ChemComm

COMMUNICATION

View Article Online
View Journal | View Issue

A reductive ligation based fluorescent probe for S-nitrosothiols†

Cite this: *Chem. Commun.*, 2014, 50, 4806

Received 19th February 2014,
Accepted 10th March 2014

DOI: 10.1039/c4cc01288g

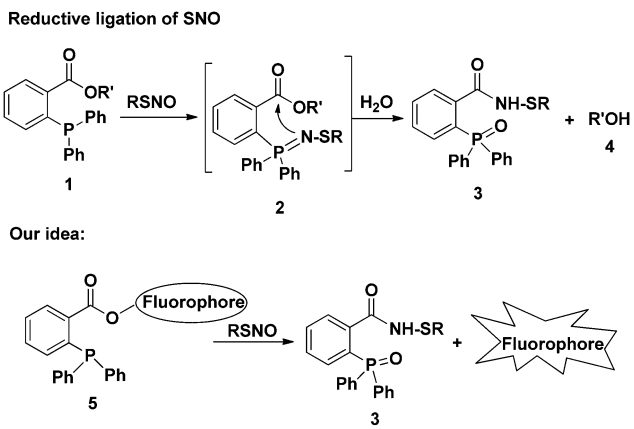
www.rsc.org/chemcomm

Di Zhang,‡[ab] Wei Chen,‡[a] Zhengrui Miao,[c] Yong Ye,*[b] Yufen Zhao,[b] S. Bruce King[c] and Ming Xian*[a]

A reductive ligation based fluorescent probe (SNOP1) for the detection of S-nitrosothiols (SNO) was developed. The probe showed good selectivity and sensitivity for SNO.

S-Nitrosation is an important post-translational modification that not only modulates biological activity of nitric oxides (NO), but also regulates protein functions.[1] The levels of S-nitrosation are finely regulated, and dysregulation is associated with the etiology of several pathologies. Currently the detection of S-nitrosation in biological systems is still a challenge due to the labiality of the products of S-nitrosation, *i.e.* S-nitrosothiols (SNO).[2] Since SNO are unstable species, most current methods (such as chemiluminescence based assays, colorimetry based assays, and biotin-switch based assays) are indirect methods, which in fact detect the decomposition products of SNO (either the S part or the NO part).[3] Careful control experiments are needed if these methods are used, otherwise false positive results could be generated. In this regard, direct methods which target the entire SNO moiety would have advantages. In the past several years our laboratory has developed a series of phosphine-based bioorthogonal reactions of SNO.[4] These reactions specifically target SNO groups and can directly convert unstable SNO to stable and detectable species. While we are continuing to work on these reactions (our goal is to utilize these reactions to develop novel reagents for directly enriching or labeling protein SNO), we realized that fluorescent probes may be developed based on these reactions. Fluorescence methods are known to have both high sensitivity and high spatiotemporal resolution for visualizing biomolecules *in vitro* and *in vivo*. Experimental operations are easy to perform. Therefore, fluorescence methods for SNO detection should be attractive. However this has not been well studied. In 2009, we reported our first generation of fluorescent probes for SNO, which was based on a SNO-mediated oxidation of phosphine substrates.[5] Although the probes showed good sensitivity for SNO *vs.* other reactive sulfur species (RSS), potential oxidation by other oxidative species such as H_2O_2 could be a problem. To solve this problem we envisioned that the reductive ligation of SNO could be useful in the development of specific SNO fluorescent probes. Here we report the design, synthesis, and evaluation of a reductive ligation-based probe for SNO.

The mechanism of reductive ligation is described in Scheme 1.[4a] SNO can react with triaryl phosphine **1** to form the azaylide intermediate **2**, which in turn undergoes a rapid intramolecular acyl transfer and hydrolysis to furnish sulfenamide **3** and R′OH. This reaction provides a unique and specific way to remove the acylated group on hydroxyl groups. It is known that acylation on many fluorophores can quench the fluorescence and de-acylation can reform the fluorescent species.[6] This strategy has been widely used in the design of many reaction based fluorescent probes.[6] Therefore we expected that if an –OH sensitive fluorophore is introduced into the triarylphosphine acylate, the resultant compound **5** would be a specific probe for SNO as it will selectively react with SNO to release the fluorophore.



Scheme 1 The design of reductive ligation-based probes for SNO.

[a] *Department of Chemistry, Washington State University, Pullman, WA 99164, USA. E-mail: mxian@wsu.edu; Fax: +1 509-335-8867*

[b] *College of Chemistry and Molecular Engineering, Zhengzhou University, Zhengzhou 450052, China. E-mail: yeyong03@tsinghua.org.cn*

[c] *Department of Chemistry, Wake Forest University, Winston-Salem, NC 27109, USA*

† Electronic supplementary information (ESI) available: Experimental procedures, characterization data, and additional spectra. See DOI: 10.1039/c4cc01288g

‡ Two authors contributed equally to this work.

To test this hypothesis, we proposed a SNO probe **SNOP1**, as shown in Scheme 2. A fluorescein (**6**) was selected as the fluorophore as it is known that bis-OH acylation of fluoresceins quenches their fluorescence.[6d-f] In addition, according to our previous experience, acylation on both OH groups usually leads to a higher level of fluorescence.[7] With two phosphine moieties in the structure, the probe could react with SNO molecules to release either the free fluorescein or the mono-acylated fluorescein, both are strong fluorescent species with the same emission wavelengths. **SNOP1** was easily prepared in one step from fluorescein and 2-(diphenylphosphino)benzoic acid (**7**). The compound was fully characterized by ^{1}H, ^{13}C, ^{31}P NMR and MS (see ESI†).

With the probe in hand, we first tested its fluorescence properties in aqueous buffers. The Tris-HCl buffer system was found to give the best results, so this system was used in all the experiments described here. As expected, the probe itself showed low fluorescence intensity. When a model SNO substrate, **8** (50 μM), was added into the solution of the probe (10 μM), significant increase of fluorescence intensity (~90 fold) was observed (Fig. 1A). The fluorescence turn-on response was found to be fast. The maximum intensity was reached in about 25 min. We also tested the response of **SNOP1** to S-nitrosoglutathione (GSNO), which is an endogenous SNO (Fig. 1B). GSNO also led to very obvious fluorescence increase, although at a smaller level (~18 fold) and slower rate (reaching the maximum at ~40 min) compared to compound **8**. GSNO is much more stable than other small molecule SNO compounds. This is probably due to its structural character which somehow protects SNO from decomposition. These properties may also cause low or slow reactivity of GSNO toward reagents like **SNOP1**, therefore it gave lower and slower fluorescence response. It should be noted that the model reaction between **SNOP1** and SNO indeed led to both free fluorescein and mono-acylated fluorescein as products (Fig. S1, ESI†), confirming the reaction mechanism proposed in Scheme 2.

To demonstrate the efficiency of the probe in determining SNO concentrations, a series of different concentrations of GSNO were treated with **SNOP1** (10 μM). For consistency, the fluorescence

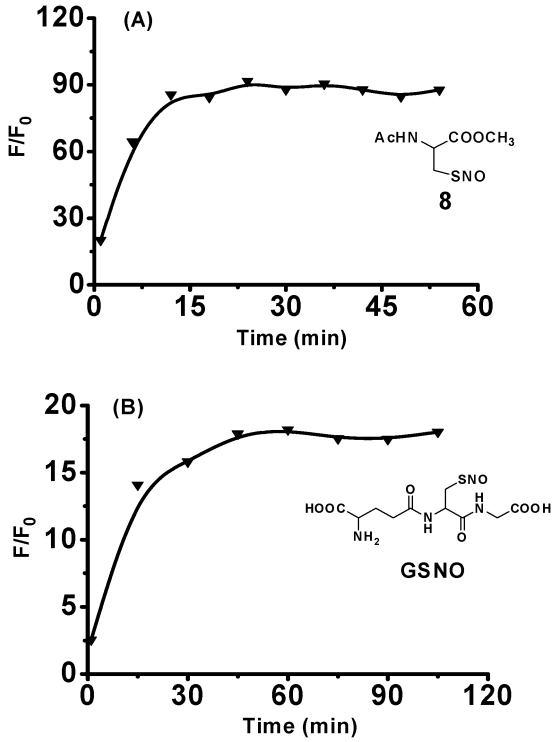
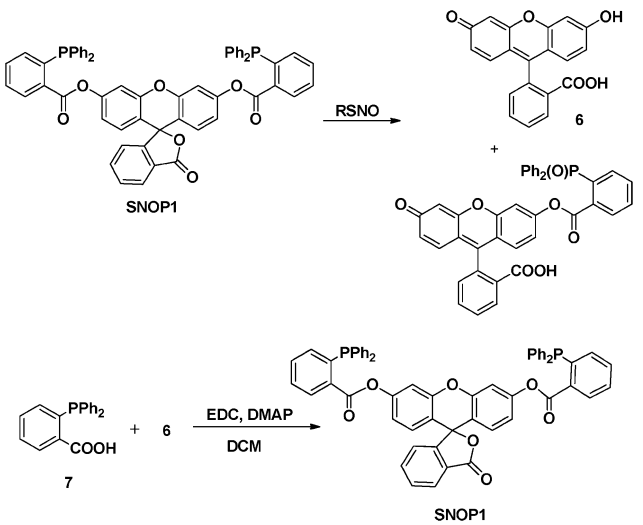


Fig. 1 Time-dependent fluorescence enhancement (F/F_0) of **SNOP1** (10 μM) to **8** (50 μM) (A) and **GSNO** (50 μM) (B). The reactions were carried out at 37 °C in Tris-HCl buffer (50 mM, pH = 7.4) with 1% DMSO ($\lambda_{ex/em}$ = 490/518 nm).

intensity was recorded after 45 min. We found that the intensities increased almost linearly in the range of 0–30 μM for GSNO (Fig. 2). The detection limits[8] were calculated to be 90 nM for GSNO, suggesting that the probe is reasonably sensitive. We also studied the effects of pH values on the reaction. The probe worked nicely at neutral pH values (6.5–7.5) (Fig. S2, ESI†). These results suggest that the probe may be suitable for detecting SNO in biological samples.

We next examined the selectivity of the probe for other reactive sulfur species and some common oxidative species (Fig. 3).



Scheme 2 The structure and preparation of **SNOP1**.

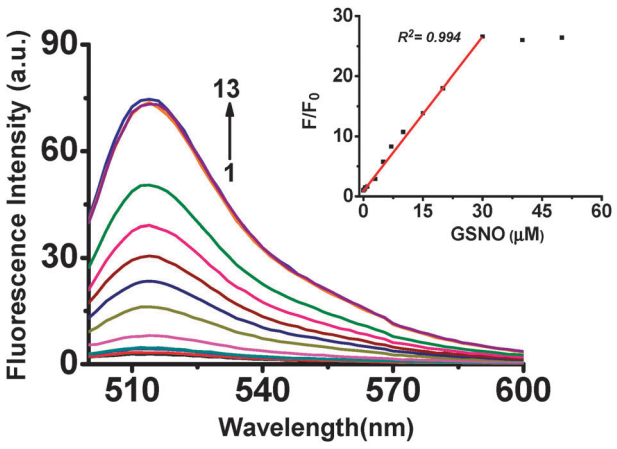


Fig. 2 Fluorescence emission spectra of **SNOP1** (10 μM) with varied concentrations of GSNO (0, 0.3, 0.5, 1, 3, 5, 7, 10, 15, 20, 30, 40, 50 μM for curves 1–13, respectively). The reactions were carried out for 45 min at 37 °C in Tris-HCl buffer (50 mM, pH = 7.4) with 1% DMSO ($\lambda_{ex/em}$ = 490/518 nm).

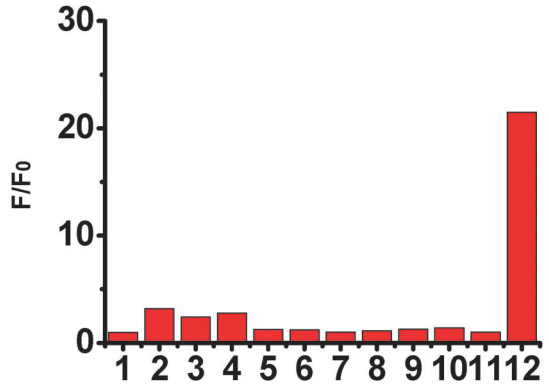


Fig. 3 Fluorescence enhancement (F/F_0) of **SNOP1** (10 μM) to various RSS or common oxides. The reactions were carried out for 45 min in Tris-HCl buffer (50 mM, pH = 7.4) with 1% DMSO at 37 °C. (1) 10 μM **SNOP1** only; (2) 1 mM GSH; (3) 100 μM Cys; (4) 100 μM Hcy; (5) 100 μM GSSG; (6) 100 μM Na_2S; (7) 100 μM $Na_2S_2O_3$; (8) 100 μM Na_2SO_3; (9) 100 μM H_2O_2; (10) 100 μM NaClO; (11) 100 μM $NaNO_2$; (12) 50 μM **GSNO**.

Sulfur containing compounds including glutathione (GSH), cysteine (Cys), homocysteine (Hcy), oxidized glutathione (GSSG), hydrogen sulfide (H_2S), thiosulfates ($S_2O_3^{2-}$), and sulfites (SO_3^{2-}) were tested and did not give any significant fluorescence enhancement. Oxidants such as hydrogen peroxide (H_2O_2), hypochlorites (ClO^-), and nitrites (NO_2^-) were found to be non-reactive either. In comparison, GSNO showed very significant fluorescent signals. To further investigate the specificity of the probe for SNO, two tertiary SNO substrates based on S-nitrosopenicillamine were also tested. These un-natural SNO showed excellent fluorescence responses to the probe (Fig. S3, ESI†). These results demonstrated good selectivity of the probe **SNOP1** for SNO.

It should be noted that the reductive ligation was also used by King *et al.* in trapping nitroxyl (HNO), although the phosphine-derived product is slightly different.[9] Recently Nakagawa and coworkers have reported a structurally similar probe which showed excellent activity for HNO, but with low response to SNO.[10] To test the activity of **SNOP1** toward HNO, we treated the probe (10 μM) with a solution of Angeli's salt (10 μM), a well known HNO donor. The fluorescence signals were recorded in 5 min in Tris-HCl buffer (50 mM, pH = 7.4, with 1% DMSO) at 37 °C. Indeed the probe showed a dramatic fluorescence increase (about 235 fold, Fig. 4). This reactivity with HNO may be a problem for using **SNOP1** for selective detection of SNO. However, it is known that HNO is a highly reactive species which quickly undergoes dimerization to hyponitrous acid followed by dehydration to give nitrous oxide (N_2O) and water.[11] We suspected that if HNO co-exists with SNO in the solution, HNO will decompose quickly and not be detected. Therefore we studied the time-dependent stability of HNO using **SNOP1**. In this study, a solution of Angeli's salt (10 μM) was prepared. At different time intervals (0–60 min) the probe was used to monitor HNO. As shown in Fig. 4, the fluorescence intensities decreased quickly. After about 10 min, there was almost no fluorescence detected, suggesting a complete decomposition of HNO. These results suggest that although **SNOP1** gives a strong fluorescence response to HNO, its signal can be observed only when it is continuously formed and is present at the time of the measurement. Because of this property, we believe **SNOP1** will be useful for selective detection of SNO, at least in some situations and might be able to distinguish SNO from HNO based on its time-dependent response. In biological systems, RSNOs exist mainly as GSNO or S-nitrosated proteins. These RSNOs are relatively stable. In contrast, HNO decomposes quickly if its generation is terminated (the biosynthesis of HNO is still unclear). We performed some model experiments to prove this hypothesis (Fig. 5). Three solutions (Angeli's salt only, GSNO only, Angeli's salt + GSNO) were prepared separately in Tris-HCl buffer (50 mM, pH = 7.4, with 1% DMSO). The solutions were incubated for 20 min at 37 °C and then tested with **SNOP1**. The solution with only Angeli's salt gave negligible fluorescence signals while the solutions with only GSNO gave strong fluorescence, at same levels of directly treating GSNO with the probe. The solutions of Angeli's salt and GSNO together produced fluorescence similar to GSNO only. These results further suggested that at the time of measurement HNO was completely gone and the fluorescence could be attributed to GSNO. Overall under these conditions HNO had no influence on the fluorescence measurement of SNO.

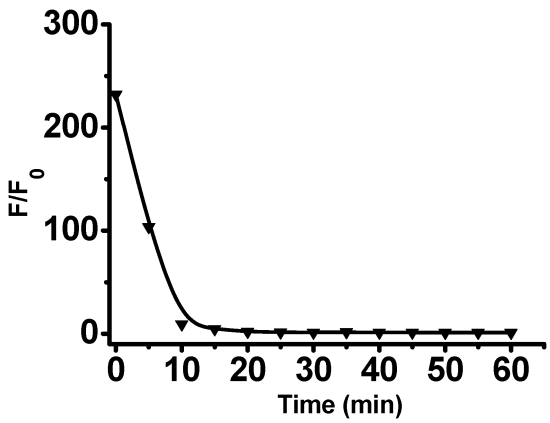


Fig. 4 Time-dependent fluorescence changes of HNO in Tris-HCl buffer (50 mM, pH = 7.4) with 1% DMSO. Angeli's salt (10 μM) was used as HNO equivalent. The solutions of Angeli's salt (10 μM) were kept at 37 °C for 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60 min, and then monitored using **SNOP1** (10 μM), respectively.

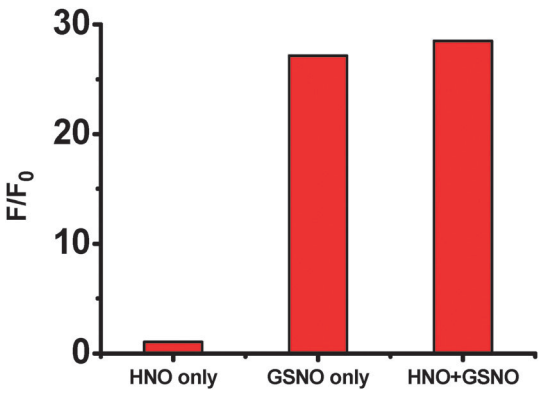


Fig. 5 Fluorescence enhancements of **SNOP1** (10 μM) to HNO only (10 μM AS), GSNO (50 μM) only, and HNO (10 μM AS) + GSNO (50 μM), respectively.

To ascertain the practical applicability of **SNOP1** for biological samples, the detection of GSNO in diluted deproteinized bovine plasma[12] was performed successfully (Fig. S4, ESI†). Upon gradual addition of GSNO (1 to 50 μM), we observed that a remarkable emission increase appeared at 518 nm in diluted deproteinized bovine plasma. An excellent linear correlation between the added GSNO concentrations and the increased fluorescence emission at 518 nm can still be obtained. These results indicated that **SNOP1** was suitable for sensitive analysis of GSNO in biological samples.

In summary, we report in this study a new fluorescent probe for the detection of SNO compounds. The design was based on SNO-mediated reductive ligation using triaryl-phosphine substrates. This probe showed good sensitivity and selectivity for some representative SNO substrates, including the endogenous GSNO.

Financial support from the NIH (R01GM088226) and NSF CAREER to M.X., NIH (R01HL62198) to S.B.K, the National Science Foundation of China (No. 21375113) and the Program for New Century Excellent Talents in University (NCET-11-0950) to Y.Y., and CSC for Graduate Student Overseas Study Program (grant number 201307040059) is greatly acknowledged.

References

1 (*a*) D. T. Hess, A. Matsumoto, R. Nudelman and J. S. Stamler, *Nat. Cell Biol.*, 2001, **3**, E46; (*b*) J. B. Mannick, A. Hausladen, L. Liu, D. T. Hess, M. Zeng, Q. X. Miao, L. S. Kane, A. J. Gow and J. S. Stamler, *Science*, 1999, **284**, 651; (*c*) M. W. Foster, T. J. McMahon and J. S. Stamler, *Trends Mol. Med.*, 2003, **9**, 160; (*d*) J. S. Stamler, S. Lamas and F. C. Fang, *Cell*, 2001, **106**, 675.

2 (*a*) J. Barrett, L. J. Fitygibbones, J. Glauser, R. H. Still and P. N. Young, *Nature*, 1966, **211**, 848; (*b*) P. D. Josephy, D. Rehorek and E. D. Janzen, *Tetrahedron Lett.*, 1984, **25**, 1685; (*c*) R. J. Singh, N. Hogg, J. Joseph and B. Kalyanaraman, *J. Biol. Chem.*, 1996, **271**, 18596.

3 For recent reviews on RSNO detection, see: (*a*) J. S. Stamler and M. Feelisch, *Methods in Nitric Oxide Res.*, 1996, p. 521; (*b*) A. Gow, A. Doctor, J. Mannick and B. Gaston, *J. Chromatogr., B*, 2007, **851**, 140; (*c*) N. J. Kettenhofen, K. A. Broniowska, A. Keszler, Y. Zhang and N. Hogg, *J. Chromatogr., B*, 2007, **851**, 152; (*d*) P. H. MacArthur, S. Shiva and M. T. Galdwin, *J. Chromatogr., B*, 2007, **851**, 93; (*e*) L. A. Palmer and B. Gaston, *Methods Enzymol.*, 2008, **440**, 157.

4 (*a*) H. Wang and M. Xian, *Angew. Chem., Int. Ed.*, 2008, **47**, 6598; (*b*) J. M. Zhang, H. Wang and M. Xian, *Org. Lett.*, 2009, **11**, 477; (*c*) H. Wang, J. M. Zhang and M. Xian, *J. Am. Chem. Soc.*, 2009, **131**, 13238; (*d*) J. M. Zhang, H. Wang and M. Xian, *J. Am. Chem. Soc.*, 2009, **131**, 3854; (*e*) D. H. Zhang, N. O. Devarie-Baez, J. Pan, H. Wang and M. Xian, *Org. Lett.*, 2010, **12**, 5674; (*f*) H. Wang and M. Xian, *Curr. Opin. Chem. Biol.*, 2011, **15**, 32.

5 J. Pan, J. A. Downing, J. L. McHale and M. Xian, *Mol. BioSyst.*, 2009, **5**, 918.

6 (*a*) X. Li, X. Gao, W. Shi and H. Ma, *Chem. Rev.*, 2014, **114**, 590; (*b*) M. M. Pires and J. Chmielewski, *Org. Lett.*, 2008, **10**, 837; (*c*) B. Tang, Y. Xing, X. Wang, Z. Chen, L. Tong and K. Xu, *Chem. Commun.*, 2009, 5293; (*d*) C. Liu, J. Pan, S. Li, Y. Zhao, L. Y. Wu, C. E. Berkman, A. R. Whorton and M. Xian, *Angew. Chem., Int. Ed.*, 2011, **50**, 10327; (*e*) W. Chen, C. R. Liu, B. Peng, Y. Zhao, A. Pacheco and M. Xian, *Chem. Sci.*, 2013, **4**, 2892; (*f*) C. Liu, B. Peng, S. Li, C. M. Park, A. R. Whorton and M. Xian, *Org. Lett.*, 2012, **14**, 2184.

7 B. Peng, W. Chen, C. Liu, E. W. Rosser, A. Pacheco, Y. Zhao, H. C. Aguilar and M. Xian, *Chem.–Eur. J.*, 2014, **20**, 1010.

8 (*a*) W. Chen, Z. Li, W. Shi and H. Ma, *Chem. Commun.*, 2012, **48**, 2809; (*b*) Z. Li, X. Li, X. Gao, Y. Zhang, W. Shi and H. Ma, *Anal. Chem.*, 2013, **85**, 3926.

9 (*a*) J. A. Reisz, E. B. Klorig, M. W. Wright and S. B. King, *Org. Lett.*, 2009, **11**, 2719; (*b*) J. A. Reisz, C. N. Zink and S. B. King, *J. Am. Chem. Soc.*, 2011, **133**, 11675.

10 K. Kawai, N. Ieda, K. Aizawa, T. Suzuki, N. Miyata and H. Nakagawa, *J. Am. Chem. Soc.*, 2013, **135**, 12690.

11 J. M. Fukuto, C. H. Switzer, K. M. Miranda and D. A. Wink, *Annu. Rev. Pharmacol. Toxicol.*, 2005, **45**, 335.

12 X. Yang, Y. Guo and M. Strongin, *Angew. Chem., Int. Ed.*, 2011, **50**, 10690.