CONFIGURATIONAL REQUIREMENTS OF AROMATIC AMINO ACID RESIDUES FOR THE ACTIVITY OF PRP-HEXAPEPTIDE

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Dedicated to the memory of Dr Karel Bláha.

The three analogues with D-amino acid substituents at position 1 and 5 of PRP-hexapeptide were synthesized and tested for its biological activity to check the influence of the spacial orientation of aromatic rings on the immune response. One of the analogs (Tyr-Val-Pro-Leu-D-Phe-Pro) was found to have the immunoregulatory activity.

It has been previously found that the proline-rich polypeptide (PRP)* isolated from ovine colostrum[1,2] has a regulatory activity in the immune response and increases the permeability of skin vessels[3]. It also affects maturation of thymocytes and generation of suppressor T-cells[4]. The peptide has a molecular weight of 6 000 D pro monomer and contains a high number of proline (22%) and other hydrophobic amino acid residues.

The nonapeptide Val-Glu-Ser-Tyr-Val-Pro-Leu-Phe-Pro, obtained by chymotrypsin digestion of PRP, has the same immunoregulatory properties as PRP (ref.[5]). The structure of this fragment of PRP was confirmed by the synthesis of this compound[6]. The hexapeptide fragment of PRP Tyr-Val-Pro--Leu-Phe-Pro was also found to have the same biological activity as natural PRP (ref.[7]). C-Terminal pentapeptide fragment of this compound is also active but its activity is distinctly lower while all shorter fragments are not active at all[8]. On the basis of NMR measurements, we constructed a model of conformation

* Abbreviations used follows the published recommendations – Eur.J.Biochem. *138*, 9 (1984). PRP, proline-rich polypeptide; TEA, triethylamine; DMF, dimethylformamide; PFC, plaque forming cells; SRBC, sheep red blood cells.

of PRP-hexapeptide which dominates in polar solution[9]. The model implies the gamma bend conformation on the Leu[4] residue. Recently we reported the synthesis and biological activity of cyclic PRP-hexapeptide analog which contains the azo bridge between Tyr[1] and Phe[5] residues[10]. High biological activity of the synthesized azo-PRP-hexapeptide suggests that in the biologically active conformation of PRP-hexapeptide both aromatic rings (Tyr[1] and Phe[5]) are in a close proximity.

In the order to study the influence of configurational changes on Tyr[1] and Phe[5] residues on PRP-hexapeptide we synthesized three new peptides *I*–*III*.

I, L-Tyr-Val-Pro-Leu-D-Phe-Pro
II, D-Tyr-Val-Pro-Leu-L-Phe-Pro
III, D-Tyr-Val-Pro-Leu-D-Phe-Pro

We felt that this study may be helpful for the design of biodegradation-resistant analogues of PRP and provide further information about the bioactive conformation of this compound.

EXPERIMENTAL

The protected amino acids were prepared as described by Bodanszky and Bodanszky[11]. The synthesis of Boc-Val-Pro-OH, HCl.H-Val-Pro-Leu-Phe-Pro-OBzl and PRP-hexapeptide have been described before[6]. All solvents were distilled from proper agents, other reagents were of analytical or reagent grade. The melting points (uncorrected) were determined in a PHMK Veb Analytic (Dresden) apparatus. The optical activity was measured on Polamat (Zeiss, Jena) polarimeter. Thin layer chromatography (TLC) was carried out on silica gel plates F 254 from MERCK in the solvent systems: chloroform–methanol–water (8:3:1, v/v) (A), 1-butanol–acetic acid–water (4:1:5, v/v, upper phase) (B), 2-propanol–acetic acid–water–pyridine (10:4:4:5, v/v) (C), chloroform–methanol (24:1, v/v) (D). The plates were developed with ninhydrin reagent (0.3% in acetone) and by incubation in an iodine chamber. Column chromatography utilized MERCK silica gel 60 (70–230 mesh) packed in the appropriate solvent into glass columns (80–100 g silica gel per 1 g of compound). Elution from the column was controlled by TLC analysis. Amino acid compositions were determined on an AAA-851 analyzer (Mikrotechna, Czechoslovakia) following hydrolysis in 6 M HCl at 100°C for 12 h.

Z-D-Tyr-N_2H_3 (*IV*)

D-Tyrosine (5g, 27.5 mmol) was added to the precooled mixture of methanol (20 ml) and thionyl chloride (2.2 ml). The reaction mixture was stirred at 0–10°C for 30 min and at room temperature overnight. The solution was concentrated in vacuo and the residue was triturated with ethyl ether. The obtained ester (6.2 g) was suspended in chloroform (35 ml) and benzyl chloroformate (Z-Cl, 2.25 ml) was added at 0°C. Then 2.1 ml of Z-Cl more and the solution of sodium bicarbonate (2.74 g) in water (19 ml) was added in ten portions alternatingly. The resulting precipitate (7.9 g) was collected on a filter, washed with hexane and dissolved in methanol (100 ml). Aqueous solution

(80%) of hydrazine hydrate (15 ml) was added and the reaction mixture was kept at room temperature overnight. The separated hydrazide was collected by filtration and washed with water, methanol and with ethyl ether. The crystalline hydrazide was dried over P_2O_5. Overall yield 6.78 g (75%), m.p. 209–212°, $[\alpha]_D^{21}$ 70.1° (*c* 1, methanol), R_F 0.87 (A), 0.78 (B), 0.89 (C). (Ref.[12] for Z-L-Tyr-N_2H_3; m.p. 220–221°C.)

Boc-D-Phe-Pro-OBzl (*V*)

To the cooled (−20°C) solution of Boc-D-Phe-OH (13.26 g, 50 mmol) in chloroform (100 ml) and triethylamine (TEA) (6.97 ml, 50 mmol) ethyl chloroformate (4.77 ml, 50 mmol) was added dropwise. The reaction mixture was cooled for 20 min and the precooled (−20°C) solution of HCl.H-Pro-OBzl (12.1 g, 50 mmol) and TEA (6.97 ml, 50 mmol) in chloroform was added. The reaction mixture was stirred at −10°C for 3 h and at room temperature overnight. The solution was concentrated in vacuo and the residue was dissolved in ethyl acetate and washed with 5% solution of citric acid, water, saturated solution of sodium bicarbonate and brine. The solution was dried over $MgSO_4$, filtered and evaporated in vacuo. The solid residue was triturated with hexane. Yield 20.8 g, (92%), m.p. 105–106°C, $[\alpha]_D^{19}$ −50° (*c* 1.7, methanol), R_F 0.75 (A), 0.82 (B), 0.84 (C). For $C_{26}H_{32}N_2O_5$ (452.6) calculated: 69.01% C, 7.13% H, 6.19% N; found: 69.3% C, 7.1% H, 6.3% N.

HCl.H-D-Phe-Pro-OBzl (*VI*)

Compound *V* (20 g, 44 mmol) was dissolved in an excess of the solution of 4 M HCl in dioxane and left at room temperature for 2 h. The solvent was removed in vacuo and the residue was triturated with hexane. Yield 13 g (76%), m.p. 158–160°C, $[\alpha]_D^{21}$ 105° (*c* 1, methanol), R_F 0.7 (A), 0.50 (B), 0.81 (C). For $C_{21}H_{25}ClN_2O_3$ (388.9) calculated: 64.86% C, 6.48% H, 7.20% N; found: 64.5% C, 6.3% H, 7.1% N.

Boc-Leu-D-Phe-Pro-OBzl (*VII*)

The product was obtained from Boc-Leu-OH and HCl.H-D-Phe-Pro-OBzl in the same fashion as compound *V*. Yield 84.7%, m.p. 64–66°C, $[\alpha]_D^{18}$ −54.3° (*c* 1.7, methanol), R_F 0.87 (A), 0.58 (B), 0.80 (C). For $C_{32}H_{43}N_3O_6$ (565.7) calculated: 67.94% C, 7.66% H, 7.43% N; found: 67.8% C, 7.4% H, 7.2% N.

HCl.H-Leu-D-Phe-Pro-OBzl (*VIII*)

Compound *VII* was deprotected with 4 M HCl in dioxane in the same fashion as compound *V*. Yield 84%, m.p. 93–96°C, $[\alpha]_D^{21}$ −31.9° (*c* 1.6, methanol), R_F 0.67 (A), 0.79 (B), 0.64 (C). For $C_{27}H_{36}ClN_3O_4$ (502.1) calculated: 64.59% C, 7.23% H, 8.37% N; found: 64.0% C, 7.3% H, 8.0% N.

Boc-Val-Pro-Leu-D-Phe-Pro-OBzl (*IX*)

The compound *IX* was obtained from Boc-Val-Pro-OH and HCl.H-Leu-Phe-Pro-OBzl in the same fashion as compound *V*. Yield 73%, m.p. 74–76°C; $[\alpha]_D^{21}$ −69° (*c* 1, methanol); R_F 0.88 (A), 0.64 (B), 0.89 (C). For $C_{42}H_{59}N_5O_8$ (762.0) calculated: 66.21% C, 7.80% H, 9.19% N; found: 65.9% C, 7.6% H, 8.8% N.

HCl.H-Val-Pro-Leu-D-Phe-Pro-OBzl (*X*)

Compound *IX* was deprotected with 4 M HCl in dioxane in the same fashion as compound *V*. Yield 95%, m.p. 117–118 C, $[\alpha]_D^{18}$ −79.4 (*c* 1, methanol). R_F 0.64 (A) 0.49 (B), 0.70 (C). For $C_{37}H_{52}ClN_5O_6$ (698.3) calculated: 63.64% C, 7.50% H, 10.03% N; found 63.1% C, 7.6% H, 9.6% N. Amino acid analysis: Val 1.0, Pro 2.1, Leu 1.1, Phe 1.0.

Z-(D or L)-Tyr-Val-Pro-Leu-(D or L)-Phe-Pro-OBzl

The solution of Z-(D or L)-Tyr-N_2H_3 (1.72 g, 5.18 mmol) in DMF (5 ml) was cooled to −20 C and freshly distilled amyl nitrite (0.72 ml, 5.2 mmol) in DMF (0.71 ml) was added followed by the addition of 4.5 M HCl in dioxane (2.34ml, 10.36 mmol). After 20 min the reaction mixture was cooled to −30 C and adjusted to pH 7.5 with N-ethylmorpholine. The mixture of HCl.H-Val-Pro-Leu-(D or L)-Phe-Pro-OBzl (4 g, 5.7 mmol) and N-ethylmorpholine (0.72 ml, 5.2 mmol) in DMF (3 ml) was added dropwise and the mixture was kept in a refrigerator for 20 h followed by 5 h at room temperature. The solvents were evaporated in vacuo and the solid residue was dissolved in ethyl acetate and washed with 1 M HCl, water, saturated solution of $NaHCO_3$ and brine. The solvent was dried over $MgSO_4$, filtered and evaporated to dryness. The product was chromatographed on a column of silica gel (90 g). The purified material was eluted with 40:1 mixture of chloroform and methanol.

Z-D-Tyr-Val-Pro-Leu-Phe-Pro-OBzl (XI). Yield 73%, m.p. 94–98 C, $[\alpha]_D^{24}$ −75 (*c* 0.6, methanol), R_F 0.88 (A), 0.75 (B), 0.83 (C), 0.16 (D). For $C_{54}H_{66}N_6O_{10}$ (959.2) calculated: 67.62% C, 6.94% H, 8.76% N; found: 66.9% C, 6.9% H, 8.5% N. Amino acid analysis: Tyr 1.0, Val 0.9, Pro 1.9, Leu 1.0, Phe 1.2.

Z-D-Tyr-Val-Pro-Leu-D-Phe-Pro-OBzl (XII). Yield 69%, m.p. 108–111 C, $[\alpha]_D^{20}$ −85 (*c* 0.7, methanol), R_F 0.88 (A), 0.75 (B), 0.34 (D). Amino acid analysis: Tyr 1.0, Val 0.9, Pro 1.8, Leu 1.0, Phe 1.1. Elemental analysis – found: 67.8% C, 6.7% H, 8.5% N.

Z-Tyr-Val-Pro-Leu-D-Phe-Pro-OBzl (XIII). Yield 70%, m.p. 97–100 C, $[\alpha]_D^{20}$ −74.0 (*c* 1, methanol), R_F 0.88 (A), 0.83 (B), 0.24 (D). Amino acid analysis: Tyr 0.9, Val 1.0, Pro 1.9, Leu 1.0, Phe 1.1. Elemental analysis – found: 67.8% C, 6.7% H, 8.4% N.

H-(D or L)-Tyr-Val-Pro-Leu-(D or L)-Phe-Pro-OH

The blocked hexapeptides (compounds *XI*–*XIII*) (0.4 g, 0.42 mmol) were dissolved in methanol (15 ml) and hydrogenated for 7 h with 10% Pd C. The solvent was evaporated in vacuo and the product was crystallized from methanol–ethyl ether.

H-D-Tyr-Val-Pro-Leu-D-Phe-Pro-OH (XIV). Yield 63%, m.p. 160–175 C, $[\alpha]_D^{24}$ −70.3 (*c* 1, methanol), R_F 0.1 (A), 0.19 (B), 0.79 (C). For $C_{39}H_{54}N_6O_8$ (734.9) calculated: 63.74% C, 7.41% H, 11.44% N; found: 63.5% C, 7.1% H, 10.9% N. Amino acid analysis: Tyr 1.0, Val 0.9, Pro 2.1, Leu 1.0, Phe 1.0.

H-D-Tyr-Val-Pro-Leu-Phe-Pro-OH (XV). Yield 52%, m.p. 176–181 C, $[\alpha]_D^{23}$ −86.5 (*c* 1, methanol). Elemental analysis – found: 63.2% C, 7.6% H, 11.1% N. Amino acid analysis: Tyr 0.9, Val 1.0, Pro 2.0, Leu 1.0, Phe 1.1.

H-Tyr-Val-Pro-Leu-D-Phe-Pro-OH (XVI). Yield 58%, m.p. 178–185 C, $[\alpha]_D^{24}$ −106.4 (*c* 1, methanol). Amino acid analysis: Tyr 1.0, Val 1.0, Pro 2.1, Leu 1.2, Phe 1.1. Elemental analysis – found: 63.4% C, 7.5% H, 11.5% N.

Biological Tests

Six to eight week old CBA female mice were purchased from the Animal Breeding Center of the Institute of Immunology and Experimental Therapy, Wrocław, Poland. RPMI medium was a product of Grand Island Biological Company, Grand Island, New York, U.S.A. Fetal calf serum (FCS) and sheep erythrocytes (SRBC) were obtained from the Laboratory of Biopreparates of the Institute of Immunology (Wrocław). Guinea pig complement was our product and Hepes was purchased from Sigma, U.S.A.

Mice were primed with a dose of 0.1 ml of 1 % SRBC suspension, intravenously, 4 days before cell preparation. Whole spleen cell suspension was prepared from spleens of SRBC-primed mice. The organs were minced, pressed through a plastic screen into 0.83% NH_4Cl buffered with 0.017 M Tris and incubated at room temperature for 10 min. The cells free of erythrocytes, were then suspended in Hepes buffer and filtered through cotton wool to remove dead cells[13]. Finally, the cells were washed twice with PBS and cell suspensions were prepared in RPMI medium containing 10% FCS.

The splenocytes were placed into 24 well flat botton tissue culture plates (Linbro) at a concentration of $6 \cdot 10^6$ ml^{-1} well^{-1} in RPMI medium supplemented with 10% FCS and antibiotics. The antigen (SRBC) was added to the cell cultures at a dose of 0.05 ml of 0.01%

TABLE I
The effect of the PRP peptides modified with D-amino acids on the humoral immune response of CBA mice to SRBC in vivo

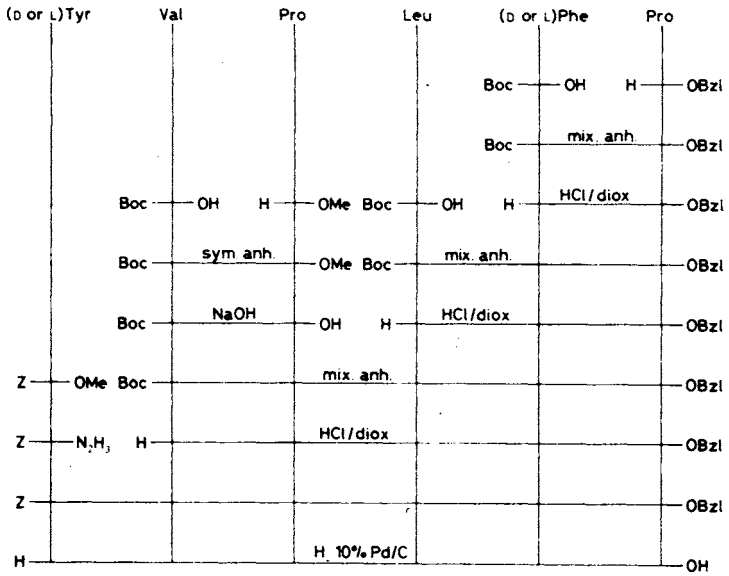
Compound	Dose μg per mouse	Mean value of PFC 10^6 splenocytes $n = 6$	± SE	p
None (control)		653	125	
L-Tyr-Val-Pro-Leu-L-Phe-Pro	1	1 572	189	$< 10^{-2}$
	10	1 709	280	$< 10^{-2}$
	100	2 457	464	$< 10^{-3}$
D-Tyr-Val-Pro-Leu-L-Phe-Pro	1	783	159	NS
	10	838	203	NS
	100	755	105	NS
L-Tyr-Val-Pro-Leu-D-Phe-Pro	1	1 222	175	< 0.05
	10	2 503	492	$< 10^{-3}$
	100	2 538	193	$< 10^{-3}$
D-Tyr-Val-Pro-Leu-D-Phe-Pro	1	451	76	NS
	10	900	139	NS
	100	926	63	NS

CBA mice were immunized ip with 0.2 ml of 10 % SRBC suspension. The preparations were administered ip 3 hours before immunization. After 4 days the splenocytes were isolated and the number of PFC was determined. The data represent a mean from 6 mice (determinations) ± standard error.

suspension per well. The peptides under investigation were added to a dose of 0.1 μg, 1 μg or 10 μg per well. After 4 days of incubation at 37°C, 5% CO_2 and 100% humidity the cells were harvested and the number of cells producing anti-SRBC antibodies of IgM class was determined using the method of Mishell and Dutton[14]. In the in vivo system mice were immunized ip with 0.2 ml of 10 % SRBC. Peptides were administrated ip to a dose of 1 μg, 10 μg or 100 μg per mouse 3 h before immunization. After 4 days the splenocytes were isolated and the numbers of PFC were determined.

RESULTS AND CONCLUSIONS

Using standard methodology, we prepared a series of hexapeptides systematically substituted at the aromatic amino acid residues with D-amino acids according to the Scheme 1. The blocked hexapeptides were obtained



SCHEME 1

according to [1 + 5] procedure via azide condensation, by Honzl and Rudinger method[11], of Z-(D or L)-Tyr-N_2H_3 with proper pentapeptide benzyl esters. The phenolic hydroxyl group of tyrosine remained unprotected throughout this synthesis. All pentapeptide fragments were synthesized by a [2 + 3] condensation via mixed anhydride method of blocked dipeptide and tripeptide benzyl esters, obtained by either ethyl carbonate mixed anhydride method or by symmetrical anhydride preactivation. The risk of a partial racemization in the coupling steps

was minimalized by building up fragments stepwise with Boc-protected amino acids and by fragment coupling to the proline residue.

The synthesized analogs were investigated for the acivity in the regulation of the immune response, assayed by measurement of PFC. The tests were carried out in two experimental systems. In the in vivo system we investigated the effect of the PRP peptides on the humoral immune response in CBA mice, determined by the number of splenocytes producing antibodies against SRBC. The results show (Table I) that the peptide L-Tyr-Val-Pro-Leu-D-Phe-Pro shows biological activity comparable with the activity of PRP-hexapeptide (L-Tyr-Val-Pro-Leu--L-Phe-Pro). The PRP-hexapeptide analogs containing D-tyrosine instead of L-tyrosine have no immunoactivity at all. Similar result was obtained in the in vitro system, where the peptides were added into the cell culture together with antigen (SRBC) (Table II). Based on this we can conclude that the configuration of the phenylalanine is not significant for biological acivity of PRP, while the L-configuration of tyrosine (Tyr^1) plays a crucial role in it.

TABLE II

The effect of the PRP peptides modified with D-amino acids on the humoral immune response of CBA mice to SRBC in vitro

Compound	Dose μg per well	Mean value of PFC 10^6 splenocytes $n = 4$	±SE	p
None (control)		1 530	68	
L-Tyr-Val-Pro-Leu-L-Phe-Pro	0.1	4 624	552	$< 10^{-3}$
	1	7 125	1 205	$< 10^{-3}$
	10	6 800	313	$< 10^{-3}$
D-Tyr-Val-Pro-Leu-L-Phe-Pro	0.1	2 210	384	NS
	1	1 987	254	NS
	10	2 137	231	NS
L-Tyr-Val-Pro-Leu-D-Phe-Pro	0.1	5 487	304	$< 10^{-3}$
	1	6 000	801	$< 10^{-3}$
	10	7 783	946	$< 10^{-3}$
D-Tyr-Val-Pro-Leu-D-Phe-Pro	0.1	1 748	93	NS
	1	2 202	282	NS
	10	1 927	164	NS

CBA mice were immunized with 1 % suspension of SRBC (0.2 ml, iv), 4 days before isolation of splenocytes. Into $5 . 10^6$ splenocytes 0.1 ml of 0.01 % of SRBC was added plus the PRP preparations (0.1, 1, and 10 μg/ml). After 4 days the cells were harvested and the number of cells producing anti-SRBC antibodies was determined. The data represent a mean from 4 determinations (cultures) ± standard error.

REFERENCES

1. Janusz M., Lisowski J., Franek F.: FEBS Lett. *49*, 267 (1974).
2. Janusz M., Starościk K., Zimecki M., Wieczorek Y., Lisowski J.: Biochem. J. *199*, 9 (1981).
3. Wieczorek Z., Zimecki M., Janusz M., Starościk K., Lisowski J.: Immunology *36*, 875 (1979).
4. Zimecki M., Lisowski J., Hraba T., Wieczorek Z., Janusz M., Starościk K.: Arch. Immun. Ther. Exp. *32*, 191 (1984).
5. Starościk K., Janusz M., Zimecki M., Wieczorek Z., Lisowski J.: Mol. Immunol. *20*, 1277 (1983).
6. Kubik A., Szewczuk Z., Siemion I.Z., Janusz J., Wieczorek Z., Spiegel K., Lisowski J.: Pol. J. Chem. (1987), in press.
7. Janusz M., Wieczorek Z., Spiegel K., Kubik A., Szewczuk Z., Siemion I.Z., Lisowski J.: Mol. Immunol. *24*, 1029 (1987).
8. Kubik A., Kliś W.A., Szewczuk Z., Siemion I.Z., Janusz M., Starościk K., Zimecki M., Lisowski J., Wieczorek Z. in: *Peptides 1984. Proc. 18th Eur. Pept. Symp.* (U. Ragnarson, Ed.), p. 457. Almquist & Wiksell, Stockholm 1984.
9. Siemion I.Z., Pędyczak A., Szewczuk Z.: Bull. Pol. Acad. Sci. *35*, 171 (1987).
10. Szewczuk Z., Kubik A., Siemion I.Z., Wieczorek Z., Spiegel K., Zimecki M., Janusz M., Lisowski J.: Int. J. Pept. Protein Res. in press.
11. Bodanszky M., Bodanszky A.: *The Practice of Peptide Synthesis*. Springer Verlag, Berlin 1984.
12. Harington C.R., Pitt-Rivers R.V.: Biochem. J. *38*, 417 (1944).
13. Boehmer H., Shortman K.: J. Immunol. Meth. *2*, 293 (1973).
14. Mishell R.I., Dutton R.W.: J. Exp. Med. *126*, 423 (1967).
15. Honzl J., Rudinger J.: Collect. Czech. Chem. Commun. *26*, 2333 (1961).