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Thiol activated prodrugs of sulfur dioxide (SO_2) as MRSA inhibitors

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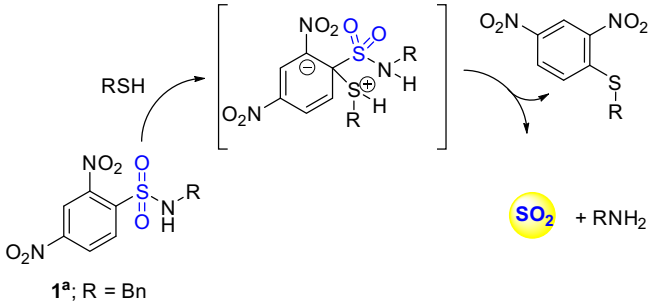
ABSTRACT

Drug resistant infections are becoming common worldwide and new strategies for drug development are necessary. Here, we report the synthesis and evaluation of 2,4-dinitrophenylsulfonamides, which are donors of sulfur dioxide (SO_2), a reactive sulfur species, as methicillin-resistant *Staphylococcus aureus* (MRSA) inhibitors. *N*-(3-Methoxyphenyl)-2,4-dinitro-*N*-(prop-2-yn-1-yl)benzenesulfonamide (**5e**) was found to have excellent in vitro MRSA inhibitory potency. This compound is cell permeable and treatment of MRSA cells with **5e** depleted intracellular thiols and enhanced oxidative species both results consistent with a mechanism involving thiol activation to produce SO_2.

Although the advent of modern antibiotics has stemmed the number of deaths due to bacterial infections, the increased occurrence of drug-resistant bacterial infections is a major global health concern. *Staphylococcus aureus* (*S. aureus*) is a Gram-positive bacterium that can be effectively treated with a number of drugs including the methicillin class of antibiotics.[1,2] However, the emergence of methicillin-resistant *S. aureus* (MRSA) is a source of concern with millions diagnosed with MRSA infections each year. Although efforts to develop new drug candidates with novel mechanisms of action have intensified, the growing number of multidrug resistant MRSA infections has further accentuated the problem of antibiotic resistance necessitating new strategies to target such drug-resistant bacteria.[1–7]

Sulfur dioxide (SO_2) is a gaseous environmental pollutant produced during volcanic eruptions and fossil fuel combustion.[8] At elevated levels, SO_2 is toxic to cells and among the various modes for cellular stress induction by SO_2, perturbation of redox homeostasis through generation of oxidative species has been reported.[9,10] For example, during auto-oxidation of hydrated forms of SO_2, sulfite and bisulfite to sulfate, a number of radical species are produced.[11–15] Hence, introduction of this reactive sulfur species intracellularly might result in irreversible change to the redox equilibrium in cells.[16] The resultant oxidative stress might be difficult for the pathogen to overcome.[17–19] The widespread use of sulfites as preservatives and anti-bacterial agents in the food industry suggests a favorable toxicological outcome as well.[10,20,21] Furthermore, sulfites are normally well tolerated and human exposure at levels of 450 mg/kg is permissible.[10,20]



1[a]; R = Bn

Scheme 1. 2,4-Dinitrosulfonamides react with thiols to produce sulfur dioxide.

Our laboratory has recently developed thiol-activated sulfur dioxide (SO_2) donors which were found to inhibit *Mycobacterium tuberculosis* (*Mtb*) growth.[22,23] A mechanism based on attack of thiol on the aromatic ring to produce a Jackson–Meisenheimer complex, which decomposed to produce SO_2, benzylamine and 2,4-dinitrophenylthioether was proposed (Scheme 1).[22,23] *N*-Benzyl-2,4-dinitrosulfonamide (**1a**) was found to be the most potent in vitro inhibitor with a low micromolar minimum inhibitory concentration (MIC).[22,23] Having established the potential for SO_2 to inhibit *Mtb*, here, we proposed to test the hypothesis that organic sources of SO_2 might be capable of inhibiting growth of

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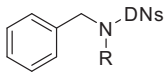
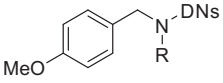
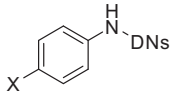
other Gram-positive bacteria as well including drug-resistant strains.



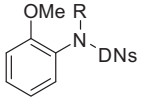
1a; R = H
1b; R = Me
1c; R = Propargyl
1d; R = $CH_2CH_2CH_2OH$



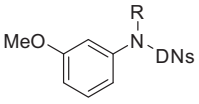
2a; R = H
2b; R = Propargyl



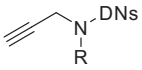
3a; X = H
3b; X = CN
3c; X = F
3d; X = OMe



4a; R = H
4b; R = Me
4c; R = Et
4d; R = ^{n}Pr
4e; R = ^{n}Bu
4f; R = Allyl
4g; R = Propargyl
4h; R = Bn



5a; R = H
5b; R = Me
5c; R = Et
5d; R = Allyl
5e; R = Propargyl

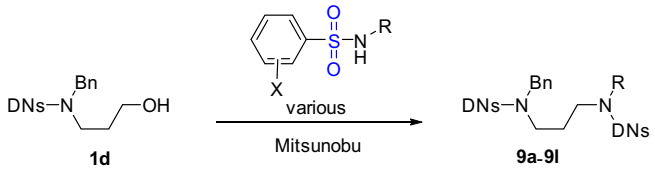


8a; R = Me
8b; R = Et
8c; R = 3-OMeBn

A library of 2,4-dinitrobenzenesulfonyl (DNs) derivatives were prepared using reported methods.[22,23] In addition, bis(2,4-dinitrophenylsulfonamides) **9a–9i** which are expected to produce twice the number of moles of sulfur dioxide generated per mole of compound were synthesized from Mitsunobu reaction of **1d** (Table 1). These compounds would be suitable for testing the effect of increasing the payload of SO_2 on inhibitory activity. Compounds **12a–12c** which contained one nitro group on the arylsulfonamide ring were synthesized from benzylamine; Mitsunobu reaction conditions gave **9j–9l** in moderate yields (Table 1).

Next, sulfur dioxide produced during thiol-mediated decomposition of these compounds was studied. Sulfur dioxide in basic pH is converted to sulfite, which is quantified using an ion chromatograph attached with a conductivity detector. Using this reported protocol,[23] SO_2 yields from compounds synthesized in this study in the presence of 10 equiv cysteine after 30 min incubation were recorded (Table 2). We find that a majority of the compounds in the **1–8** series containing one DNs group were capable of generating >80% yield of SO_2. The compounds **3b** and **3c**, which contained an electron withdrawing group gave diminished yields of SO_2 in comparison with **3d** which contained an electron donating group (Table 2, entries 8–10).[23] This result is consistent with our previously described mechanism for SO_2 generation (see Supporting information, Table S1 and Fig. S3).[22]

Table 1
Synthesis of **9a–9l**



R	X	Compd	Product	% yield
$PhCH_2$	2,4-Dinitro	**1a**	**9a**	15
2-OMe$PhCH_2$	2,4-Dinitro	**10a**	**9b**	14
4-OMe$PhCH_2$	2,4-Dinitro	**2a**	**9c**	15
2,4-Dimethoxy$PhCH_2$	2,4-Dinitro	**11a**	**9d**	19
Ph	2,4-Dinitro	**3a**	**9e**	17
2-FPh	2,4-Dinitro	**3e**	**9f**	14
2-OMePh	2,4-Dinitro	**4a**	**9g**	14
3-OMePh	2,4-Dinitro	**5a**	**9h**	15
4-OMePh	2,4-Dinitro	**3d**	**9i**	10
$PhCH_2$	2-Nitro	**12a**	**9j**	29
$PhCH_2$	3-Nitro	**12b**	**9k**	36
$PhCH_2$	4-Nitro	**12c**	**9l**	20

Table 2
Sulfur dioxide yields, calculated partition coefficients and MICs determined against *S. aureus*

Entry	Compd	% SO_2 yield[a]	clog*P*[b]	MIC, μg/mL[c]
1	**1a**	100	2.86	>16
2	**1b**	100	2.41	8
3	**1c**	100	3.01	4
4	**1d**	87	2.19	16
5	**2a**	84	2.78	>16
6	**2b**	85	2.93	8
7	**3a**	55	2.76	8
8	**3b**	5	2.28	>16
9	**3c**	55	2.92	16
10	**3d**	80	2.69	>16
11	**4a**	86	2.69	8
12	**4b**	77	2.05	16
13	**4c**	86	2.58	8
14	**4d**	Quant	3.11	>32
15	**4e**	Quant	3.64	>32
16	**4f**	83	2.90	8
17	**4g**	91	2.65	4
18	**4h**	96	3.82	>32
19	**5a**	76	2.69	16
20	**5b**	97	2.12	8
21	**5c**	98	2.65	8
22	**5d**	99	2.90	8
23	**5e**	89	2.72	4
24	**8a**	Quant	1.24	8
25	**8b**	97	1.77	16
26	**8c**	95	2.30	16
27	**9a**	77[d]	5.41	>16
28	**9b**	100[d]	5.33	>32
29	**9c**	quant[d]	5.33	>32
30	**9d**	99[d]	5.06	>16
31	**9e**	87[d]	4.96	>16
32	**9f**	98[d]	5.01	>32
33	**9g**	100[d]	5.08	>32
34	**9h**	quant[d]	5.08	>32
35	**9i**	quant[d]	5.42	>32
36	**9j**	45[d]	5.67	>32
37	**9k**	51[d]	5.85	>32
38	**9l**	47[d]	5.85	>16
39	Vancomycin			0.5–1

[a] 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 eq. of cysteine in pH 7.4 phosphate buffer.
[b] Calculated using Chembiodraw Ultra.
[c] MIC was determined using a broth dilution method.
[d] 50 μM of compound was used for determination of SO_2 yield.

Among the bis(2,4-dinitrophenylsulfonamides) **9a**–**9i**, all compounds gave nearly 2 mol of SO_2 per mol of compound (Table 2, entries 27–35). Consistent with previous observations that both nitro groups were necessary for decomposition, we found that **9j**–**9l** gave 1 mol of SO_2 per mol of compound (Table 2, entries 36–38).[22,23]

Using a standard microbroth dilution protocol, the DNs derivatives synthesized in this study were tested for their inhibitory activity against methicillin sensitive *S. aureus* (MSSA) and MICs were recorded (Table 2).[24] The SO_2 donor **1a** which had high potency against *Mtb* was inactive against MSSA at 16 μg/mL (Table 2, entry 1). Other derivatives containing a primary amine DNs functional group were similarly inactive or were found to be moderate or good inhibitors of MSSA with MICs of ⩽8 μg/mL (Table 2, entries 2, 3, 6, 7, 11, 13, 16, 17 and 20–24). The best inhibitors of MSSA were found to be **1c**, **4g** and **5e** all containing a propargyl substituent (Table 2, entries 3, 17 and 23). The MICs of the remaining compounds in this series were all ⩾8 μg/mL (Table 2). None of the bis(2,4-dinitrophenylsulfonamides) synthesized in this study were found to have significant inhibitory activity against MSSA (Table 2, entries 27–35). Similarly compounds **9j**–**9l** were incapable of inhibiting MSSA growth (Table 2, entries 36–38). The presence of a propargyl group appears to enhance efficacy. For example, in the cases of the benzylamine derivative **1a**, a significant increase in potency was seen when a propargyl group (**1c**) was introduced (Table 2, entries 1 and 3). A similar result was recorded with **2a** and **2b**; **5a** and **5e** (Table 2, entries 5, 6, 19 and 20). The presence of the allyl group on the other hand no significant effect on the efficacy (Table 2, entries 11, 13, 19 and 22). The partition coefficients were calculated ($c\log P$) as an estimate of permeability of these compounds. However, no significant trend between $c\log P$ and MIC was observed in this series. Together, these data indicate no observable correlation between the ability of the compound to generate SO_2 in a test tube (see Table S1, Supporting information), or its calculated partition coefficient ($c\log P$) and its inhibitory potency but show some sensitivity to introduction of key substituents such as the propargyl group.

Table 3
MICs against MRSA, *E. faecalis* and *E. coli*

Entry	Compd	MIC[a], MRSA	MIC[a], *E. faecalis*	MIC, *E. coli*
1	**1c**	4	16	>32
2	**1d**	8	32	>32
3	**2b**	8	16	>32
4	**4a**	8	16	>32
5	**4b**	16	16	>32
6	**4c**	8	>32	>32
7	**4d**	>32	>32	>32
8	**4e**	>32	>32	>32
9	**4f**	4	>32	>32
10	**4g**	8	8	>32
11	**4h**	>32	>32	>32
12	**5a**	32	>32	>32
13	**5b**	4	8	>32
14	**5c**	8	8	>32
15	**5d**	8	16	>32
16	**5e**	4	8	>32
17	**8a**	8	>32	>32
18	**8b**	16	>32	>32
19	**9b**	>32	>32	>32
20	**9c**	>32	>32	>32
21	**9d**	>32	>32	>32
22	Linezolid	1	2	—
23	Ciprofloxacin	0.25	—	⩽0.00075
24	Vancomycin	2	—	—
25	Meropenem	16	—	—
26	Tobramycin	>32	—	—

[a] MIC was determined using a broth dilution method and are reported in μg/mL.

When tested against MRSA, we found that **5b** and **5e** were excellent inhibitors with an MIC of 4 μg/mL (Table 3, entries 13 and 16). In order to determine if these compounds had a broad-spectrum antibacterial activity, this library was tested against *Enterococcus faecalis* (*E. faecalis*) and *Escherichia coli* (*E. coli*). A majority of the compounds in the **1**–**8** series tested were found to have moderate MICs against *E. faecalis* but none of the compounds were capable of inhibiting *E. coli*. Compounds **4g**, **5b** and **5e** were found to be good inhibitors of *E. faecalis* with MICs of 8 μg/mL (Table 3, entries 10, 13 and 16). Based on these results, **5e** was identified as the lead compound and further studies were directed toward this SO_2 donor.

Our hypothesis was that the antibacterial activity of SO_2 prodrugs is mediated by compound's entry into cells followed by activation by thiols to generate SO_2, which can possibly induce stress through enhanced oxidative species. If the SO_2 donor permeated bacterial cells, depletion of intracellular thiols might result by reaction with the 2,4-dinitroaryl group. In addition, thiols are primary responders to oxidative stress and are converted to disulfides. Depletion of free thiols might thus occur as a response to enhanced oxidative species as well. A monobromobimane (mBBr) protocol was used to assess levels of free thiols within cells.[25] Here, the weakly fluorescent mBBr reacts with thiols and is converted to a highly fluorescent adduct. Hence, increased fluorescence response in this assay is an indicator of greater free thiol levels inside cells and vice versa. When MRSA cells were incubated with varying concentrations of **5e**, we found a nearly dose-dependent decrease in fluorescence, compared to untreated control indicating that **5e** is capable of depleting thiols in bacteria (Fig. 1).

In a similar experiment, *N*-ethylmaleimide (NEM) a known thiol depleting agent was also used and we find a diminished fluorescence that is consistent with decreased thiol levels. Under these conditions, **8b** and **9d**, both with poor MRSA inhibitory activity (Table 3, entries 18 and 21), we find diminished capacity to deplete thiols (see Supporting information Fig. S1). These data indicate that the capacity of the compound to permeate cells to deplete free thiols played a significant role in the observed inhibitory potency.

SO_2 is known to generate oxidative species resulting in biomacromolecular damage.[11,15,26,27] We tested the ability of **5e** to produce oxidative species intracellularly using a reported 2,7-dichlorodihydrofluorescein-diacetate (DCFH2-DA) assay.[28,29] We found increased fluorescence levels intracellularly in *S. aureus* upon incubation with **5e** (Fig. 2). This result is indicative of production of oxidative species and perhaps, induction of oxidative stress[18,19,30–32] is a mechanism of action.[33,34] The results of this assay are also consistent with the thiol depletion assay described previously.

Next, we exploited the presence of a propargyl group in **5e** to independently determine if the compound permeated cells. A fluorescent azide was synthesized and using a copper-catalyzed

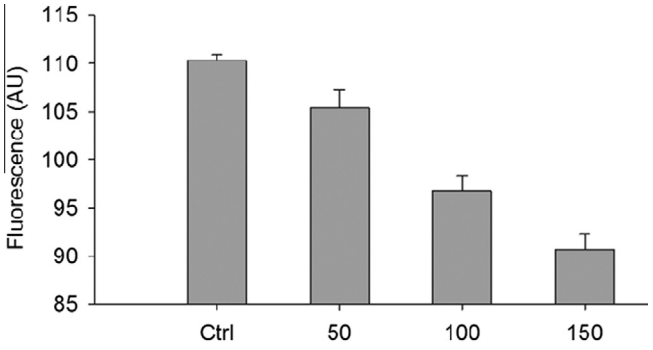


Figure 1. Intracellular thiol depletion in *S. aureus* upon treatment with the **5e** (0, 50, 100 and 150 μM) measured after 60 min of incubation at 37 °C using monobromobimane (mBBr) based fluorescence assay.

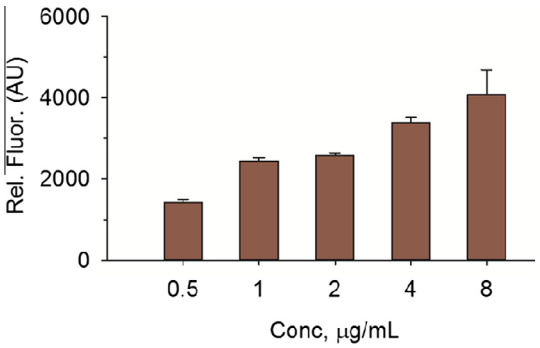


Figure 2. The dichlorofluorescein-diacetate ($DCFH_2$-DA) fluorescence assay was used to estimate the levels of oxidative species generated intracellularly in *S. aureus* upon exposure to **5e**.

Table 4
MICs of **5e** determined against patient-derived MRSA strains and reference MICs for the strain

Compd	MRSA 7419	B19506	MRSA K-1	MRSA 7425	MRSA 7386
5e	4	2	4	2	2
Vancomycin	1	1	1	1	2
Linezolid	2	4	2	4	2
Ciprofloxacin	>4	32	0.5	>4	>4
Meropenem	16	1	0.5	4	>32
Tobramycin	>32	>32	8	1	>32

[a]MIC is expressed in μg/mL.

1,3-dipolar cycloaddition reaction or 'click' reaction and a HPLC with a fluorescence detector, we provide evidence for the permeability of this compound in MRSA (see Supporting information, Fig. S2). The results of this experiment together with the previous experiments support that **5e** permeated cells to react with thiols to enhance oxidative species.

The ability of **5e** to inhibit A549 lung carcinoma cells was tested and the GI_{50} was found as 27 μM and a selectivity index (GI_{50}/MIC) of 5.2 was determined, which is encouraging. Finally, we determined the ability of **5e** to inhibit growth of patient-derived strains of methicillin-resistant *S. aureus* (MRSA). We found excellent inhibitory potency with several strains being inhibited at concentrations of 2 μg/mL (Table 4). For example, the MIC of **5e** against MRSA 7386, which was resistant to Ciprofloxacin, Meropenem and Tobramycin, was 2 μg/mL.

Taken together, we report several thiol-activated prodrugs of sulfur dioxide that were capable of inhibiting MRSA growth at low micromolar concentrations (MIC of 2 μg/mL ≈ 5 μM). Amongst structural analogs, cell permeability to react with thiols played an important role in determining efficacy. Induction of redox stress through enhancement of reactive oxygen species (ROS)[29,35] or reactive nitrogen species (RNS) has been considered as a possible mechanism for developing new anti-bacterials.[36–39] Ours is the first report of MRSA being sensitive to sulfur dioxide, a reactive sulfur species. While such approaches lead to a better mechanistic understanding of bacterial responses to redox stress, these strategies might be limited by off-target effects. Our current focus is on modulating structure in order to address concerns of cytotoxicity, maximize efficiency of delivery of SO_2[15,40] and using these prodrugs in combination with other clinical antibiotics.

Acknowledgments

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Supplementary data

Supplementary data (general procedures, characterization data for all new compounds, and biological assay procedures) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2015.04.046.

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