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This article is dedicated to Professor Satoshi Ōmura in celebration of his 2015 Nobel Prize.

Note

Antimalarial Phenanthroindolizine Alkaloids from *Ficus septica*

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During the screening of antimalarial substances, MeOH extract from the twigs of *Ficus septica* was shown to have potent antimalarial activity. Bioassay-guided fractionation of a methanol extract of the twigs of *F. septica* led to the isolation of a new *seco*-phenanthroindolizine alkaloid and three known phenanthroindolizine alkaloids. Their structures were elucidated on the basis of NMR analysis. All isolated compounds were tested against *Plasmodium falciparum*. Compounds 2–4 displayed antimalarial activity against the 3D7 strain of *P. falciparum* with IC₅₀ values 0.028–0.42 μM, whereas a new compound 1 exhibited a moderate antimalarial activity.

Key words *Ficus septica*; phenanthroindolizine; alkaloid; antimalarial activity

Malaria is caused by *Plasmodium* parasites. The parasites are spread to people through the bites of infected female *Anopheles* mosquitoes, called “malaria vectors.” There are five parasite species that cause malaria in humans. *P. falciparum* remains the most dangerous species and causes the most lethal form of malaria. In 2015, about 3.2 billion people, almost half of the world’s population, were at risk of malaria. According to the latest WHO report, malaria infections impacted an estimated 214 million people across 97 different countries, resulting in an estimated 438000 death.¹⁾ Early diagnosis and treatment of malaria reduces disease and prevents death. The best available treatment, particularly for *P. falciparum* malaria, is artemisinin based combination therapy (ACT).²⁾ However, the incidence of malaria is now increasing because of the appearance of multi-drug resistant *P. falciparum*, therefore new pharmacophore and more effective antimalarial drugs are urgently required. Natural products remain important potential sources of new and selective substances for the treatment of malaria.

Ficus septica is a small, arboreous, and evergreen plant belonging to the family *Moraceae* that is distributed widely throughout the tropical and subtropical regions of the Western Pacific area.³⁾ This plant has been used in folk medicine to treat colds, fevers, headaches, gastralgia, and fungal and bacterial diseases,^{4–6)} and has been reported to contain phenanthroindolizine-type alkaloids, triterpenoids, and phenolic compounds.^{7–12)} Among these components, the phenanthroindolizine-type alkaloids have important biological effects, including anti-inflammatory, antitumor, antifungal, and antibacterial activities.^{13–17)} In our search for natural products with antimalarial activity, we assayed the anti-*P. falciparum* effects of 250 crude extracts of plants in our laboratory. As a result, we found that a MeOH extract of the twigs of *F. septica* showed a significant *in vitro* antiplasmodial activity

on the 3D7 of *P. falciparum* with IC₅₀ value of 2.0 μg/mL. No reports have been published on antimalarial activities of this plant and phenanthroindolizine-type alkaloids. The MeOH extract was suspended in H₂O and extracted with chloroform and *n*-BuOH. The chloroform-soluble portion showed the most potent antimalarial activity. Bioassay-guided fractionation resulted in the isolation of a new *seco*-phenanthroindolizine-type alkaloid, compound 1, along with three previously known compounds, dehydrotylophorine (2),¹¹⁾ dehydroantofine (3),¹⁸⁾ and tylophoridine D (4).¹⁹⁾ In this paper, we report the structures of 1 and antimalarial activities of all four isolated compounds (Fig. 1).

Compound 1, gave the molecular formula, C₂₃H₂₄NO₃, as deduced from the high resolution (HR)-FAB-MS at *m/z*

Fig. 1. Structures of Compounds 1–4

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Table 1. ^1H -(500MHz) and ^{13}C -(125MHz) NMR Spectral Data of **1** (CD_3OD)

Position	δ_{C}	δ_{H}
1	114.3	6.73 (d, $J=1.6\text{Hz}$)
2	150.3	
3	152.4	
4	112.8	6.98 (d, $J=8.6\text{Hz}$)
4a	124.1	7.00 (dd, $J=8.6, 1.6\text{Hz}$)
4b	132.2	7.18 (d, $J=8.8\text{Hz}$)
5	115.5	6.95 (d, $J=8.8\text{Hz}$)
6	161.9	
7	115.5	6.95 (d, $J=8.8\text{Hz}$)
8	132.2	7.18 (d, $J=8.8\text{Hz}$)
8a	128.1	
8b	139.1	
9	142.0	8.82 (s)
11	59.7	4.86 (2H, t, $J=7.6\text{Hz}$)
12	22.8	2.57 (2H, quin, $J=7.6\text{Hz}$)
13	32.9	3.58 (2H, t, $J=7.6\text{Hz}$)
13a	157.7	
14	125.5	8.04 (s)
14a	157.9	
14b	129.5	
OCH_3 -2	56.2	3.53 (3H, s)
OCH_3 -3	56.4	3.80 (3H, s)
OCH_3 -6	55.9	3.84 (3H, s)

362.1751 $[\text{M}]^+$, which suggested the presence of 13 degrees of unsaturation. The ^1H - and ^{13}C -NMR data of **1** (Table 1) indicated the presence of three methylenes [δ_{H} 2.57 (2H, q, $J=7.6\text{Hz}$); δ_{C} 22.8 (C-12), δ_{H} 3.58 (2H, t, $J=7.6\text{Hz}$); δ_{C} 32.9 (C-13), δ_{H} 4.86 (2H, t, $J=7.6\text{Hz}$); δ_{C} 59.7 (C-11)], three methoxy groups [δ_{H} 3.53, 3.80, 3.84], a 1,4-disubstituted benzene ring [δ_{H} 6.95 (2H, d, $J=8.8\text{Hz}$), 7.18 (2H, d, $J=8.8\text{Hz}$), and a 1,2,4-trisubstituted benzene ring [δ_{H} 6.73 (d, $J=1.6\text{Hz}$); δ_{C} 114.3 (C-1), δ_{H} 6.98 (d, $J=8.6\text{Hz}$); δ_{C} 112.8 (C-4), δ_{H} 7.00 (dd, $J=8.6, 1.6\text{Hz}$); δ_{C} 124.1 (C-4a)], and an aromatic moiety with tetrasubstituents [δ_{H} 8.04 (s); δ_{C} 125.5, δ_{H} 8.82 (s); δ_{C} 142.0]. These ^1H -NMR data of **1** was similar to those of dehydroantofine (**3**) except for substituted patterns of two benzene rings A and B in **3**. In the case of **3**, the two benzene rings corresponding to the rings A and B in **1** are fused between C-4a and C-4b to form a phenanthrene ring, whereas the NMR data and the molecular formula of **1** showed that two benzene rings A and B in **1** (Fig. 2) are not linked and independent of each other. This disclosed that **1** should be a 4a,b-*seco* type of **3**. The positions of two phenolic methoxy groups on the aromatic ring A and one methoxy group on the aromatic ring B were determined to be at C-2 and C-3, and C-6, respectively, by heteronuclear multiple bond connectivity (HMBC) and nuclear Overhauser effect (NOEs) (H-1/ OCH_3 -2, H-4/ OCH_3 -3, and H-7/ OCH_3 -6). According to the HMBC correlations of H-9/C-11, 8b, and 14a, and H-14/C-13, and 14a and the HMBC correlations of H-11/C-9 and C-13a, H-12/C-13a, and H-13/C-13a, **1** turned out to have the same trihydroindolizidinium skeleton as that of **3**. In addition, the HMBC correlations of H-1/C-14a and H-9/C-8a and NOESY correlations between H-1/H-14, H-14/H-13 and H-8/H-9, H-9/H-11 suggested that the two benzene rings A and B were located at the C-14a and C-8b positions, respectively. Hence, compound **1** was assigned

Fig. 2. COSY, HMBC, and NOESY Correlations of **1**Table 2. Antimalarial and Cytotoxic Activity of Compounds **1**–**4**

Compound	<i>P.f.</i> 3D7 ^{a)} (μM)	L929 (μM)	SI ^{b)} (L929/3D7)
1	4.0	>56	>14
2	0.42	8.2	19.5
3	0.028	>55	>1964
4	0.058	>56	>966
Artemisinin ^{c)}	0.025	N.T. ^{d)}	—
Chloroquine ^{c)}	0.036	N.T. ^{d)}	—
Tafenoquine ^{c)}	N.T. ^{d)}	7.1	—

a) *Plasmodium falciparum* 3D7 strain. b) Selective index. c) Positive control. d) Not tested.

as 4a,b-*seco*-dehydroantofine. Compound **1** has been reported as a synthetic substance,²⁰⁾ but it is the first report on isolation from natural plant extracts.

All isolated compounds **1**–**4** were evaluated for *in vitro* antiplasmodial activity against a chloroquine-sensitive strain (3D7) of *P. falciparum* by the modified plasmodium lactate dehydrogenase (pLDH) method,^{21–23)} and were tested for cytotoxicity against mouse fibroblast cells L929. Chloroquine, tafenoquine, and artemisinin were used as the reference drugs in all experiments. The *in vitro* antiplasmodial activities of these compounds, as indicated by their IC_{50} values, are summarized in Table 2. All isolated compounds showed antiplasmodial activity against the 3D7 *P. falciparum* strains. Compound **1** showed moderate antimalarial activity with IC_{50} 4.0 μM . Compound **2** also showed toxicity against L929 cells (IC_{50} 8.2 μM , selectivity index=19.5), demonstrating low selectivity for the malaria parasite. However, both **3** and **4** showed potent antimalarial activities comparative to those of artemisinin and chloroquine, but were inactive against L929, which displayed a selectivity index of >966. Therefore, cytotoxicity was not considered to be an issue for **3** and **4**. Especially, compound **3** exhibited relatively strong activity against chloroquine-sensitive strain of *P. falciparum* and was about 2000-fold selective toward *P. falciparum* in comparison to its activity against L929 cells.

In conclusion, we investigated the MeOH extract of the twigs of *Ficus septica* that exhibited potent antimalarial activ-

ity, resulting in the isolation of a new *seco*-phenanthroindolizine alkaloid **1** and known phenanthroindolizine alkaloids **2–4**. Compounds **3** and **4** showed a strong growth inhibiting activity on malaria plasmodium. These have superior antimalarial activity and highly selective toxicity on *P. falciparum*. To the best of our knowledge, antimalarial activity of the phenanthroindolizine alkaloid has not been investigated. These results suggest that the phenanthroindolizine alkaloid may be a therapeutic lead of the new pharmacophore of anti-malarial drugs.

Experimental

General Procedure The NMR experiments were performed on a Varian 500MHz NMR spectrometer. Chemical shifts are given as δ (ppm) and deuterated solvent peaks as references for ^1H - and ^{13}C -NMR spectra. HR-FAB-MS was taken on a MStation JMS-700. Silica gel column chromatography (CC) was carried out on Wako C-300, Silica gel 60N (Kanto), and Sephadex LH-20. HPLC was performed on a JASCO PU-1580 pump equipped with a JASCO UV-1575 detector, and all peaks were detected at 254nm. TLC was carried out with silica gel 60F₂₅₄ and PR-18F₂₅₄ plates.

Plant Material The twigs of *Ficus septica* were collected on Yaeyama Island, Japan and a voucher specimen (745TW) was deposited at the Institute of Pharmacognosy, Tokushima Bunri University.

Extraction and Isolation The twigs of *F. septica* (1.5 kg) were powdered and extracted with MeOH (5 L) at room temperature for one month to give 37 g of crude extract, followed by solvent removal. The MeOH extract (17.6 g) was suspended in H₂O (200 mL) and extracted with chloroform and *n*-BuOH. The chloroform-soluble portion was subjected to silica gel column chromatography using the stepwise solvent system of chloroform–methanol (100:0→3:1→1:3) to yield 18 fractions (1–18). Active fraction 18 (98.4 mg) was chromatographed on a Sephadex LH-20 column eluted with methanol–chloroform (2:3) to yield 5 fractions (19–23). Fraction 22 (46.3 mg) was purified by reversed-phase HPLC (Cosmosil 5C₁₈-ARII, ϕ 10×250 mm, flow rate 2.0 mL/min) eluted with MeOH–H₂O (60:40) containing 3% trifluoroacetic acid (TFA) to give **1** (8.8 mg), **2** (5.8 mg), **3** (8.0 mg), and **4** (3.0 mg).

seco-Dehydroantofine (**1**)

Yellow oil; IR ν_{max} (attenuated total reflectance (ATR)) cm^{-1} : 1678, 1609, 1519, 1494, 1257, 1201, 1130 cm^{-1} ; ^1H -NMR (500 MHz, CD₃OD): Table 1; ^{13}C -NMR (125 MHz, CD₃OD): Table 1; HR-FAB-MS m/z : 362.1751 [M]⁺ (Calcd for C₂₃H₂₄NO₃: 362.1756).

Antiplasmodial Assay RPMI-1640 was supplemented with 25 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES), 50 $\mu\text{g}/\text{mL}$ hypoxanthine, 0.4% glucose and 5 $\mu\text{g}/\text{mL}$ gentamicin. NCTC-135 and Iscove's modified Dulbecco's medium (IMDM) were supplemented with sodium bicarbonate. These media were mixed in the ratio of 2:1:1 (RPMI-1640:NCTC-135:IMDM) to prepare reference nutrient intakes (RNI) incomplete medium. In addition, the medium was supplemented with heat inactivated 2.5% human serum and 1.1% Albumax II to make RNI complete medium. *Plasmodium falciparum* (3D7, strain) was routinely cultured at 37°C under reduced-oxygen condition (5% CO₂ and 5% O₂), in RNI complete medium supplemented with human erythrocytes to achieve a 3% hematocrit. Compounds were dissolved and

diluted in dimethyl sulfoxide (DMSO), and tested in triplicate. *P. falciparum* culture was prepared at 2% hematocrit and 0.5% parasitemia, and was added to diluted compounds so that 0.5% DMSO in 96-well micro titer plate. Test plates were incubated at 37°C under reduced-oxygen condition for 3 d. Anti-plasmodial activity was analyzed by the modified pLDH method of Makler *et al.* Briefly, 50 μL of Malstat reagent was dispensed into new micro titer plate. Ten microliters of *P. falciparum* culture were transferred into the plate, followed by 10 μL of nitro blue tetrazolium (NBT) (0.1%)–polyethersulfone (PES) (0.005%) mixture was added.²² After 2 h, absorbance was read at 650 nm using microplate reader. Artemisinin was used as positive controls, while DMSO was the negative (vehicle) control.

Cytotoxicity Assay For evaluation of cytotoxicity, L929 cells were cultured in D-MEM was supplemented with FBS at 37°C, 5% CO₂. L929 cultures were prepared at 2×10^5 cells/mL and were added to diluted compounds so that 1% DMSO in 96-well micro titer plate. The plates were incubated at 37°C for 2 d. Cytotoxicity was measured by 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. Ten microliters of MTT solution (2.5 mg/mL) were added to 100 μL of L929 cultures. After 4 h, absorbance was read the plate that MTT formazan were dissolved in DMSO at 650 nm using microplate reader.

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Conflict of Interest The authors declare no conflict of interest.

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