Bioorganic & Medicinal Chemistry 21 (2013) 7853–7857

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Transnitrosation of alicyclic *N*-nitrosamines containing a sulfur atom

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ARTICLE INFO

Article history:
Received 14 September 2013
Revised 8 October 2013
Accepted 9 October 2013
Available online 22 October 2013

Keywords:
Alicyclic *N*-nitrosamine
S-Nitrosoglutathione
Transnitrosation
N-Nitrosoproline
N-Nitrosothioproline

ABSTRACT

Aromatic and aliphatic nitrosamines are known to transfer a nitrosonium ion to another amine. The transnitrosation of alicyclic *N*-nitroso compounds generates *S*-nitrosothiols, which are potential nitric oxide donors in vivo. In this study, certain alicyclic *N*-nitroso compounds based on non-mutagenic *N*-nitrosoproline or *N*-nitrosothioproline were synthesised, and the formation of *S*-nitrosoglutathione (GSNO) was quantified under acidic conditions. We then investigated the effect of a sulfur atom as the substituent and as a ring component on the GSNO formation. In the presence of thiourea under acidic conditions, GSNO was formed from *N*-nitrosoproline and glutathione, and an *N*-nitroso compound containing a sulfur atom and glutathione produced GSNO without thiourea. The quantity of GSNO derived from the reaction of the *N*-nitrosamines containing a sulfur atom and glutathione was higher than that from the *N*-nitrosoproline and glutathione plus thiourea. Among the analogues that contained a sulfur atom either in the ring or as a substituent, the thiazolidines produced a slightly higher quantity of GSNO than the analogue with a thioamide group. A compound containing sulfur atoms both in the ring and as a substituent exhibited the highest activity for GSNO formation among the alicyclic *N*-nitrosamines tested. The results indicate that the intramolecular sulfur atom plays an important role in the transnitrosation via alicyclic *N*-nitroso compounds to form GSNO.

1. Introduction

Many *N*-nitrosodialkylamines exert potent mutagenic and carcinogenic effects through an α-hydroxylation via cytochrome P450, whereas denitrosation by cytochrome P450 has been reported as a detoxification pathway.[1,2] The biological events involving *N*-nitrosodialkylamines under enzymatic systems are well understood. In addition to denitrosation, photodegradation[3] and transnitrosation[4–6] are known chemical properties of *N*-nitroso compounds.

Nitric oxide (NO) has many potent biological activities and is present in plasma at extremely low levels.[7] *S*-Nitrosothiols (RSNO) represent a circulating endogenous NO reservoir due to NO's prolonged half-life.[8] The RSNOs are involved in signalling pathways, immune responses, and nitrovasodilation.[9,10] Furthermore, RSNOs have been shown to modulate the activity of numerous enzymes by participating in *S*-nitrosation reactions. Thus, some compounds that form RSNOs, such as *S*-nitrosoglutathione (GSNO), *S*-nitrosocysteine, and *S*-nitrosoalbumin, may serve as potential NO donors.[11,12]

Aromatic and aliphatic nitrosamines reportedly transfer a nitrosonium ion (NO^+) to other amines to form new *N*-nitroso compounds. Tanno and co-worker reported that *N*-aryl-*N*-nitrosoureas can transfer an NO group in organic solvents,[13,14] and Singer et al. demonstrated that alicyclic nitrosamines, that is, *N*-nitrosopiperazines or *N*-nirosomorpholine, can produce other *N*-nitrosamines via transnitrosation under acidic conditions such as those in mammalian stomachs.[6,15,16] Therefore, as NO donors, *N*-nitrosamines can transnitrosate a sulfur atom in proteins to form *S*-nitrosothiols. To investigate the effects of intramolecular *N*-nitrosamine sulfur atoms on GSNO formation, novel alicyclic *N*-nitroso compounds were synthesised with one or two sulfur atoms in the *N*-nitrosoproline 1 structure (Fig. 1). The quantity of GSNO formed in the reaction mixture was then determined via HPLC, and the structure–activity relationship for the transnitrosation was investigated.

2. Results

2.1. Chemistry

Non-carcinogenic *N*-nitrosoproline **1** is formed in our body and is a useful marker for investigating nitrosation in the human body.[17–21] Because transnitrosation from *N*-nitrosamines is accelerated in the presence of thiourea,[22,23] we designed alicyclic *N*-nitroso compounds containing sulfur atom(s) **2**–**5** based on the structures of **1** and thiourea. The 1-nitrosopyrrolidine-2-thiocarboxamide **2** was formed by replacing a carboxylate group with a thioamide group

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http://dx.doi.org/10.1016/j.bmc.2013.10.008

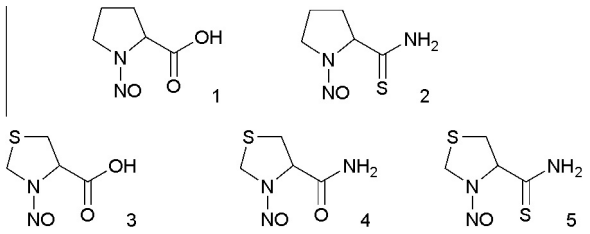


Figure 1. Structure of the alicyclic *N*-nitrosamines used in this study.

in **1**. The *N*-nitrosothioproline **3** has a sulfur atom incorporated into the five-membered ring of **1**. Like **1**, **3** reportedly forms in the body and, in the *Salmonella typhimurium* strains, is not mutagenic regardless of whether it contains an S9 mix.[17–19,24] The 3-nitroso-1,3-thiazolidine-4-carboxamide **4** maintains the structure of **3** but includes a substituted amide group. The 3-nitroso-1,3-thiazolidine-4-thiocarboxamide **5** contains two sulfur atoms, one in the five-membered ring and one as a substituent.

Compounds **2**, **4**, and **5** were synthesised as novel compounds; **1** and **3** were synthesised according to the method of Lijinsky et al.[25] Compound **2** was synthesised from **1**, whereby the carboxyl group was first converted to an amide group using diphenylphosphoryl azide and an aqueous ammonia solution;[26] the amide was then ultrasonicated with P_4S_{10} to convert it to a thioamide[27] (Scheme 1).

To obtain **4** and **5**, methyl 1,3-thiazolidine-4-carboxylate was prepared via a reaction between **1** and $SOCl_2$ in methanol (Scheme 2). An aqueous ammonia solution was added directly to the methyl 1,3-thiazolidine-4-carboxylate to obtain 1,3-thiazolidine-4-carboxamide, which was then nitrosated by $NaNO_2$ under acidic conditions to produce **4**. Compound **5** was synthesised via the nitrosation of 1,3-thiazolidine-4-thiocarboxamide, which was prepared from the thioamidation of 1,3-thiazolidine-4-carboxamide using P_4S_{10}[27] (Scheme 2).

2.2. Transnitrosation activity evaluated using GSNO formation

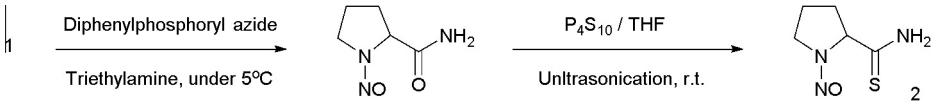
The transnitrosation activity of the compounds was evaluated in terms of their capacity to form GSNO. The pseudo-first-order rate constants (k_{obs}) were calculated using the slope of the linear relationship between the GSNO formation and the time (Fig. 2). The transnitrosation results are summarised in Table 1 with k_{obs} values ordered as follows: **1** + thiourea < 2 ≲ 3 ≒ 4 ≪ 5. After adding thiourea to the reaction mixture containing compound **1** and GSH, GSNO was detected in a low yield under the conditions. The yield of the GSNO depended on a concentration of **1**, thiourea or GSH (data not shown). Compounds **2**–**4**, which incorporate a sulfur atom into 1, exhibited higher GSNO formation activity than that of compound **1** plus thiourea. Among **2**–**4**, the k_{obs} values for **3** and **4** were slightly higher than that of **2**. Compound **5**, which contains two sulfur atoms, exhibited the highest activity of the compounds tested.

The GSNO yields were calculated by dividing the [GSNO formation] by the [*N*-nitrosamine consumption]. The GSNO yields in 3–5 were excellent and were approximately 90%, whereas the yield from 2 was low.

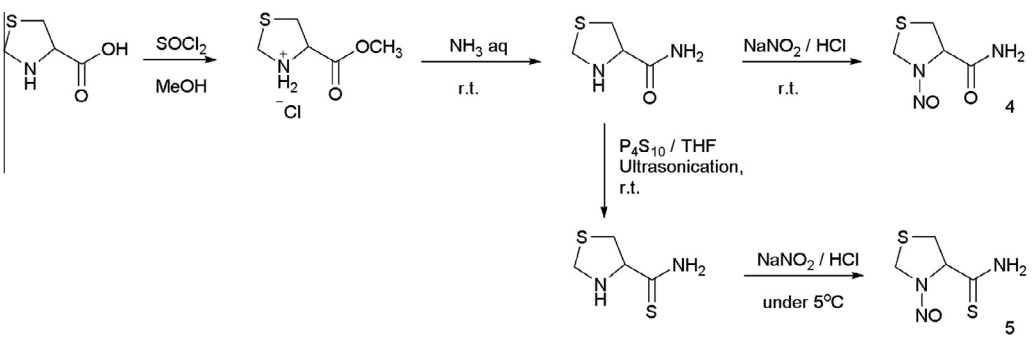
3. Discussion

Transnitrosation by aromatic and alicyclic nitrosamines can reportedly generate other *N*-nitrosamines. Tanno and co-worker reported that certain *N*-arylnitrosoureas generate nitric oxide in organic solvents,[13,14] and Singer et al. demonstrated that *N*-nitrosopiperazines or *N*-nitrosomorpholine transnitrosate in dilute acid solutions with nucleophilic catalysts. Thiocyanate ions or thioureas can be used as nucleophilic catalysts to accelerate the transnitrosation by *N*-nitrosamines under acidic conditions,[21,22] with thiourea exhibited a greater nucleophilicity than the thiocyanate ion. Recently Ohwada and co-workers demonstrated that some bicyclic nitrosamines and GSH can generate GSNO without releasing NO.[28,29] Current investigations of *S*-nitrosothiols focus on their biological activity in vivo.[10] In this study, we synthesised alicyclic nitrosamines based on non-carcinogenic **1** or **3** and evaluated the capacity of transnitrosation for GSNO formation.

The *N*-nitrosoproline **1** did not act as an NO donor itself but can transnitrosate GSH when thiourea is added to the reaction mixture, indicating that thiourea can promote the transnitrosation reaction. Compound **2** was designed and synthesised as a derivative of **1** by attaching a thioamide. The thiazolidines **3**, **4**, and **5** incorporate a sulfur atom as a ring component. Compounds **3** and **4** have carboxyl and amide substituents, respectively. Compounds **2**–**5** formed GSNO in the presence of GSH without thiourea, indicating that the transnitrosation was accelerated by the presence of an intramolecular sulfur atom. The k_{obs} values for **3** and **4** were slightly higher than that for **2**. The data indicate that a sulfur atom in a five-membered ring was favoured for transnitrosation over one in a substituent. Compound **5** had an additional sulfur atom incorporated into the thioamide group, and its k_{obs} value was the highest among the compounds tested.



Scheme 1. Synthesis of **2**.



Scheme 2. Syntheses of **4** and **5**.

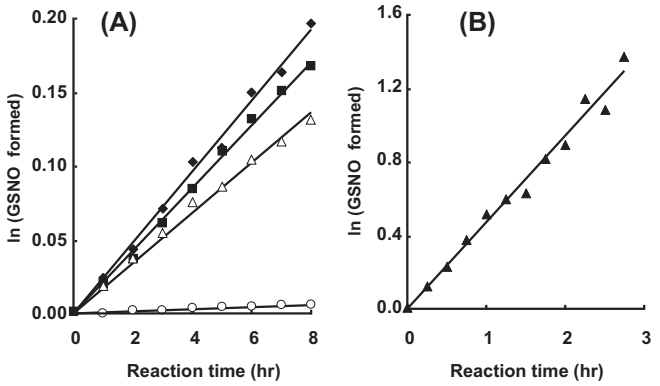


Figure 2. Formation of GSNO by the reaction of *N*-nitrosamines (**1**–**5**) with GSH. (A) The reaction conditions are as follows: *N*-nitrosamine **1** (○), 0.45 mM + thiourea, 4.5 mM; **2** (△), **3** (♦), or **4** (■), 0.45 mM; GSH, 5.0 mM; pH 1.5;37 °C. (B) The reaction conditions are as follows: *N*-nitrosamine **5** (▲), 0.45 mM; GSH, 5.0 mM; pH 1.5;37 °C.

Table 1
k_{obs} and GSNO yield

N-Nitrosamine	k_{obs}[c] ($\times 10^{-7}$/s)	GSNO[d] (mM)	*N*-Nitrosamine consumption[d] (mM)	GSNO yield[d] (%)
1 + thiourea[a]	2	0.007	Not detected	—
2[b]	42	0.10	0.16	63
3[b]	67	0.17	0.17	98
4[b]	59	0.17	0.19	89
5[c]	1301	0.43	0.45	95

[a] The reaction conditions are as follows: *N*-nitrosamine, 0.45 mM; thiourea, 4.5 mM; GSH, 5.0 mM, pH 1.5;37 °C; in the dark.
[b] The reaction conditions are as follows: *N*-nitrosamine, 0.45 mM; GSH, 5.0 mM; pH 1.5;37 °C; in the dark.
[c] The k_{obs} for GSNO formation was calculated from the slope in ln{[$GSNO_\infty$]/([$GSNO_\infty$] − [GSNO])} versus time, where GSNO and $GSNO_\infty$ refer to the GSNO concentration at the time and the final concentration (0.45 mM), respectively. The k_{obs} were determined by the method of least-squares.
[d] Data were acquired after 24 h.

The GSNO yield after 24 h was determined using the GSNO formed divided by the *N*-nitrosamine consumed, with the percentage obtained demonstrating the selectivity of *S*-nitrosation for GSNO formation. The yields of GSNO in the cases of **3**–**5** were approximately 90%, revealing a high selectivity for *S*-nitrosation. Compound **2** demonstrated a 63% selectivity for *S*-nitrosation, which may be due to the resonance stabilisation of the intermediate by the formation of a five-membered transition state between the nitrogen atom in the nitroso group and the sulfur atom in the thioamide group. Thus, the intramolecular sulfur atom(s) accelerated the transnitrosation by the *N*-nitrosamines.

4. Conclusion

S-Nitrosoglutathione was formed from GSH and the *N*-nitroso compound **1** with thiourea or **2**–**5** via transnitrosation under acidic conditions. Although the transnitrosation did not proceed from compound **1**, the reaction was promoted by the addition of thiourea. The transnitrosation of the compounds containing intramolecular sulfur atoms was efficiently accelerated. Among the compounds containing sulfur atoms, the one with two sulfur atoms exhibited the highest activity for GSNO formation. This result indicates that the intramolecular sulfur atom(s) play(s) a critical role in transnitrosation by alicyclic *N*-nitroso compounds.

5. Experimental

5.1. Chemicals

The L-thioproline was obtained from Tokyo Kasei Kogyo Co., Ltd (Tokyo, Japan). Diphosphorous pentasulfide was purchased from Sigma–Aldrich Co., Inc. (St. Louis, MO, USA). Diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid (DTPA) was acquired from Dojindo Laboratories (Kumamoto, Japan). Other reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan).

5.2. General procedure

The reaction progress was monitored using thin-layer chromatography (TLC) on silica gel 60 F_{254} (0.25 mm, Merck) and aluminium oxide 150 F_{254} neutral (Merck) plates. Column chromatography was performed using silica gel 60 (0.01–0.063 mm, Merck) or aluminium oxide 90 active neutral (0.063–0.200 mm, Merck). Melting points were determined using a Yanaco micro-melting-point apparatus without correction. The HPLC was performed using a Shimadzu LC system [SPD-20A UV spectrometric detector, Shiseido Capcell Pak column (5 μm, 250 × 4.6 mm)]. The NMR spectra were recorded with a JEOL JNM-LA400 spectrometer (Tokyo, Japan). The chemical shifts were expressed in ppm downfield shifted from TMS. The high-resolution mass spectra were collected using a JEOL JMS-SX102A mass spectrometer. The GSNO was synthesised using the method of Hart[30] [λ_{max} (H_2O); 335 nm (ε = 918) (lit.:λ_{max}; 336 nm (ε = 922)].[30]

5.3. Preparation of alicyclic *N*-nitrosamines

Compounds **1** and **3** were prepared using the method of Lijinsky et al.[25] (mp observed for **1**; 106 °C, **3**; 101 °C).

5.3.1. 1-Nitrosopyrrolidine-2-thiocarboxamide (2)

A solution of $NaNO_2$ (760 mg; 11 mmol) in 10 mL distilled water was dropped slowly into a solution of L-proline (1.2 g; 10.0 mmol) in 7 mL of 2 M HCl in an ice bath; the mixture was stirred for 1 h. The reaction mixture was extracted four times with 60 mL ethyl acetate. The combined organic phase was dried over Na_2SO_4, filtered, and evaporated to yield a yellow solid (1.1 g). Triethylamine (2.0 mL; 14.6 mmol) and diphenylphosphoryl azide (2.3 g; 2.4 mmol) were added to a solution of the yellow solid in 4 mL distilled DMF in an ice bath, and the mixture was stirred for 3.5 h. Then, a 25% aqueous NH_3 solution (1.0 mL; 15.1 mmol) was added dropwise to the mixture and stirred for 40 min. Brine (60 mL) was added to the mixture and extracted with ethyl acetate using a Soxhlet extractor. The organic phase was dried over Na_2SO_4 and filtered, and the organic solvent was evaporated to yield a tan-yellow oil. The residue was purified via column chromatography [silica gel, 5% MeOH–$CHCl_3$, UV 254 nm] to afford a tan-yellow solid (960 mg; yield 70.8%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.17 (br, 1H, *E*-CONH_2), 6.88 (br, 1H, *Z*-CONH_2), 6.66 (br, 1H, *E*-CONH_2), 6.36 (br, 1H, *Z*-CONH_2), 5.21 (m, 1H, *E*-N-C*H*-CO), 4.54 (dd, *J* = 5.1, 8.1 Hz, 1H, *Z*-N-C*H*-CO), 4.42 (m, 1H, *Z*-N-CH_2-C), 4.29 (m, 1H, *Z*-N-CH_2-C), 3.58 (m, 2H, *E*-N-CH_2-C), 2.37 (m, 2H, E_1, Z_1-C(CO)-CH_2-C), 2.26 (m, 2H, E_1, Z_1-C(CO)-CH_2-C), 2.06 (m, 4H, E_2, Z_2-C(N)-CH_2-C).

Diphosphorous pentasulfide (67 mg; 0.15 mmol) was added to a solution of 1-nitrosopyrrolidine-2-carboxamide (43 mg; 0.3 mmol) in 3 mL distilled THF, and the mixture was ultrasonicated for 1 h. This step was repeated, and the reaction mixture was filtered after adding acetone. Water (10 mL) was added to the filtrate and extracted three times with 10 mL ethyl acetate. The combined organic phase was dried over Na_2SO_4 and filtered. Because the desired product decomposed after being concentrated with excess P_4S_{10}, a small

quantity of silica gel was added to the filtrate, and the mixture was evaporated. The residue adsorbed onto the silica gel was purified via column chromatography [silica gel, 10% MeOH–CH_2Cl_2, UV 254 nm] to afford a yellow solid (9 mg; yield 19.2%). Mp 112 °C (decomp.); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.72 (br, 2H, *E*-CSNH_2), 7.58 (br, 2H, *Z*-CSNH_2), 5.51 (dd, *J* = 3.4, 10.0 Hz, 1H, *E*-N-C*H*-CS), 5.04 (m, 1H, *Z*-N-C*H*-CS), 4.58 (m, 1H, *Z*-N-CH_2-C), 4.32 (m, 1H, *Z*-N-CH_2-C), 3.73 (m, 2H, *E*-N-CH_2-C), 2.74 (m, 1H, *E*-C(CS)-CH_2-C), 2.64 (m, 1H, *E*-C(CS)-CH_2-C), 2.47 (m, 1H, *Z*-C(CS)-CH_2-C), 2.30 (m, 2H, *Z*-C(CS)-CH_2-C, *Z*-C-CH_2-C), 2.04 (m, 3H, E_2, Z_1-C-CH_2-C); ^{13}C NMR (100 MHz, $CDCl_3$) δ 204.8 (*E*-CSNH_2), 203.5 (*Z*-CSNH_2), 68.9 (*E*-N-C*H*-CS), 64.1 (*Z*-N-C*H*-CS), 50.9 (*Z*-N-CH_2-C), 46.7 (*E*-N-CH_2-C), 32.1 (*E*-C(CS)-CH_2-C), 30.5 (*Z*-C(CS)-CH_2-C), 22.9 (*Z*-C-CH_2-C), 21.0 (*E*-C-CH_2-C); IR (cm^{-1}, neat) 3270, 3100, 1660, 1640, 1440, 1140; HRMS (FAB) 160.0550 (calcd for $C_5H_9ON_3S$ 160.0545).

5.3.2. 3-Nitroso-1,3-thiazolidine-4-carboxamide (4)

Thionyl chloride (5.2 mL; 73.4 mmol) was dropped slowly into 20 mL distilled methanol in an ice bath below 5 °C under nitrogen gas; the reaction mixture was stirred for 10 min. L-Thioproline (2.7 g; 20.0 mmol) was added to the reaction mixture and stirred overnight. The organic solvent was evaporated three times after adding methanol, and the residue was washed twice with diethyl ether and decanted. This step was repeated. After evaporating the remaining solvent, methyl 1,3-thiazolidine-4-carboxylate hydrochloride was obtained (3.7 g; 99.3% yield) as a white solid. ^{1}H NMR (400 MHz, MeOH-d_4) δ 4.81 (t, *J* = 7.3 Hz, 1H, N-C*H*-CO), 4.46 (d, *J* = 9.2 Hz, 1H, S-CH_2-N), 4.40 (d, *J* = 9.8 Hz, 1H, S-CH_2-N), 3.88 (s, 3H, COOCH_3), 3.52 (dd, *J* = 7.3, 12.2 Hz, 1H, S-CH_2-C), 3.41 (dd, *J* = 6.1, 12.2 Hz, 1H, S-CH_2-C).

A 25% aqueous NH_3 solution (15 mL; 0.22 mol) was added dropwise to 1.7 g methyl 1,3-thiazolidine-4-carboxylate hydrochloride (9.4 mmol) in an ice bath and stirred for 5 h at room temperature. The reaction mixture was extracted three times with CH_2Cl_2 (30 mL), dried over anhydrous Na_2SO_4, filtered, and evaporated to give a white solid (1.1 g). The crude product was purified several times via silica gel column chromatography [silica gel, 3–5% MeOH–CH_2Cl_2, I_2 vapour], [aluminium oxide, 2–10% MeOH–$CHCl_3$, I_2 vapour], [aluminium oxide, 1–5% MeOH–CH_2Cl_2, I_2 vapour] to afford a white solid (960 mg; yield 77.3%) as 1,3-thiazolidine-4-carboxamide. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.93 (br, 1H, CONH_2), 5.55 (br, 1H, CONH_2), 4.26 (d, *J* = 10.4 Hz, 1H, S-CH_2-N), 4.17 (dd, *J* = 4.3, 7.3 Hz, 1H, N-C*H*-CO), 4.02 (d, *J* = 9.8 Hz, 1H, S-CH_2-N), 3.46 (dd, *J* = 6.3, 11.0 Hz, 1H, S-CH_2-C), 3.12 (dd, *J* = 7.3, 11.0 Hz, 1H, S-CH_2-C).

Sodium nitrite ($NaNO_2$; 78 mg; 1.1 mmol) in 1 mL distilled water was added slowly to a solution of 1,3-thiazolidine-4-carboxamide (132 mg; 1.0 mmol) in 0.6 mL 2 M HCl, and the mixture was stirred for 2 h. Water (5 mL) was added to the mixture, and the mixture was extracted three times with ethyl acetate (20 mL). The combined organic phase was dried over Na_2SO_4, filtered, and evaporated to yield a tan-yellow solid (154 mg; 95.6% yield). Mp 149 °C (decomp.); ^{1}H NMR (400 MHz, acetone-d_6) δ 7.30 (br, 1H, *E*-CONH_2), 7.08 (br, 1H, *Z*-CONH_2), 6.91 (br, 1H, *E*-CONH_2), 6.65 (br, 1H, *Z*-CONH_2), 5.77 (d, *J* = 10.5 Hz, 1H, *Z*-S-CH_2-N), 5.72 (dd, *J* = 2.9, 6.6 Hz, 1H, *E*-N-C*H*-CO), 5.24 (m, 1H, *Z*-N-C*H*-CO), 4.89 (d, *J* = 12.0 Hz, 1H, *Z*-S-CH_2-N), 4.86 (m, 1H, *E*-S-CH_2-N), 4.45 (m, 1H, *E*-S-CH_2-N), 3.50 (m, 3H, E_2, Z_1-S-CH_2-C), 3.30 (m, 1H, *Z*-S-CH_2-C); ^{13}C NMR (100 MHz, acetone-d_6) δ 171.0 (*E*-CONH_2), 169.2 (*Z*-CONH_2), 66.3 (*E*-N-C*H*-CO), 61.2 (*Z*-N-C*H*-CO), 53.7 (*Z*-S-CH_2-N), 47.3 (*E*-S-CH_2-N), 33.4 (*E*-S-CH_2-C), 32.7 (*Z*-S-CH_2-C); IR (cm^{-1}, neat) 3340, 3190, 1700, 1670, 1630, 1430; HRMS (FAB) 162.0347 (calcd for $C_4H_7O_2N_3S$ 162.0337).

5.3.3. 3-Nitroso-1,3-thiazolidine-4-thiocarboxamide (5)

Diphosphorous pentasulfide (P_4S_{10}; 1.0 g; 2.3 mmol) was added to a solution of 460 mg 1,3-thiazolidine-4-carboxamide (3.5 mmol) in 40 mL freshly distilled THF and ultrasonicated for 1 h at room temperature. This step was repeated, and the mixture was filtered after adding acetone. Because the desired product decomposed after concentrating with excess P_4S_{10}, a small quantity of aluminium oxide was added to the filtrate, and the mixture was evaporated. The residue adsorbed onto the aluminium oxide was purified several times via column chromatography [aluminium oxide, CH_2Cl_2:MeOH = 90:10, UV 254 nm + I_2 vapour], [silica gel, Et_2O:CH_2Cl_2 = 60:40 CH_2Cl_2 CH_2Cl_2:MeOH = 90:10, UV 254 nm + I_2 vapour] to afford 1,3-thiazolidine-4-thiocarboxamide as a white solid (135 mg; 26.3% yield). Mp 110 °C (decomp.); ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.84 (br, 1H, –CSNH_2), 9.40 (br, 1H, –CSNH_2), 4.21 (d, *J* = 8.5 Hz, 1H, –S-CH_2-N), 4.06 (d, *J* = 8.5 Hz, 1H, –S-CH_2-N), 3.93 (t, *J* = 6.1 Hz, 1H, –N-C*H*-CS), 3.10 (dd, *J* = 7.3, 9.7 Hz, 1H, –S-CH_2-C), 2.96 (dd, *J* = 7.3, 9.7 Hz, 1H, –S-CH_2-C); IR (cm^{-1}, neat) 3320, 3260, 3150, 1590, 1220; Elemental analysis: C, 32.23; H, 5.53; N, 18.82 (calcd for $C_4H_8N_2S_2$: C, 32.41; H, 5.44; N, 18.90).

Sodium nitrite (15 mg; 0.2 mmol) was added to a solution of 1,3-thiazolidine-4-thiocarboxamide (31 mg; 0.2 mmol) in 10 mL CH_2Cl_2, and 2.2 mL 0.1 M HCl (0.2 mmol) was added dropwise to the solution in an ice bath. The starting material disappeared immediately after the addition of HCl, as confirmed via TLC [aluminium oxide, 10% MeOH–$CHCl_3$, UV 254 nm + I_2 vapour]. The reaction mixture was extracted twice with CH_2Cl_2 (5 mL). The combined organic phase was dried over Na_2SO_4 and filtered. Because the desired product decomposed after concentrating the filtrate, a small quantity of silica gel was added to the filtrate, and the mixture was evaporated. After the filtrate was adsorbed onto silica gel, the residue was purified via column chromatography [silica gel, 5% MeOH–$CHCl_3$, UV 254 nm + I_2 vapour] to afford a tan-yellow solid (24 mg; 63.4% yield). Mp 122 °C (decomp.); ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.07 (br, 1H, *E*-CSNH_2), 9.79 (br, 1H, *Z*-CSNH_2), 9.46 (br, 1H, *E*-CSNH_2), 9.39 (br, 1H, *Z*-CSNH_2), 5.87 (dd, *J* = 3.7, 7.3 Hz, 1H, *E*-N-C*H*-CS), 5.83 (d, *J* = 11.0 Hz, 1H, *Z*-S-CH_2-N), 5.26 (d, *J* = 11.0 Hz, 1H, *Z*-S-CH_2-N), 4.98 (t, *J* = 8.0 Hz, 1H, *Z*-N-C*H*-CS), 4.85 (d, *J* = 12.2 Hz, 1H, *E*-S-CH_2-N), 4.48 (d, *J* = 12.2 Hz, 1H, *E*-S-CH_2-N), 3.62 (dd, *J* = 7.3, 12.2 Hz, 1H, *E*-S-CH_2-C), 3.57 (dd, *J* = 7.9, 12.2 Hz, 1H, *Z*-S-CH_2-C), 3.41 (dd, *J* = 3.7, 11.6 Hz, 1H, *E*-S-CH_2-C), 3.20 (dd, *J* = 7.9, 11.6 Hz, 1H, *Z*-S-CH_2-C); ^{13}C NMR (100 MHz, DMSO-d_6) δ 202.4 (*E*-CSNH_2), 200.6 (*Z*-CSNH_2), 70.7 (*E*-N-C*H*-CS), 66.2 (*Z*-N-C*H*-CS), 53.5 (*Z*-S-CH_2-N), 47.8 (*E*-S-CH_2-N), 35.2 (*E*-S-CH_2-C), 34.8 (*Z*-S-CH_2-C); IR (cm^{-1}, neat) 3280, 3120, 1650, 1400, 1260; HR-FAB-MS: 177.0031 (cacld 177.00305) ; Elemental analysis: C, 27.04; H, 4.14; N, 23.68 (calcd for $C_4H_7N_3OS_2$: C, 27.11; H, 3.98; N, 23.71).

5.4. Reaction of alicyclic *N*-nitrosamines and GSH

An L-phenylalanine (45 mM) internal standard, GSH (50 mM), and DTPA (24 μM) were dissolved in 0.1 M sodium phosphate buffer (pH 7.4). Compound 1 (9.0 mM), compounds **2**–**4** (4.5 mM), and TU (90 mM) were dissolved in acetonitrile. The stability of GSNO under acidic conditions was confirmed via HPLC (data not shown), with L-phenylalanine used as an internal standard. The reaction mixture was contaminated with transition metals; therefore, DTPA was added as a transition metal ion chelator to stabilise the GSNO.[31] All experiments were conducted in the dark due to the GSNO light instability.[31]

5.4.1. Reaction of 1 with GSH in the presence of thiourea

Aliquots of L-phenylalanine (300 μL, 4.5 mM), GSH (300 μL, 5.0 mM), DTPA (150 μL, 1.2 μM), and TU (150 μL, 4.5 mM) were mixed, and the pH was adjusted to 1.5 with 0.1 M HCl for a total volume of 2.85 mL. The reaction was initiated by the addition of 1 (150 μL, 0.45 mM) at 37 °C. Aliquots were collected at specified

intervals, and the GSNO yield was determined via HPLC using a Shiseido UG120 (5 μm, 250 × 4.6 mm) column with acetonitrile/0.05% trifluoroacetic acid (0.5:99.5) as the eluent at 1.2 mL/min at 335 nm.

5.4.2. Reaction of 2, 3, or 4 with GSH

Aliquots of L-phenylalanine (300 μL, 4.5 mM), GSH (300 μL, 5.0 mM), and DTPA (150 μL, 1.2 μM) were mixed, and the pH was adjusted to 1.5 with 0.1 M HCl for a total volume of 2.7 mL. The reaction was initiated by adding **2**–**4** (300 μL, 0.45 mM) at 37 °C. Aliquots were collected at specified intervals, and the GSNO yield was determined via HPLC using a Shiseido UG120 (5 μm, 250 × 4.6 mm) column with acetonitrile/0.05% trifluoroacetic acid (7:93) as the eluent at 1.0 mL/min for **2** and methanol/0.05% trifluoroacetic acid (5:95) as the eluent at 1.0 mL/min for **3** and **4** at 335 nm.

5.4.3. Reaction of 5 with GSH

Aliquots of GSH (300 μL, 5.0 mM) and DTPA (150 μL, 1.2 μM) were mixed, and the pH was adjusted to 1.5 with 0.1 M HCl for a total volume of 2.7 mL. The reaction was initiated by adding **5** (300 μL, 0.45 mM) at 37 °C. Aliquots were collected at specified intervals, and the GSNO yield was determined via HPLC using a Shiseido UG120 (5 μm, 250 × 4.6 mm) column with methanol/0.05% trifluoroacetic acid (5:95) as the eluent at 1.0 mL/min at 335 nm.

5.5. Kinetic analysis of transnitrosation reactions

The reaction rate constants were determined by measuring the formation of GSNO at 37 °C. The pseudo-first-order rates (k_{obs}) were determined via graphical analysis of the initial linear portion of the curve obtained from a plot of ln $\{[GSNO_\infty]/([GSNO_\infty] - [GSNO])\}$ versus t; the theoretical value for $GSNO_\infty$ was the initial concentration of the *N*-nitroso compound. All rate values were calculated using a least-squares method, and each experiment was conducted in triplicate.

5.6. GSNO yield

The quantity of *N*-nitroso remaining was quantified simultaneously with the GSNO formation. The yield of *S*-transnitrosation from *N*-nitrosamines at 24 h was calculated by dividing the percentage of GSNO formed by the percentage of *N*-nitroso compound consumed.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.10.008.

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