Synthesis of small cyclic peptides containing the disulfide bond

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Abstract: Four cyclopeptides containing disulfide linkages were efficiently synthesized using DCC as coupling reagent under highly dilute conditions.

Keywords: Cyclic peptides, disulfide bond

Introduction

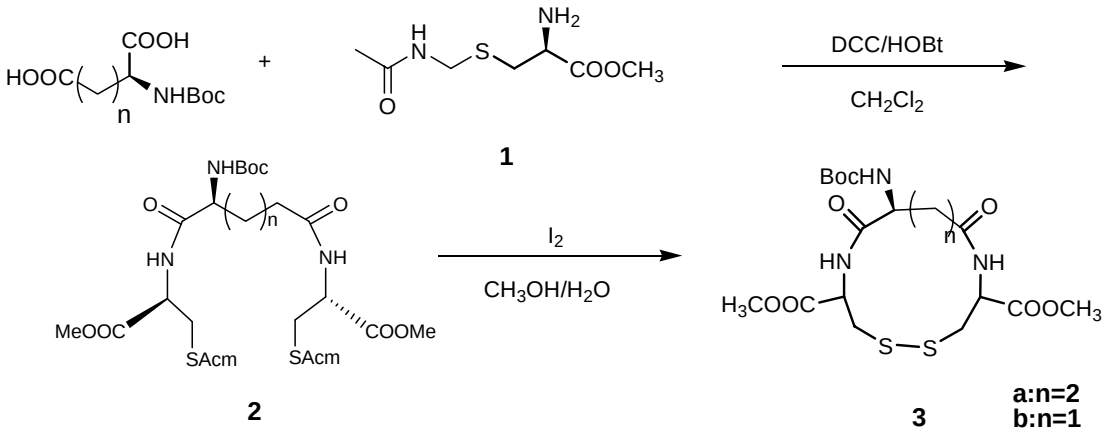
Cyclic peptides have been characterized in many natural environments, and show a wide spectrum of biological activity.[1] Bis-cystine cyclic peptides are new kinds of molecules with potential use as transporters or antagonists of target ligands.[2] Cyclization via a disulfide bridge, which plays a role in stabilizing the structure and folding of proteins by decreasing the entropy and providing a favorable local interaction[3], is a common strategy to design bis-cystine cyclic peptides.[4] It is well established that peptides containing more than one cysteine residue tend to form intermolecular disulfide linkages under high dilution and this oxidation is facilitated under basic conditions.

In the synthetic strategy, the covalent cross-linking of two nonadjacent cysteine residues in a peptide chain results in the formation of well-defined loops of the type $\overline{\text{Cys- (X)n-Cys}}$. There is considerable conformational flexibility possible within the loop.[5] For example, Karle et al. have designed amino acid-based cyclic bisureas, which were constructed by a single-step procedure to form 16-, 18- and 24-membered rings.[6] Considerable effort has been directed toward the conformational analysis of larger-membered cyclic peptide disulfides. [7] When the number of X groups separating the two Cys residues is small, the cyclic structures formed will have significantly less conformational freedom and well-defined geometries may be obtained for the cyclic disulfide segments.[5] However, very little attention has been paid to smaller ring peptide disulfides.[6, 8] Herein, we reported a novel, simple method for the synthesis of smaller ring

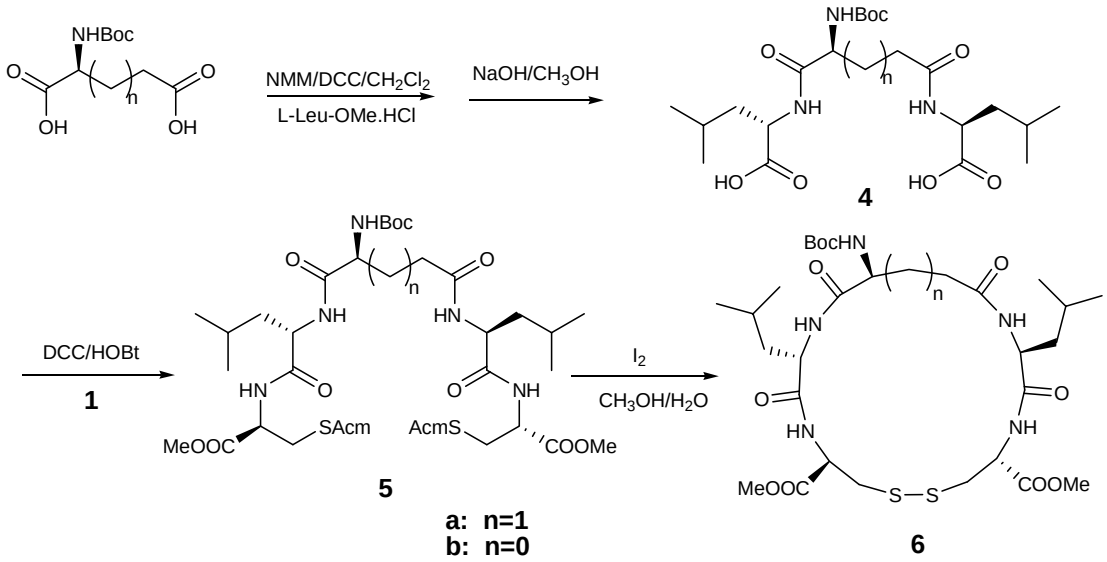
cyclotripeptides Cys-(N-Boc)-Glu-Cys **3a,** Cys-(N-Boc)-Asp-Cys **3b** and cyclopentapeptides Cys-Leu-(N-Boc)-Glu-Leu-Cys **6a**, Cys-Leu-(N-Boc)-Asp-Leu-Cys **6b**.

Results and Discussion

There are many reports on the synthesis of cyclic peptides[9], but few examples include the use of functional groups in the side chain of the amino acid to build the ring. In this paper, we use L-glutamic acid and L-aspartic acid as starting materials to synthesize some novel cyclic pseudopeptides. (The synthetic route is shown in **Schemes 1** and **2**).



Scheme 1



Scheme 2

The acetamidomethyl group (Acm) was used to protect the –SH functionality. The reactions gave the desired cyclopeptides using DCC/HOBt as a coupling reagent in fairly high yield. However, column chromatography was necessary to obtain the peptides in good purity. The linear peptides were converted to cyclic peptides to provide a large variety of cystine-based macrocyclic bisureas through 1+1 cyclization. Disulfide bond formation was effected in one step by cleavage of the Acm protective groups and subsequent oxidation of the free thiols with iodine under highly dilute conditions. The concentration was very important, almost 10^{-4} mol/L, for the conversions of **2** to **3** and **5** to **6**. The concentration of I_2 and the rate of addition also were controlled rigorously.

The cyclization products were invariably accompanied by polymeric products arising from linear oligomerization. The products were purified on a short column of silica gel using gradient elution with either petroleum ether/ethyl acetate or $CHCl_3$/MeOH as solvents. Electrospray mass spectrometry confirmed their cyclic monomeric (1+1) nature. All cyclotripeptides with L-amino acids are almost impossible to construct as the corresponding cyclodimers, the cyclohexapeptides, are the main products[10], but we found cyclomonomers as major products.

In conclusion, we have synthesized smaller ring cyclotripeptides Cys-(N-Boc)-Glu-Cys **3a,** Cys-(N-Boc)-Asp-Cys **3b** and cyclopentapeptides Cys-Leu-(N-Boc)-Glu-Leu-Cys **6a**, Cys-Leu-(N-Boc)-Asp-Leu-Cys **6b** in a novel, simple way.

Experimental Section

General Procedures. ^{1}H NMR spectra were recorded on a Varian INOVA 400 (MHz) NMR spectrometer. The chemical shifts are reported in ppm downfield to $CDCl_3$ resonance (δ=7.27) for ^{1}H NMR. ^{13}C NMR data were collected on a Varian INOVA (200MHz) NMR spectrometer with complete proton decoupling. The chemical shifts are reported in ppm downfield to the central $CDCl_3$ resonance (δ=77.0) for ^{13}C NMR. Coupling constants in ^{1}H NMR are in Hz. IR spectra were recorded on a Shimadzu FTIR-4200 spectrometer as KBr pellets or thin films on KBr plates. Melting points were measured on a XRC-1 melting point apparatus and are uncorrected. ESI mass spectra were performed on a Finnigan LCQ^{DECA} and High-Resolution MS spectral date were recorded on a Bruker Daltonics Bio TOF.

General procedure

(S-Acm)-L-Cys-OMe (1). (S-Acm)-L-Cys-OMe·HCl can be synthesized by literature procedure.[11] To a cold solution of saturated Na_2CO_3 (25 mL) was added (S-Acm)-L-Cys-OMe·HCl (2.425 g, 10 mmol). The mixture was stirred for 10 min at 0 ^oC and kept at pH 9. The mixture was filtered and the residue was extracted with CH_2Cl_2. The organic layer was washed

until neutral pH with brine then dried over Na_2SO_4. The solvent was evaporated in vacuo to yield (S-Acm)-L-Cys-OMe **1** as a colorless oil.

MeO-(S-Acm)Cys-(N-Boc)-Glu-γ-(S-Acm)Cys-OMe (2a). 1 (0.989 g, 4.8 mmol), Boc-L-Glu-OH (0.494 g, 2 mmol) and HOBt (0.54 g, 4 mmol) were placed in THF (30 mL) at 0 ^oC. DCC (0.865 g, 4.2 mmol) was then added and the mixture was stirred at 0 ^oC for 2 h and then warmed to room temperature over 20 h under nitrogen. DCU was filtered off on celite and the solvent was evaporated under reduced pressure. The residue was taken up in CH_2Cl_2, the organic layers were washed successively with 0.5 M HCl, saturated $NaHCO_3$, brine and finally dried over Na_2SO_4 and concentrated in vacuo to yield a light yellow solid. The residue was purified by column chromatography (ethyl acetate/petrol ether = 3/1) ($CHCl_3$/MeOH = 15/1) to give compound **2a** (1.02 g) as a white powder in 82% yield. M. p. 116-118 ^oC. ^1H NMR (δ, $CDCl_3$, TMS): 7.81 (d, 1H, Cys-N-H), 7.69 (d, 1H, Cys-N-H), 7.20 (s, 1H, Acm-N-H), 6.84 (s, 1H, Acm-N-H), 5.44 (d, 1H, Glu-N-H), 4.85-4.96 (m, 2H, Cys-αH), 4.47 (s, 1H, Glu-αH), 4.17-4.32 (m, 4H, Acm-CH_2), 3.79 (s, 3H, -$COOCH_3$), 3.78 (s, 3H, -$COOCH_3$), 2.93-3.21 (m, 4H, Cys-βH), 2.39-2.45 (m, 2H, Glu-γH), 2.04 (s, 3H, -$COCH_3$), 2.20 (s, 3H, -$COCH_3$), 1.74 (s, 2H, Glu-βH), 1.44 (s, 9H, Boc-CH_3). ESI-MS: m/z=624.7 $[M+1]^+$, 646.1 $[M+Na]^+$.

MeO-(S-Acm)Cys-(N-Boc)-Asp-γ-(S-Acm)Cys-OMe (2b) was obtained according to the procedure leading to **2a**. Yield: 0.99 g (81%). M. p. 97-99 ^oC. ^1H NMR (δ, $CDCl_3$, TMS): 7.41 (d, 1H, Cys-N-H), 7.17 (d, 1H,Cys-N-H), 6.81 (s, 1H,Acm-N-H), 6.70 (s, 1H, Acm-N-H), 5.83 (d, 1H, Asp-N-H), 4.77- 4.78 (m, 2H,Cys-αH), 4.56 (s, 1H, Asp-αH), 4.28-4.50 (m, 4H, Acm-CH_2), 3.76 (s, 3H, -$COOCH_3$), 3.74 (s, 3H, -$COOCH_3$), 2.72-3.05 (m, 4H, Cys-βH), 2.04 (s, 3H, -$COCH_3$), 2.01 (s, 3H, -$COCH_3$). 1.79 (s, 2H, Asp-βH), 1.46 (s, 9H, Boc-CH_3), ESI-MS: m/z=610.3 $[M+1]^+$, 632.1.1 $[M+Na]^+$.

Cys-(N-Boc)-Glu-Cys (**3a**): **2a** (62.3 mg, 0.1 mmol) was dissolved in 500 mL methanol-water (v/v = 75%). A solution of iodine (1.55 g, 6 mmol) in freshly distilled methanol (60 mL) was added over 2h with vigorous mechanical stirring. Stirring was continued at ambient temperature for another 4h. The solvent was evaporated in vacuo below 40 ^oC and the residue was dissolved in CH_2Cl_2 to give a yellow solution. The organic layer was washed with 1% $Na_2S_2O_3$ until the color of the methanol solution had completely disappeared. The solvent was dried over Na_2SO_4 and evaporated in vacuo to give a yellow solid, the residue was purified on silica gel using $CHCl_3$/EtOAc = 2/1, $CHCl_3$/MeOH = 20/1 for gradient elution. The target product **3a** (24.9 mg) was obtained as a white powder in 52% yield. M. p. 145-147 ^oC. IR (KBr, cm^{-1}): 3313, 3058, 2934, 1744, 1655, 1537, 1437, 1368, 1250, 1168, 1092, 1028, 863, 670, 468. ^1H NMR (δ, $CDCl_3$, TMS): 7.31 (d, J=8Hz, 1H, Cys-N-H), 7.00 (s, 1H, Glu-N-H), 6.52 (d, J=6.8Hz, 1H, Cys-N-H), 4.93 (m, 1H, Cys-αH), 4.78 (m, 1H, Cys-αH), 4.51 (m, 2H, Glu- αH), 3.80 (s, 3H, -$COOCH_3$), 3.75 (s, 3H, -$COOCH_3$), 3.41-3.51, 2.92-3.00 (m, 4H, Cys-βH), 2.60 (m, 2H, Glu- γH), 2.02-2.37 (m, 2H, Glu-βH), 1.49 (s, 9H, Boc-CH_3). ^{13}C NMR (δ, $CDCl_3$, TMS): 173.0, 172.1, 171.5, 170.9, 155.8, 80.2, 54.8, 53.5, 53.0, 52.8, 52.7, 33.3, 32.1, 30.9, 28.2, 23.1. ESI-MS: m/z=479.7 $[M]^+$, 502.1 $[M+Na]^+$. HRMS: $C_{18}H_{29}N_3NaO_8S_2$: calcd 502.1288, found 502.1299.

Cys-(N-Boc)-Asp-Cys **(3b)** was obtained according to the procedure used for **3a**. Yield: 21.9 mg (47%). M. p. 155-157 °C. IR (KBr, cm^{-1}): 3317, 2952, 2931, 1739, 1693, 1650, 1525, 1249, 1209, 1174, 1032, 656. ^{1}H NMR (δ, $CDCl_3$, TMS): 7.48(s, 1H, Asp-N-H), 6.81 (d, J=7.2Hz, 1H, Cys-N-H), 6.29 (d, J=7.2Hz, 1H, Cys-N-H), 4.78-4.83 (m, 2H, Cys-αH), 4.48 (s, 1H, Asp-αH), 3.78 (s, 6H, -$COOCH_3$), 3.10-3.25, 2.71-2.77 (m, 4H, Cys-βH), 2.46-2.50 (m, 2H, Asp-βH), 1.49 (s, 9H, Boc-CH_3). ^{13}C NMR (δ, $CDCl_3$, TMS): 170.9, 170.2, 169.8, 169.6, 155.8, 80.7, 53.3, 53.0, 52.7, 51.5, 33.9, 30.9, 28.2. ESI-MS: m/z= 464.1 $[M-1]^-$, 500.1 $[M+Cl]^-$. HRMS: $C_{17}H_{27}N_3NaO_8S_2$: calcd 488.1132, found 488.1146.

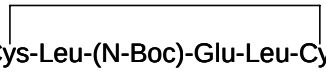
HO-Leu-(N-Boc)-Glu-γ-Leu-OH (4a). A solution of L-Leu-OMe·HCl (8.712 g, 48 mmol) in CH_2Cl_2 (120 mL) was stirred for 30 mins at 0 °C, whereupon NMM (5.3 mL, 48 mmol) was added. The solution was then stirred until completely dissolved and Boc-L-Glu (4.940 g, 20 mmol) was added. The mixture was stirred at 0 °C for 30 min and then DCC (8.652 g, 42 mmol) was added. The mixture was stirred for a further 2h at 0 °C. The temperature was then increased to r. t. and stirring was continued overnight. The solvent was evaporated under reduced pressure and DCU was filtered off on celite. The residue was taken up in CH_2Cl_2 (150 mL). The organic layers were washed successively with 1M HCl, saturated $NaHCO_3$, brine and finally dried over Na_2SO_4 and concentrated in vacuo to yield a white powder. The product was used for the next step without further purification. The white powder was dissolved in CH_3OH (30 mL) and 2 M NaOH (40 mL) was added. The reaction was monitored by TLC and after 1.5 h, it was neutralized to pH 7-8 by 1 M HCl. Methanol was removed in vacuo. And the pH was adjusted to 3-4 by addition of 0.5 M HCl. The resulting precipitate was filtered and washed with CH_3OH and water. The colorless product **4a** (6.91 g) was obtained in 73% yield. M. p. 116~118 °C, ^{1}H NMR (δ, $CDCl_3$, TMS): 8.20 (s, 1H, Leu-N-H), 7.77 (s, 1H, Leu-N-H), 5.40 (s, 1H, Glu-N-H), 4.58 (s, 1H, Leu-αH), 4.46 (s, 1H, Leu-αH), 4.16 (s, 1H, Glu-αH), 3.21-3.82 (br, 2H, Leu-OH), 2.08-2.16 (m, 2H, Glu-γH), 1.92 (m, 2H, Glu-βH), 1.77-1.56 (m. 4H, -C*H*$_2$-CH$(CH_3)_2$), 1.55-1.62 (m, 2H, -C*H*$(CH_3)_2$), 1.41 (s, 9H, Boc-CH_3), 0.92 (m, 12H, -CH(C*H*$_3)_2$). ESI-MS: m/z=474.1 $[M+H]^+$.

HO-Leu-(N-Boc)-Asp-γ-Leu-OH (4b). It was obtained according to the procedure used for **4a**. Yield: 6.52 g (71%). M. p. 180~182 °C. ^{1}H NMR (δ, $CDCl_3$, TMS): 7.94 (d, 1H, Leu-N-H), 7.77 (d, 1H, Leu-N-H), 6.85 (d, 1H, Asp-N-H), 4.04-4.21 (m, 3H, αH), 3.81-4.36 (br, 2H, -COOH), 2.41 (m, 2H, Asp-βH), 1.58-1.68 (m, 2H, -C*H* $(CH_3)_2$), 1.42-1.51 (m, 4H, -C*H*$_2$-CH $(CH_3)_2$), 1.36 (s, 9H, Boc-CH_3), 0.85 (m, 12H, -CH (C*H*$_3)_2$). ESI-MS: m/z= 460.1 $[M+H]^+$.

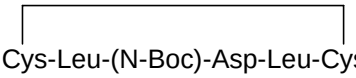
MeO-(S-Acm)Cys-Leu-(N-Boc)-Glu-γ-Leu-(S-Acm)Cys-OMe (5a). 1 (0.989 g, 4.8 mmol), **4a** (0.946 g, 2 mmol) and HOBt (0.54 g, 4 mmol) were placed in THF (30 mL) at 0 °C, whereupon DCC (0.865 g, 4.2 mmol) was added. The mixture was stirred at 0 °C for 2h and then warmed to r. t. for 20 h under nitrogen. DCU was filtered off on celite and the solvent was evaporated under reduced pressure. The residue was taken up in CH_2Cl_2 (100 mL), The organic layers were washed successively with 0.5 M HCl, saturated $NaHCO_3$, brine and finally dried over Na_2SO_4 and concentrated in vacuo to afford a light yellow solid. The residue was purified by column

chromatography (ethyl acetate/petrol ether = 3/1 and $CHCl_3$/MeOH = 15/1) to give compound **5a** (1.44 g) as a colorless powder in 85% yield. M. p. 142-144 ^oC, ^1H NMR (δ, $CDCl_3$, TMS): 9.07 (d, 1H, Cys-N-H), 8.67 (s, 1H, Leu-N-H), 7.97 (s, 1H, Leu-N-H), 7.84 (d, 1H, Cys-N-H), 7.01 (s, 2H, Acm-N-H), 5.07 (d, 1H, Glu-N-H), 4.95 (m, 1H, Leu-αH), 4.85 (m, 1H, Leu-αH), 4.39-4.54 (m, 4H, Acm-CH_2), 4.49 (m, 1H, Cys-αH), 4.31 (m, 1H, Cys-αH), 4.00 (s, 1H, Glu-αH), 3.77 (s, 3H, -$COOCH_3$), 3.75 (s, 3H, -$COOCH_3$), 3.18 (d, 2H, Cys-βH), 3.02 (d, 2H, Cys-βH), 2.06-2.27 (m, 2H, -C*H*$(CH_3)_2$), 2.03 (d, 6H, Acm-CO-CH_3), 1.94 (m, 2H, Glu-γH), 1.75-1.90 (m, 2H, Glu-βH), 1.54-1.71 (m, 4H, Leu-βH), 1.39 (s, 9H, Boc-CH_3), 0.96 (m, 12H, -CH(C*H*$_3)_2$). ESI-MS: m/z= 850.1 $[M+H]^+$.

MeO-(S-Acm)Cys-Leu-(N-Boc)-Asp-γ-Leu-(S-Acm)Cys-OMe (5b) was obtained according to the procedure used for **5a.** Yield: 1.35 g (81%). M. p. 126~129 ^oC. ^1H NMR (δ, $CDCl_3$, TMS): 7.93 (d, 1H, Leu-N-H), 7.71 (d, 1H, Leu-N-H), 7.56 (d, 1H, Cys-N-H), 7.27 (d, 1H,Cys-N-H), 7.15 (d, 2H, Acm-N-H), 5.75 (s, 1H, Asp-N-H), 4.79 (s, 2H, Cys-αH), 4.32-4.57 (m, 4H, Acm-CH_2), 4.15 (d, 1H, Leu-αH), 3.67 (d, 6H, -$COOCH_3$), 3.48 (m, 1H, Leu-αH), 3.33 (m, 1H, Asp-αH), 3.00-3.12 (m, 4H, Cys-CH_2), 2.59-2.71 (m, 2H, Asp-βH), 1.89-2.05 (m, 6H, Acm-CH_3), 1.68 (m, 2H, -C*H*$(CH_3)_2$), 1.58-1.67 (m, 4H, Leu-βH), 1.43 (s, 9H, Boc-CH_3), 0.94 (m, 12H, -CH(C*H*$_3)_2$). ESI-MS: m/z=836.1$[M+H]^+$, 858.1 $[M+Na]^+$.



(6a). 5a (85 mg, 0.1 mmol) was dissolved in 500 mL methanol-water (v/v=75%). A solution of iodine (64.6 mg, 0.25 mmol) in freshly distilled methanol (200 mL) was added over 2 h with vigorous mechanical stirring. Stirring was continued at ambient temperature for another 4 and the solvent was evaporated in vacuo below 40 ^oC to about 100 mL. White solid appeared and was filtered. The residue was dissolved in CH_2Cl_2 to give a yellow solution. The organic layer was washed with 1% $Na_2S_2O_3$ until the color of the methanolic solution had completely disappeared. The solvent was dried over Na_2SO_4 and evaporated in vacuo to give yellow solid. The residue was purified on silica gel using $CHCl_3$/EtOAc = 2/1, $CHCl_3$/MeOH = 20/1 for gradient elution and the target product **6a** (30.3 mg) was obtained as a colorless powder in 43% yield. M. p. 166~168 ^oC, ^1H NMR (δ, $CDCl_3$, TMS): 7.45 (s, 1H, Leu-N-H), 7.14 (d, 1H, Cys-N-H), 6.83 (d, 1H, Cys-N-H), 6.61 (s, 1H, Leu-N-H), 4.85 (s, 1H, Leu-αH), 4.52-4.61 (m, 1H, Cys-αH), 4.48 (s, 1H, Glu-αH), 4.34 (s, 1H, Leu-αH), 4.07-4.17 (m, 1H, Cys-αH), 3.74 (s, 6H, -$COOCH_3$), 3.19-3.29 (m, 2H, Cys-βH), 2.98-3.19 (m, 2H, Cys-βH), 2.30-2.11 (m, 2H, -C*H*$(CH_3)_2$), 1.91 (m, 2H, Glu-γH), 1.65-1.77 (m, 4H, -C*H*$_2$-CH$(CH_3)_2$), 1.48-1.63 (m, 2H, Glu-βH), 1.40 (s, 9H, Boc-CH_3), 0.91 (m, 2H, -CH(C*H*$_3)_2$). ESI-MS: m/z=704.2 $[M-1]^-$, 740.1 $[M+Cl]^-$. HRMS: $C_{30}H_{51}N_5NaO_{10}S_2$: calcd 728.2975, found 728.2997.



(6b) was obtained according to the procedure used for **6a**. Yield: 24.9 mg (36%). M. p. 140~142 ^oC. ^1H NMR (δ, $CDCl_3$, TMS): 7.58 (s, 1H, Leu-N-H), 7.15 (d, 1H, Cys-N-H), 6.84 (d, 1H, Cys-N-H), 6.41 (s, 1H, Leu-N-H), 5.69 (s, 1H,Glu-N-H), 4.81 (m, 1H, Leu-αH), 4.38-4.66 (m, 2H, Cys-αH), 4.16 (m, 1H, Leu-αH), 3.78 (d, 6H, -$COOCH_3$), 3.49 (s, 1H, Glu-αH), 3.09-3.23 (m, 2H, Cys-βH), 2.64-2.78 (m, 2H, Cys-βH), 1.96 (d, 2H, Asp-βH),

1.77-1.84 (m, 2H, -C*H*(CH_3)$_2$), 1.58-1.74 (m, 4H, -C*H*$_2$-CH(CH_3)$_2$), 1.45 (s, 9H, Boc-CH_3), 0.96 (m, 12H, -CH(C*H*$_3$)$_2$). ESI-MS: m/z=690.1 $[M-1]^-$, 726.1 $[M+Cl]^-$. HRMS: $C_{29}H_{49}N_5NaO_{10}S_2$: calcd 714.2819, found 714.2845.

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