Inhibitory Effects and Active Constituents of *Alisma* Rhizomes on Vascular Contraction Induced by High Concentration of KCl

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The acetone extract of *Alisma* rhizomes were found to possess inhibitory effects on vascular contraction induced by high concentration of KCl. With the aids of bioassay, the effective components were pursued by purification with chromatographies, resulting in the characterization of six protostane type triterpenes (alisol C and its analogues) and a known sesquiterpene, alismoxide as the active compounds.

The rhizomes of *Alisma plantago-aquatia* L. var. *orientale* Samuelsson (Alismataceae) are widely utilized as a Chinese folk medicine[1] and their chemical components have been searched from medicinal viewpoint, resulting in the isolation of a series of protostane triterpenes, alisols A and B and monoacetates of alisols A, B and C.[2] These triterpenes were reported to have lipotropic, diuretic and cholesterol suppression activities.[2,3] Yamahara's and Hikino's groups have independently reported the isolation of sesquiterpenes, alismol and alismoxide[4] from the same species. The alismol showed the inhibition activity toward high KCl-induced vascular contractions and caused an increase of $^{45}Ca^{2+}$ retention induced by 50 mM KCl.[5] We have continued to explore the rest of the active constituents of the acetone extract toward the vascular contraction inhibition.

Results and Discussion

By the aids of bioassay,[6] the active compounds **1**—**7** were isolated from the acetone extract of *Alisma* rhizomes. The separation was achieved by the application of solvent participation followed by column and high performance liquid chromatographies. The compound **1** has a molecular formula of $C_{32}H_{48}O_6$. The complete structure of **1** was established by X-ray analysis as depicted in Fig. 1. It reveals that **1** has a *trans-cisoid-trans* configuration of A/B/C ring, existing as unusual boat/boat conformation of A and B rings in the solid state. The structure is fully in accord with the ^1H and ^{13}C NMR spectra. Thus, **1** is found to be identical with the known alisol C monoacetate.[2a] Treatment of **1** with sodium hydrogencarbonate in methanol gave the deacetyl derivative which was identical with compound **2**. Reaction of **1** with potassium hydroxide in methanol afforded the deacetylated and dehydrated derivative **8**. In addition to the molecular ion at m/z 528, the mass spectrum of **1** shows at m/z 353 caused by dehydration of 11-hydroxyl group and bond fission between C-20 and 22 position.

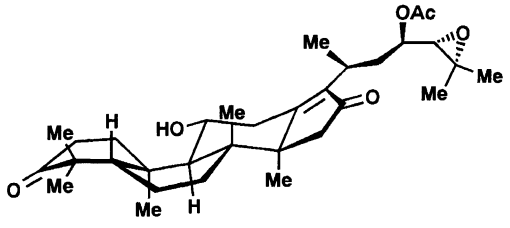

Fig. 1. Molecular structure of alisol C monoacetate (**1**).

In Table 1 are summarized the molecular formulas, mass fragment ions and UV absorption maxima of the isolated triterpenes **1**—**6** (Fig. 2). The fragment ion at m/z 353 was also detected in the mass spectra of all the isolated compounds **1**—**5** excepting **6**. The absorption maximum at 245 nm due to the conjugated enone moiety was observed in the UV spectra of **1**, **2**, **4**, and **5** (Table 1). This evidence indicates that **4** and **5** have the same carbon skeleton as compound **1**, differing the side chain structure. The dehydrated derivative **8** shows the UV absorption maximum at 287 nm. Since compound **3** has the UV absorption maximum at the same position, **3** may possess the conjugated dienone chromophore. This deduction was supported by the ^1H NMR spectra of **3** and **8**, exhibiting the 11 and 12 protons at $\delta=6.75$ (dd, $J=4$ and 10 Hz) and $\delta=6.15$ (dd, $J=2.5$ and 10 Hz), respectively. Since UV spectrum of **6** shows no absorption maximum at 246 nm due to the conjugated enone group, **6** was deduced to be 16-deoxo derivative of compound **2**. This deduction was supported by ^{13}C NMR spectrum which shows no carbonyl signal of 16-carbon as summarized in Table 2. Compound **6** afforded the corresponding diacetate **9** by the action of acetic anhydride. Physical evidence of **6** and **9** suggests that **6** is the known alisol B.[2a]

The detailed analyses of ^1H NMR spectra including H–H COSY experiments and ^{13}C NMR signals in Table 2 lead to the structure of the side chain. The absolute configuration of the triterpenes **1**, **2**, and **6** are deduced from those of alisols B and C, which have been established to possess 20*R*, 23*S*, 24*R* configurations by heavy atom method of X-ray crystallographic analysis and their chemical correlation reactions.[2a] The 23*S* and 24*R* configurations of **3** and **5** were infered from those of alisol A which corresponds to 16-deoxo derivative of compound **5**. Compound **3** might be derived from **5** by

Table 1. Molecular Formula, Fragment Ions in Mass Spectra and Absorption Maxima in UV Spectra of Triterpenes **1**—**6**

Compounds	Mol. Formula	Fragment Ions (m/z)	UV max nm ($\log\varepsilon$)
1	$C_{32}H_{48}O_6$	528(M), 510(M−H_2O), 353	245(4.23)
2	$C_{30}H_{46}O_5$	486(M), 468(M−H_2O), 353	246(4.07)
3	$C_{30}H_{46}O_5$	486(M), 468(M−H_2O), 354	287(4.13)
4	$C_{30}H_{48}O_5$	488(M), 470(M−H_2O), 353	246(3.90)
5	$C_{30}H_{48}O_6$	504(M), 486(M−H_2O), 353	246(4.10)
6	$C_{30}H_{48}O_4$	472(M), 456(M−H_2O), 339	—

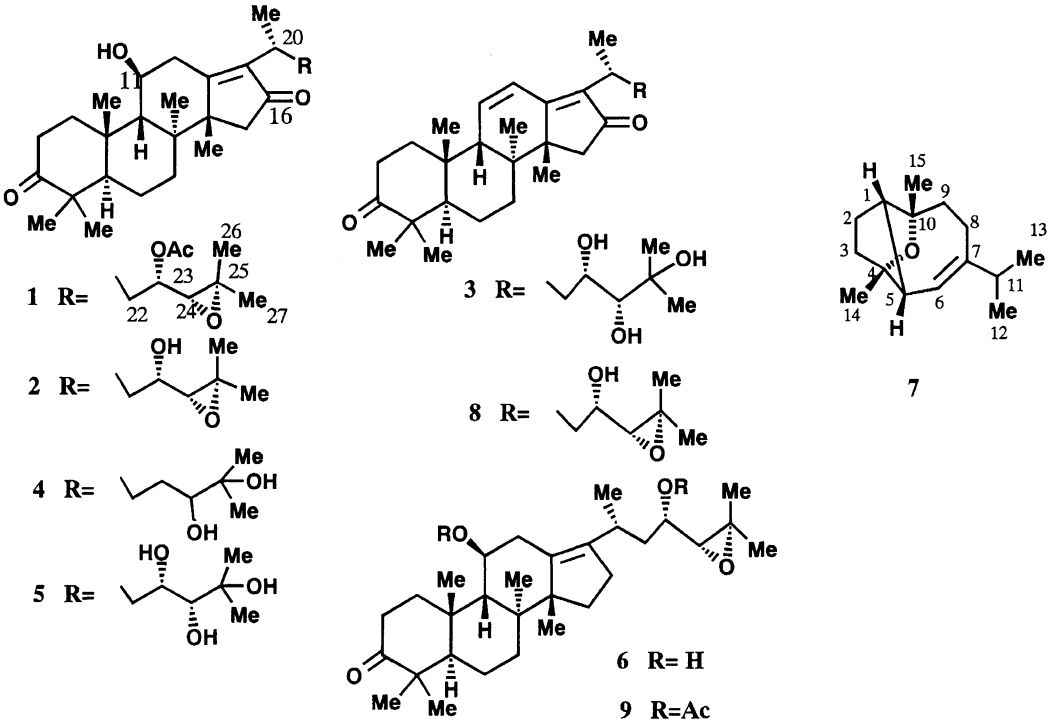

Fig. 2. Structure of triterpenes and alismoxide.

Table 2. ^{13}C NMR Spectra of Triterpenes **1**—**6**[a)]

	Compounds	**1**	**2**	**3**	**4**	**5**	**6**
Carbons	11	70.0(d)	69.8(d)	138.6(d)	69.4(d)	69.8(d)	70.1(d)
	12	NA	NA	122.4(d)	NA	NA	NA
	16	208.6(s)	208.8(s)	208.9(s)	209.7(s)	210.2(s)	NA
	23	72.1(d)	69.7(d)	69.3(d)	42.8(t)	69.5(d)	69.3(d)
	24	65.1(d)	67.9(d)	77.5(d)	77.4(d)	77.5(d)	68.1(d)
	25	58.8(s)	59.4(s)	73.7(s)	73.7(s)	74.1(s)	59.4(s)

a) NA means chemical shifts were not assignable.

dehydration of 11-hydroxyl group. The stereochemistry at 24 position of **4** remains beyond conjecture.

Compound **7** has a molecular formula of $C_{15}H_{24}O$. IR spectrum indicates the absence of hydroxyl and carbonyl groups. Detailed analysis of ^1H and ^{13}C NMR spectra indicates that **7** is identical with the known alismoxide.[4b)]

The inhibitory activity of vascular contraction of **1**—**7** was summarized in Table 3. All the compounds show weak but clear activity at relatively high concentration.[2b)] Comparison of the activities in Tables 3 and 4 indicates that the activities of pure components **1**—**7** are less than those of the corresponding crude fractions, from which pure components were isolated. As generally accepted in the field of folk medicine,[7)] this evidence suggests that the activity increases when individual component having weaker activity is mixed by each other.

Thus, the present study led to the characterization of six triterpenoids **1**—**6** and the known sesquiterpene, alismoxide **7**. Among the triterpenes, **1** and **2** were identical with the reported alisol C monoacetate and

Table 3. Effects of Compounds **1**—**7** on the Vascular Contraction[a)]

	Concentration	Relative vascular contraction (%)	
		10^{-4}M	10^{-5}M
Compounds	**1**	69.4±5.2[b)]	73.1±9.0[c)]
	2	90.5±2.4[b)]	—
	3	50.0±2.3	99.9±5.3
	4	43.2±4.3	99.4±9.4
	5	79.4±3.3	100.1±4.7
	6	10.9±0.6	45.5±2.0
	7	48.8±9.4	85.3±8.7

a) The vascular contraction was examined in the presence of each compound **1**—**7** at the concentration of 10^{-4} and 10^{-5} M in 0.1% DMSO solution excepting b) and c). The vascular contraction without any compound (control experiment) was expressed as 100%. b) Concentration at 10 $\mu g\,ml^{-1}$; c) Concentration at 20 $\mu g\,ml^{-1}$.

Table 4. The Relative Vascular Contraction (%) in the Presence of Each Fraction[a)]

Fraction	Contraction (%)	Fraction	Contraction (%)
Benzene insoluble	66.3±2.5	Ether insoluble	85.3±1.8
Benzene soluble	68.2±4.9	Ether soluble	47.3±3.1
Acidic[b)]	80.4±6.6	Acidic[c)]	68.3±3.0
Neutral[b)]	55.9±3.0	Neutral[c)]	26.7±3.0

a) Concentration of each fraction was 10 $\mu g\,ml^{-1}$ 0.1% DMSO solution; Vascular contraction without any fraction was expressed as 100% (control experiment). b) Obtained from benzene soluble fraction. c) Obtained from ether soluble fraction.

alisol C while **6** corresponds to alisol B.[8)] All of the isolated compounds show weak but clear inhibitory effects on vascular contraction induced by high concentration of KCl.

Experimental

Inhibitory Effects Assay on Vascular Contraction Induced by KCl. Male wistar rats weighing about 250 g were bled to death by severing both carotid arteries and the thoracic aorta (TA) were removed and cut into helical strips (2 mm×15—20 mm). Physiological salt solution contained (mM, 1 M=1 mol dm^{-3}): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25.0, $MgSO_4$ 1.2, glucose 10.0. It was aerated with a 95% O_2–5% CO_2 gas mixture and kept at 37 °C and pH 7.4. To investigate the mechanical response, each preparation was suspended in an organ bath (25 ml) and subjected to an initial load of about 1 g. A 1.5 h equilibration period was allowed before initial of the experiment. During this period, the solutions were replaced every 30 min. Contractions were recorded isometrically via a force-displacement transducer (Nihon Denki Sanei, Tokyo, Japan). Statistical analysis was performed by Student-t method. Results are expressed or plotted as the mean±s.e.

The maximum contractions induced by KCl (50 mM) in the preparation in a bathing medium were taken as 100% contractile force. The tissues were then washed 3 times with physiological salt solution at 10 min intervals and pretreated with each test samples for 10 min, then KCl (50 mM) was added to the bathing medium. The test samples were dissolved in a small amount of dimethyl sulfoxide (DMSO) and then added to the bath. The final concentration of DMSO in the bath did not exceed 0.1% and had no effect on muscle contraction.

Isolation of Active Constituents. Acetone extracts (32 g) of *Alisma* rhizomes were roughly devided into benzene soluble and insoluble parts by stirring the extracts with 800 ml of benzene three times at room temperature. The benzene insoluble portion was then stirred with ether (300 ml×3) to obtain ether soluble (1.1 g) and insoluble (1.2 g) parts. The benzene and ether soluble portions were independently shaken with 0.1 M KOH solution to obtain neutral (28 g) and acidic (1.5 g) parts from benzene portion and neutral (610 mg) and acidic (320 mg) parts from ether portions, respectively. The neutral part of benzene soluble portion (28 g) was then stirred with 200 ml of hexane three times to devide into hexane soluble (7.5 g) and insoluble (benzene soluble) (20 g) parts. All of the above parts were assayed toward inhibitory effect on vascular contraction and the results were summarized in Table 4. The bioassay revealed that the active components were distributed in the benzene and ether soluble parts.

The hexane insoluble (benzene soluble) part was stirred with MeOH (200 ml×2) and MeOH insoluble powder (1.1 g) was removed by filtration. The MeOH solution was concentrated in vacuo and the MeOH soluble residue (18.8 g) was subjected to chromatography over silica gel (200 g) eluting with CH_2Cl_2–MeOH by changing the ratios from 50 : 1, 20 : 1, 10 : 1, and then 5 : 1. The column was finally eluted with MeOH. From 50 : 1 and 10 : 1 fractions the triterpenes (**1**—**4**) and sesquiterpene (**7**) were isolated after repeats of HPLC with UV at 254 nm and RI detectors [column;

μ-BONDA SPHERE C-18 (19×150 mm), solvent; H_2O–MeOH 3:7. column; μ-PORASIL SiO_2 (8×100 mm), solvent; CH_2Cl_2–MeOH 25:1]. Compound **6** was isolated from the MeOH eluant of the silica-gel column chromatography by repeats of HPLC with RI detector. The active neutral portion (610 mg) of the ether soluble part was also submitted to silica-gel column chromatography with CH_2Cl_2–MeOH and the compound **5** was isolated by C-18 HPLC purification. The yields from 32 g of acetone extracts are as follows: **1**, 71 mg; **2**, 193 mg; **3**, 46 mg; **4**, 29 mg; **5**, 28 mg; **6**, 100 mg, and **7**, 34 mg.

Compound 1: Colorless pillars from MeOH, mp 232—233 °C; $[\alpha]_D^{20}$ +100.0° ($CHCl_3$; *c* 3.85) (Lit,[2a] mp 232—233 °C; $[\alpha]_D^{24}$ +102.9°) IR ($CHCl_3$) 3500, 1750, 1705, 1694, 1650 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz) δ=0.89 (3H, s), 1.07 (3H, s), 1.08 (3H, s), 1.09 (3H, s), 1.19 (3H, d, *J*=10.9 Hz, H-21), 1.22 (3H, s), 1.29 and 1.32 (each 3H, s, H-26, H-27), 1.77 (1H, d, *J*=6.2 Hz, H-9), 1.77 and 2.32 (each 1H, H-22a and 22b), 2.35 and 2.89 (each 1H, H-12a and 12b), 2.05 (3H, s, Ac), 2.60 (1H, m, H-20), 2.72 (1H, d, *J*=8.4 Hz, H-24), 3.99 (1H, ddd, *J*=6.2, 10.6, 16.5 Hz, H-11), 4.51 (1H, ddd, *J*=2.2, 8.4, 11.0 Hz, H-23). ^{13}C NMR ($CDCl_3$, 50 MHz) δ=220.2 (s), 208.6 (s), 177.4 (s), 170.6 (s), 138.9 (s), 58.8 (s), 49.9 (s), 47.1 (s), 40.2 (s), 37.0 (s), 72.1 (d), 70.0 (d), 65.1 (d), 48.9 (d), 48.5 (d), 26.7 (d), 45.8 (t), 35.7 (t), 35.1 (t), 34.9 (t), 33.6 (t), 30.9 (t), 20.0 (t), 29.6 (q), 25.6 (q), 24.7 (q), 23.2 (q), 23.1 (q), 21.2 (q), 20.1 (q), 19.8 (q), 19.3 (q). HRMS: Found: *m/z* 528.3450. Calcd for $C_{32}H_{48}O_6$: M, 528.3452.

Compound 2: Colorless plates from Et_2O, mp 204—205 °C; $[\alpha]_D^{20}$ +108.7° ($CHCl_3$; *c* 0.115). IR ($CHCl_3$) 3500, 1700, 1690, 1650 cm^{-1}. ^1H NMR ($CDCl_3$, 200 MHz) δ=0.91 (3H, s), 1.07 (3H, s), 1.08 (3H, s), 1.11 (3H, s), 1.18 (3H, d, *J*=11.8 Hz, H-21), 1.25 (3H, s), 1.29 (6H, s), 1.87 (1H, H-9), 2.68 (1H, d, *J*=8.1 Hz, H-24), 2.92 (1H, m, H-20), 3.06 (1H, H-23), 4.04 (1H, H-11). ^{13}C NMR ($CDCl_3$, 25 MHz) δ=220.3 (s), 208.8 (s), 177.4 (s), 139.6 (s), 59.5 (s), 49.9 (s), 47.1 (s), 40.0 (s), 37.0 (s), 69.8 (d), 69.7 (d), 67.9 (d), 48.7 (d), 48.5 (d), 26.3 (d), 45.7 (t), 37.8 (t), 35.7 (t), 35.0 (t), 33.6 (t), 31.0 (t), 20.0 (t), 29.6 (q), 25.6 (q), 25.0 (q), 23.6 (q), 23.3 (q), 20.1 (q), 19.4 (q), 19.2 (q). HRMS: Found: *m/z* 486.3354. Calcd for $C_{30}H_{46}O_5$: M, 486.3347.

Compound 3: Amorphous. $[\alpha]_D^{20}$ +130.5° ($CHCl_3$; *c* 0.39). ^1H NMR ($CDCl_3$, 100 MHz) δ=6.15 (1H, dd, *J*=2.5, 10.0 Hz, H-12), 6.75 (1H, dd, *J*=4.0 10.0 Hz, H-11). ^{13}C NMR ($CDCl_3$, 67.8 MHz) δ=219.1 (s), 209.2 (s), 172.0 (s), 139.4 (s), 138.8 (d), 122.4 (d), 77.3 (d), 73.6 (s), 69.2 (d), 48.4 (s), 47.6 (d), 47.1 (s), 46.0 (d), 43.9 (t), 40.6 (t), 39.5 (s), 35.9 (s), 33.3 (t), 32.2 (t), 31.2 (t), 29.2 (q), 26.9 (q), 26.3 (q), 25.4 (d), 24.7 (q), 23.9 (q), 21.7 (q), 19.3 (t), 19.3 (q), 19.1 (q). HRMS: Found: *m/z* 468.3278. Calcd for $C_{30}H_{44}O_4$: M−H_2O, 468.3241.

Compound 4: Amorphous. $[\alpha]_D^{20}$ +84.4° ($CHCl_3$; *c* 0.33). ^{13}C NMR ($CDCl_3$, 23 MHz) δ=219.3 (s), 209.7 (s), 180.3 (s), 140.1 (s), 77.4 (d), 73.7 (s), 69.4 (d), 50.4 (s), 48.0 (d), 47.0 (s), 45.5 (t), 42.8 (d), 40.4 (s), 40.1 (t), 36.2 (s), 34.5 (t), 33.6 (t), 31.7 (t), 29.3 (q), 27.0 (t), 26.3 (t), 25.6 (t), 24.9 (d), 23.6 (q), 23.3 (q), 22.2 (q), 22.0 (q), 19.9 (q), 19.7 (q), 19.5 (q). HRMS: Found: *m/z* 488.3482. Calcd for $C_{30}H_{48}O_5$: M, 488.3503.

Compound 5: Mp 109—112 °C (MeOH). $[\alpha]_D$ not measured. IR ($CHCl_3$) 3688, 3612, 1696, 1636 cm^{-1}. ^1H NMR ($CDCl_3$+D_2O, 200 MHz) δ=0.91 (3H, s), 1.08 (3H, s), 1.09 (3H, s), 1.12 (3H, s), 1.17 (3H, s), 1.22 (3H, d, *J*=6.5 Hz, H-21), 1.24 (3H, s), 1.34 (3H, s), 1.91 (1H, d, *J*=10.1 Hz, H-9), 3.05 (1H, d, *J*=1.2 Hz, H-24), 2.47 and 3.21 (each 1H, H-12a and 12b), 3.62 (1H, ddd, *J*=0.9, 2.0, 10.3 Hz, H-23), and 4.05 (1H, m, H-11). ^{13}C NMR ($CDCl_3$, 50 MHz) δ=220.1 (s), 210.2 (s), 177.8 (s), 140.8 (s), 74.1 (s), 50.3 (s), 47.1 (s), 40.1 (s), 37.0 (s), 77.5 (d), 69.8 (d), 69.5 (d), 48.6 (d), 48.5 (d), 25.7 (d), 45.4 (t), 40.1 (t), 36.3 (t), 35.0 (t), 33.6 (t), 31.0 (t), 20.0 (t), 29.6 (q), 27.1 (q), 26.4 (q), 25.5 (q), 23.6 (q), 23.2 (q), 20.1 (q), 19.5 (q). HRMS: Found: *m/z* 504.3448. Calcd for $C_{30}H_{48}O_6$: M, 504.3452.

Compound 6: Mp 90—92 °C (AcOEt); $[\alpha]_D^{20}$ +99.2° ($CHCl_3$; *c* 3.09) (Lit,[2a] mp 166—168 °C; $[\alpha]_D$ +130°); IR (KBr) 3420, 1690 cm^{-1}. ^1H NMR ($CDCl_3$, 200 MHz) δ= 0.98 (3H, s), 1.02 (3H, d, *J*=7.3 Hz), 1.05 (6H, s), 1.07 (3H, s), 1.11 (3H, s), 1.23 (3H, s), 1.31 (3H, s), 1.74 (1H, d, *J*=10.5 Hz, H-10), 2.70 (1H, d, *J*=8.3 Hz, H-24), 3.19 (1H, ddd, *J*=2.4, 8.2, 10.5 Hz, H-23), 3.86 (1H, dt, *J*=5.8, 10.5 Hz, H-11). ^{13}C NMR ($CDCl_3$, 50 MHz) δ=220.9 (s), 138.5 (s), 135.3 (s), 59.4 (s), 57.2 (s), 47.1 (s), 40.7 (s), 37.5 (s), 70.1 (d), 69.3 (d), 68.1 (d), 49.8 (d), 48.7 (d), 27.8 (d), 38.9 (t), 34.5 (t), 34.4 (t), 33.8 (t), 31.1 (t), 30.7 (t), 29.2 (t), 20.1 (t), 29.6 (q), 25.6 (q), 25.0 (q), 24.1 (q), 23.4 (q), 20.3 (q), 20.1 (q), 19.2 (q). HRMS: Found: *m/z* 454.3442. Calcd for $C_{30}H_{46}O_3$: M−H_2O, 454.3450.

Diacetate 9: Mp 129—130 °C (AcOEt); $[\alpha]_D^{20}$ +112.5° ($CHCl_3$, *c* 1.0) (Lit,[2a] mp 143—145 °C (MeOH); $[\alpha]_D^{23}$ + 117.5°). ^1H NMR ($CDCl_3$, 90 MHz) δ=2.00 (6H, s, OAc×2), 4.54 (1H, ddd, *J*=2.0, 4.5, 5.2 Hz), 4.89 (1H, sext).

Compound 7: Yellow oil. $[\alpha]_D$ not measured. ^1H NMR ($CDCl_3$, 200 MHz) δ=0.97 and 0.98 (each 3H, d, *J*=6.8 Hz), 1.21 (3H, s), 1.27 (3H, s), and 5.50 (1H, d, *J*=3.1 Hz); ^{13}C NMR ($CDCl_3$) δ=150.10 (s), 121.66 (d), 80.45 (s), 75.51 (s), 50.80 (d), 50.43 (d), 42.72 (t), 40.57 (t), 37.40 (d), 25.14 (t), 22.59 (q), 21.57 (t), 21.48 (q), 21.42 (q), 21.24 (q). HRMS: Found: *m/z* 220.1824. Calcd for $C_{15}H_{24}O$: M, 220.1828.

Reaction of Compound 1 with $NaHCO_3$. A mixture of **1** (5 mg), 0.2 M $NaHCO_3$ (0.2 ml) and MeOH (6 ml) was stirred at room temperature overnight. The reaction mixture was extracted with ether. The ether solution was washed with brine and dried over $MgSO_4$. The ether extracts were analyzed with HPLC [column; μ-BONDA SPHERE (4×150 mm), solvent; H_2O–MeOH (35:65), detector; UV at 254 nm], showing a 29:71 mixture of **1** and **2**.

Reaction of Compound 1 with KOH: A mixture of **1** (13 mg) and 0.1 M KOH–MeOH (3 ml) was stirred at room temperature for 2 d, then diluted with ether. The ether solution was washed with brine and dried over $MgSO_4$. The ether extracts were purified with HPLC [column; μ-BONDA SPHERE (9×150 mm), solvent; H_2O–MeOH (3:7)] to isolate the dehydrated product **8** (8.6 mg).

8: $[\alpha]_D^{20}$ +114.6° ($CHCl_3$; *c* 0.48). UV (MeOH) nm (log ε) 287 (4.06). ^1H NMR ($CDCl_3$, 100 MHz) δ=0.96 (6H, s), 1.07 (3H, s), 1.10 (6H, s), 1.16 (3H, s), 1.20 (3H, d, *J*=6 Hz), 1.30 (3H, s), 6.12 (1H, dd, *J*=2.5 and 10.0 Hz, H-12) and 6.70 (1H, dd, *J*=4.0 and 10.0 Hz, H-11). HRMS: Found: *m/z* 468.3256. Calcd for $C_{30}H_{44}O_4$: M, 468.3241.

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8) Direct comparison of **6** and alisol B was not carried out for the lack of available data or sample.