Synthesis and Some Pharmacological Properties of [1-β-Mercapto-β,β-diethylpropionic acid,2-(3,5-dibromo-L-tyrosine)]oxytocin†

Edwin O. Lundell,* Clark W. Smith, and Martha F. Ferger

Department of Chemistry, Cornell University, Ithaca, New York 14853. *Received May 25, 1975*

The synthesis of the protected polypeptide precursor of [1-β-mercapto-β,β-diethylpropionic acid,2-(3,5-dibromo-L-tyrosine)]oxytocin was performed in a stepwise manner by solution techniques. This analog of oxytocin has two modifications, each of which taken alone gives analogs which inhibit some of the pharmacological responses to oxytocin. The *S*-ethylcarbamoyl protecting groups of β-Mpa(β-Et_2)(Ec)-Dbt-Ile-Gln-Asn-Cys(Ec)-Pro-Leu-Gly-NH_2 were removed in refluxing liquid NH_3, and the resulting disulfhydryl compound was oxidatively cyclized in H_2O–MeOH with ICH_2CH_2I. Purification was effected by partition chromatography and gel filtration. The analog possesses antioxytocic (pA_2 = 7.08) and antiavian vasodepressor (pA_2 = 7.38) activities but has neither agonist nor antagonist activity in the rat pressor assay. These potencies are close to those exhibited by [1-β-mercaptopropionic acid,2-(3,5-dibromo-L-tyrosine)]oxytocin but different from those of [1-β-mercapto-β,β-diethylpropionic acid]oxytocin.

The introduction of two alkyl substituents on the β carbon at position 1 of oxytocin

1 2 3 4 5 6 7 8 9
Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2

or deamino-oxytocin[2] ([1-β-mercaptopropionic acid]oxytocin) has been shown to generate analogs which are potent competitive inhibitors of the oxytocic and avian vasodepressor (AVD) activities of oxytocin.[3-5] Three of these analogs ([1-β-mercapto-β,β-dimethylpropionic acid]oxytocin, [1-β-mercapto-β,β-diethylpropionic acid]oxytocin, and [1-β-mercapto-β,β-pentamethylenepropionic acid]oxytocin) have also been shown to be weak inhibitors of the rat pressor effect of 8-lysine-vasopressin.[5,6] In addition, several analogs of oxytocin with modifications at position 2 have been prepared which have inhibitory properties. Among these are analogs with *p*-alkoxyphenylalanine or *p*-alkylphenylalanine residues in this position[7] and analogs in which one or more of the hydrogen atoms of the aromatic ring of the tyrosine residue has been formally replaced by iodine[8] or bromine.[9] These halogen-containing analogs, [2-o-iodotyrosine]oxytocin and [1-β-mercaptopropionic acid,2-(3,5-dibromotyrosine)]oxytocin, are potent inhibitors with antioxytocic potencies approaching that of [1-β-mercapto-β,β-diethylpropionic acid]oxytocin, which is one of the most potent reversible inhibitors studied in this laboratory.

In order to determine the effect of incorporating into one analog two of these potent inhibitory modifications, the synthesis of [1-β-mercapto-β,β-diethylpropionic acid,2-(3,5-dibromotyrosine)]oxytocin ([β-Mpa(β-Et_2)^1,Dbt^2]-oxytocin) was undertaken. The required intermediate, β-Mpa(β-Et_2)(Ec)-Dbt-Ile-Gln-Asn-Cys(Ec)-Pro-Leu-Gly-NH_2, was prepared by the stepwise elongation of H-Ile-Gln-Asn-Cys(Ec)-Pro-Leu-Gly-NH_2,[10] using Boc-Dbt-$OPhCl_3$[11] followed by β-(*S*-ethylcarbamoylmercapto)-β,β-diethylpropionic acid [β-Mpa(β-Et_2)(Ec)] with dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HBt).[12] The *S*-ethylcarbamoyl groups were removed in refluxing liquid NH_3, and the resulting disulfhydryl peptide was oxidized to the cyclic disulfide with diiodoethane.[13] The analog was purified by partition chromatography[14] and gel filtration.[15]

The highly purified [β-Mpa(β-Et_2)^1,Dbt^2]oxytocin was found to produce inhibition (Table I) of the oxytocic and AVD responses to synthetic oxytocin in the standard assay systems (see Experimental Section). Its inhibitory potencies in those assays are close to those of [β-Mpa^1,Dbt^2]oxytocin but significantly ($p < 0.01$) less than those of [β-Mpa(β-Et_2)^1]oxytocin. In the rat pressor assay, no agonist or antagonist activity was observed for [β-Mpa(β-Et_2)^1,Dbt^2]oxytocin in doses up to 18 μg/100 g of body weight. [β-Mpa^1,Dbt^2]oxytocin also lacks agonist or antagonist activity in the pressor assay, while [β-Mpa(β-Et_2)^1]oxytocin possesses a weak antagonist activity. Thus in this study, the inhibitory potencies of the disubstituted analog are limited to the potencies of the less potent analog containing only one inhibitory modification, [β-Mpa^1,Dbt^2]oxytocin.

Experimental Section

All melting points were determined in open capillary tubes and are corrected. Thin-layer chromatography was performed on precoated glass plates of silica gel GF 254 (0.25 mm, E. Merck) in the following solvent systems: (A) $CHCl_3$–MeOH–AcOH (3:1:1); (B) BuOH–AcOH–H_2O (3:1:1); (C) BuOH–pyridine–AcOH–H_2O (15:10:3:12); (D) BuOH–pyridine–H_2O (20:10:11); (E) acetone–AcOH–H_2O (8:1:1). The load size was 10–30 μg, and chromatogram lengths were 100–150 mm. Detection was made by chlorination followed by NaI–starch treatment. In all cases, unless otherwise noted, single symmetrical spots were observed for purified material. Progress of the condensation reactions was followed by the quantitative ninhydrin test.[6,16] Amino acid analysis was performed on a Beckman Model 116 amino acid analyzer using a single column system.[8,17] Optical rotations were determined on a Perkin-Elmer Model 141 polarimeter. Where analyses are indicated only by the symbols of the elements, analytical results obtained for the elements were within ±0.4% of the theoretical values. Where compounds are formulated as containing solvent, this does not necessarily imply that they are defined solvates; particularly in the case of amorphous products, solvent retention may be due to mild drying conditions (24 hr at 22°/0.005 Torr over P_2O_5).

Boc-Dbt-Ile-Gln-Asn-Cys(Ec)-Pro-Leu-Gly-NH_2 (1). Z-Ile-Gln-Asn-Cys(Ec)-Pro-Leu-Gly-NH_2[10] (474 mg, 0.50 mmol) was treated for 1.5 hr at room temperature with 3.3 *N* HBr–AcOH (8 ml). The peptide salt was precipitated by the addition of Et_2O (30 ml), isolated and washed three times with Et_2O by centrifugation, and dried in vacuo. The residue was dissolved in dimethylformamide (DMF) (6 ml) and neutralized to pH 7.5 (Fisher Indicator Solution) with diisopropylethylamine (*i*-Pr_2NEt). Boc-Dbt-$OPhCl_3$[11] (340 mg, 0.55 mmol) was added. After 24 hr the pH was adjusted back to 7.5 with *i*-Pr_2NEt. After 48 hr the product was precipitated by the addition of EtOAc (30 ml), isolated, washed by centrifugation with EtOAc and Et_2O, and dried in vacuo: 525 mg (85%). The crude product was reprecipitated from DMF with H_2O, isolated, washed by centrifugation with H_2O, EtOH, and Et_2O, and dried in vacuo: 436 mg (83% recovery); mp 252–255°; $[\alpha]^{20}D$ −32.9° (*c* 0.98, DMF); TLC (A) 0.73 with two trace impurities (0.84 and 0.57).

Dicyclohexylammonium β-(*S*-Ethylcarbamoylmercapto)-β,β-diethylpropionate (2). β-(*S*-Benzylmercapto-β,β-diethylpropionic acid[4] (1.00 g, 4.00 mmol) was dissolved in liquid NH_3 (100 ml, freshly distilled from Na). Na (200 mg, 8.7 mmol) was added and

†The symbols β-Mpa(β-Et_2) and Dbt are used to indicate β-mercapto-β,β-diethylpropionic acid and 3,5-dibromo-L-tyrosine, respectively. All other symbols follow the recommendations (1971) of the IUPAC-IUB Commission on Biochemical Nomenclature.[1] All optically active amino acids are of the L configuration.

*Address correspondence to this author at Union Carbide Corp., Bound Brook, N.J. 08805.

Table I. Inhibitory Properties of Certain Oxytocin Analogs

Analog	Antioxytocic		Antiavian vasodepressor		Antipressor	
	$\times 10^8\ \overline{M}$[a]	pA_2[a]	$\times 10^8\ \overline{M}$	pA_2	$\times 10^7\ \overline{M}$	pA_2
[β-Mpa(β-Et_2)^1,Dbt^2]oxytocin	8.2 (19), σ = 1.8	7.08	4.2 (18), σ = 2.4	7.38	No activity	
[β-Mpa^1,Dbt^2]oxytocin	8.9 (16),[b] σ = 0.2	7.05[b]	3.6 (16),[b] σ = 1.0	7.44	No activity[b]	
[β-Mpa(β-Et_2)^1]oxytocin	5.8 (9),[c] σ = 0.12	7.24[c]	0.78 (8),[c] σ = 0.17	8.11[c]	5.7 (20),[d] σ = 2.7	6.24[d]

[a] pA_2 values [H. O. Schild, *Br. J. Pharmacol.*, 2, 189 (1947)] represent the negative log to the base 10 of the average molar concentration ($\overline{M}$) of an antagonist which will reduce the response to $2x$ units of pharmacologically active compound (agonist) to the response to x units of agonist. The number of individual determinations is given in parentheses and σ is the standard deviation. Specific details of these assays are described by Vavrek et al.[4] and by Dyckes et al.[6] Synthetic oxytocin was the agonist used in the antioxytocic and antiavian vasodepressor assays and synthetic LVP in the antipressor assays. Concentrations of antagonists were calculated on the basis of a 10-ml tissue bath (oxytocic) or assumed blood volumes of 150 ml for the chicken and 6.7 ml/100 g in the rats. [b] Lundell and Ferger.[9] [c] Vavrek et al.[4] [d] Dyckes et al.[6]

the persistent blue color of excess Na was discharged with AcOH. The NH_3 was removed by evaporation and lyophilization and the residue was dissolved in H_2O (100 ml). The pH was adjusted to 1 with concentrated HCl and the solution extracted with EtOAc. The EtOAc solution was dried with Na_2SO_4 and evaporated to dryness. The colorless oil was dissolved in DMF (6 ml) and treated with freshly distilled ethyl isocyanate (0.35 ml, 4.4 mmol). After 48 hr the solution was evaporated (oil pump) to a colorless oil which had NMR ($CDCl_3$) proton ratios and chemical shifts consistent with expected values. The oil was dissolved in EtOAc (10 ml), and dicyclohexylamine (DCHA) (0.8 ml, 4 mmol) was added. The resulting crystalline precipitate was collected, washed with EtOAc, and dried in vacuo: 1.42 g (86%). The crude product was recrystallized from EtOH–EtOAc with 93% recovery: mp 128–129°. Anal. ($C_{22}H_{42}N_2O_3S$) C, H, N.

β-Mpa(β-Et_2)(Ec)-Dbt-Ile-Gln-Asn-Cys(Ec)-Pro-Leu-Gly-NH_2 (3). The Boc group of 1 (296 mg, 0.24 mmol) was removed by treatment with trifluoroacetic acid (TFA) (2 ml) for 30 min at room temperature. The peptide salt was precipitated by the addition of Et_2O, and the precipitate was isolated, washed by centrifugation with Et_2O, and dried in vacuo. An EtOH–EtOAc solution of 2 (150 mg, 0.36 mmol) was treated with excess HCl–EtOAc (4.5–5 *N*). The precipitated DCHA·HCl was removed by filtration, and the solvents were removed from the filtrate by rotary evaporation leaving a colorless oil. The oil was dissolved in 1.00 ml of DMF and 0.20 ml was withdrawn and set aside. To the remaining 0.80 ml was added HBt (60 mg, 0.44 mmol). The solution was cooled to 0° and DCC (60 mg, 0.29 mmol) was added. After 1 hr at 0° and 1 hr at room temperature the preactivation solution was added through a fritted funnel to a solution of the peptide salt of 1 in DMF (1.2 ml) which had been adjusted to pH 6.5 with *i*-Pr_2NEt (41 μl, 0.24 mmol) along with 0.4 ml of DMF. The pH was maintained at 6–6.5 with additional aliquots of *i*-Pr_2NEt. After 36 hr the reaction had stopped, with the ninhydrin test indicating approximately 30% of the free amino groups remaining. The remaining 0.20 ml of DMF solution of β-Mpa(β-Et_2)(Ec) was preactivated with DCC in the presence of Hbt as described above and added to the reaction mixture. After 52 hr the product was precipitated by the addition of 95% EtOH (20 ml). The precipitate was isolated, washed by centrifugation with EtOH, EtOAc, and Et_2O, and dried in vacuo: 162 mg (50%). The filtrate was evaporated to dryness and the residue dissolved in boiling 95% EtOH (7 ml). The precipitate which formed upon cooling was collected: 116 mg (36%); $[\alpha]^{22}D$ −43.5° (*c* 1.08, DMF). Both crops were identical by TLC (A, 0.79) and melting point (242° dec). Anal. ($C_{53}H_{83}N_{13}O_{14}S_2Br_2 \cdot 2H_2O$) C, H, N.

[1-β-Mercapto-β,β-diethylpropionic acid,2-(3,5-dibromotyrosine)]oxytocin. A solution of 3 (65.1 mg, 47.6 μmol) in liquid NH_3 (freshly distilled from Na) was refluxed for 4 hr. The NH_3 was removed by evaporation and lyophilization and the residue dissolved in H_2O (50 ml) and MeOH (40 ml) under an N_2 atmosphere. A solution of ICH_2CH_2I (14.1 mg, 50 μmol) in 10 ml of MeOH was added and the disappearance of the sulfhydryl groups was followed by the Ellman method.[18] The reaction was complete in 5 min, AcOH (5 ml) was added, and the solvents were removed by rotary evaporation. The residue was lyophilized from AcOH. The lyophilizate was dissolved in 5 ml of the upper phase and 0.5 ml of the lower phase of the system BuOH–C_6H_6–H_2O (1:1:2 aqueous phase, 1.5% pyridine, and 3.5% AcOH) and subjected to partition chromatography[14] on a 2.2 × 53 cm column of Sephadex G-25 (100–200 mesh) which had been equilibrated with both phases of the solvent system. The product was eluted with upper phase at a flow rate of 11 ml/hr and collected in fractions of 1.9 ml. Peptide material was detected by the Folin–Lowry method.[19] The product emerged at the void volume of the column as a single tailing peak. TLC (B) showed that the product composition was the same across the entire peak area and consisted of a major component at R_f 0.51 with a faint leading impurity. The fractions comprising the peak were pooled, the solvents were removed by rotary evaporation, and the residue was lyophilized from AcOH: 41.0 mg (71%). This material was dissolved in 2 ml of 50% aqueous AcOH and further purified by gel filtration on a 2.82 × 67 cm column of Sephadex G-25 (200–270 mesh) equilibrated with 20% AcOH. The product was eluted from the column with 20% AcOH at a flow rate of 27 ml/hr and collected in fractions of 3.6 ml. Peptide material was detected by reading the absorbency of the eluate at 280 nm. The product emerged as a sharp symmetrical peak with a maximum at 70% of the column volume preceded by and resolved from a small peak at 56% of the column volume. The fractions comprising the major peak were pooled and diluted with two volumes of H_2O, and the product was isolated by lyophilization: 34.6 mg (59%); $[\alpha]^{21}D$ −11.5° (*c* 0.46, 50% AcOH); TLC (A) 0.47, (B) 0.51, (C) 0.71, (D) 0.73, (E) 0.79. Amino acid analysis[20] following 24-hr hydrolysis in deaerated 6 *N* HCl at 110° gave the following ratios: Asp 1.01; Glu, 1.04; Pro, 1.03; Gly, 0.96; Ile, 1.00; Leu, 0.99; Dbt, 1.00; NH_3, 2.88. Following hydrolysis as above of a performic acid oxidized sample[21] the ratios were Cys(SO_3H), 1.08; Asp, 1.00; Glu, 1.03; Pro, 0.96; Gly, 0.94; Ile, 0.96; Leu, 0.99; NH_3, 2.96. Anal. ($C_{47}H_{71}N_{11}O_{12}S_2Br_2 \cdot 3H_2O$) C, H, N.

Bioassays. Oxytocic assays were performed on isolated uteri from virgin rats in natural estrus according to the method of Holton[22] as modified by Munsick[23] with the use of Mg-free van Dyke–Hasting's solution as bathing fluid. AVD assays were performed on conscious chickens by the method of Coon,[24] as described in the U.S. Pharmacopeia,[25] as modified by Munsick, Sawyer, and van Dyke.[26] Pressor assays were carried out on anesthetized male rats as described in the U.S. Pharmacopeia.[27]

Acknowledgments. The authors thank Ms. Nina Smith for the bioassays and Dr. Louis L. Nangeroni, New York State Veterinary College at Cornell University, for the use of his laboratory for the bioassay work. Particular appreciation is expressed to Professor Vincent du Vigneaud for his advice and support during the course of this investigation. This work was supported in part by Grant HL-11680 to Professor du Vigneaud from the National Heart and Lung Institute, U.S. Public Health Service.

References and Notes

(1) J. Biol. Chem., 247, 977 (1972).
(2) D. B. Hope, V. V. S. Murti, and V. du Vigneaud, J. Biol. Chem., 237, 1563 (1962); B. M. Ferrier, D. Jarvis, and V. du Vigneaud, ibid., 240, 4264 (1965).
(3) H. Schulz and V. du Vigneaud, J. Med. Chem., 9, 647 (1966); W. Y. Chan, H. Schulz, and V. du Vigneaud, IIIrd International Pharmacology Congress, Sao Paulo, Brazil, 1966, abstract 449; W. Y. Chan, R. Fear, and V. du Vigneaud, Endocrinology, 81, 1267 (1967).
(4) R. J. Vavrek, M. F. Ferger, G. A. Allen, D. H. Rich, A. T. Blomquist, and V. du Vigneaud, J. Med. Chem., 15, 123 (1972).
(5) J. J. Nestor, Jr., M. F. Ferger, and V. du Vigneaud, J. Med. Chem., 18, 284 (1975).
(6) D. F. Dyckes, J. J. Nestor, Jr., M. F. Ferger, and V. du Vigneaud, J. Med. Chem., 17, 250 (1974).
(7) H. D. Law and V. du Vigneaud, J. Am. Chem. Soc., 82, 4579 (1960); K. Jošt, J Rudinger, and F. Šorm, Collect. Czech. Chem. Commun., 26, 2496 (1961); 28, 1706 (1963); Z. Beránková, I. Rychlík, K. Jošt, J. Rudinger, and F. Šorm, Collect. Czech. Chem. Commun., 26, 2673 (1961); I. Krejčí, I. Poláček, and J. Rudinger, Br. J. Pharmacol. Chemother., 30, 506 (1967); A. L. Zhuze, K. Jošt, E. Kasafírek, and J. Rudinger, Collect. Czech. Chem. Commun., 29, 2648 (1964); J. Rudinger, Prog. Endocrinol., Proc. Int. Congr. Endocrinol., 2nd, Part II, 1202 (1965).
(8) P. Marbach and J. Rudinger Experientia, 30, 696 (1974).
(9) E. O. Lundell and M. F. Ferger, J. Med. Chem., 18, 1045 (1975).
(10) St. Guttmann, Helv. Chim. Acta, 49, 83 (1966).
(11) E. O. Lundell and M. F. Ferger, Bioorg. Chem., in press.
(12) W. König and R. Geiger, Chem. Ber., 103, 788 (1970).
(13) F. Weygand and G. Zumach, Z. Naturforsch., B, 17, 807 (1962).
(14) D. Yamashiro, Nature (London), 201, 76 (1964); D. Yamashiro, D. Gillessen, and V. du Vigneaud, J. Am. Chem. Soc., 88, 1310 (1966).
(15) J. Porath and P. Flodin, Nature (London), 183, 1657 (1959).
(16) E. Kaiser, R. L. Colescott, C. D. Bossinger, and P. I. Cook, Anal. Biochem., 34, 595 (1970).
(17) C. W. Smith, M. F. Ferger, and W. Y. Chan, J. Med. Chem., 18, 822 (1975).
(18) G. L. Ellman, Arch. Biochem. Biophys., 82, 70 (1959).
(19) O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, J. Biol. Chem., 193, 265 (1951).
(20) D. H. Spackman, W. H. Stein, and S. Moore, Anal. Chem., 30, 1190 (1958).
(21) S. Moore, J. Biol. Chem., 238, 235 (1963).
(22) P. Holton, Br. J. Pharmacol. Chemother., 3, 328 (1948).
(23) R. A. Munsick, Endocrinology, 66, 451 (1960).
(24) J. M. Coon, Arch. Int. Pharmacodyn. Chemother., 62, 79 (1939).
(25) "The Pharmacopeia of the United States of America", 18th revision, Mack Publishing Co., Easton, Pa., 1970, p 469.
(26) R. A. Munsick, W. H. Sawyer, and H. B. van Dyke, Endocrinology, 66, 860 (1960).
(27) Reference 25, p 771.

3-Acyloxymethyl-7-(2-thienylacetamido)-3-cephem-4-carboxylic Acids. An Improved Synthesis and Biological Properties

David A. Berges

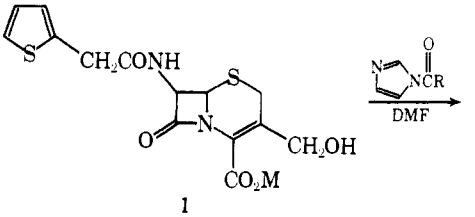
Research and Development Division, Smith Kline & French Laboratories, Philadelphia, Pennsylvania 19101. *Received July 17, 1975*

3-Acyloxymethyl-7-(2-thienylacetamido)-3-cephem-4-carboxylic acids have been prepared by an improved method utilizing the acylation of desacetylcephalothin with acid imidazolides. Their in vitro and in vivo antibacterial activities have been determined and compared with those of cephalothin.

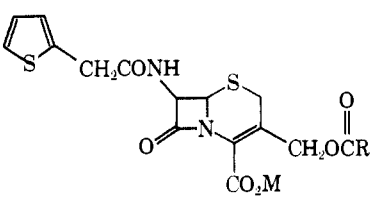
Cephalothin (2, R = CH_3), a commercially important cephalosporin, has good in vitro activity against a broad spectrum of gram-positive and gram-negative bacteria, but its in vivo activity is diminished by enzymatic hydrolysis of the acetate group to give the less active 3-hydroxymethyl compound 1.[1] Testing the hypothesis that increasing the bulk of the R group in 2 would decrease susceptibility to enzymatic hydrolysis, Kukolja[2] found only a small increase in in vivo activity relative to cephalothin for a series of cephalosporins in which R ranged from ethyl to cyclobutyl. Van Heyningen[3] reported a significant drop in in vitro activity when R was an aromatic ring. Nevertheless, when R was 2-thienyl, the in vivo activity in mice was superior to that of cephalothin. With an interest in derivatives which possess in vitro activity equivalent to that of cephalothin but with greater metabolic stability, the variation of R (in particular, the introduction of heteroatoms) has been examined further.

It has been reported that aroyl chlorides but not aliphatic acid chlorides acylate 7-acylaminodesacetylcephalosporanic acids.[3] Aliphatic acid derivatives have been made by acylation of desacetylcephalosporanic acid esters with subsequent regeneration of the 4-carboxyl[4] and by acylating 2-cephem acids followed by double bond isomerization.[2]

In contrast to the acid chlorides, acylation of sodium 3-hydroxymethyl-7-(2-thienylacetamido)-3-cephem-4-carboxylate (1) with aliphatic acid imidazolides[5] in an inert solvent (DMF) produced respectable yields[6] of the 3-acyloxymethyl compounds 2. Lactonization and double bond migration, reported to be serious problems with the acid chloride acylations,[7] were not observed with the acid imidazolides.



1



2

The new cephalosporins, which are summarized in Table I, were characterized by elemental analysis and ir and NMR spectroscopy.