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The N-(2-hydroxybenzyl) Protecting Group for Amide Bond Protection in Solid Phase Peptide Synthesis

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Abstract: Backbone amide protection using the N-(2-hydroxybenzyl) group to overcome chain aggregation during solid phase peptide synthesis is described. This is illustrated by the preparation of the notoriously difficult decapeptide acyl carrier protein (65-74). The N-(2-hydroxybenzyl) group is stable to trifluoroacetic acid/dichloromethane (1:1, v/v) but cleaved readily by trifluoromethanesulphonic acid.

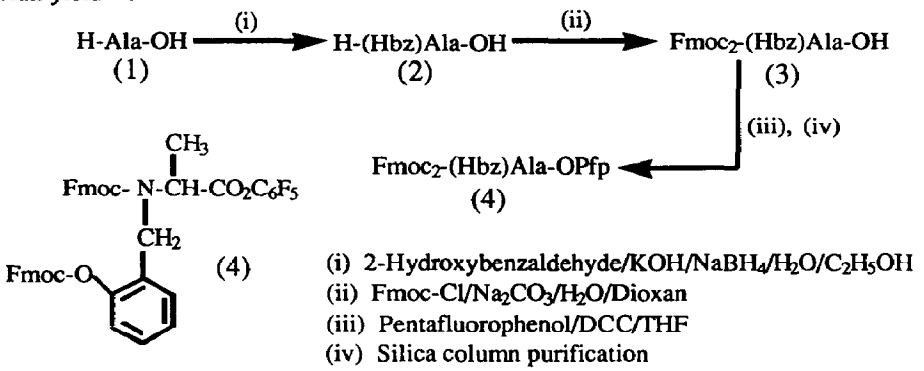
It was recognised over 20 years ago that some peptide sequences posed special problems when synthesised by the solid phase technique[1]. These special cases have since become known as 'difficult sequences'[2] and are believed to arise from the association of intermediate resin-bound peptide chains into extended β-sheet type structures[3]. Massive steric hindrance commonly results, leading to reduced reagent penetration and significantly reduced reaction rates in both acylation and deprotection steps[4]. No purification of peptide intermediates in solid phase peptide synthesis is carried out. Any deletion or truncated peptides are carried through the course of the synthesis until the final cleavage step where they contaminate the final target peptide. Even using the most modern powerful chromatographic techniques, isolation of a target peptide from other closely related structures cannot be guaranteed[2a]. Thus it is vital that all reactions on the solid phase are forced as near to completion as possible.

The occurence of chain association is a phenomenon that is encountered when both polystyrene[5] and polydimethylacryl-amide[6] based supports are used. "Difficult sequences" are easily detected in continuous flow Fmoc solid phase peptide synthesis. Onset of aggregation can be readily observed by spectophotometric monitoring of the release of the fluorene derivative into the reagent stream[7]. For unhindered release, a characteristic elution profile ('bell-shaped') is obtained that, on chain association, becomes flattened and broadened (slowing of deprotection rate)[8].

Recently we have described a practical solution for overcoming chain aggregation for peptides synthesised on the solid phase[9]. This entails reversible N-substitution of peptide amide bonds by the N-(2-hydroxy-4-methoxybenzyl) (**Hmb**) group and arose out of observations that replacement of secondary by tertiary amide bonds prevented hydrogen bonded association of peptide chains[8]. For Boc/benzyl protocols, modification of the Hmb group is necessary to reduce its acid lability[9]. The essential feature of the Hmb group is the 2-hydroxy moiety. During coupling to resin-bound Hmb derivatised amino acid residues, initial reaction occurs via an internally base catalysed mechanism to give O-acylation followed by O to N intramolecular acyl transfer[9]. This mechanism is essential for quantitative reaction as N-substitution of

amino acid residues results in massive steric hindrance at the nitrogen (except for glycine). In orienting studies, derivatives of both 2 and 4-methoxybenzyl amino acids were prepared and found to be stable to TFA but labile to TFMSA (Johnson, Quibell and Owen, unpublished). Thus, by analogy, we considered that the 2-hydroxybenzyl (**Hbz**) group would also be TFA-stable and TFMSA-labile, but maintain the essential feature of base catalysed acyl transfer.

Bis-Fmoc-N-(2-hydroxybenzyl) alanine pentafluorophenyl ester was prepared as shown in scheme 1 in 30% overall yield[10].



Scheme 1

The value of this derivative in preventing chain association was demonstarated by the synthesis of the well known difficult decapeptide sequence, acyl carrier protein (65-74).

H-Val-Gln-Ala-Ala-Ile-Asp-Tyr-Ile-Asn-Gly-OH
65 68 74

Chain aggregation occurs upon deprotection of the penultimate Gln residue[8]. Coupling of the final Val residue is strongly hindered, usually 10-15% incomplete under Fmoc-polyamide continuous flow conditions (Pfp ester-HOBt couplings, 45 min in DMF) that are completely adequate for insertion of the earlier residues[11]. The N-Hbz-alanine residue was inserted at position 68. This had earlier been shown to completely inhibit chain aggregation[9]. Fmoc-alanine was coupled to resin-bound Hbz-alanine using (i) 10 fold excess of symmetrical anhydride in dichloromethane or, (ii) 10 fold excess of urethane N-carboxy anhydride (UNCA) in dichloromethane. Both reactions were allowed to proceed for 3 hours, followed by addition of the final two residues (Gln, Val) under standard continuous flow conditions. Hplc analysis of both syntheses (data not shown) indicated that two products were present (90 + 10% respectively). Isolation and amino acid analysis of the latter revealed it to be missing Val-Gln-Ala, i.e it arose from the failure to completely couple (or O to N transfer) activated Fmoc-Ala onto resin-bound Hbz-Ala. Analogous coupling of Fmoc-Ala (symmetrical anhydride or UNCA) to resin-bound Hmb-Ala proceeds within 1 hour[9]. Presumably, the absence of the electron withdrawing *m* -OMe group (with respect to the hydroxy function) in the Hbz

system lowers its reactivity and leads to a larger than anticipated effect on the overall coupling rate. Synthesis was repeated except Fmoc-Ala-UNCA was coupled for 16 hours. Hplc analysis of the crude cleaved product (fig. 1a) shows no evidence for substantial des-Val peptide and FAB-MS confirmed the presence of the Hbz group[12]. The crude Hbz-peptide was suspended in TFA/dichloromethane (1:1, v/v) for 24 hours. No change in Hplc profile was observed on re-analysis (data not shown). The crude peptide was treated with 78% TFA, 4% EDT, 8% thioanisole, 10% TFMSA for 2 hours to remove the Hbz group. Hplc analysis (fig. 1b) of a small quantity of gel filtered material showed *ca.*90% cleavage of the Hbz group. The remaining crude peptide was purified by preparative Hplc to give ACP (65-74) (fig. 1c) in 75% yield.

We conclude that amide backbone protection by the Hbz group would be suitable for use in Boc/benzyl based solid phase peptide synthesis in order to prevent the occurence of chain association. However, it would appear that simply applying the coupling principles developed for the Hmb group[9] may prove to be insufficient as coupling to the Hbz group occurs at a slower rate.

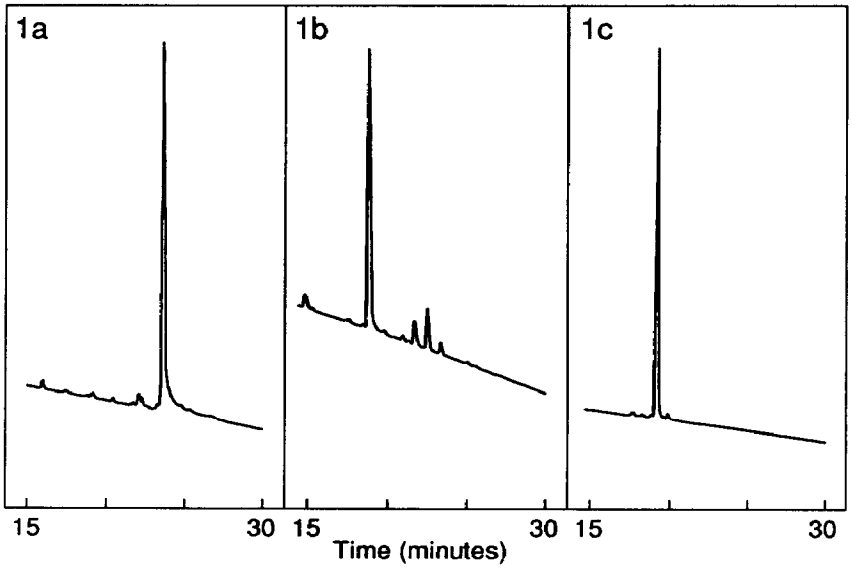


Figure 1: Analytical HPLC (Aquapore RP-300 C8 column; 15% B in A to 60% B in A over 40 min; A=0.1% aq. TFA, B=90% CH_3CN, 10% A; 1.5 ml min^{-1} flow rate; monitoring at 215 nm; **1a** crude ACP (65-74)(Hbz)Ala[68]; **1b** Crude ACP(65-74); **1c** Purified ACP(65-74)

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10. Compound (3): $C_{40}H_{33}NO_9.H_2O$, required (found), %C 73.05 (72.77), %H 5.36 (4.90), %N 2.13 (2.01); FAB-MS (+ve m/z), required=639.6, found=662.5 $[M+Na]^+$, 640.4 $[M+H]^+$; mp=120-121 (shrinks), 165-172 (melts); $[\alpha]D^{20}$= -2.3° (c=1, DMF); Tlc (10% MeOH/$CHCl_3$), Rf=0.7.

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12. **ACP (65-74) (Hbz-Ala^{68})**: Mass expected, 1169.3; FAB-MS, found $[M+H^+]$, 1169.8, $[M+Na^+]$, 1191.9; $[M+K^+]$, 1208.0. Amino acid analysis;(theory) found; Asp(2), 2.01; Glu(1), 1.05; Gly(1), 1.08; Ala(2). 1.21$^{\$}$; Val(1), 0.98; Ile(2), 1.91; Tyr(1), 1.00(ref): $-(Hbz)Ala, incomplete hydrolysis.

ACP (65-74): Mass expected, 1063.2; FAB-MS, found $[M+Na^+]$, 1086.17; Amino acid analysis;(theory) found; Asp(2), 2.03; Glu(1), 1.06; Gly(1), 1.03; Ala(2). 2.03; Val(1), 1.00; Ile(2), 1.94; Tyr(1), 1.00(ref)

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