SYNTHESIS AND IMMUNOSTIMULANT ACTIVITY OF THE COORDINATION COMPOUNDS OF SOME TRYPTOPHAN-CONTAINING DIPEPTIDES WITH METAL IONS

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A promising way to increase the specific activity of a parent compound and reduce its side effects is to form coordination compounds with metal ions [1]. Potential ligands are offered by tryptophan-containing dipeptides with the general formula H–X–Trp–OH (X = Glu, Asp, Leu, Ile) possessing immunostimulant activity [2 – 4].

We have synthesized a series of coordination compounds with Fe^{2+} and Zn^{2+} ions and studied their immunological activity. The dipeptides H–Val–Trp–OH and H–Ile–Trp–OH were synthesized using activated (pentafluorophenyl) esters obtained with the aid of di(pentafluorophenyl)carbonate. When the condensation reaction with pentafluorophenyl esters is conducted by conventional method, it is necessary to introduce amino components with protected α-carboxy groups and use pentafluorophenyl esters of N-protected amino acids in the individual form. This implies additional stages for the isolation and purification of pentafluorophenyl esters of N-protected amino acids, as well as for the introduction and removal of the protection from the carboxy group of tryptophan.

The α-carboxy groups of amino acids were protected by carbobenzoxy groups. The pentafluorophenyl esters of N-protected amino acids were were obtained with the aid of di(pentafluorophenyl)carbonate. Reactions between triethylammonium salts of the N-protected amino acids (10 mmole) and di(pentafluorophenyl)carbonate (10.5 mmole) were conducted under conventional conditions for 30 min in the presence of triethylamine (10.5 mmole) using ethyl acetate as the solvent [5].

However, the subsequent stages differed from the conventional pathway. Upon replacing ethyl acetate by dimethylformamide (DMF), the condensation process was conducted by adding tryptophan sodium salt (amino component) to the solution of a mixture of the pentafluorophenyl ester of the N-protected amino acid and the triethylammonium salt of pentafluorophenol. The pentafluorophenol liberated in the course of this reaction formed a sodium salt. The products were treated with potassium bisulfate and purified on a chromatographic silica gel column to obtain the protected dipeptides Z–Val–Trp–OH and Z–Ile–Trp–OH with a yield of 69,0 and 74.5%, respectively. Immediately upon evaporating eluate, the protected dipeptides were deblocked by catalytical hydrogenation in the presence of a 10% Pd/C catalyst and purified by precipitation from methanol with ether. The final dipeptides H–Val–Trp–OH and H–Ile–Trp–OH were obtained with a yield of 67,3 and 71.8%, respectively.

The proposed structures of the synthesized compounds were confirmed and their purity checked by TLC, specific optical rotation measurements, and amino acid analysis.

Thus, we have developed a method for the synthesis of tryptophan-containing dipeptides which allows tryptophan with free indole and carboxy groups to be used in the condensation reaction, thus reducing the number of stages in the synthesis process.

The compounds of dipeptides with metal ions were obtained by the reaction between equimolar amounts of metal salts [$FeSO_4$ or $Zn(CH_3COO)_2$] and the corresponding peptides in dilute aqueous solutions on heating without access to oxygen from air (to avoid oxidation of the indole group of tryptophan and of the Fe^{2+} ions to Fe^{3+}). The process was followed by monitoring changes in the UV spectra of dipeptides at 230 – 300 nm.

EXPERIMENTAL BIOLOGICAL PART

The immunostimulant activity of peptides and their compounds with metal ions was evaluated from the level of enhanced antibody genesis in calves immunized with an antitheileriasis vaccine obtained from the All-Russia Research Institute of Experimental Veterinary.

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The vaccine was introduced at a dose of 1 ml per animal. Three to five days after the postvaccinal reaction to the antitheileriasis preparation (15 – 17 days after immunization) the test calves were subcutaneously injected in the middle neck region with the synthesized peptide preparations in the form of aqueous solutions. Immunized calves not treated with peptides served as control. The antitheileriasis antibody titer in the blood serum was determined by immunoenzymometric assay in both test and control animals 35 days upon immunization.

The experimental data summarized in Table 1 show that the H–Ile–Trp–OH dipeptide (exhibiting a 100% activity in the E-rosetting cell test) produces a double increase in the antitheileriasis antibody titer. In contrast, the H–Val–Trp–OH dipeptide (inactive in the E-rosetting cell test) possesses no activity *in vivo*, leading to no increase in the antitheileriasis antibody titer in the blood serum of immunized animals. At the same time, the coordination compound of H-Val-Trp-OH with Fe^{2+} ion produces a double increase, and the compound of H–Ile–Trp–OH dipeptide with Fe^{2+} ion, a 4 – 8-fold increase in the antitheileriasis antibody titeras compared to that in the control group of animals.

Thus, interaction of the tryptophan-containing dipeptides with metal ions increases the immunostimulant activity of these peptides.

EXPERIMENTAL CHEMICAL PART

The syntheses were performed using amino acids of the *L*-series and their derivatives purchased from Reanal (Hungary) or synthesized by conventional methods [5, 6].

The compositions and purity of the synthesized compounds were checked by TLC on Silufol UV-254 (Chemapol, Czech Republic) and Merck (Germany) eluted in the following solvent systems: *n*-butanol – pyridine – acetic acid – water, 5 : 5 : 1 : 4 (A); chloroform – methanol – 32% aqueous acetic acid, 60 : 45 : 20 (B); chloroform – ethyl acetate – methanol – acetic acid, 9 : 3 : 1 : 0.3 (C); *n*-butanol – pyridine – acetic acid – water, 30 : 20 : 6 : 24 (D); acetic acid – water – methanol – chloroform, 7 : 3 : 1 : 1 (E). The chromatograms were developed either by spraying with a 0.05% ninhydrin solution in acetone or by chlorination followed by treating with a mixture of equal volumes of a 0.5% KI solution and a saturated solution of benzidine in a 2% aqueous acetic acid.

TABLE 1. The Immunostimulamt Activity of Tryptophan-Containing Dipeptides and Their Coordination Compounds

Compound	Dose, μg	Antibody titer (immunoassay)	E-rosetting cell test activity, % [2]
H–Val–Trp–OH	100	1 : 50 – 1 : 800	0
H–Val–Trp–OH + Fe^{2+}	700	1 : 100 – 1 : 1600	–
H–Ile–Trp–OH	100	1 : 100 – 1 : 1600	100
H–Ile–Trp–OH + Fe^{2+}	260	1 : 400 – 1 : 2800	–
Control	–	1 : 50 – 1 : 800	–
H–Ile–Trp–OH + Zn^{2+}	400	1 : 400 – 1 : 800	–
Control	–	1 : 50 – 1 : 200	

The protecting groups were removed by catalytical hydrogenation in the presence of a catalyst representing 10% Pd supported on activated charcoal (Fluka, Germany). The melting points (uncorrected) were determined on a microscopic heating table of the Boetius type (Germany). The specific optical rotation was measured with a Perkin-Elmer Model 241 automated digital polarimeter (USA) using 1-dm-long cells ($c = 1$; methanol). The reaction products were purified by column chromatography using silica gel L-100/160 (Chemapol, Czech Republic). The solvents were evaporated in vacuum at a temperature not exceeding 40°C. The amino acid analysis of the synthesized peptides was performed upon their acid hydrolysis with 3 M *p*-toluenesulfonic acid for 48 h at 110°C.

Valyltryptophan (H-Val-Trp-OH). To a mixture of 2.51 g (10 mmole) of carbobenzoxyvaline and 1.45 ml (10.5 mmole) of triethylamine in 20 ml of ethyl acetate was added 4.0 g (10.5 mmole) of di(pentafluorophenyl)carbonate and the mixture was stirred at room temperature for 30 min. Then ethyl acetate was evaporated and the residue was dissolved in 20 ml of DMF. To this solution was added 2.26 g (10 mmole) of tryptophan sodium salt and the mixture was stirred at room temperature for 3 h. Upon termination of the reaction, DMF was evaporated in vacuum and the residue was dissolved in a mixture of 40 ml of ethyl acetate and 40 ml of a 3% potassium bisulfate containing 1.2 g (10 mmole) of the salt. Upon stirring for 5 min, the aqueous layer was separated and the ethyl acetate phase was washed with 20 ml (3 × 6.5 ml) of a 0.5% sodium bisulfate solution (a 5% salt excess with respect to peptide). The ethyl acetate solution of the protected peptide was dried over anhydrous sodium sulfate and the solvent (ethyl acetate) was evaporated. The final product was purified by chromatography on a 2 × 50 cm column filled with an L-100/160 silica gel. The product was first eluted with chloroform (100 ml) and then with an ethyl acetate – benzene 3 : 2 mixture (350 ml). The eluate was collected in 5 ml fractions and each fraction was analyzed by TLC for the presence of the main product. The fractions containing protected peptide were combined and evaporated to obtain Z–Val–Trp–OH; yield, 3.72 g (71.2%); R_f 0.76 (system A), 0.69 (system B).

The isolated protected peptide was dissolved in 50 ml of methanol and hydrogenated for 4 h in the presence of a 10% Pd/C catalyst. After hydrogenation, the catalyst was separated by filtration and the filtrate was evaporated. The residue was dissolved in an *n*-butanol – water mixture, the aqueous layer was separated, and the solvent was evaporated to obtain the free dipeptide H–Val–Trp–OH; yield, 2.04 g (67.3% as calculated for the initial amino acids); R_f 0.53 (system D), 0.78 (system E); $[\alpha]_D^{20}$, –18.34° ($c = 1$; methanol); amino acid analysis: Val, 0.98; Trp, 0.95.

Isoleucyltryptophan (H–Ile–Trp–OH). H-Ile-Trp-OH was obtained by a procedure analogous to that described above, proceeding from 2.65 g (10 mmole) of carbobenzoxy-isoleucine, 1.45 ml (10.5 mmole) of triethylamine, 4.0 g (10.5 mmole) of di(pentafluorophenyl)carbonate, and 2.26 g (10 mmole) of tryptophan sodium salt. The protected dipeptide Z–Ile–Trp–OH: yield, 3.36 g (74.5%); R_f 0.81 (system B), 0.65 (system C); $[\alpha]_D^{20}$, – 10.82° ($c = 1$; ethyl acetate). The free dipeptide H–Ile–Trp–OH: yield, 2.28 g (71.8% as calculated for the initial amino acids); R_f 0.13 (system A), 0.44 (system D), 0.7 (system E); $[\alpha]_D^{20}$, – 31.14° ($c = 1$; methanol); amino acid analysis: Ile, 0.97; Trp, 0.94.

Dipeptide compounds with metal ions. Dipeptide (1.133×10^{-4} mmole) was dissolved in 100 ml of distilled water heated to 100°C. To this solution was added at pH 6.0 and constant stirring 1.133×10^{-4} mole of a metal salt solution. The reaction vessel was hermetically closed, heated to 110 – 120°C, kept at this temperature for 1 h in the dark, and cooled to room temperature.

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