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SYNTHESIS OF PYROGLUTAMYLASPARAGINE AMIDE DIPEPTIDE FRAGMENT OF VASOPRESSIN, AND THE STEREOSELECTIVITY OF ITS MNEMIC EFFECT

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Four diastereomers of pGlu-Asn-NH_2 were synthesized. They were considered on one hand as a peptide analogue of piracetam, and on the other as an N-terminal fragment of the major metabolite of vasopressin [$pGlu^4$, Cyt^6] AVP(4 – 9). The influence of these diastereomers on the memory of rats was studied in the passive avoidance conditioned reflex test. It was shown that L-pGlu-L-Asn-NH_2 and D-pGlu-D-Asn-NH_2 in the same doses facilitate the training of rats; D-pGlu-D-Asn-NH_2 is inactive, and L-pGlu-D-Asn-NH_2 has amnestic activity. These facts suggest a receptor mechanism for the action of the dipeptide pGlu-Asn-NH_2 and are the basis for a hypothesis for the topology of the nootropic receptor binding site.

We previously advanced a hypothesis [1] that the brain has special nootropic receptors controlling training and memory processes. We also postulated that the well known synthetic nootropic agent 1-carbamoylmethyl-2-pyrrolidone (Nootropil, piracetam) is an exogenic ligand of the receptors, while its structural analogs of the peptide type with an N-terminal pyrrolidone-containing (pyroglutamic) amino acid are endogenic ligands.

In this connection, we designed and synthesized a number of pyroglutamyl dipeptides that exhibited nootropic activity in an experiment [1, 2, 10]. We obtained chemical and pharmacological evidence for a receptor mechanism for the action of these peptides. First, they exhibit activity in small doses (1 – 0.01 mg / kg intraperitoneally) that are from 100 to 100,000 times lower than for a dose of piracetam itself. Second, the dipeptides display high physiological specificity. They act only on training and memory without affecting motor activity or exhibiting other kinds of physiological activity. Third, minor structural changes in the nootropic dipeptides result in inversion of the nootropic effect, i.e., in the appearance of amnesic activity instead of mnemic activity. Fourth, the nootropic peptides act on the memory stereoselectively, the effect depending on the configuration of the α-carbon atom of the pyroglutamic acid [1].

We believe all this indicates that nootropic dipeptides function by a receptor mechanism and are close in structure to the endogenic ligands of what are assumed to be nootropic receptors.

The nootropic dipeptide pyroglutamylasparagine amde has attracted special attention . Even in a dose of 0.01 mg / kg, it facilitates passive avoidance in rats and increases the amplitude of the transcallosal response [2]. The dipeptide is the most probable candidate for the role of an endogenic ligand of the nootropic receptors, first of all because it is identical to the N-terminal fragment of the major metabolite of vasopressin [$pGlu^4$, Cyt^6]AVP(4 – 9). The nonapeptide vasopressin regulates both peripheral and central functions [9]. The regulating role of vasopressin in the central nervous system is also exhibited in its influence on training and memory [5]. In the proteolysis of vasopressin, the hexapeptide pGlu-Asn-Cyt-Pro-Arg-Glu-NH_2 forms the major metabolite. It has a more powerful and selective action on the memory than vasopressin itself [6, 7].

Kovacs, et al. [11] consider vasopressin to be the precursor of a number of short peptides acting specifically on various phases of training and memory. Although several such peptides were discovered, namely, [$pGlu^4$, Cyt^6]AVP(4 – 9), [$pGlu^4$, Cyt^6]AVP(4 – 8), [Cyt^6]AVP(5 – 8), a short di- or tripeptide with an N-terminal pyroglutamic acid has not been found to date. Moreover, some authors [15] negate the importance of the pyroglutamic acid residue for the appearance of mnemic activity in the vasopressin metabolites because detachment of this residue diminishes the mnemic activity insignificantly.

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Our data on the mnemic activity of the dipeptide pyroglutamylasparagine amide point definitely to the important role of pyroglutamic acid as an N-terminal fragment of the major metabolite of vasopressin in regulating training and memory. They also point to the existence of a special subtype of vasopressin receptors binding precisely to the N-terminal fragment of the major metabolite. We cannot exclude the possibility of the existence of short endogenic peptide regulators of training and memory containing the sequence pGlu-Asn as the N-terminal fragment. Pyroglutamylasparagine amide can also be such a peptide.

In this work, we studied the stereospecificity of the mnemic effect of a pyroglutamylasparaginеamide.

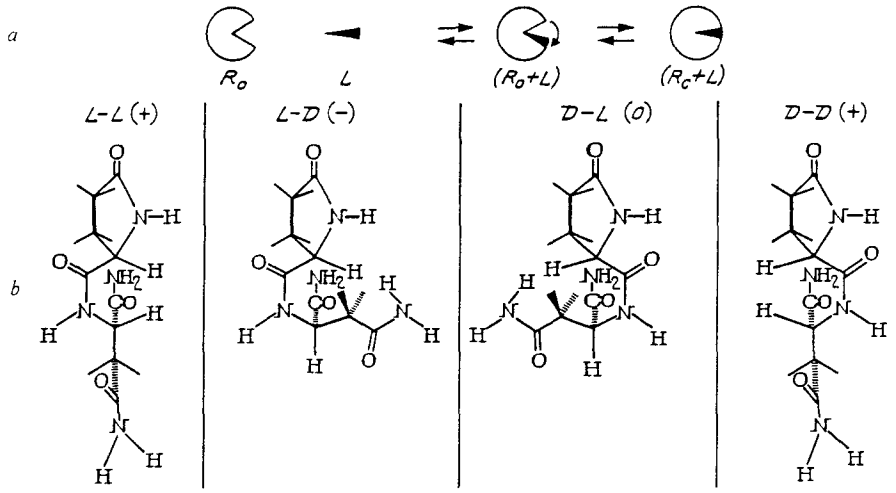

Fig. 1. Reaction of diastereomers pGlu-Asn-NH_2 with a presumed nootropic receptor: *a*) two-stage formation of a ligand receptor complex, *b*) presumed conformations of diastereomers of pyroglutamylasparaginеamide in a complex with a receptor

Its diastereomers were synthesized by the activated esters method from the pentachlorophenyl ester of pyroglutamic acid and asparagine amide in DMF:

$$\text{Z-Asn-OH} + \text{DCC} \cdot 3\text{Pcp-OH} \rightarrow [\text{Z-Asn-OPcp}] \xrightarrow{NH_3}$$

$$\text{Z-Asn-NH}_2 \xrightarrow{H_2Pd} \text{Asn-NH}_2$$

$$\text{Asn-NH}_2 + \text{pGlu-OPcp} \rightarrow \text{pGlu-Asn-NH}_2,$$

where DCC is dicyclohexylcarbodiimide, Pcp-OH is pentachlorophenol, and Z is benzyloxycarbonyl.

The high diastereomeric purity of the substance (over 98%) was confirmed by PMR (250 MHz). Synthesis of the dipeptide pGlu-Asn-NH_2 by the same activated esters method, but starting from $Asp(OEt)_2$ with subsequent ammonolysis [2] leads to a mixture of diastereomers with respect to the second amino acid in the ratio 60 : 40 (PMR 250 MHz).

The study of how the synthesized diastereomers affects training and memory in the passive avoidance conditioned reflex test revealed that the diastereomer L-pGlu-L-Asn-NH_2 hving the natural configuration, in a dose of 0.1 mg/kg intraperitioneally improves the training of the animals by 44% of the maximum possible effect. The diastereomer L-pGlu-D-pGlu-L-Asn-NH_2 is not active. We were surprised that the diastereomer D-pGlu-D-Asn-NH_2 exhibited the same activity as L-pGlu0L-Asn-NH_2 (see Table 1). These facts can be explained as follows (see Fig. 1*a* and *b*). The active receptor center is a "slot" in a protein molecule [14]. A ligand reacts with the receptor in two stages (see Fig. 1*a*). In the first stage, the ligand is sorbed on one side of the receptor slot that is in an open conformation (R_o + L). In the second stage, a conformational transition occurs in the receptor molecule that is accompanied by shutting of the slot. This leads to a definite biological response. The ligand molecule is now in a complex with the receptor in a closed conformation (R_c + L) and reacts with both sides of the slot.

The possible conformations of the diastereomer of pyroglutamylasparagine amide with a cisoid peptide bond [3] are shown in Fig. 1*b*. The conformation of the inactive diastereomer D-Asn-NH_2 differs from the conformation of the active L-pGlu-L-Asn-NH_2 in the arrangement of the side radical of the asparagine residue relative to the pyroglutamic acid residue and the C-terminal amide group (in Fig. 1*b* this radical is directed to the left of the vertical axis of the molecule). We can assume that it creates steric hindrance for binding with a receptor in an open conformation R_o. At the same time, in L-pGlu-L-Asn-NH_2, the side asparagine radical is directed to the right of the molecular axis and does not hinder the formation of a primary complex with a receptor. It creates steric

TABLE 1. Mnemic Activity of Pyroglutamyldipeptides in Passive Avoidance Conditioned Reflex Test

Compound	Dose, mg / kg	Activity A_t, %	Authenticity, p_t
L-pGlu-L-Asn-NH_2	0.1	44.0	0.01
D-pGlu-L-Asn-NH_2	0.1	0.2	Not authentic
L-pGlu-D-Asn-NH_2	0.1	−36.0	0.01
D-pGlu-D-Asn-NH_2	0.1	49.0	0.05
L-pGlu-Glu-NH_2	0.5	33.0 [1]	0.01
D-pGlu-Glu-NH_2	0.5	−39.0 [1]	0.05
L-pGlu-D-Ala-NH_2	1.0	51.0 [1]	0.025

Note: A_t is the activity according to the average time spent by the animals in the dark compartment (t), p_t is the authenticity of the effect according to the parameter *t* calculated by the Wilcoxon – Mann – Whitney method; the minus sign indicates amnesic activity.

hindrance in the stage of slot closing. This explains its amnestic activity. In D-pGlu-D-Asn-NH_2, the side radical of asparagine is directed along the axis of the molecule as in L-pGlu-L-Asn-NH_2. The distinctions between the conformations of these two diastereomers relating to the position of the peptide bond are apparently not important for reaction with the receptor.

This reasoning also holds for a transoid peptide bond because here also the active diastereomers can form molecules with a linear arrangement of the above radicals, whereas the inactive diastereomer and the diastereomer with amnestic activity produce only bent conformers, of course with a fixed arrangement of the N- and C-terminal groups.

It was shown earlier that the very simple peptide analog of piracetam L-pGlu-Glu-NH_2 has mnemic activity. Replacement of the glycine residue with D-Ala does not change the sign of the effect. Indeed, both the molecule L-pGlu-Glu-NH_2 and the molecule L-pGlu-D-Ala-NH_2 can be combined with the molecules L-pGlu-L-Asn-NH_2 and D-pGlu-D-Asn-NH_2, affecting animal training positively. Consequently, the dipeptide pyroglutamylasparagineamide acts on the memory stereospecifically, and its activity depends on the conformation of the molecule due to the configuration of the α-carbon atoms of both amino acids. Our results undoubtedly suggest a receptor mechanism for the action of this dipeptide.

As a whole, our experimental results (i) are a definite argument supporting the existence of specific receptors that control the memory and react with the N-terminal dipeptide fragment of the major metabolite of vasopressin, and (ii) suggest that pyroglutamylasparagine amide is an endogenic ligand of nootropic receptors.

CHEMICAL EXPERIMENTAL PART

We determined the melting point in open capillaries and did not correct it. We registered the PMR spectra on an AC-250 spectrometer (Bruker, Germany) in solutions of DMSO-d_6, using TMS as an internal standard. The specific optical rotation was measured on a Perkin-Elmer 214 polarimeter. Thin-layer chromatography was performed on DC-Alufolien kieselgel $60F_{254}$ plates, and the spots were developed in iodine vapor. Elemental analysis of all the compounds confirmed their composition. The solvents used were purified and dried by standard techniques.

N-Carbobenzoxy-D-asparagine (Z-D-Asn-OH). To a suspension of 6.6 g of D-asparagine and 4.3 g of magnesium oxide in 50 ml of water, we slowly added 17 ml of benzylchloroformate (a 50% solution in toluene) with cooling (0 °C) and stirring. We stirred the mixture for one hour at 20 °C, acidified it to a weakly acidic reaction (using universal litmus paper) and filtered off the precipitate. We washed it with water and then ether, and then recrystallized it from methanol. The yield was 7 g of a product melting at 166 – 168 °C; $[\alpha]_D^{20}$ – 8.1° (c, 2% in CH_3COOH).

N-Carbobenzoxy-L-asparagine is obtained in a similar way. It melts at 165 – 166 °C; $[\alpha]_D^{20}$ – 7.9° (c, 2% in CH_3COOH). Published data [4]: m. p. 165 °C; $[\alpha]_D^{20}$ + 7.6° (c, 1.6% in CH_3COOH).

N-Carbobenzoxy-D-asparagine amide (Z-D-Asn-NH_2). To a solution of 5 g (5 mmole) of the complex DCC•3Pcp-OH [12] in 10 ml of dry DMF we added 1.33 g (5 mmole) of Z-D-Asn-OH and left it overnight at 20 °C. The next day, we filtered off the precipitate of dicyclohexylcarbamide and passed dry NH_3 (gas) through the filtrate with cooling (0 °C) for 15 min. We then let it stand for three hours at 20 °C. We filtered off the precipitate, washed it with a small amount of DMF and ether, and obtained 0.3 g of a white crystalline substance melting at 232 – 233 °C. We added dry ether to the mother liquor and additionally separated 0.4 g of Z-D-Asn-NH_2 melting at 230 – 233 °C. The total yield was 0.7 g (53%).

N-Carbobenzoxy-L-asparagine amide is obtained in a similar manner. It melts at 214 – 215 °C; Published data [13], 208 – 214 °C.

D-Asparagine amide (D-Asn-NH_2). We passed hydrogen through a suspension of 400 mg of Z-D-Asn-NH_2 and 1 g of 10% Pd/C in 20 ml of methanol with stirring. After two hours, we filtered off the catalyst, evaporated the solvent, and obtained 200 mg of a white crystalline substance melting at 136 – 137 °C (from ethanol), $[\alpha]_D^{20}$ – 12.8° (c, 0.1% in DMF), R_f 0.18 (kieselgel, butanol–acetic acid-water, 4 : 1 : 1).

L-Asn-NH_2 is produced similarly. It melts at 138 °C, $[\alpha]_D^{20}$ +14.0° (c, 0.1% in DMF). Literature data [13]: m. p. 136 – 138 °C (from ethanol), $[\alpha]_D^{20}$ +13.4°.

L-Pyroglutamyl-L-asparagibne amide (L-pGlu-L-Asn-NH_2). We left a mixture of 260 ml (2 mmole) of L-asparagine amide and 750 mg (2 mmole) of the pentachlorophenyl ester of L-pyroglutamic acid [2] in 15 ml of DMF overnight at 20 °C. We evaporated the solvent under vacuum, washed the solid residue with hot ethanol, then with ether, and obtained 350 mg (73%) of a product melting at 229 °C (with decomposition), R_f 0.5 (kieselgel, dioxane–water, 10:1), R_f 0.31 (kieselgel, butanol–acetic acid–water, 4 : 1 : 1), $[\alpha]_D^{20}$ –14.0° (c, 1% in water). PMR spectrum in DMSO-d_6, δ, ppm: 1.77 – 2.35 (m, $C^{\beta}H_2C^{\gamma}H_2$, pGlu, 4H), 2.3 – 2.5 (m, $C^{\beta}H_2$Asn, 2H), 4.02 (d.d, $C^{\alpha}H$ pGlu, 1H), 4.47 (d. t, $C^{\alpha}H$ Asn, 1H), 6.91, 7.12, 7.26, 7.36 (each s, NH_2, 4H) 7.81 (s, NH pGlu, 1H), 8.11 (d, NH Asn, 1H).

D-Pyroglutamyl-D-asparagine amide (D-pGlu-D-Asn-NH_2) is obtained in a similar manner. It melts at 229 – 230 °c, $[\alpha]_D^{20}$ + 14.3° (c, 1% in water). The PMR spectrum is similar to that of the enantiomer pGlu-L-Asn-NH_2.

D-Pyroglutamyl-L-asparagine amide (D-pGlu-L-Asn-NH_2). We left a suspension of 80 mg (0.61 mmole) of L-asparagine amide and 229 mg (0.61 mmole) of the pentachlorophenyl ester of D-puroglutamic acid in 10 ml of DMF overnight at 20 °C. In two hours after the reaction begins, the reaction mixture transforms into a transparent solution, and a precipitate forms on the next day. We filter off the precipitate, wash it with ethanol and ether, and obtain 50 mg of a product melting at 232 °C. The total yield is 104 mg (71%).

R_f 0.20 (kieselgel, butanol-acetic acid-water, 3 : 1 : 1), $[\alpha]_D^{20}$ – 5.6° (c, 1% in water). PMR spectrum in DMSO-d_6, δ, ppm: 1.76 – 2.30 (m, $C^{\beta}H_2C^{\gamma}H_2$ pGlu, 4H), 2.3 – 2.5 (m, $C^{\beta}H_2$Asn, 2H close to the solvent signal), 4.04 (d.d, $C^{\alpha}H$ pGlu, 1H), 4.47 (m, $C^{\alpha}H$ Asn, 1H), 6.90, 7.12, 2.34 (each s, NH_2, 4H), 7.77 (s, NHpGlu, 1H), 8.13 (d, NH Asn, 1H).

L-Pyroglutamyl-D-asparagine amide (L-pGlu-D-Asn-NH_2) is obtained in a similar fashion. It melts at 245 °C (with decomposition), $[\alpha]_D^{20}$ + 5.3 (c, 1% in water). The PMR spectrum is identical to that of D-pGlu-L-Asn-NH_2.

Ammonolysis of the diethyl ether of L-pyroglutamyl-L-aspartic acid. We left a solution of 1.2 g (4 mmole) of L-pGlu-L-Asp $(OEt)_2$ [2] in 40 ml of methanol saturated with gaseous ammonia overnight at 20 °C. We filtered off the white precipitate, washed it with methanol and ether, and dried it in a dessicator over P_2O_5. We obtained 0.8 g (83%) of pyroglutamylasparagine amide melting at 213 – 214 °C, $[\alpha]_D^{20}$ – 8.0 ° (c, 2% in water). PMR spectrum in DMSO-d_6, δ, ppm: 1.76 – 2.35 (m, $C^{\beta}H_2C^{\gamma}H_2$ pGlu, 4H), 2.3 – 2.5 (m, $C^{\beta}H_2$ Asn, 2H), 4.04 (d.d, $C^{\alpha}H$ pGlu, 60%, 1H), 4.04 (d.d, $C^{\alpha}H$ pGlu, 40%, 1H), 4.47 (m, $C^{\alpha}H$ Asn, 1H), 6.90, 6.91, 7.12, 7.24, 7.26, 7.34, 7.36 (each s, NH_2, 4H), 7.81 (s, NH pGlu, 60% 1H), 7.77 (s, NH pGlu, 40% 1H), 8.11 (d, NH Asn, 60% 1H), 8.13 (d, NH Asn, 40% 1H).

PHARMACOLOGICAL EXPERIMENTAL PART

The influence of the preparations on training and memory was studied on a modified model of the passive avoidance conditioned reflex in rats [8].

We studied mongrel males with a mass of 180 – 200 g. The preparation being tested (0.2 ml per 100 g of mass) was injected intraperitoneally 15 min before training. Animals of the control group received an injection of a 0.9% NaCl solution. Next a rat was put into the light compartment of a two-sectional chamber. After 180 sec, a painful electrical shock was applied to the rat in the dark compartment through the floor. It consisted of five consecutive a. c. shocks (50 V, 1 sec each, 2 sec between shocks). Retention of the passive avoidance conditioned reflex was determined in 24 h. For this purpose, the animal was put in the light compartment, and the total time it spent in the dark compartment during 180 sec was measured. The change in the training of the rats under the influence of the preparations was determined by the formula

$$A_t = \frac{t_{test} - t_{con}}{t_{test}},$$

where A_t is the activity, t_{test} is the average time spent by the test animals in the dark compartment, and t_{con} is the same for the control animals.

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