128.4, 130.2 (4*d*, arom. C); 135.6 (*s*, arom. C); 137.4 (*s*, arom. C); 145.0 (*d*, C(6)); 155.2 (*d*, C(2)); 158.6 (s, arom. C); 169.92 (*s*, C(4)). ESI-MS: 546.29 (100, $[M + H]^+$).

N^2-*Acetyl-5'*-O-*(4,4'-dimethoxytrityl)isocytidine* (**60**).

A soln. of crude **59** (2.30 g, ca. 4.2 mmol) in Py (18 ml) was treated with TMS-Cl (2.6 ml, 21.0 mmol). After 90 min at 4°, DMAP (250 mg, 2.1 mmol) and AcCl (0.3 ml, 4.2 mmol) were added. After 14 h at r.t., and work-up, the residue was treated with Bu_4NF (1M) in THF (20 ml) at r.t. for 5 min. Addition of aq. Na-phosphate buffer (0.1M, 100 ml, pH 7), workup and CC (SiO_2 (60 g), CH_2Cl_2/acetone 9:1 → 1:1) gave **60** (1.81 g, 63% based on **58**). Yellow foam. TLC (CH_2Cl_2/MeOH 19:1): R_f 0.23. ^{1}H-NMR (400 MHz, $CDCl_3$): 2.24 (*s*, MeCO); 3.29–3.33 (*m*, $H_2C(5')$); 3.80 (*s*, 2 MeO); 3.37–3.46 (*m*, H–C(3'), H–C(4')); 4.40–4.43 (m, H–C(2'), OH); 5.62 (*d*, *J* = 7.8, H–C(5)); 6.02 (*s*, H–C(1')); 6.84 (*d*, *J* = 7.0, 4 arom. H); 7.24–7.38 (*m*, 9 arom. H); 7.92 (*d*, *J* = 8.6, H–C(6)); 8.62 (*d*, *J* = 3.9, HN–C(2)). ^{13}C-NMR (100 MHz, $CDCl_3$): 24.5 (*q*, *Me*CO)); 55.7 (*q*, MeO); 59.4 (*t*, C(5')); 63.4 (*d*, C(2')); 72.7 (*d*, C(3')); 86.7 (*d*, C(4')); 87.6 (*s*, arom. C); 93.5 (*d*, C(1')); 105.7 (*d*, C(5)); 113.7 (*d*, arom. C); 124.2, 127.6, 128.4, 128.5, 130.4, (5*d*, arom. C); 135.4, 135.5 (2*s*, arom. C); 139.9 (*d*, C(6)); 144.5 150.1 (2*s*, arom. C); 153.6 (*d*, C(2)); 159.1 (*s*, arom. C); 159.7 (*s*, C(4)); 184.7 (*s*, Me*C*O). ESI-MS: 588.36 (100, $[M + H]^+$).

N^2-*Acetyl-5'*-O-*(4,4'-dimethoxytrityl)-2'*-O-*{[(triisopropylsilyl)oxy]methyl}isocytidine* (**61**).

A soln. of **60** (3.50 g, 5.9 mmol) and $^{i}Pr_2NEt$ (3.50 ml, 20.8 mmol) in $ClCH_2CH_2Cl$ (35 ml) was treated with $^{t}Bu_2SnCl_2$ (1.99 g, 6.6 mmol), stirred for 30 min at r.t., treated with TOM-Cl (1.46 g, 6.6 mmol) and stirred at for 25 min r.t. Workup and CC (SiO_2 (35 g), hexane/AcOEt 9:1 → 1:1) gave **61** (552 mg, 12%). Light yellow foam. TLC (hexane/AcOEt 1:1): R_f 0.62. ^{1}H-NMR (400 MHz, $CDCl_3$): 1.00–1.22 (*m*, $^{i}Pr_3Si$); 2.20 (*s*, MeCO); 3.02 (*d*, *J* = 7.8, OH–C(3')); 3.59 (br. *s*, $H_2C(5')$); 3.82 (*s*, 2 MeO); 4.09 (*d*, *J* = 7.8, H–C(4')); 4.35 (*d*, *J* = 3.9, H–C(2')); 4.51 (*d*, *J* = 4.7, H–C(3')); 5.26 (*d*, *J* = 3.9, OCH_2O); 5.29 (*d*, *J* = 3.9, OCH_2O); 5.35 (*d*, *J* = 7.8, H–C(5)); 6.27 (*s*, H–C(1')); 6.86 (*d*, *J* = 8.6, 4 arom. H); 7.29–7.45 (*m*, 9 arom. H); 8.29 (*d*, *J* = 8.5, H–C(6)); 13.0 (br. *s*, NH–C(2)). ^{13}C-NMR (100 MHz, $CDCl_3$): 12.3 (*d*, Me_2CH); 18.2 (*q*, Me_2CH); 28.8 (*q*, *Me*CO)); 55.7 (*q*, MeO); 61.5 (*t*, C(5')); 68.6 (*d*, C(2')); 82.3 (*d*, C(3')); 84.0 (*d*, C(4')); 87.6 (*s*, arom. C); 89.4 (*d*, C(1')); 90.4 (*t*, OCH_2O); 105.6 (*d*, C(5)); 113.7 (*d*, arom. C); 127.6, 128.4, 128.6, 130.5, 130.6 (5*d*, arom. C); 135.4 (*s*, arom. C); 140.2 (*d*, C(6)); 144.7 (*s*, arom. C); 152.4 (*d*, C(2)); 159.2 (*s*, arom. C); 160.2 (*s*, C(4)); 185.4 (*s*, Me*C*O). ESI-MS: 774.29 (100, $[M + H]^+$).

N^2-*Acetyl-5'*-O-*(4,4'-dimethoxytrityl)-2'*-O-*{[(triisopropylsilyl)oxy]methyl} isocytidine 3'-(2-Cyanoethyl Diisopropylphosphoramidite)* (**62**).

As described for **42**, with **61** (100 mg, 0.13 mmol), CH_2Cl_2 (2 ml), $^{i}Pr_2NEt$ (0.05 ml, 0.32 mmol) and cyanoethyl diisopropylphosphoramidochloridite (43 mg, 0.32 mmol). CC (SiO_2 (3 g), hexane/AcOEt 9:1 → 4:6 (+ 3% Et_3N)): **66** (60 mg, 50%, 1:1 mixture of diastereoisomers). Colorless foam. TLC (hexane/AcOEt 1:1): R_f 0.64. ^{1}H-NMR (400 MHz, $CDCl_3$): 0.98–1.07 (*m*, $^{i}Pr_3Si$); 1.09, 1.18 (2*d*, *J* = 7.0, $(Me_2CH)_2N$); 2.20, 2.21 (2*s*, MeCO); 2.45 (*t*, *J* = 5.7, 1 H, CH_2CN); 2.67 (*q*, *J* = 6.3, 1 H, CH_2CN); 3.44–3.69 (*m*, 3.5 H, $POCH_2$, $(Me_2CH)_2N$, H–C(5')); 3.81 (*s*, 2 MeO); 3.94 (*m*, 1 H, $POCH_2$); 4.25 (*d*, *J* = 6.2, 0.5 H, H–C(4')); 4.30 (*d*, *J* = 6.2, 0.5 H, H–C(4')); 4.34–4.47 (*m*, H–C(2'), H–C(3')); 5.17 (br. *s*, OCH_2O); 5.28 (*dd*, *J* = 4.6, 11.2, OCH_2O); 5.34, 5.40 (2*d*, *J* = 8.1, H–C(5)); 6.54, 6.59 (2*d*, *J* = 3.1, H–C(1')); 6.81–6.88 (*m*, 4 arom. H); 7.32–7.44 (*m*, 9 arom. H); 8.17, 8.23 (2*d*, *J* = 8.6, H–C(6)); 13.03, 13.06 (2br. *s*, NH–C(4)). ^{31}P-NMR (162 MHz, $CDCl_3$): 151.2, 151.6. MALDI-MS: 974.30 (100, $[M + H]^+$).

N^6-*Isobutyrylisoguanosine* (**63**).

Carefully dried (0.01 mbar, 24 h at 50°) isoguanosine (2.83 g, 10 mmol) was suspended in Py (50 ml) containing MS 4Å, stirred 2 h at r.t., treated with tms-Cl (10 ml, 80 mmol) and stirred

for 2 h at 4°. Then, DMAP (980 mg, 7 mmol) and isobutyryl chloride (1.2 ml, 11 mmol) were added. After 12 h at r.t., workup and evaporation, the yellow oil was dissolved in MeOH:AcOH 9:1 (50 ml) and stirred for 3 h at r.t. Evaporation gave crude **63** (3.41 g) as white solid. TLC (CH_2Cl_2/MeOH 1:1): R_f 0.10. ^{1}H-NMR (400 MHz, DMSO): 1.12 (*d*, *J* = 6.8, *Me*$_2$CH); 3.00 (*sept*, *J* = 6.8, Me$_2$C*H*CO); 3.55–3.65 (*m*, H$_2$C(5')); 3.92 (*dd*, *J* = 6.7, 3.4, H–C(4')); 4.10 (*dd*, *J* = 8.2, 4.7, H–C(3')); 4.48 (*dd*, *J* = 5.8, 11.2, H–C(2')); 5.18 (*d*, *J* = 4.8, OH–C(3')); 5.25 (*m*, OH–C(5')); 5.47 (*d*, *J* = 5.9, OH–C(2')); 5.72 (*d*, *J* = 5.9, H–C(1')); 8.32 (*s*, H–C(8)); 11.92 (br. *s*, HN). ^{13}C-NMR (100 MHz, DMSO): 22.0 (*q*, *Me*$_2$CHCO); 35.1 (*d*, Me$_2$*C*HCO); 62.3 (*t*, C(5')); 71.3 (*d*, C(3')); 74.1 (*d*, C(2')); 86.6 (*d*, C(4')); 88.3 (*d*, C(1')); 110.0 (*s*, C(5)); 139.6 (*d*, C(8)); 152.2 (*s*, C(4)), 154.7 (*s*, C(6)); 157.0 (*s*, C(2)); 179.9 (*s*, NHCO). ESI-MS: 354.29 (100, $[M + H]^+$).

5'-O-*(4,4'-Dimethoxytrityl)*-O^2*-diphenylcarbamoyl*-N^6*-isobutyrylisoguanosine* (**64**).

A soln. of crude **63** (5.63 g, ca. 15.9 mmol) in Py (250 ml) was treated at r.t. with ^{i}Pr$_2$NEt (3.0 ml, 17.5 mmol) and then with *N*,*N*-diphenylcarbamoyl chloride (4.06 g, 17.5 mmol). After 30 min at r.t., the reaction mixture was treated with (MeO)$_2$TrCl (5.40 g, 15.9 mmol). Workup after 1 h at r.t. and CC (SiO_2 (90 g), AcOEt → AcOEt/MeOH 99:1) afforded **64** (6.09 g, 45% from isoguanosine). Yellow foam. TLC (CH_2Cl_2/MeOH 17:3): R_f 0.88. ^{1}H-NMR (400 MHz, $CDCl_3$): 1.28–1.32 (*d*, *J* = 7.3, *Me*$_2$CH); 3.23–3.33 (*m*, Me$_2$C*H*CO, OH–C(3'), H–C(5')); 3.40 (*dd*, *J* = 3.7, 11.0, H'–C(5')); 3.77 (*s*, 2 MeO); 4.37 (*d*, *J* = 3.0, H–C(3')); 4.43 (br. *s*, H–C(4')); 4.77 (br. *s*, H–C(2')); 5.32 (br. *s*, OH–C(2')); 6.01 (*d*, *J* = 5.1, H–C(1')); 6.75–6.79 (*m*, 4 arom. H); 7.16–7.39 (*m*, 19 arom. H); 8.12 (*s*, H–C(8)). ^{13}C-NMR (100 MHz, $CDCl_3$): 19.1 (*q*, *Me*$_2$CHCO); 35.8 (*d*, Me$_2$*C*HCO); 55.2 (*q*, MeO); 63.5 (*t*, C(5')); 72.4 (*d*, C(2')); 75.9 (*d*, C(3')); 76.9 (*d*, C(4')); 85.7 (*d*, C(1')); 90.1 (*s*, arom. C); 113.2 (*d*, arom. C); 120.5 (*s*, C(5)); 126.6, 126.7, 126.8, 126.9, 127.0, 127.1, 127.8, 127.9, 128.2, 129.2, 135.4, 136.6 (12*d*, arom. C); 141.2 (*d*, C(8)); 144.3 (*s*, C(4)); 150.3 (*s*, arom. C); 152.0 (*s*, CO); 155.5 (*s*, C(6)); 158.6 (*s*, C(2)); 176.3 (*s*, NHCO). ESI-MS: 851.36 (100, $[M + H]^+$).

5'-O-*(4,4'-Dimethoxytrityl)*-O^2*-diphenylcarbamoyl*-N^6*-isobutyryl-2'*-O-*{[(triisopropylsilyl)oxy]methyl}isoguanosine* (**65**).

A soln. of **64** (5.90 g, 6.9 mmol) and ^{i}Pr$_2$NEt (4.2 ml, 24.3 mmol) in ClCH$_2$CH$_2$Cl (28 ml) was treated with Bu_2SnCl_2 (2.32 g, 7.6 mmol), stirred 10 min at r.t., heated to 75°, treated with TOM-Cl (1.85 g, 8.3 mmol) and heated for 25 min at 75°. Workup and CC (SiO_2 (150 g), hexane/AcOEt 9:1 → 3:2) gave **65** (1.64 g, 23%). Light yellow foam. TLC (hexane/AcOEt 1:1): R_f 0.27. ^{1}H-NMR (400 MHz, $CDCl_3$): 0.92–1.21 (*m*, ^{i}Pr$_3$Si); 1.32 (*d*, *J* = 7.3, *Me*$_2$CH);

3.19 (*s*, OH–C(3')); 3.35–3.51 (*m*, Me_2CHCO); 3.74–3.82 (*m*, H–C(5')); 3.82 (*s*, 2 MeO); 3.99 (*d*, *J* = 13.2, H'–C(5')); 4.38 (*s*, H–C(3')); 4.58 (*d*, *J* = 4.4, H–C(4')); 4.84 (*d*, *J* = 5.1, OCH_2O); 4.86–4.89 (*m*, H–C(2')); 5.04 (*d*, *J* = 5.1, OCH_2O); 5.93 (*d*, *J* = 7.4, H–C(1')); 6.85 (*d*, *J* = 4.4, 4 arom. H); 7.10–7.45 (*m*, 19 arom. H); 8.57 (*s*, H–C(8)). ^{13}C-NMR (100 MHz, $CDCl_3$): 11.8 (*d*, Me_2CH); 17.8 (*q*, Me_2CH, Me_2CHCO); 35.8 (*d*, Me_2CHCO); 55.3 (*q*, MeO); 63.3 (*t*, C(5')); 72.0 (*d*, C(2')); 81.9 (*d*, C(3')); 86.5 (*d*, C(4')); 87.8 (*d*, C(1')); 89.6 (*s*, arom. C); 90.8 (*t*, OCH_2O); 113.2 (*d*, arom. C); 121.9 (*s*, C(5)); 126.6, 126.7, 126.8, 126.9, 127.0, 127.1, 127.8, 127.9, 128.2, 130.0, 130.1, 135.7, 136.6 (12*d*, arom. C); 139.5 (*d*, C(8)); 143.2 (*s*, C(4)); 150.7 (*s*, arom. C); 151.3 (*s*, CO), 155.4 (*s*, C(6)); 158.6 (*s*, C(2)); 176.4 (*s*, NHCO). ESI-MS: 1037.36 (100, $[M + H]^+$).

5'-O-*(4,4'-Dimethoxytrityl)*-O^2*-diphenylcarbamoyl*-N^6*-isobutyryl-2'*-O-*{[(triisopropylsilyl) oxy]methyl}isoguanosine 3'-(2-Cyanoethyl Diisopropylphosphoramidite)* (**66**).
As described for **42**, with **65** (100 mg, 0.096 mmol), CH_2Cl_2 (0.7 ml), $^iPr_2NEt$ (0.04 ml, 0.24 mmol) and cyanoethyl diisopropylphosphoramidochloridite (34 mg, 0.14 mmol). CC (SiO_2 (3 g), hexane/AcOEt 9:1 → 1:1 (+ 3% Et_3N)): **66** (70 mg, 56%, 1:1 mixture of diastereoisomers). Colorless foam. TLC (hexane/AcOEt 1:1): R_f 0.49. ^{1}H-NMR (400 MHz, $CDCl_3$): 0.85–1.00 (*m*, $^iPr_3Si$); 1.05 (1*d*, *J* = 6.2, $(Me_2CH)_2N$); 1.12–1.29 (*m*, $(Me_2CH)_2N$); 1.33 (1*d*, *J* = 7.0, (Me_2CH); 2.34 (*t*, *J* = 7.0, 0.5 H, CH_2CN); 2.48–2.70 (*m*, 1.5 H, CH_2CN); 3.32 (*dd*, *J* = 5.9, 10.2, 0.5 H, H–C(5')); 3.44–3.73 (*m*, 4.5 H, $(MeCH)_2N$, $(MeCH)_2CO$, H–C(5'), $POCH_2$); 3.78, 3.79 (2*s*, 2 MeO); 3.82–3.92 (*m*, 1 H, $POCH_2$); 4.30 (*d*, *J* = 3.1, 0.5 H, H–C(4')); 4.35 (*d*, *J* = 3.9, 0.5 H, H–C(4')); 4.57–4.64 (*m*, 1 H, H–C(3')); 4.85–5.02 (*m*, 3H, OCH_2O, H–C(2'), H–C(3')); 6.23 (*t*, *J* = 4.7, H–C(1')); 6.78–6.83 (*m*, 4 arom. H); 7.20–7.42 (*m*, 9 arom. H); 8.14, 8.19 (2*s*, H–C(8)). ^{31}P-NMR (162 MHz, $CDCl_3$): 151.7, 151.8. MALDI-MS: 1237.83 (100, $[M + H]^+$).

CHAPTER III

N^4*-Desmethyl-5'*-O-*(4,4'-dimethoxytrityl)- 2'*-O-*{[(triisopropylsilyl)oxy]methyl}wyosine* (**71**).
A soln. of **1** (1.00 g, 1.3 mmol) in DMF (12 ml) was treated with K_2CO_3 (197 mg, 1.4 mmol) and stirred at r.t. for 1 h. This suspension was cooled to -15° and successively treated with KI (107 mg, 0.6 mmol), bromoacetone (178 mg, 1.3 mmol) and stirred for 8 h. The reaction mixture was poured on a cold 10% soln. of ammonium chloride (150 ml) and AcOEt (150 ml). The aqueous layer was extraction twice with (2 x 150 ml) of AcOEt and the combined

organic layers, dried over $MgSO_4$. After evaporation of the solvent, the yellow oily residue **2** was redissolved in CH_2Cl_2 (10 ml) and stirred overnight on 4A molecular sieve. Filtration, evaporation and CC (SiO_2 (30 g), CH_2Cl_2/Acetone 0:1 → 2:8) offered **71** (588 mg, 56%) as green oil and (199 mg, 20%) of **1**. TLC (CH_2Cl_2/MeOH 9:1): R_f 0.53. ^{1}H-NMR (400 MHz, $CDCl_3$)): 1.04–1.11 (*m*, $^iPr_3Si$); 2.26 (*s*, CH_3–C(6)); 3.15 (br. *s*, HO–C(3')); 3.35 (*dd*, *J* = 3.4, 10.3, H–C(5')); 3.51 (*dd*, *J* = 3.2, 10.3, H'–C(5')); 3.77, 3.79 (2*s*, 2 MeO); 4.29 (*dd*, *J* = 2.6, 3.2, H–C(4')); 4.64 (*dd*, *J* = 2.2, 4.4, H–C(3')); 4.95 (*d*, *J* = 4.7, OCH_2O); 5.08 (*t*, *J* = 5.7, H–C(2')); 5.13 (*d*, *J* = 4.7, OCH_2O); 6.02 (*d*, *J* = 6.2, H–C(1')); 6.80–6.82 (*m*, 4 arom. H); 7.26–7.49 (*m*, 9 arom. H); 7.81 (br. *s*, H–C(6)). ^{13}C-NMR (100 MHz, $CDCl_3$): 11.3 (*q*, CH_3–C(6)); 12.2 (*d*, Me_2CH); 18.2 (*q*, Me_2CH); 55.7 (*q*, MeO); 64.0 (*t*, C(5')); 71.7 (*d*, C(4')); 81.7 (*d*, C(2')); 84.4 (*d*, C(3')); 86.7 (*s*, arom. C); 86.9 (*d*, C(1')); 91.4 (*t*, OCH_2O); 104.6 (*d*, C(7)); 113.4 (*d*, arom. C); 117.7 (*s*, C(9a)); 127.3, 128.3, 128.8, 130.7 (4*d*, arom. C); 136.1 (*s*, arom. C); 137.9 (*d*, C(2)); 145.1 (*s*, C(6)); 145.9 (*s*, C(4a)); 150.2 (*s*, C(3a)); 152.4 (*s*, C(9)); 158.9 (*s*, arom. C). ESI-MS: 810.30 (100, $[M + H]^+$).

N^4-*Desmethyl-5'*-O-*(4,4'-dimethoxytrityl)*-N^5-*methyl-2'*-O-*{[(triisopropylsilyl)oxy]methyl} wyosine* (**74**).

A soln. of **71** (1.00 g, 1.2 mmol) in DMF (15 ml) in a flask prevented from light, was successively treated with K_2CO_3 (198 mg, 1.4 mmol), MeI (0.08 ml, 1.3 mmol) and stirred at r.t. for 1.5 h. Workup and evaporation gave **74** (1.00 g, 98%) as yellow brown wax. No further purification has been necessary. TLC (CH_2Cl_2/MeOH 9:1): R_f 0.47. ^{1}H-NMR (400 MHz, $CDCl_3$)): 1.02–1.14 (*m*, $^iPr_3Si$); 2.32 (*s*, CH_3–C(6)); 3.15 (*d*, *J* = 3.9, HO–C(3')); 3.45 (br. *s*, H–C(5'), CH_3–N(5)); 3.78 (br. *s*, 2 MeO, H'–C(5')); 4.28 (*dd*, *J* = 1.5, 5.1, H–C(4')); 4.61 (*dd*, *J* = 4.0, 8.2, H–C(3')); 4.84 (*t*, *J* = 5.4, H–C(2')); 4.97 (*d*, *J* = 4.7, OCH_2O); 5.16 (*d*, *J* = 4.7, OCH_2O); 6.16 (*d*, *J* = 5.5, H–C(1')); 6.76–6.81 (*m*, 4 arom. H); 7.17–7.34 (*m*, 9 arom. H); 7.43 (*d*, *J* = 7.0, H–C(7)); 7.80 (br. *s*, H–C(2)); ^{13}C-NMR (100 MHz, $CDCl_3$): 10.6 (*q*, CH_3–C(6)); 12.3 (*d*, Me_2CH); 18.2 (*q*, Me_2CH); 28.8 (*q*, CH_3–N(5)); 55.7 (*q*, MeO); 64.1 (*t*, C(5')); 71.6 (*d*, C(4')); 82.4 (*d*, C(2')); 84.2 (*d*, C(3')); 86.5 (*s*, arom. C); 86.9 (*d*, C(1')); 91.3 (*t*, OCH_2O); 103.9 (*d*, C(7)); 113.5 (*d*, arom. C); 117.4 (*s*, C(9a)); 127.0 (*s*, C(6)); 127.3, 128.3, 128.6, 130.5 (4*d*, arom. C); 136.0, 136.2 (2*s*, arom. C); 137.1 (*s*, C(2)); 144.9 (*s*, C(4a)); 146.0 (*s*, arom. C); 150.3 (*s*, C(3a)); 152.3 (*s*, C(9)); 158.9 (*s*, arom. C). ESI-MS: 824.30 (100, $[M + H]^+$).

N^4-*Desmethyl-5'*-O-*(4,4'-dimethoxytrityl)*-N^5-*methyl-2'*-O-*{[(triisopropylsilyl)oxy]methyl} wyosine 3'-(2-Cyanoethyl Diisopropylphosphoramidite* (**75**).

As described for **42**, with **74** (200 mg, 0.24 mmol), CH_2Cl_2 (0.8 ml), $^iPr_2NEt$ (0.1 ml, 0.6 mmol) and cyanoethyl diisopropylphosphoramidochloridite (67 mg, 0.29 mmol). CC (SiO_2 (2 g), hexane/AcOEt 1:1 → 0:1 (+ 3% Et_3N)): **75** (192 mg, 73%; 1:1 mixture of diastereoisomers). Yellow foam. TLC (hexane/AcOEt 1:9): R_f 0.42. ^{1}H-NMR (400 MHz, $CDCl_3$): 0.91–0.93 (*m*, $^iPr_3Si$); 1.06–1.32 (4*d*, *J* = 6.2, $(Me_2CH)_2N$); 2.32 (*s*, CH_3–C(6)); 2.35–2.39 (*m*, 1 H, CH_2CN); 2.65–2.72 (*m*, 1 H, CH_2CN); 3.33–3.52 (*m*, 3.5 H, CH_3–N(5), H–C(5')); 3.53–3.69 (*m*, 4.0 H, $(MeCH)_2N$, H–C(5'), $POCH_2$); 3.78 (*s*, 2 MeO); 3.86–4.01 (*m*, 1 H, $POCH_2$); 4.10–4.28 (*m*, 0.5 H, $POCH_2$); 4.35 (br. *d*, *J* ≈ 2.7, 0.5 H, H–C(4')); 4.41 (br. *d*, *J* ≈ 3.2, 0.5 H, H–C(4')); 4.56–4.67 (*m*, 1 H, H–C(3')); 4.92 (*d*, *J* = 4.9, 0.5 H, OCH_2O); 4.97 (br. *s*, 1.5 H, OCH_2O); 5.00–5.08 (*m*, 1 H, H–C(2')); 6.12 (*d*, *J* = 6.3, 0.5 H, H–C(1')); 6.17 (*d*, *J* = 6.2, 0.5 H, H–C(1')); 6.78–6.80 (*m*, 4 arom. H); 7.18–7.55 (*m*, 9 arom. H, H–C(7)); 7.80 (*s*, H–C(2)). ^{31}P-NMR (162 MHz, $CDCl_3$): 150.6, 150.9. MALDI-MS: 1023.87 (100, $[M + H]^+$).

N^4-*Desmethyl-2'*-O-*{[(triisopropylsilyl)oxy]methyl}wyosine* (**76**)

A soln. of **71** (823 mg, 1.0 mmol) in CH_2Cl_2 (10 ml) was treated with dichloroacetic acid (0.4 ml, 6.0 mmol), stirred at r.t. After 15 min, the reaction mixture was quenched with MeOH (2.6 ml) on a 10% cold soln. of ammonium bicarbonate (50 ml) and CH_2Cl_2 (50 ml). The aqueous layer was extracted twice with (2 x 150 ml) of AcOEt and the combined organic layers was washed with brine. CC (SiO_2 (15 g), hexane/AcOEt 1:1 → 0:1) offered **76** (431 mg, 85%). Light green foam. TLC (CH_2Cl_2/MeOH 9:1): R_f 0.50. ^{1}H NMR (400 MHz, $CDCl_3$): 1.01-1.07 (*m*, $^iPr_3Si$); 2.30 (br. *s*, CH_3–C(6)); 3.05–3.60 (br. *s*, HO–C(3'), HO–C(5')); 3.83 (*d*, *J* = 11.7, H–C(5')); 4.01 (*d*, *J* = 12.5, H'–C(5')); 4.29 (br. *s*, H–C(4')); 4.57 (*d*, *J* = 4.2, OCH_2O); 4.88–4.95 (*m*, H–C(2'), OCH_2O); 5.08 (*d*, *J* = 4.6, H–C(3')); 5.89 (*d*, *J* = 7.3, H–C(1')); 7.76 (br. *s*, H–C(6)); 11.50 (br. *s*, H–N(5)). ^{13}C NMR (100MHz, $CDCl_3$): 11.3 (*q*, CH_3–C(6)); 12.2 (*d*, Me_2CH); 18.1 (*q*, Me_2CH); 63.7 (*t*, C(5')); 72.2 (*d*, C(3')); 81.2 (*d*, C(2')); 87.2 (*d*, C(4')); 89.8 (*d*, C(1')); 90.9 (*t*, OCH_2O); 104.5 (*d*, C(7)); 118.1 (*s*, C(9a)); 126.5 (*s*, C(6)); 139.4 (*d*, C(2)); 145.4 (*s*, C(4a)); 148.6 (*s*, C(3a)); 152.1 (*s*, C(9)). ESI-MS: 508.32 (100, $[M + H]^+$).

3',5'-Di-O-*acetyl* -N^4-*desmethyl-2'*-O-*{[(triisopropylsilyl)oxy]methyl}wyosine* (**77**).

A soln. of **76** (360 mg, 0.7 mmol) in Py (5 ml) was treated with Ac_2O (0.4 ml, 6.0 mmol) and stirred at r.t. for 2 h. After workup, the solvent was removed under reduced pressure and stirred for 2 h Py/H_2O/MeOH 1:1:1 (6 ml). Workup, evaporation and coevaporation with benzene (2 ml) offered **77** (382 mg, 91%). Yellow foam. No further purification has been

necessary. TLC (CH_2Cl_2/MeOH 9:1): R_f 0.42. ^{1}H-NMR (400 MHz, $CDCl_3$): 0.90–1.10 (*m*, $^iPr_3Si$); 2.09, 2.20 (2*s*, 2 MeCO); 2.38 (*s*, CH_3–C(6)); 4.45–4.47 (*m*, $H_2C(5')$); 4.50–4.60 (*m*, H–C(4')); 4.84 (*d*, *J* = 4.6, OCH_2O); 4.89 (*d*, *J* = 4.7, OCH_2O); 5.23 (*t*, *J* = 5.6, H–C(2')); 5.53 (*dd*, *J* = 3.3, 5.0, H–C(3')); 6.01 (*d*, *J* = 6.3, H–C(1')); 7.38 (*d*, *J* = 5.5, H–C(7)); 7.74 (br. *s*, H–C(2)); 9.77 (br. *s*, H–N(5)). ^{13}C-NMR (100 MHz, $CDCl_3$): 11.5 (*q*, CH_3–C(6)); 12.1 (*d*, Me_2CH); 18.0 (*q*, Me_2CH); 21.2 (*q*, *Me*CO); 21.3 (*q*, *Me*CO); 64.4 (*t*, C(5')); 72.2 (*d*, C(3')); 76.0 (*d*, C(2')); 80.7 (*d*, C(4')); 88.4 (*d*, C(1')); 89.9 (*t*, OCH_2O); 104.7 (*d*, C(7)); 118.0 (*s*, C(9a)); 125.6 (*s*, C(6)); 137.9 (*d*, C(2)); 146.1 (*s*, C(4a)); 149.9 (*s*, C(3a)); 152.3 (*s*, C(9)); 170.6, 171.6 (2*s*, Me*C*O). ESI-MS: 592.80 (100, $[M + H]^+$).

3',5'-Di-O-*acetyl-2'*-O-*{[(triisopropylsilyl)oxy]methyl}wyosine* (**78**).

A light prevented soln. of diiodomethane (1.2 ml, 14.9 mmol) in dry Et_2O (9 ml) was treated with a 1M soln. of diethylzinc in hexane (7.5 ml, 7.5 mmol) and stirred for 30 min at r.t. The clear soln. gave a cloudy white suspension upon addition of DME (0.8 ml, 7.5 mmol). After 30 min, the reaction mixture was cooled to 4° and a soln. of starting material **77** (0.424g, 0.71 mmol) in CH_2Cl_2 (1 ml) was quickly added. After 3 min of stirring, we poured the reaction mixture on an ice cold 1M aqueous soln. of ammonium carbonate (50 ml) and CH_2Cl_2 (50 ml). The aqueous layer was extracted twice with (2 x 150 ml) of AcOEt and the combined organic layers was washed with (100 ml) of a 0.1M soln. of thiosulfate. Drying over $MgSO_4$, evaporation and CC (SiO_2 (15 g), CH_2Cl_2/Acetone 99:1 → 95:5 (+ 1% Et_3N)) offered **78** (300 mg, 70%). Yellow foam. TLC (CH_2Cl_2/MeOH 9:1): R_f 0.38. ^{1}H-NMR (400 MHz, $CDCl_3$): 1.00–1.08 (*m*, $^iPr_3Si$); 2.10, 2.23 (2*s*, 2 MeCO); 2.35 (*s*, CH_3–C(6)); 4.20 (*s*, CH_3–N(4)); 4.23 (*dd*, *J* = 2.6, 12.3, H–C(5')); 4.32 (*dd*, *J* = 3.1, 12.1, H'–C(5')); 4.51 (*dd*, *J* = 2.2, 4.2, H–C(4')); 4.90 (*dd*, *J* = 4.6, 10.4, OCH_2O); 4.93 (*dd*, *J* = 4.6, 10.4, OCH_2O); 4.97 (*t*, *J* = 4.8, H–C(2')); 5.42 (*dd*, *J* = 2.1, 4.9, H–C(3')); 6.20 (*d*, *J* = 6.8, H–C(1')); 7.47 (br. *s*, H–C(7)); 7.92 (br. *s*, H–C(2)). ^{13}C-NMR (100 MHz, $CDCl_3$): 12.2 (*d*, Me_2CH); 14.7 (*q*, CH_3–C(6)); 18.2 (*q*, Me_2CH); 21.1 (*q*, *Me*CO); 21.2 (*q*, *Me*CO); 34.3 (*q*, CH_3–N(4)); 63.8 (*t*, C(5')); 71.6 (*d*, C(3')); 72.1 (*d*, C(2')); 77.2 (*d*, C(4')); 81.8 (*d*, C(1')); 89.7 (*t*, OCH_2O); 107.1 (*d*, C(7)); 117.3 (*s*, C(9a)); 134.2 (*d*, C(2)); 138.4 (*s*, C(6)); 140.1 (*s*, C(3a)); 142.9 (*s*, C(4a)); 152.6 (*s*, C(9)); 170.4 (*s*, 2 Me*C*O). ESI-MS: 606.83 (100, $[M + H]^+$).

5'-O-tert-*Butyldimethylsilyl-2'*-O-*{[(triisopropylsilyl)oxy]methyl}wyosine* (**80**).

A methanolic (0.5 ml) soln. of **79** (81 mg, 0.13 mmol) was treated with a satd. soln. of NH_3 in MeOH (2 ml) and stirred for 3 h at r.t. After evaporation to dryness, the residue was dissolved in a CH_2Cl_2/DMF 2:1 (1.1 ml) and treated with imidazole (26 mg, 0.32 mmol). After 5 min,

the reaction mixture was cooled to 4° and tBDMS-Cl (26 mg, 0.15 mmol) was added followed by stirring at 4° for 1 h. Workup and CC (SiO_2 (2 g), CH_2Cl_2/Acetone 1:1 → 0:1) offered **80** (85 mg, 98% from **78**). Light yellow oil. TLC (CH_2Cl_2/MeOH 1:9): R_f 0.75. ^{1}H-NMR (400 MHz, $CDCl_3$): 0.25, 0.26 (2*s*, ^{t}Bu*Me*$_2$Si); 1.00–1.29 (*m*, ^{i}Pr$_3$Si, ^{t}*Bu*Me$_2$Si); 2.47 (br. *s*, CH_3–C(6)); 2.60 (br. *s*, HO–C(3')); 3.98 (*dd*, *J* = 1.6, 11.5, H–C(5')); 4.06 (*dd*, *J* = 1.9, 11.5, H'–C(5')); 4.29 (*s*, CH_3–N(4)); 4.48 (br. *s*, H–C(4')); 4.58–4.62 (*m*, H–C(2'), H–C(3')); 5.00 (*d*, *J* = 5.1, OCH_2O); 5.28 (*d*, *J* = 5.1, OCH_2O); 6.39 (*d*, *J* = 6.3, H–C(1')); 7.58 (br. *s*, H–C(7)); 8.17 (br. *s*, H–C(2)). ^{13}C-NMR (100 MHz, $CDCl_3$): –5.6, –5.5 (2*q*, *Me*$_2$Si); 11.9 (*d*, Me$_2$*C*H); 14.3 (*q*, CH_3–C(6)); 17.7 (*q*, *Me*$_2$CH); 18.4 (*s*, Me$_3$*C*); 26.0 (*q*, *Me*$_3$C); 34.3 (*q*, CH_3–N(4)); 63.8 (*t*, C(5')); 71.2 (*d*, C(3')); 84.9 (*d*, C(2')); 86.2 (*d*, C(4')); 86.9 (*d*, C(1')); 90.9 (*t*, OCH_2O); 106.6 (*d*, C(7)); 116.6 (*s*, C(9a)); 134.7 (*d*, C(2)); 137.9 (*s*, C(6)); 139.6 (*s*, C(3a)); 142.6 (*s*, C(4a)); 152.3 (*s*, C(9)). ESI-MS: 636.37 (100, $[M + H]^+$).

5'-O-tert-*Butyldimethylsilyl-2'*-O-*{[(triisopropylsilyl)oxy]methyl}wyosine 3'-(2-Cyanoethyl Diisopropylphosphoramidite* (**81**).

As described for **42**, with **80** (83 mg, 0.13 mmol), CH_2Cl_2 (1.0 ml), ^{i}Pr$_2$NEt (0.06 ml, 0.32 mmol) and cyanoethyl diisopropylphosphoramidochloridite (40 mg, 0.16 mmol). CC (SiO_2 (3 g), hexane/AcOEt 4:1 → 1:4 (+ 3% Et$_3$N)): **81** (75 mg, 70%; 1:1 mixture of diastereoisomers). Colorless foam. TLC (hexane/AcOEt 3:7): R_f 0.50. ^{1}H-NMR (400 MHz, $CDCl_3$): 0.09–0.13 (*m*, ^{t}BuSi); 0.91–1.00 (*m*, ^{i}Pr$_3$Si); 1.17–1.25 (*m*, (*Me*$_2$CH)$_2$N); 2.34 (*s*, CH_3–C(6)); 2.66 (br. d, *J* = 5.1, 2 H, CH_2CN); 3.65–3.99 (*m*, 6 H, (MeC*H*)$_2$N, H$_2$–C(5'), $POCH_2$); 4.15, 4.18 (2*s*, 3 H, CH_3–N(4)); 4.34 (br. s, 1 H, $POCH_2$); 4.53–4.61 (*m*, 1 H, $POCH_2$); 4.75–4.81 (*m*, 1 H, H–C(4')); 4.88–4.90 (*m*, 0.5 H, H–C(3')); 4.96–4.98 (*m*, *J* = 4.9, 1.5 H, OCH_2O, H–C(3')); 5.03 (*d*, *J* = 4.8, 1 H, OCH_2O); 5.06 (br. *s*, 1 H, H–C(2')); 6.22–6.25 (*m*, 1 H, H–C(1')); 7.46 (br. *s*, H–C(7)); 8.04 (*s*, H–C(2)). ^{31}P-NMR (162 MHz, $CDCl_3$): 149.9, 151.6. MALDI-MS: 836.81 (100, $[M + H]^+$).

3',5'-Di-O-*acetyl*-N^4*-desmethyl*-C^7*-iodo*-N^5*-methyl-2'*-O-*{[(triisopropylsilyl)oxy]methyl} wyosine* (**85**).

A soln. of **77** (0.290 g, 0.44 mmol) in DMF (7 ml) in a flask prevented from light, was successively treated with K_2CO_3 (71 mg, 0.50 mmol), MeI (0.04 ml, 0.50 mmol) and stirred at r.t. for 1.5 h. Workup and evaporation gave crude **83**. The residue was dissolved in CH_2Cl_2 (1.6 ml) and the flask prevented from light. The soln. was successively treated with a 10% soln. of $NaHCO_3$ in water (0.4 ml), N-Iodosuccinimide (109 mg, 0.49 mmol) and stirred at r.t. for 3 h. After workup the organic layers were washed with a 10% soln. of sodium

thiosulfate in water (10 ml). Drying over $MgSO_4$ and CC (SiO_2 (6 g), CH_2Cl_2/Acetone 1:0 → 4:6) offered **85** (191 mg, 60%, from **85**). Yellow foam. TLC (CH_2Cl_2/MeOH 19:1): R_f 0.42. ^{1}H-NMR (400 MHz, $CDCl_3$): 0.92–1.02 (*m*, $^iPr_3Si$); 2.09, 2.20 (2*s*, 2 MeCO); 2.39 (*s*, CH_3–C(6)); 3.71 (*s*, CH_3–N(5)); 4.32 (*dd*, *J* = 5.5, 11.8, H–C(5')); 4.44 (*dd*, *J* = 5.5, 10.2, H–C(4')); 4.51 (*dd*, *J* = 4.7, 11.6, H'–C(5')); 4.87 (*d*, *J* = 5.1, OCH_2O); 4.96 (*d*, *J* = 5.0, OCH_2O); 5.25 (*t*, *J* = 5.2, H–C(2')); 5.59 (*t*, *J* = 5.0, H–C(3')); 6.03 (*d*, *J* = 4.9, H–C(1')); 7.69 (br. *s*, H–C(2)). ^{13}C-NMR (100 MHz, $CDCl_3$): 11.8 (*q*, CH_3–C(6)); 12.2 (*d*, Me_2CH); 18.0 (*q*, Me_2CH); 21.2 (*q*, 2 *Me*CO); 30.5 (*q*, CH_3–N(5)); 64.2 (*t*, C(5')); 72.1 (*d*, C(3')); 76.4 (*d*, C(2')); 80.1 (*d*, C(4')); 88.6 (*d*, C(1')); 90.2 (*t*, OCH_2O); 108.6 (*s*, C(9a)); 117.9 (*s*, C(7)); 132.4 (*s*, C(6)); 137.9 (*d*, C(2)); 146.7 (*s*, C(3a)); 148.3 (*s*, C(4a)); 154.3 (*s*, C(9)); 170.5 (*s*, Me*C*O); 171.0 (*s*, Me*C*O). ESI-MS: 732.32 (100, $[M + H]^+$).

3',5'-Di-O-*acetyl*-C^7-*iodo-2'*-O-*{[(triisopropylsilyl)oxy]methyl}wyosine* (**84**).

A light prevented emulsion of **83** (141 mg, 0.23 mmol) in CH_2Cl_2 (0.8 ml) and a 10% soln. of $NaHCO_3$ in water (0.2 ml) was treated with N-Iodosuccinimide (57 mg, 0.26 mmol) and stirred at r.t. for 3 h. After workup the organic layers were washed with a 10% soln. of sodium thiosulfate in water (10 ml). Drying over $MgSO_4$ and CC (SiO_2 (6 g), hexane/AcOEt 1:1 → 0:1 (+ 1% Et_3N)) offered **84** (113 mg, 67%). Yellow oil. TLC (hexane/AcOEt 1:9): R_f 0.55. ^{1}H-NMR (400 MHz, $CDCl_3$): 1.00–1.20 (*m*, $^iPr_3Si$); 2.23, 2.35 (2*s*, 2 MeCO); 2.47 (*s*, CH_3–C(6)); 4.27 (*s*, CH_3–N(4)); 4.43 (*dd*, *J* = 2.8, 12.6, H–C(5')); 4.48 (*dd*, *J* = 2.9, 12.5, H'–C(5')); 4.63 (*dd*, *J* = 2.5, 5.2, H–C(4')); 5.02 (*dd*, *J* = 5.6, 6.3, H–C(2')); 5.05 (*d*, *J* = 4.9, OCH_2O); 5.10 (*d*, *J* = 4.9, OCH_2O); 5.54 (*dd*, *J* = 2.5, 5.2, H–C(3')); 6.30 (*d*, *J* = 6.7, H–C(1')); 8.01 (br. *s*, H–C(2)). ^{13}C-NMR (100 MHz, $CDCl_3$): 14.6 (*q*, CH_3–C(6)); 12.2 (*d*, Me_2CH); 18.2 (*q*, Me_2CH); 21.1, 21.2 (2*q*, 2 *Me*CO); 34.4 (*q*, CH_3–N(4)); 63.7 (*t*, C(5')); 71.6 (*d*, C(3')); 77.3 (*d*, C(2')); 81.8 (*d*, C(4')); 87.2 (*d*, C(1')); 89.8 (*t*, OCH_2O); 117.6 (*s*, C(7)); 125.4 (*s*, C(9a)); 134.3 (*d*, C(2)); 139.5 (*s*, C(6)); 144.2 (*s*, C(3a)); 145.2 (*s*, C(4a)); 153.3 (*s*, C(9)); 170.4 (*s*, Me*C*O); 170.5 (*s*, Me*C*O). ESI-MS: 732.32 (100, $[M + H]^+$).

2',3',5'-Tri-O-*acetyl*-N^4-*desmethyl*-N^5-*methyl*-C^7-*propenitrile-wyosine* (**95**).

In a light prevented flask, a soln. of **86** (50 mg, 0.09 mmol) prepared according to (Glemarec et al. 1988) in DMF (1 ml) was successively treated with $NaHCO_3$ (18 mg, 0.21 mmol), NBu_4Cl (24 mg, 0.09 mmol), Palladium(II) acetate (2 mg, 0.01 mmol) and acrylonitrile (18 mg, 0.17 mmol) followed by stirring at r.t. for 6 h. The reaction mixture has been poured on a mixture of H_2O/AcOEt 1:1 (50 ml) and extracted with AcOEt. The combined organic layers were washed with brine, dried over Na_2SO_4 and the solvent evaporated to dryness giving

crude **88** as a mixture of cis:trans isomers. TLC (CH_2Cl_2/MeOH 9:1): R_f 0.73. ESI-MS: 513.32 (100, $[M + H]^+$).

N-Methylbut-3-enamide (**89**).

To a cold (4°) 2.6 M soln. of $MeNH_2$ in THF (50 ml, 130 mmol) was added dropwise 3-butenoic acid chloride (5.0 g, 48 mmol) followed by stirring at 4° for 5min. The precipitate filtered and the mother liquids evaporated to dryness, the oily residue has been submitted to CC (SiO_2 (60 g), CH_2Cl_2/Acetone 1:0 → 8:2) and offered **89** (2.7 g, 57%). Yellow oil. TLC (CH_2Cl_2/Acetone 1:1): R_f 0.58. ^{1}H-NMR (400 MHz, $CDCl_3$): 2.76 (*d*, *J* = 6.7, CH_3–N); 2.97 (*d*, *J* = 7.1, CH_2CO); 5.13–5.19 (*m*, H_2C=C); 5.83–5.95 (*m*, *H*C=CH_2); 6.29 (br. *s*, NH). ^{13}C-NMR (100 MHz, $CDCl_3$): 26.7 (*q*, CH_3–N); 41.8 (*t*, H_2C–CO); 119.8 (*t*, H_2C=C); 131.9 (*d*, H*C*=CH_2); 171.9 (*s*, *C*O). EI-MS: 99 (100, $[M]^+$).

N-*Methyl*-N'-*propen-2-ylurea* (**92**).

To a 11 M soln. of MeNCO **93** in Toluene (5 ml, 55 mmol) was added dropwise allylamine (3.6 ml, 48 mmol) followed by stirring at r.t. overnight. The solvent has been removed under reduced pressure and the oily residue has been submitted to CC (SiO_2 (10 g), hexane/AcOEt 1:1 → 0:1) and offered **92** (5.2 g, 95%). Light brown wax. TLC (CH_2Cl_2/Acetone 1:1): R_f 0.41. ^{1}H-NMR (400 MHz, $CDCl_3$): 2.78 (*s*, CH_3–N); 2.97 (br. *d*, *J* = 4.7, H_2C–CH); 5.11 (*dd*, *J* = 1.0, 11.0, H–CH=CH); 5.20 (*dd*, *J* = 1.7, 17.7, H'–CH=CH); 5.88 (*ddd*, *J* = 5.3, 10.6, 15.7, *H*C=CH_2). ^{13}C-NMR (100 MHz, $CDCl_3$): 27.5 (*q*, CH_3–N); 43.4 (*t*, H_2C–CH); 116.9 (*t*, H_2C=CH); 135.9 (*d*, H*C*=CH_2); 159.7 (*s*, *C*O). ESI-MS: 115.34 (100, $[M + H]^+$).

3',5'-Di-O-*acetyl*-N^4-*desmethyl*-N^5-*methyl*-C^7-*(*N-*Methyl*-N'-*propen-2-ylurea) -2'*-O-*{[(tri isopropylsilyl)oxy]methyl}wyosine* (**95**).

In a light prevented flask, a soln. of **84** (80 mg, 0.11 mmol) in DMF (2 ml) was successively treated with $NaHCO_3$ (28 mg, 0.33 mmol), NBu_4Cl (32 mg, 0.12 mmol), Palladium(II) acetate (4 mg, 0.02 mmol) and **92** (25 mg, 0.22 mmol) followed by stirring at r.t. for 6 h. The reaction mixture has been poured on a mixture of H_2O/AcOEt 1:1 (50 ml) and extracted with AcOEt. The combined organic layers were washed with brine and dried over Na_2SO_4. CC (SiO_2 (6 g), CH_2Cl_2/MeOH 1:0 → 19:1 (+1% Et_3N)) offered **95** (50 mg, 63%) as cis:trans mixture. Light yellow foam. TLC (CH_2Cl_2/MeOH 19:1): R_f 0.50. ESI-MS: 954.23 (100, $[M + H_2O+H]^+$).

N-*Methylpent-4-enamide* (**97**).

To a 33% ethanolic soln. of $MeNH_2$ (1.5 ml, 5.4 mmol) was added dropwise pent-4-enoic acid chloride (0.2 ml, 1.8 mmol) followed by stirring at r.t. for 24 h. The solvent has been removed under reduced pressure giving a light yellow solid which has been extensively washed with acetone. The mother liquids have been evaporated to dryness offering **97** (174 mg, 85%). Light orange oil. ^{1}H-NMR (400 MHz, $CDCl_3$): 2.26 (*t*, *J* = 6.3, CH_2CO); 2.37 (*q*, *J* = 7.0, CH_2–CH); 2.77 (*d*, *J* = 4.7, CH_3–N); 4.98 (*d*, *J* = 10.2, *H*–CH=CH); 5.01 (*d*, *J* = 17.2, *H*'–CH=CH); 5.83–5.95 (*m*, *H*C=CH_2); 6.07 (br. *s*, HN). ^{13}C-NMR (100 MHz, $CDCl_3$): 26.6 (*q*, CH_3–N); 30.1 (*t*, H_2C–HC=); 36.1 (*t*, H_2C–CO); 115.8 (*t*, H_2C=CH); 137.5 (*d*, HC=CH_2); 173.6 (*s*, CO). ESI-MS: 114.32 (100, $[M + H]^+$).

3',5'-Di-O-*acetyl*-N^4*-desmethyl*-N^5*-methyl*-C^7*-(*N-*Methylpent-4-enamide)-2'*-O-*{[(triisopropylsilyl)oxy]methyl}wyosine* (**98**).

In a light prevented flask, a soln. of **84** (200 mg, 0.28 mmol) in DMF (3 ml) was successively treated with $NaHCO_3$ (70 mg, 0.82 mmol), NBu_4Cl (96 mg, 0.30 mmol), Palladium(II) acetate (12 mg, 0.06 mmol) and **97** (64 mg, 0.54 mmol) stirred at r.t. for 6 h. Thus, the reaction mixture was poured on a H_2O/AcOEt 1:1 (100 ml) and extracted twice with AcOEt (2 x 50 ml). The combined organic layers were washed with brine and dried over Na_2SO_4. CC (SiO_2 (6 g), CH_2Cl_2/MeOH 1:0 → 19:1) offered **98** (140 mg, 71%) as cis:trans 7:10 mixture. Light yellow foam. TLC (CH_2Cl_2/MeOH 9:1): R_f 0.63. ESI-MS: 938.23 (100, $[M + H]^+$).

3',5'-Di-O-*acetyl*-N^4*-desmethyl*-N^5*-methyl*-C^7*-(*N-*Methylpentamide)-2'*-O-*{[(triisopropyl silyl)oxy]methyl}wyosine* (**100**).

To a well-stirred suspension of activated 5% supported Palladium on charcoal (200 mg) in EtOH (3 ml) under H_2 (balloon) was added a soln. of **98** (140 mg, 0.18 mmol) in EtOH (2 ml). After 24 h of stirring at r.t., the supernatant was collected by centrifugation and the solid suspended in MeOH (5 ml). After centrifugation the supernatant was collected and the combined organic layers were evaporated to dryness. The residue was treated with a satd. soln. of NH_3 in MeOH (6 ml) and stirred for 6 h at r.t. After evaporation to dryness, the residue dissolved in Py (4 ml) and treated with $(MeO)_2TrCl$ (135 mg, 0.4 mmol) for 12 h at r.t. Workup and CC (SiO_2 (8 g), hexane/AcOEt 1:1 then CH_2Cl_2/MeOH 19:1 → 1:9) gave **100** (50 mg, 28% from **96**). Yellow oil. TLC (CH_2Cl_2/MeOH 19:1): R_f 0.25. ^{1}H-NMR (400 MHz, Acetone): 0.91–1.07 (*m*, ^{i}Pr$_3$Si); 1.69 (*m*, CH_2–CH_2 or 2 CH_2); 2.19 (*t*, *J* = 7.3, CH_2–C(7)); 2.29 (*s*, CH_3–C(6)); 2.72 (*d*, *J* = 4.6, CH_3–NH); 3.17 (*m*, H_2C–CO or linker number);

3.38–3.44 (*m*, H–C(5'), CH_3–N(5)); 3.52 (*dd*, *J* = 5.6, 10.1, H'–C(5')); 3.77, 3.78 (2*s*, 2 MeO); 4.08 (*d*, *J* = 4.7, HO–C(3')); 4.28 (*q*, *J* = 4.5, H–C(4')); 4.68 (*q*, *J* = 4.3, H–C(3')); 5.11 (br. *s*, OCH_2O); 5.20 (*t*, *J* = 5.3, H–C(2')); 6.12 (*d*, *J* = 5.4, H–C(1')); 6.75–6.89 (*m*, 4 arom. H); 7.04 (br. *s*, HN); 7.15–7.49 (*m*, 9 arom. H); 7.87 (*s*, H–C(2)); ^{13}C-NMR (100 MHz, Acetone): 7.7 (*d*, CH_3–C(6)); 12.1 (*d*, Me_2CH); 17.6 (*q*, Me_2CH); 24.2 (*t*, CH_2–C(7)); 25.3 (*t*, CH_2); 25.5 (*t*, CH_2); 31.0 (*t*, CH_2–CO); 35.9 (*q*, CH_3–NH); 55.0 (*q*, MeO); 64.4 (*t*, C(5')); 70.9 (*d*, C(4')); 79.1 (*d*, C(2')); 84.5 (*d*, C(3')); 86.4 (*s*, arom. C); 87.3 (*d*, C(1')); 90.1 (*t*, OCH_2O); 113.3 (*d*, arom. C); 117.8 (*s*, C(9a)); 119.9 (*s*, C(7)); 123.3 (*s*, C(6)); 127.0, 128.0, 128.5, 130.5 (4*d*, arom. C); 136.3, 136.4 (2*s*, arom. C); 137.5 (*s*, C(2)); 145.6 (*s*, C(4a)); 146.3 (*s*, arom. C); 149.7 (*s*, C(3a)); 154.4 (*s*, C(9)); 159.1 (*s*, arom. C); 172.9 (*s*, *C*O-NH). ESI-MS: 937.35 ($[M + H]^+$).

Preparation of RNA sequences

RNA-sequence r(GGUGGGAG-MeW-CGUCCCACC) (**S2**).

The assembly was carried out on a Gene Assembler Plus (*Pharmacia*) from 60 mg solid support (loading 32 μmol/g) under standard conditions according to (Pitsch et al. 2001). Conventional and modified **75** 2'-*O*-TOM-protected ribonucleoside phosphoramidites have been employed. For the oxidation steps, the conventional iodine reagent was replaced with a soln. of tBuOOH (1.1M soln. in acetonitrile) and oxidation was carried out with a flow 0.5 ml/min during 6 min. Cleavage from the solid support and deprotection was carried out with a 12M aqueous soln. of NH_3 in EtOH 1:1 (1ml) for 14h at 55°. By centrifugation, the supernatant soln. was separated from the solid support and evaporated, and the residue was dissolved in 1M $Bu_4NF{\cdot}3H_2O$ soln. in THF (1 ml). After 14h at 20°, 1M TrisHCl buffer (pH 7.4, 1ml) was added and the soln. concentrated to half volume. The remaining soln. (1 ml) was applied on NAP-10 cartridge (*Pharmacia*) and eluted with H_2O. The first 1.5 ml soln. was purified by ion-exchange HPLC: AE-HPLC (0 - 50% *B* in 60 min) flow 2.5 ml/min; eluent *A*: 12 mM *Tris*·HCl (pH 7.4), 6M urea; eluent *B*: 12 mM *Tris*·HCl (pH 7.4), 0.5M $NaClO_4$, 6M urea; (detection at 260 nm, elution at 85°): t_R 38 min. LC-MS (ESI): *m*/*z* = 5'854 (calc. 5'854).

DNA-sequence d(GGUGGGACG-_-CUCCCA) (**S7**).

The assembly of the sequence was carried out under standard conditions (Pitsch et al. 2001). 2'-deoxy phosphoramidites from (*Glen Research*) and propyl phosphoramidite linker prepared according to (Seela and Kaiser 1987) were used. LC-MS (ESI): *m*/*z* = 4'731 (calc. 4'731).

RNA-sequence r(GGUGGGAG-X-CGUCCCACC) (**S8-S12**, **S15**).

The assembly of RNA sequences was carried out on a Gene Assembler Plus (*Pharmacia*), from 60 mg solid support (loading 32 μmol/g) and 2'-*O*-TOM-protected ribonucleoside phosphoramidites according to (Pitsch et al. 2001). The modified nucleotides were introduced conventionally from the 2'-*O*-TOM-protected ribonucleoside phosphoramidites X = m^1G **54**, I **56**, $m^2{}_2G$ **43**, m^6A **53** or 2'-deoxyadenosine dA (*Glen Research*). After the assembly, the solid supports were treated with a mixture of 12M $MeNH_2$ in H_2O / 8M $MeNH_2$ in EtOH (1 ml) for 4h at 35°. Then, the same procedure as described for **S2** was applied to the RNA sequences **S8-S12** and **S15**. LC-MS (ESI): *m*/*z* for X = m^1G (calc. 5'816, found 5'816) (**S8**), I (calc. 5'787, found 5'787) (**S9**), G (calc. 5'801, found 5'801) (**S10**), $m^2{}_2G$ (calc. 5'830, found 5'830) (**S11**), dA (calc. 5'770, found 5'770) (**S12**), m^6A (calc. 5'800, found 5'800) (**S15**).

RNA-sequence r(GGUGGGAGACGU-X-CCACC) (**S13,S14**).

The RNA sequences were prepared as previously described for RNA sequences **S8-S12** and **S15** from 2'-*O*-TOM, 2'-*O*-Me (*Glen Research*) and 2'-deoxy (*Glen Research*) phosphoramidite building blocks. LC-MS (ESI): *m*/*z* for X = dC (calc. 5'770, found 5'770) (**S13**), Cm (calc. 5'800, found 5'800) (**S14**).

RNA-sequence r(GGUGGGA-I-ACGUCCCACC) (**S16**).

The RNA sequence was prepared as previously described for RNA sequences **S9**. LC-MS (ESI): *m*/*z* = 5'787 (calc. 5'787).

RNA-sequence r(GGUGGGAG) (**S4**).

The RNA sequence was prepared under conditions as previously described for **S8-S12** and **S15**. The remaining soln. (1 ml) was applied on NAP-10 cartridge (*Pharmacia*) and eluted with H_2O. The first 1.5 ml soln. was purified by ion-exchange HPLC: AE-HPLC (0 - 50% *B* in 30 min) flow 2.5 ml/min; eluent *A*: 12 mM *Tris*·HCl (pH 7.4), 6M urea; eluent *B*: 12 mM *Tris*·HCl (pH 7.4), 0.5M $NaClO_4$, 6M urea; (detection at 260 nm, elution at 85°). However, the fraction containing pure product were treated with 1M aq. $Et_3N{\cdot}H_2CO_3$ to a final 0.1M concentration and applied to a *Sepak*-cartridge (conditioned by washing with MeCN (10 ml) and 0.1M aq. $Et_3N{\cdot}H_2CO_3$ (10ml)). The cartridge was washed with 20mM $Et_3N{\cdot}H_2CO_3$ (10 ml) and the RNA sequence was eluted with MeCN/H_2O 1:1 (4ml). In order to completely remove the remaining $Et_3N{\cdot}H_2CO_3$, 0.5 ml H_2O were added to the residue, followed by

lyophilisation (this procedure was carried out twice). MALDI MS (neg mode): *m*/*z* = 2'645 (calc. 2'645).

RNA-sequences 5'-monophosphate-r(X-CGUCCCACC) (**S17-S20**).

The RNA sequences were prepared under conditions as previously described for **S8-S12** and **S15** from the conventional 2'-*O*-TOM phosphoramidite building blocks and phosphorylating reagent [3-(4,4'-dimethoxytrityloxy)-2,2-dicarboxyethyl]propyl-(2-cyanoethyl)-(*N*,*N*-diisopropyl)-phosphoramidite (*Glen Research*). The crude sequences were purified by AE-HPLC (0 - 50% *B* in 30 min) flow 2.5 ml/min; eluent *A*: 12 mM *Tris*·HCl (pH 7.4), 6M urea; eluent *B*: 12 mM *Tris*·HCl (pH 7.4), 0.5M $NaClO_4$, 6M urea; (detection at 260 nm, elution at 85°). LC-MS (ESI): *m*/*z* for X = A (calc. 3'159, found 3'159) (**S17**), C (calc. 3'135, found 3'135) (**S18**), G (calc. 3'174, found 3'174) (**S19**), U (calc. 3'135, found 3'135) (**S20**).

RNA-sequence r(imG-CGUCCCACC) (**S6**).

The RNA sequence was prepared as previously described for RNA sequences **S9** but employing the 2'-O-TOM wyosine phosphoramidite building block **81**. Furthermore, a 2'-O-TOM H_2N-C(2) unprotected guanosine phosphoramidite building block, prepared according to (Stutz et al. 2000), has been introduced by performing a double coupling cycle. For the wyosine building block, the oxidation step has been adapted. The final oxidation was achieved with a 1.1M soln. of tBuOOH in MeCN (flow 0.5 ml/min during 18min). The solid support was washed with $(^{i}Pr)_2NH$/MeCN 1:9 for 20 min (flow-rate 2.5 ml/min). Cleavage from the solid support and deprotection was carried out with 12M NH_3 in MeOH (1ml) for 14h at 20°. The supernatant was removed by centrifugation and evaporated to dryness; the residue was treated with a THF soln. (1 ml) of $Bu_4NF{\cdot}3H_2O$ (1M) for 14h at 20°, diluted with aq. *Tris*·HCl (1 ml, 1M, pH 7.4) and evaporated to a volume of 1 ml. After desalting on a NAP cartridge, the crude product was purified by ion-exchange HPLC: AE-HPLC (0 - 40% *B* in 40 min) flow 2.5 ml/min; eluent *A*: 12 mM *Tris*·HCl (pH 7.4), 6M urea; eluent *B*: 12 mM *Tris*·HCl (pH 7.4), 0.5M $NaClO_4$, 6M urea; detection at 260 nm, elution at 85°.): t_R 30.0 min. LC-MS (ESI): *m*/*z* = 3'147 (calc. 3'147).

RNA-sequence 5'-monophosphate-r(imG-CGUCCCACC) (**S5**).

The oligonucleotide was dissolved to a final concentration of 20 µM for a volume of reaction of 60µl. The phosphorylation reaction was performed according the instructions from the supplier (*Fermentas*). The reaction was performed for 1.5 h at 37°. The crude reaction mixture was purified by AE-HPLC. (0 - 40% *B* in 40 min) flow 2.5 ml/min; eluent *A*: 12 mM

Tris·HCl (pH 7.4), 6M urea; eluent *B*: 12 mM *Tris*·HCl (pH 7.4), 0.5M $NaClO_4$, 6M urea; detection at 260 nm, elution at 85°.): t_R 30 min. LC-MS (ESI): *m/z* = 3'226 (calc. 3'226).

Analytical HPLC for kinetic monitoring Control: AE-HPLC. (0 - 50% *B* in 30 min) flow 1.0 ml/min; eluent *A*: 12 mM *Tris*·HCl (pH 7.4), 6M urea; eluent *B*: 12 mM *Tris*·HCl (pH 7.4), 0.5M $NaClO_4$, 6M urea; detection at 260 nm, elution at 85°. *r(imG-CGUCCCACC)*: t_R 20.3 min, *5'-monophosphate-r(imGCGUCCCACC)*: t_R 21.8 min.

Ligation

RNA-sequence r(GGUGGGAG-imG-CGUCCCACC) (**S3**).

The 8mer **S4**, 10mer **S5** and 16mer template **S7** were mixed to a final concentration of 20 µM for the substrates and 30 µM for the template (1.5eq). After the addition of PEG 6000 (3µl of 50% PEG stock soln.) and water (q.s.p. 54 µl), the mixture was heated at 95° for 4 minutes and subsequently cooled down to 40° by steps of 0.1° per second and finally to 4° within 1.5min. After the addition of ligation buffer (40mM TrisHCl, 2mM $MgCl_2$, 10mM DTT, 0.5mM ATP) and 8 units of ribonuclease inhibitor (*Fermentas*), the reaction was initiated by adding T4 DNA ligase (8 Weiss units) and kept at 37°. For the ligation kinetic, aliquots were taken and diluted in 1mL of water and injected into an analytical AE-HPLC. AE-HPLC. (0 - 60% *B* in 36 min) flow 1.0 ml/min; eluent *A*: 12 mM *Tris*·HCl (pH 7.4), 6M urea; eluent *B*: 12 mM *Tris*·HCl (pH 7.4), 0.5M $NaClO_4$, 6M urea; detection at 260 nm, elution at 85°.). *r(GGUGGGAG)*: t_R 17.7 min (**S4**). *5'-monophosphate-r(imG-CGUCCCACC)*: t_R 21.4 min (**S5**). *d(GGUGGGACG-_-CUCCCA)*: t_R 24.9 min (**S7**). *r(GGUGGGAG-imG-CGUCCCACC)*: t_R 26.0 min (**S3**). LC-MS (ESI): *m/z* = 5'854 (calc. 5'854).

Preparative ligation for NMR analysis

160 ligation batches for sequence **S3** of 60 µl as described above were performed, purified and pooled. The Et_3NH^+-form of the sequence was transformed into its sodium salt. (0.35 µmoles, 18% yield) (determined by UV-spectroscopy, ε (260 nm) = 180'900 $l \cdot mol^{-1} \cdot cm^{-1}$). ^{1}H-NMR (400 MHz, D_2O, *c* = 100 µM).

NMR Analysis of sequences S2 and S8-S16

The Et_3NH^+-form of the sequences were transformed into their sodium salt. Typically 1.56 µmoles. ^{1}H-NMR (400 MHz, D_2O, *c* = 156 µM).

CHAPTER IV

Computational Methods

All calculations were carried out using density functional theory or the MP2 method as implemented in the Gaussian03 package (Frisch et al. 2003). The geometry optimisation was performed with B3LYP/6-31+G(d,p) (Becke 1993) (Becke 1988) and the single point calculation including BSSE correction were achieved with MP2/6-31+G(d,p).

The initial coordinates were reconstructed from the NMR refinements of a human $tRNA^{Lys}_3$ backbone (pdb: 1FL8) (bases $\mathbf{t^6A}$37 and **A**36) and by introduction of an A·U base pair taken from a base pair within the tRNA (Sundaram et al. 2000). Finally, the geometries have been adapted using Materials Studio (*Accelrys*). Furthermore, in order to reduce time consumption of calculations, we limited the system to the solely bases since they provide the π-stacking interactions and the hydrogen bond pattern. The sugar-base backbone cut was saturated with hydrogen atoms and the corresponding N(9) was kept frozen during the calculation. To keep adjacent bases parallel during the geometry optimisation, we kept always three alternating atoms fixed in a series of subsequent geometry optimisations. In this way, we relaxed all degrees of freedom but kept the backbone and the "π-stacking" frozen. The geometries and wave functions were optimized by starting from the NMR refinement structure of **1** or modifications thereof. The energetic of the **AI**·**U**36 base pair in the complex ($\mathbf{t^6A}$37/**U**36·**AI**) has been estimated and compared to the (**A**37/**U**36·**AI**) complex, revealing the strong contribution of this additional hydrogen bonding to the stability. Whereas the (**A**37/**U**36·**AI**) complex exhibits an energy of

$$\mathbf{E_{A/UA} = 14.49\ kcal/mol},$$

the t^6A containing complex presents an energy of

$$\mathbf{E_{t6A/UA} = 22.98\ kcal/mol}$$

giving a stabilization energy of

$$\mathbf{8.49\ kcal/mol\ (E_{Stab} = E_{t6A/UA} - E_{A/UA})}.$$

This value, including the contribution of the stacking interaction and of the hypothetical additional hydrogen bond, permits the adjacent A·U base pair energy to reach the energy of a G·C base pair (22.98 against 24.05 kcal/mol for a G·C base pair).

It can be noticed that the energy of an isolated A·U base pair calculated in a analogous manner gives an energy of E_{UA} = 12.63 kcal/mol. Consequently, the energy of stacking provided by the adjacent adenosine is

$$\mathbf{E_{Stab(\pi)} = E_{A/UA} - E_{UA} = 14.49\text{-}12.63 = 1.86\ kcal/mol}$$

This value is quite low in comparison to the stabilization provided by the presence of t^6A nucleobase. The best descriptor of the pi-stacking energy being the surface area overlap, the stacking provided by the t6A modification does not, in our model system, exceed those from a simple adenosine. The extended ureidyl motif is nearly excluded from the surface of the base pair and we can therefore estimate the energetical contribution of the solely hydrogen bond, to be almost equal to

$$\mathbf{E_{Stab(H\text{-}bond)} = E_{Stab(\pi+H\text{-}bond)} - E_{Stab(\pi)} = (E_{t6A/UA} - E_{A/UA}) - (E_{A/UA} - E_{UA}) =}$$
$$\mathbf{(22.98\text{-}14.49) - (14.49\text{-}12.63) = 6.63\ kcal/mol}$$

which is the same order of magnitude than some already reported values (Steiner 2002). For example the stabilization provided by hydrogen bond in the case of CO_2H dimer in gas phase is E = 7.5 kcal/mol.

CHAPTER V

5'-O-*(4,4'-Dimethoxytrityl)*- N^2*-methoxyacetylguanosine* (**104**).

Guanosine hydrate (1.0 g, 3.32 mmol), dried by twice coevaporation with Py, was suspended in Py (25 ml) and CH_2Cl_2 (75 ml), cooled to 4° and treated with trimethylchlorosilane (3.8 ml, 29.87 mmol) followed by stirring at r.t. for 2 h. Then, the reaction mixture, was cooled again at 4° and methoxyacetylchloride (0.26 ml, 3.65 mmol) added followed by stirring at 4° for 4 h. Workup and evaporation gave a yellow oil which has been dissolved in a mixture of (AcOH:MeOH) (1:9) and stirred at r.t. for 3 h. Evaporation to dryness and coevaporation with toluene gave a light orange oil. The residue was dissolved in Py (100 ml) and treated with $(MeO)_2TrCl$ (1.35 g, 3.98 mmol). After 12 h at r.t., workup and CC (SiO_2 (30 g), hexane/AcOEt 1:1 → 0:1 then CH_2Cl_2/MeOH 19:1 → 4:1) offered **104** (0.85 g, 39%, from guanosine). Light colourless foam. TLC (CH_2Cl_2/MeOH 9:1): R_f 0.31. ^{1}H-NMR (400 MHz, $CDCl_3$): 3.28–3.42 (*m*, H_2–C(5'), MeO); 3.58 (br. *s*, OH–C(2')); 3.76 (*s*, MeO); 3.99 (*s*, CH_2CO); 4.31 (br. *s*, H–C(2')); 4.43 (br. *s*, H–C(3')); 4.87 (*t*, *J* = 5.1, H–C(4')); 5.91 (*d*, *J* = 6.3, H–C(1')); 6.06 (br. *s*, HO–C(3')); 6.79 (*d*, *J* = 7.8, 4 arom. H); 7.13–7.44 (*m*, 9 arom. H); 7.68 (*s*, H–C(8)); 9.30 (br. *s*, HN–C(2)); 11.82 (br. *s*, H–N(1)). ^{13}C-NMR (100 MHz, $CDCl_3$): 55.9 (*q*, MeO); 60.1 (*q*, *Me*OCH_2); 61.1 (*t*, C(5')); 71.7 (*t*, *C*H_2OMe); 72.4 (*d*, C(2')); 77.9 (*d*, C(3')); 85.4 (*d*, C(4')); 87.1 (*d*, C(1')); 89.5 (*t*, OCH_2O); 114.0 (*d*, arom. C); 121.6 (*d*, C(5)); 127.5, 128.6, 128.8, 131.0 (4*d*, arom. C); 136.3, 136.4 (2*s*, arom. C); 139.0 (*d*, C(8)); 145.3 (*s*,

arom. C); 147.0 (*s*, C(2)); 148.7 (*s*, C(4)); 155.9 (*s*, C(6)); 159.2 (*s*, arom. C); 172.5 (*s*, *C*OCH_2). ESI-MS: 658.32 (100, $[M + H]^+$).

N^2*-,3'*-O-*Bismethoxyacetyl-5'*-O-*(4,4'-dimethoxytrityl)-2'*-O-*{[triisopropylsilyl)oxy]methyl}guanosine* (**105**).

To a cold (4°) soln. of **1** (780 mg, 1.0 mmol) in Py (10 ml) has been added methoxyacetylchloride (120 mg, 1.1 mmol) followed by stirring at r.t. for 3 h. Then, the reaction mixture, cooled to 4°, was treated with trimethylchlorosilane (163 mg, 1.5 mmol) and stirred for 1 h. Then, methoxyacetylchloride (120 mg, 1.1 mmol) has been added and the reaction mixture stirred at 4° for 2 h. Workup and CC (SiO_2 (10 g), hexane/AcOEt 3:2 → AcOEt) offered **105** (732 mg, 80%). Light yellow foam. TLC (hexane/AcOEt 1:9): R_f 0.58. ^{1}H-NMR (400 MHz, $CDCl_3$): 0.90–1.00 (*m*, ^{i}Pr$_3$Si); 3.32–3.40 (*m*, MeO, H–C(5')); 3.41–3.48 (*m*, MeO, H'–C(5')); 3.79 (*s*, 2 MeO); 3.97 (*s*, CH_2CON); 4.08 (*d*, *J* = 16.6, OCH_2O); 4.15 (*d*, *J* = 16.6, OCH_2O); 4.29 (*dd*, *J* = 2.8, 5.6, H–C(4')); 4.88 (*s*, CH_2COO); 5.12 (*dd*, *J* = 4.8, 6.8, H–C(2')); 5.62 (*dd*, *J* = 2.8, 4.8, H–C(3')); 5.97 (*d*, *J* = 6.7, H–C(1')); 6.80 (*d*, *J* = 8.6, 4 arom. H); 7.20–7.44 (*m*, 9 arom. H); 7.84 (*s*, H–C(8)); 8.78 (br. *s*, HN–C(2)); 11.80 (br. *s*, H–N(1)). ^{13}C-NMR (100 MHz, $CDCl_3$): 11.7 (*d*, Me_2CH); 17.6 (*q*, Me_2CH); 55.3 (*q*, *Me*OCH_2); 59.4 (*q*, MeO); 63.1 (*t*, C(5')); 69.5 (*t*, *C*H_2OMe); 70.6 (*t*, *C*H_2OMe); 72.2 (*d*, C(2')); 82.2 (*d*, C(3')); 86.1 (*s*, C(4')); 86.8 (*d*, C(1')); 89.9 (*t*, OCH_2O); 113.3 (*d*, arom. C); 122.3 (*d*, C(5)); 127.0, 128.0, 128.1, 130.0 (4*d*, arom. C); 135.5 (*s*, arom. C); 138.0 (*d*, C(8)); 144.3 (*s*, arom. C); 146.3 (*s*, C(2)); 148.1 (*s*, C(4)); 155.3 (*s*, C(6)); 158.7 (*s*, arom. C); 169.5 (*s*, *C*OCH_2); 170.7 (*s*, *C*OCH_2). ESI-MS: 916.32 (100, $[M + H]^+$).

5'-O-*(4,4'-Dimethoxytrityl)-* N^2*-methoxyacetyl-3'*-O-*trimethylsilyl-2'*-O-*{[triisopropylsilyl) oxy]methyl}guanosine* (**107**).

^{1}H-NMR (400 MHz, $CDCl_3$): 0.10 (*s*, Me_3Si); 0.97–1.30 (*m*, ^{i}Pr$_3$Si); 3.29 (*dd*, *J* = 3.9, 10.8, H–C(5')); 3.40–3.50 (*m*, MeO, H'–C(5')); 3.80 (*s*, 2 MeO); 4.05 (*s*, CH_2CO); 4.17 (br. *t*, *J* = 3.8, H–C(4')); 4.41 (*t*, *J* = 4.5, H–C(3')); 4.71 (*t*, *J* = 5.0, H–C(2')); 4.89 (*d*, *J* = 5.0, OCH_2O); 5.00 (*d*, *J* = 4.9, OCH_2O); 6.07 (*d*, *J* = 5.3, H–C(1')); 6.82 (*d*, *J* = 6.8, 4 arom. H); 7.21–7.45 (*m*, 9 arom. H); 7.92 (*s*, H–C(8)); 8.84 (br. *s*, HN–C(2)); 11.81 (br. *s*, H–N(1)). ^{13}C-NMR (100 MHz, $CDCl_3$): 0.10 (*q*, Me_3Si); 14.6 (*d*, Me_2CH); 18.2 (*q*, Me_2CH); 55.7 (*q*, *Me*OCH_2); 60.8 (*q*, MeO); 63.3 (*t*, C(5')); 71.1 (*t*, *C*H_2OMe); 71.4 (*d*, C(2')); 78.3 (*d*, C(3')); 85.1 (*d*, C(4')); 86.9 (*d*, C(1')); 89.4 (*t*, OCH_2O); 113.7 (*d*, arom. C); 122.7 (*d*, C(5)); 127.4, 128.3, 128.6, 130.5 (4*d*, arom. C); 136.0 (*s*, arom. C); 138.2 (*d*, C(8)); 144.8 (*s*, arom. C);

146.5 (*s*, C(2)); 148.3 (*s*, C(4)); 155.8 (*s*, C(6)); 159.0 (*s*, arom. C); 171.0 (*s*, *C*OCH_2). ESI-MS: 916.31 (100, $[M + H]^+$).

5'-O-*(4,4'-Dimethoxytrityl)*-N^2-*methoxyacetyl-2'*-O-*{[triisopropylsilyl)oxy]methyl}guanosine* (**106**).

To a cold (4°) soln. of **1** (1.2 g, 1.56 mmol) in Py (16 ml) has been added, over 2 min. trimethylchlorosilane (0.6 ml, 4.67 mmol) followed by stirring at r.t. for 2 h. Then, the reaction mixture, cooled to 4°, was treated with methoxyacetylchloride (0.16 ml, 1.71 mmol) and stirred at 4° for 8 h. Workup and evaporation gave a yellow oil which has been dissolved in a mixture of (AcOH:CH_3CN:H_2O) (1:50:50) and stirred at r.t. for 5 h. Workup and CC (SiO_2 (10 g), hexane/AcOEt 1:4 → 0:1 then AcOEt/MeOH 98:2 → 4:1) offered **106** (1.1 g, 84%). Light yellow foam. TLC (CH_2Cl_2/MeOH 9:1): R_f 0.50. ^{1}H-NMR (400 MHz, $CDCl_3$): 1.02–1.18 (*m*, ^{i}Pr$_3$Si); 3.07 (br. *s*, HO–C(3')); 3.34–3.39 (*m*, MeO, H–C(5')); 3.45 (*dd*, *J* = 3.4, 10.6, H'–C(5')); 3.79 (*s*, 2 MeO); 4.00 (*s*, CH_2CO); 4.28 (br. *d*, *J* = 2.6, H–C(4')); 4.53 (*m*, H–C(3')); 4.71 (*t*, *J* = 5.5, H–C(2')); 4.95 (*d*, *J* = 4.7, OCH_2O); 5.15 (*d*, *J* = 4.7, OCH_2O); 6.04 (*d*, *J* = 7.0, H–C(1')); 6.82 (*d*, *J* = 6.8, 4 arom. H); 7.21–7.46 (*m*, 9 arom. H); 7.86 (*s*, H–C(8)); 8.85 (br. *s*, HN–C(2)); 11.78 (br. *s*, H–N(1)). ^{13}C-NMR (100 MHz, $CDCl_3$): 12.2 (*d*, Me_2CH); 18.2 (*q*, *Me*$_2$CH); 55.7 (*q*, *Me*OCH_2); 59.8 (*q*, MeO); 64.1 (*t*, C(5')); 71.4 (*t*, *C*H_2OMe); 71.6 (*d*, C(2')); 83.2 (*d*, C(3')); 84.7 (*d*, C(4')); 87.1 (*d*, C(1')); 91.4 (*t*, OCH_2O); 113.6 (*d*, arom. C); 122.5 (*d*, C(5)); 127.4, 128.4, 128.5, 130.5 (4*d*, arom. C); 136.0 (*s*, arom. C); 138.0 (*d*, C(8)); 144.9 (*s*, arom. C); 146.8 (*s*, C(2)); 148.6 (*s*, C(4)); 155.8 (*s*, C(6)); 159.0 (*s*, arom. C); 171.1 (*s*, *C*OCH_2). ESI-MS: 844.39 (100, $[M + H]^+$).

5'-O-*(4,4'-Dimethoxytrityl)*-N^2-*Methoxyacetyl-2'*-O-*{[(triisopropylsilyl)oxy]methyl}guanosine 3'-(2-Cyanoethyl Diisopropylphosphoramidite* (**108**).

As described for **42**, with **106** (800 mg, 0.95 mmol), CH_2Cl_2 (5 ml), ^{i}Pr$_2$NEt (0.4 ml, 2.4 mmol) and cyanoethyl diisopropylphosphoramidochloridite (269 mg, 1.14 mmol). CC (SiO_2 (25 g), CH_2Cl_2/Acetone 99:1 → 19:1 (+ 3% Et_3N)): **106** (714 mg, 72%; 1:1 mixture of diastereoisomers). Colorless foam. TLC (CH_2Cl_2/Acetone 4:1): R_f 0.73. ^{1}H-NMR (400 MHz, $CDCl_3$): 0.90–1.00 (*m*, ^{i}Pr$_3$Si); 1.02–1.33 (4*d*, *J* = 6.6, (*Me*$_2$CH)$_2$N); 2.36 (*t*, *J* = 6.4, 1 H, CH_2CN); 2.68–2.83 (*m*, 1 H, CH_2CN); 3.25–3.45 (*m*, 4 H, *Me*OCH_2, H–C(5')); 3.46–3.71 (*m*, 2.5 H, (Me_2CH)$_2$N, $POCH_2$); 3.79 (*s*, 2 MeO); 3.85–4.02 (*m*, 3 H, C*H*$_2$OMe, $POCH_2$); 4.10–4.26 (*m*, 1.5 H, $POCH_2$); 4.26–4.32 (*m*, 0.5 H, H–C(4')); 4.37–4.41 (*m*, 0.5 H, H–C(4')); 4.49–4.55 (*m*, 1 H, H–C(3')); 4.87–5.00 (*m*, 3 H, OCH_2O, H–C(2')); 6.01 (*d*, *J* = 6.3, 0.5 H, H–C(1')); 6.06 (*d*, *J* = 7.0, 0.5 H, H–C(1')); 6.78–6.85 (*m*, 4 arom. H); 7.19–7.50 (*m*, 9 arom.

H); 7.89, 7.91 (2*s*, H–C(8')); 8.83 (br. *s*, HN–C(2)); 11.79 (br. *s*, H–N(1)). ^{31}P-NMR (162 MHz, $CDCl_3$): 151.6, 151.9. MALDI-MS: 1044.32 (100, $[M + H]^+$).

5'-O-*(4,4'-Dimethoxytrityl)*-N^2*-methoxyacetyl-2'*-O-*{[triisopropylsilyl)oxy]methyl}guanosine 3'-(4-Nitrophenyl Heptanedioate)* (**109**).

A soln. of **106** (170 mg, 0.2 mmol) in Py (2 ml) was treated with DMAP (12 mg, 0.1 mmol) and bis(4-nitrophenyl)heptanedioate (490 mg, 1.2 mmol) followed by stirring at r.t. for 14 h. Then, the reaction mixture has been evaporated to dryness and co-evaporated twice with toluene. CC (SiO_2 (4 g), hexane/AcOEt 1:1 → 0:1) offered **109** (127 mg, 57%). Light yellow foam. TLC (CH_2Cl_2/MeOH 19:1): R_f 0.69. ^{1}H-NMR (400 MHz, $CDCl_3$): 0.88–1.06 (*m*, ^{i}Pr$_3$Si); 1.46–1.54 (*m*, CH_2); 1.70–1.85 (*m*, CH_2); 2.40–2.50 (*m*, CH_2); 2.64 (*t*, *J* = 7.0, CH_2); 3.38 (br. *s*, MeO, H–C(5')); 3.40–3.45 (*m*, H'–C(5')); 3.80 (*s*, 2 MeO); 3.99 (*s*, CH_2CO); 4.17 (br. *d*, *J* = 3.1, H–C(4')); 4.89 (br. *s*, OCH_2O); 5.11 (*t*, *J* = 5.5, H–C(2')); 5.55 (br. *d*, *J* = 3.2, H–C(3')); 6.00 (*d*, *J* = 6.3, H–C(1')); 6.81 (*d*, *J* = 9.4, 4 arom. H); 7.21–7.43 (*m*, 11 arom. H); 7.85 (*s*, H–C(8)); 8.28 (*d*, *J* = 9.4, 2 arom. H); 8.78 (br. *s*, HN–C(2)); 11.81 (br. *s*, H–N(1)). ^{13}C-NMR (100 MHz, $CDCl_3$): 12.2 (*d*, Me_2CH); 17.9 (*q*, Me_2CH); 24.7, 24.9, 28.9, 30.1, 34.2 (5*t*, CH_2); 55.7 (*q*, $MeOCH_2$); 59.8 (*q*, MeO); 63.7 (*t*, C(5')); 71.4 (*t*, CH_2OMe); 72.2 (*d*, C(2')); 77.6 (*d*, C(3')); 83.0 (*d*, C(4')); 86.5 (*d*, C(1')); 90.2 (*t*, OCH_2O); 113.6 (*d*, arom. C); 122.8 (*d*, C(5)); 125.6, 127.4, 128.4, 128.5, 129.5, 130.5 (6*d*, arom. C); 135.8, 135.9 (2*s*, arom. C); 138.3 (*d*, C(8)); 144.8 (*s*, arom. C); 146.7 (*s*, C(2)); 148.6 (*s*, C(4)); 155.7 (*s*, C(6)); 159.0 (*s*, arom. C); 171.0 (*s*, $COCH_2$). ESI-MS: 1107.33 (100, $[M + H]^+$).

Preparation of RNA sequences

RNA-sequence 5'-monophosphate-r(GGGGCUAUAGCUCAGCGGGAU) (**S31-S33**).

The sequence was assembled from 60 mg of solid support (loading 30 μmol/g) using the standard conditions for the assembly of 2'-*O*-TOM-protected ribonucleoside phosphoramidites (Pitsch et al. 2001), but employing a combination of 2'-O-TOM H_2N-C(2) unprotected guanosine phosphoramidite building block unprotected guanosine and N^2-methoxyacetyl-protected guanosine **108** instead of the standard N^2-acetyl-protected guanosine. The tetra-G motif has been assembled as described in Chapter V. Furthermore, the capping step at the end of each coupling cycle was performed with methoxyacetic acid anhydride (Ackermann and Pitsch 2002). After the final detritylation, he solid support was washed with (^{i}Pr)$_2$NH/MeCN 1:9 for 20 min (flow-rate 2.5 ml/min). Cleavage from the solid support and deprotection was carried out with 12M NH_3 in MeOH (1ml) for 14h at 20°. The

supernatant was removed by centrifugation and evaporated to dryness; the residue was treated with a THF soln. (1 ml) of $Bu_4NF{\cdot}3H_2O$ (1M) for 14h at 20°, diluted with aq. *Tris*·HCl (1 ml, 1M, pH 7.4) and evaporated to a volume of 1 ml. After desalting on a NAP cartridge, the main peak of the crude product was isolated by ion-exchange HPLC for MS analysis: AE-HPLC (15 - 60% *B* in 30 min) flow 2.5 ml/min; eluent *A*: 12 mM *Tris*·HCl (pH 7.4), 6M urea; eluent *B*: 12 mM *Tris*·HCl (pH 7.4), 0.5M $NaClO_4$, 6M urea; detection at 260 nm, elution at 85°.): t_R 25.6 min. LC-MS (ESI): *m*/*z* = 6'890 (calc. 6'890),

RNA-sequence r(GGGGCUAUAGCUCAGCDGGGAGAGCGCUUGCAUCUA) (**S28**).
The sequence was assembled from 60 mg of solid support (loading 30 μmol/g) as described above for sequence **S33** (See Chapter V) using the standard conditions for the assembly of 2'-*O*-TOM-protected ribonucleoside phosphoramidites (Pitsch et al. 2001), The dihydrouridine D at position 17 was incorporated with the phosphoramidite building block **46**. The crude RNA-sequence was analyzed by AE-HPLC (20 - 80% *B* in 40 min): t_R 21.3 min (flow 1.0 ml/min). The purification was performed by AE-HPLC (20 - 80% *B* in 60 min): t_R 25.4 min (flow 2.5 ml/min). LC-MS (ESI): *m*/*z* = 11'624 (calc. 11'623).

RNA-sequence r(imG-AGCAAGAGGUCAGCGGUUCGAUCCCGCUUAG) (**S30**).
The sequence was assembled from 60 mg of solid support (loading 30 μmol/g) as described above for sequence **S28** and the wyosine building block was incorporated as described for the preparation of the 10mer wyosine-containing RNA sequence **S6**. The crude RNA-sequence was analyzed by AE-HPLC (20 - 80% *B* in 40 min): t_R 20.6 min (flow 1.0 ml/min). The purification was performed by AE-HPLC (20 - 80% *B* in 60 min): t_R 23.5 min (flow 2.5 ml/min). LC-MS (ESI): *m*/*z* = 10'370 (calc. 10'370). The phosphorylation of the sequence was performed as described above for sequence **S5** LC-MS (ESI): *m*/*z* = 10'450 (calc. 10'450).

2'-O-*Methoxy RNA-sequence m(CCUCUUGCU-_-UAGAUGCA)* (**S34**).
The assembly of the sequence was carried out on a Gene Assembler Plus (*Pharmacia*) with the 2'-*O*-methoxy phosphoramidites from (*Glen Research*) and propyl phosphoramidite linker prepared according to (Pitsch et al. 2001).

Ligation

RNA-sequence r(GGGGCUAUAGCUCAGCDGGGAGAGCGCUUGCAUCUA-imG-AGCAAGAGGUCAGCGGUUCGAUCCCGCUUAG) (**S26**).

The 32mer **S29**, 36mer **S28** and 18mer template **S34** were mixed to a final concentration of 20 µM for the substrates and 30 µM for the template (1.5eq). After the addition of PEG 6000 (3µl of 50% PEG stock soln.) and water (q.s.p. 54 µl), the mixture was heated at 95° for 4 minutes and subsequently cooled down to 40° by steps of 0.1° per second and finally to 4° within 1.5min. After the addition of ligation buffer (40mM TrisHCl, 2mM $MgCl_2$, 10mM DTT, 0.5mM ATP) and 8 units of ribonuclease inhibitor (*Fermentas*), the reaction was initiated by adding T4 DNA ligase (8 Weiss units) and kept at 37°. For the ligation kinetic, aliquots were taken and diluted in 1mL of water and injected into an analytical AE-HPLC. AE-HPLC. (20 - 100% *B* in 29 min) flow 1.0 ml/min; eluent *A*: 12 mM *Tris*·HCl (pH 7.4), 6M urea; eluent *B*: 12 mM *Tris*·HCl (pH 7.4), 0.5M $NaClO_4$, 6M urea; detection at 260 nm, elution at 85°). *m(CCUCUUGCU-_-UAGAUGCA)*: t_R 12.8 min (**S34**). *5'-monophosphate-r(imG-AGCAAGAGGUCAGCGGUUCGAUCCCGCUUAG)*: t_R 16.2 min (**S29**). *r(GGGGCUAUAGCUCAGCDGGGAGAGCGCUUGCAUCUA)*: t_R 16.6 min (**S28**). *r(GGGGCUAUAGCUCAGCDGGGAGAGCGCUUGCAUCUA-imG-AGCAAGAGGUCAGC GGUUCGAUCCCGCUUAG)*: t_R 19.0 min (**S26**) LC-MS (ESI): *m*/*z* = 22'056 (calc. 22'055)

CHAPTER VI

General

The thiophenyl phenylalaninate (H-Phe(SPh), **116**) was prepared according (Ryan and Chung 1981).

Reversed-phase HPLC (RP-HPLC): *Waters* Xterra RP_{18}, 5µm (4.6x250mm), flow 1 ml/min, eluent A: 0.1M triethylammonium phosphate in H_2O (pH 3.5) or 0.1M triethylammoniumacetate in H_2O (pH 5.5); eluent B: MeCN; elution at 25°, detection at 260 nm; unless otherwise stated, a gradient of A→A/B 1:1 (30 min) was used. Ion-exchange HPLC: Pharmacia Source 15Q (4.6x100 mm), flow 1 ml/min, eluent A: 10mM HOAc/NaOAc (pH 5.0) in MeOH/H_2O 1:1; eluent B: HOAc/NaOAc, 1M NaCl (pH 5.0) in MeOH/H_2O 1:1, elution at 25°, detection at 260 nm; a gradient of A→A/B 1:1 (30 min) was used.

2'-Acetylthio-N^6*-Benzoyl-2'-deoxy-3',5'-*O*-(1,1,3,3-tetra-isopropyldisiloxane-1,3-diyl) adenosine* (**108**).

A soln. of **107** (1.15 g, 1.88 mmol, prepared according to (Marriott et al. 1991)) was dissolved in DMSO (8 ml), treated with KSAc (1.08 g, 9.4 mmol) and stirred for 10 min at 20°. Addition of AcOEt (100 ml) and extraction (1. 10% aq. citric acid, 2. satd. aq. $NaHCO_3$) gave crude **108** (1.26 g) as yellow foam (TLC (hexane/AcOEt 1:1): R_f 0.28).

N^6-*Benzoyl-2'-(*tert-*butyldithio)-2'-deoxy-3',5'*-O-*(1,1,3,3-tetra-isopropyldisiloxane-1,3-diyl)adenosine* (**110**).

A soln. of crude **108** (717 mg, 1.07 mmol) in THF/MeOH 5:4 (12 ml) was degassed (15 min.) by a stream of argon and treated at 0° with aq. 2M NaOH (1.3 ml) for 5 min and thus neutralized with AcOH (156 mg, 2.6 mmol). After work-up (10% citric acid/CH_2Cl_2) we obtained crude **109** (TLC (hexane/AcOEt 1:1): R_f 0.39). For the next step, a soln. of sulfurylchloride (0.086 mL, 1.07 mmol) in Et_2O (5 ml) was treated with an equimolar mixture of 2-methylpropanethiol (0.12 mL, 1.07 mmol) and Py (85 mg, 1.07 mmol) in Et_2O (1 ml) at -78°. After 45 min at -78°, the reaction mixture was treated with a soln. of crude 2'-thioadenosine derivative **109** (obtained above) and Py (85 mg, 1.07 mmol) in THF (2.5 ml) and stirred for 15 min at -78°. Workup and CC (SiO_2 (20 g), hexane/AcOEt 4:1 → 1:4) gave **110** (290 mg, 38%). Light brown foam. TLC (hexane/AcOEt 1:1): R_f 0.31. ^{1}H-NMR (400 MHz, $CDCl_3$): 1.03–1.18 (*m*, ^{i}Pr$_2$Si); 1.23 (*s*, *Me*$_3$C); 4.04–4.16 (*m*, H$_2$C(5')); 4.16–4.23 (*m*, H–C(4')); 4.34 (*dd*, *J* = 3.1, 7.2, H–C(2')); 5.27 (*t*, *J* = 7.3, H–C(3')); 6.40 (*d*, *J* = 3.2, H–C(1')); 7.47–7.63 (*m*, 5 arom. H); 8.05 (*d*, *J* = 7.4, 2 arom. H); 8.23 (*s*, H–C(8)); 8.78 (*s*, H–C(2)); 9.16 (br. *s*, HN-C(6)). ^{13}C-NMR (100 MHz, $CDCl_3$): 13.1, 13.3, 13.5, 13.9 (4*d*, Me$_2$*C*H); 17.3, 17.4, 17.5, 17.6, 17.7, 17.8, 17.9, 18.0 (8*q, Me*$_2$CH); 30.2 (*q*, *Me*$_3$C); 48.8 (*s*, Me$_3$*C*); 57.6 (*d*, C(2')); 62.2 (*t*, C(5')); 72.1 (*d*, C(3')); 84.2 (*d*, C(4')); 90.9 (*d*, C(1')); 128.3 (*s*, C(5)); 129.3 (*d*, arom. C); 133.2 (*d*, arom. C); 134.1 (*s*, arom. C); 142.6 (*d*, C(8)); 150.0 (*s*, C(6)); 151.5 (*s*, C(4)); 153.1 (*d*, C(2)); 165.0 (*s*, Ph*C*O). ESI-MS: 718.32 ([*M*+H]$^+$).

N^6-*Benzoyl-2'-(*tert-*butyldithio)-2'-deoxy-5'*-O-*(4,4'-dimethoxytrityl)adenosine* (**112**).

A soln. of **110** (1.0 g, 1.4 mmol) in CH_2Cl_2 (10 ml) was treated with a soln. of 35% HF in Py (60 ml, prepared from 70% HF/Py (30 ml) and Py (30 ml)) for 1h at 20°. After workup and evaporation, the residue was dissolved in Py (40 ml) and treated with $(MeO)_2$TrCl (520 mg, 1.5 mmol) for 1h at 20°. Workup and CC (SiO_2 (30 g), hexane/AcOEt 9:1 → 7:3) gave **112** (460 mg, 40%). Colourless foam. TLC (hexane/AcOEt 1:9): R_f 0.74. ^{1}H-NMR (400 MHz, $CDCl_3$): 1.15 (*s*, *Me*$_3$C); 2.69 (br. *s*, OH); 3.42 (*dd*, *J* = 3.8, 10.3, H–C(5')); 3.56 (*dd*, *J* = 4.7, 10.4, H'–C(5')); 3.81 (*s*, 2 MeO); 4.36 (br. *s*, H–C(4')); 4.54 (*dd*, *J* = 5.1, 8.7, H–C(2')); 4.69 (br. *d*, *J* ≈ 4.2, H–C(3')); 6.21 (*d*, *J* = 8.7, H–C(1')); 6.83 (*d*, *J* = 8.8, 4 arom. H); 7.20–7.37 (*m*,

10 H); 7.45 (*d*, *J* = 7.8, 2 arom. H); 7.56 (*t*, *J* = 7.6, 1 arom. H); 7.64 (*t*, *J* = 7.3, 1 arom. H); 8.05 (*d*, *J* = 7.7, 2 arom. H); 8.21 (*s*, H–C(8)); 8.71 (*s*, H–C(2)); 9.01 (s, NH). ^{13}C-NMR (100 MHz, $CDCl_3$): 30.1 (*q*, *Me*$_3$C); 48.7 (*s*, Me$_3$*C*); 55.6 (*q*, MeO); 59.8 (*d*, C(2')); 64.0 (*t*, C(5')); 73.5 (*d*, C(3')); 86.1 (*d*, C(4')); 88.2 (*d*, C(1')); 113.4 (*d*, arom. C); 123.6 (*s*, C(5)); 127.5, 128.2, 128.7, 129.2, 130.4 (5*d*, arom. C); 133.1, 135.7 (2*s*, arom. C); 142.5 (*d*, C(8)); 144.5 (*s*, arom. C); 149.9 (*s*, C(6)); 152.0 (*s*, C(4)); 153.1 (*d*, C(2)); 158.8 (*s*, arom. C); 164.9 (*s*, Ph*C*O).ESI-MS: 798.29 ([*M*+H]$^+$).

N^6*-Benzoyl-2'-(*tert*-butyldithio)-2'-deoxy-5'*-O-*(4,4'-dimethoxytrityl)adenosine 3'-(4-Nitrophenylheptane dioate)* (**113**).

A soln. of **112** (170 mg, 0.2 mmol) in Py (4 ml) was treated with DMAP (20 mg, 0.16 mmol) and bis(4-nitrophenyl)heptanedioate (472 mg, 1.2 mmol) for 14 h at 20. Evaporation and CC (SiO_2 (4 g), hexane/AcOEt 2:3 → 1:9) gave **113** (160 mg, 76%). Colorless foam. TLC (hexane/AcOEt 3:7): R_f 0.63. ^{1}H-NMR (400 MHz, $CDCl_3$): 1.16 (*s*, *Me*$_3$C); 1.50–1.67 (*m*, CH_2); 1.70–1.89 (*m*, 2 CH_2); 2.42–2.57 (*m*, CH_2); 2.66 (*t*, *J* = 7.3, CH_2); 3.50 (*m*, H_2C(5')); 3.80 (*s*, 2 MeO); 4.27 (*m*, H–C(4')); 4.57 (*dd*, *J* = 5.7, 8.7, H–C(2')); 5.82 (br. *d*, *J* ≈ 5.4, H–C(3')); 6.30 (*d*, *J* = 8.7, H–C(1')); 6.83 (*d*, *J* = 8.7, 4 arom. H); 7.20–7.31 (*m*, 6 arom. H); 7.35 (*d*, *J* = 8.7, 2 arom. H); 7.45 (*t*, *J* = 7.4, 1 arom. H); 7.64 (*t*, *J* = 7.6, 2 arom. H); 7.66 (*t*, *J* = 7.6, 1 arom. H); 8.07 (*d*, *J* = 7.4, 2 arom. H); 8.21 (*s*, H–C(8)); 8.27 (*d*, *J* = 9.0, 2 arom. H); 8.73 (*s*, H–C(2)); 9.09 (br. *s*, NH). ^{13}C-NMR (100 MHz, $CDCl_3$): 24.7, 24.9, 28.9 (3*t*, *C*H_2); 29.8 (*q*, *Me*$_3$C); 34.3, 34.4 (2*t*, *C*H_2); 48.4 (*s*, Me$_3$*C*); 55.7 (*q*, MeO); 58.3 (*d*, C(2')); 63.6 (*t*, C(5')); 75.3 (*d*, C(3')); 84.3 (*d*, C(4')); 87.3 (*d*, C(1')); 89.4 (*s*, arom. C); 113.7 (*d*, arom. C); 122.8 (*s*, C(5)); 125.6, 127.5, 128.4, 128.6, 129.3, 130.4, 130.5, 133.2 (8*d*, arom. C); 135.8, 135.9 (2*s*, arom. C); 142.2 (*d*, C(8)); 144.7, 145.7 (2*s*, arom. C); 149.9 (*s*, C(6)); 153.2 (*s*, C(4)); 153.2 (*d*, C(2)); 155.8 (*s*, arom. C); 159.0 (*s*, arom. C); 164.9, 172.6, 184.5 (3*s*, *C*O). ESI-MS: 1061.32 ([*M*+H]$^+$).

N^6*-Benzoyl-2'-(*tert*-butyldithio)-2'-deoxy-5'*-O-*(4,4'-dimethoxytrityl)adenosine 3'-[6-(CPG-Amino)heptanoate]* (**114**).

A suspension of LCAA-CPG (1.3 g, 500Å, *Millipore*), **113** (100 mg, 0.10 mmol) and ^{i}Pr$_2$NEt (0.4 ml) in DMF (4 ml) was shaken for 16 h at 20°. After filtration, the solid was washed with DMF and CH_2Cl_2, suspended in pyridine (1.2 ml) and Ac_2O (0.8 ml), and shaken for 2h at 20°. After filtration, the solid was washed with DMF and CH_2Cl_2, and dried to give **114**: loading: 38 μmol/g.

*RNA-sequence 5'-monophosphate-r(CCCCACC-[2'-(*tert-*butyldithio)-2'-deoxy]A* (**S35**).

The sequence was assembled from 60 mg of **114** using the standard conditions for the assembly of 2'-*O*-TOM-protected ribonucleoside phosphoramidites (Pitsch et al. 2001), but employing a modified oxidizing reagent: 20 mM I_2 in THF/ Py/H_2O 7:2:1. The solid-support was subsequently removed from the cartridge and treated with a 1:1 mixture of 12M $MeNH_2$ in H_2O and 8M $MeNH_2$ in EtOH (4 ml) for 6 h at 20°. By centrifugation, the supernatant soln. was separated from the solid support, evaporated to dryness, and the residue dissolved in 1M $Bu_4NF{\cdot}3H_2O$ soln. in THF (4 ml). After 14 h at 30°, 1M *Tris*·HCl buffer (pH 7.4, 4 ml) was added. The soln. was concentrated to 4 ml and desalted on a *NAP*-column (*Pharmacia*) according to the manufacturer's instructions. The crude RNA-sequence was purified by ion-exchange HPLC: Pharmacia Source 15Q (4.6x100 mm), flow 2.5 ml/min; eluent *A*: 12 mM *Tris*·HCl (pH 7.4), 6M urea; eluent *B*: 12 mM *Tris*·HCl (pH 7.4), 2M NaCl, 6M urea; detection at 260 nm, elution at 25°. AE-HPLC (10 - 30% *B* in 30 min, 3 injections): t_R 17.8 min. The fractions, containing pure **S35** were pooled (→ 30 ml), treated with 1M aq. $Et_3N{\cdot}AcOH$ (pH 7, 5 ml) and applied to a *Sepak*-cartridge (*Waters*): after elution of the salts with 0.1M aq. $Et_3N{\cdot}AcOH$ (pH 7, 10 ml), followed by 10 10mM aq. $Et_3N{\cdot}H_2CO_3$ (pH 8.4, 15 ml), **S35** (Et_3NH^+-form) was eluted with MeCN/H_2O 1:1 (5 ml): 40 oD of pure **S35** (33% yield based on **114**). LC-MS (ESI): *m*/*z* = 2'611 (calc. 2'611).

N^6-*Benzoyl-2'-deoxy-2'-(phenylthio)-3',5'*-O-*(1,1,3,3-tetra-isopropyldisiloxane-1,3-diyl) adenosine* (**117**).

A soln. of crude **109** (260 mg, 0.39 mmol) was dissolved in CH_2Cl_2 (0.79 ml) and added to a mixture of thiophenol (0.08 ml, 0.79 mmol) and N-chlorosuccinimide (105 mg, 0.79 mmol) in CH_2Cl_2 (6.2 ml). After 10 min at 20°, workup and CC (SiO_2 (2 g), CH_2Cl_2 → CH_2Cl_2/AcOEt 3:1) gave **117** (60 mg, 21% from **109**). TLC (CH_2Cl_2/AcOEt 3:7): R_f 0.68. ^{1}H-NMR ($CDCl_3$): 1.19–1.10 (m, ^{i}Pr$_2$Si); 4.00–4.10 (*m*, H_2C(5')); 4.16–4.24 (*m*, H–C(4')); 4.38 (*dd*, *J* = 4.3, 7.5, H–C(2')); 5.20 (*t*, *J* = 7.1, H–C(3')); 6.34 (*d*, *J* = 4.2, H–C(1')); 7.17 (*d*, *J* = 7.1, 1 arom. H); 7.22 (*t*, *J* = 7.1, 2 arom. H); 7.34 (*d*, J = 7.3, 2 arom. H); 7.55 (*t*, *J* = 7.5, 2 arom. H); 7.63 (*t*, *J* = 7.5, 2 arom. H); 7.97 (*s*, H–C(8)); 8.05 (*d*, *J* = 7.8, 2 arom. H); 8.7 (s, H–C(2)); 9.10 (br. *s*, NH). ESI-MS: 738.29 ([*M*+H]$^+$).

N^6-*Benzoyl-2'-deoxy-5'*-O-*(4,4'-dimethoxytrityl)-2'-(phenylthio)adenosine* (**119**).

A soln. of **117** (600 mg, 0.8 mmol) in CH_2Cl_2 (6.4 ml) was treated with a soln. of 35% HF in Py (6.4 ml, prepared from 70% HF/Py (3.2 ml) and Py (3.2 ml)) for 1h at 20°. After workup

and evaporation, the residue was dissolved in Py (4 ml) and treated with $(MeO)_2TrCl$ (340 mg, 1.0 mmol) for 1h at 20°. Workup and CC (SiO_2 (16 g), hexane/AcOEt 7:3 → 3:7) gave **119** (320 mg, 51%). White foam. TLC (hexane/AcOEt 3:7): R_f 0.34. ^{1}H-NMR ($CDCl_3$): 3.15 (br. *s*, OH); 3.39 (*dd*, *J* = 4.0, 10.4, H–C(5')); 3.49 (*dd*, *J* = 4.4, 10.4, H'–C(5')); 3.79 (*s*, 2 MeO); 4.30 (*t*, *J* = 3.0, H–C(4')); 4.70–4.75 (*m*, H–C(2'), H–C(3')); 6.28 (*d*, *J* = 8.6, H–C(1')); 6.80 (*d*, *J* = 8.0, 4 arom. H); 7.18–7.34 (*m*, 10 arom. H); 7.42 (*dd*, *J* = 1.2, 8.1, 2 arom. H); 7.56 (*t*, *J* = 7.4, 1 arom. H); 7.64 (*t*, *J* = 7.3, 1 arom. H); 7.98 (*s*, H–C(8)); 8.06 (*d*, *J* = 7.3, 2 arom. H); 8.62 (*s*, H–C(2)); 9.06 (*s*, NH). ^{13}C-NMR (100 MHz, $CDCl_3$): 55.7 (*q*, MeO); 61.0 (*d*, C(2')); 64.1 (*t*, C(5')); 73.6 (*d*, C(3')); 85.6 (*d*, C(4')); 88.6 (*d*, C(1')); 113.6, 113.7 (2*d*, arom. C); 123.8 (*s*, C(5)); 127.4, 127.5, 128.3, 128.4, 128.6, 129.3, 129.6, 130.5 (7*d*, arom. C); 133.2, 134.1, 135.9, 136.0, 136.1, 139.9 (5*s*, arom. C); 142.6 (*d*, C(8)); 144.9 (*s*, arom. C); 150.0 (*s*, C(6)); 152.3 (*s*, C(4)); 153.1 (*d*, C(2)); 159.0 (*s*, arom. C); 165.0 (*s*, Ph*C*O). ESI-MS: 798.29 ([*M*+H]$^+$).

N^6*-Benzoyl-2'-deoxy-5'*-O-*(4,4'-dimethoxytrityl)-2'-(phenylthio)adenosine 3'-(4-Nitrophenyl heptane dioate)* (**120**).

A soln. of **119** (320 mg, 0.4 mmol) in Py (4 ml) was treated DMAP (6 mg, 0.04 mmol) and bis(4-nitrophenyl)heptanedioate (950 mg, 2.4 mmol) for 14h at 20°. Evaporation and CC (SiO_2 (4 g), hexane/AcOEt 9:1 → 1:1) gave **120** (230 mg, 54%). Colorless foam. TLC (hexane/AcOEt 3:7): R_f 0.53. ^{1}H-NMR ($CDCl_3$): 1.50–1.61 (*m*, CH_2); 1.75–1.90 (*m*, 2 CH_2); 2.42–2.57 (*m*, CH_2); 2.67 (*t*, *J* = 7.4, CH_2); 3.45–3.54 (*m*, H–C(5'), H'–C(5')); 3.79 (*s*, MeO); 4.22–4.27 (*m*, H–C(4')); 4.78 (*dd*, *J* = 5.5, 9.1, H–C(2')); 5.77 (*dd*, *J* = 0.8, 5.6, H–C(3')); 6.36 (*d*, *J* = 9.1, H–C(1')); 6.80 (*d*, *J* = 8.9, 4 arom. H); 7.04–7.10 (*m*, 3 arom. H); 7.10–7.16 (*m*, 2 arom. H); 7.21–7.33 (*m*, 9 arom. H); 7.41 (*dd*, *J* = 1.5, 8.2, 2 arom. H); 7.56 (*t*, *J* = 7.2, 1 H); 7.93 (*s*, H–C(8)); 8.07 (*d*, *J* = 7.5, 2 arom. H); 8.26 (*td*, *J* = 2.0, 9.0, 2 arom. H); 8.64 (s, H–C(2)); 9.05 (br. *s*, NH). ^{13}C-NMR (100 MHz, $CDCl_3$): 24.8, 24.9, 29.0 (3*t*, *C*H_2); 34.4, 34.5 (2*t*, *C*H_2); 55.8 (*q*, MeO); 62.0 (*d*, C(2')); 64.3 (*t*, C(5')); 73.8 (*d*, C(3')); 85.9 (*d*, C(4')); 88.5 (*d*, C(1')); 113.5, 113.6 (2*d*, arom. C); 124.0 (*s*, C(5)); 127.3, 127.5, 128.1, 128.5, 128.6, 129.0, 129.4, 130.2 (7*d*, arom. C); 132.9, 134.0, 135.5, 136.2, 136.3, 139.7 (5*s*, arom. C); 142.4 (*d*, C(8)); 144.5 (*s*, arom. C); 151.0 (*s*, C(6)); 152.4 (*s*, C(4)); 153.0 (*d*, C(2)); 155.6, 159.0 (2*s*, arom. C); 164.4, 172.2, 185.0 (3*s*, *C*O). ESI-MS: 1061.32 ([*M*+H]$^+$).

N^6-*Benzoyl-2'-(butyldithio)-2'-deoxy-5'*-O-*(4,4'-dimethoxytrityl)adenosine 3'-[6-(CPG-Amino)heptanoate]* (**121**).

A suspension of LCAA-CPG (0.5 g, 500Å, *Millipore*), **120** (50 mg, 0.05 mmol) and $^{i}Pr_2NEt$ (0.2 ml) in DMF (2 ml) was shaken for 16 h at 20°. After filtration, the solid was washed with DMF and CH_2Cl_2, suspended in pyridine (1 ml) and Ac_2O (0.4 ml), and shaken for 2h at 20°. After filtration, the solid was washed with DMF and CH_2Cl_2, and dried to give **121**: loading: 34 μmol/g.

N^6-*Benzoyl-2'*-S-*[(4-methoxy)benzyl)]-3',5'*-O-*(1,1,3,3-tetra-isopropyldisiloxane-1,3-diyl)-2'-thioadenosine* (**122**).

At –15°, a soln. of **107** (260 mg, 0.44 mmol, prepared according to (Marriott et al. 1991)) in CH_2Cl_2/Py (1ml + 1 ml) was treated with $(CF_3SO_2)_2O$ (0.11 ml, 0.60 mmol). Workup after 5h at 10° gave the crude 2'-*O*-trifluormethanesulfonyl derivative of **107** (330 mg, TLC (hexane/AcOEt 1:9): R_f 0.50) as brown-orange foam, which was dissolved in DMSO (8 ml). For the next step, a suspension of 50% NaH in mineral oil (106 mg, ca. 2.2 mmol) and (4-methoxy)benzylthiol (343 mg, 2.2 mmol) were added to DMSO (7 ml). After 10 min at 25°, the reaction mixture was cooled to 10°, treated with the soln. of the crude 2'-*O*-trifluormethanesulfonyl derivative of **107** (330 mg, obtained above) in DMSO (8 ml) and stirred for 10 min at 10°. After addition of AcOEt (100 ml), extraction (1. 10% aq. citric acid, 2. satd. aq. $NaHCO_3$) and CC (SiO_2 (10 g), hexane/AcOEt 9:1 → 1:1) **122** (300 mg, 91%) was obtained as yellow foam. TLC (hexane/AcOEt 1:9): R_f 0.71. ^{1}H-NMR ($CDCl_3$): 0.99–1.12 (*m*, $^{i}Pr_2Si$); 3.75 (*s*, MeO); 3.78-3.92 (*m*, $H_2C(5')$); 4.01–4.19 (m, H–C(4'), H–C(2')); 4.93 (*t*, *J* = 6.9, H–C(3')); 5.31 (*s*, CH_2S); 6.22 (*d*, *J* = 3.2, H–C(1')); 6.71 (*d*, *J* = 8.5, 1 arom. H); 7.12 (*t*, *J* = 8.5, 2 arom. H); 7.54 (*t*, *J* = 7.6, 2 arom. H); 7.62 (*t*, *J* = 7.4, 1 arom. H); 8.05 (*d*, *J* = 7.6, 2 arom. H); 8.14 (*s*, H–C(2)); 8.80 (*s*, H–C(8)); 9.22 (br. *s*, NH). ^{13}C-NMR (100 MHz, $CDCl_3$): 13.0, 13.3, 13.5, 13.8 (4*d*, Me_2CH); 17.3, 17.4, 17.5, 17.7, 17.8, 17.9 (6*q*, Me_2CH); 35.5 (*t*, CH_2S); 52.4 (*d*, C(2')); 55.7 (*q*, MeO); 61.6 (*t*, C(5')); 70.8 (*d*, C(3')); 84.6 (*d*, C(4')); 90.6 (*d*, C(1')); 114.2 (*d*, arom. C); 123.8 (*s*, C(5)); 128.3, 129.3 (2*d*, arom. C); 129.7 (*s*, arom. C); 130.2, 133.2 (2*d*, arom. C); 134.2 (*s*, arom. C); 141.9 (*d*, C(8)); 149.9 (*s*, C(6)); 151.3 (*s*, C(4)); 153.1 (*d*, C(2)); 159.1 (*s*, MeO*C*), 165.1 (*s*, Ph*C*O). ESI-MS: 750.31 ([*M*+H]$^+$).

N^6-*Benzoyl-2'-(butyldithio)-2'-deoxy-3',5'*-O-*(1,1,3,3-tetra-isopropyldisiloxane-1,3-diyl)adenosine* (**123**). Simultaneously, two soln.s, one containing BuSCl (250 mg, 2 mmol, prepared according to (Thea and Cevasco 1988)) in CH_2Cl_2 (10 ml), and the other **122** (300

mg, 0.4 mmol) in CH_2Cl_2 (10 ml), respectively, were added dropwise to a cooled (4°) mixture of CH_2Cl_2/AcOH 1:1 (30 ml). After addition, the ice-bath was removed and the mixture stirred for 48h at 25°. Workup and CC (SiO_2 (10g), hexane/AcOEt 4:1 → 1:4) gave **123** (250 mg, 86%) as a light yellow foam. TLC (AcOEt/CH_2Cl_2 1:4): R_f 0.59. ^{1}H-NMR (400 MHz, $CDCl_3$): 0.84 (*t*, *J* = 7.0, *Me*CH_2); 1.02–1.17 (*m*, $^i Pr_2Si$); 1.20–1.40 (*m*, CH_2); 1.51–1.63 (*m*, CH_2); 2.63 (*t*, *J* = 7.4, CH_2S); 4.01 (*dd*, *J* = 3.5, 12.5, H–C(5')); 4.12 (*dd*, *J* = 3.5, 12.5, H'–C(5')); 4.16–4.23 (*m*, H–C(4')); 4.31 (*dd*, *J* = 3.2, 7.3, H–C(2')); 5.23 (*t*, *J* = 7.3, H–C(3')); 6.43 (*d*, *J* = 3.2, H–C(1')); 7.55 (*t*, *J* = 7.3, 2 arom. H); 7.63 (*t*, *J* = 7.3, 1 arom. H); 8.05 (br. *d*, *J* = 7.4, 2 arom. H); 8.24 (*s*, H–C(8)); 8.79 (*s*, H–C(2)); 9.14 (br. *s*, HN-C(6)). ^{13}C-NMR (100 MHz, $CDCl_3$): 13.1 (*q*, *Me*CH_2); 13.2, 13.5, 13.8, 13.9 (4*d*, Me_2CH); 17.3, 17.5, 17.6, 17.8, 17.9, 18.0, 18.1, 18.2 (8*q*, Me_2CH); 21.9 (*t*, Me*C*H_2); 31.3 (*t*, *C*H_2CH_2); 38.7 (*t*, CH_2S); 56.7 (*d*, C(2')); 62.1 (*t*, C(5')); 71.9 (*d*, C(3')); 84.3 (*d*, C(4')); 90.5 (*d*, C(1')); 128.3 (*s*, C(5)); 129.3 (*d*, arom. C); 133.2 (*d*, arom. C); 134.1 (*s*, arom. C); 142.5 (*d*, C(8)); 150.1 (*s*, C(6)); 151.6 (*s*, C(4)); 153.1 (*d*, C(2)); 165.0 (*s*, Ph*C*O). ESI-MS: 718.32 ([*M*+H]$^+$).

N^6-*Benzoyl-2'-(butyldithio)-2'-deoxy-5'*-O-*(4,4'-dimethoxytrityl)adenosine* (**125**).

A soln. of **123** (206 mg, 0.28 mmol) in CH_2Cl_2 (2.2 ml) was treated with a soln. of 35% HF in Py (2 ml, prepared from 70% HF/Py (1 ml) and Py (1 ml)) for 1h at 20°. After workup and evaporation, the residue was dissolved in Py (1.4 ml) and treated with $(MeO)_2TrCl$ (114 mg, 0.34 mmol) for 1h at 20°. Workup and CC (SiO_2 (6g), CH_2Cl_2 → CH_2Cl_2/AcOEt 1:1) gave **125** (134 mg, 60%). Colorless foam. TLC (hexane/AcOEt 7:3): R_f 0.34. ^{1}H-NMR (400 MHz, $CDCl_3$): 0.80 (*t*, *J* = 7.3, *Me*CH_2); 1.17–1.30 (*m*, CH_2); 1.38–1.40 (*m*, CH_2); 2.37–2.54 (*m*, CH_2S); 3.15 (br. *d*, *J* ≈ 2.2, OH); 3.39 (*dd*, *J* = 4.0, 10.4, H–C(5')); 3.49 (*dd*, *J* = 4.4, 10.4, H'–C(5')); 3.79 (*s*, 2 MeO); 4.30 (*t*, *J* = 3.0, H–C(4')); 4.70–4.75 (*m*, H–C(2'), H–C(3')); 6.28 (*d*, *J* = 8.6, H–C(1')); 6.80 (*d*, *J* = 8.0, 4 arom. H); 7.18–7.34 (*m*, 8 arom. H); 7.42 (*dd*, *J* = 1.2, 8.1, 2 arom. H); 7.56 (*t*, *J* = 7.4, 1 arom. H); 7.64 (*t*, *J* = 7.3, 1 arom. H); 7.98 (*s*, H–C(8)); 8.06 (*d*, *J* = 7.3, 2 arom. H); 8.62 (*s*, H–C(2)); 9.06 (s, NH). ^{13}C-NMR (100 MHz, $CDCl_3$): 13.9 (*q*, *Me*CH_2); 21.9 (*t*, Me*C*H_2); 31.1 (*t*, *C*H_2CH_2S); 39.3 (*t*, CH_2S); 55.7 (*q*, MeO); 59.7 (*d*, C(2')); 63.9 (*t*, C(5')); 73.6 (*d*, C(3')); 85.7 (*d*, C(4')); 88.3 (*d*, C(1')); 113.6 (*d*, arom. C); 123.8 (*s*, C(5)); 127.4, 128.3, 128.6, 129.3, 130.5 (5*d*, arom. C); 133.2, 135.9 (2*s*, arom. C); 142.6 (*d*, C(8)); 144.8 (*s*, arom. C); 150.0 (*s*, C(6)); 152.3 (*s*, C(4)); 153.2 (*d*, C(2)); 159.0 (*s*, arom. C); 165.0 (*s*, Ph*C*O). ESI-MS: 778.30 ([*M*+H]$^+$).

N^6-*Benzoyl-2'-(butyldithio)-2'-deoxy-5'*-O-*(4,4'-dimethoxytrityl)adenosine 3'-(4-Nitrophenylheptane dioate)* (**126**).

A soln. of **125** (134 mg, 0.18 mmol) in Py (2 ml) was treated with DMAP (12 mg, 0.08 mmol) and bis(4-nitrophenyl)heptanedioate (420 mg, 1.04 mmol) for 14h at 20°. Evaporation and CC (SiO_2 (10g), hexane/AcOEt 3:2 → 1:9) (hexane/AcOEt 3:7) gave **126** (122 mg, 68%). Colorless foam. TLC (hexane/AcOEt 7:3): R_f 0.53. ^{1}H-NMR (400 MHz, $CDCl_3$): 0.80 (*t*, *J* = 7.4, *Me*CH_2) 1.16–1.29 (*m*, CH_2); 1.38–1.45 (*m*, CH_2); 1.50–1.57 (*m*, CH_2); 1.74–1.87 (*m*, 3 CH_2); 2.29–2.51 (*m*, 2 CH_2); 2.66 (*t*, *J* = 7.3, CH_2S); 3.45–3.54 (*m*, H_2C(5')); 3.79 (*s*, 2 MeO); 4.22–4.27 (*m*, H–C(4')); 4.78 (*dd*, *J* = 5.5, 9.1, H–C(2')); 5.77 (*dd*, *J* = 0.8, 5.6, H–C(3')); 6.36 (*d*, *J* = 9.1, H–C(1')); 6.80 (*d*, *J* = 8.9, 4 arom. H); 7.04–7.10 (*m*, 3 arom. H); 7.10–7.16 (*m*, 2 arom. H); 7.21–7.33 (m, 9 arom. H); 7.41 (*dd*, *J* = 1.5, 8.2, 2 arom. H); 7.56 (*t*, *J* = 7.2, 1 arom. H); 7.93 (*s*, H–C(8)); 8.07 (*d*, *J* = 7.5, 2 arom. H); 8.26 (*td*, *J* = 2.0, 9.0, 2 arom. H); 8.64 (*s*, H–C(2)); 9.05 (br. *s*, NH). ^{13}C-NMR (100 MHz, $CDCl_3$): 13.9 (*q*, *Me*CH_2); 21.8 (*t*, Me*C*H_2); 24.7, 24.8, 28.9, 31.1, 34.2, 34.4 (6*t*, *C*H_2); 39.3 (*t*, CH_2S); 55.6 (*q*, MeO); 57.3 (*d*, C(2')); 63.8 (*t*, C(5')); 75.5 (*d*, C(3')); 84.4 (*d*, C(4')); 87.4 (*d*, C(1')); 88.9 (*s*, arom. C); 113.7 (*d*, arom. C); 122.8 (*s*, C(5)); 125.6, 127.5, 128.3, 128.4, 128.6, 129.3, 130.5, 133.2 (9*d*, arom. C); 134.0, 135.8 (2*s*, arom. C); 142.2 (*d*, C(8)); 144.8, 145.7 (2*s*, arom. C); 150.1 (*s*, C(6)); 152.5 (*s*, C(4)); 153.3 (*d*, C(2)); 155.8 (*s*, arom. C); 159.1 (*s*, arom. C); 164.9, 171.4, 172.6 (3*s*, *C*O). ESI-MS: 1041.29 ([*M*+H]$^+$).

N^6-*Benzoyl-2'-(butyldithio)-2'-deoxy-5'*-O-*(4,4'-dimethoxytrityl)adenosine 3'-[6-(CPG-Amino)heptanoate]* (**126**).

A suspension of LCAA-CPG (1.3 g, 500Å, *Millipore*), **126** (122 mg, 0.12 mmol) and ^{i}Pr$_2$NEt (1.3 ml) in DMF (5.2 ml) was shaken for 16 h at 20°. After filtration, the solid was washed with DMF and CH_2Cl_2, suspended in pyridine (1.2 ml) and Ac_2O (0.8 ml), and shaken for 2h at 20°. After filtration, the solid was washed with DMF and CH_2Cl_2, and dried to give **127**: loading: 30 μmol/g.

RNA-sequence 5'-monophosphate-r(CCCCACC-[2'-(butyldithio)-2'-deoxy]A) (**S39**).

The sequence was assembled from 60 mg of **127** using the standard conditions for the assembly of 2'-*O*-TOM-protected ribonucleoside phosphoramidites (Pitsch et al. 2001), but employing a modified oxidizing reagent: 20 mM I_2 in THF/Py/H_2O 7:2:1. After the final detritylation, the solid support was washed with (^{i}Pr)$_2$NH/MeCN 1:9 for 20 min (flow-rate 2.5 ml/min). Cleavage from the solid support and deprotection was carried out with 12M NH_3 in MeOH (1ml) for 6h at 20°. The supernatant was removed by centrifugation and evaporated to

dryness; the residue was treated with a THF soln. (1 ml) of $Bu_4NF \cdot 3H_2O$ (1M) and AcOH (0.5M) for 3h at 20°, diluted with aq. Tris-HCl (1 ml, 1M, pH 7.4) and evaporated to a volume of 1 ml. After desalting on a NAP cartridge, the crude product was purified by ion-exchange HPLC: Pharmacia Source 15Q (4.6x100 mm), flow 2.5 ml/min; eluent *A*: 12 mM *Tris*·HCl (pH 7.4), 6M urea; eluent *B*: 12 mM *Tris*·HCl (pH 7.4), 2M NaCl, 6M urea; detection at 260 nm, elution at 25°. AE-HPLC (10 - 30% *B* in 30 min, 3 injections): t_R 17.8 min. The product containing fractions were concentrated to ca. 40% of their initial volume and purified by RP HPLC (pH 5.5, A/B 9:1→A/B 7:3 (30 min). The pooled product containing fractions were evaporated to 1/3 of the volume and finally desalted on a Sepak cartridge: 30 oD of pure **S39** (25% yield based on 6). RP HPLC (pH 3.5): t_R 19.8 min (100%); LC-MS (ESI): *m*/*z* = 2'611 (calc. 2'611).

Experiments in Table VI.1.

General procedure for deprotection of **S39** *and aminoacylation of* **S36**: To an aq. soln. of **S39** (0.19 mM, 27 µl, concentration determined spectrophotometrically at 260 nm) was added an aliquot of the indicated buffer (c = 0.1M, 27 µl) followed by an aq. soln. of TCEP (6 µl, c = 10, 25 or 100 mM, resulting in 1.0, 2.5 or 25 mM soln.s, resp., see *Table*, prior to the addition, the pH of these TCEP stock soln.s was adjusted with aq. NaOH) in the corresponding buffer (c = 50 mM) and incubated at 25°. After 30 min, an aliquot of 20 µl was withdrawn and analyzed by RP HPLC (elution at pH 3.5). To the remaining reaction mixture, a soln. of **116** in DMF (4 µl, c = 10, 5 or 2.5 mM, resulting in 12, 6 or 3 equivalents, resp., see *Table*) was added and incubated at the indicated temperature. Aliquots of 20 µl were withdrawn after 10 and 50 min and analysed by RP-HPLC (pH 3.5). For experiments at 37°, the crude **S36** was pre-incubated at 37° before the addition of **116**. Identical experiments with the parent RNA sequence $^{=}O_3PO$-r(CCCCACCA) were carried out; according to HPLC and ESI-MS analyses, no aminoacylated or other products were formed, respectively.

Isolation and characterization of **S36**: To an aq. soln. of **S39** (0.19 mM, 81 µl), an aq. AcOH-NaOH soln. (0.1M, 81 µl, pH 5.0) was added, followed by an aq. soln. of TCEP (25 mM, 18 µl, in 0.05M AcOH-NaOH soln., pH 5.0). After 30 min incubation at 25°, the product soln. was desalted on a NAP cartridge. Characterization of **S36**: RP HPLC (pH 3.5): t_R 14.0 min (100%, *Fig. VI.6b*); ESI MS (neg mode, *Fig. VI.6f*): *m*/*z* = 2523 (calc. 2523).

Experiment in Figure VI.7.

To an aq. soln. of **S39** (0.19 mM, 27 µl) was added aq. Tris-HCl buffer pH 7.4 (c = 0.1M, 27 µl), followed by an aq. soln. of TCEP (10 mM, 6 µl, in 0.05M Tris-HCl buffer, pH 7.4). After 30 min at 25°, an aliquot of 20 µl was withdrawn and analyzed by RP HPLC (elution at pH 3.5). To the remaining reaction mixture, a soln. of **116** in DMF (10 mM, 4 µl) was added and the soln. was incubated at 37°. Aliquots were taken after 2, 10, 20, 40, 50, 80, 120 and 180 min, respectively, and analyzed by RP-HPLC (pH 3.5, see *Fig. VI.7b* for an example obtained after a reaction time of 2 min). By integration, the ratio of **S36**, **S38** and **S37** was determined in each chromatogram and the ratio of (**S38**+**S37**) / (**S36**+**S38**+**S37**) was plotted against the reaction time (*Fig. VI.7a*).

Hydrolysis studies in Figure VI.8.

a) Hydrolysis of **S38**: An aq. soln. of pure **S38** (c = 8 µM, 900 µl, obtained by HPLC purification and desalting as described above) was incubated for 2 min at 25° or 37°, resp. Then, an aq. Tris-HCl soln. (1M, pH 7.4, 100 µl) was added and the incubation was continued at 25° or 37°. At different time intervals, aliquots were withdrawn, treated with AcOH (→ pH 3) and analyzed by RP HPLC (pH 3.5). The integral ratios between signals of **S38** and **S36** were translated into individual pseudo-first order rate constants (*Fig. VI.8b*): k (37°) = 0.075 min^{-1}; k (25°) = 0.02 min^{-1}.

b) Hydrolysis of **S37**: A desalted 3:1 mixture of **S37** and **S38** (total c = ca. 8 µM, 900 µl, obtained at pH 5.0 as described above) was incubated for 2 min at 37°. Then, an aq. Tris-HCl soln. (1M, pH 7.4, 100 µl) was added and the incubation was continued at 37°. At different time intervals (2, 4, 8, 12 and 24 min), aliquots were withdrawn, treated with AcOH (→ pH 3) and analyzed by RP HPLC (pH 3.5). The disappearance of the signal of **S37** was translated into an pseudo-first order rate constant (*Fig. VI.8b*): k (37°) = 0.15 min^{-1}.

Graph shown in Fig. VI.8c: From the rates of hydrolysis k_1(**S37**→**S38**) = 0.15 min^{-1} and k_2(**S38**→**S36**) = 0.075 min^{-1}, the line graphs shown in *Fig. VI.8c* were obtained according to the following equations: $[\mathbf{S37}]_t = [\mathbf{S37}]_0 * \exp(-k_1 * t)$; $[\mathbf{S38}]_t = [([\mathbf{S37}]_0 * k_1)/(k_1 - k_2)] * [\exp(-k_2 * t) - \exp(-k_1 * t)]$; $[\mathbf{S36}]_t = [\mathbf{S37}]_0 * (1 + [k_1 * \exp(-k_2 * t) - k_2 * \exp(-k_1 * t)]/[k_2 - k_1])$. The experimentally determined amounts of **S36**, **S38** and **S37**, obtained at different times (see above), are also shown in the graph (as points).

Hydrolysis of **116**: A soln. of **116** in DMF (c = 10 mM, 50 µl) was added to an aq. Tris-HCl soln. (0.1M, pH 7.4, 950 µl, pre-incubated at 25° or 37°). At different time intervals, aliquots were withdrawn, treated with AcOH (→ pH 3) and analyzed by RP HPLC (pH 3.5, gradient

A→B in 30 min, full loop injection). The dissapearance of **116** (t_R = 19.3 min) was translated into individual pseudo-first order rate constants: k (25°) = 0.007 min^{-1}, k (37°) = 0.07 min^{-1}.

Experiment in Figure VI.9.

*Simultaneous deprotection of **S39** and aminoacylation of **S36**; selective cleavage of **S37** to **S38**; isolation and characterization of **S38***: To an aq. soln. of **S39** (0.19 mM, 81 µl), an aq. AcOH-NaOH soln. (0.1M, 81 µl, pH 5.0) was added and the mixture was incubated at 37° for 2 min. Then, a soln. of **116** in DMF (4 mM, 18 µl) was added, followed by an aq. soln. of TCEP (25 mM, 18 µl, in 0.05M AcOH-NaOH soln., pH 5.0). After 30 min incubation at 37°, a 20 µl aliquot was analyzed by RP HPLC (pH 3.5, see *Fig. VI.9a*). To the remaining soln., an aq. soln. of NaN_3 (1.5 mM, 480 µl) was added and incubated for 30 min at 25°. The reaction mixture was diluted with H_2O (340 µl), desalted on a NAP cartridge and characterized by RP HPLC (pH 3.5, *Fig VI.9b*). The product **S38** was obtained in pure form by RP HPLC purification at pH 3.5, concentration of the product containing fraction to ca. 60% of its initial volume, desalting on a NAP cartridge, followed by immediate addition of AcOH (1% final concentration) and lyophilization. Characterization of **S38**: RP HPLC (pH 3.5): t_R 16.9 min (100%); ESI MS (neg mode, *Fig. VI.6g*): m/z = 2671 (calc. 2671).

Isolation and characterization of **S37**: To an aq. soln. of **S39** (0.19 mM, 81 µl), an aq. AcOH-NaOH soln. (0.1M, 81 µl, pH 5.0) was added. Then, a soln. of **116** in DMF (25 mM, 18 µl) was added, followed by an aq. soln. of TCEP (25 mM, 18 µl, in 0.05M AcOH-NaOH soln., pH 5.0). After 30 min incubation at 25°, a 20 µl aliquot was analyzed by RP HPLC (pH 3.5), which showed **S37** and **S38** in a 9:1 ratio. The remaining product soln. was desalted on a NAP cartridge and stabilized immediately by addition of AcOH (final concentration 1%). Characterization of **S37**: RP HPLC (pH 3.5): t_R 16.9 min (**S38**, 10%) and 18.0 min (**S37**, 90%); ESI MS (neg mode, *Fig. VI.6h*): m/z = 2818 (**S37**, 90%, calc. 2819) and 2671 (**S38**, 10%, calc. 2671).

S-phenyl-N^{α}*-Boc-thiobiocytinate, trifluoroacetate salt.* (**129**).

A soln. of N(α)-Boc-biocytin (470 mg, 1.0 mmol) in a mixture of dry DMF (10 mL) on MS was treated successively with $^{i}Pr_2NEt$ (130 mg, 1.0 mmol), BOP [(Benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate] (440 mg, 1.0 mmol), thiophenol (120 mg, 1.1 mmol) and stirred for 10 min at 20°. Workup and CC (SiO_2 (20 g), CH_2Cl_2/MeOH 19:1 → 4:1). The fractions containing the product were collected and evaporated (bath 20°) under stream of Argon. (oxidation observed during first attempt) gave

129 (440 mg, 78%). Colourless solid. TLC (CH_2Cl_2/MeOH 1:9): R_f 0.29. ^{1}H-NMR (400 MHz, CD_3OD): 1.39–1.79 (*m*, 7 CH_2, 3 CH_3); 2.19 (*t*, *J* = 7.3, H_2CCO)); 2.72 (*d*, *J* = 12.7, SCH_2); 2.93 (*dd*, *J* = 4.9, 12.7, SC*H*); 4.22 (*dd*, *J* = 4.6, 9.6, H–C(α)); 4.30 (*dd*, *J* = 4.7, 7.8, H–CNH); 4.48 (*dd*, *J* = 4.8, 7.7, H–CNH); 7.35–7.48 (m, 5 arom. H). ^{13}C-NMR (100 MHz, CD_3OD): 23.3 (*t*, CH_2); 25.9 (*t*, CH_2); 27.9 (*q*, *Me*$_3$C); 28.5, 28.8, 29.0, 31.5, 35.9, 39.0 (6*t*, CH_2); 40.1 (*t*, *C*H_2S); 48.5, 48.7 (2*t*, CH_2); 55.6 (*d*, *C*HS); 61.4, 62.4 (2*d*, CH_2*C*H–NHCONH–*C*H); 80.1 (*d*, C(α)); 128.3 (*s*, arom. C); 129.3, 129.4, 134.9 (3*d*, arom. C); 157.1, 165.1, 175.0, 201.1 (4*s*, *C*O). ESI-MS: 566.34 ([*M*+H]$^+$).

S-phenyl-thiobiocytinate, trifluoroacetate salt. (**130**).

A soln. of **129** (440 mg, 0.78 mmol) in CH_2Cl_2 (8 mL) was treated with TFA (1.6 mL, 20.8 mmol) and stirred for 2h at 20°. The solvent has been evaporated to dryness and the residue crystallized from Et_2O gave **130** (442 mg, 98%). White solid. ^{1}H-NMR (400 MHz, DMF): 1.30–1.81 (*m*, 7 CH_2); 2.19 (*t*, *J* = 7.3, H_2CCO)); 2.72 (*d*, *J* = 12.7, SCH_2); 2.93 (*dd*, *J* = 4.9, 12.7, SC*H*); 2.00–2.23 (*m*, CH_2); 2.68–2.82 (*m*, CH_2); 3.16–3.25 (*m*, CH_2); 3.64 (br. *s*, NH_3^+) 4.22 (*dd*, *J* = 4.6, 9.6, H–C(α)); 4.27–4.32 (*m*, CH_2); 4.49 (br. *t*, *J* ≈ 7.0, H–CNH); 4.63 (*t*, *J* = 6.3, H–CNH); 7.55–7.60 (m, 5 arom. H); 8.90 (br. *s*, 2 NH). ^{13}C-NMR (100 MHz, DMF): 47.5, 51.2, 54.0, 55.7, 55.9, 56.9 (6*t*, CH_2); 61.1 (*t*, *C*H_2S); 63.8, 65.7 (2*t*, CH_2); 81.4 (*d*, *C*HS); 84.8, 85.5 (2*d*, CH_2*C*H–NHCONH–*C*H); 87.1 (*d*, C(α)); 151.5, 155.2 (2*d*, arom. C); 155.7 (*s*, arom. C); 160.3 (*d*, arom. C); 188.5, 197.9, 221.2 (3*s*, *C*O). ESI-MS: 466.34 ([*M*-TFA+H]$^+$).

References of Introduction

(S. R. Adams et al. 1989) S. R. Adams, J. P. Y. Kao, R. Y. Tsien. Biologically useful chelators that take up calcium(2+) upon illumination. *J. Am. Chem. Soc.* **1989**, *111*, 7957-7968.

(P. F. Agris 2004) P. F. Agris. Decoding the genome: a modified view. *Nucleic Acids Res.* **2004**, *32*, 223-238.

(S. S. Ashraf et al. 1999) S. S. Ashraf, E. Sochacka, R. Cain, R. Guenther, A. Malkiewicz, P. F. Agris. Single atom modification (O→S) of tRNA confers ribosome binding. *RNA*, **1999**, *5*, 188-194.

(S. S. Ashraf et al. 2000) S. S. Ashraf, R. H. Guenther, G. Ansari, A. Malkiewicz, E. Sochacka, P. F. Agris. Role of modified nucleosides of yeast tRNAPhe in ribosomal binding. *Cell Biochem. Biophys.* **2000**, *33*, 241-252.

(P. J. Beuning and K. Musier-Forsyth 1999) P. J. Beuning and K. Musier-Forsyth. Transfer RNA recognition by aminoacyl-tRNA synthetases. *Biopolymers* **1999**, *52*, 1-28.

(M. H. L. de Bruijn and A. Klug) M. H. L. de Bruijn and A. Klug. A model for the tertiary structure of mammalian mitochondrial transfer RNAs lacking the entire 'dihydrouridine' loop and stem. *EMBO J.* **1983**, *2*, 1309-1321.

(M. Buck et al. 1981) M. Buck, E. Griffiths. Regulation of aromatic amino acid transport by tRNA: role of 2-methylthio-N^6-(delta^2-isopentenyl)-adenosine. *Nucleic Acids Res.* **1981**, *9*, 401-414.

(J. H. Cate et al. 1999) J. H. Cate, M. M. Yusupov, G. Zh. Yusupova, T. N. Earnest, H. F. Noller. X-ray crystal structures of 70S ribosome functional complexes. *Science* **1999**, *285*, 2095-2105.

(F. Chapeville et al. 1962) F. Chapeville, F. Lipman, G. V. Ehrenstein, B. Weisblum, W. J. Jr. Ray, S. Benzer. Role of soluble ribonucleic acid in coding for amino acids. *Proc. Natl. Acad. Sci. U.S.A.* **1962**, *48*, 1086-1092.

(E. Chargaff et al. 1951) E. Chargaff, R. Lipschitz, C. Green, M. E. Hodes. The composition of the deoxyribonucleic acid of salmon sperm. *J. Biol. Chem.* **1951**, *192*, 223-230.

(W. E. Cohn and E. Volkin 1951) W. E. Cohn, E. Volkin. Nucleoside-5'-phosphates from ribonucleic acid. *Nature* **1951**, *167*, 483-484.

(S. Commans and A. Bock 1999) S. Commans, A. Bock. Selenocysteine inserting tRNAs: an overview. *FEMS Microbiol. Rev.* **1999**, *23*, 335-351.

(F. H. C. Crick 1966) F. H. C. Crick. Codon-anticodon pairing: the wobble hypothesis. *J. Mol. Biol.* **1966**, *19*, 548-555.

(F. F. Davis and F. W. Allen 1957) F. F. Davis, F. W. Allen. Ribonucleic acid from yeast, which contain a fifth nucleotide. *J. Biol. Chem.* **1957**, *227*, 907-915.

(E. Dawson and S. B. H. Kent 1997) E. Dawson, S. B. H. Kent. Protein synthesis by chemical ligation of unprotected peptides in aqueous solution. *Methods Enzymol.* **1997**, *289*, 266-298.

(R. E. Dickerson et al. 1982) R. E. Dickerson, H. R. Drew, B. N. Conner, R. M. Wing, A. V. Fratini, M. L. Kopka. The anatomy of A-, B-, and Z-DNA. *Science* **1982**, *216*, 475-485.

(W. M. Jr. Clemons et al. 1999) W. M. Jr. Clemons, J. L. May, B. T. Wimberly, J. P. McCutcheon, M. S. Capel, V Ramakrishnan. Structure of a bacterial 30S ribosomal subunit at 5.5 A resolution. *Nature* **1999**, *400*, 833-840.

(A. E. Dahlberg 1989) A. E. Dahlberg. The functional role of ribosomal RNA in protein synthesis. *Cell* **1989**, *57*, 525-529.

(S. Doonan 2004) *Nucleic Acids*. The Royal Society of Chemistry **2004**

(S. R. Eddy 2001) S. R. Eddy. Non-coding RNA genes and the modern RNA world. *Nature Reviews Genetics* **2001**, *2*, 919-929.

(S. M. Elbashir et al. 2001) S. M. Elbashir, J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, T. Tuschl. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **2001**, *411*, 494-498.

(J. A. Ellman et al. 1992) J. A. Ellman, D. Mendel, S. Anthony-Cahill, C. J. Noren, P. G. Schultz. Biosynthetic method for introducing unnatural amino acids site-specifically into proteins. *Methods Enzymol.* **1992**, *202*, 301-336.

(A. R. Ferré-D'Amaré 2003) A. R. Ferré-D'Amaré. RNA-modifying enzymes. *Curr. Opin. Struct. Biol.* **2003**, *13*, 49-55.

(C. Florentz et al. 2003) C. Florentz, B. Sohm, P. Tryoen-Toth, J. Putz, M. Sissler. Human mitochondrial tRNAs in health and disease. *Cell Mol. Life Sci.* **2003**, *60*, 1356-1375.

(C. A. Frederick et al. 1989) C. A. Frederick, G. J. Quigley, M.-K. Teng, M. Coll, G. A. van der Marel, J. H. van Boom, A. Rich, A. H.-J. Wang. Molecular structure of an A-DNA decamer d(ACCGGCCGGT). *Eur. J. Biochem.* **1989**, *181*, 295-307.

(T. Fujiwara et al. 2001) T. Fujiwara, M. Kimoto, H. Sugiyama, I. Hirao, S. Yokoyama. Synthesis of 6-(2thieny)purine nucleoside derivatives that form unnatural base pairs with pyridine-2-one nucleosides. *Bioorg Med. Chem. Lett.* **2001**, *11*, 2221-2223.

(M. Gu and C. D. Lima 2005) M. Gu, C. D. Lima. Processing the message: structural insights into capping and decapping mRNA. *Curr. Opin. Struct. Biol.* **2005**, *15*, 99-106.

(R. Giegé and M. Frugier 2003) R. Giegé, M. Frugier. Transfer RNA structure and identity. http://www.ncbi.nlm.nih.gov/books/bv.fcgi?rid=eurekah.chapter.20315

(A. C. Goldstrohm et al. 2001) A. C. Goldstrohm, A. L. Greenleaf, M. A. Garcia-Blanco. Co-transcriptional splicing of pre-messenger RNAs: considerations for the mechanism of alternative splicing. *Gene* **2001**, *277*, 31-47.

(H. Grosjean and R. Benne 1998) H. Grosjean, and R. Benne, (eds), *Modification and Editing of RNA*. ASM Press, Washington, DC. **1998**

(H. Grosjean. 2005) H. Grosjean. Modification and editing of RNA: historical overview and important facts to remember. *Top. Curr. Genet.* **2005**, *12*, 1-22.

(T. G. Heckler et al. 1984) T. G. Heckler, L. H. Chang, Y. Zama, T. Naka, M. S. Chorghade, S. M. Hecht. T4 RNA ligase mediated preparation of novel "chemically misacylated" $tRNA^{Phe}$s. *Biochemistry* **1984**, *23*, 1468-1473.

(T. G. Heckler et al. 1983) T. G. Heckler, Y. Zama, T. Naka, S. M. Hecht. Dipeptide formation with misacylated tRNAPhes. *J. Biol. Chem.* **1983**, *258*, 4492-4495.

(T. G. Heckler et al. 1988) T. G. Heckler, J. R. Roesser, C. Xu, P. I. Chang, S. M. Hecht. Ribosomal binding and dipeptide formation by misacylated tRNAPhe's. *Biochemistry* **1988**, *27*, 7254-7262.

(M. Helm et al. 1998) M. Helm, H. Brulé, F. Degoul, C. Cepanec, J.-P. Leroux, R. Giegé, C. Florentz. The presence of modified nucleotides is required for cloverleaf folding of a human mitochondrial tRNA. *Nucleic Acids Res.* **1998**, *26*, 1636-1643.

(M. Helm et al. 1999) M. Helm, R. Giegé, C. Florentz. A Watson-Crick base-pair-disrupting methyl group (m1A9) is sufficient for cloverleaf folding of human mitochondrial tRNALys. *Biochemistry* **1999**, *38*, 13338-13346.

(S. Hendler et al. 1970) S. Hendler, E. Fürer, P. R. Srinivasan. Synthesis and chemical properties of monomers and polymers containing 7-methylguanine and an investigation of their substrate or template properties for bacterial deoxyribonucleic acid or ribonucleic acid polymerases. *Biochemistry* **1970**, *9*, 4141-4153.

(I. Hirao et al. 2002) I. Hirao, T. Ohtsuki, T. Fujiwara, T. Mitsui, T. Yokogawa, T. Okuni, H. Nakayama, K. Takio, T. Yabuki, T. Kigawa, K. Kodama, T. Yokogawa, K. Nishikawa, S. Yokoyama. An unnatural base pair for incorporating amino acid analogs into proteins. *Nat. Biotechnol.* **2002**, *20*, 177-182.

(T. Hohsaka et al. 1999) T. Hohsaka, Y. Ashizuka, H. Sasaki, H. Murakami, M. Sisido. Incorporation of two different nonnatural amino acids independently into a single protein through extension of the genetic code. *J. Am. Chem. Soc.* **1999**, *121*, 12194-12195.

(T. Hohsaka and M. Sisido 2002) T. Hohsaka, M. Sisido. Incorporation of non-natural amino acids into proteins. *Curr. Opin. Chem. Biol.* **2002**, *6*, 809-815.

(H. R. Kalhor et al. 2005) H. R. Kalhor, M. Penjwini, S. Clarke. A novel methyltransferase required for the formation of the hypermodified nucleoside wybutosine in eukaryotic tRNA. *Biochem. Biophys. Res. Commun.* **2005**, *334*, 433-440.

(D. Kiga et al. 2002) D. Kiga, K. Sakamoto, K. Kodama, T. Kigawa, T. Matsuda, T. Yabuki, M. Shirouzu, Y. Harada, H. Nakayama, K. Takio, Y. Hasegawa, Y. Endo, I. Hirao, S. Yokoyama. An engineered *Escherichia coli* tyrosyl-tRNA synthetase for site specific

incorporation of an unnatural amino acid into proteins in eukaryotic translation and its application in a wheat germ cell-free system. *Proc. Natl. Acad. Sci U.S.A.* **2002**, *99*, 9715-9720.

(S. H. Kim et al. 1973) S. H. Kim, G. J. Quigley, F. L. Suddath. Three dimensional structure of yeast phenylalanine transfer RNA. Folding of the polynucleotide chain. *Science* **1973**, *179*, 285-288.

(S. H. Kim et al. 1974) S. H. Kim, F. L. Suddath, G. J. Quigley. Three dimensional tertiary structure of yeast phenylalanine transfer RNA. *Science* **1974**, *185*, 435-440.

(A. M. Knapinska et al. 2005) A. M. Knapinska, P. Irizarry-Barreto, S. Adusumalli, I. Androulakis, G. Brewer. Molecular mechanisms regulating mRNA stability: physiological and pathological significance. *Curr. Genomics* **2005**, *6*, 471-486.

(A. K. Kowal et al. 2001) A. K. Kowal, C. Kohrer, U. L. RajBhandary. Twenty-first aminoacyl-tRNA synthetase-suppressor tRNA pairs for possible use in site-specific incorporation of amino acid analogues into proteins in eukaryotes and in eubacteria. *Proc. Natl. Acad. Sci U.S.A.* **2001**, *98*, 2268-2273.

(N. C. Lau et al. 2001) N. C. Lau, L. P. Lim, E. G. Weinstein, D. P. Bartel. An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans. *Science* **2001**, *294*, 858-862.

(N. Lee et al. 2000) N. Lee, Y. Bessho, K. Wei, J. W. Szostak, H. Suga. Ribozyme-catalyzed tRNA aminoacylation. *Nat. Struct. Biol*. **2000**, *7*, 28-33.

(D. R. Liu et al. 1997) D. R. Liu, T. J. Magliery, M. Pastrnak, P.G. Schultz. Engineering a tRNA and aminoacyl-tRNA synthetase for the site specific incorporation of unnatural amino acids proteins in vivo. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 10092-10097.

(R. B. Loftfield and D. Vanderjagt 1972) R. B. Loftfield, D. Vanderjagt. The frequency of errors in protein biosynthesis. *Biochem. J.* **1972**, *128*, 1353-1356.

(M. J. Moore 2005) M. J. Moore. From birth to death: the complex lives of eukaryotic mRNAs. *Science* **2005**, *309*, 1514-1519.

(N. Muranaka et al. 2002) N. Muranaka, T. Hohsaka, M. Sisido. Photoswitching of peroxidase activity by position-specific incorporation of a photoisomerizable non-natural amino acid into horsereadish peroxidase. *FEBS Lett.* **2002**, *510*, 10-12.

(K. Nagai et al. 2001) K. Nagai, Y. Muto, D. A. Pomeranz Krummel, C. Kambach, T. Ignjatovic, S. Walke, A. Kuglstatter. Structure and assembly of the spliceosomal snRNPs. *Biochemical Society transactions* **2001**, *29*, 15-26.

(J. H. A. Nagel and C. W. A. Pleij 2002) J. H. A. Nagel, C. W. A. Pleij. Self-induced structural switches in RNA. *Biochimie* **2002**, *84*, 913-923.

(K. Ninomiya et al. 2004) K. Ninomiya, T. Minohata, M. Nishimura, M. Sisido. In situ chemical aminoacylation with amino acid thioesters linked to a peptide nucleic acid. *J. Am. Chem. Soc.* **2004**, *126*, 15984-15989.

(H. F. Noller 2005) H. F. Noller. RNA structure: reading the ribosome. *Science* **2005**, *309*, 1508-1514

(F. C. Oberstrass et al. 2005) F. C. Oberstrass, S. D. Auweter, M. Erat, Y. Hargous, A. Henning, P. Wenter, L. Reymond, B. Amir-Ahmady, S. Pitsch, D. L. Black, F. H.-T. Allain. Structure of PTB Bound to RNA: Specific Binding and Implications for Splicing Regulation. *Science* **2005**, *309*, 2054-2057.

(L. E. Orgel 2004) L. E. Orgel. Prebiotic chemistry and the origin of the RNA world. *Crit. Rev. Biochem. Mol. Biol.* **2004**, *39*, 99-123.

(A. Patchornik et al. 1970) A. Patchornik, B. Amit, R. B. Woodward. Photosensitive protecting groups. *J. Am. Chem. Soc.* **1970**, *92*, 6333-6335.

(S. Pitsch et al. 2001) S. Pitsch. Towards a total synthesis of aminoacylated t-RNAs. *Chimia* **2001**, *55*, 60-64.

(S. Pitsch et al. 2001) S. Pitsch, P. A. Weiss, L. Jenny, A. Stutz, X. Wu. Reliable chemical synthesis of oligoribonucleotides (RNA) with 2'-O-[(triisopropylsilyl)oxy]methyl(2'-O-tom)-protected phosphoramidites. *Helv. Chim. Acta* **2001**, *84*, 3773-3795.

(S. Pitsch et al. 2005) S. Pitsch, D. Ackermann, C. Denarie, F. Meylan, M. Meyyappan, E. Muller, A. Peer, S. Porcher, L. Reymond, A. Stutz, P. Wenter. Preparation of modified RNA sequences for biological research. *Chimia* **2005**, *59*, 808-811.

(A. Rich 1962) A. Rich. *Horizons in Biochemistry*. Eds. M. Kasha and B. Pullmann, Academic Press, New York, **1962**, p103

(M. Rigourd et al. 2003) M. Rigourd, G. Beg, P. Benas, S. F. Le Grice, B. Ehresmann, C. Ehresmann, R. Marquet. Effects of $tRNA_3^{Lys}$ aminoacylation on the initiation of HIV-1 reverse transcription. *Biochimie* **2003**, *85*, 521-525.

(S. A. Robertson et al. 1991) S. A. Robertson, J. A. Ellman, P. G. Schultz. A general and efficient route for chemical aminoacylation of transfer RNAs. *J. Am. Chem. Soc.* **1991**, *113*, 2722-2729.

(J. D. Robertus et al. 1974) J. D. Robertus, J. E. Ladner, J. T. Finch. Structure of yeast phenylalanine tRNA at 3Å resolution. *Nature* **1974**, *250*, 546-551.

(J. R. Roesser et al. 1986) J. R. Roesser, M. S. Chorghade, S. M. Hecht. Ribosome-catalyzed formation of an abnormal peptide analog. *Biochemistry* **1986**, *25*, 6361-6365.

(W.-D. Schubert et al. 2002) W.-D. Schubert, J. Moser, S. Schauer, D. W. Heinz, D. Jahn. Structure and function of glutamyl-tRNA reductase, the first enzyme of tetrapyrrole biosynthesis in plants and prokaryotes. *Photosynth. Res.* **2002**, *74*, 205-215.

(C. D. Stout et al. 1974) C. D. Stout, H. Mizuno, J. Rubin. Atomic coordinates and molecular conformation of yeast phenylalanyl tRNA. An independent investigation. *Nucleic Acids Res.* **1976**, *3*, 1111-1123.

(A. Stutz 2003) DISS. ETH Nr. 15141. *Methoden zur Ligation von Oligoribonukleotiden und ihre Anwendung bei der Totalsynthese aminoacylierter tRNAs.* ETH Zürich 2003.

(R. C. Thompson and A. M. Karim 1982) R. C. Thompson and A. M. Karim. The accuracy of protein biosynthesis is limited by its speed: High fidelity selection by ribosomes of aa-tRNA ternary complexes containing GTP[γS]. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 4922-4926.

(U. vonAhsen et al. 1997) U. vonAhsen, R. Green, R. Schroeder, H. F. Noller. Identification of 2'-hydroxyl groups required for interaction of a tRNA anticodon stem-loop region with the ribosome. *RNA*, **1997**, *3*, 49-56.

(L. Wang et al. 2001) L. Wang, A. Brock, B. Herbrich, P.G. Schultz. Expanding the genetic code of Escherichia coli. *Science* **2001**, *292*, 498-500.

(L. Wang, P. G. Schultz 2002) L. Wang, P. G. Schultz. Expanding the genetic code. *Chem. Commun.* **2002**, *7*, 1-11.

(L. Wang and P. G. Schultz 2005) L. Wang, P. G. Schultz. Expanding the genetic code *Angew. Chem. Int. Ed.* **2005**, *44*, 34–66.

(J. D. Watson, F. H. Crick 1953) J. D. Watson, F. H. Crick. Molecular structure of nucleic acids; a structure for deoxyribose nucleic acid. *Nature* **1953**, *171*, 737-738.

(R. Wing et al. 1980) R. Wing, H. Drew, T. Takano, C. Broka, S. Tanaka, K. Itakura, R. E. Dickerson. Crystal structure analysis of a complete turn of B-DNA. *Nature* **1980**, *287*, 755-758.

(W. C. Winkler and R. R. Breaker 2003) W. C. Winkler, R. R. Breaker. Genetic control by metabolite-binding riboswitches. *ChemBioChem* **2003**, *4*, 1024-1032.

(D. R. Wolstenholme et a. 1994) D. R. Wolstenholme, R. Okimoto and J. L. Macfarlane. Nucleotide correlations that suggest tertiary interactions in TV-repalecement loop-containing mitochondrial tRNAs of the nematodes, *Caenorhabditis elegans* and *Ascaris suum*. *Nucleic Acid Res*. **1994**, *22*, 4300-4306.

(X. Wu and S. Pitsch 1998) X. Wu, S. Pitsch. Synthesis and pairing properties of oligoribonucleotide analogues containing a metal-binding site attached to β-D-allofuranosyl cytosine. *Nucleic Acids Res.* **1998**, *26*, 4315-4323.

(C. Yarian et al. 2000) C. Yarian, M. Marszalek, E. Sochacka, A. Malkiewicz, R. Guenther, A. Miskiewicz, P. F. Agris. Modified nucleoside dependent Watson±Crick and wobble codon binding by tRNALys UUU species. *Biochemistry* **2000**, *39*, 13390-13395.

(C. Yarian et al. 2002) C. Yarian, H. Townsend, W. Czestkowski, E. Sochacka, A. J. Malkiewicz, R. Guenther, A. Miskiewicz, P. F. Agris. Accurate translation of the genetic code depends on tRNA modified nucleosides. *J. Biol. Chem.* **2002**, *277*, 16391-16395.

(M. M. Yusupov et al. 2001) M. M. Yusupov, G. Z. Yusupova, A. Baucom, K. Lieberman, T. N. Earnest, J. H. Cate, H. F. Noller. Crystal structure of the ribosome at 5.5 Å resolution. *Science* **2001**, *292*, 868-869.

(S. Zhou et al. 2004) S. Zhou, D. Sitaramaiah, K. R. Noon, R. Guymon, T. Hashizume, J. A. McCloskey. Structures of two new ‘‘minimalist’’ modified nucleosides from archaeal tRNA *Bioorg. Chem.* **2004**, *32*, 82–91.

References of Chapter I

(P. F. Agris et al. 1995) P. F. Agris, A. Malkiewicz, A. Kraszewski, K. Everett, B. Nawrot, E. Sochacka, J. Jankowska, R. Guenther. Site-selected introduction of modified purine and pyrimidine ribonucleosides into RNA by automated phosphoramidite chemistry. *Biochimie* **1995**, *77*, 125-134.

(Y. Apeloig and M. Nakash 1994) Y. Apeloig, M. Nakash. Reversal of stereoselectivity in the reduction of gem dichlorides by tributyltin hydride and tris(trimethylsilyl)silane. Synthetic and mechanistic implications. *J. Am. Chem. Soc.* **1994**, *116*, 10781-10782.

(J. Armengaud et al. 2004) J. Armengaud, J. Urbonavicius, B. Fernandez, G. Chaussinand, J. M. Bujnicki, H. Grosjean. N^2-Methylation of guanosine at position 10 in tRNA is catalyzed by a THUMP domain-containing, *S*-adenosylmethionine-dependent methyltransferase, conserved in archaea and eukaryota. *J. Biol. Chem.* **2004**, *279*, 37142-37152.

(J. G. Arnez et al. 1994) J. G. Arnez, T. A. Steitz. Crystal structure of unmodified $tRNA^{Gln}$ complexed with glutaminyl-tRNA synthetase and ATP suggests a possible role for pseudo-uridines in stabilization of RNA structure. *Biochemistry* **1994**, *33*, 7560-7567.

(P. Auffinger and E. Westhof 1998) P. Auffinger, E. Westhof. Effects of pseudouridylation on tRNA hydration and dynamics: A theoretical approach. In *Modification and Editing of RNA* (H. Grosjean, R. Benne, eds.). **1998** pp. 103-112, ASM Press, Washington, D.C.

(A. C. Bajii and D. R. Davis 2000) A. C. Bajii, D. R. Davis. Synthesis and biophysical characterization of tRNA(Lys, 3) anticodon stem-loop RNAs containing the mcm(5)s(2)U nucleoside. *Org. Lett.* **2000**, *2*, 3865-3868.

(M. Ballestri et al. 1991) M. Ballestri, C. Chatgilialoglu, K. B. Clark, D. Griller, B. Giese, B. Kopping. Tris(trimethylsilyl)silane as a radical-based reducing agent in synthesis. *J. Org. Chem.* **1991**, *56*, 678-683.

(H. F. Becker et al. 1997) H. F. Becker, Y. Motorin, M. Sissler, C. Florentz, H. Grosjean. Major identity determinants for enzymatic formation of of ribothymidine and pseudouridine in the TΨ-loop of yeast tRNAs. *J. Mol. Biol.* **1997**, *274*, 505-518.

(F. Bergmann and W. Pfleiderer 1994) The 2-Dansylethoxycarbonyl; (=2-{[5-Dimethylamino)naphtalen-1-yl]sulfonyl}ethoxycarbonyl; Dnseoc) group for protection of the 5'-hydroxy function in oligoribonucleotide synthesis. *Helv. Chim. Acta* **1994**, *77*, 481-501.

(A. D. Berry et al. 2004) A. D. Berry, K.-Y. Jung, S. D. Wise, A. D. Sercel, W. H. Pearson, H. Mackie, J B. Randolph, R. L. Somers. Pyrrolo-dC, pyrrolo-C: fluorescent analogs of cytidine and 2'-deoxycytidine for the study of oligonucleotides. *Tetrahedron Lett.* **2004**, *45*, 2457-2461.

(G. R. Björk 1995) G. R. Björk, *Biosynthesis and Function of Modified Nucleosides*, American Society for Microbiology, Washington DC 20005 **1995**.

(G. R. Björk et al. 1989) G. R. Björk, P. M. Wikström, A. S. Byström. Prevention of translational frameshifting by the modified nucleoside 1-methylguanosine. *Science* **1989**, *244*, 986-989.

(V. Boudou et al. 2000) V. Boudou, J. Langridge, A. Van Aerschot, C. Hendrix, A. Millar, P. Weiss, P. Herdewijn. Synthesis of the anticodon hairpin $^{t}RNA_{f}^{Met}$ containing N-{[9-(*β*-D-Ribofuranosyl)-9*H*-purin-6-yl]carbamoyl}-L-threonine (=N^6-{{[(1S,2R)-1-carboxy-2-hydroxypropyl]amino}carbonyl}adenosine, t6A). *Helv. Chim. Acta* **2000**, *83*, 152-161.

(D. Brown et al. 1968) D. Brown, M. G. Burdon, R. P. Slatcher. A synthesis of pseudouridine and of 5-β-D-ribofuranosyl-uridine. *J. Chem. Soc. C* **1968**, *9*, 1051-1053.

(A. S. Byström et al. 1983) A. S. Byström, K. J. Hjalmarsson, P. M. Wikström, G. R. Björk. The nucleotide sequence of an E.Coli operon containing genes for the tRNA(m1G)methyltransferase, the ribosomal proteins S16 and L19 and a 21-K polypeptide. *EMBO J.* **1983**, *2*, 899-905.

(J. Cabello-Villegas et al. 2002) J. Cabello-Villegas, M. E. Winkler, E. P. Nikonowicz. Solution conformations of unmodified and $A_{37}N^6$-dimethylallyl modified anticodon stem-loops of Escherichia coli $tRNA^{Phe}$. *J. Mol. Biol.* **2002**, 319, 1015-1034.

(M. Charette and M. W. Gray 2000) M. Charette, M. W. Gray. Pseudouridine in RNA: What, Where, How, and Why. *Life* **2000**, *49*, 341-351.

(C. Chatgilialoglu 1992) C. Chatgilialoglu. Organosilanes as radical-based reducing agents in synthesis. *Acc. Chem. Res.* **1992**, *25*, 188-194.

(G. B. Chheda et al. 1972) G. B. Chheda, C. I. Hong, C. F. Piskorz, G. A. Harmon. Biosynthesis of N-(6-purin carbamoyl)-L-threonine riboside. Incorporation of L-threonine in vivo into modified nucleoside of transfer ribonucleic. *Biochem. J.* **1972**, *127*, 515-519.

(H. M.-P. Chui et al. 2002a) H. M.-P. Chui, M. Meroueh, S. A. Scaringe, C. S. Chow. Synthesis of a 3-methyluridine phosphoramidite to investigatethe role of methylation in a ribosomal RNA hairpin. *Bio. Med. Chem.* **2002**, *10*, 325-332.

(H. M.-P. Chui et al. 2002b) H. M.-P. Chui, J.-P. Desaulniers, S.A. Scaringe, C.S. Chow. Synthesis of helix 69 of *Escherichia coli* 23S rRNA containing its natural modified nucleosides, $m^3\psi$ and ψ. *J. Org. Chem.* **2002**, *67*, 8847-8854.

(S. A. Connolly et al. 2004) S. A. Connolly, A. E. Rosen, K. Musier-Forsyth, C. S. Francklyn. G1:C73 recognition by an arginine cluster in the active site of Escherichia coli Histidyl-tRNA synthetase. *Biochemistry* **2004**, *43*, 962-969.

(J. F. Curran 1998) J. F. Curran. Modified nucleosides in translation. In *Modification and Editing of RNA* (H. Grosjean, R. Benne, eds.). **1998** pp. 493-516. ASM Press, Washington, D.C.

(A. S. Delk et al. 1980) A. S. Delk, D. P. Nagle, J. C. Rabinowitz. Methylenetetrahydrofolate dependent biosynthesis of ribothymidine in transfer RNA of Streptococcus faecalis. Evidence for reduction of the 1-carbon unit by $FADH_2$. *J. Mol. Biol.* **1986**, *255*, 4387-4390.

(L. Droogmans and H. Grosjean 1991) L. Droogmans, H. Grosjean. 2'-O-methylation and inosine formation in the wobble position of anticodon-substituted tRNAPhe in a homologous in vitro system. *Biochimie* **1991**, *73*, 1021-1025.

(P. C. Durant and D. R. Davis 1999) P. C. Durant, D. R. Davis. Stabilization of the anticodon stem-loop of $tRNA^{Lys,3}$ by an A^{+}-C base pair and by pseudouridine. *J. Mol. Biol.*, **1999**, *285*, 115-131.

(F. F. Davis and F. W. Allen 1957) F. F. Davis, F. W. Allen. Ribonucleic acid from yeast, which contain a fifth nucleotide. *J. Biol. Chem.* **1957**, *227*, 907-915.

(D. R. Davis and C. D. Poulter 1991) D. R. Davis, C. D. Poulter. ^{1}H-^{15}N NMR studies of *Escherichia coli* $tRNA^{Phe}$ from *hisT* mutants: A structural role for pseudouridine. *Biochemistry*, **1991**, *30*, 4223-4231.

(D. R. Davis et al. 1998a) D. R. Davis, C. A. Veltri, L. Nielsen. An RNA model system for investigation of pseudouridine stabilization of the codon-anticodon interaction in $tRNA^{Lys}$, $tRNA^{His}$, $tRNA^{Tyr}$. *J. Biomol. Struct. Dyn.* **1998**, *15*, 1121-1132.

(D. R. Davis 1999) D. R. Davis. Stabilization of RNA stacking by pseudouridine. *Nucleic Acids Res.*, **1999**, *27*, 3543-3549.

(D. R. Davis 1998) D. R. Davis. Biophysical and conformational properties of modified nucleosides in RNA. In *Modification and Editing of RNA* (H. Grosjean, R. Benne, eds.). **1998** pp. 85-102, ASM Press, Washington, D.C.

(J. J. Dalluge et al. 1996) J. J. Dalluge, T. Hashizume, A. E. Sopchik, J. A. Mac Closkey, D.R. Davis. Conformational flexibility in RNA: the role of dihydrouridine. *Nucleic Acids Res.* **1996**, *24,* 1073-1079.

(J. Edqvist et al. 1994) J. Edqvist, K. Blomqvist, K. Straeby. Structural Elements in Yeast tRNAs Required for Homologous Modification of Guanosine-26 into Dimethylguanosine-26 by the Yeast Trm1 tRNA-Modifying Enzyme. *Biochemistry* **1994**, *33*, 9546-9551.

(J. Edqvist et al. 1992) J. Edqvist, H. Grosjean, K. B. Straeby. Identity elements for N2-dimethylation of guanosine-26 in yeast tRNAs. *Nucleic Acids Res.* **1992**, *20*, 6575-6581.

(B. N. Elkins et al. 1974) B. N. Elkins, E. B. Keller. The enzymatic synthesis of N-(purin-6-ylcarbamoyl)threonine, an anticodon-adjacent base in transfer ribonucleic acid. *Biochemistry*, **1974**, *76*, 4622-4628.

(S. R. Ellis et al. 1986) S. R. Ellis, M. Morales, J. M. Li, A. K. Hoper, N. C. Martin. Isolation and characterization of the TRM1 locus, a gene essential for the N^2,N^2-dimethylguanosine modification of both mitochondrial and cytoplasmic tRNA in Saccharomyces cerevisiae. *J. Mol. Biol.* **1986**, 26, 9703-9709.

(D. Flockerzi et al. 1981) D. Flockerzi, G. Silber, R. Charubala, W. Schlosser, R.S. Varma, F. Creegan, W. Pfeiderer. Synthese and eigenschaften von 2'-O- und 3'-O-(tert-butyldimethylsilyl)-5'-O-(4-methoxytrityl)- sowie 2',3'-bis-O-(tert-

butyldimethylsilyl)ribonucleosiden – Ausgangssubstanzen für oligoribonucleotid-Synthesen. *Liebigs Ann. Chem*. **1981**, 1568-1585.

(G. A. Garcia et al. 1998) G. A. Garcia, D. M. Goodenough-Lashua. In *Modification and Editing of RNA* (H. Grosjean, R. Benne, eds.). **1998** pp. 152-154, ASM Press, Washington, D.C.

(D. Gasparutto et al. 1992) D. Gasparutto, T. Livache, H. Bazin, A.-M. Duplaa, A. Guy, A. Khorlin, D. Molko, A. Roget, R. Téoule. Chemcial synthesis of a biologically active natural tRNA with its minor bases. *Nucleic Acids Res.* **1992**, *20*, 5159-5166.

(R. Green et al. 1991) R. Green, J. W. Szostak, S. A. Benner, A. Rich, N. Usman. Synthesis of RNA containing inosine: analysis of the sequence requirements for the 5' splice site of Tetrahymena group I intron. *Nucleic Acids Res.* **1991**, *19*, 4161-4166.

(P. J. Grohar and C. S. Chow 1999) P. J. Grohar, C. S. Chow. Practical synthesis of the modified RNA nucleoside pseudouridine. *Tetrahedron Lett.* **1999**, *40*, 2049-2052.

(H. Grosjean et al. 1995a) H. Grosjean, F. Constantinesco, D. Foiret, N. Benachenhou. A novel enzymic pathway leading to 1-methylinosine modification in Haloferax volcanii tRNA *Nucleic Acids Res.* **1995**, *23*, 4312-4319.

(H. Grosjean et al. 1996a) Grosjean, H.; Auxilien, S.; Constantinesco, F.; Simon, C.; Corda, Y.; Becker, H. F.; Foiret, D.; Morin, A.; Jin, Y. X.; et al. Enzymic conversion of adenosine to inosine and to N1-methylinosine in transfer RNAs: a review. *Biochimie* **1996**, *78*, 488-501.

(H. Grosjean et al. 1996b) H. Grosjean, J. Edqvist, K. B. Straby, R. Giege. Enzymatic formation of modified nucleosides in tRNA: dependence on tRNA architecture. *J. Mol. Biol.* **1996**, *255*, 67-85.

(H. Grosjean and R. Benne 1998) *Modification and Editing of RNA* (H. Grosjean, R. Benne, eds.). **1998** ASM Press, Washington, D.C.

(C. Guthrie and J. Abelson 1982) C. Guthrie, J. Abelson. Organisation and expression of tRNA genes in Saccharomyces cervisiae p487-528. In J. N. Strathern, E. W. Jones, J. R. Broach (ed.), The molecular biology of the yeast Saccharomyces. Metabolism and gene expression. Cold Spring Harbor Laboratory Press, **1982**, Cold Spring Harbor, N. Y.

(S. Hanessian and R. Machaalani 2003) S. Hanessian, R. Machaalani. A highly stereocontrolled and efficient synthesis of α- and β-pseudouridines. *Tetrahedron Lett.* **2003**, *44*, 8321–8323.

(K. M. Harrigton et al. 1993) K. M. Harrigton, I. A. Nazarenko, D. B. Dix, R. C. Thompson, O. C. Uhlenbeck. In vitro analysis of translation rate and accuracy with an unmodified tRNA. *Biochemistry*, **1993**, *32*, 7617-7622.

(E. Haumont et al. 1984) E. Haumont, M. Fournier, S. deHeneau, H. Grosjean. Enzymatic conversion of adenosine to inosine in the wobble position of yeast tRNAAsp ; the dependence on the anticodon sequence. *Nucleic Acids Res.* **1984**, *12*, 2705-2715.

(K. J. Hjalmarsson et al. 1983) K. J. Hjalmarsson, A. S. Byström, G. R. Björk. Purification and characterization of transfer RNA(Guanine-1)methyltransferase from E.coli. *J. Biol. Chem.* **1983**, *258*, 1343-1351.

(M. Helm et al. 1998) M. Helm, H. Brulé, F. Degoul, C. Cepanec, J.-P. Leroux, R. Giegé, C. Florentz. The presence of modified nucleotides is required for cloverleaf folding of a human mitochondrial tRNA. *Nucleic Acids Res.* **1998**, 26, 1636-1643.

(M. Helm et al. 1999) M. Helm, R. Giegé, C. Florentz. A Watson-Crick base-pair-disrupting methyl group (m1A9) is sufficient for cloverleaf folding of human mitochondrial tRNALys. *Biochemistry* **1999**, *38*, 13338-13346.

(C. Hoebartner et al. 2003) C. Hoebartner, C. Kreutz, E. Flecker, E. Ottenschläger, W. Pils, K. Grubmayr, R. Micura. The synthesis of 2'-O-[(tri-isopropylsilyl)oxy]methyl (TOM) phosphoramidites of methylated ribonucleosides (m^1G, m^2G, $m^2{}_2G$, m1I, m^3U, m^4C, m^6A, $m^6{}_2A$) for use in automated RNA solid-phase synthesis. *Monatsh. Chem.* **2003**, *134*, 851-873.

(C. Hoebartner et al. 2005) C. Hoebartner, R. Rieder, C. Kreutz, B. Puffer, K. Lang, A. Polonskaia, A. Serganov, R. Micura. Syntheses of RNAs with up to 100 nucleotides containing site-specific 2'-methyl-seleno labels for use in X ray crystallography. *J. Am. Chem. Soc.* **2005**, *127*, 12035-12045.

(Y. H. Ji et al. 1990) Y. H. Ji, W. Bannwarth, B. Luu. Application of the phosphoramidite-phosphite triester approach for the synthesis of combinations between oxygenated sterols and nucleoside analogs linked by phosphodiester bonds. *Tetrahedron* **1990**, *46*, 487-502.

(H.-Q. Jiang et al. 1997) H.-Q. Jiang, Y. Motorin, Y.-X. Jin, H. Grosjean. Pleiotropic effects of intron removal on base modification pattern of yeast tRNAPhe : an in vitro study. *Nucleic Acids Res.* **1997**, *25*, 2694-2701.

(P. F. Johnson and J. Albeson 1983) P. F. Johnson, J. Abelson. The yeast $tRNA^{Tyr}$ gene intron is essential for correct modification of its tRNA product. *Nature* **1983**, *302*, 681-687.

(J. M. Keith et al. 1980) J. M. Keith, E. M. Winters, B. Moss. Purification and characterization of a HeLa cell transfer RNA (cytosine-5-)methyltransferase. *J. Biol. Chem.* **1980**, *255*, 4636-4644.

(E. Kierzek and R. Kierzek 2001) E. Kierzek, R. Kierzek. Influence of N6-isopentenyladenosine (i^6A) on thermal stability of RNA duplexes. *Biophys. Chem.* **2001**, *91*, 135-140.

(E. Kierzek and R. Kierzek 2003) E. Kierzek, R. Kierzek. The synthesis of oligoribonucleotides containing N^6-alkyladenosines and 2-methylthio-N^6-alkyladenosines via post-synthetic modification of precursor oligomers. *Nucleic Acids Res.* **2003**, *31*, 4461-4471.

(E. Kierzek and R. Kierzek 2003) E. Kierzek, R. Kierzek. The thermodynamic stability of RNA duplexes and hairpins containing N6-alkyladenosines and 2-methylthio-N6-alkyladenosines. *Nucleic Acids Res.* **2003**, *31*, 4472-4480.

(A. K. Kowal et al. 2001) A. K. Kowal, C. Kohrer, U.L. RajBhandary. Twenty-first aminoacyl-tRNA synthetase-suppressor tRNA pairs for possible use in site-specific incorporation of amino acid analogues into proteins in eukaryotes and in eubacteria. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 2268-2273.

(R. K. Kumar and D. R. Davis 1997) R. K. Kumar, D. R. Davis. Synthesis and studies on the effect of 2-thiouridine and 4-thiouridine on sugar conformation and RNA duplex. *Nucleic Acids Res.* **1997**, *25*, 1272-1280.

(D. R. Liu et al. 1997) a) D.R. Liu, T.J. Magliery, M. Pastrnak, P.G. Schultz. Engineering a tRNA and aminoacyl-tRNA synthetase for the site specific incorporation of unnatural amino acids proteins in vivo. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 10092-10097.

(J. Mengel-Jørgensen and F. Kirpekar 2002) J. Mengel-Jørgensen, F. Kirpekar. Detection of pseudouridine and other modifications in tRNA by cyanoethylation and MALDI mass spectrometry. *Nucleic Acids Res.* **2002**, *30*, e135.

(R. Micura et al. 2001) R. Micura, W. Pils, C. Höbartner, K. Grubmayr, M.-O. Ebert, B. Jaun. Methylation of the nucleobases in RNA oligonucleotides mediates duplex-hairpin conversion. *Nucleic Acids Res.* **2001**, *29*, 3997-4005.

(S. N. Mikhailov et al. 2002) S. N. Mikhailov, J. Rozenski, E. V. Efimtseva, R. Busson, A. Van Aerschot, P. Herdewijn. Chemical incorporation of 1-methyladenosine into oligonucleotides. *Nucleic Acids Res.* **2002**, *30*, 1124-1131.

(E. G. Mueller 2002) E. G. Mueller. Chips off the old block. *Nat. Struct. Biol.* **2002**, 9, 320-322.

(S. Muller et al. 2004) S. Muller; J. Wolf; S. A. Ivanov. Current strategies for the synthesis of RNA. *Curr. Org. Synthesis* **2004**, *1*, 293-307.

(J. M. Neumann et al. 1980) J. M. Neumann, J. M. Bernassau, M. Gueron, S. Tran-Dinh. Comparative conformations of uridine and pseudouridine and their derivatives. *Eur. J. Biochem.*, **1980**, *108*, 457-463.

(K. N. Nobles et al. 2002) K. N. Nobles, C. S. Yarain, G. Liu, R. H. Guenther, P. F. Agris. Highly conserved modified nucleosides influence Mg^{2+}-dependent tRNA folding. *Nucleic Acids Res.* **2002**, *30*, 4751-4760.

(L. Pichat et al. 1971) L. Pichat, J. Deschamps, B. Massé, P. Dufay. Comparaison de l'influence de groupes trimethylsilyloxy, ou dimethylsilyloxy et t-butoxy sur la reactivité de pyrimidines-lithium. Préparation de thymine (methyl ^{14}C). *Bull. Soc. Chim. Fr.* **1971**, *6*, 2110-2114.

(U. Pieles et al. 1994) U. Pieles, B. Beijer, K. Bohmann, S. Weston, S. O'Loughlin, V. Adam, B. S. Sproat. New and convenient protection system for pseudouridine, highly suitable solid-phase oligoribonucleotide synthesis. *J. Chem. Soc., Perkin Trans. I* **1994**, 3423-3429.

(S. Pitsch et al. 1999) S. Pitsch, P. A. Weiss, X. Wu, D. Ackermann, T. Honegger. Fast reliable automated synthesis of RNA and partially 2'-O-protected precursors ("caged RNA")

based on two novel, orthogonal 2'-O-protecting groups. *Helv. Chim. Acta* **1999**, *82*, 1753-1761.

(S. Pitsch et al. 2001) S. Pitsch, P. A. Weiss, L. Jenny, A. Stutz, X. Wu. Reliable chemical synthesis of oligoribonucleotides (RNA) with 2'-O-[(triisopropylsilyl)oxy]methyl (2'-O-tom)-protected phosphoramidites. *Helv. Chim. Acta* **2001**, *84*, 3773-3795.

(D. M. Powers and A. Peterkofsky 1972) D. M. Powers, A. Peterkofsky. Biosynthesis and specific labelling of N-(purin-6-ylcarbamoyl)threonine of Escherichia coli transfer RNA. *Biochem. Biophys. Res. Commun.*, **1972**, *46*, 831-838.

(M. P. Reinhart et al. 1986) M. P. Reinhart, J. M. Lewis, P. S. Leboy. A single tRNA (guanine)-methyltransferase from Tetrahymena with both mono- and di-methylating activity. *Nucleic Acids Res.* **1986**, *14*, 1131-1148.

(I. Ryu et al. 1997) I. Ryu, K. Nagahara, A. Kurihara, M. Komatsu, N. Sonoda Silylcarbonylation of 1,5-dienes accompanied by acyl radical cyclization. *J. Organomet. Chem.* **1997**, *548*, 105-107.

(M. Sekine and T. Satoh 1991) M. Sekine, T. Satoh. A convenient method for the synthesis of N^2,N^2-dimethylguanosine by reductive C-S bond cleavage with tributyltin hydride. *J. Org. Chem.* **1991**, *56*, 1224-1227.

(C. J. Spedaliere et al. 2004) C. J. Spedaliere, J. M. Ginter, M. W. Johnston, E. G. Mueller. The pseudouridine synthases: revisiting a mechanism that seemed settled. *J. Am. Chem. Soc.* **2004**, *126*, 12758-12759.

(F. Skoog and D. J. Armstrong 1970) F. Skoog, D. J. Armstrong. Cytokinins. *Annu. Rev. Plant Physiol.* **1970**, *21*, 359-384.

(D. Söll et al. 1995) D. Söll and U. RajBhandary, *tRNA: Structure, Biosynthesis and function* **1995**, 207-223, *American Society for Microbiology* and references cited therein.

(M. Sprinzl et al. 1998) M. Sprinzl, C. Horn, M, Brown, A. Loudovitch and S. Steinberg. Compilation of tRNA sequences and sequences of tRNA genes. *Nucleic Acids Research*, **1998**, *26*, 148-153.

(A. Stutz et al. 2000) A. Stutz, C. Höbartner, S. Pitsch. Novel fluoride-labile nucleobase protecting groups for the synthesis of 3'(2')-*O*-aminoacylated RNA sequences. *Helv. Chim. Acta* **2000**, *83*, 2477-2503.

(D. V. Santi and L. W. Hardy 1987) D. V. Santi, L. W. Hardy. Catalytic mechanism and inhibition of tRNA-(uracil-5-)methyltransferase: evidence for covalent catalysis. *Biochemistry* **1987**, *26*, 8599-8606.

(A. Stutz and S. Pitsch 1999) A. Stutz, S. Pitsch. Automated RNA-synthesis with photocleavable sugar and nucleobase protecting groups. *Synlett* **1999**, (Spec.), 930-934.

(M. Sundaram et al. 2000) M. Sundaram, P.C. Durant, D.R. Davis. Hypermodified nuclosides in the anticodon of tRNALys stabilize a canonical U-turn. *Biochemistry* **2000**, *39*, 12575-12584.

(D. Sung and H. Kang 2003) D. Sung, H. Kang. Prokaryotic and eukaryotic translational machineries respond differently to the frameshifting RNA signal from plant or animal virus. *Virus Res.* **2003**, *92*, 165-170.

(L. Wang et al. 2001) L. Wang, A. Brock, B. Herbrich, P.G. Schultz. Expanding the genetic code of Escherichia coli. *Science* **2001**, *292*, 498-500.

(L. Wang and P. G. Schultz 2005) L. Wang, P. G. Schultz. Expanding the genetic code *Angew. Chem. Int. Ed.* **2005**, *44*, 34–66.

(P. Wenter and S. Pitsch 2003) P. Wenter, S. Pitsch. Synthesis of selectively ^{15}N-labeled 2'-O-{[(triisopropylsilyl)oxy]methyl}(=tom)-protected ribonucleoside phosphoramidites and their incorporation into a bistable 32mer RNA sequence. *Helv. Chim. Acta* **2003**, *86*, 3955-3974.

(X. Wu and S. Pitsch 1998) X. Wu, S. Pitsch. Synthesis and pairing properties of oligonucleotide analogs containing a metal binding site attached to β-D-allofuranosyl cytosine. *Nucleic Acids Res.* **1998**, *26*, 4315-4323.

(F. Xing et al. 2004) F. Xing, S. L. Hiley, T. R. Hughes, E. M. Phizicky. The specificities of four yeast dihydrouridine synthases for cytoplasmic tRNAs. *J. Biol. Chem.*, **2004**, *279*, 17850-17860.

References of Chapter II

(J. D. Bain et al. 1992) J. D. Bain, C. Switzer, A. R. Chamberlin, S. A. Benner. Ribosome-mediated incorporation of a non-standard amino acid into a peptide through expansion of the genetic code. *Nature* **1992**, *356*, 537-539.

(X. Chen et al. 2001) X. Chen, R. Kierzek, D. H. Turner. Stability and structure of RNA duplexes containing Isoguanosine and Isocytidine. *J. Am. Chem. Soc.* **2001**, *123*, 1267-1274.

(I. Hirao et al. 2002) I. Hirao, T. Ohtsuki, T. Fujiwara, T. Mitsui, T. Yokogawa, T. Okuni, H. Nakayama, K. Takio, T. Yabuki, T. Kigawa, K. Kodama, T. Yokogawa, K. Nishikawa, S. Yokoyama. An unnatural base pair for incorporating amino acid analogs into proteins. *Nat. Biotechnol.* **2002**, *20*, 177-182.

(T. Fujiwara et al. 2001) T. Fujiwara, M. Kimoto, H. Sugiyama, I. Hirao, S. Yokoyama. Synthesis of 6-(2thieny)purine nucleoside derivatives that form unnatural base pairs with pyridine-2-one nucleosides. *Bioorg Med. Chem. Lett.* **2001**, *11*, 2221-2223.

(S. C. Jurczyk et al. 1998) S. C. Jurczyk, J. T. Kodra, D. Rozzell, S. A. Benner, T. R. Battersby. Synthesis of oligonucleotides containing 2'-deoxyisoguanosine and 2'-deoxy-5-methylisocytidine using phosphoramidite chemistry. *Helv. Chim. Acta* **1998**, *81*, 793-811.

(M. M. P. Ng et al. 1994) M. M. P. Ng, F. Benseler, T. Tuschl, F. Eckstein. Isoguanosine substitution of conserved adenosines in the hammerhead ribozyme. *Biochemistry* **1994**, *33*, 12119-12126.

(S. Pitsch et al. 2001) S. Pitsch, P. A. Weiss, L. Jenny, A. Stutz, X. Wu. Reliable chemical synthesis of oligoribonucleotides (RNA) with 2'-O-[(triisopropylsilyl)oxy]methyl (2'-O-tom)-protected phosphoramidites. *Helv. Chim. Acta* **2001**, *84*, 3773-3795.

(S. Pitsch et al. 2005) S. Pitsch, D. Ackermann, C. Denarie, F. Meylan, M. Meyyappan, E. Muller, A. Peer, S. Porcher, L. Reymond, A. Stutz, P. Wenter, X. Wu. Preparation of modified RNA sequences for biological research. *Chimia* **2005**, *59*, 808-811.

(A. Rich. 1962) A. Rich. In "*Horizons in Biochemistry*"; M. Kasha, B. Pullmann, Eds; Academic Press, New York **1962**, p103-126;

(C. Roberts et al. 1997) C. Roberts, R. Bandaru, C. Switzer. Theoretical and experimental study of isoguanine and isocytosine: base pairing in an expanded genetic system. *J. Am. Chem. Soc.* **1997**, *119*, 4640-4649.

(F. Seela et al. 1997) F. Seela, C. Wei. Oligonucleotides containing consecutive 2'-deoxyisoguanosine residues : synthesis, duplexes with parallel chain orientation, and aggregation. *Helv. Chim. Acta* **1997**, 80, 73-85.

(S. A. Strobel et al. 1994) S. A. Strobel, T. R. Cech, N. Usman, L. Beigelman. The 2,6-diamine purine riboside 5-methylisocytidine wobble base pair: an isoenergetic substitution for the study of G·U pairs in RNA. *Biochemistry* **1994**, *33*, 13224-13835.

(S. A. Strobel et al. 1996) S. A. Strobel, T. R. Cech. Exocyclic amine of the conserved G·U pair at the cleavage site of the *tetrahymena* ribozyme contributes to 5'-splice site selection and transition state stabilization. *Biochemistry* **1996**, *35*, 1201-1211.

(C. Y. Switzer et al. 1993) C. Y. Switzer, S. E. Moroney, S. A. Benner. Enzymatic recognition of the base pair between isocytidine and isoguanosine. *Biochemistry* **1993**, *32*, 10489-10496.

(J. S. Vyle et al. 1998) J. S. Vyle, K. J. Young, J. A. Grasby. Incorporation of methylated analogs into RNA. *Tetrahedron Lett.* **1998**, *39*, 5093-5096.

(L. Wang and P. G. Schultz 2005) L. Wang, P. G. Schultz. Expanding the genetic code *Angew. Chem. Int. Ed.* **2005**, *44*, 34–66.

References of Chapter III

(P. F. Agris 2004) P. F. Agris. Decoding the genome: a modified view. *Nucleic Acids Res.* **2004**, *32*, 223-238.

(S. S. Ashraf et al. 1999) S. S. Ashraf, E. Sochacka, R. Cain, R. Guenther, A. Malkiewicz, P. F. Agris. Single atom modification (O→S) of tRNA confers ribosome binding. *RNA* **1999**, *5*, 188-194.

(S. S. Ashraf et al. 2000) S. S. Ashraf, R. H. Guenther, G. Ansari, A. Malkiewicz, E. Sochacka, P. F. Agris. Role of modified nucleosides of yeast tRNAPhe in ribosomal binding. *Cell Biochem. Biophys.* **2000**, *33*, 241-252.

(H. Bazin et al. 1987) H. Bazin, X.-X. Zhou, C. Glemarec, J. Chattopadhyaya. An efficient synthesis of Y nucleoside (wyosine) by regiospecific methylation of N^4-desmethylwyosine using organozinc reagent. *Tetrahedron Lett.* **1987**, *28*, 3275-3278.

(J. Boryski and T. Ueda 1985) J. Boryski, T. Ueda. A new simple synthesis of 2-methylguanosine and its analogues via derivatives of 4-desmethylwyosine (Nucleosides & Nucleotids. Part 63). *Nucleosides & Nucleotides*, **1985**, *4*, 595-606.

(J. V. Burda et al. 1997) J. V. Burda, J. Šponer, J. Leszczynski, P. Hobza. Interaction of DNA base pairs with various metal cations (Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}, Cu^{+}, Ag^{+}, Au^{+}, Zn^{2+}, Cd^{2+}, and Hg^{2+}): Nonemperical ab initio calculations on structures, energies, and nonadditivity of the interaction. *J. Phys. Chem.* **1997**, *101*, 9670-9677.

(B. A. Carlson et al. 2001) B. A. Carlson, J. F. Mushinski, D. W. Henderson, S.-Y. Kwon, P. F. Crain, B. J. Lee, D. L. Hatfield. 1-Methylguanosine in place of Y base at position 37 in phenylalanine tRNA is responsible for its shiftiness in retroviral ribosomal frameshifting. *Virology* **2001**, *279*, 130-135.

(C. Chaix et al. 1989) C. Chaix, A. M. Duplaa, D. Molko, R. Teoule. Solid phase synthesis of the 5'-half of the initiator t-RNA from *B.subtilis*. *Nucleic Acids Res.* **1989**, *17*, 7381-7393

(H. P. M. de Leeuw et al. 1980) H. P. M. de Leeuw, C. A. G. Haasnoot, C. Altona. Empirical correlations between conformational parameters in β-D-furanoside fragments derived from from a statistical survey of crystal structures of nucleic acid cnstituents. Full description of nucleoside molecular geometries in terms of four parameters. *Isr. J. Chem.* **1980**, *20*, 108-126.

(P. J. Farabaugh 2000) P. J. Farabaugh. Translational frameshifting: Implications for the mechanism of translational frame maintenance. *Prog. Nucleic Acid Res. Mol. Biol.* **2000**, *64*, 131-170.

(C. Glemarec et al. 1988) C. Glemarec, J.-C. Wu, G. Remaud, H. Bazin, M. Oivanen, H. Lönnberg, J. Chattopadhyaya. Structure and reactivity of wyosine (Y-nucleoside) and its derivatives. Chemical, kinetic and spectroscopic studies. *Tetrahedron* **1988**, *44*, 1273-1290.

(C. Goffin et al. 1987) C. Goffin, V. Bailly, W. G. Verly. Nicks 3' or 5'AP sites or to mispaired bases, and one-nucleotide gaps can be sealed by T4 DNA ligase. *Nucleic Acids Res.* **1987**, *15*, 8755-8771.

(B. Golankiewicz and W. Folkman 1983) B. Golankiewicz, W. Folkman. Methylation of desmethyl analogue of Y nucleosides. Wyosine from guanosine. *Nucleic Acids Res.* **1983**, *11*, 5243-5255.

(K. Guckian et al. 2000) K. Guckian, B. A. Schweitzer, R. X.-F. Ren, C. J. Shells, D. C. Tahmassebi, E. T. Kool. Factors contributing to aromatic stacking in water: Evaluation in the context of DNA. *J. Am. Chem. Soc.* **2000**, *122*, 2213-2222.

(Y. Hayakawa et al. 1986) Y. Hayakawa, M. Uchiyama, R. Noyori. Non aqueous oxidation of nucleoside phosphate to the phosphates. *Tetrahedron Lett.* **1986**, *27*, 4191-4194.

(T. Itaya et al. 1988) T. Itaya, M. Shimomichi, M. Ozasa. Access to the synthesis of wybutosine, the first tricyclic fluorescent nucleoside isolated from phenylalanine transfer ribonucleic acids. *Tetrahedron Lett.* **1988**, *29*, 4129-4132

(T. Itaya and T. Harada 1984) T. Itaya, T. Harada. Hydrolysis of nucleosides related to wyosine. *J. Chem. Soc., Chem. Commun.* **1984**, 858-859.

(H. Kasai et al. 1976) H. Kasai, M. Goto, K. Ikeda, M. Zama, Y. Mizuno, S. Takemura, S. Matsuura, T. Sugimoto, T. Goto. Structure of Wye (Yt Base) and Wyosine (Yt) from *Torulopsis utilis* phenylalanine transfer ribonucleic acid. *Biochemistry* **1976**, *15*, 898-904.

(C.-H. Kim, I. Tinoco, Jr. 2000) C.-H. Kim, I. Tinoco, Jr. A retroviral RNA kissing complex containing only two G·C base pairs. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 9396–9401.

(R. K. Kumar, D. R. Davis 1995) R. K. Kumar, D. R. Davis. Synthesis of oligoribonucleotides containing 2-thiouridine. Incorporation of 2-thiouridine phosphoramidite without base protection. *J. Org. Chem.* **1995**, *60*, 7726-7727.

(P. Liu et al. 2004) P. Liu, A. Burdzy, L. C. Sowers. DNA ligase ensure fidelity by interrogating minor groove contacts. *Nucleic Acids Res.* **2004**, *32*, 4503-4511.

(J. March 1992a) J. March. Advanced Organic Chemistry. "Reactions Mechanisms and Structure". Fourth Edition. John Wiley & Sons **1992**. p1206-1238.

(J. Mengel-Jørgensen and F. Kirpekar 2002) J. Mengel-Jørgensen, F. Kirpekar. Detection of pseudouridine and other modifications in tRNA by cyanoethylation and MALDI mass spectrometry. *Nucleic Acids Res.* **2002**, *30*, e135.

(F. Meylan 2006) F. Meylan. Ph.D student 2002-2006

(M. de Oliveira Neto et al. 1998) M. de Oliveira Neto, M. Segre de Giambiagi, M. Giambiagi. Influence of the hypermodified Y base on the A·U pairing in codon-anticodon interaction. *Chemcial Physics Letters*, **1998**, *290*, 205-210.

(S. Pitsch et al. 2001) S. Pitsch, P. A. Weiss, L. Jenny, A. Stutz, X. Wu. Reliable chemical synthesis of oligoribonucleotides (RNA) with 2'-O-[(triisopropylsilyl)oxy]methyl (2'-O-tom)-protected phosphoramidites. *Helv. Chim. Acta* **2001**, *84*, 3773-3795.

(O. Pongs, E. Reinwald 1973) O. Pongs, E. Reinwald. Function of Y in codon-anticodon interaction of $tRNA^{Phe}$. *Biochem. Biophys. Res. Commun.* **1973**, *50*, 357-363.

(F. Seela and K. Kaiser 1987) F. Seela, K. Kaiser. Oligodeoxyribonucleotides containing 1,3-propanediol as nucleoside substitute. *Nucleic Acids Res.* **1987**, *15*, 3113-3129.

(D. Söll and U. RajBhandary 1995) D. Söll and U. RajBhandary, *tRNA: Structure, Biosynthesis and function* **1995**, 207-223, *American Society for Microbiology*.

(M. Sprinzl et al. 1998) M. Sprinzl, C. Horn, M, Brown, A. Loudovitch and S. Steinberg. Compilation of tRNA sequences and sequences of tRNA genes. *Nucleic Acids Research*, **1998**, *26*, 148-153.

(J. W. Stuart et al. 2003) J. W. Stuart, K. M. Koshlap, R. Guenther and P. F. Agris. Naturally-occurring modification restricts the anticodon domain conformational space of tRNAPhe. *J. Mol. Biol.* **2003**, *334*, 901–918.

(H. Sierzputowska-Gracz, et al. 1991) H. Sierzputowska-Gracz, R. H. Guenther, W. Folkman, B. Golankiewicz, P. F. Agris. Structure and conformation of the hypermodified purine nucleoside wyosine and its isomers: a comparison of coupling constants and distance geometry solutions. *Magn. Reson. Chem.* **1991**, *29*, 885-892.

(D. Sung and H. Kang 2003) D. Sung, H. Kang. Prokaryotic and eukaryotic translational machineries respond differently to the frameshifting RNA signal from plant or animal virus. *Virus Res.* **2003**, *92*, 165-170.

(A. Stutz et al. 2000) A. Stutz, C. Höbartner, S. Pitsch. Novel fluoride-labile nucleobase protecting groups for the synthesis of 3'(2')-*O*-aminoacylated RNA sequences. *Helv. Chim. Acta* **2000**, *83*, 2477-2503.

(M. Sundaram et al. 2000) M. Sundaram, P. F. Crain, D. R. Davis. Synthesis and characterization of the native anticodon domain of *E. coli* tRNALys: simultaneous incorporation of modified nucleosides mnm5s2U, t6A, and pseudouridine using phosphoramidite chemistry. *J. Org. Chem.* **2000**, *65,* 5609-5614.

(L. A. Tsoi et al. 1983) L. A. Tsoi, A. D. Salimbaeva, B. D. Abiyurov. Synthesis and structure of 2-aminooxazoline derivatives. *Zh. Org. Khim.* **1983**, *19*, 2605-2609.

(D. H. Turner et al. 1988) D. H. Turner, N. Sugimoto, S. M. Freier. RNA structure prediction. *Ann. Rev. Biophys. Chem.* **1988**, *17*, 167-192.

(I. Ugi and F. Beck 1961) I. Ugi, F. Beck. Solvolysis of carboxylic acid derivatives. Reaction of carboxylic acid chlorides with water and amines. *Chem. Ber.* **1961**, *94*, 1839-1850.

(D. Venkateswarlu et al. 1999) D. Venkateswarlu, K. E. Lind, V. Mohan, M. Manoharan, D. M. Ferguson. Structural properties of DNA:RNA duplexes containing 2'-O-methyl and S-2'-methyl substitutions: a molecular dynamics investigation. *Nucleic Acids Res.* **1999**, *27*, 2189-2195.

(U. vonAhsen et al. 1997) U. vonAhsen, R. Green, R. Schroeder, H. F. Noller. Identification of 2'-hydroxyl groups required for interaction of a tRNA anticodon stem-loop region with the ribosome. *RNA* **1997**, *3*, 49-56.

(A. S. Werneck et al. 1998) A. S. Werneck, M. de O. Neto, E. R. Maia. The hypermodified Y base electrostatic contribution to the energetics of the codon-anticodon pairing in $tRNA^{Phe}$. *J. Mol. Struct.* **1998**, *427*, 15-23.

(C. R. Woese 1990) C. R. Woese, O. Kandler, M. L. Wheelis. Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 4576–4579.

(C. Yarian et al. 2000) C. Yarian, M. Marszalek, E. Sochacka, A. Malkiewicz, R. Guenther, A. Miskiewicz, P. F. Agris. Modified nucleoside dependent Watson-Crick and wobble codon binding by tRNALys UUU species. *Biochemistry* **2000**, *39*, 13390-13395.

(C. Yarian et al. 2002) C. Yarian, H. Townsend, W. Czestkowski, E. Sochacka, A. J. Malkiewicz, R. Guenther, A. Miskiewicz, P. F. Agris. Accurate translation of the genetic code depends on tRNA modified nucleosides. *J. Biol. Chem.* **2002**, *277*, 16391-16395.

(S. Zhou et al. 2004) S. Zhou, D. Sitaramaiah, K. R. Noon, R. Guymon, T. Hashizume, J. A. McCloskey. Structures of two new ''minimalist'' modified nucleosides from archaeal tRNA *Bioorg. Chem.* **2004**, *32*, 82–91.

(K. Ziomek et al. 2002) K. Ziomek, E. Kierzek, E. Biała, R. Kierzek. The influence of various modified nucleotides placed as 3'-dangling end on thermal stability of RNA duplex. *Biophys. Chem.* **2002**, *97*, 243-249.

References of Chapter IV

(P. F. Agris 2004) P. F. Agris. Decoding the genome: a modified view. *Nucleic Acids Res.* **2004**, *32*, 223-238.

(H. T. Allawi and J. SantaLucia Jr. 1998) H. T. Allawi, J. SantaLucia Jr. Nearest-neighbor thermodynamics of internal A·C mismatches in DNA: Sequence dependence and pH effects. *Biochemistry* **1998**, *37*, 9435-9444.

(Y. Andachi et al. 1989) Y. Andachi, F. Yamaho, A. Muto, S. Osawa. Codon recognition patterns as deduced from sequences of the complete set of transfer RNA species in *Mycoplasma capricolum*: resemblance to mitochondria. *J. Mol. Biol.* **1989**, *209*, 37-54.

(J. F. Atkins et al. 1990) J. F. Atkins, R. B. Weiss, R. F. Gesteland. Ribosome gymnastics-Degree of difficulty 9.5, style 10.0. *Cell* **1990**, *62*, 413-423.

(C. J. Barnett and L. M. Grubb 2000) C. J. Barnett, L. M. Grubb. Total synthesis of Q base (queuine). *Tetrahedron* **2000**, 56, 9221-9225.

(H. Beier and M. Grimm 2001) H. Beier, M. Grimm. Misreading of termination codons in eukaryotes by natural nonsense suppressor tRNAs. *Nucleic Acids Res.*, **2001**, *29*, 4767-4782

(S. Benzer and S. P. Champe 1962) S. Benzer, S. P. Champe. A change from nonsense to sense in the genetic code. *Proc. Natl. Acad. Sci. U.S.A.* **1962**, *48*, 114-1121.

(G. R. Björk 1995b) G. R. Björk. Biosynthesis und function of modified nucleosides, p165-205. In D. Söll and U. L. RajBhandary (ed.), tRNA: Structure, Biosynthesis and Function. American Society for Microbiology, 1995, Washington D.C.

(G. R. Björk 1995c) G. R. Björk. Stable RNA modification, p861-886. In F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik,W. S. Reznikoff, M. Riley, M. Schaechter, H. E. Umbarger (ed.). Escherichia coli and Salmonella: Cellular and Molecular Biology, 2nd ed. ASM Press, 1995, Washington D. C.

(G. R. Björk 1998) G. R. Björk. Appendix 6: Modified nucleosides at position 34 and 37 of tRNAs and their predicted coding capacities. In *Modification and editing of RNA* (eds. Grojean H. and Benne R.), **1998** pp. 577-581. ASM Press, Washington, DC.

(A. Björnsson and L. A. Isaksson 1993) A. Björnsson, L. A. Isaksson. UGA codon context which spans three codons. Reversal by ms2i6A37 in tRNA, mutation in rpsD(S4) or streptomycin. *J. Mol. Biol.* **1993**, *232*, 1017–1029.

(F. Bouadloun et al. 1986) F. Bouadloun, T. Srichaiyo, L. A. Isaksson, G. R. Björk. Influence of modification next to the anticodon in tRNA on codon context sensitivity of translational suppression and accuracy. *J. Bacteriol.* **1986**, *166*, 1022–1027.

(Y. Boulard et al. 1995) Y. Boulard, J. A. H. Cognet, J. Gabarro-Arpa, M. Le Bret, C. Carbonnaux, G. V. Fazakerley. Solution structure of an oncogenic DNA duplex, the K-*ras* gene and the sequence containing a central C·A or A·G mismatch as a function of pH: Nuclear Magentic Resonance and molecular dynamics studies. *J. Mol. Biol.* **1995**, *246*, 194-208.

(J. Cabello-Villegas et al. 2002) J. Cabello-Villegas, M. E. Winkler and E. P. Nikonowicz. Solution conformations of unmodified and A37N6-dimethylallyl modified anticodon stem-loops of Escherichia coli $tRNA^{Phe}$. *J. Mol. Biol.* **2002**, *319*, 1015–1034.

(M. Cassan and J.-P. Rousset, 2001) M. Cassan, J.-P. Rousset. UAG readthrough in mammalian cells: Effect of upstream and downstream stop codon contexts reveal different signals. *Mol. Biol.* **2001**, 2-3.

(G. B. Chheda et al. 1972) G. B. Chheda, C. I. Hong, C. F. Piskorz, G. A. Harmon. Biosynthesis of N-(6-purin carbamoyl)-L-threonine riboside. Incorporation of L-threonine in vivo into modified nucleoside of transfer ribonucleic. *Biochem. J.*, **1972**, *127*, 515-519.

(W.-M. Ching 1986) W.-M. Ching. Characterization of selenium-containing tRNAGlu from clostridium sticklandii. *Arch. Biochem. Biophys.* **1986**, *244*, 137-146.

(D. M. Connolly and M. E. Winkler 1989) D. M. Connolly, M. E. Winkler. Genetic and physiological relationships among the miaA gene, 2-methylthio-N6-(D2-isopentenyl)-adenosine tRNA modification, and spontaneous mutagenesis in Escherichia coli K-12. *J. Bacteriol.* **1989**, *171*, 3233–3246.

(F. H. C. Crick 1966) F. H. C. Crick. Codon-anticodon pairing: the wobble hypothesis. *J. Mol. Biol.* **1966**, *19*, 548-555.

(J. F. Curran 1998) J. F. Curran, *Modification and Editing of RNA*, Grosjean, H.; Benne, R., Eds.; ASM Press: Washington, **1998**. pp. 493-516.

(I. Diaz et al. 1987) I. Diaz, S. Pedersen, C. G. Kurland. Effects of miaA on translation and growth rates. *Mol. Gen. Genet.* **1987**, *208*, 373–376.

(I. Diaz and M. Ehrenberg 1991) I. Diaz, M. Ehrenberg. ms2i6A Deficiency enhances proofreading in translation. *J. Mol. Biol.* **1991**, *222*, 1161-1171.

(S. K Dube et al. 1968) S. K Dube, K. A. Marker, B. F. C. Clark, S. Cory. Nucleotide sequence of N-formyl-methionyl-transfer RNA. *Nature*, **1968**, *218*, 232-233.

(P. C. Durant et al. 2005) P. C. Durant, A. C. Bajji, M. Sundaram, R. K. Kumar, D. R. Davis. Structural effects of hypermodified nucleosides in the Escherichia coli and human RNALys anticodon loop: the effect of nucleosides s^2U, mcm^5U, mcm^5s^2U, mnm^5s^2U, t^6A, and ms^2t^6A. *Biochemistry* **2005**, *44*, 8078-8089.

(S. Eiler et al. 1999) S. Eiler, A. Dock-Bregeon, L. Moulinier, J. C. Thierry, D. Moras. Synthesis of aspartyl-tRNA(Asp) in Escherichia coli-a snapshot of the second Stepp. *EMBO J.* **1999**, *18*, 6532-6541.

(J. U. Ericson et al. 1991) J. U. Ericson, G. R. Björk. tRNA anticodons with the modified nucleoside 2-methylthio-N6-(4-hydroxyisopentenyl)adenosine distinguish between bases 30 of the codon. *J. Mol. Biol.* **1991**, *218*, 509–516.

(B. Esberg and G. R. Björk 1995) B. Esberg, G. R. Björk. The methylthio group (ms^2) of N6-(4-hydroxyisopentenyl)-2-methylthioadenosine (ms^2io^6) present next to the anticodon contributes to the decoding efficiency of tRNA. *J. Bacteriol.* **1995**, *177*, 1967-1975.

(Farkas W. R. 1980) W. R. Farkas. Effect of diet on the queuosine family of tRNAs of germ free mice. *J. Biol. Chem.* **1980**, *255*, 6832-6835.

(A. Garen and O. Siddiqi 1962) A. Garen, O. Siddiqi. Suppression of mutations in the alkaline phosphatase structural cistron of E. coli. *Proc. Natl. Acad. Sci. U.S.A.* **1962**, *48*, 1121-1127.

(Goodman H. M. et al. 1968) Goodman H. M., J. Abelson, A. Landy, S. Brenner, J. D. Smith. Amber suppression: A nucleotide change in the anticodon of a tyrosine transfer RNA. *Nature* **1968**, *217*, 1019–1024.

(H. J. Grosjean et al. 1978) H. J. Grosjean, S. de Henau, D. M. Crothers. On the physical basis for ambiguity in genetic coding interactions. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 610-614.

(H. Grosjean et al. 1998) H. Grosjean, C. Houssier, P. Romby, R. Marquet, Modification *and Editing of RNA*, H. Grosjean, R. Benne, Eds.; ASM Press: Washington, **1998**.

(H. Grosjean and G. R. Björk 2004) H. Grosjean, G. R. Björk. Enzymatic conversion of cytidine to lysine in anticodon of bacterial $tRNA^{Ile}$-an alternative way of RNA editing. *Trends Biochem. Sci.* **2004**, *29*, 165-168.

(T. G. Hagervall et al. 1984) T. G. Hagervall, J. U. Ericson, B. Esberg, J.-N. Li, G. R. Björk. Undermodification in the first position of the anticodon of *supG*-tRNA reduces translational efficiency. *Mol. Gen. Genet.* **1984**, *196*, 194-200.

(T. G. Hagervall et al. 1987) T. G. Hagervall, C. G. Edmonds, J. A. McCloskey, G. R. Björk. Transfer RNA (5-methylaminomethyl-2-thiouridine from E.Coli K-12 has two enzymatic activities. *J. Biol. Chem.* **1987**, *262*, 8488-8495.

(T. G. Hagervall et al. 1990) T. G. Hagervall, J. U. Ericson, , B. Esberg, , L. Ji-nong, G. R. Björk. Role of tRNA modification in translational fidelity. *Biochim. Biophys. Acta* **1990**, *1050*, 263–266.

(T. G. Hagervall et al. 1998) T. G. Hagervall, S. C. Pomerantz, J. A. McCloskey. Reduced misreading of asparagine codons by *Escherichia coli* $tRNA^{Lys}$ with hypermodified derivatives of 5-methylaminomethyl-2-thiouridine in the wobble position. *J. Mol. Biol.* **1998**, *284*, 33-42.

(E. Haumont et al. 1987) E. Haumont, L. Droogmans, H. Grosjean. Enzymatic formation of queuosine and galactosyl queuosine in yeast tRNAs microinjected into Xenopus laevis oocytes. *Eur. J. Biochem.* **1987**, *168*, 219–225.

(W. Hillen et al. 1978) W. Hillen, E. Egert, H. J. Lindner, H. G. Gassen. Restriction or amplification of wobble recognition: the structure of 2-thio-5-methylaminomethyluridine and the interaction of odd uridines with the anticodon loop backbone. *FEBS Lett.* **1978**, *94*, 361-364.

(C. Houssier and H. Grosjean 1985) C. Houssier, H. Grosjean. Temperature jump relaxation studies on the interactions between transfer RNAs with complementary anticodons: the effect of modified bases adjacent to the anticodon triplet. *J. Biomol. Struct. Dyn.* **1985**, *3*, 387-408.

(N. Karino et al. 2001) N. Karino, Y. Ueno, A. Matsuda. Synthesis and properties of oligonucleotides containing 5-formyl-2'-deoxycytidine: *in vitro* DNA polymerase reactions on DNA templates containing 5-formyl-2'-deoxycytidine. *Nucleic Acids Res.* **2001**, *29*, 2456-2463.

(H. Kasai et al. 1975) H. Kasai, Y. Kuchino, K. Nihei, S. Nishimura. Distribution of the modified nucleoside Q and its derivatives in animal and plant transfer RNAs. *Nucleic Acids Res.* **1975**, *2***,** 1931–1939.

(H. Kasai et al. 1976) H. Kasai, K. Nakanishi, R. D. MacFarlane, D. F. Torgerson, Z. Ohashi, J. A. McCloskey, J. H. Gross, S. Nishimura. The structure of Q nucleoside isolated from rabbit liver transfer ribonucleic acid. *J. Am. Chem. Soc.* **1976**, *98*, 5044–5046.

(J. R. Katze et al. 1977) J. R. Katze, M. H. Simonian, R. D. Mosteller. Role of methionine in the synthesis of nucleoside Q in Escherichia coli transfer RNA. *J. Bacteriol.* **1977**, *132*, 174–179.

(J. R. Katze et al. 1982) J. R. Katze, B. Basile, J. A. McCloskey. Queuine, a modified base incorporated posttranscriptionally into eukaryotic transfer RNA: Wide distribution in nature. *Science* **1982**, *216*, 55–56.

(G. Kawai et al. 1994) G. Kawai, T. Yokogawa, K. Nishikawa, T. Ueda, T. Hashizume, J. A. McCloskey, S. Yokoyama, K. Watanabe. *Nucleosides Nucleotides* **1994**, *13*, 1189-1199.

(M. Kawakami et al. 1979) M. Kawakami, K. Nishio, S. Takemura, T. Kondo, T. Goto. 5-(Carboxy-hydroxymethyl)uridine, a new modified nucleoside located in the anticodon of tRNA2Gly from posterior silk glands of Bombyx mori. *Nucleic Acids Symp. Series* **1979**, *6*, s53-s55.

(M. Kawakami et al. 1980) M. Kawakami, P. A. Tsonis, K. Nishio, S. Takemura. Abnormal codon recognition of glycyl-tRNA from the posterior silk glands of Bombyx mori. *J. Biochem.* **1980**, *88*, 1151-1157.

(M. Kawakami et al. 1988) M. Kawakami, S. Takemura, T. Kondo, T. Fukami, T. Goto. Chemical structure of a new modified nucleoside located in the anticodon of Bombyx mori glycine tRNA2. *J. Biochem.* **1988**, *104*, 108-111.

(T. Kazuyuki and Y. Shigeyuki 2003) T. Kazuyuki, Y. Shigeyuki. Roles of 5-substituents of tRNA wobble uridines in the recognition of purine-ending codons. *Nucleic Acids Res.* **2003**, *31*, 6383-6391.

(H. Kersten and W. Kersten 1990) H. Kersten, W. Kersten. Biosynthesis and function of queuine and queuosine tRNAs. *J. Chromatogr., B* **1990**, *45B*, B69-B108.

(E. Kierzek and R. Kierzek 2001) E. Kierzek, R. Kierzek. Influence of N6-isopentenyladenosine i^6A on thermal stability of RNA duplexes. *Biophys. Chem.* **2001**, *91*, 135-140.

(E. Kierzek and R. Kierzek 2003) E. Kierzek, R. Kierzek. The thermodynamic stability of RNA duplexes and hairpins containing N^6-alkyladenosines and 2-methylthio-N^6-alkyladenosines. *Nucleic Acids Res.* **2003**, *31*, 4472-4480.

(M. K. Krüger et al. 1998) M. K. Krüger, S. Pedersen, T. G. Hagervall, M. A. Sørensen. The modification of the wobble base of $tRNA^{Glu}$ modulates the translation rate of glutamic acid codons in vivo. *J. Mol. Biol.* **1998**, *284*, 621-631.

(C. J. LaFrancois et al. 1998) C. J. LaFrancois, J. Fujimoto, L. C. Sowers. Synthesis and Characterization of isotopically enriched pyrimidine deoxynucleoside oxidation damage products. *Chem. Res. Toxicol.* **1998**, *11*, 75-83.

(R. Leipuviene et al. 2004) R. Leipuviene, Q. Qian, G. R. Björk. Formation of thiolated nucleosides present in tRNA from *Salmonella enterica* serovar Typhimurium occurs in two principally distinct pathways. *J. Bacteriol.* **2004**, *186*, 758–766.

(J.-N. Li et al. 1997) J.-N. Li, J. F. Curran, G. R. Björk. Three modified nucleosides present in the anticodon stem and loop influence the *in vivo* aa-tRNA selection in a tRNA-dependent manner. *J. Mol. Biol.* **1997**, *271*, 209-221.

(V. I. Lim 1994) V. I. Lim. Analysis of action of wobble nucleoside modifications on codon-anticodon pairing within the ribosome. *J. Mol. Biol.* **1994**, *240*, 8-19.

(C. Marck and H. Grosjean 2002) C. Marck, H. Grosjean. tRNomics: analysis of tRNA genes from 50 genomes of Eukarya, Archaea and bacteria reveals anticodon-sparing strategies and domain-specific features. *RNA* **2002**, *8*, 1189-1232.

(J. Matsugi et al. 1996) J. Matsugi, K. Murao, H. Ishikura. Characterization of a *B. subtilis* minor isoleucine tRNA deduced from tDNA having a methionine anticodon CAT. *J. Biochem.* **1996**, *119*, 811-816.

(J. A. McDowell et al. 1997) J. A. McDowell, L. He, X. Chen, D. H. Turner. Investigation of the structural basis for thermodynamic stabilities of tandem GU wobble pairs: NMR structures of $(rGGAGUUCC)_2$ and $(rGGAUGUCC)_2$. *Biochemistry* **1997**, *36*, 8030-8036.

(H. Mizuno and M. Sundaralingam 1978) H. Mizuno, M. Sundaralingam. Stacking of Crick wobble pair and Watson-Crick pair : stability rules of G-U pairs a tends of helical stems in tRNAs and the relation to codon-anticodon wobble interaction. *Nucleic Acids Res.* **1978**, *5*, 4451-4461.

(M. C. Mok et al. 2000) M. C. Mok, R. C. Martin, D. W. S. Mok. Cytokinins: Biosynthesis, metabolism and perception. *Plant* **2000**, *36*, 102–107.

(J. Moriya et al. 1994) J. Moriya, T. Yokogawa, K. Wakita, T. Ueda, K. Nishikawa, P. F. Crain, T. Hashizume, S. C. Pomerantz, J. A. McCloskey, G. Kawai. A novel modified

nucleoside found at the first position of the anticodon of methionine tRNA from bovine liver mitochondria. *Biochemistry* **1994**, *33*, 2234-2239.

(R. C. Morris et al. 1999) R. C. Morris, K. G. Brown, M. S. Elliott. The effect of queusine on tRNA structure and function. *J. Biomol. Struct. Dyn.* **1999**, *16*, 757-774.

(R. C. Morris and M. S. Eliott 2001) R. C. Morris, M. S. Eliott. Queuosine modification of tRNA: a case for convergent evolution. *Mol. Genet. Metab.* **2001**, *74*, 147-159.

(Y. Motorin et al. 1997): Y. Motorin, G. Bec, R. Tewari, H. Grosjean. Transfer RNA recognition by the *Escherichia coli* Δ^2 isopentenyl-pyrophosphate: tRNA Δ^2 isopentenyl-transferase: dependence on the anticodon arm structure. *RNA* **1997**, *3*, 721-733.

(T. Muramatsu et al. 1988) T. Muramatsu, S. Yokoyama, N. Horiea, A. Matsudal, T. Ueda, Z. Yamaizumi, Y. Kuchino, S. Nishimura, T. Miyazawa. A novel Lysine-substituted nucleoside in the first position of the anticodon of minor Isoleucine tRNA from *Escherichia coli*. *J. Biol. Chem.* **1988**, *263*, 9261-9267.

(F. V. Murphy IV et al. 2004) F. V. Murphy IV, V. Ramakrishnan, A. Malkiewicz, P. F. Agris. The role of modifications in codon discrimination by $tRNA^{Lys}_{UUU}$, *Nat. Struct. Biol.* **2004**, *11*, 1186-1191.

(S. J. Näsvall et al. 2004) S. J. Näsvall, P. Chen, G. R. Björk. The modified wobble nucleoside uridine-5-oxyacetic acid in $tRNA^{Pro}_{cmo}{}^{5}_{UGG}$ promotes reading of all four praline codons in vivo. *RNA* **2004**, *10*, 1662-1673.

(B. Nawrot and A. Malkiewicz 1989) B. Nawrot, A. Malkiewicz. The tRNA "wobble position" uridines. III. The synthesis of 5-[S-methoxycarbonyl(hydroxy)methyl] uridine and its 2-thio analog. *Nucleosides Nucleotides* **1989**, *8*, 1499-1512.

(R. A. Newmark, C. R. Cantor 1968) R. A. Newmark, C. R. Cantor. Nuclear magnetic resonance study of the interactions of guanosine and cytidine in dimethyl sulfoxide. *J. Am. Chem. Soc.* **1968**, *90*, 5010-5017.

(S. Nishimura 1979) S. Nishimura. Modified nucleosides in tRNA, p57-79. In P. Schimmel. P. R. D. Söll, J. N. Abelson (ed.), *Transfer RNA: Structure, Properties and Recognition.* **1979** *Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.*

(S. Nishimura 1983) S. Nishimura. Structure, biosynthesis, and function of queuosine in transfer RNA. *Prog. Nucleic Acids Res. Mol. Biol.* **1983**, *28*, 49-73.

(N. Okada et al. 1977) N. Okada, N. Shindo-Okada, N. Nishimura. Isolation of mammalian tRNA(asp) and tRNA(tyr) by lectin-sepharose affinity chromatography. *Nucleic Acids Res.* **1977**, *4*, 415–423.

(M. de Oliviera Neto et al. 1998) M. de Oliviera Neto, M. S. Gambiagi, M. de Giambiagi. Influence of the hypermodified Y base on the A·U pairing in the codon–anticodon interaction. *Chem. Phys. Lett.* **1998**, 290, 205-210.

(S. J. Oxenford et al. 2004) S. J. Oxenford, P. O'Brien, M. R. Shipton. Chiral base route to functionalised cyclopentenyl amines: formal synthesis of the cyclopentene core of nucleoside Q. *Tetrahedron Lett.* **2004**, *45*, 9053-9055.

(B. Pan et al. 1998) B. Pan, S. N. Mitra, M. Sundaralingam, Structure of a 16-mer duplex r(GCAGACUUAAAUCUGC)$_2$ with wobble C·A$^+$ mismatches. *J. Mol. Biol.* **1998**, *283*, 977-984.

(R. Parthasarathy et al. 1977) R. Parthasarathy, J. M. Ohrt, G. B. Chheda. Modified nucleosides and conformation of anticodon loops: crystal structure of t^6A and g^6A. *Biochemistry*, **1977**, *16*, 4999-5008.

(W. T. Pedersen and J. F. Curran 1991) W. T. Pedersen, J. F. Curran. Effects of the nucleotide 3' to an amber codon on ribosomal selection rates of suppressor tRNA and release factor-1. *J. Mol. Biol.* **1991**, *219*, 231-241.

(H. R. B. Pelham 1978) H. R. B. Pelham. Leaky UAG termination codon in tobacco mosaic virus RNA. *Nature*, **1978**, *272*, 469-471.

(R. Percudani 2001) R. Percudani. Restricted wobble rules for eukaryotic genomes. *Trends Genet.* **2001**, *17*, 133-135.

(B. C. Persson et al. 1998) B. C. Persson, Ó. Ólafsson, H. K. Lungren, L. Hederstedt, G. R. Björk. The ms2io6A37 modification of tRNA in *Salmonella typhimurium* regulates growth on citric acid cycle intermediates. *J. Bacteriol.* **1998**, *180*, 3144-3151.

(L. A. Petrullo et al. 1983) L. A. Petrullo, P. J. Gallagher, D. Elseviers. The role of 2-methylthio-N6-isopentenyladenosine in readthrough and suppression of nonsense codons in Eshcerichia coli. *Mol. Gen. Genet.* **1983**, *190*, 289–294.

(D. W. Phillipson et al. 1987) D. W. Phillipson, C. G. Edmonds, P. F. Crain, D. L. Smith, D. R. Davis, J. A. McCloskey. Isolation and structure elucidation of an epoxide derivative of the hypermodified nucleoside queuosine from Escherichia coli transfer RNA. *J. Biol. Chem.* **1987**, *262*, 3462–3471.

(C. D. Poulter et al. 1976) C. D. Poulter, D. M. Satterwhite, H. C. Rilling. Prenyltransferase: the mechanism of the reaction. *J. Am. Chem. Soc.* **1976**, *98*, 3376-3377.

(C. D. Poulter and D. M. Satterwhite 1977) Mechanism of the prenyl-transfer reaction. Studies with (E)- and (Z)-3-trifluoromethyl-2-buten-1-yl pyrophosphate. *Biochemistry* **1977**, *16*, 5470-5478.

(D. M. Powers, A. Peterkofsky 1972) D. M. Powers, A. Peterkofsky. Biosynthesis and specific labelling of N-(purin-6-ylcarbamoyl)threonine of Escherichia coli transfer RNA. *Biochem. Biophys. Res. Commun.*, **1972**, *46*, 831-838.

(E. Prinsen et al. 1997) E. Prinsen, M.Kamínek, H.A. van Onckelen. Cytokinin biosynthesis: a black box? *Plant Growth Reg.* **1997**, *23*, 3-15.

(G. Remaud et al. 1988) G. Remaud, C. J. Welsh, X.-X. Zhou, J. Chattopadhyaya. An assessment of electronic properties of pyrimidine and purine nucleosides by ^{15}N-NMR spectroscopy. *Nucleosides Nucleotides* **1988**, *7*, 167-179.

(Reyniers J. P. et al. 1981) J. P. Reyniers, J. R. Pleasants, B. S. Wostmann, J. R. Katze, W. R. Farkas, Administration of exogenous queuine is essential for the biosynthesis of the queuosine-containing transfer RNAs in the mouse. *J. Biol. Chem.* **1981**, *256*, 11591-11594.

(J. M. Robertson, W. Wintermeyer 1981) J. M. Robertson, W. Wintermeyer. Effect of translocation on topology and conformation of anticodon and D loops of tRNA^{Phe}. *J. Mol. Biol.* **1981**, *151*, 57-79.

(R. Rossignol et al. 2003) R. Rossignol, B. Faustin, C. Rocher, M. Malgat, J.-P. Mazat, T. Letellier. Mitochondrial threshold effects. *Biochem. J.* **2003**, *370*, 751-762.

(J. Rozenski et al. 1999) J. Rozenski, P. F. Crain, J. A. McCloskey. The RNA modification database: update. *Nucleic Acids Res.* **1999**, *27*, 196-197.

(W. Saenger 1988) W. Saenger. Chapter 5: Physical properties of nucleotides: charge densities, pK values, spectra, and tautomerism. In *Principles of Nucleic Acid Structure* (Series editors Cantor R.), **1988**. pp. 105-115. Springer-Verlag, New York.

(R. S. Schwartz and J. F. Curran 1997) R. S. Schwartz, J. F. Curran. Analyses of frameshifting at UUU-pyrimidine sites. *Nucleic Acids Res.* **1997**, *25*, 2005-2011.

(M. P. Schweizer et al. 1970) M. P. Schweizer, K. McGrath, L. Baczynskyj. Isolation and characterization of N-[(9β-D-ribofuranosyl-9H-purin-6-yl)carbamoyl)]glycine from yeast transfer RNA. *Biochem. Biophys. Res. Commun.*, **1970**, *40*, 1046-1052.

(F. Seela and H. Driller 1989) F. Seela, H. Driller. Alternating d(G-C)$_3$ and d(G-C)$_3$ hexanucleotides contaning 7-deaza-2'-deoxyguanosine or 8-aza-7-deaza-2'-deoxyguanosine in place of dG. *Nucleic Acids Res.* **1989**, *17*, 901-910.

(J. Seidi et al. 1986) [60] J. Seidi, M. Borchert, I. Ziegler, Phorbol ester causes short trm and transient accumulation of pteridines in T cell and in cell lines. *Biochem. Biophys. Res. Commun.* **1986**, *141*, 494-501

(T. Sekiya et al. 1969) T. Sekiya, K. Takeishi, T. Ukita. Specificity of yeast glutamic acid transfer RNA for codon recognition. *Biochim. Biophys. Acta* **1969**, *518*, 530-534.

(V. N. Shibaev et al. 1975) V. N. Shibaev, G. I. Eliseeva, N. K. Kochetkov. Interaction of uridine diphosphate glucose analogs with calf liver uridine diphosphate glucose deshydrogenase. Influence of substituents at C-5 of pyrimidine nucleus. *Biochim. Biophys. Acta* **1975**, *403*, 9-16.

(D. Söll and U. RajBhandary 1995) D. Söll and U. RajBhandary, *tRNA: Structure, Biosynthesis and function* **1995**. pp. 207-223. ASM Press.

(A. Soma et al. 2003) A. Soma, Y. Ikeuchi, S. Kanemasa, K. Kobayashi, N. Ogasawara, T. Ote, J.-I. Kato, K. Watanabe, Y, Sekine, T. Suzuki. An RNA-modifying enzyme that governs

both the codon and amino acid specificities of isoleucine tRNA. *Mol. Cells* **2003**, *12*, 689-698.

(U. B. Sonavane et al. 2002) U. B. Sonavane, K. D. Sonawane, R. Tewari. Conformational preferences of the base substituent in hypermodifed nucleotide queuosine 5'-monophosphate 'pQ' and protonated variant 'pQH+'. *J. Biomol. Struct. Dyn.* **2002**, *20*, 473-485.

(K. D. Sonawane et al. 2002) K. D. Sonawane, U. B. Sonavane, R. Tewari. Conformational preferences of anticodon 3'-eadjacent hypermodified nucleic acid base *cis*-or *trans*-zeatin and its 2-methylthio derivative, *cis*-or *trans*-ms^{2}zeatin. *J. Biomol. Struct. Dyn.* **2002**, *19*(4), 637-648.

(L. C. Sowers et al. 1986) L. C. Sowers, G. V. Fazakerley, H. Kim, L. Dalton, M. F. Goodman. Variation of nonexchangeable proton resonance chemical shifts as a probe of aberrant base pair formation in DNA. *Biochemistry* **1986**, *25*, 3983-3988.

(M. Sprinzl et al. 1998) M. Sprinzl, C. Horn, M, Brown, A. Loudovitch and S. Steinberg. Compilation of tRNA sequences and sequences of tRNA genes. *Nucleic Acids Research*, **1998**, *26*, 148-153. http://www.uni-bayreuth.de/departments/biochemie/trna/

(T. Steiner 2002) T. Steiner. The hydrogen bond in solid state. *Angew. Chem. Int. Ed. Eng.* **2002**, *41*, 48-76.

(L. Stern, and L. H. Schulman 1978) L. Stern, L. H. Schulman. The role of the minor base N^4-acetylcytidine in the function of the *Escherichia coli* non initiator methionine transfer RNA. *J. Biol. Chem.* **1978**, *253*, 6132-6139.

(M. Sundaram et al. 2000) M. Sundaram, P. C. Durant, D. R. Davis. Hypermodified nucleosides in the anticodon of tRNALys stabilize a canonical U-turn structure. *Biochemistry* **2000**, *39*, 12575-12584.

(K. Takai et al. 1999) K. Takai, S. Okumura, K. Hosono, S. Yokoyama, and H. Takaku. A single uridine modification at wobble position of an artificial tRNA enhances wobbling in an *Escherichia coli* cell-free translation system. *FEBS Lett.* **1999**, *447*, 1-4.

(K. Takai and S. Yokoyama 2003) K. Takai, S. Yokoyama. Roles of 5-substituents of tRNA wobble in the recognition of purine-ending codons. *Nucleic Acids Res.* **2003**, *31*, 6383-6391.

(R. C. Thompson and A. M. Karim 1982) R. C. Thompson, A. M. Karim. The accuracy of protein biosynthesis is limited by its speed: High fidelity selection by ribosomes of aa-tRNA ternary complexes containing GTP[γS]. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 4922-4926.

(G. Werner-Felmayer et al. 2002) G. Werner-Felmayer, G. Golderer, E. R. Werner. Tetrahydrobiopterin biosynthesis, utilization and pharmalogical effects. *Curr. Drug Metabol.* **2002**, *3*, 159-173.

(C. Yarian et al. 2002) C. Yarian, H. Townsend, W. Czestkowski, E. Sochacka, A. J. Malkiewicz, R. Guenther, A. Miskiewicz, P. F. Agris. Accurate translation of the genetic code depends on tRNA modified nucleosides. *J. Biol. Chem.* **2002**, *277*, 16391-16395.

(S. Yokoyama et al. 1979) S. Yokoyama, T. Miyazawa, Y. Iitaka, Z. Yamaizumi, H. Kasai, S. Nishimura. Three-dimensional structure of hyper-modified nucleoside Q located in the wobbling position of tRNA. *Nature* **1979**, *282*, 107-109.

(S. Yokohama et al. 1985) S. Yokohama, T. Watanabe, K. Murao, H. Ishikura, Z. Yamaizumi, S. Nishimura, T. Miyazawa. Molecular mechanism of codon recognition by tRNA species with modified uridine in the first position of the anticodon. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 4905-4909.

(S. Yokohama and S. Nishimura 1995) S. Yokohama, S. Nishimura. Chapter 12: Modified nucleosides and codon recognition. In *tRNA: Structure, Biosynthesis and Function.* (eds. Söll D. and RajBhandary U. L.) **1995** pp. 207-224. ASM Press, Washington, DC.

(P. Zikmanis and R. Kruce 1990) P. Zikmanis, R. Kruce. Indolylacetic acid and N6-(Δ2-isopentenyl)adenine affect NADH binding to yeast alcohol deshydrogenase and inhibit in vitro the enzymatic oxidation of ethanol. *Biofactors* **1990**, *2*, 237-240.

References of Chapter V

(J. G. Arnez and T. A. Steitz 1994) J. G. Arnez, T. A. Steitz. Crystal structure of unmodified $tRNA^{Gln}$ complexed with glutaminyl-tRNA synthetase and ATP suggests a possible role for pseudouridines in stabilization of RNA structure. *Biochemistry* **1994**, *33*, 7560–7567.

(D. Ackermann and S. Pitsch 2002) D. Ackermann, S. Pitsch. Synthesis and pairing properties of 3'-deoxyribopyranose (4' → 2')-oligonucleotides ('p-DNA'). *Helv. Chim. Acta* **2002**, *85*, 1443-1462.

(W.-C. Chu, and J. Horowitz 1991) W.-C. Chu, J. Horowitz. Recognition of Escherichia coli valine transfer RNA by its cognate synthetase: a fluorine-19 NMR study. *Biochemistry* **1991**, *30*, 1655–1663.

(C. Denarie 2006) C. Denarie. Ph.D student 2001-2006

(W. B. Derrick, and J. Horowitz 1993) W. B. Derrick, J. Horowitz. Probing structural differences between native and in vitro transcribed Escherichia coli valine transfer RNA: Evidence for stable base modification-dependent conformers. *Nucleic Acids Res*. **1993**, *21*, 4948–4953.

(Y. Fan et al. 2004) Y. Fan, B. L. Gaffney, R. A. Jones. Transient silylation of the guanosine O^6 and amino groups facilitates *N*-acylation. *Organic Lett.* **2004**, *6*, 2555-2557.

(D. Gasparutto et al. 1992) D. Gasparutto, T. Livache, H. Bazin, A.-M. Duplaa, A. Guy, A. Khorlin, D. Molko, A. Roget, R. Téoule. Chemical synthesis of a biologically active natural tRNA with its minor bases. *Nucleic Acids Res.* **1992**, *20*, 5159-5166.

(R. Giege 1990) R. Giegé. Conformation in solution of yeast tRNA(Asp) transcripts deprived of modified nucleotides. *Biochimie* **1990**, *72*, 735–744.

(K. B. Hall et al. 1989) K. B. Hall, J. R. Sampson, O. C. Uhlenbeck, A. G. Redfield. Structure of an unmodified tRNA molecule. *Biochemistry* **1989**, *28*, 5794–5801.

(M. Helm et al. 1998) M. Helm, H. Brulé, F. Degoul, C. Cepanec, J.-P. Leroux, R. Giegé, C. Florentz. The presence of modified nucleotides is required for cloverleaf folding of a human mitochondrial tRNA. *Nucleic Acids Res.* **1998**, *26*, 1636–1643.

(M. Helm et al. 1999) M. Helm, R. Giegé, C. Florentz. A Watson-Crick base-pair-disrupting methyl group (m1A9) is sufficient for cloverleaf folding of human mitochondrial tRNALys. *Biochemistry* **1999**, *38,* 13338-13346.

(R. P. Iyer 2000) R. P. Iyer. Nucleobase protection of deoxyribo- and ribonucleosides. In *Current Protocols in Nucleic Acid Chemistry* / Editorial Board, S. L. Beaucage, D. E. Bergstrom, P. Herdewijn, A. Matsuda, G. D. Glick, R. A. Jones, E. W. Harkins. **2000**, *1*, Unit 2.1.1-2.1.17. John Wiley & Sons, Inc.

(F. Meylan 2006) F. Meylan. Ph.D student 2002-2006

(S. N. Mikhailov et al. 2002) S. N. Mikhailov, J. Rozenski, E. V. Efimtseva, R. Busson, A. Van Aerschot, P. Herdewijn. Chemical incorporation of 1-methyladenosine into oligonucleotides. *Nucleic Acids Res.* **2002**, *30*, 1124–1131.

(T. Muramatsu et al. 1988) T. Muramatsu, K. Nishikawa, F. Nemoto, Y. Kuchino, S. Nishimura, T. Miyazawa, S. Yokoyama. Codon and amino-acid specificities of a transfer RNA are both converted by a single post-transcriptional modification. *Nature* **1988**, *336*, 179–181.

(E. Ohtsuka et al. 1981) E. Ohtsuka, S. Tanaka, T. Tanaka, T. Miyake, A. F. Markham, E. Nakagawa, T. Wakabayashi, Y. Taniyama, S. Nishikawa, R. Fukumoto, H. Uemura, T. Doi, T. Tokunaga. M. Ikehara. Total synthesis of a RNA molecule with sequence identical to that of Escherichia coli formylmethionine tRNA. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 5493-5497.

(T. Ohtsuki et al. 1996) T. Ohtsuki, G. Kawai, Y. I. Watanabe, K. Kita, K. Nishikawa, K. Watanabe. Preparation of biologically active Ascaris suum mitochondrial tRNAMet with a TV-replacement loop by ligation of chemically synthesized RNA fragments. *Nucleic Acids Res*. **1996**, *24*, 662–667.

(V. Perret et al. 1990) V. Perret, A. Garcia, J. D. Puglisi, H. Grosjean, J.-P. Ebel, C. Florentz, R. Giegé. Conformation in solution of yeast tRNA(Asp) transcripts deprived of modified nucleotides. *Biochimie* **1990**, *72*, 735–744.

(J. Putz et al. 1994) J. Putz, C. Florentz, F. Benseler, R. Giegé. A single methyl group prevents the mischarging of a tRNA. *Nat. Struct. Biol.* **1994**, *1*, 580-582.

(J. R. Sampson and O. C. Uhlenbeck 1988) J. R. Sampson, O. C. Uhlenbeck. Biochemical and physical characterization of an unmodified yeast phenylalanine transfer RNA transcribed in vitro. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 1033–1037.

(J. C. Schulhof et al. 1987) J. C. Schulhof, D. Molko, R. Teoule. Facile removal of new base protecting groups useful in oligonucleotide synthesis. *Tetrahedron Lett.* **1987**, *28*, 51-54.

(A. Shaabani et al. 2002) A. Shaabani, A. R. Karimi, A. R. Alamshahi. Selective removal of silyl protecting groups from hydroxyl functions with ammonium chloride in aqueous acetonitrile. *Indian J. Chem., Sect. B* **2002**, *41B*, 1972-1974.

(A. Stutz et al. 2000) A. Stutz, C. Höbartner, S. Pitsch. Novel fluoride-labile nucleobase-protecting groups for the synthesis of 3'(2')-O-amino-acylated RNA sequences. *Helv. Chim. Acta.* **2000**, *83*, 2477-2503.

(L. Wang and P. G. Schultz 2005) L. Wang, P. G. Schultz. Expanding the genetic code *Angew. Chem. Int. Ed.* **2005**, *44*, 34–66.

(Y. Wang 1984) Y. Wang. A total synthesis of yeast alanine transfer RNA. *Acc. Chem. Res.* **1984**, *17*, 393-397.

(P. Wenter et al. 2006) P. Wenter, B. Fürtig, A. Hainard, H. Schwalbe, S. Pitsch. A caged uridine for the selective preparation of an RNA fold and determination of its refolding kineics by real-time NMR. *In press*

(D. Yue et al. 1994) D. Yue, A. Kintanar, J. Horowitz. Nucleoside modifications stabilize Mg2+ binding in Escherichia coli tRNAVal: An imino proton NMR investigation. *Biochemistry* **1994**, *33*, 8905–8911.

(H. Zhao et al. 2003) H. Zhao, Z. Jiang, D. Dou, T. Wu, W. Wang, X. Du, S. Yao. Pulse radiolysis of one-electron oxidation of rare tricyclic nucleoside derivative. *Sci. China, Ser. B* **2003**, *46*, 144-151.

References of Chapter VI

(J. D. Bain et al. 1989) J. D. Bain, E. S. Diala, C. G. Glabe, T. A. Dix, A. R. Chamberlin. Biosynthetic site-specific incorporation of a non-natural amino acid into a polypeptide. *J. Am. Chem. Soc.* **1989**, *111*, 8013-8014.

(J. W. Chin et al. 2002a) J. W. Chin, S. W. Santoro, A. B. Martin, D. S. King, L. Wang, P. G. Schultz. Addition of *p*-azido-L-phenylalanine to the genetic code of *Escherichia coli*. *J. Am. Chem. Soc.* **2002**, *124*, 9026-9027.

(J. W. Chin et al. 2002b) J. W. Chin, A. B. Martin, D. S. King, L. Wang, P. G. Schultz. Addition of a photocrosslinking amino acid to the genetic code of *Escherichia coli*. *Proc. Natl. Acad. Sci U.S.A.* **2002**, *99*, 11020-11024.

(S. Chladek and M. Sprinzl 1985) S. Chladek, M. Sprinzl. The 3' terminus of tRNA and its role in protein biosynthesis. *Angew. Chemie* **1985**, *97*, 377-398.

(P. E. Dawson et al. 1994) P. E. Dawson, T. W. Muir, I. Clark-Lewis, S. B. H. Kent. Synthesis of proteins by native chemical ligation. *Science* **1994**, *266*, 776-779.

(C. Denarie 2006) C. Denarie Ph.D student 2001-2006

(G. Derbesy and D. N. Harpp 1994) G. Derbesy, D. N. Harpp. A simple method to prepare unsymmetrical di- tri- and tetrasulfides. *Tetrahedron Lett.* **1994**, *35*, 5381-5384.

(A. Eleuteri et al. 1996) A. Eleuteri, C. B. Reese, Q. Song. Synthesis of 3',5'-dithiothymidine and related compounds. *J. Chem. Soc., Perkin Trans. 1*, **1996**, 2237-2240.

(T. G. Heckler et al. 1983) T. G. Heckler, Y. Zama, T. Naka, S. M. Hecht. Dipeptide formation with misacylated $tRNA^{Phe}$s. *J. Biol. Chem.* **1983**, *258*, 4492-4495.

(T. G. Heckler et al. 1984) T. G. Heckler, L. H. Chang, Y. Zama, T. Naka, S. M. Hecht. Preparation of 2'(3')-O-acyl-pCpA derivatives as substrates for T4 RNA ligase mediated "chemical aminoacylation". *Tetrahedron* **1984**, *40*, 87-94.

(T. Hohsaka and M. Sisido 2002) T. Hohsaka, M. Sisido. Incorporation of non-natural amino acids into proteins. *Curr. Opin. Chem. Biol.* **2002**, *6*, 809-815.

(D. Kiga et al. 2002) D. Kiga, K. Sakamoto, K. Kodama, T. Kigawa, T. Matsuda, T. Yabuki, M. Shirouzu, Y. Harada, H. Nakayama, K. Takio, Y. Hasegawa, Y. Endo, I. Hirao, S. Yokoyama. An engineered *Escherichia coli* tyrosyl-tRNA synthetase for site specific incorporation of an unnatural amino acid into proteins in eukaryotic translation and its application in a wheat germ cell-free system. *Proc. Natl. Acad. Sci U.S.A.* **2002**, *99*, 9715-9720.

(C. Kohrer et al. 2001) C. Kohrer, L. Xie, S. Kellerer, U. Varshney, U. L. RajBhandary. Import of amber and ochre suppressor tRNAs into mammalian cells : a general approach to site-specific insertion of amino acid analogues into proteins. *Proc. Natl. Acad. Sci U.S.A.* **2001**, *98*, 14310-14315.

(A. K. Kowal et al. 2001) A. K. Kowal, C. Kohrer, U. L. RajBhandary. Twenty-first aminoacyl-tRNA synthetase-suppressor tRNA pairs for possible use in site-specific incorporation of amino acid analogues into proteins in eukaryotes and in eubacteria. *Proc. Natl. Acad. Sci U.S.A.* **2001**, *98*, 2268-2273.

(M. Lodder et al. 1998) M. Lodder, S. Golovine, A. L. Laikhter, V. A. Karginov, S. M. Hecht. Misacylated transfer RNAs having a chemically removable group. *J. Org. Chem.* **1998**, *63*, 794-803.

(J. H. Marriott et al. 1991) J. H. Marriott, M. Mottahedeh, C. B. Reese. Synthesis of 2'-thioadenosine. *Carbohydr. Res.* **1991**, *216*, 257-269.

(K. Ninomiya et al. 2004) K. Ninomiya, T. Minohata, M. Nishimura, M. Sisido. In situ chemical aminoacylation with amino acid thioesters linked to a peptide nucleic acid. *J. Am. Chem. Soc.* **2004**, *126*, 15984-15989.

(T. Mukaiyama and K. Takahashi 1968) T. Mukaiyama, K. Takahashi. A convenient method for the preparation of unsymmetrical disulfides by the use of diethyl azodicarboxylate. *Tetrahedron Lett.* **1968**, *56*, 5907-5908.

(L. E. Overman et al. 1974) L. E. Overman, J. Smoot, J. D. Overman. The reduction of aryl disulfides with triphenylphosphine in water. *Synthesis* **1974**, 59-60.

(S. Pitsch et al. 2001) S. Pitsch, P. A. Weiss, L. Jenny, A. Stutz, X. Wu. Reliable chemcial synthesis of oligonucleotides (RNA) with 2'-*O*-[{triisopropylsilyl}oxy]methyl (2'-*O*-tom)-protected phosphoramidites. *Helv. Chim. Acta* **2001**, *84*, 3773-3795.

(B. H. Rietman et al. 1994) B. H. Rietman, R. F. R. Peters, G. I. Tesser. A facile method for the preparation of S-(alkylsulfenyl)cysteines. *Synth. Commun.* **1994**, *24*, 1323-1332.

(S. A. Robertson et al. 1989) S. A. Robertson, C. J. Noren, S. J. Anthony-Cahill, M. C. Griffith, P. G. Schultz. The use of 5'-phospho-2 deoxyribocytidylylriboadenosine as a facile route to chemical aminoacylation of tRNA. *Nucleic Acids Res.* **1989**, *17*, 9649-9660.

(J. W. Ryan and A. Chung 1981) J. W. Ryan, A. Chung. Antihypertensive agents. Eur. Patent EP 38117 A1 19811021, **1981**.

(M. Sisido and T. Hohsaka 1999) M. Sisido, T. Hohsaka. Extension of protein functions by the incorporation of nonnatural amino acids. *Bull. Chem. Soc. Jpn.* **1999**, *72*, 1409-1425.

(A. Stutz et al. 2000) A. Stutz, C. Höbartner, S. Pitsch. Novel fluoride-labile nucleobase protecting groups for the synthesis of 3'(2')-*O*-aminoacylated RNA sequences. *Helv. Chim. Acta* **2000**, *83*, 2477-2503.

(S. Thea and G. Cevasco 1988) S. Thea, G. Cevasco. A mild and convenient preparation of sulfenyl chlorides from thiolacetates. *Tetrahedron Lett.* **1988**, *29*, 2865-2866.

(T. J. Wallace and A. Schriesheim 1962) T. J. Wallace, A. Schriesheim. Solvent effects in the base-catalyzed oxidation of mercaptans with molecular oxygen. *J. Org. Chem.* **1962**, *27*, 1514-1517.

(L. Wang et al. 2000) L. Wang, T. J. Maglliery, D. R. Liu, P. G. Schultz. A new functional suppressor tRNA/aminoacyl-tRNA synthetase pair for the *in vivo* incorporation of unnatural amino acids into proteins. *J. Am. Chem. Soc.* **2000**, *122*, 5010-5011.

(L. Wang et al. 2001) L. Wang, P. G. Schultz. A general approach for the generation of orhogonal tRNAs. *Chem. Biol.* **2001**, *8*, 883-890.

(L. Wang et al. 2002) L. Wang, Brock A., P. G. Schultz. Adding L-3-(2-naphtyl)alanine to the genetic code of *E.coli*. *J. Am. Chem. Soc.* **2002**, *124*, 1836-1837.

(L. Wang, P. G. Schultz 2002) L. Wang, P. G. Schultz. Expanding the genetic code. *Chem. Commun.* **2002**, *7*, 1-11.

(L. Wang and P. G. Schultz 2005) L. Wang, P. G. Schultz. Expanding the genetic code *Angew. Chem. Int. Ed.* **2005**, *44*, 34–66.

References of Experimental section

(D. Ackermann and S. Pitsch 2002) D. Ackermann, S. Pitsch. Synthesis and pairing properties of 3'-deoxyribopyranose (4'→2')-oligonucloeitdes (‚p-DNA') *Helv. Chim. Acta* **2002**, *85*, 1443-1462.

(A. D. Becke 1988) A. D. Becke. Density-functional exchange-energy approximation with correct asymptotic behaviour. *Phys. Rev. A: At., Mol., Opt. Phys.* **1988**, *38*, 3098-3100.

(A. D. Becke 1993) A. D. Becke. Density-functional thermochemistry. III. The role of exact exchange. *J. Chem. Phys.* **1993**, *98*, 5648-5652.

(V. Boudou et al. 2000) V. Boudou, J. Langridge, A. Van Aerschot, C. Hendrix, A. Millar, P. Weiss, P. Herdewijn. Synthesis of the anticodon hairpin $^{t}RNA_{f}^{Met}$ containing N-{[9-(*β*-D-Ribofuranosyl)-9*H*-purin-6-yl]carbamoyl}-L-threonine (=N^6-{{[(1S,2R)-1-carboxy-2-hydroxypropyl]amino}carbonyl}adenosine, t6A). *Helv. Chim. Acta* **2000**, *83*, 152-161.

(D. Flockerzi et al. 1981) D. Flockerzi, G. Silber, R. Charubala, W. Schlosser, R.S. Varma, F. Creegan, W. Pfeiderer. Synthese and eigenschaften von 2'-O- und 3'-O-(tert-butyldimethylsilyl)-5'-O-(4-methoxytrityl)- sowie 2',3'-bis-O-(tert-butyldimethylsilyl)ribonucleosiden – Ausgangssubstanzen für oligoribonucleotid-Synthesen. *Liebigs Ann. Chem*. **1981**, 1568-1585.

(M. J. Frisch et al. 2003) M. J. Frisch, G. W. Trucks, H. B. Schlegel, G. E. Scuseria, M. A. Robb, J. R. Cheeseman, J. A., Jr. Montgomery, T. Vreven, K. N. Kudin, J. C. Burant, J. M. Millam, S. S. Iyengar, J. Tomasi, V. Barone, B. Mennucci, M. Cossi, G. Scalmani, N. Rega, G. A. Petersson, H. Nakatsuji, M. Hada, M. Ehara, K. Toyota, R. Fukuda, J. Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H. Nakai, M. Klene, X. Li, J. E. Knox, H. P. Hratchian, J. B. Cross, C. Adamo, J. Jaramillo, R. Gomperts, R. E. Stratmann, O. Yazyev, Austin, A. J. R. Cammi, C. Pomelli, J. W. Ochterski, P. Y. Ayala, K. Morokuma, G. A. Voth, P. Salvador, J. J. Dannenberg, V. G. Zakrzewski, S. Dapprich, A. D. Daniels, M. C. Strain, O. Farkas, D. K. Malick, A. D. Rabuck, K. Raghavachari, J. B. Foresman, J. V. Ortiz, Q. Cui, A. G. Baboul, S. Clifford, J. Cioslowski, B. B. Stefanov, G. Liu, A. Liashenko, P. Piskorz, I. Komaromi, R. L. Martin, D. J. Fox, T. Keith, M. A. Al-Laham, C. Y. Peng, A. Nanayakkara, M. Challacombe, P. M. W. Gill, B. Johnson, W. Chen, M. W. Wong, C. Gonzalez, J. A. Pople. *Gaussian 03*, revision B.03; Gaussian, Inc.:
Pittsburgh, PA, 2003.

(D. Gasparutto et al. 1992) D. Gasparutto, T. Livache, H. Bazin, A.-M. Duplaa, A. Guy, A. Khorlin, D. Molko, A. Roget, R. Téoule. Chemcial synthesis of a biologically active natural tRNA with its minor bases. *Nucleic Acids Res.* **1992**, *20*, 5159-5166.

(C. Glemarec et al. 1988) C. Glemarec, J.-C. Wu, G. Remaud, H. Bazin, M. Oivanen, H. Lönnberg, J. Chattopadhyaya. Structure and reactivity of wyosine (Y-nucleoside) and its derivatives. Chemical, kinetic and spectroscopic studies. *Tetrahedron*, **1988**, *44*, 1273-1290.

(J. H. Marriott et al. 1991) J. H. Marriott, M. Mottahedeh, C. B. Reese. Synthesis of 2'-thioadenosine. *Carbohydr. Res.* **1991**, *216*, 257-269.
(S. Pitsch et al. 1999) S. Pitsch, P. A. Weiss, X. Wu, D. Ackermann, T. Honegger. Fast reliable automated synthesis of RNA and partially 2'-O-protected precursors ("caged RNA")

based on two novel, orthogonal 2'-O-protecting groups. *Helv. Chim. Acta* **1999**, *82*, 1753-1761.

(S. Pitsch et al. 2001) S. Pitsch, P. A. Weiss, L. Jenny, A. Stutz, X. Wu. Reliable chemical synthesis of oligoribonucleotides (RNA) with 2'-O-[(triisopropylsilyl)oxy]methyl (2'-O-tom)-protected phosphoramidites. *Helv. Chim. Acta* **2001**, *84*, 3773-3795.

(J. W. Ryan and A. Chung 1981) J. W. Ryan, A. Chung. Antihypertensive agents. Eur. Patent EP 38117 A1 19811021, **1981**.

(F. Seela and K. Kaiser 1987) F. Seela, K. Kaiser. Oligodeoxyribonucleotides containing 1,3-propanediol as nucleoside substitute. *Nucleic Acids Res.* **1987**, *15*, 3113-3129.

(T. Steiner 2002) T. Steiner. The hydrogen bond in solid state. *Angew. Chem. Int. Ed. Eng.* **2002**, *41*, 48-76.

(A. Stutz et al. 2000) A. Stutz, C. Höbartner, S. Pitsch. Novel fluoride-labile nucleobase protecting groups for the synthesis of 3'(2')-*O*-aminoacylated RNA sequences. *Helv. Chim. Acta* **2000**, *83*, 2477-2503.

(M. Sundaram et al. 2000) M. Sundaram, P.C. Durant, D.R. Davis. Hypermodified nuclosides in the anticodon of tRNALys stabilize a canonical U-turn. *Biochemistry* **2000**, *39*, 12575-12584.

(S. Thea and G. Cevasco 1988) S. Thea, G. Cevasco. A mild and convenient preparation of sulfenyl chlorides from thiolacetates. *Tetrahedron Lett.* **1988**, *29*, 2865-2866.

(J. S. Vyle et al. 1998) J. S. Vyle, K. J. Young, J. A. Grasby. Incorporation of methylated analogs into RNA. *Tetrahedron Lett.* **1998**, *39*, 5093-5096.

(P. Wenter and S. Pitsch 2003) P. Wenter, S. Pitsch. Synthesis of selectively ^{15}N-labeled 2'-O-{[(triisopropylsilyl)oxy]methyl}(=tom)-protected ribonucleoside phosphoramidites and their incorporation into a bistable 32mer RNA sequence. *Helv. Chim. Acta* **2003**, *86*, 3955-3974.

LIST OF PUBLICATIONS

S. Porcher, S. Pitsch. Synthesis of 2'-*O*-[(triisopropylsilyl)oxy]methyl (=tom)-protected ribonucleoside phosphoramidites containing various nucleobase analogues. *Helv. Chim. Acta* **2005**, *88*, 2683-2704.

S. Porcher, M. Meyyappan, S. Pitsch. Spontaneous aminoacylation of a RNA sequence containing a 3'-terminal 2'-thioadenosine. *Helv. Chim. Acta* **2005**, *88*, 2897-2909.

S. Pitsch, D. Ackermann, C. Denarie, F. Meylan, M. Meyyappan, E. Muller, A. Peer, S. Porcher, L. Reymond, A. Stutz, P. Wenter, X. Wu. Preparation of modified RNA sequences for biological research. *Chimia* **2005**, *59*, 808-811.

PORCHER Sébastien
Chemin de la Colline 11
1007 LAUSANNE, SUISSE
Tél. : 0041 21 624 75 02
Tél. Prof .: 0041 21 693 94 46
Tèl. France : 02 54 22 46 06
Ou 06 07 34 72 96

e-mail : sebastien.porcher@epfl.ch

30 ans, Célibataire
Permis de conduire

INGENIEUR CHIMISTE ENSC Mulhouse

➢ EXPERIENCES PROFESSIONNELLES

EPFL (Ecole Polytechnique Fédérale Lausanne, Suisse) Doctorat chimie de l'ARN

(4 ans) **2001-2005** "Développement d'analogues de tRNA"
Responsable : Pr. S. Pitsch

Développement du sujet: *Utilisation des techniques courantes de synthèse et purification (RMN, HPLC, MS (MALDI, ESI)) pour la préparation d'oligonucléotides.*

- *rationalisation et systématisation des méthodes existantes*
- *élaboration de chemins de synthèse innovants : réduction du nombre d'étapes*
- *incorporation de bases modifiées sensibles aux agents de déprotection : la Wyosine*

Mise en place du laboratoire: *(créé en 2001)*

- *définition des places de travail*
- *planification des besoins et création des aménagements*
- *entretien avec les fournisseurs d'équipements*
- *démarchage auprès des services de l'EPFL pour leur réalisation*

Charge d'assistanat :

- *encadrement des étudiants lors de travaux pratiques (police scientifique)*
- *fonction de tuteur d'apprentissage*

Responsable sécurité :

- *interlocuteur auprès des autorités de tutelle : mise en conformité du laboratoire. Quatre inspections ont rendu un avis favorable prévenant ainsi une éventuelle fermeture de laboratoire.*
- *compte –rendu et information auprès des collaborateurs*
- *estimation des risques, recherche de solutions et mise en place des actions correctrices*

ROCHE (Industrie pharmaceutique: Basel, Suisse) Stagiaire diplômant en recherche

(8 mois) **2000** "Synthèse de composé d'intérêt pharmaceutique"
Responsable : Dr. M. Boehringer
Synthèse d'un composé de référence (LEAD) en vue de l'amélioration de qualités biologiques d'autres molécules actives développées par la compagnie
Le produit a été obtenu dans les délais et les quantités exigées

BRANCHER (Encres d'imprimerie: Vélizy, France) Stagiaire R&D formulation

(2 mois) **1999** "Investigation de photoinitiateurs pour encres UV"
Responsable : Ing. F. Monquin
La rapidité de séchage des encres séchant sous rayonnement UV permet d'augmenter la cadence d'impression. Pour répondre au marché concurrentiel, j'ai été chargé de créer des formulations originales d'encres, en vue d'améliorer leur séchage tout en évaluant leurs qualités (séchage, odeur, solidité lumière, tenue à la rayure), permettant ainsi un gain d'environ 10% de la rapidité de séchage. Les fournisseurs de réactifs ont aussi été évalués.

LAB (Contrôle Antidopage Equin: Chatenay-M., France) Stagiaire responsable analytique

(5 mois) **1998** "Recherche de dopant dans les poils de chevaux"
Responsable : Dr. M. Popot
Mise au point de méthodes de détection pour un stéroïde spécifique. Remplacement du responsable analytique durant ses congés pour l'identification des positifs et rédaction partielle du rapport d'analyse pour le tribunal.

ICMO (Institut de Chimie Moléculaire d'Orsay, France) Technicien stagiaire

(2 mois) **1997** " Synthèse de composé chimique (ligand chiral)"
Responsable : Pr. C. Fiaud
Soumis à brevet pour Rhône-Poulenc.

CNRS (Laboratoire d'Analyse Spectroscopique Infrarouge et Raman de Vitry-Thiais, France) Technicien stagiaire
Responsable : Dr. M. Pasquier
(3 mois) **1996** "Etude spectrophotométrique RAMAN de transition de phase dans des cristaux biréfringents"

➢ COMPETENCES

- **Formation ENSCMu** : Synthèse organique, chimie des polymères, formulation, génie des procédés
- Maîtrise des **méthodes de purification** (utilisation, entretien, maintenance) et utilisation des appareils courants d'analyse spectrométrique: IR, RMN, HPLC, MS (MALDI, ESI), RAMAN.
- Synthèse, purification, analyse **d'oligonucléotides classiques**: ARN/ADN, et **modifiés** de l'ARN de transfert.
- **Encadrement d'étudiants, et tutorat d'apprentissage**
- **Responsable sécurité** au sein du groupe de recherche

➢ FORMATION CONTINUE

- **Management de l'Innovation Technologique :** Cours MINT EPFL **2004**
- **Technique du vide :** Séminaire Vacuubrand **2003**

➢ AUTRES EXPERIENCES

- **Professeur à domicile** : Mathématiques Physique et Chimie (Legendre (Paris)) **2000**
- Obligations militaires dans l'infanterie (10 mois) **2000-2001**
- Emplois de **cariste** et **vendeur**. Grossiste alimentaire METRO S.A. **1995-1997**
- **Garde-malade** **1994-1999**

➢ ETUDES

Diplôme d'Etudes Approfondies (Société Roche Basel Suisse) avec mention **2000**
Ingénieur chimiste (Ecole Nationale Supérieure de Chimie de Mulhouse ENSCMu) **2000**
Maîtrise de chimie organique (Université d'Orsay Paris XI) **1998**
Diplôme de Technicien Chimiste (Institut Universitaire de Technologie d'Orsay) **1996**
Baccalauréat : Lycée EPIN (Vitry sur Seine) : Mention bien (14/20) **1994**

➢ LANGUES

- Français : langue maternelle
- Anglais : lu, parlé, écrit
- Allemand : lu, parlé, écrit (optionnel au baccalauréat : 16/20)

➢ COLLOQUES ET COMMUNICATIONS

- Intitulé : *Synthesis of phosphoramidite building block of a Wyosine analogue and its incorporation into RNA sequences*: Présentation orale :
  -Champéry Septembre 2003, Séminaire Hors-ville
  -Session de Posters : 8th Annual Meeting of the RNA Society July 2003 Vienna, Austria
- Publications: S. Porcher, S. Pitsch. *Helv. Chimica Acta.* **2005**, *88*, 2683
  S. Porcher, M. Meyyappan, S. Pitsch. *Helv. Chimica Acta.* **2005**, *88*, 2897
- Brevet: Examen en cours

➢ LOISIRS

Photographie noir et blanc, dessin : illustration du T-shirt de l'ENSCMu (Ecole de chimie) pour le Rallye TIC 1999 (manifestation sportive nationale), lecture, théâtre (Lycée : figurant et acteur dans trois pièces, Ecole de chimie (ENSCMu) : responsable éclairage).