Synthesis of *C*-Terminal Glycopeptides from Resin-Bound Glycosyl Azides via a Modified Staudinger Reaction

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The solid-phase synthesis of glycopeptides containing the sugar at the *C*-terminus is reported. The method is demonstrated on a model, the endogenous antinociceptive peptide Leu-enkephalin. 2,3,4-Tri-*O*-acetyl-1-azido-1-deoxy-*β*-D-glucopyranuronic acid was synthesized and immobilized onto a variety of derivatized resins. Conjugation of the first amino acid was accomplished by reaction of the resin-bound glycosyl azide with an activated amino acid, in one step, via a modified Staudinger reaction. Standard solid-phase peptide synthesis then resulted in the desired amide-linked glycopeptide. Reaction conditions and reagents for the glycosylation were varied to optimize the yield and purity of the product. The optimum conditions were found to be the use of a 4-fold molar excess of activated amino acid and 3-fold excess of tri-*n*-butylphosphine in tetrahydrofuran. This methodology is generally applicable to most peptide sequences and is compatible with both Boc- and Fmoc- synthetic strategies on a variety of resins.

Introduction

Glycoproteins and glycopeptides are of significant biological interest due to their widespread roles in cell recognition and cell adhesion.[1,2] In addition, they can improve the absorption of poorly bioavailable drugs and peptides by enhancing membrane transport[3] or by utilizing specific transport systems, such as the Na^+ dependent glucose transporter.[4]

Glycopeptides are routinely synthesized in solution,[5] commonly using glycosylamines, which are readily obtained by reducing their azide precursors.[6] Standard coupling procedures can then be employed to couple this amine to an appropriately protected amino acid residue using carbodiimides, preformed anhydrides, or active esters. We sought to apply this methodology to the synthesis of *C*-terminally modified glycopeptides on a solid support. Whereas an immobilized glycosyl azide can be reduced on-resin,[7,8] we preferred to investigate the possibility of coupling the first amino acid residue to the azide in one step, thus avoiding the additional reduction step and the use of unpleasant reagents such as propane-1,3-dithiol.

The Staudinger reaction[9] involves the formation of an amide bond from the reaction between an azide and a carboxylic acid in the presence of a trialkyl- or triarylphosphine.[10,11] Modified Staudinger reactions, using an activated carboxylic acid, have become established methods for the solution-phase preparation of glycosyl amides,[10−12] and we were interested in transposing this reaction onto a solid-phase system.

We employed the pentapeptide Leu-enkephalin[13] (H-YGGFL-OH), a centrally acting endogenous antinociceptive agent, as a model peptide, and we describe a series of experiments leading to an optimized set of conditions in which to couple an activated amino acid to a resin-bound glycosyl azide in one step, to yield an amide-linked glycopeptide.

Results and Discussion

The synthesis of *N*-terminal glycopeptides is easily achieved on solid phase via established methods. The appropriately protected carbohydrate building block can be prepared and conjugated to the deprotected *N*-terminal α-amino function, either by a direct path or via a spacer, to produce glycopeptides with a variety of linkages. Preparation of *C*-terminal glycopeptides represents a greater challenge since the carbohydrate building block must have a functional group by which it can be immobilized onto the solid support, as well as a group to which the first amino acid can be conjugated. Previous

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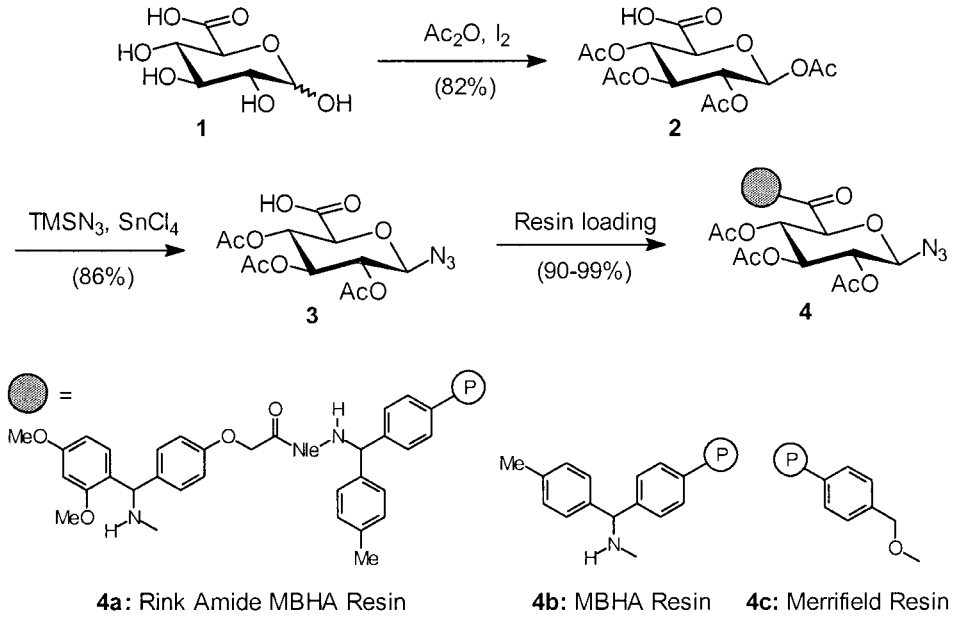
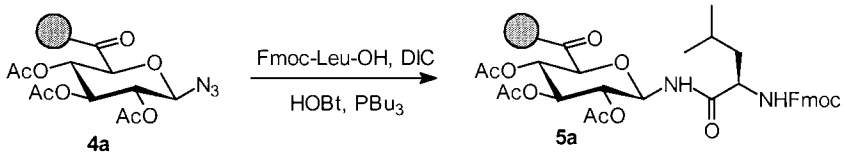
Scheme 1. Synthesis and Resin Loading of Glycosyl Azide



Nle = norleucine, P = polymeric support

Scheme 2. Coupling of First Amino Acid via Staudinger Reaction



work[7,14] in our laboratory has employed 2,3,4-tri-*O*-acetyl-1-azido-1-deoxy-β-D-glucopyranuronic acid, which is easily immobilized onto a variety of derivatized resins through the carboxyl function. The azido group can then act as a masked amino group to which the protected *C*-terminal amino acid of the peptide sequence can be coupled. Our previous approach involved reduction of the azido function to generate the glycosylamine in situ and subsequent coupling of the first amino acid using standard peptide synthetic methods. In this study, the resin-bound glycosyl azide was used directly in a modified Staudinger reaction, varying the resin, reaction conditions, and reagents, to optimize the yield and purity of the Leu-enkephalin model glycopeptide.

2,3,4-Tri-*O*-acetyl-1-azido-1-deoxy-β-D-glucopyranuronic acid (**3**) was prepared from D-glucuronic acid (**1**) using a method slightly modified from that previously reported.[14] Azide **3** was then immobilized onto Rink Amide 4-methylbenzylhydrylamine (MBHA) resin in good yield (Scheme 1).

The original reaction conditions involved activation of a 4-fold excess (over resin substitution) of N^{α}-9-fluorenylmethoxycarbonyl (Fmoc) protected leucine using an equimolar amount of *N*,*N*-diisopropylcarbodiimide (DIC) and 1-hydroxybenzotriazole (HOBt) in anhydrous tetrahydrofuran (THF). The solution of the activated amino acid was then added to the resin-bound azide followed by a 3-fold excess of tri-*n*-butylphosphine and the reaction allowed to proceed for 18 h (Scheme 2).

A variety of different reaction conditions and reagents were employed in an attempt to optimize the yield and purity of the peptides. The reaction was carried out in a variety of solvents (THF, CH_2Cl_2, MeCN, dioxane) using either DIC or dicyclohexylcarbodiimide (DCC) with HOBt to activate the amino acid. The tertiary phosphine was also varied between tri-*n*-butyl-, trimethyl-, and triphenylphosphines, as was the excess of reagents over resin substitution (4 equiv of acid with 3 equiv of phosphine or 3 equiv of acid with 2 equiv of phosphine). Finally, the derivatized resin (Scheme 1) onto which the glycosyl azide was loaded was changed from Rink Amide MBHA (Fmoc strategy synthesis of peptide amides) to MBHA or Merrifield (Boc strategy synthesis of peptide amides or peptide acids, respectively).

After initial amino acid conjugation and removal of the N^{α}-protecting group (20% (v/v) piperidine in dimethylformamide (DMF) for Fmoc, trifluoroacetic acid (TFA) for Boc), the peptide synthesis was continued. All couplings were carried out in DMF using in situ activation with 2-(1-*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), HOBt, and *N*,*N*-diisopropylethylamine (DIEA). Coupling and deprotection steps were monitored using the Kaiser[15] test. If the test was negative after the first deprotection (no free amine groups present), the first amino acid conjugation reaction was assumed unsuccessful and the synthesis not continued any further. After deprotection of the final amino acid, the peptides were removed from the resin, extracted, and lyophilized.

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Scheme 3. Cleavage and Deacetylation of Glycopeptide

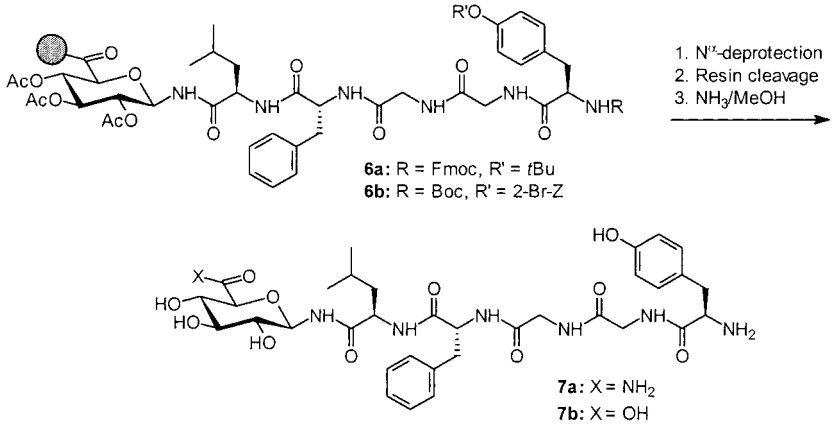

Table 1. Yields and Purities of Glycopeptides

entry	loaded resin	cleaved product	reagents[a]	PR_3	solvent	yield[b] (%)	purity[c] (%)
1	4a	7a	A	PBu_3	THF	79	72
2	4a	7a	A	PBu_3	CH_2Cl_2	n/a	n/a
3	4a	7a	A	PBu_3	MeCN	56	44
4	4a	7a	A	PBu_3	dioxane	51	66
5	4a	7a	A	PMe_3	THF	n/a	n/a
6	4a	7a	A	PPh_3	THF	n/a	n/a
7	4a	7a	B	PBu_3	THF	68	73
8	4a	7a	C	PBu_3	THF	77	76
9	4a	7a	D	PBu_3	THF	60	72
10	4a	7a	A	PBu_3	THF	81	72
11	4b	7a	A	PBu_3	THF	72	83[d]
12	4c	7b	A	PBu_3	THF	70	69[d]

[a] A: 4 equiv of DIC, HOBt; 3 equiv of PR_3. B: 3 equiv of DIC, HOBt; 2 equiv of PR_3. C: 4 equiv of DCC, HOBt; 3 equiv of PR_3. D: 3 equiv of DCC, HOBt; 2 equiv of PR_3. [b] Isolated yield of crude glycopeptide based on resin loading. N/a indicates the coupling of the first amino acid was unsuccessful. [c] Determined by HPLC. All compounds exhibited satisfactory FAB and ES MS. [d] Confirmed by isolation.

Carbohydrate *O*-acetyl protecting groups were then removed using methanolic ammonia, and the final product was lyophilized (Scheme 3). In one case (Table 1, entry 10), the *O*-acetyl groups were removed using methanolic hydrazine prior to peptide cleavage. Peptides were analyzed using fast atom bombardment (FAB) and electrospray (ES) mass spectrometry, and their purities, determined using reversed-phase (RP) HPLC. Reaction conditions, yields, and purities are summarized in Table 1. The final yield and purity are representative of the success of the Staudinger reaction, since the following peptide synthesis was always carried out using the same steps and reaction conditions. All amino acid couplings proceeded to greater than 99.8% completion.

Conclusions. Of the various combinations of conditions and reagents tried, the optimum was the use of 4 equiv (over resin substitution) of activated amino acid and 3 equiv of tri-*n*-butylphosphine in THF. The polystyrene resins had adequate swelling properties in THF, and in addition it was a good solvent for HOBt and the large majority of protected amino acids. Using MeCN or dioxane as solvent resulted in reduced yield and purity of the glycopeptide, whereas the amino acid coupling reaction surprisingly did not proceed in CH_2Cl_2 at all.

The use of DCC instead of DIC to activate the amino acid resulted in comparative yield and purity of the desired product. However, DIC was more convenient since its diisopropyl urea byproduct had considerably better solubility in organic solvents than dicyclohexyl urea (DCU), which required extensive postreaction washing to dissolve and separate from the resin particles. The use of a reduced molar excess of reactants over the resin resulted in a slightly reduced yield of glycopeptide of comparable purity.

Using tertiary phosphines other than tri-*n*-butylphosphine also resulted in failure of the coupling reaction. We propose that the reaction with the highly reactive trimethylphosphine at ambient temperature favored the elimination of nitrogen to form the trimethyliminophosphorane, which did not readily react with the activated amino acid to form a glycosyl amide. The glycosyl azide was unreactive toward triphenylphosphine at ambient temperature. These results were in agreement with the solution-phase work of Inazu et al.[11,12]

Finally, removal of the carbohydrate *O*-acetyl protecting groups was accomplished successfully prior to or after cleavage from the resin, resulting in glycopeptides of similar yield and purity. Removal prior to cleavage was more convenient since the product had only to be lyophilized once, and precipitation of the fully deprotected glycopeptide after cleavage using ether was generally easier.

This reaction was compatible with both Boc and Fmoc solid-phase peptide synthesis strategies, on a variety of resins, and should be equally applicable to the synthesis of glycopeptides of different sequence by reaction with other, appropriately protected amino acid residues.

Experimental Section

Moisture-sensitive reactions were carried out under a nitrogen atmosphere using oven-dried glassware. Solvents were dried according to established practices and were freshly distilled prior to use. Reactions were monitored by TLC on silica gel visualizing by charring with an ethanolic solution of sulfuric acid. After workup, organic solutions were dried over $MgSO_4$ and the solvents removed under reduced pressure. Analytical RP-HPLC was carried out on an analytical Vydac C_4 Protein column (25.0 cm × 4.6 mm). Separation was achieved using a linear gradient at a flow rate of 1.2 mL min^{-1} effected by a Waters 600S controller and 616 pump running solvent A (0.1% (v/v) TFA), solvent B (0.1% (v/v) TFA in 90% (v/v) MeCN), 0% B to 70% B over 20 min, and then 70% B to 0% B over 5 min. Separation was monitored with a Waters 486 absorbance detector at 214 nm. Retention and purity were determined using Waters Millennium Chromatography Manager software. Mass spectra were recorded on a VG Analytical ZAB-SE instrument using fast atom bombardment (FAB) ionization or on a Finnigan MassLab Navigator quadrupole

instrument using electrospray (ES) ionization. ^{1}H NMR (500 MHz) spectra were recorded at room temperature in $CDCl_3$ solutions.

1,2,3,4-Tetra-*O*-acetyl-β-D-glucopyranuronic Acid (2). D-Glucuronic acid (**1**) (6.0 g, 31 mmol) was suspended in 85 mL of acetic anhydride and stirred at 0 °C. Iodine (425 mg, 2 mmol) was added slowly and stirring continued at 0 °C for 2 h and then a further 1 h at 25 °C. The solution was cooled to 0 °C, 30 mL of dry MeOH added dropwise, and the solution allowed to stand for 18 h at 25 °C. The solution was concentrated and taken up in 100 mL of CH_2Cl_2, extracted twice with 1 M $Na_2S_2O_3$, dried, filtered, and concentrated. The white residue was taken up in a mixture of ether, hexane, and $CHCl_3$ and concentrated again. Addition of ether, filtering, and washing afforded the title compound as a fine white powder (9.2 g, 82%). FAB MS *m*/*z* (%): 303 $[M - OAc]^+$ (34), 325 $[M - OAc + Na]^+$ (64), 385 $[M + Na]^+$ (86), 407 $[M + 2Na]^+$ (100). ^{1}H NMR: δ 2.02, 2.03, 2.04, 2.11 (4s, 12H, 4OAc), 4.24 (m, 1H, H-5), 5.13 (m, 1H, H-2), 5.29 (m, 2H, H-3 and H-4), 5.79 (d, 1H, H-1, $J_{1,2} = 7.3$ Hz). Anal. Calcd for $C_{14}H_{18}O_{11}$: C, 46.41; H, 4.97. Found: C, 46.24; H, 5.01.

2,3,4-Tri-*O*-acetyl-1-azido-1-deoxy-β-D-glucopyranuronic Acid (3). To a solution of **2** (2.0 g, 5.5 mmol) in dry CH_2Cl_2 (40 mL) was added trimethylsilyl azide (1.87 mL, 13.8 mmol) and tin(IV) chloride (0.32 mL, 2.8 mmol). The reaction was allowed to proceed for 18 h at 25 °C under N_2. The solution was diluted with CH_2Cl_2, extracted twice with 1 M $KHSO_4$, dried, filtered, and concentrated to yield the title compound as a white foam (1.64 g, 86%). FAB MS *m*/*z* (%): 303 $[M - N_3]^+$ (35), 368 $[M + Na]^+$ (100), 390 $[M + 2Na]^+$ (22). ^{1}H NMR: δ 2.02, 2.04, 2.07 (3s, 9H, 3OAc), 4.17 (d, 1H, H-5), 4.75 (d, 1H, H-1, $J_{1,2} = 8.8$ Hz), 4.96 (m, 1H, H-2), 5.28 (m, 2H, H-3 and H-4). Anal. Calcd for $C_{12}H_{15}O_9N_3$: C, 41.74; H, 4.35; N, 12.17 Found: C, 41.65; H, 4.40; N, 12.19.

Immobilization of Compound 3 onto Rink Amide MBHA Resin (4a). Rink Amide MBHA resin (1.5 g, 0.64 mmol g^{-1} loading, 0.96 mmol) was washed and swelled in dry DMF for 30 min in a solid-phase reaction vessel. The resin was treated with a 20% (v/v) solution of piperidine in DMF (2 × 10 min) to remove Fmoc protection and was washed well with dry DMF. To a solution of **3** (0.83 g, 2.4 mmol) and HOBt (0.37 g, 2.4 mmol) in dry CH_2Cl_2 at 0 °C was added DIC (0.38 mL, 2.4 mmol) and the solution stirred for 10 min at 0 °C. The solution was concentrated and the residue taken up in dry DMF. This solution and DIEA (0.42 mL, 2.4 mmol) were added to the resin, and the suspension was mixed using a mechanical shaker for 18 h. The resin was drained, washed with DMF, CH_2Cl_2, and then 50% (v/v) MeOH in CH_2Cl_2, and dried in vacuo over KOH to constant weight (1.44 g, 90%, 0.63 mmol g^{-1} loading).

Immobilization of Compound 3 onto MBHA Resin (4b). MBHA resin (1.96 g, 0.59 mmol g^{-1} loading, 1.2 mmol) was washed and swelled in dry DMF for 30 min in a solid-phase reaction vessel. To a solution of **3** (1.21 g, 3.5 mmol) and HOBt (0.54 g, 3.5 mmol) in minimal dry DMF at 0 °C was added DIC (0.55 mL, 3.5 mmol) and the solution stirred for 10 min at 0 °C. The solution and DIEA (0.61 mL, 3.5 mmol) were added to the resin, and the suspension was mixed using a mechanical shaker for 3 h. The resin was drained, washed with DMF, CH_2Cl_2, and then 50% (v/v) MeOH in CH_2Cl_2, and dried in vacuo over KOH to constant weight (2.33 g, 99%, 0.50 mmol g^{-1} loading).

Immobilization of Compound 3 onto Merrifield Resin (4c). To a solution of **3** (0.85 g, 2.5 mmol) in MeOH (20 mL) at 0 °C was added a solution of cesium carbonate (0.40 g, 1.2 mmol) in 80% (v/v) MeOH (8 mL) dropwise over 10 min. After standing for a further 30 min, the solution was concentrated to dryness and the residue taken-up in dry DMF. The solution of the cesium salt of **3** was added to Merrifield resin (1.99 g, 0.72 mmol g^{-1} loading) which had previously been washed and swelled in dry DMF. The suspension was warmed to 50 °C and stirred for 18 h, then transferred to a solid-phase reaction vessel, drained, and washed (DMF, 50% (v/v) aqueous DMF, DMF, CH_2Cl_2, and then 50% (v/v) MeOH in CH_2Cl_2). The resin was dried in vacuo over KOH to constant weight (2.30 g, 95%, 0.59 mmol g^{-1} loading).

Example Procedure for First Amino Acid Attachment. Dried resin **4a** (100 mg, 0.63 mmol g^{-1} loading, 63 μmol) was transferred to a solid-phase reaction vessel, washed, and swelled in dry DMF under N_2 for 30 min. The resin was then washed several times with dry THF and left as a slurry. To a solution of Fmoc-Leu-OH (89 mg, 0.25 mmol) and HOBt (39 mg, 0.25 mmol) in dry THF at 0 °C was added DIC (40 μL, 0.25 mmol) and the solution stirred at 0 °C for 10 min. The solution was then added to the resin, followed by tri-*n*-butylphosphine (47 μL, 0.19 mmol), and the suspension mixed for 18 h under N_2. The resin was drained and washed well with DMF.

Peptide Synthesis. Peptide sequence elongation was accomplished manually using a stepwise solid-phase procedure. All couplings were carried out in DMF using a 2.5-fold excess (over resin loading) of protected amino acid, activated with an equimolar amount of HBTU and HOBt, in the presence of a 5-fold excess (over resin loading) of DIEA. Each coupling was repeated and completion monitored using the Kaiser[15] test for free amines. N^α-Fmoc protection was removed using 20% (v/v) piperidine in DMF (2 × 10 min). N^α-Boc protection was removed using TFA (2 × 1 min). Tyrosine side-chain protecting groups were *tert*-butyl for Fmoc strategy and 2-bromobenzyloxycarbonyl (2-Br-Z) for Boc strategy. The peptide−resin was washed very thoroughly with DMF after each coupling and deprotection step.

Peptide Cleavage. After removal of the terminal N^α-protecting group, the resin was washed well with DMF, CH_2Cl_2, and then 50% (v/v) MeOH in CH_2Cl_2 and dried in vacuo over KOH to constant weight. Peptides produced by Fmoc strategy were removed from the resin with simultaneous side-chain deprotection by acidolysis using TFA containing 2.5% (v/v) water and 2.5% (v/v) triisopropylsilane (TIS) for 2 h at 25 °C. Peptides produced by Boc strategy were cleaved using anhydrous HF containing ∼10% (w/v) *p*-cresol for 1 h 45 min at −5 to 0 °C. Crude peptides were precipitated and washed thoroughly with anhydrous ether, then extracted into 95% (v/v) acetic acid, and lyophilized.

Removal of *O*-Acetyl Protecting Groups. Solution-phase de-*O*-acetylation was achieved by suspending the lyophilized peptide in 5 mL of ∼5% (w/w) NH_3 in MeOH. The reaction was allowed to proceed for 2 h at 25 °C, monitoring completion by RP-HPLC. The solvent was then removed under reduced pressure, and the peptide was taken up in 95% (v/v) acetic acid and lyophilized. Solid-phase de-*O*-acetylation was carried out after removal of the terminal N^α-protecting group, but prior to peptide cleavage. The drained resin−peptide was suspended in 2 mL of 12.5% (v/v) hydrazine hydrate in MeOH and the suspension mixed for 18 h at 25 °C. The resin was then drained and washed well with DMF, before being prepared for cleavage as previously. Compound **7a**. HRMS. Calcd for $C_{34}H_{47}N_7O_{11}$-Na: 752.3231. Found: 752.3198. Compound **7b** HRMS. Calcd for $C_{34}H_{46}N_6O_{12}Na$: 753.3071. Found: 753.3022.

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