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Limonoids from *Citrus nippokoreana*

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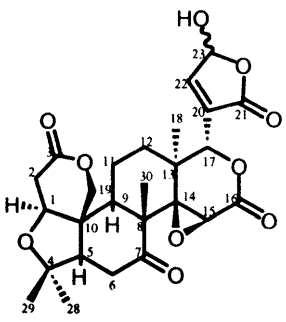
Limonexic acid and its isomer were been isolated from the whole fruit of cheongkyool (*Citrus nippokoreana*) as its main limonoids and their structures were confirmed by X-ray and NMR analysis.

Keywords: limonoid; limonexic acid; *Citrus nippokoreana*; cheongkyool; Rutaceae; X-ray; 2D NMR

Cheongkyool (*Citrus nippokoreana*) is a local species of citrus growing in Cheju Island, Korea. Its fruit each with five seeds has a strong acid taste, and the peel of unripe fruit has been used as an important medicinal resource in Korea.[1] However, there is no report on chemical and pharmacological studies.

As bitter principles in citrus, many limonoids, such as limonin, limonexic acid and so on, have been reported.[2] Preparation of limonexic acid from limonin by photosensitized air oxidation has been reported.[3] They suggested structural relationships between limonexic acid and limonin, that limonexic acid should have two isomeric structures, and that limonexic acid might be an artifact produced from limonin during its isolation process by air oxidation, and not a true metabolic product in citrus. Further their detail spectral data have not been reported. Therefore the explanation of this case must be more clearly advanced by additional evidence.



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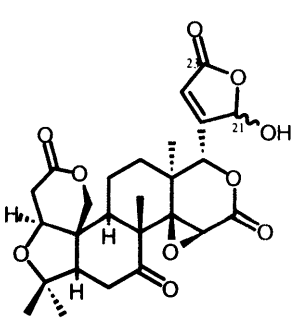

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The main purpose of the present research is to isolate limonoids in cheongkyool and to establish the three dimensional structures of limonexic acid (**1**) and its isomer (isolimonexic acid **2**) by X-ray and NMR analysis.

RESULTS AND DISCUSSION

The methanolic extract of fruit of *Citrus nippokoreana* was suspended in distilled water and then partitioned with *n*-hexane, ethyl acetate, and *n*-butanol, successively. The ethyl acetate extract was subjected to silica gel column chromatography eluting with a *n*-hexane - AcOEt and AcOEt - MeOH gradient system. Finally each fraction was purified by HPLC using a reversed phase column to give two limonoids (**1**: 0.21%; **2**: 0.016% from MeOH extract).

The major limonoid **1** obtained as colorless needles, mp 290 - 291° (dec.), had the molecular formula $C_{26}H_{30}O_{10}$, which was identical with that of limonexic acid (limonoic acid, 21,23-dihydro-23-hydroxy-21-oxo-di-δ-lactone).[4] Complete ^{1}H and ^{13}C NMR signal assignments in DMSO-d_6 conducted by the 2D NMR methods were as shown in Table 1. The stereochemistry was verified by both NOESY spectra in solution and single crystal X-ray analysis. As shown in Fig. 1, the configuration of 23-position of **1** was shown to be *S* by X-ray crystallographic analysis. However, pair signals were observed for the 17-, 22- and 23-carbons

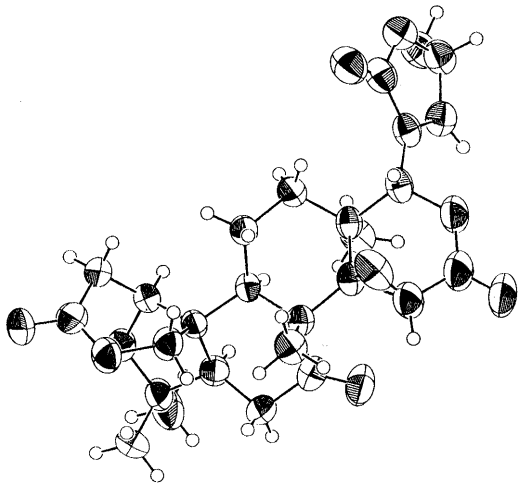

Fig. 1 A perspective view of X-ray crystallographic structure of **1**

Table 1. ^{1}H and ^{13}C NMR Signal Assignments of **1** and **2** in DMSO-d_6 at 320K.

position	δ_H [int. mult, J(Hz)]		δ_C	
	1	**2**	**1**	**2**
1	4.13 (1H, d, 3.5)	4.12 (1H, d, 3.8)	78.39	78.81
2a	2.65 (1H, dd, 3.5, 15.8)	2.67 (1H, dd, 3.8, 16.5)	35.47	36.09
2b	2.78 (1H, d, 15.8)	2.82 (1H, d, 16.5)		
3			169.83	169.48
4			79.43	79.95
5	2.52 (1H, dd, 3.3, 15.1)	2.48 (1H, dd, 2.9, 15.1)	57.33	58.68
6α	2.29 (1H, dd, 3.3, 15.1)	2.28 (1H, dd, 2.9, 15.1)	36.00	36.60
6β	3.08 (1H, t, 15.1)	3.16 (1H, t, 15.1)		
7			207.60	208.26
8			49.76	51.12
9	2.62 (1H, dd, 3.4, 12.4)	2.54 (1H, br d, 11.6)	45.80	46.84
10			45.06	45.70
11α	1.74 (1H, m)	1.69-1.77 (2H, m)	17.03	18.08
11β	1.93 (1H, m)			
12α	1.32 (1H, m)	1.69-1.77 (2H, m)	27.05	29.12
12β	1.79 (1H, m)			
13			38.44	38.27
14			66.88	66.53
15	4.25 (1H, s)	4.06 (1H, s)	54.08	53.46
16			166.50	166.55
17	5.27 (1H, s)	5.18 (1H, s)	74.73, 74.96	78.17
18	1.19 (3H, s)	1.10 (3H, s)	18.50	20.16
19a	4.51 (1H, d, 13.1)	4.47 (1H, d, 13.0)	64.53	65.31
19b	4.88 (1H, d, 13.1)	4.95 (1H, d, 13.0)		
20			130.61	164.22
21		6.08 (1H, s)	169.36	98.61
22	7.51 (1H, s)	6.29 (1H, s)	152.22, 152.86	122.44
23	6.19 (1H, br s)		97.37, 97.75	170.57
28	1.21 (3H, s)	1.19 (3H, s)	29.62	30.08
29	1.05 (3H, s)	1.03 (3H, s)	21.15	21.86
30	1.05 (3H, s)	0.98 (3H, s)	17.37	16.88
23-OH	7.79 (1H, br s)			

(Table 1). Accordingly, **1** was considered to be a mixture of 23*S* and 23*R* compounds.

The minor limonoid **2** obtained as white powder, had the molecular formula $C_{26}H_{30}O_{10}$, which is the same as that of **1**. The NMR signals in DMSO-d_6 indicated that **2** was an isomer of **1** at γ-hydroxybutenolide moiety linkage. Complete assignments of NMR signals, summarized in Table 1, indicated that **2** was identical with isolimonexic acid (limonoic acid, 21,23-dihydro-21-hydroxy-23-oxo-di-δ-lactone), which has already been reported as a natural product[5] and as a major oxidation product[6] of limonin. The three pair carbon signals observed in the NMR spectrum of **1** were not observed in **2**, but **2** was considered to be a mixture of 21*R* and 21*S* from the reason related to **1**.

A compound named substance X was isolated first by Emerson *et al.* in 1948 as a very minor constituent from seeds of immature Valencia orange.[7] In 1957, Arigoni *et al.* suggested that limonexic acid should have two isomers[8] on the basis of structural relationships between limonexic acid and limonin. Dryer *et al.* confirmed the structure of limonexic acid on the basis of NMR spectra.[9] It is remained to be solved that whether limonexic acid is an artifact derived from limonin, or a true metabolic product in citrus.

In previous studies, we isolated limonexic acid from mature whole fruit of cheongkyool and as a major limonoid in the peels and seeds of immature fruit, in which limonin was not be detected by TLC. These facts may suggest that these oxidized limonoids exist in nature, and not artifacts.

EXPERIMENTAL

General IR spectra were recorded on JASCO A-302 spectrometer, optical rotations on a JASCO DIP-4 spectrometer with the $[\alpha]_D$ values given in 10^{-1}deg cm^2g^{-1} and FAB mass spectra on a VG Autospec spectrometer. High-performance liquid chromatography (HPLC) was performed with an Inertsil PREP-ODS column (20mm i.d. × 250mm, GL Science Inc.) packed with 10 μm ODS. All NMR spectra were measured on Bruker AM400, AM500 and Varian Unity 400 spectrometer. The spectra were recorded at 320K in DMSO-d_6. The phase sensitive ROESY experiments were acquired with mixing times of 300 msec. The values of the delay to optimize one-bond correlation in the HMQC spectrum and suppress them in the HMBC spectrum was 150 msec and the evolution delay for long-range couplings in the HMBC spectrum was set to 90 msec.

Plant Material Mature fruits of cheongkyool were collected in the field of Cheju Citrus Research Institute of Rural Development Administration (R.D.A.), Korea in November 1996. The plant was identified by Dr. Han-Yong KIM, Breeding Division, Cheju Citrus Research Institute R.D.A., Korea.

Extraction and Isolation The green-peels of mature fruit were extracted three times with 99% MeOH. The filtrate was concentrated to give a MeOH extract (360 g), which was treated with *n*-hexane, AcOEt, *n*-BuOH, and H_2O, successively. The AcOEt soluble fraction (41 g) was subjected to silica gel column chromatography using *n*-hexane - AcOEt (1:1 - 0:1) and AcOEt-MeOH (9:1 - 1:1) gradient systems to give 9 fractions (A-I). Fr. D (11.3 g) was further subjected to silica gel column chromatography eluting with $CHCl_3$-AcOEt-MeOH (8:0.9:0.6) and finally purified by ODS HPLC with a 25% CH_3CN-H_2O eluting system to give **1** (750 mg). Fr. F (1.7g) was purified by ODS HPLC with an CH_3CN-H_2O system to give **2** (58 mg).

Limonexic acid (Limonoic acid, 21,23-dihydro-23-hydroxy-21-oxo-di-δ-lactone 1): Colorless needles, mp. 290°C (dec.), $[\alpha]_D$ -65° (*c* 0.10, MeOH); *m/z* 503 $[M+H]^+$; ν_{max} (KBr)/cm^{-1} 3400 (OH), 1730 and 1700 (br. C=O).

Isolimonexic acid (Limonoic acid, 21,23-dihydro-21-hydroxy-23-oxo-di-δ-lactone 2): Colorless powder, $[\alpha]_D$ -140° (*c* 0.10, MeOH); *m/z* 503 $[M+H]^+$; ν_{max} (KBr)/cm^{-1} 3400 (OH) and 1710 (br. C=O).

X-ray Crystallographic Analysis of 1. Crystal data were as follows: $C_{26}H_{30}O_{10}$, Mr=502.00, orthorhombic, $P2_12_12_1$, a=6.906(1), b=12.6570(8), c=27.688(3) Å, V=2420.2(5)$Å^3$, Z=4, D_x=1.378 g/cm^3. **1** was mounted on a Mac Science DIP2000 diffractometer with graphite-monochromated MoKα radiation (λ=0.71073Å) at 23°C. The structure was determined by the direct method using the SIR program[10] and the refinement was carried out by the full-matrix least-squared method. The final R value was 0.049 (R_w=0.056). The molecular structure determined by this method is illustrated in Fig. 1. The refined fractional atomic coordinates, bond lengths, bond angles, hydrogen-atom coordinates and thermal parameters have been deposited at the Cambridge Crystallographic Data Center (CCDC).

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