Microbiological Conversion of Lapachol by Various Microorganisms

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Oxydative cyclization of lapachol was attempted by feeding this compound into the culture broth of *Beauveria sulfurescens* and two ionophore-producing strains of *Streptomyces albus* and *Streptomyces griseus*. As a result, three compounds were obtained, lomatiol, lomatic acid and lomatiol acetate, only with *B. sulfurescens* and *S. albus*. The structures of these products were determined by NMR and mass spectrometry. The stereochemistry is given by a comparison with butene analogs.

In previous work,¹⁾ we showed that linalol **1** could be converted into a mixture of linalol oxides **2** by *Beauveria sulfurescens* (ATCC 7159).

This cyclization can also be achieved by other microorganisms, particularly *Strepto-*

myces-producing ionophorous antibiotics such as *Streptomyces albus* (NRRL B. 1865), which produces nigericin.²⁾ This has also been carried out chemically.³⁾ Whichever method was used, the intermediate epoxide **3** was assumed to be involved, though it has only

been isolated in the case of the chemical conversion.

We report here the results of the action of various microorganisms on another γ -unsaturated hydroxyl compound, lapachol **4**.

Lapachol is a naturally-occurring naphthoquinone present in the heart-wood of several higher plants, in bacteria, in fungi and in certain animals.⁴⁾ This compound and its derivatives have antibiotic,⁶⁾ antitumoral⁵⁾ and anti-malarial properties,⁷⁾ and lapachol itself is commercially available in some countries as an anti-tumor agent.⁸⁾

Otten and Rosazza^{9,10)} have studied the microbiological conversion of lapachol. In small-scale screening experiments with 48 microorganisms, they identified 20 cultures which actively metabolized lapachol to one or more metabolites. The most interesting result was obtained with *Curvularia lunata*, which gave dehydro- α -lapachone **5** with a yield of 50%.

The other microorganisms almost solely gave the ketol **6**.

Cunninghamella echinulata gave other metabolites in very small amounts, including lomatiol and lomatiol lactate.¹⁰⁾ Adams and Lewis¹¹⁾ obtained the cyclized compound **7** chemically by treating lapachol with metachloroperbenzoic acid.

Compounds **5** and **7** were presumably formed *via* the intermediate ($\pm$) epoxide **8**.

The enzyme reaction involved attack at the tertiary carbon(1) giving **5**, whereas the chemical pathway involved attack at the secondary carbon(2) giving **7**.

It thus seemed likely, given the previous results with linalol, that one of these conversions would be obtained by the action of *B. sulfurescens* and ionophorous antibiotic-producing strains on lapachol.

MATERIALS AND METHODS

Melting-points were measured with a REICHERT hot-plate microscope and are uncorrected. Infra-red spectra were recorded with a Perkin-Elmer 327 spectrometer in KBr. NMR spectra were recorded on a Bruker W2 200

SY instrument at 200 MHz for the ¹H spectra and at 50.33 MHz for the ¹³C spectra. ¹³C NMR spectra were also recorded on a JEOL FX 60 instrument at 15.03 MHz. In all cases, TMS was used as the internal standard. Analytical thin-layer chromatography was carried out on Schleicher and Schull F 1550/LS 254 silica gel plates, visualization being made with a UV lamp at 254 nm. Column chromatography was carried out using the flash-chromatography technique¹²⁾ with Merck 60 230~400 mesh silica gel. Either benzene-acetic acid 95:5 or benzene-ethyl acetate-acetic acid (80:10:10) was used as the eluent.

Chemical and microorganisms. The lapachol was of commercial grade (Aldrich). The microorganisms used were *B. sulfurescens* (ATCC 7159), *S. albus* (NRRL B. 1865) and *S. griseus* (LAB 2142).¹³⁾

Culture conditions. All cultures were grown by two-stage fermentation procedures in different media. Slant cultures were inoculated into 100 ml of seed medium in a 500 ml flask and the flask incubated at 27°C for 72 hr with shaking at 200 rpm. 5 ml of the resulting vegetative growths were transferred to 500 ml flasks containing 100 ml of culture medium.

The different media used (in g/liter tap water) were *B. sulfurescens*: glucose 10, cornsteep solid 12, pH adjusted to 5 with 10 N NaOH. *S. albus*: glucose 10, casaminoacids 4, yeast extract 1, meat extract 1. *S. griseus*: sucrose 30, soyabean meal 10, peptone 4, K₂HPO₄ 1, CaCO₃ 5.

Bioconversion conditions. Fermentations were carried out under the same conditions as the seed cultures. After shaking for 24 hr, lapachol dissolved in DMSO was added at 50 mg/flask. After the fermentation period, the culture was centrifuged, acidified to pH 2 with 6 N HCl and continuously extracted with AcOEt (16 hr). The extracts were then dried and evaporated to dryness, the residue being analyzed by thin-layer chromatography. Controls consisted of fermentation without lapachol and with lapachol added to non-seeded media shaken under the same conditions.

Isolation of products. The various metabolites produced were isolated by flash column chromatography. The following products were obtained:

Lomatiol 9. mp 114~117°C (lit. mp 119~123.5°C).¹⁴⁾ IR $\nu_{\max}$ cm⁻¹: 3360, 1640, 1590, 1360 and 725. ¹H NMR δ (CDCl₃, ppm): 1.79 (s, 3H), 3.32 (d, 2H), 3.91 (s, 2H), 5.48 (m, 1H), 7.82 (m, 2H), 8.03 (m, 2H), 9.45 (s, 2H disappearing with D₂O).

Lomatic acid 10. Melting point: decomposed between 140 and 170°C. IR $\nu_{\max}$ cm⁻¹: 3250, 1630, 1590, 1345. ¹H NMR δ (DMSO-*d*₆, ppm): 1.90 (s, 3H), 3.36 (d, 2H), 3.76 (s, 2H disappearing with D₂O), 6.63 (m, 1H), 7.82 (m, 2H), 7.48 (m, 2H). Mass spectrum *m/z* (%): 256 (9.9), 238 (23),

227 (100), 213 (16.6), 159 (5.3), 105 (13.4), 82 (8.3), 77 (16.7).

Lomatol acetate 11. mp 109~112°C. ^1H NMR $\delta(\text{acetone-}d_6, \text{ppm})$: 1.82 (s, 3H), 1.98 (s, 3H), 3.35 (s, 2H), 4.41 (s, 2H), 5.57 (m, 7H), 7.79 (m, 2H), 8.03 (m, 2H), 9.55 (s, 1H disappearing with D_2O).

RESULTS AND DISCUSSION

The first bioconversion experiments were carried out with *B. sulfureus*. The reaction was first monitored at regular intervals by sampling, extraction and analyzing the extracted residue by thin layer chromatography. This showed that a first metabolite was rapidly formed, followed by two other metabolites as the reaction proceeded. The lapachol disappeared progressively and had completely vanished after 6 to 7 days of reaction. The concentrations of the two main metabolites peaked after 3 days of reaction and

that of the 3rd metabolite peaked after 5 days of reaction. In order to identify each of these metabolites, the bioconversion was carried out preparatively on 1 g of lapachol with a reaction time of 3 days. The products were separated by column chromatography to obtain the metabolites shown below (Scheme 1).

The most abundant metabolite (45% yield) was lomatol **9**. This was the first product to appear. Its structure was determined from ^1H and ^{13}C NMR spectra.

The ^1H NMR spectrum of lapachol had 2 singlets (1.68 and 1.79 ppm) corresponding to the 14 and 15 methyl groups, whereas that of compound **9** had a single signal at 1.79 ppm (3H) and a new signal at 3.91 ppm corresponding to a hydroxy-methylene group. The ^{13}C NMR spectrum showed a single peak for the 14- and 15-methyl groups of **4** at 14.71 ppm and a new signal appeared at 67.2 ppm corresponding to a hydroxyl-bearing carbon in **9**.

SCHEME 1.

Lomatic acid **10** represented from 5 to 10% of the reaction mixture, its structure also being determined from spectral data. Its ^1H NMR spectrum showed the following differences compared with that of **9**: a shift of a methyl attached to a double bond downfield from 1.79 ppm to 1.90 ppm, disappearance of the signal at 3.91 ppm and a shift of the signal due to the double-bond proton from 5.48 ppm to 6.63 ppm. These differences show that the hydroxyl function had been replaced by a more polar one. Analogous differences can be seen in the ^{13}C spectrum. The CH_2OH signal at 67.2 ppm disappeared and a new peak at 169.9 ppm appeared corresponding to a carbon bearing an acid function. Mass spectra provided further confirmation of the structure ascribed to **10**, with peaks at $m/z = 227$ ($\text{M} - \text{CO}_2\text{H}$ 100%) and 213 ($\text{M} - \text{CH}_2 - \text{CO}_2\text{H}$).

The third and last metabolite was obtained in very small amount (about 1%). Its ^1H NMR spectrum showed two signals due to methyl groups at 1.79 and 1.82 ppm. The signal of lomatiol at 3.91 ppm ($\text{CH}_2 - \text{OH}$) was deshielded and appeared at 4.41 ppm, due to the effect of the acetate group.

The ^{13}C NMR spectrum confirmed the structure attributed to **11**. Two additional signals occurred, one at 21.6 ppm (CH_3) and the other at 171.1 ppm ($\text{O} - \overset{\text{O}}{\underset{\text{O}}{\text{C}}} -$). In addition, the

signal due to the hydroxyl-bearing carbon of lomatiol was deshielded and moved downfield from 67.2 to 69.9 ppm. Using coupling constant modulation spectra (J -mod) and the available literature data, we were able to assign all the carbons in lapachol and its 3 metabolites.

Assigned values of chemical shift are given in Table I.

The stereochemistry of the double bond in the three metabolites (E or Z) remained to be determined. Rosazza *et al.*¹⁰⁾ proposed E stereochemistry for lomatiol obtained using *Cunninghamella echinulata* by comparing the values of the ^{13}C NMR signals for the *gem*-dimethyl group of lapachol with those for 2-methyl-2-butene. We compared the ^{13}C NMR

TABLE I. ^{13}C SPECTRA OF LAPACHOL **4** AND ITS METABOLITES DETERMINED FROM J -MOD SPECTRA

Carbon	Chemical shifts in ppm ($\text{DMSO}-d_6$), TMS			
	Lapachol 4	Lomatiol 9	Lomatic acid 10	Lomatiol acetate 11
C-1	182.1	182.1	181.9	182.1
C-2	156.0	156.2	157.0	156.7
C-3	124.0	123.9	121.6	122.8
C-4	185.1	185.2	185.0	184.8
C-5	126.6	126.7	126.7	126.6
C-6	135.4	135.4	135.5	135.4
C-7	134.1	134.2	134.2	134.0
C-8	126.6	126.7	126.7	126.6
C-9	133.0	130.9	131.0	131.8
C-10	133.0	133.0	132.9	133.0
C-11	23.1	22.6	23.7	22.6
C-12	121.8	120.6	138.8	126.0
C-13	130.9	137.5	129.5	130.9
C-14	18.7	14.7	13.3	14.8
C-15	26.5	67.2	169.9	69.9
C-1'				171.1
C-2'				21.6

spectra of **4**, **9** and **10** with those of their analogs E and Z 2-methyl-2-butene, 2-methyl-2-buten-1-ol and 2-methyl-2-butenic acid. The ^{13}C chemical shift values of the relevant carbons are shown in Scheme 2.

In lapachol and 2-methyl 2-butene, the difference in value between the two methyls of the *gem*-dimethyl group was about the same, roughly 8 ppm, the *cis* methyl being more deshielded. Comparison of the value for lomatiol and the two analogous alcohols in the 2-methyl 2-butene series showed that the former had E stereochemistry. The value of the methyl signal in the Z isomer was too high and that of the hydroxymethylene signal was too low. Similarly, inspection of the values for lomatic acid and its two analogs showed that the former must be E . Thus the metabolites obtained here using *B. sulfurescens* had the same E stereochemistry as those obtained by Rosazza *et al.*

The action on lapachol of *Streptomyces albus*, which produced the antibiotic nigericin **12a**,¹⁶⁾ was subsequently studied. Analytical

SCHEME 2.

trials showed that reaction occurred faster than with *B. sulfurescens*. After three days, all the lapachol had disappeared. Only two metabolites were formed, lomatiol **9** and lomatic acid **10**, lomatiol being the more abundant product. Both had the same stereochemistry as the products of the reaction with *B. sulfurescens*.

Finally, a reaction with *Streptomyces griseus* (LAB 2142), which produced the antibiotic grisorixin (**12b**), was tried. With this microorganism, no reaction had occurred even after 15 days and the lapachol was recovered intact.

Comparison between the structures of the two antibiotics **12a** and **12b**, indicates that they differ only in a substituent of the last ring (C-30). In grisorixin, this substituent is a methyl group, whereas in nigericin it is a hydroxymethylene group. Robinson *et al.*¹⁸⁾ in the course of a study on the biosynthesis of monensin (the last two cycles of which are the same as in nigericin) showed that the oxygen of the hydroxymethylene group did not come from the acetate precursor, but from the air. The results reported here of the action of *S. albus* and *S. griseus* on lapachol are consistent with this finding and with the fact that *S. griseus* pro-

duces **12b** rather than **12a**. The results with *B. sulfurescens* are consistent with the known hydroxylating properties of this microorganism.¹⁷⁾

To conclude, although no formation of cyclic compounds such as **5** and **7** was evident, the reaction with *B. sulfurescens* and *S. albus* of lapachol is of some interest as it enables a methyl group to be hydroxylated with an excellent yield. This conversion is not readily achieved chemically.

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REFERENCES

- 1) L. David and H. Veschambre, *Agric. Biol. Chem.*, to be published.
- 2) L. David and H. Veschambre, *Tetrahedron Lett.*, **25**, 543 (1984).
- 3) D. Felix, A. Melera, J. Seibl and E. Kovats, *Helv. Chim. Acta*, **46**, 1513 (1963).
- 4) R. H. Thomson, *Naturally Occurring Quinones*, 2nd Ed., Academic Press, New York, 1974.
- 5) J. S. Driscoll, G. F. Hazard, Jr., H. B. Wood, Jr. and A. Goldin, *Cancer Chemother. Rep., Part. 2*, **4**(2), 1 (1974).

- 6) O. Goncalves de Lima, J. S. de B. Coelho, I. L. D'Albuquerque, J. F. de Mello, D. G. Martins, A. L. Lacerda and M. A. de Moraes de Souza, *Rev. Inst. Univ. Fed. Pernambuco Recife*, **11**, 21 (1971).
 - 7) L. F. Fieser *et al.*, *J. Am. Chem. Soc.*, **70**, 3151 (1948).
 - 8) M. de C. F. Linares, M. A. de Oliveira and M. R. P. Sampoio, *J. Med. Chem.*, **18**, 1959 (1975).
 - 9) S. Otten and J. P. Rosazza, *Appl. Environ. Microbiol.*, **18**, 311 (1979).
 - 10) S. Otten and J. P. Rosazza, *J. Nat. Prod.*, **44**, 562 (1981).
 - 11) J. H. Adams and J. R. Lewis, *J. Chem. Res. (S)*, 3 (1978); *J. Chem. Res. (M)*, 189 (1978).
 - 12) W. C. Still, M. Kahn and A. Mitra, *J. Org. Chem.*, **43**, 2923 (1978).
 - 13) P. Gachon, A. Kergomard, T. Staron and C. Esteve, *J. Antibiot.*, **28**, 345 (1975).
 - 14) a) I. A. McDonald, J. J. Simpson and A. F. Sierakowski, *Aust. J. Chem.*, **30**, 1727 (1977).
b) E. Pretsch, T. Clerc, J. Seibl and W. Simon, "Tables of Spectral Data for Structure Determination of Organic Compounds," Springer Verlag, Berlin, 1983.
 - 15) H. Brouwer and J. B. Stothers, *Can. J. Chem.*, **50**, 601 (1972); *ibid.*, **50**, 1361 (1972).
 - 16) L. David and G. Kergomard, to be published.
 - 17) A. Archelas, Thèse Docteur-Ingénieur, Université de Marseille, 1981.
 - 18) A. A. Ajaz and A. Robinson, *J. Chem. Soc., Chem. Commun.*, 679 (1983).
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