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PAPER

An efficient protocol for the solid-phase synthesis of glycopeptides under microwave irradiation†

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A standardized and smooth protocol for solid-phase glycopeptides synthesis under microwave irradiation was developed. Double activation system was proved to allow for highly efficient coupling of Tn-Ser/Thr and bulky core 2-Ser/Thr derivatives. Versatility and robustness of the present strategy was demonstrated by constructing a Mucine-1 (MUC1) fragment and glycosylated fragments of tau protein. The success of this approach relies on the combination of microwave energy, a resin consisting totally of polyethylene glycol, a low excess of sugar amino acid and the "double activation" method.

Introduction

Glycopeptides play a crucial role in many biological processes and drug discovery as antibiotics, antiviral and transport vehicles.[1] This class of glycans displays a high diversity of branched and non-branched (linear) carbohydrates, but such moieties are strictly related to their biological function.[2] The efficient synthesis of glycopeptides is of considerable interest because of their wide range of applications, such as understanding their roles and drug discovery research.[3] Two main approaches are considered to obtain chemically synthetic glycopeptides. The first method directly attaches the glycosyl amino acid to the sequence while the second incorporates the glycan moiety onto the properly protected full-length peptide. Usually, the first method is preferred due to the difficulty of direct glycosylation on peptides,[4] and recently more glycosylated amino acids can be obtained.[5]

There is vast literature on the stepwise synthesis of glycopeptides, denoting the interest in these substances while verifying the use of diverse strategies to obtain them.[6] It is clear that coupling reactions of glycosylated-amino acids in the synthetic scheme become a key bottleneck in glycopeptide synthesis. Steric hindrance is the most troublesome and causes lower reactivity in combination with non-preferable excess of precious glycosylated amino acid. The most used strategy is solid-phase approach including the use of polystyrene (PS)-type resin, usually applying 1.5 to 3.0 equiv. excess of the glycosyl amino acids. The glycosyl amino acids have been incorporated using different coupling reagents, such as carbodiimides and phosphonium salts; usually the reaction time is much longer than normal Fmoc amino acids due to their poor reactivity.[7] Other methods incorporate the use of more hydrophilic polyethylene glycol (PEG)-containing resins with an excess of glycosyl amino acid (1.3 to 2.5 equiv.).[8] Aminium salts are also employed as coupling agents, and in some cases, heating, including microwave irradiation, improved yields.[9–11]

Results and Discussion

The goal was to establish a general method to provide simple access to complex glycopeptides and their related compounds (Fig. 1). This strategy may consist of a combination of different approaches focusing on the key elements of synthesis, with special emphasis on the coupling of the glycosylated amino acids. Previously, we developed microwave-assisted solid-phase glycopeptide synthesis to improve coupling efficiency, which resulted in successful syntheses of heavily *O*-glycosylated MUC1 analogues.[10,11]

Comparison of different coupling reagent systems

In general, the synthesis of glycopeptides is more difficult to achieve than the equivalent naked peptides, so further elements should be considered for the suitable coupling method of these moieties.

The preferred coupling reagents in peptide synthesis are mainly the aminium and phosphonium salts.[12] They are very strong coupling activators and each group has diverse classes involving changes in the structure with differences in the reactivity. Aminium salts (as HBTU or HATU) are the most powerful

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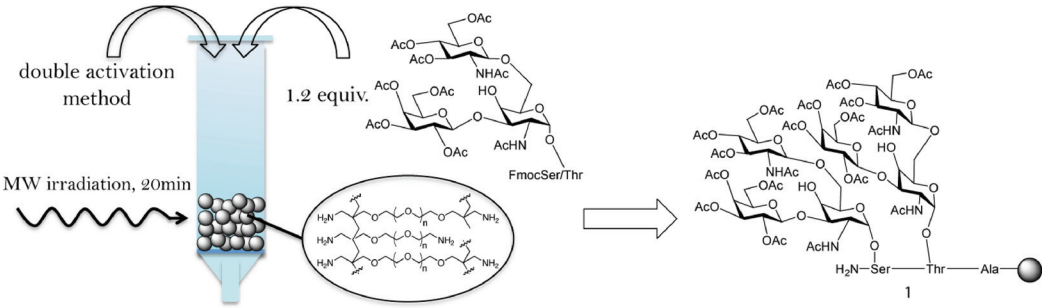


Fig. 1 The present synthetic strategy for glycopeptides.

Table 1 Extensive conversion in the preparation of **2** by applying different coupling reagent systems

#[a]	Coupling method[b]	Initial loading	Yield [Thr(Tn)]**3**[c] (%)[d]	Yield [Ser(Tn)]**4**[c] (%)[d]	Total Yield [**2**] (%)
1	HBTU/HOBt	0.26	90.9	84.6	76.4
2	HATU/HOAt	0.26	92.1	93.3	85.9
3	PyBOP/HOBt	0.26	96.0	80.8	77.6
4	PyBOP/HOBt "× 2"	0.26	95.4	85.0	81.1
5	PyAOP/HOAt	0.26	94.4	90.1	85.0
6	PyAOP/HOAt "× 2"	0.26	97.2	92.5	89.9
7	Totally PEG resin, PyAOP/HOAt "× 2"	0.37	98.1	98.0	96.1

[a] Use of 50 mg of solid support. The average of three syntheses was reported. [b] The reaction time was 20 min assisted by MW energy (0–50 W; 50 °C). [c] Loading calculated by Fmoc photometric test. UV measurement was performed in duplicate, and results were averaged. [d] Individual coupling reaction yield.

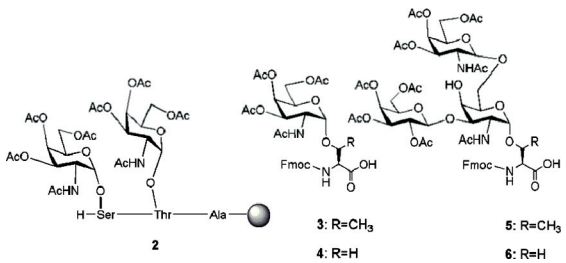


Fig. 2 Key glycosylated amino acids derivatives employed in this study.

salts, but the phosphonium salts (as PyBOP and PyAOP) are also widely used. It seems that potassium salts produce pyrrolidine (*e.g.* 0.5%, w/w), which can react with free carboxylic acid to form a corresponding pyrrolidine analogue.[13] However, a more critical effect is observed when using aminium salts due to rendering non-beneficial guanydilation on a free amine and truncating synthesis.[14] These reagents were put to the test to evaluate the efficiency with glycosylated amino acids. To fully understand the effect of these different reagents on the glycosylated amino acid coupling step, several conditions were tested using the tripeptide MUC1 sequence as a model [H-Ser(GalNAcα)-Thr(GalNAcα)-Ala-NH_2, **2**] (Fig. 2, Table 1).

In the first approach, the syntheses were carried out on Tentagel resin (Table 1, entries #1–#6), which consists of crosslinked PS core polymer grafted with PEG chains. In all cases, we applied only 1.2 equiv. of the glycosylated amino acids, such as **3** and **4**, and general coupling reagents for 20 min under microwave irradiation. We tested HBTU and HATU as the aminium salts and the phosphonium reagents, PyBOP and PyAOP. The reactions were monitored by measuring the UV absorbance at 290 nm of the Fmoc-adduct after Fmoc group removal. As anticipated, a slight improvement in the yields was observed for HATU salts compared to the phosphonium equivalent (Table 1, #2 *vs.* #5). In addition, the aza-type coupling reagents were more easily achieved than their counterparts (entry #1 *vs.* #2, entry #3 *vs.* #4). Although the use of HATU/HOAt (entry #2) and PyAOP/HOAt (entry #5) gave satisfactory improved yields in comparison with non aza-type, it is obvious that the efficiency in the yield of this coupling reaction still seems to be lower than that of common Fmoc-amino acids and should directly influence the overall yield of the glycopeptide synthesis. We hypothesized that the short life of the activated glycosylated amino acids becomes the rate-limiting step in the coupling reaction rather than the steric effect by carbohydrate, since it is well known that double coupling using some sterically hindered Fmoc amino acids would achieve a quantitative yield in any step. In light of the limitations and the assumption that not all glycosyl amino acids reacted, the double activation method was tested by adding the same amounts of coupling reagent and additive after half the reaction time. For this purpose, the aminium salt coupling reagents were not chosen due to the risk of guanydilation, especially with the hindered glycosylated amino acids as in our case study (Fig. 3). Thus, the double activation protocol was tested using phosphonium salts as they cannot form a non-desirable product and prevent the elongation.

Fig. 4 illustrates the method of "double-activation". Entries #4 and #6 show the effect of the double activation reactions using phosphonium salts; in both cases, the yield was much higher than the simple approach, and coupling was improved even when aminium reagents were used without any side reactions.

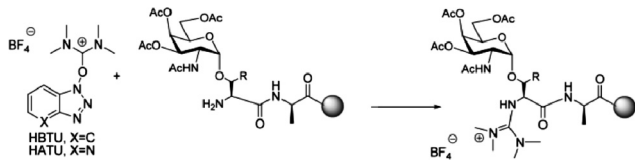


Fig. 3 Partial truncation of the synthesis due to the guanydilation secondary reaction caused by aminium salts.

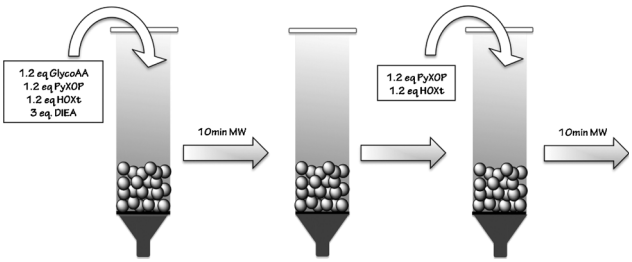
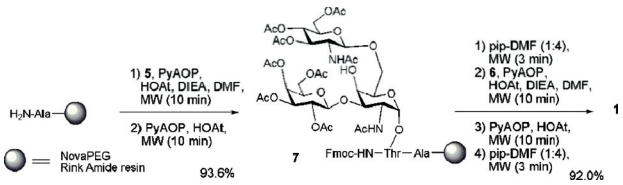


Fig. 4 Illustration of the "double activation" approach.



Scheme 1

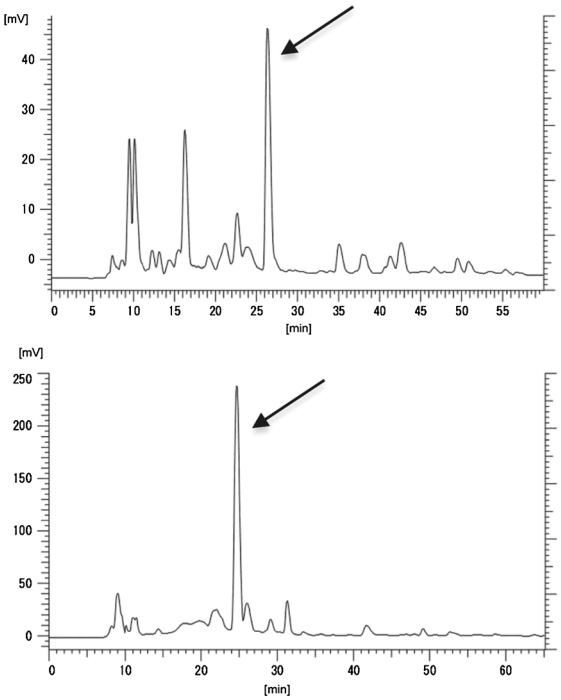


Fig. 5 (Above) HPLC chromatogram of **9** after cleavage, synthesis with the conventional protocol. (Below) Crude **10** glycopeptide HPLC chromatogram by using the "double-activation" method to incorporate Thr(core 1).

Influence of the solid support

Once the coupling conditions were established for standard synthesis of glycopeptides, the next step was to improve the method for evaluating the influence of the solid support. The nature of the resin is a crucial feature to obtain the desired compounds.[15] During the past few years, a new generation of totally polyethylene glycol (PEG)-type supports has been successfully employed for the synthesis of complex peptides.[16] The amphipathic nature of the PEG-based solid support could make it ideal for the synthesis of glycopeptides, since this kind of highly swelling polymers appears to be suitable for incorporation of the glycosylated amino acids bearing bulky sugar cores. Therefore, we tested the effect of a higher loading PEG solid support in combination with the "double activation" protocol. As shown in entry #7, the PEG-based resin produced the highest yield for the incorporation of sugar amino acids onto the loaded resin. In comparison with the Tentagel resin (entries #1 to #6), the incorporation of Fmoc-Thr(GalNAcα)-OH **3** onto the PEG resin resulted in a better performance and enhanced coupling yield. Furthermore, coupling of the second sugar residue (Fmoc-Ser(GalNAcα)-OH, **4**) provided further evidence of the method's efficiency, achieving the desired compound **2** in high yield even in challenging couplings and a higher loading of the support. As highlighted in previous studies,[17] these results confirmed the nature of the resin as a key factor in the solid-phase synthesis of peptides and their derivatives.

Synthesis of core-2 containing peptide

Optimizing the conditions for the incorporation of the simple glycosylated amino acids **3** and **4**, the method was further examined by including more architecturally complex glycans. The synthetic protocol chosen for evaluation was the cumbersome Ser(core 2)-Thr(core 2)-Ala structure on the resin (**1**, Fig. 1).

With incorporation of the representative core 2-based trisaccharide, [Galβ1,3(GlcNAcβ1,6)GalNAcα1] (Fig. 2, **5** and **6**), was chosen due to the presence of a branching point and as a consequence of the steric hindrance of the glycan moiety. As suspected, coupling of amino acids bearing these bulky core 2-type residues is more intricate than the previous Tn-type model but even this approach maintained a satisfactory yield (**1**, 86% overall yield, Scheme 1).

Considering the steric nature of these moieties and only using 1.2 equiv., this protocol is generally suitable for incorporation of bulky sugar amino acids in only 20 min with minimal excess of reagents leading to a high yield.

Synthesis of mucin glycopeptide

Certain interesting cancer antigens are the mucin-derived glycosylated peptides. In tumor cells, the mucin protein undergoes the aberrant *O*-glycosylation and exposes novel epitopes compared to normal cells.[18]

In the interest of getting mucin glycopeptides containing the repetitive Thr(core 1)[19] using **8**, we first tested the conventional method to obtain the tripeptide Thr(core1)-Thr(core1)-Val, **9** on the PS-PEG resin. The RP-HPLC chromatogram of the tripeptide showed a peak (t_R 26.5 min, 30.1% purity and overall yield:

Table 2 Obtaining glycosylated fragments of tau protein

Glycopeptide sequence[a]	Purity[b]	MS exact/found $[M+H]^+$, $[M+Na]^+$
HGAEIVYKSPVVSGDTSPRH (**11**)[c]	81%	2338.1/2338.4, 2360.4
LSNVSSTGSIDMVDsPQLATLA (**12**)[c]	73%	2406.2/2407.8, 2429.8
SGDRSGYSSPGSPGTPGSRS (**13**)[c]	74%	2301.0/2302.4, 2324.4
SGDRSGYSSPGSPGTPGSRS (**13**)[d]	29%	2301.0/2300.1, 2322.1

[a] An underline indicates the glycosylated amino acid residue, Ser(GlcNAcβ). [b] Purity calculated by RP-HPLC peak area percentage. [c] Coupling under the present double activation method (PyBOP/totally PEG resin/MW irradiation, 2 × 10 min). [d] Result obtained by using a conventional protocol (HBTU/totally PEG resin/MW irradiation, 20 min).

14%) as the desired product, but many byproducts were obtained (Fig. 5 above). It seems that the consecutive Thr's bearing core 1 [Galβ(1,3)-GalNAcα1] rendered a low yield and purity under the conventional method, even in a short glycopeptide. In our group, we were interested in having access to longer core 1-containing peptides [Ac-TTST(core 1)T(core 1)VSTT-NH_2, **10**], so the novel method was used to test these desired compounds with the PS-PEG resin. In contrast, the "double activation" protocol performed more smoothly to finally obtain the high yield and purity product (Fig. 5 below, t_R 24.5 min, 63.2% purity and overall yield: 26%).

Synthesis of tau glycoprotein fragments

Finally, to assess the versatility of this novel protocol, tau protein fragments were selected as a case study, since this glycoprotein has recently been of emerging interest in medicinal chemistry due to its relationship with Alzheimer disease.[20] It is broadly believed that the tau pathogenic form is of extreme importance during the early stage of the disease. In fact, tau protein forms aberrant aggregates that are toxic to neurons. Understanding the mechanisms of glycosylation and phosphorylation of this protein may lead to future tau-related treatment of neurodegenerative diseases. As a challenging application of our strategy, the syntheses of some glycosylated sequences of the tau protein were performed, and these results are listed in Table 2.

Three different sequences of different lengths, polarities and glycosylation sites with GlcNAcβ were synthesized. The first case study is glycopeptide His^{388} to His^{407} (**11**) containing 20 amino acid residues. The glycosylation site is located at the midpoint of the peptide. The Ser(GlcNAcβ) is incorporated after the cumbersome sequence Pro-Val-Val. Attachment to this fragment is thought to be difficult due to the presence of two consecutive β-branched amino acids and direct incorporation onto a secondary amino group. However, this strategy yielded the desired glycopeptide in a high purity. Similarly, a larger fragment **12** (Leu^{408} to Ala^{429}) was also successfully prepared with 22 residues and a sugar moiety after incorporation of seven residues and consecutively to a Pro.

As a further example, the third chosen fragment includes Ser^{191} to Ser^{210} (**13**), containing two glycosylated serine residues. The two glycosides are both located after the Pro residues and are only two residues apart. Characterization by RP-HPLC and MALDI-TOF MS confirmed the desired product in 74% purity. Judging from the result that a conventional protocol provided the same **13** only in 29% purity with another byproduct due to Pro^{200}-Ser^{210}. This truncated fragment corresponded to the peptide before the incorporation of the second glycosylated amino acid, with the impossibility of coupling the bulky residue by the conventional method. This comparative result demonstrates the high efficiency of the present approach to long peptides with sugars.

Further, some studies revealed racemization *via* azalactone on common amino acids when an excess of coupling reagents is employed. For the incorporation of glycosylated amino acids, racemization was not detected. This observation will be described in future studies.

Conclusions

We developed a novel method for the attachment of glycosylated amino acids in solid-phase based on the synergy of different approaches. Whereas other methods are tedious and time consuming, the protocol described herein is fast, efficient and uses a minimal quantity of the sugar amino acids. It is a generally suitable approach, since it can be used on the simplest and the most hindered sugar moieties, *i.e.* the complex architecture sugar core 2. The validity of this approach for longer tau glycosylated peptides was also demonstrated. We opened the possibility to employ "double activation" reactions to any hindered related amino acids in a low excess. This novel method accelerates a new path for interested glycopeptides in several biology and therapeutic areas whereas these intermediates can be subjected to further modification procedures on the basis of enzyme-assisted automated synthesis.

Experimental

General remarks

Commercially available reagents and compounds were used without further purification. The Tentagel resins were obtained from Rapp Polymere and all the PEG resins are NovaPEG from Novabiochem. The ^1H NMR and ^{13}C NMR spectra were recorded at an operating frequency of 500 or 600 MHz and a temperature of 300 K. Chemical shifts are reported in parts per million (δ) relative to TMS or to solvent as the internal standard. Thin layer chromatography was performed on silica gel. Detection was accomplished by irradiation using a UV lamp or staining with ninhydrin or anis. The chromatographic separations were achieved on silica gel columns.

Synthesis

Microwave-assisted synthesis. Reactions with assisted microwave energy were performed in a closed reaction vessel. The manufacturer of the microwave system is IDX Corporation. The system was fixed to a maximum of 50 °C, and wattage rating was 0–50 W.[10,11]

Glycosylated amino acids. Compounds **3–6**, and **8** were synthesized according to the methods reported previously[10,21] and characterized by ^{1}H NMR as follows:

Fmoc-Thr(Tn)-OH **3**: ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.91 (2H, d, J = 7.5 Hz), 7.73 (2H, m), 7.44 (2H, t, J = 7.5 Hz), 7.34 (2H, t, J = 7.5 Hz), 5.30 (1H, d, J = 3.0 Hz), 5.03 (1H, dd, J = 11.5 Hz), 4.80 (1H, d, J = 3.5 Hz), 4.46 (2H, m), 4.30 (2H, t, J = 6.0 Hz), 4.17 (3H, m), 4.01 (2H, m), 3.48 (1H, m), 2.11 (3H, s), 2.01 (3H, s), 1.90 (3H, s), 1.82 (3H, s), 1.16 (3H, d, J = 6.5 Hz).

Fmoc-Ser(Tn)-OH **4**: ^{1}H NMR (500 MHz, DMSO-d_6): δ 7.89 (2H, d, J = 7.0 Hz), 7.70 (2H, t, J = 7.0 Hz), 7.43 (2H, t, J = 7.0 Hz), 7.35 (2H, J = 7.0 Hz), 5.27 (1H, s), 5.01 (1H, dd, J = 11.5, 2.5 Hz), 4.79 (1H, d, J = 2.5 Hz), 4.23 (5H, m), 3.95 (2H, m), 3.86 (1H, s), 3.80 (1H, m), 3.74 (1H, m), 2.10 (3H, s), 1.89 (6H, s), 1.83 (3H, s).

Fmoc-Thr(core2)-OH **5**: ^{1}H NMR (500 MHz, DMSO-d_6): δ 7.90 (2H, m), 7.75 (2H, t, J = 7.5 Hz), 7.43 (2H, ddd, J = 15.0, 7.5, 3.0 Hz), 7.33 (2H, dt, J = 12.5, 7.5 Hz), 5.24 (2H, dd, J = 8.0, 4.0 Hz), 5.03 (2H, m), 4.82 (2H, m), 4.72 (1H, d, J = 8.0 Hz), 4.63 (1H, d, J = 4.0 Hz), 4.57 (1H, d, J = 8.5 Hz), 4.53 (1H, m), 4.44 (1H, m), 4.31 (1H, m), 4.19 (2H, m), 4.11–3.95 (7H, m), 3.91 (1H, m), 3.83 (2H, m), 3.75 (2H, m), 2.09 (3H, s), 2.02–1.95 (15H, m), 1.91 (3H, s), 1.90 (3H, s), 1.82 (3H, s), 1.76 (3H, s), 1.10 (3H, d, J = 6.5 Hz).

Fmoc-Ser(core 2)-OH **6**: ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.90 (2H, d, J = 7.0 Hz), 7.73 (2H, d, J = 7.0 Hz), 7.43 (2H, t, J = 7.0 Hz), 7.34 (2H, m), 5.23 (2H, br), 5.05 (2H, m), 4.82 (4H, m), 4.64 (1H, s), 4.60 (1H, d, J = 8.0 Hz), 4.40 (2H, br), 4.23 (4H, m), 4.11–3.92 (8H, m), 3.74–3.47 (4H, m), 2.10 (3H, s), 2.01 (6H, s), 1.99–1.96 (9H, s), 1.91 (6H, s), 1.81 (3H, s), 1.74 (3H, s).

Fmoc-Thr(core 1)-OH **8**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.76 (2 H, d, J = 7.4 Hz), 7.49 (2 H, t, J = 7.3 Hz), 7.41 (2 H, td, J = 3.7 Hz and 7.4), 7.29–7.36 (2 H, m), 7.05 (1 H, d, $J_{2,NH}$ = 9.7 Hz, NH), 6.85 (1 H, d, $J_{NH,CHa}$ = 7.4 Hz, Thr-NH), 5.45 (1H, d, $J_{1,2}$ = 2.9 Hz, 1-H), 5.39 (1 H, d, $J_{3',4'}$ = 3.2 Hz, 4′-H), 5.10 (1 H, br. s, 4-H), 5.08 (1 H, m, 2′-H), 4.94 (1 H, dd, $J_{3',4'}$ = 3.4 Hz and $J_{2',3'}$ = 10.5 Hz, 3′-H), 4.68 (2 H, m, Fmoc CH_2), 4.55 (1 H, d, $J_{1',2'}$ = 7.7 Hz, 1′-H), 4.35 (1H, q, $J_{CH\alpha,CH\beta}$ = 6.3 Hz, Thr-CHβ), 4.29–4.18 (4H, m, 3-H, 6- and 6′-Ha, Thr-CHα), 4.17–3.99 (4 H, m, Fmoc CH, 6- and 6′-Hβ), 3.90 (1 H, dd, $J_{2,3}$ = 11.0 Hz, 2-H), 3.79–3.88 (2 H, m), 2.18, 2.17, 2.11, 2.09, 2.04, 2.01 and 2.01 (21 H, 7 s, 7 $COCH_3$) and 1.08 (3 H, d, Thr-CHγ).

Synthesis of glycopeptides

Manual solid-phase peptide elongation and other solid-phase manipulations were carried out in polypropylene syringes, each fitted with a porous disk. Solvents and soluble reagents were removed by suction. Previous washings of the Tentagel resin were performed with CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min), in the case of totally PEG resin a previous described washing protocol was performed.[16] Washings between deprotection, coupling and subsequent deprotection steps were carried out at room temperature with DMF (3 × 1 min), CH_2Cl_2 (3 × 1 min) and DMF (3 × 1 min). Fmoc removal was carried out with pip–DMF (1 : 5) (1 × 3 min, MW assisted). The coupling of non-glycosylated amino acids (4 equiv.) was carried out with HBTU–HOBt–DIEA (4 : 4 : 6) in DMF for 10 min assisted by MW energy. The coupling and deprotection were analyzed by the ninhydrin test. Glycosyl amino acids were achieved following the double activation protocol (Fig. 4) by employing 1.2 equiv. of glycosylated amino acids. The system PyAOP–HOAt–DIEA or PyBOP–HOBt–DIEA (1.2 : 1.2 : 1.8) was performed in DMF. After 10 min reaction assisted by MW energy, without filtering, more PyXOP–HOXt (1.2 equiv.) was added and reacted for other 10 min.

The glycopeptides were cleaved from the resin with simultaneous removal of side-chain protecting groups by treatment with TFA–H_2O–TIS (95 : 2.5 : 2.5) for 1 h or 2 h at room temperature. A ratio of 100 μL of cleavage cocktail per mg of resin was performed. After cleavage reaction, peptides were precipitated by adding cold *tert*-buthylmethyl ether. Following centrifugation, the solution was decanted and this process was repeated twice. Finally, peptides were dissolved in H_2O–CH_3CN (1 : 1) and lyophilized. For the removal of the acetyl protecting groups of the glycosyl moieties, glycopeptides were dissolved in MeOH (5 mL). To the solution was added 1 N NaOH, keeping the solution at pH 13. Deacetylation reaction was followed by MS spectroscopy. After completion, the reaction mixture was neutralized by addition of AcOH. The solvents were evaporated *in vacuo*. The deprotected glycopeptides were dissolved in H_2O and characterized by RP-HPLC. Reverse phase analytical high performance liquid chromatography (RP-HPLC) was carried out with a Hitachi instrument [detector L-2400 (220 nm) and pump L-2130]. The conditions are described for each compound. For the NMR spectra, peptides were purified to a >95% purity by RP-HPLC using a semi-preparative column [Inertsil ODS-3 (ϕ 20 × 250 mm) (GL Science Inc.)], flow rate 5 mL min^{-1}. Gradient conditions depending on each peptide (see ESI†). Mass spectra were obtained on an Ultraflex MALDI-TOF instrument. The matrix used is a solution of DHB in a 1 : 1 mixture of H_2O–CH_3CN in 0.1% TFA. NMR characterization of peptides is performed on Bruker 600 MHz or Varian 500 MHz spectrometer. The peptide was concentrated to 2mM on H_2O–D_2O (9 : 1), and pH was adjusted to 4–6, unless other is indicated. Product **1** after cleavage from resin was characterized by RP-HPLC and main peak corresponds to desired compound (RP-HPLC, linear gradient from 0.3 : 9.7 to 2.5 : 7.5 of CH_3CN (0.1% TFA) in H_2O (0.1% TFA) for 40 min, 1.0 mL min^{-1}). After the deacetylation step, MALDI-TOFMS confirms the main product as the desired compound (*m*/*z* calcd for $C_{54}H_{92}N_8O_{35}$, 1412.57; found, 1435.51 $[M + Na]^+$). Product **2** (H-Ser(Ac3-α-GalNAc)-Thr(Ac3-α-GalNAc)-Ala-NH_2) after cleavage from resin was characterized by RP-HPLC and main peak corresponds to desired compound (RP-HPLC, linear gradient from 0.3 : 9.7 to 2.5 : 7.5 of CH_3CN (0.1% TFA) in H_2O (0.1% TFA) for 40 min,

1.0 mL min^{-1}). MALDI-TOFMS confirms the product as the desired compound (*m/z* calcd for $C_{38}H_{58}N_6O_{21}$, 934.37; found, 957.21 $[M + Na]^+$, 973.19 $[M + K]^+$) (NMR spectra and assignments. Spectra and chromatograms are available in the ESI†).

Abbreviations

"× 2"	"double-activation"
Ac	acetyl
ACN, CH_3CN	acetonitrile
AcOH	acetic acid
core-2	Galβ1,3(GlcNAcβ1,6)GalNAcα1
DCM	CH_2Cl_2, dichloromethane
DIEA	*N*,*N*-diisopropylethylamine
DHB	dihydroxybenzoic acid
DMF	*N*,*N*-dimethylformamide
equiv.	equivalent/s
Fmoc	9-fluorenyl-methyloxycarbonyl
HOBt	1-hydroxybenzotriazole
HOAt	7-aza-1-hydroxybenzotriazole
HATU	*N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-*b*] pyridin-1-yl-methylene)-*N*–methylmethanaminium hexafluorophosphate *N*–oxide
HBTU	*N*-[(1*H*-benzotriazol-1-yl)-(dimethylamino) methylene]-*N*-methylmethanaminium hexafluorophosphate *N*–oxide
MALDI	matrix-assisted laser desorption ionization
MeOH	methanol
MUC1	mucine-1
MS	mass
MW	microwave
NMR	nuclear magnetic resonance
PEG	polyethylene glycol
pip	piperidine
PS	polystyrene
PyAOP	azabenzotriazol-1-yl-*N*-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate
PyBOP	benzotriazol-1-yl-*N*-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate
RP-HPLC	reverse phase high pressure liquid chromatography
TIS	triisopropylsilane
TFA	trifluoroacetic acid
Tn	GalNAcα
TOF	time-of-flight
UV	ultraviolet

Amino acids and peptides are abbreviated and designated following the rules of the IUPAC-IUB Commission of Biochemical Nomenclature [*J. Biol. Chem.*, 1972, **247**, 977–983].

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