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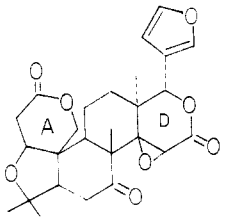
Received for review April 2, 1982. Accepted September 21, 1982. This work was supported in part by a grant-in-aid from the Ministry of Education, Science and Culture of Japan (No. 56560094).

Reduction of Nomilin Bitterness in Citrus Juices and Juice Serum with *Arthrobacter globiformis* Cells Immobilized in Acrylamide Gel

Nomilin debittering of citrus juices and juice serum was successfully demonstrated with *Arthrobacter globiformis* cells immobilized in acrylamide gel. When juice serum was passed through a column packed with immobilized cells, nomilin was converted to nonbitter 17-dehydronomilinoate A-ring lactone by two enzymes, limonin D-ring lactone hydrolase and limonoate dehydrogenase. The enzyme system in the immobilized cells was as effective toward nomilin as it was toward limonin.

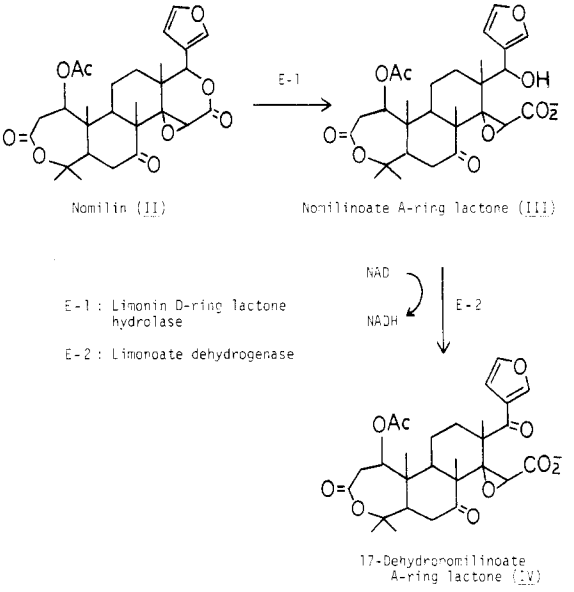
Limonoids are a group of chemically related triterpene derivatives found in Rutaceae and Maliaceae families. Limonin (I) is an intensely bitter limonoid that occurs



widely in *Citrus*. The development of bitterness in certain citrus juices due to I, sometimes referred to as delayed bitterness, is one of the primary determinants for juice acceptability and has significant ecomomic impact on the industry. We have recently developed a I debittering process that employs limonoid-metabolizing bacterial cells immobilized in acrylamide gel (Hasegawa et al., 1982). The limonoid-metabolizing enzyme system in the immobilized *Arthrobacter globiformis* cells very effectively converted I to a nonbitter metabolite, 17-dehydrolimonoate A-ring lactone, in navel orange juice and juice serum. The system could be used many times without losing its effectiveness.

Twenty-nine limonoids, 18 neutral and 11 acidic, have been isolated from *Citrus* and *Citrus* hybrids. Four of them, I, nomilin (II), ichangin, and nomilinic acid, are bitter (see Scheme I). Compound I is the major limonoid present in certain citrus juices and is the primary cause of delayed bitterness. Recently, the II content of citrus juices was reported (Rouseff et al., 1981; Hashinaga and Itoo, 1981). We have also observed the presence of II in commercial orange juices (Hasegawa and Patel, 1980), and

Scheme I. The Nomilin-Metabolizing Enzyme System in *A. globiformis* Immobilized in Acrylamide Gel



it, also, appears to play a role in the development of delayed bitterness. Organoleptic tests of Hashinaga et al. (1977) estimated that II is twice as bitter as I and II bitterness threshold is about 3 ppm. Therefore, we have tested our I debittering process to determine whether it also reduces the II content of citrus juice and juice serum.

EXPERIMENTAL SECTION

Orange juices and juice sera were prepared, *A. globiformis* cells were grown, and bacterial cells were immo-

Table I. Reduction of Nomilin and Limonin Contents in Valencia Orange Juice Sera with *A. globiformis* Cells Immobilized in Acrylamide Gel[a]

time after immobilization, days	run no.	nomilin content			limonin content		
		control, ppm	treated, ppm	reduction, %	control, ppm	treated, ppm	reduction, %
36	1				14.4	3.7	74
37	2	3.6	0.6	89			
38	3				9.1	0.6	95
39	4	16.3	5.8	64			
40	5				12.8	2.2	83
43	6	13.3	4.4	67			
44	7				13.1	3.8	71
45	8	13.1	5.1	61			
46	9				13.2	4.6	65
47	10	12.8	2.3	82			
50	11				14.2	4.6	67
51	12	19.2	8.6	55			
53	13				21.4	8.5	60
54	14	17.7	8.1	54			
59	15				23.3	12.2	48
60	16	17.7	9.4	47			
61	17				22.2	13.3	40
61	18	20.0	9.1	55			
64	19				22.2	11.1	50

[a] 30 mL of the serum was passed through a 1.5-cm diameter column packed with 1.5 g of cells immobilized (15-mL bed volume) at a rate of 1 mL/min at room temperature.

bilized in acrylamide gel by the procedures described in a previous publication (Hasegawa et al., 1982). A crude preparation of limonin D-ring lactone hydrolase was obtained from *A. globiformis* cells and assayed by the procedures reported previously (Hasegawa, 1976).

The major metabolite in treated samples with immobilized cells was isolated, methylated, and identified by the thin-layer chromatography (TLC) method in a manner similar to that used for identification of 17-dehydrolimonoate A-ring lactone (Hasegawa et al., 1972). Juice sera were treated with immobilized cells by the procedure of Hasegawa et al. (1982). Limonoids of treated juices and juice sera were analyzed by the TLC method of Maier and Grant (1970) after extraction with CH_2Cl_2.

RESULTS AND DISCUSSION

In a previous publication (Hasegawa et al., 1982) we reported that *A. globiformis* cells immobilized in acrylamide gel converted limonin (I) to nonbitter 17-dehydrolimonoate A-ring lactone via limonoate A-ring lactone in citrus juices and juice sera by the catalytic action of two enzymes, limonin D-ring lactone hydrolase and limonoate dehydrogenase.

The results of a substrate specificity study showed that the limonin D-ring lactone hydrolase preparation attacked II at a rate faster than that for I. It also attacked deacetylnomilin but was inactive toward deoxylimonin and deoxylimonic acid. The relative rates of hydrolysis were 100, 113, and 110 for I, II, and deacetylnomilin, respectively. Since limonoate dehydrogenase isolated from *A. globiformis* attacked nomilinoate A-ring lactone (III) more rapidly than limonoate A-ring lactone (Hasegawa et al., 1972), the coupled action of these two enzymes should convert II to nonbitter 17-dehydronomilinoate A-ring lactone (IV).

When 100 mL of navel orange juice, which contained 18 ppm of II, were treated for 2 h with 2 g of immobilized cells in a flask with constant stirring, 95% of II disappeared and approximately 50% was recovered as the major metabolite. This major metabolite was acidic and negative to Ehrlich's reagent (Dreyer, 1965), and its methyl ester had the same mobility as that of the authentic methyl ester of IV in three TLC systems.

Table I shows the results of treatment of Valencia orange juice sera with immobilized *A. globiformis* cells. The immobilized cells very effectively reduced the II content of the serum at rates similar to those for I reduction. Approximately 10 ppm of II was reduced per run, suggesting that this particular column appears to be capable of reducing the II content to below the 3-ppm threshold level since we have never observed II content in citrus juices higher than 10 ppm.

These results show that bitterness due to II in citrus juices can be reduced with immobilized *A. globiformis* cells as effectively as bitterness due to I. This process could also reduce the levels of other bitter limonoids such as ichangin and nomilinic acid, but debittering experiments for these compounds were not performed because amounts of these compounds in citrus juices are negligible. In an earlier experiment it was shown that the treatment has no significant effect on juice pH, total acids, total soluble solids, and sugars (Hasegawa et al., 1982). This process has potential for practical application.

ACKNOWLEDGMENT

We express our appreciation to Dr. V. P. Maier of this laboratory for his helpful comments in the preparation of this manuscript.

Registry No. I, 1180-71-8; II, 1063-77-0; III, 84028-36-4; IV, 84028-37-5; limonin D-ring lactone hydrolase, 9031-17-8; limonoate dehydrogenase, 37325-58-9; deacetylnomilin, 3264-90-2.

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Received for review March 22, 1982. Revised manuscript received October 4, 1982. Accepted November 6, 1982. This work was supported in part by the Citrus Product Technical Committee.

Mass Spectra of Some Alkyloxazoles

The mass spectra of 50 alkyloxazoles have been measured and the major peaks tabulated. The compounds were synthesized by general methods and purified by gas chromatography. The major mass spectral fragmentations of the alkyloxazoles are β scission and β scission with hydrogen rearrangement.

The analysis of volatile flavor constituents of foods by GC–MS frequently results in large numbers of mass spectra which cannot be interpreted because of the lack of published spectra. The publication of mass spectra of families of food compounds, such as alkylpyrazines by Bondarovich et al. (1967), bicyclic pyrazines by Pittet et al. (1974), and alkylthiazoles by Buttery et al. (1973) and Vitzthum and Werkhoff (1974), has allowed other researchers to identify such compounds in various food products.

In order to interpret some unknown mass spectra, we synthesized and obtained mass spectra of a number of alkyloxazoles. As there are a limited number of published mass spectra of alkyloxazoles available (Bowie et al., 1968; Vitzhum and Werkhoff, 1974; Ho and Tuorto, 1981), we report their spectra here in the hope that they may be of use to other researchers. Alkyloxazoles have been found in several foods, including roasted coffee (Stoffelsma and Pypker, 1968; Vitzthum and Werkhoff, 1974), roasted cocoa (Vitzthum et al., 1975), roasted barley (Harding et al., 1978), baked potato (Coleman et al., 1981), roasted peanuts (Lee et al., 1981), and meat products (Chang and Peterson, 1977; Mussinan and Walradt, 1974).

EXPERIMENTAL SECTION

Materials. Aliphatic ketones, aliphatic aldehydes, bromine, amides, acids were obtained from Aldrich Chemical Co.

Synthesis of α-Bromo Ketones. These were synthesized by the method described by Catch et al. (1948), which involves essentially direct bromination of the appropriate ketone. With unsymmetrical ketones, two bromoketones are formed. In all cases, the mixture of the two bromides was taken through the oxazole synthesis and the two isomeric oxazoles were separated by GC.

Synthesis of α-Bromo Aldehydes. These were synthesized by the method of Bedoukian (1944), which involves conversion of the corresponding saturated aldehyde to its enol acetate followed by the addition of bromine and then conversion to the dimethyl acetal and hydrolysis to the α-bromo aldehyde.

Synthesis of Alkyloxazoles. These were all synthesized by the method of Theilig (1953). One equivalent of α-bromo ketone or α-bromo aldehyde was allowed to react with 2 equiv of amide. Yields were all generally quite satisfactory at about 50%. The distilled products were purified by GC. Gas chromatography was performed on a Beckman GC-55 gas chromatograph, fitted with a 12 ft. long × $^1/_8$ in. o.d. stainless steel column packed with 10% SP-1000 on 80–100-mesh Chromosorb W. The flow rate was 30 mL/min. The column temperature was programmed from 50 to 230 °C at a rate of 5 °C/min. The purified oxazoles were then subjected to GC–MS analysis.

Table I. Alkyloxazoles Synthesized

2,4-diethyloxazole	2,5-dipropyl-4-methyloxazole
2-methyl-5-propyloxazole	2-propyl-4-butyloxazole
2-propyl-4-methyloxazole	2-butyl-4-methyl-5-ethyloxazole
5-pentyloxazole	
2-methyl-5-butyloxazole	2-butyl-4-ethyl-5-methyloxazole
2,4-dimethyl-5-propyloxazole	2-butyl-4-propyloxazole
2-methyl-4-butyloxazole	2-pentyl-4,5-dimethyloxazole
2,4-diethyl-5-methyloxazole	2-pentyl-4-ethyloxazole
	2-hexyl-4-methyloxazole
2-propyl-4,5-dimethyloxazole	2-ethyl-4-methyl-5-pentyloxazole
2-propyl-4-ethyloxazole	2,4-diethyl-5-butyloxazole
2-isopropyl-4,5-dimethyloxazole	2-ethyl-4-pentyl-5-methyloxazole
2-isopropyl-4-ethyloxazole	2-propyl-4-methyl-5-butyloxazole
2-butyl-4-methyloxazole	
2-methyl-5-pentyloxazole	2-propyl-4-pentyloxazole
2-methyl-4-propyl-5-ethyloxazole	2-pentyl-4-methyl-5-ethyloxazole
2-ethyl-5-butyloxazole	2-pentyl-4-ethyl-5-methyloxazole
2-propyl-4-methyl-5-ethyloxazole	2-hexyl-4,5-dimethyloxazole
2-propyl-4-ethyl-5-methyloxazole	2-hexyl-4-ethyloxazole
	2-heptyl-4-methyloxazole
2,4-dipropyloxazole	2-pentyl-4-methyl-5-propyloxazole
2-isopropyl-4-ethyl-5-methyloxazole	2-pentyl-4-butyloxazole
2-butyl-4,5-dimethyloxazole	2-hexyl-4-methyl-5-ethyloxazole
2-pentyl-4-methyloxazole	2-hexyl-4-ethyl-5-methyloxazole
2-methyl-4-ethyl-5-butyloxazole	2-hexyl-4-propyloxazole
2-ethyl-5-pentyloxazole	2-heptyl-4-ethyloxazole
2,5-diethyl-4-propyloxazole	2-octyl-4-methyloxazole

Mass Spectra. Mass spectrometry was performed on a Du Pont 21-490 mass spectrometer with a jet separator interfaced to a Varian Moduline 2700 gas chromatograph fitted with an FID detector and a $^1/_8$ in. o.d. × 12 ft. stainless steel column packed with 10% OV-101 on 80–100-mesh Chromosorb W. The ionization voltage was 70 V.

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