SYNTHESIS AND BIOLOGICAL ACTIVITY OF C-TERMINAL FRAGMENTS OF VASOPRESSIN

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In a study of vasopressin proteolysis in cerebral synaptic membranes several linear fragments of this hormone which contain a cysteine residue were discovered [5]. It turned out that the peptides of the 4-8 and 4-9 sequence that contain a disulfide bond exhibit a very high level of neurotropic activity which exceeds by hundreds of times the activity of the whole hormone in tests involving a conditional passive avoidance reflex [6]. It is recognized that behavioral activity is also manifested by other fragments of vasopressin's amino acid sequence, including the C-terminal tripeptide, the cyclic dipeptide c(PR), and the six-member cyclic peptide with a disulfide bond.

In order to find new compounds exhibiting neurotropic activity among the metabolites of vasopressin we synthesized the peptides of its C-terminal sequence that contain a cysteine residue, i.e., the amide Cys-Pro-Arg-Gly (CPRGa) and Cys-Pro-Arg (CPR).

In designing our pattern for synthesizing these compounds we tried to use a minimum number of protective groups. When such groups were essential, such as in the blocking of cysteine's sulfhydryl function and N^{α}-amino groups, we employed protective groups which were easily removed under comparably moderate conditions. This enabled us to unblock peptides at any stage of synthesis.

The C-terminal fragments of vasopressin were synthesized without the use of N^{ω}-protected derivatives of arginine. The guanidine group at all stages of the synthesis was protected by protonation. The dipeptide Boc-Proc-Arg (Boc-PR) was obtained by adding the succinamide ester of tert-butyloxycarbonylproline (Boc-P-OSu) to arginine whose guanidine group was protected by forming an internal salt with the carboxyl. The amide Boc-Pro-Arg-Gly (Boc-PRGa) was obtained by the condensation of this dipeptide with glycylamide. The protonation of the guanidine group here was provided by a proton exchange with the amino group of glycylamide HCl which takes place as a result of the significant difference in the basicity of these groups. Dicyclohexylcarbodiimide (DCHC) with an addition of oxybenzotriazole (OBT) was used as the condensing agent. The high level of the tripeptide Boc-PRGa at this stage significantly simplifies the separation of the chromatographically uniform product.

The use of DCHC and OBT as the base in the condensation method was also employed in the subsequent enlargement of the peptide chain. The sulfhydryl group of cysteine was blocked through a trityl protection which has a number of doubtless advantages over other S-protective groups inasmuch as detritylation takes place under comparatively mild conditions. In addition, the trityl group can be combined with other protective groups which can be selectively split off.

The protected tetrapeptide N,S-di-Trit-CPRGa was obtained by condensing dityrityl cysteine with PRGa. The presence of hydrophilic and hydrophobic terminals in this tripeptide results in its low solubility both in organic solvents and water which significantly simplifies purification of the compound since admixtures of the unreacted starting compounds are easily removed by washings.

The desglycylamide analog of the C-terminal tetrapeptide, the tripeptide CPR, was obtained by the addition of the succinimide ester N^{α}-tert-butyloxycarbonyl-S-tritylcysteine to Pro-Arg (PR) followed by the unblocking of the protected tripeptide.

The protective groups were completely removed by treating the peptide solutions in methylene chloride with a 10 N aq. solution of HCl. The unblocked peptide solutions were

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TABLE 1. Effect of CPRGa on Haloperidol-Disrupted Active Avoidance at a Dose of 0.01 mg/kg

Group of animals	Number of animals in a group	Number of completed conditional reflexes as a ratio of the total number of combinations on the experiment days	
		1st day	2nd day
Control	15	0,32	0,65
Administration of haloperidol, 0.6 mg/kg	16	0,26	0,49*
Administration of haloperidol and peptide	16	0,28	0,61**

Note. Reliable difference from the control group: *) at $P < 0.05$, **) at $P < 0.01$ (in comparison to haloperidol).

neutralized by an Amberlite IRA-743 ion exchange resin in the OH^- form. After the aqueous solutions were evaporated and reprecipitated from methanol with ether, the unblocked cysteine-containing peptides of the vasopressin sequence were obtained.

EXPERIMENTAL (CHEMICAL)

Amino acid derivatives manufactured by the Reanal firm (Hungary) were used for synthesis. The melting points were determined in open capillary tubes (recorded without evaporation). The solutions were concentrated on a rotary vacuum vaporizer at a residual pressure of 10-15 mm Hg and a temperature which did not exceed 40°C. The uniformity of the resultant compounds was checked by TLC on Silufol plates (Czechoslovakia), employing the following systems: n-butanol—acetic acid—water, 4:1:1 (A); chloroform—methanol—25% aq. ammonia, 60:45:15 (B); chloroform—methanol—25% aq. ammonia—acetic acid, 60:45:15:3 (C); n-butanol—pyridine—acetic acid—water, 15:10:3:6 (D). Substances were detected on chromatograms with the aid of a chlorobenzidine probe and Kirby—Berry reagent. The specific rotation of the tested compounds was measured on a J-20 Jasco spectropolarimeter (Japan).

N^{α}-tertbutyloxycarbonyl-prolyl-arginine (Boc-PR). A solution of 4.36 g (14 mmole) of Boc-P-OSu in 9 ml of dioxane was added to a solution of 1.74 g (10 mmole) of arginine in 8.5 ml of water. The mixture was then stirred for 3 h at 20°C and evaporated. A 10 ml portion of dimethylformamide (DMFA) was added to the residue after which this mixture was stirred for 3 h at 20°C, and cooled to 5°C. The precipitate was separated by filtration, washed on a cold water filter, and dried on a vacuum dessicator until a constant weight was obtained. Yield of Boc-PR was 2.98 g (88%), mp 154-155°C, $[\alpha]_D^{20}$ -49° (s 1, methanol), R_f 0.38 (A), 0.71 (B).

N^{α}-tertbutyloxycarbonyl-prolyl-arginyl-glycylamide chlorohydrate (Boc-PRGa•HCl). A mixture of 0.74 g (2 mmole) of Boc-PR and 0.22 g (2 mmole) of glycylamide chlorohydrate in 4.5 ml of DMFA was stirred for 2 h at 20°C, then cooled to —5°C after which 0.3 g (2.2 mmole) of OBT and 0.43 g of DCHC was added to the mixture which was then stirred for 1 h at —2° to —5°C and 6 h at 20°C. The precipitate of the dicyclohexylurea (DCHU) was filtered and washed with 1 ml of DMFA. A 20 ml portion of ether was added to the combined dimethylformamide solution and after the precipitate was condensed the solvent layer was decanted and the precipitate was reprecipitated from methanol with ether after which it was filtered off and dried over P_2O_5. Yield of Boc-PRGa•HCl was 0.86 g (92.5%), mp 85-87°C, $[\alpha]_D^{20}$ -51° (s 1, methanol), R_f 0.39 (C), 0.51 (D).

N,S-dityrityl-cysteyl-prolyl-arginyl-glycylamide (di-Trit-CPRGa). a) Prolyl-arginyl-glycylamide Dichlorohydrate (PRGa•2HCl). A 10 ml portion of 2.30 N HCl solution in ethyl acetate (EA) was added to a suspension of 1.18 g (2.5 mmole) of Boc-PRGa•HCl in 4 ml of EA. After the mixture was stirred at 20°C for 40 min 10 ml of ether was added and the precipitate

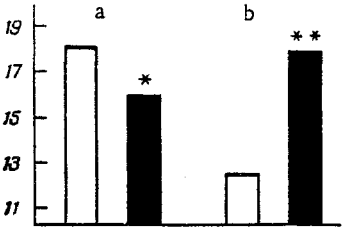

Fig. 1. Effect of CPRGa on orientation-search response components when administered at a dose of 0.01 mg/kg 1 hr prior to the placement of the animals into a mink chamber. a — Vertical activity; b — number of inspected apertures. Clear column represents control and the black column represents the peptide. *) $P < 0.05$, **) $P < 0.01$.

was separated by filtration, washed with EA and ether, and vacuum-dried over P_2O_5. The yield was 1.17 g (96%) of chromatographically uniform PRGa•2HCl in the form of a solvate with 1 molecule of EA.

b) N,S-dityrityl-cysteyl-prolyl-arginyl-glycylamide (di-Trit-CPRGa). A solution of 0.49 g (1 mmole) of PRGa•2HCl•EA and 0.13 ml (1.2 mmole) of N-methylmorpholine cooled to 5°C in DMFA was stirred for 10 min after which 0.66 g (1.1 mmole) of ditritylcysteine, 0.16 g (1.2 mmole) of OBT, and 0.23 g (1.1 mmole) of DCHC was added. The mixture was stirred for 1 h at 3-5°C and for 6 h at 20°C, The DCHU precipitate was separated and 20 ml of ether was added to the solution. After the precipitate was condensed the solvent layer was decanted and the precipitate was washed with EA, vacuum-dried, transferred to a filter and washed with cold water. After drying over P_2O_5 the yield of di-Trit-CPRGa was 0.71 g (78%), mp 196-197°C, $[\alpha]_D^{20}$ +33° (s 1, methanol), R_f 0.45 (A) 0.64 (B).

Cysteyl-prolyl-arginyl-glycylamide Chlorohydrate. A 1.2 ml portion of 10 N HCl solution was added to a solution of 0.091 g (0.1 mmole) of di-Trit-CPRGa in 5 ml of methylene chloride. After stirring the mixture for 50 min it was diluted with 4 ml of water, transferred to a separatory funnel and the aqueous and organic layers were separated. The aqueous layer was neutralized by the addition of Amberlite IRA-743 (OH^-) to pH 2.0. After the anionite was separated the solution was evaporated and the dry residue in the form of a white powder was reprecipitated from methanol with ether. The yield of CPRGa•HCl was 0.04 g (82%). $[\alpha]_D^{20}$ −42° (s 1, methanol), R_f 0.26 (C), 0.31 (D).

N^{α}-tertbutyloxycarbonyl,S-trityl-cysteyl-prolyl-arginine (Boc-Trit-CRP). a) Prolyl-arginine (PR). A suspension of 0.37 g (1 mmole) of Boc-PR in 2 ml of EA and 5 ml of a 2.3 N HCl solution in EA was stirred for 40 min after which the precipitate was separated by filtration and washed with EA. The PR•2HCl vacuum dried product was dissolved in water and placed into an Amberlite IRA-743 (OH^-) column and the resin was washed with 40 ml of water. The aqueous solution was evaporated and the residue was vacuum dried. The yield was 0.21 g (77%) of chromatographically uniform PR.

b) N^{α}-tertbutyloxycarbonyl,S-trityl-cysteyl-prolyl-arginine [Boc-C(Trit)PR]. A 0.42 g (0.75 mmole) portion of N^{α}-tertbutyloxycarbonyl, S-tritylcysteine succinimide was added to a solution of 0.14 g (0.5 mmole) of PR in 3 ml of DMFA. The reaction mixture was stirred for 14 h at 20°C, diluted with 30 ml of ether, and the resultant precipitate was filtered off. After its reprecipitation from methanol and a 1:1 mixture of EA—ether, the yield of Boc-C(Trit)PR was 0.21 g (59%). $[\alpha]_D^{20}$ +16° (s 0.5, methanol), R_f 0.49 (A), 0.55 (B).

Cysteyl-prolyl-arginine chlorohydrate (CPR•HCl). Boc-C(Trit)PR was unblocked by the method described for CPRGa•HCl. A yield of 0.051 g (69%) of CPR•HCl was obtained from 0.143 g (0.2 mmole) of Boc-C(Trit)PR. $[L]_D^{20}$ −55° (s 0.5, methanol), R_f 0.20 (A), 0.25 (D).

EXPERIMENTAL (BIOLOGICAL)

The effect of both synthesized peptides was tested on behavioral responses of rats. The experiments employed nonpedigree male white rats weighing 150-200 g.

Three methods were used to evaluate the behavioral effects of CPRGa: 1) motor activity was measured in an Opto-Varimex instrument (USA), 2) orientation-search response in a mink chamber was examined, and 3) the development of the active avoidance reflex (AAR). The peptide was administered ip in an aqueous solution at a dose of 1 ml/kg.

Motor activity was measured by injecting the test substance immediately before placing the animal into the instrument chamber and recording the readings for 30 min. This enabled us to establish the horizontal and vertical components of motor activity. The mink chamber constituted a cube shaped box with 40 cm sides and plexiglas walls. Thirteen holes 25 mm in diameter were drilled into the chamber's wooden floor. The animal remained in the chamber for 3 min. During that time we recorded the number of times the animals inspected the holes and the number of groomings.

The active avoidance reflex was designed by a previously described method [3] for two consecutive days on which 15 combinations were presented each day. The peptide was administered the first day immediately after a learning session since the vasopressin had a pronounced effect on eliciting a conditional reflex during that period [4]. The reliability of differences between the groups was judged by employing the χ^2 criterion.

DISCUSSION OF RESULTS

The motor activity of the animals was not affected by the peptide CPRGa at a dose of 0.01 mg/kg. The differences between the readings of the experimental and control groups (10 animals in each group) were statistically unreliable.

However, one hour after the peptide at the same dose was administered we observed a significant and reliable increase in search activity (see Fig. 1) which was manifested by a significant and reliable increase in the number of times the holes were inspected concomitant with a small but significant reduction in vertical activity. The peptide did not alter grooming behavior. When the compound was administered at one-tenth of the original dose there were no observable changes in behavior.

CPRGa did not elicit the AAR in intact animals, but was effective when that reflex was experimentally disrupted. Such disruptions were induced by the administration of haloperidol at a dose of 0.6 mg/kg, administered immediately after a learning session. Moreover, the number of completed responses on the test day decreased reliably (see Table 1). The conditional reflex was normalized when the peptide was administered simultaneously with the haloperidol.

We also examined the effect of the tripeptide CPR on search behavior by employing the mink chamber method. In contrast to the action of vasopressin [1] neither search nor vertical activity was reliably altered by the administration of the peptide at a dose of 0.01 mg/kg 1 h before the start of testing, nor was grooming affected by the peptide.

The results of our study show that the fragment CPRGa partially retains the physiological activity characteristic of vasopressin, i.e., it exhibits the typical hormone action of increasing search activity [1] and enhances the active avoidance reflex [4]. However, the effectiveness of the fragment was significantly lower than in the whole hormone since the fragment required a dose which in molar terms was 20 times greater than the effective dose of vasopressin that is required to bring about search behavior. Moreover, even at that dose the fragment did not elicit the active avoidance reflex in the intact animals. Such an effect necessitated a disruption of the learning process. As was demonstrated previously [3], the effectiveness of vasopressin analogs increases in such a case.

It is characteristic that CPRGa does not induce the lowered motor activity that is typically produced by vasopressin in spite of the fact that the dose we used in our experiments was four times greater in molar terms than the dose required for the entire hormone for this effect [2]. In view of the fact that the hypodynamics is apparently due to the peripheral action of vasopressin, one might assume that the fragment does not possess its hormonal activity.

Apparently the tripeptide CPR either lacks any behavioral effects or exhibits an extremely low level of such influence since it did not elicit the characteristic increase in search behavior when it was administered at a dose which was 30 times greater than the effective dose of vasopressin.

In consideration of the fact that the CPRGa fragment lacks the negative effect on motor activity that is characteristic of vasopressin, but yet retains its effect on exploratory activity and the ability to correct learning disruption induced by haloperidol, its continued study for possible medical applications would seem warranted.

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PSYCHOTROPIC PROPERTIES OF AZA-15-CROWN-5 DERIVATIVES WITH PHARMACOPHORIC GROUPS

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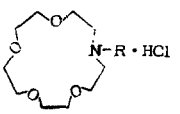
The pharmacological properties of diaza-18-crown-6 derivatives that contain amino acid residues as pharmacophoric groups have been previously synthesized and studied [3]. It was shown that these compounds have a broad spectrum of psychotropic activity that includes nootropic, anticonvulsant, and tranquilizing effects [3, 5]. However, their high toxicity has excluded their practical application. At the same time, with respect to their antiamnesiac and antihypoxic activity, derivatives of azacrown ethers surpassed piracetam, which is a nootropic agent that is widely used in clinical practice.

In order to search for new less toxic derivatives of azacrown ethers we synthesized aza-15-crown-5 (I), N-(β-alanyl)aza-15-crown-5 (II), N-(γ-aminobutyrylglycyl)aza-15-crown-5 (III), and N-(ε-aminohexanoyl)aza-15-crown-5 (IV) hydrochlorides and studied their psychotropic properties.



R = H (I), $CO(CH_2)_2NH_2$ (II), $COCH_2NHCO(CH_2)_3NH_2$ (III), $CO(CH_2)_3NH_2$ (IV).

EXPERIMENTAL (CHEMICAL)

The IR spectra were recorded with a Perkin–Elmer 580 B spectrometer (USA). The PMR spectra were obtained with a Tesla BS 467 spectrometer (Czechoslovakian SSR) with an operating frequency of 60 MHz with tetramethylsilane as the internal standard.

Aza-15-crown-5 Hydrochloride (I). A stream of dry HCl was passed with stirring and cooling through a solution of 2.2 g of aza-15-crown-5 [4] in absolute ether until the mixture was saturated. The resulting precipitate was removed by filtration, washed with absolute ether, and dried to give 2.4 g (94%) of I with mp 69-70°C. PMR spectrum ($CDCl_3$, δ, ppm): 2.75 t, 3.55 t, 7.1 s.

N-(β-Alanyl)aza-15-crown-5 Hydrochloride (II). A 2.25 g (0.011 mole) sample of dicyclohexylcarbodiimide was added with cooling (with ice water) and vigorous stirring to a solution of 2.5 g (0.011 mole) of N-carbobenzoxy-β-alanine and 2.2 g (0.01 mole) of aza-15-

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