THE STRUCTURE OF LUTEINE

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Luteine — a base without a tropolone ring isolated from the epigeal part of *Colchicum luteum* Baker [1] — has the composition $C_{19}H_{25}O_4N$, mp 228-230°C, $[\alpha]_D$ +93° (c 1.5; methanol). Its UV spectrum has absorption maxima at 210 and 285 nm (log ε 4.7, 3.7). The addition of a 0.1 N solution of caustic soda led to a bathochromic shift of the absorption maximum by 12 nm, which shows the presence of a phenolic hydroxy group in its molecule. The IR spectrum of the base has absorption bands of a hydroxy group (3470 cm^{-1}), of the stretching vibrations of the C=C bonds of an aromatic ring (1590 cm^{-1}), and of methylene groups (1470 cm^{-1}).

The PMR spectrum of luteine (Fig. 1) is characterized by a group of highly overlapping signals of the protons of the methylene and methine groups in the 3.80-2.00 ppm region and by three signals of the singlet type: The protons of a N-methyl group resonate at 2.35 ppm, those of an O-methyl group in an alicyclic ring at 3.25 ppm, and the proton of an aromatic ring at 6.47 ppm. At 4.7 ppm there is a greatly broadened signal ($\Delta\omega_{1/2} \sim 75$ Hz) relating to the hydroxy groups of the base. In the mass spectrum there are peaks of principal ions with m/e 331 (M^+, 43%), 330 $(M-1)^+$ 100%, 316, 300, 288 (M–43), 284, 270, 230, 228, 215, 201.

On the basis of its spectral characteristics, luteine can be assigned to the group of homoproaporphine alkaloids [2-4]. Its developed formula, $C_{17}H_{17}(-O-)(OCH_3)(OH)_2(NCH_3)$, is identical with that of kesselringine [5] and of regelamine [6], but their physical constants and spectral characteristics differ. On this basis, we assumed that the positions of the substituents in their spirocyclohexane rings are different. To determine the nature of the functional groups we studied the hydrolysis and methanolysis of luteine. When it was heated in dilute mineral acids, the methoxy group of the base was readily hydrolyzed and norluteine (II) was formed. Its stability to solutions of alkalis, like that of kesselringine, in-

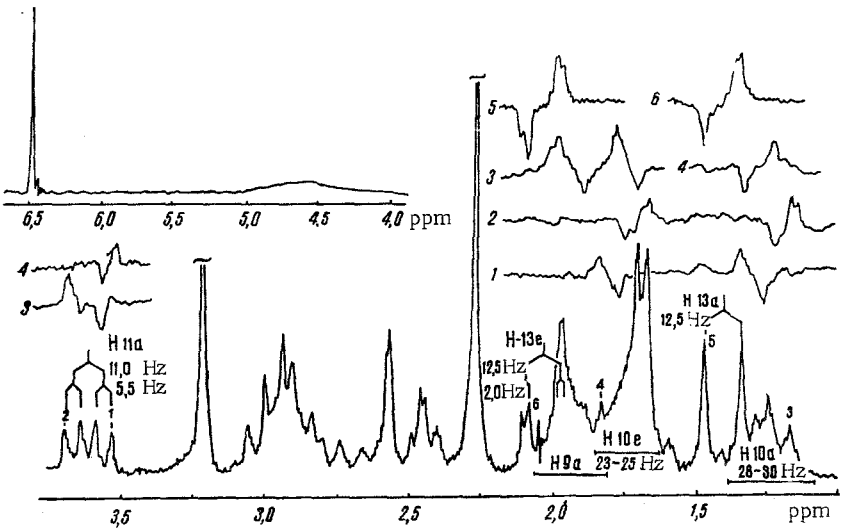

Fig. 1. PMR and INDOR spectra of luteine (in $CDCl_3$).

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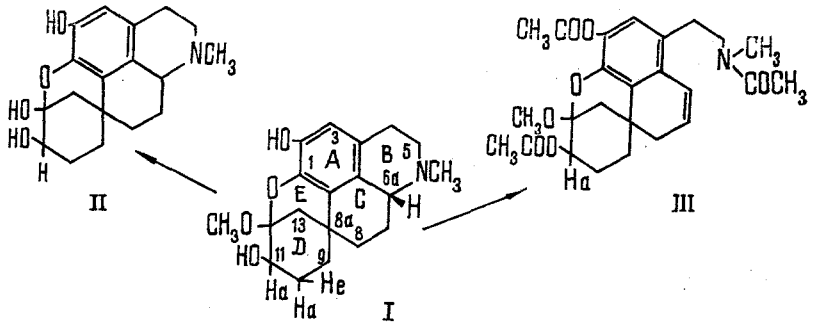
dicates the acetal nature of this methoxy group. The fact that luteine was incapable of undergoing a methanolysis reaction showed the alcoholic nature of one of its hydroxy groups, which is probably present in the spirocyclohexane ring D.



II I III

Under the action of acetic anhydride, luteine forms a O,O,N-triacetyl derivative (III), showing acetylation of the tertiary nitrogen atom with the opening of the heterocyclic ring of the isoquinoline nucleus and the presence of phenolic and alcoholic hydroxy groups.

On the basis of the facts given, and with the aid of the INDOR method we have established the structure of ring D of luteine, including the position of the acetal methoxy group and the secondary alcoholic hydroxy group, and also the spirocarbon atom. Below we give the results of an INDOR investigation of the structure of ring D of the base.

In the low-field region of the PMR spectrum of luteine there is an isolated one-proton signal at 3.64 ppm obviously corresponding to the methine proton at C_{11} of ring D. The downfield shift of this signal can be explained by the influence of the oxygen-containing geminal substituent — a hydroxy group. The doublet-doublet splitting of the signal of this proton shows two spin—spin coupling constants, J = 11.0 and 5.5 Hz. The first of these constants indicates the axial—axial arrangement of the methine proton with one of its vicinal partners, and the second shows the axial—equatorial arrangement with the other vicinal proton. The splitting of the signal of the C_{11} proton with two coupling constants shows the presence of only two protons in the α positions to it.

The positions in the spectrum of the signals of the vicinal partners of the C_{11} proton were determined by methods of internuclear double resonance — INDOR. The INDOR spectra 1 and 2 show the localization in the spectrum of the vicinal partners of the H_{11} proton. The equatorial partner resonates at 1.75 ppm. From the responses in the INDOR spectrum for this proton we established the existence of a second coupling constant, J = 10.7 Hz, and the width of the signal in the PMR spectrum $\Delta\omega$ = 23-25 Hz. The width of the signal exceeds the sum of the two coupling constants known for it (J = 5.5 and 10.7 Hz), which shows additional splitting in this case with a total contribution ΣJ = 7-9 Hz. This shows the presence of vicinal partners other than the H_{11} proton.

The axial proton of the H_{11} proton forms a broad signal in the $\sim$1.25-ppm region. The width of the signal ($\Delta\omega \geqslant$ 28-30 Hz) shows the presence of three large coupling constants in it. The latter are revealed by INDOR spectrum 3 with respect to one of the components of the signal of this axial proton. The INDOR responses in the $\sim$3.65-ppm region confirm its coupling with the H_{11} proton. The signals in INDOR spectrum 3 at $\sim$1.75 ppm show the interaction of this proton with a coupling constant J = 10.7 Hz with the equatorial partner of the H_{11} proton. Consequently, the equatorial and axial partners of the H_{11} proton form a geminal pair and may be assigned to the C_{10} position.

The second axial partner of the H_{10a} proton appears in the INDOR response in the 1.97-ppm region and forms a coupling constant J $\approx$ 10.0 Hz. On the basis of the results of a comparison of the width of the signal of the H_{10a} proton with the coupling constants revealed by the INDOR spectra it is possible to estimate the value of its quaternary coupling constant with the H_{9e} proton, J $\leqslant$ 4-5 Hz. The interaction of the H_{10e} proton with H_{11a} and H_{10a} is confirmed by INDOR spectrum 4. Additional splitting of the signal of the H_{10e} proton is caused by interaction with the geminal pair of protons in the C_9 position.

The presence of protons in the C_9 position is also confirmed by an investigation of the signals of the geminal pair of protons in position C_{13} (INDOR spectra 5 and 6). The H_{13a} proton forms a doublet with a geminal coupling constant J = 12.5 Hz at 1.40 ppm. The H_{13e}

proton resonates at 2.02 ppm. The signal of the equatorial proton shows two coupling constants — geminal (J = 12.5 Hz) and long-range (J = 2.0 Hz) with the H_{9e} proton. The absence of additional coupling constants for the geminal pair of protons in the C_{13} position and the presence of only two coupling constants for the signal of the C_{11a} proton shows the absence of hydrogen in the C_{12} position.

In the nature and distribution of the signals, the PMR spectrum of luteine is similar to that of kesselringine. As compared with the latter, the spectrum of luteine show a slight axial shift of some signals: the signal of the O-methyl group by 0.07 ppm, of the H_{11} protons by 0.07 ppm, and of the H_{13a} proton by 0.10 ppm. A small downfield shift can be detected for the H_{13e} and H_{10e} protons. The most fundamental difference between the spectra of kesselringine and luteine is the change in the shape of the signal of the C_{11} proton. In the spectrum of the former, this proton gives a signal of the pseudosinglet type with a half width $\Delta\omega_{1/2}$ = 5-6 Hz. The absence of a larger splitting constant for the H_{11} signal ($J \geqslant$ 5-6 Hz) shows its equatorial position in ring D. In this case, the splitting of the signal is masked by the natural width of the individual lines of the multiplet and the signal acquires a singlet form. The small change in the chemical shifts of the signals of the protons of ring D is also in harmony with a change in the orientation of the substituents in the C_{11} position.

As in kesselringine, the methoxy group in ring D of luteine is oriented equatorially and the ether bond axially.

On the basis of the facts given above, for luteine we propose the structure of 2,11-dihydroxy-12-methoxy-1,12-epoxyhexahydrohomoproaporphine (I) as an epimer of kesselringine with R configuration at the C_{6a} atom.

EXPERIMENTAL

The purity of the substances was checked by thin-layer chromatography in type LS 5/40 μ silica gel with chloroform—benzene—methanol—25% aqueous ammonia (20:6:4:2) (System 1) and by paper chromatography in the n-butanol—5% acetic acid (50:50) (System 2).

The UV spectra were taken in methanol on a Beckman model 25 spectrophotometer, the IR spectra on a UR-10 double-beam spectrometer, the PMR spectra on an XL-100 instrument, and the mass spectra on an MAT-311 high-resolution spectrometer.

The luteine formed a white crystalline substance with mp 228-230°C (acetone), $[\alpha]_D$ +93° (c 1.5; methanol), R_f 0.47 (system 1). It was readily soluble in methanol and chloroform, less readily in acetone, and insoluble in water and ether.

Norluteine. A mixture of 30 mg of luteine and 3 ml of 7% sulfuric acid was heated at 100°C for 2 h. After cooling, the solution was made alkaline with ammonia to pH 8 and was extracted with chloroform. The solvent was distilled off to give norluteine; R_f 0.22 (system 1).

PMR spectrum: 2.36 ppm (3 H, s, NCH_3); 6.48 ppm (1 H, s, ar.).

O,O,N-Triacetylluteine. A solution of 20 mg of luteine in 2 ml of acetic anhydride was mixed with 50 mg of freshly fused sodium acetate and the mixture was heated at 50-60°C for 24 h. Then, with the addition of methanol, the reaction mixture was evaporated by blowing with air. The dry residue was dissolved in water and the reaction product was extracted with chloroform. After the solvent had been distilled off, an amorphous powder of the acetyl derivative of luteine, having a neutral character, was obtained. R_f 0.88 (system 1).

IR spectrum: 1680, 1790 cm^{-1} ($NCOCH_3$, $2OCOCH_3$).

SUMMARY

On the basis of the results of chemical reactions and spectral characteristics, the structure of 2,11-dihydroxy-12-methoxy-1,12-epoxyhexahydrohomoproaporphine is proposed for luteine.

The structure of ring D of the base has been confirmed by a study of NMR and INDOR spectra.

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QUATERNARY STRUCTURE OF COTTONSEED TRIACETINASE

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A method of isolating cottonseed triacetinase, an enzyme catalyzing the hydrolysis of short-chain triglycerides that are substrates of esterases [2] has been described previously [1]. We give the results of an investigation of the subunit structure of this enzyme.

Esterases are oligomeric proteins [2]. By the titration of mammalian esterases with organophosphorus compounds [3-8] and carbamates [5, 6, 9] it has been established that there are two active centers in the esterase molecule. Under various conditions the dissociation of each of the esterases into two active half-molecules has been observed: a) on far-reaching dilution [7-9]; b) in an acid medium at pH 4.5 [7]; and c) at high concentration of salts (0.5 M NaCl; 0.5 M LiCl) [7]. When an esterase was incubated in a 0.2% solution of sodium dodecyl sulfate (SDS) [3], however, or in solutions of dissociating agents, it split into four polypeptide chains. The presence of two active centers in the molecule of the enzyme presupposes that the subunits are heterogeneous, but up to the present time there has been no proof of this statement.

The triacetinase was isolated by a modification of the previous method [1] in the following way: an acetone powder of cotton seeds was extracted with 0.1 M phosphate buffer, pH 7.4, and the extract was fractionated with ammonium sulfate and, after dialysis, it was chromatographed twice on DEAE-cellulose. The homogeneity of the preparations obtained was checked by disc electrophoresis and with respect to the N-terminal amino acid.

The active fraction (A) (Fig. 1) eluted from the DEAE-cellulose at a concentration of NaCl of 0.8 M [1] was dialyzed against 0.01 M phosphate buffer, pH 7.4 and was rechromatographed on DEAE-cellulose. Thereupon, in addition to fraction A, another active fraction, B, eluted at an NaCl concentration of 0.2 M was found (see Fig. 1), and in a series of experiments it was observed that the amount of protein in fraction A decreased with the simultaneous increase in the amount of protein in fraction B, and that the specific activity of A and B was approximately constant, amounting to 4500 activity units with respect to tributyrin. Similar results were obtained in an investigation of the esterase from bovine liver [8].

It is natural to assume that fraction B is formed from fraction A. The molecular weight of A determined by ultracentrifugation was 37,000 and by gel filtration 38,500, which agrees satisfactorily with literature information [1]. It was shown by ultracentrifugation and by gel filtration on Sephadex G-100 that the molecular weight of B is 20,000, which is approximately half the molecular weight of fraction A. The markers in gel filtration were

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