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Amino acid-based H_2S donors: *N*-thiocarboxyanhydrides that release H_2S with innocuous byproducts†

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A library of *N*-thiocarboxyanhydrides (NTAs) derived from natural amino acids with benign byproducts and controlled H_2S-release kinetics is reported. Minimal acute *in vitro* toxicity was observed in multiple cell lines, while longer-term toxicity in cancer cells was observed, with slow-releasing donors exhibiting the greatest cytotoxic effects.

Three endogenously produced signaling gases, nitric oxide (NO), hydrogen sulfide (H_2S), and carbon monoxide (CO), recognized as gasotransmitters, are enzymatically generated, can penetrate cell membranes, and have specific cellular targets and physiological functions.[1] The most recently recognized gasotransmitter, H_2S, directly interacts with or initiates major signaling pathways linked to various disease states, including cardiovascular disorders, neurological disorders, and other conditions including diabetes, chronic kidney disease, and cancer.[2–9] In some disease indications, endogenous H_2S production is disrupted, and administration of exogenous H_2S in animal models can alleviate symptoms and improve outcomes. Exogenous H_2S administration is also needed for *in vitro* and *in vivo* studies focused on better understanding the physiological roles of this important gas. To conduct these studies, H_2S-releasing molecules (often called H_2S donors) are employed, with a goal of mimicking or supplementing endogenous H_2S production to better understand H_2S physiology and explore its therapeutic potential.[10,11]

Controlled delivery of H_2S is challenging due to its gaseous nature, toxicity at high concentrations, and short half-life in a biological setting.[12] To address these challenges, researchers have developed several small and macromolecular synthetic H_2S donors with appreciable control over dosage and release rates.[13–17] One way to deliver H_2S that has gained interest recently is *via* the delivery of carbonyl sulfide (COS),[18,19] which is effectively converted into H_2S in eukaryotic organisms by the action of the enzyme carbonic anhydrase (CA).[20] Several groups have developed and studied thiocarbamate-based COS donors that decompose to release COS in response to specific stimuli, such as reactive oxygen species (ROS), enzyme activity, and light (Fig. 1A).[21–25] For example, Pluth and coworkers have developed enzyme-triggered, COS-releasing small molecule thiocarbamates, and our group recently described a poly(thiourethane) that releases on average 7 equivalents of COS after triggering, also relying on a thiocarbamate linkage.[26,27]

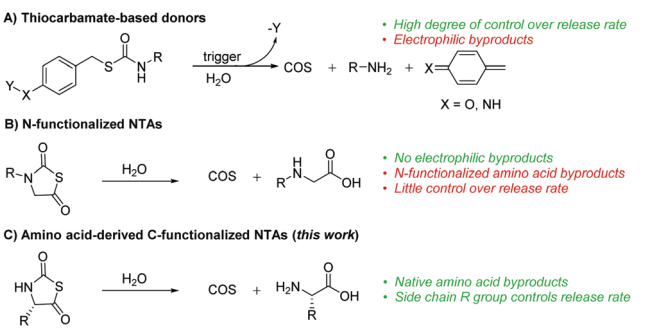

Fig. 1 Classes of COS donors. (A) Thiocarbamate-based donors (and related donors such as thionoesters and thioureas) are structurally tunable but release electrophilic byproducts. (B) N-functionalized NTAs release COS and an *N*-alkyl amino acid upon hydrolysis with little control over release rate. (C) C-functionalized NTAs, described here, release amino acid byproducts, and the R group can influence COS release rate.

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These triggerable, self-immolative, thiocarbamate-based H_2S donors present highly tunable COS/H_2S release. However, the byproducts (*e.g.*, (aza)quinone methides) associated with the activation of a 1,6-elimination reaction are electrophilic, potentially toxic, and complicate experiments when studying the chemical biology of COS/H_2S.[28] For example, an esterase-triggered thiocarbamate donor reduced viability at much higher levels than commonly used H_2S donors (NaSH and GYY4137) in human bronchial epithelium (BEAS 2B) cells and in HeLa cells by inhibiting mitochondrial respiration pathways.[29] The observed toxicity in thiocarbamates was believed to be the result of rapid COS/H_2S release from these donors rather than the byproduct, leading to a buildup of gas in toxic concentrations. A detailed follow-up study on similar thiocarbamates with varying steric bulk of the ester group suggested that toxicity depended on the rate of ester cleavage, with compounds generating COS most quickly exhibiting the highest toxicity; however, by product toxicity could not be fully ruled out.[30] This complication has led our group and others to identify COS donors that do not generate electrophilic byproducts,[31–33] with the goal of disentangling the direct chemical biology of COS/H_2S from associated byproducts.

One class of COS-mediated H_2S donors with benign byproducts are the *N*-thiocarboxyanhydrides (NTAs). Our first report was on an NTA derived from sarcosine, an *N*-alkyl amino acid that releases COS along with a sarcosine-containing byproduct (Fig. 1B).[31] More recently, we also described a series of N-substituted NTAs with bio-orthogonal handles for easy access to polymeric and peptidic COS/H_2S-releasing materials.[26] However, lack of tunability of the rate of H_2S release from these N-functionalized NTAs led us to design a series of C-substituted NTAs based solely on natural amino acids (Fig. 1C). We envisioned that hydrolysis of the peptide-derived NTAs would produce COS while regenerating the original amino acid as the only byproduct. We also hypothesized that the steric bulk of the substituent on the α-carbon could influence the rate of hydrolytic ring-opening and subsequent COS release. Therefore, this series of NTAs could (1) enable the ability to tune the rate of COS/H_2S release from NTAs, and (2) facilitate investigations into how COS release rate influences cytotoxicity because COS is the only potentially toxic product.

An interesting similarity among all small molecule N-substituted NTAs is that their H_2S release peaking times, as measured by an H_2S-sensitive electrochemical probe, were all measured at around 40 min.[26] We speculate that this similarity is related to the mechanism of the nucleophilic ring-opening reaction. The ring-opening of an NTA likely involves nucleophilic addition to the C-5 carbonyl carbon to form a tetrahedral intermediate, followed by reformation of the carbonyl in the ring-opening step, and finally release of COS from the resulting thiocarbamate (Fig. 2A). Substituents present on the N atom of the ring are therefore likely too far from the C-5 carbonyl carbon to affect the rate of nucleophilic addition. For this work, we hypothesized that it would be possible to control the rate of nucleophilic addition at the C-5 carbonyl carbon, and subsequently the rate of COS release, by substituting the

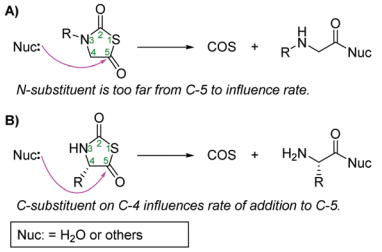

Fig. 2 Schematic representation of nucleophilic ring-opening of (A) N-substituted and (B) C-substituted NTAs.

adjacent C-4 carbon atom (Fig. 2B). We hypothesized that bulky substituents at the C-4 carbon atom (the α carbon in the original amino acid) would impede the approach of the nucleophile, slowing down COS release. Further, we were also interested in studying the effects of ring size on the rate of COS release, and therefore we attempted to synthesize rings with $n > 5$, specifically 6 and 7-membered rings.

In order to test our hypothesis, we synthesized nine different NTAs from natural L-amino acids in a two-step process (Fig. 3A). Among these, seven NTAs were derived from canonical amino acids (Fig. 3B): Gly-NTA, Ala-NTA, Val-NTA, Leu-NTA, Ile-NTA, Phe-NTA, and Pro-NTA. We also prepared two additional NTAs: one with two methyl groups on C-4 derived from 2-aminoisobutyric acid (Aib-NTA), and one that included a 6-membered NTA ring derived from β-alanine (β-Ala-NTA). We also attempted to synthesize a 7-membered NTA derived from 4-aminobutanoic acid, but the 5-membered heterocycle *O*-ethyl 2-oxopyrrolidine-1-carbothioate formed instead (for details see ESI†). This might be the result of a higher energy penalty associated with the formation of a 7-membered cyclic NTA compared to 5- or 6-membered rings. As a result, we did not attempt to make NTAs with larger ring sizes.

With nine NTAs in hand, next we measured H_2S release from the NTAs using an H_2S sensitive electrochemical probe. To test our hypothesis, we chose Gly-NTA, Val-NTA, and Ile-NTA and studied their hydrolytic ring-opening in 5% DMSO-PBS

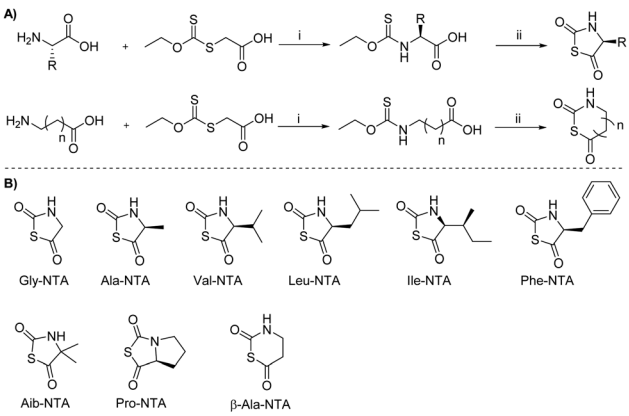

Fig. 3 (A) General scheme for synthesis of N-substituted NTAs used in this work; conditions: (i) NaOH, methanol/H_2O (ii) PBr_3, EtOAc. (B) Chemical structures of nine NTAs used in this work.

(10 mM) in the presence of 300 nM CA (Fig. S1, ESI†). For sake of simplicity, we only evaluated hydrolysis here and did not include any additional nucleophiles. The H_2S peaking times showed a clear trend: the smaller Gly-NTA showed a sharp profile with a peaking time of 51 min, while the NTAs with larger substituents at C-4 (*i.e.*, the α-carbon atom) exhibited more gradual release profiles with peaking times of 96 min (Val-NTA) and 104 min (Ile-NTA) (Table S1, ESI†). While the electrochemical probe provides a real-time analysis of H_2S release, it is difficult to use this method to determine a rate constant or compare donors with variable release profiles because the released H_2S is constantly oxidizing and volatilizing. To measure and compare pseudo-first-order H_2S release half-lives among all 9 NTAs, we used the methylene blue assay. In our experiments, water was used as the nucleophile, and we included CA in the reaction vials to quickly convert COS generated during the NTA ring-opening reaction into H_2S. Fitting the data to a pseudo-first-order kinetics model, as we have done previously using the methylene blue assay,[34] allowed us to determine half-lives of H_2S release (Fig. 4A).

Half-life values determined by the methylene blue method for Gly-NTA, Val-NTA, and Ile-NTA were 1.7, 18, and 20 h, respectively (Fig. 4B and Table S2, ESI†). The methylene blue kinetics data revealed a ~10-fold difference in release rate between Gly-NTA and NTAs with bulky substituents, highlighting the strength of this method compared to the electrochemical probe method, where only a 2-fold difference in peaking time was observed despite drastically different release profiles. Across all nine NTAs, H_2S release half-lives varied from a shortest half-life of 1.1 h for β-Ala-NTA to 20 h for Val-NTA. Looking more closely, the H_2S release half-lives could be divided into three separate groups. The first group with the shortest half-lives, ranging from 1–2 h, consists of Gly-NTA, β-Ala-NTA, and Ala-NTA; the second group is Leu-NTA, Phe-NTA, and Pro-NTA with half-lives ranging from 4–7 h. Finally, the third group consists of Aib-NTA, Leu-NTA, and Val-NTA with half-lives ranging from 10–20 h.

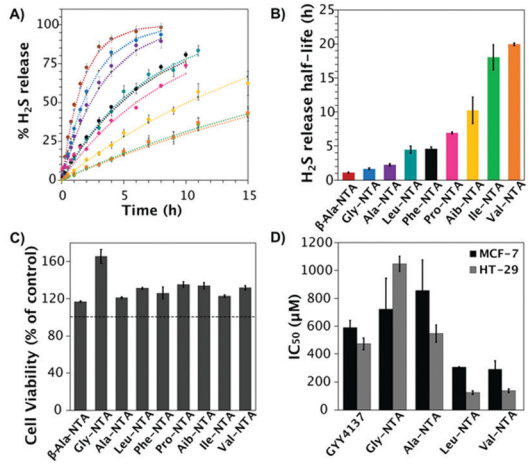

Fig. 4 (A) H_2S release from NTAs (100 μM) measured *via* the methylene blue assay in 10 mM PBS solution with 5% DMSO and 300 nM CA. Data points are solid circles, and dotted lines show pseudo-first-order kinetics fits; (B) H_2S release half-lives for NTAs determined from the pseudo-first-order kinetics fits. (C) Cell viability data for MCF-7 breast cancer cells treated with 100 μM NTA solution with 0.25% DMSO in PBS (pH 7.4) for 1.5 h. (D) IC_{50} data for selected NTAs and GYY4137 after 72 h incubation time in MCF-7 and HT-29 cancer cells.

The H_2S release half-lives of these three groups can be rationalized by considering the steric bulk near the reactive C-5 carbonyl in each set. Gly-NTA and β-Ala-NTA have no ring substitutions and therefore, little steric bulk to limit nucleophilic addition to the C-5 carbonyl. The fact that there is little difference in the H_2S release half-lives between these two NTAs (1.1 h for β-Ala-NTA and 1.7 h for Gly-NTA) indicates that the difference in ring size did not play a substantial role in controlling the rate of COS generation. Therefore, their H_2S release half-lives are the fastest of all, about 2-fold faster than Ala-NTA (half-life of 2.2 h), which has a methyl group on C-4. The third group with longest half-lives consists of NTAs with dialkyl groups present either on C-4 itself (Aib-NTA) or on the carbon next to C-4 (Ile-NTA and Val-NTA), which is the β-carbon in relation to the reactive C-5 carbonyl. Aib-NTA with gem-dimethyl groups at the α-carbon atom had a half-life of 10.2 h, whereas Ile-NTA and Val-NTA with dialkyl substituents on the β-carbon showed twofold longer half-lives (18 and 20 h, respectively), indicating that dialkyl groups on the β-carbon may be more effective in impeding nucleophilic approach at the C-5 carbonyl than dialkyl groups directly on C-4. The middle group with half-lives in between those of first and third groups consists of NTAs without dialkyl substitution on C-4 or the β-carbon. For example, Leu-NTA with a dimethyl group at the γ-carbon atom had a ~4-fold shorter half-life of 4.5 h compared to Ile-NTA and Val-NTA, further suggesting that a disubstituted β-C atom slows down the ring-opening reaction. Similarly, Phe-NTA (half-life of 4.6 h), with an aromatic ring at γ-carbon atom, has a similar half-life to Leu-NTA. Finally, Pro-NTA has a unique structure with a 5-membered ring fused with the NTA ring. This bicyclic structure appears to make accessibility of a nucleophile to the C-5 carbonyl group in between that of a C-4 dimethyl (Aib-NTA) and a phenyl group (Phe-NTA), exhibiting an intermediate half-life of 6.9 h.

Overall, these observations support our hypothesis that side chain substitution on the C-4 carbon plays a role in slowing down nucleophilic addition at the C-5 carbonyl. The data further suggest that dialkyl groups on the side chain β-carbon atom are more effective in slowing down this reaction than fused rings or mono- or di-substituents present at other positions. These results may inform design of other COS/H_2S-releasing NTAs.

We hypothesized that the fast-releasing NTAs would exhibit greater cytotoxicity compared to slow-releasing NTAs based on reports detailing acute cytotoxicity within 1.5 h due to rapid COS/H_2S accumulation in esterase-triggered thiocarbamates.[30] To test acute toxicity, we treated estrogen-positive human breast cancer MCF-7 cells with 100 μM NTA for a period of 1.5 h, and viability was measured against untreated cells using the CCK-8 cytotoxicity assay. In this cell line, none of the NTAs were cytotoxic, and increases in the CCK-8 signal compared

with controls were observed in all treatment groups, likely due to reported effects of H_2S-induced cell proliferation (Fig. 4C).[35] Additionally, β-Ala-NTA, the fastest H_2S donor of the group, showed no cytotoxicity under these conditions. Next, we tested the two fastest H_2S-releasing NTAs, Gly-NTA and β-Ala-NTA, on a different cell line, H9C2 cardiomyocytes. Again, we observed no acute cytotoxicity after 1.5 h up to a concentration of 300 μM (Fig. S3, ESI†). These results are in contrast to fast esterase-triggered thiocarbamates, which had a similar release profile to these two fastest NTA-based donors but were cytotoxic to HeLa cells at concentrations as low as 25 μM upon incubation for the same time period.[30] Therefore, this work suggests that COS/H_2S is not acutely toxic, at least in these cell lines.

Next, we examined longer-term inhibitory effects of selected NTAs from group 1 (Gly-NTA and Ala-NTA), group 2 (Leu-NTA), and group 3 (Val-NTA), comparing them with GYY4137, a slow releasing H_2S donor widely used in biological studies. Cell growth inhibition in both MCF-7 cells and human colon adenocarcinoma HT-29 cells was tested by incubating each cell line with various concentrations of each of the four NTAs for 72 h, allowing us to determine an IC_{50} value for each NTA (Fig. 4D and Table S3, ESI†). In the case of MCF-7 cell line, the IC_{50} values for Gly-NTA and Ala-NTA were higher (723 and 857 μM, respectively) compared to GYY4137 (592 μM), whereas Val-NTA and Leu-NTA (IC_{50} values of 289 and 308 μM, respectively) were more cytotoxic than GYY4137. In other words, the slow-releasing NTAs were more cytotoxic than the fast-releasing NTAs. However, we did not observe a significant difference in the cytotoxicity of Val-NTA and Leu-NTA. Similar trends were observed in the HT-29 cell line, with Val-NTA and Leu-NTA (IC_{50} values near 100 μM) showing nearly 10-fold greater cytotoxicity compared to fast-releasing Gly-NTA and 5-fold greater toxicity than Ala-NTA and GYY4137. Overall, the inhibition assays in both cell lines showed similar trends, suggesting that slow H_2S-releasing NTAs are significantly more potent cancer cell growth inhibitors over 72 h compared to fast H_2S-releasing NTAs.

In summary, the reported library of NTA COS/H_2S donors, derived from natural amino acids, exhibited varying H_2S release half-lives in aqueous solutions without electrophilic byproducts. All NTAs exhibited negligible cytotoxicity in MCF-7 and H9C2 cells over 1.5 h, indicating that COS is not acutely toxic to either cell line. In contrast, slow-releasing NTAs were effective growth inhibitors in MCF-7 and HT-29 cancer cells over 72 h, suggesting that slow H_2S delivery may inhibit cancer cell growth. We speculate that the slow donors are more effective than fast donors because they maintain toxic H_2S concentrations in the cells over a longer period of time. Because these NTAs release only amino acid byproducts, the observed *in vitro* effects can be directly linked to COS/H_2S. Therefore, these C-substituted NTAs based on α-amino acids enable molecular-level control over H_2S release rates, making them potentially useful donors to help unravel the (patho)-physiological effects of COS from the side products accompanied with most COS donors, and more broadly, to understand the physiological effects of COS and/or H_2S.

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Conflicts of interest

There are no conflicts of interest to declare.

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