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ORIGINAL ARTICLE

In vitro and *in vivo* antimalarial activity of puberulic acid and its new analogs, viticolins A–C, produced by *Penicillium* sp. FKI-4410

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In the course of screening for antimalarial agents, five tropolone compounds were isolated from the culture broth of *Penicillium* sp. FKI-4410. Two were known compounds, puberulic acid and stipitatic acid. Three were new analogs of puberulic acid, designated viticolins A–C. Among them, puberulic acid exhibited potent antimalarial inhibition, with IC_{50} values of 0.01 μg ml^{-1} against chloroquine-sensitive and -resistant *Plasmodium falciparum* strains *in vitro*. Furthermore, puberulic acid showed weak cytotoxicity against human MRC-5 cells, with an IC_{50} value of 57.2 μg ml^{-1}. The compound also demonstrated a therapeutic effect *in vivo*, which compared well against the currently used antimalarial drugs, and thus shows promise as a leading candidate for development into a new antimalarial compound.
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Keywords: antimalarial activity; *Penicillium* sp.; puberulic acid; viticolin

INTRODUCTION

Malaria, caused by *Plasmodium* species, remains a major global health problem, generating over 243 million clinical cases and causing 863 000 deaths in 2008.[1] Many antimalarial agents have been developed, but resistance to them develops quickly and is now widespread. Currently, the World Health Organization (WHO) recommends artemisinin combination therapy for antimalarial treatment. However, resistance to the recently introduced artemisinin class of drugs has been reported.[2] Therefore, the development of new, safe and potent antimalarial drugs, with new modes of action and structural features, is urgently required.

In the course of our screening program to discover antimalarial drugs from metabolites of microorganisms, which are active against drug-resistant parasites *in vitro* and *in vivo*, we have discovered various microbial metabolites with potent antimalarial properties.[3–8] Recently, we have isolated some tropolone compounds from a culture broth of *Penicillium* sp. FKI-4410. These compounds are puberulic acid (**1**)[9,10] and stipitatic acid (**2**),[11] along with three new analogs, designated viticolins A–C (**3**–**5**) (Figure 1).

Puberulic acid (**1**) exhibited potent and selective antimalarial activity *in vitro* and *in vivo*, whereas the others showed moderate or weak activity *in vitro*. We report herein, the fermentation, isolation, structure elucidation and antimalarial profiles of **1** and its analogs and compared with those of the clinically used antimalarial drugs, artemisinin, artesunate and chloroquine. We also report some significant observations with respect to the structure–activity relationship of **1**.

RESULTS

Taxonomy of the producing strain FKI-4410

The producing organism, strain FKI-4410, was considered as a new species belonging to the genus *Penicillium* and was designated as *Penicillium viticola* sp. nov. The sequence of β-tubulin and calmodulin genes of this new species was deposited at the DNA Data Bank of Japan, with accession numbers AB540174 and AB540173, respectively. Taxonomic details will be reported elsewhere.[12]

Fermentation

The typical time course of fermentation for production of puberulic acid (**1**) and its analogs in a 30-l jar fermenter is shown in Figure 2. All compounds were produced only in the sup. Production of **1** was detected at day 3, reaching a max. (75 mg l^{-1}) at day 6, slowly decreasing thereafter to 30 mg l^{-1}. In comparison, production of stipitaitc acid (**2**) and viticolin A (**3**) increased gradually, reaching a peak of 26 and 40 mg l^{-1} at days 10 and 9, respectively. Other minor components, such as viticolins B (**4**) and C (**5**), could not be detected under this condition.

Isolation

The isolation procedure for **1**–**5** is summarized in Scheme 1. The 10-day-old culture broth (15 l) was centrifuged. The sup. was passed through a Sepabeads SP207 column (100 $\phi \times 160$ mm, Mitsubishi Chemical, Tokyo, Japan) previously equilibrated with H_2O containing 0.1% TFA. After washing with 40% MeOH aq. soln with 0.1%TFA

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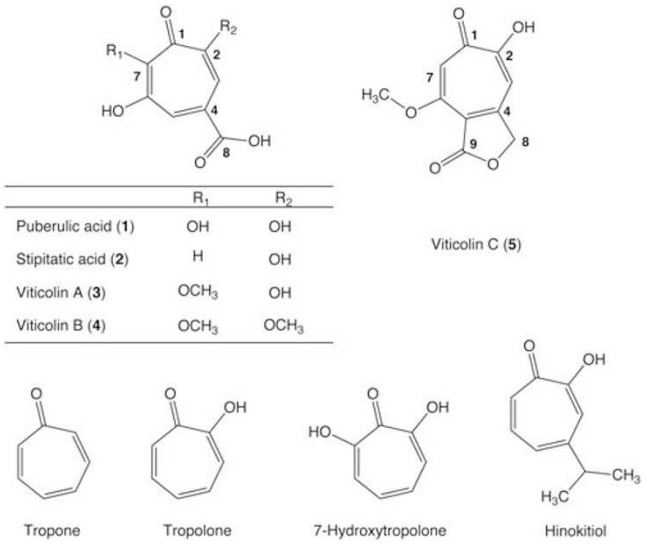
(3.5 l), the active materials were eluted with a mixture of 100% MeOH containing 0.1% TFA (3.5 l). The whole eluate was concd *in vacuo* and dried by blowing N_2 gas to give a brown material (8.0 g). The material was dissolved in a small amount of H_2O containing 0.1% TFA and passed through an ODS column (40 ϕ×160 mm, Senshu Scientific, Tokyo, Japan) previously equilibrated with H_2O containing 0.1% TFA. After washing with H_2O containing 0.1% TFA (100 ml), the active materials were eluted with 10% CH_3CN aq. soln containing 0.1% TFA (60 ml × 10). The sixth eluate fraction (60 ml) was crystallized at 4 °C and the crude crystals (197 mg) were separated by decantation. We purified 105 mg of the crude crystal by HPLC using a Pegasil ODS column (20 ϕ×250 mm, Senshu Scientific) with 25% MeOH aq. soln with 0.1% TFA at 8 ml min^{-1} detected by UV at 270 nm. The retention times of puberulic acid (**1**), stipitatic acid (**2**) and viticolins A–C (**3**–**5**) were 32, 47, 53, 39 and 22 min, respectively (Figure 3). Each active fraction was concd and freeze-dried to give **1** (5.3 mg), **2** (5.6 mg), **3** (7.7 mg), **4** (3.1 mg) and **5** (3.0 mg) as yellow powders.



	R_1	R_2
Puberulic acid (**1**)	OH	OH
Stipitatic acid (**2**)	H	OH
Viticolin A (**3**)	OCH_3	OH
Viticolin B (**4**)	OCH_3	OCH_3

Figure 1 Structures of puberulic acid (**1**), stipitatic acid (**2**), viticolins A–C (**3**–**5**) and other tropones.

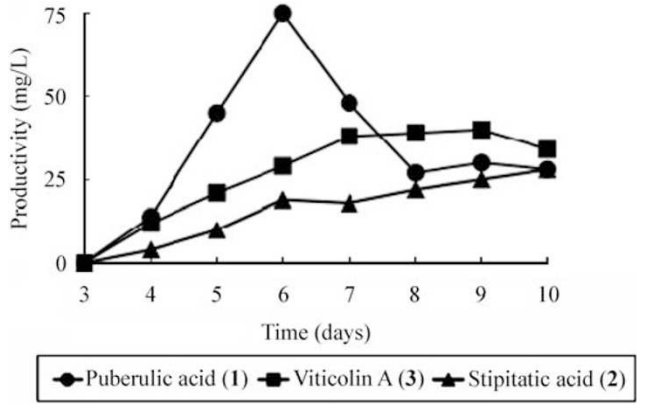


Figure 2 Typical time course of fermentation of *Penicillium* sp. FKI-4410.

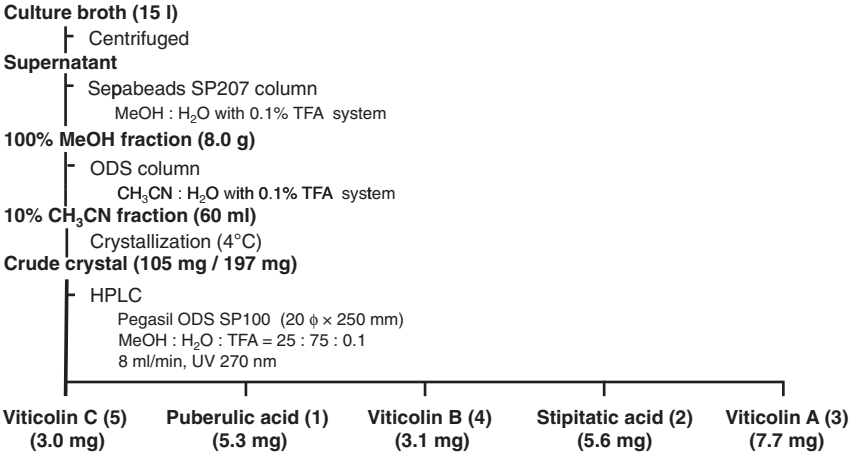
Structure elucidation

The physico-chemical properties of the isolated compounds **1**–**5** are summarized in Table 1. They are readily soluble in MeOH or DMSO but insoluble in $CHCl_3$. The characteristic absorption at UV 270 nm and the IR absorptions at 1260–1294, 1535–1581, 1593–1633 and 3410–3433 cm^{-1} suggested the presence of a hydroxytropolone moiety.[13] The strong IR absorption of **1**–**4** at 1699–1720 cm^{-1} suggested they contain carboxyl groups.

The molecular formulae of **1** and **2** were elucidated by HR-FAB-MS to be $C_8H_6O_6$ and $C_8H_6O_5$, respectively. The NMR spectral data of **1** revealed a symmetrical hydroxytropolone, consisting of four resolved aromatic carbon and one carboxyl carbon signals (Table 2). In comparison with reported data, **1** was identified as puberulic acid[11] and **2** as stipitatic acid.[14]

The structure elucidation of the other compounds was carried out by comparison of spectroscopic data obtained for **1**. The molecular formula of **3** was elucidated by HR-ESI-MS to be $C_9H_8O_6$, indicating that **3** has one additional methyl unit compared with **1**. From comparison of the ^1H and ^{13}C NMR spectra of **3** and **1,** the signals of one methoxy group appeared in **3** (Table 2) and the HMBC correlations from the methoxy proton (δ_H 3.94) and sp^2 methine proton of 5-H (δ_H 7.83) to the oxygenated sp^2 quaternary aromatic carbon of C-7 (δ_C 152.0) proved that **3** was a new 7-*O*-methyl analog of **1,** as shown in Figure 4, and now designated as viticolin A.

The molecular formula of **4** was elucidated by HR-EI-MS to be $C_{10}H_{10}O_6$, indicating that **4** has two additional methyl units when compared with **1**. From comparison of the ^1H and ^{13}C NMR spectra



Scheme 1 Isolation procedure of **1**–**5**.

of **4** and **1,** the signals of two methoxy groups appeared in **4** (Table 2). The 10 resolved carbon signals indicated that **4** might be either an unsymmetrical 2,7-*O*-dimethyl or 6,7-*O*-dimethyl analog of **1**, but not a symmetrical 2,6-*O*-dimethyl or 1,7-*O*-dimethyl analog. The HMBC correlations from a methoxy proton (δ_H 3.97) and sp^2 methine proton of 3-H (δ_H 7.55) to the oxygenated sp^2 quaternary aromatic carbon of C-2 (δ_C 162.4) and from a methoxy proton (δ_H 3.87) and sp^2 methine proton of 5-H (δ_H 7.85) to the oxygenated sp^2 quaternary aromatic carbon of C-7 (δ_C 151.0) revealed that **4** is a new 2,7-*O*-dimethyl analog of puberulic acid, as shown in Figure 1, and now designated as viticolin B (Figure 4).

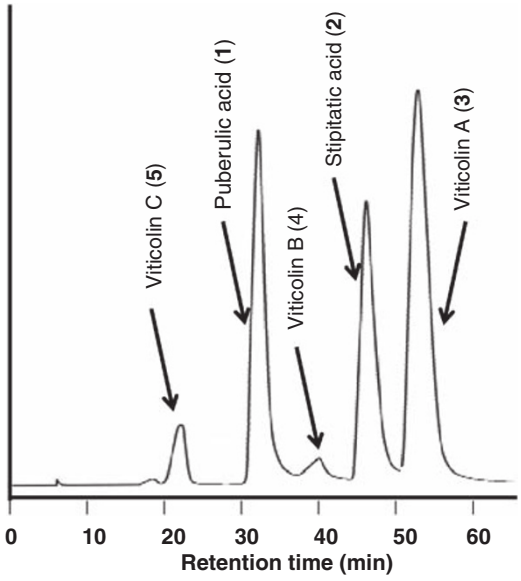


Figure 3 Purification of **1**–**5** by HPLC. The detailed conditions of HPLC are described in 'Results'.

The molecular formula of **5** was elucidated by HR-FAB-MS to be $C_{10}H_8O_5$, requiring seven degrees of unsaturation. The IR absorption at 1753 cm^{-1} suggested the presence of a lactone ring. The ^1H and ^{13}C NMR spectral data of **5** are listed in Table 2. The ^{13}C NMR, HMQC and HMBC spectra indicated 10 carbons, which were classified into seven aromatic carbons of a tropolone skeleton, one ester carbonyl carbon at δ_c 170.1, one oxygenated sp^3 methylene carbon at δ_c 70.3 and one methoxy carbon at δ_c 57.1, thus, accounting for six degrees of unsaturation. Therefore, the remaining degree of unsaturation should be because of a ring structure. The HMBC correlations from the oxygenated sp^3 methylene proton of 8-H_2 (δ_H 5.10) to the sp^2 quaternary aromatic carbon signal of C-4 at δ_C 158.2 and C-5 at δ_C 110.2 and ester carbonyl carbon of C-9 (δ_C 170.1) confirmed the presence of an α, β-unsaturated γ-lactone ring. The HMBC correlations from the sp^2 methine proton of 7-H (δ_H 6.59) to two oxygenated sp^2 quaternary aromatic carbons of C-6 (δ_C 169.1) and C-2 (δ_C 163.3) and the sp^2 quaternary aromatic carbon signal of C-5, from the sp^2 methine proton of 3-H (δ_H 6.47) to one oxygenated sp^2 quaternary aromatic carbons of C-2, the sp^2 quaternary aromatic carbon signal of C-5 and the sp^3 methylene carbon signal of C-8 (δ_C 70.3) and from a methoxy proton (δ_H 3.75) to C-6 revealed that an α, β-unsaturated γ-lactone ring was fused with a 6-*O*-methytropolone ring, as shown in Figure 4. This structure was confirmed by the NOE correlations between the methoxy proton and H-7, and between H-3 and H_2-8. Thus, the structure of **5** was determined to be another new analog of **1**, designated as viticolin C.

Antimalarial activity *in vitro*

Table 3 shows the *in vitro* antimalarial activities of the isolated compounds, together with some other known tropone compounds and some std antimalarial drugs. Puberulic acid (**1**) showed significant potent and

Table 1 Physico-chemical properties of viticolins A–C (3–5), puberulic acid (1) and stipitatic acid (2)

	*Viticolin A (**3**)*	*Viticolin B (**4**)*	*Viticolin C (**5**)*	*Puberulic acid (**1**)*	*Stipitatic acid (**2**)*
Appearance	Yellow powder	Yellow powder	Yellow powder	Yellow powder	Yellow powder
Molecular formula	$C_9H_8O_6$	$C_{10}H_{10}O_6$	$C_{10}H_8O_5$	$C_8H_6O_6$	$C_8H_6O_5$
MW	212	226	208	198	182
EI-MS (*m/z*)		226 [M^+]			
FAB-MS (*m/z*) positive			209 [M+H]$^+$		
FAB-MS (*m/z*) negative				197 [M–H]$^-$	181 [M–H]$^-$
ESI-MS (*m/z*) negative	211 [M–H]$^-$				
HR-MS (m/z)					
Calcd.	211.0243	226.0478	209.0450	197.0087	181.0144
Found	211.0235	226.0478	209.0447	197.0085	181.0137
UV λ_{max}^{MeOH} nm (ε)	270 (19720), 345 (5300)	270 (10620), 350 (3840)	252 (18510), 349 (6650)	268 (49500), 368 (10300)	270 (13320), 350 (4140)
IR ν_{max}^{KBr} cm^{-1}	3431, 1711, 1622, 1574, 1529, 1385, 1294, 1248, 1194	3423, 1718, 1629, 1581. 1442, 1385, 1260, 1209	3431, 1753, 1686, 1620, 1601, 1554, 1541, 1483, 1427, 1367, 1350, 1279, 1225, 1171	3410, 1699, 1593, 1535, 1392, 1348, 1290, 1217	3433, 1720, 1633, 1579, 1454, 1383, 1288, 1211, 1072
Solubility					
Soluble	MeOH	MeOH	DMSO	MeOH, DMSO	MeOH
Slightly soluble	CH_3CN, acetone	CH_3CN	H_2O, MeOH, CH_3CN	H_2O, CH_3CN, acetone	CH_3CN
Insoluble	$CHCl_3$	$CHCl_3$	$CHCl_3$	$CHCl_3$	$CHCl_3$

Table 2 ^{1}H and ^{13}C NMR spectral data of viticolins A–C (3–5), puberulic acid (1) and stipitatic acid (2)

	*Viticolin A (**3**)*[a]		*Viticolin B (**4**)*[a]		*Viticolin C (**5**)*[b]		*Puberulic acid (**1**)*[c]		*Stipitatic acid (**2**)*[a]	
Position	^{13}C	^{1}H *(J in Hz)*	^{13}C	^{1}H *(J in Hz)*	^{13}C	^{1}H *(J in Hz)*	^{13}C	^{1}H *(J in Hz)*	^{13}C	^{1}H *(J in Hz)*
1	170.5	—	172.3	—	171.2	—	159.4	—	176.6	—
2	163.8	—	162.4	—	163.3	—	155.4	—	165.9[d]	—
2-*O* CH_3	—	—	56.9	3.97 (3H, s)	—	—	—	—	—	—
3	111.0	7.72 (1H, s)	109.8	7.55 (1H, s)	110.1	6.47 (1H, s)	119.3	7.96 (1H, s)	113.4	7.62 (1H, s)
4	133.0	—	130.2	—	158.2	—	128.4	—	139.1	—
5	122.5	7.83 (1H, s)	126.3	7.85 (1H, s)	110.2	—	119.3	7.96 (1H, s)	124.2	7.62 (1H, s)
6	161.0	—	158.0	—	169.1	—	155.4	—	169.3[d]	—
6-*O* CH_3	—	—	—	—	57.1	3.75 (3H, s)	—	—	—	—
7	152.0	—	151.0	—	114.2	6.59 (1H, s)	159.4	—	117.4	6.95 (1H, s)
7-*O*CH_3	58.8	3.94 (3H, s)	60.1	3.87 (3H, s)	—	—	—	—	—	—
8	167.0	—	168.8	—	70.3	5.10 (2H, s)	167.4	—	168.5	—
9	—	—	—	—	170.1	—	—	—	—	—

[a]Measured in CD_3OD.
[b]Measured in DMSO-d_6 + 5% CD_3OH.
[c]Measured in acetone-d_6.
[d]Exchangable.

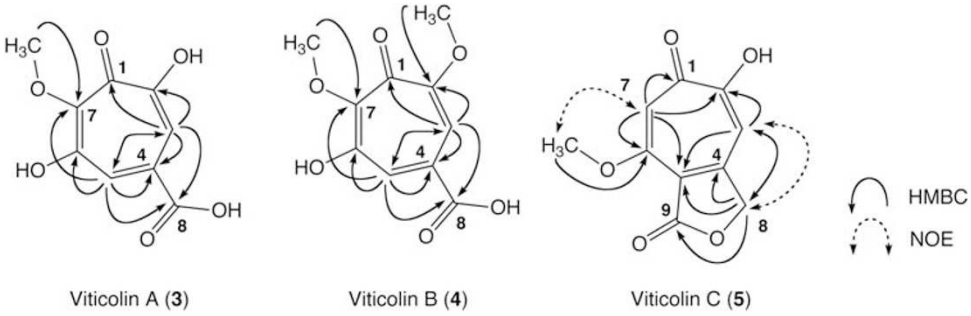


Figure 4 HMBC and NOE correlations in viticolins A–C (**3**–**5**).

Table 3 *In vitro* antimalarial activity against *Plasmodium falciparum* K1 and FCR3 strains, and cytotoxicity against MRC-5 cells of puberulic acid (1), stipitatic acid (2), viticolins A-C (3–5), selected tropone compounds and some commonly-used antimalarial drugs

	IC_{50} (ng/ml)				
	Antimalarial activity			*Selectivity index (SI)*	
Compound	*K1 strain*[a]	*FCR3 strain*[b]	*Cytotoxicity (MRC-5)*	*MCR-5/K1*	*MCR-5/FCR3*
Puberulic acid (**1**)	10	10	57 200	5720	5720
Viticolin A (**3**)	9920	ND	>100 000	>10	—
Viticolin B (**4**)	840	540	6790	8.1	12.60
Stipitatic acid (**2**)	7070	ND	>100 000	>14	—
Viticolin C (**5**)	>12 500	ND	ND	—	—
Tropone	9420	ND	>100 000	>10	—
Tropolone	>12 500	ND	ND	—	—
7-Hydroxytropolone	890	1170	240	0.27	0.21
Hinokitiol	4920	ND	5800	1.20	—
Artemisinin[c]	6	6	45 170	7528	7528
Artesunate[c]	4	1	14 224	3556	14 224
Chloroquine[c]	184	15	18 572	101	1238

Abbreviation: ND, not determined.
[a]Chloroquine-resistant strain.
[b]Chloroquine-sensitive strain.
[c]Drugs commonly used to treat malaria.

uniform activity against both the chloroquine-resistant K1 strain and the chloroquine-sensitive FCR3 strain of *P. falciparum*. Antimalarial activity against both K1 and FCR3 strains was identical (0.01 μg ml^{-1}). The activity of **1** against the K1 strain was of the same order as that of artemisinin or artesunate. The antimalarial properties of all other compounds tested were relatively weak. Against the K1 strain, viticolin B (**4**) and 7-hydroxytropolone showed IC_{50} values of 0.84 and 0.89 μg ml^{-1}, respectively. Viticolin A (**3**), stipitatic acid (**2**), tropone and hinokitiol had even weaker antimalarial activity, in the range of 5–10 μg ml^{-1}. Viticolin C (**5**) and tropolone did not show any antimalarial activity.

Among the tropolones tested, Barnard *et al.*[15] have previously reported the IC_{50} values of hinokitiol and tropolone against a clone NC-1 of the FCB strain of *P. falciparum* were 0.5 and 3.7 μg ml^{-1}, respectively. Explanation for the differences between the two sets of results may be because of either the different assay conditions or the different *Plasmodium* strains used.

The cytotoxicities of the tested compounds are also depicted in Table 3. The isolated compounds and tropones had weak (IC_{50}= 5–60 μg ml^{-1}) or no cytotoxicity, except 7-hydroxytropolone (IC_{50}=0.24 μg ml^{-1}). To evaluate the combined antimalarial activities and cytotoxicities, we introduced a selectivity index (cytotoxicity (IC_{50} for the MRC-5 cells)/ antimalarial activity (IC_{50} for the K1 strain or the FCR-3 strain)), as depicted in Table 3. Puberulic acid (**1**) showed a relatively high selectivity index of 5720, irrespective of parasite strain, significantly greater than that shown by chloroquine, indicating that the compound holds significant promise as an antimalarial lead. Of all the other compounds tested, none exhibited a favorable selectivity index.

Antimalarial activity *in vivo*

Preliminary *in vivo* antimalarial activities of **1** and the std antimalarial drugs, injected s.c., were measured in a mouse model, using the rodent malaria *P. berghei* N strain, which is chloroquine-sensitive. A dose of 2 mg kg^{-1} of **1** suppressed 69% of malaria parasites. Under the same experimental conditions, the ED_{50} values of artesunate and chloroquine were 1.7 and 1.5 mg kg^{-1}, respectively This initial finding that the *in vivo* s.c. antimalarial activity of **1** is similar to both artesunate and chloroquine confirms that puberulic acid shows substantial promise as a lead antimalarial compound.

DISCUSSION

We isolated puberulic acid (**1**), stipitatic acid (**2**) and structurally related new compounds, viticolins A–C (**3**–**5**). The *in vitro* antimalarial and cytotoxic studies of these five compounds, together with some other known tropone compounds, provided valuable insight into structure–activity relationships.

The hydroxy group at C-7 of puberulic acid appears to be an important moiety for antimalarial activity. Compounds **3** and **4,** possessing a methoxy group at C-7, and **2,** which lacks a hydroxy group at C-7, were 1,000-, 80- and 700-fold less active than **1**, respectively. Moreover, 7-hydroxytropolone, which has a hydroxy group at C-7, was 14-fold more active than tropolone. The carboxylic group at C-4 of puberulic acid appears to be important with respect to selectivity. Compounds **1** and **2**, each with a carboxylic group at C-4, showed a better selectivity index than 7-hydroxytropolone and hinokitiol. The methoxy group at C-2 of **3** also seems to improve antimalarial activity. Compound **3** has a hydroxy group at C-2 and is 12-fold less active than **4**. This might indicate that a 2-*O*-methyl analog of **1** would have more potent antimalarial activity than **1**. Further studies are necessary to understand more comprehensively the detailed structure-activity relationships of **1** and its analogs.

It is known that **1** possesses inhibitory activity against Gram-positive bacteria.[16] In general, the natural and synthetic tropolones have been reported to show antibacterial, antifungal, insecticidal, antiviral and antitumor activities, as well as inhibiting enzymes such as aminoglycoside-2″-*O*-adenyltransferase,[17] metalloprotease[18] and HIV-1 reverse transcriptase-associated ribonuclease H.[19] The inhibitory mechanisms of tropolones were thought to reflect their ability to form a complex with divalent cations.[20] Among these compounds, hinokitiol and its related synthetic derivatives have been reported to show antimalarial activity, whereas the synthetic benzotropolone derivatives[15,21,22] and a dihydrotroplone antibiotic, cordytropolone,[23] have also been reported to show moderate or weak antimalarial activities *in vitro*. However, this paper represents the first report of the antimalarial activity of carboxytropolones, such as puberulic acid.

The above results indicate that puberulic acid is a promising lead compound for development of a new antimalarial drug. Further investigation, including extensive *in vivo* testing, of the antimalarial potential of **1** is in progress.

METHODS

General experiment and compounds

NMR spectra were measured on a Varian XL-400 spectrometer (Varian, Palo Alto, CA, USA) with ^{1}H NMR at 400 MHz and ^{13}C NMR at 100 MHz or a Varian Inova 600 spectrometer with ^{1}H NMR at 600 MHz and ^{13}C NMR at 150 MHz. The chemical shifts were expressed in p.p.m. and were referenced to the solvent, $(CD_3)_2CO$ (2.05 p.p.m.), CD_3OD (3.30 p.p.m.) or $(CD_3)_2SO$ (2.50 p.p.m.) in the ^{1}H NMR spectra and referenced to the solvent, $(CD_3)_2CO$ (29.8 p.p.m.), CD_3OD (49.0 p.p.m.) or $(CD_3)_2SO$ (39.5 p.p.m.) in the ^{13}C NMR spectra. FAB-MS and ESI-MS spectra were measured on a JEOL JMS AX-505 HA-MS (JEOL, Akishima, Japan) and a JEOL AccuTOF apparatus. IR spectra (KBr) were taken on a Horiba FT-210 FT IR spectrometer (Horiba, Kyoto, Japan). UV spectra were measured with a Beckman DU640 spectrophotometer (Beckman, Fullerton, CA, USA). Tropone group compounds, tropone, tropolone and hinokitiol, were purchased from Sigma (Sigma-Aldrich, St Louis, MO, USA). The compound 7-hydroxytropolone was provided by Dr Shinichi Kondo (Bioscience Associates, Tokyo, Japan).

Fermentation

Strain FKI-4410, isolated from a fruit of grape produced in Yamanashi, Japan, was grown and maintained on an agar slant consisting of 0.1% glycerol, 0.08% KH_2PO_4, 0.02% K_2HPO_4, 0.02% $MgSO_4{\cdot}7H_2O$, 0.02% KCl, 0.2% $NaNO_3$, 0.02% yeast extract and 1.5% agar (adjusted to pH 6.0 before sterilization). A loopful of spores of the strain was inoculated into 100 ml of the seed medium consisting of 2.0% glucose, 0.5% Polypepton (Nihon Pharmaceutical, Tokyo, Japan), 0.2% yeast extract, 0.2% KH_2PO_4, 0.05% $MgSO_4{\cdot}7H_2O$ and 0.1% agar (adjusted to pH 6.0 before sterilization) in each of two 500-ml Erlenmeyer flasks. The flasks were incubated on a rotary shaker (210 r.p.m.) at 27°C for 3 days. For production of **1** and its analogs, a 200-ml portion of the seed culture was transferred to a 30-l jar fermenter containing 15 l of production medium (3.0% sucrose, 3.0% soluble starch, 2.0% malt extract, 0.3% Ebios, 0.5% KH_2PO_4 and 0.5% $MgSO_4{\cdot}7H_2O$ (adjusted to pH 6.0 before sterilization)), and fermentation was carried out at 27 °C for 10 days with aeration (8 l min^{-1}). The time courses of productivity from the sup.(s) of **1**–**3** were measured by HPLC analysis during fermentation.

Assay of antimalarial activity *in vitro* and *in vivo*

In vitro activities against *Plasmodium falciparum* strains K1 (chloroquine resistant) and FCR3 (chloroquine sensitive) and cytotoxicity against human diploid embryonic cell line MRC-5 were measured, as described previously.[3] A mouse model using a malaria-derived strain of *P. berghei* N (chloroquine sensitive) was used to assess *in vivo* antimalarial activity, as described previously.[3,4] Test compounds were solubilized in 10% DMSO-Tween 80 aq. soln and administered s.c. to mice 2 h after infection with parasites (day 0). The individual compound was then successively administered (s.c.) to the infected mice once a day for 3 days (days 1–3). One day after the last treatment (day 4),

thin blood films were made from the tail blood of the mice and parasitaemia was determined, as described previously.[4]

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