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The Study of Stability of Proline-Containing Derivatives of Dopamine and Serotonin in the Biological Media in vitro Experiments

K. V. Shevchenko[a], *, L. A. Andreeva[a], I. Yu. Nagaev[a], V. P. Shevchenko[a], and N. F. Myasoedov[a]

[a] *Institute of Molecular Genetics of the Russian Academy of Sciences, pl. Kurchatova 2, Moscow, 123182 Russia*

**e-mail: ATCarma@mail.ru*

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Abstract—The peptides Boc-Gly-Pro-DP, Z-Gly-Pro-DP, LA-Gly-Pro-DP, Boc-Gly-Pro-Srt, Z-Gly-Pro-Srt have been synthesized for the first time. The study of their stability in the presence of leucine aminopeptidase, carboxypeptidase Y, carboxypeptidase B, and proline endopeptidase (PEP) has shown that the synthesized peptides are stable in the presence of aminopeptidases and carboxypeptidases. In the presence of PEP, dopamine (DP) and serotonin (Srt) have been cleaved from these substances. Thus, the originally synthesized proline derivatives of Srt and DP may be considered as the resources, from which Srt and DP can be gradually released. This creates the possibility of a prolonged action of these biologically active compounds on cells and, consequently, on the whole body.

Keywords: proline-containing dopamine and serotonin derivatives, peptide degradation, leucine aminopeptidase, carboxypeptidase, proline endopeptidase (PEP), blood plasma enzymes

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INTRODUCTION

Biologically active peptide compounds can be administered in the body by using various methods (intravenously, intranasally, etc.). Therefore, it is very important to determine their stability in the presence of aminopeptidases and carboxypeptidases. In addition, it is interesting to elucidate, which metabolites can be formed during enzymatic transformation (as they can exhibit their own biological activity). For example, the use of different routes of Pro-Gly-Pro-Leu administration can result in almost 3-fold difference in the content of the resultant Gly-Pro dipeptide [1]. This cannot be ignored due to the fact that Gly-Pro has a pronounced pharmacological effect: it prevents an increase in anxiety and a decrease in the level of research activity [2].

Synthetic peptides are known to be stabilized by their modification with proline residues [3]. Such proline-containing compounds are highly resistant to the proteolytic action of the most common proteases [4–6]. Living organisms also contain prolidases. These enzymes are present in the brain [dipeptidyl peptidase II (EC 3.4.14.2; DPP-II)] [7], blood [prolidase (EC 3.4.13.9; peptidase D, iminopeptidase)] [8] and are involved in the cleavage of proline-containing peptides [dipeptidyl peptidase IV, (EC 3.4.14.5; DPP-IV)] [9], as well as in other processes [8, 10]. Their action on biologically active fragments linked to a proline residue results in a gradual cleavage of such prodrug and release of the biologically active fragment, which will cause a certain cell response. In other words binding a biologically active compound to a proline-containing peptide can result in formation of a drug substance characterized by prolonged life-time in the body; such approach will reduce the dosage of the drug at the unchanged positive effect and, therefore, will reduce negative side-effects.

In this study, we have investigated stability of proline derivatives of DP and Srt under the action of leucine aminopeptidase (EC 3.4.11.2), carboxypeptidase Y (EC 3.4.16.1), carboxypeptidase B (EC 3.4.17.2) and proline endopeptidase (EC 3.4.21.26).

The aim of this work was to synthesize Boc-Gly-Pro-DP, Z-Gly-Pro-DP, LA-Gly-Pro-DP, Boc-Gly-Pro-Srt, Z-Gly-Pro-Srt and to study their enzymatic hydrolysis in experiments in vitro. Semax (Met-Glu-His-Phe-Pro-Gly-Pro) was used as a reference compound.

MATERIALS AND METHODS

Microsomal leucine aminopeptidase (Type VI) from pig kidney (9.2 U/mg), carboxypeptidase Y from

Abbreviations used: AcOH—acetic acid; Boc—*tert*-butoxycarbonyl group; Bzl—benzyl group; DCCD—N, N-dicyclohexylcarbodiimide; DMF—dimethylformamide; DMSO—dimethyl sulfoxide; DP—dopamine; Et_3N—triethylamine; HPLC—high performance liquid chromatography; LA—lauric acid; MeOH—methanol; PEP—proline endopeptidase; Srt—serotonin; Su—N-oxysuccinimide; TFA—trifluoroacetic acid; Z—benzyloxycarbonyl group.

baker's yeast (17 U/mg), carboxypeptidase B from pig pancreas (70 U/mg), and proline endopeptidase from flavobacteria (55 U/mg), as well as catalysts and reagents were obtained from Sigma-Aldrich (USA). Solvents, acids, etc. are obtained from the company "Khimmed" (Russia).

Boc-Gly-Pro was synthesized at the Institute of Molecular Genetics of the Russian Academy of Sciences by condensation of Boc-Gly and Pro-OBzl followed by removal of the benzyl group by hydrogenation. Blood plasma (31 mg protein/mL) was obtained from male Wistar rats (200 g). Blood (5 mL) collected in test tube containing 20 μL of heparin (5000 U/mL) was centrifuged at 1500 *g* for 10 min. Protein concentration in blood plasma samples was determined by the Hartree-Lowry method. In vitro studies were carried out according to the previously described methods [11, 12].

In the case of experiments with commercially available enzymes, reaction mixtures were analyzed without preliminary purification and the range of values during parallel measurements did not exceed 5%. In the case of experiments with plasma samples it was necessary to perform preliminary purification by solid-phase extraction. Therefore, measurements were usually repeated at least 5 times. The range of values obtained in this case did not exceed 10–15%.

Analysis of Reaction Mixtures

Reaction mixtures were analyzed at 210 nm, 220 nm, 230 nm, 240 nm, 254 nm on a Milichrom-A02 chromatograph (Russia) using a ProntoSIL-120-5-C_{18} AQ DB-2003 column (Bishcoff Chromatography, Germany, 2 × 75 mm, particle size 5 μm), in a gradient of methanol and buffer (0.2 M $LiClO_4$ + 0.005 M $HClO_4$, pH 2.24) for 12 min at 35°C. Analysis of Z-Gly-Pro-DP, Boc-Gly-Pro-DP, Z-Gly-Pro-Srt and Boc-Gly-Pro-Srt was carried out by increasing the methanol concentration from 5% to 100% (system 1). The analysis of LA-Gly-Pro-DP was carried out by increasing the concentration of methanol from 5% to 45% in 5 min and from 85% to 100% in 6.0–12.0 min (system 2). The flow rate of the eluent was 0.2 mL/min. The retention times of the test compounds are shown in Table 1.

Compounds were analyzed using mass spectrometric data obtained on a LCQ Advantage MAX instrument (Thermo Electron Corp., USA) with electrospray ionization by direct injection of a sample solution (10 μg/mL in 0.1% acetic acid) and further fragmentation of the molecular peak in the analyzer by the ion collision method at 35 eV (Table 1).

Table 1. Chromatography-mass spectrometry analysis of compounds

Compound	τ, min	$[M + 1]^+$
Z-Gly-Pro-DP	8.13*	441
Boc-Gly-Pro-DP	7.80*	407
Z-Gly-Pro-Srt	8.30*	464
Boc-Gly-Pro-Srt	7.96*	430
LA-Gly-Pro-DP	8.80**	489
Z-Gly-Pro	7.62*	305
Boc-Gly-Pro	7.08*	271
Dopamine	2.25*	153
Serotonin	4.07*	176

* System 1; ** system 2; τ—retention time on the chromatographic column; $[M + 1]^+$—molecular mass.

Synthesis of Boc-Gly-Pro-DP

At room temperature 1.4 mg 1-hydroxybenzotriazole (0.01 mmol) and 20.8 mg DCCD (0.1 mmol) were added under stirring to a solution of 15 mg Boc-Gly-Pro (0.055 mmol) in 1 mL of dichloromethane. After 10 min, 20 μL of Et_3N and a DP solution (9.9 mg (0.064 mmol) in 0.5 mL of DMF) were added and stirring was continued for 4 h. DMF was removed by lyophilization. Solid phase extraction of Boc-Gly-Pro-DP, applied onto Diapak C16, was carried out by five solvent systems. Initially, 5 mL 5% methanol was passed, then 10 mL of 20% methanol, 6 mL of 30% methanol, 6 mL of 95% methanol, and 5 mL of 100% methanol were passed. The ratio of the content of the desired product in the fractions was 0 : 1 : 5 : 20 : 0.01. The purity of the resultant preparation increased threefold. Preparative purification of Boc-Gly-Pro-DP was carried out using a Reprosil-Pur C18aq column (Maisch Gmbh, Germany, 20 × 150 mm, particle size 10 μm) in the system MeOH–AcOH–TFA (45 : 0.1 : 0.01). The flow rate of the eluent was 20 mL/min. Retention time was 3.95 min. The yield of Boc-Gly-Pro-DP was 58% and the chemical purity was 98%.

Synthesis of Boc-Gly-Pro-Srt

A solution of 24 mg Boc-Gly-Pro (0.088 mmol), 7 mg 1-hydroxybenzotriazole (0.05 mmol), and 29 mg DCCD (0.141 mmol) in 1 mL of DMF was stirred for 20 min at room temperature. After addition of 20 μL Et_3N (0.14 mmol) and stirring for 10 min and then addition of 15 mg Srt (0.085 mmol) it was stirred for 4 h. DMF was removed by lyophilization and preparative purification was performed as above. Retention time was 5.02 min. The yield of Boc-Gly-Pro-Srt was 64%.

Synthesis of Z-Gly-Pro-DP

At room temperature 10 mg 1-hydroxybenzotriazole (0.071 mmol) and 130 mg DCCD (0.63 mmol) were added under stirring to a solution of 170 mg of

Z-Gly-Pro (0.56 mmol) in 0.3 mL DMF. After 10 min, 0.2 mL Et_3N and a solution of 100 mg DP (0.65 mmol) in 0.3 mL DMF were added. Stirring was continued for 4 h. DMF was removed by lyophilization and preparative purification was performed as described above. Retention time was 5.53 min. The yield of Z-Gly-Pro-DP was 66%.

Synthesis of Z-Gly-Pro-Srt

A solution of 28.5 mg Z-Gly-Pro (0.093 mmol), 7 mg 1-hydroxybenzotriazole (0.05 mmol), and 21 mg DCCD (0.102 mmol) of 0.5 mL of DMF was stirred for 20 min at room temperature. After addition of 20 mg Srt (0.113 mmol) in 0.5 mL DMF and 25 μL Et_3N (0.179 mmol) the resultant solutions was stirred for 4 h. Preparative purification was carried out as described above. Retention time was 5.95 min. The yield of Z-Gly-Pro-Srt was 65–70%.

Synthesis of DP and Srt Derivatives Based on the Use of Boc-Gly-Pro-Su and Z-Gly-Pro-Su

After addition of 214 mg DCCD (1.039 mmol) to the solution of 1 mmol Boc- or Z-amino acid and 115 mg N-oxysuccinimide (1 mmol) in 5 mL dry dioxane, the mixture was stirred for 2 h at 15°C and then left under stirring overnight at room temperature. The urea was filtered off, the filtrate was washed with 2 mL dioxane, and the solvent was evaporated. The precipitate was dissolved in 6 mL ethanol. 1 mmol DP or Srt was dissolved in 2 mL ethanol with 0.15 mL Et_3N. Then, 0.5 mmol of Boc-Pro-Su or Z-Gly-Pro-Su in 3 mL of ethanol was mixed with 0.5 mmol of DP or Srt solution in 1 mL ethanol. The solutions were stirred overnight at room temperature. Preparative purification was performed as described above. The yield of Boc-Gly-Pro-DP, Z-Gly-Pro-DP, Z-Gly-Pro-Srt, and Boc-Gly-Pro-Srt was 82%, 86%, 81%, and 79%, respectively.

Synthesis of Boc-Gly-ProOMe and Boc-Gly-ProOBzl

A solution of 875 mg Boc-Gly (5.57 mmol) in 5 mL chloroform was treated with 1444 mg DCCD (7 mmol) for 20 min at room temperature and after addition of 0.7 mL Et_3N (5.04 mmol) it was stirred for 10 min. Then a solution of 778 mg $ProOCH_3$ (5.37 mmol) in 4 mL of chloroform was added and the mixture was stirred for 3 h at room temperature and then 72 h at 5°C. The precipitate was filtered off and chloroform was evaporated. Boc-Gly-ProOMe was dissolved in 10 mL of ethyl acetate, washed with 5 mL 1 M HCl and 5 mL water. Ethyl acetate was evaporated, the residue was lyophilized. Boc-Gly-ProOBzl was synthesized in a similar manner. The analysis was performed on a Milichrome A-02 chromatograph in the gradient of 0.1% CH_3COOH–methanol from 30% to 100% in 12.5 min. The flow rate of the eluent was 0.2 mL/min. The retention time of Boc-Gly-ProOMe and Boc-Gly-ProOBzl was 6.53 min and 7.11 min, respectively.

Preparative purification of Boc-Gly-ProOMe and Boc-Gly-ProOBzl was performed using a Reprosil-Pur C18aq column (20 × 150 mm, particle size 10 μm) in the system MeOH–H_2O–AcOH (50 : 50 : 0.1). The retention times of Boc-Gly-ProOMe and Boc-Gly-ProOBzl were 6.53 min and 7.11 min, respectively. The yield of Boc-Gly-ProOMe and Boc-Gly-ProOBzl was about 70%.

Synthesis of LA-Gly-ProOMe and LA-Gly-ProOBzl

(a) A solution of 200 mg LA (1 mmol) in 3 mL of absolute benzene was mixed with 321 mg thionyl chloride (2.70 mmol). The solution was boiled for 2.5 h. Benzene and the excess thionyl chloride were evaporated and the solution evaporated twice more after addition of 5 mL absolute benzene. LA chloroanhydride was used without additional purification.

Boc-protection with Boc-Gly-ProOMe and Boc-Gly-ProOBzl was removed by treating a solution of 100 mg of the protected peptide with a mixture of 1 mL chloroform and 0.5 mL of TFA for 1 h at room temperature. Then the solution was evaporated and after addition of 5 mL methanol was evaporated twice more. The residue was lyophilized and used without additional purification.

A solution of Gly-ProOMe in 1 mL chloroform was treated under stirring with 0.4 mL Et_3N and 0.35 mmol LA chloroanhydride. After 2 h, the reaction mixture was treated with methanol and lyophilized. A similar reaction was carried out with Gly-ProOBzl. The analysis was performed on a Milichrome A-02 chromatograph in a gradient of 0.1% acetic acid-methanol from 10% to 100% in 10 min, then methanol for 2.5 min. The flow rate of the eluent was 0.2 mL/min. The retention time of LA-Gly-ProOMe and LA-Gly-ProOBzl was 10.30 min and 10.96 min, respectively.

Preparative purification of LA-Gly-ProOBzl and LA-Gly-ProOMe was performed using a Reprosil-Pur C18aq column (20 × 150 mm, particle size 10 μm) in the system MeOH–H_2O–AcOH–TFA (90 : 10 : 0.1 : 0.01). The flow rate of the eluent was 20 mL/min. The retention times of the compounds were 2.94 min and 2.71 min, respectively. The yields of substances were 87% and 79%, respectively.

(b) After addition of 245.3 mg DCCD (1.19 μmol) to a solution of 216 mg LA (1.08 mmol) and 124.2 mg N-oxysuccinimide (1.08 mmol) in 5 mL of dry dioxane, the solution was stirred for 2 h at 15°C. The urea was filtered off, the filtrate was washed with dioxane (2 mL × 2), and the solvent was evaporated. The precipitate was dissolved in 1 mL dioxane and treated with a solution of 250.5 mg Gly-Pro-OBzl in 2 mL ethanol with 0.2 mL of Et_3N. After 21 h, the solution

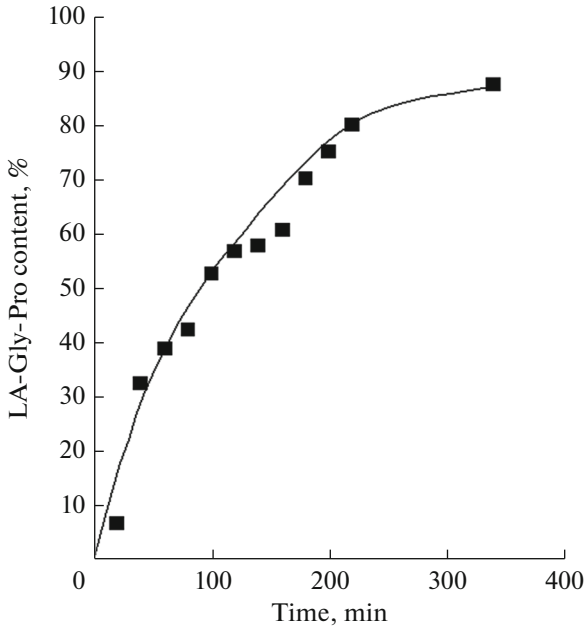

Fig. 1. Kinetics of alkaline hydrolysis of LA-Gly-ProOCH_3.

was evaporated, dissolved in 5 mL ethyl acetate, washed twice with 1 mL water. The aqueous layers were combined and extracted with 1 mL ethyl acetate. Ethyl acetate was evaporated. The yield of LA-Gly-ProOBzl was 52%.

Synthesis of LA-Gly-Pro

After addition of 0.5 mL of 2 M NaOH to a solution of 110 mg LA-Gly-ProOCH_3 in 4 mL methanol under stirring and incubation for 7 h (Fig. 1) the solution was acidified with 1 M HCl and evaporated. The residue was dissolved in 3 mL ethyl acetate and washed with water until neutral pH. The solution was evaporated, lyophilized and used without additional purification. The yield of LA-Gly-Pro was 90%.

Benzyl protection of LA-Gly-ProOBzl was removed in methanol by hydrogenation in the presence of 5% $PdO/BaSO_4$ (30 mg) for 90 min. The catalyst was filtered off, the solution was evaporated, and the residue was lyophilized. The resultant substance was used without additional purification. The yield of LA-Gly-Pro was 98%.

The analysis was performed on a Milichrome A-02 chromatograph in a gradient of 0.1% acetic acid–methanol from 10% to 100% in 10 min, then in methanol for 2.5 min. The flow rate of the eluent was 0.2 mL/min. The retention time of the LA-Gly-Pro was 10.10 min.

Synthesis of LA-Gly-Pro-DP

60 mg DCCD (0.29 mmol) was added, to a solution of 90 mg (0.26 mmol) of LA-Gly-Pro in 0.15 mL of DMF under stirring. After 10 min, 150 μL Et_3N and then a solution of 50 mg DP (0.327 mmol) in 0.3 mL DMF were sequentially added. Stirring was continued for another 4 h. After addition of 4 mL chloroform stirring was continued overnight. DMF was removed by lyophilization. The residue was dissolved in ethyl acetate (6 mL), and then 2 mL water and 0.1 mL of acetic acid were added. After centrifugation, water and urea were separated. The aqueous fraction was extracted again with ethyl acetate (4 mL) and centrifuged. Preparative purification of LA-Gly-Pro-DP was performed using a Reprosil-Pur C18aq column (20 × 150 mm, particle size 10 μm) in the system MeOH–H_2O–AcOH–TFA (85 : 15 : 0.1 : 0.01). Retention time was 2.86 min. The yield of LA-Gly-Pro-DP was 46%.

Analysis of the Effect of the Presence of Ethanol or DMSO in the Incubation Medium on the of Leucine Aminopeptidase and Carboxypeptidase Y Functioning

The effect of the presence of ethanol or DMSO on the enzyme activity in the incubation medium was analyzed using the following procedure. The effect of ethanol or DMSO was investigated using 100 μL of phosphate-buffered saline (PBS; 27.4 mM NaCl, 0.4 mM KCl, 2 mM Na_3PO_4 in 100 mL H_2O, pH 7.4) containing 0.068 μmol of Semax and 0.0039 U of leucine aminopeptidases or 0.935 U of carboxypeptidase Y. The ratio PBS to ethanol or DMSO ranged from 100 : 0 to 50 : 50 (Table 2). The incubation mixture was stirred and thermostated at 30°C; aliquots (15 μL) were taken at certain time intervals. Enzymatic hydrolysis was stopped by adding an equal volume of methanol to the samples taken.

Evaluation of Stability of Peptide Derivatives in the Presence of Leucine Aminopeptidases, Carboxypeptidases Y and B, and PEP

In the case of leucine aminopeptidases, carboxypeptidases, and PEP, conditions of enzymatic hydrolysis were optimized by studying degradation of the investigated peptides at different substrate : enzyme ratios. For subsequent work, the ratio of 1 μmol/U was used for leucine aminopeptidases, 0.092 μmol/U for carboxypeptidase Y, 0.022 μmol/U for carboxypeptidase B, 10, 1 and 0.10 μmol/U for PEP.

Enzymatic hydrolysis of proline derivatives of DP and Srt was carried out according to the following procedure. A solution of 0.5 μmol of a proline-containing derivative in 260 μL PBS (27.4 mM NaCl, 0.4 mM KCl, 2 mM Na_3PO_4 in 100 mL H_2O, pH 7.4) was treated with 0.47 U of leucine aminopeptidases or 5.45 U of carboxypeptidase Y or 22.46 U of carboxy-

Table 2. The effect of ethanol and DMSO on the enzymatic hydrolysis of Semax (the reaction was carried out for 180 min)

Enzyme	Organic solvent	Percent of ethanol and DMSO					
		0.0	1.0	1.5	5.0	20.0	50.0
Leucine aminopeptidase	Ethanol	1.2*	1.8	3.0	5.0	54.5	97.5
Carboxypeptidase Y		14.6	19.2	21.3	27.5	88.0	97.7
Leucine aminopeptidase	DMSO	1.2	2.1	9.2	17.1	76.9	98.1
Carboxypeptidase Y		14.6	23.4	29.3	44.0	96.3	97.9

* Residual Semax content in the incubation solution, %.

peptidases B in the same buffer (PBS). For different proline derivatives of DP and Srt, 0.05 U, 0.5 U, or 5.0 U of PEP were added. The incubation mixture was stirred and thermostated at 30°C and aliquots (20 μL) were taken at certain time intervals. Enzymatic hydrolysis was stopped by adding an equal volume of methanol to the samples taken.

Evaluation of Stability of Peptide Derivatives in the Presence of Blood Plasma

A solution of 0.74 μmol of a peptide derivative in 820 μL of PBS (27.4 mM NaCl, 0.4 mM KCl, 2 mM Na_3PO_4 in 100 mL H_2O, pH 7.4) was mixed with 380 μL of blood plasma (31 mg protein/mL). The incubation mixture was stirred and thermostated at 30°C; aliquots (100 μL) were taken at certain time intervals. The obtained samples were purified by reverse phase solid phase extraction: the peptide fraction was applied onto a cartridge packed with a reverse phase Lichroprep RP-18, followed by elution of the peptides with methanol with 0.1% TFA. The mixture was then evaporated and dissolved in 150 μL of a mixture of methanol : water (5 : 95). The mixture was analyzed by HPLC.

RESULTS AND DISCUSSION

The N-terminal residue is especially vulnerable peptide fragment in the compounds Boc-Gly-Pro-DP, Z-Gly-Pro-DP, LA-Gly-Pro-DP, Boc-Gly-Pro-Srt, Z-Gly-Pro-Srt and therefore, in the compounds used, its amino group was protected (Boc, Z, fatty acid). The idea of amino group protection with fatty acid was inspired by the results obtained in [13].

The study of the resistance of Pro-Gly-Pro-Leu and its acylated analogues to leucine aminopeptidase has shown that the degradation of the original peptide was much higher than that of its N-acyl derivatives and, therefore, the physiological effect of the latter can be longer [13]. Consequently, the use of N-protected peptides usually leads to an increase in their resistance to degradation in biological media [13, 14].

Synthesis of Boc-Gly-Pro-DP, Z-Gly-Pro-DP, LA-Gly-Pro-DP, Boc-Gly-Pro-Srt, Z-Gly-Pro-Srt was carried out using known methods [15, 16]. In the experiments we used DCCD, thionyl chloride, various protective groups. The Boc group was removed under acidic conditions, benzyl group was removed using hydrogen gas in the presence of a catalyst, while methyl group was removed under alkaline conditions. Saponification of methyl ester occurred rather slowly: 90% of the reaction was completed only after 6 h (Fig. 1).

Two main methods were used to obtain the above substances. The first method involved the use of DCCD for the condensation of compounds; the second method used DCCD and N-oxysuccinimide to obtain the corresponding esters, which were subsequently used to obtain desired substances. The first method was more promising for synthesis of compounds containing LA, while the second method was preferable for reactions with DP and Srt.

All substances were characterized by chromatographic methods (HPLC) and using mass spectroscopy.

The difficulty of in vitro experiments with Boc-Gly-Pro-DP, Z-Gly-Pro-DP, LA-Gly-Pro-DP, Boc-Gly-Pro-Srt, Z-Gly-Pro-Srt consisted in their insufficient solubility in water. Therefore, we first evaluated the effect of ethanol or DMSO, which were added to increase solubility of the DP and Srt derivatives, on the enzymatic hydrolysis of peptides. In these experiments Semax was used as a reference compound.

These experiments have shown that the ethanol or DMSO content less than 5% did not exhibit any noticeable decrease in the efficiency of the enzymatic hydrolysis of Semax (Table 2).

Results demonstrate preferential use of ethanol. Solutions of DP and Srt derivatives were prepared by dissolving Boc-Gly-Pro-DP, Z-Gly-Pro-DP, Boc-Gly-Pro-Srt, Z-Gly-Pro-Srt in 25% ethanol. After addition of the necessary aliquot to the incubation medium, the ethanol concentration did not exceed 1.5%. In the case of LA-Gly-Pro-DP, the concentration of ethanol or DMSO in the incubation medium had to be increased to 3%. Therefore, under such conditions, the effect of ethanol on the efficiency of enzymes was minimal.

Table 3. Stability of Boc-Gly-Pro-DP, Z-Gly-Pro-DP, LA-Gly-Pro-DP, Boc-Gly-Pro-Srt, Z-Gly-Pro-Srt in the presence of amino- and carboxypeptidases

Substrate	Enzyme	Time, min					
		2	30	60	180	1440	4320
Boc-Gly-Pro-DP	Leucine aminopeptidase	100*	89.3	79.6	67.4	55.1	33.7
Z-Gly-Pro-DP		100	77.3	73.2	53.6	48.1	33.2
LA-Gly-Pro-DP		100	83.5	67.1	40.1	33.7	27.2
Boc-Gly-Pro-Srt		100	99.3	99.0	98.3	93.8	92.4
Z-Gly-Pro-Srt		100	94.7	94.7	89.5	79.0	73.7
Boc-Gly-Pro-DP	Carboxypeptidase B	100	96.6	88.2	79.8	65.7	36.5
Z-Gly-Pro-DP		100	85.0	76.7	61.6	58.8	30.0
LA-Gly-Pro-DP		100	86.7	73.4	55.6	34.4	29.5
Boc-Gly-Pro-Srt		100	100	100	100	100	100
Z-Gly-Pro-Srt		100	100	100	94.4	88.9	83.3
Boc-Gly-Pro-DP	Carboxypeptidase Y	100	92.5	89.7	88.7	80.3	54.0
Z-Gly-Pro-DP		100	98.4	93.3	91.7	83.2	62.6
LA-Gly-Pro-DP		100	92.5	85.0	73.3	73.0	40.5
Boc-Gly-Pro-Srt		100	100	99.6	98.4	97.2	96.8
Z-Gly-Pro-Srt		100	100	100	100	94.4	88.9

* The initial content of the substance in the incubation solution, %.

Besides commercially available enzymes listed in Table 2, experiments with of DP and Srt derivatives were performed using carboxypeptidase B and PEP (Tables 3, 4).

As can be seen from the presented data, the stability of Srt derivatives in the presence of leucinaminopeptidase was higher than that of the corresponding DP derivatives. The same tendency was observed in the presence of carboxypeptidases B and Y. Compounds containing Boc protection in the presence of leucine aminopeptidases and carboxypeptidases B were more stable than compounds with Z protection or containing lauric residue. In the presence of carboxypeptidase Y, compounds with Z-protection appeared to be more stable than compounds with Boc-protection and containing lauric residue. In general, it can be concluded that all synthesized compounds are highly resistant to both amino and carboxypeptidases. In contrast to Semax, the initial compounds remain in the incubation mixture for many hours; such stability is obviously explained by the presence of a proline residue and the presence of protective groups on the N-terminal amino acid.

Table 4. Optimization of the substrate-PEP ratio (the reaction was carried out for 60 min)

Substrate	Substrate-enzyme ratio, μmol/U				
	0.05	0.49	5.7	58	597
Z-Gly-Pro-DP	0.0*	0.0	28.0	94.1	100
Boc-Gly-Pro-DP	0.0	17.5	92.4	100	100

* The initial content of the substance in the incubation solution, %.

Different rates of decrease in the content of DP and Srt derivatives observed in the presence of the peptidases listed above indicate that this process is determined not by hydrolysis of the proline-containing fragment of these derivatives, but by the degradation of the dopamine or serotonin moieties of these compounds. This is also indicated by the fact that during mass spectrometric analysis of the reaction mixtures, molecular peaks that could have formed from a proline-containing fragment were not found.

Experiments with PEP required optimization of its concentration. Initially, the substrate-enzyme ratio was used, as was the case with other enzymes. However, under these conditions degradation of DP and Srt derivatives in the presence of PEP occurred very quickly. Thus, we had to decrease PEP concentration during the kinetic study of the enzymatic hydrolysis of the synthesized compounds (Table 4).

As can be seen from the above data, stability of compounds with Z-protection in the presence of PEP was lower than that of compounds with Boc-protection. At the substrate-enzyme ratio of 5.7 μmol/U, the remaining content of Z-Gly-Pro-DP after 60-min incubation was 30%, while the content of Boc-Gly-Pro-DP was about 90%. At the substrate-enzyme ratio of 0.49 μmol/U Z-Gly-Pro-DP underwent complete degradation; in the case of Boc-Gly-Pro-DP about 17% remained. Therefore, the study on time-dependence of stability of Boc-Gly-Pro-DP, Z-Gly-Pro-

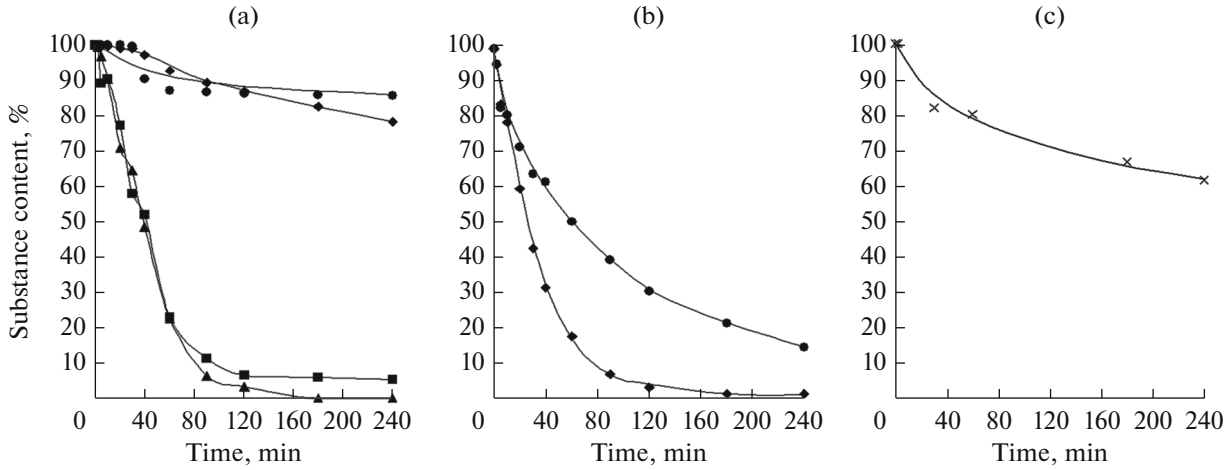

Fig. 2. Stability of Z-Gly-Pro-DP (■), Z-Gly-Pro-Srt (▲), Boc-Gly-Pro-DP (◆), Boc-Gly-Pro-Srt (●), LA-Gly-Pro-DP (×) used at various concentrations in the presence of PEP: (a) 10 μmol/U; (b) 1 μmol/U; (c) 0.1 μmol/U.

DP, LA-Gly-Pro-DP, Boc-Gly-Pro-Srt, Z-Gly-Pro-Srt in the presence of PEP was carried out for compounds with Z- and Boc-protection at different substrate-enzyme ratios (Fig. 2).

As can be seen from the presented results, the shape of the kinetic curves for all compounds was identical. Enzymatic hydrolysis of derivatives with Z-protection occurred much faster than compounds with Boc-protection (Fig. 2a). The kinetics of LA-Gly-Pro-DP hydrolysis could be determined only after 10-fold increase in the enzyme concentration (Fig. 2c). The DP and Srt residues had a minor contribution to the stability of compounds at the low enzyme content. A decrease in the substrate-enzyme ratio to 1 μmol/U was accompanied by a higher stability of the Srt derivatives (Fig. 2b). The experiment performed for several days has shown that at the lowest substrate-enzyme ratio (0.1 μmol/U), only LA derivatives (LA-Gly-Pro-DP) can be detected (Table 5). At the ratio 10 μmol/U significant amounts of compounds with Boc protection were detected, while compounds with Z protection were basically absent in the incubation medium or a few percents of the initial value could be detected (Table 5). At the substrate enzyme ratio of 1 μmol/U compounds with Z-protection and Boc-protection were completely hydrolyzed in one day (Table 5).

The kinetic study of the reaction product formation under the PEP action was performed both for compounds with Z-protection and for compounds with Boc-protection. It was shown that enzymatic hydrolysis occurred at the bond between DP / Srt and proline residue (Fig. 3, Table 6).

Thus, under the PEP action, a gradual release of DP and Srt occurs. Maximum values (about 90%) were obtained in a few hours. Consequently, it is reasonable to expect a longer exposure to DP and Srt, formed from these compounds, on processes occurring both in cells and in the whole body.

A comparison of the data obtained using commercially obtained enzymes and the enzyme system existing in rat blood plasma showed that persistence of the main patterns (Table 7).

As can be seen from the data obtained (Table 7), among the DP derivatives the compound containing LA was the most stable as compared with the derivatives with Boc protection and, especially with derivatives containing Z protection. As already emphasized above the overall stability of compounds with the same protective group was determined by the stability of dopamine or serotonin fragments. It can be concluded that in the presence of rat blood enzymes in experi-

Table 5. PEP-based enzymatic hydrolysis of substances during reaction time for more than one day

Substrate	C_{PEP}, μmol/U	Time			C_{PEP}, μmol/U	Time		
		24 h	72 h	120 h		24 h	72 h	120 h
Z-Gly-Pro-DP	10	0.0*	0.0	0.0	1	0.0	0.0	0.0
Boc-Gly-Pro-DP		72.46	34.30	23.70		0.0	0.0	0.0
Z-Gly-Pro-Srt		5.23	2.83	2.40		0.0	0.0	0.0
Boc-Gly-Pro-Srt		84.54	68.42	56.40		0.5	0.0	0.0
LA-Gly-Pro-DP		–	–	–	0.1	49.1	44.3	43.1

* The initial content of the substance in the incubation solution, %.

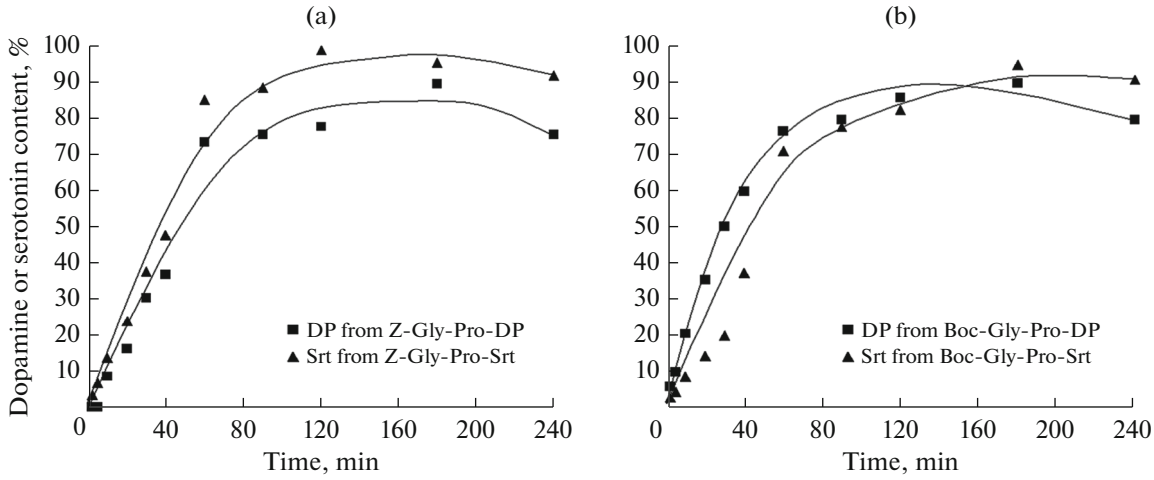

Fig. 3. Formation of DP and Srt from their peptide derivatives at various substrate-enzyme (PEP) ratios: (a) 10 μmol/U; (b) 1 μmol/U.

ments in vitro, derivatives of DP and Srt are stable. Therefore, the synthesized DP and Srt derivatives can be the sources of DP and Srt for several hours. It is reasonable to suggest that they may be drugs that can be used for a long-term effect on pathological processes with the aim of their localization and treatment.

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COMPLIANCE WITH ETHICAL STANDARDS

All animal experiments were carried out in accordance with the International Recommendations of the "European Convention for the Protection of Vertebrate Animals Used for Experiments or for Other Scientific Purposes" (The European Convention, 1986).

Table 6. Formation of DP and Srt from their peptide derivatives during their 5-day with PEP (the substrate-enzyme ratio for Z-derivatives was 10 μmol/U, for Boc-derivatives—1 μmol/U)

Source of DP	Time, days			Source of Srt	Time, days		
	1	3	5		1	3	5
Z-Gly-Pro-DP	66.8*	16.2	12.9	Z-Gly-Pro-Srt	88.5	74.9	61.3
Boc-Gly-Pro-DP	75.7	54.9	19.8	Boc-Gly-Pro-Srt	56.7	50.1	26.3

* The content of the enzymatic hydrolysis product in the incubation solution, %.

Table 7. Stability of Boc-Gly-Pro-DP, Z-Gly-Pro-DP, LA-Gly-Pro-DP, Boc-Gly-Pro-Srt, Z-Gly-Pro-Srt in the presence of rat plasma

Compound	Time, min									
	10	30	60	120	180	300	420	1440	2880	4320
Z-Gly-Pro-DP	99.0 ± 0.7*	87.1 ± 4.9	79.1 ± 4.8	48.8 ± 6.9	30.8 ± 4.3	20.0 ± 2.6	10.3 ± 1.5	0	0	0
Boc-Gly-Pro-DP	100	97.4 ± 1.5	96.0 ± 1.4	56.6 ± 3.3	50.0 ± 4.1	29.0 ± 4.2	14.5 ± 2.0	1.6 ± 0.2	1.1 ± 0.2	0
LA-Gly-Pro-DP	99.0 ± 0.5	95.9 ± 2.1	88.8 ± 3.9	81.6 ± 4.7	70.4 ± 4.4	67.4 ± 2.2	66.3 ± 0.9	60.2 ± 2.9	46.3 ± 4.8	23.5 ± 3.3
Z-Gly-Pro-Srt	100	100	80.0 ± 8.5	60.0 ± 9.2	40.0 ± 6.6	24.9 ± 3.5	13.4 ± 1.9	0	0	0
Boc-Gly-Pro-Srt	100	100	98.7 ± 0.7	82.9 ± 8.6	65.8 ± 5.8	65.8 ± 4.2	64.5 ± 4.6	63.2 ± 4.7	61.8 ± 5.1	59.2 ± 4.5

* The initial content of the substance in the incubation solution, %.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest

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