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Bond-Breaking Bio-orthogonal Chemistry Efficiently Uncages Fluorescent and Therapeutic Compounds under Physiological Conditions

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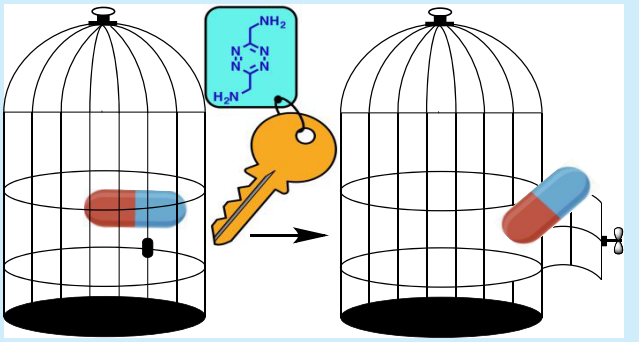
ABSTRACT: Bond-breaking bio-orthogonal chemistry, consisting of a "click" reaction between *trans*-cyclooctene and tetrazine, followed by an intramolecular cyclization-driven uncaging step is described. The two-step process allows activation of caged compounds in biological media at neutral pH. The feasibility of this chemistry has been illustrated using NMR, while kinetics and pH-dependence were studied by fluorescence spectroscopy using caged coumarin. The practicality of the strategy is illustrated by activation of an anticancer drug, etoposide.



Prodrug activation strategies are valuable chemical tools that facilitate delivery of caged cycototoxic compounds at large doses, thereby enhancing their therapeutic index. Traditionally, prodrug activation strategies relied on endogenous enzymatic reactions.[1−3] During the past decade, bond-breaking bio-orthogonal chemistry has evolved into a powerful new approach for uncaging both small therapeutic molecules as well as biomacromolecules, such as proteins and nucleic acids, inside live cells as well as *in vivo*.[4−6] Unlike the traditional strategy, the latter does not rely on endogenous factors and offers temporal control over prodrug activation.[7,8]

In recent years, we have witnessed a rapid expansion of chemical methodologies that achieve bio-orthogonal prodrug activation. Notably, Franzini and co-workers reported a [4 + 1] cycloaddition reaction between isonitriles and tetrazines (Tz) capable of in situ activation of doxorubicin, SN-38, and mitomycin C.[9] Wang et al. explored a [4 + 2] cycloaddition reaction between cyclooctynes and Tz capable of activating two therapeutic molecules upon a single "click" step.[10] Taran et al. described a [3 + 2] cycloaddition reaction between iminosydnones and cycloalkynes.[11] Inverse-electron-demand Diels−Alder (IEDDA) chemistry between *trans*-cyclooctene (TCO) and Tz has also gained popularity due to rapid reaction kinetics and biocompatibility.[12−14] It will be the main focus of the present work.

In their 2013 report, Robillard and co-workers showed that allylic TCO carbamates can be uncaged via a so-called "click-to-release" process upon conjugation to Tz and subsequent tautomerization (Scheme 1A).[13] The authors have since extended this strategy to bio-orthogonal activation of TCO carbonates, esters, and ethers.[15] However, further mechanistic investigation of the "click-to-release" chemistry revealed a number of critical challenges. It has been determined that electron-withdrawing groups attached at the 3- and 6-positions of Tz accelerate the IEDDA step by lowering the diene's LUMO. However, these Tz's have been found to be inefficient at the uncaging step.[13,16] Meanwhile, electron-donating groups have a completely opposite effect—slower "click", but more efficient "release". The most effective "click-to-release" Tzs have been reported to be asymmetric, containing one electron-withdrawing and one electron donating alkyl group.[17] The release kinetics has been shown to be also buffer and pH-dependent.[12,16] In physiologically relevant phosphate-buffered saline (PBS) media at neutral pH, the release step is slow and low yielding. It is partly due to head-to-tail orientation of the "click" step that results in the intermediate **4**. It has been demonstrated that **4** is slow to tautomerize to **5** in PBS at pH 7. Instead, a number of competing processes can lead to "nonreleasable" biproducts. To address these shortcomings, we designed an alternative prodrug uncaging strategy, illustrated in Scheme 1B. In our design, prodrug uncaging is triggered by an intramolecular cyclization, resulting in either δ- or ε-lactam. We envisioned using a symmetric Tz to eliminate the potential issues associated with orientation of the "click" step.

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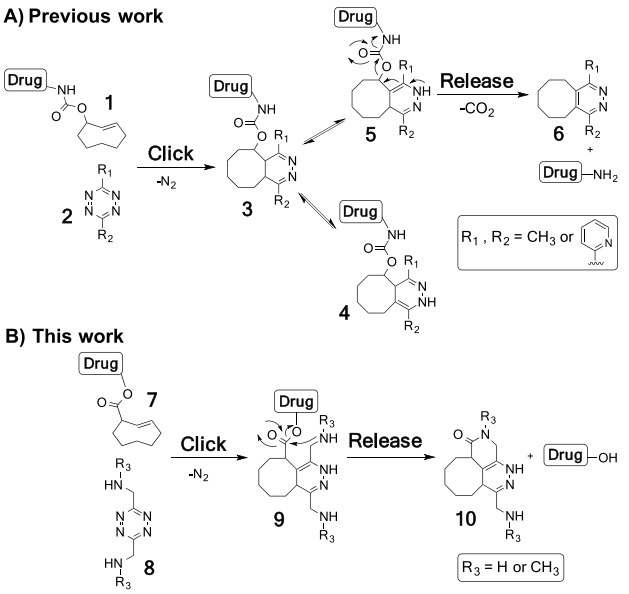
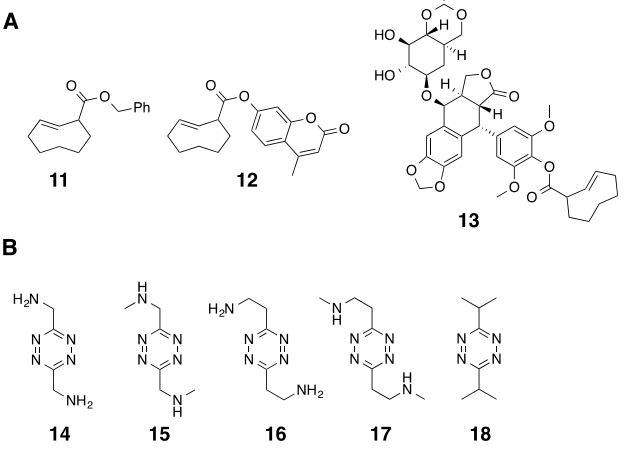
Org. Lett. XXXX, XXX, XXX−XXX

Scheme 1. Tz-Mediated Activation of Caged Drugs: (A) Previously Reported "Click-to-Release" Chemistry Where the Release Step Is Triggered by Spontaneous Tautomerization;[13] (B) Bond-Breaking Bio-orthogonal Chemistry, Where the Uncaging Step Is Caused by Intramolecular Cyclization



To assess the feasibility of the prodrug activation strategy illustrated in Scheme 1B, we synthesized a model compound **11**, shown in Scheme 2A. To examine the scope of intramolecular

Scheme 2. Compounds Described in This Work: (A) Compounds Caged by TCO; (B) Tetrazines That Were Explored for the Uncaging Reactions



cyclization, we also synthesized five different tetrazines, which are shown in Scheme 2B. Detailed syntheses of these compounds are described in the SI. Reactivity of **11** and Tz **14** was analyzed by NMR (Figure 1). The experiment was done in CD_3OD, in the presence of 2 equiv of Et_3N. Within the first hour, we observed formation of benzyl alcohol, which is the expected release product. This was confirmed by the standard spectrum of benzyl alcohol, shown in Figure 1A. Benzyl alcohol continued to form during the next 24 h. ESI-MS analysis of the reaction mixture detected the intramolecular cyclization product **10** (Figure S3). *cis*-Cyclooctene (CCO) is known to react ~10^6

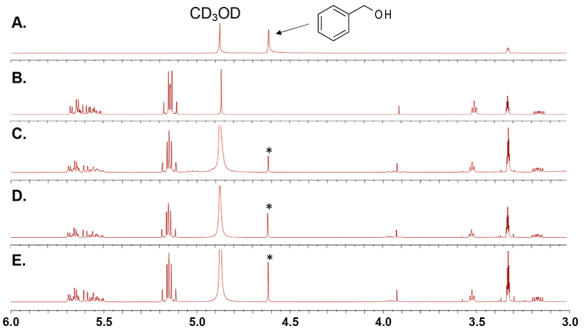


Figure 1. (A) Reference ^{1}H NMR spectrum of benzyl alcohol. Reaction of **11** and **14** monitored by ^{1}H NMR after (B) 5 min; (C) 1 h; (D) 6 h; (E) 24 h. The asterisk indicates the signature peak corresponding to methylene protons of benzyl alcohol. All spectra were acquired in CD_3OD.

times slower than TCO with Tz.[18] Control experiments involving CCO-benzyl ester **20** and Tz **14** did not produce any benzyl alcohol within the experimental time frame (Figure S1), confirming that the "click" is a necessary first step in the uncaging process. Reaction between **11** and Tz **18** also did not produce any benzyl alcohol, suggesting that the amine group is necessary for the "release" step.

To examine the kinetics of uncaging, we synthesized a fluorescent model compound **12**. Modification of the coumarin's 7-hydroxyl group typically results in quenching of fluorescence.[16,19] Compound **12** was found to be almost 200 times less fluorescent than 7-hydroxy-4-methylcoumarin in PBS over a pH range of 6−9 (Figure S2). Thus, the chromophore provides a functional handle to study the kinetics of the uncaging chemistry in a high-throughput manner. Activation of coumarin by the Tz **14**−**18** was studied in duplicate using fluorescence spectroscopy in the physiologically relevant pH range of 6−9.

The results of the coumarin uncaging studies are illustrated in Figure 2. Caged coumarin **12** (50 μM) was combined with a 10-fold excess Tz **14**-**17** in PBS (pH 6, 7, 8 and 9) at 37 °C. The CCO isomer **26** was also tested as a control. Their reactivity was monitored by fluorescence spectroscopy for 24 h. Each reaction was carried out in duplicate. Efficient uncaging of 7-hydroxy-4-methyl coumarin has been observed in all reactions, except those involving CCO. With few exceptions, fluorescence intensity reached a plateau within the first 6 h. Unlike the "click-to-release" chemistry shown in Scheme 1A, the observed coumarin uncaging was very efficient at neutral pH. The fluorescent payload release proceeded efficiently with primary and secondary amines and appeared to be slightly slower at pH 6 due to the lower nucleophilicity of the partially protonated amines. Over 80% of coumarin was uncaged by Tz **14** at pH 7. Equivalent reactions using the CCO analogue **26** proceeded at a considerably slower pace due to the inefficient "click" step. Release of coumarin did not occur in the case of Tz **18**, which lacks an amine group.

Activation of coumarin was also investigated in mouse blood plasma to understand if the uncaging strategy can be applied for in vivo studies. Compound **12** was reacted with 10 equiv of Tz **14** in mouse blood plasma at 37 °C. As a control, the CCO **26** was reacted with the Tz **14** under identical conditions. Each reaction was carried out in duplicate. The reactions were monitored using fluorescence spectroscopy, and the results are illustrated in Figure S4. Addition of **14** to **12** resulted in a rapid enhancement of fluorescence, indicative of uncaging of

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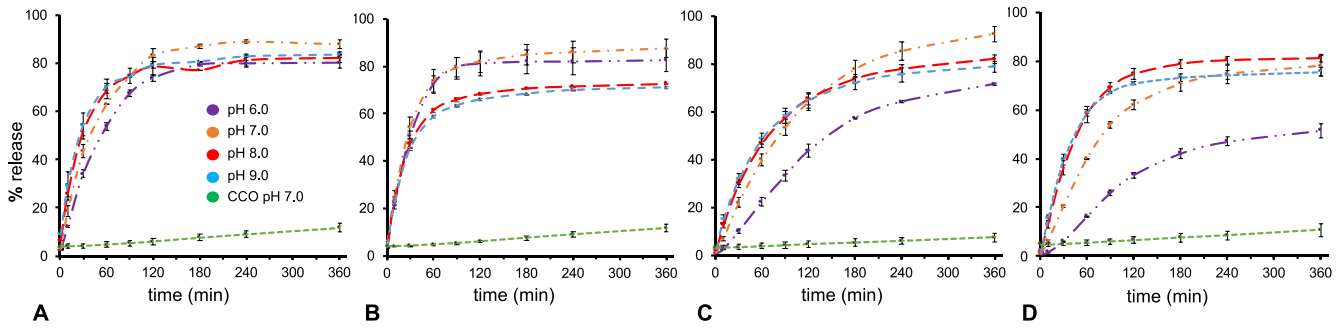

Figure 2. Uncaging of coumarin **12** (50 μM) (and its CCO analogue **26**) using Tz (500 μM) **14**–**17** monitored by fluorescence spectroscopy (λ_{ex} = 380 nm) over a pH range of 6–9 at 37 °C. **12**/Tz ratio 1:10: (A) **12** + Tz **14**; (B) **12** + Tz **15**; (C) **12** + Tz **16**; (D) **12** + Tz **17**.

coumarin. The fluorescence intensity reached a plateau in 90 min, followed by a slow decline. We believe that this behavior is due to slow decomposition of coumarin in blood plasma. As expected, the CCO analogue **26** did not produce any fluorescence enhancement upon addition of **14**. Instead, a slow decline of fluorescence intensity has been observed over time.

To illustrate a potential application of the described bond-breaking bio-orthogonal chemistry, we caged an FDA-approved anticancer drug etoposide. Etoposide is a topoisomerase II inhibitor that causes DNA damage.[20] Etoposide has been approved for treatment of testicular, lung, and ovarian cancers as well as leukemia and lymphoma. Coupling of TCO resulted in a caged etoposide, **13**.

Uncaging of **13** by the Tz **14** in PBS (pH 6, 7, 8, and 9) at 37 °C was analyzed by HPLC and evaluated using the etoposide standard. We chose the Tz that was the most efficient at uncaging coumarin. The results of the study are shown in Figure 3. Prodrug activation began shortly after addition of the Tz **14**. As illustrated in Figure 3A, the HPLC peak at 9.5 min, corresponding to etoposide, continued to grow for 24 h. Uncaging efficiency, determined upon integration of the 9.5 min peak, is shown in Figure 3B. The prodrug was effectively activated under the physiologically relevant pH range within the first several hours. This is very important, as etoposide is known to isomerize under physiological conditions to an inactive "*cis*-etoposide", but on a considerably slower time scale. The isomerization process at 37 °C and pH 7.4 has a half-life of 2 days.[21]

In conclusion, we have shown a prodrug activation strategy that is based on IEDDA chemistry between TCO and Tz. The uncaging step is triggered by intramolecular cyclization, which proceeds efficiently in PBS in physiologically relevant pH range. We have shown that the uncaging strategy can be applied toward activation of fluorescent payloads, as well as therapeutic compounds. Translation of the technology for in-cell prodrug activation is dependent on the stability of the caged compound to esterase hydrolysis. There are a number of reported prodrugs with caged ester bonds that show remarkable esterase stability.[22] In our future studies, we plan to utilize the described strategy for materials-based in vivo prodrug activation.[23] The described bifunctional Tz will be immobilized on a biocompatible material, such as polyalginate, and utilized for in vivo activation of systemically administered prodrugs.

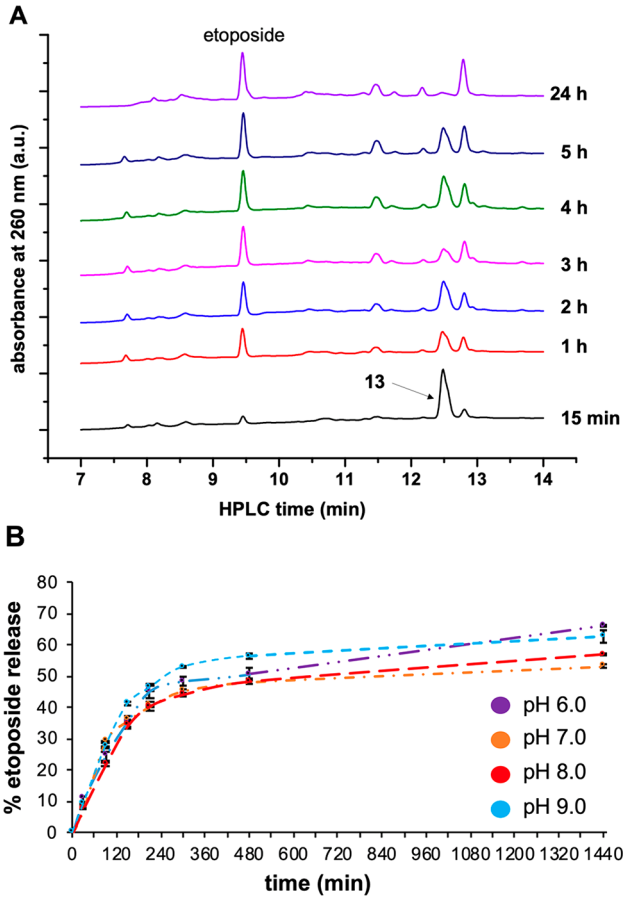

Figure 3. (A) HPLC analysis of uncaging of the prodrug **13** (50 μM) by Tz **14** (500 μM) in PBS, pH 7 at 37 °C. **13**/Tz ratio 1:10. (B) Summary of uncaging of the prodrug **13** by Tz **14** in PBS over a pH range of 6–9.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.orglett.0c02129.

Experimental details, synthetic procedures, and fluorescence and NMR spectra (PDF)

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Potter, P. M.; Wadkins, R. M. Carboxylesterases–detoxifying enzymes and targets for drug therapy. *Curr. Med. Chem.* **2006**, *13* (9), 1045−54.

(2) Liederer, B. M.; Borchardt, R. T. Enzymes involved in the bioconversion of ester-based prodrugs. *J. Pharm. Sci.* **2006**, *95* (6), 1177−95.

(3) Qi, H.; Lu, J.; Li, J.; Wang, M.; Xu, Y.; Wang, Y.; Zhang, H. Enhanced Antitumor Activity of Monophosphate Ester Prodrugs of Gemcitabine: In Vitro and In Vivo Evaluation. *J. Pharm. Sci.* **2016**, *105* (9), 2966−2973.

(4) Rossin, R.; Versteegen, R. M.; Wu, J.; Khasanov, A.; Wessels, H. J.; Steenbergen, E. J.; Ten Hoeve, W.; Janssen, H. M.; van Onzen, A.; Hudson, P. J.; Robillard, M. S. Chemically triggered drug release from an antibody-drug conjugate leads to potent antitumour activity in mice. *Nat. Commun.* **2018**, *9* (1), 1484.

(5) Li, J.; Jia, S.; Chen, P. R. Diels-Alder reaction-triggered bioorthogonal protein decaging in living cells. *Nat. Chem. Biol.* **2014**, *10* (12), 1003−5.

(6) Czuban, M.; Srinivasan, S.; Yee, N. A.; Agustin, E.; Koliszak, A.; Miller, E.; Khan, I.; Quinones, I.; Noory, H.; Motola, C.; Volkmer, R.; Di Luca, M.; Trampuz, A.; Royzen, M.; Mejia Oneto, J. M. Bio-Orthogonal Chemistry and Reloadable Biomaterial Enable Local Activation of Antibiotic Prodrugs and Enhance Treatments against Staphylococcus aureus Infections. *ACS Cent. Sci.* **2018**, *4* (12), 1624−1632.

(7) Khan, I.; Seebald, L. M.; Robertson, N. M.; Yigit, M. V.; Royzen, M. Controlled in-cell activation of RNA therapeutics using bond-cleaving bio-orthogonal chemistry. *Chem. Sci.* **2017**, *8* (8), 5705−5712.

(8) Robertson, N. M.; Yang, Y.; Khan, I.; LaMantia, V. E.; Royzen, M.; Yigit, M. V. Single-trigger dual-responsive nanoparticles for controllable and sequential prodrug activation. *Nanoscale* **2017**, *9* (28), 10020−10030.

(9) Tu, J.; Xu, M.; Parvez, S.; Peterson, R. T.; Franzini, R. M. Bioorthogonal Removal of 3-Isocyanopropyl Groups Enables the Controlled Release of Fluorophores and Drugs in Vivo. *J. Am. Chem. Soc.* **2018**, *140* (27), 8410−8414.

(10) Zheng, Y.; Ji, X.; Yu, B.; Ji, K.; Gallo, D.; Csizmadia, E.; Zhu, M.; Choudhury, M. R.; De La Cruz, L. K. C.; Chittavong, V.; Pan, Z.; Yuan, Z.; Otterbein, L. E.; Wang, B. Enrichment-triggered prodrug activation demonstrated through mitochondria-targeted delivery of doxorubicin and carbon monoxide. *Nat. Chem.* **2018**, *10* (7), 787−794.

(11) Bernard, S.; Audisio, D.; Riomet, M.; Bregant, S.; Sallustrau, A.; Plougastel, L.; Decuypere, E.; Gabillet, S.; Kumar, R. A.; Elyian, J.; Trinh, M. N.; Koniev, O.; Wagner, A.; Kolodych, S.; Taran, F. Bioorthogonal Click and Release Reaction of Iminosydnones with Cycloalkynes. *Angew. Chem., Int. Ed.* **2017**, *56* (49), 15612−15616.

(12) Carlson, J. C. T.; Mikula, H.; Weissleder, R. Unraveling Tetrazine-Triggered Bioorthogonal Elimination Enables Chemical Tools for Ultrafast Release and Universal Cleavage. *J. Am. Chem. Soc.* **2018**, *140* (10), 3603−3612.

(13) Versteegen, R. M.; Rossin, R.; ten Hoeve, W.; Janssen, H. M.; Robillard, M. S. Click to release: instantaneous doxorubicin elimination upon tetrazine ligation. *Angew. Chem., Int. Ed.* **2013**, *52* (52), 14112−6.

(14) Blackman, M. L.; Royzen, M.; Fox, J. M. Tetrazine ligation: fast bioconjugation based on inverse-electron-demand Diels-Alder reactivity. *J. Am. Chem. Soc.* **2008**, *130* (41), 13518−9.

(15) Versteegen, R. M.; Ten Hoeve, W.; Rossin, R.; de Geus, M. A. R.; Janssen, H. M.; Robillard, M. S. Click-to-Release from trans-Cyclooctenes: Mechanistic Insights and Expansion of Scope from Established Carbamate to Remarkable Ether Cleavage. *Angew. Chem., Int. Ed.* **2018**, *57* (33), 10494−10499.

(16) Sarris, A. J. C.; Hansen, T.; de Geus, M. A. R.; Maurits, E.; Doelman, W.; Overkleeft, H. S.; Codee, J. D. C.; Filippov, D. V.; van Kasteren, S. I. Fast and pH-Independent Elimination of trans-Cyclooctene by Using Aminoethyl-Functionalized Tetrazines. *Chem. - Eur. J.* **2018**, *24* (68), 18075−18081.

(17) Fan, X.; Ge, Y.; Lin, F.; Yang, Y.; Zhang, G.; Ngai, W. S.; Lin, Z.; Zheng, S.; Wang, J.; Zhao, J.; Li, J.; Chen, P. R. Optimized Tetrazine Derivatives for Rapid Bioorthogonal Decaging in Living Cells. *Angew. Chem., Int. Ed.* **2016**, *55* (45), 14046−14050.

(18) Thalhammer, F.; Wallfahrer, U.; Sauer, J. Reaktivität einfacher offenkettiger und cyclischer dienophile bei Diels-Alder-reaktionen mit inversem elektronenbedarf. *Tetrahedron Lett.* **1990**, *31* (47), 6851−6854.

(19) Cao, D.; Liu, Z.; Verwilst, P.; Koo, S.; Jangjili, P.; Kim, J. S.; Lin, W. Coumarin-based small-molecule fluorescent chemosensors. *Chem. Rev.* **2019**, *119*, 10403−10519.

(20) Pommier, Y.; Leo, E.; Zhang, H.; Marchand, C. DNA topoisomerases and their poisoning by anticancer and antibacterial drugs. *Chem. Biol.* **2010**, *17* (5), 421−33.

(21) Mader, R. M.; Steger, G. G.; Moser, K.; Rainer, H.; Krenmayr, P.; Dittrich, C. Instability of the anticancer agent etoposide under inn vitro culture conditions. *Cancer Chemoth. Pharm.* **1990**, *27*, 354−360.

(22) Ji, X.; Aghoghovbia, R. E.; De La Cruz, L. K. C.; Pan, Z.; Yang, X.; Yu, B.; Wang, B. Click and Release: A High-Content Bioorthogonal Prodrug with Multiple Outputs. *Org. Lett.* **2019**, *21* (10), 3649−3652.

(23) Mejia Oneto, J. M.; Khan, I.; Seebald, L.; Royzen, M. Vivo Bioorthogonal Chemistry Enables Local Hydrogel and Systemic Pro-Drug To Treat Soft Tissue Sarcoma. *ACS Cent. Sci.* **2016**, *2* (7), 476−82.