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Transnitrosation of Thiols from Aliphatic *N*-Nitrosamines: *S*-Nitrosation and Indirect Generation of Nitric Oxide

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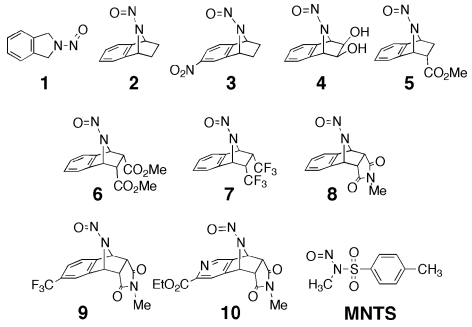
S-Nitrosothiols and heme nitrosyl species[1] are nitric oxide (NO)-derived metabolites that provide an endogenous reservoir of NO[2] and also play roles in protein *S*-nitrosation, that is, transnitrosation of thiols (or thiolates) in proteins,[3−6] thereby regulating protein functions and signal transduction pathways.[7,8] Intriguingly, endogenous *N*-nitrosamines are present in similar abundance to *S*-nitrosothiols,[9] and though they are thought to play similar physiological roles to *S*-nitrosothiols,[6] their transnitrosation reactivities and their contribution to biological events are little understood. Aromatic *N*-nitrosamines, *N*-nitrosotryptophan derivatives, can generate *S*-nitrosothiols[10] and can also act as direct NO donors,[11] but little is known about the *S*-transnitrosation reactivities of *aliphatic N*-nitrosamines, such as proline derivatives. Herein we describe the *S*-transnitrosation reaction of *aliphatic N*-nitroso derivatives of 7-azabicyclo[2.2.1]heptanes (**2**−**10**), which resemble conformationally constrained proline derivatives,[12] and its chemical features, that is, reactivity and chemoselectivity.



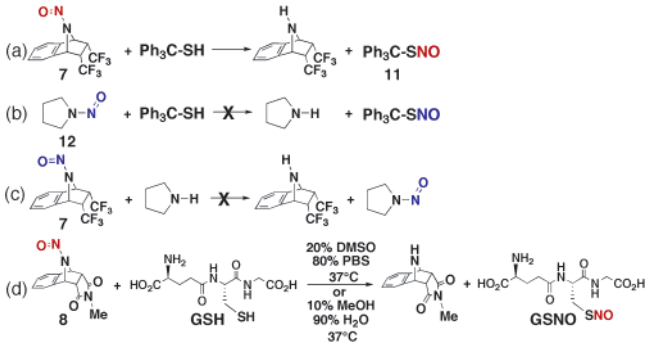
These *N*-nitroso derivatives of 7-azabicyclo[2.2.1]heptanes do not act as NO donors themselves, but can transnitrosate thiols. On the basis of the calculated activation energies of transnitrosation and the aorta smooth-muscle relaxation activities of these *N*-nitrosamines, we present a possible scenario of *S*-transnitrosation from aliphatic *N*-nitrosamines, leading to indirect generation of NO.

The N−NO bond of the *N*-nitroso derivatives of 7-azabicyclo-[2.2.1]heptanes tends to be weak,[12] and this is reflected in reduced rotational barriers of the N−NO bonds in solution and nitrogen-pyramidal structures of the *N*-nitroso group in the solid state. To increase the reactivity and hydrophilicity of these compounds, we synthesized the benzo derivatives **2**−**10** (see Supporting Information).

The reactivity of the N−NO bond of *N*-nitrosamines, or in other words, the propensity to release NO^+ (or NO) in acidic solution, can be estimated with the Griess method.[13] Well-studied nitrosating reagents, such as *N*-nitrososulfonamides (e.g., *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (MNTS)) were used as positive controls of transnitrosation.[14] All the bicyclic *N*-nitrosamines **2**−**10** were positive in the Griess assay (Figures S1 and S2) whereas *N*-nitrosamine **1** did not generate any dye at all.[12] It was confirmed that no NO was generated from the *N*-nitroso derivatives of 7-azabicyclo[2.2.1]heptanes at physiological pH (7.4, in PBS buffer) by means of an ESR spin trap experiment (Figure S5).[15] Thus, these aliphatic *N*-nitrosamines are not direct NO donors under neutral conditions.[12]

To examine the *N*-nitroso compound-dependent formation of *S*-nitrosothiols from thiols, we studied the UV−visible spectroscopic change upon transnitrosation in an aprotic solvent. We choose triphenylmethylthiol as a model thiol (*S*-nucleophile), because *S*-nitrosotriphenylmethylthiol (**11**) is sufficiently stable to be well characterized.[16] To study the chemoselectivity of transnitrosation, pyrrolidine, a mimic of proline, was used as a model secondary amine (*N*-nucleophile).

The bicyclic *N*-nitrosamine derivative **7** undergoes transnitrosation reaction with triphenylmethylthiol to afford the *S*-nitrosothiol **11** (eq a).[17] This is consistent with the relevant spectral changes (Figure 1b and the MNTS case (see Supporting Information, Figure



S3b)) and was also confirmed by ESI mass spectrometry (Figure S4e). In contrast, the monocyclic aliphatic *N*-nitrosopyrrolidine **12**, a mimic of *N*-nitrosoproline derivatives, did not nitrosate triphenylmethylthiol in chloroform even during 50 h at 37 °C (eq b and Figure S3d). These activities are consistent with the Griess assay results. In contrast, no *N*-nitrosopyrrolidine was formed from pyrrolidine by the action of **7** (Figure 1d (arrow) and eq c). Thus, the transnitrosation reaction of **7** exhibited chemoselectivity, that is, *S*-transnitrosation was preferred over *N*-transnitrosation. Similar chemoselectivity was observed for the bicyclic *N*-nitrosamine **8** (data not shown). In aqueous solution (methanol/water (20:80) or DMSO/PBS buffer (20:80)), the transnitrosation reaction of **8** also occurred with glutathione (GSH), a ubiquitous endogenous thiol, to form *S*-nitrosoglutathione (GSNO) (eq d), as indicated by the absorption spectral change (Figure S3f) and the detection of the formed GSNO by ESI mass spectroscopy (Figure S4f).

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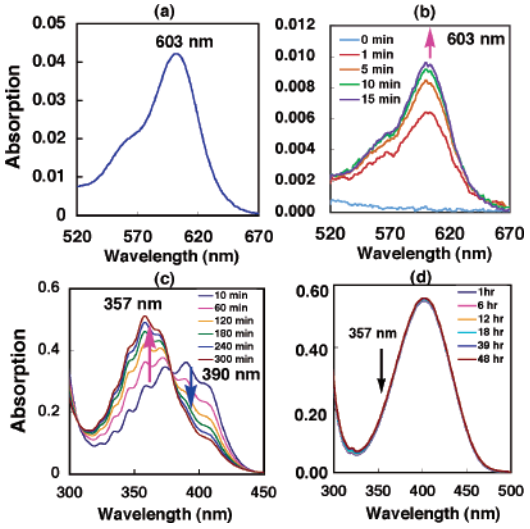
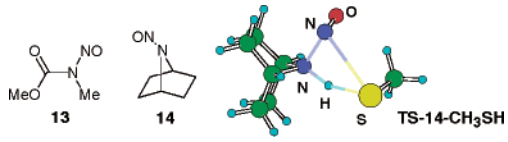


Figure 1. Absorption spectral changes in the presence of NO-donating agent. (a) *S*-Nitrosotriphenylmethylthiol **11** (authentic, 1 mM, $CHCl_3$, 37 °C); (b) **7** + triphenylmethylthiol in $CHCl_3$, 37 °C; (c) MNTS + pyrrolidine in $CHCl_3$, 37 °C; (d) **7** + pyrrolidine in $CHCl_3$, 37 °C.

To shed light on the feasibility of transnitrosation reaction of the *N*-nitroso derivatives (**2**, **12**, **13** (a model of **MNTS**), and **14**) DFT calculations were carried out, assuming an S_N2-like mechanism,[4,14] because no transnitrosation reaction occurred in the absence of a potent nucleophile, such as thiols. The calculated reaction energies at the DFT levels are summarized in Figure S8 and Table S1. The calculated transition states (TSs) for transnitrosation have a four-membered cyclic arrangement of the atoms, involving proton transfer from sulfur to nitrogen (e.g., **TS-14-CH$_3$SH**).



A model *S*-nucleophile (methylthiol) and *N*-nucleophile (methylamine) are used. The present computational results, i.e., magunitude of activation energies and overall reaction energies (endotherm/exotherm) are consistent with the experimentally observed high transnitrosation reactivities and *S*-nucleophile selectivity of the present bicyclic *N*-nitrosamines.[17]

To investigate the ability of the bicyclic nitrosamines to induce biological events, experiments were carried out with isolated rat aortic strips. The tested compounds exhibited concentration-dependent relaxing of aorta strips preconstricted with norepinephrine (Figure 2). The *N*-nitroso derivatives of 7-azabicyclo[2.2.1]heptanes (**5**−**8**) were effective, and **7** was as potent as sodium nitroprusside (SNP), a positive control vasodilator. The monocyclic *N*-nitrosamine **1** essentially did not relax the aorta, while the vehicle, DMSO, itself showed some relaxing activity. Both hemoglobin, a typical NO scavenger, and methylene blue and LY83583, guanylate cyclases inhibitors, inhibited the relaxation induced by compounds **7** (10 μM) and **8** (100 μM). These results indicate that the bicyclic *N*-nitrosamine-dependent relaxation is mediated by activation of guanylate cyclase via the induction of NO (see Figures S6 and S7).

In summary, we showed here that aliphatic *N*-nitroso derivatives of 7-azabicyclo[2.2.1]heptanes, which are not direct NO donors under physiological conditions (pH and temperature), exhibit *S*-transnitrosation reactivity. These *N*-nitrosamines showed a smooth muscle-relaxing effect, presumably through indirect generation of NO via *S*-transnitrosation. Thus, the present compounds may provide a lead for developing a new class of slow-release NO donors, which generate NO indirectly through *S*-transnitrosation.

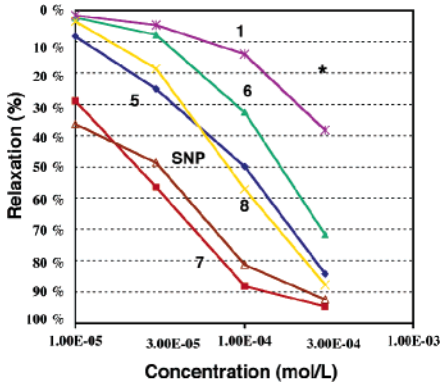


Figure 2. Relaxation of rat aorta smooth-muscle strips with *N*-Nitrosamines (n = 3∼4); dose-dependent increase of relaxation. Asterisk indicates the maximum relaxation induced by 3% DMSO.

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Supporting Information Available: Detailed discussions and experimental and calculation details. This material is available free of charge via the Internet at http://pubs.acs.org.

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(17) The second-order rate constant for *N*-nitrosamine **6** and triphenylmethylthiol in chloroform was $k_{obs} \approx (1.1 \pm 0.25) \times 10^{-1}$ M^{-1} s^{-1} at 20 °C, under a single component approximation of a second-order reaction. In the case of **2**, an Arrhenius plot of the rate constants of the reaction with triphenylmethylthiol provided the activation energy, E_a = 22.0 ± 1.8 kcal/mol, which is in good agreement with the DFT calculated values [21.4 kcal/mol (BB1K/6-31+G(d,p)); 27.5 kcal/mol (B3LYP/6-31+G-(d)), in the case of a model thiol, methylthiol] (see Supporting Infomation).

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