Polypeptides. Part XIV.[1] A Comparative Study of the Stability towards Enzymes of Model Tripeptides containing α-Aza-amino-acids, L-Amino-acids, and D-Amino-acids

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The stability of three model tripeptides, L-lysyl-α-aza-phenylalanyl-L-leucine, L-lysyl-L-phenylalanyl-L-leucine, and L-lysyl-D-phenylalanyl-L-leucine, towards aminopeptidase, carboxypeptidase, thermolysin, and trypsin has been investigated. These tripeptides were synthesised by the classical method of peptide synthesis. The α-aza-phenylalanine-containing tripeptide was stable to the first three of these enzymes, but the L-lysyl–α-aza-phenylalanine bond was readily cleaved by trypsin. The tripeptide containing D-phenylalanine was stable to all these enzymes, whereas the tripeptide with L-phenylalanine was completely cleaved in 72 h.

GENERAL methods for the synthesis of α-aza-amino-acid derivatives and their incorporation into peptides are described in an earlier publication.[1] One of our objectives in incorporating an aza-amino-acid residue into a biologically active peptide was to increase the stability of the peptide to specific peptidases, thus increasing the duration of its activity. α-Aza-amino-acid residues are not protein constituents, and their inclusion in a peptide may change its conformation,[2] thus making it less easily recognisable by specific peptidases. Such a replacement in a part of the peptide not essential for biological activity should therefore result in increased biological activity, as observed by Niedrich *et al.*[2-4] The duration of the decrease in guinea-pig blood pressure brought about by [5-α-aza-asparagine]-eledoisin-(4—11)-octapeptide (3 to 4 times more active than the parent octapeptide) was 4 to 5 times greater than that due to the parent. When incubated with liver homogenate this peptide was inactivated 4 to 5 times more slowly than the parent.[3] Amino-acid residues in [5-α-aza-asparagine]-eledoisin-(4—11)-octapeptide [2] and [5-α-aza-asparagine]-oxytocin [5] situated just before the aza-amino-acid were removed easily by aminopeptidase treatment but amino-acids present after the aza-amino-acid residue were removed only slowly.

In other attempts to increase the biological life of peptides L-amino-acid or glycine residues have been replaced by D-amino-acids or by ' unnatural ' amino-acid residues (*e.g.* sarcosine, β-alanine, *etc.*). Although several such analogues of adrenocorticotrophin,[6-8] luteinising hormone-releasing hormone,[9,10] and eledoisin-(4—11)-octapeptide [2-4] have been synthesised, no systematic study of the relative stabilities of the linkages thus introduced has yet been reported. Our results of a quantitative study of the relative stabilities of the peptide linkages involving α-aza-amino-acids, L-amino-acids, and D-amino-acids are reported here. Three model tripeptides, L-lysyl-α-aza-phenylalanyl-L-leucine, L-lysyl-L-phenylalanyl-L-leucine, and L-lysyl-D-phenylalanyl-L-leucine were synthesised, and their stability towards aminopeptidase, carboxypeptidase, thermolysin, and trypsin was investigated. Since the rate of tryptic cleavage is known to be low when a positive charge is present near the susceptible peptide bond,[11] *N*-t-butoxycarbonyl derivatives of the model tripeptides were used for the experiments with trypsin.

L-Lysyl-α-aza-phenylalanyl-L-leucine was synthesised as follows. 1-Benzyl-2-t-butoxycarbonylhydrazine [1] reacted with *N*-carbonyl-L-leucine methyl ester to give *N*-t-butoxycarbonyl-α-aza-phenylalanyl-L-leucine methyl ester. Removal of the t-butoxycarbonyl group and condensation of the product with N^{α}-t-butoxycarbonyl-N^{ϵ}-benzyloxycarbonyl-L-lysine by the dicyclohexylcarbodi-imide–hydroxybenzotriazole method [12] gave N^{α}-t-butoxycarbonyl-N^{ϵ}-benzyloxycarbonyl-L-lysyl-α-aza-phenylalanyl-L-leucine methyl ester. Saponification with aqueous sodium hydroxide followed by hydrogenation over 5% palladised carbon gave N^{α}-t-butoxycarbonyl-L-lysyl-α-aza-phenylalanyl-L-leucine, and the t-butoxycarbonyl group was then removed.

L-Lysyl-L-phenylalanyl-L-leucine was synthesised from L-leucine methyl ester. Coupling with *N*-benzyloxycarbonyl-L-phenylalanine by the mixed anhydride method, with ethyl chloroformate and *N*-methylmorpholine, gave the protected dipeptide. Debenzyloxycarbonylation followed by a mixed anhydride coupling with N^{α}-t-butoxycarbonyl-N^{ϵ}-benzyloxycarbonyl-L-lysine gave N^{α}-t-butoxycarbonyl-N^{ϵ}-benzyloxycarbonyl-L-lysyl-L-phenylalanyl-L-leucine methyl ester. Successive saponification with aqueous sodium hydroxide and hydrogenation over 5% palladised carbon

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[4] S. Katzwinkel, H. Niedrich, and P. Oehme, Proceedings of the 12th European Peptide Symposium, Reinhardsbrunn Castle, German Democratic Republic, 1972, ed. H. Hanson and H-D. Kajubke, North-Holland Publishing Company, Amsterdam, 1973, p. 416.
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[11] B. F. Erlanger, N. Kokowsky, and W. Cohen, *Arch. Biochem. Biophys.*, 1961, **95**, 271.
[12] W. Konig and R. Geiger, *Chem. Ber.*, 1970, **103**, 788.

then gave N^{α}-t-butoxycarbonyl-L-lysyl-L-phenylalanyl-L-leucine, the t-butoxycarbonyl group of which was removed with hydrogen chloride in acetic acid. L-Lysyl-D-phenylalanyl-L-leucine was synthesised similarly except that *N*-benzyloxycarbonyl-D-phenylalanine was used.

The model peptides were treated with aminopeptidase in 0.1M-ammonium hydrogen carbonate buffer and the products were subjected to amino-acid analysis after various times (Table 1). L-Lysyl-L-phenylalanyl-L-leucine was completely cleaved in 72 h to give equal amounts of L-lysine, L-phenylalanine, and L-leucine.

Thermolysin, an enzyme which cleaves the peptide bonds formed by the amino-group of amino-acids having hydrophobic side chains, cleaved L-lysyl-L-phenylalanyl-L-leucine in 72 h. Amounts of L-lysyl-L-phenylalanine, L-leucine, L-lysine, and L-phenylalanyl-L-leucine liberated were estimated by amino-acid analysis (Table 3). In contrast, cleavage of L-lysyl-D-phenylalanyl-L-leucine and of L-lysyl-α-aza-phenylalanyl-L-leucine was very slow; after 72 h only 0.52 and 0.26% of leucine was liberated, respectively.

Since tryptic cleavage was studied with *N*-t-butoxycarbonyl derivatives of the model peptides, samples were

TABLE 1

Cleavage of model tripeptides by aminopeptidase

	Lys liberated (nmol)					Phe liberated (nmol)			Leu liberated (nmol)		
Peptide *	2 h	6 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
L-Lys-L-Phe-L-Leu	3.43	7.75	28.5	33.1	41.0	13.2	23.0	35.0	13.0	26.0	36.0
L-Lys-D-Phe-L-Leu			0.066	0.142	0.24						
L-Lys-Azphe-L-Leu			0.164	0.34	0.61						

* Total quantity loaded on amino-acid analyser 40 nmol.

TABLE 2

Cleavage of model tripeptides by carboxypeptidase

	Leu liberated (nmol)						Phe liberated (nmol)						Lys liberated (nmol)					
Peptide *	1 h	2 h	6 h	24 h	48 h	72 h	1 h	2 h	6 h	24 h	48 h	72 h	1 h	2 h	6 h	24 h	48 h	72 h
L-Lys-L-Phe-L-Leu	33.5	33.2	34.0	34.5	34.5		34.8	33.7	34.6	34.5			30.0	32.4	33.8	34.1	34.5	
L-Lys-D-Phe-L-Leu			3.1	5.7	6.7	7.2			0.090	0.175	0.185	0.51			0.092	0.173	0.189	0.48
L-Lys-Azphe-L-Leu			0.74	1.4	1.7	1.8									0.076	0.14	0.16	0.31

* Total amount of peptide loaded on amino-acid analyser 40 nmol.

TABLE 3

Cleavage of model peptides by thermolysin

	Lys-Phe liberated (nmol)					Leu liberated (nmol)					Lys liberated (nmol)					Phe-Leu liberated (nmol)				
Peptide *	3 h	6 h	24 h	48 h	72 h	3 h	6 h	24 h	48 h	72 h	3 h	6 h	24 h	48 h	72 h	3 h	6 h	24 h	48 h	72 h
L-Lys-L-Phe-L-Leu	4.5	10.0	36.0	43.1	56.2	4.2	10.2	36.2	44.0	58.3	8.6	18.2	53.9	67.5	84.5	8.4	18.8	51.6	65.1	84.1
L-Lys-D-Phe-L-Leu		0.15	0.30	0.45	0.71		0.18	0.32	0.44	0.73	} No detectable Lys or Phe-Leu									
L-Lys-Azphe-L-Leu							0.10	0.15	0.21	0.36										

* Total amount loaded on amino-acid analyser 150 nmol.

L-Lysyl-D-phenylalanyl-L-leucine and L-lysyl-α-aza-phenylalanyl-L-leucine were only partially cleaved after 72 h, liberating only 0.6 and 1.5% of lysine, respectively. No phenylalanine or leucine was liberated from either. Wergin [13] has reported a slow cleavage of leucylsemicarbazide (leucyl-aza-glycine amide, K_m 0.72×10^{-3} mol l^{-1}) in comparison with leucine amide and leucine hydrazide (K_m 3.1×10^{-3} mol l^{-1}) by leucine aminopeptidase.

Amounts of leucine, phenylalanine, and lysine liberated from the model tripeptides by the action of carboxypeptidase (A + B) are summarised in Table 2. Cleavage of L-lysyl-L-phenylalanyl-L-leucine was almost complete in 1 h but L-lysyl-D-phenylalanyl-L-leucine and L-lysyl-α-aza-phenylalanyl-L-leucine were only partially cleaved even after 72 h. L-Lysyl-D-phenylalanyl-L-leucine gave 18% leucine and 1.2% lysine, and L-lysvl-α-aza-phenylalanyl-L-leucine gave 4.5% leucine and 0.77% lysine, thus indicating that peptide bonds formed with α-aza-phenylalanine are much more stable than those formed with L-phenylalanine. The azatripeptide was a little more stable than the D-phenylalanine-containing tripeptide.

treated with trifluoroacetic acid before amino-acid analysis (Table 4). *N*-t-Butoxycarbonyl-L-lysyl-D-phenylalanyl-L-leucine was completely stable to trypsin during 48 h but *N*-t-butoxycarbonyl-L-lysyl-L-phenylalanyl-L-leucine and the aza-analogue were completely

TABLE 4

Cleavage of model peptides by trypsin

	Lys liberated (nmol)				
Peptide *	1.5 h	3.5 h	5.5 h	24 h	48 h
Boc-L-Lys-L-Phe-L-Leu	2.2	3.7	5.2	11.8	18.1
Boc-L-Lys-D-Phe-L-Leu					
Boc-L-Lys-Azphe-L-Leu	4.2	9.4	9.6	19.3	19.5

* Total amount loaded on amino-acid analyser 20 nmol.

cleaved in that time. The cleavage of the L-lysyl–aza-phenylalanine bond was faster than that of the L-lysyl–L-phenylalanine bond. Complete cleavage of the aza-phenylalanyl–L-leucine bond was achieved in 24 h but cleavage of the L-phenylalanyl–L-leucine bond was almost complete only after 48 h. Such an increased rate of splitting of the hydrazide linkage has been

[13] A. Wergin, *Naturwiss*, 1965, **52**, 34.

reported.[14] The lysyl–hydrazine bond of 1-(L-alanyl-L-lysyl)-2-benzyloxycarbonylhydrazine was completely cleaved in 30 min whereas the *C*-terminal amide bond of *N*-benzoyl-L-arginine amide was totally cleaved only after 6½ h. The cleavage of L-alanyl-L-lysine hydrazide was much slower (50% in 7 h). The above results are not strictly comparable because amide and hydrazide derivatives of different amino-acids were used. To investigate this point further we have studied the cleavage of *N*-t-butoxycarbonyl-L-lysine hydrazide, *N*-t-butoxycarbonyl-L-lysine amide, and 1-t-butoxycarbonyl-2-(*N*$^{\alpha}$-t-butoxycarbonyl-L-lysyl)hydrazine with trypsin (Table 5).

TABLE 5

Cleavage of *N*-t-butoxycarbonyl-L-lysine amide and hydrazides by trypsin

	Lys liberated (nmol)			
Compound *	1 h	7.5 h	24 h	55.5 h
Boc-Lys-NH_2	5.2	5.9	8.2	9.4
Boc-Lys-NH·NH_2	50.7	54.7	56.7	66.2
Boc-Lys-NH·NH-Boc	7.3	12.9	23.8	40.0

* Total amount loaded on amino-acid analyser 100 nmol.

The cleavage of the two hydrazides (40.0 and 66.2% in 55 h) was much faster than that of the amide (9.5% in 55 h). In contradiction to the results of Blanot and Bricas,[14] the cleavage of *N*$^{\alpha}$-t-butoxycarbonyl-L-lysine hydrazide (66.2% in 55 h) was even faster than that of 1-t-butoxycarbonyl-2-(*N*$^{\alpha}$-t-butoxycarbonyl-L-lysyl)-hydrazine (40% in 55 h).

Our results demonstrate that α-aza-replacement may markedly decrease the susceptibility of peptides to proteolytic enzymes, but that the reverse may apply in the case of enzymes with trypsin-like specificity. In particular, the stability conferred towards carboxypeptidase seems greater, and the stability towards aminopeptidase and thermolysin appears similar to that resulting from change to D-configuration at the point of replacement.

However, the generality of our conclusions should not be overemphasised. The overall conformation of a large peptide depends, in addition to its primary structure, on several other forces, *e.g.* hydrogen-bond, van der Waals, and charge-transfer interactions. α-Aza-substitution may change local conformation, but at the same time the other forces may restore the conformation to its original form. The small model peptides used in this study have a relatively small number of conformations available, and α-aza-substitution might be expected to have a considerable effect on overall conformation (especially with regard to orientation of the α-side-chain). This change in conformation is probably the most likely reason for the stability to enzymes like aminopeptidase, carboxypeptidase, and thermolysin which require an L-amino-acid residue as substrate.

To explain the increased susceptibility towards trypsin it may be argued conversely that the change in conformation makes the aza-analogue a better substrate for trypsin. The presence of the t-butoxycarbonyl group would have some effect on conformation, but it is assumed that effects are similar in all the cases. However, since trypsin is an acyl group transfer enzyme, the presence of azaphenylalanine in the tripeptide could effect the rate of cleavage in two other ways: it could either increase the rate of formation of the acyl enzyme or change the rate of regeneration of the enzyme. At present we have no evidence to support either of these possibilities.

EXPERIMENTAL

Ascending thin-layer chromatograms were run on Keiselgel G plates. Details of solvent mixtures and spray reagents are given in Part XIII.[1] Optical rotations were determined with a Bendix NPL 143C automatic polarimeter with 154C digital converter. Evaporations were carried out under reduced pressure, usually below 40 °C in a rotary evaporator.

Enzymic Hydrolysis.—Aminopeptidase M (Rohm, GMBH Chemische Fabrik), carboxypeptidase A and B (Sigma Chemical Co.), trypsin — TPCK (essentially free of chymotrypsin, Worthington Biochemical Corp.), and thermolysin (Daiwa Kaseki K.K., Osaka, Japan) were used.

To a solution of the peptide (5 mg) in 0.1M-ammonium hydrogen carbonate buffer (4.5 ml), calcium chloride (0.01M; 0.4 ml), and enzyme (0.1 ml, substrate–enzyme ratio 100 : 1) were added and the solution was incubated at 37 °C for 72 h. Samples were taken at intervals, diluted with pH 2.2 buffer [prepared by dissolving citric acid (21 g), sodium hydroxide (8.4 g), concentrated hydrochloric acid (16 ml), sodium octanoate (0.1 g), and thiodiethanol (5 ml) in distilled water (1 l)], and stored at −20 °C. The amounts of amino-acids and dipeptides liberated were estimated by amino-acid analysis. In case of trypsin, where *N*-t-butoxycarbonyl-tripeptides were used, the sample (0.1 ml) was treated with trifluoroacetic acid (0.9 ml) for 1 h at room temperature. Trifluoroacetic acid was removed *in vacuo*, the residue was dissolved in pH 2.2 buffer, and the amount of lysine was estimated by amino-acid analysis.

N-t-Butoxycarbonyl-α-aza-phenylalanyl-L-leucine Methyl Ester.—1-Benzyl-2-t-butoxycarbonylhydrazine [1] (11.1 g, 50 mmol) was dissolved in tetrahydrofuran (50 ml), *N*-carbonyl-L-leucine methyl ester [1] (8.55 g, 50 mmol) was added, and the mixture was left overnight at room temperature. The solution was then evaporated and the residue dissolved in ethyl acetate; this solution was washed with 10% citric acid solution and water, dried (Na_2SO_4), and evaporated. Recrystallisation of the solid from ethyl acetate–light petroleum gave the *azadipeptide* (18 g, 92.4%), m.p. 91—92°, $[\alpha]_D^{22}$ −23.2° (*c* 2.3 in MeOH), R_{FD} 0.80, R_{FE} 0.68, R_{FF} 0.70, R_{FH} 0.70, R_{FK} 0.93 (Found: C, 61.1; H, 7.7; N, 10.7. $C_{20}H_{31}N_3O_5$ requires C, 61.2; H, 7.9; N, 10.7%).

N$^{\alpha}$*-t-Butoxycarbonyl-N*$^{\epsilon}$*-benzyloxycarbonyl-L-lysyl-α-aza-phenylalanyl-L-leucine Methyl Ester.*—The preceding compound (3.93 g, 10 mmol) was treated with 2N-hydrochloric acid in ethyl acetate (15 ml, 30 mmol) at room temperature for 1 h. Ethyl acetate was evaporated off; the residue had solidified to a foam after 1 h *in vacuo*. The hydrochloride, dissolved in dimethylformamide (25 ml), was added to a solution of *N*$^{\alpha}$-t-butoxycarbonyl-*N*$^{\epsilon}$-benzyloxycarbonyl-L-

[14] D. Blanot and E. Bricas, Proceedings of the 12th European Peptide Symposium, Reinhardsbrunn Castle, German Democratic Republic, ed. H. Hanson and H-D. Jakubke, North-Holland Publishing Company, Amsterdam, 1973, p. 420.

lysine [15] (3.8 g, 10 mmol) and triethylamine (1.4 ml) in dimethylformamide (10 ml), followed by dicyclohexylcarbodi-imide (2.1 g, 10 mmol), and 1-hydroxybenzotriazole (1.35 g, 10 mmol). The mixture was stirred overnight at room temperature, dicyclohexylurea was filtered off, and the filtrate was evaporated to dryness. The residue was taken up in ethyl acetate and the remaining dicyclohexylurea was filtered off. The solution was washed with water, 10% citric acid solution, saturated sodium hydrogen carbonate solution, and water, dried (Na_2SO_4), and evaporated to dryness. Recrystallisation from aqueous methanol gave the *tripeptide* derivative (3.4 g, 52%), m.p. 119—121°, $[\alpha]_D^{22}$ −16.4° (*c* 2.1 in MeOH), R_{FA} 0.86, R_{FB} 0.94, R_{FC} 0.91, R_{FD} 0.78, R_{FE} 0.68, R_{FF} 0.69, R_{FH} 0.74, R_{FK} 0.96 (Found: C, 62.2; H, 7.7; N, 10.7. $C_{34}H_{49}N_5O_8$ requires C, 62.2; H, 7.5; N, 10.6%).

N$^{\alpha}$-*t-Butoxycarbonyl*-N$^{\epsilon}$-*benzyloxycarbonyl*-L-*lysyl*-α-*aza*-*phenylalanyl*-L-*leucine*.—The preceding tripeptide derivative (3.28 g, 5 mmol) was dissolved in methanol (30 ml) and 1N-sodium hydroxide (7.5 ml, 7.5 mmol) was added. The saponification was complete in 90 min. Methanol was evaporated off *in vacuo* and the residue taken up in water (300 ml) and extracted with ethyl acetate; the aqueous solution was acidified with citric acid. The precipitated *tripeptide* derivative was collected, washed with water, and dried; yield 2.4 g (75%), m.p. 105—107°, $[\alpha]_D^{22}$ −17.1° (*c* 1.6 in MeOH), R_{FA} 0.78, R_{FB} 0.72, R_{FC} 0.53, R_{FD} 0.60, R_{FE} 0.16, R_{FF} 0.68, R_{FH} 0.52, R_{FK} 0.91 (Found: C, 61.7; H, 7.1; N, 10.9. $C_{33}H_{47}N_5O_8$ requires C, 61.7; H, 7.3; N, 10.9%).

N$^{\alpha}$-*t-Butoxycarbonyl*-L-*lysyl*-α-*aza*-*phenylalanyl*-L-*leucine*.—The above tripeptide derivative (2.1 g, 3.4 mmol) was hydrogenated in aqueous 80% ethanol (100 ml) over 5% palladium–charcoal (0.5 g) for 90 min. The catalyst was filtered off, the filtrate evaporated to dryness, and the azapeptide triturated with ether and collected; yield 1.5 g (90%), m.p. 183—185°, $[\alpha]_D^{23}$ −7.3° (*c* 2 in MeOH), amino-acid ratios (16 h acid digest): Leu 1.0, Lys 0.96, R_{FA} 0.52, R_{FB} 0.72, R_{FC} 0.27, R_{FD} 0.47, R_{FF} 0.21, R_{FH} 0.11, R_{FK} 0.78 (Found: C, 59.1; H, 8.2; N, 13.4. $C_{25}H_{41}N_5O_6$ requires C, 59.1; H, 8.1; N, 13.7%).

L-*Lysyl*-α-*aza*-*phenylalanyl*-L-*leucine Dihydrochloride*.—A solution of the preceding peptide (102 mg, 0.2 mmol) in glacial acetic acid was treated with 2N-hydrochloric acid in ethyl acetate (0.5 ml) for 1 h. The *dihydrochloride* was precipitated with an excess of ether, collected, washed with ether, and dried; yield 88 mg (92%), m.p. 218—220° (decomp.), $[\alpha]_D^{21}$ +11.8° (*c* 1.8 in MeOH), R_{FA} 0.38, R_{FB} 0.70, R_{FC} 0.35, R_{FX} 0.29 (Found: C, 50.0; H, 7.1; N, 14.4. $C_{20}H_{35}Cl_2N_5O_4$ requires C, 50.0; H, 7.3; N, 14.5%).

N$^{\alpha}$-*t-Butoxycarbonyl*-N$^{\epsilon}$-*benzyloxycarbonyl*-L-*lysyl*-L-*phenylalanyl*-L-*leucine Methyl Ester*.—To a stirred solution of N$^{\alpha}$-t-butoxycarbonyl-N$^{\epsilon}$-benzyloxycarbonyl-L-lysine [15] (3.28 g, 8.6 mmol) and *N*-methylmorpholine (0.95 ml, 8.6 mmol) in tetrahydrofuran (40 ml), ethyl chloroformate (0.77 ml, 8.17 mmol) was added at −20 °C, and after stirring for 2 min a precooled solution of L-phenylalanyl-L-leucine methyl ester [16] (prepared by catalytic hydrogenation of the benzyloxycarbonyl derivative [16,17]) in dimethylformamide (10 ml) was added. The mixture was stirred at room temperature for *ca.* 60 h, filtered to remove *N*-methylmorpholine hydrochloride, and evaporated to dryness. The residue, in ethyl acetate, was washed with water, aqueous 20% citric acid, saturated sodium hydrogen carbonate solution, and water, dried, and evaporated. Recrystallisation from aqueous methanol yielded the *tripeptide* (4.22 g, 75%), m.p. 138°, $[\alpha]_D^{22}$ −32.2° (*c* 2.2 in MeOH), R_{FD} 0.78, R_{FE} 0.62, R_{FF} 0.71, R_{FH} 0.72 (Found: C, 63.8; H, 7.6; N, 8.9. $C_{35}H_{50}N_4O_8$ requires C, 64.2; H, 7.7; N, 8.6%).

N$^{\alpha}$-*t-Butoxycarbonyl*-N$^{\epsilon}$-*benzyloxycarbonyl*-L-*lysyl*-L-*phenylalanyl*-L-*leucine*.—A solution of the preceding methyl ester (1.96 g, 3.0 mmol) in methanol (40 ml) was treated with N-sodium hydroxide (4.5 ml, 4.5 mmol) for 3 h at room temperature. Methanol was evaporated off *in vacuo* and the residue taken up in water (250 ml) and acidified with citric acid at 0 °C. The product, which separated as an oil, was extracted into ethyl acetate; the extract was washed twice with water, dried (Na_2SO_4), and evaporated to leave the *tripeptide* acid as a white solid (1.84 g, 96%), m.p. 94° (decomp.), $[\alpha]_D^{23}$ −24.9° (*c* 2.3 in MeOH), R_{FA} 0.74, R_{FB} 0.74, R_{FC} 0.63, R_{FD} 0.60 (Found: C, 63.2; H, 7.4; N, 8.5. $C_{34}H_{48}N_4O_8$ requires C, 63.0; H, 7.6; N, 8.7%).

N$^{\alpha}$-*t-Butoxycarbonyl*-L-*lysyl*-L-*phenylalanyl*-L-*leucine*.—The preceding tripeptide derivative (1.6 g, 2.5 mmol) was dissolved in 80% ethanol (50 ml) and hydrogenated for 6 h over 5% palladium–charcoal. Some of the product precipitated out during hydrogenation. The mixture was warmed to 60 °C, the catalyst filtered off, and the filtrate evaporated to dryness to leave a white *solid* (1.2 g, 95%), m.p. 223° (decomp.), $[\alpha]_D^{22}$ −36.1° (*c* 2.1 in MeOH), R_{FA} 0.62, R_{FB} 0.68, R_{FC} 0.42, R_{FD} 0.52, R_{FK} 0.88 (Found: C, 61.4; H, 8.2; N, 11.0. $C_{26}H_{42}N_4O_6$ requires C, 61.6; H, 8.4; N, 11.1%)

L-*Lysyl*-L-*phenylalanyl*-L-*leucine Dihydrochloride*.—5N-Hydrochloric acid in ethyl acetate (0.8 ml, 4.0 mmol) was added to a solution of the preceding peptide (0.507 g, 1.0 mmol) in acetic acid (4 ml). After 30 min at room temperature anhydrous ether (20 ml) was added and the product collected, washed with ether, and dried to yield the *tripeptide dihydrochloride* (0.383 g, 69.5%), m.p. 278° (decomp.), $[\alpha]_D^{22}$ +15.5° (*c* 1.6 in MeOH), R_{FA} 0.39, R_{FB} 0.62, R_{FC} 0.21, R_{FD} 0.12, R_{FK} 0.40 (Found: C, 52.3; H, 7.5; N, 11.6. $C_{21}H_{34}Cl_2N_4O_4$ requires C, 52.6; H, 7.6; N, 11.7%).

N$^{\alpha}$-*t-Butoxycarbonyl*-N$^{\epsilon}$-*benzyloxycarbonyl*-L-*lysyl*-D-*phenylalanyl*-L-*leucine Methyl Ester*.—D-Phenylalanyl-L-leucine methyl ester, obtained by catalytic hydrogenation of the *N*-benzyloxycarbonyl derivative [16] (3.66 g, 8.6 mmol) was coupled with N$^{\alpha}$-t-butoxycarbonyl-N$^{\epsilon}$-benzyloxycarbonyl-L-lysine (3.28 g, 8.6 mmol) by the mixed anhydride method [with ethyl chloroformate (0.77 ml, 8.17 mmol) and *N*-methylmorpholine (0.95 ml, 8.6 mmol)] as described earlier for N$^{\alpha}$-t-butoxycarbonyl-N$^{\epsilon}$-benzyloxycarbonyl-L-lysyl-L-phenylalanyl-L-leucine methyl ester. Two recrystallisations from aqueous methanol gave the protected *tripeptide* as a white powder (4.22 g, 75%), m.p. 138°, $[\alpha]_D^{22}$ −7.52° (*c* 2 in MeOH), R_{FD} 0.85, R_{FE} 0.68, R_{FF} 0.80, R_{FH} 0.78 (Found: C, 64.2; H, 7.6; N, 8.5. $C_{35}H_{50}N_4O_8$ requires C, 64.2; H, 7.7; N, 8.6%).

N$^{\alpha}$-*t-Butoxycarbonyl*-N$^{\epsilon}$-*benzyloxycarbonyl*-L-*lysyl*-D-*phenylalanyl*-L-*leucine*.—1N-Sodium hydroxide (3 ml, 3.0 mmol) was added to a solution of the preceding methyl ester (1.81 g, 2.0 mmol) in methanol (15 ml) and the mixture was stirred for 4 h at room temperature. Methanol was evaporated off *in vacuo* and the residue was dissolved in water (100 ml) and acidified with citric acid at 0 °C. The

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precipitated *tripeptide acid* was collected, washed with water, and dried *in vacuo* (P_2O_5); yield 1.28 g (100%), m.p. 97° (decomp.), $[\alpha]_D^{22}$ −2.83° (*c* 2.1 in MeOH), R_{FA} 0.80, R_{FB} 0.76, R_{FC} 0.64, R_{FD} 0.64 (Found: C, 63.5; H, 7.5; N, 8.7. $C_{34}H_{48}N_4O_8$ requires C, 63.7; H, 7.6; N, 8.7%).

Nα-t-Butoxycarbonyl-L-lysyl-D-phenylalanyl-L-leucine.—The preceding peptide (0.96 g, 1.5 mmol) was hydrogenated in 80% ethanol over 5% palladium–charcoal (200 mg) for 5 h. The catalyst was filtered off and the filtrate evaporated to dryness. The residue was triturated with ether; the solid was then filtered off, washed with ether, and dried to give the *tripeptide* derivative (0.703 g, 92.5%), m.p. 177° (decomp.), $[\alpha]_D^{22}$ −1.32° (*c* 2.1 in MeOH), R_{FA} 0.62, R_{FB} 0.68, R_{FC} 0.31, R_{FD} 0.56, R_{FK} 0.78 (Found: C, 61.7; H, 8.2; N, 11.0. $C_{26}H_{42}N_4O_6$ requires C, 61.6; H, 8.4; N, 11.1%).

L-Lysyl-D-phenylalanyl-L-leucine Dihydrochloride.—A solution of the preceding peptide (253 mg, 0.5 mmol) in glacial acetic acid (1.2 ml) was treated with 5N-hydrochloric acid in ethyl acetate (0.4 ml, 2.0 mmol). Anhydrous ether (150 ml) was added after 1 h and the product was collected, washed with ether, and dried to yield the *tripeptide dihydrochloride* (0.186 g, 77.5%), m.p. 260° (decomp.), $[\alpha]_D^{22}$ +1.82° (*c* 2.3 in MeOH), R_{FA} 0.42, R_{FB} 0.71, R_{FC} 0.70, R_{FK} 0.21 (Found: C, 52.5; H, 7.8; N, 11.5. $C_{21}H_{36}Cl_2N_4O_4$ requires C, 52.6; H, 7.6; N, 11.7%).

Nα-t-Butoxycarbonyl-Nε-benzyloxycarbonyl-L-lysine Amide.—Ethyl chloroformate (0.45 ml, 4.75 mmol) was added to a stirred solution of *Nα*-t-butoxycarbonyl-*Nε*-benzyloxycarbonyl-L-lysine [15] (1.90 g, 5.0 mmol) and *N*-methylmorpholine (0.55 ml, 5.0 mmol) in dry tetrahydrofuran (50 ml) at −15 °C. Stirring was continued for 5 min at −15 °C and then dry ammonia was bubbled through the mixture for 5 min. After stirring for 2 h at room temperature the tetrahydrofuran was evaporated off and the residue taken up in ethyl acetate. The solution was washed with water, saturated sodium hydrogen carbonate solution, citric acid solution, and water, dried (Na_2SO_4), and evaporated. Recrystallisation from ethyl acetate gave the *amide* (1.30 g, 69%), m.p. 142°, $[\alpha]_D^{22}$ −27.8° (*c* 2 in MeOH), R_{FA} 0.81, R_{FB} 0.87, R_{FC} 0.85, R_{FD} 0.72, R_{FE} 0.44, R_{FF} 0.68, R_{FH} 0.68 (Found: C, 60.3; H, 7.8; N, 11.4. $C_{19}H_{29}N_3O_5$ requires C, 60.2; H, 7.7; N, 11.1%).

Nα-t-Butoxycarbonyl-L-lysine Amide.—The above lysine derivative (1.13 g, 3.0 mmol) was hydrogenated for 5 h over 5% palladium–charcoal in 80% ethanol (100 ml). The catalyst was filtered off and the filtrate evaporated to leave an oil which solidified to give the amide (0.589 g, 80%) on trituration with ether; m.p. 75—76° (decomp.), $[\alpha]_D^{23}$ −53.7° (*c* 2 in MeOH), R_{FA} 0.67, R_{FB} 0.70, R_{FC} 0.66, R_{FD} 0.61, R_{FK} 0.76 (Found: C, 53.7; H, 9.3; N, 17.2. $C_{11}H_{23}N_3O_3$ requires C, 53.8; H, 9.5; N, 17.1%).

1-Benzyloxycarbonyl-2-(Nα-t-butoxycarbonyl-Nε-benzyloxycarbonyl-L-lysyl)hydrazine.—To a stirred solution of *Nα*-t-butoxycarbonyl-*Nε*-benzyloxycarbonyl-L-lysine [15] (1.90 g, 5.0 mmol) and *N*-methylmorpholine (0.55 ml, 5.0 mmol) in dry tetrahydrofuran, ethyl chloroformate (0.45 ml, 4.75 mmol) was added at −20 °C and stirring was continued for 2 min. A precooled (−20 °C) solution of benzyloxycarbonylhydrazine [18] (0.83 g, 5 mmol) in tetrahydrofuran (5 ml) was then added, and the mixture was stirred for 2 h at room temperature. The solvent was removed *in vacuo* and the residue, in ethyl acetate, was washed with 20% citric acid, saturated sodium hydrogen carbonate solution, and water, and dried. Evaporation left the substituted *hydrazide* as a light yellow oil (2.45 g, 93%), $[\alpha]_D^{22}$ −29.1° (*c* 2.1 in MeOH), R_{FA} 0.84, R_{FB} 0.90, R_{FC} 0.87, R_{FD} 0.81, R_{FE} 0.62, R_{FF} 0.74, R_{FH} 0.76 (Found: C, 61.3; H, 7.1; N, 10.4. $C_{27}H_{36}N_4O_7$ requires C, 61.5; H, 6.8; N, 10.6%).

Nα-t-Butoxycarbonyl-L-lysine Hydrazine.—The preceding compound (2.28 g, 4.33 mmol) was hydrogenated over 5% palladium–charcoal (0.9 g) in 80% ethanol for 4 h. The catalyst was removed and the filtrate evaporated to leave an oil, purified by silica gel (200 g) column chromatography. The eluants were chloroform (200 ml), 5% methanol–chloroform (250 ml), 10% methanol–chloroform (250 ml), and 15% methanol–chloroform, which eluted the *hydrazide* (0.71 g, 64%), m.p. 127° (decomp.), $[\alpha]_D^{22}$ −27.6° (*c* 2.5 in MeOH), R_{FA} 0.62, R_{FB} 0.64, R_{FC} 0.58, R_{FD} 0.57, R_{FK} 0.68 (Found: C, 50.8; H, 9.3; N, 21.7. $C_{11}H_{24}N_4O_3$ requires C, 50.8; H, 9.2; N, 21.5%).

1-t-Butoxycarbonyl-2-(Nα-t-butoxycarbonyl-Nε-benzyloxycarbonyl-L-lysyl)hydrazine.—Ethyl chloroformate (0.45 ml, 4.75 mmol) was added to a stirred solution of *Nα*-t-butoxycarbonyl-*Nε*-benzyloxycarbonyl-L-lysine (1.90 g, 5.0 mmol) and *N*-methylmorpholine in tetrahydrofuran (25 ml) at −20 °C. After 5 min a solution of t-butoxycarbonylhydrazine [19] (0.66 g, 5.00 mmol) in tetrahydrofuran (5 ml) was added and stirring was continued for 3 h at room temperature. *N*-Methylmorpholine hydrochloride was filtered off and the filtrate was evaporated to dryness. The residue was taken up in ethyl acetate and the solution was washed with 20% citric acid, saturated sodium hydrogen carbonate solution, and water, dried (Na_2SO_4), and evaporated to give the *hydrazide* as an oil (2.42 g, 98%), $[\alpha]_D^{22}$ −24.4° (*c* 2 in MeOH), R_{FD} 0.76, R_{FE} 0.63, R_{FF} 0.72, R_{FH} 0.71 (Found: C, 57.9; H, 7.9; N, 11.2. $C_{24}H_{38}N_4O_7$ requires C, 58.2; H, 7.7; N, 11.3%).

1-t-Butoxycarbonyl-2-(Nα-t-butoxycarbonyl-L-lysyl)hydrazine.—A solution of the preceding compound (1.0 g, 2.02 mmol) in 80% ethanol (50 ml) was hydrogenated over 5% palladium–charcoal for 6 h. The catalyst was filtered off and the filtrate was evaporated to leave the *hydrazide* derivative as an oil (0.49 g, 68%), $[\alpha]_D^{22}$ −21° (*c* 2.2 in MeOH), R_{FA} 0.50, R_{FB} 0.64, R_{FC} 0.17, R_{FK} 0.10 (Found: C, 53.6; H, 8.6; N, 15.4. $C_{16}H_{32}N_4O_5$ requires C, 53.3; H, 8.9; N, 15.5%).

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