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New Anti-Malarial Peroxides with In Vivo Potency Derived from Spongean Metabolites

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Abstract—The structure–activity relationship of the anti-malarial substance **3** having a 6-carbomethoxymethyl-3-methoxy-1,2-dioxane structure was studied. The ester portion of the peroxide **3**, showing little in vivo efficacy in malaria-infected mice in spite of the potent in vitro activity, was hydrolyzed in serum to afford an inactive free acid **4**. The amide analogues (**8** and **9**) robust to mouse serum were disclosed to exhibit in vivo anti-malarial potency.

Today, malaria is one of the most deadly diseases on earth and the leading cause of sickness and death in the developing world. Annually, an estimated 300 million cases of malaria occur throughout the world. Mortality associated with the disease is estimated at over one million per year.[1] About 40% of the world population live in malaria endemic countries. Due to the spreading resistance to the conventional anti-malarials, there is an urgent demand to search for new anti-malarial principles.[2] In this context, we have been engaged in exploration for new anti-malarial candidates originating in natural resources.[3,4]

Previously, methyl esters (**1** and **2**) of spongean peroxides,[5] peroxyplakoric acids, were shown to display potent in vivo anti-malarial activity. Furthermore, the facile construction of their core skeleton was established, and the new, readily accessible anti-malarial peroxide **3** with higher selectivity index than that of **1** and **2** was found.[6] However, the promising candidate **3** was shown to exert little in vivo potency because of lability in mouse serum. On the basis of the analysis for the metabolized portion in **3**, the acquirement of stability in serum resulted in the discovery of new anti-malarial peroxides **8** and **9** with in vivo potency. This paper deals with the in vivo anti-malarial peroxides utilizing **1** and **2** as scaffolds (Fig. 1).

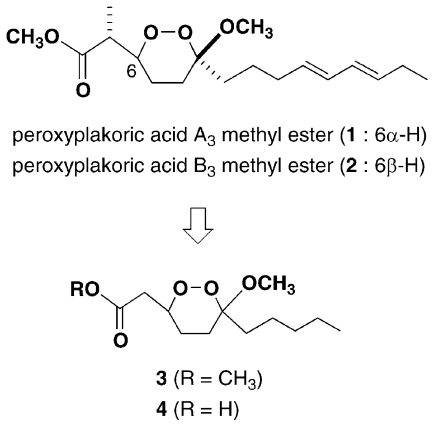

Figure 1.

When the peroxide **3** was examined by an in vivo system using a mouse model, **3** showed little anti-malarial potency. This undesired finding indicated that the ester function in **3** may be metabolized in serum. Therefore, stability of **3** in mouse serum was examined.[7] Treatment of **3** with fresh mouse serum afforded the corresponding carboxylic acid **4** in 85% yield involving complete disappearance of **3** within 1.5 h. The carboxylic acid **4** was almost stable after 24 h exposure to mouse serum, while the anti-malarial activity (IC_{50} $>1.2\,\mu M$) of **4** was significantly reduced. The malaria parasite converts toxic-free heme to nontoxic hemozoin by oxidative polymerization. In a mechanistic study of

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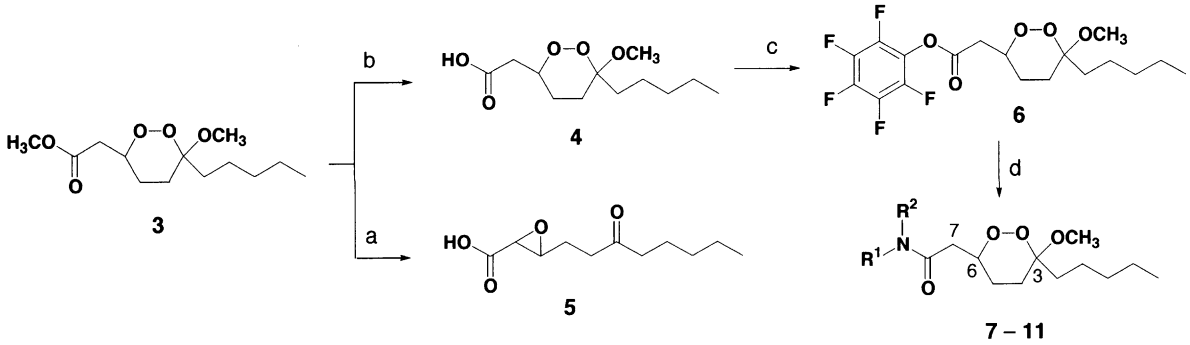

Scheme 1. Reagents and conditions: (a) LiOH·H_2O, DMSO-*d*, quant; (b) esterase (from porcine liver), phosphate buffer (0.2 M, pH 7.4), 89%; (c) pentafluorophenol, EDCI·HCl, pyridine, 84%; (d) $MeNH_2$·HCl, pyridine, 92% for **7**; $EtNH_2$·HCl, pyridine, 96% for **8**; $PrNH_2$, pyridine, 98% for **9**; Me_2NH·HCl, pyridine, 93% for **10**; Et_2NH, pyridine, 63% for **11**.

the action of anti-malarial peroxides, the formation of the heme-peroxide adducts was proposed to inhibit this detoxication pathway.[8] The formation of the heme-peroxide adducts was shown to take place through the production of a radical species in the food vacuole, an acidic organelle characteristic of *Plasmodium falciparum*.[9] Thus, the poor accumulation of the carboxylic acid **4** into the food vacuole was presumed to be a cause of the reduced anti-malarial activity. Taking the stability against esterase in serum and their neutral property into account, the amide analogues were designed.

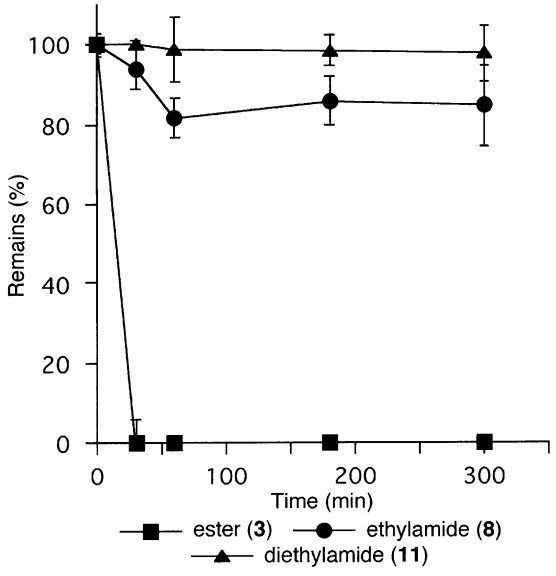

Figure 2. Stability of ester and amides in mouse serum.

Preparation of the amide analogues was conducted as illustrated in Scheme 1. Alkaline hydrolysis of **3** with LiOH in DMSO conclusively provided an epoxy carboxylic acid **5** by concomitant cleavage of the dioxane ring quantitatively. On the other hand, the treatment of **3** with commercially available esterase from porcine liver in a phosphate buffer (pH 7.4) furnished **4** in 89% yield. Conversion from the carboxylic acid **4** to amides analogues was carried out via an activated pentafluorophenyl ester.[10] The carboxylic acid **4** was coupled with pentafluorophenol in the presence of *N*-ethyl-*N*′-3-dimethylaminopropylcarbodiimide hydrochloride (EDCI·HCl) in pyridine to give an ester **6** in 84% yield. In the first instance, the monoethyl and diethyl amides (**8** and **11**)[11] were prepared in order to evaluate their stability in mouse serum. Treatment of **6** with $EtNH_2$·HCl or Et_2NH in pyridine afforded the corresponding amide in 96 and 63% yields, respectively. As a result of assessment for stability in mouse serum, both analogues were shown to be free from metabolism for 5 h (Fig. 2). This encouraging biological property directed us to synthesize several amide analogues. In expectation of potency in a mouse model, we further designed three analogues (**7**, **9**, **10**) under the guidance of appropriate clogPs.[12,13] ClogP is a parameter correlated to the permeability of drugs, and is thus believed to be an important index in predicting biological activity in animal models. In general, clogP values larger than 4 tend to reduce in vivo pharmacological efficacy significantly regardless of a

Table 1. Anti-malarial activity of amide analogues

Compd	R^1	R^2	cLogP	IC_{50} (μM)		Selectivity index	ED_{50} (mg/kg)	T/C[a]
				P. falciparum	KB 3-1		*P. berghei*	
7	H	Me	2.06	0.52	5.2	10	n.d.[b]	118
8	H	Et	2.59	0.54	14	25	11	125
9	H	Pr	3.12	0.31	12	39	9.3	138
10	Me	Me	2.45	0.31	4.0	13	13	95
11	Et	Et	3.15	0.49	13	27	20	118
3			2.17	0.12	43	360	>30	81
Artemisinin							5.0	145

[a]Dose **3**, **7**–**11**: 10 mg/kg; artemisinin: 5 mg/kg. T/C is the quotient of the survival days of the treated animals (T) and those of the control animals (C). T/C values of >120 are considered to be active.
[b]Some mice died by treatment with 30 mg/kg of **7**, then ED_{50} could not be determined.

good in vitro biological score. The analogues (**7**, **9**, **10**) were synthesized from **6** in the same fashion as the preparation of **8** and **11** (Scheme 1) and all the analogues were evaluated for anti-malarial activity in vitro.[14] The resulting biological outcome is summarized in Table 1. Although all of the amide analogues reduced in vitro inhibitory activity for proliferation of *P. falciparum* as compared with **3**, selectivity indices of more than 10 were observed in all compounds. Hence, anti-malarial potency was tested by a conventional 4-day suppressive test using mice infected by *P. berghei* in regard to all the analogues.[15] Among them, the monoethyl and monopropyl analogues (**8** and **9**[16]) showed in vivo anti-malarial potency (T/C > 120) by intraperitoneal administration.

In conclusion, we have developed new anti-malarial peroxides **8** and **9** with in vivo potency by modifying the ester portion of **3** to the amide function robust to mouse serum. Further biological potency of **8** and **9** by use of a complete-cure model with higher dosages is under investigation.

Acknowledgements

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7. Each sample (30 μL of 0.1 mg/mL solution) was treated with fresh mouse serum (300 μL) and incubated at 37 °C for 0, 30, 60, 180, 300 min, respectively. After extraction of the mixture with EtOAc, each extract was concentrated under reduced pressure. The residue was dissolved with 150 μL of *n*-hexane–EtOAc (3:2), then an aliquot (25 μL) of the solution was analyzed by SiO_2-phase HPLC (column: YMC-Pack SIL-06 4.6 mm i.d. × 150 mm, mobile phase: **3**; *n*-hexane/EtOH = 200:1, **8** and **11**; *n*-hexane/EtOH = 90:10, flow rate: 0.5 mL/min, detection: UV 220 nm) to determine the remaining amounts of the test samples by the absolute calibration method in triplicate.
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11. In the transformation from **3** (*syn*/*anti* = 8.3:1) into the amides, the ratio of stereoisomers was slightly changed. The amides **8** and **11** were furnished as a mixture of *syn* and *anti* isomers in a ratio of 8.7:1 and 11:1, respectively. The physicochemical data were determined as a mixture. In the ^{1}H NMR spectra, only the signals due to H-7 and OCH_3 were definitely separated between the two isomers. **8**: colorless solid. IR υ_{max} (KBr) cm^{-1}: 1650, 1550. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 0.88 (3H, t, J = 7.4 Hz, $(CH_2)_4C\underline{H}_3$), 1.13 (3H, t, J = 7.3 Hz, $NCH_2C\underline{H}_3$), 1.25–1.35 (6H, m), 1.50–1.84 (4H, m), 1.86–1.90 (2H, m), 2.31 (1H, dd, J = 15.9, 3.7 Hz, Ha-7), 2.35 (1H, dd, J = 15.9, 7.3 Hz, Hb-7, *syn*), 2.92 (1H, dd, J = 14.7, 8.5 Hz, Hb-7, *anti*), 3.20–3.33 (2H, m), 3.26 (3H, s, OCH_3, *syn*), 3.28 (3H, s, OCH_3, *anti*), 4.38 (1H, m, H-6), 5.96 (1H, brs, NH). FAB-MS m/z: 296 $(M+Na)^+$. FAB-HR MS m/z: calcd for $C_{14}H_{27}NO_4Na$: 296.1838, found: 296.1837; **11**: colorless oil. IR υ_{max} (KBr) cm^{-1}: 1640. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 0.87 (3H, t, J = 7.2 Hz, $(CH_2)_4C\underline{H}_3$), 1.10 (3H, t, J = 7.2, $NCH_2C\underline{H}_3$), 1.15 (3H, t, J = 6.9 Hz, $NCH_2C\underline{H}_3$), 1.25–1.45 (6H, m), 1.50–1.85 (6H, m), 2.27 (1H, dd, J = 15.4, 5.7 Hz, Ha-7), 2.54 (1H, dd, J = 15.4, 5.9 Hz, Hb-7, *syn*), 2.94 (1H, dd-like, J = ca. 14, 8 Hz, Hb-7, *anti*), 3.19–3.27 (2H, m, $NC\underline{H}_2C\underline{H}_3$), 3.24 (3H, s, OCH_3, *syn*), 3.28 (3H, s, OCH_3, *anti*), 3.31 (2H, q-like, J = ca. 7 Hz, $NC\underline{H}_2CH_3$), 4.54 (1H, m, H-6). FAB-MS m/z: 324 $(M+Na)^+$. FAB-HR MS m/z: calcd for $C_{16}H_{31}O_4NNa$: 324.2151, found: 324.2169.
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14. A strain of *P. falciparum* (FCR3, cycloguanil-resistant from Gambia) was used in sensitivity test. After synchronization by sorbitol treatment, 50 μL of a parasite culture at the ring stage (0.55% parasitemia and 2% hematocrit) was added to each well in 96-well microculture plates. The test samples were dissolved in DMSO and diluted to the appropriate concentration using complete medium, then 50 μL of each sample solution was inoculated. The final concentration of DMSO in the culture is 1%. After incubation at 37 °C for 48 h, the proliferation of *P. falciparum* was assessed by Giemsa-stained smear by observing 10,000 erythrocytes per one thin blood film in triplicate. Quinine was used as a reference anti-malarial. In this anti-malarial assay, quinine inhibited the proliferation of *P. falciparum* in a concentration-dependent manner with IC_{50} of 40 ng/mL and IC_{90} of 90 ng/mL. Cytotoxic potency was evaluated by the colorimetric MTT assay, in which mitomycin C used as a positive control showed the IC_{50} of 0.1 μg/mL.
15. In vivo anti-malarial activities of the compounds were determined by the 4-day suppressive test using mice infected with *P. berghei* (NK 65 strain). Five-week-old ddY female mice obtained in sterile containers from Charles River Breeding Laboratories Inc. (Yokohama, Japan) weighing 24–27 g were used. They were housed under a natural day–night (12 h each) cycle at 25 °C. The mice were randomly assigned to treated groups and housed in cages each containing five individuals. Parasites are collected by cardiac puncture from a donor mouse harbouring about 15% parasitemia. The blood is diluted with one-seventh volume of 3.2% trisodium citrate solution, then a final concentration of the infected erythrocytes was adjusted to 5×10^6 by adding 0.9% NaCl solution. Initially, each mouse was inoculated intravenously in the tail vein with 1×10^6 parasitized erythrocytes in 0.2 mL of the infected suspension. Test compounds were prepared at doses of 1, 3, 10, and 30 mg/kg in dimethylsulfoxide and administrated by 0.1 mL once a day from day 0 to day 3. The first administration of the test compound started intraperitoneally 2 h after parasite inoculation. Parasitemia levels were determined on day 4. To evaluate the anti-malarial activity of the compounds, we

prepared tail blood smears and stained them with Giemsa (E. Merck, Germany). Total 1×10^4 erythrocytes per one thin blood film were examined under microscopy. On day 4, parasitemia of control mice were between 30 and 35%. The suppression of parasitemia was calculated by the formula: [(average of parasitemia for control−average of parasitemia for treated mice)/average parasitemia for control]×100. Five infected and dimethylsulfoxide-dosed mice were used as a control. The data are determined from the five individuals in duplicate.

16. As for the amide **9**, only the *syn* isomer was obtained. **9**: colorless solid. IR υ_{max} (KBr) cm^{-1}: 1650, 1560. ^1H NMR (500 MHz, $CDCl_3$) δ: 0.88 (3H, t, $J=6.7$ Hz), 0.91 (3H, t, $J=7.3$ Hz) [$(CH_2)_4C\underline{H}_3$ and $NCH_2\underline{CH}_3$)], 1.27–1.45 (6H, m), 1.55–1.70 (6H, m), 1.81–1.91 (2H, m), 2.33 (1H, dd, $J=15.3$, 2.4 Hz, Ha-7), 2.37 (1H, dd, $J=15.3$, 6.1 Hz, Hb-7), 3.14–3.30 (2H, m), 3.25 (3H, s, OCH_3), 4.38 (1H, m, H-6), 6.03 (1H, brs, NH). FAB-MS *m*/*z*: 310 $(M+Na)^+$. FAB-HR MS *m*/*z*: calcd for $C_{15}H_{29}NO_4Na$: 310.1995, found: 310.2008.