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Beilstein Journal of Organic Chemistry

Synthesis in the glycosciences

Thisbe K. Lindhorst

Editorial

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All cells are coated in carbohydrates and glycoconjugates. Today, after decades where sugars were regarded mainly as a means of energy storage or simply as molecular material, it is now known that carbohydrates are deeply involved in cellular communication. This awareness of the biological importance of carbohydrates has led to glycosciences becoming an intriguing and fascinating field of interdisciplinary research. However, the structural diversity found in the carbohydrate regime is unparalleled [1] which makes the biological study of carbohydrate recognition and understanding the processes involved rather complicated. In addition, the multivalent nature of most carbohydrate ligands constitutes a special challenge in glycoscience.

Since the isolation of those complex glycans which are active in cellular communication is problematic, oligosaccharide synthesis is an important area of research. Moreover, what Professor Hans Paulsen, one of the greatest exponents of glycoside synthesis, observed in 1982 [2] still holds true today: "*Although we have now learned to synthesize oligosaccharides, it should be emphasized that each oligosaccharide synthesis remains an independent problem, whose resolution requires considerable systematic research and a good deal of know-how. There are no universal reaction conditions for oligosaccharide syntheses*".

It is therefore not surprising that the majority of contributions collected in this Thematic Series deal with methods, both chemical and enzymatic, for oligosaccharide synthesis.

One approach to deal with the problem of glycoside synthesis is the preparation of so-called glycomimetics. This is a strategy where the glycosidic linkage is substituted by another type of ligation, or where glycosidations are limited to rather simple reactions whilst complexity and multivalency of a complicated target structure are introduced by alternative methods. Examples of such a versatile approach are also presented in this series. Furthermore, carbohydrate chemistry is presented in the context of chemical biology together with organic chemistry making use of known chemical reactions as well as the stereochemical advantages of saccharides to construct novel molecules with unique properties.

It has been a joy to direct this stimulating collection of research from the many areas of the exciting field of the glycosciences. I would like to thank all authors for their excellent contributions. Enjoy reading them!

Thisbe Lindhorst

Kiel, February 2010

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Beilstein Journal of Organic Chemistry

Convergent syntheses of Le^X analogues

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Full Research Paper

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Abstract

The synthesis of three Le^X derivatives from one common protected trisaccharide is reported. These analogues will be used respectively for competitive binding experiments, conjugation to carrier proteins and immobilization on gold. An *N*-acetylglucosamine monosaccharide acceptor was first glycosylated at O-4 with a galactosyl imidate. This coupling was performed at 40 °C under excess of $BF_3{\cdot}OEt_2$ activation and proceeded best if the acceptor carried a 6-chlorohexyl rather than a 6-azidohexyl aglycon. The 6-chlorohexyl disaccharide was then converted to an acceptor and submitted to fucosylation yielding the corresponding protected 6-chlorohexyl Le^X trisaccharide. This protected trisaccharide was used as a precursor to the 6-azidohexyl, 6-acetylthiohexyl and 6-benzylthiohexyl trisaccharide analogues which were obtained in excellent yields (70–95%). In turn, we describe the deprotection of these intermediates in one single step using dissolving metal conditions. Under these conditions, the 6-chlorohexyl and 6-azidohexyl intermediates led respectively to the *n*-hexyl and 6-aminohexyl trisaccharide targets. Unexpectedly, the 6-acetylthiohexyl analogue underwent desulfurization and gave the *n*-hexyl glycoside product, whereas the 6-benzylthiohexyl analogue gave the desired disulfide trisaccharide dimer. This study constitutes a particularly efficient and convergent preparation of these three Le^X analogues.

Introduction

Our group is involved in the design of new anti-cancer vaccines based on the Tumor Associated Carbohydrate Antigen (TACA) dimeric Le^X (dimLe^X) [1-6]. This tumor specific antigen consists of a hexasaccharide that displays the Le^X trisaccharide antigen linked to O-3″ of the galactose residue of another Le^X trisaccharide. Since it was first characterized [7,8], the Le^X antigenic determinant, β-D-Gal*p*(1,4)[α-LFuc*p*(1,3)]-D-GlcNAc*p*, has been found on numerous cells and tissues such as kidney tubules, gastrointestinal epithelial cells, and cells of the spleen and brain [9-11]. Thus, there are numerous reports in the literature that deal with the chemical [12-36] or chemoenzymatic [37,38] preparation of Le^X analogues as well as that of Le^X intermediate building blocks to be further converted into the Sialyl Le^X tetrasaccharide. The chemical syntheses usually follow one of three synthetic schemes: 1. a stepwise approach involving the successive galactosylation then fucosylation of a glucosamine acceptor [12-28]; 2. a stepwise approach in which the sequence of glycosylation of the glucosamine acceptor is reversed, i.e. the fucosylation is followed by the galactosylation [28-34]; 3. a block approach in which a lactosamine derivative

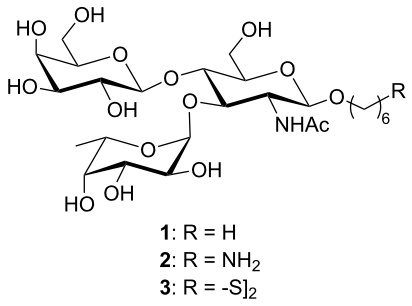


Figure 1: Structure of Le^x analogues **1**–**3**.

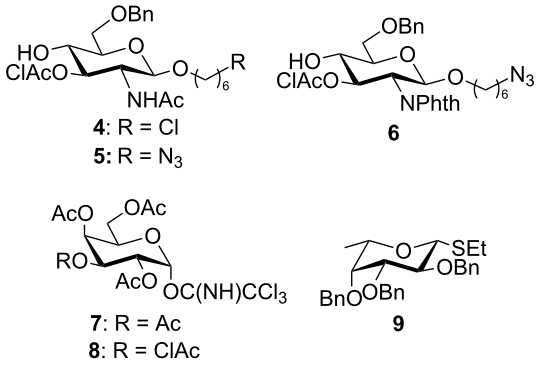


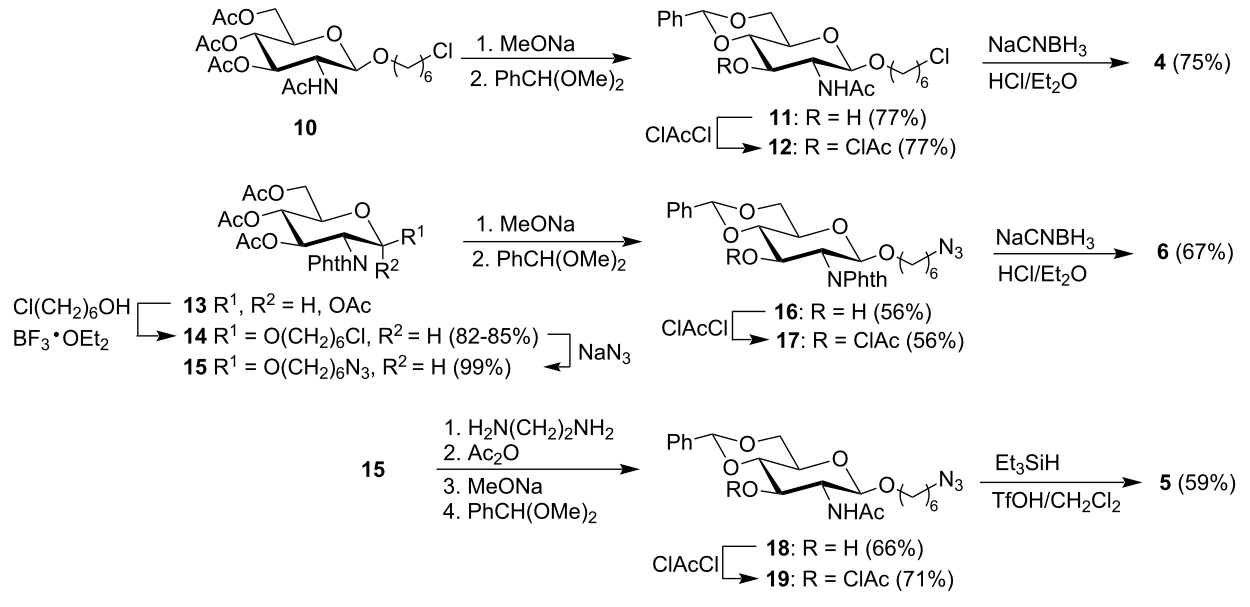
Figure 2: Monosaccharide glycosyl acceptors (**4**–**6**) and donors (**7**–**9**) used in this study.

prepared from lactose is subjected to fucosylation at O-3 [35,36]. Whereas these reports usually describe the preparation of one compound to be used in a specific experiment, we describe here the convergent synthesis of the three Le^x derivatives **1**–**3** (Figure 1) from one common protected trisaccharide intermediate. These three Le^x analogues (**1**–**3**) will be used respectively for competitive binding experiments (**1**), conjugation to carrier proteins (**2**) and immobilization to a gold plate (**3**).

Results and Discussion

Our synthetic approach to prepare these Le^x derivatives began with the galactosylation at O-4 of glycosyl acceptor **4** with the known [39-41] galactosyl donor **7** followed by deprotection at O-3 of the glucosamine residue and fucosylation of the resulting disaccharide with the known [42] ethylthioglycoside **9**. Since in addition to the Le^x trisaccharide we are also interested in preparing fragments of the dimLe^x antigen, we examined the glycosylation at O-4 of glucosamine glycosyl acceptors with galactosyl donor **8**, which is chloroacetylated rather than acetylated at O-3. Finally, we also investigated the reactivity towards glycosylation of the *N*-acetylated and phthalimido acceptors **5** and **6**, respectively, that both carry a 6-azidohexyl aglycon (Figure 2).

Synthesis of monosaccharide building blocks. The 6-chlorohexyl acceptor **4** was prepared in four steps from the known [43] chlorohexyl glucoside **10** (Scheme 1). Thus, peracetate **10** was deacetylated (NaOMe/MeOH) and converted to the benzylidene acetal **11** by reaction with benzaldehyde dimethyl acetal under camphorsulfonic acid (CSA) catalysis. Chloroacetylation of alcohol **11** gave the intermediate **12** which was converted to acceptor **4** via the reductive opening of the



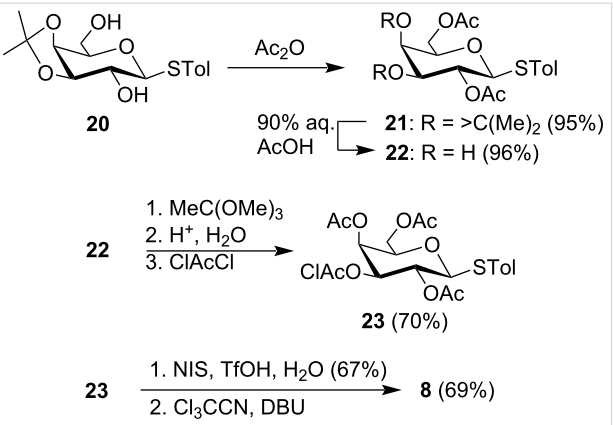
Scheme 1: Synthesis of monosaccharide glycosyl acceptors **4**–**6**.

benzylidene acetal using $NaCNBH_3$ and $HCl{\cdot}Et_2O$ in anhydrous THF at 0 °C.

Both 6-azidohexyl acceptors **6** and **5** were prepared from the anomeric mixture of the known tetraacetate **13** [44]. Thus, tetraacetate **13** was reacted with 6-chlorohexanol (4 equiv) in the presence of $BF_3{\cdot}OEt_2$ (5 equiv). To promote coupling, the reaction mixture was either stirred for 1 h at 50 °C in an oil bath (Supporting Information File 1, Method A) or submitted to microwave irradiation for 5 min at 50 °C (Supporting Information File 1, Method B). After acetylation of the excess chlorohexanol to ease its removal, pure glycoside **14** was isolated in excellent yield whether method A or B was followed. Thus, these syntheses of glycoside **14** constitute efficient alternatives to that reported by Nitz et al. in which the starting material was the corresponding anomeric bromide [45]. Nucleophilic displacement of the chlorine atom in glycoside **14** (NaN_3, DMF, 80 °C) gave the known [46] 6-azidohexyl glycoside **15** quantitatively. Zemplén deacetylation of triacetate **15** followed by conversion of the triol to the 4,6-benzylidene acetal (**16**) and then chloroacetylation at O-3 gave intermediate **17** that was submitted to reductive opening of the benzylidene group ($NaCNBH_3$, $HCl{\cdot}Et_2O$) to yield acceptor **6**.

The triacetate **15** was also converted in seven steps to acceptor **5**. The phthalimido group was first removed (ethylenediamine, EtOH) and the free amine acetylated. Zemplén deacetylation was followed by conversion of the triol to the 4,6-benzylidene acetal **18** which was chloroacetylated at O-3 to give the fully protected intermediate **19**. Finally, the benzylidene acetal in compound **19** was reductively opened with Et_3SiH and TfOH in CH_2Cl_2 at −30 °C to give acceptor **5**.

The trichloroacetimidate glycosyl donor **8** was prepared from the *p*-thiotolyl glycoside **20** [47] (Scheme 2). Diol **20** was first acetylated to the diacetate **21** which was then treated with 90% AcOH at 70 °C to remove the isopropylidene group affording diol **22**. The diol **22** was selectively acetylated at O-4 by converting it to the corresponding cyclic methylorthoacetate and opening the orthoacetate in situ by adding water to the reaction mixture. The resulting triacetate was chloroacetylated at O-3 and the resulting fully protected thioglycoside **23** was converted to the corresponding hemiacetal that was, in turn, treated with trichloroacetonitrile and DBU to give the α-trichloroacetimidate galactosyl donor **8**.

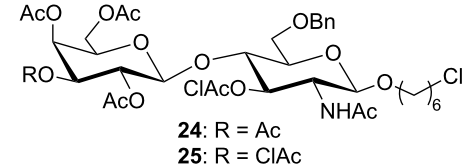


Scheme 2: Synthesis of the galactosyl donor **8**.

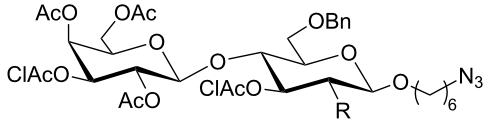
Glycosylation at O-4 of glucosamine acceptors. It is well known that the hydroxyl group at C-4 of *N*-acetylglucosamine is a poor nucleophile and has reduced reactivity towards glycosylation when compared to other acceptors [48-50]. However, we have recently reported the successful O-4 glucosylation of an *N*-acetylglucosamine monosaccharide acceptor using a peracetylated glucopyranose α-trichloroacetimidate donor under activation with 2 equiv of $BF_3{\cdot}OEt_2$ at room temperature [51]. We applied similar conditions: 2 equiv $BF_3{\cdot}OEt_2$, 5 equiv of donor, 1 h at 40 °C for the coupling of donors **7** and **8** with the acceptors **4**-**6** (Table 1). As can be seen in Table 1 the 6-chlorohexyl glycoside acceptor **4** was easily glycosylated with either donors **7** or **8**, affording the desired disaccharides **24** and **25** in about 70% yield for both reactions (entries 1 and 2).

In contrast, the coupling of donor **8** with the 6-azidohexyl glycoside acceptor **5** did not proceed well (entry 3). Monitoring of the reaction by TLC showed degradation of the acceptor, and isolation of the desired disaccharide required both silica gel

Table 1: Glycosylation at O-4 of glucosamine acceptors **4**–**6**[a].



24: R = Ac
25: R = ClAc

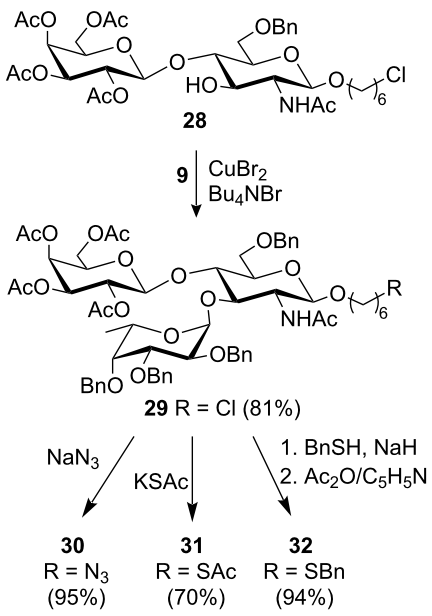


26: R = NHAc
27: R = NPhth

Entry	Donor	Acceptor	Product (%)
1	**7**	**4**	**24** (69%)
2	**8**	**4**	**25** (72%)
3	**8**	**5**	**26** (27%)[b]
4	**8**	**6**	**27** (11%)

[a]Reagents and conditions: $BF_3{\cdot}OEt_2$ (2 equiv), donor (5 equiv), CH_2Cl_2, 40 °C, 1 h.
[b]Contaminated with degraded acceptor.

chromatography and RP-HPLC. Indeed, despite our efforts, and even though its structure was confirmed by NMR and HR-ESI mass spectrometry, disaccharide **26** could not be isolated free of degraded acceptor and/or disaccharide. To further test if the *N*-acetyl group was impacting negatively the glycosylation of acceptor **5**, we attempted to couple trichloroacetimidate **8** with the phthalimido acceptor **6**. However as can be seen in Table 1, entry 4, this glycosylation also gave disappointing results: TLC showed a considerable amount of degraded products and the isolation of the desired disaccharide from the reaction mixture required both silica gel chromatography and RP-HPLC. In this case, the disaccharide **27** could be obtained pure albeit in very low yield. These last two reactions suggest that the presence of the azido group on the hexyl aglycon carried by acceptors **5** and **6** is not compatible with the glycosylation conditions that we have established previously [51] for the glycosylation at O-4 of glucosamine acceptors. The disaccharide **24** was further used in the preparation of the Le^x analogues **1**–**3**.



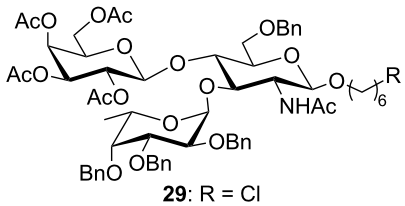
Scheme 3: Convergent synthesis of trisaccharides **29**–**32**.

Preparation of protected Le^x analogues. The chloroacetate in disaccharide **24** was removed with thiourea (C_5H_5N/EtOH, 70 °C) to give the acceptor disaccharide **28** (61%), which was then fucosylated with the thioethyl glycoside **9** under copper (II) bromide–tetrabutylammonium bromide activation (Scheme 3). The desired Le^x trisaccharide **29** was obtained in excellent yield and the α-configuration of the newly formed fucosidic bond was confirmed by ^{1}H NMR ($J_{H\text{-}1',H\text{-}2'}$ = 3.7 Hz). The 6-chlorohexyl trisaccharide glycoside **29** was in turn used as a precursor to the 6-azidohexyl, 6-acetylthiohexyl and 6-thiobenzylhexyl trisaccharides **30**–**32** (Scheme 3).

Thus, nucleophilic displacement of the chloride with sodium azide or potassium thioacetate was carried out in DMF at 80 °C and provided the 6-azidohexyl and 6-acetylthiohexyl trisaccharides **30** and **31**, respectively. The introduction of the azido or thioacetyl groups into trisaccharides **30** and **31** was confirmed by HR-ESI mass spectrometry and by NMR. Indeed, the signals assigned to the methylene CH_2Cl in trisaccharide **29** (^{1}H NMR δ 3.50 ppm, ^{13}C NMR δ 44.9 ppm) were no longer observed in trisaccharides **30** and **31**. The methylene CH_2N_3 in trisaccharide **30** gave signals at 3.20 and 54.3 ppm in the ^{1}H and ^{13}C NMR spectra, respectively, whereas the methylene CH_2SAc in trisaccharide **31** gave signals at 2.81 and around 28.5 ppm, in the ^{1}H and ^{13}C NMR spectra, respectively. In addition, signals corresponding to the thioacetyl group in trisaccharide **31** were identified at 2.29 ppm and 30.6 ppm in the ^{1}H and ^{13}C NMR spectra, respectively. Since, as will be described below, the deprotection of trisaccharide **31** under dissolving metal conditions did not provide the desired trisaccharide **3**, the 6-benzylthiohexyl glycoside **32** was also prepared from the 6-chlorohexyl glycoside **29**. Thus, the chloride **29** was allowed to react for 16 h with excess benzylthiol (15 equiv) and sodium hydride (15 equiv) in DMF at 80 °C. These reaction conditions led to the displacement of the chloride as well as to some deacetylation of the galactose residue. Thus, after acetylation of the crude product, the desired 6-benzylthiohexyl trisaccharide **32** was isolated in excellent yield (Scheme 3). It is important to point out that the 6-chlorohexyl glycoside **29** and the 6-benzylthiohexyl glycoside **32** co-eluted on silica gel and that only a very careful analysis of the NMR data recorded for the product could confirm the absence of unreacted starting material. Indeed, the large excess of benzylthiolate used to displace the chloride in trisaccharide **29** was essential for its complete conversion to the desired 6-benzylthiohexyl glycoside **32**. The structure of trisaccharide **32** was confirmed by HR-ESI MS as well as by NMR. The methylene CH_2SBn gave signals at 2.36 and 31.3 ppm, in the ^{1}H and ^{13}C NMR spectra, respectively whereas the *S*-benzyl group gave additional signals in the aromatic regions as well as signals corresponding to the SCH_2Ph methylene around 3.70 and 36.3 ppm in the ^{1}H and ^{13}C NMR spectra, respectively.

Deprotection of trisaccharides 29–32 under dissolving metal conditions. As reported by Seeberger et al. [52], the removal of *O*- and *S*-benzyl groups as well as that of *O*-acetyl groups can be accomplished in one step and concurrently with the reduction of azido groups to the corresponding amines, using Birch reduction conditions. Thus we embarked on the one step deprotection of trisaccharides **29**–**32** with sodium in ammonia (Table 2).

Table 2: One step deprotection of trisaccharides **29**–**32**[a].



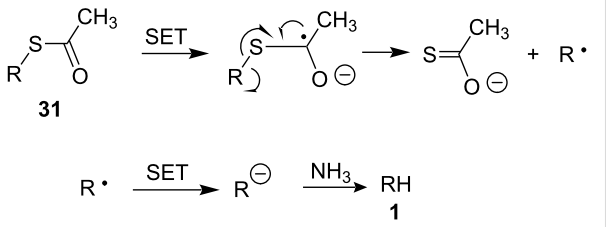
29: R = Cl
30: R = N_3
31: R = SAc
32: R = SBn

Entry	Trisaccharide	Product	Yield (%)
1	**29**	**1**	82
2	**30**	**2**	59
3	**31**	**1**	73
4	**32**	**3**	70

[a]Reagents and conditions: Na/$NH_{3(l)}$, −78 °C, 50 min.

Treatment of trisaccharides **29** and **30** with sodium in liquid ammonia at −78 °C followed by neutralization of the reaction mixtures with AcOH gave the desired trisaccharides **1** and **2** (entries 1 and 2) that were isolated pure after chromatography on a Biogel P2 column eluted with water for compound **1**, and 0.05 M ammonium acetate for the 6-aminohexyl compound **2**. Whereas the structure of trisaccharide **1** was confirmed by HR-ESI mass spectrometry and NMR, the structure of the 6-aminohexyl glycoside **2** was confirmed by comparing its analytical data to that previously reported [31]. To our surprise, treatment of the 6-acetylthiohexyl trisaccharide **31** under Birch reduction conditions did not lead to the desired corresponding thiol or disulfide product but produced the hexyl glycoside **1**. The mechanism proposed to explain this reductive desulfurization is shown in Scheme 4. It involves first a single electron transfer to the thioacetyl group that is followed by the cleavage of the carbon sulfur bond giving a thioacetate salt and an alkyl radical. The alkyl radical is then converted to the corresponding anion by a second electron transfer and the resulting anion is protonated by ammonia giving trisaccharide **1**.

In contrast to the thioacetate **31**, treatment of the 6-benzylthiohexyl glycoside **32** under Birch reduction conditions did not lead to desulfurization and gave the disulfide trisaccharide dimer **3**. Under these reductive conditions, and based on the work by Seeberger et al. [52], we did not expect the formation of the disulfide dimer as the major product but rather that of the corresponding thiol. However, the structure and homogeneity of disulfide dimer **3** was unequivocally confirmed by HR-ESI mass spectrometry and NMR. Interestingly this dimer gave a well resolved ^{1}H NMR spectrum in D_2O that did not support



Scheme 4: Proposed mechanism for the desulfurization of thioacetate **31** under dissolving metal conditions.

the formation of intramolecular Le^x–Le^x interactions such as those reported by de la Fuente and Penadés for a similar analogue [33]. Following published procedures, the disulfide dimer **3** will be reduced immediately prior to its conjugation to proteins [53] or immobilization on gold surface or gold nanoparticules [34].

In conclusion, we have reported above the efficient and convergent preparation of three Le^x derivatives (**1**–**3**) from one common protected trisaccharide (**29**). Our results seem to indicate that glycosylation at O-4 of a glucosamine monosaccharide acceptor under excess $BF_3{\cdot}OEt_2$ activation at 40 °C is compatible with a chlorinated aglycon but not with an aglycon carrying an azido group. We have also established that the fully protected precursors could be deprotected in one single step to give the final target compounds using dissolving metal conditions. However, we observed that a thioacetylated derivative will undergo an undesired reductive desulfurization. This study constitutes a particularly efficient convergent preparation of analogues that can each be used for a specific biochemical application.

Experimental

General Methods: ^{1}H (600.14, 400.13 or 300.13 MHz) and ^{13}C NMR (150.9, 100.6 or 75.5 MHz) spectra were recorded for compounds solubilized in $CDCl_3$ (internal standard, for ^{1}H: residual $CHCl_3$ δ 7.24; for ^{13}C: $CDCl_3$ δ 77.0) or D_2O [external standard 3-(trimethylsilyl)-propionic acid-d_4, sodium salt (TSP) for ^{1}H δ 0.00, for ^{13}C δ 0.00]. Chemical shifts and coupling constants were obtained from a first-order analysis of one-dimensional spectra. Assignments of proton and carbon resonances were based on COSY and ^{13}C–^{1}H heteronuclear correlated experiments. Mass spectra were obtained under electron spray ionization (ESI) on a high resolution mass spectrometer. TLC were performed on precoated aluminum plates with Silica Gel 60 F254 and detected with UV light and/or charred with a solution of 10% H_2SO_4 in EtOH. Compounds were purified by flash chromatography with Silica Gel 60 (230–400 mesh) unless otherwise stated. Solvents were distilled and dried according to

standard procedures [54], and organic solutions were dried over Na_2SO_4 and concentrated under reduced pressure below 40 °C. HPLC purifications were run with HPLC grade solvents.

***n*-Hexyl 2-acetamido-2-deoxy-3-*O*-(α-L-fucopyranosyl)-4-*O*-(β-D-galactopyranosyl)-β-D-glucopyranoside (1).** Trisaccharide **29** (20 mg, 0.017 mmol) or trisaccharide **31** (19 mg, 0.016 mmol) were dissolved in THF (5 mL) and liquid ammonia (20 mL) was condensed into the solution at −78 °C. Na (74 mg, 3.2 mmol) was added and the mixture was stirred for 50 min at −78 °C. The reaction was quenched with MeOH (5 mL) and the ammonia was allowed to evaporate at room temp. The remaining solution was neutralized with acetic acid (203 μL, 3.5 mmol), the solvent was evaporated and the residue was passed twice through a Biogel P2 column (100 × 1 cm) eluted with Milli-Q water to give the trisaccharide **1** (8.5 mg, 82% from **29**; 7.0 mg, 73% from **31**) as a white amorphous powder after lyophilization. $[\alpha]_D$ = −47 (*c* 0.5, MeOH), ^{1}H NMR (400 MHz, D_2O): δ 5.12 (d, 1H, *J* = 4.5 Hz, H-1′); 4.83 (m, 1H, H-5′); 4.53 (d, 1H, *J* = 7.5 Hz, H-1); 4.46 (d, 1H, *J* = 7.5 Hz, H-1″); 4.00 (dd, 1H, *J* = 12.0, 1.0 Hz, H-6a); 3.83–3.95 (m, 7H, H-2, H-3, H-4, H-6b, H-3′, H-4″, OC*H*HCH$_2$); 3.78 (d, 1H, *J* = 3.0 Hz, H-4′); 3.73 (m, 2H, H-6a″, H-6b″); 3.70 (m, 1H, H-2′); 3.66 (m, 1H, H-3″); 3.60 (m, 3H, H-5, H-5″, OCH*H*CH$_2$,); 3.59 (m, 1H, H-2″); 2.03 (s, 3H, CH$_3$CO); 1.55 (m, 2H, OCH$_2$C*H*$_2$); 1.24–1.37 (m, 6H, OCH$_2$CH$_2$C*H*$_2$C*H*$_2$C*H*$_2$); 1.17 (d, 3H, *J* = 6.0 Hz, H-6′); 0.88 (t, 3H, *J* = 6.6 Hz, CH$_2$C*H*$_3$). ^{13}C-NMR (100 MHz, D_2O): 174.17 (C=O); 101.81 (C-1″); 100.91 (C-1); 98.61 (C-1′); 75.32 (C-5); 74.94, 74.88 (C-3, C-5″); 73.36 (C-4); 72.44 (C-3″); 71.89 (C-4′); 71.02 (C-2″); 70.66 (O*C*H$_2$CH$_2$); 69.19 (C-3′); 68.32 (C-4″); 67.68 (C-2′); 66.68 (C-5′); 61.47 (C-6″); 59.76 (C-6); 55.84 (C-2); 30.67, 28.53, 24.77, 22.00 (OCH$_2$*C*H$_2$*C*H$_2$*C*H$_2$*C*H$_2$); 22.23 (*C*H$_3$CO); 15.27 (C-6′); 13.30 (CH$_2$*C*H$_3$). HRESIMS Calcd for $C_{26}H_{48}NO_{15}$ $[M+H]^+$ 614.3024, found 614.3035.

6-Aminohexyl 2-acetamido-2-deoxy-3-*O*-(α-L-fucopyranosyl)-4-*O*-(β-D-galactopyranosyl)-β-D-glucopyranoside (2). The azidotrisaccharide **30** (19 mg, 0.16 mmol) was deprotected in the same conditions as described above for the deprotection of trisaccharide **29**. After work up, the residue was passed twice through a Biogel P2 column (100 × 1 cm) eluted with 0.05 M ammonium acetate and after repeated lyophilization from Milli-Q water (3 × 10 mL) the known [31] trisaccharide **2** (6.5 mg, 59%) was obtained as the acetate salt in the form of a white amorphous powder. $[\alpha]_D$ = −54 (*c* 0.9, H_2O), lit. [31]: $[\alpha]_D$ = −54.3 (*c* 1, H_2O), ^{1}H NMR (400 MHz, D_2O): δ 5.12 (d, 1H, *J* = 4.5 Hz, H-1′); 4.83 (m, 1H, H-5′); 4.53 (d, 1H, *J* = 7.5 Hz, H-1); 4.46 (d, 1H, *J* = 7.5 Hz, H-1″); 4.00 (dd, 1H, *J* = 12.0, 1.0 Hz, H-6a); 3.83–3.95 (m, 7H, H-2, H-3, H-4, H-6b, H-3′, H-4″, OC*H*HCH$_2$); 3.78 (d, 1H, *J* = 3.0 Hz, H-4′); 3.73 (m, 2H, H-6a″, H-6b″); 3.70 (m, 1H, H-2′); 3.66 (m, 1H, H-3″); 3.60 (m, 3H, H-5, H-5″, OCH*H*CH$_2$); 3.59 (m, 1H, H-2″); 2.99 (t, 2H, *J* = 7.0 Hz, C*H*$_2$NH$_2$); 2.03, 2.01 (s, 6H, CH$_3$CO); 1.57, 1.67 (m, 4H, OCH$_2$C*H*$_2$, C*H*$_2$CH$_2$NH$_2$); 1.30–1.42 (m, 4H, OCH$_2$CH$_2$C*H*$_2$C*H*$_2$); 1.17 (d, 3H, *J* = 6.0 Hz, H-6′). ^{13}C-NMR (100 MHz, D_2O): 173.96 (C=O); 101.64 (C-1″); 100.81 (C-1); 98.45 (C-1′); 75.16 (C-5); 74.73 (C-3, C-5″); 73.17 (C-4); 72.28 (C-3″); 71.70 (C-4′); 70.85 (C-2″); 70.30 (O*C*H$_2$CH$_2$); 69.01 (C-3′); 68.15 (C-4″); 67.51 (C-2′); 66.53 (C-5′); 61.31 (C-6″); 59.57 (C-6); 55.65 (C-2); 39.21 (CH$_2$NH$_2$); 28.18, 26.46, 25.05, 24.45 [OCH$_2$(*C*H$_2$)$_4$]; 22.05 (*C*H$_3$CO); 15.10 (C-6′). HRESIMS calcd for $C_{26}H_{48}N_2O_{15}$ $[M+H]^+$ 629.3133, found 629.3121.

6,6′-Dithio-bis(hexan-1,6-diyl)-bis[2-acetamido-2-deoxy-3-*O*-α-L-fucopyranosyl-4-*O*-(β-D-galactopyranosyl)-β-D-glucopyranoside] (3). The 6-benzylthiohexyl trisaccharide **32** (30 mg, 0.024 mmol) was deprotected in the same conditions as described above for the deprotection of trisaccharide **29**. After work up, the residue was passed through a Biogel P2 column eluted with water to give the trisaccharide **3** (10.6 mg, 70%) as white amorphous powder after lyophilization. $[\alpha]_D$ = −57 (*c* 0.7, MeOH), ^{1}H NMR (600 MHz, D_2O): δ 5.05 (d, 1H, *J* = 3.8 Hz, H-1′); 4.82–4.75 (m, 1H, H-5′); 4.47 (d, 1H, *J* = 7.7 Hz, H-1); 4.39 (d, 1H, *J* = 7.9 Hz, H-1″); 3.95 (d, 1H, *J* = 10.9 Hz, H-6a); 3.90–3.76 (m, 7H, H-2, H-3, H-4, H-6b, H-3′, H-4″, OC*H*HCH$_2$); 3.75–3.71 (m, 1H, H-4′); 3.70–3.56 (m, 4H, H-2′H-3″, H-6a″, H-6b″); 3.55–3.49 (m, 3H, H-5, H-5″, OCH*H*CH$_2$); 3.44 (t, 1H, *J* = 8.1 Hz, H-2″); 2.70 (t, 2H, *J* = 7.1 Hz, CH$_2$S); 1.98 (s, 3H, CH$_3$CO); 1.68–1.58 (m, 2H, SCH$_2$C*H*$_2$); 1.54–1.43 (m, 2H, OCH$_2$C*H*$_2$); 1.41–1.21 (m, 4H, OCH$_2$CH$_2$C*H*$_2$C*H*$_2$CH$_2$CH$_2$S); 1.12 (d, 3H, *J* = 6.6 Hz, H-6′. ^{13}C-NMR (150 MHz, D_2O): 174.09 (C=O); 101.83 (C-1″); 100.93 (C-1); 98.63 (C-1′); 75.35 (C-5); 74.96, 74.90 (C-3, C-5″); 73.41 (C-4); 72.48 (C-3″); 71.91 (C-4′); 71.05 (C-2″); 70.45 (O*C*H$_2$CH$_2$); 69.22 (C-3′); 68.34 (C-4″); 67.72 (C-2′); 66.71 (C-5′); 61.48 (C-6″); 59.81 (C-6); 55.86 (C-2); 38.16 (CH$_2$S); 28.44, 28.30, 27.15, 24.67 (OCH$_2$*C*H$_2$*C*H$_2$*C*H$_2$*C*H$_2$); 22.35 (*C*H$_3$CO); 15.30 (C-6′). HRESIMS Calcd for $C_{59}H_{92}N_2O_{30}S_2Na$ $[M+Na]^+$ 1311.5074, found 1311.5065.

6-Chlorohexyl 2-acetamido-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-6-*O*-benzyl-3-*O*-(chloroacetyl)-2-deoxy-β-D-glucopyranoside (24). $BF_3{\cdot}Et_2O$ (150 μL, 1.19 mmol, 2.0 equiv) was added to a solution of the acceptor **4** (300 mg, 0.59 mmol) and glycosyl donor **7** (1.46 g, 2.96 mmol, 5.0 equiv) [39-41] in anhyd CH_2Cl_2 (15 mL) at 40 °C. The reaction mixture was stirred for 1 h at 40 °C. The reaction was quenched with Et_3N (170 μL, 1.22 mmol) and the solvent was evaporated. Flash chromatography of the residue (EtOAc–hexanes, 1:1 to

6:4) gave the disaccharide **24** (341 mg, 69%) as colorless oil. $[\alpha]_D$ = −5 (*c*1.0, $CHCl_3$), ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.40–7.26 (m, 5H, Ar); 5.72 (d, 1H, *J* = 9.2 Hz, NH); 5.24 (bd, 1H, *J* = 3.4 Hz, H-4′); 5.11 (dd, 1H, *J* = 10.0, 8.9 Hz, H-3); 4.95 (dd, 1H, *J* = 10.4, 8.0 Hz, H-2′); 4.78 (dd, 1H, *J* = 10.4, 3.5 Hz, H-3′); 4.72 (d, 1H, *J* = 12.0 Hz, PhC*H*H); 4.50–4.41 (m, 3H, H-1, PhCH*H*); 4.39 (d, 1H, *J* = 8.0 Hz, H-1′); 4.15–4.01 (m, 4H, H-6a′, H-6b′, $ClCH_2CO$); 4.01–3.88 (m, 2H, H-2, H-4); 3.86–3.78 (m, 1H, OC*H*H); 3.73–3.65 (m, 2H, H-6a, H-6b); 3.62 (t, 1H, *J* = 6.5 Hz, H-5′); 3.53–3.38 (m, 4H, H-5, OCH*H*, CH_2Cl); 2.10, 2.04, 1.93, 1.92 (4 s, 15H, CH_3CO); 1.77–1.67 (m, 2H, C*H*$_2$$CH_2Cl$); 1.61–1.49 (m, 2H, OCH_2CH_2); 1.44–1.27 (m, 4H, $OCH_2CH_2CH_2CH_2$). ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.30, 170.16, 169.96, 168.96, 167.34 (C=O); 137.64, 128.57, 128.07, 127.97 (Ar); 100.87 (C-1); 100.12 (C-1′); 74.48, 74.40, 74.25 (C-3, C-4, C-5); 73.62 (Ph*C*H_2); 70.75, 70.60 (C-3′, C-5′); 69.27 (CH_2O); 69.09 (C-2′); 67.35 (C-6); 66.81 (C-4′); 61.02 (C-6′); 53.45 (C-2); 44.97 (CH_2Cl); 40.80 (Cl*C*H_2CO); 32.40 (*C*$H_2$$CH_2Cl$); 29.21 ($OCH_2CH_2$); 26.44, 25.14 ($OCH_2CH_2CH_2CH_2$); 23.25, 20.64, 20.58, 20.48, 20.48 (CH_3CO). HRESIMS Calcd for $C_{37}H_{52}Cl_2NO_{16}$ $[M+H]^+$ 836.2663, found 836.2634.

6-Chlorohexyl 2-acetamido-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-6-*O*-benzyl-2-deoxy-β-D-glucopyranoside (28). Thiourea (162 mg, 2.13 mmol, 6.0 equiv) was added to a solution of the disaccharide **24** (298 mg, 0.356 mmol) in a mixture of pyridine and EtOH (2:1, 15 mL). The solution was stirred for 10 h at 70 °C, the solvents removed by evaporation and the residue co-concentrated with toluene (2 × 10 mL). The crude residue was dissolved in CH_2Cl_2 (20 mL) and washed sequentially with 2 M HCl (10 mL), saturated aq $NaHCO_3$ (10 mL) and brine (10 mL). The aq phases were re-extracted with CH_2Cl_2 and the combined organic layers were dried and concentrated. Flash chromatography of the residue (EtOAc–hexanes, 6:4) gave the pure disaccharide **28** (165 mg, 61%) as a white amorphous powder. $[\alpha]_D$ = +1 (*c* 1.3, $CHCl_3$), ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.37–7.26 (m, 5H, Ar); 5.62 (d, 1H, *J* = 7.7 Hz, NH); 5.32 (bd, 1H, *J* = 3.4 Hz, H-4′); 5.13 (dd, 1H, *J* = 10.4, 8.0 Hz, H-2′); 4.90 (dd, 1H, *J* = 10.4, 3.4 Hz, H-3′); 4.74 (d, 1H, *J* = 8.2 Hz, H-1); 4.68 (d, 1H, *J* = 12.1 Hz, PhC*H*H); 4.47 (d, 1H, *J* = 12.1 Hz, PhCH*H*); 4.45 (d, 1H, *J* = 8.0 Hz, H-1′); 4.13–4.05 (m, 2H, H-6a′, H-6b′); 4.04–3.96 (m, 1H, H-3); 3.96–3.92 (bs, 1H, OH); 3.90–3.79 (m, 2H, H-5′, OC*H*H); 3.69–3.57 (m, 3H, H-4, H-6a, H-6b); 3.53–3.41 (m, 4H, H-5, OCH*H*, CH_2Cl); 3.41–3.31 (m, 1H, H-2); 2.12, 2.03, 1.97, 1.95 (4 s, 15H, CH_3CO); 1.78–1.69 (m, 2H, C*H*$_2$$CH_2Cl$); 1.62–1.50 (m, 2H, OCH_2CH_2); 1.46–1.29 (m, 4H, $OCH_2CH_2CH_2CH_2$). ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.36, 170.07, 169.98, 169.91, 169.15 (C=O); 138.02, 128.48, 127.86, 127.78 (Ar); 101.13 (C-1′); 99.96 (C-1); 80.98 (C-4); 73.92 (C-5); 73.59 (Ph*C*H_2); 71.34 (C-3); 71.08 (C-5′); 70.67 (C-3′); 69.31 (CH_2O); 68.73 (C-2′); 68.05 (C-6); 66.81 (C-4′); 61.31 (C-6′); 57.05 (C-2); 44.99 (CH_2Cl); 32.44 (*C*$H_2$$CH_2Cl$); 29.28 ($OCH_2CH_2$); 26.49, 25.19 ($OCH_2CH_2CH_2CH_2$); 23.58, 20.65, 20.56, 20.53, 20.47 (CH_3CO). HRESIMS Calcd for $C_{35}H_{51}ClNO_{15}$ $[M+H]^+$ 760.2947, found 760.2928.

6-Chlorohexyl 2-acetamido-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-6-*O*-benzyl-3-*O*-(2,3,4-tri-*O*-benzyl-α-L-fucopyranosyl)-2-deoxy-β-D-glucopyranoside (29). A solution of the disaccharide acceptor **28** (100 mg, 0.132 mmol) and fucosyl donor **9** (189 mg, 0.395 mmol, 3.0 equiv) [42] in a mixture of CH_2Cl_2 and DMF (1:1, 8 mL) containing activated powdered MS 4Å (400 mg) was stirred at room temp for 30 min. Cu(II)Br_2 (88 mg, 0.394 mmol, 3.0 equiv) and Bu_4NBr (131 mg, 0.409 mmol, 3.1 equiv) were added and the reaction mixture was stirred for 20 h at room temp. The reaction mixture was filtered over Celite® and the solids were washed with CH_2Cl_2 (5 mL). The filtrate was diluted with CH_2Cl_2 (60 mL) and washed sequentially with brine (50 mL) and saturated aq $NaHCO_3$ (6 × 50 mL). The aq layers were re-extracted with CH_2Cl_2 (50 mL) and the combined organic layers were dried and concentrated. Flash chromatography of the residue (EtOAc–hexanes, 3:7) gave the trisaccharide **29** as colorless oil (126 mg, 81%). $[\alpha]_D$ = −19 (*c* 0.5, $CHCl_3$), ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.45–7.24 (m, 20H, Ar); 6.02 (bs, 1H, NH); 5.29 (d, 1H, *J* = 3.2 Hz, H-4″); 5.09 (d, 1H, *J* = 3.7 Hz, H-1′); 5.04 (dd, 1H, *J* = 10.4, 8.2 Hz, H-2″); 4.98 (d, 1H, *J* = 11.9 Hz, PhC*H*H); 4.92–4.85 (m, 2H, H-1, PhCH*H*); 4.85–4.79 (m, 3H, H-3″, Ph*C*H_2); 4.75 (d, 1H, *J* = 11.9 Hz, PhC*H*H); 4.71 (d, 1H, *J* = 11.8 Hz, PhCH*H*); 4.69 (d, 1H, *J* = 12.0 Hz, PhC*H*H); 4.58 (d, 1H, *J* = 8.2 Hz, H-1″); 4.45 (d, 1H, *J* = 12.0 Hz, PhCH*H*); 4.43–4.38 (m, 1H, H-5′); 4.19 (t, 1H, *J* = 7.6 Hz, H-3); 4.16–4.11 (m, 2H, H-2′, H-6a″), 4.02 (dd, 1H, *J* = 10.9, 5.9 Hz, H-6b″); 3.97–3.90 (m, 2H, H-4, H-3′); 3.85–3.74 (m, 3H, H-6a, H-6b, OC*H*HCH_2); 3.68 (s, 1H, H-4′); 3.58 (t, 1H, *J* = 7.0 Hz, H-5″); 3.55–3.45 (m, 4H, H-2, H-5, CH_2Cl); 3.44–3.38 (m, 1H, OCH*H*CH_2); 2.03, 2.02, 1.98, 1.84, 1.78 (5 s, 15H, CH_3CO); 1.76–1.69 (m, 2H, C*H*$_2$$CH_2Cl$); 1.58–1.47 (m, 2H, OCH_2CH_2); 1.44–1.27 (m, 4H, $OCH_2CH_2CH_2CH_2$); 1.18 (d, 3H, *J* = 6.5 Hz, H-6′). ^{13}C NMR (150 MHz, $CDCl_3$): δ 170.45, 170.00, 169.88, 169.85, 169.21 (C=O); 138.72, 138.68, 138.47, 137.78, 128.46, 128.40, 128.30, 128.24, 128.11, 127.90, 127.73, 127.69, 127.61, 127.49, 127.28, 127.00 (Ar); 99.38, 99.34 (C-1, C-1″); 97.33 (C-1′); 79.82 (C-3′); 76.79 (C-4′); 76.31 (C-2′); 74.23 (Ph*C*H_2); 74.21 (C-5); 74.03 (C-4); 73.58, 73.35 (Ph*C*H_2); 73.32 (C-3); 72.38 (Ph*C*H_2); 70.52 (C-3″); 70.27 (C-5″); 69.23 (O*C*$H_2$$CH_2$); 68.73 (C-2″); 68.33 (C-6); 66.61 (C-4″); 66.39 (C-5′); 60.22 (C-6″); 56.20 (C-2); 44.92 (CH_2Cl); 32.38 (*C*$H_2$$CH_2Cl$); 29.12 ($OCH_2CH_2$); 26.45, 25.08 ($OCH_2CH_2CH_2CH_2$); 22.95, 20.65, 20.52, 20.49, 20.44

(CH_3CO). HRESIMS Calcd for $C_{62}H_{79}ClNO_{19}$ $[M+H]^+$ 1176.4935, found 1176.4933.

6-Azidohexyl 2-acetamido-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-6-*O*-benzyl-3-*O*-(2,3,4-tri-*O*-benzyl-α-L-fucopyranosyl)-2-deoxy-β-D-glucopyranoside (30). NaN_3 (17 mg, 0.26 mmol, 8.2 equiv) was added to a solution of the trisaccharide **29** (38 mg, 0.032 mmol) in anhyd DMF (2.5 mL) and the reaction mixture was heated at 80 °C for 36 h. The solvent was evaporated, the residue was dissolved in CH_2Cl_2 (50 mL) and washed with water (2 × 10 mL). The aq phases were re-extracted with CH_2Cl_2 and the combined organic layers were dried and concentrated. Flash chromatography of the residue (EtOAc-hexanes, 6:4) afforded the trisaccharide **30** as a clear glass (36 mg, 95%). $[\alpha]_D = -47$ (*c* 1.0, CH_2Cl_2), ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.41–7.20 (m, 20H, Ar); 5.81 (d, 1H, *J* = 7.6 Hz, NH); 5.26 (d, 1H, *J* = 3.0 Hz, H-4″); 5.06 (d, 1H, *J* = 3.8 Hz, H-1′), 5.00 (dd, 1H, *J* = 10.4, 8.2 Hz, H-2″); 4.94 (d, 1H, *J* = 11.8 Hz, PhC*H*H); 4.91–4.83 (m, 2H, H-1, PhCH*H*); 4.82–4.74 (m, 3H, H-3″, PhCH_2); 4.73–4.62 (m, 3H, PhCH_2, PhC*H*H); 4.54 (d, 1H, *J* = 8.1 Hz, H-1″); 4.43–4.34 (m, 2H, H-5′, PhCH*H*); 4.20–4.05 (m, 3H, H-3, H-2′, H-6a″); 3.98 (dd, 1H, *J* = 10.8, 5.9 Hz, H-6b″); 3.95–3.87 (m, 2H, H-4, H-3′); 3.83–3.68 (m, 3H, H-6a, H-6b, OC*H*HCH$_2$); 3.65 (d, 1H, *J* = 1.4 Hz, H-4′); 3.57–3.44 (m, 2H, H-5, H-5″); 3.43–3.31 (m, 2H, H-2, OCH*H*CH$_2$); 3.20 (t, 2H, *J* = 6.9 Hz, CH_2N_3); 1.99, 1.98, 1.93, 1.89, 1.70 (5s, 15H, CH_3CO); 1.58–1.43 (m, 4H, CH_2CH$_2$N$_3$, OCH$_2$CH_2); 1.33–1.21 (m, 4H, OCH$_2$CH$_2$CH_2CH_2); 1.15 (d, 3H, *J* = 6.5 Hz, H-6′). ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.09, 170.06, 169.96, 169.91, 169.20 (C=O); 138.88, 138.77, 138.57, 137.89, 128.53, 128.47, 128.38, 128.32, 128.19, 127.97, 127.77, 127.75, 127.66, 127.56, 127.36, 127.08 (Ar); 99.45 (C-1, C-1″); 97.44 (C-1′); 79.97 (C-3′); 76.90 (C-4′); 76.42 (C-2′); 74.19 (C-5, C-4); 73.70 (Ph*C*H$_2$); 73.43 (C-3, Ph*C*H$_2$); 72.46 (Ph*C*H$_2$); 70.62 (C-3″); 70.35 (C-5″); 69.29 (O*C*H$_2$CH$_2$); 68.80 (C-2″); 68.43 (C-6); 66.69 (C-4″); 66.42 (C-5′); 60.28 (C-6″); 56.60 (C-2); 51.32 (CH$_2$N$_3$); 29.23, 28.73, 26.41, 25.43 (OCH$_2$*C*H$_2$*C*H$_2$*C*H$_2$*C*H$_2$N$_3$); 23.16, 20.70, 20.59, 20.56, 20.51 (*C*H$_3$CO); 16.71 (C-6′). HRESIMS Calcd for $C_{62}H_{79}N_4O_{19}$ $[M+H]^+$ 1183.5339, found 1183.5325.

6-Acetylthiohexyl 2-acetamido-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-6-*O*-benzyl-3-*O*-(2,3,4-tri-*O*-benzyl-α-L-fucopyranosyl)-2-deoxy-β-D-glucopyranoside (31). KSC(O)CH_3 (26 mg, 0.22 mmol, 10 equiv) was added to a solution of the trisaccharide **29** (27 mg, 0.023 mmol) in anhyd DMF (1.5 mL) and the reaction mixture was heated at 80 °C for 16 h. Work up and chromatography (EtOAc–hexanes, 6:4), as described above for compound **30** gave the trisaccharide **31** as colorless glass (19 mg, 70%). $[\alpha]_D = -43$ (*c* 0.7, CH_2Cl_2), ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.40–7.20 (m, 20H, Ar); 5.85 (d, 1H, *J* = 7.7 Hz, NH); 5.25 (d, 1H, *J* = 3.0 Hz, H-4″); 5.07 (d, 1H, *J* = 3.8 Hz, H-1′), 5.01 (dd, 1H, *J* = 10.4, 8.2 Hz, H-2″); 4.98 (d, 1H, *J* = 11.8 Hz, PhC*H*H); 4.90–4.83 (m, 2H, H-1, PhCH*H*); 4.82–4.74 (m, 3H, H-3″, PhCH_2); 4.74–4.62 (m, 3H, PhCH_2, PhC*H*H); 4.54 (d, 1H, *J* = 8.2 Hz, H-1″); 4.44–4.35 (m, 2H, H-5′, PhC*H*H); 4.18 (t, 1H, *J* = 7.7 Hz, H-3); 4.14–4.05 (m, 2H, H-2′, H-6a″); 3.97 (dd, 1H, *J* = 10.8, 5.9 Hz, H-6b″); 3.94–3.85 (m, 2H, H-4, H-3′); 3.83–3.68 (m, 3H, H-6a, H-6b, OC*H*HCH$_2$); 3.65 (d, 1H, *J* = 2.7 Hz, H-4′); 3.53 (t, 1H, *J* = 6.8 Hz, H-5″); 3.50–3.44 (m, 1H, H-5); 3.43–3.30 (m, 2H, H-2, OCH*H*CH$_2$); 2.81 (t, 2H, *J* = 7.2 Hz, CH$_2$S); 2.29 (s, 3H, SCOCH_3); 1.99, 1.97, 1.93, 1.89, 1.71 (5s, 15H, CH_3CO); 1.59–1.41 (m, 4H, CH_2CH$_2$S, OCH$_2$CH_2); 1.33–1.20 (m, 4H, OCH$_2$CH$_2$CH_2CH_2); 1.15 (d, 3H, *J* = 6.5 Hz, H-6′). ^{13}C NMR (100 MHz, $CDCl_3$): δ 195.99, 170.16, 170.06, 169.97, 169.92, 169.19 (C=O); 138.86, 138.80, 138.59, 137.90, 128.53, 128.47, 128.37, 128.31, 128.19, 127.97, 127.80, 127.77, 127.67, 127.56, 127.34, 127.09 (Ar); 99.45, 99.42 (C-1, C-1″); 97.40 (C-1′); 79.94 (C-3′); 76.94 (C-4′); 76.40 (C-2′); 74.33 (C-5); 74.31 (Ph*C*H$_2$); 74.21 (C-4); 73.66, 73.44 (Ph*C*H$_2$); 73.36 (C-3); 72.49 (Ph*C*H$_2$); 70.64 (C-3″); 70.34 (C-5″); 69.39 (O*C*H$_2$CH$_2$); 68.81 (C-2″); 68.40 (C-6); 66.71 (C-4″); 66.40 (C-5′); 60.29 (C-6″); 56.60 (C-2); 30.61 (SCO*C*H$_3$); 29.40, 29.40, 29.20, 28.96, 28.43, 25.36 (O*C*H$_2$*C*H$_2$*C*H$_2$*C*H$_2$*C*H$_2$*C*H$_2$S); 23.18, 20.70, 20.59, 20.56, 20.51 (*C*H$_3$CO); 16.71 (C-6′). HRESIMS Calcd for $C_{64}H_{82}NO_{20}S$ $[M+H]^+$ 1216.5151, found 1216.5151.

6-Benzylthiohexyl 2-acetamido-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-6-*O*-benzyl-3-*O*-(2,3,4-tri-*O*-benzyl-α-L-fucopyranosyl)-2-deoxy-β-D-glucopyranoside (32). $PhCH_2SH$ (60 μL, 0.44 mmol, 15 equiv) and NaH (21 mg, 0.44 mmol, 15 equiv) were added to a solution of the trisaccharide **29** (36 mg, 0.030 mmol) in anhyd DMF (3.0 mL) at room temp. After 10 min the reaction mixture was heated to 80 °C for 16 h, the solvent was evaporated and the residue was dissolved in Ac_2O and pyridine (5 ml, 1:1). After 18 h the reaction mixture was co-concentrated with toluene (3 × 20 ml), the residue was dissolved in CH_2Cl_2 (30 mL) and the solution was washed with water (2 × 10 mL). The aq phases were re-extracted with CH_2Cl_2 and the combined organic layers were dried and concentrated. Flash chromatography of the residue (EtOAc–hexanes, 1:1) gave the trisaccharide **32** (35.6 mg, 94%) as a white solid. $[\alpha]_D = -28$ (*c* 1.0, CH_2Cl_2), ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.44–7.13 (m, 25H, Ar); 5.79 (d, 1H, *J* = 7.6 Hz, NH); 5.25 (d, 1H, *J* = 3.0 Hz, H-4″); 5.05 (d, 1H, *J* = 3.8 Hz, H-1′), 5.00 (dd, 1H, *J* = 10.5, 8.2 Hz, H-2″); 4.94 (d, 1H, *J* = 11.8 Hz, PhC*H*H); 4.90–4.74 (m, 5H, H-1, H-3″, PhCH_2, PhCH*H*); 4.73–4.64 (m, 3H, PhC*H*H, PhCH_2); 4.54 (d, 1H, *J* = 8.2 Hz, H-1″); 4.43–4.36 (m, 2H, H-5′, PhC*H*H); 4.17 (t, 1H, *J* = 7.7 Hz, H-3); 4.14–4.06 (m, 2H, H-2′, H-6a″); 4.01–3.95 (m,

1H, H-6b″); 3.93–3.87 (m, 2H, H-4, H-3′); 3.79–3.64 (m, 6H, H-6a, H-6b, H-4′, SCH_2Ph, OCHHCH_2); 3.56–3.45 (m, 2H, H-5, H-5″); 3.41–3.31 (m, 2H, H-2, OCHHCH_2); 2.36 (t, 2H, J = 7.3 Hz, CH_2SBn); 1.99, 1.98, 1.94, 1.90, 1.70 (5s, 15H, CH_3CO); 1.52–1.42 (m, 4H, CH_2CH_2S, OCH$_2$CH_2); 1.33–1.28 (m, 4H, OCH$_2$CH$_2$CH_2CH_2); 1.15 (d, 3H, J = 6.4 Hz, H-6′). ^{13}C NMR (150 MHz, $CDCl_3$): δ 170.14, 170.10, 170.02, 169.96, 169.20 (C=O); 140.06, 138.91, 138.81, 138.61, 137.93, 137.63, 130.14, 129.02, 128.97, 128.53, 128.49, 128.30, 128.00, 127.89, 127.73, 127.60, 127.39, 126.89 (Ar); 99.48, 99.41 (C-1, C-1″); 97.47 (C-1′); 80.05 (C-3′); 76.86 (C-4′); 76.42 (C-2′); 74.34 (C-5); 74.31 (PhCH$_2$); 74.25 (C-4); 73.77, 73.46 (PhCH$_2$); 73.35 (C-3); 72.47 (PhCH$_2$); 70.67 (C-3″); 70.33 (C-5″); 69.49 (OCH$_2$CH$_2$); 68.81 (C-2″); 68.39 (C-6); 66.70 (C-4″); 66.41 (C-5′); 60.28 (C-6″); 52.07 (C-2); 36.30 (S-CH$_2$Ph); 31.30 (CH$_2$SBn); 29.29, 29.13, 29.06, 25.53 (OCH$_2$$CH_2$$CH_2$$CH_2$$CH_2CH_2$S); 23.23, 20.75, 20.64, 20.61, 20.57 (CH$_3$CO); 16.76 (C-6′). HRESIMS Calcd for $C_{69}H_{86}NO_{19}S$ $[M+H]^+$ 1264.5515, found 1264.5509.

Supporting Information

Supporting Information File 1

Experimental procedures and characteristics for compounds **4**–**6**, **8**, **11**, **12**, **14**–**19**, **21**–**23**, **25**–**27**.
[http://www.beilstein-journals.org/bjoc/content/supplementary/1860-5397-6-17-S1.pdf]

Supporting Information File 2

^{1}H and ^{13}C NMR spectra for compounds **1**–**6**, **8**, **11**, **12**, **16**–**19**, **21**–**32**, ^{1}H NMR data for known compounds **14**, **15**.
[http://www.beilstein-journals.org/bjoc/content/supplementary/1860-5397-6-17-S2.pdf]

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Chemical synthesis using enzymatically generated building units for construction of the human milk pentasaccharides sialyllacto-*N*-tetraose and sialyllacto-*N*-neotetraose epimer

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Full Research Paper

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Abstract

α,2-3- and α,2-6-sialylated lactosaminide precursor structures obtained by various enzymatic procedures could be used for glycosylations employing triflic acid/*N*-iodosuccinimide. Easily accessible selectively protected lactoside derivatives served as acceptor disaccharides to give the corresponding human milk pentasaccharides in good yields. These were characterized by spectroscopic means in the form of their peracetylated derivatives.

Introduction

From an inspection of contemporary syntheses of biologically and medicinally relevant oligosaccharides, it is evident that the majority is performed either by classical chemical methods or exclusively by enzymatic procedures. Even although considerable progress has been reported during the recent decades, every synthesis of a complex heterooligosaccharide still represents a challenge. To arrive at an oligosaccharide structure with specific patterns of substitution and defined regio- and stereochemical layout, all the presently available procedures need to be checked for efficiency with respect to not only all the above points, but also the efforts required at the purification steps as well as the yields. Whilst both approaches can be employed advantageously in certain cases, in others this is certainly not so.

For instance, for structures that contain glycosamino units, it has been demonstrated that these units can be introduced by classical methods in high yields and with good to excellent stereochemical control. In case of glucosylations and galactosylations, and in particular for β-galactosylations, classical and enzymatic methods are almost equal in terms of stereoselectivity and transfer efficiency. By contrast, for syntheses of

sialylated structures, enzymatic procedures are still considerably superior to classical chemical sialylations with respect to both stereochemical outcome and preparative input.

The use of both procedures in a synergistic mode should also be considered. One of the general approaches ideally suited in such cases is the block synthesis method. Moreover, in recent years a number of combined chemical and chemoenzymatic syntheses have been reported [1-4].

As a proof of principle, we were interested to employ some trisaccharide building units previously obtained by enzymatic routes in block syntheses *en route* to interesting structures. To this end, two human milk pentasaccharides of prominent importance, sialyllacto-*N*-tetraose (**1**) and an epimer of sialyllacto-*N*-neotetraose (**2**) (Figure 1) were selected as target molecules. Both these pentasaccharides, Neu5Acα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc (**1**) and Neu5Acα2-6Galβ1-4GlcNAcβ1-4Galβ1-4Glc (**2**), are dominant constituents of complex human milk oligosaccharides (Figure 1). They are considered to play a major role in immuno defense against bacterial and viral infections in the gastrointestinal tract of infants [5]. It is thought that they effectively inhibit bacterial adhesion to epithelial surfaces and so block the first stages of infection processes. Thus, these human milk oligosaccharides are considered as soluble receptor analogues of epithelial cell surfaces [6].

Results and Discussion

Previously, we reported the chemoenzymatic synthesis of the 3-sialylated lactosamine derivative **3** obtained by the enzymatic β-galactosylation of the 2-azidothioglucoside with *p*-nitrophenyl β-galactopyranoside and β-galactosidase (*Bovin testes*). The subsequent transsialylation was carried out with *p*-nitrophenyl sialoside (pNp-αNeu5Ac) and either sialidase from *Salmonella typhimurium* or from Newcastle disease virus [7]. Recently, a more effective higher yielding transfer has been reported in which sialylation with recombinant transsialidase (*Trypanosoma cruzi*) gave the trisaccharide **3** in 32% yield [8]. Treatment of **3** with methanol and acidic ion exchange resin led to the methyl ester (for the method cf. lit. [9]) which was then peracetylated to give trisaccharide **4** as the donor building block.

For formation of the disaccharide acceptor **6**, a straight-forward three-step standard reaction sequence was used [10]. Methyl β-lactoside was isopropylidenated at 3′,4′-position with dimethoxypropane and *p*-toluene sulfonic acid in DMF/acetone. Peracetylation (Ac_2O/Py) and subsequent cleavage of the isopropylidene group with 80% acetic acid at 80 °C gave the diol acceptor **6**. Since it is known that in galactopyranosyl structures the nucleophilicity of 3-OH considerably exceeds that of the 4-OH-group, further protecting group manipulations were not required.

Glycosylation of **4** by **6** catalyzed by *N*-iodosuccinimide and trifluoromethane sulfonic acid (as introduced by van Boom et al. [11]) gave the β,1-3-linked pentasaccharide **7** in 61% yield. About 5% of the corresponding α,1-3-linked compound and ca. 7% of the bis (β,1-3- and β,1-4-) linked octasaccharide were observed as side products and separated by chromatography but these were not further characterized. Reduction of the 2‴-azido

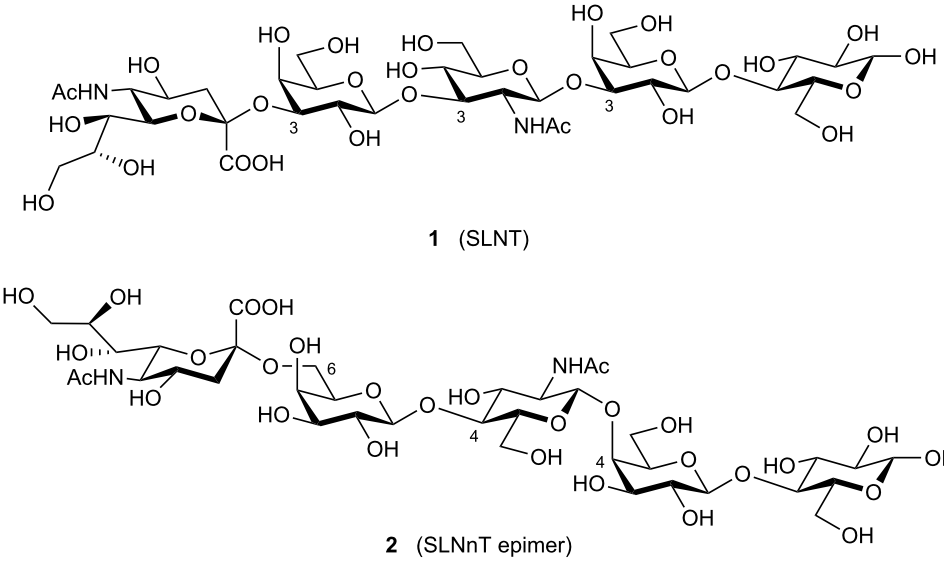
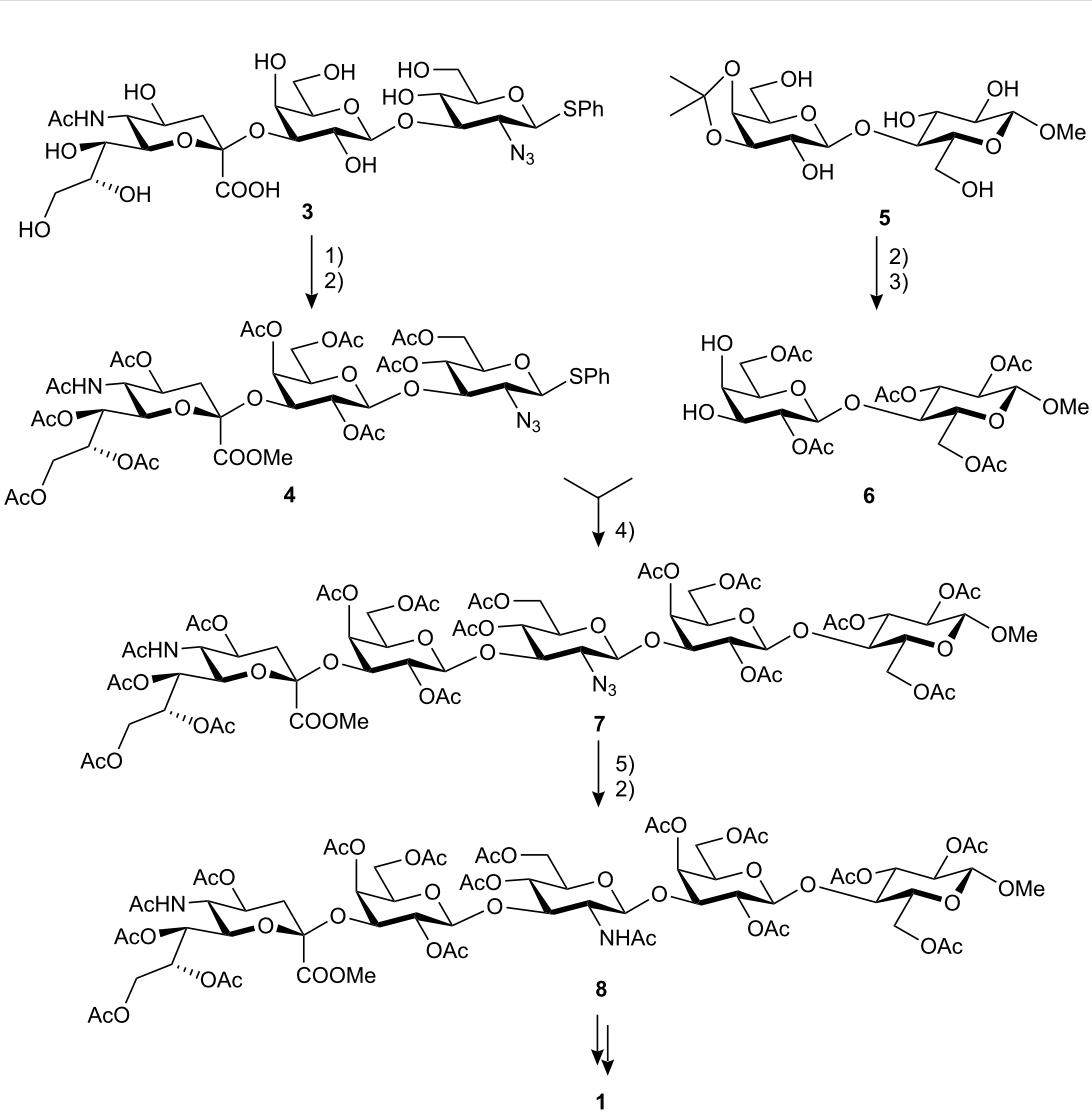


Figure 1: Structures of pentasaccharides **1** and **2**.



Scheme 1: Preparation of pentasaccharide **8**. 1) MeOH, acidic ion exchange resin; 2) Ac_2O, pyridine; 3) 80% HOAc, 90 °C; 4) NIS, CF_3SO_3H, 61%; 5) $NiCl_2$•$6H_2O$, H_3BO_3, EtOH, then $NaBH_4$, EtOH and acidic workup.

to the 2‴-amino group with nickel boride [12,13] followed by peracetylation gave **8** in 81% yield (Scheme 1). ^{1}H NMR spectrum contained a doublet for H-1″ at δ 4.96 ($J_{1''2''}$ = 8.0 Hz) and a down field shifted doublet for H-4′ at δ 5.37 ($J_{3'4'}$ = 2.9 Hz).

A similar approach was employed for the synthesis of the protected epimer of sialyllacto-*N*-neotetraose **14**. β-Galactosylation of 2-azidothioglucoside with *p*-nitrophenyl β-galactopyranoside and β-galactosidase (*Bacillus circulans*) gave the β,1-3-linked isolactosamine derivative. Further sialylation at position 3′-OH with pNp-αNeu5Ac and either sialidase from *Vibrio cholerae* or *Clostridium perfringens* afforded the α,2-6-sialylated trisaccharide **9** exclusively [7]. Later studies showed that **9** could be obtained in an enhanced yield of 32% by transsialylation with recombinant transsialidase (*Trypanosama cruzi*) [8]. Formation of the methyl ester and peracetylation led to the trisaccharide donor building block **10**.

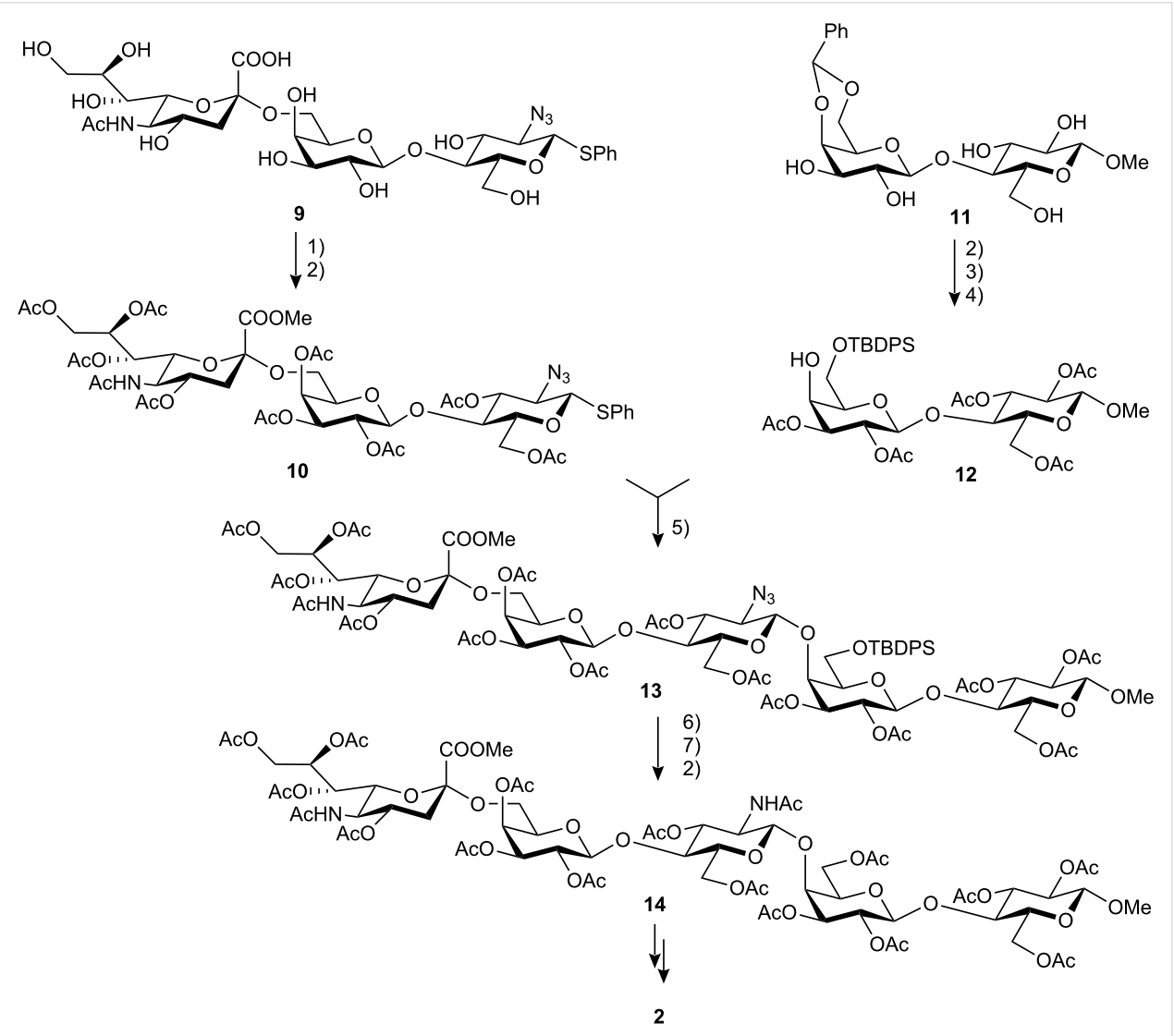
Synthesis of the disaccharide acceptor in this case started from methyl β-lactoside, which was transformed into its 4′,6′-benzylidene-protected derivative **11** in almost quantitative yield by transacetalization with benzaldehyde dimethylacetal in acetonitrile under *p*-toluenesulfonic acid catalysis. Subsequent peracetylation with acetic anhydride/pyridine, selective cleavage of the benzylidene group with 80% acetic acid at 90 °C and finally treatment with *tert*-butyldiphenylsilyl chloride and imidazole in DMF afforded the disaccharide **12** (cf. references [14,15]).

After activation of the trisaccharide donor **10** with *N*-iodosuccinimide and trifluoromethanesulfonic acid, the disaccharide acceptor unit **12** could be glycosylated to give the β,1-4-linked pentasaccharide derivative **13** in 53% yield. In addition, the corresponding α,1-4-linked pentasaccharide was obtained in 8% yield.

Finally, the azido group was reduced by the nickel boride method with sodium borohydride, nickel chloride and boric acid [12,13]. During this step partial cleavage of the *tert*-butyldiphenylsilyl groups was also observed. Complete removal was achieved with trifluoroacetic acid in dichloromethane. For characterization purposes, peracetylation was carried out to give the completely protected pentasaccharide **14** in 67% yield (Scheme 2). As evident from a comparison of the ^{1}H NMR data of **14** with the precursor tri- and disaccharide units **10** and **12**, the novel characteristic doublet for the anomeric H-1″ of the β-GlcNAc unit at δ 5.12 ($J_{1''2''}$ = 8.2 Hz) as well as the downfield shift Δδ 0.15 of H-4′ to δ 4.14 compared to **12** were in accord with structure of the target pentasaccharide.

Conclusion

In this contribution chemoenzymatically generated sialyl α,2-3- and sialyl α,2-6-glycosylated thiophenol 2-azido-lactose derivatives were employed as precursors for sialylated lactosaminide donor substituents in triflic acid/*N*-iodosuccinimide glycosylations. With easily accessible selectively unprotected lactose acceptor glycosides the pentasaccharide structures sialyllacto-*N*-tetraose and the epimer of sialyllacto-*N*-neotetraose could be obtained in good yields, and subsequently transformed into their



Scheme 2: Preparation of pentasaccharide **14**. 1) MeOH, acidic ion exchange resin; 2) Ac_2O, pyridine; 3) 80% HOAc, 90 °C; 4) TBDPSCl, imidazole, DMF; 5) NIS, CF_3SO_3H, 53%; 6) $NiCl_2$•$6H_2O$, H_3BO_3, EtOH, then $NaBH_4$, EtOH, then acidic workup; 7) CF_3CO_2H, CH_2Cl_2.

peracetylated derivatives for structure elucidation. Thus, a combination of enzymatic and purely chemical procedures was shown to be advantageous in the preparation of complex oligosaccharides.

Experimental

For general methods cf. reference [16]. The NMR data for the saccharide rings in the pentasaccharides **7**, **8**, **13** and **14** are denoted according to the Roman numberals I-V from the reducing end, as depicted for compounds **8** and **14** (Figure 2):

Methyl *O*-(methyl-5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-α-D-glycero-D-galacto-2-nonulopyranosylonate)-(2-3)-*O*-(2,4,6-tri-*O*-acetyl-β-D-galactopyranosyl)-(1-3)-*O*-(4,6-di-*O*-acetyl-2-azido-2-deoxy-β-D-glucopyranosyl)-(1-3)-*O*-(2,4,6-tri-*O*-acetyl-β-D-galactopyranosyl)-(1-4)-2,3,6-tri-O-acetyl-β-D-glucopyranoside (7): Glycosylation was carried out as described for the synthesis of compound **13** from compound **4** (95 mg, 83 μmol) as donor and compound **6** (50 mg, 86 μmol) as acceptor. The pentasccharide derivative **7** was obtained as a colorless amorphous solid; 83 mg (61%); $[\alpha]_D^{20} = -3.5$ (*c* 0.1, $CHCl_3$); ^{1}H NMR (500 MHz, CDCl3) δ 5.75 (dt, 1 H, *H*-8^V), 5.72 (d, 1 H, NH), 5.39 (d, $J_{3,4}$ = 2.8 Hz, 1 H, *H*-4^{II}), 5.36 (dd, $J_{7,8}$ = 9.3 Hz, 1H, *H*-7^V), 5.15 (dd, $J_{2,3} = J_{3,4}$ = 9.4 Hz, 1 H, *H*-3^I), 5.06 (dd, $J_{1,2}$ = 8.0 Hz, $J_{2,3}$ = 10.2 Hz, *H*-2^{IV}), 4.99–4.97 (m, 2 H, *H*-2^{II}, *H*-4^{III}), 4.96 (dd, $J_{1,2}$ = 7.8 Hz, $J_{2,3}$ = 9.6 Hz, 1 H, *H*-2^I), 4.91 (dd, $J_{1,2}$ = 8.0 Hz, $J_{2,3}$ = 10.2 Hz, 1 H, *H*-1^{IV}), 4.87 (d, $J_{3,4}$ = 2.6 Hz, *H*-4^{IV}), 4.83 (dt, $J_{3eq,4}$ = 4.4 Hz, $J_{3ax,4} = J_{4,5}$ = 12.1 Hz, 1 H, *H*-4^V), 4.75 (dd, $J_{5,6}$ = 10.1 Hz, $J_{6,7}$ = 2.2 Hz, *H*-6^V), 4.62 (dd, 1 H, *H*-3^{III}), 4.57 (dd, $J_{2,3}$ = 10.0 Hz, $J_{3,4}$ = 2.8 Hz, 1 H, *H*-3^{IV}), 4.51 (d, $J_{1,2}$ = 7.8 Hz, 1 H, *H*-1^I), 4.49 (dd, 1 H, *H*-6^V), 4.39 (d, $J_{1,2}$ = 8.0 Hz, 1 H, *H*-1^{II}), 4.37 (d, $J_{1,2}$ = 8.1 Hz, 1 H, *H*-1^{III}), 4.29 (dd, $J_{9a,9b}$ = 12.4 Hz, $J_{8,9b}$ = 2.4 Hz, *H*-9b^V), 4.24 (dd, $J_{6a,6b}$ = 12.6 Hz, $J_{5,6a}$ = 6.6 Hz, 1 H, *H*-6a^{III}), 4.17 (dd, $J_{5,6}$ = 10.2 Hz, *H*-5^V), 4.13 (d, 1 H, NH), 3.75 (s, 3 H, $COOCH_3^V$), 4.00 (dd, $J_{8,9a}$ = 5.8 Hz, 1 H, *H*-9a^V), 3.72 (bt, 1 H, *H*-5^{IV}), 3.59 (m, 1 H, *H*-5^I), 3.47–3.44 (m, 2 H, *H*-3^{II}, *H*-6a^{IV}), 3.39 (dd, $J_{6a,6b}$ = 11.0 Hz, $J_{5,6b}$ = 7.8 Hz, *H*-6b^{IV}), 2.61 (dd, $J_{3ax,3eq}$ = 12.4 Hz, $J_{3eq,4}$ = 4.8 Hz, 1 H, *H*-3eq^V), 2.17–1.33 (15s, 45 H, 14 OAc, 1 NAc), 1.91 (dd, $J_{3ax,4}$ = 11.8 Hz, 1 H, *H*-3ax^V). $C_{67}H_{92}N_4O_{43}$ (1641.45): Found C, 49.33; H, 5.59; N, 3.62. Calculated C; 49.02; H, 5.65; N, 3.41. MALDI-TOF: 1664.44 $(M+Na)^+$; 1680.59 $(M+K)^+$.

Methyl *O*-(methyl-5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-α-D-glycero-D-galacto-2-nonulopyranosylonate)-(2-3)-*O*-(2,4,6-tri-*O*-acetyl-β-D-galactopyranosyl)-(1-3)-*O*-(4,6-di-*O*-acetyl-2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1-3)-*O*-(2,4,6-tri-*O*-acetyl-β-D-galactopyranosyl)-(1-4)-2,3,6-tri-O-acetyl-β-D-glucopyranoside (8): Reduction and peracetylation of compound **7** was carried out as described for **14**. Thus, from 80 mg (49 μmol) of **7**, 65 mg (81%) of **8** was obtained as a colorless amorphous solid; $[\alpha]_D^{20} = -12.7$ (*c* 0.5, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.74 (dt, 1 H, *H*-8^V), 5.72 (d, 1 H, NH), 5.38 (d, $J_{3,4}$ = 2.8 Hz, 1 H, *H*-4^{II}), 5.33 (dd, $J_{7,8}$ = 9.3 Hz, 1H, *H*-7^V), 5.12 (dd, $J_{2,3} = J_{3,4}$ = 9.6 Hz, 1 H, *H*-3^I), 5.05 (dd, $J_{1,2}$ = 8.0 Hz, $J_{2,3}$ = 10.2 Hz, *H*-2^{IV}), 4.99 (dd, $J_{1,2}$ = 8.0 Hz, $J_{2,3}$ = 9.7 Hz, 1 H, *H*-2^{II}), 4.97 (m, 2 H, *H*-4^{III}), 4.95 (dd, $J_{1,2}$ = 8.0 Hz, $J_{2,3}$ = 9.8 Hz, 1 H, *H*-2^I), 4.91 (dd, $J_{1,2}$ = 7.9 Hz,1 H, *H*-1^{IV}), 4.86 (dt, $J_{3eq,4}$ = 4.6 Hz, $J_{3ax,4} = J_{4,5}$ = 12.0 Hz, 1 H,

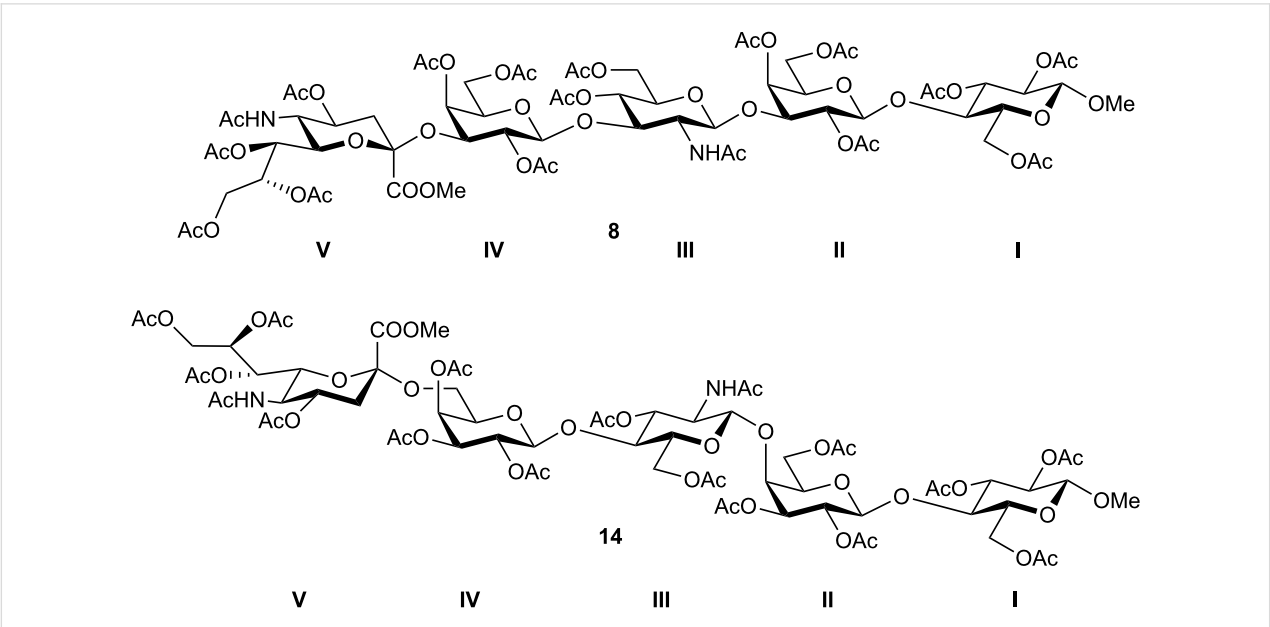


Figure 2: Roman numbering of saccharide units in all pentasaccharides for NMR assignment.

H-4^{V}), 4.83 (d, $J_{3,4}$ = 2.6 Hz, *H*-4^{IV}), 4.75 (dd, $J_{5,6}$ = 9.9 Hz, $J_{6,7}$ = 1.8 Hz, *H*-6^{V}), 4.61 (dd, 1 H, *H*-3^{III}), 4.57 (dd, $J_{2,3}$ = 9.8 Hz, $J_{3,4}$ = 2.6 Hz, 1 H, *H*-3^{IV}), 4.53 (dd, 1 H, *H*-6^{I}), 4.51 (d, $J_{1,2}$ = 8.0 Hz, 1 H, *H*-1^{I}), 4.39–4.37 (m, 2 H, *H*-1^{II}, *H*-1^{III}), 4.32 (dd, $J_{9a,9b}$ = 12.3 Hz, $J_{8,9b}$ = 2.1 Hz, *H*-9b^{V}), 4.26 (dd, $J_{6a,6b}$ = 12.2 Hz, $J_{5,6a}$ = 6.1 Hz, 1 H, *H*-6a^{III}), 4.13 (dd, $J_{5,6}$ = 9.8 Hz, *H*-5^{V}), 4.13 (d, 1 H, NH), 4.00 (dd, $J_{8,9a}$ = 5.8 Hz, 1 H, *H*-9a^{V}), 3.75 (s, 3 H, COOCH$_3$^{V}), 3.71 (bt, 1 H, *H*-5^{IV}), 3.55 (m, 1 H, *H*-5^{I}), 3.46–3.44 (m, 2 H, *H*-6a^{IV}, *H*-3^{II}), 3.43 (dd, $J_{6a,6b}$ = 11.0 Hz, $J_{5,6b}$ = 7.8 Hz, *H*-6b^{IV}), 2.59 (dd, $J_{3ax,3eq}$ = 12.2 Hz, $J_{3eq,4}$ = 4.6 Hz, 1 H, *H*-3eq^{V}), 2.21–1.35 (17s, 51 H, 15 OAc, 2 NAc), 1.93 (dd, $J_{3ax,4}$ = 12.0 Hz, 1 H, *H*-3ax^{V}). $C_{69}H_{96}N_2O_{44}$ (1657.49): Found C, 49.90; H, 5.69; N, 1.57. Calculated C, 50.00; H, 5.84; N, 1.69. MALDI-TOF: 1680.48 $(M+Na)^+$; 1696.59 $(M+K)^+$.

Methyl *O*-(methyl-5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-α-D-glycero-D-galacto-2-nonulopyranosylonate)-(2-6)-*O*-(2,3,4-tri-*O*-acetyl-β-D-galactopyranosyl)-(1-4)-*O*-(3,6-di-*O*-acetyl-2-azido-2-deoxy-β-D-glucopyranosyl)-(1-4)-*O*-(2,3-di-*O*-acetyl-6-*O*-*tert*-butyldiphenylsilyl-β-D-galactopyranosyl)-(1-4)-*O*-2,3,6-tri-*O*-acetyl-β-D-glucopyranoside (13): A solution of trisaccharide **10** (68 mg, 60 μmol) and disaccharide **12** (56 mg, 70 μmol) in anhydrous toluene (2 mL) was cooled to −40 °C. *N*-Iodosuccinimide (20 mg, 94 μmol), molecular sieves (4 Å, 200 mg) were added, and after cooling a saturated solution of trifluoromethane sulfonic acid in CCl_4 (ca. 2 M, 50 μL) was added with vigorous stirring. The mixture was gradually warmed over 2.5 h to −10 °C. Ethyl acetate (20 mL) was added and the reaction quenched by addition of a saturated aqueous $NaHCO_3$ solution (10 mL). After filtration through Celite, the phases were separated. The organic phase was washed with aqueous $Na_2S_2O_3$ solution (10 mL), dried over $MgSO_4$, evaporated and the residue purified by flash chromatography with petroleum ether/ethyl acetate 2:1. Compound **7** was obtained as a colorless amorphous solid; 58 mg (53%). $[\alpha]_D^{20}$ = −21.6 (*c* 0.3, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.70–7.24 (m, 10, Ph), 5.74 (dd, 1 H, *H*-4^{IV}), 5.70 (ddd, 1 H, *H*-8^{V}), 5.61 (dd, $J_{2,3}$ = 10.2 Hz, 1 H, *H*-2^{IV}), 5.44 (dd, $J_{7,8}$ = 9.0 Hz, 1 H, *H*-7^{V}), 5.26 (dd, 1 H, *H*-3^{IV}), 5.22 (t, $J_{2,3}$ = $J_{3,4}$ = 9.8 Hz, 1 H, *H*-3^{I}), 5.10 (dd, $J_{1,2}$ = 8.0 Hz, $J_{2,3}$ = 9.9 Hz, 1 H, *H*-2^{II}),4.93 (t, $J_{2,3}$ = 9.8 Hz, 1 H, *H*-2^{I}), 4.87 (2d, 2 H, *H*-1^{IV}, *H*-3^{III}), 4.85 (ddd, $J_{4,5}$ = 10.2 Hz, 1 H, *H*-4^{V}), 4.65 (dd, 1 H, *H*-3^{II}), 4.61 (d, $J_{3,4}$ = 4.0 Hz, *H*-4^{II}), 4.59 (dd, $J_{9a,9b}$ = 12.2 Hz, 1 H, *H*-9a^{V}), 4.51 (d, 1 H, *H*-1^{III}), 4.49 (d, $J_{1,2}$ = 8.0 Hz, 1 H, *H*-1^{II}), 4.48 (dd, 1 H, *H*-6b^{III}), 4.46 (d, $J_{1,2}$ = 10.0 Hz, 1 H, *H*-1^{I}), 4.42 (dd, 1 H, *H*-6b^{I}), 4.34 (dd, 1 H, *H*-9b^{V}), 4.32 (d, 1 H, N*H*^{V}), 4.29 (ddd, $J_{5,6}$ = 10.6 Hz, 1 H, *H*-5^{V}), 4.20 (dd, 1 H, *H*-6b^{III}), 4.13 (m, 2 H, *H*-6^{V}, *H*-6b^{II}), 4.10 (dd, $J_{5,6a}$ = 5.6 Hz, $J_{6a,6b}$ = 12.4 Hz, 1 H, *H*-6a^{I}), 4.08 (dd, $J_{5,6a}$ = $J_{6a,6b}$ = 6.0 Hz, 1 H, *H*-6a^{II}), 4.06 (dd, 1 H, *H*-6b^{IV}), 3.88 (ddd, 1 H, *H*-5^{IV}), 3.83 (m, 2 H, *H*-5I, *H*-4^{III}), 3.78 (s, 3 H, OCH_3), 3.77 (t, $J_{3,4}$ = 9.9 Hz, 1 H, *H*-4^{I}), 3.62 (ddd, $J_{4,5}$ = 10.0 Hz, $J_{5,6a}$ = 5.5 Hz, $J_{5,6b}$ = 2.0 Hz, 1 H, *H*-5^{I}), 3.61 (dd, 1 H, *H*-6b^{IV}), 3.59 (ddd, 1 H, *H*-5^{III}), 3.56 (dd, $J_{2,3}$ = 10.2 Hz, 1 H, *H*-2^{III}), 3.33 (s, 3 H, C*H*$_3$^{V}), 2.69 (dd, $J_{3eq,3ax}$ = 12.7 Hz, $J_{3eq,4}$ = 4.6 Hz, 1 H, *H*-3$_{eq}$^{V}), 2.15–1.36 (14s, 42 H, 13 OAc, 1 NAc), 2.03 (dd, $J_{3ax,4}$ = 12.0 Hz, 1 H, *H*-3$_{ax}$^{V}), 1.01 (s, 9 H, SiCCH$_3$). $C_{81}H_{108}N_4O_{42}Si$ (1837.81): Found C, 53.89; H, 6.34; N, 2.66. Calculated C, 52.94; H, 5.92; 3.04. MALDI-TOF: 1860.80 $(M+Na)^+$; 1876.91 $(M+K)^+$. The α,1^{III}-4^{II}-anomer of **7** was obtained as colorless syrup (9 mg, 8%) and not further characterized.

Methyl *O*-(methyl-5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosylonate)-(2-6)-*O*-(2,3,4-tri-*O*-acetyl-β-D-galactopyranosyl)-(1-4)-*O*-(3,6-di-*O*-acetyl-2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1-4)-*O*-(2,3,6-tri-*O*-acetyl-β-D-galactopyranosyl)-(1-4)-*O*-2,3,6-tri-O-acetyl-β-D-glucopyranoside (14): Compound **13** (53 mg, 29 μmol), $NiCl_2 \cdot 6H_2O$ (105 mg, 450 μmol) and boric acid (55 mg, 900 μmol) were dissolved in ethanol (3 mL). Under vigorous stirring a suspension of sodium borohydride (28 mg, 750 μmol) in ethanol (1 mL) was added with the temperature maintained at 20 °C. After 30 min ethanol (6 mL) and acetic acid (3 mL) were added. Then the mixture was co-distilled three times with toluene (5 mL each), and then the residue dissolved in dichloromethane (10 mL). After washing with diluted aqueous $KHSO_4$ solution (5 mL), saturated aqueous $NaHCO_3$ solution (5 mL), and water (5 mL), the organic phase was dried ($MgSO_4$) and evaporated to dryness. The resulting material was treated with dichloromethane/trifluoroacetic acid (9:1, 2 mL) for 1 h at room temperature, then co-distilled three times with toluene (5 mL each) and dried under high vacuum. The residue was treated with acetic anhydride (1 mL) and pyridine (5 mL) for 10 h, then co-distilled three times with toluene (5 mL each). Purification by flash chromatography (toluene/acetone 3:1) gave **14** (35 mg, 67%) as a colorless amorphous solid; $[\alpha]_D^{20}$ = −31.2 (*c* 0.4, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.72–7.25 (m, 10, Ph), 5.72 (ddd, 1 H, *H*-8^{V}), 5.70 (dd, 1 H, *H*-4^{IV}), 5.59 (dd, $J_{2,3}$ = 9.9 Hz, 1 H, *H*-2^{IV}), 5.43 (dd, $J_{7,8}$ = 8.8 Hz, 1 H, *H*-7^{V}), 5.32 (d, $J_{3,4}$ = 4.0 Hz, *H*-4^{II}), 5.23 (dd, 1 H, *H*-3^{IV}), 5.21 (t, $J_{2,3}$ = $J_{3,4}$ = 10.2 Hz, 1 H, *H*-3^{I}), 5.11 (dd, $J_{1,2}$ = 7.8 Hz, $J_{2,3}$ = 9.8 Hz, 1 H, *H*-2^{II}), 4.90 (t, $J_{2,3}$ = 10.0 Hz, 1 H, *H*-2^{I}), 4.88 (d, 1 H, *H*-1^{IV}), 4.86 (m, 2 H, *H*-3^{III}, *H*-4^{V}), 4.63 (dd, 1 H, *H*-3^{II}), 4.59 (dd, $J_{9a,9b}$ = 11.9 Hz, 1 H, *H*-9a^{V}), 4.53 (d, $J_{1,2}$ = 8.0 Hz, 1 H, *H*-1^{II}), 4.50 (d, 1 H, *H*-1^{III}), 4.47 (m, 2 H, *H*-6b^{III}, *H*-1^{I}), 4.39 (dd, 1 H, *H*-6b^{I}), 4.32 (d, 1 H, N*H*), 4.31 (dd, 1 H, *H*-9b^{V}), 4.27 (ddd, $J_{5,6}$ = 10.3 Hz, 1 H, *H*-5^{V}), 4.18 (dd, 1 H, *H*-6b^{III}), 4.11 (dd, 1 H, *H*-6^{V}), 4.09 (dd, $J_{5,6a}$ = 5.8 Hz, $J_{6a,6b}$ = 12.2 Hz, 1 H, *H*-6a^{I}), 4.01 (dd, 1 H, *H*-6b^{IV}), 3.92 (ddd, 1 H, *H*-5^{IV}), 3.87 (dd, $J_{5,6a}$ = $J_{6a,6b}$ = 6.0 Hz, 1 H, *H*-6a^{II}), 3.85 (dd,

1 H, *H*-6b^{II}), 3.82 (m, 2 H, *H*-5^{II}, *H*-4^{III}), 3.77 (s, 3 H, OCH_3), 3.75 (t, $J_{3,4}$ = 10.1 Hz, 1 H, *H*-4^{I}), 3.65 (ddd, $J_{4,5}$ = 9.8 Hz, $J_{5,6a}$ = 5.6 Hz, $J_{5,6b}$ = 1.8 Hz, 1 H, *H*-5^{I}), 3.59 (m, 2 H, *H*-6b^{IV}, *H*-5^{III}), 3.57(dd, $J_{2,3}$ = 10.0 Hz, 1 H, *H*-2^{III}), 3.31 (s, 3 H, CH_3^{V}), 2.70 (dd, $J_{3eq,3ax}$ = 12.5 Hz, $J_{3e,4}$ = 4.4, 1 H, *H*-3eq^{V}), 2.17–1.33 (16s, 48 H, 14 OAc, 2 NAc), 2.01 (dd, $J_{3ax,4}$ = 12.0 Hz, 1 H, *H*-3ax^{V}), 1.00 (s, 9 H, $SiCCH_3$). $C_{69}H_{96}N_2O_{44}$ (1657.49): Found C, 49.86; H, 5.77; N, 1.65. Calculated C, 50.00; H, 5.84; N, 1.69. MALDI-TOF: 1680.39 $(M+Na)^+$; 1696.59$(M+K)^+$.

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Benzyne arylation of oxathiane glycosyl donors

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Preliminary Communication

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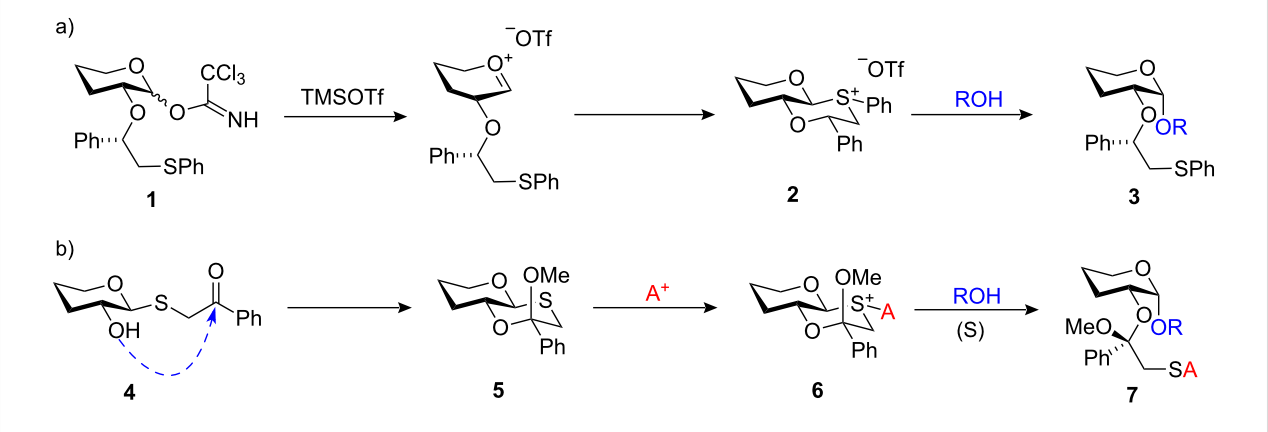
Abstract

The arylation of bicyclic oxathiane glycosyl donors has been achieved using benzyne generated in situ from 1-aminobenzotriazole (1-ABT) and lead tetraacetate. Following sulfur arylation, glycosylation of acetate ions proceeded with high levels of stereoselectivity to afford α-glycosyl acetates in a 'one-pot' reaction, even in the presence of alternative acceptor alcohols.

Introduction

Carbohydrates play important roles in many biological processes including tumour metastasis [1,2], bacterial and viral recognition [3-5], and the immunological response [6-8]. In order to obtain pure samples of oligosaccharides for biological studies, carbohydrate chemists must overcome the myriad challenges presented by their complex synthesis. The most important challenge is control over the stereoselectivity of reactions at the anomeric centre; in particular for the stereoselective synthesis of 1,2-*cis*-glycosides [9]. This area has been the subject of much fervent study in the last two decades, and has led to many significant developments [10,11]. However, despite these advances, modern synthetic carbohydrate chemistry has still to provide a general method for the efficient synthesis of 1,2-*cis*-α-glycosidic linkages.

In 2005 Boons and co-workers reported an elegant chiral auxiliary-based glycosylation protocol for the synthesis of 1,2-*cis*-α-glycosides [12]. Completely stereoselective glycosylation was achieved when a thiophenyl-containing chiral auxiliary was attached to *O*-2 of an imidate glycosyl donor **1** (Scheme 1a). Low temperature ^{1}H NMR spectroscopy studies confirmed the formation of a quasi-stable *trans*-decalin intermediate **2**, which was able to cause glycosylation to take place from the α-face of the glycosyl donor. We sought to improve this strategy and recently reported a novel class of bicyclic oxathiane ketal donors **5** containing an inbuilt α-directing group (Scheme 1b) [13]. The principal objective of our approach was to develop a thioglycoside donor that could mimic the key *trans*-decalin intermediate **2** by using the sulfur-containing auxiliary as both the anomeric leaving group and α-directing participating group. An efficient synthesis of the key bicyclic intermediate was achieved starting from a simple thioglycoside **4** where the essential β-sulfur linkage was already installed, followed by a regio and stereoselective cyclisation onto the *O*-2 position to afford oxathiane glycosyl donor framework **5**. The oxathiane ketal donor **5** is then already pre-organised to give a 1,2-*cis* directing group upon activation, and afford 1,2-*cis*-glycosides **7** on alcohol addition.



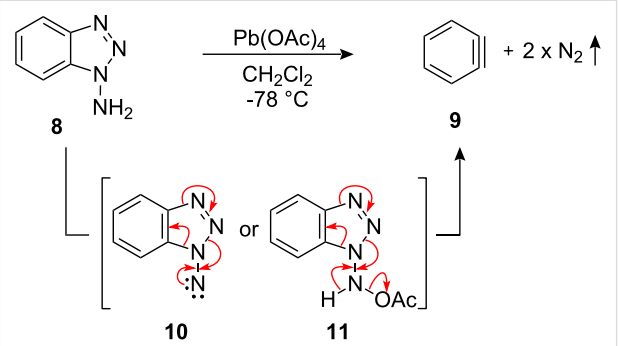
Scheme 1: a) Boons' chiral auxiliary-based approach to α-stereoselective glycosylations. b) Modified strategy for stereoselective glycosylations using oxathiane ketal glycosyl donors **5**.

Following the synthesis of the oxathiane ketal glycosyl donors **5**, activation of the β-thioglycoside linkage was necessary to form the key *trans*-decalin sulfonium ion **6**, and turn the α-directing participating group into an anomeric leaving group reactive enough to partake in glycosylations. Thioglycosides are widely used as glycosyl donors [14,15], and many different reagents are available for their activaton including *N*-iodosuccinimide (NIS)/TMSOTf [16], dimethyl(methylthio)sulfonium trifluoromethanesulfonate (DMTST) [17,18], PhSeOTf [19], MeS-SMe/Tf_2O [20], and MeOTf [21-23]. However, in order to recreate the reactive sulfonium ion used by Boons, it would be necessary to activate the anomeric sulfur with a phenyl group. Herein we describe our synthetic endeavours to achieve this goal and the first use of benzyne as an activating agent for thioglycosides [24].

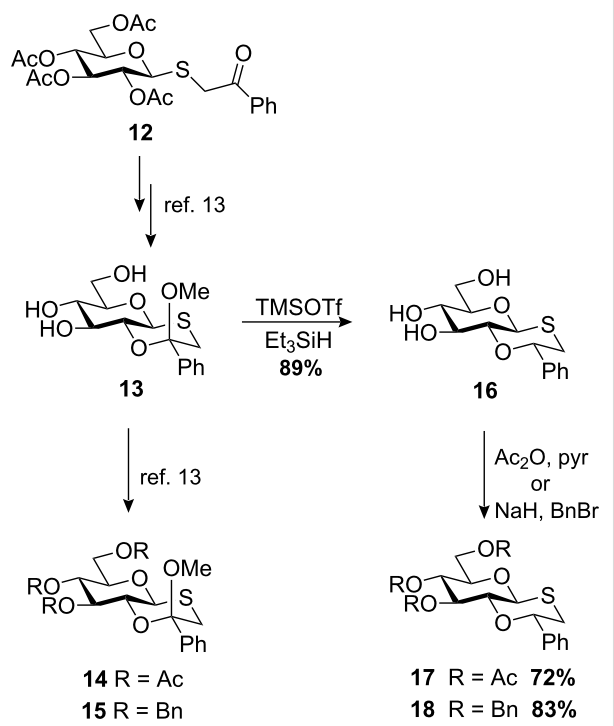
Results and Discussion

The method chosen for in situ benzyne (**9**) generation was the reaction of 1-aminobenzotriazole (1-ABT) (**8**) with lead tetraacetate by the procedure pioneered by Rees and co-workers (Scheme 2) [25-28]. The low reaction temperature was expected to be compatible with stereoselective glycosylation. Following the reaction of 1-ABT (**8**) with lead tetraacetate, benzyne (**9**) formation is believed to occur via degradation of either an *N*-nitrene intermediate **10** or an *N*-acetate substituted intermediate **11** the driving force for which is the release of di-nitrogen [29].

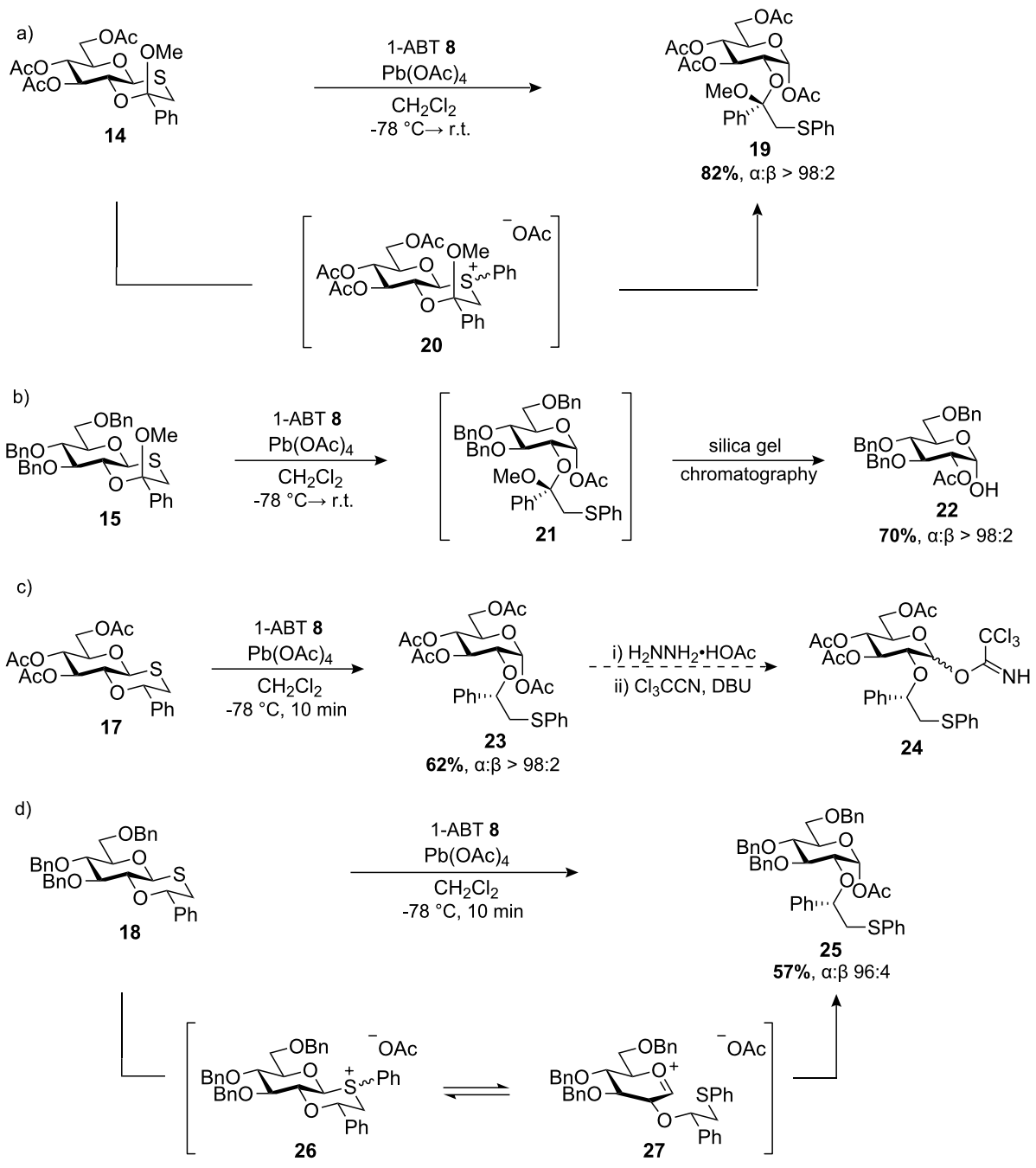
The synthesis of oxathiane ketal **13** was achieved in two steps from thioglycoside **12** as previously reported, followed by protection to afford the acetylated oxathiane ketal **14** or the benzylated oxathiane ketal **15** (Scheme 3) [13]. Ketal **13** was also reduced to the novel oxathiane ether **16** in 89% yield, and



Scheme 2: Benzyne generation from 1-ABT.



Scheme 3: Oxathiane donor synthesis.



Scheme 4: Arylation/acetate glycosylation of oxathiane glycosyl donors.

protected to afford acetylated oxathiane ether **17** and benzylated oxathiane ether **18** in yields of 72% and 83%, respectively.

With the oxathiane ketal and ether donors in hand, initial studies focussed on benzyne arylation in the absence of any alcohol acceptor. Unexpectedly, when activated under the reaction conditions, acetylated oxathiane ketal **14** afforded α-glycosyl acetate **19** stereoselectively in 82% yield (Scheme 4a). Presumably, the mechanism proceeds via sulfur arylation with benzyne to afford putative phenyl sulfonium ion **20**, followed by glycosylation of the acetate anion. The very high α-stereoselectivity (α:β > 98:2) of the reaction was in line with selectivities previously observed for intermediate oxathiane sulfonium ions [13]. When the reaction was repeated in the absence of 1-ABT, the starting material was unchanged, thus precluding the possibility of initial sulfur activation by lead tetraacetate.

Arylation of benzylated oxathiane ketal **15** under identical conditions also afforded α-glycosyl acetate **21** as the sole crude product as evidenced by ^{1}H NMR spectroscopy (Scheme 4b). However, only α-hemiacetal **22** was isolated in 70% yield following purification by flash silica chromatography [30]. Cleavage of the acyclic *O*-2 ketal on glycosyl acetate **21**, followed by acetyl transfer from *O*-1 to *O*-2 could account for this transformation [31].

Arylation/acetate glycosylation using oxathiane ether donor **17** also occurred readily to give the α-glycosyl acetate **23** in 62% yield with complete anomeric control (Scheme 4c). It is of interest to note that glycosyl acetate **23** has been reported previously by Boons and co-workers as an advanced intermediate in their synthesis of trichloroacetimidate donor **24** bearing a 2-*O*-(1*S*)-phenyl-2-(phenylsulfanyl)ethyl group (Scheme 4c) [12]. Although the benzyne arylation method does not allow us to access α-glycosides directly, it could be beneficial as an alternative route to glycosyl donors bearing the Boons' participating group. This strategy has the advantage of utilising the inherent chirality of the sugar to determine the stereochemistry of the key benzylic centre in the chiral auxiliary, and also facilitates regioselective attachment of the auxiliary group to *O*-2.

The benzylated oxathiane ether **18** also afforded a glycosyl acetate **25** in 57% yield but on this occasion as a 96:4 (α:β) mixture of anomers (Scheme 4d). This slight drop in stereoselectivity is consistent with the increased reactivity of benzylated relative to acetylated thioglycoside donors [13,32], commonly attributed to greater stabilisation of the developing positive charge on an oxacarbenium intermediate **27** on the reaction pathway. Overall, reactions using the oxathiane ether donors proceeded more rapidly than those with the oxathiane ketal donors, indicating that the methoxy substituent moderates the reactivity of the glycosyl donors.

Attempts to intercept putative phenyl sulfonium ions such as **20** and **26** in glycosylation reactions with other acceptors prior to acetate glycosylation were in vain, presumably due to the high effective concentration of acetate anions in solution. Therefore, alternative oxidising agents for benzyne formation were also investigated in the hope that glycosylation with external alcohols would be easier to achieve if the phenyl sulfonium ion was formed with a less reactive counter ion. However, oxidation of 1-ABT in the presence of ketal **14** with NIS [33], or hypervalent iodine (III) with either bis(acetoxy)iodobenzene [$PhI(OAc)_2$] [34] or bis(trifluoroacetoxy)iodobenzene [$PhI(OCOCF_3)_2$] were unsuccessful [35], resulting in at best only trace amounts of phenyl sulfonium ion formation. Under these reaction conditions, nitrogen evolution and presumably benzyne formation, was much slower than when using lead tetraacetate as the oxidising agent. Further studies using the more reactive Zefirov's reagent (μ-oxobis[(trifluoromethanesulfonato)(phenyl)iodine]) [36,37] were also undertaken. Preliminary results were promising yielding simple α-glycosides and a full study will be reported in due course. Unfortunately, attempted extension of the arylation/acetate glycosylation methodology to conventional thiophenyl glycosyl donors was disappointing, as experiments either did not proceed to completion, or were hampered by oxidation of the thiophenyl group in the presence of lead tetraacetate [38].

Conclusion

In conclusion, it has been demonstrated that benzyne arylation of novel oxathiane glycosyl donors can be achieved using a combination of 1-ABT and lead tetraacetate. Following arylation, glycosylation with an acetate anion takes place with a high degree of stereoselectivity to afford 1,2-*cis*-α-acetates.

Supporting Information

Supporting Information File 1 features full experimental data for the synthesis of compounds **16–19**, **22**, **23** and **25**.

Supporting Information File 1

Experimental data for the synthesis of compounds **16–19**, **22**, **23** and **25**.
[http://www.beilstein-journals.org/bjoc/content/supplementary/1860-5397-6-19-S1.pdf]

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(Pseudo)amide-linked oligosaccharide mimetics: molecular recognition and supramolecular properties

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Review

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Abstract

Oligosaccharides are currently recognised as having functions that influence the entire spectrum of cell activities. However, a distinct disadvantage of naturally occurring oligosaccharides is their metabolic instability in biological systems. Therefore, much effort has been spent in the past two decades on the development of feasible routes to carbohydrate mimetics which can compete with their *O*-glycosidic counterparts in cell surface adhesion, inhibit carbohydrate processing enzymes, and interfere in the biosynthesis of specific cell surface carbohydrates. Such oligosaccharide mimetics are potential therapeutic agents against HIV and other infections, against cancer, diabetes and other metabolic diseases. An efficient strategy to access this type of compounds is the replacement of the glycosidic linkage by amide or pseudoamide functions such as thiourea, urea and guanidine. In this review we summarise the advances over the last decade in the synthesis of oligosaccharide mimetics that possess amide and pseudoamide linkages, as well as studies focussing on their supramolecular and recognition properties.

Review

Among the major classes of biomolecules, carbohydrates are characterized by nearly unlimited structural diversity. Monosaccharide units can combine to produce oligosaccharides in a number of permutations that increases rapidly with the number of units present, more so than is the case with other biomolecules such as polypeptides or oligonucleotides. This is determined by the stereochemical identity of the monosaccharide units present (e.g. glucose, galactose, mannose), their glycosidic linkage positions (e.g. 1→4, 1→6), the nature at anomeric centres (α or β), the presence of additional substituents such as sulfate or acyl groups and the overall degree of branching. The molecular diversity of oligosaccharide offers a valuable tool for drug discovery in the areas of biologically important oligosaccharides, glycoconjugates and molecular scaffolds by investigating their structural and functional impact. Currently, oligosaccharides are known to have functions in a

broad variety of cell–cell interactions related to invading bacteria, viruses and cancer cells [1-4] and to play central roles in post-translational modifications of proteins [5-7], cell–cell communication [8] and immune response to pathogens [7,9-11]. However, the application of oligosaccharides as potential therapeutic agents has its main drawback in the low instability of natural carbohydrates in biological systems. In addition, solid phase synthesis of oligosaccharides is not yet efficient enough for generating oligosaccharide-based libraries that may be useful in the future for the discovery of new therapeutic drugs. Taking into account the increasing importance of glycobiology and the difficulties associated to the synthesis of carbohydrate-based libraries, several approaches based on the assembly of sugar building blocks through amide and pseudoamide linkages have been developed by different research groups over the last few years. Sugar aminoacids (SAAs, Figure 1) have extensively been used in the development of a large variety of molecules. In the past 10 years, some reviews on SAAs have summarised the different synthetic methods used to obtain such molecules [12-14] as well as their applications to access to a diversity of linear, branched and cyclic pseudooligosaccharides and glycomimetics often referred to as carbopeptoids [13-17]. In addition, SAAs have been widely used in the field of material sciences as carbohydrate-derived monomers for the design of novel chiral polyamides [18]. Nevertheless, these review articles mainly focus on amide-linked sugars and do not pay too much attention to other types of pseudosugars. A notable exception is the contribution by Wessel and Dias Lucas, who recently published an interesting review where oligosaccharide mimetics which deviate from the natural linking pattern were discussed [19]. This review also included pseudoamide-linked sugars (Figure 1).

In this current review we have tried to highlight the recent advances in the synthesis of oligosaccharide mimetics. We have focussed on amide and pseudoamide-linked oligosaccharides, and their molecular recognition properties. We intend this survey to be wide-ranging and cover the most recent trends in the field.

Amide-linked sugars: carbopeptoids

The replacement of the glycosidic oxygen atom by an amide group leads to amide-linked sugars (Figure 1), which are synthesised by the sequential coupling of SAAs to form a class of compounds commonly referred to as carbopeptoids. The use of the amide functional group to connect sugar building blocks is inspired by the structure of peptides and the potential of carbohydrates to reproduce the structural features and biological properties of these polymers [12,13,17]. Poor bioavailability and metabolic stability of peptides have resulted in significant limitations as drug candidates. Another general problem is the loss of the original conformation in the isolated peptide fragments present in natural proteins, where the rest of molecule fixes a specific spatial disposition. Furthermore, short linear peptides cannot be restricted to adopt a particular conformation that enables effective interaction with a receptor [20]. As a consequence, a wide variety of methods to restrict the conformational freedom has been developed. One approach to get round this problem is the isosteric replacement of the amide bond in the peptide with a suitable mimetic to induce a specific secondary structure. Recent investigations have demonstrated that incorporation of SAAs in peptide structures can circumvent the adverse properties of bioactive natural systems. Thus, SAAs containing amino and carboxylic functional groups, and a rigid ring system (pyran, furan, oxetane) have emerged as versatile and conformationally constrained building blocks for the formation of peptide and oligosaccharide mimetics using standard peptide coupling techniques in solution or in the solid phase. Exploitation of the rigidity and diversity of the sugar backbone should permit subtle modifications and the rational design of oligomeric derivatives with tendency to adopt specific compact conformations (foldamers).

Linear naturally-occurring SAA homo-oligomers

SAAs occur extensively in nature as subunits of oligosaccharides in cell walls such as *N*-acetylneuraminic **1** and muramic acids **2**, as well as in some nucleoside antibiotics (e.g., gougerotin **3** and aspiculamycin **4**) (Figure 2).

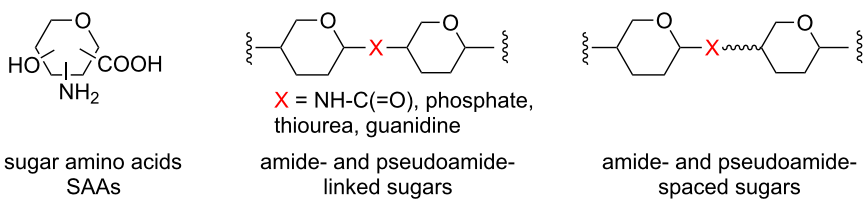


Figure 1: Schematic representation of sugar aminoacids (SAAs) and (pseudo)amide oligosaccharide mimetics.

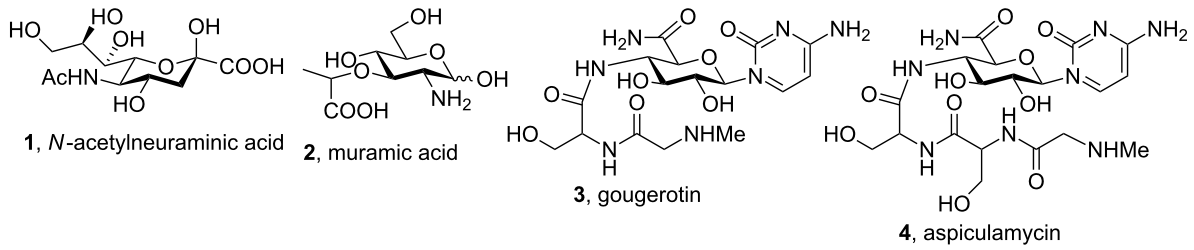


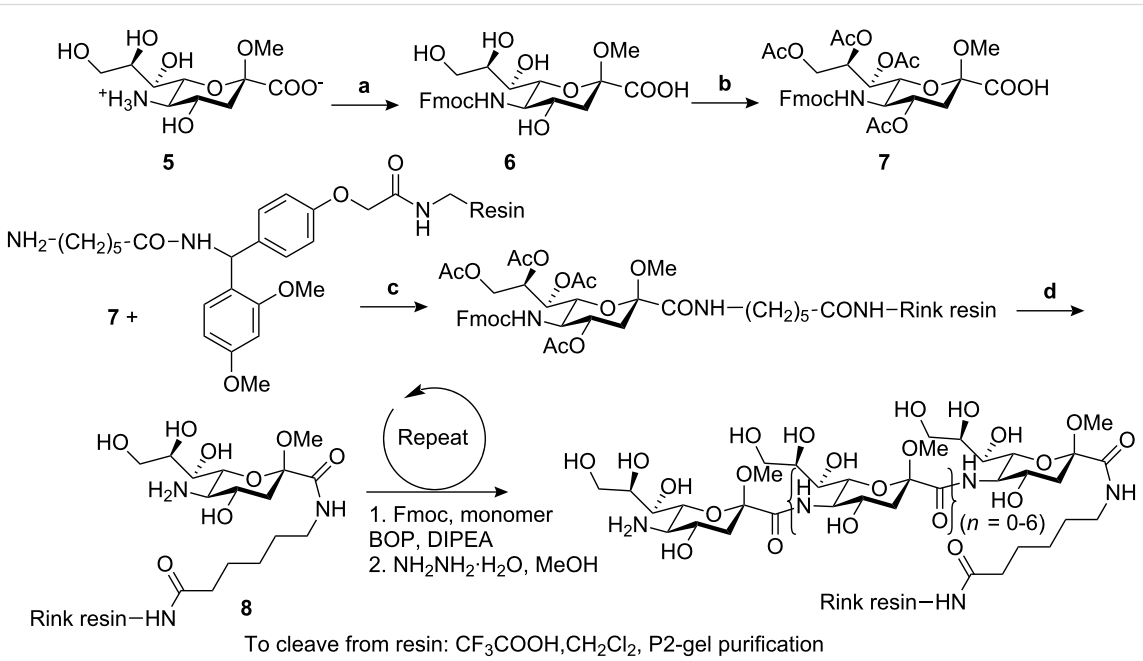
Figure 2: Natural SAAs structures and natural nucleosidic antibiotics.

N-Acetylneuraminic acid is a readily available naturally occurring δ amino acid. By applying solid phase peptide methods on Rink resin with Fmoc protecting group chemistry [21,22], Gervay and co-workers have reported a series of (1→5) amide-linked sialooligomers ranging from dimeric to octameric species (Scheme 1) [23,24]. All of these were soluble in water, DMSO and methanol, which allowed NH/ND exchange NMR experiments and circular dichroism (CD) studies to be carried out. Combined structural studies showed that a tetramer is required for an ordered secondary structure [23,25].

Linear non-naturally-occurring SAA homo-oligomers

The first examples of amide-linked SAA-pyranose oligomers were reported as long ago as 1975 by Fuchs and Lehman [26,27]. In 2002, Ichikawa and co-workers developed a synthetic methodology to access oligomers of glycoamino acids, a family of non-natural SAAs with a carboxyl group at C-1 position and an amino group replacing one of the hydroxyl groups at either the C-2, 3, 4 or 6 positions (Figure 3).

Glucose-type building blocks were prepared and used to construct β(1→2), β(1→3), β(1→4) and β(1→6)-linked homo-oligomers [28-30] that were found to form rigid secondary structures predetermined by the linkage position as evidenced from CD and NMR measurements. Moreover, conformational analysis by molecular modelling calculation on the β(1→2)-linked decamer **9** supported a helical arrangement, stabilised by a intramolecular hydrogen bonding in the form of a 16-membered ring, characteristic for β-peptides (Figure 4) [29]. Analogously, NMR and IR data of a β(1→6)-linked unsaturated glycamino acid tetramer showed a turn-like structure in chloroform solution [31].



Scheme 1: Synthetic route to the target amide-linked sialooligomers. (a) Fmoc-Cl, $NaHCO_3$, H_2O, dioxane, 0 °C. (b) Ac_2O, Pyridine, 0 °C. (c) BOP, DIPEA. (d) $NH_2NH_2{\cdot}H_2O$, MeOH.

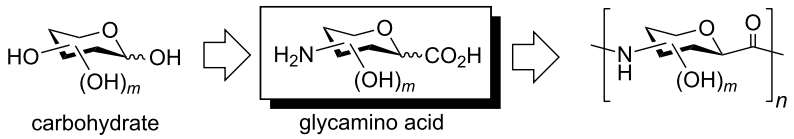


Figure 3: The general structure of glycoamino acids and their corresponding oligomers.

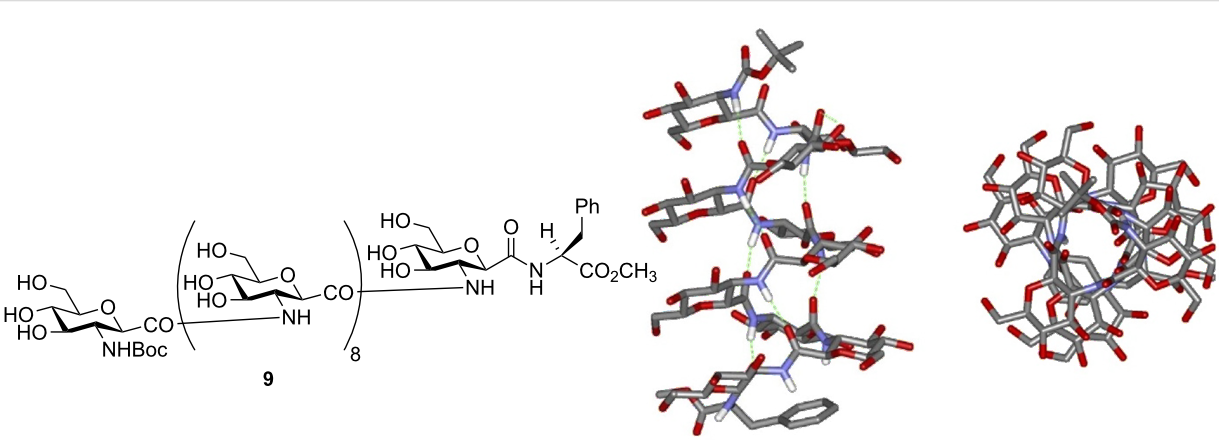


Figure 4: Conformational analysis of the β(1→2)-amide-linked glucooligomer **9**.

Glycoamino acid-based carbopeptoids are resistant to glycosidases and exhibit interesting biological activities. Thus, the *O*-sulfated β(1→2) and β(1→6)-linked homo-oligomers effectively inhibit L-selectin-mediated cell adhesion, HIV infection and heparanase acitivity in a linkage-specific manner [28].

SAAs can have a wide range of structural diversity as a consequence of differing on ring sizes, structural manipulation (such as chain branching, deoxygenation, hydroxyl protection, unsaturation), relative position between amine and carboxylic function, and, perhaps most importantly, the spatial arrangement of the amine and acid moieties. The limitations of stereocontrol of glycosidic linkages can be circumvented by their replacement by an amide function. Furthermore, as a result of the presence of amine and acid moieties, SAAs are suitable for conventional combinatorial synthesis in both solid and solution phase. Many of these aspects have been extensively studied by Fleet's group over the last 10 years. Efficient synthetic procedures for protected and unprotected homo-oligomeric derivatives of D-*arabino*- (Figure 5) [32-34] , D-*talo*- [35], L-*allo*- [35], D-*galacto*- [36], D-*allo*- [37], D-*lyxo*- [38], L-*xylo*- [39] and L-*ribo*-configured [39] tetrahydrofuran (THF) amino acids were optimised in order to study the influence of ring configuration and protecting groups on the secondary structure in these carbopeptoids.

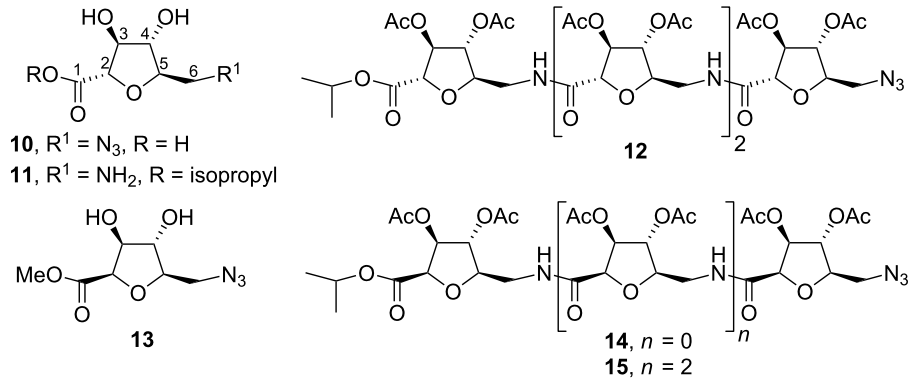


Figure 5: Short oligomeric chains of *C*-glycosyl D-*arabino* THF amino acid oligomers.

Short oligomeric chains of *C*-glycosyl β-D-*arabino* THF amino acids **14** and **15** (where the C-2 and C-5 substituents of the THF ring are *cis* to each other) exhibit a well defined repeating turn secondary structure stabilised by inter-residual hydrogen bonds, whereas the epimeric α-D-*arabino*furanose oligomer **12** (with the C-2 and C-5 substituents in *trans* relative disposition) do not show any secondary structure in solution [34]. NMR and IR studies on D-*galacto*- [36] and D-*allo*-configured [37] oligomeric carbopeptoids demonstrated that the inversion of a single stereocentre on the THF ring can have more pronounced effects for solution conformations than the presence or absence of different protecting groups. Thus, 2,4-*cis*-THF-L-ribonate oligomers adopt hydrogen bond stabilised conformations whereas 2,4-*trans*-THF-L-xylonate oligomers do not. Additionally, a number of structurally related THF aminoacid oligomers were examined by chiroptical spectroscopy to aid interpretation of their conformational preferences. The use of CD, in addition to NMR and solution IR, enabled the classification of the conformations adopted by carbopeptoids as hydrogen bonded regular, non-hydrogen bonded regular and non-hydrogen bonded irregular [40]. "Regular" is used to define a conformation that is either hydrogen bonded or non-hydrogen bonded and not in equilibrium with other multiple conformations. If conformational exchange operates, the term "irregular" is used. For example, these studies demonstrated that an octameric chain of *C*-glycosyl α-D-*lyxo* furanose amino acids **16** adopts a regular hydrogen bonded conformation similar to an π-helix (Figure 6) [38], whereas α-D-*lyxo* [38], D-*talo* and L-*lyxo* tetramers have regular non-hydrogen bonded conformations [40]. Also, based on these techniques, Andreini et al. [41] have recently demonstrated that homo-oligomers of β-SAA (β-*N*-mannofuranosyl-3-ulosonic acid) adopt eight-membered ring hydrogen bonded double-turn regular conformations in solution.

Chakraborty's group has also prepared protected and unprotected homo-oligomeric derivatives of D-*manno*- [42] and D-*gluco*-configured [43] tetrahydrofuran (THF) amino acids and studied their conformational properties by chiroptical and NMR techniques. Although both hydroxyl-protected linear oligomers displayed well-defined turn structures, their unprotected counterparts resulted in a less robust and regular conformation. However, in some cases the studies of the conformational preference in carbopeptoids is quite challenging and must be argued by computational methods [38,44] and infrared ion-dip spectroscopy [45] in order to interpret the data from CD, IR and NMR experiments reliably.

To complement its research programme on foldamer design and because of the absence of structural investigations on linear homo-oligomers constrained by 4-membered rings, Fleet and co-workers undertook the synthesis of carbopeptoids based on the oxetane template (Figure 7) [46-48]. Conformational analysis was carried out for two oxetane β-SAA hexamers with the 6-deoxy-L-*altro* and D-*arabino* configurations by means of detailed NMR and IR studies in combination with molecular mechanics [49]. These studies identified a left-handed helical secondary structure for the 6-deoxy-L-*altro*-oxetane hexamer and a right-handed helical structure for the D-*arabino*-configured oxetane hexamer **21** (having the opposite absolute configuration at C-2 and C-3) stabilised in both instances by 10-membered ring hydrogen bond arrangement (Figure 8).

Similar conformational studies combined with CD spectroscopy have been carried out for homo-oligomers of silyl-protected δ-2,4-*trans*-oxetane-SAAs with different configurations. The results showed that 6-deoxy-L-altronate, D-fuconate and 6-deoxy-D-gulonate oligomers are characterised by irregular non-hydrogen bonded conformations whereas L-rhamnoate and D-lyxonate oligomers have regular conformations governed by sterical interactions [50]. In contrast to the δ-2,4-*trans*-oxetane-SAA oligomers, the δ-2,4-*cis*-oxetane-L-ribonate tetramer and hexamer adopted a repeating β-turn structure [51]

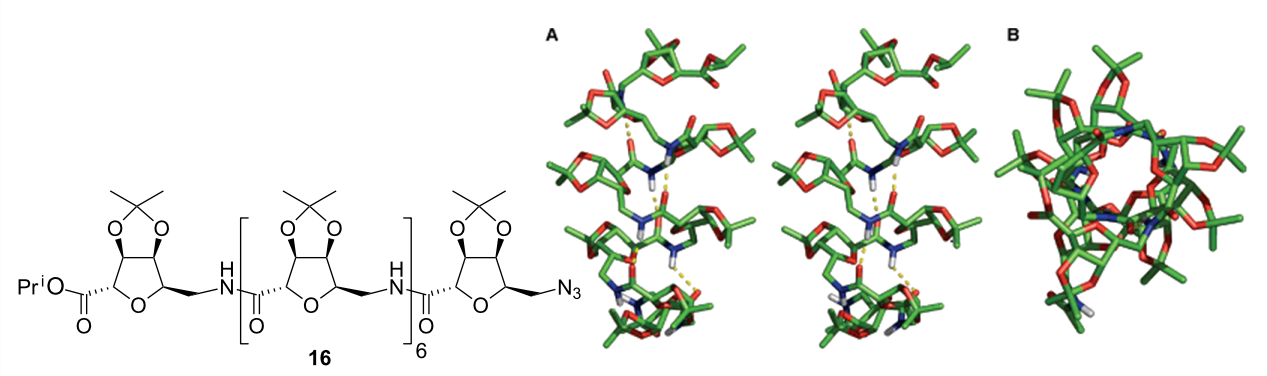


Figure 6: (A) Stereoview of the minimized structure of compound **16** (produced by a 500 ps simulation) that most satisfies the 27 NOE derived distance constraints. (B) View looking from *C* to *N* terminus. Hydrogen bonds are indicated by dotted lines.

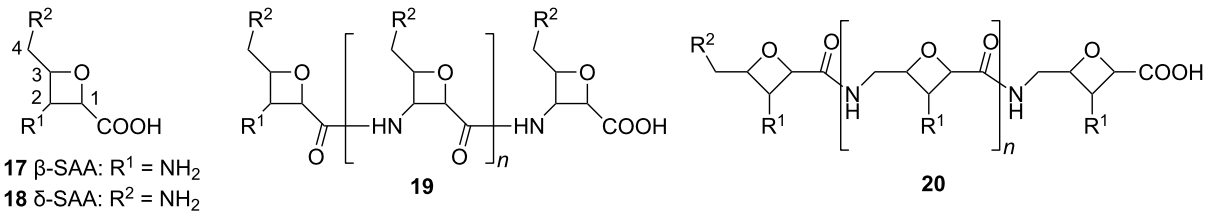


Figure 7: Structures of linear oxetane-β- and δ-SAA homo-oligomers **19**–**20**.

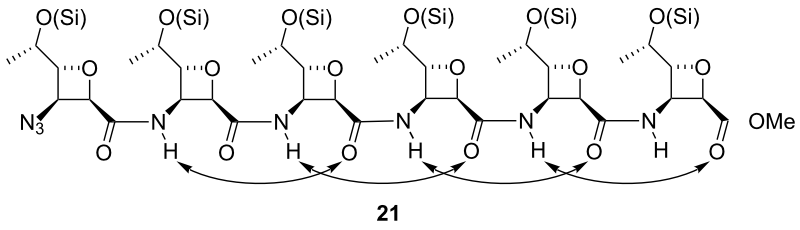


Figure 8: 10-Membered ring H-bonds in compound **21** consistent with NMR and modelling investigations.

dictated by internal 10-membered hydrogen bonded rings. Such conformations are similar to those reported for the δ-2,5-*cis*-oxetane-THF oligomers [37].

D'Onofrio et al. [52] have carried out solid phase synthesis of oligonucleotides conjugated at the 3′ terminus with (1→6)-amide-linked oligosaccharide mimics (Figure 9). The presence of the saccharide unit at the 3′-end of 18-mers significantly enhanced the stability of the oligonucleotides in bovine fetal serum, non-negatively interfering with their ability to form stable duplexes with complementary DNA strands, as evaluated by UV thermal denaturation studies.

Orthogonally protected sugar diamino acids (SDA) were first synthesised by Sicherl and Wittmann [53] and used to form linear and branched amide-linked mimetics (Figure 10).

Due to the various possibilities with which SDAs can be connected to each other, a high degree of diversity can be achieved by employing only a small set of different SDAs. Moreover, oligomeric SDAs with unprotected groups represent a novel type of aminoglycoside mimetics with potential recognition properties towards new RNA targets emerging in the post-genome era.

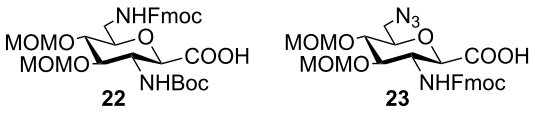


Figure 10: Protected derivatives of 2,6-diamino-2,6-dideoxy-β-D-glucopyranosyl carboxylic acid **22** and **23**.

Cyclic SAA homo-oligomers

A cyclic array of desired ring size and defined secondary structure of alternating carbohydrate moieties and amide groups might conceivably lead to exquisite specificity of recognition and catalysis. One application of this concept is the mimicry of

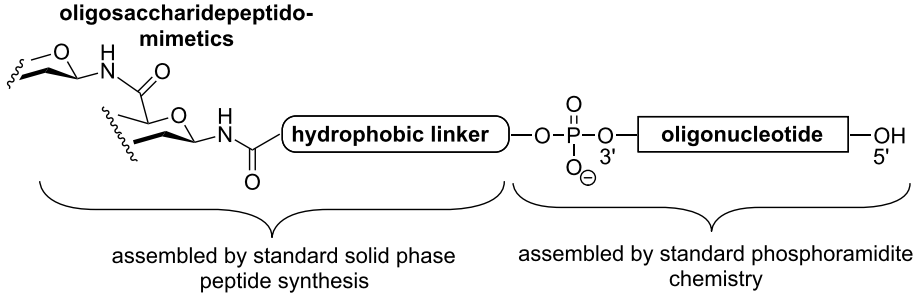


Figure 9: General structure of carbopeptoid-oligonucleotide conjugates.

cyclodextrin inclusion complexes. Thus, Kessler and co-workers have reported the synthesis of cyclic oligomers containing glucopyranosyluronic acid by exploiting standard solid and solution phase coupling procedures [54]. These cyclic homo-oligomers of SAAs behave as host molecules that form inclusion complexes with *p*-nitrophenol and benzoic acid. Following the same objective, Xie's group prepared orthogonally protected cyclic homo-oligomers with two to four SAA units that can be selectively or fully deprotected to afford macrocycles that can undergo further functionalisation (Figure 11) [55].

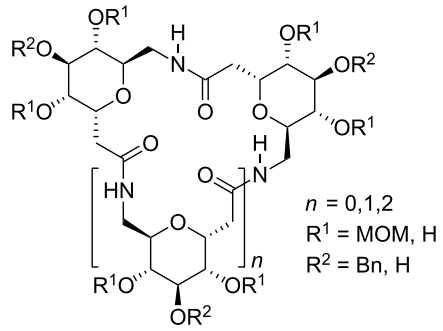
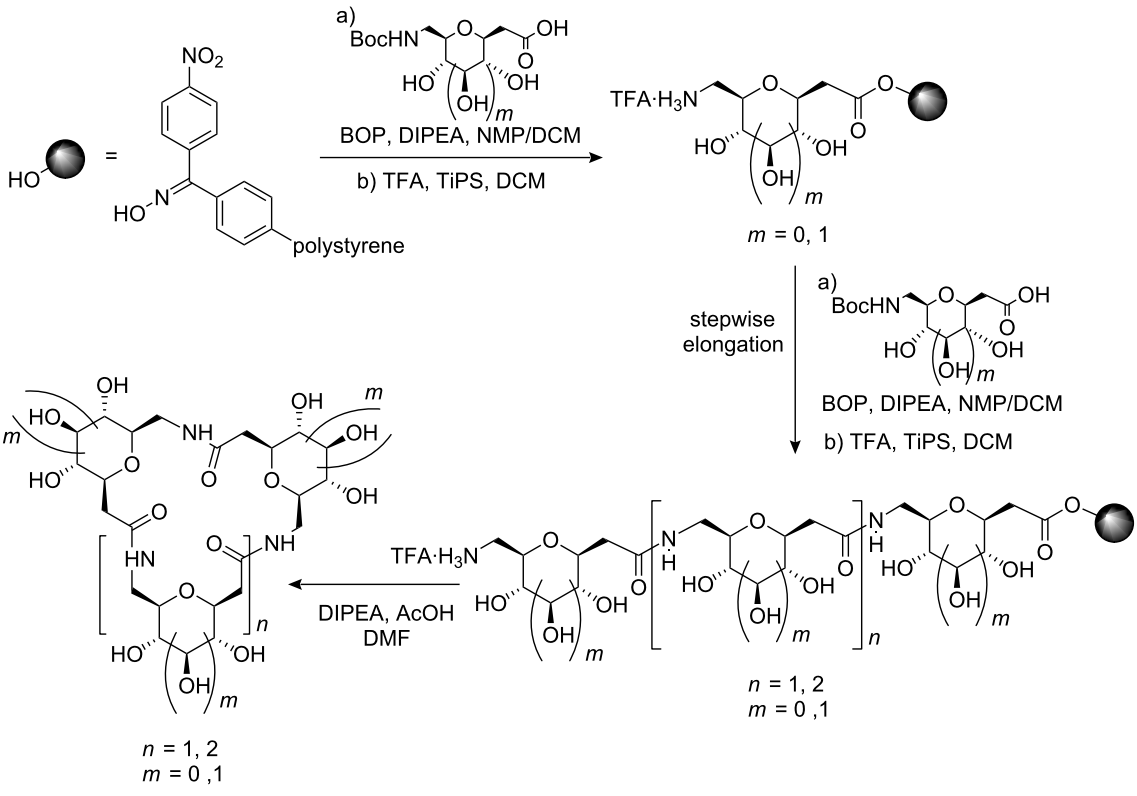


Figure 11: Cyclic homo-oligomers containing glucopyranoid-SAAs.

Conformational analysis by molecular modelling showed that the unprotected cyclic trimers and tetramers preferred a $^4C_1$ chair conformation with the oxygen atoms of the sugar ring located in the interior of the cavity and the secondary hydroxyl groups outside. This conformation is in agreement with the capacity of these cyclic carbopeptoids to form inclusion complexes with aromatic guest molecules [54] and Cu(II) ion [56]. Additionally, van Well et al. have described a cyclization/cleavage strategy for solid phase synthesis of cyclic trimers and tetramers containing pyranoid δ-SAAs (Scheme 2) and reported their structural analysis from ROESY data in combination with molecular dynamics calculations [57]. The results showed that the furanoid and the pyranoid SAA trimers adopt well-defined structures, although the trimer composed of pyranoid SAAs is less flexible than its furanoid counterpart.

Chakraborty and co-workers have also synthesised cyclic homo-oligomers of mannose- and glucose-derived furanoid SAAs [58] in order to constrain their conformational degrees of freedom and to induce desirable structural elements essential for their biological activities, such as tubular structures for transporting ions or molecules across membranes. Detailed ROESY, temperature coefficient ($\Delta\delta/\Delta T$) measurements of amide protons and constrained MD simulations revealed that all the



Scheme 2: Strategy for solid-phase synthesis of cyclic trimers and tetramers containing pyranoid δ-SAAs.

cyclic oligomers had symmetrical structures, although none of the unprotected cyclic oligomers displayed any ability to transport ions across model membranes according to ion flux studies.

Cyclic tetramers of L-*rhamno*- and D-*gulo*-configured oxetane-SAAs **24** and **25** (Figure 12) have been synthesised by Fleet et al. [59] as mimics of naturally occurring cyclic peptides and cyclodextrins, although no further conformational and recognition studies on these compounds have currently been published.

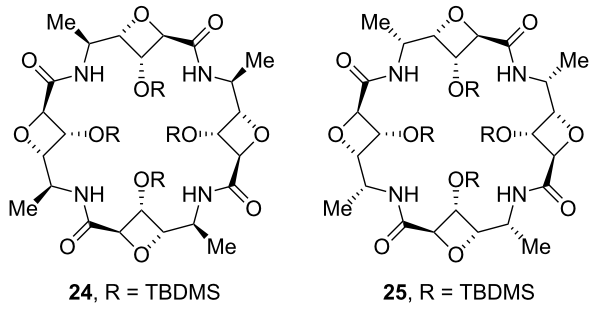


Figure 12: Cyclic tetramers of L-*rhamno*- and D-*gulo*-configured oxetane-SAAs.

Urea, carbamate and thiourea-linked sugars

The replacement of amide groups by pseudoamide (NH–C = X)–Y; X, Y = O, N, S) has been widely used in peptide chemistry to induce well-defined secondary structures. In carbohydrate chemistry, the incorporation of pseudoamide-type intersaccharide linkages has an additional interest because these functional groups are isosteric with phosphodiesther groups occurring in oligoglycosylphosphates and nucleotides.

Some examples of natural compounds having saccharide units connected by pseudoamide linkage are known. For example, the family of glycocinnamoylspermidine antibiotics **26**–**28**, whose structures were determined by Ellestad and co-workers [60] by NMR spectroscopy and X-ray diffraction, are characterised by the presence of a glycosylurea linkage (Figure 13). In 2005, Ichikawa and co-workers described the total synthesis of the glycocinnasperemicin D **29**, a broad-spectrum antibiotic against Gram-negative organisms, which contains two highly functionalised aminosugars connected by an urea linkage [61]. While exploring the synthesis of this target molecule, this group established a new method for the stereoselective synthesis of novel β-urea-linked pseudooligosaccharides, which involves the reaction of amine-glycosides with Steyemark-type gluco- and galactopyranosyl oxazolidinones [62,63].

Another example of natural disaccharide analogue containing a pseudoamide linkage is trehazolin **33** [64], which possesses a cyclic isourea functionality between the α-D-glucose and aminocyclopentitol rings. Trehazolin is a potent trehalase inhibitor in which an aminocyclopentitol ring replaces the glucopyranosyl cation postulated as an intermediate in the enzymatic hydrolysis of α,α-trehalose. Although several synthetic methodologies had successfully been applied to prepare this inhibitor, Chiara and co-workers have described a novel complementary approach in which the oxazoline ring is generated by S_N2 nucleophilic displacement reaction from the β-hydroxyurea **30** via the triflate intermediate **31** (Scheme 3) [65].

A similar methodology was used by the same authors to prepare the trehazolin analogue **37** with an isothiourea group instead of an isourea group, which is also a potent trehalase inhibitor [66,67]. D-*Gluco* trehazolin analogues have also been synthesised by means of novel methodology based on the coupling of *O*-unprotected β-D-glucopyranosyl isothiocyanate with different aminosugars followed by treatment with yellow mercury (II) oxide [68].

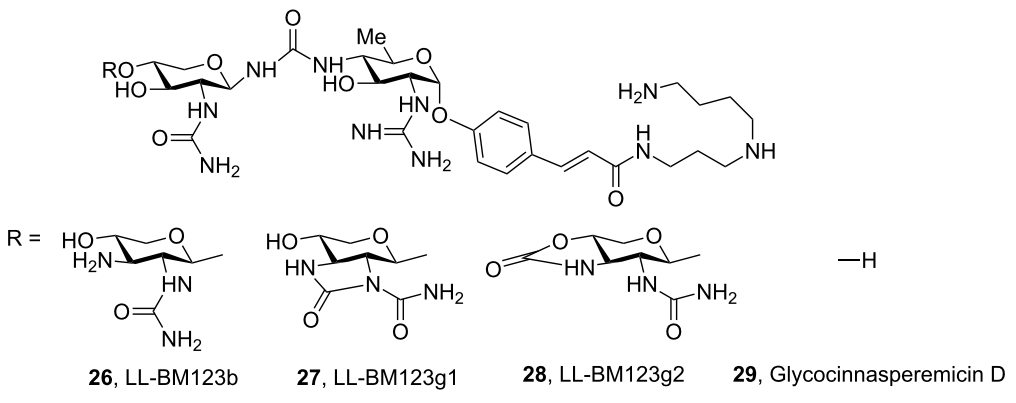
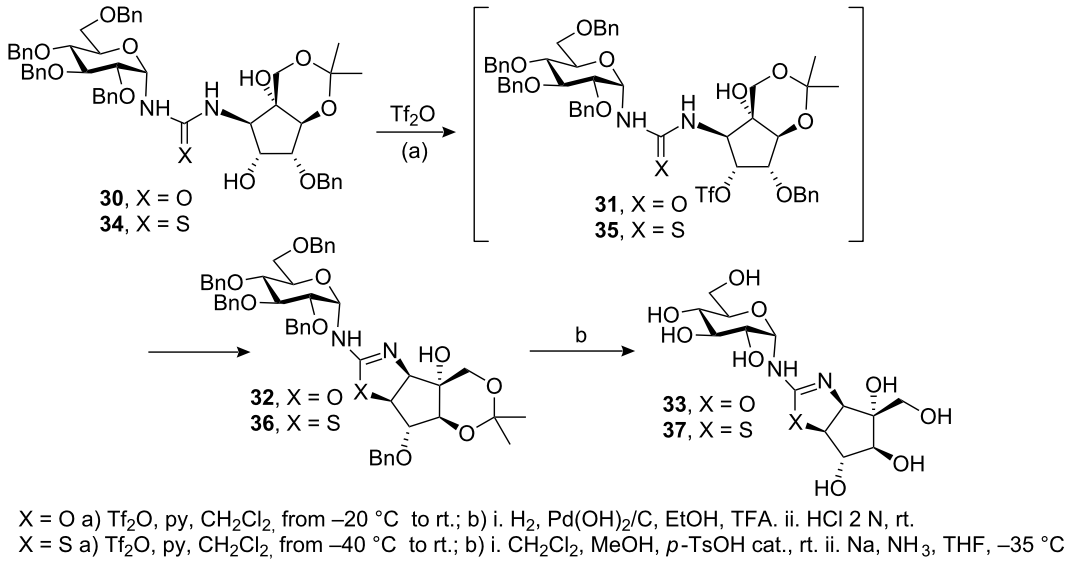


Figure 13: Aminoglycosidic antibiotics of the glycocinnamoylspermidine family.



Scheme 3: Synthesis of (thio)trehazoline, via triflate, from β-hydroxy(thio)urea.

There are two general strategies to access pseudoamide-type oligosaccharide mimics: i) nucleophilic addition of sugar derivatives to carbohydrate isocyanates, isothiocyanates or isocyanides; and ii) conversion of sugar azides into glycosyl carbodiimides via a tandem Staudinger-aza-Wittig type reaction with triphenylphosphine and an isothiocyanate, followed by the addition of a nucleophile (H_2O, H_2S, NH_3, amines) (Figure 14). Although this second methodology is more straightforward and versatile than the first, only a few examples of pseudooligosaccharides having cyanamide, urea and thiourea-linkages via the Staudinger reaction have been reported [69,70].

In our group, the carbodiimide approach was used to prepare calystegine B_2 analogues with the urea-linked disaccharide structure (Figure 15). These compounds, however, did not show inhibitory activities, probably due to the presence of a hydrophilic sugar substituent at the nitrogen atom of the *nor*-tropane ring [71,72].

Several groups have shown that disaccharide ureas and carbamates with different bridging positions as α/β-anomers are readily accessible by the coupling reaction of aminosugars or sugars, respectively, and glycosyl-isocyanates [73,74] or isocyanides [75]. However, the experimental difficulties in handling isocyanates has led to the preferential use of sugar carbodiimides as key intermediates for the preparation of glycosylureido sugars [76-79].

From the large number of functional groups that can be employed as surrogates for the natural amide and phosphodi-

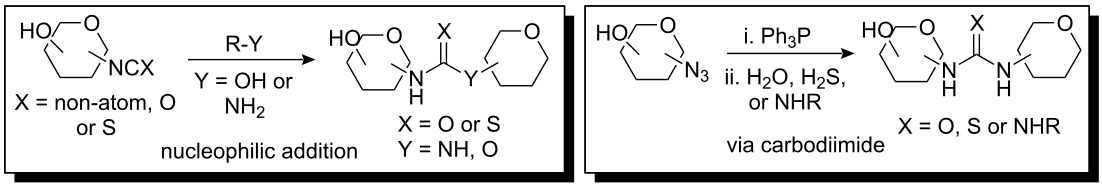


Figure 14: Approaches to access pseudoamide-type oligosaccharide mimics.

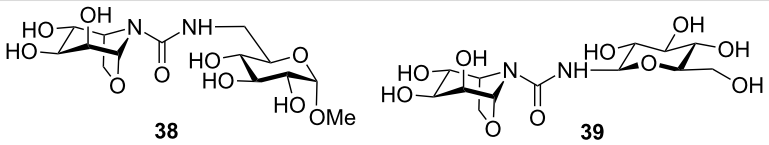


Figure 15: Calystegine B_2 analogues **38** and **39** with urea-linked disaccharide structure.

ester linkages, the thiourea functionality ranks among the most popular. Firstly, it can generally be generated in high yield and are ideally suited for solid phase and combinatorial approaches. Secondly, thioureas can be easily transformed into carbodiimides and thus serve as key intermediate in the synthesis of other pseudoamide functionalities (urea, guanidine, etc.) [70-72,76-79]. Thirdly, thiourea bridges provide efficient anchoring points for bidentate hydrogen-bonding recognition, which can give rise to defined secondary structures or be exploited in molecular recognition. Conformational studies by temperature coefficient measurements and ROESY experiments showed that thioureidodisaccharides adopt the *Z,E* conformation at N–(C=S) bonds, which is stabilised by intramolecular hydrogen bonding with the formation of a 7-membered ring (e.g. **40**) [80]. This conformation changes to the *Z,Z*-conformation in aqueous solution [70] or in the presence of carboxylate ligands due to the formation of a bidentate hydrogen bond (Figure 16) [80].

Examples of thiourea-connected saccharides have been typically limited to compounds where the tethering reaction involves only two moieties, with no possibility of chain elongation [68,70-72,81], except in cases where there are spacer substituents [82]. Interestingly, a thiourea-linked dinucleotide was elongated by solid-phase synthesis to incorporate positively charged isothiouronium inter-nucleoside linkages into otherwise negatively charged DNA (Figure 17) [83,84]. The binding of these artificial DNAs (DNmt or DNT) to its complementary DNA strand occurs as with the unmodified DNA·DNA duplex but with enhanced nuclease resistance.

In order to design longer thiourea-linked glycooligomers with linear dendritic and branched architectures, our group has recently reported an efficient synthetic strategy based on the use of **AB**, **AB$_2$** and **ABC**-type monosaccharide building blocks containing isothiocyanate (**A**), azido (**B**) or carbamate groups (**C**) (Scheme 4) [85,87]. An iterative and efficient three-step reaction sequence was described for the assembly of monosaccharide units that involves: (i) a thiourea-forming reaction; (ii) deprotection of the hydroxy groups in the adducts and (iii) the generation of a new amino group in the growing chain. Branching points are incorporated by inserting building blocks

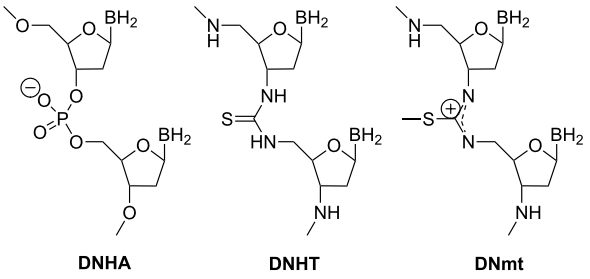


Figure 17: Nucleotide analogues with thiourea and *S*-methylisothiouronium linkers.

bearing orthogonal amine functionalities at specific locations in the chain [86]. Conformational studies by dynamic NMR spectroscopy of β-(1→3)- and β-(1→6)-linked diglucosylthioureas (e.g. **41**) detected the presence of *Z,Z* and *Z,E* conformers at the N–(C=S) bond (Figure 18).

β-(1→6)-Linked thioureido-di- and trisaccharides were used to obtain the corresponding ureido- and guanidine-linked oligomers. These compounds were evaluated as phosphate binders in water [76]. Association constants (K_{as}) for the binding of dimethyl and, especially, phenyl phosphate were obtained from NMR titration experiments for both series of glucooligomers. The binding affinity of the thiourea and guanidine oligomers was stronger than in the case of the urea analogues.

Stimulated by the interesting supramolecular properties and applications of cyclodextrins (CDs), a range of novel host molecules with differently shaped internal cavities has been obtained by replacement of the natural glycosidic bonds by urea and thiourea linkages. Thus, our group developed an efficient and modular strategy for the synthesis of cyclopseudooligosaccharide receptors relying on alternating α,α-trehalose motifs and semi-rigid thiourea segments (cyclotrehalans, CTs) [77-79,88,89]. Molecular diversity was introduced at the inter-saccharide connectors by exploiting the chemistry of macrocyclic carbodiimides [77-79,88,89] as well as by varying the size of the macrocycle (Figure 19).

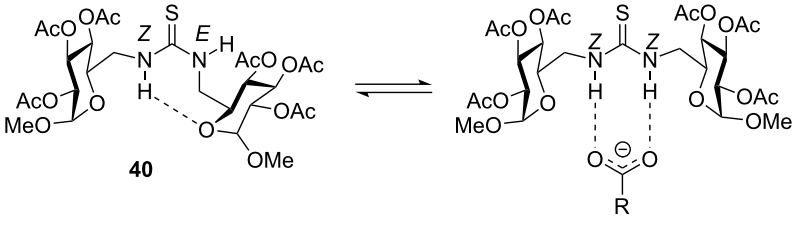
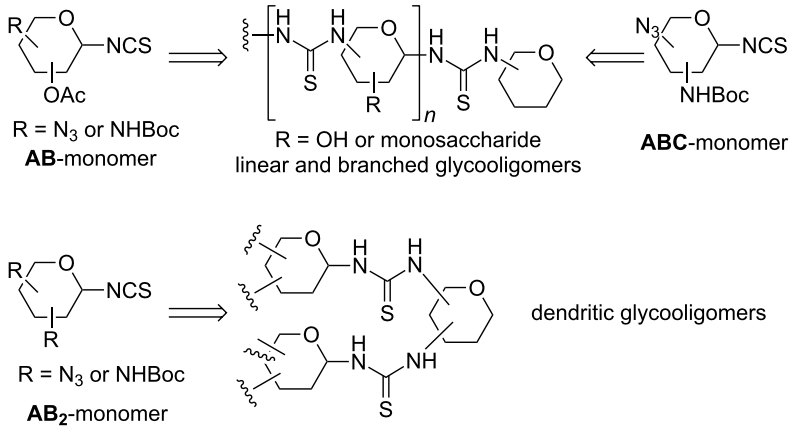


Figure 16: Rotameric equilibrium shift of **40** by formation of a bidentate hydrogen bond.



Scheme 4: Retrosynthetic approach to synthesize thiourea-linked glycooligomers.

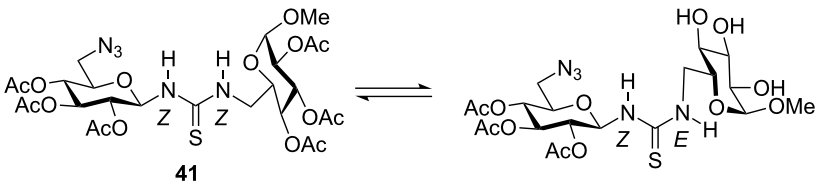


Figure 18: Rotameric equilibria for β-(1→6)-thiourea-linked glucodimer **41**.

Molecular modelling predicts the existence of a flexible, relatively hydrophobic cavity suited for molecular inclusion of non-polar guests [79,88]. NMR supports this fact and confirms the high symmetry of these hosts, where all glucopyranosyl units are magnetically equivalent as in the case of cyclodextrins. Determination of the inclusion capabilities towards a series of structurally diverse guests demonstrated that large-ring CTs are well suited for forming supramolecular complexes in water [77,79,88].

Guanidine-linked sugars

The use of the guanidine functional group to connect monosaccharide units in glycooligomers is particularly attractive with regard to molecular recognition processes. Like thioureas and ureas, guanidines can also form bidentate hydrogen bonds. In addition, because of their positively charged character, guanidines can exert strong electrostatic interactions with negatively charged functional groups such as phosphate or carboxylate groups in DNA, RNA and proteins.

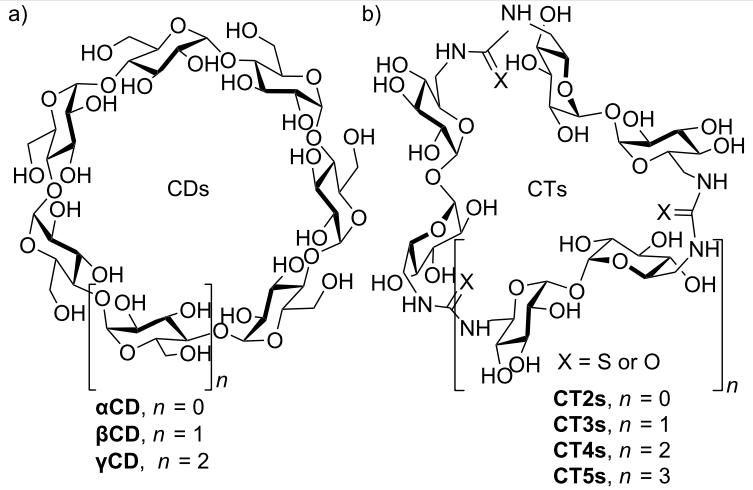
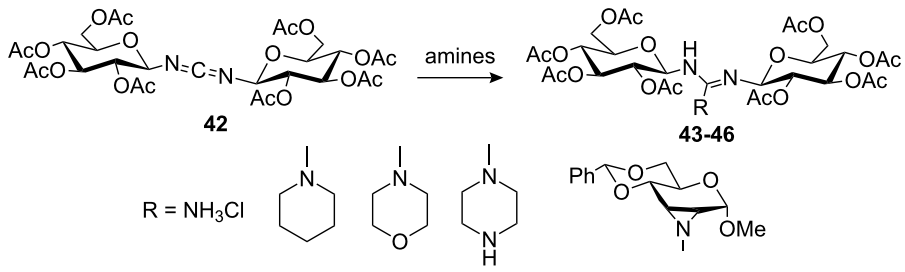


Figure 19: Schematic representation of (a) cyclodextrin (CDs) and (b) cyclotrehalan (CTs) family members.



Scheme 5: Synthesis of guanidine-linked pseudodisaccharides via carbodiimide.

Tóth and co-workers [90] synthesised a series of guanidine-linked sugars as a new class of oligosaccharide mimics to study their interactions with proteins. Thus, reaction of the carbodiimide-linked pseudooligosaccharide **42** with different secondary amines gave the corresponding guanidines **43**–**46** (Scheme 5) and their conformational behaviour and tautomerism was studied by NMR spectroscopy.

β(1→6)-Linked pseudodi- (**47**) and pseudotrisaccharides (**48**) incorporating guanidine intersaccharide bridges have been prepared and evaluated as phosphate binders in water (Figure 20) [76]. Association constants (K_{as}) for binding with dimethyl and phenyl phosphate were obtained from NMR titration experiments for both compounds. The results indicated that the binding strength depends strongly on the acidity of the NH protons and on solvating properties. The guanidine derivatives showed higher K_{as} values than the isosteric thiourea and urea analogues.

Ortiz Mellet's group has reported the synthesis of *N*-benzylguanidine-linked dimeric cyclotrehalans (CT2s, **50**) [78] via carbodiimide **49** by reaction with benzylamine hydrochloride and subsequent deacetylation (Scheme 6). Structural and conformational studies on **50** were carried out by NMR, which showed that the structure was stabilised by two anti-parallel seven-membered ring intramolecular hydrogen bonds, resulting in relatively high rotational barriers for the *Z,E:E,Z/E,Z:Z,E* equilibrium. The cavity collapses due to the presence of these intramolecular hydrogen bonds thus preventing the formation of inclusion complexes.

Most examples of linear pseudoamide-linked glycooligomers correspond to oligonucleotide analogues. The "antisense" strategy to regulate gene expression in living cells has required the development of modified oligonucleotides as potential therapeutic agents. A key goal in the design of such agents include increasing binding affinity while maintaining sequence specificity, resistance to degradation by nucleases and improved membrane permeability [83,84]. Numerous structural analogues of DNA/RNA designed to be effective antisense/antigene agents have been reported. An interesting approach involves replacing the negatively charged phosphodiester linkages of RNA/DNA by positively charged guanidinium linkages. The guanidinium linkage is resistant to nucleases [91,92] and the positive charge may give rise to cell membrane permeability through electrostatic attraction with the negatively charged groups of the proteoglycans at the cell surface. Using this strategy, Bruice and co-workers synthesised ribonucleic guanidine (RNG) and deoxyribonucleic guanidine (DNG) as analogues of RNA and DNA, respectively (Figure 21).

The methodology developed to generate internucleoside guanidinium linkages can be used in both solution phase and solid phase synthesis [91-94]. It involves the abstraction of the

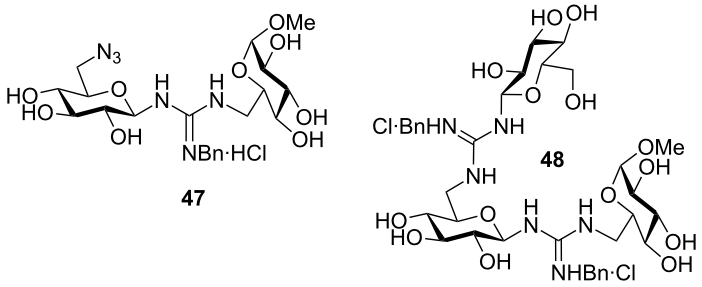
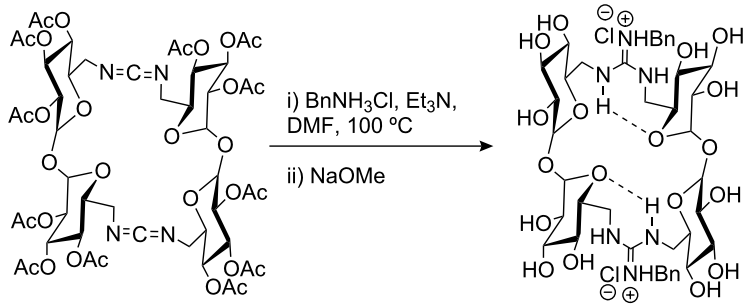


Figure 20: β(1→6)-Guanidine-linked pseudodi- and pseudotrisaccharides **47** and **48**.



Scheme 6: Synthesis of *N*-benzylguanidine-linked CT2 **50**.

sulphur atom from a (Fmoc)-protected thiourea by Hg^{2+} to provide an activated carbodiimide which can react with a free amino group to give the protected guanidine (Figure 22).

Homo-oligomeric RNG sequences, containing trimeric and pentameric uridyl [95] and adenyl moieties (**52**-**55**) (Figure 23), and a mixed-base pentamer [96] (**56**, 5′-AUAUA-3′), were synthesised using this methodology (Figure 24).

Binding studies of uridyl ribonucleic guanidine **52** to DNA [97] and RNA [98] showed that it binds pentameric adenyl DNA and RNA with a 1:1 stoichiometry. The RNG **52** dimeric complex with DNA is thermodynamically favoured in comparison to RNA·DNA and DNA·DNA duplexes and was able to discriminate between complementary and non-complementary base pairs. These results suggest that **52** is a good candidate for an antisense agent.

A 21 base pair RNG/DNA chimera containing both anionic phosphodiester linkages of DNA and cationic guanidine linkages of RNG has been synthesised [99]. Phosphoramidite **56** (Figure 24) was used as a building block to introduce guanidinium linkages at desired positions in the chimeric oligonucleotides. The biological properties were evaluated using the bcr-abl oncogene (the cause of chronic myeloid leukaemia) as the target. The results showed that the binding of a 21-mer RNG/DNA chimera containing six guanidinium linkages is more than 104-fold stronger than the binding of its 21-mer DNA counterpart.

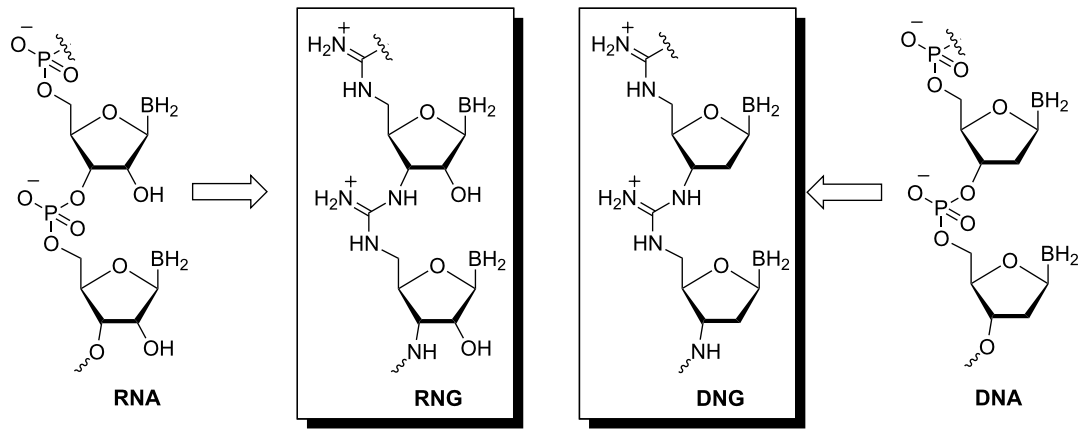


Figure 21: Structure of **RNG** and **DNG**.

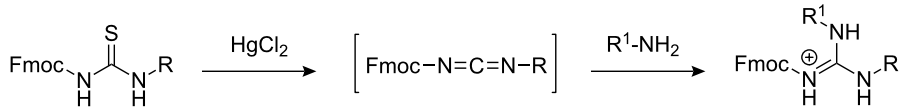


Figure 22: Preparation of Fmoc-guanidinium derivatives.

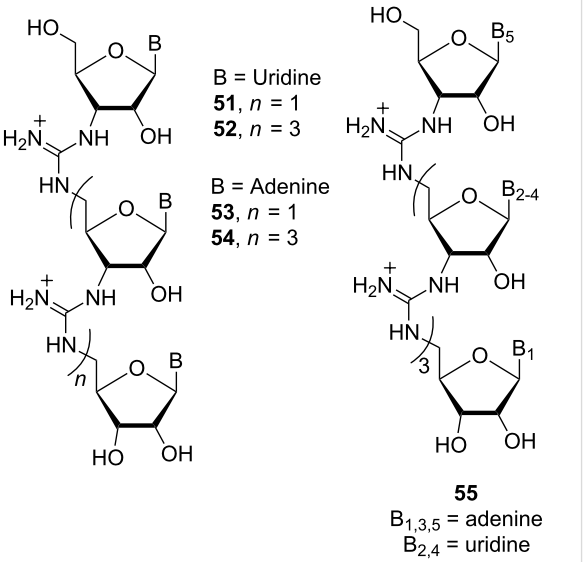


Figure 23: Structures of the homo-oligomeric RNG derivatives **51**–**55**.

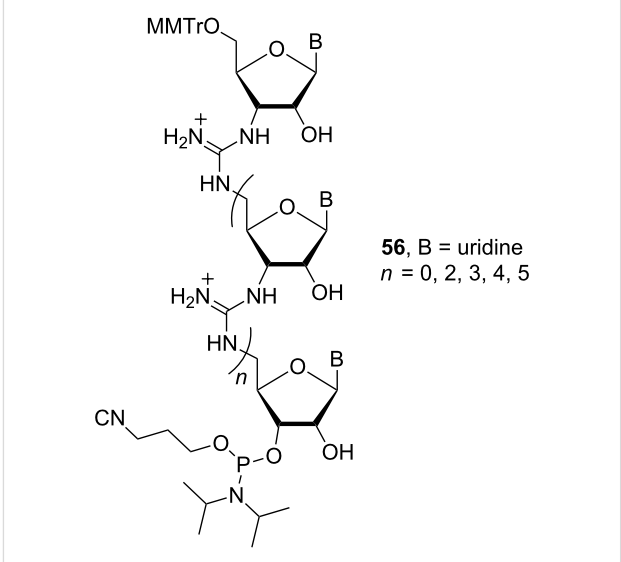


Figure 24: Phosphoramidite building block **56**.

Deoxyribonucleic guanidine homo-oligomeric sequences, consisting of trimeric and octameric thymidyl [100], octameric cytidyl [101] and trimeric 7-deazaguanidyl moieties (**57**–**60**) [102] and a mixed-base tetramer [103] and hexamer [104] (**61**–**63**) were synthesised using the same strategy as noted above for RNG synthesis (Figure 25).

Binding studies of homo-oligomers **57** and **58** to complementary DNA oligomers showed a 1:2 DNG:DNA stoichiometry, similar to homo-polymeric thymidyl and adenyl DNA strands, which can form a triple helical structure. On the other hand, octameric cytidyl DNG **59** and mixed-based oligomers **61**–**63** form 1:1 complexes with complementary DNA oligomers. The trimeric 7-deazaguanidyl **60**, in which

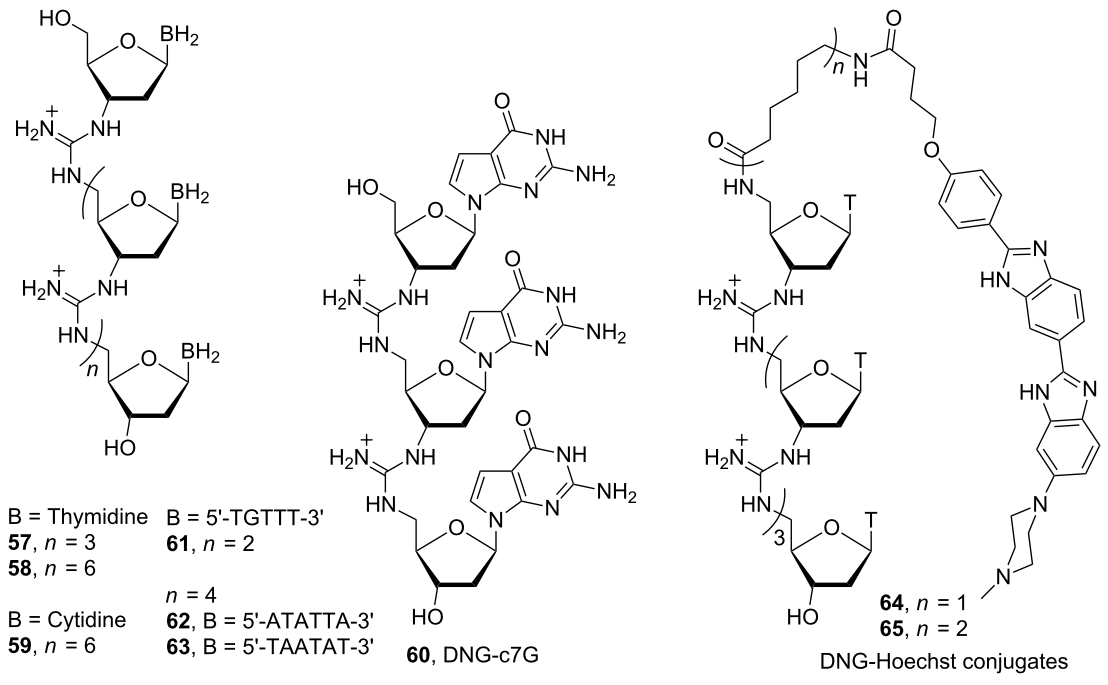


Figure 25: Structures of DNGs **57**–**65**.

guanine is replaced by 7-deazaguanine (c^7G), forms a 1:1 complex with pentameric adenyl DNA. Deazaguanine nucleobase was chosen because of the unique glycoside bond stability and its ability to prevent G-quartet formation.

To increase the already strong binding of DNG to DNA, DNG was combined with a ligand capable of binding specifically to the minor groove of the DNA. A pentameric thymidyl DNG incorporating bis-benzimidazole (Hoechst 33258) ligand (**64**) was synthesised [105]. The stability of DNG-Hoechst conjugates **64** and **65** with a 30-mer double-strand DNA (dsDNA) and single-strand DNA (ssDNA) were evaluated. Fluorescent emission studies showed that hybridization of DNG-Hoechst conjugates **64** and **65** to dsDNA enhances the stability of the triple helix through simultaneous minor groove binding by the tethered Hoechst 33258 ligand. Furthermore, Hoechst 33258 is able to enhance the stability of the duplex DNG·DNA with a flexible minor groove. As in the case of RNG, a 20 base pair DNG/DNA chimera has been synthesised [106].

Phosphoramidite **66** (Figure 26) was used as the starting material for the introduction of guanidinium linkages at desired positions in the chimeric oligonucleotides. The biological properties were evaluated using the juvenile esterase gene as the target. The results showed that binding of a 20-mer DNG/DNA hexameric (ATATAT) chimera containing guanidinium linkages is more than $10^{5.7}$ times stronger than the binding of the corresponding 20-mer of DNA. The hexameric DNG binds to DNA in a 1:1 ratio and is able to discriminate between complementary and non-complementary base pairs. In addition, the DNG·DNA complex is more stable than the DNA·DNA duplex.

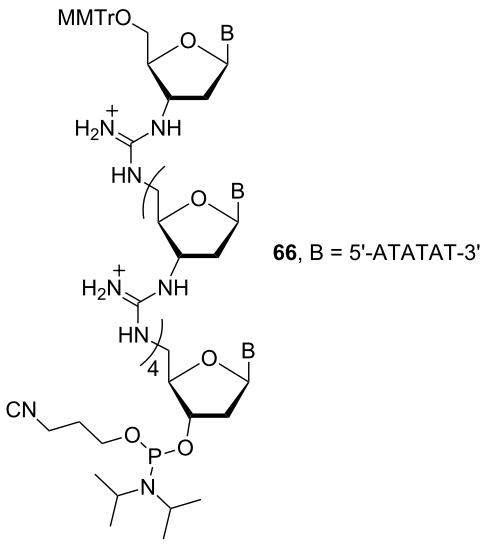


Figure 26: Structure of the phosphoramidite building block **66**.

Cancer cell immortality is due to relatively high concentrations of telomerase enzyme which maintains the telomer sequence during cell division. One approach to prevent telomer replacement in cancer cells is by inhibiting the telomerase enzyme by blocking the telomeric RNA 11-base template (5′-CUAACCCAAC-3′). For this purpose, Bruice and co-workers [107] prepared complementary DNG (5′-GATTGGGATTG-3′) to telomeric RNA complex. Binding studies demonstrated that the DNG/telomeric RNA complex is favoured over the telomer substrate, and could be of use as an anticancer agent.

Conclusion

Over the last 10 years considerable efforts have been expended in the synthesis of novel linear and cyclic pseudoamide-linked oligosaccharide mimics. In particular, important advances in synthetic methodologies for thioureido and ureido glycooligomers have made thus enabling the preparation of virtually any oligomeric structure (linear, branched, dendritic and cyclic). The secondary structures adopted by these pseudooligosaccharides have been extensively studied by current NMR and molecular modelling techniques. The results have shown that, in many cases, the three-dimensional structure of naturally occurring biomolecules can be mimicked by carbopeptoids and pseudoamide-linked oligomeric surrogates. Nevertheless, further investigations are required to clarify the structure-activity relationships in order to design novel biologically active analogues of potential therapeutic value.

Acknowledgements

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Synthesis of lipophilic 1-deoxygalactonojirimycin derivatives as D-galactosidase inhibitors

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Abstract

N-Alkylation at the ring nitrogen of the D-galactosidase inhibitor 1-deoxygalactonojirimycin with a functionalised C_6 alkyl chain followed by modification with different aromatic substituents provided lipophilic 1-deoxygalactonojirimycin derivatives which exhibit inhibitory properties against β-glycosidases from *E. coli* and *Agrobacterium* sp. as well as green coffee bean α-galactosidase. In preliminary studies, these compounds also showed potential as chemical chaperones for GM1-gangliosidosis related β-galactosidase mutants.

Introduction

Iminosugars such as compounds **1**–**4** (Figure 1) have been shown to be potent glycosidase inhibitors and useful tools for the study of glycoside-hydrolysing enzymes. These sugar mimetics have been found to have anti-viral, anti-cancer, anti-diabetes, anti-infective, as well as insect anti-feedant and plant growth regulatory effects. Because of their diverse properties, iminosugars have enjoyed continuous interest since their discovery in the 1960s. Consequently, many different

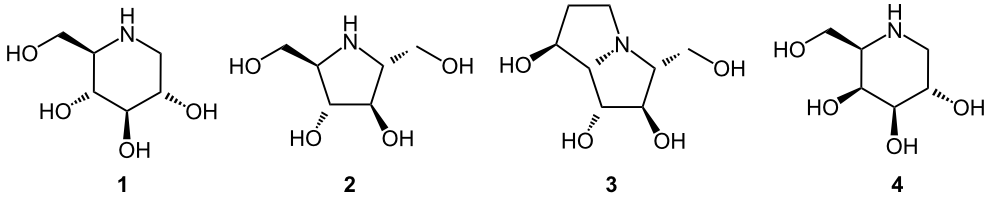


Figure 1: Typical representatives of iminosugars.

derivatives have been prepared for biological evaluations via a wide range of synthetic approaches and have been used for various medicinal and biomolecular applications [1-9].

Recently, iminosugars were found to have potential to serve as pharmacological chaperones for the treatment of lysosomal storage diseases in chaperone mediated therapy (CMT) [10]. In contrast to enzyme replacement therapy (ERT), where recombinant enzyme is given to the patient at regular intervals, the iminosugars used for CMT (recently called pharmacological chaperone therapy, PCT) are able to cross the blood brain barrier. This gives the opportunity also to treat types of lysosomal storage diseases involving the central nervous system. Furthermore, CMT is a cost-efficient alternative to ERT. In this context, *N*-alkylated derivatives of 1-deoxynojirimycin [11] such as **5** and **6** (Figure 2) as well as *N*-substituted D-glucono-δ-lactams **7** (Figure 2) [12] have been shown to be highly potent pharmacological chaperones for the potential treatment of Gaucher [13] and Pompe [14] diseases by 'rescuing' the related mutant enzymes. Both Wong [15] and Overkleeft [16] have shown that a rather large lipophilic substituent such as the adamantyl group (**8**, Figure 2) attached via an alkyl chain with a chain length from C_3 up to C_9 to the ring nitrogen of 1-deoxynojirimycin and isofagomine respectively, can increase the interaction with the lysosomal glycosphingolipid glucocerebrosidase. Interestingly, 5-*N*, 6-*X*-(*N*′-alkyliminomethylene)nojirimycin derivatives where X is O, NH or S such as in structure **9** (Figure 2) also have chaperone activity for Gaucher related mutations [17].

1-Deoxygalactonojirimycin (**4**) was shown to be a candidate for the treatment of Fabry disease, an X-linked inherited lysosomal storage disorder caused by the deficiency of α-galactosidase A activity resulting in the accumulation of globotriaosylceramide, thereby affecting the lysosomes of vascular endothelial cells. Iminosugar **4** can increase α-galactosidase A levels 1.5 to 28 fold in cultured Fabry patient cell lines (baseline α-Gal A levels range from 0–52%) after incubation for five days, as was observed for 49 different missense mutant forms [18-21]. It can also reduce tissue globotriaosylceramide levels in a mouse model [22].

Suzuki and co-workers found, that *N*-octyl-4-epi-β-valienamine (**10**) (Figure 3), a competitive inhibitor of lysosomal β-galactosidase, when orally administered to GM1-gangliosidosis model mice, is able to enter the brain through the blood-brain barrier and thereby enhancing β-galactosidase activity, reduce substrate storage, and clinically improve neurological deterioration [23].

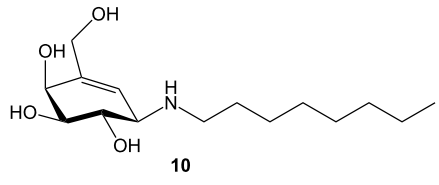


Figure 3: Structure of NOEV **10**.

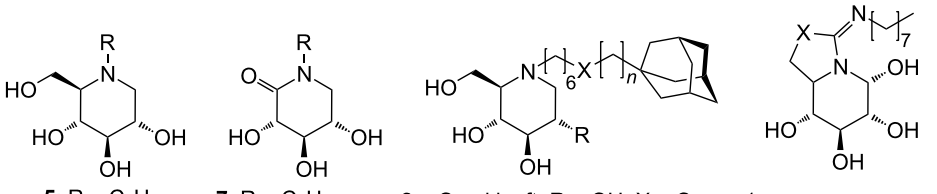
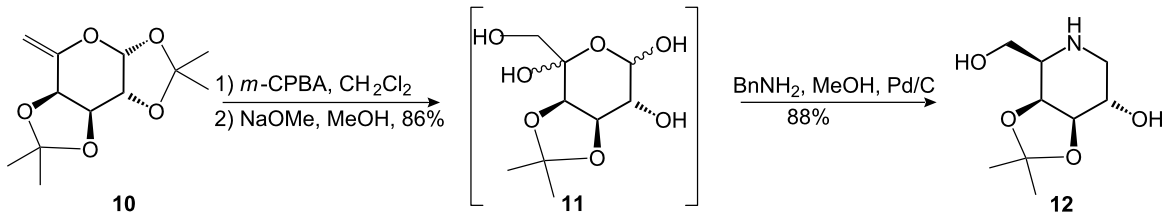


Figure 2: *N*-Modified iminosugars **5**–**9** as potential pharmacological chaperones.



Scheme 1: Three-step-synthesis of partially protected 1-deoxy-D-galactonojirimycin derivative **12** from **10** via L-*arabino*-hexos-5-ulose **11**.

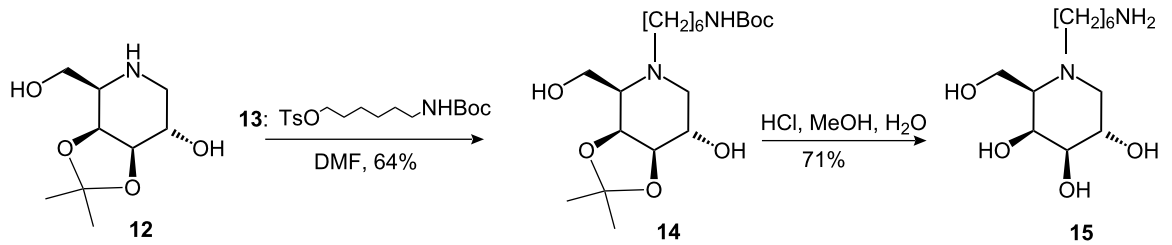
Our studies revealed that 1-deoxy-D-galactonojirimycin-lysine hybrids, when carrying an aromatic substituent, such as a dansyl moiety, in its nature a lipophilic aromatic substituent, are potent D-galactosidase inhibitors and also show activity with human lysosomal β-galactosidase, exhibiting improvements of the enzyme activity in mutant cell lines [24]. In the course of this work, we became interested in the influence of other lipophilic aromatic substituents on the biological activity of such compounds. Different aromatic acid derivatives were prepared by coupling to the free amine at the terminus of the C_6 alkyl chain in compound **15**, which is anchored to the ring nitrogen of 1-deoxygalactonojirimycin, to yield derivatives **16**–**19** and **22**. The spacer length of six carbon units has been proven suitable for enzyme recognition in previous studies [25] and was kept constant to compare the different aromatic substituents. Additionally, Wong [15] as well as Suzuki [23] have shown from computational studies, that in case of *N*-substitution on compounds **8b** and **10**, the iminosugar and carbasugar units respectively, were found to interact with the active site of the corresponding enzymes whereas the alkyl chains were located in the distinctly hydrophobic entrance region to the active site. Thus, for comparison, a lipophilic aliphatic *tert*-butyl group in compound **20** was included in this study. In addition to the synthetic approaches, the influence of the lipophilic substituents of the new *N*-modified 1-galactonojirimycin derivatives on their biological interaction with respective glycoside hydrolases are described.

Results and Discussion

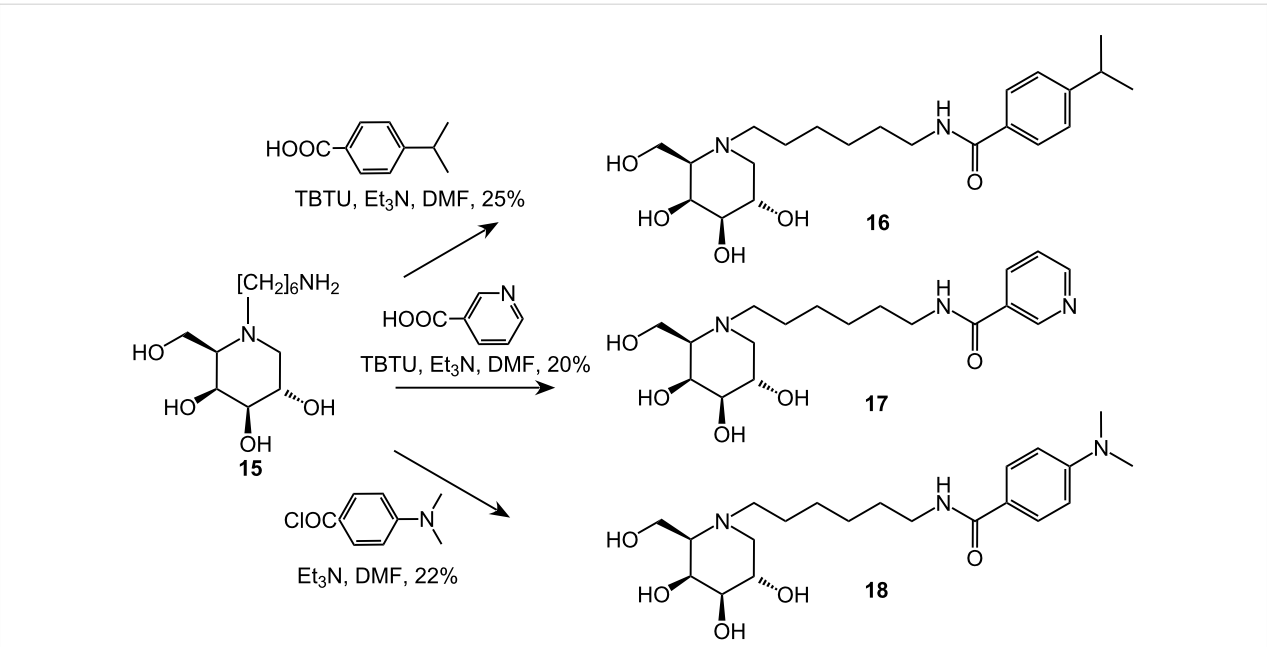
The key intermediate for the synthesis of *N*-modified lipophilic 1-deoxygalactonojirimycin derivatives **16**–**20** as well as **22** was the 3,4-*O*-isopropylidene iminosugar **12**. Starting from enol ether **10** [26,27], treatment with *m*-chloroperbenzoic acid gave the 5-*O*-chlorobenzoic ester via the corresponding 5,6-epoxide. This ester underwent hydrolysis under basic conditions to afford the L-*arabino*-hexos-5-ulose **11**, which was immediately used for the next step after brief silica gel purification. The reductive amination and *N*-deprotection of **11** was carried out under an atmosphere of H_2 with benzylamine in methanol and Pd/C as catalyst to produce 3,4-*O*-isopropylidene-1-deoxy-D-galactonojirimycin (**12**) in an overall yield of 75% (Scheme 1).

Compound **12** underwent *N*-substitution upon treatment with 1-*O*-tosyl-6-*N*-(*tert*-butoxycarbonyl)-6-aminohexanol (**13**) [28] in DMF to give the 1-deoxygalactonojirimycin derivative **14** in 64% yield. The isopropylidene and *tert*-butoxycarbonyl protecting groups were simultaneously removed under standard conditions to afford the desired free amine **15** [25], the key building block for further modifications (Scheme 2).

The chemoselective acylation of the free amine **15** was conducted with three different benzoic acid derivatives in order to investigate the influence of the potential basicity of an additional nitrogen at the aromatic substituent. For the synthesis of compound **16**, 4-isopropylbenzoic acid was reacted with the primary amine under amide coupling conditions with *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoro-borate (TBTU) as the coupling reagent in DMF and triethylamine. Likewise, nicotinic acid under the same conditions gave compound **17** in 20% yield. Reaction of **15** with 4-(dimethylamino)benzoyl chloride in DMF and triethylamine afforded derivative **18** in 22% yield (Scheme 3). These unusu-



Scheme 2: Synthesis of *N*-(6-aminohexyl)-1-deoxygalactonojirimycin (**15**) from **12** via **14**.

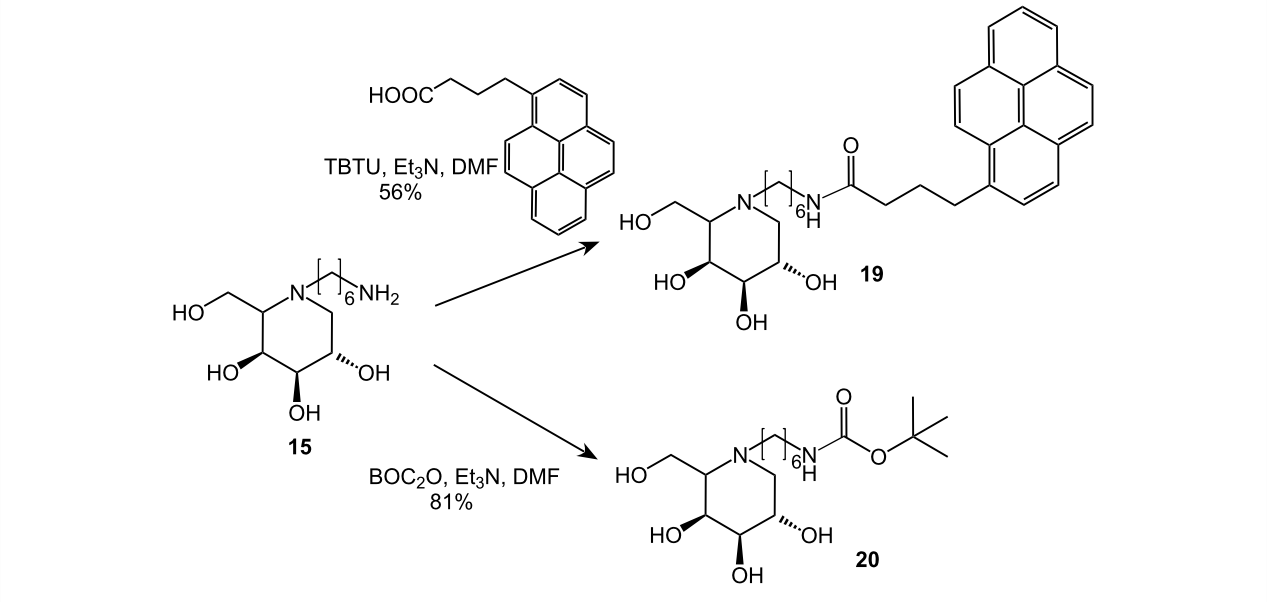


Scheme 3: Synthesis of lipophilic 1-deoxy-D-galactonojirimycin derivatives **16**–**18** by chemoselective acylation of **15**.

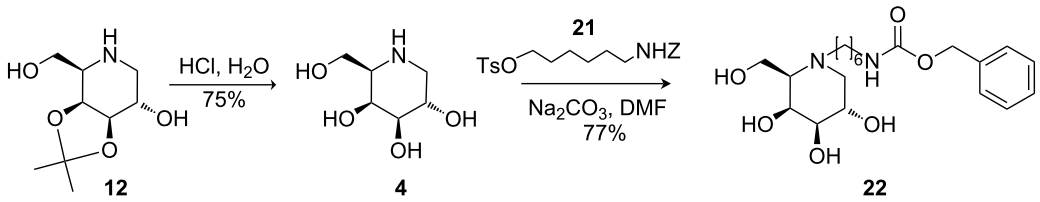
ally low yields for the standard coupling reactions were due to the formation of very polar side products as well as material losses during column chromatography.

For increased lipophilicity as well as for analytical purposes, compound **15** was also coupled to 1-pyrenebutyric acid with TBTU in DMF in the presence of triethylamine to give compound **19** in 56% yield. The pyrenyl substituent was chosen because of its potential to serve as diagnostic tool. Conventional BOC-protection of amine **15** gave derivative **20** in 81% yield (Scheme 4).

Aromatic derivative **22** was synthesised from **4** [29-42] (conveniently obtained by deprotection of compound **12** under acidic conditions), in 77% yield by ring nitrogen alkylation with tosylate **21** (Scheme 5) [43].



Scheme 4: Synthesis of compounds **19** as well as **20** from primary amine **15**.



Scheme 5: Synthesis of compound **22**.

Inhibition constants of the compounds synthesised are presented in Table 1. The 1-deoxygalactonojirimycin analogues were tested as inhibitors of *Agrobacterium* sp. β-glucosidase/galactosidase and *E. coli* β-galactosidase as well as green coffee bean α-galactosidase (Table 1). All new compounds inhibited the *Agrobacterium* sp. enzyme better than the parent iminosugar **4**. The pyrenyl substituted compound **19** with an extended aromatic system turned out to be the most active inhibitor with a K_i value of 60 nM against *Agrobacterium* sp. β-glucosidase/galactosidase and 0.25 μM against *E. coli* β-galactosidase. However, the toxicity of this compound clearly requires further evaluation. In general, *N*-substitution does not dramatically affect the inhibitory properties of the derivatives against β-galactosidase from *E. coli*, with K_i values distributed in the range of the parent iminosugar **4**. No particular trend could be observed in a comparison of compounds **16**–**19** as regards the presence or absence of the additional nitrogen at the aromatic substituent. Iminosugars **16**–**20**, as well as **22**, were less active than the parent compound with α-galactosidase from green coffee beans. However, the K_i values are still in the low μM range and thus, suitable for use as chemical chaperones. Gratifyingly, compounds **20** and **22** exhibited IC_{50} values of 10.9 μM (K_i = 2.0 μM) and 3.26 μM (K_i = 0.72 μM), respectively, with human lysosomal β-galactosidase.

Table 1: Inhibitory activities of compounds **16**–**20** and **22** with β-glycosidases from *Agrobacterium* sp. (β-glu/gal Abg), *E. coli* (β-gal *E. coli*) as well as with the α-galactosidase from green coffee beans (α-gal GCB).

Compound	K_i [μM] β-glu/gal Abg	K_i [μM] β-gal *E. coli*	K_i [μM] α-gal GCB
4	100	13	0.013[a]
16	13	1.3	2.6
17	1.5	0.83	2.2
18	3.8	1.1	0.49
19	0.06	0.25	7.0
20	3.0	2.4	4.3
22	6.0	0.4	2.2

[a]See reference [7].

In preliminary studies compounds **17** as well as **22** served as chemical chaperone and increased the enzyme activity of a β-galactosidase mutant feline fibroblast cell line up to 5.5 fold when applied at a concentration of 100 μM. Compound **18** was a significantly better chemical chaperone for this mutant increasing the relative enzyme activity 4.8 fold at a concentration of 2 μM.

Conclusion

We have synthesised new 1-deoxygalactonojirimycin derivatives **16**–**20**, as well as **22**, which feature a C_6 chain anchored to the ring nitrogen. Different lipophilic aromatic and aliphatic substituents at the *N*-alkyl chain were introduced resulting in an interesting K_i-value profile against β-galactosidases from *Abg* and *E. coli*, respectively, as well as with α-galactosidase from green coffee beans. The K_i values with human lysosomal β-galactosidase and preliminary data for chaperone activity in a cat fibroblast model of the new compounds suggest that such iminosugar derivatives have interesting potential in the chaperone-mediated therapy of lysosomal storage diseases, such as, for example, GM1 gangliosidosis as well as Morquio B disease, possibly also Fabry's disease. Further bio-medicinal evaluation and toxicity studies are currently in progress.

Supporting Information

Supporting Information File 1

Full experimental details and characterisation data
[http://www.beilstein-journals.org/bjoc/content/supplementary/1860-5397-6-21-S1.pdf]

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Beilstein Journal of Organic Chemistry

Bis(oxazolines) based on glycopyranosides – steric, configurational and conformational influences on stereoselectivity

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Full Research Paper

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Abstract

In previous studies we found that the asymmetric induction of bis(oxazolines) based on D-glucosamine strongly depended on the steric demand of the 3-*O*-substituents. To further probe the impact of the 3-position of the pyranose scaffold, we prepared 3-epimerised and 3-defunctionalised versions of these ligands as well as a 3-*O*-formyl derivative. Application of these new ligands in asymmetric cyclopropanation revealed strong steric and configurational effects of position 3 on asymmetric induction, further dramatic effects of the pyranose conformation were also observed.

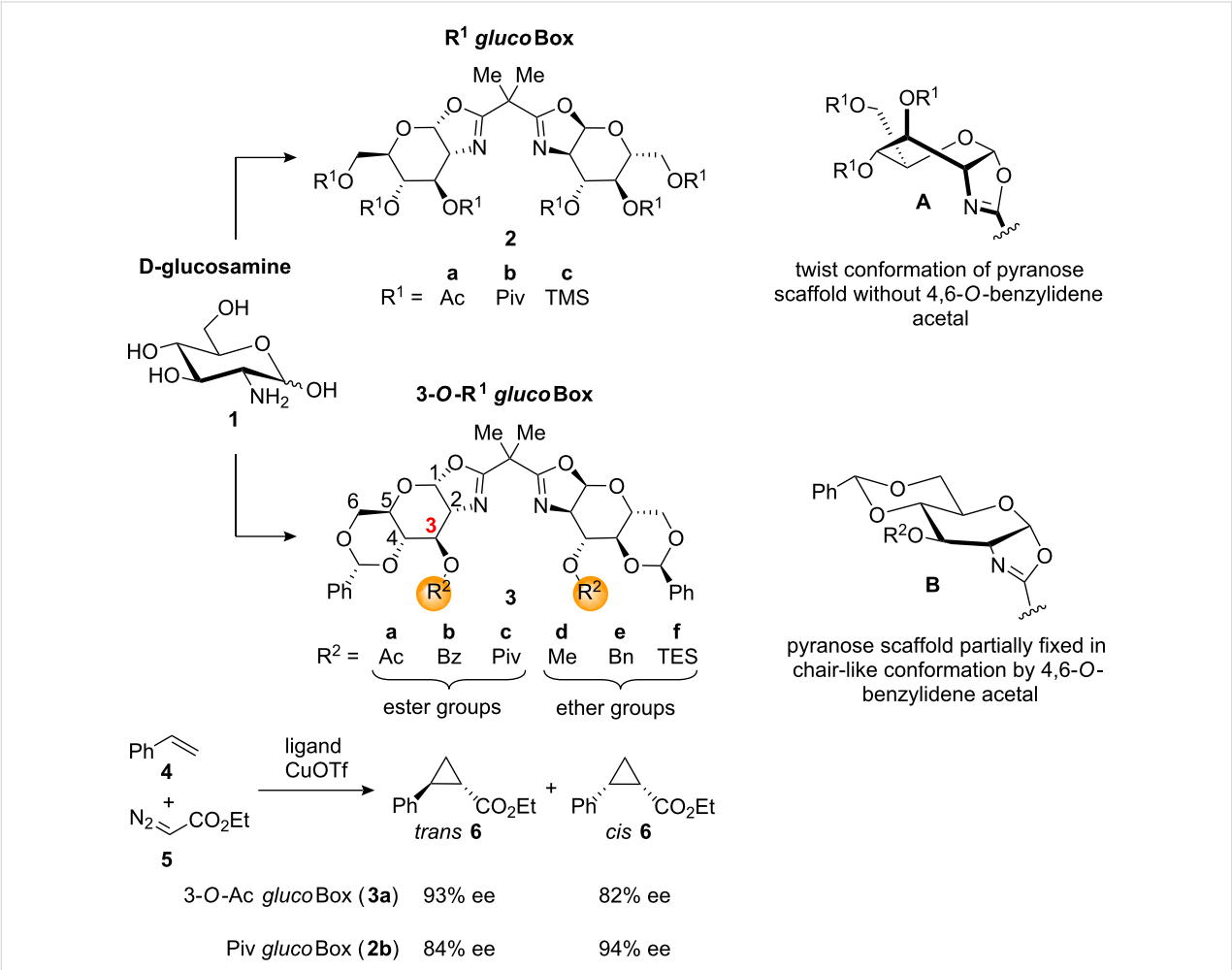
Introduction

The design and optimisation of chiral ligands for metal catalysed transformations is of crucial importance for stereoselective synthesis and is therefore an active field of research. In this context, carbohydrates are interesting, even if comparatively rarely used as starting materials for the preparation of new chiral ligand structures. Today, 30 years after the first reports on carbohydrate-based ligands [1-4], the potential of saccharide compounds in this area is more and more appreciated [5-12].

Chiral bis(oxazolines) (Box) are very efficient ligands for many asymmetric transformations [13,14]. Even though N-acylated derivatives of D-glucosamine easily form bicyclic carbohydrate oxazolines, until recently only a few examples of mono(oxazoline) ligands [15-17] and the corresponding bis(oxazolines) [18] based on this monosaccharide have appeared in the literature. In the course of our work we have introduced new glucosamine-derived bis(oxazolines) **2a**–**c** with uniform protective groups on all oxygen functions [19-21] and derivatives **3a**–**f** with cyclic 4,6-*O*-benzylidene protection as well as various other 3-*O*-substituents that differ in steric demand and electronic nature [20,21].

These ligands were subsequently employed in the asymmetric cyclopropanation [22,23] of styrene (**4**) with ethyl diazoacetate (**5**). Our results revealed a strong dependence of

the enantioselectivity on both the steric bulk and electronic nature of the *O*-substituents in ligands **2a**–**c** and **3a**–**f**. Furthermore, the conformation of the pyranose scaffold – a twist conformation for ligands **2a**–**c** without 4,6-*O*-benzylidene protection (Scheme 1, conformer **A**) and a partially chair-like conformation for ligands **3a**–**f** (Scheme 1, conformer **B**) fixed by the annulated 4,6-*O*-benzylidene group – has a direct impact on the enantioselectivity of the reaction. For ligands **3a**–**f** with cyclic protection, a decrease in the bulk of the 3-*O*-residues led to an improvement in stereoselectivity, while the opposite trend



Scheme 1: New glucosamine-based bis(oxazoline) ligands with their pyranose conformation and application in asymmetric cyclopropanation.

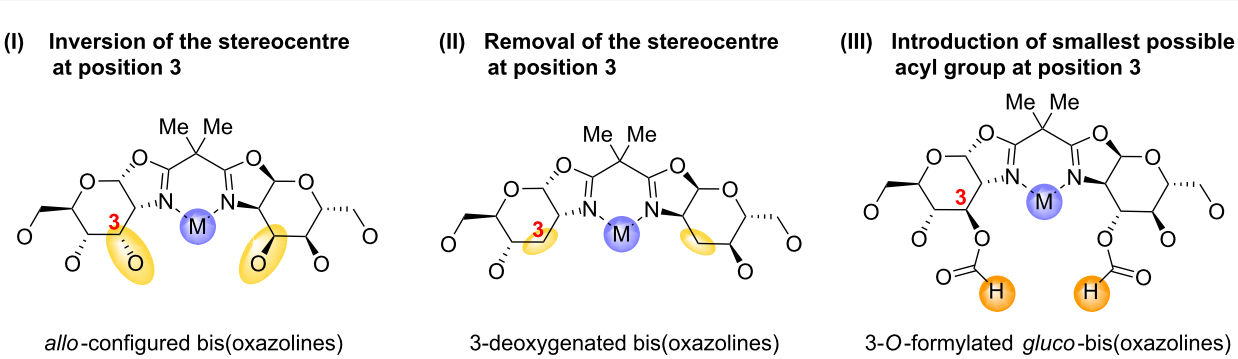
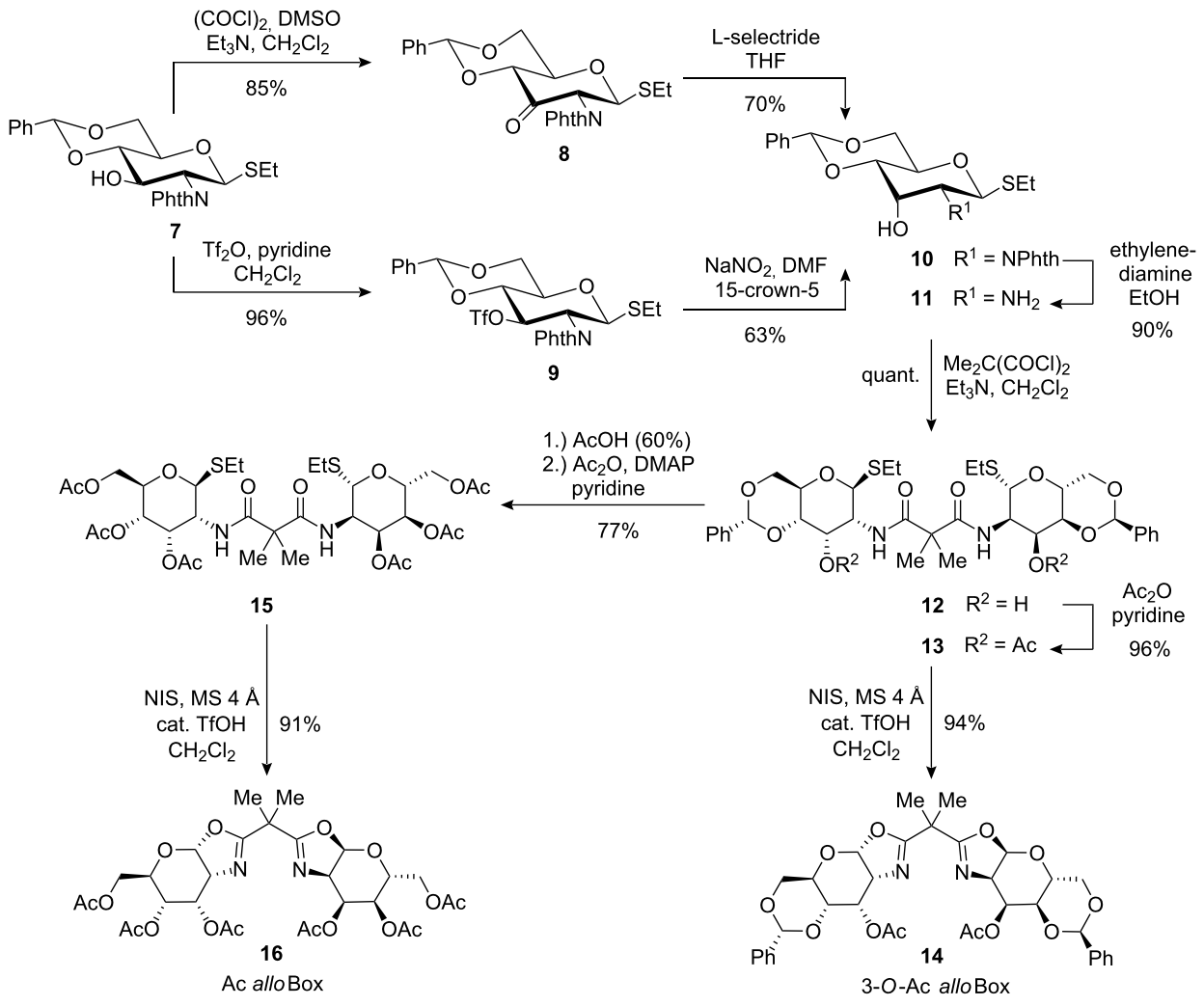


Figure 1: Planned modifications at pyranose position 3 of carbohydrate bis(oxazolines).

was observed for counterparts **2a**–**c** with acyclic 4,6-*O*-protection. Moreover, ester modified ligands **2a**, **2b** and **3a**–**c** led to higher stereoselectivity than the corresponding ether-modified compounds **2c** and **3d**–**f**. The best results were obtained with 3-*O*-Ac *gluco*Box **3a** that combines a small 3-*O*-acyl residue with cyclic 4,6-*O*-protection, and with bulky ligand Piv *gluco*Box (**2b**) without any cyclic protection. These findings are summarised in Scheme 1.

Because of the strong impact of the pyranose position 3 in ligands **3a**–**f** on the stereoselectivity, we became interested in elucidating the influence of the stereochemistry at this position by both 3-epimerisation and 3-defunctionalisation. Inversion of the configuration at position 3 to give an *allo*-configured ligand scaffold, will bring the 3-*O*-substituent into a *syn*-relationship with the oxazoline nitrogen atom and therefore into very close proximity to a coordinated metal centre (Figure 1, **I**). Deoxygenation of the 3-postion on the other hand will lead to a ligand with comparably little steric shielding of metal centres coordinated by the oxazoline nitrogen atoms (Figure 1, **II**). As the stereoselectivity of the model reaction for ligands **3a**–**f** improved with decreasing steric demand of the 3-*O*-substituent and since the best results were obtained with acyl-modified ligands, we also set out to prepare a corresponding ligand with a formyl group as the smallest possible acyl residue at the 3-*O*-position. In this paper we describe the synthesis of new 3-epimerised and 3-deoxygenated carbohydrate bis(oxazolines), the preparation of a 3-*O*-formate analogue of ligands **3** as well as the testing of these new ligands in stereoselective cyclopropanation.

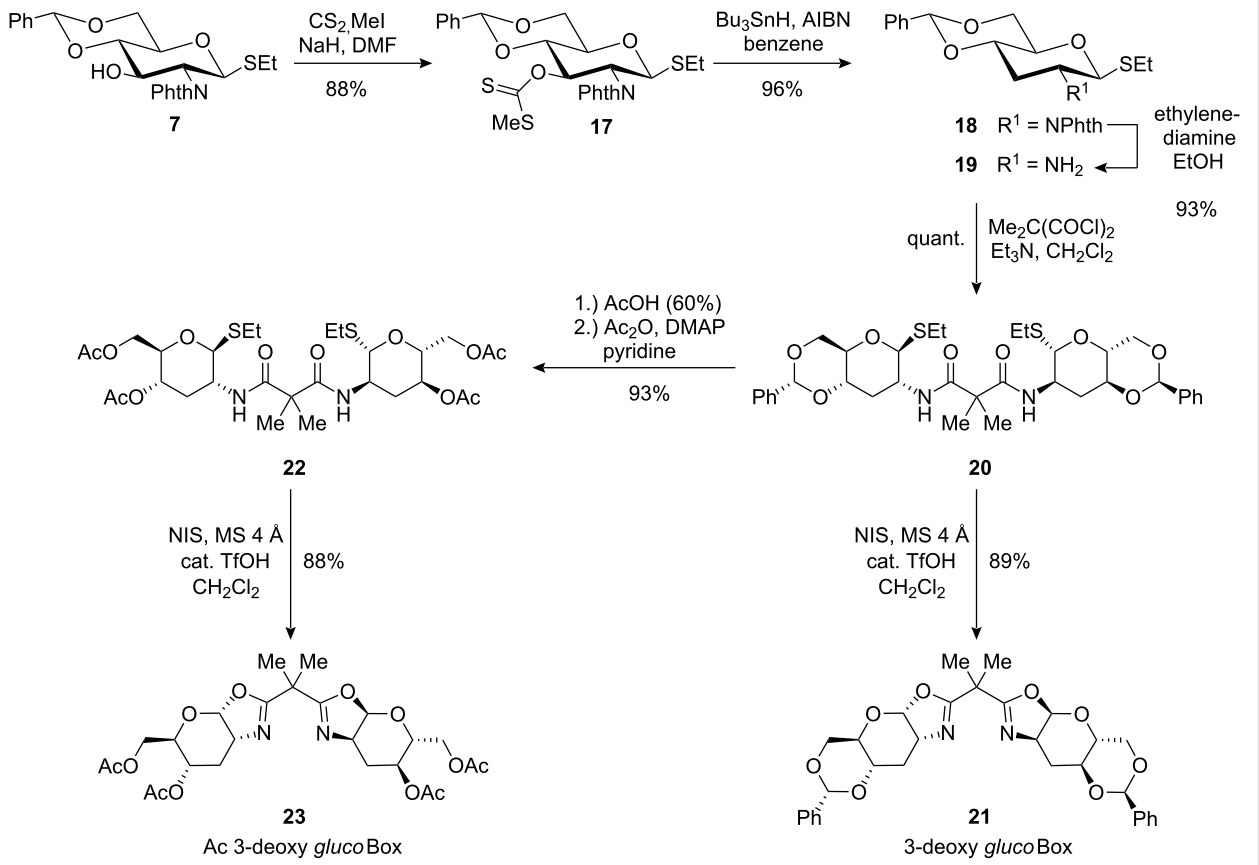


Scheme 2: Synthesis of *allo*-configured bis(oxazolines) 3-*O*-Ac *allo*Box (**14**) and Ac *allo*Box (**16**) from thioglucoside **7** as the key intermediate.

Results and Discussion

The synthesis of all new ligands started from the known thioglucoside **7** [24] which was also employed as key intermediate for the preparation of ligands **3a**–**f** [20,21] and is accessible from D-glucosamine in 5 steps and 57% overall yield. To prepare an *allo*-configured precursor for ligand synthesis, we decided first to use a previously described epimerisation sequence for **7** featuring Swern oxidation and subsequent reduction with sodium borohydride [25]. In our hands this method led to an inseparable product mixture in the second step however, on switching to L-selectride for the stereoselective reduction [26], the allosamine derived thioglycoside **10** was obtained in good overall yield. For an alternative route, **7** was transformed into the 3-*O*-triflate **9** and then subjected to nucleophilic displacement with sodium nitrite in the presence of 15-crown-5 [27,28] to afford **10** in similar yields as the oxidation-reduction sequence (Scheme 2). After deprotection of the phthalimide (phthN) [29], the free amine **11** was transformed into the 4,6-*O*-benzylidene protected ligand by our standard protocol for the preparation of carbohydrate bis(oxazoline) ligands [20,21]: Formation of bis(amide) **12** with dimethylmalonyl chloride, 3-*O*-acetylation and subsequent activation of the thioethyl moieties of **13** with NIS [30] for the double cyclisation step, led to benzylidene protected ligand 3-*O*-Ac *allo*Box **14** in excellent yield. As noted previously, the presence of a 4,6-*O*-benzylidene group has a pronounced influence on the conformation adopted by the pyranose scaffold in *gluco*-configured ligands (Scheme 1, conformers **A** and **B**), which in turn has a direct influence on the stereoselectivity in the model reaction. In order to ascertain if a similar conformative effect is also in operation for *allo*-configured bis(oxazolines), we prepared ligand Ac *allo*Box **16** with acyclic 4,6-*O*-protection by the removal of the benzylidene groups from **12** under acidic conditions and per-*O*-acetylation in a one-pot reaction followed by NIS-mediated cyclisation of resulting bis(amide) **15**.

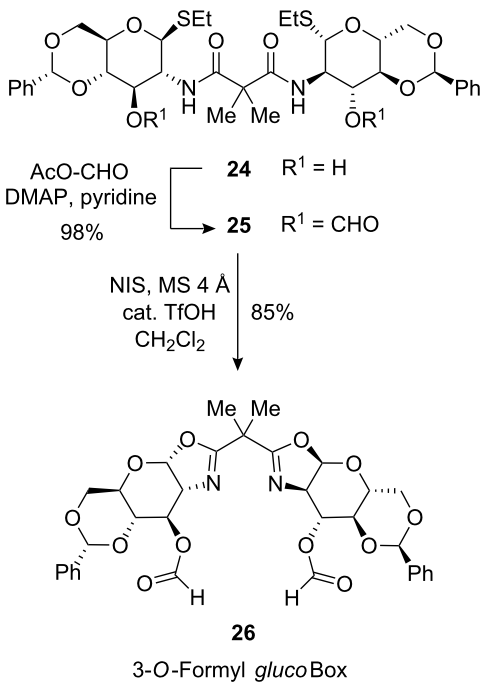
For the preparation of 3-deoxygenated ligands, we planned a defunctionalisation of the key intermediate **7**. Surprisingly, a thorough search of the literature revealed only one example of the 3-deoxygenation of a glucosamine-derived thioglycoside, reported by Herdewijn et al. in 2006 [31]. Because the Barton–McCombie deoxygenation [32] failed on their *N*-Troc protected thio aminoglucoside under various conditions, Herdewijn et al. used a sequence via a 3-iodide derivative. To



Scheme 3: Preparation of ligands 3-deoxy *gluco*Box (**21**) and Ac 3-deoxy *gluco*Box (**23**) from key intermediate **7**.

avoid the rather complicated preparation of a 3-iodo derivative, we tried the Barton–McCombie reaction on our phthalimido protected precursor **7** (Scheme 3). Introduction of the 3-xanthogenate with carbon disulfide and methyl iodide yielded **17**, which was cleanly deoxygenated in high yield by tributyltin hydride under standard conditions [32,33]. From the resulting compound **18**, the ligands 3-deoxy *gluco*Box **21** with benzylidene groups and Ac 3-deoxy *gluco*Box **23** with acyclic 4,6-*O*-protection were prepared in high overall yields (Scheme 3).

The 3-*O*-formate analogue of *gluco*-configured ligands **3** was obtained by treatment of bis(amide) **24** [20,21] with formyl acetate [34] to yield **25** which was then cyclised to the desired ligand **26** with NIS (Scheme 4).



Scheme 4: Preparation of ligand 3-*O*-Formyl *gluco*Box (**26**) from bis(amide) **24**.

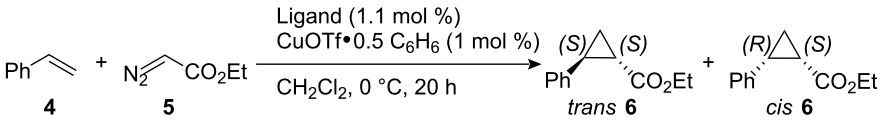
The five new ligands **14**, **16**, **21**, **23** and **26** were now employed in the copper(I) catalysed asymmetric cyclopropanation of styrene (**4**) with diazoacetate (**5**) under known conditions [19,21,22] (Table 1). All ligands led to formation of the products *trans* **6** and *cis* **6** in good to excellent yields and the *trans*/*cis* ratio was in the typical range (around 70:30) obtained with bis(oxazoline) ligands [22]. However, the enantioselectivities differed dramatically for the new ligands and revealed once again the strong influence of position 3 and the pyranose conformation on the efficiency of the asymmetric induction. The best results were obtained with 3-*O*-formyl *gluco*Box **26** which gave *trans* **6** and *cis* **6** in 95% ee and 94% ee respectively (Table 1, entry 5).

Figure 2 gives a summary of the results obtained with the new ligands as well as a comparison with the previously reported ligands **2a** and **3a**. Both, benzylidene-protected ligands 3-*O*-Ac *allo*Box **14** and 3-deoxy *gluco*Box **21** gave only racemic products while their counterparts Ac *allo*Box **16** and Ac 3-Deoxy *gluco*Box **23** lacking cyclic 4,6-*O*-protection led to substantial asymmetric induction. This demonstrates that the dramatic conformational effect of the pyranose scaffold on stereoselectivity, which was first observed for *gluco*-configured ligands **2** and **3**, is also in operation in *allo*- and 3-deoxy *gluco*-ligands. However, while benzylidene protection in 3-*O*-Ac *gluco*Box **3a** led to improved asymmetric induction in comparison to ligand Ac *gluco*Box **2a** lacking cyclic protection, the opposite was observed for the *allo*- and 3-deoxy-ligands. The strong influence of the configuration of pyranose position 3 on stereoselectivity becomes apparent by a comparison of ligand 3-*O*-Ac *gluco*Box **3a** to its 3-epimerised and 3-defunctionalised counterparts **14** and **21**: Both modifications, inversion of the configuration in *allo*-ligand **14** and 3-defunctionalisation in **21** result in a complete loss of stereoselectivity in the model reaction, whilst 3-*O*-Ac *gluco*Box **3a** provides the products in 93% ee and 82% ee respectively. Finally, *gluco*-configured ligand **26** with a 3-*O*-formyl residue led to higher stereoselectivities (95% ee and 94% ee for *trans* **6** and *cis* **6** respectively) than 3-*O*-acetylated ligand **3a**. This confirms the trend we initially observed for *gluco*-configured ligands. A decrease in steric bulk of 3-*O*-acyl substituents results in improved asymmetric induction of the ligand in the cyclopropanation reaction: ee for 3-*O*-Piv **3c** < 3-*O*-Bz **3b** < 3-*O*-Ac **3a** < 3-*O*-Formyl **26**. Thus, of all carbohydrate-derived bis(oxazolines) prepared by us, ligand **26** led to the best enantioselectivities for cyclopropanes *trans* **6** and *cis* **6**.

Conclusion

We have prepared new derivatives of *gluco*-configured bis(oxazoline) ligands **2** and **3** with 3-epimerisation or 3-defunctionalisation in the pyranose scaffold. Application in stereoselective cyclopropanation as a model reaction highlighted the strong impact of modifications at the pyranose position 3 on the asymmetric induction exerted by carbohydrate-based ligands. Furthermore, the previously observed conformational effect of cyclic 4,6-*O*-benzylidene protection on stereoselectivity is also in operation in the new derivatives. Introduction of a 3-*O*-formate in *gluco*Box ligands led to improved stereoselectivities compared to the corresponding 3-*O*-acetate. This underlines our previous findings that the best results for *gluco*-configured ligands are obtained with small acyl-based 3-*O*-substitutents.

Table 1: Cyclopropanations with *allo*-configured ligands **14** and **16**, 3-deoxygenated ligands **21** and **23** and 3-*O*-formylated, *gluco*-configured ligand **26**.



Entry	Ligand	Yield [%][a]	*trans*/*cis*[b]	ee *trans* [%][b]	ee *cis* [%][b]
1	3-*O*-Ac *allo*Box (**14**)	75	66:34	rac.	rac.
2	Ac *allo*Box (**16**)	79	70:30	71	87
3	3-deoxy *gluco*Box (**21**)	86	69:31	rac.	rac.
4	Ac 3-deoxy *gluco*Box (**23**)	75	74:26	78	72
5	3-*O*-formyl *gluco*Box (**26**)	95	71:29	95	94

[a]Isolated yield after chromatography.
[b]Determined by GC on a chiral stationary phase.

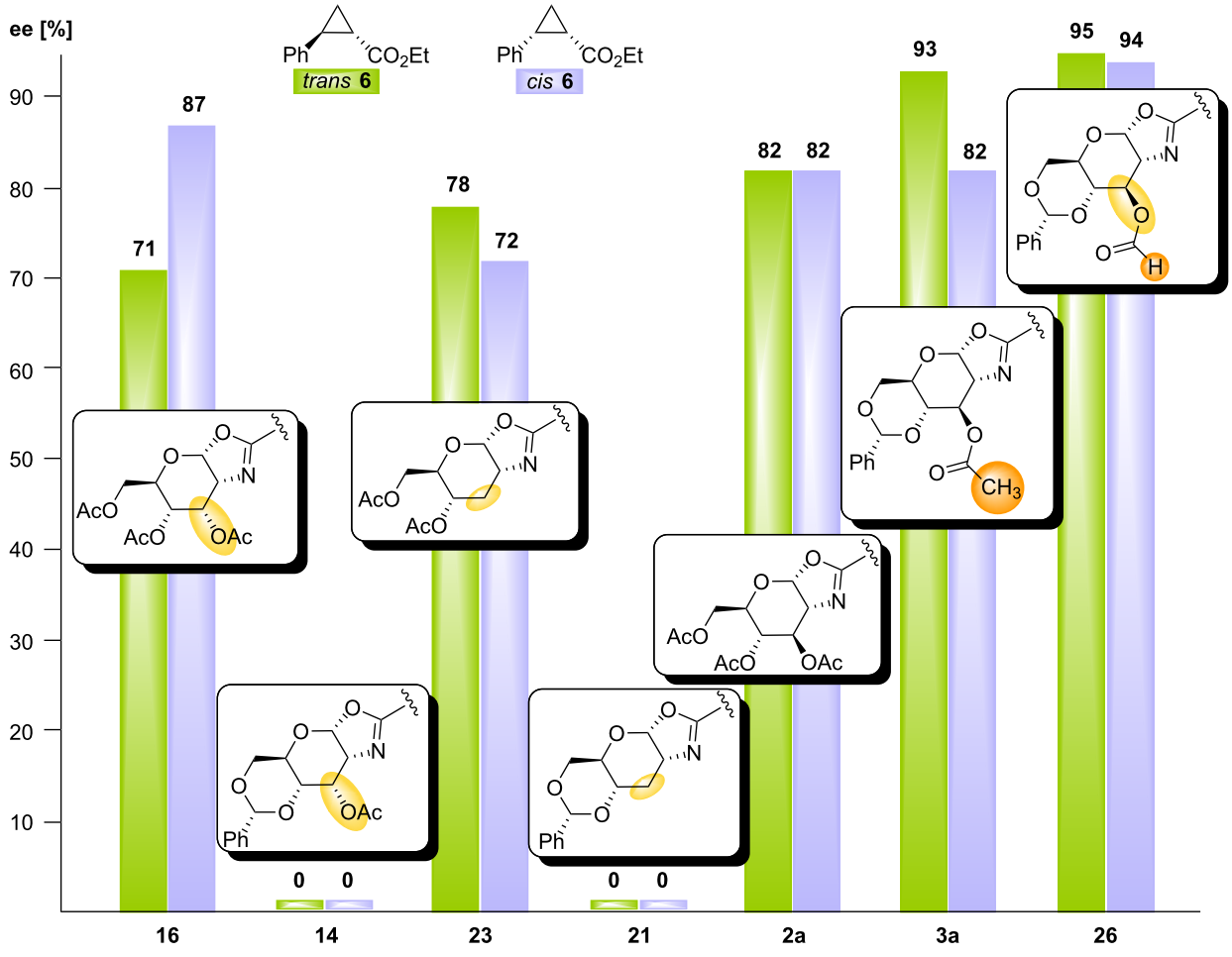


Figure 2: Impact of structural ligand modifications on the stereoselectivity of cyclopropanations.

The observed steric, configurational and conformational effects are as yet not fully understood and investigations to elucidate their origins are currently under way.

Supporting Information

Supporting information contains full experimental details for the preparation of all new ligands and general conditions for cyclopropanations using *gluco*Box ligands and copper(I) triflate.

Supporting Information File 1

Experimental details.
[http://www.beilstein-journals.org/bjoc/content/supplementary/1860-5397-6-23-S1.pdf]

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Bioorthogonal metabolic glycoengineering of human larynx carcinoma (HEp-2) cells targeting sialic acid

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Full Research Paper

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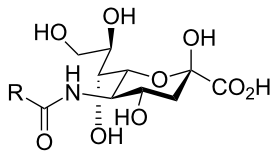
Abstract

Sialic acids are located at the termini of mammalian cell-surface glycostructures, which participate in essential interaction processes including adhesion of pathogens prior to infection and immunogenicity. Here we present the synthesis and bioorthogonal metabolic incorporation of the sialic acid analogue *N*-(1-oxohex-5-ynyl)neuraminic acid (Neu5Hex) into the cell-surface glycocalyx of a human larynx carcinoma cell line (HEp-2) and its fluorescence labelling by click chemistry.

Introduction

The surface of eukaryotic cells is heavily covered with glycan structures of various types forming the individual, dynamic glycocalyx of each cell type. These glycolipids and glycoproteins often carry sialic acids, in humans *N*-acetylneuraminic acid (Neu5Ac, **1**, Scheme 1), at their terminal position which mediate cell-cell recognition and signal transduction processes involved in infection, inflammation or tumor formation [1]. Recent studies have shown that the surface of a T-cell line (Jurkat), Chinese hamster ovary (CHO) cells, cervical adenocarcinoma (HeLa) cells as well as many other cell types can be labelled with bioorthogonal, that is metabolically inert, functionalized carbohydrates both in vitro and in vivo [2,3]. Acetylated monosaccharides, for example 2-azidoacetylamino-2-deoxy-1,3,4,6-tetraacetyl-β-D-glucopyranoside (Ac_4GlcNAz, **16**), are believed to permeate the cell membrane by diffusion processes [4]. Recently, it was reported that neuraminic acid analogues enter the cell by pinocytosis and are incorporated into the cellular glycosylation machinery by active transporter systems [5]. In other mammals *N*-glycolylneuraminic acid (Neu5Gc, **2**, Scheme 1) corresponds to Neu5Ac **1** found in humans. Although the human gene for the synthesis of Neu5Gc **2** is inactive, small amounts of Neu5Gc **2** are also found in the human metabolism presumably dietary derived from carbohydrate salvage pathways [5,6]. The efficient uptake and incorporation of sialic acid modified in positions C-5 and C-9 into human B-lymphoma cells (BJA-B), Jurkat and others including primary cells has been demonstrated [3,7]. The sialic acid modifications influence the interaction with sialic acid binding immunoglobulin-like lectin (Siglec)-2 and infection processes of BJA-B cells by the B-lymphotrophic papovavirus [8]. It was

further shown that the uptake and incorporation of alkynylated *N*-acetylmannosamine (1,3,4,6-tetraacetyl-*N*-(4-pentynoyl)mannosamine) into six different kinds of cells was more efficient than the incorporation of its azido derivative (1,3,4,6-tetraacetyl-*N*-azido-acetylmannosamine) [3]. In the current study, metabolic glycoengineering of human larynx carcinoma (HEp-2) cells with *N*-(1-oxohex-5-ynyl)neuraminic acid (Neu5Hex, **3**) is demonstrated. The bioorthogonal modification, that is the introduction of hexyne, was carried out at the sialic acid acetyl residue at position C-5 which is prone to mammalian evolution processes [5,6].



1 R = -CH_3, Neu5Ac, human
2 R = -CH_2OH, Neu5Gc, mammals
3 R = -$CH_2(CH_2)_2CCH$, Neu5Hex

Scheme 1: The natural forms of sialic acids, human *N*-acetylneuraminic acid (Neu5Ac, **1**) and mammalian *N*-glycolylneuraminic acid (Neu5Gc, **2**). *N*-(1-oxohex-5-ynyl)neuraminic acid (Neu5Hex, **3**) is used for bioorthogonal metabolic labelling of human larynx carcinoma (HEp-2) cells.

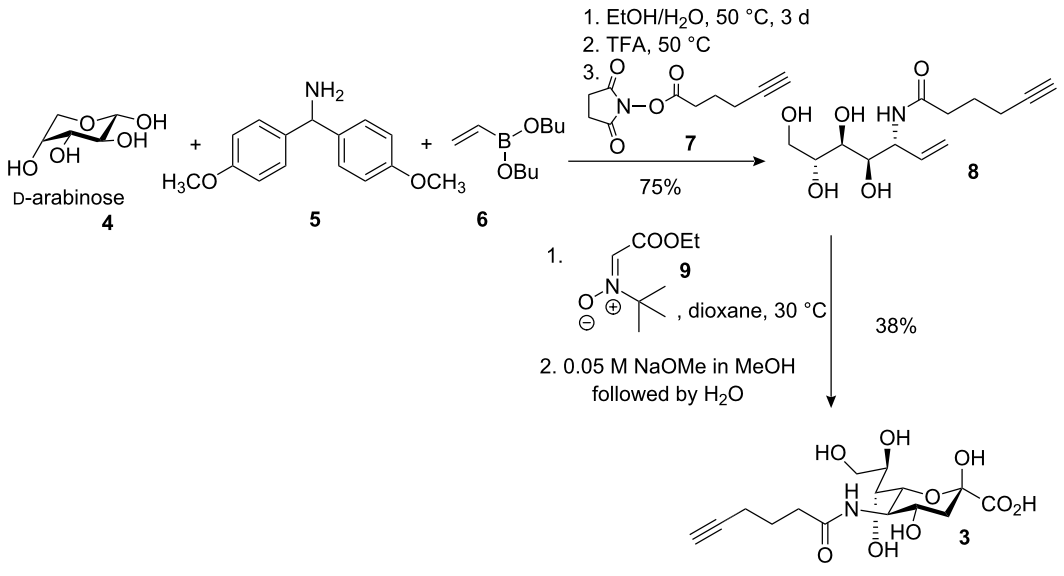
Results and Discussion

Synthesis of the sialic acid analogue *N*-(1-oxohex-5-ynyl)neuraminic acid (Neu5Hex, **3**)

The bioorthogonality of *N*-(1-oxohex-5-ynyl)neuraminic acid (**3**) was exploited to incorporate it into human larynx carcinoma (HEp-2) cells by metabolic glycoengineering. The synthesis of *N*-(1-oxohex-5-ynyl)neuraminic acid (Neu5Hex, **3**) was achieved by a previously described route [9]. The Petasis coupling was performed starting from D-arabinose (**4**), the secondary amine **5** and dibutyl vinyl boronic acid ester **6**. In situ hydrolysis of the bis(4-methoxyphenyl)methyl group with a catalytic amount of trifluoroacetic acid (TFA), followed by *N*-acylation with the activated ester **7** led to the alkyne **8** in a yield of 75% based on D-arabinose. A [3+2] cycloaddition reaction between *N*-*tert*-butyl nitrone **9** and **8** and subsequent base-catalyzed ring-opening and hydrolysis afforded *N*-(1-oxohex-5-ynyl)neuraminic acid (Neu5Hex, **3**) in 38% yield (Scheme 2).

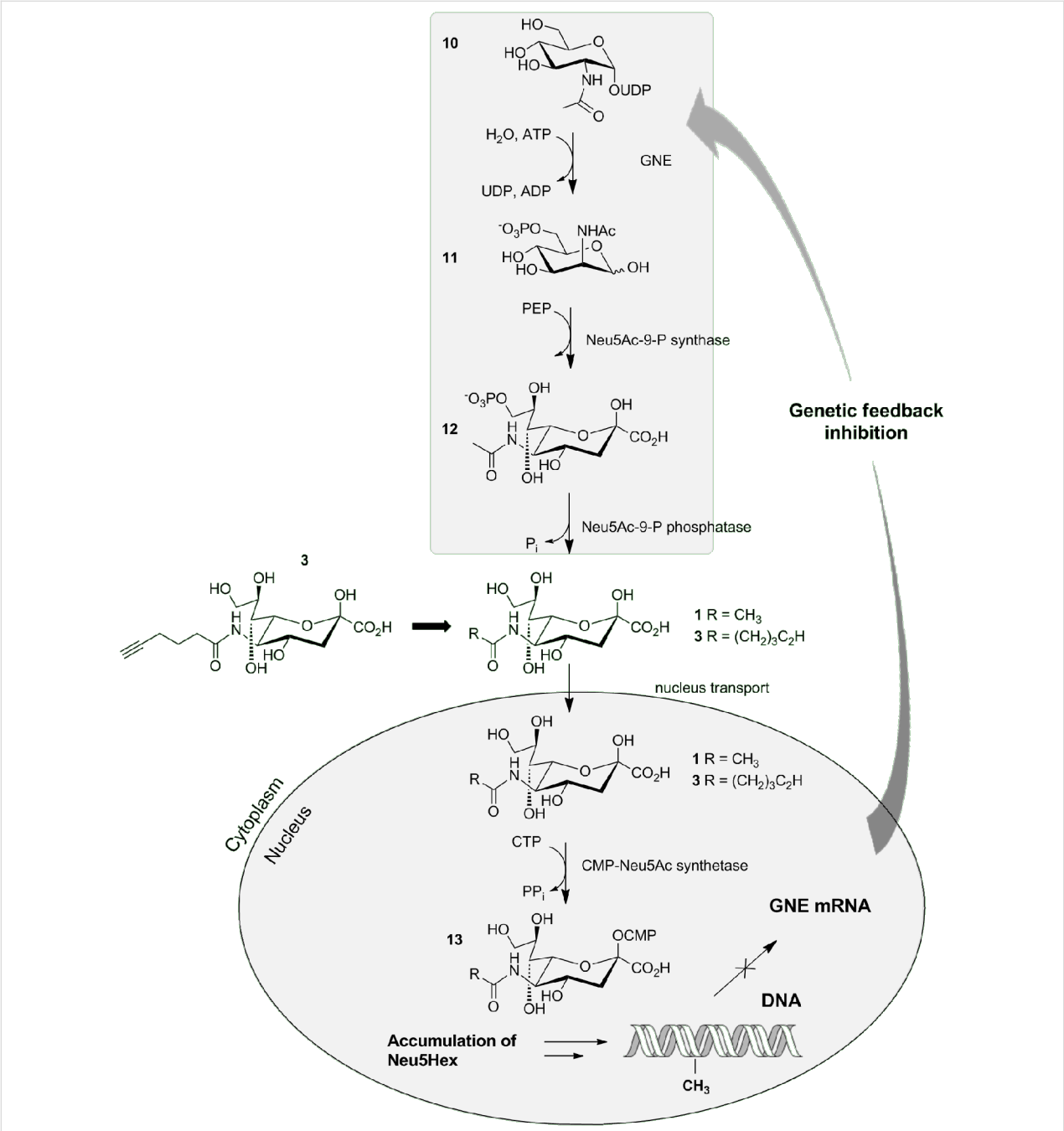
Metabolic glycoengineering of human larynx carcinoma (HEp-2) cells by incorporation of *N*-(1-oxohex-5-ynyl)neuraminic acid (Neu5Hex, **3**)

The metabolic labelling of human larynx carcinoma (HEp-2) cell surfaces was carried out in order to study and characterize the influence of sialic acid in cell signalling and cell-cell interactions. HEp-2 cells were investigated because of their metabolic capability to incorporate 2-azidoacetylamino-2-deoxy-(1,3,4,6)-tetraacetyl-β-D-glucopyranoside (Ac_4GlcNAz, **16**). The internalization of this acetylated monosaccharide was described previously as a diffusion process through the membrane of eukaryotic cells [3]. Neu5Hex (**3**) is a new substrate for metabolic glycoengineering which is proposed to be incorporated into the cell surface glycan structures. It was shown that carbohydrates in growth media contribute to alterations in glycosylation patterns in human cells [8,10]. The bifunctional enzyme UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase (GNE) is the key enzyme in sialic

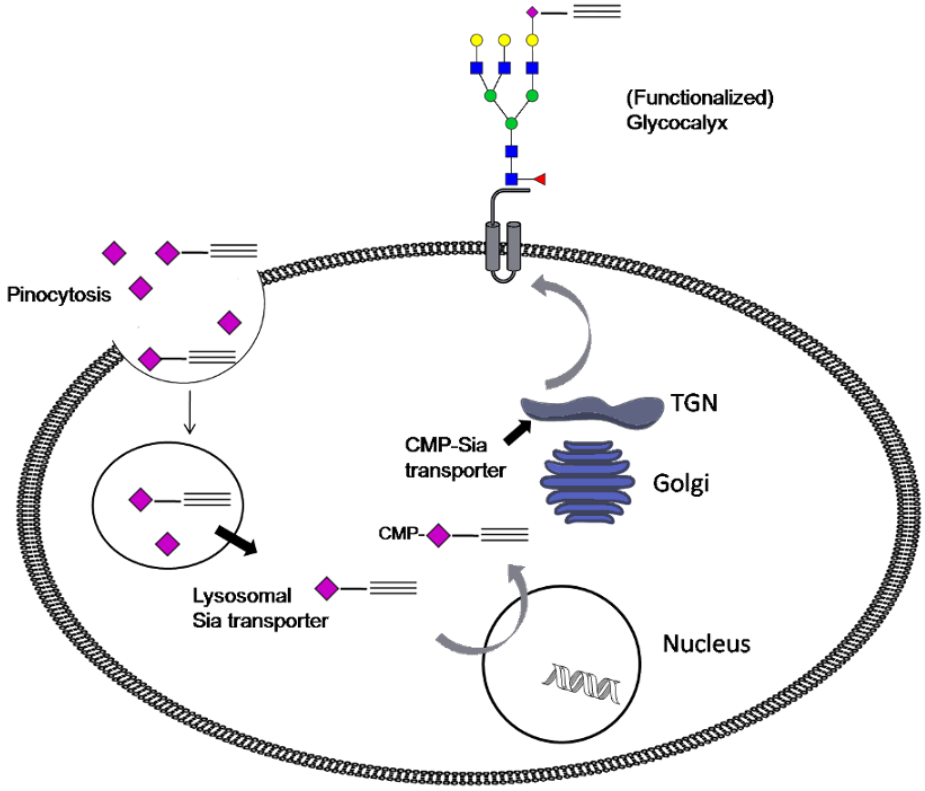


Scheme 2: Synthesis of N-(1-oxohex-5-ynyl)neuraminic acid (Neu5Hex **3**).

acid biosynthesis. The inhibitory effect of the sialic acid concentration towards the UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase (GNE) by allosteric effects is known [11]. Recently, the regulation of UDP-GlcNAc 2-epimerase/ManNAc kinase expression on the transcriptional level by DNA methylation was demonstrated [12] and a genetic feedback regulation for this process was proposed (Scheme 3) [13]. Ac_4GlcNAz **16** or Neu5Hex **3**, respectively, were incubated with HEp-2 cells. Ac_4GlcNAz **16** is believed to enter the cell by diffusion through the membrane, to undergo deacetylation in the cytoplasm and then incorporated into the cell surface glycoproteins and glycolipids. Alternatively, it is metabolically converted to Neu5Az [14].



Scheme 3: Metabolic pathway of Ac_4GlcNAz and the genetic control of Neu5Ac **1** synthesis by feedback inhibition. The accumulation of Neu5Hex **3** is proposed by incubation of **3** with the target cell line as the synthesis of Neu5Ac **1** is down-regulated [11,13]. UDP: uridine diphosphate; GNE: UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase; ATP: adenosine triphosphate; PEP: phosphoenolpyruvate; CTP: cytidine triphosphate; PP_i: pyrophosphate; DNA: deoxyribonucleic acid; mRNA: messenger ribonucleic acid.



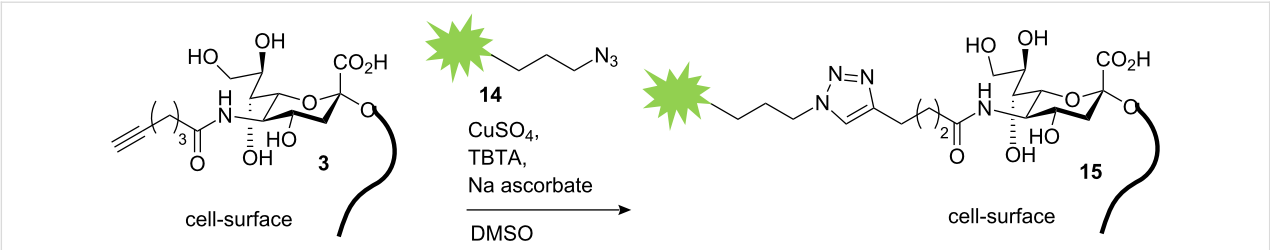
Scheme 4: Proposed metabolic pathway of Neu5Hex **3** based on known mechanisms of Neu5Gc **2** uptake [5]. TGN: trans-Golgi network, CMP: cytidine monophosphate, Sia: sialic acid.

Neu5Hex may enter the cell by the previously described pinocytosis processes (Scheme 4) or by an, as yet, unknown internalization mechanism [5]. It is believed that Neu5Hex enters the nucleus and enhances the genetic feedback control of the GNE coding gene which blocks the synthesis of natural Neu5Ac [11,13]. Alkyne- or azide-functionalized carbohydrates in the glycocalyx are specifically addressed by complementary functionalized fluorescence agents 9-[2-carboxy-4-[(2-propyn-1-ylamino)carbonyl]phenyl]-3,6-bis(dimethylamino)xanthylium, alkynylated TAMRA or benzoic acid 2-[6-(3-azidopropanyloxy)-3-oxo-3*H*-xanthen-9-yl] 3-azidopropanyl ester, azido-fluorescein (**14**).

For the metabolic labelling of eukaryotic cells, HEp-2 cells were incubated in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS). At 80% confluence they were split into 6-well plates with DMEM containing the functionalized carbohydrates (Ac_4GlcNAz **16** or Neu5Hex **3**, 25 μM, 48 h). HEp-2 cells were harvested with a cell scraper, not trypsin, in order to preserve the partially protein-coupled glycocalyx. To highlight the successful incorporation of the azide and alkyne functionalities into the glycocalyx of HEp-2 cells, the fluorescence labelling reaction was performed according to a modified protocol of the [3+2] triazole cycloaddition [15,16]. The appropriate functionalized fluorescent detection molecule and the conditions for the click reaction ($CuSO_4$, sodium ascorbate and tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine, TBTA) were applied in dimethylsulfoxide (DMSO) (Scheme 5). After one hour, the cells were analyzed by microscopy (phase contrast) at the appropriate wavelength for fluorescence imaging. Although the incubation of HEp-2 in DMSO and in the presence of copper ions is cytotoxic, the fluorescence in the labelled glycocalyx was clearly detectable. In order to analyze the natural background fluorescence of HEp-2, one sample was incubated without any additional carbohydrates. The cells were analyzed by fluorescence microscopy (580 nm for TAMRA staining and at 525 nm for fluorescein). At either wavelength, the negative control does not show any significant background fluorescence (Figure 1). In both the Neu5Hex fed HEp-2 and the incubation with Ac_4GlcNAz a clear staining of the cellular glycocalyx at the expected wavelengths was observed.

Conclusion

Sialic acids are prominent sugars which are located in the terminal position on cell-surface glycans. Although it has been known for many years that sialic acids are involved in myriads of interaction processes including viral infections such as the



Scheme 5: Labelling of alkynylated neuraminic acid by azido-fluorescein.

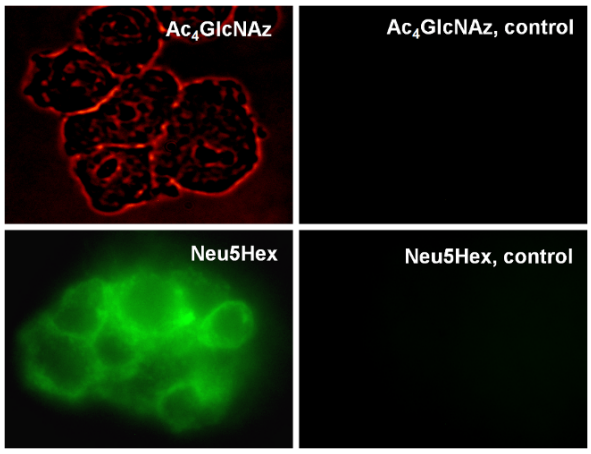


Figure 1: Top left: HEp-2 cells incorporated with Ac_4GlcNAz **16,** labelled with alkynylated TAMRA at 580 nm. Bottom left: HEp-2 cells with incorporated NeuNHex **3**, labelled with azido-fluorescein **14**, at 525 nm. On the right: background and unspecific staining controls at the same wavelengths as the corresponding pictures on the left.

emerging flu variants, their biological role on cell surfaces of different cell lines and at different development states remains unclear. As new techniques for probing glycans have evolved only relatively recently, more information about the fundamental biological functions of carbohydrate structures can be obtained. Therefore we introduced metabolic glycoengineering of the human larynx carcinoma cell line HEp-2. The incorporation and cell surface presentation of Ac_4GlcNAc **16** as well as the new substrate Neu5Hex **3** was successful. The copper-catalyzed [3+2] triazole formation ("click reaction") proved very useful for the cell surface labelling because of its bioorthogonality. The incubation of HEp-2 cells with the sialic acid analogue Neu5Hex **3** guarantees its direct incorporation into the cell surface glycan patterns bypassing metabolic bottlenecks. Furthermore, the described genetic feedback inhibition by sialic acid leading to an accumulation of the fed Neu5Hex **3** ensures an efficient integration into the cell surface glycocalyx. A drawback of the reaction parameters and compounds used for the click reaction is the cytotoxicity of DMSO and copper. But this problem for in vivo labelling can be overcome by different reaction conditions and different detection molecules. For example, the strain-promoted click reaction with difluorinated cyclooctyne (DIFO) and cell-surface azido-glycans introduced recently has been proven to be suitable for in vivo labelling [14,17,18].

Experimental

2-azidoacetylamino-2-deoxy-1,3,4,6-tetraacetyl-β-D-glucopyranoside (**16**) was synthesized as described previously [9]

N-(1*R*,2*R*,3*S*,4*R*)-Hex-5-yonic acid (2,3,4,5-tetrahydroxy-1-vinyl-pentyl)-amide (**8**)

A solution of D-arabinose (1.09 g, 5.73 mmol, **4**), 4,4'-dimethoxybenzhydrylamine (1.39 g, 5.73 mmol, **5**) and vinyl boronic acid dibutyl ester (2.51 g, 11.46 mmol, **6**) in aqueous ethanol (60 mL, ethanol/H_2O = 4:1) was stirred at 50 °C for 72 h. TFA (1.79 ml) was added and the reaction mixture stirred for a further 16 h. The solvent was evaporated and the residue dissolved in MeOH (30 mL). Sodium bicarbonate (974 mg, 11.59 mmol) and 5-hexynoic acid, 2,5-dioxo-1-pyrrolidinyl ester [15] (950 mg, 8.5 mmol) was added and the solution stirred for 1 h at room temperature. The solids were removed by filtration, the filtrate was dried and the residue purified by flash chromatography on silica gel (eluted with CH_2Cl_2 and MeOH) to afford **8** as white solid in 75% yield. R_f = 0.34 (CH_2Cl_2/MeOH, 7:1); $[\alpha]_D^{20}$: +19.8 (*c* 1, MeOH); ^{1}H NMR (400 MHz, CD_3OD) δ = 6.05 (ddd, *J* = 17.29, 10.53, 5.66 Hz, 1H, 1"-H), 5.27 (td, *J* = 17.29, 1.52 Hz, 1H, 2"-H), 5.22 (td, *J* = 10.54, 1.52 Hz, 1H, 2"-H), 4.56 (m, 1H, 1-H), 3.84–3.50 (m, 5H, 5-H_2, 4-H_2, 3-H, 2-H), 2.50 (t, *J* = 7.26 Hz, 2H, 2'-H_2), 2.30–2.20 (m, 3H, 6'-H, 4'-H_2), 1.90–1.70 (m, 2H, 3'-H_2); ^{13}C NMR: (100 MHz, CD_3OD) δ = 175.29 (CO), 137.04 (C-1"), 116.65 (C-2"), 84.12 (C-5'), 72.46, 72.11, 71.55 (C-4, C-3, C-2), 70.34 (C-6'), 64.94 (C-5), 55.11 (C-1), 35.89 (C-2'), 25.77 (C-3'), 18.58 (C-4'); MS (ESI): *m/z* $[M+Na]^+$ calculated for $C_{13}H_{20}NO_5[Na]^+$, 294.14, found 294.1.

Synthesis of *N*-(hex-5'-ynoyl)neuraminic acid (1"*S*,2"*R*,3"*S*,4"*R*)-2-*tert*-butyl-5-(1"-(hex-5'-ynoyl)amino-2",3",4",5"-tetrahydroxy-pentyl)-isoxazolidine-3-carboxylic acid ethyl ester

Polyhydroxy olefin (1.50 g, 5.53 mmol, **8**) and nitrone (2.01 g, 11.6 mmol, **9**) in dioxane (100 mL) were stirred at 30 °C for 14 d. After complete conversion of the starting material as

monitored by TLC, the solvent was removed at reduced pressure. The residue was purified by normal silica gel chromatography (MeOH/CH_2Cl_2, 1:10 to 1:5) to afford the ester as colourless oil (2.06 g, 4.51 mmol) in 82% yield. R_f = 0.42 (CH_2Cl_2/MeOH, 7:1); $[\alpha]_D^{20}$ = +7.2 (*c* 1, MeOH); ^{1}H NMR (400 MHz, CD_3OD) δ = 4.66–4.61 (dt, *J* = 8.44, 1.56 Hz, 1H, 5-H), 4.14–4.08 (dq, *J* = 7.16, 1.54 Hz, 2H, OCH_2CH$_3$), 3.91–3.87 (t, *J* = 8.52 Hz, 1H, 1''-H), 3.86–3.47 (m, 5H, 5''-H_2, 4''-H, 3''-H, 2"-H), 3.32–3.28 (dd, *J* = 8.70, 0.83 Hz, 1H, 3-H), 3.23–3.20 (m, 1H, 6'-H), 2.65–2.57 (m, 1H, 4-H_a), 2.40–2.34 (t, *J* = 7.18 Hz, 2H, 4'-H_2), 2.18–2.06 (m, 3H, 4-H_b, 2'-H_2), 1.79–1.71 (m, 2H, 3'-H_2), 1.20–1.15 (t, *J* = 7.12 Hz, 3H, OCH$_2$CH_3), 1.05 (s, 9H, C(CH$_3$)$_3$); ^{13}C NMR (75 MHz, CD_3OD) δ = 177.07 (CONH), 174.22 (COO), 84.19 (C-5'), 77.34 (C-5), 72.15, 71.42, 70.63 (C-4'', C-3'', C-2"), 70.35 (C-6'), 65.27 (C-5''), 62.50 (OCH$_2$CH$_3$), 62.23 (C-3), 61.21 (*C*(CH$_3$)$_3$, 53.71 (C-1''), 39.07 (C-4), 35.92 (C-2'), 25.91 (C-4'), 25.91 (C(*C*H$_3$)$_3$), 25.85 (C-3'), 14.45 (OCH$_2$*C*H$_3$); MS (ESI): *m/z* [M+Na]$^+$ calculated for $C_{21}H_{36}N_2O_8$[Na]$^+$ 467.2, found 467.2.

N-(Hex-5'-ynoyl)neuraminic acid (**3**)

Isoxazoline (2.50 g, 5.63 mmol) and NaOMe (0.74 mL of 5.4 M solution in MeOH) in anhydrous MeOH (100 mL) were stirred at room temperature overnight. Water (100 mL) was added and the solution stirred for further 24 h. The mixture was then neutralized with acidic ion exchange resin containing formate ions (Amberlyte). The solvent was removed under reduced pressure and the crude product subjected for size exclusion chromatography with Biogel P2 (Bio-Rad) to afford pure **3** (934 mg) in 46% yield. R_f = 0.29 (CH_2Cl_2/MeOH, 5:2); $[\alpha]_D^{20}$ = -19.04 (*c* 1, H_2O); ^{1}H NMR (300 MHz, CD_3OD), β-anomer: δ = ppm 4.09–4.02 (m, 1H, 4-H), 4.03–4.00 (d, *J* = 10.74 Hz, 1H, 6-H), 3.87–3.81 (t, *J* = 10.29 Hz, 1H, H-5), 3.81–3.79 (dd, *J* = 11.47, 2.74 Hz, 1H, 9-H_a), 3.74–3.89 (m, 1H, 8-H), 3.64–3.60 (dd, *J* = 11.21, 5.60, 1H, 9-H_b), 3.52–3.49 (d, *J* = 9.35, 1H, 7-H), 3.23–3.20 (m, 1H, 6'-H), 2.42–2.38 (t, *J* = 7.35 Hz, 2H, 4'-H_2), 2.26–2.20 (m, 4H, 4-H_a, 4-H_b, 2'-H_2), 2.17–2.11 (dd, *J* = 12.83, 4.87, H-3eq), 1.86–1.80 (m, 3H H-3ax, 3'-H_2); ^{13}C NMR (75 MHz, CD_3OD), δ = 177.00 (2 × CONH), 173.49 (COOH), 96.49 (C-1), 84.05 (C-5'), 72.03 (C-8), 71.55 (C-6), 70.08 (C-7), 70.03 (C-6'), 67.63 (C-4), 64.68 (C-9), 53.94 (C-5), 40.94 (C-3), 35.67 (C-2'), 25.64 (C-3'), 18.49 (C-4'); MS (ESI): *m/z* [M-H]$^-$ calculated for $C_{14}H_{21}NO_9$[H]$^-$ 360.13, found 360.2.

Benzoic acid 2-[6-(3-azidopropanyloxy)-3-oxo-3*H*-xanthen-9-yl] 3-azidopropanyl ester, azido-fluorescein (**14**)

Iodopropyl azide (210 mg, 26 mmol) was added to a solution of fluorescein (1g, 2.6mmol) in a mixture of distilled THF/MeOH (1:1, 25 mL) and the reaction mixture stirred overnight. The crude mixture was concentrated under reduced pressure, diluted with water and extracted with EtOAc (3 × 25 mL). The combined organic layers were dried, concentrated followed and purified by flash chromatography to afford the pure required product (1.1 g, 2.2 mmol) in 84% yield. R_f = 0.34 (EtOAc), ^{1}H NMR (400 MHz, $CDCl_3$), δ = 8.18 (dd, *J* = 7.80, 1.38 Hz, 1H), 7.65 (m, 2H, 4-H, 5-H), 7.25 (dd, *J* = 7.55, 1.23 Hz, 1H, 3-H), 6.90 (d, *J* = 2.44 Hz, 1H, 5'''-H), 6.82 (d, *J* = 8.91 Hz, 1H, 8'''-H), 6.78 (d, *J* = 9.71 Hz, 1H, 1'''-H), 6.68 (dd, *J* = 8.91, 2.44 Hz, 1H, 7'''-H), 6.47 (dd, *J* = 9.71, 1.97 Hz, 1H, 2'''-H), 6.38 (d, *J* = 1.97 Hz, 1H, 4'''-H), 4.10 (t, *J* = 5.95 Hz, 2H, OCH_2), 4.01 (m, 2H, OCH_2), 3.47 (t, *J* = 6.50 Hz, 2H, CH_2N$_3$), 2.99 (m, 2H, CH_2N$_3$), 2.03, 1.55 (2m, 4H, 2''-H_2, 2'''-H_2). ^{13}C NMR (101 MHz, $CDCl_3$), δ = 185.56 (C-3'''), 165.23 (C-1'), 163.10 (C-6'''), 158.70 (C-4a'''), 154.12 (C-5a'''), 149.67 (C-9a'''), 134.15 (C-2), 132.78, 131.31, 130.49, 130.11, 130.08, 129.72, 128.90 (C-3, C-4, C-5, C-6, C-1''', C-7''', C-8'''), 130.27 (C-9'''), 117.71, 114.91 (C-1, C-8a'''), 113.55 (C-5'''), 105.88 (C-2'''), 100.93 (C-4'''), 65.38 (C-1''''), 62.37 (C-1''), 47.92 (C-3''''), 47.77 (C-3''), 28.45 (C-2''''), 27.76 (C-2'').

Cultivation and metabolic labelling of HEp-2 cells

Human larynx carcinoma (HEp-2) cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) at 37 °C under a 5% CO_2 atmosphere. At 80% confluence the medium was discarded and the cells washed with PBS buffer (Gibco). After the addition of 1.5 ml of a trypsin/EDTA mixture, the cells were detached for 5 min at 37 °C. They were supplied with 8.5 ml of fresh medium and split in a ratio of 1:10.

For the metabolic labelling, HEp-2 cells were cultivated as described above. Subsequently, at 80% confluence they were seeded into 6-well dishes and incubated in 2 ml of the medium described above. The medium contained 25 μM of the modified carbohydrate to be incorporated (Ac$_4$GlcNAz **16** or Neu5Hex **3**). The incubation time was 48 hours. The cells were detached using a cell scraper in order to retain the glycocalyx. 150 μL from each well was transferred into an 8-well microscopy cultivation slide and filled with 150 μL of the fresh medium. The cells were cultivated at the described growth conditions until reattachment. The medium was discarded and the cells were washed several times with PBS buffer (Gibco). The labelling reaction was performed in the dark with 2 mM of the complementary labelling molecule 9-[2-carboxy-4-[(2-propyn-1-ylamino)carbonyl]phenyl]-3,6-bis(dimethylamino)xanthylium, alkynylated TAMRA or azido-fluorescein **14**) with 2 mM $CuSO_4$, 10 mM sodium ascorbate and 2 mM Tris-[(1-benzyl-1*H*-1,2,3-triazol-4-yl) methyl]amine (TBTA) in DMSO. After

1 h each well was washed several times with DMSO/water (1:1) and subsequently examined by fluorescence microscopy.

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Synthesis of glycosylated β^3-homo-threonine conjugates for mucin-like glycopeptide antigen analogues

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Full Research Paper

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Abstract

Glycopeptides from the mucin family decorated with tumour-associated carbohydrate antigens (TACA) have proven to be important target structures for the development of molecularly defined anti-cancer vaccines. The strategic incorporation of β-amino acid building blocks into such mucin-type sequences offers the potential to create pseudo-glycopeptide antigens with improved bioavailability for tumour immunotherapy. Towards this end, T_N and TF antigen conjugates *O*-glycosidically linked to Fmoc-β^3-homo-threonine were prepared in good yield via Arndt–Eistert homologation of the corresponding glycosyl α-amino acid derivative. By incorporation of T_N-Fmoc-β^{3}hThr conjugate into the 20 amino acid tandem repeat sequence of MUC1 using sequential solid-phase glycopeptide synthesis, a first example of a mixed α/β-hybrid glycopeptide building block was obtained. The latter is of interest for the development of novel glycoconjugate mimics and model structures for anti-cancer vaccines with increased biological half-life.

Introduction

Glycosylation is the predominant co- and post-translational modification in higher organisms responsible for tailoring and fine-tuning of the activity of proteins involved in fundamental biological recognition events of cell adhesion, cell differentiation and cell growth [1-3]. As a consequence, synthetic oligosaccharides and their conjugates are recognised as important tools for the expanding field of chemical biology [4]. Aberrant glycosylation of cell surface glycoproteins is associated with various pathological incidents, e.g., autoimmune and infectious diseases and cancer. In the latter case, unusual glycan structures composed of truncated *O*-linked oligosaccharides of carcinoma-derived mucin glycoproteins can be used as markers of the tumourigenic process and as target structures for cancer immunotherapy [5]. Over the last years, mucin-type glycopeptides decorated with tumour-associated carbohydrate antigens (TACA) have been shown to trigger strong humoral immunity within molecularly defined vaccine prototypes [6-10]. However, the limited metabolic stability of the

glycopeptide conjugates represents a major obstacle for the development of efficient carbohydrate-based vaccines. Various strategies towards the incorporation of non-natural hydrolysis resistant carbohydrate analogues into vaccine constructs have been pursued. For instance, stable TACA mimics comprising C-glycosides [11-14], S-glycosides [15-19] and deoxyfluoro sugars [20] have been used to circumvent hydrolytic degradation by endogenous glycosidases.

In principle, antigenicity of the artificial TACA derivatives should be enhanced by minor structural modifications assuming that the conformations remain similar to those of the natural antigens. In this respect, hybrid peptides in which β^3-homo-amino acids are used to strategically replace α-amino acids might be of interest as platforms for carbohydrate-based vaccines. That is because such mixed α/β-peptides adopt stable secondary structures closely related to those of natural α-peptides [21,22]. Moreover, inclusion of a single β-amino acid into an α-peptidic sequence already augments local and/or general stability against proteolytic degradation in vitro and in vivo; thus enabling the development of diverse peptidomimetics for an increasing number of applications [22-25]. Therefore we contemplated the use of mucin-derived α/β-hybrid glycopeptides as stable mimetics of naturally occurring glycocopeptide antigens for cancer vaccines. We were surprised to see how little precedence was available for this approach. Besides a recent report on α/δ-hybrid peptides derived from Neu2en and L-Glu [26], representing potentially immunogenic mimics of α-2,8-linked polysialic acid, only a few β-glycopeptides comprising *N*-acetylglucosamine [27-29] and *N*-acetylgalactosamine [30,31] (T_N antigen) linked to β^3-homo-serine are known. Despite their importance as specific tumour antigens, conjugates of Fmoc-β^3hSer and Fmoc-β^3hThr carrying larger TACA structures such as the Thomsen–Friedenreich antigen (TF) or its sialylated variants (α2-6sTF and α2-3sTF) have not been reported to date.

By presenting orthogonally protected T_N and TF antigen conjugates of Fmoc-β^3hThr (Figure 1) as well as a first α/β-hybrid glycopeptide analogue comprising the 20 amino acid tandem repeat sequence of the human mucin MUC1, we describe preliminary results of our synthetic efforts towards the preparation of mucin-type glycopeptide mimetics.

Results and Discussion

Initial attempts to directly link the carbohydrate entity to the β-side chain of a preformed Fmoc-β^3hThr conjugate were unsuccessful due to rapid lactonisation [29]. Similarly, despite the use of various glycosyl donors and reaction conditions, glycosylation of the corresponding dipeptide precursor Fmoc-β^3hThr(OH)-Ala-OBn failed completely. Therefore we encountered the strategy of Arndt–Eistert homologation in the synthesis of the target Fmoc-β^3hThr(αAc_3GalNAc) and Fmoc-β^3hThr(β(Ac_4Gal(1–3))α(Ac_3GalNAc)) conjugates **2a** and **4**, respectively, as reported by Norgren et al. [29]. T_N antigen derivative Fmoc-Thr(αAc_3GalNAc)-OH (**1a**) was prepared according to published procedures [32,33] and converted into the corresponding diazo ketone upon treatment with isobutyl chloroformate in the presence of *N*-methylmorpholine (NMM) and diazomethane (Scheme 1). Without further purification, the latter was subjected to a silver-promoted Wolff-rearrangement, again using NMM as the base, providing the spectroscopically pure T_N antigen analogue Fmoc-β^3hThr(αAc_3GalNAc)-OH (**2a**) in 60% yield over two steps after aqueous work-up.

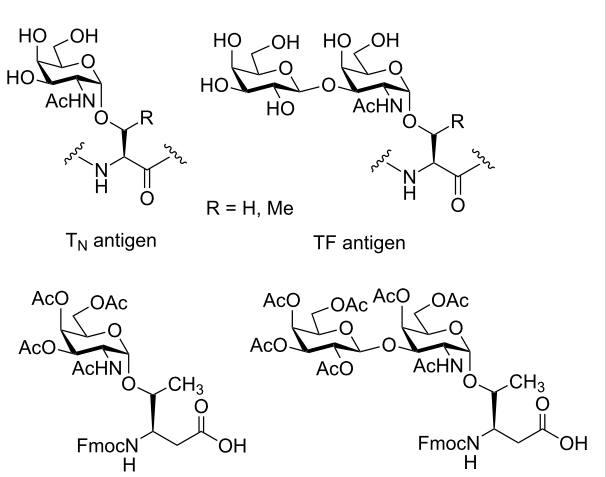
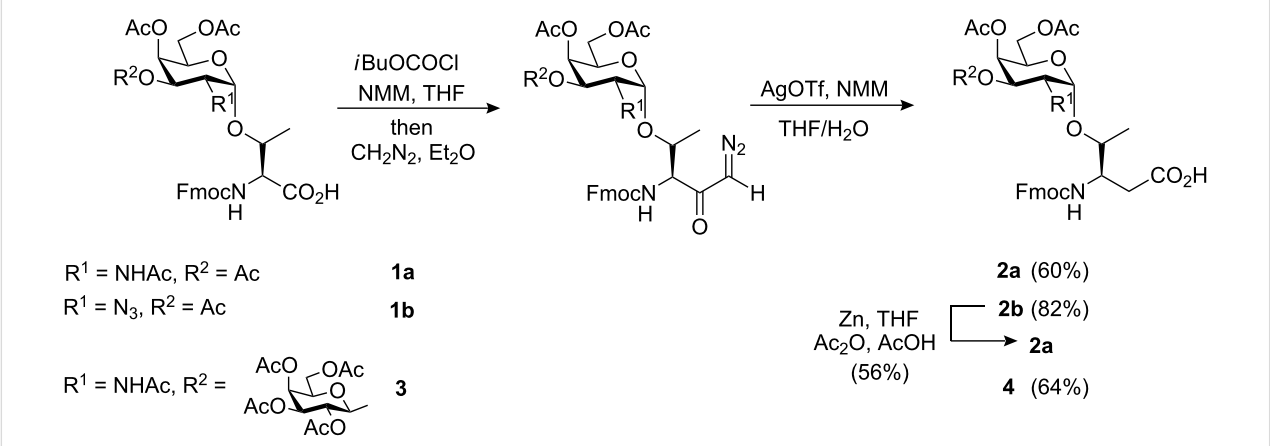


Figure 1: Structures of the naturally occurring T_N and TF antigens and the targeted Fmoc-β^3hThr analogues.

Compound **2a** was also accessible from a direct synthetic precursor of T_N derivative **1a** in which the 2-acetamido substituent was masked by an azido group. Thus, upon subjection of Fmoc-Thr(αAc_3GalN_3)-OH (**1b**) [32] to the same homologation sequence as before, the corresponding β^3hThr analogue **2b** was obtained in 82% yield. Subsequent zinc-mediated reduction and acetylation led to the formation of conjugate **2a** in 56% yield.

During biosynthesis T_N antigen acts as an immediate precursor of the TF antigen. As a consequence, a biomimetic approach towards larger TACA structures via stepwise assembly of the glycan chain has been pursued in various antigen syntheses [33,34]. By applying chemical or enzymatic 3β-galactosylation, the 3-OH deprotected conjugate **2b** could be converted into the desired antigen derivative Fmoc-β^3hThr(β(Ac_4Gal(1–3))α(Ac_3GalNAc))-OH (**4**). While this strategy certainly requires the use of optimised protecting group manipulations and glycosylation protocols, the alternative route



Scheme 1: Synthesis of Fmoc-β^{3}hThr antigen conjugates by Arndt–Eistert homologation.

to compound **4** via Arndt–Eistert homologation would benefit from an established and reliable synthesis of key building block **3** [32,33]. To our delight, the homologation reaction of glycosyl amino acid **3** again proceeded smoothly to afford the desired TF-β^{3}hThr conjugate **4** in spectroscopically pure form and good chemical yield after aqueous work-up (Scheme 1).

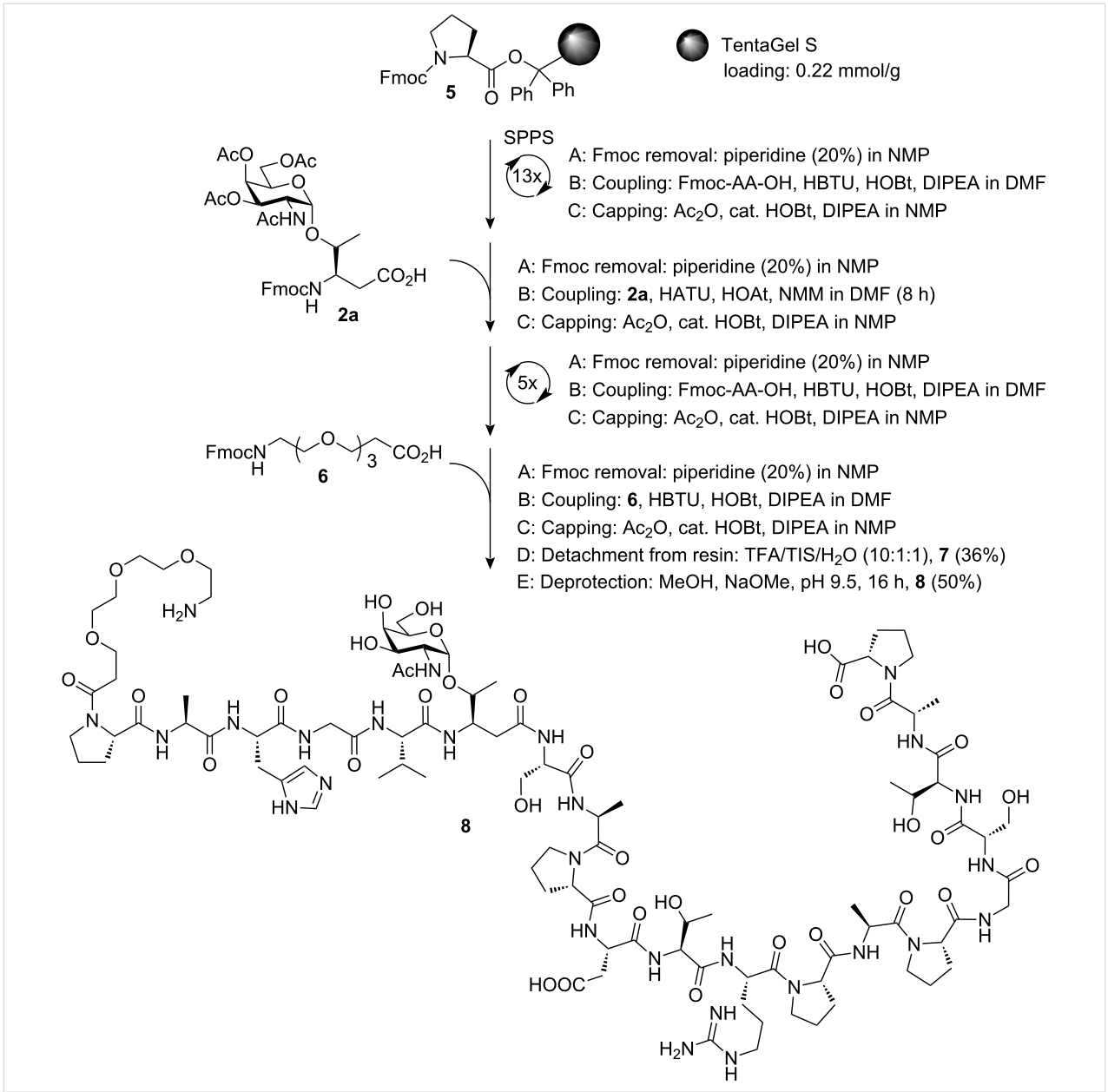
To demonstrate the usefulness of the novel glycosylated β^{3}hThr conjugates as antigen mimics, T_N antigen analog **2a** was incorporated into an α/β-hybrid glycopeptide **7** comprising a full tandem repeat sequence of the epithelial mucin MUC1 and an *N*-terminal non-immunogenic triethylene glycol spacer. The latter can be used for further conjugation to immunostimulants (e.g., BSA [35] or tetanus toxoid [36]) and for immobilisation onto microarray platforms [37] within functional immunological studies. The MUC1 pseudo-glycopeptide was assembled in an automated synthesiser by the Fmoc-strategy on a TentaGel S resin **5** equipped with a bulky trityl linker [38] to avoid diketopiperazine formation and pre-loaded with Fmoc-proline (Scheme 2). The first 13 amino acids of the MUC1 sequence were coupled under standard conditions using piperidine in *N*-methylpyrrolidone (NMP) to remove the temporary Fmoc protecting group followed by coupling of excess (10 equiv) Fmoc-amino acid activated by HBTU/HOBt [39] and diisopropylethylamine (DIPEA) in DMF. Unreacted amino acids were capped after each cycle with Ac_2O in the presence of DIPEA and catalytic amounts of HOBt in NMP. The sterically demanding glycosylated β^{3}hthreonine building block **2a** (1.5 equiv), was coupled over an extended reaction time of 8 h employing the more reactive reagents HATU/HOAt [40] with *N*-methylmorpholine (NMM) in NMP for activation. After the final five Fmoc-amino acids of the TR-sequence were coupled according to the standard protocol, a triethylene glycol spacer **6** [41] (10 equiv) was attached using the standard coupling procedure, again. Simultaneous detachment of the glycopeptide from the resin and cleavage of the acid-labile amino acid side chain protective groups was achieved upon treatment with a mixture of TFA, triisopropylsilane and water. The resulting partially deblocked glycopeptide **7** was isolated after purification by semi-preparative RP-HPLC in a yield of 36%, based on the loaded resin **5**. The final de-*O*-acetylation of the glycan portion was accomplished upon prolonged treatment with catalytic amounts of NaOMe in methanol at pH 9.5 to afford glycopeptide **8** in 18% yield (based on the loaded resin) after semi-preparative RP-HPLC.

Conclusion

Two novel tumour-associated carbohydrate antigen analogues with T_N and TF determinants *O*-glycosidically linked to the side chain of Fmoc-β^{3}hThr-OH have been prepared by Arndt–Eistert homologation of the corresponding glycosylated α-amino acids **1a** and **1b**. The resulting β^{3}hThr glycoconjugates are valuable antigen mimetics with potentially enhanced chemical and metabolic stability. They might serve as precursors for the preparation of further mucin-type antigen structures, e.g., sialylated ones or those based on core2 structures. In addition, the preparation of a first MUC1 pseudo-glycopeptide comprising the modified glycosyl amino acid Fmoc-β^{3}hThr(αGalNAc)-OH at position Thr15 has been accomplished using solid-phase peptide synthesis. By appropriate conjugation, the resulting α/β-hybrid glycopeptide conjugate could be used as an antigen surrogate to elucidate the effects of chemically modified antibody determinants on the immunological properties of glycopeptide antigen analogues.

Experimental

General remarks: DMF (amine-free, for peptide synthesis) and NMP were purchased from Roth and Ac_2O in p.a. quality from Acros. Fmoc-protected amino acids were purchased from



Scheme 2: Solid-phase synthesis of the tumour-associated MUC1 α/β-hybrid glycopeptide analogue **8** carrying the T_N antigen glycan. NMP = *N*-methylpyrrolidone, HBTU = *O*-(1*H*-benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, HOBt = *N*-hydroxybenzotriazole, DIPEA = diisopropylethylamine, HATU = *O*-(7-azabenzotriazole-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, HOAt = *N*-hydroxy-7-azabenzotriazole, NMM = *N*-methylmorpholine, TIS = triisopropylsilane.

Orpegen Pharma. For solid-phase synthesis, pre-loaded TentaGel S resin (Rapp Polymere) was employed. Reactions were monitored by TLC with pre-coated silica gel 60 F_{254} aluminium plates (Merck KGaA, Darmstadt). HPLC analyses were performed on a JASCO-HPLC system with Phenomenex Luna C18(2) (250 × 4.6 mm, 10 μm) and Phenomenex Jupiter C18(2) (250 × 4.6 mm, 10 μm) columns at a flow rate of 1 mL min^{-1}. Preparative RP-HPLC separation was carried out on a JASCO-HPLC System with Phenomenex Luna C18(2) (250 × 30 mm, 10 μm) and Phenomenex Jupiter C18(2) (250 × 30 mm, 10 μm) columns at a flow rate of 20 mL min^{-1} or 10 mL min^{-1}. ^1H, ^{13}C and 2D NMR spectra were recorded on a Bruker AC-300 or a Bruker AM-400 spectrometer. The chemical shifts are reported in ppm relative to the signal of the deuterated solvent. Multiplicities are given as: s (singlet), br s (broad singlet), d (doublet), t (triplet) and m (multiplet). In the case of known compounds, all spectra obtained were consistent with the literature. HR-ESI-mass spectra were recorded on a Micromass Q TOF Ultima 3 spectrometer and optical rotations were measured at 546 nm with a Perkin-Elmer polarimeter 241.

General procedure for the synthesis of diazomethane in ethereal solution

Caution: Diazomethane is toxic, highly-volatile, cancerogenic and explosive. Its generation and handling thus requires special precautions [42]. With regard to the free acids, 10 equiv of *N*-methyl-*N*-nitroso-urea were added to a solution of 50% KOH in H_2O which was layered on top with Et_2O. The organic layer was decanted and replaced by a new portion of Et_2O until the organic layer was no longer coloured. The united organic phases were dried over KOH at −25 °C for 3 h.

General procedure (GP1) for the synthesis of diazo ketones: The free acid (1 equiv) was dissolved in 2 mL of dry THF under an argon atmosphere. At −25 °C, 1 equiv of NMM and 1 equiv of isobutyl chloro formate were added subsequently and the resulting suspension was stirred for 20 min at this temperature. The mixture was allowed to reach 0 °C and the diazomethane solution in Et_2O was added. The yellow solution was stirred 20 min at 0 °C before it was allowed to reach room temperature and stirred for further 16 h. Excess of diazomethane was destroyed by adding a few drops of acetic acid to the solution until no further nitrogen formation was observed. The solvents were removed under reduced pressure and the residue was dissolved in 20 mL Et_2O. The organic layer was washed twice with saturated aqueous $NaHCO_3$, saturated aqueous NH_4Cl and brine. The organic layer was dried over Na_2SO_4, filtered, and the solvents were removed under reduced pressure. The resulting diazo ketones were used without further purification.

General procedure (GP2) for the Wolff-rearrangement: The diazo ketone (1 equiv) was dissolved in a mixture of THF/H_2O (9:1) and was cooled to 0 °C. A solution of 0.11 equiv silver trifluoroacetate in 2.3 equiv NMM was added, and the mixture was stirred for 16 h while it was allowed to warm to room temperature. After evaporation of THF, the aqueous layer was diluted with saturated aqueous $NaHCO_3$ and Et_2O was added. The organic layer was extracted three times with saturated aqueous $NaHCO_3$. The aqueous phases were collected, cooled to 0 °C and acidified to pH 1. The resulting suspension was extracted five times with Et_2O. The organic layer was dried over Na_2SO_4 and evaporated in vacuo.

N*-(9*H*-fluoren-9-yl)methoxycarbonyl-[α-3,4,6-tri-*O*-acetyl-2-acetamido-2-deoxy-galactopyranosyl]-β^3-homo-threonine 2a:** ***Procedure A: The synthesis followed the general procedures GP1 and GP2. Amounts: 150 mg (0.22 mmol) **1a**. Yield: 90 mg (0.13 mmol), 60%, colourless amorphous solid. Analytical RP-HPLC (Luna, MeCN–H_2O + 0.1% TFA, 20:80 → 60:40, 40 min, t_R = 29.8 min). ***Procedure B:*** To a stirred solution of **2b** (860 mg, 0.48 mmol) in a mixture of THF–Ac_2O–AcOH (3:2:1, 36 mL) activated zinc dust (0.6 g, 9.1 mmol) was added. Activation was achieved by suspension in aq. 2% solution of $CuSO_4$, followed by subsequent washings with water, EtOAc and Et_2O. The reaction mixture was stirred for 16 h at room temperature, diluted with 50 mL THF and filtered through Hyflo Supercel®. The filtrate was concentrated in vacuo and co-evaporated five times with toluene and CH_2Cl_2. The residue was dissolved in 50 mL CH_2Cl_2, washed with saturated aqueous $NaHCO_3$ and brine, dried (Na_2SO_4) and concentrated in vacuo. Yield: 328 mg (0.48 mmol), 56%, colourless amorphous solid; $[\alpha]_D^{23}$ = 42.6 (*c* = 1.00, $CHCl_3$); ^{1}H NMR (COSY, HSQC, 400 MHz, CD_3OD), δ (ppm) = 7.81 (d, 2 H, *J* = 7.2 Hz, Fmoc-H4, Fmoc-H5), 7.66, 7.65 (2d, 2 H, *J* = 7.6 Hz, Fmoc-H1, Fmoc-H8), 7.39 (pt, 2 H, Fmoc-H3, Fmoc-H6), 7.32 (t, 2 H, *J* = 7.6 Hz, Fmoc-H2, Fmoc-H7), 5.44 (d, 1 H, *J* = 2.4 Hz, GalNAc-H4), 5.10 (dd, 1 H, *J* = 3.2 Hz, 11.6 Hz, GalNAc-H3), 4.98 (d, 1 H, *J* = 3.6 Hz, GalNAc-H1), 4.56 (pdd, 1 H, Fmoc-CH_2), 4.45 (dd, 1 H, *J* = 3.6 Hz, 11.6 Hz, GalNAc-H2), 4.41–4.35 (m, 1 H, Fmoc-CH_2), 4.31 (pt, 1 H, GalNAc-H5), 4.21 (pt, 1 H, Fmoc-H9), 4.15–4.07 (m, 2 H, GalNAc-H6), 4.05–4.02 (m, 1 H, hT$^\beta$), 3.83 (pdd, 1 H, hT$^\gamma$), 2.55 (pdd, 1 H, hT$^\alpha$), 2.38 (pdd, 1 H, hT$^\alpha$), 1.76 (d, 3 H, *J* = 6.4 Hz, hT$^\delta$); ^{13}C NMR (DEPT, HSQC, 100.6 MHz, CD_3OD), δ (ppm) = 175.1, 172.2, 170.7, 170.7, 170.6 (C=O), 157.0 (C=O-urethane), 144.0, 143.8 (Fmoc-C1a, Fmoc-C8a), 141.3, 141.3 (Fmoc-C4a, Fmoc-C5a), 127.4 (Fmoc-C3, Fmoc-C6), 126.8, 126.7 (Fmoc-C2, Fmoc-C7), 124.7, 124.6 (Fmoc-C1, Fmoc-C8), 119.6, 119.5 (Fmoc-C4, Fmoc-C5), 99.1 (GalNAc-C1), 77.6 (hT$^\gamma$), 68.3 (GalNAc-C3), 67.4 (GalNAc-C4), 66.6 (GalNAc-C5), 65.9 (Fmoc-CH_2), 61.8 (GalNAc-C6), 52.6 (hT$^\beta$), 47.6 (GalNAc-C2), 47.3 (Fmoc-C9), 36.8 (hT$^\alpha$), 21.3 (CH_3-NHAc), 19.3, 19.2, 19.1 (CH_3-Ac), 16.7 (hT$^\delta$); HR-ESI-MS (positive, *m/z*): 707.2433 ([M+Na]$^+$, calc.: 707.2428).

***N*-(9*H*-fluoren-9-yl)methoxycarbonyl-[α-3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-galactopyranosyl]-β^3-homo-threonine 2b:** The synthesis followed the general procedures GP1 and GP2. Amounts: 156 mg (0.23 mmol) **1b**. Yield: 130 mg (0.19 mmol), 82%, colourless amorphous solid; $[\alpha]_D^{23}$ = 56.0 (*c* = 1.00, $CHCl_3$); ^{1}H NMR (400 MHz, COSY, HSQC, CD_3OD), δ (ppm) = 7.79 (d, 2 H, *J* = 7.6 Hz, Fmoc-H4, Fmoc-H5), 7.66 (d, 2 H, *J* = 7.6 Hz, Fmoc-H1, Fmoc-H8), 7.39 (t, 2 H, *J* = 7.2 Hz, Fmoc-H3, Fmoc-H6), 7.32 (t, 2 H, *J* = 7.2 Hz, Fmoc-H2, Fmoc-H7), 5.44 (d, 1 H, *J* = 2.0 Hz, GalNAc-H4), 5.33 (dd, 1 H, *J* = 3.2 Hz, 11.2 Hz, GalNAc-H3), 5.14 (d, 1 H, *J* = 3.6 Hz, GalNAc-H1), 4.41–4.32 (m, 3 H, Fmoc-CH_2, Fmoc-H9), 4.21 (pt, 1 H, GalNAc-H5), 4.14–4.06 (m, 3 H, GalNAc-H6, hT$^\beta$), 3.91–3.86 (m, 2 H, GalNAc-H2, hT$^\gamma$), 2.76 (dd, 1 H, *J* = 5.2 Hz, 16 Hz, hT$^\alpha$), 2.55 (dd, 1 H, *J* = 8.8 Hz, 16.4 Hz, hT$^\alpha$), 2.14, 2.02, 1.98 (3s, 9 H, 3 × CH_3-Ac); 1.76 (d, 3 H, *J* = 6.4 Hz, hT$^\delta$); ^{13}C NMR (101 MHz, DEPT, HSQC, CD_3OD), δ (ppm) =

173.5, 170.7, 170.6, 170.1 (C=O), 157.1 (C=O-urethane), 144.0, 143.9 (Fmoc-C1a, Fmoc-C8a), 141.2 (Fmoc-C4a, Fmoc-C5a), 127.4 (Fmoc-C3, Fmoc-C6), 126.8, 126.7 (Fmoc-C2, Fmoc-C7), 124.9 (Fmoc-C1, Fmoc-C8), 119.5 (Fmoc-C4, Fmoc-C5), 98.6 (GalNAc-C1), 77.5 (hT$^{\gamma}$), 69.0 (GalNAc-C3), 67.7 (GalNAc-C4), 66.8 (GalNAc-C5), 66.4 (Fmoc-CH_2), 58.4 (GalNAc-C6), 52.0 (hT$^{\gamma}$), 47.6 (GalNAc-C2), 47.3 (Fmoc-C9), 34.8 (hT$^{\alpha}$), 19.3, 19.2, 19.1 (CH_3-Ac), 16.4 (hT$^{\delta}$); HR-ESI-MS (positive, *m/z*): 691.2232 ([M+Na]$^+$ calc.: 691.2227).

***N*-(9*H*-fluoren-9-yl)methoxycarbonyl-[(β-2,3,4,6-tetra-*O*-acetylgalactopyranosyl)-(1–3)-(α-2-acetamido-4,6-di-*O*-acetyl-2-deoxy-galactopyranosyl)]-β^3-homo-threonine 4:** The synthesis followed the general procedures GP1 and GP2. Amounts: 200 mg (0.21 mmol) **3**. Yield: 130.3 mg (0.13 mmol), 64%, colourless amorphous solid; $[\alpha]_D^{23}$ = 29.9 (*c* = 0.50, $CHCl_3$); ^{1}H NMR (400 MHz, COSY, HSQC, CD_3OD), δ (ppm) = 7.85 (d, 2 H, *J* = 7.6 Hz, Fmoc-H4, Fmoc-H5), 7.69 (d, 2 H, *J* = 7.6 Hz, Fmoc-H1, Fmoc-H8), 7.44 (t, 2 H, *J* = 7.6 Hz, Fmoc-H3, Fmoc-H6), 7.36 (t, 2 H, *J* = 7.6 Hz, Fmoc-H2, Fmoc-H7), 5.43 (d, 1 H, *J* = 3.4 Hz, GalNAc-H4), 5.39 (m, 1 H, Gal-H3), 5.04–5.02 (m, 2 H, Gal-H4, Gal-H2), 4.98 (under H_2O-peak, GalNAc-H1), 4.71 (m, 1H, Gal-H1), 4.51 (d, 2 H, *J* = 6.2 Hz, Gal-H6), 4.44 (dd, 1 H, *J* = 3.5 Hz, 11.5 Hz, GalNAc-H2), 4.28–4.23 (m, 2 H, Gal-H5, Fmoc-H9), 4.23–4.21 (m, 3 H, Fmoc-CH_2, GalNAc-H6), 4.09–3.91 (m, 4 H, GalNAc-H5, Fmoc-CH_2, hT$^{\beta}$, GalNAc-H3), 3.86 (dd, 1 H, *J* = 2.7 Hz, 6.2 Hz, hT$^{\gamma}$), 2.71 (dd, 1 H, *J* = 6.3 Hz, 15.5 Hz, hT$^{\alpha}$), 2.55 (dd, 1 H, *J* = 8.7 Hz, 15.6 Hz, hT$^{\alpha}$), 2.17, 2.15, 2.06, 2.06, 2.05, 2.04, 1.97 (7s, 21 H, 6 × CH_3-Ac, CH_3-AcNH), 1.22 (d, 3 H, *J* = 6.6 Hz, hT$^{\delta}$); ^{13}C NMR (101 MHz, DEPT, HSQC, CD_3OD), δ (ppm) = 174.7, 173.0, 172.5, 172.2, 171.6, 171.2 (C=O), 158.5 (C=O-urethane), 145.5, 145.3 (Fmoc-C1a, Fmoc-C8a), 142.8, 142.8 (Fmoc-C4a, Fmoc-C5a), 129.0, 128.4 (Fmoc-C3, Fmoc-C6), 126.3, 126.2 (Fmoc-C2, Fmoc-C7), 121.2 (Fmoc-C1, Fmoc-C8, Fmoc-C4, Fmoc-C5), 102.6 (Gal-C1), 100.6 (GalNAc-C1), 78.2 (hT$^{\gamma}$), 74.9 (GalNAc-C3), 72.2 (Gal-C4), 71.8 (GalNAc-C5), 71.2 (GalNAc-C4), 70.1 (Gal-C2), 68.8 (Gal-C5), 68.5 (Gal-C3), 67.4 (Gal-C6), 64.3 (Fmoc-CH_2), 62.3 (GalNAc-C6), 53.6 (hT$^{\beta}$), 50.3 (GalNAc-C2), 36.5 (hT$^{\alpha}$), 23.0 (CH_3-NHAc), 20.8, 20.8, 20.7, 20.5 (CH_3-Ac), 18.0 (hT$^{\delta}$); HR-ESI-MS (positive, *m/z*): 1159.3216 ([M+Na]$^+$, calc.: 1159.3210).

Amino-4,7,10-trioxadodecanylamido-*N*-L-prolyl-L-alanyl-L-histidyl-L-glycyl-L-valyl-*O*-[α-3,4,6-tri-*O*-acetyl-2-acetamido-2-deoxy-galactopyranosyl]-homo-β^3-threonyl-L-seryl-L-alanyl-L-prolyl-L-aspartyl-L-threonyl-L-arginyl-L-prolyl-L-alanyl-L-prolyl-L-glycyl-L-seryl-L-threonyl-L-alanyl-L-proline 7: The synthesis was carried out in an Applied Biosystems ABI 433A peptide synthesiser (standard program Fastmoc 0.1 mmol) using pre-loaded Fmoc-Pro-Trt-TentaGel S resin (455 mg, 0.10 mmol; loading: 0.22 mmol/g). For the coupling reactions, the amino acids Fmoc-Ala-OH, Fmoc-Arg(Pmc)-OH, Fmoc-Asp-OH, Fmoc-Gly-OH, Fmoc-His(Trt)-OH, Fmoc-Pro-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Thr(*t*Bu)-OH, and Fmoc-Val-OH were employed. In every coupling cycle, the *N*-terminal Fmoc group was removed by treatment of the resin with a solution of piperidine (20%) in NMP for at least 3 × 2.5 min. The coupling of the amino acids (1 mmol or 10 equiv based on the loaded resin) was carried out with HBTU (1 mmol), HOBt (1 mmol) and DIPEA (2 mmol) in DMF (20–30 min vortex). After every coupling step, unreacted amino groups were capped by treatment with a mixture of Ac_2O (0.5 M), DIPEA (0.125 M) and HOBt (0.015 M) in NMP (10 min vortex). Coupling of the glycosylated β^{3}hThr building block **2a** (102.70 mg, 0.15 mmol) was performed using HATU (1.2 equiv with respect to the glycosyl amino acid), HOAt (1.2 equiv) and NMM (4 equiv) for activation (8 h vortex). After coupling of the remaining five amino acids by the standard procedure, the triethylene glycol spacer (1 mmol, 10 equiv based on the loaded resin) was coupled using HBTU (1 mmol), HOBt (1 mmol) and DIPEA (2 mmol) in DMF (20–30 min vortex) and the *N*-terminal Fmoc group was removed by piperidine (20%) in NMP. Detachment from the resin and simultaneous removal of all side chain protecting groups was performed in a Merrifield glass reactor by shaking with TFA (10 mL), TIS (1.0 mL) and H_2O (1.0 mL) for 3 h. The solution was filtered, the resin was washed with CH_2Cl_2 (5 × 10 mL) and the combined solutions were concentrated in vacuo to a volume of 0.5 mL. After co-evaporation with toluene (3 × 10 mL), the crude product was dissolved in H_2O and subjected to lyophilisation. The crude product was purified by RP-HPLC (Luna, MeCN–H_2O + 0.1% TFA, 5:95 → 100:0, 50 min, t_R= 25.2 min). Yield: 88.3 mg (0.04 mmol), 36%, colourless amorphous solid; $[\alpha]_D^{23}$ = −79.9 (*c* = 1.00, H_2O); ^{1}H NMR (400 MHz, COSY, HSQC, TOCSY, D_2O), δ (ppm): 8.50 (m, 1 H, H$^{\varepsilon}$), 7.19 (m, 1 H, H$^{\delta}$), 5.31–5.28 (m, 1 H, GalNAc-H4), 5.08 (dd, 1 H, *J* = 3.1 Hz, 11.7 Hz, GalNAc-H3), 5.01 (d, 1 H, *J* = 3.5 Hz, GalNAc-H1), 4.64–4.54 (m, 2 H, D$^{\alpha}$ {4.61}, H$^{\alpha}$ {4.57}), 4.54–4.37 (m, 5 H, R$^{\alpha}$ {4.52}, A_2^{α} {4.47}, A_3^{α} {4.46}, A_4^{α} {4.45}, S_1^{α} {4.39, t, *J* = 5.5 Hz}), 4.36–4.20 (m, 10 H, S_2^{α} {4.33}, hT$^{\beta}$ {4.27}, A_1^{α} {4.29}, GalNAc-H5 {4.32}, T_1^{α} {4.30}, T_2^{α} {4.24}, P_{1-4}^{α} {4.29} {4.28} {4.27} {4.24}), 4.19 (d, 1 H, *J* = 4.5 Hz, V$^{\alpha}$), 4.15–3.89 (m, 6 H, GalNAc-H2 {4.12}, T_1^{β}, T_2^{β} {4.09} {4.07}, GalNAc-H6 {4.02}, P_5^{α} {3.94}), 3.89–3.73 (m, 7 H, G_1^{α} {3.86}, G_2^{α} {3.83}, G_1^{α} {3.80}, G_2^{α} {3.77}, hT$^{\gamma}$ {3.77}, S_2^{β} {3.76}), 3.73–3.59 (m, 11 H, P_{1-5}^{δ} {3.67}, S_1^{β} {3.65}, 2 × CH_2-spacer {3.64} {3.62}), 3.59–3.28 (m, 13 H, 2 × CH_2-spacer {3.57}, 2 × CH_2-spacer {3.54}, P_{1-5}^{δ} {3.52}), 3.18 (dd, 1 H, *J* = 5.2 Hz, 15.1 Hz, H$^{\beta}$), 3.11–3.01 (m, 5 H, CH_2-spacer {3.08}, H$^{\beta}$ {3.07}, R$^{\delta}$ {3.07}),

2.84 (dd, 1 H, *J* = 6.5 Hz, 16.8 Hz, D^{β}), 2.75 (dd, 1 H, *J* = 6.5 Hz, 17.3 Hz, D^{β}), 2.67–2.40 (m, 4 H, CH_2-spacer {2.61} {2.55}, hT^{α} {2.59} {2.46}), 2.23–2.05 (m, 8 H, P_{1-5}^{β} {2.20} {2.17} {2.14}, CH_3-Ac {2.07}), 2.01–1.66 (m, 29 H, 4 × CH_3-Ac {1.94} {1.92} {1.89} {1.88}, P_{1-5}^{γ} {1.95–1.83}), P_{1-5}^{β} {1.92–1.71}, V^{β} {1.86}, R^{β} {1.70}), 1.65–1.46 (m, 3 H, R^{β} {1.61}, R^{γ} {1.54}), 1.28–1.17 (m, 12 H, A_{1-4}^{β} {1.24} {1.20}), 1.07–1.01 (m, 9 H, T_1^{γ} {1.07}, hT^{δ} {1.06}, T_2^{γ} {1.04}), 0.76 (d, 3 H, *J* = 5.8 Hz, V^{γ}), 0.71 (d, 3 H, *J* = 6.4 Hz, V^{γ}); ^{13}C NMR (101 MHz, DEPT, HSQC, D_2O), δ (ppm) = 176.6, 175.7, 174.9, 174.4, 174.3, 173.9, 173.43, 173.4, 173.1, 172.9, 172.8, 172.6, 172.4, 172.3, 171.9, 171.5, 171.3, 171.2, 171.1, 170.9, 170.8, 170.7 (C=O), 163.3, 163.0, 162.6, 162.2 (TFA), 156.7 (C=NH), 133.5 (H^{β}), 128.4 (H^{ε}), 117.3 (H^{δ}), 98.2, 98.0 (GalNAc-C1), 77.2, 76.9 (hT^{γ}), 69.6, 69.5, 69.4, 69.4 (CH_2-Spacer), 68.4 (GalNAc-C3), 68.0 (GalNAc-C2), 67.0 (T_1^{β}, T_2^{β}), 66.3, 66.0 (CH_2-spacer), 62.5 (GalNAc-C6), 61.5, 61.1 (S_1^{β}, S_2^{β}), 60.8, 60.5, 60.0, 59.7, 58.7 (P_{1-5}^{α}), 59.6, 59.3 (T_1^{α}, T_2^{α}), 58.9 (V^{α}), 55.5, 54.9, 52.24 (H^{α}), 51.1 (R^{α}), 50.5 (hT^{β}), 50.0 (D^{α}), 49.6 (GalNAc-C2), 48.0, 47.9, 47.7, 47.4 (P_{1-5}^{δ}), 47.8, 47.8, 47.6, 47.6 (A_1^{α}, A_2^{α}, A_3^{α}, A_4^{α}), 42.4, 42.3 (G_1^{α}, G_2^{α}), 40.5 (CH_2-spacer), 39.0 (R^{δ}), 36.2 (hT^{α}), 34.9 (D^{β}), 34.0 (CH_2-spacer), 29.9, 29.8 (V^{β}), 29.6, 29.3, 29.2, 29.2, 28.1 (P_{1-5}^{β}), 27.4 (R^{β}), 26.2 (H^{β}), 24.7, 24.6, 24.5, 24.3 (P_{1-5}^{γ}), 23.9 (R^{γ}), 21.8 (CH_3-AcNH), 20.3, 20.0, 20.0 (CH_3-Ac) , 18.8, 18.7 (T_1^{γ}, T_2^{γ}), 18.3, 17.7 ($V^{\gamma\alpha}$ $V^{\gamma\beta}$), 16.6 (hT^{δ}), 16.2, 15.4, 15.2, 15.1 (A_{1-4}^{β}); HR-ESI-MS: 1217.0941 ($[M+2H]^+$, calc.: 1217.0932).

Amino-4,7,10-trioxadodecanylamido-*N*-L-prolyl-L-alanyl-L-histidyl-L-glycyl-L-valyl-*O*-[α-2-acetamido-2-deoxy-galactopyranosyl]-homo-β^3-threonyl-L-seryl-L-alanyl-L-prolyl-L-aspartyl-L-threonyl-L-arginyl-L-prolyl-L-alanyl-L-prolyl-L-glycyl-L-seryl-L-threonyl-L-alanyl-L-proline 8: Peptide **7** was dissolved in 10 mL of methanol (HPLC grade). A fresh solution of sodium methanolate in methanol (0.5 g Na in 25 mL methanol (HPLC grade)) was added drop wise until pH 9.5 was reached. The reaction mixture was stirred over night and neutralised with a few drops of acetic acid. The solvent was removed in vacuo and the residue was dissolved in H_2O and subjected to lyophilisation. The crude product was purified by RP-HPLC (Luna, MeCN–H_2O + 0.1% TFA, 5:95 → 100:0, 60 min, t_R = 15.3 min). Yield: 44 mg (0.02 mmol), 50% (18% based on the loaded resin **5**), colourless amorphous solid; $[\alpha]_D^{23}$ = −83.7 (*c* = 1.00, H_2O); ^{1}H NMR (400 MHz, COSY, HSQC, D_2O), δ (ppm) = 8.50 (d, 1 H, *J* = 1.6 Hz, H^{δ}), 7.20 (d, 1 H, *J* = 1.3 Hz, H^{ε}), 4.89 (d, 1 H, *J* = 3.9 Hz, GalNAc-H1), 4.62 (t, 1 H, *J* = 6.8 Hz, D^{α}), 4.58 (dd, 1 H, *J* = 6.7 Hz, 8.6 Hz, H^{α}), 4.55–4.49 (m, 1 H, R^{α}), 4.50–4.43 (m, 3 H, A_{1-3}^{α} {4.48} {4.47} {4.45}), 4.40 (t, 1 H, *J* = 5.4 Hz, S_1^{α}), 4.34–4.21 (m, 7 H, S_2^{α} {4.32}, P_{1-5}^{α} {4.29} {4.28} {4.28} {4.26} {4.26}, T_1^{α} {4.24}), 4.20 (d, 1 H, *J* = 4.5 Hz, V^{α}), 4.16–4.10 (m, 1 H, A_4^{α}), 4.11–4.05 (m, 2 H, T_1^{β}, T_2^{β}), 4.00 (dd, 1 H, *J* = 3.8 Hz, 11.1 Hz, GalNAc-H2), 3.95–3.89 (m, 2 H, T_2^{α} {3.93}, GalNAc-H5 {3.89}), 3.89–3.82 (m, 4 H, G_1^{α} {3.87}, G_1^{α} {3.84}, G_2^{α} {3.84}, GalNAc-H4 {3.84}), 3.82–3.74 (m, 3 H, GalNAc-H3 {3.80}, G_2^{α} {3.80}, S_1^{β} {3.79}), 3.77–3.74 (m, 1 H, S_1^{β}), 3.74–3.61 (m, 11 H, P_{1-5}^{δ} {3.70} {3.68} {3.68} {3.68} {3.67}, GalNAc-H6 {3.65}, 2 × CH_2-spacer {3.65} {3.62}), 3.61–3.31 (m, 15 H, S_2^{β} {3.59}, 4 × CH_2-spacer {3.58}, 4 × CH_2-spacer {3.54}, P_{1-5}^{δ} {3.58–3.48}), 3.19 (dd, 1 H, *J* = Hz, H^{α}), 3.11–3.02 (m, 5 H, CH_2-spacer {3.09}, R^{δ} {3.07}, H^{α} {3.07}), 2.85 (dd, 1 H, *J* = 6.6 Hz, 17.4 Hz, D^{β}), 2.76 (dd, 1 H, *J* = 6.6 Hz, 17.0 Hz, D^{β}), 2.69–2.38 (m, 4 H, CH_2-spacer {2.62}, hT^{α} {2.57}, CH_2-spacer{2.55}, hT^{α} {2.43}), 2.26–2.07 (m, 5 H, P_{1-5}^{β} {2.20} {2.18} {2.17} {2.16} {2.14}), 2.00–1.48 (m, 23 H, P_{1-5}^{γ} {1.98–1.80}, P_{1-5}^{β} {1.92–1.70}, CH_3-AcNH {1.93}, V^{β} {1.85}, R^{β} {1.71}, R^{β} {1.61}, R^{γ} {1.54}), 1.29–1.18 (m, 12 H, A_{1-4}^{β} {1.26}, {1.24}, {1.21}, {1.21}), 1.10–1.02 (m, 9 H, T_1^{γ}, T_2^{γ}, hT^{δ} {1.06}, {1.04}, {1.04}), 0.76 (d, 3 H, *J* = 6.7 Hz, V^{γ}), 0.72 (d, 3 H, *J* = 6.7 Hz, V^{γ}); ^{13}C NMR (101 MHz, DEPT, HSQC, D_2O), δ (ppm) = 175.9, 174.9, 174.4, 174.4, 173.9, 173.5, 173.5, 173.1, 172.9, 172.8, 172.6, 172.5, 172.4, 172.4, 172.0, 171.5, 171.3, 171.2, 171.1, 170.9, 170.8 (C=O), 163.1, 162.7 (TFA), 156.7 (C=NH), 133.4 (H^{β}), 128.4 (H^{ε}), 117.3 (H^{δ}), 98.0 (GalNAc-C1), 76.3 (hT^{γ}), 69.6, 69.5, 69.5, 69.4 (CH_2-Spacer), 71.3 (GalNAc-C5), 67.4 (GalNAc-C3), 61.5 (GalNAc-C6), 61.2, 61.1 (S_1^{β}, S_2^{β}), 60.8, 60.5, 60.0, 59.7, 58.7 (P_{1-5}^{α}), 59.6, 59.3 (T_1^{α}, T_2^{α}), 58.9 (V^{α}), 55.5 (S_2^{α}), 54.9 (S_1^{α}), 52.3 (H^{α}), 51.1 (R^{α}), 50.5 (hT^{β}), 50.1 (D^{α}), 50.0 (GalNAc-C2), 49.6 (A_4^{α}), 48.0, 47.9, 47.7, 47.4 (P_{1-5}^{δ}), 47.8, 47.6, 47.6 (A_1^{α}, A_2^{α}, A_3^{α}), 42.4, 42.3 (G_1^{α}, G_2^{α}), 40.5 (CH_2-spacer), 39.0 (R^{δ}), 36.1 (hT^{α}), 35.0 (D^{β}), 34.0 (CH_2-spacer), 29.9 (V^{β}), 29.6, 29.3, 29.2, 29.1, 28.7 (P_{1-5}^{β}), 27.4 (R^{β}), 26.2 (H^{β}), 24.7, 24.6, 24.5, 24.3 (P_{1-5}^{γ}), 23.9 (R^{γ}), 21.9 (CH_3-AcNH), 18.8, 18.7 (T_1^{γ}, T_2^{γ}), 18.3, 17.7 ($V^{\gamma\alpha}$, $V^{\gamma\beta}$), 16.6 (hT^{δ}), 16.2, 15.4, 15.1, 15.0 (A_{1-4}^{β}); HR-ESI-MS: 1154.0817 ($[M+2H]^{2+}$, calc.: 1154.0774).

Supporting Information

Supporting Information File 1

NMR spectra of compounds **2a**, **2b**, **4**, **7**, **8** and HPLC chromatogram of compound **2a**.
[http://www.beilstein-journals.org/bjoc/content/supplementary/1860-5397-6-47-S1.pdf]

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Beilstein Journal of Organic Chemistry

Preparation of aminoethyl glycosides for glycoconjugation

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Full Research Paper

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Abstract

The synthesis of a number of aminoethyl glycosides of cell-surface carbohydrates, which are important intermediates for glycoarray synthesis, is described. A set of protocols was developed which provide these intermediates, in a short number of steps, from commercially available starting materials.

Introduction

The chemical conjugation of carbohydrates through the anomeric centre to biomolecules such as peptides, proteins, lipids, metabolites and to array surfaces is an important synthetic challenge [1-5]. A diverse range of linkers and spacers has been described in the literature [2-12], among which aminoalkyl glycosides have become the most popular, in particular the aminoethyl linker. This linker has been tested in a large number of arrays and appears to be biocompatible in array screening [2,3,7]. Given that aminoethyl glycosides are conveniently conjugated to surfaces containing activated carboxylates, they have become a useful generic anomeric functional group for glycoconjugation. The importance of this linker merits efforts into finding a robust synthetic method than can be used by scientists who are not experienced in carbohydrate synthesis. Here we describe a systematic study with the aim of finding such robust and efficient methods for a number of commonly used mono- and disaccharides starting from commercially available reagents and with a minimal number of steps. In our studies no such general aminoethylation method that was applicable to all targets was found, which rather suggests that protocols need to be tailored for each sugar.

Results and Discussion

Coupling reactions

Aminoethyl glycosides have previously been generated in a number of ways. Free sugars have been glycosylated with 2-chloroethanol under acid catalysis, followed by peracetylation, nucleophilic substitution with azide and finally, reduction

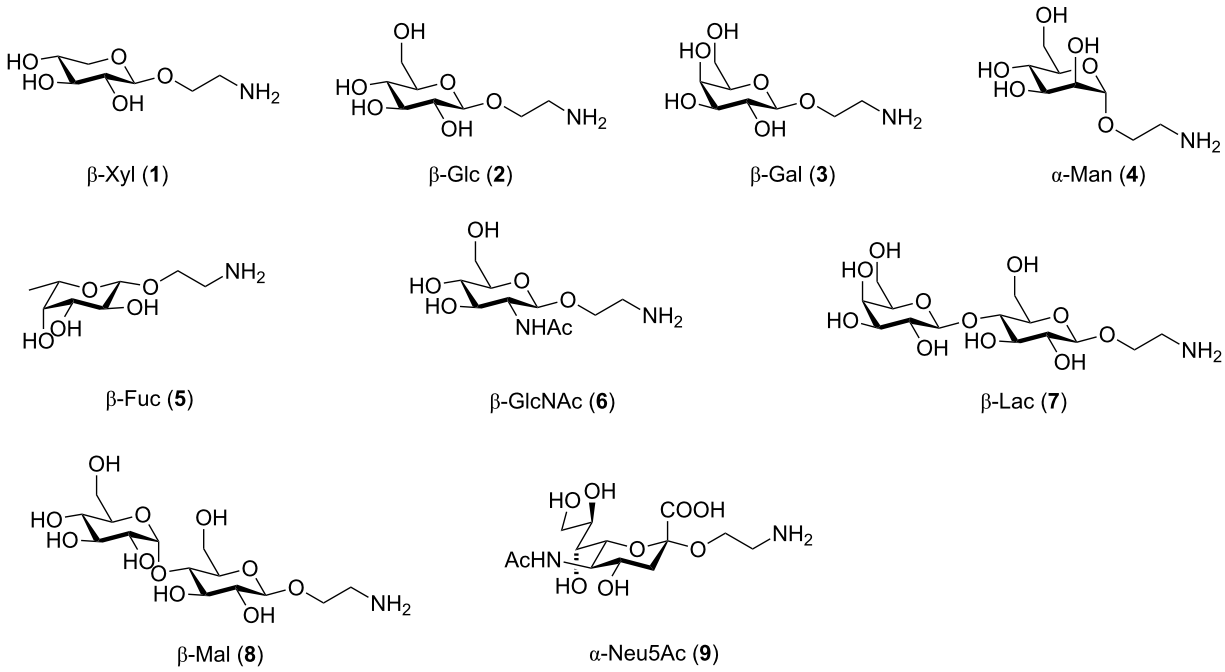


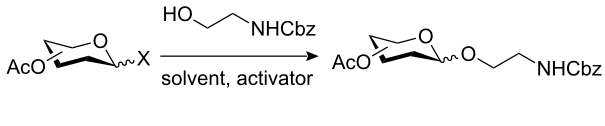
Figure 1: Aminoethyl glycosides (**1**–**9**) which were synthesised in this study.

of the azido group [13,14]. Alternatively, the carbohydrate was first activated as the trichloroacetimidate or bromide followed by glycosylation with *N*-Cbz-aminoethanol [15], bromoethanol [16] or azidoethanol [17] and subsequently transformed into the amine.

In the interest of finding fast reliable methods, we have investigated two general aminoethylation protocols: First, the direct glycosylation of peracetylated sugars, which can be either purchased or easily prepared from free sugars and can be used without purification. Where these proved to be unreactive, the anomeric acetates were converted to glycosyl bromides, usually in quantitative yields, and products were used without further purification. Where possible, *N*-Cbz-protected aminoethanol was used as the glycosyl acceptor because it is commercially available, crystalline and can be easily deprotected in one step avoiding use of azides. Figure 1 lists the target aminoethyl glycosides (**1**–**9**) generated in this study (q.v. Scheme 1 and Table 1).

The key glycosylation step is shown in Scheme 1 and the results of the different glycosylation reactions are summarised in Table 1.

Fully protected xylopyranoside **10** could be prepared both from the corresponding bromide as well as the acetate (the β-anomer was prepared from xylose with sodium acetate in acetic anhydride) in similar yields. In each case both anomers were formed



Scheme 1: General reaction scheme for generation of aminoethyl glycosides. X = OAc, Br or Cl.

with moderate selectivity and from the reaction mixture the pure β-anomer (>95:5) was isolated by column chromatography.

The glucoside **11**, galactoside **12** and mannoside **13** were prepared in moderate to good yield directly from the acetate (X = OAc in Scheme 1) giving rapid access to these monosaccharide derivatives.

The fucoside **14** was generated both from the acetate and bromide. Higher alpha selectivity was observed with the bromide (α/β ratio of crude product 82:18).

N-Acetylglucosamine **15** was successfully prepared from the β-acetate using $SnCl_4$ (method D). When starting from the α-acetate, no reaction was observed and only starting material could be recovered. Under microwave conditions (method B or using other Lewis acids as $Yb(OTf)_3$ in DCM, 90 °C, 30 min, 200 W) the reaction was not reproducible, giving low yields and leading to decomposition products.

Table 1: Results of glycosylation reactions as shown in Scheme 1.

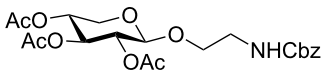
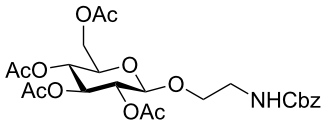
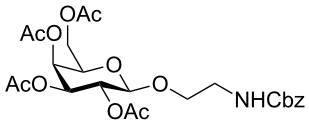
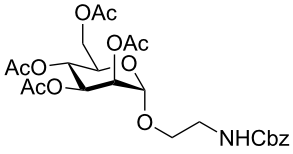
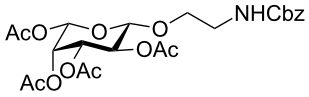
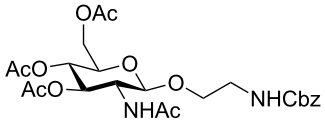
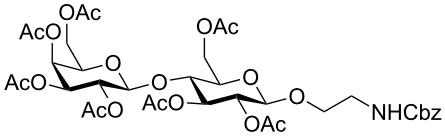
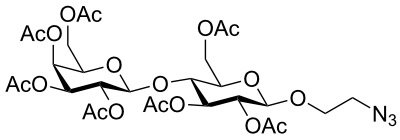
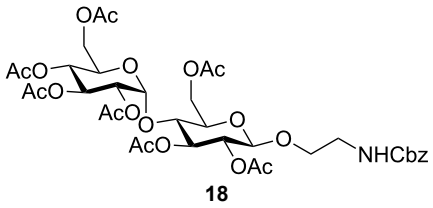
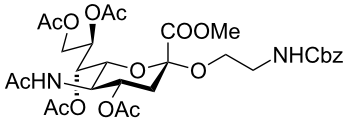
Entry	Product	X	Method	α/β[c]	Yield[d]
1	 **10**	Br	A	17:83	67 + 23[e]
2	**10**	Br	B	23:77	58 + 19[e]
3	**10**	OAc	C	22:78	52 + 17[e]
4	 **11**	OAc	C	15:85	36
5	 **12**	OAc	C	8:92	62
6	 **13**	OAc	C	>95:5	57
7	 **14**	Br	A	14:86	75 + 19[e]
8	**14**	Br	B	15:85	75 + 20[e]
9	**14**	OAc	C	35:65	n.d.
10	 **15**	OAc	D	>5:95	61
11	 **16**	Br	A	10:90	56
12	**16**	Br	B	10:90	86
13	**16**	Br	A[a]	31:68	30
14	**16**	Br	B[b]	30:70	73[e]
15	**16**	Br	E	37:63	59
16	 **17**	Br	E	10:90	88

Table 1: Results of glycosylation reactions as shown in Scheme 1. (continued)

17	 **18**	Br	B	14:86	47
18	 **19**	Cl	F	10:90	70

[a]The reaction was performed overnight at r.t. [b]The reaction was performed in CH_2Cl_2. [c]determined by ^{13}C NMR from crude reaction mixture. [d]Yield of pure major anomer (>95:5) after column chromatography. [e]Mixture of both anomers. Method A: $Hg(CN)_2$, CH_3CN, 60 °C, 2–4 h. Method B: $Hg(CN)_2$, CH_3CN, 90 °C, microwave, 200 W, 15 min. Method C: $BF_3{\cdot}Et_2O$, CH_3CN, 0 °C, 1 h, r.t., overnight. Method D: $SnCl_4$, CH_3CN, 60 °C, 16 h. Method E: $Hg(CN)_2$, $HgBr_2$, CH_3CN, r.t., overnight. Method F: Ag_2CO_3, CH_2Cl_2, r.t., overnight.

Lactose is both cheap and readily available. It is an important component of glycoprotein glycans and also a substrate for sialyltransferases to generate biologically important sialyllactosides. The aminoethyl lactoside **16** was prepared in greatest yield from the bromide and attempts to prepare **16** directly from the acetate using $BF_3{\cdot}Et_2O$ as the activator only resulted in decomposition.

A number of reaction conditions for generating **16** from the bromide with *N*-Cbz-aminoethanol were investigated. With Ag_2CO_3 in dichloromethane at room temperature low yields of product **16** along with a number of side-products (orthoester, elimination or hydrolysis) were observed and the product was difficult to separate from starting materials, in particular the *N*-Cbz-aminoethanol. With $Hg(CN)_2$, or the more reactive $Hg(CN)_2/HgBr_2$-mixture, in dichloromethane or acetonitrile, glycosylation was more successful, but both anomers were generated. Given the problems previously encountered with purification, the glycosylation with $Hg(CN)_2$ was further optimised by increasing both the temperature and the amount of acceptor. The problem of separation of the alcohol from the product was solved by acetylation of the crude reaction mixture to lower the polarity of the free alcohol. Attempts to speed up the reaction by heating led to the observation that in acetonitrile predominantly one (β) anomer is formed, but anomerisation occurs with longer reaction times. In dichloromethane both anomers were formed. The best reaction conditions were combined to give Method A. The success of Method A led to attempts to improve the method further and to use microwave irradiation as in method B. Method B also works well for monosaccharides and maltoside.

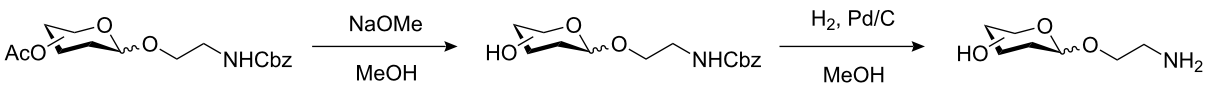
Given the problems with purification, the use of azidoethanol as a glycosyl acceptor was also investigated. This reaction (Table 1) was much more successful and produced mainly the beta anomer **17**.

Maltoside **18** was generated by the same microwave-mediated glycosylation as developed for lactoside **16** (Method B) and in reasonable yield.

N-Acetyl neuraminic acid (sialic acid) is an important component of cell surfaces and chemical glycosylation procedures involving sialic acid are generally challenging. In our hands activation as the chloride (prepared from Neu5Ac in 3 steps) using silver carbonate (Method F) gave reasonable yields of **19**.

Deprotection reactions

The general deprotection for compounds **10**–**18** is shown in Scheme 2. Acetates were cleaved using NaOMe followed by hydrogenation to generate **1**–**8** in good yields.



Scheme 2: Deprotection protocols.

Deprotection was also successful when the hydrogenation was performed first, but in some cases migration of acetate to the aminoethyl linker was observed. However, this can be avoided by using palladium hydroxide on charcoal as the hydrogenation catalyst, with short reaction times, followed by the immediate use of the resulting amine in further coupling [18].

Sialoside **19** was deprotected by treatment with NaOMe, followed by LiOH and subsequent hydrogenation to give **9**.

Conclusion

We have described rapid and convenient methods for the synthesis of a range of aminoethyl glycosides (**1**–**9**) of common mono- and disaccharides. Although some of the glycosylation reactions could be improved by using alternative glycosylation methods (such as trichloroacetimidates, thiols), these would require more steps with chromatographic purifications and less overall yields. These aminoethyl glycosides are now readily accessible for incorporation into glycan arrays.

Supporting Information

A Supporting Information containing all experimental details and analytical data of all compounds described in the article as well as their precursors is available.

Supporting Information File 1

Experimental procedures and analytical data
[http://www.beilstein-journals.org/bjoc/content/supplementary/1860-5397-6-81-S1.pdf]

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Beilstein Journal of Organic Chemistry

Synthesis of 6-PEt*N*-α-D-Gal*p*NAc-(1→6)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*, a *Haemophilus influenzae* lipopolysaccharide structure, and biotin and protein conjugates thereof

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Full Research Paper

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Abstract

Background: In bacteria with truncated lipopolysaccharide structures, i.e., lacking the O-antigen polysaccharide part, core structures are exposed to the immune system upon infection and thus their use as carbohydrate surface antigens in glycoconjugate vaccines can be considered and investigated. One such suggested structure from *Haemophilus influenzae* LPS is the phosphorylated pentasaccharide 6-PEt*N*-α-D-Gal*p*NAc-(1→6)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*.

Results: Starting from a spacer-containing lactose derivative a suitably protected lacto-*N*-neotetraose tetrasaccharide structure was constructed through subsequential couplings with two thioglycoside donors, a glucosamine residue followed by a galactose derivative, using NIS/AgOTf as promoter. Removal of a silyl protecting group at the primary position of the non-reducing end residue afforded an acceptor to which the terminal α-galactosamine moiety was introduced using a 2-azido bromo sugar and halide assisted coupling conditions. Global deprotection afforded the non-phosphorylated target pentasaccharide, whereas removal of a silyl group from the primary position of the non-reducing end residue produced a free hydroxy group which was phosphorylated using H-phosphonate chemistry to yield the phosphoethanolamine-containing protected pentasaccharide. Partial deprotection afforded the phosphorylated target pentasaccharide with a free spacer amino group but with a protected phosphoethanolamino group. Conjugation of

the spacer amino group to biotin or dimethyl squarate followed by deprotection of the phosphoethanolamino group and, in the case of the squarate derivative, further reaction with a protein then afforded the title conjugates.

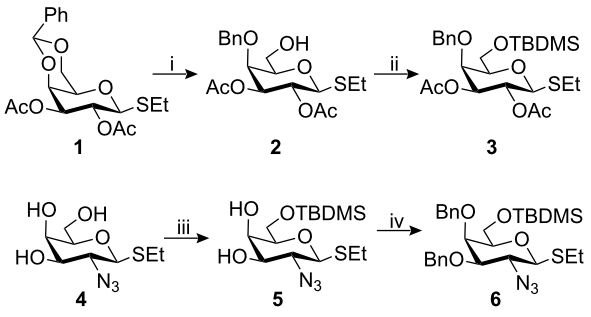
Conclusion: An effective synthesis of a biologically interesting pentasaccharide structure has been accomplished. The target pentasaccharide, an α-GalNAc substituted lacto-*N*-neotetraose structure, comprises a phosphoethanolamine motif and a spacer aglycon. Through the spacer, biotin and protein conjugates of the title compound have been constructed to allow further use in biological experiments.

Introduction

Haemophilus influenzae are Gram-negative bacteria divided into six serotypes, a–f, related to the structure of the capsular polysaccharide usually surrounding the bacterium [1]. Among these serotypes, type b is the cause of the most severe diseases, i.a. meningitis and pneumonia. However, there are now several commercial vaccines against this serotype that have proven to be highly effective [2]. These vaccines are glycoconjugate vaccines, based on capsular poly- or oligosaccharide structures, either native or synthetic [3,4], linked to a carrier protein. The lipopolysaccharide (LPS) of *H. influenzae* shows a huge structural variety and hence non-capsulated bacteria are referred to as non-typable *H. influenzae* (NTHi) [5,6]. This structural diversity is a good defence mechanism against the human acquired immune system, and NTHi often cause repetitive infections. Another way used by *H. influenzae* to avoid the human immune system seems to be molecular mimicry, i.e. the bacteria express common human carbohydrate structures on their surface that the host recognises as self-structures and do not produce antibodies against. Construction of a glycoconjugate vaccine against NTHi is thus quite complex. Carbohydrate structures that are both exposed on the surface of most bacteria and also immunogenic in humans must be found and produced. To tackle this problem we are presently pursuing two possible routes. A common conserved LPS inner-core pentasaccharide structure has been identified, and efforts to produce this structure, i.a. through synthesis, are on-going [7-9]. Furthermore, analysis of the LPS of NTHi strains to find frequent non-human outer-core structures is continuously performed [5,6]. One candidate recently suggested is a lacto-*N*-*neo*tetraose structure substituted with a PEt*N*-GalNAc residue (Figure 1) [10]. Herein we describe the synthesis of this structure and its conjugation to biotin and a carrier protein to form glycoconjugates that can be used in biological experiments to evaluate the immunological properties of the title structure.

Results and Discussion

The target structure is, as noted above, a substituted lacto-*N*-neotetraose structure that we have experience of from, i.a., former synthetic work related to *Streptococcus pneumoniae* type 14 CPS structures [11]. To allow for the introduction of the GalNAc residue at the 6^{IV}-position and the subsequent phosphorylation of the 6^{V}-hydroxy group, two new galactosyl donors were designed and synthesised (Scheme 1). Reductive ring opening, with BH_3/Bu_2BOTf [12], of the benzylidene acetal in the known ethyl thioglycoside **1** [13] gave the



Scheme 1: i. BH_3, Bu_2BOTf, THF/CH_2Cl_2, 85%; ii. TBDMSCl, pyridine, CH_2Cl_2, 90%; iii. TBDMSCl, pyridine, 92%; iv. BnBr, NaH, DMF, 85%.

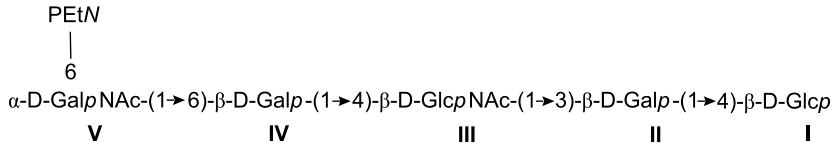
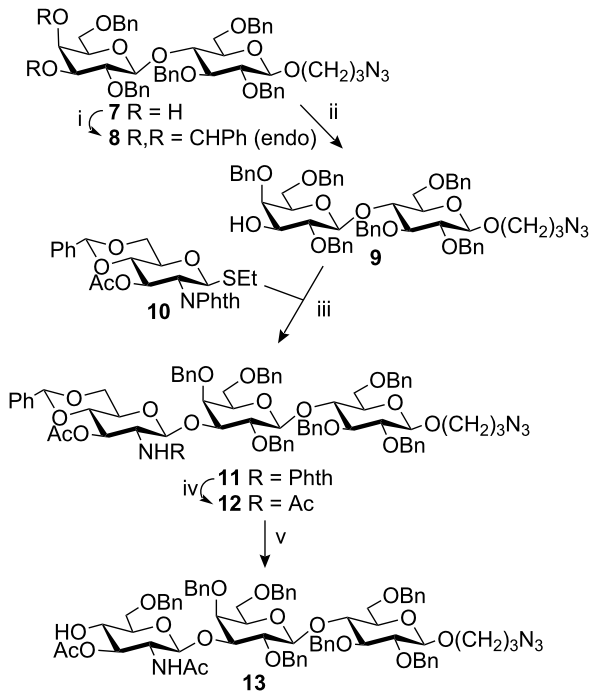


Figure 1: The *H. influenzae* outer core target structure.

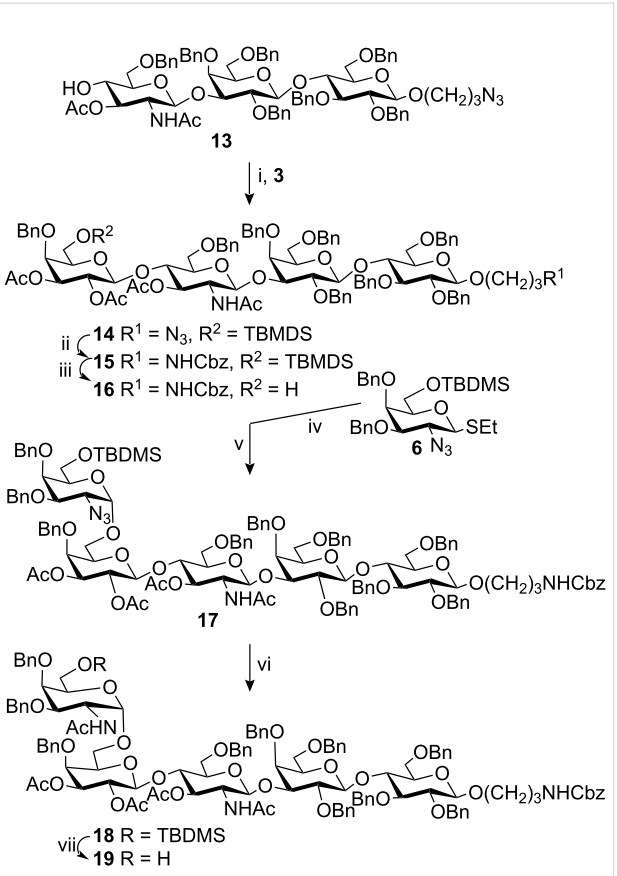
6-hydroxy derivative **2** (85%), which was then silylated to afford donor **3** (90%). Regioselective silylation of the 2-azido-galactose ethyl thioglycoside **4** [14] yielded the 6-*O*-silylated compound **5** (92%), benzylation of which gave donor **6** (85%). A benzyl group in the 4-position was preferred to an ester group to avoid the risk of acyl migration during subsequent reactions; desilylation, glycosidation and phosphorylation.

$3^{II},4^{II}$-Diols of lactose are often used as acceptors for regioselective glycosidations in the 3-position. However, the selectivity is dependent on the donor, promoter and conditions employed [15], e.g., it has been shown that donors containing a 4,6-*O*-benzylidene acetal can give a mixture of products [16]. Hence, the diol **7** [17] was transformed using benzaldehyde dimethyl acetal and camphorsulfonic acid stereo-selectively into the corresponding 3,4-*endo*-benzylidene derivative **8** which was in turn converted to the 3-hydroxy derivative **9** by $NaBH_3CN$/HCl-mediated reductive opening of the acetal ring [18] (80% overall yield from **7**, Scheme 2). NIS/AgOTf-promoted glycosidation of this acceptor with donor **10** [19] (1.4 equiv) then efficiently gave the β-linked trisaccharide **11** (83%). At this stage the phthalimido group was removed by aminolysis and the resulting amino compound acetylated to yield **12** (93%) with the target acetamido function in place. Once more reductive opening of a benzylidene acetal ($NaBH_3CN$/HCl) gave a new mono-hydroxy compound, the acceptor **13** (81%).



Scheme 2: i. $PhCH(OMe)_2$, CSA; ii. $NaBH_3CN$, HCl/Et_2O, THF, 80%; iii. NIS/AgOTf, CH_2Cl_2, 83%; iv. a) NaOMe, MeOH; b) $NH_2(CH_2)_2NH_2$, EtOH; c) Ac_2O, pyridine, 93%; v. $NaBH_3CN$, HCl/Et_2O, THF, 81%.

The 4-hydroxy group in GlcNAc derivatives is known to be quite unreactive towards glycosylations, which, i.a., has led to development of new protecting group patterns to improve the reactivity [20,21]. Here, however, NIS/AgOTf-promoted glycosylation of acceptor **13** with donor **3** proceeded without difficulties to produce the tetrasaccharide **14** in high yield (77%, Scheme 3). Before the introduction of the azido-galactose residue, the spacer azido function was reduced to an amino group, which was protected as a benzyl carbamate (→ **15**, 91%). Removal of the TBDMS group with TBAF then gave the tetrasaccharide acceptor **16** (89%). To obtain complete α-selectivity with 2-azido-galactose donors is not trivial. A 4,6-silyl acetal has been suggested as one way to improve the selectivity [22]. We have earlier tried halide-assisted conditions [23], which is not that common due to the incompatibility between the mild coupling conditions and the low reactivity of 2-azido donors, with good results when simple spacer alcohols were used as aglycons [24]. This approach also worked well here, although extended reaction time was necessary. A halide-assisted coupling between acceptor **16** and the bromosugar,

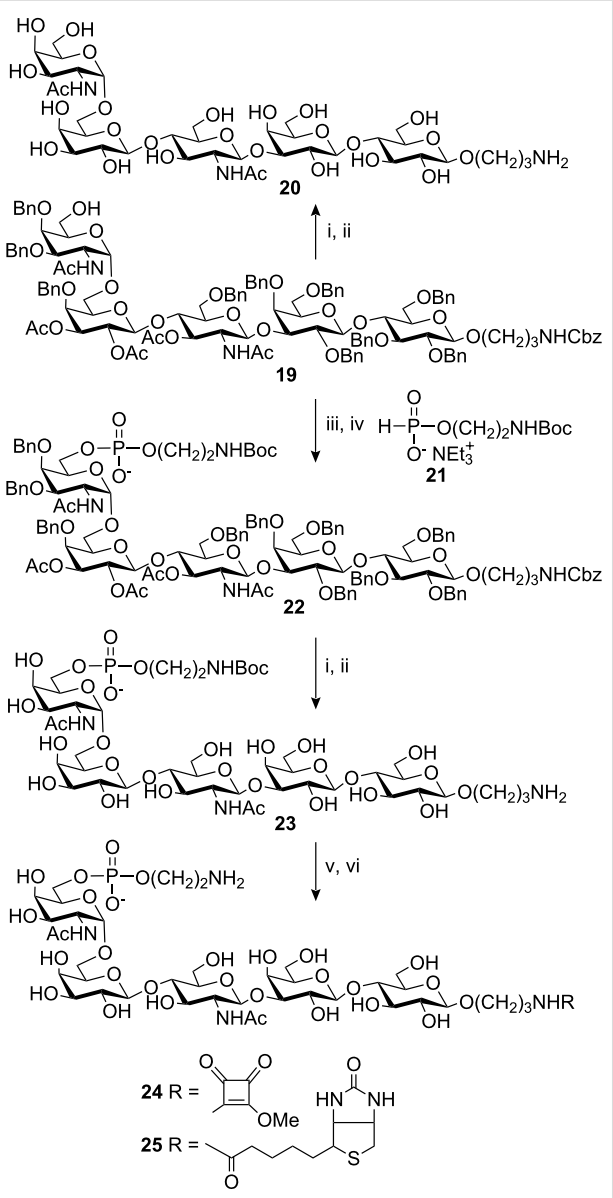


Scheme 3: i. NIS/AgOTf, CH_2Cl_2, 77%; ii. a) H_2S, pyridine, Et_3N; b) CbzCl, pyridine, CH_2Cl_2, 91%; iii. TBAF, THF, 89%; iv. Br_2, CH_2Cl_2; v. Et_4NBr, DMF, CH_2Cl_2, 79%; vi. a) H_2S, pyridine, Et_3N; b) Ac_2O, pyridine, CH_2Cl_2, 91%; vii. TBAF, THF, 85%.

obtained from thioglycoside **6**, gave (after 11 days) the α-linked pentasaccharide **17** in 79% yield. Due to the excess of donor used and the long reaction time required the acetamido carbonyl oxygen also partly behaved as a nucleophile and gave a pseudo-hexasaccharide acetimidate side product according to MALDI-TOF and NMR. Similar side products have earlier been described [25-28]. Mild acid treatment of the glycosylation mixture prior to purification led to cleavage of the formed imidate and afforded the desired pentasaccharide in the above noted yield.

Reduction of the azido group in **17** and subsequent acetylation afforded compound **18** (91%). Removal of the TBDMS group, again using TBAF, gave derivative **19** (85%), ready for the introduction of the phosphoethanolamine. Compound **19** was also completely deprotected by sodium methoxide treatment followed by catalytic hydrogenolysis to give the non-phosphorylated target structure **20** (66%, Scheme 4), to be used as a reference in biological experiments. Earlier we used the Cbz-protected ethanolamine H-phosphonate monoester as a reagent in the formation of phosphoethanolamines [29]. Since the amino group in the spacer was already Cbz-protected and we wanted to be able to differentiate between the two amino groups during conjugation, a Boc-protected H-phosphonate monoester **21** was synthesised and used in the phosphorylation step. Activation of **21** with pivaloyl chloride in the presence of **19** afforded the H-phosphonate diester, which was oxidised with I_2/pyridine in water to afford the phosphate diester **22** [30]. Deprotection with sodium methoxide followed by catalytic hydrogenolysis then afforded the still Boc-protected phosphoethanolamine pentasaccharide **23** (44% from **19**) ready for conjugation via the free spacer amino group.

The protein conjugations were carried out using squarate ester methodology [31,32]. Reaction of compound **23** with dimethyl squarate at neutral pH afforded the monomethyl ester squarate amide of **23**, from which the Boc-group was removed by acid hydrolysis to afford derivative **24**. Protein conjugations were performed by reaction of **24** (20 equiv) with human serum albumin (HSA). Compound **20** was similarly activated with dimethyl squarate and conjugated to HSA. MALDI-TOF MS of the HSA-conjugates of compounds **20** and **24** showed a loading of 16 oligosaccharides/protein molecule and 7 oligosaccharides/protein molecule, respectively. Since derivative **24** contains a free amino group there is a possibility of oligomerisation in addition to protein conjugation. To what extent this happened was not examined. Compound **23** was also biotinylated for use in ELISA-screening of antibodies raised against native LPS. Reaction with the commercial NHS-activated ester of biotin followed by TFA-treatment to remove the Boc-group afforded derivative **25**.



Scheme 4: i. NaOMe, MeOH; ii. H_2, Pd/C, MeOH/H_2O; iii. **21**, PivCl, pyridine, MeCN; iv. I_2, H_2O, pyridine; v. Dimethyl squarate or NHS-biotin, buffer pH 7; vi. TFA (10% aqueous).

Supporting Information

Supporting Information File 1

Experimental Section

[http://www.beilstein-journals.org/bjoc/content/supplementary/1860-5397-6-80-S1.pdf]

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Beilstein Journal of Organic Chemistry

A bivalent glycopeptide to target two putative carbohydrate binding sites on FimH

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Abstract

FimH is a mannose-specific bacterial lectin found on type 1 fimbriae with a monovalent carbohydrate recognition domain (CRD) that is known from X-ray studies. However, binding studies with multivalent ligands have suggested an additional carbohydrate-binding site on this protein. In order to prove this hypothesis, a bivalent glycopeptide ligand with the capacity to bridge two putative carbohydrate binding sites on FimH was designed and synthesized. Anti-adhesion assays with the new bivalent ligand and type 1-fimbriated bacteria have revealed, that verification of the number of carbohydrate binding sites on FimH with a tailor-made bivalent glycopeptide requires further investigation to be conclusive.

Introduction

Bacterial adhesion is a phenomenon which occurs on the surface of host cells as well as on the surface of surgical implants, where it can lead to the formation of persistent biofilms. In all cases of bacterial adhesion and of biofilm formation severe health problems can result for the host organism [1,2]. A number of microbial adhesins are known, that co-operate in the adhesion process [3], such as the fimbriae, which are long filamentous adhesive organells on the surface of many bacteria, comprising carbohydrate-binding sub-units [4-6]. The type 1 fimbriae, for example, which are widely spread among the Enterobacteriaceae are terminated with the mannose-specific protein FimH. FimH is structured in the form of two domains, a carbohydrate-specific adhesin domain and a pilin domain, which is required for fimbriae assembly [7]. The FimH adhesin domain features a carbohydrate binding site at its tip, called the carbohydrate recognition domain (CRD), which is known from X-ray studies [8-11]. It is a monovalent binding site, which can accommodate one α-D-mannosyl moiety, for example, the terminal mannoside residues of high-mannose-type glycoproteins of the glycocalyx [12]. However, the precise nature of the ligand-receptor interactions is not fully understood. For example, when multivalent carbohydrate ligands were tested as ligands of FimH and type 1 fimbriated bacteria, respectively [13,14], multivalency effects were observed in many cases. In addition, concentration-dependent inhibitory and stimulating allosteric effects on adhesion have also been

reported with certain carbohydrate ligands [15], which might be explained by the existence of allosteric binding sites.

Thus, it has been suggested that there could be multiple binding sites on the FimH adhesin [16] but this hypothesis has so far neither been proven nor disproven. It is interesting to note, that although the sequence of the FimH adhesin is highly conserved, studies by Sokurenko and collegues [17-20] have indicated that allelic variation in FimH is correlated with different carbohydrate-binding profiles. None of the allelic variations giving rise to differences in mannose-binding occurs within, or even close to, the FimH mannose-binding pocket. Additional sugar-binding sites dispersed throughout the lectin domain are a possible explanation for this finding. This feature could aid in recognising large and multivalent carbohydrate receptors respectively, on the host surface.

In order to look for possible additional carbohydrate-binding sites in the FimH lectin, the surface of the lectin domain was probed by computational docking studies [16]. Three new potential carbohydrate binding cavities on the surface of the FimH lectin domain, in addition to the mannose pocket at the tip of the domain, were identified which have a marked preference for the same subset of high-mannose trisaccharide substructures, mainly α-D-Man-(1→3)-[α-D-Man-(1→6)]-D-Man. By employing site directed mutagenesis, it was found that mutations in one of these cavities significantly reduces binding, indicating that this could be a second carbohydrate binding site, relevant for ligand binding [21]. Thus, it was our goal to design a bivalent carbohydrate ligand so shaped that it could concomitantly occupy the mannose binding site at the tip of the adhesin domain and the putative second carbohydrate binding site on the receptor. Based on the published structure of FimH, we have estimated the distance between the known CRD at the tip of FimH and the suggested second binding site, which is a more extended region on the protein (Figure 1). Docking using FlexX [22-24] recommended a spacer of 10 to 15 amino acids to ligate the two different carbohydrate ligand portions of a bivalent glycoconjugate. This corresponds to a linker length of between 30 and 40 Å. While the known CRD accommodates exactly one α-D-mannosyl residue in the binding pocket, with the aglycone of the mannoside sticking out of the binding site, the postulated second binding site could rather interact with a carbohydrate of the size of a mannotrioside. Hence, a monomeric mannoside and the trisaccharide α-D-Man-(1→3)-[α-D-Man-(1→6)]-D-Man were selected as carbohydrate ligands and azidoethyl aglycone moieties were chosen to allow their ligation via an oligoglycine spacer of an appropriate length.

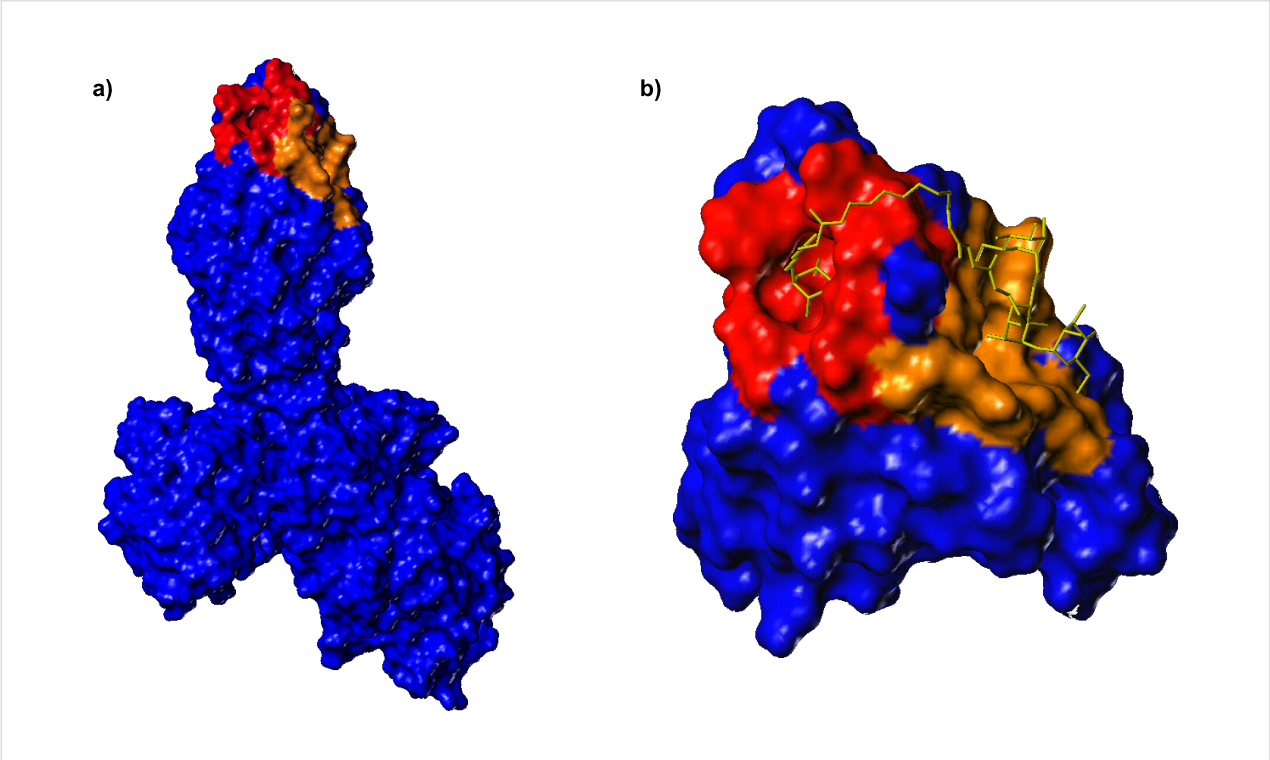


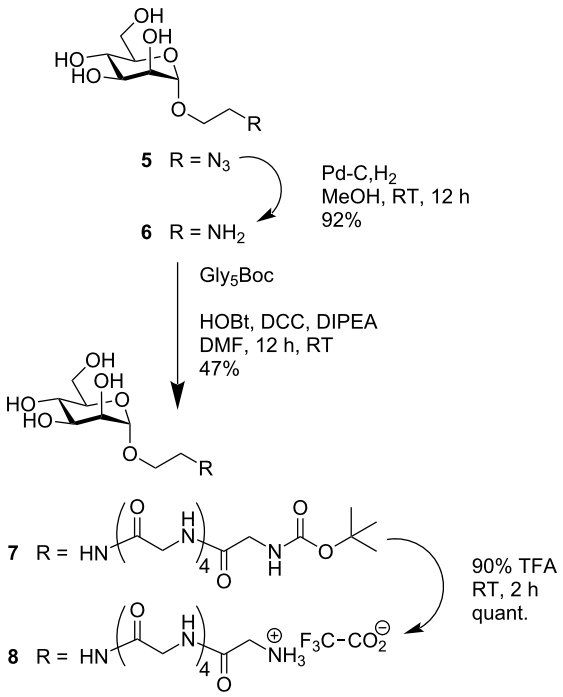
Figure 1: a) Connolly surface of FimH in complex with FimC [8]. The CRD known from X-ray structures at the tip of FimH is coloured in red and can accommodate one α-D-mannosyl residue. A second hypothetical carbohydrate binding region on the protein, as suggested by modeling studies [16], is coloured in brown and represents a more extended area on the protein. **b)** Docking studies were used to estimate the length of a linker that is required to bridge the putative two binding sites.

The well-known squaric acid diester linkage strategy [25] was applied to connect the monosaccharide and the trisaccharide part of the bivalent glycopeptide target structure **1** (Figure 2). Accordingly, retrosynthetic analysis of **1** leads to the 2-azidoethyl glycosides **2** and **5**, with the azido group masking an amino function; two pentaglycine spacer molecules (**3**) and squaric acid diethyl ester (**4**, DES). The synthetic assembly relies on peptide coupling chemistry and the squaric acid diester to link two different amines in two subsequent steps.

Results and Discussion

Synthesis of the eastern part of target molecule **1** started from the known azidoethyl mannoside **5**, which can be prepared from mannose pentaacetate in three simple steps [26]. Catalytic hydrogenation led to the amine **6** [27], which was subjected to peptide coupling with *N*-Boc-protected pentaglycine (Gly_5Boc) under standard reaction conditions (Scheme 1). This led to the *N*-Boc-protected glycopeptide **7** and removal of the Boc protecting group with TFA gave amine **8** as its TFA salt.

To prepare the western part of target molecule **1**, the trisaccharide azide **2** was required by analogy to the synthetic pathway leading to glycopeptide **8**. To establish the protecting



Scheme 1: Synthesis of the eastern part of target molecule **1**.

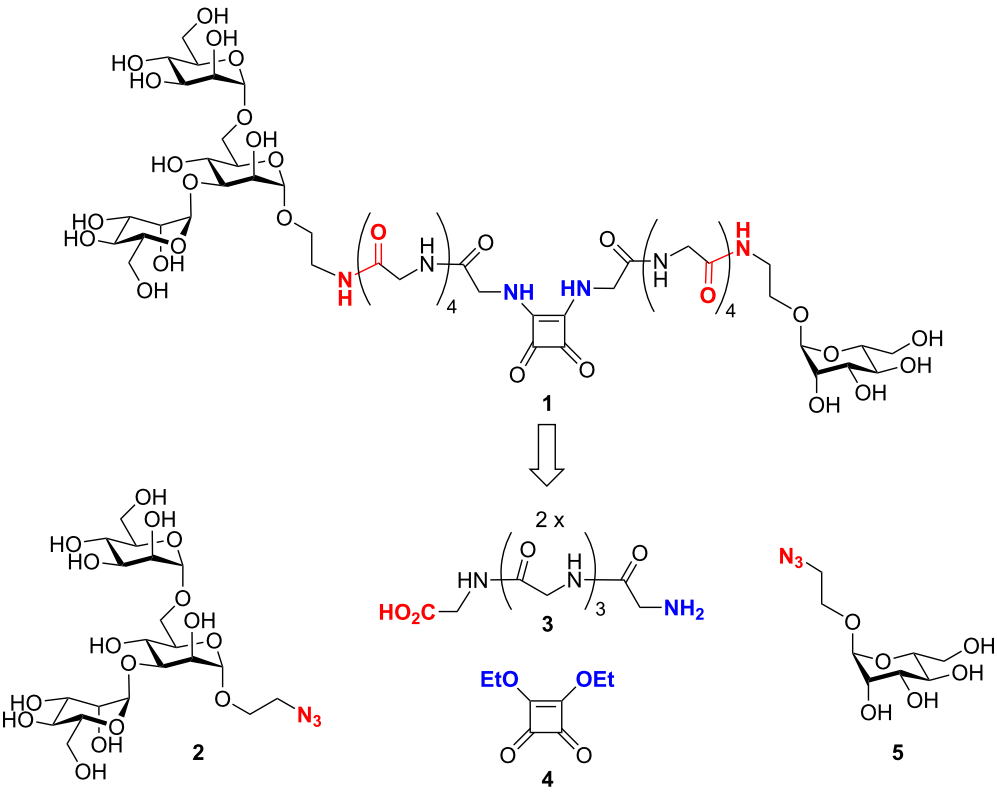
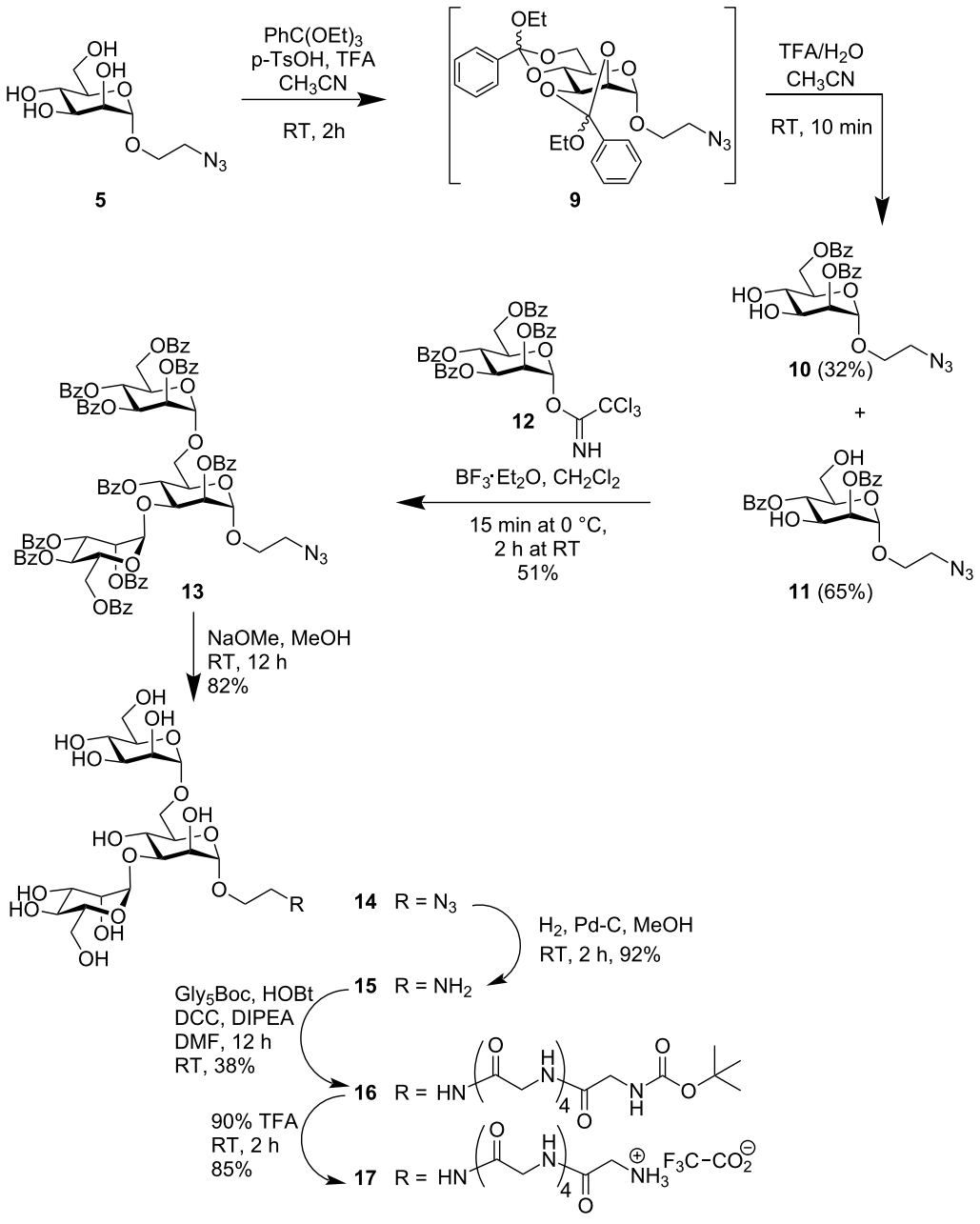


Figure 2: The bivalent glycopeptide **1** is the target molecule to test the hypothesis of two carbohydrate binding sites on FimH. Its retrosynthesis delivers the azido-functionalized mannotrioside **2** as the western part of the target structure and 2-azidoethyl mannoside **5** as its eastern portion. Squaric acid diethyl ester (DES, **4**) can link both parts via two pentaglycine spacers (**3**).

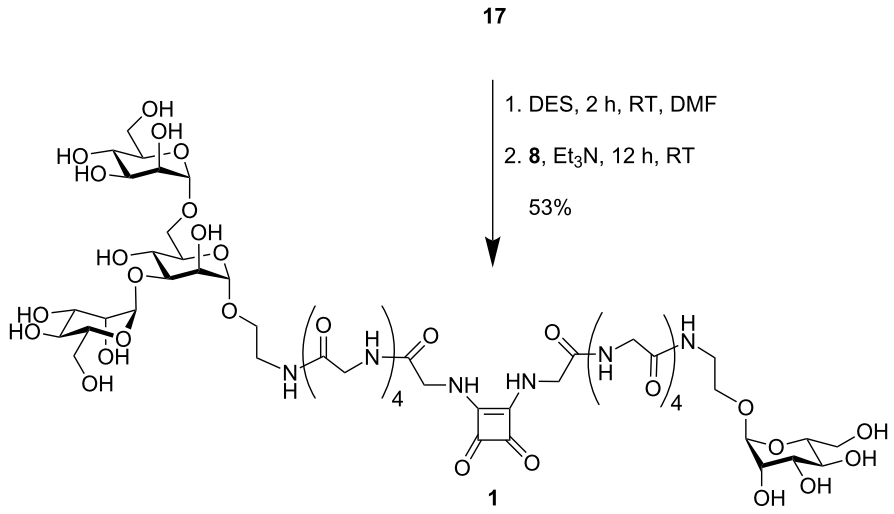
group pattern that is required for 3,6-bis-mannosylation, the azidoethyl mannoside **5** was converted into the bis-orthoester **9**, which was further converted in situ under acidic conditions to yield the required 2,4-dibenzoate **10** together with the isomeric 2,6-dibenzoate **11** as a byproduct, according to a known literature procedure (Scheme 2) [28,29].

Glycosylation of the acceptor diol **11** with the trichloroacetimidate **12** [30] led to mannotrioside **13** with the required azidoethyl aglycone. Reduction of the azido group gave amine **15** and subsequent peptide coupling reaction with Gly_5Boc with inexpensive coupling reagents led to the glycopeptide **16**. Boc-deprotection gave the required mannotrioside peptide **17** in the form of its TFA salt.

The glycopeptides **8** and **17** were finally ligated via squaric acid employing DES for ligation (Scheme 3). Control of the pH-value during ligation allows sequential substitution [31]: the



Scheme 2: Synthesis of the western part of target molecule **1**.



Scheme 3: Synthesis of the target molecule **1** employing squaric acid diethylester (DES).

first amine reacts at neutral pH, whereas addition of base is required to couple a second amine to the intermediate squaric acid monoester. Thus, the bivalent glycopeptide **1** was obtained in pure form, albeit after laborious gel permeation chromatography.

Testing of the bivalent glycopeptide 1 in type 1 fimbriae-mediated bacterial adhesion

The synthesis of the bivalent glycopeptide **1** was not optimized in order to improve all the individual yields, because it has been the primary goal of this study to test the anti-adhesive properties of this type of ligand. An ELISA was performed as reported earlier [32,33], employing type 1 fimbriated *E. coli* bacteria and mannan-coated microtiter plates. In this assay a mannosidic ligand competes with the polysaccharide mannan adsorbed on a polystyrene microtiter plate for FimH-mediated binding to the type 1 fimbriated bacteria. The inhibitory ligand is employed in serial dilutions. This leads to inhibition curves, from which IC_{50}-values can be deduced. The IC_{50}-values reflect the inhibitor concentration that causes 50% inhibition of bacterial binding to mannan. ELISA measurements typically lead to IC_{50}-values covering a broad range of absolute values. Therefore, highly reproducible relative inhibitory potencies (RIP-values) are usually reported in order to compare different inhibitors. Here, RIPs were based on the IC_{50}-value determined for MeMan on the same plate, which was defined as IP ≡ 1. In addition to the bivalent glycopeptide **1**, the disaccharides allyl 3-*O*-α-D-mannosyl-α-D-mannoside (α-D-Man-(1→3)-D-Man-α-allyl) (Table 1, entry 3) and allyl 6-*O*-α-D-mannosyl-α-D-mannoside (α-D-Man-(1→6)-D-Man-α-allyl) (Table 1, entry 4) as well as the branched mannotrioside allyl 3,6-di-*O*-(α-D-mannosyl)-α-D-mannoside (α-D-Man-(1→3)-[α-D-Man-(1→6)]-D-Man-α-allyl) (Table 1, entry 5) were compared to MeMan, as reported earlier (Table 1) [34].

Table 1: Inhibitory potencies in mannose-specific *E. coli* adhesion of the bivalent glycopeptide **1** in comparison with reference ligands, as determined by ELISA. IC_{50}-values are average values from three independent assays. *S*: standard deviation; RIP: relative inhibitory potency based on methyl α-D-mannopyranoside (MeMan) with IP(MeMan) ≡ 1.

Entry	Tested Ligand	IC_{50} [μmol] (*S*)	RIP
1	MeMan	2900 (890)	1
2	**1**	1500 (360)	2
3	allyl Man(1,3)Man		7[a]
4	allyl Man(1,6)Man		0.5[a]
5	allyl Man(1,3)[Man(1,6)]Man		20[a]

[a][34].

Conclusion

From the results collected in Table 1 it is obvious that the new glycopeptide **1** does not lead to a significant increase in the inhibition of type 1 fimbriae-mediated bacterial adhesion in comparison to the standard inhibitor MeMan. Unexpectedly, the bivalent ligand **1** performs just twice as good as MeMan. Consequently, it can hardly be argued that **1** is bridging two binding sites on FimH, or is executing any cooperative effect. Strikingly, ligand **1** is clearly a weaker ligand than the trisaccharide allyl Man(1,3)[Man(1,6)]Man (Table 1, entry 5), even although it contains the same trisaccharide as a partial structure.

There are several possible explanations for our findings with the new bivalent ligand **1**: i. a., (i) ligand **1** does not have the appropriate dimensions to match the two binding sites; (ii) the entropic penalty, that is experienced by the rather extended structure of **1** upon complexation with FimH leads to overall weak binding (high IC_{50}-values); (iii) a coherent conformational disadvantage of ligand **1** turns it into an even worse ligand than the branching mannotrioside Man(1,3)[Man(1,6)]-Man; (iv) ligand **1** might bind to a different site or to a different protein of the fimbrial shaft, thus lowering its effective concentration.

Thus, the hypothesis of multiple binding sites on FimH could not be conclusively supported by testing the new bivalent ligand **1**, likewise, neither can our findings be taken as counter-evidence. In light of very recent findings, our observations as well as results with other multivalent ligands, might be well explained by an allosteric model of FimH-mediated adhesion [35]. It has been reported that interdomain allosteric regulation can lead to a catch bond mechanism of adhesion in which the adhesive interaction becomes stronger with increased tensile force. The crystal structure of FimH in its native conformation -integrated into fimbrial tips- revealed that the binding domain of FimH is twisted and compressed by interaction with the pilin domain, thus loosening the adhesin mannose-binding pocket. This leads to a low-affinity state of the protein, whereas upon interaction with mannose, the domains separate and the binding domain untwists and elongates into a tight mannose-binding pocket. It will be important to investigate, how complex multivalent ligands function in this allosteric regulation of the ligand-receptor interaction.

Experimental

Docking studies

Computer-aided modeling to estimate the spacer lengths of a bivalent glycopeptide ligand to allow bridging of two putative binding sites on FimH was carried out using FlexX flexible docking and consensus scoring, implemented in Sybyl 6.8, as previously described [36]. Docking was based on the published X-ray structure of the FimH [8].

ELISA

An ELISA protocol was used to determine the IC_{50}-value of the bivalent target glycopeptide **1** in comparison with monovalent reference ligands, employing mannan-coated microtiter plates and type 1 fimbriated *E. coli* (HB101pPKL4) [37] as described earlier [34] Optical densities (ODs) were measured on an AMP 400 COM ELISA reader at 405 nm (reference wavelength 492 nm). The percentage inhibition was calculated as $\{[OD(nI)-OD(I)] \times 100 \times [OD(nI)]^{-1}\}$ (nI: no inhibitor, I: with inhibitor). The IC_{50}-values were determined where the sigmoidal fit of a set of measured inhibitions crosses an imaginary 50%-line. F-shaped 96-well microtiter plates from Sarstedt were used, mannan from *Saccharomyces cerversiae* was purchased from Sigma and used in 50 mM aq Na_2CO_3 solution (1 mg × ml^{-1}; pH 9.6). Peroxidase-conjugated goat anti-rabbit antibody (IgG, H+L) was purchased from Dianova.

General

All solvents were distilled prior to use. Commercially available starting materials, reagents and anhydrous DMF were used without further purification unless otherwise noted. Air- and/or moisture-sensitive reactions were carried out under an atmosphere of nitrogen or argon. Thin layer chromatography was performed on silica gel plates (GF 254, Merck). Detection was effected by UV irradiation and subsequent charring with 10% sulfuric acid in EtOH followed by heat treatment. Flash chromatography was performed on silica gel 60 (230–400 mesh, particle size 0.040–0.063 mm, Merck). Preparative MPLC was performed on an apparatus of BÜCHI Labortechnik GmbH using a LiChroprep RP-18 (40–60 μm, Merck) column for reversed-phase silica gel chromatography. Gel permeation chromatography (GPC) was carried out on Sephadex LH-20 or on Biogel P4 (bio-Rad), if not otherwise stated. ^1H and ^{13}C spectra were recorded on a Bruker DRX-500 (500 MHz for ^1H and 125.76 MHz for ^{13}C) instrument with Me_4Si ($\delta = 0$) as the internal standard. Optical rotations were determined with a Perkin Elmer 241 polarimeter (Na-D-line: $\lambda = 589$ nm, length of cell: 1 dm). ESI-MS measurements were recorded on a Mariner ESI-TOF 5280 (Applied Biosystems) instrument and MALDI-MS measurements on a MALDI-TOF-MS-Biflex III (Bruker) instrument.

2-[*N*-(*N*$^{\omega}$-*tert*-Butyloxycarbonyl-pentaglycyl)]-amidoethyl α-D-mannopyranoside (7)

$Glyc_5Boc$ (300 mg, 0.74 mmol) was dissolved in dry DMF (10 mL) and DCC (170 mg, 0.83 mmol), HOBt (110 mg, 0.81 mmol), and DIPEA (100 μL) were added at 0 °C. The reaction mixture was stirred for 30 min and then the amine **6** (150 mg,

0.67 mmol) was added. Stirring was continued at 0 °C for another 30 min and then overnight at RT. The mixture was filtered through celite, the solvent removed in vacuo and the residue purified by MPLC-RP chromatography (MeOH:H_2O = 1:4 → 2:3) to yield the title compound as a white lyophilisate (190 mg, 0.31 mmol, 47%).

^{1}H NMR (500 MHz, D_2O): δ = 4.78 (d, 1H, $J_{1,2}$ = 1.8, H-1), 3.95 (m_c, 6H, 2 NH-CH_2-CO, manOCH_2CH_2N), 3.93 (s, 2H, NH-CH_2-CO), 3.87 (m_c, 1H, $J_{2,3}$ = 3.5, H-2), 3.86 (s, 2H, NH-CH_2-CO), 3.82 (m, 2H, manOCH$_2$CH_2N), 3.81 (dd, 1H, $J_{6a,6b}$ = 12.1 Hz, H-6a), 3.77 (s, 2H, NH-CH_2-CO), 3.72 (m, 1H, $J_{3,4}$ = 8.9, H-3), 3.59–3.50 (m, 3H, H-4, H-5, H-6b), 1.39 (s, 9H, C(CH_3)$_3$) ppm; ^{13}C NMR (125.76 MHz, D_2O): δ = 175.96 (NH-*C*(O)O), 174.60, 174.36, 173.74 (5 NH-CH$_2$-*C*O), 101.98 (C-1), 84.15 (*C*(CH$_3$)$_3$), 75.11 (C-5), 72.80 (C-3), 72.32 (C-2), 69.10 (C-4), 68.02 (manOCH$_2$*C*H$_2$N), 63.25 (C-6), 45.82 (manO*C*H$_2$CH$_2$N), 44.81 (4 NH-*C*H$_2$-CO), 41.27 (NH-*C*H$_2$-CO), 29.89 (C(*C*H$_3$)$_3$) ppm; MALDI-TOF-MS: *m/z* 631.1, [M + Na]$^+$ (631.3 calcd. for $C_{23}H_{40}N_6O_{13}$ + Na).

2-[*N*-(Pentaglycyl)]-amidoethyl α-D-mannopyranoside hydrotrifluoroacetate (8)

The Boc-protected mannoside **7** (190 mg, 0.31 mmol) was dissolved in acetonitrile (5 mL) and treated with TFA (90% in water, 100 μL) for 2 h at RT. Then the solvent was removed in vacuo, the residue dissolved in water and lyophilized to yield the title compound as a white lyophilisate (193 mg, 0.31 mmol, quant.).

^{1}H NMR (500 MHz, D_2O): δ = 4.79 (d ≈ m, 1H, H-1), 3.97 (m_c, 2H, NH-CH_2-CO), 3.95 (m_c, 4H, NH-CH_2-CO, manOCH_2CH_2N), 3.87, 3.86 (each m_c, each 2H, 2 NH-CH_2-CO), 3.80–3.38 (m, 10H, H-2, H-3, H-4, H-5, H-6a, H-6b, manOCH$_2$CH_2N, NH-CH_2-CO) ppm; MALDI-TOF-MS: *m/z* 531.1 [M + Na]$^+$ (531.2 calcd. for the free amine $C_{18}H_{32}N_6O_{11}$ + Na).

2-Azidoethyl 2,4-di-*O*-benzoyl-3,6-di-*O*-(2,3,4,6-tetra-*O*-benzoyl-α-D-mannopyranosyl)-α-D-mannopyranoside (13)

The 2,4-di-*O*-benzoyl-protected mannoside **11** [28] (250 mg, 0.55 mmol) and the trichloroacetimidate **12** [30] (950 mg, 1.2 mmol) were dissolved in dichlormethane (20 mL) under an argon atmosphere. Molecular sieves (4 Å, 100 mg) and BF_3-etherate (200 μL) were added at 0 °C. The reaction mixture was stirred at this temperature for 30 min and then at RT overnight. Water was added (200 μL), the solvent removed and the residue co-distilled with toluene in vacuo. Purification of the residue by GPC on Sephadex LH-20 (MeOH/dichlormethane, 1:1) led to a white amorphous solid (451 mg, 279 mmol, 51%).

^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.32, 8.16, 7.84, 7.76, 7.72 (each m_c, 20H, *o*-aryl-H), 7.60–7.10 (m, 30H, *m*- and *p*-aryl-H), 6.10, 6.02, 5.88 (each dd ≈ t, 3H, *J* = 9.9 and 10.0 Hz, H-3, H-3', H-3''), 5.74, 5.72, 5.69 (each dd, 3H, J_3 = 3.4 and 3.5 Hz, H-2, H-2', H-2''), 5.36 (d, 1H, d, J_2 = 1.7 Hz, H-1), 5.19, 5.15 (each d, 2H, *J* = 1.8 Hz, H-1', H-1''), 4.67–4.60 (m_c, 3H, H-6a, H-6a', H-6a''), 4.36 (each dd, 3H, *J* = 9.6, H-6b, H-6b', H-6b''), 4.27 (m_c, 3H, H-4, H-4', H-4'') 4.14 (m_c, 3H, *J* = 10.8 and 10.9 Hz, H-5, H-5', H-5''), 3.79 (ddd, 2H, *J* = 5.7 and 9.7 Hz, manOCH_2CH$_2$N$_3$), 3.54 (ddd, 2H, manOCH$_2$CH_2N$_3$) ppm; ^{13}C NMR (125.76 MHz, $CDCl_3$): δ = 166.3–161.7 (10 C=O), 134.4–132.8 (10 CH, *p-a*ryl-C), 131.8–127.6 (20 CH, *o*-aryl-C), 127.2–126.3 (20 CH, *m*-aryl-C), 99.8, 97.5, 97.3 (C-1, C-1', C-1''), 73.5-64.5 (C-2, C-2', C-2'', C-3, C-3', C-3'', C-4, C-4', C-4'', C-5, C-5', C-5''), 63.3 (manOCH$_2$*C*H$_2$N$_3$), 61.6, 61.4, 61.9 (C-6, C-6', C-6''), 48.9 (manO*C*H$_2$CH$_2$N$_3$) ppm; MALDI-TOF-MS: *m/z* 1637.39 [M + Na]$^+$ (1636.45 calcd. for $C_{90}H_{75}N_3O_{26}$).

2-Azidoethyl 3,6-di-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside (14)

The protected mannotrioside **13** (400 mg, 0.24 mmol) was dissolved in dry MeOH (20 mL) and treated with sodium methanolate solution (1 M, 400 μL) at RT. After stirring overnight, the reaction mixture was neutralized by addition of ion exchange resin (Amberlite IR-120), filtered, the filtrate evaporated and the residue purified by reversed-phase chromatography on silica gel (H_2O:MeOH = 1:5) to yield the title compound as a colourless lyophilisate (113 mg, 0.20 mmol, 82%).

^{1}H NMR (500 MHz, D_2O): δ = 5.09, 4.89, 4.87 (each d, 3H, *J* = 1.8 and 1.9 Hz, H-1, H-1', H-1''), 4.12, 4.05, 3.98 (each dd, 3H, J_3 = 3.0, 3.4, and 3.5 Hz, H-2, H-2', H-2''), 3.87 (m_c, 9H, H-3, H-3', H-3'', H-4*, H-4'*, H-5, H-6a,* H-6b*, H-6b'*), 3.73 (m_c, 5H, H-5', H-5'', H-6a'*, H-6''*, manOCH_2CH$_2$N$_3$), 3.65 (m_c, 3H, H-4''*, H-6b''*) 3.51 (2 H, m_c, manOCH$_2$CH_2N$_3$) ppm; ^{13}C NMR (125.76 MHz, D_2O): δ = 104.1, 101.33, 100.73 (C-1, C-1', C-1''), 74.98, 74.93, 74.39, 73.76, 72.68, 72.47, 72.13, 71.39, 68.79, 68.53, 67.51, 67.32 (C-2, C-2', C-2'', C-3, C-3', C-3'', C-4, C-4', C-4'', C-5, C-5', C-5''), 66.7 (manOCH$_2$*C*H$_2$N$_3$), 65.8, 64.3, 64.1 (3 C-6), 51.3 (manO*C*H$_2$CH$_2$N$_3$) ppm; assignments indexed with * are interchangable. MALDI-TOF-MS: *m/z* 595.88 [M + Na]$^+$ (596.19 calcd. for $C_{20}H_{35}N_3O_{16}$ + Na).

2-Aminoethyl 3,6-di-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside (15)

The azide **14** (100 mg, 0.17 mmol) was dissolved in dry methanol (10 mL) and Pd on charcoal (10%, 10 mg) added. Hydrogenation with vigorous stirring for 3 h led to the title

amine, which was obtained after filtration through celite, evaporation and reversed-phase chromatography on silica gel (H_2O:MeOH = 1:1) as a colourless lyophilisate (86 mg, 0.16 mmol, 92%).

^{1}H NMR (500 MHz, D_2O): δ = 5.05, 4.82, 4.79 (each d, 3H, $J_{1,2}$ = 1.7, 1.8, and 1.9 Hz, H-1, H-1', H-1''), 4.03, 3.96, 3.89 (each dd, 3H, J_3 = 3.0 and 3.4, H-2, H-2', H-2''), 3.83–3.71 (m, 9H, H-3, H-3', H-3'', H-5, H-5', H-5'', H-6a, H-6a', H-6a''), 3.68–3.60 (m, 6H, H-4, H-4', H-4'', H-6b, H-6b', H-6b''), 3.40 (m_c, 4H, manOCH_2C$H_2$$NH_2$) ppm; ^{13}C NMR (125.76 MHz, D_2O): δ = 104.0, 102.3, 101.9 (C-1, C-1', C-1''), 83.2, 82.9, 82.7, 81.6, 79.9, 78.6, 78.2, 75.3, 74.9, 74.8, 72.8, 71.7 (C-2, C-2', C-2'', C-3, C-3', C-3'', C-4, C-4', C-4'', C-5, C-5', C-5''), 70.2 (manOCH$_2$C$H_2$$NH_2$), 69.7, 67.6, 67.4 (C-6, C-6', C-6''), 52.3 (manOCH$_2$C$H_2$$NH_2$) ppm; MALDI-TOF-MS: *m/z* 570.24 $[M + Na]^+$ (570.20 calcd. for $C_{20}H_{37}NO_{16}$ + Na).

2-[*N*-(*N*$^{\omega}$-*tert*-Butyloxycarbonyl-pentaglycyl)]-amidoethyl 3,6-di-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside (16)

The mannotrioside **15** (250 mg, 0.46 mmol) and $Glyc_5Boc$ (220 mg, 0.55 mmol) were dissolved in dry DMF (10 mL) and DCC (125 mg, 0.60 mmol), HOBt (80 mg, 0.59 mmol), and DIPEA (100 µL) added at 0 °C. The reaction mixture was stirred for 30 min at 0 °C, followed by overnight stirring at RT. The mixture was filtered through celite, the solvent reduced in vacuo and the residue purified by MPLC-RP chromatography (MeOH:H_2O = 1:4 → 2:3) to yield, after lyophilisation, the title glycopeptide (162 mg, 0.17 mmol, 38%).

^{1}H NMR (500 MHz, CD_3OD): δ = 5.14 (d, 1H, $J_{1,2}$ = 1.5 Hz, H-1), 4.93 (s, 1H, $J_{1',2'}$ = 1.6 Hz, H-1'), 4.77 (d, 1H, $J_{1'',2''}$ = 1.9 Hz, H-1''), 4.09, 4.05, 4.02 (each dd, each 1H, $J_{2,3}$ = 3.1, $J_{2',3'}$ = 3.3, $J_{2'',3''}$ = 3.3 Hz, H-2, H-2', H-2''), 3.98 (m, 8H, 3 N-CH_2-CONH, manOCH_2CH$_2$), 3.92–3.84 (m, 6H, H-3, H-3', H-3'', H-6a), 3.81 (m, 4H, 2 N-CH_2-CONH), 3.76 (m, 2H, manOCH$_2$CH_2), 3.68–3.30 (m, 9H, H-4, H-4', H-4'', H-5, H-5', H-5'', H-6b, H-6b', H-6b''), 1.40 (s, 9H, C(CH$_3$)$_3$) ppm; MALDI-TOF-MS: *m/z* 956.1 $[M + Na]^+$ (955.4 calcd. for $C_{35}H_{60}N_6O_{23}$ + Na).

2-[*N*-(Pentaglycyl)]-amidoethyl 3,6-di-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside hydrotrifluoroacetate (17)

The Boc-protected amine **16** (70 mg, 75.0 µmol) was stirred in aqueous TFA (90%, 1 mL) for 2 h and then co-evaporated with toluene. The residue was dissolved in water and lyophilized to yield the deprotected glycoamino acid as its TFA salt (62 mg, 64 µmol, 85%).

^{1}H NMR (500 MHz, CD_3OD): δ = 5.05 (d, 1H, $J_{1,2}$ = 1.6 Hz, H-1), 4.84 (d, 1H, $J_{1',2'}$ = 1.6 Hz, H-1'), 4.79 (s, 1H, H-1''), 4.05 (1-H, m, H-2), 4.02 (2H, s, NH-CH_2-CO), 4.01 (1-H, m, H-2'), 3.98, 3.96 (each 2H, s, 2 NH-CH_2-CO), 3.93 (1H, dd, H-2''), 3.88–3.86 (m, 4H, NH-CH_2-CO), 3.85–3.60 (m,19H, H-3, H-3', H-3'', H-4, H-4', H-4'', H-5, H-5', H-5'', H-6a, H-6a', H-6a'', H-6b, H-6b', H-6b'', manOCH_2CH$_2$, manOCH$_2$CH_2) ppm; MALDI-TOF-MS: *m/z* 855.8 $[M + Na]^+$ (855.8 calcd for the free amine $C_{30}H_{52}N_6O_{21}$ + Na).

Bivalent target glycopeptide 1

The glycopeptide **17** (15 mg, 16 µmol) was dissolved in DMF (1 mL) and neutralized with triethylamine (8 µL). DES (3.4 µL, 23 µmol) was then added and the reaction mixture stirred at RT. According to MALDI-TOF-MS monitoring, the first ligation reaction was complete after 2 h. Then, mannoside **8** (20 mg, 32 µmol) and triethylamine (100 µL) were added and the basic reaction mixture was stirred at RT overnight. The solvent was removed under reduced pressure and the residue purified by GPC (Bio-Gel P4, water as eluent) to yield, after lyophilisation, the title compound (12 mg, 8.46 µmol, 53% based on **17**).

^{1}H NMR (500 MHz, D_2O, water suppression): δ = 4.88 (d, 1H, $J_{1,2}$ = 1.6 Hz, H-1*), 4.83 (d, 1H, $J_{1',2'}$ = 1.3 Hz, H-1'*), 4.80 (d, 1H, $J_{1'',2''}$ = 1.5 Hz, H-1''*), 4.72 (d, 1H, $J_{1,2}$ = 1.1 Hz, H-1'''*), 4.03, 4.01, 4.00, 3.97, 3.96 (each s, each 2H, 5 NH-CH_2-CO), 3.91 (m, 2H, H-2, H-2'), 3.93 (s, 2H, NH-CH_2-CO), 3.88–3.81 (m, 10H, H-2'', H-2''', H-3, H-3', H-3'', H-3''', H-6a, H-6a', H-6a'', H-6a'''), 3.76–3.68 (m, 28H, 4 NH-CH_2-CO, H-3, H-3', H-3'', H-3''', H-4, H-4', H-4'', H-4''', H-5, H-5', H-5'', H-5''', H-6b, H-6b', H-6b'', H-6b''', 2 manOCH$_2$CH_2, 2 manOCH_2CH$_2$) ppm; ^{13}C NMR (125.76 MHz, D_2O): δ = 185.45, 178.85 (C=O_{SA}), 176.04, 175.43, 174.68, 174.61, 174.58, 174.44, 174.41, 174.33, 174.27, 173.82, 173.76, (C=O), 104.83, 102.08, 102.09, 101.80 (C-1, C-1', C-1'', C-1'''), 75.74, 75.22, 75.12, 74.67 (C-5, C-5', C-5'', C-5'''), 73.33, 73.12, 73.01, 72.92 (C-3, C-3', C-3'', C-3'''), 72.55, 72.50, 72.44, 72.41 (C-2, C-2', C-2'', C-2'''), 69.22, 69.16 (C-4, C-4', C-4'', C-4'''), 68.10 (manOCH$_2$CH$_2$), 67.62 (manOCH$_2$CH_2), 64.19, 64.06, 63.69, 63.34 (C-6, C-6', C-6'', C-6'''), 48.69 (manOCH$_2$CH$_2$), 46.07 (manOCH$_2$CH$_2$), 45.87 (CH$_2$), 45.24 (CH$_2$), 45.18 (CH$_2$) ppm; assignments indexed with * are interchangable; MALDI-TOF-MS: *m/z* = 1442.2 $[M + Na]^+$ (1441.5 calcd. for $C_{52}H_{82}N_{12}O_{34}$ + Na); ESI-MS: *m/z* 1441.1 $[M + Na]^+$ (1441.5 calcd. for $C_{52}H_{82}N_{12}O_{34}$ +Na; ESI-HRES-MS: *m/z* 1441.4901 (1441.4954 calcd. for $C_{52}H_{82}N_{12}O_{34}$ + Na).

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En route to photoaffinity labeling of the bacterial lectin FimH

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Abstract

Mannose-specific adhesion of *Escherichia coli* bacteria to cell surfaces, the cause of various infections, is mediated by a fimbrial lectin, called FimH. X-ray studies have revealed a carbohydrate recognition domain (CRD) on FimH that can complex α-D-mannosides. However, as the precise nature of the ligand–receptor interactions in mannose-specific adhesion is not yet fully understood, it is of interest to identify carbohydrate recognition domains on the fimbrial lectin also in solution. Photoaffinity labeling serves as an appropriate methodology in this endeavour and hence biotin-labeled photoactive mannosides were designed and synthesized for photoaffinity labeling of FimH. So far, the photo-crosslinking properties of the new photoactive mannosides could be detailed with the peptide angiotensin II and labeling of FimH was shown both by MS/MS studies and by affino dot–blot analysis.

Introduction

Photoaffinity labeling is a technique by which ligand binding sites of a receptor protein can be identified in solution. It requires a photoactive ligand derivative, which can form a reactive species upon photo-excitation. Thus, incubation of the photoprobe with a protein followed by irradiation can result in a photo-crosslinked product, that provides structural information on the binding site of the protein (Figure 1) [1].

It has become our goal to utilize this methodology for the investigation of carbohydrate binding of the bacterial lectin FimH. The protein FimH is found on the tips of type 1 fimbriae, long adhesive filamentous appendages on the surface of many bacteria, such as *Escherichia coli* [2-5]. In X-ray studies a carbohydrate recognition domain (CRD), which can complex one α-D-mannosyl residue, has been clearly identified [6-8].

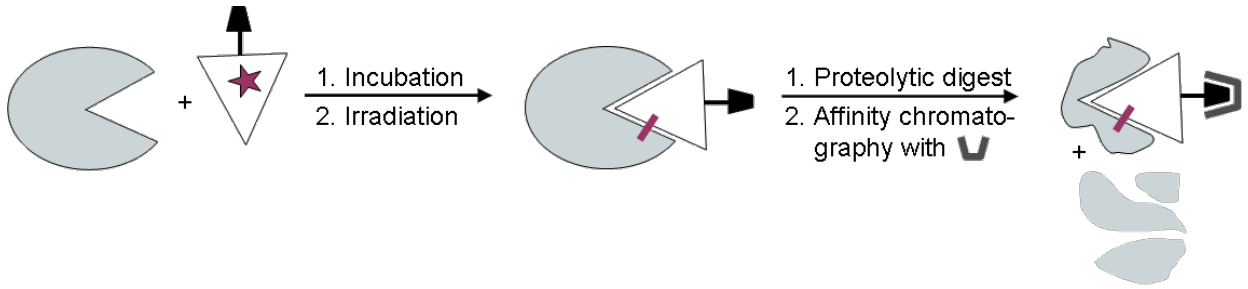


Figure 1: The photoaffinity technique allows the identification of ligand binding sites of a receptor protein after photo-crosslinking, proteolysis and affinity chromatography.

However, other binding experiments performed with a multitude of synthetic as well as natural mannosides and oligomannosides are not in complete agreement with just one monovalent carbohydrate binding site on the FimH protein [9-13]. Thus, we decided to employ photoactive ligands to probe carbohydrate binding to the known CRD in solution and to identify possibly auxiliary, so far unknown, binding sites on bacterial lectin FimH [2].

The known FimH CRD was taken as the lead structure for the design of an appropriate photoactive ligand. Inspection of the available X-ray data clearly shows that α-D-mannosides are complexed in the CRD with the aglycone moiety sticking out of the binding pocket. The entrance of the CRD is flanked by the tyrosine residues Tyr48 and Tyr137 ('tyrosine gate') that can form favorable interactions with appropriate mannosidic aglycone moieties, such as π-π-stacking with the phenyl group of benzyl mannosides [14]. In a preceding work, we synthesized the three corresponding photoactive α-D-mannosides, **1**, **2**, and **3** (Figure 2a) for photolabeling of the bacterial lectin FimH [15]. They differ in their photoactive functional groups, which are part of the aglycone moiety. Upon irradiation, the aryl azide **1** and the diazirine **2** expel nitrogen to yield a reactive nitrene and a carbene intermediate, respectively. Irradiation of the benzophenone **3**, on the other hand, delivers a reactive triplet diradical.

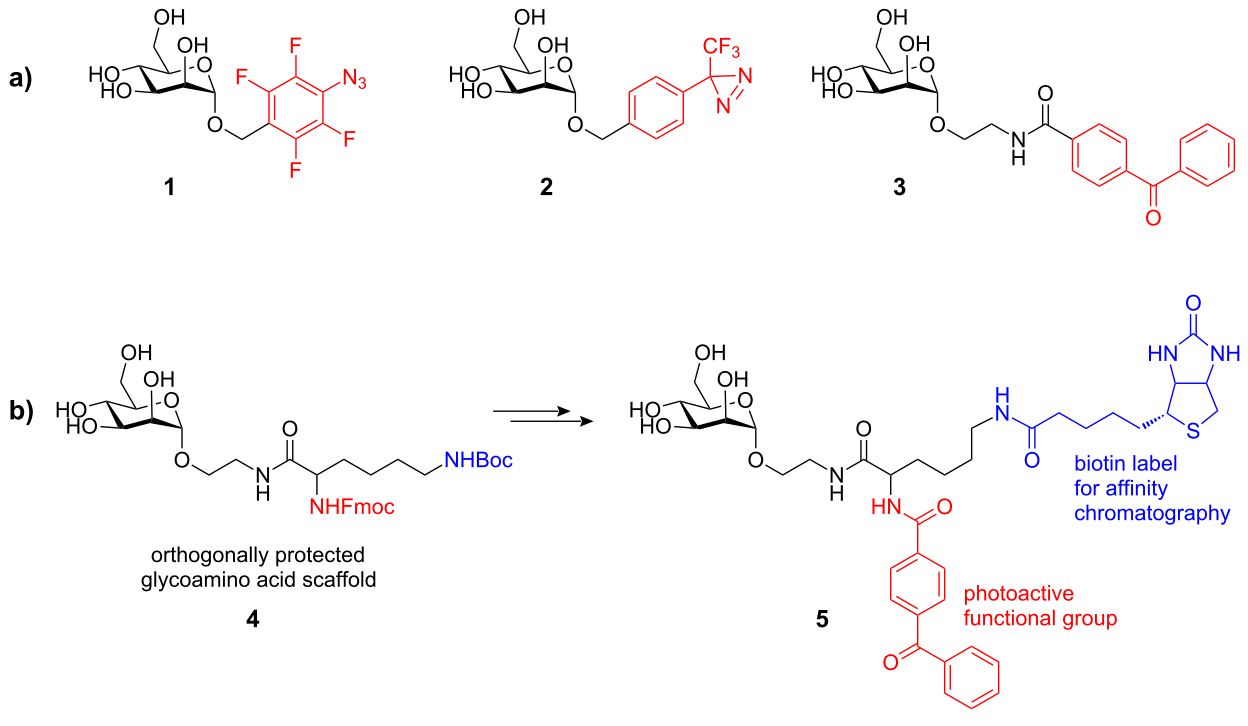


Figure 2: a) Three known photolabile α-D-mannosides that differ in the nature of the photoactive functional group (in red); **b)** the known bifunctional mannoside ligand **5** is based on the orthogonally protected glycoamino acid **4** and well suited for photoaffinity labeling.

In addition, in order to combine a photoactive functional group with an affinity label within the same mannoside, the orthogonally protected glycoamino acid scaffold **4** was synthesized and used for the preparation of the biotin-labeled photophore **5**, which is well suited for the streptavidine-based photoaffinity labeling, relying on the extraordinary high affinity of the protein streptavidine for biotin (Figure 2b) [16].

Results and Discussion

In order to learn more about the ligand properties of the previously prepared photoactive mannosides **1**, **2**, **3**, and **5**, we have performed computer-aided docking studies using FlexX [17-19] to get an idea about their binding to the bacterial lectin FimH, as reported earlier [20]. FlexX produces so-called scoring values for each docked ligand, which can be regarded as a rough estimate of its free binding energy. Low (more negative) scores correlate with high affinities, whilst higher scores reflect diminished binding potency (Table 1). Docking was based on two different X-ray structures. In the first case, crystals with an open tyrosine gate were taken as the basis [7], whilst in the second case, a closed-gate conformation of FimH was used for the modeling [8]. This led to somewhat different predictions, as expected, however, the same trends were revealed for ligands **1**–**3** and **5** in comparison with the well-known standard ligands methyl α-D-mannoside (MeMan) and *p*-nitrophenyl α-D-mannoside (pNPMan), respectively (Table 1).

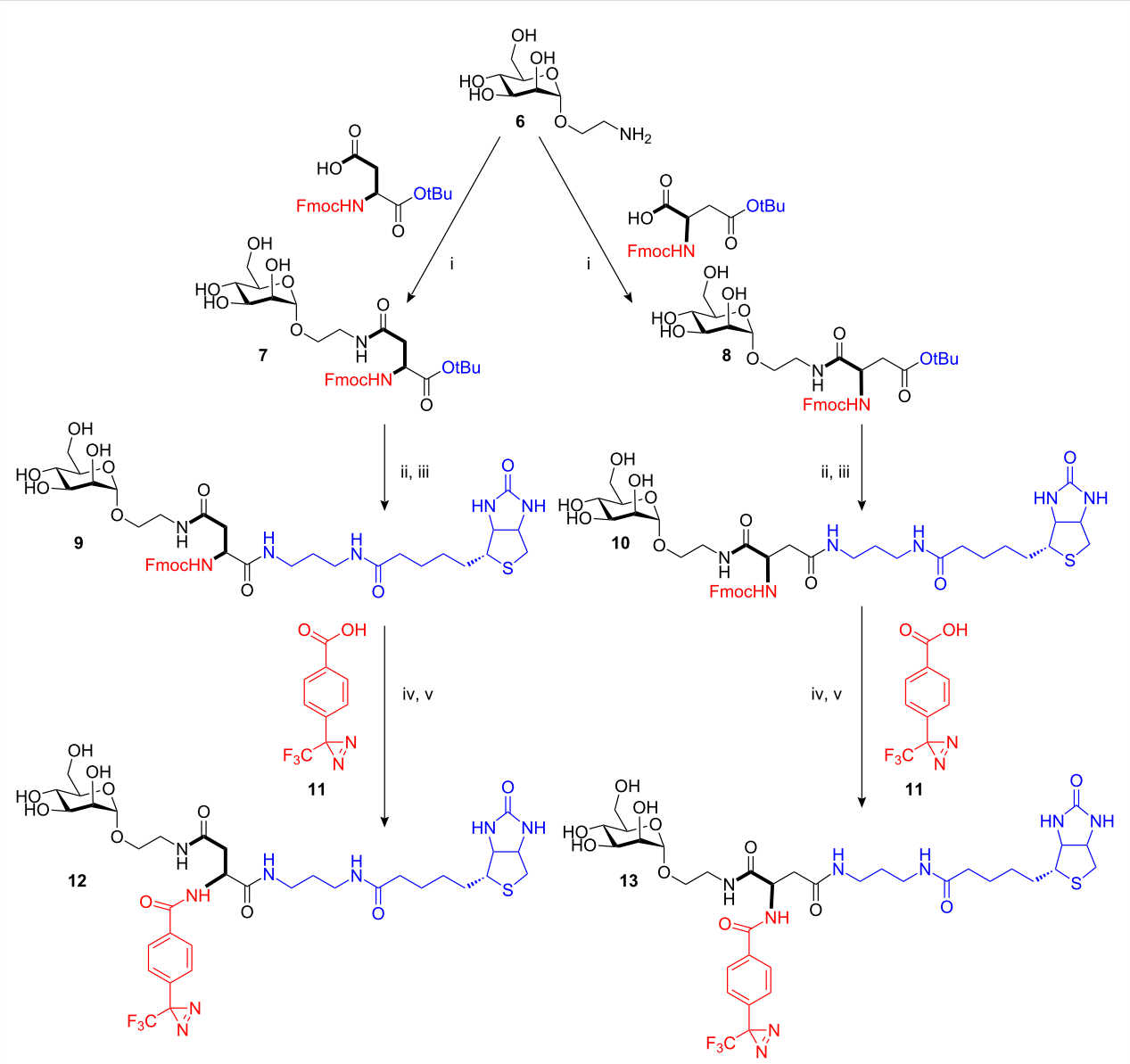
When these mannosides were tested as inhibitors of type 1 fimbriae-mediated bacterial adhesion to a mannan-coated surface in an ELISA [21,22], IC_{50}-values were obtained, which reflect the concentration of the derivative employed, that leads to 50% inhibition of bacterial adhesion. Three independent measurements resulted in high standard deviations, a typical characteristic of this assay, whereas the relative trend in a series of tested ligands can be verified with high reproducibility. Hence, the inhibitory potencies of the tested ligands were referenced to an internal standard inhibitor, MeMan (inhibitory potency ≡ 1), to deduce relative inhibitory potencies, so-called RIP-values (Table 1). This uniform referencing shows, that all photoactive mannosides surpass the inhibitory potency of MeMan, and that mannosides **1** and **2** are more powerful inhibitors than **3** and **5**. Consequently, the synthetic photoactive mannosides are suited as ligands for the bacterial lectin FimH. Comparison of the measured IC_{50}-values with the theoretical docking results, discloses a somewhat limited value of the computer-aided predictions in this case. Docking suggested that mannosides **2**, **3** and **5** are the most potent ligands of FimH in the tested series, which was not confirmed experimentally. On the other hand, it has to be kept in mind that the employed ELISA is not based on pure FimH, but rather on type 1-fimbriated bacteria, a dissimilar more complex system.

The binding studies showed that mannosides with a typical photolabel in the aglycone moiety serve as ligands for FimH, and that even the more complex glycoamino acid derivative **5** is a suitable ligand. Encouraged by these results, we set out to improve the photochemical properties of **5** in order to facilitate later photoaffinity-labeling of FimH. Our earlier work suggested that diazirines are more useful photoactive groups than aryl azides and benzophenones [15]. Therefore, the synthesis of a biotin-labeled daizirine-functionalized mannoside was our next target. In this synthesis, aspartic acid was utilized as the scaffold molecule in two different ways in order to allow fine-tuning of the spacing between the mannoside ligand and the photoactive group. Thus, the amino acid derivatives Fmoc-Asp-O*t*Bu and Fmoc-Asp(O*t*Bu)-OH were employed in two analogous synthetic pathways, starting with peptide coupling to the known 2-aminoethyl mannoside **6** (Scheme 1) [23]. This led

Table 1: Binding of mannoside ligands to FimH was predicted by computer-aided docking (FlexX) and measured by ELISA utilizing type 1-fimbriated *E. coli*. IC_{50}-values are averaged from three independent experiments.

mannoside	FlexX Scores "open-gate"[a]	FlexX Scores "closed-gate"[b]	IC_{50} (SD)[c] [mmolar]	RIP[d]
MeMan	−22.5	−23.3	1.84 (1.32)	1
pNPMan	−24.9	−27.4	0.04 (0.01)	46
1	−23.6	−24.0	0.06 (0.01)	31
2	−28.3[e]	−29.3[e]	0.12 (0.09)	15
3	−30.2	−29.8	0.20 (0.10)	9
5	−31.9	−36.0	0.63 (0.42)	3

[a]based on open-gate structure of FimH, PDB ID: 1KLF [7].
[b]based on closed-gate structure of FimH, PDB ID: 1UWF [8].
[c]SD: standard deviation.
[d]RIP: relative inhibitory potency.
[e]To facilitate docking, the diazirine ring was substituted by a cyclopropyl ring.



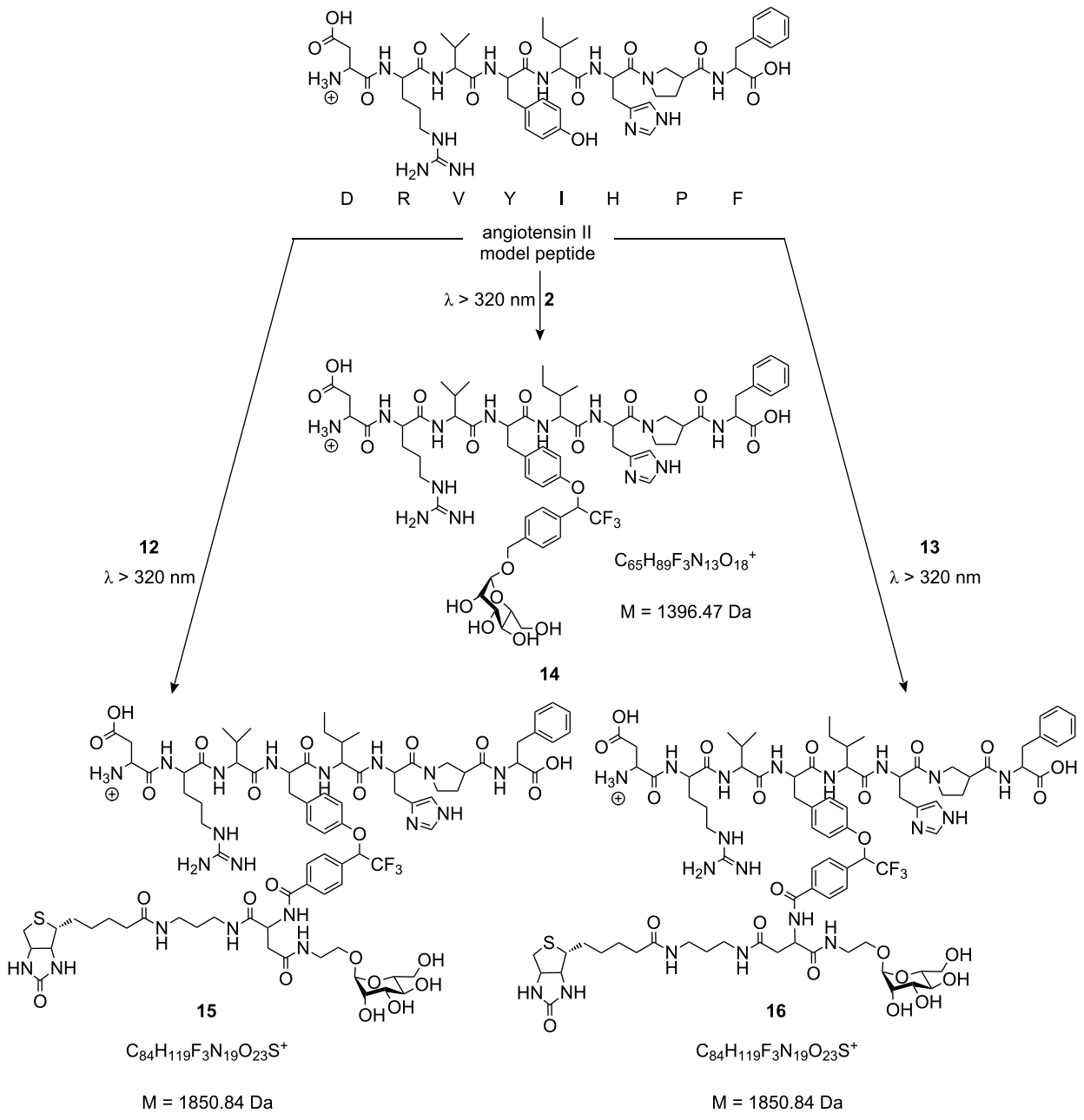
Scheme 1: Synthesis of photoactive glycoamino acids **11** and **12**. i) Fmoc-Asp-O*t*Bu (for **7**), Fmoc-Asp(O*t*Bu)-OH (for **8**), HATU, DIPEA, dry DMF, N_2, RT, 95% for **7** and 96% for **8**; ii) 80% aq TFA, RT; iii) (+)-biotinylamidopropylammonium trifluoroacetate, HATU, DIPEA, dry DMF, N_2, RT, 48% (two steps) for **9** and 26% (two steps) for **10**; (iv) 20% piperidine, dry DMF, RT; v) HATU, DIPEA, dry DMF, Ar, RT, 81% (two steps) for **12** and 98% (two steps) for **13**.

to the orthogonally protected mannoside amino acid *tert*-butyl esters **7** and **8**. The *tert*-butyl ester groups were then cleaved under acidic conditions and the resulting acids ligated with the biotin derivative biotinylamidopropylammonium trifluoroacetate. These two steps gave the Fmoc-protected biotin-labeled glycoamino acids **9** and **10**, respectively. Fmoc-cleavage and peptide coupling with the diazirine **11** led to the target molecules **12** and **13** in good yield.

To test the prepared photoactive mannosides in crosslinking reactions, the model peptide angiotensin II (DRVYIHPF) was first employed. It was irradiated with the three different diazirine-functionalized mannosides **2**, **12**, and **13**. From our earlier work [15] it was known that irradiation of the diazirine-functionalized mannoside **2** with angiotensin II led to a photo-crosslinked product with *m/z* = 698.82. This double positively charged ion correlates with a 1:1-photoaddition product of peptide and photolabel with the molecular formula $C_{65}H_{90}F_3N_{13}O_{18}^{2+}$ and a monoisotopic mass of 1397.47 Da. We have now carried out MS/MS experiments to analyze the structure of this photo-crosslinked adduct and unequivocally shown that insertion of the carbene, which results after irradi-

ation of **2**, occurs at the hydroxyl group in the side chain of the angiotensin tyrosine (Y) (see Experimental Part). This crosslinking reaction leads to the ether **14** (Scheme 2). Analogously, the photo-crosslinked products, obtained from irradiation of angiotensin II with either **12** or **13**, can be correlated with the structures of **15** and **16**, respectively, both showing a peak at *m/z* = 1851.1 in their mass spectra, corresponding to a molecular formula of $C_{84}H_{119}F_3N_{19}O_{23}S^+$ (monoisotopic mass M = 1850.84 Da).

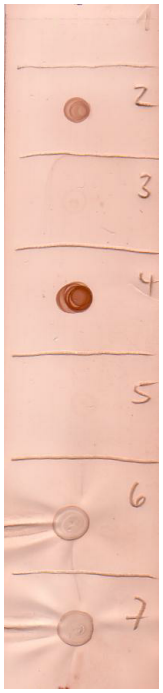
The photo-crosslinking experiments with angiotensin II demonstrated, that the synthesized diazirines are well suited as photoprobes for the labeling of this peptide, with a preference for the tyrosine side chain. Thus, the photoactive mannosides were next investigated with the bacterial lectin FimH. For the irradiation experiments a FimH truncate, $FimH_{tr}$, which resembles the adhesin domain of the complete FimH, was used [24]. $FimH_{tr}$ comprises of amino acids 1-160 of FimH and is terminated by a histidine tag His_6. $FimH_{tr}$ has the same carbohydrate binding



Scheme 2: Photo-crosslinking experiments with the model peptide angiotensin II and the photoactive mannosides **2**, **12**, and **13**, respectively (see Experimental Part).

properties as FimH. Mass spectrometric analysis of $FimH_{tr}$ revealed *m/z* = 17839 (calcd. *m/z* = 17845). Solutions of $FimH_{tr}$ and the photophores **2**, **12**, and **13** were applied in a ratio of 5:1. Samples were incubated at 37 °C to allow formation of the lectin-ligand complex and then irradiated at $\lambda \geq 320$ nm. This led to 1:1-photo-crosslinked products with the corresponding masses (Table 2).

In addition to the mass spectrometric analysis, dot–blots were performed with $FimH_{tr}$ and the photoactive mannosides. Affinity staining was carried out with a streptavidine–HRP conjugate and the chromogene 3,3'-diaminobenzidine (DAB). The biotin-labeled mannosides **12** and **13** gave violet spots on the nitrocellulose membrane when tested. Affino dot–blot with mannoside **2**, that does not contain a biotin moiety, was negative, as predicted. In addition, control experiments were carried out, leading to the expected results in all cases. Interestingly, photoaffinity probes **12** and **13** seem to exhibit unequal affinity to $FimH_{tr}$ as suggested by the different intensity of color of the respective precipitates (Figure 3). Affinity staining using western blots led to analogous results (not shown).



Lane 1: $FimH_{tr}$, irradiated

Lane 2: $FimH_{tr}$, **13**, irradiated

Lane 3: $FimH_{tr}$, **12**, not irradiated

Lane 4: $FimH_{tr}$, **12**, irradiated

Lane 5: $FimH_{tr}$, **13**, not irradiated

Lane 6: **13**, not irradiated

Lane 7: **12**, not irradiated

Figure 3: Affino dot–blot with $FimH_{tr}$ and photoactive mannosides applied to nitrocellulose disks. It was irradiated, incubated with streptavidine–HRP conjugate and stained by addition of the chromogene 3,3'-diaminobenzidine. Photoaffinity probes **12** (lane 4) and **13** (lane 2) led to different color intensities, suggesting that ligand **12** is bound more tightly to $FimH_{tr}$ than ligand **13**.

Conclusion

In conclusion, we have demonstrated the synthesis of new biotin-labeled photoactive mannosides for photoaffinity-labeling of the bacterial lectin FimH. The target molecules **12** and **13** were selected after docking studies based on the structure of FimH and according to binding studies employing type 1-fimbriated *E. coli*. Photo-crosslinking was tested with the model peptide angiotensin II and the regiochemistry of the insertion reaction could be solved by MS/MS studies. Furthermore, photoaffinity-labeling of $FimH_{tr}$ was successful and could be demonstrated by mass spectrometric studies as well as dot–blot analysis.

The overall goal of this study is to identify mannose binding sites on the bacterial lectin FimH in solution. After photo-crosslinking of the lectin with photoactive, biotin-labeled mannosidic ligands, proteolytic digestion of the products of photo-crosslinking, followed by affinity chromatography and mass-spectrometric analysis of the fragments, should allow the identification of the critical amino acid residues on FimH, according to the photoaffinity methodology. However, so far we

Table 2: Results of mass spectrometric analysis of photo-crosslinked products, obtained from irradiation of $FimH_{tr}$ with photoactive mannoside ligands **2**, **12**, and **13**.

$FimH_{tr}$[a] incubated with	$FimH_{tr}$[b] measured mass *m/z*	crosslinked product measured mass *m/z*	Δ mass *m/z* ($FimH_{tr}$) - *m/z* (crosslinked product)	Δ mass expected
2	17858 $[M + H]^+$	18200[c] $[M + H]^+$	342	350[e]
12	17905 $[M + H]^+$	18672[d] $[M + H]^+$	767	804[f]
13	17697 $[M + H]^+$	18500[d] $[M + H]^+$	803	804[f]

[a]For control experiments $FimH_{tr}$ was irradiated similarly without addition of photophore. Mass spectrometric analysis revealed that the protein FimH survived the conditions of irradiation without any damage.
[b]Calcd. mass M = 17845 Da; measured values are acceptable within accuracy of the measurement.
[c]Measured on 4700 Proteomics Analyzer (Applied Biosystems).
[d]Measured on Bruker Biflex III.
[e]Based on the carbene resulting from irradiation of **2**: $C_{15}H_{17}F_3O_6$ (M = 350 Da); measured Δm-values acceptable within accuracy of the measurement.
[f]Based on the carbene resulting from irradiation of **12** and **13**, respectively: $C_{34}H_{47}F_3N_6O_{11}S$ (M = 804 Da); measured Δm-values acceptable within accuracy of the measurement.

have not been successful in an unequivocal mass-spectrometric analysis of any proteolytic digest, we have obtained so far. Thus, this study is currently continued in our laboratory.

Experimental

Docking studies

Computer-aided modeling to predict binding of the various FimH ligands was carried out using FlexX flexible docking and consensus scoring as implemented in Sybyl 6.8 as described earlier [20]. Docking was based on published X-ray structures of the FimH CRD. This CRD was held fixed during the minimization, whereas the sugar ligand was allowed to change its conformation freely under the influence of the force field.

ELISA

ELISAs to determine IC_{50}-values of the various FimH ligands were carried out with *E. coli* bacteria of strain HB101pPKL4 and mannan-coated microtiter plates as described earlier [21,22].

Mass spectrometry

For mass spectrometric analyses of the photo-crosslinked products, a Bruker Biflex III instrument (MALDI-TOF-MS, Prof. Th. K. Lindhorst, CAU), or a Bruker Biflex II instrument (ESI-FT-ICR-MS/MS, group of PD Dr. B. Lindner at the Research Center Borstel), or the 4700 Proteomics Analyzer mass spectrometer (Applied Biosystems MALDI-TOF/TOF-MS, group of Prof. M. Leippe, CAU) were used. Mass spectra were acquired using standard experimental sequences as provided by the manufacturer. Analysis of photo-crosslinking with angiotensin II is exemplified with diazirine **2** and presented in Figure 4.

FimH truncate

The amino acid sequence of the FimH truncate [24], $FimH_{tr}$, used in the photoaffinity labeling studies is as follows:

FACKTANGT AIPIGGGSAN VYVNLAPVVN VGQN-
LVVDLS TQIFCHNDYP ETITDYVTLQ RGSAYGGVLS
NFSGTVKYSG SSYPFPTTSE TPRVVYNSRT DKPWP-
VALYL TPVSSAGGVA IKAGSLIAVL ILRQTNNYNS
DDFQFVWNIY ANNDVVVPT GGHHHHHH

Affino dot–blot

Samples (2 µL, 1.15 mmolar) were applied on a nitrocellulose membrane, incubated with streptavidine-HRP conjugate and stained with 3,3'-diaminobenzidine.

Methods and materials for synthesis

Reactions were monitored by TLC on silica gel GF_{254} (Merck) with detection under UV light and by charring with 10% sulfuric acid in ethanol or using anisaldehyde and subsequent heating. Flash column chromatography was performed on silica gel 60 (40–63 µm, Merck) and for RP-MPLC a Merck Licroprep RP-18 column (Büchi) was used. Preparative HPLC was accomplished on a Shimadzu LC-8a machine (LiChrosorb RP-8, HIBAR). NMR spectra were recorded on Bruker AMX 400, Bruker DRX 500 or Bruker Avance 600 instruments. Chemical shifts are relative to TMS or the solvent peaks of $CDCl_3$ (7.24 ppm for ^1H, 77.0 ppm for ^{13}C) or MeOD (3.35 ppm and 4.78 ppm for ^1H, 49.3 ppm for ^{13}C). Where necessary, assignments were based on 2D experiments (COSY, HSQC, HMBC or NOESY). IR spectra were taken with a Perkin Elmer FT IR Paragon 1000 (KBr). Optical rotations were measured with a Perkin-Elmer polarimeter (22 °C, 589 nm, length of cuvette: 1 dm). For MS analysis of the synthetic products MALDI-TOF mass spectra were measured with a Bruker Biflex III with 19 kV acceleration voltage. 4-Hydroxy-α-cyanocinnamic acid (HCCA) was used as matrix, either as a saturated solution in a solvent mixture (33% MeCN/ double distilled water and 0.1% TFA) or as saturated solution in acetone. Ionisation was effected with a nitrogen laser at 337 nm. ESI-MS spectra of the synthesized derivatives were measured with an Applied Biosystems Mariner ESI-TOF 5280 and millipore C_{18}-pipette tips were used for ZipTipping®.

N-(Fluoren-9-ylmethoxycarbonyl)-4-[2-(α-D-mannopyranosyloxy)ethylamido]-L-aspartic acid *tert*-butyl ester (7)

The aminoethyl mannoside **6** (200 mg, 0.90 mmol), HATU (320 mg, 0.90 mmol) and Fmoc-Asp-O*t*Bu (400 mg, 0.98 mmol) were dried under vacuum for 10 min and then dissolved in dry DMF (12 mL) under a nitrogen atmosphere. DIPEA (300 µL, 2.25 mmol) was added and the reaction mixture stirred for 18 h at RT. The solvent was removed in vacuo and the residue purified by flash chromatography (ethyl acetate:MeOH:H_2O = 6:2:1). Pure product fractions were pooled, filtered, concentrated and the residual taken up in water. Lyophylisation gave the title compound (531 mg, 0.86 mmol, 95%); R_f = 0.86 (ethyl acetate:MeOH:H_2O = 6:2:1).

^1H NMR (600 MHz, D_4-MeOH): δ = 7.84 (d, 2H, *J* = 7.6 Hz, aryl-Fmoc), 7.71 (d, 2H *J* = 7.5 Hz, aryl-Fmoc), 7.43 (t, 2H, *J* = 7.5 Hz, aryl-Fmoc), 7.35 (t, 2H, *J* = 7.5 Hz, aryl-Fmoc), 4.81 (d, 1H, *J* = 1.5 Hz, H-1man), 4.50 (dd, 1H, *J* = 7.2 Hz, *J* = 5.6 Hz, Hα-asp), 4.40 (dd, 1H, *J* = 10.5 Hz, *J* = 7.2 Hz, Fmoc-CH*H*), 4.35 (dd, 1H, *J* = 10.5 Hz, *J* = 7.0 Hz, Fmoc-C*H*H), 4.28 (t, 1H, *J* = 7.0 Hz, Fmoc-C*H*-CH_2), 3.88 (dd, 1H, *J* = 11.6 Hz, *J* = 2.2 Hz, manOCH_2CH*H*), 3.87 (dd, 1H, *J* = 3.5 Hz, *J* = 1.8 Hz, H-2man), 3.79 (dd, 1H, *J* = 10.5 Hz, *J* = 4.5 Hz, manOCH*H*CH_2), 3.75 (dd, 1H, *J* = 11.7 Hz, *J* = 5.9 Hz, manOCH_2C*H*H), 3.74 (dd, 1H, *J* = 9.3 Hz, *J* = 3.4 Hz,

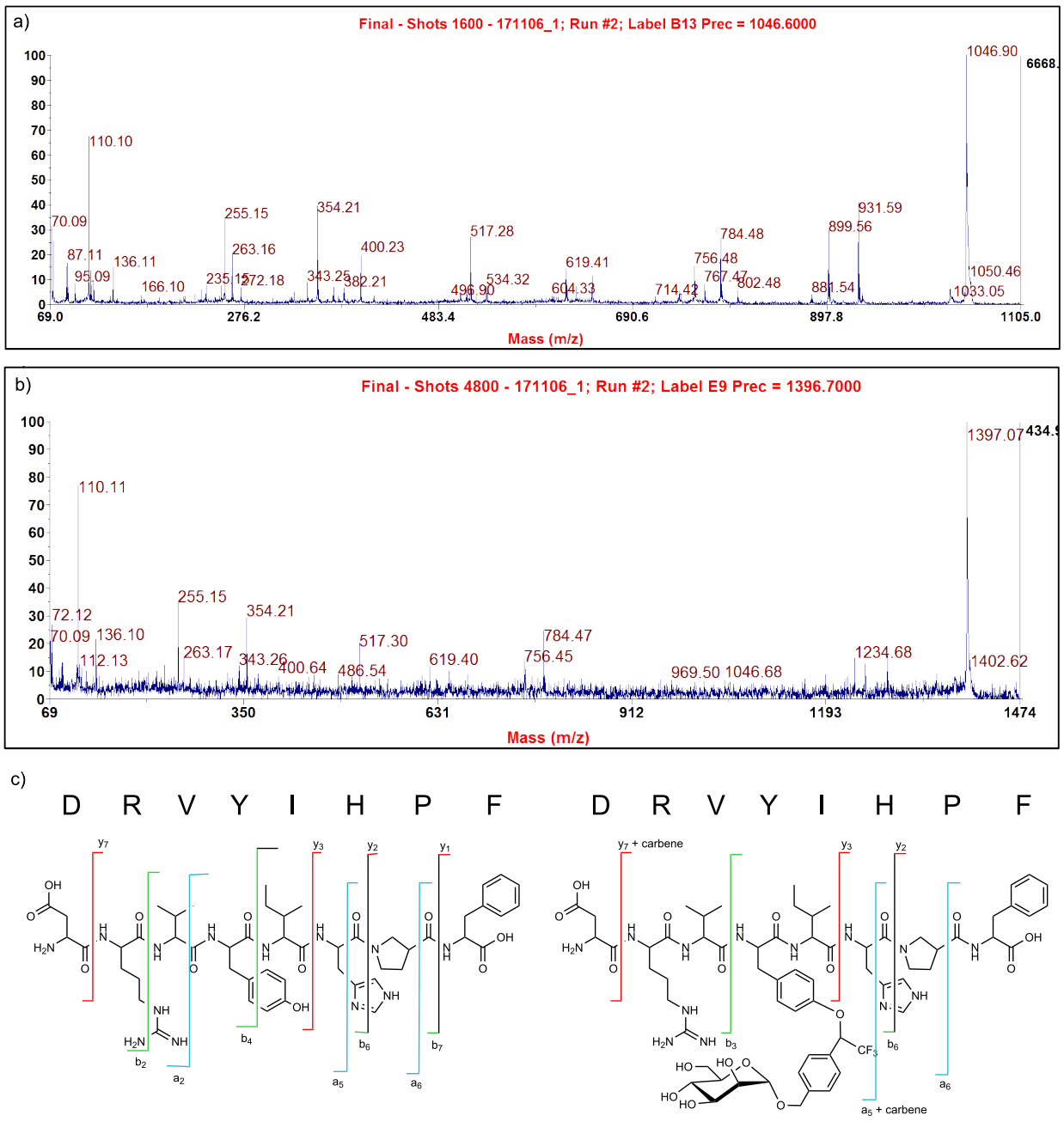


Figure 4: MS/MS spectra of angiotensin II (**a**) and of angiotensin II, photo-crosslinked with diazirine **2** (**b**), recorded on 4700 Proteomics Analyzer mass spectrometer (Applied Biosystems); (**c**) fragments of angiotensin II and photo-crosslinked angiotensin II according to Biemann's nomenclature (Table 3).

H-3man), 3.65 (t, 1H, J = 9.5 Hz, H-4man), 3.58 (ddd, 1H, J = 9.7 Hz, J = 5.7 Hz, J = 2.1 Hz, H-5man), 3.57 (dd, 1H, J = 10.2 Hz, J = 4.3 Hz, manOC*H*HCH$_2$), 3.47 (dd, 1H, J = 14.2 Hz, J = 6.2 Hz, H-6man), 3.04 (dd, 1H, J = 14.2 Hz, J = 4.7 Hz, H-6'man), 2.78 (dd, 1H, J = 15.1 Hz, J = 5.4 Hz, Hβ-asp), 2.69 (dd, 1H, J = 15.2 Hz, J = 7.3 Hz, Hβ'-asp), 1.49 (s, 9H, O*t*Bu) ppm; ^{13}C NMR (150.9 MHz, D$_4$-MeOH): δ = 170.92 (Fmoc-C=O), 170.63 (O*t*Bu-C=O), 156.92 (C-γ), 143.81, 141.15, 127.40, 126.79, 124.88, 119.54 (aryl-Fmoc), 100.28 (C-1man), 81.63 (O*C*(CH$_3$)$_3$), 74.78 (C-5man), 72.53 (C-3man), 72.05 (C-2man), 68.64 (C-4man), 68.12 (Fmoc-CH$_2$), 67.17 (manO*C*H$_2$CH$_2$), 66.71 (manOCH$_2$*C*H$_2$), 51.63 (C-α), 48.30 (CH-Fmoc), 40.35 (C-6), 38.55 (C-β), 28.21 (OC(*C*H$_3$)$_3$) ppm.

Table 3: Assignment of peptide fragment ion pattern according to Biemann [25]; left two columns: angiotensin; right two columns photo-crosslinked product of angiotensin and diazirine **2** (Figure 4).

MS-MS experiment with angiotensin II *m/z*	corresponding fragment	photo-crosslinking of angiotensin II and 2; MS-MS experiment *m/z*	corresponding fragment
1046.90	$[MH]^+$	1397.07	$[MH]^+$
931.59	y_7	1281.47	$[MH - D]^+$
899.56	$b_7 - H_2O$	1046.68	$[MH - carbene]^+$
881.54	b_7	969.50	$[a_5 + carbene]$
802.48	$b_6 - H_2O$		
784.48	b_6	784.47	b_6
767.47	$b_6 - NH_3$	756.45	a_6
756.48	a_6	619.40	a_5
619.41	a_5	517.30	$b_4 - NH_3$
534.32	b_4	486.54	[Y + carbene]
517.28	$b_4 - NH_3$	400.64	y_3
400.23	y_3		
354.21	$b_3 - NH_3$	354.21	$b_3 - NH_3$
343.25	a_3	343.26	a_3
272.18	b_2		
263.16	y_2	263.17	y_2
255.15	$b_2 - NH_3$	255.15	$b_2 - NH_3$
235.12	HP		
166.10	y_1		
136.11	Y	136.10	Y
110.10	H	110.11	H
87.11	R	72.12	V
70.09	P	70.01	P

MALDI-TOF-MS: *m/z* = 639.2 $[M + Na]^+$, 656.2 $[M + K]^+$; ESI-MS: *m/z* = 639.26 $[M + Na]^+$ (616.66 calcd. for $C_{31}H_{40}N_2O_{11}$).

N-(Fluoren-9-ylmethoxycarbonyl)-4-(*tert*-butyl ester)-L-aspartic acid [2-(α-D-mannopyranosyloxy) ethyl]-amide (8)

The aminoethyl mannoside **6** (200 mg, 0.90 mmol), HATU (320 mg, 0.90 mmol) and Fmoc- Fmoc-Asp(O*t*Bu)-OH (400 mg, 0.98 mmol) were dried under vacuum for 10 min and then dissolved in dry DMF (12 mL) under a nitrogen atmosphere. DIPEA (300 µL, 2.25 mmol) was added and the reaction mixture stirred for 18 h at RT. The solvent was removed in vacuo and the residue purified by flash chromatography (ethyl acetate:MeOH:H_2O = 6:2:1). Pure product fractions were pooled, filtered, concentrated and the residue taken up in water. Lyophylisation gave the title compound (536 mg, 0.87 mmol, 96%); R_f = 0.75 (ethyl acetate:MeOH:H_2O = 6:2:1).

^{1}H NMR (500 MHz, D_4-MeOH): δ = 7.82 (dd, 2H, *J* = 7.6 Hz, *J* = 3.5 Hz, aryl-Fmoc), 7.71 (t, 2H, *J* = 7.7 Hz, aryl-Fmoc), 7.42 (dd, 2H, *J* = 7.5 Hz, *J* = 3.4 Hz, aryl-Fmoc), 7.34 (dd, 2H, *J* = 6.9 Hz, *J* = 1.1 Hz, aryl-Fmoc), 4.81 (d, 1H, *J* = 1.4 Hz, H-1man), 4.52 (dd, 1H, *J* = 8.7 Hz, *J* = 5.2 Hz, Hα-asp), 4.48 (dd, 1H, *J* = 10.5 Hz, *J* = 7.0 Hz, Fmoc-CH*H*), 4.39 (dd, 1H, *J* = 10.7 Hz, *J* = 6.6 Hz, Fmoc-C*H*H), 4.27 (t, 1H, *J* = 7.0 Hz, Fmoc-CH), 3.88 (dd, 1H, *J* = 12.1 Hz, *J* = 2.2 Hz, manOCH$_2$CH*H*), 3.86 (dd, 1H, *J* = 1.4 Hz, *J* = 3.0 Hz, H-2man), 3.79 (dd, 1H, *J* = 10.7 Hz, *J* = 6.4 Hz, manOC*H*HCH$_2$), 3.73 (dd, 1H, *J* = 8.8 Hz, *J* = 3.0 Hz, H-3man), 3.75 (dd, 1H, *J* = 11.8 Hz, *J* = 5.8 Hz, manOCH$_2$C*H*H) 3.65 (t, 1H, *J* = 9.6 Hz, H-4man), 3.57 (ddd, 1H, *J* = 10.3 Hz, *J* = 7.1 Hz, *J* = 4.7 Hz, H-5man), 3.56 (dd, 1H, *J* = 10.8 Hz, *J* = 5.8 Hz, manOCH*H*CH$_2$), 3.48 (dd, 1H, *J* = 13.9 Hz, *J* = 7.6 Hz, H-6man), 3.42 (dd, 1H, *J* = 13.9 Hz, *J* = 5.0 Hz, H-6'man), 2.82 (dd, 1H, *J* = 16.1 Hz, *J* = 5.1 Hz, Hβ-asp), 2.60 (dd, 1H, *J* = 16.1 Hz, *J* = 8.9 Hz, Hβ'-asp), 1.47 (s, 9H, OC(CH$_3$)$_3$) ppm; ^{13}C NMR (150.92 MHz, D_4-MeOH): δ = 173.48 (O*t*Bu-C=O), 172.48 (Fmoc-C=O), 171.39 (NH-(C=O)$_{asp}$), 145.22, 142.59, 128.79, 128.17, 126.23, 120.91 (aryl-Fmoc), 101.64 (C-1man), 82.43 (O*C*(CH$_3$)$_3$), 74.67 (C-5man), 72.52 (C-3man), 72.05 (C-2man), 68.67 (C-4man), 68.19 (Fmoc-CH$_2$), 67.06 (manO*C*H$_2$CH$_2$), 62.84

(manOCH$_2$*C*H$_2$), 53.32 (C-α), 48.34 (CH-Fmoc), 40.42 (C-6man), 38.74 (C-β), 28.33 (C(*C*H$_3$)$_3$) ppm.

MALDI-TOF-MS: *m/z* = 639.2 [M + Na]$^+$; ESI-MS: *m/z* = 639.26 [M + Na]$^+$ (616.66 calcd. for $C_{31}H_{40}N_2O_{11}$).

N-(Fluoren-9-ylmethoxycarbonyl)-4-[2-(α-D-mannopyranosyloxy)ethyl]-1-[(+)-biotinylamidopropyl]-L-aspartic acid diamide (9)

The glycoamino acid **7** (220 mg, 0.36 mmol) was dissolved in 80% aq TFA (5 mL) and stirred at RT for 60 min to cleave the *tert*-butyl ester. When the deprotection reaction was complete (TLC control in ethyl acetate:MeOH:H$_2$O = 6:2:1) the solvent was removed in vacuo and the residue suspended in MeOH, filtered and the filtrate concentrated under reduced pressure. The resulting crude product was combined with HATU (150 mg, 0.43 mmol), (+)-biotinylamidopropylammonium trifluoroacetate (170 mg, 0.41 mmol) and dried for 30 min under vacuum. DMF (5 mL) and DIPEA (350 µL, 2.63 mmol) were then added under a nitrogen atmosphere. The reaction mixture was stirred overnight at RT, the solvent removed in vacuo and the residue purified by HPLC (MeCN-H$_2$O gradient: 0–5 min 100% H$_2$O, 5–20 min 60% H$_2$O, 20–30 min 40% H$_2$O, 30–40 min 0% H$_2$O, 40–50 min 0% H$_2$O, 50–60 min 100% H$_2$O). The title compound was obtained after lyophylisation (145 mg, 0.17 mmol, 48%); R_f = 0.47 (ethyl acetate:MeOH:H$_2$O = 6:2:1).

^{1}H NMR (500 MHz, CD$_3$CN:D$_2$O = 1:1): δ = 7.85 (d, 2H, *J* = 7.6 Hz, aryl-Fmoc), 7.71 (dd, 2H, *J* = 6.8 Hz, *J* = 4.2 Hz, aryl-Fmoc), 7.45 (t, 2H, *J* = 7.4 Hz, Fmoc-H), 7.37 (t, 2H, *J* = 7.5 Hz, *J* = 4.1 Hz, Fmoc-H), 4.80 (d, 1H, *J* = 1.6 Hz, H-1man), 4.51 (dd, 1H, *J* = 7.6 Hz, *J* = 5.8 Hz, biotin-NHC*H*CH$_2$S), 4.42 (dd, 1H, *J* = 11.8 Hz, *J* = 6.8 Hz, manOC*H*HCH$_2$), 4.38 (dd, 1H, *J* = 11.8 Hz, *J* = 7.3 Hz, manOCH*H*CH$_2$), 4.27 (d, 1H, *J* = 7.2 Hz, biotin-NHC*H*CHalkyl), 4.24 (t, 1H, *J* = 6.8 Hz, Hα-asp), 3.86 (dd, 1H, *J* = 3.4 Hz, *J* = 1.7 Hz, H-2man), 3.77 (dd, 1H, *J* = 12.1 Hz, *J* = 2.2 Hz, H-6man), 3.69 (dd, 1H, *J* = 9.6 Hz, *J* = 3.9 Hz. H-3man), 3.68 (dd, 1H, *J* = 12.2 Hz, *J* = 3.6 Hz, H-6'man), 3.65 (dd, 1H, *J* = 11.9 Hz, *J* = 6.9 Hz, Fmoc-CH*H*), 3.59 (t, 1H, *J* = 9.6 Hz, H-4man), 3.53 (ddd, 1H, *J* = 9.9 Hz, *J* = 5.5 Hz, *J* = 2.2 Hz, H-5man), 3.50 (dd, 1H, *J* = 11.2 Hz, *J* = 6.7 Hz, Fmoc-CH*H*), 3.41 (dd, 1H, *J* = 14.1 Hz, *J* = 5.3 Hz, manOCH$_2$CH*H*), 3.28 (ddd, 1H, *J* = 14.2 Hz, *J* = 5.7 Hz, manOCH$_2$C*H*H), 3.16 (dd, 1H, *J* = 9.3 Hz, *J* = 6.9 Hz, NH(C=O)C*H*H-CH$_2$-CH$_2$-CH$_2$-biotin), 3.15 (t, 1H, *J* = 7.4 Hz, biotin-NHCHC*H*alkyl), 3.12 (dd, 1H, *J* = 9.5 Hz, *J* = 6.6 Hz, NH(C=O)CH*H*-CH$_2$-CH$_2$-CH$_2$-biotin), 2.87 (dd, 1H, *J* = 12.8 Hz, *J* = 5.1 Hz, biotin-NHCHC*H*HS), 2.69 (dd, 1H, *J* = 12.9 Hz, *J* = 4.9 Hz, biotin-NHCHCH*H*S), 2.67 (dd, 1H, *J* = 13.7 Hz, *J* = 7.5 Hz, HN-C*H*H-CH$_2$-CH$_2$-NH), 2.59 (dd, 1H, *J* = 13.9 Hz, *J* = 7.8 Hz, HN-CH*H*-CH$_2$-CH$_2$-NH), 2.56 (dd, 1H, *J* = 14.2 Hz, *J* = 7.2 Hz, HN-CH$_2$-CH$_2$-C*H*H-NH), 2.53 (dd, 1H, *J* = 14.0 Hz, *J* = 6.8 Hz, HN-CH$_2$-CH$_2$-CH*H*-NH), 2.15 (t, 1H, *J* = 7.3 Hz, CH-Fmoc), 2.13 (d, 1H, *J* = 6.8 Hz, Hβ-asp), 2.11 (d, 1H, *J* = 6.5 Hz, Hβ'-asp), 1.66 (dd, 1H, *J* = 14.3 Hz, *J* = 7.6 Hz, NH(C=O)CH$_2$-CH$_2$-CH$_2$-C*H*H-biotin), 1.64 (dd, 1H, *J* = 13.9 Hz, *J* = 7.0 Hz, HN-CH$_2$-C*H*H-CH$_2$-NH), 1.62 (dd, 1H, *J* = 14.0 Hz, *J* = 7.2 Hz, HN-CH$_2$-CH*H*-CH$_2$-NH), 1.59 (dd, 1H, *J* = 14.5 Hz, *J* = 7.0 Hz, NH(C=O)CH$_2$-CH$_2$-CH$_2$-CH*H*-biotin), 1.54 (dd, 1H, *J* = 15.3 Hz, *J* = 8.0 Hz, NH(C=O)CH$_2$-CH$_2$-C*H*H-CH$_2$-biotin), 1.49 (dd, 1H, *J* = 15.3 Hz, *J* = 8.1 Hz, NH(C=O)CH$_2$-CH$_2$-CH*H*-CH$_2$-biotin), 1.33 (dd, 1H, *J* = 15.2 Hz, *J* = 7.5 Hz, NH(C=O)CH$_2$-C*H*H-CH$_2$-CH$_2$-biotin), 1.28 (dd, 1H, *J* = 15.0 Hz, *J* = 7.3 Hz, NH(C=O)CH$_2$-CH*H*-CH$_2$-CH$_2$-biotin) ppm; ^{13}C NMR (125.75 MHz; CD$_3$CN:D$_2$O = 1:1): δ = 180.86 (C-γ), 176.52 (C$_{asp}$(O)-NH-propyl), 173.26 (C$_{biotin}$(O)-NH-propyl), 172.17 ((NH)$_2$C=O), 165.62 (Fmoc-C=O), 144.81, 142.0, 128.93, 128.36, 126.25 (aryl-Fmoc), 100.73 (C-1man), 73.82 (C-5man), 71.68 (C-2man), 71.12 (C-3man), 67.86 (Fmoc-CH$_2$), 67.69 (C-4man), 66.75 (manO*C*H$_2$CH$_2$), 63.0 (biotin-NH*C*HCHalkyl), 62.0 (C-6man), 61.07 (biotin-NH*C*HCH$_2$S), 56.30 (biotin-NHCH*C*Halkyl), 53.14 (C-α) 47.81 (CH-Fmoc), 40.79 (biotin-CH$_2$), 39.89 (manOCH$_2$*C*H$_2$), 38.45, 37.64 (HN-*C*H$_2$-CH$_2$-*C*H$_2$-NH), 37.59 (C(O)-*C*H$_2$-CH$_2$-CH$_2$-CH$_2$-biotin), 36.52 (C-β), 29.29 (HN-CH$_2$-*C*H$_2$-CH$_2$-NH), 29.09 (C(O)-CH$_2$-CH$_2$-CH$_2$-*C*H$_2$-biotin), 28.80 (C(O)-CH$_2$-CH$_2$-*C*H$_2$-CH$_2$-biotin), 26.27 (C(O)-CH$_2$-*C*H$_2$-CH$_2$-CH$_2$-biotin) ppm.

MALDI-TOF-MS: *m/z* = 843.5 [M + H]$^+$, 865.4 [M + Na]$^+$, 881.4 [M + K]$^+$, (842.96 calcd. for $C_{40}H_{54}N_6O_{12}S$); ESI-MS: *m/z* = 865.44 [M + Na]$^+$ (842.96 calcd. for $C_{40}H_{54}N_6O_{12}S$).

N-(Fluoren-9-ylmethoxycarbonyl)-4-[(+)-biotinylamidopropyl]-1-[2-(α-D-mannopyranosyloxy)ethyl]-L-aspartic acid diamide (10)

The glycoamino acid **8** (250 mg, 0.41 mmol) was dissolved in 80% aq TFA (6 mL) and stirred at RT for 60 min to cleave the *tert*-butyl ester. When the deprotection reaction was complete (TLC control in ethyl acetate:MeOH:H$_2$O = 6:2:1) the solvent was removed in vacuo and the residue suspended in MeOH, filtered and the filtrate concentrated under reduced pressure. The resulting crude product was combined with HATU (98.1 mg, 0.28 mmol) and (+)-biotinylamidopropylammonium trifluoroacetate (109 mg, 0.26 mmol), and dried for 30 min under vacuum. DMF (4 mL) and DIPEA (220 µL, 1.65 mmol) were then added under a nitrogen atmosphere. The reaction mixture was stirred overnight at RT, the solvent removed in vacuo and the residue purified by HPLC (MeCN-H$_2$O gradient: 0–5 min 100% H$_2$O, 5–20 min 60% H$_2$O, 20–30 min 40% H$_2$O,

30–40 min 0% H_2O, 40–50 min 0% H_2O, 50–60 min 100% H_2O). The title compound was obtained after lyophylisation (90.4 mg, 0.11 mmol, 26%); R_f = 0.54 (ethyl acetate:MeOH:H_2O = 6:2:1).

^{1}H NMR (600 MHz, CD_3CN:D_2O, 1:1): δ = 7.85 (d, 2H, *J* = 7.6 Hz, Fmoc-H), 7.67 (d, 2H, *J* = 7.5 Hz, Fmoc-H), 7.45 (t, 2H, *J* = 7.4 Hz, Fmoc-H), 7.38 (td, 2H, *J* = 7.5 Hz, *J* = 0.9 Hz, Fmoc-H), 4.77 (d, 1H, *J* = 1.7 Hz, H-1man), 4.45 (dd, 1H, *J* = 6.7 Hz, *J* = 4.5 Hz, biotin-NHC*H*CH_2S), 4.43 (t, 1H, *J* = 6.8 Hz, Hα-asp), 4.40 (dd, 1H, *J* = 12.2 Hz, *J* = 5.5 Hz, Fmoc-C*H*H), 4.37 (dd, 1H, *J* = 12.0 Hz, *J* = 5.3 Hz, Fmoc-CH*H*), 4.29 (dd, 1H, *J* = 5.5 Hz, *J* = 4.4 Hz, CH-Fmoc), 4.27 (dd, 1H, *J* = 6.5 Hz, *J* = 4.7 Hz, biotin-NHC*H*CHalkyl), 3.83 (dd, 1H, *J* = 3.2 Hz, *J* = 1.6 Hz, H-2man), 3.77 (dd, 1H, *J* = 12.2 Hz, J = 6.5 Hz, manOC*H*HCH_2), 3.69 (dd, 1H, *J* = 9.7 Hz, *J* = 3.2 Hz, H-3man), 3.66 (dd, 1H, *J* = 12.1 Hz, *J* = 6.9 Hz, manOCH*H*CH_2), 3.64 (dd, 1H, *J* = 12.0 Hz, *J* = 5.3 Hz, H-6man), 3.58 (t, 1H, *J* = 9.8 Hz, H-4man), 3.51 (ddd~dd, 1H, *J* = 9.8 Hz, *J* = 5.5 Hz, H-5man), 3.50 (dd, 1H, *J* = 11.9 Hz, *J* = 5.6 Hz, H-6'man), 3.43 (dd, 1H, *J* = 14.4 Hz, *J* = 6.5 Hz, manOCH_2CH*H*), 3.33 (dd, 1H, *J* = 13.9 Hz, *J* = 6.1 Hz, manOCH_2C*H*H), 3.17 (td, 1H, *J* = 7.1 Hz, *J* = 6.9 Hz, biotin-NHCHC*H*alkyl), 3.12, 3.10 (each dd, each 1H, HN-CH_2-CH_2-CH_2-NH), 3.09, 3.07 (each dd, each 1H, NH(C=O)CH_2-CH_2-CH_2-CH_2-biotin), 2.66, 2.54 (each dd, each 1H, HN-CH_2-CH_2-CH_2-NH), 2.11, 2.09 (each dd, each 1H, 2Hβ-asp), 1.61, 1.58 (each dd, each 1H, HN-CH_2-CH_2-CH_2-NH), 1.52 (dd, 1H, *J* = 13.9 Hz, *J* = 6.7 Hz, NH(C=O)CH_2-CH_2-CH_2-C*H*H-biotin), 1.50 (dd, 1H, *J* = 14.0 Hz, *J* = 6.8 Hz, NH(C=O)CH_2-CH_2-C*H*H-CH_2-biotin), 1.48 (dd, 1H, *J* = 13.9 Hz, *J* = 7.2 Hz, NH(C=O)CH_2-CH_2-CH_2-CH*H*-biotin), 1.45 (dd, 1H, *J* = 14.2 Hz, *J* = 7.0 Hz, NH(C=O)CH_2-CH_2-CH*H*-CH_2-biotin), 1.30, 1.27 (each dd, each 1H, NH(C=O)CH_2-CH_2-CH_2-CH_2-biotin) ppm; ^{13}C NMR (150.92 MHz, CD_3CN:D_2O = 1:1): δ = 175.93 (NH-$(C=O)_{asp}$), 172.84 (NH-*C*(O)-$(CH_2)_4$-biotin), 171.53 (C-γ), 165.08 ($(NH_2)_2$C=O), 157.25 (Fmoc-C=O), 144.24, 144.19, 129.51, 128.51, 128.40, 127.83 (aryl-Fmoc), 100.1 (C-1man), 73.22 (C-5man), 71.12 (C-3man), 68.07 (C-2man), 67.38 (Fmoc-CH_2), 67.13 (C-4man), 66.01 (C-6man), 62.25 (biotin-NH*C*HCHalkyl), 61.36 (manO*C*H_2CH_2), 60.50 (biotin-NH*C*HCH_2S), 57.67 (biotin-NHCH*C*Halkyl), 52.61 (C-α), 47.22 (Fmoc-CH), 39.35 (biotin-NHCH*C*H_2S), 38.36 (manOCH_2*C*H_2), 37.07, 36.84 (HN-*C*H_2-CH_2-*C*H_2-NH), 35.95 (C(O)-*C*H_2-CH_2-CH_2-CH_2-biotin), 28.69 (C-β), 28.52 (HN-CH_2-*C*H_2-CH_2-NH), 28.22 (C(O)-CH_2-CH_2-CH_2-*C*H_2-biotin), 25.66 (C(O)-CH_2-CH_2-*C*H_2-CH_2-biotin), 25.53 (C(O)-CH_2-*C*H_2-CH_2-CH_2-biotin) ppm.

MALDI-TOF-MS: *m/z* = 865.3 $[M + Na]^+$; ESI-MS: *m/z* = 865.34 $[M + Na]^+$ (842.96 calcd. for $C_{40}H_{54}N_6O_{12}S$).

N-[*p*-(Trifluoromethyl-diazirinyl)-benzoyl]-4-[2-(α-D-mannopyranosyloxy)ethyl]-1-[(+)-biotinylamido-propyl]-L-aspartic acid diamide (12)

The glycoamino acid derivative **9** (25.0 mg, 29.7 μmol) was dissolved in piperidine (20% in DMF, 3.0 mL) and stirred at RT for 60 min to remove the Fmoc protecting group. The solvent was removed in vacuo and the resulting crude product combined with HATU (11.3 mg, 29.7 μmol) and the diazirine **11** (7.2 mg, 31.0 μmol), and dry DMF (1.5 mL) added under a nitrogen atmosphere. DIPEA (0.03 mL, 87.0 μmol) was then added and the reaction mixture stirred overnight at RT. The solvent was removed under reduced pressure and the residue purified by flash chromatography with the exclusion of light (ethyl acetate:MeOH:H_2O = 6:2:1) to yield, after lyophilisation, the title compound (20.1 mg, 24.1 μmol, 81%); R_f = 0.51; $[\alpha]_D^{20}$ +23.0 (*c* 0.12 mM, MeCN:H_2O = 1:1); UV–Vis (*c* 0.12 mM, MeCN:H_2O = 1:1): $\lambda_{max}(1)$ = 353.3 nm, ε(1) = 6000 $Lmol^{-1}cm^{-1}$; $\lambda_{max}(2)$ = 303.5 nm, ε(2) = 10000 $Lmol^{-1}cm^{-1}$; $\lambda_{max}(3)$ = 285.2 nm, ε(3) = 24000 $Lmol^{-1}cm^{-1}$; $\lambda_{max}(4)$ = 251.6 nm, ε(4) = 37000 $Lmol^{-1}cm^{-1}$; FT-IR (KBr): $\tilde{\nu}$ = 3447.5 cm^{-1}, 2928.6 cm^{-1}, 2381.0 cm^{-1}, 1654.2 cm^{-1}, 1560.1 cm^{-1}, 1267.9 cm^{-1}, 1136.9 cm^{-1}, 1101.9 cm^{-1}, 1059.5 cm^{-1}, 767.9 cm^{-1}.

^{1}H NMR (600 MHz, CD_3CN:D_2O = 1:1): δ = 7.90 (dt, 2H, *J* = 8.7 Hz, *J* = 2.0 Hz, aryl-H), 7.39 (d, 2H, *J* = 8.2 Hz, aryl-H), 4.81 (dd, 1H, *J* = 7.7 Hz, *J* = 6.1 Hz, Hα-asp), 4.75 (d, 1H, *J* = 1.6 Hz, H-1man), 4.50 (dd, 1H, *J* = 7.9 Hz, *J* = 4.9 Hz, biotin-NH_2C*H*CHalkyl), 4.31 (dd, 1H, *J* = 7.9 Hz, *J* = 4.5 Hz, biotin-NH_2C*H*CH_2S), 3.82 (dd, 1H, *J* = 3.4 Hz, *J* = 1.7 Hz, H-2man), 3.76 (dd, 1H, *J* = 12.1 Hz, *J* = 2.2 Hz, manOC*H*HCH_2), 3.69 (dd, 1H, *J* = 9.3 Hz, *J* = 3.4 Hz, H-3man), 3.67 (dd, 1H, *J* = 12.0 Hz, *J* = 2.4 Hz, manOCH*H*CH_2), 3.58 (t, 1H, *J* = 9.8 Hz, H-4man), 3.52 (ddd, 1H, *J* = 9.8 Hz, *J* = 5.3 Hz, *J* = 2.2 Hz, H-5man), 3.49 (dd, 1H, *J* = 5.5 Hz, *J* = 4.2 Hz, H-6man), 3.42 (dd, 1H, *J* = 5.7 Hz, *J* = 2.3 Hz, H-6'man), 3.39 (dd, 1H, *J* = 6.7 Hz, *J* = 12.3 Hz, manOCH_2CH*H*), 3.30 (dd, 1H, *J* = 6.4 Hz, *J* = 12.3 Hz, manOCH_2C*H*H), 3.27, 3.24 (each dd, each 1H, HN-CH_2-CH_2-CH_2-NH), 3.20 (dd, 1H, *J* = 7.7 Hz, *J* = 6.8 Hz, biotin-NH_2CHC*H*alkyl), 3.13, 3.12 (each dd, each 1H, NH(C=O)CH_2-CH_2-CH_2-CH_2-biotin), 2.91 (dd, 1H, *J* = 13.0 Hz, *J* = 5.0 Hz, biotin-*N*H_2CHC*H*HS), 2.82, 2.72 (each dd, each 1H, HN-CH_2-CH_2-CH_2-NH), 2.70 (dd, 1H, *J* = 13.1 Hz, *J* = 6.4 Hz, biotin-NH_2CHCH*H*S), 2.16, 2.15 (each dd, each 1H, NH(C=O)CH_2-CH_2-CH_2-CH_2-biotin), 1.68, 1.67 (each dd, each 1H, NH(C=O)CH_2-CH_2-CH_2-CH_2-biotin), 1.63 (dd, 1H, *J* = 13.3 Hz, *J* = 6.7 Hz, NH(C=O)CH_2-C*H*H-CH_2-CH_2-biotin), 1.57 (dd, 1H, *J* = 15.1 Hz, *J* = 7.4 Hz, HN-CH_2-C*H*H-CH_2-NH), 1.53 (dd, 1H, *J* = 13.1 Hz, *J* = 6.9 Hz, NH(C=O)CH_2-CH*H*-CH_2-CH_2-biotin), 1.51 (dd, 1H, *J* = 13.9 Hz, *J* = 6.8 Hz, Hβ-asp), 1.36 (dd, 1H, *J* = 15.5 Hz, *J* = 7.2 Hz, HN-CH_2-CH*H*-

CH_2-NH), 1.30 (dd, 1H, J = 13.4 Hz, J = 6.9 Hz, Hβ'-asp) ppm; ^{13}C NMR (150.90 MHz, CD_3CN:D_2O = 1:1): δ = 176.60 (aryl-C=O), 172.78 (NH-(C=O)$_{asp}$), 172.37 (C-γ), 168.61 (NH-*C*(O)-CH_2-$(CH_2)_3$-biotin), 165.66 ($(NH_2)_2$C=O), 135.57, 133.14, 129.11, 127.73 (aryl-C), 124.44 (q, $J_{C,F}$ = Hz, CF_3), 100.72 (C-1man), 87.77 (N=N-*C*CF_3) 73.82 (C-5man), 71.68 (C-3man), 71.10 (C-2man), 67.70 (C-4man), 66.73 (C-6man), 62.81 (biotin-NH_2*C*HCHalkyl), 61.95 (manO*C*H_2CH_2), 61.09 (biotin-NH_2*C*HCH_2S), 56.29 (biotin-NH_2CH*C*Halkyl), 52.36 (C-α), 40.78 (biotin-NH_2CH*C*H_2S), 39.89 (manOCH_2*C*H_2), 38.13 (HN-*C*H_2-CH_2-CH_2-NH), 37.68 (HN-CH_2-CH_2-*C*H_2-NH), 37.36 (NH-C(O)-*C*H_2-$(CH_2)_3$-biotin), 36.49 (C-β), 29.71 (HN-CH_2-*C*H_2-CH_2-NH), 29.24 (NH-C(O)-$(CH_2)_3$-*C*H_2-biotin), 28.78 (NH-C(O)-CH_2-CH_2-*C*H_2-CH_2-biotin), 26.24 (NH-C(O)-CH_2-*C*H_2-CH_2-CH_2-biotin) ppm.

MALDI-TOF-MS: m/z = 827.3 $[M - N_2 + Na]^+$; ESI-MS: m/z = 855.29 $[M + Na]^+$, 827.29 $[M - N_2 + Na]^+$ (832.85 calcd. for $C_{34}H_{47}F_3N_8O_{11}S$).

N-[*p*-(Trifluoromethyl-diazirinyl)-benzoyl]-4-[(+)-biotinylamidopropyl]-1-[2-(α-D-mannopyranosyloxy)-ethyl]-L-aspartic acid diamide (13)

The glycoamino acid derivative **10** (19.6 mg, 23.2 μmol) was dissolved in piperidine (20% in DMF, 1.5 mL) and stirred at RT for 60 min to remove the Fmoc protecting group. The solvent was removed in vacuo and the resulting crude product combined with HATU (14.0 mg, 36.8 μmol) and the diazirine **11** (7.2 mg, 30.9 μmol), and dry DMF (1.5 mL) added under a nitrogen atmosphere. DIPEA (0.05 mL, 145 μmol) was then added and the reaction mixture stirred overnight at RT. The solvent was removed under reduced pressure and the residue purified by flash chromatography with the exclusion of light (ethyl acetate:MeOH:H_2O = 6:2:1) to yield, after lyophilisation, the title compound (19 mg, 22.8 μmol, 98%); R_f = 0.33; $[\alpha]_D^{20}$ +30.0 (*c* 0.12 mM, MeCN:H_2O = 1:1); UV-Vis (*c* 0.12 mM, MeCN:H_2O = 1:1): $\lambda_{max}(1)$ = 335.9 nm, ε(1) = 9833.3 $Lmol^{-1}cm^{-1}$; $\lambda_{max}(2)$ = 278.2 nm, ε(2) = 18333.3 $Lmol^{-1}cm^{-1}$; $\lambda_{max}(3)$ = 233.5 nm, ε(3) = 23333.3 $Lmol^{-1}cm^{-1}$; FT-IR (KBr): $\tilde{\nu}$ = 3447.7 cm^{-1}, 2940.5 cm^{-1}, 2380.9 cm^{-1}, 1684.7 cm^{-1}, 1654.2 cm^{-1}, 1636.6 cm^{-1}, 1560.1 cm^{-1}, 1438.3 cm^{-1}, 1400.2 cm^{-1}, 1279.8 cm^{-1}, 1202.4 cm^{-1}, 1113.1 cm^{-1}, 761.9 cm^{-1}.

^{1}H NMR (600 MHz, CD_3CN-D_2O, 1:1): δ = 8.23 (dd, 2H, J = 8.5 Hz, J = 1.4 Hz, aryl-H), 7.38 (dd, 2H, J = 8.8 Hz, J = 1.3 Hz, aryl-H), 4.86 (dd, 1H, J = 8.6 Hz, J = 5.3 Hz, Hα-asp), 4.76 (d, 1H, J = 1.5 Hz, H-1man), 4.49 (dd, 1H, J = 7.7 Hz, J = 4.4 Hz, biotin-NHC*H*CH_2S), 4.30 (dd, 1H, J = 8.0 Hz, J = 4.4 Hz, biotin-NHC*H*CHalkyl), 3.80 (dd, 1H, J = 3.3 Hz, J = 1.7 Hz, H-2man), 3.73 (dd, 1H, J = 12.2 Hz, J = 2.3 Hz, manOC*H*HCH_2), 3.69 (dd, 1H, J = 11.0 Hz, J = 4.3 Hz, H-6man), 3.68 (dd, 1H, J = 9.7 Hz, J = 3.2 Hz, H-3man), 3.66 (dd, 1H, J = 12.2 Hz, J = 3.3 Hz, manOCH*H*CH_2), 3.59 (t, 1H, J = 9.8 Hz, H-4man), 3.52 (dd, 1H, J = 10.6 Hz, J = 4.4 Hz, H-6'man), 3.49 (dd, 1H, J = 10.0 Hz, J = 4.1 Hz, H-5man), 3.44 (dd, 1H, J = 6.8 Hz, J = 4.1 Hz, manOCH_2C*H*H), 3.32 (dd, 1H, J = 6.1 Hz, J = 4.0 Hz, manOCH_2CH*H*), 3.20 (dd, 1H, J = 8.8 Hz, J = 5.4 Hz, biotin-NHCHC*H*alkyl), 3.19 (dd, 1H, J = 13.7 Hz, J = 6.9 Hz, HN-C*H*H-CH_2-CH_2-NH), 3.16, 3.10 (each dd, each 1H, NH(C=O)CH_2-CH_2-CH_2-CH_2-biotin), 3.05 (dd, 1H J = 13.4 Hz, J = 7.0 Hz, HN-CH*H*-CH_2-CH_2-NH), 2.90 (dd, 1H, J = 13.0 Hz, J = 5.1 Hz, biotin-NHCHCH*H*S), 2.77 (dd, 1H, J = 14.7 Hz, J = 5.3 Hz, HN-CH_2-CH_2-C*H*H-NH), 2.69 (dd, 1H, J = 12.6 Hz, J = 4.8 Hz, biotin-NHCHC*H*HS), 2.68 (dd, 1, J = 14.9 Hz, J = 5.8 Hz, HN-CH_2-CH_2-CH*H*-NH), 2.16 (dd, 1H, J = 15.3 Hz, J = 7.7 Hz, Hβ-asp), 2.12 (dd, 1H, J = 14.9 Hz, J = 7.5 Hz, Hβ'-asp), 1.66 (ddd, 1H, J = 13.8 Hz, J = 7.1 Hz, J = 6.2 Hz, NH(C=O)CH_2-CH*H*-CH_2-CH_2-biotin), 1.64 (dd, 1H, J = 15.3 Hz, J = 6.2 Hz, NH(C=O)CH_2-CH_2-CH*H*-CH_2-biotin), 1.57 (ddd, 1H, J = 13.3 Hz, J = 6.8 Hz, J = 6.3 Hz, NH(C=O)CH_2-C*H*H-CH_2-CH_2-biotin), 1.55 (dd, 1H, J = 15.5 Hz, J = 6.8 Hz, NH(C=O)CH_2-CH_2-C*H*H-CH_2-biotin), 1.53, 1.52 (each dd, each 1H, NH(C=O)CH_2-CH_2-CH_2-CH_2-biotin), 1.34, 1.27 (each dd, each 1H, J = 15.3 Hz, J = 7.0 Hz, HN-CH_2-CH_2-CH_2-NH) ppm; ^{13}C NMR (150.90 MHz, CD_3CN:D_2O = 1:1): δ = 176.86 (C-γ), 172.58 (NH-(C=O)$_{asp}$), 171.47 (NH(*C*=O)-$(CH_2)_4$-biotin), 168.28 (aryl-C=O), 165.16 ($(NH_2)_2$C=O), 149.11, 139.43 (aryl-C), 136.19 (q, $J_{C,F}$ = Hz, CF_3), 129.08, 128.99 (aryl-C), 100.67 (C-1man), 84.21 (N=N-*C*CF_3), 73.78 (C-5man), 71.69 (C-2man), 71.12 (C-3man), 67.74 (C-4man), 66.57 (C-6man), 62.85 (biotin-NH*C*HCHalkyl), 61.92 (manO*C*H_2CH_2), 61.09 (biotin-NH*C*HCH_2S), 56.32 (biotin-NHCH*C*Halkyl), 52.34 (C-α), 40.78 (biotin-NHCH*C*H_2S), 39.97 (manOCH_2*C*H_2), 38.47, 37.63 (HN-*C*H_2-CH_2-*C*H_2-NH), 37.39 (NH(C=O)*C*H_2-$(CH_2)_3$-biotin), 36.50 (C-β), 29.21 (C- HN-CH_2-*C*H_2-CH_2-NH), 29.08 (NH(C=O)-$(CH_2)_3$-*C*H_2-biotin), 28.79, 26.23 (NH(C=O)-CH_2-*C*H_2-*C*H_2-CH_2-biotin) ppm.

MALDI-TOF-MS: m/z = 855.3 $[M + Na]^+$; ESI-MS: m/z = 827.31 $[M - N_2 + Na]^+$; 855.31 $[M + Na]^+$ (832.85 calcd. for $C_{34}H_{47}F_3N_8O_{11}S$).

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Design and synthesis of a cyclitol-derived scaffold with axial pyridyl appendages and its encapsulation of the silver(I) cation

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Letter

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Abstract

Conversion of a *myo*-inositol derivative into a *scyllo*-inositol-derived scaffold with C_{3v} symmetry bearing three axial pyridyl appendages is presented. This pre-organized hexadentate ligand allows complexation of silver(I). The crystal structure of the complex was established.

Introduction

Despite its recognized potential for nuclear medicine applications [1], the β^- emitter ^{111}Ag ($t_{1/2}$ = 7.47 d), which can be produced carrier-free and with high specific activity [2,3], has not yet found widespread uses [4,5]. This is due in part to the lack of suitable Ag complexing agents and in this work the synthesis of an cyclitol-derived scaffold designed to sequester Ag is presented. Interaction of *myo*-inositol-derived podands [6] and crown ethers [7] with Ag salts has been shown to depend on the relative spatial orientation of the binding sites moreover, in mono-orthoesters of *scyllo*-inositol the three axial hydroxy groups can be used to link substituents in a pre-organized manner [8-10]. Thus the introduction of pyridine groups (known to bind Ag(I) efficiently [11-14]) on a *scyllo*-inositol orthoester was considered, which led to the design of scaffold **1** (Figure 1). Indeed, upon complexation with participation of three oxygen atoms in the binding, no substantial rearrangement of this semi-rigid structure is to be expected. In addition, reductive cleavage of one of the ortho-ester C–H benzylic bonds, which is known to yield a free hydroxy group [15,16], would allow ligation of this scaffold.

Results and Discussion

Chemistry

The synthesis of **1** was accomplished in 6 steps from the readily available *myo*-inositol orthobenzoate (**2**) [17] (Figure 2). As *scyllo*-inositol derivatives are derived from *myo*-inositols by an

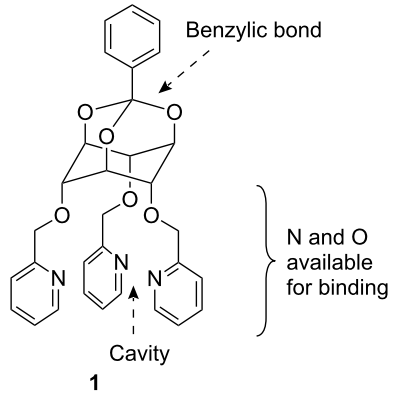


Figure 1: spatial representation of structure **1**.

oxidation/reduction sequence [8,18,19], selective protection (*tert*-butyldimethylsilyl chloride, 2,6-lutidine, DMF, 80 °C, 2 days) of the equatorial hydroxy group of **2** was first performed, to give the silyl ether **3** (70%) and subsequent picolylation (NaH, 2-picolyl chloride, DMF) of the remaining free hydroxy groups gave **4** (65%). The equatorial hydroxy group then was deblocked (tetrabutylammonium fluoride in THF) to afford **5** (86%). Inversion of configuration was accomplished via Swern oxidation [20] to furnish ketone **6** (95%), which was then reduced ($NaBH_4$, CH_3OH) to **7** (99%). Although a configurational assignment did not prove possible from ^{1}H NMR data (in epimers **5** and **7** the dihedral angles of the proton geminal to the hydroxy group with vicinal protons are similar), the ^{1}H- and ^{13}C NMR spectra of **7** clearly differ from those of its epimer. Compounds **5** and **7** were also acetylated (to **8** and **9**, respectively) and the chemical shift of the acetyl methyl group of **9** (δ = 1.7 ppm) displayed an upfield shift compared to that of **8** (δ = 2.1 ppm) due to ring-current effects from through-space interactions with the pyridyl groups. That a *scyllo*-inositol derivative had been obtained was confirmed after the introduction of the third pyridyl group (NaH, 2-picolyl chloride). In the resulting tri-picolyl derivative **1** (71%) a C_{3v} axis of symmetry is now present, which leads to simplification of NMR spectra. The structure of **1** was subsequently confirmed (see below) by X-ray diffraction of a single crystal of its Ag-complex.

Complexation

Incremental addition of silver(I) trifluoromethanesulfonate to a solution of **1** in 1:1 $CD_3OD/CDCl_3$ was monitored by NMR. A single set of resonances was observed at all concentrations. That the pyridine rings and the "oxygenated cavity" were involved in silver binding was shown by the relevant shifts in both the ^{1}H- and ^{13}C-NMR spectra (see Supporting Information File 1). However, in the absence of characteristic signals for Ag(I)-complex (even at low temperature: −50 °C) NMR methods cannot be used for the determination of the complex stability

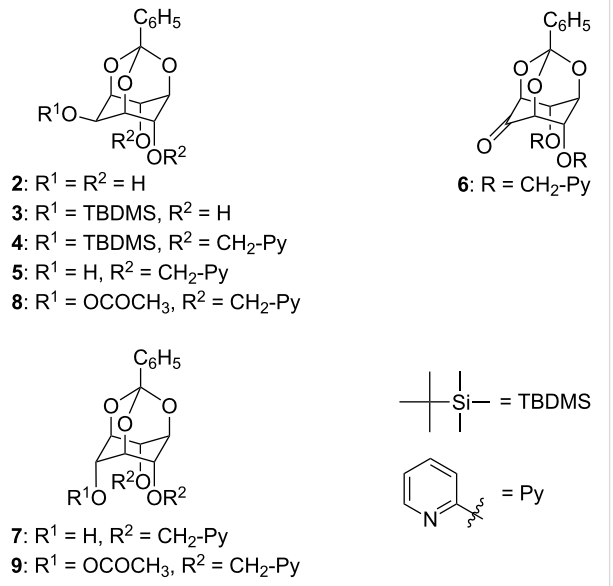


Figure 2: structures of compounds **2**–**9**.

constant. The electrospray mass spectrum (from the very solution contained in the NMR tube) revealed a 1:1 complex, with the expected ^{107}Ag/^{109}Ag isotopic ratio. The stability constant of the complex could be determined by spectrophotometric titration, which was carried out in 1:1 aqueous methanol (the complex is not water soluble) and its value (log $K_{\mathbf{1}\cdot Ag}$ = 3.8 ± 0.2) is in agreement with that of a previously described Ag(I) complex in a N_3O_3 environment [13].

Study of the molecular structure of the complex was made possible as a single crystal could be grown by slow evaporation of a methanol/ethanol solution of stoichiometric amounts of **1** and silver trifluoromethanesulfonate. The complex crystallises in the triclinic system, with a pseudo-ternary axis of symmetry. Its coordination sphere involves all 3 nitrogen atoms as well as the 3 oxygen atoms which are implied in the linkage of the pyridines (Figure 3). Bond distances and angles (see Supporting Information File 1) are in accord with those of known silver complexes which are hexa-coordinated with pyridyl groups and oxygen atoms [13,14].

Conclusion

A rigid scaffold with C_{3v} symmetry bearing three appendages in a pre-organized manner, **1**, could be synthesized in 6 steps from *myo*-inositol orthobenzoate and in 25% overall yield. Noteworthy is that the synthetic scheme may allow variation of heterocyclic substituents, which need not be the same since they are introduced at different stages of the synthesis. The complex formed with silver cation (log $K_{1\cdot Ag}$ = 3.8 ± 0.2) was shown to involve N_3O_3 coordination with the Ag(I) ion lying within the

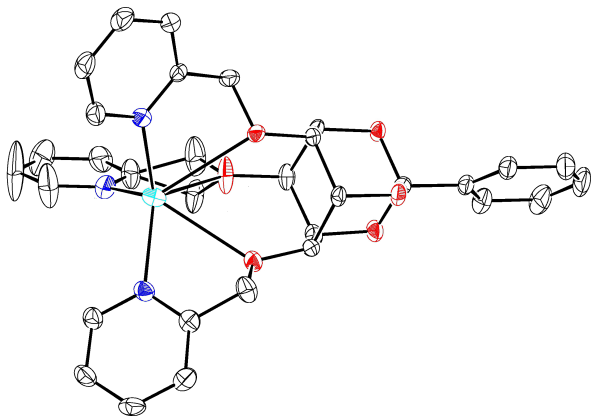


Figure 3: ORTEP drawing for the **1**·Ag(I) cation complex (ellipsoids are drawn at the 50% probability level and H atoms are omitted for clarity).

cage. However, the complex is not water soluble (which precludes its use for biomedical applications) although the grafting of hydrophilic appendages to aromatic residues could increase its hydrophilicity.

Supporting Information

Supporting Information features experimental procedures, ^{1}H NMR and ^{13}C NMR spectra; electrospray mass spectrum and the crystal structure of the Ag complex.

Supporting Information File 1

Synthesis and characterisation data for compounds **1**–**9** and **1**·Ag(I).
[http://www.beilstein-journals.org/bjoc/content/supplementary/1860-5397-6-115-S1.pdf]

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