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METABOLIC INTERMEDIATES IN THE BIOLOGICAL OXIDATION OF LANOSTEROL TO EBURICOIC ACID

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Abstract—Eburicodiol and eburical as well as previously reported eburicol have been isolated from the neutral chloroform extracts of *Fomes officinalis*. A triterpenic acid, identified as sulphurenic acid, has also been isolated.

INTRODUCTION

THE ROLE of lanosterol (I) and lanosterol precursors as intermediates in the biosynthesis of eburicoic acid (II) has been confirmed by a number of experiments using isotopically labeled precursors.[1–4] Other tetracyclic triterpenic acids closely related to eburicoic acid, such as sulphurenic acid (III)[5] and tumulosic acid (IV),[6] undoubtedly arise from these same precursors.

We wish to report the isolation from the wood rotting fungus, *Fomes officinalis*, of two, previously unreported as naturally occurring, triterpenoids: 24-methylene-lanost-8-ene-3β, 21-diol (V), (eburicodiol) and 24-methylene-lanost-8-ene-3β-ol-21-al (VI), (eburical) and propose that these new triterpenoids are indeed metabolic intermediates in the biosynthesis of eburicoic acid.

RESULTS

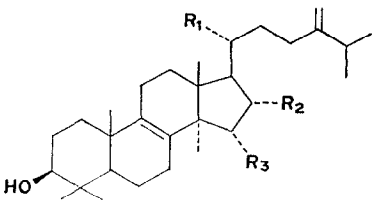
The chloroform extracts of the whole fungus were separated into strong and weak acids and saponifiable and nonsaponifiable neutrals by the usual methods. The nonsaponifiable fraction was subjected to an involved separational scheme described in detail in the experimental section. After removal of dehydroeburicoic acid*, a new compound was isolated, 24-methylene-lanost-8-ene-3β, 21-diol, by chromatography on Florisil and subsequent crystallization from methanol. This compound, V, was shown to be identical in all respects to 24-methylene-lanost-8-ene-3β, 21-diol prepared by reduction of eburicoic acid.[7]

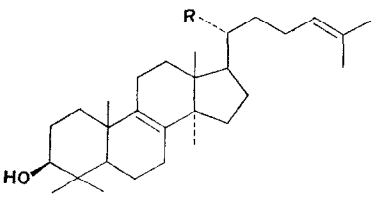
A second batch of neutral material which had not been saponified but had been subjected to urea complexing in order to remove fatty compounds was treated in a similar manner to the above. Chromatography, first on Florex then on Florisil, yielded two compounds of significance. The first compound was shown to be eburicol (VII)† by comparison to an

* This compound is an unusually weak acid which makes it very difficult to quantitatively remove from the neutral extracts of *F. officinalis*.

† This compound has been previously reported as a constituent of *F. officinalis*.[8]

[1] W. G. DAUBEN and J. H. RICHARDS, *J. Am. Chem. Soc.* **78**, 5329 (1956).
[2] W. G. DAUBEN, Y. BAN and J. H. RICHARDS, *J. Am. Chem. Soc.* **79**, 968 (1957).
[3] W. LAWRIE, J. MCLEAN, P. L. PAUSON, and J. WATSON, *Chem. Commun.* **24**, 623 (1965).
[4] D. H. R. BARTON, D. M. HARRISON, G. P. MOSS and D. A. WIDDOWSON, *J. Chem. Soc.* (C), 775 (1970).
[5] J. FRIED, P. GRABOWICH, E. SABO and I. A. COHEN, *Tetrahedron* **20**, 2297 (1964).
[6] L. F. FIESER and M. FIESER, *Steroids*, p. 386, Reinhold, New York, (1959).
[7] F. W. LAHEY and P. H. A. STRASSER, *J. Chem. Soc.* 873 (1951).
[8] W. W. EPSTEIN and G. VAN LEAR, *J. Org. Chem.* **31**, 3434 (1966).





II $R_1 = CO_2H$, $R_2 = H$, $R_3 = H$

III $R_1 = CO_2H$, $R_2 = H$, $R_3 = OH$

IV $R_1 = CO_2H$, $R_2 = OH$, $R_3 = H$

V $R_1 = CH_2OH$, $R_2 = H$, $R_3 = H$

VI $R_1 = CHO$, $R_2 = H$, $R_3 = H$

VII $R_1 = CH_3$, $R_2 = H$, $R_3 = H$

I $R = CH_3$

VIII $R = CO_2H$

IX $R = CH_2OH$

3 - Keto

authentic sample, and the second, isolated in small quantities, a new compound, 24-methylene-lanost-8-ene-3β-ol-21-al. When each of these compounds was analysed by TLC (Silica gel G) and the plates developed with methanolic–sulfuric acid followed by heating, the spots were colored, VII producing a dull orange color and VI a yellow color, which was useful in the final identification.

The structure of VI was unambiguously established by consideration of the following data. Acetylation of VI with acetic anhydride–pyridine followed by crystallization from methanol yielded small plates, m.p. 154–156°, which had a mass spectrum and R_f on TLC identical to an authentic sample of VI acetate prepared from eburicoic acid. No depression of m.p. was observed on admixture of the acetate of VI with authentic material. $LiAlH_4$ reduction of VI acetate yielded a compound with an R_f identical to that of V. Wolf-Kishner reduction of VI acetate yielded hydrolysed and C-20 epimerized VI plus a compound which was identical to VII on TLC and produced a dull orange spot on developing with methanolic–sulfuric acid followed by heating.

The relative ease of epimerization at C-20 of VI under basic as well as acidic conditions was demonstrated by the following experiments.

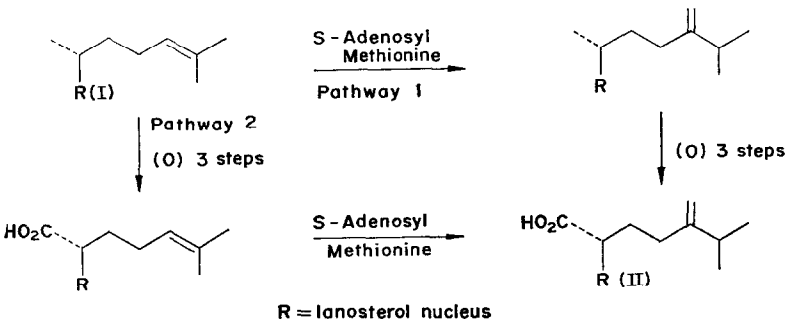
When the acetate of VI was prepared by CrO_3 oxidation in acetic acid of eburicodiol-3-acetate, according to the procedure of Lahey and Strasser,[7] we isolated a compound (30%) which had a m.p. and optical rotation identical to those reported by the authors (m.p. 140–144°, $[\alpha]_D^{24}$ +53°). This compound was shown, however, to be a mixture of the C-20 epimers of VI acetate by TLC, the unnatural 20-R epimer having a slightly smaller R_f. When the experiment was repeated using Jones Reagent as the oxidant we isolated the pure 20-S epimer (65%), which had a m.p. and optical rotation of 154–156° and $[\alpha]_D^{28}$ +57° (c = 1·4) respectively. NMR, IR, and mass spectral data were consistent with the structure of this compound.

The acetate of VI was not as sensitive to alkaline conditions since it could be hydrolysed to VI in ethanolic KOH (0·08 N, 24 hr, 25°) with negligible epimerization. However, when the product of hydrolysis, VI, was allowed to reflux for 5 hr in 0·08 N ethanolic KOH, we observed a change in optical rotation and the appearance of a second, slightly more polar compound on TLC, indicating that epimerization had occurred.

The above results suggest that eburicol (VII), prepared from VI acetate by Wolf-Kishner reduction (strongly alkaline conditions), is a mixture of 20-S and 20-R epimers indistinguishable by TLC.

THEORETICAL CONSIDERATIONS

Eburicoic acid can be envisaged as arising from lanosterol by one or both of the following pathways (Scheme 1). Pathway 1 involves alkylation of the lanosterol side chain by S-adenosyl methionine[9,10] to yield 24,25-dihydro-24-methylene-lanosterol (VII), (eburicol). The C-21 methyl group is then oxidized presumably in a stepwise process to finally yield eburicoic acid. Barton, *et al.*[4] have in fact shown that isotopically labelled VII is efficiently converted into eburicoic acid by the fungus *P. sulphureus*, lending support to this pathway.



SCHEME 1. POSSIBLE PATHWAYS OF EBURICOIC ACID BIOSYNTHESIS.

Pathway 2 describes the reverse sequence of reactions, that is, oxidation of the C-21 methyl group followed by alkylation of the intermediate acid. The isolation of acids such as trametenolic acid B (VIII)[11] and alcohols such as lanost-8,24-diene-3-one-21-ol (IX)[12] from natural sources lends support to the suggestion that oxidation of the C-21 methyl group can occur without prior alkylation of the side chain. Of course, the possibility that alkylation can occur during any one of the oxidation steps cannot be excluded.

The isolation of the two new compounds, eburicodiol and eburical along with the previously isolated compounds, eburicol and eburicoic acid from the extracts of *F. officinalis* taken in conjunction with the results of Barton's experiments utilizing radioactive precursors adds support for the involvement of Pathway 1 and provides a rather more complete picture of the biosynthesis of eburicoic acid in this organism. Intermediates analogous to eburicodiol and eburical in Pathway 2 have not been isolated from *F. officinalis*, lending further support for the involvement of Pathway 1.

ADDENDUM

In conjunction with the above work, a second study was conducted on the $CHCl_3$ soluble weak acids of *Fomes*. This study resulted in the isolation of a metabolic product closely related to eburicoic acid, sulphurenic acid (III)-15α-hydroxyeburicoic acid. This

[9] L. W. PARKS, *J. Am. Chem. Soc.* **80**, 2023 (1958).
[10] L. J. GOAD, A. S. A. HAMMAM, A. DENNIS and T. W. GOODWIN, *Nature, Lond.* **210**, 1322 (1966).
[11] G. OURISSON, P. CRABBÉ, and O. R. RODIG, *Tetracyclic Triterpenes*, p. 142, Holden-Day, San Francisco (1964).

triterpenic acid was previously reported as a constituent of *P. sulphureus* by Fried *et al.*[5] The identity of the compound was confirmed by direct comparison of the methyl-ester with authentic material.*

EXPERIMENTAL

Unless otherwise mentioned, optical rotations were measured in $CHCl_3$ solution on a Perkin–Elmer Model 141 Polarimeter; m.ps (uncorrected) were determined in sealed, evacuated capillaries; IR spectra recorded in CCl_4 solution on a Beckman IR-5a; NMR recorded on a Varian A-60 spectrometer; mass spectra determined on a Perkin–Elmer Model 270 using PFK as a standard; and Silica gel G sprayed plates used for TLC (benzene–EtOAc, 9:1). When three solvent systems were used to determine the purity of a compound on TLC, they were as follows: benzene–EtOAc (9:1), $CHCl_3$–acetone (9:1), and cyclohexane–isopropyl ether (3:1).

Preliminary treatment of fungus extract. Described under results section.

Isolation of 24-*methylene-lanost-8-ene-3β,21-diol* (V). The nonsaponifiable neutrals (37·5 g) were chromatographed on 2 kg of Florex (Floridin Co). The benzene fractions (9·7 g) were rechromatographed on 500 g of Florex and the benzene–ether (1:1) eluents (710 mg) found to be a two component mixture. Chromatography 40 g on Florisil (Floridin Co) separated the mixture and the ether eluents yielded 56 mg of pure V as needles after crystallization from MeOH: m.p. 211–212°; M^+ 456; $[\alpha]_D^{28}$ +57° (c = 1·2); diacetate, m.p. 132–133°; $[\alpha]_D^{28}$ +46° (c = 1·0). V was identical in all respects to 24-methylene-lanost-8-ene-3β,21-diol prepared by $LiAlH_4$ reduction of methyl eburicoate.

Isolation of 24,25-*dihydro-24-methylene-lanosterol* (VII) *and* 24-*methylene-lanost-8-ene-3β-ol-21-al* (VI). A second batch of neutrals (15 g) which had not been saponified was treated under complexing conditions with urea to remove fatty substances. The defatted material (13·9 g) was chromatographed on 700 g of Florex and the benzene fraction (3·2 g) rechromatographed on Florisil (200 g). Benzene–Et_2O (8:2) eluted 650 mg of a two component mixture. The benzene–EtOAc (95:5) fractions from rechromatography of the mixture on Florisil (25 g) proved to be 24,25-dihydro-24-methylene lanosterol (VII), (635 mg) by comparison to an authentic sample. The second component (10 mg) eluted with benzene–EtOAc (9:1) had the same R_f and yellow color on TLC (3 solvents) as 24-methylene-lanost-8-ene-3β-ol-21-al (VI).

Acetate of VI. The residue (10 mg) from above was dissolved in 0·5 ml of pyridine and 1·0 ml of Ac_2O added. The resulting solution was allowed to stand 24 hr and worked up in the usual manner. The crude product crystallized from MeOH to yield 7 mg of small plates, m.p. 154–155°. This compound had a mass spectrum and R_f on TLC (3 solvents) identical to an authentic sample of VI prepared from eburicoic acid.

Reduction of acetate of VI. To 15 mg of $LiAlH_4$ in 2 ml of anhydrous Et_2O under N_2 was added 3·5 mg of the acetate of VI in 3 ml of Et_2O. The solution was stirred for 2·5 hr, the aluminium salts hydrolysed and the product extracted into Et_2O. TLC analysis (3 solvents) of the extract proved it to be the expected eburicodiol (V).

Wolf–Kishner reduction of the acetate of VI. The acetate of VI (3·5 mg) was dissolved in 5 ml of hydrazine hydrate (90%) and 2 ml of diethylene glycol and one KOH pellet added. The solution was refluxed for 4 hr under N_2, the condenser turned off and the temperature allowed to rise to 200° for 1 hr. TLC analysis (3 solvents) of the extracted product indicated it to be a mixture of hydrolysed and C-20 epimerized starting material and eburicol (VII).

Preparation of VI *acetate from eburicodiol-3-acetate using Jones reagent.* To a solution of 230 mg of eburicodiol-3-acetate in 100 ml of reagent grade acetone was added 1·7 mole equivalents of Jones Reagent. The solution was stirred at 28° for 20 min, diluted with a large volume of H_2O and the products extracted into Et_2O. Removal of the Et_2O *in vacuo* and crystallization of the resulting residue from MeOH yielded 149 mg of pure VI acetate: m.p. 154–156°; $[\alpha]_D^{24}$ +57° (c = 1·4); M^+ 496; NMR (CCl_4) broad 1 proton singlet at 9·58 δ; IR, 2849 (shoulder) (w), 2740 (w) 1725 cm^{-1} (s).

Hydrolysis of VI *acetate.* Eburicalyl acetate (46 mg) was dissolved in 30 ml of 0·08 N ethanolic KOH solution and the reaction set aside under N_2 for 24 hr. The solution was diluted with a large volume of H_2O and the products extracted into Et_2O. Removal of the Et_2O *in vacuo* and crystallization of the residue from MeOH yielded 34 mg of pure eburical (VI) as needles; m.p. 177–179°; $[\alpha]_D^{26}$ +56° (c = 1·25); M^+ 454; IR 2850 (shoulder), 2741 (w), 1725 cm^{-1} (s); NMR ($CDCl_3$) 1 proton singlet at 9·55 δ.

Epimerization of VI *at* C-20 *under alkaline conditions.* Eburical (VI), (25 mg) was dissolved in 30 ml of 0·08 N ethanolic KOH solution and the solution refluxed under N_2 for 5 hr. The reaction was worked up as described above and an optical rotation taken. The epimeric products had an optical rotation of 51° compared to +56° for pure VI. TLC analysis indicated the crude reaction product to be approximately a 70:30 mixture of 20-S and 20-R epimers, respectively.

* The authentic sample of methyl sulphurenate was graciously provided by Dr. Josef Fried of the Ben May Laboratory for Cancer Research.

Isolation of sulphurenic acid (III). The $CHCl_3$ extracts of the whole fungus were separated into neutrals, strong and weak acids by the usual methods. The weak acid fraction (15 g) after removal of the $CHCl_3$ *in vacuo* was then taken up in MeOH and allowed to stand for about 1 week resulting in the crystallization of 10·7 g of eburicoic acid. The MeOH was removed *in vacuo* and the resulting residue converted to methyl-esters with CH_2N_2. Chromatography of the methyl-esters (4·5 g) on 250 g of Florisil separated the mixture and benzene–Et_2O (95:5) eluents yielded 2·4 g of pure methyl sulphurenate as long needles after crystallization from MeOH: m.p. and mixed m.p. 191–192°; $[\alpha]_D^{28}$ +65° (c = 0·75); M^+ 500. These properties and others were identical in every respect to those of authentic material.

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