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Piperidine is Preferable to Morpholine for Fmoc Cleavage in Solid Phase Synthesis of *O*-Linked Glycopeptides

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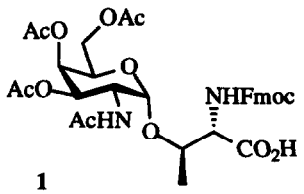
Abstract: Morpholine was found to give slow and incomplete Fmoc removal resulting in substantial by-product formation in solid phase synthesis of an *O*-linked HIV-related glycopeptide. A significant improvement was obtained by replacement of morpholine with piperidine and, in contrast to common belief, β-elimination was not observed.

The diverse and important biological functions of the carbohydrate chains of glycoproteins[1] has stimulated considerable efforts towards the synthesis of glycopeptides[2]. Solid phase peptide synthesis based on N^{α}-Boc protection requires conditions for the final deprotection and cleavage of the peptide from the resin which are incompatible with acid labile glycosidic linkages. Therefore the N^{α}-fluoren-9-ylmethoxycarbonyl (Fmoc) group[3] is predominantly used in solid phase synthesis of glycopeptides since mild acidic conditions can then be used for the final deprotection and cleavage. *O*-linked glycopeptides can undergo β-elimination on treatment with base and this reaction is used to cleave *O*-glycosylserine and -threonine linkages in glycoproteins. In some early syntheses[4] of *O*-linked glycopeptides piperidine was used for Fmoc cleavage but fear of β-elimination during the synthesis[2a,c] has since lead to replacement of piperidine with the weaker base morpholine.

As part of a study[5] of the functions of the carbohydrate moieties of the HIV envelope glycoprotein gp120 we were interested in the preparation of glycopeptide **2**. The synthesis was performed on a polystyrene-polyoxyethylene resin functionalized with the Rink amide linker[6] using a fully automatic



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Ac_3-α-D-GalNAc*p*

Ac-Gly-Arg-Ala-Phe-Val-Thr-Ile-Gly-Lys-

Ile-Gly-Asn-Met-Arg-Gln-Ala-Cys-NH_2 **2**

continuous flow peptide synthesizer constructed in this laboratory. N^{α}-Fmoc amino acids (4 equiv.) and the glycosylated threonine **1**[7] (2.4 equiv.) were activated in the synthesizer with 1,3-diisopropylcarbodiimide and 1-hydroxybenzotriazole in DMF and the *N*-acylations were monitored on the column using the acid-base indicator Bromophenol Blue[8]. Spectrophotometric monitoring of the dibenzofulvene-morpholine adduct in the column effluent at 350 nm revealed that the cleavages of the Fmoc groups of Gly^1-Thr^6 with 50% morpholine in DMF were slow. Most likely this reflects internal aggregation[9] of the peptide chains during the synthesis and results in incomplete Fmoc removal. Consequently the crude glycopeptide **2**, obtained after cleavage from the resin and side chain deprotection, was found to contain several by-products that were difficult to remove by preparative HPLC (Figure 1a). When the synthesis was repeated using 20% piperidine (instead of morpholine) in DMF fast and complete Fmoc removal was observed and crude **2** of high purity was obtained (Figure 1b). Purification by preparative reversed-phase HPLC was now facile and **2**[10] was obtained in 50% yield, based on the resin capacity. The two by-products eluting immediately before **2** (Figure 1b) were shown by FAB MS to originate from incomplete incorporation of one glycine and oxidation of **2**, respectively.

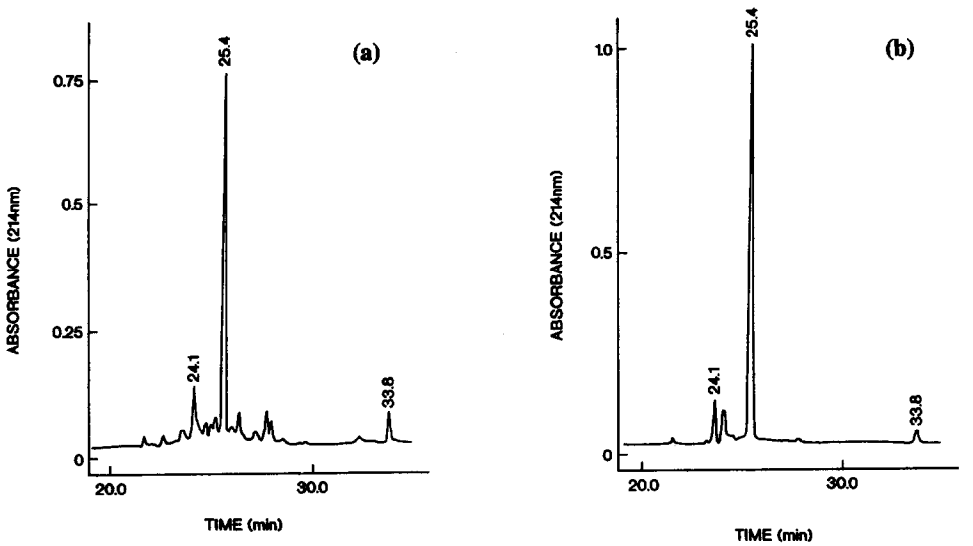

Figure 1. HPLC chromatograms of the crude glycopeptide **2** (25.4 min) prepared with cleavage of the N^{α}-Fmoc groups of Gly^1-Thr^6 with **(a)** 50% morpholine or **(b)** 20% piperidine in DMF. HPLC conditions: Kromasil C-8 column. Gradient of 0-80% B in A over 60 min with a flow rate of 1.5 mL/min. A = 0.1% aqueous TFA, B = 0.1% TFA in CH_3CN.

β-Elimination of threonine or racemization[11] were thus not observed in the synthesis of glycopeptide 2 when Fmoc-removal was effected with piperidine instead of the commonly used morpholine. We have also found this to be the case in syntheses of the mucin glycopeptides Leu-Ser-Glu-Ser-Thr-Thr-Gln-Leu-Pro-Gly and Pro-Thr-Thr-Thr-Pro-Ile-Ser-Thr-Thr-Met (all Ser and Thr carried α-D-GalNAc*p* residues), as well as further *O*-linked glycopeptides related to gp120 of the HIV-virus[12]. The fear of β-elimination and racemization in synthesis of glycopeptides therefore seems to have been exaggerated and we are now investigating the extent of these side reactions under the basic conditions used for removal of acetyl and benzoyl protective groups from the carbohydrate moiteis of glycopeptides. Finally, it should be noted that the more efficient Fmoc removal obtained with piperidine, in place of morpholine, can result in significant improvements in yield and purity of longer glycopeptides that are prone to internal aggregation during the synthesis. This observation is especially important since the recent rapid development of synthetic methodology[2] has put large glycopeptides, and even small glycoproteins, within reach of chemical solid phase synthesis.

Acknowledgment

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10. Compound **2**. FAB MS: $(M+H)^+$ 2192 (Calc. 2191). Amino acid analysis: Ala 2.00 (2), Arg 1.98 (2), Asp 1.02 (1), Cys 1.05 (1), Glu 0.98 (1), Gly 2.94 (3), Ile 1.97 (2), Lys 1.06 (1), Met 0.98 (1), Phe 1.01 (1), Thr 0.97 (1), Val 1.03 (1).
500 MHz ^{1}H-NMR in DMSO-d_6 at 300 K (solvent δ=2.50 ppm as internal reference); δ (ppm) Ac_3-α-D-GalNAc*p* 7.09 (AcN*H*), 5.30 (bd,2.9Hz,H-4), 4.98 (dd,11.4 and 2.9Hz,H-3), 4.82 (d,3.4Hz,H-1), 4.25 (H-5), 4.22 (H-2), 4.03 (H-6,6'), 1.83 (CH_3CONH); Gly^1 8.12 (NH), 3.70 (Hα,α'), 1.84 (CH_3CONH); Arg^2 7.98 (NH), 7.48 (NHδ), 4.28 (Hα), 3.07 (Hδ,δ'), 1.64 (Hβ,β'), 1.43 (Hγ,γ'); Ala^3 8.00 (NH), 4.25 (Hα), 1.25 (CH_3); Phe^4 8.02 (NH), 7.22 (H-arom), 4.56 (Hα), 3.02 (Hβ), 2.82 (Hβ'); Val^5 7.82 (NH), 4.39 (Hα), 1.97 (Hβ), 0.88 (CH_3β,β'); Thr^6 7.99 (NH), 4.51 (Hα), 4.20 (Hβ), 1.16 (CH_3); Ile^7 7.96 (NH), 4.15 (Hα), 1.71 (Hβ), 1.11 (Hγ,γ'), 0.85 (CH_3β,γ); Gly^8 8.05 (NH), 3.91 (Hα), 3.53 (Hα'); Lys^9 7.93 (NH), 7.67 (NH_3^+), 4.34 (Hα), 2.76 (Hε,ε'), 1.65 (Hβ,β'), 1.53 (Hδ,δ'), 1.29 (Hγ,γ'); Ile^{10} 7.88 (NH), 4.16 (Hα), 1.72 (Hβ), 1.46 (Hγ), 1.09 (Hγ'), 0.84 (CH_3β,γ); Gly^{11} 8.15 (NH), 3.80 (Hα), 3.68 (Hα'); Asn^{12} 8.08 (NH), 7.51 and 6.99 ($CONH_2$), 4.58 (Hα), 2.58 (Hβ), 2.47 (Hβ'); Met^{13} 8.16 (NH), 4.28 (Hα), 2.48 (Hγ,γ'), 1.96 (Hβ), 1.83 (Hβ'); Arg^{14} 8.06 (NH), 7.52 (NHδ), 4.20 (Hα), 3.10 (Hδ,δ'), 1.73 (Hβ,β'), 1.58 (Hγ), 1.51 (Hγ'); Gln^{15} 7.90 (NH), 7.25 and 6.79 ($CONH_2$), 4.19 (Hα), 2.12 (Hγ,γ'), 1.90 (Hβ), 1.78 (Hβ'); Ala^{16} 8.00 (NH), 4.25 (Hα), 1.25 (CH_3); Cys^{17} 7.83 (NH), 7.68 (SH), 7.35 and 7.21 (αCONH$_2$), 4.29 (Hα), 2.78 (Hβ), 2.26 (Hβ').

11. 500 MHz ^{1}H-NMR spectroscopy of **2** did not reveal any impurities, and D-alloisoleucine was not detected in the amino acid analysis.

12. J. Kihlberg, S. Roy, and T. Vuljanic, to be published.

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