[2,4-Diisoleucine]-oxytocin. An Analog of Oxytocin with Natriuretic and Diuretic Activities[1a,b]

VICTOR J. HRUBY AND VINCENT DU VIGNEAUD[1c]

Department of Chemistry, Cornell University, Ithaca, New York 14850

AND W. Y. CHAN[1d]

Department of Pharmacology, Cornell University Medical College, New York, New York 10021

Received October 29, 1969

[2,4-Diisoleucine]-oxytocin has been synthesized from the requisite protected nonapeptide intermediate which was prepared by the stepwise *p*-nitrophenyl ester method. In this analog of oxytocin, isoleucine residues replace the tyrosine and glutamine residues at the 2 and 4 positions of the hormone, and thus the isoleucine residue occurs successively at positions 2, 3, and 4. This compound possesses a very low level of avian vasodepressor, oxytocic, and pressor activities, and no antidiuretic activity. However, [2,4-diisoleucine]-oxytocin has a pronounced natriuretic activity and a mild diuretic effect.

A recent communication[2] reported that the replacement of the tyrosine residue at position 2 of oxytocin (Figure 1) by an isoleucine residue afforded an analog ([2-isoleucine]-oxytocin) that exhibited much higher levels of oxytocic, avian vasodepressor, and milk-ejecting activities than did [2-leucine]-oxytocin.[3,4] It was also found that [2-valine]-oxytocin[4] is somewhat more potent in these activities than [2-leucine]-oxytocin, though considerably less active than [2-isoleucine]-oxtyocin. [2-Glycine]-oxytocin possesses negligible oxytocic and avian vasodepressor activities.[5]

In studies involving replacement of the glutamine residue at position 4 of oxytocin by a variety of aliphatic amino acids including isoleucine,[6] alanine,[7] valine,[8] α-aminobutyric acid,[9] and leucine,[6] analogs were obtained that possessed varying degrees of avian vasodepressor, oxytocic, and milk-ejecting activities depending on the nature of the aliphatic side chain. [4-Valine]-oxytocin was found to be the most active, and [4-isoleucine]-oxytocin was found to be more potent than [4-leucine]-oxytocin in these activities. [4-Glycine]-oxytocin[5] had also been synthesized and was found to possess very low avian vasodepressor, oxytocic, and milk-ejecting activities and no pressor and antidiuretic activities. All of the analogs containing aliphatic side chains at position 4 possessed small or negligible antidiuretic and pressor activities. [4-Leucine]-oxytocin possesses considerable diuretic and natriuretic effects, as well as anti-ADH activity.[6,10]

The aforementioned results led us to prepare [2,4-diisoleucine]-oxytocin, in which the tyrosine residue at position 2 and the glutamine at position 4 are both formally replaced by isoleucine. This highly lipophilic analog contains isoleucine residues at the 2, 3, and 4 positions of oxytocin, since in the natural hormone the residue at position 3 is that of isoleucine.

For the synthesis of [2,4-diisoleucine]-oxytocin the protected heptapeptide, *N*-benzyloxycarbonyl-isoleucylisoleucylasparaginyl-*S*-benzylcysteinylprolyl-leucylglycinamide, used in the synthesis of [4-isoleucine]-oxytocin,[6] served as starting material. Following removal of the *N*-benzyloxycarbonyl group with HBr in AcOH and neutralization of the heptapeptide salt, the next residue, isoleucine, was added as *p*-nitrophenyl *N*-benzyloxycarbonylisoleucinate.[11] The entire procedure was repeated and the free octapeptide was treated with *p*-nitrophenyl *N*-benzyloxycarbonyl-*S*-benzylcysteinate[11] to give the desired protected nonapeptide, *N*-benzyloxycarbonyl-*S*-benzylcysteinylisoleucylisoleucylisoleucylasparaginyl-*S*-benzylcysteinylprolylleucylglycinamide. The nonapeptide was treated with Na in liquid NH_3[12] to cleave the benzyloxycarbonyl and *S*-benzyl protecting groups, and the resulting disulfhydryl compound was oxidized in aqueous solution with $K_3Fe(CN)_6$.[13] The analog was purified as described in the Experimental Section by partition chromatography on Sephadex G-25[14] in two different solvent systems and by gel filtration[15] on Sephadex G-25.

The [2,4-diisoleucine]-oxytocin was assayed for avian vasodepressor,[16] oxytocic,[17] pressor,[18] and anti-

(1) (a) This work was supported in part by Grants HE-11680 (VduV) and HE-09795 (WYC) from the National Heart Institute, U. S. Public Health Service; (b) All optically active amino acid residues are of the L variety; (c) To whom reprint requests should be addressed; (d) Recipient of Career Development Award, 1-K4-HE-38,849 USPHS.

(2) L. A. Branda, V. J. Hruby, and V. du Vigneaud, *Mol. Pharmacol.*, **3**, 248 (1967).

(3) K. Jošt, J. Rudinger, and F. Šorm, *Collect. Czech. Chem. Commun.*, **28**, 1706 (1963).

(4) V. J. Hruby and V. du Vigneaud, *J. Med. Chem.*, **12**, 731 (1969).

(5) S. Drabarek, *J. Amer. Chem. Soc.*, **86**, 4477 (1964).

(6) V. J. Hruby, G. Flouret, and V. du Vigneaud, *J. Biol. Chem.*, **244**, 3890 (1969).

(7) S. Guttmann and R. A. Boissonnas, *Helv. Chim. Acta*, **46**, 1626 (1963).

(8) V. du Vigneaud, G. Flouret, and R. Walter, *J. Biol. Chem.*, **241**, 2093 (1966).

(9) V. du Vigneaud, G. S. Denning, Jr., S. Drabarek, and W. Y. Chan, *ibid.*, **238**, PC1560 (1963); **239**, 472 (1964).

(10) W. Y. Chan, V. J. Hruby, G. Flouret, and V. du Vigneaud, *Science*, **161**, 280 (1968).

(11) M. Bodanszky and V. du Vigneaud, *Nature*, **183**, 1324 (1959); *J. Amer. Chem. Soc.*, **81**, 5688 (1959).

(12) R. H. Sifferd and V. du Vigneaud, *J. Biol. Chem.*, **108**, 753 (1935).

(13) D. B. Hope, V. V. S. Murti, and V. du Vigneaud, *ibid.*, **237**, 1563 (1962).

(14) D. Yamashiro, *Nature*, **201**, 76 (1964); D. Yamashiro, D. Gillessen, and V. du Vigneaud, *J. Amer. Chem. Soc.*, **88**, 1310 (1966).

(15) J. Porath and P. Flodin, *Nature*, **183**, 1657 (1959).

(16) Avian vasodepressor assays were performed on conscious chickens according to the method of R. A. Munsick, W. H. Sawyer, and H. B. van Dyke, *Endocrinology*, **66**, 860 (1960).

(17) Oxytocic assays were performed according to the method of P. Holton, *Brit. J. Pharmacol.*, **3**, 328 (1948), as modified by R. A. Munsick, *Endocrinology*, **66**, 451 (1960), on isolated uteri from rats in natural estrus with the use of magnesium-free van Dyke–Hastings solution.

(18) Pressor assays were carried out on anesthetized male rats as described in "The Pharmacopeia of the United States of America," 17th rev, Mack Publishing Co., Easton, Pa., 1965, p 749.

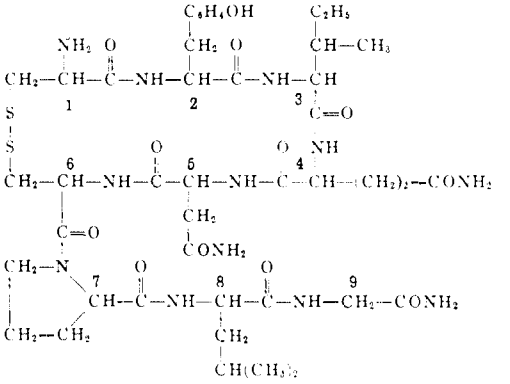


Figure 1.—Structure of oxytocin, with numbers indicating the position of the individual amino acid residues.

diuretic[19] activities against the USP Posterior Pituitary Reference Standard. Because of the low order of activities of the analog, it was difficult to carry out a precise assay. Thus only rough estimates of activities were obtained. The analog was found to possess about 3 units/mg of avian vasodepressor activity, negligible oxytocic activity (~0.5 unit/mg) and pressor activity (~0.05 unit/mg), and no detectable antidiuretic activity. However, [2,4-diisoleucine]-oxytocin was found to have marked natriuretic and diuretic activities in rats under experimentally induced water diuresis.

The renal effects of [2,4-diisoleucine]-oxytocin are of particular interest. It appears that in this analog the natriuretic activity is enhanced to such an extent that the antidiuretic activity characteristic of the parent compound is either masked or absent. Oxytocin itself can cause natriuresis in rats,[20] but it is also an active antidiuretic agent. On the other hand, [4-leucine]-oxytocin is a potent natriuretic–diuretic agent.[8,10] The renal effects of [2,4-diisoleucine]-oxytocin, however, differ from that of [4-leucine]-oxytocin both quantitatively and qualitatively. On a weight basis, [2,4-diisoleucine]-oxytocin is less potent than [4-leucine]-oxytocin in promoting urinary Na^+ excretion. In rats under water diuresis, the average dose for a 50% to 100% increase in Na^+ excretion was 0.7 μg/100 g iv for [2,4-diisoleucine]-oxytocin and 0.5 μg/100 g iv for [4-leucine]-oxytocin. The diuretic response to [2,4-diisoleucine]-oxytocin was mild and could be accounted for by the natriuretic activity. The diuretic response to [4-leucine]-oxytocin was marked and resulted in part from the anti-ADH activity as well as from the natriuretic activity possessed by this analog.[10]

The natriuretic effects of [2,4-diisoleucine]-oxytocin and [4-leucine]-oxytocin are direct renal effects. Neither analog has an effect on the systemic blood pressure or the renal glomerular filtration rate, at the dose level employed in these experiments. Furthermore, the renal effects of [2,4-diisoleucine]-oxytocin and [4-leucine]-oxytocin suggest that the natriuretic and antidiuretic actions of oxytocin and vasopressin involve two different receptor mechanisms and that each possesses a fair degree of structural specificity.

Experimental Section[21]

N-Benzyloxycarbonylisoleucylisoleucylisoleucylasparaginyl-S-benzylcysteinylprolylleucylglycinamide.—A solution of 1.20 g (1.25 mmol) of N-benzyloxycarbonylisoleucylisoleucylasparaginyl-S-benzylcysteinylprolylleucylglycinamide[8] in 12 ml of anhydrous AcOH was stirred with 15 ml of 5.4 N HBr in glacial AcOH for 1 hr at room temperature. Anhydrous Et_2O (150 ml) was added, and the colorless precipitate was filtered off, washed (Et_2O), and dissolved in 175 ml of MeOH. This solution was poured through a column of Rexyn ROH (OH cycle) (12 g) followed by two 60-ml portions of MeOH. The combined MeOH solutions were evaporated to dryness *in vacuo*. The free heptapeptide so obtained was dissolved in 18 ml of DMF. Then 0.50 g (1.25 mmol) of *p*-nitrophenyl N-benzyloxycarbonylisoleucinate[10] was added, and the mixture was stirred for 18 hr at room temperature. The product was triturated with 150 ml of EtOAc, cooled, filtered off, and washed with 50 ml of EtOAc and two 50-ml portions of EtOH. It was then dissolved in boiling 85% EtOH, precipitated by cooling to −20°, filtered off, washed with two 50-ml portions of EtOAc, and dried *in vacuo* to give 1.15 g of white powder, mp 279–281° dec, $[\alpha]^{26}D$ −92.2° (*c* 0.5, AcOH). *Anal.* ($C_{58}H_{89}N_{13}O_{16}S$) C, H, N.

N-Benzyloxycarbonyl-S-benzylcysteinylisoleucylisoleucylisoleucylasparaginyl-S-benzylcysteinylprolylleucylglycinamide. A solution of 0.43 g (0.4 mmol) of the preceding protected octapeptide in 12 ml of anhydrous AcOH was stirred with 12 ml of 5.4 N HBr in AcOH for 1 hr at room temperature. Then 125 ml of anhydrous Et_2O was added, and the precipitate was filtered off and washed with three 75-ml portions of Et_2O. The powder (0.47 g) was dried *in vacuo* and dissolved in 15 ml of DMF. The pH was adjusted to 6.0 with Et_3N, and 0.20 g (0.4 mmol) of *p*-nitrophenyl N-benzyloxycarbonyl-S-benzylcysteinate[11] was added with stirring. After the mixture was stirred for 24 hr, the slurry was triturated with 100 ml of EtOAc and cooled. The precipitate was filtered off and washed with two 25-ml portions of EtOAc, 25 ml of EtOH, two 25-ml portions of 50% aqueous EtOH, 25 ml of EtOH, and 25 ml of EtOAc. The product was dried *in vacuo* to give 0.42 g of white powder, mp 271–273° dec, $[\alpha]^{25}D$ −89.2° (*c* 0.5 AcOH). *Anal.* ($C_{68}H_{91}N_{13}O_{18}S_2$) C, H, N.

[2,4-Diisoleucine]-oxytocin. A solution of 252 mg (0.2 mmol) of the preceding protected nonapeptide in 175 ml of boiling, freshly distilled NH_3 was treated with Na until a blue coloration persisted for 1 min. The NH_3 was removed by distillation and lyophilization and the residue was dissolved in 500 ml of deaerated H_2O containing 0.5 ml of F_3CCO_2H. The pH of the solution was adjusted to 8.2 with dilute NH_4OH and F_3CCO_2H, and 35.0 ml of 0.01 N $K_3Fe(CN)_6$ was added. After the mixture was stirred for 15 min, no SH was detected (Ellman determination[22]). The pH was adjusted to 6.8 with dilute F_3CCO_2H and the excess ferricyanide and ferrocyanide ions were removed by treatment with ion-exchange resin AG3-X4 (trifluoroacetate cycle). The mixture was stirred for 20 min, the resin was filtered off, and the solution was lyophilized. The powder was dissolved in 18.8 ml of the upper phase of the solvent system 3.5% aqueous AcOH (containing 1.5% pyridine)–1-BuOH–CaH_6 (5:4:1), and placed on a 2.8 × 65-cm column of Sephadex G-25 (100–200 mesh) that had been equilibrated with both lower and upper phases according to the method of Yamashiro.[13] Eighty 9.6-ml fractions were collected and the fractions corresponding to the major peak (R_f 0.67) as determined by plotting of the Folin–Lowry color values[23] were pooled. Then 250 ml of glass-distilled H_2O was added, and the mixture was concentrated to 30 ml *in vacuo* and lyophilized. The resulting powder (70.1 mg) was again subjected to partition chromatography on Sephadex G-25 using the solvent system 3.5% aqueous AcOH (containing 1.5% pyridine)–1-BuOH–CaH_6 (17:5:2), and 61.7 mg of product was isolated from the major peak (R_f 0.56). This powder was dissolved in 15 ml of 0.2 N AcOH and placed on a 2.8 × 65-cm column of Sephadex G-25 (200–270 mesh) in 0.2 N AcOH for gel filtration.[15] Eighty 6.8-ml fractions were collected, and the fractions corresponding to the major peak (tubes 39–50) as determined by plotting of the Folin–Lowry color values were

(19) Assays for antidiuretic activity were performed on anesthetized male rats according to the method of W. A. Jeffers, M. M. Livezey, and J. H. Austin, *Proc. Soc. Exptl. Biol. Med.*, **50**, 184 (1942) as modified by W. H. Sawyer, *Endocrinology*, **63**, 694 (1958).

(20) W. Y. Chan, *Ibid.*, **77**, 1097 (1965).

(21) Capillary melting points were determined and are corrected. Where analyses are indicated only by symbols of the elements, analytical results obtained for the elements were within ±0.4% of the theoretical values.

(22) G. L. Ellman, *Arch. Biochem. Biophys.*, **82**, 70 (1950).

(23) O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

pooled and lyophilized to give 45.75 mg of [2,4-diisoleucine]-oxytocin as a white powder, $[\alpha]^{25}D$ −35.7° (*c* 0.47, 1 *N* AcOH). *Anal.* ($C_{41}H_{72}N_{11}O_{10}S_2$) C, H, N.

The sample was hydrolyzed for 90 hr in 6 *N* HCl at 110° and analyzed on a Beckman/Spinco amino acid analyzer according to the method of Spackman, Stein, and Moore.[24] The molar ratios obtained with glycine taken as 1.0 were: aspartic acid, 1.0; proline, 1.1; glycine, 1.0; cystine, 0.95; isoleucine, 3.0; leucine, 1.0; and NH_3, 2.0. Prolonged hydrolysis was necessitated by the difficulty in the hydrolysis of an isoleucyl-isoleucine peptide bond.[2,6,25]

Acknowledgments.—The authors wish to thank Mr. H. L. Aanning for the preparation of *N*-benzyloxycarbonylisoleucylasparaginyl-*S*-benzylcysteinylprolylleucylglycinamide, Mr. Joseph Albert for the elemental analyses, and Mr. Roger Sebbane for the amino acid analysis.

(24) D. H. Spackman, W. H. Stein, and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).

(25) S. Moore and W. H. Stein, *Methods Enzymol.*, **6**, 819 (1963).

Selective Modifications of the α^4-Position of Pyridoxol. I. Extension and Branching of the 4-Side Chain[1]

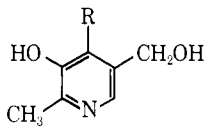
W. KORYTNYK AND B. PAUL

Department of Experimental Therapeutics, Roswell Park Memorial Institute, Buffalo, New York 14203

Received June 23, 1969

To develop general methods for modifying the 4-position, various blocking groups have been introduced into the α^5- and 3-*O*-positions of pyridoxol. Starting with 3,α^5-*O*-dibenzylpyridoxol, we have synthesized a homolog of pyridoxol with a 3-C side chain in the 4-position. α^4-Methylpyridoxol has also been synthesized. The method appears to be of considerable promise for introducing various modifications into the 4-position. In some cases, however, certain deblocking procedures give anomalous results. Thus deblocking of 3,α^5-dibenzyl-α^4-phenylpyridoxol with HCl gives a cyclic derivative, whereas hydrogenolysis gives α^4-phenyl-4-deoxypyridoxol.

Pyridoxol analogs obtained by modification of the 4-position (I, R = CH_2OH) have been of considerable



I

interest in enzymatic and pharmacological studies. 4-Deoxypyridoxol (4-DOP; I, R = CH_3) is a potent antagonist of vitamin B_6 in a number of systems, and its antitumor effects have been studied extensively.[2b] α^4-*O*-Methylpyridoxol ("4-methoxypyridoxol"; I, R = CH_2OCH_3) was also found to be a potent antagonist of vitamin B_6 in some mammalian systems,[3] but in some tissues was subject to demethylation.[4] Replacement of the 4-methyl H's in 4-DOP with F (I, R = CF_3) renders the compound less active in various systems,[5] and replacement of the entire 4-side chain with H (I, R = H)[6] or with OH[7] considerably reduces inhibitory potency (test organism: *Saccharomyces carlsbergensis*). On the other hand, replacement of the aldehydic oxygen of pyridoxal with bulky nitrogenous groups, such as hydroximino, azino, and various hydrazone groups (I, R = CH=NHNHR), makes them powerful inhibitors of pyridoxal phosphokinase *in vitro*.[8a] Compounds of this type have also been found to be of some biological interest as inhibitors of human neoplastic cells *in vitro*[9a] and retarders of S-180 tumor growth.[9b]

Some pyridoxol analogs that have the 5-CH_2OH unchanged, such as in I, have been found to be susceptible to phosphorylation catalyzed by pyridoxal phosphokinase,[8] and the phosphorylated analogs are capable of effective competition with the cofactor pyridoxal phosphate for the same site on the apoenzyme.[2a]

In this study we have developed methods for the selective modification of the 4-position. A suitable intermediate was required which would parallel the general utility of α^4,3-*O*-isopropylidenepyridoxol (II) for modifying the 5-CH_2OH group.[10] Pyridoxal (I, R = CHO) or pyridoxic acid (I, R = CO_2H) could not be used because of the tendency of these compounds to form a hemiacetal or a lactone, respectively.[11] Thus at least the α^4-OH of pyridoxol had to be blocked.[12] A suitable blocking group was benzyl, which was introduced by either one of the methods outlined in Scheme I to give α^5-*O*-benzylpyridoxol (V).

It was also desirable to block the phenolic OH in V with a suitable blocking group in order to prevent it from interfering with the substitution reactions and to make the intermediate soluble in organic solvents. Benzylation of the phenolic OH of 5-*O*-benzylpyridoxol was readily accomplished with dimethylphenylbenzylammonium hydroxide ("leucotrope"),[13] which re-

(1) (a) Pyridoxine Chemistry. XXII. Preceding paper in this series: H. Ahrens and W. Korytnyk, *Anal. Biochem.*, **30**, 413 (1969). (b) Brief reports of this study have appeared: E. E. Snell, A. E. Braunstein, E. S. Severin, and Yu. M. Torchinsky, Ed., "Pyridoxal Catalysis: Enzymes and Model Systems," Interscience, New York, N. Y., 1968, p 615; Abstracts of the 150th National Meeting of the American Chemical Society, Atlantic City, N. J., Sept 1965, p 9P.

(2) (a) E. E. Snell, *Vitamins Hormones*, **16**, 77 (1958); (b) F. Rosen, E. Milich, and C. A. Nichol, *ibid.*, **22**, 609 (1964).

(3) W. H. Ott, *Proc. Soc. Exptl. Biol. Med.*, **66**, 215 (1967); D. A. Karnofsky, C. C. Stock, L. P. Ridgway, and P. A. Patterson, *J. Biol. Chem.*, **182**, 471 (1950).

(4) C. C. Porter, I. Clark, and R. H. Silber, *ibid.*, **167**, 573 (1947).

(5) J. L. Green, Jr., and J. A. Montgomery, *J. Med. Chem.*, **6**, 294 (1963).

(6) L. A. Perez-Medina, R. P. Mariella, and S. M. McElvain, *J. Amer. Chem. Soc.*, **69**, 2574 (1947).

(7) W. Korytnyk and B. Paul, *J. Heterocycl. Chem.*, **2**, 144 (1965).

(8) (a) D. B. McCormick and E. E. Snell, *J. Biol. Chem.*, **236**, 2085 (1961); (b) J. Hurwitz, *ibid.*, **217**, 513 (1955).

(9) (a) E. Testa, A. Bonati, and G. Pagani, *Chimia*, **15**, 314 (1961); (b) R. H. Wiley and G. Irick, *J. Med. Pharm. Chem.*, **5**, 49 (1962).

(10) W. Korytnyk, *ibid.*, **8**, 112 (1965).

(11) H. Ahrens and W. Korytnyk, *J. Heterocycl. Chem.*, **4**, 625 (1967).

(12) R. P. Singh and W. Korytnyk [*J. Med. Chem.*, **8**, 116 (1965)] have been using benzoyl groups for selective blockage of the α^5-hydroxyl group. This approach had serious limitations because of the instability of the group and other factors.

(13) H. M. Wuest, J. A. Bigot, Th. J. deBoer, and J. P. Wibaut, *Koninkl. Ned. Akad. Wetenschap. Proc., Ser. B*, **61**, 150 (1958).