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GRANDIS BANHAKUYU

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## BITTER LIMONOIDS FROM THE SEEDS OF *CITRUS GRANDIS* BANHAKUYU

by  
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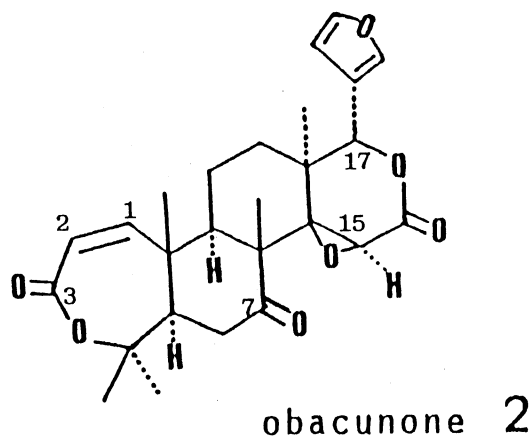
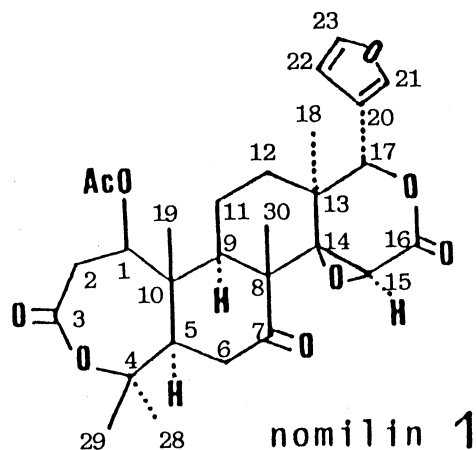
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### Abstract

Four known limonoids, nomilin, obacunone, limonin and deacetylnomilin, have been isolated as bitter components from the seeds of *Citrus grandis* Osbeck sorma Banhakuyu. Their structures were identified by spectral and chemical evidences.

### Introduction

Meliaceae and Lutaceae plants are rich sources of limonoids. In the course of our studies on the biologically active limonoids, we previously reported the isolation and structures of many limonoids, trichilins,<sup>1,2)</sup> Trs<sup>3)</sup> and glaucins<sup>4,5)</sup> from *Trichilia roka* (Meliaceae) and *Evodia glauca* Miq. (Rutaceae). Recently, some interesting biologically active limonid glucosides were first isolated from citrus fruits.<sup>6)</sup> *Citrus grandis* Osbeck sorma Banhakuyu, originated in Malayan Peninsula, is being cultivated at Yatsushiro district in Kumamoto prefecture. Its fruits present strong bitter taste and the bitter flavonoid constituents, naringin etc., have been well studied<sup>7)</sup> but there are no minute reports on limonoid. In this paper we report the isolation of four known limonoids, nomilin (1), obacunone (2), limonin (3) and deacetylnomilin (4), as bitter components of the seeds.



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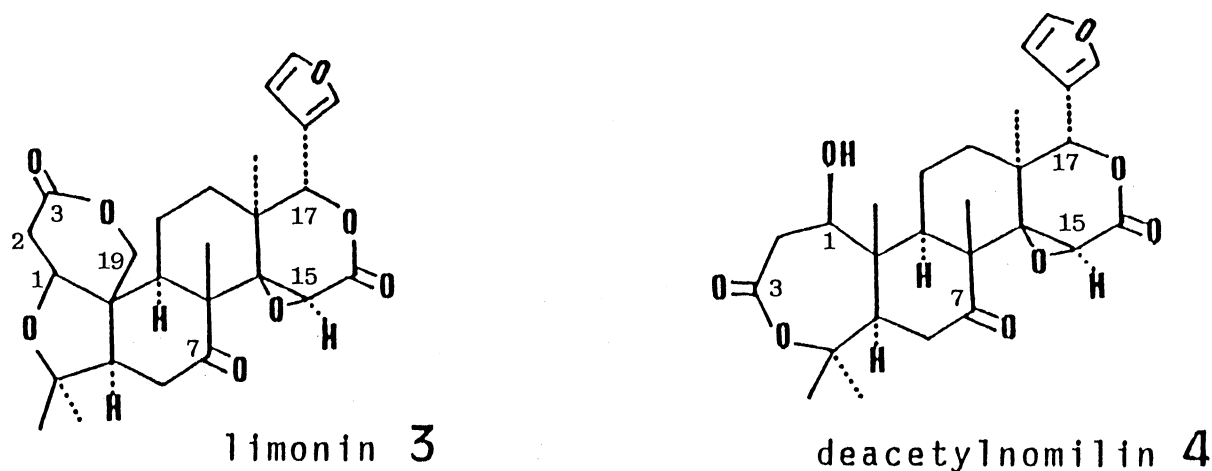


Fig. 1

These limonoids are directly involved in main limonoid biosynthetic pathway in *Citrus*,<sup>8)</sup> but, to my regret, obcunoic acid and isoobacunoic acid assumed to be intermediates to limonin and any limonid glycoside were not isolated in this work.

### Results and Discussion

The MeOH extract of the seeds (150 g) showed the presence of at least six limonoids which were detected by the characteristic color with Ehrlich's reagent on TLC. The extract (8.6 g) was separated into two fractions by using  $\text{CHCl}_3$  solution containing 20% MeOH: Fraction I (soluble part); 2.7 g and Fraction II (insoluble part); 5.9 g. Further fractionation of Fraction I (940 mg) by extensive silica-gel column chromatography followed by a TLC separation and the final HPLC purification on a reverse phase column using  $\text{H}_2\text{O}/\text{MeOH}$  solvent system gave four limonoids: 1 (8 mg), 2 (7 mg), 3 (14 mg) and 4 (14 mg).

Limonoid 1, needles from MeOH; mp  $270^\circ\text{C}$ , exhibited the following spectral data. SI-MS:  $m/z$  515 ( $M+1$ )<sup>+</sup> corresponding to  $\text{C}_{28}\text{H}_{34}\text{O}_9$ ;  $[\alpha]_{\text{D}}^{20} -109^\circ$  ( $\text{CHCl}_3$ ); UV (MeOH); 212 nm ( $\epsilon$  2000,  $\pi-\pi^*$  of furan). The IR spectrum showed the presence of lactone and ester ( $1740\text{ cm}^{-1}$ ) and ketone groups ( $1710\text{ cm}^{-1}$ ). The  $^1\text{H}$  NMR (Table 1.) spectrum showed signals of five tertiary methyl groups at  $\delta$  1.09, 1.18, 1.33, 1.47 and 1.55, one acetyl singlet at  $\delta$  2.02, a sharp singlet at  $\delta$  3.80 attributable to H-15, and a broad signal at  $\delta$  5.45 assigned to H-17, weakly coupling with furan protons<sup>9)</sup> at  $\delta$  7.40 (H-21), 6.33 (H-22) and 7.40 (H-23). One proton signal at  $\delta$  5.02 (dd) under an acetoxy group only coupled to two geminal proton signals at  $\delta$  3.10 (dd) and 3.20 (dd) coupled with 7.3 and 1.3 Hz, attributable to the methylene group adjacent to carbonyl group. The partial structure of  $-\text{CO}-\text{CH}_2-\text{CHOAc}-\dot{\text{C}}-$  was attributed to the A-ring and it also deduced the  $\epsilon$ -lactone structure on biogenetic grounds, because some limonoids possess the same substitution pattern.<sup>3)</sup> The  $\beta$  orientation of the 1-OAc group was revealed by the coupling constants of 7.3 and 1.3 Hz between H-1 $\alpha$  and H-2 $\beta$  and  $-2\alpha$ . Geminal protons observed at  $\delta$  2.66 (dd) and 2.77 (t),

coupled to H-5 $\alpha$  with 3.7 and 15 Hz, were assigned to 6-methylene protons because all the limonoids possess O-function at C<sub>7</sub>.

2D-Homonuclear J-correlation (COSY) spectrum clarified the coupling protons on the linked carbons (C-1 and -2, C-5 and -6, and C-9, -11 and -12). These <sup>1</sup>H NMR spectral features and physical properties revealed this compound **1** to be nomilin.<sup>10)</sup>

Limonoid **2**, [ $\alpha$ ]<sub>D</sub><sup>20</sup> -34° (acetone); prisms from MeOH; mp 234°C, showed a molecular ion at m/z 455 (M+1)<sup>+</sup> corresponding to C<sub>26</sub>H<sub>30</sub>O<sub>7</sub> by SI-MS. The IR spectrum, as well as compound **1**, showed absorptions at 1740 and 1705 cm<sup>-1</sup>. On the other hand, the UV absorption at 230 nm ( $\epsilon$  4300) suggested the presence of conjugated enone system, which was also confirmed by the appearance of two olefinic proton signals at  $\delta$  5.96 (d, J=11.7 Hz) and 6.51 (d, J=11.7 Hz) in the <sup>1</sup>H NMR spectrum of **2**, instead of the acetyl signal in the spectrum of **1**. Since the olefinic proton signal at  $\delta$  5.96 showed a W-type long range coupling with 19-methyl signal at  $\delta$  1.45, the proton was assigned to H-1, that is to say, the conjugated system belongs to the A-ring, and steric requirements for the W-type coupling deduced its  $\epsilon$ -lactone structure and a comprehensive conformation (figure 2).

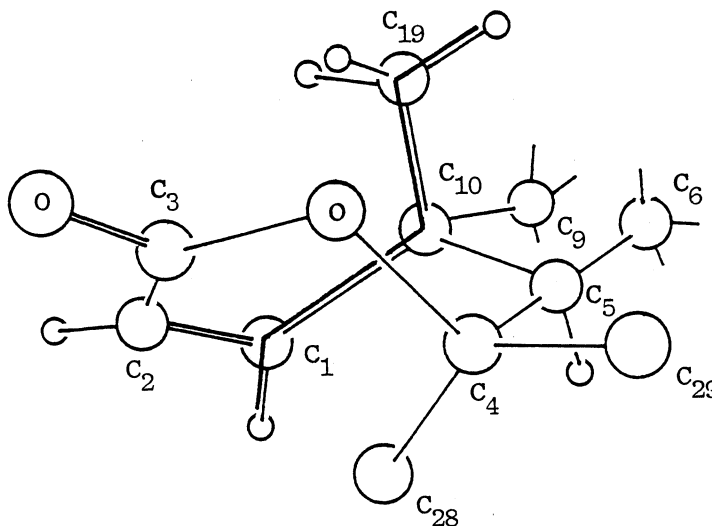


Fig. 2 Stereochemistry of ring-A

These observations indicated strongly that the compound **2** had to be obacunone,<sup>11)</sup> which was identified by direct comparison (IR, <sup>1</sup>H NMR and mixed mp.) with an authentic sample.

Limonoid **3**, [ $\alpha$ ]<sub>D</sub><sup>20</sup> -136° (acetone); prisms from MeOH; mp 290°C, was assigned the molecular formula C<sub>26</sub>H<sub>30</sub>O<sub>8</sub> from EI-MS (m/z 470[M]<sup>+</sup>). Its UV, IR and CD spectra (see Experimental) showed the presence of furan,  $\delta$ -lactone and ketone groups. The <sup>1</sup>H NMR spectrum of **3** showed signals of four tertiary methyl groups at  $\delta$  1.07, 1.18, 1.18 and 1.30, and a characteristic AB quartet due to 19-methylene protons at  $\delta$  4.46 (1H, d, J=13 Hz) and 4.77 (1H, br d, J=13 Hz), the lower signal of which exhibited a specific W-type long range

Table 1.  $^1\text{H}$  NMR Data of Limonoids 1, 2, 3 and 4

H	1			2			3			4 <sup>†</sup>		
	$\delta$ (J/Hz)	Mult	Coupled to	$\delta$ (J/Hz)	Mult	Coupled to	$\delta$ (J/Hz)	Mult	Coupled to	$\delta$ (J/Hz)	Mult	Coupled to
1	5.02 (7.3, 1.3)	dd	2 $\beta$ , 2 $\alpha$	5.96 (11, 7)	d	2,(19)*	4.03	m	2 $\alpha$ , 2 $\beta$ (19 $\alpha$ )	3.94 (8, 1.5)	dd	2 $\beta$ , 2 $\alpha$
2 $\alpha$	3.20 (15.6, 1.3)	dd	2 $\beta$ , 1	6.51 (11.7)	d	1	2.68 (17, 2)	dd	2 $\beta$ , 1	3.35 (15, 1.5)	dd	2 $\beta$ , 1
2 $\beta$	3.10 (15.6, 7.3)	dd	2 $\alpha$ , 1				2.98 (17, 4)	dd	2 $\alpha$ , 1	2.82 (15, 8)	dd	2 $\alpha$ , 1
5	2.58 (15, 3.7)	dd	6 $\beta$ , 6 $\alpha$	2.30 (14, 5)	dd	6 $\beta$ , 6 $\alpha$	2.23 (16, 3.5)	dd	6 $\beta$ , 6 $\alpha$	2.45 (14.5, 4)	dd	6 $\beta$ , 6 $\alpha$
6 $\alpha$	2.60 (15, 3.7)	dd	6 $\beta$ , 5	2.60 (14, 5)	dd	6 $\beta$ , 5	2.47 (14.5, 3.5)	dd	6 $\beta$ , 5	2.65 (14.5, 4)	dd	6 $\beta$ , 5
6 $\beta$	2.77 (15)	t	5, 6 $\alpha$	2.98 (14)	t	5, 6 $\alpha$	2.86 (16, 14.5)	dd	5, 6 $\alpha$	3.02 (14.5)	t	5, 6 $\alpha$
9	2.49 (10.3, 2.8)	dd	11 $\alpha$ , 11 $\beta$	2.15 (9, 3.5)	dd	11 $\alpha$ , 11 $\beta$	2.55 (12, 2.5)	dd	11 $\alpha$ , 11 $\beta$	2.90 (11, 1)	dd	11 $\alpha$ , 11 $\beta$
11 $\alpha$	1.61	m	9, 11 $\beta$ 12 $\alpha$ , 12 $\beta$	1.89	m	9, 11 $\beta$ 12 $\alpha$ , 12 $\beta$	1.83	m	9, 11 $\beta$ 12 $\alpha$ , 12 $\beta$	1.90	m	9, 11 $\beta$ 12 $\alpha$ , 12 $\beta$
11 $\beta$	1.64	m	9, 11 $\alpha$ 12 $\alpha$ , 12 $\beta$	1.65	m	9, 11 $\alpha$ 12 $\alpha$ , 12 $\beta$	1.90	m	9, 11 $\alpha$ 12 $\alpha$ , 12 $\beta$	1.61	m	9, 11 $\alpha$ 12 $\alpha$ , 12 $\beta$
12 $\alpha$	1.53	m	11 $\alpha$ , 11 $\beta$ 12 $\beta$	1.50	m	11 $\alpha$ , 11 $\beta$ 12 $\beta$	1.52	m	11 $\alpha$ , 11 $\beta$ 12 $\beta$	1.45	m	11 $\alpha$ , 11 $\beta$ 12 $\beta$
12 $\beta$	1.79 (14, 7.2, 2.6)	ddd	12 $\alpha$ , 11 $\alpha$ 11 $\beta$	1.89	m	11 $\alpha$ , 11 $\beta$ 12 $\alpha$	1.78	m	11 $\alpha$ , 11 $\beta$ 12 $\alpha$	1.85	m	11 $\alpha$ , 11 $\beta$ 12 $\alpha$
15	3.80	s		3.66	s		4.04	s		3.81	s	
17	5.45	br s	(21), (22) (23)	5.46	br s	(21), (22) (23)	5.48	br s	(21), (22) (23)	5.48	br s	(21), (22) (23)
18	1.18	s		1.24	s		1.18	s		1.21	s	
19 $\alpha$							4.46	d	19 $\beta$			
19 $\beta$	1.47	s		1.45	br s	(1)	(13)			1.39	s	
							4.77 (13)	br d	19 $\alpha$ , (1)			
21	7.40 (1.4)	br d	22, (23) (17)	7.43 (1.8, 0.8)	dd	23, 22 (17)	7.42 (1.5, 1)	dd	23, 22 (17)	7.61 (1.5, 1)	dd	22, 23 (17)
22	6.33 (1.4)	t	21, 23 (17)	6.37 (1.8, 0.8)	dd	23, 21 (17)	6.34 (2, 1)	dd	23, 21 (17)	6.50 (1.5, 1)	dd	21, 23 (17)
23	7.40 (1.4)	br d	22, (21) (17)	7.40 (1.8)	t	21, 22 (17)	7.40 (2, 1.5)	dd	22, 21 (17)	7.51 (1)	t	21, 22 (17)
28	1.55	s		1.50	s		1.30	s		1.56	s	
29	1.33	s		1.50	s		1.18	s		1.33	s	
30	1.09	s		1.12	s		1.07	s		1.12	s	
OAc	2.02	s										

Measured in  $\text{CDCl}_3$  at 500 MHz. <sup>†</sup> in acetone- $d_6$ .

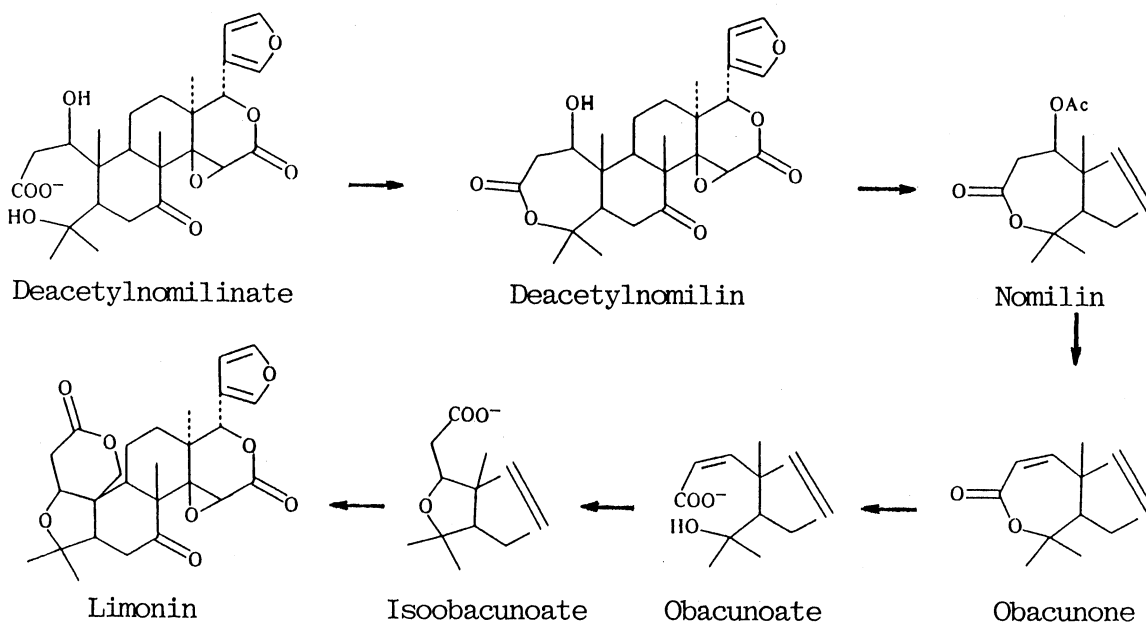
\* Distinguished couplings are listed and numerals in parenthesis denote small coupling.

coupling with a signal (H-1) at  $\delta$  4.03.

These observations and a specific low field shift of H-15 to  $\delta$  4.04<sup>12)</sup> indicated the compound **3** to be limonin.<sup>13)</sup> Direct comparison with an authentic sample clarified the structure **3**.

Limonoid **4**,  $[\alpha]_D^{20} -83^\circ\text{C}$  (acetone); prisms from MeOH; mp  $268^\circ\text{C}$ , was assigned the molecular formula  $\text{C}_{26}\text{H}_{32}\text{O}_8$  from SI-MS ( $m/z$  473  $[\text{M}+1]^+$ ). The IR spectrum showed absorptions due to hydroxyl ( $3420\text{ cm}^{-1}$ ), two ester carbonyl ( $1735$  and  $1720\text{ cm}^{-1}$ ) and ketone ( $1708\text{ cm}^{-1}$ ) groups. The  $^1\text{H}$  NMR spectrum showed five tertiary methyl signals at  $\delta$  1.12, 1.21, 1.33, 1.39 and 1.56, and it well resembled to that of the compound **1** except for the absent of acetyl group (see Table 1.). One proton under hydroxyl group observed at  $\delta$  3.94 only coupled to methylene protons adjacent to carbonyl group observed at  $\delta$  2.82 and 3.35 and so it was assigned to H-1. The coupling constants of 8 and 1.5 Hz indicated that the configuration of H-1 was  $\beta$ . From these data, the compound **4** was presumed to be deacetylnomilin (**4**),<sup>14)</sup> which was confirmed by getting nomilin (**1**) from **4** on acetylation.

During the past few years, substantial progress has been made in the field of limonoid biochemistry in *Citrus*. The conversion of nomilin to abacunone,<sup>15)</sup> obacunone to obacunoate<sup>16)</sup> and obacunoate to limonin<sup>17)</sup> have been demonstrated. Based on these studies and the work on deacetylnomilinate to nomilin, possible biosynthetic pathways of limonoids in *Citrus* have been proposed by S. Hasegawa and Z. herman (Scheme 1).<sup>8)</sup> Four limonoids isolated in this work, are directly involved in the limonoid biosynthetic pathways, and so the isolation of other minor limonoids, such as obacunoic acid and isoobacunoic acid is now in progress.



Scheme 1. Biogenetic pathways for the formation of limonoids in *Citrus*.

The authors are indebted to Mr. Kuwabata and Mr. Isobe (Kagoshima and Kumamoto Fruit Tree Experimental Station) for kindly providing of the samples investigated. We also wish to thank Dr. H. Naoki and Dr. T. Iwashita (Suntory Institute for Bioorganic Research) for MS and NMR measurements.

### Experimental

*Plant Material.* The fruits were offered from Kumamoto Fruit Tree Experimental Station.

*Extraction and Isolation.* The seeds (150 g) of the fruits were extracted with MeOH to get an extract (8.6 g), which was separated into two fractions by using  $\text{CHCl}_3$  soln containing 20% MeOH: Fraction I (soluble part); 2.7 g and Fraction II (insoluble part); 5.9 g. The fraction I (940 mg) was chromatographed on  $\text{SiO}_2$  with MeOH/ $\text{CHCl}_3$  solvent system to give two limonoid fractions, each of which was separated on prep. TLC to give four compounds. Each compound was purified by HPLC,  $\text{C}_{18}$  column, using 30–35%  $\text{H}_2\text{O}$ –MeOH as the solvent to give 1 (8 mg), 2 (7 mg), 3 (14 mg) and 4 (2 mg).

*Nomilin (1).* Needles from MeOH; mp  $270^\circ\text{C}$ ;  $[\alpha]_{\text{D}}^{20}$  (*c* 0.08,  $\text{CHCl}_3$ ):  $-109^\circ$ ; SI-MS:  $m/z$  515 $[\text{M}+1]^+$ ; UV (acetone):  $\lambda_{\text{max}}$  212 nm ( $\epsilon$  2000); IR (Nujol):  $\nu_{\text{max}}$  1740, 1710 and  $880\text{ cm}^{-1}$ .

*Obacunone (2).* Prisms from MeOH; mp  $234^\circ\text{C}$ ;  $[\alpha]_{\text{D}}^{20}$  (*c* 0.09, acetone):  $-34^\circ$ ; SI-MS:  $m/z$  455 $[\text{M}+1]^+$ ; UV ( $\text{CHCl}_3$ ):  $\lambda_{\text{max}}$  230 nm ( $\epsilon$  4300); IR (Nujol):  $\nu_{\text{max}}$  1740, 1705 and  $880\text{ cm}^{-1}$ .

*Limonin (3).* Prisms from MeOH; mp  $290^\circ\text{C}$ ;  $[\alpha]_{\text{D}}^{20}$  (*c* 0.4, acetone):  $-136^\circ$ ; EI-MS:  $m/z$  470 $[\text{M}]^+$ ; UV (MeOH):  $\lambda_{\text{max}}$  208 nm ( $\epsilon$  7400); IR (Nujol):  $\nu_{\text{max}}$  1760, 1705 and  $878\text{ cm}^{-1}$ ; CD (MeOH):  $\Delta_\epsilon$   $-4.9$  (230 nm) and  $-2.3$  (293 nm).

*Deacetylnomilin (4).* Prisms from MeOH; mp  $268^\circ\text{C}$ ;  $[\alpha]_{\text{D}}^{20}$  (*c* 0.03, acetone):  $-83^\circ$ ; SI-MS:  $m/z$  473 $[\text{M}+1]^+$ ; UV (MeOH):  $\lambda_{\text{max}}$  209 nm ( $\epsilon$  3500); IR (Nujol):  $\nu_{\text{max}}$  3420, 1735, 1720, 1708 and  $876\text{ cm}^{-1}$ .

*Acetylation of Deacetylnomilin (4).* Compound 4 (3 mg) was acetylated in pyridine with  $\text{Ac}_2\text{O}$  to give a monoacetate (1 mg, mp  $273^\circ\text{C}$ ), which was identified as nomilin 1 by TLC and  $^1\text{H}$  NMR spectrum.

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