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Aza-dibenzocyclooctynes for fast and efficient enzyme PEGylation *via* copper-free (3 + 2) cycloaddition†

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A strained aza-dibenzocyclooctyne was prepared *via* a high-yielding synthetic route. Copper-free, strain-promoted click reaction with azides showed excellent kinetics, and a functionalised aza-cyclooctyne was applied in fast and efficient PEGylation of enzymes.

The process of PEG conjugation to either peptides or proteins, known as PEGylation, is a procedure of growing interest in both biotechnology and therapeutical science.¹ PEGylation of proteins improves their *in vivo* applicability by reducing aggregation, shielding critical protein binding sites and improving the water solubility.² As a result, a large variety of methods for the conjugation of PEG to either peptides or proteins is currently available.³ In most cases, however, a large excess of PEG and prolonged reaction times are required, and nevertheless generally result in suboptimal conversions.⁴ With the recent discovery of the Cu(I)-catalysed Huisgen cycloaddition reaction,⁵ a powerful new methodology was added to the field of bioconjugation⁶ with the particular benefits that the reaction is selective, fast and high-yielding. The Cu(I)-catalysed 1,3-dipolar cycloaddition reaction has become of great use in labelling studies, the development of new therapeutics and in protein modification.⁷ In addition, the Cu(I)-catalysed 1,3-dipolar cycloaddition reaction has also been applied in the synthesis of PEG–protein bioconjugates.⁸ A disadvantage of this reaction in PEGylation is the potential presence of residual copper in the product. Not only is Cu(I) toxic to cells, it can also bind to the active site of enzymes, thereby blocking or reducing biological activity.^{9,10} Furthermore, Cu(I) is easily disproportionated in an aqueous environment, thus reducing the rate of the reaction. Therefore, interest is growing in methods involving Cu-free 1,3-dipolar cycloaddition reactions.¹¹ Several strain-promoted systems, such as oxanorbornadienes,¹² cyclooctynes,¹⁰ and dibenzocyclooctynes¹³ (Fig. 1) have recently been developed for the fast and selective reaction with azide-containing biomolecules and have found application in *e.g.* tumour imaging,¹⁴ glycan labelling,^{10,13} *in vivo* imaging,¹⁵ and surface modification.¹⁶ However, to

the best of our knowledge, these systems have not yet been employed in the PEGylation of proteins.

Inspired by the dibenzocyclooctyne derivative (DIBC, structure **D** in Fig. 1) developed by Boons *et al.*¹³ and aza-dimethoxycyclooctyne (DIMAC, structure **C** in Fig. 1) synthesised by Bertozzi *et al.*,¹⁷ we set out to develop the hybrid structure aza-dibenzocyclooctyne (DIBAC, structure **E** in Fig. 1). The aza-dibenzocyclooctyne was designed to combine the favourable kinetics of DIBC (**D**)¹³ with the increased hydrophilicity of DIMAC (**C**).¹⁷ With respect to the latter, the nitrogen atom in our designed analogue should allow straightforward functionalisation of the aniline moiety and modification of the system, *e.g.* by sulfonation. At the same time, we set ourselves the goal to develop a straightforward, high-yielding and fast synthetic route. Herein we report the facile synthesis of aza-dibenzocyclooctyne and derivatives thereof for the quantitative PEGylation of proteins.

The synthetic route towards key-intermediate dihydro-dibenzo-azocine (**5**) is shown in Scheme 1, and is based on a Sonogashira cross-coupling reaction and a reductive amination ring-closing step. Whereas the Sonogashira reaction under standard inert atmosphere initially resulted in Glaser coupling, performing the reaction under an N₂/H₂-atmosphere¹⁸ effectively nihilated Glaser coupling and led to the Sonogashira coupling product **1** in quantitative yield. Next, Boc-protection, generating compound **2**, was found to be essential, in order to make partial hydrogenation of the acetylene to the *Z*-alkene possible, yielding **3** (95%). Next, Dess–Martin oxidation led to aldehyde **4**, the precursor for the reductive amination. Boc-deprotection under acidic conditions resulted in immediate and exclusive formation of a cyclic imine, which was not isolated but subjected to NaBH₄ reduction, generating the free secondary amine (**5**) in quantitative yield over the two steps. *Via* this high-yielding

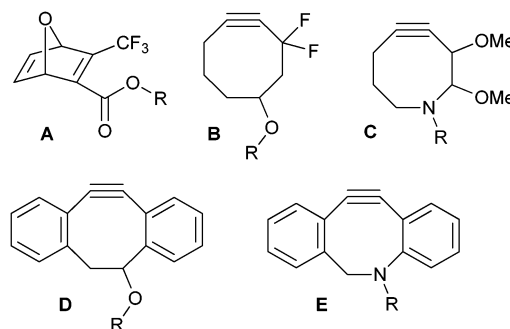


Fig. 1 Strain-promoted systems for Cu-free click reactions.

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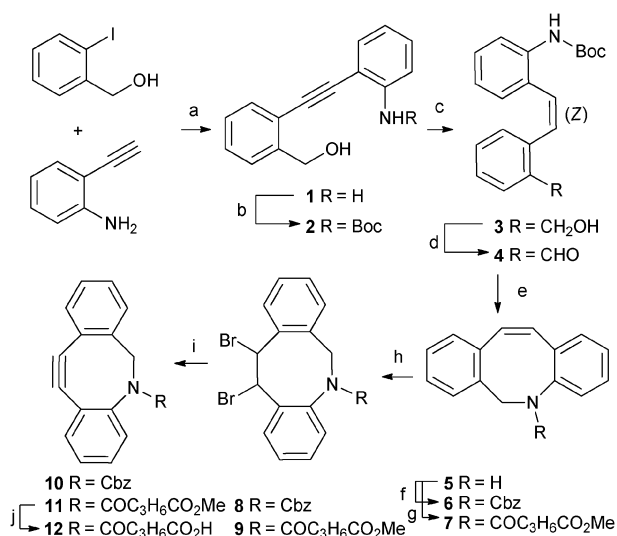
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Scheme 1 Reagents and conditions: (a) PdCl₂(PPh₃)₂, CuI, Et₃N, THF, N₂/H₂-atmosphere, r.t., 4 h (99%); (b) Boc₂O, THF, 70 °C, 2 d (83%); (c) 10% Pd/BaSO₄, quinoline, H₂, MeOH, r.t., 1.5 h (95%); (d) Dess–Martin periodinane, NaHCO₃, CH₂Cl₂, r.t., 40 min (90%); (e) (1) 2 M HCl in EtOAc, r.t., 1 h; (2) NaBH₄, H₂O, r.t., o.n. (100%); (f) CbzCl, Na₂CO₃, H₂O, CH₂Cl₂, r.t., 3 h (86%); (g) ClCOC₃H₆CO₂Me, Et₃N, CH₂Cl₂, r.t., 1.5 h (94%); (h) Br₂, CH₂Cl₂, 0 °C, 2 h (8: 67%, 9: 81%); (i) KO^tBu, THF, 0 °C → r.t., o.n. (10), –40 °C, 2 h (11) (10: 87%, 11: 84%); (j) LiOH, THF, H₂O, r.t., 3 h (92%).

protocol the key aza-cyclooctene intermediate **5** was synthesised in an excellent yield of 70% over five steps.

Unfortunately, direct bromination of dihydrodibenzazocine **5** resulted in the intramolecular substitution of a bromide, leading to an indoline, thus requiring protection of the amine. Consequently, a Cbz-group was introduced generating compound **6** in good yield (86%). The alkene moiety could then be smoothly converted into an alkyne *via* successive bromination (67%) and elimination (87%). Elimination was performed using KO^tBu since other bases such as LDA, *n*-BuLi, NaOH (6 or 12 M) all failed to give the desired aza-dibenzocyclooctyne **10**. Compound **10**, although fulfilling our aim to prepare an aza-cyclooctyne, is clearly lacking a handle for further functionalisation. Unfortunately, deprotection of the Cbz-group, using either acidic or basic conditions, did not yield the free amine (*i.e.* E, R = H, Fig. 1). Instead, 6*H*-isoindolo[2,1-*a*]indole (**13**) was formed, presumably *via* a 5-endo-dig cyclisation, thereby relieving ring-strain with formation of the indole. Consequently, *N*-functionalisation was required prior to alkyne formation. To this end, **5** was equipped with a small functionalised linker, leading to compound **7** in 94% yield (Scheme 1). Subsequent bromination (81%) and elimination with KO^tBu (84%) cleanly resulted in alkyne **11**. A 1 M solution of KO^tBu in THF was used since the use of solid KO^tBu resulted in hydrolysis of the methyl ester, which gave rise to undesired side-reactions. Finally, a functionalizable probe (**12**) was prepared *via* hydrolysis of the methyl ester. The above reported reaction sequence yielded the desired strained aza-cyclooctyne probe, with a functional handle, in a good overall yield of 41% over 9 steps.

Next, the kinetics in the 1,3-dipolar cycloaddition of the Cbz-protected and acid-functionalised DIBACs (**10** and **12**,

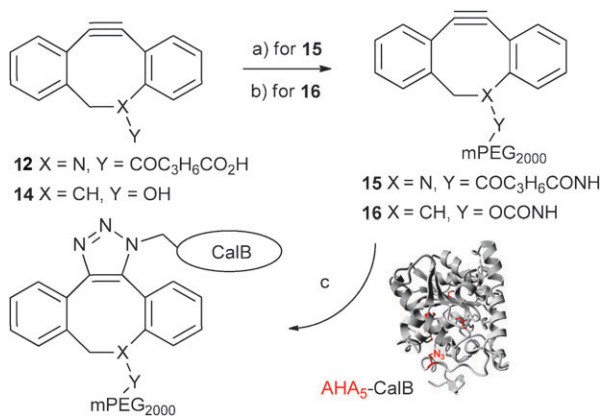
Table 1 Rate constants of the different cyclooctyne systems

Entry	Compound	Solvent	<i>k</i> (M ⁻¹ s ⁻¹)
1	DIBAC (10)	CD ₃ OD	0.29 ± 0.01
2	DIBAC (12)	CD ₃ OD	0.31 ± 0.02
3	DIBAC (12)	D ₂ O	0.36 ^a

^a Reaction was performed under basic conditions with 1.1 equiv. of **12**.

respectively) with benzyl azide were determined in CD₃OD, giving rate constants of 0.29 M⁻¹ s⁻¹ and 0.31 M⁻¹ s⁻¹, respectively. Cycloaddition of DIBAC **12** and 2-azidopropanoic acid was also investigated by performing the reaction in basic D₂O, since addition of a small amount of 2 M NaOH was necessary to dissolve **12**. In D₂O cycloaddition was calculated to proceed with a rate constant of 0.36 M⁻¹ s⁻¹, slightly faster than in CD₃OD. The results of the kinetic experiments are summarised in Table 1. Much to our satisfaction, the kinetic parameters of our new system proved slightly better than DIBC (*k* = 0.17 M⁻¹ s⁻¹)¹³ and DIFO (*k* = 0.076 M⁻¹ s⁻¹)¹⁰ and it reacted approximately 100-fold faster than the hydrophilic DIMAC-system (*k* = 3 × 10⁻³ M⁻¹ s⁻¹).¹⁷

To investigate the applicability of the Cu-free 1,3-dipolar cycloaddition in the conjugation of polyethylene glycol to proteins, the aza-dibenzocyclooctyne analogue **12** was functionalised with H₂N-PEG₂₀₀₀-OMe *via* an EDC-coupling, yielding DIBAC-mPEG₂₀₀₀ (**15**, Scheme 2). For comparison, DIBC-mPEG₂₀₀₀ (**16**) was also prepared *via* conversion of the alcohol into a 4-nitrophenyl carbonate¹³ followed by substitution with H₂N-PEG₂₀₀₀-OMe. Initially, azide-containing CalB (AHA-CalB)¹⁹ was used for the conjugation studies. This modified enzyme, obtained by recombinant expression in autotrophic *E. coli*, contains five azidohomoalanine residues, four of which are concealed inside the protein. Consequently, only one azido residue is exposed on the exterior of the enzyme, readily available for ligation. We have previously reported that this enzyme underwent PEGylation selectively with the most accessible residue by applying the Cu(I)-catalysed (3 + 2) cycloaddition reaction. However, full conversion could never be reached, despite the use of 20 equivalents of acetylene-functionalised acetylene-PEG₅₀₀₀ and stirring for 1–3 days.¹⁹ Now, PEGylation of AHA-CalB was pursued by mixing AHA-CalB (1 μg/μL, corresponding to ~30 μM) with DIBAC-mPEG₂₀₀₀ **15** or DIBC-mPEG₂₀₀₀ **16** (one, two and five equiv). After three hours the reaction was quenched with benzyl azide and analysed by SDS-PAGE (Fig. 2). As becomes clear, with five equivalents of DIBAC-mPEG₂₀₀₀ (**15**) full conversion was observed within three hours (Fig. 2, lane 2). Interestingly, not only was the enzyme fully functionalised, but it appears that one of the less accessible azides also reacted, generating di-PEGylated CalB, confirming the excellent reactivity of aza-dibenzocyclooctyne towards azides. The higher reactivity of the DIBAC system is further demonstrated by the fact that a higher degree of PEGylation is observed than with DIBC-mPEG₂₀₀₀. In both cases, the small amount of ‘unreacted’ CalB observed is not caused by failure to react, but can be ascribed to the fact that AHA-CalB always contains a small amount of non-modified enzyme.¹⁹ Performing the PEGylation with either one or two equivalents of DIBAC **15**, approximately 50% conversion to the single PEGylated



Scheme 2 Reagents and conditions: (a) H₂N-PEG₂₀₀₀-OMe, EDC, DMAP, CH₂Cl₂, 2 d, r.t.; (b) (1) (4-NO₂C₆H₄)OCOCI, pyridine, CH₂Cl₂, 4 h, r.t.; (2) H₂N-PEG₂₀₀₀-OMe, CH₂Cl₂, 1 d, r.t.; (c) PBS-buffer (pH 8.5, 30 μM), 3 h, r.t.

product was observed (see lanes 3 and 4). Using DIBC 16 under the same conditions resulted in somewhat lower conversions (see lanes 6 and 7).

The fast and quantitative ligation of both reagents to AHA-CalB shows the high potential of these systems. To investigate the reactivity and efficiency of these reagents towards other enzymes, HRP was modified *via* diazotransfer as recently developed in our group.²⁰ The resulting HRP-N₃ was then reacted with either 15 or 16 following the same procedure as applied to AHA-CalB, giving almost identical results (See ESI, Fig. S1†). The aza-dibenzocyclooctyne is in both cases more reactive than the dibenzocyclooctyne, especially when only one or two equivalents were used.

Nevertheless, in comparison to the Cu(I)-catalysed cycloaddition reaction, both DIBCs show fast and efficient modification of both enzymes, clearly showing the power and applicability of these copper-free systems.

In conclusion, we have developed a new, highly efficient reagent for copper-free (3+2) cycloaddition reactions. The aza-dibenzocyclooctyne is easily accessible *via* a versatile and high-yielding synthetic route, and allows straightforward functionalisation *via* the nitrogen atom. In addition, the outstanding reaction rate constant ($k = 0.3 \text{ M}^{-1} \text{ s}^{-1}$) marks the suitability of this probe for bioconjugation purposes, as

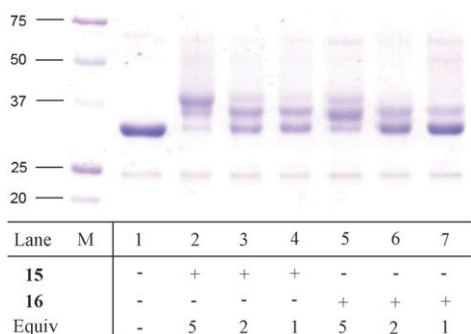


Fig. 2 SDS-PAGE gel of PEGylated AHA-CalB (1 μg/μL) using various equivalents of cyclooctyne 15 or 16.

was underlined by the effective PEGylation of enzymes. The functionalisation, further derivatisation and application of aza-dibenzocyclooctynes in bioconjugation are topics currently under investigation in our laboratories.

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