Bioactive Naphthoquinone Derivatives from *Diospyros maritima* BLUME

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From the fresh fruits of *Diospyros maritima* BLUME (Ebenaceae) three new naphthoquinones, 3-bromoplumbagin (1), ethylidene-6,6'-biplumbagin (2), and 3-(2-hydroxyethyl)plumbagin (3), were isolated, in addition to six known naphthoquinones, 3-chloroplumbagin (4), 3-methylplumbagin (5), plumbagin (6), droserone (7), elliptinone (8), and maritinone (9). The structures of the new compounds were established by chemical and spectroscopic means.

The 21 compounds isolated from this plant were examined for ichthyotoxic activity, germination inhibitory activity, and antifungal activity. The naphthoquinone derivatives from the fruits of *Diospyros maritima* showed a variety of biological activities, especially plumbagin (6), which showed strong activity in these three bioassays.

Key words *Diospyros maritima*; naphthoquinone; plumbagin; 3-bromoplumbagin; ethylidene-6,6'-biplumbagin; 3-(2-hydroxyethyl)plumbagin

Diospyros maritima BLUME (Ebenaceae) (ryukyugaki in Japanese) is a shrub growing in southeast Asia, and it bears a fruit of 2—3 cm diameter in the spring. The fruit is known to be poisonous, and is said to be used as a pesticide in the Ryukyu islands (Okinawa). From the bark and the roots of this plant the isolation of plumbagin, elliptinone, maritinone, isoshinanolone, scopoletin, lupeol, betulin, and betulinic acid has been reported.^{1,2)}

As a part of our search for biologically active substances from subtropical plants in the Ryukyu islands, we have so far studied the constituents of *Diospyros maritima*. Some of the results have already been reported in preliminary communications.^{3,4)} This paper reports further work on the constituents of the plant and their biological activities such as ichthyotoxic activity, germination inhibitory activity, and antifungal activity.

Results and Discussion

Structure Elucidation of the Isolates The ethanol extract of the fresh fruits afforded, after column chromatographic separation, six known naphthoquinone derivatives, 3-chloroplumbagin (4),⁵⁻⁷⁾ 3-methylplumbagin (5),⁸⁾ plumbagin (6),²⁾ droserone (7),⁹⁾ elliptinone (8),²⁾ maritinone (9),²⁾ and three new quinones 1, 2, and 3, besides abbeokutone (10),¹⁰⁻¹²⁾ 3 α ,16 α ,17-trihydroxykaurane (11),¹³⁾ lupenone (12), lupeol (13), betulin (14), betulinic acid (15), friedelin (16), oleanolic acid (17), and scopoletin (18).

The new quinone 1, orange needles of mp 121—122 °C, has a molecular formula, C₁₁H₇BrO₃, based on elemental analysis and MS spectral data. The IR [$\nu_{\max}$ (KBr) cm⁻¹: 1655, 1631, 1593] and UV [$\lambda_{\max}$ (CHCl₃) nm (log ϵ): 241 (3.89), 287 (4.13), 429 (3.69)] spectra showed the characteristics of juglone (5-hydroxy-1,4-naphthoquinone) derivatives. The MS spectrum exhibited a molecular ion peak at m/z 266 with a satellite peak at m/z 268. The relative intensity of M⁺ + 2 to M⁺ was 97%, which indicates the presence of one bromine atom in the quinone 1. The ¹H-NMR spectrum revealed the presence of one hydrogen-bonded hydroxyl [δ 11.78 (1H, s)], one quinonoid methyl [δ 2.38 (3H, s)], and three aromatic protons [δ 7.26 (1H, dd, J = 1.5, 8.0 Hz, H-6), δ 7.62 (1H, t, J = 8.0 Hz, H-7), δ 7.66 (1H, dd, J = 1.5, 8.0 Hz, H-8)] in the

quinone 1. Since the absence of the allylic coupling in the quinonoid methyl group indicates that the 3-position is occupied by a substituent, the new quinone 1 must be 3-bromoplumbagin. The final assignment of the structure has been accomplished by the following synthesis. The bromination of plumbagin (6) with bromine in tetrachlorocarbon afforded a dibromide (22), which is assumed to be the racemate of *trans*-2,3-dibromide. The dehydrobromination of the dibromide (22) with sodium acetate in acetic acid afforded 3-bromoplumbagin, and it was identical with the natural product. The isolation of brominated compounds from non-marine sources is extremely rare¹⁴⁾ and the quinone 1 is the first example of a brominated naphthoquinone isolated as a higher plant constituent.

The new quinone 2, orange-yellow needles of mp 193—195 °C, has a molecular formula, C₂₄H₁₈O₆, based on elemental analysis and MS spectral data. The IR [$\nu_{\max}$ (KBr) cm⁻¹: 1660, 1638, 1602] and UV [$\lambda_{\max}$ (CHCl₃) nm (log ϵ): 247 sh (4.35), 263 (4.41), 437 (4.04)] spectra showed the characteristics of juglone derivatives. The ¹H-NMR spectrum revealed the presence of two hydrogen-bonded hydroxyls [δ 12.43 (2H, s)], two quinonoid methyls [δ 2.14 (6H, d, J = 1.5 Hz)], two quinonoid protons [δ 6.73 (2H, q, J = 1.5 Hz)], two pairs of *ortho*-coupled aromatic protons [δ 7.49, 7.57 (each 2H, d, J = 8.0 Hz)], and one ethylidene group [δ 1.64 (3H, d, J = 6.5 Hz, >CHCH₂), δ 4.97 (1H, q, J = 6.5 Hz, >CHCH₂)] in the quinone 2. This ¹H-NMR spectrum indicates that the quinone 2 is a symmetrical dimer of plumbagin (6) linked by an ethylidene bridge between the two benzene rings. The position of the dimeric linkage can be established by the downfield shift of the signals of the carbons in the ¹³C-NMR spectrum.¹⁵⁾ The symmetrical dimeric linkage of the quinone 2 is reflected in its ¹³C-NMR spectrum which exhibits eleven lines for the 22 carbons, except for the ethylidene carbons. Ten of these lines correspond closely to those of plumbagin (6) in chemical shift. The eleventh line corresponding to C-6 (δ 124.1) in the ¹³C-NMR spectrum of plumbagin (6) is replaced by a line at δ 140.5 due to substitution. Thus, a 6-6' dimeric linkage through an ethylidene bridge is obvious, and the quinone 2 must be ethylidene-6,6'-biplumbagin. The assigned

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Chart 1

structure of the quinone **2** was fully supported by the heteronuclear multiple bond correlation (HMBC) spectrum of the quinone **2** (Fig. 1). The linkage by an ethylidene bridge between two naphthoquinone rings is quite rare in naturally occurring quinones. So far, only two compounds with an ethylidene bridge have been isolated and these are from the pigments of *Spatangus purpureus* (sea urchin).¹⁶⁾ The quinone **2** is the first example isolated as a plant constituent.

The new quinone **3**, orange needles of mp 121–123 °C, has a molecular formula, C₁₃H₁₂O₄, based on elemental analysis and MS spectral data. The IR [$\nu_{\max}$ (KBr) cm⁻¹: 1654, 1628, 1603] and UV [$\lambda_{\max}$ (CHCl₃) nm (log ϵ): 245 (3.97), 277 (4.12), 422 (3.67)] spectra showed the characteristics of juglone derivatives. The ¹H-NMR spectrum revealed the presence of one hydrogen-bonded hydroxyl [δ 12.11 (1H, s)], one quinonoid methyl [δ 2.24 (3H, s)], one 2-hydroxyethyl [δ 2.19 (1H, brs, -CH₂CH₂OH)], δ 2.95 (2H, t, J =6.5 Hz, -CH₂CH₂OH), δ 3.86 (2H, t, J =6.5 Hz, -CH₂CH₂OH)], and three aromatic protons [δ 7.23 (1H, dd, J =1.0, 8.0 Hz, H-6), δ 7.58 (1H, t, J =8.0

Fig. 1. HMBC Correlations for Compounds **2** and **3**

Table 1. Ichthyotoxic, Germination Inhibitory, and Antifungal Activities of the Compounds Isolated from *Diospyros maritima*

Compound	MLC ^{a)} (ppm)	Germination inhibitory ratio (%) ^{b)} Concentration (ppm)			Growth inhibitory zone (mm) ^{c)}
		100	10	1	
3-Bromoplumbagin (1)	0.2	4			8
Ethylidene-6,6'-biplumbagin (2)	3.0	0			NT ^{d)}
3-(2-Hydroxyethyl)plumbagin (3)	5.3	100	8		10
3-Chloroplumbagin (4)	0.2	4			6
3-Methylplumbagin (5)	3.2	10			5
Plumbagin (6)	0.4	100	58	38	15
Droserone (7)	> 10	96	2		0
Elliptinone (8)	2.7	4			0
Maritinone (9)	2.0	4			0
Abbeokutone (10)	> 20	10			0
3 α ,16 α ,17-Trihydroxykaurane (11)	> 50	6			0
Lupenone (12)	> 20	2			0
Lupeol (13)	> 50	4			0
Betulin (14)	> 50	4			0
Betulinic acid (15)	> 50	6			0
Friedelin (16)	> 20	2			0
Oleanolic acid (17)	2.7	4			0
Scopoletin (18)	> 20	2			0
Glutanol (19)	> 50	0			0
β -Amyrin (20)	> 50	8			0
Vanillic acid (21)	> 20	2			0

a) MLC: minimum lethal concentration. b) Control = 0. Complete inhibition = 100. c) Figures denote (radius of inhibition circle)—(radius of disc). Concentration: 250 μ g/disc. d) NT: not tested.

Hz, H-7), δ 7.62 (1H, dd, J = 1.0, 8.0 Hz, H-8)] in the quinone 3. These spectral data show that the new quinone 3 must be 3-(2-hydroxyethyl)plumbagin or 2-(2-hydroxyethyl)-3-methyljuglone, the former being favoured by analogy with the congeners. The position of the methyl group in the quinone 3 was determined by the HMBC spectrum of the quinone 3, which showed long-range correlations between the methyl protons and C-1 and between H-8 and C-1 (Fig. 1). On the basis of this evidence, the quinone 3 was characterized as 3-(2-hydroxyethyl)-plumbagin.

From the ethanol extract of the fresh leaves and the twigs of this plant, eleven compounds 10, 11, 13–18, glutinol (19), β -amyrin (20), and vanillic acid (21) and eight compounds 10, 11, and 13–18 were isolated, respectively. No sign of the presence of naphthoquinone derivatives was seen.

Biological Activities of the Isolates Three kinds of bioassays, ichthyotoxicity, seed germination, and antifungal test, were performed with the 21 compounds isolated from *Diospyros maritima*. The results are shown in Table 1. The ichthyotoxicity test was carried out using guppies (*Poecilia (Lebites) reticulata* PETERS). Strong activity was observed with the naphthoquinone derivatives except for the quinone 7. Contrary to expectation, the triterpenoid 17 (oleanolic acid) showed relatively strong activity. The seed germination test was carried out using lettuce seeds (*Lactuca sativa* L. var. Great Lakes). Strong activity was observed with the quinone 6. Moderate activity was observed with the quinones 3 and 7. The antifungal test was carried out using *Penicillium citrinum*. Strong activity was observed with the quinones 3 and 6. Mild activity was observed with the quinones 1, 4, and 5.

The naphthoquinone derivatives from *Diospyros mar-*

itima showed a variety of biological activities, especially plumbagin (6) showed strong activity in the three bioassays. The toxicity of the fruit of this plant is attributable to the quinone 6. The quinone 6 has been reported to show ecdysis inhibition against the larva of pink bollworm (*Pectinophora gossypiella*)¹⁷⁾ and nematocidal activity against the larva of dog roundworm (*Toxocara canis*).¹⁸⁾ Thus, the quinone 6 is expected to be applied to medicine and agricultural chemicals. *Diospyros maritima* contains a large quantity of the quinone 6 in the fruit, and hence is thought to be a valuable plant as a biological resource in the subtropics.

Experimental

General Procedures Melting points were measured on a Yanagimoto micro melting point apparatus MP-S3 and are uncorrected. Spectral data were obtained using the following instruments: IR on a JASCO A-302, UV on a Hitachi 100-50, EI-MS on a Hitachi RMU-6L (70 eV, direct inlet system), and NMR on a Hitachi R-24 (¹H, 60 MHz), a JEOL JNM-FX-100 (¹³C, 25 MHz), and JEOL α 500 (two dimensional (2D) NMR). Chemical shifts are given on a δ (ppm) scale with tetramethylsilane as an internal standard. The symbols s, d, t, q, m, and brs denote singlet, doublet, triplet, quartet, multiplet, and broad singlet, respectively. Assignments were based on the heteronuclear multiple quantum coherence (HMQC) and HMBC spectra. Thin layer chromatography (TLC) was performed on a precoated Kieselgel 60 F₂₅₄ plate (Merck) and column chromatography (CC) was carried out with Wakogel C-300 (Wako Pure Chemical).

Extraction of the Fruits The fresh fruits (26 kg) of *Diospyros maritima*, collected at Chinen, Okinawa, in March 1985, were immersed in 95% EtOH for 3 months. The extract was evaporated to dryness and the residue was partitioned between CHCl₃ and H₂O. The CHCl₃ solution was evaporated and the residue (347 g) was heated with benzene. The benzene-soluble fraction was chromatographed on a silica gel column. The column was eluted with benzene–EtOAc (gradient) and each fraction was further purified by CC and/or preparative TLC. The following compounds were separated in order of elution: 1 (15 mg), 4 (770 mg), 5 (45 mg), lupenone (12, 30 mg), 6 (33.9 g), friedelin (16, 120 mg), 2 (50 mg),

lupeol (**13**, 400 mg), **8** (225 mg), **7** (670 mg), **9** (80 mg), betulin (**14**, 550 mg), **3** (370 mg), betulinic acid (**15**, 2.64 g), scopoletin (**18**, 30 mg), oleanolic acid (**17**, 1.20 g), abbeokutone (**10**, 4.85 g), and 3 α ,16 α ,17-trihydroxykaurane (**11**, 85 mg).

3-Bromoplumbagin (**1**): Orange needles (hexane), mp 121–122°C. *Anal.* Calcd for C₁₁H₇BrO₃: C, 49.47; H, 2.64. Found: C, 49.59; H, 2.54. IR $\nu_{\max}$ (KBr) cm⁻¹: 1655, 1631, 1593, 1451, 1284, 1235, 1194, 1159, 826, 744, 730, 691. UV $\lambda_{\max}$ (CHCl₃) nm (log ϵ): 241 (3.89), 287 (4.13), 429 (3.69). ¹H-NMR (CDCl₃) δ : 2.38 (3H, s, Me), 7.26 (1H, dd, J = 1.5, 8.0 Hz, H-6), 7.62 (1H, t, J = 8.0 Hz, H-7), 7.66 (1H, dd, J = 1.5, 8.0 Hz, H-8), 11.78 (1H, s, OH). ¹³C-NMR (CDCl₃) δ : 182.5 (C-4), 181.1 (C-1), 161.7 (C-5), 149.8 (C-2), 137.9 (C-3), 136.6 (C-7), 131.4 (C-9), 124.3 (C-6), 120.1 (C-8), 114.0 (C-10), 17.9 (Me). MS m/z (%): 268 (M⁺ + 2, 74), 266 (M⁺, 76), 240 (5), 238 (5), 187 (100), 159 (51), 131 (21), 103 (25), 92 (17), 77 (43), 63 (35), 51 (27), 39 (46).

Ethylidene-6,6'-biplumbagin (**2**): Orange-yellow needles (hexane), mp 193–195°C. *Anal.* Calcd for C₂₄H₁₈O₆: C, 71.64; H, 4.51. Found: C, 71.43; H, 4.49. IR $\nu_{\max}$ (KBr) cm⁻¹: 1660, 1638, 1602, 1424, 1361, 1327, 1255, 1240, 1211. UV $\lambda_{\max}$ (CHCl₃) nm (log ϵ): 247 sh (4.35), 263 (4.41), 437 (4.04). ¹H-NMR (CDCl₃) δ : 1.64 (3H, d, J = 6.5 Hz, >CHCH₃), 2.14 (6H, d, J = 1.5 Hz, Me-2,2'), 4.97 (1H, q, J = 6.5 Hz, >CHCH₃), 6.73 (2H, q, J = 1.5 Hz, H-3, 3'), 7.49 (2H, d, J = 8.0 Hz, H-7, 7'), 7.57 (2H, d, J = 8.0 Hz, H-8, 8'), 12.43 (2H, s, OH-5, 5'). ¹³C-NMR (CDCl₃) δ : 190.7 (C-4, 4'), 184.8 (C-1, 1'), 159.3 (C-5, 5'), 149.8 (C-2, 2'), 140.5 (C-6, 6'), 135.6 (C-3, 3'), 134.2 (C-7, 7'), 130.6 (C-9, 9'), 119.2 (C-8, 8'), 114.9 (C-10, 10'), 32.6 (>CHCH₃), 18.7 (>CHCH₃), 16.7 (Me-2, 2'). MS m/z (%): 402 (M⁺, 100), 384 (41), 369 (26), 356 (4), 341 (5), 328 (2), 313 (4), 214 (19), 201 (20), 189 (11), 115 (17).

3-(2-Hydroxyethyl)plumbagin (**3**): Orange needles (CHCl₃ hexane), mp 121–123°C. *Anal.* Calcd for C₁₃H₁₂O₄: C, 67.23; H, 5.21. Found: C, 66.99; H, 5.32. IR $\nu_{\max}$ (KBr) cm⁻¹: 3475, 1654, 1628, 1603, 1453, 1383, 1360, 1329, 1308, 1293, 1268, 1201, 1052, 1032, 758. UV $\lambda_{\max}$ (CHCl₃) nm (log ϵ): 245 (3.97), 277 (4.12), 422 (3.67). ¹H-NMR (CDCl₃) δ : 2.24 (3H, s, Me), 2.19 (1H, br s, -CH₂CH₂OH), 2.95 (2H, t, J = 6.5 Hz, -CH₂CH₂OH), 3.86 (2H, t, J = 6.5 Hz, -CH₂CH₂OH), 7.23 (1H, dd, J = 1.0, 8.0 Hz, H-6), 7.58 (1H, t, J = 8.0 Hz, H-7), 7.62 (1H, dd, J = 1.0, 8.0 Hz, H-8), 12.11 (1H, s, OH-5). ¹³C-NMR (CDCl₃) δ : 190.2 (C-4), 184.2 (C-1), 161.1 (C-5), 146.5 (C-2), 143.6 (C-3), 136.0 (C-7), 132.0 (C-9), 123.9 (C-6), 119.0 (C-8), 114.7 (C-10), 61.3 (-CH₂CH₂OH), 30.0 (-CH₂CH₂OH), 13.1 (Me). MS m/z (%): 232 (M⁺, 34), 214 (83), 203 (100), 189 (27), 174 (16), 173 (18), 121 (23), 120 (17), 115 (18), 92 (21), 63 (16).

3-Chloroplumbagin (**4**)⁵: Orange-yellow leaflets (hexane), mp 123–125°C.

3-Methylplumbagin (**5**)⁸: Orange needles (hexane), mp 123–124°C.

Plumbagin (**6**)²¹: Orange needles (hexane), mp 75–76°C. ¹³C-NMR (CDCl₃) δ : 190.1 (C-4), 184.5 (C-1), 161.0 (C-5), 149.5 (C-2), 136.0 (C-7), 135.3 (C-3), 131.9 (C-9), 124.1 (C-6), 119.1 (C-8), 115.0 (C-10), 16.5 (Me).

Droserone (**7**)⁹: Orange needles (acetone–pet. ether), mp 180–182°C.

Elliptinone (**8**)²: Orange needles (benzene), mp 290–300°C (dec.).

Maritunone (**9**)²¹: Orange-red needles (hexane), mp 199–200°C.

Compounds **10** and **11** were identified by their spectral data and the identification of **12**–**18** was carried out by direct comparisons with authentic samples.

Extraction of the Leaves The fresh leaves (7.0 kg) of *Diospyros maritima*, collected at Chinen, Okinawa, in May 1985, were immersed in 95% EtOH for 3 months. The extract was evaporated to dryness and the residue was partitioned between CHCl₃ and H₂O. The CHCl₃ solution was evaporated and the residue (235 g) was heated with benzene. The benzene-soluble fraction was chromatographed on a silica gel column. The column was eluted with benzene–EtOAc (gradient) and each fraction was further purified by CC and/or preparative TLC. The following compounds were separated in order of elution: **16** (20 mg), glutinol (**19**, 15 mg), **13** (865 mg), β -amyrin (**20**, 270 mg), **14** (480 mg), **15** (3.41 g), **18** (80 mg), **17** (3.04 g), vanillic acid (**21**, 70 mg), **10** (43.6 g), and **11** (210 mg).

Compounds **19**–**21** were identified by direct comparisons with authentic samples.

Extraction of the Twigs The fresh twigs (3.8 kg) of *Diospyros maritima*, collected at Chinen, Okinawa, in May 1985, were immersed in 95% EtOH for 3 months. The extract was evaporated to dryness and the residue was partitioned between CHCl₃ and H₂O. The CHCl₃ solution was evaporated and the residue (65.4 g) was heated with benzene. The benzene-soluble fraction was chromatographed on a silica gel

column. The column was eluted with benzene–EtOAc (gradient) and each fraction was further purified by CC and/or preparative TLC. The following compounds were separated in order of elution: **16** (60 mg), **13** (1.60 g), **14** (300 mg), **15** (880 mg), **18** (25 mg), **17** (600 mg), **10** (3.80 g), and **11** (35 mg).

Synthesis of 3-Bromoplumbagin (1) Bromine (0.45 g) in CCl₄ (5 ml) was added gradually to **6** (0.5 g) in CCl₄ (5 ml), and then the reaction mixture was allowed to stand at room temperature for 24 h. After addition of H₂O, the reaction mixture was extracted with CHCl₃. The extract was washed with H₂O and dried over Na₂SO₄. After evaporation of the solvent, the residue was chromatographed on a silica gel column using benzene as eluent. The dibromide **22**, 550 mg, was obtained.

2,3-Dibromo- β -dihydroplumbagin (**22**): Colorless plates (benzene), mp 92–93°C. *Anal.* Calcd for C₁₁H₈Br₂O₃: C, 37.97; H, 2.32. Found: C, 38.03; H, 2.24. IR $\nu_{\max}$ (KBr) cm⁻¹: 1700, 1650, 1600, 1450, 1238, 1161, 1055, 885, 827, 701, 663, 505. UV $\lambda_{\max}$ (CHCl₃) nm (log ϵ): 245 (4.04), 273 sh (3.75), 368 (3.75). ¹H-NMR (CDCl₃) δ : 2.18 (3H, s, Me), 5.01 (1H, s, H-3), 7.2–7.8 (3H, m, Ar H), 11.30 (1H, s, OH). MS m/z (%): 350 (M⁺ + 4, 15), 348 (M⁺ + 2, 31), 346 (M⁺, 17), 269 (61), 267 (71), 240 (2), 238 (2), 188 (100), 187 (63), 173 (24), 160 (42), 159 (43), 131 (45), 120 (40), 103 (26), 92 (82), 77 (42), 63 (72).

To a solution of **22** (0.26 g) in AcOH (5 ml), NaOAc (0.2 g) was added, and then the mixture was allowed to stand at 80°C for 12 h. After addition of H₂O, the reaction mixture was extracted with CHCl₃. The extract was washed with H₂O and dried over Na₂SO₄. After evaporation of the solvent, the residue was chromatographed on a silica gel column using benzene as eluent. The dehydrobromination product, 3-bromoplumbagin (**1**), orange needles of mp 121–122°C from hexane, 210 mg, was obtained, and it was identical with the natural product.

Bioassays Ichthyotoxicity Test: Male guppies (*Poecilia (Lebites) reticulata* PETERS) were used for this assay. A MeOH solution (1 ml) of a test sample was added to 150 ml of distilled water in a 200 ml beaker. Five guppies were placed in the beaker. After 24 h, the dead fishes were counted. Minimum value of the concentration at which all 5 guppies were killed was determined.

Seed Germination Test: Lettuce seeds (*Lactuca sativa* L. var. Great Lakes) was used for this assay. A MeOH solution (1 ml) of a test sample was poured on a filter paper disc in a Petri dish (10 cm). After evaporation of the solvent, distilled water (10 ml) was poured into the dish. Fifty seeds were placed on the filter paper. The dish was placed in the dark for 3 d, and the number of seeds which germinated was counted. The activities were expressed as a percentage compared to the control.

Antifungal Test: *Penicillium citrinum* was used for this assay. Plates were prepared by mixing the fungus in distilled water with potato dextrose agar medium (Eiken Chemical). Each sample (250 μ g) in MeOH–CH₂Cl₂ (3:2) (25 μ l) was applied to a paper disc (8 mm). After air-drying, the disc was placed on the agar plate and incubated at 26°C for 3 d. The diameter of the growth-inhibition circle was then measured.

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