FEATURE ARTICLE

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Chemoselective ligation in glycochemistry

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This feature article describes chemoselective techniques for the assembly of neoglycopeptides and oligosaccharide mimics. Chemoselective ligation, allowing the use of aqueous environments and non-protected substrates, provides rapid access to complex glycoconjugates. The role of these molecules in recognition, signal transduction pathways and other events of fundamental biomedical significance is an object of study in the emerging field of chemical glycomics.

The specific molecular recognition by protein receptors of oligosaccharides covalently linked to lipids and proteins at cell surfaces play a key role in a number of physiological and pathological events such as cell adhesion, inflammation, metastasis, and embryonic development.[1] A major challenge in cell biology is to decipher the sugar code, that is to define the interactions between cell-coating sugars and proteins and work out how they recognize each other. Advancement in the understanding of these molecular events will provide in the near future new molecular targets for drug development as well as fundamental answers in the area of functional genomics.

From a pharmaceutical point of view, natural saccharides are not good therapeutic agents because they are rapidly degraded by enzymes and are unstable in the strongly acidic gastric environment. In addition, polysaccharides are usually difficult to synthesize by conventional chemical techniques, though the growing availability of glycosidases and glycosyl-transferases has made enzymatic approaches more feasible in recent years.[2] New methodologies and technologies have also decreased the difficulty of chemical synthesis.[3] For these reasons, it is frequently desirable to design compounds that mimic carbohydrates or glycoconjugates associated with important biological events, and that can be prepared in a simpler and more efficient way. Moreover, carbohydrate mimics present advantages over their parent structures as therapeutic agents. They can be designed so that they are more stable towards degradative enzymes, have improved bioavailability and reduced clearance rates and possibly have a higher affinity and selectivity for their cognate receptors.

In this feature article, the development of chemoselective methods for the synthesis of glycopeptide analogs (neoglycopeptides) and oligosaccharide mimics is summarized.

Chemoselective assembly of neoglycopeptides

A variety of methods have been developed for the synthesis of natural *O*- and *N*-linked glycopeptides and glycoproteins: sugars are generally introduced into peptides during solid phase synthesis (SPPS) by means of glycosyl amino acid building blocks.[4] This "linear" synthetic approach is generally laborious, requires extensive use of protecting groups and allows the preparation of one glycoform per synthesis. The alternative "convergent" approach is based either on the enzymatic elongation of the oligosaccharide chain of a pre-synthesized glycopeptide or glycosyl amino acid,[5] or on the conjugation of a fully elaborated, complex saccharide to short synthetic peptides.[6]

Chemoselective ligation, first described by protein chemists as the coupling of two mutually and uniquely reactive groups in an aqueous environment, also provides access to complex neoglycoconjugates in an elegant and convergent way. In this technique, two uniquely reactive couples of functional groups (generally an electrophile and a nucleophile) are introduced into the peptide and sugar fragments giving rise to selective covalent bond formation even in the presence of an array of other unprotected functionalities (Fig. 1).

The chemoselective techniques so far developed for neoglycoconjugate synthesis fall into two broad categories: the first characterized by the reaction of a carbonyl group (ketone or aldehyde) with strong nucleophiles, and the second by the addition

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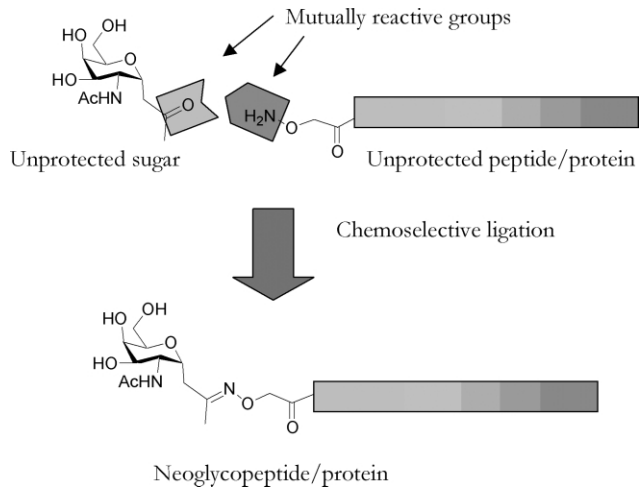


Fig. 1 Schematic representation of neoglycopeptide assembly by chemoselective ligation.

of sulfur nucleophiles to a variety of electrophiles.[7] The absence of aldehydes and ketones on the side chains of the naturally occurring amino acids inspired a set of chemoselective reactions based on the condensation of the anomeric carbon of a reducing sugar (aldehyde group in the cyclic hemiacetal form) with a variety of non-natural nucleophile groups introduced into the peptide chain. Mutter and coworkers first demonstrated that the somatostatin analog RC-160 bearing an oxyamino group (compound **1**, Fig. 2), can be

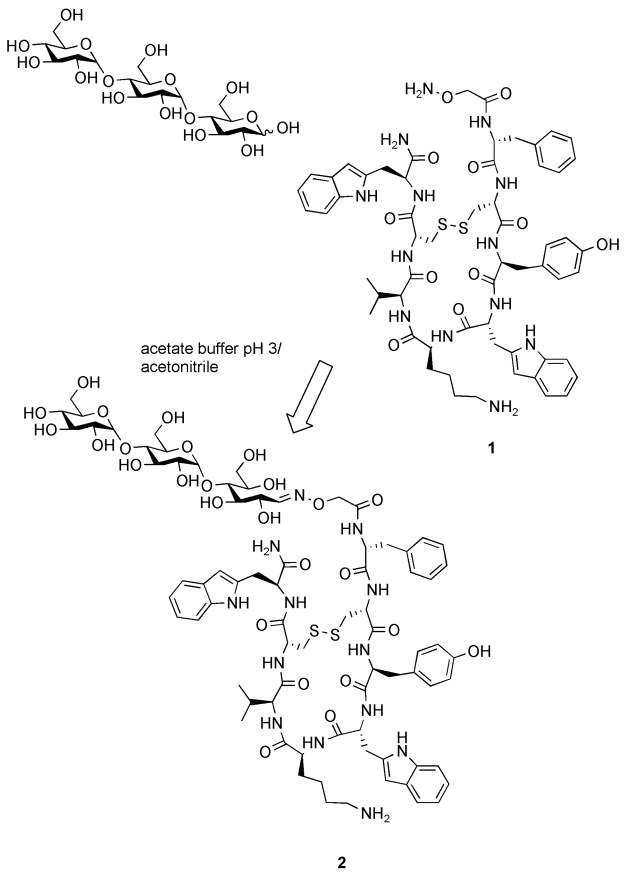


Fig. 2 Chemoselective ligation between free maltotriose and amino-oxy-acetylated somatostatin analog RC-160 (**1**) with formation of neoglycopeptide **2**.

glycosylated in a chemoselective way after SPPS with a variety of unprotected mono- and oligosaccharides, obtaining oxime-linked neoglycopeptides with increased bioavailability (compound **2**).[8] The same ligation strategy was employed to glycosylate with lactose the *N*-terminal segment of P-selectin glycoprotein ligand 1 (PSGL-1), in order to mimic the sialyl Lewis x glycosylated threonine that is fundamental for efficient adhesion to P-selectin. The neoglycopeptide was further decorated by the reaction of a cysteine side chain with iodoacetylated biotin (Fig. 3).[9] The main

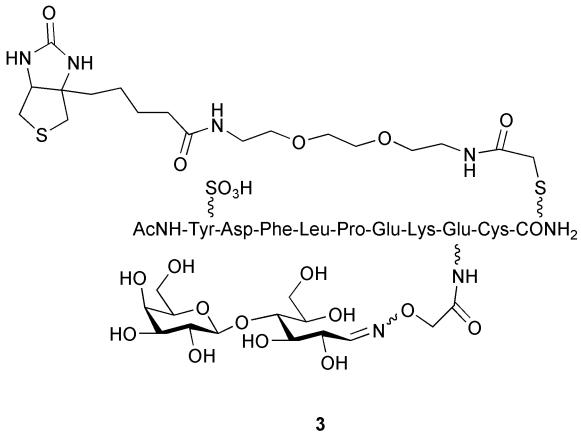


Fig. 3 Mimic of the *N*-terminal segment of PSGL-1: Thr[57](sialyl Lewis x) has been replaced by an oxime-linked lactosyl amino-oxy-acetyl lysine.

drawback of these methods is the formation of the oxime linear form, quite different from the natural pyranose form, of the first attached sugar. This problem was addressed with functionalization of the peptide moiety with an *N*-methylated oxyamino group. After condensation of peptide with unprotected mono- and oligosaccharides, the cyclic pyranose form of the first attached sugar was maintained in the obtained neoglycopeptide (Fig. 4).[10] The glycoside linkage of the first attached sugar was formed exclusively in the β-configuration in the case of D-glucose and *N*-acetylglucosamine, allowing the preparation of mimetics structurally similar to their parent *N*-linked glycopeptides.[11]

The mechanism we propose to explain the diastereoselection in the coupling reaction consists of the formation of an intermediate oxy-iminium ion by reaction of an *N,O*-disubstituted hydroxylamine group with the aldehyde of the open-chain sugar (Scheme 1).[10] The preference for the β-anomer can be explained in terms of stabilization of the β conformation of the oxy-iminium intermediate through a reverse anomeric effect that is particularly relevant when a positive charged nitrogen atom is linked to the anomeric position.[12]

Chemoselective ligation with oxime bond formation proved to be an efficient and versatile tool for the preparation of biologically active molecules presenting several carbohydrate recognition domains. Multivalency is generally required to have high affinity in the sugar–receptor interaction in accordance with cluster effects.[13] Dendritic cell (DC)-targeted synthetic vaccines that present one or two copies of a sugar epitope have been synthesized with this method. In order to induce a DC-mediated immunological response against the tumour-associated antigen T_n antigen (GalNAc-Ser/Thr), one or two copies of the C-glycoside analog of *N*-acetyl-D-galactosamine (GalNAc) were conjugated by means of oxime bonds to the immunogenic peptide OVA$^{(327–339)}$, obtaining compounds **5** and **6** (Fig. 5).[14] In these molecules, the C-glycoside mimics the α-anomeric configuration of the natural T_n epitope. It was demonstrated *in vitro* that compound **7** is more efficiently internalized by DCs and consequently presented to T cells than its analog **6** containing only one sugar unit, indicating that the C-glycoside epitope GalNAc either acts as an internalization agent by binding specific DCs surface sugar receptors, or promotes receptor clustering that is essential for DC activation.[15] *In vivo* assays on C57BL/6 mice indicated that specific T and B cell responses against the tumour were induced by immunization with molecule **7** and antibody production was higher than that obtained using **6**.

An array of DC-targeted synthetic vaccines was generated by chemoselective ligation, presenting different peptide epitopes

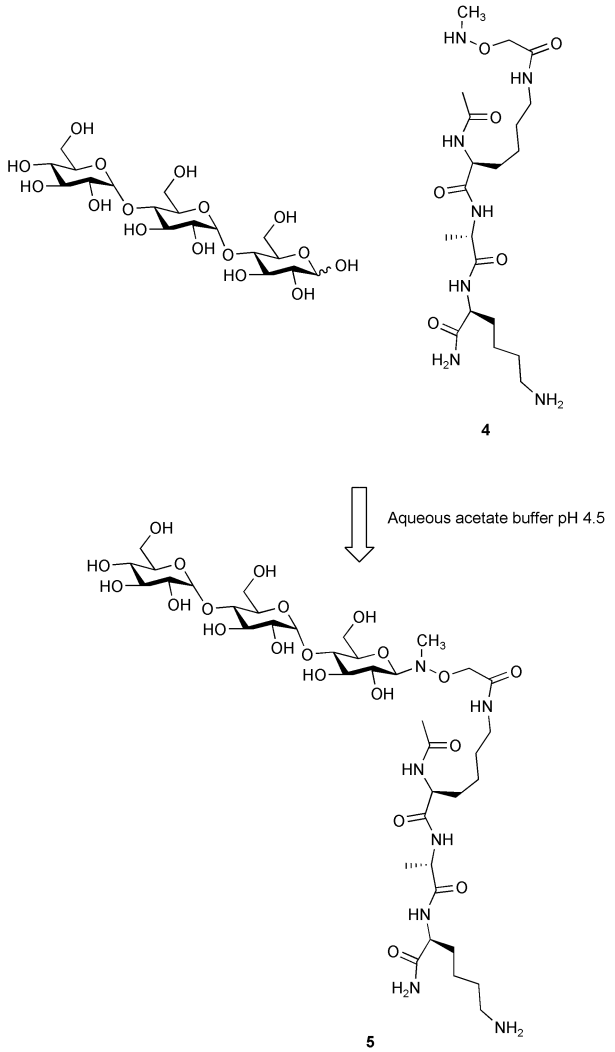
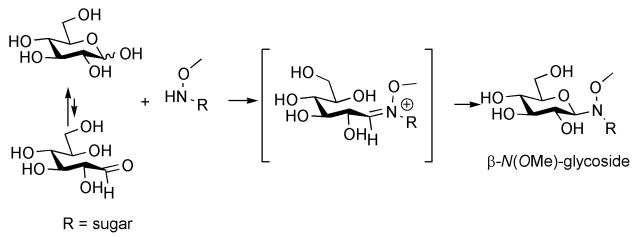


Fig. 4 Chemoselective ligation of free maltotriose with methylamino-oxy peptide **4**.



Scheme 1 Mechanism of reaction between glucose and *O*,*N*-disubstituted hydroxylamines.

linked to a poly-mannose cluster (Fig. 6).[16] This synthetic approach, based on the combination of orthogonal hydrazone and thioether methods, is one elegant example of double ligation strategy, one reaction being used for sugar–peptide, the other for peptide–peptide conjugation.

In a similar chemoselective approach, the keto group of the unnatural amino acid (2*S*)-aminolevulinic acid was used as an electrophile for chemoselective reaction with sugars presenting anomeric substituents with nucleophile residues, thus obtaining analogs of the *O*-linked glycopeptide drosocin (Fig. 7).[17] It was also proved that the oxime-linked neoglycopeptide possessed activity as an inhibitor of bacterial growth similar to that of the native glycopeptide.[18] The chemoselective strategy was extended to the formation of sugar–sugar linkages in the synthesis of drosocin analogs: the glycosyl amino acid Thr/Ser(Ac_3-D-GalNAc) was incorporated into the peptide by solid phase synthesis, then the

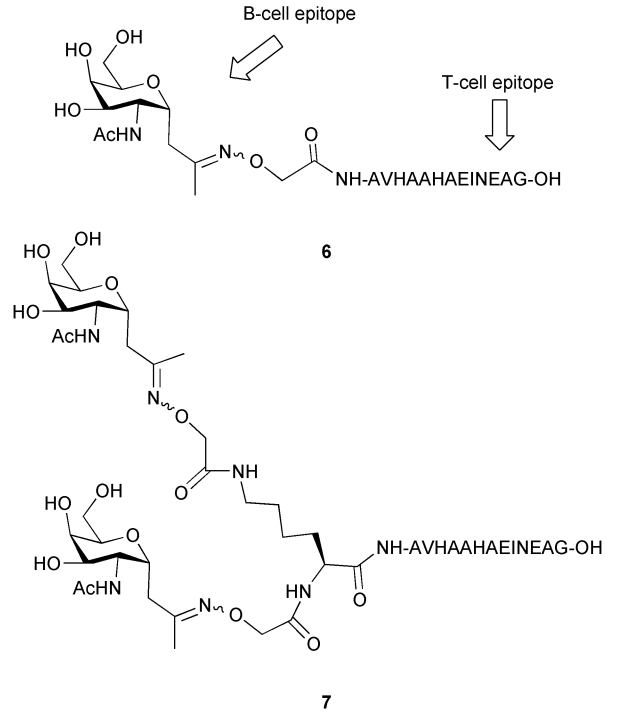


Fig. 5 Synthetic vaccine assembled by chemoselective synthesis: one and two copies of the C-saccharide analogue of the T_n epitope were conjugated by means of oxime bonds to the immunogenic peptide $OVA^{(327–339)}$.

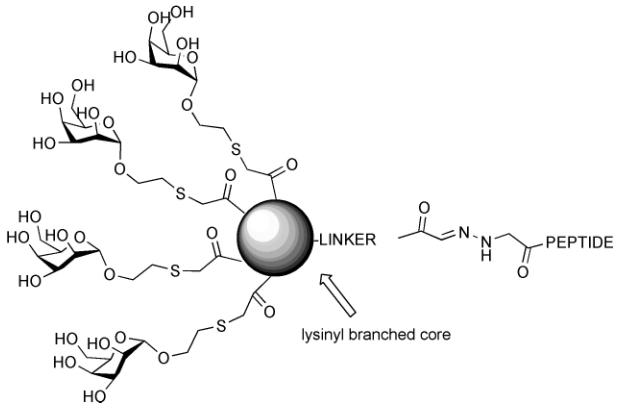


Fig. 6 Clustered mannoside–antigen conjugate obtained by one-pot orthogonal chemoselective ligation.

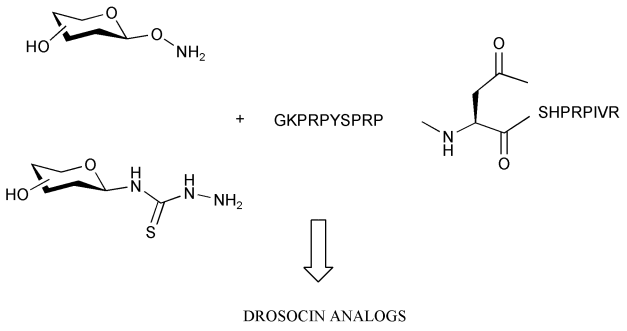


Fig. 7 β-oxyamino and β-thiohydrazide glycosides reacted selectively with the peptide bearing a ketone group giving drosocin analogues.

sugar was deprotected and selectively oxidized to the corresponding C-6 aldehyde by treatment with the enzyme galactose oxidase. Finally, the glycopeptide was conjugated through reactions with mono- and disaccharides bearing amino-oxy groups at the anomeric position (Fig. 8).[19] Chemoselectively glycosylated analogs were found to be 3- to 4- fold more potent in blocking bacterial growth than unglycosylated drosocin.

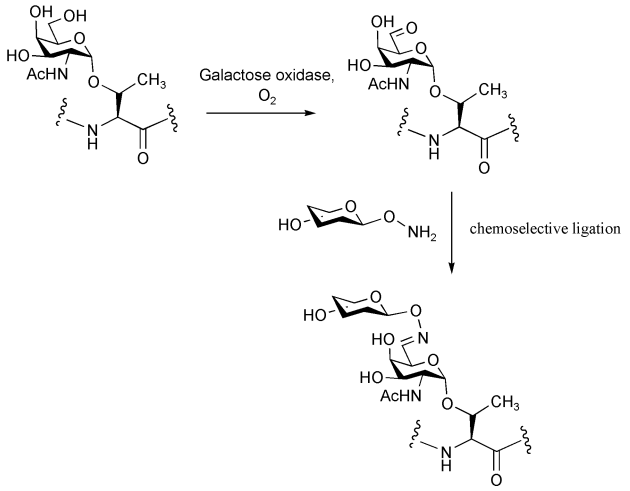
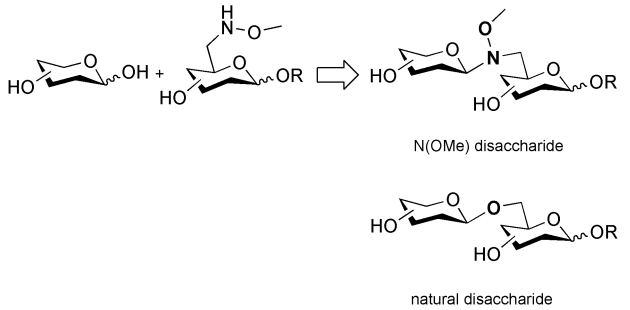


Fig. 8 Synthesis of oxime-linked drosocin analogues by chemoselective extension of the saccharide part.

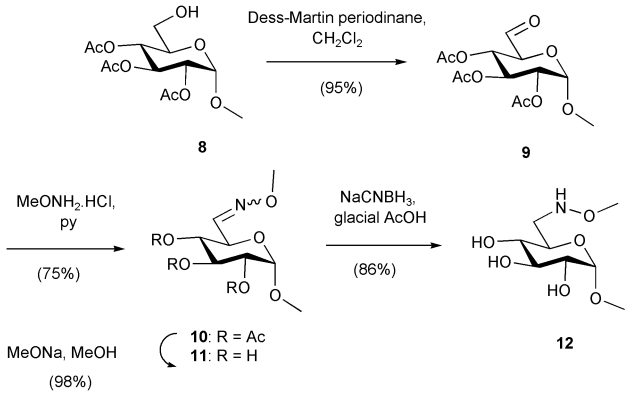
Chemoselective synthesis of oligosaccharide analogs

Inspired by this last example, we aimed to extend chemoselective ligation to the assembly of oligosaccharide mimetics. The synthesis of oligosaccharides and their analogs is laborious and challenging, requiring an extensive use of orthogonal protecting groups and strictly anhydrous conditions in the glycosylation reaction; for these reasons it is still far from routine, both in solution[20] and in the solid phase.[21] The use of chemoselective strategies could alleviate some of these challenges: an anhydrous environment is not required, protecting groups and activating agents are not necessary. These are ideally optimal conditions for the development of solid phase synthesis and subsequent automation. In analogy with sugar–peptide ligation, reacting 6-deoxy-6-methoxyamino glycosides with reducing sugars, isosteric mimics of (1–6)-linked oligosaccharides could be obtained (Scheme 2). To prove this hypothesis, monosaccharide **12**, bearing a methoxyamino group at C-6, was prepared according to the synthetic route depicted in Scheme 3.[22] Compound **12** was then reacted with D-glucose, D-galactose and *N*-acetylglucosamine affording, respectively, disaccharide analogs **13**, **14** and **15** (Scheme 4). The coupling reactions were carried out in aqueous media and proceeded with good yields and stereoselectivity. In the case of D-glucose and *N*-acetylglucosamine only β(1–6) disaccharides were formed.

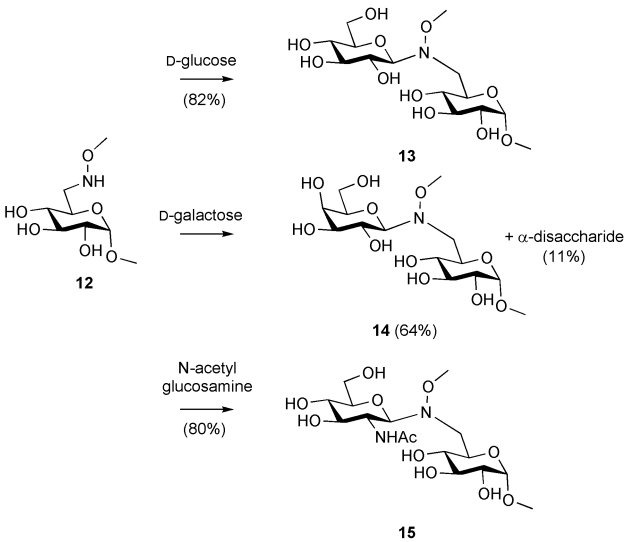


Scheme 2 β(1–6)-linked *N*(*O*Me) glycosides as isosteric mimics of natural β(1–6) oligosaccharides.

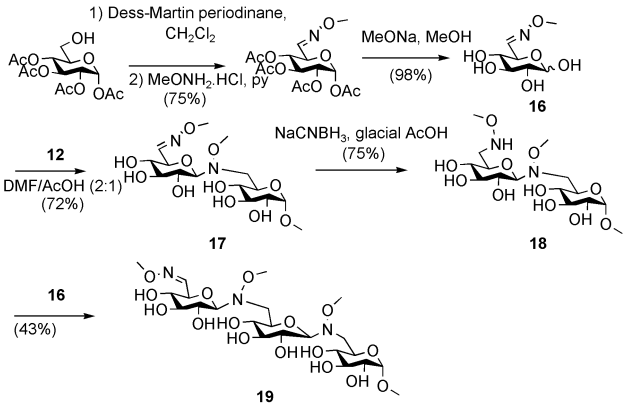
We investigated the possibility of using the chemoselective approach iteratively for the synthesis of linear oligosaccharide mimetics linked through a β(1–6)-methoxyamino bond. To accomplish this task, we synthesized monosaccharide **16** which presents the two complementary functionalities required for chemoselective ligation, the aldehyde and the aminomethoxy group, this last being masked as *O*-methyloxime. Compound **16** was reacted with **12** to afford disaccharide **17** (Scheme 5). Upon reduction of the oxime group of **17** with $NaCNBH_3$, disaccharide **18** was obtained, the methoxyamino group of which was used for a further chemoselective coupling with **16** affording trisaccharide mimic **19**. Further elongation is possible upon reduction followed by chemoselective ligation.



Scheme 3 Synthesis of 6-deoxy-6-methoxyamino glucoside **12**.



Scheme 4 Chemoselective assembly of *N*(*O*Me) disaccharides.



Scheme 5 Iterative procedure for the synthesis of linear β(1–6)*N*(*O*Me) oligosaccharides.

Future directions

The chemoselective ligation techniques developed so far allow the efficient formation of sugar–sugar and sugar–peptide bonds with non-natural architectures. Our current work on oligosaccharide analogs is focussed on the extension of chemoselective chemistry to the synthesis of branched structures with increased diversity by

varying the regio- and stereochemistry of the attachment of monosaccharide units.

For neoglycoprotein synthesis, the major limitation lies in the need to insert artificial amino acids at the desired site of conjugation. This can be accomplished by residue incorporation during SPPS, but the size of a protein normally exceeds the possibilities of SPPS that is currently executed in a routine fashion for peptides of around 60 amino acids or less. The development of highly productive techniques for *in vivo* incorporation of unnatural amino acids into proteins[23] would provide an extremely powerful and general method to install desired reacting groups at precise sites for chemoselective glycosylation.

Another important future target would be the extension of the ligation chemistry to the synthesis of glycolipid analogs; even though the extreme lipophilicity of these molecules discourages the use of aqueous media for their preparation, convergent strategies could greatly enhance the production of diversity in drug development processes.

Acknowledgement

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Notes and references

1 (*a*) Y. C. Lee and R. T. Lee, *Biomol. Sci.*, 1997, **3**, 221; (*b*) *Glycosciences: Status and perspectives*, ed. H.-J. Gabius and S. Gabius, Chapman & Hall, London, UK, 1997; (*c*) *Essentials of glycobiology*, ed. A. Varki, R. Cummings, J. Esko, H. Freeze, G. Hart and J. Marth, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1999.

2 (*a*) For reviews, see: C.-H. Wong and G. M. Whitesides, *Enzymes in Synthetic Organic Chemistry*, Pergamon, New York, 1994; (*b*) X. Chen, K. Przemyslaw and G. Peng, *Curr. Opin. Drug Disc. Dev.*, 2000, **3**, 756.

3 For reviews, see: (*a*) S. Danishefsky and M. T. Bilodeau, *Angew. Chem., Int. Ed. Engl.*, 1996, **35**, 1380; (*b*) K.-H. Jung, M. Müller and R. R. Schmidt, *Chem. Rev.*, 2000, **100**, 4423; (*c*) P. H. Seeberger and W.-C. Haase, *Chem. Rev.*, 2000, **100**, 4349.

4 O. Seitz, *ChemBioChem*, 2000, **1**, 214.

5 (*a*) O. Blixt, K. Allin, L. Pereira, A. Datta and J. C. Paulson, *J. Am. Chem. Soc.*, 2002, **124**, 5739; (*b*) D. Ramos, P. Rollin and W. Klaffke, *Angew. Chem., Int. Ed.*, 2000, **39**, 396.

6 E. Meinjohanns, M. Meldal, H. Paulsen, R. A. Dwek and K. Bock, *J. Chem. Soc., Perkin Trans. 1*, 1998, 549.

7 For a review see: H. C. Hang and C. R. Bertozzi, *Acc. Chem. Res.*, 2001, **34**, 727.

8 S. E. Cervigni, P. Dumy and M. Mutter, *Angew. Chem., Int. Ed. Engl.*, 1996, **35**, 1230.

9 P. Durieux, J. Fernandez-Carneado and G. Tuchscherer, *Tetrahedron Lett.*, 2001, **42**, 2297.

10 F. Peri, P. Dumy and M. Mutter, *Tetrahedron*, 1998, **54**, 12269.

11 M. R. Carrasco, M. J. Nguyen, D. R. Burnell, M. D. MacLaren and S. M. Hengel, *Tetrahedron Lett.*, 2002, **43**, 5727.

12 (*a*) R. U. Lemieux and A. R. Morgan, *Can. J. Chem.*, 1965, **43**, 2205; (*b*) C. L. Perrin and K. B. Armstrong, *J. Am. Chem. Soc.*, 1993, **115**, 6825.

13 (*a*) Y. C. Lee and R. T. Lee, *Acc. Chem. Res.*, 1995, **28**, 321; (*b*) M. Mammen, S.-K. Choi and G. M. Whitesides, *Angew. Chem.*, 1998, **110**, 2908; M. Mammen, S.-K. Choi and G. M. Whitesides, *Angew. Chem., Int. Ed.*, 1998, **37**, 2754.

14 F. Peri, L. Cipolla, M. Rescigno, B. La Ferla and F. Nicotra, *Bioconj. Chem.*, 2001, **12**, 325.

15 L. Cipolla, M. Rescigno, A. Leone, F. Peri, B. La Ferla and F. Nicotra, *Bioorg. Med. Chem.*, 2002, **10**, 1639.

16 (*a*) C. Grandjean, C. Rommens, H. Gras-Masse and O. Melnyk, *Angew. Chem., Int. Ed.*, 2000, **39**, 1068; (*b*) C. Grandjean, H. Gras-Masse and O. Melnyk, *Chem. Eur. J.*, 2001, **7**, 230.

17 E. C. Rodriguez, L. A. Marcaurelle and C. R. Bertozzi, *J. Org. Chem.*, 1998, **63**, 7134.

18 L. A. Marcaurelle, E. C. Rodriguez and C. R. Bertozzi, *Tetrahedron Lett.*, 1998, **39**, 8417.

19 E. C. Rodrigues, K. A. Winans, D. S. King and C. R. Bertozzi, *J. Am. Chem. Soc.*, 1997, **119**, 9905.

20 K. H. Jung, M. Müller and R. R. Schmidt, *Chem. Rev.*, 2000, **100**, 4423.

21 P. H. Seeberger and W.-C. Haase, *Chem. Rev.*, 2000, **100**, 4349.

22 F. Peri, A. Deutman, B. La Ferla and F. Nicotra, *Chem. Commun.*, 2002, 1505.

23 L. Wang, A. Brock, B. Herberich and P. G. Schultz, *Science*, 2001, **292**, 498.