A Concise Synthetic Approach to Highly Reactive Click-to-Release *Trans*-Cyclooctene Linkers

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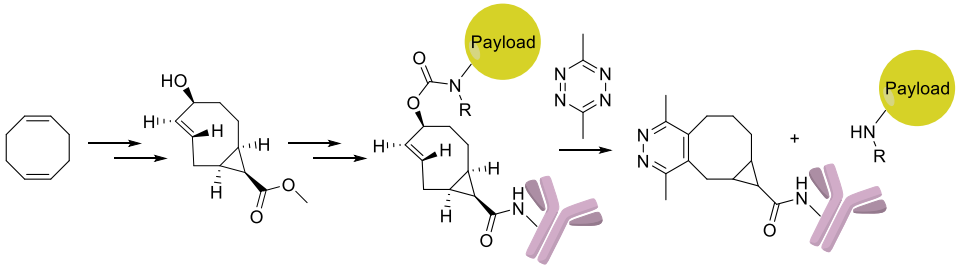
Abstract
An increase of the click-to-release reaction rate between cleavable *trans*-cyclooctenes (TCO) and tetrazines would be beneficial for drug delivery applications. In this work, we developed a short and stereoselective synthesis route towards highly reactive sTCOs that serve as cleavable linkers, affording quantitative tetrazine-triggered payload release. In addition, the 5-fold more reactive sTCO exhibited the same in vivo stability as the parent TCO linker when used as antibody linker in circulation in mice.



Bioorthogonal cleavage reactions enable the controlled release or activation of a wide range of constructs in chemical and biological settings.[1-3] Among these, the high reactivity and selectivity of the IEDDA pyridazine elimination reaction between tetrazine and *trans*-cyclooctene (TCO)-linked conjugates has led to many *in vivo* applications,[4] such as tetrazine-triggered cleavage and activation of TCO-containing antibody-drug conjugates (ADCs),[5,6] prodrugs[7,8] and proteins.[9,10] This so called click-to-release reaction is derived from the fastest and widely used click-conjugation reaction, the IEDDA between TCO and tetrazine, forming a 4,5-dihydropyridazine, which can then tautomerize to a 1,4- and a 2,5-dihydropyridazine.[1] The IEDDA pyridazine elimination retains the favorable properties of the parent reaction regarding speed, stability, and selectivity, and in addition affords the release of a carbamate, ester or ether-linked payload from the allylic position of the TCO upon formation of the 1,4-dihydropyridazine.[4,11]

However, the allylic substituent does decrease the click conjugation rate ca. 20-fold *vs.* unsubstituted TCO.[4] An increase of this reaction rate would be beneficial for a number of applications. For example, complete *in vivo* activation of a target-localized protein or ADC requires the intravenous administration of an excess of tetrazine activator. A higher click reaction rate may allow a lower dose of the activator, which would facilitate clinical translation and may open up other prodrug approaches such as targeting the activator to the tumor instead of or in addition to the prodrug.

One approach to improve reaction kinetics is to increase the ring strain of the TCO linker. Fox

and co-workers demonstrated that *cis*-fusion of a cyclopropane to the TCO forces the TCO ring to adopt a highly strained "half-chair" conformation as opposed to a crown conformation.[12] This (E)-bicyclo[6.1.0]non-4-ene (sTCO) is 160 times more reactive towards 3,6-diphenyl-s-tetrazine in methanol solution than the parent TCO and more reactive than other *cis*-fused TCOs that have since then been developed, such as d-TCO and aza-TCO (Figure 1).[13,14] While the sTCO and other half-chair TCOs were so far only developed as click-conjugation tags, their excellent reactivity, especially that of the sTCO, warrant their development into a click-to-release linker.

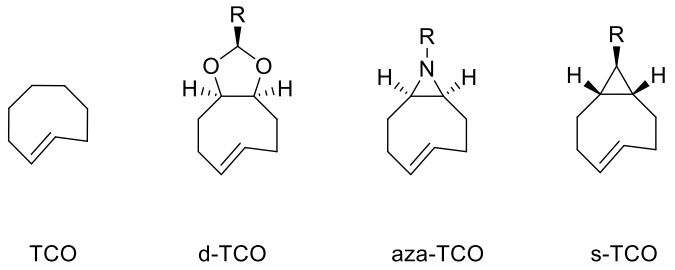
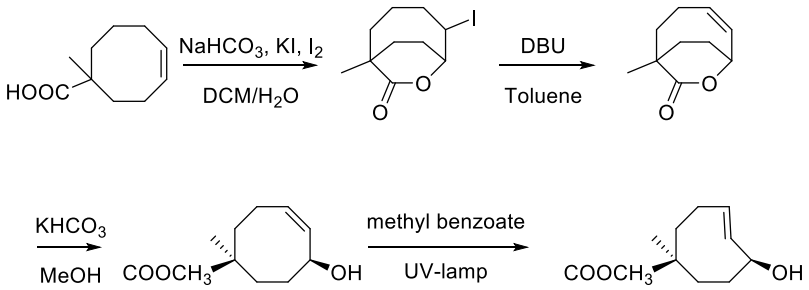


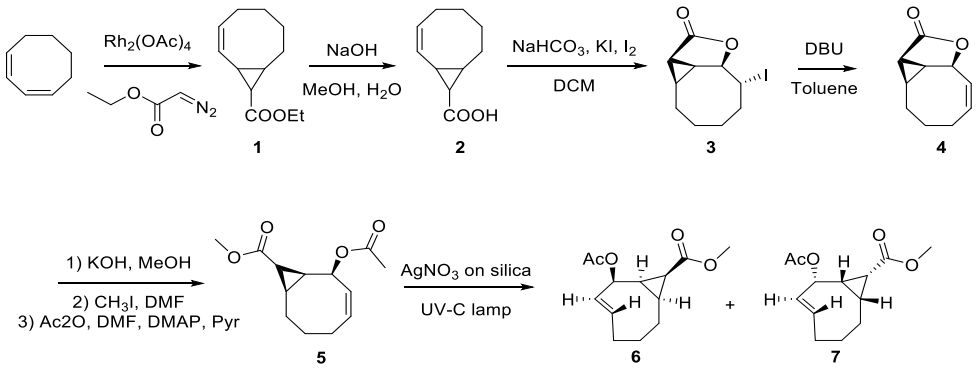
Figure 1. Structure of TCOs.

To obtain an sTCO that can release a carbamate-linked compound, a hydroxyl group is needed at the allylic position of the sTCO. In our previous work on the synthesis of releasing TCO linkers, a carboxylic acid substituent on the 5-position of the cyclooctene was enlisted to intramolecularly install a hydroxyl adjacent to the alkene in a stereoselective manner.[5] This was achieved by an iodolactonization reaction, converting the olefin to a iodolactone, thus forming a favored 6- and 8-membered bicyclic ring (Scheme 1). Subsequent elimination of hydrogen iodide and hydrolysis of the lactone produced the olefinic bond together with the allylic hydroxyl group in *cis* position relative to the carboxylic acid. This was followed by *cis-trans* alkene photoisomerization and separation of the axial and equatorial hydroxyl-substituted TCO diastereomers.



Scheme 1. Stereoselective strategy towards allylic hydroxyl-containing *trans*-cyclooctene via iodolactonisation and elimination reactions.

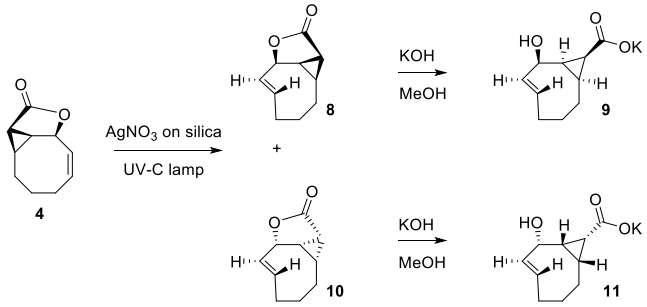
We hypothesized that this stereoselective and efficient method could also be used to prepare cleavable sTCO linkers using a *syn*-positioned carboxylic acid substituent on the fused cyclopropane of a bicyclo[6.1.0]non-2-ene, forming a 5- and 10-, and a 7- and 8-membered bicyclic lactone ring starting from 1,3-cyclooctadiene and 1,5-cyclooctadiene respectively (Scheme 2 and 4). During the completion of this manuscript, Mikula[15] and Rutjes[16] recently published elegant alternative approaches to stereoselective preparation of cleavable d-TCO and sTCO linkers.



Scheme 2. Synthesis of releasing sTCO from 1,3-cyclooctadiene.

Starting from 1,3-cyclooctadiene, following the method of Fox and co-workers,[12] we used a Rh-catalyzed reaction of ethyl diazoacetate to form bicyclo[6.1.0]non-2-ene-9-carboxylate **1** as a mixture of *anti*- and *syn*-isomers. After saponification of the ethyl ester, iodolactonization of **2** gave **3** as a single isomer. NMR results confirmed that only the *syn*-isomer was able to generate a lactone, allowing efficient removal of the *anti*-isomer. The following hydrogen iodide elimination resulted in olefinic lactone **4**. However, upon hydrolysis of lactone **4** the resulting free hydroxyl was unstable, as it readily re-formed lactone **4** with the carboxylic acid or its methyl ester. Therefore, the hydroxyl as well as the carboxyl groups had to be rapidly protected to prevent this. Indeed, one-pot lactone hydrolysis followed by methylation of the carboxyl group and acetylation of the hydroxyl group gave the desired compound **5**, with the acetoxy and methylester groups positioned *cis* as expected. The subsequent photoisomerization of **5** generated a mixture of two sTCO isomers, in which the acetoxy groups were located in axial (**6**) and equatorial (**7**) position, respectively.

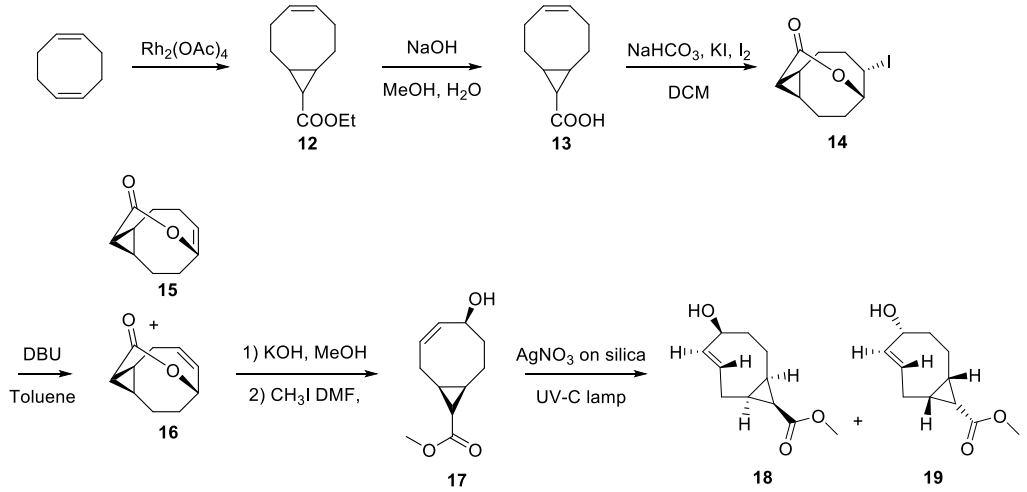
To bypass the unstable intermediates, we assessed whether it would be possible to photoisomerize lactone **4** directly (Scheme 3), to form the *trans*-cyclooctene-lactone **8** and **10**, or if ring strain would prohibit its formation or isolation. To our delight, photoisomerization of the lactone indeed afforded **8** and **10** as a mixture of its axial and equatorial isomers. Hydrolysis of the lactone generated the axial and equatorial isomers 2-hydroxybicyclo[6.1.0]nonene **9** and **11**, as the potassium salts.



Scheme 3. Photoisomerization and hydrolysis of lactone **4**.

Next, we evaluated whether the iodolactonization strategy would also work when starting from 1,5-cyclooctadiene. Reaction of 1,5-cyclooctadiene with ethyl diazoacetate and hydrolysis of the ethyl group, afforded ethyl bicyclo[6.1.0]non-4-ene carboxylate **12**. Hydrolysis of the ethyl ester then yielded carboxylic acid **13**. Also here, the iodolactonization was successful, forming iodolactone **14** from the *syn*-configuration. Subsequent elimination of hydrogen iodide to form the olefinic bond of the cyclooctene gave the desired cyclooctene lactone **16** in 67 % yield, alongside byproduct **15**, resulting from elimination of hydrogen iodide from the tertiary carbon. Hydrolysis to **17** and photoisomerization generated two *trans*-cyclooctene isomers with the

hydroxyl groups located at the axial (**18**) and equatorial (**19**) positions, respectively. The stereochemistry of **18** was determined by NMR (Figure S1 in Supplementary Information).



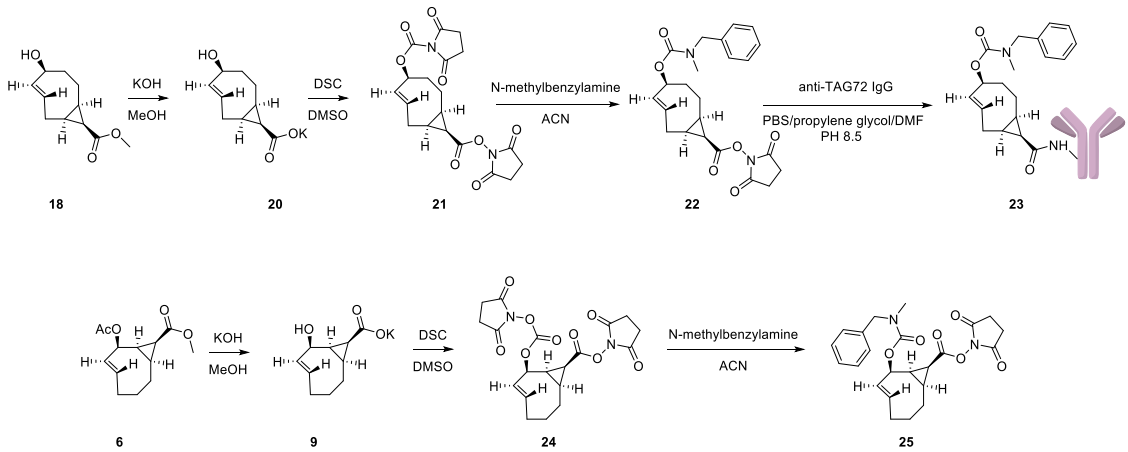
Scheme 4. Synthesis of releasing sTCO from 1,5-cyclooctadiene.

We next examined the reactivity of the resulting sTCOs. Their second-order rate constants were determined with 3,6-dimethyl-1,2,4,5-tetrazine in ACN solvent at 20 °C (Table 1). As expected the equatorial isomers were less reactive for both sTCOs.[4] The axial isomers **18** and **9** were chosen for the further evaluation as a cleavable linker. In order to verify the linker stability, reactivity and release efficacy we set out to prepare conjugatable NHS-ester derivatives of sTCO **18** and **6** functionalized with *N*-methylbenzylamine as a model for an attached drug (Scheme 5). After saponification of the methyl ester, the carboxylic acid salt and hydroxyl of resulting **20** and **9** were activated to obtain bis-NHS TCO derivatives **21** and **24**. Similar to our work on our previous bis-NHS-activated TCO linker,[5] we reasoned that the NHS-carbonate could be more reactive than the NHS-ester allowing selective functionalization, as the latter is *syn*-positioned above the plane of the TCO ring and may therefore be sterically shielded. Indeed, when **21** and **24** were reacted with 1 equivalent of *N*-methylbenzylamine we observed selective reaction with the NHS-carbonate forming compounds **22** and **25**. The reactivity of both compounds towards 3,6-dimethyl-1,2,4,5-tetrazine was determined in ACN, affording second-order rate constants of 2.9 M^{-1} s^{-1} and 8.0 M^{-1} s^{-1} respectively (Table 1), ca. 5- and 15-fold higher than the reactivity (0.54 M^{-1} s^{-1}) of the parent TCO with a similar allylic benzylamine substituent.[4] The *in vitro* payload release triggered by 3,6-dimethyl-1,2,4,5-tetrazine was evaluated in 20% ACN/PBS at 20 °C, and LC-MS analysis after 30 minutes showed that *N*-methylbenzylamine had been completely released from **22** and **25** (Figure S2 and S3 in Supplementary Information).

Table 1. Reactivity of sTCO derivatives towards tetrazine 3,6-dimethyl-1,2,4,5-tetrazine (ACN, 20 °C).

Compound	k_2 (M^{-1} s^{-1})
9	35.0
11	4.0
18	15.0
19	3.3
22	2.9
25	8.0

The NHS-TCO-benzylcarbamate **22** was subsequently conjugated to antibody CC49 affording CC49-TCO-benzylamine **23** with a drug-to-antibody ratio (DAR) of ca. 3. The conjugate was stable when stored in PBS at 4 °C over one year as indicated by DAR analysis. In contrast, the 1,3-sTCO-derived CC49 conjugate was surprisingly unstable under these conditions, with TCO linker deactivation occurring within days.



Scheme 5. Preparation of the sTCO derivatives.

Proceeding with CC49-TCO-benzylcarbamate **23** we evaluated its tetrazine-triggered payload release *in vitro.* The antibody conjugate was reacted with 40 equivalents of 3,6-dimethyl-1,2,4,5-tetrazine in 25% propyleneglycol in PBS at 37 °C, and *N*-methylbenzylamine release was monitored with LC-MS, showing 87% release after 1h (Figure 2).

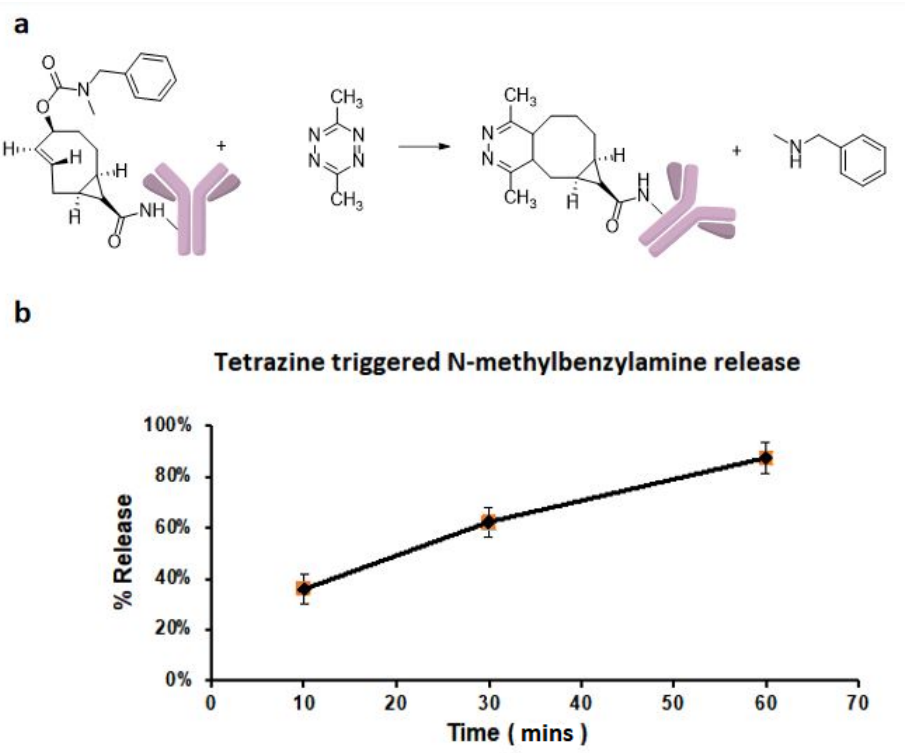


Figure 2. (a) Cleavage reaction of CC49-sTCO-*N*-methylbenzylcarbamate (**23**) triggered by 3,6-dimethyl-1,2,4,5-tetrazine; (b) Tetrazine triggered *N*-methylbenzylamine release from **23**.

Finally we questioned if and to what extent the increased TCO reactivity results in decreased linker stability *in vivo*. Earlier studies have shown that TCO constructs and their protein conjugates can be isomerized to the unreactive *cis*-isomer by interaction with copper-containing proteins such as albumin and that the isomerization rate increases with increasing TCO reactivity.[17] To evaluate this for the new and more reactive TCO linker, conjugate **23** was labeled with iodine-125 and administered to nude mice (n=3), and blood samples were withdrawn at several timepoints.[17] The radioactivity levels in blood showed typical mAb

pharmacokinetics with a 2.8h $t_{1/2,\alpha}$ (54.3%) and a 64.4h $t_{1/2,\beta}$ for the mAb-conjugate (Figure 3a), demonstrating that the conjugation of linker-payload did not adversely affect the protein. Following *ex vivo* reaction of the blood samples with an excess of ^{111}In-labeled tetrazine,[17] the In-111/I-125 cpm ratio in the samples was plotted *vs* time demonstrating an *in vivo* TCO deactivation half-life of 5.6 days (Figure 3b), which is comparable to that of the currently used TCO linker that has a 5-fold lower reactivity.[5,6] In an earlier study, an antibody-conjugated sTCO tag lacking an allylic substituent showed much faster *in vivo* deactivation ($t_{1/2}$ = 0.67 days), most likely due to higher reactivity and/or lower steric hindrance making it more prone to interact with Cu-containing serum proteins in circulation.[17]

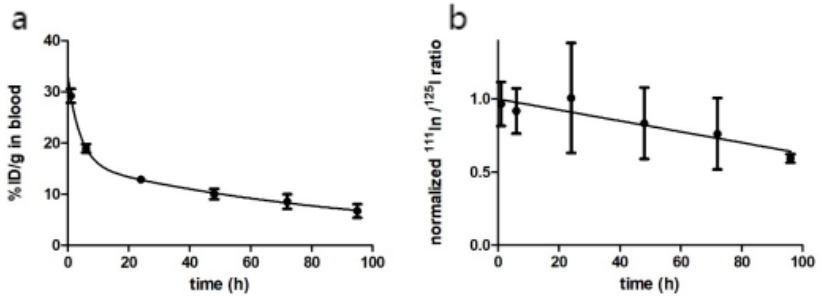


Figure 3. (a) Blood clearance profile of ^{125}I-CC49-sTCO-*N*-methylbenzylcarbamate (**23**) in tumor-free mice; (b) *In vivo* sTCO linker stability measured by reacting blood aliquots with ^{125}I-CC49-sTCO-*N*-methylbenzylcarbamate *ex vivo* with ^{111}In-labeled tetrazine. Data represent the mean with one SD (n = 3).

In conclusion, we synthesized sTCO linkers from 1,3-cyclooctadiene and 1,5-cyclooctadiene via a concise stereoselective strategy affording bifunctional sTCOs with an allylic hydroxyl positioned *cis* relative to a carboxylic acid group, which can selectively be derivatized. Treatment with tetrazine resulted in efficient payload release from the sTCOs *in vitro*. Compared with the parent TCO linker,[5] the sTCO linker derived from 1,5-cyclooctadiene showed a 5-fold higher reactivity combined with maintained *in vivo* stability as antibody linker, supporting future applications with low reagent concentrations or limited timeframes. As such, the payload releasing sTCO described herein expands the TCO/tetrazine click-to-release reaction toolbox.

Acknowledgments
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References

1) Blackman, M. L., Royzen, M., & Fox, J. M. (2008). J. Am. Chem. Soc., 130(41), 13518-13519.
2) Oliveira, B. L., Guo, Z., & Bernardes, G. J. L. (2017). Chem. Soc. Rev., 46(16), 4895-4950.
3) Scinto, S. L., Bilodeau, D. A., Hincapie, R., Lee, W., Nguyen, S. S., Xu, M., ... & Fox, J. M. (2021). Nat. Rev. Methods Primers, 1(1), 1-23.
4) Versteegen, R. M., Rossin, R., Ten Hoeve, W., Janssen, H. M., & Robillard, M. S. (2013). Angew. Chem. Int. Ed., 125(52), 14362-14366.
5) Rossin, R., Van Duijnhoven, S. M., Ten Hoeve, W., Janssen, H. M., Kleijn, L. H., Hoeben, F. J., ... & Robillard, M. S. (2016). Bioconjugate Chem., 27(7), 1697-1706.
6) Rossin, R., Versteegen, R. M., Wu, J., Khasanov, A., Wessels, H. J., Steenbergen, E. J., ... & Robillard, M. S. (2018). Nat. Commun., 9(1), 1-11.
7) Czuban, M., Srinivasan, S., Yee, N. A., Agustin, E., Koliszak, A., Miller, E., ... &

Mejia Oneto, J. M. (2018). ACS Cent. Sci., 4(12), 1624-1632.
8) Yao, Q., Lin, F., Fan, X., Wang, Y., Liu, Y., Liu, Z., ... & Gao, Y. (2018). Nat. Commun., 9(1), 1-9.
9) Li, J., Jia, S., & Chen, P. R. (2014). Nat. Chem. Biol., 10(12), 1003-1005.
10) Zhang, G., Li, J., Xie, R., Fan, X., Liu, Y., Zheng, S., ... & Chen, P. R. (2016). ACS Cent. Sci., 2(5), 325-331.
11) Versteegen, R. M., Ten Hoeve, W., Rossin, R., & De Geus, M. A. R.; Janssen, H. M.; Robillard, M. S. (2018). Angew. Chem. Int. Ed., 57(33), 10494-10499.
12) Taylor, M. T., Blackman, M. L., Dmitrenko, O., & Fox, J. M. (2011). J. Am. Chem. Soc., 133(25), 9646-9649.
13) Darko, A., Wallace, S., Dmitrenko, O., Machovina, M. M., Mehl, R. A., Chin, J. W., & Fox, J. M. (2014). Chem. Sci., 5(10), 3770-3776.
14) Siegl, S. J., Vázquez, A., Dzijak, R., Dračínský, M., Galeta, J., Rampmaier, R., ... & Vrabel, M. (2018). Chem. - Eur. J., 24(10), 2426-2432.
15) Kuba, W., Sohr, B., Keppel, P., Svatunek, D., Humhal, V., Stöger, B., ... & Mikula, H. (2022). Chem. - Eur. J., e202203069.
16) Sondag, D., Maartense, L., De Jong, H., De Kleijne, F. F., Bonger, K. M., Löwik, D. W., ... & Rutjes, F. P. (2022). Chem. - Eur. J., e202203375.
17) Rossin, R., Van Den Bosch, S. M., Ten Hoeve, W., Carvelli, M., Versteegen, R. M., Lub, J., & Robillard, M. S. (2013). Bioconjugate Chem., 24(7), 1210-1217.