# Effect of obligatory replacement and conformational restriction on psychotropic activity of thyroliberin analogs

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Easy lactamization of Gln(Asn)—Pro—$NH_2$ with the formation of cyclic dipeptides with the diketopiperazine structure (mimetics of the conformational fragments of linear tripeptides with the X—Pro *trans*-bond) was observed in the synthesis of tripeptide Glp—Gln—Pro—$NH_2$ modified by the replacement of histidine with obligatory similar glutamine in thyroliberin (Glp—His—Pro—$NH_2$, TRH) and in the synthesis of its structural analog [$Asn^2$]TRH. Ion peaks corresponding to the Glp and Pro amino acid residues were revealed in the mass spectra of the peptides synthesized. The biological properties of the compounds obtained were determined indicating that the obligatory replacement resulted in an increased physiological specificity of [$Gln^2$]TRH. The enhanced activity of conformationally restricted cyclic peptides compared to linear ones suggests that the biologically active conformation responsible for the antidepressant activity of linear TRH analogs is the conformation with X—Pro *trans*-bond.

**Key words:** thyroliberin, structural modification, obligatory replacement; glutaminyl(asparaginyl)prolylamide, lactamization; cyclodipeptides, antidepressant activity, biologically active conformation.

The use of natural peptides in medicine is associated with several difficulties due to such disadvantages as fast metabolism, restricted penetration into brain, and biological multifunctionality. Therefore, the majority of commercially available drugs of peptide nature are modified analogs of natural peptides.

The results of clinical and pharmacological studies indicate that the action of the natural hormone thyroliberin Glp—His—Pro—$NH_2$ (TRH) on the central nervous system is multifunctional.[1,2] To study the possibility of narrowing the spectrum of neurotropic activity of TRH, we modified its structure and studied the influence of the latter on the biological activity of the analogs obtained.[3—5] In this work, of numerous different variants of modification of natural peptides, we used the method proposed previously: replacement of obligatory similar amino acids in peptide.[6,7] Therefore, histidine in thyroliberin was replaced by obligatory similar glutamine and asparagine structurally similar to the latter.

The condensation of prolylamide with *p*-nitrophenyl *N*-*tert*-butyloxycarbonylglutaminate in the presence of *N*-hydroxybenzotriazole (HOBt) gave dipeptide, which after acidolytic deprotection reacted with pentafluorophenyl pyroglutaminate (Scheme 1, $n = 2$).

In addition to the pyroglutaminylprolylamide (**1**) expected, the reaction mixture contained 3-(1,4-dioxooctahydropyrrolo[1,2-*a*]pyrazin-3-yl)propanamide (**2**) and pyroglutamylprolylamide (**3**), whose compositions and structures indicate that their formation is most likely related to intramolecular cyclization of glutaminylprolylamide after deprotonation of its primary amino group (Scheme 2).

It can be assumed that mild conditions of secondary reactions forming diketopiperazine (**2**) and pyrrolidone (**3**) structures are the result of the intramolecular activation of carboxamide groups of glutaminyl and prolylamide residues. The analysis of the molecular models indicates that the α-amino group can be involved simultaneously in two hydrogen bonds (see Scheme 2).[8] In the quasi-cluster structure that formed, the electrophilicity of both carboxamide carbon atoms and that of the nitrogen atom of the amino group are increased, due to which the ring is closed at room temperature.

In the synthesis of the structural analog of tripeptide **1**, pyroglutamylasparaginylprolylamide (**4**) (see Schemes 1 and 2, $n = 1$), by the reaction of asparaginylprolylamide with activated ester of pyroglutamic acid, the ring is also readily closed to form 2-(1,4-dioxooctahydropyrrolo[1,2-*a*]pyrazin-3-yl)acetamide (**5**) as the by-product.

The peptides synthesized were identified and their structures were studied by electron impact (EI) and fast atom bombardment (FAB) mass spectrometry and $^1H$ and $^{13}C$ NMR spectroscopy.

The FAB mass spectra of compounds **1**, **3**, and **4** exhibit, along with peaks of protonated molecular ions, intense (100%) peaks of ions with $m/z$ 84 and peaks of

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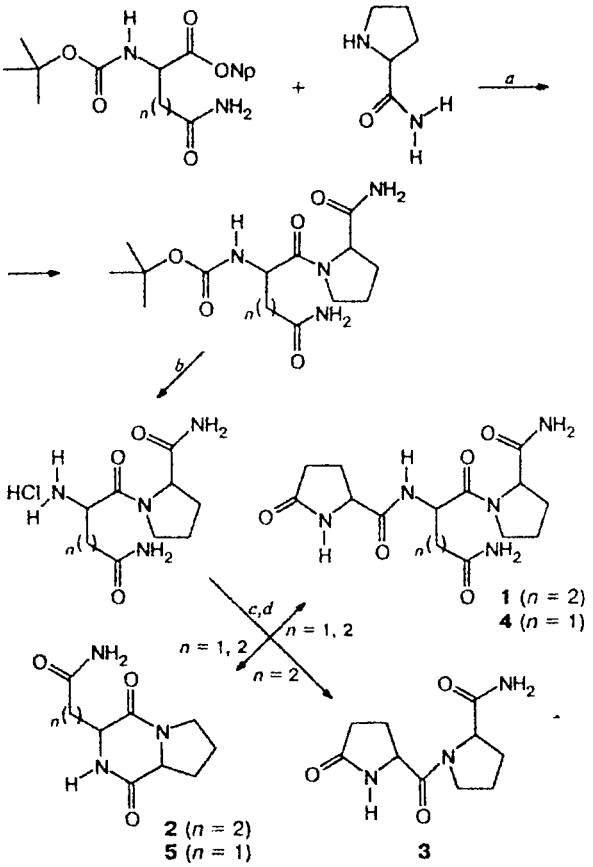
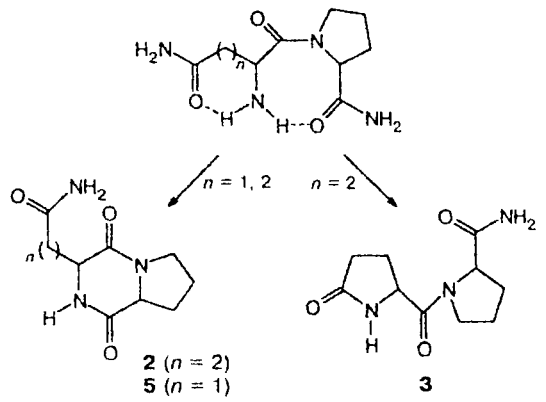
**Scheme 1**



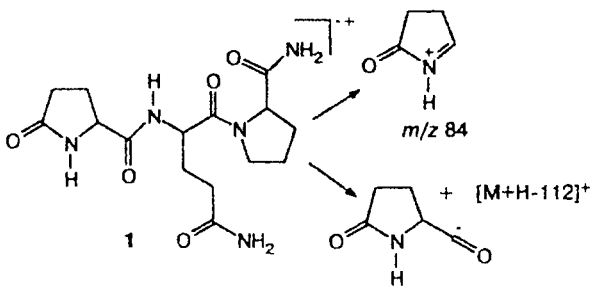

**Reagents and conditions:** *a*. HOBt; *b*. 4 *N* HCl in dioxane; *c*. $NEt_3$; *d*. GlpOPfp.

**Scheme 2**




ions $[M+H-112]^+$. This suggests two main directions of the decomposition associated with the presence of the terminal pyroglutamine fragment in molecules of these peptides (Scheme 3).

**Scheme 3**




Ions $[M+H-112]^+$ are formed due to the cleavage of the peptide bond and elimination of the residue of pyroglutamic acid with localization of the positive charge on the remaining part of the molecule. The localization of the charge on the pyroglutamine fragment and cleavage of the bond at the α-C atom of pyroglutamic acid are accompanied by the formation of the α-pyrrolidonium cations (ions *m/z* 84), *i.e.*, the peaks of ions $[M+H-112]^+$ and *m/z* 84 are caused by the presence of the terminal pyroglutamine fragment in molecules of compounds **1**, **3**, and **4** and can serve as its analytical property in the mass spectra.

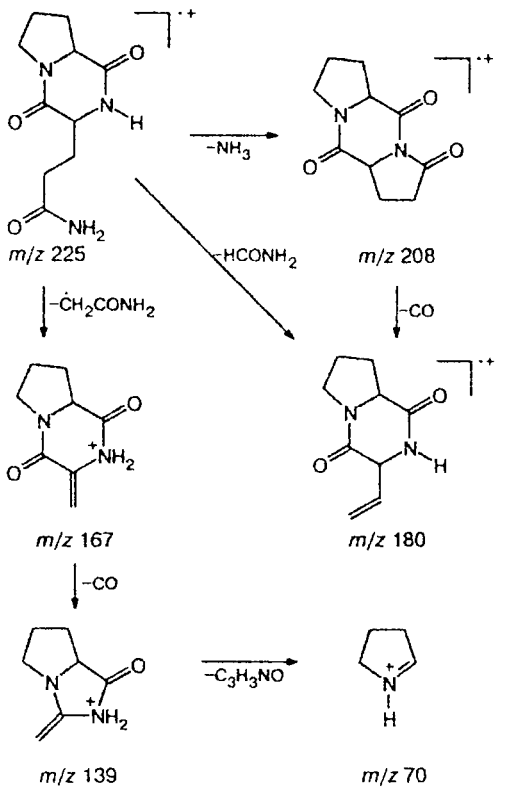
Cyclic dipeptides **2** and **5** are volatile, which made it possible to obtain their EI spectra along with FAB mass spectra. Since diketopiperazines **2** and **5** are homologs, they were identified and their structures were studied using compound **2** as the example. Exact masses were measured and molecular formulas were determined for the molecular and main fragmentation ions.

The FAB mass spectrum of cyclodipeptide **2** contains intense peaks of ions *m/z* 226 ($[M+H]^+$). The EI mass spectra of compounds **2** and **5** are close to the FAB spectra by the main fragmentation directions. The exact mass of the molecular ions of cyclodipeptide **2** (225.1114) corresponds to the molecular formula $C_{10}H_{15}N_3O_3$ and degree of unsaturation $R = 5$.

The analysis of the spectra of metastable ions (scanning technique of accelerating voltage) and the data of high-resolution mass spectrometry suggest that the main primary processes of fragmentation of molecular ions of compound **2** include the elimination of $NH_3$, $NH_2CHO$, and $^{\cdot}CH_2CONH_2$ species, which are fragments of the lateral chain (Scheme 4). In other words, the main routes of fragmentation are related to shortening of the lateral chain, the fragmentation of which results in the formation of ions with *m/z* 167. The further fragmentation of these ions occurs with opening of the six-membered cycle *via* the directions typical of 2,5-diketopiperazines.[9] One of these directions results in the formation of ions *m/z* 70, which appear in the mass spectrum as a peak with the maximum intensity. The exact mass of these ions corresponds to the molecular formula $C_4H_8N$, which indicates the presence of the pyrrolidone cycle (fragment of proline) in the molecule and suggests that the presence of the peak of ions *m/z* 70

in the spectrum is the analytical property of the latter, *i.e.*, the mass spectrometric data agree entirely with the structure presented above for cyclic dipeptide **2** and its homolog **5**.

**Scheme 4**




Thus, the study of the mass spectra of linear and cyclic analogs of thyroliberin confirmed the structure of the compounds obtained, the main directions of fragmentation of their molecular ions were determined, and the peaks of ions, which are the analytical properties of the residues of pyroglutamic acid and proline, were revealed in the mass spectrum.

The signals in the $^1H$ and $^{13}C$ NMR spectra were assigned on the basis of the published data[10] and using methods of double homonuclear resonance and comparison of spectra of related compounds.

It follows from the NMR data that the analog of thyroliberin [Gln$^2$]TRH in a solution of DMSO-$d_6$ has two preferred conformations: with the *trans*-peptide Gln—Pro bond (population of the conformation 80%) and *cis*-peptide Gln—Pro bond (population of the conformation 20%). A similar isomerism of the peptide His—Pro bond is observed for TRH in DMSO-$d_6$, and only 6% molecules are in the conformation with the *cis*-configuration of the bond.[11]

In the $^1H$ NMR spectra of compounds **2** and **5** in solutions of DMSO-$d_6$, the signals of δ-H of the Pro residue are shifted upfield compared to the corresponding signals of linear tripeptides **1** and **4** (Table 1). The signals of the other protons of $CH_2$ are superimposed and appear as a complex multiplet at 1.7—2.4 ppm. These protons give separated signals in the spectrum of prolylglutaminyldiketopiperazine **2** in pyridine due to magnetic anisotropy of the pyridine ring. The signals were assigned by double homonuclear resonance.

The $^{13}C$ NMR spectrum of compound **2** contains three signals in the region of carbonyl groups (Table 2). The signal from the carbon nucleus of the α-CO group of the Gln residue is considerably shifted upfield as compared to the signals of these nuclei in compound **1**. This

**Table 1.** Chemical shifts (δ) of signals of protons of peptidomimetics and linear peptides studied (solutions in DMSO-$d_6$)

| Compound | Amino acid residue | $NH_2$ | NH | α-H | β-H | γ-H | δ-H |
|---|---|---|---|---|---|---|---|
| 1 | Glp | — | 7.86 | 4.11 | 1.82—1.90; 2.23—2.33 | 2.10—2.15 | — |
| 1 | Gln | 7.38; 6.85 | 8.28 | 4.51 | 1.69—1.80; 1.90—2.05 | 2.15—2.20 | — |
| 1 | Pro | 7.40[a]; 6.96[a]; 7.60[b]; 7.28[b] | — | 4.26 | 1.75—1.85; 2.00—2.10 | 1.85—2.00 | 3.65 |
| 2 | Pro | — | — | 4.10 | 1.8—2.0; 2.1—2.4 | 1.8—2.0 | 3.35—3.50 |
| 2 | Gln | 7.33; 6.86 | 8.20 | 4.21 | 1.7—2.0; 1.9—2.1 | 2.1—2.4 | — |
| 2 | Pro[c] | — | — | 4.11 | 2.00—2.15; 2.15—2.30 | 1.5—1.7 | 3.35—3.55 |
| 2 | Gln[c] | 8.54; 7.90 | 9.41 | 4.25 | 2.45—2.62; 2.62—2.70 | 2.87 | — |
| 3 | Glp | — | 8.17 | 4.05 | 1.8—2.3 | 1.8—2.3 | — |
| 3 | Pro | 7.30; 6.82 | — | 4.17 | 1.8—2.3 | 1.8—2.3 | 3.37 |
| 4 | Glp | — | 7.86 | 4.08 | 1.85—2.00; 2.20—2.30 | 2.0—2.2 | — |
| 4 | Asn | 7.26; 7.03 | 8.38 | 4.81 | 2.70; 2.45 | — | — |
| 4 | Pro | 7.66; 7.09 | — | 4.22 | 1.8—2.0; 2.0—2.2 | 1.8—2.0 | 3.70 |
| 5 | Pro | — | — | 4.26 | 1.8—2.0; 2.1—2.2 | 1.8—2.0 | 3.35—3.50 |
| 5 | Asn | 7.48; 6.98 | 8.04 | 4.52 | 2.77; 2.36 | — | — |

[a] *trans*-Configuration.
[b] *cis*-Configuration.
[c] Solution in pyridine.

**Table 2.** Chemical shifts of signals of carbon nuclei (δ) of some peptides (solutions in DMSO-$d_6$)

| Compound | Amino acid residue | α-CO | γ-CO | α-C | β-C | γ-C | δ-C |
|---|---|---|---|---|---|---|---|
| 1 | Glp | 172.18 | 177.26 | 55.25 | 24.81 | 29.03 | — |
| 1 | Gln | 173.53 | 173.83 | 49.94 | 27.05 | 30.90 | — |
| 1 | Pro | 169.68 | — | 59.28 | 29.03 | 24.29 | 46.62 |
| 2 | Pro | 169.87 | — | 58.38 | 27.46 | 22.12 | 44.64 |
| 2 | Gln | 165.68 | 174.13 | 54.05 | 24.55 | 30.63 | — |

shift can be explained by conjugation of the unshared electron pair of the N atom of the Pro residue with π-electrons of this carbonyl groups. A substantial electron density redistribution on the carbon nuclei of proline in compound **2** is also indicated by the upfield shift of their signals in the $^{13}C$ NMR spectrum as compared to the corresponding signals of compound **1**, and the maximum effect is observed for γ-C nuclei of the Pro residue.

Antidepressant activity was studied by the Porsolt "forced swimming" test.[12] Following Borsini's recommendations,[13] which were formulated on the basis of the results shown in this test for the known antidepressants, we should consider a compound to be efficient if the time of immobilization decreases by at least 20% with respect to the standard. Using this criterion, we can state that compounds **2**, **4**, and **5** possess antidepressant activity in different doses depending on the structure (Table 3).

The introduction of the obligatory replacement favors an increase in selectivity of the action: tripeptide **1** exhibits no antidepressant properties (see Table 3), but retains the antiamnesial activity of thyroliberin.[7]

Among the compounds studied, cyclodipeptide **2** exhibits maximum antidepressant properties. The pronounced effect of this compound and the short time of its achievement (10 min), which is 3—5 times faster (by this test) than the corresponding value for antidepressants used in clinical practice, indicate that the further study of prolylglutaminyldiketopiperazine **2** as a rapidly acting antidepressant is promising. When glutamine in compound **2** is replaced by asparagine (diketopiperazine **5**), some decrease in the biological activity is observed. At the same time, cyclodipeptides **2** and **5** possess a higher antidepressant activity than the corresponding linear tripeptides **1** and **4** (see Table 3).

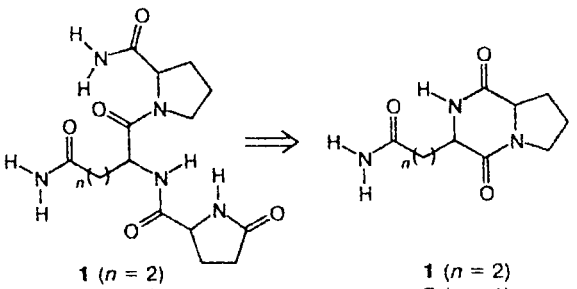
**Table 3.** Antidepressant activity of synthesized compounds according to the "forced swimming" test

| Compound | Immobilization time (%) at $C$/mg kg$^{-1}$ | | | |
|---|---|---|---|---|
| | 1.0 | 0.1 | 0.01 | 0.001 |
| TRH | 82±7(10) | 80±10(9) | — | — |
| 1 | 128±7(17)[a] | 114±8(17) | 132±7(8)[a] | 95±18(8) |
| 2 | 57±9(7)[b] | 75±12(7) | 109±18(7) | 81±11(7) |
| 3 | 135±11(8)[c] | 132±13(9)[c] | — | — |
| 4 | 80±12(11) | 99±16(10) | — | — |
| 5 | 76±8(10)[d] | 93±8(9) | — | — |

*Note.* The (M±m) data are presented in % with respect to the standards accepted as zero. The number of rats in the group is indicated in parentheses. Confidence of the difference between the standard and experiment according to the Student t-criterion. [a] Confidence increase $-p < 0.01$. [b] Confidence decrease $-p < 0.01$. [c] Confidence increase $-p < 0.05$. [d] Confident decrease $-p < 0.05$.

Analyzing the data obtained, we can assume that the disappearance of the antidepressant activity of [Gln$^2$]TRH is related to a decrease (to 80%, compared to 94% for TRH) of the population of conformations of Glp—Gln—Pro—$NH_2$ molecules with the *trans*-configuration of the Gln—Pro bond. In fact, diketopiperazine **2** mimics the fragment of the conformation of tripeptide **1** with the *trans*-Gln—Pro bond (Scheme 5). Diketopiperazine **5** can also be considered to mimic the corresponding fragment of tripeptide **4** with the *trans*-Asn—Pro bond.

**Scheme 5**



**1** ($n$ = 2)
**4** ($n$ = 1)

**1** ($n$ = 2)
**5** ($n$ = 1)

Thus, the conformation with the *trans*-X—Pro bond is the biologically active conformation responsible for the antidepressant properties of linear analogs of the TRH, and the higher antidepressant activity of cyclodipeptides compared to that of linear ones may be due to the advantage of the conformational restriction.

## Experimental

EI mass spectra were obtained on an MX-1321 mass spectrometer with an ionizing voltage of 70 V (temperature of the ion source 200 °C) using the system of direct introduction of samples. Exact masses were measured with a resolution ability of 8000 using perfluorokerosene as the standard.

FAB mass spectra were recorded on an MI 1201-E mass spectrometer with bombardment of a glycerol solution of samples with argon atoms with an energy of 5 keV.

$^1H$ and $^{13}C$ NMR spectra were obtained on a Bruker AM-250 spectrometer attached to an Aspect-3000 computer in the regime of Fourier-transformation with a working frequency of 250.13 and 62.896 MHz, respectively. Stabilization was performed on deuterium nuclei of DMSO-$d_6$ used as the solvent. $^1H$ NMR spectra of 0.05 *M* solutions of thyroliberin and its analogs in DMSO-$d_6$ were recorded at 30 °C. Chemical shifts of signals in the spectra were measured relative to the signal of the solvent and recalculated to the TMS scale with an error ±0.005 ppm. $^{13}C$ NMR spectra were obtained with a broad-band suppression of spin-spin coupling with protons. Chemical shifts were calculated from the signal of the solvent (39.6 ppm in the TMS scale) with an absolute error ±0.01 ppm.

Chemical purity of the compounds obtained was controlled by TLC on Silufol (Kavalier) and Kieselgel F-254 (Merck) plates in the systems of solvents (A): chloroform—ethyl acetate—methanol—acetic acid, 9 : 3 : 2 : 1; (B): butanol—acetic acid—water, 4 : 1 : 1, and by HPLC on a DuPont 8800 liquid chromatograph using a Zorbax C8 analytical column and a methanol—water (4 : 1) mixture as the mobile phase. Compounds on chromatographic plates were determined by chlorotoluidine and ninhydrin reagents. Amino acids were analyzed on an amino-acid AAA 339 Mikrotechna analyzer (Prague, Czech Republic) with preliminary hydrolysis of peptides in 6 *N* HCl at 110 °C for 24 h. The data of amino-acid analysis corresponded to the amino-acid composition of peptides. Specific rotation of substances was determined on a Perkin—Elmer 241 MC spectropolarimeter. *L*-Amino acids and their derivatives available from Merck (Switzerland) and Reanal (Hungary) were used for the synthesis of peptides.

Tripeptides **1** and **4** were obtained previously.[14] The structures of the side reaction products, cyclodipeptides **2** and **5**, formed due to lactamization were refined and the formation of peptide **3** was proved by mass spectrometry and $^1H$ and $^{13}C$ NMR spectroscopy. A 4 *N* solution (10 mL) of HCl in dioxane was added to Boc—Gln—Pro—$NH_2$[14] (2.74 g, 8 mmol). Half an hour after, the solvent was evaporated, the residue was dissolved in DMF (20 mL), and $Et_3N$ (1.12 mL, 8 mmol) and GlpOPfp (2.35 g, 8 mmol) were added. One day after, the mixture was filtered off, DMF was evaporated *in vacuo*, and the residue was purified by flash chromatography using ethyl acetate—methanol (9 : 1) and (2 : 1). The following compounds **1**, **2**, **3** were successively isolated.

**3-(1,4-Dioxooctahydropyrrolo[1,2-*a*]pyrazin-3-yl)propanamide (2)** (1.15 g, 60%), m.p. 238—241 °C, $R_f$ 0.25 (B), $[\alpha]^{20}_{578}$ −122.5° (*c* 1, $H_2O$). MS, *m/z*, EA: 225 $[M^+]$. Found (%): C, 52.99; H, 6.31; N, 18.83. $C_{10}H_{15}N_3O_3$. Calculated (%): C, 53.32; H, 6.71; N, 18.65.

**Pyroglutamylprolylamide (3)** (0.23 g, 12%), m.p. 240 °C (decomp.), $R_f$ 0.24 (A), $[\alpha]^{20}_{578}$ −68.2° (*c* 0.5, MeOH). MS, *m/z*, FAB: 226 $[M+H]^+$. Found (%): C, 53.01; H, 6.80; N, 18.39. $C_{10}H_{15}N_3O_3$. Calculated (%): C, 53.32; H, 6.71; N, 18.65.

**Pyroglutamylglutaminylprolylamide (1)** (0.45 g, 16%), m.p. 145 °C (decomp.), $R_f$ 0.18 (B), $[\alpha]^{20}_{578}$ −56.3° (*c* 1, MeOH). MS, *m/z*, FAB: 354 $[M+H]^+$. Found (%): C, 50.71; H, 6.37; N, 19.98. $C_{15}H_{23}N_5O_5$. Calculated (%): C, 50.99; H, 6.56; N, 19.82.

The following compounds **4** and **5** were obtained similarly from Boc—Asn—Pro—$NH_2$ and Glp—OPfp:

**Pyroglutamylasparaginylprolylamide (4)**, yield 25%, hygr., $R_f$ 0.12 (B), $[\alpha]^{20}_{578}$ −51.6° (*c* 1, MeOH). MS, *m/z*, FAB: 340 $[M+H]^+$. Found (%): C, 49.27; H, 5.82; N, 20.83. $C_{14}H_{21}N_5O_5$. Calculated (%): C, 49.55; H, 6.24; N, 20.64.

**2-(1,4-Dioxooctahydropyrrolo[1,2-*a*]pyrazin-3-yl)ethanamide (5)**, yield 50%, m.p. 205—207 °C, $R_f$ 0.29 (B), $[\alpha]^{20}_{578}$ −89.6° (*c* 1, MeOH). MS, *m/z*, EI: 211 $[M^+]$. Found (%): C, 50.95; H, 5.87; N, 19.75. $C_9H_{13}N_3O_3$. Calculated (%): C, 51.18; H, 6.16; N, 19.89.

Substances were tested on white mongrel rats with a weight of 140—230 g using intraperitoneal injections. In all cases, the introduction was performed 10 min before the beginning of the experiment. Thyroliberin was used as the standard.

Antidepressant activity was studied by the "forced swimming" test[12] in the following modification. The experimental animals were placed in a cylinder 30 cm in diameter and 50 cm high. The cylinder was filled with water up to 2/3 high (temperature of the water 19—21 °C). The activity of the compound was estimated by the time of poses of immobilization of the animals in water. The animals were observed for 5 min, and recording began immediately after they were placed in the cylinder.

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