Renin Substrates. Part 1. Liquid-phase Synthesis of the Equine Sequence with Benzotriazolyloxytris(dimethylamino)phosphonium Hexafluorophosphate (BOP)

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The tetradecapeptide Ac-Asp[1]-Arg[2]-Val[3]-Tyr[4]-Ile[5]-His[6]-Pro[7]-Phe[8]-His[9]-Leu[10]-Leu[11]-Val[12]-Tyr[13]-Ser[14]-OH has been synthesized by a three-segment coupling strategy. All the coupling steps were carried out with benzotriazolyloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP reagent). Histidine was incorporated in good yield by using Boc(N^{im}-Boc)histidine (DCHA).

The highly specific enzyme renin acts upon the Leu-Leu bond of its plasma protein substrate (angiotensinogen) to produce angiotensin I which is further converted by a conversion enzyme into angiotensin II, a key hormone involved in arterial blood pressure regulation. The active segment of angiotensinogen (from horse plasma) is represented by a tetradecapeptide whose sequence has been determined by Skeggs *et al.*[1] Further work has shown that the amino acid sequence in the *C*-terminal region of the cleavage site is species dependent after the tenth residue.[2]

Human substrate: Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile-His

Ratine——:	——Angiotensin I	——Leu-Tyr-Lys
Porcine——:	——Angiotensin I	——Leu-Val-Tyr
Equine——:	——Angiotensin I	——Leu-Val-Tyr-Ser

Although a large number of solid-phase syntheses of equine tetradecapeptide have been described in the literature,[3] since 1958 only one solution synthesis has been reported.[4] Two assays have been used to test the renin activity. The free peptide may be assayed by RIA with angiotensin I as a standard,[5] or the *N*-acetylated peptide may be quantified using a fluorometric assay of the liberated tetrapeptide.[6]

In order to demonstrate the usefulness of benzotriazolyloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP) in peptide synthesis,[7,8] we report here a liquid-phase synthesis of the equine free and *N*-acetylated tetradecapeptides renin substrates using BOP exclusively as a coupling reagent.

Synthesis.—We decided to attempt the preparation of a hydrogenolysable protected tetradecapeptide using a *C*-terminal benzyl ester, benzyl ether protection of tyrosine, a β-benzyl ester on aspartic acid, and ω-nitroarginine.

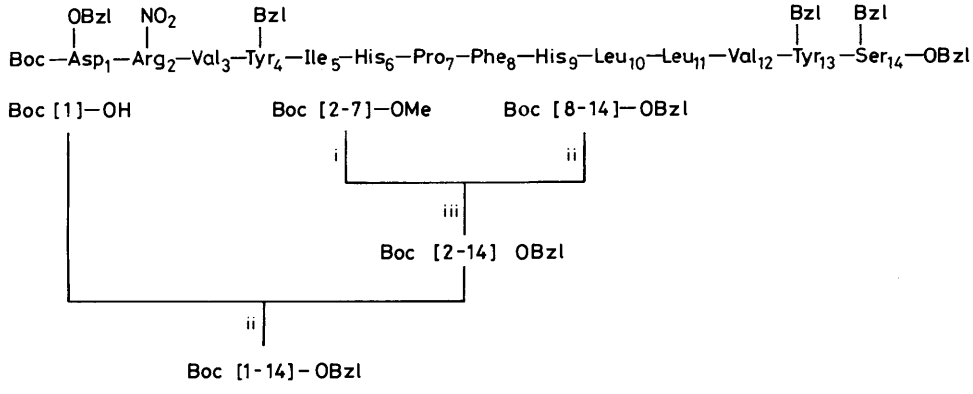
The strategic position of the non-racemizable proline allows the logical segmentation of the tetradecapeptide sequence into two equal parts (Scheme 1) and the methyl ester was chosen to protect the proline *C*-terminus. As transposition of the β-benzyl ester aspartic acid might readily occur during saponification,[9] the aspartyl residue was incorporated after the coupling of Arg(NO_2)-Val-Tyr(Bzl)-Ile-His-Pro with Phe-His-Leu-Leu-Val-Tyr(Bzl)-Ser(Bzl)-OBzl had been accomplished. *N*-Terminal acetylation was then performed using BOP and acetic acid.

Three types of protection have been tested for histidine. With 2,4-dinitrophenyl (DNP) as an imidazole-protecting group[10] coupling reactions gave the expected good results, but unexpected side reactions during His-6-DNP group thiolysis reduced the efficiency of the synthesis. Indeed this protecting group seemed to be incompatible with the saponification conditions as brownish tars were formed during this step.

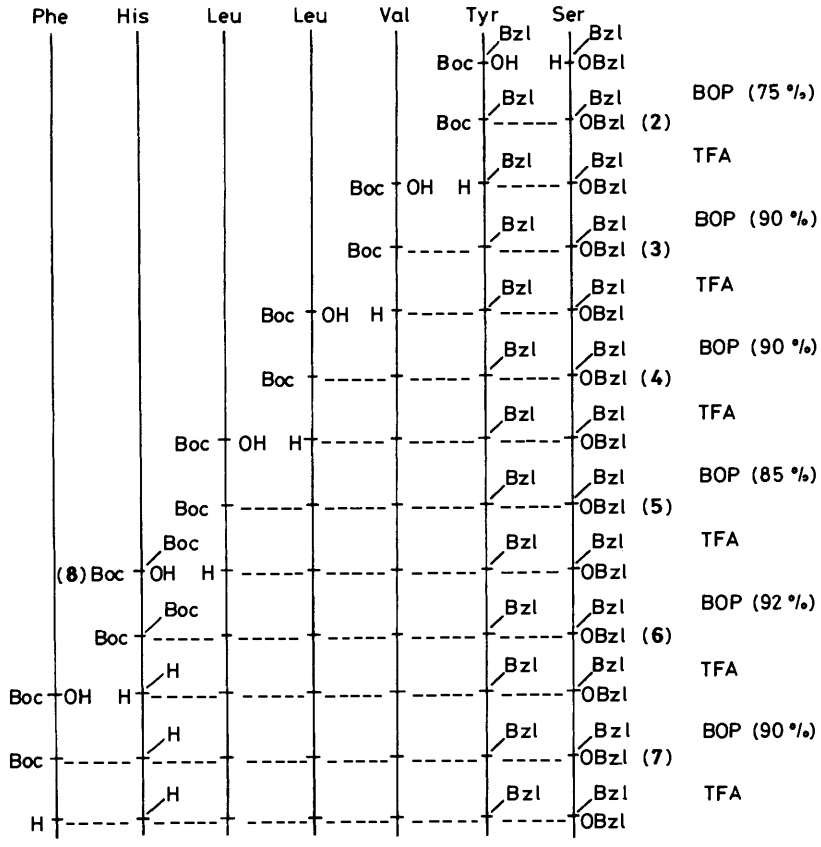
A second attempt using the benzyloxymethyl (π-Bom) derivative[11] gave excellent coupling yields; however the final hydrogenolysis appeared to be exceedingly slow, and we therefore decided to use bis-Boc-histidine.[12]

N^{im} and N^{α}-Boc Groups were cleaved simultaneously during the next TFA deprotection step. Hence histidine protection was achieved only during the critical coupling step. Thus, the protected tetradecapeptide was obtained with free-imidazole histidines and there were no problems at the deprotection stage.

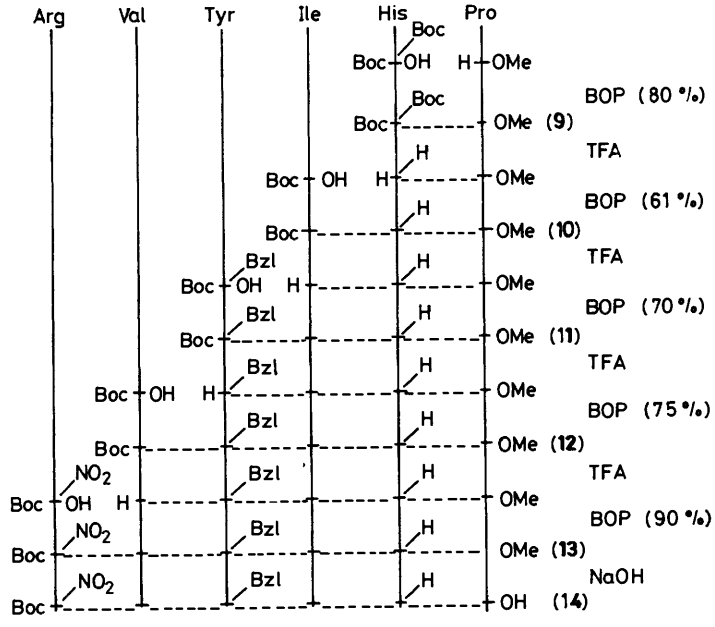
As shown in Schemes 2 and 3, coupling with BOP reagents generally gave good results. Yields after purification approached 85%, except in the case of Boc-Ile-His-Pro-OMe where the use of methyl ester protection for proline resulted in the ready formation of dioxopiperazine[13–15] on neutralization of the His-Pro-OMe dipeptide trifluoroacetate. However, we found that this undesirable reaction, which is in competition with the coupling reaction, is considerably depressed when BOP is used and if all the reactants are mixed in the solvent except DIEA



Scheme 1. General strategy for the synthesis of the protected [1-14] segment. *Reagents:* i, $^{-}$OH; ii, TFA; iii, BOP



Scheme 2. Synthesis of protected [8-14] segment



Scheme 3. Synthesis of protected [2-7] segment

which is then added slowly, dropwise; thus, the deprotonated dipeptide and the active ester are simultaneously formed and react at once. In this way, the yield of this very difficult coupling was raised to more than 60%.

Purification and Bioassay.—The hydrogenolysed tetradecapeptides were run on a G15 Sephadex column in H_2O–AcOH (50:50)[3] (Figure 1). The major peak was collected, the solvents were removed under reduced pressure, and the peptide was

Table. 360 MHz ^{1}H N.m.r. spectral results for the *N*-acetylated tetradecapeptide renin substrate (**18**)

Residue (number)	NH	$C_\alpha H$	$C_\beta H$	$C_\gamma H$	$C_\delta H$	Others
Arg	8.23	4.30	1.42	1.45	3.06	7.45 (ε-NH)
Asp	8.23	4.52	2.61—2.44			
Tyr (13)	7.90	4.52	2.95—2.67			7.0—6.6 (Aromatic)
(4)	7.96	4.46	2.81—2.61			
Phe	8.23	4.49	2.97—2.93			7.20 (Aromatic)
His (3)	8.19	4.61	3.06—2.95			8.85—8.76 (C-2)
(5)	8.29	4.75	2.93—2.81			7.30—7.20 (C-4)
Val (12)	7.58	4.11	1.90	0.74		
(13)	7.55	4.11	1.90	0.74		
Pro		4.30	1.97—1.76	1.76	3.45	
Leu (10)	8.07	4.25	1.45	1.56	0.74	
(11)	7.99	4.25	1.45			
Ile	7.79	4.11	1.63	1.35—1.03	0.78	0.86 (α-CH_3)
Ser	8.07	4.30	3.70—3.60			
						Ac: 1.83 (Methyl)

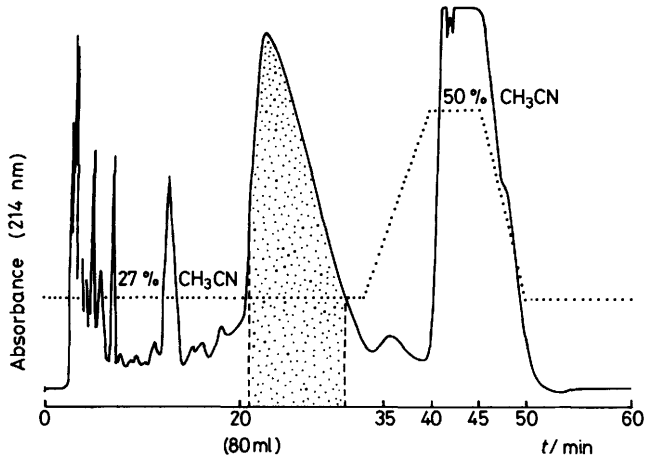


Figure 1. Acetyltetradecapeptide purification by repetitive semi-preparative h.p.l.c. The peptide (stippled peak) elution was performed in the isocratic mode with 27% aqueous acetonitrile and 0.1% TFA. A gradient up to 50% of aqueous acetonitrile (dotted line) allowed a rapid regeneration of the column before the following injection [8 × 300 mm μBondapak column; 4 ml/min; 214 nm u.v. detection, 2 AUFS (Absorbance Unit Full Scale)]

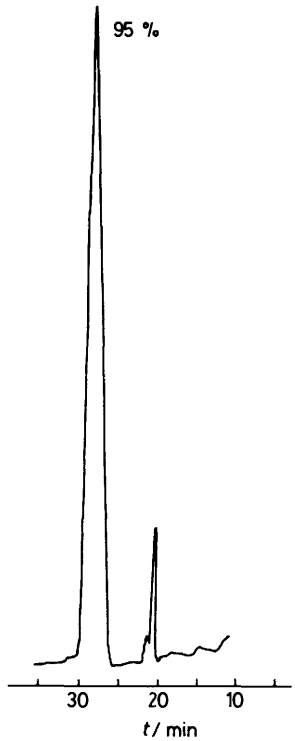


Figure 2. H.p.l.c. analysis of tetradecapeptide renin substrate. 4 × 250 mm Waters C18 μBondapak column. Elution: 76% triethylammonium phosphate buffer (pH 3) + 24% CH_3CN, 2 ml/min. Detection: 210 nm

lyophilized as the acetate and analysed by h.p.l.c. (Figure 2). Integration of the tetradecapeptide peak (identified by comparison with a commercial reference and by amino acid analysis) shows a high degree of purity (*ca.* 95%). In some experiments, the crude sample was purified by semipreparative h.p.l.c. and the peptide was obtained as a trifluoroacetate salt. The assignments of the NH and CH-α protons in the 360 MHz ^{1}H n.m.r. spectrum are given in the Table.

Biological assays were carried out with mouse submaxillary gland renin.[16] Enzyme incubations (30 min) were performed at 37 °C in 0.5M-citrate–phosphate buffer at pH 6.0. The renin concentration was 10^{-5}M. The value of the Michaelis constant (K_M) was determined according to the Lineweaver–Burk plot by radioimmunoassay ($K_M = 0.12 \times 10^{-6}$M).

Thus we have obtained a synthetic tetradecapeptide renin substrate with high purity and biological activity. This successful synthesis demonstrates the usefulness of BOP reagent in peptide synthesis. Coupling reactions can be carried out under easily obtained conditions; amine residues are coupled without a previous neutralization step and either chlorohydrate or trifluoroacetate salts can be used. Furthermore, dicyclohexylamine salts of carboxylic acids can directly be coupled, as we have shown in the preparation of compound (**6**).

Experimental

BOP Reagent was obtained from SEMPA-CHIMIE* and all the coupling steps were carried out at room temperature.

All amino acids derivatives used were of the L form and were purchased commercially from BACHEM or FLUKA. T.l.c. analyses were performed on Kieselgel F 254 (Merck) precoated plates and peptides were revealed with ninhydrin or by charring (sulphuric acid–ammonium sulphate). Optical rotations were

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measured with a Schmidt and Haensch apparatus (Polartronic D) in a 10-cm cell in ethanol or dimethylformamide. M.p.s were determined on a Kofler plate. ^{1}H N.m.r. data were recorded on a Bruker 360 MHz instrument. The spectra were plotted in deuteriated chloroform or dimethyl sulphoxide, and tetramethylsilane was used as internal standard. The spectra were assigned by the usual spin decoupling method and 2D COSY and relayed COSY (RELSY). Microanalyses were performed by the Laboratoire Central de Microanalyse du CNRS (Lyon, France). H.p.l.c. analyses were carried out using a Varian 5000 instrument, a Waters C-18 μBondapak column, and a pH 3 triethylamine–phosphate buffer–acetonitrile elution system. The products were purified by chromatography on silica gel (Merck, 0.05—0.2 mm), gel filtration on Sephadex G 15 (AcOH 50%), or semi-preparative h.p.l.c. The semi-preparative runs were performed with Waters equipment consisting of two pumps (6000A and M45), an automatic injector (WISP), a programmer (720), two photometers (model 440 for 254 nm and model 441 for 214 nm), a two channel recorder (Houston), a guard-column and a semi-preparative (8 × 300 mm) C18 μBondapak column. The fractions were automatically collected with a Pharmacia Frac 100 collector.

Amino acid analyses were performed on a Beckman 119 B amino acid analyser.

N$^{\alpha}$-*t-Butoxycarbonyl-O-benzylserine Benzyl Ester* (**1**).—*N*$^{\alpha}$-t-Butoxycarbonyl-*O*-benzylserine (4.8 g, 16.5 mmol) was dissolved in ethanol (10 ml). The solution was cooled to 5 °C and slowly neutralized with 1M-caesium hydroxide. Ethanol was then evaporated under reduced pressure and water was removed by a series of co-evaporations with toluene to dryness. The caesium salt was then dissolved in dimethylformamide (DMF) (30 ml) and treated with benzyl bromide (2.83 g, 16.5 mmol) with stirring. After 3 h, the caesium bromide precipitate was removed by filtration, DMF was evaporated off, and the product was extracted with ethyl acetate (500 ml) and washed twice with saturated sodium hydrogen carbonate ($NaHCO_3$) (50 ml) and twice with water (50 ml). The organic phase was dried ($MgSO_4$), filtered and concentrated by rotary evaporation.

A silica gel chromatographic column using the mixture hexane–ethyl acetate 80:20 (v/v) as eluant gave a transparent oil which was dried under vacuum overnight (6.2 g, 15.8 mmol, 96%), R_F 0.80 [hexane–ethyl acetate 4:1 (v/v)]; $[\alpha]_D$ −40.2° (*c* 2 in ethanol); δ($CDCl_3$) 1.42 [s, 9 H, $(CH_3)_3C$], 3.65 and 3.86 (2dd, $J_1 = J'_1 = 3$ Hz, $J_2 = J'_2 = 9$ Hz, 2 H, β-CH_2, Ser), 4.2—4.6 (br m, 1 H, α-CH Ser), 4.37 and 4.44 (2d, $J = J' = 12$ Hz, 2 H, CH_2 Ph ester), 5.08 and 5.20 (2d, $J = J' = 12$ Hz, 2 H, CH_2Ph ether), 5.50 (d, *J* 9 Hz, 1 H, NH Ser), 7.1—7.3 (br m, 10 H, Ph ester + Ph ether) (Found: C, 68.4; H, 7.1; N, 3.60. Calc. for $C_{22}H_{27}N_1O_5$: C, 68.55; H, 7.06; N, 3.63%).

N$^{\alpha}$-*t-Butoxycarbonyl-O-benzyltyrosyl-O-benzylserine Benzyl Ester* (**2**).—Compound (**1**) (5.77 g, 15 mmol) was treated with methylene dichloride–trifluoroacetic acid [20 ml; 50:50 (v/v)]. After 30 min, the solvents were evaporated under reduced pressure. The remaining acid was thoroughly removed by 5 co-evaporations with hexane. After being dried under vacuum overnight, the intermediate product was dissolved in acetonitrile (100 ml) and Boc-Tyr(Bzl)-OH (5.56 g, 15 mmol) and BOP (6.63 g, 15 mmol) were added. Coupling was initiated by addition of di-isopropylethylamine (DIEA) (4.55 g, 35 mmol). After 2 h, the reaction mixture was reduced under reduced pressure to 20 ml and the product extracted with ethyl acetate (300 ml). The organic phase was then washed with 3M-hydrochloric acid, saturated sodium hydrogen carbonate, and saturated sodium chloride, dried ($MgSO_4$) and evaporated under reduced pressure. Preparative chromatography on silica gel using a PREP 500 Waters apparatus and AcOEt–hexane [50:50 (v/v)] as eluant gave a solid product (7.4 g, 11.6 mmol, 75%). An aliquot was recrystallized from diethyl ether, R_F 0.57 [AcOEt–hexane 50:50 (v/v)], $[\alpha]_D$ −3° (*c* 1.5 in ethanol); m.p. 106 °C; δ($CDCl_3$) 1.40 [s, 9 H, $(CH_3)_3C$], 3.04 (d, *J* 6 Hz, 2 H, β-CH_2Tyr), 3.68 and 3.86 (2dd, $J_1 = J'_1 = 3$ Hz, $J_2 = J'_2 = 9.5$ Hz, 2 H, β-CH_2 Ser), 4.2—4.5 (m, 1 H, α-CH Ser), 4.36 and 4.44 (2d, $J = J' = 12$ Hz, 2 H, CH_2 Ph ester), 4.73 (m, 1 H, α-CH Tyr), 4.97 (s, 2 H, CH_2 Ph ether Tyr), 5.06 (d, 1 H, α-NH Tyr), 5.16 (d, *J* 2.5 Hz, 2 H, CH_2 Ph ether Ser), and 7.21—7.61 (br m, 20 H, 3Ph + PhTyr + α-NH Ser) (Found: C, 71.4; H, 6.55; N, 4.15. Calc. for $C_{38}H_{42}N_2O_7$: C, 71.45; H, 6.64; N, 4.39%).

N$^{\alpha}$-*t-Butoxycarbonylvalyl-O-benzyltyrosyl-O-benzylserine Benzyl Ester* (**3**).—Compound (**2**) (6.38 g, 10 mmol) was dissolved in CH_2Cl_2–TFA [20 ml; 50:50 (v/v)] and the mixture was stirred for 30 min. The trifluoroacetate salt was then obtained as described above. Coupling was carried out within 2 h in CH_3CN (50 ml) by addition of Boc-Val-OH (2.17 g, 10 mmol), BOP (4.42 g, 10 mmol), and DIEA (3.22 g, 25 mmol). The product was extracted and washed following the procedure described above. It was then chromatographed on a silica gel column with AcOEt–hexane (60:40) as eluant to yield the product (6.63 g, 9 mmol, 90%). An aliquot was recrystallized from diethyl ether; R_F 0.93 (AcOEt–hexane 60:40), $[\alpha]_D$ −30.2° (*c* 1.5 in ethanol), m.p. = 152 °C, δ($CDCl_3$) 0.82 and 0.88 (2d, $J = J' = 6.5$ Hz, 6 H, γγ′-CH_3 Val), 1.44 [s, 9 H, $(CH_3)_3C$], 2.1 (m, 1 H, β-CH Val), 3.01 (d, *J* 6 Hz, 2 H, β-CH_2 Tyr), 3.59 and 3.85 (2dd, $J_1 = J'_1 = 3.5$ Hz, $J_2 = J'_2 = 9$ Hz, 2 H, β-CH_2 Ser), 3.98 (m, 1 H, α-CH Val), 4.34 and 4.43 (2d, $J = J' = 12$ Hz, 2 H, CH_2Ph ester), 4.68 (m, 1 H, α-CH Ser), 4.72 (d, *J* 8 Hz, 1 H, α-NH Val), 4.81 (s, 2 H, CH_2Ph ether Tyr), 5.09 (m, 1 H, α-CH Tyr), 5.15 (d, *J* 2.5 Hz, 2 H, CH_2Ph ether Ser), 6.85 (d, *J* 9 Hz, 2 H, α-NH Tyr + α-NH Ser), and 7.06—7.66 (br m, 19 H, 3Ph + Ph-Tyr) (Found: C, 70.1; H, 6.85; N, 5.7. Calc. for $C_{43}H_{51}N_3O_8$: C, 69.99; H, 6.97; N, 5.69%).

N$^{\alpha}$-*t-Butoxycarbonyl-leucylvalyl-O-benzyltyrosyl-O-benzylserine Benzyl Ester* (**4**).—Compound (**3**) (5.89 g, 8 mmol) was treated with CH_2Cl_2–TFA (20 ml; 1:1) for 30 min. The solvents were evaporated under reduced pressure and the trifluoroacetate salt precipitated by addition of diethyl ether. It was then collected on a fine glass filter, washed with diethyl ether, and dried *in vacuo* over potassium hydroxide.

The trifluoroacetate salt was then dissolved in CH_3CN–DMF (75:25; 40 ml), Boc-Leu-OH·H_2O (1.99 g, 8 mmol), BOP (3. 53 g, 8 mmol), and DIEA (2.58 g, 20 mmol) were added. After 2 h, the solvents were evaporated and the reaction product extracted as described for compound (**2**). Chromatography on a silica gel column yielded the product (6.1 g, 7.2 mmol, 90%). An aliquot was recrystallized from diethyl ether; R_F 0.90 (AcOEt–hexane 60:40), $[\alpha]_D$ −18° (*c* 0.5 in ethanol), m.p. 160 °C; δ($CDCl_3$) 0.80—0.92 (m, 12 H, δ CH_3 Leu + γ-CH_3 Val), 1.45 [s, 9 H, $(CH_3)_3C$], 1.62 (m, 2 H, β-CH_2 Leu), 2.02 (m, 1 H, β-CH Val), 2.38 (m, 1 H, γ-CH Leu), 2.96 (d, *J* 7 Hz, 2 H, β-CH_2 Tyr), 3.56 and 3.82 (2dd, $J_1 = J'_1 = 3.5$ Hz, $J_2 = J'_2 = 9$ Hz, 2 H, β-CH_2 Ser), 4.28 and 4.40 (2d, $J = J' = 12$ Hz, 2 H, CH_2Ph ester), 4.62 (m, 1 H, α-CH Leu), 4.84 (s, 2 H, 2CH_2Ph ether Tyr), 5.10 and 5.18 (2d, $J = J' = 12.5$ Hz, 2 H, CH_2Ph ether Ser), 5.74 (d, *J* 8.5 Hz, 1 H, α-NH Leu), and 7.00—7.54 (br m, 19 H, 3Ph + PhTyr) (Found: C, 68.9; H, 7.3; N, 6.7. Calc. for $C_{49}H_{62}N_4O_9$: C, 69.15; H, 7.35; N, 6.58%).

N$^{\alpha}$-*t-Butoxycarbonyl-leucyl-leucylvalyl-O-benzyltyrosyl-O-benzylserine Benzyl Ester* (**5**).—Compound (**4**) (5.1 g, 6 mmol) was deprotected as described above. The trifluoroacetate salt was dissolved in CH_3CN–DMF (30 ml; 66:34), and coupling was carried out by addition of Boc-Leu-OH·H_2O (1.49 g, 6 mmol), BOP (2.65 g, 6 mmol), and DIEA (1.93 g, 15 mmol). After

2 h, the solvents were evaporated and the product precipitated with diethyl ether. The precipitate was collected and chromatographed on a silica gel column (eluant AcOEt–hexane 60:40) to give the product (4.9 g, 5.08 mmol, 85%). An aliquot was recrystallized from ethanol–diethyl ether, R_F 0.81 (AcOEt–hexane, 60:40), $[\alpha]_D$ −21° (*c* 1 in DMF), m.p. 172 °C (Found: C, 68.5; H, 7.5; N, 6.9. Calc. for $C_{55}H_{73}N_5O_{10}$: C, 68.50; H, 7.64; N, 7.26%).

N$^{\alpha}$-*t-Butoxycarbonyl*-N^{im}-*t-butoxycarbonylhistidyl-leucyl-leucylvalyl*-O-*benzyltyrosyl*-O-*benzylserine Benzyl Ester* (**6**).—Compound (**5**) (4.8 g, 4.9 mmol) was deprotected as described for the synthesis of the product (**4**). The trifluoroacetate salt was dissolved in DMF (10 ml) and a solution of Boc(Nim-Boc)His (2.7 g, 5 mmol), dicyclohexylamine salt (DCHA) was added. After 10 min of stirring BOP (2.21 g, 5 mmol) and DIEA (0.64 g, 5 mmol) were added. After 2 h, the reaction mixture was filtered over Celite to remove the salt precipitate. The solvents were then evaporated. The crude product was precipitated out with water, collected on a fine glass filter, and washed successively with 10% citric acid solution, saturated sodium hydrogen carbonate, water and finally diethyl ether. It was then dried *in vacuo* overnight, yield 5.4 g (4.5 mmol, 92%). An aliquot was recrystallized from ethanol–diethyl ether, R_F 0.54 (CH_2Cl_2–MeOH 95:5), $[\alpha]_D$ −8° (*c* 1 in DMF), m.p. 180 °C (Found: C, 64.0; H, 7.1; N, 8.6. Calc. for $C_{66}H_{88}N_8O_{13}\cdot 2H_2O$: C, 64.05; H, 7.50; N, 9.05%).

N$^{\alpha}$-*t-Butoxycarbonylphenylalanylhistidyl-leucyl-leucylvalyl*-O-*benzyltyrosyl*-O-*benzylserine Benzyl Ester* (**7**).—Compound (**6**) (3.6 g, 3 mmol) was deprotected by treatment with trifluoroacetic acid (10 ml) for 30 min. The trifluoroacetate salt was directly precipitated by addition of diethyl ether. After the usual work-up, the salt was dissolved in DMF (100 ml), and Boc-phenylalanine (0.79 g, 3 mmol), BOP (1.33 g, 3 mmol), and DIEA (1.03 g, 8 mmol) were added. After 2 h of stirring, the product was precipitated by addition of water. It was collected on a fine glass filter and washed successively with 1M-hydrochloric acid, saturated sodium hydrogen carbonate, water, and diethyl ether to give the crude product (3.36 g, 2.7 mmol, 90%). An aliquot (500 mg) was chromatographed and recrystallized from ethanol–diethyl ether, R_F 0.65 (CH_2Cl_2–MeOH, 90:10), $[\alpha]_D$ −21° (*c* 1 in DMF), m.p. 220 °C (Found: C, 67.2; H, 6.9; N, 9.25. Calc. for $C_{70}H_{89}N_9O_{12}$: C, 67.33; H, 7.19; N, 10.10%). Amino acid ratios in acid hydrolysate: Ser 1.01, Val 1.00, Leu 2.04, Tyr 0.81, His 1.02.

N$^{\alpha}$-*t-Butoxycarbonyl*-N^{im}-*t-butoxycarbonylhistidine Dicyclohexylamine Salt*. (**8**).—To a suspension of histidine (31 g, 200 mmol) and triethylamine (60.6 g, 600 mmol) in water (100 ml), a solution of di-t-butyl dicarbonate (BOC_2O) (110 g, 500 mmol) in dioxane (100 ml) was slowly added. The reaction temperature was maintained between 30 and 45 °C. After the mixture had been stirred overnight, water (200 ml) was added and the aqueous phase was washed three times with 50-ml portions of diethyl ether and three times with 50-ml portions of ethyl acetate. The total volume was increased to 1 l with ethyl acetate and the solution acidified with 3M-sulphuric acid until it reached pH 3. The aqueous phase was poured off and extracted twice with ethyl acetate (300 ml). The organic phases were pooled, washed three times with saturated sodium chloride, and dried ($MgSO_4$). The solvent was evaporated under reduced pressure and the oily residue dissolved in 1 l of diethyl ether. DCHA (36 g, 200 mol) was added and the mixture was stored in the cold overnight. Crystallized Boc(Nim-Boc)His-OH·DCHA (80.4 g, 150 mmol, 75%) was collected on a fine glass filter, washed with ether and dried *in vacuo* over diphosphorus pentaoxide, m.p. 166 °C, $[\alpha]_D$ +27° (*c* 1 in methanol), +37° (*c* 2 in chloroform) {lit.,[12] m.p. 157—159 °C, $[\alpha]_D$ +17.6° (*c* 2 in chloroform)}.

N$^{\alpha}$-*t-Butoxycarbonyl*-N^{im}-*t-butoxycarbonylhistidylproline Methyl Ester* (**9**).—Compound (**8**) (10.7 g, 20 mmol) was dissolved in methylene dichloride (20 ml), then mixed with a solution of proline methyl ester hydrochloride (2.79 g, 20 mmol) in methylene dichloride (20 ml). The mixture was stirred for 15 min and filtered over Celite to remove the salt precipitate and BOP (8.84 g, 20 mmol) was added to the filtrate. DIEA was added dropwise until the solution reached pH 8 (2.6 g, 20 mmol) and the mixture was allowed to react for 2 h. Methylene dichloride was then evaporated under reduced pressure. The reaction product was dissolved in ethyl acetate (100 ml) and stored in a refrigerator overnight. The crystallised dipeptide (5.6 g, 12 mmol, 60%) was collected on sintered glass, washed with diethyl ether and dried over KOH pellets. The filtrate was recovered and washed twice with 10% citric acid, saturated sodium hydrogen sulphate, and saturated sodium chloride and dried ($MgSO_4$). Purification by silica gel column chromatography (eluant: AcOEt–hexane 50:50) gave an additional product (1.9 g, 4 mmol). The two portions showed identical characteristics. Total yield 7.5 g (16 mmol, 80%); R_F 0.37 (AcOEt–hexane 50:50), $[\alpha]_D$ −32° (*c* 1 in methanol), m.p. 149 °C; δ($CDCl_3$) 1.40 [s, 9 H, $(CH_3)_3C$, N$^{\alpha}$-His)], 1.61 [s, 9 H, $(CH_3)_3C$, N^{im}His], 1.98 and 2.19 (2 m, 4 H, β-CH_2 + γ-CH_2 Pro), 2.85 and 3.04 (2dd, J_1 5 Hz, J'_1 8 Hz, $J_2 = J'_2$ = 15 Hz, 2 H, β-CH_2His), 3.70 (s, 3 H, OCH_3), 3.73 (m, 2 H, δ-CH_2 Pro), 4.53 (dd, *J* 6 Hz, *J'* 8 Hz, 1 H, α-CH Pro), 4.74 (m, 1 H, α-CH His), 5.41 (d, *J* 8 Hz, 1 H, α-NH His), 7.22 (s, 1 H, δ-H His), and 8.00 (s, 1 H, ε-H His) (Found: C, 56.8; H, 7.35; N, 11.8. Calc. for $C_{22}H_{34}N_4O_7$: C, 56.63; H, 7.35; N, 12.00%).

N$^{\alpha}$-*t-Butoxycarbonylisoleucylhistidylproline Methyl Ester* (**10**).—The dipeptide (**9**) (6.99 g, 15 mmol) was stirred in CH_2Cl_2–TFA (30 ml; 50:50) for 30 min. The solvents were then evaporated under reduced pressure and the remaining acid was thoroughly removed by five co-evaporations with hexane. Trituration of the oily trifluoroacetate salt with diethyl ether afforded a solid compound which was washed on a fine glass filter with diethyl ether and dried *in vacuo* (KOH pellets).

The hygroscopic trifluoroacetate salt was then dissolved in acetonitrile (30 ml), Boc-Ile-OH (3.96 g, 16.5 mmol), and H_2O, and BOP (7.3, 16.5 mmol) was added. DIEA was then added dropwise until the solution reached pH 8 (5.16 g, 40 mmol) and the mixture stirred for 2 h.

Acetonitrile was evaporated under reduced pressure and the residue was dissolved in ethyl acetate (300 ml), washed with 1M-hydrochloric acid (1 × 30 ml), saturated sodium hydrogen carbonate (2 × 30 ml), and saturated sodium chloride (2 × 30 ml) and dried ($MgSO_4$). The solvent was then evaporated under reduced pressure and the residue purified by silica gel column chromatography (eluant CH_2Cl_2–MeOH 95:5) to give the product (4.3 g, 9.22 mmol, 61%), R_F 0.28 (CH_2Cl_2–MeOH 95:5), $[\alpha]_D$ −68° (*c* 1 in ethanol), m.p. >90 °C (dried gum), δ($CDCl_3$) 0.94 (t, *J* 7.5 Hz, 3 H, δ-CH_3 Ile), 1.03 (d, *J* 6.5 Hz, 3 H, γ'-CH_3 Ile), 1.41 [s, 9 H, $(CH_3)_3C$], 1.96 and 2.18 (2m, 4 H, β-CH_2 + γ-CH_2 Pro), 2.95 and 3.09 (2dd, J_1 6.5 Hz, J'_1 5 Hz, $J_2 = J'_2$ = 15 Hz, 2 H, β-CH_2 His), 3.73 (s, 3 H, OCH_3), 4.05 (m, 1 H, α-CH Ile), 4.91 (m, 1 H, α-CH His), 5.4 (d, *J* 7.5 Hz, 1 H, α-NH Ile), 6.90 (s, 1 H, δ-H His), 7.49 (d, *J* 7.5 Hz, 1 H, α-NH His), and 7.69 (s, 1 H, ε-H His) (Found: C, 56.05; H, 7.8; N, 14.1. Calc. for $C_{23}H_{37}N_5O_6\cdot CH_3OH$: C, 56.33; H, 8.09; N, 13.69%).

N$^{\alpha}$-*t-Butoxycarbonyl*-O-*benzyltyrosylisoleucylhistidylproline Methyl Ester* (**11**).—Compound (**10**) (5.8 g, 8 mmol) was treated with CH_2Cl_2–TFA (30 ml; 50:50) for 30 min. The solvent and

acid were removed and the trifluoroacetate salt isolated by trituration as described above.

Coupling with Boc-Tyr(Bzl)-OH (2.98 g, 8 mmol) was carried out in acetonitrile (40 ml) with addition of BOP (3.53 g, 8 mmol) and DIEA (3.09 g, 24 mmol). After being stirred overnight, the reaction mixture was concentrated under reduced pressure and the product purified by silica gel column chromatography, yield 4.09 g (5.6 mmol, 70%), R_F 0.38 (CH_2Cl_2–MeOH 95:5), $[\alpha]_D$ −50° (*c* 1 in ethanol, m.p. >111 °C (dried gum).

N$^{\alpha}$-*t-Butoxycarbonylvalyl-O-benzyltyrosylisoleucylhistidyl-proline Methyl Ester* (**12**).—Compound (**11**) (2.95 g, 4 mmol) was treated with CH_2Cl_2–TFA (10 ml; 50:50) for 30 min. The solvents were evaporated under reduced pressure and the trifluoroacetate salt precipitated with diethyl ether, then dissolved in DMF (10 ml), and Boc-Val-OH (0.87 g, 4 mmol), BOP (1.77 g, 4 mmol), and DIEA (1.54 g, 12 mmol) were added. After 2 h of stirring, the reaction product was precipitated by the addition of diethyl ether. The precipitate was collected on a fine glass filter, washed with 1M-hydrochloric acid, saturated sodium hydrogen carbonate, and saturated sodium chloride and recrystallized from ethanol–diethyl ether, yield 2.6 g (3.5 mmol, 75%), R_F 0.41 (CH_2Cl_2–MeOH 95:5), $[\alpha]_D$ −33° (*c* 1 in DMF), m.p. 201 °C (Found: C, 62.9; H, 7.2; N, 11.5. Calc. for $C_{44}H_{61}N_7O_9$: C, 63.51; H, 7.39; N, 11.78%).

N$^{\alpha}$-*t-Butoxycarbonyl-ω-nitroarginylvalyl-O-benzyltyrosylisoleucylhistidylproline Methyl Ester* (**13**).—The pentapeptide (**12**) (2.5 g, 3 mmol) was treated with TFA (10 ml) for 30 min. The mixture was then concentrated and the trifluoroacetate salt was precipitated and washed with diethyl ether. The resulting salt was dissolved in DMF (10 ml) and Boc-Arg(ωNO$_2$)-OH (0.96 g, 3 mmol), BOP (1.32 g, 3 mmol), and DIEA (1.16 g, 9 mmol) were added. After 2 h, the reaction mixture was concentrated under reduced pressure and the product was precipitated with diethyl ether. The precipitate was collected, washed with 1M-HCl, saturated $NaHCO_3$, and saturated NaCl. Recrystallization from ethanol–diethyl ether yielded a white solid (2.8 g, 2.7 mmol, 90%), R_F 0.41 (CH_2Cl_2–MeOH 9:10), $[\alpha]_D$ −32° (*c* 1 in DMF), m.p. 225 °C (Found; C, 58.2; H, 6.9; N, 16.3. Calc. for $C_{50}H_{72}N_{12}O_{12}$: C, 58.18; H, 7.03; N, 16.27%). Amino acid analysis: Val 1.04, Ile 1.01, Tyr 0.96, His 0.88, Arg 0.94, Pro 0.98.

N$^{\alpha}$-*t-Butoxycarbonyl-ω-nitroarginylvalyl-O-benzyltyrosyl-isoleucylhistidylproline* (**14**).—The hexapeptide (**13**) (2.6 g, 2 mmol) was dissolved in dimethylformamide (10 ml), and 2M-NaOH (2 ml) and water (2 ml) were added. The reaction was monitored by thin layer chromatography. After 1.5 h, the mixture was acidified to pH 3 with 1M-HCl. The saponified hexapeptide was precipitated with H_2O and collected on a glass filter. It was washed with diethyl ether and recrystallized from ethanol, yield 1.73 g (1.7 mmol, 85%), $[\alpha]_D$ −31° (*c* 1 in DMF), m.p. 232 °C (Found: C, 56.7; H, 6.5; N, 15.9. Calc. for $C_{49}H_{70}N_{12}O_{12}\cdot 2H_2O$: C, 56.74; H, 7.00; N, 16.20%).

N$^{\alpha}$-*t-Butoxycarbonyl-ω-nitroarginylvalyl-O-benzyltyrosyl-isoleucylhistidylprolylphenylalanylhistidyl-leucyl-leucylvalyl-O-benzyltyrosyl-O-benzylserine Benzyl Ester* (**15**).—The heptapeptide (**7**) (1.87 g, 1.5 mmol) was treated with TFA (10 ml) for 30 min. The trifluoroacetate salt was precipitated and isolated as described previously, then dissolved in DMF (10 ml), and the hexapeptide (**14**) (1.52 g, 1.5 mmol), BOP (0.66 g, 1.5 mmol), and DIEA (0.58 g, 4.5 mmol) were added. The mixture was stirred for 4 h and the reaction product was precipitated with diethyl ether, collected on a glass filter and washed with 1M-HCl, saturated $NaHCO_3$, and saturated NaCl, yield 3.05 g (1.4 mmol, 95%). A portion (1 g) was purified by silica-gel column chromatography (eluant CH_2Cl_2–MeOH 90:10) and recrystallized from ethanol, R_F 0.49 (CH_2Cl_2–MeOH 85:15), m.p. 210 °C. Amino acid analysis: Ser 0.75, Leu 1.80, Phe 0.90, His 1.64, Arg 0.75, Val 1.00, Tyr 1.64, Ile 0.80, Pro 1.10.

N$^{\alpha}$-*t-Butoxycarbonyl-O-benzylaspartyl-ω-nitroarginylvalyl-O-benzyltyrosylisoleucylhistidylprolylphenylalanylhistidyl-leucyl-leucylvalyl-O-benzyltyrosyl-O-benzylserine Benzyl Ester* (**16**).—The tridecapeptide (**15**) (2.14 g, 1 mmol) was treated with TFA (5 ml) for 30 min. The resulting trifluoroacetate salt was isolated as described previously. Coupling with Boc-Asp(OBzl)-OH (0.37 g, 1 mmol) was carried out in DMF (10 ml) by addition of BOP (0.44 g, 1 mmol) and DIEA (0.38 g, 3 mmol). After 2 h, the product was precipitated with diethyl ether, then purified by silica gel column chromatography (eluant CH_2Cl_2–MeOH 90:10), yield 2.1 g (0.9 mmol, 90%). An aliquot was recrystallized from ethanol, R_F 0.53 (CH_2Cl_2–MeOH 85:15), m.p. 190 °C (Found: C, 62.9; H, 6.8; N, 12.5. Calc. for $C_{125}H_{160}N_{22}O_{24}\cdot 2H_2O$: C, 62.79; H, 6.92; N, 12.88%). Amino acid analysis: Asp 0.91, Arg 0.85, Val 1.71, Ile 0.90, Leu 2.09, Tyr 1.64, Phe 0.94, His 1.67, Ser 1.00, Pro 1.08.

N$^{\alpha}$-*Acetyl-O-benzylaspartyl-ω-nitroarginylvalyl-O-benzyltyrosylisoleucylhistidylprolylphenylalanylhistidyl-leucyl-leucylvalyl-O-benzyltyrosyl-O-benzylserine Benzyl Ester* (**17**).—Compound (**16**) (588 mg, 0.25 mmol) was treated with trifluoroacetic acid (5 ml) for 30 min. The trifluoroacetate salt was precipitated and isolated as described previously, then dissolved in DMF (1.5 ml) and mixed with a solution containing acetic acid (60 mg, 1 mmol), BOP (442 mg, 1 mmol) and DIEA (129 mg, 1 mmol) in DMF (1 ml). The mixture was brought to pH 8 with DIEA (260 mg, 2 mmol) and stirred overnight. The acetylated tetradecapeptide was precipitated with diethyl ether and collected on a fine glass filter. It was washed with 1M-HCl, saturated $NaHCO_3$, and saturated NaCl, and recrystallized from ethanol, yield 510 mg (0.22 mmol, 88%), R_F 0.92 (CH_2Cl_2–MeOH 85:15), m.p. 210 °C (Found: C, 62.9; H, 7.0, N, 13.0. Calc. for $C_{122}H_{154}N_{22}O_{23}\cdot 2H_2O$: C, 62.79; H, 6.92; N, 12.88%).

N$^{\alpha}$-*Acetylaspartylarginylvalyltyrosylisoleucylhistidylprolyl-phenylalanylhistidyl-leucyl-leucylvalyltyrosylserine* (**18**).—The acetylated peptide (**17**) (100 mg, 0.043 mmol) was dissolved in acetic acid (10 ml) and hydrogen was bubbled through the solution at atmospheric pressure for 20 h with palladium black 10% (50 mg) as catalyst. The catalyst was removed by filtration over Celite. The solvent was evaporated under reduced pressure and the product purified by G15 Sephadex column chromatography with AcOH–H_2O (50:50). The major peak was collected and lyophilized to give the product (60 mg, 0.033 mmol, 77%). T.l.c. showed a unique spot revealed by charring [20 g $SO_4(NH_4)_2$ + 4 ml H_2SO_4 + 100 ml H_2O]; the R_F value was identical with that of a commercial product (Bachem). R_F in three solvent systems: 0.45 in n-butanol–pyridine–acetic acid–H_2O (30:20:6:24); 0.15 in n-butanol–acetic acid–H_2O (40:10:10); 0.57 in ethyl acetate–pyridine–acetic acid–H_2O (50:40:10:30). Amino acid analysis: Asp 1.13, Ser 0.66, Val 1.97, Ile 1.09, Leu 1.88, Tyr 1.91, Phe 0.95, His 1.88, Arg 1.04, Pro 1.01.

Highly purified samples have also been obtained by semi-preparative h.p.l.c. Some preliminary analytical runs (10 μg) allowed a check on the best conditions. 27% Acetonitrile acidified by 0.1% TFA as eluant yielded 5 mg of tetradecapeptide from each 20 mg of crude hydrogenate injection. We obtained well-resolved 360 ^{1}H n.m.r. spectra from these samples. Based on the 214/254 nm absorption ratio, an impurity, slightly more lipophilic than the preceding peptide, could be isolated and identified as the corresponding *N*-ω-nitroarginylpeptide which was rather difficult to hydrogenate.

Aspartylarginylvalyltyrosylisoleucylhistidylprolylphenyl-alanylhistidyl-leucyl-leucylvalyltyrosylserine (**19**).—The non-

acetylated peptide (**16**) was treated with TFA and hydrogenated in the same way. The crude product was directly purified by semi-preparative h.p.l.c. with 43% MeOH as eluant; 7 mg of highly pure peptide was isolated from each 27 mg injected. R_F in three solvent systems: 0.39 in n-butanol–pyridine–acetic acid–H_2O (30:20:6:24); 0.10 in n-butanol–acetic acid–H_2O (40:10:10); 0.48 in ethyl acetate–pyridine–acetic acid–H_2O (50:40:10:30). Amino acid analysis: Asp 1.07, Ser 0.89, Val 1.98, Ile 0.85, Leu 2.06, Tyr 1.84, Phe 0.96, His 1.98, Arg 1.92.

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