Synthesis of DNA-encoded disulfide- and thioether-cyclized peptides

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KEYWORDS

DNA-encoded libraries, cyclic peptide, cysteine, thiol protection group, disulfide, thioether

ABSTRACT

DNA-encoded chemical library technologies enable the screening of large combinatorial libraries of chemically and structurally diverse molecules, including short cyclic peptides. A challenge in the combinatorial synthesis of cyclic peptides is the final step, the cyclization of linear peptides that typically suffers from incomplete reactions and a large variability between substrates. Several efficient peptide cyclization strategies rely on the modification of thiol groups, such as the formation of disulfide or thioether bonds between cysteines. In this work, we have established a strategy and reaction conditions for the efficient chemical synthesis of cyclic peptide-DNA conjugates based on linking the side chains of cysteines. We tested two different thiol-protecting groups and found that *tert*-butylthio (*S*-*t*Bu) works best for incorporating a pair of cysteines, and we show that the DNA-linked peptides can efficiently be cyclized through disulfide- and thioether bond formation. In combination with established procedures for DNA encoding, the strategy for incorporation of cysteines may readily be applied for the generation and screening of disulfide- and thioether-cyclized peptide libraries.

INTRODUCTION

DNA-encoded chemical libraries (DECLs) have emerged as a powerful tool for hit identification in both, the pharmaceutical industry and academia.[1–3] In these libraries, each component is linked to a piece of DNA that serves as an amplifiable genetic barcode. Using combinatorial synthesis principles, libraries containing millions to billions of DNA-encoded compounds can be produced. The resulting libraries are screened in a single test tube against proteins of pharmaceutical interest, and hits can be identified by PCR amplification of the DNA barcodes and subsequent high-throughput sequencing. Ligands with good affinities, isolated from DECLs, were reported from both academic laboratories and pharmaceutical companies to a wide range of targets.[3]

An interesting application of DECLs is the development of macrocyclic compounds with a molecular weight below 1 kDa, that can potentially be transformed into orally available and/or cell permeable ligands. The circular structure of macrocycles reduces the conformational flexibility that allows binding to "challenging" targets to which no potent small molecule ligands could be identified.[4] Several macrocycles isolated from natural sources have shown excellent therapeutic activity which demonstrate the enormous potential of macrocyclic compounds.[5] Given that nature offers macrocyclic ligands to only a fraction of the currently identified therapeutic targets, large combinatorial libraries of synthetic macrocycles may offer an attractive new source of such ligands, and DNA-encoding technology could provide an efficient way to screen these synthetic libraries. First successes have been achieved in this direction in recent years, such as Liu and co-workers who have applied DNA-templated chemistry to generate combinatorial libraries of cyclic peptides,[6,7] Neri and co-workers who have generated a DNA-encoded macrocycle library by decorating a DNA-linked macrocyclic scaffold with chemical elements,[8] and teams at GlaxoSmithKline as well as the group of Gillingham who have independently generated DNA-encoded macrocycles by split-and-pool peptide synthesis and subsequent macrocyclization.[9–11]

The most difficult step in the synthesis of combinatorial libraries of cyclic peptides is the final step – the macrocyclization.[12] The connection of the two ends of a linear molecule reduces its conformational flexibility, which is energetically unfavorable. For short peptides, there may also be a steric hindrance involved impairing the cyclization reaction. For the generation of DNA-encoded cyclic peptides, Liu and co-workers developed a strategy based on the Wittig cyclization.[6] The group of Neri had elegantly bypassed the need for macrocyclization by conjugating a pre-cyclized macrocyclic scaffold to DNA.[8] The teams at GlaxoSmithKline had cyclized the DNA-encoded peptides by ruthenium-catalyzed ring-closing metathesis[9] or copper-catalyzed azide-alkyne cycloaddition,[10] and the group of Gillingham had cyclized the DNA-encoded peptides by amide bond formation.[11]

Many efficient peptide cyclization strategies are based on cysteines. A pair of cysteines can be most easily connected through disulfide bond formation using oxidizing agents or even spontaneously. Thiol groups of two or three cysteines in peptides can also be efficiently ligated by reagents that contain an equal number of thiol-reactive groups. A wide range of such reagents are applied to cyclize peptides with yields of more than 90% and high selectivity, for example several different halogeno-derivatives, vinylsulfone or perfluoroaromatic reagents efficiently cyclize peptides via the side chains of cysteines.[13–17] Some of these cyclization reagents, such as 1,3,5-tris(bromomethyl)benzene are routinely used to generate bicyclic peptides by phage display.[18] Alternatively, a reaction based on the linkage of one cysteine to chloroacetamide is used for the *in vitro* evolution of cyclic peptides by mRNA display.[19,20]

In this work, we have established a strategy and reaction conditions for the efficient chemical synthesis of DNA-encoded peptides cyclized by a disulfide or thioether bridge. The main challenges were i) the incorporation of two cysteine residues into DNA-linked peptides and ii) the cyclization of the peptide moiety in presence of the DNA. The incorporation of cysteines was not trivial because the removal of the thiol protecting groups required conditions that do not modify DNA, which excluded many reagents that are common in peptides synthesis. The peptide cyclization was challenging because the bis-electrophile reagents applied for linking two thiol groups could potentially react with nucleophilic groups of the DNA bases.

RESULTS AND DISCUSSION

Strategy for synthesizing peptide-DNA conjugates containing two cysteines

Several efficient strategies for the synthesis of DNA-encoded peptides are used.[21–23] Most protocols rely on Fmoc-protected amino acids and the water soluble coupling reagent 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM). A challenge in the synthesis of peptide-DNA conjugates with cysteines was the removal of the cysteine side chain protecting groups. Cysteine building blocks commonly used in solid-phase peptide synthesis (SPPS), such as Fmoc-Cys(Trt)-OH, Fmoc-Cys(4-methoxytrityl)-OH, and Fmoc-Cys(Acm)-OH require harsh conditions such as strong acids or oxidizing agents for the recovery of the free thiol group, that would damage the DNA. Cysteine building blocks with alternative thiol protecting groups labile under mild conditions have been developed for orthogonal cysteine sidechain protection, allowing the synthesis of peptides containing multiple disulfide bonds with correct disulfide connectivity.[24] We chose to test two such Fmoc-cysteine building blocks, the Fmoc-Cys(S-TMP)-OH that is protected at the thiol group with trimethoxyphenylthio (S-TMP), and Fmoc-Cys(*S*-*t*Bu)-OH that is protected with *tert*-butylthio (*S*-*t*Bu). The free thiol groups are recovered by reducing the disulfide bridges using reagents such as thiols or phosphines that are compatible with reactions in presence of DNA. The amino acid Fmoc-Cys(S-TMP)-OH has been developed to allow particularly fast and efficient removal of the thiol protecting group by reduction,[25] and has successfully been applied for the synthesis of a range of peptides containing multiple disulfide bridges. The Fmoc-Cys(*S*-*t*Bu)-OH is broadly used in solid-phase peptide synthesis and has previously been applied to generate cysteine-DNA conjugates in order to subsequently link DNA to proteins by expressed protein ligation.[26,27] While the *S*-*t*Bu protecting group could efficiently be removed from the cysteine-DNA conjugates, it has been reported in other studies that elimination of S-*t*Bu can require long reaction times of several hours[25,28,29]. In a study by Halpin and co-workers, Fmoc-Cys(*S*-*t*Bu)-OH was used to synthesize peptide-DNA conjugates on solid phase and they had reported problems to recover free thiol-containing peptide.[30] Given the challenges reported in removing the *S*-*t*Bu protecting group, we tested first the Fmoc-Cys(S-TMP)-OH reagent.

Application of trimethoxyphenylthio (S-TMP) as thiol protecting group

We conjugated Fmoc-Cys(*S*-TMP)-OH to the single stranded example DNA 5'-GCCTCAGCCAGGATAG-3' via an amino group that was linked to the 5' end via a hexyl tether (Figure 1a). For this and subsequent peptide bond formation reactions, amino-DNA conjugate was incubated at a final concentration of 714 μM with an 80-fold molar excess of amino acid and DMT-MM in 285 mM borate buffer, pH 9.4 for one hour at 30°C. A white, small precipitate was observed which was presumed to be the activated ester. HPLC analysis of the reaction showed a good yield of 84% (Figure 1a). A small peak eluting before the desired product was identified as dehydroalanine-DNA (6%) that was generated by elimination of the *S*-TMP protected thiol group (Figure S1). The desired Fmoc-Cys(*S*-TMP)-DNA product was purified by RP-HPLC.

In a next step, we incubated the Fmoc-Cys(*S*-TMP)-DNA conjugate with 10% piperidine in an aqueous solution and monitored the reaction by HPLC analysis after 10, 20, 30 minutes and 12 hours. The Fmoc protecting group was completely removed in 20 minutes at room temperature, affording the desired product in 85% yield but two side products were also observed (Figure 1b). Longer reaction times favored the formation of these side products; the yield of desired product was only 31% after 12 hours. The molecular mass of the major side product was of 5206 Da as determined by MALDI mass spectrometry but we could not determine the structure of this product. The side products were likely originating from a reaction of the *S*-TMP-protected cysteine side chain, as they were not found in a conjugation reaction of Fmoc-Gly-OH with the same DNA. For this reason, the shorter reaction time of 20 minutes was chosen, and the desired product was subsequently purified by RP-HPLC before conjugating a glycine residue to the cysteine. Fmoc removal at the N-terminus of Fmoc-Gly-Cys(*S*-TMP)-DNA in aqueous piperidine again yielded two side products that eluted earlier on the HPLC than the desired product (Figures 1c and S2), indicating that the *S*-TMP-protected thiol groups in cysteine-DNA conjugates are not entirely stable during the Fmoc removal with piperidine. Cleavage with smaller concentrations of piperidine reduced the side products but did not fully remove the Fmoc group (data not shown). While Fmoc-Cys(*S*-TMP)-OH is suited for solid-phase peptide synthesis, we concluded that it was not an ideal building block for the synthesis of cysteine-rich DNA-conjugated peptides.

Application of tert-butylthio (S-tBu) as thiol protecting group

As indicated above, the Fmoc-Cys(S-*t*Bu)-OH amino acid has previously been conjugated to DNA in order to subsequently link the DNA to proteins by expressed protein ligation,[26,27] and to synthesize peptide-DNA conjugates on solid phase.[30] The bulky *tert*-butyl group of *S*-*t*Bu renders this protecting group more stable under basic conditions such as during Fmoc removal with piperidine. At the same time, we expected that the reductive cleavage of Cys(*S*-*t*Bu) would be more difficult as the removal of S-*t*Bu was reported to require reaction times of several hours.[25,28,29]

We conjugated Fmoc-Cys(*S*-*t*Bu)-OH to DNA using the same coupling agent, DMT-MM, and conditions as above, which yielded 78% product, as analyzed by HPLC (Figure 2a). We were delighted to find that the Fmoc protecting group was quantitatively removed by incubation in 10% aqueous piperidine for 20 minutes at room temperature (Figure 2a) and the resulting Cys(*S*-*t*Bu)-DNA product was isolated by simple centrifugal concentration under vacuum at 30°C. Even upon incubation for up to 12 hours, the formation of side product was detected in only trace amounts (3%). Given the efficient synthesis requiring HPLC purification only after the amino acid coupling reaction, the conjugate Fmoc-Cys(*S*-*t*Bu)-Gly-Cys(*S*-*t*Bu)-DNA could be synthesized on a single day (Figure 2b).

The free thiol group of Cys(*S*-*t*Bu) is typically accessed by exposition to a base such as *N*-methylmorpholine and reducing agents such as dithiothreitol (DTT) or β-mercaptoethanol (βME). The reaction is normally performed at a high temperature and for an extended time period. We hypothesized that treatment of the peptide-DNA conjugate with piperidine and a reducing agent should allow the removal of both, the Fmoc and *S*-*t*Bu protecting groups, in a single step. Incubation of Fmoc-Cys(*S*-*t*Bu)-Gly-Cys(S-*t*Bu)-DNA in an aqueous solution containing 10% (v/v) piperidine and 5% (v/v) DTT efficiently led to the free amino-dithiol product after one hour (Figure 3). HPLC analysis also showed that most of the peptide was oxidized to the disulfide-cyclized peptide (Figure 3). The amino-dithiol peptide-DNA conjugate was subsequently purified by RP-HPLC. Incubation of the purified fraction overnight at room temperature resulted, without addition of an oxidizing agent, in conversion to the cyclic product. While Gauthier and co-workers have found that peptides of the format Cys-Xaa-Cys form preferentially disulfide-linked dimers,[31] the absence of a side chain in the middle amino acid (glycine) has likely led to the formation of disulfide-cyclized monomer in our study.

Cyclization of DNA-encoded peptides with chemical linkers

Next, we established the cyclization of the Cys-Gly-Cys-DNA conjugate using functionally diverse linkers. As described in the introduction, a wide range of reagents containing two thiol-reactive electrophiles, can efficiently cyclize peptides that are terminally flanked with cysteines. Previous work with phage display peptide libraries has shown that peptides with cysteines spaced by only one amino acid (Cys-Xaa-Cys) can efficiently be cyclized with bis-electrophile reagents.[32] A potential challenge was that such reagents could theoretically also react with the DNA, in particular when the reagents had reacted with a first thiol group and the reaction with DNA would happen intramolecularly. In a pilot experiment, we tested two reagents, 1,3-bis(bromomethyl)benzene (DBMB) and divinylsulfone (DVS) (Figure 4). After complete reduction of the disulfide bridges of the oxidized Cys-Gly-Cys-DNA with tris(2-carboxyethyl)phosphine (TCEP), the peptide-DNA conjugate was incubated at a concentration of 100 μM with a 16-fold molar excess of DBMB or DVS. HPLC and mass spectrometric analysis showed that both reagents efficiently cyclized the peptide, DBMB with an 88% yield and DVS with a 73% yield. No reaction with other nucleophiles, such as the bases of the DNA, was detected.

For the generation of structurally diverse macrocycle libraries, it is important to vary the backbone of the macrocycles, in addition to varying the amino acid side chains. An efficient strategy for generating a large skeletal diversity is to cyclize the peptides with many different bis-electrophile reagents. We therefore tested three additional reagents that we had previously used for cyclizing phage-encoded peptide libraries. The reagents 1,3-bis(bromomethyl)pyridine, 1,4-bis(bromomethyl)benzene and bis(vinylsulfonyl)methane

cyclized the Cys-Gly-Cys-DNA conjugate with yields ranging from 76 to 81% and thus as efficiently as the first two reagents DBMB and DVS (Figure 4).

Synthesis of DNA-encoded peptides with variable sequences and cyclization of a peptide mixture

We further tested if the cyclic peptide-DNA synthesis strategy also works efficiently with different peptide sequences, which was important in view of the planned application of the synthesis strategy to combinatorial libraries (Figure 5a). We synthesized one peptide containing a proline between the cysteines (Cys-Pro-Cys-DNA) and one that contained a β-amino acid (Cys-βAla-Cys-DNA). The two amino acids were expected to position the two cysteine side chains differently relative to each other, which could potentially influence the cyclization reaction. An additional peptide sequence contained two amino acids between the cysteines instead of only one (Cys-Phe-Gly-Cys-DNA). All three peptides could be synthesized and the yields for the individual amino acid coupling reactions were comparable to those in the synthesis of the Cys-Gly-Cys-DNA conjugate. The *S*-*t*Bu protecting groups could be removed efficiently and the reaction appeared not to be sequence dependent. The reaction with DBMB yielded the desired cyclic products, wherein the yields were even better than for the cyclization of Cys-Gly-Cys-DNA with DBMB (> 90%). The efficient cyclization observed for different peptide-DNA conjugates is in line with our previous observation that the cyclization efficiency of phage-displayed cysteine-rich peptides is to a large extent independent of the amino acid sequences.

Finally, we tested if the peptide-DNA conjugates can be cyclized as a mixture, which mimicked the condition used for split-and-pool library synthesis. We mixed the four conjugates Cys-Gly-Cys-DNA (A), Cys-Pro-Cys-DNA (B), Cys-βAla-Cys-DNA (C), and Cys-Phe-Gly-Cys-DNA (D) at identical ratios and reacted the mixture with the bis-electrophile reagent DBMB. All four cyclic peptide-DNA products were obtained efficiently as followed by RP-HPLC and mass spectrometric analysis (Figure.5b).

CONCLUSIONS

We have established an efficient approach for the synthesis of disulfide- and thioether cyclized DNA-encoded peptides. Key steps in this work were the identification of a suitable Fmoc cysteine building block for the synthesis of linear peptide-DNA conjugates, and the cyclization of the peptides in presence of the DNA. We found that Fmoc-Cys(S-*t*Bu)-OH is suited for the rapid synthesis of peptide-DNA conjugates containing two cysteines, but not Fmoc-Cys(S-TMP)-OH. Specifically, we found that the amino acid is stable throughout peptide bond formation, Fmoc removal, and HPLC purification, and that the S-*t*Bu protecting group can be removed efficiently. We furthermore show that a DNA-linked peptide can efficiently be cyclized through disulfide bond formation or reaction with symmetrical bis electrophiles forming thioether bonds via nucleophilic substitutions and 1,4-addition processes. Importantly, no reactions with other nucleophiles such as DNA bases, were detected. The efficient and clean incorporation of cysteine into DNA-encoded peptides and the quantitative peptide macrocyclization reaction are an optimal basis for the synthesis of large combinatorial libraries of encoded cyclic peptides. The protocols for the synthesis of DNA-encoded libraries, and specifically, the DNA-encoding procedures, are well established. In combination with the herein presented synthesis strategy, they may be readily applied for the synthesis and screening of large combinatorial libraries of disulfide or thioether cyclized peptides.

EXPERIMENTAL PROCEDURES

Representative procedure for on-DNA amide bond formation

To 1 μL of DNA oligomer (10 nmol, to achieve a final concentration of 714 μM) 5 μL of borate buffer pH 9.4 was added (800 mM, to achieve a final concentration of 285 mM), 4 μL of Fmoc-Cys(*S*-*t*Bu)-OH (200 mM in DMF, 800 nmol, to achieve a final concentration of 57 mM) and 4 μL of DMT-MM (200 mM in water, 800 nmol, to achieve a final concentration of 57 mM). The reaction was kept at 30°C for 1 hour. The white precipitate formed during the reaction was removed and the DNA-conjugate was isolated by NH_4OAc precipitation. The solid residue was dissolved in water and purified by RP-HPLC.

Fmoc removal of peptide-DNA conjugates

To an aqueous solution of 500 μM DNA-conjugate was added 0.1 volume of piperidine. The mixture was kept at room temperature for 30 min. Volatile residues were removed by centrifugation under vacuum (250 g, 30°C, 30 minutes), delivering the desired DNA-conjugate without further purification.

Full cleavage of Fmoc-Cys(S-tBu)-Gly-Cys(S-tBu)-DNA and disulfide cyclization

To a solution of Fmoc-Cys(*S*-*t*Bu)-Gly-Cys(*S*-*t*Bu)-DNA (11.8 nmol) in 9.4 μL of water, aqueous DTT (10% v/v in water, 11.8 μL) and piperidine (2.4 μL) was added. After one hour at room temperature, the reaction mixture was purified by RP-HPLC. The collected fractions were kept at room temperature overnight. Volatile residues were removed by centrifugation under vacuum, delivering the DNA-conjugated disulfide peptide, cyclo(Cys-Gly-Cys)-DNA.

Macrocyclization of Cys-Gly-Cys-DNA with chemical linkers

Cys-Gly-Cys-DNA (7 nmol) was dissolved in 35 μL NH_4HCO_3 buffer (60 mM, pH 8), followed by the addition of 5.6 μL of TCEP (10 mM in water, 56 nmol). The mixture was kept at room temperature for one hour. Next, 11.2 μL of 10 mM of the chemical linker in MeCN (112 nmol), 2.8 μL MeCN, and 15.4 μL of 60 mM NH_4HCO_3 buffer at pH 8 were added. The reaction mixture was kept at 30°C for two hours, then directly purified by RP-HPLC.

ASSOCIATED CONTENT

Supporting Information.

Supplementary materials and methods are provided along with two supplementary figures showing the RP-HPLC and MS analysis of peptide-DNA conjugates and the Fmoc removal on Fmoc-Gly-Cys(S-TMP)-DNA.

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Author Contributions

M.V.P and C.H. designed the project, M.B.B. established HPLC and MS techniques for peptide-DNA conjugate analysis, M.V.P. performed all experiments, M.V.P and C.H. wrote the manuscript.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

βME, β-mercaptoethanol; DBMB, 1,3-bis(bromomethyl)benzene; DECLs, DNA-encoded chemical libraries; DMT-MM, 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride; DTT, dithiothreitol; DVS, divinylsulfone; Fmoc, fluorenylmethyloxycarbonyl; HPLC, high-performance liquid chromatography; MALDI, matrix-assisted laser desorption/ionization; mRNA, messenger RNA; PCR, polymerase chain reaction; RP-HPLC, reversed-phase high-performance liquid chromatography; SPPS, solid-phase peptide synthesis; *S*-TMP, trimethoxyphenylthio; *S*-*t*Bu, tert-butylthio; TCEP, tris(2-carboxyethyl)phosphine.

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FIGURES

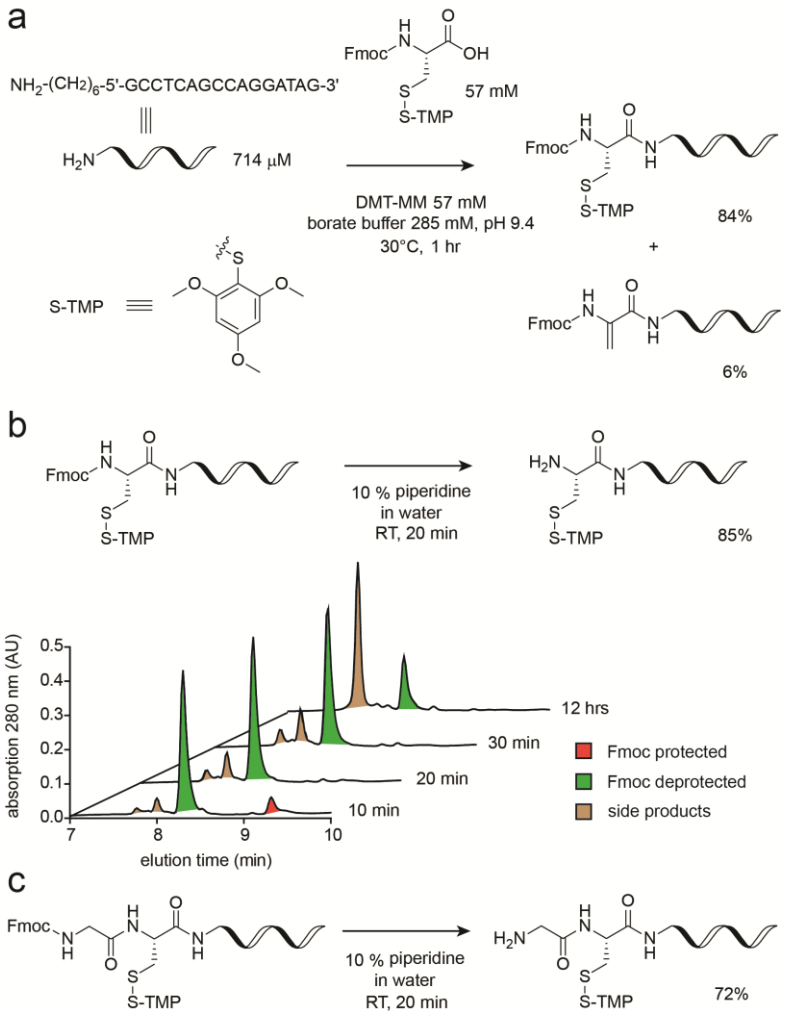


Figure 1. Synthesis of a DNA-conjugated cysteine using *S*-TMP as a thiol protecting group. (a) Conjugation of Fmoc-Cys(S-TMP)-OH to NH_2-$(CH_2)_6$-DNA. (b) Fmoc removal on Fmoc-Cys(*S*-TMP)-DNA with 10% piperidine. Two side products with shorter elution times are formed. (c) Fmoc removal on Fmoc-Gly-Cys(*S*-TMP)-DNA with 10% piperidine. For the di-peptide-DNA substrate, two side products eluting earlier are also formed (Figure S1).

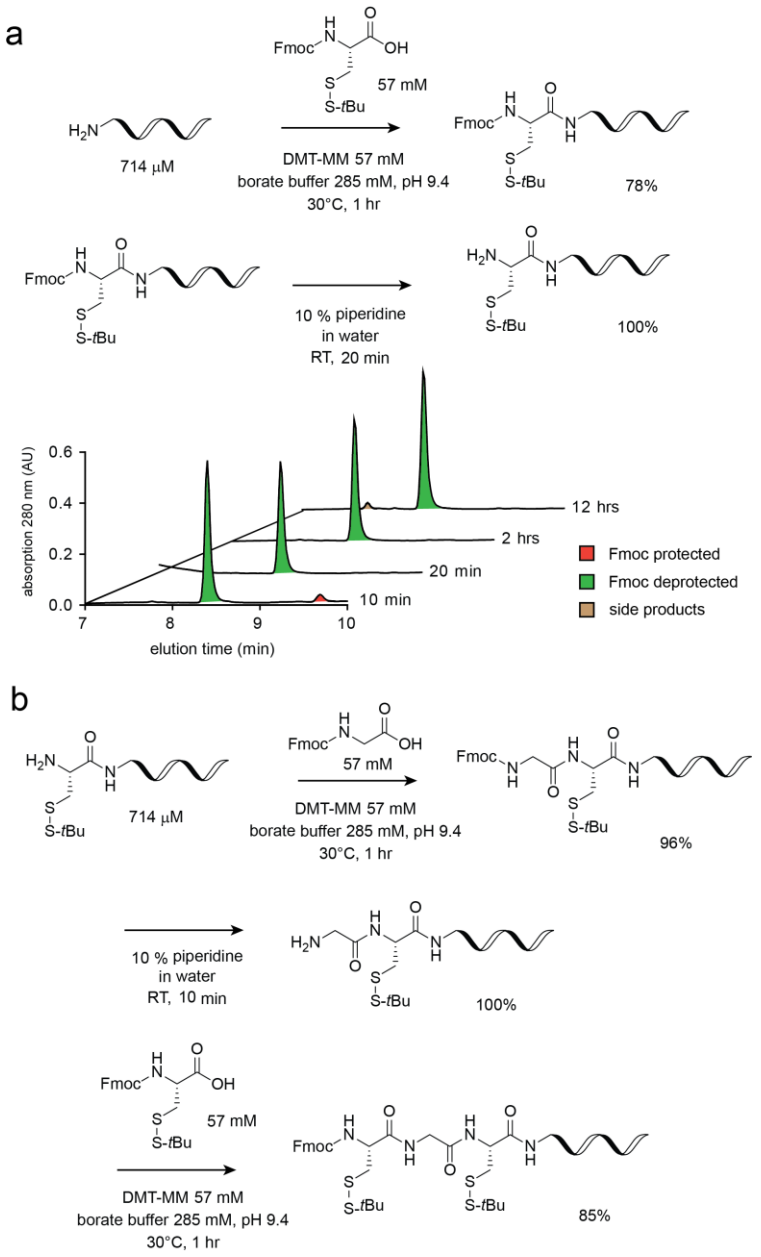


Figure 2. Synthesis of a DNA-encoded disulfide-cyclized peptide using *S*-*t*Bu as a thiol protecting group for the incorporation of cysteines. (a) The conjugation of Fmoc-Cys(*S*-*t*Bu)-OH to NH_2-$(CH_2)_6$-DNA and the Fmoc removal on Fmoc-Cys(*S*-*t*Bu)-DNA with 10% piperidine. Chromatographic analysis of the Fmoc cleavage reaction after different time points shows the efficient removal of Fmoc. (b) Stepwise synthesis of Fmoc-Cys(*S*-*t*Bu)-Gly-Cys(S-*t*Bu)-DNA.

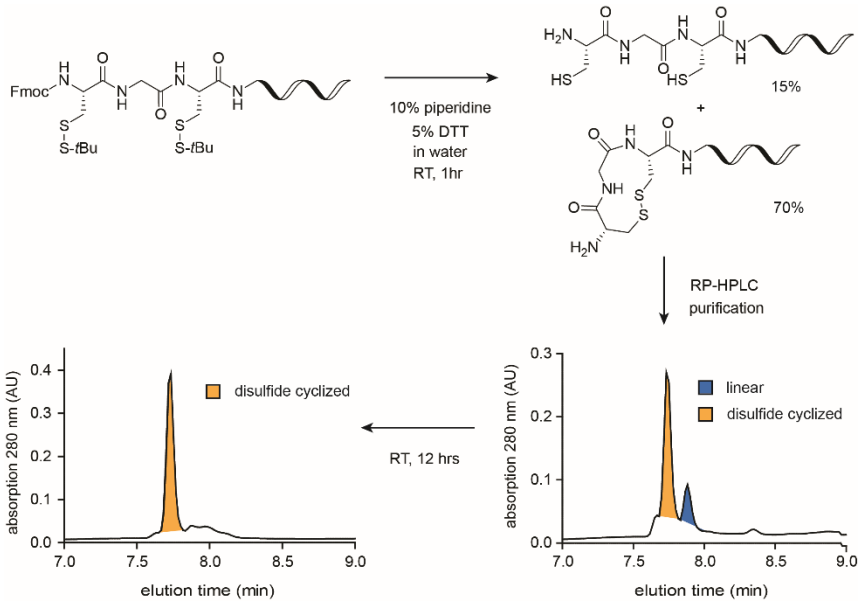


Figure 3. Elimination of the Fmoc and *S*-*t*Bu protecting groups in a single step using piperidine and DTT. HPLC analysis shows efficient removal of protecting groups and that a majority of the product contains oxidized thiol groups that form a disulfide bridge.

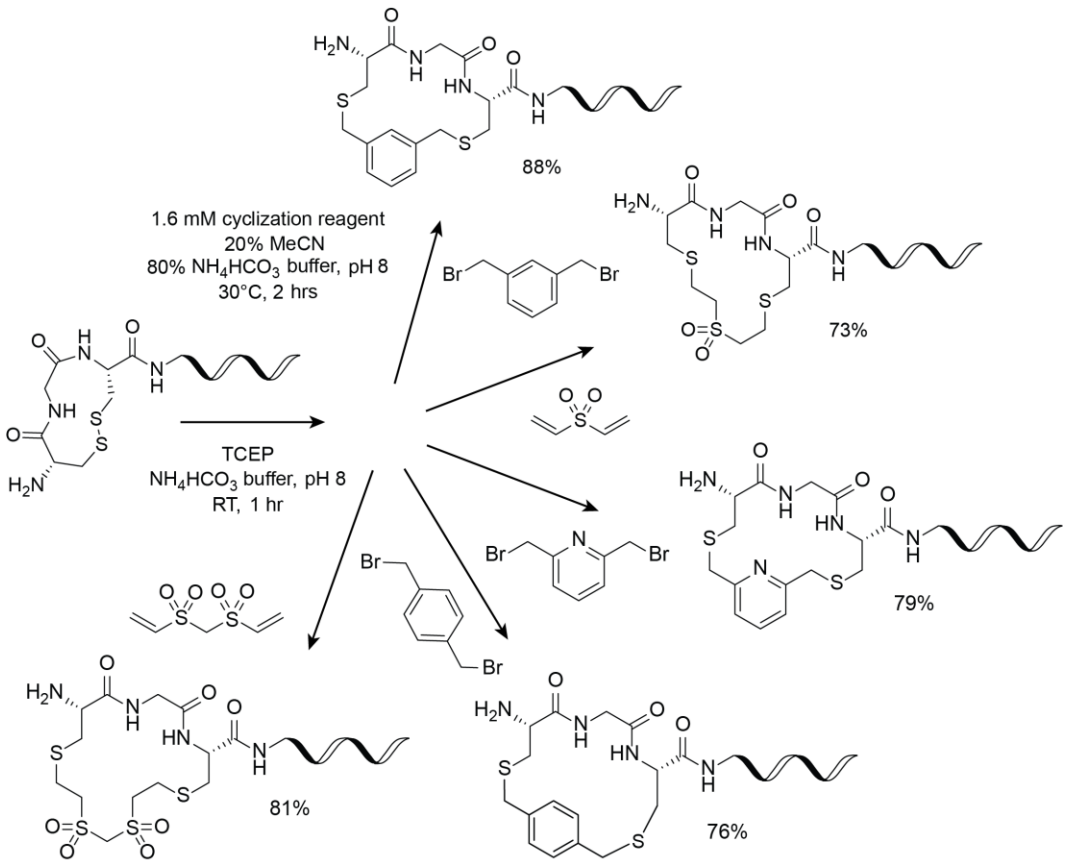


Figure 4. Synthesis of thioether-cyclized DNA-encoded peptides. The disulfide-cyclized peptide-DNA conjugates are reduced with TCEP and cyclized with the indicated bis-electrophile reagents.

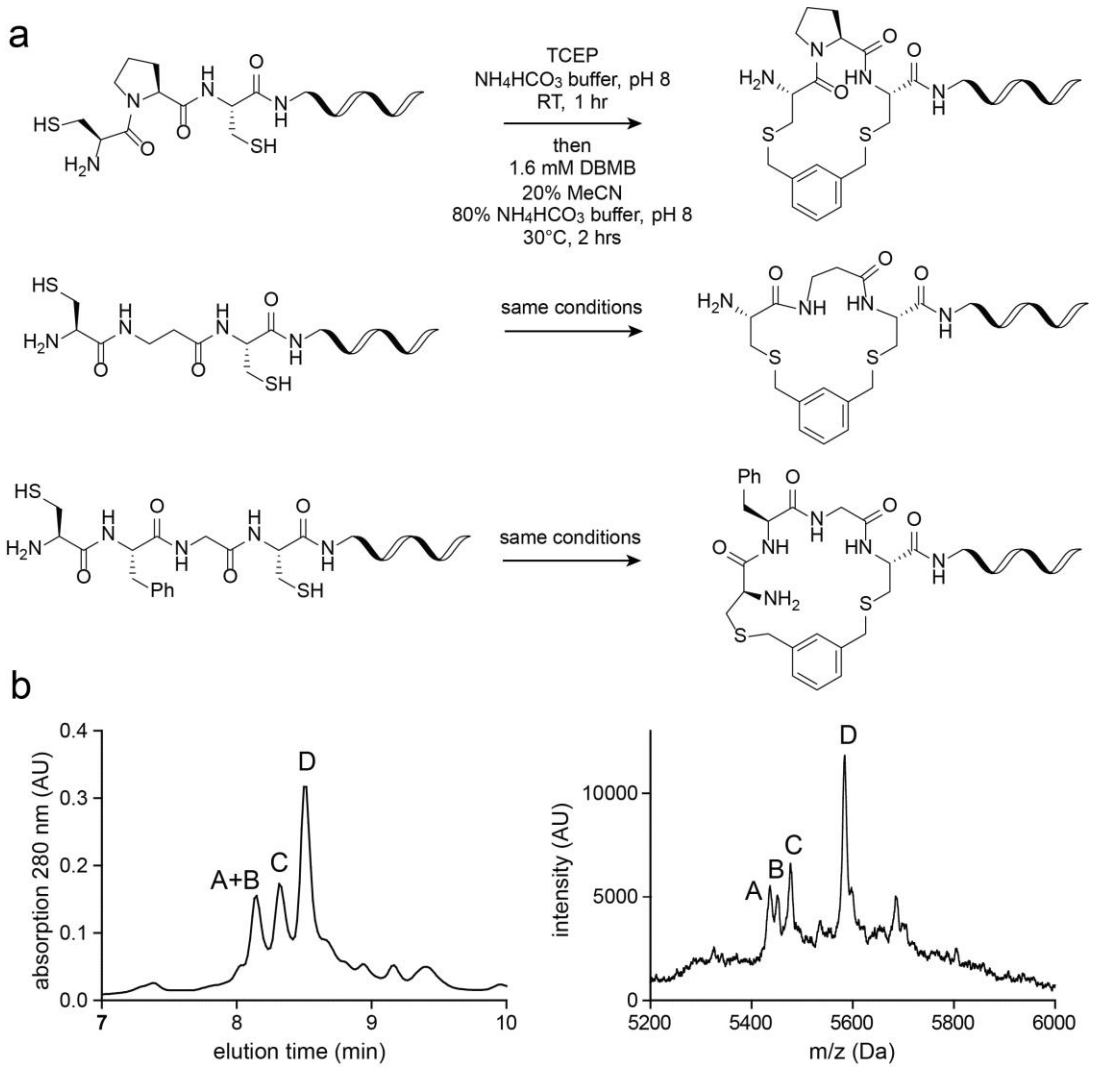
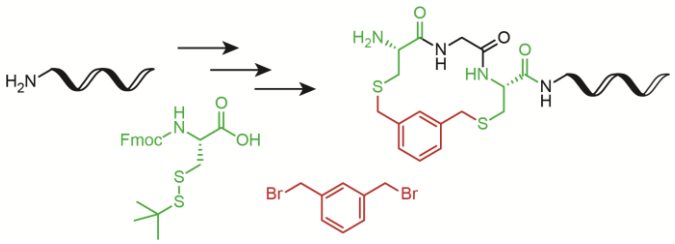


Figure 5. Synthesis of DNA-encoded cyclic peptides with variable amino acid sequences and cyclization of a mixture of four peptide-DNA conjugates. (a) Three different peptides are synthesized and cyclized with DBMB. (b) Cyclization of a mixture of four different peptide-DNA conjugates by DBMB. The mixture was analyzed by RP-HPLC (left panel) and mass spectrometry (right panel). A: Cys-Gly-Cys-DNA, B: Cys-Pro-Cys-DNA, C: Cys-βAla-Cys-DNA, D: Cys-Phe-Gly-Cys-DNA.

TOC Figure



SUPPORTING INFORMATION

Supplementary Materials and Methods

Materials and instruments

All reagents and solvents were purchased from commercial sources and used as received. C6-amino-modified 16-mer oligonucleotide (5'-NH_2-C6-GCCTCAGCCAGGATAG-3') was purchased from Iba Lifesciences. HPLC purification and analysis of DNA-conjugates were performed by ion-pairing RP-HPLC on a Waters XBridge® Oligonucleotide BEH C18 column (130 Å, 2.5 μm, 4.6 mm × 50 mm) using a gradient elution with a mobile phase composed of filtered HPLC-grade methanol (eluent B) and filtered MilliQ water (eluent A) both containing 8 mM of triethylamine and 100 mM 1,1,1,3,3,3-hexafluoropropan-2-ol (HFIP). The detection wavelength was fixed at 280 nm. MS analysis of DNA-conjugates was performed by a Bruker AutoFlex speed MALDI-TOF spectrometer in linear positive mode and given in m/z. The yield of on-DNA reactions was determined by the area under the peak of the product in relation to the area of all peaks of DNA conjugates. The isolated yield of on-DNA reactions was determined from the concentration of purified product in MilliQ water measured on a Nanodrop 8000 spectrophotometer.

NH_4OAc precipitation of peptide-DNA conjugates

To an aqueous DNA solution was added one volume of cold NH_4OAc (10 M in water). The mixture was kept at -20°C for 20 minutes and then centrifuged (9000 G, 4°C, 30 minutes) to pellet the precipitated DNA conjugate. The supernatant was discarded and the solid residue was dissolved in water for subsequent analysis or purification.

Supplementary Figures

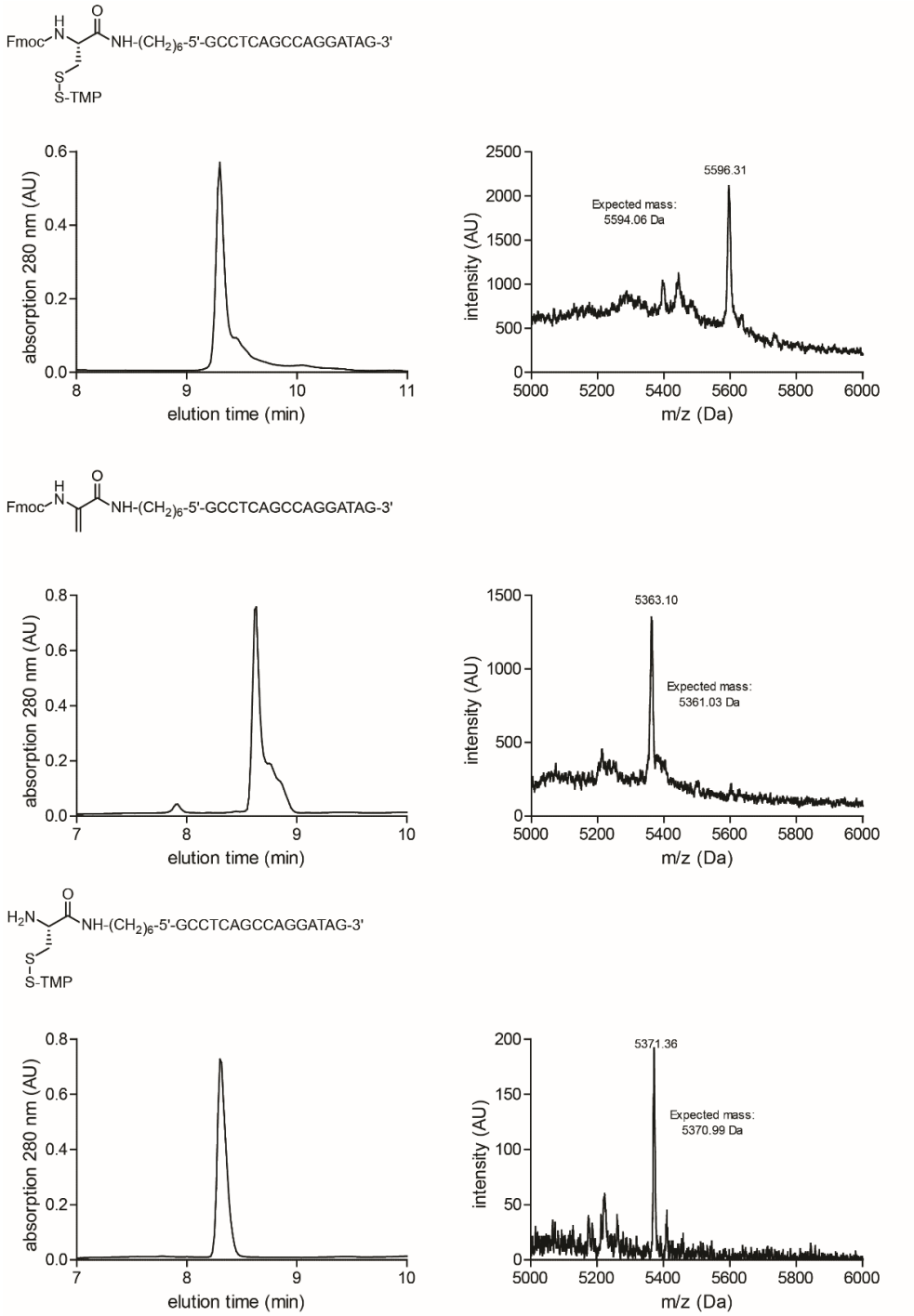
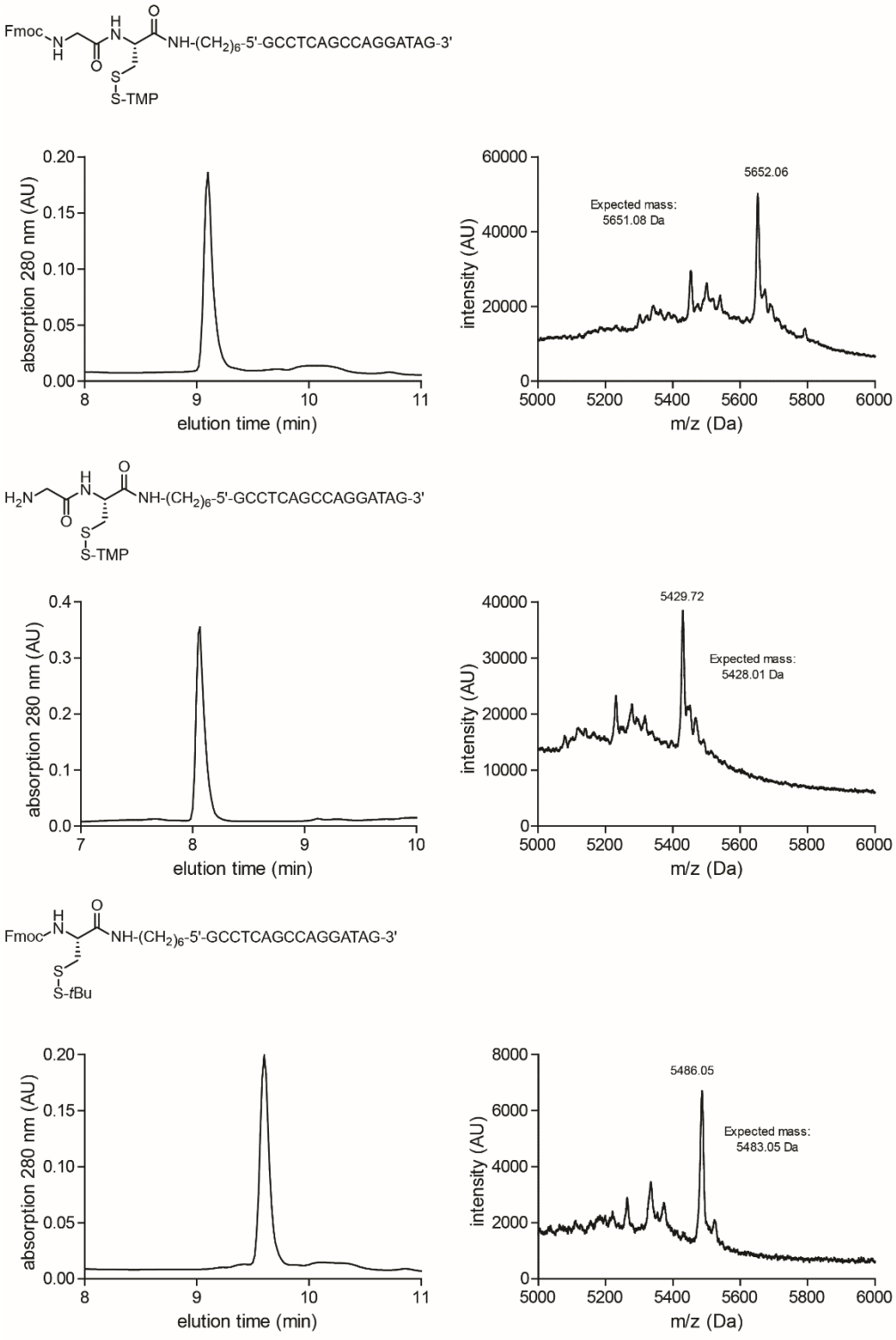


Figure S1. RP-HPLC and MS analysis of peptide-DNA conjugates.



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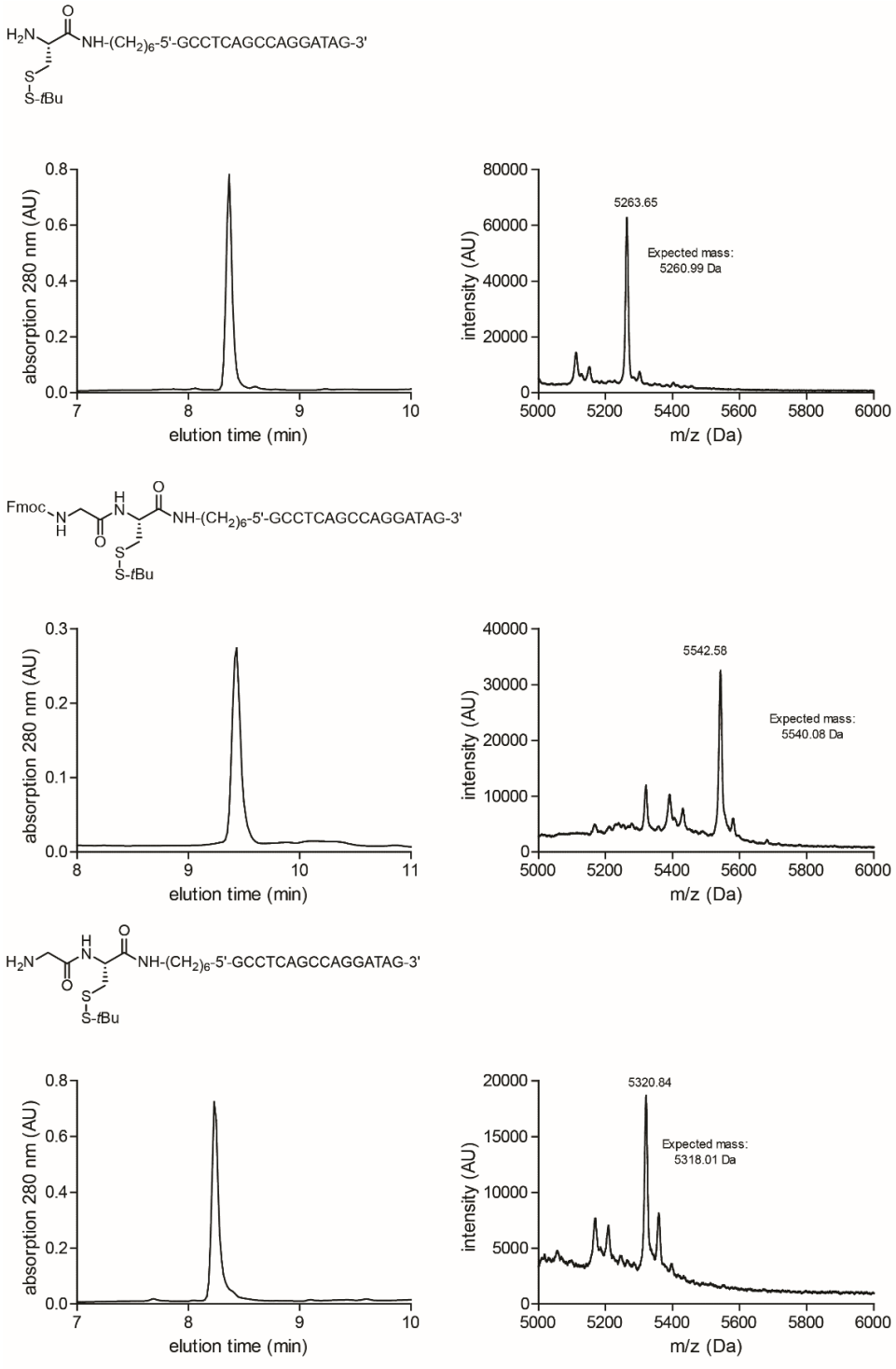

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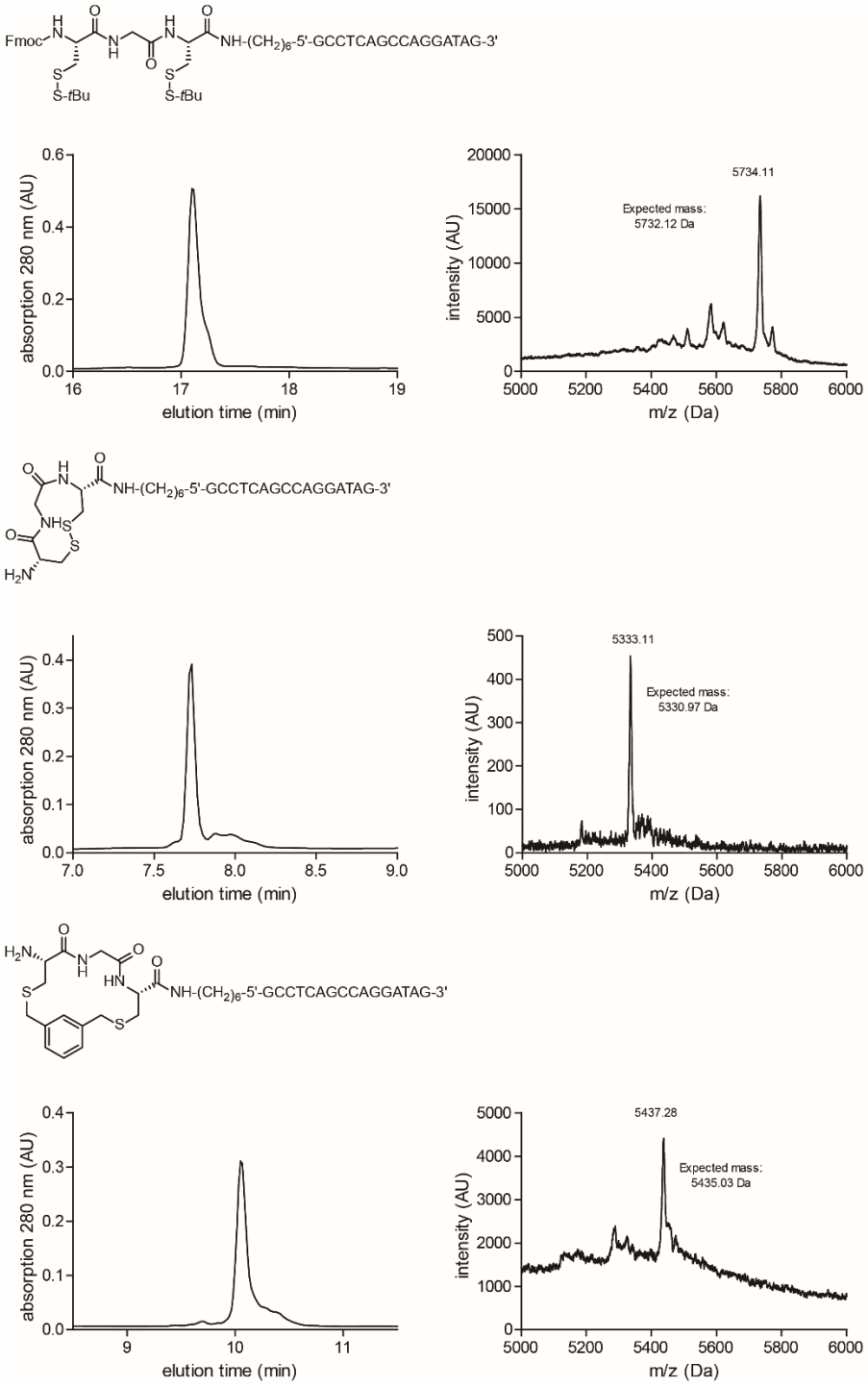

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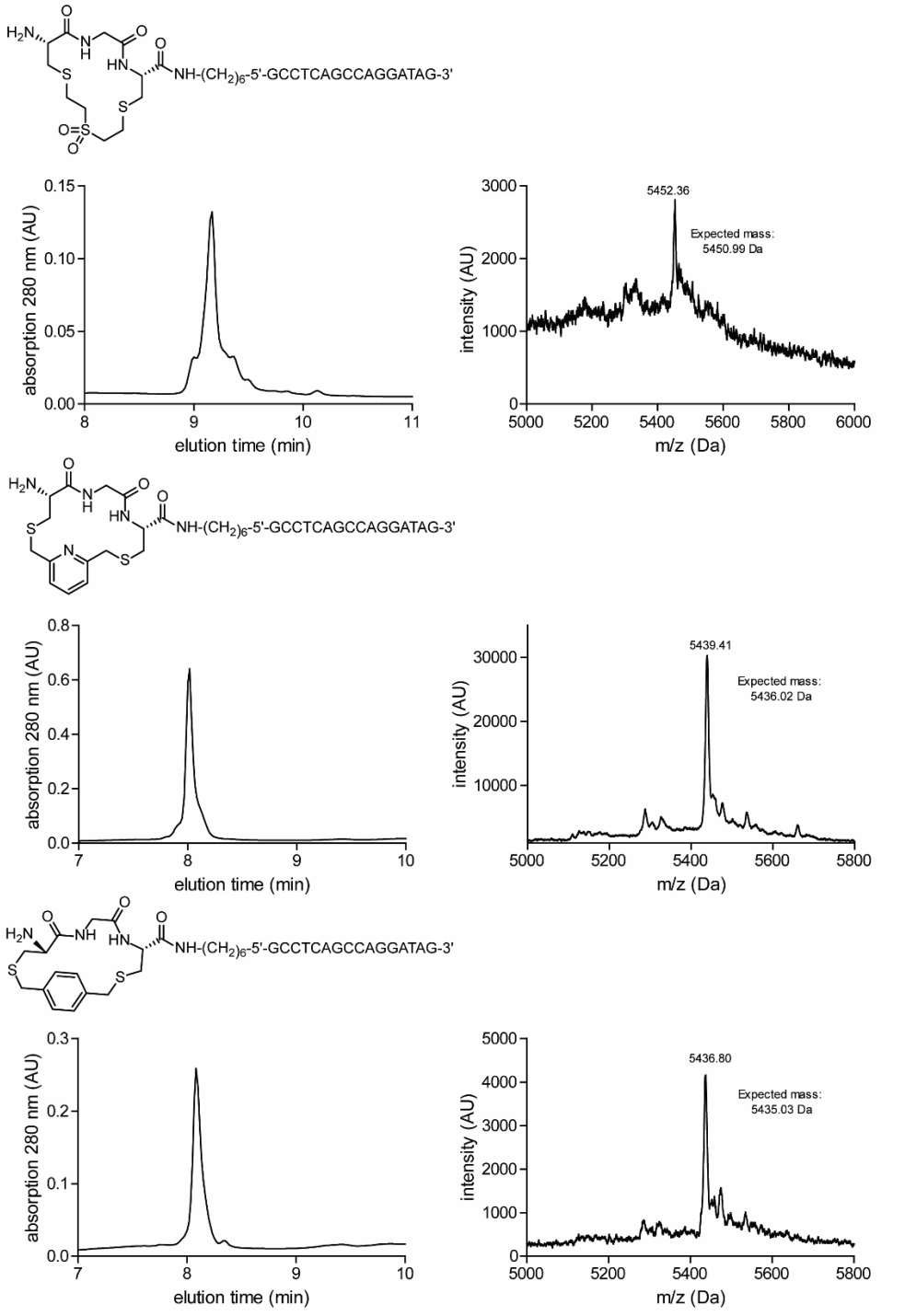

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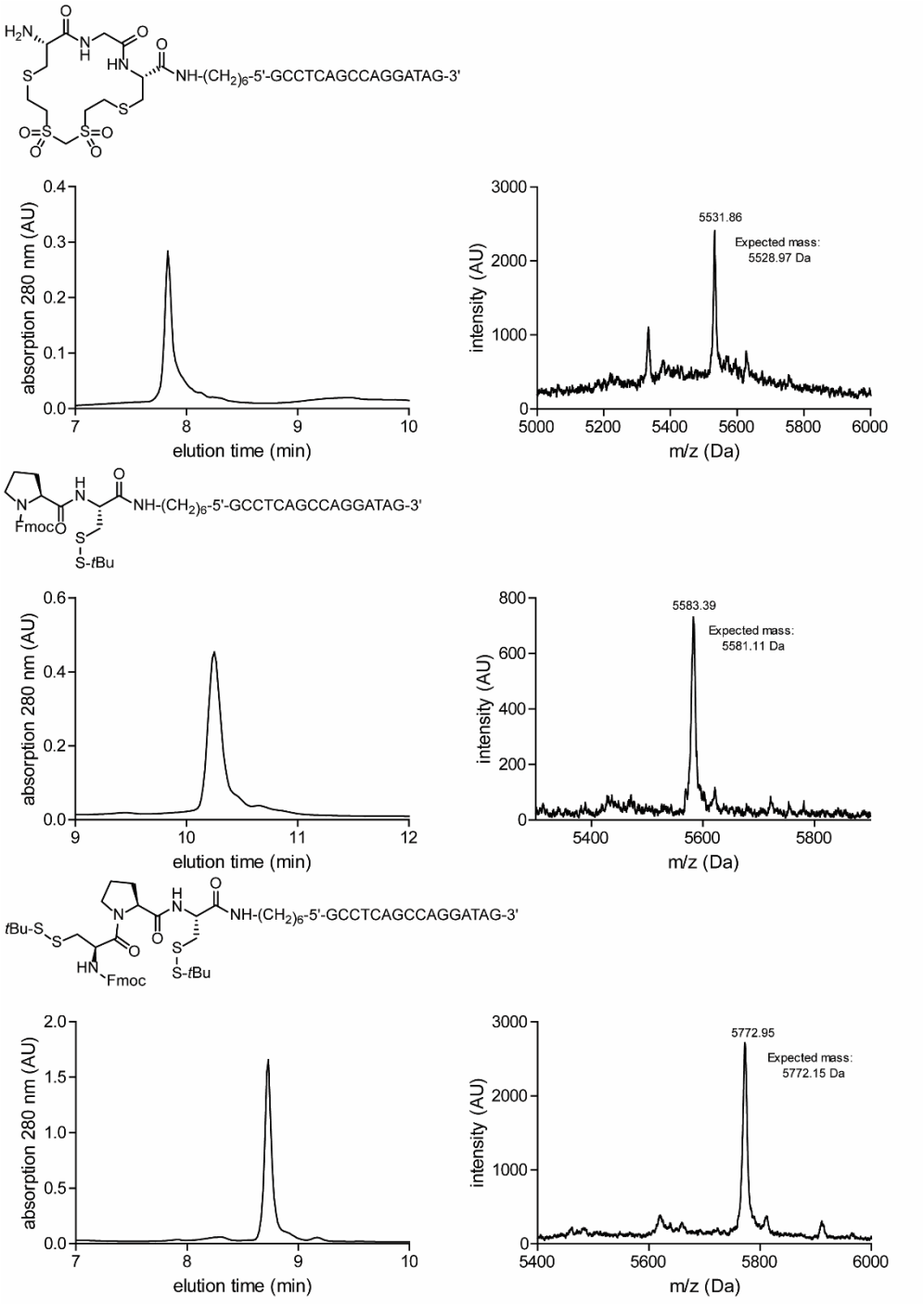

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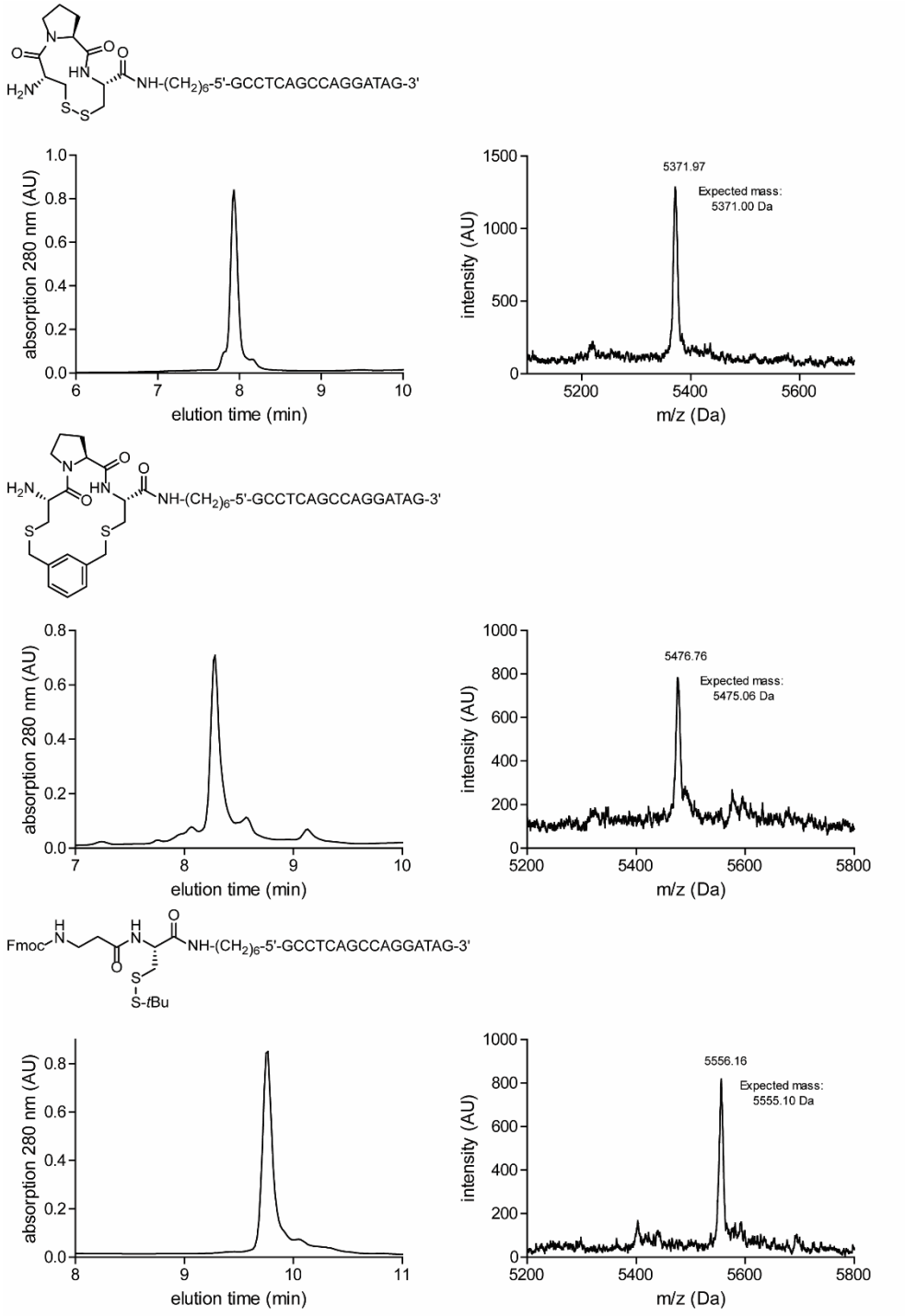

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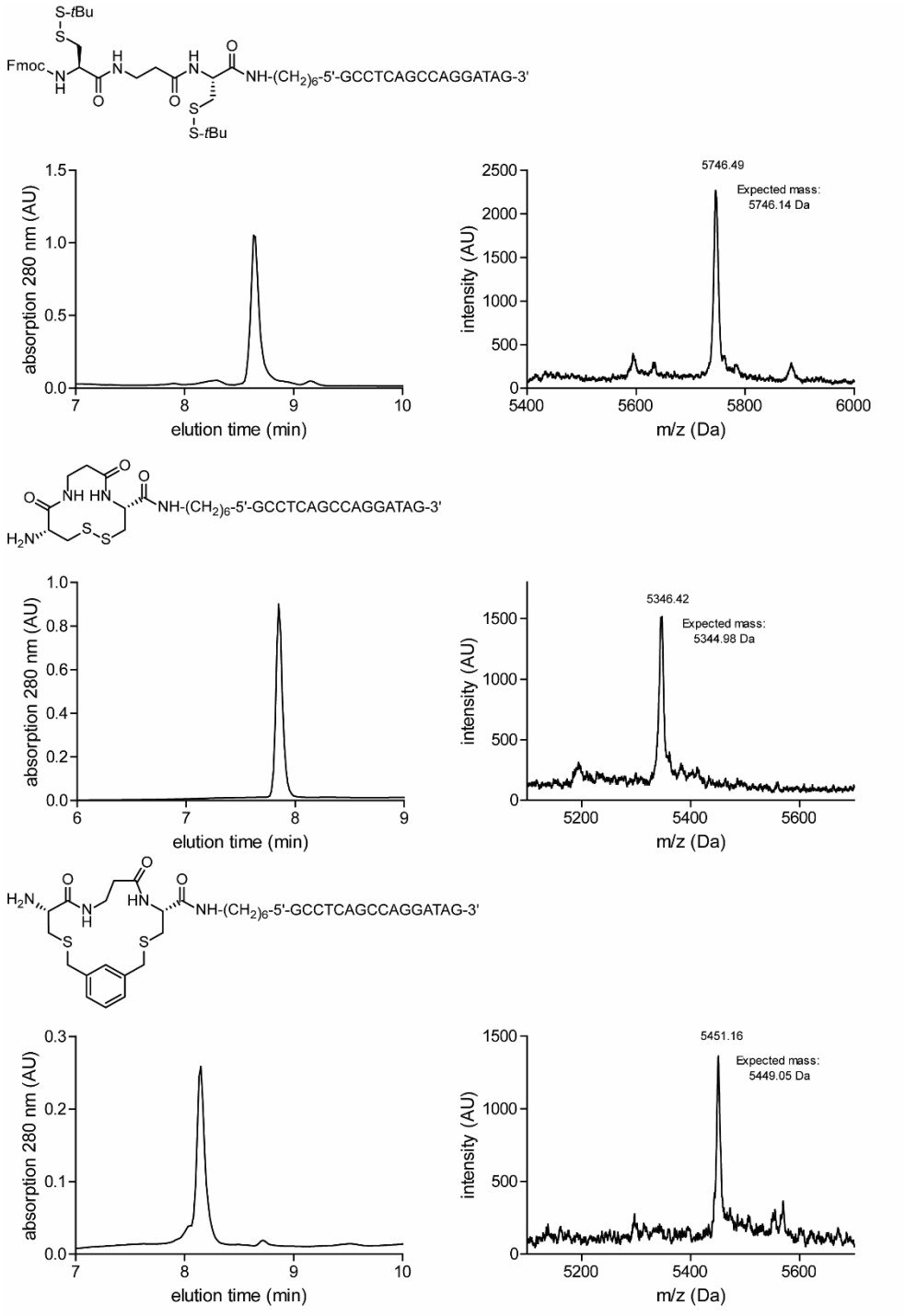

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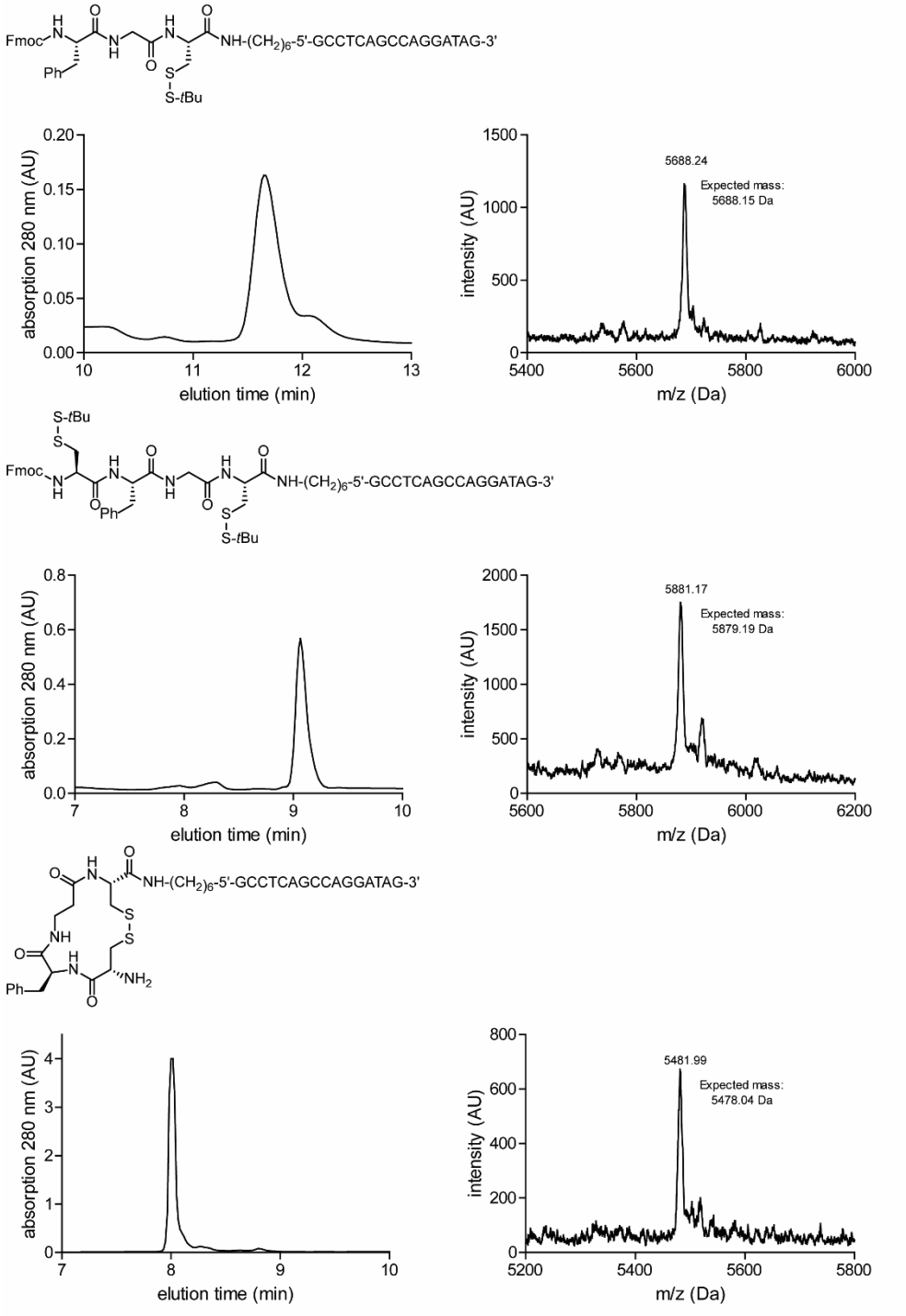

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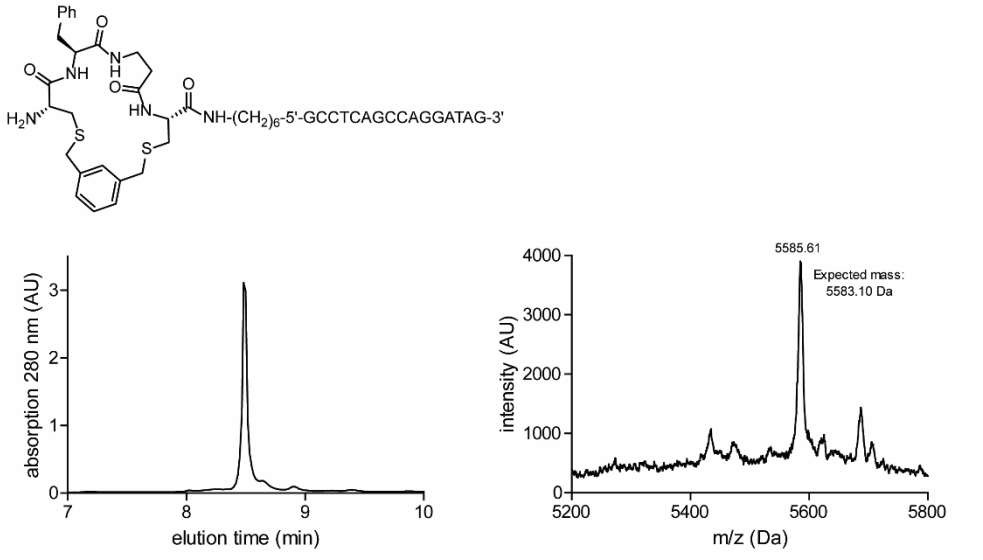

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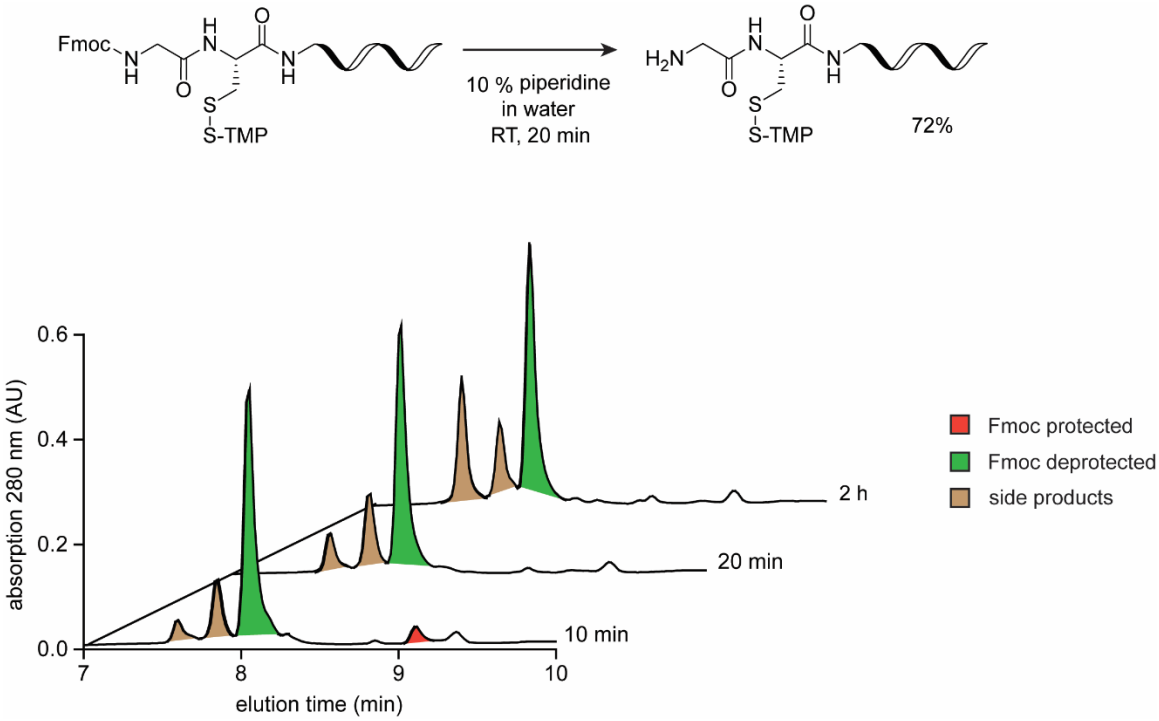

Figure S2. Fmoc removal on Fmoc-Gly-Cys(*S*-TMP)-DNA with 10% piperidine. Two side products with shorter elution times are formed.