



Minireview

Getting a grip on glycans: A current overview of the metabolic oligosaccharide engineering toolbox

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ABSTRACT

This review discusses the advances in metabolic oligosaccharide engineering (MOE) from 2010 to 2016 with a focus on the structure, preparation, and reactivity of its chemical probes. A brief historical overview of MOE is followed by a comprehensive overview of the chemical probes currently available in the MOE molecular toolbox and the bioconjugation techniques they enable. The final part of the review focusses on the synthesis of a selection of probes and finishes with an outlook on recent and potential upcoming advances in the field of MOE.

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1. Introduction

The field that studies glycobiology has been in existence for well over a century and was first properly defined over 25 years ago [1]. Since then the intimate and complex involvement of carbohydrates, also called glycans, in a vast number of health- and disease-associated biological communication processes has been further substantiated and many new connections have been discovered. One can even consider them a companion to nucleic acids & proteins as key players in the storage and transfer of information in virtually all organisms. In stark contrast to these established 'central dogma' biomolecules, however, stands our understanding of the relationship between glycan molecular structure and their biology activity. The reason we are still almost literally in the dark is that studying glycans poses unique challenges when compared with proteins and nucleotides, related to their highly complex, often branched structures. An additional challenge is that the assembly of glycans – their structure – is not template-driven (encoded in genes), and instead occurs via complex and dynamic processing and trimming of the glycan chain under the action of a series of competitive glycosyltransferase and glycosidase enzymes. The study of how glycans are assembled by glycosyltransferases, catabolised by glycosidases, recognised by lectins, and how or when taken together this enables the dynamic storage and transfer of information via their molecular structure is therefore at the frontier of science. The field that uses a chemistry-based approach to answers these largely unanswered questions about glycobiology has been coined 'Chemical Glycobiology'. The 15 years that have passed since it was first defined as such by Carolyn Bertozzi have seen it blossoming and many excellent reviews cover all these advances [2–4].

The current review focusses on a chemical glycobiology strategy that uses the glycan salvage pathways that most organisms possess. These pathways allow a cell to convert monosaccharides, liberated from complex glycans or originating from the extracellular space, into their nucleotide-activated counterparts that are substrates for glycosyltransferases located in the endoplasmic reticulum and Golgi apparatus. This eventually results in the monosaccharide becoming part of complex cellular glycan structures, e.g. *N*-glycans and *O*-glycans on proteins and glycolipids (Fig. 1). In the 1990's it was discovered that the enzymes that participate in these glycan salvage pathways sometimes allow for unnatural modifications in the structure of their monosaccharide-based substrate [5,6]. This enabled the design of tailor-made monosaccharide-based chemical probes that are labelled with a small unnatural chemical handle that can be addressed after incorporation under specific conditions at a predefined moment. Processing of these probes via the glycan salvage pathways allows for direct profiling of cellular glycans. This technique is nowadays often called **metabolic oligosaccharide engineering** (MOE) and it complements other approaches to profile cellular glycans, like tracing incorporation of isotope (radio) labeled glycans, indirect profiling using (chemical) lectins or direct chemical tagging of native glycans, e.g. the use of sodium periodate-mediated generation of taggable aldehydes from neuraminic acid. For a comprehensive overview of all the applications developed thus far with MOE we direct the reader to various recent reviews [7–18]. This review focusses on another integral part of MOE, namely the fact that all chemical probes evaluated for MOE have to be designed and synthesised. We here provide an up-to-date comprehensive overview of the current toolbox of chemical probe structures implemented in MOE, i.e. the chemical handles employed to enable (bioorthogonal) bioconjugation reactions for

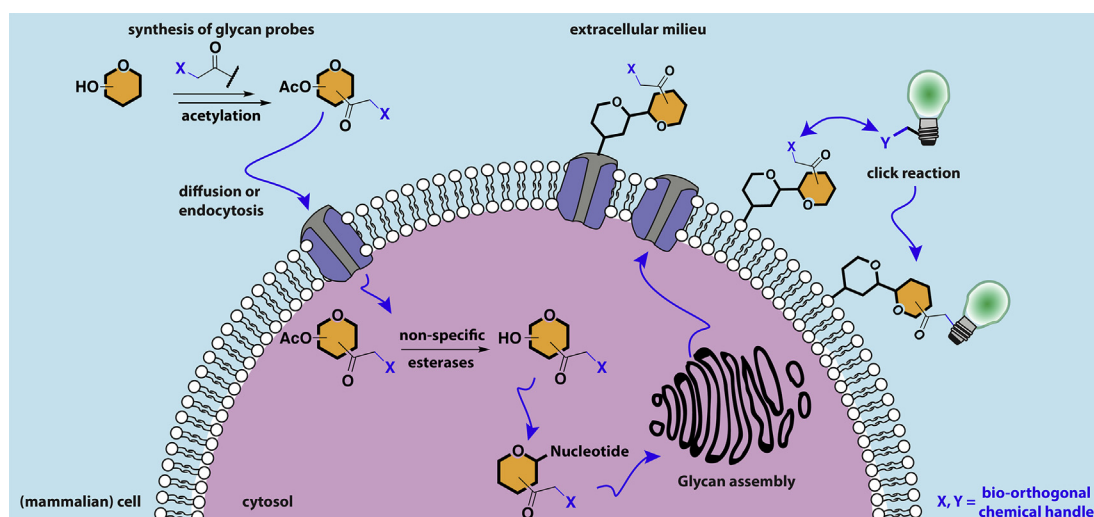


Fig. 1. Schematic overview of metabolic oligosaccharide engineering.

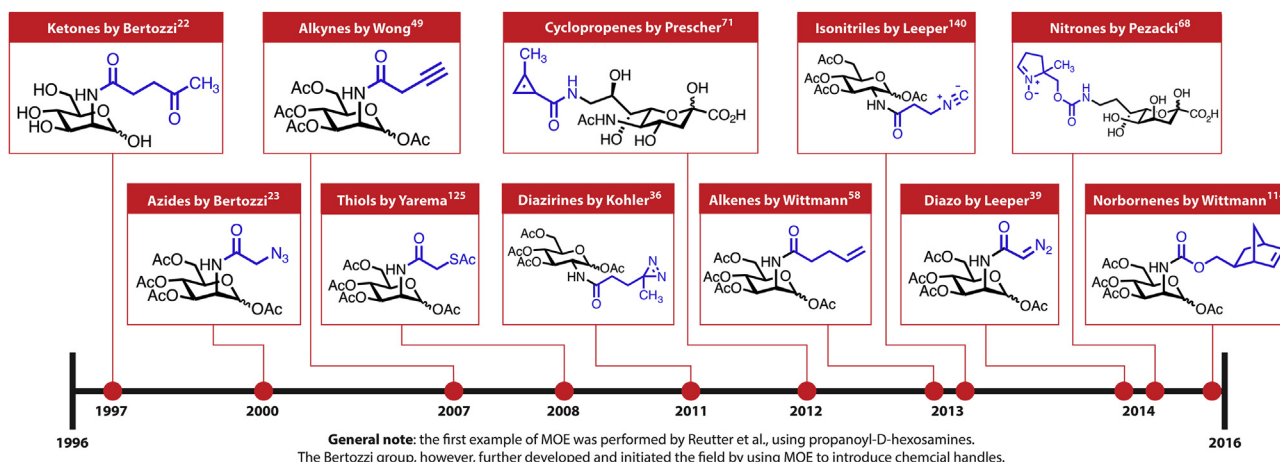


Fig. 2. Timeline of the history of metabolic oligosaccharide engineering probes.

profiling labeled glycans. We illustrate their use with selected recent examples of applications in various organisms. Additionally we provide a selected overview of various synthetic routes that are nowadays used to obtain these probes. The review finishes with an outlook that highlights some recent and prospective advances in the application of MOE.

2. Historical overview of MOE and bioconjugation reactions

Key to the success of any metabolic oligosaccharide engineering approach is the ability of the labelled glycans to be selectively tagged after incorporation via a bioconjugation reaction. The requirements for bioconjugate reactions suitable for use with MOE are largely known, such as compatibility with complex biological mixtures, stability of the bioconjugate, high reaction rates, and ideally no interference with native biochemical processes (known as bio-orthogonality) [17]. Many of the currently available bioconjugation reactions have already been applied in MOE (see Table 1), and many efforts to further extend and improve the application and capabilities of bioconjugation reactions are ongoing. In this section we limit ourselves to a brief summary; the development, reaction kinetics, and general handling of these reactions are highlighted in several recent reviews [13,16,17,19–21].

Among the first bioconjugation reactions to be applied for MOE were aldehyde/ketone condensation reactions with hydrazides and aminoxy groups to give hydrazones and oximes, respectively (Fig. 2) [22]. With the discovery of azide and alkyne conjugations, the field of metabolic labelling exploded [23]. Azides are reduced under Staudinger conditions and trapped intramolecularly with a biocompatible phosphine reagent as discovered by the Bertozzi group [23]. In addition, azides are easily used in the Copper-catalysed Azide Alkyne Cycloaddition (CuAAC) reaction [24]. In the presence of copper(I) ions, azide and alkyne substrates react rapidly to form stable triazoles. This hallmark reaction in click chemistry is nowadays still the most used technique. In order to exclude toxic copper from biological systems two methods were developed. The first method uses amine-triazole based ligands to accelerate the CuAAC reaction and limit the required amount of copper, while the other method uses strained alkynes to improve the reaction kinetics in a copper-free manner [7]. The use of strained alkynes in biological systems is known as the Strain-Promoted Azide Alkyne Cycloaddition (SPAAC) reaction [25]. Despite the exclusion of copper, the SPAAC reaction is not always superior over the CuAAC reaction, since some strained alkyne reagents are known to have a higher background labelling due to side reactions with cellular thiols [26]. Several recent advances in the

Table 1
Overview of bioconjugation reactions.

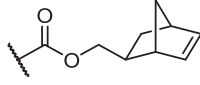
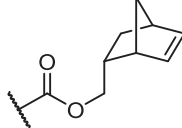
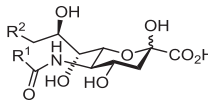
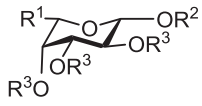
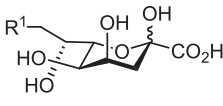
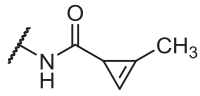
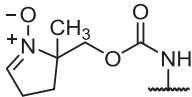
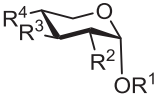
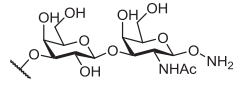
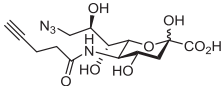
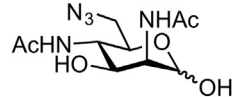
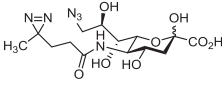
Name	Reactant 1	Reactant 2	Remarks	Average reaction rate [30]	Ref
Staudinger-Bertozzi ligation	azide	phosphine	phosphazene trapped intramolecularly as amide	$k \sim 10^{-3} \text{ M}^{-1} \text{ s}^{-1}$	[23]
CuAAC	alkyne	azide	Cu-catalysed with ascorbic acid Cu(I)-regeneration	$k \sim 10 - 200 \text{ M}^{-1} \text{ s}^{-1}$	[31,32]
SPAAC	strained alkyne	azide	Ring strain is driving force of the reaction	$k \sim 10^{-2} - 1 \text{ M}^{-1} \text{ s}^{-1}$	[25]
Hydrazide ketone ligation	ketone	hydrazide	product is hydrazone	$k \sim 10^{-3} \text{ M}^{-1} \text{ s}^{-1}$	[5,6]
Oxime ligation	ketone	amino-oxo	product is oxime	unknown	[33]
invDA	(strained/terminal) alkene	tetrazine	dihydropyrazine product	$k \sim 1 - 10^4 \text{ M}^{-1} \text{ s}^{-1}$	[34]
1,3-dipolar cycloaddition	(strained/terminal) alkene	nitrile imine	nitrile imines are formed <i>in situ</i> after UV activation of tetrazoles	$k \sim 890 \text{ M}^{-1} \text{ s}^{-1}$	[35]
Photo-activated 1,3-cycloaddition	terminal alkene	tetrazole	product is pyrazole	$k \sim 4.41 \pm 0.34 \times 10^{-2} \text{ M}^{-1} \text{ s}^{-1}$	[27]
Photo-activated diazirines	diazirine		UV activation needed	unknown	[36]
Isonitrile ligation	isonitrile	tetrazine	stable product for tertiary isonitrile	$k \sim 57.5 \pm 1.5 \times 10^{-2} \text{ M}^{-1} \text{ s}^{-1}$	[37]
Hetero Diels Alder	vinyl sulfide	diol	product is thioacetal	$k \sim 1.5 \pm 0.1 \times 10^{-3} \text{ M}^{-1} \text{ s}^{-1}$	[38]
Diazo ligation	diazo	strained alkyne	product is pyrazole	$k \sim 0.04 \text{ M}^{-1} \text{ s}^{-1}$	[39]
SPOQC	strained alkyne	quinone	among fastest known bioconjugation reactions	$k \sim 496 \pm 70 \text{ M}^{-1} \text{ s}^{-1}$	[40]
SPANC	Alkyne	Nitrone		$k \sim 39 \text{ M}^{-1} \text{ s}^{-1}$	[41]

Table 2

Overview of glycan-based chemical probes for metabolic oligosaccharide engineering.

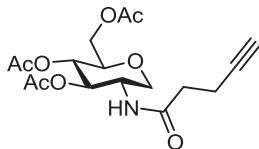
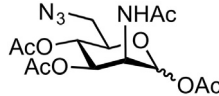
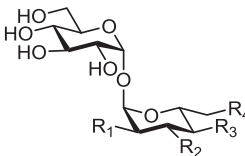
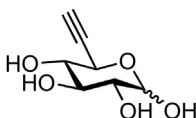
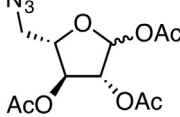
Label	Mannosamine	REF	Glucosamine	REF	Galactosamine	REF
Natural glycans	ManNAc $R^1 = \text{CH}_3$ $R^2 = \text{OR}^3 = \text{H}$	[6]	GlcNAc $R^1 = \text{CH}_3$ $R^2 = \text{H}$	[6]	GalNAc $R^1 = \text{CH}_3$ $R^2 = \text{H}$	[6]
Aliphatic analogues	ManNPro $R^1 = \text{CH}_2\text{CH}_3$ $R^2 = \text{OR}^3 = \text{H}$	[6]	GlcNPro $R^1 = \text{CH}_2\text{CH}_3$ $R^2 = \text{H}$	[6]	GalNPro $R^1 = \text{CH}_2\text{CH}_3$ $R^2 = \text{H}$	[6]
Ketones	ManNLev $R^1 = (\text{CH}_2)_2\text{COCH}_3$ $R^2 = \text{OR}^3 = \text{H}$	[22]	GlcNLev $R^1 = (\text{CH}_2)_2\text{COCH}_3$ $R^2 = \text{H}$	[22]	GalNLev $R^1 = (\text{CH}_2)_2\text{COCH}_3$ $R^2 = \text{H}$	[22]
Azides	Ac₄ManNAz $R^1 = \text{CH}_2\text{N}_3$ $R^2 = \text{OR}^3 = \text{Ac}$	[23]	Ac₄GlcNAz $R^1 = \text{CH}_2\text{N}_3$ $R^2 = \text{Ac}$	[23]	Ac₄GalNAz $R^1 = \text{CH}_2\text{N}_3$ $R^2 = \text{Ac}$	[23]
	ManNAz $R^1 = \text{CH}_2\text{N}_3$ $R^2 = \text{OR}^3 = \text{H}$	[23]	GlcNAz $R^1 = \text{CH}_2\text{N}_3$ $R^2 = \text{H}$	[23]	GalNAz $R^1 = \text{CH}_2\text{N}_3$ $R^2 = \text{H}$	[23]
	1,3,4-O-Bu₃ManNAz $R^1 = \text{CH}_2\text{N}_3$ $R^2 = R^3 = \text{Bu}$	[46]				
	Ac₄ManN2Azaryl $R^1 = \text{CH}_2\text{PhN}_3$ $R^2 = \text{OR}^3 = \text{Ac}$	[47]				
	Ac₃-4-Az-ManNAc $R^1 = \text{OAc}$ $R^2 = \text{Ac}$ $R^3 = \text{N}_3$	[48]				
Alkynes	Ac₄ManNAIk $R^1 = (\text{CH}_2)_2\text{C}\equiv\text{CH}$ $R^2 = \text{OR}^3 = \text{Ac}$	[49,50]	Ac₄GlcNAIk $R^1 = (\text{CH}_2)_2\text{C}\equiv\text{CH}$ $R^2 = \text{Ac}$	[51]	Ac₄GalNAIk $R^1 = (\text{CH}_2)_2\text{C}\equiv\text{CH}$ $R^2 = \text{Ac}$	[52]
	Ac₄ManPoc $R^1 = \text{OCH}_2\text{C}\equiv\text{CH}$ $R^2 = \text{OR}^3 = \text{Ac}$	[53]	Ac₄GlcPoc $R^1 = \text{OCH}_2\text{C}\equiv\text{CH}$ $R^2 = \text{Ac}$	[53]	Ac₄GalPoc $R^1 = \text{OCH}_2\text{C}\equiv\text{CH}$ $R^2 = \text{Ac}$	[53]
Thiols	Ac₄ManNTGc $R^1 = \text{CH}_2\text{SAC}$ $R^2 = \text{OR}^3 = \text{Ac}$	[54]	Ac₄GlcNTGc $R^1 = \text{CH}_2\text{SAC}$ $R^2 = \text{Ac}$	[54]	Ac₄GalNTGc $R^1 = \text{CH}_2\text{SAC}$ $R^2 = \text{Ac}$	[54]
Cyclo-propene	Ac₄ManNCyc $R^1 =$ 	[55]				
	$R^2 = \text{OR}^3 = \text{Ac}$ Ac₄ManNCyoc $R^1 =$ 	[56]	Ac₄GlcNCyoc $R^1 =$ 	[57]	Ac₄GalNCyoc $R^1 =$ 	[57]
	$R^2 = \text{OR}^3 = \text{Ac}$		$R^2 = \text{Ac}$		$R^2 = \text{Ac}$	
Terminal alkenes	Ac₄ManNPtl $R^1 = (\text{CH}_2)_2-\text{CH}=\text{CH}_2$ $R^2 = \text{OR}^3 = \text{Ac}$	[58]				
	Ac₄ManNHxl $R^1 = (\text{CH}_2)_3-\text{CH}=\text{CH}_2$ $R^2 = \text{OR}^3 = \text{Ac}$	[58]				
	Ac₄ManNALoc $R^1 = \text{OCH}_2-\text{CH}=\text{CH}_2$ $R^2 = \text{OR}^3 = \text{Ac}$	[59]	Ac₄GlcNALoc $R^1 = \text{OCH}_2-\text{CH}=\text{CH}_2$ $R^2 = \text{Ac}$	[59]		
	Ac₄ManNBeoc $R^1 = \text{O}(\text{CH}_2)_2-\text{CH}=\text{CH}_2$ $R^2 = \text{OR}^3 = \text{Ac}$	[59]	Ac₄GlcNBeoc $R^1 = \text{O}(\text{CH}_2)_2-\text{CH}=\text{CH}_2$ $R^2 = \text{Ac}$	[59]		
	Ac₄ManNPeoc $R^1 = \text{O}(\text{CH}_2)_3-\text{CH}=\text{CH}_2$ $R^2 = \text{OR}^3 = \text{Ac}$	[59]	Ac₄GlcNPeoc $R^1 = \text{O}(\text{CH}_2)_3-\text{CH}=\text{CH}_2$ $R^2 = \text{Ac}$	[59]		
	Ac₄ManNHeoc $R^1 = \text{O}(\text{CH}_2)_4-\text{CH}=\text{CH}_2$ $R^2 = \text{OR}^3 = \text{Ac}$	[59,60]	Ac₄GlcNHeoc $R^1 = \text{O}(\text{CH}_2)_4-\text{CH}=\text{CH}_2$ $R^2 = \text{Ac}$	[58,59]		

Table 2 (continued)

Label	Mannosamine	REF	Glucosamine	REF	Galactosamine	REF
Diazirines	Ac₄ManNDaz(3Me) R ¹ = (CH ₂) ₃ -1-methyldiaziriny R ² = OR ³ = Ac	[61]	Ac₄GlcNDaz(3Me) R ¹ = (CH ₂) ₃ -1-methyldiaziriny R ² = Ac	[61]	Ac₄GalNDaz(2Me) R ¹ = (CH ₂) ₂ -1-methyldiaziriny R ² = Ac	[62]
	Ac₄ManNDaz(4Me) R ¹ = (CH ₂) ₄ -1-methyldiaziriny R ² = OR ³ = Ac	[61]	Ac₄GlcNDaz(4Me) R ¹ = (CH ₂) ₄ -1-methyldiaziriny R ² = Ac	[61]		
	Isonitrile Ac₄ManN-<i>n</i>-Iso R ¹ = (CH ₂) ₂ -N≡C R ² = OR ³ = Ac	[63]	Ac₄GlcN-<i>n</i>-Iso R ¹ = (CH ₂) ₂ -N≡C R ² = Ac	[63]	Ac₄GalN-<i>n</i>-Iso R ¹ = (CH ₂) ₂ -N≡C R ² = Ac	[63]
	Ac₄ManN-<i>t</i>-Iso R ¹ = C(CH ₃) ₂ CH ₂ -N≡C R ² = OR ³ = Ac	[63]	Ac₄GlcN-<i>t</i>-Iso R ¹ = C(CH ₃) ₂ CH ₂ -N≡C R ² = Ac	[63]	Ac₄GalN-<i>t</i>-Iso R ¹ = C(CH ₃) ₂ CH ₂ -N≡C R ² = Ac	[63]
Diazo-ketones	Ac₄ManDiaz R ¹ = CHN ₂ R ² = OR ³ = Ac	[52]			Ac₄GalDiaz R ¹ = CHN ₂ R ² = Ac	[39]
Norbor-nene	Ac₄ManNNorbo_c_{exo} R ¹ =  R ² = Ac Ac₄ManNNorbo_c_{endo} R ¹ =  R ² = Ac					
	Sialic acid	REF	Fucose	REF	KDO	REF
	 Neu5Hex R ¹ = (CH ₂) ₃ C≡CH R ² = OH	[64]	 FucAl or 6-alkynyl Ac₄fucose R ¹ = C≡CH R ² = Ac R ³ = Ac	[65]	 9Al-KDO R ¹ = C≡CH	[66]
	SiaNAI R ¹ = (CH ₂) ₂ C≡CH R ² = OH	[67]			KDO-alkyne R ¹ = NHCO(CH ₂) ₂ C≡CH	[68]
	SiaNProc R ¹ = OCH ₂ C≡CH R ² = OH	[69]	Ac₄FucAz R ¹ = CH ₂ N ₃ R ² = R ³ = OAc	[70]	KDO-N₃ R ¹ = N ₃	[66]
	9-Cp-NeuAc R ¹ = CH ₃ R ² = 	[71]	FucAz R ¹ = CH ₂ N ₃ R ² = GDP R ³ = H	[70,72]	KDO-HMMPO R ¹ = 	[68]
	9BA-Neu5Ac R ¹ = OH R ² = NHCH ₂ COC ₆ H ₄ COH	[73]				
Xylose	REF	Miscellaneous	REF	Two functionalities	REF	
		 Hydrazine	[74]		[75]	
UDP-2-XylAz	[76]	 6-azido-2,4-diacetamido-2,4,6-trideoxy-d-mannopyranose	[77]		[75]	
R ¹ = UDP R ² = N ₃ R ³ = OAc						

(continued on next page)

Table 2 (continued)

Xylose	REF	Miscellaneous	REF	Two functionalities	REF
$R^4 = \text{OAc}$ UDP-3-XylAz	[76]		[51]		
$R^1 = \text{UDP}$ $R^2 = \text{OH}$ $R^3 = \text{N}_3$ $R^4 = \text{OH}$ UDP-3-XylAz	[76]		[45]		
$R^1 = \text{UDP}$ $R^2 = \text{OH}$ $R^3 = \text{OH}$ $R^4 = \text{N}_3$					
Trehalose	REF	Glucose	REF		
 2-TreAz: $R^1 = \text{N}_3$; $R^2 = \text{OH}$; $R^3 = \text{OH}$; $R^4 = \text{OH}$ 3-TreAz: $R^1 = \text{OH}$; $R^2 = \text{N}_3$; $R^3 = \text{OH}$; $R^4 = \text{OH}$		 6dAG	[78]		
		Arabinose	REF		
		 Ac₃ArabAz	[156]		
4-TreAz: $R^1 = \text{OH}$; $R^2 = \text{OH}$; $R^3 = \text{N}_3$; $R^4 = \text{OH}$ 6-TreAz: $R^1 = \text{OH}$; $R^2 = \text{OH}$; $R^3 = \text{OH}$; $R^4 = \text{N}_3$	[79] [79]				

field have introduced photoactivation to provide spatial and temporal control in bioconjugation reactions. The Boons laboratory introduced a photochemically triggered SPAAC click reaction with a dibenzocyclooctyne derivative [27]. A photocontrolled version of the Staudinger-Bertozzi ligation has also been reported and successfully applied in living organisms such as zebrafish [28]. A recent reinvestigation of the photo-induced 1,3-dipolar cycloaddition between an alkene and tetrazole has questioned its bio-orthogonality and alkene selectivity of the reactive intermediate nitrile imine [29]. This bioconjugation reaction has to the best of our knowledge not yet been applied in MOE.

Other photochemical activation reactions have also been used in combination with MOE. Photoactivation by UV irradiation of aryl azides, diazirines, and benzophenone results in the formation of reactive nitrene, carbene and radical intermediates, respectively, that react with almost any structure in close proximity, even aliphatic C–H bonds [42]. These photo-active probes can be taken up by the cell without significant toxic effects; however, UV irradiation can of course damage the cells. The generated reactive intermediate will react with proteins in the near vicinity of the labelled glycan, and thereby provide fruitful insights in protein-glycan interactions in biological pathways.

A recently discovered bioconjugation reaction is the inverse

electron demand Diels Alder reaction (invDA) [34,43]. The reaction is fast and irreversible, and most tetrazines are easy to make and stable in aqueous media. For MOE, the dienophile can be a terminal alkene, cyclopropene, diazo, or isonitrile moiety. The invDA reactions are superior over the CuAAC or SPAAC reaction in terms of reaction kinetics and biocompatibility. A photoactivatable tetrazine for use in invDA was recently reported by Fox and co-workers [44]. Dihydropyridazines were oxidised to tetrazines upon irradiation by red light (660 nm) in the presence of methylene blue. Upon addition of strained alkenes (e.g. trans-cyclooctene (TCO)), the generated tetrazine reacts rapidly with these. The same dihydropyridazine precursors could also be chemo-enzymatically activated using a peroxidase. Beside the three main classes of bioconjugation, azide-alkyne, tetrazine invDA ligation and photo-chemical activation, other methods are also being explored for use in MOE, but not widely applied. Several recent developments are discussed in the outlook.

3. Overview of chemical probes their applications and tagging-mechanism

The tagging of labelled glycans after a MOE experiment can be performed with a wide variety of different chemical handles.

Azides, alkynes, and cyclopropenes are among the most popular nowadays. Other frequently used chemical handles are diazirines, diazo, isonitriles, thiols, and nitrones. The chemical synthesis of these chemical handles varies from basic amide couplings to more complex synthetic strategies. In most routes the endpoint of the chemical synthesis is a fully acetylated monosaccharide-based chemical probe. The overall *modus operandi* of MOE is that acetylated monosaccharide-based chemical probes are fed to target cells or an organism, as the acetyl esters facilitate the passive uptake of the probe [5,6]. An additional beneficial result of acetylated probes is the easier purification thereof by standard procedures such as flash column chromatography. Once inside the cell, the acetyl groups are hydrolysed by nonspecific cytosolic esterases and the probe is processed by the glycan salvage pathway and transformed into nucleotide-activated donor substrates for glycosyltransferase enzymes in the glycosylation pathways. These glycosylation pathways take place in the endoplasmic reticulum and Golgi apparatus of the cell. There, the labelled-donors are incorporated into the many complex cellular glycan structures, e.g. *N*-glycans and *O*-glycans on proteins and glycolipids. Finally, the small chemical handle can be addressed to directly profile the targeted monosaccharide in the cells complex glycans.

Since its inception in 1997 an extensive toolbox of chemical probes has been developed for use in MOE. Table 2 provides a comprehensive overview of the glycan-based chemical probes that have thus far been used in MOE and tested in a variety of organisms from mammalian cell lines to zebrafish and bacteria, and more recently also in plants. The table is organised per glycan and functional group. In general, the ManNAc, GlcNAc, and GalNAc salvage pathways are most tolerant towards unnatural glycans with small or sometimes even substantial chemical handles, as can be seen by their overwhelming presence in Table 2. Especially acyl substituents on the 2-position of these 2-deoxy amino glycans are well tolerated. In early work by the Bertozzi group, 2-deoxy 2-azido glycans were found to be either toxic or not converted to cell-surface glycans, indicating that the *N*-acyl group itself is essential for enzymatic recognition and conversion of ManNAc analogues [45]. The overview of Table 2 is followed by sections, subdivided on the basis of the chemical handles in these probes, that discuss their development, mode of action and selected applications.

3.1. Azides

MOE with azido-labelled glycans is still the most widely adopted variant, and is used in a broad range of applications. Azido-containing MOE probes have been successfully used for metabolic labelling in various organisms and tissues, such as pancreatic cells [46,80], mice [81], and primary hippocampus neuron cells [82]. Several recent reviews can be found that specifically cover this topic [17,83,84]. Perhaps their most famous application, the labelling of neuraminic acid in complex glycans, is covered in these reviews. This section focusses on their recent application in glycan labelling in *C. elegans*, zebrafish, bacteria, and plants.

Currently, all azido probes are *O*-acetylated when used in MOE. Despite this being the current standard method, investigating other esters or groups to mask the probe and facilitate uptake by the target organism or cell might be a way to further optimize MOE. Yarema et al. have shown that *O*-butyrate probes are also successfully taken up and recognised by the cellular metabolic machinery [86]. Since the early discovery that azido-acyl groups on amino sugars are well tolerated and recognised by the enzymatic machinery in organisms, no further changes have been made to this golden standard chemical handle. The tolerance for bigger chemical handles such as cyclopropenes and diazirine suggests that the design of the azido acetyl groups might still be further explored.

***C. elegans*:** The efficiency of metabolic incorporation of azido-sugars in *C. elegans* was initially unknown. Therefore, Bertozzi and co-workers first determined the ability for metabolic incorporation of Ac₄ManNAz, Ac₄GalNAz, and Ac₄GlcNAz into *C. elegans* glycans and glycoconjugates [87]. This revealed that *C. elegans* is unable to incorporate Ac₄ManNAz and Ac₄GlcNAz, but labelling with Ac₄GalNAz worked. The azido-glycans were first incubated in *E. coli* and the bacteria were afterwards consumed by *C. elegans*. It is therefore unknown whether *C. elegans* can actually take up these azido-glycans itself or if *E. coli* metabolises them and *C. elegans* obtains them by metabolism of *E. coli* glycans. It should also be noted that MOE in *E. coli* with Ac₄GalNAz or Ac₄GlcNAz has never been reported, while it has been reported for other gram-negative bacteria [88,89]. Recently, labelling of glycoconjugates using Ac₄GlcNAz was also observed in primary embryonic *C. elegans* cells [90].

Zebrafish: The zebrafish is an excellent organism to study *O*-glycosylation and *N*-glycosylation with MOE and the fact that it is a transparent organism makes fluorescent imaging easier. The research of MOE in zebrafish started in the group of Bertozzi in 2008 [91]. Ac₄GalNAz was the only studied substrate and showed incorporation in *O*-glycans at concentrations higher than 25 μM, as was visualised with DIFO-488. Sialylated glycans were studied in developing zebrafish using Ac₄ManNAz. Here MOE clearly proved superior over the previous performed work on sialylated glycans using aldehyde ligation techniques by Baskin et al. [92].

In later studies fucosylation was studied in developing zebrafish. The fucose salvage pathway is active in zebrafish embryos, but efficient processing of azide-modified fucose was poor. Azido-fucose labelling strategies in zebrafish are therefore limited to fucosylated glycoproteins active during the development. In addition, it has been reported that in certain cases azido-fucose can be toxic for mammalian cells [49,93]. For this reason, the use of alkynyl-fucose analogues in mammalian cell lines has since been the preferred approach.

Bacteria: Glycans are important for the viability of bacteria and almost all bacteria require glycans as a carbon source for growth. A wide variety of glycans is exclusively found in microbes, and the uniqueness of these bacterial glycans makes them ideal targets for the development of new antibiotics that interfere with the bacterial metabolism [94]. For this reason, metabolic labelling of bacterial glycans is a rapidly growing field of research. Both pathogenic bacteria - like *Haemophilus ducreyi*, [95] *Campylobacter jejuni*, [96] and *Helicobacter pylori* - and non-pathogenic bacteria - like *Escherichia coli*, [66] and *Bacteroides fragilis*, [89,97] have been successfully labelled in this way.

In bacteria, probe incorporation and competing probe degradation via catabolic processes can also play an important role and is highly dependent on the type of bacteria. Rather high concentrations of azido-glycans are usually added in MOE experiments with bacteria, compared to other organisms or cell lines, to compensate for this factor. These rather high concentrations have, however, not shown significant toxicity in bacteria. Even the copper used for the CuAAC reaction, which is toxic for most organisms, is better tolerated by bacteria. If needed, a biocompatible CuAAC reagent, e.g. the triazole ligand BTAA, can be used to further reduce copper toxicity as shown by Wu and co-workers [97]. Since many SPAAC reagents have become commercially available (e.g. BCN-rhodamine), both SPAAC and CuAAC have been successfully applied in MOE on bacteria. The wide variety of non-common glycans present in bacteria requires extensive and complex synthetic routes to systematically generate the probes needed to label specific rare microbial glycans. Several synthetic strategies to these unique bacterial glycans have, however, been reported, and a selection thereof is detailed in the next section

[96,98–103]. One interesting example is the metabolic incorporation of azido-Pseudaminic acid (Az-Pse), a bacterial glycan belonging to the class of sialic acids [96]. Mutant *C. jejuni* cells deficient in Pse pathway were fed with the azido-Pse precursor, 6-deoxy-AltdiNAz. This resulted in the decoration of the bacterial flagella with azido-Pse. The presence of azido-Pse on the flagella restored the motility of *C. jejuni*. Subsequently, the azido-glycan was visualised using biotin-Staudinger reagent and later visualised using streptavidin which was separated on SDS-PAGE. The single labelling in whole-cell flagellin lysates, as shown by SDS-PAGE, indicates that flagellin proteins are the only cell-surface-associated proteins that are glycosylated with Pse. The study of these bacterial glycans is therefore of interest as a potential new antibiotic target.

Another gram-negative bacterium which has been evaluated with MOE, using Ac₄GlcNAz and GlcNAz, is *H. pylori*. Both azido-glycans were tested to check the need for O-acetylation, to investigate the presently still unknown mechanism of uptake by bacteria, and to probe for the presence of esterase activity inside the bacteria. The per-acetylated GlcNAz was easily incorporated into N-glycans of *H. pylori* [104], but incorporation in O-linked glycans was not seen. Furthermore, the non-acetylated GlcNAz was not metabolically consumed and installed on cell surface glycoconjugates. Recently, an extensive study with a panel of azido-glycans was recently performed by Kasper and co-workers on human-gut related pathogenic and non-pathogenic bacteria in gram-negative and gram-positive bacteria [89]. Prior to this work, Seibel and co-workers looked into MOE of *S. aureus* with GlcNAz [105].

As mentioned, many bacteria use microbe-specific glycans to construct their cell wall, and the disaccharide trehalose is such a glycan. Bertozzi and co-workers introduced an azide on this disaccharide for metabolic labelling in mycobacteria, a class of bacteria that includes the pathogen *Mycobacterium tuberculosis* [79]. Four different trehalose-azide analogues were synthesised to study a recycling process that might be conserved across mycobacterial species. All four trehalose-azide analogues showed labelling in a variety of mycobacteria. In a next step this method can therefore be used to study glycolipid distribution, trafficking, and dynamics of trehalose biosynthesis, which in turn might provide clues on how to target these bacteria with new classes of antibiotics.

Another relevant microbe-specific glycan is 3-deoxy-D-mannooctulosonic acid (KDO), an essential and characteristic part of lipopolysaccharides in gram-negative bacteria [106]. To characterise gram-negative bacteria, metabolic labelling with KDO-based probes is thus highly relevant. Vauzeilles and co-workers synthesised a 9Az-KDO glycan (see Table 2), and incubated this together with several classes of bacteria [66]. Successful labelling was achieved in *E. coli* K12, *E. coli* O86, *S. typhimurium*, and *L. pneumophila*.

Azido-glycans in combination with MOE are proving to be a valuable tool to label glycans in many types of bacteria. Increasing our insight into bacterial glycobiology through among other MOE and finding ways to perturb bacterial glycans in pathogenic bacteria will hopefully provide new leads for the development of much needed next-generation antibiotics.

Plants: Metabolic oligosaccharide engineering is relatively new to plant sciences. The first implementation of MOE in plants was reported in 2012 by Somerville and co-workers [65]. They successfully incorporated FucAl into *Arabidopsis thaliana* and subsequent CuAAC labelling allowed fluorescence imaging of fucosylated glycans in the cell wall. Imaging indicated that the fucose-alkyne containing glycans were initially incorporated to locations on the cell surface that are likely involved in exocytic vesicle fusion.

Recently, azido-KDO was metabolically incorporated into the RG-II glycan of the pectin-rich cell wall of *Arabidopsis thaliana* and *Nicotiana tabacum* [107]. Successful labelling via CuAAC ligation with Alexa Fluor[®] 488-alkyne was observed. Dual labelling using Fuc-Al and KDO-N₃ was performed by taking advantage of the two bio-orthogonal reactions. This allowed simultaneous imaging of Fuc-Al in RG-I and KDO-N₃ in RG-II. Chen and coworkers have reported on the metabolic incorporation and imaging of N-linked glycans in Arabidopsis with Ac₄GlcNAz. Through MOE and direct fluorescent imaging of N-glycans they showed that protein N-glycosylation in *Arabidopsis thaliana* root tissue possess distinct patterns of distribution in the various developmental zones [108]. We recently also reported on imaging of plant glycans in Arabidopsis with both Ac₄GlcNAz and GlcNAz, the latter probably via active uptake. In our study we observed that Arabidopsis can interestingly also metabolically incorporate Ac₄GalNAz, probably via an as of yet unidentified enzymatic GalNAc to GlcNAc epimerase activity. We also observed successful MOE in Arabidopsis with Ac₄FucAz and the novel L-arabinofuranose probe, Ac₃ArabAz. Finally we also demonstrated the successful application of strain-promoted azide-alkyne cycloaddition (SPAAC) and inverse electron demand Diels-Alder (invDA) click reactions to allow for improved imaging of MOE in live plants [156].

3.2. Alkynes

Alkynes were among the first handles used in metabolic oligosaccharide engineering. The virtual absence of alkynes in nature, their relative small size, their fast reactivity with azides under CuAAC conditions, and their stability makes them very suitable for metabolic labelling. An alkyne derivative of N-acetylmannosamine (25 μM) and 6-alkynyl Ac₄fucose (200 μM) were tested in Jurkat cells back in 2007 by Chi-Huey Wong and co-workers [49]. In mammals, N-acetylmannosamine is the precursor for the sialic acid, N-acetylneuraminic acid (Neu5Ac), and the alkynylated probe versions therefore result in alkynylated sialic acids on glycoconjugates. The fucose alkyne was able to label fucosylated glycans *in vivo*.

A more advanced study that compared Ac₄ManNAI with Ac₄ManNAz in six different cell lines at a concentration of 50 μM was reported by Bertozzi et al., in 2009 [109]. In all six cell lines metabolic labelling of Neu5Ac in glycans with the alkynyl-glycans was more efficient than with the azido-glycans. In a next step, *in vivo* analysis of the alkynyl glycan incorporation in mice showed that after a daily injection of Ac₄ManNAI for seven days, organs were harvested to show metabolic labelling in all tissues.

MOE in bacteria with alkyne glycans has been performed in the gram-negative class *bacteroides*. MOE was achieved with alkyne-fucose analogues in *Bacteroides fragilis* and *Parabacteroides distasonis*. A wide variety of glycoproteins proved enriched with alkyne-fucose analogues [97]. However, the rising popularity of the SPAAC reaction has made the alkynyl glycans that require the generally toxic copper for CuAAC less popular for metabolic labelling. This trend is despite the discovery of bio-compatible copper reagents. The advantage of these reagents is the reduced amount of free Cu(I) ions which is beneficial for labelling in living organisms [110].

McClosky et al. have developed a 6-deoxy-alkynyl glucose analogue for metabolic glucose labelling experiments in *Arabidopsis thaliana* roots [78]. They based their choice for the alkyne handle on the unreported assumption that azido analogues of fucose are less well incorporated in the cell wall of plants, compared to their alkyne analogues. Acetylated 6-deoxy-alkynyl glucose successfully labelled glycans in *Arabidopsis thaliana* roots. Other relevant alkynyl-functionalised analogues of glucose, rhamnose, mannose,

and sucrose were also tested, but did not show any incorporation. Whether 6-deoxy-alkynyl glucose was selectively incorporated in the cell wall or also in other cellular components was not yet investigated, but would of course be highly interesting. These initial examples of the application of MOE in plants highlights the potential for the further development of this technique to study glycobiology in plants.

3.3. Cyclopropenes, norbornenes, and alkenes

Alkene-functionalised glycans have also been successfully used for MOE. The loss in reaction speed for alkene-tetrazine conjugation compared to CuAAC conjugation is compensated by the stability and easy synthesis of alkene carbohydrates. Wittmann and co-workers synthesised a variety of alkene-equipped *N*-acetylmannosamine analogues [58]. The length of the linker did not influence the successful labelling in HEK293T and Hela cells [58]. In later studies, the length of the linker going from ManNAloc to ManHeoc showed an increase in second-order rate constant for the invDA with tetrazines [59].

New possibilities in MOE emerged with the advent of strained alkenes (or alkynes) that react extremely fast under physiological conditions via an inverse electron demand Diels-Alder reaction (invDA). Early investigations with *trans*-cyclooctene (TCO) and bicyclononyne (BCN) showed the potential of this new ligation technique in cellular environments [111].

Cyclopropenes are strained alkenes that are ideal for MOE with respect to their bio-orthogonal reactivity, and small size (similar to diazirines). Obtaining sufficient stability is essential for the reactive cyclopropenes probes, as it is known that they are prone to polymerisation, and can be reactive towards thiols and other nucleophiles present in cells [112]. Substituents on the cyclopropene ring, such as a methyl, showed a big improvement in stability, while retaining their reactivity [71,111]. A few years later a carbamate linker instead of an amide linker to the glycan further improved the stability and reactivity [57,113]. The groups of Devaraj and Prescher worked independently on cyclopropene-linked glycan analogues for MOE [55,71,111]. After Devaraj proved the biocompatibility of cyclopropenes in phospholipid labelling, the Prescher group showed the first application of MOE with a cyclopropene-linked sialic acid analogue (9-Cp-Neu5Ac) [71,111]. Flow cytometry analysis with different concentrations of the probe showed efficient metabolic incorporation, although the optimum was found at the relative high concentration of 1 mM. In a second step, the compatibility during dual labeling with an azido sialic acid probe was tested. Both cyclopropene and azido sialic acids were added to Jurkat cells and incubated for 24 h. Successful labelling with corresponding fluorescent dyes showed the orthogonality of tetrazine click with cyclopropene glycans and the SPAAC click with azido glycans. In subsequent years, the groups of Devaraj, Prescher, and Wittmann have reported even more successful examples of cyclopropenes glycans [55,57,113]. The utilised concentrations nowadays vary between 10 and 100 μ M. The independent investigations of cyclopropene glycans resulted in different names for the same glycan analogues. Two distinct classes, the amide-linked

cyclopropenes and the carbamate-linked cyclopropenes have been developed. The amide-linked cyclopropenes are known as NMCP or NCyc for the 3-methylated cyclopropene glycan; the carbamate-linked cyclopropenes are named NCyoc or CCp.

In recent years, the effect of either an amide or carbamate linker between the glycan and cyclopropene moiety has been investigated. Different probes based on glucosamine, galactosamine, and mannosamine with the different linkers have been synthesised and tested in various cell lines. Prescher et al. took a closer look at the effect of the carbamate linker in comparison to the amide linker for mannose-based chemical probes [57]. The invDA-reactions are approx. 100 times faster with carbamates at C3 compared to their amide derivative. A clear 130-fold increase in fluorescence signal was obtained by flow cytometry when the two linkers were directly compared. The disappearance of fluorescence output after addition of the natural *N*-acetylmannosamine substrate suggested that the cyclopropene-ManNAc used the sialic acid biosynthetic pathway.

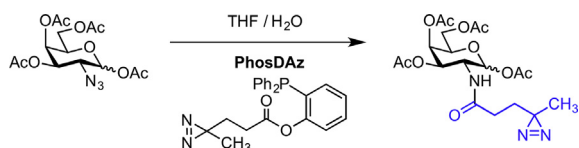
Cyclopropene-labelled glycans are attractive probes for use in MOE. They display fast enough reaction kinetics, short syntheses for their preparation and different variants (amide and carbamate scaffolds) are available. Over the last years, the relative high concentration of tetrazine counterpart needed for post incorporation tagging has also been reduced and is now in the range of 50–100 μ M, further enhancing the potential of this method [56].

Another alkene variant was recently reported by Wittmann and co-workers [114]. Norbornene-modified mannosamine derivatives were used for MOE and successfully applied in HEK 293 T cells. *Exo* and *endo* norbornene-modified mannosamine derivatives were made. The reaction kinetics of *exo* norbornene mannosamine proved faster than those of the *endo* norbornene mannosamine when reacted in a invDA reaction with a tetrazine.

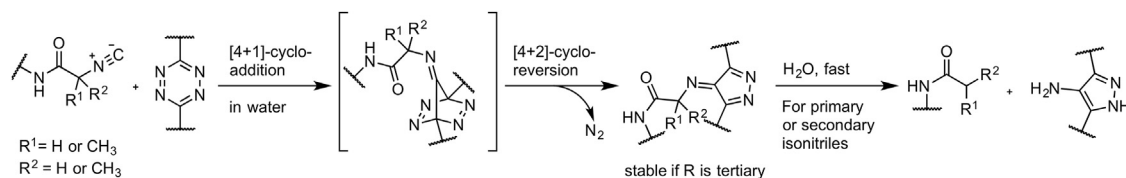
3.4. Diazirine

Diazirines are small highly strained heterocycles. The metabolic labelling step is followed by UV activated crosslinking of the incorporated diazirine with nearby biomolecules. When the cell is irradiated at a 254–400 nm wavelength N_2 will be expelled leaving a highly reactive carbene on the chemical handle attached to the glycan, which will react very rapidly with a wide variety of different functional groups inside or on the outside of the cell. The advantage of this photo-crosslinking is that it can be initiated in intact cells. Furthermore, various processes surface-glycans participate in can be studied (e.g. sialidases activity or lectin interactions). The use of photo-crosslinking is not limited to cell-surface glycans but has of course also successfully applied to other biomolecules [42]. A disadvantage of diazirines as chemical handles in probes is their lack of selectivity. Once the carbene is generated it will react with everything in close proximity.

Before diazirines were explored as metabolic labelling functionalities Paulson et al. reported in 2005 the use of aryl azides on Neu5Ac (known as 9-AAz-NeuAc) as a labelling agent for cell-surface glycoproteins or glycolipids [115]. A benefit of aryl azides is the possibility of using the Staudinger-Bertozzi ligation as an extra option to address the labelled glycans. Both tagging methods proved successful in labelling cell surface glycans. The use of diazirines in MOE was developed further by the group of Kohler [42]. Different types of glucosamine, mannosamine, and galactosamine *N*-acyl-diazirine derivatives were synthesised and tested in a wide variety of cell types. The effect of *N*-acyl chain length of diazirines on MOE was investigated by Kohler and co-workers in 2011 [61]. Diazirine-modified *N*-Acetylmannosamine analogues with 2, 3 or 4 methylene groups in between the *N*-acyl chain and diazirine were synthesised and tested in BJA-B, Daudi, and Jurkat cells. All probes were incorporated on sialylated glycoprotein or sialylated



Scheme 1. A traceless Staudinger-Bertozzi reagent to install diazirines onto azido-glycans.



Scheme 2. Tetrazine ligation with primary, secondary, or tertiary isonitriles.

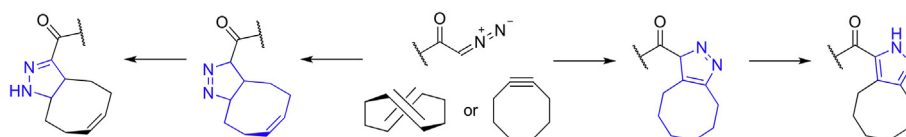


Fig. 3. Mechanisms [3 + 2] cycloaddition of diazo substrates with strained alkenes or alkynes.

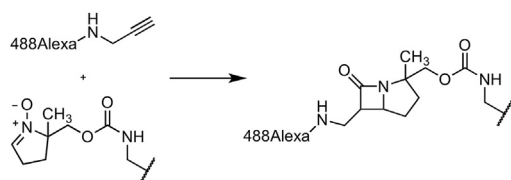
glycolipids to some extent. It was, however, clearly seen that the short linker (2Me) was incorporated most efficiently, and this resulted in effective cross-linking after photo-irradiation.

A combination of the Staudinger-Bertozzi ligation and UV irradiation was performed by Jewett and co-workers [62]. They developed a reagent that uses the traceless Staudinger-Bertozzi ligation to convert azides on glycans into an acyl-diazirines handle (Scheme 1). The method has not yet been tested on azido-glycans inside cells yet, but it looks promising.

3.5. Isonitrile group

Isonitriles, also known as isocyanides, are overall neutral molecules that can undergo a [4 + 1] cycloaddition with tetrazines or strained alkenes. Although isonitriles have been found in some natural products, they are only weak nucleophiles and show excellent stability in biological systems [116]. Hence, isonitriles do not react with common electrophiles like ketones, aldehydes or imines. Although isonitrile-containing probes themselves are stable, their imine-containing products after a [4 + 1] cycloaddition can decompose over time (Scheme 2). Inside cells, cycloaddition products of primary or secondary isonitriles are easily hydrolysed into amines. Products from tertiary isonitriles are more stable but bigger and therefore harder to incorporate into glycoconjugates. Primary isonitriles with an additional methylene group tautomerise after the click to a more stable α,β -unsaturated imine. Primary isonitrile-containing glycans were used at high concentration (200 μ M) to test for toxicity, but none was observed [37].

After initial experiments with isonitrile labelling on proteins proved successful, the Leeper group recently showed the potential of isonitriles as new metabolic oligosaccharide probes for the *in vitro* labelling of mammalian cells [37,63]. Three different probes based on *N*-acylated mannosamine, galactosamine, and glucosamine were synthesised and successfully tested on Lewis lung carcinoma cells. A two-step labelling method with tetrazine–biotin followed by neutravidin–DyLight680 provided the best signal to noise ratio and flow cytometry was used to quantify incorporation.



Scheme 3. Nitron-alkyne cycloaddition.

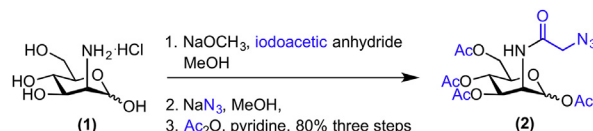
This study showed that these isonitrile glycan probes were successfully incorporated into glycans and are stable over the time period of labelling. Interestingly, when comparing incorporation of the glucosamine- and mannosamine-based probes they show a reversed trend in comparison with azido-glycans. It is well known that compared to ManNAz, GlcNAz is poorly incorporated into glycoconjugates, but Ac₄GlcN-*n*-Iso was efficiently incorporated, whereas Ac₄ManN-*n*-Iso showed poor incorporation [117]. However, overall azido glycans appear to be incorporated slightly better than isonitrile glycans.

In conclusion, isonitrile-containing glycans have been successfully applied in MOE, but for not yet fully understood reasons they need relatively high concentration (200 μ M) compared to azido glycans (50 μ M). Similar to click reactions with alkene/cyclopropene versus azides, isonitriles show orthogonal reactivity to azides, which enables future use in dual-labelling MOE experiments.

3.6. Diazo group

In 2014 the Leeper group reported a new bioconjugation reaction with the diazoacetyl group [39]. This group is smaller than its azide counterpart and reacts under physiological conditions with strained alkenes or alkynes [118]. Furthermore, the diazoacetyl group can be used in parallel with an alkyne-based chemical handle to enable dual bio-orthogonal labelling strategies.

The reaction of diazo compounds with strained alkenes or alkynes has been known for many years. For instance, their reactivity with cycloalkynes like DIBO and DIFO has been reported by Raines et al. and indicated their potential as chemical handles in metabolic probes [118]. The Leeper and Raines groups independently investigated the reaction kinetics of various diazo compounds with cyclo-alkynes and alkenes (for both ± 0.04 M⁻¹ s⁻¹), solvent and stability [39,118]. The Leeper group reported the first *in vivo* labelling with diazo glycans [39]. A two-step [3 + 2] cycloaddition with simple diazo building blocks was performed. In the first step an invDA reaction was performed with the highly strained (*E,E*)-1,5-cyclooctadiene (COD) (Fig. 3). In the second step a [3 + 2]



Scheme 4. Synthesis of Ac₄ManNAz (2).

cycloaddition isomerisation results in stable adducts. Imaging the incorporation of a diazoacetyl-GalNAc derivative (Ac₄GalNDiaz) was achieved in Lewis lung carcinoma (LL2) cells via a two-step method using an initial click reaction with biotin-TMDIBO, a stable dibenzocyclo-octyne, and subsequent visualization with avidin or neutravidin-appended fluorescent dyes. This two-step labelling procedure provided a better signal-to-background ratio compared to a one-step labelling method.

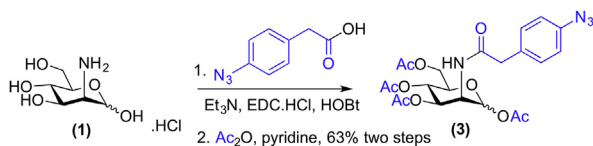
Although successful, the level of labelling achieved with Ac₄GalNDiaz, as analysed by flow cytometry, was significantly lower when compared with the Ac₄GalNAz. The exact reason(s) for this low level of incorporation or subsequent labelling is still not known, but one reason might be that the diazo-glycan is a poor substrate for the enzymes involved in the galactosamine salvage pathway or for the associated glycosyltransferases.

A peracetylated diazo-mannose glycan (Ac₄ManDiaz) has been successfully incorporated in Jurkat, CHO K1, HEK293T and HeLa cell lines by the Raines group [52]. This matches the fact that the enzymes involved in the conversion of *N*-acetylmannosamine into Neu5Ac have a high tolerance for alternatively *N*-acylated derivatives [85]. However, also here Ac₄ManDiaz was incorporated less efficiently compared to ManNAz as shown by flow cytometry. It is known that diazo compounds are prone to degradation at low pH via C-protonation and subsequent hydrolysis. So if endosomes are involved in the uptake of diazo-glycans, this could also explain the general lower incorporation level.

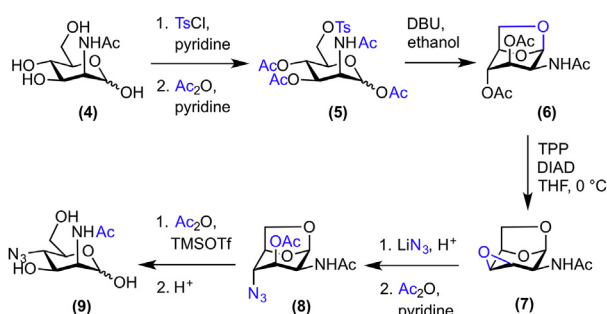
Toxicity of diazo glycans was only observed after prolonged labelling times of 60 min at 10 μM concentration. Diazo compounds are not affected by the CuAAC reaction and a successful dual-labelling strategy with azido-glycans has been explored [52]. Analysis by flow cytometry and microscopy showed that both click reactions could be performed without cross-reactivity and are independent of the labelling order. No cross-linking was observed for diazo- and azido-glycans, which is an undesired known phenomenon for alkyne and azido glycan combinations. This clearly illustrates the potential of dual-labelling techniques with diazo-glycans.

3.7. Nitron-alkyne cycloaddition followed by rearrangement

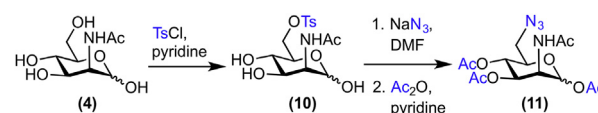
A new copper-catalysed bioconjugation reaction was discovered by Pezacki and co-workers in 2014, involving coupling a terminal alkyne and nitron to form a β-lactam ring (Scheme 3) [119]. This



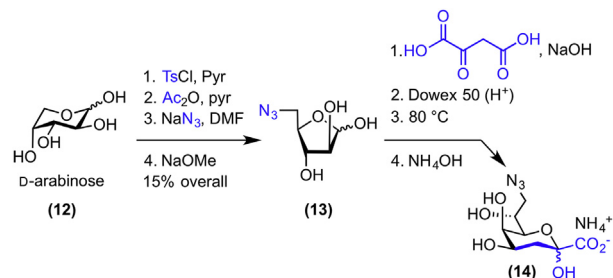
Scheme 5. Synthesis of Ac₄Man-2-arylAz (3).



Scheme 6. Synthesis of 4-azido ManNAc (9).



Scheme 7. Synthesis of 6-azido Ac₃-*N*-acetylmannosamine (11).



Scheme 8. Synthesis of 8-Az-KDO.

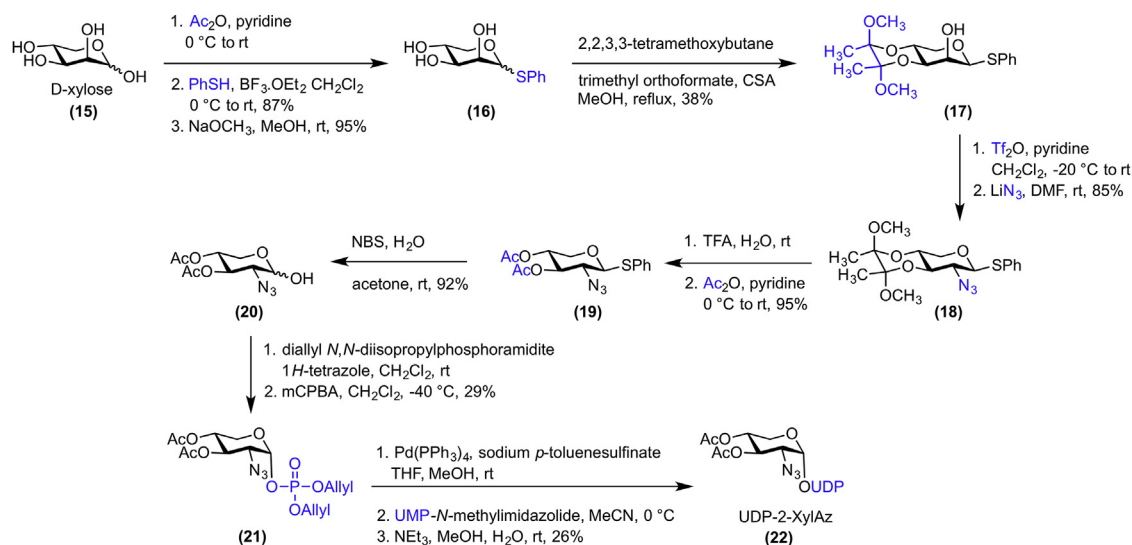
reaction is fast and both starting materials and product are stable in aqueous media. The copper-catalysed nitron-alkyne cycloaddition followed by rearrangement (CuANCR) has been successfully performed in living cells, like Huh-7 cells, and *E. coli*. For the labelling in *E. coli* a nitron-bearing KDO derivative (HMMPO) was synthesised. *E. coli* was incubated overnight in M9 minimal medium containing 4 mM KDO with or without an alkyne or nitron chemical handle. KDO-HMMPO and KDO-alkyne were successfully incorporated into the bacterial LPS as shown by SDS-PAGE analysis. The alkyne nitron cycloaddition can also be performed with strained alkynes (SPANC), and is compatible with cyclic or acyclic nitrones [120].

4. Synthetic strategies for selected members from the MOE toolbox

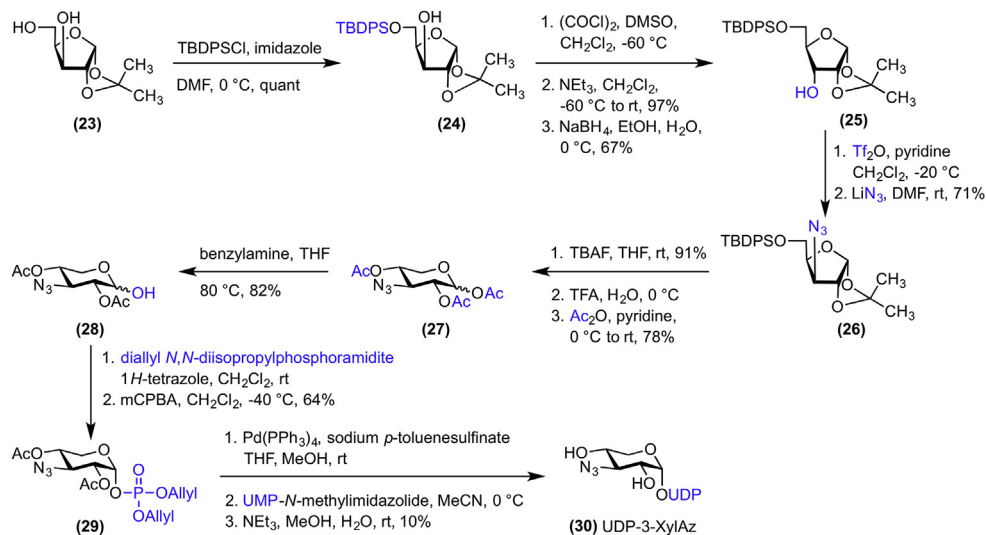
The MOE toolbox is filled with unnatural glycan derivatives and most of these are not yet commercially available. Therefore most studies that are undertaken using MOE still start with the synthesis of the required probes using a combination of carbohydrate chemistry and organic chemistry. In this section we provide an overview of synthesis strategies used for a diverse selection of glycan-based chemical probes used in MOE. In general, the same synthetic strategies apply for the construction of all three of the much used *N*-acylated mannosamine, galactosamine, and glucosamine-based chemical probes. For that reason we here only discuss several representative examples for the synthesis of the mannosamine-based chemical probes. A comprehensive overview of chemical probes used for MOE can be found in the previous section in Table 2.

4.1. Azido-labelled glycans

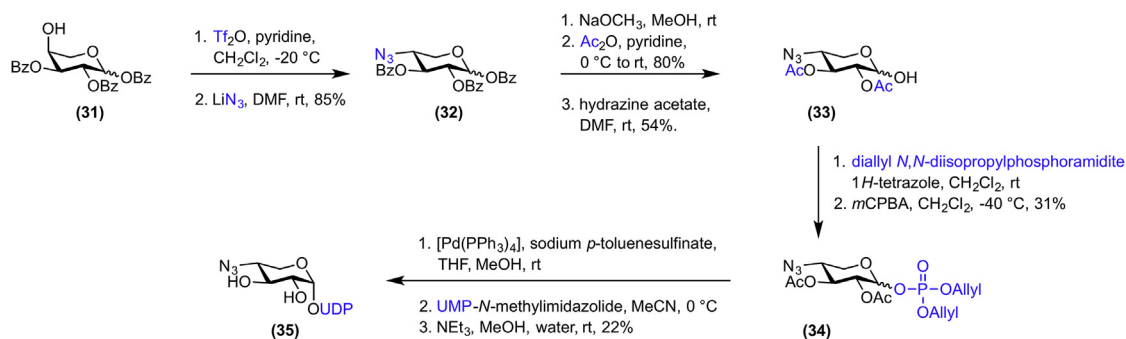
The first azido-glycans to be reported were the mannosamine, galactosamine, and glucosamine-based *N*-azidoacetyl glycans. These per-acetylated glycans are easily prepared via standard carbodiimide-mediated amide bond formation with azido acetic acid and the respective amino sugar (Scheme 4) [23,45]. Alternatively, iodoacetic anhydride or iodoacetic acid can be coupled to mannosamine, followed by nucleophilic displacement with sodium azide to provide the *N*-azidoacetyl glycan. The final step includes acetylation of all free hydroxyl groups under standard conditions. Aldolases inside mammalian cells are able to convert Ac₄ManNAz (2), once deacetylated, towards a sialic acid, to yield azido-labelled



Scheme 9. Synthesis of UDP-2-XylAz (22).



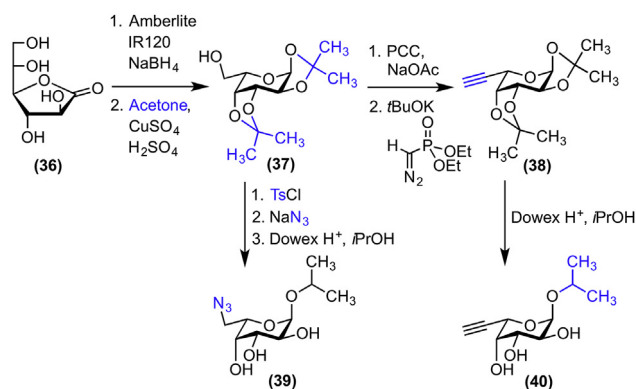
Scheme 10. Synthesis of UDP-3-XylAz (30).



Scheme 11. Synthesis of UDP-4-XylAz (35).

neuraminic acid (Neu5Az) [23]. Neuraminic acid is the most abundant sialic acid, and known to be important for many cellular communication and processes. Additional neuraminic acid-based chemical probes for MOE are known and will be discussed later.

Photoactivatable phenyl azide-based chemical probes have also been made via a similar route. 2-(4-Azidophenyl)acetic acid was coupled to mannosamine hydrochloride (1), and subsequent acetylation provided the $\text{Ac}_4\text{Man-2-arylAz}$ (3), which is a precursor for



Scheme 12. Synthesis of 6-Az fucose (39) and 5-alkyne fucose (40).

Sia-5-arylAz (Scheme 5) [47].

Other azido-substituted mannosamine derivatives have been synthesised as well. For example, 4-azido *N*-acetylmannosamine derivative (9) shown in Scheme 6 is made from a 1,6-anhydrosugar intermediate (6) that itself is made in three steps from *N*-acetylmannosamine (4) [121]. A Mitsunobu reaction on the 1,6-anhydro intermediate (6) was performed to give a 3,4-epoxide (7). Selective axial opening of the epoxide (7) with lithium azide provided, after opening of the 1,6-anhydrosugar (8) a di-amino glycan (9). This di-amino mannosamine glycan (9) has been used for metabolic labelling in HEK293 cells lacking the key enzyme for sialic acid biosynthesis to improve incorporation rate into glycans [48]. For membrane permeability glycan 9 was peracetylated before it was used in MOE.

6-azido-*N*-acetylmannosamine (11) is converted inside the cell to 9-azido-*N*-acetylneuraminic acid. The 6-azido glycan (11) can be synthesised from *N*-acetylmannosamine (4) by selectively tosylating the 6-OH (10) (Scheme 7) [45]. Nucleophilic substitution with sodium azide at 50 °C for 3 days gave, after acetylation, the 6-azido Ac₃-*N*-acetylmannosamine (11).

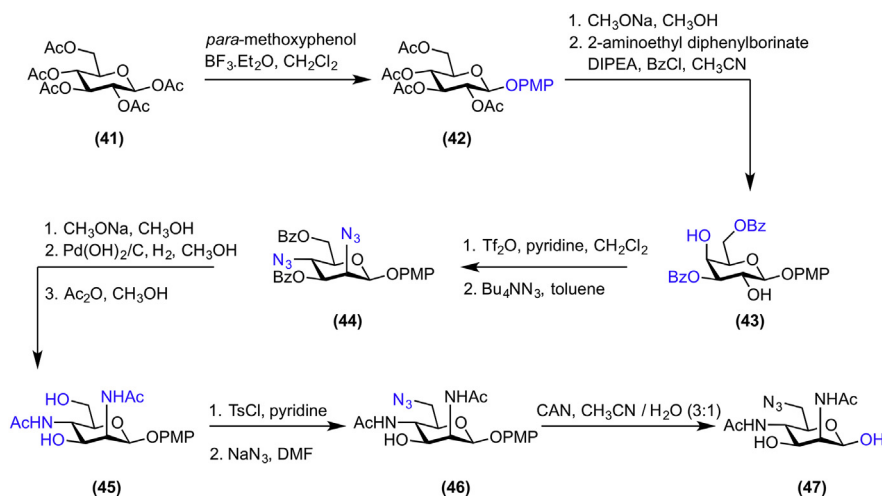
3-Deoxy-*D*-manno-octulosonic Acid (KDO) is a subclass of sialic acids having an eight-carbon skeleton. It is highly abundant and essential component in the outer-cell membrane of lipopolysaccharide gram negative bacteria and also found in the RG-II pectin of plant cell walls [107]. Human gut bacteria like *H. pylori* are known

to have KDO in their LPS, and Dumont and co-workers imaged this via metabolic labelling with KDO probes [66]. The 8-azido KDO (14) is readily synthesised from *D*-arabinose (12) (Scheme 8). After selective tosylation of the primary alcohol, the remaining free alcohols are acetylated, and the resulting mixture is then treated with NaN₃ in DMF. In the final step sodium methoxide gives the 5-Az-5-deoxy-*D*-arabinofuranose (13) in an overall yield of 15%. 8-Azido KDO (14) is made by the reaction between 5-Az-5-deoxy-*D*-arabinofuranose (13) and sodium oxaloacetate under acidic conditions, followed by a decarboxylation.

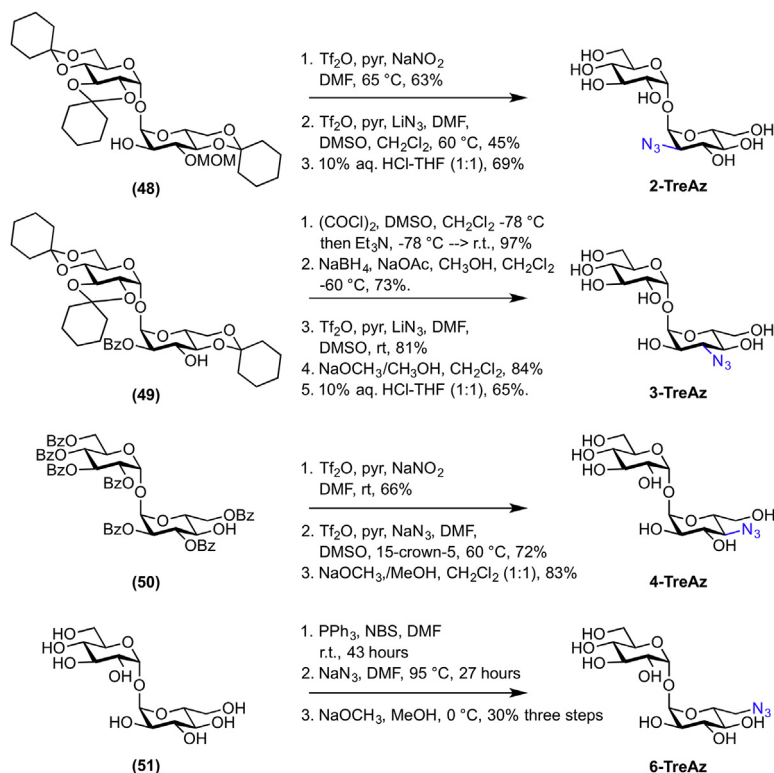
Metabolic oligosaccharide engineering can also be a highly valuable tool to study the action or inaction of glycosyltransferases as recently shown by Bertozzi et al. with the use of UDP-azido glycans as metabolic chain inhibitors [76]. We here discuss the synthesis of three different UDP azido-glycans and the outlook discusses their application in MOE. UDP-2-XylAz (22) was synthesised from *D*-xylose (15) as shown in Scheme 9. After protection of the anomeric center (16), selective protection of the 3- and 4-OH with a cyclohexane-1,2-diacetal 'Ley' protecting group made functionalisation of the 2-position possible (17) [76]. After activation of the 2-OH with Tf₂O in pyridine, nucleophilic displacement was performed with LiN₃. The UDP-donor was prepared by first selectively deprotecting the anomeric center using *N*-bromosuccinimide. The phosphate analogue was made by treatment with diallyl-*N,N*-diisopropylphosphoramidite. UMP-*N*-methylimidazolid was added, after Pd-catalysed isomerisation and cleavage of the allyl groups, to finally provide UDP-2-XylAz (22).

The synthesis of UDP-3-XylAz (30) was performed in 15 steps starting from 1,2-*O*-isopropylidene- α -*D*-xylofuranose (23) (Scheme 10) [76]. To install the azide functionality on the 3-position, the hydroxyl on this position was first converted from an axial to an equatorial hydroxyl group by oxidation and subsequent stereoselective reduction (25). Thereafter, conversion to the 3-OTf and its substitution with LiN₃ provided the 3-azido glycan (26). The UDP group was installed in the same manner as for the 2-XylAz glycan (22).

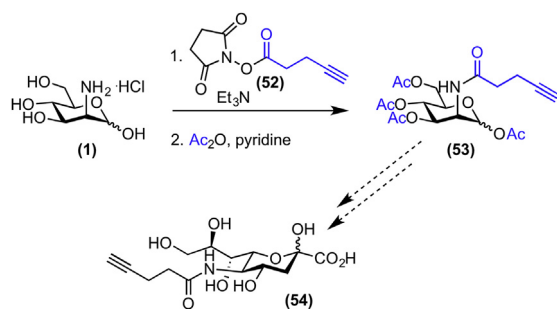
4-OH Benzoylated *L*-arabinose (31) proved a perfect starting point for the synthesis of UDP-4-XylAz (35) (Scheme 11) [76,122]. The 4-OH was reacted with triflic anhydride followed by substitution of the triflate with LiN₃ starting product is easily synthesised from *L*-arabinose. Deprotection of all benzoyl groups followed by acetylation, and selective anomeric deprotection with hydrazine acetate gave acetylated 1-OH 4-XylAz (33). Installation of the allyl



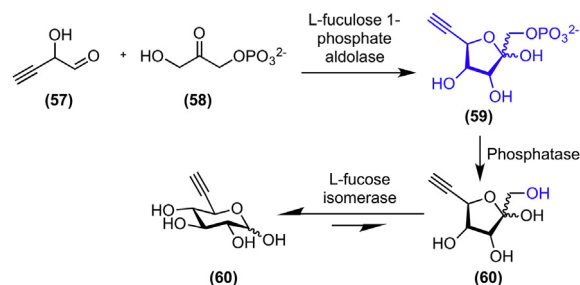
Scheme 13. Synthesis of 6-azido Leg hexose (47).



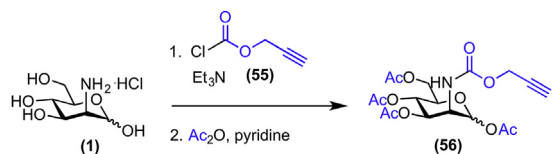
Scheme 14. Synthesis of 2-TreAz, 3-TreAz, 4-TreAz, and 6-TreAz.



Scheme 15. Synthesis of mannosamine alkyne glycans (53).



Scheme 17. Synthesis of D-glucose alkynes (60).

Scheme 16. Synthesis of $\text{Ac}_4\text{ManIPoc}$ (56).

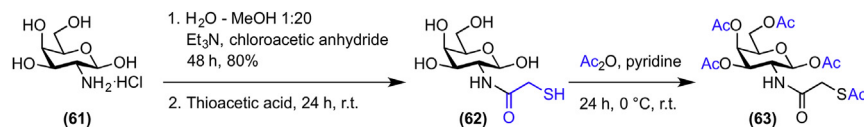
phosphodiester analogue proceeded in moderate to low yield. The UDP group was finally installed by removal of the allyl esters followed by coupling with UMP-*N*-methylimidazole.

Purification of synthesised UDP-glycans is a challenging step. The described UDP-glycans were for example purified in a HPLC C-18 column with tributylammonium bicarbonate and methanol as mobile phases. Once the crude product eluted from the column it was further purified by size exclusion chromatography and sodium ion exchange resin.

L-Galactonic acid γ -lactone (36) was selected as a starting point

for the synthesis of azido fucose (39) (FucAz) and alkyne fucose (40) (FucAl) analogues (Scheme 12) [70]. A reduction of the lactone with sodium borohydride followed by acetonide protection gave the 6-OH glycan (37). The 6-OH was activated and nucleophilic displacement using NaN_3 followed by acidic deprotection gave the 6-azido fucose (39) as the isopropyl protected anomeric center. FucAl (40) was made as follows; The 6-OH intermediate (37) was oxidised to the aldehyde with PCC followed by treatment with the Seyferth/Gilbert reagent [123]. Acid deprotection provided FucAl (40) in excellent yield. For MOE the isopropyl acetal was cleaved and the hydroxyl groups were acetylated. These azide and alkyne fucose analogues were also used to make their GDP-donor counterparts for use in MOE.

As mentioned, the microbial sialic acid, legionaminic acid, is a relevant target for metabolic labelling to study its glycobiology in human associated pathogenic bacteria. An 6-azido hexose-precursor (47) was synthesised starting from β -D-glucose pentaacetate (41) (Scheme 13) [77]. A glycosylation reaction with *para*-methoxyphenol followed by Zemplén deacetylation and subsequent selective benzoylation gave the 2,4-dihydroxyl glucose



Scheme 18. Synthesis of thio-N-acetylgalactosamine probe (63).

derivative (43). This intermediate was treated with triflic anhydride and reacted with tetrabutylammonium azide to install the two azides (44). Removal of the benzoyl groups and reduction of the azides followed by controlled acetylation gave the 3,6-dihydroxy intermediate (45). Selective tosylation of the primary alcohol, followed by nucleophilic displacement with sodium azide and oxidative deprotection of the anomeric PMP group provided the probe (47).

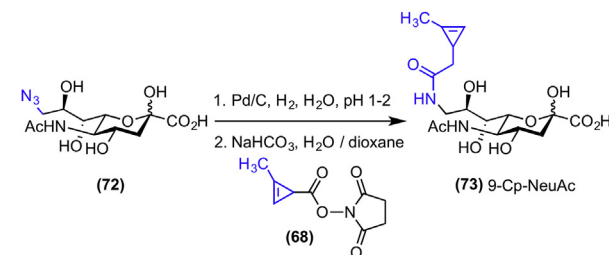
The earlier discussed trehalose azido sugars were synthesised from a series of known differentially protected trehalose building blocks (48–51) (Scheme 14) [79]. Their synthesis involved alcohol activation, followed by nucleophilic substitution with an azide source and complete deprotection to provide the azido-trehalose library (2-TreAz, 3-TreAz, 4-TreAz, and 6-TreAz).

4.2. Alkyne-labelled glycans

Terminal alkynes on carbohydrates were among the first probes used for metabolic oligosaccharide engineering. Their easy synthesis and stability made it possible to synthesise a variety of different alkyne-glycans (see Table 1). Alkyne-labelled *N*-acetylmannosamine glycans are made from *N*-mannosamine hydrochloride (1) in a two-step procedure (Scheme 15). Under basic conditions the amine group is reacted with a NHS activated alkyne (52) [49]. Subsequent acetylation with acetic anhydride and pyridine provided the probe (53). The alkyne-labelled *N*-acetylmannosamine (53) can either be directly used for metabolic labelling or first converted to alkyne-labelled *N*-acetylneuraminic acid (54) using an enzymatic reaction or the chemical equivalent using a Barbier alkylation and ozonolysis.

For the synthesis of the carbamate-linked alkyne probes, reported by Pratt et al., mannosamine, galactosamine, or glucosamine hydrochloride were reacted with propargylchloroformate (55) (Scheme 16) [53]. Subsequent acetylation under standard conditions gave the peracetylated propargyl alkyne glycans (56).

Glucose alkynes (60) can be made via an enzymatic procedure starting from α -hydroxylated aldehyde (57) and dihydroxyacetone phosphate (58) (Scheme 17) [124].



Scheme 20. Synthesis of 9-Cp-Neu5Ac (73).

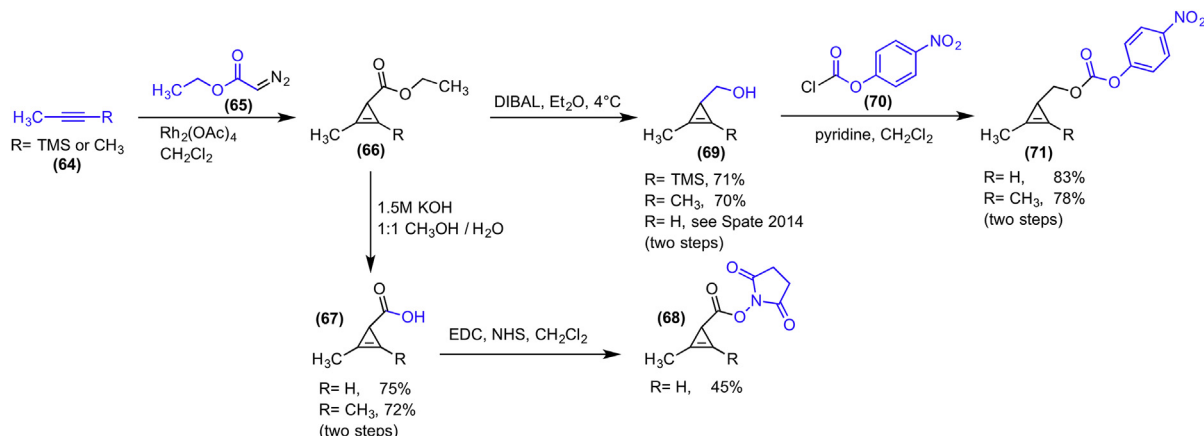
4.3. Thiol-labelled glycans

Thiol-containing *N*-acetyl amino sugars (63) are made from commercially available glucosamine, galactosamine, or mannosamine derivatives. Thioacetic acid is coupled to provide, after full acetylation, the thio acetate probe (63) that can be used for MOE (Scheme 18) [54,125].

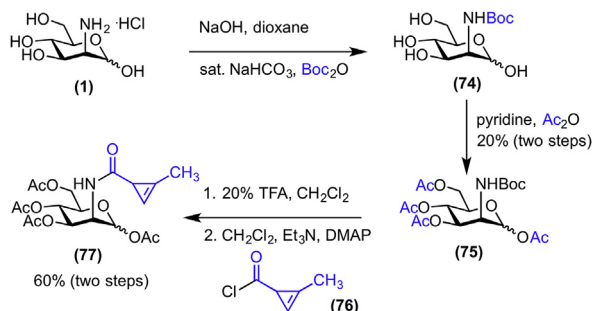
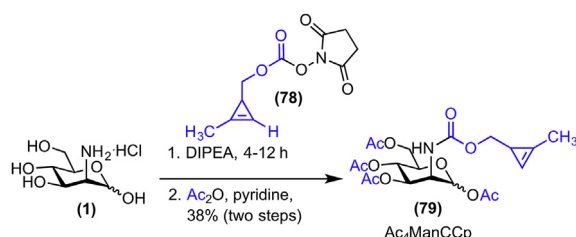
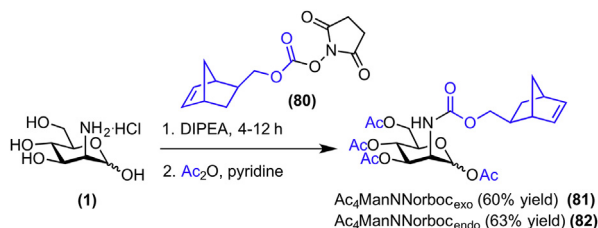
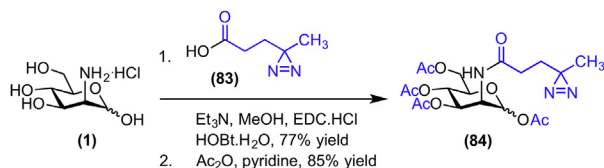
The thioacetyl handle has not been reported yet for the traditional metabolic labelling via click chemistry, but the thiols are biocompatible and have been successfully incorporated onto surface glycans [126].

4.4. Cyclopropene-labelled glycans

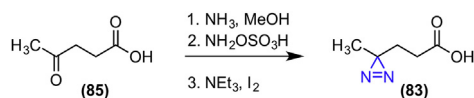
Highly strained cyclopropenes react very fast with tetrazines making them excellent candidates for MOE [55]. The synthesis of cyclopropenes is not trivial, however, and yields below 50% for the activated cyclopropenes are the standard (Scheme 19). Also, the amide formation to attach them to amino sugars is a low yielding step [71]. The synthesis of 2-methyl cyclopropenes, in which the methyl substituent is important for stability, starts with a rhodium-catalysed cyclopropanation of the appropriate alkyne (64) with ethyl diazoacetate (65), followed by a reduction of the ethyl ester [57]. The resulting cyclopropene-alcohol products (69) are known to be volatile and prone to polymerisation. Installation of the cyclopropene handle onto the amino sugar is achieved via the NHS



Scheme 19. Synthesis of activated cyclopropenes handles (68 and 71).

Scheme 21. Synthesis of $\text{Ac}_4\text{ManNCyc}$ (77).Scheme 22. Synthesis of Ac_4ManCCp (79).Scheme 23. Synthesis of $\text{Ac}_4\text{ManNNorboC}_{\text{exo}}$ and $\text{Ac}_4\text{ManNNorboC}_{\text{endo}}$.

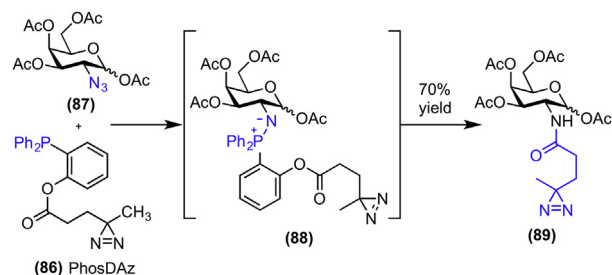
Scheme 24. Synthesis of mannosamine-diazirines derivatives (84).



Scheme 25. Synthesis of diazirine building block (83).

(68) or *p*-nitrophenyl carbonate activated intermediate (71).

To improve the stability and reaction yields another route to the activated cyclopropene handles was developed. A TMS protected alkyne is reacted with ethyl diazoacetate under catalytic amounts of rhodium tetraacetate. (Scheme 19) [56]. Reduction of the ester to the alcohol with DIBAL was performed. Next, desilylation and subsequent activation was performed in a one-pot reaction using Bu_4NF to deprotect and *p*-nitrophenyl chloroformate to activate the alcohol (69). This crystalline activated cyclopropene (71; $\text{R} = \text{CH}_3$) can easily be stored and used for the installation of the cyclopropene group onto amino sugars. 9-Azido *N*-acetylneuraminic



Scheme 26. Synthesis of galactosamine-diazirine (89) via Staudinger reduction.

acid (72) is an ideal starting point for the synthesis of 9-cyclopropenyl neuraminic acid (73) (Scheme 20). After Pd-catalysed hydrogenation, NHS-activated cyclopropene (68) is added to give the final product [71].

The mannosamine cyclopropenes ($\text{Ac}_4\text{ManNCyc}$ and Ac_4ManCCp) are made from $\text{D-mannosamine}^*\text{HCl}$ (1). When $\text{D-mannosamine}^*\text{HCl}$ (1) was directly reacted with the acid chloride of a cyclopropene handle (76), it yielded several products making the synthesis low yielding and the purification difficult (Scheme 21). A three-step procedure with a Boc protection, followed by acetylation, and Boc deprotection gave the acetylated mannosamine (75). This *O*-protected amino sugar could then be converted into the cyclopropene probe (77) in good yield (60% over two steps) [55,127].

The less reactive NHS-ester of a cyclopropene handle (78) was directly coupled to $\text{D-mannosamine}^*\text{HCl}$ (1), and subsequent acetylation produced Ac_4ManCCp (79) in 38% yield (Scheme 22) [56,57].

The same route was followed to install norbornene-derived mannosamine probes (81 and 82). For that a succinimide activated norbornene derivative (80) was synthesised in three steps (Scheme 23).

4.5. Diazirine-labelled glycans

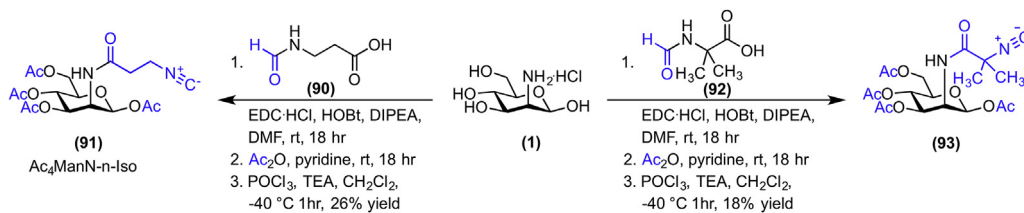
Diazirine-based chemical probes (84) are synthesised from mannosamine, glucosamine, or galactosamine via an amide coupling with the diazirine building block (83) under standard amide-coupling techniques (Scheme 24). Similar synthesis with longer linkers and other amino sugars are known [61].

The diazirine building block (83) is commercially available or can be made from levulinic acid (85) (Scheme 25). Levulinic acid (85) is converted into the diazirine handle (83) in a two-step reaction with hydroxylamine-*O*-sulfonic acid and ammonia, followed by triethylamine and iodine in methanol (Scheme 25) [36]. With this building block in hand, Kohler and co-workers made the $\text{Ac}_4\text{ManNDAz}$ (3Me) (84) and $\text{Ac}_4\text{GlcNDAz}$ (3Me) [61].

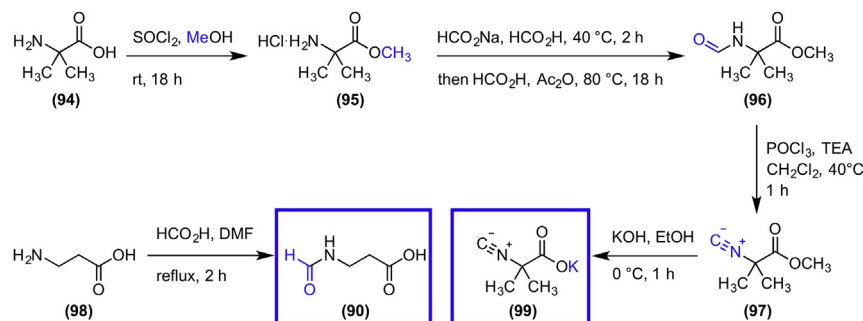
Jewett and co-workers recently reported an elegant alternative way to install diazirines on azide-containing carbohydrate derivatives using a traceless Staudinger reaction [62]. They successfully reduced a C-2 azide, for example glucosamine and galactosamine (87), with the phosphine reagent PhosDAz (86), trapping the formed aza-ylide by an intramolecular reaction (88) with the ester-appended diazirine (89) (Scheme 26).

4.6. Isonitrile-labelled glycans

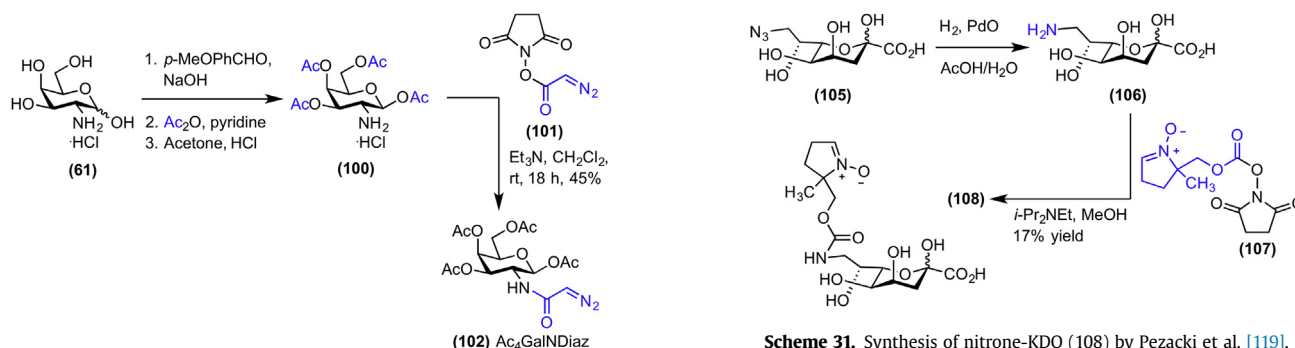
In 2013 Leeper and co-workers developed isonitrile-labelled probes for MOE (Scheme 27) [63]. The amide coupling of 2-deoxy 2-amino glycans (e.g. $\text{D-mannosamine}^*\text{HCl}$ (1)) with a β -alanine formamide (90) under standard conditions gives the formamide-functionalised carbohydrate. Subsequent acetylation followed by



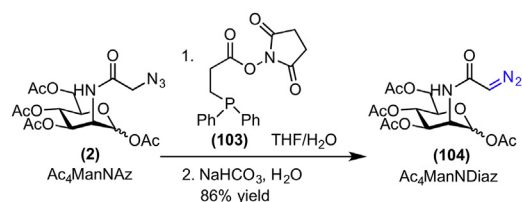
Scheme 27. Synthesis of primary (91) and tertiary isonitrile (93) mannosamine glycans.



Scheme 28. Synthesis of formamide building block (90) and tertiary isonitrile building block (99).

Scheme 29. Synthesis of $\text{Ac}_4\text{GalNDiaz}$ (102) by Leeper et al. [39].

Scheme 31. Synthesis of nitron-KDO (108) by Pezacki et al. [119].

Scheme 30. Synthesis of $\text{Ac}_4\text{ManNDiaz}$ (104) by Raines et al. [52].

formamide dehydration with POCl_3 gives the isonitrile-containing probe (91). Isonitriles are stable under neutral conditions and can be purified by column chromatography or HPLC. The amide couplings can also be performed with the isonitrile beta alanine carboxylic acid in an one step procedure. Amide couplings with formamide or isonitriles are challenging and generally result in low yields (<30%). Dehydration of formamides can be performed via different procedures. The most common procedure uses POCl_3 , but this rather harsh condition can lead to decomposition of the product. Other methods for dehydration exist, for example, Burgess reagent is able to perform the same dehydration step under much milder conditions [128].

Tertiary isonitriles can also be synthesised in a direct coupling

between the amino sugar and the isonitrile using potassium 2-isocyano-2-methylpropanoate (99) (Scheme 28). 2-Amino-2-methylpropanoic methyl ester (95), made from the corresponding acid (94), is exposed to *in situ* formed acetic formic anhydride (96) and dehydration of the formamide intermediate with POCl_3 gives the methyl-protected tertiary isonitrile (97). Saponification of the methyl ester provides the isonitrile (99) ready for coupling to an amino sugar.

4.7. Diazo-labelled glycans

The diazo derivative of *N*-acetylmannosamine or *N*-acetylgalactosamine (61), ManDiaz or GalDiaz, respectively, are easily synthesised via the route published by the Leeper group (Scheme 29) [39]. Starting from galactosamine (61), $\text{Ac}_4\text{GalDiaz}$ (102) can be synthesised anomerically pure in a three-step process involving transient protection of the amine as its imine with anisaldehyde, acetylation of the hydroxyls, imine hydrolysis, followed by installing the diazoacetyl group via its NHS ester derivative (101).

Another method to synthesise $\text{Ac}_4\text{ManDiaz}$ (104) was reported by the Raines group (Scheme 30) [52]. A deimidogenation of the parent azido-glycan, via an acyl triazenophosphonium salt intermediate, gave the diazo-glycan in high yields [129]. Both diazo-glycans proved to be bio-orthogonal as incubation at pH 7.4 at

37 °C showed no reaction of diazo acetyl glycans with biologically relevant functional groups such as thiols, carboxylic acids, and amines. Diazo-acetyl glycans are stable compounds and did not show any degradation during synthesis or purification.

4.8. Nitron-labelled glycans

Very recently, the use of nitron-labelled glycans in MOE was reported for the first time (Scheme 31) [119]. Nitron-labelled KDO (108) was made from the earlier discussed 8-azido KDO precursor (105). The azido glycan was reduced using PdO, H₂ under slightly acidic conditions followed by amide coupling with an activated nitron-succinimide (107). The resulting product was purified by preparative HPLC before it was used for metabolic labelling. Metabolic labelling with these nitron glycans were performed in *E. coli* and Huh-7 cells. The CuANCR reaction was also successfully applied to synthesise unnatural amino acids which were incorporated into *E. coli*, *L. innocua*, and *L. lactis* [122].

5. Outlook

In this final section some exciting recent advances are discussed that might impact MOE in the near future, as well as several MOE-related developments that might advance the field of chemical glycobiology in general.

5.1. Selective exo-enzymatic labelling

A promising complementary method to MOE for the incorporation of labelled glycans is Selective Exo-Enzymatic Labelling (SEEL), introduced in 2013 by Boons and co-workers [130]. In this technique, intact cells are exposed to extracellularly added recombinant glycosyltransferases, for example sialyltransferases, and incubated together with labelled donor derivatives, for instance C-5 or C-9 azido-modified CMP-sialic acids. The recombinant sialyltransferase enzymes still recognize and transfer sialic acid donors with an azide introduced on these positions. As a result, an azide-labelled sialic acid was introduced exclusively on the cell surface glycans that displayed the appropriate terminal acceptor glycans. SEEL thus has several advantages over MOE, for example the possibility of selectively labelling glycans on the cell surface and the benefit of not interfering with the intracellular metabolic pathways. The main disadvantage is the need to synthesise the valuable labelled-glycan donors and prepare and/or add the required glycosyltransferases.

5.2. Metabolically generated glycosyltransferases inhibitors

Glycosyltransferases have, with the exception of glucosylceramide synthase that can be reliably targeted by lipophilic imino-sugars (like Zavesca and AMP-DNM), remained elusive towards a general small molecule design principle for their inhibition. We clearly lack structural insight in glycosyltransferases when compared to their metabolic counterpart, glycosidases, and hence hardly any effective, bioavailable glycosyltransferase inhibitor currently exists. Many different approaches to glycosyltransferases

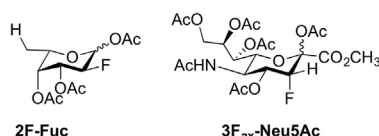


Fig. 4. Two glycosyltransferases inhibitors developed by Paulson et al. [137].

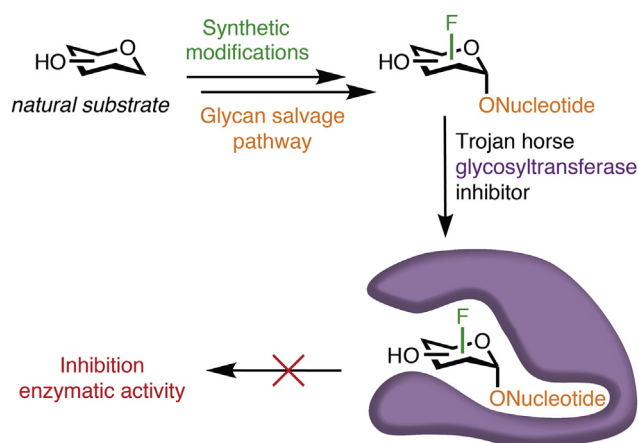


Fig. 5. Overview of principle behind MOE-generated trojan horse glycosyltransferase inhibitors.

inhibitors are thus known and discussed in a recent review by Gloster and Vocadlo [131]. However, it has recently been shown that the principles that drive MOE can also be used to perturb the activity of glycosyltransferases. The two MOE-related strategies that make use of the cell's glycan salvage pathways are trojan horse donor inhibitors and metabolic chain inhibitors.

The Paulson group developed a new technique to tune intracellular glycosyltransferase activity through the metabolic generation of transferase inhibitors from unnatural glycans derivatives – the trojan horse – by hijacking the salvage pathway (Fig. 4) [115,131–137]. Fluorinated analogues of fucose and sialic acid were shown to be taken up and metabolised by cells into fluorinated donors that still tightly bind their corresponding glycosyltransferase, but are no longer transferred to an acceptor (Fig. 5) [137]. This is caused by the fact that the required active site oxocarbenium ion transition state is destabilised by the neighbouring fluorine atom and is thus no longer formed. The hijacking of the salvage pathway via this trojan horse strategy also causes a reduced production of the natural nucleotide sugar donors. The Vocadlo group published a similar strategy that instead uses glycan derivative in which the endocyclic oxygen is replaced by a thio-group [138]. These are similarly metabolised into glycosyltransferase substrates that still bind the active site, but are no longer transferred.

An elegant example of metabolic chain inhibitors is the work on azido-xylose analogues by Bertozzi and co-workers [76]. The azido-xylose glycans were used as chain-terminating metabolic inhibitors of glycosaminoglycans (GAG) synthesis in zebrafish. The lack of a salvage pathway that could convert azido-xylose glycans to UDP-activated forms gave an extra synthetic challenge. The synthesis of the 2-, 3- and 4-azido UDP-xylose was discussed in section 4.1. The UDP-azido-xylose analogues were added to the zebrafish embryos via microinjection for further study. After development, a SPAAC reaction with difluorocyclooctyne-AlexaFluor 488 was performed on the embryos. Successful labelling was only observed with UDP-4-XylAz as confirmed by confocal microscopy and flow cytometry. The mechanism of action, hence the reason why only UDP-4-XylAz showed successful labelling, is not known yet.

5.3. Dual labelling

Dual labeling is an area of MOE that is gaining increasing attention. Two different types of dual-labelling strategies are currently being investigated. The first strategy uses two

metabolically incorporated glycan-based chemical probes with different bio-orthogonal reactive groups, while the second strategy uses two bio-orthogonal reactive groups within the same molecule. A recent example of the first approach is the successful simultaneous incorporation of Ac₄GalNAz and Ac₄GlcN-n-Iso and the simultaneous labeling of both these glycans in a single step using SPAAC and invDA. Other reported examples used nitron- and azido-labeled glycans for bioconjugation via SPANC and SPAAC, and norbornene or cyclopropene-labelled glycans in combination with azido-labeled glycans for dual invDA and SPAAC labelling in HEK 293 T cells [56,114,139,140]. An example of the second strategy is the successfully metabolic incorporation of a sialic acid derivative containing both an azide and a photocleavable diazine group [75].

5.4. What's next for MOE?

Besides the already impressive toolbox of probes and associated bioconjugation methods that are currently known for use in MOE, several new bioconjugation reactions have been reported recently that might also end up as part of MOE toolbox. Four highly interesting cases are: a) the reaction of *N*-oxides with boron reagents, as developed by Bertozzi; b) the reaction between cyclopropenones and functionalised phosphines; c) 2*H*-azirines that form after ring opening nitrile ylides, and react spontaneously with alkene dipolarophiles to produce stable δ^1 -pyrrolines; and d) the reaction between strained cycloalkynes and sulfenic acids [68,141–144]. On the other hand, the 'old' established oxime ligation reaction [33], between an aldehyde and oxy-amine, is also still a powerful ally for bioconjugation reactions in MOE, especially in view of the recent finding that oximes are formed faster in water at neutral pH at -20°C [145]. One of the fastest known click reactions at the moment is the Strain-Promoted Oxidation-Controlled Cyclooctyne–1,2-Quinone Cycloaddition (SPOCQ) reaction, and its speed might offer chances with respect to competing biological processes and allow specific labelling [40]. The compatibility of these reactions in polar media suggest future possibilities for their use in metabolic oligosaccharide engineering.

The CuAAC and SPAAC are still by far the most popular click reaction currently used for MOE. Many studies have focussed on the improvement of the reactivity of the agents by changing the characters of the alkyne moiety. Such changes involve the ring strain in the cyclic molecule (SPAAC reagents), the lipophilicity of SPAAC reagents (introduction of polar groups), CuAAC-accelerating ligands, and the use of iodo-alkynes [24]. Interestingly, changing the electronic character of the azide was not investigated until recently. One method uses sulfonyl azides as reactive precursor for a variety of applications [24]. In another method, van Delft and co-workers have shown the potential of difluoro azido glycans to improve the speed of SPAAC. They synthesised a difluoro azido acetic acid precursor, which can be installed via peptide chemistry to glucosamine, galactosamine, or mannosamine glycans [146]. This reactive probe has not been used for MOE yet.

Most of the methods described for the labelling of glycans can also be applied to other biomolecules such as lipids and proteins. A complete overview of the possibilities and achievements in this field is highlighted in other reviews [40,147,148].

In the past 15 years many different mammalian cell lines have been successfully used in metabolic oligosaccharide engineering studies. Furthermore, different organisms like zebrafish, *C. elegans*, and bacteria have also been studied. A promising new target for the study of glycans with MOE are plants. At this point, only a few studies have been published that report the use of MOE in plants [65,78,107,108,149,156]. These studies alone have already provided valuable insights in the spatiotemporal dynamics of plant glycans, but many questions in plant glycobiology are still unanswered.

Metabolic oligosaccharide engineering uses the glycan salvage pathway of cells. As a consequence labelled glycans end up in all biomolecules that contain these glycans, e.g. *N*- and *O*-glycans on proteins and glycolipids [150]. This can be a drawback, as it is difficult to distinguish exactly where the incorporated glycans end up and what the ratio of incorporation is across the various glycoconjugates. To further advance the application of MOE and the information that can be obtained from its use, methods should be developed that either allow for more selective MOE or accurate identification and quantification of the various labelled glycan populations. One method that show promise in this area is the combination of fluorescence resonance energy transfer (FRET) and MOE which allows for intracellular detection of glycosylation of a protein of interest [151–155].

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