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Reductive Ligation Mediated One-Step Disulfide Formation of *S*-Nitrosothiols

Jiming Zhang,[†] Sheng Li,[‡] Dehui Zhang,[†] Hua Wang,[†] A. Richard Whorton,[‡] and Ming Xian*,[†]

Department of Chemistry, Washington State University, Pullman, Washington 99164, and Department of Pharmacology & Cancer Biology, Duke University Medical Center, Durham, North Carolina 27710

mxian@wsu.edu

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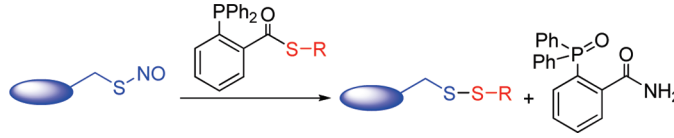
ABSTRACT



A one-step reductive ligation mediated disulfide formation of *S*-nitrosothiols was developed. This reaction involves the reaction of the *S*-nitroso group with phosphine-thioesters to form sulfenamide and thiolate intermediates, which then undergo a fast intermolecular disulfide formation to form stable conjugates. This reaction can be used to design new biosensors of S-nitrosated proteins.

S-Nitrosation is an important post-translational modification that can affect protein activity, localization, or stability. This reversible modification is suggested to occur as a result of biological nitric oxide (NO) formation and has been thought of as a mechanism by which NO can transmit signals both within and between cells and tissues.[1] However, the detection of protein S-nitrosation is still problematic because the nitrosation products, i.e., *S*-nitrosothiols (RSNOs), are very labile moieties.[2] As a unique functional group, SNO is expected to have distinct reactivity from other biological functional groups. If new reactions which specifically target SNO and convert unstable SNO to stable products under physiological conditions can be developed, such reactions would hold considerable promise in applications for the detection of protein S-nitrosation.

In 2008, our group reported a fast reductive ligation of RSNOs, which can selectively convert SNO to a relatively stable sulfenamide product under very mild conditions (Scheme 1, eq 1).[3] This reaction proceeds through a Staudinger-ligation type mechanism.[4] Recently, in the study of a "traceless" version of the reductive ligation, we discovered an unexpected bisligation, which led to the formation of stable disulfide-iminophosphorane products from primary RSNOs (Scheme 1, eq 2).[5] In this process, the thiolate intermediate undergoes an intramolecular substitution with the pseudo-sulfenamide linkage to form the disulfide-iminophosphorane product in excellent yields.

On the basis of the high reactivity of the sulfenamide toward thiolate observed in bisligation, we envisioned that phosphine-

[†] Washington State University.
[‡] Duke University Medical Center.

(1) (a) Lancaster, J. R., Jr. *Nitric Oxide* **2008**, *19*, 68–72. (b) Zhang, Y.; Hogg, N. *Free Radical Biol. Med.* **2005**, *38*, 831–838. (c) Hess, D. T.; Matsumoto, A.; Kim, S.-O.; Marshall, H. E.; Stamler, J. S. *Nat. Rev. Mol. Cell. Biol.* **2005**, *6*, 150–166. (d) Foster, M. W.; McMahon, T. J.; Stamler, J. S. *Trends Mol. Med.* **2003**, *9*, 160–168.

(2) For a recent RSNO detection method, see: (a) Bechtold, E.; Reisz, J. A.; Klomsiri, C.; Tsang, A. W.; Wright, M. W.; Poole, L. B.; Furdui, C. M.; King, S. B. *ACS Chem. Biol.* **2010**, *5*, 405–414. For selected reviews on RSNO detection, see: (b) Gow, A.; Doctor, A.; Mannick, J.; Gaston, B. *J. Chromatogr. B* **2007**, *851*, 140–151. (c) Kettenhofen, N. J.; Broniowska, K. A.; Keszler, A.; Zhang, Y.; Hogg, N. *J. Chromatogr. B* **2007**, *851*, 152–159. (d) MacArthur, P. H.; Shiva, S.; Gladwin, M. T. *J. Chromatogr. B* **2007**, *851*, 93–105. (e) Jaffrey, S. R. *Methods Enzymol.* **2005**, *396*, 105–118. For deficiencies of current methods, see: (f) Giustarini, D.; Milzani, A.; Dalle-Donne, I.; Rossi, R. *J. Chromatogr. B* **2007**, *851*, 124–139. (g) Gladwin, M. T.; Wang, X.; Hogg, N. *Free Radical Biol. Med.* **2006**, *41*, 557–561.

(3) Wang, H.; Xian, M. *Angew. Chem., Int. Ed.* **2008**, *47*, 6598–6601.

(4) (a) Saxon, E.; Bertozzi, C. R. *Science* **2000**, *287*, 2007–2010. (b) Lin, F. L.; Hoyt, H. M.; van Halbeek, H.; Bergman, R. G.; Bertozzi, C. R. *J. Am. Chem. Soc.* **2005**, *127*, 2686–2695. (c) Wang, H.; Zhang, J.; Xian, M. *J. Am. Chem. Soc.* **2009**, *131*, 13238–13239.

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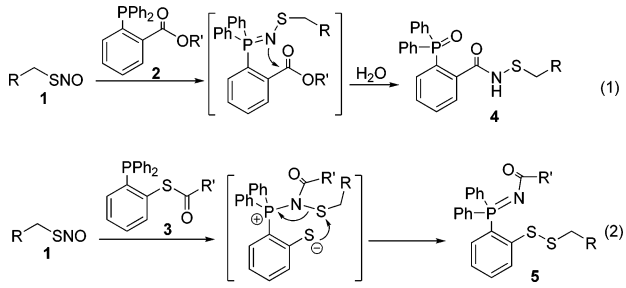
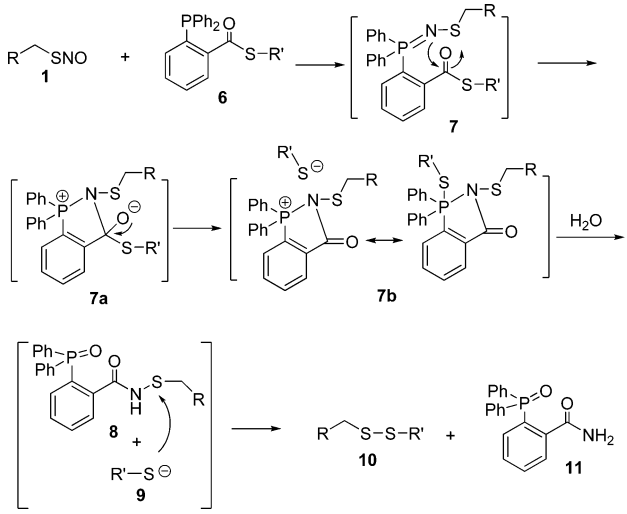
Scheme 1



thioester substrates like **6** should undergo a reductive ligation mediated one-step disulfide formation with RSNOs (Scheme 2):

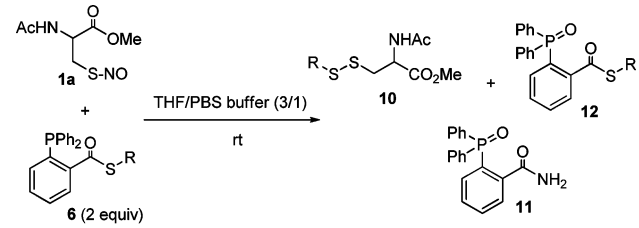
Scheme 2



the reaction between RSNO **1** and phosphine **6** should generate azaylide **7** first. Then, an intramolecular acyl transfer should occur to provide the intermediate **7b**. Upon hydrolysis in aqueous buffer, **7b** should be converted to the sulfenamide product **8** and thiolate **9**. Finally, the intermolecular reaction between **8** and **9** could proceed spontaneously to provide a stable disulfide **10** and liberate the phosphine oxide **11**. As the thioesters are better leaving groups than esters, we expect that substrates like **6** should facilitate the reductive ligation process. In addition, the formation of simple disulfide products, without the bulky phosphine adducts, would be attractive for the applications in protein systems.

With this idea in mind, a series of phosphine-thioester substrates were prepared and tested with a RSNO model compound, **1a** (Table 1). As expected, the desired disulfide products were obtained in a mixture solvent THF/PBS buffer (pH 7.4). Other byproducts isolated were corresponding phosphine-oxide **12** and amide **11**. With primary thiol-based thioester substrates (**6a** and **6b**), the disulfide products were isolated in excellent yields. Thiophenol-based thioester substrates (**6c** and **6d**) also resulted in good yields of disulfide products. However, a moderate yield (43%, entry 5) of disulfide was observed when

Table 1. Reaction between RSNO and Phosphine-thioesters



6a: R = *n*-Bu; **6b**: R = $PhCH_2$; **6c**: R = Ph; **6d**: R = 4-$MeOC_6H_4$; **6e**: R = *t*-Bu

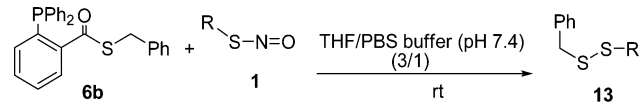
entry	6	yield of **10** [%][a]
1	**6a**	79
2	**6b**	82
3	**6c**	63
4	**6d**	51
5	**6e**	43

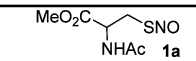
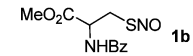
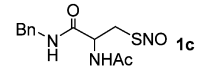
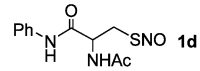
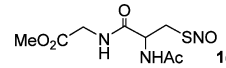
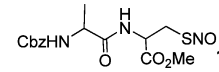
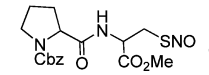
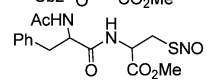
[a] Yield of isolated product.

1a was treated with a tertiary thiol-based substrate **6e**. Presumably the *t*-butylthiolate generated in the reductive ligation was less reactive toward the sulfenamide intermediate, due to the steric hindrance. In all examples, the disulfide formation proved to be a fast reaction which usually completed within 1 h.

To explore the generality of this disulfide formation, we next tested the reaction of substrate **6b** with a series of *S*-nitrosocysteine derivatives (**1a**−**1h**). As shown in Table 2, the desired

Table 2. One-Step Disulfide Formation of RSNO



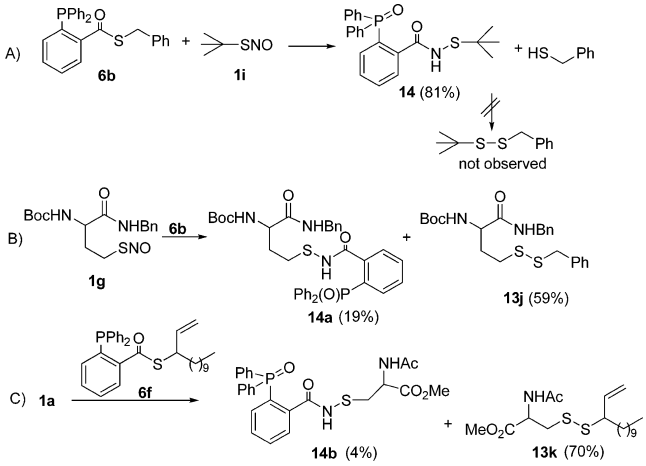
entry	RSNO	yield of **13** [%][a]
1	 **1a**	82
2	 **1b**	85
3	 **1c**	68
4	 **1d**	66
5	 **1e**	74
6	 **1f**	73
7	 **1g**	72
8	 **1h**	70

[a] Yield of isolated product.

disulfide products were obtained in good yields in all cases. It should be mentioned that the formation of the sulfenamide intermediate was not observed with these substrates (by TLC). This suggested that the disulfide formation was a faster reaction than the formation of sulfenamide intermediate.

To probe the formation of the sulfenamide intermediates as shown in the mechanism proposal, we carried out the experiment using some different substrates (Scheme 3). When the

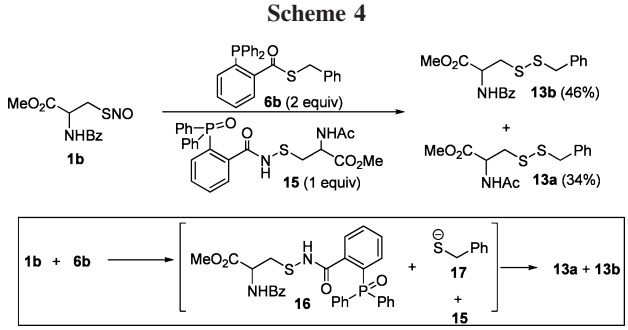
Scheme 3



tertiary RSNO **1i** was treated with **6b** (eq A), corresponding sulfenamide **14** was isolated in good yield, while the disulfide product was not observed. This result is consistent with previous observations[3] that sulfenamides generated from tertiary RSNOs are much more stable than the ones generated from primary RSNOs. In addition, when a homocysteine SNO derivative **1g** was treated with **6b** and when substrate **1a** was treated with a phosphine-thioester (**6f**) prepared from a secondary thiol, we observed the formation of the sulfenamide intermediates (**14a** and **14b**, eqs B and C). Therefore, the results shown in Scheme 3 clearly demonstrated that sulfenamides were the intermediates in the reaction between RSNOs and phosphine-thioesters.

To further prove the presence of the sulfenamide intermediate in this reaction, we designed a crossover experiment (Scheme 4). When the reaction between RSNO **1b** and phosphine substrate **6b** was carried out, a sulfenamide compound **15** was

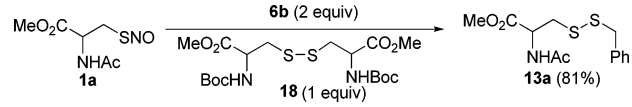
Scheme 4



also added into the reaction mixture. We anticipated that the reductive ligation between **1b** and **6b** should generate sulfenamide **16** and thiolate **17** quickly. Due to the presence of **15** in the reaction, thiolate **17** could react with both **15** and **16**. Indeed, we isolated the desired product **13b** in 46% yield, as well as the crossover product **13a** in 34% yield.

If this reaction will be applied for labeling protein SNOs, a concern is that the thiolate intermediate may react with disulfides in proteins to give false positive linkage. To address this question, we tested the reaction between **1a** and **6b** in the presence of a disulfide compound **18** (Scheme 5).

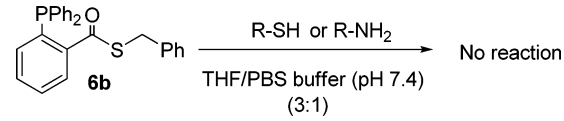
Scheme 5



However, only the desired product **13a** was obtained from the reaction. No crossover product with **18** was observed.

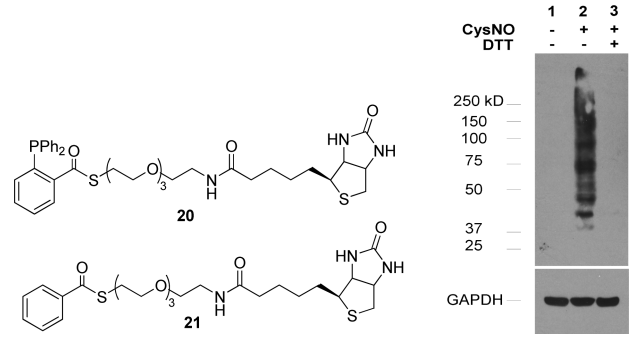
To address the bio-orthogonality of the phosphine-thioester substrates, we tested the reactions between **6b** and potential cellular nucleophiles such as thiols and amines. **6b** appeared to be quite stable toward lysine derivatives and cysteine derivatives as no reaction was observed (Scheme 6, see Supporting Information for experimental details).

Scheme 6



To explore the application of this new reaction in the detection of S-nitrosated proteins, experiments shown in Scheme 7 were carried out. Briefly, COS-7 cells were first treated with *S*-nitrosocysteine (CysNO). It is known that the treatment of cells with CysNO will result in the S-nitrosation

Scheme 7



on proteins.[6] The cells were then washed, fixed, and permeablized. After blocking free thiols with NEM, the cells were incubated with a biotin-linked phosphine substrate **20**. Cellular proteins that were S-nitrosated should react with **20** to form a stable disulfide linkage with biotin. The labeled proteins were detected by NeutrAvidin-HRP and visualized by ECL Plus Western Blotting Detection System. As expected, we observed a number of labeled proteins on SDS-PAGE from CysNO treated cells (lane 2). In the control experiment, non-CysNO treated cells were carried through the same process, and no labeled proteins were observed on SDS-PAGE (lane 1). We also found that the signals of CysNO treated cells were reversible by DTT (lane 3). This result confirmed the formation of disulfide linkages between the proteins and biotin. The GAPDH bands shown in the lower panel exhibited equal protein loading in these experiments.

In this biotin-labeling experiment, one concern was that reagent **20** may react with cellular nucleophiles (such as amines or thiols) to release biotin-linked thiolate, which might react with the disulfides in proteins to form false positive biotin conjugates. However, experiment results shown in Schemes 5 and 6 and more importantly the fact of lacking any observed biotin-labeled proteins in the control lane (lane 1, Scheme 7) should rule out that possibility. To further clarify this question, a structurally related thioester compound **21** was prepared and used in CysNO treated cells. No biotin labeling was observed with **21** (even in the presence of PPh_3, see Figure S1 in the Supporting Information). These results clearly demonstrated the efficiency and specificity of the phosphine-mediated disulfide formation on SNO moieties.

In summary, a reductive ligation mediated disulfide formation between RSNOs and phosphine-thioester substrates has been developed. This reaction can selectively convert unstable RSNOs to stable disulfides in one step under very mild conditions. We also demonstrated that this reaction can be used to identify SNO proteins in cell extracts. Further explorations of this reaction for the detection of protein S-nitrosation continue in our laboratory.

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Supporting Information Available: Synthetic procedures, spectroscopic data, and experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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