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# Structure of D-Prephenyllactate

## A CARBOXYCYCLOHEXADIENYL METABOLITE FROM *NEUROSPORA CRASSA**

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**A novel natural product structurally related to prephenate and arogenate was isolated from a mutant of *Neurospora crassa*. This D-β-(1-carboxy-4-hydroxy-2,5-cyclohexadiene-1-yl)-lactic acid is herein given the trivial name of D-prephenyllactate. The new metabolite is even more acid labile than is prephenate and is quantitatively converted to phenyllactate at mildly acidic pH. The structure characterization of prephenyllactate was performed using spectroscopic techniques (ultraviolet, $^1$H NMR, $^{13}$C NMR, two-dimensional heteronuclear experiments and mass spectrometry). Circular dichroism proved conclusively the *R* configuration of the asymmetric carbon at C-8 of prephenyllactate. Enzymatic utilization of prephenyllactate by cyclohexadienyl dehydratase and by cyclohexadienyl dehydrogenase from *Klebsiella pneumoniae* was demonstrated.**

In 1953 the first natural product with a carboxycyclohexadienyl moiety, prephenate (*PPA*, Fig. 1) was characterized (1). Its function as a precursor in the biosynthesis of phenylalanine and tyrosine has been known for some time (2). In 1980 the structure of a second carboxycyclohexadienyl metabolite named arogenate (*AGN*, Fig. 1) was determined (3), and it has been shown to be a common but not inevitable precursor of phenylalanine and/or tyrosine in microorganisms (4) and plants (5). Spiro-arogenate, an unusual spiro-γ-lactam (*SPN*, Fig. 1) possessing the same cyclohexadienyl moiety as arogenate was discovered in 1983 (6, 7). It seems to be a dead-end metabolite formed from L-arogenate (8).

We have recently isolated prephenyllactate, another carboxycyclohexadienyl derivative (*PPL*, Fig. 1), from culture supernatants of *Neurospora crassa*. Like other members of the family of carboxycyclohexadienyl molecules, prephenyllactate is acid labile. Thus, acidic conditions which convert prephenate to phenylpyruvate, or which convert arogenate and spiroarogenate to phenylalanine, also convert prephenyllactate to phenyllactate. The probable structure of prephenyllactate deduced from a combination of several chemical and biochemical techniques described herein was proven by analysis of NMR and mass spectral data. The D-configuration (*R*) of the asymmetric carbon at C-8 was established by circular dichroism. Enzymological studies of the ability of prephenyllactate to mimic prephenate or L-arogenate as substrate for aromatic-pathway enzymes are also presented.

### MATERIALS AND METHODS

*Instrumentation*—UV absorption data were recorded on Cary 118 and Perkin-Elmer 559A UV/VIS spectrophotometers. The Fab mass spectrum of the sample was obtained on a ZAB HS instrument under the following conditions: glycerol matrix, Xe atoms of 8 kV energy, and 1 mA beam current with an initial resolving power of 1,000. The NMR spectra were obtained in $D_2O$ on a Varian XL-300 spectrometer operating at 300 MHz for protons and 75.4 MHz for $^{13}$C or on a Bruker WH 400 operating at 400 MHz for protons. The samples (2–5 mg of the potassium salt) were dissolved in $D_2O$ or $Me_2SO$, and the spectra were recorded at ambient temperature (22 °C). The NMR conditions used with the Varian XL-300 were the following: (i) the carbon-13 spectrum was obtained with a spectral window of 16,500 Hz, a pulse angle of 40° (7.6 μs) and 32 K data points. The regular decoupled carbon-13, the DEPT and the two-dimensional heteronuclear correlation experiments are the result of three overnight runs of 15 h each, (ii) for the two-dimensional heteronuclear spectra, 128 free induction decays were acquired at regular time increments to provide a 1 K × 512 matrix after double Fourier transform process. Line broadening was applied along both domains, and (iii) the proton NMR spectra were acquired with a spectral window of 4,000 Hz, 32 K data points, 96 transients and a 45° pulse. The $^1$H NMR spectrum was obtained on the Bruker WH-400 with 3-s preirradiation of the water peak. The spectrum width is 4,500 Hz, the pulse width is ~80°, the number of transients is ~360, and the data points are 32 K. The circular dichroism measurements were carried out using a JASCO model J-5006 spectropolarimeter.

HPLC[1] anion exchange separations were accomplished on an Alltech SAX (4.6 × 250 mm) column using 15 mM sodium phosphate buffer, pH 7.0, as mobile phase. Reverse-phase separations were performed on an Alltech $C_{18}$ column (4.6 × 250 mm) with 10 mM sodium phosphate, pH 6.9, with 0 or 5% (v/v) methanol as mobile phase. Altex model 110A metering pumps maintained flow rates of 1 ml/min. Prephenyllactate was detected by its UV absorbance at 215 nm on a Gilson Holochrom model HM spectrophotometer. Further HPLC anion exchange purifications were performed using a Whatman SAX analytical column (4.6 × 250 mm) or a Whatman SAX semipreparative column (9.4 × 500 mm) with 10 mM potassium phosphate buffer, pH 7.0, as mobile phase. Reverse-phase purifications were also done on a Waters $C_{18}$ analytical column (3.9 × 300 mm) with 10 mM sodium phosphate buffer, pH 7.0, as mobile phase or with a Whatman ODS-3 semipreparative column (9.4 × 250 mm) with 2 mM potassium phosphate buffer, pH 7.0, as mobile phase. A Waters Delta Prep 3000 instrument was used for these HPLC purifications.

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[1] The abbreviations used are: HPLC, high performance liquid chromatography; PEP, phosphoenolpyruvate; EPPS, *N*-[2-hydroxyethyl]-piperazine-*N*′-3-propanesulfonic acid; DEPT, distortionless enhancement by polarization transfer.

*Biochemicals*

Sodium phosphoenolpyruvate (PEP), EPPS, PEP carboxylase, malate dehydrogenase, oxaloacetate, L-phenyllactic acid, nicotinamide adenine dinucleotide $(NAD)^+$ and NADH were obtained from Sigma. DE52 was purchased from Whatman. Prephenate was isolated from the culture supernatants of a tyrosine auxotroph of *Salmonella typhimurium* as described by Dayan and Sprinson (9). L-Arogenate was isolated from a triple auxotroph of *N. crassa* as described previously (3).

*Decarboxylation of Prephenyllactate and Prephenate*

A coupled enzymatic assay was employed to detect $CO_2$ liberated upon aromatization of prephenyllactate and prephenate in aliquots of unheated or heated solutions:

$$\text{PEP} + CO_2\ (HCO_3^-) \xrightarrow[Mg^{2+}]{\text{PEP carboxylase}} \text{oxaloacetate}$$

$$\text{oxaloacetate} + \text{NADH} \xrightarrow{\text{malate dehydrogenase}} \text{malate} + NAD^+$$

Reaction mixtures containing the above enzymes, 50 mM EPPS buffer, pH 8.3, 3.6 mM $MgCl_2$, 2.6 mM PEP, and 0.6 mM NADH were placed in cuvettes held at 28–30 °C while extraneous $CO_2$ was consumed. Aliquots (10–30 μl) of heated (70 °C, pH 8.1–8.3, 10 min) and unheated solutions containing 10 mM prephenyllactate in 200 mM $NH_4Br$ or 5 mM prephenate in 50 mM $NH_4Br$ were transferred to the cuvettes, and the decrease in absorbance at 340 nm (due to oxidation of NADH) was monitored with time on a Gilford 260 UV/VIS spectrophotometer. A control containing only 50 mM $NH_4Br$ was also included.

*Microbiological Aspects*—The media, growth regimen, and protocol for accumulation of prephenyllactate in the triply blocked mutant of *N. crassa* (ATCC 36373) were the same as described for the accumulation of L-arogenate (3). *Klebsiella pneumoniae* ATCC 25304 was obtained from American Type Culture Collection, Rockville, MD. The growth conditions, preparation of crude extract, and DE52 column chromatography were performed exactly as described previously (10). For activity profiles of prephenate dehydratase and prephenate dehydrogenase, refer to Ref. 2.

*Isolation of Prephenyllactate*—After 5–6 days of growth and accumulation, the mycelial mass was captured by filtration through cheesecloth, and the pH of the supernatant was adjusted to 10.7–11.0 with NaOH. Precipitated hydroxide salts were removed by suction filtration through Whatman No. 1 filter paper. All other operations were performed at 4 °C. The supernatant was loaded onto a 4.5 × 15.0 cm column of Dowex 1-X8 (50–100 mesh; $Cl^-$ form) at a flow rate of 5 ml/min. The column was washed with 300 ml of 0.1% *N*-ethylmorpholine, pH 8.5. Solutes were eluted by a linear gradient consisting of 1.5 liters of 0.05 M $NH_4Br$ in 0.1% *N*-ethylmorpholine, pH 8.5, and 1.5 liters of 1.0 M $NH_4Br$ in 0.1% *N*-ethylmorpholine, pH 8.5, at a flow rate of 1.5 ml/min. Nine-ml fractions were collected. Prephenyllactate eluted between fractions containing arogenate (110–135) and prephenate (160–195), overlapping somewhat with prephenate. Fractions containing prephenyllactate (and prephenate) were diluted to a final volume of 4 liters with water, and the pH was adjusted to 8.5. This was then loaded onto a Dowex 1 × 8 (100–200 mesh, $Cl^-$ form) anion-exchange column (3.0 × 18.0 cm) at a flow rate of 4 ml/min. The column was washed with 200 ml of 1 mM $NH_4OH$, and the solutes were eluted by a linear gradient consisting of 600 ml of 0.01 M $NH_4Br$, pH 8.5, and 600 ml of 0.8 M $NH_4Br$, pH 8.5. Five-ml fractions were collected at a flow rate of 1 ml/min. Prephenyllactate eluted in about 10 fractions before prephenate. The prephenyllactate-containing fractions were combined and treated with 0.2 g of $BaBr_2$, 2 volumes of chilled methanol, and 3 volumes of chilled 1-propanol. The mixture was allowed to stand overnight. The precipitated barium prephenyllactate was recovered by centrifugation, washed with methanol/1-propanol (2:3, v/v), and then with ethyl ether. The precipitate was dried under vacuum. Purity was calculated by using a molecular formula of Ba prephenyllactate·$2H_2O$ ($M_r$ = 380), a state of hydration analogous to that used for the prephenate molecule (1).

*Purification of Prephenyllactate*—The prephenyllactate was initially purified by HPLC using a Whatman ODS-3 MAG-9 semipreparative column (9.4 × 250 mm) at a flow rate of 3 ml/min with 2 mM potassium phosphate, pH 7.0, as eluant. The UV detector was set at 204 nm. The retention time of prephenyllactate was 2.2 min and of phenyllactate was 5.9 min. As prephenyllactate was collected, the pH was raised to 10 by the addition of 1 N KOH. The prephenyllactate solution was lyophilized overnight. An aliquot of this prephenyllactate sample was examined on a Whatman analytical SAX (strong anion-exchange) column (4.6 × 250 mm) which was eluted at 1 ml/min with 10 mM potassium phosphate buffer, pH 7.0, with UV detection at 204 nm. Four minor impurities which were acid stable were present at retention times of 6.7, 7.5, 10.6, and 15.5 min. The retention time of prephenyllactate was 8.6 min, and the retention time of phenyllactate produced by hydrolysis was 4.4 min. Therefore, a second HPLC purification was carried out using a Whatman semipreparative SAX column (9.4 × 500 mm) which was eluted with 10 mM potassium phosphate, pH 7.0, with a flow rate of 4 ml/min and UV detection at 204 nm. The fractions collected were kept at pH 10–11 by addition of 1 N KOH. The retention time for prephenyllactate was 16.0 min. The fractions collected were then lyophilized. The prephenyllactate sample obtained was very pure judging from HPLC analysis, NMR data confirming this conclusion. An aliquot of the purified prephenyllactate sample was acidified and injected onto a reverse-phase column (Waters 15–20 μm $C_{18}$, 3.9 × 300 mm, 1 ml/min, 10 mM sodium phosphate, pH 7.0, λ = 215). A single symmetrical peak having an identical retention time (14.0 min) as standard phenyllactate (Sigma) was recovered. The yield of prephenyllactate in *N. crassa* is of the same order of magnitude as the yield of L-arogenate.

*Enzyme Assays*—Prephenyllactate dehydrogenase from *K. pneumoniae* was assayed in the temperature controlled compartment of a spectrophotofluorometer by the method used for prephenate dehydrogenase except that prephenyllactate replaced prephenate as a substrate (excitation λ = 340 nm, emission λ = 460 nm). The enzyme was recovered after DE52 column chromatography of the crude extracts (2, 10). The reaction mixture in a final volume of 200 μl contained 50 mM potassium phosphate buffer, pH 7.0, containing 1 mM dithiothreitol, 0.5 mM $NAD^+$, 2 mM potassium-prephenyllactate (the barium salt was converted to the potassium salt before use), and enzyme. The enzyme was incubated with the cofactor to exhaust any background reduction of the cofactor. The substrate was then added, and the continuous formation of NADH was followed spectrophotofluorometrically at 37 °C. The formation of 4-hydroxyphenyllactate was confirmed by HPLC. The enzyme was incubated with 50 mM potassium phosphate buffer, pH 7.0, containing 1 mM dithiothreitol, 0.4 mM $NAD^+$, and 2 mM prephenyllactate in a final volume of 275 μl at 34 °C for 60 min. The tubes were transferred to ice, and the contents were injected into a 20-μl loop and swept onto a $C_{18}$ column (4.6 × 250 mm) that was equilibrated and eluted with 10 mM potassium phosphate buffer, pH 7.0, at a flow rate of 1 ml/min. The absorbance of the eluate was monitored at 219 nm. The appearance of 4-hydroxyphenyllactate was confirmed by the identification of a peak at a retention time characteristic of authentic 4-hydroxyphenyllactate.

Prephenate dehydratase isozymes from *K. pneumoniae* were assayed with prephenyllactate as substrate by HPLC monitoring of phenyllactate appearance. The isozymes were recovered after DE52 column chromatography of crude extracts (2). The reaction mixture in a final volume of 200 μl contained 50 mM potassium phosphate, pH 7.0, containing 1 mM dithiothreitol, 2 mM prephenyllactate, and enzyme. The reaction was carried out at 34 °C for 60 min. Controls were included in which only enzyme was incubated, only substrate was incubated, or the complete reaction mixture was injected without incubation. After incubation the tubes were transferred to ice, and the contents were injected into a 20-μl loop and swept onto a $C_{18}$ column (4.6 × 250 mm), equilibrated, and eluted with 10 mM potassium phosphate, pH 7.0, at a flow rate of 1 ml/min. The absorbance of the eluate was monitored at 219 nm. The appearance of phenyllactate was confirmed by establishing the exact correlation of the increment of peak area at the phenyllactate position with the decreased peak area corresponding to prephenyllactate. The identity of the product as phenyllactate was also confirmed by comparison of the specific retention time with that of authentic phenyllactate.

*Detection and Quantitation of Prephenyllactate*—Prephenyllactate was detected and quantitated by HPLC after acid conversion to phenyllactate. The acidified samples (5–10 μl HCl, 100 μl of sample) were incubated at 37 °C for 15 min. They were then injected into a 20-μl sample loop and swept through a 4.6 × 250 mm $C_{18}$ reversed-phase column (Alltech). The compound was eluted with a solvent system consisting of 95% 10 mM potassium phosphate buffer, pH 7.0, and 5% methanol (v/v) using a flow rate of 1 ml/min. Phenyllactate was detected by measuring its absorbance at 215 nm (end absorption).

Prephenyllactate concentrations were determined by comparing the absorbance (peak height or peak area) of phenyllactate (the acid-converted product) to a standard curve relating authentic phenyllactate concentration to $A_{215}$.

*Chemical Syntheses of L- and D-Phenyllactate*—L- and D-phenyllactate were obtained from L- and D-phenylalanine, respectively, by a diazotization procedure according to Ref. 11. The L- and the D-phenyllactate were identical in all spectroscopic properties except for the circular dichroism measurements. The Cotton effect of the synthesized L-phenyllactate was positive and identical to the L-phenyllactate purchased from Sigma. As expected, the Cotton effect of the synthesized D-phenyllactate was negative. The melting point of synthesized L-phenyllactate was 122.5–123 °C (literature 124–126 °C); the melting point of synthesized D-phenyllactate was 122.5–123° C (literature 124–125° C), and the melting point of synthesized DL-phenyllactate was 96.5–97 °C (literature 98 °C).

## RESULTS AND DISCUSSION

*Metabolic Imbalance Causing Prephenyllactate Accumulation*—The mutant blocks present in *N. crassa* ATCC 36373 create biochemical interruptions whereby abnormally high levels of prephenate accumulate under conditions of end product starvation (to avoid early pathway regulation of metabolite flow). Fig. 1 illustrates the appearance of two new dead-end paths that do not ordinarily function in wild type. In one case, prephenate is transaminated to initiate the arogenate/spiroarogenate sequence. In the second case, now described in this paper, the side chain of prephenate is reduced to produce prephenyllactate.

*Purification of Prephenyllactate*—As specified under "Materials and Methods," prephenyllactate was purified by several steps of reverse-phase and anion-exchange HPLC. Purity of prephenyllactate was assessed by acidification of an aliquot, whereby the entire peak corresponding to prephenyllactate disappears with *concomitant* formation of a new peak corresponding to authentic phenyllactate on reverse-phase HPLC.

*Stability of Prephenyllactate*—Anion-exchange HPLC was used to follow the rates of thermally induced aromatization of prephenyllactate and prephenate (Fig. 2). Prephenyllactate and prephenate in approximately equal concentrations in 0.2 M $NH_4Br$ were heated together at 70 °C, pH 7.5. Aliquots taken at time 0 (*a*), 5 min (*b*), and 10 min (*c*) were injected onto an analytical SAX column. The retention times of phenyllactate and phenylpyruvate are very similar and overlap ~4.8 min. Prephenyllactate ($R_t$ = 11.1 min) is easily separated from prephenate ($R_t$ = 13.2 min). From the HPLC chromatograms in Fig. 2, it is apparent that prephenyllactate is more thermally labile than is prephenate.

*The Structure of Prephenyllactate*—Useful clues about the structure of the new metabolite present in accumulation supernatants of *N. crassa* were obtained from anion-exchange and reverse-phase HPLC, UV spectra, and enzymological detection of $CO_2$ evolved upon acidification and/or heating of solutions containing purified prephenyllactate. Anion-exchange HPLC and reverse-phase HPLC showed that acidification yielded a peak eluting at the same retention time as authentic phenyllactate. Following anion-exchange HPLC, the new metabolite was found to possess a retention time in the range of compounds with −2 charge, whereas the acidification product was in the range of −1 charged compounds. The UV spectra of prephenyllactate showed a large end absorption. After 15 min at 37 °C and pH 2, the UV spectrum showed the typical B band pattern of an aromatic ring. The tertiary carboxylate group in the new metabolite (prephenyllactate) was indicated by using a coupled enzymatic assay involving phosphoenolpyruvate carboxylase and malate dehydrogenase to detect $CO_2$ and $HCO_3^-$ in solution as described under "Materials and Methods." Values listed in Table I (left column) are the differences in absorbance changes between reaction mixtures containing aliquots of heated and unheated solutions. From these data the concentration changes of NADH, and thus the amount of $CO_2$ evolved, can be calculated. Heating of ~10 mM prephenyllactate was found to produce four times as much $CO_2$ as heating of 5 mM prephenate. Although the initial concentrations of prephenyllactate and prephenate were not equal, this result qualitatively confirms that prephenyllactate is somewhat more thermally labile than is prephenate (as was demonstrated above by HPLC). Evolution of $CO_2$ from prephenyllactate, taken together with the identification of phenyllactate as its acid-catalyzed decomposition product, indicated the structure depicted for prephenyllactate in Fig. 1.

*$^{13}C$ NMR Analysis of Prephenyllactate, DEPT, and Two-dimensional Heteronuclear Experiments*—The $^{13}C$ NMR spectra of prephenyllactate, a DEPT experiment and a two-dimensional heteronuclear shift correlation experiment enables assignment of the signals observed. These $^{13}C$ shifts, along with the chemical shifts of arogenate (3), are given in Table II which shows that the chemical shifts of the two compounds are very similar. They differ significantly only in the shift of carbon C-8, which in the case of L-arogenate is influenced by an $\alpha$-$NH_2$ group ($\delta$ = 54.8 ppm) while in the prephenyllactate, this group is replaced by an −OH ($\delta$ = 71.8 ppm). The $^{13}C$ NMR analysis was taken in $D_2O$ with the potassium salt of prephenyllactate. Under these conditions, the compound seems to form aggregates which broaden somewhat the C-3/C-5 signals. This aggregate formation is also consistent with the proton NMR spectrum as will be discussed in the next section. The two-dimensional heteronuclear shift correlation experiment confirmed unambigously that the signals observed at 134.4, 132.2, 128.5, and 127.9 ppm represent carbons that are uniquely correlated with the vinylic protons (Fig. 3). In addition, it shows the correlation between C-4/H-4 and C-8/H-8. The two-dimensional heteronuclear experiment has allowed a more precise correlation of the C-3/C-5 (134.2, 134.4 ppm) signals with the downfield part of the complex vinylic proton signals at 5.87–5.90 ppm (for H-5), as well as with half of the proton signal at 5.82 ppm (for H-3) (almost an $A_2$ pattern) (the assignment for H-3 and H-5 protons can be interchanged). Similarly, the C-2/C-6 signals (128.5, 127.9 ppm) are correlated with the high field vinylic protons at 5.74 ppm (for H-6) and with the $A_2$ signal observed at 5.82 ppm (for the H-2 proton) (The assignment for the H-2 and H-6 protons presented here can be interchanged).

*$^1H$ NMR Analysis of Prephenyllactate*—The $^1H$ NMR spectrum obtained at 300 MHz for the potassium salt of prephenyllactate in $D_2O$ is shown in Fig. 4*a*, along with a decoupled experiment(*b*). The multiplets observed for H-7 and H-8 represent an ABX pattern very similar to those observed in the case of L-arogenate and of spiroarogenate (3, 6, 7). This ABX spin system is characteristic of a $CH_2$ group with nonequivalent protons coupled to a neighboring CH group at C-8 (the analysis is presented in Table III). The vicinal coupling constants show similar behavior as observed for the L-arogenate; the lowest field H-7 proton (H-7a, 1.937 ppm) is characterized by a small vicinal coupling (2.9 Hz in prephenyllactate and 3.0–3.5 Hz in arogenate), while the higher field H-7 proton (H-7b, 1.814 ppm) is characterized by a larger coupling (8.8 Hz in prephenyllactate and 8.5 Hz in arogenate). These similar coupling values indicate similar conformation. The H-4 signal, practically overlapping with the strong $D_2O$ gave rise to a broad complex multiplet. This proton is coupled to H-5 and H-3 (vicinal coupling) and to H-2/H-6 (long range coupling). Upon decoupling of this signal, the H-2/H-6 and H-3/H-5 vinylic protons simplify as shown in Fig. 4*b*. The signals

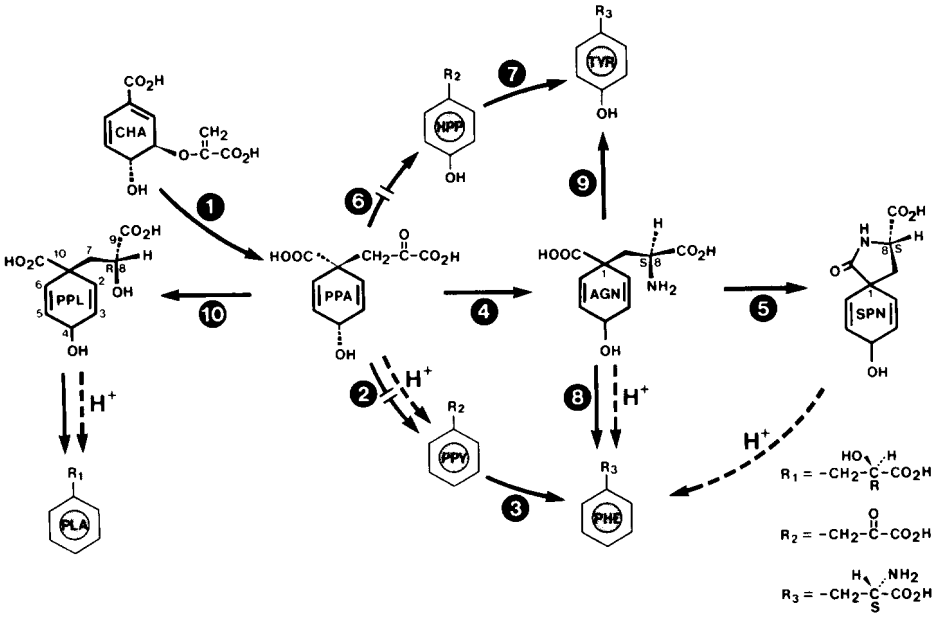


FIG. 1. **Conversion to aromatic compounds of cyclohexadienyl metabolites produced in a triple mutant of *N. crassa* deficient in enzymes (2, 6) and anthranilate synthase.** The abbreviations are: chorismate (*CHA*), prephenate (*PPA*), L-arogenate (*AGN*), spiroarogenate (*SPN*), prephenyllactate (*PPL*), 4-hydroxyphenylpyruvate (*HPP*), phenylpyruvate (*PPY*), L-phenylalanine (*PHE*), L-tyrosine (*TYR*), and phenyllactate (*PLA*). Enzymes shown are: 1, chorismate mutase; 2, prephenate dehydratase; 3, phenylpyruvate aminotransferase; 4, prephenate aminotransferase; 5, a postulated arogenate "spirase"; 6, prephenate dehydrogenase; 7, 4-hydroxyphenylpyruvate aminotransferase; 8, arogenate dehydratase; 9, arogenate dehydrogenase; and 10, a postulated D-prephenyllactate dehydrogenase. For brevity, cofactors and cosubstrates are not shown for dehydrogenase and aminotransferase reactions. The *dotted arrows* show the acid-catalyzed conversions obtained *in vitro*.

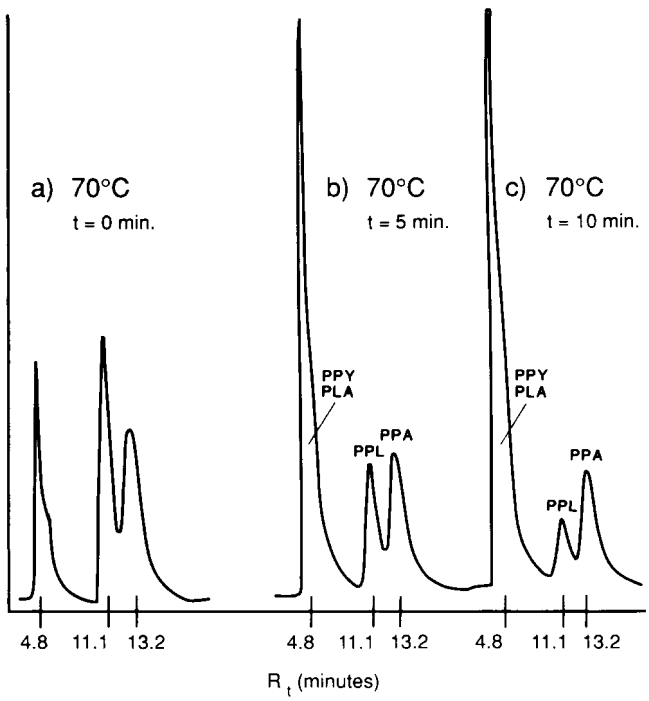


FIG. 2. **Comparison of the thermal stabilities of prephenate and prephenyllactate.** The mixture of prephenate and prephenyllactate was incubated at 70 °C, pH 7.0, for zero min (*a*), 5 min (*b*), and 10 min (*c*) followed by HPLC separation of the reaction components on a SAX analytical column (4.6 × 250 mm; 15 mM sodium phosphate buffer at pH 7.0; elution 1 ml/min, UV detector at 214 nm).

TABLE I

*Carbon dioxide produced by heating solutions of prephenyllactate or prephenate*

Approximately 10 mM prephenyllactate in 200 mM $NH_4Br$, pH 8.3, and 5 mM prephenate in 50 mM $NH_4Br$, pH 8.1, were heated at 70 °C for 10 min and then assayed as described under "Materials and Methods."

| Sample | $\Delta A_{340}$[a] | $CO_2$ |
|---|---|---|
| | | *mM* |
| Prephenyllactate (~10 mM) | +0.57 | 1.14 |
| Prephenate (5 mM) | +0.14 | 0.28 |
| $NH_4Br$ (50 mM) | −0.04 | — |
| $NaHCO_3$ | +0.77 (unheated) | 1.54 |

[a] $\Delta A_{340}$ = $\Delta A_{340}$ (heated) − $\Delta A_{340}$ (unheated). A value of 0.1 corresponds to 0.02 mM NADH.

TABLE II

*$^{13}C$ NMR chemical shifts observed for prephenyllactate and L-arogenate*

The multiplicity indicated in parentheses comes from a DEPT experiment.

| Carbon no. | Prephenyllactate | L-Arogenate |
|---|---|---|
| C-9 | 182.8/182.0 (*S*) | 182.3/183.7 |
| C-10 | 182.0/182.8 (*S*) | 183.7/182.3 |
| C-3 | 134.4/134.2 (D) | 132.5/131.9 |
| C-5 | 134.2/134.4 (D) | 131.9/132.5 |
| C-2 | 128.5/127.9 (D) | 129.6/129.0 |
| C-6 | 127.9/128.5 (D) | 129.6/129.9 |
| C-4 | 62.7 (D) | 62.1 |
| C-8 | 71.8 (D) | 54.8 |
| C-1 | 51.5 (*S*) | 51.1 |
| C-7 | 45.4 (T) | 45.0 |

observed for the olefinic protons appear to be much more complex than in the case of L-arogenate. The presence of an asymmetric carbon at C-8 render the two olefinic groups (H-2/H-3 and H-5/H-6) nonequivalent as observed in the case of L-arogenate. The H-2 and H-3 protons (or H-5/H-6), characterized by similar chemical shift, form an $A_2$ spin system ($\delta$ = 5.82 ppm) coupled to the H-4 proton and to the H-5/H-6 protons. On the other hand, the H-5 and H-6 olefinic protons (or H-2/H-3) appear at two different chemical shift positions ($\delta$ = 5.90 ppm and $\delta$ = 5.737 ppm, respectively). These coupled protons are both coupled to H-4 and to H-2/H-3 protons and give rise to a very complex coupling pattern. Upon decoupling the H-4 proton, the olefinic region for H-5 and H-6 simplify somewhat into two AB patterns of different ratio (3:1), further coupled to protons H-2 and H-6 (Fig. 4*c*). The presence of two AB patterns of different ratios for only one olefinic group led us to postulate the presence of aggregates in the $D_2O$ solution. To confirm the presence of aggregates, the sample was run in another solvent. In dimethyl sulfoxide the olefinic region sharpened up into the expected four-multiplet pattern for the H-2, H-6, H-3, and H-5 protons (Fig. 4*d*). At 400 MHz

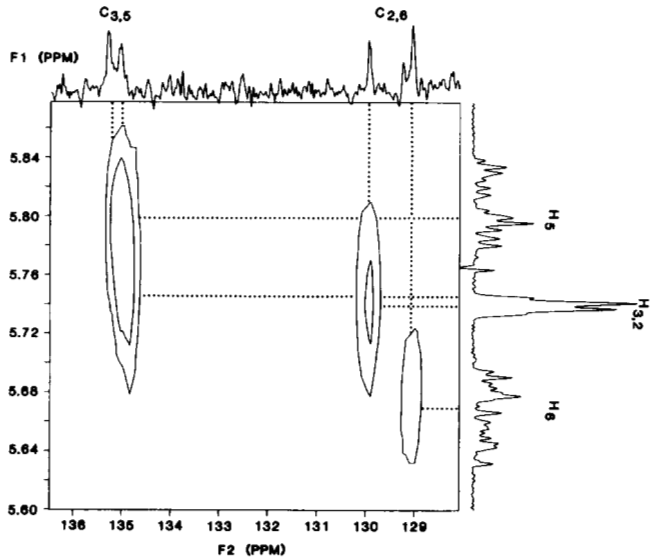


FIG. 3. **Two-dimensional heteronuclear shift correlation spectra of prephenyllactate-vinylic expansion.** The C-3/C-5 signals are correlated with H-3/H-5 protons while the C-2/C-6 signals are correlated with the H-2/H-6 protons. (The H-5/H-3 signals can be interchanged as well as the signals observed for H-2/H-6.)

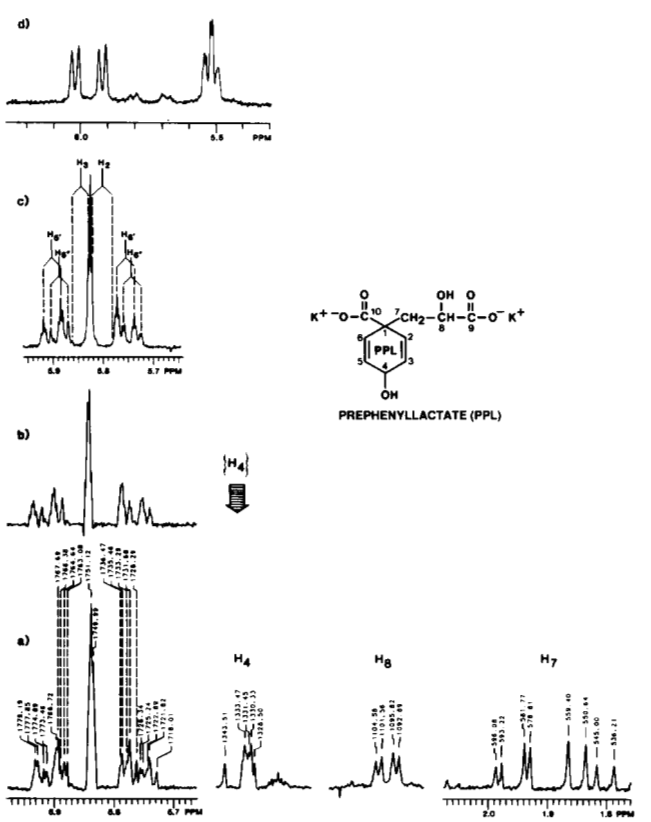


FIG. 4. **NMR analysis of prephenyllactate.** *a*, 300 MHz $^1H$ NMR spectrum of potassium salt of prephenyllactate in $D_2O$. *b*, olefinic region with H-4 decoupling. *c*, expanded olefinic region, with H-4 decoupling showing the presence of a two AB pattern for the H-5 and H-6 protons. *d*, 400 MHz $^1H$ NMR spectrum of potassium salt of prephenyllactate in the $Me_2SO$-$d_6$-olefinic region.

TABLE III

*$^1H$ NMR assignments of prephenyllactate*

| Proton no. | δ | Multiplicity | J |
|---|---|---|---|
| | *ppm* | | *Hz* |
| H-7A | 1.937 | dd | $J_{7A\text{-}B} = 2.9$ $J_{7A\text{-}7B} = -14.3$ |
| H-7B | 1.814 | dd | $J_{7B\text{-}8} = 8.8$ $J_{7A\text{-}7B} = -14.3$ |
| H-8 | 3.647 | dd | $J_{7A\text{-}8} = 2.9$ $J_{7B\text{-}8} = 8.8$ |
| H-4 | 4.426 | m | |
| H-6 | 5.737 | dd | $J_{2\text{-}3} = 10.4$ $J_{2\text{-}4} = 5.1$ |
| Minor | | | |
| H-6 | | ddt | $J_{2\text{-}3} = 10.4$ $J_{2\text{-}4} = 3.8$ |
| Major | | | $J_{2\text{-}5} = J_{2\text{-}6} = 1.2$ |
| H-2 | 5.82 | m | |
| H-3 | 5.82 | | |
| H-5 | 5.87 | dd | $J_{2\text{-}3} = 10.4$ $J_{3\text{-}4} = 1.5$ |
| Minor | | | |
| H-5 | 5.90 | dq | $J_{2\text{-}3} = 10.3$ |
| Major | | | $J_{3\text{-}4} = J_{3,6} = 1.5$ |

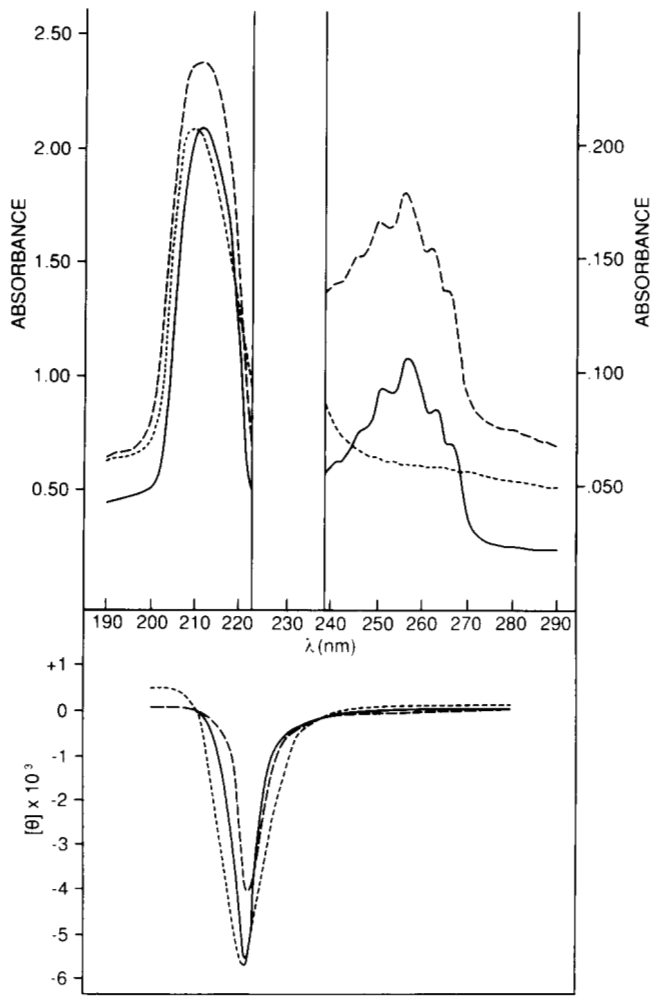


FIG. 5. **Ultraviolet and circular dichroism results proving the configuration of prephenyllactate at C-8 to be *R*.** Ultraviolet and circular dichroism spectra were done on prephenyllactate at pH 9.0 (-----), on the acid-converted product of prephenyllactate (– – –), on D-phenyllactate synthetic (——). The *top* part shows the UV spectra of these samples whereas the *bottom* part shows the negative Cotton effects obtained from the respective compounds.

we observed two doublets for H-2 and H-6 (6.02 and 5.93 ppm) at low field and further upfield, two overlapping and broader doublets (5.52 and 5.51 ppm), for protons H-3 and H-5. It, thus, seems that the more complex nature of the olefinic pattern observed in $D_2O$ solution is indeed due to aggregate formation that modifies slightly the shift of one of the olefinic groups. The presence of aggregates was also observed in the $^{13}C$ NMR spectrum as a broadening of signals due to C-3 and C-5.

*Mass Spectrum of Prephenyllactate*—The Fab mass spec-

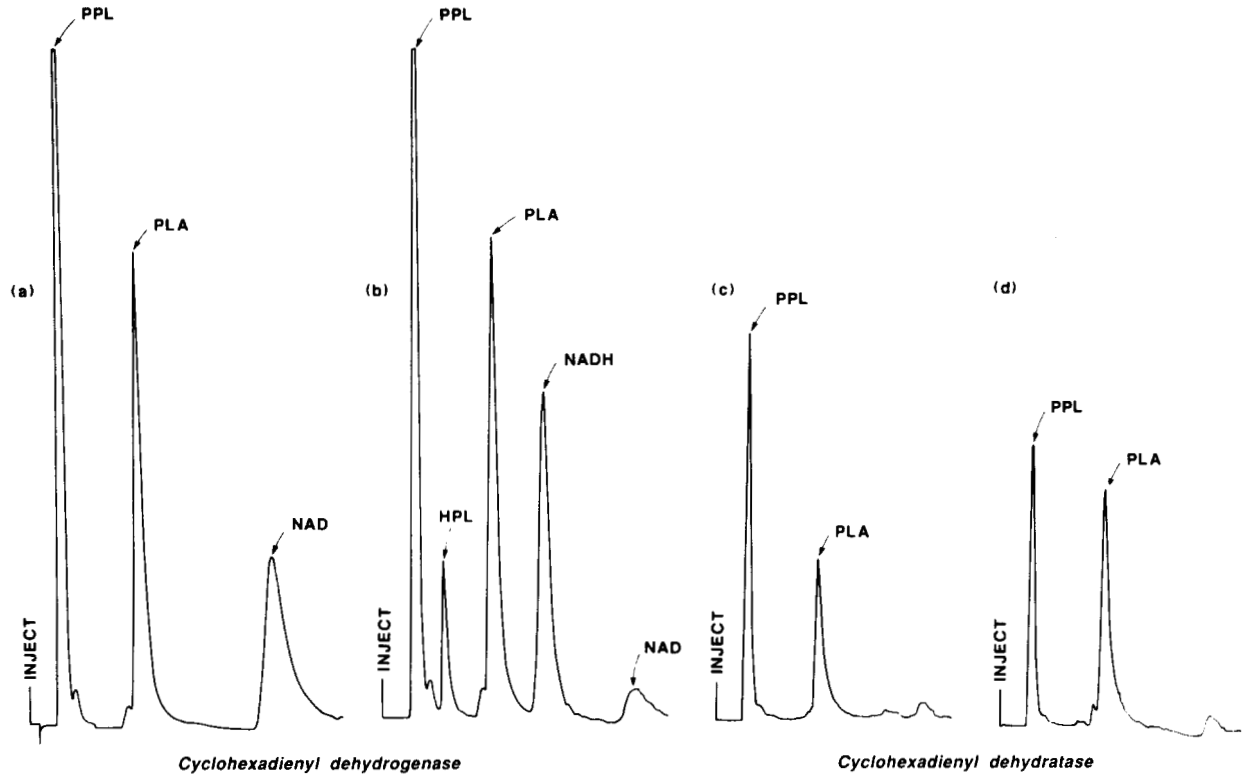


FIG. 6. **Identification by HPLC of 4-hydroxyphenyllactate (*HPL*) (*a* and *b*) and phenyllactate (*PLA*) (*c* and *d*) as reaction products in the prephenyllactate dehydrogenase and prephenyllactate dehydratase reactions, respectively, from *K. pneumoniae*.** The enzyme activities were assayed, and the reaction components were separated on a $C_{18}$ column as described under "Materials and Methods." *a*, control reaction mixture for prephenyllactate dehydrogenase in which only enzyme was incubated and the substrates added later (identical to data obtained from other controls where only substrates were incubated and the enzyme was included just before injection, or where both the substrates and enzyme were mixed at zero time just before injection). *b*, complete reaction mixture showing the appearence of two new peaks which eluted at the same retention time as authentic samples of HPL and NADH. *c*, control reaction mixture for prephenyllactate dehydratase showing the presence of background phenyllactate in the prephenyllactate substrate preparations. *d*, the formation of phenyllactate in the complete reaction mixture as evidenced by the increase in the peak height corresponding to phenyllactate (the basal level represents initial levels present in the original prephenyllactate preparation) as well as by the corresponding decrease in the peak height corresponding to prephenyllactate. The phenyllactate peak was identified by comparing its retention time with that of an authentic sample of phenyllactate.

trum was taken of the dipotassium salt of prephenyllactate. As expected, few fragmentation peaks were observed. The M + $H^+$ ion ($m/z$ 305) was manually peak matched (resolution: 4000) against the $m/z$ 277 ion of the glycerol matrix and was found to have an exact mass of 304.98291 (calculated for $C_{10}H_{11}O_6K_2$:304.98298). The close fit between the observed M + 1 peak, and the calculated value ensures the validity of the structure assigned to prephenyllactate.

*Circular Dichroism of Prephenyllactate*—Prephenyllactate has three asymmetric carbons at C-1, C-4, and C-8. Prephenyllactate is probably derived enzymatically from prephenate, and therefore the C-4 OH and C-1 COOH substituents are undoubtedly cis. In order to prove the configuration at C-8, we ran the circular dichroism of a buffered solution (pH 9.0) of prephenyllactate, of its acid-converted product (phenyllactate) as well as of the buffered solutions of L-phenyllactate (Sigma), L-phenyllactate (synthetic), and D-phenyllactate (synthetic). The circular dichroism of the acid-converted product of prephenyllactate showed a negative Cotton effect identical to the negative Cotton effect of D-phenyllactate (Fig. 5). The configuration at C-8 of prephenyllactate is therefore *R* and is opposite to the *S* configuration obtained for C-8 in arogenate and spiroarogenate.

*Enzymatic Basis for Prephenyllactate Formation*—It seems likely that the formation of prephenyllactate from prephenate occurs via catalysis by a stereospecific D-lactate dehydrogenase of broad substrate specificity. If so, it may be a membrane-bound pyridine nucleotide-independent type since we did not find a soluble NAD(P)H-dependent species in *Neurospora* extracts. *Lactobacillus confusus* possesses a NADH-dependent D-lactate dehydrogenase that is almost as efficient in the stereospecific reduction of phenylpyruvate ($K_m$ = 3.0 mM) as in the reduction of pyruvate ($K_m$ = 0.68 mM) according to Hummell *et al.* (12). It seems very likely that this enzyme would also function well with prephenate as substrate.

*Enzymatic Conversions of Prephenyllactate*—Since prephenyllactate structurally resembles prephenate and arogenate, we considered the possibility that prephenyllactate might be utilized as substrate by enzymes that use prephenate and/or arogenate. Prephenate dehydratase and arogenate dehydratase convert prephenate and L-arogenate to phenylpyruvate and L-phenylalanine, respectively; prephenate dehydrogenase and arogenate dehydrogenase convert prephenate and L-arogenate to 4-hydroxyphenylpyruvate and tyrosine, respectively. In some microorganisms these enzyme activities exhibit substrate ambiguity and can utilize either compound as substrate. In such cases the enzymes are denoted as cyclohexadienyl dehydratases or as cyclohexadienyl dehydrogenases (13).

The enzymatic conversions of prephenyllactate were shown utilizing enzyme activities isolated from *K. pneumoniae*. *K. pneumoniae* possesses a specific prephenate dehydratase (part of the bifunctional P-protein), a second monofunctional cyclohexadienyl dehydratase, and a cyclohexadienyl dehydrogenase (part of the bifunctional T-protein) (2, 10). The cyclohexadienyl dehydrogenase from *K. pneumoniae* utilized prephenyllactate as substrate with $NAD^+$ as cofactor, leading to the formation of 4-hydroxyphenyl lactate. The product of the dehydrogenase reaction was identified as 4-hydroxylphenyl lactate by HPLC (Fig. 6*b*). The prephenate dehydratase component of the bifunctional P-protein did not utilize prephenyllactate. However, the cyclohexadienyl dehydratase utilized prephenyllactate, converting it to phenyllactate. The formation of phenyllactate was confirmed by the increase in the peak height of phenyllactate (already present as an impurity in prephenyllactate substrate preparation) identified by HPLC following enzymic catalysis (Fig. 6*d*).

*D-Prephenyllactate as a Natural Product*—Similar to spiroarogenate, prephenyllactate has no known function as a normal metabolite. In *N. crassa* it is formed under aberrant conditions where abnormally high levels of prephenate are accumulated behind mutant blocks (6). The relatively large amounts of prephenyllactate formed indicate the latent catalytic potential of an unknown enzyme. In addition, intracellular redox conditions during accumulation must be appropriate to drive reduction of prephenate strongly. Since prephenyllactate is able to mimic prephenate and/or L-arogenate as substrate in some cases, the basic structure of prephenyllactate has potential in applications for development of microbial antimetabolites or herbicides.

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REFERENCES

1. Davis, B. D. (1953) *Science* **118,** 251–252
2. Cotton, R. G. H., and Gibson, F. (1965) *Biochim. Biophys. Acta* **100,** 76–88
3. Zamir, L. O., Jensen, R. A., Arison, B. H., Douglas, A. W. Albers-Schongerg, G., and Bowen, J. R. (1980) *J. Am. Chem. Soc.* **102,** 4499–4504
4. Jensen, R. A. (1985) *Mol. Biol. Evol.* **2,** 92–108
5. Jensen, R. A. (1986) *Rec. Adv. Phytochem.* **20,** 57–82
6. Zamir, L. O., Tiberio, R., Jung, E., and Jensen, R. A. (1983) *J. Biol. Chem.* **258,** 6486–6491
7. Zamir, L. O., Tiberio, R., Fiske, M., Berry, A., and Jensen, R. A. (1985) *Biochemistry* **24,** 1607–1612
8. Zamir, L. O., and Devor, K. A. (1986) *Can. J. Microbiol.* **32,** 215–218
9. Dayan, J., and Sprinson, D. B. (1970) *Methods Enzymol.* **17,** 559–561 (abstr.)
10. Ahmad, S., and Jensen, R. A. (1986) *FEBS Lett.* **216,** 133–139
11. Morin, C., and Sawaya, G. (1987) *Synthesis* **5,** 479–480
12. Hummel, W., Schütte, H., and Kula, M. R. (1983) *Eur. J. Microbiol. Biotechnol.* **18,** 75–85
13. Jensen, R. A., and Fischer, R. (1987) *Methods Enzymol.* **142,** 479–487