

Isoepoxydon, a New Metabolite of the Patulin Pathway in *Penicillium urticae*

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A patulin-negative mutant (J1) of *Penicillium urticae* (N.R.R.L. 2159A) was known to accumulate about 100 mg per litre quantities of the 5,6-epoxygentisyl quinone, (-)-phyllostine and another metabolite (UIII). Both were derived from acetate and hence were polyketides. Purified UIII (m.p. 53°C, $[\alpha]_D^{25} + 206^\circ$, $\lambda_{\text{max}}^{\text{methanol}}$ 240 nm; ϵ 3806 litre·mol⁻¹·cm⁻¹) was characterized as a partially reduced derivative of (-)-phyllostine and was found to be a diastereoisomer of the known phytotoxin, (+)-epoxydon. Hence its designation as (+)-iso- or epi-epoxydon. From ¹H n.m.r. and c.d. data the stereochemistry of the epoxide ring in (+)-isoepoxydon was determined to be identical with that in (+)-epoxydon (i.e. *R,R*) but the configuration of the secondary alcohol at C-4 was *S* rather than *R* as in (+)-epoxydon. Isoepoxydon (compound UIII) is therefore (4*S*,5*R*,6*R*)-5,6-epoxy-4-hydroxy-2-hydroxymethylcyclohex-2-en-1-one. The boat conformation in which the C-4 hydroxy group is axial is preferred. In the range of 1 mm to 5 mm, the antibiotic activity of (+)-isoepoxydon against *Bacillus subtilis* sp. was 56% of that obtained with patulin. Over a period of 1 to 3 h, [¹⁴C]isoepoxydon was efficiently converted into patulin by a shake culture of the parent strain of *P. urticae*. The precursor relationship of isoepoxydon to patulin was confirmed by feeding unlabelled isoepoxydon (1 mM) to a washed-cell suspension of a mutant (J2) in which, over a period of 3 to 5 h, a better than 60% conversion into patulin was attained. The enzymic relationship between isoepoxydon and phyllostine and their positions in the late portion of the patulin biosynthetic pathway are discussed.

Patulin is a well-studied antibiotic (Florey *et al.*, 1949), and mycotoxin (Scott, 1974) that is produced by a variety of *Penicillium*, *Aspergillus*, *Nectria* and *Byssoschlamys* sp. Interest in the biosynthesis of patulin dates back to the speculations of Birkinshaw (1953), and gained momentum when its biosynthesis from acetate via 6-methylsalicylic acid (Bu'Lock & Ryan, 1958; Tanenbaum & Bassett, 1959) led to its classification as a polyketide; that is as a metabolite synthesized from acetyl-CoA via an enzyme-bound poly- β -oxomethylene chain. Since then both the patulin pathway (Forrester & Gaucher, 1972) and its enzymology (Murphy & Lynen, 1975, and references therein) have been intensively studied in *Penicillium urticae*. *P. urticae* is a relatively common soil microbe (Smith, 1969) and has been shown to produce significant quantities of patulin in the soil (Norstadt & McCalla, 1969). Patulin production is not, however, essential for survival and hence this metabolite along with thousands of others is classified as a secondary metabolite. The role of such compounds in nature has been the subject of a long-standing debate (Demain, 1974; Zähler, 1977; Swain, 1977) which is yet to be resolved. In a study designed

to test the hypothesis that patulin production in *P. urticae* might be a pre- or co-requisite to asexual sporulation (Sekiguchi & Gaucher, 1977), a series of patulin-minus mutants was isolated. One of the mutants (J1) accumulated three unknown metabolites, two of which (UII and UIII) were shown to be derived from acetate and from the late-patulin-pathway metabolite gentisaldehyde (Sekiguchi & Gaucher, 1978). In the same study compound UII was shown to be 5,6-epoxygentisylquinone or phyllostine, a previously characterized metabolite of a *Phyllosticta* sp. (Sakamura *et al.*, 1971). We have also shown that phyllostine is an efficient precursor of patulin in all strains of *P. urticae* that possess an intact post-gentisaldehyde section of the pathway. Thus the terminal steps of patulin biosynthesis are not few in number, and are not initiated by a bacterium-like ring cleavage of gentisaldehyde, as was long assumed. These findings have also served to emphasize that very similar pathways occur in a variety of non-patulin-producing microbes (Read *et al.*, 1969; Al-Rawi *et al.*, 1974; Nabeta *et al.*, 1975).

As part of our continuing effort to unravel the terminal portion of the patulin pathway, the present paper details the structure and absolute configuration of compound UIII, a new secondary metabolite and patulin precursor.

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Materials and Methods

Organism and culture conditions

P. urticae (N.R.R.L. 2159A) and two patulin-minus mutants (J1 and J2) that were derived from this strain (Sekiguchi & Gaucher, 1977) were used in the present study. These two mutants possess pathway blocks that have been characterized as occurring before (mutant J2) and after (mutant J1) the new post-gentisaldehyde intermediate, phyllostine (Sekiguchi & Gaucher, 1978).

A vegetative-cell inoculum was prepared from 24 h-old shake cultures (50 ml) grown in a glucose/nitrate/yeast-extract medium as previously described (Sekiguchi & Gaucher, 1978). Unfiltered portions (5 ml) of this seed culture were added to 500 ml Erlenmeyer flasks containing 50 ml of a glucose/yeast-extract medium (D-glucose, 50.0 g; yeast extract (Difco), 2.5 g; KH_2PO_4 , 1.0 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g; KCl, 0.5 g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1.9 mg; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 4.5 mg; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.23 mg; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.15 mg; and double-deionized water to 1 litre, pH 6.5) and the flasks were incubated at 28°C on a rotary shaker (NBS model G-10; 250 rev./min; 2.54 cm stroke) for various times.

Isolation and analysis of isoepoxydon (compound UIII)

Twenty 50 ml shake-flask cultures of mutant J1 were cultivated for 48 h as described above. The combined filtrate (1 litre) was acidified to pH 2 with 1 M-HCl and extracted with 2 vol. of ethyl acetate. After drying over anhydrous Na_2SO_4 the extract was concentrated by rotary evaporation and streaked on preparative thin-layer plates (20 cm × 20 cm; 1 mm Woelm silica gel GF₂₅₄) that had been activated for 2 h at 110°C and equilibrated at room temperature without desiccation. Development with chloroform/ethyl acetate/diethyl ether/methanol (7:7:2:0.5, by vol.) resulted in the separation of compound UIII (R_F 0.39) from the other metabolites except for a trace of gentisyl alcohol (R_F 0.50). The band corresponding to compound UIII, which was clearly visible under u.v. light, was scraped from the plate(s), extracted with ethyl acetate and rechromatographed with toluene/ethyl acetate/formic acid (6:3:1, by vol.) to yield separate bands for compound UIII (R_F 0.23) and gentisyl alcohol (R_F 0.40). The compound UIII obtained from this second chromatogram was then rechromatographed once more with the first solvent. Isoepoxydon (compound UIII) was crystallized from 1,2-dichloroethane to yield approx. 70 mg of colourless needles (m.p. 53°C). Analytical thin-layer chromatograms of this compound exhibited a single light-brown spot when sprayed with 3-methylbenzothiazolin-2-one hydrazone hydrochloride as previously described (Sekiguchi & Gaucher, 1977).

Melting points were determined by using a Leitz hot-stage microscope and the elemental analysis was carried out by Schwarzkopf Microanalytical Laboratory, Woodside, NY, U.S.A. U.v. and i.r. spectra were recorded on Beckman DB-G and Perkin-Elmer 467 spectrophotometers respectively. Mass and ^1H n.m.r. spectra were obtained by using a Varian MAT CH5 Massenspektrometer and a Varian HA-100 n.m.r. spectrometer respectively. O.r.d. and c.d. curves were recorded on a Durrum-Jasco model ORD/UV5 spectropolarimeter equipped with a Sproul SS-20 modification.

Acetylation of isoepoxydon

A solution of crystalline compound UIII (121 mg) and anhydrous sodium acetate (180 mg) in acetic anhydride (6 ml) was refluxed in a boiling-water bath for 1 h as described by Sakamura *et al.* (1969) and then poured into approx. 30 ml of an ice/water mixture. The reaction mixture was left overnight and then extracted with chloroform and the concentrated extract was subjected to preparative t.l.c. (chloroform/ethyl acetate/diethyl ether, 5:2:1, by vol.). Compound UIII was not present, but a minor (R_F 0.40) and a major (R_F 0.76) band were visualized under u.v. light. This major band was scraped from the plate, extracted with chloroform, and evaporated to yield 109 mg of acetylated derivative. Crystallization from ethanol/methanol yielded 32 mg of colourless plates (m.p. 92°C).

Preparation of [^{14}C]isoepoxydon

Six 50 ml shake cultures of mutant J1 were cultivated in glucose/yeast-extract medium as described above. At 32 h 50 μl of a sodium [^{14}C]acetate solution in ethanol (1 $\mu\text{Ci}/\mu\text{l}$; sp. radioactivity 57.8 mCi/mmol; New England Nuclear) was added to each culture. After 12 h all six cultures were harvested by filtration and extracted. The extract was subjected to preparative t.l.c. 3 times as described above for isoepoxydon (compound UIII), to yield 6.4 mg of [^{14}C]isoepoxydon (sp. radioactivity 2.2×10^5 c.p.m./mg). The scintillation-counting procedure was as previously described (Sekiguchi & Gaucher, 1977).

*Conversion of [^{14}C]isoepoxydon into patulin by *P. urticae* (N.R.R.L. 2159A)*

At 34 h, 20 ml of a 500 ml shake culture grown in glucose/yeast extract medium, as described under 'Organism and culture conditions', was transferred to a 200 ml Erlenmeyer flask containing 1.9 mg of [^{14}C]isoepoxydon and shaken as before. Culture samples (2 ml) obtained at various times after transfer were filtered, and 0.5 ml of each filtrate was acidified (pH 2) and extracted twice with 3 ml of ethyl acetate.

After concentration in a test tube (Buchler Evapo-Mix), the entire extract was spotted onto a 250 μm -thick silica gel (Woelm, GF₂₅₄) plate (5 cm \times 20 cm) and developed with chloroform/acetic acid (4:1, v/v). After examination under u.v. light, 2 mm wide slices of each chromatogram were scintillation-counted for radioactivity as previously described (Sekiguchi & Gaucher, 1978).

Conversion of unlabelled isoepoxydon (compound UIII) into patulin by a cell suspension of P. urticae mutant J2

After 32 h of cultivation in glucose/yeast extract medium, the cells from two 50 ml shake cultures were harvested by filtration, washed with water and resuspended in 100 ml of 0.05 M-phosphate buffer (pH 6.5). Half of this suspension was transferred to each of two 500 ml flasks and pure isoepoxydon was added to one of these flasks to yield a final concentration of 1 $\mu\text{mol/ml}$. Shaking at 28°C was resumed and 5 ml samples were withdrawn from each flask at various times (5 min, 30 min, 1 h, 2 h, 3 h and 5 h). A portion (0.5 ml) of each sample was extracted and chromatographed as described above, except that the solvent used was chloroform/ethyl acetate/diethyl ether (5:2:1, by vol.). The metabolites on the t.l.c. plate were developed as coloured spots (e.g. patulin, yellow; compound UIII, brown) as previously described (Sekiguchi & Gaucher, 1978).

A quantitative assessment of the patulin produced in this experiment was obtained by using previously described antibiotic (Sekiguchi & Gaucher, 1978) and g.l.c. (Ehman & Gaucher, 1977) assays. For the g.l.c. assay, filtrates (2 ml) from both the experimental and control suspensions were extracted with diethyl ether, evaporated to dryness, and silylated in the presence of *p*-bromophenol as an internal standard, with chlorotrimethylsilane and hexamethyldisilazane in pyridine. Portions (2 μl) were then injected into a 10% QF-1 column (1.88 m \times 2.16 mm) and chromatograms were obtained by using an attenuation of 16. Patulin had a retention time of about 24 min. To further verify the identity of the patulin produced after 5 h, crystalline patulin (0.2 mg) was added to 2 ml of filtrate and the sample was extracted and assayed.

Results and Discussion

Identification of compound UIII as (+)-isoepoxydon

Crystalline compound UIII (isoepoxydon) that had been purified from the culture filtrate of *P. urticae* mutant J1 as described in the Materials and Methods section had m.p. 53°C; $[\alpha]_D^{25} + 206^\circ$ (c 0.17 in methanol); $\lambda_{\text{max}}^{\text{methanol}}$ (ϵ) 212 (5756), 240 (3806), 329 nm (36.7 litre \cdot mol⁻¹ \cdot cm⁻¹); i.r., $\nu_{\text{max}}^{\text{KBr}}$ 3390(s), 2930, 2880, 1680(s), 1400, 1240, 1145, 1110, 1075, 1030(s), 1010(s), 890, 870, 830, 815, 777, 720 cm⁻¹; *m/e* (relative abundance) 138 (*M*⁺ - H₂O, 2.0), 127 (3.1),

121 (2.7), 110 (*M*⁺ - H₂O - CO, 33), 97 (21), 81 (37), 71 (40), 53 (94), 39 (98), 29 (100); (Found: C, 53.7; H, 5.2; O, 41.2; C₇H₈O₄ (mol.wt. 156) requires C, 53.8; H, 5.1; O, 41.1%).

Given the biosynthetic relationship of compound UIII to the epoxyquinone phyllostine (C₇H₆O₄), the elemental analysis and mass spectrum suggested that compound UIII might arise from a simple reduction of phyllostine. The resulting partially reduced epoxyquinone would be expected to possess an *R*_F (t.l.c.) that is significantly less than that of phyllostine (Sekiguchi & Gaucher, 1978), and a λ_{max} close to that calculated (Timmons, 1972) for a disubstituted $\alpha\beta$ -enone (i.e. 237 nm). The fungal metabolite (+)-epoxydon (Closse *et al.*, 1966) has a structure identical with that proposed for compound UIII, but a comparison of properties clearly showed that compound UIII could be a diastereoisomer of epoxydon, but not (+)- or (-)-epoxydon. Thus (+)-epoxydon (C₇H₈O₄) had m.p. 40–45°C, $[\alpha]_D^{25} + 93^\circ$ (c 0.29 in methanol), $\lambda_{\text{max}}^{\text{ethanol}}$ (ϵ) 240 nm (5129 litre \cdot mol⁻¹ \cdot cm⁻¹), a similar i.r. spectrum except for the 800–1100 cm⁻¹ region and an almost identical mass spectrum [cf. *m/e* 138 (2.8), 127 (3.9), 121 (3.1), 110 (43), 97 (25), 81 (43), 71 (46), 53 (98), 39 (100), 29 (99)]. These latter mass-spectral data for (+)-epoxydon were obtained from an authentic sample supplied by Sakamura *et al.* (1969).

As described in the Materials and Methods section, compound UIII was exhaustively acetylated to yield a product (tetra-*O*-acetyl-2,3,5-trihydroxybenzyl alcohol, m.p. 92°C, $\lambda_{\text{max}}^{\text{ethanol (95%)}}$ (ϵ) 269 nm (694 litre \cdot mol⁻¹ \cdot cm⁻¹); ¹H n.m.r. δ (p.p.m.) (²H]chloroform) 2.09, 2.28, 2.30, 2.33 (4 \times 3 H, S, CH₃CO), 5.06 (2H, S, CH₂O), 7.07, 7.12 (2H, pair d, *J* 2.5 Hz, aromatic), which was identical with that obtained from (+)-epoxydon by Closse *et al.* (1966). This compound's i.r. ($\nu_{\text{max}}^{\text{KBr}}$ 1775, 1740, 1690, 1605, 1435, 1370, 1316, 1219, 1192, 1160, 1126, 1052, 1017, 986, 960, 910, 882, 850, 813 cm⁻¹) and mass spectrum [*m/e* (relative abundance) 324 (*M*⁺, 1.21), 282 (9.6), 266 (7.9), 240 (16), 222 (5.3), 198 (3.5), 180 (39), 155 (5.5), 138 (96), 43 (100)] were identical to those reported by Sakamura *et al.* (1969). Since any 5,6-epoxy-4-hydroxy-2-hydroxymethylcyclohex-2-en-1-one should yield this product regardless of its stereochemistry, this evidence is consistent with compound UIII being a diastereoisomer of (+)-epoxydon; hence our designation of compound UIII as (+)-isoepoxydon. The detailed analysis of the ¹H-n.m.r. spectrum (Fig. 1) of this metabolite was not compatible with any other structure. The closely related fungal metabolites (+)-epoxydon (Closse *et al.*, 1966) and (-)-panepoxydon (Kis *et al.*, 1970) possess virtually identical chemical shifts for the epoxide protons H⁶ and H⁵ [cf. δ (p.p.m.) 3.40, 3.43, and 3.77, 3.75 respectively], as well as for protons H⁴ and H³ [cf. δ (p.p.m.) 4.72, 4.6 and 6.42, 6.65 respectively]. These

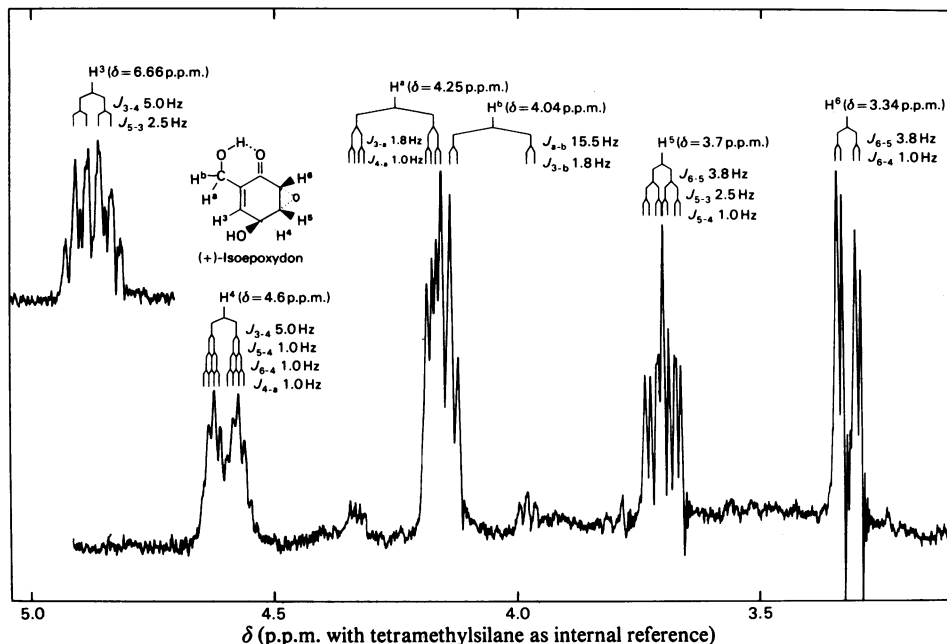


Fig. 1. ^1H n.m.r. spectrum of pure isoepoxydon (compound UIII)
The solvent was $[\text{2H}]$ acetone and the sweep width was 250Hz.

previous structural assignments are strongly supported by more recent ^{13}C n.m.r. data on epoxydon (Nabeta *et al.*, 1975) and phyllostine (Sekiguchi & Gaucher, 1978).

The complexity of the ^1H n.m.r. spectrum of (+)-isoepoxydon is due to favourable long-range coupling between protons and to the non-equivalence of the methylene protons H^a and H^b . This latter observation is supported by the H-bonded O-H stretching frequency (3400cm^{-1}) observed in a thin-film i.r. spectrum of this compound. Although a detailed analysis of proton-proton coupling constants cannot distinguish between mirror images, it can often be used to distinguish between diastereoisomers and conformers since the degree of coupling is strongly affected by the spatial relationship of one proton to another. As indicated in Fig. 2 a partially reduced epoxyquinone can exist as *cis*- or *trans*-diastereoisomers and in turn as *a*-OH or *e*-OH conformers. With the additional possibility of mirror images, a total of eight structures are possible. A comparative analysis of short- and long-range- (i.e. via three and four or five bonds respectively) coupling constants in Table 1 indicates that the $\text{C}_{(4)}$ -OH and epoxide moieties of (+)-isoepoxydon (compound UIII) have a *trans* orientation. Furthermore the identity between the J values for (+)-isoepoxydon, (\pm)-epiepoxydon, and (-)-panepoxydon is striking and suggests that not only are they all *trans* isomers, but that they also

possess the same conformation (Fig. 2). A careful analysis of molecular models and their predicted coupling constants supported this conclusion. Thus as indicated in Table 2 the calculated ratio of the short-range coupling constants J_{4-3} and J_{5-4} varies dramatically for the four structural possibilities given in Fig. 2. From Table 1 *iso*-, *epi*- and *pan*-epoxydon all possess a ratio of 5 and hence must be either the (+)- or (-)-*trans* (*a*-OH) isomer. In contrast, the ratio of 1.0 for (+)-epoxydon suggests either the *cis* (*a*-OH) isomer or a 1:1 mixture of the *a*-OH and *e*-OH conformers. Long-range couplings are often of even greater stereochemical significance (Read & Ruiz, 1970). Thus couplings through four sigma bonds arranged in an approximately planar all-*trans* or 'w' conformation exhibit J values of approx. 1-3 Hz, whereas non-planar arrangements yield values close to zero (McFarlane, 1972). For the *trans*-isomers, only the *a*-OH conformer possesses a semi-planar or planar conformation of the four bonds that join H^6 to H^4 and H^5 to H^3 respectively. The values of J_{6-4} 1 Hz and J_{5-3} 2.5 Hz are in accord with this prediction. For the *cis*-isomers, values of 0 and 2.5 Hz for the same two coupling constants suggest that the *e*-OH conformer predominates. Homoallylic long-range coupling through four sigma bonds and a central double bond can yield coupling constants in the range 0-4 Hz if neither proton is in the plane of the intervening four carbon atoms (McFarlane, 1972).

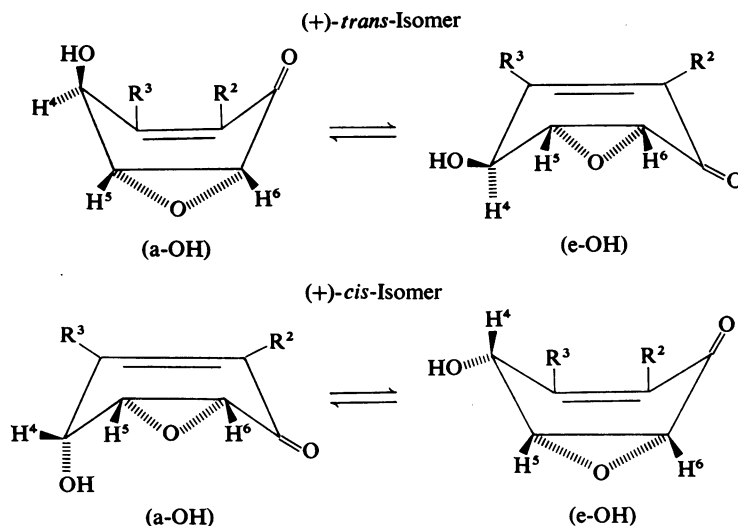


Fig. 2. Principal conformers of the *trans*- and *cis*-isomers of a partially reduced epoxyquinone

The designations *trans* and *cis* refer to the relative orientation of the epoxide and $C_{(4)}$ -OH groups, whereas the conformers are distinguished by the axial (a) or equatorial (e) position of the $C_{(4)}$ -OH group. Each of the above structures can also exist as its mirror image (–) to yield four other structures. The substituents R^2 and R^3 are respectively, for epoxydon and iso- or epi-epoxydon, $-\text{CH}_2\text{OH}$ and $-\text{H}$, for panepoxydon, $-\text{CHOH}-\text{CH}=\text{C}(\text{CH}_3)_2$ and $-\text{H}$, for terremutin and epiterremutin, $-\text{CH}_3$ and $-\text{OH}$, and for isopanepoxydon, $-\text{H}$ and $-\text{CH}=\text{CH}-\text{COH}(\text{CH}_3)_2$.

Table 1. ^1H n.m.r. coupling constants for isoepoxydon and related partially reduced epoxyquinones

The structures and nomenclature for these compounds are given in the legend to Fig. 2. For the complete spectrum of isoepoxydon see Fig. 1. Some values have not been determined (?), whereas others are absent for structural reasons (–).

	$J(\text{H}_2)$					
	Short-range coupling			Long-range coupling		
	$^3J_{6-5}$	$^3J_{5-4}$	$^3J_{4-3}$	$^4J_{6-4}$	$^4J_{5-3}$	$^5J_{4-a}$
<i>trans</i> -Isomers						
(+)-Isoepoxydon (compound UIII)	3.8	1.0	5.0	1.0	2.5	1.0
(±)-Epiepoxydon*	3.5	?	5.0	1.0	2.5	?
(–)-Panepoxydon†	3.5	1.0	5.0	1.0	2.5	<1.0
(–)-Terremutin‡	3.9	1.4	–	1.1	–	0
<i>cis</i> -Isomers						
(+)-Epoxydon§	4.0	2.5	2.5	0	2.5	?
(±)-Epiterremutin‡	4.5	2.7	–	0	–	0.75
(+)-Isopanepoxydon†	4.0	3.0	–	?	–	–

* In ^2H acetone (Ichihara *et al.*, 1976).

† In ^2H chloroform (Kis *et al.*, 1970).

‡ In dimethyl sulphoxide/ $^2\text{H}_2\text{O}$ (Read & Ruiz, 1970).

§ In ^2H dimethyl sulphoxide (Closse *et al.*, 1966; Kis *et al.*, 1970).

The magnitude of constant J_{4-a} (Table 1) indicates that such coupling does occur. The expected differences between conformers are, however, of uncertain diagnostic value.

Isoepoxydon (compound UIII) also possessed o.r.d. spectra-{positive Cotton effects; $[\alpha]_D^{32}+245^\circ$, $[\alpha]_{369}^{32}+3370^\circ$; $[\alpha]_{319}^{32}-1920^\circ$; $[\alpha]_{340}^{32}0^\circ$ (c 0.0756 in dioxan);

$[\alpha]_D^{32}+206^\circ$; $[\alpha]_{363}^{32}+2875^\circ$; $[\alpha]_{313}^{32}-1760^\circ$; $[\alpha]_{336}^{32}0^\circ$ (c 0.1696 in methanol)} and c.d. spectra $[\Delta\epsilon_{396} 0$, $\Delta\epsilon_{342}+2.30$, $\Delta\epsilon_{279} 0$, $\Delta\epsilon_{245}+8.75$, $\Delta\epsilon_{227} 0$, $\Delta\epsilon_{216}-4.06$ (c 0.0756 in dioxan at 32°C); $\Delta\epsilon_{395} 0$, $\Delta\epsilon_{339}+2.05$, $\Delta\epsilon_{282}+0.185$, $\Delta\epsilon_{244}+7.67$, $\Delta\epsilon_{227} 0$, $\Delta\epsilon_{216}-4.29$ litre \cdot mol $^{-1}$ \cdot cm $^{-1}$ (c 0.1696 in methanol at 32°C)] that were distinct from those of known partially reduced epoxyquinones

Table 2. Short-range ^1H n.m.r. coupling constants (3J) predicted from the dihedral angles (Φ) of Dreiding models of the partially reduced epoxyquinones depicted in Fig. 2

The approximation of Karplus (1959) for a saturated H-C-C-H fragment was used: $^3J_{\text{H-H}} = 8.5 \cos^2\Phi - 0.28$ for $0^\circ \leq \Phi \leq 90^\circ$ and $^3J_{\text{H-H}} = 9.5 \cos^2\Phi - 0.28$ for $90^\circ \leq \Phi \leq 180^\circ$.

	Approximate dihedral angle (degrees)		Calculated J (Hz)		
	H ⁵ -H ⁴	H ⁴ -H ³	J_{5-4}	J_{4-3}	J_{4-3}/J_{5-4}
<i>trans</i> (a-OH)	60	20	1.85	7.2	3.89*
<i>trans</i> (e-OH)	140	100	5.3	0.01	0.002*
<i>cis</i> (a-OH)	25	20	6.7	7.2	1.08†
<i>cis</i> (e-OH)	60	100	1.85	0.01	0.005†

* A 1:1 mixture of both *trans*-conformers would yield a ratio of 1.01.

† A 1:1 mixture of both *cis*-conformers would yield a ratio of 0.85.

Table 3. O.r.d. and c.d. data for isoepoxydon and related partially reduced epoxyquinones

The structures and nomenclature for these compounds are given in the legend to Fig. 2. C.d. data were obtained in dioxan unless otherwise indicated.

	$[\alpha]_D^{20-32}$ (solvent) ^o	R-Band $\Delta\epsilon$ (litre·mol ⁻¹ ·cm ⁻¹) [λ (nm)]	K-Band $\Delta\epsilon$ (litre·mol ⁻¹ ·cm ⁻¹) [λ (nm)]
<i>trans</i> -Isomers			
(+)-Isoepoxydon (compound UIII)	+206 (methanol)	+2.30 (342)	+8.75 (245)
(-)-Panepoxydon †	-61 (dichloromethane)	-1.81 (341)	-4.85 (246)
(-)-Terremutin ‡	-269 (methanol)	-1.64 (318)	-0.42 (265)
<i>cis</i> -Isomers			
(+)-Epoxydon§	+93 (methanol)	+4.70 (341)	-5.86 (245)
(+)-Isopanepoxydon†	+163 (methanol)	+5.04 (345)*	-1.48 (240)*

* C.d. data ($\Delta\epsilon$) were obtained in ethanol.

† Data from Kis *et al.* (1970).

‡ Data from Miller (1968).

§ Data from Closse *et al.* (1966).

(Table 3) and that yielded invaluable structural information. Correlations of the c.d. spectra and configurations of α -epoxyketones have shown that the sign of the R-band at approx. 340 nm is dictated by the octant in which the epoxy oxygen atom lies, if an inverse octant rule is used (Crabbé, 1965; Snatzke & Snatzke, 1973). This empirical epoxyketone rule is also known to take precedence over $\alpha\beta$ -enone or $\alpha\beta$ - $\beta\gamma$ -dienone moieties, as well as other substituents (Kis *et al.*, 1970). As shown in Fig. 3, the application of this rule to the reduced epoxyquinones in Table 3 clearly predicts the correct sign for the R-band of these compounds. Stated differently, a positive R-band indicates that an α -epoxyketone moiety will have the configuration shown in Fig. 1, 3(a) or 3(c). Although this rule decreases the eight possibilities to four (i.e. Fig. 3a or 3c) no distinction between the (+)-*cis*- and (+)-*trans*-isomers, nor the a-OH or e-OH conformers is possible. If, however, one considers the sign of the K-band at about 245 nm, it would appear that this sign is predicted by the inverse octant position of the $\alpha\beta$ -enone chromophore (Fig. 3). Thus two possible structures (Fig. 3a) for isoepoxydon remain (Table 3).

One of these is the *trans* (a-OH) structure identified by the proton-proton coupling constants. This K-band correlation seems reasonable (Crabbé, 1965; Snatzke & Snatzke, 1973), but empirical rules for the K-band have not been well established. Despite this uncertainty the combination of ^1H n.m.r. and c.d. spectra clearly established the absolute configuration and probable conformation of isoepoxydon (compound UIII) to be that of the (+)-*trans* (a-OH) isomer (Fig. 2).

Antibiotic activity

By using a standard agar plate diffusion assay (Sekiguchi & Gaucher, 1978), a relative measure of the antibiotic activity of isoepoxydon against *Bacillus subtilis* sp. showed that at equal concentrations in the range 1-5 mm, phyllostine and isoepoxydon exhibit about 81 and 56% of the activity obtained with patulin respectively. Alternatively, to obtain an inhibition equal to that of 1 mm-patulin, phyllostine and isoepoxydon must be present at concentrations of 3 and 10 mm respectively.

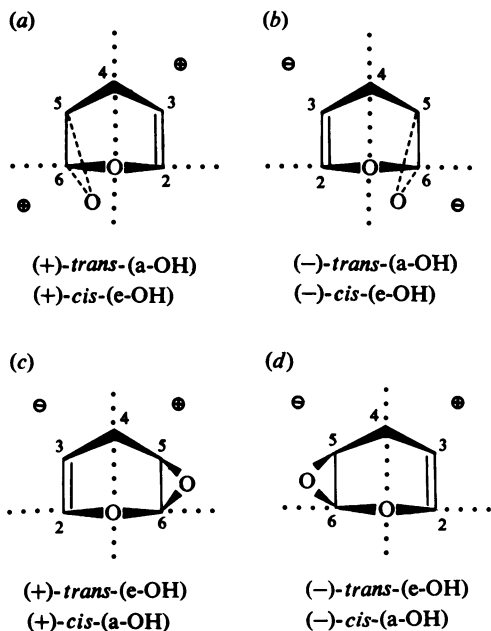


Fig. 3. Octant-rule projections of the eight stereo and conformational isomers of the partially reduced epoxy-quinones (see Fig. 2)

The signs of the rear quadrants are in accord with the inverse octant rule. Figs. 3(a) and 3(b) are mirror images, as are Figs. 3(c) and 3(d), whereas Figs. 3(a) and 3(c), and Figs. 3(b) and 3(d) are conformer pairs. Note that the configuration at C₍₄₎ does not influence the c.d. spectrum, but does determine the designation *trans* or *cis* and a-OH or e-OH.

Conversion of unlabelled isoepoxydon into patulin by *P. urticae* mutant J2

The observation that isoepoxydon is a patulin precursor was confirmed by employing a mutant that accumulates gentisyl metabolites, but not patulin or the epoxides phyllostine and isoepoxydon. Mutant J2 is, however, efficient in converting phyllostine into patulin (Sekiguchi & Gaucher, 1978). Thus the metabolism of isoepoxydon (1 $\mu\text{mol/ml}$) by a washed-cell suspension of mutant J2 in phosphate buffer (pH 6.5) was examined qualitatively and quantitatively as described in the Materials and Methods section. A careful t.l.c. analysis showed that isoepoxydon (compound UIII) was lost over a 5 h period, whereas patulin was clearly present after 30 min and continued to accumulate thereafter. The conversion was a clean one since the only other metabolites that appeared (toluquinol > gentisyl alcohol \gg *m*-hydroxybenzyl alcohol) were excreted by the control in the absence of any added isoepoxydon. A quantitative assessment of this conversion (Fig. 5) showed that in a period of 3–5 h a better than 60% conversion of isoepoxydon into patulin was obtained. This quantification was confirmed by a g.l.c. analysis that indicated a patulin concentration of 0.56 mM at 5 h after the addition of isoepoxydon, and a complete absence of patulin in the control. This technique was also used to show that the patulin produced from isoepoxydon co-chromatographed with an authentic sample of the antibiotic.

General Discussion

The new fungal metabolite (+)-isoepoxydon is similar to its co-metabolite (-)-phyllostine in a number of respects. Both are efficient patulin precursors, both are not normally extracellular metabolites, and both are probably obligatory rapidly turning over intermediates involved in the oxidative fission of the aromatic ring of either gentisyl alcohol or gentisaldehyde. This latter suggestion is strongly supported by the chiral α -epoxy-ketone moiety that both compounds possess. At present, published (Sekiguchi & Gaucher, 1978) and unpublished observations (J. Sekiguchi & G. M. Gaucher) suggest that isoepoxydon is an obligatory precursor of phyllostine rather than a rapidly equilibrated side product of phyllostine. Its exact position in the patulin pathway is, however, yet to be proven.

The structures of these two epoxides suggests that they are metabolically interconverted by a simple alcohol dehydrogenase-mediated redox reaction. In fact cell-free extracts of *P. urticae* (N.R.R.L. 2159A) possess an active NADP⁺-isoepoxydon dehydrogenase, which has a high substrate specificity, since it would not oxidize an authentic sample of epoxydon,

Conversion of [¹⁴C]isoepoxydon into patulin by the parent strain of *P. urticae*

When added to a shake culture of *P. urticae*, [¹⁴C]isoepoxydon (compound UIII) was readily incorporated into patulin (Fig. 4). Thus after an incubation of 30 min (Fig. 4a) and 1 h (Fig. 4b), a radiochromatogram of an extracellular extract showed radioactivity to be present first in compound UV (*R_f* 0.69), and then in patulin (*R_f* 0.42). After 2 and 3 h (Figs. 4c and 4d) more than 90% of the ¹⁴C on each chromatogram was present in patulin. Since not even a transient incorporation of ¹⁴C into any other known pathway metabolite was observed, it was clear that as a patulin precursor, isoepoxydon must be positioned past the gentisyl metabolites in the pathway, as is the related metabolite phyllostine (Sekiguchi & Gaucher, 1978). Since phyllostine is a normally undetectable transient intermediate, its position in the pathway relative to isoepoxydon cannot be ascertained by these results.

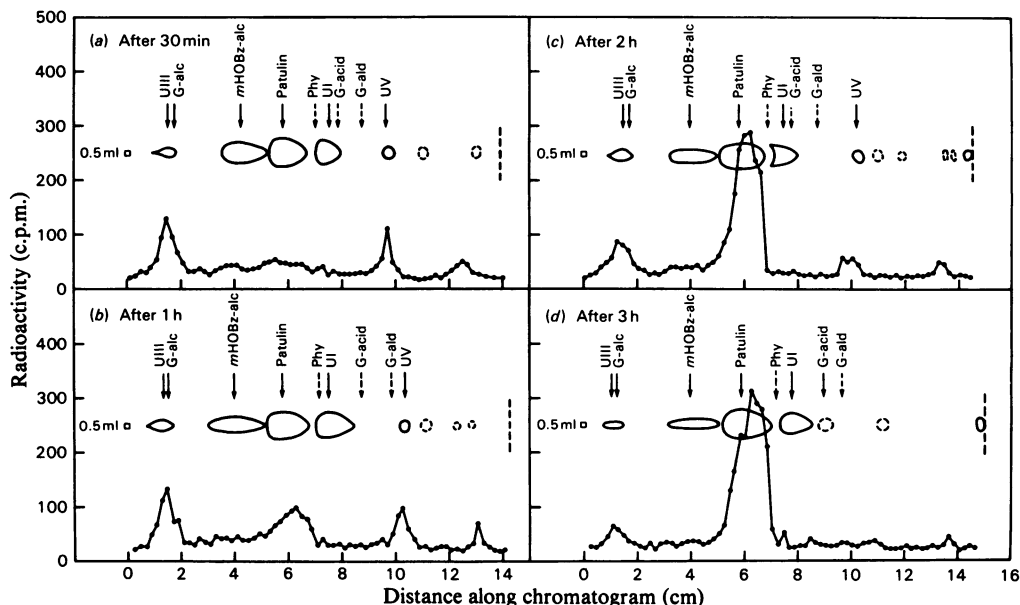


Fig. 4. Thin-layer radiochromatograms of extracts obtained at various times after addition of [^{14}C]isoeopoxydon (compound UIII) to a 34 h-old shake culture of *P. urticae* (N.R.R.L. 2159A)

The entire extract from a 0.5 ml sample of culture filtrate was spotted onto each t.l.c. plate. The chromatogram, as seen under u.v. light, is given above each radiochromatogram. A 5 min extract gave a chromatogram with a uniform background (approx. 25 c.p.m.) except for a peak (approx. 120 c.p.m.) corresponding to isoeopoxydon (compound UIII). Isoeopoxydon and patulin had R_F values of 0.09 and 0.42 respectively. The abbreviations for the various metabolites are for gentisyl alcohol, gentisic acid and gentisaldehyde, G-alc, G-acid and G-ald respectively, for *m*-hydroxybenzyl alcohol, *m*HOBz-alc, and for phyllostine, Phy.

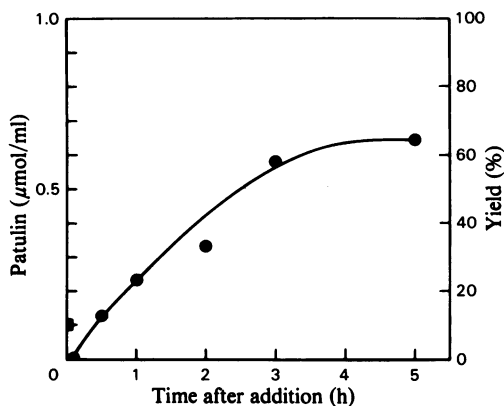


Fig. 5 Quantitative assessment of the conversion of unlabelled isoeopoxydon (compound UIII) into patulin by a washed-cell suspension of *P. urticae* mutant J2

The time course of this conversion was determined by measuring the antibiotic activity of the patulin produced as described in the text. The initial 1 mM solution of isoeopoxydon had an antibiotic activity equivalent to that of a 0.1 mM solution of patulin (■).

the (+)-*cis*-isomer (Fig. 2) of its natural substrate isoeopoxydon (J. Sekiguchi & G. M. Gaucher, unpublished work). Structurally similar epoxyquinone and partially reduced epoxyquinone pairs co-occur in a number of other fungi. A *Phyllosticta* sp. (Sakamura *et al.*, 1969, 1970) produces the pair (–)-phyllostine and (+)-epoxydon, in which the stereochemistry of the epoxyketone moiety is identical with that found in *P. urticae*, whereas the configuration of the chiral hydroxy group in the partially reduced quinone is opposite (see Table 3). Similar pairs of metabolites that possess an epoxyketone moiety of opposite chirality (Kis *et al.*, 1970) are produced by *Aspergillus terreus* [i.e. (–)-terreic acid and (–)-terremutin] and by *Panus radis* and *Panus conchatus* [i.e. (+)-panepoxydion and (–)-panepoxydion]. Except for this latter pair of metabolites, the others have been shown to be synthesized from acetate via 6-methylsalicylic acid (Read *et al.*, 1969; Nabeta *et al.*, 1975).

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