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Synthesis and Some Pharmacological Activities of [2-L-Valine]-oxytocin and [2-L-Leucine]-oxytocin[1a,b]

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[2-Valine]-oxytocin and [2-leucine]-oxytocin have been synthesized from the requisite protected nonapeptide intermediates, which were prepared by the stepwise *p*-nitrophenyl ester method. The analogs were isolated by partition chromatography and gel filtration on Sephadex and then tested for a number of pharmacological activities. [2-Valine]-oxytocin possesses approximately 7 units/mg of avian vasodepressor, 2 units/mg of oxytocic, 15 units/mg of milk-ejecting, 0.05 unit/mg of pressor, and less than 0.01 unit/mg of antidiuretic activities. [2-Leucine]-oxytocin has approximately 3 units/mg of avian vasodepressor, less than 0.5 unit/mg of oxytocic, about 7 units/mg of milk-ejecting, 0.05 unit/mg of pressor, and negligible antidiuretic activities. Both of these analogs are much less active than [2-isoleucine]-oxytocin.

It was recently reported[2] that [2-isoleucine]-oxytocin, an analog in which the aromatic tyrosine residue at position 2 of oxytocin (Figure 1) is replaced by the aliphatic amino acid isoleucine, possesses a considerable degree of avian vasodepressor, oxytocic, and milk-ejecting activities. This finding was surprising in view of the extremely low activities reported for [2-leucine]-oxytocin by Jošt, *et al.*[3] The striking difference in the biological activities of two such similar analogs of oxytocin has led us to synthesize [2-valine]-oxytocin and examine its pharmacological properties.

To make possible the comparison of the pharmacological activities of [2-isoleucine]-oxytocin and [2-valine]-oxytocin with those of [2-leucine]-oxytocin under the same assay conditions, the [2-leucine]-oxytocin was also prepared in this laboratory. The stepwise *p*-nitrophenyl ester method as used in the synthesis of oxytocin[4] and [2-isoleucine]-oxytocin[2] was utilized for the preparation of the requisite protected nonapeptide intermediates for [2-valine]-oxytocin and [2-leucine]-oxytocin. The preparation of the protected nonapeptide intermediate for the synthesis of [2-leucine]-oxytocin by Jošt, *et al.*,[5] was accomplished by means of the azide method with the use of N-tosyl-S-benzylcysteinylleucine hydrazide and isoleucylglutaminylasparaginyl-S-benzylcysteinylprolylleucylglycinamide.

In our synthesis of [2-valine]-oxytocin and [2-leucine]-oxytocin, the protected nonapeptides N-benzyloxycarbonyl-S-benzylcysteinylvalylisoleucylglutaminylasparaginyl-S-benzylcysteinylprolylleucylglycinamide and N-benzyloxycarbonyl-S-benzylcysteinylleucylisoleucylglutaminylasparaginyl-S-benzylcysteinylprolylleucylglycinamide were treated with Na in liquid NH_3[6] to remove the protecting groups, and the resulting disulfhydryl peptides were oxidized to the oxytocin analogs by treatment with potassium ferricyanide.[7] The [2-valine]-oxytocin and [2-leucine]-oxytocin were purified by partition chromatography[8] followed by gel filtration on Sephadex G-25[9] with the use of the solvent systems given in the Experimental Section.

The four-point assay design[10] was used for measurement of the pharmacological activities against the USP posterior pituitary reference standard. Avian vasodepressor assays were performed on conscious chickens according to the procedure employed by Munsick, *et al.*[11] Oxytocic assays were performed on isolated uteri from rats in natural estrus according to the method of Holton,[12] as modified by Munsick,[13] with the use of magnesium-free van Dyke–Hastings solution as the bathing fluid. Milk-ejecting activity was measured on anesthetized rabbits by the method of Cross and Harris,[14] as modified by van Dyke, *et al.*,[15] and by Chan.[16] Rat pressor assays were carried out on anes-

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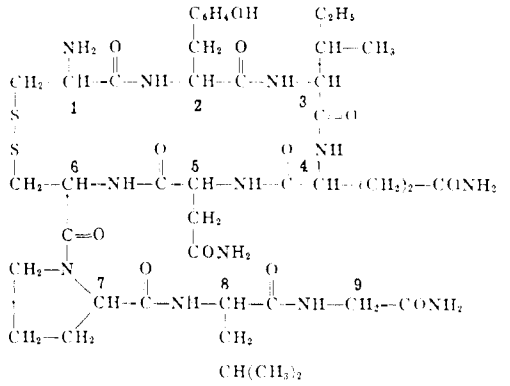

Figure 1.—Structure of oxytocin with numbers indicating the position of the individual amino acid residues.

thetized male rats as described in the U. S. Pharmacopeia.[17] Assays for antidiuretic activity were performed on anesthetized male rats according to the method of Jeffers, *et al.*,[18] as modified by Sawyer.[19]

[2-Valine]-oxytocin was found to possess 6.8 ± 0.5 units/mg of avian vasodepressor, 2.1 ± 0.1 units/mg of oxytocic, 15.6 ± 0.6 units/mg of milk-ejecting, ~0.05 unit/mg of pressor, and less than 0.01 unit/mg of antidiuretic activities. Our preparation of [2-leucine]-oxytocin was found to possess 3.3 ± 0.3 units/mg of avian vasodepressor, less than 0.5 unit/mg of oxytocic, about 7 units/mg of milk-ejecting, about 0.05 unit/mg of pressor, and negligible antidiuretic activities. These results on the avian vasodepressor, oxytocic, and antidiuretic activities for [2-leucine]-oxytocin are similar to those reported by Jošt, *et al.*[3] It is not possible to compare the milk-ejecting potencies reported for this analog from the two laboratories since our assay was performed on the rabbit, while their assay results were obtained on the guinea pig.

Although [2-valine]-oxytocin exhibits higher avian vasodepressor, oxytocic, and milk-ejecting activity than [2-leucine]-oxytocin, both of these analogs are much less active than [2-isoleucine]-oxytocin. As noted previously,[2] [2-isoleucine]-oxytocin is also far more potent than several other analogs possessing nonaromatic amino acid residues at position 2. The isoleucine analog possesses approximately seven times the avian vasodepressor, ten times the oxytocic, and three times the milk-ejecting activities exhibited by the valine analog. Further investigation will be required to ascertain the underlying reason for the much higher potencies of the 2-isoleucine analog of oxytocin compared to those of the 2-valine and 2-leucine analogs. In earlier studies[20] of the effect on pharmacological properties of the replacement of the *glutamine* residue at *position 4* of oxytocin by these same three amino acid residues, the [4-valine]-oxytocin was found to be far more potent with respect to the activities mentioned than the 4-isoleucine and 4-leucine analogs.

Experimental Section[21]

N-Benzyloxycarbonylleucylisoleucylglutaminylasparaginyl-S-benzylcysteinylprolylleucylglycinamide.—A solution of 1.93 g of N-benzyloxycarbonylisoleucylglutaminylasparaginyl-S-benzylcysteinylprolylleucylglycinamide[4] in 22 ml of anhydrous AcOH was stirred with 22 ml of 5.3 *N* HBr in AcOH for 1 hr at room temperature. Then 200 ml of Et_2O was added and the resulting colorless precipitate was filtered off, washed with two 100-ml portions of Et_2O, and dissolved in 150 ml of MeOH. The solution was passed through a short column containing Rexyn RG1 (OH cycle). The column was washed with two 80-ml portions of MeOH, the eluates were combined, and the solvents were removed *in vacuo*. After the resulting powder was dissolved in 15 ml of DMF, 0.80 g of *p*-nitrophenyl N-benzyloxycarbonylleucinate[4] was added. The mixture was stirred at room temperature for 20 hr and 250 ml of EtOAc was added. The mixture was cooled and the solid residue was filtered off and washed with two 50-ml portions of EtOAc, two 75-ml portions of EtOH, and 75 ml of EtOAc. The resulting gelatinous powder was dried *in vacuo* to give 2.00 g of white powder, mp 256–258°, $[\alpha]^{26}D$ −46.6° (*c* 1, DMF). *Anal.* ($C_{56}H_{77}N_{11}O_{18}S$) C, H, N.

N-Benzyloxycarbonyl-S-benzylcysteinylleucylisoleucylglutaminylasparaginyl-S-benzylcysteinylprolylleucylglycinamide.—A solution of 0.87 g of the preceding protected octapeptide in 10 ml of anhydrous AcOH was treated with 10 ml of 5.3 *N* HBr in AcOH for 1 hr and the resulting hydrobromide was isolated and converted to the free octapeptide by the usual procedure as described in the preceding section. The colorless powder was dissolved in 15 ml of DMF, 0.38 g of *p*-nitrophenyl N-benzyloxycarbonyl-S-benzylcysteinate[4] was added, and the mixture was stirred for 20 hr at room temperature. After addition of 100 ml of EtOAc, the mixture was cooled and the precipitate that formed was filtered off and washed with 50 ml of EtOAc, two 50-ml portions of EtOH, and 50 ml of EtOAc. The product was dried *in vacuo* to give 1.00 g of white powder, mp 257–259° dec, $[\alpha]^{24}D$ −55.3° (*c* 0.50, DMF). *Anal.* ($C_{62}H_{89}N_{13}O_{19}S_2$) C, H, N.

[2-Leucine]-oxytocin.—A solution of 256 mg of the preceding protected nonapeptide in 175 ml of boiling anhydrous NH_3 was treated with Na until a blue coloration persisted for a few minutes. The NH_3 was removed by lyophilization and the salt was dissolved in 500 ml of deaerated water containing 0.50 ml of trifluoroacetic acid. The pH was adjusted to 8.2 with 1 *N* NH_4OH, 40.6 ml of 0.01 *N* $K_3Fe(CN)_6$ was added, and the mixture was stirred for 15 min. The pH was adjusted to 6.5 with dilute trifluoroacetic acid and the solution was stirred with a small amount of ion-exchange resin AG3-X4 (trifluoroacetate cycle) for 15 min. The polymer was removed by filtration, and the solution was lyophilized. The resulting solid was dissolved in 15 ml of the upper phase of the solvent system 3.5% aqueous AcOH (containing 1.5% pyridine)–1-BuOH (1:1) and added to a 2.15 × 110 cm column of Sephadex G-25 (100–200 mesh) which had been equilibrated with both lower and upper phases according to the method of Yamashiro.[8] One hundred 9.6-ml fractions were collected and the fractions corresponding to the major peak (R_f 0.30), as determined by plotting of the Folin–Lowry color values,[22] were pooled. H_2O (250 ml) was added and the solution was evaporated to 40 ml *in vacuo* and lyophilized. The colorless powder was dissolved in 5 ml of 0.2 *N* AcOH and placed on a 2.8 × 65-cm column of Sephadex G-25 (200–270 mesh) for gel filtration.[9] The column was then eluted with 0.2 *N* AcOH and one hundred 5-ml fractions were collected. The eluates corresponding to the major peak (fractions 52–63), as determined by plotting of the Folin–Lowry color values, were pooled and lyophilized to give 129.7 mg of [2-leucine]-oxytocin as a white powder, $[\alpha]^{25}D$ −37.3° (*c* 0.53, 1 *N* AcOH). *Anal.* ($C_{49}H_{68}N_{12}O_{12}S_2$) C, H, N.

A sample was hydrolyzed for 22 hr in 6 *N* HCl at 110° and subjected to amino acid analysis[23] on a Beckman/Spinco amino acid analyzer. The following molar ratios were obtained with the value of glutamic acid taken as 1.0: aspartic acid, 1.0; glutamic

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acid, 1.0; proline, 1.1; glycine, 0.9; cystine, 1.0; isoleucine, 1.0; leucine, 2.0; and ammonia, 3.0.

N-Benzyloxycarbonylvalylisoleucylglutaminylasparaginyl-S-benzylcysteinylprolylleucylglycinamide.—A solution of 1.67 g of crystalline isoleucylglutaminylasparaginyl-S-benzylcysteinylprolylleucylglycinamide[24] in 20 ml of DMF was stirred at room temperature with 0.75 g of *p*-nitrophenyl N-benzyloxycarbonylvalinate[25] for 20 hr, 300 ml of EtOAc was added, and the slurry was cooled to −20°. The precipitate was filtered off and washed with 75 ml of EtOAc, two 75-ml portions of EtOH, and 75 ml of EtOAc. The powder was dried *in vacuo* to give 2.04 g of white powder, mp 252–253°, $[\alpha]^{26}D$ −53.9° (*c* 0.40, DMF). *Anal.* ($C_{51}H_{75}N_{11}O_{12}S$) C, H, N.

N-Benzyloxycarbonyl-S-benzylcysteinylvalylisoleucylglutaminylasparaginyl-S-benzylcysteinylprolylleucylglycinamide.—A solution of 0.85 g of the preceding protected octapeptide in 10 ml of AcOH was converted to the free octapeptide in the usual manner. The product was dissolved in 15 ml of DMF, 0.38 g of *p*-nitrophenyl N-benzyloxycarbonyl-S-benzylcysteinate[4] was added, the mixture was stirred for 20 hr at room temperature, and 150 ml of EtOAc was added. The slurry was cooled and the precipitate was filtered off, washed with EtOAc (50 ml), two 50-ml portions of EtOH, and 50 ml of EtOAc and dried *in vacuo* to give 0.85 g of white powder, mp 261.5–262.5°, $[\alpha]^{25}D$ −46.9° (*c* 0.49, DMF). *Anal.* ($C_{61}H_{86}N_{12}O_{13}S_2$) C, H, N.

[2-Valine]-oxytocin.—The preceding protected nonapeptide (252 mg) was converted to [2-valine]-oxytocin, and the resulting preparation was subjected to partition chromatography on a 2.85 × 54 cm column of Sephadex G-25 according to the procedures used for [2-leucine]-oxytocin. One hundred 9.6-ml fractions from the partition chromatography were collected, the fractions corresponding to the principal peak (R_f 0.24) (determined by plotting of the Folin–Lowry color values) were pooled, and 300 ml of H_2O was added. The mixture was concentrated to about 40 ml and the solution was lyophilized to give a pale yellow hygroscopic glass which was dissolved in 10 ml of 0.2 *N* AcOH and subjected to gel filtration on Sephadex G-25. One hundred 5-ml fractions were collected and the eluates corresponding to the major peak (fractions 48–57) were pooled, concentrated to 40 ml, and lyophilized to give 196 mg of a hygroscopic powder, which was again subjected to partition chromatography as before and worked up in the usual way to give 119 mg of a white powder. Subjection of this material to gel filtration as before afforded 104 mg of [2-valine]-oxytocin as a white powder, $[\alpha]^{25}D$ −33.5° (*c* 0.51, 1 *N* AcOH). *Anal.* ($C_{39}H_{66}N_{12}O_{11}S_2 \cdot C_2H_4O_2$) H, N; C: calcd, 49.0; found, 48.5.

The analog was hydrolyzed in 6 *N* HCl at 110° for 22 hr and the following molar ratios of amino acids and ammonia were found with glutamic acid taken as 1.0: aspartic acid, 1.0; glutamic acid, 1.0; proline, 1.0; glycine, 1.0; cystine, 1.0; valine, 0.72; isoleucine, 0.72; leucine, 1.0; and ammonia, 2.7. A 45-hr hydrolysis under the same conditions gave the following results with glutamic acid taken as 1.0: aspartic acid, 1.0; glutamic acid, 1.0; proline, 1.1; glycine, 1.0; cystine, 1.05; valine, 0.9; isoleucine, 0.9; leucine, 1.0; and NH_3, 3.0. The prolonged hydrolysis was necessitated by the difficulty in the hydrolysis of a valyl-isolencine peptide bond.

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Angiotensin II Analogs. I. Synthesis and Biological Evaluation of [Gly¹,Gly²,Ile⁵]-angiotensin II, [Ac-Gly¹,Gly²,Ile⁵]-angiotensin II, and [Gly¹,Gly²,Ile⁵,His(Bzl)⁶]-angiotensin II[1]

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[Gly¹,Gly²,Ile⁵]-angiotensin II has been synthesized by fragment condensation and also in better yield and purity by solid-phase synthesis. This peptide showed 16–20% of the pressor activity of [Asn¹,Val⁵]-angiotensin II in the rat and the dog, while its N-acetyl derivative showed an activity of 0.4%. These results show that only a single basic group is necessary in the N-terminal dipeptide for good pressor potency. This essential basic group may be correlated with either the terminal amino group or the guanido group of arginine in angiotensin II. The low activity of the acetylated peptide shows that extension of the peptide backbone from six to eight amino acids does not alone contribute measurably to the potency of natural angiotensin II. The synthetic intermediate [Gly¹,Gly²,Ile⁵,His(Bzl)⁶]-angiotensin II showed a pressor activity of 0.3% indicating the importance of the free imidazole ring.

The octapeptide tissue hormone angiotensin II, Asp-Arg-Val-Tyr-Ile- (or Val-) His-Pro-Phe,[2] has been the subject of numerous studies on the relationship between chemical structure and biological activity because of its possible relationship to hypertension. Most of this work has been reviewed by Law[3] and by Schröder and Lübke.[4] The portion of the molecule which has been most thoroughly studied is the N-terminal dipeptide, Asp-Arg-. The analogs described by Schwyzer,[5] Khosla, *et al.*,[6] and Arakawa, *et al.*,[7] listed in Table I show that none of the functional groups in this portion of the molecule is essential for significant pressor activ-

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