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Synthesis of glycocluster peptides

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Abstract—A new type of glycoconjugate mimetic is introduced that combines a glycocluster head group with a peptide part. These 'glycocluster peptides' are designed to serve as mimetics of glycocalyx constituents. A convergent synthetic scheme was followed, consisting of (i) the synthesis of a clustered carbohydrate head group carrying an amino acid at the focal point, and (ii) the solid phase synthesis of the peptide moiety. Finally, peptide coupling on resin furnished two prototype glycocluster peptides, which each exposes three α-mannosyl residues in the form of a dendritic wedge, with different conformational features. Extensive purification and NMR studies were necessary to characterize the target compounds and the results of these investigations are reported here together with the synthesis.

Keywords: Glycoconjugate mimetics; Cluster mannosides; Mannosylation; Solid phase synthesis; Peptide coupling

1. Introduction

Eukaryotic cells are covered with a highly diverse and complex glyco-environment, which is called the 'glycocalyx'. [1] Hundreds of reports clearly demonstrate the importance of the glycocalyx components in key events in cell biology[2] such as in cell signalling, cell differentiation and many normal as well as pathological states of cell development.[3] Although the biological relevance of the glycoconjugates making up the glycocalyx is beyond dispute, researchers are still seeking for appropriate methods to unravel the secrets of glycocalyx function.

Indispensable for the investigation of glycocalyx biochemistry are synthetic glycoconjugates, as the relevant compounds cannot be obtained by isolation from natural material in sufficient amounts and purity. However, the synthesis of the desired molecules is highly laborious,[4] and thus glycoconjugate mimetics[5] have been introduced, as they allow for a more rapid preparation and a larger variation of molecular diversity and functional properties. Glycoconjugate mimetics have been shown to serve as valuable tools in glycobiology[6] and thus our research has focused on the design of further representatives of this advantageous class of molecules.

It has been an interesting approach to design glycopeptide mimetics in which the peptide part serves to scaffold carbohydrate epitops.[7] Here we would like to introduce a different type of glycopeptide mimetic, combining a glycocluster wedge with a peptide chain in order to eventually mimic transmembrane glycoproteins. With this approach it has been our goal to extend a successful molecular design for the preparation of oligosaccharide mimetics, which relies on the principles of dendrimer chemistry,[8] coupled with the highly flexible solid phase synthesis of peptide chains. This led us to the so-called 'glycocluster peptides', which mimic a portion of glycosylated membrane proteins that are constituents of the glycocalyx (Fig. 1).

Our approach to glycocluster peptides combines a number of favourable properties, such as (i) easy variation of carbohydrate multiplicity of the branched glycosylated head moiety of the conjugate according to the

Abbreviations: Fmoc, fluorenylmethoxycarbonyl; Gly, glycine; Ser, serine; DIC, diisopropylcarbodiimide; HATU, *O*-(7-azabenzotriazol-1-yl)- *N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HBTU, *O*-(benzotriazol-1-yl)- *N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; DIPEA, *N*-ethyl- *N*-diisopropylamine

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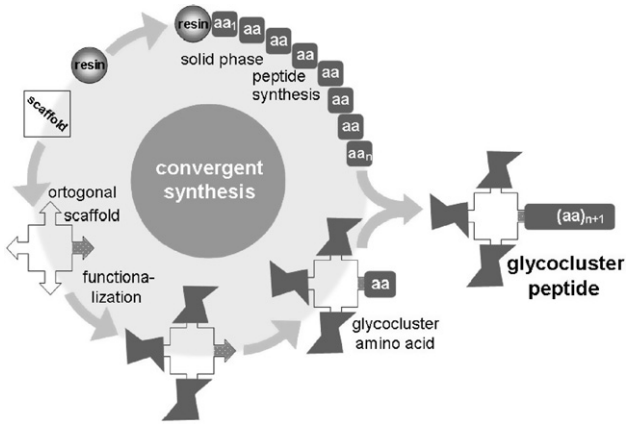


Figure 1. This cartoon highlights the chosen approach to glycocluster peptides. The convergent synthesis relies, on one hand, on solid phase peptide synthesis, and on the other hand on the orthogonal derivatization of an appropriate tetrafunctional scaffold molecule. Three carbohydrate epitopes can be attached to this scaffold, which in turn is further functionalized with an amino acid (aa) to furnish a glycocluster amino acid. This is eventually combined with a desired peptide on solid phase to yield the target glycoconjugate mimetic.

principles of dendrimer chemistry; (ii) variation of the peptide part as the synthetic plan is convergent and thus independent of the peptide; and (iii) orthogonal functionalization of the peptide portion thus enabling biolabelling, which is often a requirement for biological assaying. Here we describe the synthesis of a model peptide on the solid phase, the synthesis of two different trivalent cluster mannosides carrying an aspartic acid residue at the focal point, and finally the convergent assembly of the glycocluster building block and the peptide on solid phase to yield the two prototype glycocluster peptides **20** and **21**, both exposing three terminal α-mannosyl residues in their glycosylated head group.

2. Results and discussion

2.1. Solid phase synthesis of model octapeptide

Solid phase peptide synthesis is a well-established method, which allows multiple steps of peptide coupling reactions without any purification. We synthesized a model octapeptide with the sequence SSGGGGGG using Wang resin, following a conventional Fmoc strategy. The resin was first loaded with Fmoc-Gly-OH by esterification using DIC, HOBt and DIPEA, followed by capping of unreacted resin surface to obtain **1** (Scheme 1). Analysis of **1** revealed a resin load of 0.60 mmol/g. Fmoc-protected **1** was treated under nitrogen atmosphere with 20% piperidine in DMF to deprotect the terminal amino group, which was then coupled to Fmoc-Gly-OH using HBTU together with HOBt and DIPEA as coupling reagents to provide **2**. Complete peptide coupling was assured by the 'Kaiser test'.[9]

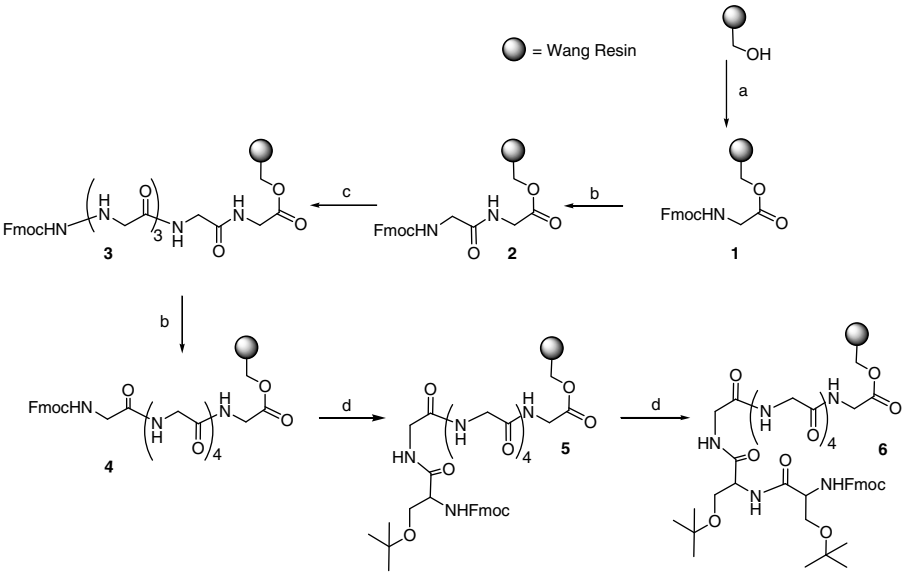
All following peptide coupling steps were carried out in a manner analogous to the first. Thus, peptide **2** was further elongated with Fmoc-protected triglycine to give **3**, followed by an additional coupling to Fmoc-Gly-OH to yield the resin-bound hexapeptide **4**. This approach to the hexapeptide was a result of careful optimization as coupling of a hexaglycine building block was inefficient and afflicted with solubility problems. Also, coupling of the dipeptide Fmoc-Gly-Gly-OH to **1** was unfavourable due to poor solubility of the diglycine derivative. Finally, **4** was coupled to two molecules Fmoc-Ser-O*t*-Bu-OH in two subsequent steps to yield **5** and the target octapeptide **6**, respectively. Cleavage of the peptide from 50 mg of resin revealed a final resin load of 0.46 mmol/g.

2.2. Synthesis of glycocluster amino acid 13

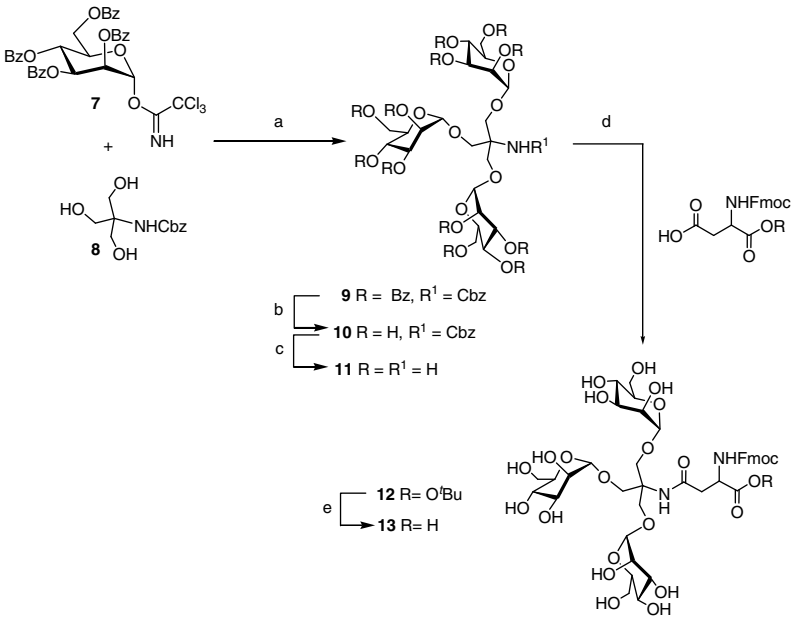
In a second part of our work, we targeted trivalent branched cluster mannosides carrying suitable functionalization for coupling to peptide **6** on solid phase. A well-known molecule, which can serve as an appropriate scaffold in the synthesis of our target molecules, is the trihydroxy amine TRIS (tris(hydroxymethyl)aminomethane), which can be easily obtained in its Cbz-protected form **8**[10] (Scheme 2). This triol **8** was mannosylated using the *O*-benzoyl-protected mannosyl trichloroacetimidate **7**[11] to yield the protected trivalent cluster mannoside **9** in a $BF_3{\cdot}Et_2O$-catalyzed reaction.

TRIS has been used earlier in the synthesis of cluster glycosides;[12] however, its glycosylation is not trivial. Frequently, environmentally hazardous mercury salts such as $Hg(CN)_2$ were employed as promoters to effect complete O-glycosylation of TRIS.[13] Mannosylation of TRIS, delivering **9** and **10**, has been reported[14] employing protected mannosyl bromide and silver salts according to a Koenigs–Knorr protocol.[15] Here we report a successful procedure leading to **9** without the use of heavy metal salts according to the Schmidt glycosylation technology.[16] In addition, no orthoester formation was observed in the mannosylation reaction of **8**, which is often problematic in multi-mannosylation reactions.[17]

De-O-benzoylation of the fully protected glycocluster **9** was accomplished according to Zemplén and Pacsu[18] to give rise to pure **10** after purification by size exclusion chromatography on LH-20 gel to remove the methyl benzoate by-product of the deprotection reaction. Then, the Cbz-protected amino group of **10** was deprotected via Pd-catalyzed hydrogenation to give amine **11** in nearly quantitative yield. As the cluster mannoside **11** has been designed for coupling to a peptide, our synthetic approach included elongation of the amino group at the focal point of **11** to avoid steric hindrance in the following peptide coupling step. Thus, **11** was functionalized as glycocluster amino acid employing an orthogonally protected aspartic acid derivative (Scheme 2).



Scheme 1. Synthesis of model octpeptide **6** on Wang resin. (a) (i) Fmoc-Gly-OH, DIC, HOBt, DIPEA, DMF; (ii) Ac_2O, pyridine; (b) (i) 20% piperdine in DMF; (ii) Fmoc-Gly-OH, HBTU, HOBt, DIPEA; (c) (i) 20% piperdine in DMF; (ii) Fmoc-Gly-Gly-Gly-OH, HBTU, HOBt, DIPEA; (d) (i) 20% piperidine in DMF; (ii) Fmoc-Ser-O*t* -Bu-OH, HBTU, HOBt, DIPEA.

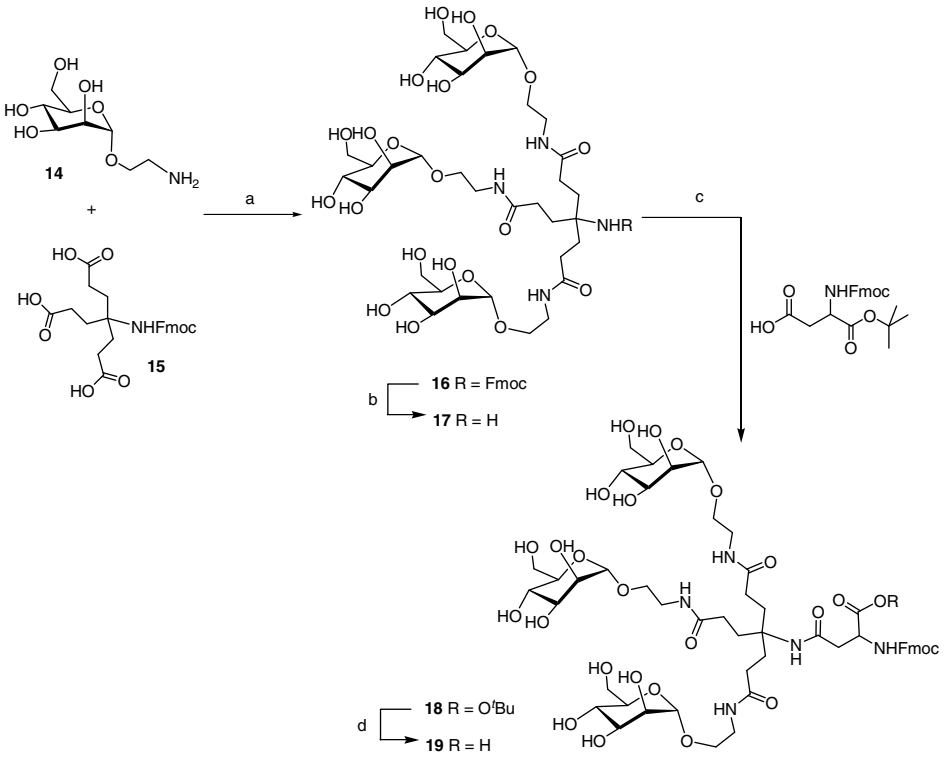


Scheme 2. Synthesis of glycocluster amino acid **13**. (a) $BF_3{\cdot}Et_2O$, CH_2Cl_2, 0 °C→rt, overnight, 66%; (b) MeONa, MeOH, rt, 4 h, 96%; (c) Pd–C, H_2, MeOH, rt, overnight, 95%; (d) HATU, DIPEA, DMF, 0 °C→rt, 5 h, 37%; (e) TFA–H_2O (7:3), rt, 1.5 h, 94%.

HATU-mediated peptide coupling to the side chain carboxylic group afforded glycocluster **12** in good yield. Purification of compound **12** was quite tedious, and involved gel chromatography to remove low molecular mass impurities and subsequent HPLC employing a water–acetonitrile gradient. This lengthy purification protocol might account for the moderate yield in which the protected glycocluster amino acid **12** was finally obtained after lyophilization. Lastly, treatment of **12** with a TFA–H_2O mixture (7:3) gave the carboxylic acid **13** in excellent yield.

2.3. Synthesis of glycocluster amino acid 19

Conformational availability, as well as epitope flexibility is an important parameter in molecular recognition.[19] Therefore, we have also focused on the preparation of cluster mannosides with a more flexible architecture



Scheme 3. Synthesis of glycocluster amino acid **19**. (a) HATU, DIPEA, DMF, 0 °C→rt, 56%; (b) morpholine, DMF; (c) HATU, DIPEA, DMF, 0 °C→rt, 36%; (d) TFA–H_2O (7:3), 95%.

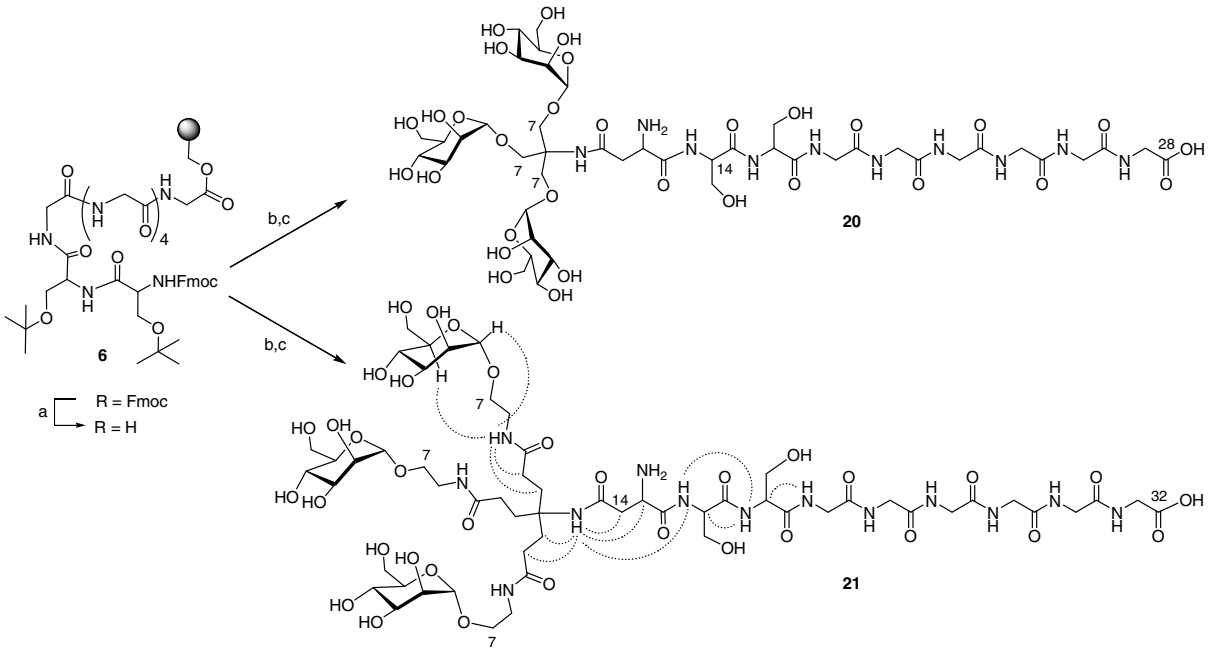
within the carbohydrate head group. By employing spaced analogues of **7** and **8**, a conformationally varied glycocluster analogue of **11** was constructed. For this purpose the unprotected aminoethyl mannoside **14**[20] could be utilized together with the well-known triacid **15**[21] (Scheme 3). Peptide coupling of the 'Newkome-type' [22] trivalent wedge **15** and mannoside **14** was successful using HATU and DIPEA to yield the known cluster mannoside **16**,[21] which carries an Fmoc-protected amino group at its focal point. Deprotection of Fmoc was accomplished with morpholine to get the free amine **17**, which was used in the next step without further purification.

In analogy to the approach exemplified in Scheme 2, **17** was elongated at the focal point by peptide coupling to the side chain carboxylic group of the aspartic acid derivative Fmoc-Asp-O*t*-Bu-OH (Scheme 3) to give **18**. Purification of compound **18** required a lengthy protocol similar to **12**. Again subsequent GPCs and HPLC on reversed phase silica gel accounted for yield losses. Next, the *t*-butyl ester was cleaved using TFA–H_2O giving rise to glycocluster amino acid **19** in pleasant 90% yield.

2.4. Coupling of glycocluster amino acids 13 and 19 to peptide 6 on solid phase: synthesis of target glycocluster peptides 20 and 21

With the unprotected cluster mannosides amino acids **13** and **19** in hand, we set out to prepare the target glycocluster peptides on the solid phase. First, peptide **6** was treated with 20% piperidine in DMF to remove the terminal Fmoc-group. Then, after repeated washing with DMF, the glycocluster amino acids, **13** or **19**, were attached to the peptide using HATU and DIPEA for peptide coupling (Scheme 4). Owing to the high value of **13** and **19**, just 1.5 equiv of the respective glycocluster amino acid were employed in the peptide coupling step, contrary to all other peptide coupling reactions performed at an earlier stage of the synthetic route, where 4 equiv of the amino acids were employed. The coupling reaction was allowed to run overnight and after 12 h reaction time the subsequent Kaiser test was negative, indicating the absence of unreacted free terminal amino groups on the resin. Then, the glycopeptide was cleaved from the resin employing acidic conditions (TFA–CH_2Cl_2, 4:1). The *t*-butyl ether protecting groups on both serine side chains were removed in the same step. After the TFA was evaporated, the glycopeptide was dissolved in H_2O and filtered through a syringe filter to remove insoluble impurities and to allow isolation of glycopeptides **20** and **21** in excellent yields.

The structures of both glycopeptides were determined by several NMR experiments. In the ^1H NMR spectra of both **20** and **21**, α-protons of all six glycine residues overlap at 3.76 ppm. The amide protons of the peptide are reasonably well dispersed, and resonate between 7 and 8 ppm. They were sequentially assigned



Scheme 4. Synthesis of glycocluster peptides **20** and **21**. (a) 20% piperdine in DMF; (b) HATU, DIPEA, DMF, **13** (for **20**) or **19** (for **21**), respectively, overnight; (c) release from resin according to general procedure 4.2.3: 82% (**20**), 93% (**21**) (Numbering as depicted here was used for assignment of NMR data; NOEs are shown with dotted lines.).

by comparison of COSY and NOESY spectra. In the latter, sequential $d_{\alpha N}(i, i+1)$ NOEs were observed between the first and second serine residues, and between the second serine and the following first glycine.

Additionally, a high quality NOE data set on **21** provided further expected NOEs between 'spacer NH' to the spatially proximal protons, which facilitated the complete characterization of the linker structure. An analysis of the chemical shifts of the structurally sensitive H α-protons of all amino acids indicates that these shifts are nearly identical to random coil chemical shifts,[23] which is suggestive of a lack of defined secondary structure. Notably though, a sequential d_{NN}-NOE is observed between the aspartic acid γ-carboxyamide proton (HN at the focal point) and the first serine HN in **21**. This is unexpected due to the separation between this aspartic acid γ-carboxyamide and the serine, and may indicate a conformational preference in this compound. However, the interpretation of this NOE requires more detailed NMR-spectroscopic studies, which are also warranted in light of putative effects of the spacer length on the cluster structure. In particular, in the somewhat more crowded compound **20**, the peptide conformation and the relative orientation of the carbohydrate moieties may be affected by steric hindrance. NMR spectroscopy will be ideally suited for analysis of such local interactions, due to the availability of techniques to assess not only spatial proximity, but also mobility, rotational restriction and partial alignment.[24] These studies are currently under way in our laboratory.

3. Conclusion

We have elaborated a convergent and highly flexible approach to the so-called glycocluster peptides, which were designed to serve as glycoconjugate mimetics. Along the chosen synthetic route we have demonstrated convenient synthesis of glycocluster amino acids **13** and **19**, including a metal-free mannosylation of TRIS furnishing the trivalent cluster mannoside **11**. Peptide coupling of **13** and **19** to a chosen model peptide of the sequence SSGGGGGG on solid phase led us to the first two prototype glycocluster peptides **20** and **21** after cleavage from the resin.

The approach introduced herein allows for easy variation of carbohydrate valency as well as variation of the conformational circumstances within the glycocluster head group. In addition, we can easily modify peptide sequences due to the solid phase protocol employed. Here we have chosen an octapeptide sequence containing two serine residues, which allow further modification of the side chain hydroxyl groups to further develop the target glycocluster peptides to even more sophisticated membrane mimetics. For biological and biophysical reasons, the conformational and three-dimensional properties of the herein presented glycomimetics and their change upon interaction with sugar-specific lectins will be of foremost interest. The NMR studies we have performed so far with **20** and **21** revealed interesting NOEs between Ser 1 and the linker moiety of **21** and thus this molecule together with representatives of an analogous type will be subject to in-depth structural

characterization including their supramolecular chemistry in different solvents.

4. Experimental

4.1. General methods

TLC was performed on silica gel plates GF_{254} (detection with 10% ethanolic sulfuric acid solution and charring), and flash chromatography was performed on silica gel 60 (Merck, particle size 40–63 μm, 230–400 mesh). For the size exclusion chromatography Sephadex LH-20 was used with MeOH as the eluent. The organic solutions were concentrated using a rotary evaporator at bath temperatures <45 °C. Aqueous solutions were concentrated by lyophilization on a freeze dryer. ^1H and ^{13}C NMR spectra were recorded at 298 K, 500 MHz (for ^1H, 125.75 MHz for ^{13}C NMR) and 600 MHz (for ^1H, 150.90 MHz for ^{13}C NMR). Chemical shifts are given in ppm relative to internal TMS (0.00 ppm for ^1H and ^{13}C NMR). Two-dimensional ^1H–^1H and ^1H–^{13}C COSY (HMQC) experiments were performed for complete signal assignments wherever necessary. The sugar residues were numbered from 1 to 6 with the anomeric position being number 1, the remaining part of the molecules were numbered along their longest chains. MALDI-ToF MS were recorded on a Bruker Biflex and ESIMS on a Finnigan MAT 95. For MALDI-ToF measurements the samples were prepared as solutions in MeOH–water, with a concentration of 1 mg/mL solution. Compounds were co-crystallized with either 2,5-dihydroxy benzoic acid (DHB) or α-cyano-4-hydroxycinnamic acid (CCA). The mass peaks obtained with all the samples were calibrated in reference to the $[M+H]^+$ peaks of angiotensin II (1046.54), angiotensin I (1296.69), bombesin (1619.82), and to the $[2M+H]^+$ peak of CCA (380.02). High resolution ESI mass spectra were measured with an Applied Biosystems Mariner ESI-ToF 5280. The DMF (peptide synthesis grade), DIPEA, HATU and Fmoc-Gly-OH, were purchased from Fluka. The other amino acids, Fmoc-Asp-O*t*-Bu-OH and Fmoc-Ser-O*t*Bu-OH were purchased from Novabiochem. The Wang resin (loading capacity 0.9 mmol/g) was purchased from Advanced Chemtech.

4.2. General procedures

4.2.1. General coupling procedure for peptides. Before each peptide coupling step the resin was treated with a solution of piperidine in DMF (20%, 5 mL) for 10 min. The same treatment was repeated to assure complete cleavage of Fmoc protection and then the resin was repeatedly washed with DMF (five times with 5 mL). For peptide coupling, the respective amino acid derivatives (4 equiv relative to the peptide on resin), HBTU (3.6 equiv) and HOBT (4 equiv), were dissolved in DMF (2–3 mL) and DIPEA (4 equiv) was added. This solution was shaken for 5 min and then the activated amino acid derivative was transferred to the resin. This mixture was kept under N_2 bubbling for 3–4 h at room temperature. Completion of the coupling reaction was confirmed by the 'Kaiser test'. Kaiser's test is negative, in the absence of any blue dye on the resin.

4.2.2. General procedure for *t*-butyl deprotection. The *t*-butyl ester to be cleaved is dissolved in a mixture of TFA and H_2O (7:3). The reaction mixture is stirred at room temperature for 1–1.5 h, then concentrated in vacuo and lyophilized to give the corresponding carboxylic acid which can be employed without further purification.

4.2.3. Release of unprotected glycocluster peptides 20 and 21 from resin. For Fmoc deprotection the resin was first treated with piperidine in DMF (20%, 5 mL) for 20 min and was then subsequently washed with DMF (5 mL, three times) and CH_2Cl_2 (5 mL, three times). The resin was slurried in a mixture of TFA and CH_2Cl_2 (4:1, 5 mL) and kept for 30 min. Then the resin was filtered through a sintered funnel and washed with TFA–CH_2Cl_2 (4:1, 3 mL three times). The combined washings were evaporated in vacuo, then dissolved in H_2O and passed through a syringe filter to remove insoluble impurities. Lyophilization furnished the respective glycocluster peptide.

4.3. Synthesis of peptide 6 on solid support

4.3.1. Loading the resin with Fmoc-Gly-OH (1). In a round-bottomed flask Wang resin (2 g) was suspended in a mixture of CH_2Cl_2 and DMF (9:1, 30 mL). In a separate flask Fmoc-Gly-OH (1.34 g, 2.5 equiv relative to resin) was dissolved in DMF (4 mL) along with HOBt (610 mg, 2.5 equiv relative to resin). This solution was added to the resin, followed by addition of DIC (700 μL, 1.0 equiv relative to Fmoc-Gly-OH) and DMAP (218 mg, 0.1 equiv relative to Fmoc-Gly-OH) which were dissolved in DMF (2 mL). The flask was equipped with a drying tube and the reaction mixture was agitated on a mechanical shaker for 3 h at room temperature. Then, Ac_2O (140 μL, 2 equiv relative to the resin) and pyridine (170 μL, 2 equiv relative to the resin) were added and shaking was continued for an additional 30 min to complete end-capping of unreacted hydroxyl groups. The resin was filtered on a sintered funnel and washed three times with DMF (5 mL each), three times with CH_2Cl_2 (5 mL each) and then three times with MeOH (5 mL each). Then the resin was dried in vacuo to a constant weight. The amount of resin was found to be 2.66 g of which 666 mg (corresponding to

0.45 mmol of Fmoc-Gly-OH load) was used for the following peptide synthesis.

4.3.2. Preparation of peptide 6. The dry resin, loaded with Fmoc-Gly was swelled for 1 h in DMF. Fmoc-Gly-OH (535 mg, 4 equiv relative to the peptide on resin), HOBt (243 mg, 4 equiv) and HBTU (614 mg, 3.6 equiv) were dissolved in DMF (2 mL) and DIPEA (310 μL) was added. This mixture was stirred for 5 min and was then transferred to the swelled resin. Peptide coupling was carried out as described in Section 4.2.1. This first peptide coupling step led to **2**, then a mixture of Fmoc-Gly-Gly-Gly-OH (740 mg, 4 equiv relative to the peptide on resin), HOBt (243 mg, 4 equiv) and HBTU (614 mg, 3.6 equiv) in DMF (2 mL) and DIPEA (310 μL) was added after 5 min of stirring. This coupling step led to **3**. The next glycine was attached by adding a mixture of Fmoc-Gly-OH (535 mg, 4 equiv relative to the peptide on resin), HOBt (243 mg, 4 equiv) and HBTU (614 mg, 3.6 equiv) in DMF (2 mL) and DIPEA (310 μL) after 5 min of stirring. The resulting hexapeptide **4** was then subjected to two subsequent peptide coupling steps employing a mixture of Fmoc-Ser-O*t*-Bu-OH (383 mg, 4 equiv relative to the peptide on resin), HOBt (243 mg, 4 equiv) and HBTU (614 mg, 3.6 equiv) in DMF (2 mL) and DIPEA (310 μL) in each step, which had been stirred for 5 min. Thus, the target peptide **6** was obtained.

4.4. Synthesis of glycocluster amino acid 13

4.4.1. Protected clustermannoside 9. In a Schlenk flask compound **8** (231 mg, 0.90 mmol) and mannosyl trichloroacetimidate **7** (5.03 g, 6.70 mmol) were dissolved in absolute CH_2Cl_2 (50 mL) under inert atmosphere. The reaction mixture was cooled to 0 °C and $BF_3{\cdot}Et_2O$ (800 μL, 7.30 mmol) was added drop-wise to the solution. Then the reaction mixture was stirred at room temperature overnight. A saturated aq solution of $NaHCO_3$ was added and the aqueous phase was extracted with CH_2Cl_2 (100 mL) for three times. The combined organic phases were washed with brine, dried over $MgSO_4$ and concentrated in vacuo after filtration. Flash chromatography (30% ethyl acetate in cyclohexane) gave **9** (1.19 g, 0.60 mmol, 66%) as a colorless foam. ^{1}H NMR (600 MHz, $CDCl_3$, TMS): δ 8.13 (dd, 6H, Ar-H), 7.97 (dd, 6H, Ar-H), 7.86 (dd, 6H, Ar-H), 7.75 (dd, 6H, Ar-H), 7.53 (m_c, 6H, Ar-H), 7.43–7.36 (m, 12H, Ar-H), 7.32–7.25 (m, 11H, Ar-H), 7.19 (dd, 6H, Ar-H), 7.10 (dd, 6H, Ar-H), 6.20 (dd ~ t, 3H, $J = 10.10$ Hz, 3H-4), 5.94 (dd, 3H, $J_{2,3} = 3.22$, $J_{3,4} = 10.12$ Hz, 3H-3), 5,79 (dd, 3H, $J_{2,3} = 3.22$, $J_{1,2} = 1.76$ Hz, 3H-2), 5.30 (d, 3H, $J_{1,2} = 1.82$ Hz, 3H-1), 5.18 (d, 2H, $J = 2.31$ Hz, H-10), 4.80–4.77 (m, 3H, 3H-6) 4.61–4.56 (m, 6H, 3H-6′, 3H-5), 4.47 (d, 3H, $J = 10.34$ Hz, H-7), 4.08 (d, $J = 10.38$ Hz, H-7′); ^{13}C NMR (150.91 MHz, $CDCl_3$, TMS): δ 166.15, 166.48, 166.44, 166.24, (Ar-C), 155.26 (C-9), 136.07, 133.28, 133.16, 133.04, 132.91, 130.08, 129.94, 129.84, 129.76, 129.72, 129.39, 129.10, 128.94, 128.59, 128.49, 128.42, 128.26, 128.22 (Ar-H), 99.77 (C-1), 70.35 (C-3), 70.20 (C-2), 69.66 (C-5), 67.49 (C-7), 67.13 (C-10), 66.54 (C-4), 62.67 (C-6) ppm. ESIMS m/z $[M+Na]^+$ calcd for $C_{114}H_{95}NO_{32}$: 2012.5735. Found: 2013.5849.

4.4.2. Unprotected clustermannoside 11. Zemplén deprotection of **9** led to the OH-free cluster mannoside **10**[14a] as reported in the literature. The Cbz-protected cluster **10** (246 mg, 0.33 mmol) was dissolved in absolute MeOH (10 mL) and a catalytic amount of Pd on activated charcoal was added. The round-bottomed flask was equipped with a H_2 balloon and allowed to stir at room temperature for 12 h. The reaction mixture was filtered through Celite and concentrated in vacuo. After lyophilization with H_2O the title compound **11** (192 mg, 0.31 mmol, 95%) was isolated as a white foam. ^{1}H NMR (500 MHz, DMSO-d_6, TMS): δ 4.66 (q, 7H, 7 OH), 4.54 (d, 3H, $J_{1,2} = 1.33$ Hz, 3H-1), 4.48 (d, 3H, 3 OH), 4.38 (t, 3H, 1 OH, NH_2), 3.71 (d, $J = 9.69$ Hz, 3H-7), 3.63–3.59 (m, 6H, 3H-2, 3H-6), 3.49 (m_c, 6H, $J = 9.84$ Hz, 3H-6, H-7′), 3.44 (dd, 3H, $J_{2,3} = 5.7$, $J_{3,4} = 8.17$ Hz, 3H-3), 3.41 (dd, 3H, $J = 9.27$ Hz, 3H-4), 3.34 (m, 3H, H-5); ^{13}C NMR (125.75 MHz, DMSO-d_6, TMS): δ 100.70 (C-1), 73.71 (C-5), 70.96 (C-3), 70.17 (C-2), 66.80 (C-4), 65.28 (C-7), 60.90 (C-6), 58.02 (C-8) ppm. ESIMS m/z $[M+Na]^+$ calcd for $C_{22}H_{41}NO_{18}$: 630.2221. Found: 630.2171.

4.4.3. Glycocluster amino acid *t*-butyl ester 12. In a Schlenk flask, **11** (75 mg, 0.12 mmol), Fmoc-Asp-O*t*-Bu (103 mg, 0.25 mmol) and HATU (95 mg, 0.25 mmol) were dissolved in absolute DMF (10 mL). The solution was cooled to 0 °C for 10 min. Then DIPEA (42 μL, 0.25 mmol) was added and the reaction mixture was allowed to stir at room temperature for 4 h. The solvent was evaporated in vacuo and the residue was subjected to gel chromatography. Further purification by HPLC gave compound **12** (46 mg, 0.045 mmol, 37%) as white lypophilisate. HPLC: $t_R = 44.93$ min [250/4 LiChrosorb 7 μm C8, A = water, B = acetonitrile, 20% B → 50% B, 75 min, 1 mL/min]. ^{1}H NMR (600 MHz, CD_3OD, TMS): δ 7.82 (d, 2H, Ar-H), 7.73–7.71 (m, 2H, Ar-H), 7.43 (t, 2H, Ar-H), 7.35 (t, 2H, Ar-H), 4.79 (d, 3H, $J_{1,2} = 1.57$ Hz, 3H-1), 4.45–4.41 (m, 2H, 1H of H-13 and 1H of H-11), 4.36 (t, 1H, H-13), 4.30 (t, 1H, H-14), 3.99 (d, 3H, $J = 9.57$ Hz, H7), 3.90–3.87 (m, 6H, 3H-2, 3H-6), 3.84 (d, 3H, $J = 9.74$ Hz, H-7′), 3.77–3.74 (m, 3H, $J = 11.8$ Hz, 1H of H-6′), 3.74–3.72 (dd, 3H, $J = 3.40$, $J = 9.23$ Hz, 3H-3), 3.65–3.62 (m, 6H, H-5, H-4), 2.79 (dd, 1H, H-10), 2.73 (dd, 1H, H-10′), 1.50 (s, 9H, CCH_3); ^{13}C NMR (150.91 MHz,

CD_3OD, TMS): δ 172.36 (C-9), 172.13 (C-15), 158.42 (C-12), 145.25 (Ar-C), 142.59 (Ar-C), 128.79, 128.21, 126.36, 120.91 (Ar-C), 102.42 (C-1), 83.16 (C-16), 74.89 (C-5), 72.75 (C-3), 71.97 (C-2), 68.89 (C-4), 68.31 (C-13), 67.35 (C-7), 62.88 (C-6), 60.77 (C-8), 53.10 (C-11), 38.93 (C-10), 28.34 (CCH_3) ppm. MALDI-ToF m/z $[M+K]^+$ calcd for $C_{45}H_{67}N_2O_{23}$: 1039.35. Found: 1040.70. ESIMS m/z $[M+Na]^+$ calcd for $C_{45}H_{67}N_2O_{23}$: 1023.3798. Found: 1023.3806.

4.4.4. Glycocluster amino acid 13. The *t*-butyl ester **12** (57 mg, 0.056 mmol) was deprotected according to Section 4.2.2 to give **13** (50 mg, 0.052 mmol, 94%). ^{1}H NMR (600 MHz, CD_3OD, TMS): δ 7.82 (d, 2H, Ar-H), 71 (d, 2H, Ar-H), 7.42 (t, 2H, Ar-H), 7.35 (t, 2 H, Ar-H), 4.79 (d, 3H, $J_{1,2} = 1.70$ Hz, 3H-1), 4.55 (m, 1H, H-11), 4.43–4.40 (m, 1H, H-13), 4.35 (dd ∼ t, 1H, H-13′), 4.30 (dd ∼ t, 1H, H-14), 4.02 (d, 3H, $J = 7.87$ Hz, H-7), 3.88 (m $_c$, 6H, 3H of H-2, 3H of H-6), 3.80–3.73 (m, 9H, 3H-3, 3H-6′ and 3H-7′), 3.66–3.63 (m, 6H, 3H-4 and 3H-5), 2.85 (m_c, 1H, H-10), 2.79 (m_c, 1H, H-10′); ^{13}C NMR (150.91 MHz, CD_3OD, TMS): 174.66 (C-15), 172.41 (C-9), 145.27 (Ar-C), 142.57 (Ar-C), 128.78, 128.21, 126.37, 120.89 (Ar-C), 102.38 (C-1), 74.82 (C-5), 72.70 (C-3), 71.97 (C-2), 68.89 (C-4), 68.33 (C-13), 67.09 (C-7), 62.88 (C-6), 60.77 (C-8), 52.1 (C-11), 48.36 (C-14), 39.06 (C-10) ppm;. MALDI-ToF m/z $[M+Na]^+$ calcd for $C_{41}H_{56}N_2O_{23}$: 967.32. Found: 967.46.

4.5. Synthesis of glycocluster amino acid 19

4.5.1. Glycocluster amino acid *t*-butyl ester 18. In a round-bottomed flask, Fmoc-protected cluster mannoside **16**[21] (172 mg, 0.138 mmol) was dissolved in absolute DMF (10 mL) under inert atmosphere. Then morpholine (260 μL) was added and the mixture was stirred at room temperature for 2 h before the solvent was evaporated in vacuo to get amine **17** (ESIMS m/z $[M+Na]^+$ calcd for, $C_{37}H_{62}N_4O_{21}$: 862.8703. Found: 885.3754). Without further purification compound **17** was mixed with Fmoc-Asp-O*t*-Bu-OH (142 mg, 0.345 mmol) and HATU (131 mg, 0.345 mmol) and this mixture was dissolved in absolute DMF (15 mL) under inert atmosphere and cooled to 0 °C. Then DIPEA (58 μL, 0.345 mmol) was added and the reaction mixture was allowed to stir at room temperature for 4 h. The solvent was evaporated under reduced pressure and the residue was subjected to gel chromatography. Further purification by HPLC resulted in the title compound (63 mg, 0.05 mmol, 36%) as white lypophilisate. HPLC: t_R = 41.98 min [250/4 LiChrosorb 7 μm C8, A = water, B = acetonitrile, 20% B → 50% B, 75 min, 1 mL/min]. ^{1}H NMR (600 MHz, CH_3OD, TMS): δ 7.82 (d, 2H, Ar-H), 7.71 (m_c, 2H, Ar-H), 7.43 (t, 2H, Ar-H), 7.35 (m_c, 2H, Ar-H), 4.79 (d, 3H, $J_{1,2} = 1.58$ Hz, 3H-1), 4.45 (dd, 1H, H-15), 4.41 (dd, 1H, H-17), 4.36 (m_c, 1H, H-17′), 4.26 (m_c, 1H, H-18), 3.87 (dd, 3H, $J_{6,6'} = 2.33$, $J_{5,6} = 11.74$ Hz, 3H-6), 3.85 (dd, 3H, $J_{1,2} = 1.71$, $J_{2,3} = 3.38$ Hz, 3H-2), 3.78 (m_c, 3H, 3H-7), 3.74 (m_c, 6H, 3H-3 and 3H-6′), 3.64 (dd ∼ t, $J_{3,4} = 9.7$, $J_{4,5} = 9.3$ Hz, 3H-4), 3.59–3.54 (m, 6H, 3H-5 and 3H-7′), 3.46–3.42 (m, 3H, H-8), 3.38 (m_c, 3H, 3H-8′), 2.78 (dd, 1H, H-14), 2.63 (dd, 1H, H-14′), 2.22 (m, 6H, H-10), 2.01 (m, 6H, H-11), 1.5 (s, 9H, CCH_3); ^{13}C NMR (150.90 MHz, CD_3OD, TMS): δ 175.99 (C-9), 172.31 (C-13), 171.72 (C-19), 158.45 (C-16), 145.25, 142.59, 128.85, 128.24, 126.23, 120.97 (Ar-C), 101.71 (C-1), 83.28 (C-20), 74.84 (H-5), 72.58 (C-3), 72.08 (C-2), 68.79 (C-4), 68.22 (C-17), 67.26 (C-7), 63.05 (C-6), 59.35 (C-12), 53.41 (C-15), 48.37 (C-18), 40.42 (C-8), 39.54 (C-14), 32.03 (C-11), 31.40 (C-10), 28.32 (C–CCH_3). ESIMS m/z $[M+Na]^+$ calcd for $C_{57}H_{85}N_5O_{26}$: 1278.5380. Found: 1278.5633.

4.5.2. Glycocluster amino acid 19. The *t*-butyl ester **18** (40 mg, 0.031 mmol) was deprotected according to Section 4.2.2 to give **19** (36 mg, 0.029, 95%). ^{1}H NMR (600 MHz, CH_3OD, TMS): δ 7.78 (d, 2H, Ar-H), 7.76 (m_c, 2H, Ar-H), 7.38 (m_c, 2H, Ar-H), 7.31 (m_c, 2H, Ar-H), 4.76 (d, 3H, $J_{1,2} = 1.54$ Hz, 3H-1), 4.49 (m_c, 1H, H-15), 4.34 (m_c, 2H, H-17, H-17′), 4.23 (m_c, 1H, H-18), 3.83 (dd, 3H, $J = 1.30$, $J_{5,6} = 11.74$ Hz, 3H-6 and 3H-2), 3.76–3.69 (m, 9H, 3H-6′, 3 H-3 and 3H-7), 3.61 (dd ∼ t, $J = 9.40$ Hz, H-4), 3.56–3.49 (m, 6H, 3H-5 and 3H-7′), 3.42–3.32 (m, 6H, H-8), 2.76 (m, 1H,H-14), 2.18 (m, 1H, H-14′), 2.18 (m, 6H, H-10), 2.96 (m, 6H, H-11); ^{13}C NMR (150.90 MHz, MeOD): δ 145.27, 142.56, 128.82, 128.25, 126.34, 120.93 (Ar-C), 101. 70 (C-1), 74.82 (C-5), 72.55 (C-3), 72.08 (C-2), 68.80 (C-4), 67.20 (C-7), 63.02 (C-6), 59.35 (C-12), 55.87 (C-15), 48.36 (C-18), 40.40 (C-8, C-14), 31.99 (C-11), 31.40 (C-10). ESIMS m/z $[M+Na]^+$ calcd for $C_{53}H_{77}N_5O_{26}$: 1222.4754. Found: 1222.4847.

4.6. Synthesis of glycocluster peptides 20 and 21

4.6.1. Synthesis of 20. Resin-bound peptide **6** (30 mg, 0.014 mmol) was swelled in DMF for 1 h. In a round-bottomed flask, glycocluster **13** (27 mg, 0.028 mmol) and HATU (11 mg, 0.028 mmol) were dissolved in DMF (2 mL) and DIPEA (5 μL) was added. The mixture was shaken for 5 min and added to the resin. After 24 h the glycocluster peptide was cleaved using standard procedure 4.2.3 to give of **20** (14 mg, 0.011 mmol, 82%). ^{1}H NMR (600 MHz, DMSO-d_6, TMS): δ 8.64 (d, 1H, C-12-NH), 8.24 (d, 1H, C-14-NH), 8.13 (m_c, 6H, C-16-NH, C-18-NH, C-20-NH, C-22-NH, C-24-NH, C-26-NH), 7.81 (s, 1H, C-8-NH), 4.56 (s, 3H, 3H-1), 4.47 (m_c, 1H, H-13), 4.32 (m_c, 1H, H-15), 4.13 (m_c, 1H,

H-11), 3.77 (m_c, 12H, 1H-19, 2H-21, 2H-23, 2H-25, 2H-27, 3H-7), 3.63 (m_c, 14H, 3H-2, 3H-6, 3H-7′, 1 C-13-CH_2, 2 C-15-CH_2, 2H-17, 1H-19), 3.53–3.41 (m, 10H, 3H-3, 3H-4, 3H-6′, 1 C-13-CH_2), 3.31 (m, 5H, 3H-5, 2 C-11-NH_2), 2.77 (m_c, 1H, H-10), 2.59 (m_c, 1H, H-10′); ^{13}C NMR (150.90 MHz, DMSO-d_6): δ 170.98 (C-28), 170.23, 169.69, 169.05, 169.0 (C-9, C-12, C-14, C-16, C-20, C-22, C-24, C-26), 100.73 (C-1), 73.76 (C-5), 70.98 (C-3), 70.08 (C-2), 66.92 (C-4), 65.48 (C-7), 61.64, 61.71 (C-13-*C*H$_2$, C-15-*C*H$_2$), 60.96 (C-6), 59.20 (C-8), 55.39 (C-15), 54.74 (C-13), 49.08 (C-11), 42.14, 41.98, 41.70, 40.55 (C-17, C-19, C-21, C-23, C-25, C-27), 36.15 (C-10). MALDI-ToF *m*/*z* $[M+Na]^+$ calcd for $C_{44}H_{72}N_{10}O_{31}$: 1261.44. Found: 1261.50.

4.6.2. Synthesis of 21. Resin-bound **6** (32 mg, 0.015 mmol) was swelled in the glass apparatus used for peptide coupling. In a round-bottomed flask glycocluster **19** (27 mg, 0.022 mmol) and HATU (8 mg, 0.022 mmol) were dissolved in of DMF (2 mL) and DIPEA (5 μL) was added. The mixture was shaken for 5 min and then added to the resin. After 24 h the glycocluster peptide was cleaved using standard procedure 4.2.3 to give **21** (21 mg, 0.014 mmol, 93%). ^{1}H NMR (600 MHz, DMSO-d_6, TMS): δ 8.58 (m_c, 1H, C-16-NH), 8.16 (t, 1H, C-13-NH), 8.15 (m_c, 5H, C-18-NH, C-22-NH, C-24-NH, C-26-NH, C-28-NH), 7.95 (t, 1H, C-20-NH), 7.80 (t, 3H, C-8-NH), 7.62 (s, 1H, C-12-NH), 4.62 (s, 3H, H-1), 4.45 (m_c, 1H, H-17), 4.36 (m_c, 1H, H-19), 4.16 (m_c, 1H, H-15), 3.76 (m_c, 12H, 2H-21, 2H-23, 2H-25, 2H-27, 2H-29, 2H-31), 3.67–3.62 (m, 6H, 3H-2, 3H-6), 3.58–3.44 (m, 13H, 3H-3, 3H-6′, 2 C-17-CH_2, 2 C-19-CH_2, 3H-7), 3.40–3.36 (m, 6H, 3H-7′, 3H-4), 3.32 (m_c, 3H, 3H-5), 3.25 (m, 3H, 3H-8), 3.18 (m, 3H, 3H-8′) 2.67 (m_c, 1H, H-14), 2.55 (m_c, 1H, H-14′), 2.04 (m_c, 6H, H-10), 1.83 (m_c, 6H, H-11); ^{13}C NMR (150.90 MHz, DMSO-d_6): δ 169.65, 169.13, 169.09, 169.01, 168.80, 168.80 (C-9, C-13, C-16, C-18, C-20, C-22, C-24, C-26, C-28, C-30), 99.89 (C-1), 73.91 (C-5), 70.87 (C-3), 67.00 (C-4), 65.31 (C-7), 61.64 (C-17′, C-19′), 61.24 (C-6), 57.49 (C-12), 53.51 (C-17), 53.37 (C-19), 49.24 (C-15), 41.70 (C-21), 40.68 (C-23, C-25, C-27, C-29, C-31), 38.45 (C-8), 34.29 (C-14), 30.06 (C-10), 29.45 (C-11). MALDI-ToF *m*/*z* $[M+Na]^+$ calcd for $C_{56}H_{95}N_{13}O_{34}$: 1516.60. Found: 1516.97.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2008.04.015.

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