Phytochemistry, Vol 27, No 5, pp 1543-1545, 1988
Printed in Great Britain

0031-9422/88 $3.00+0.00
Pergamon Press plc

ICHANGENSIN: A NEW CITRUS LIMONOID

RAYMOND D. BENNETT, ZAREB HERMAN and SHIN HASEGAWA

USDA, ARS, Fruit and Vegetable Chemistry Laboratory, 263 South Chester Avenue, Pasadena, California 91106, U.S.A.

(*Received* 27 *July* 1987)

Key Word Index—*Citrus ichangensis*; Rutaceae, limonoids, ichangensin, methyl 1-hydroxyisoobacunoate

Abstract—A new limonoid, ichangensin, was isolated from seeds of *Citrus ichangensis*. It is probably a metabolite of deacetylnomilinic acid. In chloroform solution the compound formed an equilibrium mixture of isomers, a ketone and an intramolecular hemiketal, while in aqueous solution and in the solid state it was entirely in the hemiketal form. Ichangensin was prepared by synthesis from methyl deacetylnomilinate

INTRODUCTION

We have been investigating the biosynthesis of the citrus bitter substance limonin (**1**) in conjunction with our efforts to develop methods for debittering citrus juices [1, and references therein]. Dreyer [2] previously reported that seeds of *Citrus ichangensis* and its hybrids contain several unidentified limonoids. He isolated and determined the structure of one of these compounds, ichangin. Since one or more of them could be intermediates in limonin biosynthesis, we have further investigated the limonoid constituents of *C. ichangensis*. A new limonoid (**2**) was isolated and its structure determined. It appears to be part of a unique limonoid biosynthetic pathway which has implications for debittering research.

RESULTS AND DISCUSSION

A nonpolar limonoid was isolated by chromatography from an extract of *C. ichangensis* seeds. Although it appeared to be pure by TLC in three solvent systems, the ^{1}H NMR spectrum in $CDCl_3$ showed the presence of two components, in a ratio of 2.4:1. After several crystallizations from different solvents both components were still present in the same ratio. This suggested that we were dealing with an equilibrating mixture of isomers. In the ^{13}C NMR spectrum separate peaks could be observed for most carbons of the two components. One significant difference was the presence of an additional ketone carbonyl resonance in the spectrum of the minor component. Likewise, the major component showed a peak at 105 ppm absent in the spectrum of the minor component, ascribable to a carbon bonded to two oxygens. These observations suggested an equilibrium between a ketone and a hemiketal, the latter being formed by intramolecular bonding with a hydroxyl group.

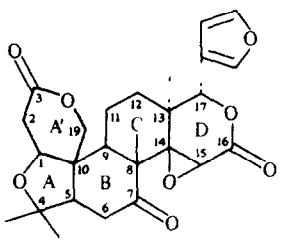
All of the known citrus limonoids contain either a singly bonded oxygen or a 1,2-double bond at C-1, and therefore a signal for H-1 is always present in their ^{1}H NMR spectra. Since no signals attributable to H-1 were observed for either isomer in the ^{1}H NMR spectrum of the *C. ichangensis* compound, the most likely site of the keto group was C-1. The hemiketal could then be formed by reaction with a 4-hydroxyl group to yield a 5-membered ring.

The ^{1}H and ^{13}C NMR spectra were consistent with normal limonoid B, C, and D rings. However, no signals were observed for the C-3 carbonyl, normally a lactone or free acid, in the ^{13}C NMR spectrum. A limonoid containing a C-1 keto group and a C-3 free acid would be a β-keto acid and would therefore be expected to readily decarboxylate, C-2 would then become a methyl group. All citrus limonoids except those oxygenated at C-19, like limonin, contain five C-methyl groups. The *C. ichangensis* compound showed no evidence of oxygenation at C-19 in its NMR spectra. Thus, if it was formed by decarboxylation as postulated above, it should contain six C-methyl groups. The ^{1}H NMR spectrum was too crowded in the methyl region to allow an accurate count. However, the ^{13}C NMR spectrum, with the help of a DEPT pulse sequence with proton decoupling [3] to invert methylene carbons in the same region of the spectrum, clearly showed six methyl signals for each isomer. Therefore we assign the structures **2a** and **2b** to the ketone and hemiketal forms, respectively, of the isolated compound, and we have named it ichangensin.

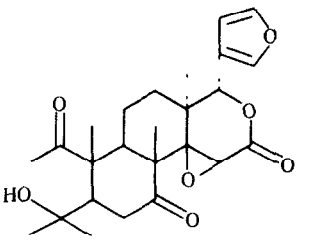
To confirm these structures we have prepared the compound by synthesis from methyl deacetylnomilinate (**3b**). Oxidation of **3b** (CrO_3) gave the 1-ketone, which immediately cyclized to the hemiketal, methyl 1-hydroxyisoobacunoate (**4**). The ^{1}H NMR spectrum of **4** showed no signals for the keto isomer. Both the ^{1}H and ^{13}C NMR spectra were very similar to those of **2b**. Hydrolysis of the ester with base followed by acidification gave the free acid, which was quite stable, presumably because it too was almost entirely in the hemiketal form. However, prolonged heating resulted in decarboxylation, and the product was identical with ichangensin. The ratio of the two isomers in the ^{1}H NMR spectrum was also the same as that observed for ichangensin.

A similar limonoid, clausenolide (**5**), was previously isolated from *Clausena heptaphylla* [4]. Clausenolide is, in fact, the 11-hydroxy derivative of **2b**. In *C. ichangensis* seeds ichangensin (**2**) is the major limonoid present, whereas the seeds of most other citrus species contain limonin **1** as the predominant limonoid [5].

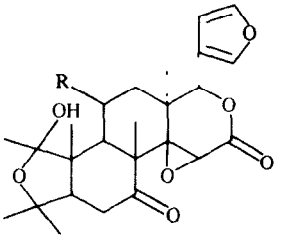
In citrus leaves, stems, and fruits the D-ring lactone of the limonoids is open, which renders them water-soluble, whereas in seeds the D-ring is closed and the limonoids are insoluble. When the D-ring of **2** was opened by



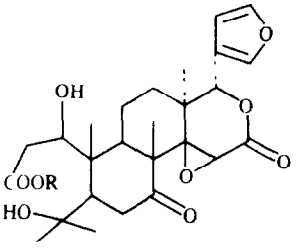
Limonin **1**



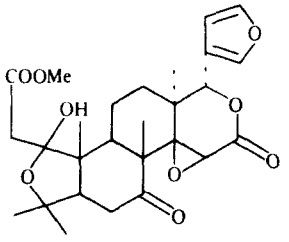
Ichangensin (ketone) **2a**



R = H Ichangensin (ketal) **2b**
R = OH Clausenolide **5**



R = H Deacetylnomilinic acid **3a**
R = Me Methyl deacetylnomilinate **3b**



Methyl 1 - hydroxyisoobacunoate **4**

treatment with 3% potassium hydrogen carbonate in 70% methanol, the ^{13}C NMR spectrum in D_2O showed carbon signals for only one component This was the hemiketal form, as evidenced by a signal at 108 ppm for C-1 and only one ketone carbonyl signal (C-7), at 217 ppm Likewise, the solid state ^{13}C NMR spectrum of **2** was that of a single constituent, and again this was the hemiketal form, with peaks for C-1 at 105 ppm and C-7 at 210 ppm Thus, it seems likely that *in vivo* **2** is entirely in the hemiketal form

During the past few years the biosynthetic pathways of the major citrus limonoids have been well established [6–10] Compound **3a** has been shown to be a precursor of all of the known limonoids of citrus and its hybrids. Nomilin is formed directly from **3a**, and limonin (**1**) is biosynthesized from nomilin via obacunone and obacunoate [7, 8] Compound **2** is probably formed by dehydrogenation of **3a**, followed by decarboxylation The latter reaction undoubtedly occurs under enzyme catalysis, considering the difficulty experienced in carrying it out in the laboratory

Since all known citrus limonoids contain a C-3 carbonyl group, the conversion of **3a** to **2** must represent an alternative biosynthetic pathway of limonoids Apparently this pathway is the major one in *C. ichangensis* and is unique to this species, since we have not detected **2** in seeds of other citrus species If this pathway could be transferred genetically to citrus species which have a limonoid bitterness problem, **3a** might be largely converted to the nonbitter **2**, and subsequently less nomilin and limonin (**1**) might accumulate in fruit tissues This could represent a possible solution to the limonin bitterness problem.

EXPERIMENTAL

Materials and methods *C ichangensis* seeds were obtained from the University of California at Riverside citrus groves Silica gel plates for TLC were developed with solvent systems (a) cyclohexane–EtOAc (2 3); (b) CH_2Cl_2–MeOH (49 1), (c) EtOAc–CH_2Cl_2 (2 3) Solid state ^{13}C NMR spectra were run at 67 8 MHz with spinning speeds of about 3000 Hz under CPMAS conditions and referenced to the methyl resonance of hexamethylbenzene (17 4 ppm)

Isolation of ichangensin Limonoids were extracted from 40 g of *C ichangensis* seeds by the procedure previously described

[5] The extract was fractionated by preparative TLC with system (a) to give 61 mg of chromatographically pure **2**. After crystallization from *iso*-PrOH, MeOH, and CH_2Cl_2–hexane the mp was 175–178° (uncorr.) ^{1}H NMR (270 MHz, $CDCl_3$). ketone form (**2a**). δ1.14, 1.16, 1.19, 1.25, 2.26 (18H, 6*s*, C-Me), 3.74 (1H, *s*, H-15), 5.40 (1H, *s*, H-17), 6.33 (1H, *d*, J=1 Hz, β-furan), 7.40 (2H, *d*, J=1 Hz, α-furans). (The sixth methyl was probably at δ1.28, under a larger signal of the isomer); ketal form (**2b**): δ1.12, 1.21, 1.22, 1.28, 1.30, 1.46 (18H, 6*s*, C-Me), 4.53 (1H, *s*, H-15), 5.60 (1H, *s*, H-17), 6.33 (1H, *d*, J=1 Hz, β-furan), 7.38 (2H, *d*, J=1 Hz, α-furans), ^{13}C NMR (67.8 MHz, $CDCl_3$): ketone form (**2a**) δ15.4 (*q*, Me), 16.4 (*q*, Me), 18.4 (*t*, C-11), 20.6 (*q*, Me), 25.8 (*q*, Me), 27.8 (*t*, C-12), 28.4 (*q*, Me), 32.9 (*q*, Me), 37.2 (*s*, C-13), 38.5 (*t*, C-6), 46.9 (*d*, C-9), 52.4 (*s*, C-8), 53.4 (*d*, C-15), 54.2 (*s*, C-10), 57.1 (*d*, C-5), 65.4 (*s*, C-14), 72.9 (*s*, C-4), 78.1 (*s*, C-17), 110.0 (*d*, β-furan), 120.3 (*s*, β-furan), 141.2 (*d*, α-furan), 143.0 (*d*, α-furan), 166.9 (*s*, C-16), 208.5 (*s*, C-7), 214.9 (*s*, C-1); ketal form (**2b**): δ14.8 (*q*, Me), 16.7 (*t*, C-11), 19.2 (*q*, Me), 19.6 (*q*, Me), 23.5 (*q*, Me), 24.8 (*q*, Me), 27.8 (*t*, C-12), 31.9 (*q*, Me), 36.8 (*t*, C-6), 39.6 (*s*, C-13), 41.2 (*d*, C-9), 49.3 (*s*, C-10), 50.2 (*s*, C-8), 54.2 (*d*, C-5), 55.7 (*d*, C-15), 69.1 (*s*, C-14), 78.1 (*s*, C-17), 79.8 (*s*, C-4), 105.9 (*s*, C-1), 110.0 (*d*, β-furan), 120.7 (*s*, β-furan), 141.2 (*d*, α-furan), 143.0 (*d*, α-furan), 167.9 (*s*, C-16), 208.8 (*s*, C-7)

Synthesis of methyl 1-hydroxyisoobacunoate (**4**) A soln of 40 mg of **3b** [11] in 2 ml of Me_2CO was cooled to 10° and treated slowly with Jones' reagent over a period of 20 min, until the orange colour of the reagent persisted. The excess reagent was destroyed by adding a drop of *iso*-PrOH and the ppt. removed by centrifuging. The supernatant was concentrated to 0.5 ml in vacuum and 2 ml of EtOAc added. The soln was washed with 1 ml of H_2O and evapd *in vacuo*. The residue (37 mg) was purified by silica gel CC with CH_2Cl_2, giving 15 mg of **4**, ^{1}H NMR (270 MHz, $CDCl_3$) δ1.11, 1.19, 1.24, 1.30, 1.32 (15H, 5*s*, C-Me), 3.74 (3H, *s*, Me ester), 3.58 (1H, *s*, H-15), 5.46 (1H, *s*, OH), 5.60 (1H, *s*, H-17), 6.32 (1H, *d*, J=1 Hz, β-furan), 7.40 (2H, *d*, J=1 Hz, α-furans), ^{13}C NMR (67.8 MHz, $CDCl_3$): δ13.9 (*q*, Me), 16.5 (*t*, C-11), 19.3 (*q*, Me), 19.4 (*q*, Me), 23.4 (*q*, Me), 27.3 (*t*, C-12), 31.5 (*q*, Me), 36.5 (*t*, C-6), 39.6 (*s*, C-13), 39.8 (*t*, C-2), 40.7 (*d*, C-9), 49.8 (*s*, C-10), 49.8 (*s*, C-8), 52.0 (*s*, Me ester), 53.4 (*d*, C-5), 55.7 (*d*, C-15), 69.1 (*s*, C-14), 77.9 (*s*, C-17), 80.0 (*s*, C-4), 104.3 (*s*, C-1), 109.8 (*d*, β-furan), 120.4 (*s*, β-furan), 141.0 (*d*, α-furan), 142.7 (*d*, α-furan), 167.7 (*s*, C-16), 172.7 (*s*, C-3), 208.4 (*s*, C-7)

Conversion of **4** *to ichangensin.* A soln of 5 mg of **4** in 100 μl of DMSO was treated with 25 μl of 5 M KOH at 37° for 1 hr to open the D-ring lactone and render the compound H_2O-soluble. After addition of 700 μl H_2O and 200 μl of 5 M KOH the soln was kept at 25° overnight to hydrolyse the Me ester. The soln was acidified with 3 M HCl and the ppt. was separated by centrifuging, washed with H_2O, and dried in vacuum, giving 4 mg of crude acid. This material was dissolved in 0.5 ml EtOAc and heated at 75°. The reaction was followed by TLC with system (a), after 3 days conversion was complete. The product was identical (TLC, NMR) with the compound isolated from *C. ichangensis*.

REFERENCES

1. Hasegawa, S., Herman, Z., Orme, E. and Ou, P. (1986) *Phytochemistry* **25**, 2783
2. Dreyer, D. L. (1966) *J. Org. Chem.* **31**, 2279
3. Doddrell, D. M., Pegg, D. T. and Bendall, M. R. (1982) *J. Magn. Reson.* **48**, 323
4. Chakraborty, D. P., Bhattacharyya, P., Bhattacharyya, S. P., Bordner, J., Hennessee, G. L. A. and Weinstein, B. (1979) *J. Chem. Soc. Chem. Commun.* 246.
5. Hasegawa, S., Bennett, R. D. and Verdon, C. P. (1980) *J. Agric. Food Chem.* **28**, 922
6. Hasegawa, S., Bennett, R. D. and Maier, V. P. (1984) *Phytochemistry* **23**, 1601
7. Hasegawa, S. and Herman, Z. (1985) *Phytochemistry* **24**, 1973
8. Herman, Z. and Hasegawa, S. (1985) *Phytochemistry* **24**, 2911
9. Hasegawa, S., Herman, Z. and Ou, P. (1986) *Phytochemistry* **25**, 542
10. Hasegawa, S. and Herman, Z. (1986) *Phytochemistry* **25**, 2523
11. Bennett, R. D. (1971) *Phytochemistry* **10**, 3065