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A highly convergent approach to O- and N-linked glycopeptide analogues

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Deprotected C-glycopyranosyl-ketones have been conjugated by a chemoselective approach to a peptide or an amino acid bearing an aminooxy group on the N-terminus or on the side-chain, respectively. The coupling reaction, performed in aqueous media, does not require protecting groups on the peptide or saccharide moieties, nor auxiliary coupling reagents.

Keywords: **glycomimetics, C-glycosides, glycopeptides, chemoselective ligation**

Abbreviations: **Aoa, aminooxyacetyl; Boc, *tert*-Butoxycarbonyl; Dpr, α,β-L-Diaminopropionic acid; DIPEA, Diisopropylethylamine; DMF, *N,N*-Dimethylformamide; Fmoc, 9-Fluorenylmethoxycarbonyl; PyBOP, Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate**

Introduction

Protein glycosylation plays a crucial role in the biological functions of proteins by modulating their folding and stability and serving as a recognition signal in cell-cell, cell-matrix and cell-pathogen interactions [1–5]. For a better understanding of the complex interplay between sugar and peptide parts in the physiological events mediated by glycopeptides and glycoproteins, and in order to obtain structures with useful antigenic properties, many efforts have been devoted to the chemical synthesis of these complex natural structures [6] and their unnatural analogues, generally defined as neoglycopeptides [7]. In particular, recently we [8,9] and Bertozzi et al. [10–12] have synthesised glycopeptide analogues exploiting the chemoselective ligation procedure through oxime bond formation. However, a problem that remains unsolved is the sensitivity of the O- or N-glycosidic linkage to chemicals and glycosidases. To overcome this problem, efforts have been recently devoted to the synthesis of analogues of glycopeptides in which the O-glycosidic linkage is replaced by the more stable C-glycosidic bond. Towards this aim, protected C-glycosyl aminoacids, to be incorporated into a peptide chain, have been synthesised by many groups [13–23]. Alternatively *C*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glycopyranosyl) methylamine, a protected C-glycoside with a primary amino group at the C-glycosidic appendage, has been condensed with the carboxylic function of the asparagine residue of a peptide, affording a protected C-glycopeptide [24]. It is noteworthy that all these approaches, which are not chemoselective, require the use of protected sugar and peptide moieties in the condensation step, needing a careful final deprotection of the conjugate.

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Materials and methods

General

All protected amino acids were purchased from Calbiochem-Novabiochem (Läufelfingen, CH). Reagents and solvents were purchased from Fluka and used without further purifications. HPLC was performed on Waters equipment using column packed with Vydac Nucleosil 300 Å 5 μm C_{18} particles. The analytical column (250 × 4.6 mm) was operated at 1 mL/min and the preparative column (250 × 21 mm) at 18 mL/min, monitoring at 214 nm. Solvent A consisted of 0.09% TFA in water and solvent B of 0.09% TFA in acetonitrile/water 9:1. Mass spectra of compounds **11** and **12** were obtained by Electron Spray Ionization (ESI-MS) on a Finningan MAT SSQ 710C; mass spectra of **13** and **14** were obtained on a MALDI TOF spectrometer (Kompact Alpha, Kratos Analitica, Shimadzu Group). NMR spectra were recorded on Bruker AC 300 and Varian XL 200 spectrometers for solutions in $CDCl_3$, unless otherwise stated. $[\alpha]_D$ values were measured at 20 °C on a Perkin-Elmer 241 polarimeter. Column chromatography was performed with the flash procedure using Merck silica gel

60 (230–400 mesh), TLC was performed on Merck silica gel 60 F-254 plates and visualized by spraying with a solution containing H_2SO_4 (31 ml), ammonium molibdate (21 g) and $Ce(SO_4)_2$ (1 g) in water (500 ml) and then heating at 110 °C for 5 min.

General procedure for conversion of allyl-C-glycosides into ketones

The allyl C-glycosides (1 eq.) were dissolved in a mixture THF/H_2O 3:2 then $Hg(OAc)_2$ (1 eq.) was added and the reaction was stirred at r.t. until the disappearance of the starting material (determined by TLC). THF was added to a 3:1 ratio of THF/H_2O (3 mL), then Na_2PdCl_4 (1 eq.) was added and the reaction mixture warmed to 60 °C for 2 hrs. After filtration over a Celite pad and dilution with CH_2Cl_2, the organic phase was washed with water, dried (Na_2SO_4), filtered and evaporated under reduced pressure. The crude products were purified by flash chromatography on silica gel (eluent: hexane/AcOEt, 2:1).

General procedure for deprotection of benzyl ethers by catalytic hydrogenation

A solution of benzylated ketones in AcOEt/EtOH 1:1 was submitted to hydrogenation with $Pd(OH)_2$ (10% w/w). After 20 hrs, filtration over a Celite pad and evaporation afforded the pure deprotected derivatives.

*1-(2′,3′,4′,6′-tetra-O-benzyl-α-D-glucopyranosyl)-2-propanone (**2**) [25]*

*1-(α-D-glucopyranosyl)-2-propanone (**3**)*

Debenzylation of **2** afforded ketone **3** in quantitative yield. Oil, $[\alpha]_D$ + 4.0 (c = 0.8, MeOH); ^{1}H-NMR (400 MHz, D_2O): 2.17 (s, 3H, H—3); 2.82–2.85 (m, 2H, H—1); 3.26 (dd, 1H, J = 9.2, 0.5, H—2′); 3.40–3.45 (m, 1H, H—5′); 3.48 (t, 1H, J = 9.3); 3.57 (dd, 1H, J = 12.0, 5.3, H—6′$_a$); 3.64 (t, 1H, J = 9.0); 3.67 (dd, 1H, J = 12.2, 2.3, H—6′$_b$); 4.47 (m, 1H, H—1′). ^{13}C-NMR (50.29 MHz, D_2O): 32.5 (q, C—3); 48.6 (t, C—1); 63.7 (t, C—6′); 72.3, 76.5, 78.8, 80.2, 82.5 (d, C—1′, C—2′, C—3′, C—4′, C—5′); 211.4 (s, C—2). MS: m/z = 220 (M), 203 (M-17), 185 (M-35).

*1-(2′,3′,4′,6′-tetra-O-acetyl-α-D-glucopyranosyl)-2-propanone (**5**)*

Compound **4** was hydroxymercuriated following the general procedure, crude product was purified by flash chromatography (eluent: hexane/AcOEt, 2:1) affording pure **5** as oil in 80% yield. $[\alpha]_D$ + 55.0 (c = 1, $CHCl_3$). ^{1}H-NMR (200 MHz, $CDCl_3$): 2.00–2.10 (4s, 12H, 4CH_3 of acetyl groups); 2.22 (s, 3H, H—3); 2.75 (dd, 1H, J = 16.2, 6.9, H—1$_a$,); 2.83 (dd, 1H, J = 16.2, 6.9, H—1$_b$); 3.87 (ddd, 1H, J = 3.0, 5.3, 8.6 H—5′); 4.11 (dd, 1H, J = 12.1, 3.0, H—6′$_a$); 4.24 (dd, 1H, J = 12.1, 5.3, H—6′$_b$); 4.74 (dt, 1H, J = 4.0, 6.9, H—1′); 4.97 (t, 1H, J = 8.6, H—4′); 5.11 (dd, 1H, J = 8.6, 4.0, H—2′); 5.26 (t, 1H, J = 8.6, H—3′). ^{13}C-NMR (75.43 MHz, $CDCl_3$): 20.55 (q, CH_3COO); 30.21 (q, C—3); 41.64 (t, C—1); 62.09 (t, C—6′); 68.45, 68.62, 69.49, 70.12, 70.30 (d, C—1′, C—2′, C—3′, C—4′, C—5′); 169.10, 169.91, 170.02, 171.25 (s, CO); 212.29 (s, C—2). MS: m/z = 386 (M).

*1-(β-D-glucopyranosyl)-2-propanone (**6**)*

A suspension of compound **5** (170 mg, 0.5 mmol) in a saturated aqueous bicarbonate solution was heated at 80 °C overnight. The solvent was then evaporated *in vacuo* and the residue purified by flash chromatography on silica gel (eluent: AcOEt/MeOH 85:15), obtaining **6** as a colourless oil with 90% yield. $[\alpha]_D$ + 7.1 (c = 0.8, MeOH). ^{1}H-NMR (300 MHz, D_2O): 2.41 (s, 3H, H—3); 2.86 (dd, 1H, J = 16.6, 9.0, H—1$_a$); 3.15 (dd, 1H, J = 16.6, 3.3, H—1$_b$); 3.37 (t, 1H, J = 9.2); 3.52–3.59 (m, 2H); 3.63 (t, 1H, J = 9.2); 3.80 (dd, 1H, J = 12.4, 4.8, H—6′$_a$); 3.94 (td, 1H, J = 9.2, 3.3, H—1′); 3.98 (dd, 1H, J = 12.4, 2.2, H—6′$_b$). ^{13}C-NMR (50.29 MHz, D_2O): 32.75 (q, C—3); 49.58 (t, C—1); 63.73 (t, C—6′); 72.70, 76.01, 79.20, 80.17, 82.47 (d, C—1′, C—2′, C—3′, C—4′, C—5′); 216.17 (s, C—2). MS: m/z = 203 (M-17), 185 (M-35).

*1-(2′-Acetamido-3′,4′,6′-tri-O-benzyl-2′-deoxy-β-D-glucopyranosyl)-2-propene (**8**)*

To a solution of compound **7** [26] (0.54 g, 1 mmol) in dry CH_2Cl_2 (10 mL), dry pyridine (0.24 mL, 3 mmol), acetic anhydride (0.19 mL, 2 mmol) and DMAP (catalytic) were added and the solution was stirred at r.t. for 4 hrs. The reaction mixture was washed with aqueous HCl (5%), then with water, dried over Na_2SO_4, filtered and evaporated under reduced pressure. Purification by flash chromatography (eluent: hexane/AcOEt 4:6) afforded pure 7 as white solid (yield 97%).

$[\alpha]_D$ + 27.1 (c = 1, $CHCl_3$); m.p. = 149 °C; ^{1}H-NMR (300 MHz, $CDCl_3$): 1.81 (s, 3H, CH_3CO); 2.20–2,42 (m, 2H, H—1); 3.31–3.44 (m, 2H, H—1′ and H—5′); 3.58–3.75 (m, 5H, H—2′, H—3′, H—4′, H—6′); 4.54–4.66 (m, 4H, CH_2OPh); 4.78–4.86 (m, 2H, CH_2OPh); 5.02–5.08 (m, 3H, H—3 and NH); 5.80–5.94 (m, 1H, H—2), 7.18–7.40 (m, 15 H, H$_{arom}$). ^{13}C-NMR (75.43 MHz, $CDCl_3$): 23.46 (q, CH$_3$CONH); 36.52 (t, C—1); 54.97 (d, C—2′); 69.03 (t, C—6′); 73.40, 74.34, 74.75 (t, 3 C_2Ph); 78.37, 78.96, 79.17, 83.18 (d, C—1′, C—3′, C—4′, C—5′); 116.73 (t, C—3); 127.48–128.45 (d, C$_{arom}$); 134.69 (d, C—2); 138.10, 138.34, 138.51 (s, C$_{arom\ quat}$); 170.04 (s, *C*ONH). MS: m/z = 517 (M).

*1-(2′-Acetamido-3′,4′,6′-tri-O-benzyl-2′-deoxy-β-D-glucopyranosyl)-2-propanone (**9**)*

Compound **8** (0.15g, 0.26 mmol) was converted into ketone **9** following the general procedure. Purification by flash chromatography (eluent: hexane/AcOEt 2:8), afforded **9** as white solid (83% yield). $[\alpha]_D$ + 14.36 (c = 1, CHCl3); m.p. = 142–144 °C. ^{1}H-NMR (300 MHz, $CDCl_3$): 1.78 (s, 3H, H—3); 2.12 (s, 3H, CH_3CONH); 2.64 (dd, 1H, J = 16.0, 4.3, H—1$_a$); 2.79 (dd, 1H, J = 16.0, 7.0, H—1$_b$); 3.44 (dt, 1H, J =

9.8, 2.9, H—5′); 3.50 (t, 1H, J = 9.6, H—3′); 3.65–3.68 (m, 3H, H—4′ and 2H—6′); 3.71–3.78 (m, 1H, H—1′); 3.91 (q, 1H, J = 9.6, H—2′); 4.48–4.85 (m, 6H, CH_2OPh); 5.42 (d, 1H, J = 9.6, NH), 7.24–7.44 (m, 15 H, H_{arom}). ^{13}C-NMR (75.43 MHz, $CDCl_3$): 23.33 (q, CH_3CONH); 31.19 (q, C—3); 46.50 (t, C—1); 54.64 (d, C—2′); 68.94 (t, C—6′); 73.42, 74.51, 74.87 (3t, CH_2Ph); 75.27, 78.73, 79.08, 83.24 (d, C—1′, C—3′, C—4′, C—5′); 127.67–128.58 (C_{arom}); 138.07, 138.41 (C_{arom}); 170.41 (s, CONH); 207.45 (s, C—2). MS: m/z = 533 (M).

*1-(2′-Acetamido-β-D-glucopyranosyl)-2-propanone (***10***)*

Compound **9** (0.117 mg) was hydrogenated affording **9** as white solid (96% yield). $[\alpha]_D$ −8.1 (c = 1, MeOH); m.p. = 135–137 °C. ^1H-NMR (200 MHz, MeOD): 1.96 (s, 3H, H—3); 2.16 (s, 3H, CH_3CONH); 2.62–2.65 (m, 2H, H—1); 3.19–3.85 (m, 7H, H—1′, H—2′, H—3′, H—4′, H—5′, H—6′), 4.20 (d, 1H, J = 5.7, NH). ^{13}C-NMR (75.43 MHz, D_2O): 22.93 (CH_3CONH); 30.65 (q, C—3); 46.55 (t, C—1); 55.85 (d, C—2′); 61.62 (t, C—6′); 70.86, 75.02, 75.88, 80.39 (d, C—1′, C—3′, C—4′, C—5′); 175.31 (s, CONH), 213.38 (s, C—2). Ms: m/z = 261 (M).

*Solid-phase synthesis of peptide Lys-Leu-Phe-Ala-Val-Ala-Lys-Ile-Thr-Tyr-Lys-Asp-Thr (***11***)*

Peptide **11** was synthesized manually following a standard Fmoc solid-phase synthetic protocol. The resin was swollen in CH_2Cl_2 for about 30 min before starting the synthesis. Commercial DMF was degassed for several hours with nitrogen. In the coupling steps, Nα-Fmoc amino acids (1.5 eq.) activated *in situ* with PyBOP (1.5 eq.) in the presence of DIPEA (3 eq.) were used. The Nα-Fmoc deprotection was carried out by treatment with piperidine (20% v/v in DMF, 1 × 5 min and 2 × 10 min) and assessed by UV analysis (at λ = 301 nm) of the effluents. The Nα-Fmoc amino acids were protected on the side chains functional groups as follows: *tert*-Butyl for Thr, Tyr and Asp, Boc for Lys. After removal of the Fmoc group from the N-terminal residue, the aminooxy functionality was introduced by solid-phase condensation of N-hydroxysuccinimide activated Boc-aminooxyacetic acid (2 eq.) in the presence of diisopropylethylamine (6 eq.). After cleavage from the resin (5% TFA in CH_2Cl_2), purification by Et_2O precipitation and preparative reverse-phase HPLC (Vydac C_{18} 218TP1002 column, gradient: 20 to 55% of MeCN in water, 0.1%TFA, in 30 min) and liophylization, pure peptide **11** was receovered in 85% overal yield based on the loading of the resin.

Peptide 11: *ESI-MS: m/z: 822.6 [M+2/2] and m/z 548.7 [M+3/3]*

Glycopeptide **12**

Peptide **11** was dissolved in 15 mL of a 50% v/v mixture of acetonitrile and an aqueous sodium acetate buffer at about pH 4, then the sugar **3** (1.5 eq.) was added and the solution s stirred for 4 hrs at r.t. Evaporation of the solvent and preparative HPLC (gradient, 20 to 50% MeCN in water, 0.1% TFA in 50 min) gave pure glycopeptide **12** (85% yield). After lyophylization glycopeptide **12** was recovered as a white solid more than 95% pure (HPLC); ESI-MS: *m/z* 922.5 [M+2/2] and *m/z* 615.8 [M+3/3].

Compounds 13 *and* 14

Commercially available Fmoc-Dpr(BocAoa)-OH (100 mg, 0.20 mmol) was dissolved in 2 mL of a 50% CH_2Cl_2/TFA mixture and stirred at r.t. for about 2 hrs until the Boc group was cleaved (determined by TLC; eluent: AcOEt/MeOH/water 5:2:0.1). The solvents were evaporated in vacuo, and the Fmoc-Dpr(Aoa)-OH directly reacted with C-glycosil ketones **6** or **10** (1.1 eq) in 2 mL of methanol. Reaction crudes were purified by flash chromatography on silica gel (eluent: AcOEt/MeOH/water 5:2:0.1), and pure glycoconjugates **13** and **14** were recovered as colorless oils in 90% and 93% yields, respectively.

Compound **12**. *MALDI-TOF MS: 601.3 [M+1], 623.3 [M+23], 639.3 [M+39]*

Compound **13.** *MALDI-TOF MS: 664.2 [M+23], 680.2 [M+39]*

Results and discussion

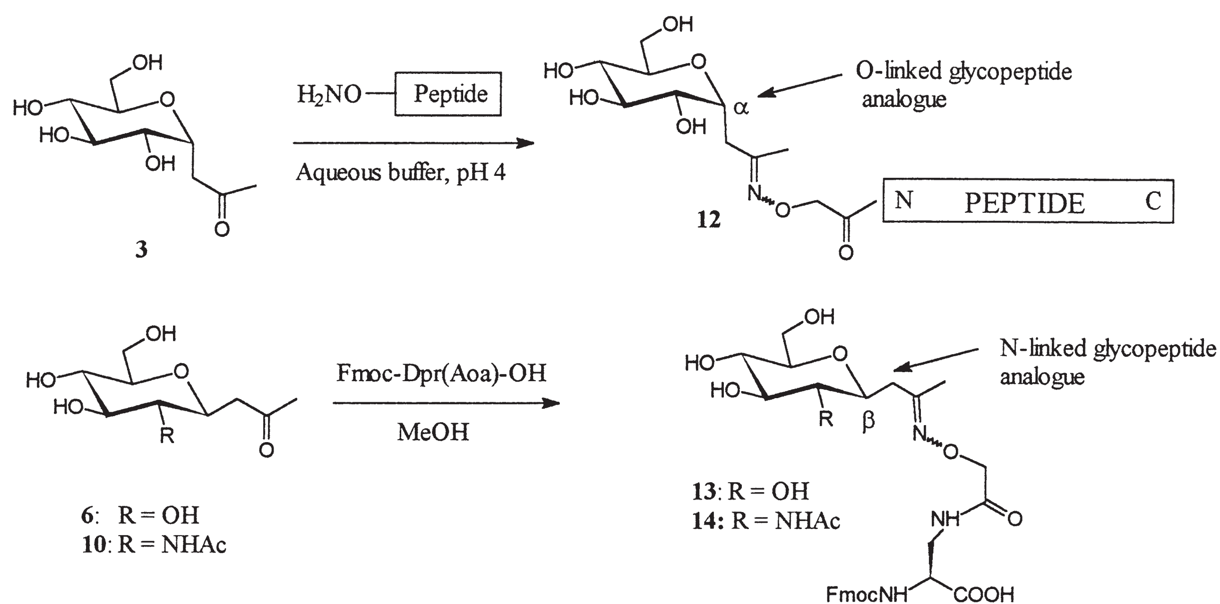
We describe our preliminary results on a chemoselective approach that allows the easy and convergent synthesis of C-glycopeptides, in aqueous solution, by direct conjugation of a deprotected C-glycosyl ketone with a deprotected peptide bearing an oxyamino group. By this approach, the C-saccharide can be covalently linked to an aminooxy acetyl residue at the N-terminal end of a peptide sequence as well as to the side chain of a residue internal to the sequence as exemplified in the case of the condensation with the *N*α-Fmoc,*N*β-aminooxyacetyldiaminopropionic acid residue (Scheme 1). The resulting glycoconjugates can be considered as analogues of the O-linked glycopeptides with sugar in the α-anomeric configuration (as in the case of compound **12**) or of the N-linked glycopeptides with a β-anomeric configuration (compounds **13** and **14**).

The C-glycosyl ketones **3, 6** and **10** were prepared according to the synthetic steps depicted in Scheme 2.

The allyl C-glycosides **1** [27] and **8** were hydroxymercuriated [$Hg(OAc)_2$ 1.1 eq., acetone-water 1:1, then NaCl], and the crude intermediates were directly converted into the methylketones, respectively **2** and **9** (Na_2PdCl_4 1.1 eq., THF, 60 °C). Catalytic hydrogenation of **2** and **9** (H_2, $Pd(OH)_2$, AcOEt/EtOH) afforded the desired deprotected C-glycosyl ketones **3** and **10.**

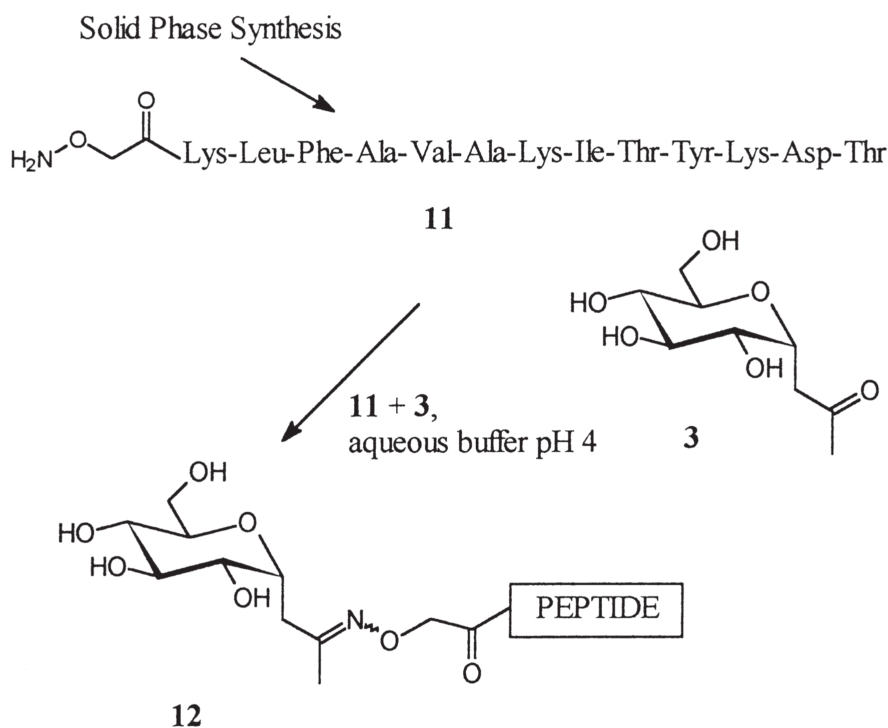
Compound **4** [28] was converted into derivative **5** using the same procedure, then deprotection and anomerization gave compound **6** (aqueous $NaHCO_3$, 80 °C).

The peptide sequence KLFAVAKITYKDT correspond-



Scheme 1. Chemoselective condensation step for the conjugation of sugars to an aminooxy peptide and to the side chain of the aminooxy-functionalized residue *N*α-Fmoc,*N*β-aminooxyacetyldiaminopropionic acid [Fmoc-Dpr(Aoa)-OH].

Scheme 2. *Reagents and conditions:* i, $Hg(OAc)_2$, THF/H_2O; ii, Na_2PdC1_4 1.1 eq., 60 °C; iii, H_2, $Pd(OH)_2$, AcOEt/EtOH; iv, aqueous $NaHCO_3$, 80 °C.



Scheme 3. Chemoselective glycosylation of the epitope-T sequence.

ing to a T-cell epitope of the VP1 protein of poliovirus type 1, was assembled on Sasrin resin by stepwise manual solid phase synthesis using the Fmoc standard procedure [29]. The aminooxy functionality was introduced on the N-terminal end on solid phase and finally peptide **11** was cleaved from the solid support and simultaneously deprotected. The conjugation of **11** with the unprotected C-glycosyl ketone **3** was carried out in a buffer solution (50% v/v mixture of MeCN and an aqueous sodium acetate buffer at pH about 4) and gave glycopeptide **12** in excellent yield and purity (Scheme 3).

Commercially available Fmoc-Dpr(BocAoa)-OH [30] was deprotected on the side-chain aminooxy functionality and then reacted with compounds **6** and **10** in MeOH, affording glycoconjugates **13** and **14** in quantitative yields. The two isomeric forms (*syn-anti* isomerism) of the oxime bond of **12, 13** and **14** resulted interconvertible at r.t. All of these glycoconjugates are stable at r.t. in aqueous buffers within 2 to 12 pH range, thus indicating that the oxime bond is a relatively resistant covalent linkage between sugar and peptide moieties.

In conclusion, we developed a highly convergent strategy for the covalent assembly of glycopeptides that paves the way for a straightforward synthesis of artificial vaccines where a carbohydrate B-cell epitope is linked to a peptide T-epitope. The proposed chemoselective strategy, allows the use of unprotected sugars and peptides in aqueous media, avoiding the extensive use of protecting groups and the group activation for the conjugation. Furthermore, this ligation technique may be applied in combinatorial chemistry for a rapid and efficient synthesis of new chimeric glycopeptide libraries as a source of lead compounds of pharmacological interest.

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