Ichthyotoxic Phloroglucinol Derivatives from *Dryopteris fragrans* and Their Anti-tumor Promoting Activity

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Two new ichthyotoxic compounds, aspidin PB (8) and dryofragin (9), along with three known phloroglucinol derivatives (1–3) and five terpenoids, were isolated from the whole herbs of *Dryopteris fragrans* by toxicity-directed fractionation using *Oryzias latipes* (Japanese name; medaka). The structures of the new compounds were determined by spectroscopic methods including 2D NMR techniques. Amongst the isolates, aspidin PB (8), dryofragin (9), and 1–5 exhibited potent ichthyotoxic activity against medaka with a median tolerance limit (TLm after 24 h) of 1.2–4.3 $\mu\text{g}/\text{ml}$. These compounds which are toxic to fish also had a potent inhibitory effect on the activation of Epstein–Barr virus early-antigen (EBV-EA) induced by tetradecanoyl phorbol 13-acetate, which is an *in vitro* short-term assay for anti-tumor promoting agents. Aspidin BB (2) and albicanol (4), which exhibited strong inhibitory effects on the EBV-EA activation, significantly suppressed an *in vivo* two-stage carcinogenesis on mouse skin.

Key words *Dryopteris fragrans*; Aspidiaceae; phloroglucinol derivative; dryofragin; ichthyotoxic activity; anti-tumor promoter

Naturally occurring ichthyotoxic compounds are reported to exhibit a variety of other biological activities, e.g., an anti-tumor promoting effect¹⁾ for rotenoids, antibacterial activity²⁾ for 5-*O*-methylembeline, a tumor promoting effect³⁾ for phorbol diesters and anti-tumorigenic activity⁴⁾ for buddledin A.⁵⁾ We recently reported the isolation and characterization of several iridal-type triterpenoids with ichthyotoxic activity from *Iris*⁶⁾ and *Belamcanda*⁷⁾ species of the family Iridaceae, employing bioassay-guided fractionation. These active isolates contained compounds reported to exhibit a remarkable anti-ulcer activity in stress ulceration of experimental animals,⁸⁾ and an *in vitro* and *in vivo* tumor-promoting activity.⁹⁾ Therefore, the ichthyotoxic test provides a convenient primary assay method for searching out new biologically active compounds in nature. In our ongoing survey of bioactive substances using this assay method, *n*-hexane extract of *Dryopteris fragrans* (L) SCOTT. (Aspidiaceae) was found to be toxic to killifish (*Oryzias latipes*; Japanese name, medaka). Extensive phytochemical studies^{10–14)} on the *Dryopteris* species have revealed that these ferns produce phloroglucinol derivatives with diverse structures, which possess a variety of biological properties including anthelmintic,¹⁵⁾ antibacterial,^{16–18)} anti-tumor¹⁹⁾ and uterotonic effects.¹⁹⁾ *D. fragrans* has been used as a remedy for skin diseases in Northeast China. However, there is only one report of the isolation of phloroglucinol derivatives, namely aspidin BB, albaspidin BB, butyrylfilicin acid and aspidinol, from the plant.²⁰⁾ We describe herein the characterization of ichthyotoxic constituents (1–5, 8, 9) including two new compounds named aspidin PB (8) and dryofragin (9)²¹⁾ isolated from this plant, and their inhibitory effects on the activation of Epstein–Barr virus early-antigen (EBV-EA) induced by 12-*O*-tetradecanoyl phorbol 13-acetate (TPA). In addition, aspidin BB (2) and albicanol (4), potent inhibitors of EBV-EA activation, were subjected to an *in vivo* assay for two-stage carcinogenesis on mouse skin.

Results and Discussion

Isolation and Characterization of the Constituents A *n*-hexane extract of *D. fragrans*, which was toxic to killifish, was subjected to column chromatography over silica gel. Upon ichthyotoxicity-guided fractionation, testing was performed in deionized H₂O that was sufficiently aerated in advance, and fractions (40 ppm each) were applied to 200 ml beakers containing five fish, in 100 ml H₂O. Positive fractions were further purified by repeated chromatography on silica gel, Sephadex LH-20 and preparative HPLC to afford two new toxic compounds, 8 and 9, and eight known compounds. The known compounds were identified by comparison of their ¹H- and ¹³C-NMR data with values reported in the literature as the phloroglucinol derivatives, aspidin AB (1),²²⁾ BB (2),²²⁾ and aspidinol (3)²²⁾; the sesquiterpenoids, albicanol (4),²³⁾ albicanyl acetate (5),²⁴⁾ and α -cadinene (6); and a triterpene, β -sitosterol.

The structure of compound 7 was deduced on the basis of NMR analyses including HMQC and HMBC techniques, and was found to be identical with that proposed for conicumol, which was isolated from the liverwort, *Conocephalum conicum*.²⁵⁾

Aspidin PB (8) was obtained as colorless prisms, mp 134–136 °C. The molecular formula of C₂₄H₃₀O₈ was determined by high resolution (HR) FAB-MS, which gave a quasi-molecular ion peak at *m/z* 477.2019 [M+H]⁺. The UV spectrum of 8 showed maxima at 216, 316 and 352 nm similar to those of 1 and 2, indicating it to be an aspidin analogue. The ¹H-NMR spectrum displayed signals due to five methyl groups, two of which are primary (δ 0.99, 1.17) and three tertiary (δ 1.45, 1.53, 2.14), as well as four methylene groups [δ 1.72 (sext, *J*=7 Hz), 3.08 (t, *J*=7 Hz), 3.20 (q, *J*=7 Hz), 3.57 (br s)]. The spectrum also showed the presence of four hydroxyl groups at δ 18.5, 15.6, 10.7 and 10.0 (each s). Among them, a remarkably downfield-shifted signal (δ 18.5) was characteristic of an enolic proton of the β -triketone system as found in the acylated filicin acid unit of the aspidins. These proton signals were accompanied by two small peaks

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in a ratio of approximately 6:1:1 (estimated from the hydroxyl proton signal intensities) due to a mixture of keto-enol tautomers (**8a**–**8c**)²⁶ at the filicinic acid moiety. These findings along with ¹³C-NMR data (see Experimental) were consistent with those of aspidin BB (**2**) except for the lack of a methylene group in **8** (Table 1), suggesting that **8** is an analog of **2** being different only in the structures of side chains. The occurrence of propynyl and butyryl chains was inferred from the multiplicities of the methylene signals assigned by ¹H–¹H shift correlation spectroscopy (COSY) and two ketonic carbon resonances at δ 206.8 and 207.4. The allocation of these side chains was possible with nuclear Overhauser enhancement spectroscopy (NOESY) measurement of **8**. The propynyl methyl (δ 1.17, H-10') signal showed an NOE to the strongly hydrogen-bonded hydroxyl proton at δ 18.5 which is assigned to 4'-OH of the acylfilicinic acid moiety (see Fig. 1). On the other hand, the butyryl methylene signal [δ 3.08 (t, $J=7$ Hz), H-9] displayed an NOE with a methoxyl signal which also showed a clear NOE to toluene methyl protons (δ 2.14, H-12), thus establishing the location of the side chains as depicted in the formula. An isolated (bridged) methylene group (δ 3.57, H-7') showed NOEs with the more upfield-shifted hydroxyl protons at δ 10.0 and 10.6, the former of which also exhibited an NOE correlation with the C-5 methyl signal, allowing its assignment to the 6-hydroxyl proton. However, no NOE was detected between the hydroxyl proton signal at δ 10.6 and H-11' or H-12'. This might be due to the unusually diffused signals of the *gem*-dimethyl group, which are caused by the presence of the tautomers (**8a**–**8c**) and a slow rotation around the bridged methylene bond at C-1'.²⁶ This anomaly was released in the pentaacetyl derivative (**8d**), which had an enol acetyl group at the propynyl moiety, as shown by sharp singlet due to the *gem*-dimethyl group (see NMR in Experimental). The NOESY measurement of **8d** gave definite NOEs between two acetyl and *gem*-dimethyl groups, substantiating the predominant

Fig. 1. NOE Correlations of **8**Table 1. ¹H-NMR Spectral Data of **2**, **4**, **8** and **9** (500 MHz, CDCl₃)

	2	8	4	9
H-1, 2, 3, 5, 6			1.1–1.8	1.1–1.7
H-7	3.73 (3H, s)	3.73 (3H, m)	2.02 (1H, m)	5.64 (1H, br s)
			2.45 (1H, m)	
H-9	3.08 (2H, m)	3.08 (2H, t, $J=7$ Hz)	1.97 (1H, br s, $J=6.5$ Hz)	2.13 (1H, m)
H-10	1.71 (2H, m)	1.72 (2H, sext, $J=7$ Hz)		
H-11	1.01 ^a (3H, t, $J=7$ Hz)	0.99 (3H, t, $J=7$ Hz)	3.79 (2H, m)	4.08 (1H, d, $J=11$ Hz)
				3.71 (1H, dd, $J=9, 11$ Hz)
H-12	2.14 (3H, s)	2.14 (3H, s)	4.94 (1H, br s)	2.13 (2H, m)
			4.63 (1H, br s)	
H-13			0.71 (3H, s)	0.73 (3H, s)
H-14			0.87 (3H, s)	0.88 (3H, s)
H-15			0.80 (3H, s)	0.88 (3H, s)
H-7'	3.57 (2H, br s)	3.57 (2H, br s)		2.13 (2H, m)
H-9'	3.08 (2H, m)	3.20 (2H, q, $J=7$ Hz)		2.98 (2H, dt, $J=7$ Hz)
H-10'	1.71 (2H, m)	1.71 (3H, t, $J=7$ Hz)		1.66 (2H, sext, $J=7$ Hz)
H-11'	0.99 ^a (3H, t, $J=17$ Hz)	1.45 ^c (3H, br s)		0.99 (3H, t, $J=7$ Hz)
H-12'	1.44 ^b (3H, br s)	1.53 ^c (3H, br s)		1.35 ^d (3H, s)
H-13'	1.53 ^b (3H, br s)			1.34 ^d (3H, s)
2-OH	15.6 (1H, s)	15.6 (1H, s)		
6-OH	10.0 (1H, s)	10.0 (1H, s)		
4'-OH	10.7 (1H, s)	18.5 (1H, s)		19.1 (1H, s)
6'-OH	18.6 (1H, s)	10.6 (1H, s)		

a)–d) Values are interchangeable.

Table 2. ^{13}C -NMR Spectral Data of **1**, **2**, **4**, **8** and **9** (126 MHz, CDCl_3)

Carbons	1	8	2	4	9
C-1	112.4	112.4	112.4	39.7	35.3
C-2	162.8	162.8	162.8	19.1	18.8
C-3	107.6	107.5	107.5	41.8	35.8
C-4	159.6	159.6	159.6	33.1	33.0
C-5	109.4	109.4	109.0	55.0	49.6
C-6	160.3	160.2	160.2	24.1	25.2
C-7	61.4	61.5	61.5	33.9	124.8
C-8	206.7	207.4	206.8	147.6	136.6
C-9	44.3	42.9	44.2	58.9	55.0
C-10	18.2	18.2	18.0 ^{a)}	38.8	39.6
C-11	13.8	13.9	13.9	58.5	63.0
C-12	9.1	9.2	9.2	106.3	22.3 ^{e)}
C-13				15.4	14.3
C-14				33.5	33.2
C-15				21.6	22.0
C-1'	110.9	110.9	110.9		105.5
C-2'	198.8	198.0	198.6		197.8
C-3'	108.4	108.0	108.0		106.8
C-4'	187.4	187.5	187.4		189.0
C-5'	44.1	44.2	44.2		42.1
C-6'	172.0	171.8	171.8		175.6
C-7'	17.1	17.1	17.1		29.7 ^{e)}
C-8'	203.3	206.8	206.5		203.7
C-9'	29.1	34.8	42.9		41.9
C-10'	24.6 ^{a)}	8.4	18.1 ^{c)}		18.5
C-11'	25.2 ^{a)}	24.3 ^{b)}	13.9		14.0
C-12'		25.3 ^{b)}	24.2 ^{d)}		23.6 ^{f)}
C-13'			25.2 ^{d)}		24.5 ^{f)}

a)–f) Values are interchangeable.

Chart 2

tautomeric form to be **8a**. The structure of aspidin PB was thus represented as **8**.

Although aspidin analogues with a diverse combination of acyl side chains have been reported,^{12–14)} to our knowledge, aspidin PB is a new compound.

Dryofragin (**9**), obtained as colorless needles, mp 180–181 °C, showed a quasi-molecular ion peak at m/z 459.3127 $[\text{M}+\text{H}]^+$ in HRFAB-MS, indicating that its molecular formula is $\text{C}_{28}\text{H}_{42}\text{O}_5$. The UV (λ_{max} 222 and 354 nm) and ^1H -NMR spectra, which exhibited a hydroxyl resonance at δ 19.12, strongly suggested the presence of a filicinic acid moiety in the molecule, like in **8**. The occurrence of a butyryl chain as well as a *gem*-dimethyl group (δ 1.34, 1.35) was indicated by multiplicities of methylene protons at δ 2.98 (t, $J=7$ Hz) and 1.66 (sext, $J=7$ Hz), and methyl protons at δ 0.99 (t, $J=7$ Hz), which showed sequential couplings in ^1H - ^1H COSY. The ^{13}C -NMR data substantiated that **9** contained a butyryl filicinic acid moiety identical to that found

Fig. 2. HMBC and NOE Correlations of **9a**

Chart 3

in **2** (Table 2). The rest of the molecule, $\text{C}_{16}\text{H}_{27}\text{O}$, had three quaternary methyl groups [δ 0.73 (3H), 0.88 (6H)], a methanol protons (δ 3.71, 4.08) and an olefinic proton (δ 5.64, br s), which are indicative of an albicanol skeleton. Comparison of the ^1H - and ^{13}C -NMR spectra between **9** and **4** (Tables 1 and 2) revealed that **9** is composed of the butyrylfilicinic acid and drimenol (**10**, a double bond isomer of **4**) moieties, which are linked through a methylene bridge. Conclusive evidence for the structure **9** was obtained by spectral analysis of an acetyl derivative (**9a**). The ^1H -NMR spectrum of **9a** showed signals due to four acetyl methyl protons and a newly formed olefinic proton (δ 5.37, t, $J=7$ Hz), indicating that enolization followed by acetylation occurred at the butyryl side chain as in the acetylation of **8**. In the HMBC spectrum, H-12 displayed three-bond correlations with C-7 and C-1', while the methylene protons (H-7') coupled with H-12 showed two- or three-bond correlations with C-1', C-6' and a ketone carbonyl signal (C-2') as illustrated by arrows in Fig. 2, establishing the position of the bridged ethylene group. The structure of the filicinic acid moiety as represented by **9** was confirmed by the HMBC correlations of *gem*-dimethyl protons with C-4' and C-6', respectively, and also by NOEs between the acetyl and *gem*-dimethyl groups in NOESY. A *cis*-arrangement of the methylene and methyl groups at C-9 and C-10, respectively, was apparent from their NOE correlations. The absolute configuration at the drimenol moiety as shown in the formula was indicated by the same sign of the specific rotation ($[\alpha]_{\text{D}} -30.1^\circ$) of **9** as that reported for **10** ($[\alpha]_{\text{D}} -20^\circ$).²⁷⁾ Consequently, the structure **9** was assigned to dryofragin.

The most plausible biogenetic route of **9** would involve the intermediacy of an *o*-quinone methide **11** formally derivable from an alkylated phloroglucinol precursor, which reacts in an electrophilic addition reaction at the activated exomethylene carbon of **4**, in a way similar to the formation¹⁵⁾ of aspidins (Chart 3).

Although diverse phloroglucinol derivatives linked with a sesquiterpenoid through a C–C bond have been isolated from seaweed,²⁸⁾ ferns²⁹⁾ and higher plants,³⁰⁾ dryofragin is, to our knowledge, the only filicinic acid-sesquiterpene conjugate.

Ichthyotoxic Activity Among the compounds isolated in

this study, all of the phloroglucinol derivatives (**1**–**3**, **8**, **9**) exhibited potent ichthyotoxic activity against medaka, in the evaluation of the median tolerance limit (TLm)⁵⁾ after 24 h (see Table 3). With TLm values of 1.2–2.4 $\mu\text{g/ml}$, they were comparable in potency to buddledin B and iripallidal which were isolated from *Buddleja davidii*⁵⁾ and *Iris germanica*,³¹⁾ respectively. The toxicity of the aspidin series increases as the C-3 side chain becomes longer. Albicanol (**4**) and its acetate (**5**) were less toxic to fish with a TLm of 3.7 and 4.3 $\mu\text{g/ml}$, respectively. Albicanol derivatives (**12**,³²⁾ **13**,³²⁾ **14**), which were prepared in order to examine structure–activity relationships, showed weaker toxicity to fish than the parent compound (**4**) (see Table 3), suggesting that a hy-

Table 3. Ichthyotoxic Activity of the Compounds from *D. fragrans* and Albicanol Derivatives

Compounds	TLm ($\mu\text{g/ml}$) ^{a)}
Aspidin AB (1)	2.4
Aspidin BB (2)	1.2
Aspidinol (3)	1.5
Albicanol (4)	3.7
Albicanyl acetate (5)	4.3
Aspidin PB (8)	1.4
Dryofragin (9)	1.6
Albicanol epoxide (12)	11.8
Driman-8, 11-diol (13)	18.4
Albicanyl succinate (14)	15.1

a) TLm: median tolerance limit after 24 h.

droxyl and/or exomethylene group in the drimane skeleton plays an important role in the ichthyotoxicity.

In Vitro and *In Vivo* Anti-tumor Promoting Activity

Activation of EBV-EA is recognized to be induced by *in vivo* tumor-promoters and inhibited by anti-tumor promoters.³³⁾ An inhibition test of the activation using EBV genome-carrying lymphoblastoid cells (Raji cells) has thus been used as a convenient primary *in vitro* screening in the search for anti-tumor promoters in nature. Ichthyotoxic substances often exhibit an anti-tumor and/or related activity, though relationships among these activities are not clear, as mentioned earlier. Therefore, the compounds that were toxic to fish in the present study were assessed for inhibitory effects on the activation of EBV-EA induced by TPA. As shown in Table 4, all the compounds except for **9** showed inhibitory effects on EBV-EA activation, much stronger than that of a positive control, (–)-epigallocatechin gallate (main polyphenol of green tea), at a concentration of 100 mol ratio/TPA. Interestingly, **4** and its epoxide (**12**), which were less toxic to fish than the phloroglucinol derivatives, showed stronger activity than **2** and **8**. Notably, the compounds **2**, **4**, **5** and **8** showed 100% inhibition, preserving the high viability of Raji cells at a concentration of 500 mol ratio/TPA.

Aspidin BB (**2**) and albicanol (**4**), which showed potent inhibition in the *in vitro* assay, were then subjected to the *in vivo* two-stage carcinogenesis assay on mouse skin using 7, 12-dimethylbenz[a]anthracene (DMBA) as an initiator and TPA as a promoter. The results are shown in Fig. 3. The con-

Table 4. Relative Ratio of EBV-EA Activation with Respect to Positive Control (100%) in the Constituents of *D. fragrans* and Albicanol Derivatives

Compounds	Relative Ratio of EBV-EA Activation ^{a)}			
	1000	Concentration (mol ratio/TPA) ^{b)} 500	100	10
Aspidin BB (2)	0.0±0.0 (50) ^{c)}	0.0±0.3 (70)	30.2±1.4	94.1±0.7
Albicanol (4)	0.0±0.0 (40)	0.0±0.4 (60)	19.5±1.2	71.3±1.1
Abicanyl acetate (5)	0.0±0.0 (50)	0.0±0.4 (70)	26.9±1.4	78.8±1.6
Aspidin PB (8)	0.0±0.0 (60)	0.0±0.5	26.8±2.1	77.6±1.5
Dryofragin (9)	22.5±0.2 (60)	41.9±1.0	65.0±2.0	88.4±1.1
Albicanol epoxide (12)	0.0±0.0 (40)	0.0±0.7 (60)	16.3±1.6	71.0±1.9
Albicanyl succinate (14)	0.0±0.3 (60)	19.4±0.5	42.7±2.0	86.9±0.8
Epigallocatechin gallate (EGCG)	6.4±0.5 (70)	34.9±1.1	68.1±1.7	87.7±1.4

a) Values represent relative percentages to the positive control value (100%). b) TPA concentration was 20 ng (32 pmol)/ml. c) Values in parentheses are viability percentage of Raji cells; unless otherwise stated, the viability percentage of Raji cells was more than 80%.

Fig. 3. Inhibition of TPA-induced Tumor Promotion by Multiple Application of **2** and **4**

All mice were initiated with DMBA (390 nmol) and promoted with 1.7 nmol of TPA given twice weekly starting 1 week after initiation. A: percentage of mice bearing papillomas. B: average number of papillomas per mouse. ●, control TPA alone; ▲, TPA+85 nmol of **2**; ■, TPA+85 nmol of **4**. $p<0.01$, Papillomas per mouse of **2** and **4** treatment significantly different from positive control at 20 weeks after promotion.

trol animals showed a 100% incidence of papillomas at 10 weeks after promotion, while treatment with **2** and **4** along with initiator and promoter remarkably reduced the percentage of tumor-carrying mice to 20% and 30%, respectively, after 10 weeks, and 65% and 85%, respectively, even over a 20-week period, at 85 nmol of test compounds (Fig. 3A). The average number of papillomas per mouse in the experimental groups was also reduced to about 40% and 55%, respectively, relative to the control group in week 20 (Fig. 3B).

Albicanol (**4**), a representative drimane sesquiterpene, was reported to be a pungent principle³⁴ and antifeedant²⁴ agent against fish and worm. On the other hand, as described earlier, aspidins are known to have anthelmintic¹⁵ and antibacterial properties.^{16–18} Therefore, the present study provided additional evidence that fractionation guided by toxicity to medaka can lead to the isolation of compounds which may have anti-tumor promoting activity and/or the above mentioned biological properties as well as ichthyotoxicity.

Experimental

¹H- and ¹³C-NMR spectra were recorded on a Varian VXR-500 instrument (500 MHz for ¹H and 126 MHz for ¹³C) and the chemical shifts are given in δ (ppm) values relative to that of residual CHCl₃ (δ_{H} 7.26; δ_{C} 77.0). Optical rotations were measured with a Jasco DIP-1000 polarimeter. UV spectra were measured with a Hitachi U-2000 spectrophotometer. EI-, FAB- (matrix agent: 3-nitrobenzyl alcohol) and ESI-MS (solvent: 50% aqueous MeOH containing 0.1% AcONH₄; flow rate: 0.02 ml/min) were recorded on a Micromass AutoSpec OA-Tof mass spectrometer. Column chromatography was carried out on silica gel (Silica gel 60, 70–230 mesh, Merck Co., Germany) and Sephadex LH-20 (Pharmacia, Sweden). TLC was performed on a pre-coated Silica gel 60 F₂₅₄ (0.25 mm, Merck Co. Germany), and spots were visualized under UV light and by spraying 5% H₂SO₄-EtOH followed by heating. Normal phase HPLC was conducted on a YMC-Pack SIL A-003 (YMC Co., Ltd.) column (4.6 mm i.d. × 250 mm) developed with *n*-hexane-EtOAc-AcOH (80:20:5) (flow rate: 1.0 ml/min; detection: 254 nm) at room temperature. Reversed phase HPLC was performed on a YMC-Pack ODS AQ-302 (YMC Co., Ltd.) column (4.6 mm i.d. × 150 mm) developed with MeOH-H₂O-AcOH (90:5:5) and MeCN-H₂O-AcOH (85:10:5) (flow rate: 1.0 ml/min; detection: 254 nm) at 40 °C. Solvents were evaporated under reduced pressure below 40 °C.

Plant Materials *D. fragrans* was collected in January, 1995 in the suburbs of Harbin, China and identified by Prof. Liu Ming Yuan of the Department of Biology, Heilongjiang University of Education. A voucher specimen has been deposited in the herbarium of Heilongjiang Commercial College, Harbin, China.

Extraction and Isolation The dried whole herbs of *D. fragrans* (1 kg) were soaked in *n*-hexane (20 l), and filtered. The *n*-hexane extract (34 g), which showed potent ichthyotoxic activity to medaka, was chromatographed over silica gel with *n*-hexane-acetone. The toxic fractions were subjected to a combination of column chromatography over silica gel (CHCl₃-MeOH and/or *n*-hexane-EtOAc) and Sephadex LH-20 (CHCl₃-MeOH), and preparative HPLC [YMC-GEL ODS A-324 (10 mm i.d. × 300 mm) (YMC Co. Ltd.) using MeCN-H₂O-AcOH (90:50:5)] to give β -sitosterol (21 mg), aspidin AB (**1**) (114 mg), aspidin BB (**2**) (111 mg), aspidinol (**3**) (13 mg), albicanol (**4**) (125 mg), albicanyl acetate (**5**) (24 mg), α -cadiene (**6**) (14 mg), conicumol (**7**) (146 mg), aspidin PB (**8**) (59 mg), and dryofragin (**9**) (179 mg).

The plant residues after extraction with *n*-hexane were further homogenized in 70% aqueous acetone (2 l). The concentrated homogenate was extracted with *n*-hexane (9 l), EtOAc (8 l) and 1-butanol (6 l), successively. The toxic *n*-hexane extract (7 g) was purified in a way similar to that described above to furnish additional amounts of β -sitosterol (82 mg), **2** (3 mg), **3** (3 mg), **4** (51 mg), **7** (20 mg) and **8** (3 mg).

Conicumol (7) A colorless oil, $[\alpha]_{\text{D}}^{20} -30.1^\circ$ ($c=1.0$, CHCl₃). EI-MS m/z : 222 (M)⁺. ¹H-NMR (500 MHz, CDCl₃) δ : 0.78, 0.89 (each 3H, s, CH₃), 0.91 (3H, d, $J=7$ Hz, CH₃), 1.19 (3H, s, tertiary CH₃), 1.20, 1.39 (CH₂), 1.46 (CH₂), 1.6 (CH), 1.6, 1.73 (CH₂), 1.73, 1.96 (CH₂). ¹³C-NMR (126 MHz, CDCl₃) δ : 15.3 (secondary CH₃), 19.7 (CH₂), 22.0 (tertiary CH₃), 28.4 (tertiary CH₃), 29.4 (CH₂), 30.9 (tertiary CH₃), 36.3 (CH), 39.2 (CH₂), 40.2 (C), 42.1 (CH₂), 45.8 (C), 51.3 (CH), 58.9 (CH₂), 63.4 (CH), 84.8 (C).

Aspidin PB (8) Colorless prisms, mp 134–136 °C (MeOH). FAB-MS m/z : 447 (M+H)⁺. HR FAB-MS m/z : 447.2019 (M+H)⁺, Calcd for C₂₄H₃₀O₈+H, 447.2019. UV λ_{max} (EtOH) nm (log ϵ): 216 (4.47), 316 sh (4.12), 352 (4.25). ¹H-NMR (CDCl₃): see Table 1. ¹³C-NMR (CDCl₃): see Table 2.

Acetylation of 8 To a solution of **8** (10 mg) in pyridine (1 ml) was added acetic anhydride (1 ml), and the mixture was left to stand overnight at room temperature. After removal of the solvent, the reaction mixture was subjected to preparative TLC with *n*-hexane-acetone (3:1) to give penta-acetate (**8d**) (9 mg). **8d**: A colorless oil, ESI-MS m/z 657 (M+H)⁺. ¹H-NMR (500 MHz, CDCl₃) δ : 5.49 (1H, q, $J=7$ Hz, H-9'), 3.69 (3H, s, H-7), 3.47 (2H, br s, H-7'), 2.79 (2H, t, $J=7$ Hz, H-9), 2.24, 2.22, 2.19, 2.16, 2.12 (each 3H, s, OAc × 5), 2.01 (3H, s, H-12), 1.66 (2H, sext, $J=7$ Hz, H-10), 1.63 (3H, d, $J=7$ Hz, H-10'), 1.22 (6H, s, H-11', 12'), 0.96 (3H, t, $J=7$ Hz, H-11).

Dryofragin (9) Colorless fine needles, mp 180–181 °C (MeOH). $[\alpha]_{\text{D}}^{20} -30.1^\circ$ ($c=1.7$, CHCl₃). FAB-MS m/z : 459 (M+H)⁺. HR FAB-MS m/z : 459.3127 (M+H)⁺, Calcd for C₂₆H₄₂O₅+H, 459.3111. UV λ_{max} (EtOH) nm (log ϵ): 222 (4.31), 354 (4.28). ¹H-NMR (CDCl₃): see Table 1. ¹³C-NMR (CDCl₃): see Table 2.

Acetylation of 9 A solution of **9** (20 mg) in pyridine (2 ml) was treated with acetic anhydride (2 ml) overnight at room temperature. After removal of the solvent, the residue was purified by preparative TLC (*n*-hexane-acetone, 3:1) to yield a tetra-acetate **9a** (14 mg). **9a**: A colorless oil, ESI-MS m/z 627 (M+H)⁺. ¹H-NMR (500 MHz, CDCl₃) δ : 5.41 (1H, br s, H-7), 5.37 (1H, t, $J=7$ Hz, H-9'), 4.28 (1H, dd, $J=3, 12$ Hz, H-11), 4.08 (1H, dd, $J=5.5, 12$ Hz, H-11), 2.40 (1H, m, H-7), 2.29, 2.24 (each 3H, s, OAc × 2), 2.20 (1H, m, H-12), 2.16 (1H, br s, H-9), 2.11 (3H, s, OAc), 2.08 (4H, m, H-12, 10'), 2.04 (3H, s, OAc), 1.30, 1.27 (each 3H, s, H-12', 13'), 1.01 (3H, t, $J=7$ Hz, H=11'), 0.88 (3H, s, H-14), 0.85 (3H, s, H-15), 0.80 (3H, s, H-13). ¹³C-NMR (126 MHz, CDCl₃) δ : 183.8 (C-2'), 164.3 (C-4'), 162.3 (C-6'), 136.3 (C-8'), 135.7 (C-8), 127.8 (C-1'), 126.9 (C-9'), 124.4 (C-7), 124.3 (C-3'), 63.2 (C-11), 51.6 (C-9), 49.6 (C-5), 43.4 (C-5'), 42.2 (C-3), 39.9 (C-10), 36.1 (C-1), 33.3 (C-14), 32.9 (C-4), 32.5 (C-12 or 7'), 24.1 (C-6), 23.9 (C-7' or 12), 23.2, 23.6 (C-12', 13'), 22.0 (C-15) 19.7 (C-2), 18.8 (C-10'), 14.4 (C-13), 13.3 (C-11').

Epoxidation of 4 To a solvent of **4** (10 mg) in dry CH₂Cl₂ (10 ml) was added *m*-chloroperbenzoic acid (20 mg) at 0 °C, and the mixture was stirred at room temperature for 5 h. Ether was added to the reaction mixture and the organic layer was washed with saturated NaHCO₃ solution and brine, dried over anhydrous MgSO₄, and evaporated *in vacuo*. The residue was purified by preparative TLC (*n*-hexane-acetone, 3:1) to give an epoxide (**12**) (10 mg). **12**: A white amorphous powder. $[\alpha]_{\text{D}}^{20} -28.8^\circ$ ($c=1.0$, CHCl₃). ESI-MS m/z 256 (M+NH₄)⁺, 221 (M-H₂O+H)⁺. ¹H-NMR (500 MHz, CDCl₃) δ : 3.63 (1H, dt, $J=3, 11$ Hz, H-12), 3.41 (1H, t, $J=11$ Hz, H-11), 3.21 (1H, m, H-12), 3.11 (1H, br d, $J=10$ Hz, 11-OH), 2.71 (1H, d, $J=4$ Hz, H-12), 1.88 (1H, dd, $J=3, 10.5$ Hz, H-9), 0.90 (3H, s, H-14), 0.84 (3H, s, H-15), 0.83 (3H, s, H-13).

Preparation of Succinate (14) of 4 A solution of **4** (10 mg) in dry CH₂Cl₂ (10 ml) containing 4-dimethylaminopyridine (30 mg) and succinic anhydride (30 mg) was stirred at room temperature for 5 h. The reaction mixture was poured into ice water, and extracted with EtOAc. The organic layer was washed with brine and dried over anhydrous MgSO₄, and the solvent was evaporated *in vacuo*. The resulting product was subjected to preparative TLC developed with *n*-hexane-acetone (3:1) to yield a succinate (**14**) (13 mg), as colorless oil. **14**: $[\alpha]_{\text{D}}^{20} +24.5^\circ$ ($c=1.3$, CHCl₃). ESI-MS: m/z 340 (M+NH₄)⁺, 323 (M+H)⁺. ¹H-NMR (500 MHz, CDCl₃) δ : 4.84 (1H, s, H-12), 4.50 (1H, s, H-12), 4.38 (1H, dd, $J=3.5, 11$ Hz, H-11), 4.21 (1H, dd, $J=9.5, 11$ Hz, H-11), 2.39 (1H, m, H-7 β), 2.04 (1H, dd, $J=5.5, 11$ Hz, H-9), 0.88 (3H, s, H-14), 0.81 (3H, s, H-15), 0.75 (3H, s, H-13).

LiAlH₄ Reduction of Albicanol Epoxide (12) A mixture of **12** (6 mg) and LiAlH₄ (6 mg) in dry ether (4 ml) was stirred overnight at room temperature. To the reaction mixture was added carefully ice water and then 1N HCl. The reaction mixture was diluted with ether, washed with brine and dried over anhydrous MgSO₄. The organic layer was evaporated *in vacuo*. The residue was purified by preparative TLC (*n*-hexane-acetone, 3:1) to give driman-8, 11-diol (**13**) (6 mg). **13**: A white amorphous powder. $[\alpha]_{\text{D}}^{20} +0.7^\circ$ ($c=1.0$, CHCl₃). ESI-MS: m/z 481 (2M+H)⁺, 223 (M-H₂O+H)⁺, 205 (M-2H₂O+H)⁺. ¹H-NMR (500 MHz, CDCl₃) δ : 3.92 (2H, s, H-11), 3.91 (1H, s, H-11), 1.88 (1H, dt, $J=3.5, 12.5$ Hz, H-7 β), 1.34 (3H, s, 8-CH₃), 0.87 (3H, s, H-14), 0.78 (6H, s, H-13, 15).

Ichthyotoxic Assay A test solution was prepared by adding an acetone solution (0.5 ml) of test compounds of known concentration into aerated water (100 ml). The median tolerance limit (TLm) after 24 h was evaluated

for 5 fish/group by straight-line graphical interpolation. A control experiment was carried out under the same conditions, adding acetone (0.5 ml) alone.

EBV-EA Assay EBV-EA positive serum from a patient with nasopharyngeal carcinoma (NPC) was a gift from the Department of Biochemistry, Oita Medical University. EBV genome-carrying lymphoblastoid cells (Raji cells derived from Burkitt's lymphoma) were cultured in 10% FBS RPMI-1640 medium (Nissui). Spontaneous activation of EBV-EA in our subline of Raji cells was less than 0.1%. The inhibition of EBV-EA activation was assayed using Raji cells (virus non-producer type) as described previously.³⁵⁾ The indicator cells (Raji, 1×10^6 /ml) were incubated at 37 °C for 48 h in 1 ml of medium containing *n*-butyric acid (4 mmol) and TPA [32 pmol=20 ng in dimethylsulfoxide (DMSO), 2 μ l] as inducer, and various amounts of test compounds in 5 μ l of DMSO. Smears were made from the cell suspension, and the activated cells that were stained by EBV-EA positive serum from NPC patients were detected by an indirect immunofluorescence technique.³⁶⁾ In each assay, at least 500 cells were counted, and the number of stained cells (positive cells) present was recorded. Triplicate assays were performed for each compound. The average EBV-EA induction of the test compounds was expressed as a relative ratio to the control experiment (100%) which was carried out only with *n*-butyric acid (4 mmol) plus TPA (32 pmol). EBV-EA induction was ordinarily around 35%. The viability of treated Raji cells was assayed by the trypan-blue staining method.

In Vivo Two-Stage Carcinogenesis Assay Specific pathogen-free female ICR mice (6 weeks old) were obtained from Japan SLC Inc. (Shizuoka, Japan), and were housed, 5 per polycarbonate cage, in a temperature-controlled room at 24 ± 2 °C and given food and water *ad libitum* throughout the experiment. Animals were divided into three experimental groups of 15 each. The back of each mouse was shaved with surgical clippers, and the mice were topically treated with DMBA (100 μ g, 390 nmol) in acetone (0.1 ml) as an initiating treatment. One week after the initiation, papilloma formation was promoted twice a week by the application of TPA (1 μ g, 1.7 nmol) in acetone (0.1 ml) to the skin. One hour before each treatment, the mice were treated with the samples (85 nmol) in acetone (0.1 ml). The incidence of papillomas was examined weekly during 20 weeks.

Acknowledgements The authors are grateful to the SC-NMR Laboratory of Okayama University for the NMR experiments. This work was financially supported by a Grant-in-Aid for Scientific Research (No. 10557207) from the Ministry of Education, Science, Sports and Culture, Japan.

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