Novel synthetic route for antimalarial benzo[a]phenoxazine derivative SSJ-183 and two active metabolites

Yuki Mizukawa,^{a,b} Jian-Feng Ge,^{a,c} Abu Bakar Md.,^{a,d} Isamu Itoh,^{a,b} Christian Scheurer,^{e,f} Sergio Wittlin,^{e,f} Reto Brun,^{e,f} Hiroyuki Matsuoka,^g Masataka Ihara^{a,*}

^a Drug Discovery Science Research Center, Hoshi University, 2-4-41 Ebara, Shinagawa, Tokyo 142-8501, Japan.

^b Synstar Japan Co., Ltd., Orbic Bldg 3F, 2-9-46 Sakaecho, Odawara 250-0011, Japan

^c College of Chemistry, Chemical Engineering and Materials Science, Soochow University, Suzhou 215006, China

^d Center for Advanced Research in Sciences, University of Daka, Bangladesh

^e Swiss Tropical and Public Health Institute, Socinstrasse 57, 4002 Basel, Switzerland

^f University of Basel, CH-4003 Basel, Switzerland

^g School of Medicine, Department of Infection and Immunity, Division of Medical Zoology, Jichi Medical University, 3311-1 Yakushiji, Shimotsuke-shi, 329-0498, Japan

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ABSTRACT

A productive synthesis of benzo[a]phenoxazine derivative SSJ-183 (**1**), a promising lead for antimalarial agents, was developed using a one pot procedure. Furthermore, *N*-Deethylated metabolite **3** and bis-*N,N*-deethylated metabolite **4** were synthesized by the application of the method. The metabolites **3** and **4** showed comparable and ~2 fold increased activities against drug-sensitive and drug-resistant *Plasmodium falciparum* parasites.

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1. Introduction

The prevalence of malaria is one of the most serious challenges to public health all over the world. According to recent reports, malaria is accounting for about 1,000,000 deaths per year.^{1,2} To date malaria remains a major health care problem in many parts of the world. The disease control is hampered by lack of an effective vaccine and the occurrence of multidrug resistant strains of *Plasmodium falciparum*. Malaria endemic regions worldwide are now facing an unprecedented situation wherein affordable treatment options are losing therapeutic efficacy and are developing resistance whether used in monotherapy or in combination. Of the nearly 1,400 drugs registered worldwide in the last quarter of the 20th century, only four were antimalarials.³

High costs and, most of all, the spread of resistance have dramatically minimized the choice of effective medicines in endemic countries and stressed the urgency of developing new therapeutic agents.

SSJ-183 (**1**), a benzo[a]phenoxazine-based antimalarial molecule, has recently been discovered through a focused medicinal chemistry program.⁴ The reported in vitro IC₅₀ (half-maximal inhibitory concentration) value of 7.6 nM against the parasite strain K1 and a selectivity index of >7000 revealed the specific antimalarial potency of the compound. Accordingly, this was reflected by in vivo data obtained from NMRI mice infected with *P. berghei* ANKA strain, which were cured by oral doses of 100 mg/kg given for three consecutive days.^{4,5} Further in vitro and in vivo studies demonstrated that the molecule **1** is a promising new lead compound with an attractive antimalarial profile and a half-life of about 10 hours in mice infected with rodent malaria. It has a fast and strong inhibitory effect on the

* Corresponding author. Tel./fax: +81-3-5498-6391; e-mail: ihara@hoshi.ac.jp

growth of all *P. falciparum* blood stages. At low concentrations the compound shows a tendency for stage-specific action on ring forms.⁶ This observation is similar to findings reported for Methylene Blue (2), which was considered as a useful antimalarial drug but exhibited haemolysis to subjects with certain forms of inherited glucose-6-phosphate dehydrogenase (G6PD) deficiency.^{7,8} SSJ-183 showed comparable in vitro activity and a better in vivo efficacy compared to Methylene Blue. No haemolysis was detected in blood from G6PD deficient patients.⁹ These findings support the new molecule as a promising candidate for further profiling studies. Here we would like to report a novel synthetic route for SSJ-183 applicable to large-scale production as well as the synthesis of two metabolites 3 and 4 possessing potent antimalarial activity.

Figure 1. Structures of SSJ-183 (1) and Methylene Blue (2)

2. Results and discussion

2.1. Metabolites of SSJ-183

As described earlier,⁶ an *N*-deethylated metabolite 3, a bis-*N,N*-deethylated metabolite 4 and more than one oxygenated metabolite of SSJ-183 were identified in human, rat and dog liver microsomal incubations in the presence and absence of UDPGA (glucuronidation cofactor). The structures of two putative metabolites were determined by LC-MS analysis, respectively. The *N*-deethylated metabolite (3, $M^+ - 28$) was found to be the predominant metabolite for all species.

Figure 2. Structures of two metabolites

2.2. Novel Improved Method for Synthesis of SSJ-183

Prior to synthesis of two putative metabolites 3 and 4, coupling reaction of the free base 5 of commercially available Nile blue A and 4-chloropyridine was examined under various reaction conditions. The synthesis of 1 could be performed by the application of Buchwald's method¹⁰ for the coupling of 5 with 4-chloropyridine in the presence of $\text{Pd}_2(\text{dba})_3$ (2 mol%) and BINAP (8 mol%) and NaO^tBu (2.1 eq.) in toluene at 100 °C for 24 h under Ar atmosphere (Scheme 1). Although the desired compound 1 could be obtained, the yield was variable. Particularly, the transformation of the commercially available Nile Blue A, the corresponding sulfate of 5, to the free base

required a tedious procedure. Furthermore, the chromatographic removal of Pd from the product was a serious problem.

Scheme 1. Synthesis of SSJ-183 (1) from the free base 5 of Nile Blue A using Pd complex

We then considered that the amine 6, formed by the reduction of Nile Blue A, would exhibit a good nucleophilicity, and that the subsequent substitution reaction with 4-chloropyridine could afford 1. Based on this idea, reduction of Nile Blue A was investigated utilizing various reagents. The use of NaHSO_3 and $\text{Na}_2\text{S}_2\text{O}_4$ as reducing agents gave unsatisfactory results. When reduction was carried out with Zn or Fe in the presence of acids as acetic acid or pyridine hydrochloride, the amine 6 was smoothly formed. Without isolation of the formed amine 6, the treatment with 4-chloropyridine under Ar caused the addition-elimination reaction and the objective 1 was obtained by further treatment under atmospheric air conditions. Since the sulfate of 5 is commercially available as Nile Blue A at a low price, the sulfate is the preferred starting material as compared with the corresponding chloride. The practical synthesis of SSJ-183 from the commercially available Nile Blue A was thus established as shown in Scheme 2. The optimized experimental procedure is recorded in the experimental section. The present method could be applicable for the large-scale production of SSJ-183.

Scheme 2. Practical one-pot synthesis of SSJ-183 (1)

2.3. Synthesis of Two Metabolites

Two putative metabolites **3** and **4** were synthesized by the application of the above method as shown in scheme 3. Thus, the commercially available Darrow Red (**7**) was subjected to the reduction using Zn and pyridine hydrochloride, followed by the coupling reaction with 4-chloropyridine and the subsequent oxidation reaction to produce **8** as a crude product. Reduction of **8** with sodium bis(2-methoxyethoxy)aluminum hydride in dioxane gave the *N*-deethylated metabolite **3** in 42.9% overall yield from **7**.

Conversion of **7** into the bis-*N,N*-deethylated metabolite **4** was achieved in successive four steps. Namely, one-pot procedure, reduction of **7** with Zn and pyridine hydrochloride, coupling reaction, hydrolysis with concentrated hydrochloric acid and the sequential oxidation, afforded **4** in 35% overall yield from **7**. Identities of products **3** and **4** confirmed the proposed structures of metabolites formed by incubation of SSJ-183 with liver microsomes by LC-MS analysis.

Scheme 3. Synthesis of two metabolites **3** and **4** from Darrow Red (**7**)

2.4. Antimalarial Activity of Metabolites

In vitro IC₅₀ (half-maximal inhibitory concentration) value of two metabolites **3** and **4** against two strains, K1, resistant to chloroquine and pyrimethamine, and NF54, drug-sensitive, of *P. falciparum* are recorded together with the activities of SSJ-183, Methylene Blue (**2**) and Chloroquine in Table 1. It is noteworthy that the metabolites showed comparable (metabolite **3**) and ~2 fold increased (metabolite **4**) IC₅₀s against *P. falciparum* strains K1 and NF54.

Table 1

In vitro inhibition against *P. falciparum* K1 and NF54

Compound	IC ₅₀ (nM) ^a	
	K1	NF54
SSJ-183 (1) Maleate	6.5	11.0
<i>N</i> -Deethylated Metabolite (3)	5.0	7.5
Bis- <i>N,N</i> -deethylated Metabolite (4)	17.1	24.0
Methylene Blue (2)	5.2	4.2
Chloroquine	198.0	11.0

^a Average of 2-3 independent assays.

3. Conclusion

A novel and practical methodology for the synthesis of the benzo[*a*]phenoxazine derivative SSJ-183 (**1**), a promising new lead compound with an attractive antimalarial profile, was developed using a one pot procedure. By the application of this method, two putative metabolites **3** and **4** could also be prepared. Importantly, the two metabolites showed also potent activities against two *P. falciparum* strains.

4. Experimental Section

¹H-NMR spectra were recorded on a Bruker-400 spectrometer; TMS was used as an internal standard for ¹H NMR. LC-MS (ESI⁺) spectra were taken with a Shimadzu LCMS-2010EV spectrometry. The purities (>98%) of products were determined by the HPLC spectrometer using TMS-gel 100V 5.0 □m, 4.6 mm I.D. x 150 mm eluting with 1.0 mL/min of 40% MeOH (0.1% AcOH + 0.1% NEt₃): 60% H₂O (0.1% AcOH + 0.1% NEt₃), binary gradient, 50 min, at 40 °C, and detecting with UV at 254 nm.

4.1. *N,N*-Diethyl-5-(pyridin-4-ylimino)-5*H*-benzo[*a*]phenoxazin-9-amine (**1**)

To the suspension of Nile Blue A (source: Apollo Scientific, UK, 18.3 g, 0.05 mol) and Fe (4.2 g, 0.075 mol) in ethylene glycol (100 mL) was added acetic acid (9.0 g, 0.15 mol) under Ar. After being heated at 90 °C for 5 h, 4-chloropyridine hydrochloride (11.25 g, 0.075 mol) was added to the mixture at the same temperature. The resulting mixture was further heated at 130 °C for 6 h under Ar and then cooled to room temperature. After addition of MeOH (100 mL), followed by stirring at 80 °C for 1h, the mixture was filtered through celite. To the filtrate were added H₂O (50 mL), NaHCO₃ (25 g, 0.3 mol) and *N,N*-dimethylacetamide (100 mL) and the mixture was stirred at 70 °C for 30 min under atmospheric air. Filtration, followed by evaporation of the solvents and the subsequent treatment with MeOH, afforded **1**⁴ (10.46 g, 53.0%) as a purple-violet solid with 98% purity on LC-MS analysis.

4.2. 9-*N*-Ethylamino-5-(pyridin-4-ylimino)-5*H*-benzo[*a*]phenoxazine (**3**)

Zn powder (1.57 g, 0.024 mol) was added to a stirred mixture of Darrow Red (**7**) (3.4 g, 0.01 mol) and pyridine hydrochloride (4.62 g, 0.04 mol) in ethylene glycol (35 mL) at 85 – 90 °C under Ar. The resulting mixture was stirred for 1 h at 80 – 90 °C under Ar. To the mixture was added 4-chloropyridine hydrochloride (3.0 g, 0.02 mol) at 100 – 110 °C and the mixture was further stirred at 100 – 110 °C for 3 h. After cooling to room temperature, MnO₂ (1 g) was added and the mixture was stirred at room temperature for 1 h under atmospheric air. The reaction mixture was poured into a solution of NaHCO₃ (5 g) in H₂O (500 mL). The formed solid was subjected to silica gel column chromatography. Elution with CHCl₃-MeOH (10:1 v/v) gave **8** (1.81 g, about 47.7%) as a crude product. To a stirred mixture of the product **8** (1.2 g, 3.15 mmol) in dry dioxane (50 mL) at 85 °C was added a toluene solution of NaAl(OCH₂CH₂OMe)₂H₂ (3.6 M sol., 5.3 mL, 18.9 mmol) and the mixture was stirred at 85 °C for 1 h. After slow addition of MeOH (5 mL), the resulting mixture was partitioned between a saturated aqueous solution of Na₂SO₄ and CHCl₃. The organic layer was dried over Na₂SO₄ and then filtered. Evaporation of solvents gave a solid, which was subjected to silica gel chromatography. The eluate with CHCl₃-MeOH (2:1 v/v) provided **3** (1.04 g, 90% from the crude **8**) as a purple solid: ¹H NMR (400 Mz, CDCl₃) δ 8.65-8.63 (1H, m), 8.56-8.51 (3H, m), 7.69-7.65 (2H, m), 7.49 (1H, d, *J* = 8.8 Hz), 6.87-6.85 (2H, m), 6.49 (1H, dd, *J* = 8.8 and 2.4 Hz), 6.25 (1H, d,

$J = 2.4$ Hz), 6.15 (1H, s), 4.20 (1H, s), 3.22 (2H, q, $J = 7.2$ Hz), 1.30 (3H, t, $J = 7.2$ Hz); MS (ESI⁺) m/z 367 ($M^+ + 1$). Due to the scarce solubility of **3** in any solvent, ¹³C NMR data are not available.

4.3. 9-*N*-Amino-5-(pyridin-4-ylimino)-5*H*-benzo[*a*]phenoxazine (**4**)

Darrow Red (**7**) (1.7 g, 5 mmol) was reduced with Zn in the presence of pyridine hydrochloride and then reacted with 4-chloropyridine hydrochloride as above. After addition of conc. HCl (4.3 mL), the mixture was stirred at 75 °C for 4 h and then diluted with MeOH (100 mL). Treatment of the reaction mixture with NaHCO₃ (100 g), followed by filtration through celite and evaporation of solvents, gave a solid, which was subjected to chromatography on silica gel. Elution with CHCl₃-AcOEt (2:1 v/v) and CHCl₃-AcOEt (1:1 v/v) and AcOEt gave **4** (0.59 g, 35%) as a purple solid: ¹H NMR (400 Mz, DMSO-*d*₆) δ 8.53-8.50 (3H, m), 8.49 (1H, dd, $J = 8.0$ and 1.2 Hz), 7.79-7.69 (2H, m), 7.43 (1H, d, $J = 8.8$ Hz), 6.91-6.90 (2H, m), 6.58 (1H, dd, $J = 8.6$ and 1.2 Hz), 6.46 (2H, s), 6.36 (1H, d, $J = 2.0$ Hz), 5.99 (1H, s); MS (ESI⁺) m/z 339 ($M^+ + 1$). Due to the scarce solubility of **4** in any solvent, ¹³C NMR data are not available.

4.4. Malaria In Vitro Screening

Stock drug solutions were prepared in 100% DMSO at 10 mg/mL, and heated or sonicated if necessary to dissolve the sample. After use, the stocks were kept at -20 °C. For the assays, the compounds were further diluted in DMSO and finally to the appropriate concentration in complete medium without hypoxanthine. The DMSO concentration in the wells with the highest drug concentration was 0.5%. Assays were performed in sterile 96-well microtiter plates, each well containing 200 μ L of parasite culture (0.3% parasitemia, 1.25% hematocrit) with or without serial drug solutions. Seven 2-fold dilutions were used. Each drug was tested in duplicate. After 48 h of incubation at 37 °C, 0.5 μ Ci ³H-hypoxanthine was added to each well. Cultures were incubated for a further 24 h before being harvested onto glass-fiber filters and washed with distilled water. The radioactivity was counted using a liquid scintillation counter. The results were recorded as counts per minute per well at each drug concentration and expressed as percentage of the untreated

controls. IC₅₀ values were calculated from the sigmoidal inhibition curves.

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Supplementary data

Supplementary data (¹H NMR spectra of new compounds **3** and **4**) associated with this article can be found, in the online version, at

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IC_{50} values against *Plasmodium falciparum* K1