Aza-dibenzo[cyclooctynes for fast and efficient enzyme PEGylation via copper-free (3 + 2) cycloaddition†

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A strained aza-dibenzo[cyclooctyne 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-dibenzo[cyclooctyne (DIBAC, structure E in Fig. 1). The aza-dibenzo[cyclooctyne 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-dibenzo[cyclooctyne 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.](image-url)
Instead, resulted in alkyne (d) Dess–Martin periodinane, NaHCO₃, CH₂Cl₂, r.t., 40 min (90%); 2 d (83%); (c) 10% Pd/BaSO₄, quinoline, H₂, MeOH, r.t., 1.5 h (95%); THF, N₂/H₂-atmosphere, r.t., 4 h (99%); (b) Boc₂O, THF, 70 °C, 2 h (95%), (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) ClCOC₃H₆-Bu, CO₂Me, Et₃N, CH₂Cl₂, r.t., 1.5 h (94%); (h) Br₂, CH₂Cl₂, 0 °C, 2 h (87%, 9:81%); (i) KO'Bu, 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%).

The reaction of compound 5 with 2 M HCl in EtOAc is crucial for the alkyne formation, 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'Bu 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, 6H-isoidolo[2,1-α]indole (13) was formed, presumably via an 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'Bu (84%) cleanly resulted in alkyne 11. A 1:1 solution of KO'Bu in THF was used since the use of solid KO'Bu 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, respectively) with benzyl azide were determined in CD₂OD, 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 CD₂OD. 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-dibenzo[cyclooctyne 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 carbonate¹² followed by substitution with H₂N-PEG₂₀₀₀-OMe. Initially, azide-containing CalB (AHA-CalB)¹⁰ was used for the conjugation studies. This modified enzyme, obtained by recombinant expression in auxotrophic E. coli, contains five azidohomoalanine residues, four of which are concealed inside the protein. Consequently, only one azide 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-dibenzo[cyclooctyne 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

<table>
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<th>Entry</th>
<th>Compound</th>
<th>Solvent</th>
<th>k (M⁻¹ s⁻¹)</th>
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<tr>
<td>1</td>
<td>DIBC (10)</td>
<td>CD₂OD</td>
<td>0.29 ± 0.01</td>
</tr>
<tr>
<td>2</td>
<td>DIBC (12)</td>
<td>CD₂OD</td>
<td>0.31 ± 0.02</td>
</tr>
<tr>
<td>3</td>
<td>DIBC (12)</td>
<td>D₂O</td>
<td>0.36°</td>
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Table 1 Rate constants of the different cyclooctyne systems

*Reaction was performed under basic conditions with 1.1 equiv. of 12.*
was underlined by the effective PEGylation of enzymes. The functionalisation, further derivatisation and application of aza-dibenzo-cyclooctynes in bioconjugation are topics currently under investigation in our laboratories.

Notes and references


