

## ANALOGUES OF OXYTOCIN AND VASOPRESSIN WITH AN N<sup>α</sup>-METHYLAMINO ACID RESIDUE IN POSITION 8\*

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The syntheses of two analogues of neurohypophysial hormones are described, in which the residue in sequence position 8 was replaced by an N<sup>α</sup>-methylamino acid: [8-N-methylleucine]oxytocin and [8-N<sup>α</sup>-methylarginine]deamino-vasopressin. Alkylation of the α-nitrogen atom at this position resulted in a moderate to marked reduction in some biological actions of the parent substances in terms of potency (particularly with antidiuresis and contraction of vascular smooth muscle) but what activity remained (*e.g.* natriuretic) was prolonged. Circular dichroic spectra were recorded for these substances, and it is suggested that changes observed in the Cotton effect may be related to the changes in molecular conformation.

The accumulation of data on sites of initial enzymic cleavage — and therefore of inactivation — of neurohypophysial nonapeptide hormones was the starting point of attempts, by synthetic alteration, to prolong the very short biological half-life of these molecules (or of their responses). One of the first principles to be employed was substitution of a D isomer for the natural L form at various positions near to cleavage sites.\*\* Most of these attempts succeeded in providing greater biochemical stability of the molecules, but at the price of a drastic loss in activity<sup>2</sup>. A striking exception to this are analogues of vasopressin with a basic D isomer in sequence position 8 (*ref.*<sup>3,4</sup>) which show very high and prolonged antidiuretic activity, but have either lost their action on smooth muscle or have become partial antagonists to vasopressin at this receptor type<sup>5,6</sup>. A further theoretical possibility of preventing peptide bond cleavage is introduction of an N<sup>α</sup>-methyl group directly onto the backbone<sup>7-9</sup>. Thus far, neurohypophysial nonapeptides have been modified in this manner in position 1 (N-methylcysteine<sup>10,11</sup> and sarcosyl-cysteine<sup>10</sup>), 2 (N-methyltyrosine<sup>12,13</sup>) and 9 (sarcosine<sup>14,15</sup>). In all of the latter examples there were drastic losses of activity. Since the C-terminal tripeptide is known to be critical for most

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\*\* Amino acids used in the present work were all in the L-configuration. Terminology and symbolics follow published suggestions<sup>1</sup>. Mpr 3-mercaptopropionic acid.

of the known biological activities of vasopressin and oxytocin<sup>4,16,17</sup> and one steric alteration at sequence position 8 (L- for D-arginine residue) has already been found to be highly useful, the N<sup>α</sup>-methylation approach has been attempted at the same position.

Synthesis of [8-N-methylleucine]oxytocin utilised 4-toluenesulphonylleucine converted according to Brenner and coworkers<sup>18</sup> to tert-butyl ester *I* and then methylated with dimethyl sulphate at alkaline pH to the corresponding N-methyl derivative *II*. After hydrolysis with trifluoroacetic acid and reaction with dicyclohexylamine, the resulting dicyclohexylammonium salt of N-4-toluenesulphonyl-N-methylleucine was condensed with glycylamide hydrobromide by the action of N,N'-dicyclohexylcarbodiimide in the presence of 1-hydroxybenzotriazole<sup>19</sup> to give the protected dipeptide amide *III*. After removal of the N-4-toluenesulphonyl protecting group with calcium in liquid ammonia<sup>20</sup> the free dipeptide amide was isolated in the form of a di(4-toluenesulphonyl)imide salt<sup>21</sup>. Using dicyclohexylcarbodiimide again, the dipeptide was condensed with benzyloxycarbonylproline in the presence of 1-hydroxybenzotriazole to yield protected tripeptide amide *IV*. Further prolongation of the peptide chain involved attachment of N-tert-butyloxycarbonyl-S-benzylcysteine<sup>22</sup> to the free tripeptide amide obtained from hydrogenolysis of *IV*, using N,N'-dicyclohexylcarbodiimide in the presence of 1-hydroxybenzotriazole. This gave protected tetrapeptide amide *V*, and after removal of the N-tert-butyloxycarbonyl protecting group with trifluoroacetic acid and sequential condensation with 2-nitrobenzenesulphenylasparagine and 2-nitrobenzenesulphenylglutamine (as the 2,4,5-trichlorophenyl ester<sup>23</sup>) we obtained substances *VI* and *VII*. In both cases the amino-protecting groups were removed by hydrogen chloride in ether. The hexapeptide amide hydrochloride obtained from *VII* was acylated by the azide of N-4-toluenesulphonyl-S-benzylcysteinyl-tyrosyl-isoleucine (prepared from the corresponding hydrazide<sup>24</sup>) to give protected nonapeptide amide *VIII*. The latter was reduced by sodium in liquid ammonia and the disulphide bridge was closed by oxidation with air. Purification of analogue *IX* was carried out by counter-current distribution and gel filtration.

For the preparation of N<sup>α</sup>-methylarginine, starting amino acid in the synthesis of [8-N<sup>α</sup>-methylarginine]deamino-vasopressin, we had available the isopropyl ester of N<sup>α</sup>-4-toluenesulphonyl-N<sup>β</sup>-acetylornithine<sup>25</sup>, which was methylated in the same manner as substance *II*, and the resulting N<sup>α</sup>-methyl derivative *X* was hydrolysed in 4M-HCl to N<sup>α</sup>-4-toluenesulphonyl-N<sup>α</sup>-methylornithine (*XI*). Guanidation of the latter with S-methylisothiourea at an alkaline pH resulted in the corresponding N<sup>α</sup>-methylarginine derivative *XII* which was converted to N<sup>α</sup>-methylarginine (*XIII*) itself by boiling in 42% hydrobromic acid in the presence of phenol<sup>25</sup> with freeing up of the N<sup>α</sup>-methylamino acid from the hydrobromide on Amberlite IRA 410. By reaction with benzyloxycarbonyl chloride followed by 4-toluenesulphonyl chloride



to only 0.5 IU/mg in nephrectomised rats<sup>31</sup> and 2.5 IU/mg in despinalised rats<sup>32</sup>. In dosages up to 10 µg/kg there were no haemodynamic changes in the myocardium, gut or uterus of the rat<sup>33</sup>. Antidiuretic activity determined in trained, unanaesthetised rats<sup>4</sup> was less than 50 IU/mg. When injected *i.v.* into chloralosed cats<sup>34</sup>, doses of 5–10 µg/kg resulted in the same peak increments in sodium excretion into the urine as the parent hormone, but the half-life of the response was prolonged by a factor of 3.5.

Thus, with the possible exception of natriuretic activity, all other measured biological actions of both analogues were moderately to markedly decreased, and in general actions on smooth muscle were more affected than actions on other receptor categories. In the case of the one activity which remained to a considerable degree, the originally hoped-for effect of N<sup>8</sup>-methylation at sequence position 8 — prolongation of effect — was in fact observed. The differential activity losses speak in favour of the view<sup>4,33,35</sup> that smooth muscle receptors for neurohypophysial cyclic nonapeptides have much higher conformational requirements for binding and triggering than other receptor types for the same agonist species (*e.g.* antidiuretic and plasminogen-activator-release). It has already been suggested elsewhere<sup>4,33</sup> that these activity decrements with alkylation of the α-nitrogen atom of the peptide chain at position 8 can be related to conformational changes in the C-terminal tripeptide portion of the molecule, and for this reason both analogues were subjected to measurement of CD spectra, and compared with the appropriate parent unmethylated molecules<sup>36,37</sup>, in an attempt to find a physical chemical correlate for the observed effects.

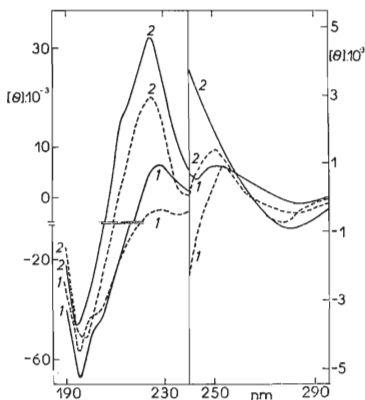


FIG. 1  
CD Spectrum of Oxytocin (—) and [8-N-Methylleucine]oxytocin (----) in Buffered Neutral Water (1) and in Hexafluoroacetone Trihydrate (2)

As can be seen in Figs 1 and 2, the general shape of the CD curves in both neutral water and "conformation-breaking" hexafluoroacetone trihydrate was similar for each pair of 8-N<sup>α</sup>-methylated and unmethylated variants, with one exception: the 8-N<sup>α</sup>-methylated variants showed a decrease in ellipticity of the positive Cotton effect which includes the  $B_{1u}$  band of the tyrosine residue and the  $n-\pi^*$  transition of the amide group<sup>36,37</sup>. This decrease of ellipticity is evident in neutral water for methylated oxytocin *vs* oxytocin, and even more clearly — both in neutral water and in hexafluoroacetone trihydrate — for methylated deamino-arginine-vasopressin *vs* deamino-arginine-vasopressin. Since most of the information from CD spectra concerns interactions of the aromatic side-chains near the N-terminus of the molecule<sup>36,37</sup> and NMR evidence has suggested that the C-terminal tripeptide interacts with the N-terminus<sup>38</sup>, it can be suggested that methylation of the  $\alpha$  nitrogen atom in the peptide chain at position 8 interferes with this same interaction. It would therefore follow that the conformation of the C-terminal tripeptide has been altered in the analogues, and it can be suggested that this is related to the changes in the spectrum of biological activities. This is particularly so since activity at smooth muscle receptors is most affected by 8-N<sup>α</sup>-methylation — while antidiuretic receptors were least influenced by the same molecular change. If the arguments given above prove to have substance, it would follow that a "natriuresis-vasopressin receptor system" has even lower conformational requirements than the antidiuretic receptor, but more will have to be known concerning the site and mechanism of action of the natriuretic

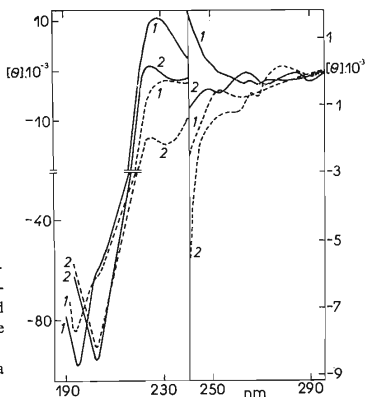


FIG. 2

CD Spectrum of Deamino-arginine-vasopressin (—) and [8-N<sup>α</sup>-Methylarginine]-deamino-vasopressin (----) in Buffered Neutral Water (1) and Hexafluoroacetone Trihydrate (2)

The full curves are based on the data published in ref.<sup>37</sup>.

activity before any such point could be established. The prolonged duration of the natriuretic activity, however, provides evidence that 8-N<sup>α</sup>-methylation does in fact increase the metabolic stability of the vasopressin analogue to enzymic degradation, regardless of receptor-related phenomena.

## EXPERIMENTAL

Melting points were determined on a Kofler block. Samples for elemental analysis were dried for several hours at room temperature and 1 Torr over P<sub>2</sub>O<sub>5</sub>. Evaporation was carried out in a rotary evaporator (water pump, temperature of water bath 35–40°C). Mixtures containing dimethylformamide were evaporated with a vacuum pump at 1 Torr. Optical rotations were determined with a Perkin Elmer 141 MCA instrument. Electrophoresis was carried out on Whatman 3 MM paper for 45 min at a potential drop of 20 V/cm in a 1M acetic acid (pH 2.4) and a pyridine-acetate buffer (pH 5.7) with ninhydrin detection. The guanidine group of derivatives of N<sup>α</sup>-methylarginine was detected by the Sakaguchi method<sup>39,40</sup>.

Continuous free-flow electrophoresis was carried out on a modified version<sup>41</sup> of the Hannig instrument<sup>42</sup>. Qualitative chromatography on Kieselgel G (Merck) plates was carried out in the following systems: S<sub>1</sub> (2-butanol–25% ammonia–water 85 : 7.5 : 7.5), S<sub>2</sub> (2-butanol–99% formic acid–water 75 : 12.3 : 12.7), S<sub>3</sub> (1-butanol–acetic acid–water 4 : 1 : 1), S<sub>4</sub> (1-butanol–pyridine–water–acetic acid 15 : 10 : 6 : 3). Preparative chromatography on thin-layer silica gel was carried out on fluorescent silica gel of particle size 30 μ; column chromatography, unless stated otherwise, was carried out on silica gel of particle size 30–60 μ. Counter-current distribution was performed on the instrument produced by Quickfit & Quartz. Ltd., Stone, Staffordshire, UK. Amino acid analyses first involved hydrolysis in 6M-HCl for 20 h at 105°C, followed by detection on the automatic analyser 6020 A produced by the Development Workshops of this Academy. The IR spectrum of substance *II* was measured on the UR 20 instrument produced by Zeiss, Jena, German Democratic Republic.

### 4-Toluenesulphonylleucine Tert-butyl Ester (*I*)

N-4-Toluenesulphonylleucine (15 g, 53 mm) was shaken under pressure with isobutylene (150 ml) in dichloromethane (200 ml) in the presence of H<sub>2</sub>SO<sub>4</sub> (2 ml) for 12 h at room temperature. The solution was diluted with ethyl acetate and washed with water, 5% NaHCO<sub>3</sub> and water, dried over Na<sub>2</sub>SO<sub>4</sub> and evaporated. To the residue we added light petroleum and filtered off the solid remnant with a yield of 17.5 g, m.p. 128–131°C. Recrystallisation from ethyl acetate and light petroleum gave a yield of 16.5 g (91%), m.p. 131–133°C;  $[\alpha]_D^{23} = 31.9^\circ$  (*c* 0.47; dimethylformamide). For C<sub>17</sub>H<sub>27</sub>NO<sub>4</sub>S (341.5) calculated: 59.80% C, 7.97% H, 4.10% N, 9.39% S; found: 59.87% C, 7.95% H, 4.28% N, 9.41% S.

### N-4-Toluenesulphonyl-N-methylleucine Tert-butyl Ester (*II*)

To substance *I* (17.2 g; 50 mm) in 4M-NaOH (33 ml) we added detergent (1.5 ml) with mixing and, at 0°C, dimethyl sulphate (23 ml, 250 mm). The mixture was stirred at room temperature. Then over a 4-h period we added 4M-NaOH (6 × 3.3 ml), the mixture was washed with ethyl acetate, the pooled ethyl acetate extracts were washed with 4M-NaOH, saturated Na<sub>2</sub>SO<sub>4</sub>, and then dried with Na<sub>2</sub>SO<sub>4</sub>. After evaporation of solvents there was an oily product (15.1 g; 85%) which crystallised at 3°C and thawed to 20°C. The IR spectrum of this product did not show a band corresponding to valence vibrations of an NH group.  $[\alpha]_D^{22} = 31.9^\circ$  (*c* 0.52; dimethylformamide).

For  $C_{18}H_{29}NO_4S$  (355.5) calculated: 60.82% C, 8.22% H, 3.94% N, 9.02% S; found: 61.10% C, 8.24% H, 3.79% N, 8.62% S.

#### N-4-Toluenesulphonyl-N-methylleucyl-glycinamide (*III*)

Substance *II* (17.8 g; 50 mm) was dissolved in trifluoroacetic acid (20 ml) and after 2 h at room temperature the mixture was evaporated. The remainder was evaporated 3× with water, then with benzene, and after solution of the residue in ethanol (15 ml) and ether (50 ml) we added dicyclohexylamine (9 ml) by drops at 0°C. When a salt began to crystallize we added 150 ml ether, and after 2 h at -5°C the product was filtered off and washed with ether, with a yield of 21.2 g of the dicyclohexylammonium salt of N-4-toluenesulphonyl-N-methylleucine, m.p. 144–147°C. Recrystallisation from ethanol and ether gave a yield of 19.4 g (81%), m.p. 147–148°C;  $[\alpha]_D^{22} - 18.3^\circ$  (c 0.48; dimethylformamide). For  $C_{26}H_{47}N_2O_4S$  (480.7) calculated: 64.97% C, 9.23% H, 5.83% N, 6.67% S; found: 65.41% C, 9.28% H, 5.71% N, 6.24% S.

This product (33.5 g; 70 mm) was added to a solution of glycinamide hydrobromide (16 g; 105 mm), 1-hydroxybenzotriazole (10.4 g; 77 mm) and dicyclohexylcarbodiimide (15.9 g; 77 mm) in dimethylformamide (200 ml) at -12°C with mixing which continued for 1 h. The mixture was then left for 20 h at 0°C and the dicyclohexylurea which separated out was filtered off. The solvent was distilled off and the oily residue was dissolved in ethyl acetate and the solution washed with 1M-HCl, 5%  $NaHCO_3$ , water, dried with  $Na_2SO_4$  and the solvent distilled off. The product crystallised when light petroleum was added, with a yield of 23 g, m.p. 115–118°C. Recrystallisation from ethyl acetate and light petroleum gave a yield of 21.5 g (86%), m.p. 121–122°C;  $[\alpha]_D^{20} + 18.6^\circ$  (c 0.49; dimethylformamide). For  $C_{16}H_{25}N_3O_4S$  (355.5) calculated: 54.07% C, 7.09% H, 11.82% N, 9.02% S; found: 54.52% C, 7.28% H, 11.81% N, 8.98% S.

#### Benzyloxycarbonylprolyl-N-methylleucyl-glycinamide (*IV*)

Protected dipeptide amide *III* (35.5 g; 10 mm) was dissolved in freshly redistilled ammonia (1500 ml) and reduced with calcium (13.5 g) with stirring at room temperature until the blue colour had become constant (35 min). After 1 min we added by drops methanol (2 ml) and the ammonia was distilled off. The residue was dissolved in ice water (1000 ml), saturated with  $CO_2$  and with addition of solid  $CO_2$  until  $CaCO_3$  precipitated. The latter was filtered off through a Hyflo filter and the filtrate was evaporated. The oily residue was dissolved in ethanol (20 ml) and while hot we added a solution of di(4-toluenesulphonyl)imide (32.5 g; 100 ml) in ethanol (25 ml); this mixture was left overnight at 0°C. The salt which separated out was filtered off to yield 42.9 g of product, m.p. 188–191°C. Recrystallisation from ethanol and ether gave 41.5 g (79%) of the di(4-toluenesulphonyl)imide salt of the dipeptide amide, m.p. 190–192°C;  $[\alpha]_D^{20} + 17.3^\circ$  (c 0.51; dimethylformamide). For  $C_{23}H_{34}N_4O_6S_2$  (526.7) calculated: 52.45% C, 6.51% H, 10.64% N, 12.17% S; found: 52.14% C, 6.50% H, 10.85% N, 12.24% S.

This salt (22 g; 41.5 mm) and N-ethylpiperidine (5.7 ml) were dissolved in dimethylformamide (50 ml) and added at -12°C to a solution of benzyloxycarbonylproline (7.7 g; 31 mm), 1-hydroxybenzotriazole (4.6 g; 34 mm) and dicyclohexylcarbodiimide (6.5 g; 34 mm) in dimethylformamide (80 ml) (the latter solution had been stirred at -12°C for 30 min previously). The mixture was stirred for 1 h at the same temperature, left for 26 h at 7°C, stirred for 18 h at 20°C and then processed as for *III*. The yield was 9.2 g of a syrup which was chromatographed on silica gel (300 g) in a system 1% methanol in chloroform, to yield 5.7 g (46%) of a pure product as a foam.  $R_F$  in  $S_1$  0.59;  $S_2$  0.55;  $S_3$  0.57;  $S_4$  0.66; in 10% methanol in chloroform 0.89. Amino acid analysis: Gly 1.0; MeLeu 1.0; Pro 0.9.  $[\alpha]_D^{23} - 92.0^\circ$  (c 0.50; dimethylformamide). For  $C_{22}H_{32}N_4O_5$  (432.5) calculated: 61.09% C, 7.45% H, 12.95% N; found: 60.70% C, 7.57% H, 12.92% N.

## N-Tert-butyloxycarbonyl-S-benzylcysteinyl-prolyl-N-methylleucyl-glycinamide (V)

Protected tripeptide amide III (3.7 g; 8.5 mm) in ethanol (200 ml) was hydrogenated at room temperature and atmospheric pressure in the presence of 5% PdO/BaSO<sub>4</sub> (6.0 g) for 90 min and after filtration through Hyflo filter and evaporation of the filtrate we had a yield of 2.54 g (100%) of prolyl-N-methylleucyl-glycinamide as a foam which was homogeneous on electrophoresis:  $E_{2,4}^{\text{Gly}}$  1.1;  $E_{5,7}^{\text{His}}$  0.76. A solution of this substance (2.53 g; 8.5 mm) in dimethylformamide (4 ml) was added to a mixture of N-tert-butyloxycarbonyl-S-benzylcysteine (2.38 g; 7.6 mm), 1-hydroxybenzotriazole (1.07 g; 8.0 mm) and dicyclohexylcarbodiimide (1.65 g; 8.0 mm) in dimethylformamide (10 ml) (previously mixed for 30 min at -12°C). The mixture was stirred for 1 h at -12°C, left for 14 h at 0°C and stirred 12 h at 20°C, and processed as for III, except that instead of 1M-HCl for washing we used 10% citric acid. The yield was 4.5 g foam which was chromatographed on silica gel (270 g) in 1% methanol in chloroform to yield 3.6 g (80%) of protected tetrapeptide amide V as foam. On chromatography: in S<sub>1</sub> 0.66; S<sub>2</sub> 0.52; S<sub>3</sub> 0.65; S<sub>4</sub> 0.77; in 6% methanol in chloroform 0.95. Amino acid analysis: Pro 1.0; Gly 1.0; Cys(Bzl) 0.9. (With regard to low colour constant (ninhydrine) of N<sup>α</sup>-methylamino acids, the peak of MeLeu was not well detectable and it could not be precisely calculated at this and next N-methylleucine-containing peptides.)  $[\alpha]_D^{20}$  -82.4° (c 0.41; dimethylformamide). For C<sub>29</sub>H<sub>45</sub>N<sub>3</sub>O<sub>6</sub>S.1/2 H<sub>2</sub>O (600.8) calculated: 57.97% C, 7.71% H, 11.65% N, 5.33% S; found: 58.09% C, 7.59% H, 11.64% N, 4.98% S.

## 2-Nitrobenzenesulphenylasparaginyl-S-benzylcysteinyl-prolyl-N-methylleucyl-glycinamide (VI)

Protected amide V (2.01 g; 3.4 mm) was dissolved in trifluoroacetic acid (11 ml). After 20 min at room temperature the solution was evaporated, the residue extracted with ether and after filtration and drying of the resulting powder over P<sub>2</sub>O<sub>5</sub> there was a yield of 2.05 g of trifluoroacetate. Electrophoretic values:  $E_{2,4}^{\text{Gly}}$  0.86;  $E_{5,7}^{\text{His}}$  0.60.

This product was dissolved in dimethylformamide (15 ml) and to the solution was added by drops N-ethylpiperidine (0.48 ml; 3.4 mm) and then the trichlorophenyl ester of 2-nitrobenzenesulphenylasparagine (6.3 g; 13.6 mm). The mixture was stirred for 60 h at room temperature, dimethylformamide was distilled off, the residue was dissolved in ethyl acetate (80 ml) and the solution washed with 0.05M-H<sub>2</sub>SO<sub>4</sub>, 5% NaHCO<sub>3</sub>, water, dried with Na<sub>2</sub>SO<sub>4</sub> and solvent was distilled off. The resulting syrup was chromatographed on silica gel (260 g) first in chloroform, then in 20% methanol in chloroform, with a yield of 1.61 g (65%), m.p. 110-113°C. Chromatographic values for substance VI: R<sub>F</sub> in S<sub>2</sub> 0.52; S<sub>3</sub> 0.53; S<sub>4</sub> 0.67; in 15% methanol in chloroform 0.37. Amino acid analysis: Gly 1.0; Pro 1.0; Cys(Bzl) + 1/2 Cys 0.7; Asp 1.1.  $[\alpha]_D^{23}$  -113.8° (c 0.38; dimethylformamide). For C<sub>34</sub>H<sub>48</sub>N<sub>8</sub>O<sub>9</sub>S<sub>2</sub> (767.9) calculated: 53.17% C, 6.03% H, 14.59% N, 8.35% S; found: 52.91% C, 6.05% H, 14.76% N, 8.33% S.

## 2-Nitrobenzenesulphenylglutamyl-asparaginyl-S-benzylcysteinyl-prolyl-N-methylleucyl-glycinamide (VII)

To a solution of protected amide VI (1.46 g; 1.9 mm) in dimethylformamide (4 ml) we added 2.5M-HCl in ether (2.5 ml) and after 6 min an excess of ether. The product was decanted, extracted with ether (5 × 50 ml) until it separated as a powder which was filtered off, washed with ether and dried over P<sub>2</sub>O<sub>5</sub>. The yield was 1.23 g (98%) of pentapeptide amide hydrochloride with electrophoretic values:  $E_{2,4}^{\text{Gly}}$  0.75;  $E_{5,7}^{\text{His}}$  0.51.

To a solution of the latter hydrochloride (1.23 g; 1.88 mm) in dimethylformamide (7 ml) we dropped in N-ethylpiperidine (0.27 ml; 1.88 mm) and then added the trichlorophenyl ester of 2-nitrobenzenesulphenylglutamine (4.78 g; 10 mm). The mixture was stirred for 40 h at room tem-



perature and then processed as for VI. The powder product was chromatographed on TLC silica gel in 35% methanol in chloroform, with a yield of 1.22 g (71%), m.p. 126–128°C,  $R_F$  in  $S_2$  0.34;  $S_3$  0.40;  $S_4$  0.66. Amino acid analysis: Gly 1.0; Pro 1.0; Cys(Bzl) + 1/2 Cys 0.6; Asp 1.0; Glu 1.0.  $[\alpha]_D^{25} - 76.6^\circ$  ( $c$  0.37; dimethylformamide). For  $C_{39}H_{54}N_{10}O_{10}S_2 \cdot 21/2 H_2O$  (932.1) calculated: 50.25% C, 6.38% H, 15.02% N, 6.88% S; found: 50.20% C, 6.26% H, 15.14% N, 7.01% S.

N-4-Toluenesulphonyl-S-benzylcysteinyl-tyrosyl-isoleucyl-glutaminyl-asparaginyl-S-benzylcysteinyl-prolyl-N-methylleucyl-glycinamide (VIII)

To protected hexapeptide amide VII (0.75 g; 0.81 mm) in dimethylformamide (2 ml) we added by drops 2M hydrogen chloride in ether (2 ml) and after 6 min an excess of ether. The product was decanted, ground up with ether until it was in powder form, filtered off, washed with ether and dried over  $P_2O_5$ . The yield was 0.67 g of hexapeptide amide hydrochloride with the following electrophoretic parameters:  $E_{2,4}^{Gly}$  0.68;  $E_{5,7}^{His}$  0.46; 0.23 g (0.28 mm) of the latter in dimethylformamide (0.8 ml) were added to a solution of N-4-toluenesulphonyl-S-benzylcysteinyl-tyrosyl-isoleucine azide, prepared by dissolving the corresponding hydrazide (0.17 g; 0.27 mm) in 3.9M hydrochloride in tetrahydrofuran (0.68 ml) over 30 min at room temperature, with addition of butyl nitrite (0.035 ml in 0.35 ml tetrahydrofuran) over 15 min at  $-10^\circ C$  and adjustment of pH of the azide solution to 7.0 by addition of N-ethylpiperidine (0.35 ml) at  $-15^\circ C$ . The mixture was stirred for 93 h at 0 to  $-5^\circ C$  and the solvent was distilled off. On addition of water to the residue it separated out as a powder which was filtered and washed with 1M-HCl, water, 5%  $NaHCO_3$ , water and ether. The yield was 0.28 g, m.p. 175–180°C. The product was precipitated by ether from a solution of acetic acid with a yield of 0.23 g, m.p. 180–183°C. For  $C_{65}H_{88} \cdot N_{12}O_{14}S_3 \cdot 31/2 H_2O$  (1420) calculated: 54.95% C, 6.73% H, 11.83% N; found: 54.95% C, 6.23% H, 12.08% N.

[8-N-Methylleucine]oxytocin (IX)

Protected nonapeptide amide VIII (0.2 g; 0.14 mm) was dissolved in freshly redistilled ammonia (200 ml) and with stirring at room temperature reduced by sodium to a constant blue colour, which was eliminated after 30 s by addition of  $NH_4Cl$ . The ammonia was distilled off, the residue dissolved in 2% HCl (200 ml) at pH 3.5, the solution washed with ethyl acetate (200 ml), pH adjusted with 0.01M-NaOH to 6.9, and the solution was stirred for 2 h at this pH, which was maintained constant by addition of 0.01M-NaOH. After completion of the oxidation, pH was again adjusted to 3.5 with 2% HCl and analogue IX was freeze-dried.

Analogue IX was purified by counter-current distribution in 2-butanol–0.05M acetic acid 1 : 1 (300 transfers of the upper phase, 50 of the lower) and had a partition coefficient  $K_{6,7}$  of 0.5. This was followed by gel filtration on Biogel P4 in 1M acetic acid. The yield was 17.8 mg of an electrophoretically homogeneous product:  $E_{2,4}^{Gly}$  0.63;  $E_{5,7}^{His}$  0.41, which was also homogeneous on chromatography.  $R_F$  in  $S_1$  0.38,  $S_3$  0.44,  $S_4$  0.74. Amino acid analysis: Gly 1.0; Pro 0.9; 1/2 Cys 1.9; Asp 1.0; Glu 1.0; Ile 0.8; Tyr 0.8;  $[\alpha]_D^{22} - 26.7^\circ$  ( $c$  0.27 1M acetic acid). For  $C_{44}H_{68} \cdot N_{12}O_{12}S_2 \cdot C_2H_4O_2 \cdot 6 H_2O$  (1189) calculated: 46.45% C, 7.11% H, 14.13% N; found: 46.51% C, 6.91% H, 13.98% N.

N $^{\alpha}$ -4-Toluenesulphonyl-N $^{\alpha}$ -methyl-N $^{\delta}$ -acetylornithine Isopropyl Ester (X)

The isopropyl ester of N $^{\alpha}$ -4-toluenesulphonyl-N $^{\delta}$ -acetylornithine $^{25}$  (18.5 g; 50 mm) was methylated using dimethyl sulphate (23 ml; 250 mm) in the presence of 4M-NaOH (in all 53 ml) and de-

tergent (1.5 ml) using the procedure described for *II*. The result was an oily *X* (18.1 g, 94% yield);  $[\alpha]_D^{22} - 19.9^\circ$  (*c* 0.38; dimethylformamide). For  $C_{18}H_{28}N_2O_5S$  (384.5) calculated: 56.23% C, 7.34% H, 7.29% N, 8.34% S; found: 55.97% C, 7.38% H, 7.02% N, 8.63% S.

#### $N^\alpha$ -4-Toluenesulphonyl- $N^\alpha$ -methylornithine (*XI*)

A solution of substance *X* (50.5 g; 131.3 mm) in 4M-HCl (850 ml) was boiled 4 h and then extracted with ethyl acetate — the aqueous phase was evaporated. The oily residue was evaporated 3× with water, 3× with benzene, dissolved in a small amount of water, pH was adjusted to 6.5 by drops of ammonia, and crystals separated out. Ethanol (500 ml) was added to the product and after 2 h at 3°C the latter was filtered off, washed with ethanol and ether, with a yield of 30.2 g, m.p. 175–182°C. Recrystallisation from aqueous ethanol yielded 28 g (71%), m.p. 180–182°C.  $R_F$  in  $S_1$  0.11;  $S_2$  0.53;  $S_3$  0.48;  $S_4$  0.59. Electrophoresis:  $E_{2,4}^{Gly}$  0.96;  $E_{5,7}^{His}$  0.18.  $[\alpha]_D^{22} - 50.5^\circ$  (*c* 0.42; dimethylformamide). For  $C_{13}H_{20}N_2O_4S$  (300.4) calculated: 51.98% C, 6.71% H, 9.33% N, 10.67% S; found: 51.47% C, 6.67% H, 9.36% N, 10.47% S.

#### $N^\alpha$ -4-Toluenesulphonyl- $N^\alpha$ -methylarginine (*XII*)

To a solution of substance *XI* (6.0 g; 20 mm) in 2M-NaOH (30 ml; 60 mm) we added the sulphate of S-methylisothiouraea (5.57 g; 40 mm) and after 24 h a further portion of the same (2.78 g; 20 mm). After 48 h the pH of the mixture was adjusted to 5.5 with acetic acid (about 5 ml) and temperature was dropped to 0°C. The resulting crystals were filtered off, washed in ice water, ethanol and ether. The yield was 3.2 g; m.p. 225–228°C. Recrystallisation from 4% acetic acid gave 2.95 g (45%), m.p. 227–230°C of *XII* with homogeneous electrophoretic behaviour:  $E_{2,4}^{Gly}$  0.94;  $E_{5,7}^{His}$  0.19, and homogeneous chromatographically:  $R_F$  in  $S_1$  0.23;  $S_2$  0.60;  $S_3$  0.54;  $S_4$  0.65;  $[\alpha]_D^{22} - 16.1^\circ$  (*c* 0.49; 5M-HCl). For  $C_{14}H_{22}N_4O_4S$  (342.4) calculated 49.11% C, 6.48% H, 16.36% N, 9.37% S; found: 48.92% C, 6.40% H, 16.38% N, 9.42% S.

#### $N^\alpha$ -Methylarginine (*XIII*)

A solution of substance *XII* (15.2 g; 44.5 mm) in 42% HBr (76 ml) in the presence of phenol (15.2 g) was boiled for 30 min, and then washed with ether, the aqueous layer was evaporated and the residue evaporate 3× with water and 3× with benzene. The resulting  $N^\alpha$ -methylarginine hydrobromide was dissolved in water and put onto a column of Amberlite IRA 410 (400 ml).  $N^\alpha$ -Methylarginine was eluted from the column with nitrogenated water (ninhydrin detection) and after evaporation of the water the residue was transferred in ethanol onto a filtre, washed with ethanol and ether, with a yield of 6.84 g of electrophoretically and chromatographically ( $E_{2,4}^{Gly}$  1.49;  $E_{2,4}^{His}$  0.90;  $E_{5,7}^{His}$  0.96;  $R_F$  in  $S_2$  0.13;  $S_3$  0.12;  $S_4$  0.26) homogeneous product.  $[\alpha]_D^{22} + 28.3^\circ$  (*c* 0.51; 3M-HCl). Recrystallisation from water gave 6.27 g (75%), m.p. 260–262°C;  $[\alpha]_D^{22} + 31.9^\circ$  (*c* 1.0; 6M-HCl). For  $C_7H_{16}N_4O_2$  (188.2) calculated: 44.67% C, 8.57% H, 29.76% N; found: 44.80% C, 8.76% H, 29.49% N.

#### $N^\alpha$ -Benzyloxycarbonyl- $N^\alpha$ -methylarginine (*XIV*)

To a solution of *XIII* (1.88 g; 10 mm) in water (8.4 ml) at 0°C we added by drops, with stirring over 30 min, benzyloxycarbonyl chloride (2.2 ml) and a solution of  $Na_2CO_3$  (2.94 g in 11.2 ml of water) in such a manner that the pH did not exceed 9. The mixture was stirred for 2 h at 0°C, and during this period we added a further aliquot of  $Na_2CO_3$  solution (0.92 g in 3.5 ml of water). The solution was then washed 3× with ether, the aqueous layer was acidified with acetic acid

to pH 5.5 and the solid material which separated out was decanted with ice water ( $2 \times 20$  ml). The remainder was dissolved in methanol and the solution evaporated. The residue was distilled  $3 \times$  with methanol,  $3 \times$  with ether and after drying over  $P_2O_5$  there was a syrupy yield of 1.75 g. The product dissolved in a minimum of methanol, was placed on a column of silica gel (60 to 120  $\mu$ , 120 g, dispersed in benzene). The more mobile impurities were eluted with a 1 : 1 mixture of methanol and benzene, and then the product per se with methanol. The yield was 1.62 g (50%) of *XIV* which showed homogeneous electrophoretic and chromatographic behaviour ( $E_{2,4}^{Gly}$  0.96;  $E_{2,4}^{His}$  0.62;  $E_{5,7}^{His}$  0.22).  $R_F$  in  $S_1$  0.11;  $S_2$  0.63;  $S_3$  0.49;  $S_4$  0.64.  $[\alpha]_D^{22} - 15.2^\circ$  ( $c$  0.71; 1M-HCl). For  $C_{15}H_{22}N_4O_4$  (322.4) calculated: 55.89% C, 6.88% H, 17.38% N; found: 55.46% C, 7.11% H, 17.26% N.

#### $N^\alpha$ -Benzyloxycarbonyl- $N^\alpha$ -methyl- $N^G$ -4-toluenesulphonylarginine (*XV*)

To a solution of *XIV* (1.62 g; 50 mm) in water (6 ml) and acetone (25 ml) at  $0^\circ C$ , pH 11.5, we added by drops a solution of 4-toluenesulphonyl chloride (2.37 g) in acetone (4 ml) over 30 min. pH was then adjusted to 7.0 by addition of 1M-HCl and acetone was evaporated at room temperature. The residue was dissolved in water (10 ml), and the solution washed with ether ( $3 \times 10$  ml). The aqueous layer was cooled to  $0^\circ C$  and acidified with 6M-HCl to pH 3, with separation of a syrup from which the aqueous layer was poured off, saturated with NaCl and extracted with ethyl acetate ( $3 \times 10$  ml). The syrup was dissolved in the pooled ethyl acetate extracts and the solution washed with 1M-HCl ( $5 \times 20$  ml) and water to neutrality and dried with  $Na_2SO_4$ . After evaporation of the solvent the yield was 1.85 g of foam. This substance dissolved in a minimum of ethyl acetate was placed on column of silica gel (60–120  $\mu$ , 100 g, dispersed in ether) and the column was washed with a 9 : 1 mixture of ether and ethyl acetate. The yield was 1.62 g (68%) of *XV*.  $R_F$  in  $S_1$  0.24;  $S_2$  0.82;  $S_3$  0.78;  $S_4$  0.73;  $[\alpha]_D^{22} - 11.2^\circ$  ( $c$  0.22; dimethylformamide). For  $C_{22}H_{28}N_4O_6S$  (476.6) calculated: 55.44% C, 5.92% H, 11.76% N, 6.73% S; found: 55.41% C, 5.82% H, 12.01% N, 6.62% S.

#### $N^\alpha$ -Benzyloxycarbonyl- $N^\alpha$ -methyl- $N^G$ -4-toluenesulphonylarginyl-glycinamide (*XVI*)

To a solution of glycinamide hydrobromide (0.58 g; 3.7 mm) in dimethylformamide (8 ml) at  $0^\circ C$  we added by drops *N*-ethylpiperidine (0.51 ml) and with mixing 1-hydroxybenzotriazole (0.34 g; 2.5 mm), dicyclohexylcarbodiimide (0.52 g; 2.5 mm) and *XV* (1.01 g; 2.1 mm), and the mixture was left at  $0^\circ C$  for 60 h. Then it was filtered to remove the dicyclohexylurea and the filtrate was evaporated. The oily residue was extracted with ether (20 ml) and ethyl acetate (20 ml) and was then dissolved in chloroform (50 ml) and this solution was washed with 1M-HCl ( $2 \times 20$  ml), 5%  $NaHCO_3$  ( $2 \times 20$  ml), water, dried with  $Na_2SO_4$ , the solvent was distilled off and the yield was 1.03 g of a foamy substance with homogeneous chromatographic behaviour ( $R_F$  in  $S_1$  0.48;  $S_2$  0.58;  $S_3$  0.61;  $S_4$  0.72).  $[\alpha]_D^{22} - 13.4^\circ$  ( $c$  0.21; dimethylformamide). For  $C_{24}H_{32}N_6O_6S$  (533.6) calculated: 54.02% C, 6.04% H, 15.75% N, 6.01% S; found: 54.15% C, 6.01% H, 16.03% N, 5.87% S.

#### Benzyloxycarbonylpropyl- $N^\alpha$ -methyl- $N^G$ -4-toluenesulphonylarginyl-glycinamide (*XVII*)

Amide *XVI* (1.03 g; 1.9 mm) was hydrogenated in methanol (30 ml) at room temperature and atmospheric pressure in the presence of 5% PdO/ $BaSO_4$  (2.1 g) for 2 h. The mixture was filtered through Hyflo, the filtrate was evaporated and the yield was 0.71 g of dipeptide amide as a foam.  $E_{2,4}^{Gly}$  1.03;  $E_{2,4}^{His}$  0.66;  $E_{5,7}^{His}$  0.70.  $R_F$  in  $S_1$  0.25;  $S_2$  0.22;  $S_3$  0.33;  $S_4$  0.46. This substance (0.23 g; 0.59 mm) in dimethylformamide (0.4 ml) was added to benzyloxycarbonylproline (0.08; 0.41 mm),

1-hydroxybenzotriazole (0.06 g; 0.44 mm) and dicyclohexylcarbodiimide (0.09 g; 0.44 mm) in dimethylformamide (0.4 ml) and the mixture was stirred at  $-7^{\circ}\text{C}$  for 1 h and at  $10^{\circ}\text{C}$  for 17 h. Dicyclohexylurea was filtered off, solvent was distilled off and the residue was dissolved in methanol and put onto TLC silica gel in 10% methanol in chloroform. The band with  $R_F$  0.26 was separated and after elution of the silica gel with methanol and distillation of the latter there was a yield of 0.12 g (47%) of pure product *XVII*.  $[\alpha]_D^{25} - 48.2^{\circ}$  ( $c$  0.25; dimethylformamide).  $R_F$  in  $S_1$  0.45;  $S_2$  0.46;  $S_3$  0.55;  $S_4$  0.68. Amino acid analysis: Pro 1.0; Gly 1.0. ( $N^{\alpha}$ -Methylarginine could not be determined at the same time because of the same reason as in the case of the determination of MeLeu. In addition, the small peak of MeArg was overlapped with peak of ammonia.) For  $C_{29}H_{39}N_7O_7S$  (629.7) calculated: 55.32% C, 6.24% H; 15.57% N, 5.09% S, found: 55.18% C, 6.22% H, 15.88% N, 5.01% S.

3-Benzylthiopropionyl-tyrosyl-phenylalanyl-glutaminy-asparaginy-S-benzylcysteinyl-prolyl- $N^{\alpha}$ -methyl- $N^G$ -4-toluenesulphonylarginyl-glycinamide (*XVIII*)

Protected tripeptide amide *XVII* (0.13 g; 0.21 mm) was hydrogenated under the same conditions as *XVI* with a yield of 0.11 g of prolyl- $N^{\alpha}$ -methyl- $N^G$ -toluenesulphonylarginyl-glycinamide.  $E_{2,4}^{Gly}$  0.90;  $E_{2,4}^{His}$  0.56;  $E_{5,7}^{His}$  0.69.  $R_F$  in  $S_1$  0.10;  $S_2$  0.22;  $S_3$  0.19;  $S_4$  0.49. A solution of this substance (0.11 g) in dimethylformamide (0.72 ml) was added by drops to a solution of the azide of 3-benzylthiopropionyl-tyrosyl-phenylalanyl-glutaminy-asparaginy-S-benzylcysteine, prepared from the corresponding hydrazide (0.16 g; 0.14 mm) dissolving latter in dimethylformamide (1.24 ml), and adding 4.3M-HCl in dioxane (0.39 ml), followed addition of butyl nitrite (0.02 ml) in dioxane (0.22 ml) at  $-10^{\circ}\text{C}$  over 15 min, cooling of the solution to  $-15^{\circ}\text{C}$  and adjustment of pH to 7 by addition of *N*-ethylpiperidine (about 0.22 ml). The mixture was stirred for 90 h at  $5^{\circ}\text{C}$  and after evaporation of the solvent, addition of water to the residue resulted in separation of the product, which was ground to a powder and then washed with 1M-HCl, 5%  $\text{NaHCO}_3$ , water and ether. The yield was 0.27 g, m.p.  $176-181^{\circ}\text{C}$ . The compound *XVIII* was dissolved in dimethylformamide and reprecipitated by addition of water, filtered and dried over  $P_2O_5$ , with a yield of 0.21 g, m.p.  $182-184^{\circ}\text{C}$ . Amino acid analysis: Tyr 0.9; Phe 1.0; Glu 1.0; Asp 1.1; Cys(Bzl) + 1/2 Cys 0.9; Pro 0.9; Gly 1.0.  $N^{\alpha}$ -Methylarginine was not determined at the same time.

[8- $N^{\alpha}$ -Methylarginine]deamino-vasopressin (*XIX*)

Protected octapeptide amide *XVIII* (0.15 g) was reduced with sodium in liquid ammonia in the same manner as nonapeptide *VIII* and oxidation was carried out after adjustment of pH to 6.9 with 0.01M-NaOH in a nitrogen atmosphere with constant stirring, and addition by drops of 0.02M- $K_3[Fe(CN)_6]$  (10.6 ml). During oxidation pH was maintained in the range 6.8-7.0 by addition of 0.1M-NaOH. After oxidation the yellow solution was acidified with 2% HCl to pH 2.5 and put onto a column of Amberlite IR 4B (30 ml) in the  $Cl^-$  cycle. The analogue was eluted from the column with water (300 ml), the solution was concentrated at room temperature to 30 ml and freeze-dried. Desalting was carried out by dissolving the powder in water, acidifying the solution to pH 4 with 2% HCl, and ion-exchanging through a column of Amberlite IRC-50 (XE-64) in the  $H^+$  cycle. The salts were eluted by 0.25% acetic acid (200 ml) and then the analogue in 50% acetic acid (200 ml). This solution was diluted 1 : 1 with water, freeze-dried, and the yield was 0.04 g. The product was purified by continuous free-flow electrophoresis in 0.05M acetic acid at pH 2.4 for 60 min, with a final yield of 35 mg analog *XIX*,  $E_{2,4}^{Gly}$  0.58;  $E_{2,4}^{His}$  0.34;  $E_{5,7}^{His}$  0.38.  $R_F$  in  $S_2$  0.14;  $S_3$  0.48;  $S_4$  0.65. Amino acid analysis: Tyr 0.9; Phe 1.0; Asp 1.0; Glu 1.0; Pro 1.0; Gly

1.0; 1/2 Cys 0.9.  $[\alpha]_D^{25} - 57.1^\circ$  (*c* 0.15; 1M acetic acid). For  $C_{47}H_{66}N_{14}O_{12}S_2 \cdot 2 C_2H_4O_2 \cdot 6 H_2O$  (1311) calculated: 46.71% C, 6.61% H, 14.95% N; found: 46.59% C, 6.65% H, 15.11% N.

### Pharmacological Methods

a) Uterotonic activity<sup>26,27</sup> was assayed on rat uterus horn strips 24 h after oestradiol administration (10 µg/kg, *s.c.*). Van Dyke-Hastings solution<sup>43</sup> was used in a 5 ml organ bath at pH 7.4 at 30°C. Isometric contractions were measured with a permanent magnet feedback transducer<sup>44</sup> and recorded on a strip-chart recorder.

b) Pressor activity: Two pressor assays were used: nephrectomised, ganglion-blocked rats in urethane anaesthesia<sup>31</sup> and despinalised Wistar rats<sup>32</sup>. In both cases, activities were calculated from threshold values extrapolated from pressor-effect-log doses dependence, using synthetic lysine-vasopressin as standard.

c) Antidiuretic activity: Two antidiuretic assays were used: ethanol anaesthetised rats with an oral tap water load maintained at 6–8% body weight, with urine flow rate measured as drop counts recorded automatically, and continuous measurement of urine conductivity<sup>28,29</sup>. The second method was a modification of the Burn assay in which trained, unanaesthetised rats, body weight 150–180 g, received an oral tap water load of 2% body weight, and T-1/2 of excretion of the load, in comparison with untreated controls run at the same time, was measured in min<sup>4</sup>. Peptides were given *s.c.* at the time of oral loading. Special metabolic cages were used to ensure quantitative collection of spontaneously voided urine with no faecal contamination, the surface of the plexiglass funnels being pre-treated with Bryj 35 to make the surface more non-wettable. In both cases activity was taken as threshold from extrapolation of 3–4 point curves, using synthetic lysine-vasopressin as a standard. The total pharmacological activity was measured as intensity times duration. In the latter assay, the rats were trained to the assay situation at least one week before starting to measure, and all rats were rotated through all doses of all peptides and non-treated (*s.c.* injection of saline alone) controls.

d) Galactogogic activity was measured by changes in milk duct pressure in the mammary glands of lactating rats<sup>30</sup>.

e) Natriuretic activity was measured as described in detail elsewhere<sup>34</sup>, in chloralosed male cats under conditions of moderate osmotic diuresis.

### Spectral Measurements

Circular dichroic spectra were measured on the Roussel-Jouan Dichrograph CD 185, Model II using cells with an optical pathway of 0.01–1.0 cm at 22 to 25°C. Concentration of the solutions was calculated from the weighed amount of dry substance (0.6–0.9 mg/ml). Spectral data presented in Figs 1 and 2 are given in values of molar ellipticity  $[\theta]$  (deg. cm<sup>2</sup>. d mol<sup>-1</sup>) and are not corrected for the refractive index of the solvent. Spectral measurements were carried out in phosphate buffer (pH 7.1) and hexafluoroacetone trihydrate (Hynes Chemical Research Co.) used without further purification.

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## REFERENCES

1. IUPAC-IUB Commission: *Biochem. J.* **126**, 773 (1972).
2. Jošt K., Rudinger J.: *Colloques Internationaux du C.N.R.S.* **117**, 13 (1969).
3. Zaoral M., Kolc J., Šorm F.: *This Journal* **32**, 1250 (1967).
4. Cort J. H., Schück O., Stříbrná J., Škopková J., Jošt K., Mulder J. L.: *Kidney Int.* **8**, 292 (1975).
5. Sawyer W. H., Acosta M., Balaspiri L., Judd J., Manning M.: *Endocrinology* **94**, 1106 (1974).
6. Cort J. H., Frič I., Carlsson L., Gillesen D., Bystrický S., Škopková J., Gut V., Studer R. O., Mulder J. L., Bláha K.: *Mol. Pharmacol.* **12**, 313 (1976).
7. Andreatta R. H., Scheraga H. A.: *J. Med. Chem.* **14**, 489 (1971).
8. Bergmann M., Fruton J. S.: *J. Biol. Chem.* **117**, 189 (1937).
9. Fujino M., Hatanaka C., Nishimura O.: *Chem. Pharm. Bull.* **18**, 1288 (1970).
10. Jošt K., Rudinger J., Šorm F.: *This Journal* **28**, 202 (1963).
11. Yamashiro D., Aanning H. L., Branda L. A., Cash W. D., Murti V. V. S., du Vigneaud V.: *J. Amer. Chem. Soc.* **90**, 4141 (1968).
12. Jošt K., Rudinger J., Šorm F.: *This Journal* **26**, 2496 (1961).
13. Huguenin R. L., Boissonnas R. A.: *Helv. Chim. Acta* **44**, 213 (1961).
14. Meienhofer J., du Vigneaud V.: *J. Amer. Chem. Soc.* **83**, 142 (1961).
15. Cash W. D., McCulloch-Mahaffey L., Buck A. S., Nettleton D. E., Romas C., du Vigneaud V.: *J. Med. Pharm. Chem.* **5**, 413 (1962).
16. Servitová L., Barth T.: *This Journal* **40**, 215 (1975).
17. Glass J. D., Schwartz I. L., Walter R.: *Proc. Nat. Acad. Sci. U.S.A.* **63**, 1426 (1969).
18. Brenner M., Huber W.: *Helv. Chim. Acta* **36**, 1109 (1953).
19. König W., Geiger R.: *Chem. Ber.* **103**, 788 (1970).
20. Rudinger J.: *This Journal* **19**, 375 (1954).
21. Runge F., Engelbrecht H. J., Franke H.: *Chem. Ber.* **88**, 533 (1955).
22. Schnabel E.: *Justus Liebigs Ann. Chem.* **702**, 188 (1967).
23. Poduška K.: Unpublished results.
24. Honzl J., Rudinger J.: *This Journal* **20**, 1190 (1955).
25. Gut V., Poduška K.: *This Journal* **36**, 3470 (1971).
26. Holton P.: *Brit. J. Pharmacol.* **3**, 328 (1948).
27. Munsick R. A.: *Endocrinology* **66**, 451 (1960).
28. Jeffers W. A., Livezey M. M., Austin J. H.: *Proc. Soc. Exp. Biol. Med.* **50**, 184 (1942).
29. Pliška V., Rychlík I.: *Acta Endocrinol. (Copenhagen)* **54**, 129 (1967).
30. Bisset G. W., Clark B. J., Haldar J., Harris M. C., Lewis G. P., Rocha e Silva M.: *Brit. J. Pharmacol. Chemother.* **31**, 537 (1967).
31. Schaechtelin G., Walter R., Salomon H., Jelinek J., Karen P., Cort J. H.: *Mol. Pharmacol.* **10**, 57 (1974).
32. Krejčí I., Kupková B., Vávra I.: *Brit. J. Pharmac. Chemother.* **30**, 497 (1967).
33. Cort J. H., Albrecht I., Nováková J., Mulder J. L., Jošt K.: *Eur. J. Clin. Invest.* **5**, 165 (1975).
34. Sedláková E., Prusík Z., Škopková J., Barth T., Klugh I., Cort J. H.: *Eur. J. Clin. Invest.* **4**, 285 (1974).
35. Cort J. H., Jošt K., Bláha K., Mulder J. L., Cash J. D.: *Proc. XIV Europ. Peptide Symp., Wépion, Belgium, 1976*, p. 479. Presses Universitaires, Bruxelles, 1976.
36. Frič I., Kodíček M., Jošt K., Bláha K.: *This Journal* **39**, 1271 (1974).
37. Frič I., Kodíček M., Flegel M., Zaoral M.: *Eur. J. Biochem.* **56**, 493 (1975).
38. Urry D. W., Walter R.: *Proc. Nat. Acad. Sci. U.S.A.* **68**, 956 (1971).
39. Sakaguchi S.: *J. Biochem. (Tokyo)*, **37**, 231 (1950).

40. Fraenkel-Conrat H., Harris J. I., Levy A. L.: *Methods Biochem. Analys.* 2, 377 (1955).
41. Prusik Z., Sedláková E., Barth T.: *Hoppe-Seyler's Z. Physiol. Chem.* 353, 1837 (1972).
42. Hannig K.: *Fresenius' Z. Anal. Chem.* 181, 244 (1961).
43. Munsick R. A. in the book: *Experimental Pharmacology* (B. Berde, Ed.), Vol. 3, p. 443. Springer, Berlin 1968.
44. Ženišek K., Barth T.: *Česk. Fysiol.* 23, 67 (1974).

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