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Preparation of sialylated oligosaccharides employing recombinant trans-sialidase from *Trypanosoma cruzi*

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Terminally sialylated oligosaccharides were synthesised employing recombinant trans-sialidase from *Trypanosoma cruzi*. Regio- and stereoselectively Sia-α(2–3)-Gal-βR derivatives could be obtained in respectable yields, using combined chemical and enzymatic methodologies. An array of different disaccharide precursors such as Gal-β(1–3)-GalNAc-αSer/Thr, lactosides and lactosamide derivatives were sialylated and successfully purified by facile isolation procedures. Depending on the acceptor structure isolated, yields for trans-sialylation products were between 20 and 60%.

Introduction

Neuraminic acid (Neu5Ac) plays an important role in nature as a major constituent of a variety of glycoconjugates (such as oligosaccharides, glycoproteins and gangliosides) occurring in animals and several pathogens.[1] The terminal position of neuraminic acid in these glycoconjugates implies that it exerts a significant influence in cell biology.[2] The biology of Neu5Ac is dominated by its dual role in either masking recognition sites or, in contrast, as a biological target, recognised by a receptor protein.[3,4] Most frequently, neuraminic acid is found attached α(2–3) or α(2–6) to galactose or *N*-acetyl-galactosamine in oligosaccharide scaffolds which constitute the glycocalyx. Further, Neu5Ac is also found attached α(2–8) and α(2–9) to Neu5Ac units to form polysialylic acids (PSA).

There is a demand for alternative strategies towards regio- and stereoselective syntheses of sialylated oligosaccharides in sufficient quantities required to investigate structure–function relationships. Isolation from natural sources appears to be less effective and excludes access to non-natural derivatives.

In recent years, the interest in novel syntheses of structurally-defined sialylated oligosaccharides has increased. Despite considerable progress, classical methods still lack experimental simplicity and often stereospecificity.[5] As an alternative, various enzymes were introduced as synthetic tools to prepare stereo- and regiospecifically sialylated glycostructures. Several sialyltransferases and sialidases were employed in this chemoenzymatic approach, although some disadvantages associated with the method remain unresolved.

Sialyltransferases were used for the synthesis of a wide range of complex sialyl oligosaccharides in excellent yields.[6–8] They became the enzymes of choice for the synthesis of sialylated oligosaccharides, despite their complex donor selectivity and the modest availability of the donor CMP-Neu5Ac.

The application of the reversible nature of glycosidases in the synthesis of various oligosaccharides presents a facile glycosylation procedure.[9,10] Sialidases from several sources were exploited in trans-sialylations since their acceptance of artificial donor glycosides such as pNP-αNeu5Ac and MU-αNeu5Ac has been recognized.[11–13] In contrast to transferase-mediated reactions, this pathway lacks comparable yields, generally barely exceeding 30%, depending on the type and source of the enzyme.

The gap between both types of enzymes is closed by a rare but efficient enzyme, exhibiting only transferase character in the presence of a suitable acceptor. Herein, we report the application of trans-sialidase (TS) from *Trypanosoma cruzi* to the preparation of α(2–3)-linked sialylated oligosaccharides.

Trans-sialidase from *Trypanosoma cruzi*

According to WHO data *Trypanosoma cruzi* (*T. cruzi*), the pathogen responsible for Chagas disease, causes up to 18 Mio infections and 50.000 deaths per year. Upon infection the parasitic trans-sialidase catalyzes an unusually efficient sialyltransferase reaction, scavenging Neu5Ac molecules from sialo glycoconjugates in the external milieu of the host's glycocalix to sialylate its own surface mucins and thus mask its own epitopes.[14–17]

Due to the importance of TS during the infection, its mechanistic aspects are still under investigation in order to better understand the pathogenic properties and pathways finally expected to lead to suitable inhibitors, as well as the development of prophylactic or therapeutic agents against *T. cruzi*.

Based on its primary structure, TS belongs to glucosidase family 33, which also includes multiple bacterial sialidases.[18–20] In contrast to these various sialidases, no suitable TS inhibitor is available so far, and several investigations were performed to improve the understanding of the catalytic mechanism of its transferase activity.[21–23] Trans-sialidase from *T. cruzi* is closely related to the sialidase from *Trypanosoma brucei* (*T. brucei*), which exhibits a similar amino acid sequence of the globular core (70% homology). The latter enzyme was used as a model to study the nature of the binding pocket and the transglycosylation mechanism.[21–23] The latest kinetic and crystallographic studies support this assumption regarding the mechanism, and emphasize the importance of the amino acid Tyr342, which participates in a covalent glycosyl–enzyme interaction.[23]

The present work is intended to demonstrate that the alternative TS from *T. cruzi* is eminently suitable for the highly regio- and stereoselective synthesis of α(2–3)-linked sialylated oligosaccharides containing a preterminal Galβ-R residue. Depending on the choice of acceptors, the accessible oligosaccharides can vary between naturally occurring and artificial structures.

Results and discussion

According to the donor specificity of trans-sialidase, non-natural sialyl donor substrates, such as pNP-αNeu5Ac (**1**) and MU-αNeu5Ac (**2**), could be used and led to α(2–3)-linked sialylated oligosaccharides. Despite the much lower activity of

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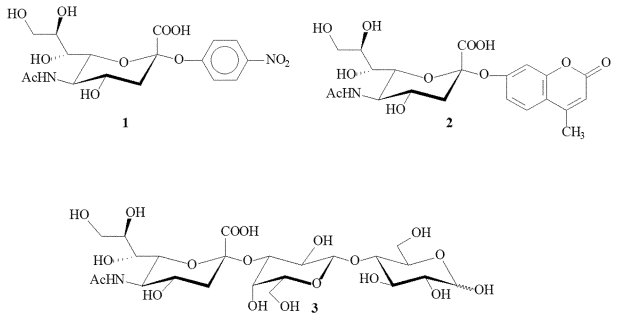
pNP-αNeu5Ac and MU-αNeu5Ac compared to Neu5Acα(2–3)Galβ(1–4)Glc (**3**, sialyl lactose), both phenolic donor glycosides could be used for the efficient synthesis of sialylated oligosaccharides. Due to the amphiphilic character of sialyl lactose the reaction reached an equilibrium of sialyl lactose and product. In contrast, the non-natural donor structures possess the advantage that strict kinetic control was not necessary, since the free phenolic compounds pNP-OH and MU-OH are not suitable acceptors[24] (Scheme 1). pNP-αNeu5Ac and MU-αNeu5Ac were synthesised by phase transfer glycosylation in acceptable overall yields of about 40 to 50%.[25]



Scheme 1 Donor substrates: pNP-αNeu5Ac (**1**), MU-αNeu5Ac (**2**) and Neu5Acα(2–3)Galβ(1–4)Glc (**3**).

Currently, TS is poorly available, however, we received a clone[26] which allowed us to express the recombinant enzyme in *E. coli* in high concentration and purity. The activity of TS is difficult to measure and the assay would usually require radioactively labelled material. Therefore, we determined the quantity *via* the concentration of the pure enzyme using the same concentration for each test, and this allowed us to compare the yields of different trans-sialylations, following a procedure established by R. Field *et al.*[26]

Synthesis of sialylated oligosaccharides

Employing the activated Neu5Ac donors, various disaccharide acceptor substrates containing a terminal Galβ-R residue could be glycosylated. The subterminal residues of the acceptors could bear different regio- and stereochemical properties. Eleven potential acceptors were tested for chemoenzymatic trans-sialylations including naturally occurring structures, *e.g.* epitopes of the T-antigen but also non-natural oligosaccharides such as potential building-block saccharides.

The subterminal domain of the acceptor saccharide generally consisted of a Gal, GalNAc, Glc or GlcNAc moiety. The Galβ(1–4)-R linked acceptors were slightly preferred compared to the Galβ(1–3)-R linked components and trans-sialylations led to the corresponding oligosaccharides. It was further observed that L-Fuc residues directly or closely linked to the terminal Gal residue decreased the trans-sialidase transfer dramatically, which disabled the formation of sialyl Lewis^X saccharides.[27]

The transfer activity depended strongly on incubation conditions, such as pH, temperature and time. In contrast to sialidases requiring long incubation times and a high excess of acceptor substrates,[13] transglycosylation with TS took place with equimolar amounts of substrates and much shorter reaction times. Furthermore, TS catalysed only the transfer reaction, which caused less side-products. This allowed us to monitor the process of the reaction not only by HPLC, but by TLC; no hydrolysis of donor substrates or the formed oligosaccharides could be observed in the transglycosylation with TS (in contrast to the sialidase-mediated glycosylations).[13]

Common TS incubation conditions applied a slight excess (1.1 equivalents) of donor substrate at low concentrations, approximately 20 μmol ml^{-1}, in a cacodylate–HCl buffer at pH 6.9 and 37 °C. During these tests, TS exhibited a high sensitivity to its environment, especially towards change of pH and temperature. Higher enzyme activities, as well as increased yields, were achieved under modified conditions. The buffer system was changed to Tris–HCl buffer at pH 7.5 and the temperature was reduced to 23 °C. Under these conditions, even after prolonged incubation times, no generation of Neu5Ac by hydrolysis of the donors was observed by TLC and HPLC.

Further advantages of this reaction were the simple purification of the sialylated oligosaccharides. Since there was no formation of Neu5Ac as a side product and due to the different polarity of the reaction compounds, isolation of the products was facile by reverse phase chromatography with a RP-18 column instead of size exclusion chromatography, *e.g.* with Biogel P-2. Using RP-18 with water as eluent reduced the amount of the required solid phase drastically and led to better and faster separation of the products.

As already mentioned a considerable variety of different acceptor glycosides could be sialylated. Preparation of the acceptor structures was achieved following either a purely chemical synthesis, *e.g.* for lactose derivatives or a chemoenzymatic route giving iso-lactose and Gal–GalNAc derivatives. The *N*-acetyl lactosaminide acceptor disaccharide **8** was synthesised *via* the oxazoline precursor, the subsequent mild acid glycosylation of which led to the corresponding methyl glycoside. The allyl lactoside **7** was prepared following a slightly modified Koenigs–Knorr glycosylation.[28]

As in expected from nature, Galβ(1–4)Glc derivatives were regio- and stereoselectively sialylated in rather good yields. According to NMR data only α-sialosides with a 2–3-glycosidic linkages were synthesised. The stereochemistry of the linkage was assigned by a characteristic shift of H-3$_{eq}$ and H-3$_{ax}$ of Neu5Ac due to the anisotropy of the carbonyl group. Further, the regiochemistry of the linkage could be determined by the chemical shift of C-3′. After sialylation this carbon showed a clear downfield shift of approximately 3 ppm compared to the non-sialylated disaccharides.

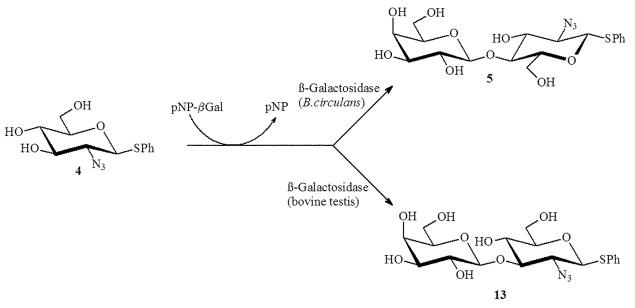
Interestingly, in corresponding transglycosylation methods, allyl β-lactoside (**7**) afforded better yields of product **11** than the α/β-anomeric mixture of free lactose (**6**) to give the sialylated product **10**. It may be assumed that the α-anomer and the open chain form of the subterminal carbohydrate residue are less favoured for transfer reactions, whereas especially the defined β-lactoside led to oligo-saccharides of the type Neu5Acα(2–3)Galβ(1–4)Glc-βR in excellent yields.

LacNAc-sialosides are found on mammalian cell surfaces and play an important role in cell communication. Preparation of these compounds in a defined and pure manner is of considerable importance and sialylation with trans-sialidase provided an easy access in convincing yields.

Apparently the LacNAc methyl glycoside exhibited a lower polarity than the Lac derivative, and thus the conversion to the corresponding oligosaccharides gave lower yields. Still the chemoenzymatic approach with trans-sialidase represented an excellent and comfortable alternative to the chemical synthesis of sialylated LacNAc derivatives.

In addition to the preparation of naturally occurring sialo-oligosaccharides, non-natural building blocks such as the thiophenyl trisaccharide **9**, capable of further derivatisation, were synthesised. For this purpose, following the preparation of the acceptor monosaccharide **4**, an enzymatic pathway employing galactosidase from *B. circulans* and pNP-βGal as donor substrate gave the disaccharide **5**.[29,30] The acceptor disaccharide **13** was prepared from **4** analogous to **5**, following an enzymatic pathway with bovine testis β-galactosidase to form the β,1–3 linkage (Scheme 2).

Both acceptor disaccharides were sialylated, despite their rather unpolar substituents at the anomeric center, to give trisaccharides **9** and **14**, respectively. As expected, yields decreased



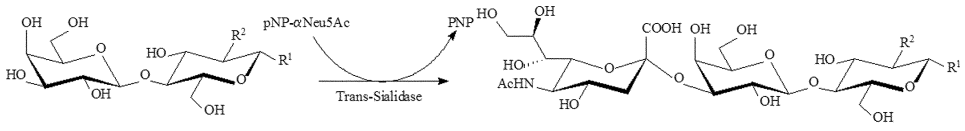
Scheme 2 Transglycosylation with β-galactosidase from *B. circulans* and bovine testis.

to about 30% (Schemes 3 and 4). However, in comparison to alternative syntheses this is quite convincing, and a respectable amount of product was isolated in a comfortable manner.

The structures **19** and **20** have received increasing attention since they were identified as tumour-associated antigens.[31,32] Acceptor compounds **15**–**18** were prepared by regioselective transglycosylation employing β-galactosidase from different sources in acceptable yields.[13,33] The resulting disaccharides represent useful donor substrates for trans-sialylation. According to NMR data the α(2–3) transfer of Neu5Ac with trans-sialidase took place regio- and stereoselectively (Scheme 5). Apparently, the structural differences in the subterminal β(1–3)-linkage are responsible for a reduced transfer resulting in lower yield. All these trisaccharides were prepared following the cacodylate buffered procedure.[34] Both higher temperature and repeated purification of the product with Biogel P-2 reduced the isolated amount of trisaccharides. Unexpectedly, the different polarities of the residues in the acceptor substrates **15**–**18** showed no significant influence on the result of the reactions. Sialylated glycopeptide epitopes **19** and **20** and sialylated building blocks with 2-deoxy or 2-azido functions **21** and **22** could be synthesised from the corresponding disaccharide acceptors **15**–**18** in moderate yields. The preparation of the β1–4 linked galactobiose compound **23** was accomplished by employing β-galactosidase from *Bacillus circulans*.[13] Trans-sialylation at 37 °C in cacodylate buffer afforded **24** in 25% (Scheme 6). The improved yield in comparison to **21** may indicate a slight preference for the β1–4 linkage in the acceptor substrate.[35]

Conclusion

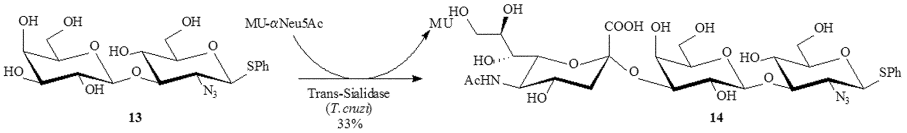
The sialylation of an array of various oligosaccharides was demonstrated employing trans-sialidase from *Trypanosoma cruzi*. The derived trisaccharides were isolated in different yields depending on the structure and the polarity of the acceptor substrates as well as on the incubation conditions and purification. Trans-sialidase exhibited a higher preference for Galβ(1–4)-linked acceptor disaccharides than for the corresponding Galβ(1–3) linked derivatives. Therefore, lactoside derivatives were sialylated in excellent yields, revealing an efficient alternative especially in comparison to sialyltransferase mediated reactions. Also Galβ(1–3)-linked derivatives are suitable acceptors



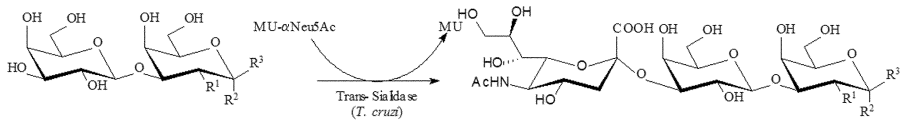
	R^1	R^2		Yield
5	SPh	N_3	**9**	32 %
6	OH	OH	**10**	49 %
7	OAll	OH	**11**	61 %
8	OMe	NHAc	**12**	42 %

[a] Prepared by method B

Scheme 3 Trans-sialylation of lactose and modified derivatives employing trans-sialidase.

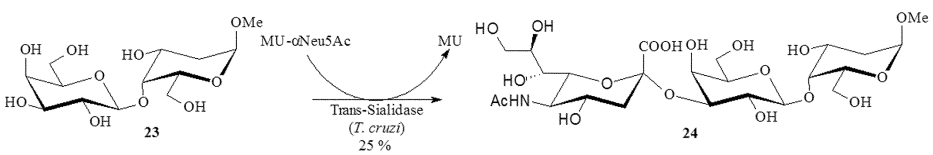


Scheme 4 Sialylation of a Galβ(1–3)Glc building block.



	R^1	R^2	R^3		Yield
15	NHAc	Thr	H	**19**	25 %
16	NHAc	Ser	H	**20**	22 %
17	H	OMe	H	**21**	16 %
18	N_3	H	OAll	**23**	31 %

Scheme 5 Trans-sialylations of Galβ(1–3)-Gal-R derivatives employing trans-sialidase.



Scheme 6 Trans-sialylation of a Galβ(1–4) derivative employing trans-sialidase.

for sialylation showing reduced transfer. Also the synthesis of naturally occurring sialylated oligosaccharides such as the sialyl T-antigen was accomplished in respectable yield.

Experimental

General

Commercially available starting materials were used without further purification. Solvents were dried according to standard methods. Purification of the products was carried out by flash chromatography (FC) using Merck silica gel 60 (230–400 mesh) and reverse phase chromatography (RPC) using Merck silica gel 60 RP-18 (40–63 mesh). The enzymatic reactions were incubated in a Thermomixer comfort (Merck) at 600 rpm. The nuclear magnetic resonance spectra were recorded on Bruker AMX-400 (100.62 MHz for ^{13}C) or DRX-500 (125.83 MHz for ^{13}C). All chemical shifts (δ) are quoted in ppm downfield from TMS or referred to the characteristic signals of the used solvents $CHCl_3$ in $CDCl_3$ (7.24 ppm), d_3-MeOH in d_4-MeOH (3.35 ppm) or HDO in D_2O (4.63 ppm). Mass spectra were recorded on Bruker MALDI-Tof Biflex III.

Expression and purification of trans-sialidase from *T. cruzi*

Recombinant TS was expressed in *E. coli* M15 (pREP4) cells (Qiagen, Hilden) in "terrific broth" medium overnight at 18 °C. Induction was performed with 0.5 mM IPTG. Cells were lysed in 20 mM Tris (pH 8.0), 0.5 M NaCl, 0.05% Lubrol (ICN, Eschwege) with lysozyme and benzoase (Merck, Darmstadt) and 5 cycles of sonication. After centrifugation, the supernatant was applied on a Ni–NTA superflow column (Qiagen, Hilden). The eluent protein was dialysed against 20 mM Tris (pH 8.0) and further purified with a Poros HQ/M column (Roche, Mannheim) in the same buffer with a linear NaCl gradient.

Trans-sialylation with trans-sialidase

Method A. A solution of donor (50 μmol) and acceptor (45 μmol) in 2.5 ml degassed incubation buffer (50 mM sodium cacodylate–HCl, pH 6.9, 50 μg BSA (ultra clean), 0.02% NaN_3O was incubated with 1 U recombinant trans-sialidase at 37 °C. The reactions were monitored by HPLC (LiChrospher NH_2–amino column, 5 μm, 4 × 250 mm, eluent: water) or TLC (butanol : acetic acid : water, 5 : 2 : 2). After completion of the reaction, the enzyme was denatured and centrifuged before the supernatant was lyophilised. The dry residue was dissolved in water and purified by biogel chromatography (P-2 Biogel, 16 × 900 mm).

Method B. A solution of *p*-NP-Neu5Ac (23 μmol) and acceptor (46 μmol) in 1 ml degassed incubation buffer (100 mM Tris/HCl, pH 7.5, 50 μg BSA (ultra clean), 0.02% NaN_3) was incubated with recombinant TS (100 μg) at 23 °C. The reaction was monitored by TLC (butanol : acetic acid : water, 5 : 2 : 2). After completion of the reaction the enzyme was denatured with ethanol, centrifuged and the supernatant was lyophilised. The dry residue was dissolved in water and purified by reverse phase chromatography (RP-18, Merck, 10 × 16 mm).

Phenyl 2-azido-2-deoxy-4-(β-D-galactopyranosyl)-1-thio-β-D-glucopyranoside (5)

Phenyl 2-azido-2-deoxy-4-*O*-β-D-galactopyranoside (**4**) (244 mg, 0.82 mmol) and pNP-βGal (740 mg, 2.46 mmol) were dissolved in 3 ml buffer (50 mM NaOAc, pH 5.0) and incubated with galactosidase from *Bacillus circulans* (1 U) for 36 h at 37 °C. Subsequently, the enzyme was denatured by heating of the mixture to 100 °C for 2 min. The crude product was purified by biogel chromatography (P-2 Biogel), yielding **5** (24%, 90 mg) as a white amorphous solid.

$[\alpha]_D^{20} = 6.7$ (0.6, H_2O); calc.: C 47.05, H 5.48, N 9.14; found: C 46.99, H 5.41, N 9.09%; ^{1}H-NMR (D_2O): $\delta = 7.58$ (m, 2 H, Ph), 7.33 (m, 3 H, Ph), 4.95 (d, 1 H, H-1, $J_{1,2} = 8.3$ Hz), 4.46 (d, 1 H, $J_{1',2'} = 8.2$ Hz, H-1′), 3.97 (m, 1 H, H-6a), 3.93 (dd, 1 H, H-4′, $J_{3',4'} = 4.0$ Hz, $J_{4',5'} = 1.0$ Hz), 3.86 (m, 1 H, H-6b), 3.81 (m, 1 H, H-2), 3.75 (m, 1 H, H-3), 3.74 (m, 1 H, H-4), 3.74–3.71 (m, 2 H, H-6a′, H-6b′), 3.65 (dd, 1 H, H-3′, $J_{2',3'} = 9.8$ Hz, $J_{3',4'} = 3.0$ Hz), 3.61 (m, 1H, H-5), 3.61 (m, 1 H, H-5′), 3.47 (dd, 1 H, H-2′) ppm. ^{13}C-NMR (D_2O): $\delta = 133.27$–128.92 (Ph), 103.76 (C-1′), 103.51 (C-1), 79.11 (C-4), 76.22 (C-5′), 75.31 (C-5), 73.35 (C-3′), 73.05 (C-3), 71.69 (C-2′), 69.31 (C-4′), 61.85 (C-6′), 60.79 (C-6), 55.97 (C-2) ppm.

Phenyl (5-acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosylonic acid)-(2–3)-(β-D-galactopyranosyl)-(1–4)-2-azido-2-deoxy-1-thio-β-D-glucopyranoside (9)

Following method A, acceptor 5 and MU-αNeu5Ac were incubated with TS for 14 h yielding compound **13** (10.6 mg, 32%); amorphous colourless solid; $[\alpha]_D^{20} = -1.8$ (0.6, H_2O); calc. C 47.28, H 5.75, N 5.70; found C 47.21, H 5.83, N 5.64%; ^{1}H-NMR (D_2O): $\delta = 7.62$–7.38 (m, 5H, Ph), 5.10 (m, 1 H, H-1), 4.54 (d, 1 H, H-1′, $J_{1',2'} = 7.6$ Hz), 4.12 (m, 1H, H-3′), 3.97 (dd, 1H, H-4′, $J_{4',5'} = 1.0$ Hz), 3.90 (m, 3H, H-8″, H-6a, H-6b), 3.87 (m, 1H, H-9a″), 3.85 (dd, 1 H, H-5″, $J_{5'',6''} = 10.2$ Hz), 3.82 (m, 1 H, H-3), 3.73–3.58 (m, 11 H, H-2, H-2′, H-4, H-4″, H-5, H-5′, H-6a′, H-6b′, H-6″, H-7″, H-9b″), 2.77 (dd, 1 H, H-3_{eq}″, $J_{3eq,\,3ax} = 12.0$ Hz, $J_{3eq,\,4''} = 4.6$ Hz), 1.80 (dd, 1 H, H-3_{ax}″, $J_{3ax,4} = 12.0$) ppm; ^{13}C-NMR (D_2O): $\delta = 175.79$ ($COCH_3$″), 174.75 (C-1″)133.14–128.83 (Ph), 103.56 (C-1′), 100.68 (C-2″), 87.24 (C-1), 76.33 (C-3′), 76.02 (C-5′), 75.54 (C-5), 74.22 (C-4), 73.75 (C-6″), 73.91 (C-3), 72.65 (C-8″), 70.18 (C-2′), 69.24 (C-4″), 69.05 (C-7″), 68.34 (C-4′), 63.47 (C-9″), 61.85 (C-6′), 60.26 (C-6), 56.89 (C-2), 52.62 (C-5″), 40.65 (C-3″), 22.84 ($COCH_3$) ppm.

(5-Acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosylonic acid)-(2–3)-(β-D-*galacto*pyranosyl)-(1–4)-D-glucopyranose (10)

Following method A, lactose and pNP-αNeu5Ac were incubated with TS for 14 h yielding **10** (13.9 mg, 49%) as an amorphous colourless solid. The physical and analytical data are in accordance with the literature.[13]

Allyl (5-acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosylonic acid)-(2–3)-(β-D-galactopyranosyl)-(1–4)-β-D-glucopyranoside (11)

Following method B, allyl lactoside **7** and pNP-αNeu5Ac were incubated with TS for 12 h yielding **11** (9.4 mg, 61%); colourless amorphous solid; $[\alpha]_D^{20} = -2.6$ (0.2, H_2O); MALDI-TOF: 696.49 [M + Na^+]; ^{1}H-NMR (D_2O): $\delta = 5.77$–5.85 (m, 1H, H_{allyl}), 5.25 (dd, 1H, CH=CH, $J_{gem} = 15.9$ Hz, $J_{CH=CH} = 3.2$ Hz), 5.12 (dd, 1H, CH=CH, $J_{CH=CH} = 2.3$ Hz), 4.38 (d, 1H, H-1, $J_{1,2} = 8.1$ Hz), 4.36 (d, 1H, H-1′, $J_{1',2'} = 7.9$ Hz), 4.23 (dd, 1H, OCH_{allyl}, $J_{gem} = 12.61$ Hz, $J_{OCH,CH=CH} = 5.68$ Hz), 4.07 (dd, 1H, OCH_{allyl}, $J_{OCH,CH=CH} = 6.47$ Hz), 3.91 (dd, 1H, H-3′, $J_{2',3'} = 9.7$ Hz), 3.84–3.80 (m, 2H, H-4′, H-6a), 3.70–3.42 (m, 14 H, H-3, H-4, H-4″, H-5, H-5′, H-5″, H-6b, H-6a′, H-6b′, H-6″, H-7″, H-8″, H-9a″, H-9b″), 3.40 (dd, 1H, H-2′, $J_{2',3'} = 9.0$ Hz), 3.18 (dd, 1H, H-2, $J_{2,3} = 8.8$ Hz), 2.55 (dd, 1H, H-3eq″, $J_{3eq'',3ax''} = 12.1$ Hz, $J_{3eq'',4''} = 4.4$ Hz), 1,90 (s, 3H, NAc), 1.60 (dd, 1H, H-3ax″, $J_{3ax'',4''} = 12.0$ Hz) ppm. ^{13}C-NMR (D_2O): $\delta = 176.02$ (C-1″), 132.43 (CH_{allyl}), 119.96 ($CH_{2\text{-}allyl}$), 103.49 (C-1′), 102.8 (C-1), 78.67, 77.96, 76.40, 75.90, 75.54, 75.11, 74.92, 73.87, 73.22 (C-3, C-4, C-4″, C-5, C-5′, C-5″, C-6″, C-7″, C-8″), 76.49 (C-3′), 73.19 (C-2), 70.01 (C-2′), 68.24 (C-4′), 61.41, 60.89, 60.20 (C-6, C-6′, C-9″), 54. 67 (OCH_2), 38.91 (C-3″), 21.99 ($NCOCH_3$) ppm.

Phenyl (5-acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosylonic acid)-(2–3)-(β-D-galactopyranosyl)-(1–3)-2-azido-2-deoxy-1-thio-β-D-glucopyranoside (14)

Following method A, compound **13** and MU-αNeu5Ac were incubated with TS for 12 h yielding compound **14** (10.6 mg, 32%); colourless amorphous solid; $[\alpha]_D^{20} = -28$ (0.4, H_2O); calc. C 47.28, H 5.75, N 5.70; found C 47.73, H 5.66, N 5.73%; ^{1}H-NMR (D_2O): $\delta = 7.61$–7.36 (m, 5H, Ph), 4.86 (d, 1H, H-1, $J_{1,2} =$ 8.1 Hz), 4.50 (d, 1H, H-1′, $J_{1',2'} = 8.1$ Hz), 4.19 (nr, 1H, H-3), 4.12 (dd, 1H, H-4, $J_{3,4} = 4.0$ Hz, $J_{4,5} = 1.6$ Hz), 4.08 (nr, 1H, H-3′), 3.93 (m, 1H, H.4′), 3.87–3.84 (m, 3H, H-8″, H-9a″, H-5″), 3.76 (m, 2H, H-6a′, H-6b′), 3.70–3.62 (m, 6H, H-5′, H-4″, H-6″, H-9b″, H-6a, H-6b), 3.59 (nr, 1H, H-7″), 3.54 (m, 2H, H-2, H-2′), 2.75 (dd, 1H, H-3_{eq}″ $J_{3eq'',3ax''} = 12.0$ Hz, $J_{3eq'',4''} = 4.6$ Hz), 2.03 (s, 3H, NAc) 1.78 (dd ≈ t, 1H, H-3_{ax}, $J_{3eq'',4''} = 12.0$ Hz) ppm;^{13}C-NMR (D_2O): δ = 175.78 ($COCH_3$″), 174.59 (C-1″), 133.10–128.75 (Ph), 104,25 (C-1′), 100.39 (C-2″), 90.14 (C-1), 80.42 (C-3), 76.36 (C-3′), 75.82 (C-5′), 75.22 (C-5), 73.61 (C-6″), 72.57 (C-8″), 69.86 (C-2′), 69.11 (C-4″), 68.81 (C-7″), 68.35 (C-4), 68.12 (C-4′), 63.32 (C-9″), 62.88 (C-2), 61.89 (C-6′), 61.18 (C-6), 52.44 (C-5″), 40.62 (C-3″), 22.87 ($COCH_3$″) ppm.

Methyl (5-acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosylonic acid)-(2–3)- (β-D-galactopyranosyl)-(1–4)-2-acetamido-2-deoxy-β-D-glucopyranoside (12)

Following method B, the acceptor **8** and pNP-αNeu5Ac were incubated with TS for 12 h yielding **12** (7.8 mg, 49%); colourless amorphous solid; MALDI-TOF: 711.09 [M + Na^+]; physical and NMR data are in accordance with the literature.[36]

(5-Acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosylonic acid)-(2–3)-(β-D-galactopyranosyl)-(1–3)-(2-acetamido-2-deoxy-α-D-galactopyranosyl)-(1–3)-L-threonine (19)

Acceptor **15** and MU-αNeu5Ac were incubated for 14 h with TS following method A, giving compound **19** (7.7 mg, 22%). The analytical data were identical to published results.[37]

(5-Acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosylonic acid)-(2–3)-(β-D-galactopyranosyl)-(1–3)-(2-acetamido-2-deoxy-α-D-galactopyranosyl)-(1–3)-L-serine (20)

Acceptor substrate **16** and MU-αNeu5Ac were incubated for 14 h with TS following method A, giving compound **20** (6.8 mg, 20%); colourless amorphous solid; physical and NMR data are in accordance with the literature.[38]

Methyl (5-acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosylonic acid)-(2–3)-(β-D-galactopyranosyl)-(1–3)-2-deoxy-α-D-lyxo-pyranoside (21)

Acceptor **17** and MU-αNeu5Ac were incubated for 14 h with TS following method A, giving compound **21** (4.5 mg, 16%); colourless amorphous solid; $[\alpha]_D^{20} = 52$ (0.5, H_2O), calc. C 45.67, H 6.54, N 2.22; found C 45.59, H 6.52, N 2.28%; ^{1}H-NMR (D_2O): $\delta = 5.12$ (dd, 1H, H-1, $J_{1,2ax} = 4.2$ Hz, $J_{1,2eq} = 1.0$ Hz), 4.54 (d, 1H, H-1′, $J_{1',2'} = 7.9$ Hz), 4.15–4.11 (m 2 H, H-3, H-4), 4.08 (nr, 1H, H-3′), 4.03 (nr, 1H, H-5), 3.96 (nr, 1H, H-4′), 3.88–3.83 (m, 3H, H-8″, H-9b″, H5″), 3.79–3.65 (m, 8H, H-6a, H-6b, H.6a′, H-6b′, H-5″, H-6″, H-4″, H-9b″), 3.62 (nr, 1H, H-7″), 3.53 (s, 3H, OCH_3), 3.51 (m, 1H, H-2′), 2.77 (dd, 1H, H-3_{eq}″, $J_{3eq'',3ax''} = 12.2$ Hz, $J_{3eq'',4''} = 4.9$ Hz), 2.03–1.97 (m, 2H, H-2eq, H-2ax), 1.80 (dd ≈ t, 1H, H-3ax″, $J_{3ax',4'} = 12.2$ Hz) ppm. ^{13}C-NMR (D_2O): $\delta = 175.86$ ($COCH_3$), 174.63 (C-1″), 104.68 (C-1′), 100.65 (C-2″), 98.54 (C-1), 76.26 (C-3′), 75.97 (C-5′), 74.02 (C-3), 73.81 (C-6″), 72.74 (C-8″), 71.22 (C-5), 70.31 (C-2′), 69.25 (C-4″), 68.85 (C-7″), 68.44 (C-4′), 67.87 (C-6), 66.45 (C-4), 63.57 (C-9″), 61.97 (C-6′), 54.71 (OCH_3), 52.49 (C-5″), 40.63 (C-3″), 31.49 (C-2), 22.83 ($COCH_3$″) ppm.

Allyl (5-acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosylonic acid)-(2–3)-(β-D-galactopyranosyl)-(1–3)-2-azido-2-deoxy-β-D-galactopyranoside (22)

Acceptor **18** and MU-αNeu5Ac were incubated for 13 h with TS following trans-sialylation procedure A to give compound **22** (9.3 mg, 31%); colourless amorphous solid; physical and NMR data are in accordance with the literature.[13]

Methyl (5-acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosylonic acid)-(2–3)-(β-D-galactopyranosyl)-(1–4)-2-deoxy-α-D-lyxopyranoside (24)

Acceptor **23** and MU-αNeu5Ac were incubated for 13 h with TS following method A to giving compound **24** (6.9 mg, 25%); colourless amorphous solid; physical and NMR data are in accordance with the literature.[13]

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References

1 A. P. Corfield, and R. Schauer, in *Sialic Acids – Chemistry, Metabolism and Function*, ed. R. Schauer, Springer, Wien, 1982, pp. 5–43.
2 R. Schauer, *Glycoconjugate J.*, 2000, **17**, 485–499.
3 R. Schauer, *Trends Biochem. Sci.*, 1985, **10**, 357–360.
4 S. D. Kelm, and R. Schauer, in *Int. Rev. Cytology*, ed. K. W. Jeron, J. W. Jarwik, 1997, 175, pp. 137–240.
5 G. Boons and A. V. Demchenko, *Chem. Rev.*, 2000, **100**, 4539–4565.
6 J. Thiem and W. Treder, *Angew. Chem., Int. Ed. Engl.*, 1986, **25**, 1096–97.
7 V. Kren and J. Thiem, *Angew. Chem., Int. Ed. Engl.*, 1995, **34**, 893–895.
8 O. Blixt, K. Allin, L. Pereira, A. Datta and J. C. Paulson, *J. Am. Chem. Soc.*, 2002, **124**, 5739–46.
9 V. Kren and J. Thiem, *Chem. Soc. Rev.*, 1997, **26**, 463–474.
10 T. Murata and T. Usui, *Biosci. Biotechnol. Biochem.*, 1997, **61**, 1059–66.
11 J. Thiem and B. Sauerbrei, *Angew. Chem., Int. Ed. Engl.*, 1991, **30**, 1503–6.
12 Y. Makimura, H. Ishida, A. Kondo, A. Hasegawa and M. Kiso, *J. Carbohydr. Chem.*, 1998, **17**, 975–979.
13 D. Schmidt, B. Sauerbrei and J. Thiem, *J. Org. Chem.*, 2000, **65**, 8518–8526.
14 A. C. Frasch, *Parasitol. Today*, 2000, **16**, 282–286.
15 L. C. Pontes de Carvalho, S. Tomlinson, F. Vandekerckhove, E. J. Bienen, A. B. Clarkson, G. W. Hart and V. Nussenzweig, *J. Exp. Med.*, 1993, **177**, 465–474.
16 S. Schenkmann, D. Eichinger, M. E. Pereira and V. Nussenzweig, *Annu. Rev. Microbiol.*, 1994, **48**, 499–523.
17 S. Schenkmann, M. S. Jiang, G. W. Hart and V. Nussenzweig, *Cell*, 1991, **65**, 1117–25.
18 O. Campetella, A. Uttaro, A. J. Parodi and A. C. Frasch, *Mol. Biochem. Parasitol.*, 1994, **64**, 337–340.
19 B. Herissat and A. Bairoch, *Biochem. J.*, 1993, **293**, 781–788.
20 B. Henrissat, *Biochem. Soc. Trans.*, 1998, **26**, 153–156.
21 A. Buschiazzo, G. A. Tavares, O. Campetella, S. Spinelli, M. L. Cremona, G. Paris, M. F. Amaya, A. C. Frasch and P. M. Alzari, *EMBO J.*, 2000, **19**, 16–24.
22 A. Buschiazzo, M. L. Cremona, G. Paris, M. F. Amaya, A. C. Frasch and P. M. Alzari, *Mol. Cell*, 2002, **10**, 757–768.
23 A. G. Watts, I. Damager, M. F. Amaya, A. Buschiazzo, P. M. Alzari, A. C. Frasch and S. G. Withers, *J. Am Chem. Soc.*, 2003, **125**, 7532–3.
24 P. Scudder, J. P. Doom, M. Chuenkova, I. D. Manger and M. E. A. Pereira, *J. Biol. Chem*, 1993, **268**, 9886–9891.

25 J. Rothermel and H. Faillard, *Carbohydr. Res.*, 1990, **196**, 29–40.
26 W. B. Turnbull, J. A. Harrison, A. K. R. Kartha, S. Schenkmann and R. A. Field, *Tetrahedron*, 2002, **58**, 3207–12.
27 H. D. Ly, B. Lougheed, W. W. Wakarchuk and S. Withers, *Biochemistry*, 2002, **41**, 5075–5085.
28 W. Koenigs and E. Knorr, *Ber. Dtsch. Chem. Ges.*, 1901, 957–981.
29 P. B. Alper, S.-C. Hung and C.-H. Wong, *Tetrahedron Lett.*, 1996, **37**, 6029–32.
30 A. Vasella, C. Witzig, J.-L. Chiara and M. Martin-Lomas, *Helv. Chim. Acta*, 1991, **74**, 273–281.
31 G. F. Springer, *Science*, 1984, **224**, 1198–1206.
32 S. Hakomori, in *Glycoproteins and Disease*, ed. J. Montreuil, J. F. G. Vliegenthart and H. Schachter, Elsevier Science B. V., Oxford, 1996, pp. 243–276.
33 U. Gambert and J. Thiem, *Carbohydr. Res.*, 1997, **299**, 85–89.
34 A. R. Todeschini, L. Mendonca-Previato, J. O. Previato, A. Varki and H. van Halbeck, *Glycobiology*, 2000, **10**, 213–221.
35 F. Vandekerckhove, S. Schenkmann, L. Pontes de Carvalho, S. Tomlinson, M. Kiso, M. Yoshida, A. Hasegawa and V. Nussenzweig, *Glycobiology*, 1992, **2**, 541–548.
36 S. Sabesan and J. C. Paulson, *J. Am. Chem. Soc.*, 1986, **108**, 2068–80.
37 U. Gambert and J. Thiem, *Eur. J. Org. Chem.*, 1999, 107–110.
38 H. Iijima and T. Ogawa, *Carbohydr. Res.*, 1988, **172**, 183–193.