The naphthoquinones lapachol and dichloroallyl lawsone readily undergo oxidative ring fission when incubated with several fungi and streptomycetes. *Penicillium notatum* was employed to produce the ring fission product of dichloroallyl lawsone which was isolated and characterized by spectral analyses and chemical synthesis. The mechanism of oxidative ring fission of lapachol was studied by growing *P. notatum* cultures in an \(^{16} \text{O}_2\) atmosphere. Mass spectral analysis of the isolated and labeled metabolite indicates that ring fission occurs via a monoxygenase pathway most probably involving an epoxide intermediate.

Naturally occurring naphthoquinones such as lapachol (Fig. 1, 1) are widely distributed in nature and have been found in bacteria, fungi, higher plants, and animals (Thomson, 1974). Naphthoquinones are frequently involved in cellular respiration and photosynthesis, and many of them are biologically active. Lapachol and its analogs possess antitumor (Rao et al., 1979), hydroxylation, glucosylation, and esterification (Otten et al., 1969), antibiotic (Goncalves de Lima et al., 1971; Gilbert et al., 1970), and antimalarial (Fieser et al., 1948) activities.

Little is known about the metabolism of naphthoquinones in microorganisms or in mammals. Studies concerned with the microbial metabolism of lapachol have been conducted, and previous reports describe oxidative ring fission (Otten and Rosazza, 1978), oxidative cyclization (Otten and Rosazza, 1979), hydroxylation, glucosylation, and esterification (Otten and Rosazza, 1981) of lapachol. Oxidative ring cleavage is a common and major pathway of microbial metabolism of lapachol. This report describes the results of investigation on the mechanism of oxidative ring fission of lapachol by *Penicillium notatum* and demonstrates that dichloroallyl lawsone (2), a synthetic derivative of lapachol, is metabolized in an analogous manner (Fig. 1).

**EXPERIMENTAL PROCEDURES**

**Materials**

Lapachol was purchased from Aldrich, and its characteristics were published (Linardi et al., 1975; Otten and Rosazza, 1978). Dichloroallyl lawsone (National Screening Center 126771) was obtained from the National Cancer Institute and was characterized as follows: m.p. 158-159 °C; UV (EtOH) \(\lambda_{max}\) 259 (15,801), 252 (15,570), 276 (26,225), 333 (2,908), 386 (1,410); nmr (acetone-d\(_6\)) \(\delta 3.48\) (d, 2H, J \(8 \) Hz), \(6.08\) (6H, CH\(_3\)), 6.06 (t, 1H, J 8 Hz — CH\(_2\)-), 7.82 (m, 2H, aromatic), 8.07 (m, 2H, aromatic); mass spectrum, m/e (relative intensity) 282 (0.01), 247 (0.58), 183 (42), 105 (29), 77 (100), 76 (84), 63 (45), 51 (91), 50 (85), 36 (31), 28 (41). Oxygen 18 (90.8 atom % \(^16\)O) was obtained from Miles Laboratory, Inc., Elkhart, IN. \(\text{H}_2\text{O}(95 \text{ atom}\% \(^{18}\)O) was obtained from Merck and Co., Inc., Rahway, NJ.

**Instruments**

Melting points were determined in open-ended capillary tubes with a Fisher-Jones-Hoover capillary melting point apparatus and were corrected. Proton magnetic resonance spectra were obtained with a Varian Associates model T-90 spectrometer, using tetramethylsilane as an internal standard. Low resolution mass spectra were obtained with a Finnigan model 3200 mass spectrometer. High resolution mass spectral data were obtained through the courtesy of the Chemistry Department of the Massachusetts Institute of Technology, Cambridge, MA. Infrared spectra were obtained with a Beckman IR-4240 spectrophotometer, and ultraviolet spectra were obtained with a Pye-Unicam SP-1800 spectrophotometer.

**Chromatography**—Thin layer chromatography was performed on 0.25-mm thick layers of Silica G\(_254\) (Merck) buffered with 8% (w/v) potassium phosphate monobasic. Layers were prepared on glass plates with a Quickfit Industries Spreader (Quickfit Industries, London, England) and were activated for 30 min at 120 °C before use. Chromatograms were developed in hexane:acetone:acetic acid (58:40:5, by volume) and were visualized by fluorescence quenching with ultraviolet light. Chromatograms were also visualized by spraying with ceric ammonium sulfate (1% Ce(NH\(_4\))\(_2\) (SO\(_4\))\(_3\) in 50% H\(_2\)PO\(_4\)) and warming sprayed plates with a heat gun. Silica gel for column chromatography was prepared by making a thick slurry of silica gel (Baker 3404) with 8% (w/v) potassium phosphate monobasic and drying overnight at 120 °C. Columns were well packed in the developing solvent and fractions were collected in a Fractomette 200 instrument.

**Fermentation Procedures**— Cultures used in this work were stored at 4 °C in sealed screw-capped tubes on Sabouraud maltose agar slants. Microorganisms were grown according to the previously described two-stage fermentation procedure (Betts et al., 1974) in soybean meal-glucose medium. Screening experiments were conducted in 125-ml cotton-plugged Erlenmeyer flasks containing 25 ml of medium. Dichloroallyl lawsone (10 mg in 0.1 ml of dimethyl formamide) was added to 24-h Stage II cultures, and samples were withdrawn at 24, 48, 72, and 144 h. Samples (4 ml) were acidified to pH 2 with 6 N HCl and extracted with 1 ml of ethyl acetate. Approximately 30 ml of the ethyl acetate extracts were examined by TLC.

Controls consisted of fermentations without dichloroallyl lawsone and solutions of dichloroallyl lawsone in buffers, including 0.1 M citric acid (pH 2.1), 0.1 M sodium phosphate (pH 6.5), and 0.1 M Tris (pH 8.7), all of which were incubated with shaking for the duration of normal fermentations (144 h).

**Transformation of Dichloroallyl Lawsone (2) to (4) by P. notatum** (U1 1952)—All of the cultures in Table I converted dichloroallyl lawsone to a common polar metabolite (4) in screening experiments. *P. notatum* (U1 1952) was selected for the preparative scale conversion of dichloroallyl lawsone to this metabolite for purposes of isolation and identification. Stage II fermentations of *P. notatum* were grown according to the usual fermentation procedure in two 1-liter Erlenmeyer flasks each containing 200 ml of culture. Dichloroallyl lawsone (100 mg/ml of N,N-dimethylformamide) was added to each Stage II culture flask, and the flask contents were incubated with shaking. TLC
P. notatum were grown by the usual procedure in 125-ml Erlenmeyer flasks. The cells were resuspended in 75 ml of potassium phosphate buffer (0.1 M, pH 7.0). The combined filtrate was adjusted to pH 4.2 with 6 N HCl and extracted with ethyl acetate. The combined ethyl acetate extract was dried over anhydrous Na$_2$SO$_4$ and concentrated to dryness.

The residue was dissolved in chloroform:methanol (9:1, v/v) and applied to the top of a silica gel column (2 g of silica gel buffered with 0.16 g of KH$_2$PO$_4$, 15 × 0.5 cm) which was eluted first with chloroform (7 ml) and then with methanol (13 ml) at a flow rate of 1 ml/min while eight 2.5-ml fractions were collected. Fractions containing the metabolite (fractions 4 and 5) were concentrated to dryness under nitrogen. The residue was further purified by preparative TLC (chloroform:methanol, 9:1, v/v) followed by acidification with methanol-washed Dowex 50W resin to yield the metabolite (3) as the free acid (1 mg). The purified metabolite was identical with authentic 3 based on its mass spectral properties, and these are summarized in Table II.

An experiment was conducted to determine the possible exchangeability of the oxygen atoms in the metabolite (3). Unlabeled 3 (15 mg) was dissolved in tetrahydrofuran (2 ml) and 0.5 N HCl (0.25 ml) in H$_2$O (95 atom %) was added. This mixture was held for 24 h at room temperature, and the solution was concentrated to dryness under vacuum and subjected to mass spectral analysis. By direct comparison to authentic 3, no $^{18}$O was found in any of the fragments observed in the mass spectrum of the treated metabolite.

**RESULTS AND DISCUSSION**

Earlier experiments had shown that lapachol (1) was metabolically converted into the ring fission product 3 by *P. notatum* and other microorganisms (Otten and Rosazza, 1978). As an extension of this work, dichloroallyl lawsone (2) was examined as a substrate for *P. notatum* and other organisms. Those capable of successfully metabolizing 2 to a major and common metabolite are listed in Table I. In these experiments, *P. notatum* (UI 16902) utilized all of the substrate (2) within 24 h, and this organism was selected for preparative scale conversion to the metabolite.

Following isolation and purification by column chromatography, the metabolite was identified as the ring fission product 4. It was soluble in saturated NaHCO$_3$, indicating the presence of a carboxylic acid functional group. A broad OH stretching absorption band centered at 3500 to 2400 cm$^{-1}$ and a carbonyl peak at 1720 cm$^{-1}$ in the infrared spectrum confirmed the presence of the carboxyl group. The infrared spectrum also contained a carboxyl absorption band at 1700 cm$^{-1}$, which suggested the presence of a conjugated ketone. The compound spectrum gave a molecular weight of 316, consistent with a molecular formula of C$_9$H$_{18}$O$_2$Cl$_2$. Peaks in the mass spectrum at m/e 271, 207, and 162 could be readily explained by the loss of COOH and/or CH$_2$=CH=C(OH)$_2$ fragments from the molecular ion. The spectral data suggested that the metabolite resulted from oxidative cleavage of the naphthoquinone ring of dichloroallyl lawsone to produce a molecule like 4. This compound was prepared from dichloroallyl lawsone by treatment with H$_2$O$_2$ under alkaline conditions (Fieser and Fieser, 1948). The synthetic material was fully characterized and gave infrared and mass spectra which were completely identical with those of the metabolite 4. In addition, the mixture melting point of the metabolite and the synthetic material was undepressed. This spectral and physical evidence confirms the structure of the *P. notatum* metabolite of dichloroallyl lawsone as the ketol derivative 4.

**Mechanism of Oxidative Ring Fission of Lapachol by *P. notatum***—The present work and earlier studies with lapachol would suggest that ring fission of these structurally similar naphthoquinones occurs by a common mechanism. Since the

**Table I**

**Dichloroallyl lawsone-metabolizing microorganisms**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cunninghamella bainieri</td>
<td>UI 3065</td>
<td>*</td>
</tr>
<tr>
<td>Cunninghamella blakesleeanae</td>
<td>ATCC 8688a</td>
<td>*</td>
</tr>
<tr>
<td>Cunninghamella ochinita</td>
<td>NRRL 3655</td>
<td>*</td>
</tr>
<tr>
<td>Heliocystis piriforme</td>
<td>NM 6945</td>
<td>*</td>
</tr>
<tr>
<td>Penicillium notatum</td>
<td>UI 1602</td>
<td>*</td>
</tr>
<tr>
<td>Stemphylium commune</td>
<td>Wice 4136</td>
<td>*</td>
</tr>
<tr>
<td>Streptomyces punkalis</td>
<td>NRRL 3529</td>
<td>*</td>
</tr>
<tr>
<td>Streptomyces',' risinosus</td>
<td>ATCC 23955</td>
<td>*</td>
</tr>
</tbody>
</table>

* UI, University of Iowa, College of Pharmacy Culture Collection; ATCC, American Type Culture Collection, Rockville, MD; NRRL, Northern Regional Research Laboratories, Peoria, IL; QM, Quartermaster Culture Collection, US Army Laboratories, Natick, MA; Wisc, University of Wisconsin, School of Pharmacy Culture Collection.

**FIG. 1**

Structures of lapachol (1) and dichloroallyl lawsone (2) and the ketol metabolites derived from them, 3 and 4, respectively.
cleavage of naphthoquinone rings is not a well known oxidative process in nature, an examination of the mechanism of ring cleavage was undertaken. Two plausible mechanisms of ketol (3) formation from lapachol (1) would involve monooxygenase or dioxygenase pathways as illustrated in Fig. 2. In both pathways, oxygen atoms introduced into the ketol metabolites 3 and 4 would derive from molecular oxygen. In the monooxygenase pathway, the initial epoxide 5 could undergo acid- or base-catalyzed opening of the epoxide ring to form 3 containing the epoxide oxygen atom in either the hydroxyl or carboxyl group. This pathway would represent the biochemical equivalent of the Hooker oxidation of lapachol (Otten and Rosazza, 1978; Fieser and Fieser, 1948). The dioxygenase pathway results in the incorporation of both atoms of molecular oxygen into a cyclic endoperoxy intermediate 8 which presumably would undergo hydride- or acid-catalyzed opening to provide 3 with molecular oxygen incorporated into both the carboxylic acid and hydroxyl groups of the ketol.

Some *P. notatum* strains and other fungi catalyze epoxidation reactions with quinoid substrates (Kieslich, 1976), and attempts were made to prepare the proposed epoxide intermediate (5) by chemical methods. All attempts to form this intermediate were unsuccessful with reactions providing intractable mixtures containing numerous products. Thus, it was impossible to directly test the intermediacy of 5 in the reaction sequence.

The mechanism of metabolite (3) formation was investigated by conducting the metabolic transformation reaction in an \(^{18}\text{O}_{2}\) atmosphere. The location of heavy oxygen atom(s) in the metabolite structure was made relatively simply by use of the well defined fragmentation pattern of 3 which was derived from high resolution mass spectral analysis (Fig. 3). Several useful and relatively intense peaks are evident in the mass spectrum of 3 including those at \(m/e\) 231 derived by loss of the carboxyl group from the prominent molecular ion which occurs at \(m/e\) 276 and the fragment at \(m/e\) 178 which derives from the loss of the dimethylallyl side chain, including the alcohol functional group. These two fragments provide a direct means for measuring the presence or absence of \(^{18}\text{O}\) in the carboxyl group or in the secondary alcohol group of 3. Additional useful information concerning the positioning of molecular oxygen label in the metabolite could be obtained from the intensity ratio of the molecular ion and its \((M+2)/M\) ratio. The availability of empirical formulae from the high resolution mass spectrum facilitated the assignments of structures to the fragment ions.

To obtain the metabolite, incubations were conducted using resting cells of *P. notatum* in an \(^{18}\text{O}_{2}\) atmosphere. Considerable effort was required to establish appropriate conditions to conduct the incubation reaction in a closed system. Resting
cells of P. notatum were employed since the isolation of metabolite from buffer versus complex culture medium would be facilitated. The fungal cells were relatively fragile in that overdrying by suction filtration resulted in loss of desired metabolic activity. Following incubation, the metabolite was isolated and purified by chromatography and compared with authentic 3 prepared synthetically and biosynthetically in our laboratories (Otten and Rosazza, 1978). Major peaks obtained in the high resolution mass spectrum of the isolated metabolite are presented in Table II, and these may be compared with important portions of the fragmentation pattern shown in Fig. 3.

The molecular ion of unlabeled ketol (3) at m/e 276 is accompanied by m/e 278 and 280 peaks. The very low intensity of the m/e 280 peak (1.36%) is very close to the theoretical value for natural abundance 16O based on the observed intensity of 60.76% for the peak at m/e 278. This clearly indicates that the metabolite contains only one atom of 18O. The ratio of intensities of m/e 278/276 peaks was 1.35, and this result demonstrates that 57% of the oxygen incorporated into the metabolite was derived from 16O2. Isolated metabolite (3) bearing unlabeled oxygen was most likely produced from residual molecular oxygen retained within filtered fungal cells or the incubation medium.

Loss of the carboxyl group from the molecular ion gave an m/e 233/231 peak intensity ratio of 1.37. This demonstrates that 18O label was not present in the COOH moiety of 3. The lack of heavy oxygen in the carboxyl group was confirmed by fragment ions existing at m/e 165/163 (peak intensity ratio of 1.36) and m/e 177/175 (peak intensity ratio of 1.38). When the fragment CH(OH)=CH—CH═C(CH3)2 is lost from the molecular ion, an m/e 178 peak is observed with no accompanying M+2 peak. This demonstrates that the 18O label is located in the side chain hydroxyl group. Ions at m/e 133 and 105 confirm this conclusion.

The results support the introduction of molecular oxygen into lapachol via the monoxygenase pathway as shown in Fig. 2. It would appear that the formation of 3 proceeds by the initial formation of an epoxide (5) which undergoes either acid- or base-catalyzed opening. In this case, the epoxide oxygen atom is finally located as the secondary alcohol of 3. We investigated the possibility that our isolation procedures could have resulted in the exchange of labeled carboxyl group oxygen atoms with those of water during extraction or workup. Others have shown that carboxyl oxygen atoms are exchangeable under acidic conditions (Llewellyn and O’Connor, 1964). Thus, a nonlabeled sample of 3 was incubated in H218O under strongly acidic conditions for 24 h. Mass spectral analysis of 3 isolated from this mixture indicated that no oxygen 18 was incorporated into the ketol and that it was unlikely that oxygen exchange occurred between the 18O labeled metabolite and water under the experimental conditions employed. Further elaboration of the mechanism by which P. notatum achieves the oxidative transformation of lapachol will require the establishment of stable cell-free enzyme preparations.

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REFERENCES


Oxidative ring fission of the naphthoquinones lapachol and dichloroallyl lawsone by *Penicillium notatum*.

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