Metabolism of Limonoate via a *trans*-19-Hydroxyobacunoate Pathway

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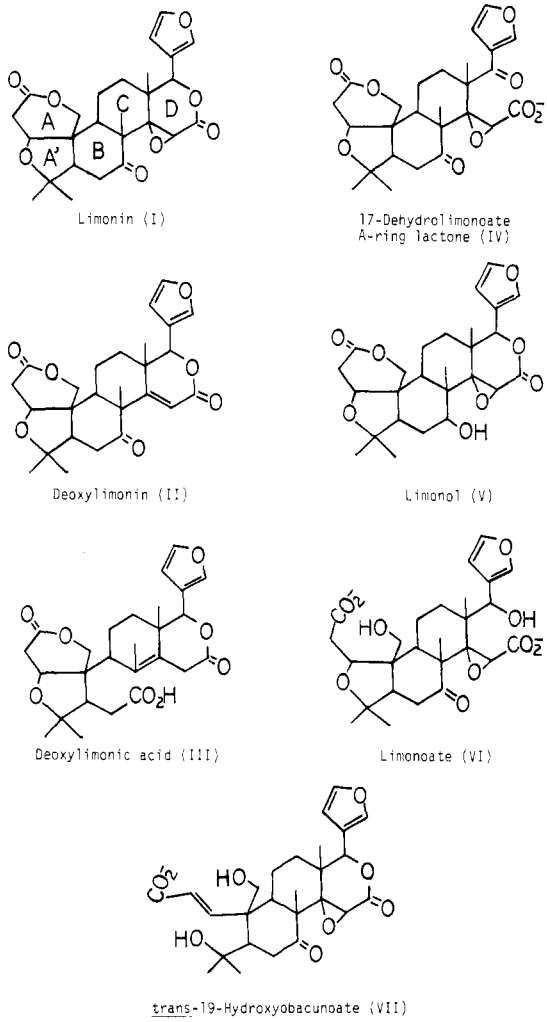
The metabolite of limonoate produced by *Corynebacterium fascians* was isolated from its growth medium and its structure was determined as *trans*-19-hydroxyobacunoate. The metabolism via *trans*-19-hydroxyobacunoate is the fourth metabolic pathway of limonoids found in bacteria.

Bitterness due to limonin (I) in certain citrus juices is



one of the major problems of the citrus industry worldwide and has significant economic impact. Substantial progress has been made in studies of the bacterial degradation of limonoids since deoxylimonin (II) and deoxylimonic acid (III) were isolated as the first proven bacterial metabolites of limonoids in 1972 (Hasegawa et al., 1972a). On the basis of the metabolites and enzymes involved, three metabolic pathways of limonoids have been established. The first one is via deoxylimonoids like deoxylimonin (Hasegawa et al., 1972a; Hasegawa and Kim, 1975; Vaks and Lifshitz, 1981), the second one via 17-dehydrolimonoids like 17-dehydrolimonoate A-ring lactone (IV) (Hasegawa et al., 1972b), and the third one via 7α-hydroxylimonoids like limonol (V) (Hasegawa et al., 1983; Hasegawa and Pelton, 1983).

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Recently, a fourth metabolic pathway was found in *Corynebacterium fascians* (Hasegawa and King, 1983). The organism metabolized limonoate (VI) mainly through the 17-dehydrolimonoid pathway, but careful analyses of its growth medium on limonoate showed the presence of three minor metabolites which differed from any of those of the known pathways. This paper reports the isolation of one of those minor metabolites and its structure determination.

MATERIALS AND METHODS

Silica gel G plates were used for TLC. Plates were developed with toluene–EtOH–H_2O–HOAc (200:47:15:1), cyclohexane–EtOAc (2:3), and CH_2Cl_2–MeOH (97:3). Chromatograms were revealed by spraying with 50% H_2SO_4 and heating or by spraying with Ehrlich's reagent and exposing to HCl gas (Dreyer, 1965). ^1H NMR spectra were run at 100 MHz and ^{13}C NMR spectra at 15 MHz. ^{13}C NMR spectral assignments were made on the basis of single-frequency off-resonance decoupled spectra and comparisons with spectra of related limonoids for which assignments had previously been made (Dreyer et al., 1976; Bennett and Hasegawa, 1980, 1981).

Isolation of Metabolite. In a 2.8-L Fernbach flask, 500 mL of 0.2% limonoate–mineral salt medium was inoculated with 10 mL of a 48-h culture of *C. fascians* grown on the same medium. The mixture was incubated on a shaker at 25 °C for 3 days where the growth reached maximum level. The supernatant from the culture was acidified with HCl to pH 2 and extracted twice with CH_2Cl_2. The extracts were combined and evaporated to give 235 mg. TLC analyses showed two major components; one was limonin (I) [from the acidification of limonoate (VI)] and the other was 17-dehydrolimonoate A-ring lactone (IV). There were three minor, unidentified metabolites which were acidic and Ehrlich positive. The extract was therefore dissolved in CH_2Cl_2 and extracted twice with 5% $KHCO_3$. The $KHCO_3$ extracts were combined, washed with CH_2Cl_2, acidified with HCl, and extracted with CH_2Cl_2. The CH_2Cl_2 extract was washed with H_2O and evaporated to give 135 mg. This acidic fraction contained mainly 17-dehydrolimonoate A-ring lactone (IV) and about 25 mg of Erhlich-positive metabolites. The fraction was methylated with CH_2N_2 and chromatographed on a silica gel column. The column was eluted, stepwise, by increasing concentrations of EtOAc in hexane.

A column fraction (15 mg) was shown by TLC to contain two Erhlich-positive compounds, in a ratio of about 3:1. The mixture was separated by chromatography on a 2-g column of silica gel (Polygosil 60-2540, Macherey-Nagel). The column was eluted with a continuous gradient formed from 10 mL of C_6H_6–EtOAc (60:40) and 10 mL of C_6H_6–EtOAc (20:80), and the major component was obtained in chromatographically pure form (9.5 mg): ^1H NMR ($CDCl_3$) δ 7.38 (2 H, t, J = 1 Hz, α-furans), 7.05 (1 H, d, J = 16 Hz, H-1), 6.35 (1 H, d, J = 1 Hz, β-furan), 5.98 (1 H, d, J = 16 Hz, H-2), 5.40 (1 H, s, H-17), 4.42 (1

Table I. Limonoid-Metabolizing Bacteria and Their Limonoid Metabolic Pathways[a]

bacteria	enzymes	pathways	references
Arthrobacter globiformis	induced	17-dehydrolimonoids	Hasegawa et al. (1972b)
Pseudomonas 321-18	induced	deoxylimonoids	Hasegawa et al. (1972a)
		17-dehydrolimonoids	Hasegawa et al. (1974b)
Bacterium 342-152-1	induced	17-dehydrolimonoids	Hasegawa and Kim (1975)
		deoxylimonoids	
Acinetobacter sp.	induced	deoxylimonoids	Vaks and Lifshit (1981)
Arthrobacter globiformis II	induced	17-dehydrolimonoids	Hasegawa et al. (1983)
		7α-hydroxylimonoids	
Corynebacterium fascians	constitutive	17-dehydrolimonoids	Hasegawa and King (1983)
		trans-19-hydroxyobacunoic acid	Hasegawa and Bennett (1982)

[a] Refer to the original literatures for substrates used.

H, d, J = 11 Hz, H-19), 3.85 (1 H, d, J = 11 Hz, H-19), 3.74 (3 H, s, OMe), 3.66 (1 H, s, H-15), 1.43, 1.29, 1.15, 1.09 (12 H, 4 s, quaternary Me); ^{13}C NMR ($CDCl_3$) δ 209.5 (C-7), 167.4 and 167.2 (C-3 and C-16), 155.9 (C-1), 143.1 (C-23), 141.1 (C-21), 120.4 (C-20), 120.1 (C-2), 109.9 (C-22), 78.3 (C-17), 73.4 (C-4), 65.4 (C-14), 59.5 (C-19), 59.4 (C-5), 53.0 (C-15), 52.6 (C-8), 51.8 (OMe), 49.8 (C-10), 48.5 (C-9), 38.1 (C-6), 37.3 (C-13), 33.3 (C-12), 32.1 (quaternary Me), 29.5 (quaternary Me), 21.1 (quaternary Me), 19.6 (C-11), 16.7 (quaternary Me).

RESULTS AND DISCUSSION

Six species of bacteria, each capable of metabolizing limonoids, have been isolated from soil and used for studies of metabolic pathways of limonoids, enzymes involved in the pathways and development of biological processes for limonin (I) debittering of citrus juices (Table I). Unlike others, *C. fascians* produces constitutive limonoid-metabolizing enzymes (Hasegawa and King, 1983). This has significance from a practical viewpoint because cells which possess limonoid-metabolizing enzymes can be produced relatively cheaply and conveniently with inexpensive carbon sources.

During the course of investigation of *C. fascians*, we observed that the organism produced metabolites in its limonoate (VI) growth medium which differed from any of those of known metabolic pathways of limonoids, in addition to the metabolites of the 17-dehydrolimonoid pathway (Hasegawa and King, 1983). The major metabolite was therefore isolated, as the methyl ester, by chromatography on silica gel and its structure was determined. The ^{1}H NMR spectrum showed furan and H-17 resonances indicative of a normal D-ring lactone and also a single methyl ester resonance. The ^{13}C NMR spectrum showed three carbonyl resonances, two of which may be attributed to the D-ring lactone and the methyl ester and the third having approximately the same chemical shift as the B-ring keto group in limonin. All of the original carbon atoms of the substrate could be accounted for in the ^{13}C NMR spectrum of the metabolite, so no degradation of the molecule had occurred. Thus, the methyl ester carbonyl can most reasonably be assigned to C-3, which was the A-ring lactone carbonyl in limonin (I) and became a free carboxyl in the substrate, limonoate. The ^{13}C chemical shift of this ester carbonyl (167 ppm) suggested that it was conjugated to a double bond. Other limonoid methyl esters having this structural feature show chemical shifts in the range 165–167 ppm, while nonconjugated methyl esters have chemical shifts greater than 170 ppm (Dreyer et al., 1976; Bennett and Hasegawa, 1981). The presence of such a double bond was confirmed by the ^{1}H NMR spectrum, which showed a widely separated olefinic AB quartet, with a coupling constant (16 Hz) characteristic of a trans double bond. The C-6, C-7, C-8, C-11, C-12, C-13, C-14, C-15, C-16, C-17, and furan ^{13}C resonances had chemical shifts approximately the same as for limonoids whose B, C, and D rings are the same as in limonin but lack the A and A′ rings of the latter (Dreyer et al., 1976; Bennett and Hasegawa, 1981). Thus, the NMR evidence indicated that conversion of limonoate (IV) to the metabolite did not involve changes in the B, C, and D rings but a trans double bond was introduced into the 1,2-position. Signals for the quaternary oxygen function at C-4 and the methylene oxygen function at C-19 were both present in the ^{13}C NMR spectrum, and the latter function was also observed as a characteristic AB quartet in the ^{1}H NMR spectrum. These oxygen functions must either both be hydroxyl or be part of a C-4 to C-19 cyclic ether system, since there are no other oxygenated carbon resonances unaccounted for. However, the corresponding cyclic ether has previously been prepared (Bennett and Hasegawa, 1980) from isolimonic acid, and its ^{1}H NMR spectrum was quite different from that of the metabolite. Therefore, hydroxyl groups must be present in both the 4- and 19-positions, which leads to the structure VII for the metabolite.

This newly found metabolite, *trans*-19-hydroxyobacunoate (VII), is apparently produced by the action of a transeliminase (lyase). The enzyme has not been isolated yet, but this type enzymic action is seen in the breakdown of pectic substances by pectin transeliminase (Hasegawa and Nagel, 1966). In this pathway, the attack is initiated on the A′ ring of the limonoate (VI) molecule, whereas in the deoxylimonoid and 17-dehydrolimonoid pathways, the attack is initiated on the D ring. On the other hand, in the 7α-hydroxylimonoid pathway, the attack is initiated on the B ring.

Metabolites of three previously proven bacterial pathways have also been isolated from *Citrus* (Dreyer, 1965; Hsu et al., 1973; Bennett and Hasegawa, 1982), but those of *trans*-19-hydroxyobacunoate (VII) have not been found yet in *Citrus*, suggesting that this newly found pathway may not be operative in *Citrus*. Both the 17-dehydrolimonoid and deoxylimonoid pathways have been shown to be present in *Citrus* (Hasegawa et al., 1974a, 1980).

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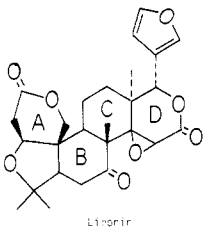
A Species of Bacterium-Producing Constitutive Enzymes for Limonoid Metabolism

Shin Hasegawa* and A. Douglas King, Jr.

A species of bacterium, capable of metabolizing limonoids, was isolated from soil by enrichment with 3-furoic acid as a single carbon source. It was classified as *Corynebacterium fascians* (NRRL B-15096). Unlike other limonoid-metabolizing bacteria identified thus far, this organism produced constitutive enzymes for limonoid metabolism. Cells grown on carbon sources other than limonoids such as fructose and galactose produced limonoate dehydrogenase (limonoate:NAD oxidoreductase).

Bitterness due to limonin in certain citrus juices is one



Limonin

of the major problems of the citrus industry worldwide and has significant economic impact. In recent years, several processes have been developed for reduction of limonin bitterness of citrus juices. Such processes are (1) a metabolic debittering process which involves exposure of intact fruit to 20 ppm of ethylene gas for 3 h and then holding them for several days in air (Maier et al., 1973), (2) a selective adsorption of limonin from the juice using cellulose acetate beads (Chandler and Johnson, 1979), (3) the use of limonin bitterness suppressing agents like neodiosmin in the juice (Guadagni et al., 1976), or (4) the use of enclosure complex forming agents with limonin like β-cyclodextrin in the juice (Misaki et al., 1981). There is also a preharvest approach—spraying citrus trees with inhibitors of limonoid biosynthesis (Hasegawa et al., 1977; Casas et al., 1980). However, none of them is totally satisfactory.

Recently, limonin-debittering processes using immobilized bacterial cells have been developed (Vaks and Lifshitz, 1981; Hasegawa et al., 1982; Hasegawa and Pelton, 1983). These processes have several advantages over the others and have potential for practical applications. For the biological processes, several species of bacteria, each capable of metabolizing limonoids, namely, *Arthrobacter globiformis* (Hasegawa et al., 1972b), *Pseudomonas* 321-18 (Hasegawa et al., 1974b), *Bacterium* 342-152-1 (Hasegawa and Kim, 1975), and *Acinetobacter* sp. (Vaks and Lifshitz, 1981), have been isolated from soil. The limonoid-metabolizing enzymes produced by these species of bacteria are all induced enzymes. They can be induced by the presence of limonoids in the growth media.

We have recently isolated from soil a species of bacterium which produces constitutive enzymes for limonoid metabolism. This paper shows the production of limonoid-metabolizing enzyme activity in this bacterium grown on carbon sources other than limonoids.

MATERIALS AND METHODS

Isolation and Characterization of the Bacterium. The bacterium was isolated from soil by enrichment with 3-furoic acid as a single carbon source. After purification the organism was classified as *Corynebacterium fascians* (NRRL B-15096) by following standard procedures (Buchanan and Gibbons, 1974; Lennette et al., 1974) and the use of the API 20 E Gram-negative bacteria identification system (Analytab Products, Plainview, NY).

Growth of Cells. The substate, 500 mL of a mineral salt medium (Hasegawa et al., 1972b), 0.2% nutrient broth, and 0.4% carbon source of interest, was placed in a 2.8-L Fernbach flask and inoculated with 10 mL of a 48-h culture of *C. fascians*. Incubation was at 25 °C on a shaker, and growth was followed by measuring the increase in optical density of the medium at 600 nm. After incubation, cells were harvested by centrifugation, washed with 0.5 M phosphate buffer at pH 7.0, and frozen until used for enzyme analysis.

Identification of Metabolites. Silica gel G plates were used for thin-layer chromatography (TLC). Plates were developed with toluene–EtOH–H_2O–HOAc (200:47:15:1, upper layer), cyclohexane–EtOAc (2:3), and CH_2Cl_2–MeOH (97:3). Chromatograms were revealed by spraying with 50% H_2SO_4 and heating or by spraying with Ehrlich's reagent and exposing to HCl gas (Dreyer, 1965). NMR spectra were run at 100 MHz in $CDCl_3$ with a JEOL JNN-PS-100 spectrometer.

The supernatant from a culture of *C. fascians* which had been incubated with 1 g of sodium limonoate in 500 mL

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