ENZYMATICALLY CATALYZED SYNTHESIS OF DIPEPTIDES CONTAINING O^4-SULFOTYROSINE AND O^4-PHOSPHOTYROSINE*

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Received June 2nd, 1986

Conditions for the thermolysin- or papain-catalyzed peptide bond formation between tert-butyloxycarbonyl-O^4-sulfotyrosine and leucine or methionine phenylhydrazide have been found. Acylation of O^4-sulfotyrosine phenylhydrazide with tert-butyloxycarbonylphenylalanine methyl ester was catalyzed by α-chymotrypsin. Tert-butyloxycarbonyl-O^4-phosphotyrosyl-leucine phenylhydrazide was prepared by thermolysin-catalyzed reaction.

In spite of a number of methodical studies, the synthesis of peptides containing O^4-sulfotyrosine or O^4-phosphotyrosine** represents a still not completely solved problem. The preparation of cholecystokinin and caerulin fragments and analogues is usually based on synthesis of the corresponding peptide with free tyrosine residue which in the last step is sulfated with various reagents (for a review see refs[3,4]). Similar sulfation[5] or phosphorylation[6] has been described with simple peptides. According to another method[3,4,7], the peptide chain is built using active esters or hydrazide of protected O^4-sulfotyrosine or O^4-phosphotyrosine (no experimental details[7] are given).

This paper describes utilization of proteolytic enzymes in the preparation of dipeptides with O^4-sulfotyrosine or O^4-phosphotyrosine as one of the amino acid components. The starting tyrosine derivatives were prepared essentially according to the described procedures. Reaction of sulfuric acid with free tyrosine gave O^4-sulfotyrosine[5,8,9] which was converted into its tert-butyloxycarbonyl derivative *Ia* (as the dipotassium salt) by treatment with ditert-butyl dicarbonate. The numerical value of its optical rotation ($[\alpha]_D$ +51·7°) agreed with that described[4] for the sodium salt ($[\alpha]_D$ −52·4°); it was, however, of the opposite sign. Therefore, we converted our potassium salt into the sodium salt which exhibited $[\alpha]_D$ +51·9°. The same (positive) sign of rotation was also found for the O^4-phosphotyrosine derivative and we assume that the published[4] negative value is incorrect. The corresponding methyl ester *Ib*

* Part CXCVIII in the series Amino Acids and Peptides; Part CXCVII: Int. J. Pept. Protein Res., in press.

** The nomenclature and symbols of amino acids, peptides and protecting groups obey the published recommendations[1,2]. The amino acids used in this study have the L-configuration.

was prepared by treatment of tert-butyloxycarbonyltyrosine methyl ester with pyridinium acetylsulfate[10,11]; the same method was used in the preparation of the phenylhydrazide *Ic*. This compound was obtained also by the papain-catalyzed condensation of tert-butyloxycarbonyl-O^4-sulfotyrosine with phenylhydrazine, albeit in lower yield caused by isolation difficulties. O^4-Phosphotyrosine was prepared by treatment of free tyrosine with a mixture of phosphorus pentoxide and phosphoric acid[12] and was purified by chromatography on a sulfonate cation exchanger[13,14]. The compound was acylated again with ditert-butyl dicarbonate and the product *V* was obtained as the potassium salt.

The peptide bond between tert-butyloxycarbonyl-O^4-sulfotyrosine (*Ia*) and leucine phenylhydrazine was formed in the presence of thermolysin in an aqueous buffer pH 7. The product *II* precipitated in the reaction medium as the leucine phenylhydrazidium salt. The dipeptide *IIIa* was obtained from *II* by chromatography on an ion exchanger. Under the same reaction conditions we prepared the dipeptide *IIIb* which is a part of the CCK-8 sequence. The compound *II* was also obtained from the derivative *Ia* at pH 4·8 with papain as catalyst; however, the yield was lower and the product was contaminated by an unidentified side-product, chromatographically non-identical with compound *VII*. Using papain or thermolysin, we prepared under analogous reaction conditions also the dipeptide *VII*. In both cases the product precipitated from the reaction mixture shifting the chemical equilibrium and increasing the yield of the dipeptide. In the syntheses of dipeptides with O^4-sulfotyrosine and O^4-phosphotyrosine, the higher solubility of the products in the reaction medium makes the equilibrium shift unfavourable. A favourable effect occurs only when the ionization of the sulfo and phospho groups in the arising product is compensated by salt formation with the corresponding amino component. A very fast reaction was observed in the papain-catalyzed synthesis of the dipeptide *IIIa* from *Ib* at pH 9. As found by HPLC, the starting *Ib* was almost quantitatively consumed during 30 min. Attempted catalysis of this reaction with α-chymotrypsin at pH 10 completely failed, although a parallel synthesis of the dipeptide *VII* from tert-butyloxycarbonyltyrosine methyl ester gave 52% yield of the product. We studied also the enzymatically catalyzed acylation of O^4-sulfotyrosine phenylhydrazide, obtained from compound *Ic* by treatment with trifluoroacetic acid. Of the enzymes mentioned, only α-chymotrypsin catalyzed the reaction of tert-butyloxycarbonylphenylalanine methyl ester with O^4-sulfotyrosine phenylhydrazide in an aqueous medium at pH 10. According to HPLC, only 30% of product was formed and the product *IV* was isolated by preparative HPLC in low yield. The thermolysin-catalyzed synthesis of dipeptide *VI* from compound *V* afforded a lower yield than the synthesis of the analogous compound *II*.

On the basis of our experiments and data in the literature we can conclude that the presence of a strongly acidic functional group in the side-chain of some amino acids in position P_1 does not hinder the activity of thermolysin and papain in the

peptide bond synthesis. This agrees also with our previous experiments in papain-catalyzed synthesis of peptides containing γ-carboxyglutamic acid[15] in position P_1. The enzymatic synthesis of peptide bonds with substrates containing acidic coded

Boc-Tyr(SO_3X)-Y
Ia, X = K, Y = OK
Ib, X = Na, Y = OMe
Ic, X = Na, Y = N_2H_2-C_6H_5

Boc-Tyr(SO_3H)-Leu-N_2H_2-C_6H_5 . Leu-N_2H_2-C_6H_5
II

Boc-Tyr(SO_3X)-Xaa-N_2H_2-C_6H_5
IIIa, X = K, Xaa = Leu
IIIb, X = NH_4, Xaa = Met

Boc-Phe-Tyr(SO_3Na)-N_2H_2-C_6H_5
IV

Boc-Tyr(PO_3K_2)-OH
V

Boc-Tyr(PO_3H_2)-Leu-N_2H_2-C_6H_5 . (Leu-N_2H_2-C_6H_5)$_2$
VI

Boc-Tyr-Leu-N_2H_2-C_6H_5
VII

amino acids (Asp, Glu) in the position P_1 is described for papain[16–19] as well as thermolysin[16,20]. However, sulfation of the tyrosyl residue (in position P_1) hindered completely the peptide bond formation with α-chymotrypsin in the synthesis of dipeptide *IIIa*. An opposite conclusion can be drawn if O^4-sulfotyrosine is in position P_1'. In such case only α-chymotrypsin exhibited the affinity to this residue. The synthesis of the dipeptide *IV* with α-chymotrypsin resulted in only low conversion, similarly to the acylation of glutamic acid α-amide[21]. Obviously, the presence of an acidic amino acid in position P_1' decreases the nucleophilic reactivity[22] in the α-chymotrypsin-catalyzed synthesis of peptide bonds.

EXPERIMENTAL

Melting points were determined on a Kofler block and are uncorrected. The reaction mixtures were evaporated on a rotatory evaporator at bath temperature 30°C. Analytical samples were dried over phosphorus pentoxide at room temperature and 150 Pa. Thin-layer chromatography (TLC) was performed on silica gel plates (Silufol, Kavalier, Czechoslovakia) in the following systems: 2-butanol–98% formic acid–water (75 : 13·5 : 11·5) (S1), 2-butanol–25% aqueous ammonia–water (85 : 7·5 : 7·5) (S2), 1-butanol–acetic acid–water (4 : 1 : 1) (S3), 1-butanol–pyridine–acetic acid–water (15 : 10 : 3 : 6) (S4). Electrophoresis was carried out in moist chamber on a Whatman 3MM paper (20 V/cm, 1 h) in 1 mol l^{-1} acetic acid (pH 2·4) and a pyridine–acetate buffer (pH 5·7). The mobility values (*E*) refer to compounds obtained after deprotection of the α-amino group. The compounds were detected by ninhydrin or by the chlorination method. Samples for the amino acid analysis were hydrolyzed with 6 mol l^{-1} HCl at 110°C for 20 h and with 0·2 mol l^{-1} $Ba(OH)_2$ for 20 h at the same temperature[23]. The barium hydroxide was removed by treatment with gaseous CO_2. The analyses were performed on an AAA 339 (Mikrotech-

na) analyzer. Optical rotations were determined on a Perkin–Elmer 141 MCA polarimeter. High performance liquid chromatography (HPLC) was performed on a Spectra Physics SP 8700 instrument equipped with an SP 8400 UV detector and an SP 4100 integrator. Analytical HPLC was carried out on a 15 × 0·4 cm column packed with Separon SIX C-18; flow rate 42 ml/h, detection at 220 nm, mobile phase: mixtures of methanol with 0·05% aqueous trifluoroacetic acid (the amount of methanol in vol.% is given as a subscript with the k' values). Preparative HPLC was carried out on a 25 × 0·8 cm column packed with the same stationary phase as above; flow rate 180 ml/h, mobile phase: mixtures of methanol with water. Trifluoroacetates of the amino acid and peptide derivatives were prepared by treatment with trifluoroacetic acid and their homogeneity was checked by paper electrophoresis and HPLC. The course of the enzymatic reactions was monitored by HPLC in order to determine the end or to follow side reactions. Thermolysin and α-chymotrypsin were Serva (F.R.G.) products, papain was purchased from Sigma (U.S.A.).

O-Sulfotyrosine and O-Phosphotyrosine

O-Sulfotyrosine was prepared[5] by treatment of tyrosine with sulfuric acid; the sulfation should be performed at the described temperature (below −5°C) since reaction at higher temperatures led to sulfonation of the benzene nucleus (*cf.* ref.[9]). O-Phosphotyrosine was obtained by reaction of tyrosine with a mixture of phosphorus pentoxide and 85% H_3PO_4[12] and subsequent purification[13,14] on Dowex-50 (H^+-form). For the hemihydrate $C_9H_{12}NO_6.0{\cdot}5\,H_2O$ (271·0) calculated: 39·88% C, 4·79% H, 5·17% N, 11·43% P; found: 39·52% C, 4·87% H, 5·13% N, 11·84% P. M.p. 236–237°C, $[\alpha]_D$ −4·2° (*c* 1, 2 mol l^{-1} HCl). Reported m.p. 226–227°C and $[\alpha]_D^{28}$ −7·8° (ref.[12]), m.p. 227°C and $[\alpha]_D^{20}$ −8·8° (ref.[6]), m.p. 253°C and $[\alpha]_D^{20}$ −2·0° (ref.[24]), m.p. 224–225°C and $[\alpha]_D$ −9·19° (ref.[25]), and m.p. 246–247°C (ref.[13]).

Under conditions of alkaline hydrolysis (0·2 mol l^{-1} $Ba(OH)_2$ 20 h, 110°C), 88% of O-phosphotyrosine was converted into tyrosine. In the reaction with L-amino acid oxidase under conditions leading to 72% oxidation of tyrosine, O-phosphotyrosine was cleaved to the extent of 83% and O-sulfotyrosine quantitatively. The physico-chemical properties of both these tyrosine derivatives are given in Table I.

Dipotassium Salt of Tert-butyloxycarbonyl-O-sulfotyrosine (*Ia*)

Ditert-butyl dicarbonate (0·4 g) was added to a solution of potassium salt of O-sulfotyrosine (0·4 g) in a mixture of dioxane (1·6 ml) and 1 mol l^{-1} KOH (1·6 ml). After stirring at room temperature for 4 h, dioxane was evaporated and the aqueous solution was washed with ether and filtered through a column of Dowex-50 (12 ml). The eluate was neutralized with 0·5 mol l^{-1} KOH to pH 5 and taken down. The residue was triturated with ether and crystallized from ethanol–ether to give 0·465 g (79%) of the product, m.p. 285–288°C. R_F 0·59 (S1), 0·10 (S2), 0·54 (S3), 0·51 (S4); k'_{40} = 1·73; $[\alpha]_D$ +51·7° (*c* 0·3, dimethylformamide). For $C_{14}H_{18}K_2NO_8S.3\,H_2O$ (491·6) calculated: 34·20% C, 4·71% H, 2·85% N; found: 34·59% C, 4·45% H, 2·48% N. A part of the product was converted to its sodium salt by means of an ion exchanger; $[\alpha]_D$ +51·9° (*c* 0·3, dimethylformamide), ref.[4] reports $[\alpha]_D$ −52·4° (*c* 1, dimethylformamide).

Sodium Salt of Tert-butyloxycarbonyl-O-sulfotyrosine α-Methyl Ester (*Ib*)

Pyridinium acetylsulfate (880 mg) was added to a solution of tert-butyloxycarbonyltyrosine methyl ester (300 mg) in a mixture of dimethylformamide (5 ml) and pyridine (5 ml) and the solution was incubated at 35°C for 20 h. The solvents were evaporated, the residue was dissolved in water, adjusted to pH 7 with 0·1 mol l^{-1} NaOH and washed with ethyl acetate. The aqueous solution was evaporated, the residue triturated with methanol, filtered and the filtrate evaporated.

The residue was triturated with dioxane and filtered. Evaporation of the filtrate and crystallization from ether gave 195 mg (48%) of the product, m.p. 105–107°C; R_F 0·60 (S1), 0·29 (S2), 0·52 (S3), 0·62 (S4); k'_{40} = 3·05; $[\alpha]_D$ −14·5° (*c* 0·5, dimethylformamide); $E^{2,4}_{Gly} = 0{\cdot}0$, $E^{5,7}_{Gly} = 0{\cdot}0$. For $C_{15}H_{20}NNaO_8S.1{\cdot}5\ H_2O$ (424·4) calculated: 42·45% C, 5·46% H, 3·30% N; found: 42·16% C, 5·05% H, 3·48% N.

Sodium Salt of Tert-butyloxycarbonyl-O-sulfotyrosine α-Phenylhydrazide (*Ic*)

A) Pyridinium acetyl sulfate (880 mg) was added at 35°C to a solution of tert-butyloxycarbonyltyrosine phenylhydrazide (380 mg) in a mixture of dimethylformamide (5 ml) and pyridine (5 ml) and the solution was incubated at 35°C for 20 h. After evaporation of the solvents, the residue was dissolved in water, neutralized with 1 mol l^{-1} NaOH to pH 7 and washed with ethyl acetate. The aqueous solution was taken down, the residue triturated with methanol, filtered and the filtrate evaporated. The residue was triturated with dioxane, filtered, the solvent evaporated and the residue crystallized from ether. Yield 290 mg (60%) of the product, m.p. 164–172°C, containing about 5% of a side product (according to TLC, HPLC and paper electrophoresis). An analytical sample was obtained by HPLC; m.p. 166–168°C, R_F 0·67 (S1), 0·35 (S2), 0·60 (S3), 0·63 (S4); k'_{40} = 4·78; $E^{2,4}_{Gly}$ = 0·13, $E^{5,7}_{Gly}$ = 0·0; $[\alpha]_D$ 0° (*c* 0·3, dimethylformamide), $[\alpha]_D$ 0° (*c* 0·3, methanol); however, the optical activity was proved by a quantitative CD measurement. For $C_{20}H_{24}N_3.NaO_7S.H_2O$ (491·5) calculated: 48·87% C, 5·33% H, 8·54% N; found: 48·54% C, 5·68% H, 8·45% N.

B) Ethylenediaminetetraacetic acid (0·3 mg) and cysteine hydrochloride (1 mg) were added to a solution of *Ia* (40 mg) and phenylhydrazine (20 µl) in a 0·2 mol l^{-1} acetate buffer pH 4·8 (1 ml). The mixture was adjusted to pH 4·8 and after addition of papain (4 mg) incubated at 38°C for 25 h. Methanol (0·5 ml) was added and the mixture was filtered through a column of Dowex 50 (2 ml) in 20% aqueous methanol. The eluate was neutralized to pH 7 with 0·1 mol l^{-1} NaOH, and after evaporation of methanol freeze-dried. The lyophilizate (57 mg) was purified by HPLC and the corresponding fraction freeze-dried. Trituration of the residue with ether afforded 10 mg (20%) of *Ic* of the same m.p., R_F and k' as described in the experiment *A*).

TABLE I

Properties of O^4-sulfotyrosine and O^4-phosphotyrosine

Parameter		Tyr(PO_3H_2)	Tyr(SO_3K)	Reference amino acid
R_F[a]	S1	0·07	0·24	0·44 (Tyr)
	S2	0·00	0·00	0·45 (Tyr)
	S3	0·00	0·03	0·46 Tyr)
	S4	0·02	0·15	0·34 (Tyr)
E[b]	pH 2·4	0·49	0·63	Cys(O_3H)
	pH 5·7	0·57	0·66	Cys(O_3H)
RT[c]		12·3′	12·6′	15·3′ (taurine)
				58·2′ (Tyr)
k'_{10}[d]		0·51	1·58	3·28 (Tyr)

[a] TLC; [b] paper electrophoresis; [c] retention time (min) on amino acid analyzer; [d] phase capacity ratio (HPLC).

Leucine Phenylhydrazidium Salt of Tert-butyloxycarbonyl-O-sulfotyrosyl-leucine α-Phenylhydrazide (*II*)

Calcium chloride (0·5 mg) was added to a solution of dipotassium salt of tert-butyloxycarbonyl--O-sulfotyrosine (40 mg) and leucine phenylhydrazide trifluoroacetate (84 mg) in a mixture of ethanol (0·1 ml) and 0·2 mol l^{-1} TRIS–maleate buffer, pH 7 (0·9 ml). The solution was adjusted to pH 7 and after addition of thermolysin (4 mg) incubated at 38°C for 20 h. The precipitate was filtered off, washed with a small amount of water, 0·1 mol l^{-1} HCl and water, dried and washed with ether to give 28 mg (44%) of the salt *II*, m.p. 189–190°C; k'_{60} = 1·48 (Leu-$N_2H_2C_6H_5$) and 1·83 (*IIIa*); $[\alpha]_D$ −20·1° (*c* 0·3, methanol). Amino acid analysis: Leu 2·20, Tyr 1·00. For $C_{38}H_{55}N_7O_9S$ (786·0) calculated: 58·06% C, 7·05% H, 12·48% N; found: 58·14% C, 6·92% H, 12·79% N.

Potassium Salt of Tert-butyloxycarbonyl-O-sulfotyrosyl-leucine α-Phenylhydrazide (*IIIa*)

A) A solution of the salt *II* (25 mg) in 50% aqueous methanol was filtered through a column of Dowex 50 (1 ml) in 50% aqueous methanol and the filtrate was titrated with 0·1 mol l^{-1} KOH to pH 7. After evaporation of methanol, the residue was lyophilized and triturated with ether to afford 15 mg (76%) of the product, m.p. 164–166°C; R_F 0·64 (S1), 0·35 (S2), 0·62 (S3), 0·66 (S4); k'_{60} = 1·83; $[\alpha]_D$ −21·3° (*c* 0·25 ,methanol). Amino acid analysis: Leu 1·05, Tyr 1·00; after alkaline hydrolysis: Tyr(SO_3^-) 1·00, Leu 1·14, no trace of Tyr. For $C_{26}H_{35}KN_4O_8S$ (602·8) calculated: 51·80% C, 5·85% H, 9·29% N; found: 51·90% C, 5·60% H, 9·41% N.

B) Ethylenediaminetetraacetic acid (0·3 mg) and cysteine hydrochloride (1 mg) were added to a solution of *Ib* (50 mg) and leucine phenylhydrazide trifluoroacetate (28 mg) in a mixture of ethanol (0·2 ml) and 0·2 mol l^{-1} carbonate–bicarbonate buffer pH 9 (0·8 ml). The solution was adjusted to pH 9 and, after addition of papain (4 mg), stirred at 30°C for 30 min. The mixture was diluted with methanol (1 ml) and filtered through a column of Dowex 50 (3 ml) in 50% aqueous methanol. The filtrate was titrated with 0·1 mol l^{-1} KOH to pH 7 and methanol was evaporated. Freeze-drying afforded 71 mg of product which was purified by preparative HPLC. The product fraction was freeze-dried and triturated with ether to afford 19 mg (27%) of *IIIa*, m.p. 166–168°C, which had the same R_F and k' values as the product obtained by procedure *A*).

Ammonium Salt of Tert-butyloxycarbonyl-O-sulfotyrosyl-methionine α-Phenylhydrazide (*IIIb*)

Calcium chloride (0·5 mg) was added to a solution of dipotassium salt of tert-butyloxycarbonyl--O-sulfotyrosine (90 mg) and methionine phenylhydrazide trifluoroacetate (180 mg) in a mixture of ethanol (0·2 ml) and 0·2 mol l^{-1} TRIS–maleate buffer pH 7 (0·8 ml). The mixture was adjusted to pH 7 and after addition of thermolysin (4 mg) incubated at 38°C for 20 h. The formed oil was separated by decanting, washed with small amount of water, 0·1 mol l^{-1} HCl and water, and dissolved in 50% aqueous methanol (2 ml). The solution was filtered through a column of Dowex 50 (2 ml) in 50% aqueous methanol, the filtrate was neutralized with 0·1 mol l^{-1} NH_4OH to pH 7 and after evaporation of methanol freeze-dried. Trituration of the residue with ether afforded 31 mg (26%) of the product, m.p. 170–173°C. An analytical sample was obtained by HPLC, lyophilization of the corresponding fraction and trituration with ether; m.p. 173–175°C; R_F 0·62 (S1), 0·35 (S2), 0·61 (S3), 0·66 (S4); k'_{60} = 1·09; $[\alpha]_D$ − 12·7° (*c* 0·3, methanol). Amino acid analysis: Met 1·00, Tyr 1·03; after alkaline hydrolysis: Tyr(SO_3^-) 1·00, Met 0·83, Met(O_2) 0·33, no trace or Tyr. For $C_{25}H_{37}N_5O_8S_2 \cdot 2{\cdot}5\ H_2O$ (644·8) calculated: 46·56% C, 6·56% H, 10·86% N; found: 46·41% C, 5·92% H, 10·76% N.

Sodium Salt of Tert-butyloxycarbonylphenylalanyl-O-sulfotyrosine α-Phenylhydrazide (*IV*)

Tert-butyloxycarbonylphenylalanine methyl ester (110 mg) in methanol (0·4 ml) was added to a solution of sodium salt of O-sulfotyrosine α-phenylhydrazide trifluoroacetate (90 mg) in a mixture of methanol (0·7 ml) and 0·2 mol l^{-1} carbonate–bicarbonate buffer, pH 10·5 (3 ml) and the mixture was adjusted to pH 10. α-Chymotrypsin (5 mg) was added and the mixture was stirred at 30°C for 2 h, the pH being rendered constant. After acidification with acetic acid to pH 3, the product was taken up in ethyl acetate, then water and the aqueous solution was freeze-dried. The residue was dissolved in water, titrated with 0·1 mol l^{-1} NaOH to pH 7 and again freeze-dried. The residue (19 mg) was finally purified by HPLC. Freeze-drying of the pertinent fraction and trituration with ether gave 7 mg (6%) of the product, m.p. 170—172°C; R_F 0·62 (S1), 0·35 (S2), 0·64 (S3), 0·65 (S4); k'_{60} = 1·96; $[\alpha]_D$ −5·5° (*c* 0·2, methanol). Amino acid analysis: Tyr 1·00, Phe 0·98; after alkaline hydrolysis: Tyr(SO_3^-) 0·90, Phe 1·00, no traces of Tyr. For $C_{29}H_{33}N_4.NaO_8S.H_2O$ (638·7) calculated: 54·53% C, 5·52% H, 8·77% N; found: 54·31% C, 5·75% H, 8·86% N.

Dipotassium Salt of Tert-butyloxycarbonyl-O-phosphotyrosine (*V*)

A solution of ditert-butyl dicarbonate (0·4 g) in dioxane (2 ml) was added to a solution of O-phosphotyrosine (0·35 g) in a mixture of dioxane (3 ml) and 1 mol l^{-1} KOH (3 ml). The solution was stirred at room temperature for 4 h and rendered at pH 10—10·5. After evaporation of dioxane, the aqueous solution was washed with ether and filtered through a column of Dowex 50 (12 ml). The eluate was adjusted to pH 5·1 with 1 mol l^{-1} KOH and the solution was freeze-dried. The residue was dissolved in ethanol and the product (0·57 g; 59%) precipitated with ether; m.p. 166—168°C; R_F 0·13 (S1), 0·00 (S2), 0·06 (S3), 0·20 (S4); k'_{40} = 1·89; $[\alpha]_D$ +57·9° (*c* 0·2, dimethylformamide). For $C_{14}H_{18}K_2NO_8P.H_2O$ (455·5) calculated: 36·91% C, 4·43% H, 3·07% N, 6·80% P; found: 37·20% C, 4·54% H, 3·09% N, 6·61% P.

Bis-(Leucine Phenylhydrazidium) Salt of Tert-butyloxycarbonyl-O-phosphotyrosyl-leucine α-Phenylhydrazide (*VI*)

Calcium chloride (0·5 mg) was added to a solution of dipotassium salt of tert-butyloxycarbonyl--O-phosphotyrosine (47 mg) and leucine phenylhydrazide trifluoroacetate (100 mg) in a mixture of ethanol (0·1 ml) and 0·2 mol l^{-1} TRIS–maleate buffer, pH 7, (0·9 ml) and the solution was adjusted to pH 7. After addition of thermolysin (4 mg), the mixture was incubated at 38°C for 20 h. The precipitate was filtered, washed with a small amount of water, 0·1 mol l^{-1} HCl, again with water, and dried. Washing with ether afforded 14 mg (13%) of the product, m.p. 184—186°C. On chromatography, the compound gave two spots (or two peaks): one due to the free dipeptide: R_F 0·81 (S1), 0·03 (S2), 0·64 (S^3), 0·47 (S4) and the other due to Leu-$N_2H_2C_6H_5$: R_F 0·41 (S1), 0·57 (S2), 0·55 (S^3), 0·65 (S4); k'_{60} = 1·48 (Leu-$N_2H_2C_6H_5$) and 2·72 (free dipeptide); $[\alpha]_D$ −11·8° (*c* 0·15, dimethylformamide). Amino acid analysis: Leu 2·82, Tyr 1·17. For $C_{50}H_{75}N_{10}O_{10}P.2\,H_2O$ (1 043) calculated: 57·57% C, 7·63% H ,13·43% N; found: 57·40% C, 7·92% H, 13·49% N.

Tert-butyl oxycarbonyltyrosyl-leucine Phenylhydrazide (*VII*)

Calcium chloride (0·5 mg) was added to a solution of tert-butyloxycarbonyltyrosine (56 mg) and leucine phenylhydrazide trifluoroacetate (100 mg) in a mixture of ethanol (0·2 ml) and 0·2 mol. . l^{-1} TRIS–maleate buffer, pH 7 (0·8 ml). After adjusting to pH 7 and addition of thermolysin (4 mg) the mixture was incubated at 38°C for 7 h. The precipitate was filtered, washed with water, 20% citric acid, water, 0·5 mol l^{-1} sodium hydrogen carbonate, water, and dried. Crystallization

from ethyl acetate afforded 78 mg (80%) of the product, m.p. 217–218°C; R_F 0·88 (S1), 0·80 (S2), 0·86 (S3), 0·85 (S4); $k'_{60} = 6·68$; $[\alpha]_D$ −25° (*c* 0·3, dimethylformamide). Amino acid analysis: Leu 1·01, Tyr 1·00. For $C_{26}H_{36}N_4O_5.0·5\ H_2O$ (493·6) calculated: 63·26% C, 7·56%H, 11·35%N; found: 63·37% C, 7·36% H, 11·42% N.

We are indebted to Mr J. Zbrožek for carrying out the amino acid analyses, to Mrs Z. Ledvinová for determination of optical rotations and to Dr I. Frič for measurement of the CD *spectrum. The elemental analyses were performed in the Analytical Laboratory of this Institute (Dr V. Pechanec, Head).*

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Translated by M. Tichý.