SYNTHESIS AND PROPERTIES OF ANALOGUES OF VASOPRESSIN WITH 1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID IN POSITION 9*

Zdenko PROCHÁZKA[a], Juris E. ANCANS[a]**, Jiřina SLANINOVÁ[a], Alena MACHOVÁ[b], Tomislav BARTH[a], Jana ŠKOPKOVÁ[a], Miloš BUDĚŠÍNSKÝ[a], Františka PAVLÍKOVÁ[c] and Michal LEBL[a]

[a] *Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, 166 10 Prague 6,*
[b] *Research Institute for Pharmacy and Biochemistry, 194 09 Prague 9 and*
[c] *Prague Institute of Chemical Technology, 166 28 Prague 6*

Received June 2nd, 1988
Accepted July 23rd, 1988

Dedicated to the memory of Dr Karel Bláha.

Solid phase methodology on benzhydrylamine resin was used for the synthesis of three analogues of vasopressin with non-coded amino acid, 1-aminocyclopropane-1-carboxylic acid, in position 9. Two analogues of lysine-vasopressin ([Lys^8, Acc^9]vasopressin (*I*) and Gly$_3$-[Lys^8, Acc^9]vasopressin (*II*)) and one analogue of arginine-vasopressin ([Arg^8, Acc^9]vasopressin (*III*)) have been synthesized. The dubious value of the biological activity of [Lys^8, D-Ala^9]vasopressin was reevaluated and [Lys^8, L-Ala^9]vasopressin was also synthesized and tested for the comparison. Differences in solution conformation of these two analogues were studied by ^{1}H and ^{13}C NMR spectroscopy. Biological activities of all analogues were either significantly lowered or almost completely eliminated. Analogues *I–III* were found to be completely inactive in analgesia and the CNS activities tested (active and passive avoidance).

During the years of structure–activity studies, the extent of attention devoted to different positions in the peptide chain of vasopressin varied. As far as the position 9 is concerned, even though some analogues modified in this position were among the first which had been prepared, the position 9 was not studied very thoroughly. This was probably due to the low biological activities of the analogues obtained[1–3]. For a long time it was supposed that this position cannot be modified without a loss of peripheral activities. However, lately it was

* Part CCVII in the series Amino Acids and Peptides; Part CCVI: Collect. Czech. Chem. Commun. *53*, 2542 (1988).

** Visiting scientist, Institute of Organic Synthesis, Latvian Academy of Sciences, Riga, USSR.

found that this hypothesis is not correct[4], e.g. analogues of vasopressin* having L- or D-Ala in position 9 display high antidiuretic activity[4,6], though lower than the parent hormones. In the case of [8-arginine]vasopressin the activities are higher than those of [8-lysine]vasopressin. Besides, D-alanine substitution in both LVP and AVP conserves certain pressor activity (about 50 times lower than that of the parent hormone), in the L-alanine substituted analogues the pressor activity is practically zero. Interestingly, the "design"[7] of analogues (described[7] and patented[8], but probably never synthesized[9]) selectively influencing the production of factor VIII was based just on the reported[2] low activity of [Lys[8], D-Ala[9]]VP. This analogue was later synthesized and biologically evaluated either by Gazis[4,6] and by us (see Table I) and found to be antidiuretically rather potent. Since there is no doubt about its structure (verified by ^1H and ^{13}C NMR) and purity (TLC, HPLC), the originally reported

TABLE I
Biological activities (rat, I.U./mg) of vasopressin analogues with the modification in the position 9

Compound	Uterotonic (in vitro)	Galactogogic (in situ)	Pressor	Antidiuretic[a]		Ref.
				S	B	
LVP	5[b]	65[c]	285[b]	260[b]	260[d]	–
[Gly-OH[9]]LVP	4.8	–	1.27[e]	–	–	14
[Gly-NHMe[9]]LVP	0.4	–	1.5	–	–	3
[Gly-NMe_2 [9]]LVP	0.4	–	0.06	–	–	3
[$NHC_2H_4NH_2$ [9]]LVP	0.2	–	0.002	2	–	2
[Ala[9]]LVP	0.08	–	0.05	2	–	2
	–	–	0.1	4.3	–	[c]
[D-Ala[9]]LVP	0.03	–	0.01	0.8	–	2
	–	–	3.7	22	–	6,[c]
[β-Ala[9]]LVP	0.04	–	[e]	–	–	14
[Sar[9]]LVP	0	–	0.45	–	–	1
AVP	17[b]	95[c]	412[b]	465[b]	450[f]	–
[Gly-OH[9]]AVP	–	–	0.3	4.7	–	16
[Ala[9]]AVP	–	–	<0.4	104	–	4,6
[D-Ala[9]]AVP	–	–	7.5	189	–	4,6
I	0.2 (0.11)[g]	0.36	<0.02	2.3	9.8	[c]
II	0.71	<0.02	<0.01	0.06	2	[c]
III	0.71 (0.04)[g]	0.12	<0.01	21	10	[c]

[a] First value determined according to Sawyer (S) (ref.[10]), the second according to Burn (B) (ref.[11]); [b] according to[12]; [c] this paper; [d] ref.[13]; [e] inhibition; [f] ref.[15]; [g] in situ.

* All the chiral amino acids, mentioned in this work, are of the L-series. The nomenclature and symbols of the amino acids and peptides obey the published recommendations [5]: Acc denotes the 1-aminocyclopropane-1-carboxylic acid moiety.

activities must be considered wrong. Regarding these results we decided to synthesize also the analogue containing L-alanine in position 9, originally synthesized together with [Lys^8, D-Ala^9]VP. In this case, the activities found with the newly synthesized analogue are very well comparable with the activities reported earlier[2].

The differences in biological activities of analogs with D- or L-alanine in position 9 prompted us to study the influence of this substitution on the conformation of the cyclic part of the molecule. We have measured ^{1}H and ^{13}C NMR spectra of both analogs in deuterated dimethylsulfoxide (Tables II and III). The comparison of ^{13}C chemical shifts didn't reveal any significant differences between these two compounds. However, in the ^{1}H NMR spectrum the differences in chemical shifts were found for α-CH of Phe (0.09 ppm), β-CH_2 of Phe (0.07 and 0.09 ppm), β-CH_2 of Asn (0.07 ppm) and α-CH of Cys^6 (0.15 ppm). The most important seems to be the difference in magnetic nonequivalence of β-protons of phenylalanine and asparagine and the difference in NH proton chemical shift temperature dependence of these two residues.

The temperature dependence of asparagine NH, which has usually very small or even positive coefficient of temperature dependency[19], is slightly lowered in [Lys^8, L-Ala^9]VP, but is of approximately average value in [Lys^8, D-Ala^9]VP. On the other hand, NH-proton of phenylalanine in [Lys^8, D-Ala^9]VP is significantly shielded from the interaction with the solvent. All these findings support the idea that the substitution of glycine in position nine influences very importantly the conformation of the cyclic region of the molecule, especially the phenylalanine residue which is known to be important for the expression of vasopressin-like activities.

With the growing interest in the central effects of vasopressin (for the review see ref.[20]), the position 9 gained in significance. The application of CNS activities of the neurohypophyseal hormones is complicated by the high peripheral activities. Therefore, analogues with eliminated classical (antidiuretic, pressor) activities and unchanged potential in influencing the CNS are valuable. Rather successful in this direction is des-Gly-$NH_2{}^9$-[Lys^8]vasopressin which displays low peripheral acivities and still retains high degree of central effects. Analogues synthesized up to now with the modification in position 9 are reported to lose their activities, especially the pressor activity. There are no reports about their CNS activities.

In order to study more thoroughly the effect of the changes in position 9, we have synthesized analogues of vasopressin containing a non-coded amino acid, 1-aminocyclopropane-1-carboxylic acid (Acc) in this position. This amino acid was already used in the syntheses of enkephalin[21,22], oxytocin[23], and tuftsin[24] analogues. Acc is achiral as glycine, however, sterically more demanding and it may influence the whole conformation of the C-end of the

vasopressin molecule. The question, how Acc will be recognized by the living organism was also of interest. It is well known that 1-aminocyclopropane-1--carboxylic acid is an intermediate in methionine metabolism to ethylene[25,26] and, moreover, that it is recognized by the appropriate enzyme as an L-amino acid[27].

TABLE II
Proton NMR parameters of [L-Ala^9]LVP and [D-Ala^9]LVP in hexadeuteriodimethylsulfoxide

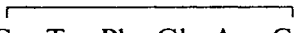
Residue	Parameter	[L-Ala^9]LVP[a]		[D-Ala^9]LVP[a]	
1. Cys	NH_2 ($\Delta\delta NH_2/\Delta T$)	8.27	(−1.9)	8.27	(−0.1)
	αCH	4.04		4.09	
	βCH_2 ($J\alpha,\beta$)	3.05	(7.0)	3.05	(8.4)
		[b]	(5.4)	[b]	(4.7)
2. Tyr	NH ($\Delta\delta NH/\Delta T$)	8.23	(−4.0)	8.21	(−4.7)
	αCH (JN,α)	4.24	(6.3)	4.24	(7.1)
	βCH_2 ($J\alpha,\beta$)	2.92	(10.1)	2.90	(10.3)
		3.19	(5.1)	3.20	(4.8)
	arom.H	6.95, 6.64		6.95, 6.63	
	OH ($\Delta\delta OH/\Delta T$)	9.21	(−4.6)	9.16	(−4.9)
3. Phe	NH ($\Delta\delta NH/\Delta T$)	8.68	(−2.5)	8.89	(−1.1)
	αCH (JN,α)	4.42	(8.1)	4.33	(8.1)
	βCH_2 ($J\alpha,\beta$)	2.62	(9.3)	2.69	(9.1)
		2.84	(5.2)	2.75	(5.5)
	arom.H	7.19−7.31		7.19−7.30	
4. Gln	NH ($\Delta\delta NH/\Delta T$)	8.40	(−2.7)	8.48	(−3.4)
	αCH (JN,α)	3.93	(5.5)	3.92	(5.1)
	βCH_2 ($J\alpha,\beta$)	~1.89	(8.5)	~1.91	(8.2)
		~1.96	(5.0)	~1.91	(5.4)
	γCH_2	~2.16		~2.18	
	$CONH_2$ ($\Delta\delta NH/\Delta T$)	[c]		[c]	
		6.79	(−4.3)	6.78	(−4.7)
5. Asn	NH ($\Delta\delta NH/\Delta T$)	7.94	(−1.7)	8.07	(−3.5)
	αCH (JN,α)	4.54	(8.0)	4.50	(8.1)
	βCH_2 ($J\alpha,\beta$)	~2.63	(6.4; 6.3)	~2.61	(6.3)
				~2.70	(6.3)
	$CONH_2$ ($\Delta\delta NH\ \Delta T$)	7.40	(−3.2)	7.42	(−3.6)
		6.90	(−4.1)	6.91	(−4.7)
6. Cys	NH ($\Delta\delta NH\ \Delta T$)	8.11	(−5.3)	8.17	(−5.4)
	αCH (JN,α)	4.74	(7.6)	4.89	(7.6)
	βCH_2 ($J\alpha,\beta$)	2.98	(7.7)	3.05	(8.0)
		3.15	(5.4)	3.20	(5.1)
7. Pro	αCH ($J\alpha,\beta$)	4.29	(10.6; 3.6)	4.25	(8.7; 3.2)
	βCH_2	~2.02		~2.00	
	γCH_2	~1.83		~1.81	
	δCH_2	~3.62		~3.64	

TABLE II
(Continued)

Residue	Parameter	[L-Ala^9]LVP^a		[D-Ala^9]LVP^a	
8. Lys	NH ($\Delta\delta$NH/ΔT)	8.02	(−3.8)	8.07	(−3.9)
	αCH (JN,α)	4.14	(7.8)	4.19	(8.1)
	βCH$_2$ (Jα,β)	1.60	(10.3)	~1.75	(9.6; 5.1)
		1.72	(4.1)		
	γCH$_2$	~1.36		~1.34	
	δCH$_2$	~1.52		~1.54	
	εCH$_2$	2.77		2.76	
	NH$_2$ ($\Delta\delta$NH$_2$/ΔT)	7.66	(−0.1)	7.69	(−0.1)
9. Xxx	NH ($\Delta\delta$NH/ΔT)	7.65	(−3.8)	7.65	(−2.5)
	αCH (JN,α)	4.16	(7.4)	4.14	(7.6)
	βCH$_3$ (Jα,β)	1.23	(7.1)	1.26	(7.2)
	CONH$_2$ ($\Delta\delta$NH/ΔT)	[c]		[c]	
		6.97	(−3.9)	6.95	(−5.6)

[a] Temperature 319.54 K; referenced to DMSO (δ 2.50); $\Delta\delta$NH/ΔT values were obtained for temperature interval 300.56 K to 319.54 K; ~ indicates the approximate value obtained usually from a cross-peak in 2D-COSY spectrum; [b] signal is overlapped with a strong residual water peak; [c] overlapped by aromatic proton signals.

Three analogues of vasopressin were synthesized containing Acc in position 9. Two of these analogues were derived from [8-lysine]vasopressin-[Lys^8, Acc^9]VP (*I*) and Gly$_3$-[Lys^8, Acc^9]VP (*II*) — and one was derived from [8-arginine]vasopressin-[Arg^8, Acc^9]VP (*III*).



Y-Cys-Tyr-Phe-Gln-Asn-Cys-Pro-X-Acc-NH$_2$

I, X = Lys, Y = H
II, X = Lys, Y = H-Gly-Gly-Gly
III, X = Arg, Y = H

Syntheses of all the three analogues were perfomed by solid phase technique on the benzhydrylamine resin. As an α-amino group protection we have used tert. butyloxycarbonyl group with the exception of Acc where benzyloxycarbonyl group was used. For the side chain protection we have used: 2-bromobenzyloxycarbonyl (Lys), tosyl (Arg), benzyl (Tyr), 4-methylbenzyl

(Cys). Protected amino acids were coupled by N,N'-dicyclohexylcarbodiimide (DCC) and N-hydroxybenzotriazole (HOBt) in dimethylformamide. Benzyloxycarbonyl group was cleaved by hydrogen bromide in acetic acid-dichloromethane mixture. Side chain protecting groups were cleaved simultaneously with the cleavage of the peptide from the resin by liquid

TABLE III
Carbon-13 NMR chemical shifts of [L-Ala^9]LVP, [D-Ala^9]LVP and LVP

Residue	Parameter	[L-Ala^9]LVP DMSO[a]	[D-Ala^9]LVP DMSO[a]	LVP DMSO[b]	LVP D_2O[c]
1. Cys	CO	166.93	166.79	?	168.7
	$C^{\alpha}H$	52.07	52.05	54.3	53.5
	$C^{\beta}H_2$	40.89	41.44	46.7	40.9
2. Tyr	CO	170.93	170.75	?	173.3
	$C^{\alpha}H$	55.46	55.28	56.6	56.5
	$C^{\beta}H_2$	36.96	37.41	38.7	37.6
	arom. C-1	127.49	127.52	129.3	129.0
	C-2,4	130.09	130.01	131.4	131.7
	C-3,5	115.27	115.19	116.5	116.8
	C-6	156.13	156.05	157.4	155.8
3. Phe	CO	171.14	171.05	?	174.0
	$C^{\alpha}H$	55.92	55.97	56.9	56.8
	$C^{\beta}H_2$	36.81	36.66	38.7	37.1
	arom. C-1	137.73	137.70	137.6	137.4
	C-2,4	128.38	128.22	129.7	130.1
	C-3,5	129.28	129.36	130.7	130.3
	C-6	126.59	126.48	127.8	128.5
4. Gln	CO	171.14	171.05	?	174.2
	$C^{\alpha}H$	54.36	54.47	55.8	56.1
	$C^{\beta}H_2$	26.59	26.49	28.9	?
	$C^{\gamma}H_2$	31.54	31.45	33.0	32.2
	$CONH_2$	171.29	171.19	?	?
5. Asn	CO	171.85	171.91	?	173.3
	$C^{\alpha}H$	49.95	50.17	51.5	51.3
	$C^{\beta}H_2$	36.09	35.90	38.7	37.6
	$CONH_2$	174.32	174.36	?	175.6
6. Cys	CO	167.94	168.37	?	170.9
	$C^{\alpha}H$	51.42	51.86	52.6	52.3
	$C^{\beta}H_2$	[d]	40.78	45.8	39.6
7. Pro	CO	172.13	172.13	?	175.4
	$C^{\alpha}H$	60.20	60.57	61.6	61.8
	$C^{\beta}H_2$	29.26	29.19	30.6	30.5
	$C^{\gamma}H_2$	24.60	24.60	26.0	25.9
	$C^{\delta}H_2$	47.01	47.18	48.6	49.1

TABLE III
(Continued)

Residue	Parameter	[L-Ala^9]LVP DMSO[a]	[D-Ala^9]LVP DMSO[a]	LVP DMSO[b]	LVP D_2O[c]
8. Lys	CO	174.30	174.06	?	175.5
	$C^{\alpha}H$	52.70	52.47	54.6	54.9
	$C^{\beta}H_2$	26.48	26.49	28.7	27.4
	$C^{\gamma}H_2$	22.35	22.42	23.9	23.2
	$C^{\delta}H_2$	30.79	30.29	32.4	31.2
	$C^{\varepsilon}H_2$	38.97	38.84	40.0	40.4
9. Xxx	$C^{\alpha}H$	48.16	48.40	43.7	43.2
	$C^{\beta}H_3$	18.45	18.10	–	–
	$CONH_2$	171.88	171.97	?	175.1

[a] Our measurement; referenced to hexadeuteriodimethylsulfoxide (δ 39.7); the assignment of signals is based on LVP data; carbonyl carbons can be interchanged; [b] data taken from ref.[17]; [c] pD 3.9, referenced to external TMS; data taken from ref.[18]; [d] signal is overlapped with strong solvent peak.

hydrogen fluoride. Sulfhydryl groups oxidation was performed by potassium ferricyanide and analogues were purified by HPLC and gel filtration. [L-Ala^9]LVP was prepared in a similar way.

Biological activities of the analogues containing Acc in position 9 are given in the Table I. According to the pressor activity, it may be stated that Acc resembles L-alanine in the position 9, which means an almost complete elimination of the activity. Analogue *I* exhibits also a comparable antidiuretic activity to L-Ala containing analogue. On the other hand, analogue of AVP with Acc in position 9 has a significantly diminished antidiuretic activity in comparison to either L- or D-Ala containing analogues. It should be noted that modification in position 9 exerts quite different influence on the activities depending on the basic amino acid in the position 8. These modifications have much more pronounced (usually activity decreasing) effect in the LVP series than in the AVP one. The influence of the substitutions in other positions of the peptide chain is usually much more similar in both series. Therefore, one can conclude that the stimulation of the appropriate receptor (mainly the V_2) is performed by the side chain of the amino acid in position 8 and the carboxy-terminal amino acid in cooperation.

In the tests for antidiuretic activity on unanaesthetized animals no differences between the LVP and AVP analogues have been found. Analogue with the backbone prolonged by three glycine residues had higher antidiuretic activity in this test than in the test on anaesthetized rats. Oxytocin-like activities of all the compounds were significantly decreased.

The new compounds did not show any analgetic or sedative activity in tail-flick or hot plate tests at doses 20 μg per mouse. Analogs *II* and *III* were tested also in the passive and active avoidance tests. No effect on memory after subcutaneous injection of 3 and 5 μg/kg was observed in rats (SPF Wistar and Long Evans).

In conclusion it may be summarized that the introduction of Acc into the position 9 of vasopressin leads to a selectively acting antidiuretic agonist with low potency and no effect on the CNS activity. Comparison of the influence of Acc substitution on pressor and antidiuretic activities in lysine- and arginine-vasopressin series suggests that the conformational restriction produced by 1-aminocyclopropane-1-carboxylic acid might be the same as that caused by L-alanine.

EXPERIMENTAL

General methods

Thin-layer chromatography (TLC) was carried out on silica gel coated plates (Silufol, Kavalier, Czechoslovakia) in the following systems: 2-butanol 98% formic acid water (10 : 3 : 8) (S1), 1-butanol acetic acid–water–pyridine (15 : 3 : 6 : 10) (S4), 1-butanol acetic acid–water (50 : 15 : 40) (S13). Paper electrophoresis was performed in a moist chamber in 1 M acetic acid (pH 2.4) and in pyridine acetate buffer (pH 5.7) on Whatman 3MM paper at 20 V cm for 60 min. Spots in TLC and electrophoresis were detected with ninhydrin or by chlorination method. Samples for amino acid analyses were hydrolyzed with 6 M HCl at 105°C for 20 h or with a mixture propionic acid hydrochloric acid (1 : 1) at 160°C for 15 min and analyzed on an Amino acid analyzer T 339 (Mikrotechna Praha, Czechoslovakia) or D-500 analyzer (Durrum Corp., U.S.A.). Optical rotations were determined on a Perkin Elmer instrument type 141 MCA (Norwalk, U.S.A.). Fast atom bombardment mass spectra were obtained on a ZAB-EQ spectrometer (VG Analytical Ltd., Manchester) with xenon at 8 kV as the bombarding gas. Proton 1D-NMR spectra, a homonuclear ^1H-^1H correlated 2D COSY spectra and a carbon-13 1D-NMR spectra were measured on a Bruker AM-400 (at 400.13 MHz resp. 100.6 MHz) instrument in hexadeuteriodimethylsulfoxide. High performance liquid chromatography (HPLC) was carried out on an SP-8700 instrument equipped with an SP-8400 detector and SP-4100 integrator (all from Spectra Physics, Santa Clara, U.S.A.). Preparative liquid chromatography was carried out on a Modulprep preparative liquid chromatograph (Jobin Yvon, Longjumeau Cédex, France). As the final purification step, the Biogel P-2 column chromatography (1 × 100 cm) in 1 M acetic acid was used. Chelaton III was added to the sample in order to remove the traces of heavy metals if present. Before use, all amino-acid derivatives were subjected to the ninhydrin test[28].

Solid-phase peptide synthesis

A cycle for incorporation of each amino acid residue into the growing peptide chain consisted of the following steps: 1, washing with dichloromethane (3 × 20 ml, 1 min/wash); 2, cleavage the Boc group by 20 ml of 45% trifluoracetic acid in dichloromethane containing 5% anisole, one treatment for 5 min, a second one for 30 min; 3, washing with dichloromethane (3 × 20 ml, 1 min/wash); 4,

washing with isopropanol (3 × 20 ml, 1 min/wash); 5, washing with dichloromethane (3 × 20 ml, 1 min/wash); 6, neutralizing by 5% diisopropylethylamine in dichloromethane (2 × 20 ml, 2 min/wash); 7, washing with dichloromethane (4 × 20 ml, 1 min/wash); 8, washing with dimethylformamide (3 × 20 ml, 1 min/wash); 9, adding the Boc-protected amino acid derivative in 20 ml of dimethylformamide followed by HOBt, followed by DCCI and stirring for 30 min – 16 h; 10, washing with dimethylformamide (3 × 20 ml, 1 min/wash); 11, washing with dichloromethane (3 × 20 ml, 1 min/wash). Between the steps 9 and 10, several milligrams of the resin were removed and used for the ninhydrin test[28] to determine the progress of the coupling. In the case of the coupling on the imino group of proline the chloranil test[29] was used. The synthesis of [L-Ala^9]LVP was monitored by the bromophenol blue ("BB") method[30].

Nonapeptide-resin (Peptide-resin A)

The benzhydrylamine resin (UCB, 0.6 mmol/g, 0.83 g) was suspended in dichloromethane and was coupled with a three-molar excess of N^{α}-benzyloxycarbonyl-1-aminocyclopropane-1-carboxylic acid[31] in the presence of N-hydroxybenzotriazole and dicyclohexylcarbodiimide in dimethylformamide. The coupling was interrupted after 2 h, the resin was washed consequently by dimethylformamide (3 × 20 ml) and dichloromethane (3 × 20 ml) and free amino groups were acetylated. The acetylation mixture I (5 ml acetanhydride, 2 ml triethylamine in 50 ml dichloromethane) was used at the beginning. Since the acetylation was not complete even after 24 h, acetylation mixture II (4 ml acetanhydride, 6 ml triethylamine in 50 ml dichloromethane) was used and the free amino groups disappeared during two hours (according to the ninhydrin test).

The benzyloxycarbonyl protecting group was cleaved from N^{α}-benzyloxycarbonyl-1-aminocyclopropane-1-carboxy-resin by 35% HBr in acetic acid in the mixture with dichloromethane (1 : 1) during 15 min. The following procedure was performed according to a general scheme (see above - starting from the point 3.). Boc-amino acids were coupled to the resin by the DCCI/HOBt procedure. All reagents were used in three-molar excess and coupling was monitored by the ninhydrin test. The protected derivatives were used in the following order: Boc-Lys(2-Br-Z)-OH[32], Boc-Pro-OH, Boc-Cys(4-Me-Bzl)-OH[33], Boc-Asn-OH, Boc-Gln-OH, Boc-Phe-OH, Boc-Tyr(Bzl)-OH and Boc-Cys(4-Me-Bzl)-OH. The Boc-Tyr(Bzl)-OH was released before use from its dicyclohexylamonium salt. In the case of proline and glutamine coupling, the reaction was not complete even after 20 h, thus one equivalent of a nucleophilic acylation catalyst (4-dimethylaminopyridine) was used. Finally, the Boc-group was cleaved from the nonapeptide-resin, resin was washed and dried. Yield: 0.85g.

[8-Lysine, 9-(1-aminocyclopropane-1-carboxylic acid)]vasopressin (*I*)

The peptide-resin A (0.42g, 0.25 mmol) was treated with liquid hydrogen fluoride (10 ml, 60 min, 0°C) in the presence of thioanisole (1 ml). The unprotected nonapeptide, together with the resin, was triturated with ethyl acetate after the evaporation of hydrogen fluoride, filtered off, washed with ethyl acetate and then free peptide was extracted successively by acetic acid, 50% acetic acid, water and lyophilized. The lyophilizate was dissolved in water (150 ml) and the pH of the solution was adjusted with 0.1 M NaOH to 7.0. Potassium ferricyanide (65 mg in 30 ml of water) was added to this solution in a course of 10 min. During the oxidation (20 min), the pH was maintained at 7.0 by addition of 0.1 M NaOH and then adjusted with acetic acid to 4.5. The solution was applied on a column of Amberlite CG-50I, the column was washed with 0.25% acetic acid and the product eluted with 50% acetic acid. After freeze-drying, the product (52.6 mg) was purified by HPLC (Separon SIX C-18, 7 μm, 25 × 0.8 cm). Elution with methanol–0.05% trifluoroacetic acid mixture (3 : 7) and lyophilization of the corresponding fractions afforded 10.5 mg of the product pure

according to HPLC (k' 5.87; methanol–0.05% trifluoroacetic acid 3 : 7). This product was filtered over the column of Biogel P-2. R_F: 0.00 (S1), 0.33 (S4), 0.36 (S13). $E_{2.4}^{Gly}$ 0.92, $E_{5.7}^{His}$ 0.67. $[\alpha]_D$ −14.5 (*c* 0.12; 1 M acetic acid). Amino acid analysis: Asp 0.99, Glu 0.99, Pro 1.15, Cys 1.15, Tyr 0.91, Phe 0.93, Lys 1.01. The Acc was not determined because of its low colour yield of the ninhydrin reaction (see[31]). For $C_{48}H_{67}N_{13}O_{12}S_2$. 2 TFA . 4 AcOH (1550.5) calculated: 46.48% C, 5.53% H, 11.74% N; found: 46.46% C, 5.45% H, 11.99% N.

N^{α}-Glycyl-glycyl-glycyl-[8-lysine, 9-(1-aminocyclopropane-1-carboxylic acid)]vasopressin (*II*)

The peptide-resin A (0.42 g, 0.25 mmol) was coupled according to the general scheme three times with Boc-Gly-OH. Finally, the Boc-group was cleaved from the dodecapeptide-resin, the resin was washed, dried, and treated with liquid hydrogen fluoride (10 ml, 60 min, 0°C) in the presence of thioanisole (1 ml). The unprotected dodecapeptide, was worked up in the same way as peptide *I*. The lyophilizate (85 mg) was dissolved in water (100 ml) and oxidized as above by potassium ferricyanide (30 mg in 30 ml of water). After the desalting (see above) and freeze-drying, the product (69 mg) was purified by HPLC (Separon SIX C-18, 7 μm, 25 × 0.8 cm). Elution with methanol–0.05% trifluoroacetic acid mixture (3 : 7) and lyophilization of the corresponding fractions afforded 15.6 mg of the product pure according to HPLC (k' 2.83; methanol–0.05% trifluoroacetic acid 36 : 64). This product was filtered through Biogel P-2. R_F: 0.00 (S1), 0.21 (S4), 0.31 (S13). $E_{2.4}^{Gly}$ 0.92, $E_{5.7}^{His}$ 0.65. $[\alpha]_D$ −44.7 (*c* 0.25; 1 M acetic acid). Amino acid analysis: Asp 1.08, Glu 1.05, Pro 1.00, Gly 2.72, Tyr 0.82, Phe 1.00, Lys 1.07. For $C_{54}H_{76}N_{16}O_{15}S_2$.5 TFA . 4.5 H_2O (1904.6) calculated: 40.36% C, 4.76% H, 11.76% N; found: 40.32% C, 4.54% H, 11.76% N.

[8-Arginine, 9-(1-aminocyclopropane-1-carboxylic acid)]vasopressin (*III*)

The benzhydrylamine resin (UCB, 0.6 mmol/g, 1.66 g) was suspended in dichloromethane and after washing with dimethylformamide it was coupled with a three-molar excess of N^{α}-benzyloxycarbonyl-1--aminocyclopropane-1-carboxylic acid[31], N-hydroxybenzotriazole and dicyclohexylcarbodiimide in dimethylformamide. The coupling was interrupted after 2 h, the resin was washed consequently with dimethylformamide (3 × 20 ml) and dichloromethane (3 × 20 ml) and free amino groups were acetylated with the mixture of 4 ml acetanhydride and 6 ml triethylamine in 50 ml dichloromethane. The free amino groups disappeared during 90 min (according to the ninhydrin test).

The benzyloxycarbonyl protecting group was cleaved from N^{α}-benzyloxycarbonyl-1-amino-cyclopropane-1-carboxy-resin by 35% HBr in acetic acid in the mixture with dichloromethane (1 : 1) during 15 min. The following procedure was the same as in the case of analogue *I* with the exception of the first step in which Boc-Arg(Tos)-OH was used instead of lysine derivative. Finally, the Boc-group was cleaved from the nonapeptide-resin, resin was washed and dried (2.2 g).

The nonapeptide-resin (1.1 g) was treated with liquid hydrogen fluoride (15 ml, 60 min, 0°C) in the presence of thioanisole (2 ml). The unprotected nonapeptide after workup (see above, 168 mg) was oxidized by potassium ferricyanide (60 mg in 20 ml of water), desalted on a column of Amberlite CG-50I and the product (93 mg) was purified by HPLC (Separon SIX C-18, 7 μm, 25 × 0.8 cm). Elution with methanol–0.05% trifluoroacetic acid mixture (27.5 : 72.5) and lyophilization of the corresponding fractions afforded 10.5 mg of the product pure according to HPLC (k' 6.75; methanol–0.05% trifluoracetic acid 3 : 7). This product was purified by filtration over the column of Biogel P-2. R_F: 0.00 (S1), 0.40 (S4), 0.42 (S13). $E_{2.4}^{Gly}$ 0.92, $E_{5.7}^{His}$ 0.65. $[\alpha]_D$ −27.5° (*c* 0.42; 1 M acetic acid). Amino acid analysis: Asp 1.11, Glu 1.06, Pro 1.03, Cys 1.95, Tyr 0.92, Phe 0.99, Arg 1.00. For $C_{48}H_{67}N_{15}O_{12}S_2$. 3 TFA . 5.5 H_2O (1551.5) calculated: 41.80% C, 5.26% H, 13.54% N; found 41.72% C, 4.82% H, 13.54% N.

[8-Lysine, 9-L-alanine]vasopressin

The benzhydrylamine resin (UCB, 0.6 mmol g, 0.67 g) was suspended in dichloromethane and after washing with 5% diisopropylethylamine in dichloromethane and with dimethylformamide it was coupled with a two-molar excess of Boc-L-Ala-OH, N-hydroxybenzotriazole and dicyclohexylcarbodiimide in dimethylformamide. The coupling was interrupted after 1.5 h, the resin was washed consequently with dimethylformamide (3 × 20 ml) and dichloromethane (3 × 20 ml) and free amino groups were acetylated with a mixture of 4 ml acetanhydride and 6 ml triethylamine in 50 ml dichloromethane. The free amino groups disappeared during 150 min (according to the ninhydrin test).

The following procedure was performed according to the general scheme (starting from the point 2.). Boc-amino acids were coupled to the resin by the DCCI/HOBt procedure. All reagents were used in a 3 molar excess and coupling was monitored by bromophenol blue method[30]. The protected derivatives were used in the following order: Boc-Lys(2-Br-Z)-OH[32], Boc-Pro-OH, Boc-Cys(4-Me-Bzl)-OH[33], Boc-Asn-OH, Boc-Gln-OH, Boc-Phe-OH, Boc-Tyr(2,6-Cl_2-Bzl)-OH[32] and Boc-Cys(4-Me-Bzl)-OH. The coupling was complete during 30 min, but it was always performed for 2 h. Finally, the Boc-group was cleaved from the nonapeptide-resin, the resin was washed and dried (1.1 g).

The nonapeptide-resin (1.1 g) was treated with liquid hydrogen fluoride (10 ml, 60 min, 0°C) in the presence of thioanisole (1 ml). The unprotected nonapeptide (300 mg) was oxidized by potassium ferricyanide (100 mg in 30 ml of water), desalted on a column of Amberlite CG-50I and the product (162 mg) was purified by HPLC (Vydac 218TP5, 5 μm, 25 × 1.0 cm). Elution by a gradient from 0 to 20% methanol in 0.05% trifluoroacetic acid and lyophilization of the corresponding fractions afforded 40.6 mg (14.6%) of the product pure according to HPLC (k' 6.51; methanol–0.05% trifluoroacetic acid 3 : 7). This product was further purified by filtration over the column of Biogel P-2. R_F: 0.00 (S1), 0.33 (S4). $E_{2,4}^{Gly}$ 0.95, $E_{5,7}^{His}$ 0.64. $[\alpha]_D$ −34.6 (*c* 0.64; 1 M acetic acid). Amino acid analysis: Asp 0.97, Glu 1.12, Pro 1.11, Ala 1.03, Cys 1.89, Tyr 0.88, Phe 0.99, Lys 0.97. For $C_{47}H_{67}N_{13}O_{12}S_2$.3 TFA.4 H_2O (1484.4) calculated: 42.89% C, 5.30% H, 12.27% N; found 43.06% C, 5.09% H, 12.23% N. FAB MS (*m/z*): 1071 ($M + H^+$).

Pharmacological Methods

All pharmacological test were performed using Wistar rats weighing 200–300 g. The uterotonic potency in vitro was evaluated using the Holton procedure[34] in Munsick[35] solution, and in vivo according to ref.[36]. The galactogogic potency in vivo was established according to refs[37,38]. The pressor activity was tested on pithed rat preparation according to refs[39,40]. The antidiuretic potency on anaesthetized rat was followed according to the method in ref.[10]. The activity on unanaesthetized rat was evaluated using the modified Burn's method[11,41]. Male rats (160–200 g), after overnight fasting with free access to water, were applied per os the water load (4 ml/100 g) and, simultaneously, a s.c. injection of the tested compound or a standard preparation was administered. The urine was collected for 5 h and its volume was read every 15 min. The time in minutes ($T^{1/2}$) during which the half of water load was excreted was taken as a measure for the delay of water diuresis. LVP was used as the standard at doses 1.0 a 5.0 mU/100 g in 0.2 ml. Analogues *I* and *III* were applied at doses from 20 to 500 ng/100 g, analogue *II* from 100 to 1250 ng 100 g. Control rats were injected with physiological solution.

The tail flick test and the hot plate test were performed according to refs[42,43]. The tests of passive and active avoidance were performed according to refs[44,45].

The authors are indebted to Mrs V. Holoubková and Mrs I.Hošková for skillful technical assistence in the case of peptide synthesis and pharmacological evaluation, to Mrs H. Farkašová and Mr J. Zbrožek for the amino acid analyses and Mrs Z. Ledvinová for the optical rotation measurements. The elemental analyses were carried out in the Analytical Laboratory of our Institute (Dr J. Horáček, Head).

REFERENCES

1. Meienhofer J., du Vigneaud V.: J. Am. Chem. Soc. *83*, 142 (1961).
2. Zaoral M., Kolc J., Korenczki F., Černeckij V.P., Šorm F.: Collect. Czech. Chem. Commun. *35*, 843 (1967).
3. Glass J.D., du Vigneaud V.: J. Med. Chem. *15*, 486 (1972).
4. Gazis D., Buku A., Schwartz I.L. in: *Peptides 1982. Proc.17th Eur.Pept.Symp.* (K. Bláha and P. Maloň, Eds), p.465. De Gruyter, Berlin 1983.
5. IUPAC – IUB Joint Commission on Biochemical Nomenclature: Eur. J. Biochem. *138*, 9 (1984).
6. Buku A., Gazis D., Schwartz I.L.: Int. J. Pept. Protein Res. *23*, 551 (1984).
7. Cort J.H., Fischman A.J., Dodds W.J., Rand J.H., Schwartz I.L.: Int. J. Pept. Protein Res. *17*, 14 (1981).
8. Cort J.H., Fischman A.J.: Eur. Pat. Appl. 0 037 516 (1981).
9. Chalmers T.C., Schwartz I.L.: Int. J. Pept. Protein Res. *23*, 558 (1984).
10. Pliška V., Rychlík I.: Acta Endocrinol. *54*, 129 (1967).
11. Burn J.H., Finney D.J., Goodwin L.G. in: *Biological Standardization*, 2nd ed, p.187. Oxford University Press, London 1950.
12. *Handbook of Neurohypophyseal Hormone Analogs* (K.Jošt, M.Lebl and F.Brtník, Eds) Vol.II, part 2, p. 134. CRC Press, Boca Raton 1987.
13. Škopová J., Hrbas P., Barth T.: Endocrinol. Exp. *15*, 129 (1981).
14. Dutta A.S., Ananad N., Srimal R.C.: Ind. J. Chem. *7*, 3 (1969).
15. Jošt K., Procházka Z., Cort J.H., Barth T., Škopková J., Prusík Z., Šorm F.: Collect. Czech. Chem. Commun. *39*, 2836 (1974).
16. Manning M., Olma A., Klis W., Kolodziejczyk A., Nawrocka E., Misicka A., Seto J., Sawyer W.H.:Nature *38*, 652 (1984).
17. Smith I.C.P., Deslauriers R., Saito H., Walter R., Gannigou-Lagrange C., McGregor H., Sarantkis D.: Ann. N.Y. Acad. Sci. *222*, 597 (1973).
18. Walter R., Prasad K.V.M., Deslauriers R., Smith I.C.P.: Proc. Natl. Acad. Sci. U.S.A. *70*, 2086 (1973).
19. Hruby V.J., Lebl M. in: *Handbook of Neurohypophyseal Hormone Analogs* (K.Jošt, M.Lebl and F.Brtník, Eds) Vol.I, part 1, p. 105. CRC Press, Boca Raton 1987.
20. Brtník F. in: *Handbook of Neurohypophysed Hormone Analogs* (K.Jošt, M.Lebl and F. Brtník, Eds) Vol.II, part 2, p. 1. CRC Press, Boca Raton 1987.
21. Stewart F.H.C.: Aust.J.Chem. *34*, 2431 (1981).
22. Mazur R.H.: Ger.Offen. DE 3 044 793; Chem. Abstr. *96*, 69 439 (1982).
23. Procházka Z., Lebl M., Barth T., Hlaváček J., Trka A., Buděšinský M., Jošt K: Collect. Czech. Chem. Commun. *49*, 642 (1984).
24. Procházka Z., Veretennikova N.I.: Unpublished results.
25. Adams D.O., Yang S.F.: Proc. Natl. Acad. Sci. U.S.A. *76*, 170 (1979).
26. Lürssen K., Naumann K., Schröder R.: Naturwissenschaften *66*, 264 (1979).
27. Hoffman N.E., Yang S.F., Ichibara A., Sakamura S.: Plant Physiol. *70*, 195 (1982).
28. Kaiser E., Colescott R.L., Bossinger C.D., Cook P.I.: Anal. Biochem. *34*, 595 (1970).

29. Christiensen T.: Acta Chem. Scand. *33*, 736 (1979).
30. Krchňák V., Vágner J., Šafář P., Lebl M.: Collect. Czech. Chem.Commun. *53*, 2542 (1988).
31. Procházka Z., Buděšinský M., Smolíková J., Jošt K., Trška P.: Collect. Czech. Chem. Commun. *47*, 2291 (1982).
32. Yamashiro D., Li C.H.: J. Am. Chem. Soc. *95*, 1310 (1973).
33. Cosand W.L., Merrifield R.B.: Proc. Natl. Acad. Sci. U.S.A. *74*, 2771 (1977).
34. Holton P.: Br. J. Pharmacol. *3*, 328 (1984).
35. Munsick R.A.: Endocrinology *66*, 451 (1960).
36. Pliška V.: Eur. J. Pharmacol. *5*, 253 (1969).
37. Bisset G.W., Clark B.J., Haldar J., Harris M.C., Lewis G.P., Rocha e Silva M.: Br. J. Pharmacol. Chemother. *31*, 537 (1967).
38. Barth T., Jošt K., Rychlík I.: Endocrinol. Exp. *9*, 35 (1975).
39. Shipley R.E., Tilden J.H.: Proc. Soc. Exp. Biol. *64*, 453 (1947).
40. Krejčí I., Kupkova B., Vávra I.: Br. J. Pharmacol. Chemother. *30*, 497 (1967).
41. Vávra I., Machová A., Krejčí I.: J. Pharmacol. Exp. Therap. *188*, 241 (1974).
42. Harris L.S., Pierson A.K.: J. Pharmacol. Exp. Therap. De Wied D.: Psychon. Sci. *29*, 46 (1972).
45. Gaffori O., De Wied D.: Psychoneuroendocrinology *10*, 439 (1985).

Translated by the author (M.L.).