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PAPER

Microwave-assisted chemical ligation of *S*-acyl peptides containing non-terminal cysteine residues†

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An efficient approach for the synthesis of a series of *S*-acyl peptides containing internal cysteine residues has been developed and the chemical long-range ligation of these *S*-acyl peptides *via* 5-, 8-, 11- and 14-membered cyclic transition states has been investigated. Our results include the first examples of successful isopeptide ligations starting from *S*-acyl peptides containing non-terminal cysteine residues and indicate that the cyclic transition states studied in this present paper are decreasingly favored in the order of their sizes 5≫14>11≫8.

Introduction

Decades of optimization have made stepwise solid-phase peptide synthesis (SPPS) a routine and reliable tool for preparing peptides up to around 25 amino acids long;[1,3] however, yields generally decrease as the length of the polypeptide increases further. Cysteine-containing peptides are valuable intermediates for the synthesis of larger peptides and proteins utilizing the chemical ligation of unprotected peptides.[2,4–6] The ligation of an unprotected peptide thioester in aqueous solution to another unprotected peptide possessing an N-terminal cysteine with formation of an amide bond is termed native chemical ligation (NCL).[7] The development of a synthetic strategy for the preparation of a polypeptide by means of native chemical ligation relies on a suitable cysteine ligation site. Unfortunately, cysteine is a relatively rare amino acid (1.3% average content)[8] and is not always available in a terminal position. Moreover, some amino acid-cysteine bonds such as Pro-Cys, Asp-Cys, Glu-Cys and Lys-Cys can be difficult to access by chemical ligation.[8] Recently, Haase and Seitz reported a chemical ligation approach utilizing internal cysteine residues to accelerate thioester-based peptide ligations compared to direct aminolysis reactions of a cysteine-lacking control peptide.[8] However, this method required relatively long reaction times (48–72 h) and the ligation products were not isolated.[8]

The classical NCL method is limited to peptides possessing an N-terminal cysteine residue. To overcome this requirement of a specifically placed cysteine residue one approach is the use of thiol ligation auxiliaries, but unfortunately, removable cysteine mimics can sterically hinder ligation and difficulties can arise at the stage of auxiliary removal.[6,8]

Other strategies to extend the repertoire of native chemical ligation include (i) conversion of cysteine residues into alanine[9–11] and serine;[12] (ii) ligation at a β-mercapto-phenylalanine residue[13] which is subsequently desulfurized to give phenylalanine; (iii) use of a γ-thiolated valine building block as an alternative precursor to valine.[14]

An useful approach for the synthesis of cysteine containing peptides is isopeptide ligation methodology. Yoshiya *et al.*[15] synthesized *S*-acyl peptides containing N-terminal cysteine residues; subsequent *S*→*N* intramolecular acyl migration then furnished native peptide bonds. Very recently, we described the chemical ligation of selectively *S*-acylated cysteine peptides to form native peptides *via* 11- and 14-membered cyclic transition states.[16,17] This method allows the synthesis of native peptides from *S*-acyl peptides with a C-terminal cysteine without utilizing auxiliaries.

An extension of our previously reported isopeptide methodology[16,17] utilizing non-terminal cysteine residues could significantly expand the applicability of this isopeptide ligation approach. We herein document the first isopeptide ligations to form native peptides from non-terminal cysteine residues: our examples involve 11- and 14-membered transition states.

Results and discussion

Study of the feasibility of *S*→*N* acyl migration *via* an 8-membered compared with a 5-membered cyclic transition state

The protected dipeptide dimer **2** was prepared in 79% yield by mixed anhydride coupling of bis-Boc-cystine **1** and H-Gly-OCH_3 according to a literature procedure.[18] To investigate the feasibility of chemical ligation through a 5-membered transition state, the Boc-protected dipeptide dimer **2** was first reacted with

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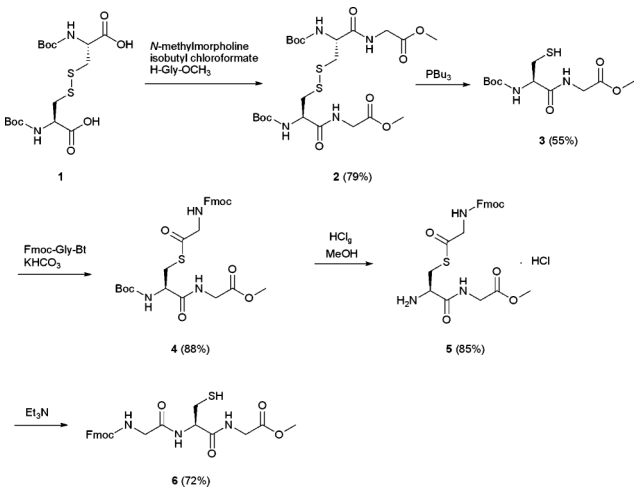
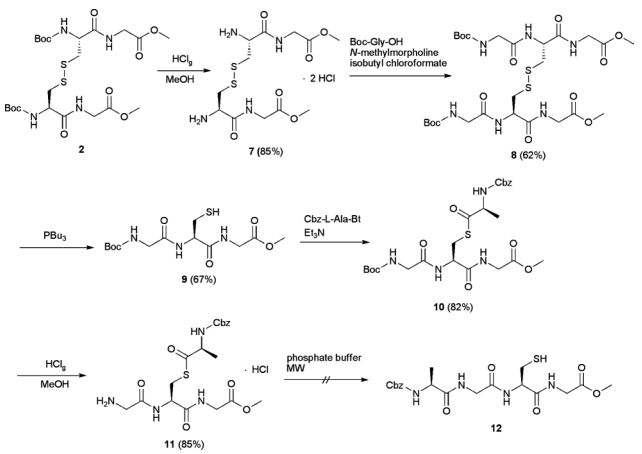
tributylphosphine to afford the dipeptide monomer **3** in 55% yield. Subsequent *S*-acylation of **3** with Fmoc-Gly-Bt[19] (Bt = benzotriazol-1-yl) furnished the *S*-acyl peptide **4** in 88% yield. Next, **4** was Boc-deprotected selectively with methanol saturated with hydrochloric acid gas. The crude HCl salt **5** was treated with triethylamine to give the corresponding free base which as expected underwent classical $S\rightarrow N$ acyl migration *via* a 5-membered transition state to furnish the native tripeptide **6** in 72% yield (Scheme 1).



Scheme 1 Chemical ligation of *S*-acyl dipeptide **5**.

The preparation of the starting material **11** for a possible $S\rightarrow N$ acyl migration *via* an 8-membered transition state is illustrated in Scheme 2. First, we deprotected the Boc-protected dipeptide dimer **2** utilizing saturated hydrochloric acid in methanol to afford the dipeptide dimer **7** as dihydrochloride (85% yield) followed by mixed anhydride coupling of **7** with 2 equiv. of Boc-glycine to furnish the novel tripeptide dimer **8** in 62% yield. Treatment of the disulfide **8** with tributylphosphine gave the tripeptide monomer **9** (67%, Scheme 2). *S*-Acylation of **9** with Cbz-L-Ala-Bt[20] in the presence of triethylamine provided the key intermediate **10** (82% yield) which on deprotection by MeOH–HCl gave the desired *S*-acyl tripeptide **11** in 85% yield (Scheme 2).

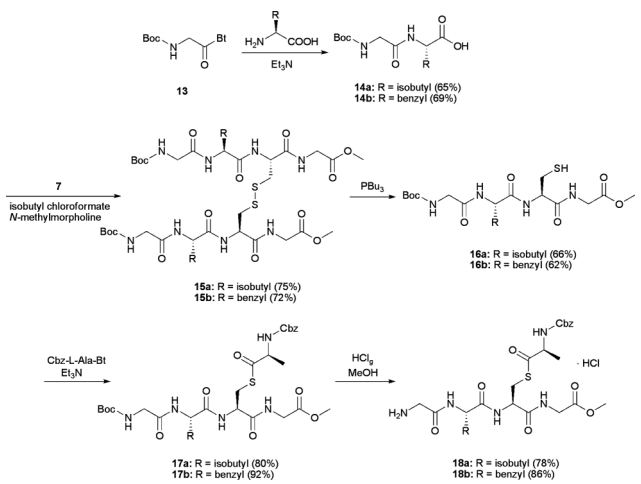


Scheme 2 Synthesis and attempted chemical ligation of *S*-acyl tripeptide **11**.

We then investigated the chemical ligation of **11** *via* an 8-membered cyclic transition state. The *S*-acyl tripeptide **11** was dissolved in a mixture 0.4 M NaH_2PO_4/Na_2HPO_4 buffer (pH = 7.8) and acetonitrile (7 : 1). Acetonitrile was used as co-solvent to dissolve the starting material. The reaction mixture was subjected to microwave irradiation for 3 h at 70 °C and 50 W (Scheme 2). Interestingly, the HPLC-MS (ESI) analysis of the reaction mixture revealed that the major component was unreacted starting material **11**. The (+)ESI-MSn spectra of the major product (*m*/*z* 455 $[M+H]^+$ ion) were nearly identical with those of **11**. Moreover, the HPLC-MS (ESI) analysis of a mixture of **11** and the product from the ligation experiment detected only one MW 454 compound, whose characteristics matched those of the *S*-acyl tripeptide **11**. Absence of the $S\rightarrow N$ acyl migration **11**→**12** suggests that the necessary $S\rightarrow N$ acyl migration *via* an 8-membered cyclic transition state for **11**→**12** is disfavored.

Study of $S\rightarrow N$ acyl migration *via* an 11-membered cyclic transition state

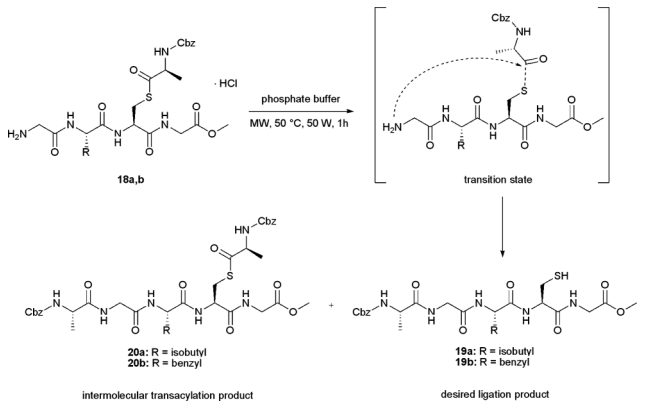
To study possible chemical ligation *via* an 11-membered cyclic transition state, we first prepared the *N*-Boc-protected dipeptides **14a,b** in 65–69% yield by peptide coupling reactions of Boc-Gly-Bt[21] with L-amino acids in partially aqueous solution in the presence of triethylamine (Scheme 3). Mixed anhydride couplings of the dipeptide dimer **7** with 2 equiv. of each of the dipeptides **14a,b** furnished the tetrapeptide dimers **15a,b** in 72–75% yield (Scheme 3). NMR analysis of **15a,b** revealed no detectable epimerization. Next, the cleavage of the disulfides **15a,b** afforded the monomer tetrapeptides **16a,b** (62–66%), which were subsequently *S*-acylated with Cbz-L-Ala-Bt in the presence of triethylamine. The Boc-protected *S*-acyl tetrapeptides **17a,b** were purified by flash column chromatography or recrystallization and then isolated in 80–92% yield. Acid-catalyzed deprotection of Boc group proceeded smoothly to form the desired *S*-acyl tetrapeptides **18a,b** (78–86%, Scheme 3).



Scheme 3 Synthesis of *S*-acyl tetrapeptides **18a,b**.

We investigated the $S\rightarrow N$ acyl migration in compounds **18a,b** *via* 11-membered cyclic transition states. Our chemical ligation experiment on **18a** was carried out under microwave irradiation at 50 °C and 50 W for 1 h. After workup, HPLC-MS (ESI) analysis

of the crude ligation mixture showed the presence of two major products in 70 : 30 ratio (88% combined crude yield[22]). The most abundant (+)ESI-MS peak was produced by the intermolecular transacylation product **20a** (MW 772) as shown by (+)ESI-MS dissociation of its *m/z* 773 $[M+H]^+$ ion. The HPLC-MS (ESI) analysis confirmed the second major product to be the desired ligation product **19a** (Scheme 4).



Scheme 4 Chemical ligation of *S*-acyl tetrapeptides **18a,b**.

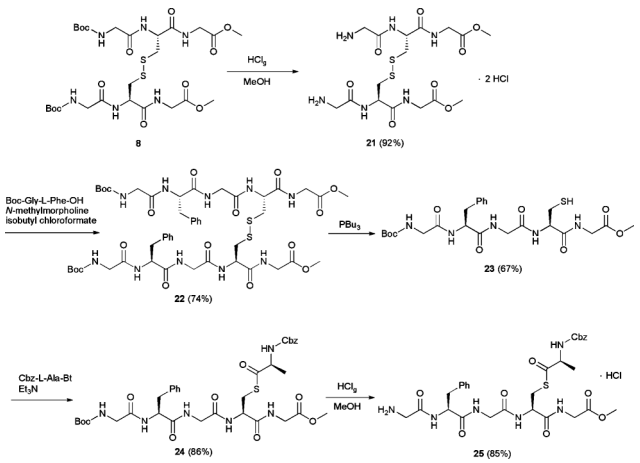
Similarly, the chemical ligation experiment of the *S*-acyl peptide **18b** under the same conditions described above provided the desired ligation product **19b**. HPLC-MS analysis revealed that this ligation experiment yielded the expected ligation product **19b** and the intermolecular transacylation product **20b** in 33 : 67 ratio (86% combined crude yield,[22] Scheme 4).

The product mixture of the ligation experiment of *S*-acyl tetrapeptide **18a** was subsequently purified by semi-preparative HPLC, which allowed the isolation of the desired ligation product **19a** as well as isolation of the intermolecular transacylation product **20a** in yields of 23% and 52%, respectively. Both products were characterized by analytical HPLC and HRMS analysis (Table 1).

The formation of the side products **20** could be explained as follows: in the first step the intramolecular $S \rightarrow N$ acyl migration provides the desired product **19**, which is later *S*-acylated by another equivalent of the starting material **18** to form the transacylation product **20** by intermolecular reaction (Scheme 4).

Study of $S \rightarrow N$ acyl migration *via* a 14-membered cyclic transition state

The synthesis of starting material **25** for a possible $S \rightarrow N$ ligation *via* a 14-membered transition state was accomplished in a five-step procedure starting from the tripeptide dimer **8**. After initial deprotection of **8** to form **21**, subsequent mixed anhydride coupling of **21** with Boc-Gly-L-Phe-OH **15b** afforded the pentapeptide dimer **22** in 74% yield. NMR analysis of **22** revealed no detectable epimerization during the peptide coupling. Cleavage of the disulfide bond in **22** furnished the monomer **23** (67%) which was *S*-acylated with Cbz-L-Ala-Bt to provide the Boc-protected *S*-acyl pentapeptide **24** (86%). Final deprotection of the Boc group in **24** afforded the desired starting material **25** (85%, Scheme 5).



Scheme 5 Synthesis of *S*-acyl pentapeptide **25**.

Chemical ligation from *S*-acyl pentapeptide **25** through a 14-membered cyclic transition state was investigated by dissolving **25** in 0.4 M phosphate buffer (pH = 7.8) and acetonitrile (7 : 1) and subjecting the mixture to microwave irradiation (50 °C, 50 W, 1h). HPLC-MS (ESI) analysis of the crude ligation mixture showed the presence of two main products in 57 : 43 ratio (87% combined crude yield[23]). The major product of this experiment was the expected ligation product **26**. Again, the intermolecular transacylation product **27** was formed as side product (Scheme 6). The subsequent separation of **26** by semi-preparative HPLC afforded the purified ligation product **26** in 41% yield, which was characterized by analytical HPLC and HRMS analysis (Table 2).

These are the first examples of successful isopeptide ligations starting from non-terminal *S*-acyl peptides and the results strongly suggest chemical long-range ligation *via* 11- and 14-membered cyclic transition states as a promising approach for the synthesis of native peptides.

Competitive ligation experiment

We repeated the chemical ligation of *S*-acyl tetrapeptide **18a** in the presence of 10 equivalents of glycinamide **28** under similar reaction conditions as described above (50 °C, 50 W, 1h). Glycinamide **28** was chosen as model compound to mimic the possible intermolecular reaction at the thioester moiety. However, our HPLC-MS

Table 1 Characterization data of isolated products **19a** and **20a**

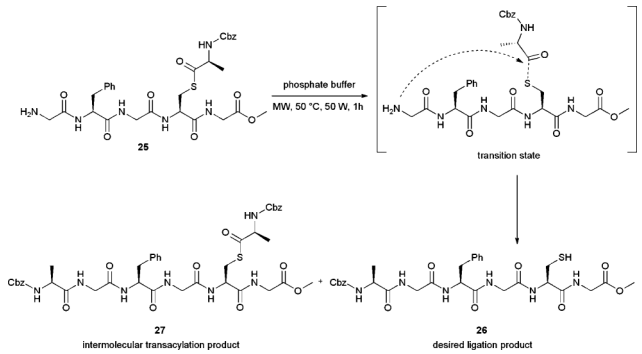
Product	Isolated yield [%]	Purity [%][b]	HRMS $[M+Na]^+$ found
19a	23	> 95	1155.4423[c,d]
20a	52[a]	> 95	795.3027[e]

[a] The isolated yield was calculated according to the following equation: isolated yield = (2 x [20a])/[18] [b] Purity based on analytical HPLC [c] Isolated as disulfide dimer [d] HRMS calc. $[M+Na]^+$: 1155.4461 [e] HRMS calc. $[M+Na]^+$: 795.2994.

Table 2 Characterization data of the isolated ligation product **26**

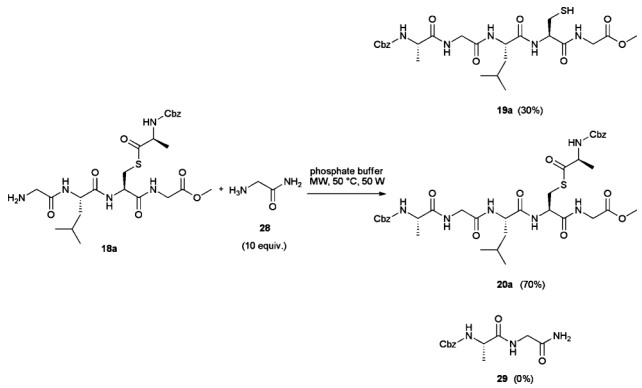
Product	Isolated yield [%]	Purity [%][a]	HRMS $[M+Na]^+$ found
26	41	> 95	1337.4629[b,c]

[a] Purity based on analytical HPLC [b] Isolated as disulfide dimer [c] HRMS calc. $[M+Na]^+$: 1337.4578.



Scheme 6 Chemical ligation experiment of *S*-acyl pentapeptide **25**.

(ESI) analysis did not detect any compound **29**, which would be the product of *N*-acylation of glycinamide **28**. Instead, the HPLC-MS (ESI) analysis of the crude ligation mixture revealed that the desired ligation product **19a** and the intermolecular transacylation product **20a** were again formed in 30 : 70 ratio (Scheme 7). The results of this competition experiment further support the intramolecular nature of our isopeptide ligation.



Scheme 7 Competitive ligation experiment of **18a** in the presence of glycinamide **28**.

Very recently, our group performed a computational investigation on the intramolecular nature of this ligation approach and found that hydrogen bonds arising in certain conformations of *S*-acyl peptides can provide additional stabilization of the cyclic transition states.[24] These hydrogen bonds clearly favor the 11-membered over the 8-membered cyclic transition state.[24]

Conclusions

In summary, we have efficiently and conveniently synthesized several novel *S*-acyl peptides containing internal cysteine residues. The chemical ligation studies of these *S*-acyl peptides *via* 5-, 8-, 11- and 14-membered cyclic transition states show that the 8-membered transition state is clearly disfavored, whereas the 11- and 14-membered transition states are relatively favored for our long-range ligation approach. These results indicate that the transition states studied in this present paper are decreasingly favored in the order of their sizes 5≫14>11≫8. Our microwave-assisted isopeptide ligation offers the following advantages: (i) short reaction times (1 h) at moderate temperature (50 °C), (ii) chemical ligation from *non-terminal* cysteine residues and (iii) avoidance of ligation auxiliaries. In contrast to the classical native chemical ligation approach, our methodology allows the isolation of the *S*-acyl peptide intermediates, which might be useful for several possible synthetic and biological applications. In order to optimize the isopeptide ligation we are currently investigating the *S*→*N* acyl migration *via* various longer and intermediate cyclic transition states.

Experimental

General procedure for Boc deprotection of peptides 4, 10, 17a,b and 24 to give the corresponding unprotected peptides 5, 11, 18a,b and 25. HCl gas was passed through a solution of peptide **4**, **10**, **17a,b** or **24** in methanol (15 mL) for 30 min. The methanol solution was concentrated under vacuum and diethyl ether (20 mL) was added. The turbid solution was left to crystallize in the freezer overnight. The solid formed was filtered and washed with dry ethyl acetate (10 mL) and diethyl ether (10 mL) and dried to give the corresponding deprotected peptide **5**, **11**, **18a,b** or **25**.

General procedure for the synthesis of peptides 9, 16a,b and 23. A mixture of tributylphosphine (0.607 g, 3 mmol) and dimer peptide **8**, **15a,b** or **22** (1.5 mmol) in MeOH : water (9 : 1, 20 mL) was stirred at rt for 2 h under argon. The solvent was evaporated and the residue was dissolved in diethyl ether (15 mL). The solution was dried over magnesium sulfate and concentrated under reduced pressure. The crude peptides were purified according to the following procedures. Peptide **9** was recrystallized from diethyl ether : hexanes. Compound **16a** was recrystallized from CH_2Cl_2 : hexanes. Compounds **16b** and **23** were recrystallized from MeOH : diethyl ether. The precipitates were washed with cold hexanes (5 mL) and CH_2Cl_2 (5 mL) and dried under reduced pressure.

General procedure for the preparation of *S*-acyl peptides 10, 17a,b and 24. Cbz-L-Ala-Bt (0.325 g, 1 mmol) was added to a mixture of **9**, **16a,b** or **23** (1 mmol) and triethylamine (0.1 g, 1 mmol) in acetonitrile (20 mL). The mixture was stirred for 3 h at rt and the solvent was removed under reduced pressure. The crude product **17a** was purified by flash column chromatography using ethyl acetate : hexanes (gradient) as eluent. The crude compounds **10**, **17b** and **24** were purified according to the following procedure. The residue was dissolved in ethyl acetate (20 mL), extracted with 2 N HCl (2 × 20 mL), water (15 mL), and brine (10 mL). The organic layer was dried over magnesium sulfate and concentrated under reduced pressure. Compound **10** was recrystallized from CH_2Cl_2 : hexanes. The *S*-acyl peptides **17b** and **24** were recrystallized from ethyl acetate : hexanes. The solids obtained were filtered, washed with diethyl ether (5 mL) and dried.

General procedure for chemical ligation of *S*-acyl peptides 11, 18a,b and 25. The respective *S*-acyl peptide hydrochloride **11**, **18a,b** or **25** (0.05 mmol) was suspended in degassed phosphate buffer (NaH_2PO_4/Na_2HPO_4) (0.4 M, pH 7.8, 7 mL) and acetonitrile (~1 mL) was added dropwise until the starting material was dissolved. The mixture was subjected to microwave irradiation (for compounds **18a,b** and **25**: 50 °C, 50 W, 1 h; for compound **11**: 70 °C, 50 W, 3 h) under argon. The reaction was allowed to cool to room temperature, acetonitrile was removed under reduced pressure and the residue was acidified with 2 N HCl to pH = 1. The mixture was extracted with ethyl acetate (3 × 20 mL), the

combined organic extracts were dried over $MgSO_4$ and the solvent was removed under reduced pressure. The ligation mixture was weighed and then a solution in methanol (1 mg mL^{-1}) was analyzed by HPLC-MS. Compounds **19a**, **20a** and **26** were subsequently isolated by semi-preparative HPLC and characterized by analytical HPLC and HRMS analysis (see ESI†).

(*R*)-1-(9*H*-Fluoren-9-yl)-3,6,10,13-tetraoxo-2,14-dioxa-7-thia-4,11-diazapentadecan-9-aminium chloride (5). White solid, 85% yield, mp 203–206 °C; Anal. Calcd for $C_{23}H_{25}N_3O_6S.HCl{\cdot}2H_2O$: C 50.78; H 5.56; N 7.72. Found: C 50.81; H 5.07; N 7.47; ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.29–3.42 (m, 2H), 3.61 (s, 3H), 3.90 (d, J = 5.4 Hz, 2H), 3.96 (d, J = 5.9 Hz, 2H), 4.08 (br s, 1H), 4.24 (t, J = 6.8 Hz, 1H), 4.34 (d, J = 6.8 Hz, 2H), 7.33 (t, J = 7.4 Hz, 2H), 7.41 (t, J = 7.4 Hz, 2H), 7.71 (d, J = 7.4 Hz, 2H), 7.88 (d, J = 7.4 Hz, 2H), 8.13 (t, J = 5.9 Hz, 1H), 8.54 (br s, 3H), 9.12 (t, J = 5.4 Hz, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 28.6, 40.9, 46.7, 50.5, 51.3, 52.0, 66.1, 120.3, 125.3, 127.2, 127.8, 140.8, 143.8, 156.6, 167.3, 169.6, 197.9.

Chemical ligation of Cys-(*S*-(Fmoc-L-Ala))-Gly-OCH$_3$ hydrochloride (5) to form native tripeptide (6). (*R*)-1-(9*H*-Fluoren-9-yl)-3,6,10,13-tetraoxo-2,14-dioxa-7-thia-4,11-diazapentadecan-9-aminium chloride (0.58 g, 1 mmol) was dissolved in a mixture of water (8 mL) and acetonitrile (24 mL). Triethylamine (0.168 mL, 1.2 mmol) was added and the mixture was stirred at room temperature for 1 h under argon. The reaction was acidified to pH 1 using 2 N HCl and extracted with ethyl acetate (2 × 10 mL). The ethyl acetate layer was washed with 2 N HCl (3 × 15 mL), sat. NaCl solution (20 mL) and dried over $MgSO_4$. The ethyl acetate solution was concentrated under vacuum and hexane was added. The turbid solution was left to crystallize in the freezer overnight. The solid formed was filtered and dried to give the corresponding native tripeptide **6**. White microcrystals, 72% yield, mp 88–92 °C; Anal. Calcd for $C_{23}H_{25}N_3O_6S$: C 58.59; H 5.34; N 8.91. Found: C 58.72; H 5.17; N 8.62; ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.89–2.97 (m, 1H), 3.02–3.07 (m, 1H), 3.64 (s, 3H), 3.70 (t, J = 7.3 Hz, 1H), 3.93–9.99 (m, 2H), 4.05–4.09 (m, 2H), 4.21–4.24 (m, 1H), 4.39 (d, J = 6.9 Hz, 2H), 5.54–5.59 (m, 1H), 6.17 (br s, 1H), 7.13–7.19 (m, 1H), 7.28 (t, J = 7.4 Hz, 2H), 7.39 (t, J = 7.4 Hz, 2H), 7.59 (d, J = 7.4 Hz, 2H), 7.75 (d, J = 7.4 Hz, 2H), 8.47 (br s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 40.8, 43.4, 46.7, 51.8, 62.2, 65.8, 74.9, 78.6, 120.1, 125.3, 127.1, 127.7, 140.7, 143.9, 156.6, 169.4, 170.0, 170.5.

(*R*)-Methyl 9-(mercaptomethyl)-2,2-dimethyl-4,7,10-trioxo-3-oxa-5,8,11-triazatridecan-13-oate (9). White microcrystals, 67% yield, mp 77–79 °C; Anal. Calcd for $C_{13}H_{23}N_3O_6S$: C 44.69; H 6.63; N 12.03. Found: C 44.29; H 6.77; N 11.67; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.45 (s, 9H), 1.85 (dd, J = 11.3, 6.5 Hz, 1H), 2.64–2.72 (m, 1H), 3.3 (br s, 1H), 3.74 (s, 3H), 3.82 (t, J = 5.2 Hz, 2H), 3.87–3.95 (m, 1H), 4.10–4.18 (m, 1H), 4.75–4.79 (m, 1H), 5.28 (br s, 1H), 7.19 (br s, 1H), 7.30 (br s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 27.1, 28.5, 41.4, 44.6, 52.5, 54.3, 80.6, 156.8, 170.3, 170.7.

(*S*)-*S*-((*R*)-2-(2-((*tert*-butoxycarbonyl)amino)acetamido)-4-((methoxycarbonyl)amino)-3-oxobutyl) 2-(((benzyloxy)-carbonyl)-amino)propanethioate (10). White microcrystals, 82% yield, mp 62–67 °C; Anal. Calcd for $C_{24}H_{34}N_4O_9S$: C 51.98; H 6.18; N 10.10. Found: C 51.70; H 5.87; N 9.75; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.44 (s, 12H), 3.37–3.41 (m, 2H), 3.72 (br s, 4H), 3.81–3.88 (m, 1H), 3.95–4.08 (m, 2H), 4.38 (t, J = 7.2 Hz, 1H), 4.66 (br s, 1H), 5.06–5.20 (m, 2H), 5.43 (d, J = 6.6 Hz, 1H), 5.55 (br s, 1H), 6.89 (d, J = 7.4 Hz, 1H), 7.18 (br s, 1H), 7.36 (br s, 5H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 18.2, 28.5, 29.7, 41.4, 44.5, 52.5, 53.4, 57.2, 67.6, 80.6, 128.3, 128.5, 128.8, 136.1, 156.3, 156.6, 170.0, 170.1, 170.8, 202.5.

(5*S*,9*R*)-methyl 9-(2-aminoacetamido)-5-methyl-3,6,10-trioxo-1-phenyl-2-oxa-7-thia-4,11-diazatridecan-13-oate hydrochloride (11). White microcrystals, 85% yield, mp 130–135 °C; Anal. Calcd for $C_{19}H_{27}ClN_4O_8S{\cdot}1.5H_2O$: C 44.06; H 5.84; N 10.82. Found: C 43.95; H 6.05; N 10.78; ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.26 (d, J = 7.0 Hz, 3H), 2.73–2.77 (m, 1H), 3.00–3.06 (m, 1H), 3.62 (br s, 5H), 3.85 (d, J = 5.2 Hz, 2H), 4.20 (t, J = 7.1 Hz, 1H), 4.54–4.56 (m, 1H), 5.02–5.11 (m, 2H), 7.37 (br s, 5H), 8.08–8.10 (m, 1H), 8.15 (br s, 3H), 8.70–8.72 (m, 1H), 8.82 (d, J = 8.5 Hz, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 17.4, 28.2, 30.3, 40.8, 51.8, 56.7, 65.8, 127.8, 128.0, 128.4, 136.8, 155.9, 166.1, 169.7, 169.9, 201.7.

Boc-Gly-L-Leu-L-Cys-Gly-OCH$_3$ (16a). White microcrystals, 66% yield, mp 152–154 °C; Anal. Calcd for $C_{19}H_{34}N_4O_7S$: C 49.34; H 7.41; N 12.11. Found: C 49.51; H 7.67; N 11.99; ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.79–0.92 (m, 6H), 1.37 (s, 9H), 1.41–1.52 (m, 2H), 1.53–1.67 (m, 1H), 2.29 (t, J = 8.5 Hz, 1H), 2.62–2.89 (m, 2H), 3.55 (d, J = 6.0 Hz, 2 H), 3.62 (s, 3H), 3.76–3.97 (m, 2H), 4.25–4.48 (m, 2H), 6.97 (t, J = 5.7 Hz, 1H), 7.88 (d, J = 8.0 Hz, 1H), 8.11 (d, J = 8.2 Hz, 1H), 8.38 (t, J = 5.8 Hz, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 21.6, 23.1, 24.0, 26.1, 28.2, 40.7, 40.8, 43.2, 50.9, 51.7, 54.8, 78.1, 155.7, 169.3, 170.1, 172.0.

Boc-Gly-L-Phe-L-Cys-Gly-OCH$_3$ (16b). White microcrystals, 62% yield, mp 158–162 °C; Anal. Calcd for $C_{22}H_{32}N_4O_7S$: C 53.21; H 6.50; N 11.28. Found: C 52.83; H 6.43; N 10.91; ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.35 (br s, 9H), 2.31 (t, J = 8.2 Hz, 1H), 2.72–2.84 (m, 3H), 3.01–3.05 (m, 1H), 3.45–3.60 (m, 2H), 3.63 (br s, 3H), 3.87 (br s, 2H), 4.42–4.44 (m, 1H), 4.58 (br s, 1H), 6.90 (br s, 1H), 7.22 (br s, 5H), 7.97 (d, J = 7.6 Hz, 1H), 8.30 (d, J = 7.6 Hz, 1H), 8.37 (br s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 26.2, 28.2, 37.4, 40.7, 43.1, 51.8, 53.8, 54.9, 78.1, 126.3, 128.0, 129.3, 137.6, 155.7, 169.3, 170.1, 171.1.

Boc-Gly-L-Leu-L-Cys(*S*-Cbz-L-Ala)-Gly-OCH$_3$ (17a). White microcrystals, 80% yield, mp 95–97 °C; Anal. Calcd for $C_{30}H_{45}N_5O_{10}S$: C 53.96; H 6.79; N 10.49. Found: C 53.67; H 6.70; N 10.30; ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.78–0.92 (m, 6H), 1.26 (d, J = 7.2 Hz, 3 H), 1.37 (s, 9H), 1.40–1.51 (m, 2H), 1.52–1.67 (m, 1H), 3.02 (dd, J = 13.3, 8.8 Hz, 1H), 3.22 (dd, J = 13.4, 5.3 Hz, 1H), 3.56 (d, J = 6.0 Hz, 2H), 3.62 (s, 3H), 3.78–3.92 (m, 2H), 4.13–4.25 (m, 1H), 4.26–4.44 (m, 2H), 4.99–5.15 (m, 2H), 6.95 (t, J = 5.9 Hz, 1H), 7.24–7.42 (m, 5H), 7.85 (d, J = 8.0 Hz, 1H), 8.07 (d, J = 7.2 Hz, 1H), 8.23 (d, J = 7.7 Hz, 1H), 8.31 (t, J = 5.6 Hz, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 17.3, 21.6, 23.1, 24.0, 28.1, 30.0, 40.7, 41.0, 43.2, 51.0, 51.7, 56.7, 65.7, 78.1, 127.7, 127.9, 128.3, 136.7, 155.7, 169.3, 169.8, 172.0, 201.9.

Boc-Gly-L-Phe-L-Cys(*S*-Cbz-L-Ala)-Gly-OCH$_3$ (17b). White microcrystals, 92% yield, mp 164–167 °C; Anal. Calcd for $C_{33}H_{43}N_5O_{10}S$: C 56.48; H 6.18; N 9.98. Found: C 56.17; H 6.20; N 9.97; ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.26 (d, J = 6.9 Hz, 3H), 1.35 (s, 9H), 2.77 (dd, J = 13.7, 9.3 Hz, 1H), 3.00–3.06 (m, 2H),

3.21 (dd, J = 13.2, 5.4 Hz, 1H), 3.63 (br s, 5H), 3.87 (br s, 2H), 4.19 (t, J = 7.3 Hz, 1H), 4.38–4.47 (m, 1H), 4.54 (br s, 1H), 5.01–5.10 (m, 2H), 6.90 (t, J = 5.4 Hz, 1H), 7.17–7.28 (m, 5H), 7.35 (br s, 5H), 7.92 (d, J = 7.7 Hz, 1H), 8.09 (d, J = 7.3 Hz, 1H), 8.38 (t, J = 5.1 Hz, 1H), 8.45 (d, J = 7.8 Hz, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 18.6, 28.9, 30.9, 38.8, 42.1, 44.7, 52.8, 54.0, 56.2, 58.4, 68.0, 80.9, 128.0, 128.9, 129.2, 129.6, 129.7, 130.5, 138.1, 138.3, 158.4, 171.5, 172.2, 172.6, 173.4, 203.6.

H-Gly-L-Leu-L-Cys(*S*-Cbz-L-Ala)-Gly-OCH$_3$ hydrochloride (18a). White microcrystals, 78% yield, mp 82–86 °C; Anal. Calcd for $C_{25}H_{38}ClN_5O_8S$: C 49.70; H 6.34; N 11.59. Found: C 49.52; H 6.41; N 11.70; ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.80–0.94 (m, 6H), 1.26 (d, J = 7.3 Hz, 3H), 1.40–1.54 (m, 2H), 1.54–1.71 (m, 1H), 3.06 (dd, J = 13.2, 8.6 Hz, 1H), 3.22 (dd, J = 13.3, 5.7 Hz, 1H), 3.59 (s, 2H), 3.62 (s, 3H), 3.84 (d, J = 5.8 Hz, 2H), 4.11–4.25 (m, 1H), 4.32–4.46 (m, 2H), 4.99–5.12 (m, 2H), 7.26–7.42 (m, 5H), 8.09 (d, J = 7.3 Hz, 1H), 8.21 (br s, 3H), 8.41 (t, J = 5.7 Hz, 1H), 8.47 (d, J = 8.2 Hz, 1H), 8.65 (d, J = 8.0 Hz, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 17.4, 21.5, 23.1, 24.1, 30.0, 40.8, 41.0, 51.3, 51.7, 56.7, 65.8, 127.8, 127.9, 128.4, 136.7, 155.8, 165.9, 169.9, 169.9, 171.6, 201.9.

H-Gly-L-Phe-L-Cys(*S*-Cbz-L-Ala)-Gly-OCH$_3$ hydrochloride (18b). White microcrystals, 86% yield, mp 172–175 °C; Anal. Calcd for $C_{28}H_{36}ClN_5O_8S{\cdot}2H_2O$: C 49.88; H 5.38; N 10.39. Found: C 50.06; H 5.70; N 10.76; ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.27 (d, J = 7.0 Hz, 3H), 2.72–2.80 (m, 1H), 3.07–3.11 (m, 2H), 3.23 (dd, J = 13.0, 5.7 Hz, 1H), 3.62 (br s, 5H), 3.86 (d, J = 5.1 Hz, 2H), 4.20 (t, J = 7.2 Hz, 1H), 4.40–4.44 (m, 1H), 4.61–4.64 (m, 1H), 5.05 (dd, J = 17.1, 12.4 Hz, 2H), 7.14–7.44 (m, 10 H), 8.11 (d, J = 7.3 Hz, 1H), 8.18 (br s, 3H), 8.49 (br s, 1H), 8.71 (d, J = 7.9 Hz, 1H), 8.79 (d, J = 8.0 Hz, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 17.4, 30.1, 37.8, 40.8, 51.8, 54.3, 56.7, 65.8, 126.4, 127.8, 127.9, 128.1, 128.4, 129.3, 136.7, 137.5, 155.8, 165.7, 169.9, 170.7, 201.9.

Boc-Gly-L-Phe-Gly-L-Cys-Gly-OCH$_3$ (23). White microcrystals, 67% yield, mp 188–193 °C; Anal. Calcd for $C_{24}H_{35}N_5O_8S_1$: C 52.07; H 6.37; N 12.65. Found: C 52.00; H 6.62; N 11.79; ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.35 (br s, 9H), 2.34 (br s, 1H), 2.68–2.83 (m, 3H), 3.00–3.05 (m, 1H), 3.62 (br s, 5H), 3.72–4.00 (m, 4H), 4.46–4.50 (m, 2H), 6.88 (br s, H), 7.23 (br s, 5H), 8.07 (d, J = 5.6 Hz, 2H), 8.41 (br s, 1H), 8.52 (br s, 1H); ^{13}C NMR (75 MHz, CD$_3$OD) δ 27.1, 28.9, 38.2, 42.1, 43.9, 44.7, 52.8, 56.8, 57.2, 81.0, 128.0, 129.7, 130.4, 138.4, 158.7, 171.8, 172.8, 174.5.

Boc-Gly-L-Phe-Gly-L-Cys(*S*-Cbz-L-Ala)-Gly-OCH$_3$ (24). White microcrystals, 86% yield, mp 126–128 °C; Anal. Calcd for $C_{35}H_{46}N_6O_{11}S$: C 55.40; H 6.11; N 11.07. Found: C 55.02; H 6.05; N 11.00; ^{1}H NMR (300 MHz, CD$_3$OD) δ (ppm) 1.36 (d, J = 7.3 Hz, 3H), 1.43 (s, 9H), 2.96 (dd, J = 13.9, 8.7 Hz, 1H), 3.17–3.24 (m, 2H), 3.43 (dd, J = 13.9, 5.1 Hz, 1H), 3.60–3.69 (m, 1H), 3.70 (br s, 5H), 3.97 (br s, 3H), 4.29 (q, J = 7.3 Hz, 1H), 4.56–4.66 (m, 2H), 5.11 (s, 2H), 7.19–7.36 (m, 10H); ^{13}C NMR (75 MHz, CD$_3$OD) δ (ppm) 18.0, 28.9, 31.0, 38.4, 42.2, 43.8, 44.7, 52.8, 54.0, 56.5, 58.4, 68.0, 81.0, 127.9, 128.9, 129.2, 129.7, 130.4, 138.2, 138.5, 158.5, 171.6, 172.5, 172.8, 174.2, 203.4.

H-Gly-L-Phe-L-Gly-Cys(*S*-Cbz-L-Ala)-Gly-OCH$_3$ hydrochloride (25). White microcrystals, 85% yield, mp 131–134 °C; Anal. Calcd for $C_{30}H_{39}ClN_6O_9S$: C 51.83; H 5.65; N 12.09. Found: C 51.78; H 5.76; N 12.26; ^{1}H NMR (300 MHz, CD$_3$OD) δ 1.40 (d, J = 7.1 Hz, 3H), 2.98–3.06 (m, 1H), 3.24–3.28 (m, 2H), 3.43–3.50 (m, 1H), 3.66–3.91 (m, 5H), 3.98–4.06 (m, 4H), 4.35 (t, J = 6.9 Hz, 1H), 4.60–4.67 (m, 2H), 5.14 (s, 2H), 7.29–7.39 (m, 10H); ^{13}C NMR (75 MHz, CD$_3$OD) δ 18.0, 31.0, 38.3, 41.8, 42.1, 43.7, 52.9, 53.9, 57.0, 58.4, 68.0, 128.0, 128.9, 129.2, 12.6, 129.7, 130.4, 138.2, 138.4, 158.5, 167.9, 171.6, 171.7, 172.5, 173.8, 203.7.

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Notes and references

1 In our peptide nomenclature the prefixes di-, tri-, tetra- etc refer to the number of amino acid residues in the main peptide chain; amino acid residues attached to sulfur are designated as *S*-acyl peptides.
2 The term isopeptide ligation is used for non-native chemical ligations from *S*-acyl peptides to furnish native peptides in a stepwise approach. A cysteine containing peptide is converted to a *S*-acyl peptide, deprotected and then transferred to a native peptide by a subsequent *S*→*N* acyl migration. In contrast to the native chemical ligation this isopeptide methodology allows the isolation and characterization of the *S*-acyl peptides and to migrate the *S*-acyl group in an independent step.
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23 The combined crude yield of **26** and **27** was calculated based on the isolated amount of the product mixture **26**:**27** according to the following equation: combined crude yield = ([26] + 2 x [27])/[25]. The *S*-deacylated pentapeptide side product was removed during the work-up.
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