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# Thiol-Activated 1,2,4-Thiadiazolidin-3,5-diones Release Hydrogen Sulfide through a Carbonyl-Sulfide-Dependent Pathway

Haley M. Smith and Michael D. Pluth*

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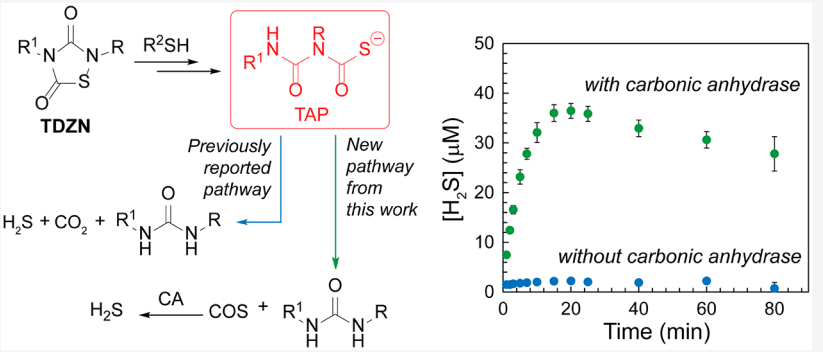


**ABSTRACT:** Recent efforts have expanded the development of small molecule donors that release the important biological signaling molecule hydrogen sulfide ($H_2S$). Previous work on 1,2,4-thiadiazolidin-3,5-diones (TDZNs) reported that these compounds release $H_2S$ directly, albeit inefficiently. However, TDZNs showed promising efficacy in $H_2S$-mediated relaxation in ex vivo aortic ring relaxation models. Here, we show that TDZNs release carbonyl sulfide (COS) efficiently, which can be converted to $H_2S$ by the enzyme carbonic anhydrase (CA) rather than releasing $H_2S$ directly as previously reported.

Hydrogen sulfide ($H_2S$) is an endogenous signaling molecule that plays important roles in biology. Such roles include vasodilation, neuromodulation, and cytoprotection against reactive oxygen species.[1−3] The need to advance our understanding of the different roles and therapeutic potential of $H_2S$ in biology has motivated the development of new investigative tools, including small molecule donor technologies. A now common approach to developing $H_2S$ donors is to design molecules that can be engineered to release carbonyl sulfide (COS),[4] which can be rapidly converted to $H_2S$ by the enzyme carbonic anhydrase (CA).[5] CA is a ubiquitous enzyme that maintains endogenous pH but can also readily convert COS to $H_2S$, which provides a simple approach for delivering $H_2S$ through the intermediate release of COS. Our group, as well as others, has developed a palette of COS-releasing $H_2S$ donors, including self-immolative thiocarbamates,[6−16] *N*-thiocarboxyanhydrides,[17−19] sulfenyl thiocarbonates,[20] dithiasuccinoyl compounds,[21,22] cyclic sulfenyl thiocarbamates,[23] and *N*-alkyl perthiocarbamates,[24] as well as other approaches (Figure 1a). These donors all have similar COS-producing moieties and proceed through common intermediates prior to COS/$H_2S$ release.

1,2,4-Thiadiazolidin-3,5-diones (TDZNs) were reported by Severino and co-workers as direct $H_2S$-releasing donors.[25] Interestingly, these donors have low $H_2S$-releasing efficiencies (<5% with 1 mM TDZN and 4 mM cysteine) but showed good activities in ex vivo aortic ring relaxation models. In the previously proposed mechanism of $H_2S$ release, initial thiol attack on the TDZN results in formation of a disulfide-containing intermediate, which can be reduced by a thiol to form a thioallophanate (TAP) intermediate (Figure 1b). Subsequent hydrolysis would release $H_2S$ followed by decarboxylation to form $CO_2$ and the diphenyl urea product. When taken together, the low $H_2S$-releasing activity, good donor efficacy in aortic ring relaxation, and similarity of the TAP intermediate to thiocarbamate and thiocarbonate anions found as common intermediates in COS-releasing molecules led us to question whether TDZN donors might release $H_2S$ by a different mechanism. Moreover, the direct release of COS, rather than $H_2S$, would still support the observed biological activity due to the presence of membrane-associated isozyme CA IV in the heart.[26] Based on the similarity of the TAP intermediate with common intermediates for established COS-releasing donors, we envisioned that dethiocarboxylation of the TAP intermediate would release COS, which would then be

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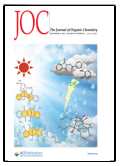




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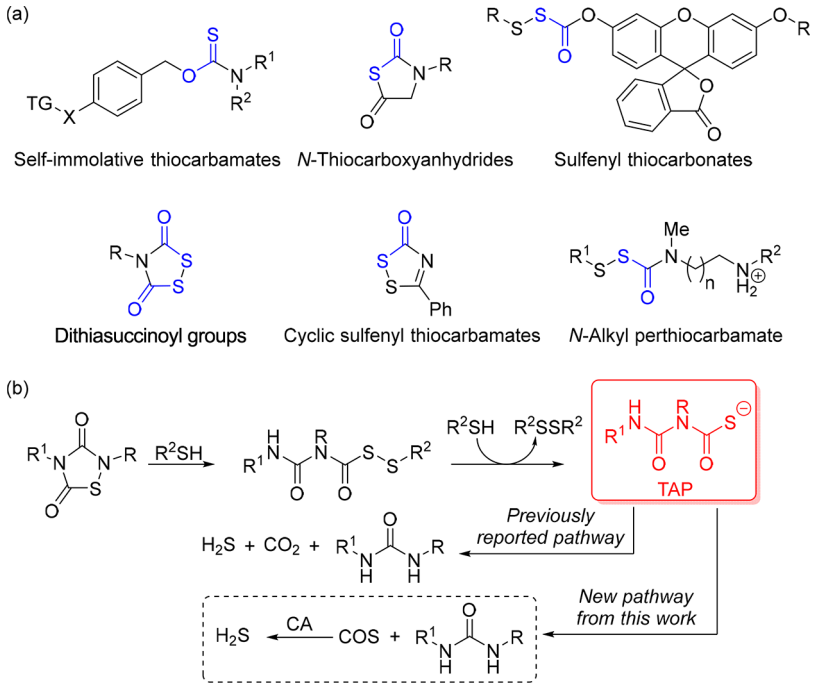
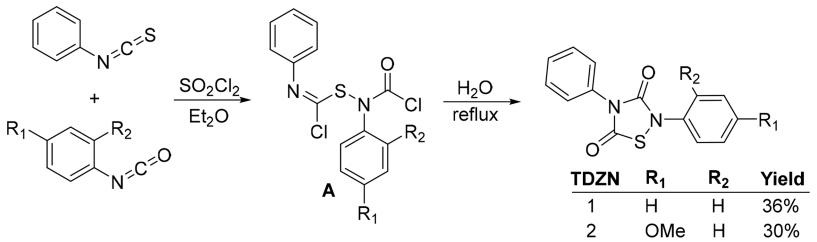



**Figure 1.** (a) Selected examples of COS-releasing $H_2S$ donors. (b) Previously reported pathway for $H_2S$ release from TDZNs and the mechanism of release determined in this work.

**Scheme 1. Synthesis of TDZNs**




| TDZN | $R_1$ | $R_2$ | Yield |
|---|---|---|---|
| 1 | H | H | 36% |
| 2 | OMe | H | 30% |
| 3 | $CF_3$ | H | 33% |
| 4 | H | $CH_3$ | 24% |

hydrolyzed to $H_2S$ by CA (Figure 1b). Using different mechanistic investigations, we report here that TDZNs release $H_2S$ through a COS-dependent pathway, which further expands the chemistry that can lead to efficacious COS/$H_2S$ release.

To investigate the mechanism of $H_2S$ release from TDZNs, we prepared four different TDZN compounds. Donors **1**–**4** were synthesized by mixing phenyl isothiocyanate, substituted phenyl isocyanates, and sulfuryl chloride in $Et_2O$ to form intermediate **A**.[25,27] This crude intermediate was subsequently cyclized by refluxing in water to afford the final TDZN compounds in low (24–36%) yields (Scheme 1). Despite this inefficient cyclization, a variety of TDZNs can be made in appreciable quantities from commercially available starting materials.

With TDZN compounds in hand, we next evaluated the COS/$H_2S$ release from **1**–**4** using the colorimetric methylene blue (MB) assay for $H_2S$. Initial attempts to reproduce the previously reported TDZN $H_2S$ release with conditions using 1 mM TDZN, 4 mM Cys, and 1% DMSO led to inhomogeneous solutions. Lowering the TDZN concentrations to 50 $\mu$M and Cys to 500 $\mu$M in phosphate-buffered saline (PBS) (pH 7.4, 10 mM) containing 1% DMSO at 25 °C resulted in homogeneous solutions, which were used for subsequent studies. Under these conditions, we observed 2.2 ± 0.10 $\mu$M of $H_2S$ release (4.4% efficiency) over 20 min, which qualitatively matched the reported efficiency of TDZN **1**. We next repeated this experiment using physiologically relevant concentrations of CA (25 $\mu$g/mL). If COS, rather than $H_2S$, was released directly from the TDZN, then we would expect to observe higher $H_2S$ efficiency in the presence of CA. Confirming our hypothesis, we observed 36.4 ± 1.5 $\mu$M (73% efficiency) of $H_2S$ release in the presence of CA, which strongly supports that COS release is a key pathway from $H_2S$ release in TDZNs (Figure 2). We next monitored the reaction progress by HPLC by measuring the release of diphenyl urea product from TDZN **1**. Under identical conditions as described above, we observed 46.5 $\mu$M (93% yield) of diphenyl urea as the sole organic product derived from TDZN **1**, which further supports the efficient conversion of TDZNs to release COS/$H_2S$ and the urea byproduct.

To further investigate whether TDZN substitution impacted direct COS versus $H_2S$ release, we next investigated COS/$H_2S$ release from TDZNs **2**–**4**, which have *p*-OMe, *p*-$CF_3$, and *o*-Me substituents, respectively. Much like for the parent TDZN compound, we only measured minimal $H_2S$ release for **2**–**4** in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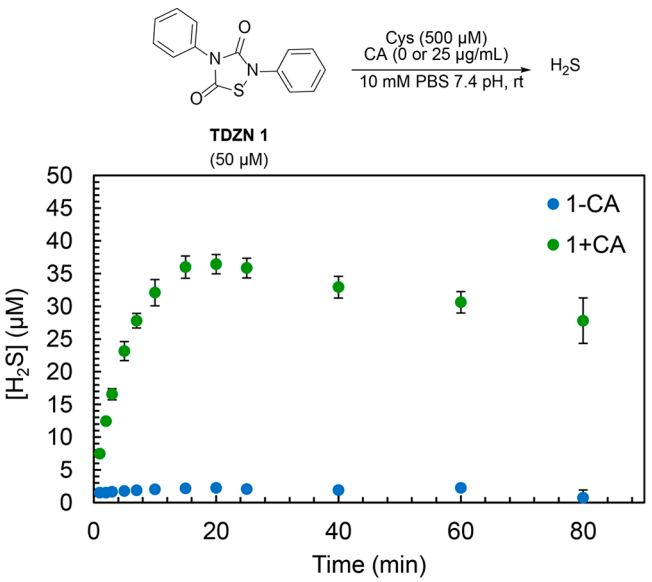


**Figure 2.** COS/$H_2S$ release from TDZN **1** (50 $\mu$M) treated with Cys (500 $\mu$M) in 10 mM PBS (pH 7.4) at room temperature, in the presence (+CA, green circle) or absence (−CA, blue triangle) of CA (25 $\mu$g/mL). The experiments were completed in triplicate, and results are expressed as mean ± SD ($n$ = 3).

the absence of CA but found that $H_2S$ generation increased significantly when CA was present. For example, treatment of TDZNs **2** and **3** with 500 $\mu$M Cys resulted in only 2.2 ± 0.23 $\mu$M $H_2S$ (4.3% efficiency) and 4.6 ± 0.82 $\mu$M (9.2%), respectively (Figure 3). By contrast, repeating the same experiments in the presence of CA (25 $\mu$g/mL) resulted in 42.4 ± 0.8 $\mu$M (84.8% efficiency) and 47.9 ± 0.3 $\mu$M (95.8% efficiency), respectively. We originally hypothesized that the electron-donating group of TDZN **2** would result in a release

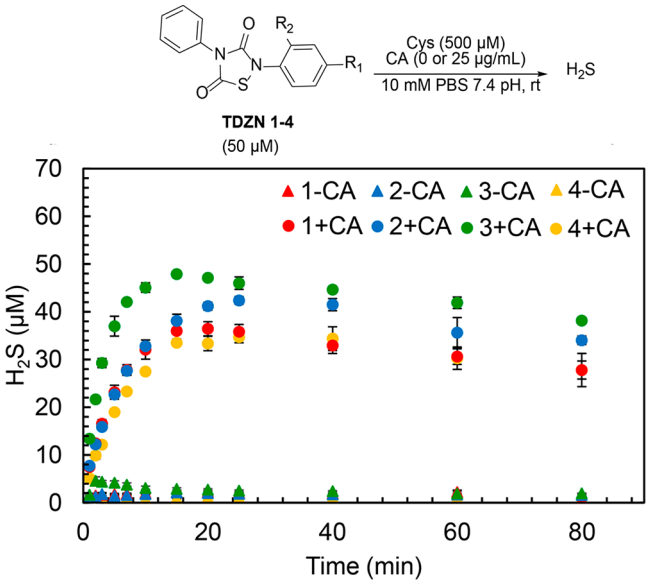


**Figure 3.** COS/$H_2S$ release from TDZNs (50 $\mu$M) **1** (red), **2** (blue), **3** (green), and **4** (gold) when treated with Cys (500 $\mu$M) in the presence (+CA, circles) or absence (−CA, triangles) of CA (25 $\mu$g/mL). The experiments were completed in triplicate in 10 mM PBS (pH 7.4) at room temperature, and results are expressed as mean ± SD ($n$ = 3).

of $H_2S$ through the COS pathway less efficient than that of TDZN **1** due to the electron-donating methoxy group; however, this was not the case. We speculated that the *p*-OMe phenyl ring may not be fully conjugated with the $H_2S$-releasing motif, which would attenuate the effective electron-donating effects of the OMe group. To further test this hypothesis, we synthesized TDZN **4** with an *o*-Me group, which should disrupt the planarity of the phenyl ring with the TDZN motif and prevent full conjugation. Consistent with this hypothesis, treatment of TDZN **4** with 500 $\mu$M Cys resulted in only 1.2 ± 0.50 $\mu$M $H_2S$ (2.3% efficiency), which increased to 34.7 ± 1.2 $\mu$M (69.4% efficiency) in the presence of CA. We also performed similar HPLC analysis of TDZNs **2**−**4** and only observed one TDZN-derived product at the end of the reaction corresponding to the urea product. Taken together, these results suggest that the electronic substitution does not significantly enhance the direct $H_2S$-releasing pathway but rather further supports that TDZN actually releases COS directly, which is then converted to $H_2S$ by CA.

Having established that TDZNs release $H_2S$ through a COS-dependent pathway, we next investigated what types of nucleophiles could activate TDZNs. For each experiment, we treated TDZN **1** (50 $\mu$M) with various nucleophiles (500 $\mu$M) in the presence of CA (25 $\mu$g/mL) and measured the $H_2S$ released using the MB assay (Figure 4). In the absence of Cys,

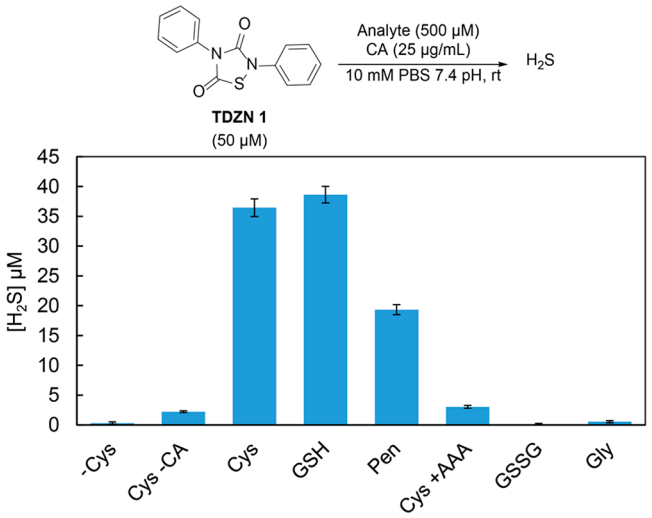


**Figure 4.** COS/$H_2S$ release from TDZN **1** (50 $\mu$M) in the presence of various analytes (500 $\mu$M) and CA (25 $\mu$g/mL, unless otherwise noted). The CA inhibitor AAA (100 $\mu$M) was used for the CA inhibition experiment. The experiments were completed in triplicate in 10 mM PBS (pH 7.4) at room temperature, and results are expressed as mean ± SD ($n$ = 3).

no $H_2S$ was generated, which confirms that CA cannot directly activate TDZNs. We next determined whether other thiols could also activate TDZNs by treating **1** with Cys, GSH, or penicillamine (Pen). In each case, significant $H_2S$ release was observed, with Cys and GSH having equal efficiencies, 36.4 ± 1.5 and 38.6 ± 1.4 $\mu$M $H_2S$, respectively, and treatment with Pen only resulting in 19.3 ± 0.8 $\mu$M $H_2S$. We also repeated the Cys experiment in the presence of the CA inhibitor acetazolamide (AAA, 100 $\mu$M), which significantly decreased $H_2S$ production, again supporting that COS is released from TDZN donors. Treatment of **1** with GSSG or Gly failed to generate $H_2S$. When taken together, these data support that


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TDZNs are stable toward nucleophiles like Gly but are activated by reduced thiols to generate COS/$H_2S$.

## CONCLUSIONS

We demonstrated that TDZN-based compounds release COS efficiently as a precursor to $H_2S$ generation, rather than release $H_2S$ directly. TDZNs are stable toward biological amine-based nucleophiles but are activated by thiols, including Cys and GSH. This work expands the list of functional groups that have been demonstrated to release COS efficiently upon activation, with further advances future applications of TDZNs as COS-based $H_2S$ donors.

## EXPERIMENTAL SECTION

**Synthesis/Spectral Details.** All reagents were used as received and were purchased from either Sigma-Aldrich, Tokyo Chemical Industry, VWR, or Cambridge Isotope Laboratories. Diethyl ether was dried with a Pure Process Technologies purification system. A Bruker 500 MHz or Bruker 600 MHz instrument was used to record $^1$H, $^{13}$C{$^1$H}, and $^{19}$F NMR spectra. Mass spectrometric measurements for TDZNs **3** and **4** were completed by the University of Illinois, Urbana—Champaign. TDZN **1** and TDZN **2** were prepared as reported previously, and the spectral data match previous reports.[25] HPLC experiments were conducted on an Agilent 1260 Infinity II HPLC with a InfinityLab Poroshell 120 EC-C18 2.7 $\mu$m, 4.6 × 100 mm column, 10 $\mu$L injections.

**$H_2S$ Detection Materials and Methods.** Buffered solutions (140 mM NaCl, 3 mM KCl, 10 mM phosphate, pH 7.4) in DI water were prepared with PBS tablets. PBS solutions were sparged with nitrogen and stored in a nitrogen-filled glovebox. An Agilent Cary 60 UV/vs spectrophometer was used for all absorbance measurements.

*$H_2S$ Quantification.* Under nitrogen, a 10 mM NaSH stock solution was prepared in PBS, and this solution was further diluted to 1.0 mM. To prepare a $H_2S$ calibration curve, individual cuvettes were charged with 0.5 mL of methylene blue cocktail, 0.5 mL of PBS containing 1% DMSO, 500 $\mu$M Cys, and CA (25 $\mu$g/mL final concentration). Each 0.5 mL aliquot of the methylene blue cocktail contains 100 $\mu$L of 1% (w/v) $Zn(OAc)_2$, 200 $\mu$L of *N*,*N*-dimethyl-*p*-phenylenediamine (20 mM) in 7.2 M HCl, and 200 $\mu$L of $FeCl_3$ (30 mM) in 1.2 M HCl. Individual aliquots of the 1.0 mM NaSH solution were added to the individual cuvettes so that the final NaSH concentrations were 10, 20, 30, 40, and 50 $\mu$M. The solutions were mixed and incubated for 60 min, and then the absorbance at 670 nm was measured.

*$H_2S$ Release Measurements from TDZN Compounds.* In a nitrogen-filled glovebox, three vials containing 20 mL of PBS and 1% DMSO were prepared and removed from the glovebox. Each vial was then charged with CA (25 $\mu$g/mL final concentration) and the desired nucleophile (500 $\mu$M final concentration) using a syringe. In the trial with AAA, sufficient AAA was injected to reach a final concentration of 100 $\mu$M. Outside of the glovebox, disposable cuvettes were charged with 0.5 mL of methylene blue cocktail. To start the experiment, the desired TDZN donor (50 $\mu$M final concentration) was injected into each vial, and 500 $\mu$L reaction aliquots were taken at the desired time points and added to the cuvettes containing the methylene blue cocktail. The $H_2S$ quantification reactions were incubated for 60 min shielded from light, after which the absorbance values at 670 nm were measured.

**TDZN HPLC Analysis Methods.** *TDZN 1.* Quantification of diphenyl urea released by TDZN **1** was completed by HPLC analysis. A vial containing 20 mL of PBS and 1% DMSO was prepared in the glovebox. Outside of the glovebox, the vial was charged with CA (25 $\mu$g/mL final concentration), Cys (500 $\mu$M final concentration), and TDZN **1** (50 $\mu$M final concentration) to start the experiment. Reaction aliquots (500 $\mu$L) were taken at desired time points and added to vials containing 500 $\mu$L of MeCN. These vials were filtered before running on the HPLC. The method used followed a flow rate of 1 mL/min with $\lambda_{detection}$ = 250 nm and a solvent system of 0.1% TFA in water with a gradient of 0−100% acetonitrile over 25 min. A calibration curve for diphenyl urea was completed under the same method. A 10 mM diphenyl urea stock solution in MeCN was prepared and diluted to 50, 40, 25, 10, and 2.5 mM. From these stock solutions, 10 $\mu$L was added to vials containing 50% MeCN/50% PBS with 1% DMSO to make final concentrations of 50, 40, 25, 10, and 2.5 $\mu$M that were filtered before HPLC analysis.

*TDZNs 2−4.* Reaction analyses for TDZNs **2**−**4** were completed by HPLC. Vials containing 20 mL of PBS and 1% DMSO were prepared in a glovebox. Stock solutions of 10 mg/mL CA, 100 mM Cys, and 50 mM TDZN were prepared. Outside of the glovebox, the reaction vial was charged with CA (25 $\mu$g/mL final concentration), Cys (500 $\mu$M final concentration), and TDZN donor (50 $\mu$M final concentration) to start the experiment. Reaction aliquots (500 $\mu$L) were removed at the desired time points and added to vials containing 500 $\mu$L of MeCN. These vials were filtered before running on the HPLC. The method used a flow rate of 1 mL/min, $\lambda_{detection}$ = 250 nm, and a gradient of 0.1% TFA in $H_2O$ to 100% MeCN over 25 min.

**Synthetic Procedures.** *TDZN 1.* TDZN **1** was synthesized as described in Method A by Severino et al.[25] with minor modifications for the isolation and purification. After refluxing with water, TDZN **1** was extracted with ethyl acetate (3 × 50 mL) The combined organic extracts were dried with $MgSO_4$ and filtered, and the solvent was reduced under pressure, leaving the crude product mixture. TDZN **1** was isolated as a white solid by column chromatography (1−20% ethyl acetate in hexanes) to yield the pure product (710 mg, 36% yield). The NMR data match those reported previously: $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ 7.59 (d, $J$ = 8.0 Hz, 2H), 7.56−7.50 (m, 2H), 7.47 (m, 5H), 7.31 (t, $J$ = 7.5 Hz, 1H); $^{13}$C{$^1$H} NMR (126 MHz, $CDCl_3$) $\delta$ 164.8, 150.5, 136.0, 132.7, 129.7, 129.6, 129.5, 127.5, 127.3, 123.7.

*TDZN 2.* TDZN **2** was synthesized as described in Method A by Severino et al.[25] and purified by column chromatography (1−20% ethyl acetate in hexanes) to yield a white solid (667 mg, 30% yield). The NMR data match those reported previously: $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ 7.53 (t, $J$ = 7.6 Hz, 2H), 7.49−7.39 (m, 5H), 6.96 (d, $J$ = 8.9 Hz, 2H), 3.83 (s, 3H); $^{13}$C{$^1$H} NMR (151 MHz, $CDCl_3$) $\delta$ 165.1, 159.1, 150.9, 132.8, 129.6, 129.5, 128.2, 127.5, 126.4, 114.9, 55.7.

*TDZN 3.* Phenyl isothiocyanate (0.88 mL, 7.4 mmol, 1 equiv) and 4-trifluoromethylphenyl isocyanate (1.0 mL, 7.4 mmol, 1 equiv) were added to $Et_2O$ (3.6 mL) at 0 °C under nitrogen. Sulfuryl chloride (0.60 mL, 7.4 mmol, 1 equiv) was dissolved in $Et_2O$ (0.1 mL) and added dropwise to the reaction mixture. The resultant reaction mixture was warmed to room temperature and stirred for 24 h. The $Et_2O$ was then removed under reduced pressure, and the isolated yellow solid was filtered. This intermediate was dissolved in 10 mL of water, refluxed for 50 min, and slowly cooled to 0 °C. TDZN **3** was isolated by filtration and purified by column chromatography in (1−20% ethyl acetate in hexanes) to yield a yellow white solid (826 mg, 33% yield): $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ 7.79−7.67 (m, 4H), 7.55 (t, $J$ = 7.5 Hz, 2H), 7.50 (t, $J$ = 7.4 Hz, 1H), 7.45 (d, $J$ = 7.2 Hz, 2H); $^{13}$C{$^1$H} NMR (126 MHz, $CDCl_3$) $\delta$: 163.9, 150.4, 139.3, 129.8, 129.7, 128.8, 128.5, 127.5, 126.9, 126.9, 124.8, 122.7; $^{19}$F NMR (471 MHz, $CDCl_3$) $\delta$ −62.52; HRMS-ESI $m/z$ [M + H]+ calcd for [$C_{15}H_{10}N_2O_2SF_3$], 339.0415; found, 339.0407.

*TDZN 4.* Phenyl isothiocyanate (0.88 mL, 7.4 mmol, 1 equiv) and *o*-tolylphenyl isocyanate (0.92 mL, 7.4 mmol, 1 equiv) were added to $Et_2O$ (3.6 mL) at 0 °C under nitrogen. Sulfuryl chloride (0.60 mL, 7.4 mmol, 1 equiv) was dissolved in $Et_2O$ (0.1 mL) and added dropwise to the reaction mixture. The resultant reaction mixture was warmed to room temperature and stirred for 24 h. The $Et_2O$ was then removed under reduced pressure, and the isolated yellow solid was filtered. The isolated intermediate was dissolved in 10 mL of water, refluxed for 50 min, and slowly cooled to 0 °C. Once at room temperature, the aqueous solution was extracted with ethyl acetate (3 × 50 mL). The combined organic extracts were dried with $MgSO_4$ and filtered. The solvent was then removed under reduced pressure to leave the crude product mixture. TDZN **4** was isolated as a white solid by column chromatography (1−20% ethyl acetate in hexanes) to yield the pure


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product (503 mg, 24% yield): $^1$H NMR (600 MHz, $CDCl_3$) $\delta$ 7.53 (t, $J$ = 7.2 Hz, 2H), 7.47−7.46 (m, 3H), 7.41 (d, $J$ = 9.2 Hz, 1H), 7.39−7.27 (m, 3H), 2.38 (s, 3H); $^{13}C\{^1H\}$ NMR (151 MHz, $CDCl_3$) $\delta$ 165.9, 151.0, 137.8, 133.5, 132.9, 131.8, 130.1, 129.6, 129.4, 128.8, 127.5, 127.4, 18.1; HRMS-ESI $m/z$ $[M + H]^+$ calcd for $[C_{15}H_{12}N_2O_2S]$, 285.0692; found, 285.0698.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.joc.2c01220.

Methylene blue data, NMR spectra, and HPLC chromatograms (PDF)

## ■ AUTHOR INFORMATION


### Corresponding Author

**Michael D. Pluth** − *Department of Chemistry and Biochemistry, Materials Science Institute, Knight Campus for Accelerating Scientific Impact, Institute of Molecular Biology, University of Oregon, Eugene, Oregon 97403, United States;* orcid.org/0000-0003-3604-653X; Email: pluth@uoregon.edu

### Author

**Haley M. Smith** − *Department of Chemistry and Biochemistry, Materials Science Institute, Knight Campus for Accelerating Scientific Impact, Institute of Molecular Biology, University of Oregon, Eugene, Oregon 97403, United States*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.joc.2c01220


### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

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