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Brief Article

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Design, Synthesis, and Evaluation of Thiol-Activated Sources of Sulfur Dioxide (SO_2) as Antimycobacterial Agents

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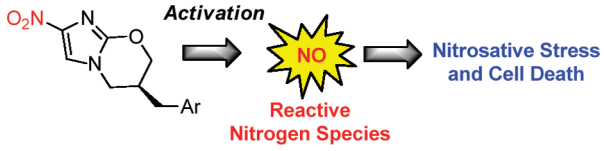
Supporting Information

ABSTRACT: Here, 2,4-dinitrophenylsulfonamides with tunable cysteine-activated SO_2 release profiles with half-lives of SO_2 release varying from 2 to 63 min are reported. *N*-Benzyl-2,4-dinitrobenzenesulfonamide (**6**), which is prepared in one step from commercial sources, had a potency (MIC = 0.15 μM) of inhibiting *Mycobacterium tuberculosis* (*Mtb*) higher than the clinical agent isoniazid (MIC = 0.37 μM).

INTRODUCTION

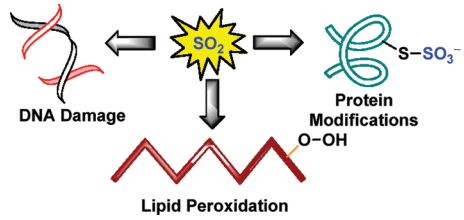
Tuberculosis affects millions each year, and coinfection with HIV is emerging as a threat of enormous proportions.[1] The first line of defense against tuberculosis, a combination of isoniazid with other antibiotics, is becoming ineffective against multi drug-resistant and extensively drug-resistant strains of *Mycobacterium tuberculosis* (*Mtb*).[2] Despite identification of molecular targets based on genomic, proteomic, and bioinformatic information relevant to *Mtb*, no new antibiotic has been approved for human use in more than four decades.[3] Thus new and novel approaches to combat this highly adaptive bacterium are necessary. The nitroimidazole (6*S*)-2-nitro-6-{[4-(trifluoromethoxy)benzyl]oxy}-6,7-dihydro-5*H*-imidazo[2,1-*b*]-[1,3]oxazine (PA-824, Scheme 1)[4] is a promising tuberculosis drug candidate in phase III clinical trials and was recently demonstrated to be a deazaflavin-dependent nitroreductase-activated prodrug of reactive nitrogen species such as nitric oxide (NO).[5] Although toxic at elevated concentrations, controlled delivery of nitric oxide by the use of reliable surrogates known as nitric oxide donors has been recognized as a potential therapeutic agent in targeting several diseases including cancer.[6]

Scheme 1. Nitroimidazole-Based Tuberculosis Drug Candidates Generate Reactive Nitrogen Species Such as NO



Like NO, sulfur dioxide (SO_2) is an environmental pollutant and is toxic at elevated concentrations.[7] Although mechanisms of its cytotoxicity are yet unclear, SO_2 at elevated concentrations is known to induce oxidative damage to biomacromolecules such as proteins, lipids, and DNA (Scheme 2).[8] Its anionic form, sulfite also reacts with dithiols to form *S*-sulfonates, which could affect thiol levels and hence redox balance in cells (Scheme 2).[9] Oxidation of SO_2 to sulfate is known to occur through radical intermediates (such as $\cdot SO_3$), which in turn can damage biomacromolecules.[8]

Scheme 2. Possible Biological Targets of Sulfur Dioxide



However, despite its well-documented cytotoxic effects, SO_2 has been routinely used as an antibiotic and antioxidant in wine-making.[10] Barring certain individual cases of allergies, sulfur dioxide is well-tolerated in humans; in certain meats that are consumed on a daily basis, SO_2 levels can reach up to 450 mg kg^{-1}.[9] Thus, it was envisaged that the susceptibility of bacteria to the deleterious effects of SO_2 could be exploited to develop new SO_2-based tuberculosis drug candidates. To tap its therapeutic potential and possibly avoid undesirable side effects, controlled delivery of SO_2 is necessary. The poor bioavailability of gaseous sulfur dioxide precludes its usage for therapeutic purposes and the use of complex inorganic sulfite mixtures, typically used to generate SO_2 in biological systems, suffers from a lack of control of rate and amount of SO_2 generated.[11] As there were no reliable SO_2 sources available, we proposed to develop organic donors of sulfur dioxide with tunable release profiles in order to evaluate their efficacy as against *Mtb*.

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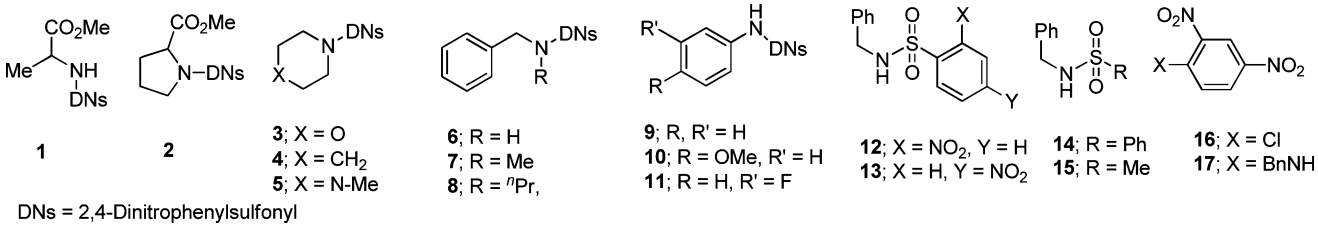
Chart 1



DNs = 2,4-Dinitrophenylsulfonyl

Fukuyama and co-workers reported that amines can be protected by the reaction with 2,4-dinitrophenylsulfonyl chloride (DNsCl) as 2,4-dinitrophenylsulfonamides.[12] Deprotection of such amides is carried out by thiols such as 2-mercaptoethanol in basic medium to produce amines in excellent yields.[12] Although not characterized in the aforementioned report, a byproduct of these deprotection reactions is sulfur dioxide.

2,4-Dinitrophenylsulfonamides have also been used in thiol-detection systems for environmental and biological applications.[13] Furthermore, variation of the groups on the amine may provide a handle for modulating SO_2 release profiles. Unlike mammalian cells, *Mtb* does not contain glutathione (GSH) but mycothiol (MSH) as the primary thiol in millimolar concentrations.[14] MSH is critical for the maintenance of redox homeostasis and alteration of thiol-levels could induce stress to *Mtb*.[15] Upon reaction with a 2,4-dinitrophenylsulfonamide, MSH is expected to be arylated to generate sulfur dioxide intracellularly. Thus this two-pronged strategy of introduction of SO_2 and a thiol-depleting agent could inhibit *Mtb* growth.

RESULTS AND DISCUSSION

2,4-Dinitrophenylsulfonamides **1**–**6** of primary and secondary amines were prepared (Chart 1).[12] To study the effect of increasing sterics on the nitrogen bearing the sulfonamide on its reactivity toward thiols, *N*-methyl (**7**) and *N*-propyl (**8**) derivatives were prepared by alkylation of **6** (Chart 1). With an aim of studying the effect of electronic modulation of the aromatic ring attached to the DNs group on the rate of SO_2 generation, aniline derivatives **9**–**11** were prepared by the reaction of the corresponding amine with DNsCl. Finally, to study the effect of removal of one or both the aryl nitro groups and replacement of the aryl ring with a methyl group, compounds **12**–**15** were prepared (Chart 1).

SO_2 from environmental and biological samples is typically quantified by estimation of sulfite, the anionic form of SO_2 in basic media (eq 1).[16] Treatment of compounds with cysteine in pH 7.4 buffer produced SO_2, which was detected using a *p*-rosaniline-based assay.[17] Next, we used an ion chromatograph (IC) equipped with a conductivity detector for quantitative SO_2 analysis as sulfite.[18] Cysteine-mediated decomposition of compounds **1**–**15** in pH 7.4 buffer was carried out. All 2,4-dinitrosulfonamides (100 μM) tested were found to generate sulfite (24–100 μM) in 30 min (Table 1, entries 1–15). The derivatives **12** and **13**, which had one nitro group on the aryl ring, did not produce any detectable levels of SO_2 even after 4 h and neither did the analogues **14** and **15** (Table 1, entries 12–15).

(1)

Table 1. SO_2 Analysis during Thiol-Mediated Decomposition, And Antimycobacterial Activity of 2,4-Dinitrosulfonamides and Related Analogues

entry	compd	SO_2 source?[a]	SO_2 yield, 30 min (μM)[b]	MIC (μg mL^{-1})[c]	MIC (μM)
1	**1**	yes	93	6.25	18.7
2	**2**	yes	83	12.5	35
3	**3**	yes	96	6.25	19.6
4	**4**	yes	91	0.78	2.5
5	**5**	yes	88	12.5	38
6	**6**	yes	100	0.05	0.15
7	**7**	yes	100	0.4	1.1
8	**8**	yes	88	3.13	8.25
9	**9**	yes	55	3.13	9.7
10	**10**	yes	79.5	1.56	4.4
11	**11**	yes	24	25	73
12	**12**	no	0	>50	>100
13	**13**	no	0	>50	>100
14	**14**	no	0	>50	>100
15	**15**	no	0	>50	>100
16	$BnNH_2$	no		>100	>250
17	BnNHMe	no		>100	>250
18	**16**	no	0	6.25	30.8
19	**17**	no	0	>100	>250
20	isoniazid			0.05	0.37

[a]Sulfur dioxide was detected using a pararosaniline-based colorimetric assay. [b]Sulfur dioxide as sulfite was quantified using an ion chromatograph equipped with a conductivity detector: yields are 30 min after treatment of compound (100 μM) with 10 equiv of cysteine in pH 7.4 phosphate buffer. [c]Minimum inhibitory concentration (MIC) is the minimum concentration of the compound required to inhibit 99% of bacterial growth and was found against *Mycobacterium tuberculosis* $H_{37}R_v$ strain.

To test the hypothesis that sulfur dioxide sources could inhibit *Mtb*, compounds **1**–**15** were screened for their antimycobacterial activity against *Mycobacterium tuberculosis* $H_{37}R_v$ and minimum inhibitory concentrations (MICs) were determined. In this assay, six compounds were found to have MICs < 10 μg mL^{-1} (Table 1, entries 1–15). The compounds **1**–**11**, which all produced SO_2 upon reaction with cysteine, were found to have greater inhibitory activity (MIC ≤ 25 μg mL^{-1}) than **12**–**15** that were unreactive to cysteine (MIC > 50 μg mL^{-1}, Table 1, entries 12–15). These results clearly indicate a correlation between the analogue's ability to release sulfur dioxide within 30 min and its *Mtb* inhibitory potency.

The best *Mtb* inhibitor in this series was the benzylamine-derivative **6** with MIC of 0.05 μg mL^{-1} (0.15 μM), which was better than the MIC of isoniazid (0.05 μg mL^{-1}, 0.37 μM) determined under similar conditions (Table 1). Hence, further studies conducted were focused on understanding the mechanisms of efficacy of this compound. Upon reaction

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with a number of biologically relevant nucleophiles such as amino acids and nucleosides in pH 7.4 buffer, a nearly quantitative recovery of **6** was observed (HPLC analysis), suggesting that **6** was selectively reactive to thiols under physiological pH (See Supporting Information). Previous reports on 2,4-dinitrosulfonamides also show the thiol-selectivity of this class of compounds under biologically relevant conditions.[13] The decomposition of **6** in the presence of thiols generates sulfur dioxide, benzylamine, and the arylated thiol.[19] Benzylamine, a decomposition product of **6** did not show a significant inhibition of growth of *Mtb* at 100 μg mL^{-1} (Table 1, entry 16); a similar observation for *N*-methylbenzylamine was recorded (Table 1, entry 17),[20] and sodium sulfite was inactive against *Mtb* at 100 μg mL^{-1}. To understand if combinations of decomposition products were responsible for *Mtb* inhibitory activity, a mixture of cysteine and **6** in pH 7.4 buffer approximately 2 h post mixing was tested at 2 μg mL^{-1}; this mixture was found to be inactive against *Mtb*. These results demonstrated that sulfur dioxide formation (and not sulfite) intracellularly contributes to the *Mtb* inhibitory activity of **6**.

At the outset, our goal of preparing sources of sulfur dioxide was 2-fold: one was to determine if SO_2 could inhibit *Mtb* growth and the other was to determine if the rate of SO_2 generation had any role in inhibitory activity. Our initial data clearly showed the potential of SO_2 to inhibit *Mtb* growth. Next, to study the effect of rate of SO_2 generation on the *Mtb* inhibitory activity, the time courses of cysteine-mediated SO_2 generation from compounds **6**–**11** (Chart 1), which have comparable estimated cell permeability (−clogP, Table 2), were

Table 2. Calculated −clogP, Kinetic Parameters for SO_2 Release, Maximum SO_2 Yields, and pK_{aH}s of Amines

entry	compd	−clogP[a]	k (min^{-1})[b]	$t_{1/2}$ (min)[c]	max SO_2 yield (μM)[d]	pK_{aH}[e]
1	**6**	2.87	*f*	2[g]	100	9.34
2	**7**	2.41	*f*	4[g]	100	9.58
3	**8**	3.47	0.1517	4.6	96	9.68
4	**9**	2.76	0.0273	25	94	4.64
5	**10**	2.69	0.0575	12	97	5.29
6	**11**	2.93	0.0106	63	86	3.38

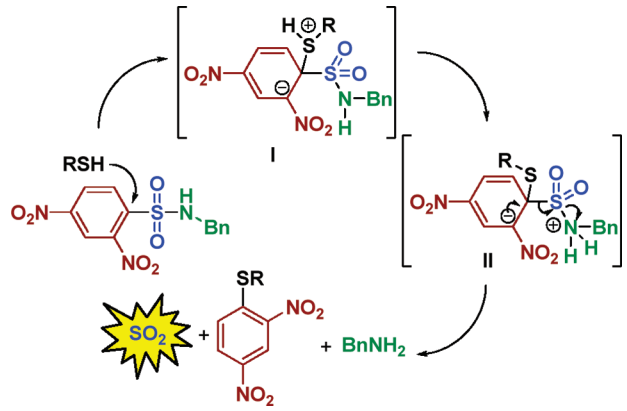
[a]Calculated using Chembiodraw Ultra. [b]Rate analysis of sulfite release from the compound (100 μM) in the presence of cysteine (10 equiv) in pH 7.4 phosphate buffer (20 mM). [c]Half-life was estimated from rate constants. [d]Maximum amount of sulfur dioxide generated the during the reaction with no further increase in total sulfur dioxide; values reported are a sum of sulfite and sulfate (minor, see Supporting Information). HPLC analysis showed complete disappearance of 2,4-dinitrophenylsulfonamide. [e]Values are for the amine without a DNs group. [f]Accurate rate constant could not be determined. [g]Approximate half-life estimated based on yields of sulfur dioxide.

determined and compared with their antimycobacterial activities.

All compounds tested were found to be excellent sources of SO_2 with maximum yields of SO_2 ranging from 86 to 100 μM (Table 2). For **6** and **7**, under the assay conditions, accurate determination of rate constant (k) for SO_2 formation was not possible; the $t_{1/2}$ for **6** was estimated to be 2 min; and for **7**, $t_{1/2}$ was 4 min (Table 2). SO_2 release during cysteine-mediated decomposition of **8**–**11** followed pseudo-first-order kinetics with half-lives ranging from 4.6 to 63 min (Table 2).

The following mechanism of SO_2 generation was considered (Scheme 3). Thiol-attack on the 2,4-dinitrophenylsulfonamide

Scheme 3. Proposed Mechanism for Thiol-Mediated SO_2 Generation from 6



produces intermediate **I**, which converts to intermediate **II** by a proton transfer; collapse of **II** produces SO_2. The proposed mechanism would predict that the rate constant for SO_2 generation (k) would depend on the stability of the transition state leading to the formation of the protonated amine intermediate **II**. Hence, the stronger the basicity (or weaker the conjugate acid), the greater would be the tendency of nitrogen bearing the 2,4-dinitrophenylsulfonyl group to get protonated and leave as the amine resulting in a higher k. As basicity data for 2,4-dinitrosulfonamides was unavailable, we compared the pK_{aH}s of benzylamine, *N*-alkylbenzylamines, aniline, 4-methoxyaniline, and 3-fluoroaniline, with the k for their corresponding DNs derivative, **6**–**11** (Table 2, entries 1–6).[21] Consistent with the proposed mechanism, a comparison of k and pK_{aH}s revealed that an increased amine pK_{aH} resulted in higher rates of SO_2 release (See Supporting Information). A comparison of estimated half-life of SO_2 generation ($t_{1/2}$) and MICs showed that the fluoro derivative **11**, which was the longest among the compounds tested, was also the least potent of the 2,4-dinitrophenylsulfonamides that we evaluated in this study (Table 1). Further analysis of data for **6**–**11**, which have comparable −clogP values, showed that a higher rate of SO_2 generation (k) correlated well with better inhibitory activity (see Supporting Information). Taken together, these observations may provide us a rational basis for further design of analogues of **6** with antimycobacterial activity. However, without data pertinent to drug uptake of these compounds in *Mtb*, it is not possible to attribute differences in MICs of **6**–**11** only to differences in rates of cysteine-mediated SO_2 generation from these compounds.

To test the selectivity of SO_2 inhibitory activity toward *Mtb*, a cell viability assay with conducted using human embryonic kidney 293 cells (HEK) cell lines and the IC_{50} for **6** was determined as 7 μM (See Supporting Information). Based on the MIC and IC_{50}, **6** was nearly 50-fold selective in inhibitory activity (SI = 47) toward *Mtb* over human embryonic kidney cells.

In addition to generation of SO_2, the reaction of **6** with thiols could also affect cellular redox balance by mycothiol depletion in *Mtb*. The possible role of thiol-depletion in the observed efficacy of **6** was determined by testing 1-chloro-2,4-dinitrobenzene (**16**, Chart 1) against *Mtb*; the MIC of **16**

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was found as 6.25 μg mL^{-1} (Table 1, entry 18). The analogue of **6** without a sulfonyl group **17** (Chart 1), which is unreactive with thiols showed no *Mtb* inhibitory activity (Table 1, entry 19). These results support a role for thiol depletion and perhaps protein *S*-arylation as a contributor to the observed antibacterial activity of **6**.

Thiol-based metabolic activation mode of **6** is similar to glutathione-activated nitric oxide prodrugs, which have shown potent tumoristatic activity in several animal models with favorable toxicological profiles.[22] In addition to thiol depletion, possible mechanisms of antimycobacterial activity of **6** include SO_2's ability to induce stress by affecting cellular redox equilibrium and causing damage to biomacromolecules such as lipids, proteins, and DNA.[8] In its diversity and multitude of cellular targets, SO_2 is comparable with NO,[5] and like NO prodrugs perhaps, organic SO_2 donors may also have broad therapeutic applications.[6] We are currently exploring new strategies to trigger SO_2 release under other metabolic conditions and evaluate the therapeutic potential of such compounds. Recently, sulfur dioxide has been shown to have vasodilatory activity similar to that of NO and in synergy with NO, suggesting that development of new donors of SO_2 may also have cardiovascular applications.[23] Future work will include synthesizing and evaluating nitric oxide−sulfur dioxide hybrid compounds for therapeutic applications. Finally, a major impediment to development of new drugs for infectious diseases is the cost of multistep synthesis to prepare potential drug candidates. The compounds prepared in this study can be obtained in one step from relatively inexpensive commercial sources, making them especially attractive for further development as potential drug candidates.[24] In summary, we provide evidence for the utility of masked sources of sulfur dioxide as antimycobacterial agents and whose *Mtb* inhibitory activity in part depended on the rate of thiol-mediated sulfur dioxide generation.

EXPERIMENTAL SECTION

General Procedure for the Synthesis of 2,4-Dinitrophenylsulfonamides. A solution of the amine in DCM containing triethylamine or pyridine was treated with a solution of DNsCl in DCM at 0 or −40 °C. The reaction mixture was warmed to RT. Work up included extraction with DCM or EtOAc followed by silica gel chromatography using mixtures of PE/EtOAc or DCM as the eluant to produce the desired compound as a yellow solid (unless otherwise stated).

Cysteine-Activated Sulfur Dioxide Release. *P*-Rosaniline assay for sulfur dioxide: The dye was prepared using a literature procedure.[17] Assay conditions: 900 μL of *p*-rosaniline-based dye was mixed with 50 μL of satd $HgCl_2$ solution and 50 μL of 0.2 mM sulfite. This solution was covered with aluminum foil for 15 min until violet color developed and the absorbance was measured. A similar protocol was used for compounds which were treated with 10 equiv cysteine in pH 7.4 phosphate buffer containing 1−5% DMSO (or ethanol).

Ion chromatography analysis: An ion chromatograph attached with a conductivity detector was used for sulfite analysis. One mM $NaHCO_3$/3.2 mM Na_2CO_3 was the eluant, and the flow rate was 0.7 mL/min. Using stock solutions of sulfite, a calibration curve was generated (R^2 = 0.9999). To 3 mL of 1 mM stock solution of compound in acetonitrile, 24 mL of phosphate buffer (pH = 7.4, 20 mM) was added and vortexed for 20 s. To this mixture, 3 mL of 10 mM cysteine solution (pH 7.4) was added, and the reaction mixture was stirred at RT under inert atmosphere. Aliquots at appropriate time intervals were analyzed by IC. Maximum sulfur dioxide yield was calculated based on completion of the reaction with no further increase in sulfite formation. In all these cases, when sulfur dioxide reached a maximum, HPLC analysis of the reaction mixture showed complete disappearance of the 2,4-dinitrophenylsulfonamide.

Antimycobacterial Activity Assay. Ten-fold serial dilutions of each test compound/drug were prepared and incorporated into Middlebrook 7H11 agar medium with OADC growth supplement. Inoculum of *Mycobacterium tuberculosis* $H_{37}R_v$ were prepared from fresh Middlebrook 7H11 agar slants with OADC growth supplement adjusted to 1 mg mL^{-1} (wet weight) in Tween 80 (0.05%) saline diluted to 10^{-2} to give a concentration of approximately 107 cfu mL^{-1}. Five μL of bacterial suspension was spotted into 7H11 agar tubes containing 10-fold serial dilutions of drugs per mL. The tubes were incubated at 37 °C, and final readings were recorded after 28 days. The minimum inhibitory concentration (MIC) is defined as the minimum concentration of compound required to completely inhibit the bacterial growth.

ASSOCIATED CONTENT

Supporting Information

General procedures, characterization data for all new compounds, decomposition studies, sulfur dioxide analysis data, and biological assay procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS USED

Mtb, *Mycobacterium tuberculosis*; DNS, 2,4-dinitrophenylsulfonyl; HPLC, high performance liquid chromatography; MIC, minimum inhibitory concentration; IC_{50}, 50% inhibitory concentration; SI, selectivity index

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