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Application of Reversible Amide-bond Protection to Suppress Peptide Segment Epimerisation

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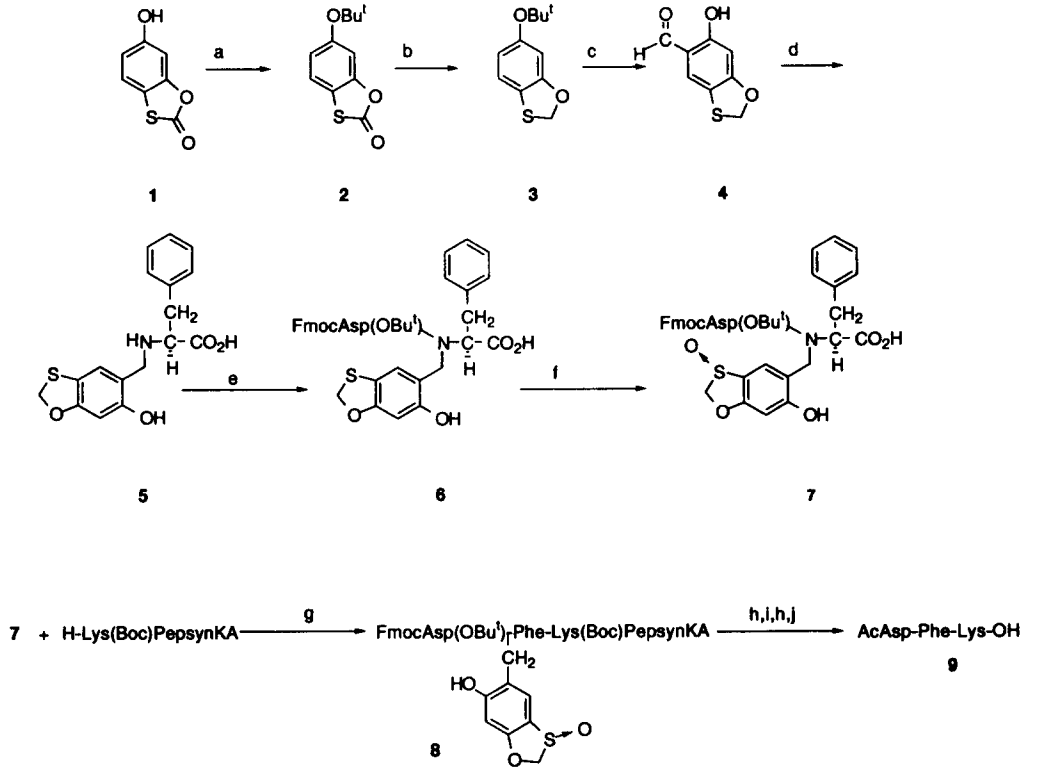
Abstract: Reversible alkylation of the C-terminal amide-bond of a protected peptide segment with 2-hydroxy-4-methoxybenzyl dramatically suppresses epimerisation during activation and coupling. However, due to the formation of a 4,5-dihydro-8-methoxy-1,4-benzoxazepin-2(3H)-one species upon activation the rate of coupling is low. A safety-catch amide-bond protecting group, 6-hydroxy-5-methyl-1,3-benzoxathiolyl, has been designed to suppress epimerisation and couple with excellent yield.

There are two conceptual approaches to the solid-phase chemical synthesis of small proteins. Stepwise synthesis using urethane protected amino acids or sequential assembly of smaller, purified fully protected segments. In practice, the vast majority of peptides up to 50 amino acids are almost routinely prepared by the former method. The use of N^{α}-urethane protection, during stepwise synthesis, protects the C^{α}-chiral integrity of the individual amino acid residues during activation and coupling[1]. However for longer peptide targets, even with near quantitative reactions, deletion sequences gradually accumulate to contaminate the final crude product and often provide a formidable purification task. Thus for target sequences up to and in excess of 100 residues the segment assembly method is an attractive alternative - any crude deletion products differ by at least one segment (typically 10-20 residues) and are more readily separable from the target. However the segment approach has a major drawback, the C-terminal amino acid no longer has urethane protection and is prone to epimerisation during activation and coupling.[2] This problem is usually avoided by choosing segments with a C-terminal glycine or proline residue,[3] however such a constraint is not always compatible with the target sequence. Thus a general chemical method for the activation and coupling of fully protected peptide segments with no epimerisation, to match the utility of N^{α}-urethane protection in stepwise synthesis, remains a major challenge in peptide chemistry. In contrast to extensive studies on the effect of C-terminal amino acid residue, side chain protection and method of activation on protected segment epimerisation, there is little work on the effect C-terminal backbone-amide substitution has on epimerisation.[4] Here we report our results on the use of C-terminal backbone amide protection to suppress epimerisation during protected segment coupling.

The concept of backbone amide protection was introduced to Fmoc/tBu SPPS after careful study of the mechanism of synthesis failure during the assembly of 'difficult sequences'.[5] Backbone protection has been used as the basis for a protected peptide segment assembly strategy.[3] In the course of these studies it was observed that the use of 2-hydroxy-4-methoxybenzyl (Hmb) substitution at the C-terminal amide bond of a fully protected peptide segment significantly reduced the rate of peptide segment coupling.[6] This reduction in coupling rate was due to the formation of a 4,5-dihydro-8-methoxy-1,4-benzoxazepin-2(3H)-one species between the activated carboxyl group and the hydroxyl function of Hmb, deactivating the peptide segment to acylation. Recently, this benzoxazepin-2(3H)-one species was observed by Nicolas *et al.*, upon the activation of N^{α}-Fmoc-N^{α}-(Hmb)amino acids.[7]



Scheme 1. a) $CCl_3C(=NH)OC(CH_3)_3$, $(C_2H_5)_2O:BF_3$/THF; r.t. 15 min. b) K_2CO_3, CH_2Br_2/butan-2-one; reflux 48 h. under N_2. c) DMF, $POCl_3$/$ClCH_2CH_2Cl$; 0°C to r.t. 12 h. d) (L)-Phenylalanine, KOH, $NaBH_4/H_2O$, C_2H_5OH; r.t. 15 min. e) $(FmocAsp(OBu^t))_2O$, Na_2CO_3/H_2O, dioxan. f) 3-Chloroperoxybenzoic acid (1.1 eq.)/CH_2Cl_2. g) see Table 1. h) 20% piperidine/DMF. i) Ac_2O/DMF; 1 h. j) NH_4I, $(CH_3)_2S$, TFA.

We considered that by modifying the Hmb skeleton by introducing an electron-withdrawing group (e.g. nitro) para to the 2-hydroxyl function of Hmb, the benzoxazepin-2(3H)-one would now be activated to acylation in a manner similar to classical *p*-nitrophenylester activation of a carboxylic acid. We reasoned that this substitution would substantially increase coupling kinetics with a concomitant reduction in the first order epimerisation process.

Segment epimerisation was studied using the model system, Ac-Asp-Phe-Lys-OH, used in a previous study from this laboratory.[8] We had previously identified optimal conditions for the suppression of epimerisation during activation and coupling using this test sequence. DIC/HOBt in DCM yielded the best results whilst BOP/HOBt/DIEA in DMF gave very poor results in agreement with others (Table 1).[9,10] Initial orienting studies used 2-hydroxy-4-methoxy-5-nitrobenzyl backbone protection **12** for activation of the benzoxazepin-2(3H)-one species. Coupling yields were excellent in comparison to unsubstituted Hmb **11** and yielded products containing substantially reduced LDL epimer compared with the use of no backbone protection **10** (Table 1). Not suprisingly, 2-hydroxy-4-methoxy-5-nitrobenzyl amide protection was stable to acidolysis by TFA and this series was not investigated further. Nevertheless, these pioneering experiments established the validity of our approach. To circumvent the irreversibility of backbone protection when an electron withdrawing group is introduced we applied the safety catch principle. The principle, that a stable bond is smoothly

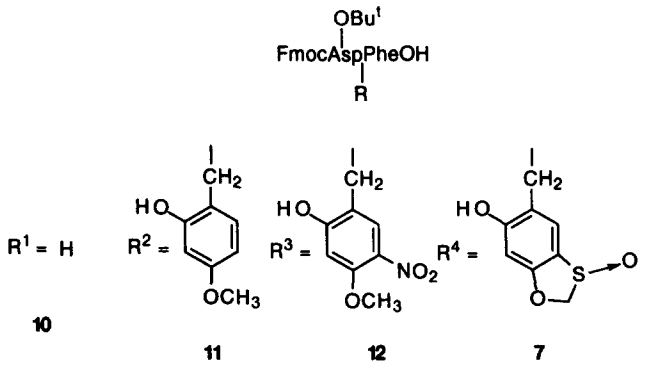


Table 1: Comparison of the effect of variation of backbone protection on coupling and epimerisation

amide substitution	conditions/solvent	coupling yield(%)	%LDL epimer
R^1	DIEA/BOP/HOBt/DMF	100	40.0
R^2	*	43	4.0
R^3	*	99	3.0
R^4	*	96	3.6
R^1	DIC/HOBt/DCM	100	0.5
R^2	*	40	<0.25
R^3	*	95	<0.25
R^4	*	94	<0.25

All couplings were carried out in parallel, with a two-fold excess (10 μmol) of segment and coupling reagents to H-Lysine(Boc)PepsynKA resin (5 μmol), for 4 h. in a minimum of solvent at room temperature. For couplings with DMF as solvent, the segment and coupling agents were dissolved in DMF at r.t., 5 min. before addition of resin; for couplings in DCM the segment and coupling agents were added to DCM at 0°C 10 min. before addition of resin. Coupling yield and Lysine substitution on resin were quantified by amino acid analysis. LDL epimer synthesised during coupling was detected by analytical HPLC, identified by co-running with standards Ac-DFK-OH containing either D or L phenylalanine synthesised by SPPS except for R^3 where standards were synthesised with backbone substitution. The limit for detection of epimerisation in this assay were estimated as 0.25%. The results are from one experiment representative of several.

converted to a labile one at a convenient point during a synthesis[11] has been applied in peptide chemistry for the development of novel linkers and protecting groups. One elegant safety catch approach has been to exploit the facile reductive conversion of a sulfoxide to sulfide,[12] conditions demonstrated to be compatible with Fmoc/tBu peptide synthesis. Application of this strategy allowed us to use a method of proven generality. In addition the sulfoxide conferred an additional level of orthogonality to backbone-amide protection[13] and suggested the extension of backbone protection to the synthesis of difficult sequences for Boc/benzyl peptide synthesis.

Synthesis of the aldehyde, 6-hydroxy-5-formyl-1,3-benzoxathiole **4**, was carried out in four steps (Scheme 1) from 6-hydroxy-1,3-benzoxathiol-2-one **1**, obtained in a single step from resorcinol.[14] The best yield of 6-butyloxy-1,3-benzoxathiol **3** (70% from **2**) was obtained by adding the benzoxathiol-one **2** to a refluxing mixture of methylene bromide, K_2CO_3 and butan-2-one under N_2. The benzoxathiol **3** was formylated to give **4** as a single product. Reactions a,b,c,e and f were monitored to completion by TLC and the product from each purified by silica gel chromatography. Backbone substituted (L)-phenylalanine derivatives were synthesised as previously described in detail for the preparation of Hmb substituted amino acids.[15] The dipeptide segments **7, 10-12** to be coupled onto H-Lys(Boc)PepsynKA resin were all synthesised by solution methods, illustrated here for the sulfoxide system **7.**

Coupling of segment **7**, under standard conditions (Table 1), was comparable to that of segment **12**, again exhibiting a dramatic improvement compared to that of segment **11**. After 1 h. the coupling yield was 81% under the conditions using DCM as solvent and 89% when using DMF. Treatment of resin-bound **8**, via

removal of Fmoc, acetylation and a second treatment with piperidine (to de-*O*-acetylate the 2-hydroxyl functionality),[13] gave upon TFA mediated cleavage a species with $[M + H]^+$ at 633 Da. This corresponds to the tripeptide Ac-Asp-(R^4)-Phe-Lys-OH. As envisaged, the sulfoxide moiety in the amide protecting group conferred stability to TFA treatment. Therefore peptide-resin **8** was treated as described above, but cleaved by addition to a cocktail of 20 eq. each of NH_4I and $(CH_3)_2S$ in TFA at O°C and left to reach r.t. over 2 h., reducing sulfoxide to sulfide[16] and consequently restoring TFA lability. Encouragingly tripeptide **9** showed substantially reduced epimerisation (Table 1).

A panacea to stop epimerisation in peptide segment activation and coupling that matches the utility of urethane protection of single amino acids has long been pursued. It maybe that the coupling conditions for a range of protected segments may be optimised for minimal epimerisation.[8] This preliminary communication suggests that another option, backbone amide substitution may now also be considered. Further work is in progress to establish its generality.

Abbreviations

Boc = t-butoxycarbonyl; BOP = benzotriazol-1-yloxytris(dimethylamino)-phosphonium hexafluorophosphate; Bu^t = t-butyl ether; OBu^t = t-butyl ester; DIC = 1,3 diisopropylcarbodiimide; DIEA = diisopropylethylamine; Fmoc = fluoren-9-ylmethoxycarbonyl; HOBt = 1-hydroxybenzotriazole; TFA = trifluoroacetic acid.

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