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Preparation of a Glycopeptide Analogue of Type II Collagen — Use of Acid Labile Protective Groups for Carbohydrate Moieties in Solid Phase Synthesis of *O*-Linked Glycopeptides

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Abstract: A glycopeptide analogue of the immunodominant T cell epitope on type II collagen has been prepared by solid phase synthesis. Preparation of a glycosylated amino acid from two monosaccharide units that carried silyl and isopropylidene protective groups and Fmoc 5-hydroxynorvaline was essential for the synthesis. After assembly of the glycopeptide the carbohydrate protective groups were removed simultaneously with acid catalyzed cleavage from the solid phase.
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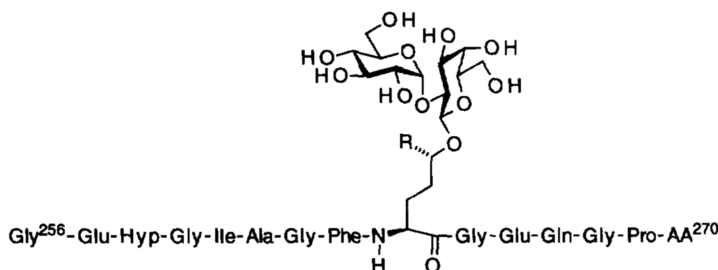
In a model for rheumatoid arthritis autoimmune T cells are elicited by antigens obtained after processing of type II collagen (CII), the major protein of cartilage. The immunodominant T cell antigen was recently revealed to be a glycopeptide containing the collagen fragment CII(256-270)[1]. CII(256-270) has two hydroxylysine (Hyl) residues glycosylated with either β-D-Gal*p*- or α-D-Glc*p*-(1→2)-β-D-Gal*p*-moieties (cf. **1**). Synthetic, nonglycosylated CII(256-270) failed to stimulate the autoimmune T-cells suggesting an important immunological role for the carbohydrate moieties of CII(256-270)[1]. As a first step in efforts to elucidate the submolecular details of the immune response to CII(256-270) we were interested in preparing the analogue **2**, in which the disaccharide moiety is linked to 5-hydroxynorvaline (Hnv) instead of hydroxylysine at the centre of the glycopeptide.

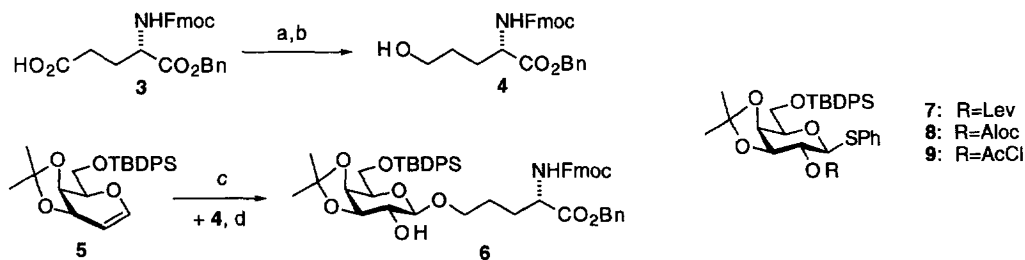


1: R = CH_2NH_2 and AA^{270} = α-D-Glc*p*-(1→2)-β-D-Gal*p*-Hyl. **2**: R = H and AA^{270} = Lys.

Solid phase synthesis using glycosylated N^{α}-Fmoc amino acids as building blocks has been demonstrated as an efficient method for preparation of glycopeptides[2]. The carbohydrate part of the glycosylated amino acids has predominantly been protected with acetyl or benzoyl groups which stabilize the glycosidic bonds towards the acidic conditions used for cleavage of the glycopeptide from the solid phase and

deprotection of the amino acid side chains[3,4]. Unfortunately the use of acetyl and benzoyl protective groups has some drawbacks. $O \rightarrow N$ acyl migration may occur during coupling of the glycosylated amino acid to the peptide-resin and leads to termination of the growing peptide chain[5]. β-Elimination of *O*-glycosylated serine and threonine residues, as well as epimerization of peptide stereocenters, has been encountered during removal of *O*-acyl protective groups with base[6,7], even though these side reactions have been found[8,9] to be less serious than previously anticipated[10]. Alternative strategies have utilized glycosylated amino acids in which the sugar hydroxyl groups were either left unprotected[7,11] or carried TMS protective groups[12]. However, the presence of hydroxyl groups that are unprotected or carry labile TMS groups severely restricts the possibilities for further synthetic manipulations of such building blocks. Assembly of the glycosylated amino acid from carbohydrate moieties carrying protective groups which are removed during acidic cleavage of the glycopeptide from the resin, but that are still sufficiently stable to withstand conditions encountered in carbohydrate and peptide synthesis, constitutes a more versatile approach that has not been investigated previously. As an example of this approach we now report the synthesis of the glycosylated amino acid **14** which has isopropylidene, *t*-butyldimethylsilyl and *t*-butyldiphenylsilyl protective groups for the carbohydrate moiety, as well as the use of **14** for preparation of the *O*-linked glycopeptide **2**.

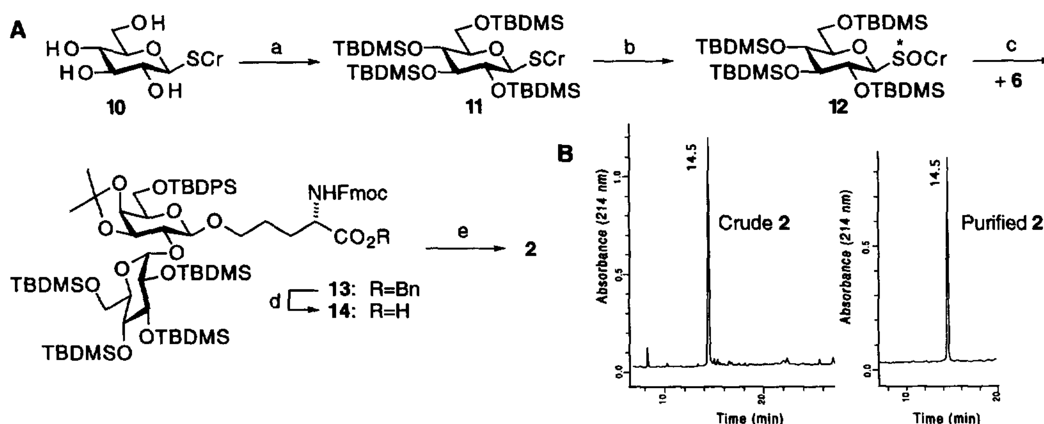
Protected 5-hydroxynorvaline **4** was prepared from N^{α}-Fmoc glutamic acid benzyl ester (**3**)[13] (Scheme 1). Conversion of **3** to a mixed carbonic anhydride by treatment with isobutyl chloroformate, and subsequent reduction[14,15] with sodium borohydride in methanol gave the alcohol **4**. Epoxidation of the galactal **5**[16] (1.2 equiv.) with dimethyldioxirane gave the corresponding 1α,2α-anhydrosugar[17] which was immediately used for glycosylation of **4** (1 equiv.) using zinc chloride as promoter. The desired β-glycoside **6**[18] (50%) was obtained together with the corresponding α-glycoside (6%), both of which had unprotected hydroxyl groups at C-2 of the galactose moiety. Synthesis of β-glycosides such as **6** is commonly performed using glycosyl donors that have participating groups at O-2. However, attempts to use the galactosyl donors **7-9**, which have levulinyl, allyloxycarbonyl and chloroacetyl protective groups at O-2, in glycosylations of **4** gave poor results with formation of < 10% of the desired β-glycosides.



Scheme 1. (a) iBuOCOCl, NMM, THF, -10°C; (b) $NaBH_4$, MeOH, 0°C, 84% (two steps); (c) dimethyldioxirane, acetone, CH_2Cl_2, 0°C; (d) $ZnCl_2$, THF, AW-300, -40°C $\rightarrow$ RT, 50% (two steps).

The α-D-glucose moiety was attached to the acceptor **6** using the TBDMS protected thioglucoside **12** as glycosyl donor (Scheme 2A). The donor **12** was prepared from the thiocresyl glucoside **10**[19] in two steps. Treatment of **10** with TBDMSOTf in the presence of catalytic amounts of DMAP in pyridine gave **11** which was oxidized with MCPBA to give a mixture of the diastereomeric sulfoxides **12**. Treatment[20] of the donor **12** with trifluoromethanesulfonic anhydride and then acceptor **6**, gave the α-glycoside **13** (28%), the corresponding β-glycoside (28%) and acceptor having a TBDMS group at O-2 of the galactose moiety (~10%). Attempted glycosylation of **6** with the donor **11**, or the corresponding chloro- or bromo sugars, under a variety of conditions gave very low yields of the desired **13**. The Fmoc group is known to be slowly cleaved

during hydrogenolysis but use of ethyl acetate as solvent and adjustment of the amount of Pd/C allowed selective removal[21] of the more reactive benzyl ester in **13** to give the building block **14**[22] (83%).



Scheme 2. **A**: (a) TBDMSOTf, DMAP, pyridine, 87%; (b) MCPBA, CH_2Cl_2, -78 → -40°C, 93%, *(diastereomeric ratio 7.8:1); (c) Tf_2O, 2,6-di-*tert*-butyl-4-methylpyridine, PhMe, -78°C, 28%; (d) H_2, 10% Pd/C, EtOAc, 83%, (e) solid phase peptide synthesis, 38% (overall). **B**: HPLC chromatograms of crude and purified **2** (Conditions: Kromasil C-8 column. Linear gradient of 0-100% B in A over 60 min with a flowrate of 1.5 mL/min. A=0.1% aqueous TFA, B=0.1% TFA in MeCN).

Synthesis of the glycopeptide **2** was performed in an automatic peptide synthesizer on a polystyrene resin grafted with polyethylene glycol spacers (TentaGel™ resin) that carried a 4-alkoxybenzyl alcohol linker. In the synthesizer Fmoc amino acids (4 equiv.) having standard side-chain protective groups (*i.e.* *t*-Boc for Lys, *t*-Bu for Glu, and Trt for Gln) were activated as benzotriazolyl esters with 1,3-diisopropyl carbodiimide in DMF. The glycosylated amino acid **14** (1 equiv.) was coupled to the peptide resin as its azabenzotriazolyl ester[23]. All couplings were monitored spectrophotometrically with bromophenol blue as an indicator[24] of unacylated amino groups and Fmoc deprotection was effected with 20% piperidine in DMF. Cleavage from the resin was performed with TFA/water/thioanisole/ethanedithiol (87.5:5:5:2.5). These conditions also removed the protective groups used for the disaccharide moiety without effecting the glycosidic bonds, and the glycopeptide **2**[25] (38%) was obtained after purification by reversed-phase HPLC (Scheme 2B).

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18. **6**: FABMS: $(M+H)^+$ 886.3987 calcd., 886.3946 obsd.; Selected ^1H NMR-data (500 MHz, $CDCl_3$): δ (ppm) 5.43 (d, 1 H, *J* 8.2 Hz, NH), 5.18 (ABd, 1 H, *J* 12.2 Hz, $PhCH_2O$), 5.14 (ABd, 1 H, *J* 12.3 Hz, $PhCH_2O$), 4.41 (ABdd, 1 H, *J* 7.1, 10.7 Hz, $OCOCH_2CH$), 4.35 (ABdd, 1 H, *J* 7.2, 10.6 Hz, $OCOCH_2CH$), 4.26 (dd, 1 H, *J* 2.1, 5.4 Hz, H-4), 4.19 (bt, 1 H, *J* 6.9 Hz, $OCOCH_2CH$), 4.10 (d, 1 H, *J* 8.3 Hz, H-1), 4.05 (dd, 1 H, *J* 5.6, 7.2 Hz, H-3), 3.97 (dd, 1 H, *J* 7.4, 9.8 Hz, H-6), 3.50 (bt, 1 H, *J* 7.8 Hz, H-2), 1.48 and 1.34 (2 s, each 3 H, CH_3), 1.05 (s, 9 H, *t*Bu).
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22. **14**: FABMS: $(M+Na)^+$ 1436.7284 calcd., 1436.7284 obsd.; Selected ^1H NMR-data (500 MHz, $CDCl_3$): δ (ppm) 5.32 (d, 1 H, *J* 7.3 Hz, NH), 5.09 (d, 1 H, *J* 3.0 Hz, H-1'), 4.30 (d, 1 H, *J* 7.0 Hz, H-1), 4.25 (dd, 1 H, *J* 1.9, 5.9 Hz, H-4), 4.23 (t, 1 H, *J* 5.8 Hz, H-3), 4.19 (bt, 1 H, *J* 7.2 Hz, $OCOCH_2CH$), 3.77 (dd, 1 H, *J* 5.8, 6.8 Hz, H-2), 1.50 and 1.32 (2 s, each 3 H, CH_3), 1.04, 0.90, 0.87, 0.87, and 0.86 (5 s, each 9 H, *t*Bu), 0.10, 0.09, 0.08, 0.08, 0.07, 0.06, 0.04, and 0.03 (8 s, each 3 H, Si-CH_3).
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25. **2**: FABMS: $(M+H)^+$ 1799 calcd., 1799 obsd.; Amino acid analysis: Ala 1.00 (1), Glu 3.00 (3), Gly 4.95 (5), Hyp 1.02 (1), Ile 1.00 (1), Lys 1.01 (1), Phe 1.01 (1), Pro 1.01 (1); ^1H NMR-data (500 MHz, H_2O/D_2O 9:1, pH 5.4, 278.2 K): δ (ppm) α-D-Glc*p* 5.26 (bs, H-1), 3.97 (H-5), 3.72 (H-6), 3.67 (H-3), 3.66 (H-6'), 3.43 (H-2), 3.37 (H-4); β-D-Gal*p* 4.44 (d, *J* 8.7 Hz, H-1), 3.85 (H-4), 3.78 (H-6), 3.71 (H-6'), 3.65 (H-3), 3.60 (H-5), 3.55 (H-2); Gly^{256} 3.76 (Hα), 3.62 (Hα'); Glu^{257} 8.73 (NH), 4.63 (Hα), 2.28 (Hγ,γ'), 2.00 (Hβ), 1.78 (Hβ'); Hyp^{258} 4.58 (Hγ), 4.47 (Hα), 3.81 (Hδ,δ'), 2.30 (Hβ), 2.02 (Hβ'); Gly^{259} 8.70 (NH), 3.89 (Hα,α'); Ile^{260} 8.07 (NH), 4.14 (Hα), 1.82 (Hβ), 1.37 (Hγ), 1.12 (Hγ'), 0.86 ($CH_3\gamma$), 0.77 ($CH_3\delta$); Ala^{261} 8.65 (NH), 4.22 (Hα), 1.32 (CH_3); Gly^{262} 7.91 (NH), 3.87 (Hα), 3.79 (Hα'); Phe^{263} 8.12 (NH), 7.26 and 7.17 (H-arom.), 4.54 (Hα), 3.04 (Hβ), 3.01 (Hβ'); Hnv^{264} 8.48 (NH), 4.22 (Hα), 3.86 (Hδ), 3.58 (Hδ'), 1.84 (Hβ), 1.64 (Hβ), 1.60 (Hγ), 1.53 (Hγ'); Gly^{265} 8.43 (NH), 3.83 (Hα,α'); Glu^{266} 8.36 (NH), 4.24 (Hα), 2.29 (Hγ,γ), 2.02 (Hβ), 1.85 (Hβ); Gln^{267} 8.63 (NH), 7.59 and 6.91 ($CONH_2$), 2.32 (Hγ,γ'), 2.09 (Hβ), 1.92 (Hβ); Gly^{268} 8.40 (NH), 4.10 (Hα), 3.91 (Hα'); Pro^{269} 4.36 (Hα), 3.54 (Hδ,δ'), 2.21 (Hβ), 1.95 (Hγ,γ'), 1.91 (Hβ'); Lys^{270} 8.19 (NH), 7.53 ($NH_3^+\varepsilon$), 4.11 (Hα), 2.92 (Hε,ε'), 1.77 (Hβ), 1.65 (Hβ'), 1.60 (Hδ,δ'), 1.36 (Hγ,γ').

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