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# Click and Release: $SO_2$ Prodrugs with Tunable Release Rates

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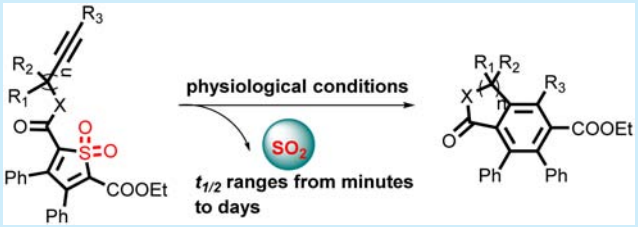
Supporting Information

**ABSTRACT:** Employing an intramolecular cycloaddition reaction, we have developed a series of $SO_2$ prodrugs with tunable release rates with half-lives ranging from minutes to days.




Sulfur dioxide ($SO_2$) has long been recognized as an air pollutant that can cause severe damage to respiratory and cardiovascular systems after long-term exposure.[1−3] However, $SO_2$ is also generated endogenously during the metabolism of sulfur-containing amino acids such as L-cysteine.[4] In the presence of cysteine oxidase, L-cysteine is first oxidized to L-cysteine sulfonate, which can be transformed into $\beta$-sulfinylpyruvate by aspartate aminotransferase. Subsequent decomposition then leads to pyruvate and $SO_2$.[5] Additionally, it was also reported that intracellular $H_2S$, which has been widely acknowledged as an important gasotransmitter, could be oxidized to sulfite or $SO_2$ by NADPH oxidase.[6]

Recently, accumulating evidence presents $SO_2$ as another gasotransmitter, especially in the cardiovascular system.[7,8] Endogenous $SO_2$ was reported to exert a negative regulation on vascular smooth muscle cell proliferation by suppressing the Erk/MAPK pathway.[9] Its sulfite and bisulfite derivatives also show endothelium-independent vasorelaxation effect partially by the PGI(2)-AC-cAMP-PKA signal pathway.[10] In addition to its physiological effects in the cardiovascular system, $SO_2$ also shows potential as a therapeutic agent with a variety of pathophysiological effects, including antihypertensive,[11] anti-atherosclerotic,[12] antioxidative,[13] and antimycobacterial effects,[14−16] as well as protective effects against cardiac ischemia-reperfusion (I/R) injury,[17] among others. However, it is still premature to conclude that endogenous $SO_2$ functions as a novel gasotransmitter with importance on par with CO, $H_2S$, and NO. Many of its biological effects and underlying mechanisms still remain to be carefully examined and require further in-depth investigations.

The majority of the biological effects observed for $SO_2$ were obtained by using gaseous $SO_2$ or its sulfite and bisulfite (3:1) derivatives, both of which, however, suffer from inherent issues. It is impractical to prepare a solution of $SO_2$ using gaseous $SO_2$ with precise concentrations due to its volatility. Although it is easy to hydrate $SO_2$ to generate sulfite and bisulfite in neutral aqueous solution,[18] it is still believed that the majority of $SO_2$ remains in the dissolved $SO_2$ form.[19] Furthermore, the biological effects observed with sulfite and bisulfite may not necessarily be associated with $SO_2$. It has been reported that biological effects obtained with these two donors sometimes contradicted each other.[20] Consequently, it is imperative to develop $SO_2$ donors/prodrugs as probes with tunable and controllable release rates to enable detailed biological studies.

The Chakrapani group was the first to report three types of $SO_2$ donors with different release mechanisms.[16,21,22] For example, benzosultines were reported to undergo cycloreversion to release $SO_2$ spontaneously upon dissolution in PBS at 37 °C.[21] Recently, Xian's group reported a water-soluble and pH-dependent $SO_2$ donor with slow ($t_{1/2}$ = 12.5 h at pH 6) and sustained $SO_2$ release, which was believed to imitate endogenous $SO_2$ generation. Moreover, this donor also demonstrated $SO_2$-related vasorelaxation activity in rat aortic rings.[23] These elegant $SO_2$ donors represent very useful tools for studying $SO_2$-associated biological effects and should help advance this field. However, due to the lack of $SO_2$ donors with tunable release rates, the important question in the field of $SO_2$ (also true for NO, $H_2S$, and CO) on how the release rate of a donor would affect the biological process still remains to be clarified. Additionally, it is also widely acknowledged that gasotransmitter prodrugs with different release profiles are needed in different biological milieu.[24−26] Consequently, it is highly desirable to devise new $SO_2$ prodrugs with tunable release rates ($t_{1/2}$ ranging from minutes to days) to serve different purposes.

Our group has had a long-standing interest in studying gasotransmitters.[24,26−29] Along this line, we have demonstrated the chemical feasibility of using a bimolecular reaction to achieve release of gasotransmitters, including $SO_2$, from their

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caged forms (Figure 1A).[27,30] However, most of the time, such bimolecular donors are not appropriate for biological

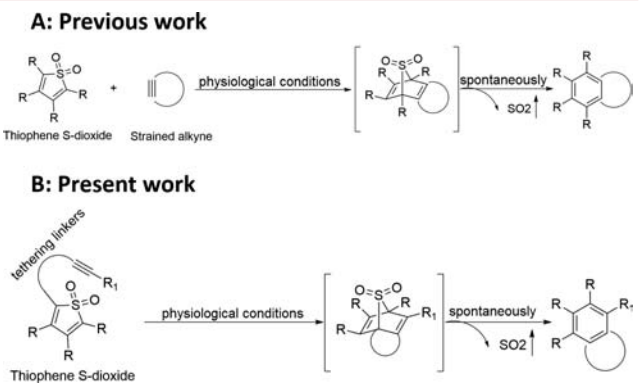


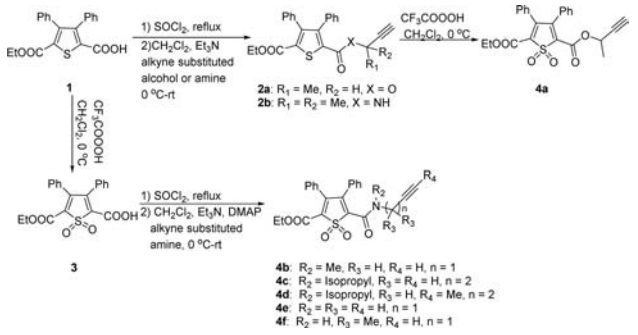

**Figure 1.** (A) Bimolecular $SO_2$ prodrug strategy; (B) unimolecular $SO_2$ prodrug strategy.

applications for two reasons. First, the reaction rate ($SO_2$ release rate) and the desired amount of $SO_2$ to be produced cannot be tuned independently because the bimolecular reaction rate is dependent on the concentrations of the reactants. Second, to synchronize the pharmacokinetic profiles of two components in a drug delivery system is a great challenge. In this contribution, we report a $SO_2$ prodrug system that combines both the dienophile (alkyne) and diene moiety (thiophene *S*-dioxide) into one molecule. Such prodrugs release $SO_2$ spontaneously under physiological conditions after the initial intramolecular inverse electron demand Diels–Alder reaction (Figure 1B). We were also able to tune the release rates with half-lives ranging from minutes to days by modifying the tethering linker, the substituents on the tether, and the electronic properties of the alkyne moiety. Furthermore, we also studied intracellular $SO_2$ release in Raw264.7 cells and its ability to cleave DNA, as described in the literature.[21]

One key issue in the design of such a unimolecular $SO_2$ prodrug system is whether it is possible to combine both the dienophile and diene into one molecule, which can be kept intact during synthesis and storage, and yet can readily undergo cycloaddition under physiological conditions to release $SO_2$. The activated dienophile used in the bimolecular system (Figure 1A, e.g., strained alkyne or *trans*-cyclooctene) cannot be used in this regard due to its high reactivity toward the diene (thiophene *S*-dioxide). We, as well as others, have shown that entropy factors can significantly accelerate reactions, sometimes by as much as a factor of $10^{13}$.[31,32] Therefore, we reasoned that by tethering a thiophene *S*-dioxide moiety to an alkyne using an appropriate linker, one may not need to employ an activated alkyne to make the intramolecular cycloaddition occur under mild conditions (e.g., physiological conditions). Consequently, as shown in Scheme 1, we designed and synthesized several potential $SO_2$ prodrugs (**4a**–**f**) with different tethering linkers. The prodrugs were expected to undergo intramolecular cycloaddition followed by $SO_2$ extrusion under physiological conditions.

Compound **1** was readily synthesized according to a similar literature method.[33] Subsequent esterification or amidation afforded **2a**,**b**. For the oxidation of the thiophene to its dioxide, we first attempted with *m*-CPBA without success. We then used a stronger oxidizing reagent, trifluoroperoxyacetic acid, which successfully oxidized **2a** to its thiophene *S*-dioxide derivative.

**Scheme 1. Synthesis of $SO_2$ Prodrugs**




However, in the case of compound **2b**, the corresponding thiophene *S*-dioxide compound was not obtained. Instead, the cyclized product was afforded, indicating facile cycloaddition reaction upon sulfur dioxide formation. One issue in this is that the oxidation reaction took several hours to achieve full conversion, giving much time for cycloaddition reaction to happen. In order to avoid this problem, compound **1** was oxidized to thiophene *S*-dioxide **3** first, which was transformed into an acyl chloride and then condensed with the corresponding alkyne substituted amine to afford the final products **4b**–**f**. Since amidation was performed at 0 °C, and finished in minutes, the thiophene *S*-dioxide products **4b**–**f** were the major product. Further purification by chromatography afforded the pure $SO_2$ prodrugs. It is worth noting that the prodrugs were designed to undergo cycloaddition and consequently to release $SO_2$ under physiological conditions, largely due to hydrophobic effects.[32] Nevertheless, the reaction could still occur in organic solvents, although at a much slower rate. Therefore, it is important to minimize the time in solution during purification. The pure products **4a**–**f** can be stored for months in a −20 °C freezer without stability issues. It is interesting to observe that **4b**–**d** existed as a mixture of rotamers around the amide bond. For example, for **4c**, two sets of protons were observed in its proton NMR at room temperature. The peaks coalesced at elevated temperature (60 °C, Figure S1), indicating that these rotamers are in thermodynamics equilibrium.

With $SO_2$ prodrugs **4a**–**f** on hand, we next investigated their $SO_2$ release profiles. Normally, the intermolecular cycloaddition between thiophene *S*-dioxide and nonactivated alkyne requires harsh conditions (e.g., reflux in toluene).[34] However, as shown in Table 1, all of the prodrugs readily underwent intramolecular cycloaddition under nearly physiological conditions with the concomitant release of 1 equiv of $SO_2$. The release of $SO_2$ was confirmed by both the elucidation of the chemical structure of the cyclized products **5b**–**f** by NMR, HRMS, and a well-accepted DTNB assay, which was widely used to detect the formation of sulfite or bisulfite (Figure S2).[35]

One can see from Table 1 that the $SO_2$ release half-life can be easily tuned from minutes to days by varying the tethering linkers. For instance, the $SO_2$ release from **4b** is very fast ($t_{1/2}$ = 7 min) due to the formation of a 5-membered ring, $\gamma$-lactam, after the cyclization. It has been reported that water or glycoprotein can accelerate the Diels–Alder reactions, much of which is ascribed to the hydrophobic interaction between reactants in hydrophilic environment.[36−38] Thus, as expected, the $SO_2$ release rate of **4b** in a mixed aqueous solution is about 3-fold faster than that in acetonitrile ($k = 0.71 \pm 0.01$ $h^{-1}$, $t_{1/2}$ = $0.98 \pm 0.01$ h). When the cycloaddition results in the formation



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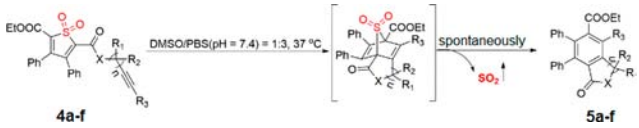
**Table 1. $SO_2$ Release Profiles for 4a−f**




| $SO_2$ prodrugs (**BW-$SO_2$−**)[a] | $k$[b] ($h^{-1}$) | $t_{1/2}$[c] (h) |
|---|---|---|
| **101** (**4a**): X = O, $R_1$ = Me | | >10 days[d] |
| **102** (**4b**): X = NMe | 4.16 ± 0.28 | 0.17 ± 0.01 |
| **103** (**4c**): X = N-*i*-Pr, *n* = 2 | 0.44 ± 0.02 | 1.6 ± 0.08 |
| **104** (**4d**): X = N-*i*-Pr, $R_3$= Me, *n* = 2 | 0.22 ± 0.06 | 3.38 ± 0.96 |
| **105** (**4e**): X = NH | 0.18 ± 0.01 | 3.86 ± 0.22 |
| **106** (**4f**): X = NH, $R_1$ = $R_2$ = Me | 2.51 ± 0.19 | 0.28 ± 0.02 |

[a]$R_1$−$R_3$ = H and *n* = 1 unless otherwise specified; [b]The cycloaddition rate constant of **4a−f**. [c]The $SO_2$ release half-lives of **4a−f**. [d]The $SO_2$ release rate was determined in DMSO-$d_6$/$D_2O$ = 1:3.

of a 5-membered lactone (e.g., **4a**), the reaction is much slower ($t_{1/2}$ >10 days) than a similar reaction leading to the formation of a lactam ring (e.g., **4b**). The reason for such a difference in reaction rate between lactam and lactone formation is multifold. First, the more rigid nature of an amide bond makes the cycloaddition more entropically favorable. Second, the *N*-methyl substitution adds further conformational constrains to favor the desired reaction. It has been reported that the propensity for an ester to stay in the transoid geometry disfavors the needed cycloaddition reaction.[39] Compared to the formation of a 5-membered ring, the formation of a 6-membered lactam ring is less entropically favorable, and hence, the $SO_2$ release rate for **4c** ($t_{1/2}$ = 1.6 h) is slower as compared to **4b** ($t_{1/2}$ = 0.17 h). Substituting the alkyne proton for a methyl group led to the formation of an internal alkyne, which disfavors the cycloaddition. As a result, the $SO_2$ release from **4d** is slower ($t_{1/2}$ = 3.38 h) than **4b**. Interestingly, **4e**, an analogue of **4b** without the *N*-methyl group, exhibited much slower $SO_2$ release with a half-life of 3.86 h as compared to 0.17 h for **4b**. In addition to the conformation constraints imposed by the presence of an *N*-methyl group, it is also possible that the amide proton in **4e** can form a hydrogen bond with the oxygen of S-dioxide, which would lead to a conformation disfavoring the intended cycloaddition (Figure S11). Introduction of a *gem*-dimethyl group[40] into the tethering linker significantly accelerates the cycloaddition rate, as exemplified by the substantial release rate difference between **4e** ($t_{1/2}$ = 3.86 h) and **4f** ($t_{1/2}$ = 0.28 h).

Having confirmed $SO_2$ release from **4a−f**, we next probed whether these prodrugs could present $SO_2$ associated biological effects as demonstrated by others. Therefore, **4f** was chosen for the pBR322 supercoiled plasmid cleavage assay.[21] As shown in Figure 2, both **4f** and the positive control sulfite/bisulfite induced pronounced DNA cleavage in comparison to the negative control using cyclized product **5f**.

Next, we probed whether these $SO_2$ prodrugs could release $SO_2$ in a biological system. Toward this end, **4f** was chosen for cell imaging studies. A literature reported $SO_2$ fluorescent probe[41] was employed to visualize the $SO_2$ release in Raw264.7 cells. As shown in Figure 3, cells cotreated with **4f** or bisulfite together with the $SO_2$ probe showed strong blue fluorescence, yet the cells treated with only **4f** or probe alone presented no or negligible blue fluorescence. Altogether, these results confirmed intracellular $SO_2$ release from **4f**.

In conclusion, a series of thiophene *S*-dioxide derivatives have been synthesized. The compounds undergo an intra-

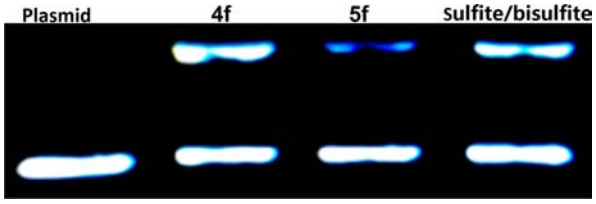



**Figure 2.** DNA cleavage assay for **4f** (100 μM), **5f** (100 μM), and sulfite/bisulfite (3:1, 100 μM).

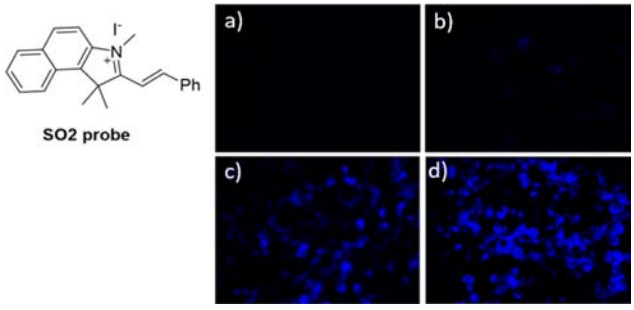



**Figure 3.** $SO_2$ release from **4f** in RAW264.7 cells: (a) **4f** (100 μM) only; (b) $SO_2$ probe only (10 μM); (c) bisulfite (100 μM) + $SO_2$ probe (10 μM); (d) **4f** (100 μM) + $SO_2$ probe (10 μM).

molecular inverse electron demand Diels−Alder reaction leading to $SO_2$ release under physiological conditions. By varying the tethering linker and the substituents on the linker, the $SO_2$ release half-lives can be easily tuned from minutes to days, which represents a more than 1000-fold difference. Since the release rate plays a vital role determining the biological effect of a gasotranmitter, prodrugs with different release profiles are very useful tools. We believe that the $SO_2$ prodrugs reported here could serve as powerful tools to enable detailed biological studies.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.6b03805.

Experimental procedure, $^1H$ and $^{13}C$ NMR spectra, DTNB assay, and the $SO_2$ release kinetics (PDF)

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### Author Contributions

[§]X.J. and E.M.E. contributed equally.


### Notes

The authors declare no competing financial interest.

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