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Identification of Phyllostine as an Intermediate of the Patulin Pathway in *Penicillium urticae*†

Junichi Sekiguchi‡ and G. Maurice Gaucher*

ABSTRACT: A patulin negative mutant (J1) of *Penicillium urticae* (NRRL 2159A) was found to accumulate large quantities (>128 mg/L culture) of a reactive, photosensitive compound, which was isolated and identified as (−)-phyllostine (5,6-epoxygentisylquinone). This epoxyquinone possessed an antibiotic activity against *Bacillus subtilis* which was ~80% of that exhibited by patulin. In separate in vivo feeding experiments, [2-^{14}C]acetate and [G-^{3}H]gentisaldehyde were readily incorporated into phyllostine by mutant J1 and [^{14}C]phyllostine was incorporated into patulin by the parent strain (NRRL 2159A). When fed to a washed-cell suspension of a second patulin negative mutant (J2) which produced gentisaldehyde but not phyllostine, unlabeled phyllostine was efficiently converted to patulin in yields of 33, 56, and 92% after 30 min, 1 and 5 h, respectively. The role of phyllostine as an intermediate of a new post-gentisaldehyde portion of the patulin biosynthetic pathway is discussed.

Patulin was first discovered as a potent antibiotic produced by *Penicillium expansum* (Van Luijk, 1938), *Aspergillus clavatus* (Wiesner, 1942), and by *P. claviforme* (Chain et al., 1942). Its present name derives from its subsequent isolation from the culture medium of *P. patulum* syn. *P. urticae* (Birkinshaw et al., 1943a). The determination of its structure (Woodward & Singh, 1949) and the identification of gentisyl alcohol (Birkinshaw et al., 1943b) and gentisic acid (Brack, 1947) as additional products of *P. urticae* provided the basis for the first speculation concerning the biosynthesis of patulin (Birkinshaw, 1953). This suggestion that ring cleavage of the then unknown metabolite, gentisaldehyde, led directly to patulin was supported by the subsequent isolation of gentisaldehyde and by the radiolabeling pattern observed for patulin which was derived from ^{14}C-labeled 6-methylsalicylic acid (Bu'Lock & Ryan, 1958; Tanenbaum & Bassett, 1959). Although various routes from 6-methylsalicylic acid to gentisaldehyde have been proposed, the most probable route is via *m*-cresol, *m*-hydroxybenzyl alcohol, and *m*-hydroxybenzaldehyde (Bassett & Tanenbaum, 1958; Bu'Lock et al., 1965; Scott & Yalpani, 1967; Forrester & Gaucher, 1972; Scott et al., 1973; Scott & Beadling, 1974; Murphy et al., 1974; Murphy & Lynen, 1975).

Although it is not obvious from its structure (see Table I), patulin is a classic example of the large group of polyacetate-derived secondary metabolites known as "polyketides" (Turner, 1971). We have chosen patulin biosynthesis in *P. urticae* (NRRL 2159A) as a model system for polyketide biosynthesis in particular and for secondary metabolite biosynthesis in general. In a continuing examination of the function as well as the regulation of patulin biosynthesis, we have recently shown that this pathway does not appear to be

† From the Biochemistry Group, Department of Chemistry, The University of Calgary, Calgary, Alberta, Canada T2N 1N4. *Received October 13, 1977.* This work was supported by National Research Council of Canada Grant A3588. A preliminary account of this work has been given (Gaucher & Sekiguchi, 1976).

‡ Present address: Department of Applied Microbial Technology, Kumamoto Institute of Technology, Kumamoto-city, Kumamoto-prefecture, Japan.

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a prerequisite to the production of viable spores by *P. urticae* (Sekiguchi & Gaucher, 1977). This conclusion was largely dependent upon the use of a series of patulin-minus mutants. One of the mutants (J1) accumulated a reactive compound which had not been previously detected in *P. urticae* cultures. This paper reports the elucidation of this compound's structure as well as evidence of its role as an intermediate in a hitherto unsuspected post-gentisaldehyde portion of the patulin biosynthetic pathway. The results suggest a ring-opening mechanism which is mediated by a monooxygenase rather than by a dioxygenase as previously postulated (Scott & Beadling, 1974).

Materials and Methods

Organism and Culture Conditions. Penicillium urticae (NRRL 2159A) and three patulin negative mutants (J1, J2, and S15) derived from this strain were used in this study. The isolation of these mutants and their characterization as mutants defective in patulin biosynthesis have been fully described (Sekiguchi & Gaucher, 1977).

Conidial suspensions were prepared from Czapek-Dox agar slants as previously described (Sekiguchi & Gaucher, 1977). Erlenmeyer flasks (500 mL) containing 50 mL of *seed culture medium* (Sekiguchi & Gaucher, 1977) were inoculated with aliquots of this suspension to yield a final concentration of 7–10 $\times 10^6$ conidia/mL, and then incubated at 28 °C on a rotary shaker (NBS Model G-10; 250 rpm; 1-in. stroke) for 24 h. This seed culture (5 or 200 mL) was used without filtration as the inoculum for 500-mL flasks or a 4-L fermentor (NBS Microferm) containing 50 mL or 3 L of *glucose-yeast extract medium* (Sekiguchi & Gaucher, 1977), respectively. The flasks were shaken as above, while the fermentor was aerated (2 L/min and 300 rpm to 18 h, then 2 L/min and 400 rpm) at 28 °C and a 10% suspension of Antifoam C (Dow Corning) was used to control foaming.

Isolation and Characterization of Phyllostine. A 48-h, 3-L fermentor culture of mutant J1 in glucose-yeast extract medium was filtered through Whatman No. 1 filter paper and the 2.8 L of filtrate (pH 4.1) was extracted with 2 equal volumes of ethyl acetate. After drying over anhydrous sodium sulfate, the extract was decanted and concentrated to 200 mL by rotary evaporation. A quarter of this concentrate was further concentrated and applied to preparative thin-layer plates (20 × 20 cm; 1 mm Woelm silica gel GF_{254}) which had been activated for 2 h at 110 °C and equilibrated at room temperature without desiccation. After development in chloroform:ethyl acetate:diethyl ether (1:1:1, v/v/v), an unknown band (UII, phyllostine, R_f 0.67), which was visible under UV light or appeared blue violet in color after reaction with 3-methyl-2-benzothiazolinone-hydrazone hydrochloride (Sekiguchi & Gaucher, 1977), was scraped from each plate and extracted with ethyl acetate. The combined extracts were evaporated to dryness and the residue was dissolved in 3 mL of hot benzene. After standing at room temperature overnight, 89 mg of colorless needles was filtered off and recrystallized to yield 40 mg of crystals (mp 56–57 °C; 1 spot on a thin-layer chromatogram).

Melting points (mp) were determined using a Leitz "hot stage" microscope and the elemental analysis was carried out by Schwarzkopf Microanalytical Laboratory, Woodside, N.Y. Ultraviolet and infrared spectra were recorded on a Beckman DB-G spectrophotometer and a Perkin-Elmer 337 grating spectrometer, while circular dichroism (CD) and optical rotatory dispersion (ORD) curves were recorded on a Durrum-Jasco Model ORD/UV5 spectropolarimeter equipped with an SS-20 (Sproul Co.) modification. The high resolution mass spectrum was taken on an AEI MS-9 mass spectrometer by Dr. A. M. Hogg, Chemistry Department, University of Alberta. Proton NMR and ^{13}C NMR spectra were obtained in $CDCl_3$ (tetramethylsilane internal standard) using a Varian HA-100 instrument, and in $(CD_3)_2CO$ (tetramethylsilane internal standard) using a Varian CFT-20 instrument, respectively.

Radiochromatographic Procedures. Duplicate radioactive samples and a standard mixture of patulin pathway metabolites were spotted onto a 250-μm thick silica gel (Woelm GF_{254}) plate (5 × 20 cm) prepared as described above for preparative plates. Unlabeled samples of gentisyl alcohol and 6-methylsalicylic acid were synthesized as described by Forrester & Gaucher (1972). Patulin was isolated from a *P. urticae* (NRRL 2159A) culture (J. W. D. Groot Wassink, unpublished procedure) in high yield and was recrystallized three times from benzene (mp 110–111 °C). All other metabolites were available commercially.

After being developed for 17–19 cm in (A) chloroform:ethyl acetate: ethyl ether (10:4:2, v/v/v) or (B) chloroform:glacial acetic acid (8:2, v/v), the pattern of spots was drawn as visualized under UV or by spraying as described by Sekiguchi & Gaucher (1977). Using the zonal scraper of Snyder & Kimble (1965), 2-mm wide slices of an unsprayed lane were consecutively scraped into scintillation vials containing 10 mL of a scintillation solution which was identical with that used by Forrester & Gaucher (1972), except that the water was omitted. After scintillation counting the cpm for each slice was plotted to yield a radiochromatogram and the previously determined elution pattern was then added.

Antibiotic Bioassay. Aliquots of pure phyllostine and patulin solutions in double deionized water and 0.05 M phosphate buffer (pH 6.5), respectively, were added to wells in an agar layer containing *Bacillus subtilis* as the sensitive bacterium (J. W. D. Groot Wassink, unpublished procedure) to obtain plots of log concentration vs. inhibition zone diameter. These standard plots were linear for concentrations of phyllostine and patulin of 0.5 to 10 mM, and 0.5–4.0 mM, respectively.

Preparation of [G-^3H]Gentisaldehyde. [G-^{3}H]Gentisaldehyde was prepared from gentisaldehyde by the method of Kirby & Ogunkoya (1965) using a reaction time of 2 h. After acidification the aqueous reaction mixture was extracted twice with equal portions of ether and this extract was in turn extracted with 0.01 M NaOH to remove any labile tritium. This NaOH extract was acidified and extracted twice with equal portions of ether and the residue after evaporation was recrystallized from chloroform to yield 30 mg (22% recovery) of crystals (2.8×10^5 cpm/mg) which exhibited a single symmetrical peak on a thin layer radiochromatogram.

Preparation of [^{14}C]Phyllostine. Six shake cultures of mutant J1 were cultivated in glucose-yeast extract medium as described above. At 32 h, 50 μL of a sodium [1-^{14}C]acetate solution in ethanol (1 μCi/μL; 57.8 mCi/mmol; New England Nuclear) was added to each culture. Twelve hours later each culture was filtered through Whatman No. 1 filter paper, and the filtrates were acidified to pH 2.0 with 4 N HCl, extracted with ethyl acetate, and dried over anhydrous Na_2SO_4. The phyllostine (UII) in this extract was partially purified by preparative thin-layer chromatography as described above (see Isolation and Characterization of Phyllostine), except that solvent A (see Radiochromatography procedures) was used as the eluent. Of the two, only just resolved bands, UII (phyllostine, R_f 0.47) and UI (R_f 0.40), the UII band was scraped from the plate(s) and extracted with ethyl acetate. A thin-layer radiochromatogram indicated that about 88% of the carbon-14 in the mixture was associated with UII. Since the UV spectrum

was essentially that of pure phyllostine, its molar extinction coefficients ($\epsilon_{214}^{MeOH} = 9270$ and $\epsilon_{260}^{MeOH} = 4190$) were used to determine the amount of phyllostine in the mixture and hence its specific activity (4.7×10^5 cpm/mg).

Addition of [2-^{14}C]Acetate to Mutant J1. At 32 h, 10 μCi of sodium [2-^{14}C]acetate (12.13 mCi/mmol, in ethanol, New England Nuclear) was added to a 50-mL shake culture (glucose-yeast extract medium) of mutant J1. Samples (7 mL) were withdrawn 5 min, 1, 3, 6, and 12 h after addition. After filtration and acidification (pH 2), 4 mL of each sample was extracted with about 2 volumes of ethyl acetate and the residue, after evaporation in a test tube evaporator (Buchler Evapo-Mix), was dissolved in 0.4 mL of chloroform. A 20-μL aliquot of this solution which corresponded to 0.2 mL of the original filtrate was then spotted onto a thin-layer plate (see Radiochromatographic procedures). The radioactivity in 0.2 mL of the 5 min, 3 and 6 hr *filtered* samples (no extraction) was 43 400, 46 500, and 42 500 cpm, respectively. Thus for the 6-h sample the recovery of radioactivity after extractions, spotting, chromatography, and scraping was 15% for the entire chromatogram.

Addition of [G-^{3}H]Gentisaldehyde to Mutant J1. At 32 h, 5 mg (1.4×10^5 cpm) of [G-^{3}H]gentisaldehyde was added to a 50-mL shake culture of mutant J1. Samples (7 mL) were withdrawn 5 min, 1, 3, 6, and 12 h after addition, and 4 mL of the culture filtrates were extracted, etc. The equivalent of 0.2 or 0.4 mL of the original filtered sample was subjected to thin-layer chromatography. The radioactivity (i.e., ^{3}H) in 0.2 mL of the 5 min, 3 and 6 h *filtered* samples (before extraction) was 3340, 4460, and 4900 cpm, respectively. Thus for the 6-h sample the radioactivity recovered after all manipulations was about 16% for the entire chromatogram and 6.4% for peak, UII.

Addition of [^{14}C]Phyllostine to the Parent Strain and to Mutant S15. At 32 h, 15 mL of a 50-mL shake culture (glucose-yeast extract medium) of *P. urticae* NRRL 2159A was transferred to a 125-mL Erlenmeyer flask containing 0.6 mg (2.8×10^5 cpm) of partially pure [^{14}C]phyllostine. This culture was shaken as described for 500-mL flasks (see Culture Conditions) and samples (2–3 mL) were withdrawn 5 min, 3 and 6 h after the transfer. Culture filtrates (0.5 mL) were extracted etc. and in each case the entire metabolite residue was spotted onto a thin-layer plate as one spot. The radioactivity in 0.2 mL of the 5 min, 3 and 6 h *filtered* samples (before extraction) was 3410, 3510, and 3490 cpm, respectively. After all manipulations the radioactivity recovered from the entire 6 h chromatogram was about 38%.

Except for the withdrawal of samples at 5 min, 6 and 12 h after the transfer, an exact duplicate of the above experiment was carried out using mutant S15. Before extraction the filtered samples contained 3470, 3400 and 3240 cpm per 0.2 mL for 5 min, 6 and 12 h, respectively. The recovery of radioactivity from the entire 6- or 12-h chromatograms averaged 29 ● 3%.

Addition of Phyllostine to a Washed-Cell Suspension of Mutant J2. After 32 h of cultivation in glucose–yeast extract medium, 20 shake cultures (50 mL) of mutant J2 were combined to yield a liter of culture. After being filtered and washed with distilled water, the cells were mixed with 1 L of 0.05 M phosphate buffer (pH 6.5) containing 154 mg of recrystallized pure phyllostine (UII) and 50-mL portions were transferred to 20 Erlenmeyer flasks (500 mL). After shaking as previously described, 5-mL samples were withdrawn from *one* flask at 5 and 30 min, and at 1, 2, 5, and 6 h, and along with a sample of the original 32-h culture filtrate, these samples were frozen immediately after filtration. Duplicate samples (0.1 mL) of each filtrate were later assayed for antibiotic activity using the bioassay described above. After extraction, the equivalent of 0.5 mL of each filtrate was also subjected to thin-layer chromatography.

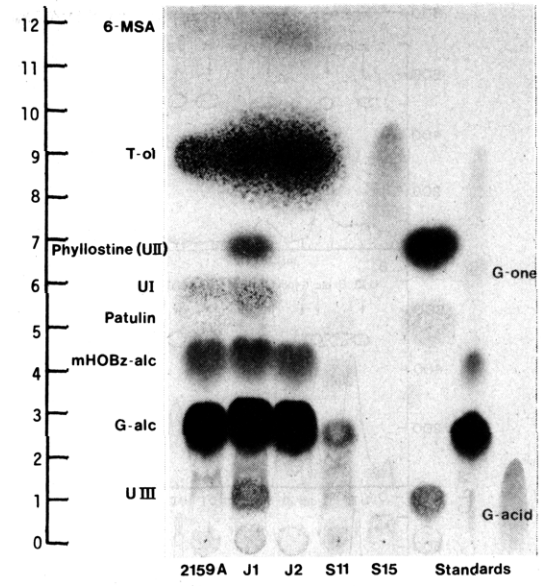


FIGURE 1: A thin-layer chromatogram of the secondary metabolites produced by the parent strain, *P. urticae* NRRL 2159A, and by the patulin negative mutants, J1, J2, S11, and S15. Ethyl acetate extracts equivalent to 40 μL of filtrate from 32-h-old, 50-mL shake cultures grown in glucose-yeast extract medium were spotted onto plates and developed in solvent A as described in the Materials and Methods section under Culture Conditions and Radiochromatographic Procedures. Three solutions of purified standards (5 μg of each) were also chromatographed: UIII (brown), patulin (yellow), and UII (violet); gentisyl alcohol (G-alc, red) and *m*-hydroxybenzyl alcohol (mHOBz-alc, purple); gentisic acid (G-acid, brown). Gentisquinone (G-one, red) was a side product of gentisyl alcohol. Standards of toluquinol (T-ol, red-orange) and 6-methylsalicylic acid (6-MSA, purple) were not used, and UI (brown) has not been isolated.

At 6 h, the 19 remaining replacement cultures were filtered and the combined filtrate was acidified to pH 2.5 with 4 N HCl and extracted twice with equal volumes of ethyl acetate. After drying over anhydrous Na_2SO_4, the concentrated extract ($\sim$ 1 mL) was applied to a preparative thin-layer plate and developed in solvent A as described above. Because the *washed* cell suspension was free of most of the normal metabolites, the patulin (41 mg) obtained from this preparative plate was pure and did not require any recrystallization prior to being analyzed. Proton and ^{13}C NMR spectra were obtained using a Brucker WH-90 NMR spectrometer.

Results

Using an improved thin-layer chromatographic system, the spectrum of secondary metabolites accumulated by the 4 patulin negative (Pat$^-$) mutants J1, J2, S11, and S15 is contrasted with that of the parent strain (2159A) in Figure 1. Since mutants J1, J2, and S11 all accumulate gentisyl alcohol and gentisic acid but no patulin, they appeared to be blocked late in the patulin pathway. In contrast, mutant S15 possessed an "early" block since it produced only 6-methylsalicylic acid (6-MSA). Three unknown compounds (UI, UII, and UIII) were immediately evident in the culture filtrate of mutant J1. Two of these (UII and UIII) were not accumulated by the parent or by any of the other mutants. Their absence in mutant J2 suggested that the block possessed by this Pat$^-$ mutant might occur before that possessed by J1. Unknown UI and a variety of "yellow" compounds were accumulated by the parent and mutant S11, respectively.

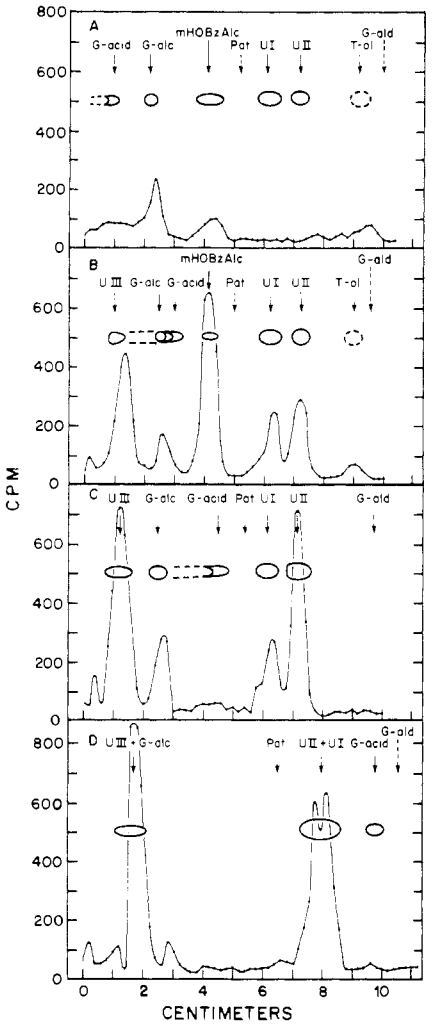


FIGURE 2: Radiochromatograms of extracts obtained in 5 min (A), 3 h (B), and 6 h (C,D) after addition of [2-^{14}C]acetate to shake cultures of *P. urticae* mutant J1. Except for gentisaldehyde (G-ald) and patulin (Pat), the abbreviations for the various metabolites are as given in the legend of Figure 1, and their positions are indicated by arrows. All extracts spotted represented 0.2 mL of culture filtrate. Chromatography solvent B was used for chromatogram D to eliminate the streaking of gentisic acid (G-acid) and to improve the separation between patulin and UI and UII. All other chromatograms were obtained using solvent A. A chromatogram from a 12-h sample was essentially the same as that for 6 h.

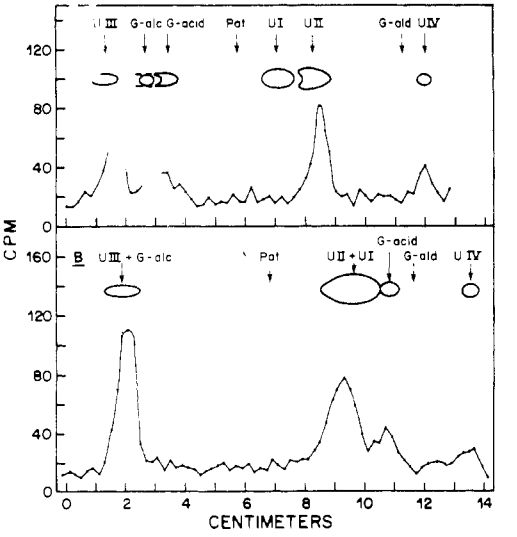


FIGURE 3: Radiochromatograms of extracts obtained 6 h after the addition of [G-^{3}H]gentisaldehyde to shake cultures of *P. urticae* mutant J1. Chromatograms A and B were developed in solvents A and B, respectively, while the extract spotted was equivalent to 0.2 and 0.4 mL of filtrate, respectively. Radiochromatograms from 3 and 12 h extracts differed only in having peak intensities which were about half and twice those for 6 h. Unknown UIV was not identified.

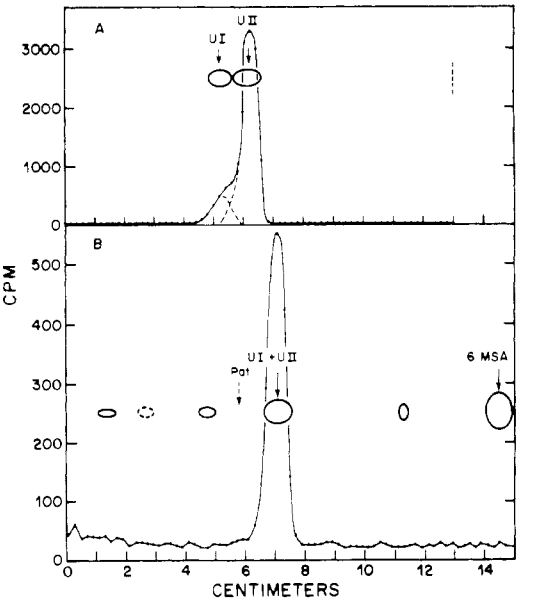


FIGURE 4: Radiochromatograms demonstrating the purity and in vivo stability of [^{14}C]phyllostine (UII). (A) Radiochromatogram (solvent A) of partially purified [^{14}C]UII which had been isolated from *P. urticae* mutant J1. The presence of a small poorly resolved peak (~12%) of [^{14}C]UI is indicated by the dashed lines. (B) Radiochromatogram(s) (solvent B) of an extract (equivalent to 0.5 mL of filtrate) obtained 6 and 12 h after the addition of [^{14}C]UII (see A), to a shake culture of *P. urticae* mutant S15. An extract obtained 5 min after the addition gave a similar chromatogram with a low background but the single peak (UI and UII) had a maximum intensity of ~1000 cpm per 0.5 mL of culture filtrate.

Radiolabeling Studies. Before engaging in the isolation and structural elucidation of any of the observed unknowns, an addition of [2-^{14}C]acetate to a shake culture of Pat^{-} mutant J1 demonstrated that all three unknowns were acetate derived (Figure 2). These radiochromatograms indicated that within 5 min some ^{14}C was incorporated into gentisyl alcohol and *m*-hydroxybenzyl alcohol; 3 h later ^{14}C was also found in UIII, UI, and UII, and after 6 h the ^{14}C resided principally in UIII and UII. The use of a different solvent system which more clearly resolved patulin and gentisic acid (Figure 2D) confirmed the absence of label in these metabolites. The rise (Figure 2B) and fall (Figure 2C) of label in *m*-hydroxybenzyl alcohol suggested that it was an intermediate, while UI, UII, UIII, and gentisyl alcohol were end products.

A similar experiment in which [G-^{3}H]gentisaldehyde was fed to the same mutant (Figure 3) showed significant incorporation into UIII and UII, minor incorporation into gentisyl alcohol, gentisic acid, and UIV, and no incorporation into UI. Incorporation into gentisic acid was confirmed by using a solvent system in which this metabolite does not streak (Figure 3B). These results suggested that UII and UIII were late (i.e., post-gentisaldehyde) products or intermediates of the patulin pathway, while UI was not.

To confirm this suggestion [^{14}C]UII was obtained by feeding [1-^{14}C]acetate to mutant J1 and by purifying the resultant extract on preparative thin layer plates. A radiochromatogram (Figure 4A) showed that, except for a small amount (~12%) of labeled UI, the preparation of [^{14}C]UII was pure. The addition of this labeled unknown to a culture of the Pat^{-}

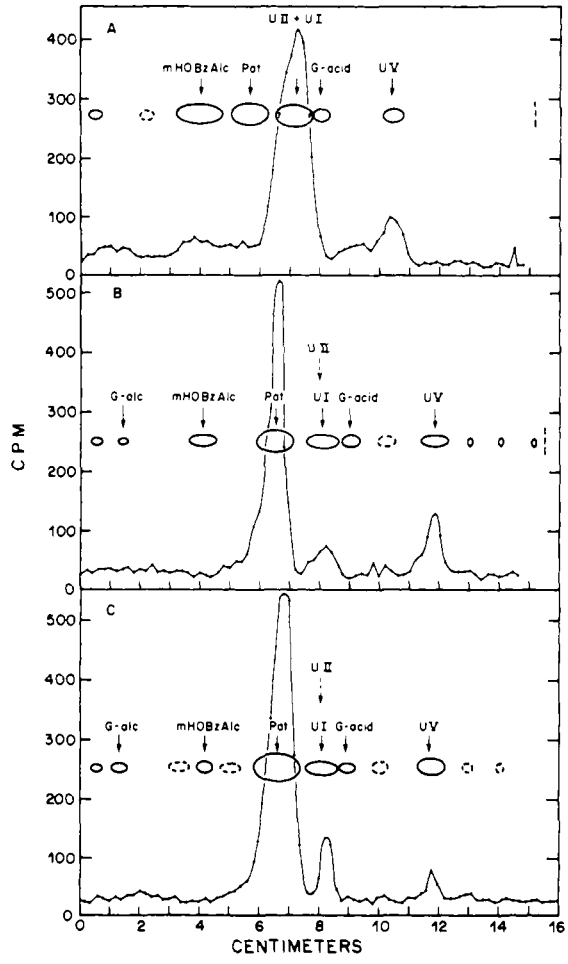


FIGURE 5: Radiochromatograms of extracts obtained 5 min (A), 3 h (B), and 6 h (C) after addition of [^{14}C]UII (see Figure 4A) to a shake culture of *P. urticae* NRRL 2159A. Each extract was equivalent to 0.5 mL of culture filtrate and chromatography solvent B was used in each case. The metabolite abbreviations are as given in the legends of Figures 1 and 2. The identity of UV was not determined.

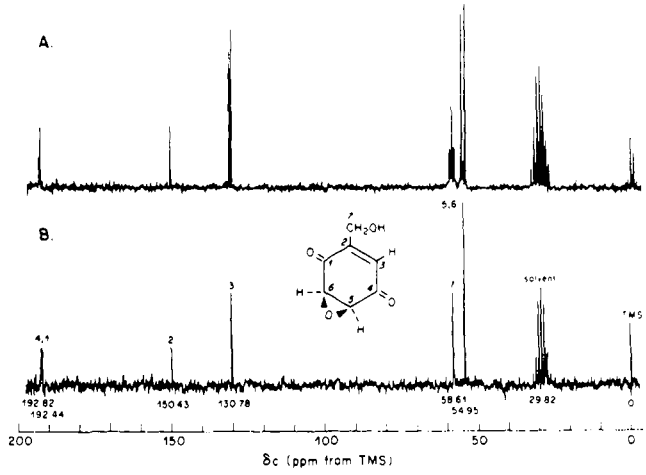


FIGURE 6: (A) Off-resonance (i.e., partial) and (B) wide band, ^{1}H-decoupled ^{13}C NMR spectra of pure phyllostine (UII) in deuterioacetone using tetramethylsilane as internal reference.

mutant which appears to be blocked immediately after the first step in the pathway (i.e., S15) served as an effective control for subsequent experiments (Figure 4B). Thus this reactive compound was not completely lost from the medium of a typical shake culture over a period of 12 h; nor was it converted into any other excreted secondary metabolites in the absence of most of the patulin pathway. These observations were in agreement with the prior observation that shake cultures of mutant J1 accumulated large amounts of UII but not a trace of patulin.

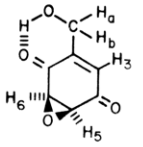
The addition of [^{14}C]UII to a shake culture of the parent clearly demonstrated the conversion of UII to patulin (Figure 5). Thus after 5 min (Figure 5A) most of the radioisotope was still associated with UII, but by 3 h (Figure 5B) its efficient transfer to patulin from UII was obvious, and no further change was observed at 6 h (Figure 5C). The small amount of radioactivity which remained at the R_f ascribed to UI and UII (Figure 5B and 5C) was not due to unconverted UII since the spot at this R_f did not possess this unknown's characteristic violet color (see Figure 1, caption). The fact that no significant radioactivity was detected in *m*-hydroxybenzyl alcohol, gentisyl alcohol, or gentisic acid at any time confirmed UII's post-gentisaldehyde position in the patulin pathway.

Identification of UII as Phyllostine. Since submerged cultures of mutant J1 produced large quantities of UII (i.e., >128 mg of crystals/L of culture medium), this photosensitive metabolite was easily purified by ethyl acetate extraction, preparative thin-layer chromatography, and crystallization from benzene. The compound was characterized by a molecular formula of $C_7H_6O_4$ (mol wt 154; Anal. Calcd: C, 54.55; H, 3.92; O, 41.53. Found: C, 54.30; H, 3.83; O, 41.87), an ORD spectrum (*c* 0.001 g/mL 95% ethanol: negative Cotton effect, $[\alpha]_D^{30} = -105°$; $[\alpha]_{404}^{30} = -1499°$; $[\alpha]_{376}^{30} = 0$; $[\alpha]_{291}^{30} = +4598°$), a CD spectrum (*c* 0.001 g/mL 95% ethanol: $\Delta\epsilon_{372} = -1.47$; $\Delta\epsilon_{296} = 0$; $\Delta\epsilon_{246} = +5.20$; $\Delta\epsilon_{230} = 0$; $\Delta\epsilon_{217} = -5.95$), an UV spectrum ($\lambda_{max}^{MeOH}$ nm (ϵ): 214 (9270), 260 (4190)); an IR spectrum ($\nu_{max}^{HCCl_3}$ cm^{-1}: 3600, 3365, 3025, 2910, 1685, 1620, 1440, 1310, 1270, 1117, 1083, 1038, 995, 910, 880, 845, 695), and a mass spectrum (154 (M^+, 0.9), 153 (0.9), 136 (1.0), 126 ($M^+ - CO$, 100), 110 (6.0), 97 ($M^+ - 2(CO + H)$, 28.4), 69 (97 − CO, 56.3). The ^{13}C NMR and ^{1}H NMR spectral assignments given in Figure 6 and Table I, respectively, were even more conclusive and unambiguously identified UII as being identical with the epoxyquinone, (−)-phyllostine, a phytotoxin produced by *Phyllosticta sp.* and shown to be the causative agent in the wilting of clover leaves by this fungus (Sakamura et al., 1970, 1971a). At 1-5 mM phyllostine possessed ~81% of the antibiotic activity of an equal concentration of patulin.

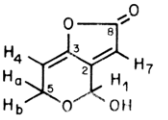
Addition of Phyllostine (UII) to Mutant J2. As noted above the Pat$^-$ mutant J2 appeared to be blocked just after gentisaldehyde and just before the two new metabolites UII and UIII. This suggestion was confirmed, by the finding that when gentisaldehyde was added to washed cells of J2 only gentisyl alcohol and gentisic acid were formed and by the results of the following experiment. When 1 μmol/mL of unlabeled phyllostine (UII) was added to a washed cell suspension of mutant J2, an antibiotic bioassay indicated that within 30 min the antibiotic activity in the medium dropped precipitously and then increased rapidly to a value which represented a 33% conversion to patulin (Figure 7a). Conversions of 56 and 92% were obtained by 1 and 5 h, respectively. This interpretation is supported by the fact that phyllostine (1 mM) possesses an antibiotic activity in this bioassay which is equivalent to about half this concentration of patulin, and it is confirmed by the thin-layer chromatogram (Figure 7b) which clearly shows that within 30 min the phyllostine in the medium is replaced by patulin.

In order to ensure that the antibiotic being produced from phyllostine in this experiment was indeed (+, −)-patulin and

TABLE I: Nuclear Magnetic Resonance Assignments.



(−)-Phyllostine



(+, −)-Patulin

^{1}H NMR data ($DCCl_3$)[a]		^{1}H NMR data ($DCCl_3$)[a]		^{13}C NMR data (D_3CCOCD_3)[j]
$\delta_3 = 6.69$ (m)[b]	$J_{3\text{-}b} = J_{3\text{-}a} = 2$ Hz	$\delta_1 = 6.07$ (d)[f]	$J_{1\text{-}OH} = 5.3$ Hz	$\delta_8 = 206.7$
$\delta_a = 4.64$, $\delta_b = 4.38$ (m)[c]	$J_{a\text{-}b} = 17$ Hz	$\delta_7 = 6.03$ (m)[g]	$J_{7\text{-}4} = 1.8$ Hz	$\delta_3 = 152.6$
		$\delta_4 = 5.96$ (m)[g]	$J_{a\text{-}4} = 2.9$ Hz	$\delta_2 = 147.1$
$\delta_{5,6} = 3.85$ (s)[d]	$J_{5,6\text{-}3} = 0.8$ Hz	$\delta_a = 4.76$, $\delta_b = 4.41$ (m)[h]	$J_{b\text{-}4} = 3.8$ Hz	$\delta_7 = 110.6$[k]
$\delta_{OH} = 1.90$ (t)[e]	$J_{OH\text{-}b} = J_{OH\text{-}a} = 6$ Hz		$J_{a\text{-}b} = 17.3$ Hz	$\delta_4 = 108.8$[k]
		$\delta_{OH} = 3.2$ (d)[i]		$\delta_1 = 89.3$
				$\delta_5 = 59.7$

[a] Chemical shifts are relative to $HCCl_3$; $\delta = 7.27$ (s). [b] Complex, poorly resolved multiplet changes to different triplets ($\delta = 6.69$) in the absence of $H_{a,b}$ or $H_{5,6}$ coupling. [c] Complex, poorly resolved multiplet ($\delta = 4.51$) changes to two pairs of doublets, or to one pair of doublets in the absence of OH, or OH and H_3 coupling, respectively. [d] Singlet changes to a multiplet ($\delta = 3.85, 3.84, 3.83$) upon exchange of OH proton (trace of H^+ added). [e] Triplet changes to a broad singlet ($\delta = 1.73$) if trace of H^+ added, and to a sharp singlet ($\delta = 1.90$) in the absence of $H_{a,b}$ coupling. [f] Changes to a sharp singlet in the absence of OH coupling. [g] Changes to a doublet in the absence of H_aH_b coupling. [h] A multiplet ($\delta = 4.59$) composed of two pairs of doublets. [i] Changes to a sharp singlet in the absence of H_1 coupling. [j] Chemical shifts are relative to TMS. [k] These shifts may be interchanged.

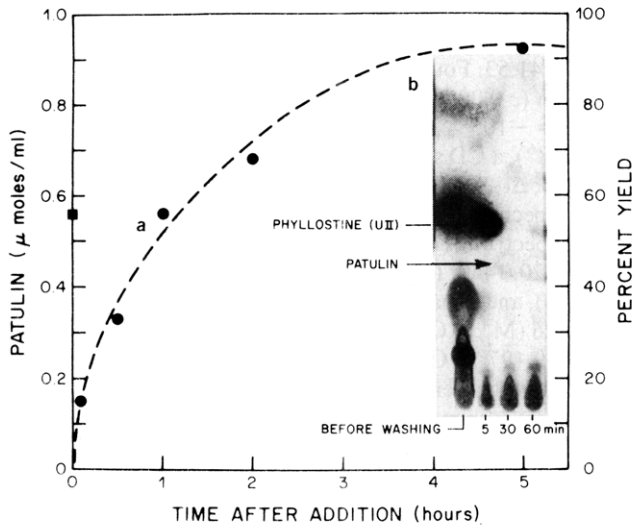


FIGURE 7: Addition of pure phyllostine (UII) to a washed cell suspension of *P. urticae* mutant J2 at an initial concentration of 1 mM. (a) Time course of patulin production as determined by an antibiotic agar plate diffusion assay of samples obtained at 5 and 30 min, and 1, 2, and 5 h after the addition. As determined by the same assay, a 1 mM solution of phyllostine would yield an antibiotic activity equivalent to that of ~0.5 μmol/mL of patulin (■). (b) Thin-layer chromatographic (solvent A) assessment of the conversion of phyllostine (violet) to patulin (yellow), 5, 30, and 60 min after the addition. The first sample was an extract of the filtrate (0.5 mL) from the 32-h-old culture (J2) which provided the cells for this experiment.

not a related compound possessing a similar R_f, color, and antibiotic activity, the patulin formed by the cell-suspension of J2 after 6 h was isolated, purified, and characterized. An absence of optical activity, and its UV ($\lambda_{max}^{HCCl_3}$ nm(ϵ): 278 (12 900)), IR ($\nu_{max}^{HCCl_3}$ cm^{-1}: 3580, 3025, 1780, 1755, 1680, 1630, 1410, 1260, 1160, 1096, 1057, 1035, 998, 970, 922, 875, 852, 826, 808) and mass (154 (M^+, 32.9), 136 (22.3), 126 (45.3), 110 (85.3), 97 (22.4), 82 (39.9), 55 (100)) spectra were in agreement with the molecular formula ($C_7H_6O_4$) and the structure of patulin. The latter has recently been confirmed by x-ray crystallography (Hubbard et al., 1977). The most unambiguous proof of structure was provided, however, by the proton and carbon-13 NMR spectra which were identical with those obtained for authentic patulin (see Table I). Thus while desoxypatulinic acid ($C_7H_8O_4$; 2,3-dihydro-4-pyrone-5-acetic acid; Scott et al., 1972) and patulin lactone ($C_7H_4O_4$; a carbonyl carbon replaces the hemiacetal carbon of patulin; Scott et al., 1973) are easily distinguishable from patulin, the hypothetical isomer, isopatulin ($C_7H_6O_4$; the OH of patulin is shifted to carbon-5, see Table I) is very much less so. The NMR spectra (Table I) would, however, clearly distinguish between patulin and isopatulin since, for example, the methylene (Ha, Hb) coupling constants (Table I) would be very different.

Discussion

The isolation of a number of Pat$^-$ mutants of *P. urticae* led to the discovery of three unknown metabolites UI, UII, and UIII. One of these unknowns (UII) was isolated from the culture medium of mutant J1 and characterized as (−)-phyllostine (5,6-epoxygentisylquinone), a previously characterized metabolite of *Phyllosticta* sp.

In *P. urticae* phyllostine was shown to be both a polyketide and a late patulin pathway metabolite. Thus in separate experiments [2-^{14}C]acetate and [G-^{3}H]gentisaldehyde both yielded radiolabeled phyllostine when fed to mutant J1. The transient labeling of *m*-hydroxybenzyl alcohol and the subsequent appearance of that carbon-14 label in phyllostine provided additional evidence for this new metabolite's association with the latter part of the patulin pathway. In contrast UI was clearly associated with the early part of the pathway.

Phyllostine was also an efficient *precursor* of patulin in all strains of *P. urticae* in which the post-gentisaldehyde portion of the patulin pathway was intact. Thus the parent strain converted [^{14}C]phyllostine to patulin, but not to *m*-hydroxybenzyl alcohol, gentisyl alcohol, or gentisic acid, and mutant J2, which converted gentisaldehyde to gentisyl alcohol and gentisic acid, but not to patulin or phyllostine, efficiently converted unlabeled phyllostine to a metabolite definitely proven to be patulin. Any nonenzymatic or fortuitous metabolic conversions of phyllostine to patulin were ruled out since mutant JI accumulated phyllostine but no patulin, while mutant S15 which is essentially devoid of the pathway, had no significant effect on [^{14}C]phyllostine.

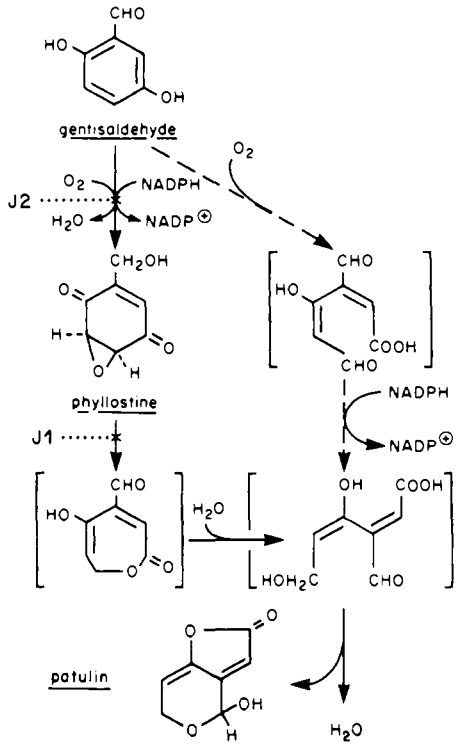


FIGURE 8: Post-gentisaldehyde portion of the patulin biosynthetic pathway. The previously postulated route (- - →) is contrasted with a proposed new route (→) which indicates the position of phyllostine as a new intermediate, as well as the location of the blocks in *P. urticae* mutants J1 and J2. The metabolites in square brackets are *postulated* intermediates.

The association of phyllostine with the patulin pathway was also supported by the co-occurrence of 6-methylsalicylic acid and gentisyl alcohol with phyllostine in the culture medium of *Phyllosticta* sp. (Sakamura et al., 1971b) and by the incorporation of [^{14}C]gentisyl alcohol and [1-^{13}C]- and [2-^{13}C]acetate into epoxydon, a reduced form of phyllostine which is also produced by *Phyllosticta* sp. (Nabeta et al., 1975). This latter observation is pertinent since we have recently characterized UIII from this study as an isomer of epoxydon.

With respect to phyllostine's position in the pathway, it is clear that this new metabolite is either a transient intermediate or an abnormal side product of the post-gentisaldehyde portion of the patulin pathway. Thus phyllostine is normally not detected in cultures of the parent strain and is never detected in cultures of the Pat$^-$ mutant, J2, which is blocked immediately after gentisaldehyde in the pathway. Yet mutant J2 rapidly converts phyllostine to patulin.

If phyllostine is indeed a required intermediate in patulin biosynthesis, then as indicated in Figure 8 this epoxyquinone must arise from an epoxidation which is mediated by a monooxygenase rather than by a dioxygenase as previously postulated (Scott et al., 1973; Scott & Beadling, 1974). Although the most obvious route to phyllostine is the oxidation of gentisyl alcohol to gentisquinone followed by epoxidation, the direct conversion of gentisaldehyde to gentisquinone via an internal redox reaction is simpler and is the basis of our proposal in Figure 8. Also postulated in Figure 8 is a simple rearrangement of phyllostine to a seven-membered lactone which upon hydrolysis yields the acyclic form of patulin. Two ring closure reactions would then yield patulin in complete agreement with the radiolabeling pattern observed by earlier workers (Bu'Lock & Ryan, 1958; Tanenbaum & Bassett, 1959). Experimental support for these proposals is presently being sought in experiments designed to identify further late intermediates of the patulin pathway.

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