SYNTHESIS, BIOLOGICAL ACTIVITY AND RECEPTOR BINDING AFFINITY OF SIX NEW ANTAGONISTS OF [8-L-ARGININE]-VASOPRESSIN

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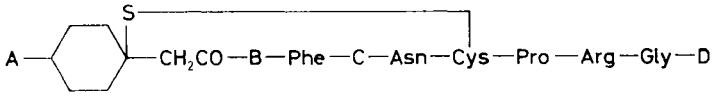
Six analogues of $d(CH_2)_5AVP$, a vasopressin inhibitor, modified in positions 1, 2, 4, and 9 were synthesized and the effect of the modifications on the inhibitory potency was followed. Bulky and lipophilic substitutions in position 1 in combination with Abu substitution in position 4 led to a slight decrease of antivasopressor potency and a strong decrease of the antiuterotonic potency. Alkylation of any type of carboxamide group at positions 4 and 9 strongly reduced the biological potency in all the tests.

Incorporation of 1-mercaptocyclohexaneacetic acid into position 1 of arginine-vasopressin (AVP)* alone or in combination with O-alkylation of the tyrosine in position 2 resulted in formation of potent and selective antagonists of the vasopressor response[2-4]. Compounds described in the present communication were prepared with the aim to investigate the influence of (i) bulky and lipophilic substitution in the cyclohexane ring in combination with 4-α-aminobutyric acid substitution and (ii) of the alkylation of the carboxamide group in position 4 and 9 upon the biological activity of some potent vasopressor inhibitors described formerly.

The following compounds have been prepared: [1-(4-tert-butyl-1-mercaptocyclohexaneacetic acid), 4-α-aminobutyric acid, 8-arginine]vasopressin (*I*); [1-(1-mercapto-4-phenylcyclohexaneacetic acid), 4-α-aminobutyric acid, 8-arginine]vasopressin (*II*); [1-(1-mercaptocyclohexaneacetic acid), 2-O-methyltyrosine, 4-glutamic acid dibutylamide, 8-arginine, 9-glycine dibutylamide]vasopressin (*III*); [1-(1-mercaptocyclohexaneacetic acid), 2-O-methyltyrosine, 4-glutamic acid monoethylamide, 8-arginine,

* Unless stated otherwise, all chiral amino acids belong to the L-series. The nomenclature and symbols of the amino acids, their derivatives and peptides obey the published IUPAC recommendations[1]. Abu, α-aminobutyric acid; CA, 1-mercaptocyclohexaneacetic acid; BCA, 4-tert-butyl-CA; PhCA, 4-phenyl-CA; $d(CH_2)_5AVP$ denotes [1-(1-mercaptocyclohexaneacetic acid), [8-arginine]vasopressin (shortened also as [Cpp^1]AVP (ref.[2])).

9-glycine monoethylamide]vasopressin (*IV*); [1-(1-mercaptocyclohexaneacetic acid), 2-O-methyltyrosine, 4-glutamic acid diethylamide, 8-arginine, 9-glycine diethylamide]vasopressin (*V*) (ref.[5]); [1-(1-mercaptocyclohexaneacetic acid), 2-O-ethyl-D-tyrosine, 4-glutamic acid diethylamide, 8-arginine]vasopressin (*VI*).



I, A = $(CH_3)_3C$; B = Tyr ; C = Abu ; D = NH_2
II, A = Ph ; B = Tyr ; C = Abu ; D = NH_2
III, A = H ; B = Tyr(Me) ; C = Glu(NBu_2) ; D = NBu_2
IV, A = H ; B = Tyr(Me) ; C = Glu(NHEt) ; D = NHEt
V, A = H ; B = Tyr(Me) ; C = Glu(NEt_2) ; D = NEt_2
VI, A = H ; B = D - Tyr(Et) ; C = Glu(NEt_2) ; D = NH_2

Biological potency of the six new analogues was determined in uterotonic, galactogogic and pressor tests and the values obtained are presented in Table I. Combination of large and lipophilic substitutients in position 1 and α-aminobutyric acid in position 4 leads to analogues with slightly decreased antiuressoric potency in comparison to the d(CH_2)$_5$AVP, however this modification strongly decreases the antiuterotonic potency (almost by two orders of magnitude) and thus improves the selectivity of the antagonist. Alkylation of any type of carboxamide group at positions 4 and 9 of the potent and selective pressor antagonist strongly reduces the biological potency in all tests.

TABLE I

Biological activities of the vasopressin analogues and their inhibition constants for displacement of [^{3}H]AVP binding to rat liver plasma membranes

Peptide	Antiuterotonic		Galactogogic IU/mg	Antivasopressor pA_2	K_i 10^{-7} mol l^{-1}
	in vitro pA_2	in vivo IU/mg			
I	6·7	0	0	8·04	3·9
II	6·3	0·45	0·7	8·00	6·0
III	5·5	n.d.	0	5·90	+
IV	6·0	0	0	0	4·9
V	6·0	n.d.	0·05	6·50	2·7
VI	6·8	n.d.	0	6·60	4·2

n.d., not determined; 0, inactive up to 2 . 10^{-2} mg per dose; +, did not displace up to concentration 10^{-4} mol l^{-1}.

All the studied modifications decrease also the analogue affinity to the rat liver plasma membrane. As can be seen from Table I, their K_i is by two orders of magnitude lower than that of AVP ($3{\cdot}2 \cdot 10^{-9}$ mol l^{-1}). Large substituents in positions 4 and 9 cause complete loss of the affinity to the receptor (analogue *III*).

EXPERIMENTAL

N,N'-Dimethylformamide (DMF) was distilled under reduced pressure, triethylamine (NEt_3) was distilled from ninhydrin. Other solvents and reagents were of analytical grade. Thin layer chromatography was carried out on silica gel plates (Merck), and the spots were visualized by ninhydrin or iodine. The following solvent systems were used: A, 1-butanol–acetic acid–water (4 : 1 : 5, v/v, upper phase); B, chloroform–methanol (7 : 3, v/v), C, 1-butanol–acetic acid–water–pyridine (15 : 3 : 3 : 19, v/v). All analogues were characterized by HPLC (Varian 5 500). A reverse phase column (μ-Bondapak C-18, 3·9 × 250 mm, Waters) was used. The mobile phases for isocratic elution were 25, 30, and 39% acetonitrile in 0·1% trifluoroacetic acid (TFA). Each of the analogues gave a single peak. The purity of all analogues was 98 to 99% as determined from the integrated areas recorded at 223 nm. For the amino acid analysis, the peptides (0·5 mg) were hydrolyzed with constantly boiling hydrochloric acid (400 μl) containing phenol (20 μl), in evacuated sealed ampoules for 18 h at 100°C. The analyses were performed on a Mikrotechna type AAA 881 analyser. Optical rotations were measured with a Hilger–Watts polarimeter with an accuracy of 0·01°. The m.p. values are uncorrected. Elemental analyses were determined on a Carlo–Erba Model 1 106 analyzer.

Synthesis of the peptides: The protected peptide intermediates required for the synthesis of analogues *I*—*VI* were prepared by solid phase method[6,7] for peptide synthesis. First, chloromethylated resin (Bio-Rad, Bio-Beads S × 1, 0·75 mmol Cl/g) was esterified with Boc-Gly to a load of 0·4 mmol/g (ref.[8]). Then BCA(Bzl)-Tyr(Bzl)-Phe-Abu-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-resin (*VII*), PhCA(Bzl)-Tyr(Bzl)-Phe-Abu-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-resin (*VIII*), CA(Bzl)-Tyr(Me)-Phe-Glu(Bzl)-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-resin (*IX*), and CA(Bzl)-D-Tyr(Et)-Phe-Glu(NEt_2)-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-resin (*X*) were prepared using solid phase methodology as described previously[6,7,9]. Coupling reactions were mediated either by DCC or DCC–HOBt method[10]. The completeness of each coupling reaction was monitored by the Kaiser test[11]. 4-Tert-butyl-1-benzyl thiocyclohexaneacetic acid[12], 4-phenyl-1-benzyl thiocyclohexaneacetic acid[12], and 1-benzyl thiocyclohexaneacetic acid[13] were each used in the final coupling steps. After the completion of the synthesis, protected acyl octapeptidyl resins were cleaved from the resin by ammonolysis (*VII*, *VIII*, *X*)[7] or using 20% solution of dibutylamine, ethylamine and diethylamine in methanol, respectively (*IX*, *X*). Following the solvent evaporation, the products were extracted into hot DMF, precipitated with boiling water and left overnight at room temperature. The peptides were collected by filtration, washed with water and dried in vacuo over P_2O_5. The products were purified by dissolving in DMF and reprecipitating with methanol–ethyl ether (1 : 3). The physico-chemical properties of these compounds (*XI*—*XVI*) are summarized in Table II.

[1-(4-Tert-butyl-1-mercaptocyclohexaneacetic acid), 4-α-aminobutyric acid, 8-arginine]vasopressin (*I*)

A solution of the protected acyl octapeptide amide *XI* (227 mg, 0·144 mmol) in sodium dried and redistilled ammonia (400 ml) was treated at boiling point and during stirring with sodium

TABLE II

Physico-chemical data for protected intermediates

Compound		R_F A / B	$[\alpha]_D$ (c = 1, DMF) M.p., °C	Formula (M.w.)	Calculated/Found % C	% H	% N
BCA(Bzl)-Tyr(Bzl)-Phe-Abu-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2	*XI*	0·70	−33·8°	$C_{82}H_{105}N_{13}O_{13}S_3$	62·4	6·7	11·5
		0·81	229–231	(1 577)	62·1	6·9	11·3
PhCA(Bzl)-Tyr(Bzl)-Phe-Abu-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2	*XII*	0·68	−36·7	$C_{84}H_{101}N_{13}O_{13}S_3$	63·2	6·4	11·4
		0·80	220–223	(1 596)	63·1	6·2	11·1
CA(Bzl)-Tyr(Me)-Phe-Glu(NBu_2)-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NBu_2	*XIII*	0·31	−36·7	$C_{89}H_{126}N_{14}O_{14}S_3$	62·4	7·4	11·4
		0·70	154–157	(1 713)	62·6	7·2	11·2
CA(Bzl)-Tyr(Me)-Phe-Glu(NHEt)-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NHEt	*XIV*	0·40	−38·1°	$C_{77}H_{126}N_{14}O_{14}S_3$	59·9	6·7	12·7
		0·69	159–162	(1 568)	60·1	6·4	12·4
CA(Bzl)-Tyr(Me)-Phe-Glu(NEt_2)-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NEt_2	*XV*[a]	0·45	−37·5°	$C_{81}H_{110}N_{14}O_{14}S_3$	60·8	6·9	12·3
		0·72	178–180	(1 600)	60·7	6·7	12·5
CA(Bzl)-D-Tyr(Et)-Phe-Glu(NEt_2)-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2	*XVI*	0·10	−24·1°	$C_{78}H_{110}N_{14}O_{14}S_3$	60·1	6·7	12·6
		0·51	172–176	(1 564)	60·3	6·7	12·3

[a] This compound was previously synthesized by Lammek et al. (ref.[5]).

from a stick of the metal inside a small bore glass tube until light-blue colour persisted in the solution for 30 s. Dry acetic acid (0·4 ml) was added to discharge the colour. The solution was evaporated, the residue dissolved in glacial acetic acid (150 ml) and the solution diluted with methanol (1 000 ml). An excess of solution of J_2 in methanol (0·1 mol/l, 2·1 ml) was added gradually during stirring. The light yellow solution was stirred for additional 2 min and then for 10 min with anion exchange resin (Amberlite IR-45, acetic form, 10 g damp weight). The reaction mixture was filtered through a bed of resin (10 g damp weight). The bed was washed with methanol (100 ml) and combined filtrate and washings were evaporated under reduced pressure. The resulting material was dissolved in 7 ml of aqueous acetic acid (50%) and desalted on a Sephadex G-15 column (120 × 2·9 cm) eluted with aqueous acetic acid (50%) at a flow rate of 6·5 ml/h. The absorbance of the fractions was monitored at 254 nm. Fractions comprising the major peak were pooled and lyophilized, and the residue (70 mg) was subjected further to gel filtration on Sephadex LH-20 column (120 × 1·4 cm) eluted with aqueous acetic acid (30%) at a flow rate of 4·5 ml/h. The peptide was eluted as a single peak. Lyophilization of the pertinent fractions gave the vasopressin analogue *I*. Yield 38 mg (26·3%) (based on the amounts of the protected peptide used in the reduction-reoxidation procedure). Physico-chemical properties of this and of the remaining five peptides *II*–*VI*, which were prepared in the same manner as described for *I*, are given in Table III.

TABLE III

Physico-chemical characteristics of AVP analogues

Peptide	R_F		$[\alpha]_D^{21}$ (c = 0·5, 1M-AcOH)	Amino acid analysis				
	A	B		Tyr Pro	Phe Arg	Glu Gly	Asp NH_3	Cys
I	0·37	0·58	−66·2°	1·02 1·01	1·03 1·04	1·03[a] 1·00	0·98 2·04	0·93
II	0·35	0·60	−68·3°	1·01 1·03	0·99 1·01	1·02[a] 1·00	0·97 2·05	0·92
III	0·30	0·50	−54·2°	1·03 0·99	1·01 1·03	1·03 1·00	0·99 1·05	0·95
IV	0·25	0·38	−50·4°	0·99 1·04	1·04 1·02	1·02 1·00	0·98 1·02	0·94
V[a]	0·20	0·31	−53·1°	1·04 1·03	1·00 1·01	1·02 1·00	1·03 1·03	0·97
VI	0·20	0·40	−62·7°	1·02 1·01	1·02 1·03	0·99 1·00	0·98 2·01	0·95

[a] Abu; [b] this analogue was previously synthesized by Lammek et al. (ref.[5]).

Pharmacological Methods

The uterotonic activity was determined both on an isolated strip of rat uterus[14,15] and on the ethanol anaesthetised rat in vivo[16]. The galactogogic activity was determined also on ethanol anaesthetised rats[17,18], pressor activity on pithed rat preparation[19]. The activity was expressed by the pA_2 value calculated from the determined effective doses[20].

Receptor binding assay: Purified plasma membranes were prepared from male Wistar rats weighing 200—250 g by partitioning in aqueous dextran-polyethylene glycol two-phase system[21]. Membrane preparations thus obtained bound approx. 3—5 pmol [^{3}H]AVP/mg protein. Non-specific binding represented approx. 12% of the total binding at the hormone concentration 1 mol . l^{-1}. Tritiated AVP was prepared by catalytic tritiation from the diiodo-derivative. Synthesis of [2-(3,5-diiodotyrosine),8-arginine]vasopressin was carried out according to ref.[22] with minor modifications. In our study Iodogen was used as a fine aqueous suspension. Reaction products were separated by reversed-phase HPLC and identified by measuring the UV spectra and biological activity. Specific radioactivity of the resulting preparation was 17 Ci/mmol. The K_i values of vasopressin and its analogues were obtained from competitive binding experiments[23]. Briefly, plasma membranes were equilibrated at 30°C with [^{3}H]AVP and varying concentrations of non-labelled peptides. After 20 min the bound and free radioactivity were separated by rapid filtration on Millipore membrane filters. Nonspecific binding of [^{3}H]AVP was determined in the presence of unlabelled vasopressin ($5 . 10^{-5}$ mol l^{-1}). Parameters of binding were determined using a weighted non-linear least-square method[24].

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