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 therapeutic 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 excess of PEG and prolonged reaction times are required, and nevertheless generally result in suboptimal conversions.5 With respect to the latter, the nitrogen atom in our designed analogue should allow straightforward functionalisation of the azoline moiety and modifications of the system, e.g. by sulphonation. 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 dihydrodibenzo-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-atmosphere 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

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† 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 azoline moiety and modification of the system, e.g. by sulphonation. 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 dihydrodibenzo-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 nihililated 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.
Instead, resulted in alkyne 11 (d) Dess–Martin periodinane, NaHCO₃, CH₂Cl₂, r.t., 40 min (90%); THF, N₂/H₂-atmosphere, r.t., 4 h (99%); (b) Boc₂O, THF, 70°C (e) (1) 2 M HCl in EtOAc, r.t., 1 h; (2) NaBH₄, H₂O, r.t., o.n. (100%); CO₂Me, Et₃N, CH₂Cl₂, r.t., 1.5 h (94%); (h) Br₂, CH₂Cl₂, 0°C, 2 h (8: 67%, 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%).

Protocol the keyaza-cyclooctene intermediate 5 was synthesised in an excellent yield of 70% over five steps.

Unfortunately, direct bromination of dihydrodibenzocycloazocine 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'Bu since other bases such as LDA, n-BuLi, NaOH (6 or 12 M) all failed to give the desiredaza-dibenzocyclooctyne 10. Compound 10, although fulfilling our aim to prepare anaza-cyclooctene, 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-ajindole (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 M 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 strainedaza-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)³ enclosed 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 carbonate¹² followed by substitution with H₂N-PEG₂₀₀₀-OMe. Initially, azide-containing CalB (AHA-CalB)¹⁶ 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-PEG₂₀₀₀ 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 DIBC-mPEG₂₀₀₀ (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 DIBC-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 DIBC 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 DIBC 15, approximately 50% conversion to the single PEGylated

Table 1 Rate constants of the different cyclooctyne systems

<table>
<thead>
<tr>
<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>DIBAC (10)</td>
<td>CD₂OD</td>
<td>0.29 ± 0.01</td>
</tr>
<tr>
<td>2</td>
<td>DIBAC (12)</td>
<td>CD₂OD</td>
<td>0.31 ± 0.02</td>
</tr>
<tr>
<td>3</td>
<td>DIBAC (12)</td>
<td>D₂O</td>
<td>0.36⁺</td>
</tr>
</tbody>
</table>

² 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[cyclooctyne]s in bioconjugation are topics currently under investigation in our laboratories.

Notes and references


![Scheme 2](image_url)

**Scheme 2** Reagents and conditions: (a) H$_2$N-PEG$_{2000}$-OMe, EDC, DMAP, CH$_2$Cl$_2$, 2 d, r.t.; (b) (1) (4-NO$_2$C$_6$H$_4$)OCOCl, pyridine, CH$_2$Cl$_2$, 4 h, r.t.; (2) H$_2$N-PEG$_{2000}$-OMe, CH$_2$Cl$_2$, 1 d, r.t.; (c) PBS-buffer (pH 8.5, 30 μM), 3 h, r.t.

A 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. The resulting HRP-N$_3$ 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-dibenzo[cyclooctyne] 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-dibenzo[cyclooctyne] 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 ($k = 0.3$ M$^{-1}$ s$^{-1}$) marks the suitability of this probe for bioconjugation purposes, as seen in both cases.

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

<table>
<thead>
<tr>
<th>Lane</th>
<th>M</th>
<th>1</th>
<th>2</th>
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