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Synthesis and Evaluation of Phosphorodithioate-Based Hydrogen Sulfide Donors

Chung-Min Park[a], **Yu Zhao**[a], **Zhaohui Zhu**[b], **Armando Pacheco**[a], **Bo Peng**[a], **Nelmi O. Devarie-Baez**[a], **Powell Bagdon**[a], **Hui Zhang**[b], and **Ming Xian**[a]

Ming Xian: mxian@wsu.edu

[a]Department of Chemistry, Washington State University, Pullman, WA 99164, United States. Fax: +1-509-335-8867; Tel: +1-509-335-6073

[b]Department of Pharmaceutical Sciences, College of Pharmacy, Washington State University, Pullman, WA 99164

Abstract

A series of *O*-aryl- and alkyl-substituted phosphorodithioates were designed and synthesized as hydrogen sulfide (H_2S) donors. H_2S released capability of these compounds was evaluated by fluorescence methods. *O*-aryl substituted donors showed slow and sustained H_2S release while *O*-alkylated compounds showed very weak H_2S release capability. We also evaluated donors' protective effects against hydrogen peroxide (H_2O_2)-induced oxidative damage in myocytes and donors' toxicity toward B16BL6 mouse melanoma cells.

Hydrogen sulfide (H_2S) was known as a toxic pollutant for years. However, since 2000 this gaseous molecule has been classified as an important cell signaling molecule, much like nitric oxide (NO). Literature published in the past few years increasingly suggests that H_2S is a mediator of many physiological and/or pathological processes.[1–5] The production of H_2S in mammalian systems has been attributed to at least three endogenous enzymes:[6–9] cystathionine β-synthase, cystathionine γ-lyase, and 3-mercaptopyruvate sulfur-transferase. These enzymes use cysteine or cysteine derivatives as substrates and convert them into H_2S within different organs and tissues. In addition to these enzymatic pathways, there are also a range of comparably simple chemical events which may liberate H_2S from the intracellular pool of 'labile' sulfur, for instance from the 'sulfane sulfur' pool (compounds containing sulfur atoms bound only to other sulfur atoms).[10] While the exact mechanisms of action of H_2S are still under investigation, some chemical and biochemical catabolic reactions of H_2S have been disclosed that may be responsible for its biological functions. For example, H_2S reacts readily with methemoglobin to form sulfhemoglobin, which might act as a metabolic sink for H_2S. H_2S is a powerful reducing agent and is likely to be consumed by endogenous oxidant species, such as peroxynitrite, superoxide, and hydrogen peroxide.[11–13] H_2S can also promote protein *S*-sulfhydration providing a possible mechanism whereby H_2S alters the functions of a wide range of proteins and modulates signaling.[14–16] It is likely that many

Correspondence to: Ming Xian, mxian@wsu.edu.

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more important reactions of H_2S are to be discovered. Nevertheless, the production of endogenous H_2S and the exogenous administration of H_2S have been demonstrated to exert protective effects in many pathologies.[1–5] For example, H_2S has been shown to relax vascular smooth muscle, induce vasodilation of isolated blood vessels, and reduce blood pressure. H_2S can also inhibit leukocyte adherence in mesenteric microcirculation during vascular inflammation in rats, suggesting H_2S is a potent anti-inflammatory molecule. Additionally, it has become evident that H_2S is a potent antioxidant and, under chronic conditions, can up-regulate antioxidant defense. These results strongly suggest that modulation of H_2S levels could have potential therapeutic values.

In H_2S research, researchers typically use sulfide salts (NaHS or Na_2S) as H_2S source. The pK_a values for the first and second dissociation steps of H_2S are 7.0 and >12.0, respectively.[17–18] In aqueous state under the physiological pH of 7.4, the ratio of HS^-/H_2S is ~3:1 as long as sulfide salt solution is prepared. Sulfide salts are therefore considered as short-lasting H_2S donors as they release H_2S quickly. The rapid release of H_2S may cause acute changes in blood pressure and may exert toxic actions. In addition, sulfide concentrations in aqueous solution can rapidly decrease due to volatilization,[19] thus significantly limiting the utility of these chemical precursors.

Due to these considerations, researchers have started to use synthetic H_2S-releasing agents (i.e., H_2S donors) to explore the biological functions of H_2S.[20–22] Currently there are six types of H_2S donors known in literature (Scheme 1): 1) a Lawesson's reagent derivative named GYY4137;[23] 2) garlic-derived organic polysulfides such as diallyl trisulfide (DATS),[24] 3) the dithiolthione moiety[25] 4) a series of *N*-(benzoyl)-thiobenzamide derivatives as thiol-activated H_2S donors,[26] 5) *S*-acylated perthiol based donors,[27] 6) amino acid-based thioacids in the presence of bicarbonate buffers.[28] Among these donors, GYY4137 is probably the most well-known donor. GYY4137 has a phosphorodithioate core structure and H_2S release from this compound is due to hydrolysis. It is considered as a slow-release donor. GYY4137 has shown some H_2S-relevant biological activities. For example, it relaxes arotas, vasodilates the preconstricted kidney, and exhibits antihypertensive activity in rats. It can also stimulate heart contraction by interaction with endogenous NO generation.[29] Although well-applied in biological studies, only one donor (i.e. GYY4137) with fixed H_2S release capability may not fulfill the requirements of different biological applications. We envisioned that the phosphorodithioate template have rich chemistry related to H_2S to be explored. Structure modifications on phosphorodithioate may lead to H_2S release capability change and in turn lead to their biological activity changes. Herein we report the synthesis and evaluation of a series of *O*-substituted phosphorodithioate-based H_2S donors.

In the structure of GYY4137, there is a phenyl-phosphorus linkage via a C-P bond. We decided to replace the C-P bond with *O*-substitution and to explore H_2S production from the resulted analogs. To this end, a four-step synthesis was developed (Scheme 2). Freshly distilled trichlorophosphine **1** was treated with 1, 2-dithioethane in 3:1 ratio in the absence of solvent and base. The desired product 2-chloro-1,3,2-dithiaphospholane **2** was obtained in almost quantitative yield by simply removing excess **1** upon distillation. Without further purification, compound **2** was treated with aniline followed by sulfurization with elemental

sulfur to provide the key intermediate: 2-amine-1,3,2-dithiaphospholan-2-sulfide **4**. Finally compound **4** was reacted with different phenols and alcohols in the presence of 1,8-diazobicyclo[5.4.0]undec-7-ene (DBU) to afford a series of phosphorodithioate analogs **5** as DBU salts. In all, five *O*-substituted compounds **5a**–**5e** were prepared and their structures are shown in Scheme 2.

With these compounds in hand, we turned to test their H_2S release capabilities. In comparison GYY4137 was also tested. Previously the measurement of H_2S release from GYY4137 was mainly done by using the standard methylene blue method.[23,30] However strong acidic conditions were involved in the methylene blue method. It is known that the hydrolysis of phosphorodithioates is affected by pH changes and hydrolysis under highly acidic conditions is much faster than under neutral pH.[31] Therefore methylene blue method is not appropriate for evaluating phosphorodithioate-based donors. Recently a number of fluorescent probes have been developed for H_2S detection[32–34] and these probes are used under neutral pH. We envisioned that such probes could be useful for evaluating H_2S donors. Dansyl azide (DNS-Az), a fluorescent probe developed by the Wang group,[35] was selected in this study due to its fast reaction rate and easy of synthesis. In our experiments, 100 μM of each donor was dissolved in a mixed MeCN/phosphate buffer solution (1:1 v/v, pH 7.4) containing DNS-Az (200 μM). The changes of fluorescence emission spectra at 535 nm (λ_{ex} = 340 nm) were then monitored for three hours at room temperature. Finally the fluorescence signals were converted to H_2S concentrations based on a reference curve obtained with a series of Na_2S standard solutions. Our results are summarized in Figure 1. GYY4137 was first tested as a reference compound. In consistent with previous reports, this donor showed slow and low H_2S release. After 3 h GYY4137 produced approximately 600 nM H_2S, which counted for ~0.6% of initial material. Using the same protocol the newly synthesized donors **5a**–**5e** were also tested. Three compounds **5a**–**5c** showed similar activities as GYY4137. They released H_2S in a slow and sustain fashion and the amounts of H_2S production were also similar to that of GYY4137. Compounds **5d** and **5e** only released trace amount of H_2S (data not shown). Presumably the *O*-alkyl substitutions led to increased stability of phosphorodithioates and therefore decrease the tendency of the hydrolysis to generate H_2S.

Having demonstrated H_2S release from phosphorodithioate-based donors in buffers, we wondered whether they could produce H_2S in cells. To address this question, we conducted cell image experiments using H9c2 myocytes. One example using donor **5a** is shown in Figure 2. H9c2 cells were incubated with donor **5a** under different concentrations (0, 100, and 200 μM) for 24 h. Then a H_2S-specific fluorescent probe, WSP-1,[36] was applied into the cells to monitor the production of H_2S. As expected, donor-treated cells (Figure 2B and 2C) shown much enhanced fluorescent signals compared to vehicle treated cells (Figure 2A). These results suggest that phosphorodithioate-based donors indeed can sustainably release H_2S in cells.

In our imaging experiments, we did not observe shape changes of the cells after the treatment with donors, which indicates the donors may not be toxic under the doses we used. To further understand phosphorodithioate-based donors' cytotoxicity, we tested H9c2 cell viability upon treating with donors **5a**–**5c**. NaHS and GYY4137 were also tested as controls.

The cells were treated with 50, 100, and 200 μM donors for 24 h and then cell viability was determined using cell counter kit (CCK)-8 assay. As shown in Figure 3, 5a, 5b and NaHS did not show significant toxicity to H9c2 cells under these doses. **5c** exhibited some toxicity when concentration increased. Interestingly GYY4137 was safe at 50 and 100 μM, but showed strongest toxicity at 200 μM.

It is known that H_2S exhibits dose-dependent improvement of cell viability following oxidative injury.[37] We hypothesized that phosphorodithioate-based H_2S donors could exert similar protective effects. **5a** and **5b** were then selected to explore their protective effects against H_2O_2-induced oxidative damage in H9c2 cells. Again GYY4137 and NaHS were tested as controls. Three concentrations (50, 100, and 200 μM) for each donors were used. For GYY4137 only two concentrations (50 and 100 μM) were tested because of its strong toxicity at 200 μM. In these experiments, cells were incubated with each donor for 24 h and then challenged with 150 μM H_2O_2 and cultured for 5 h. After that cell viability was determined by CCK-8 assay. As shown in Figure 4, cell viability dropped to about 65% if H_2S donors were absent. In the presence of donors, however, cell viability increased significantly. These results suggest that H_2S donors indeed have some protective effects against oxidative injury. It should be noted that we did not observe significant protective effects when donors were used at lower concentrations (Figure S4 in supporting information).

In 2011 Moore and co-workers showed that the slow-releasing donor GYY4137 exhibited some interesting anti-cancer activities in cells and mice xenograft.[30] We wondered if our donors would show similar activity. Therefore we tested the effects of **5a**, **5b**, and GYY4137 on cell growth and viability of mouse melanoma B16BL6 cell line. Two different concentrations (100 μM and 200 μM) were applied. Cell growth inhibitory effects were monitored for a 4-day culture period. As shown in Figure 5, 5a showed moderate toxicity at both concentrations while **5b** showed relatively strong toxicity at 200 μM. Interestingly, GYY4137 showed the strongest activity to this cell line, which may suggest the C-P linkage is crucial for potency. However, at this moment it is still uncertain if these donors' inhibitory effects on B16BL6 cells are associated with their H_2S production. Since H_2S generation is a very slow process, the inhibition may come from donor compounds themselves. Nevertheless more studies are needed to elucidate the detail mechanism.

In conclusion, here we report the design and synthesis of a series of *O*-arylated and alkylated phosphorodithioate-based H_2S donors. Their H_2S releasing capabilities were evaluated by fluorescence methods. *N*,*O*-Diarylated donors showed slow and sustainable H_2S generation which is similar to the well-known donor GYY4137, whereas *O*-alkylated donors exhibited very weak H_2S production. In addition, we explored some H_2S relevant activities of the donors. Compounds **5a** and **5b** exhibited notable protective effects against H_2O_2-induced oxidative damage in H9c2 myocytes. These donors also showed inhibitory effects against B16BL6 cancer cell proliferation but detailed mechanisms are to be explored. We are now actively studying other phosphorodithioates-based donors and their applications in H_2S research.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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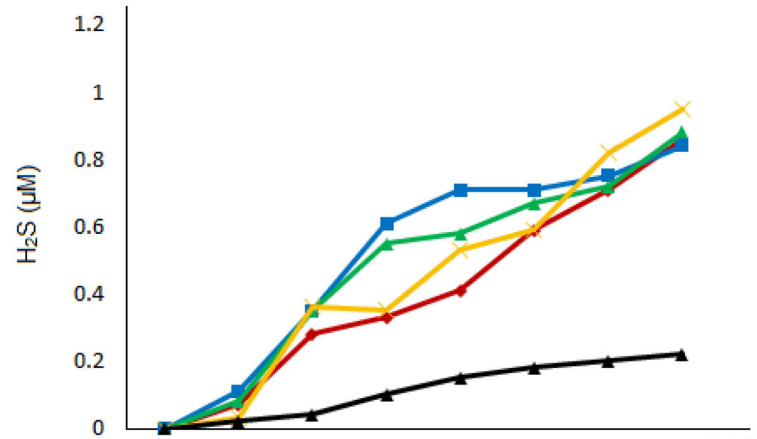
Notes and References

1. Li L, Rose P, Moore PK. Annu Rev Pharmacol Toxicol. 2011; 51:169–187. [PubMed: 21210746]
2. Wang R. Physiol Rev. 2012; 92:791–896. [PubMed: 22535897]
3. Olson KR, Donald JA, Dombkowski RA, Perry SF. Respir Physiol Neurobiol. 2012; 184:117–129. [PubMed: 22546339]
4. Fukuto JM, Carrington SJ, Tantillo DJ, Harrison JG, Ignarro LJ, Freeman BA, Chen A, Wink DA. Chem Res Toxicol. 2012; 25:769–793. [PubMed: 22263838]
5. Lavu M, Bhushan S, Lefer DJ. Clin Sci. 2011; 120:219–229. [PubMed: 21126235]
6. Kimura H. Amino Acids. 2011; 41:113–121. [PubMed: 20191298]
7. Kimura H, Shibuya N, Kimura Y. Antioxid Redox Signal. 2012; 17:45–57. [PubMed: 22229673]
8. Olson KR. Antioxid Redox Signal. 2012; 17:32–44. [PubMed: 22074253]
9. Predmore BL, Lefer DJ, Gojon G. Antioxid Redox Signal. 2012; 17:119–140. [PubMed: 22432697]
10. Jacob C, Anwar A, Burkholz T. Planta Med. 2008; 74:1580–1592. [PubMed: 18937169]
11. Whiteman M, Armstrong JS, Chu SH, Jia-Ling S, Wong BS, Cheung NS, Halliwell B, Moore PK. J Neurochem. 2004; 90:765–768. [PubMed: 15255956]
12. Chang L, Geng B, Yu F, Zhao J, Jiang H, Du J, Tang C. Amino Acids. 2008; 34:573–585. [PubMed: 18071843]
13. Geng B, Yang J, Qi Y, Zhao J, Pang Y, Du J, Tang C. Biochem Biophys Res Commun. 2004; 313:362–368. [PubMed: 14684169]
14. Mustafa AK, Gadalla MM, Sen N, Kim S, Mu W, Gazi SK, Barrow RK, Yang G, Wang R, Snyder SH. Sci Signal. 2009; 2:ra72. [PubMed: 19903941]
15. Sen N, Paul BD, Gadalla MM, Mustafa AK, Sen T, Xu R, Kim S, Snyder SH. Mol Cell. 2012; 45:13–24. [PubMed: 22244329]
16. Krishnan N, Fu C, Pappin DJ, Tonks NK. Sci Signal. 2011; 4:ra86. [PubMed: 22169477]
17. Tangerman A. J Chromatogr B: Anal Technol Biomed Life Sci. 2009; 877:3366–3377.
18. Kabil O, Banerjee R. J Biol Chem. 2010; 285:21903–21907. [PubMed: 20448039]
19. DeLeon ER, Stoy GF, Olson KR. Anal Biochem. 2012; 421:203–207. [PubMed: 22056407]
20. Hughes MN, Centelles MN, Moore KP. Free Radical Biol Med. 2009; 47:1346–1353. [PubMed: 19770036]
21. Kashfi K, Olson KR. Biochem Pharmacol. 2013; 85:689–703. [PubMed: 23103569]
22. Caliendo G, Cirino G, Santagada V, Wallace JL. J Med Chem. 2010; 53:6275–6286. [PubMed: 20462257]
23. Li L, Whiteman M, Guan YY, Neo KL, Cheng Y, Lee SW, Zhao Y, Baskar R, Tan CH, Moore PK. Circulation. 2008; 117:2351–2360. [PubMed: 18443240]
24. Benavides GA, Squadrito GL, Mills RW, Patel HD, Isbell TS, Patel RP, Darley-Usmar VM, Doeller JE, Kraus DW. Proc Natl Acad Sci U S A. 2007; 104:17977–17982. [PubMed: 17951430]
25. Wallace JL, Caliendo G, Santagada V, Cirino G, Fiorucci S. Gastroenterology. 2007; 132:261–271. [PubMed: 17241876]
26. Zhao Y, Wang H, Xian M. J Am Chem Soc. 2011; 133:15–17. [PubMed: 21142018]
27. Zhao Y, Bhushan S, Yang C, Otsuka H, Stein JD, Pacheco A, Peng B, Devarie-Baez NO, Aguilar HC, Lefer DJ, Xian M. ACS Chem Biol. 2013; 8:1283–1290. [PubMed: 23547844]

28. Zhou Z, von Wantoch Rekowski M, Coletta C, Szabo C, Bucci M, Cirino G, Topouzis S, Papapetropoulos A, Giannis A. Bioorg Med Chem. 2012; 20:2675–2678. [PubMed: 22436388]
29. Yong QC, Cheong JL, Hua F, Deng LW, Khoo YM, Lee HS, Perry A, Wood M, Whiteman M, Bian JS. Antioxid Redox Signal. 2011; 14:2081–2091. [PubMed: 21194352]
30. Lee ZW, Zhou J, Chen CS, Zhao Y, Tan CH, Li L, Moore PK, Deng LW. PLoS One. 2011; 6:e21077. [PubMed: 21701688]
31. Bode H, Arnswald W. Z Anal Chem. 1962; 185:99.
32. Chan J, Dodani SC, Chang CJ. Nat Chem. 2012; 4:973–984. [PubMed: 23174976]
33. Lin VS, Chang CJ. Curr Opin Chem Biol. 2012; 16:595–601. [PubMed: 22921406]
34. Xuan W, Sheng C, Cao Y, He W, Wang W. Angew Chem Int Ed. 2012; 51:2282–2284.
35. Peng H, Cheng Y, Dai C, King AL, Predmore BL, Lefer DJ, Wang B. Angew Chem Int Ed. 2011; 50:9672–9675.
36. Liu C, Pan J, Li S, Zhao Y, Wu LY, Berkman CE, Whorton AR, Xian M. Angew Chem Int Ed. 2011; 50:10327–10329. The probe reported in this paper is now commercially available as ‘WSP-1’ by Cayman Chemical Co.
37. Szabo G, Veres G, Radovits T, Gero D, Modis K, Miesel-Groschel C, Horkay F, Karck M, Szabo C. Nitric oxide. 2011; 25:201–210. [PubMed: 21094267]



	0	5	30	60	90	120	150	180 (min)
GYY4137	0	0.07	0.28	0.33	0.41	0.59	0.71	0.86 (μM)
5a	0	0.11	0.35	0.61	0.71	0.71	0.75	0.84
5b	0	0.08	0.35	0.55	0.58	0.67	0.72	0.88
5c	0	0.03	0.36	0.35	0.53	0.59	0.82	0.95
control	0	0.02	0.04	0.1	0.15	0.18	0.2	0.22

Figure 1.

Time dependent H_2S releasing profiles of donors **5a**–**5c** and GYY4137. Donors **5d** and **5e** released trace amounts of H_2S (data not shown).

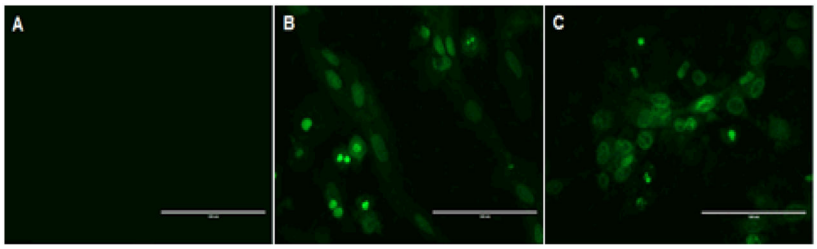


Figure 2.

H_2S production of donor **5a** in H9c2 cells. Cells were incubated with vehicle (A), 100 μM **5a** (B), and 200 μM **5a** (C) for 24 h. After removal of excess donor, 100 μM of a H_2S fluorescent probe (WSP-1) was added. Images were taken afterwards.

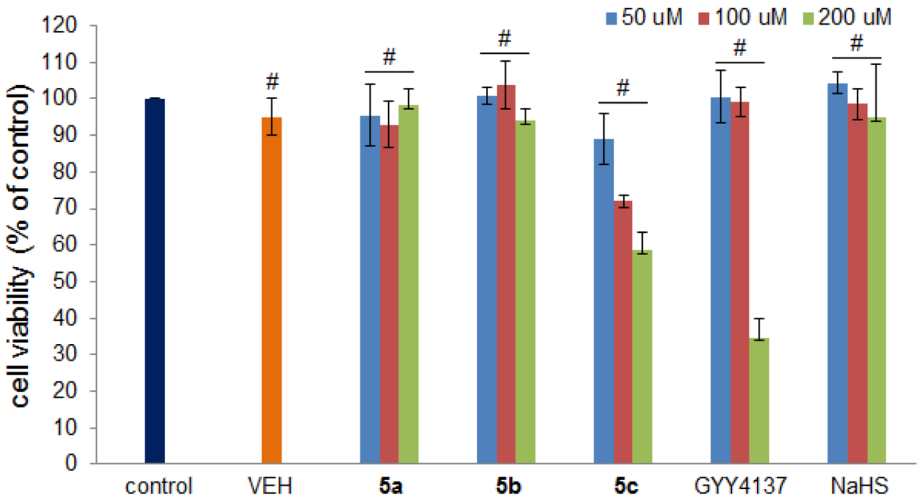


Figure 3.

Effects of H_2S donors on cell viability. H9c2 cells were treated with different concentrations of **5a**–**5c**, GYY4137, and NaHS for 24 h. CCK-8 assay was performed to measure cell viability. Data were shown as mean ± SD (n = 4). $^{\#}P < 0.01$ versus control group.

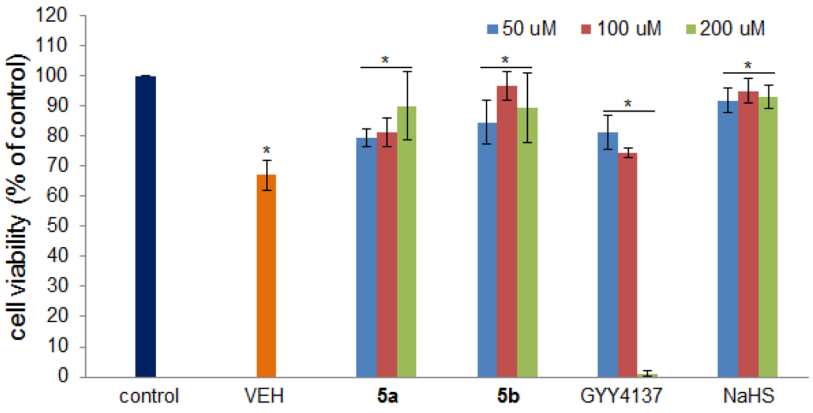


Figure 4.

H9c2 cells viability after oxidative injury by H_2O_2 in the presence of donors **5a**, **5b**, GYY4137, and NaHS. Data were shown as mean ± SD (n = 4). $^{*}P < 0.01$ versus control.

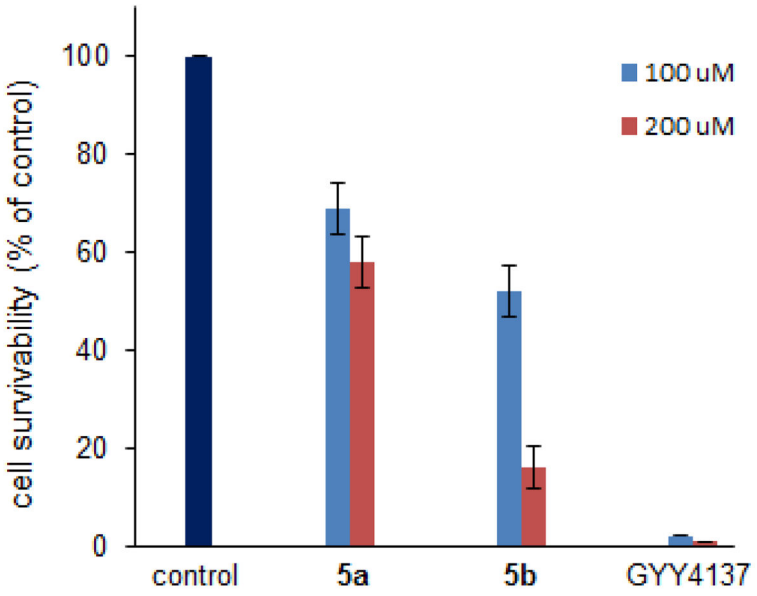
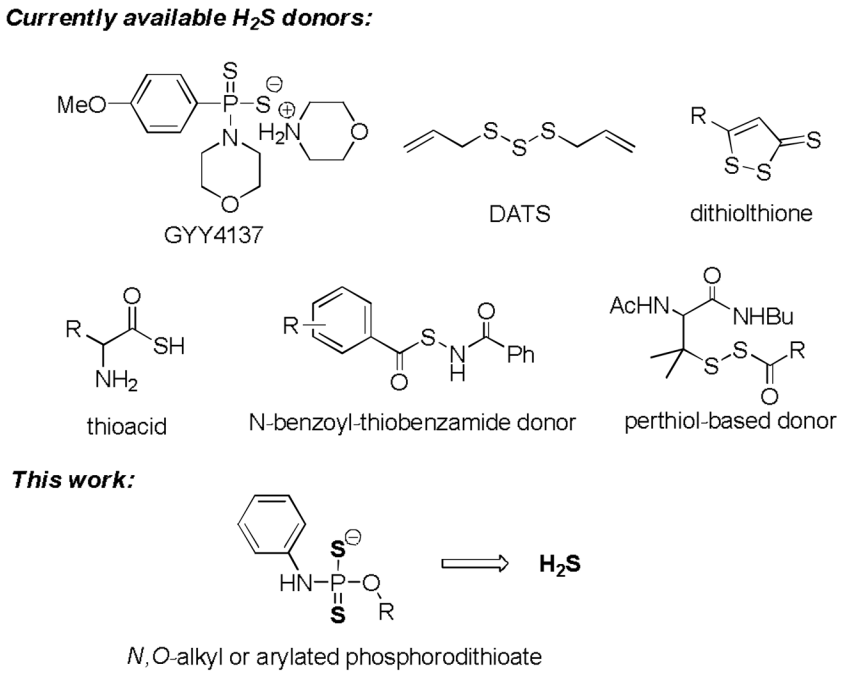
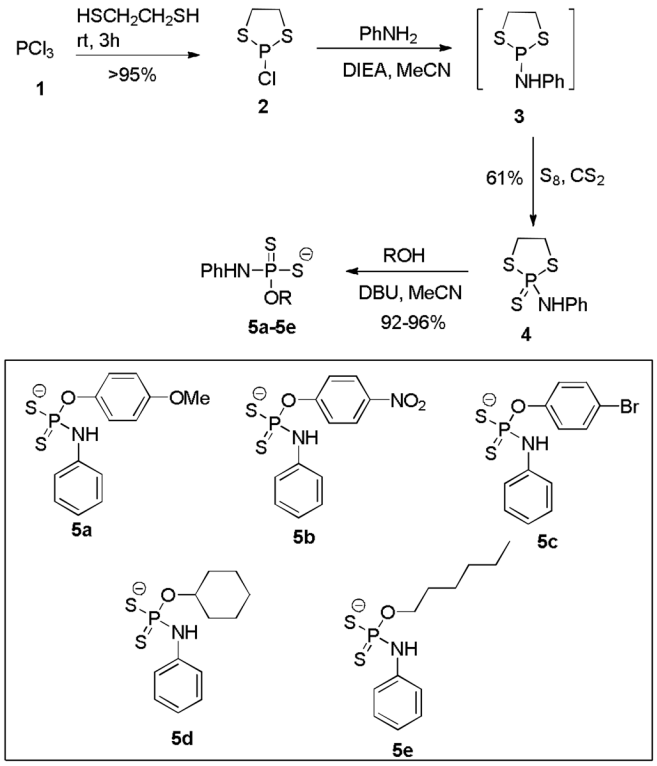


Figure 5.

Mouse melanoma B16BL6 cells were treated with indicated donors in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin, respectively. Cells were cultured for 4 days and analyzed by a cell viability analyzer.



Scheme 1.
Structures of H_2S donors.



Scheme 2.
Synthesis of phosphorodithioate-based H_2S donors.