Synthesis of Azapeptides by the Fmoc/*tert*-Butyl/Polyamide Technique

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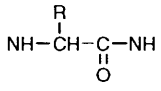
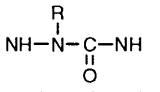
A new synthesis of azapeptides for use in the study of a proteolytic enzyme associated with Alzheimer's disease is described. The method utilizes fluoren-9-ylmethoxycarbonyl (Fmoc) amino acid carbazates and hydrazides in the Fmoc/*tert*-butyl/polyamide technique. The preparation of these compounds is presented. Reaction of Fmoc-amino acid hydrazides with an appropriate aldehyde, followed by reduction, gave fully protected amino acid carbazate dipeptide synthons. These derivatives were used to prepare aza amino acid peptide analogues by reaction with a resin-bound amino group, activated with bis-2,4-dinitrophenyl carbonate in the presence of a base. With this activation of the amino group, hydantoin is formed in a major side-reaction, but the cyclisation could be virtually eliminated by omission of the base from the activation procedure. Upon final trifluoroacetic acid-mediated cleavage of the azapeptide, trifluoroacetylation of the N-terminal serine residue was observed.

Azapeptides are peptide analogues in which the α-CH of one or more amino acid residues has been substituted by a nitrogen, bearing an appropriate side-chain. This modification of the


Amino acid residue


Aza amino acid residue

natural peptide bond, from an amide to a urea, produces marked changes in its chemistry and biochemistry. The aza-carbonyl bond is much more resistant to both chemical and enzymic hydrolysis than is the natural peptide bond.[1,2] However, several studies have shown that both the acylation and deacylation steps of enzyme-catalysed hydrolysis of aza amino acid derivatives do occur, albeit slowly.[3] This suggests that the stereochemical arrangements in aza analogues mimic those of their natural counterpart. Therefore, the introduction of an aza residue at the P_1 site[4] of a proteinase substrate is likely to yield an analogue which binds to the enzyme but which undergoes hydrolysis at a significantly diminished rate, resulting in enzymic inhibition. The prolonged activity of numerous aza analogues of biologically active peptides is probably due to this resistance to proteolytic degradation.[5-7]

Alzheimer's disease is the cause of dementia in about 70% of patients older than 65 years (for review, see ref. 8). One of the characteristic neuropathological signs of the disease is a greatly increased deposition, as compared with normally aged brains, of extracellular amyloid protein, both in cerebral blood vessels and as senile plaques.[9] Proteinaceous amyloid deposits contribute to morbidity and mortality in a wide variety of diseases, but only one characteristic protein species is confined to each disease type (reviewed in ref. 10). In Alzheimer's disease this is the β-A4 polypeptide, which is itself a fragment of a larger, precursor glycoprotein.[11] In addition to producing base levels of β-A4, the usual proteolytic processing of the precursor (to release a proteoglycan into the extracellular space) is diverted in Alzheimer's disease to yield the β-A4 fragment in abnormal abundance.[12] For this to occur, a protease cleaves the following peptide sequence between methionine and aspartate residues, and so creates the N-terminus of the β-A4 fragment:

.. Ser-Glu-Val-Lys-Met	+	Asp-Ala-Glu-Phe-Arg
(position) P_3 P_2 P_1		P_1' P_2' P_3'

Major classes of proteases, examples of which abound in brain tissue, cleave peptide bonds of the type Lys/Arg-X.[13] Thus, labelled relative to the Alzheimer-associated cleavage, the peptide linkage P_2 to P_1, above, is susceptible to these enzymes. In order to distinguish between fractions of a brain extract that contains both protease activities, we have required the following pair of azapeptides for use as mechanistic probes:

Ser-Glu-Val-azaLys-Met-Asp-Ala-Glu-Phe-Arg **1**

Ser-Glu-Val-Lys-azaMet-Asp-Ala-Glu-Phe-Arg **2**

In this paper we report the syntheses of these azapeptide analogues. They represent the first synthesis of azapeptides using the Fmoc/*tert*-butyl/polyamide technique.[14]

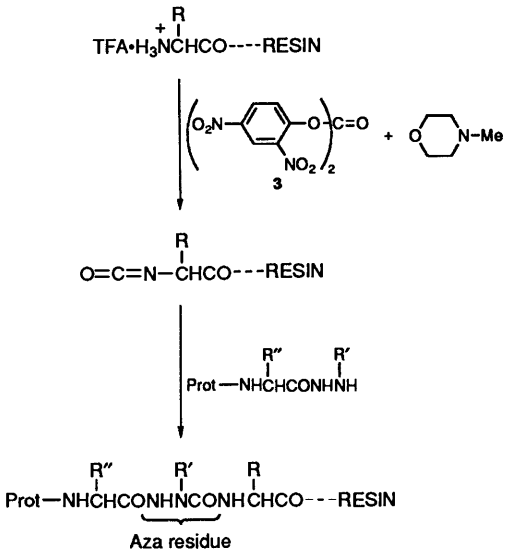
Results and Discussion

Initial Procedures.—In a recent publication by Gray *et al.*[15] the azapeptide unit was incorporated into a solid-phase scheme as shown in Scheme 1. This procedure was successfully applied using N^{α}-*tert*-butoxycarbonyl (Boc)/benzyl chemistry on an ultra-high-load, polyacrylamide-type support.[16] Resin-bound N-terminal amino groups were converted into isocyanate by using bis-2,4-dinitrophenyl carbonate[17] (**3**, Scheme 1) in the presence of base. Reaction with a protected amino acid hydrazide, bearing the appropriate side-chain group R' on the β-nitrogen (Scheme 1), then completed the aza structure. The construction of the aza unit in this way, by utilizing the protected amino acid carbazate dipeptide synthon, was shown to be the preferred route.

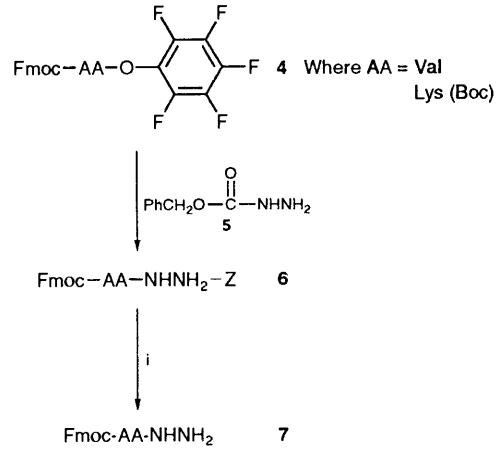
By adaptation of these techniques to a strategy for Fmoc/*tert*-butyl synthesis, the required dipeptide synthon units were prepared as shown in Schemes 2–4.

Initially, the procedure requires the synthesis of N^{α}-Fmoc-amino acid hydrazides **7**. The standard method used to prepare the equivalent N^{α}-Boc-amino acid hydrazides (hyrazinolysis of Boc-amino acid alkyl esters) was considered inappropriate, due to the lability of the Fmoc group to hydrazine.† However, reaction of Fmoc-amino acid pentafluorophenyl esters (OPfp) **4** with benzyl carbazate **5** gave quantitative yields of the

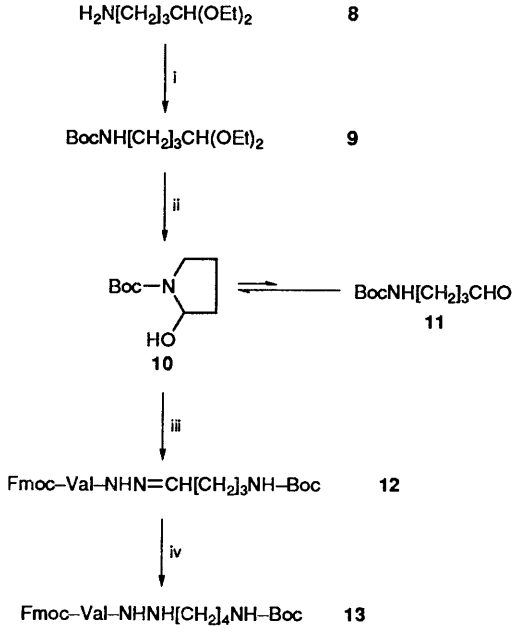
† Subsequent preliminary investigation has revealed that reaction of Fmoc-amino acid pentafluorophenyl esters with hydrazine hydrate (1 mol equiv.) also yields the Fmoc-amino acid hydrazide with little apparent cleavage of the N^{α}-Fmoc group.



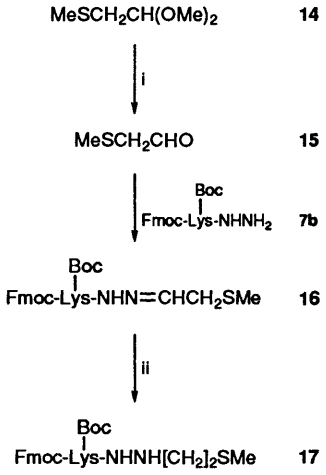
Scheme 1



Scheme 2 Synthesis of Fmoc-amino acid hydrazides. *Reagents:* i, H_2/Pd–C



Scheme 3 Synthesis of azaLys dipeptide synthon. *Reagents:* i, $(Boc)_2O$; ii, HCl; iii, Fmoc-Val-$NHNH_2$ (**7a**); iv, $NaBH_3CN$



Scheme 4 Synthesis of azaMet dipeptide synthon. *Reagents:* i, HCl; ii, $NaBH_3CN$

benzyloxycarbonyl (Z)-protected hydrazide **6**. Previously it has been shown that the Fmoc group is susceptible to cleavage by catalytic hydrogenation,[18] and, indeed, attempted removal of the Z group by catalytic transfer hydrogenation gave a mixture of products. However, it has been recently demonstrated that Z can be cleaved in the presence of Fmoc, by a carefully controlled hydrogenation using H_2 gas over a Pd–C catalyst.[19] In our hands this method worked well, with little cleavage of the N^{α}-Fmoc group, giving the fully characterized hydrazides of Fmoc-Val **7a** and Fmoc-Lys(Boc) **7b** in good yield.

For peptide sequence **1**, the side-chain required for the azaLys dipeptide synthon was prepared from the commercially available 4-aminobutyraldehyde diethyl acetal **8**, following the method of Greenlee and Thorsett.[20] Initially, the amino group of the acetal was protected with the *tert*-butoxycarbonyl group, by reaction with di-*tert*-butyl dicarbonate in chloroform. The Boc-protected diethyl acetal **9** was then treated with aq. HCl in tetrahydrofuran (THF), to give crude N^{α}-Boc-pyrrolidin-2-ol **10** in good yield. The amide carbinol exists in equilibrium with the required aldehyde **11**. Reaction between Fmoc-Val-N_2H_3 **7a** and N^{α}-Boc-pyrolidin-2-ol **10** in THF gave the hydrazone **12** as a mixture of geometrical isomers. Reduction of this crude mixture with sodium cyanoborohydride gave the required azaLys dipeptide synthon, which was purified by silica gel chromatography. This final product, *N-tert*-butoxycarbonyl-{4-[*N'*-(fluoren-9-ylmethoxycarbonyl-L-valyl)hydrazino]}-butylamine **13**, was obtained as a white solid and was fully characterized.

For sequence **2**, the side-chain required for the azaMet dipeptide synthon, (methylthio)acetaldehyde **15**, was obtained from the commercially available dimethyl acetal **14** following the method of Gassman *et al.*[21] Reaction between aldehyde **15** and Fmoc-Lys(Boc)-N_2H_3 **7b** again gave the isomeric hydrazones **16**. These were reduced with sodium cyanoborohydride to the required azaMet dipeptide equivalent 2-{*N'*-[fluoren-9-ylmethoxycarbonyl-L-(N^{ε}-Boc)lysyl]hydrazino}-1-(methylthio)ethane **17**, obtained as a white solid and fully characterized.

Prior to final synthesis of the required azapeptide, the core

H-Asp(OBu^t)-Ala-Glu(OBu^t)-Phe-Arg(Mtr)-PepSyn KA
18

sequence **18** was prepared by using pre-packed vials of pentafluorophenyl[22] (Pfp) or 3,4-dihydro-4-oxobenzotriazin-3-yl (Dhbt)[23] esters on a Biolynx continuous-flow synthesizer, using standard protocols.

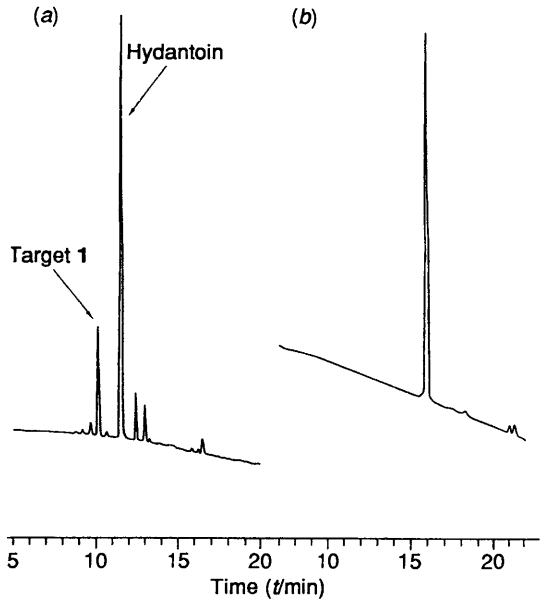


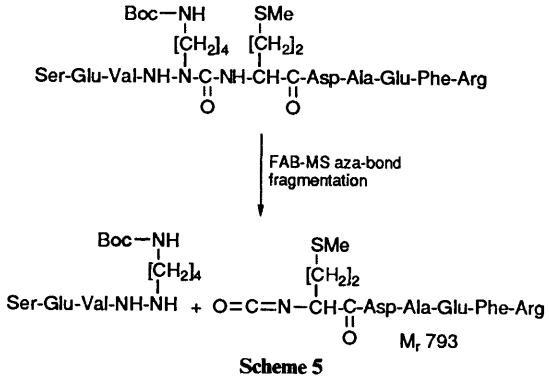
Fig. 1 (a) Analytical HPLC of crude peptide, target **1** (see Experimental section for details): gradient 10–90% B; (b) analytical HPLC of purified peptide, target **1** (see Experimental section for details): gradient 5–40% B

Table 1 FAB–MS and amino acid analysis data of the fractions obtained from the purification of target peptide **1** (see Discussion and Experimental sections for details)

Fraction	FAB–MS (+ve *m/z*)	Amino acid analysis
F1	1212.0	Asp (1) 1.00
	794.0	Ser (1) 0.69
		Glu (2) 2.06
		Ala (1) 1.04
		Val (1) 0.86
		Met (1) 1.04
		Phe (1) 1.09
F2	794.0	Arg (1) 1.04
		Asp (1) 0.17
		Glu (2) 1.04
		Ala (1) 1.24
		Met (1) 0.13
		Phe (1) 1.02
		Arg (1) 1.00

Method.—Solid-phase peptide syntheses using the dipeptide equivalents **13** and **17** were performed on functionalized and cross-linked poly(dimethylacrylamide) resin supported in macroporous Kieselguhr (Pepsyn), with an initial peptide loading of ~0.1 mmol g^{-1}.

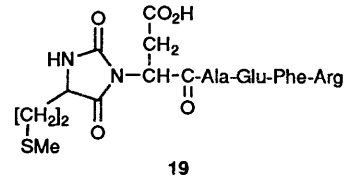
Synthesis of Peptide **1**.—The synthesis of the first target decapeptide, utilising the dipeptide synthon **13**, was attempted following the methods of Gray *et al.*[15] The core sequence **18** was extended by the next residue (Met), and the free amino terminus of the methionine was treated with bis-2,4-dinitrophenyl carbonate **3**, in the presence of *N*-methylmorpholine, with *N*,*N*-dimethylformamide (DMF) as solvent. This procedure generated the terminal isocyanate.[15] The activated terminus was then treated with carbazate **13**, again in DMF. Continuation of the synthesis on the Biolynx indicated, from the Fmoc-deprotection profile of the new terminal valine residue, that only 15% of the growing peptide chains contained a terminal valine. Addition and deprotection of the final two residues, Glu(OBu^t) and Ser(Bu^t), confirmed that only 15% of the initial peptide chains were available for reaction. It was inferred that a side-reaction had occurred, terminating peptide chains during the resin activation and coupling of the carbazate **13**.



Scheme 5

Analysis of Peptide **1**.—HPLC analysis of the cleaved, final product exhibited two principal peaks (Fig. 1a), which were isolated by preparative reversed-phase HPLC. The results of amino acid analysis (after hydrolysis for 72 h at 110 °C in 6 mol dm^{-3} aq. HCl) and fast-atom-bombardment mass spectrometry (FAB–MS) of the two isolated fractions, F1 and F2, are shown in Table 1. As the mass of the target decapeptide (**1**) is 1211.4 Da, fraction F1 was identified as the desired product. It gave the expected amino acid analysis, and an *m/z* peak of 1212.0 $[M + H]^+$.

It has been shown[1,24] that in the FAB–MS analysis of azapeptides the major fragmentation point is at the aza bond, to give carbazate and isocyanate (which may well rearrange to the hydantoin of identical relative molecular mass) peptide fragments, as shown in Scheme 5. Thus, in the fragmentation pattern of F1, the component with *m/z* 794 corresponds to the right-hand side of the aza-bond cleavage. F2 also shows a component with *m/z* 794, indicating that this is probably the hydantoin species (compound **19**) (isocyanate would decarboxylate as a consequence of the preparative HPLC isolation conditions and give an entity with *m/z* 26 units lower than that observed). Presumably, during the formation of the isocyanate of the amino terminal methionine residue, an intramolecular cyclization had occurred, terminating ~85% of the growing peptide chains. Gray *et al.*[15] describe this side-reaction only upon activation of a Gly-Gly sequence in their ultra-high load (5 mmol g^{-1}), solid-phase scheme. On the low-loading support used in the present work, the hydantoin side-reaction occurs much more readily. For this reason, in the synthesis of the second target sequence (**2**) the activation procedure was modified.



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Synthesis of Peptide **2**.—N-Terminal Asp core resin **18** was treated with bis-2,4-dinitrophenyl carbonate[17] **3** (5 mol equiv.) in DMF for 60 min, in the absence of base. This generated the *N*-2,4-dinitrophenyl-activated urethane,[25] which, although still reactive towards aza-bond formation, is much less prone to formation of a hydantoin moiety. Reaction with the azaMet dipeptide equivalent **17** in DMF overnight gave, upon cleavage of a small sample, one major peak on HPLC (Fig. 2). This result indicated that formation of the hydantoin had been reduced

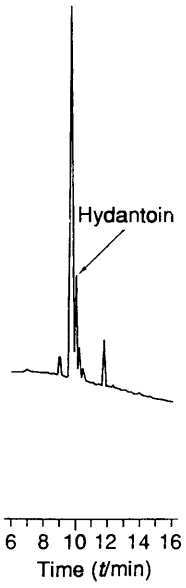

Fig. 2 Analytical HPLC of crude heptapeptide H-Lys-azaMet-Asp-Ala-Glu-Phe-Arg-OH (see Experimental section for details): gradient 10–90% B

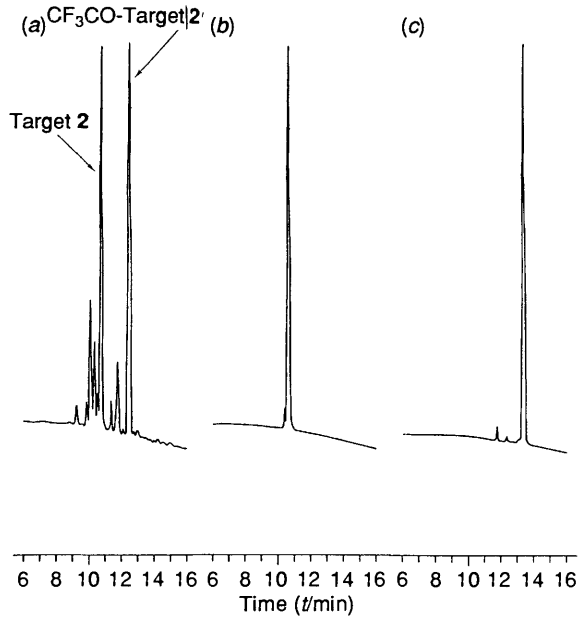

Fig. 3 Analytical HPLC of crude peptide, target **2** (see Experimental section for details): gradient 10–90% B; (b) analytical HPLC of purified peptide, target **2** (see Experimental section for details): gradient 10–90% B; (c) analytical HPLC of purified CF_3CO-target **2** (see Experimental section for details): gradient 10–90% B

more than four-fold (to ~20%, previously ~85%) by the modified activation procedure. Fmoc-deprotection profiles of the remaining three amino acids confirmed that ~80% of the initial chains were still growing.

Analysis of Peptide **2**.—HPLC of the final cleaved peptide showed, surprisingly, two major products (Fig. 3a). These were isolated and analysed; the amino acid analysis and FAB–MS results are shown in Table 2. Fraction 1 was identified as the desired decapeptide **2** (M_w 1211.4). Fraction 2 had an *m/z* top mass 97 units higher than expected, and the fragmentation of the aza bond showed this extra mass to be located in the *N*-terminal half. A side-reaction (*N*-trifluoroacetylation of a terminal hydroxy amino acid) has recently been reported by Hübener *et al.*[26] Thus, fraction 2 is probably the desired sequence, but with the *N*-terminal serine residue trifluoroacetylated. Trifluoroacetylation presumably also occurred during the deprotection and cleavage of peptide **1**. Since the full sequence represented only 10% of the crude product (the remainder being the hydantoin—see above) any trifluoroacetylated peptide would appear as a minor unidentified side-product.

Conclusions and Applications.—We have shown that Fmoc-protected fragments required for the synthesis of azapeptides can be prepared in reasonable yield. Activation procedures towards aza-bond formation required modification of the published schemes in order to minimize formation of hydantoin, which occurred much more readily on a low-loaded resin.

Preliminary results (not shown) have already enabled us to detect a protease activity in Alzheimer brain extracts that cleaves the decapeptide substrate at the P_1 to P_1' bond, and, in contrast to the fluorogenic substrate method of Wang and Krafft,[27] to distinguish this readily from other, contaminating proteases. Furthermore, we are using the azapeptides to characterize the mechanism of the enzyme, and plan to employ these analogues in subsequent preparations of the protein by affinity chromatography.

In the familial forms of Alzheimer's disease, a mutation has occurred in the gene for the β-A4 precursor.[28] Clearly, then, in these rare forms, processing of the precursor gene product is of central importance in the disease. In the much more common age-related form, the accelerated deposition of the β-A4 polypeptide is certainly of clinical importance. Thus, in all cases, inhibition of the proteolytic activity associated with Alzheimer's disease may have therapeutic value. The successful synthesis of azapeptides that mimic the substrate is a first step towards the design of potential therapeutic compounds.

Experimental

General.—Analytical HPLC was carried out on a Brownlee Aquapore RP-300 C-8 column. Reagent A, 0.1% aq. trifluoroacetic acid (TFA); reagent B, 90% acetonitrile, 10% A; gradients as below. Flow rate 1.5 $cm^3\ min^{-1}$. The effluent was monitored at 215 nm. Peptides were purified on a Brownlee Aquapore RP-300 C-8 semi-preparative column (7.8 × 240 mm), reagents as above. Flow rate 3.0 $cm^3\ min^{-1}$. Effluent monitored at 215 nm. Optical rotations were determined using a Perkin-Elmer 241 polarimeter (sodium lamp, 589 nm) at 20 °C, and are given in units of 10^{-1} deg $cm^2\ g^{-1}$. M.p.s were measured on a Gallenkamp MPD 350 melting point apparatus, and are uncorrected. TLC samples were run on aluminium sheets pre-coated with silica gel 60 F_{254} (Merck).

Solid-phase Synthesis.—General techniques are described by Atherton and Sheppard.[14] Continuous-flow syntheses utilized an LKB Biolynx model 4170 peptide synthesizer in automatic mode. Commerical Fmoc-amino acid OPfp esters (0.5 mmol vials) or ODhbt esters (0.5 mmol vials) and Fmoc-aminoacyl-PepSyn KA resins (Novabiochem, Milligen) were used. Acylation times (pentafluorophenyl ester/hydroxybenzotriazole or ODhbt active-ester chemistry) were 45 min and deprotection (20% piperidine–DMF) times 10 min. Spectroscopic acylation and deprotection monitoring was at 304 nm. All chiral amino acids used were of the L-configuration. Amino acid side-chain protection was as follows: arginine (N^G-methoxytrimethylphenylsulfonyl, Mtr), glutamic acid (*tert*-butyl ester, OBu^t), aspartic acid (*tert*-butyl ester, OBu^t), serine (*tert*-butyl ether, Bu^t), lysine (N^ε-*tert*-butoxycarbonyl, Boc).

N-Benzyloxycarbonyl-N'-(fluoren-9-ylmethoxycarbonyl-L-valyl)hydrazine **6a**.—Fmoc-Val-OPfp **4a** (5.05 g, 10 mmol) was

Table 2 FAB–MS and amino acid analysis data of the fractions obtained from the purification of target peptide **2** (see Discussion and Experimental sections for details)

Fraction	FAB–MS (+ve *m/z*)	Amino acid analysis
F1	1212.6	Asp (1) 0.95
	663.5	Ser (1) 0.87
		Glu (2) 2.07
		Ala (1) 1.02
		Val (1) 0.93
		Phe (1) 1.01
		Lys (1) 0.94
		Arg (1) 1.00
F2	1309.4	Asp (1) 0.98
	663.2	Ser (1) 0.90
		Glu (2) 2.08
		Ala (1) 0.95
		Val (1) 0.95
		Phe (1) 1.00
		Lys (1) 0.92
		Arg (1) 1.00

dissolved in vigorously stirred, dry diethyl ether (90 cm^3). Benzyl carbazate **5** (3.30 g, 20 mmol) was added portionwise over a period of 5 min to the stirred solution and the reaction was allowed to proceed for 60 h. The resulting precipitate was recovered by filtration, washed thoroughly with diethyl ether (4 × 50 cm^3), and dried *in vacuo* for 16 h (4.46 g, 92%), m.p. 182–184 °C; HPLC (10–90% B gradient during 25 min) t_R 21.6 min (99.7%); TLC [$CHCl_3$–MeOH (97.5–2.5 v/v)] R_f 0.58 (UV positive) {Found: $C_{28}H_{29}N_3O_5$, M = 487.5. FAB–MS (+ve *m/z*) 510.4 $[M + Na]^+$, 488.4 $[M + H]^+$} [$C_{28}H_{29}N_3O_5$: Found (requires): C, 68.7 (68.98); H, 6.1 (6.00); N, 8.7 (8.62)]; δ_H[270 MHz $(CD_3)_2SO$] 0.92 (6 H, m, γ- + γ'-CH'), 1.95 (1 H, m, β-CH), 3.85 (1 H, t, α-CH), 4.23 (3 H, m, Fmoc α-H_2 + Fmoc 9-H), 5.07 (2 H, s, Zα-H_2), 7.29–7.91 (14 H, m, 13 × ArH + 1 × NH), 9.22 (1 H, s, NH) and 9.89 (1 H, s, NH); $[\alpha]_D^{20}$ −21.6 (*c* 1, DMF).

Fluoren-9-ylmethoxycarbonyl-L-*valylhydrazide* **7a**.—*N*-Benzyloxycarbonyl-*N'*-(fluoren-9-ylmethoxycarbonyl-L-valyl)-hydrazine **6a** (1 g, 2.05 mmol) was dissolved in 70% aq. 1,4-dioxane (50 cm^3) containing 10% palladium-on-carbon (0.5 g). The mixture was cooled to 0 °C on an ice–salt-bath. Hydrogen gas was gently bubbled into the reaction mixture and the reaction was monitored by TLC. After 4 h, hydrogenation was stopped. TLC revealed the presence of a small amount of dibenzofulvene (R_f 0.95) and free amino group (ninhydrin-positve spot on the baseline). The reaction mixture was filtered through Celite and the insoluble material was washed with 1,4-dioxane (4 × 25 cm^3). The solvent was removed from the combined filtrate and washings by rotary evaporation under reduced pressure and the resulting powder was stirred in hexane (~150 cm^3) to remove dibenzofulvene. The powder was recovered by filtration and was dried *in vacuo* for 16 h. TLC analysis confirmed that all dibenzofulvene had been removed. The solid was dissolved in chloroform (250 cm^3) and washed successively with water (2 × 250 cm^3) followed by saturated aq. sodium chloride (2 × 250 cm^3). TLC analysis of the chloroform layer indicated that no ninhydrin-positive material was present. The chloroform layer was dried over Na_2SO_4, then was filtered, and the solvent was removed from the filtrate by rotary evaporation under reduced pressure (0.5 g, 70%), m.p. 201–202.5 °C; HPLC (10–90% B gradient during 25 min) t_R 16.6 (99%); TLC [$CHCl_3$–MeOH (97.5:2.5)] R_f 0.20 (UV positive) (Found: $C_{20}H_{23}N_3O_3$, M = 353.4), FAB–MS (+ve *m/z*) 354.5 $[M + H]^+$ [$C_{20}H_{23}N_3O_3 \cdot \frac{1}{3}H_2O$: Found (requires): C, 67.0 (66.84); H, 6.55 (6.64); N, 11.6 (11.69)]; δ_H[270 MHz; $(CD_3)_2SO$] 0.85 (6 H, m, γ- + γ'-Me), 1.90 (1 H, m, β-CH), 3.73 (1 H, t, α-CH), 4.23 [5 H, m, Fmoc α-H_2 + Fmoc 9-H + NH_2 (hydrazide)], 7.29–7.90 (9 H, m, 8 × ArH + 1 × NH) and 9.13 (1 H, s, NH); $[\alpha]_D^{20}$ +1.1 (*c* 1, DMF).

N-*Benzyloxycarbonyl*-N'-[*fluoren-9-ylmethoxycarbonyl*-(N$^\varepsilon$-tert-*butoxycarbonyl*)-L-*lysyl*]*hydrazine* **6b**.—Fmoc-Lys(Boc)-OPfp **4b** (2 g, 3.15 mmol) was dissolved in vigorously stirred, dry diethyl ether (35 cm^3) and THF (8 cm^3) was added to effect complete dissolution. Benzyl carbazate **5** (1.04 g, 6.3 mmol) was added portionwise over a period of 5 min and the reaction was allowed to proceed for 60 h. The resultant precipitate was collected by filtration, washed thoroughly with diethyl ether, and dried *in vacuo* for 16 h (1.77 g, 91%), m.p. 138 °C (shrinks), 141–144 °C (melts); HPLC (10–90% B gradient during 25 min) t_R 22.6 (99.5%); TLC [EtOAc–hexane (2:1)] R_f 0.62 (Found: $C_{34}H_{40}N_4O_7$, M = 616.6) {FAB–MS (+ve *m/z*) 639.3 $[M + Na]^+$, 617.5 $[M + H]^+$ and 517.5 (617.5 − Boc)} [$C_{34}H_{40}N_4O_7$: Found (requires): C, 66.45 (66.22); H, 6.5 (6.54); N, 8.9 (9.09)]; δ_H[270 MHz; $(CD_3)_2SO$] 1.27–1.61 (15 H, s + m, 3 × Me + β-, γ- and δ-H_2), 2.89 (2 H, m, ε-H_2), 4.01 (1 H, m, α-CH), 4.23 (3 H, s, Fmoc α-H_2 + Fmoc 9-H), 5.07 (2 H, s, Zα-H_2), 6.78 (1 H, m, NH), 7.30–7.91 (14 H, m, 13 × ArH + 1 × NH), 9.22 (1 H, s, NH) and 9.84 (1 H, s, NH); $[\alpha]_D^{20}$ −12.4 (*c* 1, DMF).

Fluoren-9-ylmethoxycarbonyl-(N$^\varepsilon$-tert-*butoxycarbonyl*)-L-*lysylhydrazide* **7b**.—*N*-Benzyloxycarbonyl-*N'*-[fluoren-9-ylmethoxycarbonyl-(N$^\varepsilon$-*tert*-butoxycarbonyl)-L-lysyl]hydrazine **6b** (0.74 g, 1.2 mmol) was dissolved in 70% aq. 1,4-dioxane (40 cm^3) containing 10% palladium-on-carbon (0.37 g) and the rapidly stirred mixture was cooled to 0 °C. Hydrogen gas was gently bubbled into the reaction mixture and the reaction was monitored by TLC. After 2 h, hydrogenation was stopped. TLC revealed the presence of dibenzofulvene and free amino group (see above). The reaction mixture was filtered through Celite and the insoluble material was washed with 1,4-dioxane (4 × 50 cm^3). The solvent was removed from the filtrate and washings by rotary evaporation under reduced pressure. The residue was dissolved in chloroform (100 cm^3) and the mixture was extracted with water (2 × 200 cm^3) followed by saturated aq. NaCl (2 × 200 cm^3). The chloroform layer was dried over Na_2SO_4, then was filtered, and the solvent was removed from the filtrate by rotary evaporation under reduced pressure. The product was stirred in hexane (150 cm^3) for 2 h. The insoluble material was recovered by filtration and was dried *in vacuo* for 16 h (0.47 g, 72%), m.p. 93–98 °C; HPLC (10–90% B gradient during 25 min) t_R 18.7 min (97%); TLC [EtOAc–hexane (2:1)] R_f 0.08 (UV positive) (Found: $C_{26}H_{34}N_4O_5$, M_r = 482.5) FAB–MS (+ve *m/z*) 483.7 $[M + H]^+$ and 383.7 [483.7 − Boc] [$C_{26}H_{34}N_4O_5$: Found (requires): C, 65.8* (64.71); H, 7.25 (7.10); N, 10.7* (11.61)]; δ_H[270 MHz; $(CD_3)_2SO$] 1.36–1.61 (15 H, s + m, 3 × Me + β- + γ- + δ-H_2), 2.89 (2 H, m, ε-H_2), 4.22 (4 H, m, α-CH, Fmoc α-H_2 and 9-H), 6.79 (1 H, t, N'-H), 7.30–7.91 (9 H, m, 8 × ArH + 1 × NH) and 10.04 (1 H, d, NH_α); $[\alpha]_D^{20}$ +15.1 (*c* 1, DMF).

4-(tert-*Butoxycarbonylamino*)*butyraldehyde Diethyl Acetal* **9**.—4-Aminobutyraldehyde diethyl acetal **8** (90% reagent; 242 mg, 1.35 mmol) was stirred in chloroform (5 cm^3) and a solution of di-*tert*-butyl dicarbonate (315 mg, 1.4 mmol) in $CHCl_3$ (5 cm^3) was added over a period of 10 min. The reaction mixture was stirred for 2 h at ambient temperature and was then

* Microanalysis affected by the presence of residual hexanes, from NMR, not removed on extensive vacuum drying.

concentrated under reduced pressure to give a crude syrup (360 mg, 102%).

N-(tert-*Butoxycarbonyl*)*pyrrolidin*-2-*ol* **10**.—Crude acetal **9** (270 mg) was dissolved in THF (6 cm^3) and the solution was stirred vigorously. Aq. HCl (0.5 mol dm^{-3}; 3 cm^3, 1.5 mmol) was added dropwise during 5 min and the mixture was stirred for 1 h. The mixture was cooled in ice, diethyl ether (8 cm^3) was added, and the aqueous layer was adjusted to pH 8 with 1 mol dm^{-3} NaOH. The organic layer was separated, the aqueous layer was washed with diethyl ether (3 × 10 cm^3), and the combined organics were dried (Na_2SO_4). Removal of the solvent under reduced pressure gave a crude syrup (220 mg, 88%).

Isomeric Hydrazones **12**.—Fmoc-Val-$NHNH_2$ **7a** (330 mg, 0.93 mmol) was suspended in THF (10 cm^3) and a solution of crude pyrrolidinol **10** (220 mg) in THF (5 cm^3) was added. The mixture was refluxed for 4 h, cooled (contains a small amount of solid), and stirred overnight. TLC (75% EtOAc–25% hexane) gave three spots, at R_f 0.15 (weak) (Fmoc-Val-$NHNH_2$), R_f 0.6 (UV very strong) and R_f 0.7–0.9 (several weak UV-positive spots).

The mixture was filtered, and the filtrate was evaporated under reduced pressure to give flakes (550 mg), HPLC (10–90% B gradient during 25 min) t_R 16.59 min (Fmoc-Val-$NHNH_2$) (65%) and t_R 24.33–25.36 (minor Fmoc-containing peaks).

The crude flakes, which consisted of a major new spot on TLC, were hydrolysed back to Fmoc-Val-$NHNH_2$ in 0.1% aq. TFA.

N-(tert-*Butoxycarbonyl*)-4-[N′-(*fluoren*-9-*ylmethoxycarbonyl*-L-*valyl*)*hydrazino*]*butylamine* **13**.—Crude compound **12** (550 mg, assuming 1.0 mmol) was partly dissolved in stirred ethanol (10 cm^3). A mixture of sodium cyanoborohydride (63 mg, 1.0 mmol) in ethanol (0.5 cm^3) was added over a period of 10 min and the mixture was stirred for a further 1 h. HPLC (10–90% B in A during 25 min), t_R 16.62 (Fmoc-Val-$NHNH_2$) (small) and t_R 20.18 min (new Fmoc peak) (major).

After the mixture had been stirred for a further 1 h, the solvents were removed under reduced pressure, and ethyl acetate (15 cm^3) was added. To this stirred, cloudy solution cooled in ice was added 0.5 mol dm^{-3} HCl (3 cm^3). The now clear solution was stirred vigorously for 15 min, then was neutralized with saturated aq. $NaHCO_3$. The organic layer was separated, the aqueous layer was washed with EtOAc (3 × 10 cm^3), and the combined organics were dried over Na_2SO_4. Removal of the solvent under reduced pressure gave crude compound **13** as a solid (480 mg), HPLC (10–90% B gradient during 25 min) t_R 20.18 min (major new Fmoc peak).

Crude compound **13** (480 mg) was purified over silica gel (75 g) packed in 75% EtOAc–25% hexane. Compound **13** was loaded onto the column in $CHCl_3$ (3 cm^3), and the column was eluted with 75% EtOAc–25% hexane (500 cm^3). A number of minor UV-positive spots were collected, and re-analysis by HPLC gave t_R 24–26 min. The solvent was then changed to 100% EtOAc and a major UV-positive fraction was collected. Evaporation under reduced pressure gave a solid, which was purified compound **13** (110 mg, 23% from **7a**), m.p. 167 °C; HPLC (10–90% B gradient during 25 min), t_R 20.10 min (>99%); TLC (EtOAC) R_f 0.50 (UV positive) (Found: $C_{29}H_{40}N_4O_5$, M_r = 524.6) FAB–MS (+ve *m*/*z*) 547.6 [M + Na]$^+$, 525.6 [M + H]$^+$ and 425.4 (525.6 − Boc) [$C_{29}H_{40}N_4O_5$·H_2O: Found (requires): C, 64.75 (64.19); H, 7.4 (7.80); N, 10.0 (10.32)]; $[\alpha]_D^{20}$ −3.9 (*c* 1, DMF).

(*Methylthio*)*acetaldehyde* **15**.—(Methylthio)acetaldehyde dimethyl acetal **14** (97% reagent; 7.0 g, 50 mmol) was refluxed in 1% aq. HCl (20 cm^3) for 30 min, then the solution was cooled to room temp. The solution was neutralized with saturated aq. $NaHCO_3$ (~4 cm^3) to pH 7, and extracted with dichloromethane (3 × 25 cm^3). The combined organics were dried (Na_2SO_4), and carefully reduced to ~10 cm^3 at 0 °C under reduced pressure. The solution was distilled, and an initial distillate of ~7 cm^3 at 40 °C was discarded. Aldehyde **15** was then collected as an oil (3.0 g, 67%), b.p. 128–132 °C (lit.,[21] 129–134 °C).

Isomeric Hydrazones **16**.—Fmoc-Lys(Boc)-$NHNH_2$ **7b** (470 mg, 0.97 mmol) was dissolved in dry THF (10 cm^3), and a solution of aldehyde **15** (90 mg, 1.0 mmol) in dry THF (2 cm^3) was added. The mixture was left at room temp. overnight. TLC (EtOAc) gave R_f 0.10 (trace **7b**) and R_f 0.80 (new Fmoc peak). The solvent was removed under reduced pressure to give pale grey flakes (550 mg, 98%).

2-{N′-[*Fluoren*-9-*ylmethoxycarbonyl*-L-(N$^{\varepsilon}$-*Boc*)*lysyl*]*hydrazino*}-1-(*methylthio*)*ethane* **17**.—Crude hydrazones **16** (550 mg, assuming 1.0 mmol) were partially dissolved in stirred ethanol (10 cm^3). A solution of sodium cyanoborohydride (63 mg, 1.0 mmol) in ethanol (0.5 cm^3) was added during 10 min and the mixture was stirred for a further 2 h. HPLC (10–90% B gradient during 25 min) t_R 18.50 min [Fmoc-Lys(Boc)-$NHNH_2$, from the hydrazone], t_R 21.63 min (new Fmoc peak).

After a further 3 h, HPLC showed no compositional change. More $NaBH_3CN$ (63 mg, 1 mol equiv.) was added and the reaction mixture was left overnight. The solvents were then removed under reduced pressure and the solid residue was partly dissolved in ethyl acetate (60 cm^3). The suspension was washed with 0.5 mol dm^{-3} HCl (3 × 50 cm^3), dried (Na_2SO_4), and evaporated under reduced pressure to give crude compound **17** as an off-white solid (370 mg). The crude solid (370 mg) was purified over silica gel (75 g) as detailed for compound **13**. Appropriate fractions were pooled, and evaporated under reduced pressure to give solid compound **17** (150 mg, 27%); m.p. 155–158 °C; HPLC (10–90% B gradient during 25 min) t_R 20.65 min (>98%); TLC (EtOAc) R_f 0.50 (UV positive) (Found: $C_{29}H_{40}N_4O_5S$, M_r = 556.6) FAB–MS (+ve *m*/*z*) 579.5 [M + Na]$^+$, 557.6 [M + H]$^+$ and 457.5 (557.6 − Boc) [$C_{29}H_{40}N_4O_5S$: Found (requires): C, 62.4 (62.57); H, 7.3 (7.24); N, 10.0 (10.06)]; $[\alpha]_D^{20}$ −3.3 (*c* 1, DMF).

Solid-phase Synthesis of Core Sequence: H-Asp(*OBu*^{t})-*Ala-Glu*(*OBu*^{t})-*Phe-Arg*(*Mtr*)-*PepSyn KA* **18**.—The title, C-terminal pentapeptide sequence was assembled by standard procedures on the Biolynx synthesizer using Fmoc-Arg(Mtr)-PepSyn KA resin (1.2 g; Milligen) and pentafluorophenyl–HOBt or ODhbt [for Arg(Mtr)] protocols [Found (peptide-resin) Asp (1), 1.00; Glu (1), 1.05; Ala (1), 1.02; Nle (1), 1.07; Phe (1), 0.99; Arg (1), 1.00].

Cleavage of the dried peptide resin (30 mg) with TFA–ethanedithiol (EDT) (95 : 5; 2 cm^3; 7 h) gave the crude peptide: HPLC (10–90% B gradient during 25 min), t_R 4.91 min (94%).

Solid-phase Synthesis of Sequence **1**.—Core sequence **18** (300 mg, 0.03 mmol) was extended a further residue by using Fmoc-Met-OPfp–HOBt and was then N^{α}-Fmoc-deprotected on the Biolynx synthesizer by using standard protocols. The dried resin was treated with bis-2,4-dinitrophenyl carbonate **3**[17] (74.1 mg, 0.15 mmol) in dry DMF (1 cm^3). After 5 min, *N*-methylmorpholine (16.6 mm^3, 0.15 mmol) was added and the mixture was left for 30 min. The excess of solution was then drawn off with a Pasteur pipette and a solution of Fmoc-Val-NHNH[CH_2]$_4$NHBoc **13** (78 mg, 0.15 mmol) in dry DMF (500 mm^3) was added. After 60 min the resin was washed successively with DMF, then with diethyl ether, and was dried *in vacuo*.

The remaining two residues were added *via* pentafluorophenyl ester–HOBt [for Glu(OBu^t)] and ODhbt ester [for Ser(Bu^t)] on the Biolynx synthesizer. The completed peptide-resin (280 mg) was cleaved with TFA–EDT (90:10; 10 cm^3) for 7 h to give crude peptide **1**: HPLC (10–90% B gradient during 25 min), t_R 10.02 min (16%), t_R 11.42 min (61%) (Fig. 1a).

The two main peaks were isolated by using multiple injections on a semi-preparative HPLC column (F1 and F2) (10–30% B gradient during 17 min) as described in the general methods.

F1; hydrolysis for 72 h [Found: (purified peptide) Asp (1), 1.00; Ser (1), 0.69; Glu (2), 2.06; Ala (1), 1.04; Val (1), 0.86; Met (1), 1.04; Phe (1), 1.09; Arg (1), 1.04] yield 1.2 μmol; HPLC (5–40% B gradient during 25 min), t_R 15.90 min (> 99%) (Fig. 1b); FAB–MS (+ve *m/z*) 1212.0 $[M + H]^+$ and 794.0 (right-hand side of aza-bond cleavage, Scheme 5).

F2; hydrolysis for 72 h [Found: (purified peptide) Asp (1), 0.17; Glu (2), 1.04; Ala (1), 1.24; Met (1), 0.13; Phe (1), 1.02; Arg (1), 1.00]; FAB–MS (+ve *m/z*) 794.0 $[M + H]^+$ (hydantoin).

Solid-phase Synthesis of Sequence **2**.—(a) *Preparation of hydantoin standard.* Core sequence **18** (100 mg, 0.01 mmol) was treated with a solution of bis-2,4-dinitrophenyl carbonate **3**[17] (247 mg, 0.05 mmol) in DMF (0.4 cm^3) for 2 min. *N*-Methylmorpholine (5.0 mg, 0.05 mmol) was added and the reaction was allowed to proceed for 60 min. Cleavage of the dried peptide-resin (30 mg) with TFA–EDT (95:5; 2 cm^3; 7 h) gave the crude hydantoin: HPLC (10–90% B gradient during 25 min), t_R 9.81 min (84%).

(b) *Completion of target sequence* **2**. Core sequence **18** (400 mg, 0.04 mmol) was treated with a solution of bis-2,4-dinitrophenyl carbonate **3**[17] (98.8 mg, 0.20 mmol) in DMF (3.0 cm^3) for 1 h. Excess of DMF was removed from the resin with the aid of a Pasteur pipette and was replaced by a solution of Fmoc-Lys(Boc)-NHNH[CH_2]$_2$SMe **17** (134 mg, 0.24 mmol) in DMF (1.5 cm^3). Reaction was allowed to proceed for 16 h. Cleavage of a small quantity (20 mg) of Fmoc-deprotected peptide-resin with TFA–EDT (95:5; 2 cm^3) gave the crude peptide: HPLC (10–90% B gradient during 25 min), t_R 9.54 min (72%); hydantoin peak t_R 9.96 min (15%) (Fig. 2) [Found: (crude peptide) Asp (1), 0.88; Glu (1), 1.06; Ala (1), 0.90; Lys (1), 0.77; Arg (1), 1.00].

The remaining residues (Ser, Glu, Val) were added *via* standard pentafluorophenyl–HOBt (Glu and Val) and ODHBt active ester (Ser) protocols on the Biolynx peptide synthesizer. Peptide-resin (300 mg) was cleaved with TFA–EDT (95:5; 10 cm^3) for 16 h to give the crude peptide: HPLC (10–90% B gradient during 25 min) (Fig. 3a), t_R 10.68 min (38%), t_R 12.4 min (35%).

The two main peaks were isolated using multiple injections on a semi-preparative HPLC column (F1 and F2) (gradient 10–30% B during 25 min) as described in the general methods.

F1 [Found: (purified peptide) Asp (1), 0.95; Ser (1), 0.87; Glu (2), 2.07; Ala (1), 1.02; Val (1), 0.93; Phe (1), 1.01; Lys (1), 0.94; Arg (1), 1.00 ref.)] yield 4.2 μmol; HPLC (10–90% B gradient during 25 min), t_R 10.70 min (> 97%) (Fig. 3b); FAB–MS (+ve *m/z*) 1212.6 $[M + H]^+$ and 663.5 (right-hand side of aza-bond cleavage).

F2 [Found: (purified peptide) Asp (1), 0.98; Ser (1), 0.90; Glu (2), 2.08; Ala (1), 0.95; Val (1), 0.95; Phe (1), 1.00; Lys (1), 0.92; Arg (1), 1.00] yield 3.0 μmol; HPLC (10–90% B gradient during 25 min), t_R 13.10 min (> 95%) (Fig. 3c); FAB–MS (+ve *m/z*) 1309.4 $[M + H]^+$ and 663.2 (right-hand side of aza-bond cleavage) (trifluoroacetylated sequence **2**).

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