[4-LEUCINE, 8-D-ARGININE]- AND [1-β-MERCAPTOPROPIONIC ACID, 4-LEUCINE, 8-D-ARGININE]-VASOTOCIN. TWO VASOTOCIN ANALOGS WITH SPECIFIC ANTIDIURETIC EFFECT*

M.ZAORAL[a], I.LAINE[b] and F.BRTNÍK[a]

[a]*Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, 166 10 Prague 6*
[b]*Institute of Organic Syntheses, Latvian Academy of Sciences, Riga, USSR*

Received March 6th, 1974

The condensation of tosyl-S-benzylcysteinyl-tyrosyl-isoleucyl-leucyl-asparaginyl-S-benzylcysteine hydrazide (*VIII*) and of β-benzylthiopropionyl-tyrosyl-isoleucyl-leucyl-asparaginyl-S-benzylcysteine hydrazide (*IX*) with prolyl-N^G-tosyl-D-arginyl-glycine amide *via* the azides afforded tosyl-S-benzylcysteinyl-tyrosyl-isoleucyl-leucyl-asparaginyl-S-benzylcysteinyl-prolyl-N^G-tosyl-D-arginyl-glycine amide (*X*) and β-benzylthiopropionyl-tyrosyl-isoleucyl-leucyl-asparaginyl-S-benzylcysteinyl-prolyl-N^G-tosyl-D-arginyl-glycine amide (*XI*). [4-Leucine, 8-D-arginine]-vasotocin (*XII*) and [1-β-mercaptopropionic acid, 4-leucine, 8-D-arginine]-vasotocin (*XIII*) were obtained from *X* and *XI*, respectively, by reduction with sodium in liquid ammonia, oxidation by potassium ferricyanide, desalting on Amberlite CG, and purification by free-flow electrophoresis. Both products showed a very specific antidiuretic effect.

The change of the configuration of the basic amino acid in position 8 of the vasopressin molecule leads[1,2], quite generally, to analogs showing a higher antidiuretic effect than the naturally occurring hormones. The magnitude of the antidiuretic effect of members of the D-series** varies inversely with the length of the side chain of the basic amino acid[3]. The situation here is opposite to that encountered[3] in the L-series[4] (Table I). Similarly to the L-series, the antidiuretic effect can be potentiated also in the D-series[5] by eliminating the amino group of the half-cystine residue in position 1 (Table I). This change, however, has led with [1-β-mercaptopropionic acid, 8-D-lysine]vasopressin*** to a decrease of both the magnitude and the specificity of its renal effect[5]. This was the first case in the vasopressin series where the elimination

* Part CXXIII in the series Amino Acids and Peptides; Part CXXII: This Journal *39*, 2835 (1974).

** Vasopressin analogs with a D-amino acid in position 8 are regarded as belonging to the D-series.

*** All the optically active amino acids are of L-configuration unless stated otherwise. The nomenclature and abbreviations used are mostly according to[6]. The original names of the vasopressin analogs prepared earlier are retained. Other abbreviations: AD antidiuretic effect, VP pressor effect, Mpr β-mercaptopropionic acid, LVP lysine vasopressin, AVT arginine vasotocin.

TABLE I

Relation between Structure of Amino Acid in Positions 1,4 and 8, and Antidiuretic and Pressor Effect of Vasopressins

[X] Vasopressin	Activity		Ref.
	AD	VP	
Length of side chain of basic amino acid			
[Dap^8]—	40—60	109	3
[Dab^8]—	120±30	149	12
[Orn^8]—	88	360	7
[Lys^8]—	209±10	243	4,5
[Hlys^8]—	~300	~250	8
Effect of length of side chain of basic amino acid. D-Series			
[D-Dap^8]—	150—170	21·2	3
[D-Dab^8]—	120±30	3·6	1
[D-Orn^8]—	50—70	0·24	3
[D-Lys^8]—	6—10	0·75	9
Effect of elimination of amino group in position 1			
[Mpr^1, Lys^8]—	301±11	162±2	4
[Mpr^1, Hlys^8]—	~10 000	~900	10
[Mpr^1, Arg^8]—	1 300±200	370±20	11
Effect of elimination of amino group in position 1. D-Series			
[Mpr^1, D-Dab^8]—	360	2·05	5
[Mpr^1, D-Lys^8]—	3.8	1·05	5
[Mpr^1, D-Arg^8]—	870±200 50 000	0·5—2	5,13
Effect of size of side chain of neutral amino acid in position 4 of LVP			
Ala^4 —	30±0·3	1·6±0·3	25
Abu^4 —	707±107	10·2±0·6	26
Leu^4 —	1—2	1·33	23

of the amino group in position 1 did not result in the usual effect. A combination of the changes in position 8 and 1 afforded an analog, [1-β-mercaptopropionic acid, 8-D-arginine]vasopressin, with a very high and specific antidiuretic activity[5]. This analog has been used with outstanding success in the substitution therapy of *diabetes insipidus*[13–16] and in the therapy of *enuresis nocturna*[17]. The use of this analog has

removed a number of difficulties and drawbacks brought about by the naturally occurring vasopressins in the application fields mentioned.*

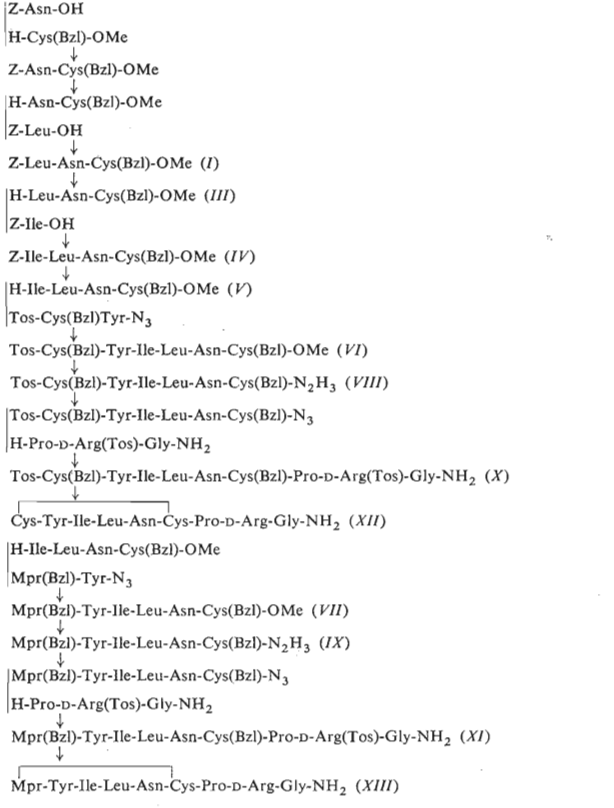
The importance of position 4 of the vasopressin molecule for the preparation of analogs with a specific antidiuretic effect was stressed recently[18,19]. It has been reported that the introduction of a lipophilic amino acid into position 4 increases the ratio of the antidiuretic to the pressor effect (AD/VP). A product[20] has been prepared by combination of the changes in position 4,1 and 8 which according to the authors possesses a somewhat higher and more specific antidiuretic effect than Adiuretin.

The arguments given in the papers mentioned above, which consider the importance of position 4 of the vasopressin molecule, are not in perfect agreement with the existing factual material. Since a correct evaluation of the role of the individual positions in the vasopressin molecule and of the structural changes is extremely important both for the understanding of the relation between chemical structure and biological effect and also for designing new analogs, we return after a certain time period[21,22] to a more detailed discussion of position 4.We wish to show that the presence of a D-amino acid in position 8 is the fundamental condition of obtaining analogs with a specific effect, and with a high and specific effect, respectively, and that the changes in position 4 are of inferior importance. The replacement of glutamine by a neutral amino acid no doubt increases the AD/VP ratio. Its effect, however, is not general. Thus, *e.g.* AD/VP for [Leu^4]-LVP (ref.[23]) is 1, for [Leu^4]-AVT (ref.[24]) even lower than 1. By contrast, a change in the configuration of the basic amino acids leads in all cases to analogs with a more specific antidiuretic effect than that of the naturally occurring hormones. This is a result which we can expect from a change affecting the three-dimensional structure of the vasopressin molecule.

Obviously it is not lipophilicity which plays a role in the effect of changes in position 4 on the AD/VP ratio. The vasopressin analogs in the order of increasing lipophilicity of the amino acid in position 4 (Ala^4-, Abu^4-, and Leu^4-LVP, ref. 25, 26, 23) are listed in Table I. The antidiuretic effect increases from the first to the second compound, then sharply decreases from the second to the third. The specificity of the antidiuretic effect decreases with its magnitude. The primary effect of the lipophilicity is excluded. The size of the side chain of the amino acid in position 4, however, increases in the series given from its first to its last member. It is obviously this very factor which affects both the magnitude and the specificity of the antidiuretic effect. An optimal length and size possesses as it appears a side chain of 3 carbon atoms. Unfortunately, we are lacking a more complete set of analogs with a neutral amino acid in position 4. We have met with a similar dependence

* Commercial names: Adiuretin-SD (Spofa, Czechoslovakia), Desurin (Ferring AB, Malmö, Sweden).

a long time ago* (ref.[3]) when studying position 8. These are results which homologization experiments of this type are expected to give, anyway.



SCHEME 1

Vertical lines at the left-hand side of the formulas denote coupling steps.

* We reported on the first result of this type already in 1959 at the IUPAC Congress in Munich.

We present by way of example – in addition to those given before which are well known – two new analogs of the vasotocin type, [Leu^4, D-Arg^8]vasotocin (*XII*) and [Mpr^1, Leu^4, D-Arg^8]vasotocin (*XIII*).

The method of preparation of *XII* and *XIII* is shown in Scheme 1. Both products were obtained by the liquid-phase synthesis. We used the usual combination[21] of protecting groups and coupling methods[21] (Scheme 1). The starting material for the synthesis of both products was the ester, H-Ile-Leu-Asn-Cys(Bzl)-OMe (*V*), prepared by stepwise synthesis from the carboxyl terminus. The latter was condensed both with Tos-Cys(Bzl)-Tyr-N_3 and with Mpr(Bzl)-Tyr-N_3. Thus we did not follow exactly the usual scheme[21] based on the condensation of tripeptide fragments. The hexapeptide ester, Tos-Cys(Bzl)-Tyr-Ile-Leu-Asn-Cys(Bzl)-OMe (*VI*), and the pentapeptide ester, Mpr(Bzl)-Tyr-Ile-Leu-Asn-Cys(Bzl)-OMe (*VII*), obtained were converted into hydrazides and condensed *via* azides with the tripeptide amide, H-Pro-D-Arg(Tos)--Gly-NH_2, synthetized by the usual procedure[5].

A few notes reporting on the complications with azide condensations of fragments 1–6 and 7–9, have appeared recently[27,28]. These notes led us to comment briefly on the 6 + 3 coupling. We have followed the 6 + 3 scheme for a longer period. At times we have met with complications also in the vasopressin series. These complications are – in our experience – of more methodical than fundamental nature. We regard the formation of amides as very little probable. We have not observed this reaction in spite of the fact that we have checked the purity of the azides spectroscopically (IR) in a number of cases. This problem has been commented on by Bodanszky[29] some time ago. Naturally, the azide synthesis is a sensitive reaction. This can be observed especially in complicated cases, such as the condensation of fragments of higher molecular weight. A number of precautions must be taken to achieve a successful synthesis in these cases. Our experience with the 6 + 3 coupling can be summarized as follows. Special attention must be devoted to the purity of solvents and reagents. Dimethylformamide, often used as a solvent in the condensation of fragments of higher molecular weight, must not contain free amines (ninhydrin test). The presence of oxidating agents must be eliminated. Hydrogen chloride must not contain free chlorine. Dioxane or tetrahydrofuran, respectively, also used as reaction media or for the preparation of hydrogen chloride solutions, must be free of peroxides. The solutions of hydrogen chloride in dioxane or tetrahydrofuran must be freshly prepared. Older solutions decrease the coupling yield even by 40%. Alkyl nitrites, used in the nonaqueous modification of the azide synthesis[30], must be freshly prepared or at least freshly distilled (aldehydes). We have very good experience with checking the preparation of the azides by the Griess reagent[31]. In case that these precautions have been taken and the results are still unsatisfactory, an improvement of the yield can be achieved by increasing the quantity of the amino component up to 40%.

We removed the protecting groups from both linear peptides, nonapeptide amide *X* and octapeptide amide *XI*, by the action of sodium in liquid ammonia[32]. We closed

the disulfide bond by oxidation with potassium ferricyanide[33], desalted the oxidation product on a column of Amberlite CG, and purified[1] it by free-flow electrophoresis[34] in 0·5M acetic acid. We have slightly modified the procedure[1] originally described. A record of the separation is shown in Figs 1 and 2. Analogs *XII* and *XIII* obtained were characterized by elemental analysis and by amino-acid analysis[35]. Their purity was checked by thin-layer chromatography and by paper electrophoresis (see Experimental).

Analogs *XII* and *XIII* were tested for antidiuretic, uterotonic, and pressor effect. Their effect on the renal secretion of sodium and potassium was also examined.

The uterotonic[36] and pressor[37] effect of both analogs was very low. The values of both activities varied in the range 0·1 – 0·3 I.U./mg. The effect of *XII* and *XIII* on the secretion of sodium and potassium was negligible.

The antidiuretic effect was determined both by the method of Burn[38] and by the method of Holeček[39]. *XII* and *XIII* showed a considerable antidiuretic activity in both tests. The determinations, however, were paralleled by complications and a considerably spread values were obtained. The renal effects of *XII* and *XIII* are being studied further and will be the subject of a separate report.

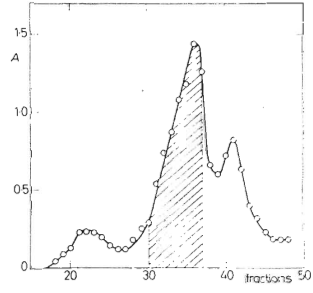


FIG. 1

Purification of [Leu^4, D-Arg^8]Vasotocin by Continuous Free-flow Electrophoresis

Electrolyte 3% acetic acid, 2600 V. Sample (5% solution in 20% acetic acid) fed at a rate of 1 ml/h. Measured at 275 nm. Fractions 1–16 were void. Fraction 30–37 (hatched area) contained a homogeneous product.

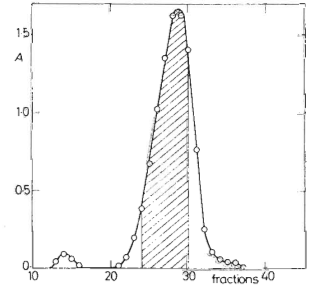


FIG. 2

Purification of [Mpr^1, Leu^4, D-Arg^8]-Vasotocin by Continuous Free-Flow Electrophoresis

Electrolyte 3% acetic acid, 3000 V. Sample (5% solution in 20% acetic acid) fed at a rate of 1 ml/h. Measured at 275 nm. Fractions 1–11 were void. Fractions 24–30 (hatched area) contained a homogeneous product.

Even though we are lacking as yet entirely reliable data on the magnitude of the antidiuretic effect of both analogs, we can regard our results as additional evidence showing that the primary condition of obtaining of vasopressin analogs with a specific and high antidiuretic effect is the change of the configuration of the basic amino acid in position 8 and that the changes in position 4 are of secondary importance only: The introduction of leucine into position 4 of arginine-vasotocin led to a drastic decrease of the antidiuretic effect and to a shift of the AD/VP ratio toward the pressor effect. A change of the configuration of arginine in position 8 led with this analog to an increase by two orders of both the antidiuretic effect and also of the AD/VP ratio. Analogs *XII* and *XIII* represent a new type of antidiuretically active products. They are the first vasotocins with a practically pure antidiuretic effect.

EXPERIMENTAL

The melting points were determined on a Kofler block and are not corrected. The optical activity was measured in Zeiss-Winkel 0·01 polarimeter and in an objective polarimeter (made in the workshops of this Institute). UV-Colorimetry was carried out in VSU-2P Spectrophotometer (Zeiss, Jena). The polarographic measurements were effected in LP 7 Polarograph (Laboratorní přístroje, Prague). The preparative-scale electrophoreses were carried out in the continuous free-flow electrophoresis apparatus (a modification of the Hanning type apparatus, constructed in the workshops of this Institute). The purity of the intermediary and final products was checked by thin-layer chromatography on aluminum sheets (Silufol, Kavalier) in the system n-butanol–acetic acid–water (4 : 1 : 1 and 4 : 1 : 5) and by paper electrophoresis (5% acetic acid and pyridine acetate buffer at pH 5·7). Unless stated otherwise, the samples for analysis were dried 8 h at 80°C and 0·1 Torr over phosphorus pentoxide.

Benzyloxycarbonylleucyl-asparaginyl-S-benzylcysteine Methyl Ester (*I*)

The solution of 13·3 g (50 mmol) of benzyloxycarbonylleucine and 7 ml (50 mmol) of N-ethylpiperidine in 75 ml of chloroform was treated with 5·5 g (50 mmol) of ethyl chloroformate at 0°C. The mixture was cooled to −10°C 5 min later and a solution of 21·0 g (50 mmol) of asparaginyl-S-benzylcysteine methyl ester hydrobromide and 7 ml (50 mmol) of N-ethylpiperidine in 120 ml of chloroform was added. The product which had precipitated after one hour of standing at room temperature was filtered off, washed with chloroform, and dried. It was subjected to repeated trituration with dilute hydrochloric acid and a solution of $NaHCO_3$, and to two-fold crystallization from aqueous acetic acid. The yield was 20·9 g (71%) of a product of m.p. 198–200°C, $[\alpha]_D^{25}$ −24·8° (*c* 0·6, dimethylformamide). Recorded[40] data: m.p. 195–197°C, $[\alpha]_D^{25}$ −28° (*c* 1, dimethylformamide).

Leucyl-asparaginyl-S-benzylcysteine Methyl Ester Hydrobromide (*II*)

A solution (80 ml) of hydrogen bromide in acetic acid (approx. 35%) was added to a solution of 19·3 g of *I* in 100 ml of acetic acid and the mixture was heated 10 min at 60°C. Leucyl-asparaginyl-S-benzylcysteine methyl ester hydrobromide (*II*) was precipitated from the reaction mixture by an excess of ether, filtered off, washed with ether, and recrystallized from methanol–ether. Yield 16·8 g (95%), m.p. 223–224°C, $[\alpha]_D^{25}$ −17·9° (*c* 0·6, dimethylformamide). For $C_{21}H_{32}N_4O_5S$.

HBr (533·5) calculated: 47·28% C, 6·24% H, 10·50% N, 6·01% S; found: 46·99% C, 6·18% H, 10·44% N, 6·02% S. The pH of a solution of 16·0 g of *II* in 100 ml of water was adjusted to 8—9 by NH_4OH. The product which had separated after 1 h of standing at 0°C was filtered off, washed with water, and dried. It was crystallized from methanol–water and methanol–ether. The yield was 13·0 g (96%) of amino ester *III* of m.p. 129—130°C, $[\alpha]_D^{25}$ —26·6 (*c* 0·7, dimethylformamide). For $C_{21}H_{32}N_4O_5S$ (452·6) calculated: 55·73% C, 7·13% H, 12·38% N, 7·08% S; found: 55·70% C, 7·18% H, 12·65% N, 6·91% S.

Benzyloxycarbonylisoleucyl-leucyl-asparaginyl-S-benzylcysteine Methyl Ester (*IV*)

A solution of 6·6 g (25 mmol) of benzyloxycarbonylisoleucine and 3·5 ml (25 mmol) of N-ethylpiperidine in 50 ml of chloroform was treated with 2·7 g (25 mmol) of ethyl chloroformate at 0°C. A solution of 11·3 g (25 mmol) of *III* in 30 ml of dimethylformamide was added 5 min later. The mixture was set aside for 1 h at room temperature and then treated with excess of ether. The solid product precipitated was filtered off, dried and repeatedly triturated with dilute hydrochloric acid and a solution of $NaHCO_3$. Three-fold crystallization from aqueous acetic acid afforded 12·0 g of *IV*, m.p. 244—245°C, $[\alpha]_D^{25}$ —27·5° (*c* 0·4, dimethylformamide). For $C_{35}H_{49}N_5O_8S$ (699·9) calculated: 60·06% C, 7·06% H, 10·01% N, 4·58% S; found: 59·79% C, 7·00% H, 9·81% N, 4·60% S.

Isoleucyl-leucyl-asparaginyl-S-benzylcysteine Methyl Ester (*V*)

The decarbobenzoxylation of *IV* was carried out as described for *I*. The yield was 8·5 g (89%) of the hydrobromide from 10·5 g of *IV*. M.p. 125—127°C after three-fold crystallization from methanol–diisopropyl ether. For $C_{27}H_{43}N_5O_6S$. HBr (646·6) calculated: 50·15% C, 6·86% H, 10·83% N, 4·96% S; found: 50·26% C, 6·98% H, 10·57% N, 4·82% S. The hydrobromide was converted into the free amino ester by treatment with ammonium hydroxide as described for *III*. Hydrobromide *V* (6·5 g) yielded 5·0 g (86%) of amino ester *V* monohydrate, m.p. 146—148°C, $[\alpha]_D^{25}$ —31·4° (*c* 0·2, dimethyl sulfoxide). For $C_{27}H_{43}N_5O_6S$. H_2O (583·7) calculated: 55·55% C, 7·43% H, 12·00% N, 5·49% S; found: 55·49% C, 7·44% H, 11·82% N, 5·45% S.

Tosyl-S-benzylcysteinyl-tyrosyl-isoleucyl-leucyl-asparaginyl-S-benzylcysteine Methyl Ester (*VI*)

A solution of 1·92 g (3·5 mmol) of the hydrazide of tosyl-S-benzylcysteinyltyrosine in 20 ml of dimethylformamide was treated with 1·75 ml of 4M solution of hydrogen chloride in dioxane free of peroxides and a solution of 0·414 g of amyl nitrite was added at —20°C. The mixture was allowed to stand 20 min at —20°C, then neutralized with N-ethylpiperidine, and treated with a solution of 2·00 g (3·5 mmol) of *V* in 10 ml of dimethyl sulfoxide. The mixture was allowed to react 12 h at 0°C and 5 h at room temperature. The solvents were distilled off under reduced pressure and the dry residue was repeatedly triturated with dilute hydrochloric acid and with a solution of $NaHCO_3$. The yield of the neutral product was 3·55 g (93%). It was twice crystallized from aqueous dimethylformamide and 3·15 g (83%) of *VI* was obtained. M.p. 240—242°C, $[\alpha]_D^{25}$ —16·3 (*c* 0·4, dimethylformamide). For $C_{53}H_{69}N_7O_{11}S_3$ (1076) calculated: 59·14% C, 6·46% H, 9·11% N; found: 58·89% C, 6·35% H, 9·10% N.

β-Benzylthiopropionyl-tyrosyl-isoleucyl-leucyl-asparaginyl-S-benzylcysteine Methyl Ester (*VII*)

The synthesis was carried out as in the preceding case. β-Benzylthiopropionyltyrosine hydrazide (1·3 g, 3·5 mmol) and *V* (2·0 g, 3·4 mmol) afforded 2·6 g (81%) of pentapeptide ester hemihydrate

VII, m.p. 242–244°C, $[\alpha]_D^{25}$ −27·1° (*c* 0·5,dimethylformamide). For $C_{46}H_{62}N_6O_9S_2 \cdot 1/2\ H_2O$ (916·1) calculated: 60·30% C, 6·93% H, 9·17% N; found: 60·30% C, 6·79% H, 9·32% N.

Tosyl-S-benzylcysteinyl-tyrosyl-isoleucyl-leucyl-asparaginyl-S-benzylcysteine Hydrazide (*VIII*)

A solution of 3·0 g of *VI* in 20 ml of dimethylformamide was treated with 2·8 ml of hydrazine hydrate (100%). The mixture was allowed to stand 24 h at room temperature. The product which had separated was filtered off, washed with water, and dried (2·4 g). It was crystallized from aqueous dimethylformamide and gave 2·0 g (67%) of a product of m.p. 250–252°C, $[\alpha]_D^{25}$ −22·6° (*c* 0·39, dimethylformamide). For $C_{52}H_{69}N_9O_{10}S_3$ (1076) calculated: 58·02% C, 6·46% H, 11·71% N; found: 57·84% C, 6·40% H, 12·11% N.

β-Benzylthiopropionyl-tyrosyl-isoleucyl-leucyl-asparaginyl-S-benzylcysteine Hydrazide (*IX*)

The product was prepared by the same procedure as the preceding product. From 2·5 g of *VII*, 1·5 g of the hydrazide hemihydrate was obtained. M.p. 257–258°C, $[\alpha]_D^{25}$ −33·5° (*c* 0·24, dimethylfomamide). For $C_{45}H_{62}N_8O_8S_2 \cdot 1/2\ H_2O$ (916·1) calculated: 58·99% C, 6·93% H, 12·23% N; found: 59·13% C, 6·81% H, 12·44% N.

Tosyl-S-benzylcysteinyl-tyrosyl-isoleucyl-leucyl-asparaginyl-S-benzylcysteinyl-prolyl-N^G-tosyl--D-arginyl-glycide Amide (*X*)

A solution of 2·0 g (1·9 mmol) of *VIII* in 20 ml of dimethylformamide and 5 ml of dimethylsulfoxide was treated with a dioxane solution of hydrogen chloride (1·1 ml, approx. 3·4M) and at −20°C with 1·11 ml of a dimethylformamide solution of amyl nitrite (0·196 g of amyl nitrite in 1 ml). The mixture was cooled to −30°C after 20 min, neutralized with N-ethylpiperidine, and then a solution of 1·3 g (2·7 mmol) of prolyl-N^G-tosyl-D-arginyl-glycinamide in 5 ml of dimethylformamide was added. The mixture was allowed to stand 12 h at 0°C and 5 h at room temperature. The solvents were distilled off under reduced pressure and the dry residue was repeatedly triturated with dilute hydrochloric acid and a solution of $NaHCO_3$. The yield was 2·55 g (90%) of the neutral product. It was crystallized from aqueous dimethylformamide and afforded 2·25 g (79·5%) of a product of m.p. 210–212°C, $[\alpha]_D^{25}$ −16·3° (*c* 0·2, dimethylformamide). For $C_{72}H_{96}N_{14}O_{15}S_4$ (1526) calculated: 56·67% C, 6·34% H, 12·86% N, 8·41% S; found: 56·54% C, 6·29% H, 12·65% N, 8·26% S. Amino-acid composition: Tyr 0·94, Ile 1·09, Leu 1·07, Asp 1·09, Pro 1·00, Arg 0·85, Gly 0·92.

β-Benzylthiopropionyl-tyrosyl-isoleucyl-leucyl-asparaginyl-S-benzylcysteinyl-prolyl-N^G-tosyl--D-arginyl-glycine Amide (*XI*)

The coupling and the treatment of the reaction mixture were carried out as described for the preceding product. From 1·3 g (1·4 mmol) of *IX* and 1·0 g (2 mmol) of prolyl-N^G-tosyl-D-arginyl--glycinamide, 1·47 g (76%) was obtained of a product of m.p. 227–230°C, $[\alpha]_D^{25}$ −21·4° (*c* 0·2, dimethylformamide). For $C_{65}H_{89}N_{13}O_{13}S_3 \cdot 1/2\ H_2O$ (1366) calculated: 57·16% C, 6·64% H, 13·33% N, 7·04% S; found: 57·12% C, 6·56% H, 13·21% N, 7·03% S. Amino-acid composition: Tyr 0·98, Ile 1·09, Leu 1·04, Asp 1·10, Pro 0·98, Arg 0·88, Gly 0·92.

[4-Leucine, 8-D-arginine]-vasotocin (*XII*)

Product *X* (0·5 g) was reduced with sodium in liquid ammonia (until a permanent blue color stable for 30 s was obtained). The solution was decolorized by the addition of acetic acid and evaporated

under reduced pressure. A 2·5% solution of acetic acid (1 liter) was added to the dry residue and a small insoluble part of the product was filtered off. The filtrates were extracted with ether (5-times) and their pH was adjusted to 6·75 by ammonium hydroxide. Subsequently they were oxidized by 0·01M solution of $K_3[Fe(CN)_6]$. The pH of the solution after oxidation was adjusted to 4 by acetic acid and the solution was desalted by filtration through an Amberlite CG column (25 ml) and washing the column with 250 ml of 0·25% acetic acid. The peptide was displaced from the column by 50% acetic acid. A total of 30 ml of effluent was collected (starting from the moment of emergence of peptide material). The effluent was made up to 50 ml by water and lyophilized. Yield 223 mg of lyophilized product (Ist lyophilisate).

The purification was effected in a modified apparatus for continuous free-flow electrophoresis according to Hannig, constructed in the workshops of this Institute. The size of the air-cooled plates was 50 × 50 cm (operating volume of the chamber between the plates approx. 90 ml). The carrier electrolyte (3% acetic acid) was pumped into the chamber by a six-fold piston pump (rate of passage of electrolyte through chamber approx. 1 cm/h). The circulation of the electrode electrolyte (6% acetic acid) was ensured by a rotary pump. The sample (5% solution of lyophilisate in 20% acetic acid) was introduced into the chamber by a peristaltic pump at a rate of approx. 1 ml/h. The purification was carried out at 2600 V (approx. 130 mA), the temperature of cooling air was −2°C. The liquid emerging from the chamber was collected in 48 vessels of the syphon type. The liquid collected was sucked off at 20 min intervals (by a vacuum system) and transported to 48 polyethylene flasks. The absorbance at 275 nm of the fractions was measured at the end of the separation (Fig. 1). The purity of the individual fractions was checked by paper electrophoresis (800 V, 6% acetic acid, 45 min, ninhydrin detection). Fractions 30—37, containing a uniform product, were pooled, filtered, and lyophilized. Yield 106·3 mg (2nd lyophilisate), $[\alpha]_D^{25}$ −7·1° (*c* 0·3, 1M-CH_3CO_2H). The analysis of the lyophilisate dried 10 h at 100°C corresponded to the content of 1 1/2 molecule of acetic acid. For $C_{44}H_{70}N_{14}O_{11}S_2 \cdot 1^1/_2\ CH_3CO_2H$ (1125) calculated: 50·02% C, 6·80% H, 17·42% N; found: 49·78% C, 6·50% H, 17·36% N. The lyophilisate behaved as a uniform product on thin-layer chromatography. Amino acid composition: Tyr 0·86, Ile 0·99, Leu 0·97, Asp 1·00, Arg 0·96, Pro 1·10, Gly 0·95, Cys 1·90. In another experiment, 516 mg of Ist lyophilisate and 154 mg of 2nd lyophilisate, showing properties described above, was obtained from 1·0 g of protected *X*.

[1-β-Mercaptopropionic Acid, 4-Leucine, 8-D-Arginine]-vasotocin (*XIII*)

Reduction, oxidation, desalting and purification were carried out as described for the preceding case except that a higher voltage (3000 V) was used. Product *XI* (0·5 g) yielded 125·1 mg of 2nd lyophilisate. $[\alpha]_D^{25}$ −58·2° (*c* 0·2, 1M-CH_3CO_2H). The analysis of the product dried as described above corresponded to monoacetate dihydrate. For $C_{44}H_{69}N_{13}O_{11}S_2 \cdot CH_3CO_2H \cdot 2\ H_2O$ (1116) calculated: 49·49% C, 6·95% H, 16·31% N; found: 49·36% C, 6·68% H, 16·57% N. The lyophilisate behaved as a uniform product when checked by paper electrophoresis and thin-layer chromatography. Amino-acid composition: Tyr 0·95, Ile 1·03, Leu 0·98, Asp 1·00, Pro 1·01, Arg 1·01, Gly 0·98. Cys and the mixed disulfide Cys-Mpr were not determined.

The authors thank Prof. J. H. Cort, Institute of Physiology, Czechoslovak Academy of Sciences, Prague, and Prof. V. Holeček, 3rd Clinic of Internal Medicine, Charles University, Prague, for the biological tests of the antidiuretic effect.

REFERENCES

1. Zaoral M., Šorm F.: This Journal *31*, 310 (1966).
2. Zaoral M., Kolc J., Šorm F.: This Journal *32*, 1242 (1967).

3. Zaoral M., Kolc J., Šorm F.: This Journal *35*, 1716 (1970).
4. Kimbrough R. D. jr, Cash W. D., Branda L. A., Chan W. Y., du Vigneaud V.: J. Biol. Chem. *238*, 1411 (1963).
5. Zaoral M., Kolc J., Šorm F.: This Journal *32*, 1250 (1967).
6. *IUPAC-IUB Commission on Biochemical Nomenclature Symbols for Amino-Acid Derivatives and Peptides Recommendations 1971.* Biochemistry *11*, 1726 (1971).
7. Jaquenoud P. - A.: Helv. Chim. Acta *48*, 1899 (1965).
8. Bodanszky M., Lindeberg G.: J. Med. Chem. *14*, 1197 (1971).
9. Zaoral M., Kolc J., Šorm F.: This Journal *31*, 382 (1966).
10. Lindeberg G., Kynčl J., Dreyfuss P., Bodanszky M.: J. Med. Chem. *15*, 629 (1972).
11. Huguenin R. L.: Helv. Chim. Acta *49*, 695 (1966).
12. Zaoral M., Šorm F.: This Journal *31*, 90 (1966).
13. Vávra I., Machová A., Holeček V., Cort J. H., Zaoral M., Šorm F.: The Lancet *1968*, 948.
14. Andersson K.- E., Arner B.: Brit. Med. J. *1971*, 111.
15. Andersson K.- E., Arner B.: Acta Med. Scand. *21*, 192 (1972).
16. Oravec D., Lichardus B., Kováč R., Sarkady H., Zaoral M.: Z. Ges. Inn. Med. *27*, 993 (1972).
17. Birkasova M., Holeček V., Skula E., Zaoral M., Birkas O.: Pediatria, in press.
18. Manning M., Coy E. J., Sawyer W. H., Acosta M.: J. Med. Chem. *16*, 463 (1973).
19. Manning M., Coy E. J., Sawyer W. H., Acosta M.: J. Med. Chem. *16*, 836 (1973).
20. Manning M., Balaspiri L., Acosta M., Sawyer W. H.: J. Med. Chem. *16*, 975 (1973).
21. Zaoral M.: This Journal *30*, 1853 (1965).
22. Zaoral M., Pliška V., Řežábek K., Šorm F.: This Journal *28*, 746 (1963); Czechoslov. Pat. No 115 178.
23. Dyckes D. F., Ferger M. F., du Vigneaud V., Chan W. Y.: J. Med. Chem. *16*, 843 (1973).
24. Pliška V., Vašák J., Rufer H., Rudinger J.: Experientia *29*, 171 (1973).
25. Boissonas R. A., Guttmann S., Huguenin R. L., Jaquenoud P. - A., Sandrin E.: Helv. Chim. Acta *46*, 2347 (1963).
26. Gillessen D., du Vigneaud V.: J. Biol. Chem. *242*, 4806 (1967).
27. Kaurov O. A., Martynov V. F., Popernackij O. A.: Ž. Obšč. Chim. *40*, 904 (1970); *43*, 909 (1973).
28. Niedrich H.: J. Prakt. Chem. *314*, 769 (1972).
29. Bodanszky M., Ondetti M. A.: *Peptide Synthesis*, pp. 80. Interscience-Wiley, New York 1966.
30. Honzl J., Rudinger J.: This Journal *26*, 2333 (1961).
31. Griess P.: Chem. Ber. *12*, 426 (1879).
32. Sifferd R. H., du Vigneaud V.: J. Biol. Chem. *108*, 753 (1935).
33. du Vigneaud V., Winestock G., Murti V. V. S., Hope D. B., Kimbrough R. D. jr: J. Biol. Chem. *235*, PC 64 (1960).
34. Hannig K.: Z. Anal. Chem. *181*, 244 (1961).
35. Spackmann D. H., Stein W. H., Moore S.: Anal. Chem. *30*, 1190 (1958).
36. Munsick R. A.: Endocrinology *66*, 451 (1960).
37. Landgrebe F. W., Macaulay M. H., Waring H.: Proc. Roy. Soc. (Edinburg) *362*, 202 (1964).
38. Burn J. H., Finney D. J., Goodwin L. D.: *Biological Standardization.* Oxford Univ. Press, Oxford 1950.
39. Holeček V., Polák H., Bláha J., Jirásek M.: Endokrinologie *32*, 39 (1954).
40. Gillesen D., Studer R. O., Rudinger J.: Experientia *29*, 170 (1973).

Translated by V. Kostka.

* *Note added in proof:* In a recent communication Lindeberg and coworkers (J. Med. Chem. *17*, 781 (1974)) report for [Mpr[1], Hlys[8]]vasopressin a value of 634 ± 54 I. U./mg.