SYNTHESIS AND PROPERTIES OF OXYTOCIN ANALOGUES WITH HIGH AND SELECTIVE NATRIURETIC ACTIVITY*

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Deamino-6-carba-oxytocin analogues with modified aromatic amino acid in the position 2 were prepared by stepwise peptide synthesis in solution and cyclization in the last step. Some of the prepared analogues exhibit high and/or specific natriuretic activity. Structural requirements for this type of biologic activity are discussed.

In addition to the typical biological effects, such as uterotonic, galactogogic, antidiuretic or pressor, neurohypophysial hormones exhibit many other activities which can be detected only under specific, suitably chosen, experimental conditions (such as effect on central nervous system, CRF-activity, fat mobilization, vasoconstrictor activity, natriuretic effect, *etc.*). In most cases, however, these effects are obscured by the "common" activities: the natriuretic effect by strong antidiuresis or the CRF activity by pressor activity. Aimed modifications of the parent structure of these hormones, based on structure-activity relationship studies, enable us to differentiate between the biological activities (*i.e.* to suppress some of them with simultaneous enhancing others).

The natriuretic activity of oxytocin was observed already many years ago[1], however, it was observable only within a narrow range of experimental conditions. Structural modifications, which would enhance this effect have been sought. The assumption that this can be achieved by introduction of a more lipophilic amino acid* into the position 4 (instead of glutamine) or by cumulation of lipophilic moieties in the amino-terminal part of the molecule proved to be erroneous (see ref.[3]). Some activity increase was observed with deamino-oxytocin[4] and [2-O-methyltyrosine]oxytocin[4,5], although the activity of the latter compound was higher only in relation to the uterotonic activity. Potentiation of the natriuretic effect was found with carba-

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* 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[2].

-analogues of oxytocin[4,6] which, however, exhibit also substantially higher other biological activities[7]. Nevertheless, we could assume that, starting from these structural modifications (replacement of the α-amino group with a hydrogen atom and of the disulfide by a thioether grouping) further suitable replacements of amino acids could bring about the desired dissociation of activities (*i.e.* to reduce other biological activities without affecting the relatively high natriuretic activity). However, such replacement, realized in the position 4 of the amino acid sequence, was again unsucccessful[3,4]. Also other tested analogues did not show any significant natriuretic effect[8,9].

We turned our attention to the position 2 since in preliminary experiments in this direction[4] showed promising results. Of the two alternative replacements of the disulfide bond we preferred the S—CH_2 grouping to the opposite one (CH_2—S) because deamino-6-carba-oxytocin exhibited a significantly higher natriuretic effect[6]. We prepared 10 analogues of deamino-6-carba-oxytocin and studied their basic pharmacological activities, together with natriuretic effect which was determined under two different experimental conditions (Table I). A part of our results was published as a preliminary communication[10].

Our synthesis of oxytocin analogues started from the largest common intermediate, amide of isoleucyl-glutaminyl-asparaginyl-S-(β-carboxyethyl)homocysteinyl-prolyl--leucyl-glycine[11] (*IIa*) with some modifications of the previously described procedure[11]. The introduction of the benzyloxycarbonyl group into S-(β-methoxycarbonylethyl)homocysteine gave invariably low yields with both benzyloxycarbonyl chloride and thiosulfate[12]. The yields were reduced by formation of the S,N-disubstituted side-product (whose structure was proved by the NMR and mass spectroscopy and elemental analysis) which, because of its low colour yield in reaction with ninhydrin, escaped detection in the purity checks by thin-layer chromatography, paper electrophoresis and amino acid analysis. High performance liquid chromatography (HPLC) revealed that in some experiments the amount of this side-product reached 30%. The pure side-product, *i.e.* S,N-bis(methoxycarbonylethyl)homocysteine, was obtained from homocysteine and methyl acrylate in liquid ammonia. It is not acylated with benzyloxycarbonyl thiosulfate and therefore is removed in the preparation of the product with protected amino group. We prepared S-(β-methoxycarbonylethyl)-homocysteine also directly by reaction of methionine and β-bromopropionic acid[13], followed by selective esterification of the ω-carboxyl. Optical purity of the product was proved with L-amino acid oxidase.

The modified aromatic amino acids were obtained either from L-phenylalanine or tyrosine by described procedures. *p*-Methylphenylalanine and *p*-ethylphenylalanine were prepared as racemates from the corresponding *p*-alkylanilines[14] and after transformation into chloroacetyl derivatives they were resolved with carboxypeptidase[14,15]. In the case of *p*-ethyl-L-phenylalanine we checked the alternative path *via* the ethyl ester which was cleaved with chymotrypsin analogously as described[16]

TABLE I

Biological activities (in rat) of oxytocin analogues (I.U./mg)

Compound	R[a]	Uterotonic			Galactogogic	Pressor	Antidiuretic	Natriuretic[b]	
		in vitro	*in situ*	I_p[c]				anesthetised	conscious
Ia		450	450	1·00	450	3	3	100	100
Ib		795	900	1·60	266·5	1·4	19	—	132
Ic		1—1·5	20—25	—	11·8	[d]	0·01	10—15	102
Id	OH	929	2 792	5·78	456	1·5	118	400—600	298
Ie	H	70	450	1·85	170	0·9	1·5	—	131
If	Me	127	450	2·96	37·4	1·0	31	582	326
Ig	Et	27	39	4·84	6·3	[e,f]	14·3	265	255
Ih	Et	17	14	—	—	[e]	0·9	—	155
Ii	OMe	3·1	75	4·84	18	[f]	—	33	143
Ij	OEt	0·001	2·9	1·90	5·6	[e]	0·005	21	31
Ik	Cl	35	307	—	140	[e]	33	243	232
Il	NH_2	13	94	1·77	6·9	[e]	2	90	87
Im	NMe_2	0·05	3·3	4·56	4·5	[e]	4	25	75
In	NHZ	0·07	[g]	—	—	[e]	0·002	—	10
Io	NO_2	0·001	19·8	2·30	10·4	[e]	0·2	44	66

[a] Substituent in the *para*-position of carba-analogues; [b] % of the activity of oxytocin; [c] index of persistence (ref.[34]), calculated for the elimination constant of the compound *Ia* = 0·237; [d] tachyfylaxis; [e] inactive up to doses $1 \cdot 10^{-2}$ mg; [f] inhibitory effect; [g] inactive up to doses $2 \cdot 10^{-2}$ mg.

for other substituted aromatic acids. With *p*-ethylphenylalanine, the reaction was slower than with tyrosine and it was necessary to use a much greater (by an order of magnitude) excess of chymotrypsin. Optical purity of the obtained amino acid was checked by HPLC on a dynamically anchored chiral phase[17] (copper complex of L-phenylalanine). The reagent of choice for the preparation of optically pure *p*-ethylphenylalanine proved to be penicillin amidohydrolase (E.C.3.5.1.11.), particularly its immobilized form[18,19]. Contrary to the previous opinions[20–22], this enzyme cleaves partially (and apparently according to the reaction conditions) also phenylacetyl-D-amino acids[23]. In the case of phenylacetyl-*p*-ethyl-DL-phenylalanine the acyl moiety is removed from the D-amino acid substantially more slowly. If the reaction is stopped at the moment of almost complete conversion of the L-form derivative, the optically pure amino acid is obtained. The reaction course was followed by liquid chromatography on a reversed phase, the arising phenylacetic acid as well as the disappearing acylated component being detected by absorption at 244 nm. The optical purity of the derived amino acid was checked by chromatography[17] and by incubation with L-amino acid oxidase.

The obtained amino acids were converted into their *o*-nitrobenzenesulfenyl derivatives (except *p*-chlorophenylalanine which was used as the Boc derivative[24]) which were characterized as such or as dicyclohexylammonium salts. For the acylation of the heptapeptide *IIa* we prepared in all cases the corresponding 2,4,5-trichlorophenyl esters. In cases when the active ester did not crystallize we performed the acylation with the oily product. Data for the amino acid derivatives are listed in Table II.

It was tempting to use chymotrypsin in acylation of the heptapeptide *IIa* because it would be possible to work with the racemic aromatic amino acid. In the case of *p*-ethylphenylalanine the rate of the concurrent removal of the terminal glycinamide was comparable with that of the desired reaction (as found by an HPLC kinetic study) and therefore this approach could not be realized. The properties of the prepared octapeptides *IIb*–*IIi* are given in Table III.

The octapeptides were cyclized after transformation of the free carboxyl into the active ester in pyridine using the high dilution technique. In accord with the literature data[25] better yields were obtained with 2,4,5-trichlorophenyl than *p*-nitrophenyl ester; we found also that it was not necessary to add N-ethylpiperidine to the cyclization solution. Good results were obtained also with cyclization of hydroxybenzotriazolyl ester in methanolic solution; the active ester was prepared, after removal of the amino-protecting group, from the octapeptide hydrochloride[26].

The cyclization products were purified by filtration through an ion exchange resin, gel filtration, free-flow electrophoresis and countercurrent distribution. Neither of these methods afforded a pure, sulfoxide-free product[27]. Liquid chromatography on a reversed phase with methanol as the mobile phase proved to be the most effective method. The compound *Im* was purified in a phosphate buffer (the product was

TABLE II

Yields, physical constants and analytical data of amino acid derivatives

Compound	Yield, % M.p., °C	$[\alpha]_D$,° (c)[a]	R_F S1 S3	R_F S2 S4	Formula (Mol.w.)	Calculated/Found % C	% H	% N
Nps-Phe(*p*-NO_2)OH.DCHA[b]	76	+13·1	0·84	0·58	$C_{27}H_{36}N_4O_6S$	59·54	6·66	10·29
	190—193	(0·06)	0·78	0·66	(544·7)	59·41	6·63	9·99
Nps-Tyr(Et)OH.DCHA[b]	39	+4·9	0·84	0·37	$C_{29}H_{41}N_3O_5S$	64·06	7·60	7·73
	163—165	(0·2)	0·84	0·67	(543·7)	63·95	7·52	7·52
Nps-Phe(*p*-NHZ)OH.DCHA[b]	78	+30·1	0·84	0·61	$C_{35}H_{44}N_4O_6S$	64·79	6·83	8·63
	170—174	(0·19)	0·78	0·73	(648·8)	64·50	6·54	8·94
Nps-Phe(*p*-NHZ)OTcp[c]	96	−5·9	0·88	0·74	$C_{29}H_{22}Cl_3N_3O_6$	53·84	3·43	6·50
	54—57	(0·20)	0·87	0·88	(646·9)	53·86	4·07	6·57
Nps-Phe(*p*-NMe_2)OH.DCHA[b]	69	+49·0	0·32	0·31	$C_{29}H_{42}N_4O_4S.H_2O$	62·11	7·90	9·99
	159—163	(0·19)	0·37	0·67	(560·8)	61·85	7·51	9·29
Nps-Phe(*p*-NMe_2)OTcp[c]	84	+21·7	0·43	0·67	$C_{23}H_{20}Cl_3N_3O_4$	51·07	3·73	7·77
	113—117	(0·11)	0·60	0·83	(540·8)	50·76	4·28	7·88
Nps-Phe(*p*-Et)OH	85	+45·3	0·81	0·61	$C_{17}H_{18}N_2O_4S.1/2\ H_2O$	57·45	5·39	7·88
	108—111	(0·15)	0·77	0·78	(355·5)	57·47	5·11	8·18
Nps-Phe(*p*-Et)OH.DCHA[b]	92	+17·2	0·81	0·61	$C_{29}H_{41}N_3O_4S$	66·00	7·83	7·96
	170—173	(0·13)	0·77	0·73	(527·7)	65·67	7·75	7·92
Nps-Phe(*p*-Me)OH.DCHA[b]	59	+11·0	0·84	0·48	$C_{28}H_{39}N_3O_4S$	65·47	7·65	8·18
	176—178	(0·20)	0·85	0·67	(513·7)	65·68	7·90	8·05
Nps-Phe(*p*-Me)OTcp[c]	75	−25·0	0·84	0·70	$C_{22}H_{17}Cl_3N_2O_4$	51·63	3·32	5·47
	127—133	(0·20)	0·87	0·86	(511·8)	51·96	3·77	5·75

[a] Dimethylformamide; [b] DCHA denotes dicyclohexylamine; [c] OTcp denotes 2,4,5-trichlorophenyl ester.

TABLE III

Some characteristics of the protected octapeptides

	$E^{His}_{5.7}$ $E^{Gly}_{2.4}$[a]	R_F S1 S3	R_F S2 S4	M.p., °C	Formula (Mol.w.)	$[\alpha]_D$ (c)[b]	Calculated/Found % C	% H	% N	Asp Ile	Glu Leu	Pro X	Gly Hcy($C_3H_5O_2$)
IIb	0·14	0·37	0·07	215—219	$C_{50}H_{71}N_{13}O_{16}S_2$	+50·1	51·14	6·09	15·51	1·05	1·04	1·05	1·07
	0·57	0·49	0·62		(1 174)	(0·18)	51·10	6·29	15·21	0·91	1·07	0·95[c]	0·92
IIc	0·10	0·40	0·17	223—225	$C_{50}N_{72}N_{12}O_{14}S_2$·	+21·6	50·72	6·64	14·19	1·05	1·01	1·01	1·04
	0·66	0·49	0·65		.3 H_2O (1 183)	(0·21)	50·69	6·38	14·31	0·93	1·02	0·96[d]	0·99
IId	0·11	0·35	0·07	220—226	$C_{51}H_{74}N_{12}O_{14}S_2$·	+13·1	52·74	6·60	14·47	1·04	1·10[e]	0·87	1·06
	0·68	0·49	0·66		.H_2O (1 161)	(0·20)	52·75	6·67	14·66	0·91	1·05	1·03[e]	0·95
IIe	0·09	0·48	0·10	222—224	$C_{52}H_{76}N_{12}O_{14}S_2$·	−12·2	52·34	6·76	14·08	1·00	1·02	0·99	1·01
	0·61	0·41	0·69		.2 H_2O (1 193)	(0·20)	52·48	6·50	13·81	0·90	1·01	1·00[f]	0·84
IIf	0·14	0·36	0·07	215—218	$C_{52}H_{76}N_{12}O_{15}S_2$·	+2·1	52·03	6·63	14·00	1·07	1·02	1·07	1·03
	0·56	0·48	0·62		.1·5 H_2O (1 200)	(0·20)	51·97	6·55	13·98	0·92	1·07	0·96[g]	0·85
IIg	0·10	0·45	0·09	214—217	$C_{49}H_{76}ClN_{11}O_{14}S$.	+24·7	52·14	6·97	13·65	0·97	0·97	1·04	1·02
	0·68	0·40	0·69		.H_2O (1 129)	(0·20)	52·26	7·20	13·44	0·93	1·07	0·99[h]	1·00
IIh	0·11	0·47	0·19	220—222	$C_{58}H_{79}N_{13}O_{16}S_2$·	+22·0	53·00	6·36	13·85	1·07	1·06	0·98	1·08
	0·65	0·57	0·69		.2 H_2O (1 315)	(0·18)	53·03	6·45	14·09	0·93	1·10	0·85[i]	0·89
IIi	0·21	0·11	0·06	201—204	$C_{52}H_{77}N_{13}O_{14}S_2$·	+ 8·2	51·69	6·75	15·07	1·08	1·01	1·10	1·06
	0·96	0·10	0·66		.2 H_2O (1 208)	(0·21)	51·81	6·56	14·98	1·00	1·07	0·74[j]	0·84

[a] After removal of the amino-protecting group; [b] dimethylformamide; [c] Phe(*p*-NO_2); [d] Phe; [e] Phe(*p*-Me); [f] Phe(*p*-Et); [g] Tyr(Et) determined as the sum Tyr + Tyr(Et); [h] Phe(*p*-Cl); [i] Phe(*p*-$NHOCOCH_2C_6H_5$) determined as Phe(*p*-NH_2); [j] Phe(*p*-NMe_2).

desalted on a small column with a reversed phase[28]) or better in a triethylammonium hydrogen carbonate buffer which was removed by freeze-drying. Use of 0·1% trifluoroacetic acid[29] did not lead to a pure product.

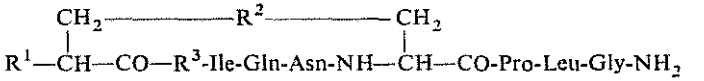
The corresponding sulfoxides arising as side-products in all the cyclizations, were separated by liquid chromatography as peaks of lower k' values. The oxidation state of sulfur in the prepared compounds was checked by liquid chromatography[30] (k' values of the analogues and their sulfoxides are given in Table IV). We did not observe any chromatographic separation of the diastereoisomeric sulfoxides (if both forms arise at all) which is not surprising for the 6-carba-analogues[30,31]. Compound *Ig* was the most interesting of the prepared analogues and we prepared for biological testing also its sulfoxide *Ih* by oxidation with sodium periodate. The product was desalted and purified by liquid chromatography on a reversed phase. On the other hand, we confirmed again that the analogue *Ig*, contaminated with its sulfoxide (*e.g.* as the result of precipitation from methanol by peroxide-containing ether) can be reduced with hydrogen bromide and acetone[32,33]. After storing *Ig* in aqueous methanol for half a year at room temperature, no sulfoxide or degradation products were detected (HPLC).

The compound *Il* was prepared by removal of the benzyloxycarbonyl protecting group from compound *In* or by reduction of the nitro group in compound *Io* with sodium in liquid ammonia. Both methods gave the same compound. The properties of the prepared analogues are given in Table IV. Synthesis of the compound *Ii* was described earlier[34].

Biological activities of the synthesized analogues and some reference compounds are given in Table I. From the structural point of view, the analogues differ only in the *para*-substituent in the aromatic amino acid in the position 2 (substituted nitrogen atom, alkylether or alkyl groups, hydrogen or chlorine atoms). A strong drop in all activities, including the natriuretic one, was observed with the analogues containing the *para*-nitrogen atom (compounds *Il* to *Io*). The same was true for the analogue *Ij* with the ethoxy group. On the other hand, analogues with hydrogen (*Ie*) or chlorine (*Ik*) atom or methoxy group (*If*) exhibit a higher natriuretic effect than oxytocin (*Ia*); however, also other activities are relatively high although they are lower than those of the parent deamino-6-carba-oxytocin (*Id*). The highest degree of dissociation between the natriuretic and other activities (with a high natriuretic activity) was found for the *para*-ethyl derivative *Ig*.* Its sulfoxide *Ih* has only about half of activity of the compound *Ig*; this is in agreement with the previous observations with 6-carba analogues[33]. Pressor activity of all the prepared analogues was very low or negligible and also the galactogogic activity (except compounds *Ie* and *Ik*) did not reach even one tenth of the oxytocin activity. Although the studied analogues increased also

* This analogue is now under study as a potential salureticum under the generic name Nacartocin.

renal excretion of potassium, the increase was relatively small compared with the natriuretic effect. The half-time estimated on the basis of antidiuresis in Burn's test was in all cases lower than for the starting compound *Id*. This means that these differences in the half-time are not caused by enzymatic cleavage but by the rate at which the compounds are removed from the receptor compartment. Also the index of persistence[35] for the uterotonic effect, related to the elimination constant of oxytocin ($k = 0{\cdot}237$) was for all the analogues lower than for the compound *Id*.



Ia, $R^1 = NH_2$, $R^2 = S{-}S$, R^3 = Tyr
Ib, $R^1 = H$, $R^2 = S{-}S$, R^3 = Tyr
Ic, $R^1 = NH_2$, $R^2 = S{-}S$, R^3 = Tyr(Me)
Id, $R^1 = H$, $R^2 = S{-}CH_2$, R^3 = Tyr
Ie, $R^1 = H$, $R^2 = S{-}CH_2$, R^3 = Phe
If, $R^1 = H$, $R^2 = S{-}CH_2$, R^3 = Phe(*p*-Me)
Ig, $R^1 = H$, $R^2 = S{-}CH_2$, R^3 = Phe(*p*-Et)
Ih, $R^1 = H$, $R^2 = SO{-}CH_2$, R^3 = Phe(*p*-Et)
Ii, $R^1 = H$, $R^2 = S{-}CH_2$, R^3 = Tyr(Me)
Ij, $R^1 = H$, $R^2 = S{-}CH_2$, R^3 = Tyr(Et)
Ik, $R^1 = H$, $R^2 = S{-}CH_2$, R^3 = Phe(*p*-Cl)
Il, $R^1 = H$, $R^2 = S{-}CH_2$, R^3 = Phe(*p*-NH_2)
Im, $R^1 = H$, $R^2 = S{-}CH_2$, R^3 = Phe(*p*-NMe_2)
In, $R^1 = H$, $R^2 = S{-}CH_2$, R^3 = Phe(*p*-$NHOCOCH_2C_6H_5$)
Io, $R^1 = H$, $R^2 = S{-}CH_2$, R^3 = Phe(*p*-NO_2)

R-Ile-Gln-Asn-Hcy($C_2H_4CO_2H$)-Pro-Leu-Gly-NH_2

IIa, R = H
IIb, R = Nps-Phe(*p*-NO_2)
IIc, R = Nps-Phe
IId, R = Nps-Phe(*p*-Me)
IIe, R = Nps-Phe(*p*-Et)
IIf, R = Nps-Tyr(Et)
IIg, R = Boc-Phe(*p*-Cl)
IIh, R = Nps-Phe(*p*-$NHOCOCH_2C_6H_5$)
IIi, R = Nps-Phe(*p*-NMe_2)

All the analogues (except compound *In*) had higher *in vivo* than *in vitro* uterotonic activities. In some cases the difference was only small but for compounds *Ij*, *Im* and *Io* it amounted several orders of magnitude. If we consider that the *in vivo* test involves many processes in which the tested compound may undergo degradation or transformation into other types of compounds (such as oxidation to sulfoxide, enzymatic inactivation, binding (possibly irreversible) to various macromolecules, reduced bioavailability *etc.*), we would expect an opposite effect, *i.e.* higher activities in the *in vitro* test. The smaller differences could be explained by different arrangements of the tests and different evaluation of the results (the *in vivo* test concerns

TABLE IV

Some characteristics of oxytocin analogues

Compound	R_F S1 S3	R_F S2 S4	Formula (Mol.w.)	Calculated/Found % C	% H	% N	Asp Ile	Glu Leu	Pro X	Gly Hcy($C_3H_5O_2$)	HPLC k'^a	System[b]
Ie	0·15 0·15	0·07 0·62	$C_{44}H_{67}N_{11}O_{11}S.2\ H_2O$ (994·2)	53·15 52·90	7·20 6·95	15·50 15·26	0·96 0·97	1·01 1·06	1·02 1·01[c]	0·97 1·01	7·00 (5·60)	B1
If	0·28 0·23	0·14 0·69	$C_{45}H_{69}N_{11}O_{11}S.4\ H_2O$ (1 044)	51·76 51·89	7·43 7·15	14·75 14·73	1·03 0·96	1·01 1·05	0·94 1·08[d]	1·03 0·90	4·15 (3·23)	B2
Ig	0·27 0·22	0·14 0·66	$C_{46}H_{71}N_{11}O_{11}S.H_2O.$ $.C_2H_4O_2$ (1 034)	54·58 54·74	7·31 7·03	14·89 14·67	1·00 0·97	1·00 1·00	1·04 1·09[e]	0·99 0·96	3·50	B3
Ih	0·25 0·20	0·14 0·66	$C_{46}H_{71}N_{11}O_{12}S.4\ H_2O$ (1 074)	51·43 51·35	7·41 7·03	14·32 14·46	1·00 0·93	1·00 1·01	1·04 1·01	1·02 0·53	2·81	B3
Ij	0·18 0·22	0·12 0·63	$C_{46}H_{71}N_{11}O_{12}S.$ $.2\ C_2H_4O_2$ (1 122)	53·51 53·25	7·10 7·22	13·73 13·75	1·07 0·97	1·04 1·07	0·94 0·89[f]	1·00 1·00	2·27 (1·85)	B2
Ik	0·28 0·22	0·14 0·66	$C_{44}H_{66}ClN_{11}O_{11}S.2\ H_2O$ (1 029)	51·38 51·09	6·86 6·57	14·98 15·13	1·02 0·92	1·00 1·03	1·03 1·01[g]	1·02 0·99	4·71 (4·05)	B4

Il	0·08	0·05	$C_{44}H_{68}N_{12}O_{11}S.2\,H_2O.$	51·67	7·16	15·72	0·96	1·10	0·96	1·02	2·23	B5
	0·05	0·57	$.C_2H_4O_2$ (1 069)	51·42	6·88	15·86	1·03	1·12	0·79[h]	0·95	(1·75)	
Im	0·10	0·32	$C_{46}H_{72}N_{12}O_{11}S.2\,H_2O.$	51·89	7·32	14·52	1·09	1·04	1·09	1·04	6·71	B6
	0·05	0·61	$.2\,C_2H_4O_2$ (1 157)	52·21	7·26	14·38	0·97	1·08	0·83[i]	0·81	(5·83)	
In	0·33	0·12	$C_{52}H_{74}N_{12}O_{13}S.3\,H_2O$	53·79	6·43	13·48	1·08	1·00	0·95	1·04	3·56	B7
	0·21	0·62	(1 161)	53·95	6·32	14·30	0·92	1·00	0·82[j]	1·00	(3·15)	
Io	0·19	0·13	$C_{44}H_{66}N_{12}O_{13}S.6\,H_2O$	47·56	7·07	15·12	1·06	1·04	0·99	1·05	4·23	B8
	0·24	0·65	(1 111)	47·58	6·63	14·80	0·95	1·04	0·89[k]	0·95	(3·34)	

[a] k' value of the sulfide; in parentheses given k' of the corresponding sulfoxide which was prepared only for analytical purposes; [b] B1, methanol–water (11 : 9); B2: methanol–0·015M sodium phosphate, pH 4·4 (3 : 2); B3: methanol–0·015M sodium phosphate, pH 4·4 (13 : 7); B4: methanol–water (3 : 2); B5: methanol–0·01M triethylammonium borate, pH 8·1 (11 : 9); B6: methanol–triethylammonium borate, pH 8·1 (3 : 2); B7: methanol–1% trifluoroacetic acid with triethylamine, pH 4·4 (9 : 5); B8: methanol– water (1 : 1); [c] Phe; [d] Phe(*p*-Me); [e] Phe(*p*-Et); [f] Tyr(Et) determined as the sum Tyr + Tyr(Et); [g] Phe(*p*-Cl); [h] Phe(*p*-NH_2); [i] Phe(*p*-NMe_2); [j] Phe(*p*-$NHOCOCH_2C_6H_5$) determined as Phe(*p*-NH_2); [k] Phe(*p*-NO_2).

the total activity whereas the *in vitro* test estimates intensity of this activity); moreover, the uterotonic *in vivo* test reflects also the protracted action of this series of compounds. However, we are of the opinion that the large activity differences found for *Ij*, *Im* and *Io* (and to some extent also for *Ii* and *Ik*) have probably other reason. We cannot *e.g.* exclude that the uterotonic activity *in vivo* is mediated by other compounds (*e.g.* by prostaglandins) whose generation is stimulated by bonding of the oxytocin analogue to the receptor responsible for its release[36]. This receptor may be stimulated both by oxytocin and the given analogue; its steric requirements, however, may somewhat differ from those of the uterotonic receptor. As the result, the given agonist may act selectively only in the *in vivo* test. The data available about the uterotonic *in vivo* tests are too scarce to decide how far this phenomenon is general. Anyway, most of the hitherto published analogues exhibited higher *in vivo* activities, although there were no differences of orders of magnitude as found by us (4 orders of magnitude in the case of *Io*). Only 1-carba-oxytocin[37] and its deamino derivative[7], [1-β-hydroxypropionic acid]oxytocin[38], [1,6-α-aminopimelic acid]-oxytocin[7], [2-phenylalanine]deamino-1-carba-oxytocin[53] and tocinamide[39] represented exceptions, showing higher *in vitro* than *in vivo* activities.

Although we have proved that compounds with substantially higher, and to significant extent selective, natriuretic effect can be prepared on the basis of oxytocin structure, the question of mechanism of this effect still remains open. It seems probable that this mechanism is identical with that of the oxytocin action, judging not only from the similar course of action of oxytocin and the prepared analogues but also from the fact that the natriuretic effect of the compound *Ig* can be blocked by a specific oxytocin inhibitor[40].

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 1M acetic acid (pH 2·4) and a pyridine–acetate buffer (pH 5·7). The compounds were detected by ninhydrin or by 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 hydrolyzed with 6M-HCl at 105°C for 20 h and analyzed on an automatic analyzer, type 6020 (Development Workshops of Czechoslovak Academy of Sciences). Countercurrent distribution was done in an all-glass instrument (Quickfit and Quartz, Stone, England) with the possibility of transfers of the upper as well as lower phases; solvent system 2-butanol–0·05% aqueous acetic acid. Preparative free-flow-electrophoresis was performed on a previously described[41,42] instrument at 2 500 V in 0·5M acetic acid; the peptidic material was detected by absorption at 250 nm. 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). Immobilized *Escherichia coli* cells of retained penicillin amidohydrolase activity[18] were used. L-Amino acid oxidase (0·3 U/mg) was a Serva product.

S-(β-Carboxyethyl)homocysteine

The title compound was prepared according to ref.[13] by refluxing methionine (5 g) and β-bromopropionic acid (5·4 g) in concentrated hydrochloric acid (67 ml) for 25 h. The solution was taken down and the residue coevaporated twice with water. An aqueous solution of the residue was applied to a column of Dowex-50 (H^+-cycle; 100 ml). The column was washed with water and the product eluted with 3% ammonium hydroxide solution. The eluates were taken down and the residue crystallized from a mixture of acetic acid (80 ml) and water (30 ml), affording 4·1 g (60%) of the product, m.p. 236–239°C; $E^{Gly}_{2,4}$ 0·58, $E^{Asp}_{5,7}$ 0·59; R_F 0·34 (S1), 0·04 (S2), 0·37 (S3), 0·27 (S4). Recrystallization of a sample from the same solvent mixture gave material, melting at 243–245°C, $[\alpha]_D$ +25·5° (*c* 0·5, 1M-HCl). Reported[13] m.p. 245–247°C; $[\alpha]_D$ +23·1° (1% solution in 1M-HCl).

S-(β-Methoxycarbonylethyl)homocysteine Hydrochloride

Methanolic 6·25M-HCl (4·3 ml), cooled to 0°C, was added at 0°C to a suspension of the above-prepared diacid (1·24 g) in methanol (18 ml). After standing for 3 h at 0°C the solution was taken down (bath temperature below 20°C), the crystalline residue triturated with ether and filtered, affording 1·48 g (96%) of product, m.p. 200–203°C, which contained very small amounts of the diacid and diester. A sample, crystallized from methanol–ether, melted at 210–211°C; $[\alpha]_D$ +20·1° (*c* 0·4, acetic acid). $E^{His}_{5,7}$ 0·00, $E^{Gly}_{2,4}$ 0·55; R_F 0·30 (S1), 0·10 (S2), 0·30 (S3), 0·45 (S4). For C_8H_{16}·.$ClNO_4S$ (257·8) calculated: 37·27% C, 6·26% H, 5·43% N; found: 37·56% C, 6·23% H, 5·11% N. Optical purity was determined by cleavage with L-amino acid oxidase and evaluation by amino acid analyzer: the product contained less than 0·1% of the D-form.

Methyl S-(β-methoxycarbonylethyl)homocysteinate Hydrochloride

A solution of S-(β-carboxyethyl)homocysteine (0·5 g) in methanolic 6·25M-HCl (10 ml) was allowed to stand at room temperature for 2 h, taken down and twice coevaporated with methanol. According to electrophoretic analysis, the material contained about 20% of the monoester. The esterification was repeated once more, the reaction time being 4 h. Crystallization from methanol–ether afforded 0·5 g (77%) of the product, m.p. 82–84°C; R_F 0·27 (S1), 0·23 (S2), 0·25 (S3), 0·70 (S4); $E^{His}_{5,7}$ 1·00, $E^{His}_{2,4}$ 0·82. $[\alpha]_D$ +46·1° (*c* 0·2, acetic acid). For $C_9H_{17}ClNO_4S$ (270·8) calculated: 39·92% C, 6·33% H, 13·10% Cl; 5·17% N, found: 39·70% C, 6·74% H, 13·06% Cl, 4·90% N.

Dicyclohexylammonium salt of Tert-butyloxycarbonyl-S-(β-methoxycarbonylethyl)homocysteine

2-(Tert-butyloxycarbonyloxyimino)-2-phenylacetonitrile (3 g) was added to a solution of S-(β-methoxycarbonylethyl)homocysteine (1·8 g) and triethylamine (1·7 ml) in aqueous acetone (1 : 1; 30 ml). The acylation was followed by thin-layer chromatography. After disappearance of the starting compound (18 h) the acetone was evaporated, the solution acidified with citric acid and the product taken up in ethyl acetate. The extract was washed with water, dried over sodium sulfate and taken down, yielding an oil which was dissolved in ethyl acetate. Dicyclohexylamine (1·5 ml)

was added and after cooling the separated crystals were collected on filter and washed with light petroleum, affording 2·69 g (54%) of product, m.p. 98—101°C. The analytical sample was crystallized from ethyl acetate; m.p. 99—101°C; $[\alpha]_D$ +10·6° (*c.* 0·2 dimethylformamide). For $C_{25}H_{46}N_2O_6S$ (502·7) calculated: 59·73% C, 9·22% H, 5·67% N; found: 59·84% C, 9·29% H, 5·40% N.

N,S-Bis-(β-methoxycarbonylethyl)homocysteine

Homocystine (1 g) was reduced with sodium in liquid ammonia (about 100 ml) until the blue coloration persisted for 8 min. The solution was decolorized with ammonium chloride, methyl acrylate (2 ml) was added and the mixture was freeze-dried. The product was de-salted by washing with water on a column of Dowex 50 (H^+ form; 40 ml) and eluted with 10% pyridine. Evaporation and precipitation from methanol and ether afforded 1·8 g of compound m.p. 213 to 217°C, which on crystallization from water melted at 217—219°C. R_F (in parentheses the values for S-(β-methoxycarbonylethyl)homocysteine): 0·27 (0·27) (S1), 0·13 (0·06) (S2), 0·25 (0·23) (S3), 0·54 (0·49) (S4). $E_{5.7}^{His}$ 0·00 (0·00), $E_{2.4}^{Gly}$ 0·31 (0·57); $[\alpha]_D$ −19·0° (*c.* 0·18, dimethylformamide). For $C_{12}H_{21}NO_6S$ (307·4) calculated: 46·89% C, 6·89% H, 4·56% N; found: 46·92% C, 7·01% H, 4·52% N. Mass spectrum: M^+ 307. ^{1}H-NMR spectrum (^2H_2O): =CH—CH_2—CH_2— 2·16 (m) 2 H, —CH_2—S—CH_2— 2·70 (m) 4 H, —CH_2—$COOCH_3$ 2·86 (m) 2 H, —CH_2—NH— 3·36 (t) J = 6·5 Hz, 2 H, —$COOCH_3$ (at the sulfur atom) 3·71 (s) 3 H, —$COOCH_3$ (at the nitrogen atom) 3·75 (s) 3 H, —NH—CH—CO— 3·76 (t) J = 6 Hz 1 H.

Ethyl *p*-Ethyl-DL-phenylalaninate Hydrochloride

A slurry of *p*-ethylphenylalanine (5·0 g) in absolute ethanol (300 ml) was saturated with hydrogen chloride at 0°C. After standing for 12 h at room temperature, the solution was several times evaporated with ethanol, affording 6·25 g (94%) of the ethyl ester, m.p. 160—163°C. R_F 0·52 (S1), 0·66 (S2), 0·53 (S3), 0·68 (S4). For $C_{13}H_{20}ClNO_2$ (257·8) calculated: 60·57% C, 7·82% H, 5·43% N; found: 60·36% C, 7·66% H, 5·22% H. Cleavage with chymotrypsin: The ethyl ester (6·2 g) was dissolved in water (200 ml), and the solution adjusted to and kept at pH 5·0 with 0·2M lithium hydroxide. After addition of chymotrypsin (220 mg) the formation of *p*-ethylphenylalanine was followed by liquid chromatography (Separon SI-C-18, methanol–phosphate buffer, pH 4 (4 : 1), ethyl ester $k' = 3·9$, free acid $k' = 1·05$). The cleavage was very slow and therefore the mixture was adjusted to pH 6·3. After 14 h only 13% of the substrate reacted. The chymotrypsin activity was checked in this stage by addition of methyl tyrosinate to a sample (100 µl) of the reaction mixture; this sample was cleaved very rapidly. Further 1·4 g of the enzyme was added to the incubation mixture and the reaction was prolonged for 10 h at 37°C. The mixture was filtered, the filtrate concentrated to a small volume and after standing in a refrigerator for two days the separated crystals were filtered, washed with water and dried, yielding 1·5 g (65%) of compound, identical (according to thin-layer chromatography and electrophoresis) with authentic *p*-ethylphenylalanine. The optical purity of the product was found to be 88% by reversed phase liquid chromatography on Separon SI-C-18, using a chiral mobile phase (0·008M phenylalanine, 0·004M $CuSO_4$ + 45% methanol) in which the k' values for the D and L forms were 6·63 and 7·62, respectively.

N-Phenylacetyl-*p*-ethyl-DL-phenylalanine

Phenylacetyl chloride (3 ml) was added portionwise at 10°C during 30 min to a suspension of *p*-ethylphenylalanine (2 g) in 2M-NaOH (7 ml). Simultaneously, 2M-NaOH was added so as

to keep the mixture at pH 12. The suspension was then stirred for 2 h at room temperature, acidified with concentrated hydrochloric acid to pH 2 and set aside overnight in a refrigerator. The product was filtered, washed with water, dried and crystallized from 30% ethanol, affording 2·65 g (92%) of compound, m.p. 146–149°C. R_F 0·93 (S1), 0·50 (S2), 0·86 (S3), 0·61 (S4). For $C_{19}H_{21}NO_3$ (311·4) calculated: 73·29% C, 6·80% H, 4·50% N; found: 72·98% C, 6·68% H, 4·32% N.

Cleavage of Phenylacetyl-*p*-ethyl-DL-phenylalanine with Penicillin Amidohydrolase (E.C.3.5.1.11)

A suspension of phenylacetyl-*p*-ethyl-DL-phenylalanine (1·1 g) in water (40 ml) was adjusted to pH 7·5 with 0·1M-NaOH (3·1 ml). To the formed solution 0·2M phosphate buffer, pH 7·5 (13 ml) and the immobilized enzyme[18] (1 g) were added and the mixture was stirred at 40°C. Course of the cleavage was followed by HPLC (Separon SI-C-18, methanol–0·05% trifluoroacetic acid 80 : 20; k' values: phenylacetic acid 1·19, phenylacetyl-*p*-ethylphenylalanine 2·11, *p*-ethylphenylalanine 2·84). After 3 h the mixture was filtered, the filtrate acidified with 1*M*-HCl (10 ml), filtered and applied to a column of Dowex 50W (60 ml). After washing with water the product was eluted with 15% pyridine. Evaporation of the solvent afforded 234 mg (68·5%) of *p*-ethylphenylalanine identical (TLC, electrophoresis) with authentic compound. The optical purity, determined by HPLC using a chiral mobile phase (*vide supra*) was >99; this value was confirmed also by incubation of the sample with L-amino acid oxidase. The product was crystallized from 1M-HCl, m.p. 206–208°C, $[\alpha]_D$ −23·9° (*c*. 0·12, water). Reported[14] m.p. 229–232°C (capillary) and $[\alpha]_D$ −23·1° (*c*. 0·12, water).

Preparation of *o*-Nitrobenzenesulfenylamino Acids

The amino acid (1·8 mmol) was dissolved in a mixture of 1M-NaOH (1·8 ml) and peroxide-free dioxane (2·5 ml). *o*-Nitrobenzenesulfenyl chloride (0·6 g) and 2M-NaOH were added at such a rate to keep the mixture at pH 8·5 (glass electrode). The reaction mixture was poured into water (0°C, 20 ml), filtered, and the filtrate acidified with 0·5M-H_2SO_4 to pH 3. The product was taken up in ethyl acetate, the organic solution washed with water, dried and taken down. If the residue did not crystallize even after trituration with light petroleum, it was dissolved in ethyl acetate, an equivalent quantity of dicyclohexylamine was added and after cooling the separated crystals were filtered, washed with ether and dried. The characteristics of the prepared derivatives are given in Table II.

Preparation of 2,4,5-Trichlorophenyl Esters of Protected Amino Acids

The protected amino acid (1 mmol) was liberated from its dicyclohexylammonium salt by addition of 0·05M sulfuric acid, extraction into ethyl acetate and evaporation of the solvent. After dissolution in dichloromethane (10 ml) or its mixture with dimethylformamide (1 : 1), addition of 2,4,5-trichlorophenol (200 mg) and cooling to −20°C, dicyclohexylcarbodiimide (230 mg) was added. The solution was stirred at −20°C for 1 h, at 0°C for another hour and at room temperature for 10 h. The mixture was filtered, taken down, washed with light petroleum and, if desirable, the product crystallized from ethanol. For data of the prepared active esters see Table II.

o-Nitrobenzenesulfenyl-*p*-nitrophenylalanyl-isoleucyl-glutaminyl-asparaginyl-
-S-(β-carboxyethyl)homocysteinyl-prolyl-leucyl-glycine Amide (*IIb*)

2,4,5-Trichlorophenol (0·29 g) and dicyclohexylcarbodiimide (0·33 g) were added to a solution of *o*-nitrobenzenesulfenyl-*p*-nitrophenylalanine (0·53 g) in a mixture of dichloromethane (15 ml) and dimethylformamide (15 ml), cooled to −10°C. After stirring at −10°C for 1 h, the mixture was stirred at room temperature for 12 h, and concentrated *in vacuo*. The separated dicyclohexylurea was filtered off, the cake on the filter washed with dichloromethane and the filtrate taken down. The residue was triturated several times with light petroleum and dissolved in dimethylformamide (12 ml). Compound *IIa* (0·8 g) was suspended in this solution and the mixture was stirred at room temperature for 135 h. The formed solution was taken down, the product which crystallized on trituration with light petroleum and ether was washed on the filter successively with water, 0·05M sulfuric acid, water and ether, affording 720 mg (63%) of compound whose characteristics are given in Table III.

[2-*p*-Nitrophenylalanine]deamino-6-carba-oxytocin (*Io*)

Bis(*p*-nitrophenyl) sulfite (0·7 g) was added to a solution of the octapeptide *IIb* (200 mg) in a mixture of dimethylformamide (7 ml) and pyridine (7 ml) under nitrogen. After stirring at room temperature for 9 h, another portion of the sulfite (0·7 g) was added and after standing for 12 h, the addition was completed by the last portion of sulfite (0·35 g). After standing for 4 h the mixture was concentrated and the product precipitated with ether, filtered and washed with ether. After drying, the product was dissolved in dimethylformamide (7 ml), the solution mixed with 2·26M hydrogen chloride in ether (0·52 ml) and after 7 min diluted with ether (100 ml). The separated precipitate was filtered, washed with ether, dried *in vacuo* and dissolved in dimethylformamide (7 ml). This solution was added at a rate 2 ml/h into a vigorously stirred mixture of pyridine (200 ml) and N-ethylpiperidine (50 µl), warmed to 50°C, into which nitrogen was bubbled. After the end of the addition the mixture was kept at 50°C for 4 h and then set aside for 12 h at room temperature. The solution was concentrated to a small volume and the product precipitated by addition of ether; yield 170 mg. A part of the product (100 mg) was dissolved in 3*M* acetic acid (4 ml) and applied on a column of Bio-Gel P-2 (100 × 1 cm). Freeze-drying of the corresponding fractions afforded 70 mg of compound which was again dissolved in 3M acetic acid (3 ml) and applied on a column of Bio-Gel P-4 (100 × 1 cm). Freeze-drying of the appropriate fractions gave 42 mg of the compound. A portion (15 mg) was dissolved in a mixture of methanol and water (2 : 3; 2 ml) and the solution applied on a column of Separon SI-C-18 (15 × 0·6 cm). The elution was carried out with a methanol–water (44 : 56) mixture (at 20 MPa). The fraction of $k' = 8{\cdot}2$ was concentrated *in vacuo* and freeze-dried, affording 6·2 mg of product. Its physical constants are given in Table IV.

o-Nitrobenzenesulfenylphenylalanyl-isoleucyl-glutaminyl-asparaginyl-
-S-(β-carboxyethyl)homocysteinyl-prolyl-leucyl-glycine Amide (*IIc*)

2,4,5-Trichlorophenyl *o*-nitrobenzenesulfenylphenylalaninate (0·8 g) was added to a suspension of the free heptapeptide *IIa* (0·8 g) in dimethylformamide (15 ml). After stirring at room temperature for 96 h the mixture was worked up as described for the preparation of the compound *IIb* affording 1 g (85%) of compound whose characteristics are given in Table III.

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

The cyclization was carried out in the same way as described for the compound *Io*. Since the product contained some ninhydrin-positive compound, it was dissolved in aqueous methanol (1 : 1) and filtered through a column of Dowex 50 (H^+ form, 5 ml). Concentration and freeze--drying afforded 125 mg of compound which was further purified by gel filtration on Bio-Gel P-4 (100 × 1 cm) in 3M acetic acid. Part of the thus-obtained material (15 mg) was chromatographed on a column of Separon SI-C-18 (15 × 0·6 cm) in a mixture of methanol and water (3 : 2). Fraction of $k' = 7{\cdot}0$ on concentration and freeze-drying afforded 4·2 mg of the product described in Table IV.

o-Nitrobenzenesulfenyl-*p*-methylphenylalanyl-isoleucyl-glutaminyl-asparaginyl--S-(β-carboxyethyl)homocysteinyl-prolyl-leucyl-glycine Amide (*IId*)

The active ester (0·65 g) was added to a suspension of the heptapeptide *IIa* (0·65 g) in dimethylformamide (13 ml). The same procedure as described for the preparation of the compound *IIb* afforded 0·83 g (96%) of product; described in Table III.

[2-*p*-Methylphenylalanine]deamino-6-carba-oxytocin (*If*)

Cyclization of the peptide *IId* (200 mg) was carried out in the same manner as described for *Io*, affording a product (180 mg) a part of which (50 mg) was purified by gel filtration on a column of Bio-Gel P-4 in 3*M* acetic acid and by repeated chromatography on a column (25 × 0·4 cm) of Separon SI-C-18 in methanol–water (3 : 2). Lyophilization of fractions of $k' = 5{\cdot}03$ gave 13·6 mg of compound, described in Table IV.

o-Nitrobenzenesulfenyl-*p*-ethylphenylalanyl-isoleucyl-glutaminyl-asparaginyl--S-(β-carboxyethyl)homocysteinyl-prolyl-leucyl-glycine Amide (*IIe*)

The heptapeptide *IIa* (4·4 g) was suspended in a dimethylformamide solution (100 ml) of the active ester, prepared from dicyclohexylammonium salt of *o*-nitrobenzenesulfenyl-*p*-ethylphenylalanine (3·3 g) analogously as described for *IIb*. The suspension was adjusted to pH 8 with N-ethylpiperidine (0·5 ml) and stirred for 24 h at room temperature. Further addition of the base (0·3 ml) was necessary. After 120 h the mixture was worked up as described for *IIb*, affording 5·1 g (83%) of compound described in Table III.

[2-*p*-Ethylphenylalanine]deamino-6-carba-oxytocin (*Ig*)

a) Bis(2,4,5-trichlorophenyl) sulfite (5 g) was added to a solution of the octapeptide *IIe* (1 g) in a mixture of pyridine (40 ml) and dimethylformamide (40 ml) into which helium was introduced. After 3 h another portion of the sulfite (5 g) was added. After 12 h the mixture was concentrated *in vacuo*, the product precipitated, washed with ether, dried and dissolved in dimethylformamide (40 ml). An ethereal 3·45M-HCl solution (1·5 ml) was added and after standing for 10 min at room temperature ether was added to precipitate the hydrochloride of the octapeptide active ester. The compound was washed with ether, dried *in vacuo*, dissolved in dimethylformamide (30 ml) and the solution added under helium at the rate 5 ml/h into stirred pyridine (900 ml) warmed to 50°C. The mixture was concentrated and the product (0·71 g) precipitated with ether. A part of the product (200 mg) was purified by gel filtration on a Sephadex LH-20 column (100 × × 2·5 cm) in dimethylformamide and on a Bio-Gel P-4 column (100 × 1 cm) in 3M acetic

acid. However, the obtained product was still not completely homogeneous (thin-layer and HPLC). Another part (300 mg) was dissolved in the upper phase (50 ml) of the system sec-butyl alcohol–0·05% aqueous acetic acid and subjected to countercurrent distribution (564 transfers of the lower phase and 198 transfers of the upper phase). The peptidic material was detected by liquid chromatography on a reversed phase, which determined directly also the purity of the desired compound in the given tube. Freeze-drying of the compound of $K = 4{\cdot}32$ (tubes 44–65) afforded 94 mg of the product which, according to HPLC was contaminated with the sulfoxide (21%). The sulfoxide was the principal part of the material of $K = 3{\cdot}64$ (tubes 25–43; 32 mg).

A further part of the crude product (100 mg) was dissolved in methanol (5 ml) and mixed with water (5 ml). The precipitate (which, according to HPLC analysis, contained only a minimum amount of the desired compound) was filtered and the filtrate (10 ml) applied on a column of Partisil ODS (50 × 0·9 cm). The product was eluted with a mixture of methanol and water (3 : 2). Concentration and freeze-drying of the fraction of $k' = 9$ afforded 22 mg of the pure compound; see Table IV. Fraction of $k' = 6{\cdot}9$ contained the corresponding sulfoxide (12 mg).

The combined fractions, containing the sulfoxide (60 mg), were suspended in acetone (2 ml), hydrogen bromide in acetic acid (0·5 ml) was added and after standing for 5 min at 0°C the solution was freeze-dried and repeatedly evaporated with acetone. The obtained material was purified by chromatography on Partisil ODS, affording 38 mg of the compound *Ig*. A part (5 mg) of the product was dissolved in 3M acetic acid (0·4 ml) and applied on a column of Bio-Gel P-2 (12 × 0·8 cm). Freeze-drying of the corresponding fractions gave 2·5 mg of product, $[\alpha]_D$ +92·1° (*c*. 0·1; 3M acetic acid). UV spectrum (water), λ_{max} (log ε): 258 (2·59), 263·5 (2·64), 272 (2·54).

b) The protected octapeptide (200 mg) was dissolved in dimethylformamide (5 ml), mixed with 3·6M-HCl in ether (0·5 ml) and allowed to stand at room temperature for 7 min. The product was precipitated with ether, dried, and dissolved in dimethylformamide (3 ml) and dioxane (1·8 ml). 1-Hydroxybenzotriazole (241 mg) was added, the mixture was cooled to 0°C and after addition of dicyclohexylcarbodiimide (265 mg) stirred at 0°C for 0·5 h and at room temperature for 2 h. The precipitate was filtered, washed with dimethylformamide and the filtrate divided into two parts. One was dissolved in a mixture of methanol (100 ml) and N-ethylpiperidine (300 µl). The solution was kept at 50°C for 2 h, concentrated and the product precipitated with ether, affording 76 mg of the crude product. The second part of the active ester was treated with pyridine (100 ml) at 50°C for 2 h. The mixture was worked up in the same manner affording 56 mg of the crude product. Preparative liquid chromatography (the same conditions as described under *a*)) afforded 13 mg and 8 mg of the pure compound from the respective first and second part. The product had the same properties as the material obtained under *a*).

[2-*p*-Ethylphenylalanine]deamino-6-carba-oxytocin Sulfoxide (*Ih*)

Sodium periodate (2·5 mg) was added to a solution of *Ig* (8 mg) in aqueous methanol (1 : 1; 1 ml). After standing for 70 min at room temperature, the solution was applied on a column of Partisil ODS (50 × 0·9 cm) and eluted with a mixture of methanol and water (3 : 2). The fraction of $k' = 6{\cdot}92$ was concentrated and freeze-dried, affording 6·4 mg of product; see Table IV.

o-Nitrobenzenesulfenyl-O-ethyltyrosyl-isoleucyl-glutaminyl-asparaginyl-S-(β-carboxyethyl)homocysteinyl-prolyl-leucyl-glycine Amide (*IIf*)

The preparation of the active ester and condensation with the heptapeptide *IIa* (0·25 g) was carried out in the same manner as described for the preparation of *IIb* and afforded 200 mg (69%) of product; see Table III.

[2-O-Ethyltyrosine]deamino-6-carba-oxytocin (*Ij*)

The cyclization was performed analogously to that described for the compound *Io* and yielded 196 mg of the crude cyclization product from 200 mg of the protected peptide. A part (30 mg) of this mixture was purified by repeated gel filtration on a column of Bio-Gel P-4 (100 × 1 cm) in 3M acetic acid, giving 8·3 mg of compound. A part (5 mg) of this product was dissolved in aqueous methanol (1 : 1; 0·5 ml) and applied on a column of Separon SI-C-18 (25 × 0·4 cm). The elution was carried out with a mixture of methanol and water (11 : 9) and the fraction of $k' = 5{\cdot}2$ was concentrated *in vacuo* and freeze-dried, affording 3·2 mg of compound, described in Table IV.

Tert-butyloxycarbonyl-*p*-chlorophenylalanyl-isoleucyl-glutaminyl-asparaginyl-
-S-(β-carboxyethyl)homocysteinyl-prolyl-leucyl-glycine Amide (*IIg*)

Tert-butyloxycarbonyl-*p*-chlorophenylalanine[24] (0·3 g) was dissolved in dichloromethane (6·5 ml) and the active ester was prepared in the same manner as described for compound *IIb*. The active ester was dissolved in dimethylformamide (12 ml) and the heptapeptide *IIa* (0·7 g) was suspended in this solution. After stirring for 80 h at room temperature the solution was worked up analogously as described for *IIb*. Yield 0·71 g (73%) of product (see Table III).

[2-*p*-Chlorophenylalanine]deamino-6-carba-oxytocin (*Ik*)

The cyclization (starting from 200 mg) and work-up procedure were the same as for *Io* except that the prepared active ester was dissolved in trifluoroacetic acid and after reaction at room temperature for 1 h the formed solution was co-evaporated with toluene. No strong base was added into the cyclization solution. The reaction gave 176 mg of product. A part (50 mg) was dissolved in 35% methanol (10 ml) and applied on a column of Partisil ODS (50 × 0·9 cm); elution with a mixture of methanol and water (11 : 9). The fraction of $k' = 10{\cdot}2$ was concentrated and freeze-dried, affording 17 mg of product; see Table IV.

o-Nitrobenzenesulfenyl-*p*-benzyloxycarbonylaminophenylalanyl-isoleucyl-glutaminyl-
-asparaginyl-S-(β-carboxyethyl)homocysteinyl-prolyl-leucyl-glycine Amide (*IIh*)

The corresponding active ester (0·8 g) was added to a suspension of the heptapeptide *IIa* (0·6 g) in dimethylformamide (15 ml). The further work-up procedure was the same as described for the compound *IIb* and afforded 0·53 g (55%) of the product described in Table III.

[2-*p*-Benzyloxycarbonylaminophenylalanine]deamino-6-carba-oxytocin (*Il*)

The cyclization (from 200 mg) was carried out in the same manner as described for the compound *Io* and afforded 156 mg of the product a part of which (40 mg) was purified by gel filtration on a column of Sephadex LH-20 (2·5 × 100 cm) in dimethylformamide and on a column of Bio-Gel P-4 (100 × 1 cm) in 3M acetic acid. Another part (30 mg) of the crude product was purified by chromatography on a column (50 × 0·9 cm) with a reversed phase (Partisil ODS-2) in methanol–water (3 : 2). Freeze-drying of the fraction containing the compound of $k' = 12{\cdot}5$ afforded 3·7 mg of compound described in Table IV.

o-Nitrobenzenesulfenyl-*p*-dimethylaminophenylalanyl-isoleucyl-glutaminyl-asparaginyl-
-S-(β-carboxyethyl)homocysteinyl-prolyl-leucyl-glycine Amide (*IIi*)

The corresponding active ester was added to a suspension of the heptapeptide *IIa* (0·3 g) in dimethylformamide. The same procedure as described for *IIb* (with omission of washing with dilute sulfuric acid) yielded 0·43 g (96%) of product (see Table III).

[2-*p*-Dimethylaminophenylalanine]deamino-6-carba-oxytocin (*Im*)

The cyclization was performed as described for the compound *Io* and afforded 190 mg of the product which was purified by gel filtration on a column of Bio-Gel P-4 in 3M acetic acid. The obtained lyophilizate (100 mg) was dissolved in 20% acetic acid and purified by free-flow electrophoresis, yielding 54 mg of a compound which was not homogeneous (HPLC). A part (10 mg) was dissolved in a mixture of methanol and 0·02*M* triethylammonium hydrogen carbonate buffer pH 8·1 (2 : 3; 250 µl) and in portions applied on a column of Separon SI-C-18 (25 × 0·4 cm); elution with a mixture of the above-mentioned solvents (1 : 1). Concentration of fractions of $k' =$ $= 12{\cdot}4$ and their repeated lyophilization from water and 1M acetic acid gave 2·4 mg of the product; see Table IV.

[2-*p*-Aminophenylalanine]deamino-6-carba-oxytocin (*Il*)

a) Sodium was added to a solution of [2-*p*-nitrophenylalanine]deamino-6-carba-oxytocin (*Io*) (3·6 mg) in liquid ammonia (5 ml) until the blue coloration persisted for 30 s. The mixture was decolorized with acetic acid, the ammonia evaporated and the residue dissolved in 3M acetic acid and applied on a column of Bio-Gel P-4. Freeze-drying of the corresponding fractions gave 2·1 mg of product; see Table IV.

b) A 35% solution of hydrogen bromide in acetic acid (1 ml) was added to a slurry of the compound *In* (30 mg) in acetone (1 ml) and the solution was set aside for 1 h at room temperature. After repeated evaporation with acetone and precipitation from methanol with ether the product was dissolved in 3M acetic acid (3 ml) and purified by gel filtration of Bio-Gel P-4. Freeze-drying afforded 8·7 mg of compound which was dissolved in a mixture of methanol and 0·1% trifluoroacetic acid (2 : 3; 1 ml) and applied on a column of Separon SI-C-18 (25 × 0·4 cm). The elution was carried out with the above-mentioned solvents (9 : 11). The fraction of $k' = 6{\cdot}3$ was concentrated and repeatedly freeze-dried from 1M acetic acid and water, affording 1·6 mg of product identical with the compound obtained by the procedure *a*).

Pharmacological Methods

The *in vitro* uterotonic assay was carried out on isolated rat uterine strips[43,44]. For the determination of the *in vivo* activity[45] oestrogenized rats in ethanol anaesthesia were used. Galactogogic activity was determined on ethanol-anaesthesized rats (4—15 days after delivery)[46,47], pressor activity on despinalized rats[48]. Antidiuretic activity[49,50] was determined on ethanol-anaesthesized rats.

Natriuretic activity was determined using two different modifications: on male Brattleboro (NDI) rats in pentobarbital narcosis and continuous water diuresis[51], and on conscious Wistar rats[4,52]. The concentration of sodium and potassium ions in the urine was determined flame-photometrically.

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