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Chemical synthesis of analogs of the glycopeptide contulakin-G, an analgetically active conopeptide from *Conus geographus*

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Abstract—Cone snails are marine predators that use immobilizing venoms for catching prey. Chemical analysis of the venoms has revealed a variety of biologically active small and intermediate size peptides rich in post-translational modifications (modified amino acids, glycosylation). The glycopeptide contulakin-G (pGlu-Ser-Glu-Glu-Gly-Gly-Ser-Asn-Ala-[β-D-Gal*p*-(1→3)-α-D-Gal*p*NAc-(1→]Thr-Lys-Lys-Pro-Tyr-Ile-Leu-OH) is a potent analgesic from *Conus geographus* venom. The in vivo activity of synthetic contulakin-G was previously found to be significantly higher compared to that of a peptide lacking the glycan. In order to further investigate the importance of the glycan, we have now synthesized analogs of contulakin-G where the glycan chain O-linked to threonine has been altered either to β-D-Gal*p*-(1→3)-β-D-Gal*p*NAc-, α-D-Gal*p*-(1→3)-α-D-Gal*p*NAc-, or β-D-Gal*p*-(1→6)-α-D-Gal*p*-NAc-. The glycopeptides were assembled on a Wang resin using commercially available Fmoc amino acids and synthetically prepared Fmoc-protected threonine derivatives carrying *O*-acetyl protected sugar chains. The final products were thoroughly characterized by NMR and mass spectroscopy.

Keywords: Contulakin-G; Toxin; Peptide; Synthesis; Glycosylated; Glycopeptide; *N*-Acetyl galactosamine; Galactose

1. Introduction

Cone snails are a group of interesting marine predators who catch their prey with the aid of immobilizing venoms. Chemical analysis of the venoms has revealed a great variety of small and intermediate size peptides, each with a distinct and very interesting neurobiological activity.[1] The peptides are rich in post-translational modifications, such as sulfation, hydroxylation (of proline), bromination (of tryptophan), γ-carboxylation (of glutamic acid) and O-glycosylation (of serine and threonine). We have recently reported[2] studies of contulakin-G, a glycopeptide from *Conus geographus* venom. Analysis and chemical synthesis confirmed the structure of contulakin-G as pGlu-Ser-Glu-Glu-Gly-Gly-Ser-Asn-Ala-[β-D-Gal*p*-(1→3)-α-D-Gal*p*NAc-(1→]Thr-Lys-Lys-Pro-Tyr-Ile-Leu-OH (Scheme 1). Contulakin-G is a potent analgesic in a number of pain models[3]. The analgetic activity of synthetic contulakin-G was found to be significantly higher compared to that of its synthetic unglycosylated counterpart. Similar observations[4–8] of differences in biological activity between glycosylated and non-glycosylated peptides or proteins have been made also in other fields. These observations all touch on the general question of the function of glycans of glycopeptides and glycoproteins in Nature. We believe that the *Conus* glycopeptides are among the best model compounds available to address this question, since they have distinct biological activities and are comparatively small molecules which can be readily prepared and modified by chemical synthesis. In addition, the synthetic products can, as has been shown[9] for contulakin-G, be subjected to conformational studies by NMR spectroscopy, which opens up possibilities to understand structure–activity relationships on a more fundamental molecular level. As part of a program directed at synthesis of glycosylated conopeptides and analogs, we have previously[10] synthesized contulakin-G analogs where the sugar chain was either shortened (to α-D-Gal*p*NAc-)

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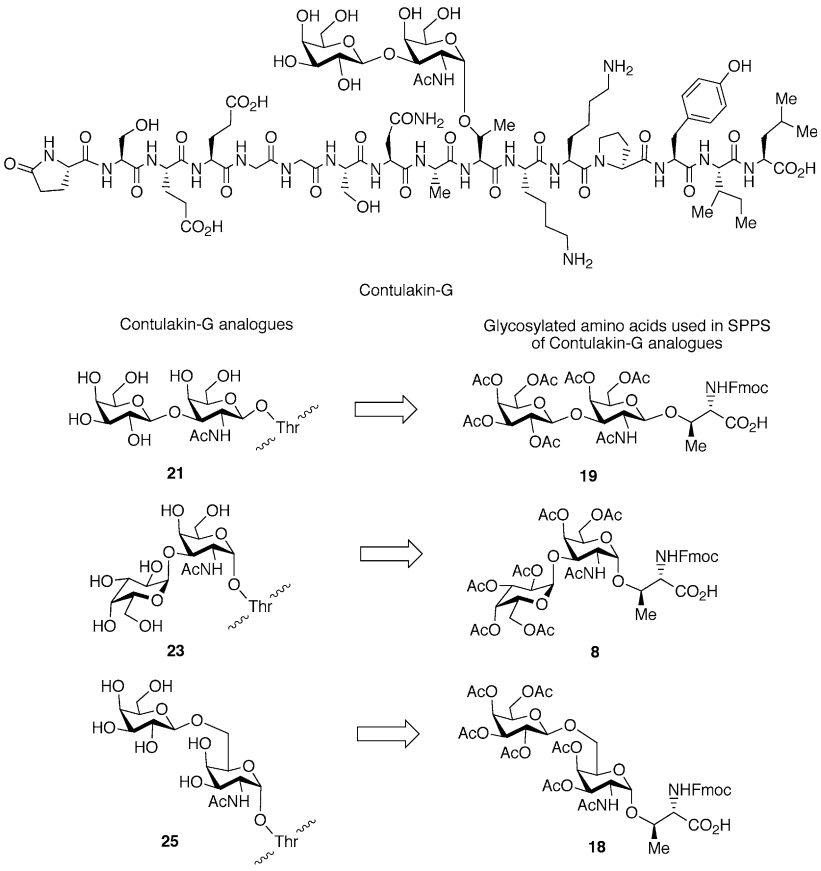


Scheme 1.

or shifted in position (to Ser 7). Both these analogs, as well as a synthetic unglycosylated analog, showed significantly lower analgetic activity in a mouse tail flick latency assay.[10] In an NMR study,[9] subtle differences in conformational preferences between these analogs and native contulakin-G were found. We have now synthesized three new analogs (**21**, **23**, and **25**) of contulakin-G where either an anomeric linkage or the intersugar glycosylation position is altered (see Scheme 1). In compound **21**, the disaccharide linkage to Thr is changed from the native α configuration to β; in compound **23** the interglycosidic linkage is changed from the native β configuration to α; and in compound **25**, the original β-(1→3) intersugar glycosylation position is changed to β-(1→6). This communication reports the chemical synthesis and spectroscopic characterization of **21**, **23**, and **25**. Biological studies and conformational analysis of the synthesized glycopeptides will be reported elsewhere in the near future.

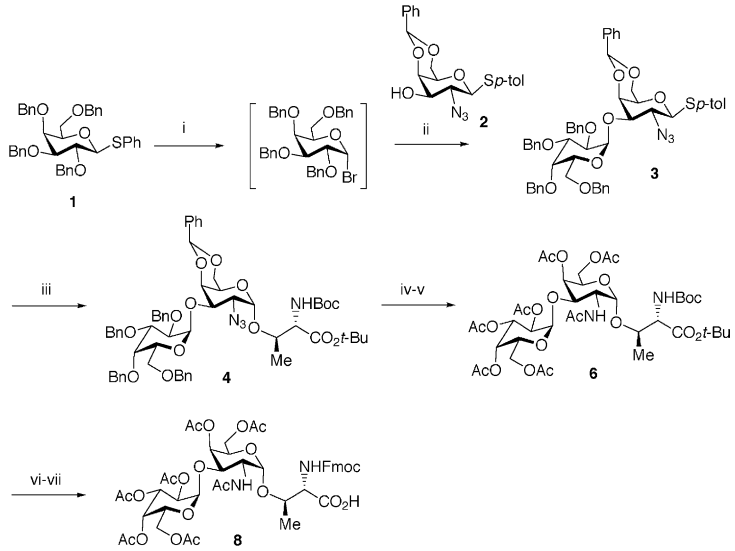
2. Results and discussion

2.1. Synthesis of glycosylated Fmoc amino acids

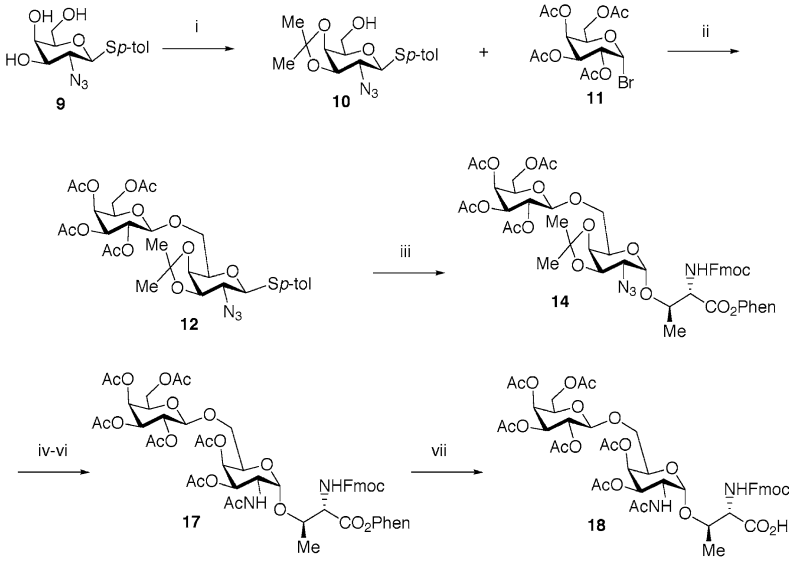
The disaccharide–Fmoc threonine derivative **19** was available from previous work[11], whereas the corresponding derivatives **8** and **18** were novel compounds, and were therefore synthesized as described below.

Derivative **8** (Scheme 2): Brief treatment of phenyl 2,3,4,6-tetra-*O*-benzyl-1-thio-β-D-galactopyranoside[12] (**1**) with bromine in dichloromethane produced the corresponding glycosyl bromide, which was reacted in situ with 4-methylphenyl 2-azido-4,6-*O*-benzylidene-2-deoxy-1-thio-β-D-galactopyranoside[11] (**2**) and tetraethylammonium bromide in the presence of molecular sieves to give the α-linked disaccharide derivative **3** (63%). Glycosylation (DMTST) of Boc-L-threonine *t*-butyl ester[13] with **3** gave, as a major product, the α-linked disaccharide–threonine derivative **4** (31%). For reasons unclear this yield could not be improved, despite several attempts. Catalytic hydrogenation (Pd/C) followed by acetylation with acetic anhydride in pyridine gave **6** (64%), which was treated with TFA in dichloromethane to give **7**, which was reacted in situ with Fmoc-OSu to give the disaccharide–threonine derivative **8** (77%), suitable for the use in peptide synthesis.

Derivative **18** (Scheme 3): Treatment of **9**[11] with 2,2-dimethoxypropane and *p*-toluenesulfonic acid in acetonitrile gave 3,4-isopropylidene acetal **10** as the major product (39%). Glycosylation of **10** with **11**[14] and silver triflate at −30 °C gave the β-linked disaccharide deriva-



Scheme 2. Reagents and conditions: (i) Br_2; (ii) Et_4NBr, 4 Å MS, CH_2Cl_2/DMF, 63%; (iii) DMTST, *N*-Boc-threonine *t*-butylester, 31%; (iv) Pd(C), H_2, EtOH; (v) Ac_2O, pyridine, 64%; (vi) TFA/CH_2Cl_2; (vii) Fmoc-OSu/Na_2CO_3/H_2O/dioxane, 77%.



Scheme 3. Reagents and conditions: (i) $Me_2C(OMe)_2$, CH_3CN, TsOH, 39%; (ii) AgOTf, toluene/CH_2Cl_2, 86%; (iii) DMTST, *N*-Fmoc-threonine phenacylester, 56%; (iv) HOAc, H_2O, 80 °C; (v) Ac_2O, pyridine; (vi) AcSH, rt, 67%; (vii) Zn, HOAc, 89%.

tive **12** (86%). Glycosylation of Fmoc-L-threonine phenacyl ester[11] with **12** promoted by DMTST[15] gave, as a major product, the α-linked disaccharide–threonine derivative **14** (56%). A compound tentatively assigned as the β-linked isomer was also isolated (20%). Treatment of **14** with successively, aq acetic acid (to remove 3,4-isopropylidene acetal), pyridine/acetic anhydride, and thioacetic acid (to convert the azido function to acetamido) gave compound **17** (67%). Finally, phenacyl ester was removed by treatment with zinc in acetic acid to give the disaccharide–threonine derivative **18** (89%), suitable for use in peptide synthesis.

2.2. Glycopeptide synthesis

Solid-phase synthesis of **21**, **23**, and **25** was carried out manually using Fmoc chemistry, with *t*-butyl ether side chain protection for tyrosine and serine, *N*-*t*-butoxycarbonyl side chain protection for lysine, and *t*-butyl ester side chain protection for glutamic acid. A small glass vessel with an upper glass stopper and a bottom sintered glass frit was used for the reactions. Agitation was by rotation of the vessel during synthesis of **21**, and by nitrogen bubbling up through the bottom frit during synthesis of **23** and **25**. It was noted that the latter agitation mode

gave considerably better total yields, mainly because of less resin leakage from the vessel. Starting with Leu-Wang resin, the next amino acid (Ileu, 5 equiv), activated with PyBOP/HOBt (5 equiv) and DIPEA (10 equiv) in DMF, was added. The Fmoc group was then removed using 20% piperidine in DMF. The Kaiser ninhydrin test[16] was performed after each coupling and after deprotection of the Fmoc group. The coupling/deprotection cycles were repeated using the appropriate amino acids until the entire peptide sequence had been assembled. Coupling of the glycosylated amino acid was performed with only 2 equiv of activated amino acid. The Kaiser tests indicated that the reactions had gone virtually to completion after 2 h except for the Asn and pGlu couplings, where longer reaction times and repetition of the couplings twice with fresh reagents had to be carried out. After the last coupling cycle, the resin was subjected to cleavage conditions (TFA–H_2O–thioanisole) and the released O-acetylated glycopeptide was preliminary purified. After O-deacetylation with sodium methoxide in methanol the material was finally purified by RP-HPLC. The total yields of pure **21**, **23**, and **25** were 9%, 37%, and 56%, respectively, calculated from the amounts of resin used. The identity of the glycopeptides was verified by mass spectroscopy as well as with ^{1}H NMR spectroscopy. The ^{1}H NMR chemical shifts measured for the amino acid residues in the glycosylated peptides **21**, **23**, and **25** are similar to those[9] in the natural peptide contulakin-G, except for signals from NH and α-H protons near or at the glycosylation site. The amino acid sequence of the peptide was confirmed by sequential assignment using the $d_{\alpha N}(i, i+1)$ NOE connectivities. The site of glycosylation of compounds **21**, **23**, and **25** was confirmed by the existence of a NOE between H-1 of Gal*p*NAc and β-H of Thr-10. The β-glycosidic bond between the glycan and Thr-10 of **21** was confirmed by an upfield chemical shift of H-1 of Gal*p*NAc as compared with native contulakin-G, as well as by the observed large (8–10 Hz) coupling constant. The 1→3 linkage of **21** was confirmed by a NOE interaction between the β-D-Gal*p* anomeric proton and H-3 of β-D-Gal*p*NAc. The glycosidic bond between H-1 of the α-D-Gal*p* and H3 of α-D-Gal*p*NAc of **23** was also verified by a NOE interaction. The downfield chemical shift as well as the small (3.7 Hz) coupling constant of H-1 of α-D-Gal*p* of **23** confirmed the presence of an α-glycosidic bond instead of the natural β. The 1→6 interglycosidic linkage in **25** was confirmed by a NOE interaction between the β-D-Gal*p* anomeric proton and H-6 of α-D-Gal*p*NAc.

3. Experimental

3.1. General methods

Compounds were concentrated under reduced pressure (bath temperature <40 °C). NMR spectra were recorded at 303 K with a Bruker DRX 400 for $CDCl_3$ solutions (internal $CHCl_3$, δ_H 7.26 ppm and δ_C 77.00 ppm at 303 K), unless otherwise stated. Only selected NMR data are reported. Assignments were corroborated by appropriate 2-D experiments. The HRMS and MALDI-MS spectra were recorded with Agilent MSD-TOF and Bruker Reflex 3 instruments, respectively. In the latter case, dihydroxybenzoic acid (DHB) was used as matrix. Optical rotations $[\alpha]_D$ were measured at room temperature (22–24 °C) with a Perkin–Elmer 241 polarimeter. TLC was performed on Silica Gel F_{254} (Merck, Darmstadt, Germany) with detection by UV-light and/or by staining with 5% sulfuric acid in ethanol. Column chromatography was performed on Matrex silica gel 60 Å (35–70 μm, Amicon) unless otherwise stated. Semipreparative HPLC were carried out using a Waters HPLC system consisting of a 600S controller, a 616 gradient pump, a 481 UV detector set at 254 nm, and a Nucleosil C-18 column (10 × 250 mm, 5 μm particle size). Acetonitrile and 0.1% aq TFA was used as solvents. Isolute cartridges (C-18 EC) were from International Sorbent Technology, Mid Glamorgan, UK. Molecular sieves (powdered 4 Å) were dried at 270 °C/0.5 Torr overnight. Dry dichloromethane was prepared by distillation from P_2O_5. Dimethyl(thiomethyl)sulfonium triflate (DMTST) was prepared essentially as previously described,[15] and was stored under dry nitrogen at −20 °C. DMF (amine-free, for peptide synthesis), *N*-hydroxybenzotriazole (HOBt), and *N*,*N*-diisopropylethylamine (DIPEA) was from Sigma–Aldrich (St. Louis, USA). Fmoc-protected amino acids were from Bachem AG (Bubendorf, Switzerland). Benzotriazole-1-yl-oxy-tris-pyrrolidinophosphonium hexafluorophosphate (PyBOP) and Leu-Wang resin were from Novabiochem/Merck Biosciences AG (Switzerland). Other reagents and solvents were purchased with high commercial quality and were used without further purification unless otherwise stated.

3.2. 4-Methylphenyl 2-azido-4,6-*O*-benzylidene-2-deoxy-1-thio-3-*O*-(2,3,4,6-tetra-*O*-benzyl-α-D-galacto-pyranosyl)-β-D-galactopyranoside (3)

Bromine (1.29 mL, 25.38 mmol) was added to a stirred and cooled (ice) mixture of **1**[12] (11.87 g, 18.75 mmol) and dichloromethane (60 mL). After 40 min at 0 °C cyclohexene (a few drops) was added until color indicated no more change and then **2**[11] (3.0 g, 7.50 mmol), molecular sieves (13 g), tetraethylammonium bromide (5.43 g), and DMF (1.50 mL) were added. The mixture was stirred at rt for 48 h. Pyridine (5 mL) was added and the mixture was filtered. The filtrate was washed with aq 1 M sulfuric acid, aq 1 M sodium hydrogen carbonate and water, dried, and concentrated. The residue was purified by column chromatography to give **3**

(4.17 g, 4.69 mmol, 63%), $[\alpha]_D$ +9 (*c* 0.1, $CHCl_3$); ^1H NMR data: δ 5.48 (1H, s, CH benzylidene), 5.17 (1H, d, $J = 3.4$ Hz, H-1′), 4.94 (1H, d, $J = 11.5$ Hz, OBn), 4.81 (1H, d, $J = 11.5$ Hz, OBn), 4.68 (1H, d, $J = 11.5$ Hz, OBn), 4.57 (1H, d, $J = 11.5$ Hz, OBn), 4.53 (1H, d, $J = 11.7$ Hz, OBn), 4.49 (3H, m, OBn), 4.35 (1H, dd, $J = 12.5$, 1.5 Hz, H-6a), 4.26 (2H, m, H-1, H-4), 4.10 (1H, dd, $J = 6.6$, 5.6 Hz, H-5′), 4.04 (2H, m, H-2′, H-3′), 3.98 (1H, dd, $J = 12.5$, 1.5 Hz, H-6b), 3.94 (1H, br d, $J < 1$ Hz, H-4′), 3.88 (1H, dd, $J = 10.0$ Hz, H-2), 3.70 (1H, dd, $J = 10.0$, 3.2 Hz, H-3), 3.60 (1H, dd, $J = 9.8$, 6.6 Hz, H-6a′), 3.47 (1H, dd, $J = 9.8$, 5.6 Hz, H-6b′), 3.26 (1H, m, H-5). HRMS: Calcd for $C_{54}H_{59}N_4O_9S$: 939.39973. Found: 939.39953 ($M+NH_4^+$).

3.3. *N*-*tert*-Butoxycarbonyl *O*-[2-azido-4,6-*O*-benzylidene-2-deoxy-3-*O*-(2,3,4,6-tetra-*O*-benzyl-α-D-galactopyranosyl)-α-D-galactopyranosyl]-L-threonine *tert*-butylester (4)

A mixture of **3** (3.65 g, 4.103 mmol), *N*-*tert*-butoxycarbonyl-L-threonine *tert*-butylester[13] (1.69 g, 6.157 mmol) and powdered 4 Å molecular sieves (13 g) in dry dichloromethane–diethyl ether–toluene (27–20–20 mL) was stirred at rt for 5 min and then DMTST (2.50 g, 9.69 mmol) was added. After 30 min, pyridine (2.69 mL) was added to quench the reaction. The mixture was diluted with diethyl ether and the solids were filtered off. The filtrate was washed with aq 2 M sulfuric acid, aq 1 M sodium hydrogen carbonate, dried (magnesium sulfate), and concentrated. Column chromatography (toluene–ethyl acetate 10:1) of the residue gave **4** (1.37 g, 1.28 mmol, 31%), $[\alpha]_D$ +114 (*c* 0.3, $CHCl_3$); ^1H NMR data: δ 5.41 (2H, m, CH benzylidene, NHThr), 5.28 (1H, d, $J = 2.9$ Hz, H-1′), 5.11 (1H, d, $J = 2.9$ Hz, H-1), 4.95 (1H, d, $J = 11.5$ Hz, OBn), 4.82 (1H, d, $J = 11.7$ Hz, OBn), 4.70 (1H, d, $J = 11.7$ Hz, OBn), 4.58 (3H, m, OBn), 4.51 (2H, abq, $J = 12.0$ Hz, OBn), 4.39 (2H, m, H-4, CHThrβ), 4.22 (1H, dd, $J = 12.7$, $J < 1$, H-6a), 4.17–4.06 (5H, m, H-3, H-2′, H-3′, H-5′, CHThrα), 4.01 (1H, m, H-4′), 3.99 (1H, m, H-6b), 3.93 (1H, dd, $J = 10.5$, 2.9 Hz, H-2), 3.62 (3H, m, H-5, H-6ab′), 1.51 (9H, s, *t*-Bu), 1.45 (9H, s, *t*-Bu), 1.24 (3H, d, $J = 6.6$ Hz, Thr-CH_3). HRMS: Calcd for $C_{60}H_{76}N_5O_{14}$: 1090.53833. Found: 1090.53684 ($M+NH_4^+$).

3.4. *N*-*tert*-Butoxycarbonyl *O*-[2-acetamido-4,6-di-*O*-acetyl-2-deoxy-3-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl)-α-D-galactopyranosyl]-L-threonine *tert*-butylester (6)

A suspension of palladium on carbon (10%, 1.50 g) in ethanol (50 mL) was added to a solution of **2** (1.20 g, 1.119 mmol) in ethanol (50 mL). The mixture was flushed first with nitrogen and then with hydrogen, and stirred for 48 h at rt under a hydrogen atmosphere. After nitrogen flushing, the solution was filtered and concentrated to give **5**. This material was dissolved in pyridine–acetic acid 2:1 (48 mL) and was stirred for 24 h. The residue was then concentrated and co-concentrated with toluene. Column chromatography (toluene–ethyl acetate 1:1) of the residue gave **6** (635 mg, 0.711 mmol, 64%). $[\alpha]_D$ +81 (*c* 0.3, $CHCl_3$); ^1H NMR data: δ 6.54 (1H, d, $J = 10.0$ Hz, NHThr), 6.14 (1H, d, $J = 10.3$ Hz, NHAc), 5.47 (1H, br d, $J = 2.9$ Hz, H-4′), 5.34 (1H, br d, $J = 2.7$ Hz, H-4), 5.30 (2H, m, H-1′, H-2′), 5.10 (1H, dd, $J = 10.5$, 2.9 Hz, H-3′), 4.86 (1H, br d, $J = 3.4$ Hz, H-1), 4.67 (1H, ddd, $J = 11.0$, 10.3, 3.4 Hz, H-2), 4.36 (1H, m, H-6a′), 4.35 (1H, m, H-5′), 4.28 (1H, m, CHThrβ), 4.25 (1H, m, CHThrα), 4.18 (1H, m, H-5), 4.10 (2H, m, H-6ab), 3.99 (1H, dd, $J = 11.0$, 2.7 Hz, H-3), 3.94 (1H, dd, $J = 9.1$Hz, H-6b′), 2.25 (3H, s, OAc), 2.19 (3H, s, OAc), 2.14 (3H, s, OAc), 2.06 (3H, s, OAc), 2.05 (3H, s, OAc), 2.00 (3H, s, NHAc), 1.94 (3H, s, OAc), 1.46 (9H, s, *t*-Bu), 1.44 (9H, s, *t*-Bu), 1.33 (3H, d, $J = 6.4$ Hz, Thr-CH_3). HRMS: Calcd for $C_{39}H_{61}N_2O_{21}$: 893.37613. Found: 893.37643 ($M+H^+$).

3.5. *N*-9-Fluoroenylmethoxycarbonyl *O*-[2-acetamido-4,6-di-*O*-acetyl-2-deoxy-3-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl)-α-D-galactopyranosyl]-L-threonine (8)

A solution of **6** (550 mg, 0.616 mmol) in dry dichloromethane (14 mL) was mixed with TFA (14 mL) and the mixture was stirred at 35 °C for 24 h, then evaporated and coevaporated with toluene to give crude **7**, which was dissolved in a mixture of dioxane (14 mL) and H_2O (14 mL). Solid Na_2CO_3 (160 mg, 1.50 mmol) was added, and the mixture was ultrasonicated for 15 min and was then cooled to 0 °C and Fmoc-OSu (252 mg, 0.747 mmol) was added and stirring was continued at rt for 6 h, after which the material was acidified to pH 1.5 with aq HCl, diluted with water and extracted three times with chloroform. Column chromatography (gradient from 100% chloroform to 20% methanol in chloroform) of the residue gave **8** (456 mg, 0.476 mmol, 77%), $[\alpha]_D$ +80 (*c* 0.4, $CHCl_3$); ^1H NMR data: δ 7.77 (2H, d, $J = 7.3$ Hz, Fmoc), 7.58 (2H, dd, $J = 7.3$, 4.9 Hz, Fmoc), 7.41 (2H, dd, $J = 7.3$ Hz, Fmoc), 7.30 (2H, ddd, $J = 7.3$, 9.5, 3.2 Hz, Fmoc), 6.88 (1H, d, $J = 9.5$ Hz, NHThr), 6.23 (1H, d, $J = 10.3$ Hz, NHAc), 5.41 (1H, br d, $J = 2.7$ Hz, H-4′), 5.36 (1H, br d, $J = 2.5$ Hz, H-4), 5.30 (2H, m, H-1′, H-2′), 5.07 (1H, dd, $J = 10.8$, 2.7 Hz, H-3′), 5.00 (1H, d, $J = 3.9$ Hz, H-1), 4.61 (1H, dd, $J = 10.8$, 6.6 Hz, FmocCH_2), 4.55 (1H, dd, $J = 11.0$, 3.9 Hz, H-2), 4.49 (1H, m, CHThrβ), 4.48 (1H, m, CHThrα), 4.42 (1H,

dd, $J = 10.8$, 6.6 Hz, FmocCH$_2$), 4.33 (1H, dd, $J = 11.0$, 4.2 Hz, H-6a′), 4.20 (3H, m, H-5, H-5′, FmocCH), 4.12 (2H, m, H-6ab), 3.97 (1H, dd, $J = 11.0$, 2.5 Hz, H-3), 3.89 (1H, dd, $J = 11.0$, 9.3 Hz, H-6b′), 3.71 (1H, s, COOH), 2.22 (3H, s, OAc), 2.17 (3H, s, OAc), 2.12 (3H, s, NHAc), 2.07 (6H, s, OAc), 2.01 (3H, s, OAc), 1.94 (3H, s, OAc), 1.29 (3H, d, $J = 6.4$ Hz, Thr-CH$_3$), ^{13}C NMR data: δ 174.79 (OAc), 172.96 (NHAc), 172.04 (COOH), 170.72 (OAc), 170.49 (OAc), 170.34 (OAc), 170.18 (OAc), 170.03 (OAc), 157.24 (NHCOO), 144.25 (FmocAr), 144.20 (FmocAr), 141.58 (FmocAr), 141.58 (FmocAr), 127.97 (FmocAr), 127.91 (FmocAr), 127.29 (FmocAr), 127.18 (FmocAr), 125.06 (FmocAr), 125.06 (FmocAr), 120.19 (FmocAr), 120.19 (FmocAr), 99.54 (H-1), 92.12 (H-1′), 76.92 (CHThrβ), 68.67 (H-3), 67.69 (H-3′), 67.64 (H-4′), 67.35 (H-5′), 66.61 (H-5), 66.46 (FmocCH$_2$), 65.59 (H-2′), 64.94 (H-4), 62.53 (H-6), 62.07 (H-6′), 58.64 (CHThrα), 47.90 (H-2), 47.61 (FmocCH), 22.44 (NHAc), 21.06 (OAc), 20.92 (OAc), 20.87 (OAc), 20.79 (OAc), 20.78 (OAc), 20.68 (OAc), 18.93 (Thr-CH$_3$). HRMS: Calcd for $C_{45}H_{55}N_2O_{21}$: 959.32918. Found: 959.32913 (M+H$^+$).

3.6. 4-Methylphenyl 2-azido-3,4-*O*-isopropylidene-2-deoxy-1-thio-β-D-galactopyranoside (10)

p-Toluenesulfonic acid (50–100 mg, enough to make the mixture acidic to indicator paper) was added to a solution of **9**[11] (2.63 g, 8.43 mmol) and 2,2-dimethoxypropane (1.45 g, 13.9 mmol) in dry acetonitrile (40 mL). After 2 h or when TLC (dichloromethane–EtOAc 9:1) indicated complete reaction, the mixture was neutralized with pyridine (0.6 mL) and concentrated to a syrup. The residue was purified by column chromatography (dichloromethane–ethyl acetate 9:1) to give **10** (1.15 g, 3.27 mmol, 39%), $[\alpha]_D$ +18 (*c* 0.1, $CHCl_3$); ^{1}H NMR data (DMSO-d_6, δ for $(CD_3)_2SO = 2.50$): δ 7.37 (2H, d, $J = 8.4$ Hz, Ph), 7.15 (2H, d, $J = 8.4$ Hz, Ph), 4.85 (1H, br t, $J = 5.4$ Hz, H-6-OH), 4.67 (1H, d, $J = 10.6$ Hz, H-1), 4.15 (2H, m, H-3, H-4), 3.86 (1H, dd, $J = 6.2$ Hz, H-5), 3.54 (2H, m, H-6), 3.33 (1H, m, H-2), 2.27 (s, 3H, S–Ph–*Me*), 1.31, 1.24 (two s, each 3H, isopropylidene Me).

3.7. 4-Methylphenyl-2-azido-3,4-*O*-isopropylidene-2-deoxy-6-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galacto-pyranosyl)-1-thio-β-D-galactopyranoside (12)

A mixture of **10** (0.81 g, 2.30 mmol), **11**[14] (0.97 g, 2.36 mmol), powdered 4 Å molecular sieves (4 g), dry toluene (7 mL), and dry dichloromethane (17 mL) was stirred at rt for 10 min, then cooled to −30 to −40 °C while a solution of silver triflate (1.3 g, 5.06 mmol) in dry toluene (26 mL) was added (during 5 min). The mixture was stirred for another 50 min at −30 to −40 °C, after which TLC (toluene–ethyl acetate 6:4) indicated no more change. Pyridine (1 mL) was added to neutralize the mixture, and the mixture was poured into diethyl ether (100 mL). The solid was filtered off and washed with diethyl ether and dichloromethane. The filtrate was washed with aq sodium thiosulfate (1 M), aq sulfuric acid (1 M), and aq sodium hydrogen carbonate (0.5 M), dried and evaporated. The residue was purified by column chromatography (toluene–ethyl acetate 7:3) to give **12** (1.35 g, 1.98 mmol, 86%), $[\alpha]_D$ +19 (*c* 0.3, $CHCl_3$); ^{1}H NMR data: δ 7.27 (2H, d, $J = 8.4$ Hz, Ph), 7.15 (2H, d, $J = 8.4$ Hz, Ph), 5.40 (1H, dd, $J = 3.4$, 1.0 Hz, H-4′), 5.22 (1H, dd, $J = 10.4$, 8.0 Hz, H-2′), 5.01 (1H, dd, $J = 10.4$, 3.4 Hz, H-3′), 4.58 (1H, d, $J = 8.0$ Hz, H-1′), 4.30 (1H, d, $J = 10.6$ Hz, H-1), 4.07 (2H, m, H-4, H-3), 3.36 (1H, dd, $J = 10.6$, 6.9 Hz, H-2), 2.35 (3H, s, S–Ph–*Me*), 2.15, 2.04, 1.98, 1.98 (each 3H, s, OAc). HRMS: Calcd for $C_{30}H_{43}N_4O_{13}S$: 699.25418. Found: 699.25534 (M+NH$_4^+$).

3.8. *N*-(9-Fluorenylmethyloxycarbonyl)-*O*-[2-azido-3,4-*O*-isopropylidene-2-deoxy-6-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-α-D-galactopyranosyl]-L-threonine phenacylester (14)

A mixture of **12** (0.673 g, 0.987 mmol), *N*-(9-fluorenylmethyloxycarbonyl)-L-threonine phenacylester[11] **13** (0.645 g, 1,40 mmol), powdered 4 Å molecular sieves (3.19 g), dry dichloromethane (6.9 mL), dry diethyl ether (5.0 mL), and dry toluene (5.0 mL) was stirred at room temperature for 5 min, then DMTST (0.753 g, 2.92 mmol) was added in portions over 5 h (until TLC indicated no more change). Pyridine (0.67 mL) was added to quench the reaction and the mixture was diluted with diethyl ether. The solid was filtered off and washed with ether and dichloromethane. The filtrate was washed with aq sulfuric acid (2 M), aq sodium hydrogen carbonate (1 M), dried, and evaporated. The residue was purified by column chromatography (toluene–ethyl acetate 7:3) to give **14** (0.561 g, 0.552 mmol, 56%), $[\alpha]_D$ +6 (*c* 0.3, $CHCl_3$); ^{1}H NMR data: 7.74–7.93 (Fmoc aromatics), 7.36–7.65 (phenacyl aromatics), 7.11–7.34 (m, Fmoc aromatics), 5.82 (1H, d, $J = 9.4$ Hz, NH-Thr) 5.55 (1H, d, $J = 16.7$ Hz, phenacyl CH$_2$), 5.38 (1H, d, $J = 3.5$ Hz, H-4′), 5.32 (1H, d, $J = 16.7$ Hz, phenacyl CH$_2$), 5.30 (1H, d, $J = 3.5$ Hz, H-1), 5.23 (1H, dd, $J = 10.2$, 7.9 Hz, H-2′), 5.01 (1H, dd, $J = 10.2$, 3.5 Hz, H-3′), 4.58 (1H, d, $J = 7.9$ Hz, H-1′), 4.53 (1H, br d, α-CH-Thr), 3.52 (1H, dd, $J = 7.9$, 3.5 Hz, H-2), 2.15, 2.04, 2.04, 1.98 (each 3H, s, OAc), 1.44 (3H, d, $J = 6.4$ Hz, CH$_3$–Thr). HRMS: Calcd for $C_{50}H_{57}N_4O_{19}$: 1017.36115. Found: 1017.35995 (M+H$^+$). A compound assigned (data not shown) as the corresponding β isomer of **14** was also isolated (0.204 g, 0.201 mmol, 20%).

3.9. *N*-(9-Fluorenylmethyloxycarbonyl)-*O*-[2-acetamido-3,4-di-*O*-acetyl-2-deoxy-6-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-α-D-galactopyranosyl]-L-threonine phenacylester (17)

A mixture of **14** (0.544 g, 0.535 mmol) and 80% acetic acid (2.5 mL) was stirred at 90 °C for 2 h, after which TLC (toluene–ethyl acetate 1:2) detected complete conversion into a slower-moving compound. The solution was concentrated and co-concentrated several times with toluene and once with pyridine. The residue (containing crude **15**) was taken up in pyridine–acetic anhydride 2:1 (2.5 mL). After allowing to stand overnight at rt, the mixture was concentrated and co-concentrated with toluene several times to give crude **16**, which was dissolved in thioacetic acid (2.5 mL) and the solution was left at rt for 48 h, after which TLC (toluene–ethyl acetate 1:2) indicated complete conversion. The mixture was purified by column chromatography (stepwise gradient: toluene, toluene–ethyl acetate 1:1, and toluene–ethyl acetate 1:2) to give **17** (0.387 g, 0.359 mmol, 67%), $[\alpha]_D$ +4 (*c* 0.2, $CHCl_3$); ^{1}H NMR data: δ 7.74–7.93 (Fmoc aromatics), 7.36–7.65 (phenacyl aromatics), 7.11–7.34 (m, Fmoc aromatics), 6.00 (1H, d, $J = 10.0$ Hz, NHAc), 5.85 (1H, d, $J = 9.1$ Hz, NH–Thr), 5.62 (1H, d, $J = 16.7$ Hz, phenacyl CH_2), 5.36 (3H, m, H-1, H-4, H-4′), 5.25 (1H, d, $J = 16,4$ Hz, phenacyl CH_2), 5.16 (2H, m, H-3, H-2′), 4.98 (1H, dd, $J = 10.5$, 3.5 Hz, H-3′), 4.64 (1H, m, H-2), 4.48 (5H, m, α,β-Thr, FmocCH$_2$, H-1′), 1.64, 1.97, 1.98, 2.04, 2.05, 2.12, 2.16, (each 3H, s, Ac), 1.46 (3H, d, $J =$ 6.1 Hz, CH_3–Thr). HRMS: Calcd for $C_{53}H_{61}N_2O_{22}$: 1077.37105. Found: 1077.37041 ($M+H^+$).

3.10. *N*-(9-Fluorenylmethyloxycarbonyl)-*O*-[2-acetamido-3,4-di-*O*-acetyl-2-deoxy-6-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-α-D-galactopyranosyl]-L-threonine (18)

A mixture of **17** (0.295 g, 0.274 mmol) and 90% acetic acid (8.6 mL) was stirred and heated to 35 °C, while activated zinc powder (1.443 g) was added in portions during 2 h, after which TLC (ethyl acetate–acetic acid 6:1) indicated complete reaction. The mixture was filtered (minding not to let the solids getting too dry on the filter), washed carefully with acetic acid and ethanol–ethyl acetate 1:1, and the filtrate was evaporated. The residue was triturated with ethyl acetate–dichloromethane 1:1 (6 mL), the mixture was filtered, the filtrate was concentrated and the residue was purified by column chromatography, eluting first with 1,2-dichloroethane and then with increasing concentrations of methanol (nine steps, from 2% to 10% and five steps, from 12% to 20%). The residue was freeze-dried from benzene to give **18** (0.234 g, 0.244 mmol, 89%), $[\alpha]_D$ +57 (*c* 0.1, $CHCl_3$); ^{1}H NMR data: δ 7.78 (2H, d, $J = 7.8$ Hz, Fmoc), 7.63 (2H, dd, $J = 7.3$, 2.5 Hz, Fmoc), 7.41 (2H, dd, $J = 7.3$ Hz, Fmoc), 7.33 (2H, dd, $J = 7.8$, 7.3 Hz, Fmoc), 6.13 (1H, d, $J = 9.1$ Hz, NHAc), 5.82 (1H, d, $J = 9.78$ Hz, NHThr), 5.37 (1H, d, $J = 3.2$ Hz, H-4′), 5.34 (1H, m, H-4), 5.17 (1H, dd, $J = 10.5$, 7.8 Hz, H-2′), 5.13 (1H, dd, $J = 10.5$, 2.9 Hz, H-3), 5.03 (1H, d, $J = 3.9$ Hz, H-1), 5.00 (1H, d, $J = 10.5$, 3.2 Hz, H-3′), 4.54 (1H, dd, $J = 10.8$, 6.6 Hz, FmocCH$_2$), 4.48 (1H, d, $J = 7.8$ Hz, H-1′), 4.46–4.34 (4H, m, H-2, CHThrα, CHThrβ, FmocCH$_2$), 4.33 (1H, dd, $J = 11.0$, 4.2 Hz, H-6a′), 4.28–4.15 (3H, m, FmocCH, H-5, H-6a′), 4.05 (1H, dd, $J = 11.3$, 6.6 Hz, H-6b′), 3.89 (1H, dd, $J = 6.6$ Hz, H-5′), 3.83 (1H, dd, $J = 10.7$, 4.4 Hz, H-6a), 3.61 (1H, dd, $J = 8.6$, 10.8 Hz, H-6b), 2.16 (3H, s, OAc), 2.13 (3H, s, OAc), 2.05 (6H, s, OAc), 2.03 (3H, s, NHAc), 2.00 (3H, s, OAc), 1.99 (3H, s, OAc), 1.31 (3H, d, $J = 6.1$ Hz, Thr-CH_3); ^{13}C NMR data: δ 172.32 (NHAc), 170.85 (COOH), 170.42 (OAc), 170.19 (OAc), 170.19 (OAc), 170.13 (OAc), 170.09 (OAc), 169.19 (OAc), 156.67 (NHCOO), 143.77 (FmocAr), 143.68 (FmocAr), 141.33 (FmocAr), 141.33 (FmocAr), 127.83 (FmocAr), 127.80 (FmocAr), 127.17 (FmocAr), 127.15 (FmocAr), 125.11 (FmocAr), 124.98 (FmocAr), 120.11 (FmocAr), 120.03 (FmocAr), 101.17 (H-1′), 99.37 (H-1), 76.91 (CHThrβ), 70.90 (H-5′), 70.82 (H-3′), 68.57 (H-2′), 68.23 (H-3), 68.19 (H-6), 67.89 (H-4), 67.74 (H-5), 67.15 (FmocCH$_2$), 66.98 (H-4′), 61.14 (H-6′), 58.57 (CHThrα), 48.09 (H-2), 47.27 (FmocCH), 22.99 (NHAc), 20.75 (OAc), 20.73 (OAc), 20.73 (OAc), 20.64 (OAc), 20.62 (OAc), 20.57 (OAc), 18.50 (Thr-CH_3). HRMS: Calcd for $C_{45}H_{55}N_2O_{21}$: 959.32918. Found: 959.32925 ($M+H^+$).

3.11. General procedures for glycopeptide synthesis

The solid-phase synthesis of **21**, **23**, and **25** was carried out manually using Fmoc chemistry, with *t*-butyl ether side chain protection for tyrosine and serine, N-*t*-butoxycarbonyl side chain protection for lysine, and *t*-butyl ester side chain protection for glutamic acid. Starting with Leu-Wang resin (loading level 0.75 mmol/g), the next amino acid (Ileu, 5 equiv), activated with PyBOP/HOBt (5 equiv) and DIPEA (10 equiv) in DMF, was added. The Fmoc group was then removed using 20% piperidine in DMF. The Kaiser ninhydrin test[16] was performed after each coupling and after deprotection of the Fmoc group. The coupling/deprotection cycles were repeated using the appropriate amino acids until the entire peptide sequence had been assembled. A small glass vessel with an upper glass stopper and a bottom sintered glass frit was used for the solid-phase reactions. Agitation was by rotation of the vessel during synthesis of **21**, and by nitrogen bubbling up through the bottom frit during synthesis of **23** and **25**. After the last coupling cycle, the resin was subjected to cleavage conditions (95% TFA, 2.5% thioanisole, and 2.5% H_2O) and the released O-acetylated glycopeptide

Table 1. ^{1}H NMR chemical shifts (ppm) of compounds **21**, **23**, and **25** at 295 K in H_2O–D_2O (95–5%)

Residue	Compound	NH	αH	βH	Others
pGlu[1]	**21**		4.43	2.12	γCH_2 2.58, 2.44
	23		4.43	2.12	γCH_2 2.58, 2.44
	25		4.42	2.10	γCH_2 2.57, 2.45
Ser[2]	**21**	8.49	4.50	3.89	
	23	8.49	4.48	3.90	
	25	8.49	4.49	3.91	
Glu[3]	**21**	8.60	4.45	2.01	γCH_2 2.49, 2.17
	23	8.59	4.44	2.01	γCH_2 2.50, 2.18
	25	8.59	4.45	2.01	γCH_2 2.51, 2.17
Glu[4]	**21**	8.44	4.42	2.01	γCH_2 2.49, 2.17
	23	8.47	4.42	2.01	γCH_2 2.50, 2.18
	25	8.46	4.41	2.01	γCH_2 2.51, 2.17
Gly[5]	**21**	8.50	4.01		
	23	8.47	4.00		
	25	8.50	4.01		
Gly[6]	**21**	8.32	4.03		
	23	8.31	4.03		
	25	8.31	4.03		
Ser[7]	**21**	8.35	4.49	3.89	
	23	8.35	4.47	3.90	
	25	8.36	4.45	3.91	
Asn[8]	**21**	8.52	4.77	2.86, 2.78	
	23	8.49	4.76	2.87, 2.79	
	25	8.51	4.75	2.87, 2.80	
Ala[9]	**21**	8.11	4.36	1.41	
	23	8.10	4.50	1.41	
	25	8.12	4.47	1.44	
Thr[10]	**21**	8.08	4.48	4.27	γCH_3 1.33
	23	8.66	4.55	4.35	γCH_3 1.33
	25	8.34	4.54	4.33	γCH_3 1.33
Lys[11]	**21**	8.09	4.35	1.77	NH_2 7.54, γCH_2 1.47, δCH_2 1.72, εCH_2 3.03
	23	8.56	4.36	1.83	NH_2 7.55, γCH_2 1.47, δCH_2 1.68, εCH_2 3.02
	25	8.40	4.37	1.71	NH_2 7.53, γCH_2 1.46, δCH_2 1.69, εCH_2 3.01
Lys[12]	**21**	8.38	4.58	1.80	NH_2 7.54, γCH_2 1.47, δCH_2 1.72, εCH_2 3.03
	23	8.40	4.41	1.85	NH_2 7.59, γCH_2 1.48, δCH_2 1.74, εCH_2 3.05
	25	8.38	4.49	1.74	NH_2 7.57, γCH_2 1.48, δCH_2 1.74, εCH_2 3.03
Pro[13]	**21**		4.41	2.25, 1.83	γCH_2 2.10, δCH_2 3.84, 3.66
	23		4.42	2.28, 1.84	γCH_2 2.04, δCH_2 3.87, 3.67
	25		4.41	2.27, 1.85	γCH_2 2.03, δCH_2 3.86, 3.64
Tyr[14]	**21**	8.14	4.59	3.02	Ar 7.13, 6.84
	23	8.24	4.59	3.02	Ar 7.13, 6.84
	25	8.25	4.58	3.00	Ar 7.13, 6.84
Ile[15]	**21**	7.96	4.15	1.81	γCH_2 1.42, 1.12, γCH_3 0.89, δCH_3 0.84
	23	7.95	4.14	1.80	γCH_2 1.41, 1.12, γCH_3 0.88, δCH_3 0.84
	25	7.95	4.14	1.80	γCH_2 1.41, 1.13, γCH_3 0.89, δCH_3 0.84
Leu[16]	**21**	8.19	4.29	1.65	γCH_2 1.64, δCH_3 0.95, 0.90
	23	8.25	4.30	1.66	γCH_2 1.65, δCH_3 0.96, 0.91
	25	8.28	4.30	1.67	γCH_2 1.64, δCH_3 0.96, 0.90

was preliminary purified. After O-deacetylation with sodium methoxide in methanol, the material was finally purified by RP-HPLC (for purification details, see below). The synthesized peptides **21**, **23**, and **25** were characterized by NMR spectroscopy using a Bruker DRX 600 MHz spectrometer equipped with a 2.5 mm microprobe. A 5 mg sample of each glycopeptide was dissolved in 0.10 mL D_2O or H_2O–D_2O (95–5%). 1-D ^{1}H

Table 2. ^{1}H NMR chemical shifts (ppm) for the glycan part of compounds **21**, **23**, and **25** at 295 K in D_2O

	Compound	H-1	H-2	H-3	H-4	H-5	H-6	NH	NAc
Gal	**21**	4.47	3.53	3.64	3.93	3.67	3.79		
	23	5.14	3.83	3.72	4.00	ND	ND		
	25	4.40	3.63	3.91	3.66	3.66	3.77		
GalNAc	**21**	4.57	4.02	3.88	4.20	3.71	3.65	8.32	2.06
	23	4.97	4.23	4.03	3.99	4.05	3.79	7.57	2.07
	25	4.93	4.12	3.94	4.05	4.22	4.09, 3.85	7.62	2.04

NMR and 2-D DQF-COSY, TOCSY, NOESY, and HSQC-DEPT were recorded at 295 K. For experiments in D_2O, the residual HDO peak was suppressed by presaturation and in H_2O–D_2O (95–5%) the WATERGATE pulse sequence was used. The 2-D spectra were recorded in phase-sensitive mode using the TIPPI method. The DQF-COSY, TOCSY, and NOESY spectra were acquired with 4K data points in t_2 and 512 increments in t_1, and a D_1 relaxation time of 1.5 s. In the TOCSY experiments mixing times of 60 and 80 ms were used and the NOESY spectra were recorded at a mixing time of 400 ms. The ^{1}H NMR chemical shifts were measured using acetone ($\delta = 2.225$) as internal reference.

3.12. β-β-Contulakin (21)

The manual solid-phase synthesis of β-contulakin **(21)** was performed starting from Fmoc-Leu-Wang resin (133 mg, 0.10 mmol). The resin was swelled with dichloromethane for 30 min and washed five times with DMF. The Fmoc group was removed using 20% piperidine in DMF (3×10 min) and washed five times with DMF. A mixture of Fmoc protected Ile (5 equiv, 177 mg, 0.5 mmol), PyBOP (260 mg, 0.5 mmol), HOBt (77 mg, 0.5 mmol), and DIPEA (10 equiv, 0.166 mL, 1.0 mmol) in DMF (2 mL) were then added to the resin and the mixture was agitated for 2 h. After complete coupling, the resin was washed five times with DMF. The Fmoc deprotections and amino acid couplings were then repeated according to the above procedure until the entire peptide sequence had been finished. Coupling of the glycosylated amino acid **19**[11] was performed with agitation for 24 h using only 2 equiv of the amino acid and coupling reagent and 4 equiv of DIPEA. The aspargine and pyroglutamate residues were coupled overnight and the couplings had to be repeated twice with fresh reagents due to sluggish reactions (according to the Kaiser test).[16] After coupling of the pyroglutamate residue, the resin was washed five times each with DMF, isopropanol, and diethyl ether and then dried overnight. The material was cleaved from the resin by addition of 3 mL cleavage cocktail (95% TFA, 2.5% thioanisole, and 2.5% H_2O) and agitation for 3 h. The resin was then filtered off and washed twice with cleavage cocktail. The filtrate was concentrated and triturated with cold diethyl ether. The solvent was decanted and the precipitate was dissolved in water and lyophilized. The residue was dissolved in water (5 mL) and applied to a C-18 Isolute cartridge (4 g, pre-washed with first 20 mL MeOH, then 100 mL of water). The cartridge was washed with 20 mL of water and then the material was eluted with 10–40% acetonitrile and lyophilized to give **20** (39 mg, 0.017 mmol). The material was treated with 0.5 M sodium methoxide (0.10 mL) in methanol (1 mL) for 1 h, neutralized with 0.05 mL TFA and was then lyophilized. The deacetylated sample was purified by semi-preparative RP-HPLC using 10–50% acetonitrile in 0.1% aq TFA to give **21** (19 mg, 0.0092 mmol, 9%). MALDI MS: Calcd for $C_{88}H_{141}N_{20}O_{37}$: 2069.98. Found: 2070.12 ($M+H^+$). ^{1}H NMR assignments are given in Tables 1 and 2.

3.13. α,α-Contulakin (23)

This compound was synthesized similarly to **21** starting with Fmoc-Leu-Wang resin (67 mg, 0.050 mmol), using glycosylated amino acid **8** (96 mg, 0.10 mmol). The glycopeptide was cleaved from resin and purified on a C-18 cartridge to give **22** (81 mg, 0.035 mmol). The material was de-O-acetylated and purified by semi-preparative RP-HPLC to give **23** (38 mg, 0.0184 mmol, 37%). MALDI MS: Calcd for $C_{88}H_{141}N_{20}O_{37}$: 2069.98. Found: 2070.36 ($M+H^+$); calcd for $C_{88}H_{140}N_{20}O_{37}Na$: 2091.96. Found: 2092.40 ($M+Na^+$) ^{1}H NMR assignments are given in Tables 1 and 2.

3.14. 1,6-Contulakin (25)

This compound was synthesized similarly to **21** starting with Fmoc-Leu-Wang resin (133 mg, 0.10 mmol), using glycosylated amino acid **18** (192 mg, 0.2 mmol). The glycopeptide was cleaved from resin and purified on a C-18 cartridge to give **24** (132 mg, 0.057 mmol). The material was de-O-acetylated and purified by semi-preparative RP-HPLC to give **25** (116 mg, 0.056 mmol, 56%). MALDI MS: Calcd for $C_{88}H_{141}N_{20}O_{37}$: 2069.98. Found: 2070.51 ($M+H^+$); calcd for $C_{88}H_{140}N_{20}O_{37}Na$: 2091.96. Found: 2092.52 ($M+Na^+$). ^{1}H NMR assignments are given in Tables 1 and 2.

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