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5-Hydroxy-5-phenyllevulinic Acid. A Novel Microbial Metabolite with Analgesic Activity

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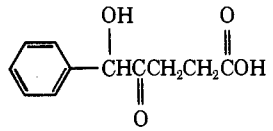
During a search for microbial metabolites with pharmacological activity, we investigated compounds produced by a fungus belonging to the *Tubercularia* species, designated in our laboratories as Z1497. When Z1497 was grown on a complex medium, the culture filtrate contained a major metabolite which could be extracted with 1-butanol. This compound (1) was readily purified by chromatography on silica gel and crystallization from benzene which yielded colorless, long-needled rosettes, mp 86–87°, $[\alpha]^{25}D$ +155°.



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The molecular formula, $C_{11}H_{12}O_4$, was deduced from the mass spectrum (parent ion *m/e* 208), elemental analyses, and the nmr spectrum (12 protons). A phenyl group was suggested by absorption near λ_{max} 260 nm with characteristic fine splitting indicating an isolated benzene chromophore. The mass spectrum had peaks at *m/e* 77, 78, and 79, indicative of a monoalkylbenzene-type structure, which is substantiated by a 5-proton singlet (δ 7.39, overlapped by two ionizable protons) in the nmr spectrum and by strong absorption at 697 and 759 cm^{-1}. The presence of a COOH group was shown by the ir spectrum (OH at 3500–2500 cm^{-1}, -C=O at 1715 cm^{-1}) and the reaction of the compound with aqueous sodium bicarbonate to evolve carbon dioxide. A positive reaction with 2,4-dinitrophenylhydrazine, the lack of an aldehyde proton in the nmr spectrum, and a shoulder near 1725 cm^{-1} indicated the presence of a keto group. There is a 4-proton absorption at δ 2.62 attributed to two almost equivalent methylene groups. The chemical shift and general appearance of this signal are very similar to those of the methylene groups of levulinic acid[1] which suggests the following unit in the Z1497 metabolite: $-C(=O)CH_2CH_2C(=O)OH$. The remaining structural feature is a >CHOH group which would be between the keto group and the phenyl ring. The base peak (*m/e* 107) of the mass spectrum is explained by fragmentation to give the ion $[C_6H_5GHOH]^+$. This structural unit also accounts for the nonionizable proton at δ 5.18 and one of the ionizable protons overlapping the absorption of the aromatic protons. The resulting novel structure (1) for the Z1497 metabolite has an asymmetric center which is consistent with the observed optical activity.

Pharmacology. Compound 1 was active by the mouse writhing syndrome.[2] In a low intensity rat tail-radiant heat experiment,[3,4] an intraperitoneal dose of 1 at 50 mg/kg induced a 75% rise in pain threshold whereas aspirin at the same dose level was inactive. By the inflamed rat paw pain test[5] the metabolite was slightly better or at least equivalent to aspirin.

The biological activity of 1 is of particular interest since few microbial metabolites with analgesic activity have been discovered.[6-8]

Experimental Section

Melting points are uncorrected. Nmr, ir, mass, and uv spectra were obtained on Varian A-60A and A-60D, Perkin-Elmer Model 21, AEI MS-9, and Cary Model 11 M spectrometers, respectively. Chemical shifts are reported as δ with tetramethylsilane as an internal reference.

Fermentation. Culture Z1497 was grown in 300-l. fermentors under standard conditions of aeration and agitation on a medium containing the following ingredients (grams per liter): glucose, 10; molasses, 20; tryptone, 10. The course of the fermentation was followed by extracting time samples of the culture filtrate with 1-butanol and analyzing the extracts by tlc on silica gel F-254 plates (Brinkmann Instruments, Inc.) developed with the system 1-butanol saturated with water. The desired product (R_f 0.5) was detected with a dinitrophenylhydrazine spray.

Isolation. The harvested mash filtrate was extracted with 175 l. of 1-butanol and the extract was evaporated to a 5-l. aqueous concentrate. The concentrate was extracted with ethyl acetate and the resulting extract was evaporated to a syrup which was redissolved in chloroform.

The chloroform solution, 200 ml, was then charged on a silica gel column (6 × 40 cm, Davison Co., 60–200 mesh). The column was eluted with chloroform at a flow rate of approximately 12 ml/min.

The manner of pooling the column fractions was determined by drying small aliquots of each one and visibly comparing the solids. Concentration of the pooled fractions corresponding to 1000–4000 ml of elute yielded crystalline material on standing. Recrystallization from warm benzene yielded 6.3 g of the crystalline metabolite.

For analytical samples, the metabolite was recrystallized from warm benzene and dried at 56°: λ_{max} (MeOH) 210 nm (ε >5000), 260 (270), 284 (270); $[\alpha]^{25}D$ +155° (*c* 0.692, MeOH); nmr ($CDCl_3$) δ 7.39 (s, 7 H, includes 2 exchangeable H's), 5.18 (s, 1 H), 2.62 (m, 4 H); ir (KBr) 3420, 3150 b, 1725 sh, 1715, 1400, 1248, 1215, 1163, 1078, 1012, 853, 828, 759, and 697 cm^{-1}; mass spectrum (70 eV) *m/e* (rel intensity) 208 (0.5), 180 (15), 107 (100), 105 (17), 101 (13), 79 (48), 51 (15), 28 (15), 18 (65).

Anal. Calcd for $C_{11}H_{12}O_4$: C, 63.46; H, 5.76; mol wt, 208. Found: C, 63.84; H, 5.86; mol wt, 208 (mass spectrum).

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[1-β-Mercapto-β,β-diethylpropionic acid]-8-lysine-vasopressin, a Potent Inhibitor of 8-Lysine-vasopressin and of Oxytocin†

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In earlier work it was found that the replacement of the half-cystine residue in position 1 of oxytocin (Figure 1) with that of L-penicillamine (L-β,β-dimethylcysteine) led to an analog, [1-L-penicillamine]oxytocin, which is a highly potent inhibitor of both the oxytocic and avian vasodepressor (AVD) activities of oxytocin.[1] The deamino analog, [1-deaminopenicillamine]oxytocin ([1-β-mercapto-β,β-dimethylpropionic acid]oxytocin),[1] was found to be more potent than the [L-penicillamine]oxytocin analog by about 20% with respect to antioxytocic activity and about 140% with respect to anti-AVD activity.[2] A further series of analogs has been prepared incorporating modifications in position 1 of [1-deaminopenicillamine]oxytocin.[2-4] Of the various analogs which were prepared,‡ [1-β-mercapto-β,β-diethylpropionic acid]oxytocin[2] ([1-β-Mpa(β-Et_2)]oxytocin) was the most potent inhibitor, possessing about twice the antioxytocic and anti-AVD activity of [deaminopenicillamine]oxytocin. We therefore decided to incorporate the β-mercapto-β,β-diethylpropionyl residue [β-Mpa(β-Et_2)] into lysine-vasopressin (Figure 1) to see whether the resulting analog, [1-β-mercapto-β,β-diethylpropionic acid]-8-lysine-vasopressin ([1-β-Mpa(β-Et_2)]LVP), would exhibit antihormonal properties.

This analog was prepared from the protected polypeptide β-Mpa(β-Et_2)(Bzl)-Tyr(Bzl)-Phe-Gln-Asn-Cys(Bzl)-Pro-Lys(Tos)-Gly-NH_2 by removal of all protecting groups with Na-NH_3[5] followed by cyclization with ICH_2CH_2I.[6] The protected polypeptide just mentioned was synthesized from Boc-Asn-Cys(Bzl)-Pro-Lys(Tos)-Gly-NH_2[7] by the stepwise *p*-nitrophenyl ester method.[8] N^{α}-Boc protection was used throughout and treatment with anhydrous trifluoroacetic acid was used for Boc removal. The hormone analog was purified by ion-exchange chromatography[9] followed by gel filtration chromatography.[10]

[1-β-Mpa(β-Et_2)]LVP was found to be inactive when tested for oxytocic, AVD, and pressor activities against the U.S.P. posterior pituitary reference standard§ but produced inhibition of the oxytocic and AVD responses to synthetic oxytocin and of the pressor response to synthetic LVP. Inhibitory potencies were determined and expressed as pA_2 values as defined by Schild.[16] This represents the negative logarithm to the base 10 of the average molar concentration ($\bar{M}$) of an antagonist which will reduce the appropriate biological response to $2X$ units of a pharmacologically active compound (agonist) to the level of response to X units of the agonist. Specific details of the antioxytocic and anti-AVD assays are described by Vavrek, *et al.*[2] Antipressor assays were performed in an exactly analogous manner, with the concentration of antagonist calculated on the basis of a blood volume of 6.7 ml per 100 g of body weight of the individual rats. The values of $\bar{M}$ for [1-β-Mpa(β-Et_2)]LVP as an antagonist of oxytocin were 1.43×10^{-7} ($\sigma = 0.37$) in the uterus and 2.34×10^{-8} ($\sigma = 0.98$) in the chicken, or $pA_2 = 6.84$ and 7.63, respectively. This means that the compound is roughly equal to [L-penicillamine]oxytocin in inhibitory potency in the oxytocic and AVD assays and one-half to one-third as potent as [1-β-Mpa(β-Et_2)]oxytocin. As an antagonist of LVP rat pressor activity, [1-β-Mpa(β-Et_2)]LVP had a value of $\bar{M} = 7.01 \times 10^{-8}$ ($\sigma = 1.15$), or $pA_2 = 7.15$. This potency is approximately eight times as great as that of [1-β-Mpa(β-Et_2)]oxytocin, which was found in the present study by identical assay techniques to have $\bar{M} = 5.73 \times 10^{-7}$ ($\sigma = 2.72$), or $pA_2 = 6.24$ in the pressor system.

Thus the substitution of the β-mercapto-β,β-diethylpropionic acid residue for the half-cystine residue in the 1 position of not only oxytocin but also of lysine-vasopressin leads to an analog possessing pronounced inhibitory properties.

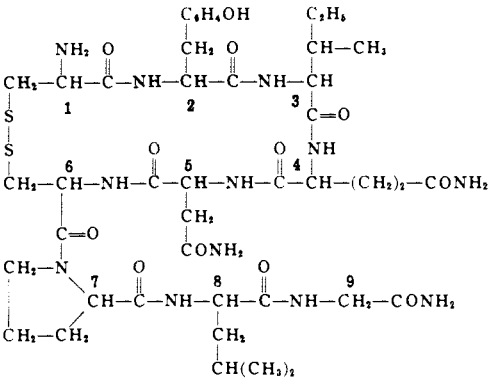

Figure 1. Structure of oxytocin with numbers indicating the position of the individual amino acid residues. Lysine-vasopressin has a phenylalanine residue instead of an isoleucine residue at position 3 and a lysine residue instead of a leucine residue at position 8. In the deamino analogs, the NH_2 at position 1 is replaced with H.

Experimental Section

Intermediate N^{α}-Boc-protected peptides were deblocked by dissolving them in cold, redistilled trifluoroacetic acid and allowing them to react at room temperature for 1 hr. The solvent was then removed *in vacuo* and the residual oil triturated with Et_2O. The resultant white powder was collected, washed with Et_2O, and dried *in vacuo*.

The disappearance of the free amine group during coupling reactions was followed by a quantitative ninhydrin color test.# The presence of excess diisopropylethylamine (*i*-Pr_2NEt) in the reaction mixture was maintained by the addition of further aliquots (10–20% of theoretical amount) of *i*-Pr_2NEt as needed. The atmosphere of the reaction vessel was periodically checked for *i*-Pr_2NEt by means of moist litmus paper.**

†This work was supported in part by Grant HL-11680 from the National Heart and Lung Institute, U. S. Public Health Service. All optically active amino acid residues are of the L configuration. The symbols for the amino acid residues follow the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature. Where analyses are indicated only by symbols of the elements, analytical results obtained for the elements were within ±0.4% of the theoretical values.

‡In ref 2, all of these analogs are discussed and a table of the antioxytocic and anti-AVD pA_2 values of some of them is given, as well as a detailed presentation of the method used to determine the pA_2 values.

§Oxytocic assays were performed on isolated uteri from virgin rats in natural estrus according to the method of Holton,[11] as modified by Munsick,[12] with the use of Mg-free van Dyke-Hastings solution as the bathing fluid. Avian vasodepressor assays were performed on conscious chickens by the method of Coon,[13] as modified by Munsick, Sawyer, and van Dyke.[14] Pressor assays were carried out on anesthetized male rats as described in the U. S. Pharmacopeia.[15]

#A standard curve for the quantitative ninhydrin color test is prepared using a 0.002 *M* DMF solution (not 0.002 m*M* as erroneously reported[17]) of the trifluoroacetate salt of the free amine compound. Aliquots (5–35 μl) of this solution are incubated for 5 min at 100° with 3 drops each of three Kaiser test reagents,[18] then diluted with 95% EtOH (1.00 ml), and read after 10 min at 590 nm against a reagent blank. During the course of the condensation reactions, appropriate aliquots of the reaction mixture are removed, tested in similar fashion, and compared to the standard curve for estimation of the amount of the free amine peptide remaining in solution.