Identification of coupling conditions proceeding with low C-terminal epimerization during the assembly of fully protected backbone-substituted peptide segments †

Martin Quibell,‡[a] Leonard C. Packman[b] and Tony Johnson*,‡[a]

[a] *MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK*
[b] *Cambridge Centre for Molecular Recognition, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, UK*

The coupling of Fmoc-Asp(OBu^t)-L-Phe-OH 1 and H-Lys(Boc)-PepsynKA 2 was used as a model to assess C-terminal epimerization in solid-phase segment condensation. A wide range of coupling reagents and reaction conditions were examined and an optimal combination of good coupling rate along with low phenylalanine epimerization was achieved by using 1-hydroxybenzotriazole-catalysed diisopropylcarbodiimide reaction in dichloromethane. Application of these conditions in the coupling of backbone protected HIV-1$_{Bru}$ *tat* protein related segments (13-mer + resin-bound 16-mer) gave quantitative reaction within 6 h, and a maximum of < 3% epimerization of C-terminal lysine.

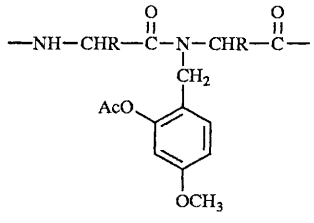
Introduction

An attractive approach towards the synthesis of peptides in excess of 100 residues is through the solid-phase assembly of smaller purified segments of approximately 10–20 residues in length.[1-4] However, this seemingly simple approach has, despite many attempts, failed to yield a *reliable* working system. Two main technical obstacles have so far hampered developments. First, fully protected peptide segments often exhibit low solubility in a variety of solvents,[1,3,5-7] leading to major difficulties during purification. Once purified, segments of low solubility and hence low concentration may give prohibitively slow reaction kinetics during coupling. Secondly, the C-terminal amino acid of a protected segment no longer contains N^{α}-urethane protection, and is thus especially prone to epimerization during activation and subsequent coupling.[8,9]

It is well recognised that segment insolubility has a structural basis, with decrease in solubility being paralleled by a transition from an unordered soluble species to an ordered inter- and intra-molecularly hydrogen-bonded insoluble species exhibiting a regular β-structure.[10,11] This hydrogen-bonded network is primarily mediated through the peptide amide backbone.[12] We have recently described a general solution to the problems posed by peptide insolubility by removing the potential for backbone hydrogen-bond formation, through the substitution of selected amides with the Fmoc/Bu^t-compatible *N*-(2-acetoxy-4-methoxybenzyl) (AcHmb) protecting group **3**.[13-17]

Using backbone amide substitution, we were readily able to purify fully protected peptide segments of up to 21 residues in length, in 100 milligram quantities, by standard reversed-phase (RP)-HPLC techniques.[18] The purified segments were extremely soluble, typically > 500 mg cm^{-3} in DCM or DMF, allowing economical and efficient solid-phase assembly of a 94-mer target sequence. Each segment was chosen to contain C-terminal glycine, so that the problem of C-terminal epimerization was not encountered. Unfortunately, this choice may not always be compatible with a desired synthetic strategy since sequences will not always contain conveniently placed glycine or proline residues.

Fmoc-Asp(OBu^t)-L-Phe-OH **1** H-Lys(Boc)-Pepsyn KA **2**



3

We have now undertaken a wide ranging study of activation and coupling conditions, in order to define a method under which fairly large segments (10–20 residues) couple efficiently (> 95%), with low C-terminal epimerization. We report here that segment activation with DIC/HOBt in DCM fulfils these criteria. In combination with soluble backbone-protected segments, this offers the first reliable and flexible solid-phase segment-assembly system.

Results

Experiments were performed to assess the effect of activation reagent, additive, isolated or *in situ* use of activated intermediates, quantity of base and reaction solvent upon the coupling and epimerization of the model reaction, Fmoc-Asp(OBu^t)-L-Phe-OH **1** + H-Lys(Boc)-Pepsyn KA **2**. A summary and brief description of procedures and results are given in Table 1.

The procedures identified from Table 1 as giving low epimerization were assessed in terms of their ability to ligate large protected segments effectively, through the coupling of fully protected 21-mer peptide **6** to 21-mer-peptide resin **7**, to give, after cleavage, the 42-mer **8** (Scheme 1). Acylation of peptide–resin **7** with DIC/HOBt-mediated activation of 2 mol equiv. of protected peptide **6** at 0 °C in DCM for 6 h (*cf.*

† Abbreviations used: dicyclohexylcarbodiimide (DCC), 4-oxo-3,4-dihydrobenzotriazin-3-yl (Dhbt), tetrahydrofuran (THF), dimethylformamide (DMF), dichloromethane (DCM), pentafluorophenol (PfpOH), (benzotriazolyloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP), 1-hydroxybenzotriazole (HOBt), diisopropylethylamine (DIEA), O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU), 7-aza-1-hydroxybenzotriazole (HOAt), isopropylchloroformate (Pr^{i}OCOCl), *N*-methylmorpholine (NMM), ethyl acetate (EtOAc), diphenylphosphinoyl chloride (Dpp-Cl), diisopropylcarbodiimide (DIC), trifluoroacetic acid (TFA), 2,2,5,7,8-pentamethylchroman-6-sulfonyl (PMC), trityl (Trt).

‡ *Present address:* Peptide Therapeutics Ltd., 321 Cambridge Science Park, Milton Road, Cambridge CB4 4WG, UK.

Table 1 Coupling and epimerization of Fmoc-Asp(OBu^t)-L-Phe-OH (2 mol equiv.) to H-Lys(Boc)-Pepsyn KA with various activation techniques

Experiment number	Activating agent/conditions	Solvent	Coupling yield (%) (by AAA ratios)	% LDL epimer
1	DCC/Dhbt	DMF	95	2
2	DCC/Dhbt	DMF/DCM/THF	97	9
3	DCC/Pfp-OH	DMF	97	16
4	BOP/HOBt/DIEA	DMF	>98	40
5	BOP/HOBt/DIEA/$CuCl_2$	DMF	90	7
6	HATU/2,4,6-collidine	DMF	90	21
7	HATU/HOAt/DIEA	DMF	>98	44
8	Pr^{i}OCOCl/NMM/Dhbt	DMF/EtOAc	75	4
9	Dpp-Cl/NMM/room temp.	DMF	85	0.5
10	Dpp-Cl/NMM/Dhbt	DMF	97	10
11	Dpp-Cl/NMM/0 °C/2 h	DMF	92	<0.1
12	Dpp-Cl/NMM/0 °C/6 h	DMF	95	<0.1
13	Dpp-Cl/NMM/1 h + Dhbt/NMM/1 h	DMF	>98	25
14	Dpp-Cl/NMM/0 °C/1 h + Dhbt/1 h	DMF	97	1.5
15	Cyanuric fluoride/pyridine (py)	DMF	95	24
16	DIC/HOAt	DMF	85	24
17	DIC/HOBt	DMF	40	19
18	DIC/HOBt	1,4-dioxane	98	3
19	DIC/HOBt	DCM	97	0.5

Standard conditions were 2 h reaction in a minimum volume of solvent at room temperature, unless otherwise stated. (1) Activation of dipeptide **1**, with equimolar amounts of DCC and Dhbt, at −5 °C in THF, isolation of the activated species by filtration and evaporation, dissolution in DMF and addition to peptide–resin **2**. (2) Activation as in (1), followed by *in situ* reaction in a 1:1:1 mixture of DCM/DMF/THF. (3) Activation of dipeptide **1**, as in (1) but with Pfp-OH, isolation and subsequent acylation of peptide–resin **2** in DMF. (4) Activation of dipeptide **1**, with equimolar amounts of BOP, HOBt and DIEA with *in situ* reaction in DMF. (5) Repeat of (4) but with the addition of mol equiv. of copper(II) chloride.[19] (6) Activation of dipeptide **1** with an equimolar amount of HATU and 2,4,6-collidine (2,4,6-trimethylpyridine) in DMF. (7) Activation and acylation with HATU, HOAt and DIEA in DMF. (8) Activation of dipeptide **1** with equimolar amounts of Pr^{i}OCOCl,[20] NMM and Dhbt in a 1:1 mixture of EtOAc/DMF. (9–14) Activation of dipeptide **1** with an equimolar amount of Dpp-Cl in DMF at 0 °C,[21] with various amounts of NMM and Dhbt: (9) Using 1 mol equiv. NMM, 2 h acylation of peptide–resin **2** at room temperature. (10) Using 1 mol equiv. NMM and 1 mol equiv. Dhbt. (11) Using 1 mol equiv. NMM, with both activation and acylation at 0 °C. (12) As in (11), but with 6 h acylation. (13) Using 1 mol equiv. NMM for 1 h, then 1 mol equiv. Dhbt and an extra 1 mol equiv. NMM for 1 h. (14) An initial reaction with 1 mol equiv. NMM at 0 °C for 1 h, followed by the addition of 1 mol equiv. Dhbt for 1 h. (15) Initial conversion of protected dipeptide **1** into the acid fluoride by use of cyanuric fluoride (2 mol equiv.) and pyridine (1 mol equiv.) in DCM.[22] Following isolation, subsequent acylation of peptide–resin **2** in DMF. (16–19) Activation of dipeptide **1** with an equimolar amount of DIC at 0 °C, with various additives and solvents: (16) Using 1 mol equiv. of HOAt in DMF, 2 h acylation of peptide–resin **2** at room temperature. (17) As in (16) but using 1 mol equiv. HOBt. (18) As in (17) but using 1,4-dioxane as reaction solvent. (19) As in (17) but using DCM as reaction solvent.

H-Asp-L-Phe-Lys-OH **4** H-Asp-D-Phe-Lys-OH **5**

Fmoc-Gly^{210}-Gln(Trt)-Val-Glu(OBu^t)-Val-Lys(Boc)-(AcHmb)Ser(Bu^t)-Glu(OBu^t)-Lys(Boc)-Leu-Asp(OBu^t)-(AcHmb)Phe-Lys(Boc)-Asp(OBu^t)-(AcHmb)Arg(Pmc)-Val-Gln(Trt)-Ser(Bu^t)-Lys(Boc)-Ile-Gly^{230}-OH **6**

+

H-Ser(Bu^t)^{231}-Leu-Asp(OBu^t)-(Hmb)Asn-Ile-Thr(Bu^t)-His(Trt)-Val-Pro-Gly-Gly-Gly-Asn(Trt)-Lys(Boc)-(Hmb)Lys(Boc)-Ile-Glu(OBu^t)-Thr(Bu^t)-His(Trt)-Lys(Boc)-Leu^{251}-R **7**

↓ i–iii

H_2N-Gly^{210}–Leu^{251}-OH **8**

Scheme 1 Fully protected peptide segments from a partial sequence of Human-2 Tau, used to assess the efficiency of an activation method in terms of large segment coupling. *Reagents and conditions:* i, coupling, room temp., 6 h; ii, 20% piperidine–DMF, 3 and 20 min; iii, 90% TFA, 5% ethane-1,2-dithiol, 3% Et_3SiH, 2% water, 8 h.

Experiment 19, Table 1), followed by reaction at room temperature gave >98% acylation. A sample of the crude 42-mer product **8** was of good quality by analytical HPLC (Fig. 1), with the expected mass from MALDITOF-MS at 4630.9 Da (required M, 4632.0 Da).

DIC/HOBt-mediated activation of a protected peptide at 0 °C in DCM was also assessed in terms of both coupling efficiency and C-terminal epimerization through the coupling of fully protected 13-mer **9**, containing C-terminal lysine, to 16-mer-peptide–resin **10**. By use of 2 mol equiv. of protected peptide **9**, >98% acylation of peptide–resin **10** was achieved by reaction at room temperature for 6 h (Scheme 2). The crude 29-mer peptide **11** was analysed by analytical HPLC and was shown to be of good quality (Fig. 2); there was a main species [Fig. 2(*a*)], with a retention time of 11.65 min (76.4%), which gave the expected mass from MALDITOF-MS at 3704.4 Da (required M, 3705.4 Da). The later eluting impurity [Fig. 2(*b*)], with a retention time of 12.24 min (23.6%), gave a mass from MALDITOF-MS at 3800.0 Da, corresponding to M + 96 Da, the N^{α}-trifluoroacetyl derivative of peptide **11** (required M, 3801.0 Da).

Chymotrypsin digest of peptide **11** (1.3 mmol; enzyme:substrate, 1:100) at 37 °C gave maximal release of hexapeptide product **13** (Scheme 3) after 1.5–2.5 h (Fig. 3). Analysis of compound **13** allowed a quantification of the extent of epimerization of the C-terminal residue of segment **9** (lysine^{28}) arising during DIC/HOBt-mediated coupling in DCM to peptide–resin **10**. HPLC comparison against separately prepared L- and D-lysine^{28} epimers of compound **13** (Fig. 4) gave a maximum value of 3% D-lysine^{28} epimer of compound **13** present in the digest sample. The value of 3% represents an estimation of epimerization based upon HPLC separation and

Fmoc-Ser(Bu^t)^{16}-Gln(Trt)-Pro-Lys(Boc)-Thr(Bu^t)-Ala-Cys(Acm)-Thr(Bu^t)-Thr(Bu^t)-Cys(Acm)-(AcHmb)Tyr(Bu^t)-Cys(Acm)-Lys(Boc)^{28}-OH **9**

+

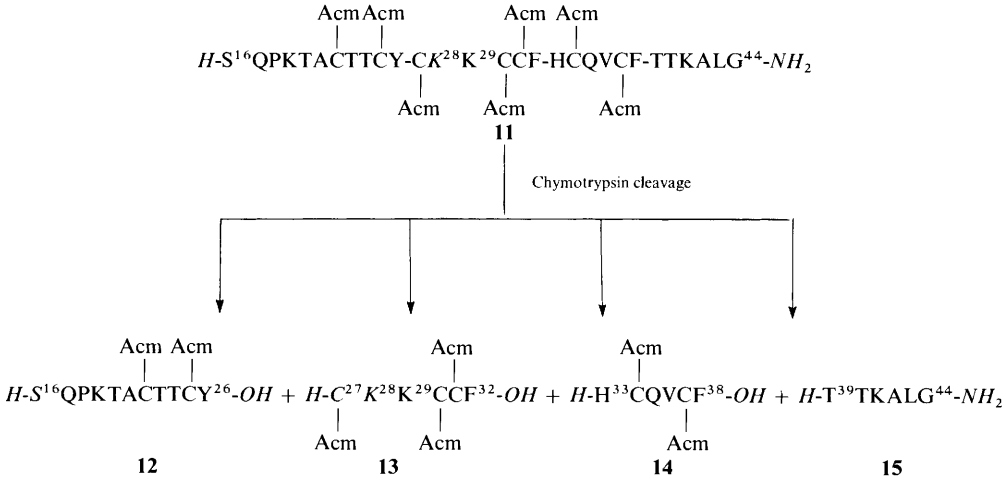
H-Lys(Boc)^{29}-Cys(Acm)-Cys(Acm)-Phe-His(Trt)-Cys(Acm)-(Hmb)Gln(Trt)-Val-Cys(Acm)-Phe-Thr(Bu^t)-Thr(Bu^t)-Lys(Boc)-(Hmb)Ala-Leu-Gly^{44}-Rink Amide Linker-Pepsyn K **10**

i–iii

AcmCys^{22,25,27,30,31,34,37}

H-Ser^{16}–Gly^{44}-NH_2 **11**

Scheme 2 Fully protected peptide segments from a partial sequence of HIV-1_{Bru} *tat* protein, used to asses the efficiency of DIC/HOBt activation in DCM, in terms of large segment coupling, and chiral C-terminal segment epimerization. *Reagents and conditions:* i, coupling, DIC/HOBt, DCM, room temp., 6 h; ii, 20% piperidine–DMF, 3 and 20 min; iii, 95% TFA, 5% Et_3SiH, 2 h.



Scheme 3 Theoretical fragmentation pattern from chymotrypsin digest of peptide **11**

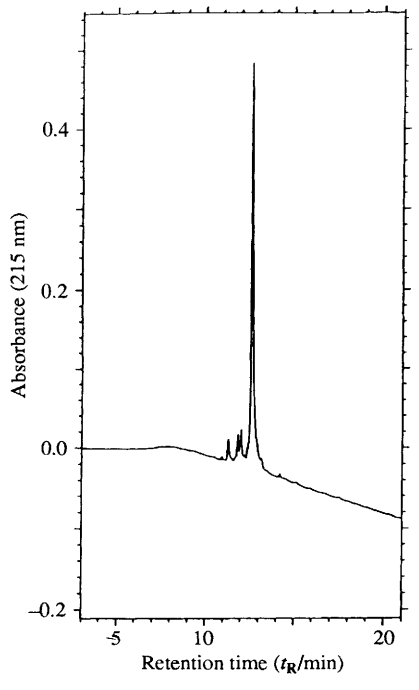


Fig. 1 Analytical HPLC of the crude 42-mer *H*-Gly^{210}–Leu^{251}-*OH* **8** from coupling of the 21-mer segment **6** onto the resin-bound 21-mer peptide-resin **7**. Coupling conditions were: 2 mol equiv. of AcHmb backbone-protected segment **6** with equimolar DIC/HOBt activation in DCM at 0 °C for 1 h, followed by 6 h acylation of **7** at room temp. HPLC conditions: Vydac C_8 (250 × 4.6 mm) column, 10–90% B in A gradient over a period of 27 min (1.5 cm^3 min^{-1} flow; 215 nm UV detection), where buffer A = 0.1% aq. TFA and buffer B = 90% acetonitrile/10% buffer A.

integration. Owing to complicating factors in the HPLC separation of the digest mixture (see Discussion section), 3% safely represents the absolute maximum limit for the D-epimer observed and the actual amount present could well be less.

Discussion

(a) Identification of optimal coupling methods

The goal of the present work was to define conditions under which peptide segments would couple efficiently with minimal C-terminal epimerization. The loss of chiral integrity of activated amino acid and peptide species can proceed through a number of possible mechanisms and has been excellently reviewed by Kemp.[23] The actual extent occurring during a specific reaction is dependent upon many factors such as the nature of the C-terminal and N-terminal residues at the site of coupling and the relative rate constants of numerous inter-related equilibria.[23] The ideal segment coupling should proceed with a rate constant for acylation by the L-carboxy-activated component that is far larger than that for epimerization and subsequent coupling of the D-carboxy component. Unfortunately, owing to the complex nature of these equilibria, no model test coupling can be used to predict exactly the extent of epimerization for any given reaction, although certain trends have been identified and are reviewed by Kovacs.[24] Here, we aimed to identify an activation procedure, from the many recently described in the literature, that is generally superior in terms of low epimerization and near quantitative coupling. In order to identify this *optimal* procedure we initially focused upon the preparation, under various conditions, of a test tripeptide H-Asp-Phe-Lys-OH through the coupling of Fmoc-Asp(OBu^t)-L-Phe-OH **1** to H-Lys(Boc)-Pepsyn KA **2**. This particular sequence was chosen after consideration of the

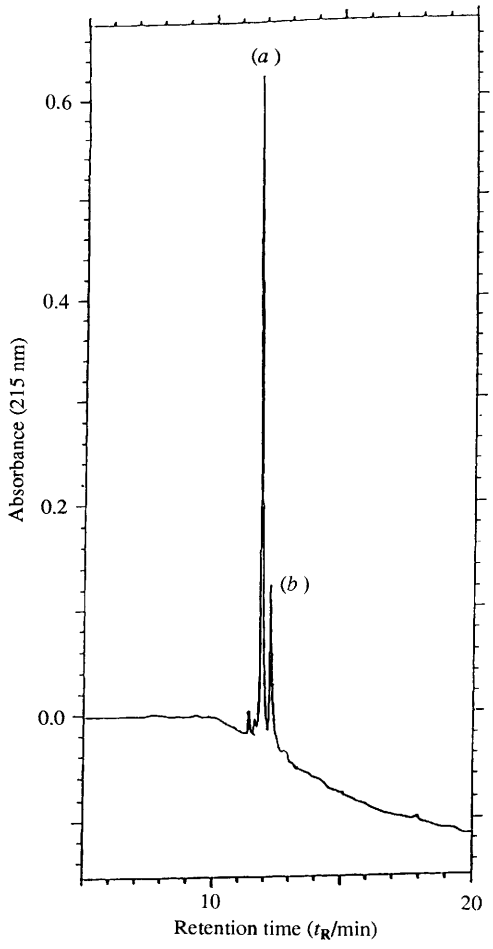


Fig. 2 Analytical HPLC of crude 29-mer *H*-Ser[16]-Gly[44]-NH_2 (AcmCys[22,25,27,30,31,34,37]) **11** from the coupling 13-mer segment **9** onto the resin-bound 16-mer peptide–resin **10**. Coupling conditions: 2 mol equiv. of AcHmb backbone-protected segment **9** with equimolar DIC/HOBt activation in DCM at 0 °C for 1 h, followed by 6 h acylation of **10** at room temp. HPLC conditions: Vydac C_8 (250 × 4.6 mm) column, 10–90% B in A gradient over a period of 27 min (1.5 cm^3 min^{-1} flow; 215 nm UV detection), where buffer A = 0.1% aq. TFA and buffer B = 90% acetonitrile/10% buffer A.

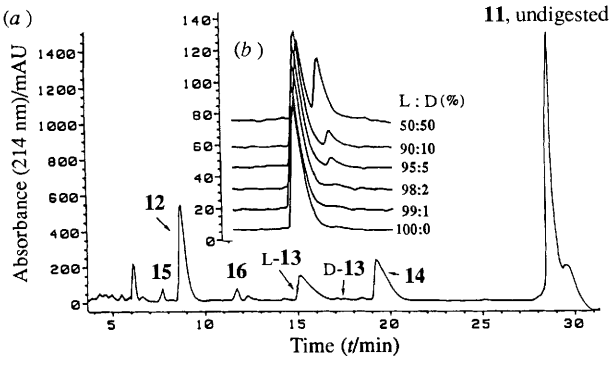


Fig. 3 (*a*) Analytical HPLC profile of the chymotrypsin digest of peptide **11**. Digestion conditions: substrate [100 mm^3; 10 mg peptide **11** cm^{-3} aq. ammonium hydrogen carbonate (100 mmol dm^{-3})]/sequence grade chymotrypsin (in 1 mmol dm^{-3} HCl), with enzyme:substrate 1:100, for 1.5 h at 37 °C. Aliquots (5 mm^3) were monitored by HPLC. HPLC condition: Hypersil C_{18}(2.1 × 100 mm) equilibrated with solvent A. Gradient 0–15% B in 2 min, 15–25% B in 23 min at 0.2 cm^3 min^{-1} and 214 nm diode array UV detection, where solvent A = 0.1% aq. TFA and B = acetonitrile/0.1% TFA. Peak identification: Each HPLC digest peak was analysed by MALDITOF-MS (see Experimental section for conditions) and peaks are labelled **11**–**15** according to Scheme 3. Peak **16** was identified as *H*-KC(Acm)C(Acm)F-*OH* from secondary digestion of the desired hexapeptide **13**. (*b*) Inset. HPLC (conditions and gradient as above) of co-injections of standard mixtures of epimeric hexapeptides *H*-Cys(Acm)-L/D-Lys-Lys-Cys(Acm)-Cys(Acm)-Phe-*OH*.

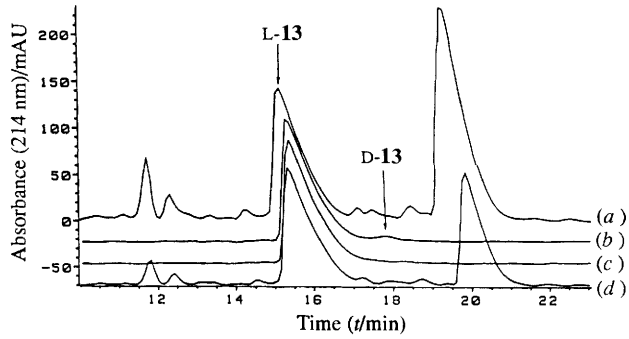


Fig. 4 Expanded HPLC from the chymotrypsin digest of peptide **11**. Conditions and gradient as for Fig. 3. (*a*) Digest trace. (*b*) Co-injection of 98% L and 2% D epimeric hexapeptide standards, showing good baseline separation. (*c*) Injection of the all-L-hexapeptide standard. (*d*) Co-injection of equal quantities of target peptide in digest aliquot and all-L-hexapeptide standard.

review of Kovacs[24] describing the prediction of epimerization and coupling rates for the 2 + 1 segment coupling of protected dipeptides containing various C-terminal amino acid residues. C-Terminal phenylalanine should offer a stringent test, being readily susceptible to epimerization, and therefore should identify reagents and conditions that will generally perform well in many different couplings.

The basic experimental method involved activation of the protected dipeptide Fmoc-Asp(OBu[t])-L-Phe-OH (2 mol equiv.), isolation of the activated intermediate (if desired) or *in situ* acylation (2 h), at room temperature, to H-Lys(Boc)-Pepsyn KA (1 mol equiv.). After coupling, a sample of the peptide–resin was analysed for amino acid ratios, giving a measurement of the extent of coupling achieved. After removal of the Fmoc group and TFA-mediated peptide–resin cleavage, the resulting two epimeric tripeptides were separated by RP-HPLC.[25] A comparison against the standard tripeptides H-Asp-L-Phe-Lys-OH **4** and H-Asp-D-Phe-Lys-OH **5** gave a good quantification of the extent of phenylalanine epimerization occurring with the various coupling procedures.

The most promising results were obtained with carbodiimide- or diphenylphosphinoyl chloride-based couplings (Table 1). The Dpp-Cl-mediated reactions 9, 11 and 12 highlighted the need for an additive during these couplings to facilitate near quantitative reaction. The quantity of base added along with Dhbt during the *in situ* reactions 10, 13 and 14 had a marked effect upon the extent of epimerization, the addition of one extra mole equivalent of base, as in Experiment 13, leading to a substantial increase. Surprisingly, Experiments 6 and 7, using the HATU/HOAt-mediated uronium salt couplings, gave poor results. These reagents have performed well in alternative tripeptide systems,[26,27] indicating that tripeptide model **3** probably offers a particularly stringent epimerization test. Experimentally, the most useful effect observed was the substantial reduction in epimerization during activation by DIC in the presence of HOBt, through the use DCM as reaction solvent. This system has rarely been considered for segment assembly due to the very poor solubility of the majority of fully protected segments in DCM. However, with the use of backbone protection, this restriction is effectively eliminated.[17,18]

(b) Efficient ligation of large protected segments

An effective segment assembly coupling method requires the efficient ligation of the protected segments together with minimal C-terminal epimerization. The preliminary results from Table 1 identified two procedures, Experiments 12 and 19, that potentially fulfil these requirements. We have recently described the coupling of fully protected 21-mer **6** to 21-mer-peptide resin **7**, to give after cleavage, 42-mer **8** (Scheme 1) during the solid-phase segment assembly of Asp[158]–Leu[251], a

94-mer from Human-2 Tau protein.[18] Here, since protected segment **6** contained a C-terminal glycine residue, coupling was mediated through BOP reagent in DMF solvent. Acylation was >98% complete in 6 h when only 2 mol equiv. of activated segment **6** was used. These conditions and results were used as a basis for comparing reactions protocols identified from Table 1 (giving low epimerization) in terms of coupling ability.

Acylation of peptide–resin **7** was performed by using DPP-Cl-mediated activation of protected peptide **6** in DMF (*cf.* Experiment 11, Table 1), followed by reaction at room temperature. Quantitative UV analysis and amino acid ratios indicated reaction to be <50%. A repeat with the addition of 1 mol equiv. of Dhbt, as in Experiment 14, Table 1, improved the reaction to >75% completion. However, no reaction conditions were found when using DPP-Cl-mediated activation of compound **6** (*e.g.*, extended reaction time), that achieved >80% acylation of compound **7**. The DPP-Cl-activation method, identified as superior in terms of minimal epimerization, appeared to give unsatisfactory reaction rates in the coupling of large segments.

Acylation of peptide–resin **7** was also repeated by using DIC/HOBt-mediated activation of protected peptide **6** in DCM (*cf.* Experiment 19, Table 1), followed by reaction at room temperature for 6 h. From quantitative UV analysis and amino acid ratios, the reaction was found to be >98% complete, to give the 42-mer product **8** of good quality (Fig. 1) with the expected mass. This activation technique efficiently couples large segments, and from the results detailed in Table 1 appears potentially attractive with reasonably low epimerization.

(c) Performance of optimal coupling procedures: C-terminal epimerization assessment

The optimal conditions involving DIC/HOBt-mediated activation of a protected peptide at 0 °C in DCM were also assessed in terms of both coupling efficiency and C-terminal epimerization through the coupling of fully protected 13-mer **9** to 16-mer-peptide resin **10**. Subsequent cleavage gave 29-mer **11**, a partial sequence from HIV-1_{Bru} *tat* protein[28] (Scheme 2).

By use of 2 mol equiv. of protected peptide **9**, >98% coupling to peptide–resin **10** was achieved by reaction for 6 h at room temperature, based upon quantitative UV analysis and amino acid ratios. The crude 29-mer **11** was of good quality (Fig. 2) with the expected mass. The later eluting impurity [Fig. 2(*b*)] is probably N-terminal trifluoroacetyl derivative of peptide **11** (required M, 3801.0 Da), a recently described side-reaction arising during the TFA-mediated cleavage of N-terminal serine peptides.[29]

It having been established that coupling of segment **9** to resin-bound segment **10** using DIC/HOBt activation and acylation in DCM proceeds in near quantitative fashion, the extent (if any) of epimerization of the C-terminal residue of segment **9**, lysine[28], had to be determined. Digestion of peptide **11** with chymotrypsin should induce chain fragmentation C-terminal of the aromatic residues phenylalanine and tyrosine.[30] Theoretically, the digestion pattern detailed in Scheme 3 should be obtained, to release compound **13**, a hexapeptide containing the potentially epimerized residue lysine[28].

H-Lys-Cys(Acm)-Cys(Acm)-Phe-*OH*
16

HPLC analysis of an optimized chymotrypsin digestion of **11** is shown in Fig. 3(*a*). A time–course study of the digestion showed that maximal release of target peptide *H*-Cys(Acm)-Lys-Lys-Cys(Acm)-Cys(Acm)-Phe-*OH* occurred after 1.5–2.5 h; beyond this point further degradation occurred, possibly by low-level tryptic activity in the commercial enzyme preparation; indeed, the peptide *H*-Lys-Cys(Acm)-Cys(Acm)-Phe-*OH* **16** was observed at 2.5 h and beyond. Each peak from the HPLC digest profile was analysed by MALDITOF-MS, and assigned accordingly. Samples of standard peptides *H*-Cys(Acm)-L/D-Lys-Lys-Cys(Acm)-Cys(Acm)-Phe-*OH*, known to separate well under the chromatographic conditions employed (see Fig. 3 legend), were injected to establish the retention times of each epimer. The relative peak sizes in an equimolar mixture and in various mixtures containing 0–10% of the D-epimer gave good baseline separation, down to 1% of D in the presence of L peptide [Fig. 3(*b*)].

The digest profile and the resolution of a similar amount of the standard mixtures of 0–2% of the D-peptide in the presence of the L-peptide are shown in Fig. 4. The L-standard and the target peak **13** from the digest differ slightly (but reproducibly) in retention time such that the expected position of the D-peptide falls between two small peaks in the digest profile [Fig. 4(*a*)]. It is therefore unclear as to which peak, if any, is the D form; the closest peak is at t_R 17.5 min and would represent 2–3% D-peptide in the digest sample. Co-injection of equal amounts of the target peptide (in the digest) and the L-standard [Fig. 4(*d*)] shows that under these conditions the standard and target now co-elute exactly and the baseline profile now becomes more uniform. Clearly there is a subtle influence of the composition of the digest mixture on the retention time of the target peptide **13**. A small peak in the digest mixture now corresponds well with the expected elution position of the D-peptide. By using the peak at t_R 19–20 min as an internal standard, the exact contributions to the area under the target peak by endogenous and added L-peptide are readily quantified and verified as being equal; the quantity of the D-peptide in the digest is calculated to be 2–3% once more.

Conclusions

An optimal combination of good protected peptide coupling rate along with reasonably low C-terminal epimerization was observed using HOBt-catalysed DIC activation and subsequent coupling in DCM. The activation and subsequent coupling of a C-terminal lysine fully protected peptide segment (13-mer) using these conditions proceeded to completion with a maximum of 3% lysine epimerization. *N*-(2-Acetoxy-4-methoxybenzyl) (AcHmb) backbone-amide-substituted fully protected segments exhibit excellent solubility in DCM, allowing coupling in this solvent when required. In contrast, the solubility of non-backbone-protected segments in DCM is unpredictable and often poor. This coupling procedure does not constitute an absolute solution to the problem of epimerization, but does in our hands appear to be the superior procedure of those currently available, and may well perform adequately in many segment couplings. In combination with our techniques for the easy preparation and purification of soluble backbone-protected segments (~10–20 residues in length), a reliable and flexible solid-phase segment-assembly system towards the preparation of small proteins is now available.

Experimental

(i) General

Continuous-flow Fmoc-polyamide methods reviewed by Atherton and Sheppard[31] were used exclusively. Fmoc amino acid pentafluorophenyl-activated esters (Novabiochem, UK) were used exclusively except for Ser(OBu^t) coupled as the dihydrooxobenzotriazine ester, and Arg(Pmc)/His(Trt) (Novabiochem, UK) which were coupled using PyBOP/HOBt/DIEA. Fmoc *N*-carboxyanhydrides (Propeptide, Vert Le Petit, France), used for coupling Asp(OBu^t) and Lys(Boc) residues to Hmb-protected amino acids, were commercially available. PolyHipe SU 500 resin§[32] (Phase Separations Ltd, Deeside, CH2 2NU, UK) was commercially available. PepSyn KR and PepSyn KA resins (Novabiochem, UK) was commercially

available. *N,O*-BisFmoc-*N*-(2-hydroxy-4-methoxybenzyl) amino acid pentafluorophenyl esters [33] were prepared in house as previously detailed. All solvents were purified as previously described.[31]

Solid-phase peptide synthesis was performed on an LKB 'Biolynx' 4170 automated synthesizer programmed to perform acylation reactions (in DMF) for 45 min, and Fmoc-deprotection reactions (in 20% piperidine/DMF v/v) for 10 min. All chiral amino acids used were of the L-configuration. Amino acid side-chain protection was as follows: lysine (N^{ε}-*tert*-butoxycarbonyl, Boc), serine and threonine (*tert*-butyl ether, Bu^t), aspartic and glutamic acid (*tert*-butyl ether, OBu^t), glutamine and asparagine (Trt), histidine (N^{im}-trityl, Trt), tyrosine (*tert*-butyl ether, Bu^t), arginine (N^G Pmc), cysteine (acetamidomethyl, Acm). Peptide hydrolyses were performed at 110 °C for 24 h in 6 mol dm^{-3} HCl containing a trace of phenol, in evacuated, sealed tubes. Analysis of hydrolysis products was performed on a Beckman 7300 analyser. Separation was obtained using ion-exchange resin with manufacturer's buffer solutions and post-column separation detection by ninhydrin. Amino acid sequencing was performed on an Applied Biosystems 470A protein sequencer, using phenylthiohydantoin (PTH) chemistry. Analytical HPLC was performed on a Brownlee Aquapore RP300 C_8 column (250 × 4.6 mm), using a 10–90% B in A gradient over a period of 25 min (1.5 cm^3 min^{-1}; 215 nm UV) unless otherwise stated, where A = 0.1% aq. TFA and B = 90% acetonitrile/10% A. Preparative HPLC was performed on a Vydac 219TP1022, diphenyl column (25 × 2.5 cm) at 10 cm^3 min^{-1} flow rate and 215 nm UV detection, solvents A and B as above. MALDITOF-MS were obtained on a Kratos MALDI III bench-top linear/reflectron mass spectrometer, operated in the linear mode with internal and external calibration by bovine insulin. α-Cyano-4-hydroxycinnamic acid matrix was deposited as a thin film from a saturated solution in acetone (0.5 mm^3) containing 4% aq. 0.1% TFA. Peptide solution was applied to the dry film and allowed to evaporate. The sample was washed with water and redried before analysis. Peptides larger than ~3000 Da were analysed using sinapinic acid matrix. The peptide sample (0.5 mm^3) was mixed with an equal volume of 10 mg cm^{-3} sinapinic acid in 50% aq. acetonitrile containing 0.1% TFA, and allowed to dry. The sample was washed with water (5 mm^3) and redried before analysis.

(iii) Preparation of fully protected segments

Fully protected peptides **1**, **6**, **9** and **10** were prepared on PolyHipe SU 500 resin,[32] through a 2-chlorotrityl linkage of the C-terminal amino acid. All couplings were performed under standard conditions,[31] except for the incorporation and subsequent acylation of Hmb-protected residues performed as previously described.[33] Fully protected peptide–resins were treated with 0.75% TFA/DCM for 45 min at room temperature. The resin was filtered off, washed with DCM (2 × 25 cm^3), and the combined organics were treated with NMM (equal volume to TFA used). The organics were then washed with saturated aq. NaCl (2 × 750 cm^3), dried (Na_2SO_4), and removed under reduced pressure to give the crude fully protected segment. The cleaved peptide–resin was washed and dried in the usual manner, and a sample was analysed for quantitative UV absorbance at 290 nm, to give >90% cleavage in all cases.

(iii) Purification of fully protected segments

The crude segment (100–500 mg) was dissolved in trifluoroethanol (TFE)/water (3:1, v/v), and purified by Vydac diphenyl preparative HPLC using a high % acetonitrile/0.1% aq. TFA gradient. Fractions were pooled, diluted with water (approximately half the pooled volume) and neutralized with 0.01 mol dm^{-3} aq. Na_2CO_3 to pH 7 (by wide range indicator paper). The neutralized solution was freeze-dried, and the lyophilised product was dissolved in chloroform (100 cm^3), washed with saturated aq. NaCl (3 × 500 cm^3), dried (Na_2SO_4), and the solution was evaporated under reduced pressure. The residue was dissolved in TFE (15 cm^3), the solution was transferred to a 50 cm^3 falcon tube, reduced to ~2 cm^3, diluted to 15 cm^3 with distilled water, and lyophilised to give the purified segment.

(iv) Segment coupling of Fmoc-Asp-L-Phe-*OH* and *H*-Lys(Boc)-KA

(a) Preparation of *H*-Asp-D/L-Phe-Lys-*OH* tripeptide standards (4, 5). Prepared from 0.1 mmol g^{-1} Fmoc-Lys(Boc)-KA (500 mg) under standard conditions.[31] Tripeptide–resins cleaved with TFA/triethylsilane (95:5, v/v, 5 cm^3) for 90 min. Crude peptides **4** and **5** were isolated by filtration, N_2 sparge, and ethereal precipitation (the 'standard method'). Peptides were dissolved in 0.1% aq. TFA and analysed by analytical HPLC to give: *H*-Asp-L-Phe-Lys-*OH*, t_R = 3.58 min. *H*-Asp-D-Phe-Lys-*OH*, t_R = 4.42 min.

(b) Assessment of activation techniques. Protected dipeptide **2** (5.59 mg, 10 μmol) was activated with the appropriate reagent (see Table 1), then *H*-Lys(Boc)-KA (50 mg, 5 μmol) was added. After completion of the appropriate reaction time, the coupled peptide–resin was filtered off, washed with DMF, and then treated with 20% piperidine–DMF for 3 and 7 min. The peptide–resin was then washed (DMF, then diethyl ether) and dried *in vacuo* over KOH pellets. A sample of the coupled resin (approximately 2 mg) was hydrolysed and analysed for amino acid ratios. The remaining coupled resin was cleaved and worked up as detailed in **iv (a)** above, and analysed by analytical HPLC.

(v) Preparation of Human-2 Tau *H*-Gly^{210}–Leu^{251}-*OH* 8

Fully protected peptide **6** (M = 4637.5 Da; 9.3 mg, 2 μmol) and HOBt (0.31 mg, 2 μmol) were dissolved in DCM (60 mm^3) and the solution was cooled in ice. DIC (0.25 mg, 10 mm^3 of a stock solution of 25.2 mg cm^{-3} DCM, 2 μmol) was added and the mixture was left at 0 °C for 1 h. Peptide–resin **7**[18] (20 mg, 1 μmol) was then added, and the reaction was continued at room temperature for 6 h. The coupled resin was then filtered off, washed successively with DMF and then diethyl ether, and dried *in vacuo*. The coupled resin was treated with 20% piperidine in DMF for 3 and 7 min and again washed and dried. An aliquot (2 mg) was analysed for amino acid proportions to give: Asp/Asn (5) 5.21, Thr (2) 2.13, Ser (3) 2.88, Glu/Gln (5) 5.27, Pro (1) 0.98, Gly (5) 5.26, Val (4) 3.93, Ile (3) 2.72, Leu (3) 3.14, Nle (1) 1.24, Phe (1) 1.00, β-Ala (1) 1.06, His (2) 1.94, Lys (7) 7.30, Arg (1) 0.98.

The remaining deprotected peptide–resin was cleaved with TFA/ethane-1,2-dithiol/triethylsilane/water (90:5:3:2, v/v/v/v; 1 cm^3) and worked up as detailed in **iv (a)** above. Analytical HPLC (Fig. 1) t_R = 12.72 min (85.7%), with observed M = 4630.9 Da; H-Gly^{210}–Leu^{251}-OH required M, 4632.0 Da.

(vi) Preparation of HIV-1$_{Bru}$ *tat* *H*-Ser^{16}–Gly^{44}-*OH* (AcmCys^{22,25,27,30,31,34,37}) 11

Fully protected peptide **10**, Fmoc-Lys(Boc)^{29}–Gly^{44}-OH (M = 3448.2; 10.4 mg, 3.03 μmol), BOP (1.34 mg, 3.03 μmol), HOBt (0.46 mg, 3.03 μmol) and DIEA (0.39 mg, 3.03 μmol) were dissolved in DMF (140 mm^3) and the solution was left for 5 min. NovaSyn KR (25 mg, 2.75 μmol, assuming 0.1 mmol g^{-1}) was added, and the reaction mixture was left overnight. The coupled resin was then filtered off, washed successively with DMF and then diethyl ether, and dried *in vacuo* over KOH pellets (28.9 mg). The coupled resin was treated with acetic anhydride (2.8 mg, 10 mol equiv.) in DMF for 2 h, then was washed and

§ A Kieselguhr-supported polystyrene polymer impregnated with a polyamide gel, containing ~0.5 mmol reactive methylsarcosine ester g^{-1} (see ref. 32).

dried over KOH pellets. The coupled and capped resin was then treated with 20% piperidine in DMF for 3 and 7 min, and again was washed and dried. A sample (2 mg) was analysed for amino acid proportions to give: Thr (2) 1.89, Glu/Gln (1) 1.09, Gly (1) 1.09, Ala (1) 1.04, Val (1) 0.95, Leu (1) 0.96, Nle (1) 2.13, Phe (2) 2.00, His (1) 0.90, Lys (2) 2.10.

Deprotected peptide–resin (2 mg) was cleaved with TFA/triethylsilane (95:5, v/v; 1 cm^3) for 2 h and worked-up as detailed in (iv)(a) above. Analytical HPLC gave t_R = 12.29 min (89.7%), with observed M = 2073.4 Da; *H*-Lys^{29}–Gly^{44}-*OH* (AcmCys^{30,31,34,37}) required M = 2073.5 Da.

Fully protected peptide **9** (M = 2657.2; 7.45 mg, 2.8 μmol) and HOBt (0.43 mg, 2.8 μmol) were dissolved in DCM (100 mm^3) and the solution was cooled in ice. DIC (0.35 mg, 10 mm^3 of a stock solution of 35.3 mg cm^{-3} DCM, 2.8 μmol) was added and the mixture was left at 0 °C for 1 h. Free amino terminal peptide–resin **10** (25 mg, 1.4 μmol) was then added, and the reaction was allowed to continue at room temperature for 6 h. The coupled resin was then filtered off, washed successively with DMF and diethyl ether, and dried *in vacuo*. The coupled resin was treated with 20% piperidine in DMF for 3 and 7 min, and again washed and dried. A sample (2 mg) was analysed for amino acid proportions to give: Thr (5) 4.63, Ser (1) 0.88, Glu/Gln (2) 2.23, Gly (1) 1.00, Ala (2) 2.00, Val (1) 0.94, Leu (1) 0.93, Nle (1) 2.09, Tyr (1) 1.00, Phe (2) 1.86, His (1) 0.92, Lys (4) 3.90.

The remaining deprotected peptide–resin was cleaved with TFA/triethylsilane (95:5, v/v; 1 cm^3) and worked up as detailed in (iv)(a) above. Analytical HPLC (Fig. 2) t_R = 11.65 min (76.4%), with observed M, 3704.4 Da; *H*-Ser^{16}–Gly^{44}-*OH* (AcmCys^{22,25,27,30,31,34,37}) required M, 3705.5 Da; second component Da; t_R = 12.24 min (23.6%), with observed M, 3800.0 Da.

(vii) Chymotrypsin digest of peptide 11

Peptide substrate **11** was dissolved in freshly made aq. ammonium hydrogen carbonate (100 cm^3; 100 mmol dm^{-3}) at a concentration of ~ 10 mg cm^{-3}. Not all the peptide was soluble and an even suspension was obtained upon sonication. Sequence-grade chymotrypsin (Boehringer) was dissolved in 1 mmol dm^{-3} HCl and added to the peptide at an enzyme : substrate ratio of 1 : 100. Digestion proceeded at 37 °C for 2.5 h, after which time a second addition of enzyme was made. The time course of the digest was monitored by removing aliquots (5 cm^3) at timed intervals. Each aliquot was acidified with 10% aq. acetic acid (10 cm^3) and monitored by HPLC on Hypersil C_{18} (2.1 × 100 mm) pre-equilibrated with 0.1% aq. TFA (solvent A). The peptides were eluted with a gradient of 0–15% B in 2 min, then 15–25% B in 23 min at 0.2 cm^3 min^{-1}, where solvent B = acetonitrile/0.1% TFA. The eluent was monitored on a Hewlett Packard 1090 liquid chromatography system with 214 nm diode array UV detection. Each HPLC digest peak was collected, and identified by MALDITOF-MS.

References

1 E. Atherton, E. Brown, G. Priestly, R. C. Sheppard and B. J. Williams, in *Peptides: Synthesis, Structure, Function*. Proceedings of the 7th American Peptide Symposium, ed. D. H. Rich and E. Gross, Pierce Chemical Company, Rockford, Illinois, 1981, pp. 163–175.
2 H. Benz, *Synthesis*, 1994, 337.
3 P. Lloyd-Williams, F. Albericio and E. Giralt, *Tetrahedron*, 1993, **49**, 11 065.
4 B. Riniker, A. Florsheimer, H. Fretz, P. Sieber and B. Kamber, *Tetrahedron*, 1993, **49**, 9307.
5 K. Barlos, D. Gatos and W. Schafer, *Angew. Chem., Int. Ed. Engl.*, 1991, **30**, 590.
6 H. Yajima and N. Fujii, *J. Chem. Soc., Perkin Trans. 1*, 1981, 789.
7 E. Giralt, F. Albericio, I. Dalcol, G. Margarida, P. Lloyd-Williams and F. Rabanal, in *Innovations and Perspectives in Solid Phase Synthesis*, Third International Symposium, ed. R. Epton, Mayflower Worldwide Limited, Birmingham, England, 1994, pp. 51–60.
8 N. L. Benoiton, in *The Peptides: Analysis, Synthesis, Biology*, ed. E. Gross and J. Meienhofer, Acedemic Press, 1983, vol. 5, p. 217.
9 N. L. Benoiton and K. Kuroda, *Int. J. Pept. Protein Res.*, 1981, **17**, 197.
10 C. Toniolo, G. M. Bonora and W. M. M. Schaaper, *Int. J. Pept. Protein Res.*, 1984, **23**, 389.
11 C. Toniolo, G. M. Bonora, E. P. Heimer and A. M. Felix, *Int. J. Pept. Protein Res.*, 1987, **30**, 232.
12 R. C. de L. Milton, S. F. C. Milton and P. A. Adams, *J. Am. Chem. Soc.*, 1990, **112**, 6039.
13 C. H. Hyde, T. Johnson, D. Owen, M. Quibell and R. C. Sheppard, in *Innovations and Perspectives in Solid Phase Synthesis*, Third International Symposium, ed. R. Epton, Mayflower Worldwide Limited, Birmingham, England, 1994, pp. 29–38.
14 T. Johnson, M. Quibell, D. Owen and R. C. Sheppard, *J. Chem. Soc., Chem. Commun.*, 1993, 369.
15 C. Hyde, T. Johnson, D. Owen, M. Quibell and R. C. Sheppard, *Int. J. Pept. Protein Res.*, 1994, **43**, 431.
16 M. Quibell, T. Johnson and W. G. Turnell, *Biomed. Pept., Proteins Nucleic Acids*, 1995, **1**, 3.
17 M. Quibell and T. Johnson, in *Peptides 1994*, Proceedings of the 23rd European Peptide Symposium, ed. H. L. S. Maia, ESCOM Science Publishers B. V., Leiden, Netherlands, 1995, pp. 173–174.
18 M. Quibell, L. C. Packman and T. Johnson, *J. Am. Chem. Soc.*, 1995, **117**, 11 656.
19 A. C. Haver and D. D. Smith, in *Peptides 1994*, Proceedings of the 23rd European Peptide Symposium, ed. H. L. S. Maia, ESCOM Science Publishers B.V., Leiden, Netherlands, 1995, pp. 199–200.
20 N. L. Benoiton, Y. C. Lee and F. M. F. Chen, in *Peptides 1994*, Proceedings of the 23rd European Peptide Symposium, ed. H. L. S. Maia, ESCOM Science Publishers B.V., Leiden, Netherlands, 1995, pp. 203–204.
21 I. J. Galpin, A. K. Mohammed and A. Patel, *Tetrahedron*, 1988, **44**, 1685.
22 L. A. Carpino, D. Sadat-Aalaee, H. G. Chao and R. H. DeSelms, *J. Am. Chem. Soc.*, 1990, **112**, 9561.
23 D. S. Kemp, in *The Peptides: Analysis, Synthesis, Biology*, ed. E. Gross and J. Meienhofer, Academic Press, vol. 1, 1979, pp. 315–383.
24 J. Kovacs, in *The Peptides: Analysis, Synthesis, Biology*, ed. E. Gross and J. Meienhofer, Academic Press, 1980, vol. 2, pp. 485–539.
25 T. Miyazawa, T. Otomatsu, T. Yamada and S. Kuwata, *Int. J. Pept. Protein Res.*, 1992, **39**, 229.
26 L. A. Carpino and A. El-Faham, *J. Org. Chem.*, 1993, **59**, 695.
27 L. A. Carpino, A. El-Faham and F. Albericio, *Tetrahedron Lett.*, 1994, **35**, 2279.
28 M. J. Churcher, C. Lamont, F. Hamy, C. Dingwall, S. M. Green, A. D. Lowe, J. G. Butler, M. J. Gait and J. Karn, *J. Mol. Biol.*, 1993, **230**, 90.
29 G. Hubener, W. Gohring, H.-J. Musiol and L. Moroder, *Pept. Res.*, 1992, **5**, 287.
30 A. Fersht, in *Enzyme Structure and Mechanism*, Freeman, Reading and San Francisco, 1977, p. 18.
31 E. Atherton and R. C. Sheppard, in *Solid Phase Peptide Synthesis: A Practical Approach*, Oxford University Press, Oxford, 1989, pp. 1–203.
32 D. P. Gregory, N. Bhaskar, R. C. Sheppard and S. Singleton, in *Innovations and Perspectives in Solid Phase Synthesis*, Second International Symposium, ed. R. Epton, Intercept, Andover, England, 1992, pp. 391–396.
33 T. Johnson, M. Quibell and R. C. Sheppard, *J. Pept. Sci.*, 1995, **1**, 11.

Paper 5/06751K
Received 11*th October* 1995
Accepted 18*th December* 1995