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Ammonium tetrathiomolybdate as a water-soluble and slow-release hydrogen sulfide donor

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ABSTRACT

Ammonium tetrathiomolybdate (TTM) was found to be a slow hydrogen sulfide (H₂S) releasing agent. Its H₂S generation capability in aqueous solutions was confirmed by UV–vis and fluorescence assays. TTM also showed H₂S-like cytoprotective effects in hydrogen peroxide (H₂O₂)-induced oxidative damage in HaCaT cells.

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Hydrogen sulfide (H₂S) is newly recognized as a nitric oxide (NO)-like gaseous transmitter that plays regulatory roles in many physiological and pathological processes.^{1–5} Endogenous production of H₂S involves both enzymatic pathways (mediated by cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase (3-MST)) and non-enzymatic pathways (from the sulfane sulfur pools). Current knowledge strongly suggests that modulation of H₂S could have potential therapeutic values for certain disease states, including vasodilation, anti-inflammation, anti-oxidation, and down regulation of cellular metabolism under stress.^{1–5} Because of this, the search for compounds that can release H₂S and mimic the beneficiary activities of H₂S has become an attractive area in medicinal chemistry.^{6–10} So far, a number of H₂S releasing compounds (also known as H₂S donors), such as GYY4137, dithiothiones, *N*-mercapto-based donors, persulfide-based donors, gem-dithiol-based donors, etc., have been reported (Fig. 1).^{6–10} These compounds are normally small organic molecules which can be triggered by certain biologically relevant or compatible reactions to release H₂S. Some of them have shown promising bioactivities.^{6–10} On the other hand, inorganic molecule-based donors, especially metal complexes, which can slowly hydrolyze to release H₂S have not been reported. Inspired by the fact that sodium nitroprusside (SNP), with the formula Na₂[Fe(CN)₅NO], is a widely used NO donor,^{11–13} we

suspected that certain sulfide-containing inorganic compounds might be able to serve as interesting H₂S donors in aqueous solutions. Like SNP, the release of H₂S from inorganic molecule-based donors is likely to be the result of simple hydrolysis. As such, the donors would be suitable for many biological studies. Herein we wish to report the discovery of ammonium tetrathiomolybdate (TTM) as an effective inorganic H₂S donor.

TTM, with the formula (NH₄)₂MoS₄, is a commonly used building block in the chemistry of molybdenum.^{14,15} As an excellent copper chelator, TTM has been used therapeutically in the treatment of copper toxicosis, especially for Wilson's disease.¹⁶ It was previously noted that under strong acidic condition (5% H₂SO₄) H₂S could be generated from TTM.¹⁷ We first wondered if TTM could produce H₂S under mild and biologically friendly conditions,

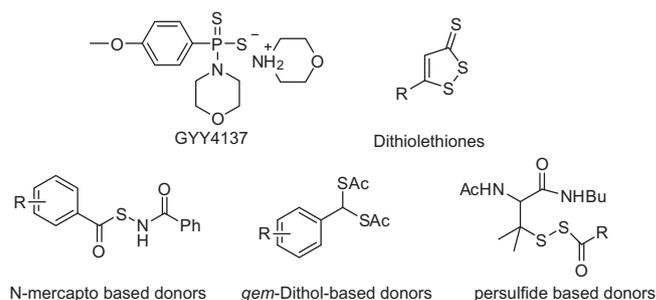


Figure 1. Representative H₂S donors.

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especially under physiological pH. To this end, we measured H₂S release from TTM in four different pH buffers (5, 6, 7.4, and 8). Normally the release of H₂S from the donors can be determined by the standard methylene blue (MB) method.¹⁸ However, a strong acidic condition was involved in this method. As it is known that acidic media would facilitate TTM hydrolysis, we envisioned that the standard MB method was not appropriate. In this study, a zinc-sulfide precipitation based MB method was used.¹⁹ This method should avoid the false positive signals caused by acid-promoted TTM hydrolysis. Briefly, the solutions of 500 μM TTM were freshly prepared in phosphate buffers under different pH. At different time intervals, aliquots were taken to Eppendorf vials containing a mixture of zinc acetate and NaOH solution. After 15 min, solid ZnS was formed and collected by centrifugation. ZnS was then treated by a MB cocktail. The resulted H₂S concentrations were obtained by UV–vis measurements and calculated based on a calibration curve. As shown in Figure 2, we observed immediate H₂S formation in all of these TTM solutions. The level of H₂S in pH 5 was significantly higher than the levels in other pH values. In all solutions we found H₂S concentrations were maintained in a stable level for a long time (up to 15 h). These results indicate TTM is a stable and slow release H₂S donor.

To further confirm the production of H₂S from TTM in buffers, a H₂S gas trapping experiment was designed. As shown in Figure 3, a solution of TTM was placed in a sealed glass vial. An Eppendorf vial containing a solution of WSP-5, a H₂S specific fluorescent probe,²⁰ was also placed in the vial to trap the evaporated H₂S from the TTM solution. After incubation at 37 °C for 3 h, the trapping solution was diluted and the fluorescence intensity (excitation at 502 nm, emission at 525 nm) was measured. Both the positive and negative controls (using the standard Na₂S solution or pure buffer) were also carried out using the same procedure for comparison. As shown in Figure 4, TTM and Na₂S led to significant fluorescence increases in the trapping solutions while the pure buffer did not cause any detectable fluorescence increase. The pH dependence of H₂S release from TTM was also noted as higher fluorescence was observed in pH 5 than in other pHs. These results further demonstrated H₂S generation from TTM in buffers.

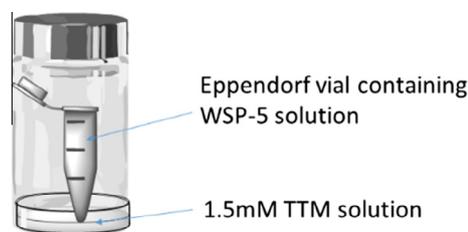


Figure 3. H₂S gas trapping set-up.

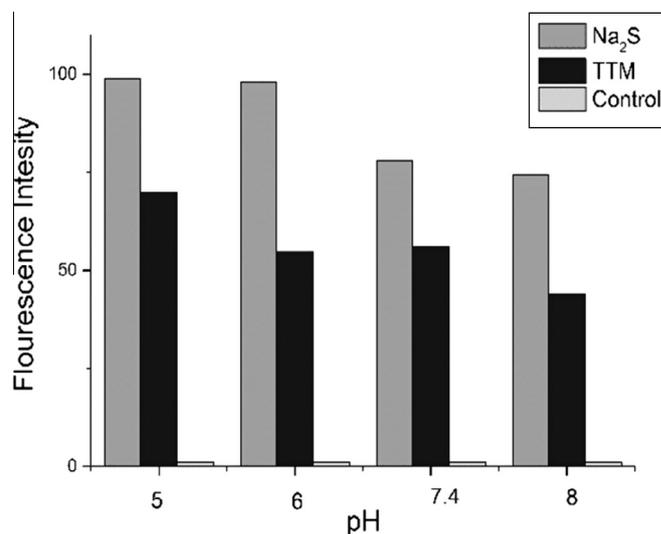


Figure 4. H₂S release of TTM detected by indirect fluorescent assay.

One of the most well-studied biological functions of H₂S is its cytoprotective effects under oxidative stress. Being a H₂S donor, TTM was expected to have similar effects. We then applied TTM

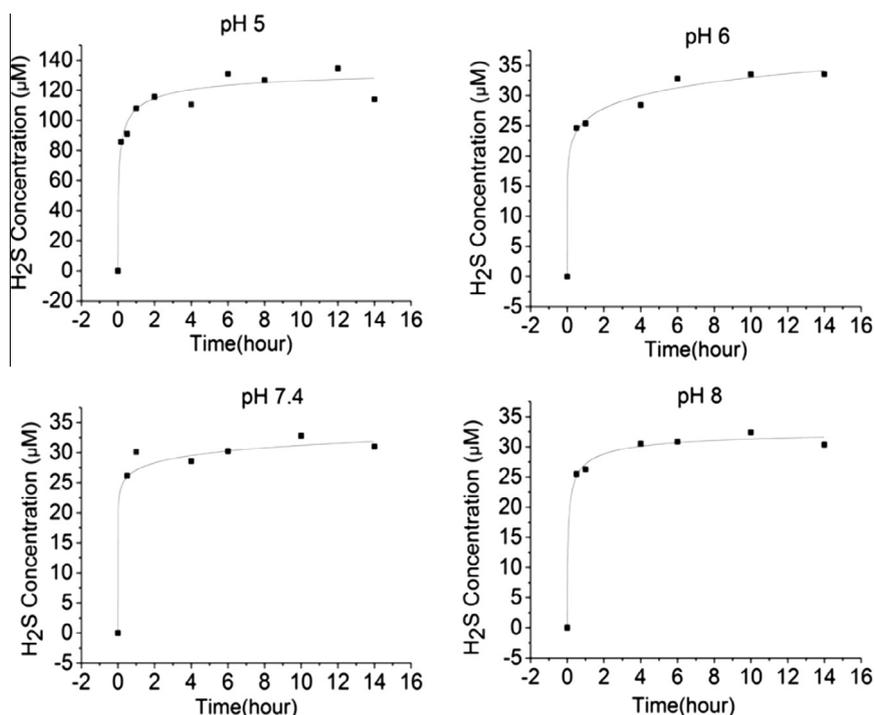


Figure 2. H₂S release from TTM under different pHs.

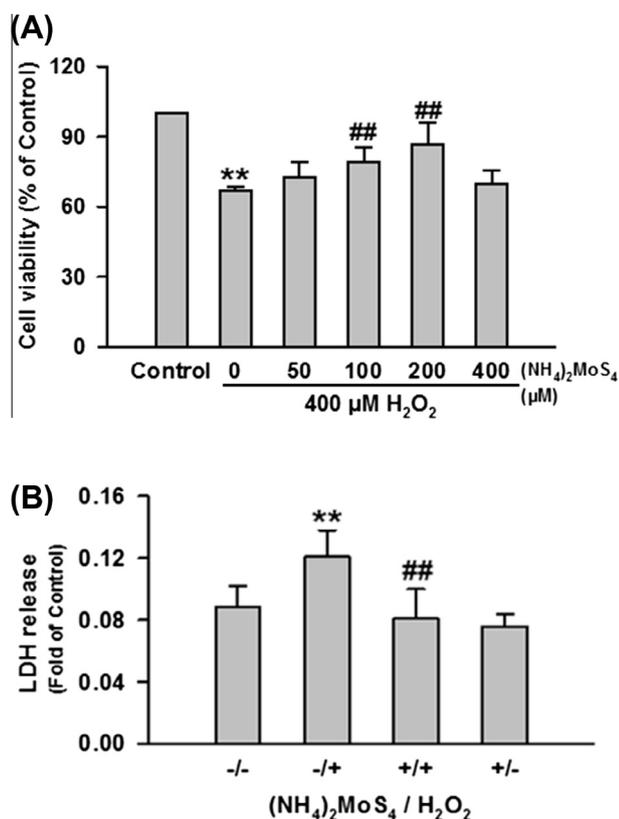


Figure 5. Effects of TTM on H₂O₂-induced cellular damage. (A) Cell viability assay under the treatment of 400 μM H₂O₂ for 5 h in the absence or presence of various concentrations of TTM for 1 h. (B) LDH release assay of cells treated with 400 μM H₂O₂ in the absence or presence of 200 μM TTM. Data were shown as the mean ± SE. ***P* < 0.01 versus Control group, ##*P* < 0.01 versus H₂O₂ alone group.

in a cellular model of oxidative damage to study its cytoprotective ability, using protocols established in our previous works.^{21,22} As shown in Figure 5A, hydrogen peroxide (H₂O₂) alone caused the decrease of cell (HaCaT) viability due to oxidative damage. When the cells were pre-treated with TTM (50, 100, 200 μM), a dose-dependent protective effect was observed. At a higher concentration (400 μM), TTM had no protection, presumably because of the slight toxicity of TTM under high concentration. In addition, lactate dehydrogenase (LDH) release assay was also carried out to validate TTM's cytoprotective effects. As shown in Figure 5B, the exposure of cells to H₂O₂ (400 μM) remarkably enhanced LDH release. However, LDH release was significantly reduced when cells were pretreated with TTM (200 μM).

Mitochondria membrane potential (MMP) usually reflects whether mitochondria are healthy, which further indicates if cells are suffering from noxious damage. Rhodamine 123 (Rh123) staining followed by fluorescence photography can be used to observe MMP. Figure 6A shows that under normal conditions the cells had bright green fluorescence. When cells were treated with H₂O₂ (400 μM), a dramatic MMP loss was noted (Fig. 6B), evidenced by decreased fluorescence, suggesting that H₂O₂ damaged cells. However, preconditioning with 200 μM TTM greatly impeded MMP loss by preserving mitochondrial functions (Fig. 6C). TTM alone did not significantly alter MMP (Fig. 6D). These results confirmed that TTM could exhibit H₂S-like cellular protection against oxidative injury in cells.

In conclusion, our results demonstrate that ammonium tetrathiomolybdate (TTM) is an effective H₂S donor in aqueous buffers. TTM's release of H₂S is pH-dependent. Under neutral pH, the release is a slow but sustained process. TTM can exhibit H₂S-like

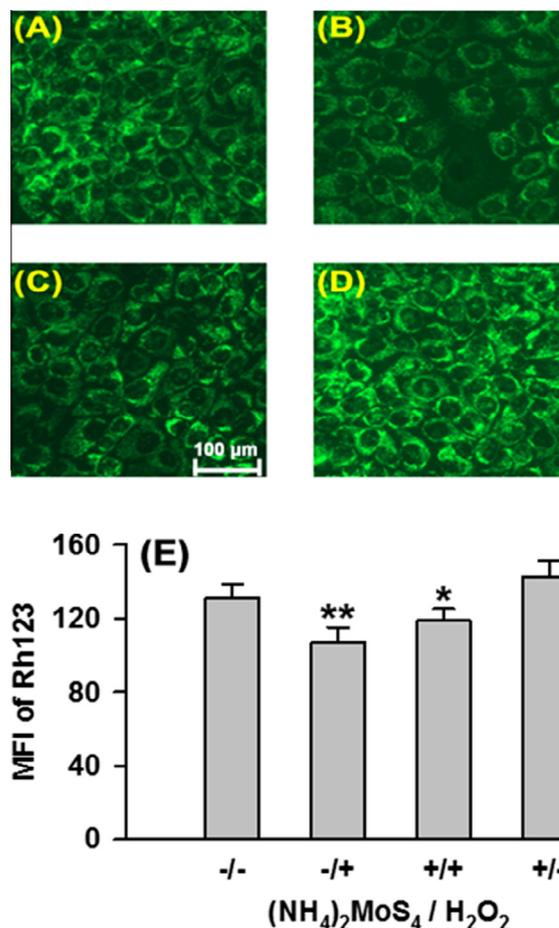


Figure 6. Effects of TTM on H₂O₂-induced MMP loss in HaCaT cells. Rh123 staining followed by photofluorography for the observation of MMP. (A) Control cells, (B) cells treated with 400 μM H₂O₂ for 5 h, (C) cells treated with 400 μM H₂O₂ combined with pretreatment with 200 μM TTM for 1 h. (D) Cells treated with TTM alone. (E) Quantitative analysis of the mean fluorescence intensity (MFI) of Rh123 in group a–d using Image J software. Data were shown as the mean ± SE. ***P* < 0.01 versus Control group, **P* < 0.05 versus H₂O₂ alone group.

cytoprotection against oxidative damage. These results should promote researchers to re-think the biological activities of TTM and apply it to other studies.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bmcl.2016.02.005>.

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