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Development and evaluation of new cyclooctynes for cell surface glycan imaging in cancer cells†

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Abstract

Two reagents have been synthesized for selective labeling of cell surface azidoglycans, an unusually stable version of a dibenzocyclooctyne (TMDIBO) and a third-generation difluorinated cyclooctyne (DIFO3). Both syntheses are efficient with minimal purification, and the dibenzocyclooctyne is stable under basic and acidic conditions. Flow cytometric measurements with azidosugar labeled cancer cells, in which these reagents were linked to the fluorophore Alexa Fluor 647, gave a signal-to-background ratio of up to 35 with TMDIBO as compared to ≈ 10 for DIFO3 and ≈ 5 for a phosphine reagent. TMDIBO-based probes should have applications in molecular imaging of cell surface glycans *in vivo*.

Introduction

Altered glycosylation patterns of cell surface proteins are a hallmark of the tumour phenotype. Bertozzi and co-workers have pioneered a novel approach to glycan imaging, where cells, or even whole animals, are exposed to peracetylated azido-labeled sugars and these get incorporated into cell surface glycans.¹ The azido group is small, stable in biological systems, and selectively reactive with phosphines such as **1** *via* Staudinger ligation² and with cyclooctynes (*e.g.* **2–5**) *via* Huisgen [3+2] cycloaddition^{3–11} (Figure 1). The Staudinger ligation suffers from a slow reaction rate and oxygen-sensitivity of the phosphine reagent. Thus cyclooctynes are more attractive for detection of azides on live cells. Although a number of cyclooctynes have already been reported,¹² there is still a need to develop (a) more efficient syntheses, (b) faster reacting yet stable cyclooctynes and (c) cyclooctyne-based imaging agents that show minimal nonspecific binding to cells and serum proteins and fast metabolic clearance in the body.¹³ *In vivo* imaging studies may require large quantities of reagent. This requires efficient synthetic routes, in which there is the flexibility to generate a variety of analogues in order to tune pharmacokinetic and other properties.

In this paper we present syntheses of two new cyclooctynes, TMDIBO **8** and DIFO3 **9**, both of which are high yielding and easily scaled up to gram quantities. The new cyclooctynes react with azides at rates equivalent to those of other commonly used cyclooctynes and TMDIBO, in particular, shows good to excellent stability to other sets of conditions (air,

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acid, base, thiols). Both cyclooctynes have been used in cell-labeling studies and show much more efficient labeling than the equivalent phosphine.

Results and Discussion

Boons and coworkers developed a synthesis of dibenzocyclooctyne **5**, in ca. 10% yield from phenylacetaldehyde.⁶ Here we sought to improve ease of synthesis, total yield and stability of the final cyclooctyne. Our new tetramethoxydibenzocyclooctyne TMDIBO (**8**) was inspired by the work of Seitz *et al.*¹⁴, who found that **7** was very stable, whereas without the methoxy groups **6** decomposed within a few days at room temperature. The synthesis of **8**, shown in Scheme 1, follows the same approach as that of **5** but is much easier and higher yielding in many of the steps. Dimerisation of aldehyde **11** to yield ether **12**¹⁵ is much more efficient than without the methoxy groups (95% in 2 h vs 50% in 7 days), as is the bromination of alkene **13** (5 min vs 12 h). It was found that the brominated product **15** is labile but can be dehydrobrominated in one pot without workup and isolation. Several bases were tried for the double dehydrobromination and 1-methylpiperazine with KO^tBu gave excellent yields of **14**, which was deprotected with TBAF. The overall yield of the synthesis is 57%, and flash column chromatography is only required after two of the steps.

TMDIBO is indeed remarkably stable. It has been stored in this laboratory at room temperature for 1.5 years without detectable decomposition. No oxidation of the triple bond to cyclooctane-dione could be detected, as observed in strained ring systems.¹⁶ In addition, no decomposition could be detected upon stirring in 0.3 M K₂CO₃ in H₂O/MeCN (1:1) at reflux for 6 hours. Its half life in 3 M HCl/MeOH/THF (1:1:1) at 70 °C is ≈12 h. Multiple products could be detected by LCMS analysis having a mass corresponding to the addition of water. These are presumably compounds where the triple bond is hydrated to the corresponding ketones, giving two possible regioisomers, both of which could form two diastereoisomeric cyclic hemiketals. Interestingly, when TMDIBO was exposed to a 0.7 M solution of mercaptoethanol (37 °C, 12 h in either DMSO or 1:1 DMSO/PBS buffer), the strained triple bond was not affected. Instead ca. 10% of the TMDIBO was converted into a product that had a molecular weight that corresponded to replacement of the -OH group by -SCH₂CH₂OH, with the remainder of the TMDIBO unaffected. TMDIBO was derivatized by generating its 4-nitrophenyl carbonate **17**, which in turn can be reacted with primary amines forming carbamates.

In addition we sought to develop a synthesis of a difluorinated cyclooctyne (DIFO) that would allow the generation of large quantities of material, with minimal purification and avoiding the use of electrophilic fluorinating agents. It was thought that a DIFO might exhibit less non-specific binding to cells than TMDIBO due to its lower lipophilicity. The reported syntheses of the first- and second-generation DIFOs proceeded in ≈1% and ≈24% yield, respectively.^{4,7} Both rely on the rather expensive Selectfluor® to introduce the difluoromethylene group and the syntheses do not provide easy access to analogues. Therefore, the development of an efficient synthesis that would improve upon these points was investigated. A suitable precursor for our target third generation DIFO3 **9** was the difluoroketone **24** synthesised by Percy and co-workers from trifluoroethanol (Scheme 2).¹⁷ The synthesis of **24** is five steps and can easily be conducted on a multigram scale, with only one step requiring column chromatography. The benzylic ether of **24** was cleanly cleaved under reductive conditions, also reducing the double bond. However, the double bond in **24** would be ideally suited for derivatisation (*e.g.* dihydroxylation), thereby allowing alteration of the PK properties. The resulting alcohol **25** was TBS-protected and the enol triflate **27** was formed using *N*-(5-chloro-2-pyridyl)triflimide. The elimination to form alkyne **28** was accomplished in good yield and the TBS-group was removed without purification of the intermediate to yield DIFO3 **9**. The synthesis was accomplished in 28% overall yield from

trifluoroethanol, requires only four purifications by flash chromatography and use of fluorinating agents is completely avoided.

The rate constants for the [3+2] cycloaddition with benzyl azide were determined by ^1H -NMR spectroscopy in CDCl_3 at 300 K to be $(9.8 \pm 0.2) \times 10^{-2} \text{ M}^{-1}\text{s}^{-1}$ for TMDIBO and $(6.8 \pm 0.1) \times 10^{-2} \text{ M}^{-1}\text{s}^{-1}$ for DIFO3. The value for DIFO3 is comparable to that determined for DIFO2 **3** $(5.2 \pm 0.2) \times 10^{-2} \text{ M}^{-1}\text{s}^{-1}$ (in CD_3CN).⁴ The rate constant for TMDIBO was also conveniently measured by UV-spectroscopy in MeOH and was found to be $(9.4 \pm 0.4) \times 10^{-2} \text{ M}^{-1}\text{s}^{-1}$, slightly greater than the rate constant found for DIBO $(5.7 \pm 0.3) \times 10^{-2} \text{ M}^{-1}\text{s}^{-1}$ in the same solvent.⁸ Thus it is clear that the stability of TMDIBO towards decomposition has not at all compromised its rate of reaction with azides.

Next, the performance of the probes for azido glycan detection was assessed. Commonly, a two-step labeling protocol is used for glycan analysis, using biotin-derivatized probes, which are then detected using a labeled avidin.^{4,6} However, it would be much more practical to label the glycans in a single step. Thus, TMDIBO and DIFO3 were directly conjugated to the far-red fluorescent AlexaFluorTM 647, chosen because it can also be used for *in vivo* imaging. Linking AlexaFluor 647 cadaverine to the cyclooctynes gave TMDIBO-AF₆₄₇ **30** and DIFO3-AF₆₄₇ **31**. The corresponding PHOS-AF₆₄₇ conjugate **32** was also produced (Figure 2). A crucial quality parameter for a one-step labeling probe is its signal-to-background ratio (SBR, in this case azido-sugar treated cells vs. untreated cells). SBR data for biotin-derivatized probes have been reported,^{3-8,10} whereas quantitative data for one-step labeling probes for azidoglycans are not available.

Small probes such as **30-32** should be more generally applicable for *in vivo* imaging than the (strept)avidin-based proteins needed to image biotinylated probes, because the small molecules should be able to diffuse to parts of the body inaccessible to proteins, which are largely restricted to the bloodstream. Furthermore the SBR achieved with biotinylated probes plus labeled (strept)avidin may be higher than for small probes because the size of the (strept)avidin may (a) prevent it reaching a probe hydrophobically bound to the plasma membrane due to obstruction by glycoproteins, *etc.*, on the cell-surface and (b) cause a biotinylated probe-(strept)avidin complex that is weakly bound to the surface to be more easily washed away in the washing steps than a small molecule would be.

In order to compare the SBRs of our new cyclooctyne and phosphine probes, Lewis lung carcinoma (LLC) cells were cultured in the presence or absence of 50 mM peracetyl N-azidoacetyl galactosamine (Ac4GalNAz) for 24 h, resulting in azido labeling of cell surface glycans, and then washed, resuspended and exposed to the probes. The extent of the labeling was measured at various time-points by flow cytometry (Figure 3). TMDIBO-AF₆₄₇ exhibited the highest fluorescence signal at all time points with labeled cells, followed by DIFO3-AF₆₄₇ and PHOS-AF₆₄₇, which correlates well with their rates of reaction with benzyl azide. TMDIBO-AF₆₄₇ also gave the lowest background signal with unlabeled cells, followed by PHOS-AF₆₄₇ and DIFO3-AF₆₄₇. This was unexpected because TMDIBO's cLogP (3.2) is higher than DIFO3's (2.7). The signal-to-background ratio for TMDIBO-AF₆₄₇ (>30) is much higher than those for DIFO-AF₆₄₇ (≈ 10) and PHOS-AF₆₄₇ (≈ 5). No probe-induced cellular toxicity was observed at cell labeling times typically used in the study (e.g. 15 min).

The labeling efficiency of TMDIBO-AF₆₄₇ was confirmed by fluorescence microscopy (Figure 4). Cells were treated as described above and automated epifluorescence laser microscopy images were analyzed for total cellular probe concentration (Figure 4). TMDIBO-AF₆₄₇ exhibited the highest signal-to-background fluorescence at 647 nm (≈ 15), followed by DIFO3-AF₆₄₇ (≈ 7) and PHOS-AF₆₄₇ (≈ 3), in agreement with flow cytometry

data (Figure 3). PHOS-AF647 showed more endocytic uptake during the incubation period of 15 minutes in the absence of Ac4GalNAz (see Figure 4) and subsequent localization of the probe to perinuclear endosomes.

Albumins are known to bind and remove small hydrophobic molecules from the circulation. A DIFO-based probe has recently shown significant binding to mouse serum albumin (MSA).¹³ The probes described here were reacted with MSA under physiological conditions and then analysed by native gel electrophoresis (Figure S3). DIFO3-AF647 and TMDIBOAF647 showed distinct binding to MSA (ca. 7- and 5-fold greater fluorescence than the untreated MSA) whereas PHOSAF647 showed hardly any binding (1.1-fold increase). Prior iodoacetamide-treatment of the MSA only slightly reduced binding of DIFO3-AF647 and TMDIBO-AF647, which suggests that the majority of the bound probe is not attached to cysteine residues on MSA and is probably non-covalently bound.

Conclusions

In conclusion, efficient scalable syntheses of a new, remarkably stable dibenzocyclooctyne (TMDIBO) and a new difluorinated cyclooctyne (DIFO3) have been developed. Both cyclooctynes were tested *in vitro* against a phosphine reagent commonly used for azidosugar detection and it was shown that TMDIBO exhibits superior fluorescent labeling and signal-to-background ratios. Both cyclooctynes showed higher levels of binding to serum albumin than the phosphine but their greater reactivity and stability would be a more important factor in many situations and give them an advantage over slower reacting, oxidation-prone phosphines. TMDIBO-based probes, in particular, show great promise for use in molecular imaging studies, including assessing cell glycosylation states *in vivo* and the dynamics of tumor glycosylation in relation to disease progression and response to therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Selected literature compounds						Compounds developed in this study	
Probe							
Compound	$\mathbf{1^{(H)}}$	$\mathbf{2^3}$	$\mathbf{R=CO_2H}$ ($\mathbf{3a^4}$) $\mathbf{R=C_6H_4CO_2H}$ ($\mathbf{3b^4}$)	$\mathbf{4^1}$	$\mathbf{R=OH}$ ($\mathbf{5^6}$) $\mathbf{R=H}$ ($\mathbf{6^{(40, 140)}}$)	$\mathbf{R=H}$ ($\mathbf{7}$) $\mathbf{R=OH}$ ($\mathbf{8}$)	$\mathbf{9}$
Reaction rate [$\text{M}^{-1} \times \text{s}^{-1}$]	2.5×10^{-2} (CDCl_3)	0.96 (MeCN)	5.2×10^{-2} (CDCl_3)	14.0×10^{-2}	5.7×10^{-2} (MeOH)	9.8×10^{-2} (CDCl_3) 9.4×10^{-2} (MeOH)	6.8×10^{-2} (CDCl_3)
Stability	Prone to oxidation, to be kept under an argon atmosphere	"stable to traditional chromatography and on the benchtop"	"Stable for many months when stored at -20 °C"	"Sufficiently stable for prolonged storage at -20 °C"	"Decomposes within a few days at room temperature" (R=H)	Has been stored unchanged for 1.5 years at room temperature under air atmosphere. Stable in aqueous systems at elevated temperatures	Long-term stability at -20 °C
yield (starting from commercially available material)	76%	18%	19% ($\mathbf{3a}$) 24% ($\mathbf{3b}$)	15% (exo) 35% (endo)	10%	57%	28%

Fig. 1. Probes for azide detection (**1-5**), previously synthesised cyclooctynes (**6, 7**) and compounds developed for this study (**8,9**).

Fig. 2.
AlexaFluorTM 647-labeled compounds.

Fig. 3.

Flow cytometry data. Lewis lung carcinoma (LLC) cells were cultured with and without 50 mM Ac₄GalNAz for 24 h, then incubated with a 30 mM solution of TMDIBO-AF647, DIFO3-AF647 or PHOS-AF647. Aliquots taken at various time-points were analysed by flow cytometry. Left: median fluorescence intensity (MFI) of viable cells that had been treated with Ac₄GalNAz. Inset: MFI of viable cells that had not been treated with Ac₄GalNAz. Right: Ratio of MFIs of treated vs untreated cells. Data points were collected in triplicate and are shown as mean $\pm$ SD (error bars lie within the symbols when not visible).

Fig. 4.

Fluorescence microscopy of LLC cell glycans (A). Cells were cultured with (+) or without (-) Ac4GalNAz (50 μ M) for 24 h and then incubated with a 5 μ M solution of TMDIBO-AF647, DIFO3-AF647 or PHOS-AF647. Red: AF647, Blue: DAPI nuclear stain. Scale bar is 25 μ m. Images were analyzed by quantitative imaging cytometry and signal-to-background ratio (B), determined from the MFI of cells cultured with (+) or without Ac4GalNAz. Signal-to-background ratios represent means of two independent measurements (ca. 10,000 events counted per well).

Scheme 1.
Synthesis of TMDIBO.