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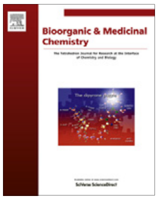

Thioglycine and L-thiovaline: Biologically active H_2S-donors

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ABSTRACT

Thioglycine and L-thiovaline are stable under acidic and basic conditions but in the presence of bicarbonate they liberate the gasotransmitter H_2S. In cells both thioamino acids were proven to enhance cGMP formation and promote vasorelaxation in mouse aortic rings. Given that H_2S is known to lower arterial hypertension, reduce oxidative stress and exhibit cardioprotective effects in preclinical models, H_2S donors hold promise as novel treatments for cardiovascular diseases.

1. Introduction

Hydrogen sulfide (H_2S) is a gas with a characteristic pungent smell of rotten eggs, mostly known for its toxic properties in living organisms.[1] More recently, H_2S has attracted the attention of biomedical researchers, as it was discovered that low amounts of this gas are endogenously produced in many mammalian tissues.[2] Increasing evidence shows that H_2S acts as a signaling molecule in cells and is now considered the third member of the gasotransmitter family along with nitric oxide and carbon monoxide.[3] Most of H_2S is produced by two enzymes cystathionine-synthase (CBS) and cystathionine γ-lyase (CSE) that use L-cysteine as a substrate and pyridoxal phosphate as a co-factor.[4] CBS is highly expressed in the central nervous system, while CSE is abundantly present in the heart, liver, kidney and to a smaller extent in the blood vessels.[2b,5] A third enzymatic system that contributes to the endogenous production of H_2S is 3MST/CAT.[4a,5]

H_2S exhibits neuroprotective and cardioprotective actions, reduces vascular tone stimulates the growth of new blood vessels (angiogenesis).[2b,4b,5,6] The role of H_2S in inflammation is less clear as both anti-inflammatory and pro-inflammatory actions of this gasotransmitter have been reported.[2a] Several disease states, including diabetes, hypertension, stroke and Alzheimer's disease, are associated with decreased H_2S production.[2] Therefore, administration of H_2S to compensate for decreased levels or to capitalize on its beneficial effects would be desirable. This, theoretically, could be achieved by delivery of H_2S gas, but such an approach would have major disadvantages that include practical hurdles associated with the delivery of gaseous molecules and lack of targeted delivery. Small molecules that can release H_2S in a slow, regulated fashion, mimicking the endogenous release of H_2S, would be preferable.

The majority of researchers use NaHS or Na_2S as a source for H_2S in their experiments.[7] Under physiological conditions (pH 7.4), ca. 28% of hydrogen sulfide ($pK_a = 7$) is undissociated and present in biological fluids as H_2S. It should, however, be noted that it is unclear whether H_2S, HS^- or both are the biologically relevant species. Exposure of biological systems to NaHS causes a burst of H_2S that does not recapitulate the low level continuous production of H_2S that occurs in vivo. In addition, NaHS is rapidly oxidized by O_2. Another approach used to increase H_2S production in cells and tissues, is to provide L-cysteine that is enzymatically converted to H_2S through the action of CSE and CBS. This would, however, not yield the expected results in conditions where the levels and/or activity of these enzymes are down-regulated by disease processes; in addition cysteine has biological effects that are not related to H_2S production. On the other hand, only a handful of H_2S-donors have been reported in the literature. These include GYY4137,[8] a synthetic derivative of the Lawesson's reagent, *N*-benzoylthiobenzamides,[9] the garlic constituents diallyl disulfide and trisulfide (DADS and DATS),[10] modified forms of clinically used agents that carry a H_2S-releasing moiety (such as sildenafil, diclofenac, etc),[7] as well as L-cysteine analogues.[11] Obvious disadvantages exist for most of the above. For example, the biological

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properties/potential toxicity of the backbone of GYY4137 that remains after H_2S release are unknown, while agents like S-diclofenac have biological activities unrelated to H_2S release that complicates the interpretation of experimental data. Moreover, *N*-benzoylthiobenzamides that release H_2S in the presence of cysteine, will not only do so when added to cells, but in addition they will also acylate biological molecules carrying nucleophilic groups.

The aim of the present study was to design, synthesize and pharmacologically characterize new safe, water-soluble, slow-releasing H_2S donors. We thought, thioaminoacids could satisfy the above mentioned criteria and could, thus, be ideal H_2S donors. In fact such amino acid derivatives have been reported in the literature and used as starting materials for the synthesis of peptides.[12] Interestingly, thioaminoacids are stable under acidic and basic conditions. However, in the presence of bicarbonate they are spontaneously transformed at mild alkaline pH values to the corresponding α-amino acid *N*-carboxyanhydrides by simultaneous liberation of H_2S. This property is described as the bicarbonate effect (Fig. 1).[12]

Taking into consideration that the bicarbonate concentration in blood at physiological pH (7.4) is about 27 mmol/l[13] we hypothesized that thioaminoacids could be optimal H_2S donors. In order to prove our hypothesis we synthesized thioglycine as outlined in Figure 2. A similar strategy was used to synthesize thiovaline. Starting from commercially available Boc-protected glycine **4a** (Fig. 2) the amino acid was activated as imidazolide and subsequently, without prior isolation, H_2S gas was bubbled through the solution according to known protocols.[14,15] Treatment of the obtained derivatives **5a,b** with trifluoroacetic acid (TFA) afforded the known free thioglycine **1a**. In an analogous way we synthesized thiovaline **1b**. Both thioaminoacids showed identical properties as described by Wieland.[16]

Thioglycine gave a positive result in the sodium nitroprusside assay[17] indicating its ability to liberate H_2S. This was confirmed additionally using a specific H_2S electrode (Fig. 3).

In addition, ^{1}H NMR-spectroscopy studies (see Supplementary data Fig. S1) were carried out, measuring the time- and temperature-dependent decomposition of thioglycine in the presence of $NaHCO_3$ to give its *N*-carboxyanhydride **3**[18] and HS^-. These experiments revealed stability of thioglycine in a 40 mM bicarbonate solution at 25 °C. However, raising the temperature at 40 °C showed a slow decomposition of thioglycine until an equilibrium was reached after 72 h. At this time the mixture consisted of 65% of thioglycine and 35% of *N*-carboxyanhydride **3**. It should be kept in mind that under these conditions (alkaline pH, closed system) complete transformation of thioglycine to *N*-carboxyanhydride **3** and HS^- is not possible because HS^- remains in the reaction mixture.

To compare the release of H_2S from thioglycine and thiovaline to the release of H_2S from known donors, we used the methylene blue method,[19] a standard method for H_2S determination. In this assay, both thioaminoacids seemed to release only minor amounts of H_2S (Fig. S2). The inability to detect larger amounts of H_2S coming from thioaminoacids relates to the fact that they are stable in the acid conditions utilized during of the methylene blue assay. Therefore we developed a new assay based on the fluoresence emitted by thiobimane in order to detect H_2S release from donor compounds that could also be used in the future to detect H_2S in biological samples. We hypothesized that dibromobimane **6** could be used as a specific reagent to detect H_2S as only **6** after reaction with H_2S/HS^- would yield fluorescent[20] thiobimane **7** (Fig. 4). Dibromobimane **6** does not show significant emission under the same conditions (excitation at 360 nm and emission at 465 nm, see also Supplementary data Fig. S3–S5). Although free thiols and aminoacids like cysteine are known to react[21] with dibromobimane the corresponding products are only weakly fluorescent under the detection conditions (Fig. S6A). These physicochemical properties make dibromobimane an ideal reagent for specific sensitive detection of H_2S. In fact we developed a sensitive fluorescence assay, which detects H_2S (HS^-, S^{2-}) at concentrations as

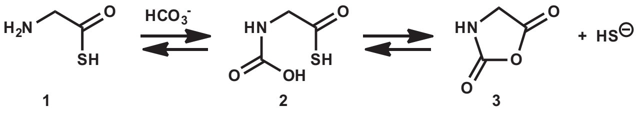

Figure 1. Proposed mechanism for the formation of glycine *N*-carboxy- anhydride 3 and release of H_2S (HS^-) in the presence of bicarbonate.

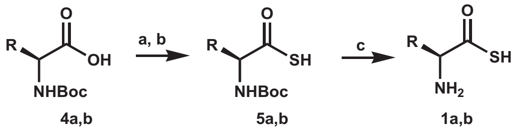

Figure 2. Synthesis of thioglycine **1a** and thiovaline **1b**. Reaction conditions: (a) 1,1′-carbonyldiimidazole, CH_2Cl_2; (b) H_2S-gas; (c) TFA, CH_2Cl_2 (1:1), 52% over two steps; Boc, *tert*-butoxy-carbonyl; H_2S, hydrogen sulfide; TFA, trifluoroacetic acid; a series R = H, b series R = *iso*-propyl.

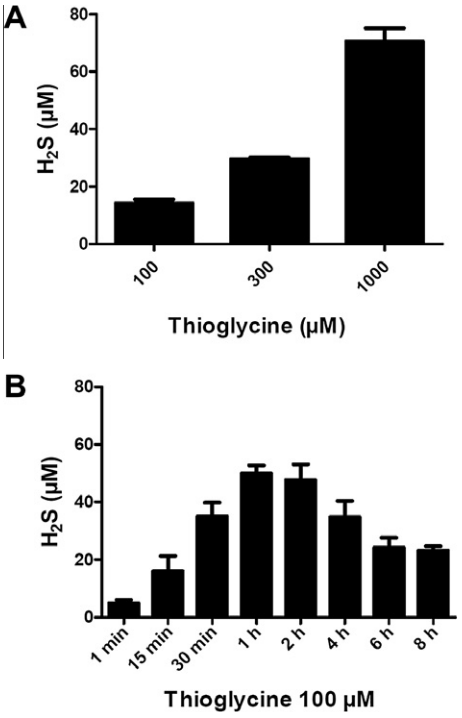

Figure 3. Thioglycine releases H_2S. H_2S released by thioglycine was detected using the H_2S specific amperometric sensor. (A) Concentration-dependent release of H_2S by thioglycine. (B) Time dependency of H_2S release by thioglycine (100 µM). (see Supplementary data for details).

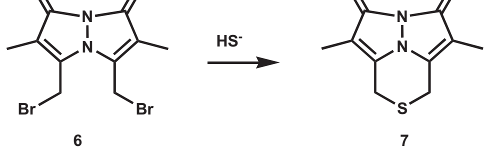

Figure 4. Transformation of dibromobimane 6 to thiobimane 7 in the presence of H_2S/HS^-.

low as 5 μM (Fig. S6 B). When this fluorescent method was used, it became evident that thioglycine and thiovaline released ample amounts of H_2S (Fig. 6S C)[22] Using the thiobimane method we observed that thioglycine released more H_2S than L-thiovaline; release of the gasotransmitter from the thioaminoacids was gradual, reaching a plateau after 60 min, in contrast to the rapid rate of H_2S generation observed with NaHS and Na_2S (Fig. S7). Thioglycine and L-thiovaline liberated more H_2S than GYY4137. Interestingly, thioamino acid addition in Hanks Buffered Salt Solution (HBSS) which contains calcium- and magnesium-ions promoted the formation of glycine *N*-carboxyanhydride and showed faster decomposition at 37 °C (data not shown).

In order to pharmacologically characterize the thioaminoacids, we initially determined their ability to increase intracellular cGMP levels. We have recently shown that NaHS increases this cyclic nucleotide in smooth muscle cells by inhibiting the activity of phosphodiesterase (PDE), the enzymes responsible for breakdown of this cyclic nucleotide.[23] Exposure of cultured rat aortic cultured smooth muscle to thioglycine or thiovaline lead to a concentration-dependent increase in cGMP levels (Fig. 5).

Glycine and valine failed to increase the levels of cGMP (Fig. S8). It is noteworthy that cGMP in response to NaHS reached a plateau at 50 μM yielding a twofold increase, while exposure to thioglycine was much more robust (approximately 10-fold increase) (Fig. 2) and increased further up to 3 mM (data not shown). Similarly, exposure to L-thiovaline increased cGMP levels to a greater extent compared to NaHS.

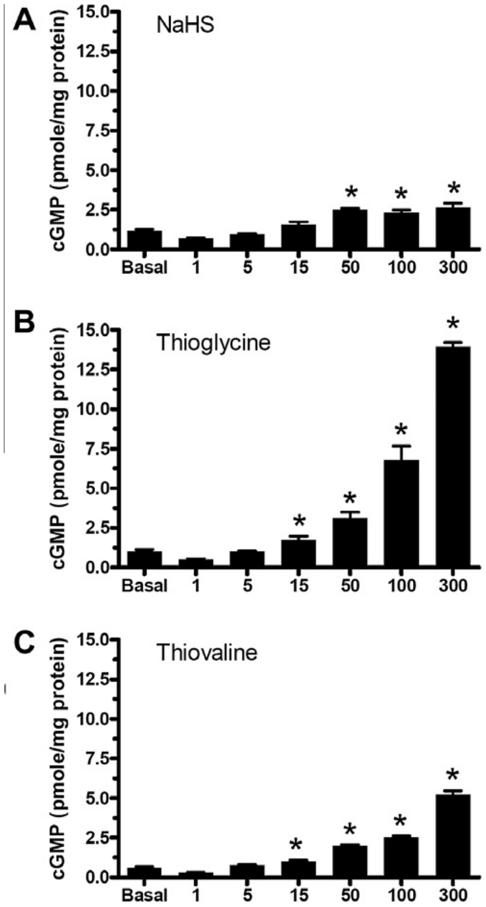

Figure 5. Thioamino acids increase cGMP levels in smooth muscle cells. Rat aortic smooth muscle cells were exposed to the indicated concentration of NaHS (A), thioglycine (B) or L-thiovaline (C) for 5 min; cellular cGMP was then extracted and measured by EIA (see supp. info for details); $n = 4$;$^{*}P$ <0.05 form control.

Increased levels of cGMP in smooth muscle are known to trigger relaxation through multiple pathways that lower intracellular calcium, cause myosin light chain dephosphorylation and activate potassium channels.[24] To further characterize the biological activities of thioglycine and L-thiovaline and to test their potential to be used as smooth muscle relaxing agents, we evaluated the ability of the thioaminoacids to cause dilatation. Mouse aortic rings were precontracted with phenylephrine and then exposed to various H_2S donors (Fig. 6A). In these experiments, we observed that L-thiovaline was more potent, but elicited the same maximal response with thioglycine. Both L-thiovaline and thioglycine were more efficacious and potent than GYY4137. The observation that L-thiovaline is more potent than thioglycine in eliciting vasorelaxation, although might be perceived to be in apparent contradiction to the lower release of H_2S by thiovaline in buffered solutions can be explained by a more extensive uptake of this thioaminoacid into cells compared to thioglycine or by faster liberation of H_2S from thiovaline promoted by biomolecules present in tissues.

To evaluate the ability of thioglycine to promote H_2S release in vivo, this aminoacid was administered to rats and plasma samples were removed at the indicated times points (Fig. 6B). Hydrogen sulfide levels increased after intraperitoneal (ip) injection of 5 mg/kg in a time-dependent manner. H_2S plasma levels first became significantly increased after 30 min and remained elevated for 8 h.

In conclusion, we have synthesized two new H_2S donors that use endogenous molecules (proteinogenic amino acids) as carrier scaffolds of the active moiety (H_2S) and are, thus, non-toxic. In contrast to the commonly used prototype H_2S donor NaHS, thioaminoacids liberate H_2S at a slow rate mimicking the sustained endogenous H_2S production. Thioglycine and thiovaline were proven to enhance cGMP formation and promote vasorelaxation. Given that H_2S is known to lower arterial hypertension, reduce oxidative stress and exhibit cardioprotective effects in preclinical

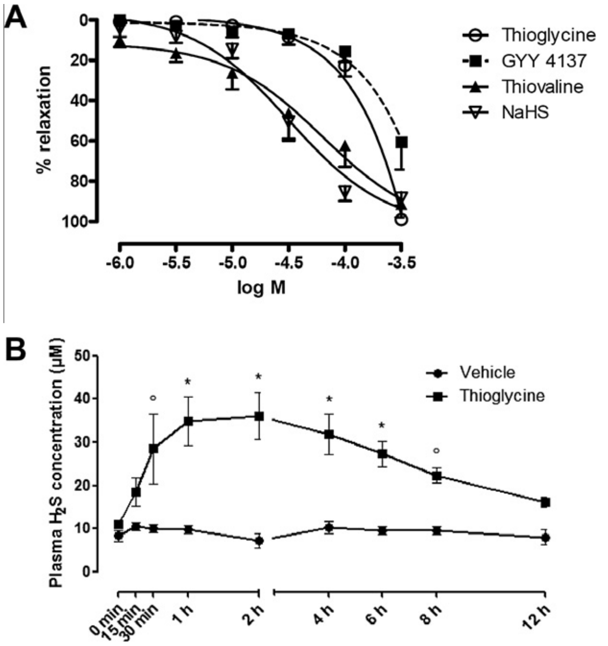

Figure 6. Biological activity of thioglycine (A) Aortic rings were denuded of endothelium and contracted with phenylephrine PE (1 μM); once the plateau was reached, cumulative-concentration response curves to GYY4137, NaHS, thioglycine or thiovaline were performed. $n = 6$, (B): Blood samples were obtained 15 min, 30 min, 1 h, 2 h, 4 h, 6 h, or 12 h after intraperitoneal injection of thioglycine (5 mg/kg) or saline.

models, H_2S donors hold promise as novel treatments for cardiovascular diseases.

Acknowledgments

In memoriam of Theo Angelopoulos (1936–2012).

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Supplementary data

Supplementary data (experimental procedures, spectral data for all unknown compounds) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2012.02.028.

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