PROLINE- AND 4-HYDROXYPROLINE-CONTAINING DIPEPTIDES*

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Dedicated to Professor F. Šantavý on the occasion of his 60th birthday.

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Diastereoisomeric linear peptides, namely, L-prolyl-4-hydroxy-L-proline and D-prolyl-4-hydroxy-L-proline have been prepared along with the corresponding cyclodipeptides. Conformation of these compounds has been investigated with the use of infrared spectroscopy and circular dichroism.

In examination of metabolic and endocrine dependent bone diseases it became indispensable to determine excretion of 4-hydroxy-L-proline (free or „bound" in the form of peptides) with urine. Differentiation inside the fraction of „bound" 4-hydroxy-L-proline could contribute to elucidation of pathogenesis of some osteopathies. L-Prolyl-4-hydroxy-L-proline (*Ia*) represents one of the low-molecular metabolites of collagen. It has been proposed[1] to determine compound *Ia* by means of a method consisting in cyclisation to cyclo(L-prolyl-4-hydroxy-L-prolyl) (*IIa*) and the subsequent gas chromatography. In connection with detailed work on this method it was necessary to synthesise the appropriate compounds which also were interesting from the stereochemical standpoint (*cf.* the earlier papers of this Laboratory on spatial relations in cyclodipeptides containing cyclic imino acids[2-6]). In the present paper, we report a synthesis of the two diastereoisomers of both the linear peptides *I* as well as the corresponding cyclodipeptides *II*; members of these pairs differ in absolute configuration of the proline residue. Preparation of these compounds has not been so far satisfactorily described in the literature in spite of some reported experiments with compounds *Ia* and *IIa* (*cf.*[7,8]).

The two linear dipeptides *I* were prepared by the following sequence of reactions. Condensation of benzyloxycarbonyl-L(or D)-proline with 4-hydroxy-L-proline methyl ester by means of N,N'-dicyclohexylcarbodiimide afforded the protected dipeptide

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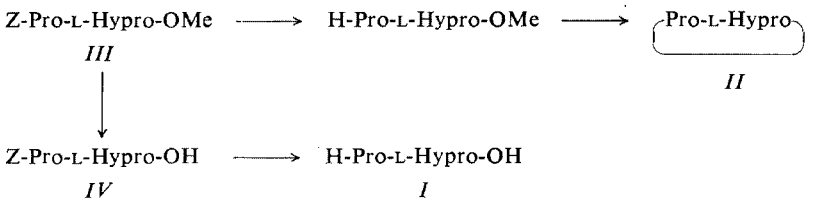
III. Hydrolysis of compound *III* by the action of 1M-NaOH to the N-protected dipeptide *IV* and the subsequent catalytic hydrogenolysis yielded the free dipeptide *I*. Removal of the benzyloxycarbonyl group in *III* by the action of hydrogen bromide in acetic acid afforded a product which was for the most part acetylated on the hydroxylic function[9]; however, the acetyloxy group was removed in the course of the alkaline hydrolysis. An analogous process was used in the preparation of cyclo(D-prolyl-4-hydroxy-L-prolyl) (*IIb*). By the action of hydrogen bromide in acetic acid, the protected dipeptide *IIIb* was converted to the dipeptide methyl ester which was then without isolation cyclised by the action of methanolic ammonia under the simultaneous solvolysis of the acetoxy group to yield the required cyclodipeptide *IIb*. Since the protected dipeptide *IIIa* was not crystalline, the cyclodipeptide *IIa* was prepared *via* *o*-nitrobenzenesulfenyl-L-prolyl-4-hydroxy-L-proline methyl ester by deblocking with methanolic hydrogen chloride and cyclisation by the action of methanolic ammonia. The thus-obtained compounds *I* and *II* were homogeneous and their properties corresponded with the required structure including fragmentation in the mass spectrometry of cyclodipeptides.



a, L-proline derivatives; *b*, D-proline derivatives

Both the cyclodipeptides were characterised by means of physical methods on comparison with the earlier investigated analogous compounds lacking the hydroxyl group, namely, cyclo(L-prolyl-L-prolyl) and cyclo(D-prolyl-L-prolyl). From the analytical standpoint there is of great value the easy separation by means of gas chromatography of both the free cyclodipeptides and their trimethylsilyl ethers. Somewhat shorter elution times of the *cis*-isomers *IIa* when compared with those of the *trans*-isomers *IIb* indicate a more compact conformation of compounds *IIa* in accordance with the idea on their boat conformation.

The attempted determination of the conformation by means of ^{1}H NMR spectroscopy resulted in poorly differentiated spectra (some difficulties are due to the low solubility of substances). Consequently, only a rough assignment of the particular peak groups was possible. With cyclodipeptides *IIa* and *IIb*, in connection with the spectrum of the O-acetyl derivative of compound *IIa* and on comparison with the earlier examined[4] spectra of cyclo(L-prolyl-L-prolyl) and cyclo(D-prolyl-L-prolyl), the tricyclic system appears to exist in the same conformation as the corresponding diastereoisomeric compounds lacking the hydroxyl group. In the case of the *trans*-disubstituted

TABLE I
Wave Numbers (cm^{-1}) of Bands in Infrared Spectra of Cyclodipeptides

Compound	Solvent	ν(C=O)	$\nu(C_{(\alpha)}—N)$	$\nu(O—H)_{free}$
IIa	CCl_4	1 676	1 425	3 610
IIa	$CHCl_3$	1 662	1 438	3 600
IIb	CCl_4	1 670	1 451	3 610
IIb	$CHCl_3$	1 660	1 461	3 600

isomer *IIb*, the central ring is planar or in a very flattened chair conformation with nonplanar amide groupings while a pronounced boat conformation may be ascribed to the *cis*-isomer *IIa*. Also the infrared spectroscopic data of compounds *IIa* and *IIb* (Table I) which are closely related to those of substances lacking the hydroxyl group are in accordance with this conclusion. Neither compound *IIa* nor the isomer *IIb* exhibits the presence of an intramolecular hydrogen bond.

The CD curves of the *cis*-isomer *IIa* and cyclo(L-prolyl-L-prolyl) are almost superimposable under identical conditions, in accordance with the idea on the same spatial relations in the chromophoric system of the two peptide groupings. From this point of view, the *trans*-isomer *IIb* is more interesting. As indicated by the negative long-wavelength and the positive short-wavelength dichroic bands, the CD curve is mainly shaped by the 4-hydroxyproline residue belonging to the L-series. The presence of

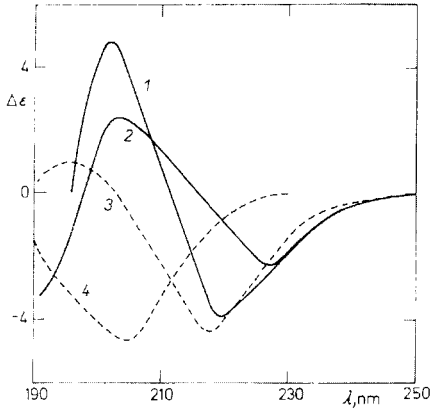


FIG. 1
CD Curves in 2,2,2-Trifluoroethanol
1 Cyclo(L-prolyl-4-hydroxy-L-prolyl) (*IIa*); *2* cyclo(D-prolyl-4-hydroxy-L-prolyl) (*IIb*); *3* L-prolyl-4-hydroxy-L-proline (*Ia*); *4* D-prolyl-4-hydroxy-L-proline (*Ib*).

the very similar D-proline residue manifests itself by a rather significant intensity compensation of both the Cotton effects but nevertheless the rotational strength of either the n-π^* or (particularly) the π-π^* transition remains considerably high when compared with the relatively low violation of the molecular symmetry due to the presence of one hydroxyl on the periphery of the molecule. The linear dipeptide *Ia* exhibits similarly to the cyclic dipeptide *IIa* a virtually identical curve to that of L-prolyl-L-proline[10]. On the other hand, the curve of the isomer *Ib* is of a completely different type; extremum of the n-π^* transition is not visible at all and dichroic band of the π-π^* transition exhibits a negative sign, (Fig. 1).

In conclusion, replacement of the proline residue by the 4-hydroxyproline residue does not affect to an appreciable extent conformation of the molecule in solution as also observed by Deber and coworkers[11] in the case of cyclo(L-prolyl-L-prolyl-L-prolyl).

EXPERIMENTAL

Methods

Melting points were taken on a heated microscope stage (Kofler block). Analytical samples were dried over phosphorus pentoxide at room temperature and 0·5 Torr for 24 h. Optical rotations were measured on a photoelectric polarimeter; concentration, 0·5 g per 100 ml; dimethylformamide, unless stated otherwise. The ^{1}H NMR spectra were recorded on a Varian 100 HA apparatus with the use of tetramethylsilane as internal standard; the data are expressed in δ (p.p.m.) values. Mass spectra were recorded on an AEI MS-902 apparatus. The purity of products was checked by thin-layer chromatography on silica gel (Kieselgel G, Merck) in the solvent systems 4 : 1 : 1 1-butanol–acetic acid–water and 3 : 1 2-butanol–3% aqueous ammonia, and by electrophoresis on paper Whatman 3 MM in a moist chamber in 6% aqueous acetic acid at a potential gradient of about 20 V/cm. Compounds with a free amino group were detected with ninhydrin. Detection of protected peptides was performed with the tolidine reagent after chlorination[12]. Substances used in further stages were pure according to these criteria. Solutions were taken down on a rotatory evaporator at 40°C/15 Torr.

Gas chromatography was performed on a Fractovap 2200 (C. Erba, Italy) apparatus in combination with the Speedomax recorder, flame-ionisation detection, argon as carrier gas, flow rate 0·70 ml s^{-1}. Glass column, 2 m of length, 0·4 cm of internal diameter, packed with 100/120 mesh Gas Chrom P, 3% (by weight) of OV-225 (methyl silicone with 25% phenyl and 25% 3-cyanopropyl groups) as the stationary phase. Column temperature, 230°C (free cyclodipeptides) and 220°C (trimethylsilyl derivatives). The trimethylsilylation was performed with trimethylchlorosilane in pyridine in the presence of hexamethyldisilazane[13]. Elution times in min (the data of trimethylsilyl derivatives are given in parentheses): *IIa* 83 (26·8), *IIb* 92 (30·0), cyclo(L-prolyl-4-acetoxy-L-prolyl) 40·8, cyclo(L-Pro-L-Pro) 11·8, cyclo(D-Pro-L-Pro) 12·8, cyclo(L-Pro-Gly) 11·4.

4-Hydroxy-L-proline Methyl Ester Hydrochloride

The free amino acid (5 g; homogeneous on paper chromatography, ^{1}H NMR spectrum, and amino acid analyser) was esterified with methanol under introduction of hydrogen chloride and the whole

mixture was evaporated. The hydrochloride was recrystallised from methanol–ether. Yield, 5·5 g (79%), m.p. 167—168°C (reported[14], m.p. 162—164°C), $[\alpha]_D$ —26·3° (*c* 0·5, methanol).

o-Nitrobenzenesulfenyl-L-prolyl-4-hydroxy-L-proline Methyl Ester

To a solution of *o*-nitrobenzenesulfenyl-L-proline dicyclohexylammonium salt (4·5 g) and 4-hydroxy-L-proline methyl ester hydrochloride (1·8 g) in chloroform (100 ml) there was added with stirring N,N′-dicyclohexylcarbodiimide (2·3 g) at —20°C. The mixture was stirred at —20°C for 1 h, kept at 0°C overnight, evaporated, the residue was triturated with ethyl acetate, and the suspension filtered. The filtrate was washed with 0·1M-H_2SO_4, water, 0·5M-$NaHCO_3$ and water again, dried over anhydrous sodium sulfate, and evaporated. Crystallisation of the residue from ethyl acetate–light petroleum yielded 3·02 g (77%) of the title ester, m.p. 117—118°C, $[\alpha]$ —139·6°. For $C_{17}H_{11}N_3O_6S$ (395·5) calculated: 10·62% N, 8·11% S; found: 10·78% N, 7·87% S.

Benzyloxycarbonyl-L-prolyl-4-hydroxy-L-proline (*IVa*)

To a suspension of benzyloxycarbonyl-L-proline (1·9 g) and 4-hydroxy-L-proline methyl ester hydrochloride (1·4 g) in chloroform there was added at —10°C dicyclohexylamine (1·47 ml) and N,N′-dicyclohexylcarbodiimide (1·75 g). The mixture was stirred at —10°C for 1 h, kept at 0°C overnight, evaporated, and the suspension of the residue in ethyl acetate filtered. The filtrate was washed with 1M-HCl, water, 0·5M-$NaHCO_3$ and water again, dried over anhydrous sodium sulfate, and evaporated. The residual oil was dissolved in acetone (20 ml), the solution treated with 1M-NaOH (8 ml), the mixture stirred at 20°C for 2 h, diluted with water, and filtered with active charcoal. The acetone was evaporated and the remaining aqueous solution acidified with hydrochloric acid. The solid was reprecipitated from a mixture of 2-propanol and ethyl acetate by the addition of ether and light petroleum. Yield, 1·1 g (40%, based on benzyloxycarbonyl-L-proline) of compound *IVa*, m.p. 223—225°C, $[\alpha]_D$ —63·1°. For $C_{18}H_{22}N_2O_6$ (362·4) calculated: 59·66% C, 6·12% H, 7·73% N; found: 59·76% C, 6·19% H, 7·85% N.

L-Prolyl-4-hydroxy-L-proline (*Ia*)

Hydrogen was passed for 1 h through a solution of the dipeptide *IVa* (0·52 g) in 50% aqueous methanol (40 ml) in the presence of a palladium catalyst (prepared from 8 ml of 10% aqueous $PdCl_2$). The mixture was filtered, the fitrate evaporated, the residue dried, and crystallised from methanol–ether. Yield, 0·32 g (94%) of compound *Ia*, m.p. 120—122°C, $[\alpha]_D$ —149·0° (*c* 0·42, 0·5M-HCl). For $C_{10}H_{16}N_2O_4 \cdot 1/2\ H_2O$ (237·3) calculated: 50·62% C, 7·22% H, 11·80% N; found, 50·61% C, 7·32% H, 11·49% N. ^{1}H NMR spectrum (trifluoroacetic acid): 1·50—2·55 m, 6 H: β-CH_2 + γ-CH_2 of proline and β-CH_2 of hydroxyproline; 3·00—3·75 m, 4 H, δ-CH_2 of both residues; 4·50 centre of a multiplet, 3 H, α-CH of both residues and γ-CH of hydroxyproline; 7·53 b, < 2 H, NH + OH.

Cyclo(L-prolyl-4-hydroxy-L-prolyl) (*IIa*)

To a solution of *o*-nitrobenzenesulfenyl-L-prolyl-4-hydroxy-L-proline methyl ester (2·5 g) in methanol (10 ml) there was added 3·7M methanolic hydrogen chloride (3·2 ml), the mixture kept at room temperature for 10 min, and evaporated. The residue was triturated with ether and finally dissolved in methanol (5 ml). The solution was filtered, the filtrate treated with 20% methanolic ammonia (2 ml), the mixture allowed to stand for 48 h, and evaporated. The residue was triturated with ether, filtered off, dried, and dissolved in 50% aqueous methanol (5 ml). The solution was

filtered through columns of Dowex 50 WX2 ion exchange resin (50 ml) and Amberlite IR-4 B resin (40 ml). The elution was performed with 50% aqueous methanol and the eluates were evaporated. The residue was dried, dissolved in methanol, and the methanolic solution precipitated with ether (this purification was repeated twice). Yield, 0·59 g (44%) of compound *IIa*, m.p. 141–143°C, $[\alpha]_D$ −115·9° (*c* 0·54, dimethylformamide). For $C_{10}H_{14}N_2O_3$ (210·2) calculated: 57·13% C, 6·71% H, 13·32% N; found: 56·83% C, 6·73% H, 13·21% N. ^{1}H NMR spectrum (deuteriochloroform): 1·75 m, 6 H, β-CH_2 + γ-CH_2 of proline and β-CH_2 of hydroxyproline residue; 3·55 centre of a multiplet, 4 H, δ-CH_2 of both residues; 4·24 m, > 1 H, α-CH of proline + OH; 4·55 m, 2 H, α-CH of proline + γ-CH of hydroxyproline.

Benzyloxycarbonyl-D-prolyl-4-hydroxy-L-proline Methyl Ester (*IIIb*)

To a suspension of benzyloxycarbonyl-D-proline (0·90 g) and 4-hydroxy-L-proline methyl ester hydrochloride (0·7 g) in chloroform (50 ml) there was added at −10°C dicyclohexylamine (0·73 ml) and N,N′-dicyclohexylcarbodiimide (0·9 g). The mixture was stirred at −10°C for 1 h, kept at 0°C overnight, and evaporated. A suspension of the residue in ethyl acetate was filtered to remove N,N′-dicyclohexylurea, the filtrate washed with 1M-HCl, water, 0·5M-$NaHCO_3$, and water again, dried, and evaporated. The residue was crystallised from ethyl acetate–light petroleum to yield 0·72 g (53%) of compound *IIIb*, m.p. 147–148°C, $[\alpha]_D$ −32·7°. For $C_{19}H_{24}N_2O_6$ (376·4) calculated: 60·63% C, 6·43% H, 7·44% N; found: 60·95% C, 6·65% H, 7·73% N.

Benzyloxycarbonyl-D-prolyl-4-hydroxy-L-proline (*IVb*)

To a solution of the dipeptide *IIIb* (0·45 g) in acetone (5 ml) there was added 1M-NaOH (1·5 ml), the mixture stirred at 20°C for 2 h, diluted with water, filtered, the acetone evaporated, and the aqueous solution acidified with hydrochloric acid to deposit a solid which was crystallised from water. Yield, 0·27 g (63%) of compound *IVb*, $[\alpha]_D$ −43·5° (*c* 0·3, dimethylformamide). For $C_{18}H_{22}N_2O_6$ (362·4) calculated: 59·66% C, 6·12% H, 7·73% N; found: 59·56% C, 6·12% H, 7·78% N.

D-Prolyl-4-hydroxy-L-proline (*Ib*)

Hydrogen was passed for 1 h through a solution of compound *IVb* (150 mg) in 50% aqueous methanol (10 ml) in the presence of a palladium catalyst (prepared from 4 ml of 10% aqueous $PdCl_2$). The catalyst was then filtered off, the filtrate evaporated, and the residue crystallised from methanol–water to yield 75 mg (80%) of compound *Ib*, m.p. 193–195°C, $[\alpha]_D$ −29·5° (*c* 0·22, 0·5M-HCl). For $C_{10}H_{16}N_2O_4.1/2\,H_2O$ (237·3) calculated: 50·62% C, 7·22% H, 11·80% N; found: 50·87%, 7·10% H, 11·55% N. ^{1}H NMR spectrum (trifluoroacetic acid): 1·65–2·60 m, 6 H, β-CH_2 + γ-CH_2 of proline and β-CH_2 of hydroxyproline; 3·05–3·65 m, 4 H, δ-CH_2 of both residues; 4·20–4·60 m, 3 H, α-CH of both residues and γ-CH of hydroxyproline; 7·48 b, 2 H, NH + OH.

Cyclo(D-prolyl-4-hydroxy-L-prolyl) (*IIb*)

A mixture of the dipeptide *IIIb* (200 mg) and 33% hydrogen bromide in acetic acid (1 ml) was kept at room temperature for 20 min and then diluted with ether. The precipitate was repeatedly triturated with ether, dried, and dissolved in methanol (1 ml). The methanolic solution was treated with 20% methanolic ammonia (1 ml), the mixture allowed to stand for 49 h, and evaporated. The residue was triturated with ether, the solid portion collected, dried, and dissolved in 80%

aqueous methanol (4 ml). This solution was passed through columns of Dowex 50 WX2 and Amberlite IR-4 B ion exchange resins (20 ml each), the columns washed with 50% aqueous methanol, the effluents evaporated, and the residue crystallised from methanol–ether. Yield, 35 mg (32%) of compound *IIb*, m.p. 195—198°C, $[\alpha]_D$ −10·2° (*c* 0·48, dimethylformamide). For $C_{10}H_{14}N_2O_3$ (210·2) calculated: 57·13% C, 6·71% H, 13·32% N; found: 57·13% C, 6·64% H, 13·48% N. ^{1}H NMR spectrum (deuteriochloroform): 1·60—2·60 m, 6 H, β-CH_2 + γ-CH_2 of proline and β-CH_2 of hydroxyproline; 3·10—3·50 m, 2 H, δ-CH_2 of proline; 3·85—4·70 m, 5 H, α-CH of proline and α-CH, γ-CH + δ-CH_2 of hydroxyproline.

Cyclo(L-prolyl-4-acetoxy-L-prolyl)

A sample of compound *IIa* was kept in 33% hydrogen bromide in acetic acid for 20 min at room temperature (*i.e.*, under usual conditions to remove the benzyloxycarbonyl group) and the mixture was then diluted with ether. The solid was collected, washed with ether and light petroleum, dried under diminished pressure, and crystallised from methanol–ether to yield the title compound, m.p. 201—204°C. ^{1}H NMR spectrum (deuteriochloroform): 1·80—2·60 m, 6 H, β-CH_2 of both residues and γ-CH_2 of proline; 2·07 s, 3 H, —$OCOCH_3$, 3·40—3·90 m, 4 H, δ-CH_2 of both residues; 4·22 bt, 1 H, α-CH of proline; 4·45 bt, 1 H, α-CH of hydroxyproline; 5·40 bt, 1 H, γ-CH of hydroxyproline. In the IR spectrum (chloroform), no stretching vibration band of the O—H bond was detected.

Spectra

Infrared spectra were measured on a Zeiss UR 10 (Jena, German Democratic Republic) apparatus with the accuracy of ± 2 cm^{-1} in tetrachloromethane (saturated solution, cell width 0·5 mm with compound *IIa* and 1·0 mm with *IIb*) and in chloroform (concentration 0·06M, cell width 0·1 mm). For the data see Table I.

Circular dichroism spectra were measured on a Roussel-Jouan CD 185-II Dichrograph in cells with optical path length of 0·01 to 0·05 cm at a temperature of 22—25°C in 2,2,2-trifluoroethanol (concentrations about 0·3 mg/ml). The values are expressed in λ nm ($\Delta\varepsilon$).

Cyclo(L-prolyl-4-hydroxy-L-prolyl) (*IIa*): 250 (0), max. 219 (−3·9), 211·5 (0), max. 202 (+4·86), end value 196 (0).

Cyclo(D-prolyl-4-hydroxy-L-prolyl) (*IIb*): 250 (0), max. 227 (−2·3), 220 (0), max. 203 (+2·40), 198 (0), end value 193 (−3·16).

L-Prolyl-4-hydroxy-L-proline (*Ia*): 260 (0), max. 218 (−4·34), 203 (0), max. 196 (+1·0), end value 190 (+0·7).

D-Prolyl-4-hydroxy-L-proline (*Ib*): 230 (0), max. 205 (−4·65), end value 190 (−1·4).

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