Patulin Biosynthesis: Epoxidation of Toluquinol and Gentisyl Alcohol by Particulate Preparations from *Penicillium patulum*†

Jeffrey W. Priest‡ and Robley J. Light*

Department of Chemistry and Institute of Molecular Biophysics, Florida State University, Tallahassee, Florida 32306

Received April 18, 1989; Revised Manuscript Received July 17, 1989

ABSTRACT: A crude extract that catalyzes the epoxidation of toluquinol and gentisyl alcohol was isolated from cultures of *Penicillium patulum*. About 60% of the activity sedimented from crude extract upon centrifugation at 105000*g* for 2 h, and at 30000*g* for 30 min after precipitation with 30% ammonium sulfate and resuspension in buffer. The quinone epoxide phyllostine, a product of gentisyl alcohol epoxidation, has previously been shown to be an intermediate in the biosynthesis of patulin [Sekiquchi, J., & Gaucher, G. M. (1978) *Biochemistry 17*, 1785–1791] and was shown to be further converted to neopatulin by the extract. The epoxide product of toluquinol, desoxyphyllostine (2-methyl-5,6-epoxy-1,4-benzoquinone), has not been reported previously from fungal cultures. Its structure was confirmed by GC–mass spectrometry and proton and ^{13}C NMR. Its CD spectrum showed the same shape and signs as that of phyllostine, indicating that it too is an enzymatic product with a similar absolute configuration. Whereas chemical epoxidation of toluquinone and gentisyl quinone occurs with hydrogen peroxide, the enzymatic epoxidation utilized oxygen and the hydroquinone. The epoxidation was inhibited by 1,10-phenanthroline, EDTA, and *p*-(chloromercuri)benzenesulfonic acid and by degassing with nitrogen, but no inhibition was observed with KCN, catalase, or CO. The apparent K_m's were similar for the two substrates (0.17 mM for toluquinol, 0.24 mM for gentisyl alcohol), with both substrates showing inhibition at 1.0 mM. The rate of desoxyphyllostine formation was more than 10 times that of phyllostine formation at equivalent substrate concentrations. Gentisaldehyde was not a substrate for the enzyme. The epoxidase was induced in late fermentor cultures of *P. patulum* with the same kinetics as *m*-hydroxybenzyl alcohol dehydrogenase, another enzyme associated with the induction of patulin biosynthesis.

Patulin is a toxin produced by a number of fungal species, notably *Penicillium* and *Aspergillus* (Scott, 1974). It is made from the aromatic polyketide precursor 6-methylsalicylic acid (Bu'Lock & Ryan, 1958; Tanenbaum & Basset, 1959). The detection of related aromatic compounds *m*-cresol, *m*-hydroxybenzoic acid, and toluquinone in fungal cultures led to the proposal of a metabolic grid of reactions involving two pathways to the intermediate gentisaldehyde, which was presumed to be the final aromatic intermediate prior to ring opening (Bu'Lock et al., 1965, 1969). The isolation of several patulin-minus mutants of *Penicillium urticae* (Sekiguchi & Gaucher, 1977) led to the identification of four postaromatic intermediates: phyllostine (Sekiguchi & Gaucher, 1978), isoepoxydon (Sekiguchi & Gaucher, 1979), neopatulin (Sekiguchi et al., 1979), and ascladiol (Sekiguchi et al., 1983). All were shown to be converted to patulin in vivo or in vitro. Several versions of a revised grid pathway have been suggested in which either the quinone epoxide phyllostine or the quinol epoxide isoepoxydon is the first nonaromatic intermediate leading to ring opening (Zamir, 1980; Gaucher et al., 1981). The nature of the epoxidation reaction has remained a matter of speculation given conflicting reports on the relative position of gentisaldehyde and gentisyl alcohol in the pathway (Forrester & Gaucher, 1972; Scott & Beadling, 1974; Iijima et al., 1986).

We report here the isolation of a cell-free extract from *Penicillium patulum* which catalyzes epoxidation of gentisyl alcohol to phyllostine and toluquinol to desoxyphyllostine, a quinone epoxide not previously reported in fungal cultures. Some of the properties of the crude epoxidase, which is associated with particulate fractions of the extract, are described. We propose that the metabolic pathway from 6-methylsalicylic acid to patulin is linear rather than a grid of alternative intermediates and that *m*-hydroxybenzaldehyde, gentisaldehyde, gentisyl quinone, and isoepoxydon are, like toluquinol, toluquinone, and desoxyphyllostine, branch products of 6-methylsalicylic acid degradation rather than obligatory intermediates in patulin biosynthesis. A preliminary account of this work has appeared (Light & Priest, 1988).

Materials and Methods

Organism and Culture Conditions. A single colony isolate of *P. patulum* (or *P. urticae*) NRRL 2159A, designated RL-17 and showing "early" production of 6-methylsalicylic acid upon transfer from a rich to poor medium (Light, 1967), was deposited with the USDA culture collection laboratory in Peoria, IL, as NRRL A14806. With continuous subculture over a period of years, this strain reverted to a mixture of the "early" and "late" phenotype in the step-down test, and single colonies with the "early" characteristic were reisolated three times over a 6-year period.

Conidia for fermentor inoculation were produced in 16-oz medicine bottles on a bed of 60 mL of malt extract agar (0.1% peptone, 2% agar, 2% malt extract, and 2% glucose). Bottles were inoculated with 1.5 mL of a suspension of at least 2.0×10^8 spores/mL in 0.05% Tween 20 (Atlas Chemicals Inc.). After growth at room temperature for 13–17 days, bottles were stored at 3 °C for up to 3 months prior to use.

Fermentor cultures were grown in 10 L of a yeast extract–glucose–manganese medium contained in a 14.0-L fermentor jar (New Brunswick Scientific). Media components were autoclaved at 121 °C separately: Difco yeast extract (50

† This work was supported in part in its early stages by Grant PCM-8213610 from the National Science Foundation.

* Author to whom correspondence should be addressed.

‡ Present address: Department of Biochemistry, University of Alabama at Birmingham, Birmingham, AL 35294.

0006-2960/89/0428-9192$01.50/0 © 1989 American Chemical Society

g) in 6.0 L of double-distilled water for 45 min in the fermentor jar; glucose (600 g) in 4 L of distilled water for 15 min; and $MnCl_2 \cdot 4H_2O$ (297 mg) in 50 mL of water for 15 min. The walls of the fermentor jar had been silanized with Prosil 28 (Specialty Chemicals) to minimize adhesion of mycelia. A conidial suspension, prepared in 100 mL of 0.05% Tween 20 from enough malt extract agar bottles (usually two) to yield at least 2.0×10^8 spores/mL, was filtered through sterile cheesecloth and used to inoculate the fermentor to a final concentration of 2.0×10^6 spores/mL. The fermentor was aerated (2 L/min and 500 rpm) at 28 °C, with 1.0 mL of Dow Corning Antifoam Emulsion C (Sigma) added at the beginning and 0.5-mL increments added as necessary after 20 h.

Mycelial samples taken at varous stages of the culture were collected by filtration, washed with 0.5 volume of 20 mM phosphate buffer, pH 7.5, suspended in the same buffer at 2.0 mL/g wet weight, lyophilized, and stored at −70 °C.

Enzyme Preparations. Lyophilized mycelium was extracted with cold acetone to facilitate cell breakage and to remove phenolic metabolites that might inhibit enzyme activity (Niehaus & Dilts, 1982). Mycelium, frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle, was stirred into 10 mL of −20 °C acetone per gram of powder, collected by suction filtration, washed with 10 mL of −20 °C ether per gram, and dried thoroughly by suction filtration. The dried powder was then ground in a cold mortar with 1 g of sand and 10 mL of cold extraction buffer (100 mM phosphate buffer, pH 7.5, 2.5 mM dithiothreitol, and 15% glycerol) per gram of powder. The resulting paste was centrifuged at 4 °C for 10 min at 10000*g* and the resulting supernatant centrifuged an additional 30 min at 3000*g*. This process usually yielded approximately 7 mL of crude extract/g dry weight at a protein concentration of 18–48 mg/mL. For ultracentrifugation, an extract made in a 5% glycerol extraction buffer was centrifuged at 5000*g* for 5 min to remove cellular debris and at 30000*g* for 30 min, then diluted 1 to 4.4 with 10% glycerol extraction buffer, and centrifuged at 105000*g* for 2 h.

Ammonium sulfate fractionation was carried out by adding solid enzyme-grade ammonium sulfate, stirring slowly for 30 min, and centrifuging at 20000*g* for 20 min. Pellets were usually stored at −20 °C as a suspension in 100 mM potassium phosphate buffer, pH 7.5, containing 15% v/v glycerol and saturated with ammonium sulfate. For assays and further purification attempts, aliquots of this suspension were centrifuged at 20000*g* for 20 min, and the pellets were resuspended in an appropriate buffer.

Enzyme Assays. Products from enzyme assays were analyzed by a Beckman HPLC[1] system consisting of two Model 114M solvent pumps, a Model 421A controller, a Model 210A sample injection valve, and a Model 160 UV detector with 280- and 254-nm filters. Peak areas were determined by a Shimadzu Model C-R3A integrator. Assay incubations were generally carried out in 5.0 mL of 100 mM potassium phosphate buffer, pH 7.5, for 60 min at 28 °C. The reactions were started by the addition of enzyme and stopped by addition of concentrated HCl to a pH of 1–2. The mixture was immediately extracted twice with 2 volumes of anhydrous ether, the combined ether extracts were dried over 10 g of anhydrous Na_2SO_4, the ether was removed in vacuo, and the residue was resolubilized in 0.50 mL of 14 mM potassium phosphate, pH 6.0, containing 20% (v/v) methanol. (Exhaustive drying should be avoided because desoxyphyllostine is slightly volatile.) Particulate material was removed by a 30-s centrifugation in an Eppendorf centrifuge, and an aliquot (usually 20 μL) was loaded onto a 0.46 × 7.5 cm, 3-mm C-18 column (Altex ODS), previously equilibrated in a methanol–water solvent mixture. The column was eluted at a flow rate of 0.35 mL/min with an elution solvent which depended upon the products being analyzed. Toluquinol epoxidase products were eluted with a 35%–100% methanol–water (v/v) gradient in 10 min; gentisyl alcohol epoxidase products were eluted in 10% methanol–water (v/v, isocratic); and *m*-hydroxybenzyl alcohol dehydrogenase products were eluted with 35% methanol–water (v/v, isocratic). Elution profiles were recorded at either 280 or 254 nm with a full-scale sensitivity of 0.04 absorbance unit.

Reaction product standards at known concentrations were analyzed under the same conditions to calibrate peak areas with quantity of product. In most cases the product quantities detected were corrected for extraction efficiency, also determined with standards as follows: toluquinol, 76 (±10%); toluquinone, 43 (±1%); desoxyphyllostine, 60 (±5%); gentisyl alcohol, 32 (±3%); gentisyl quinone, 66 (±5%); gentisaldehyde, 77 (±12%); phyllostine, 68 (±4%); *m*-hydroxybenzyl alcohol, 68 (±5%); and *m*-hydroxybenzaldehyde, 76 (±8%).

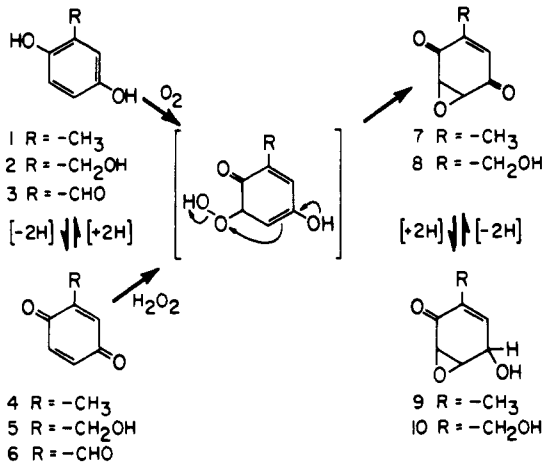
Qualitative assays were carried out by thin-layer chromatography. Residues from the ether extracts were dissolved in ethyl acetate, and half of the total product was spotted on 250-μm silica gel 60 G (E. Merck) thin-layer plates. Plates were developed with ethyl acetate–petroleum ether (bp 30–60 °C)–acetic acid, 90:60:2 v/v/v (system B), for assay of the gentisyl alcohol products, and 30:120:2 v/v/v (system A), for assay of the toluquinol products. Plates were developed by spraying with a 5 mg/mL solution of MBTH (Aldrich) and heating at 110 °C for 5–10 min (Sekiguchi & Gaucher, 1977).

Isolation and Identification of Phyllostine and Desoxyphyllostine. Preparative-scale incubations of gentisyl alcohol (30 mg) and toluquinol (30 mg) were carried out in 1.0 L of 100 mM potassium phosphate buffer, pH 7.5, and 6 mL of crude enzyme extract for 2 h at 28 °C. Reactions were acidified to pH 2–3 with concentrated HCl and extracted twice with 1-L portions of ether. The combined ether layers were equilibrated with saturated NaCl and dried over anhydrous Na_2SO_4. The ether was removed in vacuo, the dried residue was dissolved in 3–4 mL of 20% methanol (v/v)–14 mM potassium phosphate buffer, pH 6.0, and the products were separated by preparative HPLC of 0.5-mL portions on a 1.0 × 25 cm, 5-μm C-18 column (Altex ODS). The column was eluted at a flow rate of 2.0 mL/min with 17.5% methanol–water for gentisyl alcohol epoxidase and with a linear gradient of 35%–100% methanol in 30 min for toluquinol epoxidase. Phyllostine and neopatulin produced from gentisyl alcohol (retention times 11.8 and 14.3 min, respectively) were collected and analyzed for purity by analytical HPLC and by thin-layer chromatography after extraction (see enzyme assay conditions above). UV and CD spectra were determined on the collected material. The desoxyphyllostine product from toluquinone (retention time 14.0 min) was extracted with ether, the ether was equilibrated with saturated aqueous NaCl, dried over anhydrous Na_2SO_4, and evaporated, and the oily residue was extracted once in 75 μL of boiling benzene, which was decanted. The residue was then dissolved in 100 μL of deuterated chloroform (Aldrich), and aliquots were used for thin-layer chromatography, GC–mass spectrometry, and ^{13}C and proton NMR. An aliquot was also dried and dissolved in water to record the UV and CD spectra and to check purity by analytical HPLC (see enzyme assay conditions above).

UV spectra were recorded on a Varian Cary Model 219 dual-beam spectrophotometer. CD spectra were recorded on

[1] Abbreviations: HPLC, high-performance liquid chromatography; MBTH, 5-methyl-2-benzothiazolinone hydrazone hydrochloride hydrate.

Scheme I



a Jasco J-500C spectrophotometer. Proton and ^{13}C NMR spectra were obtained in $CDCl_3$ on a Brucker H-270 spectrometer. Capillary GC–mass spectral analysis was carried out on a Finnegan Model 4510 system. The product was eluted from a 25-m DB-5 capillary column with a temperature program of 50–140 °C at 15 °C/min and 140–240 °C at 5 °C/min. Desoxyphyllostine had a retention time of 3.5 min. A small peak (less than 5% of the desoxyphyllostine) with a retention time of 2.8 min had a molecular ion at 138 *m/e* and a mass fragmentation pattern similar to that of desoxyphyllostine. This minor product might be the chemically formed 2,3-epoxide isomer of desoxyphyllostine.

Preparation of Substrates. Reagent-grade toluquinol (Matheson) was recrystallized twice from chloroform, mp 124–126 °C (lit. 126–127 °C; Weast & Astle, 1982). HPLC analysis showed trace toluquinone contamination. Toluquinone was synthesized by oxidation of toluquinol with Ag_2O by the method of Willstatter and Pfannenstiel (1904). Gentisaldehyde was obtained from Aldrich and also was synthesized from *p*-hydroquinone (Matheson) by the Riemer–Tieman reaction (Hodgson & Jenkinson, 1929). Gentisyl alcohol was prepared by $NaBH_4$ reduction of gentisaldehyde, and gentisyl quinone by $NaIO_4$ oxidation of gentisyl alcohol (Bruce & Knowles, 1966). An attempt to synthesize the quinone from gentisaldehyde by oxidation of gentisaldehyde with dichlorodicyanobenzoquinone produced a sublimable product which decomposed upon exposure to air or water. A reference sample of phyllostine was kindly provided by Dr. G. M. Gaucher, University of Calgary, Canada (Sekiguchi & Gaucher, 1978). A 2 mg/mL stock solution of each compound in water was stored at −20 °C and used as substrate or chromatographic reference.

Results

Thin-layer chromatography of products formed by incubation of toluquinol (**1**; see Scheme I for structure) with a crude extract from *P. patulum* revealed four substances giving a color reaction after spraying with MBTH reagent and heating. Two of these substances cochromatographed with toluquinol (**1**) and toluquinone (**4**) (R_f = 0.26 and 0.50, respectively, solvent system A) and showed the orange color characteristic of these compounds. A third (R_f = 0.31) gave a yellow color and a fourth (R_f = 0.45) a deep purple color. The color of this latter compound was identical with that shown by the quinone epoxide phyllostine (**8**) (Sekiguchi & Gaucher, 1978), suggesting it might be the corresponding epoxide desoxyphyllostine (2-methyl-5,6-epoxy-1,4-benzoquinone, **7**).

Although Scott et al. (1973) isolated desoxyepoxydon (**9**) from cultures force-fed toluquinol in vivo, desoxyphyllostine has never been reported from any fungal cultures. We undertook a preparative-scale isolation of the material for structure determination. The preparative HPLC profile of the reaction products from a large-scale incubation of crude extract with toluquinol showed three major peaks. Material collected from the first (retention time 9.7 min) and the third (retention time 14.8 min) were shown to contain toluquinol (**1**) and toluquinone (**4**), respectively, while that from the second (retention time 14.0 min) was identified as desoxyphyllostine (**7**) by several spectral criteria. Isolated material was shown to be greater than 98% pure by analytical HPLC and TLC and to correspond to the R_f = 0.45 spot giving a deep purple color with MBTH reagent. Its UV absorption spectrum in water showed maxima at 212, 261, and 340 nm (relative ϵ = 1.0, 0.57, and 0.026), whereas phyllostine (**8**) in methanol has maxima at 214 nm (ϵ = 9270) and 260 nm (ϵ = 4190) (Sekiguchi & Gaucher, 1978). Its CD spectrum [*c* 0.16 mM, H_2O: $\Delta\epsilon_{354} = -1.03$; $\Delta\epsilon_{294} = 0$; $\Delta\epsilon_{266} = +2.63$; $\Delta\epsilon_{228} = +2.33$; $\Delta\epsilon_{219} = 0$; $\Delta\epsilon_{211} = -3.24$ rad/(M·cm)] shows the same shape and signs as that of phyllostine (Sekiguchi & Gaucher, 1978) except that phyllostine has a single positive peak at 246 nm, with a shoulder at 260 nm. Therefore, the product is optically active with the same absolute configuration as phyllostine. Its mass spectrum [138 (M^+, 80), 137 (18), 123 ($M^+ - CH_3$, 70), 110 ($M^+ - CO$, 54), 95 ($M^+ - CO - CH_3$, 7), 82 ($M^+ - 2CO$, 46), 81 ($M^+ - 2CO - H$, 30), 69 (100), 68 (47), 54 (78), 53 (42)] is consistent with the desoxyphyllostine structure (**7**), molecular formula $C_7H_6O_3$. The ^{13}C NMR spectrum had peaks at 133.3 ppm (vinyl C), 54.1 and 53.8 ppm (epoxide carbons), and 16.3 ppm (methyl carbon). The carbonyl and quaternary vinyl carbon signals were too weak to discern conclusively above background. The ^1H NMR spectrum had peaks at 6.4 ppm (multiplet, vinyl proton), 3.8 ppm (multiplet, epoxide protons), and 2.0 ppm (doublet, J = 1.64 Hz, methyl protons) with integrated intensities in a ratio of 1:2:3, respectively. [Rashid and Read (1967) reported the ^1H NMR spectrum of chemically synthesized 2-methyl-5,6-epoxy-1,4-benzoquinone as 6.48 ppm (m, 1), 3.84 ppm (s, 2), and 2.02 ppm (d, 3).]

The optical activity of the product confirmed that it was being formed enzymatically, and its clean separation from toluquinol and toluquinone on HPLC provided the basis for a quantitative enzymatic assay, by measuring the peak area of the product. For example, a 5.0-mL assay containing 90 μL of a 0–30% ammonium sulfate enzyme fraction and 250 μM toluquinol gave a linear rate of production of desoxyphyllostine (4.8 nmol/min) for at least 60 min, and the product formed in a 60-min assay was proportional to extract concentration at least to 120 μL of extract. Alternatively, thin-layer chromatography of reaction products and development of a purple spot after MBTH spray provided a more rapid but only qualitative assay for the presence of epoxidation activity.

The epoxidation of quinones by hydrogen peroxide is a well-known chemical reaction, especially under alkaline conditions (Alder et al., 1960), and presumably occurs by Michael addition of peroxide anion, forming a peroxide intermediate as shown in Scheme I. Because toluquinone (**4**) is also formed in quantity in the incubation (products from the preparative incubation, for example, contained 12% toluquinol, 15% desoxyphyllostine, and 73% toluquinone), it seemed reasonable that the quinone might be an intermediate in the enzymatic reaction as well. Hydrogen peroxide could have been formed in some redox process in the crude extract, including the one

Table I: Effect of Hydrogen Peroxide and Catalase on Epoxide Formation[a]

substrate	addition	epoxide formed (nmol) active enzyme	epoxide formed (nmol) heated enzyme
toluquinol	none	168	5
toluquinone	none	29	–[b]
toluquinol	H_2O_2 (400 μM)	–[b]	30
toluquinone	H_2O_2 (400 μM)	57	59
toluquinol	catalase (5 units)	190	–[b]
toluquinol	catalase (50 units)	211	–[b]

[a] Assays (5.0 mL) contained 0.65 mg of protein from a 0%–30% ammonium sulfate precipitate of mycelial extract from a 32-h culture, 100 mM potassium phosphate buffer, pH 7.5, 250 μM toluquinol or toluquinone, and the indicated quantity of hydrogen peroxide or catalase. Bovine liver catalase, 20000 units/mg, was obtained from Sigma. Control incubations contained protein that had been inactivated by heating at 100 °C for 10 min. After incubation at 28 °C for 60 min, products were extracted and analyzed by HPLC as described under Materials and Methods. [b] Not determined.

involving oxidation of toluquinol to toluquinone. Testing this hypothesis with a crude enzyme preparation proved to be a bit difficult, because incubation of the enzyme with toluquinone also produced some toluquinol; thus one substrate could not be tested in the complete absence of the other. Nevertheless, the experiments reported in Table I support the conclusion that the enzymatic reaction utilizes hydroquinone plus oxygen and not quinone plus hydrogen peroxide. With toluquinone as added substrate, only 17% of the amount of product found in the toluquinol control was formed, product that could have come from the toluquinol generated in the incubation. Addition of hydrogen peroxide stimulated product formation from toluquinone, but the same quantity was produced when the enzyme extract had been heat inactivated, indicating that the chemical epoxidation can occur under the temperature and pH conditions of the assay. (In the GC–mass spectral analysis of enzymatically formed desoxyphyllostine, a minor peak was observed with the same molecular ion and fragmentation pattern as desoxyphyllostine, possibly the chemically formed 2,3-epoxy isomer.) Less product was formed chemically from toluquinol than from toluquinone when hydrogen peroxide was added, presumably from quinone formed by nonenzymatic oxidation occurring in the incubation. Finally, the addition of catalase did not inhibit the reaction with toluquinol, ruling out hydrogen peroxide as a free intermediate in the enzymatic reaction. In fact, a slight stimulation by catalase was noted.

The reaction did not involve loosely bound cofactors. Activity was retained after gel filtration of crude extract and after ammonium sulfate precipitation (see below). Addition of 250 mM ATP, $NADP^+$, NADPH, NAD^+, NADH, FAD, or FMN neither stimulated nor inhibited the epoxidase activity. Activity was inhibited by the sulfhydryl reagent *p*-(chloromercuri)benzenesulfonic acid and the metal chelators EDTA and 1,10-phenanthroline, but not by cyanide nor by carbon monoxide (Table II). Addition of phenylmethanesulfonyl fluoride had no appreciable effect beyond that of the 2-propanol in which it was added, nor did added ferrous sulfate have an effect. Removing oxygen by bubbling with nitrogen inhibited the reaction completely, while bubbling with air had no effect. Therefore, the reaction appears to involve oxygen, a metal ion, and possibly a sulfhydryl group, but the crude enzyme is not deficient in iron. Cytochrome P_{450} and cytochrome oxidase, common sites of oxygen reduction, do not appear to be involved.

Activity could be precipitated from crude extract with ammonium sulfate, though the activity was spread over several

Table II: Effect of Potential Inhibitors on Toluquinol Epoxidase Activity[a]

addition	concn (mM)	rel act.[b] (%)
none		100
2-propanol	23	84
PMSF/2-propanol	1.0/23	67
PCMBS	0.7	1.0
1,10-phenanthroline	0.2	49
1,10-phenanthroline	1.0	15
EDTA	1.0	35
EDTA	5.0	35
FMN	0.02	91
$FeSO_4$	1.0	96
KCN	1.0	112
gases		
CO, 5-min preincubation		97
air, continuous addition		116
N_2, continuous addition		1.8

[a] Assays (5.0 mL) contained 0.65 mg of protein from a 0%–30% ammonium sulfate precipitate of mycelial extract from a 32-h culture, 100 mM potassium phosphate buffer, pH 7.5, 250 μM toluquinol, and the indicated quantity of inhibitor, which was added 5 min prior to the addition of substrate. For the gases, CO was bubbled into the assay tubes during the 5-min preincubation, while bubbling of air and N_2 began as a 5-min preincubation and continued during the 60-min assay. Toluquinol substrate was added at a concentration of 250 μM, assays were incubated for 60 min at 28 °C, and products were extracted and analyzed by HPLC as described under Materials and Methods. [b] The control (100%) activity corresponded to 168 nmol of desoxyphyllostine formed in the 60-min incubation.

Table III: Ammonium Sulfate Fractionation of Epoxidase[a]

ammonium sulfate fraction (% saturation)	epoxidase act.[b] (units/30 mL)	ammonium sulfate fraction (% saturation)	epoxidase act.[b] (units/30 mL)
0–10 pellet	0.47	30–40 pellet	0.77
10–20 pellet	0.85	40 supernatant	0.52
20–30 pellet	3.5		

[a] Crude extract (30 mL, containing 5.0 units of toluquinol epoxidase activity) derived from 4 g of lyophilized mycelium from a 32-h culture was brought to successively higher ammonium sulfate concentrations by the addition of solid ammonium sulfate, as described under Materials and Methods. Pellets were suspended in an equal volume of buffer. A 100-μL aliquot of each fraction was incubated in 5.0 mL of potassium phosphate buffer, pH 7.5, with 200 μM toluquinol for 60 min at 28 °C. Products were extracted and analyzed by HPLC as described under Materials and Methods. [b] Activity in each aliquot was corrected to a total volume of 30 mL. One unit of activity is 1 μmol of desoxyphyllostine formed per minute.

ammonium sulfate fractions (Table III), and it showed altered solubility properties after exposure to ammonium sulfate. Differential centrifugation of crude extract pelleted 2% of the activity after 30000*g* for 30 min and 60% of the activity after 105000*g* for 2 h, while 40% of the activity was recovered from the high-speed supernatant. After precipitation with ammonium sulfate, 60% of the recovered activity could be pelleted upon centrifugation at 30000*g* for 30 min with 40% remaining in the supernatant. Sequential extraction of the ammonium sulfate pellet with buffer containing decreasing quantities of ammonium sulfate (33%–13%) yielded epoxidase activity in every fraction. A sucrose gradient analysis of the 20%–30% ammonium sulfate fraction is shown in Figure 1. The profile shows approximately 60% of the recovered activity in the heavier fractions, corresponding to the quantity of activity pelleted in the earlier centrifugations. The supernatant from a 20-min, 20000*g* centrifugation of the 20%–30% ammonium sulfate fraction, probably corresponding to the lighter material in the sucrose gradient, was desalted on a G-25 column and applied to a 0.5 × 5.0 cm Mono-Q ion-exchange column. This column was eluted with a 30-mL linear gradient of 0–0.5 M NaCl, and all of the eluate fractions from 3 to 30 mL con-

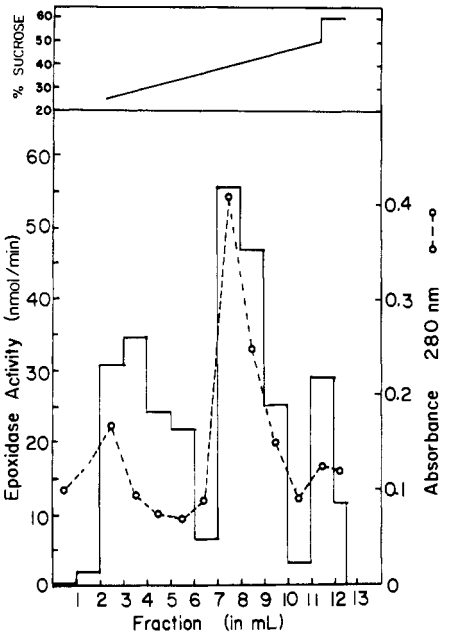


FIGURE 1: Sucrose density gradient centrifugation of toluquinol epoxidase from a 20%–30% ammonium sulfate fraction. An aliquot of a 20%–30% ammonium sulfate precipitate of mycelial extract from a 32-h culture that had been stored under saturated ammonium sulfate was washed with one-third volume of 50 mM potassium phosphate buffer, pH 7.5, and centrifuged for 20 min at 20000*g*, to remove excess ammonium sulfate. The pellet, containing all of the epoxidase activity, was then resuspended in one-third volume of the same buffer and layered in 2.3-mL aliquots on top of several 9-mL linear sucrose gradients (25%–50% sucrose in 50 mM phosphate, pH 7.5) with a 1 mL of 60% sucrose cushion. After centrifugation for 1 h at 200000*g*, tube contents were collected from the top in 1-mL fractions. A 40-μL aliquot of each fraction was assayed for toluquinol epoxidase activity as described under Materials and Methods, and another aliquot was diluted 1:100 for 280-nm absorbance determinations. (Activity values were not corrected for extraction efficiency of desoxyphyllostine, which is 60%.) Activity in fraction 11 came from a portion of opaque material in fraction 8 which floated on the denser underlying sucrose as fractions were collected.

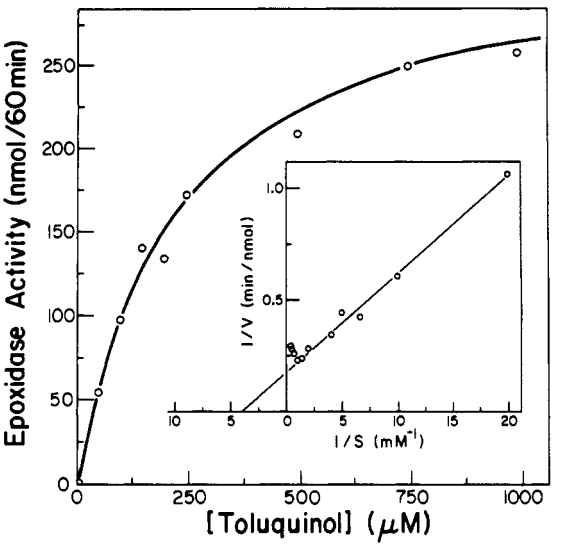


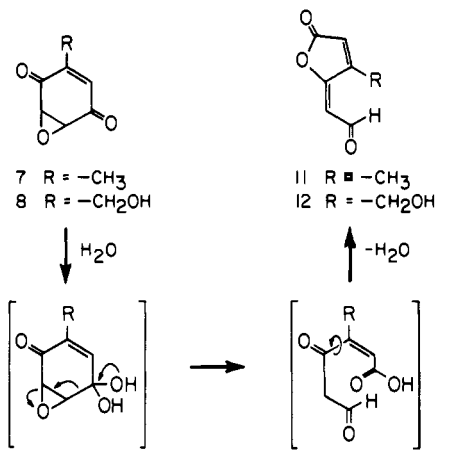
FIGURE 2: Effect of toluquinol concentration on epoxidase activity. Assays (5.0 mL) contained 0.65 mg of protein from a 0%–30% ammonium sulfate fraction and the indicated concentration of toluquinol in 100 mM potassium phosphate buffer, pH 7.5. After incubation for 60 min at 28 °C the product was extracted and analyzed by HPLC as described under Materials and Methods. The line on the reciprocal plot is a least-squares fit (correlation = 0.995) to the activity values for substrate concentrations at 1.0 mM and lower. Data for substrate concentrations above 1.0 mM are plotted on the reciprocal plot but not on the direct plot.

tained epoxidase activity (data not shown). These results indicate that even the nonsedimentable material has some heterogeneous character and may be associated with lipid or lighter membrane fractions. Purification will therefore probably require detergent solubilization.

The effect of initial toluquinol concentration on epoxide formation is shown in Figure 2. Substrate inhibition occurs at concentrations above 1.0 mM. An apparent K_m of 0.24 mM estimated from the points below 1.0 mM is probably a bit high, since substrate concentration is depleted during the reaction by quinone formation. The fraction of toluquinol converted to toluquinone during the assay, extraction, and analysis varied from 24% at concentrations of 1.0 mM and higher to 50% at 0.1 mM and 70% at 0.05 mM.

Toluquinol is a convenient and inexpensive substrate to use for isolation and characterization of the epoxidase, but its formation and metabolism are side reactions to the patulin biosynthetic pathway (Scott et al., 1973). More relevant to the pathway is the formation of phyllostine (**8**) (Sekiguchi & Gaucher, 1978) and the question of whether the substrate for that reaction is gentisyl alcohol (**2**) or gentisaldehyde (**3**). Incubation of crude extract with gentisyl alcohol and preparative HPLC of the reaction products showed five major peaks. Unreacted gentisyl alcohol (retention time 10.2 min) and its two oxidation products, gentisyl quinone (retention time 16.2 min) and gentisaldehyde (retention time 25.0 min) were identified by spectral analysis and HPLC and thin-layer

Scheme II



chromatographic comparison with standards. Phyllostine (retention time 11.4 min) had the same HPLC retention time, R_f on TLC, and color with MBTH spray reagent as a reference sample. Its UV spectrum in water had absorption maxima at 213, 258, and 342 nm (ϵ ratio of 1:0.45:0.01), and its CD spectrum [*c* 0.10 mM, 17.5% methanol: $\Delta\epsilon_{215} = -4.2$; $\Delta\epsilon_{229} = 0$, $\Delta\epsilon_{240} = +4.0$; $\Delta\epsilon_{294} = 0$; $\Delta\epsilon_{360} = -0.9$ rad/(M·cm)] was similar to though not identical with that reported by Sekiguchi and Gaucher (1978) for a solution in 100% methanol. The product from the fifth peak (retention time 14.3 min) was not optically active and had a UV absorption maximum at 268 nm in 17.5% methanol. Its R_f on thin-layer chromatography (0.38) and yellow color with MBTH spray were similar to those of patulin, but its retention time on an analytical HPLC column (8.1 min) was less than that of patulin (8.6 min). These properties are consistent with the compound neopatulin (hemiacetal form of **12**) (Sekiguchi et al., 1979, 1980, 1983), the lactone product from phyllostine ring opening. Therefore, the crude extract and ammonium sulfate fractions employed in this study also contained neopatulin synthase activity

(Scheme II). The level of this activity, together with overlap of the gentisyl alcohol and phyllostine peaks, made gentisyl alcohol a less satisfactory substrate for preliminary study of the epoxidase enzyme.

A comparison of gentisyl alcohol with gentisyl quinone and gentisaldehyde as substrates was carried out by using a total ammonium sulfate precipitate of crude extract and thin-layer chromatography as a qualitative assay. Plates were developed with MBTH spray reagent. The product from gentisyl alcohol contained phyllostine (purple, $R_f = 0.59$), neopatulin (yellow, $R_f = 0.36$), and gentisaldehyde (yellow, $R_f = 0.63$) in addition to gentisyl alcohol (red, $R_f = 0.38$) and gentisyl quinone (red, $R_f = 0.58$). These are the same products detectable by preparative HPLC. Incubation with gentisyl quinone produced only a mixture of gentisyl alcohol and gentisyl quinone, with neither phyllostine nor neopatulin observable. Incubation of heat-inactivated enzyme with gentisyl quinone and H_2O_2 produced phyllostine, showing the chemical addition of peroxide to the quinone could also occur with gentisyl quinone. Incubation with gentisaldehyde produced no observable products on the thin-layer plates. By use of a preparation devoid of gentisyl alcohol dehydrogenase activity, the 0%–30% ammonium sulfate precipitate employed in Table I, gentisaldehyde was incubated with active and heat-inactivated enzyme and the products were analyzed by HPLC. There were no detectable differences in the HPLC profiles from the two incubations.

We were unsuccessful in preparing the quinone form of gentisaldehyde for testing in either the enzymatic or chemical reactions. Oxidation of gentisaldehyde with dichlorodicyanobenzoquinone produced a sublimable product which decomposed upon exposure to air or water, so presumably the quinone form would not exist as a stable intermediate in the pathway.

The epoxidation of gentisyl alcohol and subsequent formation of neopatulin was completely inhibited by 1 mM *p*-(chloromercuri)benzenesulfonic acid and partially inhibited by 1 mM 1,10-phenanthroline (about 90%) and by 5 mM EDTA (about 50%). Accurate quantitation of product was difficult because of overlap of the phyllostine peak (retention time 6.2 min) with gentisyl alcohol (retention time 5.8 min) on the analytical column. The extent of inhibition, nevertheless, was consistent with that found for epoxidation of toluquinol reported in Table II.

The extent of conversion of phyllostine to neopatulin varied with different enzyme preparations. A 20%–30% ammonium sulfate fraction accumulated some phyllostine, while a 0%–50% ammonium sulfate fraction produced primarily neopatulin. Mixing the extracts yielded primarily neopatulin, indicating that the neopatulin synthase activity of the second extract is active with the phyllostine formed by the first. Neopatulin (retention time 8.4 min) is easier to measure quantitatively than phyllostine on the analytical column because the latter overlaps with gentisyl alcohol. Therefore, in order to study the effect of gentisyl alcohol substrate concentration on the epoxidation reaction, we utilized a preparation that gave complete conversion of phyllostine to neopatulin and used the quantity of neopatulin formed as a measure of epoxidase activity. Figure 3 shows the result of this experiment. The same 0%–30% ammonium sulfate precipitate used for toluquinol epoxidation in Figure 2 was employed in this experiirment, and it lacked gentisyl alcohol dehydrogenase activity. As with toluquinol in Figure 2, substrate inhibition is seen at high substrate concentrations, in this case above 0.5 mM. An apparent K_m estimated from the linear part of the reciprocal plot is 0.17 mM, not very different from that estimated for toluquinol. Quinone formation also occurs in this reaction, though no gentisaldehyde is formed, and the disproportionate depletion of substrate at lower substrate concentrations probably also makes this K_m estimate high. The apparent V_m's for toluquinol and gentisyl alcohol, obtained from the intercept of the linear parts of Figures 2 and 3, are 330 and 18 nmol/h per 0.65 mg of protein, respectively. Thus the rate of epoxidation of toluquinol is more than 10 times greater than the rate of epoxidation of gentisyl alcohol.

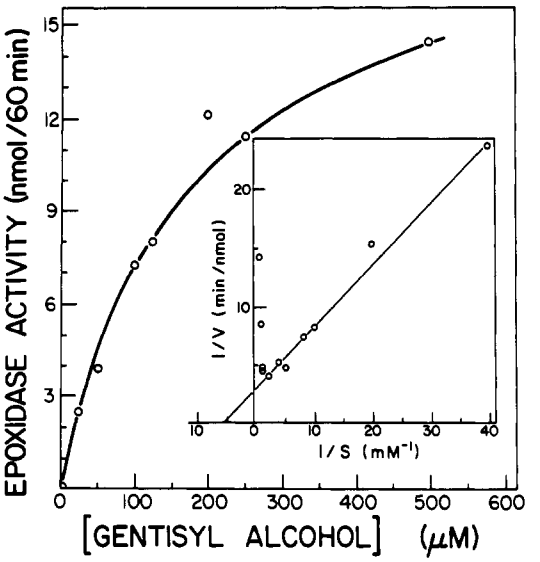


FIGURE 3: Effect of gentisyl alcohol concentration on epoxidase activity. Assays were run under the same conditions as described in Figure 2 except they contained the indicated concentration of gentisyl alcohol rather than toluquinol. Epoxidase activity was determined from the quantity of neopatulin formed as this preparation accumulated no phyllostine. The line on the reciprocal plot is a least-squares fit (correlation = 0.984) to the activity values for substrate concentrations at 0.5 mM and lower. Data for substrate concentrations above 0.5 mM are plotted on the reciprocal plot but not on the direct plot.

Figure 4 shows that the epoxidase is induced in fermentor cultures in a coordinate manner with *m*-hydroxybenzyl alcohol dehydrogenase. Neway and Gaucher (1981) have shown that this dehydrogenase appears coordinately with other pathway enzymes, though at an earlier time (20–22 h) in their fermentor cultures. Our cultures appear to be slower in reaching the "tropophase–idiophase" transition (Bu'Lock et al., 1965). We will report elsewhere a more complete description of the metabolite profile during this transition, and the coordinate induction of other pathway related enzymes, 6-methylsalicyclic acid decarboxylase and gentisyl alcohol dehydrogenase. The time of first appearance of 6-methylsalicylic acid and patulin in the culture is indicated by the letters a and b, respectively.

Discussion

Using a 0%–30% ammonium sulfate fraction that was depleted of gentisyl alcohol dehydrogenase activity, we have demonstrated a conversion of gentisyl alcohol to phyllostine and neopatulin at a rate of 0.2 nmol min^{-1} mg^{-1} at 100 μM substrate concentration (Figure 3) under conditions in which there is no conversion of 250 μM gentisaldehyde to detectable products. These results should clear up some confusion in the literature over which compound is the closest precursor to patulin. The early proposal by Birkinshaw (1953) that gentisaldehyde is the precursor made sense on structural grounds when labeling studies showed that the side-chain carbon became carbon 1 of patulin, also at the aldehyde level of oxidation (Tanenbaum & Bassett, 1959; Scott et al., 1973). The

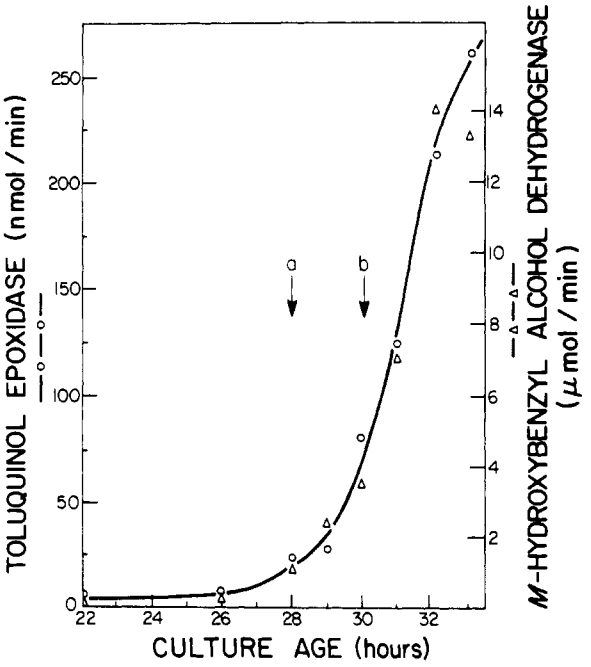


FIGURE 4: Induction of epoxidase and *m*-hydroxybenzyl alcohol dehydrogenase in a fermentor culture of *P. patulum*. Lyophilized mycelial samples harvested at the indicated times from a fermentor culture of *P. patulum* were extracted as described under Materials and Methods, and the crude extracts were assayed for enzymic activity by HPLC. Epoxidase assays contained 100 μM toluquinol in 5.0 mL of 100 mM potassium phosphate, pH 7.5, while dehydrogenase assays contained 100 μM *m*-hydroxybenzaldehyde, 100 μM NADPH, 10 μM ATP, and 1 mM $MgCl_2$ in the same buffer. After incubation at 28 °C for an appropriate time, the product was extracted and analyzed by HPLC as described under Materials and Methods. Activity plotted is that calculated for total crude extract from 0.5 g of lyophilized mycelium.

discoveries of phyllostine (Sekuguchi & Gaucher, 1978), neopatulin (Sekiguchi et al., 1979), and ascladiol (Sekiguchi et al., 1983) as intermediates in the pathway, each having an alcohol function at the corresponding position, should have negated this argument. Scott and Beadling (1974) obtained a cell-free "supernatant" which converted 0.2 μM [^{14}C]gentisaldehyde to patulin at an activity of 0.36 pmol min^{-1} mg^{-1} in the presence of NADPH (required) and ATP (stimulatory). That preparation would have contained the nonsedimentable portion of our epoxidase, as well as gentisyl alcohol dehydrogenase, which could explain the NADPH requirement. A more recent experiment by Iijima et al. (1986) employed a "particulate" cell-free preparation converting [*ring*-^{3}H]-gentisyl alcohol and not [*ring*-^{3}H]gentisaldehyde to patulin. They also showed that [1-^{14}C,$^{3}H_2$]gentisyl alcohol retained one hydrogen upon conversion to patulin by the particulate preparation, whereas both hydrogens are lost during in vivo conversion. Presumably gentisyl alcohol dehydrogenase in whole cells exchanges the other proton. While their Waring blender technique for cell breakage may not have been very effective and the rate of conversion is not easy to calculate from their data, our results are consistent with theirs. An early observation by Forrester and Gaucher (1972), that [G-^{3}H]gentisaldehyde but not [G-^{3}H]gentisyl alcohol is converted to patulin by intact cells, is probably in error, since Scott et al. (1973) also have shown in vivo conversion of ring-deuterated gentisyl alcohol into patulin.

Removing gentisaldehyde from the direct pathway to patulin is also consistent with studies by Murphy and Lynen (1975) in which ring hydroxylation of *m*-hydroxybenzyl alcohol to gentisyl alcohol could be demonstrated, but ring hydroxylation of *m*-hydroxybenzaldehyde to gentisaldehyde could not. Only *m*-hydroxybenzoic acid could be detected as a product from *m*-hydroxy[^{14}C]benzaldehyde.

Nucleophilic attack of hydrogen peroxide on a quinone and electrophilic attack of oxygen on a hydroquinone can lead to the same hydroperoxy intermediate on the way to an epoxide (Scheme I). Although the former mechanism occurs chemically (Alder et al., 1960), our data support the latter mechanism for the epoxidase. The proposed sequence is similar to that observed in synthesis of the fatty acid epoxide leukotriene A_4 (Samuelsson et al., 1987). In that case the hydroperoxide intermediate 5-HPETE is isolable, even though the enzyme 5-lipoxygenase catalyzes both reactions. Although the 5-lipoxygenase from human leukocytes is a soluble 80 000-dalton protein, it readily associates with membranes in the presence of Ca^{2+} and requires at least two cytosolic and one membrane component for optimum activity (Rouzer et al., 1986). Whether our epoxidase association with membranes will prove to be similar remains to be seen. EDTA does not solubilize the activity, although it does partially inhibit (Table III).

Inhibition by 1,10-phenanthroline suggests a metal ion involvement, and the role of the metal could be to complex both the hydroquinone and the attacking oxygen to facilitate oxygen reaction with the ring. Direct reaction of triplet oxygen with the hydroquinone in the absence of the metal ion would be a spin-forbidden process (Hamilton, 1974). The proposed hydroperoxy intermediate in Scheme I may prove to be too unstable chemically to isolate.

Specificity of the epoxidase parallels that of the microsomal ring hydroxylase (Murphy & Lynen, 1975), which utilizes the side-chain methyl (*m*-cresol) and hydroxymethyl (*m*-hydroxybenzyl alcohol) derivatives, but not the side-chain formyl derivative (*m*-hydroxybenzaldehyde). The electron-withdrawing nature of the formyl group may deactivate the ring to electrophilic attack in both cases. In contrast to the ring hydroxylase, which shows about the same activity with both substrates, the epoxidase is an order of magnitude more active with toluquinol (**1**) than with gentisyl alcohol (**2**). As with the ring hydroxylase activity, purification will be necessary to determine whether a single epoxidase or multiple epoxidases are involved.

The reaction of oxygen with a hydroquinone may be a general mechanism utilized in the synthesis of other fungal quinone epoxides such as terreic acid (Read et al., 1969), panepoxydion (Kis et al., 1970), fumigatin epoxide (Yamamoto et al., 1967), and frenolicin (Ellestead et al., 1968). Epoxide intermediates have not been ruled out in other aromatic ring opening reactions such as that leading to penicillic acid (Zamir, 1980). The combination of the epoxidase reaction (Scheme I) and the neopatulin synthase reaction (Scheme II) accomplishes the same overall ring cleavage as the "decyclizing" extradiol-cleaving dioxygenases such as metapyrocatechase and homogentisate dioxygenase (Jefford & Cadby, 1980). Some of these enzymes have been shown to be true dioxygenases by demonstrating incorporation of both atoms of oxygen (Crandall et al., 1960), but the possibility of an epoxide intermediate in others should be considered. For example, the recent report on the ring cleavage of quertin by an extract from *Aspergillus terreus* (Fujii et al., 1988) could involve reduction of the anthroquinone by NADPH followed by an epoxidase–hydrolase sequence yielding desmethylsulochrin.

The neopatulin synthase reaction shown in Scheme II is illustrated as a 2-(hydroxymethyl)-5,6-epoxybenzoquinone-4,5 hydrolase (decyclizing), followed by bond rotation and lac-

tonization. An alternative mechanism could involve attack of a nucleophilic group of the enzyme at the quinone carbonyl, 4,5 bond cleavage, and displacement of the nucleophilic group by the enol function of the rearranged molecule to form the lactone directly. In this case the enzyme should be classified as an isomerase instead of a hydrolase. We have no evidence for or against the appearance of the free acid intermediate, nor as to whether its lactonization would be spontaneous. If undetected acid did accumulate in the reaction, then our measurement of gentisyl alcohol epoxidase activity in Figure 3 would be low and might explain why the measured toluquinol epoxidase activity of the same preparation is so much higher. Clearly, both the epoxidase and the synthase must be purified much further before such questions can be addressed.

A corresponding ring-opening reaction with desoxyphyllostine (**7**) to form "desoxyneopatulin" (**11**) did not occur in a quantity readily detectable in the HPLC assays. The TLC analysis of the product from epoxidase action on toluquinol did show a yellow spot at $R_f = 0.31$ after spraying with MBTH and heating. The R_f of this material is two-thirds that of desoxyphyllostine ($R_f = 0.45$), approximately the same relationship as neopatulin ($R_f = 0.38$) to phyllostine ($R_f = 0.58$) in a more polar solvent system. Isolation and identification of this material has not yet been pursued, but its presence suggests that the ring opening of desoxyphyllostine might occur to some extent and that one should look not only for desoxyneopatulin (**11**) but also for desoxyascladiol as metabolites of a pathway parallel to the patulin pathway. The proportion of metabolite flow into this parallel pathway would be determined by the relative activities of the *m*-cresol ring and methyl hydroxylase activities. Hydroxylation of the methyl group must occur first for patulin synthesis, and that reaction has a lower K_m for *m*-cresol than does the ring hydroxylation (5 μM vs 20 μM; Murphy et al., 1974).

Three sequential reactions of the patulin pathway—methyl group hydroxylation to form *m*-hydroxybenzyl alcohol (Murphy et al., 1974), ring hydroxylation to form gentisyl alcohol (Murphy & Lynen, 1975), and the gentisyl alcohol epoxidase reported here—all involve molecular oxygen and are membrane associated. The first two hydroxylases are inhibited by carbon monoxide in a process reversible by light, suggesting involvement of cytochrome P_{450}, while the epoxidase is not. The ring-hydroxylating activity appeared in both light and heavy fractions upon sucrose density gradient centrifugation, somewhat analogous to the behavior of the epoxidase in Figure 1, while the methyl hydroxylase occurred primarily in the more slowly sedimenting enzyme fraction (Murphy & Lynen, 1975). A fourth enzyme, that catalyzing the last step of the pathway by converting ascladiol to patulin (Sekiguchi et al., 1983), has been reported to require oxygen. Its subcellular association has not been reported nor was any data presented to document the oxygen requirement.

The quinol epoxides isoepoxydon and desoxyepoxydon have also been isolated from cultures of *P. urticae*, the former by Sekiguchi and Gaucher (1979a) from the patulin-negative mutant J1 and the latter by Scott et al. (1973) from cultures force-fed toluquinol. Neway and Gaucher (1980) have isolated a dehydrogenase interconverting phyllostine and isoepoxydon and shown that it is induced along with 6-methylsalicylic acid synthase and *m*-hydroxybenzyl alcohol dehydrogenase during the onset of patulin production in fermentor cultures. Sekiguchi and Gaucher (1979b) also showed that isoepoxydon is converted via phyllostine to patulin and other pre-patulin "yellow compounds" by cell-free extracts of *P. urticae*. They concluded that isoepoxydon must therefore be the precursor of phyllostine and hence the initial product of the epoxidation reaction. We were unable to detect either isoepoxydon or desoxyepoxydon in our epoxidase assays, which required neither $NADP^+$ nor NADPH, arguing against Gaucher's proposed sequence: gentisyl alcohol → isoepoxydon → phyllostine, or toluquinol → desoxyepoxydon → desoxyphyllostine.

Isoepoxydon dehydrogenase, along with *m*-hydroxybenzyl alcohol dehydrogenase and gentisyl alcohol dehydrogenase (Scott & Beadley, 1974), now all appears to lie off the main pathway to patulin, forming side products that can equilibrate with pathway intermediates. The latter two enzymes (which have not yet been established as separate proteins) are involved in the biosynthesis of the "dead-end" metabolites *m*-hydroxybenzoic acid and gentisic acid. Yet these dehydrogenases not only have activities much higher than other pathway enzymes, they are all coordinately induced in fermentor culture along with other pathway enzymes (Figure 4; Neway & Gaucher, 1981). The evidence cited here argues against their participation in a metabolic "grid" of alternative pathways. Therefore, their role in and requirement for patulin biosynthesis remains obscure and can be clarified only by isolation of mutants defective in these activities.

Actually, no "role" in patulin biosynthesis is necessary if one takes a different viewpoint of the patulin biosynthetic pathway. Instead of viewing this complex pathway as a mechanism for making the antibiotic patulin as an "end product", one could just as well view it as an inducible catabolic pathway for degrading the aromatic polyketide 6-methylsalicylic acid. This viewpoint is plausible given that under some culture conditions a clear distinction can be made between the time of induction of 6-methylsalicylic acid synthase and other pathway enzymes and that addition of 6-methylsalicylic acid to a mutant blocked in the synthase results in enhanced induction of two later enzymes, 6-methylsalicylic acid decarboxylase and *m*-hydroxybenzyl alcohol dehydrogenase (Gaucher et al., 1981). In this view, no metabolite should be considered any more of a "dead-end" product than patulin itself. The major end product accumulating in such a catabolic situation would depend upon the evolutionary history of the organism—perhaps the toxicity of one or another metabolite providing an ecological advantage. It would not, therefore, be too surprising that multiple strategies for degradation developed in a single organism or that different organisms developed different strategies for degradation, leading to a different spectrum of products. The same argument could be applied to other polyketide families resulting from degradation of other initial polyketide synthase products.

Acknowledgments

We acknowledge the support of Hank Henricks and the Biochemical Analysis and Synthesis Service facility of the Department of Chemistry for access to HPLC equipment and of Beckman Instruments Co. for donation of an analytical HPLC column. We thank Dr. G. M. Gaucher for providing an authentic sample of phyllostine.

References

Alder, K., Flock, F. H., & Beumling, H. (1960) *Chem. Ber. 93*, 1896–1899.

Birkinshaw, J. H. (1953) *Annu. Rev. Biochem. 22*, 371–398.

Bruce, J. M., & Knowles, P. (1966) *J. Chem. Soc. C*, 1627–1634.

Bu'Lock, J. D., Hamilton, D., Hulme, M. A., Powell, A. J., Smalley, H. M., Shepherd, D., & Smith, G. N. (1965) *Can. J. Microbiol. 11*, 765–778.

Bu'Lock, J. D., & Ryan, A. J. (1958) *Proc. Chem. Soc.*, 222–223.
Bu'Lock, J. D., Shepherd, D., & Winstanley, D. J. (1979) *Can. J. Microbiol. 15*, 279–285.
Crandall, D. I., Krueger, R. C., Anon, F., Yasunobu, K., & Mason, H. S. (1960) *J. Biol. Chem. 235*, 3011–3015.
Ellestead, G. A., Kunstmann, M. P., Whalley, H. A., & Patterson, E. L. (1968) *J. Am. Chem. Soc. 90*, 1325–1332.
Forrester, P. L., & Gaucher, G. M. (1972) *Biochemistry 11*, 1102–1107.
Fujii, I., Ebizuka, Y., & Sankawa, U. (1988) *J. Biochem. 103*, 878–883.
Gaucher, G. M. (1979) *J. Food Prot. 42*, 810–814.
Gaucher, G. M., Lam, K. S., Grootwassink, J. W. D., Neway, J., & Deo, Y. M. (1981) *Dev. Ind. Microbiol. 22*, 219–232.
Hamilton, G. A. (1974) in *Molecular Mechanisms of Oxygen Activation* (Hayaishi, O., Ed.) pp 405–451, Academic Press, New York.
Hodgson, H. H., & Jenkinson, T. A. (1929) *J. Chem. Soc. 131*, 469–471.
Iijima, H., Ebizuka, Y., & Sankawa, U. (1986) *Chem. Pharm. Bull. 34*, 3534–3537.
Jefford, C. W., & Cadby, P. A. (1980) *Prog. Chem. Org. Nat. Prod. 40*, 191–265.
Kis, Z., Closse, A., Sigg, H. P., Hruban, L., & Snatzke, G. (1970) *Helv. Chim. Acta 53*, 1577–1597.
Light, R. J. (1967) *J. Biol. Chem. 242*, 1880–1886.
Light, R. J., & Priest, J. W. (1988) *J. Cell. Biol. 107* (6, part 3), 622a.
Murphy, G., & Lynen, F. (1975) *Eur. J. Biochem. 58*, 467–475.
Murphy, G., Vogel, G., Krippahl, G., & Lynen, F. (1974) *Eur. J. Biochem. 49*, 443–455.
Neway, J., & Gaucher, G. M. (1981) *Can. J. Microbiol. 27*, 206–215.
Niehaus, W. G., Jr., & Dilts, R. P., Jr. (1982) *J. Bacteriol. 151*, 243–250.
Rashid, A., & Read, G. (1967) *J. Chem. Soc. C*, 1323–1325.
Read, G., Westlake, D. W. S., & Vining, L. C. (1969) *Can. J. Biochem. 47*, 1071–1079.
Rouzer, C. A., Matsumoto, T., & Samuelsson, B. (1986) *Proc. Natl. Acad. Sci. U.S.A. 82*, 7505–7509.
Samuelsson, B., Dahlen, S. E., Lindgren, J. Å., Rouzer, C. A., & Serhan, C. N. (1987) *Science 237*, 1171–1176.
Scott, A. I., & Beadling, L. (1974) *Bioorg. Chem. 3*, 281–301.
Scott, A. I., Zamir, L., Phillips, G. T., & Yalpani, M. (1973) *Bioorg. Chem. 2*, 124–139.
Scott, P. M. (1974) in *Mycotoxins* (Purchase, I. F. H., Ed.) pp 383–403, Elsevier, Amsterdam.
Sekiguchi, J., & Gaucher, G. M. (1977) *Appl. Environ. Microbiol. 33*, 147–158.
Sekiguchi, J., & Gaucher, G. M. (1978) *Biochemistry 17*, 1785–1791.
Sekiguchi, J., & Gaucher, G. M. (1979a) *Biochem. J. 182*, 445–453.
Sekiguchi, J., & Gaucher, G. M. (1979b) *Can. J. Microbiol. 25*, 881–887.
Sekiguchi, J., Gaucher, G. M., & Yamada, Y. (1979) *Tetrahedron Lett. 1*, 41–42.
Sekiguchi, J., Gaucher, G. M., & Yamada, Y. (1980) in *Advances in Biotechnology* (Moo-Young, M., Vezino, C., & Singh, K., Eds.) Vol. 3, pp 107–112, Pergamon Press, Toronto.
Sekiguchi, J., Shimamoto, J., Yamamato, Y., & Gaucher, G. M. (1983) *Appl. Environ. Microbiol. 45*, 1939–1942.
Tanenbaum, S. W., & Bassett, E. W. (1959) *J. Biol. Chem. 234*, 1861–1866.
Weast, R. C., & Astle, M. J., Eds. (1982) *CRC Handbook of Chemistry and Physics*, CRC Press, Boca Raton, FL.
Willstatter, R., & Pfannenstiel, A. (1904) *Ber. 37*, 4744–4746.
Yamamoto, Y., Nitta, K., & Jinbo, A. (1967) *Chem. Pharm. Bull. 15*, 427–431.
Zamir, L. O. (1980) in *Biosynthesis of Mycotoxins: Study of Secondary Metabolites* (Steyn, P., Ed.) pp 223–268, Academic Press, New York.