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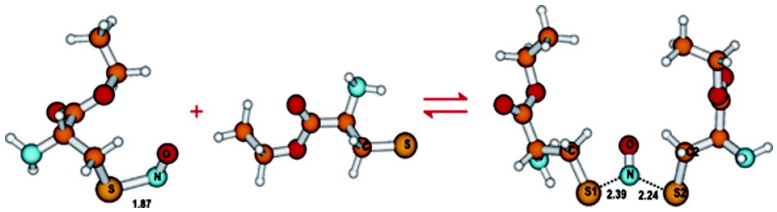
J. Am. Chem. Soc., **2005**, 127 (2), 486-487• DOI: 10.1021/ja044056v • Publication Date (Web): 23 December 2004



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Transnitrosation of Nitrosothiols: Characterization of an Elusive Intermediate

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It has been suggested that NO (nitric oxide) is stabilized and stored by a carrier molecule that prolongs its half-life and preserves its biological activity.[1−3] Low molecular weight thiols, such as cysteine and glutathione (GSH), are prime candidates for such carrier molecules because they can form (*S*)-nitrosothiols (RSNOs). These compounds are believed to be involved in many bioregulatory functions, such as NO storage, transport, and delivery. They have been detected in human airway-lining fluid plasma, platelet, and neutrophils,[4] and they are potent smooth-muscle relaxants and inhibitors of platelet aggregation.[5]

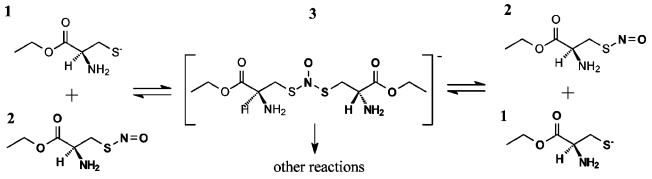
Previous work on the physiological chemistry of (*S*)-nitrosothiols indicates that they can release NO in the presence of cuprous ion,[6] ascorbate,[7] or thiols,[8] serve as a possible source of nitroxyl (NO^-) ions,[9] and can undergo transnitrosation reactions.[6,10,11] One possible reaction of this type is protein modification by cysteine S-nitrosation, an important mechanism for the regulation of protein function.[12]

Transnitrosation has been reported to be a reversible reaction,[13] first order in both thiol and nitrosothiol. Recently, a novel nitroxyl disulfide intermediate of this reaction has been proposed on the basis of electrospray ionization mass spectrometry experiments for (*S*)-nitroso-*N*-acetylpenicillamine (SNAP),[14] GSNO/GSH,[15] and SNAP/AR (aldose reductase) systems.[16] The existence of the intermediate has also been shown theoretically for a simplified model thiol.[14]

In this work, we shed light on the reaction mechanism, providing direct NMR spectroscopic and theoretical evidence of the elusive proposed intermediate. We have chosen for this study a physiologically relevant model of transnitrosation, (*S*)-nitroso-L-cysteine ethyl ester (ECySNO). This compound turned out to be a good candidate for our study because it provides a better model of a thiol group in a protein environment; it can be isolated, and it is relatively stable.

The transnitrosation reaction has been proposed to occur by the mechanism shown in Scheme 1.

Scheme 1. Reaction Mechanism



The rate-limiting step for this reaction is the nucleophilic attack of thiolate **1** to the nitrogen atom of the S−N−O moiety of **2**.[12] Therefore, the reaction rate is dependent on the reactivity of the thiol, which is directly correlated with pH, the pK_a of the sulfhydryl group, and stereoelectronic factors in the thiol.[13]

Kinetic measurements were obtained for the transnitrosation reaction in water. The obtained rate constant is $k_2 = 15.6 \pm 0.2 \times 10^{-3}$ M^{-1} s^{-1} (26 °C, pH 7.4). This value is in reasonable agreement with other experimental values of NO transfer from (*S*)-nitrosothiols to thiolates in aqueous solution.[13] The experimental activation energy is 22.7 ± 0.3 kcal/mol (Figure SI 6 in the Supporting Information). To our knowledge, this is the first reported value for activation energies of transnitrosation reactions between thiols. The activation enthalpy and entropy obtained using transition-state theory are 22.2 ± 0.9 kcal/mol and −8.5 ± 3.1 cal/K mol, respectively. Consistently with the proposed second-order rate constant, the value of the activation entropy is significantly negative.

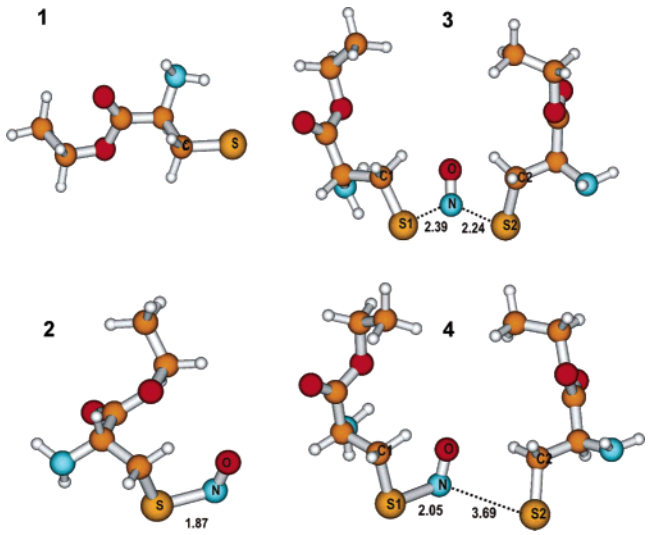


Figure 1. Optimized geometries for all species at the B3LYP/6-311+G* level of theory (selected bond lengths in Å).

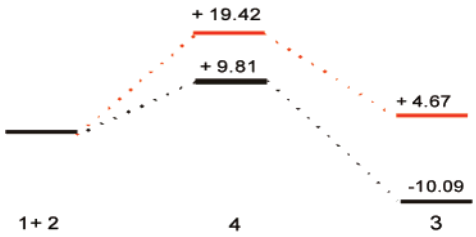


Figure 2. Energy profile (in kcal/mol) for the transnitrosation reaction between ECyS and ECySNO in vacuo (in black) and aqueous solution (in red).

We have performed density functional theory calculations using the Gaussian98 package[17] at the B3LYP[18] level with a 6-311+G* basis set for the species depicted in Figure 1. Solvent effects were modeled using the polarized continuum model (PCM) scheme.[19] The calculated energy profile for this reaction in aqueous solution and in vacuo is depicted in Figure 2. The formation of the anionic intermediate (**3**) in vacuo is exothermic, but in aqueous solution, it is less favorable. The computed energy barrier of 19.4 kcal/mol is

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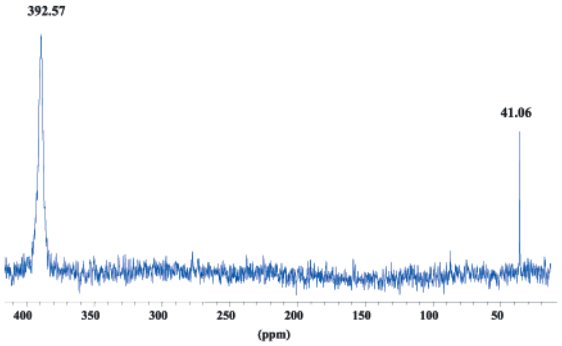

Figure 3. ^{15}N NMR spectra for the reaction of ECyS with ECySNO.

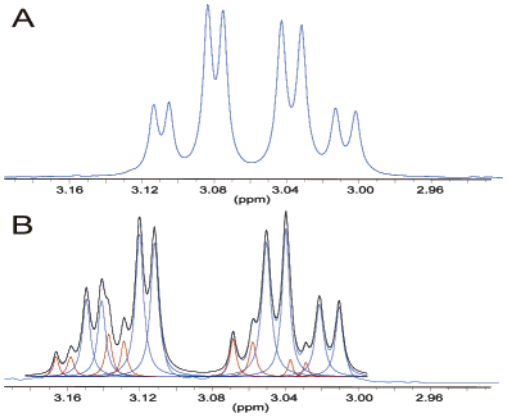

Figure 4. ^{1}H NMR spectra for ECyS (A) and for the reaction of ECySNO with ECyS (B); complete NMR spectra are shown in Figure SI 1 in the Supporting Information.

in reasonable agreement with our experimental activation energy. Our computed results suggest that the proposed transnitrosation mechanism should also be operative in less polar solvents. Moreover, the process is expected to be faster and more favorable in lipophilic environments.

Selected bond distances for species **1**−**4** are depicted in Figure 1. There is a 0.18 Å lengthening of the nitrosothiol S−N bond in the transition state and almost no change in the length of the N−O bond in **4**. The same trend was observed for methylthiol.[14] The optimized S−N bond distance in **3** is almost 0.5 Å longer than the corresponding bond in **2**. The intermediate surprisingly shows quite different lengths for both S−N bonds.

To characterize **3**, we performed ^{1}H, ^{13}C, and ^{15}N NMR experiments. Since the intermediate lifetime in aqueous solution was too short at the concentrations required for NMR experiments, we have performed the experiments in methanol. This solvent turned out to be a good choice, probably due to the absence of side reactions and because it resembles water polarity. The reaction mixture ^{15}N NMR spectrum displays a signal at 41 ppm referenced to $Na^{15}NO_2$ (Figure 3) (or −351 ppm referenced to ECySNO) assigned to **3**. We have confirmed this assignment by performing ^{15}N NMR DFT calculations, which predict that the chemical shift for **3** referenced to ECySNO should be −288 ppm.

The ^{13}C NMR spectra of the reaction mixture shows a new signal assigned to $-CH_2S$ in **3** (Figure SI 2 in the Supporting Information). ^{13}C DEPT, 2D HETCOR, and COSY NMR confirmed the assignment (Figures SI 3−5 in the Supporting Information). In the ^{1}H NMR spectra of the reaction mixture, the signal corresponding to $-CH_2S$ in **1** (Figure 4A) is shifted and its ABX pattern changes, while a second signal with a similar pattern appears and superimposes with the first one (Figure 4B). By performing signal deconvolution, we could estimate the amount of intermediate formed for various $[\mathbf{1}]_0$:$[\mathbf{2}]_0$ ratios. We found that as the concentration of **1** is increased at a fixed concentration of **2**, the concentration of **3** increases but never reaches the fixed concentration of **2** (ca. $[\mathbf{3}]$:$[\mathbf{2}]_0 = 0.9$ for $[\mathbf{1}]_0$:$[\mathbf{2}]_0 = 3{:}1$), suggesting the existence of an equilibrium among these species.

The highly relevant intermediate **3** of the transnitrosation reaction has been thoroughly characterized regarding its kinetic, thermodynamic, spectroscopic, and structural aspects in polar solvents. Our experimental and theoretical evidence confirms clearly the postulated mechanism of Scheme 1 for a physiologically relevant transnitrosation reaction between thiols.

Acknowledgment. We thank Professor Burton for helpful discussions, and ANPCyT, Antorchas, and UBA for financial support.

Supporting Information Available: NMR spectra, Arrhenius plot, computed geometries, and geometrical parameters are included. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA044056V