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# Supporting Information

# Synthesis and Antimalarial Property of Orally Active Phenoxazinium Salts

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## Chemical Experiments

**General Procedure.** All reactions were carried out under an open atmosphere. Unless otherwise described, the materials and the solvent were obtained from commercial suppliers and used without further purification. The $^1$H NMR (300 and 400 MHz) spectra were recorded on a Varian Gemini 2000 and JEOL-AL400 spectrometers, respectively, with tetramethylsilane as internal standard. Chemical shifts are given in ppm, coupling constants are in hertz, and splitting patterns are designated as follows: s, singlet; d, doublet; dd, doublet of doublets; t, triplet; q, quartet; m, multiplet. Fast atom bombardment (FAB) mass spectra were determined with a JEOL JMS DX-303 mass spectrometer. Elemental analyses were performed on Yanagimoto MT-3, and the results (C, H, N) were within ±0.4% of theoretical values. Because of deliquescence and hygroscopicity, correct elemental analyses for most of the compounds could only be obtained by factoring in partial hydration of these organic salts.

**General procedure for the synthesis of phenoxazinium salt (route A).**

To a solution of *N*,*N*-dialkyl-3-aminophenol **8** (3.0 mmol) and *N*,*N*-dialkyl-4-nitrosoaniline **9** (3.0 mmol) in EtOH (50 mL) was added 70% $HClO_4$ (5.5 mmol). Then, the mixture was refluxed for 8-24 h. After being cooled, the reaction mixture was concentrated under reduced pressure. Chromatography on silica gel ($CHCl_3$ : MeOH = 9 : 1), followed by crystallization from MeOH, gave **1** (perchlorate salt). If necessary, the counter anion was changed into chloride ion by the following procedure. The above perchlorate salt was dissolved in MeOH. After the solution was mixed with Amberlite IRA-400($Cl^-$) resin, the mixture was filtered off. The resulted solution was concentrated under reduced pressure, and the residue was recrystallized from MeOH to give **1** (chloride salt).

**General procedure for the synthesis of phenoxazinium salt (route B).**

A mixture of **10** (2.0 mmol) and $NaNO_2$ (3.0 mmol) in 10% HCl aq was stirred for 30 min at 0 °C. The resulted solution was quenched with 5% NaOH. The mixture was extracted with AcOEt three times, and the combined organic layers were washed with brine. The residue was recrystallized to give **11**, and then 11 was acidified with HCl in EtOH to give **11**•HCl. A mixture of **11**•HCl (1.4 mmol) and **8** (2.1 mmol) in $^{i}$PrOH (10 mL) was stirred for 4 h at ambient temperature. After concentration in vacuo, the resulted residue was purified with chromatography on silica gel ($CHCl_3$ : MeOH = 10 : 1), followed by crystallization, to give **1**.

**3,7-Bis(*N*,*N*-dimethylamino)phenoxazinium Perchlorate (1b)**

Compound **1b** was obtained in 40% yield by recrystallization from MeOH as dark blue solids. Mp >300 °C. $^1$H NMR (300 MHz, $CD_3OD$) δ 7.80 (d, 2H, *J* = 9.6 Hz), 7.40 (dd, 2H, *J* = 9.6, 2.8 Hz), 6.95 (d, 2H, *J* = 2.8 Hz), 3.42 (s, 12H). Anal. Calcd. For $C_{16}H_{18}ClN_3O_5$: C, 52.25; H, 4.93; N, 11.43. Found: C, 52.01; H, 5.12; N, 11.24.

**3,7-Bis(*N*,*N*-dimethylamino)phenoxazinium Chloride, Zinc Chloride double salt (1b**•$1/2ZnCl_2$**) .**

Compound **1b**•$1/2ZnCl_2$ was obtained in 44% yield as dark green solids. Mp >300 °C. $^1$H NMR (400 MHz, $CD_3OD$) δ 7.80 (d, 2H, *J* = 9.2 Hz), 7.40 (dd, 2H, *J* = 9.2, 2.8 Hz), 6.96 (d, 2H, *J* = 2.8 Hz), 3.42 (s, 12H). FAB-MS *m/z* 268 ($M^+$). Anal. Calcd. For $C_{16}H_{18}ClN_3O$•$1/2ZnCl_2$: C, 51.67; H, 4.88; N, 11.30; Found: C, 51.30; H, 5.02; N, 11.17.

**3-(*N*-Ethyl-*N*-methylamino)-7-(*N*,*N*-dimethylamino)phenoxazinium Perchlorate (1c).**

Compound **1c** was obtained in 2% yield by chromatography on silica gel with $CHCl_3$ -MeOH (9 : 1), and recrystallization from MeOH as dark blue solids. Mp 210 °C. $^1$H NMR (400 MHz, $CD_3OD$) δ 7.80 (s, 1H), 7.78 (s, 1H), 7.41 (dd, 1H, *J* = 6.8, 2.8 Hz), 7.39 (dd, 1H, *J* = 6.8, 2.8 Hz), 6.96 (d, 1H, *J*

= 2.8 Hz), 6.94 (d, 1H, *J* = 2.8 Hz), 3.81 (q, 4H, *J* = 7.2 Hz), 3.41 (s, 6H), 3.38 (s, 3H), 1.34 (t, 3H, *J* = 7.2 Hz). FABMS *m/z* 282 ($M^+$). Anal. Calcd. For $C_{17}H_{20}ClN_3O_5$: C, 53.48; H, 5.28; N, 11.01. Found: C, 53.61; H, 5.42; N, 11.18.

**3-(*N*,*N*-Diethylamino)-7-(*N*,*N*-dimethylamino)phenoxazinium Perchlorate (1d).**

Compound **1d** was obtained in 15% yield by recrystallization from MeOH as dark blue solids, Mp 229 °C. $^1$H NMR (300 MHz, $CD_3OD$) δ 7.81 (d, 1H, *J* = 0.80 Hz), 7.78 (d, 1H, *J* = 1.2 Hz), 7.40 (dd, 1H, *J* = 6.8, 2.8 Hz), 7.38 (dd, 1H, *J* = 6.8, 2.8 Hz), 6.90 (d, 1H, *J* = 2.8 Hz), 6.94 (d, 1H, *J* = 2.8 Hz), 3.79 (q, 4H, *J* = 6.8 Hz), 3.41 (s, 6H), 1.36 (t, 6H, *J* = 6.8 Hz). FAB-MS *m/z* 296 ($M^+$). Anal. Calcd. For $C_{18}H_{22}ClN_3O_5$: C, 54.62; H, 5.60; N, 10.62. Found: C, 54.23; H, 5.46; N, 10.49.

**3-(*N*,*N*-Dibutylamino)-7-(*N*,*N*-dimethylamino)phenoxazinium Perchlorate (1f).**

Compound **1f** was obtained in 5% yield by chromatography on silica gel with $CHCl_3$-MeOH (9 : 1), and recrystallization from MeOH as dark blue solids. Mp 227 °C. $^1$H NMR (300 MHz, $CD_3OD$) δ 7.79 (s, 1H), 7.76 (s, 1H), 7.39 (t, 1H, *J* = 2.8 Hz), 7.36 (t, 1H, *J* = 2.8 Hz), 6.94 (d, 1H, *J* = 2.8 Hz), 6.90 (d, 1H, *J* = 2.8 Hz), 3.71 (t, 4H, *J* = 7.6 Hz), 3.41 (s, 6H), 1.77-1.73 (m, 4H), 1.51-1.45 (m, 4H), 1.02 (t, 6H, *J* = 7.6 Hz). FAB-MS *m/z* 352 ($M^+$). Anal. Calcd. For $C_{22}H_{30}ClN_3O_5$: C, 58.47; H, 6.69; N, 9.30. Found: C, 58.29; H, 6.81; N, 8.99.

**3-(*N*,*N*-Dibutylamino)-7-(*N*,*N*-diethylamino)phenoxazinium Chloride (1g).**

Perchlorate salt of **1g** was obtained in 21% yield by chromatography on silica gel with $CHCl_3$-MeOH (9 : 1), and recrystallization from MeOH as dark blue solids. Mp 199 °C. IR (KBr) 2932, 1597, 1400, 1339, 1153 $cm^{-1}$. $^1$H NMR (400 MHz, $CD_3OD$) δ 7.77 (d, 2H, *J* = 9.8 Hz), 7.38 (dd, 1H, *J* = 9.8, 2.7 Hz), 7.35 (dd, 1H, *J* = 9.8, 2.7 Hz), 6.95 (d, 1H, *J* = 2.4 Hz), 6.88 (d, 1H, *J* = 2.4 Hz), 3.77 (q, 4H, *J* =

7.1 Hz), 3.70 (t, 4H, *J* = 7.8 Hz), 1.74 (quint, 4H, *J* = 7.8 Hz), 1.47 (sext, 4H, *J* = 7.8 Hz), 1.35 (t, 6H, *J* = 7.1 Hz), 1.02 (t, 6H, *J* = 7.3 Hz). FAB-MS *m/z* 380 ($M^+$).

Chloride salt of **1g** as dark blue amorphous was obtained in 20% by ion exchange of the above perchlorate salt and recrstallization from MeOH. $^1$H NMR (400 MHz, $CD_3OD$) δ 7.79 (d, 1H, *J* = 9.8 Hz), 7.78 (dd, 1H, *J* = 9.5 Hz), 7.39 (dd, 1H, *J* = 10.0, 2.7 Hz), 7.36 (dd, 1H, *J* =10.0, 2.7 Hz), 6.96 (d, 1H, *J* = 2.7 Hz),6.89 (d, 1H, *J* = 2.7 Hz), 3.78 (q, 4H, *J* = 7.3 Hz), 3.71 (t, 4H, *J* = 7.5 Hz), 1.74 (quint, 4H, *J* = 7.5 Hz), 1.47 (sext, 4H, *J* = 7.5 Hz), 1.35 (t, 6H, *J* = 7.3 Hz), 1.02 (t, 6H, *J* = 7.3 Hz). Anal. Calcd. For $C_{24}H_{34}ClN_3O$: C, 69.29; H, 8.24; N, 10.10. Found: C, 69.01; H, 8.45; N, 9.94.

**3-(*N,N*-Dimethylamino)-7-piperazinophenoxazinium Chloride, Hydrochloride (1h•**HCl**).**

Compound **1h•**HCl was obtained in 14% yield by chromatography on silica gel with $CHCl_3$-MeOH (9 : 1), ion exchange with Amberlite IRA-400 ($Cl^-$) resin and recrystallization from MeOH as dark blue solids. mp >300 ˚C. IR (KBr) 3374 (br), 1596, 1396, 1130, 906 $cm^{-1}$. $^1$H NMR (400 MHz, $CD_3OD$) δ 7.90 (brs, 1H), 7.84 (brs, 1H), 7.59 (d, 1H, *J* = 9.9 Hz), 7.50 (d, 1H, *J* = 9.9 Hz), 7.23 (s, 1H), 7.07 (s, 1H), 4.06 (brs, 4H), 3.52 (s, 6H), 3.46 (t, 4H, *J* = 5.3 Hz). FAB-MS *m/z* 309 ($M^+$-1). Anal. Calcd. For $C_{18}H_{22}Cl_2N_4O$: C, 56.70; H, 5.82; N, 14.69. Found: C, 56.91; H, 5.70; N, 14.77.

**1-Hydroxy-3,7-bis(*N,N*-dimethylamino)phenoxazinium Chloride (1i)**

Compound **1i** was obtained in 1% yield by chromatography on silica gel with $CHCl_3$-MeOH (9 : 1), ion exchange with Amberlite IRA-400 ($Cl^-$) resin and recrystallization from MeOH as dark blue solids. Mp >300 ˚C. IR (KBr) 3431 (br), 1604, 1338, 1286, 1180, 908 $cm^{-1}$. $^1$H NMR (400 MHz, $CD_3OD$) δ 7.77 (d, 1H, *J* = 9.5 Hz), 7.12 (dd, 1H, *J* = 9.3, 2.7 Hz), 6.75 (d, 1H, *J* = 2.4 Hz), 6.54 (d, 1H, *J* = 2.4 Hz), 6.31 (d, 1H, *J* = 2.4 Hz), 3.33 (s, 12H). FAB-MS *m/z* 284 ($M^+$). Anal. Calcd. For $C_{16}H_{18}ClN_3O_2$: C, 60.19; H, 5.67; N, 13.14. Found: C, 59.83; H, 5.81; N, 12.94.

**1-Hydroxy-7-(*N,N*-dimethylamino)-3-(4'-methylpiperazino)phenoxazinium Chloride, Hydrochloride (1j•**HCl**)**.

Compound **1j•**HCl was obtained in 33% yield by chromatography on silica gel with $CHCl_3$-MeOH (9 : 1), ion exchange with Amberlite IRA-400 ($Cl^-$) resin and recrystallization from MeOH as dark blue solids. Mp >300 ˚C. IR (KBr) 3387 (br), 2590, 1608, 1485, 1406, 1346, 1286, 1184, 907, 876, 826 $cm^{-1}$. $^1H$ NMR (400 MHz, $CD_3OD$) δ 7.86 (d, 1H, *J* = 9.8 Hz), 7.43 (dd, 1H, *J* = 9.8, 2.2 Hz), 6.94 (d, 1H, *J* = 2.2 Hz), 6.83 (d, 1H, *J* = 2.2 Hz), 6.75 (d, 1H, *J* = 2.2 Hz), 4.07 (brs, 4H), 3.47 (brs, 4H), 3.45 (s, 6H), 2.97 (s, 3H). FAB-MS *m/z* 339 ($M^+$-1). Anal. Calcd for $C_{19}H_{24}Cl_2N_4O_2$•1.2$H_2O$: C, 52.60; H, 6.16; N, 12.91. Found: C, 52.45; H, 6.02; N, 12.59.

**7-(*N,N*-Dimethylamino)-3-(4'-methylpiperazino)-1-pivaloylhydroxyphenoxazinium Chloride, Hydrochloride (1k•**HCl**)** .

To a solution of **1j** (50 mg, 0.12 mmol) and DMAP (1.5 mg, 12 μmol) in MeCN (5.0 mL) at 0 ˚C were added DBU (0.036 mL, 0.24 mmol), followed by pivaloyl chloride (30 μL, 0.24 mmol). The reaction mixture was stirred at room temperature for 1 h. After being cooled to 0 ˚C, the reaction mixture was filtered to give compound **1k•**HCl (20 mg, 34%) as dark blue solids. Mp >300 ˚C. IR (KBr) 1606, 1488, 1400, 1344, 1267, 1180, 1151, 1097, 906 $cm^{-1}$. $^1H$ NMR (400 MHz, $CD_3OD$) δ 7.73 (d, 1H, *J* = 9.3 Hz), 7.60 (m, 1H), 7.31 (d, 1H, *J* = 2.7 Hz), 7.15 (bs, 1H), 7.09 (bs, 1H), 4.54 (bs, 2H), 3.68 (bs, 2H), 3.56 (bs, 6H), 2.99 (s, 3H), 1.47 (s, 9H). FAB-MS *m/z* 423 ($M^+$-1). Anal. Calcd for $C_{24}H_{32}Cl_2N_4O_3$•2$H_2O$: C, 54.24; H, 6.83; N, 10.54. Found: C, 54.15; H, 6.71; N, 10.69.

## Elemental analyses

| compound | formula | calculated | | | found | | |
|---|---|---|---|---|---|---|---|
| | | C | H | N | C | H | N |
| **1b** | $C_{16}H_{18}ClN_3O_5$ | 52.25 | 4.93 | 11.43 | 52.01 | 5.12 | 11.24 |
| **1b**•1/2$ZnCl_2$ | $C_{16}H_{18}ClN_3O$•1/2$ZnCl_2$ | 51.67 | 4.88 | 11.30 | 51.30 | 5.02 | 11.17 |
| **1c** | $C_{17}H_{20}ClN_3O_5$: | 53.48 | 5.28 | 11.01 | 53.61 | 5.42 | 11.18 |
| **1d** | $C_{18}H_{22}ClN_3O_5$ | 54.62 | 5.60 | 10.62 | 54.23 | 5.46 | 10.49 |
| **1f** | $C_{22}H_{30}ClN_3O_5$ | 58.47 | 6.69 | 9.30 | 58.29 | 6.81 | 8.00 |
| **1g** | $C_{24}H_{34}ClN_3O$ | 69.29 | 8.24 | 10.10 | 69.01 | 8.45 | 9.94 |
| **1h**•HCl | $C_{18}H_{22}Cl_2N_4O$ | 56.70 | 5.82 | 14.69 | 56.91 | 5.70 | 14.77 |
| **1i** | $C_{16}H_{18}ClN_3O_2$ | 60.19 | 5.67 | 13.14 | 59.83 | 5.81 | 12.94 |
| **1j**•HCl | $C_{19}H_{24}Cl_2N_4O_2$•1.2$H_2O$ | 52.60 | 6.16 | 12.91 | 52.45 | 6.02 | 12.59 |
| **1k**•HCl | $C_{24}H_{32}Cl_2N_4O_3$•2$H_2O$ | 54.24 | 6.83 | 10.54 | 54.15 | 6.71 | 10.69 |

**Assay for Antimalarial Efficacy**

**General.** The care and treatment of mice were in accordance with the guidelines (No. 141, 1987) issued by the Science and International Affairs Bureau of the Japanese Ministry of Education, Culture, Science and Technology.

**Materials**. Compound **1a** (>95% purity) and **1b**-**1k** were synthesized, and the biological assay was carried out using the synthetic ones, whose purity was more than 95% (elementary analysis level). Basic blue 3 (**1a**, ~60% purity) was purchased from MP Biochemicals. Methylene blue (**2**, ~80% purity) was purchased from Wako Pure Chemical Industries. Pyronin Y (**3**, ~50% purity), Janus green B (**4**, ~65% purity), brilliant cresyl blue (**5**, ~65% purity) and gallocyanine (**6**, ~90%) were purchased from Sigma-Aldrich, Inc. Ozaxine 170 perchlorate (**7**, ~95% purity) was purchased from Fluka Chemicals.

**In Vitro Antimalarial Assay**. *Plasmodium falciparum* K1 strain (a clone originating from Thailand) was used in this study. The strain was maintained in RPMI 1640 medium with 0.36 mM hypoxanthine, supplemented with 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sufonic acid (HEPES), 25 mM $NaHCO_3$, neomycin (100 U/mL), and 5 g/L of Albumax® II (lipid-rich bovine serum albumin, GIBCO, Grand Island, NY, USA), together with 5% washed human $A^+$ erythrocytes. All cultures and assays were conducted at 37 °C under an atmosphere of 4% $CO_2$, 3% $O_2$, and 93% $N_2$. Cultures were kept in incubation chambers filled with the gas mixture. Subcultures were diluted to a parasitemia of between 0.1 and 0.5% and the medium was changed daily. Stock drug solutions were prepared in 100% DMSO at 10 mg/mL and heated or sonicated if necessary to dissolve the sample. For the assay, the compound was further diluted in serum-free culture medium and finally to the appropriate concentration in

complete medium without hypoxanthine. The DMSO concentration in the wells with the highest drug concentration did not exceed 1%.

Assays were performed in sterile 96-well microtiter plates, each well containing 200 μL of parasite culture (0.15% parasitemia, 2.5% hematocrit) with or without serial drug solutions. Seven 2-fold dilutions were used, covering a range from 5 μg/mL to 0.078 μg/mL. Each drug was tested in duplicate and the assay was repeated for active compounds showing an $EC_{50}$ below 1.0 μg/mL. After 48 h of incubation at 37 °C, 0.5 mCi 3H-hypoxanthine is 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 Betaplate™ liquid scintillation counter (Wallac, Zurich, Switzerland). The results are recorded as counts per minute per well at each drug concentration and expressed as percentage of the untreated controls. $EC_{50}$ values are calculated from the sigmoidal inhibition curves.

**In Vitro Cytotoxicity Assay.** The rat skeletal myoblast cell line (L-6 cells) was used to assess cytotoxicity in host cells. The cells were grown in RPMI 1640 medium supplemented with 1% L-glutamine (200 nM) and 10% fetal bovine serum in T-25 tissue culture flasks at 37 °C in 5% $CO_2$ in air. The cultures were subpassaged three times a week using trypsin to detach the cells and split in a 1:2 or 1:3 ratio depending on the density of the parent culture. Stock drug solutions were prepared in 100% DMSO at 10 mg/mL. For the assays, the compound is further diluted to the appropriate concentration using complete medium. The DMSO concentration in the wells with the highest drug concentration did not exceed 1%.

Assays were performed in 96-well microtiter plates, each well receiving 100 μL of culture medium with $4 \times 10^4$ cells. After 24 hours, the medium was removed from all wells and replaced by 100 μL of fresh medium in all wells except for those in row H of the plate. Fresh medium (150 μL) containing the highest drug concentration was added to wells of row H. Serial

drug dilutions are prepared by transferring 50 μL from wells of row H to wells of row G. After gentle mixing 50 μL from row G are transferred to row F, and so on. The highest concentration for the test compounds was 200 μg/mL. Seven 3-fold dilutions were used, covering a range from 200 μg/mL to 0.274 μg/mL. Each drug was tested in duplicate. After 72 h of incubation the plates were inspected under an inverted microscope to assure growth of the controls and sterile conditions. Then, 10 μL of Alamar Blue (12.5 mg resazurin dissolved in 100 mL distilled water) were added to each well and the plates were incubated for another 2 h. Then the plates were read with a Spectramax Gemini XS microplate fluorometer (Molecular Devices Cooperation, Sunnyvale, CA, USA) using an excitation wave length of 536 nm and an emission wave length of 588 nm. $EC_{50}$ values are determined using the microplate reader software Softmax Pro (Molecular Devices Cooperation, Sunnyvale, CA, USA).

**In Vivo Antimalarial Assay.** All efficacy studies were approved by the institutional animal experimentation ethics committee. In vivo antimalarial activities were determined in mice infected with *P. berghei* (NK 65 strain). Five-week-old ICR male mice obtained in sterile containers from Japan SLC, Inc. (Hamamatsu, Japan) weighing 22-25 g were used. They were housed under a natural day-night cycle at 25 °C. The mice were randomly assigned to treated groups and housed in cages each containing five individuals. The heparinized blood collected by cardiac puncture from a donor mouse with approximately 20% parasitemia was taken and diluted in saline to 5 x $10^6$ parasitized erythrocytes per mL. An aliquot (0.2 mL) of this suspension was injected intravenously (iv) into experimental groups of 5 mice and a control group of 5 mice. Test compounds were prepared at each dose in saline and administrated once a day from day 0 to 3. The first administration of the test compound started intraperitoneally or orally two hours post-infection. Test compounds were formulated in saline (0.9% NaCl) for dosing. Parasitemia levels were determined on day 4. Five infected mice and saline-dosed mice

were used as a control. To evaluate the antimalarial activity of the compounds, the tail blood smears were prepared and stained with Diff-Quik. Total 1,000 erythrocytes per thin blood film were examined under microscopy (Leica, CTR 6000). On day 4, parasitemia of control mice rose to ~40%. The difference between the mean of the control group and those of the experimental groups was calculated and expressed as a percent relative to the control group (= % suppression). Treatment was considered curative when no parasites were detected on day 30 after malaria infection.

## Pharmacokinetic Study

### Animals

Male Wistar rats, about 8 weeks old, were obtained from Nippon SLC Co. Ltd. The rats were housed under controlled environmental conditions and fed commercial feed pellets. All rats had free access to food and water.

### Pharmacokinetics studies in rats

In the orally administration studies, five or three male Wistar rats weighing 180-220 g were fasted overnight with free access to water for at least 12h. Dosing solution of basic blue 3 was prepared in saline at a drug concentration of 5 mg/mL. The rats were administrated at a dose of 10 mg/kg by an oral route. Other rats were administrated at a dose of 10 mg/kg by an intravenous route bolus via the foot vein. Then blood samples were collected via the tail vein at selected times (5, 10, 30 min, 1, 2, 4, 6, 8, 10 h) after dosing. Blood samples were kept on ice, heparinized, and centrifuged for 10 min at 6200 rpm to obtain 50 μL of plasma, and the plasma were immediately stored at –20 °C.

The plasma sample was combined with $CH_3CN$ containing trifluoroacetic acid (1mL/L), and the sample mixture was vortex-mixed well and centrifuged for 5 min at 6200 rpm. After centrifugation, the supernatants were filtered with 0.45 μm pore size filter. Then 20 μL of the solution was load onto the RP-HPLC system.

### RP-HPLC analysis

The HPLC system consisted of the Shimadzu LC-10Avp series. The separation was performed on an ODS-80$T_M$ (4.6 x 250 mm) with a pre-column filter (ODS-80$T_M$). The elution condition

was $CH_3CN$-$H_2O$ (70:30, v/v) containing trifluoroacetic acid (1 mL/L) at a flow rate of 1 ml/min with UV detection at 650 nm.