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Synthesis of symmetric disulfides as potential alternative substrates for trypanothione reductase and glutathione reductase: Part 1

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Summary. The synthesis of a series of symmetrical disulfides as potential substrates of trypanothione reductase and glutathione reductase was described. The key intermediate in the synthetic approach was the choice of S-^{t}butylmercapto-L-cysteine (1). The spermidine ring in the native substrate, trypanothione disulfide (TSST), was replaced with 3-dimethylaminopropylamine (DMAPA), while the γ-Glu moiety was replaced by phenylalanyl or tryptophanyl residues. The same modifications in the γ-Glu moiety of glutathione disulfide (GSSG) were applied.

Key words: Amino acids – Synthesis – Symmetrical disulfide – Trypanothione reductase – Glutathione reductase

Introduction

There is a continuing need to design and develop effective and inexpensive antitrypanosomal drugs to combat Chagas' disease and African sleeping sickness (Benson et al., 1992; Garforth et al., 1994). Trypanothione reductase (TR) belongs to the glutathione reductase (GR) family of enzymes, all of which are FAD and NAD (PH) dependent disulfide oxidoreductases. Reduced glutathione is involved in radical uptake and detoxification reactions. Trypanothione reductase maintains the reduced pool of trypanothione by reducing trypanothione disulfide (TSST) (Schirmer et al., 1995). Trypanothione disulphide is involved in maintaining the reduced state in the pathogen, and along with trypanothione peroxidase protects the parasite against redox damage. Trypanothione reductase, a key metabolic enzyme, is unique to the trypanosomitidae, and represents a useful target for antitrypanosomal drugs (Fairlamb, 1985).

Protein homology modelling (Benson et al., 1992), and the subsequent evaluation of the crystal structure of TR (Hunter et al., 1992) provided details of the substrate binding site of TR. Structural variation in inhibitors and

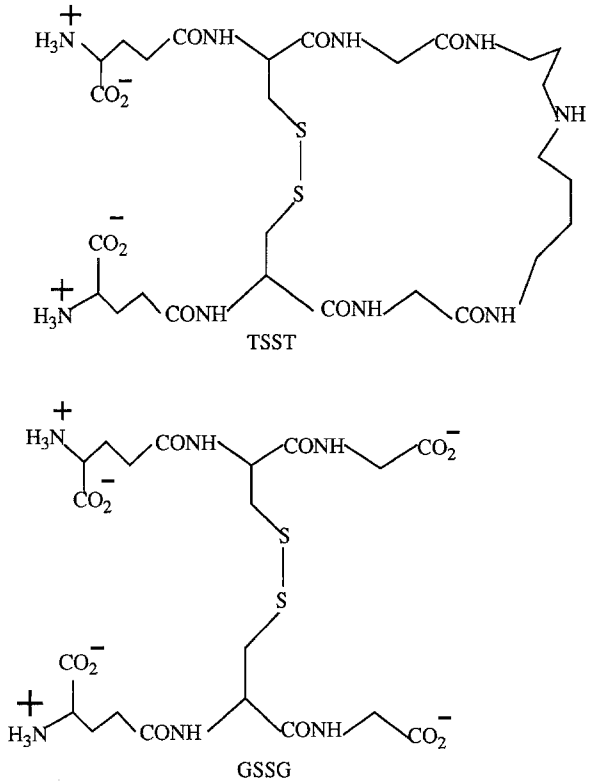


Fig. 1. Structures of trypanothione disulfide (TSST) and glutathione disulfide (GSSG)

substrate-analogues, a valuable way of determining the importance of particular substrate-binding site interactions, provides complementary information which can be rationalized with the aid of the 3-dimensional structure. Modification of the spermidine bridge of TSST has been studied (Walsh et al., 1991) and the 3-dimethylaminopropylamine (DMAPA) group was found to provide a convenient C-terminal replacement substituent of TSST (El-Waer, 1992), and was used in this sudy. We report here synthetic aspects of further modifications which have been performed including the replacement of the diionic γ-glu groups of TSST analogues by nonpolar substituents such as phenylalanyl and trytophanyl. A general synthetic route to series of novel substrate analogues of (TR) and (GR) has been established.

Materials and methods

Protected amino acids (Boc-Phe-OSu, Boc-Trp-OSu, Z-Gly-OSu) were obtained from Bachem Feinchemikalen AG, glutamic acid derivatives from Sigma, S-^{t}butylmercapto-L-cysteine from Fluka and all other chemicals from Aldrich. Media for column chromatography, aluminium oxide (active neutral) and silica gel 60, were purchased from BDH.

Reactions were usually carried out under an inert atmosphere. For coupling reactions, anhydrous reactants and dry solvents were distilled before used.

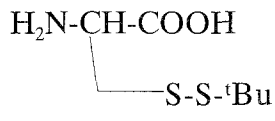
The progress of synthesis was monitored by TLC on Merck plates precoated with a layer (0.25 mm thick) of silica gel 60 F_{254} and visualised by UV absorption (model GL-58 Mineral-light UV lamp). Melting points were determined using a Gallenkamp apparatus and are uncorrected. FAB-MS were recorded by means of a Kratos-Concept instrument operating in the FAB mode (Xe beam bombardment), using m-nitrobenzyl alcohol as a

matrix. Elemental analyses were recorded on a EA1108-Elemental Analyser (Carlo Erba Instruments) by the Department of Chemistry at the University of Manchester.

NMR spectra were recorded either on Bruker WP8OSY spectrometer operating at 80.13 Mhz for ^{1}H and 20.15 Mhz for ^{13}C nmr spectroscopy or a Jeol JNM EX 270 spectrometer operating at 270 Mhz for ^{1}H nmr and 68 Mhz for ^{13}C. ^{1}H nmr spectroscopic data are reported using the following convention: chemical shift (ppm) (integrated intensity, splitting paterns are abbreviated as: s-singlet; d-doublet; t-triplet q-quartet; p-pentet; m-unresolved multiplet; bs-broad singlet. Assignments were made on the basis of one-dimensional decoupling and on two-dimensional homonuclear shift correlation spectroscopy (COSY). Exchangeable protons were characterized by their disappearance on the addition of a drop of D_2O and are indicated by *. The ^{1}H (^{13}C) heterocorrelated spectra were obtained with the pulse sequence of Bax et al. The multiplicities of the 68 Mhz ^{13}C spectral resonnances were determined by using DEPT spectra with pulse angles, $\theta = 90°$ and $\theta = 135°$.

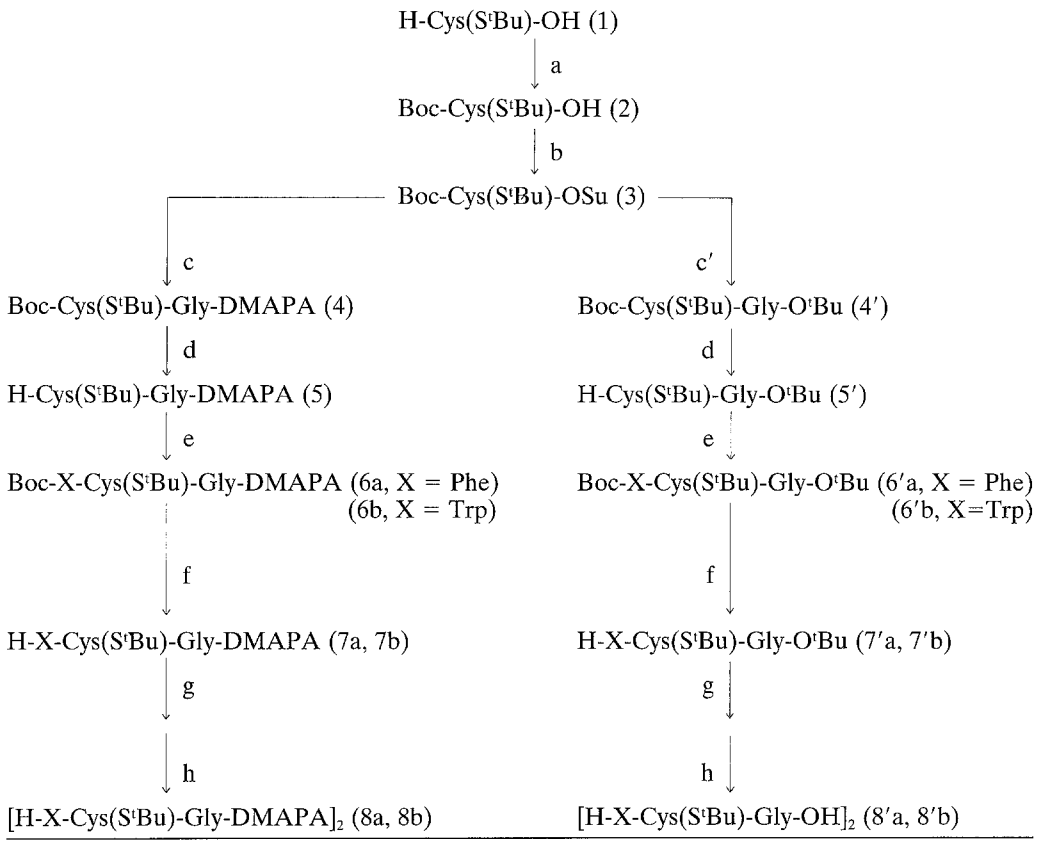
Discussions and results

The development of methods suitable for the preparation of symmetrical, open-chain cystine derivatives represents an important synthetic aspect of polypeptide chemistry, because of the necessary stepwise formation of the disulfide (Hiskey et al., 1966). The reaction requires the use of sulfur- directed protective groups that will withstand the conditions used for the coupling of various reagents. However, the sulfur protective group must be easily removed later when disulfide bond formation is required. The key intermediate in our synthetic approach was the choice of S-^{t}butylmercapto-L-cysteine (1).



(1)

This intermediate provided the basis for the synthesis of a variety of analogues of trypanothione. The characteristic features of the synthetic route (Scheme 1) were the following: (i) The preparation of an open-chain amino- and carboxy-protected peptide. For amino-group protection of L-cysteine in basic medium, 2-^{t}butoxycarbonyloxyimino-2-phenylacetonitrile (Boc-ON) is a convenient reagent (compound 2, Scheme 1), while N-hydroxysuccinimide and N-hydroxybenzotriazole hydrate are good esterification reagents in peptide chemistry (Bitter et al., 1965), because of their ready displacement in nucleophilic substitutions. The coupling reagent used was N,N′-dicyclohexylcarbodiimide (DCC) in an appropriate solvent. (compound 3). (ii) Displacement of carboxyl activating groups by the glycine derivatives, H-Gly-DMAPA (El-Waer, 1992) or H-Gly-O^{t}Bu (compounds 4 and 4′). (iii) Removal of the remaining protective groups, without disturbing either the S-protective group or the chirality of the molecule. The use of triethylsilane as a carbocation scavenger in the presence of trifluoroacetic acid and in dichloromethane led to improved selectivity for the deprotection of tertiary-butyl ester and tertiary-butoxycarbonyl groups in protected amino acids and



Scheme 1. Synthesis of tripeptide dimethylaminopropylamide (8) and carboxylate (8′) disulfides. *a* Boc-on, Et_3N, dioxane/water, 4h/rt, *b* N-hydroxysuccinimide-DCC-dioxane, 8°C/16h, *c* H-Gly-DMAPA, DMF, 3h/rt, *d* TFA/CH_2Cl_2, 1h/rt. *e* Boc-X-OSu (X = Phe, 3h/rt, X = Trp, 16h/rt), DMF, *f* TFA/CH_2Cl_2, 3h/rt, *g* DTT, *h* I_2/CH_3OH, *c'*H-Gly O^{t}Bu, HCl, DMF, Et_3N, 16/rt

peptides in the presence of other acid-sensitive protecting groups such as the tertiary-butylthio group (Mehta et al., 1992). (iv) By deprotecting selectively the amino group of the cysteine residue, the peptide chain could be lengthened from 2 to 3 or more amino acids residues by successive reactions, e.g. by coupling with N,O-protected amino acids (in this case with Boc-Phe-OSu, Boc-Trp-OSu), followed by removal of the protective group (Boc), and then coupling with either N-protected amino acid (e.g. Z-Gly-OH) or N,O-protected amino acid (e.g. Z-Gly-OSu).

The coupling of (5 and 5′) with Boc-Phe-OSu occurred at room temperature in a polar solvent to give a single clean product, which was identified as the tripeptide (6a and 6a′) by nmr spectroscopy. When the reaction was carried out with Boc-Trp-OSu two products were obtained with very close R_f values (6b). Unsuccessful attempts were made to separate them by flash chromatography on alumina. Analysis of the ^1H and ^{13}C nmr spectra showed that most of the peaks were doubled. No similar phenomenon was recorded in the case of phenylalanine instead of tryptophan. The doubling of the peaks could be due to an equilibrium between two isomers of the products, to

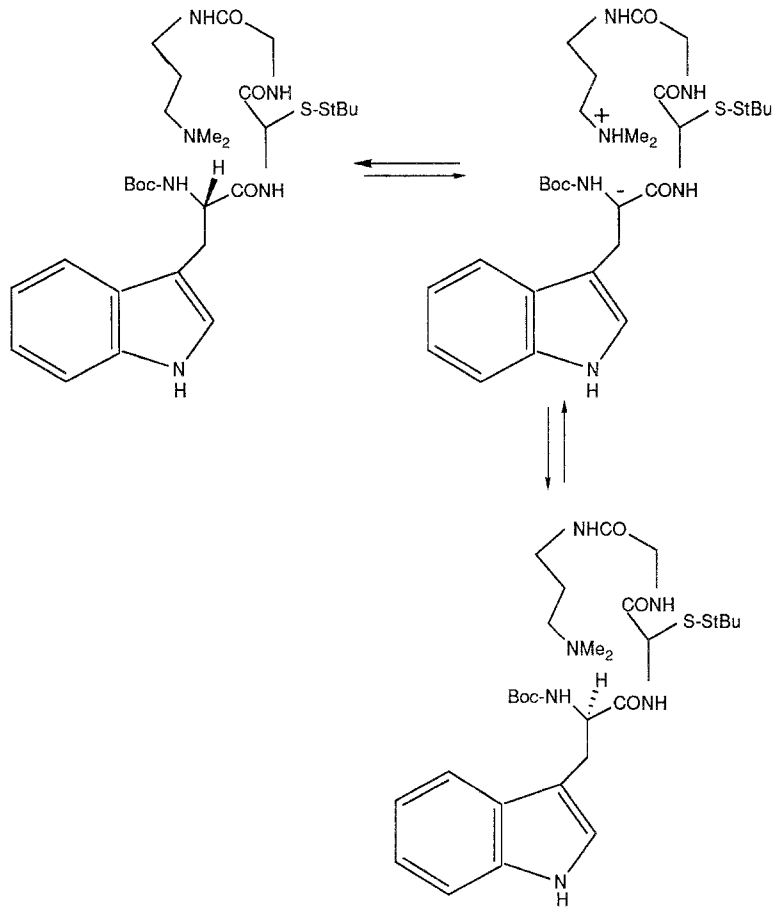


Fig. 2. A possible α-catalysed intramolecular racemisation in [Boc-Trp-Cys(S^{t}Bu)-Gly-DMAPA]

racemisation or to oxidation of the nitrogen of tryptophan. The last of these is considered unlikely as no similar observation was made on coupling Boc-Trp-OSu with H-L-Cys(S^{t}Bu)-Gly-O^{t}Bu under similar conditions and a single product (6′b) was obtained (Scheme 1). If racemisation is the explanation there must be something special about Boc-Trp-OSu, but only with the DMAPA derivative of glycine, not with the t-butyl ester as the latter did not give rise to peak doubling.

The major difference between $-CO_2Bu^t$ and $-NH(CH_2)_2NMe_2$ is that the latter has a terminal tertiary amine and in principle, this could catalyse α-H racemisation intramolecularly. (v) Dithiothreitol (DTT) was found to cleave the S-^{t}butyl group of the protected L-cysteine residue readily. Because it has a low redox potential and other convenient properties (Cleland, 1964), it is the reagent of choice for removal of the mixed disulfide class of protective groups. All the disulfides prepared in this study were treated by Ellman's reagent (5, 5′-dithiobis(2-nitrobenzoic acid) to confirm the absence of thiol traces and were obtained as iodide ammonium salts.

N-^{t}Butoxycarbonyl-(S-^{t}butylmercapto)-L-cysteine (2)
[Boc-Cys(S^{t}Bu)-OH]
S-^{t}Butylmercapto)-L-cysteine (1) (6.04 g, 28.85 mmol) and triethylamine (4.37 g, 43.28 mmol) were added to a mixture of dioxane/water (25 ml/25 ml). 2-^{t}Butoxycarbonyloxyimino-2-phenylacetonitrile (Boc-on) (7.38 g, 30 mmol) was added, the reaction mixture stirred at room temperature for 4 hours and then partitioned between ether (250 ml) and 5% aqueous sodium carbonate (250 ml). The aqueous layer was collected, acidified, with 10% citric acid solution to pH 2.5, and extracted with ethyl acetate 2 × 25 ml), the combined organic layers being dried ($MgSO_4$). After removal of solvent, the residue was triturated with hexane to give the desired compound as an off-white solid (6.6 g, 14.9 mmol) m.p. 120–122°C.

^{1}H nmr ($CDCl_3$): 1.32 (s, 9H, ^{t}Bu), 3.02–3.20(m, 2H, $C_\beta H_2$-S), 4.30–4.50(m, 1H, C_αH-Cys).

N-^{t}Butoxycarbonyl-(S-^{t}butylmercapto)-L-cysteinyl-N-oxo-succinimide ester (3)
[Boc-Cys(S^{t}Bu)-OSu]
To an ice-water cooled dioxane solution (30 ml) of Boc-Cys(S^{t}Bu)-OH (4.6 g, 14.9 mmol) and N-hydroxysuccinimide (1.71 g, 14.9 mmol), N,N′-dicyclohexycarbodiimide (3.07 g, 14.9 mmol) was added. After shaking the solution for a few minutes, a solid precipitated and the reaction mixture was kept at 8°C overnight. N,N′-Dicyclohexylurea was filtered off and the filtrate evaporated to dryness. The resulting oily residue solidified and was recrystallised from isopropanol to give (3) (5.6 g, 13.8 mmol), m.p. 80–82°C.

^{1}H nmr ($CDCl_3$); 1.28(s, 9H, ^{t}Bu), 2.85(s, 4H, OSu), 3.1–3.45(m, 2H, $C_\beta H_2$-Cys), 4.8–4.95(m, 1H, C_αH-Cys), 5.22–5.35(m, 1H, NH)*.

N-^{t}Butoxycarbonyl-(S-^{t}butylmercapto)-L-cysteinylglycyl-3-dimethylaminopropylamide (4)
[Boc-Cys(S^{t}Bu)-Gly-DMAPA*]
*DMAPA = [dimethylaminopropylamine: $(CH_3)_2NH$-C^1H_2-C^2H_2-C^3H_2NH]
A solution of (H-Gly-DMAPA) (0.8, 5 mmol) in DMF (3 ml) was added to a DMF (10 ml) solution of (3) (2 g, 4.9 mmol) and the progress of reaction was monitored by TLC (chloroform, visualised by iodine).

After stirring the reaction mixture for 4 hours at room temperature, solvent was evaporated, the residue taken up with chloroform (3 ml) and applied to a flash chromatography column of alumina (active neutral) and eluted with chloroform. Evaporation of the fractions which showed (TLC) the desired compound (4) gave a colourless oil (0.59 g, 1.31 mmol, 26.7%).

^{1}H nmr ($CDCl_3$): 1.30(s, 9H, ^{t}Bu), 1.38(s, 9H, ^{t}Bu), 1.65(p, J = 7.8 Hz, 2H, C^2H_2-DMAPA), 2.20(s, 6H, 2xCH_3-DMAPA), 2.35(t, J = 8.1 Hz, 2H, C^1H_2-DMAPA), 2.95(m, 2H, $C_\beta H_2$-Cys), 3.35(q, J = 6.3 Hz, 2H, C^3H_2-DMAPA), 3.9(m, 2H, CH_2-Gly), 4.45(m, 1H, C_αH-Cys), 5.3–5.38(m, 2H, 2xNH)*, 6.9(t, J = 6.2 Hz, 1H, NH)*.

^{13}C nmr: 26.40(C^2-DMAPA), 28.23(CH_3-S^{t}Bu), 29.97(CH_3-O^{t}Bu), 38.92(C^1-DMAPA), 39.15($C_\beta H_2$-Cys), 43.20(CH_2-Gly), 45.12(CH_3-DMAPA), 48.84(C-S^{t}Bu), 54.26(C-O^{t}Bu), 56.51(C_αH-Cys), 58.07(C^3-DMAPA), 169.53(CO-Boc), 172.04 (CO-Cys), 172.50(CO-Gly).

(S-^{t}Butylmercapto)-L-cysteinylglycyl-3-dimethylaminopropylamide (5)
[H-Cys(S^{t}Bu)-Gly-DMAPA]

Compound (4) (0.59 g, 1.31 mmol) was dissolved in anhydrous dichloromethane (4 ml). After cooling the solution in an ice-water bath, trifluoroacetic acid (TFA) (4 ml) was added and the reaction mixture was stirred for 45 minutes. Further TFA (1 ml) was added and stirring continued for another hour, after which the reaction mixture was evaporated to give an oily residue which was reconstituted in 2N NaOH (3 ml). This solution was extracted with dichloromethane as well as the aqueous layer (twice). The combined organic layers were dried, and solvent evaporated to give (5) as a viscous oil, (0.356 g, 1.02 mmol, 77%).

^1H nmr (DMSO-d_6): 1.30(s, 9H, ^{t}Bu), 1.69(p, J = 7.8 Hz, 2H, C^2H_2-DMAPA), 2.25(s, 6H, 2xCH_3-DMAPA), 2.4(t, J = 8.1 Hz, 2H, C^1H_2-DMAPA), 2.9(m, 2H, $C_\beta H_2$-Cys), 3.75(m, 2H, C^3H_2-DMAPA), 3.85(bd, 1H, C_αH-Cys), 3.95(s, 2H, CH_2-Gly), 7.4(m, 1H, NH)*, 8(m, 1H, NH)*.

^{13}C nmr: 26.40(C^2-DMAPA), 26.50(C^2 -DMAPA), 28.41(CH_3-S^{t}Bu), 38.65(C^1-DMAPA), 39.98($C_\beta H_2$-Cys), 43.10(CH_2-Gly), 45.28(CH_3-DMAPA), 48.50(C-S^{t}Bu), 56.22(C_αH-Cys), 57.50(C^3-DMAPA), 170.13 (CO-Cys), 173.15(CO-Gly)

N-^{t}Butoxycarbonyl-L-Phenylalanyl-(S-^{t}butylmercapto)-L-cysteinylglycyl-3-dimethylaminopropylamide (6a)
[Boc-Phe-Cys(S^{t}Bu)-Gly-DMAPA]

A solution of compound (5) (0.156 g, 0.45 mmol) and Boc-Phe-OSu (0.162 g, 0.45 mmol) in a mixture of THF/DMF (95 ml/5 ml) was stirred for 3 hours. On evaporation to dryness, the residue was dissolved in chloroform (3 ml) and purified by flash chromatography on neutral alumina ($CHCl_3$). After monitoring the fractions for the required product by TLC (UV and visual-isation by iodine vapour) the required fractions were combined and evaporated to afford (6a) as a yellowish oil (0.198 g, 0.332 mmol, 74%).

^1H nmr ($CDCl_3$): 1.30(s, 9H, ^{t}Bu), 1.4(s, 9H, ^{t}Bu), 1.7(p, J = 7.7 Hz, 2H, C^2H_2-DMAPA), 2.25(s, 6H, 2xCH_3-DMAPA), 2.38(t, J = 8.1 Hz, 2H, C^1H_2-DMAPA), 2.95-3.4(m, 4H, $C_\beta H_2$-Cys and $C_\beta H_2$-Phe), 3.75–4.0(AB(X), J_{AB} = 16.3 Hz, J_{NH} = 4.8 Hz, 2H, CH_2-Gly), 4.325(q, J = 6.2 Hz, 2H, C^3H_2-DMAPA), 4.62(m, 2H, C_αH-Cys and C_αH-Phe), 5.1(d, J = 3.1 Hz, 1H, NH)*, 7.1(bs, 1H, NH)*, 7.2–7.4(m, 5H, Phe).

^{13}C nmr: 26.40 (C^2-DMAPA), 28.25(CH_3-S^{t}Bu), 29.75(CH_3-O^{t}Bu), 37.52 (C^1-DMAPA), 31.2($C_\beta H_2$-Phe), 40.35($C_\beta H_2$-Cys), 43.30(CH_2-Gly), 45.23(CH_3-DMAPA), 48.74(C-S^{t}Bu), 54.25(C-O^{t}Bu), 56.51(C_αH-Cys), 56.74(C_αH-Phe), 57.34(C^3-DMAPA), 127.43, 129.0, 129.12, 135.79, (aromatic C, Phe), 162.51(CO-Boc), 168.58(Co-Gly), 169.95(CO-Phe), 172.21(CO-Cys).

L-Phenylalanyl-(S-^{t}butylmercapto)-L-cysteinyglycyl-3-dimethylaminopropylamide (7a)
[H-Phe-Cys(S^{t}Bu)-Gly-DMAPA]

To a solution of (6a) (0.192 g, 0.322 mmol) in anhydrous dichloromethane (2 ml) TFA (2 ml) was cautiously added, and the reaction mixture stirred for 3 hours. The residue obtained after evaporation of the solvent was dissolved in 1N NaOH and extracted with chloroform (2 × 25 ml). After drying the organic layer, the solvent was removed, to leave an extremely hygroscopic solid (7a) (0.158 g, 0.318 mmol, 99%).

^{1}H nmr (DMSO-d_6): 1.42(s, 9H, S^{t}Bu),, 1.85(p, J = 7.6 Hz, 2H, C^2H_2-DMAPA), 2.25(s, 6H, 2xCH_3-DMAPA), 2.35(t, J = 8.1 Hz, 2H, C^1H_2-DMAPA), 2.9–3.45(m, 6H, $C_\beta H_2$-Cys, $C_\beta H_2$ Phe and C^3H_2-DMAPA), 3.95(AB(X), J_{AB} = 16.2 Hz, J_{NH} = 4.8 Hz, 2H, CH_2-Gly), 4.22(m, 1H, C_αH-Phe), 4.65(m, 1H, C_αH-Cys), 5.1(bd, 1H, NH)*, 7.0(m, 1H, NH)*, 7.2–7.4(m, 5H, Phe).

^{13}C nmr: 26.40(C^2-DMAPA, 26.35(C^2-DMAPA), 30.21CH_3-S^{t}Bu), 29.75, 37.77(C^1-DMAPA), 41.15($C_\beta H_2$-Phe), 41.65($C_\beta H_2$-Cys), 44.65(CH_3-DMAPA), 48.87(C-S^{t}Bu), 56.32(CH-Cys), 56.75(C_αH-Phe), 57.81(C^3-DMAPA), 127.24, 129.0, 129.11, 135.35, (aromatic C, Phe), 168.51(CO-Gly), 169.91(CO-Phe), 172.13(CO-Cys).

N-^{t}Butoxycarbonyl-L-tryptophanyl-(S-^{t}butylmercapto)-L-cysteinylglycyl-3 dimethylaminopropylamide (6b)
[Boc-Trp-Cys(S^{t}Bu)-Gly-DMAPA]

A solution of compound (5) (0.327 g, 2.1 mmol) and Boc-Trp-OSu (0.866 g, 2.1 mmol) in a mixture of THF/DMF (110 ml/15 ml) was stirred for 6 hours, and then evaporated to dryness. The residue was dissolved in chloroform (3 ml) and purified by flash chromatography on neutral alumina ($CHCl_3$). After monitoring the fractions for the required product by TLC (UV and visualisation by iodine vapour) the required fractions were combined and evaporated to afford (6b)as a yellowish oil (0.894 g, 1.5 mmol, 71.5%).

^{1}H nmr ($CDCl_3$): 1.30(s, 9H, ^{t}Bu), 1.4(s, 9H, ^{t}Bu), 1.7(p, J = 7.7 Hz, 2H, C^2H_2-DMAPA), 2.25(s, 6H, 2xCH_3-DMAPA), 2.38(t, J = 8.1 Hz, 2H, C^1H_2-DMAPA), 2.95–3.4(m, 4H, $C_\beta H_2$-Cys and $C_\beta H_2$Phe). 3.75–4.0(AB(X), J_{AB} = 16.3 Hz, J_{NH} = 4.8 Hz, 2H, CH_2-Gly), 4.32(q, J = 6.2 Hz, 2H, C^3H_2-DMAPA), 4.62(m, 2H, C_αH-Cys and C_αH-Phe), 5.4(d, J = 3.5 Hz, 1H, NH)*, 7.1(t, J = 6.3 Hz, 1H, NH)*, 7.2(m, 3H, Trp), 7.39(d, J = 8.4 Hz, 1H, Trp). 7.6(d, J = 8,4 Hz, 1H, Trp), 8.55 and 8.65(m, 2H, 2xNH)*.

^{13}C nmr: 26.51 and 26.82(C^2-DMAPA), 28.45(CH_3-S^{t}Bu), 29.82 and 29.90 (CH_3-O^{t}Bu), 38.21 and 38.52(C^1-DMAPA), 38.03($C_\beta H_2$-Phe), 39.34 and 40.00($C_\beta H_2$-Cys), 40.70 and 40.89 and 41.4($C_\beta H_2$-Trp), 43.20(CH_2-Gly), 45.24 and 45.40(CH_3-DMAPA), 48.61 and 48.84(C-S^{t}Bu), 54.01 and 54.50(C-O^{t}Bu), 56.12(C_αH-Trp), 56.52(C_αH-Cys), 57.26 and 57.30(C^3-DMAPA), 109.81 and 110.34, 111.50 and 111.84, 118.55 and 118.92, 119.83 and 120.11, 122.46 and 122.82, 123.62 and 123.72, 127.26 and 127.34, 136.38 and 136.57(Trp), 169.21(CO-Boc), 170.18(CO-Cys or Trp), 172.82(CO-Gly), 173.28(CO-Cys or Trp).

L-Tryptophanyl-(S-^{t}butylmercapto)-L-cysteinylglycyl-3-dimethylaminopropylamide (7b)
[H-Trp-Cys(S^{t}Bu)-Gly-DMAPA

Compound (6b) (0.253 g, 0.4) was dissolved in anhydrous dichloromethane (4 ml) and treated with TFA (4 ml). The reaction mixture was stirred for 1.5 hour at room temperature, further TFA (0.5 ml) added and the stirring continued for 1 hour. The reaction mixture was evaporated to dryness, the residue treated with 2N NaOH (3 ml), and extracted with chloroform (2 × 50 ml). The chloroform layer was washed with brine, dried ($MgSO_4$) and evaporated to give (7b) as an off-white solid (0.21 g, 0.391 mmol, 98%), m.p. 101–102°C.

^{1}H nmr (DMSO-d_6): 1.3(s, 9H, S^{t}Bu), 1.85(p, J = 7.8 Hz, 2H, C^2H_2-DMAPA), 2.21(s, 6H, 2xCH_3-DMAPA), 2.41(t, J = 8.1 Hz, 2H, C^1H_2-DMAPA), 2.81–3.15(m, 4H, $C_\beta H_2$-Cys, $C_\beta H_2$-Trp), 3.21–3.38(m, 2H, C^3H_2-DMAPA), 3.6–3.82(AB(X), J_{AB} = 15.8 Hz, J_{NH} = 4.6 Hz, 3H, C_αH-Trp and CH_2-Gly), 4.62(m, 1H, C_αH-Cys), 6.45(t, J = 6.5 Hz, 1H, NH)*, 6.78(t, J = 6.3 Hz, 1H, NH)*, 7.25(m, 3H, Trp), 7.38(d, J = 8.3 Hz, 1H, Trp), 7.59(d, J = 8.3 Hz, 1H, Trp), 7.95(d, J = 3.5 Hz, 1H, NH)*, 8.05(m, 1H, NH)*, 8.4(s, 1H, NH)*.

^{13}C nmr: 26.05 and 26.20(C^2-DMAPA), 30.12(CH_3-S^{t}Bu), 38.60 and 38.65 (C^1-DMAPA), 41.25($C_\beta H_2$-Trp), 41.63($C_\beta H_2$-Cys), 43.43 and 43.69(CH_2-Gly), 45.20 and 45.25(CH_3-DMAPA), 48.50(C-S^{t}Bu), 53.10 and 53.35(C_αH-Trp), 55.50 and 55.92(C_αH-Cys), 57.82 and 57.93(C^3-DMAPA), 110.52, 117.33, 119.82, 120.61, 122.52, 123.87, 124.0(Trp), 168.12(CO-Cys or Trp), 171.22(CO-Gly), 175.42(CO-Cys or Trp).

N-^{t}Butoxycarbonyl-L-(S-^{t}butylmercapto)-L-cysteinylglycine O-^{t}butyl ester (4′)
[Boc-Cys(S^{t}Bu)-Gly-O^{t}Bu]

To a solution of compoud (3) (1.25 g, 3.08) in a mixture of THF (20 ml) and DMF (8 ml) was added H-Gly-O^{t}Bu, HCl (0.515 g, 3.08) and triethylamine(0.45 ml), 3.23 mmol). The reaction mixture was stirred for 16 hours then evaporated to dryness. Flash chromatography of the residue on neutral alumina (eluted with chloroform) gave (4′), monitored by TLC (chloroform, R_f = 0.85) as a yellowish, pure solid (1.12 g, 2.65 mmol, 86%) m.p. 89–91°C.

^{1}H nmr ($CDCl_3$): 1.32(s, 9H, S^{t}Bu), 1.48 and 1.49(s, 18H, O^{t}Bu), 3.1(d, J = 9.9 Hz, 4H, $C_\beta H_2$-Cys), 3.92 (AB(X), J_{AB} = 16.1 Hz, J_{NH} = 4.8 Hz, 2H, CH_2-Gly), 4.5(m, 1H, C_αH-Cys), 5.35(bs, 1H, NH)*, 6.81(bs, 1H, NH)*.

S-^{t}Butylmercapto)-L-Cysteinylglycine O-^{t}butyl ester (5′)
[H-Cys(S^{t}Bu)-Gly-O^{t}Bu]

To an anhydrous dichloromethane solution (20 ml) of (4′) (1.1 g, 2.6 mmol) was added TFA (4 ml), the solution stirred for 1 hour at room temperature, and excess solvent removed to give (5′) as a nearly colourless oil (0.7 g, 2.18 mmol, 83%), which was used without further purification.

^{1}H nmr (DMSO-d_6): 1.35 and 1.48(2s, 18H, 2^{t}Bu), 3.25(m, 2H, $C_\beta H_2$-Cys), 3.95 (AB(X), J_{AB} = 15.9Hz, J_{NH} = 4.9Hz, 2H, CH_2-Gly), 4.52(m, 1H, C_αH-Cys), 7.82(t, J = 6.5Hz, 1H, NH)*.

N-^{t}Butoxycarbonyl-L-phenylalanyl-(S-^{t}butylmercapto)-L-cysteinylglycine O^{t}butyl ester (6′a)
[Boc-Phe-Cys(S^{t}Bu)-Gly-O^{t}Bu]
A mixture of (5′) (0.4g, 1.24mmol) and Boc-Phe-OSu (5′) (0.45g, 1.24mmol) in DMF (4ml) was stirred at room temperature for 3 hours. After evaporation of the solvent, the residue was dissolved in dichloromethane (2ml) and purified by flash chromatography on neutral alumina ($CHCl_3$ elution). The fractions containing the desired product were detected by TLC (chloroform), combined and evaporated to yield (6′a) as a sticky solid (0.38g, 0.74mmol, 59.6%).

^{1}H nmr ($CDCl_3$): 1.3, 1.4, 1.42(3s, 27H, 3x^{t}Bu), 2.3(bm, 1H, NH)*, 2.95–3.2(m, 4H, $C_\beta H_2$-Cys, $C_\beta H_2$-Phe), 3.82(AB(X), J_{AB} = 16.1Hz, J_{NH} = 4.8Hz, 2H, CH_2-Gly), 4.38(q, J = 7.4Hz, 1H, C_αH-Phe), 4.75(q, J = 7.1Hz, 1H, C_αH-Cys), 5.15(bd, 1H, NH)*, 6.95(m, 1H, NH)*, 7.12–7.3(m, 5H, Phe).

L-Phenylalanyl-(S-^{t}butylmercapto)-L-cysteinylglycine (7′a)
[H-Phe-Cys(S^{t}Bu)-Gly-OH]
To an anhydrous dichloromethane solution (3ml) of (6′a) (0.38g, 0.74 mmol) was added TFA (3ml) and triethylsilane (0.2ml), the solution stirred for 1.5 hour at room temperature, and then evaporated to dryness. The residue was triturated with anhydrous ether, causing the precipitation of product (7′a) as an off-white solid (0.20g, 0.5mmol, 67%), m.p. 88–90°C.

^{1}H nmr (DMSO-d_6): 1.22(s, 9H, ^{t}Bu), 2.81–3.81–3.2(m, 4H, $C_\beta H_2$-Cys, $C_\beta H_2$-Phe), 3.78(m, 1H, CH_2-Gly), 3.92(m, 1H, C_αH-Phe), 4.58(m, 1H, C_αH-Cys), 7.2–7.38(m, 5H, Phe), 8.35(t, J = 6.4Hz, 1H, NH)*.

N-^{t}Butoxycarbonyl-L-tryptophanyl-(S-^{t}butylmercapto)-L-cysteinylglycine O-^{t}butyl ester (6′b)
[Boc-Trp-Cys(S^{t}Bu)-Gly-O^{t}Bu]
A mixture of (5′) (0.34g, 1.05mmol) and Boc-Trp-OSu (0.433g, 1.05mmol) in DMF (3ml) was stirred at room temperature for 3.5 hours. The residue obtained after evaporation of solvent was purified by flash chromatography on neutral alumina ($CHCl_3$ elution; TLC (chloroform) R_f = 0.55). The product (6′b) was obtained as a viscous oil (0.3g, 0.493mmol, 46.7%).

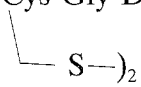
^{1}H nmr ($CDCl_3$): 1.32, 1.4, 1.42(3s, 27H, 3x^{t}Bu), 2.95–3.2(m, 4H, $C_\beta H_2$-Cys,), 3.35(AB(X), J_{AB} = 15.7Hz, J_{NH} = 4.6Hz, 2H, $C_\beta H_2$-Trp), 3.82(AB(X), J_{AB} = 16.3Hz, J_{NH} = 4.8Hz, 2H, CH_2-Gly), 4.45(q, J = 6.4Hz, 1H, C_αH-Cys),), 4.72(q, J = 6.4Hz, 1H, C_αH-Trp), 5.12(m, 1H, NH)*, 6.62–6.9(m, 1H, NH)*, 7.1–7.25(m, 3H, Trp), 7.35(d, J = 8.4Hz, 1H, Trp), 7.65(d, J = 8.4Hz, 1H, Trp), 8.31(s, 1H, NH)*.

L-Tryptophanyl-(S-^{t}butylmercapto)-L-cysteinylglycine (7′b)
[H-Trp-Cys(S^{t}Bu)-Gly-OH]

To an anhydrous dichloromethane solution (2 ml) of (6′b) (0.3 g, 0.493 mmol) was added TFA (2 ml) and triethylsilane (0.1 ml) and the mixture stirred for 1.5 hour at room temperature. Evaporation of the solvent provided a nearly colourless oil, which was triturated with ether causing the precipitation of (7′b) an off-white solid (0.2 g, 0.38 mmol, 75%), m.p. 102–103°C.

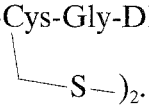
^{1}H nmr (DMSO-d_6): 1.3(s, 9H, ^{t}Bu), 2.82–3.38(m, 4H, $C_\beta H_2$-Cys, $C_\beta H_2$-Trp), 3.75(AB(X), J_{AB} = 16.2 Hz, J_{NH} = 4.9 Hz, 2H, CH_2-Gly), 4–4.15(m, 1H, C_αH-Trp),), 4.62–4.7(m, 1H, C_αH-Cys), 6.98–7.12(m, 3H, Trp), 7.2(bm, 1H, NH)*, 7.38(d, J = 8.2 Hz, 1H, Trp), 7.72(d, J = 8 Hz, Trp), 8.47(t, J = 6.3 Hz, 1H, NH)*, 9.05(bd, 1H, NH)*.

L-Phenylalanyl-L-cysteinylglycyl-3-dimethylaminopropylamide disulfide (8a)
[Phe-Cys-Gly-DMAPA]



H-Phe-Cys(S^{t}Bu)-Gly-DMAPA (7a) (0.15 g, 0.3 mmol) was dissolved in deionised water (20 ml), the slightly cloudy solution filtered, and its pH recorded as 7.6. This aqueous solution was purged for a few minutes with argon before dithiothreitol (0.155 g, 1 mmol) was added. The reaction mixture was stirred for 1.5 hour in a well-ventilated fume-cupboard (t-butylthiol was evolved). After dilution of the reaction mixture with water (30 ml), the solution was adjusted to pH 2.65 with 1N HCl, extracted with ethyl acetate (5 × 50 ml), and the aqueous layer further diluted with water (20 ml). This reaction mixture was maintained in an ice-bath under argon and a solution of iodine (78.8 mg, 0.302 mmol) in methanol (4 ml) added dropwise over 40 minutes (the solution turned from colourless through light yellow to dark red). After the addition of I_2, stirring was continued for 10 minutes and the solution extracted with ethyl acetate (300 ml). The extraction mixture formed an emulsion so the two layers were left to settle for two hours, during which time separation occurred. The collected aqueous layer was extracted with ethyl acetate (2 × 200 ml) and freeze-dried. The solid obtained was suspended in ethyl acetate, the filtrate decanted and the solid dissolved in water (20 ml) and freeze-dried again. The resulting yellow solid was dissolved in absolute ethanol and drops of anhydrous ether added until the solution turned cloudy. This mixture was kept at −20°C overnight. After removal of solvent by filtration, product (8a) was obtained (92 mg, 0.059 mmol, 19.5%) as an extremely hygroscopic, tetraammonium iodide salt (Tables 1, 2, 3).

L-Tryptophanyl-L-cysteinylglycyl-3-d imethylaminopropylamide disulfide (8b)
[Trp-Cys-Gly-DMAPA]



H-Trp-Cys(S^{t}Bu)-Gly-DMAPA (7b) (0.2 g, 0.373 mmol) was dissolved with difficulty in deionised water (25 ml) and the pH of the solution adjusted to pH

Table 1. ^{1}H nmr chemical shifts (ppm) of symmetrical tripeptide dimethylaminopropylamide (Y = DMAPA) and carboxylate (Y = OH) disulfides (270 MHz, D_2O)[a,b]

peptide	entry	X	X = Phe or Trp		Cys		Gly	DMAPA			
X-Cys-Gly-Y S)$_2$			$C_\alpha H$	$C_\beta H_2$	$C_\alpha H$	$C_\beta H_2$	$C_\alpha H_2$	C^1H_2	C^2H_2	C^3H_2	CH_3
X = Phe Y = DMAPA	(8a)	7.17–7.3 m	4.25 t J = 7.1 Hz	3.13[c] d J = 2 Hz	4.55[d] m	2.85,2.91 dd J = 8.5 Hz	3.77 s	3.05[c] t J = 7.6 Hz	1.81 p J = 7.2 Hz	3.21[c] t J = 6.7 Hz	2.75 s
X = Trp Y = DMAPA	(8b)	6.85–7.45 m	4.17 t J = 7.3 Hz	3.18 d J = 7.1 Hz	4.55–4.39[d] m	2.55–2.85 m	3.65 s	2.87 t J = 7.5 Hz	1.82 m	3.08 m	2.62 s
X = Phe Y = OH	(8′a)	7.02–7.25 m	4.18 t J = 7.2 Hz	2.95,3.1 dd J = 6.7 Hz	4.71[d] m	2.76,2.84 dd J = 8.8 Hz	3.62 s				
X = Trp Y = OH	(8′b)	6.85–7.4 m	4.11 m	3.22 bd	4.22[d] m	2.55–3.95 m	3.52 s				

[a] The signal positions are given relative to D_2O. All integrals correspond to the appropiate number of identical hydrogen. [b] Assignments of the signal are supported by H-H COSY. [c] Signals overlapping. [d] Overlapped by water signal; assignments confirmed by H-H COSY.

Table 2. ^{13}Cnmr chemical shifts of symmetrical tripeptide dimethylaminopropylamide (Y = DMAPA) and carboxylate (Y = OH) disulfides [68 MHz, D_2O, reference to dioxane][a]

peptide	entry	CO	X	X = Phe or Trp		cys		Gly	DMAPA			
			Phe or Trp $C_{aromatic}$	CαH	CβH_2	CαH	CβH_2	CβH_2	C^1H_2	C^2H_2	C^3H_2	CH_3
X-Cys-Gly-Y S)$_2$												
X = Phe Y = DMAPA	(8a)	172.01 171.72 169.8	134.24, 130.14, 129.87, 128.92	53.22	37.53	54.91	38.90	43.42	36.78	24.79	55.95	43.48
X = Trp YDMAPA	(8b)	172.8 171.52 162.45	136.4, 127.4 125.85, 123.42 120.35, 119.42 113.02, 105.82	54.45	37.82[b]	55.1	37.82[b]	44.25[b]	37.89[b]	25.09	56.2	44.3[b]
X = Phe Y = OH	(8′a)	172.5 171.24 169.82	134.51, 131.0, 129.5, 128.24	53.55	40.28	54.91	41.81	42.85				
X = Trp Y = OH	(8′b)	174.1 171.24 170.07	136.86, 127.25 125.97, 122.94 120.39, 118.66 112.76, 106.95	53.15	40.52	54.15	42.56	42.50				

[a] The signal positions are given relative to dioxane. [b] Assignments of signal are supported by C-H COSY.

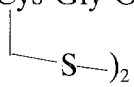
Table 3. Physical properties, FAB-MS (Xe beam) and elemental analyses of tripeptide dimethylaminopropylamide (Y = DMAPA) and carboxylate (Y = OH) disulfides

peptide X-Cys-Gly-Y S)$_2$	entry	m.p.^{0}(C)	R_f^a	(M + 1)^b (%)^c	(M/2 + 1)^b (%)^d	Formula	C	H	N	I	S
X = Phe Y = DMAPA	(8a)	125–127 (dec.)	0.45	817 (100)	409 (48)	calc. $C_{38}H_{64}N_{10}I_4O_6S_2 \cdot 2H_2O$ found	33.6 33.7	5.0 5.6	10.3 10.0	37.4 37.2	4.7 4.7
X = Trp Y = DMAPA	(8b)	175–177	0.38	895 (15)	448(18)	calc. $C_{42}H_{67}N_{12}I_4O_6S_2 \cdot 10H_2O$ found	31.7 31.6	5.4 5.3	10.6 10.5	32.0 31.9	4.0 3.9
X = Phe Y = OH	(8′a)	95–98 (dec.)	0.22	649 (20)		calc. $C_{28}H_{38}N_6I_{12}O_8S_2 \cdot 6H_2O$ found	33.2 33.4	4.9 4.8	8.3 8.2	25.0 25.8	6.3 6.7
X = Trp Y = OH	(8′b)	140–142 (dec.)	0.22	727(80)		calc. $C_{32}H_{60}N_8I_2O_8S_2 \cdot 10H_2O$ found	33.0 32.9	5.2 5.1	9.6 9.6	21.9 21.8	5.5 5.5

[a] R_f retention time, neutral alumina plate, solvent: methanol. [b] Parent ion. [c] Daughter peak. [d] Relative aboundance.

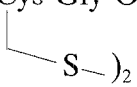
7.65 by adding a few drops of 1N HCl. The solution was filtered and the filtrate diluted with a further portion of water (15 ml). It was then purged with argon for a few minutes, DTT (0.162 g, 1.045 mol) added and the reaction mixture stirred for 2 hours, then diluted with water (25 ml) and adjusted to pH 2 with 1N HCl. The solution was then cooled in an ice-water bath and a solution of iodine (97 mg, 0.381 mmol) in methanol (6 ml) added dropwise under an inert atmosphere over 35 minutes. Stirring was maintained for a further 8 minutes before the product was extracted into ethyl acetate (5 × 100 ml) as described previously for compound (8a). The hygroscopic, yellowish product (8b) (112 mg, 0.0065 mmol, 17.5%) was obtained as the tetraammonium iodide salt (Tables 1, 2, 3).

L-Phenylalanyl-L-cysteinylglycine disulfide (8′a)
[Phe-Cys-Gly-OH]



H-Phe-Cys(S^{t}Bu)-Gly-OH (7′a) (0.207 g, 0.448 mmol) was dissolved in deionised water (100 ml), the solution adjusted to pH 7.5, filtered, the filtrate diluted with water (100 ml) and purged with argon for a few minutes before DTT (0.155 g, 1 mmol) was added. The reaction mixture was stirred for 2 hours, its pH was adjusted to 2.65 with 1N HCl and then it was extracted with ethyl acetate (5 × 75 ml). To the aqueous layer, diluted with water (20 ml) and cooled with an ice-water bath, a solution of iodine (0.127 g, 0.492 mmol) in methanol (6 ml) was added dropwise under argon over 35 minutes. The reaction mixture was diluted with water (15 ml) and the work-up carried out as described for compound (8a). An off-white solid (8′a) (85 mg, 0.083 mmol, 17%) was obtained as the diammonium iodide salt (Tables 1, 2, 3).

L-Tryptophanyl-L-cysteinylglycine disulfide (8′b)
[Trp-Cys-Gly-OH]



H-Trp-Cys(S^{t}Bu)-Gly-DMAPA (7′b) (0.312 g, 0.691 mmol) was dissolved in basified water (45 ml, pH 8.33), filtered and the filtrate purged with argon for a few minutes before DTT (0.219 g, 1.415 mmol) was added and the mixture stirred for 2 hours. The solution was adjusted to pH 2 with 1N HCl, diluted further with water (15 ml), and cooled with an ice-water bath. A solution of iodine (0.177 g, 0.695 mmol) in methanol (8 ml) was added dropwise under Argon over 35 minutes and stirring continued for a further 5 minutes. Work-up was carried out as described for compound (8a), yielding a very light off-white solid (8′b) (81 mg, 0.07 mmol, 10%) as the diammonium iodide salt (Tables 1, 2, 3).

References

Benson TJ, McKie JH, Garforth J, Borges A, Fairlamb AH, Douglas KT (1992) Rationally designed selective inhibitors of trypanothione reductase. Biochem J 286: 9–11

Bitter S, Knobler Y, Frankel M (1965) Active α-aminoacyl O derivatives of N-substituted hydroxylamine, O to N migration and a method for peptide synthesis. Tetrahedron Lett 2: 95–98

Cleland WW (1964) Dithiothreitol, a new protective reagent for SH groups. Biochemistry 3: 480–482

EL-Waer A, Douglas KT, Smith K, Fairlamb AH (1991) Synthesis of N-benzyloxycarbonyl-L-cysteinylglycine 3-dimethylaminopropylamide disulfide: a cheap and convenient new assay for trypanothione reductase. Anal Biochem 198: 212–216

Fairlamb AH (1985) Trypanothione metabolism in the chemotherapy of leishmanias and trypanosomiasis. In: Wang CC (ed) Molecular & immunological aspects of parasitism, chapter 10. pub AAAS, pp 107–121

Garforth J, McKie JH, Jaouhari R, Benson TJ, Fairlamb AH, Douglas KT (1994) Rational design of peptide-based inhibitors of trypanothione reductase as potential antitrypanosomal drugs. Amino Acids 6: 295–299

Hunter WN, Bailey S, Habash J, Harrop SJ, Helliwell JR, Aboagye-Kwarteng T, Smith K, Fairlamb AH (1992) Active site of trypanothione reductase, a target for rational drug design. J Mol Biol 227: 322–333

Itoh M, Hagiwara D, Kamiya T (1975) A new *t*-butyloxycarbonylation reagent, 2 *t*-butyloxycarbonyloxyimino-2-phenylacetonitrile. Tetrahedron Lett 49: 4393–4394

Mehta A, Jaouhari R, Benson TJ, Douglas KT (1992) Improved efficiency and selectivity in peptide synthesis: use of triethylsilane as a carbocation scavenger in deprotection of *t*-butyl esters and *t*-butoxycarbonyl-protected sites. Tetrahedron Lett 33: 5441–5444

Schirmer HR, Muller JG, Krauth-Siegel RL (1995) Disulfide-reductase as chemotherapeutic agents: the design of drugs fot trypanosomiasis and malaria. Angew Chem Int Ed 34: 141–154

Walsh C, Bradley M, Nadeau K (1991) Molecular studies of trypanothione reductase, a target for antiparasitic drugs. Trends Biochem Sci 16: 305–309

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