Pergamon

Bioorganic & Medicinal Chemistry Letters 10 (2000) 641–644

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Synthesis and Biological Evaluation of Enantiopure Thionitrites: The Solid-Phase Synthesis and Nitrosation of D-Glutathione as a Molecular Probe

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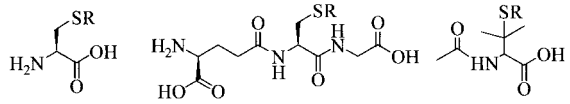
Received 13 December 1999; accepted 27 January 2000

Abstract—The D-isomer of the naturally-occurring tripeptide glutathione (γ-L-Glu-L-Cys-Gly, L-GSH) has been synthesised using the Fmoc solid phase peptide synthesis strategy. The D-GSH obtained has been nitrosated to give the D-isomer of the bioactive thionitrite, *S*-nitroso-L-glutathione. The biological activity of both enantiomers of *S*-nitrosoglutathione has been studied and compared to the activity of the D- and L-isomers of *N*-acetyl-*S*-nitrosopenicillamine (SNAP) and *S*-nitrosocysteine (CysNO).

Introduction

Within the last 10 years, the endogenously generated free radical nitric oxide (NO) has been implicated in an increasing number of essential biological events.[1] Thionitrites (also called *S*-nitrosothiols, RSNOs) are bioactive molecules and the necessary sulfydryl group is often provided by a cysteine residue within the proteins. Their important role in many physiological processes attributed to NO has been recognised.[2] Several thionitrites have been detected and quantified in vivo and, as a result, they have been postulated as endogenous carrier and storage forms of NO.[3] RSNOs circulate in human plasma mainly in the form of *S*-nitroso-albumin.[4] However, low molecular weight thiols also form *S*-nitrosothiols in biological systems, the most important being those derived from the amino-acids cysteine (L-Cys, **1**), *S*-nitrosocysteine (L-CysNO, (L)-**2**) and that with the tripeptide glutathione (γ-L-Glu-L-Cys-Gly, (L)-**3**), *S*-nitrosoglutathione ((L)-**4**). The thionitrite SNAP (**6**) derived from *N*-acetylpenicillamine (NAP, **5**) has been used for many years as a pharmacological tool due to its ability to generate NO in aqueous solution.



(*L*)-**1** R= H (*L*-Cys) (*L*)-**3** R= H (*L*-GSH) **5** R= H (NAP)
(*L*)-**2** R= NO (*L*-CysNO) (*L*)-**4** R= NO (*L*-GSNO) **6** R= NO (SNAP)

It is well established that in solution, in vitro, thionitrites liberate NO with concomitant formation of the corresponding disulfide (Scheme 1) in a process catalysed by heat, light or certain metal salts[5] (e. g. Cu^{2+} or Cu^{+}).

By way of contrast, it has been reported that in biological systems the release of NO from thionitrites could be a cell-mediated process which might therefore require stereospecific recognition by a receptor site.[6] In order to investigate this hypothesis further we have therefore prepared and examined three pairs of enantiomeric thionitrites. The corresponding L-isomers of **2** and **4** possess well characterised biological activity as does racemic SNAP (**3**).[6a,7] Hence, it was of interest to investigate if, under identical conditions, both the L- and D-isomers of these thionitrites showed equivalent activity. This was achieved by comparing the vaso-relaxant properties of each enantiomer using rat isolated aorta rings in organ bath studies (vide infra).

Both enantiomers of the thiol cysteine are commercially available and, therefore, obtaining both enantiomers of

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0960-894X/00/$ - see front matter
PII: S0960-894X(00)00060-3

$$RSNO \xrightarrow[Cu^{2+}\text{ or }Cu^{+}]{\Delta,\ h\nu\text{ or}} RSSR + NO$$

Scheme 1. Thionitrite decomposition.

2 required only nitrosation. Both enantiomers of penicillamine are also commercially available. Preparation of both enantiomers of **6** involved introduction of an acetyl group onto the amide nitrogen, followed by nitrosation. These operations were performed following literature procedures.[7,8] In contrast, only the naturally-occurring (L) isomer of glutathione (**3**) is available commercially and the D-isomer was, therefore, synthesised de novo. This was achieved by a convenient solid phase peptide synthesis (SPPS) method and subsequent nitrosation of the thiol yielded the required enantiomeric *S*-nitroso-D-glutathione, (D)-**4**.

Several syntheses of L-GSH using solution phase chemistry have been reported previously[9] many of which obtain the oxidised form of the tripeptide (GSSG) after deprotection. To our knowledge, the SPPS approach has not been previously been employed for the preparation of either enantiomer of glutathione.

Synthetic Scheme

Our approach is outlined in Scheme 2. Overall, the Fmoc strategy for SPPS was employed. The sulphur group of cysteine was protected as the trityl (Tr) thioether, the α-carboxylic acid of glutamic acid was protected as its *tert*-butyl (^{t}Bu) ester and the N-terminal group was protected as the *tert*-butyloxycarbonyl (Boc) derivative. By choosing this protecting group strategy, we anticipated that the cleavage of the peptide from the resin and deprotection of all functional groups could be achieved in a single operation through the use of acidic conditions.

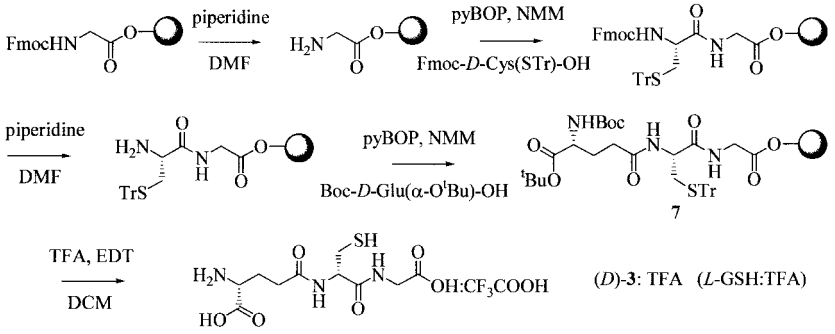
Results and Discussion

The first amino acid of the sequence (Gly) was initially protected (Fmoc) and linked to the polystyrene/polydimethylacrylamide resin (NovaSyn PA500) via the TFA-labile 4-hydroxymethylphenoxyacetic acid linker. Fmoc deprotections were carried out with 20% piperidine in *N*,*N*-dimethylformamide (DMF). The efficiency of the deprotections was qualitatively checked by the Ninhydrin (Kaiser[10]) test. The peptide chain was elongated by successive coupling of Fmoc-D-Cys(STrt)-OH and Boc-D-Glu(α-O^{t}Bu)-OH. The conveniently protected glutamic acid residue is not commercially available and was prepared in three steps from unprotected D-glutamic acid.[11] Coupling reactions were performed in DMF using pyBOP as the coupling reagent in the presence of *N*-methylmorpholine (NMM). A ratio of 2:2:2:1 of added amino acid/pyBOP/NMM/linked peptide was employed in order to ensure quantitative couplings. Completion of the couplings was qualitatively checked by the Ninhydrin test.

The cleavage of the peptidyl-resin **7** and simultaneous deprotection of all functional groups was achieved by exposure to TFA in dichloromethane (DCM) (95% v/v) with ethanedithiol (EDT) as scavenger. After filtration of the cleaved mixture, the acidic filtrate was partitioned between Et_2O and H_2O. The aqueous extract was lyophilised and the crude product purified by repeated reverse-phase-HPLC (RP-HPLC) using TFA/H_2O (0.1% v/v) as the eluent and UV-detection at $\lambda = 215$ nm (retention time 7.5 min). The pure D-tripeptide (D-**3**) was isolated as its TFA salt in an overall yield of 20%[12] and was identical by analytical HPLC to a sample of commercial L-GSH. Our TFA salt of D-GSH and that of L-GSH (prepared from commercial L-GSH and 1 equivalent of TFA) gave the following specific optical rotations: $[\alpha]_D = +8.0$ (c 0.5, H_2O) for L-GSH:TFA, $[\alpha]_D = -8.5$ (*c* 0.5, H_2O) for D-GSH:TFA.

A sample of the D-GSH:TFA salt was nitrosated to give D-GSNO (D-(**4**)) with $NaNO_2$ in H_2O at 0 °C (D-GSH:TFA/$NaNO_2$ 1:1 ratio). It should be noted that concentration appears to be a critical factor for the success of this reaction and optimised conditions involved the use of 0.5 mmole of tripeptide in 1 mL of H_2O in order to achieve the precipitation of the nitrosated peptide as a pink solid. The reaction also appears to be extremely sensitive to the presence of minor impurities and D-GSNO decomposed in situ after only a few minutes unless a very pure sample of D-GSH:TFA was used.

The D-GSNO obtained[13] was identical by analytical RP-HPLC (TFA/H_2O 0.1% v/v; retention time 14.5



Scheme 2. Solid-phase synthesis of D-glutathione.

min) to a sample of L-GSNO prepared by nitrosation of commercial L-GSH with one equivalent of $NaNO_2$ and one equivalent of acid (HCl or TFA) under the same conditions as above. The material prepared contained 10% of the corresponding disulfide.

Biological Results

The three pairs of enantiomeric thionitrites, D- and L-glutathione (**4**), D- and L-SNAP (**6**) and D- and L-CysNO (**2**) were prepared by in situ nitrosation of the corresponding thiol isomers as described above.[6,7]

Figure 1 shows concentration–response curves for relaxation of rat aortic rings by L- and D-GSNO. Significantly, the L-isomer was approximately 3-fold more potent than the D-isomer in eliciting relaxation of phenylephrine-induced tone.

In contrast, Table 1 shows the EC_{50} values (concentration of drug required to produce 50% of the maximal response) for relaxation of rat aortic rings for the L- and D- isomers of CysNO and SNAP in conjunction with those for L- and D-GSNO. Here, the L- and D-isomers of CysNO and SNAP did not differ in their ability to relax aortic smooth muscle, as indicated by similar EC_{50} values. However, the EC_{50} value for L-GSNO is approximately 3-fold lower than that for D-GSNO, confirming the greater potency of L-GSNO in eliciting vascular smooth muscle relaxation.

The pharmacological data reported herein therefore support the thesis that a stereoselective 'bioactivation' of *S*-nitrosothiols might be involved, at least in part, in the vasorelaxant actions of these compounds. However, this process appears to be specific for GSNO; the D- and L-isomers of CysNO and SNAP were equipotent in relaxing aortic smooth muscle whereas the L-isomer of GSNO was demonstrably and reproducibly more potent than the D-isomer.

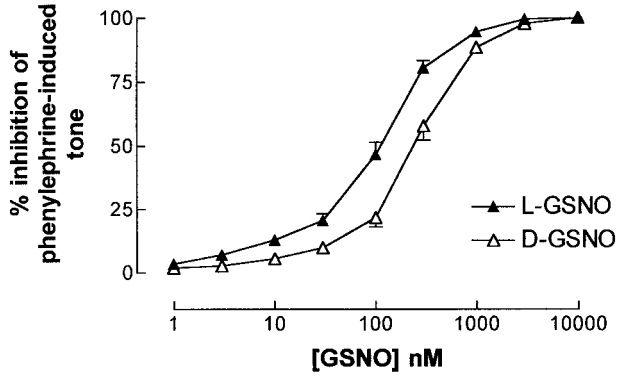


Figure 1. Concentration–response curves for relaxation of rat aortic smooth muscle by L- and D-GSNO.

Table 1. Comparative EC_{50} values for relaxation of rat aortic rings by different *S*-nitrosothiols

S-nitrosothiol	EC_{50} (nM)	
	L-isomer	D-isomer
GSNO	107.5 ± 7.6	289.9 ± 14.2
CysNO	2.0 ± 0.3	2.1 ± 0.2
SNAP	475.5 ± 19.2	492.1 ± 16.9

Acknowledgements

We are grateful to the CNRS (Centre Nationale de la Recherche Scientifique, Gif-sur-Yvette, Paris, France) for a pre-doctoral grant to M. Cavero. A. J. Hobbs is a recipient of a Wellcome Trust Research Career Development Fellowship.

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11. Glu to L-Glu(γ-OBn)-OH (Albert, R.; Danklmair, J.; Hunig, H.; Kandolf, H. *Synthesis* **1987**, 635): m.p.: 160–162 °C. ^{1}H NMR (D_2O + D_2SO_4; 400 MHz): δ 7.0–6.9 (5H, m), 4.67 (2H, s), 3.64 (1H, t, J = 6.6 Hz), 2.18 (2H, t, J = 7.8 Hz), 1.77 (2H, m). ^{13}C NMR (D_2O + D_2SO_4, 75.4 MHz): δ 174.47, 171.77, 139.82, 129.58, 128.83, 128.43, 64.99, 52.95, 30.24, 25.40. MS (FAB): *m/z* 260 (17), 238 (15), 154 (90), 136

(87), 102 (84), 91 (100), 77 (44). HRMS: found 238.1079, $C_{12}H_{16}NO_4$ (M + H) requires 238.1060. D-Glu(γ-OBn)-OH to Boc-D-Glu(α-O^{t}Bu)(γ-OBn) (Olsen, R. K.; Ramasamy, K.; Emery, T. *J. Org. Chem.* **1984**, *49*, 3527): ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.40–7.30 (5H, m), 5.14 (2H, s), 5.10 (1H, m), 4.25 (1H, m), 2.52–2.38 (2H, m), 2.23–2.15 (1H, m), 2.01–1.92 (1H, m), 1.46 (9H, s), 1.44 (9H, s). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 172.62, 171.26, 155.31, 135.76, 128.51, 128.16, 82.14, 74.72, 66.38, 53.3, 30.28, 28.25, 28.03, 27.93. MS (FAB): *m*/*z* 394 (18), 338 (18), 282 (82), 238 (67), 192 (20), 154 (50), 136 (24), 91 (100), 57 (69). HRMS: found 394.2245, $C_{21}H_{32}NO_6$ (M + H) requires 394.2230. Boc-D-Glu(α-O^{t}Bu)(γ-OBn) to Boc-D-Glu(α-O^{t}Bu)-OH (same reference as above): mp: 103–107 °C. [α] = + 30.4 (*c* 1, MeOH). ^{1}H NMR (DMSO, 400 MHz): d 7.12 (1H, d, *J* = 7.8 Hz), 4.06 (1H, m), 2.25 (2H, m), 1.85 (1H, m), 1.70 (1H, m), 1.63 (9H, s), 1.62 (9H, s). ^{13}C NMR (DMSO, 100 MHz): δ 173.86, 171.63, 155.65, 80.43, 78.18, 53.65, 30.13, 28.26, 27.72, 26.02. MS (FAB): *m*/*z* 326 (32), 304 (12), 270 (13), 248 (19), 204 (10), 148 (43), 130 (22), 84 (11), 57 (74), 92 (100). HRMS: found 326.1580, $C_{14}H_{25}O_2NNa$ (M + Na) requires 326.1570.

12. D-GSH:TFA: ^{1}H NMR (D_2O, 400 MHz): δ 4.37 (1H, t, *J* = 6.2 Hz), 3.85 (1H, t, *J* = 6.2 Hz), 3.83 (2H, s), 2.75 (2H, dd, *J* = 5.9, 2.6 Hz), 2.41 (2H, m), 2.05 (2H, m). ^{13}C NMR (D_2O, 100 MHz): δ 174.95, 173.44, 172.8, 55.94, 52.93, 41.44, 31.23, 25.85, 25.67. MS (FAB): *m*/*z* 308 (100), 273 (12), 223 (20), 207 (50), 131 (31), 115 (100). HRMS: found 308.0910, $C_{10}H_{17}SN_3O_6$ (M + H) requires 308.0916.

13. D-GSNO: [α]: + 42 (*c* 1, H_2O). ^{1}H NMR (D_2O, 300 MHz): d 4.50 (1H, t, *J* = 7.0 Hz), 3.93 (1H, m), 3.79 (1H, m), 3.75 (2H, s), 3.64 (1H, t, *J* = 6.3 Hz), 2.27 (2H, t, *J* = 7.6 Hz), 1.95 (2H, m). ^{13}C NMR (D_2O, 75.4 MHz): δ 176.59, 175.36, 173.75, 55.57, 54.46, 43.40, 32.97, 32.98, 27.81. MS (FAB): *m*/*z* 337 (25). HRMS: found 337.0828, $C_{10}H_{17}N_4SO_7$ (M + H) requires 337.0818. HPLC: 85% pure, contains 10% of the disulfide.