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Synthesis of Selenocysteine Peptides and their Oxidation to Diselenide-bridged Compounds

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Abstract: Using the Fmoc/tBu protection scheme and the *p*-methoxybenzyl derivative of selenocysteine, the synthesis of related peptides in the selenol-protected form could be optimized by operating the coupling steps in the absence of auxiliary bases and by reducing the piperidine treatment to the minimum time required for quantitative Fmoc cleavage. Under these conditions, β-elimination of the *p*-methoxybenzylselenol as the main side reaction of these syntheses, as well as epimerization of the protected selenocysteine, was largely suppressed. Conversion of the selenol- and thiol-protected bis-selenocysteine and selenocysteine, cysteine peptides into the related cyclic monomeric forms by iodine-mediated oxidation failed since a complex mixture of compounds was produced. Cleavage of the selenoether bond with mercuric acetate was found to proceed smoothly, but displacement of the heavy metal ions by treatment with excesses of thiols or hydrogen sulphide was unsuccessful since a stable Hg^{2+} diselenide complex was obtained. However, oxidation was achieved in good yields by the dimethylsulphoxide/trifluoroacetic acid procedure and the peptides were then used for determining the redox potential of the diselenide and selenide/sulphide bridge, respectively. © 1997 European Peptide Society and John Wiley & Sons, Ltd.

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INTRODUCTION

Biosynthetic replacement of methionine residues with selenomethionine in proteins is known to produce isomorphous variants [1–3]. Similar effects are expected from a substitution of cysteine residues with selenocysteine (Sec). However, the slightly differentiated electronegativities of selenium (2.55) and sulphur (2.58) [4] and their different covalence radii (Se 1.17 Å, S 1.02 Å) [5] should result in minor changes of the chemical and electrochemical properties of the selenol vs. thiol group. Accordingly, even the redox potential of selenocysteine should differ from that of cysteine as is well supported by the observation that the reduction of the diselenide group in organic compounds and peptides requires strongly reducing dithiols such as dithiotreitol (DTT) [6–8], whereas monothiols are unable to reduce diselenides to any significant extent [6, 7, 9].

We have previously determined the redox potentials of bis-cysteinyl peptides containing the characteristic Cys-Xxx-Yyy-Cys sequence motif [10]. Among the various peptides analysed, fragment 10–17 of glutaredoxin was found to exhibit a redox potential almost identical to that of the enzyme, and was thus unaffected by the three-dimensional fold of the protein. Correspondingly, we have selected this glutaredoxin fragment to determine the redox po-

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tential of selenocystine in an unconstrained diselenide-bridged linear peptide [11, 12].

In first syntheses of selenocysteine peptides the benzyl group was exclusively used for protection of the selenol function in analogy to the *S*-benzyl-cysteine derivative [13–19]. Consistently low yields were reported in the deprotection step with sodium in liquid ammonia. Since desulphuration had already been observed to occur to a significant extent during deprotection of *S*-benzyl-cysteine derivatives by this method [20], β-elimination of the benzyl selenolate most probably was the main cause of the low yields. It is now well established that benzyl selenolate represents a much better leaving group than the corresponding thiolate [21]. In more recent syntheses of selenocysteine peptides, Se-*p*-methylbenzyl [22] and Se-*p*-methoxybenzyl protection [9, 23, 24] has been proposed. Thereby better results in terms of yields were reported for the syntheses performed with *p*-methoxybenzyl (Mob) protection. In view of these findings we have chosen this protecting group for the syntheses of the mono- and bis-selenocysteine analogues of the bis-cysteinyl octapeptide 10–17 of glutaredoxin listed in Figure 1.

MATERIALS AND METHODS

Amino acid derivatives, solvents and reagents were of the highest quality commercially available. Tentagel S RAM was from Rapp Polymere GmbH (Tübingen, Germany), Rink-amide-MBHA from Novabiochem (Läufelfingen, Switzerland), Lichroprep RP18 and TLC silica gel 60 plates from Merk AG (Darmstadt, Germany).

HPLC was carried out on Waters equipment (Eschborn, Germany) on Nucleosil 300/C18 and C8 (Machery & Nagel, Düren, Germany) using a linear gradient of acetonitrile/2% H_3PO_4 from 5 : 95 to 80 : 20 in 40 min. Amino acid analysis of the acid hydrolysates (6 M HCl containing 2.5% thioglycolic acid; 110 °C; 24 h) were performed on a Biotronic amino acid analyser (LC 6001). Since Sec(Mob) residues are partially degraded during acid hydrolysis, Sec_2 ($t_R = 88$ min) is detected, but not quantified; Dha is unstable to acid hydrolysis and is not detected. For the gas chromatographic racemization test the acid hydrolysates were derivatized to pentafluoropropionyl amino acid methyl esters and analysed with a chirasil-Val glass capillary column on a Fractovap (Carlo Erba 4160). The racemization test for Cys and Sec was performed according to Siedler *et al.* [25] by CE of the Cys(4-PE) and Sec(4-PE) derivatives on a Spectra Phoresis 1000 capillary electrophoresis apparatus (TSP, Darmstadt) at 25 kV using an underivatized fused silica capillary (67 cm × 75 μm; length × ID) and 50 mM sodium borate buffer (pH 8.5). FAB-MS spectra were recorded on a Finnigan MAT 900 with *m*-hydroxybenzyl alcohol as matrix.

CD spectra were recorded on a Yobin-Yvon dichrograph Mark IV equipped with a cell holder with thermostat and connected to a data station for signal averaging and processing. All data are averages of 10 scans and the spectra were taken at 20 °C employing quartz cells of 0.1 cm optical path length in the far UV and 1 cm path length in the near UV. The spectra in the near UV are reported in terms of ellipticity units per mole of peptide ($[\theta]_M$) and in the far UV region as ellipticity units per mole of peptide residue ($[\theta]_R$). The spectra were recorded in 0.1 M phosphate buffer (pH 7.0) and concentrations were determined by weight and peptide content as determined by quantitative amino acid analysis.

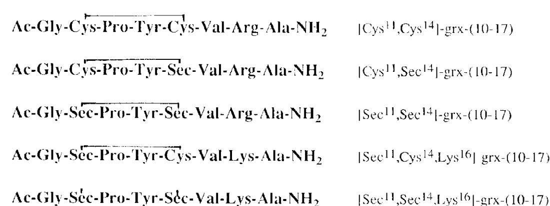

Figure 1 Selenocysteine peptides related to the active-site fragment 10–17 of glutaredoxin.

Fmoc-Sec(Mob)-OPfp

Following the procedure described by Kisfaludy and Schön [26] for the related cysteine derivative, the title compound was prepared from Fmoc-Sec(Mob)-OH [9]. Yield: 71%; homogeneous on TLC (solvent system: cyclohexane/$CHCl_3$/AcOH, 9 : 9 : 2); m.p. 155 °C; FAB-MS: $m/z = 678$ $[M + H^+]$; $M_r = 677.03$ calcd for $C_{32}H_{24}O_5NF_5Se$.

Ac-Gly-Cys(StBu)-Pro-Tyr-Sec(Mob)-Val-Arg-Ala-NH_2, (Cys(StBu)^{11},Sec(Mob)^{14})-grx-(10–17)

The synthesis was performed on Rink-amide-MBHA resin (0.47 mmole/g) following the protocol of Table 1 and using in the HBTU/HOBt/DIEA (1 : 1 : 2) coupling steps 3-fold excesses of Fmoc-amino-acids and in the pentafluorophenyl ester couplings 2-fold excesses of active esters and 1 equiv. HOBt as catalyst. Coupling efficiency was analysed with the

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Kaiser's test. Acetylation was done with 10 eq. acetanhydride and 10 eq. DIEA for 2 h. Aliquots of the resin were treated with different TFA cocktails under different conditions (see Figure 2). The crude products were purified via RP-chromatography on Lichroprep RP18 by isocratic elution with 0.1 M ammonium acetate (pH 3.8)/2-propanol/1-butanol (73 : 22 : 5, v/v) at a flow rate of 2 ml/min for 2 h, followed by a linear gradient from 73 : 22 : 5 to 50 : 40 : 10 in 12 h. The six main components were isolated and characterized as follows:

(Cys(StBu)^{11},Sec(Mob)^{14})-grx-(10–17). Homogeneous on HPLC (t_R = 26.25 min); FAB-MS: m/z = 1165.8 [M + H$^+$]; M_r = 1164.4 calcd for $C_{50}H_{76}N_{12}O_{11}S_2Se$; amino acid analysis: Pro 1.14 (1) Gly 1.00 (1) Ala 0.99 (1) Cys 0.98 (1) Val 0.95 (1) Tyr 0.93 (1) Arg 0.90 (1); peptide content: 95%.

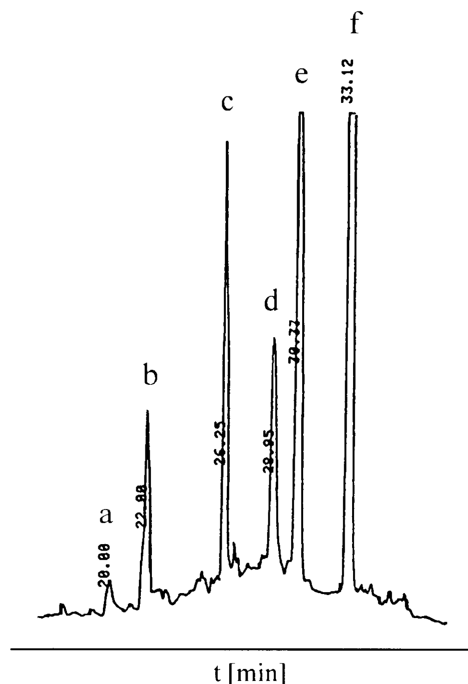

Figure 2 HPLC of the crude [Cys(StBu)^{11},Sec(Mob)^{14}]-grx-(10–17) synthesized according to the standard protocol of Table 1 upon acidolytic cleavage from the resin under optimized conditions, i.e. with TFA/H_2O/CH_2Cl_2/TIPS (89/5/5/1; v/v) at 4 °C for 1 h. The peaks were assigned upon isolation and characterization of the compounds as follows: (a) [Cys(StBu)^{11},Ala(β-piperidyl)^{14}]-grx-(10–17); (b) [Cys(StBu)^{11},Dha^{14}]-grx-(10–17); (c) [Cys(StBu)^{11},Sec(-Mob)^{14}]-grx-(10–17); (d) [Cys(StBu)^{11},Ala(β-piperidyl)^{14},Arg (Pmc)^{16}]-grx-(10–17); (e) [Cys(StBu)^{11},Dha^{14},Arg(Pmc)^{16}]-grx-(10–17); (f) [Cys(StBu)^{11},Sec(Mob)^{14},Arg(Pmc)^{16}]-grx-(10–17).

(Cys(StBu)^{11},Sec(Mob)^{14},Arg(Pmc)^{16})-grx-(10–17). Homogeneous on HPLC (t_R = 33.12 min); FAB-MS: m/z = 1431.8 [M + H$^+$]; M_r = 1430.7 calcd for $C_{60}H_{88}N_{12}O_{14}S_3Se$; amino acid analysis: Pro 1.09 (1) Gly 0.94 (1) Ala 1.00 (1) Cys 0.73 (1) Val 0.97 (1) Tyr 0.95 (1) Arg 0.94 (1); peptide content: 76%.

(Cys(StBu)^{11},Dha^{14})-grx-(10–17). Homogeneous on HPLC (t_R = 22.00 min); FAB-MS: m/z = 963.8 [M + H$^+$]; M_r = 963.2 calcd for $C_{42}H_{66}N_{12}O_{10}S_2$.

(Cys(StBu)^{11},Dha^{14},Arg(Pmc)^{16})-grx-(10–17). Homogeneous on HPLC (t_R = 30.33 min); FAB-MS: m/z = 1229.9 [M + H$^+$]; M_r = 1229.55 calcd for $C_{52}H_{79}N_{12}O_{13}S_3$; amino acid analysis: Pro 1.1 (1) Gly 1.00 (1) Ala 0.99 (1) Cys 0.76 (1) Val 0.85 (1) Tyr 0.92 (1) Arg 0.83 (1); Dha n.d.; peptide content: 81%.

(Cys(StBu)^{11},Ala(β-piperidyl)^{14}]-grx-(10–17). Homogeneous on HPLC (t_R = 20.00 min); FAB-MS: m/z = 1048.8 [M + H$^+$]; M_r = 1048.19 calcd for $C_{47}H_{77}N_{13}O_{10}S_2$.

(Cys(StBu)^{11},Ala(β-piperidyl)^{14},Arg(Pmc)^{16})-grx-(10–17). Homogeneous on HPLC (t_R = 28.95 min); FAB-MS: m/z = 1315.0 [M + H$^+$]; M_r = 1314.55, calcd for $C_{67}H_{90}N_{13}O_{13}S_3$; amino acid analysis: Pro 0.73 (1) Gly 0.78 (1) Ala 1.00 (1) Cys 0.52 (1) Val 0.79 (1) Tyr 0.73 (1) Arg 0.86 (1); Ala(β-piperidyl) detected, but not quantified; peptide content: 85%.

Ac-Gly-Sec(Mob)-Pro-Tyr-Sec(Mob)-Val-Lys-Ala-NH_2, (Sec(Mob)^{11},Sec(Mob)^{14},Lys^{16})-grx-(10–17)

The synthesis was performed on 2.0 g Rink-amide-MBHA resin (0.47 mmole/g) following the general protocol described in Table 2 and using exclusively 2-fold excesses of the pentafluorophenyl esters with 1 equiv. HOBt as catalyst in the coupling steps. Final acetylation was performed with an 8-fold excess of AcOSu/HOBt (1 : 1) for 30 h. Acidolytic cleavage was carried out with TFA/H_2O/CH_2Cl_2/TIPS (89 : 5 : 5 : 1, v/v) at 4 °C for 1 h. The crude product (1.02 g) containing according to HPLC about 90% (t_R = 26.45 min) of the desired peptide was purified by RP chromatography on a Lichroprep RP18 column (2 × 230 cm) by elution at a flow rate of 4 ml/min with 0.1 M ammonium acetate (pH 3.8)/2-propanol/1-butanol (88 : 16 : 4, v/v) in isocratic manner for 2 h, followed by a linear gradient from 88 : 16 : 4 to 57 : 34 : 9 in 12 h. Fractions containing homogeneous product were pooled and lyophilized; yield: 0.7 g; homogeneous on HPLC (t_R = 26.45 min);

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Table 1 Standard Protocol of the Synthesis of Selenocysteine Peptides on Rink-amide-MBHA Resin According to the Fmoc Strategy

Synthetic step	Reagents and solvents	Reaction time
Fmoc cleavage	20% (v/v) piperidine/NMP	15 min
Washing	20% (v/v) CH_2Cl_2/NMP	2 min (2 ×)
	CH_2Cl_2	2 min (2 ×)
	20% (v/v) CH_2Cl_2/NMP	2 min (2 ×)
Coupling	3-fold excesses of Fmoc-Ala-OH, Fmoc-Gly-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Val-OH and Fmoc-Arg(Pmc)-OH via HBTU/HOBt/DIEA (1 : 1 : 2) in 20% (v/v) CH_2Cl_2/NMP	90 min
	2-fold excesses of Fmoc-Sec(Mob)-OPfp, Fmoc-Pro-OPfp and Fmoc-Cys(StBu)-OPfp with HOBt as catalyst in 20% (v/v) CH_2Cl_2/NMP	90 min
Washing	20% (v/v) CH_2Cl_2/NMP	2 min (2 ×)
	2-propanol	1 min (2 ×)
	CH_2Cl_2	2 min (2 ×)
Acetylation	10 equiv. $(Ac)_2O$/10 equiv. DIEA 20% (v/v) CH_2Cl_2/NMP	2 h
Acidolytic cleavage	See Figure 2	

FAB-MS: $m/z = 1216.9$ [M + H$^+$]; $M_r = 1215.3$ calcd for $C_{54}H_{76}N_{10}O_{12}Se_2$; amino acid analysis: Pro 0.90 (1) Gly 1.00 (1) Ala 1.07 (1) Val 0.87 (1) Tyr 0.96 (1) Lys 0.92 (1); peptide content: 85%.

Ac-Gly-Cys(StBu)-Pro-Tyr-Sec(Mob)-Val-Lys-Ala-NH$_2$, (Cys(StBu)^{11},Sec(Mob)^{14},Lys^{16})-grx-(10–17)

The synthesis was performed as described for [Sec(Mob)^{11},Sec(Mob)^{14},Lys^{16}]-grx-(10–17) on 0.53 g Rink-amide-MBHA resin (0.47 mmole/g) and using TFA/H_2O/CH_2Cl_2/TIPS (89 : 5 : 5 : 1, v/v) at 4 °C for 1 h for the cleavage step. The crude product (0.2 g) containing, again according to HPLC, about 90% ($t_R = 27.87$ min) of the desired peptide was purified by RP chromatography on a Lichroprep RP18 column (2 × 230 cm) by elution at a flow rate of 4 ml/min with 0.1 M ammonium acetate (pH 3.8)/ 2-propanol/l-butanol (88 : 10 : 2, v/v) in isocratic manner for 2 h followed by a linear gradient from 88 : 10 : 2 to 60 : 30 : 10 in 12 h; yield: 75 mg; homogeneous on HPLC ($t_R = 27.87$ min); FAB-MS: $m/z = 1137.1$ [M + H$^+$]; $M_r = 1136.4$ calcd for $C_{50}H_{79}N_{10}O_{11}S_2Se$.

Table 2. Optimized Protocol of the Synthesis of Selenocysteine-Peptides on Rink-amide-MBHA Resin According to the Fmoc Strategy

Synthetic step	Reagents and solvents	Reaction time
Fmoc cleavage	20% (v/v) piperidine/NMP	3 min
Washing	20% (v/v) CH_2Cl_2/NMP	2 min (2 ×)
	CH_2Cl_2	2 min (2 ×)
	NMP	2 min (2 ×)
Coupling	2-fold excesses of pentafluorophenylesters/ HOBt (2 : 1) in 20% (v/v) CH_2Cl_2/NMP	90 min
Washing	20% (v/v) CH_2Cl_2/NMP	2 min (2 ×)
	CH_2Cl_2	1 min (2 ×)
	NMP	2 min (2 ×)
Acetylation	8-fold excess of *N*-acetyl-hydroxysuccinimide/ HOBT(1 : 1) in 20% (v/v) CH_2Cl_2/NMP	30 h
Acidolytic cleavage	TFA/H_2O/CH_2Cl_2/TIPS (89 : 5 : 5 : 1,v/v) 4 °C	1 h

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Ac-Gly-Sec(Mob)-Pro-Tyr-Cys(Mob)-Val-Lys-Ala-NH_2, (Sec(Mob)^{11},Cys(Mob)^{14},Lys^{16})-grx-(10–17)

The synthesis was performed as described for [Sec(Mob)^{11},Sec(Mob)^{14},Lys^{16}]-grx-(10–17) on 1 g Tentagel S RAM resin (0.26 mmole/g) following the synthetic protocol of Table 2 and using TFA/H_2O/CH_2Cl_2/TIPS (89 : 5 : 5 : 1, v/v) at 4 °C for 1 h for the cleavage step. the crude product (0.22 g) was purified by RP chromatography on a Lichroprep RP18 column (2 × 230 cm) by elution at a flow rate of 4 ml/min with 0.1 M ammonium acetate (pH 3.8)/2-propanol/l-butanol (88 : 16 : 4, v/v) in isocratic manner for 2 h followed by a linear gradient from 88 : 16 : 4 to 57 : 34 : 9 in 12 h; yield: 180 mg; homogeneous on HPLC ($t_R = 27.12$ min); FAB-MS: $m/z = 1168.7$ $[M + H^+]$; $M_r = 1168.4$ calcd for $C_{54}H_{76}N_{10}O_{12}SSe$; amino acid analysis: Pro 0.93 (1) Gly 1.00 (1) Ala 0.95 (1) Cys 0.95 (1) Val 0.88 (1) Tyr 0.92 (1) Lys 0.88 (1); peptide content: 80%.

Ac-Gly-Cys-Pro-Tyr-Sec(Mob)-Val-Lys-Ala-NH_2, (Cys^{11}, Sec(Mob)^{14},Lys^{16})-grx-(10–17)

To a solution of Ac-Gly-Cys(StBu)-Pro-Tyr-Sec(Mob)-Val-Lys-Ala-NH_2 (30 mg; 0.026 mmol) in 5 ml 95% TFE tributylphosphine (64 µl; 0.26 mmol) was added. Upon stirring the reaction mixture for 16 h at room temperature, the bulk of the solvent was evaporated and the product precipitated with methyl *tert*-butyl ether; yield: 19 mg; homogeneous on HPLC ($t_R = 23.79$ min); FAB-MS: $m/z = 1049.4$ $[M + H^+]$; $M_r = 1048.1$ calcd for $C_{46}H_{68}N_{10}O_{11}SSe$.

Ac-Gly-Sec-Pro-Tyr-Sec-Val-Lys-Ala-NH_2, (Sec^{11},Sec^{14},Lys^{16})-grx-(10–17)

Procedure (a). A solution of [Sec(Mob)^{11},Sec(Mob)^{14}, Lys^{16}]-grx-(10–17) (13 mg; 0.01 mmol) in ice-cold TFA (50 ml) was added dropwise to an ice-cold solution of DMSO (2 ml) in TFA (8 ml). The reaction mixture was stirred for 30 min at 4 °C. The TFA was evaporated and the residue was purified by preparative HPLC on a Nucleosil C18 column (2 × 25 cm) at a flow rate of 10 ml/min; eluents: 0.1% aqueous TFA (A), 0.08% TFA in acetonitrile; elution in an isocratic manner for 15 min with (A)/(B) (95 : 5) followed by a linear gradient of (A)/(B) from 95 : 5 to 70 : 30 in 2 h. Fractions containing the desired product were pooled and lyophilized; yield: 6 mg; homogeneous on HPLC ($t_R = 19.57$ min); FAB-MS: $m/z = 975.3$ $[M + H^+]$; $M_r = 974.2$ calcd for $C_{38}N_{58}N_{10}O_{10}Se_2$; amino acid analysis: Pro 0.99 (1) Gly 1.00 (1) Ala 0.94 Val 0.87 (1) Tyr 0.91 (1) Lys 0.85 (1); peptide content: 84%. An analytical sample was reduced with a 10-fold excess of tris-(carboxyethyl)phosphine in water (pH 2.0) at 65 °C for 1 h and then reacted with 1.5 equiv. of 4-vinylpyridine as described by Siedler *et al.* [25] prior to acid hydrolysis; amino acid analysis: Pro 0.82 (1) Gly 1.00 (1) Ala 1.04 (1) Val 0.83 (1) Tyr 0.90 (1) Lys 0.89 (1); Sec(4-PE) detected at 156 min, racemization test by CE: D-Sec(4-PE) 6%; racemization test by gas chromatography according to Frank *et al.* [27]: D-Ala, D-Val, D-Tyr, D-Lys and D-Pro < 0.5%.

Procedure (b). To a solution of I_2 (40.6 mg; 0.16 mmol) in MeOH (80 ml) a solution of [Sec(Mob)^{11},Sec(Mob)^{14},Lys^{16}]-grx-(10–17) (48.6 mg; 0.04 mmol) in MeOH (200 ml) is added dropwise in 10 min. The reaction mixture is stirred at room temperature for additional 20 min, then the excess I_2 is reduced with 1 M $Na_2S_2O_3$. The solution is concentrated to small volume and the peptide precipitated with methyl *tert*-butyl ether. The crude product was purified by RP chromatography on Lichroprep RP18 by elution at a flow rate of 3 ml/min with 0.1 M ammonium acetate (pH 3.8)/2-propanol/l-butanol (88 : 10 : 2) in an isocratic manner for 3 h followed by a linear gradient from 88 : 10 : 2 to 84 : 13 : 3 in 6 h. Yield: 2.5 mg; the analytical data are identical to those of procedure (a) within the limits of error of the techniques used.

Ac-Gly-Sec-Pro-Tyr-Cys-Val-Lys-Ala-NH_2, (Sec^{11},Cys^{14},Lys^{16})-grx-(10–17)

[Sec(Mob)^{11},Cys(Mob)^{14},Lys^{16}]-grx-(10–17) (59 mg; 0.05 mmol) was reacted with DMSO (8 ml) in ice-cold TFA (180 ml) and worked up and purified by HPLC as described in procedure (a) for [Sec^{11},-Sec^{14},Lys^{16}]-grx-(10–17); yield: 12 mg; homogeneous on HPLC ($t_R = 18.55$ min); FAB-MS: $m/z = 927.3$ $[M + H^+]$; $M_r = 926.1$ calcd for $C_{38}H_{58}N_{10}O_{10}SeS$; amino acid analysis: Pro 1.04 (1) Gly 1.00 (1) Ala 0.99 (1) Val 0.88 (1) Tyr 0.91 (1) Lys 0.92 (1) Cys 1.03 (1); peptide content: 77%; racemization test by CE according to Siedler *et al.* [25]: D-Cys(4-PE) 5%; D-Sec(4-PE) 6%; racemization test by gas chromatography according to Frank *et al.* [27]: D-Ala, D-Val, D-Tyr, D-Lys and D-Pro < 0.5%.

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RESULTS

Synthesis of the Se-protected Selenocysteine Peptides

For the solid-phase synthesis of the selenocysteine octapeptides the Fmoc/tBu strategy [28] was applied. Correspondingly, L-selenocystine was synthesized according to Tanaka and Soda [29] and then converted to Se-*p*-methoxybenzyl and, finally, to the related N^{α}-Fmoc derivative by known procedures [9, 23]. For the synthesis of the two arginine-containing octapeptides [Cys(StBu)^{11},Sec(Mob)^{14}]-grx-(10–17) and [Sec(Mob)^{11},Sec(Mob)^{14}]-grx-(10–17) standard synthetic protocols as listed in Table 1 were applied with HBTU/HOBt/DIEA (1 : 1 : 2) as coupling procedure [30], except for Fmoc-Sec(Mob)-OH and Fmoc-Cys(StBu)-OH for which the pentafluorophenyl esters were used in order to suppress racemization [31]. Acidolytic cleavage of the Cys(StBu)/Sec(Mob) peptide from the resin was performed under different conditions and in all cases a crude product consisting of mainly six compounds was obtained as shown in Figure 2. The single compounds were isolated and characterized. The side products, besides originating from incomplete removal of the Pmc group, were found to derive from β-elimination of the *p*-methoxybenzylselenol with formation of dehydroalanine (Dha) at extents unexpected in view of the results reported in the previous syntheses of Sec(Mob) peptides [9, 24]. A similar complex mixture of compounds was obtained upon acidolytic cleavage of the [Sec(Mob)^{11},Sec(Mob)^{14}]-grx-(10–17) octapeptide. By lowering the reaction temperature to 4 °C the rates of Arg(Pmc) cleavage are reduced, but

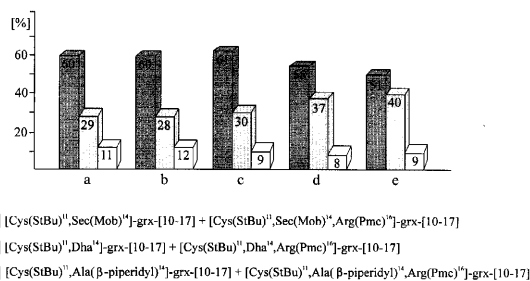

Figure 3 Product distribution upon cleavage of [Cys(StBu)^{11},Sec(Mob)^{14}]-grx-(10–17) from the resin under different conditions: (a) TFA/H_2O/CH_2Cl_2/TIPS (89 : 5 : 5 : 1, v/v) at 4 °C for 30 min; (b) TFA/H_2O/EDT/thioanisole/*m*-cresol (80 : 5 : 5 : 5 : 5, v/v) at 4 °C for 1 h; (c) TFA/H_2O/CH_2Cl_2/TIPS (89 : 5 : 5 : 1, v/v) at 4 °C for 1 h; (d) TFA/H_2O/CH_2Cl_2/TIPS (89 : 5 : 5 : 1, v/v) at 21 °C for 1 h; (e) TFA/H_2O (95 : 5, v/v) at 21°C for 1 h.

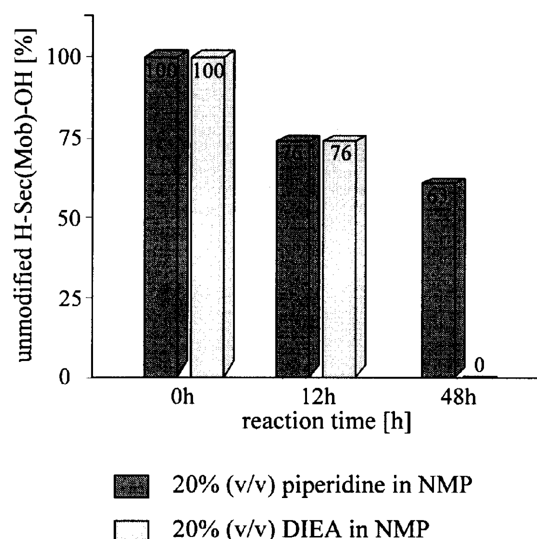

Figure 4 Effect of 20% DIEA and 20% piperidine in NMP on the stability of H-Sec(Mob)-OH as determined by amino acid analysis.

β-elimination in this acidolytic step is largely suppressed, as shown by comparing the percentages of related side products as a function of time (Figure 3). Exposure to bases in the course of the synthesis has, therefore, to represent the main source of the side reaction. This was fully confirmed by model experiments on the effect of the amines on the stability of the Sec(Mob) derivative (Figure 4). The results clearly confirmed that β-elimination is provoked mainly by the amines applied in the different synthetic steps, whereby treatment with piperidine results in an additional partial conversion of the Dha residue into piperidinoalanine, Ala(β-piperidyl).

In order to avoid the difficulty deriving from the slow cleavage of the Arg(Pmc) derivative, a new series of selenocysteine octapeptides was synthesized as [Lys^{16}] analogues. In these syntheses, particular attention was paid to reduce to a minimum the time of exposure of the Sec(Mob) residues to amines by following the optimized protocol reported in Table 2. It is based on the exclusive use of pentafluorophenyl esters without addition of auxiliary bases and on a reaction time with piperidine reduced to the minimum indispensable for quantitative Fmoc cleavage. With these precautions and performing the acidolytic cleavage step with the TFA/H_2O/CH_2Cl_2/TIPS cocktail (89 : 5 : 5 : 1, v/v) at low temperature (4 °C; 1 h), the Sec(Mob)/Sec(Mob), Sec(Mob)/Cys(Mob) and Cys(StBu)/Sec(Mob) octapeptide analogues of [Lys^{16}]-grx-(10–17) were found to represent more than 90% of the crude product (Figure 5). However,

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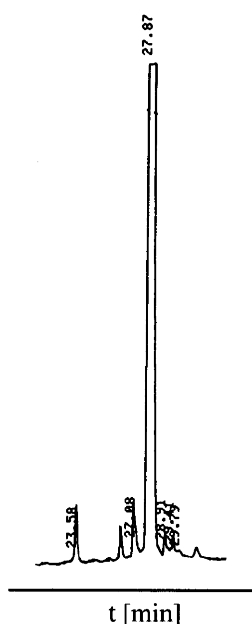

Figure 5 HPLC of the crude [Cys(StBu)[11],Sec(Mob)[14], Lys[16]]-grx-(10–17) synthesized according to the optimized protocol of Table 2 upon acidolytic cleavage from the resin with $TFA/H_2O/CH_2Cl_2/TIPS$ (89 : 5 : 5 : 1, v/v) at 4 °C for 1 h.

even under these optimized conditions the related Dha compounds still represent the main side product. By replacing Cys(StBu) with the trityl derivative the crude product resulting upon acidolytic cleavage with $TFA/H_2O/CH_2Cl_2/TIPS$ was found to contain mainly the Dha compound [Dha[11], Cys[14],Lys[16]]-grx-(10–17) besides minor amounts of the side product [Dha[11], Cys(Trt)[14],Lys[16]]-grx-(10–17) and of the desired octapeptide [Sec(Mob)[11], Cys(Trt)[14],Lys[16]]-grx-(10–17). This would suggest that β-elimination of the *p*-methoxybenzylselenol is strongly favoured even by carbocations formed in the acidolytic deprotection step.

Cleavage of the *p*-Methoxybenzyl Group and Intramolecular Oxidation to the Diselenide- and Mixed Selenide/Sulphide Peptides

Oxidation of a Sec(Mob) pentapeptide to the related dimer has been successfully performed with procedures well established in cysteine chemistry, i.e. I_2, $Tl(TFA)_3$ and DMSO/TFA [9]. Applying the I_2-mediated oxidation in the case of the octapeptide [Sec(Mob)[11],Sec(Mob)[14],Lys[16]]-grx-(10–17) at 5×10^{-4} M concentration in MeOH, a complex mixture of compounds was formed from which the desired diselenide-bridged peptide could be isolated only in about 10% yield. Similarly disappointing in terms of yields was the I_2 oxidation of [Cys[11],Sec(Mob)[14], Lys[16]]-grx-(10–17) to produce the mixed selenide/sulphide bridge.

Cleavage of the *p*-methoxybenzyl group from the Sec(Mob) residues with mercuric acetate in TFA was found to proceed as efficiently as in the case of Cys(Mob) peptides [32]. In cysteine chemistry it is well known that quantitative displacement of Hg^{2+} ions from cysteine peptides by the use of excesses of thiols is difficult to achieve. Because of the large atomic radius and thus strong tendency of selenium to form complexes, displacement of the heavy metal ion from selenocysteine peptides was expected to be even more difficult. In fact, despite flushing the solution for longer periods of time with hydrogen sulphide, only the cyclic mercuric diselenolate complex, shown in Figure 6, could be isolated as a stable and well-characterized compound (homogeneous on HPLC; FAB-MS: $m/z = 1175.4$ $[M+H^+]$; $M_r = 1174.5$ calcd for $C_{38}H_{58}N_{10}O_{10}Se_2Hg$).

The 5% DMSO/TFA (v/v) procedure has been successfully applied for cleavage of the *p*-methoxybenzyl group from Cys(Mob) peptides and concomitant oxidation to cystine peptides by Otaka *et al.* [33]. Using this procedure both the Sec(Mob)/Sec(Mob) and Sec(Mob)/Cys(Mob) peptide were converted in high yields to the cyclic monomers in the diselenide- and mixed selenide/sulphide-bridged form, respectively.

Racemization of Se-*p*-Methoxybenzyl-selenocysteine

S-protected cysteine residues have been reported to be unusually prone to racemization via the Cα-proton abstraction mechanisms in the presence of bases [34–37]. Thereby, even the thiol-protecting group was found to exert surprisingly strong effects with a rank order in the rate of epimerization of StBu < Trt < < Tacm < Acm < MeBzl. This would suggest that even the *p*-methoxybenzyl group should

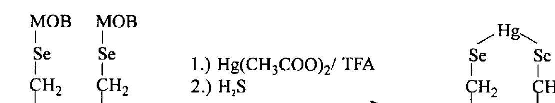

Figure 6 Deprotection of [Sec(Mob)[11],Sec(Mob)[14],Lys[16]]-grx-(10–17) with mercuric acetate in TFA followed by displacement of the Hg^2 with mercaptanes and H_2S.

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favour racemization in a manner similar to the benzyl-type protecting groups [34, 35] and in the case of selenocysteine possibly at higher rates than for cysteine residues.

We have recently elaborated a new and rapid racemization test for cysteine [25]. It is based on the reduction of the cystine residues, when present, with tris-(2-carboxyethyl)phosphine, followed by derivatization of the thiol functions with 4-vinylpyridine [38], acid hydrolysis of the derivatized cysteine peptides, and finally enantiomeric resolution of the D,L-S-β-(4-pyridylethyl)cysteine (Cys(4-PE)) by capillary zone electrophoresis (CE) exploiting the host–guest complexation with crown ethers. The acid stability of the Cys(4-PE) derivative prevents racemization via thiazoline intermediates [39], and thus allows for standardization of the hydrolysis-dependent epimerization in function of time. By applying this procedure for the selenocysteine peptides 6% D-Sec(4-PE) was detected after acid hydrolysis, a value which correlates well with the observed hydrolysis-dependent racemization of Cys(4-PE) (4–5% D-enantiomer) [25]. In view of these results the optimized protocol used in the synthesis of the selenocysteine peptides, where auxiliary bases were excluded whenever possible, proved to be well suited to suppressing both β-elimination and racemization.

Conformational Effects of Cys/Sec Replacements

The far UV CD spectrum of the disulphide-bridged grx-(10–17) in aqueous solution is characterized by a relatively strong negative band centred at 227 nm (Figure 7). It is very similar to the spectra reported by Kishore *et al.* [40] for the grx-(11–14) tetrapeptide derivative Boc-Cys-Pro-Tyr-Cys-NH-CH_3 in the oxidized form in various organic solvents. The latter spectra were assigned to a type I β-turn in equilibrium with a type II β-turn as confirmed by NMR data [40]. Similar CD spectra were also reported for model 14-membered cyclic peptide disulphides containing Pro-X sequences for which a distinction between type I and type II β-turn conformations could not readily be made from the CD data alone [41]. From NMR structural analysis of the grx-(10–17) octapeptide in DMSO we concluded that the preferred conformation of the bis-cysteinyl octapeptide in the oxidized form consists of a type II β-turn [42, 43].

In the near UV the CD spectrum of the tetrapeptide derivative Boc-Cys-Pro-Tyr-Cys-NH-CH_3 was found to depend strongly upon the protic or aprotic nature of the solvent. In fact, the long wavelength nσ* transition band at 276 nm is changing both in terms of intensity and sign, e.g. from positive values in MeOH/H_2O (1 : 1) to negative values in DMSO,

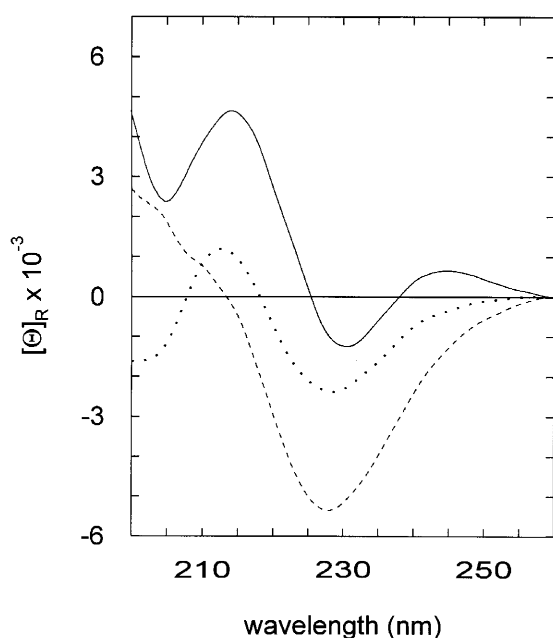

Figure 7 Far UV CD spectra of [Cys^{11},Cys^{14}]-grx-(10–17) (- - - - -), [Sec^{11},Cys^{14},Lys^{16}]-grx-(10–17) (. . . . .) and [Sec^{11},Sec^{14},Lys^{16}]-grx-(10–17) (——) in 0.1 M phosphate buffer, pH 7.0 at 20 °C.

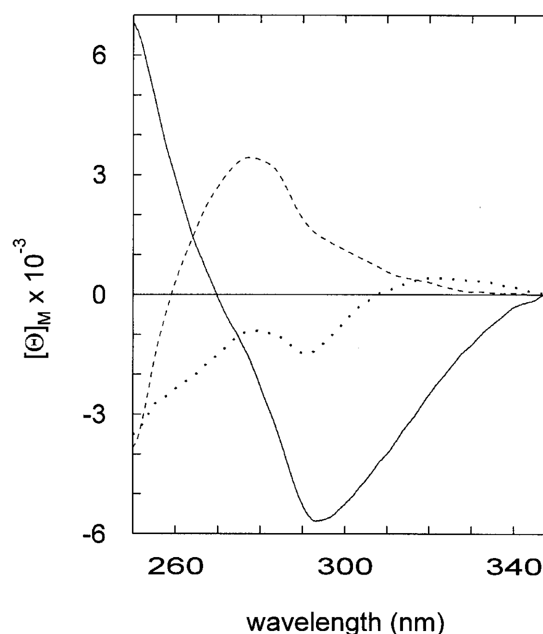

Figure 8 Near UV CD spectra of [Cys^{11},Cys^{14}]-grx-(10–17) (- - - - -), [Sec^{11},Cys^{14},Lys^{16}]-grx-(10–17) (. . . . .) and [Sec^{11},Sec^{14},Lys^{16}]-grx-(10–17) (——) in 0.1 M phosphate buffer, pH 7.0 at 20 °C.

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suggesting that different backbone conformations involving different disulphide geometries are populated in solution [40]. The positive long wavelength CD band was attributed to a right-handed chirality about the S–S bond in the tetrapeptide, and the negative maximum in DMSO to the left-handed disulphide chirality. The near UV CD spectrum of the octapeptide grx-(10–17) (Figure 8) in aqueous solution is very similar to that of the tetrapeptide in MeOH/H_2O (1 : 1) both regarding location and intensity of the positive maximum at 276–278 and of the shoulder at ~300 nm which was assigned to the phenolic chromophore [40].

Studies of model diselenide compounds have shown that the long wavelength nσ* band of diselenides is red-shifted relative to the dilsulphide band and that the signs of the maxima are identical for the same absolute configurations of diselenides and disulphides [44]. The near UV CD spectrum of the Sec,Sec peptide is characterized by a relatively strong negative maximum at 294 nm and a weak shoulder at 270–280 nm. A similar strong red-shift of the nσ* band has previously been observed upon replacement of both cysteine residues in desamino-oxytocin with selenocysteine [45]. Interestingly, both in the case of grx-(10–17) and desamino-oxytocin, the exchange of sulphur with selenium leads to inversion of the sign of the nσ* long wavelength band. This fact indicates inversion of chirality of the side-chain bridge in both peptides. Thus, the diselenide bridge in [Sec^{11},Sec^{14},Lys^{16}]-grx-(10–17) should be of left-handed chirality.

Associated with the negative long wavelength nσ* band a positive maximum was expected in the 240 nm range. The CD spectrum of the Sec,Sec-peptide exhibits such positive maximum of relatively weak intensity at 244 nm (Figure 7), whereby the overall dichroic properties in the far UV region were found to differ significantly from those of the Cys,Cys peptide. The CD pattern is more consistent with a type II β-turn for the cyclic portion of the molecule.

The CD of disulphides and correspondingly of diselenides results from coupling of the two nσ* transitions or from the imbalance in the magnitude of the two rotational strengths. The inherent imbalance of the rotational strengths of a mixed selenide/sulphide group in the [Sec^{11},Sec^{14},Lys^{16}]-grx-(10–17) octapeptide leads to a splitting of the long wavelength band into two bands of opposite sign and located at the maxima of the disulphide and diselenide, respectively (Figure 8). A very similar phenomenon was observed previously for the selenide/sulphide analogues of desamino-oxytocin where nearby chromophores, i.e. location of the selenocysteine in the sequence, were found to additionally affect the dichroic properties [45]. The overall CD spectra of [Sec^{11},Cys^{14},Lys^{16}]-grx-(10–17) in the near and far UV (Figures 7 and 8) exhibit a pattern that may be regarded as a mixture of the dichroic properties of the Cys,Cys- and Sec,Sec-peptide.

The CD spectra of both the [Sec^{11},Cys^{14},Lys^{16}]-grx-(10–17) and [Sec^{11},Sec^{14},Lys^{16}]-grx-(10–17) suggest a conformational shift in the peptide backbone due to the minor changes deriving from sulphur/selenium replacements in the side-chain bridge. In fact, the bond length of Se–Se is 2.28–2.33 Å vs. 1.8–2.1 Å for S–S; the bond length of C–Se 1.95–1.99 Å vs. 1.82 for C–S, and the dihedral angle $\chi_{Se-Se} \cong 75–88^\circ$ vs. $\chi_{S-S} \cong 90^\circ$. NMR conformational analyses are presently in progress to answer the question of whether the isosteric replacement of cysteine with selenocysteine is capable of modulating conformational preferences as suggested by the CD spectra.

DISCUSSION

In previous syntheses of selenocysteine peptides by the Fmoc strategy in combination with the *p*-methoxybenzyl group for the selenol protection, β-elimination as a serious side reaction was not reported to occur [9, 24]. In the present study this side reaction was found to take place at considerable rates, thus leading to dehydroalanine- and, at minor extents, to Ala(β-piperidyl) peptides. Applying the optimized synthetic protocol listed in Table 2 for the synthesis of the Sec(Mob)/Sec(Mob) octapeptide, this side reaction was significantly reduced, although the required five piperidine treatments sufficed for about 10% dehydroalanine formation. These results clearly show the difficulties encountered by this synthetic approach if higher molecular weight selenocysteine peptides are wanted. Our experiences do not therefore, agree with the high yields reported for the synthesis of [$Sec^{7,23}$]-*r*ANP-(7–28) according to the Fmoc strategy [24].

Besides strong bases that possibly operate via Cα-proton abstraction, even strong electrophiles favour β-elimination, as is well shown by the dehydroalanine formation in the acidolytic cleavage of the Sec(Mob)/Cys(Trt) peptide. An electrophilic attack of the trityl cation on selenium as the factor responsible for β-elimination was fully confirmed

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by the results obtained upon addition of excesses of triphenylmethanol as used to suppress cleavage of the trityl group from [Sec(Mob)^{11}, Cys(Trt)^{14},Lys^{16}]-grx-(10–17). In fact, under these conditions almost quantitative conversion to [Dha^{11}, Cys(Trt)^{14},Lys^{16}]-grx-(10–17) was observed.

Most surprising in the present study was the failure of I_2 oxidation of both [Sec(Mob)^{11}, Sec-(Mob)^{14},Lys^{16}]-grx-(10–17) and [Cys^{11},Sec(Mob)^{14}, Lys^{16}]-grx-(10–17) to produce the related cyclic monomers in acceptable yields. However, cleavage of the *p*-methoxybenzyl group and oxidation to the intramolecular diselenide and mixed selenide/sulphide bridge was successfully accomplished with the DMSO/TFA procedure in the case of the Sec(Mob)/Sec(Mob) and Sec(Mob)/Cys(Mob) peptides, but failed in an unexpected manner with the Cys/Sec(Mob) peptide.

The significantly different CD spectra of the oxidized and reduced [Sec^{11},Sec^{14},Lys^{16}]-grx-(10–17) and [Sec^{11},Cys^{14},Lys^{16}]-grx-(10–17) octapeptides, as well as the observation that mixed disulphides with the reductant are not formed, allowed the redox potential of the diselenide and mixed selenide/sulphide group, respectively, to be determined, using the strongly reducing DTT as the reference redox system [12]. The redox potential of the diselenide peptide ($E_0 = -381$ mV) was found to be remarkably lower than previously speculated for selenocystine peptides and proteins [8], and significantly more reducing than DTT ($E_0 = -323$ mV [44]), whereas the mixed selenide/sulphide peptide exhibits redox properties ($E_0 = -326$ mV) very similar to those of DTT. For comparison, the redox potential of the related Cys/Cys peptide [Cys^{11},Cys^{14})-grx-(10–17) is -180 mV relative to a glutathione ($E_0 = -205$ mV [12]). We postulated that this remarkable difference in the redox potentials of a disulphide, mixed selenide/sulphide and diselenide bridge should guarantee regioselective diselenide-binding in synthetic peptides and biosynthetic proteins even in presence of additional cysteine residues [11, 12]. This expectation was fully confirmed by model studies performed on the human [Sec^{3,11}, Cys^{1,15},Nle^{7}]-endothelin-I analogue [47].

The regioselective induction of a correct pairing of two cysteines via their replacement with selenocysteines should facilitate not only the correct oxidative refolding of multiple cysteine-containing peptides [47, 48]], but also of proteins, since specific bioincorporation of selenocysteine into two defined sequence positions via the natural translational machinery is conceivable. This approach would open a new access to isomorphous atomic mutants of proteins well suited for the study of folding pathways, but even for X-ray crystallography via the multiwavelength anomalous dispersion method [1]. For this purpose, however, it is essential that the 3D-structure of the wt-protein is fully retained in the Sec mutants. The structural shifts suggested by the CD spectra of the octapeptides could derive from the relatively free conformational space of such side-chain bridged peptides as is well supported by previous studies on the conformational changes induced by various aprotic and protic solvents of the active-site tetrapeptide of glutaredoxin [40]. In proteins or in peptides with more complex cystine frameworks, the conformational space should be significantly more restricted and the constraints imparted by the overall fold are expected to anneal the minor changes induced by a diselenide bridge on the microenvironment.

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