



SESQUITERPENOIDS FROM THE THREE JAPANESE LIVERWORTS *LEJEUNEA AQUATICA*, *L. FLAVA* AND *L. JAPONICA*

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Key Word Index—*Lejeunea aquatica*; *L. flava*; *L. japonica*; Lejeuneaceae; Hepaticae, aquaticenol, cuparane- and barbatane-type sesquiterpenoids; cuparane-type dimer; chemosystematics.

Abstract—Two new cuparane-type sesquiterpenoids, aquaticenol and 1,4-cuparenediol have been isolated from the liverwort *Lejeunea aquatica*, along with 1,2-cuparenediol, 2-cuparenol, cuparene-1,4-quinone, 1-cuparenol, deoxyhelicobasidin and β -barbatene. The cuparane-type dimer, aquaticenol was synthesized by the dimerization of 1,2-cuparenediol. Cuparene-1,4-quinone and 1,2-cuparenediol were isolated from *L. flava*. The latter compound, 1,4-cuparenediol and (–)-cuparene were also isolated from *L. japonica*. These three *Lejeunea* species are chemically closely related. © 1997 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Liverworts are rich sources of terpenoids and aromatic compounds which show interesting biological activities such as muscle relaxation, anti-microbial, antifungal, 5-lipoxygenase, cyclooxygenase and calmodulin inhibitory and anti-HIV activities [1–4]. The Lejeuneaceae are the largest family of the Hepaticae [5]. Except for a few species, most of the Lejeuneaceae liverworts are morphologically very small and their identification and classification are very difficult even by specialists. In order to clarify the taxonomical position of each subfamily belonging to the Lejeuneaceae, we have investigated the chemical constituents of three Japanese *Lejeunea* species, *L. aquatica*, *L. flava* and *L. japonica*. *L. aquatica* grows on wet rocks or rocks in rivers. It is distributed in southern Japan and Taiwan and contains ca 10–25 oil bodies per cell. *L. flava* grows on trunks or twigs of evergreen plants and has 3–15 oil bodies per cell. *L. japonica* is found on rocks, trunks or stone walls. *L. aquatica* and *L. japonica* develop a bluish-violet colour in the course of a few weeks storage. This is a very characteristic phenomenon of only two species belonging to the Lejeuneaceae so far examined. Here we report the isolation and structure elucidation of three new sesquiterpenoids and the chemosystematics of each species.

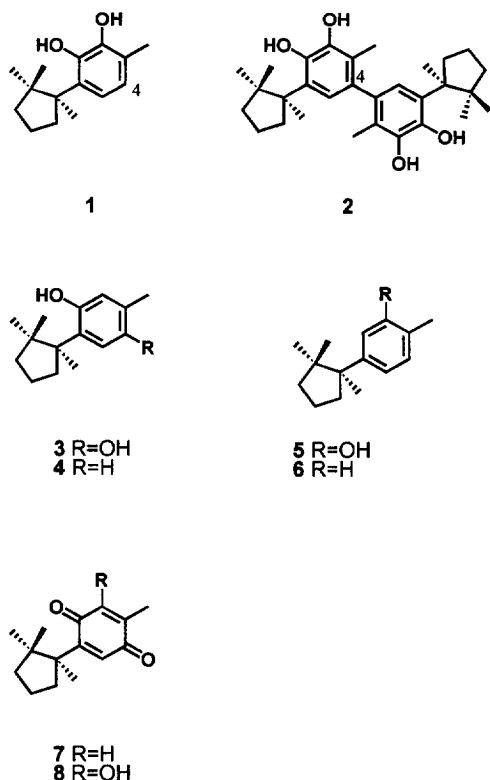
RESULTS AND DISCUSSION

The GC mass spectral analysis of the ether extract of *L. aquatica* detected 1,2-cuparenediol (**1**) as the

major constituent of this species. The extract was directly chromatographed on Sephadex LH-20 and silica gel successively and then fractions obtained were purified by HPLC to give two new sesquiterpenoids, aquaticenol (**2**) and 1,4-cuparenediol (**3**), along with seven known sesquiterpenoids, 1,2-cuparenediol (**1**) [6], 1-cuparenol (**4**), 2-cuparenol (**5**) [6], cuparene-1,4-quinone (**7**) [7], deoxyhelicobasidin (**8**) [8], 15-hydroxybarbatane (**9**) [9] and β -barbatene (**10**) [10]. The ether extract of *L. flava* was also chromatographed on Sephadex-LH 20 to give 1,2-cuparenediol (**1**) and cuparene-1,4-quinone (**7**). Treatment of the ether extract of *L. japonica* in the same manner gave two cuparenediols (**1**, **3**) and (S)-(–)-cuparene (**6**) [1].

Previously, compound **1** has been isolated from the liverwort, *Radula perrottetii* [7] but its absolute stereochemistry has not been discussed. Enzell *et al.* [11] reported the isolation of (R)-(+)-cuparene, the enantiomer of **6**, from higher plants and determined its absolute configuration by the formation of (+)-camphanic acid ($[\alpha]_D^{21} + 21^\circ$) by ozonolysis of (+)-cuparene. On the other hand, Matsuo *et al.* [12] also applied the method to confirm the absolute configuration of (–)-cuparene (**6**) isolated from the liverwort *Bazzania pompeana*, then obtained (–)-camphanic acid ($[\alpha]_D^{13} - 13^\circ$). However, the value of the specific rotation is different between (+)- and (–)-camphanic acid obtained from both (R)-(+)- and (S)-(–)-cuparenes. In order to clarify the absolute configuration of 1,2-cuparenediol (**1**) and the value of the optical rotation of camphanic acid, (S)-(–)-cuparene (**6**) which was isolated from the thalloid liverwort *Marchantia polymorpha* in good yield and **1**

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were ozonized to give camphononic acid, whose specific rotation was -12.8° from (*S*)-(-)-cuparene and -12.0° from **1**, respectively. Thus the absolute configuration of **1** was determined to be (*S*)-(-)-1,2-cuparenediol.

The structure of **2** was deduced by comparing its spectral data with those of the previously known compound **1**. While the ^1H NMR spectrum of **2** resembled those of (*S*)-(-)-1,2-cuparenediol (**1**), all the signals appeared as pairs, particularly as a 3:2 ratio for four methyl groups, as shown in Fig. 1. In the ^1H NMR spectrum of **2** in CDCl_3 , a singlet signal at δ 6.68 (and δ 6.65) was observed in place of a doublet signal at δ 6.58 (*d*, $J = 8$ Hz) and δ 6.79 (*d*, $J = 8$ Hz) in the ^1H NMR spectrum of **1**.

Temperature-dependent ^1H NMR spectra of **2** are shown in Fig. 1. When the ^1H NMR spectrum was measured in $\text{DMSO}-d_6$ at 130° , the pair signals disappeared and the equivalent signals were observed. Clearly, a conformational equilibrium between a major form and a minor form exists with the ratio 3:2. Taking account of this phenomenon, it suggested that compound **2** was a dimeric 1,2-cuparenediol and composed the mixture of conformational isomers. Furthermore, the EI-mass spectrum of **2** showed a molecular ion and base peak at m/z 466 which was a duplex M_r of **1**. The molecular ion peak at m/z 466.3091 was observed by HR-mass spectral analysis of **2**. Hence, the molecular formula was found to be $\text{C}_{30}\text{H}_{42}\text{O}_4$ (M_r , 466.3083). Attempts to clarify the positioning of linkage by an X-ray crystallographic analysis on crystals of **2** obtained from an ether solution

was thwarted by the inclusion of the solvent in the crystals. The final R value did not decrease to less than 0.125, although the linkage between C-4 and C-4' was partially solved. The above feature, and the phenolic linkage, were confirmed by the ^1H and ^{13}C NMR spectra (Table 1) and extensive 2D NMR experiments. In particular analysis of the HMQC and HMBC spectra supported the structural assignment. Correlation between H-15 and C-2 at δ 142.5 (δ 142.3) and C-3 at δ 131.7 (δ 132.1) was observed. In particular, the long range ^1H - ^{13}C correlation of H-15 with a quaternary sp^2 carbon at δ 121.6 (δ 121.5) in $\text{DMSO}-d_6$, supports the positioning of the biphenyl linkage at C-4 (C-4'). From this experiment, the structure of **2** was established to be a 1,2-cuparenediol dimer coupled with a phenyl bond between C-4 and C-4'.

In order to determine the absolute configuration of **2**, the partial synthesis of **2** was carried out. According to Sartori's method [13], compound **2** was treated with aluminium chloride in nitromethane and then with DDQ to give a dimeric compound whose spectral data and specific rotation ($[\alpha]_D -23.8^\circ$) were in good agreement with those of natural **2** ($[\alpha]_D -23.4^\circ$). Thus the absolute structure of **2** was determined to be (*7S,7'S*)-(-)-4,4'-bis-1,2-cuparenediol. Steric hindrance of a 1,1-dimethyl cyclopentane ring in the molecule of **2** may contribute to lowering the production of 5,5'-and/or 5,4'-bis-1,2-cuparenediol by the radical coupling.

Two conformational isomers (3:2), which were confirmed by the intensity of the signals of four methyl groups in the ^1H NMR spectrum at 30°C (Fig. 1) are present in **2** as mentioned above. The isomeric ratio of (*S*)-(-)- and (*R*)-(+)-aquaticeanol in structure of **2** was confirmed by the CD spectrum of **2** which showed a positive Cotton effect at 235 nm and the negative Cotton effect at 270 nm. This indicated that an excess of (*S*)-(-)-aquaticeanol existed in the mixture.

The structure of **3** was deduced by comparing its spectral data with those of previously known compound **7**. The presence of a hydroxyl group in **3** was apparent from the absorption band at 3400 cm^{-1} in the IR spectrum. The ^1H NMR spectrum of **3**, $\text{C}_{15}\text{H}_{22}\text{O}_2$ (HR-EIMS), contained signals due to four tertiary methyl groups and two singlet signals of an aromatic proton. This indicated that **3** contained a 1,3,4,6-tetrasubstituted benzene ring. When **3** was allowed to stand at room temperature, it was easily converted to a quinone whose spectral and specific optical rotation were identical to those of the previously known cuparene 1,4-quinone (**7**) [7]. Accordingly, the structure of **3** was elucidated as 1,4-cuparenediol.

Very similar herbertane-type (=isocuparene-type) sesquiterpene dimers, mastigophorenes A–D have been isolated from the liverwort *Mastigophora diclados*. These unique dimers show neurite sprouting and network formation in the primary neurite cell culture derived from the fetal rat hemisphere [14]. We

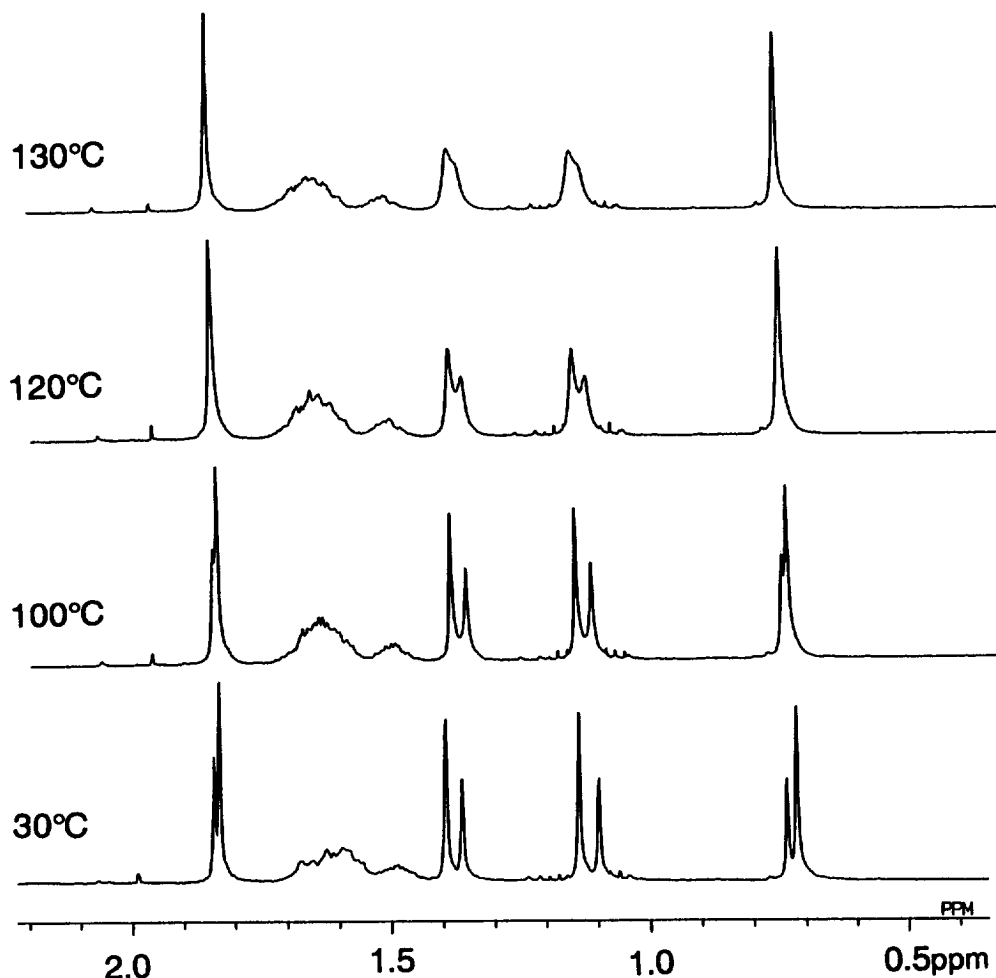


Fig. 1. 400 MHz ^1H NMR spectra of aquaticenol (**2**) at various temperatures.

are testing the biological activity of **2** to determine if it shows the same activity.

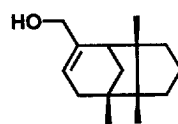
The occurrence of dimeric sesquiterpenoids in liverworts is very rare. Except for the above mastigophorenes, only eudesmane- and aromadendrane-type dimers have been found in *Frullania* [15], *Mylia* [16] and *Plagiochila* species [17, 18]. These three *Lejeunea* species are closely related chemically, because they contain basically *ent*-cuparene-type sesquiterpenoids as the major component. The GC-mass spectra analysis of the ether extract of the present three species, which were collected in various locations, showed the distribution of sesquiterpenoids presented in Table 2. The cuparene-type sesquiterpenoids are rare in the other genera of Lejeuneaceae, species of which produce pinguisane-, striatane-, labdane-type terpenoids and simple aromatic compounds [1]. Thus cuparanes are suggested to be very significant chemical markers for the subfamily Lejeuneoideae, although only three of 12 species belonging to this subfamily have been investigated. The cuparene dimer **2** and barbatane

(= gymnomitrane) (**9**) isolated from *L. aquatica* were not isolated from *L. flava* and *L. japonica*.

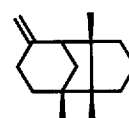
EXPERIMENTAL

General. TLC was carried out on silica gel precoated glass plates with *n*-hexane–EtOAc (1:1 and 4:1). Detection was with Godin reagent [19]. For normal phase column chromatography (CC), silica gel 60 (40–63 μm) was used. CH_2Cl_2 –MeOH (1:1) was used for CC on Sephadex LH-20 as solvent.

Spectral data. NMR spectra were recorded at 100 or 50 MHz for ^{13}C and 400 or 200 MHz for ^1H . EIMS



9



10

Table 1. ^1H NMR and ^{13}C NMR data for aquaticenol (**2**) in $\text{DMSO}-d_6$

	^{13}C	^1H
1	144.1	
2	142.5 (142.3)	
3	131.7 (132.1)	
4	121.6 (121.5)	
5	121.7 (121.8)	6.45 (6.44) (1H, s)
6	129.2 (129.5)	
7	50.49 (50.52)	
8	38.7	1.60 and 2.50 (each 1H, m)
9	20.0 (20.2)	1.67 (2H, m)
10	40.7 (40.6)	1.49 and 1.59 (each 1H, m)
11	44.40 (44.44)	
12	27.0 (26.7)	0.72 (0.74) (3H, s)
13	25.6 (25.4)	1.14 (1.10) (3H, s)
14	22.7 (23.2)	1.40 (1.36) (3H, s)
15	13.5 (13.7)	1.83 (1.84) (3H, s)

Measured at 600 MHz for ^1H and 150 MHz for ^{13}C .

The chemical shifts of a minor conformational isomer of aquaticenol (**2**) are shown in parentheses.

were measured at 70 eV. The temp. programming of GC-MS analysis performed from 50° isothermal for 3 min, then 50 – 250° at 5° min^{-1} , and finally isothermal at 250° for 15 min. Injection temp was 250° . A fused silica column coated with DB-17 (30 m $\times$ 0.25 mm i.d., film thickness 0.25 μm) was used.

Plant materials. *L. aquatica* (#95152), *L. flava* (#95098) and *L. japonica* (#95060) were collected in Inouchi, Kito-son, Naka-gun, Tokushima in July 1995, Onakazura, Kamikatsu-cho, Katsuura-gun, Tokushima, in March 1995 and Mamiya, Katsuura-cho, Tokushima, Feb. 1995, respectively. The voucher specimens were deposited with the Faculty of Pharmaceutical Sciences, TBU.

Extraction and isolation. *L. aquatica* was dried for 3 days and ground mechanically. The powder (70 g) was extracted with Et_2O for 7 days. Evapn of the solvent gave a green oil (4.73 g) which was chromatographed on Sephadex LH 20 to divide into 3 frs

(A–C). Fr. A (1.10 g) was further chromatographed on silica gel using *n*-hexane–EtOAc gradient to give **7** (772 mg) and a mixt. containing **8** and **9**. The mixt. was rechromatographed on Sephadex LH 20 and purified by HPLC using *n*-hexane–EtOAc (4:1) to give **8** (6 mg) and **9** (12 mg). Fr. B (1.20 g) was chromatographed on silica gel using *n*-hexane–EtOAc gradient afforded **1** (260 mg), **5** (61 mg), **7** (61.7 mg) and to divide 3 frs (B-1 ~ 3). Fr. B-1 (77.9 mg) was further purified by HPLC using *n*-hexane to give **10** (50.2 mg). Fr. D-2 (350 mg) was purified by HPLC using *n*-hexane–EtOAc (19:1), affording **7** (59 mg), **4** (33.6 mg) and **1** (260 mg). Fr. B-3 (192 mg) was rechromatographed on Sephadex LH-20 to give **2** (120 mg). Fr. C (1.60 g) was further chromatographed on silica gel using *n*-hexane–EtOAc gradient to divide into 3 frs (C-1 ~ 3). Fr. C-1 (1.00 g) was further purified by HPLC using *n*-hexane–EtOAc (4:1) afforded **1** (800 mg) and **5** (20 mg). Fr. C-2 (60 mg) was purified by HPLC to give **7** (5 mg), **3** (119.6 mg) and **9** (2 mg). Fr. C-3 (500 mg) was rechromatographed on silica gel using *n*-hexane–EtOAc gradient and Sephadex LH-20 to yield **2** (294 mg).

L. flava. (dry wt 3.1 g) was ground mechanically and extracted with Et_2O for 7 days and filtered to give a green oil (73 mg) which was chromatographed on Sephadex LH-20 to give **1** (5 mg) and **7** (13 mg).

L. japonica. (dry wt 450 mg) was treated in the same manner described above to give the crude extract (33.8 mg) which was divided into two frs by CC using Sephadex LH-20. Both frs were purified by HPLC to give **6** (3 mg) from the first fr. and **1** (11 mg) and **3** (5 mg) from the latter fr.

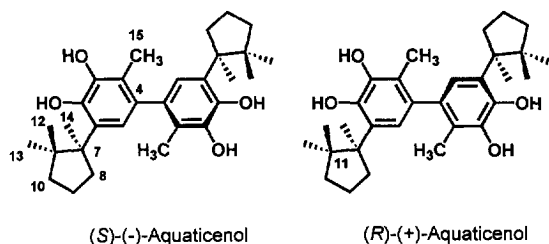
Aquaticenol (2): mp 145 – 146° ; $[\alpha]_D -23.4^\circ$ (CHCl_3 ; c 0.47); HRMS: 466.3091 $[\text{M}]^+$ Calcd for $\text{C}_{30}\text{H}_{42}\text{O}_4$, 466.3083. IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 3400–3500 (br), 2980, 1600. UV λ_{max} nm (log ϵ): 209 (4.52), 250 (3.70), 278 (3.41). ^1H NMR (600 MHz; CDCl_3): δ 0.77 (0.81) (3H, s), 1.18 (1.14) (3H, s), 1.47 (1.43) (3H, s), 1.93 (1.95) (3H, s), 1.55 (1H, m), 1.60–1.75 (4H, m), 2.58 (1H, m), 5.17 (5.15) (1H, br s), 5.60 (5.59) (1H, s), 6.68 (6.65) (1H, s); ^1H NMR ($\text{DMSO}-d_6$): Table 1; ^{13}C NMR ($\text{DMSO}-$

Table 2. The distribution of sesquiterpenoids in three species of *Lejeunea*

Liverwort	1	3	5	6	7	9	10	Collection locations
<i>L. aquatica</i> (95152)*	38.4%†	12.0%	16.4%	1.7%	5.4%	1.5%	9.1%	Kito-son, Tokushima
(96051)*	30.4	4.3	35.0	4.5	7.7		8.0	Izuhara, Nagasaki
<i>L. japonica</i> (95060)	36	15.8	19.4	2	4.2		2.2	Katsuura, Tokushima
(95062)	25.9	5	19.3		25.4		5.4	Katsuura, Tokushima
(95090)	41	3.8	13.9		22		2	Kamikatsu, Tokushima
(95092)	45	6.7	10.8		22.6			Kamikatsu, Tokushima
(95093)	30.4	14.7	15.7		25			Kamikatsu, Tokushima
<i>L. flava</i> (95098)	29.9	3.9	10.4		25.3			Kamikatsu, Tokushima

* Aquaticenol (**2**) was not detected by GC-MS analysis of the ether extracts.

† The proportion (%) of each compound was determined from total abundance value on the total ion chromatograph.



d_6): Table 1. EIMS m/z (rel. int.): 466 ($[M]^+$ 100), 396 (7), 383 (22), 369 (7), 313 (5), 273 (5), 163 (4), 111 (4), 83 (3), 61 (11).

X-ray crystallographic analysis of aquaticeanol (2). Orthorhombic space group $P2_12_12_1$, $a = 18.175(3)$, $b = 28.675(6)$, $c = 7.022(2)$ Å; $V = 3659(1)$ Å³ Z value = 4, $D_c = 1.11$ g cm⁻³, $\mu_{Cu} = 5.06$ cm⁻¹. Crystal size $0.5 \times 0.5 \times 0.2$ mm. Reflection data were measured with a Mac Science MXC 18 diffractometer using copper radiation Cu K α ($\lambda = 1.54178$). Final residuals R and R_w were 0.125, 0.147.

1,4-Cuparenediol (3): Oil; $[\alpha]_D + 171.6^\circ$ (CHCl₃; c 0.67); HRMS: m/z 234.1618 $[M]^+$ Calcd for C₁₅H₂₂O₂: 234.1620. IR ν_{max}^{KBr} cm⁻¹: 3400. UV λ_{max} nm (log ϵ): 204 (4.08), 295 (3.33). ¹H NMR (400 MHz; CDCl₃): δ 0.76, 1.16, 1.38 and 2.16 (each 3H, *s*, H-12, H-13, H-14 and H-15), 1.57 and 1.68 (each 1H, *m*, H₂-10), 1.74 (3H, *m*, H-8 and H₂-9), 2.52 (1H, *m*, H-8), 6.46 and 6.74 (each 1H, *s*, H-2 and H-5) ¹³C NMR (50 MHz; CDCl₃): δ 15.1, 20.2, 22.8, 25.4, 26.9, 39.3, 41.1, 44.6, 50.8, 116.2, 119.1, 121.9, 132.0, 146.8, 146.1 EIMS: m/z (rel. int.): 234 $[M]^+$ (99), 178 (13) 177 (25), 165 (16), 164 (70), 163 (31), 152 (35), 151 (100), 150 (12), 149 (13), 137 (22), 124 (8), 95 (9), 69 (8), 41 (8).

Ozonolysis of (S)-(-)-cuparene (6). Compound **6** was isolated from the liverwort *Marchantia polymorpha*. O₃ was bubbled into compound **6** (50 mg) in MeOH (2 ml) at room temp. overnight. To the reaction mixt. was added 5% NaOH (1 ml) and 30% H₂O₂ (1 ml), and the mixt. was refluxed for 1 hr. The resulting mixt. was neutralized with 5% HCl and extracted with Et₂O. Evapn of the solvent left a residue which was chromatographed on silica gel using an *n*-hexane–EtOAc gradient to give (S)-(-)-camphononic acid (32.6 mg). (S)-(-)-Camphononic acid; oil $[\alpha]_D - 12.8^\circ$ (CHCl₃; c 1.63) {ref. [10] + 21° (CHCl₃; c 1.30)} {ref. [11] - 13.2° (CHCl₃; c 0.4)}. ¹H NMR (200 MHz; CDCl₃): δ 0.96, 1.05, 1.18 (each 3H, *s*), 1.50 (1H, *m*), 1.60–1.80 (4H, *m*), 2.40 (1H, *m*). ¹³C NMR (50 MHz; CDCl₃): δ 19.8, 20.7, 23.9, 25.5, 34.6, 39.6, 44.2, 54.6, 184.1.

Ozonolysis of 1,2-cuparenediol (1). Compound **1** (40 mg) was treated in the same manner as described above to give (S)-(-)-camphononic acid (13 mg), whose spectral data and specific rotation were identical to those of (S)-(-)-camphononic acid derived from (S)-(-)-cuparene (**6**). (S)-(-)-Camphononic acid; $[\alpha]_D - 12.8^\circ$ (CHCl₃; c 0.6).

Partial synthesis of 2. To compound **1** (80 mg) in

MeNO₂ (5 ml) was added AlCl₃ (45 mg) and the mixt. stirred for 15 min at room temp. under N₂. Then 2,3-dichloro-5,6-dicyano-*p*-benzoquinone (DDQ) (450 mg) in MeNO₂ (10 ml) was added and the reaction mixt. was stirred for 1 hr. The resulting mixt. was filtered through a short pad column of celite and the residue, after removal of the solvent, was chromatographed on silica gel using an *n*-hexane–EtOAc gradient to give **2** (53 mg). The spectral data and specific rotation ($[\alpha]_D - 23.8^\circ$) were in good accordance with those of natural aquaticeanol (**2**).

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