Biosynthesis of Epoxydon and Related Compounds by *Phyllosticta* sp.

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The tetraketide origin of epoxydon I produced by *Phyllosticta* sp. is established by ^{13}C NMR measurement on labelled epoxydons obtained from $1\text{-}^{13}\text{C}$ - and $2\text{-}^{13}\text{C}$ -acetate. The chlorinating mechanism of epoxydon to form non-enzymatically compound IV, V and VI was also confirmed by exposing epoxydon to the culture broth.

A most abundant metabolite, epoxydon^{1,2)} (phyllosinol) (I in Scheme I) has been isolated from *Phyllosticta* sp.,¹⁾ a pathogenic fungus of red clover.³⁾ The fungus also produces 6-methylsalicylic acid (II) and gentisyl alcohol (III).⁴⁾ It has been noted that these compounds are biosynthesized via polyketide pathway in other organisms.⁵⁾ These findings let us to postulate that epoxydon are most probably biosynthesized via tetraketide intermediate, although shikimate pathway is presumptive one. The results of experiments with ^{13}C NMR measurements of labelled epoxydon incorporated with ^{13}C -sodium acetates now confirmed that epoxydon was derived from gentisyl alcohol via a tetraketide chain and reported in a preliminary form.⁶⁾ Feeding experiments of ^{14}C gentisyl alcohol, which is postulated to be an immediate or very near precursor of epoxydon, was carried out by using the well washed mycelial mats from 14 days -old culture. Gentisyl alcohol was incorporated (25%) into epoxydon. This rather low incorporation may be due to the endogeneous formation of epoxydon. In fact, it has been shown that five mycelial mats placed on the surface of each 15 ml desalted water formed *ca.* 2 mg of epoxydon, the amount of which was estimated from UV absorption. Either starvation of mycelia or elongation of the incubating period did not diminish endogeneous formation of epoxydon. These observations serve to emphasize that epoxydon was biosynthesized from gentisyl alcohol. The tetraketide origin of epoxydon

was confirmed as follows.

The ^{13}C -assignments of signals of natural epoxydon are shown in the ^1H noise decoupled FT spectrum in Figure (1a) and Table I.

TABLE I. ^{13}C NMR DATA FOR ^{13}C LABELLED EPOXYDON

a)	Shift	$2\text{-}^{13}\text{C}$ Acetate	$1\text{-}^{13}\text{C}$ Acetate
C-1	-66.5	-66.4	—
C-2	- 6.7	—	-6.9
C-3	-13.4	-13.3	—
C-4	63.3	—	62.8
C-5	73.5	73.7	—
C-6	74.6	—	74.5
C-7	69.3	69.4	—

a) Number of positions are given in Scheme 1.

Peaks at δ 63.3 (ppm) and 69.3 were ascribed to a secondary alcoholic carbon (C-4) and a primary alcoholic carbon (C-7) respectively. CW irradiation⁷⁾ at the protons of methyl iodide in MeOH gives a doublet (the residual splitting: $J=38.6$ Hz) in the former and triplet ($J=36.6$) in the later (Fig. 1(b)). Peaks at δ 73.5 and δ 74.6 were assigned to epoxy-carbon (C-6 and C-5 respectively) by CW decoupling ^{13}C -NMR. Thus the ^1H -spectrum of epoxydon in CD_3OD shows 6-H (δ 3.37 from TMS) separated further from the irradiating field at δ 2.12 (ppm from TMS) than 5-H (δ 3.77). In the CW spectrum of epoxydon, the epoxy-carbons C-6 and C-5 appear as doublets and the assignments are based on $J=44.7$, smaller residual coupling, as observed for C-6 relative

SCHEME 1. Biosynthesis of Epoxydon and Related Compounds.

to $J=46.2$ for C-5 according to Ernst⁸⁾ equation, assuming the same J_{CH} for them. Of the two tertiary centers in α , β -unsaturated ketone system, peak at $\delta -66.5$ was ascribed to carbonyl carbon and $\delta -6.7$ to α carbon (C-2). β -Carbon (C-3) resonated at $\delta -13.3$ shows doublet ($J=46.8$) in CW decoupling ^{13}C -NMR.

The epoxydon (Ia) and (Ib) biosynthesized from each 400 mg of sodium 1- ^{13}C (88%) and 2- ^{13}C -acetate (86%) gives the ^{13}C NMR spectra Fig. 1(d) and Fig. 1(c) respectively.* The former spectrum was measured by ^1H -noise decoupling with multiscan and the later, by FT system. Assignment of their signals demonstrated that C-1, C-3, C-5 and C-7 of epoxydon were derived from the methyl carbon and C-2, C-4 and C-6 from the carboxyl carbon of the acetate. The ^1H - ^{13}C satellite bands for 3-H in Ia (J_{CH} 164 Hz) and 6-H in Ib (J_{CH} 191 Hz) in the ^1H NMR spectra (90 MHz) support this labelling pattern.

This labelling pattern confirms the polyketide origin of epoxydon. Additionally chlorinated substances (IV, V and VI) have been founded in the culture broth of this fungus.⁹⁾

Sequin-Frey and Tamm suggested the hypothetical chlorination mechanism from gen-

tisyl alcohol to chlorogentisyl alcohol (VI) by the action of enzyme such as chloroperoxidase.^{10,11)} However known chemical transformation of epoxydon to chlorogentisyl alcohol⁹⁾ and rather higher concentration of chloric anion (estimated *ca.* 130 ppm) in the used culture medium indicates the possibility of non-enzymic formation of chlorogentisyl alcohol from epoxydon. Experiments without fungus inoculation as mentioned below gave a clear indication that chlorogentisyl alcohol is formed from epoxydon.

When 85 ml of concentrated potato extract including 85 mg of epoxydon was allowed to stand for 6 days, chlorogentisyl alcohol (a main component) and compound (V) in the acetone extracts were observed on TLC. Crystalline chlorogentisyl alcohol (0.7 mg) was isolated by a chromatographic separation over silicic acid and its IR spectrum was identical to that of the authentic specimen. On the other hand, when the solution mixed with 430 mg of epoxydon in 430 ml of the concentrated medium was allowed to stand for 60 hr, crystalline material (5 mg) of compound IV whose IR spectrum was identical to that of authentic specimen was isolated as a major chlorinated compound and at the same time 23 mg of epoxydon was recovered. The existence of not only chlorogentisyl alcohol but also compound IV and V indicates that chlorogentisyl

* The incorporation of ^{13}C -acetate into Ia and Ib was estimated to be 6.6% efficiency according to the primary experiment using ^{14}C -acetate.

FIG. 1.

a) ^1H noise-decoupled ^{13}C -Ft spectrum of 224 mg of epoxydon (I) in 1.2 ml of CH_3OH at 22.6 MHz; sweep time 800 sec, number of scan 200 times, pulse angle $25\ \mu\text{sec}$, filter 8 KHz.

b) CW decoupled ^{13}C NMR spectrum of epoxydon. 224 mg of epoxydon/1.2 ml of CH_3OH at 22.6 MHz; sweep time 800 sec, number of scan 6266 times, pulse angle $25\ \mu\text{sec}$, filter 8 KHz.

c) ^1H noise-decoupled ^{13}C -Ft spectrum of 97 mg of epoxydon/1.5 ml of CH_3OH biogenetically enriched with ^{13}C from $2\text{-}^{13}\text{C}$ acetate at 22.6 MHz, sweep time 800 sec, number of scan 50 times, pulse angle $25\ \mu\text{sec}$, filter 8 KHz.

d) ^1H -noise-decoupled ^{13}C -spectrum of 40 mg of epoxydon from $1\text{-}^{13}\text{C}$ acetate in 1.0 ml of CH_3OH with 1208 scans at 40 sec/scan at 22.6 MHz. Chemical shifts were measured relative to the ^{13}C signal of methanol and converted to ppm from benzene using the relationship $\delta\text{c (benzene)} = \delta\text{ (MeOH)} + 79.0\text{ ppm}$.

alcohol was formed non-enzymically in culture medium through the way established chemically and depicted in Scheme 1. This process was confirmed by the fact that by shortening of exposing time to the culture medium, compound IV was obtained as major product among the chlorinated compounds.

This experimental results indicate the most possible process concerning formation of chlorogentisyl alcohol from epoxydon summarized as Scheme 1. These results might be expanded to include the mechanism of production of chlorogentisyl alcohol by *Phoma*¹⁰⁾ sp., since this species also gives epoxydon.²⁾ It is, however, not obvious whether other halogenated compounds, such as erdin, geodin, dihydrogeodin, ascochlorin or geodoxin⁵⁾ are formed in the same manner as in case of chlorogentisyl alcohol by *Phyllosticta* sp.

EXPERIMENTAL

¹⁴C-Labelled epoxydon

Phyllosticta sp. used in this experiment was isolated by Narita. Inocula were grown on the slant of potato-glucose agar for 6 days. Growth was in 500 ml flasks holding 200 ml of potato extract (potato: water 150 g/liter) containing D-glucose (1.5%) and ¹⁴C-sodium acetate (100 mg; 250 million cpm/mg) for 20 days at 25°C. Medium from the flask was added 6 g carbon-celite (1:1) mixture, the slurry was packed in a column and then washed with water. ¹⁴C-Labelled epoxydon could be eluted with acetone (60 ml). Then purified crystalline epoxydon (1.26 mg, 3180 cpm/mg) was obtained by chromatographic separation using a silicic acid column (10 g) eluted with ethyl acetate-chloroform (2:8) followed by crystallization.

¹³C-Epoxydons (Ia and Ib)

Mycelia were grown in four 500 ml flasks containing the same potato D-glucose medium, as mentioned above, to which was added 100 mg 1-¹³C sodium acetate (88%) or 2-¹³C sodium acetate (86%) for 20 days at 25°C. The combined filtrates from four flasks were added to 30 g carbon-celite (1:1) mixture, and the slurry was packed in a column and then washed with water. ¹³C-Labelled epoxydon was obtained by elution with acetone, then chromatographic separation using a silicic acid (25 g) column with ethyl acetate-chloroform (2:8) followed by crystallization from ethyl acetate gave 1-¹³C or 2-¹³C-acetate incorporated epoxydon, (30 mg, 80 mg), mp 78°C, in a pure crystalline state.

Preparation of ¹⁴C-labelled gentisyl alcohol from epoxydon

Gentisyl alcohol was prepared in a modified method described by Sigg *et al.*¹⁾ Potassium iodide (200 mg) was added to 40 mg of radioactive epoxydon in acetic acid (30 ml). The resulting solution was allowed to stand for 40 min at 80°C. After being cooled in an ice-bath, the solution was poured into a cooled aqueous solution of sodium bisulfate (40 mg/35 ml). After six or seven seconds, the aqueous solution was extracted three times with 35 ml portions of ethyl acetate. The combined solution was dried over sodium sulfate. The solvent was removed and the residue was chromatographed over a silicic acid column eluted with ethyl acetate-chloroform (8:2). The crystalline gentisyl alcohol (6.6 mg, yielded 19%) was obtained. The crystals were mixed with non-labelled gentisyl alcohol (6.7 mg) and recrystallized from CHCl₃. Gentisyl alcohol thus obtained has 810 cpm of specific radio-activity.

Conversion of gentisyl alcohol to epoxydon

Mycelial mats from 14 days culture were grown in 500 ml flask each containing the same growing medium as employed previously. After washing with distilled water thoroughly, ten mycelial mats were placed on 15 ml of aqueous solution which contained the radioactive gentisyl alcohol (810 cpm/mg) in 0.0067% concentration of the aq. solution, and incubated at 25°C for 8 hr. After removal of mycelial mats, the incubated mixture was subjected to column chromatography with carbon-celite and acetone followed by column chromatography with silicic acid and chloroform-ethyl acetate (2:8). Pure crystals of epoxydon (2 mg, 190 cpm/mg) were obtained by recrystallization from ethyl acetate-chloroform.

Non enzymic chlorination of epoxydon

The potato extract (150 g/liter) containing 1.5% D-glucose was concentrated to one-tenth of its original volume and then autoclaved. To 85 ml of this solution, aqueous epoxydon solution, 86 mg/10 ml, sterilized by filtration through a membrane filter was added. This mixture was allowed to stand at 25°C for 6 days. In other case, epoxydon solution, 430 mg/10 ml was added to 430 ml of concentrated potato extract and the mixture was allowed to stand for 60 hr. Then, positive substances for Folin-Ciocalteu reagent from each solution were isolated by a carbon-celite column chromatography eluting with acetone, and identified as IV, V and VI.

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