New Type of Febrifugine Analogues, Bearing a Quinolizidine Moiety, Show Potent Antimalarial Activity against *Plasmodium* Malaria Parasite

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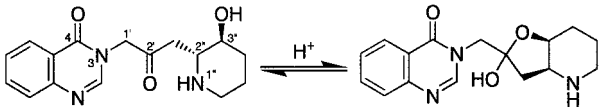
Febrifugine (**1**) and isofebrifugine (**2**), isolated from the roots of *Dichroa febrifuga* Lour. (Chinese name: Cháng Shan), are active principles against malaria. Adducts of **1** and **2** with acetone, Df-1 (**3**) and Df-2 (**4**), respectively, were obtained using silica gel and acetone. They showed high activity against *P. falciparum* malaria in vitro. Compound **3** was found to be equally effective against *P. berghei* in vivo as the clinically used drug chloroquine, whereas **4** showed only 1/24 of the activity of **3**. Metabolism studies of these compounds revealed that compound **4** is readily metabolized in mouse liver. Accordingly, the dose of **4** must be higher than that of **3** to attain blood levels sufficient for a favorable therapeutic effect.

Introduction

Malaria is by far the most important tropical parasitic disease, and it kills more people than any other communicable disease except for tuberculosis. In many developing countries, especially in Africa, malaria exacts an enormous toll in lives, in medical costs, and in days of labor lost. *Plasmodium falciparum* accounts for the majority of infections and is the most lethal. Malaria is a curable disease if promptly diagnosed and adequately treated. A number of medicines such as chloroquine and quinine are available for treatment of malaria, but the rapid development of drug resistance is a serious problem. Medicinal agents based on novel mechanisms of action are, therefore, required to overcome the emergence of resistance and to control an ever-increasing number of epidemics caused by the malaria parasite.

For centuries in China, the roots of *Dichroa febrifuga* Lour. (Chinese name: Cháng Shan), a saxifragaceous plant, have been employed against malaria fevers, and no parasite resistant to *D. febrifuga* has been reported. Febrifugine (**1**) and isofebrifugine (**2**) were isolated as active principles against malaria.[1,2] The absolute configurations of **1** and **2** were revised very recently through the asymmetric total synthesis of each stereoisomer of febrifugine by Kobayashi et al.[3] Isofebrifugine (**2**) is an isomer of febrifugine (**1**) (Scheme 1) which is said to isomerize by the mechanism suggested by Berkelhammer et al.[4] In our experiments, the isomerization of **1** to **2** and the reverse reaction were found to readily occur in a protic solvent, such as methanol. Treatment of these compounds in methanol at 50 °C for less than 12 h afforded a 1:1 mixture of each compound as judged on the basis of TLC analysis. Even at room temperature, their isomerization was found to occur slowly, whereas their hydrochloric acid salts did not isomerize under the same conditions.

Scheme 1. Structures and Isomerization of **1** and **2**



Clinical testing of **1** was not successful, and the results of clinical evaluation are not yet available. To assess the biological properties of these alkaloids in detail, we set about studying febrifugine and its analogues.

A lot of work on the antimalarial activity of many febrifugine analogues has been reported previously.[5−7] It has been demonstrated that most febrifugine analogues bearing a modified or nonmodified 4-quinazolinone moiety are active, but analogues produced through modification of the side chain attached to the N-3 position of the 4-quinazolinone ring proved to be ineffective. Furthermore, a racemic febrifugine prepared synthetically was reported to be about one-half as effective as natural febrifugine.[5] From this line of evidence, it can easily be deduced that the antipode of natural febrifugine possesses no or very poor activity against the malaria parasite. These results suggested that the 4-quinazolinone moiety, the nitrogen atom of the piperidine ring, and the hydroxyl group are necessary for the antimalarial activity and that the absolute configuration of these functional groups plays an important role.

In an investigation of the functional and conformational factors contributing to the antimalarial activity of these compounds, the isomerization and multiconformations of **1** may pose some difficulty.

Chemistry

In this study, we tried to cyclize at the nitrogen atom of the piperidine ring and the α-methylene of the keto

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Table 1. Antimalarial Activity of Febrifugine Derivatives against *P. falciparum* in Vitro

	P. falciparum FCR-3[a]		*P. falciparum* K1[b]		
compd	EC_{50} (M)	selectivity[d]	EC_{50} (M)	selectivity[d]	cytotoxicity[c] EC_{50} (M)
1	7.0×10^{-10}	243	1.2×10^{-9}	142	1.7×10^{-7}
2	3.4×10^{-9}	53	1.8×10^{-9}	100	1.8×10^{-7}
3	1.6×10^{-9}	238	3.0×10^{-9}	127	3.8×10^{-7}
4	2.8×10^{-9}	857	4.2×10^{-9}	571	2.4×10^{-6}
chloroquine	1.8×10^{-8}	1778	1.3×10^{-7}	246	3.2×10^{-5}
artemisinin	7.8×10^{-9}	1280		*e*	1.0×10^{-5}

[a] Chloroquine-sensitive strain. [b] Chloroquine-resistant strain. [c] Against FM3A mouse mammary cells. [d] Selectivity = antimalarial acitivity/cytotoxicity. [e] Not tested.

Scheme 2. Transformation of **1** and **2** into Their Acetone Adducts

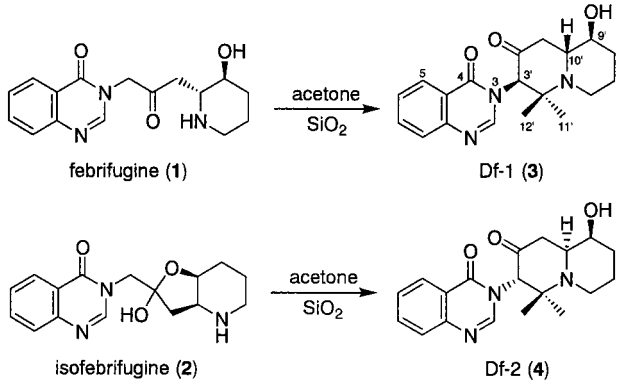
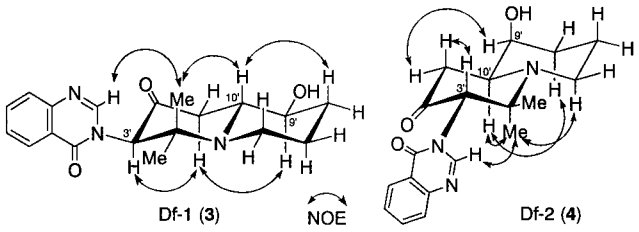


Chart 1. NOEs of **3** and **4**



group of natural febrifugine (**1**) via an acetone molecule by the Mannich reaction, so as to form a comparatively rigid conformation with the quinolizidine ring. It was hoped that this transformation would fix the conformation of the molecule and facilitate investigation of the structure−activity relationship. From our preliminary attempts, it was revealed that under the typical conditions of the Mannich reaction, in the presence of hydrochloric acid or formic acid, decomposition of the molecule occurred. Therefore, silica gel for column chromatography was adopted as a weak acid for this transformation.

Febrifugine (**1**) was applied to a silica gel column prepared with a mixture of acetone, *n*-hexane, and methanol, and the same solvent mixture was used as the eluent (Scheme 2). An adduct of **1** with acetone, Df-1 (**3**), was obtained with a yield of 74%, with 17% of recovery of the starting material. Isofebrifugine (**2**) was also transformed to its acetone adduct, Df-2 (**4**), in a yield of 22%, with 46% recovery of the starting material. Prolongation of elution resulted in decomposition of the starting materials without improvement of the yield of the adduct. The structures were elucidated by NMR spectral analysis and EI-MS, and the stereochemistry of each product was determined by NOE spectral analysis as shown in Chart 1. However, using this method, an adduct of **1** with formaldehyde was obtained with very poor yield, and unfortunately, no other aldehyde or ketone adduct was produced under the same conditions.

Table 2. Antimalarial Activities of Febrifugine Derivatives against *P. berghei* in Vivo[a]

compd	ED_{50} (μmol/kg)	ED_{90} (μmol/kg)
1	1.0	5.0
2	*b*	*b*
3	7.3	19.4
4	76	*b*
chloroquine	4.7	9.4
artemisinin	17.7	46.0

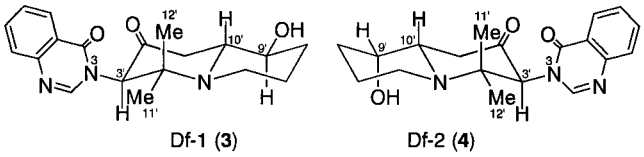
[a] *P. berghei* malaria parasite was injected iv, and samples were also administrated ip. [b] Not tested or determined.

Biological Evaluation and Discussion

Antimalarial activity against *P. falciparum* and cytotoxicity against mouse mammary FM3A cells in vitro were investigated examining the febrifugine derivatives as their hydrochloric acid salts. Table 1 demonstrates that febrifugine derivatives (**1**−**4**) showed antimalarial activity with higher potency in vitro than the clinically used drugs, chloroquine and artemisinin,[8] and the therapeutic selectivity of each of the compounds was fairly favorable. Moreover, **1**, **2**, and their derivatives (**3**, **4**) showed almost the same antimalarial activity against both the chloroquine-sensitive FCR-3 strain and the chloroquine-resistant K1 strain of *P. falciparum*, whereas the EC_{50} of chloroquine against the K1 strain was approximately 1/10 of the EC_{50} against the FCR-3 strain. Interestingly, in an in vivo study of the antimalarial activity against the mouse malaria parasite *P. berghei*, it was found that compound **3** (ED_{50}: 6.0 μmol/kg) was 24 times more potent than **4** (ED_{50}: 145 μmol/kg), though the effects of both of these highly active compounds against *P. falciparum* in vitro were almost the same (Table 2).

To examine the reason **3** and **4** showed drastically different activity in vivo, the conformations of these compounds were investigated. It was found that their structures differ only in the stereochemistry at the 3′ and 10′ positions. This evidence confirmed that Df-1 (**3**) was an antipode of Df-2 (**4**), if the stereochemistry of the hydroxyl group at the 9′ position was disregarded (Chart 2). These diastereomeric compounds may be a good model for the racemic febrifugine reported by Hewitt et al.[5] These two compounds must act on the malaria parasites in erythrocytes directly, since both are effective against *P. falciparum* in vitro. However, it was assumed that a different rate of metabolism of the samples administered intraperitoneally is responsible for the difference in their activities in vivo. This assumption was proved by a preliminary metabolic ex-

Chart 2. Conformations of **3** and **4**



periment using S-9 mix prepared from mouse liver. As expected, **4** could not be detected after incubation with S-9 mix for 20 min or longer at 37 °C, whereas **3** remained detectable for more than 40 min. In other words, **4** was immediately metabolized selectively by enzymes in the liver, and the dose of the sample must be higher than that of **3** to attain blood levels sufficient for a favorable therapeutic effect.

Summary and Conclusion

We have obtained febrifugine derivatives, Df-1 (**3**) and Df-2 (**4**), with high antimalarial potency against both chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum* with good therapeutic selectivity and found that **3** showed high activity in vivo which was comparable to that of chloroquine. These results support the view that it is a promising new lead compound of a new type of antimalarial drug. Further investigations examining the structure−activity relationships, including the participation of each functional group of molecules, and the mechanisms of action of these compounds including the metabolism are now in progress.

Experimental Section

General Methods. The melting points were measured under a microscope (Yanagimoto micro melting point apparatus) and are uncorrected. Analytical TLC was performed on silica gel 60 F_{254} (Merck) and aluminum oxide 60 F_{254} (Merck). Column chromatography was carried out on silica gel 60 (70−230 mesh, Merck) and activated alumina (300 mesh, Wako Pure Chemicals, Co., Ltd., Osaka). For the preparation of **3** and **4** Wako-gel C-200 (Wako Pure Chemicals, Osaka) was used as a silica gel. Chloroquine diphosphate (Sigma) was used as a positive control in the antimalarial assay in vitro and in vivo. IR and UV spectra were recorded using a JASCO FT-IR 5300 infrared spectrometer and a Hitachi U-3200 spectrometer, respectively. Optical rotation was measured using a JASCO DIP-370 digital polarimeter. ^{1}H and ^{13}C NMR spectra were recorded on a JEOL JNM GX-500 spectrometer (^{1}H: 500 MHz, ^{13}C: 125 MHz) and a Varian Gemini 2000 spectrometer (^{1}H: 300 MHz). Chemical shifts for ^{1}H and ^{13}C NMR are given in ppm (δ) relative to tetramethylsilane (δ_H 0.00) and $CDCl_3$ (δ_C 77.1) as internal standards, respectively, and coupling constants were reported in hertz. High-resolution EI mass spectra were measured on JEOL JMS DX-303 and JMS AX-500 mass spectrometers.

Isolation of Febrifugine (1) and Isofebrifugine (2) from *D. febrifuga* Roots. A dried root of *D. febrifuga* (20 kg) was extracted three times with 70 L of methanol at room temperature to give the extract. The methanol extract was partitioned with 0.1 M hydrochloric acid and ethyl acetate. The aqueous layer was then adjusted to pH 9−10 with ammonium hydroxide, followed by extraction with ethyl acetate to give an alkaloid fraction (18 g). The alkaloid fraction was chromatographed over silica gel, and the column was eluted with ethyl acetate−methanol mixtures by increasing polarity. A repeated fractionation of the ethyl acetate−methanol (8:2−0:10) eluting fraction using silica gel and aluminum oxide columns eluted with chloroform−methanol mixture afforded febrifugine (**1**) (0.88 g) and isofebrifugine (**2**) (1.12 g). Spectral data of these compounds were identical with those in the literature.[9]

3-[(3*R*,9*S*)-Hydroxyl-4,4-dimethyl-2-oxo-3-quinolizidyl]-4-quinazolinone (Df-1) (3). To a silica gel (5 g) column made with hexane−acetone−methanol (4:16:1) was applied a solution of **1** (46 mg) in the same solvent. The column was eluted with the same solvent until a spot of the desired product (**3**) on TLC analysis disappeared, and acetone, a mixture of acetone−methanol (9:1), and methanol were used as the following eluent. The eluted fractions were combined, and the products were purified by an aluminum oxide column chromatography using a mixture of hexane and chloroform. **3** and recovered **1** were obtained, 38 mg (74%) and 8 mg (17%), respectively.

Df-1 (3): colorless needle; mp 111−112 °C; $[\alpha]^{25}_D$ −239.3° (*c* 0.941, $CHCl_3$); UV ($CHCl_3$) λ_{max} (ϵ) 316.1 (2731), 304.5 (3465), 265.6 (7970), 225.0 (27716); IR ($CHCl_3$) ν_{max} 3470, 1726, 1680 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 8.30 (1H, dd, J = 1.4, 8.0, H-5), 8.03 (1H, s, H-2), 7.78 (1H, ddd, J = 1.4, 7.2, 8.1, H-7), 7.72 (1H, dd, J = 1.2, 8.1, H-8), 7.49 (1H, ddd, J = 1.2, 7.2, 8,0, H-6), 6.12 (1H, s, H-3′), 3.52 (1H, ddd, J = 4.3, 7.5, 10.2, H-9′), 3.12 (1H, dt, J = 11.1, 4.0, H-6′α), 3.04 (1H, dd, J = 4.0, 15.1, H-1′β), 2.76 (1H, ddd, J = 4.0, 7.5, 11.4, H-10′), 2.58 (1H, dd, J = 11.4, 15.1, H-1′α), 2.34 (1H, dt, J = 2.8, 11.1, H-6′β), 2.02 (1H, dq, J = 12.1, 4.3, H-8′α), 1.83 (1H, m, H-7′β), 1.68 (1H, m, H-7′α), 1.37 (1H, ddt, J = 4.1, 12.1, 10.2, H-8′β), 1.17 (3H, s, H-11′), 1.13 (3H, s, H-12′); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 202.7 (s, C-2′), 161.7 (s, C-4), 147.5 (s, C-8a), 146.4 (d, C-2), 134.7 (d, C-7), 127.5 (d, C-8), 127.4 (d, C-5), 127.1 (d, C-6), 121.7 (s, C-4a), 74.1 (d, C-9′), 66.3 (d, C-3′), 63.8 (s, C-4′), 61.3 (d, C-10′), 44.7 (t, C-6′), 44.1 (t, C-1′), 32.7 (t, C-8′), 26.1 (q, C-11′), 23.0 (t, C-7′), 15.6 (q, C-12′); HREI-MS *m*/*z* 341.1740; (341.1740 calculated for $C_{19}H_{23}N_3O_3$). Anal. ($C_{19}H_{23}N_3O_3$·MeOH) C, H, N.

3-[(3*S*,9*S*)-Hydroxyl-4,4-dimethyl-2-oxo-3-quinolizidyl]-4-quinazolinone (Df-2) (4). Compound **4** was prepared using **2** (674 mg) in the same manner as described for **3**. Compound **4** and recovered **2** were obtained, 168 mg (22%) and 308 mg (46%), respectively.

Df-2 (4): colorless needle; mp 176−177 °C dec; $[\alpha]^{25}_D$ +212.06° (*c* 0.232, $CHCl_3$); UV ($CHCl_3$) λ_{max} (ϵ) 316.4 (2547), 304.8 (3285), 266.0 (7711), 231.6 (14825), 226.4 (14376), 193.2 (14648); IR ($CHCl_3$) ν_{max} 3350, 1726, 1680 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 8.30 (1H, dd, J = 1.4, 8.0, H-5), 8.05 (1H, s, H-2), 7.77 (1H, ddd, J = 1.5, 7.1, 8.1, H-7), 7.72 (1H, dd, J = 1.2, 8.1, H-8), 7.49 (1H, ddd, J = 1.2, 7.1, 8.0, H-6), 6.05 (1H, s, H-3′), 3.62 (1H, br.s, w_H = 10, H-9′), 3.18 (1H, dd, J = 10.8, 15.9, H-1′β), 3.16 (1H, br. d, J = 11.4, H-6′β), 2.99 (1H, ddd, J = 1.5, 4.8, 10.8, H-10′), 2.56 (1H, dd, J = 4.8, 15.9, H-1′α), 2.32 (1H, dt, J = 3.1, 11.4, H-6′α), 1.97 (1H, br. dq, J = 13.1, 4.0, H-8′β), 1.90 (1H, br. tq, J = 4.0, 11.4, H-7′β), 1.66 (1H, m, H-7′α), 1.52 (1H, ddt, J = 2.7, 4.0, 13.1, H-8′α), 1.16 (3H, s, H-12′), 1.13 (3H, s, H-11′); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 203.4 (s, C-2′), 161.6 (s, C-4), 146.3 (s, C-8a), 146.2 (d, C-2), 134.7 (d, C-7), 127.5 (d, C-8), 127.3 (d, C-5), 127.1 (d, C-6), 121.6 (s, C-4a), 68.1 (d, C-9′), 66.1 (d, C-3′), 63.8 (s, C-4′), 59.9 (d, C-10′), 45.8 (t, C-6′), 42.2 (t, C-1′), 30.7 (t, C-8′), 25.1 (q, C-12′), 20.2 (t, C-7′), 15.1 (q, C-11′); HREI-MS *m*/*z* 341.1721 (341.1740 calculated for $C_{19}H_{23}N_3O_3$). Anal. ($C_{19}H_{23}N_3O_3$) C, H, N.

Antimalarial Assays in Vitro and in Vivo. The antimalarial activities in vitro against *P. falciparum* and in vivo against *P. berghei* were investigated as described in ref 10. Each of the samples was used as a hydrochloride.

Metabolic Experiment Using S-9 Mix. Livers from untreated male ICR mice (5-weeks old, Japan SLC) were used. The S-9 mix fraction was prepared from a 30% (w/v) liver homogenate in 100 mM phosphate buffer (pH 7.4). The homogenate was centrifuged at 9000*g* for 20 min to give S-9 mix suspension as a supernatant. The incubation mixtures contained 100 mM phospate buffer (pH 7.4), 40 μg/mL substrate, cofactor (1.3 mM NADP, 10 mM glucose-6-phosphate, 0.4 unit/mL glucose-6-phosphate dehydrogenase), 5 mM magnesium chloride, and 2.5 mg of protein/mL of S-9 mix, in a final volume of 1 mL. The incubation was performed at 37 °C and terminated by the addition of 2 mL of acetonitrile. The

samples were examined by TLC analysis using aluminum oxide plate developed with chloroform−methanol (99:1).

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