PERKIN 1

Solid-phase assembly of backbone amide-protected peptide segments: an efficient and reliable strategy for the synthesis of small proteins†

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In the preceding paper we showed that epimerization of the C-terminal residue of a fully protected peptide segment was minimized when activation (and subsequent coupling) was performed using 1-hydroxybenzotriazole-catalysed reaction with diisopropylcarbodiimide in dichloromethane (DCM). Good solubility of the segment in DCM was ensured by the incorporation of appropriately placed *N*-(2-acetoxy-4-methoxybenzyl) (AcHmb) backbone amide protection into the fully protected peptide. This low-epimerization protocol, combined with our previously described straightforward purification of AcHmb-substituted fully protected segments, provides an efficient, reliable and flexible strategy for the solid-phase assembly of small proteins. We describe here the preparation of HIV-1_{Bru} *tat* [1–72, Cys(Acm)^{22,25,27,30,31,34,37}] protein through the sequential solid-phase assembly of five backbone-protected segments (11–17 residues). Individual addition of each segment was very efficient, achieving >95% acylation using a two-fold excess with reaction for 6 h. The target 72-mer was readily purified and isolated in 38.4% overall yield.

Introduction

The solid-phase peptide synthesis principle, introduced in 1962 by Merrifield,[2] has proven to be a tremendously successful method for the rapid preparation of peptides. With finely tuned techniques,[3] the synthesis and purification of 50-residue peptides can be achieved on an almost routine basis. However, since the method is essentially a stepwise process in which single amino acids are added sequentially to a growing resin-bound peptide chain, even with optimized near-quantitative reactions at each stage, a progressive accumulation of resin-bound impurities occurs to contaminate the final product. With large target peptides, the microheterogeneous nature of these impurities being single and combination amino acid deletions provides an extremely challenging purification task.

To facilitate a general chemical synthesis of proteins, alternative approaches to the purely stepwise method have been developed. A number of chemical strategies for the ligation of fully deprotected peptides have been described giving products (occasionally in excess of 100 residues) with a native peptide bond at the ligation site.[4–6]

A potentially attractive and popular solid-phase approach to protein synthesis involves the division of a large target sequence into smaller fragments. These are prepared, purified and characterized, then sequentially combined to form the resin-bound product in the 'solid-phase segment-assembly approach'.[7–10] The essential features and benefits of the solid-phase principle are retained in that the growing sequence is anchored to a solid support and the peptide chain is again built up by the sequential addition of reagents. Here, however, the reagents are blocks of peptide segments, rigorously purified and characterized, which will intrinsically lead to a more homogeneous crude product. Deletions will now differ by at least one segment (typically 5–20 residues) and are likely to be separable chromatographically.

The solid-phase segment assembly of a number of impressive targets have recently been described;[11–13] however, the general progression of this attractive approach has been hampered by two major obstacles. First, fully protected segments may exhibit prohibitively low solubility in virtually all solvents useful for peptide synthesis.[7,14–16] This intrinsic property places severe difficulties upon the purification and characterization of protected segments, and can often preclude useful reaction kinetics in subsequent coupling reactions. Secondly, the C-terminal residue of the protected segment no longer contains N^{α}-urethane protection, often leading to unacceptable epimerization of the C^{α} chiral centre upon activation and coupling.[17,18] Thus, protected segments are often chosen to contain a C-terminal glycine (C^{α} non-chiral) or proline (negligible epimerization) residue, a constraint that is not always compatible with the desired synthetic strategy. The former problem, leading to poor coupling kinetics, compounds the first-order epimerization of chiral C-terminal segments.

We have recently described a solution to the general problem of low fully protected peptide solubility[19,20,21] by removing the potential for backbone hydrogen-bond formation (the major contributing factor leading to low solubility),[22] through the substitution of selected amides with the Fmoc/Bu^t-compatible[23] *N*-(2-acetoxy-4-methoxybenzyl) (AcHmb)[19,24] protecting group. The incorporation of AcHmb backbone amide protection gave a dramatic improvement in the protected segment's solubility, providing simple and rapid purification along with excellent coupling kinetics.[19,20] These improved

† Abbreviations for amino acids and nomenclature of peptide structures follow the recommendations given in ref. 1. All chiral amino acids were of the L-configuration. Other abbreviations are as follows: Acm = acetamidomethyl; Ac_2O = acetic anhydride; HMPAA = 4-(hydroxymethyl)phenoxyacetic acid; Boc = *tert*-butoxycarbonyl; BOP = benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate; Bu^t = *tert*-butyl ether; OBu^t = *tert*-butyl ester; DMF = *N,N*-dimethylformamide; DCM = dichloromethane; DIC = diisopropylcarbodiimide; DIEA = diisopropylethylamine; EDT = ethane-1,2-dithiol; Fmoc = fluoren-9-ylmethoxycarbonyl; HOBt = 1-hydroxybenzotriazole; HPLC = high-pressure liquid chromatography; MALDITOF-MS = matrix-assisted laser desorption time-of-flight mass spectrometry; Pfp = pentafluorophenyl; Pmc = 2,2,5,7,8-pentamethylchroman-6-sulfonyl; Trt = trityl; TFA = trifluoroacetic acid; TFE = 2,2,2-trifluoroethanol; TES = triethylsilane.

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solubility characteristics additionally allow the activation and coupling of fully protected segments in DCM. We have shown that the use of this solvent along with DIC/HOBt-mediated activation provides an excellent combination of good protected segment coupling kinetics along with low epimerization of the segment C^{α} chiral centre.[25] The combination of these two findings provides a reliable platform from which the true potential of solid-phase segment assembly may be realized.

Here we demonstrate the potential of our solid-phase segment-assembly protocols with the preparation of HIV-1_{Bru} *tat* [1–72, Cys(Acm)^{22,25,27,30,31,34,37}] protein **1**.[26] The assembly proceeded efficiently providing a total crude product of exceptional quality, purified and isolated in 38.4% overall yield.

H-Met^{1}-Glu-Pro-Val-Asp-Pro-Arg-Leu-Glu-Pro^{10}-Trp-Lys-His-Pro-Gly-Ser-Gln-Pro-Lys-Thr^{20}-Ala-Cys(Acm)-Thr-Thr-Cys(Acm)-Tyr-Cys(Acm)-Lys-Lys-Cys(Acm)^{30}-Cys(Acm)-Phe-His-Cys(Acm)-Gln-Val-Cys(Acm)-Phe-Thr-Thr^{40}-Lys-Ala-Leu-Gly-Ile-Ser-Tyr-Gly-Arg-Lys^{50}-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Pro-Pro-Gln^{60}-Gly-Ser-Gln-Thr-His-Gln-Val-Ser-Leu-Ser^{70}-Lys-Gln^{72}-*OH*

1

Results

The target sequence HIV-1_{Bru} *tat* [1–72, Cys(Acm)^{22,25,27,30,31,34,37}] **1** was divided into five smaller segments, 11–17 residues in length (see Table 1), prepared through the hyper-acid-labile 2-chlorotrityl handle[27] on the polyamide-based resin PolyHipe SU500.§[28] Crude segments were cleaved from the peptide–resin assembly, *via* the 2-chlorotrityl handle, using a single 0.75% TFA/DCM treatment for 35 min. Crude segments were easily purified by Vydac diphenyl reversed-phase preparative HPLC using high acetonitrile/0.1% aq. TFA gradients, and a summary of the crude yield, purification gradient and purified yield for each segment is presented in Table 1.

Prior to purification of the protected segment, a sample of each crude segment was stripped of protecting groups, the quality of the base peptide assessed by C_8 reversed-phase analytical HPLC, and the main species isolated and confirmed by MALDITOF-MS mass analysis. In each case the crude base peptides contained minor deletion sequences along with the full-length base peptide. After protected segment purification the above process was repeated, with each base peptide now > 98% pure by analytical HPLC. A typical set of HPLC profiles are shown in Figs. 1 and 2, illustrating the purification of fully protected segment 2. The crude base peptide [Fig. 2(*a*)] contains a quicker eluting deletion sequence {identified by mass analysis at 1857 Da, [M − 283 Da] and probably des(Arg-Lys)}. This deletion sequence is clearly separated in the crude fully protected peptide [Fig. 1(*a*)], giving an excellent, purified, fully protected segment 2 [Fig. 1(*b*)]. The base peptide from purified segment 2 no longer contains the deletion sequence [Fig. 2(*b*)].

Segment 1 was prepared containing an extended C-terminus comprising the acid-labile 4-(hydroxymethyl)phenoxyacetic acid handle[29] and an achiral β-alanine residue. This allowed purification and reattachment of segment 1 to a solid support through activation of the achiral β-alanine residue, protecting the chiral integrity of the C-terminal L-glutamine(Trt)^{72} residue. Purified segment 1 was coupled to H-Nle-NovaSyn KD [a Kieselguhr-supported poly(dimethylacrylamide)polymer containing an ethylenediamine spacer and norleucine internal reference amino acid] by using BOP[30]-mediated activation to give a loading of 0.048 mmol peptide g^{-1} peptide–resin (see Scheme 1). This provided the base assembly for the sequential addition of purified segments 2–5.

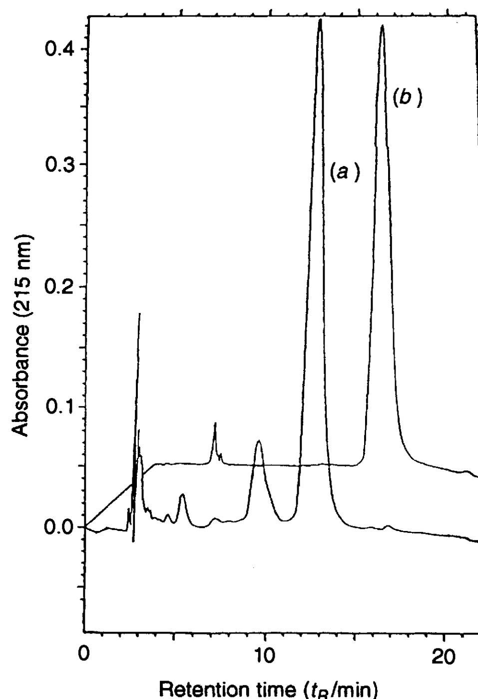

Fig. 1 Analytical HPLC of (*a*) crude and (*b*) diphenyl preparative HPLC purified, fully protected segment 2 (see Table 1). Conditions: Vydac phenyl (250 × 4.6 mm) column, 80–95% B in A gradient over a period of 27 min (1.5 cm^3 min^{-1} flow, 215 nm UV detection), where buffer A = 0.1% aq. TFA and buffer B = 90% acetonitrile/10% buffer A.

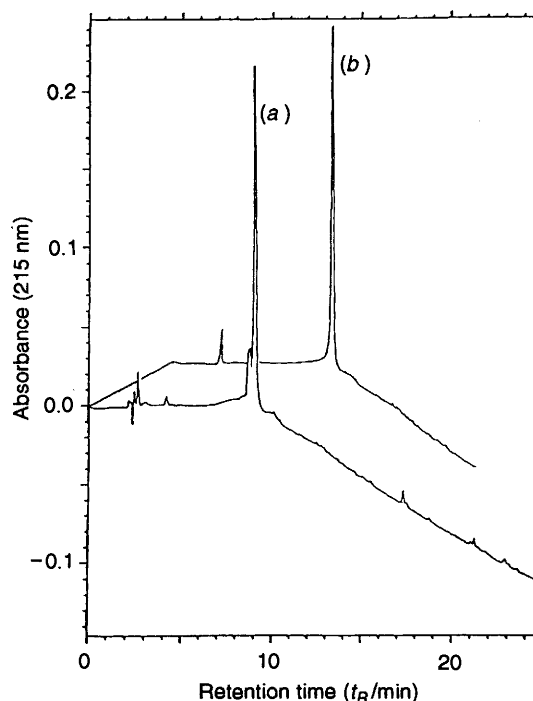

Fig. 2 Analytical HPLC of (*a*) base peptide *H*-Ile^{45}–Gly^{61}–*OH* generated from crude, fully protected segment 2 and (*b*) base peptide generated from purified, fully protected segment 2. Conditions: Vydac C_8 (250 × 4.6 mm) column, 10–90% B in A gradient over a period of 27 min (1.5 cm^3 min^{-1} flow, 215 nm UV detection), where buffer A = 0.1% aq. TFA and buffer B = 90% acetonitrile/10% buffer A.

§ A Kieselguhr-supported polystyrene polymer impregnated with a polyamide gel, containing ~0.5 mmol reactive methylsarcosine ester g^{-1} (see ref. 28).

Table 1 A summary of the crude yield, HPLC analysis, purification gradient and final isolated yields of fully protected segments spanning HIV-1$_{Bru}$ *tat*

Segment	Structure	M	Crude yield [mg (%)] HPLC (t_R/min)[a] (% main peak)	Purification gradient[b] (%)	Purified yield [mg (%)]
1	Fmoc-Ser(Bu^t)^{62}-Gln(Trt)-Thr(Bu^t)-His(Trt)-Gln(Trt)-Val-Ser(Bu^t)-(AcHmb)Leu-Ser(Bu^t)-Lys(Boc)-Gln(Trt)^{72}-Handle-βAla-OH	3170.9	500 mg (72), 34.48 (67.9)	80–95	297 (42.6)
2	Fmoc-Ile^{45}-Ser(Bu^t)-Tyr(Bu^t)-Gly-Arg(Pmc)-Lys(Boc)-(AcHmb)Lys(Boc)-Arg(Pmc)-Arg(Pmc)-Gln(Trt)-Arg(Pmc)-Arg(Pmc)-Arg(Pmc)-Pro-Pro-Gln(Trt)-Gly^{61}-OH	4934.4	1695 mg (75), 31.95 (70.3)	80–95	820 (36.5)
3	Fmoc-Lys(Boc)^{29}-Cys(Acm)-Cys(Acm)-Phe-His(Trt)-Cys(Acm)-(AcHmb)Gln(Trt)-Val-Cys(Acm)-Phe-Thr(Bu^t)-Thr(Bu^t)-Lys(Boc)-(AcHmb)Ala-Leu-Gly^{44}-OH	3448.2	600 mg (68), 29.31 (91.8)	72–88	166 (18.8)
4	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	2657.2	520 mg (89), 26.77 (89.0)	65–95	317 (54.2)
5	Fmoc-Met^1-Glu(OBu^t)-Pro-Val-Asp(OBu^t)-Pro-Arg(Pmc)-Leu-Glu(OBu^t)-Pro-Trp(Boc)-Lys(Boc)-His(Trt)-Pro-Gly^{15}-OH	2885.6	600 mg (81), 28.32 (83.4)	70–95	370 (50.0)

HPLC conditions: [a] Vydac phenyl (250 × 4.6 mm) column, 10–90% B in A gradient over a period of 27 min, then 15 min at 90% B (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. [b] Vydac diphenyl (250 × 25 mm) preparative purification. Gradient (as in Table) over a period of 25 min at 10 cm^3 min^{-1} and 215 nm UV detection. Solvents as detailed above.

Fmoc-L-Gln(Trt) — OCH_2 —C$_6$H$_4$— OCH_2 — C(=O) — β-Ala — O — C(Ph)(2-ClC$_6$H$_4$) —C$_6$H$_4$— C(=O) — Nle.EDA.SU500

90% TFA lability   0.5% TFA lability

i,ii

Fmoc-Ser^{62}(Bu^t)-Gln(Trt)-Thr(Bu^t)-His(Trt)-Gln(Trt)-Val-Ser(Bu^t)-[N-CH_2-(2-AcO-4-OCH_3-C$_6$H$_3$)]Leu-Ser(Bu^t)-Lys(Boc)-Gln^{72}(Trt)— HMPA— βAla—2-ClTrt—Nle.EDA.SU500

iii-v

Fmoc-Ser^{62}(Bu^t)-Gln(Trt)-Thr(Bu^t)-His(Trt)-Gln(Trt)-Val-Ser(Bu^t)-[N-CH_2-(2-AcO-4-OCH_3-C$_6$H$_3$)]Leu-Ser(Bu^t)-Lys(Boc)-Gln^{72}(Trt)— HMPA— βAla—Pepsyn KD

Scheme 1 Basic peptide–resin structure from which segment 1 was initially prepared, incorporating the double-handle strategy. Initial cleavage of a C-terminal elongated segment 1, through the hyperacid-labile 2-chlorotrityl handle, followed by purification and reattachment through achiral β-alanine gives the base assembly for the addition of the remaining segments. The chiral integrity of the C-terminal Gln(Trt)^{72} is protected through this scheme. *Reactions, reagents and conditions:* i, stepwise elaboration using Fmoc-AA Pfp esters to full-length segment 1; ii, Ac_2O (10 mol equiv.), DIEA (5 mol equiv.), 1 h; iii, cleavage with 0.75% TFA–DCM, room temp., 35 min; iv, Vydac diphenyl preparative HPLC purification; v, load segment 1 onto H-Nle-Pepsyn KD.

The sequential addition of segments 2, 3 and 5 (to 10 µmol of loaded *H*-Seg-1-R), each containing C-terminal glycine, was performed using a 2-fold excess (with respect to the resin-bound assembly) of the protected segment with equimolar activation using BOP/HOBt/DIEA in a minimum of DMF. Each coupling was shown to be at least 95% complete after reaction for 6 h by quantitative UV analysis of released Fmoc chromophore from a sample of the coupled peptide–resin.[19] The cumulative efficiency of the entire assembly was calculated at 84.5%. After each segment coupling, a sample of peptide–resin assembly was cleaved and the quality of the crude peptide product assessed by analytical HPLC and mass analysis (see Table 2).

Protected segment 4 containing C-terminal lysine(Boc)^{28} was coupled (2 mol equiv.) using DIC/HOBt-mediated activation in

Table 2 Data for the analysis of intermediates during the sequential assembly of purified, fully protected segments 2–5 on to *H*-Seg1-R. Coupling efficiencies were calculated by UV quantification of Fmoc release after coupling. After each coupling peptide intermediates were cleaved and characterized

Coupling reaction: protected segment + resin-bound assembly	Coupling efficiency individual (cumulative) (%)	Intermediate crude peptide for analysis	HPLC (t_R/min) (main peak %)	MALDITOF-MS observed (expected) (Da)
2 + *H*-Seg1-R	95 (95)	*H*-Ile^{45}–Gln^{72}-*OH*	9.92 (85.7)^a	3365 (3365)
3 + *H*-Seg2-1-R	94.5 (90)	*H*-Lys^{29}–Gln^{72}-*OH*, Cys(Acm)^{30,31,34,37}	19.37 (72.9)^b	5420 (5420)
4 + *H*-Seg3-2-1-R	96 (86.5)	*H*-Ser^{16}–Gln^{72}-*OH*, Cys(Acm)^{22,25,27,30,31,34,37}	19.28 (57.2)^{b,*}	7051 (7049)
5 + *H*-Seg4-3-2-1-R	98 (84.8)	*H*-Met^1–Gln^{72}-*OH*, Cys(Acm)^{22,25,27,30,31,34,37}	13.85 (71.3)^c	8819 (8818)

HPLC conditions: Vydac C_8 (250 × 4.6 mm) column, with ^{a} 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. ^{b} Vydac C_8, 10–40% B in A gradient over a period of 27 min. ^{c} 20–35% B in A gradient over a period of 27 min. * N-Terminal serine was partially N^α-trifluoroacetylated,[31] complicating HPLC analysis.

DCM. The reaction was shown to be 96% complete by quantitative UV estimation after 6 h. A direct measurement of the extent of Lys(Boc)[28] epimerization was not possible in this case. Chymotrypsin digest as previously described[25] gave overlapping species which eluted in the region of the released peptide of interest, *H*-Cys(Acm)^{27}-*Lys*^{28}-Lys-Cys(Acm)-Cys-(Acm)-Phe-*OH*, complicating a direct measurement of the relative ratios of the L- and D-Lys^{28} hexapeptide epimers. However, we have previously shown that coupling of segment 4 using DIC/HOBt in DCM to resin-bound *H*-Seg-3-R proceeds to >98% reaction in 6 h with a maximum of 3% Lys epimerization.[25] This reaction has further been shown to be 85% complete after 1 h.[32] Here, in the synthesis of the full-length protein, activated segment 4 couples to resin-bound *H*-Seg3-2-1-R at a comparable rate, being 81% complete after 1 h and 96% complete after 6 h. Since protected segment 4 has been activated in an identical manner and couples at a comparable rate in both cases, it can be inferred that the extent of epimerization (a unimolecular process steadily increasing with the time a species remains activated)[33] of Lys(Boc)[28] will be similar, at a maximum of <3%.

The full-length 72-residue assembly was cleaved with 85% TFA/10% EDT/3% triisopropylsilane/2% water for 3.5 h and the total crude peptide (82.1 mg) was shown to be of an exceptional quality for a target of this size and content of sensitive amino acid residues [Fig. 3(*a*)]. The major species was confirmed as the desired sequence *H*-Met^1-Gln^{72}-*OH*, Cys(Acm)^{22,25,27,30,31,34,37} with MALDITOF-MS at 8819 Da (theoretical M = 8818 Da), and was readily purified by C_4 preparative HPLC [Fig. 3(*b*)]. The purified protein was isolated with an overall yield of 3.54 μmol (38.4%, allowing for small-scale removals for analysis during assembly). The main quicker eluting impurity in the crude full length peptide [Fig. 3(*a*)] gave mass analysis at 8246 Da [M − 572 Da]. This is probably the des(Met^1–Asp^5) (M = 573.1) peptide from the acid-catalysed cleavage of the Asp^5-Pro^6 bond.[34] Thus, the assembly of the peptide backbone is actually better than that represented in Fig. 3(*a*), with the main impurity deriving from a post-assembly modification.

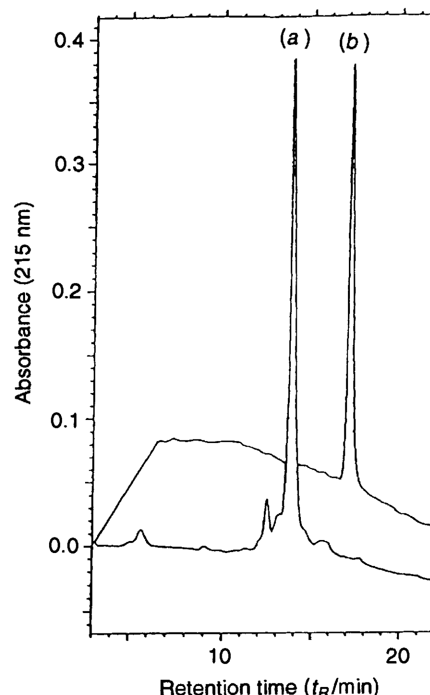

Fig. 3 Analytical HPLC of (*a*) total crude and (*b*) C_4 preparative HPLC purified HIV-1$_{Bru}$ *tat* *H*-Met^1–Gln^{72}-*OH*, Cys(Acm)^{22,25,27,30,31,34,37} prepared by the solid-phase assembly of AcHmb backbone-substituted fully protected segments 1–5 (see Table 1). Conditions: Vydac C_4 (Bu) (250 × 4.6 mm) column, 20–35% 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.

Discussion

The huge advances made in the molecular biology of protein function and interaction over the last decade necessitates the preparation of large complex structures, potentially specifically phosphorylated or glycosylated and commonly exceeding 100 amino acid residues in length. To meet these needs, on a more routine basis than can be currently achieved, requires an extension of the purely linear techniques of peptide synthesis. With this in mind we have undertaken a review of the problems associated with the potentially attractive solid-phase segment-assembly technique[7–18] and have developed a reliable and efficient convergent strategy based upon the use of backbone amide-substituted fully protected segments.[19,20,25] The potential of these improved methods for small-protein preparation are demonstrated with the synthesis of HIV-1$_{Bru}$ *tat* [1–72, Cys(Acm)^{22,25,27,30,31,34,37}] **1**. The fully deprotected *tat* protein is a potent *trans*-activator of human immunodeficiency viral gene expression and is essential for viral replication.[35] *tat* Protein interacts with the *trans*-activation-responsive RNA element (TAR)[36] and specifically activates viral transcription. Structural elucidation of the *tat*-TAR RNA complex necessitates an appreciable quantity of purified protein, and although a number of linear solid-phase preparations of this protein exist[37,38] no details concerning isolated yields were reported, prompting our segment-assembly synthesis.

An improved solid-phase segment-assembly protocol

(1) Division of the target sequence. The initial stage of a solid-phase segment-assembly scheme is the division of the target sequence into a set of smaller segments of 10–20 residues in length. The exact point of division is based upon a number of factors. An important consideration is that the resin-bound assembly needs to be in a freely solvated state[22] during all coupling reactions. This has recently been demonstrated with a

study on the segment assembly of β-amyloid (26–43).[20] It was clearly shown that the efficiency of segment coupling to a poorly solvated peptide–resin showed little correlation with the solubility of the incoming segment. Excellent peptide–resin solvation is ensured through a distribution of the β-secondary structure inhibiting tertiary amide residues[39,40] proline and AcHmb-protected amino acids. It has been shown that good solvation is achieved when tertiary amides are incorporated into a growing peptide–resin assembly at a maximum of every sixth residue,[39–41] and as a synthesis progresses this becomes more flexible. Thus, for segments of ~ 10 residues, placement of backbone protection (if proline is absent) around the segment centre should ensure an even resin-bound distribution as segments are coupled sequentially. The backbone protection of an amide bond needs to be compatible with Hmb chemistry,[24] the only real limitation being that an amide bond where the N-terminal residue is β-branched (Val, Ile, Thr) cannot be protected, unless the C-terminal residue is glycine. All other bonds may be readily protected.

The opportunity to choose C-terminal segments giving little or no epimerization upon segment coupling is a further important consideration. Where possible, segments are chosen to be C-terminal glycine or proline. When this is not compatible with the target sequence, we are now able to specify coupling conditions which have the potential to give low C-terminal epimerization.[25] Additionally, a number of recent publications[42,43] have highlighted the sometimes susceptible nature of the aspartyl amide bond, during Fmoc-based syntheses, to the repetitive base treatment used for removal of Fmoc. We have recently reported[44] that backbone protection of the aspartyl bond completely inhibits these base-catalysed transformations even in highly susceptible cases and this consideration warrants a high priority if good purity of a target is to be achieved.

On examining the primary sequence of HIV-1_{Bru} *tat* (1–72) **1** and incorporating the above considerations we divided the target into the five segments detailed in Table 1, three of which were C-terminal glycine, 1 being C-terminal lysine and the C-terminal segment itself. The entire assembly contains a good distribution of tertiary amide residues with prolines at residues 3, 6, 10, 14, 18, 58 and 59 along with AcHmb-substituted residues 26, 35, 42, 51 and 69 ensuring good peptide–resin solvation throughout the assembly.

(2) Preparation and purification of fully protected segments. The detailed preparation of AcHmb backbone-substituted fully protected segments on polyamide-based resin supports has recently been described.[19] The basic protocol used is depicted in Scheme 1. The polyamide resin is initially derivatized with N^{α}-fluoren-9-ylmethoxycarbonyl amino acid [2-chloro-4'-(carboxypentafluorophenoxy)triphenyl] methyl esters[19] prepared in solution. All couplings were performed under standard Pfp active ester conditions,[23] except for the incorporation and subsequent acylation of Hmb-protected residues, performed as previously described.[24] Crude, fully protected peptides were isolated in good yield by 0.75% TFA/DCM-mediated peptide–resin cleavage. In the simple purification of these protected segments we see probably the single most beneficial effect afforded by backbone amide protection. Simple Vydac diphenyl reversed-phase HPLC using acetonitrile/0.1% aq. TFA gradients gave excellent resolution and purification of the protected segments from contaminating minor deletion sequences, and this is exemplified in the purification of protected segment 2 (see Figs. 1 and 2).

(3) Assembly of AcHmb backbone-substituted fully protected segments. Our earlier investigations into the coupling of backbone-protected segments[19] showed that activation of AcHmb rather than Hmb-protected segments gave superior crude products. However, once incorporated, it is necessary to de-*O*-acetylate resin-bound AcHmb functions. Simultaneous removal along with that of the N^{α}-Fmoc group is achieved by piperidine-mediated reaction. These criteria are incorporated into our optimized assembly protocols which use two methods of segment C-terminal activation. If the C-terminal residue is not prone to epimerization the coupling method of choice is BOP/HOBt/DIEA in a minimum volume of DMF. Acylation of the resin-bound assembly for 6 h with 2 mol equiv. of activated segment gives excellent incorporation of the incoming segment. Reactions are typically > 95% complete, and appear to be independent of the length of resin-bound assembly or protected segment. When coupling of carboxy-terminal C^{α}-chiral segments is undertaken, the method of choice is DIC/HOBt. Of critical importance is the use of DCM as the reaction medium.[25] Again, 2 mol equiv. of activated segment gives excellent incorporation together with low C-terminal epimerization.[25] The particular example we have examined involved the activation of a C-terminal lysine(Boc) residue. Considering the low epimerization observed similar results may be obtainable upon activation of a number of other residues such as alanine, leucine and glutamic(OBu^t).[45]

Conclusions

We have described here a solid-phase assembly strategy in which fairly large (approaching 20 residues) AcHmb backbone-substituted fully protected segments are readily purified, then efficiently coupled to a growing resin-bound peptide assembly. The coupling efficiency remained high (> 95%) as the length of resin-bound peptide increased and only 2 mol equiv. of activated segment were needed. A major factor in such efficiencies is the full solvation of the growing peptide chain,[22] which is ensured by the incorporation of appropriately placed backbone protection[20,40,41] with the addition of each new segment. The problem of C-terminal epimerization of activated segments has been investigated[25] and although an absolute solution to the problem has not been found, early indications are that the DIC/HOBt reaction in DCM may well perform adequately in many segment couplings. The use of DCM as reaction solvent in DIC/HOBt-mediated activation is essential.[25] This necessitates good solubility of the protected segment in this solvent, again provided by the use of appropriately placed AcHmb protecting groups. Utilization of these new protocols in the segment assembly of HIV-1_{Bru} *tat* [1–72, Cys-(Acm)[22,25,27,30,31,34,37]] gave a crude protein of remarkable quality (Fig. 3), which was easily purified and isolated in 38.4% overall yield. Our improved segment-assembly methods have good flexibility in terms of division of the target (10–20 residue segments) and segment C-terminal residue (Gly, Pro and probably Ala, Leu, Glu and Lys) and should provide reasonable segments for almost any desired sequence. In conjunction with the recently described chemical strategies for peptide ligation,[4–6] a major tool is now available to enable the synthetic peptide chemist actively to contribute to the exciting field of protein molecular biology.

Experimental

Equipment, materials and methods

Continuous-flow Fmoc-polyamide methods reviewed by Atherton and Sheppard[23] were used exclusively. Fmoc amino acid pentafluorophenyl active esters (Novabiochem, Beeston, Nottingham, NG9 2JR, UK) were used exclusively except for Ser(OBu^t) and Thr(OBu^t) which were coupled as the oxodihydrobenzotriazine esters (Novabiochem, UK). Also, FmocHis(Trt)-OH and FmocArg(Pmc)-OH were coupled using benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) activation in the presence of HOBt and DIEA. Fmoc *N*-carboxyanhydrides (Propeptide, Vert Le Petit, France) were commercially available. *N*,*O*-BisFmoc-*N*-(2-hydroxy-4-methoxybenzyl) amino acid pentafluorophenyl esters[24] were prepared as previously detailed. Polyhipe SU 500 resin[28] was purchased from Phase Separations Ltd, Deeside,

CH2 2NU, UK. NovaSyn KD resin was purchased from Novabiochem, UK. All solvents were purchased and purified as previously detailed.[23]

Solid-phase peptide synthesis was performed on an LKB 'Biolynx' 4170 automated synthesizer programmed to perform acylation reactions (in DMF) for 45 min unless otherwise stated, and Fmoc-deprotection reactions (in 20% piperidine/DMF v/v) for 10 min. Amino acid side-chain protection was as follows: lysine (N^{ε}-Boc), serine and threonine (*tert*-butyl ether, Bu^t), aspartic and glutamic acid (*tert*-butyl ester, OBu^t), glutamine and asparagine (Trt), histidine (N^{τ}-trityl, Trt), tyrosine (*tert*-butyl ether, Bu^t), arginine (Pmc), cysteine (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 detection by ninhydrin. Analytical HPLC (600E Programmable Solvent Delivery System, 994 diode array detector, Waters Ltd, Watford, Herts, England), monitored at 215 nm, was performed using (a) Vydac 208TP54, C_8 column (250 × 4.6 mm), (b) Vydac 214TP54, C_4 (butyl) (250 × 4.6 mm) or (c) Vydac 219TP54, phenyl (250 × 4.6 mm). A 10–90% B in A gradient over a period of 25 min at (1.5 cm^3 min^{-1}) was used unless otherwise stated, where A = 0.1% aq. TFA and B = 90% acetonitrile/10% A. Preparative HPLC was performed using (a) Vydac 214TP1022, protein C_4 column (22 × 250 mm) or (b) Vydac 219TP1022, diphenyl column at 10 cm^3 min^{-1} flow rate and 215 nm UV detection, solvents A and B as above. All HPLC columns were purchased from HiChrom Ltd, Reading, Berkshire, England.

MALDITOF-MS were obtained on a Kratos MALDI III bench-top linear/reflectron mass spectrometer (Kratos Analytical, Manchester, England), using α-cyano-4-hydroxycinnamic acid or sinapinic acid matrices. In general, the sinapinic acid matrix gave superior results for the larger peptides (> 6000 Da). Matrix was dissolved in acetone containing 2% (v/v) 0.1% aq. TFA, to give a saturated solution. Matrix (0.15–0.2 mm^3) was applied to a sample slide and allowed to evaporate. The peptide sample was applied to successive matrix spots in a series of dilutions to find the quantity giving the best signal. Calibration by insulin and/or myoglobin was external or internal depending upon the accuracy required. Most analyses were performed with the instrument in the linear mode; when extra accuracy was required, the reflectron option was used. All masses were determined using positive-mode ionization; as required, the negative mode was used to confirm the mass observed.

(1) Preparation and purification of segment 1. HMPA-βAla-(2-ClTrt)-Nle-EDA-SU 500[19] (1000 mg, 0.3 mmol) was treated with the symmetric anhydride $[FmocGln(Trt)]_2O$ as follows: FmocGln(Trt)-OH (1830 mg, 3 mmol) was dissolved in dry DCM (7 cm^3) cooled to 0 °C and the solution was stirred. DIC (187 mg, 1.48 mmol) in DCM as a solution (2 cm^3) was added, and the mixture was stirred at 0 °C for 30 min, then evaporated under reduced pressure. The resultant symmetric anhydride was dissolved in DMF (6 cm^3) and added to the above peptide–resin. After 2 min, a solution of 4-(dimethylamino)pyridine (37 mg, 0.3 mmol) in DMF (1 cm^3) was added with swirling, and the mixture was left at room temperature for 2 h. The peptide–resin was then thoroughly washed and dried as usual (yield 1170 mg). Amino acid analysis (18 h hydrolysis) gave the following proportions: Glu 0.78, β-Ala 0.85, Nle 1.00. Loading of Fmoc-Gln(Trt) = 0.25 mmol peptide g^{-1} peptide–resin.

Fmoc-Gln(Trt)-HMPA-βAla-(2-ClTrt)-Nle-EDA-SU 500 (800 mg, 0.22 mmol) was elaborated with the following residues: residue (coupling time, deprotection area, deprotection height), Lys[71] (45 min, 82.4, 0.95), Ser[70] (45 min, 79.7, 1.18), (AcHmb)Leu[69] (5 h, 168.5, 2.62), Ser[68] (9 h, 89.0, 1.00), Val[67] (3 h, 75.1, 0.88), Gln[66] (3 h, 90.5, 1.09), His[65] (3 h, 89.1, 1.05), Thr[64] (3 h, 81.8, 1.13), Gln[63] (3 h, 87.0, 0.98), Ser[62] (45 min, kept Fmoc protected). The peptide–resin was treated with Ac_2O (255 mg)/DIEA (160 mg) for 1 h, and the fully assembled peptide–resin washed successively with DMF and diethyl ether, and dried *in vacuo* over KOH pellets (1200 mg). A sample was analysed for amino acid ratios: Thr (1) 0.95, Ser (3) 2.59, Glu/Gln (3) 3.19, Val (1) 0.91, Leu (1) 0.94, Nle (1) 1.08, β-Ala (1) 1.02, His (1) 0.96, Lys (1) 1.00.

Crude, fully protected segment (2 mg) was treated with 20% piperidine/DMF (~50 mm^3) for 30 min, then was diluted with ice-cold diethyl ether (25 cm^3). After cooling of the mixture in solid CO_2–acetone for 3 min, it was centrifuged for 7 min (3000 rpm). The liquid was decanted and the ethereal wash was repeated. The resultant solid was dried *in vacuo*, then was treated with TFA/EDT/TES/water (85:10:3:2 by vol.; 1 cm^3) for 2 h. The solution was sparged with nitrogen to remove volatile solvents, and was washed with diethyl ether (5 × 25 cm^3) as above. The crude, fully deprotected segment was then dissolved in 0.1% aq. TFA (500 mm^3), and a sample (50 mm^3) was analysed by C_8 analytical HPLC which found a major species at t_R = 6.63 min (74.2%), with MALDITOF-MS at 1244 Da (theoretically requires M, 1243 Da).

Crude, fully protected peptide–resin (1200 mg) was treated with 0.75% TFA/DCM (50 cm^3) for 35 min at room temperature. The resin was filtered off, washed with DCM (2 × 25 cm^3), and the combined organics were treated with *N*-methylmorpholine (375 mm^3). The organics were then washed with saturated aq. NaCl (2 × 750 cm^3), dried (Na_2SO_4), and evaporated under reduced pressure to give the crude fully protected segment 1 (500 mg, 72%). Phenyl analytical HPLC (10–90% B in A, 2–27 min, then 90% B in A for 13 min) (fully protected), t_R = 34.48 min (67.9%).

Crude, fully protected segment 1 (495 mg) was dissolved in TFE (15 cm^3)–water (5 cm^3), and purified by 20 × 1 cm^3 injections on a Vydac diphenyl preparative column using an 80–95% B in A gradient over a period of 25 min. Fractions were pooled, diluted with approximately half the pooled volume of water, and neutralized (pH indicator paper) with 0.01 mol dm^{-3} aq. Na_2CO_3. The neutralized solution was freeze-dried, and the lyophilized product was dissolved in DCM (100 cm^3); the solution was washed with saturated aq. NaCl (3 × 500 cm^3), dried (Na_2SO_4) and evaporated under reduced pressure. The residue was dissolved in TFE (15 cm^3), transferred to a 50 cm^3 falcon tube, reduced to approximately 2 cm^3, diluted to 15 cm^3 with distilled water, and lyophilized to give the purified segment (297 mg, 42.6%).

(2) Preparation of fully protected segments 2–5. Segments 2, 3 and 5 were prepared from Fmoc-Gly-(2-ClTrt)-Nle-EDA-SU 500[19] on a 0.3–0.8 mmol scale as generally detailed for segment 1. Likewise, segment 4 was prepared from Fmoc-Lys(Boc)-(2-ClTrt)-Nle-EDA-SU-500 on a 0.2 mmol scale. Samples (2 mg) of the crude, fully protected peptide–resins were stripped to the base peptide and analysed by HPLC and MALDITOF-MS as described above. Crude protected peptides were then purified as detailed for segment 1; see Table 1 for gradients and corresponding analytical data.

(*a*) *Segment 2.* Resin-bound amino acid proportions: Ser (1) 0.84, Glu (2) 2.15, Gly (2) 2.00, Pro (2) 2.25, Ile (1) 0.94, Nle (1) 1.19, Tyr (1) 0.98, Lys (2) 2.01, Arg (6) 5.37. Base peptide *H*-Ile[45]–Gly[61]-*OH* from crude fully protected peptide, C_8 analytical HPLC, t_R = 8.76 min (16.5%) with MALDITOF-MS at 1857 Da (theoretical requires M, 2140 Da), t_R = 8.95 min (78.1%) with MALDITOF-MS at 2140 Da. Base peptide from purified, fully protected peptide, C_8 analytical HPLC t_R = 8.98 min (98.2%) (Figs. 1 and 2).

(*b*) *Segment 3.* Resin-bound amino acid proportions: Thr (2) 1.95, Glu (1) 1.16, Gly (1) 1.05, Ala (1) 1.00, Val (1) 0.97, Leu (1) 1.04, Nle (1) 0.98, Phe (2) 1.96, His (1) 0.97, Lys (2) 1.93. Base peptide *H*-Lys[29]–Gly[44]-*OH*, Cys(Acm)[30,31,34,37] from crude, fully protected peptide, C_8 analytical HPLC t_R = 12.35

min (66.5%) with MALDITOF-MS at 2075 Da (theoretical requires M, 2075 Da). Base peptide from purified, fully protected peptide, C_8 analytical HPLC t_R = 12.27 min (98.7%).

(*c*) *Segment 4*. Resin-bound amino acid proportions: Thr (3) 2.49, Ser (1) 0.90, Glu (1) 1.09, Pro (1) obscured by the presence of Cys(Acm), Ala (1) 0.96, Nle (1) 1.19, Tyr (1) 1.00, Lys (2) 2.09. Base peptide *H*-Ser[16]–Lys[28]-*OH*, Cys(Acm)[22,25,27] from crude, fully protected peptide, C_8 analytical HPLC t_R = 8.46 min (62.8%) with MALDITOF-MS at 1649 Da (theoretical requires M, 1648 Da), t_R = 9.97 min (14.9%) with MALDITOF-MS at 1745 Da (N^{α}-trifluoroacetylated peptide[31] requires M, 1744 Da). Base peptide from purified, fully protected peptide, C_8 analytical HPLC t_R = 8.51 min (84.3%), t_R = 10.02 min (14.8%).

(*d*) *Segment 5*. Resin-bound amino acid proportions: Asp (1) 1.07, Glu (2) 2.15, Pro (4) 4.20, Gly (1) 1.07, Val (1) 0.97, Met (1) 0.83, Leu (1) 1.00, Nle (1) 1.16, His (1) 0.96, Lys (1) 1.04, Arg (1) 0.85. Base peptide *H*-Met[1]–Gly[15]-*OH* (this acidolytic cleavage solution contained triisopropylsilane instead of TES due to the presence of a tryptophan residue in segment 5) from crude, fully protected peptide, C_8 analytical HPLC t_R = 12.08 min (75.1%) with MALDITOF-MS at 1788 Da (theoretical requires M, 1788 Da). Base peptide from purified, fully protected peptide, C_8 analytical HPLC t_R = 11.99 min (98.1%).

(3) Assembly of segments 1–5. (*a*) *Initial loading of segment 1 onto* H-*Nle-NovaSyn KD*. To purified segment 1 (290 mg, 91.5 μmol) were added BOP (40.4 mg, 91.5 μmol), HOBt (14.0 mg, 91.5 μmol), and DIEA (11.8 mg, 91.5 μmol) in DMF (500 mm^3). After 2 min, H-Nle-NovaSyn KD (720 mg, ~0.075 mmol) was added, followed by DMF (1.5 cm^3) and the reaction mixture was left for 7 h. The peptide–resin was washed and dried, then treated with Ac_2O (30.6 mg, 0.3 mmol)/DIEA (15.5 mg, 0.12 mmol), pre-mixed in DMF (3 cm^3; 2 min), for 30 min. The peptide–resin was then thoroughly washed and dried (845 mg), loading = 0.048 mmol peptide g^{-1} peptide–resin. The loaded peptide–resin was then treated with 20% piperidine–DMF for 3 and 7 min, thoroughly washed and dried as usual.

Peptide–resin (5 mg) was treated with 20% piperidine–DMF, and after 3 min was assessed by quantitative UV analysis,[19] which showed A_{290} = 0.441, then after 30 min was washed and dried *in vacuo* as usual. Amino acid proportions: Thr (1) 1.08, Ser (3) 2.67, Glu/Gln (3) 3.04, Val (1) 0.95, Leu (1) 1.00, Nle (1) 1.31, β-Ala (1) 1.03, His (1) 0.94, Lys (1) 0.99.

Fmoc-deprotected peptide–resin (2 mg) was cleaved and worked up as detailed above with C_8 analytical HPLC: t_R 6.74 min (94.2%), MALDITOF-MS at 1243 Da (theoretical requires M, 1243 Da).

(*b*) *Addition of segment 2*. Purified segment 2 (98.7 mg, 20 μmol, 2 mol equiv.), BOP (8.84 mg, 20 μmol), HOBt (3.1 mg, 20 μmol) and DIEA (2.6 mg, 20 μmol) were dissolved in DMF (900 mm^3) and left for 5 min. *H*-Seg1-R [from 3(*a*), above; 210 mg, 10 μmol] was added, and the reaction mixture was left for 6 h. The coupled resin was then filtered off, washed successively with DMF and diethyl ether and dried *in vacuo* over KOH pellets (253 mg).

Peptide–resin (5 mg) was treated with 20% piperidine–DMF; after 3 min the mixture was assessed by quantitative UV analysis,[19] which showed A_{290} = 0.342 (equating to 95% coupling), then after 30 min was washed and dried *in vacuo* as usual. A sample (2 mg) was analysed for amino acid: Thr (1) 0.96, Ser (4) 3.53, Glu/Gln (5) 5.40, Pro (2) 1.73, Gly (2) 1.78, Val (1) 1.00, Ile (1) 0.81, Leu (1) 1.06, Nle (1) 1.27, β-Ala (1) 1.02, Tyr (1) 0.81, His (1) 1.02, Lys (3) 2.72, Arg (6) 5.36.

Deprotected peptide–resin (2 mg) was cleaved with TFA–EDT–TES–water (85:10:3:2 by vol.; 1 cm^3) for 3 h and worked up as detailed above. Analytical data are in Table 2.

The coupled resin was treated with acetic anhydride (10.3 mg) in DMF for 1 h, then was washed and dried. The coupled and capped resin was then treated with 20% piperidine in DMF for 3 and 7 min, and again was washed and dried.

(*c*) *Addition of segment 3*. Purified segment 3 (69.0 mg, 20 μmol, 2 mol equiv.), BOP (8.84 mg, 20 μmol), HOBt (3.1 mg, 20 μmol) and DIEA (2.6 mg, 20 μmol) were dissolved in DMF (1.1 cm^3) and left for 5 min. *H*-Seg2-Seg1-R [from 3(*b*), above; 240 mg] was added, and the reaction mixture was left for 9 h. The coupled resin was then filtered off, washed successively with DMF and diethyl ether and dried *in vacuo* over KOH pellets (275 mg).

Peptide–resin (5 mg) was treated with 20% piperidine–DMF, and after 3 min was assessed by quantitative UV analysis,[19] A_{290} = 0.286 (equating to 94.5% coupling), then after 30 min was washed and dried *in vacuo* as usual. A sample (2 mg) was analysed for amino acid: Thr (3) 3.16, Ser (4) 3.65, Glu/Gln (6) 6.27, Pro (2) obscured by the presence of Cys(Acm), Gly (3) 3.13, Ala (1) 0.87, Val (2) 2.00, Ile (1) 0.95, Leu (2) 2.08, Nle (1) 1.51, β-Ala (1) 1.03, Tyr (1) 0.91, Phe (2) 1.57, His (2) 1.92, Lys (5) 4.83, Arg (6) 6.81.

Deprotected peptide–resin (2 mg) was cleaved with TFA–EDT–TES–water (85:10:3:2 by vol.; 1 cm^3) for 3 h and worked up as detailed above. Analytical data are in Table 2.

The coupled resin was treated with acetic anhydride (10.3 mg) in DMF for 1 h, then was washed and dried. The coupled and capped resin was then treated with 20% piperidine in DMF for 3 and 7 min and again washed and dried.

(*d*) *Addition of segment 4*. Purified peptide 4 (53.2 mg, 20 μmol) and HOBt (3.1 mg, 20 μmol) were dissolved in DCM (1 cm^3) and the solution was cooled in ice. DIC (2.5 mg, 100 mm^3 from a stock solution of 25.2 mg cm^{-3} DCM, 20 μmol) was added and the mixture was left at 0 °C for 1 h. *H*-Seg3-Seg2-Seg1-R [from 3(*c*), above; 265 mg, 10 μmol] was then added. After 1 h, coupled peptide–resin (5 mg) was removed and washed down as usual. Quantitative UV analysis[19] gave A_{290} = 0.219 (equating to 81% coupling). The remaining coupling reaction was continued at room temperature for a further 5 h. The coupled resin was then filtered off, washed successively with DMF and diethyl ether and dried *in vacuo* over KOH pellets (280 mg).

Peptide–resin (5 mg) was treated with 20% piperidine–DMF and after 3 min was assessed by quantitative UV analysis,[19] A_{290} = 0.255 (equating to 96% coupling), then after 30 min was washed and dried *in vacuo* as usual. A sample (2 mg) was analysed for amino acid: Thr (6) 5.36, Ser (5) 4.26, Glu/Gln (7) 8.22, Pro (3) obscured by the presence of Cys(Acm), Gly (3) 2.73, Ala (2) 1.70, Val (2) 2.00, Ile (1) 0.91, Leu (2) 2.01, Nle (1) 1.71, β-Ala (1) 1.03, Tyr (2) 1.75, Phe (2) 1.65, His (2) 1.97, Lys (7) 6.46, Arg (6) 6.62.

Deprotected peptide–resin (2 mg) was cleaved with TFA–EDT–TES–water (85:10:3:2 by vol.; 1 cm^3) for 3 h and worked up as detailed above. Analytical data are in Table 2.

The coupled resin was treated with acetic anhydride (10.3 mg) in DMF for 1 h, then was washed and dried. The coupled and capped resin was then treated with 20% piperidine in DMF for 3 and 7 min and again was washed and dried.

(*e*) *Addition of segment 5*. Purified segment 5 (57.7 mg, 20 μmol, 2 mol equiv.), BOP (8.84 mg, 20 μmol), HOBt (3.1 mg, 20 μmol) and DIEA (2.6 mg, 20 μmol) were dissolved in DMF (1.8 cm^3) and left for 5 min. *H*-Seg4-Seg3-Seg2-Seg1-R [from 3(*d*), above; 270 mg] was added, and the reaction mixture was left for 6 h. The coupled resin was then filtered off, washed successively with DMF and diethyl ether and dried *in vacuo*. The coupled peptide–resin was then treated with 20% piperidine in DMF for 3 and 7 min and again was washed and dried (295 mg).

Peptide–resin (5 mg) was treated with 20% piperidine–DMF, and after 3 min was assessed by quantitative UV analysis,[19] giving A_{290} = 0.229 (equating to 98% coupling), then after 30 min was washed and dried *in vacuo* as usual. A sample (2 mg) was analysed for amino acid: Asp (1) 0.89, Thr (6) 5.52, Ser (5) 4.40, Glu/Gln (9) 10.62, Pro (7) obscured by the presence of Cys(Acm), Gly (4) 3.84, Ala (2) 1.77, Val (3) 3.00, Met (1) 0.73,

Ile (1) 0.97, Leu (3) 3.02, Nle (1) 1.74, β-Ala (1) 1.04, Tyr (2) 1.80, Phe (2) 1.58, His (3) 3.00, Lys (8) 7.60, Arg (7) 7.11.

Deprotected peptide–resin (2 mg) was cleaved with TFA–EDT–triisopropylsilane–water (85:10:3:2, v/v/v/v; 1000 mm^3) for 3 h and worked up as detailed above. Analytical data are in Table 2.

(4) Cleavage and purification of HIV-1_{Bru} *tat* [1–72, Cys(Acm)^{22,25,27,30,31,34,37}]. Peptide–resin from 3(*e*) (290 mg) was cleaved with TFA–EDT–isopropylsilane–water (85:10:3:2 by vol.; 25 cm^3) for 3.5 h and worked up as detailed above to give a solid (82.1 mg). Amino acid analysis of the post-cleaved resin gave an Asp:Nle ratio of 0.03:2.52, indicating 98% cleavage.

Crude *H*-Met^1-Gln^{72}-*OH*, Cys(Acm)^{22,25,27,30,31,34,37}, 16TFA was dissolved in 0.1% aq. TFA (5 cm^3), and aliquots (5 mm^3) were analysed by C_4 analytical HPLC [Fig. 3(*a*)]. A gradient of 20–35% B in A over a period of 25 min gave 2 main species, with t_R = 12.1 min (9.4%) with MALDITOF-MS at 8246 Da (theoretical requires M, 8818 Da), and t_R = 13.85 min (71.3%) with MALDITOF-MS at 8819 Da. The crude peptide was purified (10 × 500 mm^3) by C_4 preparative HPLC using a 20–35% B in A gradient over a period of 25 min. The desired fractions were pooled and freeze-dried, then re-dissolved in 0.1% aq. TFA (15 cm^3). C_4 Analytical HPLC [Fig. 3(*b*)] gave t_R = 13.39 min (>99%) with MALDITOF-MS = 8818.3 ± 4.1 Da (*n* = 16).

An aliquot (100 mm^3) was hydrolysed, giving the following amino acid proportions: Asp (1) 0.95, Thr (6) 5.51, Ser (5) 4.17, Glu/Gln (9) 9.84, Pro (7) obscured by the presence of Cys(Acm), Gly (4) 4.04, Ala (2) 2.01, Val (3) 3.00, Met (1) 0.84, Ile (1) 0.94, Leu (3) 2.98, Tyr (2) 1.95, Phe (2) 1.90, His (3) 3.09, Lys (8) 8.36, Arg (7) 6.84 (37.0 mg, 38.4% overall).

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Paper 5/06750B
Received 11*th October* 1995
Accepted 18*th December* 1995