Synthesis and NMR Characterization of the Trypanosomatid Metabolite, N^1,N^8-Bis(glutathionyl)spermidine Disulphide (Trypanothione Disulphide)

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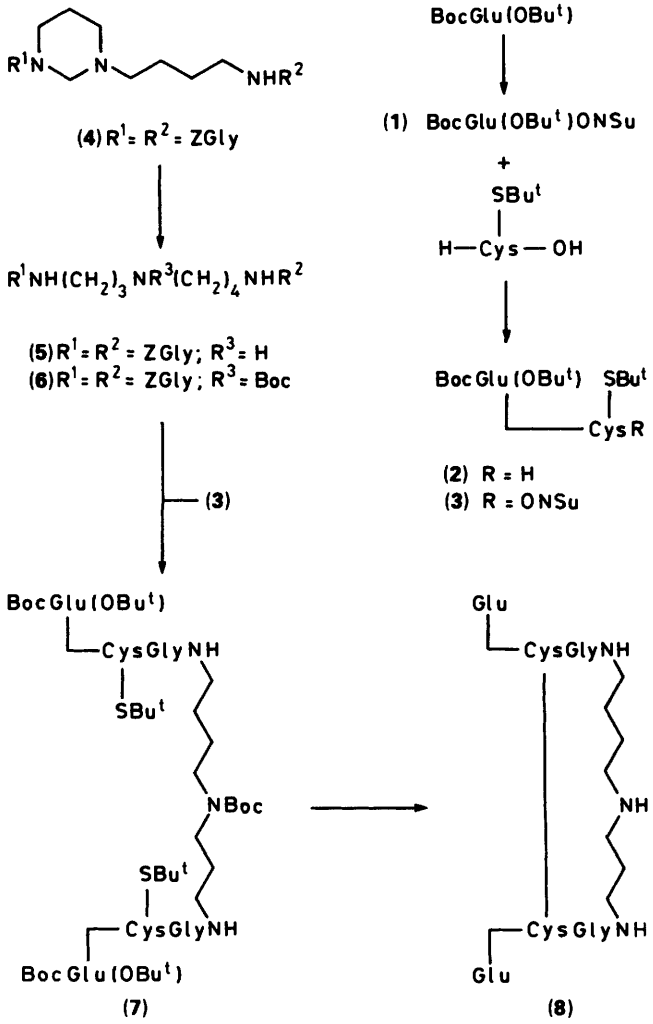
An optimized chemical synthesis of the novel trypanosomatid metabolite, N^1,N^8-bis(glutathionyl)-spermidine(trypanothione disulphide) is described, and its solution structure has been investigated by NMR spectroscopy. The ^1H, ^{13}C, and ^{15}N chemical shifts, as well as proton NOE data are compatible with two similar extended peptide segments connected by a flexible spermidine chain. The data do not support a predominant β sheet structure but indicate considerable flexibility in aqueous solution.

The tripeptide, L-γ-glutamyl-L-cysteinylglycine (glutathione, GSH) is ubiquitously distributed in both prokaryotic and eukaryotic cells.[1] GSH participates in several enzyme-mediated and non-enzymatic redox processes, which include: thiol–disulphide exchange reactions, peroxide reduction and free radical scavenging. These reactions result in the oxidation of GSH to the disulphide form (GSSG). In order to complete the cycle, GSSG is in turn reduced at the expense of NADPH by a specific flavoprotein, glutathione disulphide reductase.

Investigation of GSH metabolism in trypanosomatids has revealed that these organisms do not possess a classical GSH-based redox system. Trypanosomatids possess instead an analogous system which is based on a glutathione–spermidine conjugate, N^1,N^8-bis(glutathionyl)spermidine. This compound has been given the trivial name, trypanothione.[2,3] Recent studies indicate that trypanothione has functional roles in trypanosomatids which are equivalent to those of glutathione in other biological systems. Thus trypanothione is maintained in the reduced (dithiol) form by a novel NADPH-dependent flavoprotein, trypanothione disulphide reductase,[4] and it is also a specific hydrogen donor for trypanosomatid peroxidase activity.[5,6] The 'trypanothione system' has been detected in all trypanosomatids examined to date, including the pathogenic species responsible for African sleeping sickness, South American Chagas' disease and visceral and cutaneous leishmaniasis.[7] Trypanothione itself and the enzymes which control its biosynthesis and metabolism represent an important target for the development of new anti-parasitic drugs to treat these diseases and much recent work has focused on this objective.[8,9] We have previously communicated the chemical synthesis of trypanothione[10] and now report the chemical synthesis of this important metabolite in full. It has been shown that trypanothione and glutathione reductases display mutually exclusive specificities towards their respective substrates.[4,11] With the availability of high expression plasmids encoding native and mutant glutathione and trypanothione reductases,[12] these proteins are now available in quantity and the use of NMR spectroscopy to probe the catalytic mechanisms of these enzymes as well as the molecular basis of substrate discrimination becomes feasible. As a first step in this direction, we also report the NMR spectroscopic analysis of trypanothione disulphide in aqueous solution.



Scheme.

Results and Discussion

At present trypanothione is not available in quantity from biological sources. Consequently, a high yielding chemical synthesis of the molecule is essential in order to allow biochemical analyses to proceed. This synthetic route to trypanothione (Scheme) was based on coupling of the fully protected dipeptide fragment (3) to N^4-protected N^1,N^8-bis(glycyl)spermidine (6) to give the fully-protected trypanothione derivative (7). The conversion of (7) to trypanothione

Table. NMR data for trypanothione.[a]

δ_H/ppm[b]					
Residue	α	β	γ	δ	NH
Glu[c]	3.83	2.16	2.55		
Cys	4.75	3.28, 3.04			8.67
	4.73	3.26, 3.07			8.67
Gly	3.93, 3.85				8.76
	3.95, 3.83				8.73
Spermidine[d]	3.29	1.60	1.69	3.04	7.87
	3.36	1.90	3.05		8.10

^3J Proton coupling constants/Hz				
Residue	α,β	β,γ	γ,δ	NH,α
Glu	5.5	5.0		
Cys	4.5, 9			7
Gly				6
Spermidine				6

δ_C/ppm[b]				
Residue	α	β	γ	δ
Gln	57.0	28.9	33.9	
Cys	55.7	41.0		
Gly	46.1			
Spermidine	38.9	28.5	28.5	50.2
	40.1	28.4	47.5	

δ_N/ppm[e]	
Residue	Amide
Cys	122.9
Gly	110.5
	111.2
Spermidine	117.7
	119.6

[a] 25 °C. [b] From internal trimethylsilyl propionate. [c] Centre of overlapping signal due to two glutamyl residues. [d] The spermidine chain has been labelled as follows: $CONH\overset{\alpha}{C}H_2\overset{\beta}{C}H_2\overset{\gamma}{C}H_2\overset{\delta}{C}H_2NH\overset{\gamma}{C}H_2\overset{\beta}{C}H_2\ \overset{\alpha}{C}H_2NHCO$. [e] From anhydrous ammonia at 25 °C, measured from external nitromethane.

disulphide (**8**) was effected by sequential treatment of (**7**) with trifluoroacetic acid (TFA), dithiothreitol (DTT) and I_2. Trypanothione disulphide was then purified by fast protein liquid chromatography (FPLC). The pure peptide was isolated as a hygroscopic triacetate salt. The final product was homogeneous by HPLC analysis (co-eluting with natural material) and by NMR analysis. All synthetic steps have now been optimized and trypanothione disulphide can routinely be prepared in 50–100 mg batches by careful application of the methods described here. Synthetic trypanothione disulphide prepared by this procedure has been used to purify trypanothione reductase from *Crithidia fasciculata*[4] and *Trypanosoma cruzi*.[13]

As part of the physical characterization of synthetic trypanothione disulphide and to faciliate NMR analysis of the trypanothione–trypanothione reductase interaction, we have carried out NMR analysis of the disulphide form of the compound in aqueous solution. Space-filling models of trypanothione disulphide and the linear dithiol, (dihydrotrypanothione), indicate that both forms might assume the conformation of a β-pleated sheet. Such a solution structure might be imposed by the formation of three intramolecular hydrogen bonds (between adjacent carbonyl and amide functions) and also by two potential salt bridges between adjacent glutamate residues. Spectroscopic evidence for such a preferred structure would include: (i) large differences in chemical shift values between like residues in the two 'halves' of the molecule; (ii) large differences in chemical shift values between trypanothione residues and random coil residues; and (iii) large and small ^3J homonuclear coupling constants in the proton spectra indicating preferred, rather than averaged, rotamers.

COSY and ROESY spectra were taken in order to make assignments and obtain information on the conformation. In addition, proton detected heterocorrelated spectroscopy (HMP-COSY) was performed for both ^{13}C and ^{15}N nuclei to facilitate comparison of chemical shifts with values from linear peptides and random coil models.

All spectral data (Table) indicate the approximate symmetry of the two halves of the molecule. That is, resonances from pairs of like residues overlap, with only minor differences attributable to the actual asymmetry of the spermidine moiety. The proposed parallel β sheet structure would place the half-cystyl and glycyl amide protons of each arm of the molecule in different orientations with respect to the disulphide bridge. However, there are no significant differences in the chemical shifts. This is also the case for the amide ^{15}N chemical shifts,

which have often been shown to be sensitive to hydrogen bonding in small molecules.[14] The geminal protons of the glycyl and spermidyl residues also gave essentially one set of peaks, suggesting rotational averaging. Only the side chains of the half-cystyl residues are partially restricted since the β protons are non-equivalent and have different $^3J(H_\alpha\text{-}H_\beta)$ values.[17] In addition, comparison of proton and carbon chemical shifts with linear tetrapeptides[15] and nitrogen shifts with random coil models[16] showed no significant differences.

The data from the ROESY experiment are also compatible with a flexible molecule. No amide to amide NOEs were observed, and only the glycyl residues showed NOEs between their amide and α protons. Strong crosspeaks from the half-cystyl amide protons to β protons indicate that the former are orientated towards the disulphide bond. This would tend to direct the terminal glutamyl residues away from each other, contrary to the requirements of a terminal salt bridge. Cross-peaks were observed between the glutamyl τ protons and the cytidyl amide protons, as well as between the spermidine amide and glycyl α protons. However, potentially diagnostic cross-peaks from the half-cystyl H_α to either the cystyl or glycyl amide could not be detected. Local flexibility is consistent with observed NOEs between α and β methylene protons in both glutamyl and spermidyl residues.

Taken together, the chemical shift and coupling data provide no evidence that trypanothione disulphide adopts a preferred structure in aqueous solution at ambient temperature. On the contrary, the data indicate considerable conformational flexibility within the molecule and approximate symmetry between the two sides of the macrocycle. This information forms the basis for further NMR analysis of trypanothione disulphide within the macromolecular environment of trypanothione reductase; these experiments are now in progress.

Experimental

NMR Analysis.—Spectra of trypanothione disulphide were acquired on a GE GN500 spectrometer at 25 °C using 5 mm probes from either GE-NMRI or Cryomagnetics Inc. The data were processed with FTNMR software.[18] A sample of trypanothione disulphide (44 mmol) in 90% H_2O–10% D_2O at pH 5.0 was used for the proton-detected ^{15}N heterocorrelated spectrum, and a 5 mmol sample was used for the double-quantum filtered COSY and ROESY experiments. The hetero-correlated carbon spectrum used the 44 mmol sample that had been lyophilized, exchanged once, then redissolved in D_2O. The final pH was 6.0. Proton chemical shifts were referenced to internal TSP. The 2D spectra were acquired with 5 000 Hz spectral width in a 2 048 × 256 data matrices, using pre-saturation to reduce the water signal. The COSY was processed with shifted sinebell functions and the ROESY with Gaussian functions in both dimensions. The $^1H\{^{13}C\}$ heterocorrelated spectrum was obtained with the pulse sequence of Bax *et al.*[19] with presaturation to reduce the remaining HDO peak. The 400 scans per t_1 block were phase-cycled to obtain double quantum frequencies. The final data matrix was 2 048 × 128 with acquisition times of 682 ms and 16 ms for t_2 and t_1, respectively. The $^1H\{^{15}N\}$ heterocorrelated spectrum used a selective Redfield 214 pulse[20] to avoid excitation of the water resonance. The data matrix was 2 048 × 64 with acquisition times of 410 ms and 16 ms for t_2 and t_1, respectively. 800 scans per block gave a total acquisition time of 13 h. The nitrogen and carbon chemical shifts were calculated from internal TSP.[21]

Spectra of synthetic intermediates were acquired on a GE 360 spectrometer at 25 °C. Chemical shifts were calculated relative to internal TMS.

Syntheses: General Procedure.—M.p.s were determined with a Kofler hot-stage apparatus. Evaporation was performed by rotary evaporator under reduced pressure at or below room temperature; solutions in organic solvents were dried over magnesium sulphate.

Boc-L-glutamyl-α-t-butyl-N-oxosuccinimide Ester (**1**).—To a solution of boc-L-glutamic acid-α-t-butyl ester (350 mg, 1.155 mmol) and *N*-hydroxysuccinimide (135 mg, 1.173 mmol) in tetrahydrofuran (THF) (3 ml) at −20 °C was added dicyclohexyl carbodi-imide (DCC) (240 mg, 1.163 mmol). The mixture was allowed to warm to room temperature (rt) and stirring was continued for 12 h. Glacial acetic acid (0.1 ml) was added and stirring was continued for 1 h. The reaction mixture was diluted with ethyl acetate (EtOAc) (15 ml) and filtered. The filtrate was washed with ice-cold 5% $NaHCO_3$ (2 × 10 ml), water (10 ml) and brine (10 ml) then dried and evaporated to give the *N*-oxosuccinimide ester (**1**) (385 mg, 83%); $\delta_H(CDCl_3)$ 1.354 (9 H, s, Bu^t), 1.385 (9 H, s, Bu^t), 2.07 (2 H, m, βCH_2 Glu), 2.626 (3 H, m, $\alpha,\tau CH_2$ Glu), 2.753 (4 H, s, CH_2 NSu), and 5.212 (1 H, d, NH).

Boc-L-glutamyl-α-t-butyl-τ-(S-t-butylmercaptocysteine) (**2**).—A solution of *S*-t-butylmercaptocysteine (544 mg, 2.6 mmol) and (**1**) (1.04 g, 2.6 mmol) in dimethylformamide (DMF) (4 ml) containing triethylamine (Et_3N) (362 μl; 2.5 mmol) was stirred at rt for 8 h. The resulting mixture was diluted with 5% citric acid (20 ml) and extracted with EtOAc (2 × 20 ml). The organic extract was then washed with brine (10 ml), dried and evaporated to yield (**2**) as a colourless residue. Compound (**2**) was recrystallized from EtOAc–hexane, m.p. 101–105 °C (1.06 g; 86%); $\delta_H([^2H_6]DMSO)$ 1.251 (9 H, s, SBu^t), 1.346 (9 H, s, Bu^t), 1.356 (9 H, s, Bu^t), 2.15 (2 H, m, βCH_2 Glu), 2.462 (2 H, m, τCH_2 Glu), 3.00 (2 H, m, βCH_2 Cys), 3.74 (1 H, m, αCH Glu), 4.42 (1 H, m, αCH Cys), 7.083 (1 H, d, NH), 8.208 (1 H, d, NH), and 12.823 (1 H, brs, COOH).

Boc-L-glutamyl-α-t-butyl-τ-(S-t-butylmercaptocysteinyl)-N-oxosuccinimide Ester (**3**).—To a solution of (**2**) (8.58 g, 17.3 mmol) and *N*-hydroxysuccinimide (2 g, 17.3 mmol) in THF (10 ml) at −20 °C was added DCC (3.5 g, 17 mmol). The mixture was allowed to warm to room temperature and stirring was continued for 12 h. The resulting solution was processed as described for compound (**1**). Compound (**3**) was obtained as a colourless residue which was recrystallized from diethyl ether–hexane m.p. 102–106 °C (7.56 g; 74%); $\delta_H([^2H_6]DMSO)$ 1.273 (9 H, s, SBu^t), 1.346 (9 H, s, Bu^t), 1.357 (9 H, s, Bu^t), 2.180 (2 H, m, βCH_2 Glu), 2.555 (2 H, m, τCH_2 Glu), 2.76 (4 H, s, CH_2 NSu), 3.100 (1 H, m, βCH_2 Cys), 3.750 (1 H, m, αH Glu), 4.895 (1 H, m, αH Cys), 7.09 (1 H, d, NH), and 8.630 (1 H, d, NH).

N^1,N^8-Bis(benzyloxycarbonylglycyl)-N^1,N^4-methylenespermidine (**4**).—To a solution of N^1,N^4-methylene spermidine[17] (640 mg, 4 mmol) in THF (30 ml) was added Et_3N (568 μl, 8 mmol) and benzyloxycarbonylglycyl-*N*-oxosuccinimide ester (2.45 g, 8 mmol). The mixture was stirred at rt for 10 h and then evaporated. The residue was taken up in EtOAc (50 ml) and the solution washed with 5% $NaHCO_3$ (2 × 25 ml), water and brine and then dried and evaporated. Flash column chromatography of the resulting oil (silica G; 5% MeOH–dichloromethane) gave (**4**) as a viscous oil which solidified at 4 °C (1.77 g; 80%).

N^1,N^8-Bis(benzyloxycarbonylglycyl)spermidine (**5**).—To a solution of (**4**) (0.5 g; 0.93 mmol) in absolute EtOH (8 ml) was added pyridine (225 μl; 2.78 mmol) and malonic acid (385 mg; 3.7 mmol). The mixture was heated at reflux for 2 h. On cooling, the resulting mixture was diluted with ice–water (30 ml) and on

addition of NaOH (10M) to pH 14, compound (**5**) precipitated from solution and was recrystallized from aq. EtOH, m.p. 149–150 °C (350 mg; 72%); δ_H([$^2H_6$]DMSO) 1.305 (2 H, m, τCH_2 SPD), 2.238 (2 H, m, τCH_2 SPD), 2.305 (4 H, m, βCH_2 SPD), 2.881 (2 H, m, δCH_2 SPD), 3.141 (4 H, m, αCH_2 SPD), 3.364 (4 H, d, CH_2 Gly), 4.827 (4 H, s, $ArCH_2$), 7.156 (10 H, m, ArH).

N^1,N^8-*Bis*(*benzyloxycarbonylglycyl*)-N^4-t-*butoxycarbonylspermidine* (**6**).—To a suspension of (**5**) (588 mg, 1.1 mmol) in THF (30 ml) was added Et_3N (186 μl, 1.32 mmol) and di-t-butyl oxyformate (292 mg, 1.32 mmol). The mixture was stirred at rt for 6 h and was then partitioned between EtOAc (50 ml) and water (25 ml). The organic solution was separated and the aqueous solution extracted with EtOAc (2 × 10 ml). The combined EtOAc extracts were washed with 5% citric acid (2 × 20 ml), water and brine then dried and evaporated to give a colourless viscous oil (650 mg, 93%); δ_H([$^2H_6$]DMSO) 1.271 (9 H, s, Bu^t), 1.300 (2 H, m, τCH_2 SPD), 2.311 (4 H, m, βCH_2 SPD), 2.896 (4 H, m, τ,δ CH_2 SPD), 3.145 (4 H, m, αCH_2 SPD), 3.380 (4 H, d, CH_2 Gly), 4.827 (4 H, s, $ArCH_2$), 7.156 (10 H, m, ArH).

Fully Protected Trypanothione (**7**).—To a solution of the protected spermidine derivative (**6**) (320 mg, 0.51 mmol) in absolute EtOH (8 ml) was added 10% Pd/C (20 mg) and the misture was stirred under hydrogen for 1 h. The reaction mixture was filtered through Celite and evaporated to give a gum which was pumped under high vacuum for 3 h. The hydrogenolysis product was dissolved in DMF (10 ml) containing the hydroxysuccinimide ester (**3**) (603 mg, 1.02 mmol) and the solution was stirred under nitrogen for 12 h. The resulting solution was partitioned between EtOAc (25 ml) and water (15 ml). The aqueous layer was extracted with EtOAc and the combined EtOAc extracts were washed with 5% citric acid (2 × 25 ml), 5% $NaHCO_3$ (2 × 25 ml), water (10 ml) and brine then dried and evaporated to give a colourless gum (470 mg, 70%).

Trypanothione Disulphide (**8**).—To a solution of the fully-protected trypanothione (**7**) (107 mg, 0.113 mmol) in CH_2Cl_2 (0.5 ml) was added TFA (2 ml). The mixture was stirred at rt for 45 min and then evaporated under nitrogen. The residue was dissolved in water (5 ml), the solution was adjusted to pH 8.0 with dilute NaOH and was then filtered. To the foregoing solution, DTT (155 mg, 1 mmol) was added and the mixture was stirred under nitrogen for 1 h. The resulting solution was diluted to 15 ml with water, adjusted to pH 2.0 with HCl and extracted with EtOAc (5 × 5 ml) to remove excess DTT. The aqueous solution was diluted to 150 ml with water, adjusted to pH 7.0 with dilute NaOH, cooled to 0 °C and then oxidized by dropwise addition (0.85 ml) of a solution of I_2 in MeOH (30 mg ml^{-1}). The resulting solution was again extracted with EtOAc (2 × 25 ml) and then lyophilized to dryness. The lyophilized residue was dissolved in 0.5M AcOH (10 ml) and trypanothione disulphide was purified by FPLC as follows: solvent A, 0.5M AcOH; solvent B, 0.5M NH_4OAC. Gradient: t = 0, %B = 0; t = 20, %B = 12; t = 40, %B = 100. Under these conditions, trypanothione disulphide elutes at 30% B.

Acknowledgements

We thank Miyuki Yamaguchi and Nicholas J. Murgolo for technical assistance. This work was supported by grants from the National Institutes of Allergy and Infectious Diseases [AI 26784 (to G. B. H.), and AI 19429 (to A. C.)], and General Medicine [GM-36596 (to D. C.)]. NMR equipment was purchased with funds from NIH, NSF and the Keck Foundation.

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Paper 9/03643A
Received 25*th August* 1989
Accepted 20*th November* 1989