OXYTOCIN ANALOGUES WITH INHIBITORY PROPERTIES, CONTAINING IN POSITION 2 A HYDROPHOBIC AMINO ACID OF D-CONFIGURATION*

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Ten analogues derived from oxytocin, deamino-oxytocin and deamino-carba-oxytocin were prepared which contained a D-amino acid in the position 2 of the parent system. The following D-amino acids were introduced: tyrosine, phenylalanine, *p*-methylphenylalanine, *p*-ethylphenylalanine and O-ethyltyrosine. Combination of two structural features which alone lead to strong inhibitors (a suitable D-amino acid in position 2 and a penicillamine moiety in position 1) did not enhance the inhibitory effect. Compounds containing D-tyrosine are weak agonists in the uterotonic assay; in case of 1-carba-analogues they can be converted into sulfoxides with low inhibitory activity. Analogues with D-phenylalanine substituted in the *para*-position are the most potent antagonists of the uterotonic effect of oxytocin ($pA_2 = 8{\cdot}73$) *in vitro*.

Recently, there is a considerable interest in the preparation and study of neurohypophyseal hormone analogues with inhibitory activity towards oxytocin and vasopressin in a great variety of biological tests[1-3]. These compounds are interesting not only because of their possible use in the study of mechanism of action or spatial arrangement but they may also find use in the clinical therapeutical practice. There are several structural modifications leading to oxytocin analogues with inhibitory effects in the uterotonic test. We may mention the alkylation of the tyrosine** hydroxyl[5-7], particularly with simultaneous acylation of the primary α-amino group of cysteine in position 1 (ref.[8-11]) or its replacement by a hydrogen atom[12]. Particularly important is the attachment of two methyl groups[13,14], two ethyl groups[15] or a cyclopentamethylene group[16] to the β-carbon atom of cysteine in position 1 which might be combined with deamination in this position. Various combinations of this modification with substitution of the amino acids have been realized[2,3,14,17-24] (in positions 2, 4, 7 and 8) which gave very strong inhibitors.

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** 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 recommendations[4]. Hcy denotes homocysteine, Pen penicillamine and Mpa β-mercaptopropionic acid moieties.

In the oxytocin series, replacement of tyrosine in position 2 by its D-enantiomer leads to analogues of very low biological activity[25,26]. Introduction of a D-amino acid other than tyrosine (such as phenylalanine[27], pentafluorophenylalanine[27,28], 3-nitrotyrosine[29], leucine[30] or tryptophan[31]) gives analogues with a certain inhibitory activity. Interestingly, the compound containing O-ethyl-D-tyrosine showed[31] an intrinsic activity in the *in vitro* uterotonic test which was even higher than that of the analogue with the L-enantiomer[7] (which, contrariwise, in certain experimental arrangement had an inhibitory effect). A similar modification (introduction of O-alkyl-D-tyrosine) of [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid), 4-valine, 8-arginine]vasopressin gave analogues with inhibitory activity in the antidiuretic as well as pressor tests; however, also the corresponding analogues with the L-amino acid in position 2 showed inhibitory activity, similarly to the compound with unsubstituted D-tyrosine[32,33]. If, however, D-tyrosine is introduced into the vasopressin molecule as the only structural change, the resulting analogue ([2-D-tyrosine, 8-arginine]vasopressin) exhibits relatively high vasopressin-like activities[26]. This corresponds to the assumptions about the spatial arrangement of the tyrosine side chain in the vasopressin molecule[26,34]. [1-(β-Mercapto-β,β-cyclopentamethylenepropionic acid), 4-valine, 8-L- (or D-)arginine]vasopressin analogues containing D-phenylalanine or an aliphatic D-amino acid in position 2 showed inhibitory activity in the antidiuretic, pressor and uterotonic tests, the ratio of the inhibition constants in various assays depending on the type of the aliphatic amino acid in position 2 (ref.[33]) or in position 4 (ref.[36]).

A combination of deamino-penicillamine in position 1 with an alkyltyrosine in position 2 increases the inhibitory activity in the uterotonic test as compared with [1-deamino-penicillamine]oxytocin[18]. The same has been found[35] also with the corresponding amino derivatives: both [1-penicillamine, 2-O-methyltyrosine]oxytocin and [1-penicillamine, 2-O-methyltyrosine, 8-lysine]vasopressin are strong inhibitors in the uterotonic and pressor tests. On the other hand, acetylation of the α-amino group of penicillamine in position 1 reduced the inhibitory activity[35], regardless whether the molecule contained a tyrosine or O-methyltyrosine in the position 2 or not. Interestingly enough, the inhibitory activity increased upon introduction of leucine into the position 2 (ref.[14]). The spatial arrangement of the obtained analogue was intensively studied by physical methods and its enhanced inhibitory activity was explained[37] by a reduction of the steric strain in the side chains of the amino acids in positions 1 and 2. The steric bulk of the substituent in position 2 can be influenced by change in configuration of this amino acid. The tyrosine side chain can form hydrogen bonds and its hydroxyl is assumed[38] to be a part of the so-called active site of the oxytocin molecule. On the other hand, hydrophobic interactions of the side chains of amino acids in positions 3, 4, 7 and 8 are very important for binding to the receptor[38]. A change in configuration of the amino acid in position 2 combined with replacement of the hydrophilic hydroxyl by a hydrophobic substituent

could strengthen the bond to the receptor with simultaneous reduction or complete elimination of the intrinsic activity, *i.e.* the resulting analogue should be an inhibitor.

We prepared deamino-6-carba-oxytocin analogues containing D-tyrosine, D-phenylalanine, *p*-methyl-D-phenylalanine, *p*-ethyl-D-phenylalanine and O-ethyl-D-tyrosine in the position 2. Deamino-6-carba-oxytocin was chosen because its high uterotonic activity indicates a high affinity towards the uterotonic receptor and its possible oxidation has a substantially smaller effect on the biological activities than oxidation of the 1-carba-analogue[39,40]. To check the suitability of the 6-carba substitution combined with deamination for obtaining more potent inhibitors we synthesized oxytocin and deamino-oxytocin analogues with *p*-ethyl-D-phenylalanine in the position 2. We prepared also analogues with penicillamine in position 1 and *p*-ethylphenylalanine of D or L configuration in position 2.

The analogues were prepared by stepwise synthesis of the chain using active esters. Acylations with phenylalanine derivatives (protected with *o*-nitrobenzenesulfenyl group) were performed with the 2,4,5-trichlorophenyl ester or N-hydroxybenzotriazolyl ester prepared *in situ*[41]. Benzyloxycarbonyl-S-benzylpenicillamine was attached as the *p*-nitrophenyl ester[13]. *p*-Ethyl-DL-phenylalanine was resolved *via* its phenylacetyl derivative using penicillin amidohydrolase (E.C.3.5.1.11) (ref.[42,43]). D-Tyrosine was alkylated with diethyl sulfate as *o*-nitrobenzenesulfenyl derivative in which the acylation of the amino group as well as the alkylation at the oxygen could be carried out without isolation of the N-acyl intermediate. This alkylation required a substantially higher pH of the reaction mixture and a longer reaction time than the analogous alkylation with dimethyl sulfate and was followed by liquid chromatography.

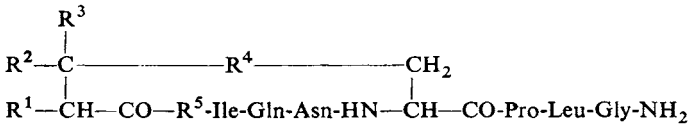
The carba-analogues were cyclized *via* N-hydroxybenzotriazolyl ester prepared from hydrochloride of the free octapeptide[44]. Disulfide-containing analogues were obtained by removal of the protecting groups with sodium in liquid ammonia and oxidation with potassium ferricyanide. The products were desalted either on Amberlite IRC-50 or using Sep-Pak C_{18} cartridges[45]. All the obtained analogues were purified by reversed phase liquid chromatography.

In syntheses of some analogues we made use of the fact that diastereoisomeric peptides can be easily separated by reversed phase chromatography[46,47]. We therefore performed the synthesis with racemic *para*-substituted phenylalanine derivatives and the formed diastereoisomers were separated only after cyclisation. Their identity was determined by incubation of the hydrolysate with L-amino acid oxidase.

In the diastereoisomeric analogues *Ie* and *If*, hydrolysis of the peptide bond D-Phe(Et)-Ile is markedly slower than the L-Phe(Et)-Ile bond, as seen from Table I.

Whereas in all the studied cases the analogue with an aromatic D-amino acid is eluted in the reversed phase chromatography later than the corresponding L-diastereoisomer, the compound containing D-tyrosine differs from its L-analogue only very little ($\alpha = 1{\cdot}05$) and is eluted first. It was particularly important to exclude

contamination of the analogue *Ib* with the L-tyrosine analogue (which is very active) and it was therefore purified *via* its sulfoxide (prepared by periodate oxidation[40]) whose capacity factor differs substantially from that of the sulfoxide of the L-tyrosine compound ($\alpha = 1{\cdot}47$). The sulfoxide was then reduced with hydrogen bromide in acetone[39,48] and the obtained analogue purified by liquid chromatography.



	R^1	R^2	R^3	R^4	R^5
Ia	NH_2	H	H	S—S	Tyr
Ib	H	H	H	S—CH_2	D-Tyr
Ic	H	H	H	S—CH_2	D-Phe
Id	H	H	H	S—CH_2	D-Phe(Me)
Ie	H	H	H	S—CH_2	Phe(Et)
If	H	H	H	S—CH_2	D-Phe(Et)
Ig	H	H	H	SO—CH_2	D-Phe(Et)
Ih	H	H	H	S—CH_2	D-Tyr(Et)
Ii	NH_2	H	H	S—S	D-Phe(Et)
Ij	H	H	H	S—S	D-Phe(Et)
Ik	NH_2	CH_3	CH_3	S—S	Phe(Et)
Il	NH_2	CH_3	CH_3	S—S	D-Phe(Et)

The biological activities of the synthesized analogues are given in Table II. A comparison with uterotonic activities of analogues containing L-amino acids in position 2 can be found in our preliminary communication[50]. As seen, both in the *in vitro*

TABLE I

Dependence of amount of liberated amino acids on the time in acid hydrolysis of compounds *Ie* and *If* (related to glycine)

Hydrolysis time h	Phe(Et)		Ile		Glu	
	If	*Ie*	*If*	*Ie*	*If*	*Ie*
5	0·57	0·72	0·39	0·44	0·72	0·76
10	0·69	0·79	0·66	0·74	0·90	0·94
20	0·87	0·97	0·87	0·90	1·02	1·00
40	0·97	0·99	0·98	0·99	0·99	1·00

and *in vivo* assays on uterus the highest inhibitory activity was found with the analogue *If* containing *p*-ethyl-D-phenylalanine and carba-substitution in the disulfide bridge. Compounds with non-modified bridge show a slightly lower inhibitory activity, confirming the assumption of higher affinity of carba-compounds towards the uterotonic receptor. The amino compound is slightly more potent inhibitor than the analogue *Ij*. A similar situation was observed also with other analogues containing penicillamine or deaminopenicillamine[22]. The combination of substitution at the β-carbon atom of cysteine in position 1 with introduction of a D-amino acid into position 2 is not of additive character; on the contrary, it reduces slightly the inhibitory activity of the analogue. A comparison of properties of compounds *Ii*, *Ik* and *Il* shows that a configurational inversion at the α-carbon of the amino acid in position 2 alone is more important than the introduction of penicillamine into the position 1.

However, a mere configurational inversion is not sufficient; very important is removal of hydroxyl from the tyrosine moiety because compounds containing D-tyrosine have their own activity. Alkylation of D-tyrosine preserves a polar oxygen atom in the molecule which, as it seems, is unfavourable for the inhibitory activity. The activity of [2-O-ethyl-D-tyrosine]oxytocin[31] (as compared with the inhibitory activity of compound *Ih*) again shows the suitability of the carba-modification for obtaining an inhibitor.

TABLE II

Some biological activities of the prepared compounds (I.U./mg or pA_2 values)

Compound[a]	Uterotonic		Galactogogic	Pressor	Antidiuretic
	in vitro	*in vivo*			
Ib [D-Tyr^2]dC^{6}OXT	28	59	40·3	4	0·12
Ic [D-Phe^2]dC^{6}OXT	$pA_2 = 8·06$	$pA_2 = 7·15$	1·25	—	0·04
Id [D-Phe(Me)^2]dC^{6}OXT	$pA_2 = 8·73$	$pA_2 = 7·51$	10·3	—	0·17
If [D-Phe(Et)^2]dC^{6}OXT	$pA_2 = 8·73$	$pA_2 = 7·82$	$pA_2 = 5·82$	$pA_2 = 6·32$	0·08
Ig [D-Phe(Et)^2]dC^{6}OXT--sulfoxide	$pA_2 = 7·91$	$pA_2 = 7·5$	$pA_2 = 6·8$	$pA_2 = 5·75$	0·002
Ih [D-Tyr(Et)^2]dC^{6}OXT	$pA_2 = 7·45$	—	0	0	—
Ii [D-Phe(Et)^2]OXT	$pA_2 = 8·15$	$pA_2 = 7·70$	1·22	—	1·5
Ij [Mpa^1, D-Phe(Et)^2]-OXT	$pA_2 = 8·06$	$pA_2 = 7·12$	0·3	$pA_2 = 6·82$	—
Ik [Pen^1, Phe(Et)^2]OXT	$pA_2 = 7·78$	$pA_2 = 5·92$	0·45[b]	$pA_2 = 7·8$	0·025
Il [Pen^1, D-Phe(Et)^2]-OXT	$pA_2 = 8·09$	$pA_2 = 7·25$	$pA_2 = 7·7$	$pA_2 = 7·4$	0·0007

[a] dC^{6}OXT means deamino-6-carba-oxytocin; [b] in some cases inhibition was observed.

R-Ile-Gln-Asn-Hcy(C_2H_4COOH)-Pro-Leu-Gly-NH_2

IIa, R = H
IIb, R = Nps-D-Tyr
IIc, R = Nps-D-Phe
IId, R = Nps-DL-Phe(Me)
IIe, R = Nps-D-Phe(Et)
IIf, R = Nps-D-Tyr(Et)

R-Ile-Gln-Asn-Cys(Bzl)-Pro-Leu-Gly-NH_2

IIIa, R = Nps
IIIb, R = Nps-DL-Phe(Et)
IIIc, R = Z-Cys(Bzl)-DL-Phe(Et)
IIId, R = Mpa(Bzl)-DL-Phe(Et)
IIIe, R = Z-Pen(Bzl)-DL-Phe(Et)

Oxidation of carba-analogues of neurohypophyseal hormones affords sulfoxides exhibiting mostly lower activities than the corresponding sulfides[39,40] (for exceptions see ref.[40,49]), the 1-carba-analogues being substantially less active than the 6-carba-analogues. We prepared the sulfoxide of compound *If* which showed about six times lower inhibitory activity than the starting sulfide.

The inhibitory activities found in the *in vivo* test on the rat uterus parallel the activities determined *in vitro*. The galactogogic activity was inhibited only in the case of compound *If* and, surprisingly, its sulfoxide *Ig* was even more potent inhibitor. On the contrary, compound *Ij* which does not contain a carba-bridge, shows no inhibition. Except compound *Ik*, the prepared analogues are not effective inhibitors of vasopressin pressor activity. This indicates that for this inhibitor type (contrary to the uterotonic inhibitor) alkylation of the β-carbon atom of cysteine in position 1 represents a much more effective structural feature than configurational inversion in position 2 (compound *Ib* retains to a high degree its own pressor activity). Except compound *Ii*, all the prepared analogues exhibit a very low antidiuretic activity.

EXPERIMENTAL

The analytical samples were dried *in vacuo* (150 Pa) over phosphorus pentoxide at room temperature. Melting points were determined on a Kofler block and are uncorrected. Thin-layer chromatography was carried out on silica gel plates (Silufol, Kavalier, Czechoslovakia) in the systems 2-butanol–98% formic acid–water (75 : 13·5 : 11·5) (S1), 2-butanol–25% aqueous ammonia–water (85 : 7·5 : 7·5) (S2), 1-butanol–acetic acid–water (4 : 1 : 1) (S3), and 1-butanol–pyridine–acetic acid–water (15 : 10 : 3 : 6) (S4). Electrophoresis was performed on a Whatman 3MM paper in moist chamber (20 V/cm) for 1 h in 1 mol l^{-1} acetic acid (pH 2·4) and a pyridine–acetate buffer (pH 5·7). The compounds were detected by ninhydrin or by the chlorination method. Solvents were evaporated on a rotatory evaporator at bath temperature 30°C; dimethylformamide was evaporated at the same temperature at 150 Pa. Samples for amino acid analyses were hydro-

lyzed with 6 mol l^{-1} HCl at 105°C for 20 h and analyzed on an automatic analyzer, type 6020 (Development Workshops of Czechoslovak Academy of Sciences). The high performance liquid chromatography (HPLC) was carried out on an SP-8700 instrument equipped with an SP-8400 detector and an SP-4100 integrator (all from Spectra-Physics, Santa Clara, USA). L-Amino acid oxidase (0·3 U/mg) was a Serva product.

Dicyclohexylammonium Salt of *o*-Nitrobenzenesulfenyl-O-ethyl-D-tyrosine

o-Nitrobenzenesulfenyl chloride (5·78g) was added to a solution of D-tyrosine (2·83 g) in 4 mol. l^{-1} NaOH (9·8 ml) and dioxane (16 ml), the pH of the mixture being kept at 10 and the temperature below 5°C. After stirring for 30 min, diethyl sulfate (6·9 g) and 4 mol l^{-1} NaOH were added so as to keep the pH at 10·7. The alkylation course was followed by reversed-phase HPLC (70% of methanol and 30% of 0·05% aqueous trifluoroacetic acid; $k'_{NpsTyr} = 2{\cdot}21$, $k'_{NpsTyr(Et)} = 9{\cdot}56$). After disappearance of the starting compound (80 min), the mixture was filtered, extracted with ether and ethyl acetate and the aqueous layer was acidified at 0°C with 0·5 mol l^{-1} H_2SO_4 to pH 3 and extracted with ethyl acetate. The organic layer was washed with water, dried over sodium sulfate, concentrated and poured into an ethereal solution of dicyclohexylamine (3·44 ml in 200 ml). After standing overnight the crystals were collected on filter and dried, affording 6·5 g (76%) of the product, m.p. 174–178°C, $[\alpha]_D$ −49° (*c* 0·19; methanol). For $C_{29}H_{41}N_3O_5S$ (543·7) calculated: 64·06% C, 7·60% H, 7·73% N; found: 64·53% C, 7·55% H, 7·86% N.

General Method of Preparation of Octapeptides *IIb*–*IIf*

The dicyclohexylammonium salt of the amino acid derivative (0·8 mmol) was suspended in ethyl acetate (30 ml) at 0°C and the amino acid derivative was liberated by addition of 0·05 mol l^{-1} H_2SO_4. The ethyl acetate solution was dried over sodium sulfate and taken down. The residue was dissolved in dimethylformamide (4 ml), N-hydroxybenzotriazole (122 mg) was added, and, after cooling to 0°C, dicyclohexylcarbodiimide (182 mg). After stirring for 1 h at 0°C and 1 h at room temperature, the mixture was filtered into a flask containing the heptapeptide *IIa* (350 mg). The solid cake was washed with dimethylformamide (3 ml) and the obtained solution was stirred for 12 h at room temperature. After filtration and evaporation of solvent, the residue was triturated with ether and water, the product was dried and an analytical sample was precipitated with ether from dimethylformamide; its properties are given in Table III.

o-Nitrobenzenesulfenyl-*p*-ethyl-DL-phenylalanyl-isoleucyl-glutaminyl-asparaginyl-S-benzylcysteinyl-prolyl-leucyl-glycine Amide (*IIIb*)

A solution of the heptapeptide *IIIa* (2·5 g) in dimethylformamide (100 ml) was mixed with 3·4 mol l^{-1} HCl in ether (1·7 ml). After standing for 5 min at room temperature, the heptapeptide hydrochloride was precipitated with ether, filtered, dried *in vacuo* and dissolved in dimethylformamide (100 ml). *o*-Nitrobenzenesulfenyl-DL-*p*-ethylphenylalanine 2,4,5-trichlorophenyl ester (1·8 g) was added, the mixture was adjusted to pH 8 (moist indicator paper) and stirred at room temperature for 130 h. The solution was concentrated to a small volume, the product was precipitated with ether, filtered, washed with ether and water and dried *in vacuo*; yield 3·1 g (89%) of *IIIb* (for data see Table III).

Benzyloxycarbonyl-S-benzylcysteinyl-*p*-ethyl-DL-phenylalanyl-isoleucyl-glutaminyl-asparaginyl-S-benzylcysteinyl-prolyl-leucyl-glycine Amide (*IIIc*)

The octapeptide *IIIb* (0·5 g) was dissolved in dimethylformamide (25 ml) and the solution

TABLE III

Some properties of protected peptides

Compound	Yield, % m.p., °C	R_F S1 S3	R_F S2 S4	Formula (mol.w.)	Calculated/Found % C	% H	% N	$[\alpha]_D$, deg (c)[a]	Asp Ile	Glu Leu	Pro X	Gly Y	$E^{His}_{5,7}$ $E^{Gly}_{2,4}$
IIb	62	0·31	0·05	$C_{50}H_{72}N_{12}O_{15}S_2$.	50·84	6·48	14·23	−33·6	1·05	1·05	0·98	0·98	0·07
	202–204	0·42	0·66	.2 H_2O	50·57	6·27	14·07	(0·18)	0·92	1·07	1·00[b]	0·93[c]	0·63
				(1 145)									
IIc	88	0·32	0·07	$C_{50}H_{72}N_{12}O_{14}S_2$.	52·75	6·46	14·77	−29·1	1·03	0·93	1·06	1·01	0·08
	214–217	0·41	0·67	.0·5 H_2O	52·49	6·41	15·09	(0·15)	0·93	1·07	0·91[d]	0·89[c]	0·63
				(1 138)									
IId	71	0·52	0·06	$C_{51}H_{74}N_{12}O_{14}S_2$.	51·94	6·67	14·25	−20·0	1·00	0·97	1·03	1·02	0·11
	222–224	0·61	0·68	.2 H_2O	51·82	6·64	13·23	(0·13)	0·94	1·05	0·97[e]	0·94[c]	0·63
				(1 179)									
IIe	83	0·53	0·12	$C_{52}H_{76}N_{12}O_{14}S_2$.	52·33	6·76	14·08	−36·4	1·01	1·00	1·05	0·98	0·08
	227–228	0·55	0·67	.2 H_2O	51·93	6·48	14·08	(0·2)	0·93	1·03	0·94[f]	0·93[c]	0·64
				(1 193)									
IIf	53	0·47	0·13	$C_{52}H_{76}N_{12}O_{15}S_2$.	53·23	6·53	14·32	−39·0	1·01	1·04	1·00	1·07	0·11
	220–222	0·51	0·67	(1 173)	52·82	6·61	13·29	(0·15)	0·92	1·06	0·94[g]	0·91[c]	0·63
IIIb	89	0·83	0·56	$C_{55}H_{76}N_{12}O_{12}S_2$	56·01	6·67	14·25	−22·6	1·02	0·98	1·06	0·96	0·33
	233–240	0·66	0·76	.H_2O	55·95	6·41	13·39	(0·17)	0·92	1·02	0·81[f]	0·89[h]	0·63
				(1 179)									
IIIc	73	0·60	0·42	$C_{67}H_{90}N_{12}O_{13}S_2$	57·91	6·96	12·10	−40·8	1·05	1·00	1·06	1·01	0·42
	225–228	0·65	0·72	.3 H_2O	58·09	6·66	12·43	(0·12)	0·95	1·07	0·91[f]	1·94[h]	0·61
				(1 390)									
IIId	69	0·54	0·36	$C_{59}H_{83}N_{11}O_{11}S_2$	59·73	7·05	12·99	−27·2	1·00	0·99	1·04	0·99	—
	217–230	0·62	0·71	(1 187)	59·89	7·59	12·97	(0·14)	0·91	1·04	0·90[f]	0·94[h]	—
IIIe	87	0·61	0·43	$C_{69}H_{94}N_{12}O_{13}S_2$	59·21	7·06	12·01	−46·4	1·06	1·06	1·00	1·06	0·42
	202–205	0·66	0·73	.2 H_2O	58·63	6·52	12·05	(0·14)	0·97	1·03	0·91[f]	1·02[h]	0·60
				(1 400)									

[a] Dimethylformamide, [b] Tyr, [c] Hcy(C_2H_4COOH), [d] Phe, [e] Phe(Me), [f] Phe(Et), [g] Tyr+Tyr(Et), [h] Cys(Bzl).

was mixed with 3·45 mol l^{-1} HCl in ether. After standing for 5 min the product was precipitated with ether, filtered, dried *in vacuo* and dissolved in dimethylformamide (23 ml). *p*-Nitrophenyl ester of benzyloxycarbonyl-S-benzylcysteine (0·8 g) was added, the mixture was adjusted to pH 8 with N-ethylpiperidine, stirred at room temperature for 125 h, and concentrated to a small volume. The product was precipitated with ether, filtered, washed consecutively with ether, 1 mol l^{-1} HCl, water, 0·5 mol l^{-1} $NaHCO_3$, water, and dried; yield 0·42 g (73%). For data see Table III.

Benzyloxycarbonyl-S-benzylpenicillaminyl-*p*-ethyl-DL-phenylalanyl-isoleucyl-glutaminyl--asparaginyl-S-benzylcysteinyl-prolyl-leucyl-glycine Amide (*IIIe*).

Benzyloxycarbonyl-S-benzylcysteine *p*-nitrophenyl ester (0·9 g) was added to a solution of the octapeptide hydrochloride prepared in the same manner as in the case of *IIIc*. The condensation and work up were carried out as described for compound *IIIc*, yielding 0·46 g of *IIIe* (Table III).

S-Benzylmercaptopropionyl-*p*-ethyl-DL-phenylalanyl-isoleucyl-glutaminyl-asparaginyl--S-benzylcysteinyl-prolyl-leucyl-glycine Amide (*IIId*)

A solution of the octapeptide hydrochloride, prepared as described for *IIIe*, was adjusted to pH 8 with N-ethylpiperidine. S-Benzylmercaptopropionic acid (95 mg), followed by N-hydroxybenzotriazole (62 mg), was added and the mixture was cooled to −20°C and treated with dicyclohexylcarbodiimide (104 mg). After stirring at −20°C for 1 h, at 0°C for 30 h, and at room temperature for 2 h, the mixture was filtered and taken down. The residue was suspended in water, filtered, washed successively with 1 mol l^{-1} HCl, water and ether and dried, affording 380 mg of the product whose properties are given in Table III.

[2-D-Phenylalanine]deamino-6-carba-oxytocin (*Ic*)

A solution of the octapeptide *IIc* (150 mg) in dimethylformamide (4 ml) was mixed with 3·2 mol . . l^{-1} HCl in ether (0·4 ml). After standing for 6 min at room temperature, the octapeptide hydrochloride was precipitated with ether. The hydrochloride was reprecipitated from dimethylformamide with ether, filtered, dried *in vacuo* and dissolved in dimethylformamide (4 ml).N--Hydroxybenzotriazole (181 mg) was added, the mixture was cooled to 0°C and treated with dicyclohexylcarbodiimide (200 mg). After stirring at 0°C for 1 h and at room temperature for 1·5 h, the separated crystals of dicyclohexylurea were filtered off and the filtrate was introduced into warm (45°C) methanol (150 ml). The mixture was adjusted to pH 8·5 with N-ethylpiperidine, kept at 45°C for 2 h and concentrated to a small volume. The product was precipitated with ether, filtered and dried *in vacuo*; yield 140 mg. A part (30 mg) was dissolved in aqueous methanol (1 : 1, 10 ml), applied on a column of Partisil ODS-2 (50 × 0·9 cm) and eluted with a mixture of 55% methanol and 0·05% aqueous trifluoroacetic acid. The product-containing fraction ($k' = 7·3$) was taken down *in vacuo* and freeze-dried, affording 6·5 mg of the product *Ic*, pure according to HPLC and TLC. Its properties are given in Table IV.

[2-D-Tyrosine]deamino-6-carba-oxytocin (*Ib*)

The octapeptide *IIb* (150 mg) was cyclized in the same manner as the analogue *Ic*. The crude product was purified on the same column using a mobile phase containing 40% of methanol. Freeze-drying of the product fractions afforded 22 mg (16%) of the product which, according to HPLC of sulfoxides prepared by periodate oxidation, contained 0·8% of the L-tyrosine analogue (compared with the authentic sulfoxide[39]). Therefore, a part of the product (10 mg) was

TABLE IV

Some characteristics of oxytocin analogues

Compound	R_F S1 S3	R_F S2 S4	Formula (mol.w.)	Calculated/Found % C	% H	% N	$[\alpha]_D$, deg (c)[a]	Asp Ile	Glu Leu	Pro X	Gly Y
Ib	0·18	0·08	$C_{44}H_{67}N_{11}O_{12}S.3\ H_2O$	51·41	7·16	14·98	−81·1	1·02	0·99	1·00	1·0
	0·14	0·60	(1 028)	51·06	7·02	14·77	(0·14)	0·94	1·06	0·97[b]	1·02[c]
Ic	0·13	0·07	$C_{44}H_{67}N_{11}O_{11}S.2\ H_2O$	53·16	7·20	15·50	−89·3	1·04	1·05	0·95	1·04
	0·14	0·62	(994·2)	52·88	7·39	15·29	(0·2)	0·94	1·05	0·98[d]	0·97[e]
Id	0·15	0·08	$C_{45}H_{69}N_{11}O_{11}S.3\ H_2O$	52·67	7·37	15·01	−91·3	1·01	1·00	1·06	1·02
	0·16	0·62	(1 026)	52·40	7·61	14·83	(0·15)	0·91	1·04	0·99[e]	0·97[c]
If	0·16	0·07	$C_{46}H_{71}N_{11}O_{11}S.4\ H_2O$	52·21	7·52	14·56	−102·6	0·97	0·97	1·08	0·98
	0·16	0·62	(1 054)	51·90	7·64	14·41	(0·07)	0·92	1·01	1·03[f]	1·03[c]
Ih	0·11	0·08	$C_{46}H_{71}N_{11}O_{12}S.4\ H_2O$	51·43	7·41	14·34	−89·9	1·03	1·02	1·01	0·99
	0·13	0·61	(1 074)	51·27	7·65	14·08	(0·09)	0·95	1·02	1·02[g]	0·96[c]
Ii	0·08	0·06	$C_{45}H_{70}N_{12}O_{11}S_2.$	50·61	7·05	15·07	−79·1	0·96	0·98	1·01	0·95
	0·03	0·51	$.2\ H_2O.C_2H_4O_2$ (1 115)	50·36	6·81	15·23	(0·05)	1·07	1·02	1·01[f]	1·76[h]
Ij	0·16	0·09	$C_{45}H_{69}N_{11}O_{11}S_2.4\ H_2O$	50·22	7·21	14·32	−38·0	1·02	0·97	0·93	1·00
	0·17	0·62	(1 076)	49·94	7·24	14·18	(0·08)	7·91	1·00	1·00[f]	0·53[h]
Ik	0·14	0·08	$C_{47}H_{74}N_{12}O_{11}S_2.$	52·31	7·17	14·93	−91·1	1·03	1·00	1·07	1·02
	0·08	0·61	$.H_2O.C_2H_4O_2$ (1 125)	51·98	7·38	14·66	(0·08)	0·93	0·96	0·96[f]	1·48[i]
Il	0·11	0·07	$C_{47}H_{74}N_{12}O_{11}S_2.$	50·67	7·29	14·47	−66·7	0·98	1·00	0·92	1·00
	0·07	0·61	$.3\ H_2O.C_2H_4O_2$ (1 161)	50·12	7·56	13·99	(0·13)	1·03	1·02	0·97[f]	1·56[h]

[a] 3 mol l^{-1} acetic acid, [b] Tyr, [c] Hcy($C_2H_4CO_2H$), [d] Phe, [e] Phe(Me), [f] Phe(Et), [g] Tyr+Tyr(Et), [h] Cys, [i] Cys+Pen.

oxidized with periodate and purified again on the above-mentioned column (elution with 35% methanol). A part of the sulfoxide, obtained by freeze-drying of the corresponding fractions, was used for biological assays, another part (7 mg) was suspended in acetone (200 µl), treated with 35% HBr in acetic acid (200 µl) at room temperature for 5 min and again purified on a reversed phase column (40% methanol) to give 4·6 mg of the compound.

[2-*p*-Methyl-D-phenylalanine]deamino-6-carba-oxytocin (*Id*)

The octapeptide *IId* (280 mg) was cyclized as described for the analogue *Ic*. A part of the crude product (100 mg) was dissolved in a mixture of methanol (4 ml) and water (6 ml) and applied on a column of Partisil ODS (50 × 0·9 cm). Elution with a mixture of methanol (55%) and 0·05% aqueous trifluoroacetic acid afforded fractions of $k' = 5·7$ (12·6 mg) and $k' = 7·6$ (13·7 mg), the former containing a compound identical with the already described [2-*p*-methyl-L-phenylalanine]-deamino-6-carba-oxytocin. The acid hydrolysate of both fractions was incubated with L-amino acid oxidase which removed *p*-methylphenylalanine from the former but not from the latter material. Properties of the analogue *Id* are given in Table IV.

[2-*p*-Ethyl-D-phenylalanine]deamino-6-carba-oxytocin (*If*)

Cyclisation of the octapeptide *IIe* (300 mg) was carried out as described for the analogue *Ic* and afforded 249 mg of the crude product. This material was dissolved in a mixture of methanol (10 ml) and water (18 ml) and the filtered solution was purified in three portions on a column of Partisil ODS (50 × 0·9 cm) using methanol—0·05% trifluoroacetic acid (56 : 44) as the mobile phase. Concentration and freeze-drying of fractions of $k' = 9·4$ gave 68 mg of the product whose properties are given in Table IV.

[2-*p*-Ethyl-D-phenylalanine]deamino-6-carba-oxytocin Sulfoxide (*Ig*)

Sodium periodate was added to a solution of the analogue *If* (4 mg) in aqueous methanol (1 : 1; 400 µl). After standing for 2 h at room temperature, the mixture was applied on a column of Partisil ODS (50 × 0·9 cm) and eluted with a methanol–water (3 : 2) mixture. Freeze-drying of the product fractions afforded 2·8 mg (64%) of the product, R_F 0·14 (S1), 0·05 (S2), 0·16 (S3), 0·62 (S4). For $C_{46}H_{71}N_{11}O_{12}S.4\,H_2O$ (1 074) calculated: 51·43% C, 7·41% H, 14·34%N; found: 51·15% C, 6·85% H, 14·26% N. Amino acid analysis: Asp 1·00, Glu 1·00, Pro 1·03, Gly 1·02, Ile 0·93, Leu 1·01, Phe(Et) 1·01, Hcy($C_3H_5O_2$) 0·53.

[2-O-Ethyl-D-tyrosine]deamino-6-carba-oxytocin (*Ih*)

The octapeptide *IIf* (130 mg) was cyclized in the same way as described for the analogue *Ic*, affording 135 mg of the crude product. A part of this material (30 mg) was dissolved in a methanol–water mixture (4 : 6), applied on a column of Partisil ODS-2 (50 × 0·9 cm) and eluted with a mixture of methanol and 0·05% trifluoroacetic acid (1 : 1). The fraction of $k' = 26·3$ was concentrated and freeze-dried, affording 6·2 mg of the product, pure according to HPLC and TLC. Its properties are given in Table IV.

[2-*p*-Ethyl-D-phenylalanine]deamino-oxytocin (*Ij*)

The peptide *IIId* (50 mg) was dissolved in liquid ammonia and reduced with a sodium rod until the blue colouration persisted for 1 min. The excess sodium was destroyed with a drop of acetic acid and the mixture was freeze-dried. The residue was dissolved in 0·1 mol l^{-1} HCl (8 ml),

the solution was diluted to 100 ml and adjusted to pH 7 with 0·1 mol l^{-1} NaOH. A solution of $K_3Fe(CN)_6$ (25 mg in 5 ml of water) was added during 20 min the pH value being kept at 7. After stirring for 40 min at room temperature, the mixture was adjusted to pH 4 by addition of acetic acid and applied on a column of Sepharon SI-C-18 (25 × 0·4 cm) by means of a high pressure pump. The column was washed with water and the peptides were eluted with a mixture of 80% methanol and 0·05% aqueous trifluoroacetic acid. The eluate was concentrated *in vacuo*, freeze-dried, the residue was again dissolved in aqueous methanol (1 : 1; 8 ml) and applied on a column of Partisil ODS (50 × 0·9 cm). Elution with a mixture of methanol and 0·05% aqueous trifluoroacetic acid (65 : 35) afforded two fractions of $k' = 10·2$ (8·1 mg; 38%) and $k' = 15·9$ (7·6 mg; 36%). The former product contained *p*-ethyl-L-phenylalanine whereas the latter contained *p*-ethyl-D-phenylalanine (according to incubation of the hydrolysate with L-amino acid oxidase). The properties of the latter compound are given in Table IV.

[2-*p*-Ethyl-D-phenylalanine]oxytocin (*Ii*)

The nonapeptide *IIIc* (200 mg) was reduced and oxidized as described for the compound *Ij*. The cyclization solution was applied on a column of Amberlite IRC-50 (30 ml), the column was washed with 0·25% acetic acid and the peptides were eluted with 50% acetic acid. The product obtained by freeze-drying was dissolved in 50% aqueous methanol (8 ml), applied on a column of Partisil ODS (50 × 0·9 cm) and eluted with a mixture of methanol (60%) with 0·1 mol l^{-1} ammonium acetate (40%), pH 7. Concentration and freeze-drying of the product fraction afforded 16·4 mg (21%) of compound, $k' = 11·6$, containing *p*-ethyl-L-phenylalanine, and 15·8 mg (20%) of compound, $k' = 23·3$, containing *p*-ethyl-D-phenylalanine (incubation with L-amino acid oxidase). The former product was identical with the sample prepared 16 years ago[7], stored in the form of lyophilisate at room temperature. For properties of the latter compound see Table IV. As side product we obtained compounds which (according to amino acid analysis) did not contain the terminal tripeptide ($k' = 7·3$ and 14·4; 5·2 and 3·8 mg, respectively).

[1-Penicillamine, 2-*p*-ethyl-D- and L-phenylalanine]oxytocin (*Ik* and *Ie*)

The nonapeptide *IIIe* (200 mg) was reduced and oxidized as described for the analogue *Ii*. The cyclization mixture was processed and purified in the same manner as in the case of *Ij* except that the elution was carried out with a mixture containing 65% of methanol. Concentration and freeze-drying of the corresponding fractions afforded 17·4 mg (22%) of product containing *p*-ethyl-L-phenylalanine ($k' = 8·05$) and 14·6 mg (19%) of compound containing *p*-ethyl-D-phenylalanine ($k' = 13·8$); for their properties see Table IV.

Pharmacological Methods

Uterotonic activity was determined on an isolated strip of rat uterus[51,52], the activity *in vivo* was estimated according to Pliška[53]. Galactogogic activity was determined on ethanol-anesthetized rats[54,55], pressor activity on despinalized rats[56] and antidiuretic potency on anesthetized rats[57,58]. Inhibitory properties were expressed by the pA_2 value; for the test *in vitro* (rat uterus) the recommendations[59] were obeyed, for the assay *in vivo* we followed the described method[15,60].

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