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Early Intermediates in the Degradation of α-Conidendrin by a *Pseudomonas multivorans**

Anne Toms and J. M. Wood

ABSTRACT: A *Pseudomonas* spp. tentatively identified as a *Pseudomonas multivorans* was isolated by elective culture with α-conidendrin as sole carbon and energy source. Two new organic compounds, 1,2,3-trihydro-6-hydroxy-7-methoxy-4-oxo-3-hydroxymethyl-2-naphthoic acid γ-lactone (I) and 4,6-dihydroxy-3-hydroxymethyl-7-methoxy-2-naphthoic acid γ-lactone (II), were isolated from culture filtrates and characterized by CH analysis, mass spectrometry, nuclear magnetic resonance, infrared spectrophotometry, and ultraviolet spectrophotometry. For compound I additional information was required in order to determine rigorously its structure owing to keto–enol tautomerization providing keto–enol mixtures. Consequently the *O*-acetyl- and 2,4-dinitrophenylhydrazone derivatives were prepared and the structure of each compound was determined by CHN analysis and mass spectrometry. Early intermediates in the oxidative degradation of α-conidendrin leading to a substituted 2-naphthoic acid γ-lactone for this *Pseudomonas* spp. are proposed.

Study of the microbial degradation of α-conidendrin can be related to the process of lignin decomposition in nature. α-Conidendrin is a lignin model compound containing two phenylpropane units and is believed to be synthesized by the condensation of dehydrogenated *trans*-ferulic acid with a quinone methide radical produced by dehydrogenation of a second molecule of coniferyl alcohol (Freudenberg and Geiger, 1963). It can also be regarded as a natural product since Erdtman (1944) succeeded in isolating it by direct extraction with acetone from spruce wood.

α-Conidendrin was first isolated by Lindsey and Tollens (1892) in the form of soft crystals in the oily residue extracted by ether from the spent liquor from a sulfite cook of spruce (*Picca abies*). Later, it was found to be readily isolated from the spent sulfite liquor of western hemlock (*Tsuga heterophylla*) (Pearl, 1945).

Several investigators (Konetzka *et al.*, 1952; Pratt *et al.*, 1953; Tabak *et al.*, 1959; Sundman, 1965) have isolated microorganisms of the genus *Pseudomonas*, *Achromobacter*, *Flavobacterium*, and *Agrobacterium* which will grow with α-conidendrin as sole source of carbon. Paper chromatog-

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raphy, ultraviolet–visible spectrophotometry, and manometry were the methods used to determine the later degradative products of α-conidendrin cultures. Sundman (1965) found that an *Agrobacterium* degraded α-conidendrin to isovanillin, whereas Konetzka *et al.* (1957) studied washed cell suspensions of a *Flavobacterium* and implicated vanillic acid, *p*-hydroxybenzoic acid, and protocatechuic acid as intermediates in α-conidendrin degradation.

Early intermediates in α-conidendrin oxidation by microorganisms have not yet been characterized. We report two new compounds which have been isolated from the culture filtrates of a *Pseudomonas* spp. tentatively identified as a *Pseudomonas multivorans*. We propose an oxidative degradation scheme for α-conidendrin by this microorganism leading to a substituted naphthalene derivative which could then be further metabolized by the naphthalene metabolism pathway similar to that proposed by Davies and Evans (1964).

Materials and Methods

Organism and Growth Conditions. A gram-negative, motile, asporogenous rod having the features of a nonfluorescent pseudomonad, was isolated from soil by elective culture on α-conidendrin as sole carbon source. This organism was tentatively identified as a *P. multivorans* according to the following tests (Stanier *et al.*, 1966). Positive growth occurred on glucose, glycollate, and eight out of the nine substrates tested which are commonly utilized by this group. The bacteria grew 30, 37, and 41°, but only slightly at 4°. They are oxidase positive. Cleavage of protocathechuic acid occurs at the 3,4 position. Cells were grown with forced aeration at 30° with α-conidendrin by sequential inoculation from 50 ml to 1 l. to 16 l. The growth medium consisted of α-conidendrin (0.6 g/l.), ammonium sulfate (1.0 g/l.), potassium dihydrogen phosphate (2.0 g/l.), and magnesium sulfate (50 mg/l.), adjusted to pH 7.2 with 5 N sodium hydroxide.

The α-conidendrin was suspended in 2 l. of distilled water and sonicated for 10 min with the maximum frequency output of a Branson sonic probe to provide an emulsion before adding it to a 16-l. carboy. Cells were harvested after complete solubilization of α-conidendrin in the late exponential growth phase (20 hr) in a Sharples air-driven centrifuge.

Isolation of Intermediates from Culture Filtrates. Compounds I and II were isolated from culture filtrates of α-conidendrin-grown cells. Culture filtrate (16 l.) was acidified with 20 ml of concentrated H_2SO_4 prior to hand extraction into 8 l. of ethyl acetate. Water was removed from the organic solvent by filtration through anhydrous sodium sulfate followed by evaporation to dryness. The residue was dissolved in 100 ml of chloroform and the chloroform-insoluble material was dissolved in 75 ml of diethyl ether. The ether-insoluble material was dissolved in 75 ml of ethyl acetate. The chloroform, ether, and ethyl acetate solutions each contained four compounds other than α-conidendrin as adjudged by thin-layer chromatography on Eastman Chromagram silica gel sheets 6065 with fluorescent indicator in benzene–dioxane–acetic acid (90:25:5). R_F values of 0.04, 0.08, 0.24, and 0.39 were recorded in addition to α-conidendrin R_F 0.48.

The compound with R_F 0.39 was separated from the mixture in the chloroform solution by chromatography on silica gel (compound I). The 100 ml of chloroform was evaporated down to 6.0 ml and the solid precipitate was filtered. The residual solution was applied to a silica gel column (26 × 2.0 cm) which was generated in chloroform and fractions of 4.6 ml were eluted from the column with chloroform. Compound I eluted in fractions 56–76. After evaporation to dryness this compound was recrystallized from hot ethanol (yield 104 mg). This compound was dried over P_2O_5 (2 mm) before CH analysis, mass spectrometry, nuclear magnetic resonance, and infrared analysis.

The compound with R_F 0.24 was obtained from the extraction with 20 l. of ethyl acetate of four 16-l. carboys each acidified with 20 ml of concentrated H_2SO_4 (compound II). The ethyl acetate was dried by filtration through anhydrous sodium sulfate followed by evaporation to dryness. The residue left after suspension in 100 ml of chloroform followed by 75 ml of diethyl ether was dissolved in 75 ml of ethyl acetate. The 75 ml of ethyl acetate was evaporated down to 25 ml, filtered, and applied to a silica gel column (26 × 2.0 cm) generated in ethyl acetate. Fractions of 5.0 ml were collected by elution with ethyl acetate, and tubes 13–26 contained compound II. After evaporation to dryness, recrystallization from pyridine–H_2O gave a yield of 62.2 mg.

Isolation of ^{18}O-Substituted Intermediate. Compound I was isolated as described above from a 25-ml culture enriched with 9% $[^{18}O]H_2O$. This ^{18}O-substituted compound was analyzed by mass spectrometry.

The isolation of I (4 mg) from a 90-ml culture grown in an atmosphere of 80% nitrogen, 20% oxygen enriched 10.92% with $^{18}O_2$ was carried out as described above. The isolated compound was analyzed by mass spectrometry.

Preparation of Derivatives. The *O*-acetyl derivative of I was synthesized by dissolving 100 mg (0.403 mmole) in 2.0 ml of dry pyridine, flushing the solution with N_2 for 3 min, and adding 0.1 ml of acetic anhydride anaerobically. This solution was stirred at room temperature for 23 hr before the reaction product was added to 10.0 ml of crushed ice. The resulting crystals were filtered off and recrystallized from hot ethanol (yield 53%). The derivative was analyzed by CH analysis, mass spectrometry, and nuclear magnetic resonance. A mono-2,4-dinitrophenylhydrazone derivative of I was synthesized by reacting 2,4-dinitrophenylhydrazine (0.2 g) dissolved in 1.0 ml of concentrated H_2SO_4 plus 1.5 ml of water followed by 5.0 ml of ethanol, with 100 mg of I dissolved in 20 ml of hot ethanol. The resulting mixture was allowed to stand at room temperature for 12 hr to allow crystallization. Crystals were filtered and recrystallized from hot ethanol and ethyl acetate (yield 45%). The derivative was analyzed by CHN analysis and mass spectrometry.

Analytical Methods. Mass spectra were obtained on an Atlas CH_4 spectrometer. Infrared spectra were taken on a Perkin-Elmer Infracord, samples being prepared as KBr disks. Melting points (uncorrected) were determined with a Thomas-Hoover capillary melting point apparatus. Nuclear magnetic resonance spectra were recorded in deuterated dimethyl sulfoxide or deuterated pyridine on a Varian A60 or Varian HA-100 spectrometer. Ultraviolet spectra were recorded on a Cary Model 14 recording spectrophotometer.

Materials. Silica gel (0.05–0.20 mm) was obtained from E. Merck, Darmstadt, Germany. α-Conidendrin was obtained from K & K Laboratories, Plainview, N. Y. This material was routinely recrystallized from acetone–water (mp 237–240°). ^{18}O-Enriched water was obtained from Bio-Rad Lab-

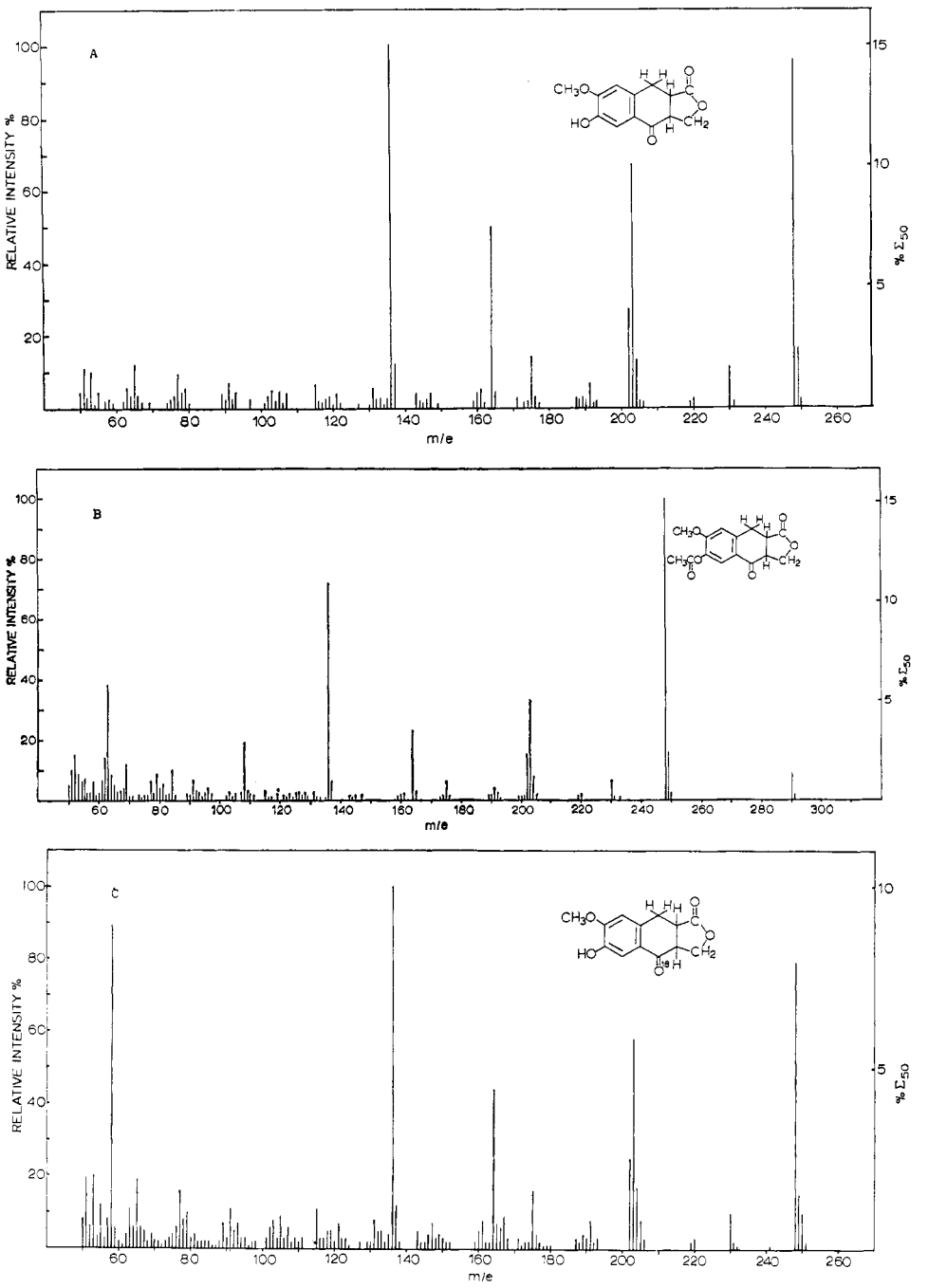


FIGURE 1: Mass spectral analysis of (A) I, (B) the monoacetate derivative of I, and (C) the ^{18}O-substituted I.

oratories, Richmond, Calif. Oxygen gas enriched with ^{18}O was purchased from Isomet Corp., Palisades Park, N. J. All other chemicals were of highest purity commercially available.

Results

Structure of I. Mass spectral analysis of I gives a molecular ion at *m/e* 248 indicating that the aromatic nucleus at C_4 has been removed in the degradation of α-Conidendrin (Figure 1A). A metastable peak at 213 indicates the loss of a neutral fragment of mol wt 18 (H_2O) probably obtained from the lactone ring which opens and extracts hydrogen from some other part of the molecule. The remaining charged fragment appears at *m/e* 230. A second metastable peak at 166 indicates the loss of neutral particles from the lactone ring of molecular weight 45 (HO and CO). The charged fragment appears at *m/e* 203. The parent peak at *m/e* 136 arises from a complete retro-Diels–Alder fragmentation from the *m/e* 164 fragment which loses 29 (CO) leaving the hydroxyl- and methoxyl-substituted aromatic ring with an extra CH_2 substituent (Budzikiewicz *et al.*, 1964). *Anal.* Calcd for: $C_{13}H_{12}O_5$: C, 62.88; H, 4.90. Found: C, 63.42; H, 4.94 (mp 213–215° dec).

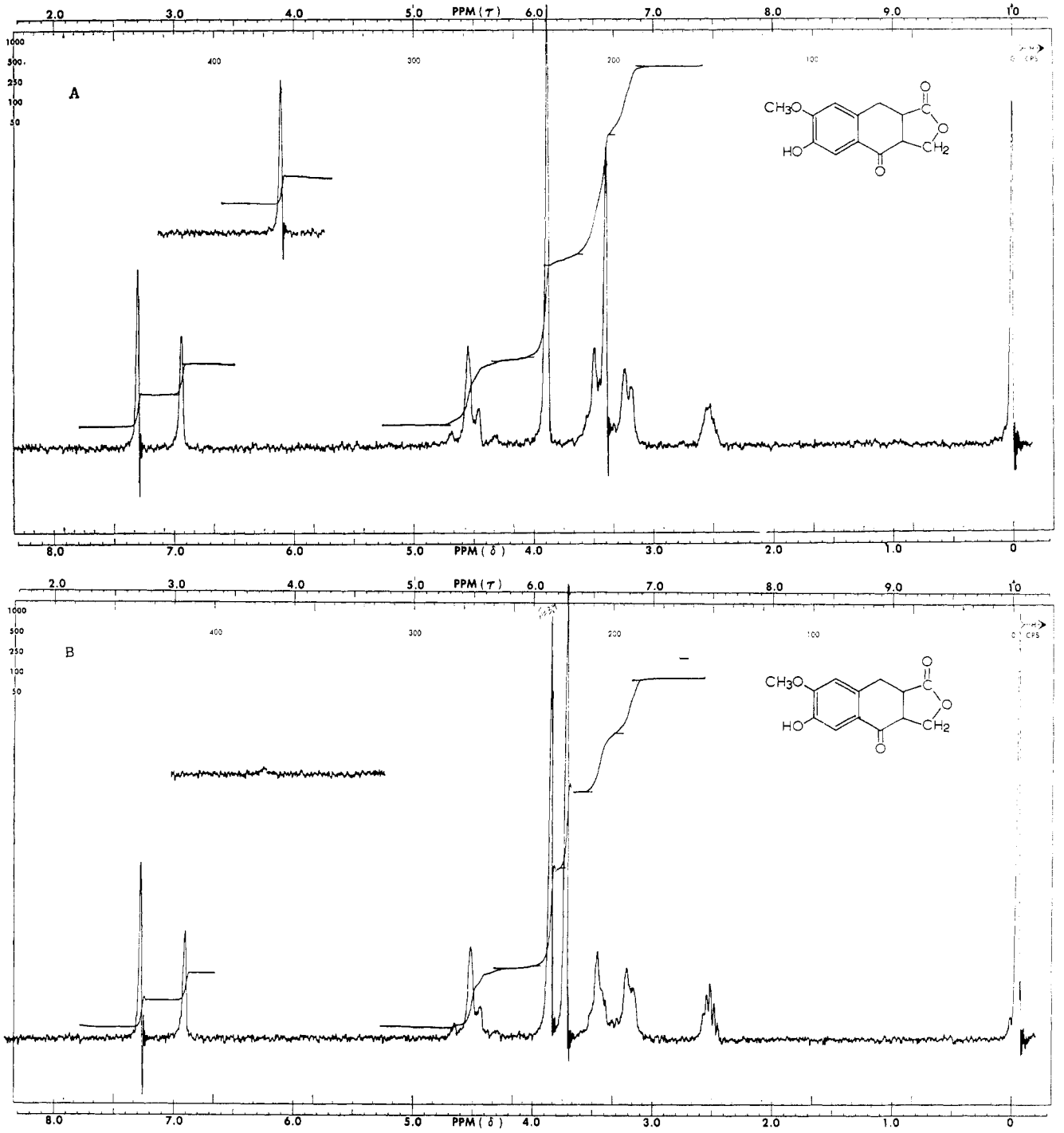


FIGURE 2: Nuclear magnetic resonance spectra of the keto form of I in d_6-dimethyl sulfoxide. (A) In the absence of D_2O and (B) in the presence of D_2O.

Infrared analysis indicates the presence of phenol (3500 cm^{-1}), lactone (1757 cm^{-1}), and methoxyl (1200 cm^{-1}) groups. Two nuclear magnetic resonance spectra were recorded, one in d_6-dimethyl sulfoxide (Figure 2A) and the other in d_5-pyridine (Figure 3A). For dimethyl sulfoxide, the methylene protons on the lactone ring are located at δ 4.5 and the other methylene protons appear at δ 3.48. Two methine protons each adjacent to a carbonyl carbon and a methylene group give a broad resonance at δ 3.25. The methoxyl protons provide a useful standard for integration (δ 3.9). The only exchangeable proton is the phenolic hydrogen (δ 9.33); this exchanges on the addition of D_2O (Figure 2B). Two aromatic protons at δ 7.38 and 6.95 account for the remaining hydrogens. When the nuclear magnetic resonance spectrum is recorded in d_5-pyridine, the compound exists as a phenolate ion (Figure 3A). The decoupling of three different protons H_a, H_b, and H_c

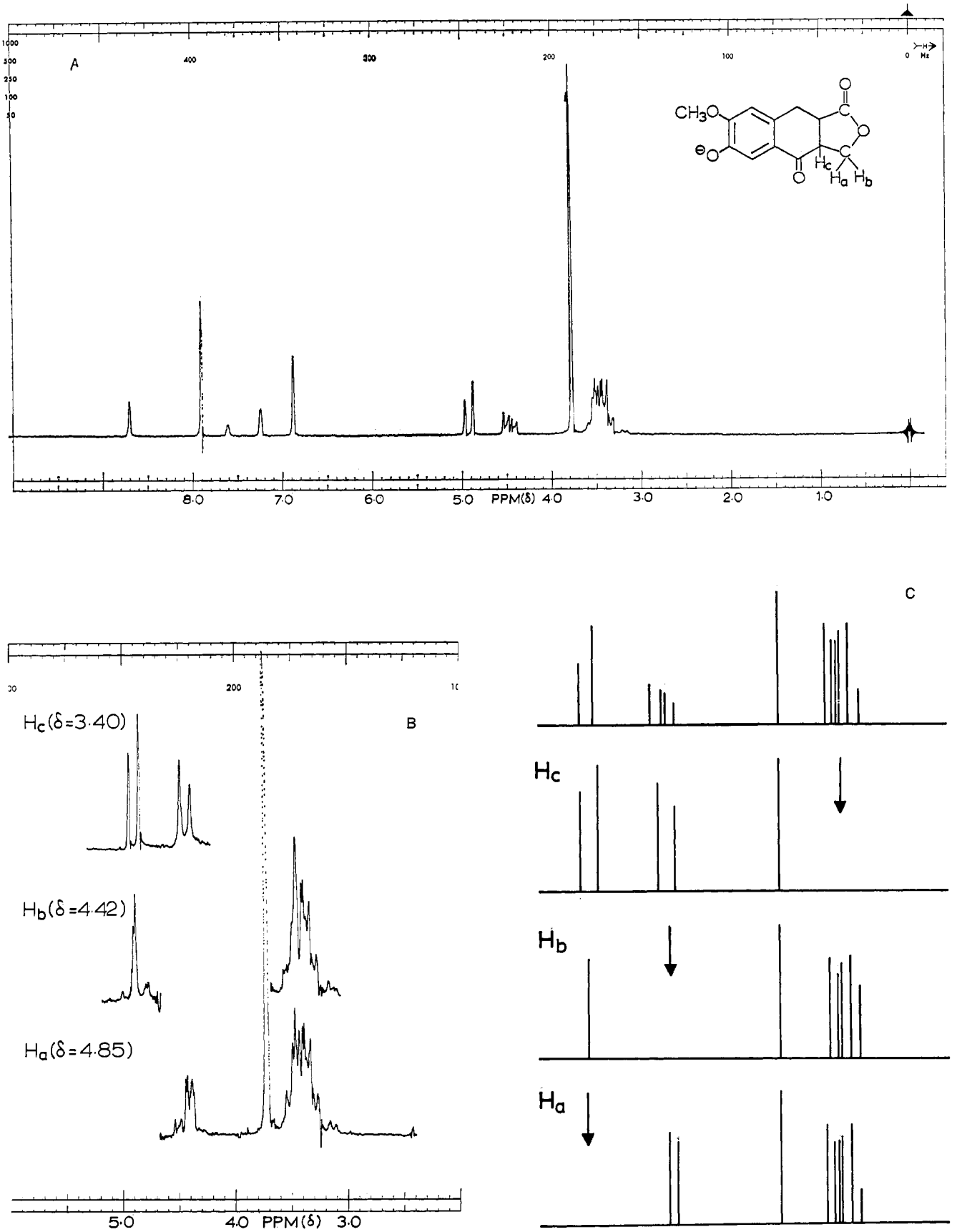


FIGURE 3: Nuclear magnetic resonance spectra of the phenolate ion of I in d_5-pyridine. (A) Complete spectrum 100 MHz, (B) decoupled protons H_a, H_b, and H_c, and (C) interpretation of decoupling experiment.

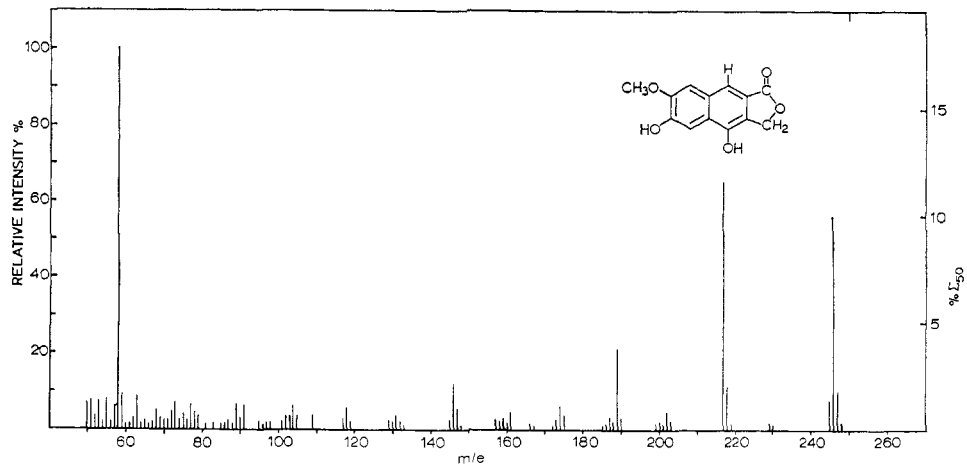


FIGURE 4: Mass spectrum of II.

is indicated in Figure 3B,C. The methylene protons in the lactone ring have different chemical shifts, δ 4.85 for H_a and δ 4.42 for H_b with a coupling constant of J_{ab} = 9 cps. Protons H_b (δ 4.42) and H_c (δ 3.40) couple with a J value of 6 cps. Decoupling H_a causes the H_b quartet to collapse to a doublet with a coupling constant of J_{bc} = 6 cps. The H_a signal becomes a singlet when H_b is decoupled. When H_c is decoupled, H_a and H_b form a quartet with J_{ab} = 9 cps. Keto–enol tautomerization is indicated by the ultraviolet–visible spectrum showing a λ_{max} of 325 nm at pH 2.0 and a λ_{max} of 365 nm at pH 12.0. The ultraviolet–visible spectrum of I in ethanol has absorption maxima for the lactone ((ϵ 15,000) 238 nm), the aromatic ring ((ϵ 10,900) 281 nm), and the ketone ((ϵ 6950) 325 nm).

Mass spectral analysis of the *O*-acetyl derivative of I gives a molecular ion at *m/e* 290 (Figure 1B). The fragmentation pattern indicates a loss of a neutral particle of mass 42 (C_2H_2O) from the acetyl ester. The remaining fragmentation is the same as for I. CH analysis for this monoacetate gave the following. *Anal.* Calcd for $C_{15}H_{14}O_6$: C, 62.05; H, 4.86. Found: C, 62.92; H, 4.82 (mp 190–192°). Mass spectral analysis of the 2,4-dinitrophenylhydrazone derivative of I gave a molecular ion of 428, indicating the formation of the monoderivative. Carbon–hydrogen–nitrogen analysis of this monohydrazone gave the following. *Anal.* Calcd for: $C_{19}H_{16}N_4O_8$: C, 53.27; H, 3.77; N, 12.75. Found: C, 53.12; H, 3.81; N, 12.75 (mp 263°). On the basis of the above data it is concluded that I is 1,2,3-trihydro-6-hydroxy-7-methoxy-4-oxo-3-hydroxymethyl-2-naphthoic acid γ-lactone, and that this compound undergoes keto–enol tautomerization to give a phenolate ion in base.

^{18}O-Substituted I. Compound I (3.7 mg) isolated from a 25-ml culture containing water enriched 9% with [^{18}O]H_2O gives a molecular ion at *m/e* 248 (Figure 1C). The peak at *m/e* 250 is 10% of the parent peak at 136. This increase at *m/e* 250 indicates an oxygen from water has been incorporated into the compound. The increase in peaks at *m/e* 205 and 166 substantiate the retro-Diels–Alder fragmentation pattern.

When I was isolated from a 90-ml culture grown under 80% nitrogen and 20% oxygen enriched with ^{18}O, the mass spectrum showed no increase in the *m/e* 250 peak.

Structure of II. Mass spectral analysis of II gives a molecular ion at *m/e* 246 (Figure 4). A metastable peak at 191 indicates the loss of a neutral fragment of mol wt 29 (CHO). The charged fragment remaining appears at *m/e* 217. Loss of 28 (CO) gives a peak at *m/e* 218. Finally, the peak at *m/e* 189 is due to loss of both neutral fragments 28 (CO) and 29 (CHO), which are usual fragments lost from phenols (Budzikiewicz *et al.*, 1964). *Anal.* Calcd for: $C_{13}H_{10}O_5$: C, 63.41; H, 4.10. Found: C, 62.82; H, 3.86 (mp above 300° with dec). Infrared analysis gave a carbonyl stretch at 1750 cm^{-1} and a methoxyl stretch at 1265 cm^{-1}. The nuclear magnetic resonance spectrum of II was recorded in d_6-dimethyl sulfoxide and shows the presence of three aromatic protons at δ 7.45, 7.55, and 7.8, indicating that aromatization to a naphthalene derivative has occurred (Figure 5A). A phenolic proton appears at δ 3.35, and this proton together with the phenolic proton resonance at δ 10.1 exchanges on the addition of D_2O (Figure 5B). A methoxyl resonance at δ 3.9 and a methylene group at δ 5.4 account for the remainder of the protons. The ultraviolet spectrum of II in ethanol has four maxima at 231 nm (ϵ 9460), 258 nm (ϵ 12,900), 268 nm (ϵ 12,800), and 323 nm (ϵ 3500). On the above basis II is characterized as 4,6-dihydroxy-3-hydroxymethyl-7-methoxy-2-naphthoic acid γ-lactone.

Discussion

Microorganisms which grow with α-conidendrin as sole source of carbon have been isolated from the genus *Pseudomonas*, *Achromobacter*, *Flavobacterium*, and *Agrobacterium* (Konetzka *et al.*, 1952; Pratt *et al.*, 1953; Tabak *et al.*, 1959; Sundman, 1965). However, little is understood about the mechanism of oxidative degradation of this lignin model compound by soil bacteria. Manometric experiments with washed cell suspensions of a *Flavobacterium*, supported by paper chromatographic analysis of products, implicated vanillic acid, *p*-hydroxybenzoic acid, and protocatechuic acid as intermediates in α-conidendrin degradation (Konetzka *et al.*, 1957). Paper chromatographic analysis can be misleading because compound I has been shown to run with an R_F value identical with *p*-hydroxybenzoate in all the solvent systems which we have employed. In addition to this preliminary evidence provided by Konetzka *et al.* (1957), Sundman (1965)

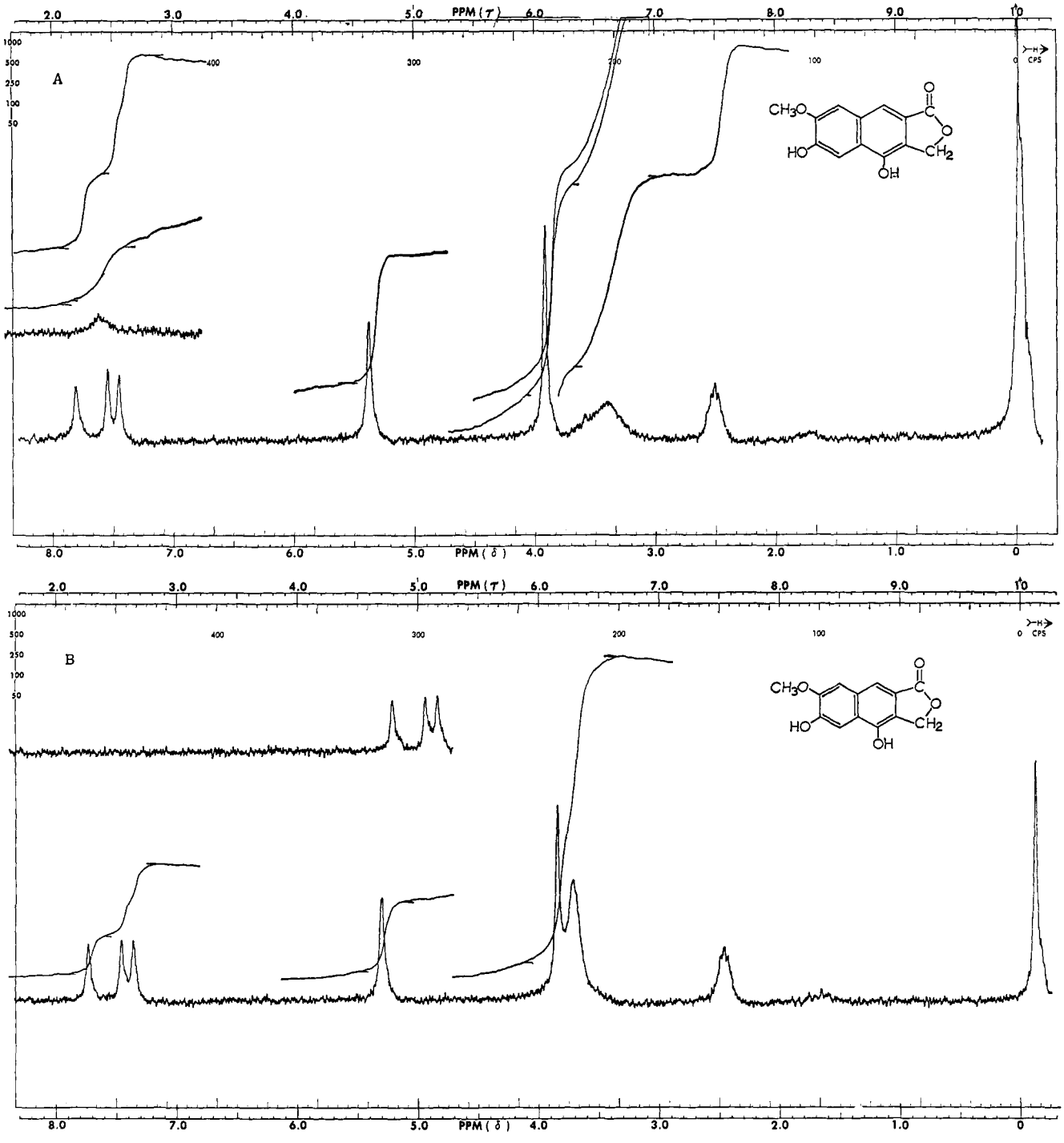
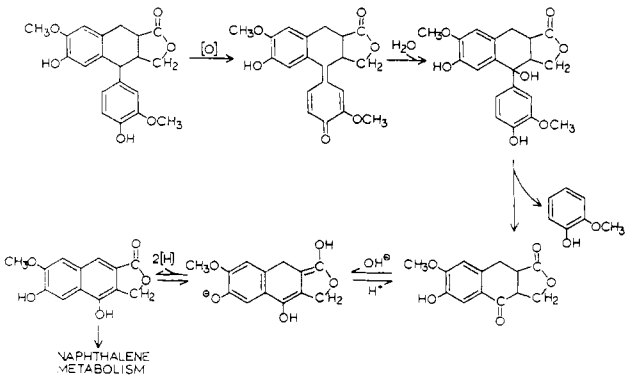


FIGURE 5: Nuclear magnetic resonance spectra of II in d_6-dimethyl sulfoxide. (A) In the absence of D_2O and (B) in the presence of D_2O.

found that a species of *Agrobacterium* would accumulate isovanillic acid when grown at the expense of α-conidendrin. Details of the structure of early intermediates in α-conidendrin oxidation are unprecedented.

The isolation of two substituted naphthalene derivatives from culture filtrates of this pseudomonad grown with α-conidendrin provides two new organic compounds and an insight into the mechanism of the oxidative degradation of this lignan. The first conclusion that can be drawn is that rapid removal of the aromatic nucleus at C_4 occurs in growing cultures. Attempts have been made to isolate this fragment, and although a phenol with similar chromatographic properties to guaiacol appears early in the growth curve, it is rapidly assimilated at an early stage in the growth cycle. The organism grows well with guaiacol as sole carbon source. We are presently searching for mutants which lack the mixed-function oxygenase enzymes necessary to convert the methoxyl-substituted derivatives into their corresponding catechols (Cart-

SCHEME I



wright and Smith, 1967; Toms and Wood, 1970). A reaction sequence for the oxidative degradation of α-conidendrin by this pseudomonad is proposed in Scheme I. The mechanism for the removal of the benzene nucleus from C_4 of α-conidendrin probably involves an oxidation step to a quinone (Turner, 1964). Hydration of the double bond between C_4 and C_1' accounts for the insertion of oxygen from water. An aldolase cleavage gives the keto form of I and quaiacol. Conversion of I into the enol form followed by a second oxidation step would yield II. Hydrolysis of the γ-lactone of II would provide a substituted naphthalene derivative which may be metabolized by a pathway identical with that reported for naphthalene metabolism by pseudomonads (Davies and Evans, 1964). Such a reaction sequence may yield vanillin, vanillic acid, and protocatechuic acid, respectively, as the ultimate aromatic products.

It is of special interest to note that these organisms have the genetic capacity to metabolize compounds such as naphthalene, phenanthrene, and anthracene when confronted with the task, because substituted naphthalene derivatives are available for metabolism in the degradation of such a predominant natural product as lignin. We feel that studies on the microbial dissimilation of complex lignins, and the isolation of the enzymes involved, may provide a useful technique to facilitate the elucidation of the structure of lignins in a novel way with full regard for the stereochemistry of these polymers.

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