Polypeptides. Part 16.[1,2] Synthesis and Biological Activity of α-Aza-Analogues of Luliberin with High Antagonist Activity

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Analogues of luliberin (luteinising hormone-releasing hormone) were synthesised where the glycine residue in position 10 was replaced by either azaglycine (–NH–NH–CO–) or aza-alanine (–NH–NMe–CO–), the histidine residue in position 2 was either omitted, or replaced by D-phenylalanine or D-tryptophan, and the glycine residue in position 6 was substituted either by D-phenylalanine or D-tryptophan. These compounds were evaluated for their ability to prevent ovulation induced by luliberin in androgen-sterilised constant-oestrus rats. Compounds with the azaglycine residue in position 10 and other modifications in positions 2 and 6 showed good antagonist activity, whereas aza-alanine replacement in position 10 together with modifications in position 2 resulted in inactive compounds. The most potent analogue, [2-D-Phe-6-D-Phe-10-Azgly]-luliberin, completely inhibited ovulation induced by luliberin (0.5 μg/rat) at a dose of 15 μg/rat.

In an earlier publication [1,2] the synthesis and biological activity of [2-de-His-10-Azgly]-luliberin (1), an antagonist of luliberin (luteinising hormone releasing hormone) was reported. It completely prevented ovulation induced by luliberin (0.5 μg/rat) in androgen-sterilised constant-oestrus rats at a dose of 250 μg/rat and partially at doses of 62.5—125 μg/rat. The corresponding non-aza-analogue, [2-de-His]-luliberin (2), has been reported to have a weak antagonist activity which was only demonstrated in an *in vitro* test system.[3] On the basis

pGlu-X-Trp-Ser-Tyr-Y-Leu-Arg-Pro-Z

(1) X = nothing, Y = Gly, Z = Azgly–NH_2
(2) X = nothing, Y = Gly, Z = Gly–NH_2
(3) X = nothing, Y = D-Phe, Z = Azgly–NH_2
(4) X = nothing, Y = D-Trp, Z = Azgly–NH_2
(5) X = D-Phe, Y = D-Phe, Z = Azgly–NH_2
(6) X = D-Phe, Y = D-Trp, Z = Azgly–NH_2
(7) X = D-Trp, Y = D-Phe, Z = Azgly–NH_2
(8) X = D-Trp, Y = D-Trp, Z = Azgly–NH_2
(9) X = nothing, Y = Gly, Z = Azala–NH_2
(10) X = D-Phe, Y = Gly, Z = Azala–NH_2
(11) X = D-Trp, Y = Gly, Z = Azala–NH_2
(12) X = D-Phe, Y = D-Phe, Z = Gly–NH_2

of the apparently higher antagonist activity of [2-de-His-10-Azgly]-luliberin primarily due to the presence of an aza-amino-acid residue in position 10, several other analogues (3)—(11) have now been synthesised with azaglycine or aza-alanine replacements in position 10 along with modifications in positions 2 and 6. A known antagonist of luliberin [2-D-Phe-6-D-Phe]-luliberin, has also been synthesised for comparison with the aza-analogues in our own test systems.[4]

Antagonists of luliberin have earlier been synthesised by (*a*) omitting the histidine residue in position 2 (ref. 3), (*b*) substituting D-amino-acid residues, *e.g.* D-Phe or D-Trp, in the 2 and 6 positions,[4,5] or (*c*) replacing the tryptophan residue in position 3 with L-proline along with D-amino-acid substitutions in positions 2 and 6.[6-8] Some of the highly active antagonists derived from the above modifications, [2-D-Phe-6-D-Phe]-luliberin, and [2-D-Phe-3-Pro-6-D-Trp]-luliberin, were shown to block ovulation in normal-cycling rats at a dose of 500 μg to 1.5 mg/rat. Highly active antagonists of luliberin may be useful as antifertility agents.[9,10]

RESULTS AND DISCUSSION

The synthesis of each of the analogues (3)—(12) was achieved by coupling three peptide fragments (Figure). The *C*-terminal fragments, N^{α}-t-butoxycarbonyl-N^{ω}-nitro-L-arginyl-L-prolyl-azaglycine or -aza-alanine amide were prepared by a stepwise coupling procedure. The coupling of *N*-benzyloxycarbonyl-L-proline with aza-glycine amide (semicarbazide) or aza-alanine amide [11] by the dicyclohexylcarbodi-imide–hydroxybenzotriazole procedure [12] (DCCI–HOBt) gave the azadipeptide derivatives. During the work-up procedure (extraction of the ethyl acetate solution of the reaction mixture with water), *N*-benzyloxycarbonyl-L-prolylazaglycine amide dissolved in water but crystallised out when the aqueous solution was concentrated. *N*-Benzyloxycarbonyl-L-prolylaza-alanine amide was soluble in ethyl acetate and could, therefore, be purified by normal extraction procedure. Removal of the benzyloxycarbonyl protecting group (catalytic reduction over 5% Pd–C) followed by coupling with N^{α}-t-butoxycarbonyl-N^{ω}-nitro-L-arginine by DCCI–HOBt gave the azatripeptide derivatives. Both these protected tripeptides, N^{α}-t-butoxycarbonyl-N^{ω}-nitro-L-arginyl-L-prolyl-azaglycine or -aza-alanine amide, were highly soluble in water and were purified by a counter-current distribution procedure using an n-butanol–5% acetic acid solvent system and ion-exchange chromatography. Removal of the t-butoxycarbonyl protecting group by treatment with HCl in ethyl acetate gave N^{ω}-nitro-L-arginyl-L-prolyl-azaglycine or -aza-alanine amide.

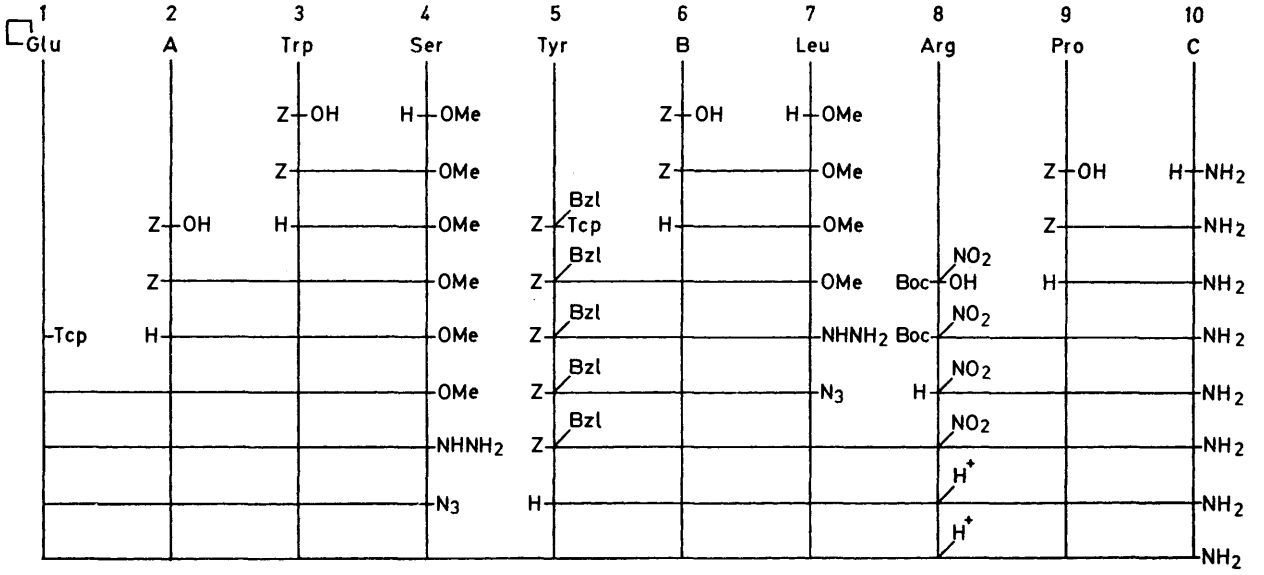
The middle tripeptide derivatives, *N*-benzyloxycarbonyl-*O*-benzyl-L-tyrosyl-B-L-leucine methyl ester (B = glycyl, D-phenylalanyl, or D-tryptophyl residue), were also prepared in a stepwise manner. L-Leucine methyl ester was coupled with either *N*-benzyloxycarbonylglycine, *N*-benzyloxycarbonyl-D-phenylalanine, or *N*-benzyloxycarbonyl-D-tryptophan using DCCI–HOBt. These dipeptides were hydrogenolysed over 5% Pd–C in the presence of 1 equiv. hydrogen chloride. The resulting peptides were reacted with *N*-benzyloxycarbonyl-*O*-benzyl-L-tyrosine 2,4,5-trichlorophenyl ester. Hydrazine hydrate treatment converted the tripeptide

methyl esters to the hydrazides which were then converted to the azides by Rudinger's method [13] before coupling to N^{ω}-nitro-L-arginyl-L-prolyl-azaglycine or -aza-alanine amide. The hexapeptide derivatives, *N*-benzyloxycarbonyl-*O*-benzyl-L-tyrosyl-B-L-leucyl-L-arginyl-L-prolyl-C-NH_2 (B = Gly, D-Phe, or D-Trp; C = Azgly or Azala) were purified either by crystallisation or by silica gel column chromatography eluting with chloroform–methanol. Catalytic hydrogenation (5% Pd–C) in the presence of 2 equiv. hydrogen chloride removed all protecting groups and the dihydrochlorides thus obtained were converted to the monohydrochlorides before coupling to the *N*-terminal tri- or tetra-peptide azides.

The synthesis of the *N*-terminal tri- or tetra-peptides was achieved as follows. *N*-Benzyloxycarbonyl-L-tryptophan was coupled with serine methyl ester by the DCCI–HOBt method to give *N*-benzyloxycarbonyl-L-tryptophyl-L-serine methyl ester. Debenzyloxycarbonylation (hydrogenolysis, 5% Pd–C) followed by coupling with either *N*-benzyloxycarbonyl-D-phenylalanine, *N*-benzyloxycarbonyl-D-tryptophan, or L-pyroglutamic acid 2,4,5-trichlorophenyl ester yielded the tripeptide derivatives. L-Pyroglutamyl-D-tryptophyl-L-serine methyl ester was converted to the hydrazide and then the azide for use in the preparation of [2-de-His]-analogues. The other tripeptides were hydrogenated and reacted with L-pyroglutamic acid 2,4,5-trichlorophenyl ester to give the desired tetrapeptide methyl esters. These were converted to hydrazides by hydrazine hydrate treatment.

Reaction of the *N*-terminal tri- or tetra-peptide derivatives with the *C*-terminal hexapeptides gave the appropriate luliberin analogues. All these analogues were purified by column chromatography on Sephadex LH-20 using DMF as solvent and then by partition chromatography on Sephadex G-25 using either n-butanol–acetic acid–water (4 : 1 : 5) or n-butanol–acetic acid–water–pyridine (5 : 1 : 5 : 1) solvent systems. The physical and chemical properties of the luliberin analogues are described in Table 1.

The biological (agonist and antagonist) activity of the analogues was measured in androgen-sterilised constant-oestrus rats.[14] Synthetic luliberin reliably induced ovulation in these rats at a dose of 0.5 μg/rat. When injected alone the analogues did not induce ovulation at dose levels of 250—2 000 μg/rat. However, when injected together with luliberin (0.5 μg/rat), compounds (3), (5), (7), and (12) blocked ovulation but the other compounds were inactive. A summary of the biological results obtained with the active analogous is recorded in Table 2. The most potent compound, [2-D-Phe-6-D-Phe-10-Azgly]-luliberin, blocked ovulation completely when injected (i.v. or s.c.) at doses of 15—250 μg/rat and partially at a dose of 8 μg/rat. When injected simultaneously (i.v. or s.c.) with luliberin the corresponding non-aza-analogue, [2-D-Phe-6-D-Phe]-luliberin, was fully active only at a dose of 125 μg/rat. The aza-analogue was also fully effective when injected (100 μg/rat, i.v.) up to 1 h before luliberin (0.5 μg/rat) and partially effective when injected up to 2 h before luliberin. The compounds containing D-tryptophan in position 2 and 6 and azaglycine in position 10 [(6) and (8)] were only tested at a maximum dose of 250 μg/rat due to the poor solubility in saline and were inactive at this dose level. The inactivity of these compounds is somewhat surprising, especially since in the non-aza-series they have been claimed to have good antagonist activity.[6-8] Aza-alanine substitution in position 10 either in the 2-de-His-series or in combination with D-amino-acid replacements in positions 2 and 6, also leads to inactive compounds. It is possible that the methyl group of the



Synthesis of luliberin analogues. A = D-Phe, D-Trp, or nothing; B = Gly, D-Phe, or D-Trp; C = Azgly (–NH–NH–CO–) or Azala [–NH–N(CH_3)–CO–]; Z = benzyloxycarbonyl; Boc = t-butoxycarbonyl; Bzl = benzyl; Tcp = 2,4,5-trichlorophenyl ester

aza-alanine residue interferes with the interaction of these analogues with the receptors. The low activity of [10-Ala]-luliberin (6% as active as luliberin in releasing LH and FSH) also indicates that a side-chain methyl group is not tolerated in this position.[15]

stable than the Pro–Gly linkage. Increased stability of the aza-linkages to enzymic degradation was also indicated from our earlier studies involving model tripeptides.[17] Although D-amino-acid replacements in position 6 and ethylamide substitution in position 10

TABLE 1

Physical and chemical characteristics of luliberin analogues

Compound	Yield (%)	Purification	R_{FA}	Paper electrophoresis R_F relative to luliberin pH 2.1	pH 6.5	Amino-acid analysis (16 h acid digest) Glu	Gly	Ser	Tyr	Phe	Leu	Arg	Pro
[2-de-His-6-D-Phe-10-Azgly]-luliberin	32.3	A,C	0.45	0.57	0.66	1.02		0.85	1.01	1.0	1.0	1.01	1.02
[2-de-His-6-D-Trp-10-Azgly]-luliberin	39.8	A,C	0.56	0.45	0.00	0.98		0.90	0.98		1.0	0.96	1.01
[2-D-Phe-6-D-Phe-10-Azgly]-luliberin	59.2	A,B	0.57	0.46	0.0	1.05		0.84	0.98	2.01	1.0	1.0	1.07
[2-D-Phe-6-D-Trp-10-Azgly]-luliberin	36.6	A,C	0.66	0.45	0.00	0.98		0.84	0.97	1.0	1.0	1.03	1.01
[2-D-Trp-6-D-Phe-10-Azgly]-luliberin	35.4	A,B	0.52	0.48	0.23	0.97		0.86	0.99	1.0	1.0	1.04	1.02
[2-D-Trp-6-D-Trp-10-Azgly]-luliberin	42.5	A,C	0.40	0.00	0.00	1.03		0.81	0.98		1.0	0.97	0.99
[2-D-Phe-6-D-Phe]-luliberin	40.3	A,C	0.42	0.51	0.48	1.02	1.01	0.88	0.96	2.02	1.0	0.96	0.96
[2-de-His-10-Azala]-luliberin	36.5	A,C	0.46	0.55	0.60	1.02	0.96	0.84	1.02		1.0	1.02	0.96
[2-D-Phe-10-Azala]-luliberin	42.3	A,C	0.40	0.47	0.64	1.04	0.97	0.85	1.07	1.04	1.0	1.01	1.01
[2-D-Trp-10-Azala]-luliberin	34.2	A,B	0.37	0.46	0.54	1.02	1.0	0.88	1.01		1.0	1.02	1.0

The results reported here and in an earlier publication [1] show that azaglycine substitution in position 10 along with other modifications in position 2 and/or 6 leads to highly active antagonists. Direct comparison of these aza-analogues with previously synthesised

have resulted in highly active agonists with increased metabolic stability, it has not been possible to synthesise good antagonists with ethylamide substitution in position 10 along with relevant modifications in the 2, 3, and 6 positions. In fact [2-de-His-6-D-Ala-10-de-Gly-NH_2-

TABLE 2

Antagonist activity of the luliberin analogues

Compound	Dose (μg/rat)	Luliberin dose (μg/rat)	Response (no. blocked/ no. treated)
[2-D-Phe-6-D-Phe-10-Azgly]-luliberin	125 (i.v.)	0.5	3/3
	62.5 (i.v. or s.c.)	0.5	11/12
	31.25 (i.v. or s.c.)	0.5	9/9
	15.2 (i.v. or s.c.)	0.5	9/9
	7.8 (i.v.)	0.5	12/18
	4.0 (i.v.)	0.5	7/15
[2-D-Phe-6-D-Phe]-luliberin	250 (i.v.)	0.5	3/3
	125 (i.v.)	0.5	3/3
	62.5 (i.v.)	0.5	2/3
[2-de-His-6-D-Phe-10-Azgly]-luliberin	250 (i.v.)	0.5	3/3
	125 (i.v.)	0.5	1/3
[2-D-Trp-6-D-Phe-10-Azgly]-luliberin	250 (i.v.)	0.5	3/3
	62.5 (i.v.)	0.5	1/3
	31.25 (i.v.)	0.5	0/3

antagonists cannot be made since the latter were tested in normal-cycling rats.

Since in our test system [2-D-Phe-6-D-Phe-10-Azgly]-luliberin (5) is about ten times more potent than [2-D-Phe-6-D-Phe]-luliberin (12), it is probable that the aza-analogue (5) is several times more potent than any of the antagonists so far described in the literature.

The high antagonist activity of the α-aza-analogues could either be due to an increased affinity for the receptor or to increased stability to enzymic degradation. In luliberin, the peptide bonds between 6-Gly-7-Leu and 9-Pro-10-Gly have been shown to be the main points of enzymic attack.[16] Results of preliminary investigations of the stability of the aza-analogues to tissue homogenates indicate that the Pro–Azgly linkage is more

9-Pro-ethylamide]-luliberin was less active than [2-de-His-6-D-Ala]-luliberin.[18] It can, therefore, be speculated that a combination of D-amino-acid replacement in positions 2 and 6, and azaglycine in position 10 generates compounds which have better affinity for the luliberin receptors and greater stability to enzymic degradation.

EXPERIMENTAL

Details of the solvent systems and spray reagents used for thin-layer chromatography are described in Part 13.[11] The phrase ' worked-up in the usual manner ' implies the following: after the reaction was over any solid material was removed by filtration and the filtrate was evaporated to dryness. The residue was taken up in ethyl acetate and washed with water, 20% citric acid solution, water, and saturated sodium hydrogencarbonate solution. The

organic layer was dried over anhydrous sodium sulphate and evaporated to dryness.

General Procedure for Coupling L-*Pyroglutamyl-A-*L-*tryptophyl-*L-*Serine Azide* (*A = Nothing,* D-*Phe, or* D-*Trp*) *with* L-*Tyrosyl-B-*L-*leucyl-*L-*arginyl-*L-*prolyl-C-Amide* (*B =* D-*Phe or* D-*Trp; C = Azgly or Azala*).—To a cooled (0 °C) and stirred suspension of L-pyroglutamyl-A-L-tryptophyl-L-serine hydrazide (0.275 mmol) in DMF (1.0 ml.) was added 5.92M hydrogen chloride in dioxan (1.1 mmol). The clear solution obtained after several minutes stirring was further cooled to −20 °C and to it was added t-butyl nitrite (0.29 mmol). Stirring was continued for 20 min at −20 °C and the solution was neutralised by the addition of triethylamine (1.1 mmol). A pre-cooled (−20 °C) mixture of L-tyrosyl-B-L-leucyl-L-arginyl-L-prolyl-C amide dihydrochloride [0.25 mmol, obtained by the hydrogenolysis of *N*-benzyloxycarbonyl-*O*-benzyl-L-tyrosyl-B-L-leucyl-(N^{ω}-nitro)-L-arginyl-C amide in aqueous methanol (80% v/v) for 40 h over Pd–C (5% w/w) in the presence of 2 equiv. hydrogen chloride] and triethylamine (0.25 mmol) in DMF (1.0 ml) was added. The mixture was stirred for 24 h at 4 °C. The reaction mixture was applied directly to a Sephadex LH-20 column using DMF as eluant (purification procedure A). The peptide was further purified by partition chromatography on Sephadex G-25 using either n-butanol–acetic acid–water–pyridine (5 : 1 : 5 : 1 v/v) (purification procedure B) or n-butanol–acetic acid–water (4 : 1 : 5) (procedure C).

N-t-*Butoxycarbonyl-*N^{ω}*-nitro-*L-*arginyl-*L-*prolylazaglycine Amide.*—A solution of N^{α}-t-butoxycarbonyl-N^{ω}-nitro-L-arginine (13.5 g, 42.3 mmol), L-prolylazaglycine amide hydrochloride (9.81 g, 47 mmol),[1] 1-hydroxybenzotriazole (11.5 g, 85 mmol), and triethylamine (6.58 ml, 47 mmol) was cooled to 0 °C and dicyclohexylcarbodi-imide (9.13 g, 44.4 mmol) was added. The reaction mixture was stirred overnight at 4 °C, filtered to remove the solid material, and the filtrate was evaporated to dryness *in vacuo*. The residue was partitioned between ethyl acetate and water by counter-current distribution (4 transfers). The aqueous phases were combined, evaporated to dryness, and the residue was partitioned between n-butanol and aqueous acetic acid (5% v/v) by counter-current distribution (12 transfers). The crude peptide obtained by evaporating the combined n-butanol phases was purified by silica gel column chromatography using methanol–chloroform (5% v/v, 10% v/v, and 20% v/v) as eluting solvents. The product-containing fractions were combined, evaporated to dryness, and an aqueous solution of the residue was passed through an anion-exchange resin (AG 1X-2) column to remove N^{α}-t-butoxycarbonyl-N^{ω}-nitro-arginine. The column was then washed with water, and the combined aqueous phases and the washings were freeze-dried, to give the azapeptide derivative (13.82 g, 69%), m.p. 135—136 °C, R_{FA} 0.49, R_{FB} 0.65, R_{FC} 0.46, R_{FD} 0.64, R_{FF} 0.35, R_{FH} 0.19, and R_{FK} 0.86 (Found: C, 43.3; H, 6.6; N, 26.5. $C_{17}H_{31}N_9O_7$ requires C, 43.1; H, 6.6; N, 26.6%).

N-*Benzyloxycarbonyl-*O-*benzyl-*L-*tyrosyl-*D-*phenylalanyl-*L-*leucine Methyl Ester.*—To a stirred solution of *N*-benzyloxycarbonyl-*O*-benzyl-L-tyrosine 2,4,5-trichlorophenyl ester (4.89 g, 8.36 mmol) and D-phenylalanyl-L-leucine methyl ester hydrochloride [17] (2.5 g, 7.6 mmol) in DMF, triethylamine (1.1 ml, 7.6 mmol) was added and the stirring was continued overnight at room temperature. Triethylamine hydrochloride was filtered off, the filtrate was evaporated to dryness, and recrystallisation of the residue from aqueous methanol gave the tripeptide derivative (3.6 g, 69.7%), m.p. 183—184 °C, R_{FD} 0.82, R_{FE} 0.69, R_{FH} 0.78, R_{FP} 0.71, and R_{FQ} 0.82 (Found: C, 70.6; H, 6.7; N, 6.2. $C_{40}H_{45}N_3O_7$ requires C, 70.6; H, 6.6; N, 6.1%).

N-*Benzyloxycarbonyl-*O-*benzyl-*L-*tyrosyl-*D-*phenylalanyl-*L-*leucine Hydrazide.*—A solution of the preceding methyl ester (3.42 g, 5.04 mmol) and hydrazine hydrate (60 mmol) in DMF (30 ml) was stirred at room temperature for 4 h, concentrated to a small volume, and the hydrazide was precipitated by the addition of water (500 ml). It was collected, washed with water, methanol–ether (1 : 4 v/v), and ether, and dried (2.94 g, 85.9%), m.p. 179—180 °C, R_{FA} 0.81, R_{FB} 0.79, R_{FC} 0.88, R_{FD} 0.69, R_{FE} 0.49, R_{FF} 0.65, R_{FH} 0.67, R_{FP} 0.25, and R_{FQ} 0.57 (Found: C, 68.0; H, 6.7; N, 10.2. $C_{39}H_{45}N_5O_6 \cdot 0.5H_2O$ requires C, 68.0; H, 6.7; N, 10.1%).

N-*Benzyloxycarbonyl-*D-*tryptophyl-*L-*leucine Methyl Ester.*—Dicyclohexylcarbodi-imide (4.87 g, 23.6 mmol) was added to a solution of *N*-benzyloxycarbonyl-D-tryptophan (7.27 g, 21.5 mmol), L-leucine methyl ester (3.12 g, 21.5 mmol), and 1-hydroxybenzotriazole (5.8 g, 43 mmol) in DMF (50 ml) at 0 °C. The reaction mixture was stirred overnight at room temperature and was worked up in the usual manner. Recrystallisation from ethyl acetate–light petroleum (b.p. 60—80 °C) gave the dipeptide derivative (9.55 g) which showed traces of impurities on t.l.c. It was purified by column chromatography on silica gel (300 g), eluting with chloroform and methanol–chloroform (5% v/v) (9.18 g, 91.7%), m.p. 151—153 °C, R_{FA} 0.84, R_{FB} 0.80, R_{FC} 0.86, R_{FD} 0.78, R_{FE} 0.61, R_{FF} 0.68, R_{FH} 0.73, R_{FP} 0.55, and R_{FQ} 0.73 (Found: C, 66.8; H, 6.8; N, 8.9. $C_{26}H_{31}N_3O_5$ requires C, 67.0; H, 6.6; N, 9.0%).

N-*Benzyloxycarbonyl-*O-*benzyl-*L-*tyrosyl-*D-*tryptophyl-*L-*Leucine Methyl Ester.*—A solution of *N*-benzyloxycarbonyl-*O*-benzyl-L-tyrosine 2,4,5-trichlorophenyl ester (11.69 g, 20 mmol), D-tryptophyl-L-leucine methyl ester (6.28 g, 19 mmol, prepared by hydrogenating the benzyloxycarbonyl derivative over 5% Pd–C) in DMF (1 000 ml) was stirred at room temperature for 60 h. The reaction mixture was worked-up in the usual manner and the residue was crystallised from ethyl acetate–light petroleum (b.p. 60—80 °C) to give the tripeptide derivative (8.52 g, 62.5%), m.p. 165—166 °C, R_{FA} 0.78, R_{FB} 0.73, R_{FC} 0.84, R_{FD} 0.80, R_{FE} 0.62, R_{FF} 0.70, R_{FH} 0.76, R_{FP} 0.58, and R_{FQ} 0.68 (Found: C, 70.0; H, 6.5; N, 7.7. $C_{42}H_{46}N_4O_7$ requires C, 70.1; H, 6.4; N, 7.7%).

N-*Benzyloxycarbonyl-*O-*benzyl-*L-*tyrosyl-*D-*tryptophyl-*L-*leucine Hydrazide.*—Hydrazine hydrate (100 mmol) was added to a solution of the above ester (7.26 g, 10.1 mmol) in a mixture of methanol (200 ml) and DMF (50 ml). After 24 h at room temperature the solution was concentrated *in vacuo* (30 ml) and the product was precipitated with water (500 ml), filtered off, washed with water, methanol–ether (4 : 1), and ether, and dried (6.86 g, 94.6%), m.p. 200—202 °C, R_{FA} 0.90, R_{FB} 0.95, R_{FC} 0.90, R_{FD} 0.74, and R_{FQ} 0.59 (Found: C, 68.1; H, 6.6; N, 11.7. $C_{41}H_{46}N_6O_6$ requires C, 68.5; H, 6.4; N, 11.6%).

N-*Benzyloxycarbonyl-*O-*benzyl-*L-*tyrosyl-*D-*phenylalanyl-*L-*leucyl-*(N^{ω}-*nitro*)-L-*arginyl-*L-*prolylazaglycine Amide.*—A solution of 6.02M hydrogen chloride in dioxan (1.83 ml, 11 mmol) was added to a solution of *N*-benzyloxycarbonyl-*O*-benzyl-L-tyrosyl-D-phenylalanyl-L-leucine hydrazide (1.86 g, 2.75 mmol) in DMF (5 ml) at −20 °C followed by t-butyl nitrite (0.33 ml, 2.89 mmol). After 2 min a pre-

cooled (−20 °C) solution of triethylamine (1.89 ml, 13.5 mmol) and N^{ω}-nitro-L-arginyl-L-prolylazaglycine amide hydrochloride (1.02 g, 2.5 mmol) in DMF (10 ml) was added and the reaction mixture was stirred overnight at 4 °C. The usual work-up gave the hexapeptide derivative which was further purified by silica gel (120 g) column chromatography, eluting with methanol chloroform (5% and 10% v/v) and chloroform–methanol–water (11 : 8 : 2 v/v) (0.74 g, 29.3%), m.p. 137—139 °C, R_{FA} 0.68, R_{FB} 0.72, R_{FC} 0.58, R_{FD} 0.62, R_{FH} 0.39, and R_{FK} 0.95 (Found: C, 59.3; H, 6.5; N, 16.3. $C_{51}H_{64}N_{12}O_{11}\cdot 0.5H_{20}$ requires C, 59.4; H, 6.3; N, 16.3%). Amino-acid ratios after 16 h acid digest: Tyr 0.98, Phe 0.98, Leu 1.0, Pro 0.97, Arg + Orn 0.98.

N-*Benzyloxycarbonyl*-O-*benzyl*-L-*tyrosyl*-D-*tryptophyl*-L-*leucyl*-(N^{ω}-*nitro*)-L-*arginyl*-L-*prolylazaglycine Amide*.—The preparation was similar to that of the corresponding D-phenylalanine-containing hexapeptide derivative. The purification was achieved by silica gel column chromatography, eluting with chloroform and methanol–chloroform (5% v/v), yield 34.4%, m.p. 139—140 °C (decomp.), R_{FA} 0.67, R_{FB} 0.72, R_{FC} 0.58, R_{FH} 0.32, and R_{FK} 0.95 (Found: C, 59.8; H, 6.3; N, 17.1. $C_{53}H_{65}N_{13}O_{11}$ requires C, 60.0; H, 6.1; N, 17.1%). Amino-acid ratio; Tyr 0.95, Leu 1.0, Pro 0.97, Arg + Orn 1.01.

N-*Benzyloxycarbonyl*-D-*phenylalanyl*-L-*tryptophyl*-L-*serine Methyl Ester*.—To a cooled (0 °C) and stirred solution of *N*-benzyloxycarbonyl-D-phenylalanine (2.99 g, 10 mmol), L-tryptophyl-L-serine methyl ester hydrochloride [1] (3.42 g, 10 mmol), triethylamine (1.40 ml, 11 mmol), and hydroxybenzotriazole (2.70 g, 20 mmol) in DMF (50 ml), was added dicyclohexylcarbodi-imide (2.27 g, 11.0 mmol) and the stirring was continued for 60 h at room temperature. The reaction mixture was worked up in the usual manner and the residue was crystallised from hot ethyl acetate, yield 4.89 g (83.4%), m.p. 195—197 °C, R_{FA} 0.86, R_{FB} 0.81, R_{FC} 0.85, R_{FD} 0.72, R_{FE} 0.37, R_{FF} 0.63, R_{FH} 0.67, R_{FP} 0.15, and R_{FQ} 0.53 (Found: C, 64.5; H, 5.9; N, 9.4; $C_{32}H_{34}N_4O_7\cdot 0.5H_2O$ requires C, 64.4; H, 5.9; N, 9.3%).

D-*Phenylalanyl*-L-*tryptophyl*-L-*serine Methyl Ester Hydrochloride*.—A solution of the preceding compound (4.5 g, 7.72 mmol) in a mixture of ethanol (50 ml) and DMF (50 ml) was hydrogenated over 5% Pd–C (0.9 g) for 5 h in the presence of 1M HCl (8.5 ml). The catalyst was removed by filtration and the filtrate was evaporated to dryness. The residue was triturated with ether, filtered off, washed with ether, and dried, yield 100%, R_{FA} 0.61, R_{FB} 0.68, R_{FC} 0.72, R_{FD} 0.57, R_{FF} 0.48, and R_{FH} 0.42.

L-*Pyroglutamyl*-D-*phenylalanyl*-L-*tryptophyl*-L-*serine Methyl Ester*.—A solution of L-pyroglutamic acid 2,4,5-trichlorophenyl ester (2.62 g, 8.5 mmol), D-phenylalalanyl-L-tryptophyl-L-serine methyl ester hydrochloride (3.75 g, 7.7 mmol), and triethylamine (1.08 ml, 7.7 mmol) in DMF (30 ml) was stirred overnight at room temperature and then concentrated to dryness *in vacuo*. The residue was triturated with ethyl acetate and water, filtered off, washed with methanol–ether (1 : 5 v/v) and ether, and dried. The crude peptide was crystallised from methanol–ether to yield the tetrapeptide derivative (2.15 g, 49.3%), m.p. 194—196 °C, R_{FA} 0.68, R_{FB} 0.76, R_{FC} 0.70, R_{FD} 0.66, R_{FH} 0.38, and R_{FQ} 0.18 (Found: C, 60.9; H, 6.0; N, 12.2. $C_{29}H_{33}N_5O_7$ requires C, 61.2; H, 5.8; N, 12.4%).

L-*Pyroglutamyl*-D-*phenylalanyl*-L-*tryptophyl*-L-*serine Hydrazide*.—A solution of L-pyroglutamyl-D-phenylalanyl-L-tryptophyl-L-serine methyl ester (2.03 g, 3.6 mmol) in DMF (20 ml) was treated with hydrazine hydrate (72 mmol). After 6 h, the solvent was removed by evaporation *in vacuo* and the residue was triturated with water, filtered off, washed with water, methanol–ether (1 : 5 v/v), and ether, and dried to yield the tetrapeptide hydrazide (1.73 g, 84.7%), m.p. 234 °C (decomp.), R_{FA} 0.58, R_{FB} 0.81, R_{FC} 0.58, R_{FD} 0.66, and R_{FK} 0.70 (Found: C, 59.3; H, 5.7; N, 17.1. $C_{28}H_{33}N_7O_6$ requires C, 59.6; H, 5.9; N, 17.3%).

L-*Pyroglutamyl*-L-*tryptophyl*-L-*serine Methyl Ester*.—A solution of L-pyroglutamic acid 2,4,5-trichlorophenyl ester (3.4 g, 11 mmol), L-tryptophyl-L-serine methyl ester hydrochloride [1] (3.42 g, 10 mmol), and triethylamine (1.42 ml, 10 mmol) in DMF (30 ml) was set aside for 48 h at room temperature. DMF was evaporated off *in vacuo* and the residue was triturated with ethyl acetate–water, collected, washed with water, methanol–ether (5 : 1 v/v), and ether, and dried, yield 1.82 g (43.6%), m.p. 198 °C, R_{FA} 0.64, R_{FB} 0.73, R_{FC} 0.63, R_{FD} 0.64, R_{FH} 0.28, and R_{FQ} 0.11 (Found: C, 58.0; H, 6.1; N, 13.4. $C_{20}H_{24}N_4O_6$ requires C, 57.7; H, 5.8; N, 13.4%).

L-*Pyroglutamyl*-L-*tryptophyl*-L-*serine Hydrazide*.—A solution of the preceding methyl ester (1.66 g, 4 mmol) in DMF (20 ml) was treated with hydrazine hydrate (20 mmol) for 24 h at room temperature. The solvent was removed *in vacuo* and the residue was crystallised from methanol to give the tripeptide hydrazide (1.09 g, 68%), m.p. 230—231 °C (decomp.) (Found: C, 54.4; H, 5.8; N, 20.3. $C_{19}H_{24}N_6O_5$ requires C, 54.7; H, 5.7; N, 20.1%).

N-*Benzyloxycarbonyl*-D-*tryptophyl*-L-*tryptophyl*-L-*serine Methyl Ester*.—This method is similar to the one described for the D-phenylalanine-containing tripeptide derivative. It was crystallised from methanol, yield 68%, m.p. 150—151 °C, R_{FA} 0.80, R_{FB} 0.78, R_{FC} 0.86, R_{FD} 0.74, R_{FE} 0.33, R_{FF} 0.69, R_{FH} 0.69, and R_{FQ} 0.35 (Found: C, 65.2; H, 5.3; N, 10.8. $C_{34}H_{35}N_5O_7$ requires C, 65.2; H, 5.5; N, 11.1%).

L-*Pyroglutamyl*-D-*tryptophyl*-L-*tryptophyl*-L-*serine Methyl Ester*.—The preparation was similar to that of L-pyroglutamyl-D-phenylalanyl-L-tryptophyl-L-serine methyl ester, yield 85%, m.p. 244—246 °C, R_{FA} 0.67, R_{FB} 0.74, R_{FC} 0.68, and R_{FD} 0.65 (Found: C, 61.4; H, 5.7; N, 13.7. $C_{31}H_{34}N_6O_7$ requires C, 61.7; H, 5.6; N, 13.9%).

L-*Pyroglutamyl*-D-*tryptophyl*-L-*tryptophyl*-L-*serine Hydrazide*.—The preparation was similar to that of L-pyroglutamyl-D-phenylalanyl-L-tryptophyl-L-serine hydrazide except that the reaction was carried out for 3 h; yield 86.1%, m.p. 234—235 °C (Found: C, 59.7; H, 5.4; N, 18.3. $C_{30}H_{34}N_8O_6$ requires C, 59.7; H, 5.6; N, 18.5%).

N-*Benzyloxycarbonyl*-L-*prolylaza-alanine Amide*.—Dicyclohexylcarbodi-imide (10.3 g, 50 mmol) was added to a solution of *N*-benzyloxycarbonyl-L-proline (9.96 g, 40 mmol), aza-alanine amide hydrochloride [1] (5.02 g, 40 mmol), triethylamine (5.72 ml. 40 mmol), and 1-hydroxybenzotriazole (10.8 g, 80 mmol) in DMF (200 ml) at 0 °C and the reaction mixture was stirred overnight at 4 °C. Working up in the usual manner gave the crude peptide which was purified by column chromatography on silica gel (500 g) eluting with chloroform (1 500 ml) and methanol–chloroform (5% v/v). The dipeptide–amide-containing fractions were combined and evaporated to dryness, yield 8.7 g (67.9%), m.p. 182—184 °C (Found: C, 56.3; H, 6.4; N, 17.5. $C_{15}H_{20}N_4O_4$ requires C, 56.2; H, 6.2; N, 17.5%).

N^{α}-*t*-*Butoxycarbonyl*-N^{ω}-*nitro*-L-*arginyl*-L-*prolylaza-alanine Amide*.—The preparation is similar to that of the azaglycine-containing tripeptide derivative; yield 70%, m.p. 169—170 °C, R_{FA} 0.48, R_{FB} 0.67, R_{FC} 0.59, R_{FD} 0.70,

R_{FE} 0.20, R_{FF} 0.60, R_{FH} 0.61, R_{FK} 0.85, and R_{FQ} 0.13 (Found: C, 42.8; H, 6.7; N, 24.8; $C_{18}H_{33}N_9O_7 \cdot H_2O$ requires C, 42.8; H, 6.9; N, 25.0%).

N-Benzyloxycarbonyl-O-benzyl-L-tyrosylglycyl-L-leucine Methyl Ester.—*N*-Benzyloxycarbonyl-*O*-benzyl-L-tyrosine 2,4,5-trichlorophenyl ester (5.84 g, 10 mmol) was allowed to react overnight with glycyl-L-leucine methyl ester (1.98 g, 10 mmol) in DMF (50 ml) at room temperature. The solvent was removed *in vacuo* and the residue was crystallised from aqueous methanol, yield 98%, m.p. 113—115 °C, R_{FA} 0.52, R_{FB} 0.63, R_{FC} 0.59, R_{FD} 0.80, R_{FE} 0.49, R_{FH} 0.63, R_{FP} 0.55, and R_{FQ} 0.65 (Found: C, 67.1; H, 6.6; N, 6.9. $C_{33}H_{39}N_3O_7$ requires C, 67.2; H, 6.7; N, 7.1%).

N-Benzyloxycarbonyl-O-benzyl-L-tyrosylglycyl-L-leucine Hydrazide.—A solution of *N*-benzyloxycarbonyl-*O*-benzyl-L-tyrosylglycyl-L-leucine methyl ester (4.28 g, 7.28 mmol) in methanol (75 ml) was treated with hydrazine hydrate (62%, 2 ml) and the solution was set aside at room temperature for 2 h, and then overnight at −20 °C. The tripeptide hydrazide was filtered off, washed with methanol and ether, and dried, yield 3.8 g (88.7%), m.p. 173—175 °C, R_{FA} 0.77, R_{FB} 0.82, R_{FC} 0.87, R_{FD} 0.67, R_{FE} 0.31, R_{FH} 0.56, R_{FP} 0.11, and R_{FQ} 0.35 (Found: C, 64.9; H, 6.5; N, 11.9. $C_{32}H_{39}N_5O_6$ requires C, 65.2; H, 6.7; N, 11.9%).

N-Benzyloxycarbonyl-O-benzyl-L-tyrosylglycyl-L-leucyl-(N$^{\omega}$-nitro)-L-arginyl-L-prolylaza-alanine Amide.—To a cooled (−20 °C) and stirred solution of *N*-benzyloxycarbonyl-*O*-benzyl-L-tyrosylglycyl-L-leucine hydrazide (1.18 g, 2.2 mmol) in DMF (10 ml) a 6.02M solution of hydrogen chloride in dioxan (1.46 ml, 8.8 mmol) was added followed by t-butyl nitrite (0.26 ml, 2.3 mmol). After 2 min a precooled (−20 °C) solution of N^{ω}-nitro-L-arginyl-L-prolylaza-alanine amide trifluoroacetate (1.0 g, 2.0 mmol) and triethylamine (1.51 ml, 10.8 mmol) in DMF was added and the reaction mixture was stirred overnight at 4 °C. The usual work-up left an oil which was dissolved in methanol, and the hexapeptide derivative was precipitated by the addition of ether, yield 1.75 g (92.8%), m.p. 150—153 °C, R_{FA} 0.58, R_{FB} 0.60, R_{FC} 0.59, R_{FD} 0.62, R_{FH} 0.31, and R_{FK} 0.94 (Found: C, 56.2; H, 6.4; N, 17.6. $C_{45}H_{60}N_{12}O_{11} \cdot H_2O$ requires C, 56.1; H, 6.4; N, 17.5%). Amino-acid ratios after 16 h acid digest; Tyr 0.98, Gly 1.0, Leu 1.0, Arg + Orn 0.96, Pro 0.97.

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