Nitrogen Oxides

DOI: 10.1002/ange.200801654

Fast Reductive Ligation of *S*-Nitrosothiols**

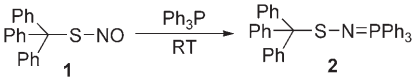
*Hua Wang and Ming Xian**

Dedicated to Professor Jin-Pei Cheng on the occasion of his 60th birthday

Nitric oxide (NO) plays many significant roles in physiology and pathophysiology.[1] The cellular response to NO is mediated by different reactions of various reactive nitrogen species (RNS), including direct reactions with metalloproteins and indirect reactions following oxidation and other metabolic processes. In particular, the reaction of RNS with cysteine residues of proteins that results in S-nitrosylation has received a great deal of attention. This is because S-nitrosylation represents an important post-translational modification that may transduce NO-dependent signals.[2] To date, a large group of proteins have been characterized as targets for S-nitrosylation, and in many cases S-nitrosylation is believed to regulate protein activity and function.[3] However, the detection of S-nitrosylation still remains a challenge because of the labile nature of *S*-nitrosothiols (RSNOs; R = substituent).[4,5] Herein, we report a novel reductive ligation reaction of RSNOs which can potentially be used as an efficient "one-step" strategy for detection of S-nitrosylation in biological systems.

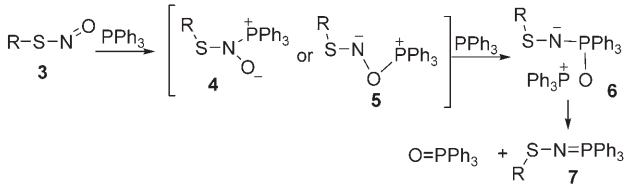
Although RSNO compounds have been known for over a century, their reactions remain limited because of their instability.[6] However, we postulated that the increased reactivity of RSNO compounds could be exploited if: 1) a reagent was developed that could react with SNO groups to form stable products (or conjugates), and 2) the reagent was compatible with other biological functionalities, especially disulfide bonds. With these considerations in mind, the 1972 report by Haake,[7] in which TrSNO (**1**; Tr = trityl) reacted with PPh_3 in benzene to provide azaylide **2** as an isolable product, attracted our attention. We revisited this reaction and found that it gave azaylides in benzene and in other organic solvents such as $CHCl_3$, THF, and CH_3CN (Table 1). In addition, this reaction proceeded nicely in water-containing systems such as CH_3CN/H_2O. The reaction proved to be rapid and was usually complete within minutes. Prolonged exposure to aqueous systems led to lower yields because of azaylide hydrolysis. Besides TrSNO, other RSNOs such as *t*BuSNO also underwent a similar process to generate the corresponding azaylide products (see the Supporting Information).

Table 1: Azaylide formation from RSNO and Ph_3P.



Entry	Solvent	Yield [%]
1	benzene	82
2	$CHCl_3$	86
3	THF	90
4	CH_3CN	88
5	CH_3CN/H_2O (1:1)	85

We noticed that two equivalents of PPh_3 were consumed in this reaction, and a plausible mechanism is proposed in Scheme 1. PPh_3 first reacts with the nitroso group to form either phosphonitroxide **4** or zwitterion **5**. Then, a second



Scheme 1. Proposed mechanism for the formation of azaylide.

molecule of PPh_3 reacts with either **4** or **5** to generate intermediate **6**, which finally leads to azaylide **7** and $Ph_3P{=}O$.

We hypothesized that azaylide formation might be general for RSNO moieties. We also envisioned that intermediates **4**–**6**, and the final azaylide **7** could be potential nucleophilic species. If a suitable electrophilic group is attached to the phosphine reagent then it could trap these intermediates and undergo spontaneous intramolecular reactions to form stable products in only one step, thus making new ligation reactions of RSNOs possible. These reactions could be used to selectively label the S-nitrosylation process in biological systems.

To test our hypothesis we studied the reactions between RSNOs and phosphine esters **8**. These phosphine compounds have been used in the well-known Staudinger ligation to selectively label azides.[8] Based on the pioneering work of the Bertozzi and Raines research groups,[8] we expected that a similar ligation process would also proceed when the azaylide intermediates (such as compound **7**, Scheme 1) were formed. The model substrate *t*BuSNO (**9**) was treated with either **8a** (R′ = Me) or **8b** (R′ = Ph) in different solvent systems

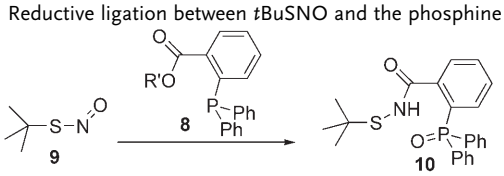
[*] Dr. H. Wang, Prof. Dr. M. Xian
Department of Chemistry
Washington State University
Pullman, WA 99164 (USA)
Fax: (+1) 509-335-6087
E-mail: mxian@wsu.edu

[**] We thank the Washington State University for supporting this work. We also thank Prof. P. Garner and Prof. R. Ronald for helpful discussions.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.200801654.

(Table 2). Organic solvents such as THF (results not shown) and CH_3CN (Table 2, entries 1 and 2) only gave a trace amount of ligation product **10**. We also screened a number of

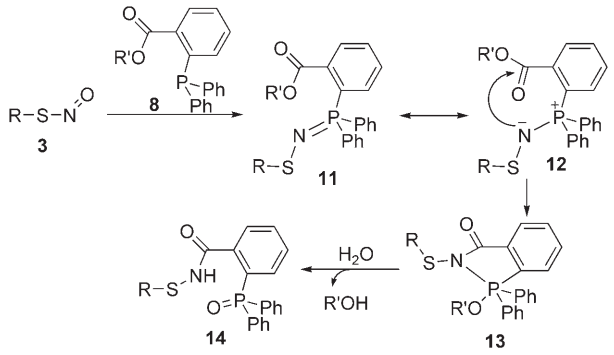
Table 2: Reductive ligation between *t*BuSNO and the phosphine esters.



Entry	Phosphine ester	Solvent	Yield [%]
1	**8a**	CH_3CN	< 5
2	**8b**	CH_3CN	< 5
3	**8a**	CH_3CN/H_2O (3:1)	50
4	**8b**	CH_3CN/H_2O (3:1)	80
5	**8b**	$CH_3CN/THF/H_2O$ (1.5:1.5:1)	93

organic solvent/water mixtures, and with CH_3CN/H_2O (3:1; Table 2, entries 3 and 4) we observed a significant amount of ligation product. When THF was added to improve the solubility of **8b** (Table 2, entry 5) we obtained the sulfenamide compound **10** in excellent yield (93%).

We believe that formation of the sulfenamide product follows a similar mechanism to that of the Staudinger ligation (Scheme 2).[8b] The azaylide intermediate **11** first forms upon

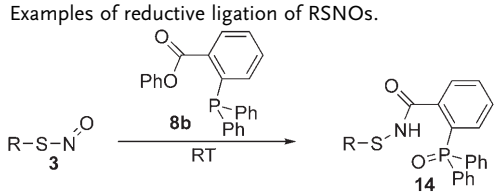


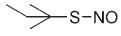
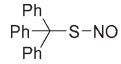
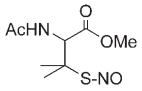
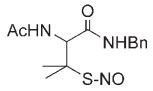
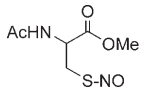
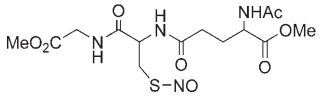
Scheme 2. Proposed mechanism of the reaction between RSNO and the phosphine ester.

treatment of RSNO with **8**. Then **11** undergoes an intramolecular reaction with the ester functionality (see **12**) to provide phosphorane **13**. Finally, hydrolysis of **13** in the presence of water leads to the final product **14**.

Next, we tested the generality of this reductive ligation process by using a series of RSNO compounds (Table 3). Under the optimized reaction conditions, the relatively stable tertiary RSNOs **3a** and **3b** gave the desired products in excellent yields (Table 3, entries 1 and 2). The steric bulk of R did not affect the reaction. This method was also used to capture extremely unstable primary RSNOs such as **3c** in good yield (Table 3, entry 3). In addition, the reactions employing amino acid and peptide derivatives (**3d**–**3g**) were also examined (Table 3, entries 4–7). In all cases the desired

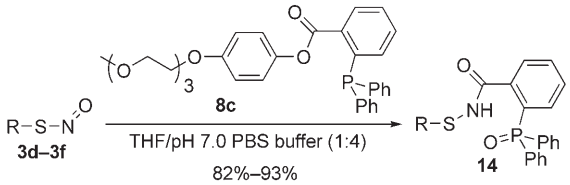
Table 3: Examples of reductive ligation of RSNOs.



Entry	RSNO		Yield [%]
1	**3a**		92
2	**3b**		90
3	**3c**		89
4	**3d**		91
5	**3e**		89
6	**3f**		84
7	**3g**		69

ligation products were obtained in good yield. Notably, the reductive ligation of RSNOs is very fast and the desired products are typically formed within a few minutes. Therefore, the possible hydrolysis of azaylide intermediates does not appear to be a problem.

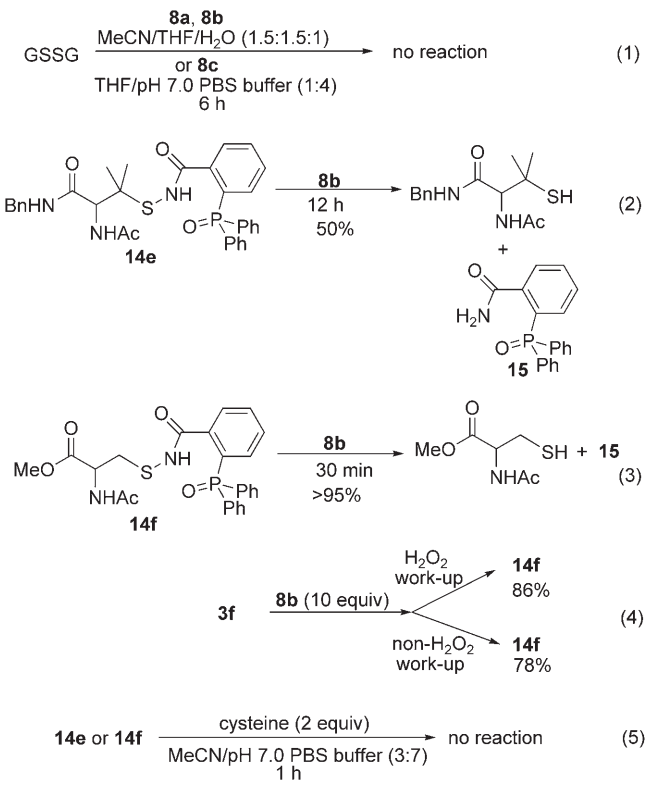
We then prepared the poly(ethylene glycol)-linked phosphine ester **8c** to further test the reductive ligation process (Scheme 3). This reagent was more water soluble than **8a** and



Scheme 3. Reductive ligation of RSNOs with **8c**.

8b, and the reactions proceeded nicely in a solvent system containing pH 7.0 PBS buffer (80%; PBS = phosphate-buffered saline) and THF (20%). Once again, the desired ligation products were obtained rapidly and in good yields (see the Supporting Information for experimental details).

To test the potential compatibility of this reaction with biological systems we carried out some control experiments (Scheme 4). Phosphine compounds are known to be mild reducing agents, which may raise the possibility of disulfide bond reduction in proteins as a potential undesirable side reaction. Previous studies have demonstrated that some triaryl phosphine compounds are safe for use in the presence of disulfide bonds.[8a] We also found that treatment of



Scheme 4. Control experiments for the reductive ligation reaction. Bn = benzyl.

compounds **8a**–**8c** with glutathione disulfide [GSSG; Eq. (1)] did not generate any detectable free thiols after 6 h.

If we apply this method to labeling SNO proteins, then the phosphine compound must be used in excess relative to the SNO. Therefore, a major concern is that the excess phosphine reagent might induce reductive fragmentation to cleave the S–N bond of the sulfenamide ligation products.[9] To address this question, ligation products **14e** and **14f** were treated with **8b** [Eqs. (2) and (3)]. Under our optimized reductive ligation reaction conditions, the tertiary sulfenamide **14e** gave only a very small amount (< 10%) of fragmentation product after 1 h, while after 12 h we obtained 50% of the decomposition product **15** and the corresponding thiol, as well as recovered starting material. In contrast, the primary sulfenamide **14f** was quite sensitive to phosphine **8b** and decomposition was complete after 30 minutes. However, since reductive ligation is a very fast process, it was possible to avoid unwanted by-products if the reaction was stopped before decomposition, or by destroying the reactivity of the phosphine reagent immediately after ligation was complete. Indeed, after mixing the primary RSNO **3f** with excess **8b** (10 equiv) for 1 minute (a disappearance of the red color accompanied the conversion of **3f**), H_2O_2 was used to quench the reaction and the desired ligation product **14f** was obtained in 86% yield [Eq. (4)]. Even without the peroxide work-up, a good yield (78%) was still obtained with a quick separation (see the Supporting Information).

Another concern in biological systems is the possibility of breaking the S–N bond of the sulfenamides with thiol-containing compounds.[9,10] No decomposition was observed after 1 hour when the reactions between **14e** (or **14f**) and cysteine were carried out in pH 7.0 and 8.0 buffer solutions [Eq. (5)], (see the Supporting Information for experimental details).

In summary, we have developed a fast reductive ligation reaction which targets SNO moieties. To the best of our knowledge, this is the first "one-step" method to convert unstable SNO groups into relatively stable conjugates. In light of the results from the control experiments, we expect that this reaction can be used to design new detection methods for S-nitrosylation in biological systems. Progress toward this goal is ongoing.

Experimental Section

General procedure for the reductive ligation of RSNOs: Compound **9** was freshly prepared from the corresponding thiol. Compounds **9** (1.0 mmol) and **8b** (2.1 mmol) were added to a solution of CH_3CN/THF/H_2O (15 mL/15 mL/10 mL) were. The reaction mixture was stirred at room temperature until the reaction was complete (usually less than 5 min, as indicated by disappearance of the green color). The mixture was then diluted with EtOAc (100 mL) and the organic phase was washed with brine, dried over $MgSO_4$, and concentrated in vacuo. Purification of the residue by column chromatography on silica gel (MeOH/CH_2Cl_2, 1:100) afforded **10** in 93% yield (see the Supporting Information for the characterization data).

Received: April 8, 2008
Published online: July 18, 2008

Keywords: nitrenes · nitrogen oxides · reductive ligation · *S*-nitrosothiols

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