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SHORT COMMUNICATION

ACIDIC LIMONOIDS OF GRAPEFRUIT SEEDS

RAYMOND D. BENNETT

Fruit and Vegetable Chemistry Laboratory,* Pasadena, California 91106, U.S.A.

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Abstract—Five acidic limonoids were isolated from a grapefruit seed extract. Two of them were identified as isoobacunoic acid and epiisoobacunoic acid, and two new compounds, nomilinic acid and deacetylnomilinic acid, were found to be hydroxy acids derived from the lactones nomilin and deacetylnomilin. Deacetylnomilinic acid may be a key intermediate in the biosynthesis of limonin.

INTRODUCTION

THE MAJOR *Citrus* limonoids can be arranged in a hypothetical biosynthetic sequence: deacetylnomilin (I)→nomilin (II)→obacunone (III)→limonin (IV).[1] At least two intermediate steps are necessary between obacunone and limonin. Obacunoic acid (V) and isoobacunoic acid (VI) have been suggested as intermediates,[1] but the former has not been isolated from *Citrus* species and the latter has not been found in any plant. However, minor acidic components would probably have escaped notice in previous work, which relied upon crystallization[2–4] or column chromatography on alumina[4, 5] for separation of *Citrus* limonoids. Acidic compounds are strongly adsorbed by alumina. Thus, the possible occurrence of V and VI, as well as other acidic limonoids, in *Citrus* fruit seemed to be an open question worth investigating. Since grapefruit (*C. paradisi*) seeds are known to be the richest source of limonin,[6] they were selected for this work.

RESULTS AND DISCUSSION

At the beginning of this work, grapefruit seed extracts were separated into neutral and acidic fractions by extraction with $KHCO_3$ solution. However, the formation of emulsions made this a tedious procedure, and it soon became apparent that prolonged contact with even as weak a base as $KHCO_3$ caused the formation of limonoid artifacts, including isoobacunoic acid. Since one purpose of this investigation was to determine if the latter were a natural constituent, a different method for separating acidic from neutral limonoids was employed. The crude extract was chromatographed on a silica gel column, and the neutral limonoids were eluted with a neutral solvent system. The acidic limonoids, which had remained at the top of the column, were then eluted with a solvent system containing acetic acid. The acidic fraction was next methylated with diazomethane, in order to facilitate separation of its components by chromatography. TLC showed the presence of five compounds giving the characteristic limonoid color reaction[4] with Ehrlich's reagent. These

* A laboratory of the Western Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture.

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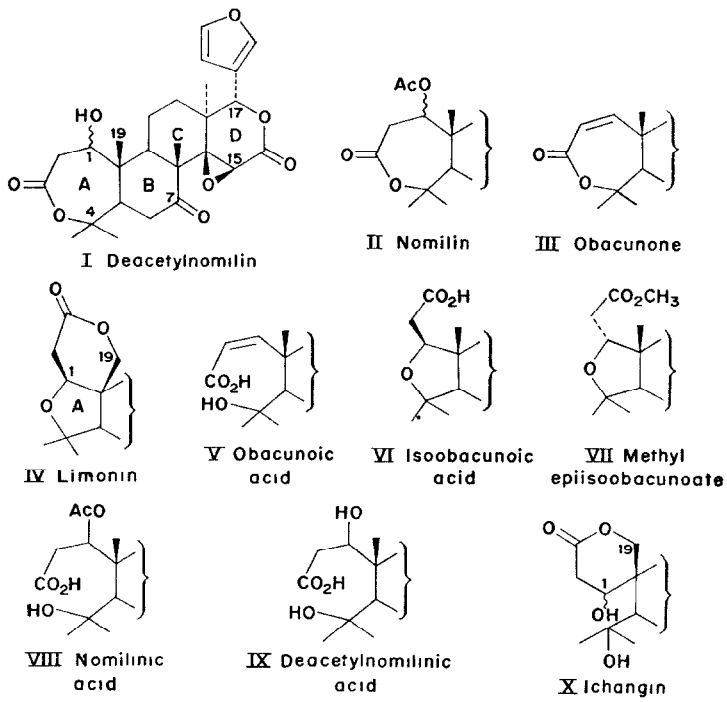
[3] O. H. EMERSON, *J. Am. Chem. Soc.* **73**, 2621 (1951).
[4] D. L. DREYER, *J. Org. Chem.* **30**, 749 (1965).
[5] D. L. DREYER, *J. Org. Chem.* **31**, 2279 (1966).
[6] D. H. R. BARTON, S. K. PRADHAN, S. STERNHELL and J. F. TEMPLETON, *J. Chem. Soc.* 255 (1961).

compounds were separated by column chromatography on silica gel, and each was further purified by preparative TLC. Two of these limonoids were identical with synthetic samples of methyl isoobacunoate and methyl epiisoobacunoate (VII). A third component could not be related to any of the known limonoids, and the amount isolated was too small for characterization.

The other two compounds, which were present in much higher concentrations than the first three, were also different from any of the known limonoids. Compound A, the less polar of the two, showed furan, H_{15}, and H_{17} NMR signals characteristic of a normal limonoid D-ring,[7] as in compounds I–VII. The presence of five tertiary methyl signals in the spectrum indicated that compound A was related to compounds I–III, rather than to limonin, in which C_{19} is oxidized. An acetate methyl resonance and a downfield one-proton quartet centred at 6·51 ppm completed the significant features of the spectrum. Compound B gave an NMR spectrum similar to that of compound A, with two exceptions. No acetate methyl signal was present, and the one-proton quartet appeared at 4·66 ppm. This suggested that compound A might be the acetate of compound B. In fact, acetylation of compound B did give compound A.

The position of the H_{15} resonances in compounds A and B indicated that they did not contain a 5-membered oxide ring as in compounds IV, VI, and VII.[7] This raised the possibility that they were derivatives of nomilin and deacetylnomilin. Treatment of the latter with KOH opened the A-ring, giving an acid which was then methylated. The methyl ester proved to be identical with compound B. Nomilin was likewise converted to compound A by acid-catalysed opening of the A-ring, followed by methylation. Thus, the parent



[7] D. L. DREYER, *Tetrahedron* **21**, 75 (1965).

TABLE 1. NMR SPECTRA OF LIMONOID ACIDS AND METHYL ESTERS*

Compound	α-Furan	β-Furan	H_{17}	H_{15}	H_1	Tertiary methyl	Other
Methyl isoobacunoate	7·41	6·35	5·54	4·40	3·91 (5,11)	1·25, 1·25, 1·21, 1·17, 1·13	Ester methyl, 3·71
Methyl epiisoobacunoate	7·42	6·36	5·51	4·14	4·24 (4,10)	1·40, 1·29, 1·21, 1·18, 1·18	Ester methyl, 3·69
Nomilinic acid	7·40	6·37	5·43	3·68	6·54 (1,9)	1·36, 1·33, 1·29 1·15, 1·11	Acetate methyl, 2·04
Methyl nomilinate	7·41	6·39	5·45	3·69	6·51 (2,10)	1·37, 1·37, 1·32, 1·16, 1·11	Ester methyl, 3·65; acetate methyl, 2·06
Deacetylnomilinic acid	7·39	6·36	5·44	3·68	4·73 (2,10)	1·37, 1·30, 1·30, 1·18, 1·11	
Methyl deacetylnomilinate	7·40	6·36	5·43	3·70	4·66 (2,10)	1·37, 1·30, 1·30, 1·18, 1·13	Ester methyl, 3·70

* Spectra were taken on a Jeolco 4H-100 instrument in $CDCl_3$, with TMS as internal standard. Chemical shifts are given in ppm (δ). Coupling constants (cycles/sec) are enclosed in parentheses.

acids of compounds A and B must have the structures VIII and IX, and accordingly they have been named *nomilinic acid* and *deacetylnomilinic acid*, respectively. VIII and IX are stable in the presence of aqueous acids, but they can be cyclized to the corresponding lactones (II and I) by treatment with anhydrous trifluoroacetic acid.

The NMR spectra of the compounds isolated in this work are summarized in Table 1. The H_1 and one of the C_4 methyl resonances of methyl epiisoobacunoate are shifted downfield from the corresponding signals of methyl isoobacunoate, presumably due to the difference in conformation of the deshielding carbomethoxy group relative to the A-ring. Surprisingly, isomerization at C_1 also affects the position of the epoxy H_{15} resonance, which is shifted 0·26 ppm upfield in methyl epiisoobacunoate. Structural changes in limonoids which distort the conformation of the B-ring, and thereby alter the angle between the 7-keto group and H_{15}, are known to affect the chemical shift of the latter proton.[7] It is not apparent from Dreiding models that a change in configuration at C_1 would have a significant effect on the B-ring conformation, but no other explanation for the observed shift seems feasible.

The H_1 resonance in nomilinic acid is found unusually far downfield, at 6·54 ppm, while the corresponding signal in nomilin appears at 5·01 ppm, within the normal range for protons attached to a carbon atom bearing an acetoxy group. The favoured conformation of the side chain in nomilinic acid must be such that H_1 is strongly deshielded by the carboxyl group. A similar effect, but of lesser magnitude, is seen in comparing the H_1 resonances of deacetylnomilinic acid (4·73 ppm) and deacetylnomilin (3·77 ppm).

The finding of isoobacunoic acid as a natural constituent of grapefruit seeds lends support to the hypothesis that this compound may be the immediate precursor of limonin.[1] Isoobacunoic acid, in turn, could be derived from obacunone via obacunoic acid, or more directly by cyclization of deacetylnomilinic acid. Our failure to detect obacunoic acid in grapefruit seeds indicates that the latter pathway may be more plausible.

Dreyer isolated ichangin (X) from ichang lemon seeds and suggested that it might be the immediate precursor of limonin on an alternative biosynthetic route.[5] Deacetylnomilinic acid could, in theory, be converted to ichangin by hydroxylation at C_{19}, followed by lactone

ring closure. Thus, deacetylnomilinic acid could act as a key intermediate in at least two biosynthetic pathways leading to limonin.

Methyl epiisoobacunoate has previously been isolated from *Vepris bilocularis*, a tree found in India.[8] Epiisoobacunoic acid is the only known limonoid having the opposite configuration to limonin at C_1, and it is therefore unlikely to be involved in limonin biosynthesis.

EXPERIMENTAL

Materials and methods. Silica gel G plates were used for TLC. All solvent systems were saturated with H_2O. Spots were revealed by spraying with 50% H_2SO_4 and heating, or by spraying with Ehrlich's reagent and exposing to HCl gas.[4] Zones on preparative plates were located by spraying with 0·01% Rhodamine 6G solution in MeOH–H_2O (20:80) and examining under short-wave UV light. Silica gel for column chromatography (particle size 0·05–0·2 mm) was obtained from Brinkmann Instruments, Westbury, New York. Acidic limonoids were isolated from extracts of fresh grapefruit seeds or, on a larger scale, from a commercial citrus seed meal. Both sources contained the same limonoids.

Isolation of limonoids. The seeds or meal were extracted and limonin removed from the extract by crystallization as previously described.[4] The mother liquor was evaporated to dryness and partitioned between $CHCl_3$ and H_2O. The $CHCl_3$ solution was then evaporated to dryness and chromatographed on a column of silica gel, packed as a slurry in CH_2Cl_2–MeOH (96·4). The neutral limonoids were eluted with the same solvent, and then CH_2Cl_2–MeOH–AcOH (94:6:1) was passed through the column to remove the acidic limonoids. The acidic fraction was methylated with excess CH_2N_2 in Et_2O and chromatographed on a silica gel column with CH_2Cl_2–MeOH (96:4). Nonpolar material was first eluted, and then a fraction containing the five limonoid methyl esters was collected. The individual limonoids were separated by column chromatography on silica gel. To achieve maximum resolution a long, narrow column was run at a slow rate.[9] Nonlimonoid components were first removed with cyclohexane–EtOAc (70:30). Development with cyclohexane–EtOAc (60.40) then eluted methyl isoobacunoate, followed by methyl epiisoobacunoate. Cyclohexane–EtOAc (50:50) yielded successively an unidentified limonoid and methyl nomilinate. Finally, methyl deacetylnomilinate was eluted by cyclohexane–EtOAc (40:60).

Methyl isoobacunoate. The material isolated from the column was further purified by preparative TLC, first with cyclohexane–EtOAc (40·60) and then with CH_2Cl_2–MeOH (96.4). The product was identical (TLC, NMR) with a sample of methyl isoobacunoate prepared by synthesis from methyl obacunoate.[10,11]

Methyl epiisoobacunoate. The column fraction was purified by preparative TLC in the same systems used for methyl isoobacunoate. Comparison of the product with methyl epiisoobacunoate prepared from obacunoic acid[11] showed identity of the two samples (TLC, NMR).

Methyl nomilinate. Preparative TLC of the column fraction with cyclohexane–EtOAc (30.70) gave chromatographically homogeneous material, which was crystallized from hexane–Et_2O, m.p. 108–110°. (Found: C, 63·9; H, 7·19. $C_{29}H_{38}O_{10}$ required: C, 63·7; H, 7·07%.)

Nomilinic acid. A solution of 20 mg of nomilin in 1·0 ml of AcOH and 1·5 ml of dioxane was treated with 0·8 ml of 1·5 N HCl. The solution was kept at 25° for 2 days, poured into 20 ml of H_2O, and the product isolated by extraction with C_6H_6. An acidic fraction (14 mg), separated by $KHCO_3$ extraction, was chromatographically homogeneous but could not be induced to crystallize. Treatment with CH_2N_2 produced methyl nomilinate, identical (TLC, NMR) with the compound isolated from the seed extract.

Methyl deacetylnomilinate. The last fraction from the column was purified by preparative TLC with cyclohexane–EtOAc and then with CH_2Cl_2–MeOH (96:4). The amorphous material thus obtained was chromatographically pure but could not be crystallized. (Found: C, 63·6; H, 7·36. $C_{27}H_{36}O_9$ required: C, 64·3; H, 7·19%.)

Deacetylnomilinic acid. A solution of 43 mg of deacetylnomilin in 2 ml of MeOH and 0·5 ml of tetrahydrofuran was treated with 0·5 ml of 5 N KOH. After 5 hr at 25° 2 ml of H_2O was added, and, after acidification the product was isolated by extraction with EtOAc. This material was fractionated by extraction with $KHCO_3$ to give 36 mg of acidic product. Attempts to crystallize the acid were unsuccessful, but CH_2N_2 converted it to methyl deacetylnomilinate, identical (TLC, NMR) with that previously isolated.

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