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Preparation of Tn and Sialyl Tn Building Blocks Used in Fmoc Solid-phase Synthesis of Glycopeptide Fragments from HIV gp120

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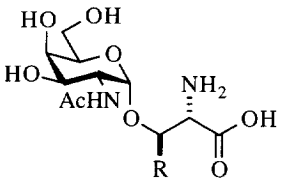
Abstract: The synthesis of novel building blocks corresponding to the Tn [α-D-GalNAc-(1→*O*)-Thr] and sialyl Tn [α-D-Neu5Ac-(2→6)-α-D-GalNAc-(1→*O*)-Thr] epitopes is described. The Tn building block was prepared from 4-methylphenyl 2-azido-2-deoxy-1-thio-β-D-galactopyranoside in four steps (42% yield) and carries *tert*-butyldimethylsilyl protective groups for the GalNAc moiety. Further conversion into a sialyl Tn building block, having acetyl protective groups for the sialic acid unit, was achieved in an additional four steps (37% yield). Both building blocks were used, in low excess (<20%), for Fmoc solid-phase synthesis of glycopeptide fragments from HIV gp120. The silyl protective groups of the Tn epitope were completely removed simultaneously with acid catalyzed cleavage of the glycopeptides from the solid phase. Hydrolysis of the sialic acid residue was not encountered during acid catalyzed cleavage of the sialyl Tn glycopeptide from the solid phase or during purification, even though glycosides of sialic acid are labile under acidic conditions.

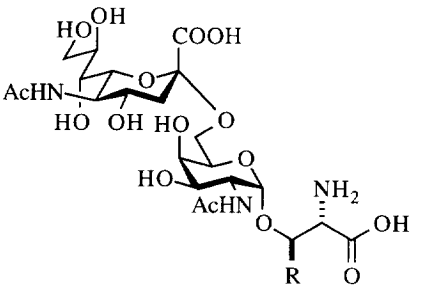
INTRODUCTION

The roles of protein-bound oligosaccharides have been investigated extensively and during the last decade information regarding their physiochemical and biological functions has started to emerge from several glycobiology laboratories (reviewed in references 1-3). The Tn [α-D-GalNAc-(1→*O*)-Ser/Thr, **1**] and sialyl Tn [α-D-Neu5Ac-(2→6)-α-D-GalNAc-(1→*O*)-Ser/Thr, **2**] epitopes are tumour-associated antigens present in glycoproteins on the surface of cancer cells,[4-6] and synthetic cancer vaccines based on the Tn epitope have been prepared.[7] The two epitopes also appear as partial structures in saccharides of mucins,[8] which are heavily

glycosylated proteins found in epithelial secretions, and on glycophorin,[9] a well characterized trans-membrane glycoprotein abundant on human red blood cells.



1 (R = H, Me)



2 (R = H, Me)

Recently Tn and sialyl Tn epitopes were discovered on the envelope glycoprotein gp120 of the human immunodeficiency virus (HIV).[10-12] Gp120 is one of the most heavily glycosylated proteins known to date, with most of its glycans being *N*-linked.[13] HIV escapes neutralization by antibodies of the infected individual by continuous mutations in the polypeptide part of gp120.[14] Furthermore, the viral *N*-linked glycans are similar to those of the host and are in general not immunogenic. In contrast, the *O*-linked Tn and sialyl Tn epitopes on gp120 may be potential targets for immune intervention since they are otherwise produced only in cancer cells as a result of aberrant glycosylation. Support for this hypothesis was recently provided by an investigation in which anti-Tn antibodies were found to cross-react with and neutralize HIV.[15] The number of *O*-linked glycans on gp120 has been estimated to be 4-5,[12] and the location of some of the glycans has been indicated by enzymatic means.[16] As a first step in efforts to locate the *O*-glycosylation sites of gp120 and to investigate the use of glycopeptides as HIV vaccines, we have recently prepared Tn glycopeptides from the V3-loop of gp120.[17]

Recent methodological developments have almost transformed the preparation of small glycopeptides carrying simple saccharides into a matter of routine and have even put glycoproteins within reach of the synthetic chemist.[18] At present glycosylation of protected oligopeptides by chemical means does not constitute a feasible route to *O*-linked glycopeptides. Instead, the most general synthetic route to both *O*- and *N*-linked glycopeptides employs glycosylated amino acids for stepwise assembly of glycopeptides, preferably on solid phase (reviewed in references 19-21). Synthetic routes to suitably protected glycosylated amino acids therefore constitute a key to success in synthesis of glycopeptides.

An early approach to solid-phase synthesis of *O*-linked glycopeptides relied on the *tert*-butyloxycarbonyl (Boc) group for α-amino group protection.[22] However, this approach requires repeated N^{α}-deprotection with trifluoroacetic acid and final cleavage from the solid phase with a strong acid, that is use of conditions which are likely to cleave glycosidic bonds. Later studies have therefore employed the N^{α}-fluoren-9-ylmethoxycarbonyl (Fmoc) group[23] which is removed by weak base, thus allowing the use of protective groups for amino acid side chains, and linkers to the solid phase, which are cleaved by a moderately stong acid such as trifluoroacetic acid.[19-21]

Several studies indicate that the *O*-glycosidic linkages of common saccharides (*e.g.* GlcNAc, GalNAc, Glc, Gal and Man) having unprotected hydroxyl groups are stable during trifluoroacetic acid catalyzed cleavage

of glycopeptides from a solid phase,[24-26] but exceptions to this rule have also been reported.[27,28] However, only a slight increase in the acid lability of the *O*-linked saccharide seems to necessitate protection of the hydroxyl groups with electron-withdrawing acetyl or benzoyl groups. For instance, acyl protection has been found to be required for glycosides of the 6-deoxysugar L-fucose,[29,30] which undergo acid catalyzed hydrolysis only 5-6 times faster than glycosides of the corresponding non-deoxygenated monosaccharide galactose.[31] In addition, 2-*O*-acyl groups are used for formation of 1,2-*trans* glycosidic bonds and as a result of the synthetic sequence acyl protective groups are commonly employed also for more stable saccharides in glycopeptides.

Cleavage of the Fmoc group and removal of saccharidic *O*-acyl protective groups must be performed under basic conditions, which may lead to side-reactions such as β-elimination of *O*-linked carbohydrates, epimerization of peptide stereocenters and aspartimide formation. During early glycopeptide synthesis this potential drawback caused considerable concern,[32,33] but recent studies have revealed that Fmoc removal from glycopeptides can be performed with piperidine just as for ordinary peptides.[34,35,17] In addition, conditions in common use for deacetylation of glycopeptides were found to cause neither β-elimination nor epimerization of the stereocenters in a model glycopeptide.[36] In spite of this, such side-reactions have occasionally been encountered indicating the need for careful choice and control of reaction conditions used for deacetylation.[30,17,37] Importantly, the more severe conditions required for removal of benzoyl groups have often been found to be accompanied by β-elimination.[38,39,25,36,40]

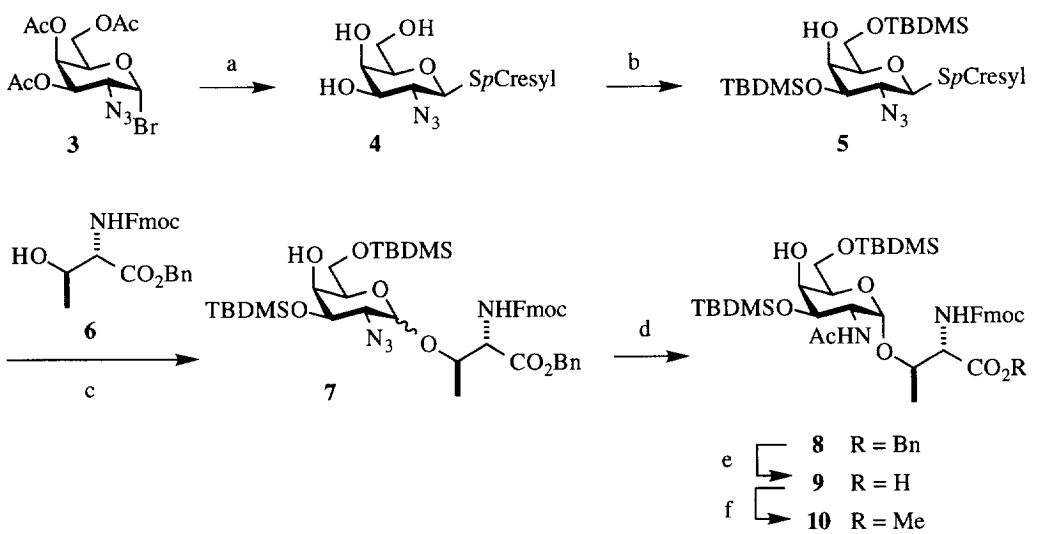
Tn building blocks described previously have all carried acetyl protective groups for the carbohydrate moiety (reviewed in references 19 and 41). To circumvent the problems that may occur during removal of *O*-acetyl protective groups from glycopeptides, we have now prepared a Tn building block carrying silyl protective groups.[42] Silyl protective groups are removed during acid catalysed cleavage of the glycopeptide from the solid phase and thus a separate base-mediated deacylation step is avoided. They have recently been emplyed for protection of the carbohydrate moieties of *N*- and *O*-linked glycopeptides prepared by solid-phase synthesis.[43-45] In addition, silyl protection allowed transformation of the Tn building block, in a few steps, into a sialyl Tn building block,[42] which carried acetyl protective groups for the sialic acid unit in order to confer stability to treatment with acid. Both building blocks were successfully employed in Fmoc solid-phase synthesis of glycopeptide fragments from HIV gp120. To the best of our knowledge this work provides the first example of a glycopeptide containing *O*-linked sialic acid being prepared on solid-phase by chemical synthesis.

RESULTS AND DISCUSSION

Formation of the α-glycosidic linkage between *N*-acetylgalactosamine and serine or threonine is a key step in the synthesis of glycosylated amino acids corresponding to the Tn and sialyl Tn epitopes. This glycosylation has until recently only been performed using glycosyl donors that have a non-participating group at C-2 of the glycosyl donor. Derivatives of 2-azido-2-deoxy-galactose, which are readily prepared by azidonitration of 3,4,6-tri-*O*-acetyl-D-galactal,[46,47] are then the donors of choice. However, during the completion of the present work it was shown that *N*-acetylgalactosamines can be used as donors in syntheses of α-glycosides if the donor carries a 4,6-*O*-benzylidene protective group.[48,49] Thioglycosides constitute one of the three types of glycosyl donors which are in common use in carbohydrate synthesis, the other two being glycosyl halides and imidates.[50] Surprisingly, thioglycosides have only been employed for synthesis of protected derivatives of the Tn epitope [α-D-GalNAc-(1→*O*)-Ser/Thr, **1**] in one previous study,[51] whereas other investigations have

predominantly relied on the use of 3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-D-galactopyranosyl halides (reviewed in references 19 and 41).

In order to enable incorporation of silyl protective groups for the hydroxyl groups of the Tn antigen we decided to base our strategy on a 2-azido-2-deoxy-galactopyranosyl thioglycoside as a key building block. The azidobromide **3** [46,47] was therefore converted, in one pot, to the *para*-thiocresyl glycoside **4**[52] by treatment with *para*-thiocresolate in a mixture of ethanol and chloroform (Scheme 1). Protection[53] of the triol **4** with *tert*-butyldimethylsilyl chloride (TBDMSCl) then gave the desired glycosyl donor **5**. Due to severe steric hindrance of the axial HO-4 in **5** it was not necessary to protect this hydroxyl group during the subsequent glycosylation. Use of the donor **5** in *N*-bromosuccinimide/tetrabutylammonium triflate (NBS/QOTf) promoted glycosylation[54] of N^{α}-Fmoc-threonine benzyl ester (**6**) gave the glycoside **7** as an inseparable mixture of α- and β-glycosides (α/β-ratio 5.2:1, 71% yield). In contrast to many other methods in use for activation of thioglycosides the NBS/QOTf promoter combination has the advantage of not generating a strong acid during the glycosylation; thus the acid labile TBDMS groups remained intact.[55]



Scheme 1. (a) *para*-thiocresol, NaOH, $CHCl_3$ + EtOH; 68% yield. (b) TBDMSCl, imidazole, DMF; 99% yield. (c) NBS, QOTf, CH_2Cl_2, -28 °C; 71% yield. (d) AcSH, pyridine; 67% yield of **8** together with 13% of the corresponding β-glycoside. (e) H_2, Pd-C, EtOAc; 90% yield. (f) $TMSCHN_2$, MeOH, CH_2Cl_2; 89% yield.

Reduction of the azido group in **7** proved to be more difficult than anticipated, and several methods commonly employed for reduction of azides failed or gave low yields of the desired product (Table 1). No conversion of **7** was obtained with $NiCl_2/NaBH_4$[56] or with H_2S/pyridine,[57] and in the latter case addition of triethylamine caused cleavage of the Fmoc group. Treatment with tertiary phosphines such as trioctyl- and tributylphosphine, and subsequent acetylation,[58] gave low and irreproducible yields of **8** and its β-isomer (42-74% total yields). With neat thioacetic acid[59] the reaction was either sluggish or required heating (50 °C), but yields were only modest (~50%). However, use of thioacetic acid[59] in pyridine was more efficient and the α-

glycoside **8** was obtained in 67% yield together with the corresponding β-glycoside (13%) after purification by normal phase HPLC. The reason for the low propensity of the azido group in **7** to undergo reduction is unknown.

Table 1. Reduction of the Azido Group in **7** to Give **8** and the Corresponding β-Glycoside

Method	Yield (%)[a]	Remarks
$NiCl_2/NaBH_4/B(OH)_3/EtOH$, rt	—	<10% conversion of **7**
H_2S/pyridine/H_2O, rt, 20 h	—	<10% conversion of **7**
H_2S/pyridine/H_2O/Et_3N, rt, 20 h	—	Decomposition[b]
Trioctylphosphine/HOAc/THF, rt[c]	53-74	
Tributylphosphine/HOAc/THF, rt[c]	42	
AcSH, rt, 10 days	50[d]	
AcSH, 50 °C, 26 h	55	
AcSH/pyridine, rt, 4 h	90	

[a]Total isolated yield of **8** and the corresponding β-glycoside. [b]Most likely cleavage of the Fmoc group occurred. [c]Reduction was followed by acetylation with Ac_2O in MeOH. [d]Estimated from TLC.

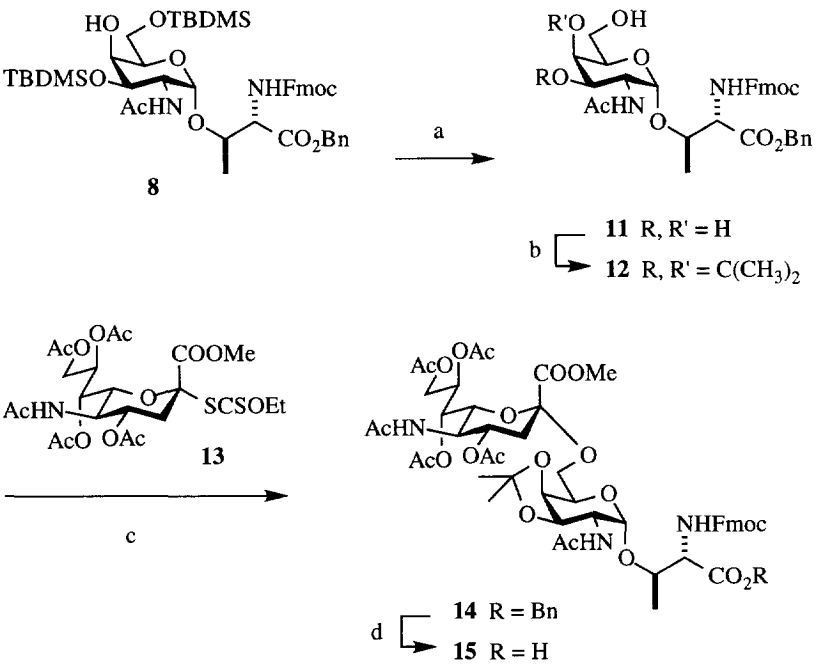
Broad peaks corresponding to ~10% of the material were observed in the ^1H NMR spectrum of **8** recorded in $CDCl_3$, in spite of careful chromatographic purification. Similar, minor peaks were also found in the spectra of the glycosylated derivatives **10**, **12**, **14** and **15**, as well as for Fmoc threonine benzyl ester (**6**). The additional peaks in the spectrum of **8**, however, became sharp when the spectrum was recorded in DMSO-d_6 instead of in $CDCl_3$. When the temperature of the DMSO-sample was gradually raised to 360 K the two sets of peaks converged to one, accompanied by some decomposition (~10%). Since minor peaks were found both for the structurally diverse glycosylated amino acids, and for Fmoc threonine benzyl ester, we suggest that the phenomenon is due to formation of rotamers about the amide bond in the Fmoc urethane, which is a structural element shared by the compounds. Such rotamers have also been reported in a previous synthesis of N^{α}-Fmoc protected Tn building blocks.[60]

The desired Tn building block **9** was then obtained in 90% yield by cleavage of the benzyl ester in **8** by catalytic hydrogenolysis. The Fmoc group is known to be cleaved slowly during hydrogenolysis but use of ethyl acetate as solvent and careful adjustment of the amount of Pd/C allowed selective removal of the more reactive benzyl ester in **8**.[61] The resolution in the ^1H NMR spectrum of **9** was found to be poor and the structure of **9** was therefore confirmed by conversion to the corresponding methyl ester **10** using trimethylsilyldiazomethane.[62]

A few reports describing syntheses of sialyl Tn building blocks have appeared,[63-65] and two of these have been used for synthesis of short glycopeptides in solution.[64,41] These sialyl Tn building blocks, however, carried protective groups unsuitable for solid phase synthesis, *i.e.* either benzyl protection was used for the sialic acid residue or the α-amino group carried a benzyloxycarbonyl group. Both protective groups are removed by hydrogenolysis, which is incompatible with peptides containing cysteine or methionine.[66] Furthermore, benzyl groups are electron donating and would destabilize the glycosidic bonds of sialic acid residues in glycopeptides during attempted cleavage from the solid phase with trifluoroacetic acid. Since acid catalyzed

hydrolysis of α-sialosides is known to be faster than hydrolysis of the common hexopyranosides,[67,68] and may be similar to that of fucosides, protection of sialic acid residues with acetyl instead of benzyl groups appeared to be a suitable precaution in solid phase glycopeptide synthesis.

In order to convert the Tn building block **8** into a sialyl Tn building block the TBDMS groups were removed from **8** to give the corresponding triol **11**. The low solubility of this triol in different organic solvents made regioselective sialylation at HO-6 impossible. Similarly, low solubility was found to be a problem in a previous synthesis of a sialyl Tn building block when an Fmoc group was employed for α-amino protection,[64] but not in a more recent study which employed a benzyloxycarbonyl group.[65] To circumvent the low solubility of **11** HO-3 and HO-4 were protected by isopropylidenation[69] and the glycosyl acceptor **12** was obtained in 85% yield from **8**.



Scheme 2. (a) HOAc + MeOH + THF, 55-60 °C; 86% yield. (b) 1) dimethoxypropane, *p*-TsOH; 2) Et_3N; 3) MeOH + H_2O, reflux; 85% yield from **8**. (c) MSB, AgOTf, 3Å MS, CH_2Cl_2 + CH_3CN, -78 °C; 49% yield. (d) H_2, Pd-C, EtOAc; 88% yield.

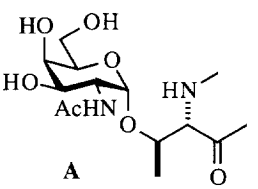
Sialylations are generally difficult to perform and only a limited number of donors give high yields and high stereoselectivity (reviewed in references 70-72). Early sialylations were predominatly carried out using sialyl chlorides and to a lesser extent bromides (reviewed in reference 70), but yields and stereoselectivity were in most cases low or at best moderate. Introduction of a participating group, such as a phenylsulfenyl group,[73,74] at C-3 of the sialyl donor inreased the yields and selectivity, but several synthetic steps were required for preparation of the donor and the subsequent removal of the participating group. The growing interest in glycobiology and conjugates of sialic acid has, however, spurred the development of new efficient

sialyl donors such as phosphites,[75,76] thioglycosides,[77] and xanthates,[78,79] which lack a participating group. Especially the xanthate **13**[78] has been found to give high yields and stereoselectivity when employed for sialylations promoted by methylsulfenyl bromide/silver triflate (MSB/AgOTf) at low temperature in mixtures of acetonitrile and dichloromethane.[80] Sialylation of the acceptor **12** with **13** according to this method, and purification with normal phase HPLC, gave the α-glycoside **14** in 49% yield together with a mixture of **14** and the corresponding β-glycoside (~10%, α:β ~1:1). The anomeric configuration of the sialoside **14** was established[81] from the coupling constant between C-1' and H-3'$_{ax}$.[82] Hydrogenolysis of the benzyl ester in **14** then furnished the target compound **15** (88% yield) which can be used directly in solid phase synthesis of sialylated glycopeptides. Use of **13** as sialyl donor thus gave a high stereoselectivity for the critical sialylation and allowed conversion of the Tn epitope **8** into a sialyl Tn building block in four steps (37% overall yield).

To prove the practicability of the novel Tn building block **9** the two Tn containing glycopeptides **16**[17] and **17** were chosen as synthetic targets, since various problems were encountered in previous syntheses of these glycopeptides. The glycopeptide **16** corresponds to amino acids 489-503[83] from HIV gp120, with a cysteine residue added at the *N*-terminus in order to allow conjugation to carrier proteins. The Tn peptide **17** is a *N*-terminally glycosylated derivative of a modelled T-cell determinant.[84]

16 Ac-Cys-Lys^{489}-Ile-Glu-Pro-Leu-Gly-Val-Ala-Pro-**A**-Lys-Ala-Lys-Arg-Arg^{503}-NH_2

17 H-**A**-Pro-Glu-Leu-Phe-Glu-Ala-Leu-Gln-Lys-Leu-Phe-Lys-His-Ala-Tyr-OH



A

R-Pro-Glu-Leu-Phe-Glu-Ala-Leu-Gln-Lys-Leu-Phe-Lys-His-Ala-Tyr-OH

18 R = H

19 R = Ac

The glycopeptides **16** and **17** were synthesized on a resin consisting of a cross-linked polystyrene backbone grafted with polyethyleneglycol chains. A custom made, fully automatic continuous flow peptide synthesizer[85] was used for the syntheses with DMF as solvent. In the synthesizer *N*α-Fmoc amino acids (4 equivalents), carrying standard side chain protective groups, were activated as benzotriazolyl esters using 1-hydroxybenzotriazole (HOBt) and diisopropylcarbodiimide, and then coupled to the peptide-resin. The Tn building block **9** (1.2 equivalents) was activated as its azabenzotriazolyl ester, since azabenzotriazole esters were recently found to be more efficient than benzotriazole esters in peptide synthesis.[86,87] Coupling of activated **9** was performed manually after removal of the peptide-resin from the synthesizer in order to allow use of a minimal volume of DMF as solvent. The *N*-acylations were monitored spectrophotometerically using the acid-base indicator Bromophenol Blue.[88] *N*α-Fmoc deprotections were performed with piperidine[17] in DMF and

were monitored using the absorbance of the dibenzofulvene-piperidine adduct.[89] After completion of the solid-phase synthesis the two glycopeptides were cleaved from the resin and all protective groups were removed by treatment with trifluoroacetic acid containing water, thioanisole and ethanedithiol as cation scavengers. Analytical reversed-phase HPLC revealed that the crude glycopeptides were of high purity (cf. HPLC trace of crude **16** in Figure 1a), and **16**[17] and **17** were obtained in 22 and 23% overall yields, based on the resin capacity, after purification by preparative HPLC. In the synthesis of **17** coupling of **9** was slow and did not reach completion and the peptide **18** (~16%) was also obtained. Both glycopeptides were characterized by FABMS and amino acid analysis, and in addition ^{1}H NMR spectroscopy was used for **17**.

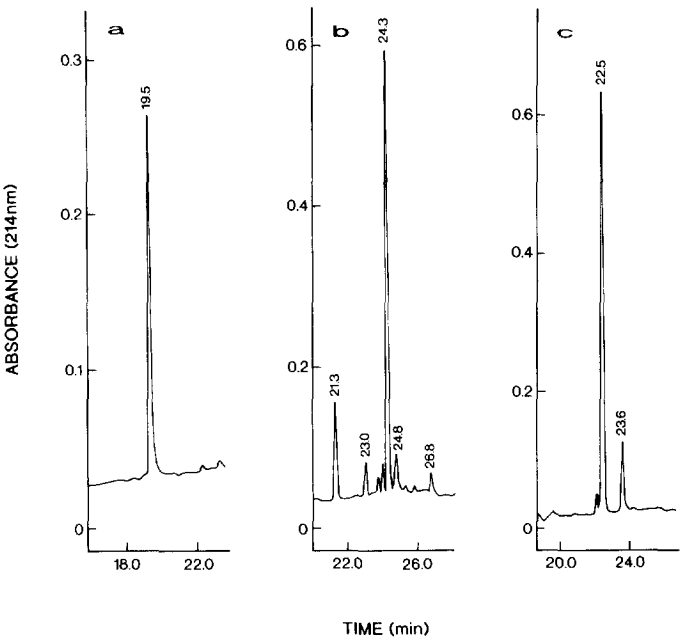


Figure 1. a: Analytical reversed-phase HPLC chromatogram of the crude Tn-glycopeptide **16**. **b** and **c:** Chromatograms of the crude protected and deprotected sialyl Tn glycopeptides **20** and **22**, respectively. The peak eluting at 21.3 min in the chromatogram of **20** (**b**) was identified as the terminated, N^{α}-acetylated peptide **24** whereas the peak at 23.6 min in the chromatogram of crude **22** (**c**) was identified as the disulfide **23**. HPLC conditions are described in the general procedure for solid-phase glycopeptide synthesis in the experimental section.

Glycopeptide **16**[17] and **17**[90] have both been prepared previously using 3 equivalents of N^{α}-(fluoren-9-ylmethoxycarbonyl)-3-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-galactopyranosyl)-L-threonine for incorporation of the Tn epitope, with other conditions being identical to those used in the present paper. In the previous synthesis of **16** attempts to remove the acetyl protective groups from the carbohydrate moiety after cleavage of the glycopeptide from the solid phase failed, presumably due to cysteine induced side reactions when saturated methanolic ammonia was used for deacetylation.[17,91] In that case, deacetylation was instead

performed before cleavage from the solid phase, an alternative which would not have been possible if **16** had been desired as a *C*-terminal acid instead of an amide. When **17** was prepared from N^{α}-(fluoren-9-ylmethoxycarbonyl)-3-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-galactopyranosyl)-L-threonine the *N*-terminus of the resin bound peptide was acetylated during the coupling of the Tn epitope; a side reaction which must have occurred by transfer of an acetyl protective group from the Tn building block.[92] As a result, **17** was only obtained in 8% overall yield after deacetylation together with terminated, *N*-acetylated peptide **19** (~14 %). *N*-Capping by acetyl transfer from glycosylated amino acids used to prepare a series of glycopeptides has been described previously.[93] Use of the silyl-protected Tn building block **9** succesfully solved these two problems and allowed **16** and **17** to be obtained in increased yields (15→22% for **16**, 8→23% for **17**), even though **9** was employed in only 20% excess as compared to the twofold excess used for the corresponding *O*-acetylated Tn building block.

No glycopeptide side-products were observed in the synthesis of **16** and **17**, revealing that the TBDMS groups had been completely removed during the final treatment with trifluoroacetic acid. We have recently described the assembly of a building block consisting of the disaccharide α-Glc-(1→2)-β-Gal linked to N^{α}-Fmoc-5-hydroxynorvaline benzyl ester which carried acid labile silyl and isopropylidene protective groups for the carbohydrate moiety.[44] This building block was used in solid-phase synthesis of analogues of glycopeptide fragments of collagen and removal of the carbohydrate protective groups occurred simultaneously with cleavage from the solid phase. Previously, trimethylsilyl protective groups have been used for the carbohydrate moieties of N-linked glycosylated amino acids incorporated in glycopeptides,[43,45] but the lability of the TMS group restricts the use of such building blocks in further synthetic transformations such as protective group manipulations and glycoside synthesis.

The sialyl Tn building block **15** was used in solid-phase synthesis of the glycopeptide **22** which consists of amino acids 312-327 from HIV gp120,[83] with a cysteine residue added at the *C*-terminus in order to allow conjugation to carrier proteins. The glycopeptide **22** was assembled in the automatic synthesizer as described for glycopeptides **16** and **17**, and one equivalent of the sialyl Tn building block **15**, relative to the capacity of the resin, was coupled manually to the peptide resin as its azabenzotriazolyl ester.[86,87] The coupling of **15** did not reach completion, as determined by Bromophenol Blue indication,[88] and unreacted amino groups were capped by addition of acetic anhydride. After completion of the solid-phase synthesis, the glycopeptide resin was treated with trifluoroacetic acid and scavengers which cleaved the glycopeptide from the solid-phase, removed the protective groups from the amino acid side-chains, and hydrolyzed the isopropylidene group of the *N*-acetylgalactosamine unit to give **20**.

Analysis of crude **20** by reversed-phase HPLC revealed that **20** was contaminated by the terminated peptide **24** and some minor by-products (Figure 1b). The Tn glycopeptide which corresponds to **20**, *i.e.* which lacks the sialic acid residue, has been prepared previously[17] and was found to elute at 22.5 min. The absence of this Tn glycopeptide in crude **20** revealed that partial hydrolysis of the *O*-acetylated sialic acid residue from **20** had not occurred during cleavage from the solid phase. Subsequent purification with reversed-phase HPLC gave the terminated peptide **24** (~14%), and the protected glycopeptide **20**. Removal of the *O*-acetyl groups from **20** (→**21**) was achieved with methanolic sodium methoxide (2.5 mM, pH ~8 on dry pH paper), and the methyl ester was then hydrolyzed with aqueous sodium hydroxide (2 mM) to give **22**. The deacetylation and the saponification were carried out under an argon atmosphere using degassed solvents in order to minimize dimerization via the cysteine residue. Purification of crude **22** (Figure 1c) with reversed-phase HPLC furnished

the target sialo glycopeptide in 12% overall yield, based on resin capacity, together with the corresponding disulfide **23** in ~1% yield. The structure of glycopeptide **22** was confirmed using FABMS, amino acid analysis and ^{1}H NMR spectroscopy.

Ac-Gly^{312}-Arg-Ala-Phe-Val-**A**-Ile-Gly-Lys-Ile-Gly-Asn-Met-Arg-Gln-Ala^{327}-Cys-NH_2

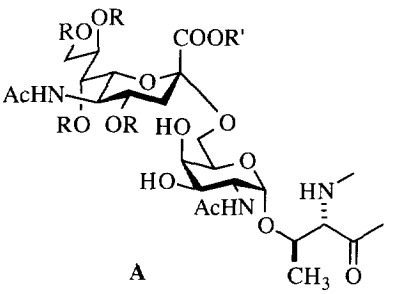
20 R = Ac, R' = Me

21 R = H, R' = Me

22 R = R' = H

(Ac-Gly^{312}-Arg-Ala-Phe-Val-**A**-Ile-Gly-Lys-Ile-Gly-Asn-Met-Arg-Gln-Ala^{327}-Cys-NH_2 with S—S)$_2$

23 R = R' = H



A

24 Ac-Ile^{318}-Gly-Lys-Ile-Gly-Asn-Met-Arg-Gln-Ala^{327}-Cys-NH_2

Acid catalyzed hydrolysis of unprotected, non-acylated α-sialosides is known to be faster than hydrolysis of the common hexopyranosides.[67,68] Purification of **22** on reversed-phase was performed at pH ≈ 1, that is under conditions which conceivably might lead to hydrolysis of the sialic acid residue. Such a side reaction, however, seems less likely since an early study revealed that a sialic acid residue linked to HO-6' of lactose was stable at pH=1 for >5 h, provided that the temperature was not raised above room temperature.[67] Analytical reversed-phase HPLC could not be used to investigate the stability of **22**, since **22** and the corresponding peptide lacking the sialic acid residue coeluted under several different chromatographic conditions. However, 500 MHz ^{1}H NMR spectroscopy revealed that free sialic acid was not present in **22**. The signals for the two protons at C-3 of sialic acid were especially diagnostic since they appeared in a non-crowded part of the spectrum, with H-3_{eq} and H-3_{ax} at 2.70 and 1.66 ppm for **22**, and at 2.16 and 1.72 ppm for sialic acid when analysed under conditions identical to those used for **22**. The synthesis of **22** thus demonstrates that glycopeptides carrying acid-labile sialic acid residues indeed can be synthesized by chemical solid-phase synthesis methodology according to the Fmoc protocol, utilizing as little as one equivalent of the sialyl Tn building block in the synthesis. Some reports on enzymatic synthesis of glycopeptides containing sialic acid have also appeared in the recent literature.[94-96]

CONCLUSIONS

Two novel glycosylated amino acid building blocks, **9** and **15**, which correspond to the Tn [α-D-GalNAc-(1→*O*)-Thr] and sialyl Tn [α-D-Neu5Ac-(2→6)-α-D-GalNAc-(1→*O*)-Thr] epitopes have been prepared and successfully used in Fmoc solid-phase glycopeptide synthesis. *tert*-Butyldimethylsilyl protective groups were employed for the carbohydrate moiety of the Tn epitope and the silyl groups were found to undergo complete removal during cleavage of the glycopeptides from the solid phase under acidic conditions. Thereby problems encountered previously when an *O*-acetylated Tn building block was used were avoided. Glycosides of sialic acid undergo acid catalyzed hydrolysis more rapidly than common hexopyranosides. However, when a glycopeptide was assembled from the sialyl Tn building block, which carried *O*-acetyl protective groups for the sialic acid unit, hydrolysis was not encountered during cleavage from the solid phase, or when the completely deprotected sialo glycopeptide was purified using reversed-phase HPLC under acidic conditions. For the first time it has thus been demonstrated that glycopeptides containing *O*-linked sialic acid can be prepared using standard solid-phase methodology according to the Fmoc protocol.

EXPERIMENTAL SECTION

General Methods and Materials.—TLC was performed on Silica Gel 60 F_{254} (Merck) with detection by UV light and charring with 10% aqueous H_2SO_4. Flash column chromatography was performed on silica gel (Matrex, 60 Å, 35-70 μm, Grace Amicon) with distilled solvents. CH_2Cl_2 and CH_3CN were dried by distillation from CaH_2 immediately before being used. Organic solutions were dried over Na_2SO_4 before being concentrated.

The ^1H and ^{13}C NMR spectra were recorded at 500 and 126 MHz, respectively, for solutions in $CDCl_3$ [residual $CHCl_3$ (δ_H 7.25 ppm) or $CDCl_3$ (δ_C 77.0 ppm) as internal standard] or CD_3OD [residual CD_2HOD (δ_H 3.33 ppm) or CD_3OD (δ_C 49.15 ppm) as internal standard] at 300 K, or in a 9:1 mixture of H_2O and D_2O [H_2O (δ_H 4.98 ppm) as internal standard] at 278 K. First-order chemical shifts and coupling constants were obtained from one-dimensional spectra and proton resonances were assigned from COSY,[97] TOCSY[98] and ROESY[99] experiments. Resonances for aromatic protons and resonances that could not be assigned are not reported. Ions for positive fast atom bombardment mass spectra were produced by a beam of Xenon atoms (6 keV) from a matrix of glycerol and thioglycerol. Ions for MALDI mass spectra were produced by a pulsed N_2-laser at 337 nm using a matrix of sinapinic acid. In the amino acid analyses, asparagine and glutamine were determined as aspartic acid and glutamic acid, respectively.

Analytical normal phase HPLC was performed on a Kromasil silica column (100 Å, 5 μm, 4.6 x 250 mm) using linear gradients of ethanol in hexane fraction, with a flowrate of 2.5 mL/min and detection at 254 nm. Preparative purifications were performed on a Kromasil silica column (100 Å, 5 μm, 20 x 250 mm) with the same eluant and a flowrate of 20 mL/min.

The preparation of 3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-α-D-galactopyranosyl bromide[46,47] (**3**), N^{α}-fluoren-9-ylmethoxycarbonyl-L-threonine benzyl ester[100] (**6**), and *O*-ethyl *S*-[methyl (5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosyl)onate] dithiocarbonate[78] (**13**) have been described previously.

General Procedure for Solid-Phase Glycopeptide Synthesis.—Glycopeptides **16**, **17**, and **22** were synthesized in a custom made, fully automatic continuous flow peptide synthesizer constructed essentially as described.[85] A resin consisting of a cross-linked polystyrene backbone grafted with polyethyleneglycol chains (TentaGel™, Rapp Polymere, Germany) was used for the syntheses. The resin was functionalized with the linker *p*-[α-(fluoren-9-ylmethoxyformamido)-2,4-dimethoxybenzyl]phenoxyacetic acid[101,102] (Novabiochem, Switzerland) for synthesis of **16** and **22**, whereas a resin carrying the *C*-terminal tyrosine on a *p*-hydroxymethylphenoxy linker (TentaGel S PHB™, Rapp Polymere, Germany) was used for **17**. *N*α-Fmoc-amino acids (Bachem, Switzerland) with the following protective groups were used: 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) for arginine; triphenylmethyl (Trt) for asparagine, cysteine, glutamine, and histidine; *tert*-butoxycarbonyl (Boc) for lysine; and *tert*-butyl for glutamic acid and tyrosine. DMF was distilled before being used.

In the synthesis of glycopeptide **16** 60 μmol of resin was used in the peptide synthesizer. The *N*α-Fmoc-amino acids and acetic acid were activated as 1-benzotriazolyl esters.[103] These were prepared in situ by reaction of the appropriate *N*α-Fmoc-amino acid or acetic acid (0.24 mmol), 1-hydroxybenzotriazole (HOBt) (0.42 mmol) and 1,3-diisopropylcarbodiimide (0.23 mmol) in DMF (1.3 mL). After 45 min bromophenol blue (45 nmol, 0.3 mL of a 0.15 mM solution in DMF) was added to the 1-benzotriazolyl ester solution which was then recirculated through the column containing the resin. The acylation was monitored[88] using the absorbance of bromophenol blue at 600 nm, and the peptide-resin was automatically washed with DMF after 1 h or when monitoring revealed the coupling to be complete. *N*α-Fmoc deprotection of the peptide resin was performed by a flow of 20% piperidine in DMF (2 mL/min) through the column for 12.5-17.5 min, and was monitored[89] using the absorbance of the dibenzofulvene-piperidine adduct at 350 nm. After completion of the *N*α-Fmoc deprotection the peptide-resin was again washed automatically with DMF. The glycosylated amino acid **9** (72 μmol) was activated separately in a minimal volume of DMF at rt during 35 min by addition of 1,3-diisopropylcarbodiimide (72 μmol) and 1-hydroxy-7-azabenzotriazole[86] (HOAt, 0.22 mmol). Compound **9** was then coupled manually to the peptide resin which had been removed from the synthesizer. The coupling of **9** was performed in a mechanically agitated reactor during 24 h, and it was monitored by bromophenol blue as described above. After coupling of **9** the glycopeptide resin was reinserted in the synthesizer and coupling of the remaining amino acids and acetic acid was performed as outlined above.

Glycopeptides **17** and **22** were synthesized essentially as described for **16** using 20 and 40 μmol of resin, respectively. In the synthesis of **17** and **22**, the glycosylated building blocks **9** (24 μmol) and **15** (42 μmol) were activated as 1-(7-azabenzotriazolyl) esters,[86] which were then coupled manually to the peptide resins during 24 h, as described above for the synthesis of **16**. After coupling of **15** unreacted amino groups were capped by addition of acetic anhydride.

After completion of the synthesis, the resins carrying the protected glycopeptides **16**, **17** and **22** were washed with CH_2Cl_2 (5 x 5 mL) and dried under vacuum. For each glycopeptide-resin the glycopeptide was then cleaved from a portion of the peptide-resin (*c.f.* details given for each glycopeptide), the amino acid side chains were deprotected, and acid-labile carbohydrate protective groups were removed, by treatment with trifluoroacetic acid-water-thioanisole-ethanedithiol (87.5:5:5:2.5, 20 mL/200 mg of glycopeptide resin) for 2-2.5 h followed by filtration. Acetic acid (10 mL) was added to the filtrate, the solution was concentrated, and acetic acid (15 mL) was added again followed by concentration. The residue was triturated with diethyl ether (10 mL)

which gave a solid, crude glycopeptide which was dissolved in a mixture of acetic acid and water (20 mL) and freeze dried. Purification by preparative HPLC gave pure **16**, **17**, and **20**. The *O*-acetyl groups of **20** were removed by treatment with methanolic sodium methoxide (2.5 mM, pH ~8 on dry pH-paper) for 75 min to give **21**. The methyl ester of **21** was hydrolyzed with aqueous sodium hydroxide (2 mM, pH ~8-9) during 65 min, and purification by preparative HPLC then gave pure **22**.

The glycopeptides were analyzed on a Kromasil C-8 column (100 Å, 5 μm, 4.6 x 250 mm) using a linear gradient of 0→80% of *B* in *A* over 60 min with a flow rate of 1.5 mL/min and detection at 214 nm (solvent systems *A*: 0.1% aqueous trifluoroacetic acid and *B*: 0.1% trifluoroacetic acid in CH_3CN). Purification of the crude glycopeptides was performed on a Kromasil C-8 column (100 Å, 5 μm, 20 x 250 mm) using the same eluant and flow rates of 10-11 mL/min.

In calculating the final yields for the glycopeptides the peptide content of the purified glycopeptide, as determined by amino acid analysis, has been taken into account. For example, 13.0 mg of **16** with a peptide content of 72% was obtained and the yield was therefore based on 9.36 mg (13.0 mg x 0.72).

4-Methylphenyl 2-azido-2-deoxy-1-thio-β-D-*galactopyranoside* (**4**).—A solution of **3**[47,46] (1.64 g, 4.16 mmol) in $CHCl_3$ (8.2 mL) was added to a solution of *para*-thiocresol (0.568 g, 4.58 mmol) and NaOH (0.250 g, 6.24 mmol) in EtOH (16.3 mL), and the mixture was stirred at room temperature for 55 min. The turbid solution was diluted with CH_2Cl_2 (40 mL) and washed with a mixture of brine and saturated aqueous $NaHCO_3$ (160 mL, 1:1). The aqueous phase was extracted with CH_2Cl_2 (2x40 mL) and EtOAc (4x40 mL), and the combined organic phases were dried, filtered, and concentrated. Flash column chromatography (EtOAc) of the residue gave **4** (0.88 g, 68%): $[\alpha]^{25}_D$ +29° (*c* 0.50, MeOH), lit.[52] +23° (*c* not given, MeOH); ^{13}C NMR data (CD_3OD) were in agreement with those published.[52]

4-Methylphenyl 2-azido-2-deoxy-3,6-di-O-*tert-butyldimethylsilyl-1-thio*-β-D-*galactopyranoside* (**5**).—Imidazole (2.49 g, 36.6 mmol), *tert*-butyldimethylsilyl chloride (5.09 g, 33.8 mmol), and **4** (1.76 g, 5.63 mmol) were stirred in freshly distilled DMF (33 mL) at rt for 1 h and then at 50 °C for a further 3 h. The mixture was diluted with CH_2Cl_2 (200 mL) and washed with saturated aqueous NH_4Cl (200 mL). The aqueous phase was extracted with CH_2Cl_2 (2x80 mL) and the combined organic phases were dried, filtered, and concentrated. Flash column chromatography (toluene) of the residue gave **5** (3.02 g, 99%): $[\alpha]^{25}_D$ -4° (*c* 0.40, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 4.33 (d, *J*=9.7 Hz, 1H, H-1), 3.90 (dd, *J*=10.3, 6.4 Hz, 1H, H-6), 3.83 (dd, *J*=10.2, 5.5 Hz, 1H, H-6'), 3.83 (1H, H-4), 3.52 (dd, *J*=9.1, 3.1 Hz, 1H, H-3), 3.47 (t, *J*=9.4 Hz, 1H, H-2), 3.42 (bt, *J*=5.9 Hz, 1H, H-5), 2.47 (t, *J*=1.3 Hz, 1H, OH), 2.33 (s, 3H, CH_3Ph), 0.91 and 0.88 (2s, each 9H, 2 *t*-Bu), 0.16, 0.12, 0.087, and 0.076 (4s, each 3H, 4 $SiCH_3$); ^{13}C NMR ($CDCl_3$) δ 138.2, 133.3, 129.7, 128.4, 87.1, 78.4, 77.2, 77.0, 76.8, 75.5, 68.5, 63.4, 62.2, 25.8, 25.7, 21.2, 18.2, 18.0, -4.8, -5.3, and -5.5; HRMS (FAB): calcd for $C_{25}H_{46}O_4N_3SSi_2$ 540.2747 ($M+H^+$), found 540.2751. Anal. Calcd for $C_{25}H_{45}O_4N_3SSi_2$: C, 55.6; H, 8.2; N, 7.8. Found: C, 55.2; H, 8.2; N, 7.7.

N$^{\alpha}$-*Fluoren-9-ylmethoxycarbonyl-3*-O-*(2-acetamido-3,6-di*-O-*tert-butyldimethylsilyl-2-deoxy*-α-D-*galactopyranosyl)*-L-*threonine benzyl ester* (**8**).—A solution of NBS (55.4 mg, 311 μmol) and tetrabutylammonium triflate[54] (24.4 mg, 62.2 μmol) in CH_2Cl_2 (1.5 mL) was added dropwise during 2.5 min to a solution of **5** (168 mg, 311 μmol) and **6** (201 mg, 467 μmol) in CH_2Cl_2 (7.5 mL) under N_2 at -28 °C.

Triethylamine (173 μL, 1.24 mmol) was added after 50 min and the solution was allowed to attain rt. Flash column chromatography of the crude reaction mixture (toluene—CH_3CN, 30:1→20:1, 0.5% triethyl amine) gave **7** as an inseparable mixture of α- and β-glycosides (186 mg, 71%). Freshly distilled thioacetic acid[59] (10 mL) was added to a solution of **7** (380 mg, 449 μmol) in pyridine (10 mL) at 0 °C, and the solution was allowed to attain rt. After 4 h toluene was added and the mixture was concentrated. The residue was concentrated twice from toluene. Flash column chromatography (heptane—EtOAc, 2:1→3:2) of the residue gave **8** as an α/β mixture (350 mg, 90%). The two anomers were separated by normal phase HPLC (gradient 0→15% EtOH in hexane fraction during 80 min) to give the β-glycoside (51 mg, 13% from **7**), and **8** (258 mg, 67% from **7**): $[\alpha]^{25}_D$ +45° (*c* 0.59, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 5.61 (d, *J*=10.1 Hz, 1H, AcHN), 5.38 (d, *J*=9.4 Hz, 1H, NH-α), 5.19 (ABd, *J*=12.0 Hz, 1H, $PhCH_2$), 5.08 (ABd, *J*=11.9 Hz, 1H, $PhCH_2$), 4.64 (d, *J*=3.4 Hz, 1H, H-1), 4.49 (d, *J*=6.8 Hz, 2H, Fmoc OCH_2CH), 4.39 (bd, *J*=10.0 Hz, 1H, H-α), 4.26 (t, *J*=6.6 Hz, 1H, Fmoc OCH_2CH), 4.13 (m, 1H, H-β), 3.68 (dd, *J*=9.9, 2.2 Hz, 1H, H-3), 2.47 (s, 1H, OH), 2.01 (s, 1H, Ac), 1.30 (d, *J*=6.3 Hz, 3H, H-γ), 0.89 and 0.88 (2s, each 9H, 2 *t*-Bu), 0.11, 0.097, 0.064, and 0.058 (4s, each 3H, 4 $SiCH_3$); ^{13}C NMR ($CDCl_3$) δ 171.2, 169.8, 156.4, 143.8, 143.6, 141.4, 134.4, 129.0, 128.9, 128.5, 127.8, 127.1, 125.0, 120.1, 100.8, 71.2, 70.8, 69.2, 67.7, 67.1, 62.5, 58.7, 49.4, 47.3, 25.9, 25.6, 23.5, 18.6, 18.3, 17.9, -4.5, -4.7, -5.3, and -5.5; HRMS (FAB): calcd for $C_{46}H_{67}O_{10}N_2Si_2$ 863.4335 ($M+H^+$), found 863.4343. Anal. Calcd for $C_{46}H_{66}O_{10}N_2Si_2$: C, 64.0; H, 7.7; N, 3.2. Found: C, 64.2; H, 7.5; N, 3.2.

N$^{\alpha}$-*Fluoren-9-ylmethoxycarbonyl-3-*O*-(2-acetamido-3,6-di-*O*-tert-butyldimethylsilyl-2-deoxy-*α*-*D*-galactopyranosyl)-*L*-threonine* (**9**).—Hydrogenolysis of **8** (63 mg, 73 μmol) was performed over 10% Pd-C (30 mg) at 1 atm in EtOAc (4 mL) for 2.5 h. The mixture was then filtered through celite which was washed with EtOAc and MeOH. The combined filtrates were concentrated and flash column chromatography (toluene—EtOH, 10:1) of the residue gave **9** (51 mg, 90%): $[\alpha]^{25}_D$ +82° (*c* 0.45, $CHCl_3$); HRMS (FAB): calcd for $C_{39}H_{61}O_{10}N_2Si_2$ 773.3864 ($M+H^+$), found 773.3873.

N$^{\alpha}$-*Fluoren-9-ylmethoxycarbonyl-3-*O*-(2-acetamido-3,6-di-*O*-tert-butyldimethylsilyl-2-deoxy-*α*-*D*-galactopyranosyl)-*L*-threonine methyl ester* (**10**).—Trimethylsilyldiazomethane[62] (14.8 μL, ~2 M in hexane, 30 μmol) was added to a stirred solution of **9** (17.6 mg, 22.8 μmol) in MeOH (0.1 mL) and CH_2Cl_2 (0.3 mL) at rt. After 40 min a few drops of acetic acid was added and the mixture was concentrated. Flash column chromatography (heptane—EtOAc, 1:1) of the residue gave **10** (16.0 mg, 89%): $[\alpha]^{25}_D$ +56° (*c* 0.27, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 5.64 (d, *J*=10.1 Hz, 1H, AcHN), 5.40 (d, *J*=9.4 Hz, 1H, NH-α), 4.74 (d, *J*=3.7 Hz, 1H, H-1), 4.50 (d, *J*=6.6 Hz, 2H, Fmoc OCH_2CH), 4.41 (dt, *J*=10.3, 3.6 Hz, 1H, H-2), 4.37 (dd, *J*=9.5, 2.3 Hz, 1H, H-α), 4.26 (t, *J*=6.6 Hz, 1H, Fmoc OCH_2CH), 4.16 (m, 1H, H-β), 3.74 (s, 3H, CO_2Me), 3.69 (dd, *J*=10.3, 2.9 Hz, 1H, H-3), 2.52 (s, 1H, OH), 2.02 (s, 3H, Ac), 1.32 (d, *J*=6.3 Hz, 3H, H-γ), 0.89 and 0.88 (2s, each 9H, 2 *t*-Bu), 0.12, 0.098, 0.073, and 0.071 (4s, each 3H, 4 $SiCH_3$); ^{13}C NMR ($CDCl_3$) δ 171.8, 169.8, 156.4, 155.9, 143.8, 143.6, 141.4, 127.8, 127.1, 124.9, 120.0, 100.8, 71.2, 70.7, 69.2, 67.1, 62.5, 58.6, 52.6, 49.4, 47.3, 25.9, 25.6, 23.4, 18.4, 18.3, 17.9, -4.5, -4.7, -5.3, and -5.5; HRMS (FAB): calcd for $C_{40}H_{63}O_{10}N_2Si_2$ 787.4022 ($M+H^+$), found 787.3997. Anal. Calcd for $C_{40}H_{62}O_{10}N_2Si_2$: C, 61.0; H, 7.9; N, 3.6. Found: C, 61.0; H, 8.0; N, 3.4.

N$^{\alpha}$-*Fluoren-9-ylmethoxycarbonyl-3*-O-*(2-acetamido-2-deoxy-3,4*-O-*isopropylidene*-α-D-*galactopyranosyl)*-L-*threonine benzyl ester* (**12**).—A solution of **8** (250 mg, 290 μmol) in acetic acid (20 mL), water (6.5 mL), and THF (6.5 mL) was stirred at 55-60 °C for 22.5 h. The solution was concentrated and residual water was removed by coevaporation with toluene. The residue was suspended in dimethoxypropane (6 mL) and *para*-toluenesulfonic acid was added until pH≈2. The mixture was stirred at rt for 65 h, triethylamine (200 μL, 1.4 mmol) was then added to the clear solution, and stirring was continued for 20 min. Toluene was added and the mixture was concentrated. Residual triethylamine was removed by coevaporation with toluene. The residue was dissolved in MeOH—water (11 mL, 10:1) and after refluxing for 3.5 h the solution was concentrated, and residual water was removed by coevaporation with toluene. Flash column chromatography (heptane—EtOAc, 3:5) of the residue gave **12** (166 mg, 85%): $[\alpha]^{25}_{D}$ +78° (*c* 0.33, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 5.69 (d, *J*=9.6 Hz, 1H, AcHN), 5.39 (d, *J*=9.1 Hz, 1H, NH-α), 5.20 (ABd, *J*=11.9 Hz, 1H, $PhCH_2$), 5.10 (d, *J*=11.8 Hz, 1H, $PhCH_2$), 4.73 (d, *J*=2.6 Hz, 1H, H-1), 4.49 (d, *J*=6.4 Hz, 2H, Fmoc O*C*H_2CH), 4.41 (bd, *J*=9.2 Hz, 1H, H-α), 4.07 (m, 1H, H-5), 3.81 (m, 1H, H-6), 2.10 (dd, *J*=8.8, 6.6 Hz, 1H, OH), 2.01 (s, 3H, Ac), 1.58 and 1.34 (2s, each 3H, isopropylidene CH_3), 1.27 (d, *J*=6.0 Hz, 3H, H-γ); ^{13}C NMR ($CDCl_3$) δ 170.8, 170.2, 156.3, 143.8, 143.6, 141.4, 134.4, 129.0, 128.9, 128.5, 127.8, 127.1, 124.9, 120.0, 110.1, 100.0, 74.5, 73.3, 68.1, 67.7, 67.1, 62.8, 58.5, 50.4, 47.2, 27.9, 26.6, 23.3, and 18.3; HRMS (FAB): calcd for $C_{37}H_{43}O_{10}N_2$ 675.2918 (M+H$^+$), found 675.2918. Anal. Calcd for $C_{37}H_{42}O_{10}N_2$: C, 65.9; H, 6.3; N, 4.2. Found: C, 65.6; H, 6.2; N, 4.0.

N$^{\alpha}$-*Fluoren-9-ylmethoxycarbonyl-3*-O-*{2-acetamido-2-deoxy-3,4*-O-*isopropylidene-6*-O-*[methyl (5-acetamido-4,7,8,9-tetra*-O-*acetyl-3,5-dideoxy*-D-*glycero*-α-D-*galacto-2-nonulopyranosyl)onate]*-α-D-*galactopyranosyl}*-L-*threonine benzyl ester* (**14**).—A mixture of **12** (124 mg, 184 μmol), **13** (219 mg, 368 μmol), and molecular sieves (180 mg, 3Å) in CH_3CN (3 mL) and CH_2Cl_2 (2 mL) was stirred under N_2 at rt for 30 min. Silver triflate (118 mg, 459 μmol) was added and the mixture was cooled to -78 °C and protected from light. Methylsulfenyl bromide (92 μL, 4 M in 1,2-dichloroethane, 368 μmol) was added and after 80 min the reaction was quenched by addition of triethylamine (450 μL, 3.2 mmol). After a further 70 min at -78 °C the mixture was allowed to attain rt. Flash column chromatography (toluene—acetone, 3:2) of the crude reaction mixture gave **14** (179 mg) which was slightly contaminated by the corresponding β-glycoside and additional minor impurities. Purification was achieved by normal phase HPLC (gradient 0→30% EtOH in hexane fraction during 110 min) to give pure **14** (103 mg, 49%): $[\alpha]^{25}_{D}$ +39° (*c* 0.25, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 5.72 (d, *J*=9.6 Hz, 1H, AcHN), 5.49 (d, *J*=9.5 Hz, 1H, NH-α), 5.36 (ddd, *J*=8.1, 6.0, 2.7 Hz, 1H, H-8'), 5.31 (dd, *J*=7.5, 1.9 Hz, 1H, H-7'), 5.20 (d, *J*=9.3 Hz, 1H, AcHN'), 5.19 (ABd, *J*=12.0 Hz, 1H, $PhCH_2$), 5.08 (ABd, *J*=11.9 Hz, 1H, $PhCH_2$), 4.90 (ddd, *J*= 12.0, 9.6, 4.8 Hz, 1H, H-4'), 4.67 (d, *J*=3.2 Hz, 1H, H-1), 4.48 (m, 2H, Fmoc O*C*H_2CH), 4.38 (bd, *J*=8.2 Hz, 1H, H-α), 4.34 (dd, *J*=12.4, 2.7 Hz, 1H, H-9'), 4.24 (t, *J*=6.6 Hz, 1H, Fmoc OCH_2CH), 4.20 (dd, *J*=6.1, 2.9 Hz, 1H, H-2), 4.11 (dd, *J*=12.4, 5.4 Hz, 1H, H-9'), 3.95 (dd, *J*=9.0, 4.8 Hz, 1H, H-3), 3.92 (dd, *J*=10.2, 6.9 Hz, 1H, H-6), 3.78 (s, 1H, CO_2Me), 3.70 (dd, *J*=10.2, 5.4 Hz, 1H, H-6), 2.59 (dd, *J*=12.9, 4.7 Hz, 1H, H-3'eq), 2.11 and 2.10 (2s, each 3H, 2 Ac), 2.01 (s, 9H, 3 Ac), 1.96 (t, *J*=12.4 Hz, $J_{H3'ax\text{-}C\text{-}1'}$=6.2 Hz, 1H, H-3'ax), 1.87 (s, 3H, Ac), 1.56 and 1.42 (2s, each 3H, isopropylidene CH_3), and 1.29 (d, *J*=6.2 Hz, 3H, H-γ); ^{13}C NMR ($CDCl_3$) δ 171.0, 170.7, 170.3, 170.2, 170.1, 167.9 ($J_{H3'ax\text{-}C\text{-}1'}$=6.2 Hz, C-1'), 156.5, 143.8, 143.6, 141.4, 134.5, 128.9, 128.9, 128.5, 127.8, 127.1, 125.0, 120.0, 109.7, 100.3, 98.8, 74.4, 72.8, 72.4, 69.2, 69.1, 67.7, 67.6, 67.1, 66.9, 63.7,

62.4, 58.7, 52.8, 50.3, 49.4, 47.3, 37.3, 27.9, 26.6, 23.4, 23.2, 21.0, 20.8, 20.8, 20.7, and 18.3; HRMS (FAB): calcd for $C_{57}H_{70}O_{22}N_3$ 1148.4451 (M+H$^+$), found 1148.4453. Anal. Calcd for $C_{57}H_{69}O_{22}N_3$: C, 59.6; H, 6.1; N, 3.7. Found: C, 59.4; H, 6.0; N, 3.6.

N$^{\alpha}$-*Fluoren-9-ylmethoxycarbonyl-3*-O-*{2-acetamido-2-deoxy-3,4*-O-*isopropylidene-6*-O-*[methyl (5-acetamido-4,7,8,9-tetra*-O-*acetyl-3,5-dideoxy*-D-*glycero*-α-D-*galacto-2-nonulopyranosyl)onate]*-α-D-*galactopyranosyl}*-L-*threonine* (**15**).—Hydrogenolysis of **14** (28.6 mg, 23.3 μmol) was performed over 10% Pd-C (10 mg) at 1 atm in EtOAc (2.5 mL) for 4 h. Additional Pd-C (5 mg) was then added and the reaction was continued for another 4.5 h. The mixture was filtered through celite which was washed with EtOAc, EtOH, and MeOH. The combined filtrates were concentrated and flash column chromatography (CH_2Cl_2—EtOH, 12:1→2:1) of the residue gave **15** (21.6 mg, 88%): $[\alpha]^{25}_D$ +51° (*c* 0.38, $CHCl_3$); ^{1}H NMR (CD_3OD) δ 5.42 (ddd, *J*=8.2, 5.5, 2.7 Hz, 1H, H-8'), 5.34 (dd, *J*=8.2, 2.1 Hz, 1H, H-7'), 4.55 (ABdd, *J*=10.7, 6.5 Hz, 1H, Fmoc OC*H*$_2$CH), 4.46 (ABdd, *J*=10.8, 6.2 Hz, 1H, Fmoc C*H*$_2$CH), 4.34 (dd, *J*=12.3, 2.7 Hz, 2H, H-9', H-β), 4.26 (t, *J*=6.3 Hz, 1H, Fmoc CH$_2$C*H*), 3.99 (t, *J*=10.5 Hz, 1H, H-5'), 3.96 (dd, *J*=9.9, 6.5 Hz, 1H, H-6), 3.83 (s, 3H, CO_2Me), 3.67 (dd, *J*=10.0, 5.8 Hz, 1H, H-6), 2.67 (dd, *J*=12.7, 4.8 Hz, 1H, H-3'eq), 2.13, 2.08, 2.00, 1.99, and 1.98 (5s, each 3H, 5Ac), 1.86 (t, *J*=12.5 Hz, 1H, H-3'ax), 1.84 (s, 3H, Ac), 1.48 and 1.33 (2s, each 3H, isopropylidene CH_3), and 1.23 (d, *J*=6.4 Hz, 3H, H-γ); ^{13}C NMR (CD_3OD) δ 173.8, 173.6, 172.5, 171.9, 171.8, 171.7, 169.5, 159.2, 145.6, 145.3, 142.8, 130.1, 129.4, 129.0, 128.9, 128.4, 128.3, 126.3, 126.2, 121.1, 110.7, 100.8, 100.2, 78.0, 75.2, 74.0, 73.6, 70.9, 70.1, 68.9, 68.0, 67.8, 65.2, 63.5, 60.6, 54.6, 53.5, 52.3, 50.3, 39.0, 28.6, 27.0, 23.2, 22.8, 21.6, 21.3, 21.0, 20.9, 20.8, and 19.5; HRMS (FAB): calcd for $C_{50}H_{64}O_{22}N_3$ 1058.3981 (M+H$^+$), found 1058.3961.

N$^{\alpha}$-*Acetyl*-L-*cysteinyl*-L-*lysyl*-L-*isoleucyl*-L-*glutam-1-yl*-L-*prolyl*-L-*leucyl-glycyl*-L-*valyl*-L-*alanyl*-L-*prolyl-3*-O-*(2-acetamido-2-deoxy*-α-D-*galactopyranosyl)*-L-*threonyl*-L-*lysyl*-L-*alanyl*-L-*lysyl*-L-*arginyl*-L-*arginine amide* (**16**).—Synthesis, cleavage of the resin-bound glycopeptide (150 mg, 21 μmol) with simultaneous deprotection, and then purification by reversed-phase HPLC (gradient 0→80% *B* in *A* during 80 min), according to the general procedure, gave **16** (13.0 mg, 72% peptide content, 22% overall yield). ^{1}H NMR data have been published previously.[17] MS (FAB): calcd 2011 (M+H$^+$), found 2011; amino acid analysis: Ala 1.96 (2), Arg 1.99 (2), Cys 1.04 (1), Glu 1.01 (1), Gly 1.00 (1), Ile 0.99 (1), Leu 1.01 (1), Lys 3.03 (3), Pro 1.91 (2), Thr 1.02 (1), and Val 1.03 (1).

3-O-*(2-acetamido-2-deoxy*-α-D-*galactopyranosyl)*-L-*threonyl*-L-*prolyl*-L-*glutam-1-yl*-L-*leucyl*-L-*phenylalanyl*-L-*glutam-1-yl*-L-*alanyl*-L-*leucyl*-L-*glutaminyl*-L-*lysyl*-L-*leucyl*-L-*phenylalanyl*-L-*lysyl*-L-*histidinyl*-L-*alanyl*-L-*tyrosine* (**17**) and L-*prolyl*-L-*glutam-1-yl*-L-*leucyl*-L-*phenylalanyl*-L-*glutam-1-yl*-L-*alanyl*-L-*leucyl*-L-*glutaminyl*-L-*lysyl*-L-*leucyl*-L-*phenylalanyl*-L-*lysyl*-L-*histidinyl*-L-*alanyl*-L-*tyrosine* (**18**).—Synthesis, cleavage of the resin-bound glycopeptide (163 mg, 20 μmol) with simultaneous deprotection, and then purification by reversed-phase HPLC (gradient 30→80% *B* in *A* during 190 min), according to the general procedure, gave **17** (12.7 mg, 77% peptide content, 23% overall yield) and **18** (7.4 mg). Data for glycopeptide **17**: ^{1}H NMR data, see Table 2; MS (FAB): calcd 2138 (M+H$^+$), found 2138; amino acid analysis: Ala 2.04 (2), Glu 3.00 (3), His 1.01 (1), Lys 2.00 (2), Leu 2.93 (3), Phe 2.00 (2), Pro 0.99 (1), Thr 1.01 (1), and Tyr 1.02 (1). MS (FAB) for peptide **18**: calcd 1834 (M+H$^+$), found 1834.

Table 2. ^{1}H NMR Data (δ, ppm) for Glycopeptide **17** in Water Containing 10% D_2O[a]

Residue	NH	H-α	H-β	H-γ	H-δ	Others
Thr[1]		4.25	4.32	1.40		GalNAcα[b]
Pro[2]		4.44	2.23, 1.78	1.91[c]	3.62, 3.54	
Glu[3]	8.46	4.02	1.86[c]	2.35, 2.22		
Leu[4]	8.49	4.20	1.48[c]	1.46	0.82, 0.76	
Phe[5]	8.42	4.42	2.98[c]			7.24, 7.14 (arom.)
Glu[6]	8.23	4.13	1.90, 1.83	2.30[c]		
Ala[7]	8.24	4.07	1.32			
Leu[8]	8.17	4.16	1.59[c]	1.56	0.84, 0.79	
Gln[9]	8.23	4.10	1.96, 1.89	2.24		7.52, 6.90 ($CONH_2$)
Lys[10][d]	8.23	4.12	1.65[c]	1.25[c]	1.56[c]	2.87[c] (Hε), 7.52 (ε-NH_2)
Leu[11]	8.05	4.21	1.48[c]	1.44	0.80, 0.74	
Phe[12]	8.20	4.50	2.99, 2.89			7.22, 7.12 (arom.)
Lys[13][d]	8.23	4.13	1.58[c]	1.25[c]	1.56[c]	2.87[c] (Hε), 7.52 (ε-NH_2)
His[14]	8.48	4.46	3.00[c]			7.01 (arom.)
Ala[15]	8.43	4.21	1.23			
Tyr[16]	8.26	4.46	3.05, 2.86			7.07, 6.72 (arom.)

[a]Obtained at 500 MHz, 278 K and pH = 2.5 with H_2O as internal standard (δ_H 4.98 ppm). [b]Chemical shifts (δ, ppm) for the *N*-acetylgalactosamine moiety: 7.79 (NH), 4.02 (H-2), 3.88 (H-4), 3.75 (H-3), 1.92 (Ac). [c]Degeneracy has been assumed. [d]The assignment of these residues may be interchanged.

N$^{\alpha}$-*Acetyl-glycyl*-L-*arginyl*-L-*alanyl*-L-*phenylalanyl*-L-*valyl-3*-O-*[2-acetamido-2-deoxy-6*-O-*(5-acetamido-3,5-dideoxy*-D-*glycero*-α-D-*galacto-2-nonulopyranosylonic acid)*-α-D-*galactopyranosyl]*-L-*threonyl*-L-*isoleucyl-glycyl*-L-*lysyl*-L-*isoleucyl-glycyl*-L-*asparaginyl*-L-*methionyl*-L-*arginyl*-L-*glutaminyl*-L-*alaninyl*-L-*cysteine amide* (**22**) *and* N$^{\alpha}$-*acetyl*-L-*isoleucyl-glycyl*-L-*lysyl*-L-*isoleucyl-glycyl*-L-*asparaginyl*-L-*methionyl*-L-*arginyl*-L-*glutaminyl*-L-*alaninyl*-L-*cysteine amide* (**24**).—Synthesis, cleavage of the resin-bound glycopeptide (100 mg, 16.8 μmol) with simultaneous deprotection, and then purification by reversed-phase HPLC (gradient 0→20% *B* in *A* during 10 min followed by 20→80% *B* in *A* during 120 min), according to general procedure, gave **20** (12.8 mg) and **24** (4.0 mg). Sodium methoxide (20 μL, 20 μmol, 1M in methanol) was added to a solution of **20** (8 mg) in degassed methanol (8 mL) and the solution (pH≈8 on dry pH paper) was stirred under Ar at rt for 77 min. Acetic acid in methanol (1:40) was added until pH≈6 and the solution was concentrated under vacuum at <30 °C to give crude **21**. The residue was dissolved in degassed water (8 mL), aqueous 0.1 M sodium hydroxide (160 μL, 16 μmol) was added, and the solution (pH≈9) was stirred under Ar at rt for 65 min. Acetic acid in water (1:40) was added until pH≈5 and the mixture was freeze dried. The crude product was purified by reversed-phase HPLC (gradient 0→15% *B* in *A* during 10 min followed by 15→80% *B* in *A* during 120 min) to give **22** (5.1 mg, 60% peptide content, 12% overall yield) and the corresponding disulfide **23** (0.5 mg). MS (FAB) for glycopeptide **20**: calcd 2539 (M+H$^+$), found 2540. MS (FAB) for glycopeptide **21**: calcd 2371 (M+H$^+$), found 2372. Data for glycopeptide **22**: ^{1}H NMR data, see Table 3; MS (FAB): calcd 2357

($M+H^+$), found 2356; amino acid analysis: Ala 1.96 (2), Arg 2.00 (2), Asp 1.02 (1), Cys 0.98 (1), Glu 1.02 (1), Gly 3.02 (3), Ile 1.95 (2), Lys 1.04 (1), Met 0.97 (1), Phe 1.03 (1), Thr 0.98 (1), and Val 1.01 (1). MS (MALDI) for glycopeptide **23**: calcd 4712 ($M+H^+$), found: 4720, 4724, and 4725 in three different analyses. MS (FAB) for peptide **24**: calcd 1232 ($M+H^+$), found 1231.

Table 3. ^1H NMR Data (δ, ppm) for Glycopeptide **22** in Water Containing 10% D_2O[a]

Residue	NH	H-α	H-β	H-γ	H-δ	Others
Gly^{312}	8.40	3.89[b]				2.01 (NAc)
Arg^{313}	8.35	4.28	1.73[b]	1.56[b]	3.14[b]	7.19 (δ-NH)
Ala^{314}	8.41	4.24	1.27			
Phe^{315}	8.26	4.61	3.08, 3.00			7.32, 7.26, 7.22 (arom.)
Val^{316}	8.16	4.23	1.98	0.89[b]		
Thr^{317}	8.68	4.55	4.19	1.27		Neu5Acα2,6GalNAcα[c]
Ile^{318}	8.42	4.12	1.88	1.48, 1.20	0.93	0.93 (β-CH_3)
Gly^{319}	8.52	3.97, 3.72				
Lys^{320}	8.44	4.30	1.78[b]	1.41[b]	1.67[b]	2.98[b] (Hε), 7.56 (ε-NH_2)
Ile^{321}	8.36	4.14	1.87	1.48, 1.20	0.90	0.90 (β-CH_3)
Gly^{322}	8.60	3.91[b]				
Asn^{323}	8.40	4.67	2.82, 2.74			7.68, 6.98 ($CONH_2$)
Met^{324}	8.47	4.44	2.08, 2.01	2.60, 2.51		2.03 (SCH_3)
Arg^{325}	8.43	4.26	1.79[b]	1.60[b]	3.16[b]	7.22 (δ-NH)
Gln^{326}	8.48	4.27	2.10, 1.96	2.36[b]		7.60, 6.94 ($CONH_2$)
Ala^{327}	8.57	4.29	1.39			
Cys	8.43	4.45	2.90[b]			7.69, 7.30 ($CONH_2$)

[a]Obtained at 500 MHz, 278 K and pH=3 with H_2O as internal standard (δ_H 4.98 ppm). [b]Degeneracy has been assumed. [c]Chemical shifts (δ, ppm) for the disaccharide moiety; GalNAc: 7.76 (NH), 4.05 (H-2), 3.82 (H-3), 1.99 (Ac); NeuNAc: 8.17 (NH), 4.08 (H-8), 3.91 and 3.54 (H-9,9'), 3.81 (H-5), 3.64 (H-4), 2.70 (H-3eq), 2.00 (Ac), 1.66 (H-3ax).

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