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Convenient synthesis of glycosylated hydroxylysine derivatives for use in solid-phase peptide synthesis

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Abstract

δ-*O*-Glycosylated 9-fluorenylmethoxycarbonyl-hydroxylysine (Fmoc-Hyl) derivatives have been conveniently prepared by introduction of a β-D-galactopyranosyl group to copper-complexed Hyl[ε-*tert*-butyloxycarbonyl (Boc)] or Hyl[ε-allyloxycarbonyl (Aloc)], followed by decomposition of the copper complex and addition of an Fmoc group to the α-amino group. Fmoc-Hyl[ε-Boc,*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)] was used in the solid-phase synthesis of a type IV collagen derived sequence.

Keywords: glycopeptides/glycoproteins; amino acids and derivatives; solid-phase synthesis.

Hydroxylysine (Hyl)[1] is the major glycosylation site within collagens. The δ-hydroxyl group may be posttranslationally modified by the monosaccharide galactose (β-D-galactopyranosyl) or the disaccharide glucose-galactose [α-D-glucopyranosyl-(1→2)-β-D-galactopyranosyl].[2] Interest in glycosylation of collagen stems from the recent reports of T-cell recognition of a glycosylated sequence within type II collagen,[3] the identification of melanoma and breast carcinoma binding sites within type IV collagen that contain glycosylated Hyl residues,[4,5] and the activation of specific tyrosine receptor kinases by glycosylated type I collagen.[6] Preparation of Hyl derivatives for incorporation by peptide synthesis has a long and troubled history. Initial attempts at synthesizing Hyl(ε-Cbz) resulted in severe solubility problems.[7] The successful solution phase synthesis of a tetrapeptide containing glycosylated Hyl[8] did not use a Hyl 'building block', but rather the lactone form of the amino acid. Use of the lactone is detrimental for efficient solid-phase synthesis. The recent preparation of glycosylated Fmoc-Hyl(ε-Boc) required seven steps, with the final derivative obtained in 21.4% yield.[3]

We sought a more direct and convenient method for the synthesis of glycosylated Fmoc-Hyl derivatives. Our previous work had shown that either the Boc or Aloc group was suitable for side-chain protection of Hyl, but Cbz was not.[9] Thus, Hyl derivatives bearing either Boc or Aloc side-chain protection were prepared (Scheme 1). L-Hyl (**1**; 0.50 g, 2.52 mmol) was dissolved in 3.75 ml H_2O, and basic cupric carbonate (1.62 g, 4.03 mmol) was added. The mixture was stirred and refluxed for 4 h. The resulting suspension was filtered and washed with hot H_2O until the filtrate was colorless. The

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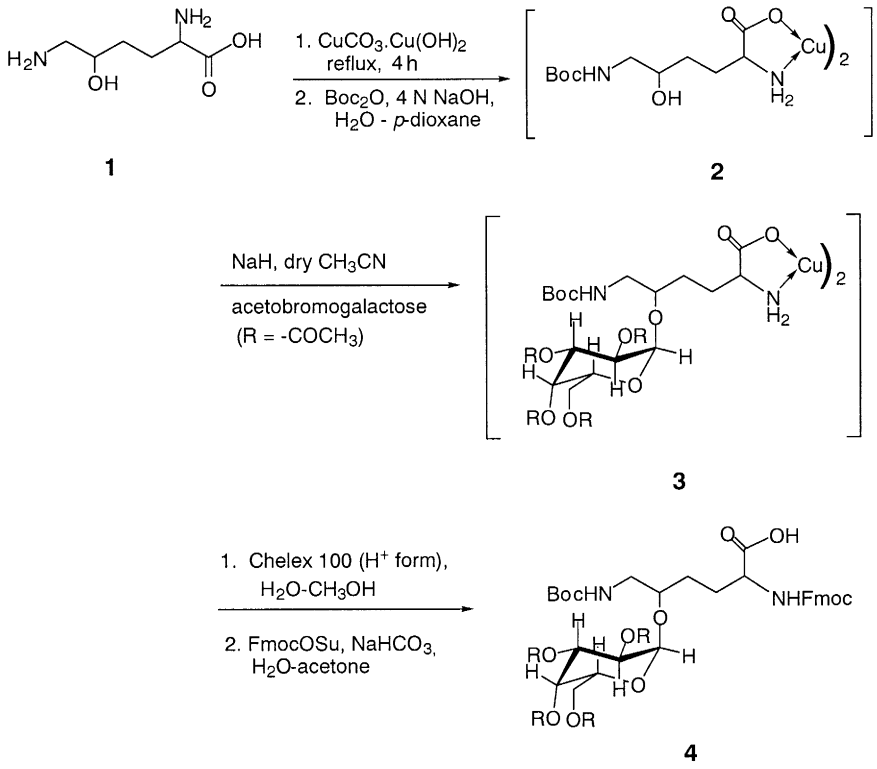

Scheme 1. Synthesis of glycosylated Fmoc-Hyl derivatives

combined filtrates were cooled to 0°C, and 150 µl of 4N NaOH was added. A solution of di-*tert*-butyl dicarbonate (0.83 g, 3.83 mmol) in 7 ml *p*-dioxane was added in four aliquots over a period of 1 h. Prior to the addition of the second, third, and fourth aliquots, 450 µl of 4N NaOH was added to maintain a pH>8. Once di-*tert*-butyl dicarbonate addition was complete the reaction proceeded overnight. The resulting precipitate was filtered, washed three times each with 3.0 ml H_2O and 3.0 ml CH_3OH, and then dried under vacuum to give copper-complexed Hyl(ε-Boc) (**2**; 1.10 g, 75% yield). The product was taken in dry CH_3CN (10 ml) under dry nitrogen atmosphere and NaH (0.2 g, 4.60 mmol) was added. The reaction mixture was refluxed for 2 h and then cooled to room temperature. 2,3,4,6-Tetra-*O*-acetyl-α-D-galactopyranosyl bromide[10] (1.88 g, 4.60 mmol) in dry CH_3CN (5 ml) was added and the reaction mixture was stirred overnight. CH_3CN was distilled off completely and the product was dried under vacuum to give copper-complexed Hyl(ε-Boc,*O*-2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl) (**3**; 1.80 g, 77% yield). The product was dissolved in 10 ml CH_3OH, stirred overnight at 20°C, and diluted with 12 ml H_2O. Na^+ Chelex 100 resin (10 g) was washed six times with 6 ml of 1N acetic acid and 15 times with 6 ml H_2O, then added to the CH_3OH–H_2O solution containing **3** and stirred for 3 h at 20°C. The resin was filtered and washed five times each with 4 ml of CH_3OH–H_2O (1:1) and 6 ml H_2O. The combined filtrates were evaporated under high vacuum at 25–30°C to provide Hyl(ε-Boc,*O*-2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl). A portion of the product (0.98 g, 1.66 mmol) and $NaHCO_3$ (0.159 g, 1.90 mmol) were dissolved in 10 ml H_2O, and a solution of 9-fluorenylmethoxycarbonyl-*N*-hydroxysuccinimide ester (FmocOSu) (0.635 g, 1.88 mmol) in 5 ml acetone was added. An additional 5 ml acetone was added, and the reaction proceeded overnight at 30°C. The solution was acidified to pH~2 with dilute HCl and the acetone was removed under high vacuum at 25–30°C. The aqueous suspension was extracted three times with 20 ml $CHCl_3$. The $CHCl_3$ layer was washed three times with 20 ml H_2O, dried over anhydrous sodium sulfate, and evaporated under high pressure at 25–30°C

to provide Fmoc-Hyl(ε-Boc,*O*-2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl) (**4**; 0.68 g, 50% yield, 29% overall yield).[11] For the synthesis of Fmoc-Hyl(ε-Aloc,*O*-2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl), allyl chloroformate (0.568 g, 4.71 mmol) in 7 ml *p*-dioxane was used instead of di-*tert*-butyl dicarbonate; product yield was 29%.[12]

Derivative **4** was used for the solid-phase synthesis of the α1(IV)1263–1277 sequence from type IV collagen (Fig. 1). This sequence promotes tumor cell binding and signal transduction,[4] and contains a glycosylated Hyl residue in position 1265.[13] α1(IV)1266–1277 was assembled on a PE-Biosystems 433A Peptide Synthesizer using Fmoc-based chemistry as described.[14] The H_2N-peptidyl-resin was then removed from the instrument, and the last three amino acids were coupled manually in an orbital shaker. Glycosylated derivative **4** was coupled using threefold molar excesses of Fmoc-amino acid and HOAt, a 2.7-fold molar excess of HATU, and a sixfold molar excess of DIEA in 10 ml DMF for 18 h. Fmoc-Val and Fmoc-Gly were coupled using fourfold molar excesses of Fmoc-amino acid and HOBt, a 3.6-fold excess of HBTU, and an eightfold molar excess of DIEA in 10 ml DMF for 1 h. Fmoc groups were removed with 20% piperidine in DMF for 1 h. Analysis of the peptide-resin by Edman degradation chemistry[15] revealed the desired sequence.[16] Peptide-resin cleavage and side-chain deprotection proceeded with H_2O–TFA (1:19) for 1.5 h.[17] The peptide was purified by preparative RP-HPLC and deacetylated with methanolic sodium methoxide[18] (2 M) for 1 h at 20°C. The product was homogeneous by analytical RP-HPLC, and MALDI-MS analysis gave the desired mass ($[M+Na]^+$ 1638.5 Da; theoretical 1637.77 Da). Treatment of the peptide with carbazole–sulfuric acid reagent[19] was positive for carbohydrate.

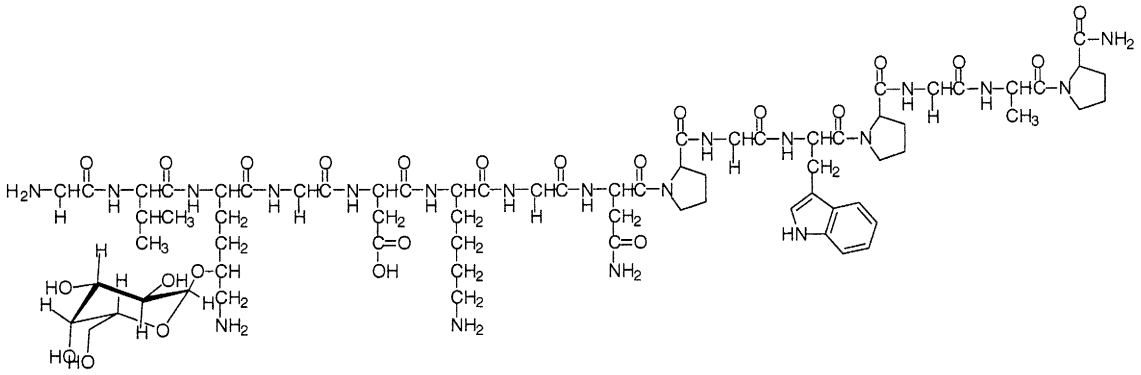

Fig. 1. Structure of the α1(IV)1266–1277 sequence

We have reported a convenient method for the preparation of glycosylated Hyl derivatives for use in Fmoc solid-phase synthesis. The procedure described herein requires only four distinct steps, as opposed to previous procedures of seven or more steps.[3,8] The preparation of glycosylated derivatives using amino acid copper complexes may also prove to be generally applicable for trifunctional compounds. The incorporation of glycosylated Hyl into peptides will allow for the further study of the effects of glycosylation on cell recognition and signalling.

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References

1. Abbreviations used are: Aloc, allyloxycarbonyl; Boc, *tertiary*-butyloxycarbonyl; Cbz, benzyloxycarbonyl; DIEA, *N,N*-diisopropylethylamine; DMF, *N,N*-dimethylformamide; Fmoc, 9-fluorenylmethoxycarbonyl; HATU, *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HBTU, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOAt, 1-hydroxy-7-azabenzotriazole; HOBt, 1-hydroxybenzotriazole; Hyl, δ-hydroxylysine; MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry; RP-HPLC, reversed-phase high-performance liquid chromatography; TFA, trifluoroacetic acid.
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11. MALDI-MS analysis indicated the desired mass (814 Da; theoretical 814.32 Da). ^{1}H NMR analysis (500 MHz, $CDCl_3$): δ 7.84–7.28 (m, 8H, Fmoc-ArH), 4.70 (d, 2H, J=7.0, 6.9 Hz, Fmoc-CH_2), 4.46 (d, 1H, J=6.8 Hz, Fmoc-CH), 5.43 (d, 1H, J=7.3 Hz, NHα), 5.41 (d, 1H, NHε), 4.46 (dt, 1H, J=5.8, 14.2 Hz, Hα), 1.78 (m, 2H, Hβ), 1.42 (m, 2H, Hχ), 3.46 (m, 1H, Hδ), 3.09 (m, 2H, Hε), 1.40 (s, 9H, Boc-CH_3); chemical shifts for the galactose moiety were 5.54 (d, 1H, J=8.0 Hz, H1), 4.98 (m, 1H, J=9.3, 8.0 Hz, H2), 5.25 (m, 1H, J=9.3 Hz, H3), 4.65 (m, 1H, J=9.7 Hz, H4), 4.78 (d, 1H, J=6.0 Hz, H5), 4.21 (d, 2H, J=12.3, 2.4 Hz, H5′), 2.01 (s, 12H, CH_3COO-). $[\alpha]_D^{21}$ 9.0° (*c* 1.0, $CHCl_3$).
12. MALDI-MS gave the desired mass (798 Da; theoretical 798.28 Da). ^{1}H NMR analysis (500 MHz, $CDCl_3$): δ 7.84–7.28 (m, 8H, Fmoc-ArH), 4.70 (d, 2H, J=7.0, 6.9 Hz, Fmoc-CH_2), 4.46 (d, 1H, J=6.8 Hz, Fmoc-CH), 5.43 (d, 1H, J=7.4 Hz, NHα), 5.41 (d, 1H, NHε), 4.46 (dt, 1H, J=5.8, 14.2 Hz, Hα), 1.78 (m, 2H, Hβ), 1.42 (m, 2H, Hχ), 3.46 (m, 1H, Hδ), 3.09 (m, 2H, Hε), 4.75 (d, 2H, J=7.0, 10.6 Hz, Aloc-CH_2), 5.89 (m, 1H, J=7.2, 10.7 Hz, Aloc-CH), 5.23–5.24 (d, 2H, J=7.4 Hz, Aloc-CH_2); chemical shifts for the galactose moiety were 5.54 (d, 1H, J=8.0 Hz, H1), 4.98 (m, 1H, J=9.3, 8.0 Hz, H2), 5.25 (m, 1H, J=9.3 Hz, H3), 4.65 (m, 1H, J=9.7 Hz, H4), 4.78 (d, 1H, J=6.0 Hz, H5), 4.21 (d, 2H, J=12.3, 2.4 Hz, H5′), 2.01 (s, 12H, CH_3COO-).
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15. Sequencing was performed on an ABI 477A Protein Sequencer; see: Fields, C. G.; VanDrisse, V. L.; Fields, G. B. *Pept. Res.* **1993**, *6*, 39–47.
16. Sequence analysis showed a derivative in cycle 3, presumably Hyl(ε-Boc,*O*-2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl), eluting at a similar position as Lys.
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