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A Study of the Use of NH_4I for the Reduction of Methionine Sulfoxide in Peptides containing Cysteine and Cystine[1]

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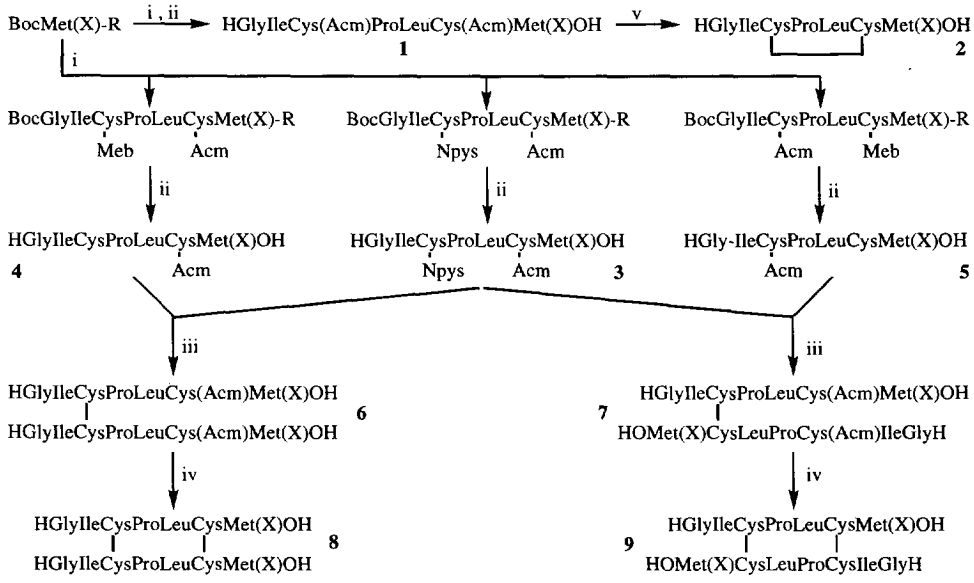
Abstract: Methionine sulfoxide reduction by NH_4I has been studied in some disulfide containing peptides. In general, this reagent has proved to be effective in neat TFA at 0°C, with the obtention of the unprotected peptides in more than 99% yields and without reduction of the disulfide bridge bond. The use of Me_2S as an additive resulted in faster reactions and disulfide scrambling was not observed. Whereas the Acm group proved to be stable to the reaction conditions, unprotected cysteine containing peptides afforded the corresponding parallel dimers.

INTRODUCTION

The main problems associated with methionine are alkylation and oxidation at the sulfur atom. Carbocation formation during deprotection steps provokes side chain t-butylation, methylation or benzylation processes to give sulfonium salts which can undergo subsequent degradation reactions.[2] Oxidation of methionine to the corresponding sulfoxide has also been detected in peptide synthesis[3] and during disulfide bridge formation.[4] We observed methionine oxidation during the photolytic cleavage[5] of one of the uteroglobin protected fragments that were designed for the convergent solid phase synthesis[6] of this protein. The presence of ten methionine residues and two disulfide bridges in Uteroglobin prompted us to consider the protection of this amino acid and its reduction after disulfide formation. Iselin et al[7a] introduced the use of methionine sulfoxide as a protected derivative to circumvent side reactions of the Met thioether and, since then, a number of methods for its reduction have been described. Many reducing reagents have proved to be suitable,[2,7] but none of them has been used enough to be considered of general application. According to the results above mentioned, methionine protection has to be considered when cysteine residues are oxidized, usually at the end of the synthesis, in peptides that contain both amino acids. In general, methionine sulfoxide reduction has been tested in peptides without disulfide bridges. Thiols have been the most widely used[2,7a-7c] but they are incompatible with disulfide containing peptides. Other reducing systems have been studied,[7a,7c-7i] but reduction has been performed using harsh conditions or reagents difficult to handle in most cases. However, iodide/$TiCl_4$[8] and iodide/TFA[9] have proved to be suitable systems for reduction of methionine sulfoxide under mild conditions and, recently, the deprotection of the amino acid with NH_4I/TFA in the presence of disulfide bridges has been described.[9c,9d] This result prompted us to get more insight on this methodology through its application to the reduction of two 14 amino acid cyclic peptides with two disulfide bridges and two methionine sulfoxide residues each (**8a** and **9a**, scheme 1). Synthetic intermediates **4a-7a** were also studied in order to explore the scope of the reaction.

RESULTS AND DISCUSSION

Peptides **8a** and **9a** were synthesized by the solid phase methodology,[10] as shown in scheme 1. Reduced peptides **8b** and **9b** were also obtained in order to provide standards for finding suitable HPLC analytical conditions for the study of the methionine sulfoxide reduction.

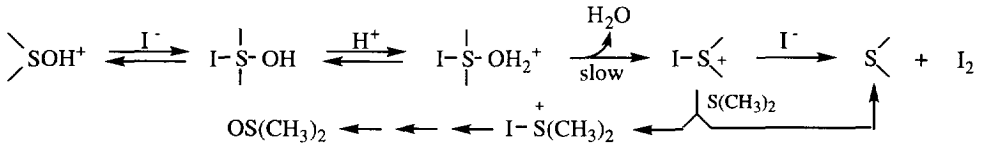


R: oxymethyl-PAM-polystyrene. X = O, methionine sulfoxide (**1a-9a**); X = none, deprotected methionine (**1b-9b**). i) Activation: Boc-amino acid, 3 eq; TBTU, 2.96 eq; DIEA, 8.5 eq; DMF, 8 min. Deprotection: neat TFA, 2+8 min. Coupling; DMF, 12 min. ii) HF (10% anisole , X=O ; 10% p-cresol , X=none), 0°C, 1h. iii) **3** , 1 eq; 10% aqueous AcOH , pH 3 , 24 h, r.t. iv) I_2 , 10 eq; X=O: 80% aqueous AcOH, 3 h; X=none: neat AcOH, 5 h. v) I_2, 32 eq, 80% aqueous AcOH, r.t. (X=O: 30 min, X=none: 40 min).

Scheme 1

The sequences of the monomers were assembled using the acid-labile Boc amino protecting group and a PAM anchoring linkage in both cases. Side-chain protection of cysteine was provided by Meb, Npys and Acm groups with the object of performing disulfide formation in a sequential way.[11] Commercially available Boc-Met(O)-OCH_2-PAM-polystyrene and Boc-Met-OCH_2-PAM-polystyrene were used as polymeric supports. Boc amino acids were coupled manually using TBTU / DIEA activation in DMF,[12] but DCC had to be used in the case of Boc-Cys(Npys)-OH because this derivative proved to be unstable in the conditions required for the activation with TBTU / DIEA. Acidolytic cleavage of the peptide-resins with HF afforded monomers **3**, **4** and **5**,[13] which were used after purification for the preparation of **8** and **9**, as shown in scheme 1 (see experimental section for details).

Sulfoxides react with halides in the presence of aqueous acid to afford sulfides when iodide is used.[14] Experimental results support a general mechanism (scheme 2) that involves nucleophilic attack of the halide at the sulfur atom of the protonated sulfoxide to afford a tetracovalent sulfur intermediate which undergoes release of water after further protonation to give an halosulfonium ion as the rate determining process. Sulfide is formed from these species with the obtention of I_2 as a by-product.



scheme 2

The use of NH_4I for methionine deprotection by reduction of its sulfoxide in methionine containing peptides was introduced by Izeboud et al[9a] but, since then, only a few more examples have been described. While the results achieved are promising, this reagent seems to be controversial when using disulfide containing peptides. The stability of disulfide in the presence of NH_4I has been questioned by Yajima et al[9c] and the use of a mixture of NH_4I and Me_2S has been proposed in order to avoid this drawback. However, Büllesbach et al[9d] have described the reduction of methionine protected human relaxin (a peptide containing three disulfide) in the absence of Me_2S. In the light of these results, we decided to focus our attention on the study of this reaction using similar conditions to those described by these authors, in order to evaluate the necessity of using Me_2S to carry out methionine sulfoxide reduction. Special attention was paid to disulfide stability during methionine sulfoxide reduction in peptides **8a** and **9a**, but other features of this reaction were explored. I_2 formation during the process moved us to check the possibility of taking advantage of that fact in order to perform *in situ* disulfide formation when Cys(Acm) and / or free-cysteine containing peptides were used. In this sense, peptides **4a**, **5a**, **6a** and **7a** were also considered in this study. The results are shown in table 1.

We had already proved the suitability of this methodology for methionine deprotection in the synthesis of the disulfide containing peptide **2b** (scheme 1). We observed that cyclization of the unprotected methionine peptide **1b** in the presence of I_2 and neat AcOH proceeded very slowly and with the formation of by-products. The use of aqueous 80% AcOH speeded up the process but partial oxidation of methionine was detected, among other products, resulting in the formation of **2a** with a 20% yield after reverse phase chromatography. These results prompted us to carry out the preparation of **2a** and further reduction of this peptide to **2b**, which was performed succesfully with NH_4I in TFA. The reaction was complete in less than one hour and no other products were observed when Me_2S was used as an additive, as shown in figure 1.

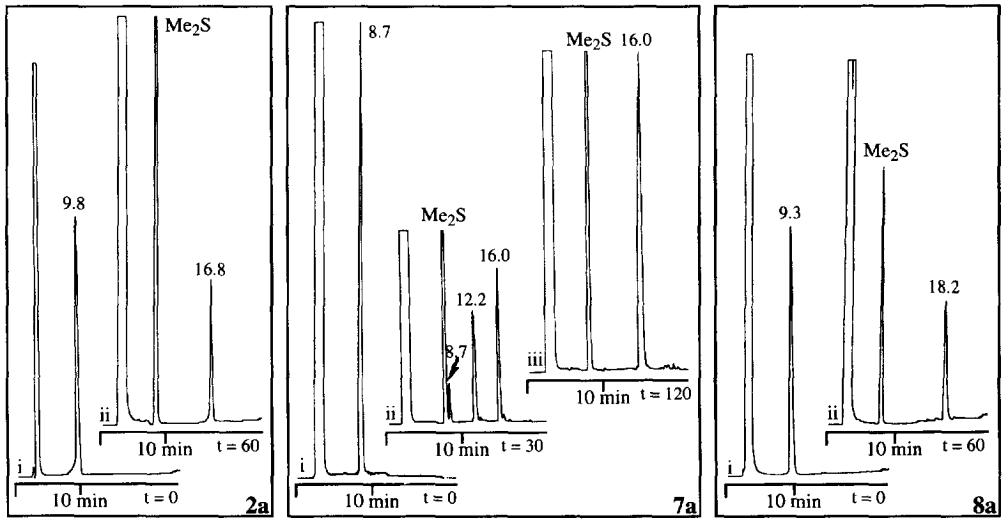
table 1

protected peptide	**1a**[c]	**2a**[c]	**4a**[d,f]	**5a**[c,f]	**6a**[c]	**7a**[d]	**8a**[c]	**9a**[e]
NH_4I[a]	54.1	21.9	37	72.5	>99	18	76	94.9
NH_4I / Me_2S[b]	>99	>99	76	>99	42.7	49.8	>99	93.9

The results are given in percentages of reduced product as determined by HPLC. a) Reaction conditions: peptide: 1.5 mM-2.2mM; NH_4I: 13-15 eq (monomers) or 5-7 eq (dimers); TFA at 0°C. b) 1:1 Molar ratio, same experimental conditions. c) 1 hour. d) 0.5 Hours. e) 26 Hours when NH_4I was used; 32 hours when the reaction was performed in the presence of Me_2S. f) Parallel dimers were formed.

In general, reductions were faster in the presence of the sulfide, which could be explained in terms of a combined effect of Me_2S and iodide (scheme 2). Oxygen exchange between sulfoxides and sulfides has been described to proceed under acidic conditions.[7(f,g),14] Kinetic studies on this process support the participation of

the sulfide as a nucleophile before or during the rate determining step.[15] Whereas the dependence of reaction rates on the acidity has been demonstrated, a catalytic effect of halide ions have been shown, especially in the case of chloride, in aqueous[16] and non-aqueous solvents.[15b] In this sense, reduction of methionine sulfoxide with Me_2S in aqueous HCl has been reported.[17] Halide interchange between halosulfonium cations as the rate limiting step could account for the experimental results in this case (see scheme 2).[16] Experiments carried out in our laboratory showed that Me_2S alone was not capable of reducing the amino acid in neat TFA. Sulfide protonation under these conditions along with the use of low concentrations of this product could explain this result. The fact that methionine sulfoxide reduction has been shown to be more effective in the presence of Me_2S and that I_2 formation has always been observed in our particular case would indicate, bearing in mind the results above mentioned, that iodide and sulfide compete for the attack to the iodosulfonium cation, resulting in faster reaction rates. However, the possibility of sulfide participation in earlier steps has to be also considered.



Chromatographic conditions: linear gradient from 20% to 40% of CH_3CN in 20 min, 1 mL/min, 220 nm. Reaction conditions: 1.56 mM of **2a** and 15 eq of NH_4I, 2.27 mM of **7a** and 5 eq of NH_4I, 1.58 mM of **8a** and 7 eq of NH_4I; TFA, 0°C; Me_2S / MH_4I, 1:1 molar ratio; reaction times are expressed in min. Retention times: **2a**, 9.8 min and **2b**, 16.8 min; **7a**, 8.7 min and **7b**, 16.0 min; **8a**, 9.3 min and **8b**, 18.2 min.

fig. 1. RP-HPLC profiles for reductions of 2a, 7a and 8a.

Peptides **6a** and **7a**, which contain two sulfoxide groups, were reduced cleanly to **6b** and **7b**, respectively. The fact that no other products were detected in the final reaction crudes established the stability of the Acm group in the experimental conditions under study. The same result was achieved when Acm diprotected peptide **1a** was reduced to **1b**. However, HPLC monitoring of reactions with dimers **6a** and **7a** showed the appearance of another product during the process with a retention time between those of the oxidized and reduced peptides in both cases (figure 1, rt = 12.2 min in the case of the conversion of **7a** into **7b**). This chromatographic behavior[18] and the fact that the compound was transformed into the desired peptide indicates that it could be a reaction intermediate such as the monosulfoxide derivative (two possible regioisomers with similar retention times in the case of **7a**).

Reductions of the two methionine sulfoxide containing parallel and antiparallel dimers **8a** and **9a** afforded the desired products; however, no intermediate was observed during the reaction in the case of the former, as shown in figure 1 for **8a**. From the point of view of reaction rates, antiparallel dimer **9a** showed different behavior. Although, in general, the reactions were carried out in less than two hours (see table 1), this peptide proved to be especially resistant to reduction, which may be explained in terms of sterical hindrance during iodide attack on the protonated sulfoxide.[16,19] The possibility of disulfide scrambling with the use of Me_2S was excluded because no reduction nor other changes were observed by HPLC when **9a** was only treated with this sulfide under the same conditions that were used for the reduction of the peptide (24 hours).

Finally, reduction of peptides **4a** and **5a** gave products different from those would be expected from methionine sulfoxide reduction as the only process. Treatment of cysteine unprotected peptide **4a** with NH_4I/Me_2S in neat TFA at 0°C resulted in simultaneous methionine sulfoxide reduction and cysteine oxidation to afford the parallel dimer **6b** as the only detected product . These results could be explained in terms of sulphydryl activation by attack of this group to any of the intermediates that are formed during the sulfoxide reduction process (see scheme 2). Similarly, the corresponding parallel dimer was formed from **5a** (not shown in scheme 1), which was identified by electrospray mass spectrometry.

In summary, the suitability of the NH_4I / TFA system for methionine sulfoxide reduction at low temperature and in the presence of disulfide bridges has been demonstrated. In general, methionine protected peptides have afforded the unprotected ones in excellent yields after a few hours (less than one hour in some cases), as shown by HPLC. Shorter reaction times have been achieved without detectable disulfide scrambling with the use of Me_2S as an additive. On the other hand, reduction of free-cysteine peptides resulted in simultaneous disulfide bridge formation to give the corresponding dimers as the only detectable products.

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EXPERIMENTAL

Boc-amino acids were supplied by Novabiochem AG (Läufelfingen, Switzerland), Bachem Feinchemikalien AG (Bubendorf, Switzerland), Advanced ChemTech (Maidenhead, England) or Propeptide (Vert-le-Petit, France). BocCys(Npys)OH was from Kokusan (Tokio, Japan). Starting resins for this work were either $BocMetOCH_2Pam$-polystyrene (0.60 mmol/g, from Novabiochem) or $BocMet(O)OCH_2Pam$-polystyrene (0.60 mmol/g, from Novabiochem or prepared in our laboratory as outlined below). TBTU, DCC, NH_4I and Me_2S were supplied by Fluka Chimie AG (Buchs, Switzerland) and were used without purification. DMF was supplied by Scharlau (Spain), and was bubbled with nitrogen to remove volatile contaminants and kept stored over activated 4Å molecular sieves. MeCN (Scharlau, Spain) was HPLC grade, DCM (Scharlau, Spain), and TFA (Solvay, Germany) were peptide synthesis grade and were used directly.

Peptide-resins were hydrolysed using 12M HCl/propionic acid (1:1) at 110°C for 48 h and peptides were hydrolysed in 6M aqueous HCl solution at 110°C for 24 h. $BocMet(O)OCH_2Pam$-polyestirene was hydrolised with 4N aqueous MSA solution at 110°C during 22 h. Amino acid analyses were performed on a Beckman System 6300 analyser. HPLC was carried out on a Shimadzu apparatus comprising two solvent delivery pumps model LC-6A, automatic injector model SIL-6B69A, variable wavelength detector model SPD-6A, system controller model SCL-6B and plotter model C-R6A. Nucleosil C_{18} Reverse-phase columns were used (25x0.5

cm, 10 μm). In general, peptides were eluted at a flow rate of 1 mL/min (A: water, 0.045% of TFA; B: CH_3CN, 0.035% of TFA) and detection was carried out at 220 nm (also 340 nm for Npys containing peptides). Reverse phase MPLC was carried out using a CFG-Prominent/Duramat pump, a 757 ABI variable wavelength detector, an automatic fraction collector model Gilson FC 203 and an Omniscribe B-5000 plotter. A glass column (2.5 cm x 26 cm) packed with reverse phase Vydac-C_{18} was used. A flow rate of 125 mL/h was utilized (A and B: mixtures of water/CH_3CN with 0.1% of TFA) and the products were detected at 220 nm. Gel filtration chromatography was performed using a LDC/ MiltonRoy pump, an LKB 2158 Uvicord SD variable wavelength detector, an automatic fraction collector model LKB Ultrorac II 2070 and a Servoscribe 1s plotter. Two glass columns (2.5 cm x 90 cm and 1.5 cm x 60 cm) packed with Sephadex G-15 (Pharmacia) were used. Peptides were eluted at 25 mL/h (0.1% of aqueous AcOH) and the detection was carried out at 206 nm in all cases.

General Procedure for the Solid-Phase Assembly of Peptides and their Acidolytic Cleavage from the Resin

Peptide syntheses were performed manually in a 20 mL polypropylene syringe fitted with a polyethylene disc. Boc-amino acids were assembled using the following protocol: 1) CH_2Cl_2, 2 x 4 min; 2) neat TFA, 1 x 2 min, 1 x 8 min; 3) DMF, 4 x 2 min; 4) Boc-amino acid (3 eq) in DMF, 12 min; 5) DMF, 4 x 2 min. Boc-amino acids were activated separately as follows: to 3 eq of Boc-amino acid were added 2.96 eq of TBTU (0.5 M solution in DMF) and 8.5 eq of DIEA consecutively; the mixture was left for 6-8 min and was added to the peptidyl-resin. Boc-Cys(Npys)-OH was coupled according to the following protocol: 1) DMF, 4 x 2 min; 2) CH_2Cl_2, 4 x 2 min; 3) 5% DIEA/CH_2Cl_2, 4 x 3 min; 4) DCM, 4 x 2 min; 5) Boc-amino acid (3 eq) and DCC (3eq), 50 min; 6) CH_2Cl_2, 4 x 2 min; 7) DMF, 4 x 2 min.

Peptides were cleaved from the resins with HF (UCAR) on a Kel-F Toho-Kasei Ltd (Tokio, Japan) apparatus. Peptidyl-resins (650 mg - 800 mg batches) were treated with 4.5 mL of HF and 500 μL of anisole (protected methionine) or p-cresol (unprotected methionine) during 1 h at 0°C. The resins were washed with 10-15 mL of Et_2O and the crude materials were dissolved with 10-20 mL of 10% aqueous AcOH and liophilyzed. Products were purified by MPLC, volatiles were removed under vacuum and the remaining solutions were lyophilized.

H-Gly-Ile-Cys(Acm)-Pro-Leu-Cys(Acm)-Met(O)-OH (**1a**). 789 mg of peptidyl-resin afforded 113.8 mg (127 μmol) of crude material (85%), which were eluted with a 10% (A, 300 mL) to 25% (B, 300 mL) convex gradient of organic component. 34.1 Mg (38 μmol, 30% recovery). HPLC: rt, 8.3 min; 10% to 100% of B over 20 min. Amino acid composition Gly: 0.99, Pro: 0.96, Cys: 0.74, Met: 0.64, Ile: 0.99, Leu: 1.00; FABMS *m/z* 916.1 $[M+Na]^+$, 894.1 $[M+1]^+$, 823.1 $[M+2\text{-Acm}]^+$, 752.1 $[M+3H\text{-}2Acm]^+$; $C_{36}H_{63}N_9O_{11}S_3$ requires M^+ 893.3.

H-Gly-Ile-Cys(Npys)-Pro-Leu-Cys(Acm)-Met(O)-OH (**3a**). 722 mg of peptidyl-resin afforded 177 mg (181 μmol) of crude material (93%), which were eluted with a 15% (A, 300 mL) to 25% (B, 300 mL) convex gradient of organic component. 79 Mg (81 μmol, 45% recovery). HPLC: rt, 10.5 min; 10% to 100% of B over 20 min. Amino acid composition Gly: 0.96, Pro: 0.99, Cys: 0.66, Met: 0.92, Ile: 0.85, Leu: 1.05; FABMS *m/z* 977.4 $[M+1]^+$, 906.4 $[M+2\text{-Acm}]^+$, 823.4 $[M+2\text{-Npys}]^+$; $C_{38}H_{60}N_{10}O_{12}S_4$ requires M^+ 976.3.

H-Gly-Ile-Cys-Pro-Leu-Cys(Acm)-Met(O)-OH (**4a**). 672 mg of peptidyl-resin afforded 152 mg (185 μmol) of crude material (95%), which were eluted with a 15% (A, 300 mL) to 20% (B, 300 mL) convex gradient of organic component. 53 Mg (64 μmol, 35% recovery). HPLC: rt, 8.7 min; 10% to 100% of B over 20 min.

Amino acid composition Gly: 0.98, Pro: 0.97, Cys: 0.68, Met: 0.80, Ile: 0.90, Leu: 1.00; FABMS *m/z* 844.9 $[M+Na]^+$, 823.6 $[M+H]^+$, 752.0 $[M+2\text{-Acm}]^+$; $C_{33}H_{58}N_8O_{10}S_3$ requires M^+ 822.3.

H-Gly-Ile-Cys(Acm)-Pro-Leu-Cys-Met(O)-OH (**5a**). 675 mg of peptidyl-resin afforded 137 mg (166 μmol) of crude material (88%), which were eluted with a 10% (A, 300 mL) to 20% (B, 300 mL) convex gradient of organic component. 41 Mg (50 μmol, 30% recovery). HPLC: rt, 9.1 min; 10% to 100% of B over 20 min. Amino acid composition Gly: 0.97, Pro: 0.97, Cys: 0.62, Met: 0.82, Ile: 0.82, Leu: 1.06; FABMS *m/z* 845.3 $[M+Na]^+$, 823.3 $[M+H]^+$, 752.0 $[M+2\text{-Acm}]^+$; $C_{33}H_{58}N_8O_{10}S_3$ requires M^+ 822.3.

H-Gly-Ile-Cys(Acm)-Pro-Leu-Cys(Acm)-Met-OH (**1b**). 745 mg of peptidyl-resin afforded 173 mg (196 μmol) of crude material (80%), which were eluted isocratically with a 20% of organic component. 73 Mg (88 μmol, 45% recovery). HPLC: rt, 10.5 min; 10% to 100% of B over 20 min. Amino acid composition Gly: 0.90, Pro: 1.01, Cys: 0.70, Met: 0.89, Ile: 0.82, Leu: 0.98; FABMS *m/z* 900.0 $[M+Na]^+$, 878.0 $[M+H]^+$, 807.0 $[M+2\text{-Acm}]^+$, 736.0 $[M+3\text{-}2Acm]^+$; $C_{36}H_{63}N_9O_{10}S_3$ requires M^+ 877.3.

H-Gly-Ile-Cys(Npys)-Pro-Leu-Cys(Acm)-Met-OH (**3b**). 716 mg of peptidyl-resin afforded 191 mg (198 μmol) of crude material (77%), which were eluted with a 20% (A: 400 mL) to 30% (B: 400 mL) convex gradient of organic component. 42 Mg (43 μmol, 22% recovery). HPLC: rt, 11.9 min; 10% to 100% of B over 20 min. Amino acid composition Gly: 0.98, Pro: 0.99, Cys: 0.60, Met: 0.75, Ile: 0.98, Leu: 0.99; FABMS *m/z* 961.4 $[M+H]^+$, 890.3 $[M+2\text{-Acm}]^+$, 807.4 $[M+2\text{-Npys}]^+$; $C_{38}H_{60}N_{10}O_{11}S_4$ requires M^+ 960.3.

H-Gly-Ile-Cys-Pro-Leu-Cys(Acm)-Met-OH (**4b**). 661 mg of peptidyl-resin afforded 134 mg (166 μmol) of crude material (74%), which were eluted with a 15% (A: 400 mL) to 25% (B: 400 mL) convex gradient of organic component. 46 Mg (56 μmol, 34% recovery). HPLC: rt, 10.3 min; 10% to 100% of B over 20 min. Amino acid composition Gly: 1.00, Pro: 0.99, Cys: 0.44, Met: 0.65, Ile: 0.90, Leu: 0.98; FABMS *m/z* 829.8 $[M+Na]^+$, 807.8 $[M+H]^+$, 736.8 $[M+2\text{-Acm}]^+$; $C_{33}H_{58}N_8O_9S_3$ requires M^+ 806.3.

H-Gly-Ile-Cys(Acm)-Pro-Leu-Cys-Met-OH (**5b**). 681 mg of peptidyl-resin afforded 132 mg (164 μmol) of crude material (73%), which were eluted with a 15% (A: 400 mL) to 20% (B: 400 mL) convex gradient of organic component. 45 Mg (55 μmol, 34% recovery). HPLC: rt, 10.6 min; 10% to 100% of B over 20 min. Amino acid composition Gly: 0.95, Pro: 1.05, Cys: 0.19, Met: 0.92, Ile: 0.74, Leu: 1.00; FABMS *m/z* 829.3 $[M+Na]^+$, 807.3 $[M+H]^+$, 736.3 $[M+2\text{-Acm}]^+$; $C_{33}H_{58}N_8O_9S_3$ requires M^+ 806.3.

General Procedure for the Obtention of Lineal Dimers, Illustrated by the Preparation of (S^3-$S^{3'}$)-[H-Gly-Ile-Cys-Pro-Leu-Cys(Acm)-Met(O)-OH]$_2$ (6a).

To a solution of 21 mg of **4a** (19 μmol) in aqueous 10% AcOH (2 mM, pH=3) were added 25 mg of **3a** (18.5 μmol). The mixture was left for 24 h at 25°C when the solvents were removed by lyophilization. The resulting crude material was chromatographied on a Sephadex G-15 column, affording 31 mg of pure peptide (18 μmol, 94.8%). HPLC: rt, 10.7 min; 20% to 40% of B over 20 min. Amino acid composition Gly: 0.95, Pro: 0.94, Cys: 0.67, Met: 0.68, Ile: 1.05, Leu: 1.04; FABMS *m/z* 1643.6 $[M+H]^+$; $C_{66}H_{114}N_{16}O_{20}S_6$ requires 1642.6.

(S^3[S^6Acm]-$S^{6'}$[$S^{3'}$Acm])-[H-Gly-Ile-Cys-Pro-Leu-Cys-Met(O)-OH]$_2$ (**7a**). 27.9 mg (26 μmol) of **5a** and 33.7 mg (26 μmol) of **3a** afforded 41 mg of **7a** (94 %). HPLC: rt, 8.7 min; 20 % to 40 % of B over 20 min. Amino acid composition Gly: 0.95, Pro: 0.99, Cys: 0.58, Met: 0.68, Ile: 0.88, Leu: 1.00; FABMS *m/z* 1643.6 $[M+H]^+$; $C_{66}H_{114}N_{16}O_{20}S_6$ requires 1642.6.

(S^3-$S^{3'}$)-[H-Gly-Ile-Cys-Pro-Leu-Cys(Acm)-Met-OH]$_2$ (**6b**). 8 mg (6.2 μmol) of **4b** and 9 mg (7.3 μmol) of **3b** afforded 10 mg of **6b** (99.9%). HPLC: rt, 16.0 min; 20% to 40% of B over 20 min. Amino acid composition Gly: 0.94, Pro: 1.01, Cys: 0.70, Met: 0.99, Ile: 0.60, Leu: 1.04; FABMS *m/z* 1611.6 $[M+H]^+$; $C_{66}H_{114}N_{16}O_{18}S_6$ requires 1610.6.

(S^3[S^6Acm]-$S^{6'}$[$S^{3'}$Acm])-[H-Gly-Ile-Cys-Pro-Leu-Cys-Met-OH]$_2$ (**7b**). 19.3 mg (24.8 μmol) of **5b** and 22.3 mg (18 μmol) of **3b** afforded 32 mg of **7b** (82 %). HPLC: rt, 16.0 min; 20 % to 40 % of B over 20 min. Amino acid composition Gly: 0.99, Pro: 0.99, Cys: 0.30, Met: 0.90, Ile: 0.60, Leu: 1.01; FABMS *m/z* 1611.6 $[M+H]^+$; $C_{66}H_{114}N_{16}O_{18}S_6$ requires 1610.6.

*General Procedure for the Synthesis of Parallel and Antiparallel Dimers, Illustrated by the Preparation of (S^3-$S^{3'}$, S^6-$S^{6'}$)-[H-Gly-Ile-Cys-Pro-Leu-Cys-Met(O)-OH]$_2$ (**8a**).*

To a solution of 16 mg of **6a** (9.1 μmol) in 31.2 mL of 80% aqueous AcOH (0.31 mM) was added I_2 (10 eq) and the resulting mixture was left for 3 h at 25°C (neat AcOH was used with a reaction time of 5 h in the case of **6b** and **7b**). 70 mL of water were added and the aqueous solution was washed with 4 x 15 mL of CCl_4. The mixture was lyophilized and the resulting crude peptide was purified by MPLC (10% [A: 300 mL] to 30% [B: 300 mL] of organic component). Volatiles were removed under vacuum and the remaining solution was lyophilized, yielding 10 mg of pure peptide (48 %). HPLC: rt, 9.8 min; 20% to 40% of B over 20 min. Amino acid composition Gly: 0.98, Pro: 0.95, Cys: 0.46, Met: 0.86, Ile: 0.73, Leu: 1.06; FABMS *m/z* 1499.8 $[M+H]^+$; $C_{60}H_{102}N_{14}O_{18}S_6$ requires 1498.5.

(S^3-$S^{6'}$, S^6-$S^{3'}$)-[H-Gly-Ile-Cys-Pro-Leu-Cys-Met(O)-OH]$_2$ (**9a**). 23.8 mg of **7a** (14 μmol) afforded 14 mg of product (50%) after purification by MPLC (15% [A: 350 mL] to 30% [B: 350 mL] of organic component). HPLC: rt, 12.5 min; 20 % to 40 % of B over 20 min. Amino acid composition Gly: 0.97, Pro: 01.01, Cys: 0.41, Met: 0.79, Ile: 0.80, Leu: 1.01; FABMS *m/z* 1499.8 $[M+H]^+$; $C_{60}H_{102}N_{14}O_{18}S_6$ requires 1498.5.

(S^3-$S^{3'}$, S^6-$S^{6'}$)-[H-Gly-Ile-Cys-Pro-Leu-Cys-Met-OH]$_2$ (**8b**). 15.7 mg of **6b** (9.2 μmol) afforded 12 mg of product (54 %) after purification by MPLC (25% [A: 300 mL] to 35% [B: 300 mL] of organic component). HPLC: rt, 18.2 min; 20% to 40% of B over 20 min. Amino acid composition Gly: 0.99, Pro: 0.97, Cys: 0.45, Met: 0.76, Ile: 0.75, Leu: 1.05; FABMS *m/z* 1467.8 $[M+H]^+$; $C_{60}H_{102}N_{14}O_{16}S_6$ requires 1466.6.

(S^3-$S^{6'}$, S^6-$S^{3'}$)-[H-Gly-Ile-Cys-Pro-Leu-Cys-Met-OH]$_2$ (**9b**). 14.4 mg of **7b** (8.5 μmol) afforded 12 mg of product (64%) after purification by MPLC (20% [A: 300 mL] to 25% [B: 300 mL] of organic component). HPLC: rt, 18.2 min; 20 % to 40 % of B over 20 min. Amino acid composition Gly: 0.94, Pro: 0.98, Cys: 0.49, Met: 0.63, Ile: 0.74, Leu: 0.96; FABMS *m/z* 1467.8 $[M+H]^+$; $C_{60}H_{102}N_{14}O_{16}S_6$ requires 1466.6.

*General Procedure for the Synthesis of Intramolecular Disulfide Containing Peptides Illustrated by the Preparation of (S^3-S^6)-[H-Gly-Ile-Cys-Pro-Leu-Cys-Met(O)-OH] (**2a**).*

To a solution of 15 mg of **1a** (16.8 μmol) in 75 mL of 80% aqueous AcOH (0.14 mM) was added I_2 (37.5 eq) and the resulting mixture was left for 40 min at 25°C. 150 mL of water were added and the aqueous solution was washed with 4 x 30 mL of CCl_4. The mixture was lyophilized and the resulting crude peptide was purified by MPLC (10% [A: 300 mL] to 25% [B: 300 mL] of organic component). Volatiles were removed under vacuum and the remaining solution was lyophilized, yielding 8.7 mg of pure peptide (69.3%). HPLC: rt, 9.3 min; 20% to 40% of B over 20 min. Amino acid composition Gly: 0.98, Pro: 0.94, Met: 0.75, Ile: 1.04, Leu: 1.02; FABMS *m/z* 771.9 $[M+Na]^+$, 750.0 $[M+1]^+$; $C_{30}H_{51}N_7O_9S_3$ requires M^+ 749.2.

(S^3-S^6)-[H-Gly-Ile-Cys-Pro-Leu-Cys-Met-OH] (**2b**). 20 mg of **1b** (22.8 μmol) afforded 2.3 mg of product (14%) after purification by MPLC (10% [A: 300 mL] to 30% [B: 300 mL] of organic component) (3.4 mg of **2a** [20%] were recovered). HPLC: rt, 16.8 min; 20 % to 40 % of B over 20 min. Amino acid composition Gly: 1.01, Pro: 0.95, Met: 0.88, Ile: 0.87, Leu: 1.03.; FABMS *m/z* 756.0 $[M+Na]^+$, 734.0 $[M+H]^+$; $C_{30}H_{51}N_7O_8S_3$ requires 733.3.

General Procedure for the Reduction of sulfoxides

Peptides were dissolved in neat TFA (**1a**, 1.54 mM; **2a**, 1.56 mM; **4a**, 1.67 mM; **5a**, 1.60 mM; **6a**, 2.20 mM; **7a**, 2.27 mM; **8a**, 1.58 mM and **9a**, 1.74 mM) and the solutions were cooled to 0°C. Then, Me_2S, when used (1:1 molar ratio respect to the reducing agent), and NH_4I (**1a**, 15 eq; **2a**, 15 eq; **4a**, 13 eq; **5a**, 14 eq; **6a**, 5 eq; **7a**, 5 eq; **8a**, 7 eq and **9a**, 7 eq) were added and the mixtures were stirred vigorously. HPLC monitoring of the reactions was performed as follows: samples were taken from the reaction mixtures and a saturated aqueous solution of ascorbic acid was added to quench the reductions and remove iodine; analyses were carried out using a linear gradient from 20% to 40% of MeCN in 20 min (1 mL/min, 220 nm). The final products were characterized by HPLC and amino acid analysis of collected samples.

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