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Functional N-substituted N-Thiocarboxyanhydrides as Modular Tools for Constructing H₂S Donor Conjugates

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Abstract

We report a synthetic route toward a family of functional COS/H₂S-releasing N-substituted N-thiocarboxyanhydrides (NTAs) with functionalities to accommodate popular conjugation reactions, including olefin cross metathesis, thiol-ene, and copper-catalyzed azide-alkyne cycloaddition. The N-substituted NTAs were attached to small molecules, polymers, and a protein to synthesize novel H₂S donors convergently. All conjugates showed sustained H₂S release kinetics.

Biology has benefitted immensely from the emergence of bioconjugation chemistry. The development of bioconjugates has led to key discoveries in measuring protein/ligand binding, developing effective MRI contrast agents, probing antibody–antigen interactions, and imaging biomolecules in vivo.¹ Expanding upon this work, many common bioconjugation reactions also find use in other areas of chemistry, including surface modification² and polymer functionalization.³ However, bioconjugation strategies have been applied only sparingly in the arena of gasotransmitter research,^{4–6} which focuses on uncovering the biological roles and exploiting the therapeutic potential of endogenously produced signaling gases. We envisioned that popular bioconjugation reactions could be employed to synthesize a wide variety of gasotransmitter donor conjugates, specifically conjugates capable of delivering hydrogen sulfide (H₂S), from a small library of functionalized donors.

As first reported by Abe and Kimura in 1996, H₂S is a gasotransmitter.⁷ Since this discovery, studies on the physiological roles of H₂S in the body have ensued.⁸ More recent discoveries suggest that alterations in endogenous H₂S production contribute to a variety of disease states, including cardiovascular disease,⁹ diabetes,¹⁰ and Parkinson's disease,¹¹

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Supporting Information

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Experimental details, synthetic procedures and compound characterization

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among others.¹² These results also indicate that exogenous delivery of H₂S may be therapeutic in certain indications by promoting wound healing,¹³ alleviating cardiovascular tissue damage in ischemia-reperfusion events,^{14, 15} and mitigating cellular damage in the central nervous system induced by reactive oxygen species (ROS).¹⁶ However, compared with existing small molecule H₂S donors, donors appended to a variety of compounds and constructs, including established drugs, polymer scaffolds, and proteins, would likely increase the therapeutic viability of exogenous H₂S delivery moving forward.

Compared with H₂S “donor salts” NaSH and Na₂S, which lead to instantaneous release, synthetic H₂S donors can incorporate specific release triggers and extend the H₂S release half-life, better mimicking endogenous H₂S production.¹⁷ These properties allow for greater sophistication in studying H₂S in biological settings and open avenues for targeted H₂S delivery. Over the past decade, various H₂S donors have been developed that respond to specific stimuli, including water, nucleophiles, light, and enzymes, among others.¹⁸⁻²⁰ Additionally, current efforts focus on extending H₂S donors to polymeric drug delivery systems as well as modifying existing drugs with H₂S-releasing moieties.²¹⁻²⁸ However, nearly all synthetic H₂S donors lack reactive chemical “handles” for use in conjugation reactions, a necessity to develop a library of donors convergently. Based on previous work in our group on donors of carbonyl sulfide (COS), which is converted into H₂S in vivo,²⁹ we aimed here to create a platform of dual COS/H₂S donors that could be readily conjugated to various scaffolds.

Previously, our lab reported on *N*-thiocarboxyanhydrides (NTAs) as a class of dual COS/H₂S donors with nontoxic, peptidic byproducts.³⁰ NTAs release COS in the presence of primary amines (e.g., glycine), and the ubiquitous enzyme carbonic anhydrase (CA) rapidly catalyzes hydrolysis of the released COS into H₂S (Scheme 1A). The initial version of NTAs as COS/H₂S donors was the NTA of sarcosine (**NTA1**) as it offered a straightforward synthesis from commercially available starting materials. However, **NTA1** lacks functional handles and is not conducive to conjugation reactions. Therefore, we sought a synthetic route to make NTAs that would tolerate the incorporation of functional groups used in common bioconjugation reactions. Functional NTAs would allow for the simple modification of existing small molecules, polymers, proteins, surfaces, or other platforms with COS/H₂S releasing NTAs, opening a range of novel H₂S donor systems. To this end, we devised a modular synthetic route to prepare NTAs with several functionalities via the same starting materials. Functional groups with high prevalence in the fields of bioconjugation and polymer chemistry such as alkynes, azides, alkenes, and norbornenes were targeted to allow access to popular conjugation reactions.

N-Functional NTAs were prepared in three steps starting from commercially available 2-((ethoxycarbonothioyl)thio)acetic acid (XAA) and iminodiacetic acid (IDAA) (Scheme 1B), affording thiocarbamate diacetic acid (TCDA). This compound was subsequently monoesterified with commercially available functionalized alcohols to give compounds **1i-v** (see Supplemental Information for synthetic routes), followed by ring-closure with phosphorous tribromide (PBr₃) to give the corresponding NTAs, labelled **NTAs 2–6**. To show the versatility of this approach, five N-substituted NTAs were prepared as alkynyl, azido, allyl, norbornyl, and benzyl esters (Scheme 1B). We aimed specifically to synthesize

alkynyl- and azido-NTAs (**NTA2** and **NTA3**) to accommodate the Huisgen [3+2] cycloaddition reaction (CuACC) between organo-azides and alkynes. Allyl-NTA (**NTA4**) was designed to accommodate olefin cross metathesis and thiol-ene reactions, owing to the prevalence of these reactions in bioconjugation and polymer chemistry. Norbornyl-NTA (**NTA5**) was synthesized to expand the NTAs available for thiol-ene reactions as norbornenes are highly reactive thiol-ene substrates.³¹ Lastly, benzyl-NTA (**NTA6**) was synthesized as a model compound to study H₂S release from NTAs under various conditions (i.e., different pH and nucleophiles) in detail. All five NTAs are solids at room temperature and stable for months on the benchtop.

To assess whether NTAs decompose under common CuAAC reaction conditions, model reactions on small molecules were conducted. The CuAAC reaction was performed using alkynyl **NTA2** and 2-azidoethyl benzoate to form compound **7** (Table 1). The best conditions found for this reaction included a THF/H₂O solvent mixture (4:1 v/v) with sonication in lieu of magnetic stirring, as sonication best facilitated the dispersion of the precipitate formed upon addition of sodium ascorbate to copper sulfate pentahydrate (CuSO₄·5H₂O). Under these conditions, we observed near quantitative conversion into the desired product by ¹H NMR spectroscopy in approximately 20 min at rt, with product isolation by flash chromatography (Table 1, compound **7**).

To further highlight the robust nature of the CuAAC reaction with **NTA2**, we extended the scope of these conditions to conjugation of **NTA2** onto an azido-functionalized lysine (Fmoc-Lys(N₃)-OH) as well as a macromolecular substrate, azide-terminated poly(ethylene glycol) (PEG-N₃, molecular weight (MW) = 5.0 kg/mol). These two substrates were chosen as analogs to peptidic and polymeric drug delivery systems. Using the same reaction conditions outlined above complete conversion to product after approximately 60 min for reactions with both Fmoc-Lys(N₃)-OH (Table 1, compound **8**) and PEG-N₃ (Table 1, compound **9**). These reactions demonstrate that a single NTA can be used to functionalize a variety of systems from small molecules to polymers under similar reaction conditions.

In order to fully encompass the scope of the CuAAC click reactions for both alkyne and azide substrates, we synthesized an azide-functionalized NTA, **NTA3**. To evaluate the reactivity of **NTA3**, we performed CuAAC on a small molecule model compound, THP-protected propargyl alcohol (Table 1, compound **10**), and an alkyne-functionalized PEG (MW = 5.0 kg/mol) (Table 1, compound **11**). Both reactions reached complete conversion within 75 min under the same CuAAC conditions described for **NTA2**.

We also investigated conjugation of NTAs using olefin cross metathesis (CM), a widely used tool in organic chemistry as a facile and mild means of forming carbon-carbon double bonds in bioconjugation,³² drug development,³³ and polymer synthesis³⁴. Grubbs and co-workers previously categorized terminal olefin CM substrates into four categories depending on their propensity to form homodimer CM products.³⁵ Ideal CM partners have mismatched reactivity (i.e., Type I + Type II or III), motivating the decision to choose methyl acrylate (Type III) as a model CM partner with allyl-NTA (**NTA4**) (Type I). CM reactions were conducted in the presence of Hoveyda-Grubbs second generation (HG2) catalyst with *p*-cresol as an additive, as reported by Tooze and coworkers.³⁶ In the presence of *p*-cresol and

2 equiv methyl acrylate, complete consumption of **NTA4** was observed in 2 h by TLC with 0.1 mol % catalyst loading. The desired NTA-methyl acrylate CM product (85:15 E/Z ratio (Figure S31)) was isolated by flash chromatography (Table 1, compound **12**).

The final class of reactions used for NTA conjugation was the radical thiol-ene reaction between thiols and electron-rich alkenes. Thiol-ene reactions can be initiated thermally or by UV light and do not require a metal catalyst, making them particularly attractive bioconjugation reactions. Thiols are commonly found in biological systems in the form of reduced cysteine or glutathione as well as cysteine residues on proteins. Additionally, polymers with thiol chain ends are readily prepared by reversible addition–fragmentation chain transfer (RAFT) polymerization followed by removal of the thio-carbonylthio species,³⁷ offering another mode for conjugation of NTAs to synthetic polymer systems.

For conjugation of NTAs to thiols via thiol-ene, (diphenylphosphoryl)(mesityl)methanone (TPO) was used as the photoinitiator. For the equimolar reaction of **NTA4** with *N*-acetyl cysteine (Ac–Cys–OH) in THF (Table 1, compound **13**), complete consumption of the allyl-NTA starting material was observed by TLC in 60 min, with isolation of the conjugate via flash chromatography. The thiol-ene conjugation reaction was then extended to a polymeric system. Water-soluble poly(acryloyl morpholine) (PACMO) was synthesized via RAFT polymerization (MW = 6.0 kg/mol, Đ = 1.08), followed by reduction of the trithiocarbonate with hydrazine to reveal a free thiol on the polymer chain end.³⁸ Reaction of this polymeric thiol for 70 min with **NTA4** under similar conditions to those described above afforded complete consumption of **NTA4**. The NTA-PACMO conjugate was easily isolated by precipitation (Table 1, compound **14**).

To evaluate the scope of thiol-ene reactions with NTAs, we performed the thiol-ene reaction between **NTA4** and bovine serum albumin (BSA), a model protein with a single reduced cysteine residue (Cys34).³⁹ To accommodate the solubility of BSA, a largely aqueous reaction media was employed. Unfortunately, there was no evidence of NTA consumption by TLC after UV irradiation for up to 2 h under these conditions.

Previous reports have demonstrated rapid and efficient light-mediated thiol-ene reactions between substituted norbornenes and thiols in aqueous media.^{31, 40} This inspired the synthesis of norbornyl-NTA (**NTA5**) to enable thiol-ene under conditions where **NTA4** does not give appreciable conversion. A small molecule model reaction between **NTA5** and benzyl mercaptan in the presence of TPO progressed smoothly, reaching full conversion in approximately 60 min (Table 1, compound **15**). Using the same reaction conditions attempted with **NTA4** and BSA, we observed complete consumption of **NTA5** by TLC after 2 h of irradiation with UV light in the presence of TPO. After isolation via precipitation, analysis by MALDI-TOF mass spectrometry revealed a shift in the center of the peak observed for pure BSA from 66,200 to 66,700 m/z (Figure S39), indicating that the conjugation reaction was successful. These results are similar to published MALDI-TOF analyses of modified BSA.⁴¹ Successful formation of NTA-functionalized BSA was also confirmed by H₂S release measurements (Figure S41).

H₂S release profiles of the functional NTAs were investigated using an H₂S-selective electrochemical probe in the presence of physiologically relevant concentrations of CA (800 nM). H₂S release profiles for the parent NTAs (**NTAs 2–6**) displayed the typical release profile for small molecule H₂S donors, with a rise to a peak concentration followed by a slow return to baseline (Figure 1A). For **NTAs 2–6** H₂S concentrations peaked between 20–35 min in PBS buffer (pH 7.4) with 100 μ M added glycine as a trigger. H₂S release from small molecule NTAs after the various functionalization reactions (compounds **7**, **8**, **10**, **12**, **13**, and **15**) did not show a change in the general shape of the H₂S release profile from their parent, N-substituted NTA, and peaking times remained within a 15–35 min timeframe (Figure 1B). Although fairly small, we attribute the differences in peaking times between non-polymeric products from the same parent NTA (e.g., compound **7** versus **8** and compound **12** versus **13**) to changes in the local environment; for example, the carboxylic acids in compounds **7** and **13** may contribute to their enhanced release rate compared with compounds **8** and **12**. Maximum concentrations of H₂S in these release profiles are related to peaking time, where a faster peaking time generally leads to a larger maximum H₂S concentration. Overall, these results demonstrate that functionalization of NTAs with small molecules does not substantially alter their H₂S release profile.

A more dramatic change in H₂S release profile was observed for the polymer-NTA conjugates, PEG-NTA (**9** and **11**) and PACMO-NTA (**14**). All of the polymer-NTA conjugates showed substantially lower maximum concentrations of H₂S relative to the small molecule NTAs at the same concentration, with peaking times occurring at 20 min for the PACMO-NTA conjugate (**14**) and approximately 2 h for the PEG-NTA conjugates (**9** and **11**). Despite the differences in peaking times, all of the macromolecular NTA conjugates showed a more gradual return to baseline H₂S concentrations relative to the small molecule NTA donors, demonstrating that polymeric donors are capable of maintaining a sustained concentration of H₂S in solution to a greater degree than the small molecule NTAs. The phenomenon of extended and sustained H₂S release from polymeric H₂S donors has been previously reported, and it likely stems from steric effects of the polymer chain in solution.
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To gather further insight into the H₂S release kinetics of N-substituted NTAs, benzyl-NTA (**NTA6**) was used as a model substrate in the presence of various biologically relevant nucleophiles and at different pH values. In the first set of experiments with **NTA6**, we explored the effect of various nucleophiles on H₂S release rate under pseudo-first-order conditions (10:1 molar ratio of nucleophile to NTA). The nucleophiles included water only (hydrolysis), glycine, Ac-Lys-OH, and Ac-Cys-OH and were chosen to provide a range of functionalities available *in vivo*. Each nucleophile tested gave a similar release profile to hydrolysis, with addition of Ac-Lys-OH leading to a moderate decrease in peaking time and a greater maximum H₂S concentration (Figure 1C) than the others, indicating a faster overall release.

Release of H₂S from **NTA6** was also measured in buffers at various pH values. Peaking times remained constant within experimental error between pH 6 and 8.8, with peaking times ranging from 20–25 min at each pH value (Figure 1D). Taken together, these results

demonstrate that NTAs provide a platform with consistent COS/H₂S release rates under a variety of physiologically relevant pH values.

Lastly, we assessed the cytotoxicity of NTA6 in the presence of H9C2 cardiomyocytes (Figure 2). NTA6 showed no effect on cell viability at concentrations 200 μ M after incubation for 24 h, relative to untreated controls. These results stand in contrast to previous reports on cell viability of other COS donors, which display significant cytotoxicity at concentrations below 100 μ M in certain cell lines..^{19, 42, 43}

In summary, we have validated functional N-substituted NTAs as a modular donor platform from which a wide variety of H₂S donor conjugates may be prepared. Herein, we have presented a synthetic route to a variety of functional NTAs, which can subsequently be used to modify existing drugs, macromolecules, or proteins to form COS/H₂S-releasing conjugates. NTAs release H₂S spontaneously in CA-containing water but at a faster rate when triggered with an amine. Synthesis of NTA conjugates in aqueous conditions was successful, which will inform preparation of H₂S-donating proteins or water-soluble polymers. An assortment of H₂S donor systems will further facilitate the study of COS/H₂S physiology and help fully realize the therapeutic benefits of exogenous H₂S delivery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A) H₂S release data from parent functional NTAs (10 μM) in the presence of Gly (100 μM) and CA (800 nM) in 1X PBS buffer (pH 7.4). B) H₂S release data from functionalized NTAs 7-15, see table 1. C) H₂S release data from benzyl NTA (10 μM) in the presence of *N*-α-acetyl-L-lysine (Ac-Lys), glycine (Gly), *N*-acetyl-L-cysteine (Ac-Cys) in 1X PBS buffer (pH 7.4). D) Plot of peaking times of benzyl NTA (10 μM) vs pH value in various buffered systems (10 mM).

Figure 2.

Viability of H9C2 cardiomyocytes treated with NTA6 at various concentrations. Viability is presented as a percentage relative to a media-only control. Quantification of viability was carried out using Cell Counting Kit-8 (CCK-8). Results are expressed as the mean $\pm$ SEM ($n = 3$ for each treatment group, where 8-10 trials were run for each independent experiment).

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Table 1.

Reactions of functional N-substituted NTAs.

Compound	N-substituted NTA	Substrate (equiv)	Product structure
7	NTA2	2-azidoethyl benzoate ^a (1)
8	NTA2	Fmoc-Lys(N ₃)-OH ^a (1)
9	NTA2	PEG-N ₃ ^a (0.8)
10	NTA3	THP-propargyl-OH (1) ^a
11	NTA3	PEG-alkyne (0.8) ^a
12	NTA4	methyl acrylate ^b (2)
13	NTA4	Ac-Cys-OH ^c (1)
14	NTA4	PACMO-SH ^d (0.5)
15	NTA5	benzyl mercaptan ^c (1)
16	NTA5	BSA ^e (0.1)

^a CuSO₄·5H₂O (0.4 equiv), sodium ascorbate (5 equiv), THF:H₂O (4:1 v/v), sonication.^b Hoveyda-Grubbs Gen. II catalyst, CH₂Cl₂, reflux, N₂ atmosphere.

^cTPO (0.1 equiv), THF, UV irradiation.

^dTPO (0.3 equiv), THF, UV irradiation, N₂ atmosphere.

^eTPO (0.05 equiv), H₂O:THF (9:1), UV irradiation.