Organic LETTERS

Letter

Cite This: Org. Lett. XXXX, XXX, XXX−XXX

pubs.acs.org/OrgLett

Click, Release, and Fluoresce: A Chemical Strategy for a Cascade Prodrug System for Codelivery of Carbon Monoxide, a Drug Payload, and a Fluorescent Reporter

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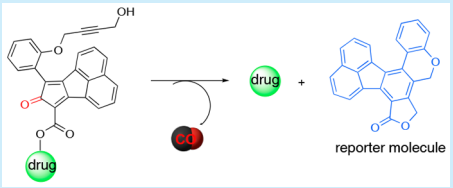
Supporting Information

ABSTRACT: A chemical strategy was developed wherein a single trigger sets in motion a three-reaction cascade leading to the release of more than one drug-component in sequence with the generation of a fluorescent side product for easy monitoring. As a proof of concept, codelivery of CO with the antibiotic metronidazole was demonstrated.



Drug combination therapy often relies on coadministration of multiple drugs. In the area of antibacterials, the use of Augmentin, which combines a β-lactam antibiotic with a β-lactamase inhibitor,[1] allows for the effective control of infections by drug-resistant strains of bacteria. Another example is the administration of a hybrid compound of sulfonamide and dihydrofolate reductase inhibitor to achieve inhibition of two enzymes on the same biosynthetic pathway leading to folic acid, which is important for bacterial survival.[2] Herein, we explore the idea of using prodrug approaches to deliver multiple components from a single chemical entity, with the aim of improved pharmacokinetic control and ease of administration. In appropriate situations, such an approach will be very useful. In demonstrating the proof of concept, we select the delivery of carbon monoxide (CO) and antibiotic metronidazole as a model drug payload.

Despite being notorious for its toxicity at high concentration, CO has recently been in the spotlight for its promising potential as a medical gas.[3] It is produced endogenously in the human body from the degradation of heme by heme-oxygenase.[4] The discovery that CO, like nitric oxide (NO), activates soluble guanylate cyclase[5] was a milestone in understanding the physiological relevance of CO and in triggering an ever-increasing level of research interest in this area.[6] Since then, mounting evidence from a multitude of studies has demonstrated that both endogenously produced and exogenously introduced CO plays important roles in inflammation,[7] cell proliferation,[8] and cellular response to pathogens[9] among other effects. Safety concerns have been addressed by human safety trials wherein it was shown that CO inhalation of 500 ppm for 1 h is well-tolerated in healthy subjects—that is CO-bound hemoglobin did not increase to more than 10% and there were no adverse events observed in the course of the experiments.[10] Due to convincing preclinical studies, CO gas is now in several clinical trials such as for treatment of idiopathic pulmonary fibrosis (ClinicalTrials.Gov identifier: NCT01214187) and for severe pulmonary arterial hypertension (ClinicalTrials.Gov identifier: NCT01523548).

Aside from direct inhalation, other routes of CO administration involve the use of metal-based CO-releasing molecules (CO-RMs)[11] including photo-CORMs,[12] boranocarbonate and its derivatives,[13] photosensitive organic CO-RMs,[14] and hemoglobin-based CO carriers.[15] Adding to this list of CO gas surrogates are a group of recently reported "spontaneously-releasing"[16] and triggered[17] organic CO prodrugs. Generally speaking, the design of such organic CO-prodrugs takes advantage of an intramolecular cycloaddition reaction between a dienone and an alkyne to form a bicycloheptenone intermediate that spontaneously undergoes a cheletropic reaction to extrude CO. The ability of this prodrug system to supply CO and to exert its biological effects has been demonstrated through the inhibition of LPS-induced TNF-α secretion in RAW 264.7 cells, and attenuation of 2,4,6-trinitrobenzensulfonic acid (TNBS)-induced experimental colitis in mice.[16b,17a,b]

Pushing forward and building on the flexibility of the design of this click-and-release organic scaffold, we were interested in exploring the possibility of coupling the release of CO to another drug payload for potential multidrug therapy applications (Figure 1). The other two gasotransmitters, nitric oxide and hydrogen sulfide, have been conjugated to compatible therapeutic agents such as anticancer, cytotoxic, and anti-inflammatory drugs to expand the therapeutic scope of these gases.[18] Based on previous reports of CO's ability to work additively and/or synergistically with other entities such as cytotoxic agents (doxorubicin),[19] antibiotics (metronidazole),[9a]

Received: October 26, 2017

DOI: 10.1021/acs.orglett.7b03348
Org. Lett. XXXX, XXX, XXX−XXX

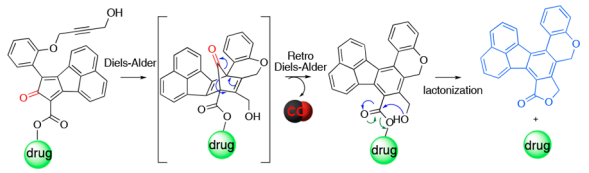


Figure 1. Cascade prodrug system for delivery of CO, another drug payload, and a fluorescent byproduct.

and anti-inflammatory agents,[20] we sought to develop a platform where compatible drugs can be easily conjugated to our previously developed CO releasing scaffold.[16b] By combining a cascade of three bioorthogonal reactions—an inverse electron-demand Diels–Alder reaction, a retro-Diels–Alder reaction/cheletropic extrusion, and lactonization, three key components are released (Figure 1). This design features a prodrug that is activated under near physiological conditions (PBS buffer, pH 7, 37 °C) wherein the rate of the inverse electron-demand Diels–Alder reaction is expected to be greatly enhanced. Then the formed norbornenone intermediate readily undergoes a cheletropic reaction to extrude CO while forming an aromatic ring. Postaromatization, the hydroxyl group is now poised to undergo an anticipated facile lactonization[21] to release the free drug. Release of both CO and free drug leaves the original scaffold as a blue fluorescent compound that can be used to track release progress (Figure 1).

To probe the potential of this design, model prodrug **10a** with benzyl alcohol as the model payload was prepared first according to the procedure detailed in the Supporting Information. The synthetic scheme was designed such that any compatible drug or agent bearing a free hydroxyl group or amino group can be easily conjugated. The latent CO is caged in the scaffold containing the carboxylic acid portion from readily available starting material, 2-hydroxyphenylacetic acid. With straightforward chemistry, compound 7 was readily obtained. A compatible payload such as the antibiotic metronidazole can be easily conjugated via amidation or esterification. Once the payload is appended, the alkyne component will then be introduced in the *ortho*-phenolic group to complete the prodrug. The modularity of this synthetic design offers possibility to synthesize prodrugs of CO that can release various drugs.

Initially, the feasibility of this design was investigated by studying CO and payload releasing capacity of a simple model compound **10a** through fluorescence spectroscopy, CO-myoglobin assay, and NMR studies. Because a fluorescent cyclized product is also produced as CO and the payload are released, the progress of cascade reaction of the prodrug can be monitored by fluorometric studies. The fluorescent cyclized product is characterized by excitation wavelength of 355 nm and emission wavelength of 460 nm (Figure S1a). Time-dependent increase in fluorescence intensity was observed, indicating that CO and the payload are released as the prodrug is incubated in aqueous solution at physiological pH, providing indirect confirmation of CO release (Figure S1b). Correlation of the data using Sigmaplot revealed that the half-life of the model prodrug is 0.66 ± 0.05 h. To supply direct proof that indeed CO is being released, a modified CO-myoglobin assay[16b] was conducted. Indeed, it was shown that incubation of deoxy-myoglobin with the prodrug produces CO-myoglobin as demonstrated by the change in the absorbance spectrum that is characteristic of CO-bound myoglobin (Figure S2).

To gain preliminary insights into the possible mechanism of CO and payload release, we decided to follow reaction progress by ^{1}H NMR experiments. A 10 mM solution of model prodrug **10a** in DMSO-d_6: PBS in D_2O was incubated at 37 °C while taking NMR spectra at indicated time points. The simplicity of the structure of prodrug **10a** allowed for easy monitoring of reaction progress. Scrutinizing the spectroscopic changes of four sets of protons from prodrug **10a** showed that three CH_2 peaks labeled **b**–**d** and the most deshielded aromatic proton labeled as **a** proved to be informative (Figure 2). As expected, the peaks

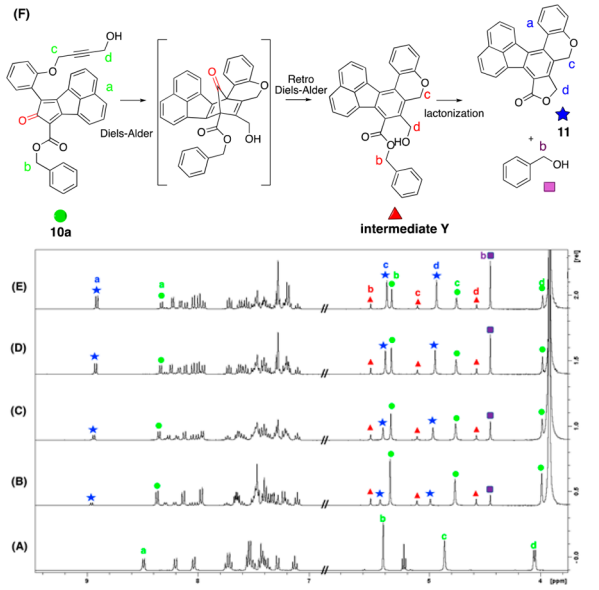


Figure 2. (A) **10a** in pure DMSO-d_6. (B) Incubation in DMSO-d_6/PBS in D_2O after 30 min, (C) 75 min, (D) 120 min, (E) 165 min. (F) Proposed mechanism of release.

corresponding to the norbornenone intermediate were not observed, confirming that the CO extrusion step is very fast[22] and the Diels–Alder reaction step is rate-limiting for CO release (Figure 1). Aside from the peaks corresponding to the intact prodrug **10a**, cyclized product **11** and benzyl alcohol, another set of peaks possibly from the prelactonization intermediate **Y** was also present. As the reaction progressed, peaks from the intact prodrug and intermediate **Y** decreased while peaks corresponding to the cyclized product **11** and benzyl alcohol steadily increased with a ratio of 1:1. After 24 h, both intact prodrug **10a** and intermediate **Y** were no longer observable and only peaks corresponding to the cyclized product **11** and benzyl alcohol were present (Figure S3).

Having confirmed the release of the two drug components from model compound **10a**, we sought to use this design to deliver CO together with a real drug payload. CO has been well-documented to exert antimicrobial effects either through directly targeting bacterial survival machinery[23] or through activating the killing potential of the host's phagosome in response to microbial invasion.[9b,24] Co-administration of CO with known antibiotics such as metronidazole, amoxicillin, and clarithromycin has been shown to enhance the efficacy of these antibiotics against *Helicobacter pylori*,[9a] the etiological agent of peptic ulcer disease or chronic atrophic gastritis.[25] Hence, we prepared prodrug **10b** such that it would release three components—CO, an antibiotic payload (metronidazole), and a fluorescent reporter molecule.

The formation of these three components following incubation of prodrug **10b** in PBS buffer was examined using RP-HPLC (Figure 3A). For reference, a mixture containing 25

DOI: 10.1021/acs.orglett.7b03348
Org. Lett. XXXX, XXX, XXX–XXX

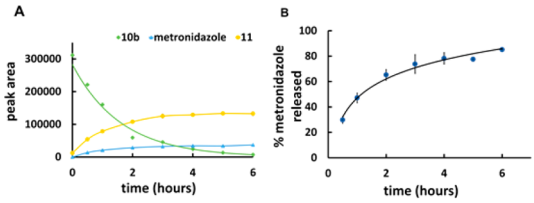


Figure 3. (A) Release profile of **10b** in PBS buffer, pH 7, 37 °C. (B) Quantification of accumulated free metronidazole released over time.

μM each of the prodrug **10b**, free drug metronidazole, and cyclized product **11** were prepared (Figure S4A). Then, a 25 μM solution of prodrug **10b** was incubated at 37 °C, pH 7. At time 0 (Figure S4B), only the peak for the prodrug was observable. After 30 min (Figure S4C), two new peaks corresponding to the cyclized product and free metronidazole emerged while the intensity of the peak for the intact prodrug decreased. As **10b** was being consumed, the free drug and **11** were in turn produced. After 6 h (Figure S4F), the peak for the intact prodrug was almost undetectable while the peaks corresponding to the free drug and cyclized byproduct appeared to level off to the same peak area as that of the 25 μM mixture shown in Figure S4A. Quantification of the release of the free drug showed that at the 6-h time point, around 85% of the free drug had already been released (Figure 3B). It is also worth noting that the prodrug fell apart in PBS buffer, pH 7 at 37 °C in a clean manner without any other discernible artifacts aside from the three components (Figure S4).

To verify CO release in biological systems, we monitored the formation of blue fluorescence of the cyclized product **11** in cell imaging studies using RAW 264.7 cells. After incubation of **10b** for 6 h, we found that the cells exhibited a dose-dependent increase (qualitative) in blue fluorescence formation (Figure S5) indicating that indeed CO was released from prodrug **10b**. To directly assay CO release in cell cultures, a CO-selective turn-on fluorescent probe COP-1[26] was used. An increase in green fluorescence (qualitative) relative to the negative control (Figure 4) was observed verifying that indeed CO was present inside the

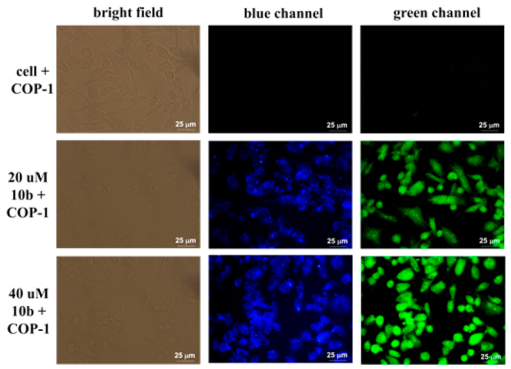


Figure 4. Concentration-dependent increase in fluorescence intensity of **11** (blue channel) and COP-1 probe (green channel) after 6 h incubation of prodrug **10b**.

cell. Certainly, these results indicate that cyclized product **11** can be used as a fluorescent reporter molecule to monitor CO and drug release from the prodrug. To further demonstrate that this cascade prodrug concept can work in living systems, we probed the ability of prodrug **10b** to deliver metronidazole and CO in *H. pylori*, and juxtaposed its growth inhibitory activity against the antibiotic metronidazole. As expected, metronidazole exhibited a dose-dependent growth inhibitory effect against *H. pylori* strain 26695 with MIC_{90} of 2.5 μg/mL. In contrast, it took only 0.31 μg/mL of prodrug **10b** to achieve greater than 90% growth inhibition against *H. pylori* (Figure 5). This is an enhancement of

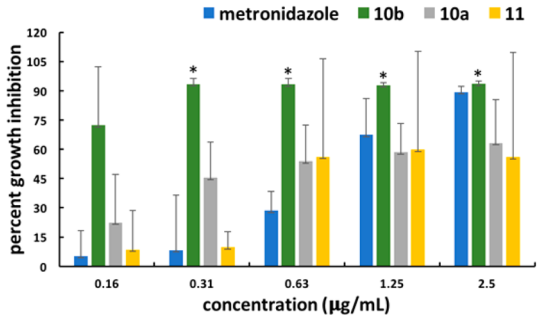


Figure 5. Inhibition of *H. pylori* growth in BHI-β-cyclodextrin medium supplemented with metronidazole, **10b**, **10a**, and **11** in 1% DMSO. Results are expressed as (mean and standard deviation) percentage of OD_{600} at 24 h for each compound compared to OD_{600} of control bottles (1% DMSO only). Growth experiments were done in triplicate. * indicates no growth.

over 8-fold in potency. Further, with metronidazole's molecular weight (171) being only less than 1/3 of that of 10b (MW = 562), the true enhancement in potency for **10b** was over 26-fold in terms of molar concentrations. As controls, prodrug **10a**, which releases CO only without metronidazole, and cyclized product **11** exhibited no or minimal inhibition at the same concentration (0.31 μg/mL). Such results are consistent with the observed bacterial sensitization effect of CO toward existing antibiotics.[9a] Collectively, these results demonstrated the feasibility of using prodrug **10b** to codeliver CO and metronidazole in a biological milieu. These results also herald the potential of combinational therapy of CO and other drugs such as metronidazole using a single prodrug entity.

The viability of this prodrug to act as an antimicrobial agent depends on its opposite toxicity profiles in microbes and in host cells. Therefore, the toxicity profiles of the prodrug (**10**) and the cyclized product (**11**) were tested in a crystal violet assay of RAW264.7 macrophage cells. No toxicity was observed at up to 100 μM of both prodrugs **10a** (50 μg/mL) and **10b** (56 μg/mL) and fluorescent byproduct **11** (36 μg/mL) after 24 h of incubation (Figure S6).

In summary, we have developed a modular platform that features a cascade reaction sequence that is set in motion once the prodrug is placed in an aqueous environment leading to the release of CO, a compatible drug payload, and a fluorescent reporter molecule. The release of both CO and drug payload was monitored by the increase in characteristic blue fluorescence of the reporter molecule byproduct. Further verification through NMR studies, RP-HPLC kinetic studies, cell imaging, and antibacterial assay provide unambiguous evidence in support of the feasibility of the cascade prodrug design.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.7b03348.

Full experimental procedure and characterization data (PDF)

DOI: 10.1021/acs.orglett.7b03348
Org. Lett. XXXX, XXX, XXX−XXX

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We gratefully acknowledge the Center for Diagnostics and Therapeutics Fellowship for L.K.C.D.L.C.

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DOI: 10.1021/acs.orglett.7b03348
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