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A program for ligation at threonine sites: application to the controlled total synthesis of glycopeptides

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ABSTRACT

A method by which to accomplish formal threonine ligation has been developed. The method accomplishes ligations of two peptide domains. We have also demonstrated the ability to successfully ligate two independent glycopeptide domains.

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1. Introduction

Glycoproteins are a class of naturally occurring biomacromolecules, which are biosynthesized through post-translational protein glycosylation. A great deal of effort has been directed toward the examination of the role that glycosylation plays in various critical protein functions, such as protein folding, proteolytic stability, and intercellular communication.[1] Of particular interest is the fact that many glycoproteins possess exploitable therapeutic activity, and may serve as promising candidates in the development of vaccines,[2] diagnostic techniques,[3] and therapeutic agents.[4] Prominent examples of therapeutically valuable glycoproteins include the erythropoietic agent, erythropoietin (EPO)[4a,5] and the fertility agent, human follicle stimulating hormone (hFSH).[6] Despite considerable interest in this class of biomacromolecules, the field of glycobiology faces a significant obstacle to the rigorous evaluation of glycoproteins: the isolation of significant quantities of *homogeneous* glycoproteins from natural sources is often prohibitively difficult, due to the fact that most naturally occurring glycoproteins are biosynthesized as heterogeneous mixtures of glycoforms.

Given the great difficulties associated with the isolation of homogeneous glycoproteins, we recognized that an opportunity for chemical synthesis might lay in the challenge of using total chemical synthesis to gain access to homogeneous glycoprotein samples.[7] The biology of such agents could then be studied in further detail. Moreover, through chemical synthesis, it would be possible to gain access to fully synthetic analog glycoproteins, possessing targeted modifications in the carbohydrate or protein domains. Of course, such targets could not necessarily be obtained through purely biologic means, since they would lack the viable biosynthetic pathways for reaching the desired structural types. In the context of our glycoprotein synthesis program, we have targeted for total synthesis the clinically relevant glycoproteins, EPO and hFSH.[8] Several years ago, at the outset of our EPO total synthesis endeavor, we identified a number of significant methodological shortcomings that would need to be addressed before a viable total synthesis effort could be launched.

In particular, we were concerned with the dearth of general techniques and protocols available for the merging of two glycopeptide fragments. To address this issue, we developed an efficient method for the coupling of two differentially glycosylated peptide fragments. This advance was predicated on the landmark breakthrough of Kent and co-workers in the field of peptide synthesis, termed native chemical ligation (NCL).[9] NCL is a widely used technique that allows for the merger of large peptide fragments, one of which presents a C-terminal thioester, and the other an N-terminal cysteine residue (Fig. 1a). Direct extension of the NCL method to the realm of glycopeptide synthesis is complicated by the difficulties associated with synthesizing pre-formed glycopeptide thioester. The solution developed in our laboratories (outlined in Fig. 1b) involves a relatively stable C-terminal *ortho*-thiophenolic ester, presented on one of the glycopeptides, and a protected N-terminal cysteine residue incorporated on the other fragment.[10] Upon simultaneous reduction of the two disulfides, the phenol moiety undergoes intramolecular O→S migration, providing an intermediate thioester, which undergoes thioester exchange with the free cysteine of the second glycopeptide. The intermediate then suffers spontaneous intramolecular transfer to yield the bidomainal glycopeptide adduct, incorporating two differentiated sites of glycosylation. The general logic of this NCL method has more recently been extended to a direct oxo-ester

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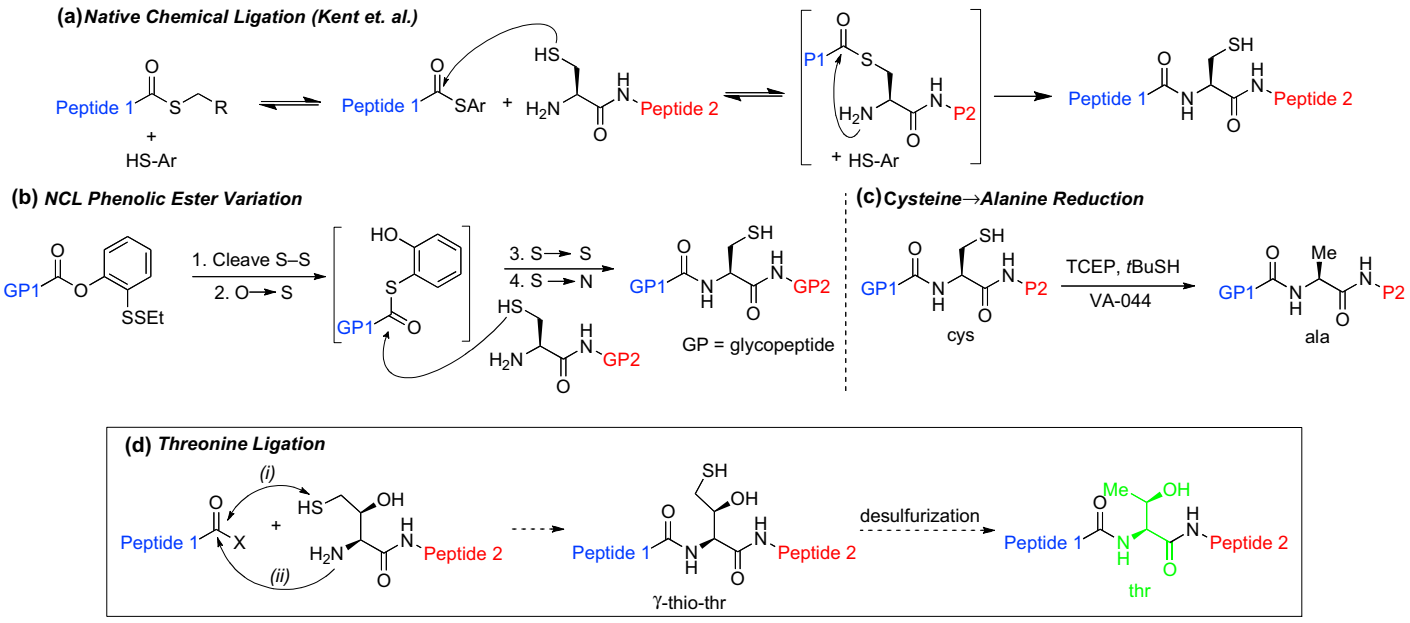


Figure 1. (a) Native chemical ligation; (b) Glycopeptide NCL; (c) Free-radical-mediated reduction of Cys→Ala; (d) Proposed native chemical ligation at threonine.

variant, in which a phenolic ester equipped with *para*-NO_2 or *para*-CN substitution, is sufficiently activated to undergo direct cysteine ligation without the need for the intermediacy of a thioester species.[11]

These glycopeptide ligation protocols do suffer from an important limitation in terms of generalizability, in that they require the presence of a cysteine residue at the ligation site. In fact, there is often a paucity of cysteine sites in naturally occurring proteins and glycoproteins. The need for a menu of efficient *cysteine-free* ligation methods thus remains quite high.

A number of useful solutions to the cysteine ligation problem have been developed, many of which depend on the use of cysteine or a thiol-containing amino acid surrogate in the ligation step. Following thiol-mediated ligation, the cysteine or surrogate residue is converted to the desired amino acid. In this way, methionine ligation has been achieved through homo-cysteine coupling, followed by post-ligational methylation.[12] Similarly, serine ligation has been accomplished through NCL followed by conversion of cysteine to serine.[13] A number of cysteine-free ligation methods make use of a two-step ligation–desulfurization sequence. For instance, metal-based post-ligational thiol reduction has been used to accomplish formal alanine[14] and phenylalanine[15] ligations. Despite the appeal of such a strategy, we noted that traditional desulfurization methods suffer from a lack of substrate generality, due to the susceptibility of many common functional groups (particularly thiol moieties) to the standard harsh reduction conditions. Based on the disclosure by Hoffmann and Walling in the 1950s,[16] we recently developed a very mild and chemoselective free-radical-mediated desulfurization strategy, which allows for the post-translational conversion of cysteine to alanine in complex glycopeptide and peptide settings (Fig. 1c).[17] This method has proven to be tolerant of a wide range of functionalities, including sulfur-containing groups—such as Thz, Cys(Acm), biotin, and thioesters—as well as amino acids, including methionine, and even complex carbohydrate moieties. This overall alanine ligation strategy has now been successfully applied in the context of the synthesis of a complex glycopeptide fragment of erythropoietin (Ala^1-Gly^{28}).[8c] In addition, our mild sulfur reduction protocol has been employed to accomplish a formal valine ligation, through the coupling and post-ligational reduction of unnatural amino acid surrogates (γ-thiol valine[18] or β-thiol valine[18,19]) to valine residues. Moreover, a dual native chemical ligation at lysine has also been achieved recently. A γ-thiol group on the N-terminal lysine mediates double chemical ligation at both α and ε amines, followed by a free-radical desulfurization.[20]

The development of a variety of methods by which to formally accomplish NCL at a diverse range of amino acid residues could be of substantial value. As noted above, due to its low frequency in nature, direct ligation at cysteine itself is of limited practical value. Furthermore, in developing a synthetic strategy toward a glycoprotein or glycopeptide target, it is often desirable to merge two pieces of relatively equal size. As the menu of amino acid ligation options expands, greater flexibility may be brought to the design of synthetic routes. Finally, the two-step ligation/reduction strategy provides the opportunity to explore the consequences of protein engineering with site-specifically modified glycoproteins.

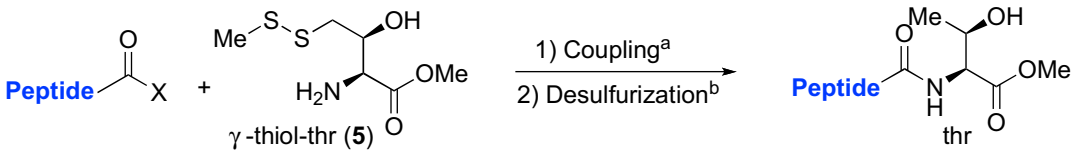
In the hopes of broadening the range of options for amino acid ligation, we sought to extend our two-step ligation/reduction protocol to the development of a formal threonine ligation. Threonine was selected for its relative abundance in nature, particularly in comparison with cysteine. We describe herein the discovery and formulation of a mild and efficient two-step formal threonine ligation protocol.

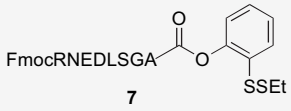
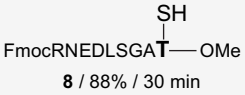
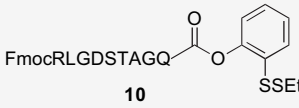
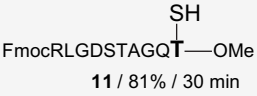
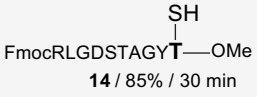
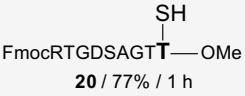
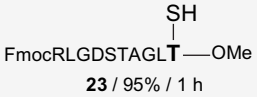
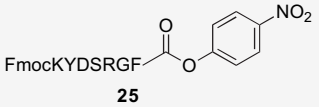
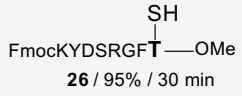
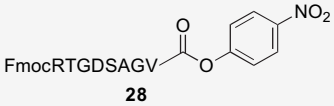
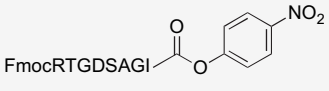
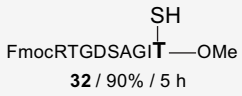
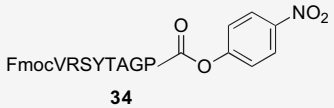
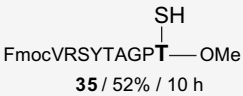
The central idea is outlined in Figure 1d. Thus, as in the case of the valine ligation,[18] a thiol-containing threonine surrogate would be incorporated at the N-terminus of Peptide 2. One could envision two possible sites at which to install a thiol group onto the threonine residue: at the β position or the γ position. We expected that the γ-thiol threonine would serve as the more productive surrogate in establishing the initial acylation event. Peptide 2, incorporating the γ-thiol threonine, was expected to undergo trans-thioesterification with Peptide 1 (presented as either a thioester or activated oxo-ester), to generate a thioester-linked intermediate, which would then undergo spontaneous intramolecular acyl transfer, generating the new amide bond. Subsequent radical-based desulfurization would serve to remove the thiol, providing the target peptide with threonine at the ligation site.

2. Results and discussion

Our first objective was to synthesize a γ-thiol threonine amino acid surrogate. To accomplish this, we drew on the earlier work of

Table 1
Threonine extension by coupling and subsequent free-radical desulfurization

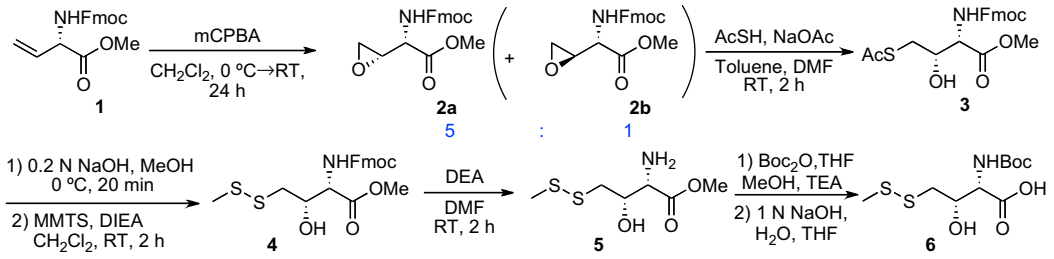


Entry	Peptide	Coupling Product/Yield/Time	Desulfurization Product/Yield/Time
1	 **7**	 **8** / 88% / 30 min	FmocRNEDLSGA**T**—OMe **9** / 92 % / 2 h
2	 **10**	 **11** / 81% / 30 min	FmocRLGDSTAGQ**T**—OMe **12** / 81% / 2 h
3	 **13**	 **14** / 85% / 30 min	FmocRLGDSTAGY**T**—OMe **15** / 89% / 2 h
4	 **16**	 **17** / 90% / 30 min	FmocRLGDSTAGW**T**—OMe **18** / 90% / 2 h
5	 **19**	 **20** / 77% / 1 h	FmocRTGDSAGT**T**—OMe **21** / 84% / 2 h
6	 **22**	 **23** / 95% / 1 h	FmocRLGDSTAGL**T**—OMe **24** / 80% / 2 h
7	 **25**	 **26** / 95% / 30 min	FmocKYDSRGF**T**—OMe **27** / 80% / 2 h
8	 **28**	 **29** / 97% / 5 h	FmocRTGDSAGV**T**—OMe **30** / 95% / 2 h
9	 **31**	 **32** / 90% / 5 h	FmocRTGDSAGI**T**—OMe **33** / 82% / 2 h
10	 **34**	 **35** / 52% / 10 h	FmocVRSYTAGP**T**—OMe **36** / 83% / 2 h

Key: (a) buffer at pH 6.5 (6.0 M Gn·HCl, 188.8 mM Na_2HPO_4), TCEP, rt (b) TCEP, VA-044, *t*BuSH, 37 °C. VA-044=2,2'-azobis-[2-(2-imidazolin-2-yl)propane] dihydrochloride. TCEP=tris(2-carboxyethyl)phosphine.

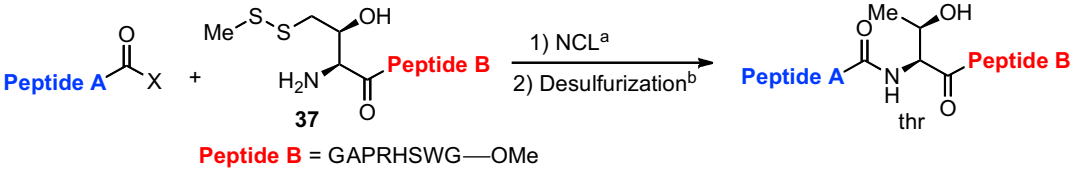
Rapoport and co-workers,[21] in which allo-threonine was obtained from D-vinylglycine. Through modification of the Rapoport route, we sought to accomplish the diastereoselective syntheses of the γ-thiol threonine derivatives, **5** and **6** (Scheme 1). Compound **5** was to be used directly for single amino acid extension studies (see Table 1), while compound **6** would be elaborated to peptide **37**, which would serve as the substrate in the threonine ligation studies at the peptide level (see Table 2). Thus, as shown in Scheme 1, vinylglycine **1**[22] was epoxidized with an excess of mCPBA to afford a 5:1 ratio of *syn* and *anti* epoxides, **2a** and **2b**, which could be separated by chromatography. The major diastereomer, **2a**, has the desired *syn* configuration, perhaps as a consequence of hydrogen bonding of mCPBA to the nitrogen functionality.[23] Upon exposure to the sodium salt of thioacetic acid, epoxide **2a** was opened to provide the acetylated thiol, **3**. The latter was transformed, in a straightforward fashion, to the target compounds, **5** and **6**.

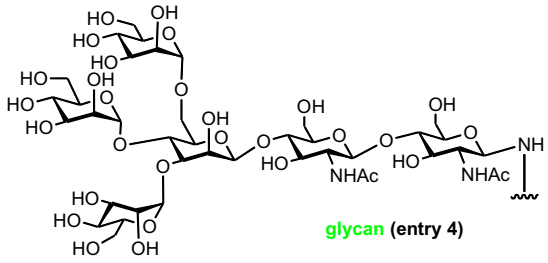
With γ-thiol threonine derivative **5** in hand, we next sought to evaluate the feasibility of the proposed threonine ligation method. We first examined the protocol in the context of a single amino acid extension of a variety of peptide substrates. As shown in Table 1, when a relatively sterically less demanding amino acid (Ala, Gln, Tyr, Trp, Phe) was presented at the C-terminus of the peptide, the amino acid extension generally proceeded very quickly and with good yield (see entries 1–4 and 7). Even when the β-branched amino acid, threonine, was incorporated at the C-terminus, the coupling was complete within 1 h (entry 5). As expected, as the C-



Scheme 1. Synthesis of γ-thiol threonine.

Table 2
NCL at threonine through ligation and subsequent free-radical desulfurization





Entry	Peptide A	Ligation Product/Yield/Time	Desulfurization Product/Yield/Time
1	FmocRLGDSTAGY(SPh) **13**	FmocRLGDSTAGY**T**GAPRHSWG—OMe (SH) **38** / 70% / 1 h	FmocRLGDSTAGY**T**GAPRHSWG—OMe **39** / 98% / 2 h
2	FmocRLGDSTAGW(SPh) **16**	FmocRLGDSTAGW**T**GAPRHSWG—OMe (SH) **40** / 73% / 1 h	FmocRLGDSTAGW**T**GAPRHSWG—OMe **41** / 85% / 2 h
3	FmocRTGDSAGI(O-4-NO2-Ph) **31**	FmocRTGDSAGI**T**GAPRHSWG—OMe (SH) **42** / 63% / 7 h	FmocRTGDSAGI**T**GAPRHSWG—OMe **43** / 96% / 2 h
4	glycan FmocRLGNSTAGQ(O-2-SSEt-Ph) **44**	glycan FmocRLGNSTAGQ**T**GAPRHSWG—OMe (SH) **45** / 82% / 2 h	glycan FmocRLGNSTAGQ**T**GAPRHSWG—OMe **46** / 96% / 2 h

Key: (a) buffer at pH 6.5 (6.0 M Gn·HCl, 188.8 mM Na_2HPO_4), TCEP, RT; (b) TCEP, VA-044, *t*BuSH, 37 °C.

terminus became more sterically hindered (Leu, Val, Ile, Pro), the reaction rate suffered. However, coupling could still be accomplished within a reasonable time frame, and in moderate to good yields (entries 6, 8–10). As shown in Table 1, a variety of different C-terminal esters participated successfully in the extension protocol, including thiophenyl ester, *ortho*-thiophenolic ester, and *para*-nitrophenyl ester. The diversity of C-terminal esters amenable to this protocol is of significance, as the *ortho*-thiophenolic ester is compatible with glycopeptide ligation, while *para*-nitrophenyl ester is particularly efficient at promoting coupling at sterically hindered ligation sites. We also note that the threonine ligation protocol is able to accommodate the presence of an unprotected lysine in the substrate peptide (**25**, entry 7). All of the coupling products obtained in Table 1 were subsequently subjected to our standard radical-based desulfurization conditions to provide the desired threonine extension products in very good yields.

We now sought to examine the two-step ligation/reduction protocol in the context of a peptide-peptide coupling. Peptide **37**, possessing the γ-thiol threonine surrogate at its N-terminus, was prepared from compound **6** (see Supplementary data for details). According to our general procedure, **37** and the peptide coupling partner were dissolved in a guanidine buffer solution. Upon addition of TCEP, the disulfide moieties were cleaved to presumably give rise to the free thiol functionalities, which then underwent the anticipated ligation reaction. As shown in Table 2, we found the ligation rate to be dependent on the nature of the C-terminal amino

acid. Thus, when a less sterically demanding amino acid—such as Tyr (**13**) or Trp (**16**)—was present at the C-terminus, ligation was complete within 1 h (entries 1 and 2). However, in the case of the more hindered amino acid, Ile (**31**), the reaction took up to 7 h to reach completion (entry 3). Once more, in each case, subsequent free-radical-based desulfurization was readily achieved in high yields through use of our mild reduction method. Finally, as shown in Table 2, entry 4, our new protocol could be readily extended to a glycopeptide ligation setting. Thus, peptide **44**, presenting an N-linked hexasaccharide domain, underwent ligation with peptide **37** to provide glycopeptide **45** in 82% yield. Upon exposure to our previously described reduction conditions, glycopeptide **46**, incorporating threonine at the ligation site, was obtained in 96% yield.

3. Conclusion

In conclusion, we have described the development of a useful new entry in the field of native chemical ligation. Through an efficient two-step ligation/reduction protocol, it is now possible to formally achieve NCL at threonine sites, in both peptide and glycopeptide settings. This methodological advance, taken in concert with the previous entries of our group and others, has served to significantly expand the NCL menu, which was originally restricted to cysteine-based ligations. Further applications and extensions of this method to the synthesis of important biologic level agents are underway in our laboratory.

4. Experimental section

4.1. General

Anhydrous THF, diethyl ether, CH_2Cl_2, toluene, and benzene were obtained from a dry solvent system (passed through column of alumina) and used without further drying. NMR spectra (^1H and ^{13}C) were recorded on a Bruker Advance DRX-500 MHz, or a Bruker DRX-600 MHz. Low-resolution mass spectral analyses were performed with a JOEL JMS-DX-303-HF mass spectrometer or Waters Micromass ZQ mass spectrometer.

HPLC: All separations of peptides and glycopeptides involved a mobile phase of 0.05% TFA (v/v) in water (solvent A)/0.04% TFA in acetonitrile (solvent B). Preparative and analytical HPLC separations were performed using a Rainin HPXL solvent delivery system equipped with a Rainin UV-1 detector. LC–MS chromatographic separations were performed using a Waters 2695 Separations Module and a Waters 996 Photodiode Array Detector equipped with X-Bridge™ C18 column (5.0 μm, 2.1×150 mm), X-Terra™ MS C18 column (3.5 μm, 2.1×100.0 mm) or Varian Microsorb C18 column (2×150 mm) at a flow rate of 0.2 mL/min. HPLC separations were performed using: X-Bridge™ Prep C18 column OBD™ (5.0 μm, 19×150 mm), a flow rate of 16 mL/min. Microsorb 100-5 C18 column at a flow rate of 16.0 mL/min or Microsorb 300-5 C4 column at a flow rate of 16.0 mL/min.

Automated peptide synthesis was performed on an Applied Biosystems Pioneer continuous flow peptide synthesizer. Peptides were synthesized under standard automated Fmoc protocols. The deblock mixture was a mixture of 100/5/5 of DMF/piperidine/DBU. Upon completion of automated synthesis on a 0.05 mmol scale, the peptide resin was washed into a peptide synthesis vessel with CH_2Cl_2. The resin cleavage was effected by treatment with AcOH/TFE/CH_2Cl_2 (2:2:6) for 2×1 h to yield peptidyl acids in good yield. The peptidyl acids were modified on *C*-terminus and/or N-terminus. The resulting peptides were subjected to a deprotection cocktail (60.0 mg of phenol, 0.2 ml of water, 0.15 ml of triisopropylsilane, and 3.0 ml TFA) for 2.0 h. TFA was removed by N_2. The oily residue was triturated with diethyl ether to give a white suspension, which was centrifuged and the ether subsequently decanted. The resulting solid was ready for HPLC purification.

4.2. Preparation and characterization of compounds 2a–6

*4.2.1. (S)-Methyl 2-(((9H-fluoren-9-yl)methoxy)carbonylamino)-2-((S)-oxiran-2-yl) acetate (**2a**).* To a stirred solution of **1** (200 mg, 0.593 mmol) in CH_2Cl_2 (6 mL) at 0 °C was added *m*-chloroperbenzoic acid (1.022 g, 5.93 mmol) and the reaction was warmed to rt. After 24 h, the mixture was filtered through a glass filter, the solids were extracted with CH_2Cl_2, and the combined organic phase was washed with 10% $NaHCO_3$, water, dried, and evaporated. The two diastereomers were then separated by chromatography (CH_2Cl_2/MeOH=300:1) to give **2a** (138 mg, 67% yield) as a white solid. ^1H NMR (500 MHz, $CDCl_3$) δ 2.59 (m, 1H), 2.76 (m, 1H), 3.46 (s, 1H), 3.81 (s, 3H), 4.20 (t, *J*=6.8 Hz, 1H), 4.40 (d, *J*=6.9 Hz, 2H), 4.70 (dd, *J*=1.5 Hz, 8.9 Hz, 1H), 5.23 (d, *J*=8.8 Hz, 1H), 7.29 (m, 2H), 7.32 (t, *J*=6.3 Hz, 2H), 7.56 (t, *J*=6.0 Hz, 2H), 7.76 (d, *J*=6.3 Hz, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 43.9, 47.1, 51.2, 53.0, 53.1, 67.2, 120.1, 125.1, 127.1, 127.1, 127.8, 141.3, 141.4, 143.6, 143.8, 156.2, 170.2; $[\alpha]_D^{20}$ −5.21 (*c* 1.08, $CHCl_3$); IR (liquid film) (ν_{max}/cm^{-1}): 3342, 3066, 3019, 2953, 2848, 1724; HRMS: *m*/*e* calcd for $C_{20}H_{19}NO_5Na^+$: 376.1161, found: 376.1153.

*4.2.2. (2S,3S)-Methyl 2-(((9H-fluoren-9-yl)methoxy)carbonylamino)-4-(acetylthio)-3-hydroxybutanoate (**3**).* Compound **2a** was dissolved in toluene (3.2 mL) and treated with a solution of sodium acetate (88 mg, 1.077 mmol) and thioacetic acid (80 μL, 1.077 mmol) in DMF (1.6 mL). The reaction mixture was stirred at rt for 2 h. The mixture was concentrated under N_2. The concentrate was dissolved in EtOAc and washed with NH_4Cl, water, and brine. The organic layer was dried, concentrated, and purified by chromatography (Hexane/EtOAc=2:1) to give **3** (72 mg, 80%) as a light yellow foam. ^1H NMR (500 MHz, $CDCl_3$) δ 2.35 (s, 3H), 3.01 (m, 3H), 3.75 (s, 3H), 4.23 (m, 2H), 4.41 (d, *J*=7.0 Hz, 2H), 4.51 (d, *J*=9.3 Hz, 1H), 5.69 (d, *J*=9.4 Hz, 1H), 7.31 (m, 2H), 7.39 (m, 2H), 7.61 (m, 2H), 7.75 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 30.6, 32.8, 47.2, 52.8, 57.3, 67.3, 71.5, 120.0, 120.0, 125.1, 125.2, 127.1, 127.8, 141.3, 141.4, 143.6, 143.8, 156.7, 170.9, 196.6; $[\alpha]_D^{20}$ 11.69 (*c* 0.68, $CHCl_3$); IR (liquid film) (ν_{max}/cm^{-1}): 3373, 3065, 3019, 2952, 2847, 1747, 1722, 1697; HRMS: *m*/*e* calcd for $C_{22}H_{23}NO_6SNa^+$: 452.1144, found: 452.1155.

*4.2.3. (2S,3S)-Methyl 2-(((9H-fluoren-9-yl)methoxy)carbonylamino)-3-hydroxy-4-(methyldisulfanyl) butanoate (**4**).* Compound **3** (47 mg, 0.11 mmol) was dissolved in MeOH (1.87 mL) and treated with 0.2 N NaOH solution (1.87 mL) at 0 °C for 20 min. The reaction mixture was carefully neutralized by the addition of 1 N HCl at 0 °C, diluted with EtOAc and washed with water and brine. The organic layer was concentrated and dried in vacuo, generating 43 mg of crude compound (*2S,3S*)-*methyl 2-(((9H-fluoren-9-yl)methoxy)carbonylamino)-3-hydroxy-4-mercaptobutanoate*, which was directly used in the next step.

S-Methyl methanethiolsulfonate (36 μL, 0.38 mmol) and DIEA (12 μL, 0.11 mmol) were added to CH_2Cl_2 (0.5 mL). The crude residue of (2*S*,3*S*)-methyl 2-(((9*H*-fluoren-9-yl)methoxy)carbonylamino)-3-hydroxy-4-mercaptobutanoate in CH_2Cl_2 (0.8 mL) was added dropwise to the above solution and stirred at rt for 2 h. The reaction mixture was concentrated and purified by chromatography (Hexane/EtOAc=2:1), to provide **4** (33 mg, 70% in two steps) as a light yellow foam. ^1H NMR (500 MHz, $CDCl_3$) δ 2.42 (s, 3H), 2.65 (dd, *J*=9.2 Hz 14.2 Hz, 1H), 2.71 (d, *J*=2.5 Hz, 1H), 2.85 (dd, *J*=3.8 Hz, 14.0 Hz, 1H), 3.79 (s, 3H), 4.22 (t, *J*=6.8 Hz, 1H), 4.43 (m, 3H), 4.51 (d, *J*=9.4 Hz, 1H), 5.58 (d, *J*=9.4 Hz, 1H), 7.29 (m, 2H), 7.37 (t, *J*=7.1 Hz, 2H), 7.60 (t, *J*=7.1 Hz, 2H), 7.75 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 22.9, 41.4, 47.2, 52.9, 57.1, 67.2, 69.9, 120.0, 120.1, 125.1, 127.1, 127.8, 141.3, 141.4, 143.6, 143.8, 156.7, 170.9; $[\alpha]_D^{20}$ 48.90 (*c* 0.27, $CHCl_3$); IR

(liquid film) (ν_{max}/cm^{-1}): 3406, 3019, 2952, 2918, 2850, 1724; HRMS: *m*/*e* calcd for $C_{21}H_{23}NO_5S_2Na^+$: 456.0915, found: 456.0909.

*4.2.4. (2S,3S)-Methyl 2-amino-3-hydroxy-4-(methyldisulfanyl)butanoate (**5**).* To a stirred solution of **4** (57 mg, 0.131 mmol) in DMF (4.3 mL) was added diethylamine (1.4 mL). The reaction mixture was stirred at rt for 2 h and the solvent was evaporated. The residue was purified by chromatography (CH_2Cl_2/MeOH=40:1) to give **5** (25 mg, 89% yield) as a clear oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.43 (s, 3H), 2.84 (dd, *J*=6.0 Hz, 13.9 Hz, 1H), 2.92 (dd, *J*=7.0 Hz, 13.9 Hz, 1H), 3.66 (d, *J*=3.1 Hz, 1H), 3.76 (s, 3H), 4.17 (m, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 23.1, 41.7, 52.5, 56.2, 70.6, 174.3; $[\alpha]_D^{22}$ 76.7 (*c* 0.3, $CHCl_3$); IR (liquid film) (ν_{max}/cm^{-1}): 3366, 3303, 2952, 2918, 1738, 1437, 1245, 1174, 1022; HRMS: *m*/*e* calcd for $C_6H_{13}NO_3S_2H^+$: 212.0415, found: 212.0413.

*4.2.5. (2S,3S)-2-(tert-Butoxycarbonylamino)-3-hydroxy-4-(methyldisulfanyl) butanoic acid (**6**).* To a solution of **5** (28 mg, 0.133 mmol) and Boc_2O (57.8 mg, 0.265 mmol) in THF (0.7 mL) and MeOH (0.5 mL) was added TEA (0.056 mL, 0.399 mmol). The mixture was stirred for 2 h at room temperature. The reaction mixture was extracted with EtOAc three times. Combined organic layers were dried and purified by chromatography (Hex/EtOAc=2:1) to give (*2S*,*3S*)-*methyl 2*-(*tert-butoxycarbonylamino*)-*3-hydroxy-4*-(*methyldisulfanyl*) *butanoate* (30 mg, 75% yield) as a clear oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.43 (s, 9H), 2.42 (s, 3H), 2.71 (m, 1H), 2.88 (m, 1H), 3.78 (s, 3H), 4.42 (d, *J*=8.8 Hz, 2H), 5.32 (d, *J*=8.4 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 22.9, 28.3, 41.6, 52.8, 56.7, 70.0, 80.4, 156.1, 171.2; $[\alpha]_D^{20}$ 59.84 (*c* 0.37, $CHCl_3$); IR (liquid film) (ν_{max}/cm^{-1}): 3407, 2979, 2918, 1752, 1716; HRMS: *m*/*e* calcd for $C_{11}H_{21}NO_5S_2Na^+$: 334.0759, found: 334.0749.

(2*S*,3*S*)-methyl 2-(*tert*-butoxycarbonylamino)-3-hydroxy-4-(methyldisulfanyl) butanoate (20 mg, 0.064 mmol) was dissolved in THF (1 mL) and treated with 1 N NaOH solution (1 mL). The mixture was stirred for 2 h at rt. The reaction mixture was washed with Et_2O and the aqueous layer was adjusted to pH 3 using 1 N HCl, and extracted with Et_2O three times. The combined organic layer was dried and purified by chromatography (CH_2Cl_2/MeOH=20:1 to 15:1) to give **6** (14 mg, 74% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.39 (s, 9H), 2.38 (s, 3H), 2,70 (m, 1H), 2.84 (m, 1H), 4.44 (m, 2H), 5.45 (d, *J*=8.8 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 23.0, 28.3, 41.4, 56.6, 70.0, 80.7, 156.4, 174.7; $[\alpha]_D^{20}$ 37.56 (*c* 0.28, $CHCl_3$); IR (liquid film) (ν_{max}/cm^{-1}): 3393, 2979, 2929, 2853, 1710, 1691; HRMS: *m*/*e* calcd for $C_{10}H_{19}NO_5S_2Na^+$: 320.0602, found: 320.0602.

4.3. General procedure for native chemical ligation at threonine site

To a solution of Peptide A (1.0 equiv, 4 μmol) and Peptide B (1.5 equiv, 6 μmol) in 0.5 mL of Guanidine buffer[‡] was added 0.5 M bond-breaker® TCEP solution (Pierce) (5.0 equiv, 20 μmol). The reaction mixture was stirred at room temperature. The reactions were monitored by LC–MS and purified directly by HPLC upon consumption of the starting material.

4.4. General procedure for desulfurization

To a solution of the peptide (0.6 mM) in 200.0 μL of water (or buffer) and 100.0 μL of CH_3CN were added 200.0 μL of 0.5 M bond-breaker® TCEP solution (Pierce), 20.0 μL of 2-methyl-2-propanethiol and 10.0 μL of radical initiator (0.1 M in water). The reaction mixture was stirred at 37 °C. The reactions were monitored by LC–MS and purified directly by HPLC upon consumption of the starting material.

*4.4.1. MS characterization of peptides **7**–**46**. Compound **7**.* ESIMS calcd for $C_{56}H_{74}N_{12}O_{17}S_2$ $[M+H]^+$ *m*/*z*=1251.48, found: 1251.60.

Compound **8**. ESIMS calcd for $C_{53}H_{75}N_{13}O_{19}S$ $[M+H]^+$ *m*/*z*=1230.51, found: 1231.12.

Compound **9**. ESIMS calcd for $C_{53}H_{75}N_{13}O_{19}$ $[M+H]^+$ *m*/*z*=1198.54, found: 1198.88.

Compound **10**. ESIMS calcd for $C_{58}H_{79}N_{13}O_{17}S_2$ $[M+H]^+$ *m*/*z*=1294.53, found: 1294.79.

Compound **11**. ESIMS calcd for $C_{55}H_{79}N_{14}O_{19}S$ $[M+H]^+$ *m*/*z*=1273.55, found: 1274.08.

Compound **12**. ESIMS calcd for $C_{55}H_{80}N_{14}O_{19}$ $[M+H]^+$ *m*/*z*=1241.58, found: 1241.91.

Compound **13**. ESIMS calcd for $C_{60}H_{76}N_{12}O_{16}S$ $[M+H]^+$ *m*/*z*=1253.53, found: 1254.20.

Compound **14**. ESIMS calcd for $C_{59}H_{81}N_{13}O_{19}S$ $[M+H]^+$ *m*/*z*=1308.56, found: 1308.92.

Compound **15**. ESIMS calcd for $C_{59}H_{81}N_{13}O_{19}$ $[M+H]^+$ *m*/*z*=1276.59, found: 1277.07.

Compound **16**. ESIMS calcd for $C_{62}H_{77}N_{13}O_{15}S$ $[M+H]^+$ *m*/*z*=1276.55, found: 1276.81.

Compound **17**. ESIMS calcd for $C_{61}H_{82}N_{14}O_{18}S$ $[M+H]^+$ *m*/*z*=1331.58, found: 1331.93.

Compound **18**. ESIMS calcd for $C_{61}H_{82}N_{14}O_{18}$ $[M+H]^+$ *m*/*z*=1299.60, found: 1300.02.

Compound **19**. ESIMS calcd for $C_{49}H_{63}N_{11}O_{15}S$ $[M+H]^+$ *m*/*z*=1078.43 $[M+2H]^{2+}$ *m*/*z*=539.72, found: 1078.58, 540.12.

Compound **20**. ESIMS calcd for $C_{48}H_{68}N_{12}O_{18}S$ $[M+H]^+$ *m*/*z*=1133.46, found: 1134.01.

Compound **21**. ESIMS calcd for $C_{48}H_{68}N_{12}O_{18}$ $[M+H]^+$ *m*/*z*=1101.49, found: 1101.83.

Compound **22**. ESIMS calcd for $C_{57}H_{78}N_{12}O_{15}S$ $[M+H]^+$ *m*/*z*=1203.55, found: 1204.02.

Compound **23**. ESIMS calcd for $C_{56}H_{83}N_{13}O_{18}S$ $[M+H]^+$ *m*/*z*=1258.58, found: 1259.00.

Compound **24**. ESIMS calcd for $C_{56}H_{83}N_{13}O_{18}$ $[M+H]^+$ *m*/*z*=1226.61, found: 1227.09.

Compound **25**. ESIMS calcd for $C_{60}H_{70}N_{12}O_{16}$ $[M+H]^+$ *m*/*z*=1215.51, $[M+2H]^{2+}$ *m*/*z*=608.26; found: 1216.04, 608.71.

Compound **26**. ESIMS calcd for $C_{59}H_{76}N_{12}O_{16}S$ $[M+H]^+$ *m*/*z*=1241.52, $[M+2H]^{2+}$ *m*/*z*=621.27; found: 1241.94, 621.63.

Compound **27**. ESIMS calcd for $C_{59}H_{76}N_{12}O_{16}$ $[M+H]^+$ *m*/*z*=1209.56, $[M+2H]^{2+}$ *m*/*z*=605.29; found: 1210.14, 605.69.

Compound **28**. ESIMS calcd for $C_{50}H_{64}N_{12}O_{17}$ $[M+H]^+$ *m*/*z*=1105.46, found: 1105.80.

Compound **29**. ESIMS calcd for $C_{49}H_{70}N_{12}O_{17}S$ $[M+H]^+$ *m*/*z*=1131.48, $[M+2H]^{2+}$ *m*/*z*=566.25; found: 1131.87, 566.81.

Compound **30**. ESIMS calcd for $C_{49}H_{70}N_{12}O_{17}$ $[M+H]^+$ *m*/*z*=1099.51, found: 1099.86.

Compound **31**. ESIMS calcd for $C_{51}H_{66}N_{12}O_{17}$ $[M+H]^+$ *m*/*z*=1119.48, found: 1120.10.

Compound **32**. ESIMS calcd for $C_{50}H_{72}N_{12}O_{17}S$ $[M+H]^+$ *m*/*z*=1145.50, $[M+2H]^{2+}$ *m*/*z*=573.26; found: 1145.93, 573.68.

Compound **33**. ESIMS calcd for $C_{50}H_{72}N_{12}O_{17}$ $[M+H]^+$ *m*/*z*=1113.52, found: 1113.87.

Compound **34**. ESIMS calcd for $C_{58}H_{72}N_{12}O_{16}$ $[M+H]^+$ *m*/*z*=1193.53, found: 1193.59.

Compound **35**. ESIMS calcd for $C_{57}H_{78}N_{12}O_{16}S$ $[M+H]^+$ *m*/*z*=1219.55, found: 1220.01.

Compound **36**. ESIMS calcd for $C_{57}H_{78}N_{12}O_{16}$ $[M+H]^+$ *m*/*z*=1187.57, found: 1188.02.

Compound **37**. ESIMS calcd for $C_{44}H_{65}N_{15}O_{12}S_2$ $[M+H]^+$ *m*/*z*=1060.45, found: 1060.44.

[‡] Guanidine buffer: To 1.0 mL of 6.0 M guanidine buffer, were added 26.8 mg of Na_2HPO_4. The pH value of resulting solution was nearly 6.5.

Compound **38**. ESIMS calcd for $C_{97}H_{133}N_{27}O_{28}S$ $[M+2H]^{2+}$ m/z=1078.99, $[M+3H]^{3+}$ m/z=719.66, found: 1079.34, 719.96.

Compound **39**. ESIMS calcd for $C_{97}H_{133}N_{27}O_{28}$ $[M+2H]^{2+}$ m/z=1063.00, $[M+3H]^{3+}$ m/z=709.00, found: 1063.35, 709.23.

Compound **40**. ESIMS calcd for $C_{99}H_{134}N_{28}O_{27}S$ $[M+2H]^{2+}$ m/z=1090.50, $[M+3H]^{3+}$ m/z=727.33, found: 1090.91, 727.57.

Compound **41**. ESIMS calcd for $C_{99}H_{134}N_{28}O_{27}$ $[M+2H]^{2+}$ m/z=1074.50, $[M+3H]^{3+}$ m/z=716.68, found: 1074.79, 716.97.

Compound **42**. ESIMS calcd for $C_{88}H_{124}N_{26}O_{26}S$ $[M+2H]^{2+}$ m/z=997.46, $[M+3H]^{3+}$ m/z=665.31, found: 997.82, 665.60.

Compound **43**. ESIMS calcd for $C_{88}H_{124}N_{26}O_{26}$ $[M+2H]^{2+}$ m/z=981.47, $[M+3H]^{3+}$ m/z=654.65, found: 981.72, 654.97.

Compound **44**. ESIMS calcd for $C_{98}H_{146}N_{16}O_{46}S_2$ $[M+2H]^{2+}$ m/z=1174.46, found: 1175.42.

Compound **45**. ESIMS calcd for $C_{133}H_{199}N_{31}O_{57}S$ $[M+2H]^{2+}$ m/z=1588.18, $[M+3H]^{3+}$ m/z=1059.12, found: 1588.27, 1059.25.

Compound **46**. ESIMS calcd for $C1_{33}H_{199}N_{31}O_{57}$ $[M+2H]^{2+}$ m/z=1572.19, $[M+3H]^{3+}$ m/z=1048.46, found: 1572.66, 1048.74.

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Supplementary data

Experimental procedures, NMR spectra, LC–MS spectra, compound characterization. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tet.2010.01.067.

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