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The Biosynthesis of Patulin

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The biosynthesis of the antibiotic patulin (II) from 6-methylsalicyclic acid (6-MSA) (I) in replacement cultures of *Penicillium patulum* has been examined with ^{2}H and $^{14}C/^{3}H$-labeled intermediates. Efficient utilization of *m*-cresol (IX), *m*-hydroxybenzyl alcohol (V), *m*-hydroxybenzaldehyde (XII), gentisaldehyde (VIII), and gentisyl alcohol (VI) could be demonstrated. Toluquinol (X), although inactive as a precursor of patulin, is converted by refloated cultures of *P. patulum* to desoxyepoxydon (XIII). *Para*-hydroxylation of *m*-cresol proceeds with loss of deuterium at the *p*-position. Side chain labeling of the aromatic precursors is lost in the conversion. A major portion of this work has been reported in preliminary form (*1*, *2*).

The mold *Penicillium patulum* produces a series of phenolic secondary metabolites derived *via* the acetate–malonate pathway. The prototype of aromatic compounds derived from polyketide metabolism, 6-methylsalicyclic acid (6-MSA) (I), forms the pivotal compound in the metabolic grid (Scheme 1) evolved by Bu'Lock as a working hypothesis to rationalize the interrelationships of the variety of compounds found in this organism. At the time of construction of the original grid (*3*) these interrelationships were confined to the experimental observations that 6-MSA biosynthesized from [1-^{14}C]acetate served as a precursor for the intriguing hemiacetal lactone, patulin (II) according to the pattern depicted in Scheme 1, and further that the metabolites 6-formylsalicyclic acid (*4*) (III), 3-hydroxyphthalic acid (*4*) (IV), *m*-hydroxybenzyl alcohol (*5*) (V), gentisyl alcohol (*6*) (VI), gentisic acid (*7*) (VII), and gentisaldehyde (*4*) (VIII) were present in the fermentation medium. The speculation (*3*) that *m*-cresol (IX) and toluquinol (X) might also form part of the grid (*8*, *9*) was later confirmed by the isolation (*1*) of these metabolites. The occurrence of triacetic lactone (TAL) (XI) in *P. patulum* has also been observed by several independent groups of investigators (*10–12*). As a preliminary to a study of the enzymology of the polyketide pathway, we have examined the incorporation of singly and multiply labeled intermediates into patulin with replacement cultures of the organism. Certain of these transformations were then chosen for study at the cell-free level (*13*).

As a fortunate consequence of the choice of this organism, the specific incorporations achieved by the replacement culture technique are sufficiently high (15–95%) to allow rapid evaluation of the pathway by direct mass spectrometric analysis following administration of deuterated intermediates.

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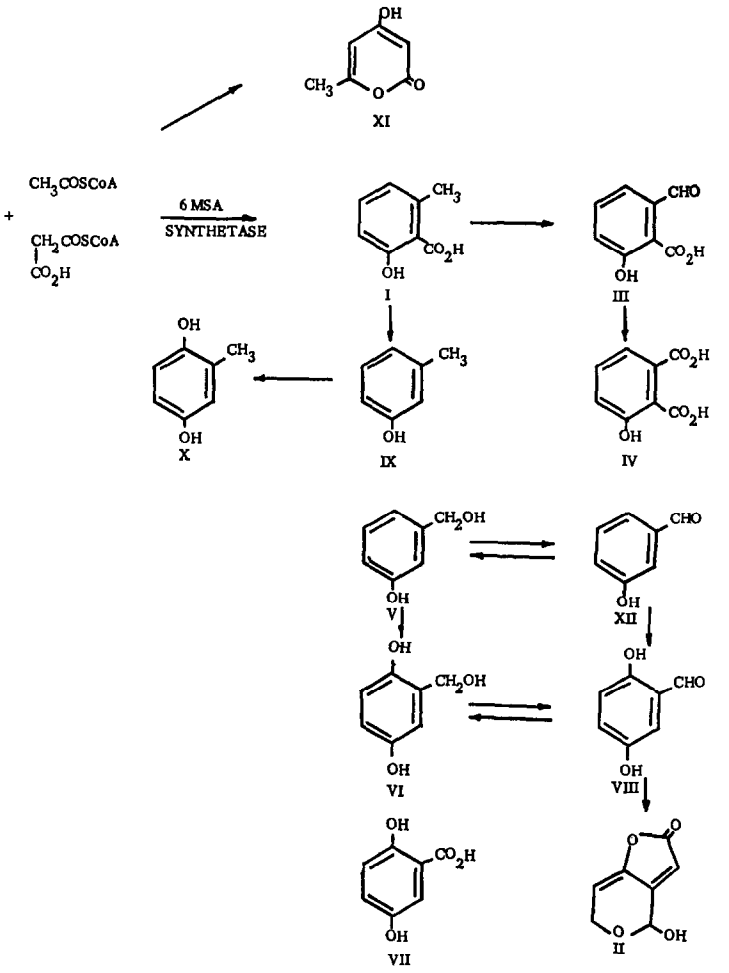
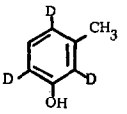
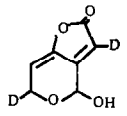


SCHEME 1. Metabolic conversion of 6-MSA to patulin.

The first intermediate in patulin biosynthesis to be discovered (*3*, *14*) was 6-MSA (I) which, as indicated in Scheme 1, is transformed to patulin by a hitherto unknown but frequently presumed sequence. With the exception of gentisic acid (VII) (which is not a precursor of patulin), none of the metabolites in Scheme 1 has been assigned other than a putative intermediary role. Based on the concept that decarboxylation is the first step in the sequence, our investigation began with the search for and discovery of *m*-cresol (IX) in normal fermentation broths, and in fact 6-MSA decarboxylase has been subsequently described (*15*). When [2,4,6-$^2H_3$]-*m*-cresol was administered to refloated cultures of *P. patulum*, the resultant [5,7-^{2}H]-patulin was enriched to the extent of 93%. The complete loss of one deuterium atom from *m*-cresol during this process is in accord with a *para*-hydroxylation mechanism in which the intermediate suffers loss of a deuterium rather than undergoing N. I. H. shift (*16*) with retention of the deuterium atom.

TABLE 1

PERCENT ENRICHMENT IN [2,4,6-^{2}H]-*m*-CRESOL FEEDING[a]

COMPOUND FED	%do	%d_1	%d_2	%d_3	% D	NMR	% Inc.
	5.1	25.7	–	69.2	77.8	96	–
COMPOUND ISOLATED							
	18.3	13.9	67.8	–	74.7	–	92.7

%d_0, d_1, d_2, d_3 represent the percent enrichment in deuterium obtained from mass spectrometry at the molecular ion (M_0), at M + 1, M + 2 and M + 3 respectively, corrected for the natural abundances at M + 1, M + 2.

%D ≡ %^{2}H mass spectrometry≡ represents the total percent enrichment in deuterium relative to the exchangeable hydrogens and therefore can be compared to the %^{2}H obtained from NMR.

$$\%\text{Inc} = \frac{\%^2\text{H in Patulin}}{\%^2\text{H in Precursor}}.$$

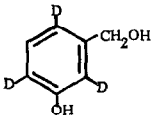
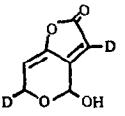
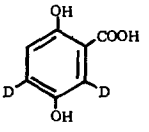
[a] Shake culture.

Similarly it was shown that [2,4,6-$^2H_3$]-*m*-hydroxybenzyl alcohol was bioconverted to [5,7-$^2H_2$]-patulin (Table 2) (60% enrichment of ^{2}H) and to gentisic acid (73%) again with loss of deuterium *para* to the phenolic hydroxyl group. When ring-labeled gentisaldehyde was fed to replacement cultures, patulin again showed enrichment corresponding to 86.6% of a d_2 species (Table 3). The foregoing experiment was carried out on a sample where the position of the label was either 2,4 or 4,5 so that the label positions in patulin (see Table 3) are not yet defined with certainty. The corresponding alcohol (VI) and acid (VII) isolated from this experiment (Table 3) however also showed remarkably high enrichment (77.7 and 100%, respectively). A possible intermediate, toluquinol (*1*) (X), was also isolated from normal fermentations. When [2,4,5-$^2H_3$]-toluquinol was fed to *P. patulum*, patulin was isolated with no enrichment above natural abundance of the M + 1 → M + 3 peaks in the mass spectrum (Table 4). However, from a large-scale incubation of toluquinol an optically active epoxide could be isolated. Its structure was assigned as (XIII) on the following evidence.

The molecular formula $C_7H_8O_3$ was confirmed by mass spectrometry. In the infrared the presence of hydroxyl (3410 cm^{-1}) and α,β unsaturated ketone (1655 cm^{-1}) were inferred. The latter assignment was also in accord with the uv spectrum λ_{max} 242 nm ($\varepsilon = 4700$). The nmr spectrum of the epoxide displayed a methyl singlet at τ 8.2, a doublet at τ 6.52 (H_2) coupled to H_3 which appears as a multiplet at τ 6.2. The H_4 is centered at τ 5.28 as a multiplet and the vinyl H_5 at τ 3.54. These assignments may be compared with the data for epoxydon (XIV), a metabolite of *Phoma* S1019 which also shows (*17*) H_2 as a doublet (τ 6.60), H_3 at τ 6.23, H_4 at τ 5.36 and H_5 at τ 3.58. The close

TABLE 2

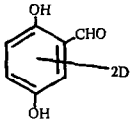
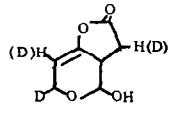
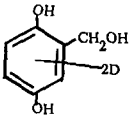
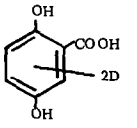
PERCENT ENRICHMENT IN [2,4,6-^{2}H]-*m*-HYDROXYBENZYL ALCOHOL FEEDING[a]

COMPOUND FED	%d_0	%d_1	%d_2	%d_3	%D	NMR	%Inc.
	5.6	10.5	–	83.9	87.4	95	–
COMPOUNDS ISOLATED							
	33.2	6.5	60.2	–	54.4	–	60
	27.1	10.1	58.6	–	66.4	–	73

[a] Shake culture.

TABLE 3

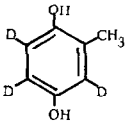
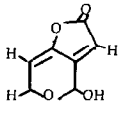
PERCENT ENRICHMENT IN DIDEUTEROGENTISALDEHYDE FEEDING[a]

COMPOUND FED	%d_0	%d_1	%d_2	%d_3	%D	NMR	%Inc.
	28.	40.4	30.6	–	51.3	53.	–
COMPOUNDS ISOLATED							
	35.2	43.1	21.7	–	43.3	–	86.6
	41.9	38.5	19.6	–	38.9	–	77.7
	7.4	59.3	33.3	–	62.9	–	100.

[a] Shake culture.

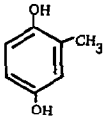
TABLE 4

PERCENT ENRICHMENT IN [2,4,6-^{2}H]-TOLUQUINOL FEEDING[a]

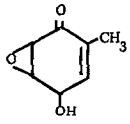
COMPOUND FED	%d_0	%d_1	%d_2	%d_3	%D	NMR	%Inc.
	5.3	12.4	16.9	70.7	76.6	83.3	—
COMPOUND ISOLATED							
	100.	–	–	–	–	–	0

[a] Shake culture.

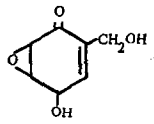
similarity of these resonances suggests that the new metabolite, a product of "forced feeding" of toluquinol to *P. patulum* is in fact desoxyepoxidon (XIII). The determination of the absolute configuration and a total synthesis of this compound will form the basis of a subsequent paper in this series.



X

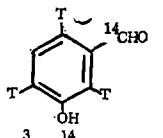


XIII



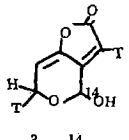
XIV

In order to confirm that the deuterated label in the above phenolic substrates was secure against exchange reactions during intermediary metabolism, or at the end of the experiment, a sample of (2,4,6-^{3}H; [^{14}C]-formyl) *m*-hydroxybenzaldehyde (XV) (^{3}H/^{14}C, 7.9) was administered to the organism. The resultant patulin (XVI) was purified to constant radioactivity and then had ^{3}H/^{14}C = 5.7. On the basis of the



^{3}H/C^{14} 7.9

XV



^{3}H/C^{14} 5.7

XVI

previously noted loss of one deuterium (or ^{3}H) atom the result agrees quite well with theory (^{3}H/^{14}C = 5.3), the slightly high ratio in patulin implying *either* an isotope effect and/or some retention (<20%) of the *para*-^{3}H in (XV). In a second experiment patulin was added to a resuspended culture in the presence of D_2O. After normal work up, mass spectrometry analysis of reisolated patulin showed that no detectable (<2%) exchange had occurred.

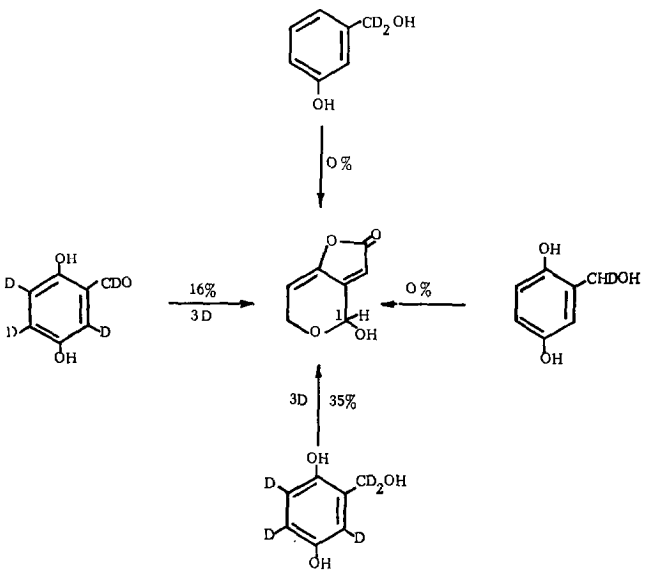
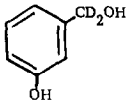
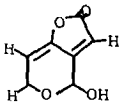
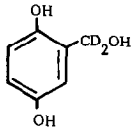


FIG. 1. Specific incorporation (from mass spectrometry) side chain deuterated compounds.

TABLE 5

PERCENT ENRICHMENT IN [1′,1′-^{2}H]-*m*-HYDROXYBENZYL ALCOHOL FEEDING[a]

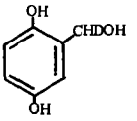
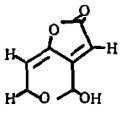
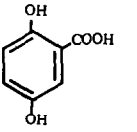
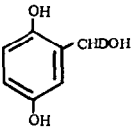
COMPOUND FED	$\%d_0$	$\%d_1$	$\%d_2$	$\%d_3$	%D	NMR	%Inc.
	10.2	–	89.8	–	90	100.	–
COMPOUNDS ISOLATED							
	93.6	1.39	–	–	0	–	0
	8.5	8.5	83.0	–	87.3	–	91.3

[a] Shake culture

With unequivocal proof for the retention of nuclear ^{2}H-labels in hand, we next examined the fate of the side chain protons in the same intermediates. The results of these experiments are shown in Tables 5–8 and are summarized in Fig. 1. The surprising

TABLE 6

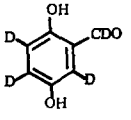
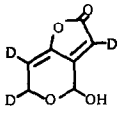
PERCENT ENRICHMENT IF [1′-^{2}H]-GENTISYL ALCOHOL FEEDING[a]

COMPOUND FED	$\%d_0$	$\%d_1$	$\%d_2$	$\%d_3$	%D	NMR	%Inc.
	29.8	70.2	–	–	70.2	68	–
COMPOUNDS ISOLATED							
	100	–	–	–	0	–	0
	98.7	–	–	–	–	–	0
	23.8	76.0	–	–	74	–	108

[a] Shake culture.

TABLE 7

PERCENT ENRICHMENT IN [2,4,5,(1′)-^{2}H]-GENTISALDEHYDE FEEDING[a]

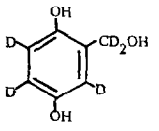
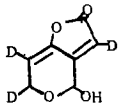
COMPOUND FED	$\%d_0$	$\%d_1$	$\%d_2$	$\%d_3$	$\%d_4$	%D	NMR	%Inc.
	11.3	5.41	38.9	29.2	15.3	57.9	57.0	–
COMPOUND ISOLATED								
	86	3.93	6.1	4.0	–	9.36	–	16.2

[a] Still culture.

result emerges that in each case studied, there is no incorporation of deuterium from the labeled side chain into the hemiacetal proton (H_1) at C_1 of patulin (Fig. 1). Differential labeling of nucleus and side chain (Tables 7, 8) give rise to patulin with three of the four

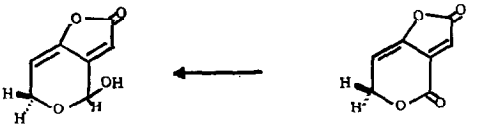
TABLE 8

PERCENT ENRICHMENT[a] IN [2,4,5,(1′,1′)-^{2}H]-GENTISYL ALCOHOL FEEDING[b]

COMPOUND FED	$\%d_0$	$\%d_1$	$\%d_2$	$\%d_3$	$\%d_4$	$\%d_5$	%D	% Inc.
	1.5	4.5	13.8	29.8	33.9	16.5	67.9	–
COMPOUND ISOLATED								
	63.2	11.1	16.4	9.2	–	–	23.9	35.2

[a] Measured at 20 cv. [b] Still culture.

original deuterons retained. On the basis of the earlier experiments with nuclear labeling, all three of the deuterium atoms (at 2,4,5) are retained. Thus in spite of the rather low incorporation shown in Tables 7 and 8, retention of label in the —CD_2OH and CHDOH side chain from *m*-hydroxybenzyl alcohol (Table 5) to gentisyl alcohol (91.3%) and the recovery of the —CHDOH label in gentisyl alcohol (Table 6) during transformations to gentisic acid and patulin suggest that this oxidative loss of deuterium is intimately involved in the final stages of the mechanism. It has been shown that patulin does not exchange the C_1 proton in D_2O under the experimental conditions and further that gentisic acid is not an intermediate (*14*). An attractive explanation of these facts invokes the equilibrium shown in Fig. 2 between patulin and the hypothetical shunt metabolite, patulin lactone (XVII). The latter compound was therefore prepared by oxidation of patulin with Jones' reagent (*18*) and exhibited the following properties.



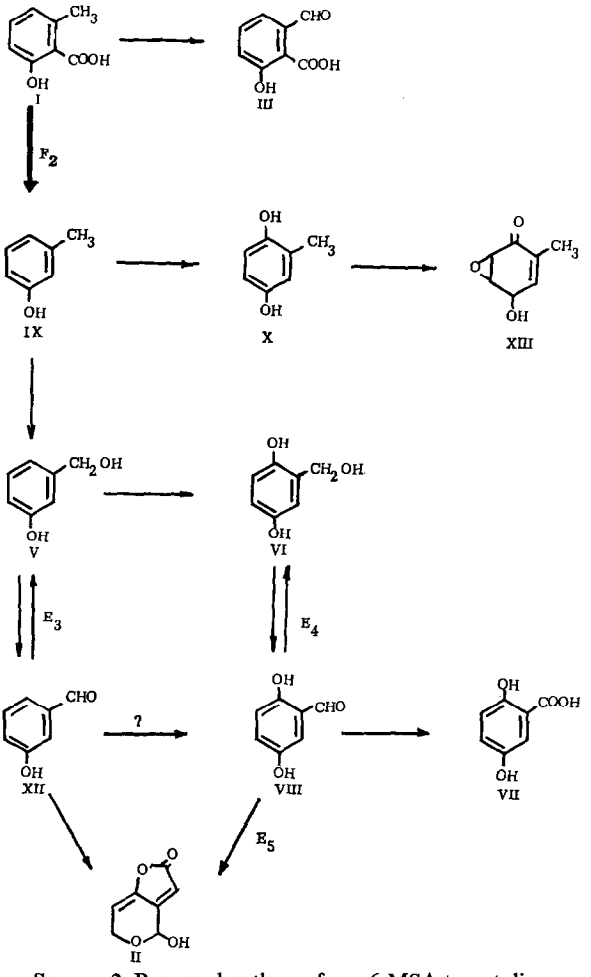
II XVII

FIG. 2

Its molecular formula from high resolution mass spectrometry is $C_7H_4O_4$, exact mass: 152.0109. In the infrared patulin lactone (mp 127.5–130°C) displays no hydroxyl frequency and the carbonyl region contains the expected $\alpha,\beta,\gamma,\delta$ unsaturated γ-lactone and cross conjugated δ lactone C=O stretching frequencies at 1730–1750 and 1770–1780 cm^{-1}. The uv spectrum is red-shifted from that of patulin (λ 278 nm) to λ_{max} 294 (ε 7920). The vinyl protons of the lactone are found in the 100 MHz nmr spectrum (d_6-acetone) at τ 3.26 (H-7) (multiplet) and τ 3.66 (H-4) (multiplet). The remaining two methylene protons occur as a complex multiplet centered at τ 4.63. Irradiation of the CH_2 group

resulted in two doublets centered at τ 3.26 and τ 3.66, the coupling constant being $J_{4,7} = 1.0$ Hz. Proof of the existence of patulin lactone in normal fermentations has not yet been obtained.

The above results allow the revision of the main pathway from 6-MSA to patulin as shown in Scheme 2. However, rigorous proof of the obligatory nature of each of the principal intermediates established in the present study must await the completion of



SCHEME 2. Proposed pathway fro 6-MSA to patulin.

our cell free experiments (*13*). Several of these have been described (*2*), but two main pathways can be considered for the oxidative cleavage of the aromatic ring in the penultimate stages of patulin biosynthesis. These are shown in Figs. 3 and 4. In the first of these (Fig. 3) *m*-hydroxybenzaldehyde (XII) is cleaved by a dioxygenase to give the species (XVIII). Direct cyclization and prototropy affords patulin in a process which requires no further adjustment of oxidation level. The second mechanism (Fig. 4) utilizes gentisaldehyde (VIII) as substrate and indeed has appeared so frequently as a hypothesis

that this latter mechanism has tended to be accepted without any experimental proof! This second mechanism also depends on oxidative cleavage (dioxygenase) followed by reduction of one of the new aldehyde functions to alcohol, lactonization and hemiacetal formation. The fact that patulin occurs as a racemate bears strongly on the latter part of this proposed pathway, for it must be assumed *either* that the final hemiacetal

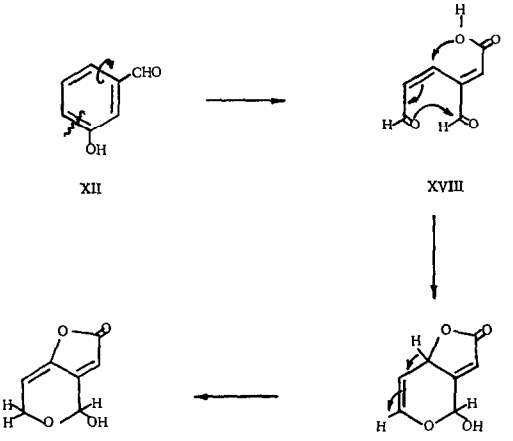


FIG. 3

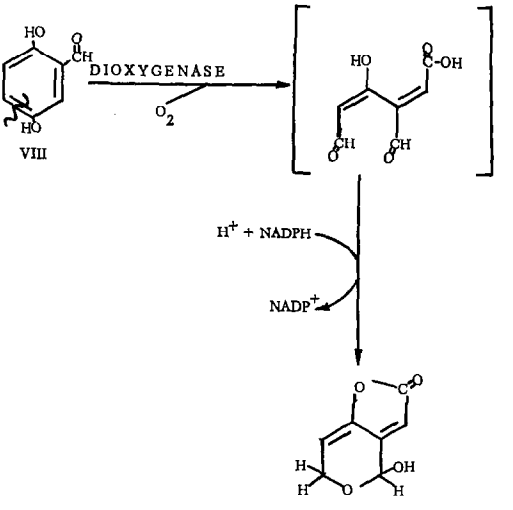


FIG. 4

closure is nonspecific *or* that racemization occurs rather easily (e.g., during work up). In this connection an interesting observation was made during those experiments in which deuterated *m*-cresol, *m*-hydroxybenzyl alcohol, and gentisaldehyde served as the source of [5,7-$^2H_2$]-patulin. Since C_5 of patulin is prochiral, the presence of deuterium at this position, which is reduced from aldehyde to alcohol (presumably by NADPH)

(Fig. 4) allows the isolation of a single diastereomer, 5-(R) or (S)-^{2}H-1-(R) or (S)-patulin (Fig. 5). The absolute configuration of this material has not been established, but in our preliminary communication (*1*) it was noted that the compound had an unusually large molecular rotation at 300 nm. Many subsequent runs with larger quantities of deuterated substrate suggest that our initial preparation was contaminated by a strongly dextrorotatory impurity. In all these subsequent runs, a reproducible value of $[\Phi]_{315}$ $-224 \pm 20°$ (corrected for optical purity) was obtained. The reasons for the earlier and quite variable ORD data for this preparation remain unexplained, but may be connected

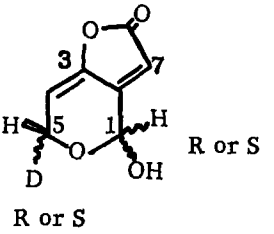


FIG. 5

with initial inexperience in working up small quantities of patulin. For example, oxidation to patulin lactone or epimerization at C_1 may well have been responsible for the previous erratic and nonreproducible values of $[\Phi]$ for [5,7-$^2H_2$]-patulin.

In summary, all of the present evidence is in accord with the revised grid (Scheme 2) and taken in conjunction with the recently demonstrated (*2, 13, 15, 19*) cell-free systems (designated E_1–E_5 in Fig. 6 and Scheme 2) provide an almost complete pathway leading

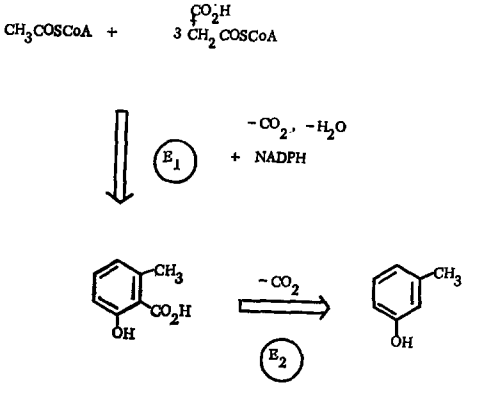


FIG. 6

to this interesting metabolite. Very recently Forrester and Gaucher (*20*) have used short-term incubation and radioassay to investigate this problem. Their results are in general qualitative agreement with those previously described in our preliminary communications (*1, 2*), although in our hands gentisyl alcohol was incorporated. Reinterpretation of the conclusions of several of these interesting short-term incubations may be necessary as further data on the obligatory role of each intermediate are obtained in the cognate enzymological studies under way in several laboratories.

EXPERIMENTAL

Materials and Methods

Analytical methods. Ultraviolet spectra (uv) were determined for spectral grade methanolic or ethereal solutions on a Beckman DBG spectrophotometer. Optical rotatory dispersion curves (ORD) were measured for methanol solution at 25°C with a Cary model 60 spectropolarimeter using 10 mm pathlength. Proton magnetic resonance (nmr) were taken on Varian HA-60 and HA-100 and Jeol MH-100 instruments. Mass spectra were taken on a Hitachi–Perkin Elmer RMU 3 mass spectrometer. Spectra were normally taken at 70 eV, unless stated otherwise, when the voltage was reduced to facilitate analyses of the peaks. It is assumed that the relative abundance ratio of the isotopes is the same in the deuterated and nondeuterated materials. The relative abundance of any deuterated species was calculated after removal of the contribution from the overlapping nondeuterated species. Melting points (mp) were determined on a Kofler "hot-stage" microscope and are uncorrected.

Thin-layer chromatograms were made on Merck Silica Gel PF_{254} containing fluorescent indicator. Chromatograms were developed with benzene:dioxan:acetic acid (90:25:4) and chloroform:acetic acid (9:1).

Organism and Growth Conditions

Penicillium patulum (NRRL 2159 A) was grown on Czapek–Dox medium containing (per liter) dextrose (40 g), sodium nitrate (3 g), potassium hydrogen phosphate (1 g), magnesium sulfate heptahydrate (0.5 g), potassium chloride (0.5 g), ferrous sulfate (0.02 g) and zinc sulfate (1 ml of a solution containing 0.1 g per liter). The pH of this medium was adjusted to 6.5 and the solution was sterilized at 15 lb psi for 15 min.

For innoculation the spore suspension from subcultures grown on Czapek–Dox agar slopes for 5 days was transferred to two 250 ml conical flasks containing 100 ml of medium and grown in shake culture on a rotary shaker at 180 rpm at 26–28°C for 4–5 days at which time patulin product was taking place. This was measured by taking the uv spectra of an ethereal extract and assaying for patulin production (λ_{max} 276 nm, ε 13,350). Alternatively the inoculum was added to 1 liter of medium and grown in still culture in 5-liter Pivotsky bottles for 8–12 days at 26–28°C.

Feeding of Deuterated Intermediates to Refloated Cultures

Cultures grown in shake flasks were washed with sterile water and suspended in an equal volume of glucose-free Czapek–Dox medium. Still cultures were similarly refloated after removal of the original medium and careful washing with glucose-free Czapek–Dox. All deuterated materials were added as aqueous solutions. Between 10–20 mg of deuterated material was fed to a shake flask containing 100 ml of medium. When still cultures were used approx 100 mg of deuterated material was administered per 1 liter of culture medium. The materials fed to shake culture and to still culture are differentiated in Results section. All compounds isolated have been previously described and were compared to synthetic samples by uv and tlc in the two aforementioned systems, and by mass spectrometry. The production of patulin was followed by uv spectroscopy and after 4–5 days the mycelium was filtered and the broth acidified to

pH 2.0, concentrated *in vacuo* and exhaustively extracted with ether or ethyl acetate. The metabolites were separated by preparative thin-layer chromatography in the aforementioned systems, eluted, and crystallized.

[*2,4,6-*^2H]-m-*Cresol.* A solution of *m*-cresol in deuterium oxide (10 ml) containing sodium metal (20 mg) was heated in a sealed tube at 130°C for 3 days. The tube was cooled and the contents acidified to pH 2.0 and extracted with ether (3 × 20 ml). The ethereal solution was washed with water (2 × 10 ml), dried (Na_2SO_4) and the ether removed by distillation. The resulting oil was distilled to give [2,4,6-^{2}H]-*m*-cresol (0.980 g), bp 102°C/20 mm.

[*2,4,6-*^2H]-m-*Hydroxybenzyl alcohol. m*-Hydroxybenzyl alcohol (0.750 g) was exchanged as described above. The product on crystallization gave [2,4,6-^{2}H]-*m*-hydroxybenzyl alcohol (0.57 g) as plates mp 68–70°C from 1,2-dichloroethane (lit.[5], mp 67°C).

m-*Hydroxybenzoic acid methyl ester.* A solution of *m*-hydroxybenzoic acid (8 g) in absolute methanol (50 ml) containing concentrated sulfuric acid (1 ml) was heated under reflux for 12 hr. the solution was cooled, neutralized with solid sodium bicarbonate, filtered and the solvent evaporated to dryness. The oily residue was triturated with ether (100 ml) and the ethereal solution washed with 1% sodium bicarbonate (3 × 100 ml), dried and the solvent evaporated. The residue was crystallized from benzene–pentane to give *m*-hydroxybenzoic acid methyl ester (6 g) needles, mp 70–71.5°C (lit.[21], mp 69–71.5°C).

m-*Hydroxybenzyl alcohol.* A solution of *m*-hydroxybenzoic acid methyl ester (5 g) in ether was added to a stirred solution (12 ml) of sodium methoxyethoxy aluminum hydride (70%) in benzene over 1 hr. The reaction mixture was stirred at room temperature for 3 hr and the resulting gel neutralized with a 10% solution of hydrochloric acid (20 ml). The ethereal layer was dried (Na_2SO_4), the solvent evaporated, and the residue crystallized from 1,2-dichloroethane to give *m*-hydroxybenzyl alcohol (2 g), mp 68–70°C.

[*1',1'-*^2H]-m-*Hydroxybenzyl alcohol.* A solution of *m*-hydroxybenzoic acid methyl ester (1 g) in ether (3 ml) was added to a stirred suspension of lithium [^{2}H]-aluminum hydride (50 mg) in ether (5 ml). The reaction mixture was stirred for 1 hr at room temperature and quenched with wet ethyl acetate, water and 1.0 *N* hydrochloric acid. The mixture was extracted with ether (3 × 20 ml), the ethereal layer dried (Na_2SO_4) and evaporated to a yellow oil which was purified by preparative thin-layer chromatography using Brinkman PF_{254} silica gel plates developed with chloroform : acetic acid (8 : 2). The compound was eluted with ether and recrystallized from 1,2-dichloroethane to give [1',1'-^{2}H]-*m*-hydroxybenzyl alcohol, mp 68–70°C.

[^2H]-*Gentisaldehyde* (*ring deuterated*). Gentisaldehyde (250 mg) was exchanged with deuterium as described for *m*-cresol. The [^{2}H]-gentisaldehyde was extracted into ethyl acetate (3 × 200 ml) and the solvent evaporated to leave a residue which on crystallization from chloroform gave [^{2}H]-gentisaldehyde (100 mg), mp 99–100.5°C (lit.[22], mp 99°C).

[*1'-*^2H]-*Gentisyl alcohol.* Sodium borodeuteride (137 mg) was added in small portions to a stirred ice-cold solution of gentisaldehyde (1 g) in tetrahydrofuran (25 ml). After 30 min when a white solid precipitates, the solvent was removed and the residue dissolved in ice-cold water (30 ml), and quickly acidified to pH 6.0. The solution was

saturated with sodium chloride and extracted with ether (3 × 30 ml) which was washed with water and dried (Na_2SO_4). The ether was removed and the resulting oil crystallized from chloroform to give [1′,1′-^{2}H]-gentisyl alcohol (360 mg) plates, mp 113–114°C (lit.[6], mp 99–100°C).

[2,3,5,6-^{2}H]-Hydroquinone. Hydroquinone (1.8 g) was added to a solution of sodium (20 mg) in deuterium oxide (30 ml) and heated in a sealed tube at 130°C for 3 days. The [2,3,5,6-^{2}H]-hydroquinone was worked up as described under [2,4,6-^{2}H]-*m*-cresol.

[2,4,5,(1′)-^{2}H]-Gentisaldehyde. This was synthezized by the method of Gatterman–Wieland (*23*). [2,3,5,6-^{2}H]-Hydroquinone (33.7 g) was added to a hot solution of sodium hydroxide (95 g) in deuterium oxide (103 ml) under a nitrogen atmosphere. The resulting solution was slowly cooled to 60°C and chloroform (42 ml) added batchwise over 1 hr to prevent foaming and left at 1.5 hr at 60°C. The solution was cooled and acidified. The solution was saturated with sodium chloride, exhaustively extracted with ether and dried (Na_2SO_4). The ether was evaporated to leave a brown solid (31.6 g) which was triturated with chloroform and filtered. The filtrate gave crude gentisaldehyde (2 g) while the remaining solid gave recovered hydroquinone (28 g). The recovered hydroquinone was recycled five times and the chloroform-soluble fractions were filtered through a pad of silica gel. The chloroform was removed and the residue crystallized from benzene to give [2,4,5,(1′)-^{2}H]-gentisaldehyde (3 g) mp 99.5–100.5°C (lit.[23], mp 99°C).

[2,4,5,(1′,1′)-^{2}H]-Gentisyl alcohol. This was prepared from [2,4,5,(1′)-^{2}H]-gentisaldehyde (1 g) and sodium borodeuteride, as described under [1′-^{2}H]-gentisyl alcohol. The preparation gave [2,4,5-(1′,1′)-^{2}H]-gentisyl alcohol (100 mg), mp 98–101°C.

[2,4,5-^{2}H]-Toluquinol. Gentisaldehyde (1 g) in ethanol (100 ml) was catalytically reduced over 10% Pd/C until 2 moles of hydrogen were taken up. The catalyst was filtered and the solvent evaporated to give an oil which on sublimation at 100°C/0.25 mm yielded toluquinol (500 mg), mp 126–217°C (lit.[7], mp 126–127°C). The toluquinol was exchanged with deuterium oxide as already described for *m*-cresol.

Attempted exchange of patulin with deuterium oxide in refloated cultures of P. patulum. The culture was grown as described previously, but washed and refloated in Czapek–Dox medium containing deuterium oxide. Patulin (20 mg per 100 ml of medium) was added and the culture grown for a further 3 days and the patulin isolated as described earlier.

[1-^{14}C]-m-Hydroxybenzaldehyde. This was synthesized from [7-^{14}C]-benzaldehyde (5.4 mCi/m*M*) by modifying the published method (*24*) to small quantities. The control of the temperature in the conversion of benzaldehyde to nitrobenzaldehyde was effected by using a calibrated thermistor ($32A_1W_9$ Victory Engineering Corp., Springfield, NJ). To 5 ml concentrated sulfuric acid and 0.43 ml nitric acid in a 25-ml three-necked flask with a thermistor and magnetic stirrer, [7-^{14}C]-benzaldehyde (1.25 g) was added dropwise keeping the temperature between 0–5°C. The reaction mixture was then warmed up to 40°C and cooled to room temperature. Ice was added slowly, the reaction mixture was extracted with ether, washed with a saturated solution of $NaHCO_3$, then with NaCl (saturated solution). After drying the solvent was removed at room temperature by rotatory evaporation. The residue was chromatographed on 30 g of silica gel and eluted with ether. The nitrobenzaldehyde recrystallized as a white solid (mp 53–57°C). The mother liquors were resubmitted to nitration and the combined

[^{14}C]-nitrobenzaldehyde (584 mg) was reduced and diazotized according to the following procedure:

In a 10-ml beaker with a small magnetic stirrer and a thermistor, 3.5 ml of Solution I (solution I: 3.6 g $SnCl_2 \cdot 2H_2O$ in 4.8 ml HCl concn) were cooled to 5°C (ice–salt bath), then 584 mg of [^{14}C]-nitrobenzaldehyde added all at once. The temperature rose to 51°C, and the solution cooled to 5°C for a further 2.5 hr. The [^{14}C]-amino benzaldehyde was then filtered on a dry-ice cooled sinter. A suspension of this compound in 3.52 ml HCl concn was cooled in an ice–salt bath and 0.88 ml of solution II (solution II: 2.3 g $NaNO_2$ in 7.5 ml H_2O) was added very slowly maintaining the temperature to 4–5°C. After the addition was complete, stirring in ice–salt was continued for 1 hr and the mixture filtered on a dry ice cooled sintered glass funnel. The damp diazonium salt was poured slowly into 10 ml of boiling water and left in the refrigerator overnight. The crude *m*-hydroxybenzaldehyde was sublimed at 70°C/0.05 mm Hg [yield: 158 mg, mp 104–105°C (lit.[24] 106°C)] and was recrystallized from benzene to constant readioactivity (14,100 dpm/μM).

[*2,4,6-^{3}H*]-m-*Hydroxybenzaldehyde.* *m*-Hydroxybenzyl alcohol (100 mg) was exchanged with T_2O as described for [2,4,6-^{2}H]-*m*-hydroxybenzyl alcohol.

To [2,4,6-^{3}H]-*m*-hydroxybenzyl alcohol in 50 ml of ether, 723 mg of MnO_2 Attenburrow reagent (*25*) freshly prepared (kept at 120°C overnight, then ground just before the reaction) were added, left at room temperature overnight with magnetic stirring. The reaction mixture was filtered over Celite, the filtrate extracted with ether and the extract washed twice with 10 ml saturated NaCl solution, dried over anhydrous sodium sulfate, and evaporated. After several sublimations and three recrystallizations, constant radioactivity was achieved (80 mg) (38.4×10^6 dpm/mg), mp 103–105°C.

Feeding of $^{14}C/^3H$-m-hydroxybenzaldehyde to replacement culture. To 24 mg of [^{14}C]-*m*-OH-benzaldehyde (115,600 dpm/mg), 12.1 mg of [^{3}H-2,4,6]-*m*-OH-benzaldehyde (2×10^6 dpm/mg) were added and recrystallized to constant ratio ($^3H/^{14}C = 7.9$).

Feeding of the radioactive precursor. The work up of the patulin and other metabolites was modified as follows. After filtration of the mycelium at the end of the incubation, the filtrate was freeze-dried, then extracted for 7 hr (soxhlet) with ether, then thin-layer chromatographed as before.

The patulin which was obtained (after adding 19.6 mg of purified patulin as carrier, mp 110.5–111°C) was recrystallized from ether to constant ratio ($^3H/^{14}C = 5.7$).

Theoretical result for loss of 1 ^3H:$^3H/^{14}C = 5.3$.

Isolation of desoxyepoxidon. Czapek–Dox medium (20 liters) were inoculated in 1-liter batches with *P. patulum* (NRRL 2159 A) and after 4 days shaking, the mycelium was refloated on Czapek–Dox medium (without glucose) to which was added toluquinol (100 mg per liter). Five days later, the mycelium was filtered off and the filtrate was concentrated to 1 liter at 40°C by climbing film distillation and left in the cold room overnight. The filtrate was then extracted with ethyl acetate and dried.

Preparative thin-layer chromatography on silica gel GF 254 [solvent $CHCl_3$:AcOH (8:2)] afforded desoxyepoxidon (12 mg) R_f 0.46 and recrystallized from benzene (7 mg), mp 89–91°C; MW 140 (mass spectrum); ir max (KBr) 3410, 2950 and 1655 cm^{-1}; uv max (MeOH) 242 mμ (ε 4700); nmr ($CDCl_3$) τ: 8.2, 3H (s); 6.52, 1H (d); 6.2, 1H (m); 5.28, 1H (m); 3.54 1H (m); 6.67, 1H (m). ORD: $[\Phi]_{365} = +114°$; $[\Phi]_{335} = 0°$; $[\Phi]_{312.5} = -70°$; $[\Phi]_{290} = 0°$; $[\Phi]_{260} = +263°$; $[\Phi]_{250} = 0°$.

Patulin lactone. Patulin (35 mg) was dissolved in 10 ml of acetone and to this solution a few drops of Jones' reagent (*18*) were added with magnetic stirring. Water was added and the mixture extracted with ether, washed twice with water and dried over anhydrous sodium sulfate. Evaporation of the etheral solution and recrystallization (MeOH) gave needles (15 mg), mp 127.5–130°C (depending of the rate of heating]. TLC/silica PF_{254}, solvent $CHCl_3$:MeOH (10:1), R_f 0.72. uv (EtOH): 294 mμ (ε 7919). ir max (KBr) 3100, 1780, 1770, 1750, 1730 cm^{-1}.

NMR (CD_3COCD_3) τ: 4.63, 2H (m); 3.66, 1H (m); 3.26, 1H (m). Upon irradiation of the methylene resonance, the vinyl region was resolved into two doublets centered at τ 3.66 and τ 3.26, $J_{4,7} = 1.0$ Hz.

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REFERENCES

1. A. I. Scott and M. Yalpani, *Chem. Commun.* 945 (1967).
2. A. I. Scott, IUPAC Meeting, Sec. 0–13, Boston, July 1971.
3. J. D. Bu'Lock and A. J. Ryan, *Proc. Chem. Soc.* **222** (1958).
4. S. W. Tanenbaum and E. W. Bassett, *Biochim. Biophys. Acta* **28**, 21 (1958).
5. M. C. Rebstock, *Arch. Biochem. Biophys.* **104**, 156 (1964).
6. J. H. Birkinshaw, A. Bracken, and H. Raistrick, *Biochem. J.* **37**, 726 (1943).
7. A. Brack, *Helv. Chim. Acta* **30**, 1 (1947).
8. J. D. Bu'Lock, D. Hamilton, M. A. Hulme, A. J. Powell, H. M. Smalley, D. Shepherd, and G. N. Smith, *Can. J. Microbiol.* **11**, 765 (1965).
9. J. D. Bu'Lock, D. Shepherd, and D. J. Winstanley, *Can. J. Microbiol.* **15**, 279 (1963).
10. T. M. Harris, C. M. Harris, and R. J. Light, *Biochim. Biophys. Acta* **121**, 420 (1966).
11. R. J. Light, T. M. Harris, and C. M. Harris, *Biochemistry* **5**, 4037 (1966).
12. P. Dimroth, H. Walter, and F. Lynen, *Eur. J. Biochem.* **13**, 98 (1970).
13. A. I. Scott and L. Beadling, unpublished results.
14. S. W. Tanenbaum and E. W. Bassett, *J. Biol. Chem.* **234**, 1861 (1959).
15. R. J. Light, *Biochim. Biophys. Acta* **191**, 430 (1969).
16. B. J. Auret, D. R. Boyd, P. M. Robinson, C. G. Watson, J. W. Daly, and D. M. Jerina, *Chem. Commun.* 1585 (1971), and references cited therein.
17. A. Closse, R. Mauli, and H. P. Sigg, *Helv. Chim. Acta* **49**, 204 (1966).
18. A. Bowers, T. G. Halsall, E. R. H. Jones, and A. J. Lemin, *J. Chem. Soc.* 2548 (1953).
19. P. I. Forrester and G. M. Gaucher, *Biochemistry* **11**, 1108 (1972).
20. P. I. Forrester and G. M. Gaucher, *Biochemistry* **11**, 1102 (1972).
21. J. B. Cohen and H. W. Dudley, *J. Chem. Soc.* **97**, 1742 (1910).
22. H. H. Hodgson and H. G. Beard, *J. Chem. Soc.* **129**, 2339 (1927).
23. Cf. H. H. Hodgson and T. A. Jenkinson, *J. Chem. Soc.* **469** (1929).
24. R. B. Woodward, "Organic Synthesis," (E. C. Horning, Ed.), Vol. 3, p. 453. Wiley, New York, 1955.
25. J. Attenburrow, A. F. B. Cameron, J. H. Chapman, R. M. Evans, B. A. Hems, A. B. A. Jansen, and T. Walker, *J. Chem. Soc.* 1094 (1952).