Organic LETTERS

Letter

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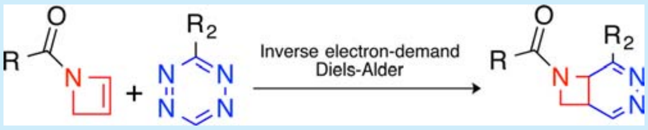
Acylazetine as a Dienophile in Bioorthogonal Inverse Electron-Demand Diels–Alder Ligation

Sander B. Engelsma, Lianne I. Willems, Claudia E. van Paaschen, Sander I. van Kasteren, Gijsbert A. van der Marel, Herman S. Overkleeft,* and Dmitri V. Filippov*

Leiden Institute of Chemistry, Leiden University, P.O. Box 9502, 2300 RA Leiden, The Netherlands

Supporting Information

ABSTRACT: A new bioorthogonal *N*-acylazetine tag, suitable for tetrazine mediated inverse electron-demand Diels–Alder conjugation, is developed. The tag is small and achiral. We demonstrate the usefulness of *N*-acylazetine-tetrazine based bioorthogonal chemistry in two-step activity-based protein profiling. The performance of the new tetrazinophile in the labeling of catalytically active proteasome subunits was comparable to that of the more sterically demanding norbornene tag.



Bioorthogonal chemistry strives to chemoselectively modify biomolecules, such as proteins, carbohydrates, and lipids, within the complex environments of cell lysates, living cells, and, ultimately, in living animals. Bioorthogonal chemistry has pushed forward various fields of research, such as activity-based protein profiling, *in vitro* and *in vivo* imaging, and modified metabolite labeling.[1] Ideally, in a bioorthogonal process a reactive group incorporated into the biomolecule of interest reacts selectively with a complementary reactive group modified with a reporter moiety, such as a fluorescent label to enable visualization of the target or an affinity probe for postlabeling purification.[2] To enable this, a bioorthogonal tag should be inert to the broad spectrum of functional groups that reside in a biological system, while exerting sufficiently fast reaction kinetics for conjugation to occur at nanomolar concentrations. Additives to promote the reaction between the tag and the reactive functionality of the reporter group are best avoided. Finally, the tag should be sterically compact to minimize unfavorable steric interactions with the biological system being studied and be synthetically accessible.

In the past decade, several bioorthogonal ligation strategies have been developed. Among these, prominent transformations are the Staudinger–Bertozzi ligation,[3] the copper-catalyzed,[4] and the strain-promoted azide–alkyne [2 + 3] cycloaddition.[5] An important recent development is the inverse-electron demand Diels–Alder reaction (IEDDA), which exhibits the fastest reaction kinetics among all commonly used bioorthogonal transformations, while being chemoselective, efficient, and additive-free.[6] In its most typical setup, IEDDA uses a strained alkene tag, while its reaction partner is a reporter-group functionalized tetrazine derivative. Well-established strained alkene handles are norbornene (Figure 1A) and *trans*-cyclooctene (Figure 1B). Although these alkenes exhibit exceptionally fast reaction kinetics, their steric bulk and lipophilicity may induce a biological response, bringing a paradoxical problem, known as the observer effect, into chemical biology. Examples of an influence of a lipophilic reporter groups, either favorable or otherwise, on the behavior of biomolecules has been noted by us[7a] and others.[7b] Hence, there is room for the development of more compact dienophiles for tetrazine mediated bioorthogonal cycloadditions. Recently, Devaraj et al. introduced the methylcyclopropene core (Figure 1C) as a dienophile for IEDDA that combines a small size ("minitag") with fast reaction kinetics.[8] Other laboratories developed related cyclopropene based bioorthogonal tags.[9] The reactivity of cyclopropenes in a normal Diels–Alder reaction has been recently highlighted as well.[10]

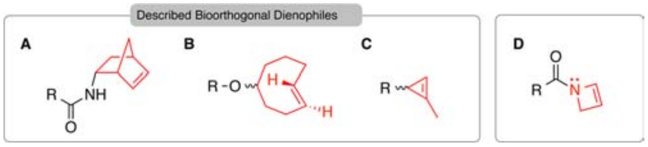


Figure 1. Known bioorthogonal dienophilic moieties (A–C) and the azetine tag (D) described here.

After perusing the literature, we observed that all dienophiles used in IEDDA-based bioorthogonal chemistry rely on ring strain to attain the desired reactivity toward tetrazines. These contemporary dienophiles—norbonene, *trans*-cylcooctene, and cyclopropene (Figure 1A–C)—are already described in the seminal 1990 report by Sauer et al. on the reactivity of electron-rich dienophiles in IEDDA processes. In the same paper, these authors also showed that alkenes activated by an electron-donating heteroatom adjacent to the double bond exhibit a higher reactivity toward tetrazine relative to their non-conjugated analogues.[11] Introduction of a heteroatom into the cyclobutene scaffold should therefore lead to a viable dienophile for IEDDA-based bioorthogonal chemistry. Following this reasoning, a 2-azetine would be the smallest viable core that could utilize this effect in addition to ring strain, with the

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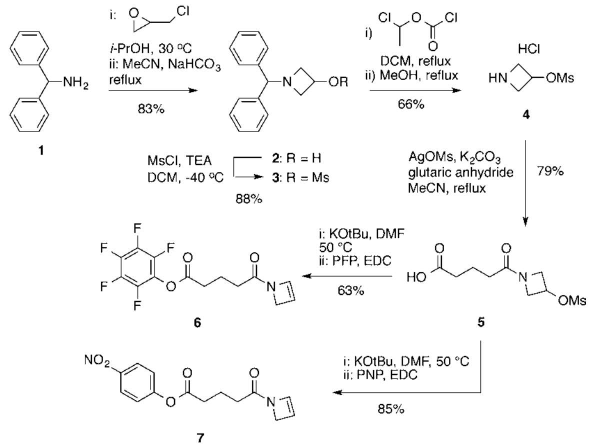
ring nitrogen amenable for substitution to yield a new and compact bioorthogonal tag. Although *N*-alkylazetines are the more electron-rich species, we considered *N*-acylazetines as more suitable candidates because of their reported stability.[12] We here report on the development of *N*-acylazetines as (Figure 1D) as IEDDA dienophiles suitable for bioorthogonal chemistry.

Scheme 1. Synthesis of Active Ester Functionalized *N*-Acylazetines 6 and 7



The synthesis of *N*-acylazetines **6** and **7**, equipped with an activated ester for further modification (Scheme 1), commenced with the preparation of the azetidine core.[13] Epichlorohydrin was reacted with benzhydrylamine in a two-step one-pot procedure to afford protected azetidine **2**. The hydroxy group of **2** was mesylated, affording methanesulfonate **3**, which was subsequently deprotected using chloroethyl chloroformate and methanol. The route from **1** to **4** was optimized in such a way that each intermediate could be purified through crystallization and washing steps (in 48% yield over three steps). With free amine **4** in hand, the ring opening of glutaric anhydride could commence. Initially this was carried out under the agency of triethylamine. However, during the reaction, a displacement reaction took place, wherein the methanesulfonate was substituted by chloride. Triethylamine proved to interfere with the column purification of the free carboxylic acid formed upon the opening of glutaric anhydride. Switching the base to potassium carbonate, and premixing **4** with silver methanesulfonate to precipitate the chloride as AgCl, solved these issues, and **5** could be obtained in 79% yield. The following elimination−esterification sequence was carried out as a one-pot procedure. First, methanesulfonic acid was eliminated with KOtBu to afford the *N*-acylazetine moiety. Next, the carboxylate was converted to either pentafluorophenol (PFP) ester (**6**) or a *para*-nitrophenyl (PNP) counterpart (**7**) via EDC mediated condensation with the appropriate alcohol. Both esters were readily obtained; however, the PFP-ester (**6**) proved to be unstable and could not be stored for extended periods of time. Another disadvantage was its gel-like consistency at rt, making it difficult to handle. On the other hand, the PNP-ester (**7**) was isolated as a crystalline solid that is stable at rt for at least 3 months.

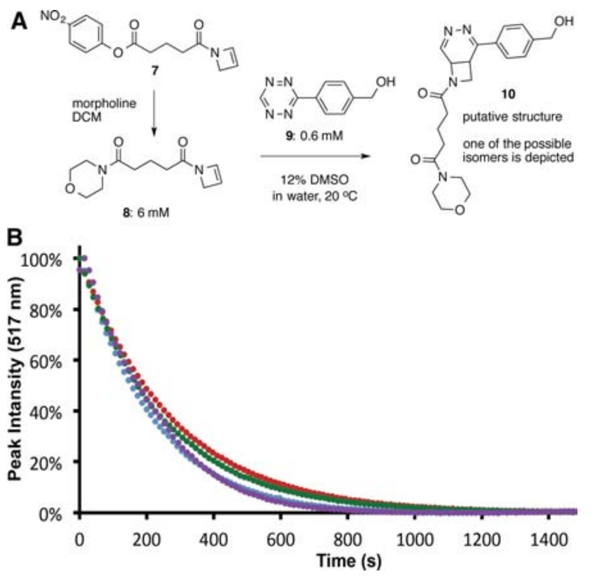
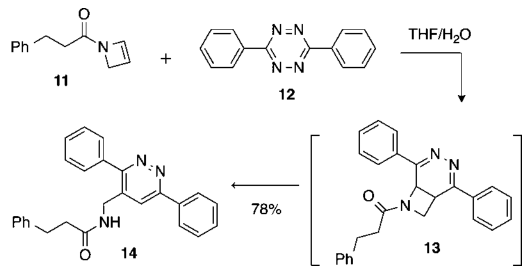


Figure 2. (A) Preparation of model acylazetine **8** and an overview of the kinetics experiment. (B) Data plots following the tetrazine absorption band over time in a reaction between *N*-acylazetine **8** (6 mM) and tetrazine **9** (0.6 mM) in 12% DMSO/water. The kinetics experiment was performed four times. Compound **10** is formed as a mixture of regioisomers and probably tautomers.

Scheme 2. A Model Cycloaddition of Simple *N*-Acylazetine 11 to Symmetric Disubstituted Tetrazine 12



To investigate the kinetics of the IEDDA reaction of *N*-acylazetine with tetrazine, **7** was coupled with morpholine to make the water-soluble *N*-acylazetine **8**. A 10-fold excess of compound **8** was reacted with tetrazine **9** (Figure 2).[14] The rate was determined by monitoring the absorbance of the tetrazine at 517 nm (Figure 2). We established the pseudo-first-order reaction rate constant to be $4.5 \times 10^{-3} \pm 8.2 \times 10^{-5}$ s^{-1} at 20 °C in a solution of 12% DMSO in water. Next, in a separate experiment, we determined a second-order rate constant k_2 to be approximately 0.39 ± 0.1 s^{-1} M^{-1} (Supporting Information (SI)). The mass-spectroscopic data of the reaction product, formed as a mixture of isomers, were fully consistent with putative structure **10**. The characterization of **10** by NMR was complicated by the two tertiary amide bonds, which appear to exist as rotamers on the NMR time scale. In order to obtain a more spectroscopically tractable product and to investigate the course of the *N*-azylazetine cycloaddition in more detail we performed the reaction of **11** with symmetrical tetrazine **12** (Scheme 2). We observed the clean formation of rearranged compound **14** that was formed as a single product via opening of the azetidine ring of the immediate IEDDA adduct **13**. We observed a trace amount of the product of the same rearrangement after the reaction of **8** with **9**, as evidenced by the resonance of the tertiary carbon of pyridazine at 123 ppm in the ^{13}C NMR spectrum of **10**.

In order to evaluate the applicability of the novel *N*-acylazetine ligation handle for biological labeling strategies, the tag was incorporated into an activity-based proteasome probe to enable two-step activity-based protein profiling (ABPP) through ligation with a fluorescently labeled tetrazine (**17**, Figure 3).[15] As a model target enzyme we selected the constitutive proteasome, a multisubunit protein complex containing three different catalytically active subunits (*β*1, *β*2, and *β*5). These *β*-subunits each have a different substrate preference and can be targeted with either subunit-selective[16] or with broad-spectrum[17] activity-based probes (ABPs). The designed ABP **15** is based on the broad-spectrum irreversible proteasome inhibitor epoxomicin[18] functionalized at the *N*-terminus with the acylazetine moiety. Compound **15** (Figure 3) was readily prepared from protected peptide epoxyketone (see SI).

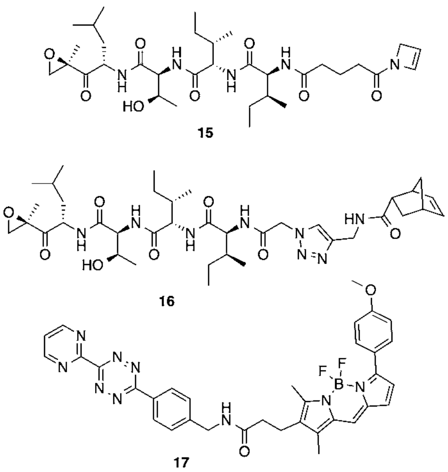


Figure 3. Structures of acylazetine- (**15**) and norbornene (**16**)-functionalized proteasome ABPs and tetrazine-BODIPY reporter reagent (**17**).

The ability of the azetine-functionalized ABP **15** to target the catalytically active proteasome *β*-subunits was confirmed by performing competition experiments against the fluorescent broad-spectrum proteasome ABP MV151 in human embryonic kidney (HEK) cell extracts (SI, Figure S1).[17] These experiments revealed that 3 *μ*M of ABP **15** was required to completely block the fluorescent labeling by MV151. Therefore, for all ensuing two-step labeling experiments a probe concentration of 5 *μ*M was used. The utility of the azetine tag for two-step labeling of proteasome activity in cell extracts was next established by exposure of HEK cell extracts to ABP **15** followed by ligation with varying concentrations of tetrazine **17** (Figure 3). As a control, the same procedure was performed using the previously reported norbornene-functionalized ABP **16**.[15] Analysis of the labeled proteins on gel with fluorescent readout (Figure 4A) revealed that tetrazine ligation of cell extracts treated with azetine-functionalized ABP **15** results in the specific fluorescent labeling of three bands which correspond to the proteasome *β*-subunits labeled by fluorescent ABP MV151. The labeling is dependent on the concentration of tetrazine in a similar manner as that for norbornene-functionalized ABP **16**. With both ABPs a similar increase in fluorescent labeling was observed when the ligation step was performed for increasing reaction times (Figure 4B).

These results demonstrate that in this experimental setup the *N*-acylazetine moiety reacts with equal efficiency as the norbornene ligation handle in the IEDDA reaction with tetrazine **17**. The absence of labeling in samples in which the proteasome activity was inhibited by an excess of epoxomicin shows that the labeling indeed reflects labeling of the catalytically active proteasome *β*-subunits. In order to test whether *N*-acylazetine in **15** may cross-react with other commonly used ligation reagents, two-step proteasome labeling procedures were performed in HEK cell extracts using alkyne-, azide-, and phosphine-functionalized reagents instead of tetrazine for the ligation step (SI, Figure S4). No proteasome labeling was detected when using Bodipy-alkyne,[19] Bodipy-azide,[15] biotin-phosphine,[20] and biotinylated dibenzocyclooctene[21] reagents, demonstrating that these do not react with the azetine-tagged probe. Together these results demonstrate the utility of the new compact *N*-acylazetine ligation handle for bioorthogonal labeling of proteins or other biomolecules via tetrazine ligation.

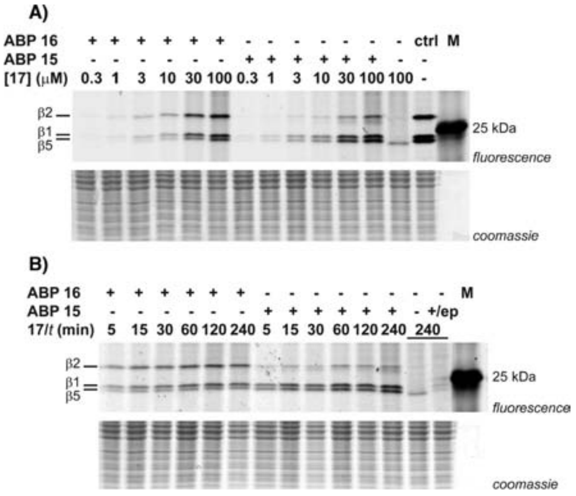


Figure 4. (A) Labeling of proteasome activity in HEK cell extracts by exposure to 5 *μ*M of ABP **15** or ABP **16** for 1 h followed by reaction with 0.3–100 *μ*M of Bodipy-tetrazine **17** for 1 h. 12.5% SDS-PAGE with fluorescent readout followed by coomassie brilliant blue staining. "ctrl": control sample labeled with 1 *μ*M MV151. Proteasome *β*-subunits are designated on the basis of reported labeling by MV151. 'M': protein marker. **B:** Labeling of proteasome activity in HEK cell extracts by exposure to 5 *μ*M of ABP **15** or ABP **16** for 1 h followed by reaction with 10 *μ*M of Bodipy-tetrazine **17** for 5–240 min. "ep": 100 *μ*M epoxomicin added to incubation with ABP **15**. Proteasome *β*-subunits are designated on the basis of reported labeling by MV151. 'M': protein marker.

In conclusion, we designed and synthesized a novel *N*-acylazetine tag for the tetrazine ligation strategy and showed its applicability in an ABPP experiment. The acylazetine was incorporated into a compact linkable tag that is accessible through an efficient five-step synthesis. Determination of the reaction kinetics between the *N*-acylazetine and tetrazine showed a reaction rate constant in line with that of the methylcyclopropene mini-tag. We functionalized epoxomicin, a broad-spectrum proteasome inhibitor, with our novel ligation handle and could successfully label the proteasome through tetrazine ligation. Based on these results, we anticipate that this small tag is a valuable addition to the current arsenal of IEDDA dienophiles for application in chemical biology research.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental details, spectra, and biological experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail: h.s.overkleeft@lic.leidenuniv.nl.
*E-mail: filippov@chem.leidenuniv.nl.

Notes

The authors declare no competing financial interest.

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