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An Improved Synthesis of a Galactosylated Hydroxylysine Building Block and its use in Solid-Phase Glycopeptide Synthesis

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Abstract—Different protective groups for (5*R*)-5-hydroxy-l-lysine were investigated in silver silicate promoted glycosylations with aceto-bromogalactose as glycosyl donor. Best results were obtained with Fmoc-Hyl(Cbz)-OAll, which was glycosylated in 80% yield. Removal of the allyl group gave a β-d-galactosylated building block which was used in solid-phase synthesis of a glycopeptide from type II collagen. Such glycopeptides are required for studies of rheumatoid arthritis in a mouse that is transgenic for HLA-DR4, i.e. the class II MHC molecule associated with rheumatism in humans.

Introduction

Collagen induced arthritis is the most common animal model for rheumatoid arthritis. In this model, symptoms identical to those displayed by patients suffering from rheumatoid arthritis are induced by immunization of mice with type II collagen from rat cartilage.[1–3] The rat type II collagen is degraded in antigen presenting cells (APCs) in the mouse to fragments presented as complexes with H-2A^q class II MHC molecules on the surface of the APC. We have recently shown that a glycopeptide fragment from type II collagen, consisting of residues 256-270 (CII256-270), stimulates the majority of the autoimmune helper T cells obtained in the mice (Fig. 1).[4] Furthermore, a β-d-galactosylated hydroxylysine at position 264 was revealed to form critical contacts with the T cell receptor, while Ile^{260} and Phe^{263} anchored the glycopeptide in the P1 and P4 pockets of the disease associated H-2A^q MHC molecule.[5]

In humans, rheumatoid arthritis is associated with HLA-DR4 class II MHC molecules.[1,2] Interestingly, mice that are transgenic for HLA-DR4 acquire rheumatoid arthritis on immunization with type II collagen.[6,7] In this case, the immunodominant epitope on type II collagen has been found to be located between residues 261 and 273 (CII261-273). The peptide is anchored into the P1 and P4 pockets of HLA-DR4 by residues Phe^{263} and Glu^{266}, respectively, while Lys^{264}, and possibly also Lys^{270}, makes

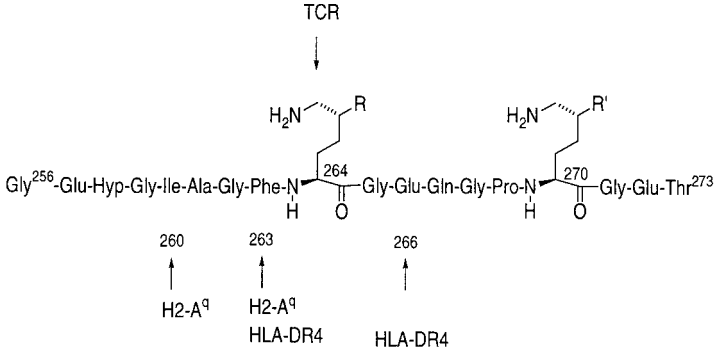

Figure 1. Immunodominant T cell epitopes on type II collagen are located between residues 256 and 273. Anchor positions to H-2A^q and HLA-DR4, as well as the known T cell contact at position 264 are indicated. In collagen, lysines may be post-translationally hydroxylated and then glycosylated.

Keywords: glycosylation; glycopeptides; solid-phase synthesis.

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important contacts with the T cell receptor. Consequently, the epitope is shifted as compared to when CII256-270 is bound by H-2A^q.

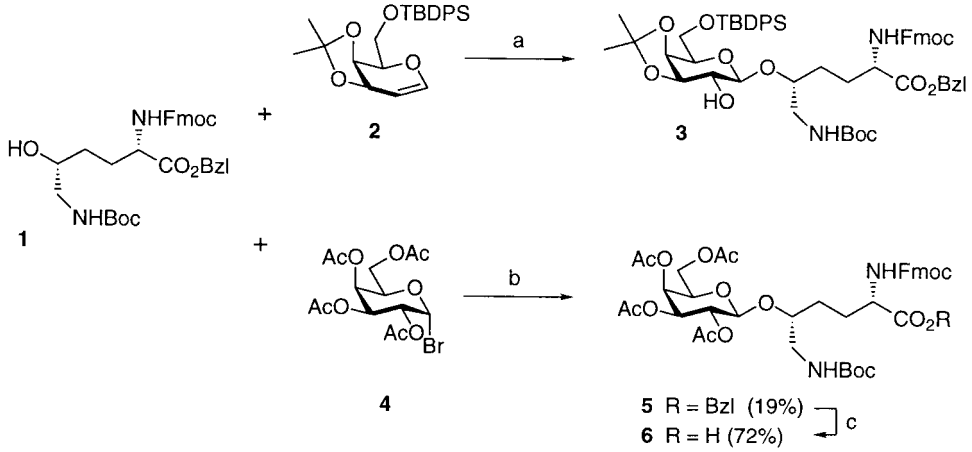
Facile access to synthetic glycopeptides from type II collagen is required in studies of the role of glycosylation for development of arthritis in HLA-DR4 transgenic mice. We have therefore evaluated different protective group patterns for (5*R*)-5-hydroxy-l-lysine and developed an improved route for attachment of a β-d-galactopyranosyl residue. The versatility of the glycosylated hydroxylysine building blocks have been demonstrated by synthesis of two glycopeptides from type II collagen using Fmoc solid-phase synthesis.

Results and Discussion

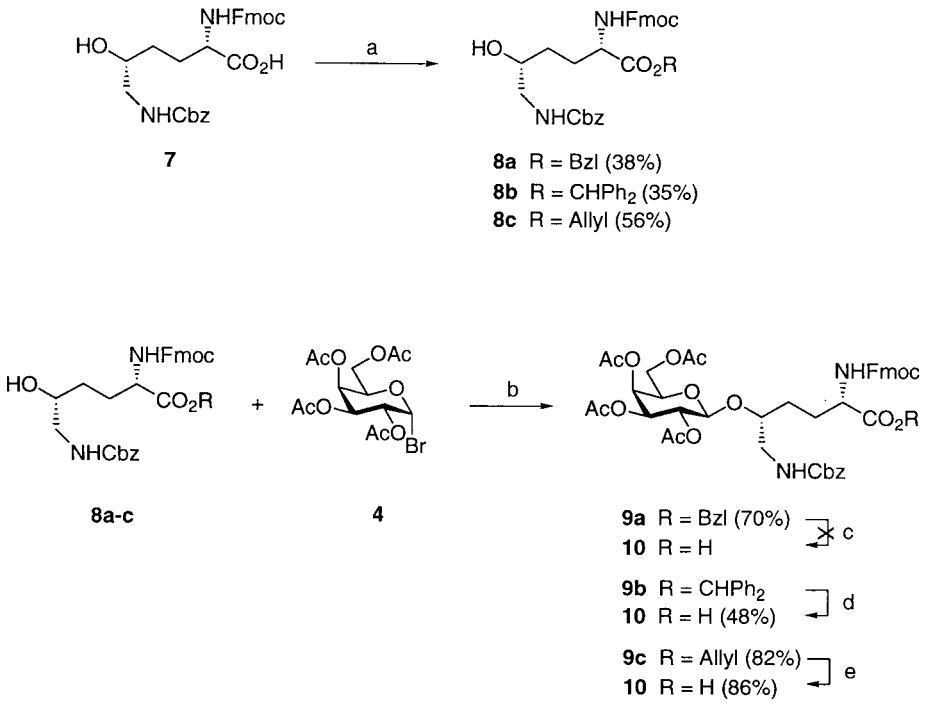
Glycopeptides having (5*R*)-5-hydroxy-l-lysine residues glycosylated[8] with β-d-galactopyranose moieties have recently been synthesized by the Fmoc-protocol using building block **3**, which was prepared from hydroxylysine **1**[9] and galactal **2** (Scheme 1).[4] This method was developed to provide, not only a 1,2-*trans*-glycosidic linkage, but also a hydroxyl group on C-2 of the galactose residue (cf. **3**) for further attachment of an α-d-glucosyl moiety. Although most efficient for this purpose,[4,10] the procedure is less suitable when a building block carrying only a single galactose moiety is desired. The reason for this is that protected glycosyl acceptor **1**, which is prepared in three steps from expensive hydroxylysine, must be used in excess (2 equiv.) over the more readily available donor[11] **2** in order to prevent further glycosylation of HO-2 in **3**. Building block **3** is therefore obtained in 19% yield based on **1** (37% based on **2**), but some unreacted **1** can be recovered after removal of a small amount of the α-anomer corresponding to **3**. Attempted improvement by glycosylation[12] of **1** with peracetylated galactosyl bromide **4** under promotion by silver silicate[13] did improve the stereoselectivity (no α-anomer detected) but not the yield (19%, Scheme 1). In this case, the low yield was explained by the inadequate stability of the N^{ϵ}-Boc protective group of acceptor **1** under the conditions of the glycosylation.[10]

In efforts to develop an improved route to β-d-galactosylated derivatives of hydroxylysine the benzyloxycarbonyl group was chosen as protective group for the side-chain of hydroxylysine, whereas benzyl, diphenylmethyl, and allyl esters were evaluated for protection of the α-carboxyl group (cf. **8a**–**c**, Scheme 2). The Cbz-group is known to be more stable than the Boc-group towards acids, but can still be removed with TFA under conditions that mediate cleavage from the solid phase and deprotection of peptides and glycopeptides prepared by the Fmoc-protocol.[10,14] The three esters were chosen since conditions for their removal which are orthogonal to Fmoc, Cbz and *O*-acetyl groups have been reported, i.e. benzyl esters can undergo selective hydrogenolysis using a palladium–ethylenediamine complex deposited on activated charcoal [Pd/C(en)],[15] diphenylmethyl esters can be hydrolysed selectively with acid, and allyl esters may be cleaved by palladium catalyzed allyl transfer.[16] The three esters were prepared by converting N^{α}-(fluoren-9-ylmethoxycarbonyl)-N^{ϵ}-benzyloxycarbonyl-5-hydroxy-l-lysine[10] (**7**) into a cesium salt which was alkylated with benzyl, diphenylmethyl, and allyl bromide, respectively, in dry DMF (Scheme 2). Spontaneous lactonization of hydroxylysine derivative **7** during the esterifications complicated isolation of **8a**–**c** and accounts for the modest yields. Lactonization is more pronounced for **8a** and **8b** since, in contrast to when using allyl bromide, a large excess of benzyl or diphenylmethyl bromide cannot be employed if repeated purification by chromatography (which also results in some lactonization) is to be avoided.

Silver silicate promoted glycosylation of hydroxylysine benzyl ester **8a** with galactosyl bromide **4** in dichloromethane at 0°C gave glycoside **9a** in 70% yield without any detectable formation of the corresponding α-anomer or orthoester. Unfortunately, in our hands, selective hydrogenolysis of the benzyl ester failed both for **9a** and the model compound Fmoc-Lys(Cbz)-OBzl. Use of different amounts of the catalyst Pd/C(en)[15] in different solvents resulted either in removal of both the N^{ϵ}-Cbz group and the benzyl ester, or in no reaction at all.[17] Attempted hydrogenolysis of **9a** with 10% Pd/C in ethyl acetate was nonselective, as expected.



Scheme 1. (a) Dimethyldioxirane, CH_2Cl_2, 0°C then **1**, $ZnCl_2$, AW-300, −50°C→room temp (cf. reference 4); (b) Silver silicate, 3 Å MS, CH_2Cl_2, 0°C; (c) H_2, Pd/C, EtOAc.



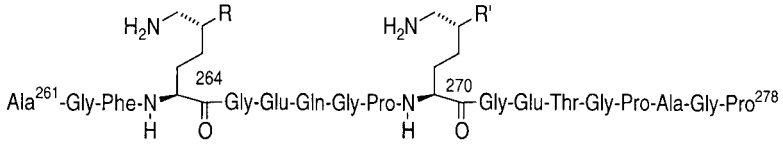
Scheme 2. (a) 30% $CsCO_3$(aq), 80% EtOH(aq), BTB then BzlBr, Ph_2CHBr or allyl bromide, DMF; (b) Silver silicate, 3 Å MS, CH_2Cl_2, 0°C; (c) H_2, Pd/C(en); (d) TFA/H_2O/CH_2Cl_2, 9:1:10, yield calculated from **8b**; (e) $(PPh_3)_4Pd(0)$, *N*-methyl-aniline, THF.

Glycosylation of diphenylmethyl ester **8b** under the same conditions as used for **8a** gave a mixture of β-glycoside **9b** and the corresponding orthoester in a 4:1 ratio, as determined by ^{1}H NMR spectroscopy. Since the two compounds could not be separated even using normal-phase HPLC, the mixture was treated with trifluoroacetic acid–water (9:1) in dichloromethane at room temperature. This led both to decomposition of the orthoester and removal of the diphenylmethyl ester, thereby affording building block **10** in 48% overall yield from hydroxylysine **8b**.

Finally, silver silicate promoted glycosylation of allyl ester **8c** with **4** gave β-glycoside **9c** in 82% yield. Under these conditions orthoester formation or lactonization of acceptor **8c**, which may be a problem during glycosylations,[10] was negligible. Deallylation of **9c** by *N*-methylaniline in THF catalysed by $(PPh_3)_4Pd(0)$[16] then gave the target glycosylated building block **10** in 83% yield (66% from hydroxylysine **8c**).

Glycosylated building blocks **6** and **10** were successfully used in solid-phase peptide synthesis of glycopeptides **12** and **11** (Fig. 2), respectively, using an automatic peptide synthesizer[18] or a mechanically agitated reactor. Synthesis was performed on a polystyrene resin grafted with polyethylene glycol spacers (Tentagel™ resin) according to the Fmoc strategy, under conditions identical to those reported previously by us.[4] Building blocks **6** and **10** were activated as azabenzotriazolyl esters[19] and coupled to the peptide–resin in a minimal volume of dry DMF during 24 h. After completion of the synthesis, treatment with a mixture of trifluoroacetic acid, water, thioanisole, and ethanedithiol (35:2:2:1) for 2–3 h liberated the glycopeptides from the resin, with simultaneous removal of all protective groups except the *O*-acetyl groups of the galactosyl moieties. This is in agreement with previous observations[10] regarding the lability of the Cbz group under acidic conditions. Deacetylation of the galactosyl moieties was performed either by treatment with a 20 mM solution of sodium methoxide in methanol, or using methanol saturated with ammonia. Purification by reversed-phase HPLC then gave glycopeptides **11** and **12** in 33 and 12% overall yields, respectively.

In conclusion, we have established a protective group pattern for hydroxylysine which allows efficient β-glycosylation of



11 R = H, R' = β-D-Gal-O-
12 R = β-D-Gal-O-, R' = H

Figure 2. Glycopeptides **11** and **12**.

the unprotected hydroxyl group. Silver silicate was found to be a suitable promoter for glycosylation in combination with acetobromogalactose as glycosyl donor. The resulting mono-galactosylated hydroxylysine building blocks were then successfully used in solid-phase synthesis of glycopeptides from type II collagen. These are now being used in investigations of the response of helper T cells generated in a mouse model for rheumatoid arthritis.

Experimental

General methods and materials

TLC was performed on Silica Gel 60 F_{254} (Merck) with detection by UV light, charring with 10% sulfuric acid or by phosphomolybdic acid/ceric sulphate in 6% aqueous sulfuric acid followed by careful heating. Flash column chromatography was performed on silica gel (Matrex, 60 Å, 35–70 μm, Grace Amicon) with solvents of HPLC grade, analytical grade or distilled technical grade. DMF was distilled and then dried over 3 Å molecular sieves. Dry THF was obtained by distilling from sodium–potassium alloy and dry CH_2Cl_2 by distilling from CaH_2.

The ^1H and ^{13}C NMR spectra of compounds **5**–**10** were recorded on a Bruker DRX-400 spectrometer at 400 MHz and 100 MHz, respectively. Chemical shifts are referenced to residual $CHCl_3$ (δ_H=7.27 ppm) and $CDCl_3$ (δ_C=77.0) for solutions in $CDCl_3$ or to CD_2HOD (δ_H=3.31 ppm) and CD_3OD (δ_C=49.0) for solutions in a 1:1 mixture of $CD_3OD/CDCl_3$. Spectra for glycopeptides **11** and **12** were recorded on Bruker DRX-600 and ARX-500 spectrometers in 40 mM phosphate buffer containing 1.5 mM NaN_3 and 10% D_2O at 278 K and pH 6.2 with H_2O as internal standard (δ_H=4.95 ppm). First-order ^1H and ^{13}C chemical shifts, as well as ^1H coupling constants, were determined from one-dimensional spectra and resonances were assigned from COSY, TOCSY, ^1H–^{13}C–HSQC, ^1H–^{15}N–HSQC, ^1H–^{13}C-HSQC-TOCSY, DEPT and NOESY experiments. Optical rotations were recorded on a Perkin–Elmer 343 polarimeter. Analytical reversed-phase HPLC was performed on a Kromasil C-8 column (250×4.6 mm, 5 μm, 100 Å), eluted with a linear gradient of MeCN in H_2O containing 0.1% TFA (a flowrate of 1.5 mL was used and detection was at 214 nm). Preparative reversed-phase HPLC was performed on a Kromasil C-8 column (250×20 mm, 5 μm, 100 Å), with the same eluent, a flowrate of 11 mL/min and detection at 214 nm. Analytical normal-phase HPLC was performed on a Kromasil silica column (250×4.6 mm, 5 μm, 100 Å), using a flowrate of 2 mL/min and with detection at 254 nm. Preparative normal-phase HPLC was performed on a Kromasil silica column (250×20 mm, 5 μm, 100 Å) using a flowrate of 15 mL/min and detection at 254 nm.

A TentalGel™ resin (Rapp Polymere, Germany) functionalized with a triphenylmethyl linker carrying an N^{α}-Fmoc-protected proline residue (TentaGel–S–Trt–Pro–Fmoc) and N^{α}-Fmoc-protected amino acids carrying triphenylmethyl (Gln), *tert*-butyl (Glu, Thr) and *tert*-butoxycarbonyl (Lys) side chain protective groups were used in the synthesis of glycopeptides **11** and **12**. In the amino acid analysis glutamine was determined as glutamic acid.

2,3,4,6-Tetra-*O*-acetyl-β-d-galactopyranosyl bromide **4** was prepared from peracetylated galactose by treatment with HBr in $HOAc/Ac_2O$. (5*R*)-N^{α}-(Fluoren-9-ylmethoxycarbonyl)-N^{ϵ}-benzyloxycarbonyl-5-hydroxy-l-lysine was prepared as described previously.[10] Silver silicate was prepared as follows: Silica–alumina (25 g, Sigma–Aldrich 35,335-4) was stirred with aqueous NaOH (1 M, 250 mL) at 90°C for 1 h. The solid was filtered off and washed with water (6×100 mL). *The remaining steps were carried out in the absence of light*. After stirring overnight at room temperature with aqueous $AgNO_3$ (0.2 M, 500 mL) the solid was filtered off and washed with water (6×100 mL) and finally with acetone (2×100 mL). Drying under high-vacuum at 105°C for 48 h gave the silver silicate as a brown powder.

(5*R*)-N^{α}-(Fluoren-9-ylmethoxycarbonyl)-N^{ϵ}-(*tert*-butoxycarbonyl)-5-hydroxy-l-lysine benzyl ester (1). Compound **1** was prepared as described previously[4] from (5*R*)-5-hydroxy-l-lysine dihydrochloride monohydrate (992 mg, 3.9 mmol) with the following modifications. After formation of the cupric chelate of hydroxylysine Boc-protection of the ϵ-amino group was found to give more reproducible results by using the following procedure. Solid $NaHCO_3$ (704 mg, 8.4 mmol) was added to the cupric chelate in water (25 mL) at 20°C. A solution of di-*tert*-butyl dicarbonate (1.23 g, 5.6 mmol) in dioxane (10 mL) was then added over 1.5 h, and the reaction mixture was stirred at 20°C. After 4 h a second portion of di-*tert*-butyl dicarbonate (520 mg, 2.4 mmol) dissolved in dioxane (5 mL) was added together with $NaHCO_3$ (50 mg), and the reaction mixture was then stirred overnight. The resulting suspension was filtered and the precipitate was washed with small portions of water. The combined filtrates were concentrated, diluted with water/THF (10 mL, 1:1), diethyl ether (2 mL) was added and the solution was cooled to 5°C. After 16 h another portion of precipitate was filtered off and washed with water. Once again, the combined filtrates were concentrated to dryness, the residue was dissolved in water/THF (10 mL, 1:1) and diethyl ether (2 mL) was added after which the solution was stored at 5°C overnight. Addition of a few crystals of the precipitate to this cooled solution accelerates the crystallization. This procedure was repeated until no appreciable amount of precipitation occurred. The combined precipitates were then converted to **1** according to the previously reported procedure. In brief the cupric chelate of N^{ϵ}-(*tert*-butoxycarbonyl)-5-hydroxy-l-lysine was dissociated using Na^+ Chelex 100 which had been converted to its H^+ form. The α-amino group was protected with an Fmoc group before formation of a cesium carboxylate salt which was finally transformed into a benzyl ester with benzyl bromide. Use of bromothymol blue as indicator during formation of the cesium carboxylate was found to reduce the amount of undesired cleavage of the Fmoc group.

(5*R*)-N^{α}-(Fluoren-9-ylmethoxycarbonyl)-N^{ϵ}-*tert*-butoxycarbonyl-5-O-(2,3,4,6-tetra-O-acetyl-β-d-galactopyranosyl)-5-hydroxy-l-lysine benzyl ester (5). Acetobromogalactose (**4**, 206 mg, 0.50 mmol) was added to a suspension of **1** (175 mg, 0.30 mmol), silver silicate

(500 mg) and crushed molecular sieves (3 Å, 360 mg) in CH_2Cl_2 (10 mL) at 0°C in the absence of light. After stirring for 2 h, Et_3N (0.15 mL) was added and the suspension was stirred for 20 min. The solid material was filtered off (Hyflo Supercel), washed with CH_2Cl_2 (35 mL) and the combined filtrates were concentrated. Flash column chromatography of the residue (Toluene/MeCN, 9:2) gave **5** (54 mg, 19%) as a white amorphous solid: $[\alpha]_d^{20}=-5$ (*c* 1.0, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 7.75 (d, *J*=7.6 Hz, 2H, Fmoc), 7.56 (d, *J*=7.3 Hz, 2H, Fmoc), 7.39 (t, *J*=7.5 Hz, 2H, Fmoc), 7.35–7.24 (m, 9H, Fmoc, OCH_2*Ph*), 5.43–5.34 (m, 2H, NH-ϵ, H-4), 5.28 (m, 1H, NH-α), 5.20–5.11 (m, 2H, OCH_2Ph, H-2), 4.98 (dd, *J*=10.5, 3.4 Hz, 1H, H-3), 4.43 (m, 2H, H-1, Fmoc), 4.35 (m, 2H, H-α, Fmoc), 4.23–4.08 (m, 3H, H-6,6, Fmoc), 3.88 (t, *J*=6.9 Hz, 1H, H-5), 3.62–3.51 (m, 1H, H-δ), 3.35–3.23 and 3.14–3.01 (2 m, each 1H, H-ϵ), 2.15 and 2.04 (2 s, each 3H, $COCH_3$), 1.97–1.94 (m, 7H, H-β, 2 $COCH_3$), 1.72–1.59 (m, 2H, H-β, H-γ), 1.58–1.47 (m, 1H, H-γ), 1.44 (s, 9H, $C(CH_3)_3$); ^{13}C NMR δ 171.9, 170.3, 170.2, 170.1, 169.4, 156.2, 156.0, 143.7, 143.6, 141.2, 135.0, 128.7, 128.6, 128.4, 127.7, 127.1, 127.0, 125.1, 120.0, 101.3, 81.3, 79.4, 70.7, 70.7, 68.7, 67.4, 67.1, 66.8, 61.3, 53.7, 47.0, 44.1, 28.5, 28.4, 28.3, 20.7, 20.6, 20.6; HRMS (FAB): calcd for $C_{47}H_{56}N_2O_{16}Na$ ($M+Na^+$) 927.3528, found 927.3521.

(5*R*)-N^{α}-(Fluoren-9-ylmethoxycarbonyl)-N^{ϵ}-*tert*-butoxycarbonyl-5-*O*-(2,3,4,6-tetra-*O*-acetyl-β-d-galactopyranosyl)-5-hydroxy-l-lysine (6). Compound **5** (49 mg, 54 μmol) in EtOAc (3.6 mL) was hydrogenated over Pd/C (49 mg) for 3 h at atmospheric pressure. The mixture was filtered (Hyflo supercel) and the filtrate concentrated. Flash column chromatography of the residue ($CHCl_3$/MeOH, 20:1→5:1) gave **6** (32 mg, 72%) as a white amorphous solid: $[\alpha]_d^{20}=-11$ (*c* 0.4, $CHCl_3$); ^{1}H NMR ($CDCl_3$/CD_3OD, 1:1) δ 7.74 (d, *J*=7.2 Hz, 2H, Fmoc), 7.58 (m, 2H, Fmoc), 7.38 (t, *J*=7.3 Hz, 2H, Fmoc), 7.30 (m, 2H, Fmoc), 5.75 (m, 1H, NH-ϵ), 5.75 (m, 1H, NH-α), 5.38 (s, 2H, H-4), 5.17 (t, *J*=11.0 Hz, 1H, H-2), 5.01 (bd, *J*=10.2 Hz, 1H, H-3), 4.50 (d, *J*=7.5 Hz, 1H, H-1), 4.35 (m, 2H, H-α, Fmoc), 4.17 (m, 4H, H-6,6, Fmoc, Fmoc), 3.90 (m, 1H, H-5), 3.63 (m, 1H, H-δ), 3.35 and 3.15 (2 m, each 1H, H-ϵ), 2.15, 2.04, 2.00 and 1.97 (4 s, each 3H, $COCH_3$), 1.75 (m, 1 H,H-β), 1.60 (m, 2H, H-γ,γ′), 1.44 (s, 9H, $C(CH_3)_3$); ^{13}C NMR ($CDCl_3$/CD_3OD, 1:1) δ 170.9, 170.7, 170.4, 170.4, 156.8, 156.7, 144.0, 143.9, 141.3, 127.7, 127.1, 125.0, 119.9, 101.2, 81.2, 79.5, 71.1, 70.5, 69.1, 67.3, 66.8, 61.3, 55.2, 47.1, 44.4, 29.6, 29.0, 28.5, 28.1, 20.3, 20.2, 20.2; HRMS (FAB): calcd for $C_{40}H_{50}N_2O_{16}Na$ ($M+Na^+$) 837.3058, found 837.3050.

(5*R*)-N^{α}-(Fluoren-9-ylmethoxycarbonyl)-N^{ϵ}-benzyloxycarbonyl-5-hydroxy-l-lysine benzyl ester (8a). A solution of crude **7**[10] (210 mg, 0.4 mmol) and bromothymol blue (BTB, <1 mg) in 80% aqueous EtOH (3 mL) at 0°C was adjusted to pH 7 with 30% Cs_2CO_3 (aq) and then concentrated. Concentration twice from 99.5% EtOH followed by drying under high vacuum overnight gave the cesium salt of **7** as a dry solid. The solid was dissolved in dry DMF (2 mL) at 0°C and benzyl bromide (72 μL, 0.6 mmol) was added. After stirring for 3.5 h at room temperature the solution was diluted with H_2O (10 mL) and extracted with diethyl ether (3×25 mL). The combined organic layers were dried (Na_2SO_4) and concentrated. Flash column chromatography of the residue (heptane/EtOAc, 1:1) gave **8a** (93 mg, 38%) as a white amorphous solid: $[\alpha]_d^{20}=-5$ (*c* 1.0, $CHCl_3$); ^{1}H NMR δ 7.76 (d, *J*=7.5 Hz, 2H, Fmoc), 7.59 (d, *J*=7.2 Hz, 2H, Fmoc), 7.39 (t, *J*=7.4 Hz, 2H, Fmoc), 7.36–7.28 (m, 14H, Cbz, Bzl, Fmoc), 5.63 (d, *J*=7.4 Hz, 1H, NH-ϵ), 5.24–5.08 (m, 5H, $N^{\epsilon}CO_2CH_2Ph$, CO_2CH_2Ph, NH-α), 4.50–4.34 (m, 3H, H-α, Fmoc, Fmoc), 4.21 (t, *J*=6.7 Hz, 1H, Fmoc), 3.73–3.62 (m, 1H, H-δ), 3.33–3.20 and 3.07–2.94 (2 m, each 1H, H-ϵ), 2.83–2.72 (m, 1H, OH), 2.07–1.96 and 1.82–1.70 (2 m, each 1H, H-β), 1.50–1.38 (m, 2H, H-γ,γ′); ^{13}C NMR δ 172.1, 157.2, 156.2, 143.8, 143.7, 141.3, 136.3, 135.2, 128.6, 128.6, 128.5, 128.4, 128.2, 128.1, 127.7, 127.1, 125.1, 120.0, 70.8, 67.3, 67.1, 66.9, 53.5, 47.1, 46.9, 29.9, 29.2; HRMS (FAB): calcd for $C_{36}H_{37}N_2O_7$ ($M+H^+$) 609.2601, found 609.2626.

(5*R*)-N^{α}-(Fluoren-9-ylmethoxycarbonyl)-N^{ϵ}-benzyloxycarbonyl-5-hydroxy-l-lysine diphenyl-methyl ester (8b). Compound **7** (104 mg, 0.2 mmol) was converted into the cesium salt of **5** as described for **8a**. The solid was dissolved in dry DMF (0.5 mL) at 0°C and diphenylmethyl bromide (250 mg, 1 mmol) dissolved in dry DMF (0.5 mL) was added. After stirring for 4 h at room temperature the solution was diluted with H_2O (10 mL) and extracted with diethyl ether (3×25 mL). The combined organic layers were dried (Na_2SO_4) and concentrated. Flash column chromatography of the residue (heptane/EtOAc, 1:1) gave **8b** contaminated by a small amount of lactone. Further purification by normal-phase HPLC (0→40% EtOH in hexane over 180 min) gave pure **8b** (48 mg, 35%) as a white amorphous solid: $[\alpha]_d^{20}=-12$ (*c* 1.0, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 7.75 (d, *J*=7.6 Hz, 2H, Fmoc), 7.58 (d, *J*=7.3 Hz, 2 H, Fmoc), 7.39–7.27 (m, 19H, Fmoc, Cbz, $CHPh_2$), 6.91 (s, 1 H, $CHPh_2$), 5.59 (bd, *J*=6.9 Hz, 1H, NH-α), 5.13–5.06 (m, 3H, $N^{\epsilon}CO_2CH_2Ph$, NH-ϵ), 4.60–4.52 (m, 1H, H-α), 4.40 (ABdd, *J*=10.6, 7.4 Hz, 1H, Fmoc), 4.38 (ABdd, *J*=10.6, 6.9 Hz, 1H, Fmoc), 3.71–3.61 (m, 1H, H-δ), 3.30–3.21 and 3.02–2.91 (2 m, each 1H, H-ϵ), 2.68 (bs, 1H, OH), 2.11–2.00 and 1.85–1.74 (2 m, each 1H, H-β(β′), 1.44–1.34 (m, 2H, H-γ,γ′); ^{13}C NMR ($CDCl_3$) δ 171.3, 157.1, 156.1, 143.8, 143.6, 141.2, 139.5, 139.2, 136.3, 128.6. 128.5, 128.5, 128.2, 128.1, 128.1, 127.7, 127.2, 127.0, 126.9, 125.0, 78.2, 70.7, 67.1, 66.9, 47.1, 46.9, 29.7, 28.9; HRMS (FAB): calcd for $C_{42}H_{41}N_2O_7$ ($M+H^+$) 685.2914, found 685.2930.

(5*R*)-N^{α}-(Fluoren-9-ylmethoxycarbnoyl)-N^{ϵ}-benzyloxycarbonyl-5-*O*-(2,3,4,6-tetra-*O*-acetyl-β-d-galactopyranosyl)-5-hydroxy-l-lysine benzyl ester (9a). A solution of **4** (69 mg, 0.16 mmol) in CH_2Cl_2 (1 mL) was added to a suspension of **8a** (63 mg, 0.10 mmol), silver silicate (155 mg) and crushed molecular sieves (3 Å, 50 mg) in CH_2Cl_2 (2 mL) at 0°C. After stirring for 2 h Et_3N (25 μL) was added and the suspension was stirred for 20 min. The solid material was filtered off (Hyflo Supercel), washed with CH_2Cl_2 (20 mL) and the combined filtrates were concentrated. Flash column chromatography of the residue (Toluene/MeCN, 8:1) gave **9a** (68 mg, 70%) as a white amorphous solid: $[\alpha]_d^{20}=1$ (*c* 1.0, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 7.76 (d, *J*=7.5 Hz, 2H, Fmoc), 7.59 (d, *J*=7.5 Hz, 2H, Fmoc), 7.40–7.28 (m, 12H, Fmoc, CO_2CH_2Ph, $N^{\epsilon}CO_2CH_2Ph$), 5.54 (t, *J*=5.5 Hz, 1H, NH-ϵ),

5.44 (d, *J*=7.9 Hz, 1H, NH-α), 5.35 (dd, *J*=3.4, 1 Hz, 1H, H-4), 5.18 (s, 2H, CO_2CH_2Ph), 5.15 (dd, *J*=10.5, 8.0 Hz, 1H, H-2), 5.12 and 5.09 (2 ABd, *J*=12.2 Hz, each 1H, $N^{\epsilon}CO_2CH_2Ph$), 4.96 (dd, *J*=10.5, 3.4 Hz, 1H, H-3), 4.47–4.30 (m, 4H, Fmoc, Fmoc, H-1, H-α), 4.20 (t, *J*=7.0 Hz, 1H, Fmoc), 4.12 (ABdd, *J*=11.4, 6.2 Hz, 1H, H-6), 4.05 (ABdd, *J*=11.2, 6.9 Hz, 1H, H-6), 3.82 (t, *J*=6.6 Hz, 1H, H-5), 3.66–3.58 (m, 1H, H-δ), 3.42–3.33 and 3.19–3.10 (2 m, each 1H, H-ϵ), 2.14 (s, 3H, $COCH_3$), 2.08–2.02 (m, 1H, H-β), 1.98, 1.98 and 1.95 (3 s, each 3H, 3 $COCH_3$), 1.74–1.43 (m, 3H, H-β, H-γ,γ′); ^{13}C NMR ($CDCl_3$) δ 171.9, 170.2, 170.1, 170.0, 169.3, 156.7, 155.9, 143.8, 143.7, 141.3, 136.5, 135.0, 129.0, 128.7, 128.6, 128.5, 128.4, 128.2, 128.2, 127.7, 127.1, 125.3, 125.0, 120.0, 101.3, 80.9, 70.8, 70.7, 68.8, 67.4, 67.1, 67.0, 66.8, 61.4, 53.7, 47.1, 44.7, 28.52, 28.3, 20.6, 20.5, 20.5; HRMS (FAB): calcd for $C_{50}H_{54}N_2NaO_{16}$ ($M+Na^+$) 961.3371, found 961.3391.

(5*R*)-N^{α}-(Fluoren-9-ylmethoxycarbonyl)-N^{ϵ}-benzyloxycarbonyl-5-*O*-(2,3,4,6-tetra-*O*-acetyl-β-d-galactopyranosyl)-5-hydroxy-l-lysine allyl ester (9c). A solution of **4** (247 mg, 0.60 mmol) in CH_2Cl_2 (1 mL) was added to a suspension of **8c** (223 mg, 0.4 mmol), silver silicate (600 mg) and crushed molecular sieves (3 Å, 50 mg) in CH_2Cl_2 (2 mL) at 0°C in the absence of light. Workup as described for **9a** followed by flash column chromatography of the residue (Toluene/MeCN, 8:1) gave **9c** (290 mg, 82%) as a white amorphous solid: $[\alpha]_d^{20}$=0 (*c* 3.0, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 7.76 (d, *J*=7.5 Hz, 2H, Fmoc), 7.60 (d, *J*=7.3 Hz, 2 H, Fmoc), 7.40 (t, *J*=7.4 Hz, 2H, Fmoc), 7.36–7.29 (m, 7H, Fmoc, Cbz), 5.96–5.85 (m, 1H, OCH_2CHCH_2), 5.59 (m, 1H, NH-ϵ), 5.45 (d, *J*=8.2 Hz, 1H, NH-α), 5.36–5.25 (m, 3 H, H-4, $OCH_2CH{=}CH_2$), 5.16 (dd, *J*=10.5, 8.0 Hz, 1H, H-2), 4.96 (dd, *J*=10.5, 3.36 Hz, 1H, H-3), 4.64 (m, 2H, $OCH_2CH{=}CH_2$), 4.48–4.43 (m, 2H, H-1, Fmoc), 4.38–4.30 (m, 2H, H-α, Fmoc), 4.22 (t, *J*=6.9 Hz, 1H, Fmoc), 4.12 (dd, *J*=11.4, 6.2 Hz, 1H, H-6), 4.05 (dd, *J*=11.2, 6.9 Hz, 1H, H-6), 3.83 (t, *J*=6.6 Hz, 1H, H-5), 3.70–3.62 (m, 1H, H-δ), 3.46–3.38 and 3.23–3.14 (2 m, each 1H, H-ϵ), 2.15 (s, 3 H, $COCH_3$), 2.05–1.93 (m, 10H, H-β, 3 $COCH_3$), 1.75–1.50 (m, 3H, H-β, H-γ,γ′); ^{13}C NMR ($CDCl_3$) δ 171.7, 170.3, 170.1, 170.0, 169.3, 156.7, 156.0, 143.7, 143.6, 141.2, 136.4, 131.3, 128.5, 128.4, 128.3, 128.2, 127.7, 127.1, 127.0, 125.0, 120.0, 119.3, 101.3, 80.9, 70.8, 70.7, 68.8, 67.0, 66.9, 66.7, 66.2, 61.5. 53.6, 53.6, 47.0, 44.8, 28.6, 28.3, 20.6, 20.5; HRMS (FAB): calcd for $C_{46}H_{52}N_2NaO_{16}$ ($M+Na^+$) 911.3215, found 911.3221.

(5*R*)-N^{α}-(Fluoren-9-ylmethoxycarbonyl)-N^{ϵ}-benzyloxycarbonyl-5-*O*-(2,3,4,6-tetra-*O*-acetyl-β-d-galactopyranosyl)-5-hydroxy-l-lysine (10). From **9c**: $(PPh_3)_4Pd(0)$ (29 mg, 25 μmol) was added to a solution of **9c** (223 mg, 0.25 mmol) and *N*-methylaniline (82 μl, 0.75 mmol) in dry THF in the absence of light. After stirring for 1.5 h at room temperature the solution was diluted with EtOAc (50 mL) and washed with saturated NH_4Cl (aq), dried, filtered and concentrated. Flash column chromatography of the residue (CH_2Cl_2→CH_2Cl_2/MeOH, 10:1) gave **10** (182 mg, 86%). From **8b**: A solution of **4** (37 mg, 87 μmol) in CH_2Cl_2 (1.5 mL) was added to a suspension of **8b** (40 mg, 58 μmol), silver silicate (87 mg) and crushed molecular sieves (3 Å, 30 mg) in CH_2Cl_2 (1 mL) at −10°C in the absence of light. After stirring for 17 h Et_3N (25 mL) was added and the suspension was stirred for 20 min. The solid material was filtered off (Hyflo supercel), washed with CH_2Cl_2 and the combined filtrates were concentrated. Flash column chromatography of the residue (Toluene/MeCN, 8:1) gave a solid which was dissolved in TFA/H_2O/CH_2Cl_2 (1 mL, 9:1:10) at 0°C. After stirring for 40 min at room temperature the solvent was removed in vacuo, toluene was added and the resulting solution was concentrated. Flash column chromatography of the residue (Toluene/EtOH, 20:1→4:1) gave **10** (24 mg, 48%) as a white amorphous solid. Compound **10** had: $[\alpha]_d^{20}$=0 (*c* 2.0, $CHCl_3$); ^{1}H NMR ($CDCl_3$/CD_3OD 1:1) δ 7.74 (d, *J*=7.5 Hz, 2H, Fmoc), 7.62 (t, *J*=6.3 Hz, 2H, Fmoc), 7.36 (t, *J*=7.5 Hz, 2H, Fmoc), 7.37–7.25 (m, 7H, Fmoc, Cbz), 5.33 (d, *J*=2.8 Hz, 1H, H-4), 5.10 (ABdd, *J*=10.5, 7.9 Hz, 1H, H-2), 5.05 (s, 2H, OCH_2Ph), 5.01 (dd, *J*=10.5, 3.3 Hz, 1H, H-3), 4.40–4.30 (m, 2H, Fmoc), 4.55 (d, *J*=7.5 Hz, 1H, H-1), 4.20 (t, *J*=6.8 Hz, 1H, Fmoc), 4.17–4.15 (m, 1H, H-α), 4.12 (dd, *J*=12.2, 7.1 Hz, 1H, H-6), 4.04 (dd, *J*=11.8, 7.5 Hz, 1H, H-6), 3.71–3.61 (m, 1H, H-δ), 3.37–3.27 and 3.22–3.14 (2 m, each 1H, 2H-ϵ), 2.11 and 2.00 (2 s, each 3H, 2 $COCH_3$), 1.97–1.93 (m, 7H, H-β, 2 $COCH_3$), 1.70–1.61 (m, 1H, H-β), 1.61–152 (m, 2 H, H-γ,γ′); ^{13}C NMR (CD_3OD/$CDCl_3$, 1:1) δ 171.5, 171.3, 171.0, 170.8, 157.8, 157.6, 144.5, 144.4, 141.9, 137.1, 129.4, 129.3, 129.0, 128.7, 128.3, 127.6, 125.6, 120.4, 101.8, 81.4, 71.6, 71.2, 69.7, 67.9, 67.4, 67.3, 62.0, 54.4, 54.0, 47.7, 45.4, 29.7, 28.5, 20.8, 20.7, 20.7; HRMS (FAB): calcd for $C_{43}H_{48}N_2NaO_{16}$ ($M+Na^+$) 871.2902, found 871.2897.

l-Alanylglycyl-l-phenylalanyl-l-lysylglycyl-l-glutam-1-yl-l-glutaminylglycyl-l-prolyl-(5*R*)-5-*O*-(β-d-galactopyranosyl)-5-hydroxy-l-lysylglycyl-l-glutam-1-yl-l-threonylglycyl-l-prolyl-l-alanylglycyl-l-proline (11). Glycopeptide **11** (Table 1) was prepared under conditions identical to those described recently.[4,10] A custom-made, fully automatic, continuous flow peptide synthesizer[18] and a TentaGel–S–Trt–Pro–Fmoc resin (480 mg, capacity 0.21 mmol/g, 0.100 mmol) was used for the synthesis. In brief, the Fmoc-amino acids (4 equiv.) were activated as benzotriazolyl esters[20] using 1,3-diisopropylcarbodiimide (DIC, 3.9 equiv.) and 1-hydroxybenzotriazole (HOBt, 6 equiv.) in DMF. The acylations were monitored[21] using the absorbance of bromophenol blue at 600 nm. N^{α}-Fmoc deprotections were performed by a flow of 20% piperidine in DMF using the absorbance of the dibenzofulvene–piperidine adduct at 350 nm for monitoring.[22] After coupling of Gly^{271} and removal of the Fmoc group, half of the resin (50 μmol) was allowed to swell in a minimal amount of DMF. Glycosylated hydroxylysine **10** (1.5 equiv.) was activated separately using DIC (1.5 equiv.) and 1-hydroxy-7-azabenzotriazole (3 equiv.) in dry DMF for 45 min before coupling to the peptide resin. The coupling was allowed to proceed for 24 h, then the resin was washed six times with DMF and transferred back to the peptide synthesizer where the remaining couplings were performed. After completion of the synthesis the resin was washed six times with DMF and 10 times with CH_2Cl_2 and dried under vacuum overnight giving 224 mg of resin. Cleavage and deprotection of the peptide moiety was performed with a mixture of TFA/H_2O/thioanisole/ethanedithiol (35:2:2:1, 10 mL) as described

Table 1. ^{1}H NMR data for glycopeptide **11**, in water containing 10% D_2O

Residue	NH	α	β	γ	Others
Ala^{261}		4.03	1.44		
Gly^{262}	8.64	3.89[a]			
Phe^{263}	8.41	4.56	3.00[a]		7.27, 7.24 and 7.20 (arom.)
Lys^{264}	8.52	4.19	1.72, 1.62	1.29[a]	1.58[a] (δ), 2.88[a] (ϵ)
Gly^{265}	7.95	3.85, 3.80			
Glu^{266}	8.38	4.21	2.20[a]	1.90, 1.84	
Gln^{267}	8.60	4.31	2.30[a]	2.07, 1.92	7.57 and 6.89 ($CONH_2$)
Gly^{268}	8.42	4.10, 3.90			
Pro^{269,c}		4.29	1.83, 1.76	2.25, 2.03	3.49 and 3.42 (δ)
Hyl^{270,b}	8.67	4.28	1.99, 1.76	1.76[a]	4.0 (δ), 2.93 and 3.13 (ϵ)
Gly^{271}	8.43	4.09, 3.89			
Glu^{272}	8.50	4.32	2.21[a]	2.01, 1.87	
Thr^{273}	8.42	4.32	4.18	1.15	
Gly^{274}	8.38	4.12, 3.99			
Pro^{275,c}		4.29	1.91, 1.86	2.13[a]	3.50[a] (δ)
Ala^{276}	8.57	4.29	1.34		
Gly^{277}	8.23	4.08, 3.92			
Pro^{278}		4.34	1.95, 1.88	2.22[a]	3.54[a] (δ)

[a] Degeneracy has been assumed.
[b] Chemical shifts (δ, ppm) for the galactose moiety: 4.40 (H-1), 3.84 (H-4), 3.69 (H-5), 3.62 (H-6,6), 3.58 (H-3), 3.46 (H-2).
[c] The assignments of Pro^{269} and Pro^{275} are uncertain.

previously.[4,10] The crude product obtained after freeze-drying was purified by reversed-phase HPLC (0→100% MeCN in 40 min) to give 12 mg of the *O*-acetylated glycopeptide. Deacetylation was performed using methanolic NaOMe (20 mM, 10 mL) at 0→20°C for 3 h (monitoring by reversed-phase HPLC, 0→100% MeCN during 60 min) and the solution was neutralized (pH paper) by addition of 10% HOAc in MeOH. Concentration, followed by purification using reversed-phase HPLC (0→100% MeCN during 50 min) gave **11** (6.8 mg, 81% peptide content, 12% overall yield) as a white amorphous solid after freeze-drying: ^{1}H NMR data, cf. Table 1; MS (FAB) calcd. 1863 ($M+H^+$), found 1862; amino acid analysis: Ala 2.03 (2), Glu 2.90 (3), Gly 6.01 (6), Hyl 1.00 (1), Lys 1.04 (1), Phe 1.01 (1), Pro 2.98 (3), Thr 1.01 (1).

l-Alanylglycyl-l-phenylalanyl-(5*R*)-5-*O*-(β-d-galactopyranosyl)-5-hydroxy-l-lysylglycyl-l-glutam-1-yl-l-glutaminylglycyl-l-prolyl-l-lysylglycyl-l-glutam-1-yl-l-threonylglycyl-l-prolyl-l-alanylglycyl-l-proline (12). Synthesis of glycopeptide **12** (Table 2) was performed in a mechanically agitated reactor on TentaGel–S–Trt–Pro–Fmoc resin (167 mg, 30 μmol) using the same conditions for synthesis and cleavage as described for glycopeptide **11**. However, the glycosylated hydroxylysine at position 264 was incorporated using building block **6** (1.3 equiv.) activated as a 7-azabenzotriazole ester. After cleavage from the solid phase, purification by reversed-phase HPLC (0→100% MeCN during 60 min) gave *O*-acetylated glycopeptide (33.5 mg). Deacetylation was performed using saturated methanolic ammonia (50 mL) at 0→20°C for

Table 2. ^{1}H NMR data for glycopeptide **12** in water containing 10% D_2O

Residue	NH	α	β	γ	Others
Ala^{261}		4.01	1.42		
Gly^{262}	8.63	3.37[a]			
Phe^{263}	8.40	4.55	2.98[a]		7.27 and 7.18 (arom)
Hyl^{264,b}	8.53	4.20	1.91, 1.66	1.51[a]	3.90 (δ), 2.88 and 3.09 (ϵ)
Gly^{265}	7.98	3.85, 3.78			
Glu^{266}	8.39	4.20	2.21[a]	1.96, 1.81	
Gln^{267}	8.61	4.29	2.29[a]	2.05, 1.76	7.56 and 6.88 ($CONH_2$)
Gly^{268}	8.39	4.06, 3.90			
Pro^{269,c}					
Lys^{270}	8.61	4.23	1.77, 1.68	1.36[a]	1.60[a] (δ), 2.9[a] (ϵ)
Gly^{271}	8.42	4.05, 3.91			
Glu^{272,d}	8.39				
Thr^{273}	8.39	4.31	4.17	1.12	
Gly^{274}	8.38	4.10, 3.98			
Pro^{275,c}					
Ala^{276}	8.54	4.23	1.32		
Gly^{277}	8.21	4.05, 3.91			
Pro^{278}					

[a] Degeneracy has been assumed.
[b] Chemical shifts (δ, ppm) for the galactose moiety: 4.38 (H-1), 3.86 (H-4), 3.70 (H-6,6), 3.64 (H-5), 3.59 (H-3), 3.46 (H-2).
[c] The assignments of Pro^{278}, Pro^{275} and Pro^{269} are uncertain. Three sets of resonances for Pro residues exist but they cannot be unambiguously identified.
[d] The remaining resonances of Glu^{272} could not be unambiguously assigned and are therefore not reported.

8 h. Concentration, followed by purification using reversed-phase HPLC (0→100% MeCN during 60 min) and freeze drying gave **12** (25 mg, 73% peptide content, 33% overall yield) as a white amorphous solid: ^{1}H NMR data, cf. Table 2; MS (FAB) calcd. 1863 ($M+H^+$), found 1862; amino acid analysis: Ala 2.03 (2), Glu 3.00 (3), Gly 5.99 (6), Hyl 1.02 (1), Lys 1.02 (1), Phe 1.01 (1), Pro 2.92 (3), Thr 1.01 (1).

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