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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 nevertheless generally result in suboptimal conversions.\(^4\) With the recent discovery of the Cu(I)-catalysed Huisgen cycloaddition reaction, a powerful new methodology was added to the field of bioconjugation\(^6\) 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.\(^3\)

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 dibenzocyclooctynes\(^13\) (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 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 N\(_2\)/H\(_2\)-atmosphere\(^18\) 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 NaBH\(_4\) reduction, generating the free secondary amine (5) in quantitative yield over the two steps. Via this high-yielding

![Fig. 1](https://chemcomm.rsc.org/content/doi/10.1039/b917797c/fig1)

**Fig. 1** Strain-promoted systems for Cu-free click reactions.
Scheme 1 Reagents and conditions: (a) PdCl\(_2\)(PPh\(_3\))\(_2\), Cul, Et\(_3\)N, THF, N\(_2\)/H\(_2\)-atmosphere, r.t., 4 h (99%); (b) Boc\(_2\)O, THF, 70 °C, 2 h (95%); (c) Dess–Martin periodinane, NaHCO\(_3\), CH\(_2\)Cl\(_2\), r.t., 40 min (90%); (d) 10% Pd/BaSO\(_4\), quinoline, H\(_2\), MeOH, r.t., 1.5 h (95%); (e) 1 M solution of KO\(_2\)-isoindolo[2,1-\(a\)]quinoline, THF, N\(_2)/\text{H}_2\)-atmosphere, r.t., 4 h (99%); (f) (1) 2 M HCl in EtOAc, r.t., 1 h; (2) NaBH\(_4\), H\(_2\)O, r.t., o.n. (100%); (g) (1) 2 M HCl in EtOAc, r.t., 1 h; (2) NaBH\(_4\), H\(_2\)O, r.t., o.n. (100%); (h) Br\(_2\), CH\(_2\)Cl\(_2\), 0 °C, 2 h (8: 67%, 9: 81%); (i) KO\(_2\)-Bu, THF, 0 °C → r.t., o.n. (10); −40 °C, 2 h (11, 12) (10: 87%, 11: 84%); (j) LiOH, THF, H\(_2\)O, r.t., 3 h (92%).

Table 1

<table>
<thead>
<tr>
<th>Entry</th>
<th>Compound</th>
<th>Solvent</th>
<th>(k) (M(^{-1}) s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DIBAC(10)</td>
<td>CD(_2)OD</td>
<td>0.29 ± 0.01</td>
</tr>
<tr>
<td>2</td>
<td>DIBAC(12)</td>
<td>CD(_2)OD</td>
<td>0.31 ± 0.02</td>
</tr>
<tr>
<td>3</td>
<td>DIBAC(12)</td>
<td>D(_2)O</td>
<td>0.36</td>
</tr>
</tbody>
</table>

\(\text{Reaction was performed under basic conditions with 1.1 equiv. of 12.}\)

respectively) with benzyl azide were determined in CD\(_2\)OD, 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 D\(_2\)O, since addition of a small amount of 2 M NaOH was necessary to dissolve 12. In D\(_2\)O cycloaddition was calculated to proceed with a rate constant of 0.36 M\(^{-1}\) s\(^{-1}\), slightly faster than in CD\(_2\)OD. 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 (\(k = 3 \times 10^{-3}\) 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 H\(_2\)N-PEG\(_{5000}\)-OMe via an EDC-coupling, yielding DIBAC-mPEG\(_{5000}\) (15, Scheme 2). For comparison, DIBC-mPEG\(_{5000}\) (16) was also prepared via conversion of the alcohol into a 4-nitrophenyl carbonate\(^{18}\) followed by substitution with H\(_2\)N-PEG\(_{5000}\)-OMe. Initially, azide-containing CalB (AHA-CalB)\(^{19}\) was used for the conjugation studies. This modified enzyme, obtained by recombination expression in auxotrophic 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 Cutt(-)catalysed (3 + 2) cycloaddition reaction. However, full conversion could never be reached, despite the use of 20 equivalents of acetylene-functionalised acetylene-PEG\(_{5000}\) 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-mPEG\(_{5000}\) 15 or DIBC-mPEG\(_{5000}\) 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-mPEG\(_{5000}\) (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-mPEG\(_{5000}\). 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
was underlined by the effective PEGylation of enzymes. The functionalisation, further derivatisation and application of azido-dibenzyclooctyne in bioconjugation are topics currently under investigation in our laboratories.

Notes and references


**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.$^{20}$ 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†).

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The aza-dibenzyclooctyne is in both cases more reactive than the dibenzyclooctyne, 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-dibenzyclooctyne 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

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