MeOH in $CHCl_3$ [$CHCl_3$ (3 L), $CHCl_3$–MeOH (100 : 1, 3 L), (50 : 1, 3 L), (30 : 1, 3 L), (20 : 1, 3 L) (10 : 1, 3 L), (5 : 1, 3 L), and (2 : 1, 3 L)], and (MeOH, 5 L). The residue (7.36 g) of the $CHCl_3$: MeOH=10 : 1 eluate obtained on silica gel CC was subsequently subjected to RPCC with a stepwise gradient elution with increasing amounts of MeOH in H_2O (30, 50, 70, 90, 100% MeOH, 300 mL containing with 0.1% TFA). The residues (190 mg) obtained from 50% MeOH eluate was purified by HPLC (ODS) with 30% acetone/0.1% TFA to afford **5** (10.3 mg) from the peak at 10.4 min (flow rate: 2.8 mL/min). The residue (5.95 g) of the $CHCl_3$: MeOH=5 : 1 eluate obtained on silica gel CC was subsequently subjected to RPCC with a stepwise gradient elution with increasing amounts of MeOH in H_2O (30, 50, 70, 90, 100% MeOH, 300 mL containing with 0.1% TFA). The residues (170 mg) obtained from 30% MeOH eluate was purified by HPLC (ODS) with 20% CH_3CN/0.1% TFA to afford **2** (4.9 mg) from the peak at 17.9 min (flow rate: 2.5 mL/min). The residues (220 mg) obtained from 50% MeOH eluate was purified by HPLC (ODS) with 30% acetone/0.1% TFA to afford **4** (7.0 mg) and **3** (5.3 mg) from the peaks at 5.2 and 14.6 min, respectively (flow rate: 2.8 mL/min).

The known compounds (**2**—**5**) were identified by comparison of spectroscopic data with those reported in the literature as follows. (*S*)-Norcorydine (**2**)[11] {ref. $[\alpha]_D^{23}$ +190 (c=0.2, $CHCl_3$)}, $[\alpha]_D^{22}$ +151 (c=0.13, $CHCl_3$), CD $\Delta\varepsilon$ (nm): −7.81 (268), +43.0 (234) ($c=3.98\times10^{-5}$ M, MeOH), (*R*)-anonaine (**3**)[12] {ref. $[\alpha]_D^{20}$ −48 (c=0.1, $CHCl_3$)}, $[\alpha]_D^{22}$ −34.9 (c=0.10, $CHCl_3$), CD $\Delta\varepsilon$ (nm): +11.4 (271), −52.5 (231) ($c=3.08\times10^{-5}$ M, MeOH), (*R*)-4′-*O*-methylcoclaurine (**4**),[13] $[\alpha]_D^{22}$ +14.5 (c=0.19, $CHCl_3$), CD $\Delta\varepsilon$ (nm): −1.09 (288), −1.75 (230) ($c=3.18\times10^{-5}$ M, MeOH), (*R*)-*O*,*O*-dimethylcoclaurine (**5**)[14] {ref. $[\alpha]_D^{25}$ +15.7 (c=0.4, $CHCl_3$)}, $[\alpha]_D^{21}$ +10.9 (c=0.16, $CHCl_3$), CD $\Delta\varepsilon$ (nm): −1.15 (288), −2.00 (232) ($c=2.59\times10^{-5}$ M, MeOH). Other chemical constituents including megastigmane glucosides have been isolated previously and published elsewhere.[15]

Annonamine (**1**): Amorphous powder; $[\alpha]_D^{22}$ −12.5 (c=1.70, MeOH); IR ν_{max} (film) cm^{-1}: 3360, 2964, 2937, 1680, 1431, 1354, 1259, 1200, 1183, 1136, 1007, 798; UV λ_{max} (MeOH) nm ($\log\varepsilon$): 310sh (3.16), 283sh (3.76), 273 (3.90), 229 (3.99), 211 (4.15); ^{1}H- and ^{13}C-NMR (CD_3OD): Table 1; CD $\Delta\varepsilon$ (nm): +0.86 (274), −6.15 (233) ($c=5.43\times10^{-5}$ M, MeOH); HR-ESI-TOF-MS (positive-ion mode) *m/z*: 296.1644 $[M]^+$ (Calcd for $C_{19}H_{22}NO_2$: 296.1645).

Cytotoxicity Assay The cytotoxicity assay was performed using human neuroblastoma cell line, SH-SY5Y by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) method. In brief, cells (5×10^3 cells/100 μL complete medium) were cultured in 96-well plates with different concentrations of the test compounds (200–6.25 μM; 1% of dimethyl sulfoxide (DMSO) was present as a vehicle in all the experiments) for 72 h at 37°C. Then the culture supernatants were replaced with 100 μL of a MTT solution comprising 0.5 mg/mL of MTT in complete medium. After 3 h incubation at 37°C, the precipitate was dissolved in 100 μL of DMSO. The optical density values for each well were measured at 520 nm with a microplate reader.

The cytotoxicity was calculated using the following equation:

$$\text{Inhibition (\%)}=[1-(A_{sample}-A_{background})/A_{DMSO}-A_{background})]\times100$$

where A_{DMSO} is the absorbance of the control reaction mixture (containing DMSO and all reagents except for the test compound).

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ORIGINAL PAPER

Three new megastigmanes from the leaves of *Annona muricata*

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Abstract Three new megastigmanes (**1–3**), named annoionols A and B (**1**, **2**) and annoionoside (**3**), were isolated from the leaves of *Annona muricata* L. (Annonaceae) together with 14 known compounds (**4–17**). Among the known compounds, annoionol C (**4**) was isolated from a natural source for the first time. The structures of all compounds were elucidated by spectroscopic and chemical analyses.

Keywords *Annona muricata* · Annonaceae · Megastigmane · Annoionoside · Annoionol

Introduction

Megastigmanes and their glycosides are a currently expanding class of compounds. In our continuing studies on sub-tropical plants collected on Okinawa, we have phytochemically investigated the leaves of *Annona muricata* L. (Annonaceae) in this paper. The plant is an evergreen tree of medium height, and is found in the Americas, Africa and Southeast Asia. The edible fruit of this plant is well-known as "soursop" and is cultivated widely in tropical and subtropical areas nowadays. However, in the Caribbean, consumption of this fruit is suggested to have a connection to an atypical form of Parkinson's disease [1]. The present study describes the isolation and structural elucidation of three new megastigmanes (**1–3**) together with 14 known compounds (**4–17**).

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Results and discussion

Air-dried leaves of *A. muricata* were extracted with MeOH three times by maceration. The combined MeOH extract was evaporated and partitioned with *n*-hexane, $CHCl_3$, EtOAc and 1-BuOH successively, to give *n*-hexane, $CHCl_3$, EtOAc and 1-BuOH soluble fractions, respectively. The $CHCl_3$ and EtOAc-soluble fractions were combined because of the similarity of their TLC patterns. The residue of the 1-BuOH-soluble fraction and the combined $CHCl_3$- and EtOAc-soluble fractions were subjected to various kinds of column chromatography to yield 17 compounds (**1–17**) (Fig. 1).

Annoionol A (**1**) was obtained as a colorless amorphous powder and its molecular formula was determined to be $C_{13}H_{26}O_3$ from its high-resolution electrospray-ionization time-of-flight mass spectrum (HR-ESI-TOF-MS) (m/z = 253.1775 $[M + Na]^+$). The IR absorption at 3382 cm^{-1} indicated the presence of a hydroxyl group. The ^{1}H-NMR spectrum exhibited the signals ascribable to two singlet (δ_H 0.88 and 0.94) and two doublet [δ_H 1.07 (d) and 1.15 (d)] methyls, and two oxygenated methines (δ_H 3.49, 3.65 and 2.78) (Table 1). The ^{13}C-NMR and DEPT spectra indicated the presence of 13 carbon signals comprising those of four methyls, three methylenes, five methines, of which three were oxygenated, and one quaternary carbon (Table 1). The proton (δ_H 2.78) was assigned to that on the highly deshielded oxygenated methine carbon (δ_C 82.4) by the HMQC spectrum. These atypically counter-shifted proton

and carbon were also observed in the case of elaeocarpionoside [2] and fruticosides A and B [3]. Considering the above together with one degree of unsaturation, a megastigmane skeleton was formulated for **1**. The ^{1}H–^{1}H correlation spectroscopy (COSY) spectrum revealed a proton coupling framework from H-2 to H_3-10 together with the connection of H_3-13 to H-5, which revealed the planar structure of **1** shown in Fig. 1. The heteronuclear multiple bond correlation (HMBC) spectrum confirmed this structure (Fig. 2a). The relative configuration of **1** was determined by considering the coupling constants (Table 1) and phase-sensitive (PS) nuclear Overhauser enhancement spectroscopy (NOESY) spectral data. The axial coupling of H-2ax [δ_H 1.21 (1H, dd, J = 13, 12 Hz)], H-3 [δ_H 3.49 (1H, ddd, J = 12, 9, 5 Hz)], H-4 [δ_H 2.78 (1H, dd, J = 10, 9 Hz)] and H-6 [δ_H 0.71 (1H, ddd, J = 11, 4, 2 Hz)], and the NOE correlations around the six-membered ring portion are in good agreement with the relative configuration shown in Fig. 2b. Finally, the absolute configuration of **1** was determined by the modified Mosher's method (Fig. 2c) [4]. The structure of annoionol A (**1**) was therefore elucidated to be (3*R*,4*R*,5*S*,6*S*,9*R*)-megastigma-3,4,9-triol.

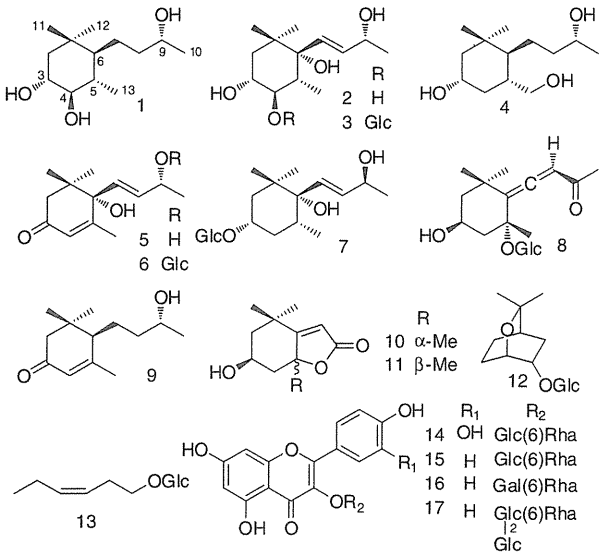

Fig. 1 Structures of the isolated compounds

Compound **2** was obtained as a colorless amorphous powder and its molecular formula was determined to be $C_{13}H_{24}O_4$ by HR-ESI-TOF-MS (m/z = 267.1562 $[M + Na]^+$). The ^{1}H and ^{13}C-NMR spectra were similar to

Table 1 ^{1}H-NMR spectral data for **1**–**4** (δ in ppm, J in Hz, in CD_3OD)

	1		2		3		4	
2	1.21 ax	dd (13, 12)	1.43 eq	dd (12, 5)	1.48 eq	dd (13, 5)	1.10 ax	dd (12, 12)
	1.62 eq	dd (13, 5)	1.81 ax	dd (12, 12)	1.82 ax	dd (13, 12)	1.65 eq	ddd (12, 3, 2)
3	3.49	ddd (12, 9, 5)	3.60	ddd (12, 9, 5)	3.71	ddd (12, 9, 5)	3.72	m
4	2.78	dd (10, 9)	3.26	dd (11, 9)	3.36	dd (11, 9)	1.03 ax	ddd (12, 12, 12)
	–		–		–		2.12 eq	m
5	1.37	m	1.77	dq (11, 7)	1.98	dq (11, 7)	1.45	m
6	0.71	ddd (11, 4, 2)	–		–		0.78	ddd (11, 5, 2)
7	1.08	m	5.55	dd (16, 1)	5.55	dd (16, 1)	1.09	m
	1.56	m	–		–		1.58	m
8	1.44	m	5.70	dd (16, 6)	5.70	dd (16, 6)	1.40	m
	1.51	m	–		–		1.50	m
9	3.65	m	4.30	qdd (6, 6, 1)	4.30	qdd (6, 6, 1)	3.66	qt (6, 6)
10	1.15	d (6)	1.25	d (6)	1.25	d (6)	1.15	d (6)
11	0.88	s	1.02	s	1.01	s	0.84	s
12	0.94	s	0.88	s	0.88	s	0.97	s
13	1.07	d (6)	0.95	d (7)	1.04	d (7)	3.42	dd (10, 7)
	–		–		–		3.70	dd (10, 3)
1′					4.311	d (8)		
2′					3.25	dd (9, 8)		
3′					3.37	dd (9, 9)		
4′					3.34	m		
5′					3.34	m		
6′					3.87	dd (12, 1)		
					3.67	dd (12, 5)		

m Multiplet or overlapping signals

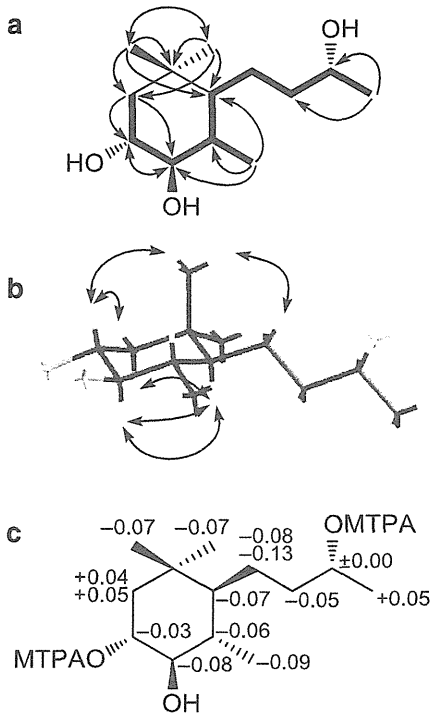

Fig. 2 Absolute structure of **1**. **a** COSY (*bold line*) and HMBC (*arrows*) correlations. **b** Important NOESY correlations. **c** Analysis by the modified Mosher's method. The values are expressed as $\Delta\delta_{S-R}$

those of **1** except for the appearance of an oxygen-bearing quaternary carbon and a *trans*-double bond. The ^{1}H–^{1}H COSY spectra revealed two proton coupling networks from H-2 to H-13 through H-5, and from H-7 to H-10, which revealed the planar structure of **2** shown in Fig. 1. The HMBC spectrum also confirmed that the structure of **2** was megastigman-7-ene-3,4,6,9-tetraol (Fig. 3). The coupling constant of 16 Hz for H-7 and H-8 indicated the geometry of the double bond to be the *E*-form. The relative configuration of **2** was determined by considering the coupling constants around the six-membered ring portion and PS NOESY spectral data. The axial coupling of H-2ax [δ_H 1.81 (1H, dd, J = 12, 12 Hz)], H-3 [δ_H 3.60 (1H, ddd, J = 12, 9, 5 Hz)], H-4 [δ_H 3.26 (1H, dd, J = 11, 9 Hz)] and H-5 [δ_H 1.77 (1H, dq, J = 11, 7 Hz)] revealed the equatorial nature of 3-OH, 4-OH and 5-Me. The relative configuration of C-6 was also determined to be as shown in Fig. 2b from the NOE correlations between H-7 and H-5ax, and between H-7 and H_3-11. Finally, the absolute configuration of **2** was determined by the modified Mosher's method (Fig. 3). Annoionol B (**2**) was therefore elucidated to be (3*R*,4*R*,5*R*,6*R*,7*E*,9*R*)-megastigman-7-ene-3,4,6,9-tetraol.

Compound **3** was obtained as a colorless amorphous powder and its molecular formula was determined to be $C_{19}H_{34}O_9$ by HR-ESI-TOF-MS (m/z = 429.2090 [M + Na]$^+$). The ^{1}H and ^{13}C NMR spectra were closely similar to those of **2**. An anomeric proton and six oxygenated carbon signals at δc 105.4, 78.1 (C × 2), 75.5, 71.6 and 62.5 indicated the presence of glucopyranose. The HMBC correlations between H-4 and C-1′, and H-1′ and C-4 confirmed the connectivity of glucose at C-4, and other two-dimensional NMR analyses confirmed the planar structure of **3** (Fig. 1). The coupling constant of the anomeric proton (8 Hz) and chiro-optical HPLC analysis of the sugar fraction following enzymatic hydrolysis of **3** revealed that the configuration of glucose was of the D-series. The relative configuration around the six-membered ring of **3** was determined to be equatorial for 3-OH, 4-OH and 5-Me by considering the axial–axial coupling constants of the corresponding protons (Table 1). The relative configuration of C-6 was also determined to be as shown in Fig. 3 from the NOE correlations between H-7 and H-5ax, and between H-7 and H_3-11. The absolute stereochemistry at C-3 was tentatively determined to be 3*R* by application of the glucosylation-induced shift-trend rule [5]. Finally, the aglycone liberated on enzymatic hydrolysis was then esterified to afford (*R*)- and (*S*)-α-methoxy-α-(trifluoromethyl)phenylacetic acid (MTPA) diesters. The ^{1}H-NMR spectral data of both the (*R*) and (*S*)-MTPA diesters were essentially identical to those of the MTPA derivatives of **2**. The absolute structure of **3** was therefore elucidated to be (3*R*,4*R*,5*R*,6*R*,7*E*,9*R*)-megastigman-7-ene-3,4,6,9-tetraol 4-*O*-β-D-glucopyranoside (Fig. 3).

Table 2 ^{13}C-NMR spectral data for **1**–**4** (δ in ppm, in CD_3OD)

	1	2 (=3a)	3	(δ_{3-3a})	4
1	36.2	40.1	39.7		36.8
2	49.0	43.9	43.0		51.9
3	72.4	72.9	71.1	(−1.8)	67.5
4	82.4	78.7	90.3	(+11.6)	40.8
5	41.6	42.1	41.6	(−0.5)	42.7
6	52.7	80.1	80.1		48.5
7	26.7	133.7	133.3		26.1
8	42.4	135.6	135.8		42.1
9	69.2	69.2	69.1		69.1
10	23.4	24.2	24.2		23.4
11	21.5	25.5	25.4		21.4
12	30.9	25.2	25.1		31.3
13	16.8	12.1	12.1		66.1
1′			105.4		
2′			75.5		
3′			78.1		
4′			71.6		
5′			78.1		
6′			62.5		

m Multiplet or overlapping signals

Compound **4** was obtained as a colorless amorphous powder and its molecular formula was revealed to be the same as that of **1**, $C_{13}H_{26}O_3$, by HR-ESI-TOF-MS (m/z = 253.1770 $[M + Na]^+$) analysis. In the ^{13}C-NMR spectrum, two methylenes (δ_C 40.8 and 66.1), of which one was oxygenated, were seen in **4** instead of the methyl (C-13 of **1**) and oxygenated methine (C-4 of **1**) groups observed in **1**. Therefore, the planar structure of **4** was assumed to be a positional isomer of **1** having a hydroxy group at C-13 (Fig. 1). The relative stereostructure of **4** was elucidated from the coupling constants and by two-dimensional NMR analyses (Fig. 4). Finally, the absolute configuration of **4** was determined by the modified Mosher's method (Fig. 4). Thus, the structure of annoionol C (**4**) was elucidated to be (3*S*,5*R*,6*S*,9*R*)-megastigman-3,9,13-triol. Annoionol C (**4**) has already been reported as the aglycone of two related glucosides, bridelionoside D [6] and rhusonoside A [7]; however, this was the first time that **4** had been isolated from a natural source.

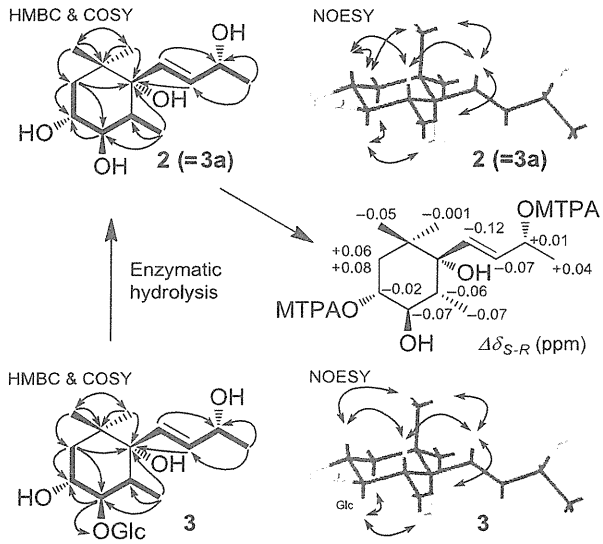

Fig. 3 Absolute structures of **2** and **3**. COSY (*bold lines*), HMBC (*arrows*), and NOESY (*arrows in 3D drawing*) correlations were indicated. The $\Delta\delta_{S-R}$ values are expressed in ppm

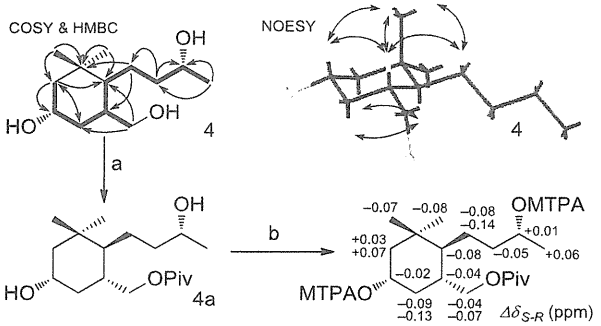

Fig. 4 Absolute structure of **4**. **a** Pivaloyl chloride/pyridine. **b** (*R*) or (*S*)-MTPA, EDC, DMAP/CH_2Cl_2. COSY (*bold line*), HMBC (*arrows*), and NOESY (*arrows in 3D drawing*) correlations are indicated. The $\Delta\delta_{S-R}$ values are expressed in ppm

It is noteworthy that the several stereoisomers for compounds **1–3** have already been isolated from various plant sources [8–11]; however the megastigmanes having (3*R*,4*R*)-diol moiety have rarely been found in nature. The remaining known compounds (**5–17**) were identified by comparison of the spectroscopic data with those reported in the literature, as follows. Vomifoliol (**5**), $[\alpha]_D^{26}$ +176.6° (*c* 0.31, MeOH) [12], roseoside (**6**), $[\alpha]_D^{25}$ +100.8° (*c* 0.54, MeOH) [13], turpinionoside A (**7**), $[\alpha]_D^{27}$ −37.4° (*c* 0.53, MeOH), t_R = 18.5 min under the same HPLC conditions as described in the literature [14, 15], citroside A (**8**), $[\alpha]_D^{27}$ −83.9° (*c* 0.73, MeOH) [16], blumenol C (**9**), $[\alpha]_D^{26}$ +49.6° (*c* 0.22, $CHCl_3$) [17], (+)-epiloliolide (**10**), $[\alpha]_D^{26}$ +22.3° (*c* 0.12, $CHCl_3$) [18], loliolide (**11**), $[\alpha]_D^{23}$ −67.9° (*c* 0.88, MeOH) [19], (1*S*,2*S*,4*R*)-*trans*-2-hydroxy-1,8-cineole β-D-glucopyranoside (**12**), $[\alpha]_D^{26}$ +0.67° (*c* 0.48, MeOH) [20], (*Z*)-3-hexenyl β-D-glucopyranoside (**13**), $[\alpha]_D^{27}$ −20.5° (*c* 0.25, MeOH) [21], rutin (**14**), $[\alpha]_D^{27}$ −10.4° (*c* 9.4, MeOH) [22], kaempferol 3-*O*-rutinoside (**15**), $[\alpha]_D^{26}$ −11.1° (*c* 0.42, MeOH) [23], kaempferol 3-*O*-robinobioside (**16**), $[\alpha]_D^{26}$ −63.1° (*c* 0.36, pyridine) [24], kaempferol 3-*O*-β-D-(2″-*O*-β-D-glucopyranosyl,6″-*O*-α-L-rhamnopyranosyl)glucopyranoside (**17**), $[\alpha]_D^{27}$ −82.2° (*c* 0.63, MeOH) [25].

Compounds **1–4** were examined for 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, and also for tumor cell growth inhibitory activity toward A549 and SBC-3 by means of a MTT assay. However, these compounds did not show any significant activity at 100 μM.

Experimental

General experimental procedures

Silica gel column chromatography (CC) was performed on silica gel 60 (Merck, Darmstadt, Germany), and reversed-phase [octadecyl silica gel (ODS)] open CC (RPCC) on Cosmosil 75C_{18}-OPN (Nacalai Tesque, Kyoto, Japan) (Φ = 5 cm, L = 20 cm). HPLC was performed on ODS (Cosmosil; Nacalai Tesque, Japan; Φ = 10 mm, L = 250 mm), and the eluate was monitored with a refractive index monitor.

Optical rotations were measured on a JASCO P-1030 polarimeter. IR spectra were measured on a Horiba FT-710 Fourier transform infrared spectrophotometer. NMR spectra were taken on a JEOL ECA 600 spectrometer at 600 MHz for ^{1}H, and 150 MHz for ^{13}C, respectively, with tetramethylsilane as an internal standard. Positive-ion

HR-ESI-TOF-MS was recorded on a Applied Biosystem QSTAR XL spectrometer. A VersaMax (Molecular Devices) was used as a microplate reader.

Plant material

Leaves of *A. muricata* were collected in Yaeyama-gun, Okinawa, Japan, in November 2004, and a voucher specimen was deposited in the Herbarium of the Department of Pharmacognosy, Graduate School of Biomedical Sciences, Hiroshima University (No. 04-AM-Okinawa-1105).

Extraction and isolation

Air-dried leaves of *A. muricata* (520 g) were extracted with MeOH (2 L) three times by maceration. The MeOH extracts were combined and evaporated to dryness to afford a viscous gummy material (78.2 g). This residue was suspended in 1.5 L of H_2O, and then extracted with equal volumes of *n*-hexane, $CHCl_3$, EtOAc and 1-BuOH successively to afford 13.4, 34.9, 1.1 and 5.0 g of fractions, respectively. The remaining H_2O layer was concentrated to furnish an H_2O-soluble fraction (23.9 g). The 1-BuOH soluble fraction (5.0 g) was subjected to silica gel column chromatography (Φ = 2.5 cm, L = 50 cm) with stepwise gradient elution with increasing amounts of MeOH in $CHCl_3$ [$CHCl_3$ (1 L), $CHCl_3$-MeOH (20:1, 750 mL), (10:1, 750 mL), (5:1, 750 mL), (3:1, 750 mL)], $CHCl_3$:MeOH:H_2O = 15:6:1, 750 mL, and MeOH, 750 mL. The combined residue (0.83 g) of the $CHCl_3$-MeOH (10:1) and (5:1) eluates obtained on silica gel CC was subsequently subjected to RPCC with stepwise gradient elution with increasing amounts of MeOH in H_2O (30, 50, 70, 90 and 100% MeOH, 300 mL each containing 0.1% TFA). The residue (470 mg) obtained from the 30% MeOH eluate was purified by HPLC (ODS) with 13% CH_3CN to afford **2** (12.7 mg), **6** (14.3 mg), **12** (4.8 mg) and **13** (7.1 mg) from the peaks at 10.8, 18.2, 23.5 and 33.0 min (flow rate: 2.5 mL/min), respectively. The residue (320 mg) obtained from the 50% MeOH eluate was purified by HPLC (ODS) with 30% acetone to afford **4** (11.1 mg) from the peak at 8.1 min (flow rate: 2.8 mL/min).

The residue (1.43 g) of the $CHCl_3$–MeOH (3:1) eluate obtained on silica gel CC was subsequently subjected to RPCC with stepwise gradient elution with increasing amounts of MeOH in H_2O (30, 50, 70, 90 and 100% MeOH, 300 mL containing 0.1% TFA). The residue (850 mg) obtained from the 30% MeOH eluate was purified by HPLC (ODS) with 13% CH_3CN to afford **3** (64.3 mg), **7** (9.7 mg) and **8** (12.8 mg) from the peaks at 10.5, 14.2 and 18.1 min (flow rate: 2.5 mL/min), respectively. The residue (620 mg) obtained from the 50% MeOH eluate was purified by HPLC (ODS) with 30% acetone to afford **15** (59.6 mg) from the peak at 14.9 min (flow rate: 2.8 mL/min).

The residue (1.13 g) of the $CHCl_3$:MeOH:H_2O = 15:6:1 eluate obtained on silica gel CC was subsequently subjected to RPCC with stepwise gradient elution with increasing amounts of MeOH in H_2O (30, 50, 70, 90 and 100% MeOH, 300 mL each containing 0.1% TFA). The residue (400 mg) obtained from the 50% MeOH eluate was purified by HPLC (ODS) with 20% CH_3CN–0.1% TFA to afford **14** (199 mg) from the peak at 11.2 min (flow rate: 3.0 mL/min).

The residue (1.05 g) of the MeOH eluate obtained on silica gel CC was subsequently subjected to RPCC with stepwise gradient elution with increasing amounts of MeOH in H_2O (30, 50, 70, 90 and 100% MeOH, 300 mL each containing 0.1% TFA). The residue (660 mg) obtained from the 50% MeOH eluate was purified by HPLC (ODS) with 22% acetone to afford **17** (12.7 mg) and **14** (14.3 mg) from the peaks at 8.9 and 15.5 min (flow rate: 2.8 mL/min), respectively.

The $CHCl_3$ and EtOAc-soluble fractions were combined and subjected to silica gel column chromatography (Φ = 5.8 cm, L = 38 cm) with stepwise gradient elution with increasing amounts of MeOH in $CHCl_3$ [$CHCl_3$ (3 L), $CHCl_3$-MeOH (100:1, 3 L), (50:1, 3 L), (30:1, 3 L), (20:1, 3 L), (10:1, 3 L), (5:1, 3 L), (2:1, 3 L) and (MeOH, 3 L)]. The residue (2.4 g) of the $CHCl_3$–MeOH (30:1) eluate obtained on silica gel CC was subsequently subjected to RPCC with stepwise gradient elution with increasing amounts of MeOH in H_2O (30, 50, 70, 90 and 100% MeOH, 300 mL each with 0.1% TFA). The residue (70 mg) obtained from the 30% MeOH eluate was purified by HPLC (ODS) with 20% CH_3CN-0.1% TFA to afford **10** (4.0 mg) from the peak at 17.2 min (flow rate: 2.5 mL/min). The residue (120 mg) obtained from the 50% MeOH eluate was purified by HPLC (ODS) with 38% acetone to afford **11** (8.8 mg) and **9** (5.0 mg) from the peaks at 8.4 and 12.1 min (flow rate: 2.5 mL/min), respectively.

The residue (4.90 g) of the $CHCl_3$–MeOH (20:1) eluate obtained on silica gel CC of the $CHCl_3$ and EtOAc soluble fractions was subsequently subjected to RPCC with stepwise gradient elution with increasing amounts of MeOH in H_2O (30, 50, 70, 90 and 100% MeOH, 300 mL each with 0.1% TFA). The residue (60 mg) obtained from the 30% MeOH eluate was purified by HPLC (ODS) with 20% CH_3CN to afford **5** (6.0 mg) from the peak at 10.8 min (flow rate: 2.5 mL/min).

The residue (7.36 g) of the $CHCl_3$–MeOH (10:1) eluate obtained on silica gel CC of the $CHCl_3$ and EtOAc-soluble fractions was subsequently subjected to RPCC with stepwise gradient elution with increasing amounts of MeOH in H_2O (30, 50, 70, 90 and 100% MeOH, 300 mL each with 0.1% TFA). The residue (190 mg) obtained from the 50%

MeOH eluate was purified by HPLC (ODS) with 30% acetone–0.1% TFA to afford **1** (1.1 mg) from the peak at 9.6 min (flow rate: 2.8 mL/min).

The residue (1.87 g) of the $CHCl_3$–MeOH (2:1) eluate obtained on silica gel CC of the $CHCl_3$ and EtOAc-soluble fractions was subsequently subjected to RPCC with stepwise gradient elution with increasing amounts of MeOH in H_2O (30, 50, 70, 90 and 100% MeOH, 300 mL each with 0.1% TFA). The residue (290 mg) obtained from the 50% MeOH eluate was purified by HPLC (ODS) with 29% acetone to afford **16** (7.1 mg) and **15** (9.9 mg) from the peaks at 10.5 and 12.2 min (flow rate: 2.8 mL/min), respectively.

Annoionol A (**1**)

Amorphous powder; $[\alpha]_D^{24}$ +1.45° (*c* 0.11, MeOH); IR ν_{max} (film) cm^{-1}: 3382, 2964, 2927, 1594, 1372, 1294, 1255, 1124, 1063; ^1H- and ^{13}C-NMR (CD_3OD): Tables 1 and 2, respectively; HR-ESI-TOF-MS (positive-ion mode) *m/z*: 253.1775 $[M + Na]^+$ (Calcd for $C_{13}H_{26}O_3Na$: 253.1774).

Annoionol B (**2**)

Amorphous powder; $[\alpha]_D^{24}$ −4.17° (*c* 1.27, MeOH); IR ν_{max} (film) cm^{-1}: 3398, 2971, 2934, 1676, 1516, 1460, 1371, 1136, 1054; ^1H and ^{13}C NMR (CD_3OD): Tables 1 and 2, respectively; HR-ESI-TOF-MS (positive-ion mode) *m/z*: 267.1562 $[M + Na]^+$ (Calcd for $C_{13}H_{24}O_4Na$: 267.1566).

Annoionoside (**3**)

Amorphous powder; $[\alpha]_D^{25}$ −15.0° (*c* 6.43, MeOH); IR ν_{max} (film) cm^{-1}: 3396, 2971, 2932, 1672, 1516, 1444, 1373, 1132, 1075, 1033; ^1H and ^{13}C NMR (CD_3OD): Tables 1 and 2, respectively; HR-ESI-TOF-MS (positive-ion mode) *m/z*: 429.2090 $[M + Na]^+$ (Calcd for $C_{19}H_{34}O_9Na$: 429.2095).

Annoionol C (**4**)

Amorphous powder; $[\alpha]_D^{26}$ −9.1° (*c* 1.11, MeOH); IR ν_{max} (film) cm^{-1}: 3374, 2931, 1672, 1370, 1201, 1125, 1044; ^1H and ^{13}C NMR (CD_3OD): Tables 1 and 2, respectively; HR-ESI-TOF-MS (positive-ion mode) *m/z*: 253.1770 $[M + Na]^+$ (Calcd for $C_{13}H_{26}O_3Na$: 253.1774).

Preparation of (*R*)- and (*S*)-MTPA diesters (**1a** and **1b**) of **1**

A solution of **1** (0.5 mg) in 1 mL of dehydrated CH_2Cl_2 was reacted with (*R*)-α-methoxy-α-(trifluoromethyl)phenylacetic acid (MTPA) (25.2 mg) in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) (23.5 mg) and 4-*N*,*N*′-dimethylaminopyridine (DMAP) (12.6 mg), followed by standing at 35°C for 12 h. After the addition of 1.0 mL each of H_2O and $CHCl_3$, the solution was washed with 1 M HCl (1.0 mL), $NaHCO_3$-saturated H_2O (1.0 mL) and saturated brine (1.0 mL) successively. The organic layer was dried over Na_2SO_4 and then evaporated under reduced pressure. The residue was purified by preparative TLC [silica gel (0.25 mm thickness, applied for 18 cm and developed with $CHCl_3$–$(CH_3)_2CO$ (20:1) for 9 cm and eluted with $CHCl_3$–MeOH (9:1)] to furnish a diester, **1a** (0.6 mg). Through a similar procedure, **1b** (0.5 mg) was prepared from **1** (0.5 mg) using (*S*)-MTPA (28.2 mg), EDC (25.6 mg) and DMAP (11.3 mg).

(*R*)-MTPA diester, (**1a**): Amorphous powder; ^1H-NMR ($CDCl_3$) δ: 7.57–7.52 (4H, m, aromatic protons), 7.43–7.39 (6H, m, aromatic protons), 5.08 (1H, m, H-9), 5.02 (1H, m, H-3), 3.56 (3H, br s, OMe), 3.53 (3H, br s, OMe), 3.17 (1H, dd, *J* = 10, 10 Hz, H-4), 1.79 (1H, dd, *J* = 12, 4 Hz, H-2eq), 1.64 (2H, m, H-8), 1.50 (1H, m, H-5), 1.49 (1H, m, H-7a), 1.29 (3H, d, *J* = 6 Hz, H_3-10), 1.27 (1H, dd, *J* = 12, 12 Hz, H-2ax), 1.12 (1H, m, H-7b), 1.08 (3H, d, *J* = 6 Hz, H_3-13), 0.91 (3H, s, H_3-12), 0.89 (3H, s, H_3-11), 0.72 (1H, br d, *J* = 11 Hz, H-6); HR-ESI-TOF-MS (positive-ion mode) *m/z*: 685.2581 $[M + Na]^+$ (Calcd for $C_{33}H_{40}O_7F_6Na$: 685.2570).

(*S*)-MTPA diester, (**1b**): Amorphous powder; ^1H-NMR ($CDCl_3$) δ: 7.56–7.52 (4H, m, aromatic protons), 7.43–7.37 (6H, m, aromatic protons), 5.08 (1H, m, H-9), 4.99 (1H, m, H-3), 3.57 (6H, br s, OMe), 3.09 (1H, dd, *J* = 10, 10 Hz, H-4), 1.83 (1H, dd, *J* = 12, 4 Hz, H-2eq), 1.59 (2H, m, H-8), 1.44 (1H, m, H-5), 1.36 (1H, m, H-7a), 1.34 (3H, d, *J* = 6 Hz, H_3-10), 1.32 (1H, dd, *J* = 12, 12 Hz, H-2ax), 1.04 (1H, m, H-7b), 0.99 (3H, d, *J* = 6 Hz, H_3-13), 0.84 (3H, s, H_3-12), 0.82 (3H, s, H_3-11), 0.65 (1H, br d, *J* = 11 Hz, H-6); HR-ESI-TOF-MS (positive-ion mode) *m/z*: 685.2581 $[M + Na]^+$ (Calcd for $C_{33}H_{40}O_7F_6Na$: 685.2570).

Preparation of (*R*)- and (*S*)-MTPA diesters (**2a** and **2b**) of **2**

Through a similar procedure, (*R*)- and (*S*)-MTPA diesters, **2a** (1.0 mg) and **2b** (0.9 mg), were prepared from **2** (1.0 mg each) with the respective reagents, (*R*)- and (*S*)-MTPA (13.6 and 12.6 mg), EDC (11.2 and 10.7 mg) and DMAP (5.9 and 6.7 mg).

(*R*)-MTPA diester, (**2a**): Amorphous powder; ^1H-NMR ($CDCl_3$) δ: 7.57–7.51 (4H, m, aromatic protons), 7.42–7.36 (6H, m, aromatic protons), 5.74 (1H, d, *J* = 16 Hz, H-7), 5.69 (1H, dd, *J* = 16, 6 Hz, H-8), 5.61 (1H, qd, *J* = 6, 6 Hz, H-9), 5.12 (1H, ddd, *J* = 12, 10, 5 Hz, H-3), 3.61

(1H, dd, J = 10, 10 Hz, H-4), 3.55 (3H, br s, OMe), 3.52 (3H, br s, OMe), 1.90 (1H, dq, J = 10, 7 Hz, H-5), 1.84 (1H, dd, J = 12, 12 Hz, H-2ax), 1.65 (1H, dd, J = 12, 5 Hz, H-2eq), 1.39 (3H, d, J = 6 Hz, H_3-10), 1.08 (3H, s, H_3-11), 0.95 (3H, d, J = 7 Hz, H_3-13), 0.812 (3H, s, H_3-12); HR-ESI-TOF-MS (positive-ion mode) *m/z*: 699.2362 $[M + Na]^+$ (Calcd for $C_{33}H_{38}O_8F_6Na$: 699.2363).

(*S*)-MTPA diester, (**2b**): Amorphous powder; ^{1}H-NMR ($CDCl_3$) δ: 7.56–7.51 (4H, m, aromatic protons), 7.42–7.36 (6H, m, aromatic protons), 5.64–5.59 (2H, m, H-7 and 8), 5.62 (1H, qd, J = 6, 6 Hz, H-9), 5.10 (1H, ddd, J = 12, 10, 5 Hz, H-3), 3.54 (1H, dd, J = 10, 10 Hz, H-4), 3.58 (3H, br s, OMe), 3.56 (3H, br s, OMe), 1.92 (1H, dd, J = 12, 12 Hz, H-2ax), 1.84 (1H, dq, J = 10, 7 Hz, H-5), 1.71 (1H, dd, J = 12, 5 Hz, H-2eq), 1.43 (3H, d, J = 6 Hz, H_3-10), 1.03 (3H, s, H_3-11), 0.88 (3H, d, J = 7 Hz, H_3-13), 0.811 (3H, s, H_3-12); HR-ESI-TOF-MS (positive-ion mode) *m/z*: 699.2368 $[M + Na]^+$ (Calcd for $C_{33}H_{38}O_8F_6Na$: 699.2363).

Enzymatic hydrolysis of **3**

Compound **3** (10.0 mg) was hydrolyzed with β-glucosidase (5.5 mg) at 37°C in 1 mL of 20 mM acetate buffer (pH 5.0) with reciprocal shaking for 12 h. The liberation of glucose was monitored by TLC analysis ($CHCl_3$:MeOH:H_2O, 15:6:1, R_f values, **3**: 0.50, aglycone **1a**: 0.76, and glucose: 0.19). The reaction mixture was concentrated and then subjected to preparative TLC (silica gel, 0.25 mm thickness, applied for 18 cm and developed with $CHCl_3$:MeOH:H_2O, 15:6:1 for 9 cm and eluted with the same solvent) to furnish an aglycone, **3a** (5.5 mg). The absolute configuration of the liberated glucose was determined to be of the D-series from the positive optical rotation sign and the retention time (8.7 min) on HPLC analysis [JASCO OR-2090 *Plus*; Optical rotation detector, Shodex Asahipak NH2P-50; Φ = 4.5 mm, L = 25 cm, 75% CH_3CN aq., 1 mL/min] of the sugar-containing fraction. Peak materials were identified by co-chromatography with authentic D-glucose. The physicochemical data for **3a** including the optical rotation value were essentially identical to those of **2**. Finally, this identity was confirmed by preparing MTPA diesters through a similar procedure to that mentioned above, (*R*)- and (*S*)-MTPA diesters, **3b** (0.6 mg) and **3c** (0.4 mg), being prepared from **3a** (0.7 mg each) with the respective reagents, (*R*)- and (*S*)-MTPA (10.5 and 11.7 mg), EDC (11.8 and 10.6 mg) and DMAP (11.1 and 10.3 mg). ^{1}H-NMR of **3b** and **3c** was essentially identical to that of **2a** and **2b**, respectively; HR-ESI-TOF-MS (positive-ion mode) of **3b** and **3c**, *m/z*: 699.2361 and 699.2358 $[M + Na]^+$, respectively (Calcd for $C_{33}H_{38}O_8F_6Na$: 699.2363).

Pivaloylation of **4**

A solution of **4** (2.4 mg) in 1.0 mL of dehydrated pyridine was reacted with pivaloyl chloride (5 μL) on ice for 3.0 h with stirring. After the addition of 0.5 mL of H_2O, the reaction mixture was concentrated under reduced pressure. The residue was purified by preparative TLC (silica gel, 0.25 mm thickness, applied for 18 cm and developed with $CHCl_3$–MeOH (10:1) for 9 cm and eluted with $CHCl_3$-MeOH (2:1) to furnish a pivaloyl ester, **4a** (1.8 mg). **4a**: Amorphous powder; ^{1}H-NMR ($CDCl_3$) δ: 4.20 (1H, dd, J = 11, 3 Hz, H-13a), 3.92 (1H, dd, J = 11, 6 Hz, H-13b), 3.79 (1H, dddd, J = 11, 11, 4, 4 Hz, H-3), 3.73 (1H, m, H-9), 2.06 (1H, m, H-4 eq), 1.72 (1H, ddd, J = 12, 4, 2 Hz, H-2eq), 1.67 (1H, m, H-5), 1.60 (1H, m, H-7a), 1.46-1.38 (2H, m, H_2-8), 1.23 (9H, s, CH_3), 1.18 (3H, d, J = 6 Hz, H_3-10), 1.13 (1H, dd, J = 12, 11 Hz, H-2ax), 1.12 (1H, ddd, J = 12, 12, 12 Hz, H-4ax), 1.09 (1H, m, H-7b), 0.98 (3H, s, H_3-12), 0.85 (1H, ddd, J = 11, 5, 3 Hz, H-6), 0.84 (3H, s, H_3-11); ^{13}C-NMR ($CDCl_3$) δ: 178.6 ($Me_3\underline{C}\underline{C}$ = O–), 68.5 (C-9), 67.0 (C-13), 66.7 (C-3), 50.9 (C-2), 47.4 (C-6), 41.1 (C-8), 40.0 (C-4), 39.0 ($Me_3\underline{C}C$ = O–), 38.6 (C-5), 35.9 (C-1), 30.6 (C-12), 27.3 ($\underline{Me_3}CC$ = O–), 24.6 (C-7), 23.6 (C-10), 20.8 (C-11); HR-ESI-TOF-MS (positive-ion mode) *m/z*: 337.2353 $[M + Na]^+$ (Calcd for $C_{18}H_{34}O_4Na$: 337.2349).

Preparation of (*R*)- and (*S*)-MTPA diesters (**4b** and **4c**) of **4a**

Through a similar procedure to that described above, (*R*)- and (*S*)-MTPA diesters, **4b** (0.73 mg) and **4c** (0.82 mg), were prepared from **4a** (0.9 mg each) with the respective reagents, (*R*)- and (*S*)-MTPA (29.2 and 23.1 mg), EDC (22.3 and 21.7 mg) and DMAP (8.1 and 10.5 mg).

(*R*)-MTPA diester, (**4b**): Amorphous powder; ^{1}H-NMR ($CDCl_3$) δ: 7.54–7.51 (4H, m, aromatic protons), 7.43–7.38 (6H, m, aromatic protons), 5.15 (1H, m, H-3), 5.05 (1H, m, H-9), 4.16 (1H, dd, J = 11, 3 Hz, H-13a), 3.93 (1H, dd, J = 11, 6 Hz, H-13b), 3.54 (3H, br s, OMe), 3.52 (3H, br s, OMe), 2.15 (1H, m, H-4 eq), 1.76 (2H, m, H-2eq and H-5), 1.61 (2H, m, H-8), 1.56 (1H, m, H-7a), 1.32 (1H, ddd, J = 12, 12, 12 Hz, H-4ax), 1.25 (1H, m, H-2ax), 1.20 (9H, s, $\underline{Me_3}CC$ = O–), 1.08 (1H, m, H-7b), 0.86 (3H, s, H_3-11), 0.92 (3H, s, H_3-12); HR-ESI-TOF-MS (positive-ion mode) *m/z*: 769.3152 $[M + Na]^+$ (Calcd for $C_{38}H_{48}O_8F_6Na$: 769.3145).

(*S*)-MTPA diester, (**4c**): Amorphous powder; ^{1}H-NMR ($CDCl_3$) δ: 7.57–7.50 (4H, m, aromatic protons), 7.43–7.38 (6H, m, aromatic protons), 5.13 (1H, m, H-3), 5.06 (1H, m, H-9), 4.12 (1H, dd, J = 11, 3 Hz, H-13a), 3.86 (1H, dd, J = 11, 6 Hz, H-13b), 3.57 (3H, br s, OMe), 3.55 (3H, br s, OMe), 2.06 (1H, m, H-4 eq), 1.79 (1H, m, H-2 eq), 1.72 (1H, m, H-5), 1.56 (2H, m, H-8), 1.42 (1H, m, H-7a), 1.33

(3H, d, $J = 6$ Hz, H_3-10), 1.32 (1H, m, H-2ax), 1.19 (1H, m, H-4ax), 1.18 (9H, s, $\underline{Me_3}CC = O$–), 1.00 (1H, m, H-7b), 0.84 (3H, s, H_3-12), 0.79 (3H, s, H_3-11), 0.79 (1H, m, H-6); HR-ESI-TOF-MS (positive-ion mode) *m/z*: 769.3149 $[M + Na]^+$ (Calcd for $C_{38}H_{48}O_8F_6Na$: 769.3145).

DPPH radical-scavenging assay

The reagents, (*S*)-(−)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and 2,2-diphenyl-1-picrylhydrazyl (DPPH), were purchased from Aldrich Chemical Co., and the DPPH radical-scavenging activities of the isolated compounds were examined according to the method previously described [26].

Human cancer cell growth inhibition assay

Growth inhibitory activities were determined using human promyelocytic leukemia cells (HL-60) and human small cell lung cancer cells (SBC-3) by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) method [27].

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SHORT COMMUNICATION

Biological Activities of Indian Celery, *Seseli diffusum* (Roxb. ex Sm.) Sant. & Wagh

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In continuation of our work on Indian celery (*Seseli diffusum* (Roxb. ex Sm.) Santapau & Wagh; Umbelliferae), the fractionation of the 80% MeOH–H_2O extract of the seeds was performed to identify the principles responsible for its folk use as an antispasmodic and diuretic. Several compounds were isolated as active components: seselin (1) and anthriscinol methyl ether (4) showed a selective cytotoxicity to some yeast strains. Compound 1 also showed spasmolytic activity. On the other hand, isopimpinellin (3) and isorutarin (5) exhibited a spasmogenic effect on the smooth muscle preparations. Compound 5 was also found to have antioxidant activity. Among them, compound 4 was isolated for the first time from this plant. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: Indian celery; *Seseli diffusum*; cytotoxicity; spasmolytic activity; spasmogenic activity; antioxidant activity.

INTRODUCTION

Previously it was reported that the celery (Indian celery) sold in southern areas of Pakistan is not the common celery, *Apium graveolens*. This was identified as *Seseli diffusum* (Umbelliferae) based on the nuclear rDNA, and ITS sequence analysis, morphological features and phytochemical compositions (Maruyama *et al.*, 2009). Seeds of *S. diffusum* are prescribed as an antispasmodic for the treatment of bronchitis, asthma, as well as in liver obstructions, intestinal debility and spleen disorders (Usmanghani *et al.*, 1997). In continuation of our work on Indian celery, 80% methanol–water extract of seeds (botanically fruits) showed some interesting biological activities, such as cytotoxicity against the yeast strains, smooth muscle relaxant and antioxidant activities. Bioassay-guided fractionation using yeast cytotoxicity assay has led to the isolation and identification of DNA damaging agents **1–2** in lipophilic extracts of *S. diffusum*. Then fractionation was performed of the extract of Indian celery to identify the compounds responsible for the biological activities, and four coumarin derivatives and one phenylpropanoid isolated as some of the active principles.

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† This publication is dedicated to the memory of the Professor Yoshisuke Tsuda (1932– 2006).

MATERIAL AND METHODS

General. Melting points (m.p.) were determined on a Yanaco MP-S3 apparatus and are uncorrected. The NMR spectra were recorded on Bruker AM-400 and AMX-500 spectrometers using a UNIX data system at 400 and 500 MHz. The ^{13}C-NMR spectra were recorded on the same instruments at 100 and 125 MHz respectively. The ^{1}H- and ^{13}C-NMR spectra were measured using solvents CD_3OD or $CDCl_3$ and referenced with respect to the residual solvent signals. The chemical shift (δ) values were reported in ppm, and coupling constants (J) were measured in Hz. Electron impact mass spectra (EI-MS) were taken at 70 eV on Finnigan MAT-112 or MAT-312 instruments, and major ions are given by m/z (%). Optical rotations were measured on a digital polarimeter Jasco DIP-360 in methanol. Infrared spectra were obtained on a Vector 22 Bruker spectrophotometer, either in KBr pellets or in chloroform, and presented in cm^{-1}. The TLC was performed on pre-coated silica gel cards (E. Merck, Germany) and the spots were observed first under UV (254 nm), and then stained with cerium (IV) sulfate spray reagent, and heated until coloration developed.

Plant material. The seeds of Indian celery were purchased from a local market, Jodia Bazar, Karachi, Pakistan, in July 2000. This was then identified as *S. diffusum* (Maruyama *et al.*, 2009). The sample was deposited in the Division of Pharmacognosy, Phytochemistry and Narcotics, National Institute of Health Sciences, Japan.

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Extraction and isolation. One hundred grams of Indian celery seeds (*S. diffusum*) was extracted successively with 400 mL of hexane, chloroform, acetone and methanol for 4 h under reflux. The extraction was performed four times for each solvent. The yields of extracts were 1.1, 2.2, 5.0 and 18 g, respectively. The silica gel column chromatography of the hexane extract was carried out with hexane–EtOAc and EtOAc–MeOH gradient elution to afford five fractions. Further chromatographic separation yielded compounds **1–3** (43, 14 and 5 mg, respectively) from the third fraction (Fr. 3, 64 mg), while compounds **1** and **4** (27 and 35 mg, respectively) were isolated from the second fraction (Fr. 2, 740 mg). Compounds **1–3** were also isolated from chloroform–acetone extracts through similar chromatographic processes. The methanol extract (water soluble part) was partitioned into EtOAc and water. The aqueous layer was evaporated and the residue was chromatographed on a silica gel column with an EtOAc–MeOH–BuOH–H_2O (80:10:0.5:0.5) elution to obtain isorutarin (**5**; 50 mg). The DNA damaging activities of fractions and pure compounds are presented in Table 1. Physical and spectral data of **1–5** (Fig. 1) were in agreement with those reported in the literature for seselin (Sattar *et al.*, 1978), bergapten (Masuda *et al.*, 1998), isopimpinellin (Elgamal *et al.*, 1979), anthriscinol methyl ether (Ikeda *et al.*, 1998) and isorutarin (Okuyama *et al.*, 1989), respectively.

Seselin (**1**). Colorless prisms from hexane, mp. 120-121 °C (119–120 °C, Murray *et al.*, 1982).

Bergapten (**2**). Colorless needles from EtOAc–hexane, mp.188–189 °C, (188 °C, Murray *et al.*, 1982).

Isopimpinellin (**3**). Colorless needles from EtOAc–hexane, mp. 151–153 °C (lit.149–150 °C, Murray *et al.*, 1982).

Anthriscinol methyl ether (**4**). Pale yellow oil. EI-MS (M^+, *m/z* 222). ^{1}H-NMR ($CDCl_3$, 400 MHz) : 6.59 (1H, d, $J_{2',6'} = 1.4$ Hz), 6.51 (1H, d, $J_{6',2'} = 1.4$ Hz), 6.46 (1H, dd, $J_{3,2} = 15.8$ Hz, $J_{3,1(a,b)} = 1.4$ Hz), 6.09 (1H, dt, $J_{2,3} = 15.8$ Hz, $J_{2,1(a,b)} = 6.0$ Hz), 5.92 (2H, s), 4.03 (2H, dd, $J_{1(a,b),2} = 6.0$ Hz, $J_{1(a,b),3} = 1.4$ Hz), 3.86 (3H, s), 3.35 (3H, s).

Isorutarin (**5**). Colorless prisms from EtOH, mp. 265–267 °C (lit. 261–263 °C, Okuyama *et al.*, 1989).

Cytotoxicity to DNA repair-deficient (rad 52Y) and repair-proficient (RAD$^+$) yeast strain. The bioassay was performed according to the method reported by Gunatilaka *et al.* (1992, 1994). This mechanism-based bioassay, employing DNA repair-deficient (*rad* 52Y) and repair-proficient (RAD$^+$) yeast strains, is a convenient method for the *in vitro* screening of potential antitumor compounds (Gunatilaka and Kingston, 1998). The two types of genetically engineered yeast strains (*Saccharomyces cerevisiae*), which were provided by Mr L. Faucette in SmithKline Beecham Pharmaceuticals (King of Prussia, Pennsylvania USA), were plated on YPD agar plates (9 × 9 cm; 7 mm layer). Each 96-well plate (6 mm diameter each) was filled for various concentrations of samples (100 μL in (1:1) DMSO–MeOH). The plates were read after 48 h at 30 °C. The activity was expressed as IC_{12} (μg/mL) (concentration required to produce an inhibition zone of 12 mm diameter). The data are presented in Table 1. Streptonigrin was used as the positive control.

Activity on isolated smooth muscle contractility. The experiments on rabbits were performed in accordance with the guidelines of the Institute of Laboratory Animal Resources, Commission on Life Sciences (National Research Council, Washington DC, USA) and were approved by the Aga Khan University's Ethics Committee for Research on Animals. The assay was carried out as described earlier by Ghayur and Gilani (2005). Briefly, segments of rabbit jejunum tissue were suspended in 10 mL tissue bath containing Tyrode's solution, aerated with a mixture of 95% O_2 and 5% CO_2 at 37 °C. A preload of 1 g was applied to each tissue, and then kept undisturbed for an equilibrium period of 30 min. Afterwards, responses to sub-maximal concentrations of acetylcholine (0.3 μM) were obtained. The tissues were presumed stable only after the reproducibility of these responses. Contractions for control and test

Table 1. Results of cytotoxicity bioassays on extracts and compounds of *Seseli diffusum*

Fraction/Compound	*rad* 52Y (mutant)[a]	RAD$^+$ (Wild)[a]	Muscle contractility	DPPH
80% MeOH–water	1000	> 1500	0.3 ± 0.05[b]	22.51[e]
Hexane extract	100	500	0.03 ± 0.002[b]	-
Chloroform extract	800	> 1000	0.12 ± 0.005	-
Acetone extract	650	> 1000	0.14 ± 0.06[b]	-
MeOH extract (water soluble)	> 1000	> 1000	0.24 ± 0.09[b]	37.55[e]
Seselin (**1**)	25	> 200	0.04 ± 0.005[b]	-
Bergapten (**2**)	50	> 200	-	-
Isopimpinellin (**3**)	100	> 200	0.1 ± 0.01[c]	-
Anthriscinol methyl ether (**4**)	20	50	-	-
Isorutarin (**5**)	40	> 100	0.3 ± 0.05[c]	79.34[d]
Streptonigrin (reference)	0.4	1.0		
Propyl gallate (reference)			92.14[d]	

- , Inactive; NT, not tested.

[a]IC_{12} in μg/mL.

[b]Spasmolytic activity, EC_{50} (mg/mL) mean ± SEM.

[c]Spasmogenic activity, EC_{50} (mg/mL) mean ± SEM.

[d]% inhibition at 1 mg/mL.

[e]200 μg/mL/1 mM.

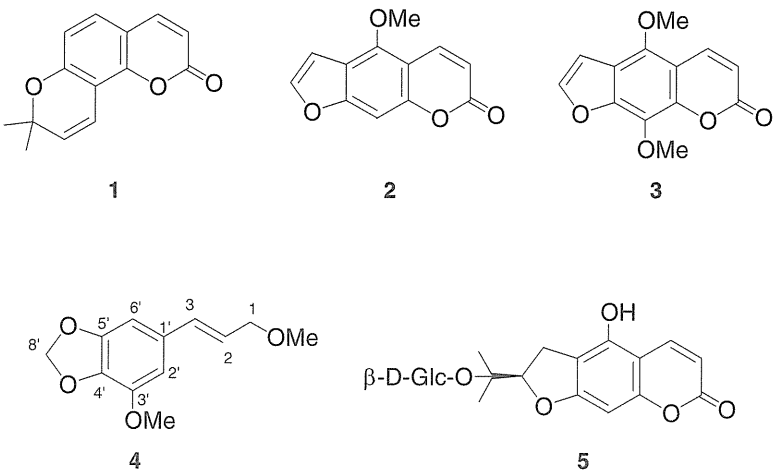

Figure 1. Structures of compounds **1–5**.

were recorded isotonically, using Harvard student oscillographs and transducers. Under these conditions, rabbit jejunum exhibited spontaneous rhythmic contractions, allowing the testing of relaxant (spasmolytic) or stimulant (spasmogenic) activities. Stock solutions of all the test extracts, fractions and compounds were made in saline or if not soluble, in 10% DMSO. All dilutions were made in saline. The final bath concentration was kept at < 0.001 mg/mL of DMSO, which had no effect on the contractions (data not shown).

DPPH Radical scavenging assay. The assay was performed according to the method developed by Lee *et al.* (1998). Briefly, reaction mixtures comprising incremental concentrations of test compounds and 300 mM of DPPH were prepared. After the incubation in a 96-well plate at 37 °C for 30 min, the absorbance at 515 nm was measured by an ELISA reader (SpectraMax plus, Molecular Devices, CA, USA). The percent radical scavenging activity was determined in comparison with the DMSO-treated control (3-*t*-butyl-4-hydroxyanisole).

RESULTS AND DISCUSSION

The 80% methanol–water extract of the Indian celery, *Seseli diffusum* (Roxb. ex. sm.) Santapau & Wagh, showed significant cytotoxicity in the mechanism-based bioassay, employing DNA repair-deficient (*rad* 52Y) and repair-proficient (RAD^+) yeast strains. This fraction also showed spasmolytic and antioxidant activities. The cytotoxicity to yeast strains was found in hydrophobic (hexane and chloroform) extracts of the seeds. In contrast, the water-soluble fraction showed a potent antioxidant activity, but a weak cytotoxic activity. Spasmolytic activity was found in both hydrophobic and hydrophilic fractions (Table 1). The hexane fraction was found to be most active in the cytotoxicity assay. Two compounds, seselin (**1**) and anthriscinol methyl ether (**4**), were mainly isolated from this fraction. The chloroform fraction yielded mostly bergapten (**2**) and isopimpinellin (**3**). The compound **4**, a known constituent from *Anthriscus sylvestris* (Ikeda *et al.*, 1998; Kozawa *et al.*, 1982), is reported for the first time from *Seseli diffusum*. The activities of the isolated compounds were also determined (Table 1). Gunatilaka *et al.* (1994) reported that in the mutant yeast assay, angular pyrano-coumarins, such as seselin (**1**), isolated from Rutaceous plants, were active, while linear furanocoumarins were inactive. Interestingly, our results demonstrated that linear furanocoumarin, bergapten (**2**) and non-cyclized compound **4**, were as active as angular pyrano-coumarins, such as seselin (**1**).

The 80% methanol–water extract of the seeds prepared for the preliminary random screening process demonstrated a dose dependent spasmolytic activity in spontaneously contracting isolated rabbit jejunum. Among the extracts prepared as a result of refluxing with various solvents, the hexane extract showed the most potent activity, followed by methanol, chloroform and acetone extracts. Among the lipophilic compounds isolated from Indian celery, compound **1** was the most potent in its spasmolytic activity, equipotent to that of its parent hexane fraction. Compounds **2** and **4** were inactive in a dose up to 0.5 mg/mL. By contrast, compound **3** showed a stimulant (spasmogenic) effect in the assay. Compound **5**, isolated from the water-soluble fraction, also showed a spasmogenic effect. However, compound **1** was much more potent in its spasmolytic effect than the spasmogenic effects of **3** and **5**. Judging from the content and potency of these compounds in Indian celery, the crude extract of the seed showed domination of spasmolytic activity. Isorutarin (**5**) also showed prominent radical scavenging activity in a DPPH assay, while the others were inactive.

It is worth mentioning here that the isolated compounds **1–5** were the major constituents in various fractions of the crude extract. This study, therefore, does not represent an account of the minor constituents contributing towards the observed bioactivities of the crude extract. A study of the spasmolytic and antioxidant constituent(s) from the water-soluble (hydrophilic) fraction will be made later.

CONCLUSION

In conclusion, the isolation study of Indian celery, guided by its biological activities, revealed that the cytotoxicity to yeast strains was mainly attributed to seselin (**1**) and anthriscinol methyl ether (**4**). Bioassay-guided

fractionation (Table 1), using antispasmodic and spasmogenic assays, have led to the identification of several active constituents. Spasmolytic activity was observed in **1**, while isopimpinellin (**3**) and isorutarin (**5**) exhibited a spasmogenic effect on the smooth muscle preparations. This study therefore rationalizes the folk usage of *Seseli diffusum* as an antispasmodic and diuretic medicine (Datta and Banerjee, 1978; Usmanghani *et al.*, 1997). Furthermore, compound **5** was found to have an antioxidant activity.

Dedication

This publication is dedicated to the memory of the Professor Yoshisuke Tsuda (1932–2006). Professor Tsuda made major contributions in the field of carbohydrate, alkaloid and saponin chemistry. He spent his life exploring the fascinating world of organic synthesis and in developing a conceptual foundation of pharmacognosy. He worked tirelessly for the promotion of science in developing countries and dedicated many years of his life to creating a scientific environment across the globe, irrespective of national boundaries. He was a great friend of Pakistan, and an admirer of the rich traditions and heritage of this ancient land.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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論　　文
Regular article

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DNA 配列解析及び形態観察に基づく *Sida* 属植物製品の実態調査

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Surveying studies on the botanical source of the herbal materials sold as the *Sida* products based on the genetic and the microscopic features

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Abstract

During the course of our study on the borderline of pharmaceuticals to non-pharmaceuticals, the morphological features and the internal transcribed spacer (ITS) sequences in the nuclear rDNA of *Sida* plants and the crude drugs/health foods so called *Sida* products were investigated. As the results, we revealed that 7 of 11 products tested contained *Sida* plants and 3 products among them included the other plant material(s) together with *Sida*. The ITS sequences of *Sida* plants observed in this study were classified into 6 genotypes. One of them is identical with that of *Sida fallax* whereas the others had no identical sequence on the international nucleotide sequence databases. On the other hand, other species including *Urena*, *Malva* and *Triumfetta* plants of the family, Malvaceae were detected from 7 products.

In field survey on Oahu Island, the state of Hawaii, USA, Malvaceus plants possessing a *Sida* like flower were observed at the same place together with *Sida* plant. This growing environment in field is likely to be one of the reasons for the contamination in the products. Simultaneously, our field survey suggests that the appearances of the flowers were not critical points for the identification of *Sida* plants. Based on microscopic observations, we found that the stellate hair on leaves and the features of mericarps were suitable for the purpose.

In conclusion, the exact identification of their botanical origin is important for regulation of *Sida* products on the borderline of pharmaceuticals to non-pharmaceuticals.

Keywords : 食薬区分、*Sida* 属植物、DNA 配列解析、形態観察
Borderline of pharmaceuticals to non-pharmaceuticals, *Sida* species, DNA sequence analysis, morphological observation

I　緒言

Sida 属は、熱帯—温帯地域を中心に、世界に 100 種以上が分布するアオイ科 (Malvaceae) 最大の属である[1-3]。*Sida* 属植物の中には、アーユルヴェーダやユナニー、中医学などの伝統医学において、リウマチや淋病治療、解熱鎮痛、利尿、消炎など、様々な目的で使用される *S. cordifolia* L., *S. rhombifolia* L., *S. acuta* Burm. f. など、強い薬理作用

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を有するものもあり、その主要成分は、エフェドリンを初めとする多種のアルカロイドであると考えられる[4-6]。アーユルヴェーダにおいて"Bala"と呼称される生薬は、*S. cordifolia*の根であるとされるが、インドの生薬市場では、他に、*S. acuta*, *S. cordata* Borss. Waalk., *S. rhombifolia*などの同属植物も"Bala"として使用されている[5]。また、中国では、*S. mysorensis* Wight et arn. 及び*S. cordifolia*の根又は葉を「黄花仔」、*S. acuta*の根又は葉を「黄花稔」、*S. rhombifolia*の全草を「黄花母」と称して、清熱、消炎、排膿などの目的で内服あるいは外用する[6]。

我が国にも、種子島以南の亜熱帯地域を中心に、*S. rhombifolia*（和名：キンゴジカ）、*S. spinosa* L.（和名：アメリカキンゴジカ）及び*S. rhombifolia* subsp. *insularis* Hatusima（和名：ハイキンゴジカ）の3種が野生あるいは帰化し、最近では、関東以西まで分布を広げている[7]。

*Sida*属植物の食薬区分上の取り扱いは、*Sida cordifolia*及び*S. mysorensis*の根及び葉が、「オウカシ」として、*S. rhombifolia*の全草が、「オウカボ」として、専ら医薬品として使用される成分本質（原材料）リストに収載されている[8]。しかし、上述の通り、*Sida*属植物は、非常に多くの種が存在し、その中には、*S. cordifolia*と同様に生薬として使用あるいは誤用されるものもある。さらに、これら同属植物にもエフェドリン、インドール及びキナゾリンアルカロイドが含まれることが報告されている[5,9]。

従って、既に専ら医薬品に指定されている3種以外の*Sida*属植物を使用した痩身目的等の健康食品が流通した場合、健康被害の発生が危惧される。また、エフェドリンは覚醒剤原料であることから、乱用目的での*Sida*属植物の使用も懸念される。

そこで本研究では、*Sida*属植物の有害性評価及び規制の範囲の検討に資する知見を得ることを目的に、まず、市場に流通する*Sida*属植物製品の実態調査を行う事とした。インターネット及び商社を通じて*Sida*属植物として販売されていた生薬あるいは健康食品を購入し、これらの試料について、形態観察及びrDNAのinternal transcribed spacer (ITS)領域の塩基配列解析による基原種の推定を行った。さらに、*Sida*属植物として販売されている生薬／健康食品には、他の植物由来のものが多く流通している事が明らかになった事から、*Sida*属植物の形態学的な特徴について情報を得るため、*Sida*属植物の自生が報告されている横浜市、茅ヶ崎市及びハワイ、オアフ島において植物採集を試み、オアフ島で得られた植物について、上記の生薬との比較検討を行った。

Ⅱ　研究方法

1. 実験材料

海外市場において、*Sida*属植物として販売されていた製品及び国内の園芸店より購入した植物試料(Sfa)の詳細をTable 1にまとめた。また、ハワイ、オアフ島での採集試料の詳細をTable 2にまとめた。これらの標本及び試料は、すべて国立医薬品食品衛生研究所、生薬部に保管されている。なお、横浜市、茅ヶ崎市では、*Sida*属植物と思われる個体を見つける事が出来なかった。

2. 形態観察

葉、分果及び種子の形態観察は、実体顕微鏡（デジタルマイクロスコープレンズ、VH-Z25; Keyence）を用いて行い、葉については上面、下面、葉柄を、分果については芒(のぎ)の上面、正面及び側面を、種子については側面をデジタル画像として記録した。

Table 1.　Details of the *Sida* products purchased in the Japanese and foreign markets

Sample no.	Source	Habitat	Procurance date	Part	Sold as
SCA-1 to 6	Through internet from Company A in USA	Unknown	2009.05	Stem and leaf	*S. cordifolia* whole plant
SCRs SCRr	Through internet from Company B in USA	Unknown	2009.05	Stem and root	*S. acuta* aerial part
Sfa	Through internet from Company C in USA	Unknown	2009.04	Alive plant	*S. fallax*
BSS-1	Through Company D in Osaka from Brazilian firm	Brazil	2009.08	Powder	*S. cordifolia* leaf
BSS-2			2009.08	Cutting leaf	*S. cordifolia* leaf
BSS-3			2009.08	Cutting leaf/stem	*S. carpinifolia* aerial part
BSS-4a, b			2009.08	Cutting leaf/stem	*S. carpinifolia* aerial part
BSS-5			2009.08	Cutting leaf	*S. rhombifolia* aerial part
ISC-1	Through Company D in Osaka from Indian firm	India	2009.10	Whole plant	*S. cordifolia* whole plant
ISC-2				Whole plant powder	*S. cordifolia* whole plant powder
ISC-3				Root powder	*S. cordifolia* root powder
Ss30	Through Company E in Hyogo from Chinese firm	China	2010.09	Whole plant	*S. acuta*（黄花稔）

These samples are deposited in Division of Pharmacognosy, Phytochemistry and Narcotics, National Institute of Health Sciences.

3. 核 rDNA の ITS 塩基配列解析

試料 10 mg を液体窒素にて凍結させた後、MM-300 (Qiagen) を用いて粉砕し、DNeasy Plant Mini Kit (Qiagen) を用いて total DNA を抽出、精製した。このものを鋳型とし、植物の rDNA に保存性の高い配列に設計したプライマー[10]を用いて、PCR を行うことにより、目的の ITS 領域を含む DNA 断片を得た。PCR は、酵素に KOD FX DNA polymerase (Toyobo) を用いて、以下の温度プログラムにより行われた：94°C 2 min; 98°C 10 sec, 50°C 30 sec, 68°C 30 sec, 40 cycles; 68°C 2 min. Montage-PCR (Millipore) により、PCR 産物を精製した後、ダイレクトシークエンスにより塩基配列を決定した。シークエンス反応は、BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) により行い、ABI Prism 3100-Avant Genetic Analyzer (Applied Biosystems) を用いて解析した。得られた配列の相同性検索は、BLASTn search プログラムを用いて行った。また、多重整列解析は、Clustal W プログラム[11]を用いて行い、分子系統樹の作成は、Kimura's two parameter により算出された遺伝距離に基づき、NJ 法[12]により行った。

Table 2. Details of plant samples collected in Oahu island

Sample no.	Habitat	Collection date	Part	Morphological type*
CMH-1	castle memorial hospital, Honolulu	2009.06.26	Aerial	Type B
CMH-2				Type A
CMH-3				Type A
CMH-4				Type A
CMH-5				Type B
CMH-6				Type B

*: type A has the triquetrous mericarp with two awns and many stellate hairs in petiole and lower side of leaf and vein. Type B has the flat reniform mericarp with an awn and many hairs, and the hairs in leaf are longer than those of type B.
These samples are deposited in Division of Pharmacognosy, Phytochemistry and Narcotics, National Institute of Health Sciences (n = 1).

Ⅲ 研究結果

全検体の遺伝子解析結果を Table 3 にまとめた。また、国際塩基配列データベース (DDBJ/EMBL/GenBank; INSD) 上の *Sida* 属植物の配列は、ITS1 と ITS2 に分けて登録されているものが多い[2]事から、*Sida* 属植物と推定された試料については、ITS1, 2 に分けて相同性検索を行い、その結果を Table 4 にまとめるとともに、*Sida* 属植物と推定された試料にデータベース上の *S. acuta*, *S. cordifolia*, *S. fallax* Walp., *S. rhombifolia*, *S. spinosa* L. の配列 (INSD Acc. No.: AJ274952/AJ251608; AJ274945/AJ251601; GQ478107; GQ478108; DQ006018; 以下同じ) 及び外群として *Sida* 属と同じアオイ亜科アオイ連に属する *Abutilon andrewsianum* W.Fitzg. の配列 (AY591807) を加えた分子系統樹を Fig. 1 に、多重整列解析の結果を Fig. 2 に示した。

Table 3. BLASTn search results of the plants found in the *Sida* products

Sample no.	most similar species (Family) INSD Acc. No.; similarity (%)
SCA-1	*Achyranthes bidentata* (Amaranthaceae) AB558157; 95.1
SCA-2	*Desmodium gangeticum* (Leguminosae) GQ413941; 100
SCA-3	not determined
SCA-4	*Urena lobata* (Malvaceae) JN407484; 99.0
SCA-5	*Urena lobata* (Malvaceae) JN407484; 99.0
SCA-6	*Sida* spp.-1
SCRs	*Triumfetta rhomboidea* (Malvaceae) EF107655; 89.8
SCRr	*Sida* spp.-2
Sfa	*Sida* spp.-2
BSS-1	*Malva sylvestris* (Malvaceae) EF419482; 99.3
BSS-2	*Sida* spp.-3
BSS-3	*Abutilon andrewsianum* (Malvaceae) AY591807; 90.2
BSS-4a	*Sida* spp.-4
BSS-4b	*Malvastrum coromandelianum* (Malvaceae) FJ204694; 97.8
BSS-5	*Sida* spp.-3
ISC-1	*Triumfetta rhomboidea* (Malvaceae) EF107655; 90.2
ISC-2	*Chenopodium ficifolium* (Amaranthaceae) HE577466; 99.2
ISC-3	*Sida* spp.-5
Ss30	*Sida* spp.-6
CMH-1	*Malvastrum coromandelianum* (Malvaceae) FJ204694; 98.2
CMH-2	*Sida* spp.-4
CMH-3	*Sida* spp.-4
CMH-4	*Sida* spp.-4
CMH-5	*Malvastrum coromandelianum* (Malvaceae) FJ204694; 98.2
CMH-6	*Malvastrum coromandelianum* (Malvaceae) FJ204694; 98.2

1. 海外市場品及び園芸市場品 (Sfa)

試料 SCA は、*Sida cordifolia* の地上部として、海外よりインターネットを通じて購入した。しかし、この製品に含まれる葉には、様々な特徴を有したものがあり、明らかに複数の植物の混合物と思われた。無作為に 6 枚の葉 (SCA-1 ～ -6) を選び、顕微鏡観察したところ、*Sida* 属植物の大きな特徴の一つである星状毛を持つものは、SCA-6 のみであった。ITS 配列解析の結果からは、SCA-1 は、*Achyranthes bidentata* Blume あるいはその近縁種、SCA-2 は、*Desmodium* 属植物、SCA-4 及び -5 は、*Urena* 属植物と推定された。なお、SCA-3 は、PCR 法による増幅が出来なかったため、解析不能だった。唯一、星状毛が確認された SCA-6 は、*S. fallax* の配列 (GQ478107) や *S. poeppigiana* (K.Schum.) Fryxell の配列 (AJ274954, AJ251610), *S. rhombifolia* の配列 (GQ478108) などと高い相同性を示し、*Sida* 属植物の一つであると推定された (Table 4, Figs. 1, 2)。

試料 SCR は、*Sida acuta* の地上部として、商社より購入した。しかし、この試料には、茎だけでなく、根と思われるものが多量に含まれていた。そこで、茎と思われるもの (SCRs) と、根と思われるもの (SCRr) とに区別して、ITS 配列の解析を行った。その結果、茎と思われたものは、アオイ科の *Triumfetta* 属 (EF107655) や *Corchorus* 属 (DQ311673) 植物の配列と相同性を示した。一方、根と思われたものは、データベース上の *S. fallax* の配列 (GQ478107) 及び園芸店より購入した *S. fallax* (Sfa) の配列と完全に一致した。

園芸店より購入した *S. fallax* (Sfa) は、*Sida* 属植物の特徴である托葉及び星状毛を持ち、黄色の五弁花を咲かせた。このものの ITS 配列は、データベース上の *S. fallax* のもの

Table 4. BLASTn search results of *Sida* plants found in the Sida products

Genotype	Order	ITS1	ITS2
		species; INSD Acc. No.; similarity (%)	species; INSD Acc. No.; similarity (%)
Sida spp.-1	1	*Sida fallax*; GQ478107; 97.6	*Sida fallax*; GQ478107; 99.5
	2	*Sida poeppigiana*; AJ274954; 96.9	*Sida poeppigian*; AJ251610; 98.5
	3	*Sida rhombifolia*; GQ478108; 93.5	*Sida rhombifolia*; GQ478108; 94.0
Sida spp.-2	1	*Sida fallax*; GQ478107; 100	*Sida fallax*; GQ478107; 100
	2	*Sida poeppigiana*; AJ274954; 96.6	*Sida poeppigian*; AJ251610; 98.5
	3	*Sida rhombifolia*; GQ478108; 93.2	*Sida rhombifolia*; GQ478108; 93.5
Sida spp.-3	1	*Sida cordifolia*; AJ274945; 97.2	*Sida cordifolia*; AJ251601; 98.7
	2	*Sida cerradoensis*; AJ274951; 96.2	*Sida cerradoensis*; AJ251607; 98.7
	3	*Sida xanti*; AJ274946; 94.8	*Sida xanti*; AJ274946; 95.6
Sida spp.-4	1	*Sida fallax*; GQ478107; 96.6	*Sida fallax*; GQ478107; 99.1
	2	*Sida poeppigiana*; AJ274954; 96.2	*Sida poeppigian*; AJ251610; 97.3
	3	*Sida rhombifolia*; GQ478108; 96.0	*Sida rhombifolia*; GQ478108; 92.5
Sida spp.-5	1	*Sida spinosa*; DQ006018; 99.3	*Sida spinosa*; DQ006018; 98.7
	2	*Sida spinosa*; AJ274962; 96.6	*Sida spinosa*; AJ251618; 98.7
	3	*Sida odorata*; AJ274960; 89.8	*Sida spinosa*; AJ251614; 98.7
Sida spp.-6	1	*Sida fallax*; GQ478107; 97.9	*Sida fallax*; GQ478107; 99.5
	2	*Sida poeppigiana*; AJ274954; 96.5	*Sida poeppigian*; AJ251610; 97.8
	3	*Sida rhombifolia*; GQ478108; 93.1	*Sida rhombifolia*; GQ478108; 92.1

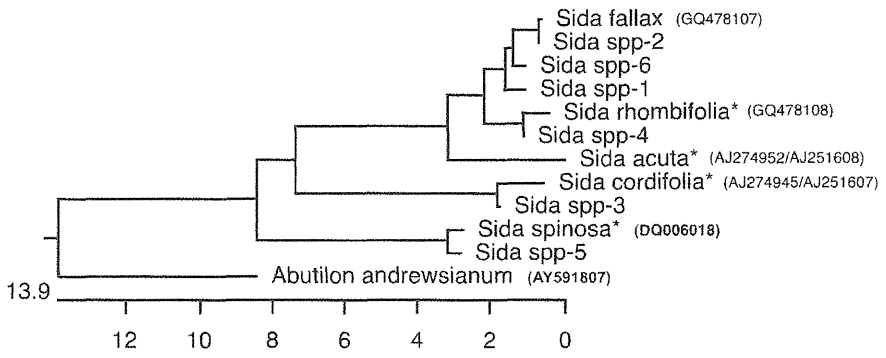

Fig. 1. NJ tree constructed from ITS sequences of the putative *Sida* species among the products used in this study *Abutilon andrewsianum* is out group. Asterisks indicate the species in which the occurrence of ephedrine alkaloids is reported. INSD accession nos. are indicated in parentheses.

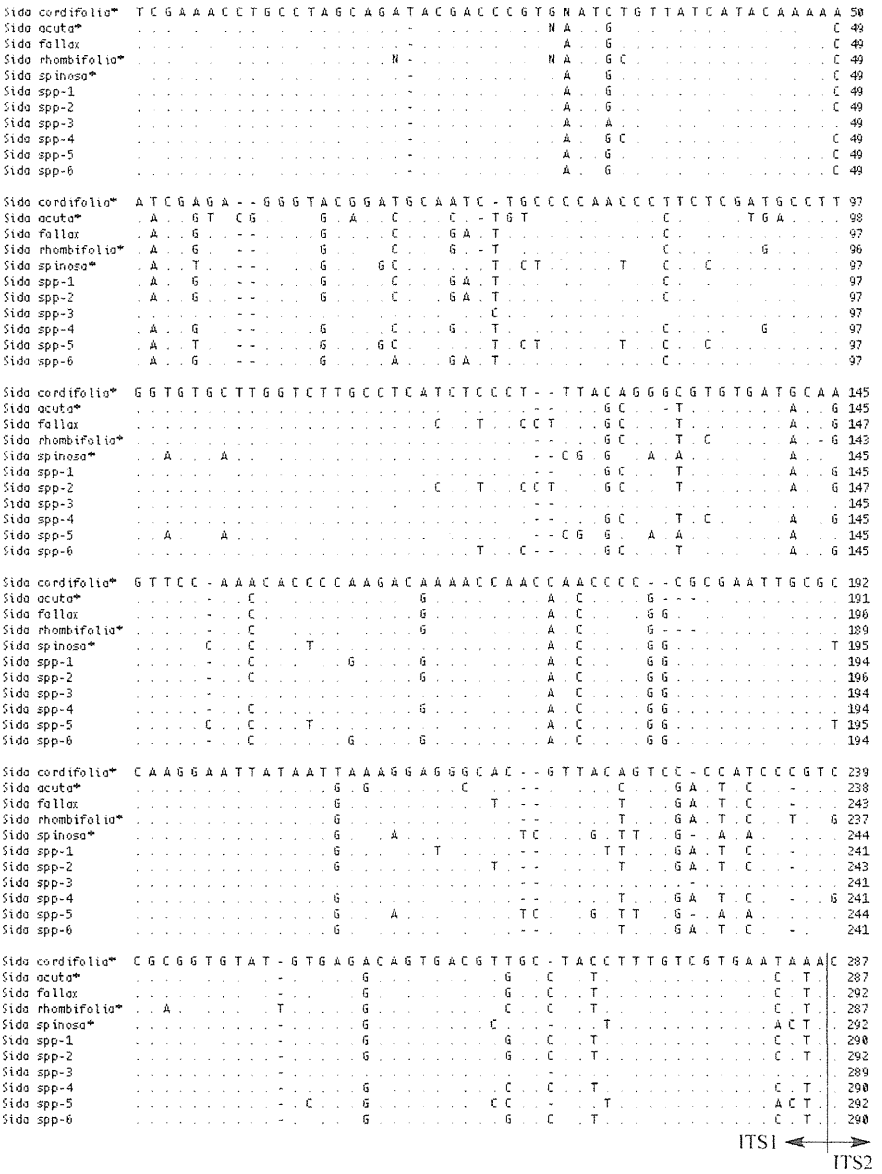

Fig. 2. ITS sequence alignment of *Sida* genotypes observed in this study

Asterisks indicate the species in which the occurrence of ephedrine alkaloids is reported.

(GQ478107) と完全に一致した。

BSS-1 から -5 及び ISC-1 から -3 は、別の商社を通じ、それぞれブラジル及びインドより購入した。この内、葉あるいは分果が含まれていた試料について形態観察を行ったところ、BSS-2 及び -5 は、*Sida* 属植物の特徴である密生した小さな星状毛が葉より確認された。これらの試料の ITS 塩基配列は、*S. cordifolia* の配列 (AJ274945, AJ251601) と高い相同性を示した。BSS-4 には外観上明らかに異なる 2 種類の分果が含まれていたことから、これらの分果を、BSS-4a, -4b と区別して扱った。BSS-4a の分果は、三稜形で、2 本の芒 (のぎ) を持ち、*Sida* 属植物の分果の特徴[7] を有していた。一方、BSS-4b の分果は、扁平な腎形を呈し、芒は 1 本、多数の毛に被われていた。このものは、文献 7 に記載のエノキアオイ *Malvastrum coromandelianum* (L.) Garcke (Malvaceae) の分果の図と良く似た特徴を有していた。これらを塩基配列解析に供したところ、BSS-4a の配列 (*Sida* spp.-4) は、SCA-6 (*Sida* spp.-1) と同じく、*S. fallax* の配列 (GQ478107) や *S. poeppigiana* の配列 (AJ274954, AJ251610), *S. rhombifolia* の配列 (GQ478108) などと高い相同性を示したが、SCA-6 の配列とはわずかな違いを示した (Figs. 1, 2)。一方、BSS-4b は、分果の形態から推察された通り、*Malvastrum coromandelianum* の配列 (FJ204694) と最も高い相同性を示した。その他の試料は、BSS-1 が、*Malva sylvestris* L. の配列 (EF419482) とほぼ一致し、BSS-3 は、*Abutilon* 属植物の配列 (AY591807, EF219369) と相同性を示した。

インドからの試料は、いずれも *S. cordifolia* として購入したが、ISC-1 は、SCRs とよく似た ITS 配列を有し、*Triumfetta* 属植物の配列 (EF107655) や *Corchorus* 属植物の配列 (DQ311673) と相同性を示した。ISC-2 は、ヒユ科の *Chenopodium ficifolium* Sm. の配列 (HE577466) と

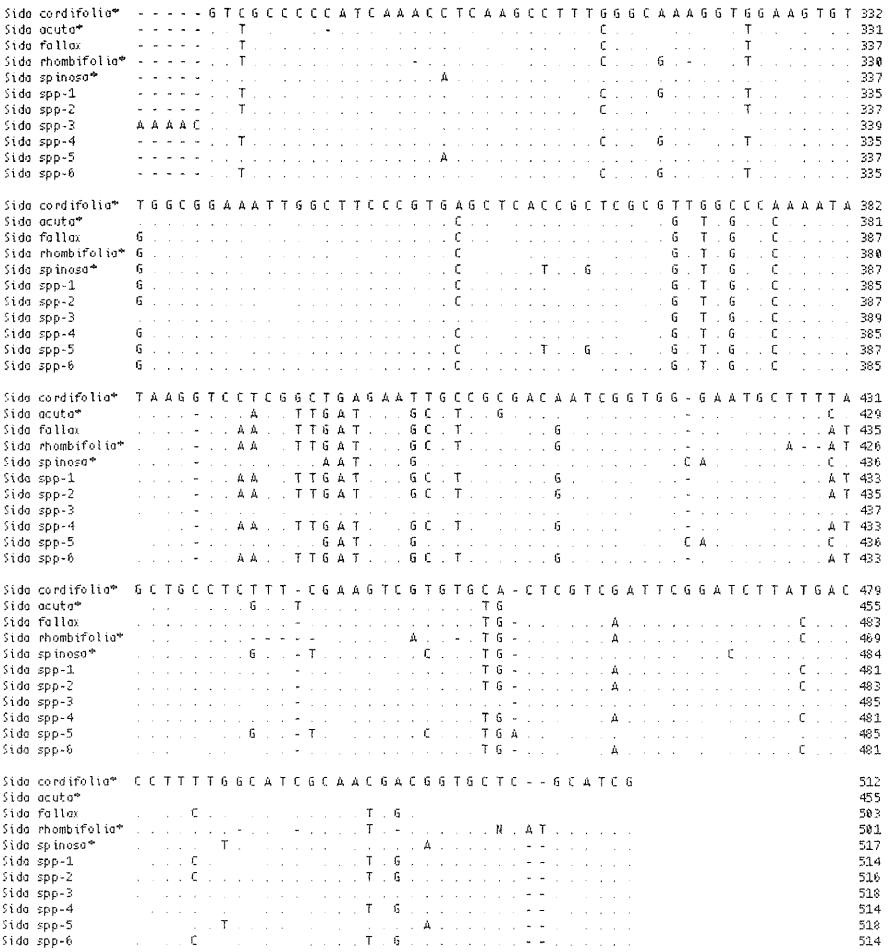

Fig. 2.　ITS sequence alignment of *Sida* genotypes observed in this study (Continued)
Asterisks indicate the species in which the occurrence of ephedrine alkaloids is reported.

高い相同性を示した。*Chenopodium* 属植物の中には、*C. ambrosioides* L. のように、インドの広い範囲に分布し、駆虫薬としてアーユルヴェーダも含め、広く世界の伝統薬として産業用途のあるものもあることから、こういったものが、市場で混入した可能性も考えられる。一方、ISC-3 は、*S. spinosa* の配列 (DQ006018) と非常に高い相同性を示し、販売名の *Sida cordifolia* とは異なるものの、*Sida* 属植物であると考えられた。

2. オアフ島採集試料

市場に流通する *Sida* 属植物製品には、他の植物由来のものが多数混入している事が明らかになった事から、*Sida* 属植物の形態学的な特徴について情報を得るため、ハワイ、オアフ島で採集した *Sida* 属植物の形態を検討した。

Sida 属植物の多くは、托葉を有し、葉に星状毛を持つ。また、花は、淡黄色—黄色のものが多く、5 枚の花弁が花冠の中央を中心に、やや渦を巻いた様な形をしている。分果は三稜形で、多くのものが、2 本の芒を持つ。これらの特徴を基に、*Sida* 属植物の探索を行い、ハワイ、オアフ島の 1 つの地点から野生品 6 個体を採集した。ただし、これらの採集試料の星状毛及び分果の特徴は、フィールド用の簡易ルーペでは確認出来なかったため、主に、托葉の存在と花の形態に基づき、*Sida* 属植物と判断した。

オアフ島採集試料の外部形態を実態顕微鏡により観察したところ、分果及び星状毛の特徴から、CMH-2, 3, 4 (タイプ A) と CMH-1, 5, 6 (タイプ B) の 2 種類に大別された。両タイプの例として、CMH-4 及び -5 の顕微鏡像を Fig. 3 に示した。タイプ A は、葉の両面及び葉柄に非常に小さな星状毛を持ち、特に葉の裏側の葉脈と葉柄に密生していた。星状毛の長さは、葉の上面のものが、43.24 μm (n=10), 下面のものが、70.33 μm (n=10) であった。また、分果は、三稜形を有し、先端に 2 本の芒を持っており、先に述べた BSS-4a の分果と同様の形状であった。このタイプに分類された 3 試料の核 rDNA の ITS 塩基配列は、全て同一の配列を示し、BSS-4a の配列と完全に一致した。タイプ B もまた、特に葉の裏側の葉脈及び葉柄に毛を有していたが、タイプ A のものと比べると直毛で長かった (上面、320.69 μm (n=5); 下面、380.36 μm (n=10))。このものの分果は、扁平な腎形で、1 本の芒と多数の毛を有しており、同じく BSS-4b の分果と同様の形状であった。このタイプに分類された 3 試料の ITS 配列も、全て同一の配列を示し、BSS-4b と同じく *Malvastrum coromandelianum* と高い相同性を示した。BSS-4b の配列、

Fig. 3. Microscopic images of plant samples collected in Oahu island

CMH-1, 5, 6 の配列ともに、同一箇所に複数の塩基を持つヘテロ型の配列を有していたが、両者の配列は、ヘテロ部位を除いて完全に一致した。

Ⅳ 考察

Sida 属植物製品として海外より購入した 11 製品 (SCA, SCR, BSS-1 ～ -5, ISC-1 ～ -3, Ss30) の内、*Sida* 属植物が含まれていたのは 7 製品であり、この内の 3 製品 (SCA, SCR, BSS-4) は、他の植物の混入が認められた。従って、*Sida* 属植物のみからなるものは、11 製品中、4 製品にとどまった。また、残る 4 製品については、主にアオイ科の別属植物を中心とした異物を原料としていた。7 製品から見出された *Sida* 属植物の遺伝子型は、6 つに分類され、この内の 1 つ (*Sida* spp.-2) は、園芸店より購入した *S. fallax* (Sfa) 及びデータベース上の同植物の配列と一致したことから、*S. fallax* であると考えられた。同植物は、かつてハワイの国花（現在は、オアフ島花）であり、レイフラワーとして使用されるが、薬用とはされず、エフェドリンをはじめとしたアルカロイドの含有も報告されていない。一方、その他の 5 つの遺伝子型については、データベース上に一致する配列が認められなかった。これらについては、今後、*Sida* 属植物の標準植物試料を揃え、慎重に同定する必要がある。

塩基配列解析の結果を基に作成した分子系統樹では、3 つの遺伝子型 (*Sida* spp.-1, 2, 6) が、*Sida fallax* と一つのクラスターを形成し、その他の 3 つの遺伝子型は、いずれもエフェドリンアルカロイドの含有が報告されている *Sida acuta*, *Sida cordifolia*, *Sida spinosa* とそれぞれ同一のクラスターに配置された。今後、これらの試料中のアルカロイドの有無を確認し、アルカロイドを含有する *Sida* 属植物種の範囲について、知見を集積し、適正な食薬区分の改訂を行うべきものと考えられる。

一方、オアフ島で採集した植物の形態観察からは、花の外観が、*Sida* 属植物の鑑別点にならず、葉の下面及び葉柄に密生した星状毛の確認あるいは分果の外観の観察が *Sida* 属植物の確認に有効であることが明らかになった。しかし、*Sida* 属植物の星状毛は非常に小さく、ルーペでの確認は不可能であることから、*Sida* 属植物の採集には、携帯型の実体顕微鏡の携行が望ましい。

Sida 属植物製品として海外より購入した植物の調査において、*Sida* 属植物とは異なるものが多数認められたが、その中