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Studies on Constituents of Medicinal Plants. XVIII.[1] Constituents of the Leaves of *Clethra barbinervis* Sieb. et Zucc. (1)

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A new triterpene acid, named barbinervic acid (I) has been isolated from the leaves of *Clethra barbinervis* Sieb. et Zucc. and barbinervic acid was elucidated as $3\alpha,19\alpha$-24-trihydroxyurs-12-en-28-oic acid by chemical and spectral evidences. Besides, rotundic acid was also isolated.

Keywords——*Clethra barbinervis* Sieb. et Zucc.; triterpene; barbinervic acid; rotundic acid; NMR

It was reported[3] that taraxerol and ceryl cerotinate were isolated from the barks of *Clethra barbinervis* Sieb. et Zucc. and ursolic acid from the fruits. Further investigation on the constituents of the leaves of this plant has led to the isolation of a new triterpene acid, named barbinervic acid and a triterpene acid (A). This paper deals with the structural elucidation of barbinervic acid. The methanolic extract of the leaves was extracted with petr-ether, benzene and ethyl acetate, successively. The ethyl acetate soluble fraction afforded barbinervic acid, $C_{30}H_{48}O_5 \cdot H_2O$ (I) of mp 298°, $[\alpha]^{31} = +18°$ (c=1.00, EtOH), Liebermann–Burchard test: red to violet, and triterpene acid (A), $C_{30}H_{48}O_5 \cdot 1/2\ H_2O$ of mp 271°, $[\alpha]^{17} = +37.5°$ (c=0.48, MeOH). The acid (A) afforded methyl ester of mp 253—255° by the methylation with diazomethane, which afforded diacetyl methylate of mp 208—210° by acetylation with acetic anhydride and pyridine. The former methylate was proved to be identical with methyl rotundate[4] by the mixed fusion and the comparison of their infrared (IR) spectra. Barbinervic acid (I) shows only an end absorption on its ultraviolet (UV) spectrum and IR bands at 3600—3200 (OH), 1710 (C=O), 1650—1620, 845 (trisubstituted double bond), 1065 (secondary OH), 1010 (primary OH) and 925 cm^{-1} (tertiary OH). The acid (I) afforded methyl ester $C_{31}H_{50}O_5$ (II) of mp 274—275° by methylation with diazomethane and II afforded diacetyl methyl barbinervate $C_{35}H_{54}O_7$ (III) of mp 211—212° by acetylation with acetic anhydride and pyridine, but triacetyl methyl barbinervate was not obtained, suggesting that I has two OH groups which are easily acetylated and a sterically hindered OH group. Methylate (II) shows IR bands at 3530 (OH), 1720 (ester), 1650—1620, 850 (trisubstituted double bond), 1065 (secondary OH), 1005 (primary OH) and 930 cm^{-1} (tertiary OH) and III at 3600—3200 (OH), 1735 (acetate), 1720 (ester), 1650—1620 (trisubstituted double bond) and 930 cm^{-1} (tertiary OH). The mass (MS) spectrum of III (M^+= 586) shows peaks at m/e 308 (A) and at m/e 278 (B). These species would result from the M^+ ion by the retro Diels–Alder fragmentation, characteristic for Δ_{12}-amyrine type triterpenoid. The diacetyl methylate (III) shows also peaks at m/e 248 and at m/e 188, which would result from the species (A) by the loss of a CH_3COOH and two CH_3COOH groups,

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TABLE I. The NMR Data (δ value, J in Hz, 100 MHz)

	$=C_{12}H$	$C_{18}H$	C_3H	$-CH_2-O-$	CO_2Me	OAc $\left(\begin{array}{c}\mathrm{Me}\ \ \mathrm{O-}\\ \times\\ \mathrm{Me}\ \ \mathrm{O-}\end{array}\right)$	CH_3 C_{23}	 C_{24}	 C_{25}	 C_{26}	 C_{27}	 C_{29}	 C_{30}
Methyl euscaphate	5.32 m, 1H	2.58 s, 1H	3.39 d, 1H		3.56 s, 3H		1.00 s, 3H	0.85 s, 3H	0.94 s, 3H	0.66 s, 3H	1.19 s, 3H	1.25 s, 3H	0.94, d, 3H, $J=6$
Diacetyl euscaphic acid	5.31 m, 1H	2.53 s, 1H	4.93 d, 1H			1.95, s, 3H 2.05, s, 3H	0.87 s, 3H	0.98 s, 3H	1.03 s, 3H	0.73 s, 3H	1.19 s, 3H	1.30 s, 3H	0.98, d, 3H, $J=6$
II	5.31 m, 1H	2.58 s, 1H	3.83, t, 1H $J=3$	3.59, AB-q, 2H $J=11$, $\delta_{AB}=19$	3.57 s, 3H		0.89 s, 3H		1.08 s, 3H	0.66 s, 3H	1.21 s, 3H	1.25 s, 3H	0.94, d, 3H, $J=6$
III	5.36 m, 1H	2.61 s, 1H	4.94, t, 1H $J=3$	4.08, AB-q, 2H $J=12$, $\delta_{AB}=22$	3.60 s, 3H	2.04, s, 3H 2.08, s, 3H	0.95[a]		0.93[a]	0.68 s, 3H	1.21 s, 3H	1.31 s, 3H	0.97, —[b]
IV	5.25 m, 1H	2.52 s, 1H	4.22, q, 1H $J=6, 10$	3.62, AB-q, 2H, $J=11$, $\delta_{AB}=17$	3.53 s, 3H	(1.34, s, 3H 1.28, s, 3H)	1.09[c] s, 3H		1.10[c] s, 3H	0.60 s, 3H	1.19 s, 3H	1.21 s, 3H	0.92, d, 3H, $J=6$
Methyl rotundate	5.38 m, 1H	2.62 s, 1H	3.69[d] m	3.56,[d] AB-q, $J=11$, $\delta_{AB}=19$	3.62[d] s			0.88 s	0.94 s	0.68 s, 3H	1.18 s, 3H	1.23 s, 3H	—[b]
Diacetyl methyl rotundate	5.31 m, 1H	2.61 s, 1H	4.74, t, 1H $J=8$	3.87, AB-q, 2H $J=15$, $\delta_{AB}=19$	3.66 s, 3H	2.03, s, 3H 2.08, s, 3H		0.85 s, 3H	0.99 s, 3H	0.70 s, 3H	1.22 s, 3H	1.25 s, 3H	0.96, d, 3H, $J=6$
Methyl rotundate acetonide	5.30 m, 1H	2.56 s, 1H	3.47,[e] m	3.47,[e] AB-q $J=11$, $\delta_{AB}=9$	3.57[e] s	(1.39, s, 3H 1.42, s, 3H)		1.05 s	0.96 s	0.67 s, 3H	1.22 s, 3H	1.25 s, 3H	0.91, —[b]

a) and *c*) The assignments of the signals might have to be reversed.
b) The signal is overlapped with other methyl signal and not identified. Abbreviation: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet.
d) and *e*) The signals are overlapped with each other.

respectively and peaks at m/e 260 and at m/e 218, which would result from the species (B) by the loss of a H_2O and a $HCOOCH_3$ groups, respectively. These spectral evidences suggest that I is trihydroxy-Δ_{12}-triterpene acid of the amyrine type, which has two OH groups on the ring A or/and the ring B, which are easily acetylated, and a OH group on the ring D or the ring E, which resists to the acetylation, and a COOH group on the ring D or the ring E. Methylate (II) afforded unstable acetonide $C_{34}H_{54}O_5$ (IV) of mp 238—240° by treatment with *p*-toluenesulfonic acid and 2,2-dimethoxypropane, suggesting that the two OH groups on the ring A or/and the ring B are in 1,2- or 1,3-diol relationship. The nuclear magnetic resonance (NMR) spectra of II, III and IV could be interpreted as follows: (Table I).

C_{12}-Proton

Methylate (II) exhibits the NMR signal (m, 1H) at 5.31 and III at 5.36, whose δ values are close to those of the signals of the C_{12}-proton of methyl euscaphate[5] at 5.32 and of diacetyl tormentic acid[6] at 5.27, but they are different from that of methyl astilbate[7] at 5.61, suggesting that I has a double bond at the C_{12}-carbon and a COOH group at the C_{17}-carbon.

C_{18}-Proton

Methylate (II) exhibits the NMR signal at 2.58 (s, 1H), III at 2.61 and IV at 2.52, whose δ values are close to those of the signals of the C_{18}-protons of methyl euscaphate at 2.58 and of diacetyl tormentic acid at 2.51, but they are different from those of methyl oleanolate at 2.75 (q, 1H)[8] and of methyl ursolate at 2.2 (d, 1H),[8] suggesting that I, as euscaphic- and tormentic-acids, is α-amyrine type triterpenoid with a methyl group and a OH group at the C_{19}-carbon and that C_{18}-proton is of β configuration.

C_3-Proton

Methylate (II) exhibits the NMR signal at 3.83 (broad, triplet-like, 1H, $J=3$ Hz), and III at 4.94 (the acetylation shift: 1.1 ppm), whose δ value is similar to that (4.96) of the signal of the C_3-proton (β, eq.) of diacetyl lycoclavanin monoacetonide.[9] But it is different from that (4.80) of the signal of the C_3-proton (β, eq.) of the diacetate of lup-20(29)-ene-3α, 23-diol[10] and that (4.74) of the signal of the C_3-proton (α, ax.) of diacetyl methyl rotundate.

Methylene Protons of the CH_2OH Group at the C_4-Carbon

Methylate (II) exhibits the NMR signal at 3.59 and III at 4.08, which could be assigned to methylene protons of a CH_2OH group and of a CH_2OAc group with no proton on the adjacent carbon, respectively, The signal of III at 4.08 is similar to the signal of the methylene protons of the CH_2OAc group (β, ax.) of the diacetate of lycoclavanin monoacetonide[9] at 4.08 (AB-q, $J=12$ Hz, $\delta_{AB}=18$ Hz), but it is different from those (α, eq.) of the diacetate of lup-20(29)-ene-3α, 23-diol[10] at 3.90 and of diacetyl methyl rotundate at 3.87.

These spectral evidences indicate that the primary alcohol group of I is located at the C_4-carbon as β (ax) and the secondary OH group (α, ax) at the C_3-carbon with one adjacent methylene group. The acetonide (IV) exhibits the NMR signal of the C_3-proton at rather low magnetic field (δ 4.22), the δ value of which is close to that (δ 4.40, $J=8.5$ Hz) of lycoclavanol acetonide,[11] the ring A of which is of boat form character, but it bears noreremblance to that (δ 3.59, $J=2$ Hz) of lup-20(29)-ene-3 α, 23-diol acetonide,[10] the ring A of which

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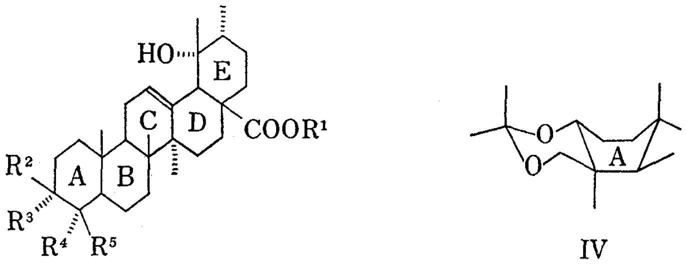
is chair form character. The acetonide (IV) exhibits also the NMR signal of the methylene protons of -CH_2-O-group at 3.62 (AB-q, 2H, $J=11$ Hz, $\delta_{AB}=17$ Hz), whose values are close to those (δ 3.72, AB-q, 2H, $J=10$ Hz, $\delta_{AB}=18$ Hz) of lycoclavanol acetonide,[11] respectively, but the signal is different from that (δ 3.43, AB-q, 2H, $J=12$ Hz, $\delta_{AB}=28$ Hz) of lup-20(29)-ene-3α, 23-diol acetonide[10] and that (δ 3.47) of the acetonide of methyl rotundate. These spectral evidences suggest that barbinervic acid (I) has diaxially arranged 3α (ax.) OH and 4β (ax.) CH_2OH groups, which in the acetonide (IV) must have changed to diequatorial arrangement by converting the ring A into energetically unfavoured boat conformation. As expected, IV was hydrolyzed into I on merely heating the compound in methanol.

Methyl Groups

The compound (II) and (III) exhibit the NMR signals of the methyl groups at δ-values as shown in Table I, each of which is nearyl close to the δ-value of the appropriate methyl group of methyl euscaphate and of diacetyl euscaphic acid,[5] respectively, except the δ-values of the methyl signals at C_{23} and C_{25}-carbons. These spectral and chemical evidences indicate that barbinervic acid (I) could be elucidated as 3α, 19α, 24-trihydroxyurs-12-en-28-oic acid as shown in Chart 1.



IV

	R^1	R^2	R^3	R^4	R^5
I	H	H	OH	CH_3	CH_2OH
II	CH_3	H	OH	CH_3	CH_2OH
III	CH_3	H	OAc	CH_3	CH_2OAc
rotundic acid	H	OH	H	CH_2OH	CH_3

Chart 1

Experimental

Melting point was taken in a Kofler-type hot plate and uncorrected. The IR spectra were taken in KBr pellet with Nippon Bunko IR-G spectrometer, the NMR spectra in $CDCl_3$ with JNM-PS-100 high resolution instrument at 100 MHz with $(CH_3)_4Si$ as internal reference and the MS spectra with JMS-01G mass spectrometer and the optical rotation at 589 nm with Nippon Bunko automatic polarimeter DIP-SL. The thin-layer chromatogram (TLC) was obtained on glass plate coated with silica gel G (Merk).

Isolation——Dried leaves (600 g) were extracted with methanol four times and the extract was concentrated to afford resinous substance (90 g), which was adsorbed on celite (100 g) and the celite was extracted with petr–ether, benzene, ethyl acetate and methanol, successively. The ethyl acetate soluble fraction was concentrated to afford powder (13 g). It was adsorbed on silica gel (65 g) and was chromatographed on silica gel (1 kg) with $CHCl_3$–MeOH (20: 1) The fraction of *Rf* 0.26 (TLC, $CHCl_3$: MeOH=10: 1) afforded colorless prisms (I) of mp 298° from MeOH. Yield: 216 mg. Mass Spectrum (*m/e*, relative intensity): $M^+=488$ (5), 470 (6, M^+-H_2O), 452 (7, M^+-2H_2O), 442 (13, $M^+-HCOOH$), 434 (4, M^+-3H_2O), 264 (31, B), 246 (73, B–H_2O), 224 (57, A), 218 (51, B–HCOOH), 206 (100, A–H_2O), 201 (44), 200 (21, *m/e* 218-H_2O). *Anal.* Calcd. for $C_{30}H_{48}O_5 \cdot H_2O$: C, 71.11; H, 9.95. Found: C, 71.38; H, 9.84. The fraction of *Rf* 0.29 (TLC, $CHCl_3$: MeOH=10: 1) afforded rotundic acid of mp 271° as crystalline solid. Yield: 25 mg. *Anal.* Calcd. for $C_{30}H_{48}O_5 \cdot 1/2H_2O$: C, 72.43; H, 9.86. Found: C, 72.52; H, 9.87. Mass Spectrum: $M^+=488$.

Methylation of I——Barbinervic acid (I) (120 mg) in methanol was methylated with diazomethane to give an oil, which was chromatographed on silica gel with $CHCl_3$–MeOH (50: 1) to afford colorless needles (II) of mp 274—275°. (ethyl acetate). *Rf*: 0.34 (TLC, $CHCl_3$: MeOH=25: 1). *Anal.* Calcd. for $C_{31}H_{50}O_5$: C, 74.06; H, 10.03. Found: C, 73.82; H, 10.13. Yield: 97 mg.

Acetylation of II——A solution of II (60 mg) in a mixture of pyridine (1.5 ml) and acetic anhydride (1.2 ml) was kept at room temperature for 15 hr and the solution was poured into dil. HCl–ice-water to afford colorless powder, which was chromatographed on silica gel with benzene–ethyl acetate (30: 1) to afford colorless needles (III) of mp 211—212° (methanol). *Rf*: 0.25 (TLC, benzene: ethyl acetate=30: 1). *Anal.* Calcd. for $C_{35}H_{54}O_7$: C, 71.64; H, 9.28. Found: C, 71.62; H, 9.42. Yield: 30 mg.

Acetonide of II——Methylate (II) (150 mg), *p*-toluenesulfonic acid (100 mg) and 2,2-dimethoxypropane (7 ml) in acetone (10 ml) was refluxed for 3.5 hr and the reaction mixture was concentrated *in vacuo* to afford an oil, which was chromatographed on silica gel with *n*-hexane–acetone (5: 2). The fraction of *Rf* 0.83 (TLC, *n*-hexane: acetone=5: 2) afforded colorless needles (IV) of mp 238—240° (*n*-hexane). Yield: 15 mg. IR ν_{max} (cm^{-1}): 3600—3200 (OH), 1720 (ester), 1650—1620, 850 (trisubstituted double bond). *Anal.* Calcd. for $C_{34}H_{54}O_5$: C, 75.23; H, 10.03. Found: C, 74.83; H, 9.97.

Methanolysis of IV——Acetonide (IV) (2 mg) in methanol (2 ml) was refluxed on a steam bath for 30 min. The solution showed a spot at 0.15 (TLC, $CHCl_3$: MeOH=25: 1) on a TLC. The *Rf* value was similar to that (*Rf* 0.15, TLC, $CHCl_3$: MeOH=25: 1) of methyl barbinervate (II). The *Rf* value of the acetonide (IV) was 0.98 (TLC, $CHCl_3$: MeOH=25: 1).

Methylation of Rotundic Acid——Rotundic acid (100 mg) in ether was methylated with diazomethane to afford triangular crystals of mp 253—255° (petr.–ether and methanol), which was proved to be identical with an authentic sample of methyl rotundate[4)] by mixed fusion and IR comparison. Yield: 80 mg. *Anal.* Calcd. for $C_{31}H_{50}O_5$: C, 74.06; H, 10.03. Found: C, 73.91; H, 10.29. IR ν_{max} (cm^{-1}): 3400 (OH), 1720 (ester), 1630, 820, 800 (trisubstituted double bond), 1230, 1145, 1040 (secondary OH), 1010 (primary OH), 930 (tertiary OH).

Acetylation of Methyl Rotundate——The methylate (28 mg) in a mixture of pyridine (0.5 ml) and acetic anhydride (0.5 ml) was kept at room temperature for 18 hr and the mixture was treated as usual to afford colorless needles of diacetyl methyl rotundate of mp 208—210° (petr.–ether and ether). (lit.[4a)] mp 105°) Yield: 15 mg. *Anal.* Calcd. for $C_{35}H_{54}O_7$: C, 71.64; H, 9.28. Found: C, 71.35; H, 9.58. IR ν_{max} (cm^{-1}): 3450 (OH), 1735, 1250, 1240 (OAc), 1720 (ester), 1640, 800 (trisubstituted double bond), 930 (tertiary OH).

Acetonide of Methyl Rotundate——A mixture of methyl rotundate (100 mg), *p*-toluenesulfonic acid (10 mg) and acetone (10 ml) was refluxed for 1.5 hr. The reaction mixture was concentrated *in vacuo* to afford an oil, which was chromatographed on silica gel with *n*-hexane–acetone (10: 1). The fraction of *Rf* 0.49 (TLC, *n*-hexane: acetone=10: 1) was crystallized from *n*-hexane to afford acetonide of mp 219—220° as colorless prisms. (lit.[4a)] mp 94°). Yield. 40 mg. *Anal.* Calcd. for $C_{34}H_{54}O_5$: C, 75.23; H, 10.03. Found: C, 75.23; H, 10.13. IR ν_{max} (cm^{-1}): 3400 (OH), 1725 (ester), 1645—1620, 820, 800 (trisubstituted double bond), 930 (tertiary OH).

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