of high molecular weight with a mass of at least 541. Other ions appear at *m/e* 449 (?), 369 (base peak) 355, 342, 341, 325, 314, 313, 249, 194, 119, 93, 91, and 77. Absence of an ion at *m/e* 105 as well as absence of pyridazine and amide absorption in the infrared spectrum indicates that extensive degradation or rearrangement of the pyrazon molecule occurred. Because of the small amounts of material available, no more attempts to identify IV were made; nor is any work on the structure of this compound contemplated.

The major product of the solar irradiation of pyrazon in water was VI, a material whose average molecular weight (determined by osmometry in pyridine solution) was greater than 9000. The infrared spectrum of VI, in addition to exhibiting the large broad bands indicative of polymeric material, was similar to the spectrum of II. Although VI has not been well characterized, it is reasonable to suggest that it is formed by the same pathway as II. A molecule of II may undergo two reactions: (a) internal cyclization to form III; or (b) trimerization. The trimer may then react to form longer chains. The ultraviolet absorption spectrum of II exhibits a maximum at 292 nm (that of I is at 286 nm) in methanol, indicating that it is capable of absorbing solar energy directly.

The results obtained by exposing I, II, and III to sunlight when absorbed on silica gel are in agreement with the above suggestion. Within 4 hr, both II and material remaining at the origin after elution were formed from pyrazon, while no III was detected. Within the same exposure period, II was also converted to material which remained at the origin after elution. Again III was not detected, a result due to the concentration effect in the solid state. It is known that concentrated systems favor polymerization over cyclization. Exposure of III under these conditions resulted in no reaction. The "dark" controls of I and II also remained unchanged.

The photolysis of pyrazon by sunlight appears to be a relatively rapid reaction when the material is dissolved in water or when absorbed on silica gel. It should be remembered that the experiment in aqueous solution, where almost half the pyrazon was lost in 1 month, was performed in a borosilicate glass flask. This means that only about 10% of the light principally responsible for energizing the reaction was transmitted (Calvert and Pitts, 1966). In a previous experiment (early November 1970), when the total amount of solar energy below 3100 Å is approximately $^1/_6$ of the total that reaches the earth's surface at this latitude (Forsythe and Christison, 1929) in April–May, trace amounts of III were detected within 6 hr.

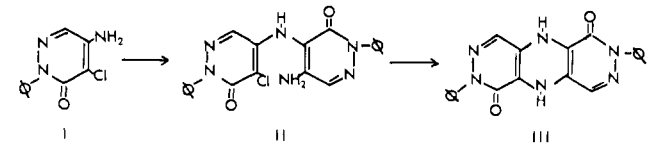

Figure 1. Structure of Compounds I, II, and III

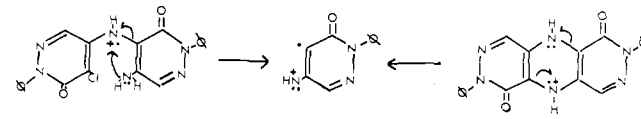

Figure 2. Possible pathways for formation of peak at *m/e* 185 from both II and III

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Metabolism of Limonin and Limonoate by Microorganisms: The Conversion to Nonbitter Compounds

A survey was made of microorganisms capable of metabolizing limonin to nonbitter compounds. It was found that several species of bacteria obtained from soil grew well on a medium containing limonin or limonoate as a single carbon source. Two nonbitter metabolites from the cultural medium were isolated and identified as deoxylimonin and deoxylimonic acid.

Limonin (Figure 1) is a bitter principle of citrus juices (Arigoni *et al.*, 1960; Emerson, 1948; Higby, 1938; Kefford, 1959). The bitterness due to limonin develops gradually in juices after extraction from certain varieties of oranges, lemons, and grapefruit. The intact fruits do not normally contain limonin but rather a nonbitter precursor, limonoate A-ring lactone (Maier and Beverly, 1968; Maier and Margileth, 1969). This nonbitter precursor converts to limonin under acidic conditions (Maier and Beverly, 1968) and the conversion is also accelerated by the action of limonoate D-ring lactone hydrolase, which has been shown to be present in citrus fruit (Maier *et al.*, 1969). In the citrus industry this phenomenon is referred to as delayed bitterness (Joslyn and Pilnik, 1961; Kefford, 1959).

In dealing with this bitterness problem, two lines of research are underway at our laboratory. The first is that of acceler-

Table I. R_f Values of Limonin Metabolites

Metabolites	Solvent system[a] A	B	C	D
Deoxylimonin	0.54	0.30	0.25	0.39
Deoxylimonic acid	0.43	0.20	0.08	
Methyl deoxylimonate			0.36	0.51

[a] Composition of solvent systems: A, ethanol; B, benzene–ethanol–water–acetic acid (200:47:15:1); C, cyclohexane–ethyl acetate (30:70); D, dichloromethane–methanol (96:4).

ated limonoid metabolism *via* preprocessing treatments, and deals with the intact fruit before the juice is extracted (Maier and Brewster, 1971). The second approach deals with the juice itself during extraction and processing. Although numerous research efforts have been made in this area, no practical solutions to this problem have been found.

At present, we are searching for enzymes capable of catalyzing the debittering of limonin in citrus juices. Efforts to isolate such an enzyme from the citrus fruit tissues themselves have thus far been unsuccessful. We have turned, therefore, to microorganisms as a possible source. The presence of such an enzyme in molds, in small but appreciable amounts, has been reported (Nomura, 1966).

Recently, we have made a general survey of microorganisms and found that several species of bacteria obtained from soil were capable of metabolizing limonin to nonbitter compounds (Bennett *et al.*, 1971). Here we report the isolation and characterization of two limonin metabolites.

EXPERIMENTAL

Sodium limonoate (Figure 1) was prepared from limonin by the methods described previously (Maier and Beverly, 1968). Media used for microbial growth consisted of mineral salts (Saunders *et al.*, 1948), 0.1% sodium limonoate, and a trace amount of vitamin B complex. Silica Gel GF plates were used for thin-layer chromatography (tlc).

A mixed culture of soil organisms was allowed to grow on a medium containing a saturated solution of limonin. All of the limonin was metabolized within 4 days. However, under these conditions, isolation of metabolites was not feasible because the low solubility of limonin in water did not permit a sufficient amount of the latter to be used.

For the isolation of metabolites, the mixed culture of soil organism was grown in a 2-l. flask containing 3 g of sodium limonoate in 500 ml of the mineral salt medium. The incubation was carried out at room temperature under aerobic conditions. After 70 hr the cultural medium was centrifuged at 20,000 × *g* for 10 min. The cell-free medium was then acidified to pH 2 with 1 *N* HCl, boiled for 10 min, and extracted with two 300-ml portions of chloroform. The extracts were combined and evaporated. The residue (700 mg) was redissolved in 30 ml of dichloromethane and extracted successively with 15 ml of 5% $KHCO_3$ and 15 ml of H_2O. The dichloromethane solution was then evaporated to give 370 mg (Neutral Fraction). The two aqueous extracts were combined, acidified to pH 2 with 3 *N* HCl, and extracted with two 15-ml portions of ethyl acetate. The extracts were each washed with 10 ml of H_2O, combined, and evaporated to give 300 mg (Acid Fraction).

The neutral fraction was examined by tlc with cyclohexane–ethyl acetate (30:70), using Ehrlich's reagent for visualization of limonoids (Dreyer, 1965). The major component was unreacted limonin, but a second minor Ehrlich-positive spot (Compound A) was also present. Most of the limonin was removed by crystallization from dichloromethane–2-propanol. The mother liquor was then evaporated to dryness, and the residue (21 mg) was subjected to preparative tlc with cyclohexane–ethyl acetate (20:80). Compound A, which was located by its strong absorption of shortwave uv light, was removed and eluted. It was further purified by preparative tlc with ether–methanol (97:3, unsaturated chamber) to give 2 mg of chromatographically homogeneous material.

Tlc of the acidic fraction with ethanol showed one major Ehrlich-positive component (Compound B). This fraction was methylated with diazomethane to facilitate purification by chromatography, and then subjected to preparative tlc with cyclohexane–ethyl acetate (20:80). Compound B methyl ester was isolated and further purified by preparative tlc with ether–water (99:1, unsaturated chamber) to give 26 mg of chromatographically homogeneous material.

As a control, sodium limonoate and limonin were subjected to the same conditions in the absence of microorganisms. All of the limonoate and limonin were recovered as limonin.

RESULTS AND DISCUSSION

The results of the survey of microorganisms showed that several species of bacteria obtained from soil grew well on a mineral salt medium containing sodium limonoate or limonin as a single carbon source. In preliminary studies we observed the formation of two Ehrlich-positive metabolites in the cultural medium. Compound A had the same mobility as deoxylimonin (Figure 1) by tlc in four solvent systems (Table I). Furthermore, its ir and nmr spectra were identical with those of authentic deoxylimonin, which has been isolated previously from grapefruit seeds (Dreyer, 1965). It can be also prepared by chemical reduction of limonin (Geissman and Tulagin, 1946).

Compound B, which was acidic, had the same mobility as deoxylimonic acid in three tlc systems (Table I). It was isolated in the form of its methyl ester, which corresponded chromatographically to methyl deoxylimonate in tlc systems (Table I). The ir and nmr spectra of compound B methyl ester were identical with those of authentic methyl deoxylimonate. Deoxylimonic acid (Figure 1) had not been found in nature previously but it had been prepared by treatment of deoxylimonin with strong base (Barton *et al.*, 1961). It seems

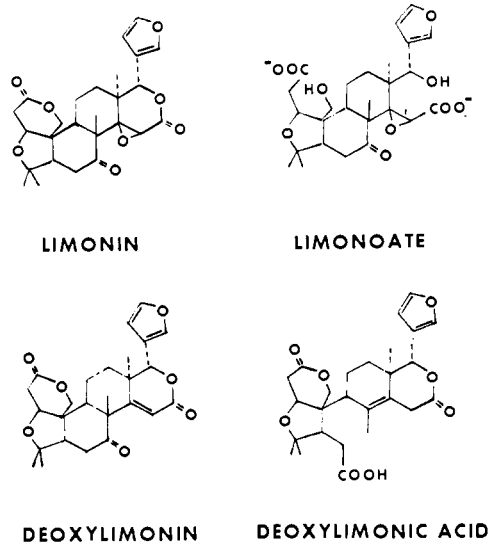

Figure 1. Structure of limonoids and their bacterial metabolites

likely that the microorganisms first convert limonin to deoxylimonin, which is then transformed to deoxylimonic acid.

In earlier work Nomura (1966) reported that *Aspergillus niger* and two species of *Penicillium* were capable of producing enzymes which metabolize limonin and nomilin. Activities of these enzymes were, however, very low, and no metabolites were isolated. The results obtained in this study show clearly the existence of enzymes capable of catalyzing debittering reactions. More important, the fact that both of these metabolites are nonbitter is of considerable practical significance.

Further investigations on characterization of limonin metabolites, isolation, and characterization of the organisms, and isolation of enzymes involved in the metabolism of limonin are in progress.

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Characterization of the Major Aroma Constituent of the Fungus *Trichoderma viride* (Pers.)

The volatile constituents of *Trichoderma viride* (Pers.) were resolved by glc. The major compound was characterized as 6-pentyl-α-pyrone by ir, ms, and nmr spectroscopy, elemental analysis, and by hydrogenation to known derivatives. The characteristic coconut-like aroma produced by this fungus is certainly due to this compound.

A commonly occurring soil fungus (*Trichoderma viride*) has been extensively studied from the aspects of its saprophytic potential and because of its antagonism and parasitism against other fungi. The fungus produces in culture a characteristic coconut-like aroma and this report is concerned with the identification of the major constituent responsible for the characteristic odor.

The organism used in this study was isolated by the authors from a soil sample collected near Coventry Lake in Coventry, Conn. The organism was grown in a potato dextrose (10%) liquid medium containing $CaCO_3$ (0.2 g/l.) and $MgSO_4$–$7H_2O$ (0.2 g/l.) in quart milk bottles containing 60 ml of the medium. After inoculation, the milk bottles were placed on their side and incubated at room temperature for 3 to 4 days. At the end of this period the surface of the medium was covered with the dark green conidia characteristic of this organism.

At the end of the growth period the cells and culture medium were steam distilled at atmospheric pressure. For the analyses reported here approximately 18 l. of medium was distilled. The aqueous distillate was saturated with NaCl and then extracted with redistilled ether. The extract was dried over Na_2SO_4 and the solvent was removed under reduced pressure, leaving 3.3 g of a yellow oil having a strong coconut-like aroma. The oil was separated into its components by glc (Hewlett-Packard Model 7620A) utilizing a flame ionization detector equipped with an effluent splitter. An 8 ft × 0.125 in. o.d. stainless steel column packed with carbowax 20M (10%) and phenyldiethanolamine (PDEAS 1%) on 80–100 mesh A.W. Chromosorb W was used. The temperature was programmed from 80–220° C at 6° C/min. The compounds were collected as they exited from the instrument by the procedure described by Halim and Collins (1970). A typical glc analysis is shown in Figure 1.

The major component of the oil (over 90%) had a retention time of approximately 24 min. The isolated compound was a colorless liquid having a strong coconut-like odor. This compound was further purified at 135° C in a 6 ft × 0.125 in. o.d. stainless steel column packed with W98 (10%) on 80–100 mesh Chromosorb W.

The infrared spectrum showed two C=O absorption bands at 1740 and 1725 cm^{-1} and two strong C=C stretching bands at 1636 and 1553 cm^{-1}, suggesting an α-pyrone ring (Nakanishi, 1962). Infrared spectra were taken as thin films contained between KBr disks (13 mm diam) on a Perkin–Elmer Model 137B Infracord Spectrometer.

The mass spectrum showed a parent ion at *m/e* 166. The molecular formula was assigned as $C_{10}H_{14}O_2$ on the basis of the mass spectral data and elemental analysis (Found: C, 72.84%; H, 8.39%; O, 18.77%; calcd: C, 72.9%; H, 8.50%; O, 19.21%). The mass spectrum also revealed a base ion at *m/e* 95 attributable to $M - C_5H_{11}$. Mass spectra were obtained on an M.S. 12 mass spectrometer using a probe inlet system, ion source, operated at 250° C.

The (nmr) showed signals at δ 0.94 (3 H, triplet), 1.4 (6 H,