Supporting Information for

Enzymatic Formation of Quinoline Alkaloids by A Plant Type III Polyketide Synthase

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Materials and Methods

Chemicals. 4-Hydroxy-1-methyl-2(1*H*)-quinolone was purchased from Aldrich. *N*-methylanthraniloyl-CoA and anthraniloyl-CoA were respectively chemically synthesized from *N*-methylanthranilic acid and anthranilic acid according to the method of Stöckigt and Zenk [1]. Thus, the two-step synthesis involved generation of the *N*-hydroxysuccinimide esters followed by a thioester exchange with Coenzyme A. Methylmalony-CoA was purchased from Sigma.

Enzyme. Recombinant *R. palmatum* benzalacetone synthase (BAS) with an additional hexahistidine tag at the C-terminal was prepared as described before [2, 3]. Thus, the *Escherichia coli* transformants harboring the plasmid DNA were cultured to an A_{600} of 0.6 in Luria-Bertani medium containing 100 μg/mL of ampicillin at 30 °C. Then, 0.4 mM isopropyl thio-β-D-galactoside was added to induce protein expression, and the culture was incubated

further at 30 °C for 16 h. The *E. coli* cells were harvested by centrifugation and resuspended in 50 mM potassium phosphate buffer, pH 8.0, containing 0.1 M NaCl. Cell lysis was carried out by the freeze-thaw method, and centrifuged at 15,000 *g* for 60 min. The supernatant was passed through a column of Pro-Bond™ resin (Invitrogen) which contained Ni^{2+} as an affinity ligand. After washing with 50 mM potassium phosphate buffer, pH 7.9, containing 0.5 M NaCl and 40 mM imidazole, the recombinant BAS was finally eluted with 15 mM potassium phosphate buffer, pH 7.5, containing 10% glycerol and 500 mM imidazole. Finally, the enzyme preparation was desalted by Bio-Gel P6DG Desalting gel. The purified enzymes showed K_M = 10.0 μM and k_{cat} = 1.79 min^{-1} for 4-coumaroyl-CoA, and K_M = 23.3 μM and k_{cat} = 1.78 min^{-1} for malonyl-CoA.

Enzyme Reaction. The standard reaction mixture contained 54 μM of *N*-methylanthraniloyl-CoA (or anthraniloyl-CoA), 108 μM of malonyl-CoA (or methylmalonyl-CoA, racemic), and 20 μg of the purified recombinant enzyme in a final volume of 500 μL of 100 mM potassium phosphate buffer, pH 8.0, containing 1 mM EDTA. Incubations were carried out at 30 °C for 12 hours, and stopped by addition of 50 μL of 20% HCl. The products were then extracted with 1 mL of ethyl acetate, and analyzed by reverse-phase HPLC and LC-ESIMS. Column, TSKgel ODS-80Ts, 4.6 x 150 mm, Tosoh, Japan; gradient elution with H_2O and MeOH, both containing 0.1% TFA: 0-5 min, 30% MeOH; 5-17 min, 30 to 60% MeOH; 17-25 min, 60% MeOH; 25-27 min, 60 to 70% MeOH; flow rate, 0.8 mL/min; monitored by a multichannel UV detector, MULTI 340, JASCO. For large-scale enzyme reactions, 2 mg of *N*-methylanthraniloyl-CoA (or anthraniloyl-CoA) and 4 mg of malonyl-CoA (or methylmalonyl-CoA, racemic) were incubated with 10 mg of purified recombinant CHS in 40

mL of 100 mM phosphate buffer, pH 8.0, containing 1 mM EDTA at 30 °C for 18 hours. After HPLC separation, pure 4-hydroxy-2(1*H*)-quinolones (ca 0.1-0.4 mg) was obtained.

On-line LC-ESIMS spectra were measured with a Agilent Technologies HPLC 1100 series coupled to a Bruker Daltonics esquire4000 ion trap mass spectrometer fitted with an ESI source. HPLC separations were carried out under the same conditions as described above. The ESI capillary temperature and capillary voltage were 320 °C and 4.0 V, respectively. The tube lens offset was set at 20.0 V. All spectra were obtained in both negative and positive mode; over a mass range of m/z 50-600, at a range of one scan every 0.2 s. The collision gas was helium, and the relative collision energy scale was set at 30.0% (1.5 eV).

The following spectroscopic data of the products were identical with those of commercially available authentic compounds, and the data reported in the literature [4].

4-Hydroxy-2(1*H*)-quinolone (**1**): HPLC: R_t = 18.3 min. LC-ESIMS: *m/z* 162 $[M+H]^+$. UV: λ_{max} 224, 272, and 312 nm. ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.92 (s, 1H), 7.77-7.68 (m, 1H), 7.47-7.42 (m, 1H), 7.27-7.20 (m, 1H), 7.18-7.01 (m, 1H), 5.30 (s, 1H).

4-Hydroxy-3-methyl-2(1*H*)-quinolone (**2**): HPLC: R_t = 18.9 min. LC-ESIMS: *m/z* 176 $[M+H]^+$. UV: λ_{max} 224, 272, 280, and 312 nm. ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.10 (s, 1H), 7.85-7.83 (m, 1H), 7.44-7.40 (m, 1H), 7.24-7.22 (m, 1H), 7.14-7.09 (m, 1H), 1.98 (s, 3H).

4-Hydroxy-1-methyl-2(1*H*)-quinolone (**3**): HPLC: R_t = 22.8 min. LC-ESIMS: *m/z* 176 $[M+H]^+$. UV: λ_{max} 224, 272, and 316 nm. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.89-7.83 (m, 1H), 7.63-7.55 (m, 1H), 7.47-7.41 (m, 1H), 7.25-7.17 (m, 1H), 5.86 (s, 1H), 3.52 (s, 3H).

4-Hydroxy-1,3-dimethyl-2(1*H*)-quinolone (**4**): HPLC: R_t = 23.5 min. LC-ESIMS: *m/z* 190

$[M+H]^+$. UV: λ_{max} 228, 276, 284, and 316 nm. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.97-7.92 (m, 1H), 7.58-7.51 (m, 1H), 7.46-7.41 (m, 1H), 7.25-7.18 (m, 1H), 3.58 (s, 3H), 2.04 (s, 3H).

Enzyme Kinetics. Steady state kinetic parameters were determined by using [2-^{14}C]malonyl-CoA (1.8 mCi/mmol) as a substrate. The experiments were carried out in triplicate using five concentrations (from 6.5 to 117.8 μM) in the assay mixture, containing 2 μg of purified enzyme, 1 mM EDTA, in a final volume of 500 μL of 100 mM Tris-HCl buffer, pH 7.0. Incubations were carried out at 30 °C for 15 min. The reaction products were extracted and separated by TLC (Merck Art. 1.11798 Silica gel 60 F_{254}; ethyl acetate/hexane/AcOH = 63:27:5, v/v/v). Radioactivities were quantified by autoradiography using a bioimaging analyzer BAS-2000II (FUJIFILM). Lineweaver-Burk plots of data were employed to derive the apparent K_M and k_{cat} values (average of triplicates) using EnzFitter software (BIOSOFT).

Table 1 Steady-State Kinetic Parameters for Enzyme Reactions.

Product	Yield[a] (%)	*Starter*			*Extender*		
		K_M (μM)	k_{cat} (min^{-1})	k_{cat} / K_M (min^{-1} μM^{-1})	K_M (μM)	k_{cat} (min^{-1})	k_{cat} / K_M (min^{-1} μM^{-1})
Benzalacetone (4-coumaroyl-CoA/malonyl-CoA)	92	10.0	1.79	0.179	23.3	1.78	0.076
4-hydroxy-2(1*H*)-quinolone (**1**) (anthraniloyl-CoA/malonyl-CoA)	4	ND[b]	ND[b]	ND[b]	ND[b]	ND[b]	ND[b]
4-hydroxy-3-methyl-2(1*H*)-quinolone (**2**) (anthraniloyl-CoA/methylmalonyl-CoA)	12	ND[b]	ND[b]	ND[b]	ND[b]	ND[b]	ND[b]
4-hydroxy-1-methyl-2(1*H*)-quinolone (**3**) (*N*-methylanthraniloyl-CoA/malonyl-CoA)	80	20.5	0.23	0.011	49.0	0.74	0.015
4-hydroxy-1,3-dimethyl-2(1*H*)-quinolone (**4**) (*N*-methylanthraniloyl-CoA/methylmalonyl-CoA)	86	23.7	1.48	0.062	31.2	0.56	0.018

[a.] Yield (%) of the product under the standard assay condition.
[b.] Not determined because of the weak enzyme activity.

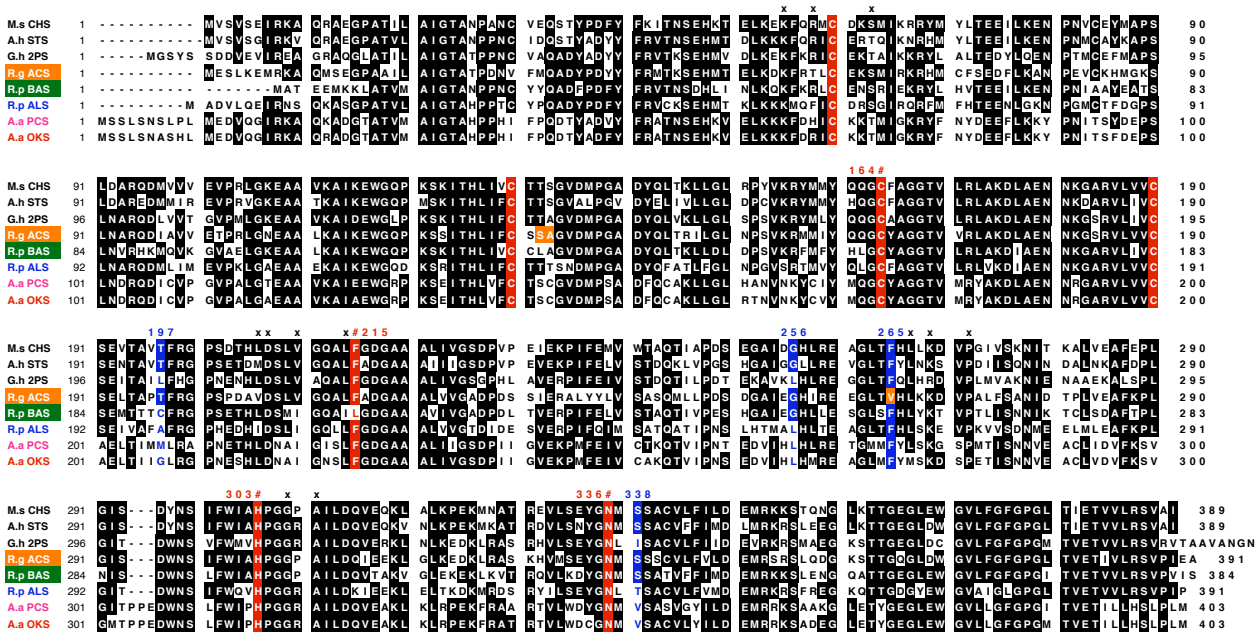

Fig. 1 Comparison of primary sequences of *Rheum palmatum* BAS and other CHS-superfamily enzymes. M.s CHS, *Medicago sativa* CHS; A.h STS, *Arachis hypogaea* stilbene synthase; G.h 2PS, *Gerbera hybrida* 2-pyrone synthase; R.g ACS, *Ruta gravenolens* acridone synthase; R.p BAS, *Rheum palmatum* benzalacetone synthase; R.p ALS, *R. palmatum* aloesone synthase; A.a PCS, *Aloe arborescens* pentaketide chromone synthase; A.a OKS, *A. arborescens* octaketide synthase. The active-site residues conserved in the CHS-superfamily enzymes (Cys164, Phe215, His303, and Asn336, numbering in *M. sativa* CHS) were marked with #, and residues for the CoA binding with x. Amino acid residues conserved in CHS-superfamily enzymes but absent in *A. arborescens* PCS are marked with *.

References

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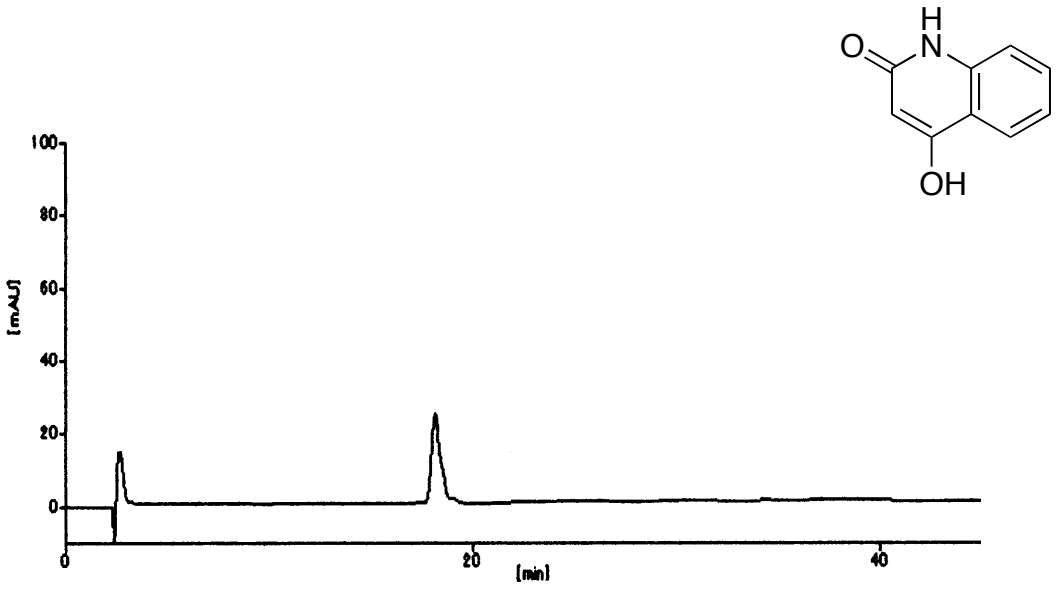
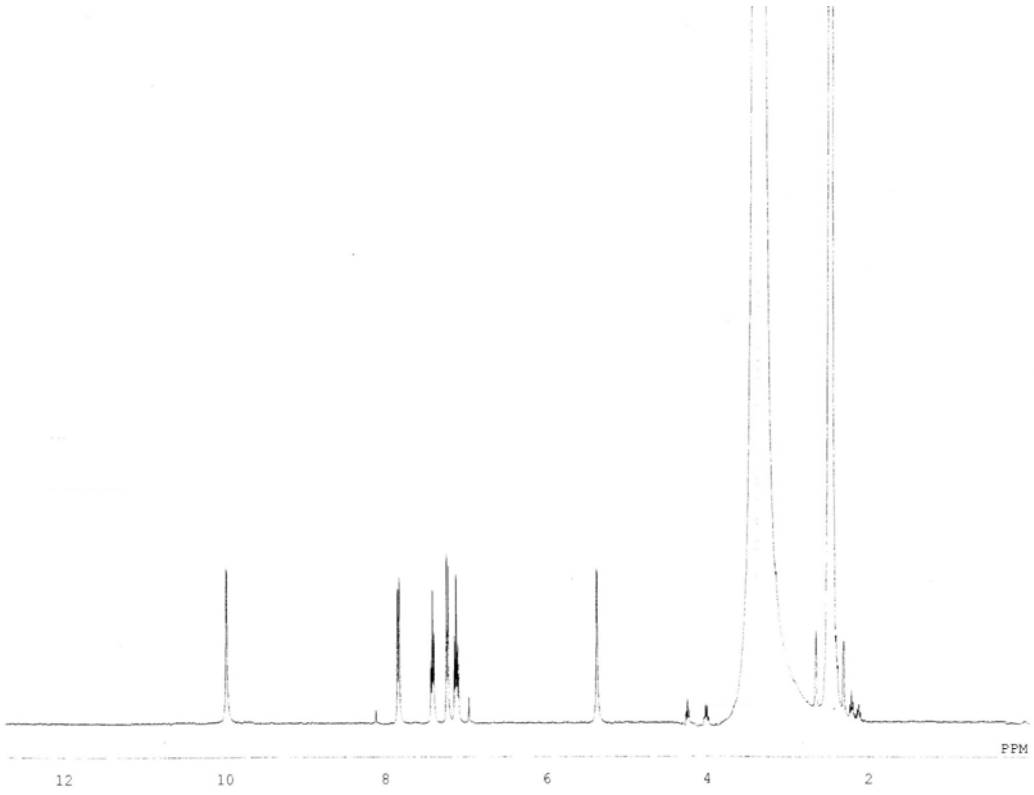
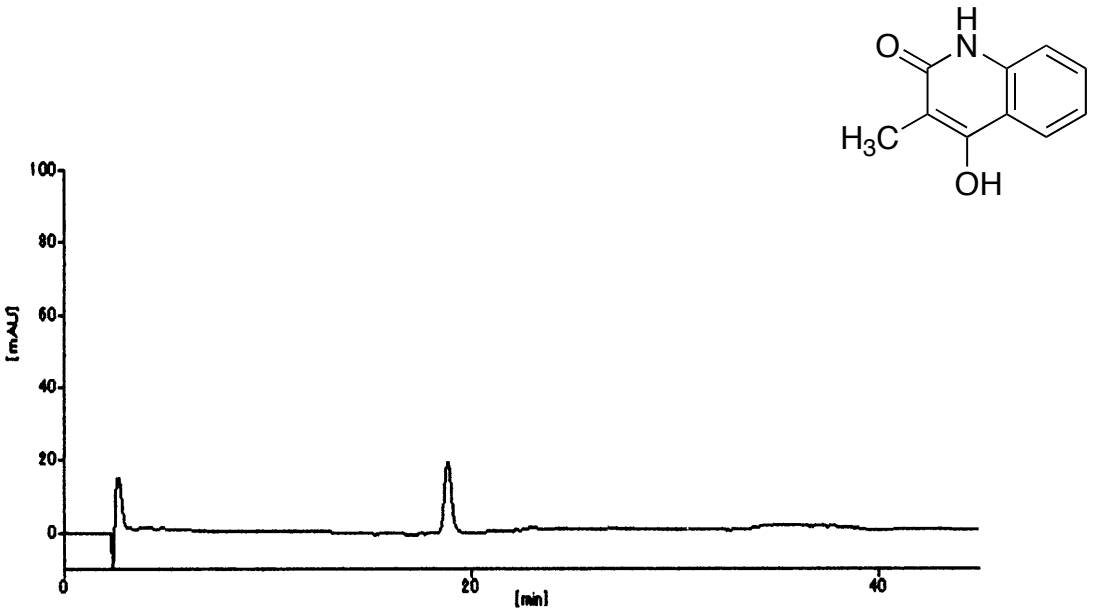
HPLC profile of 4-hydroxy-2(1*H*)-quinolone (**1**)



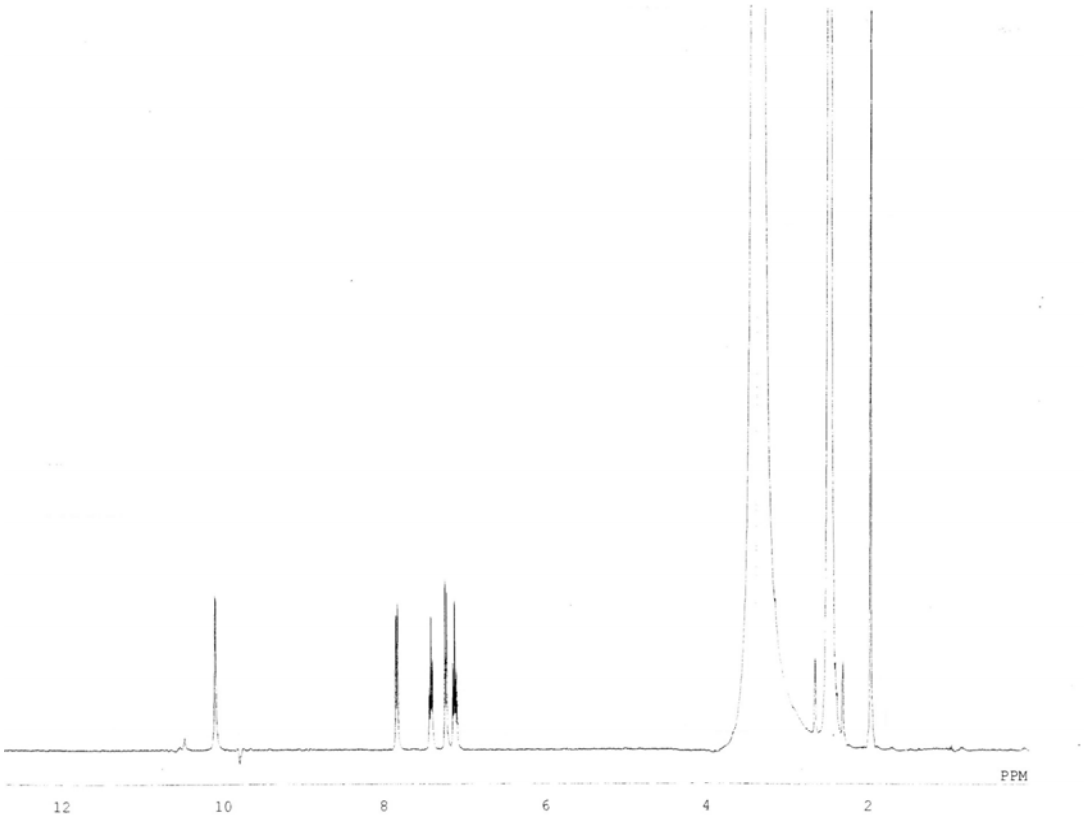
^{1}H NMR (400 MHz, DMSO-d_6) of 4-hydroxy-2(1*H*)-quinolone (**1**).



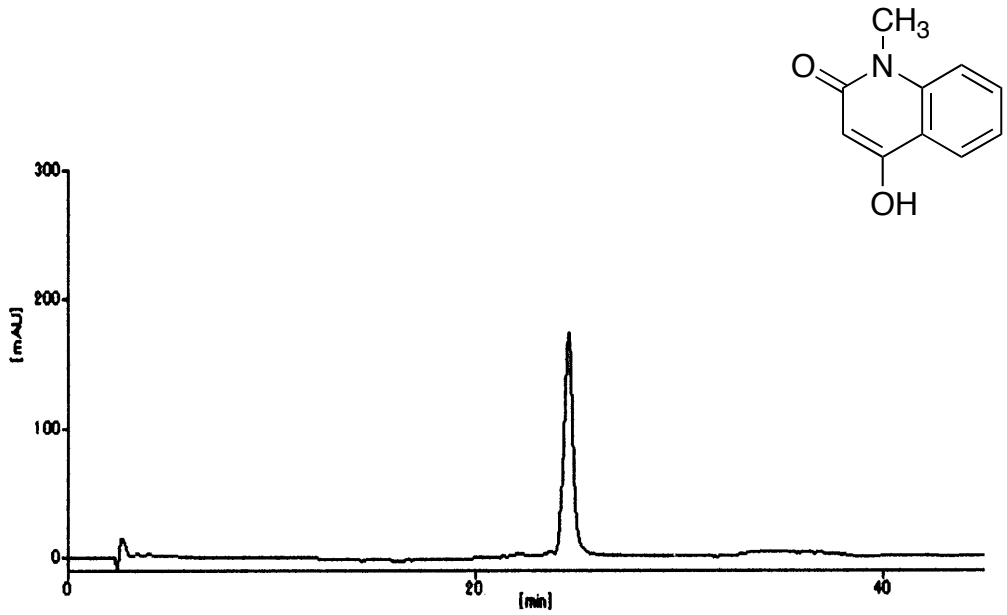
HPLC profile of 4-hydroxy-3-methyl-2(1*H*)-quinolone (**2**)



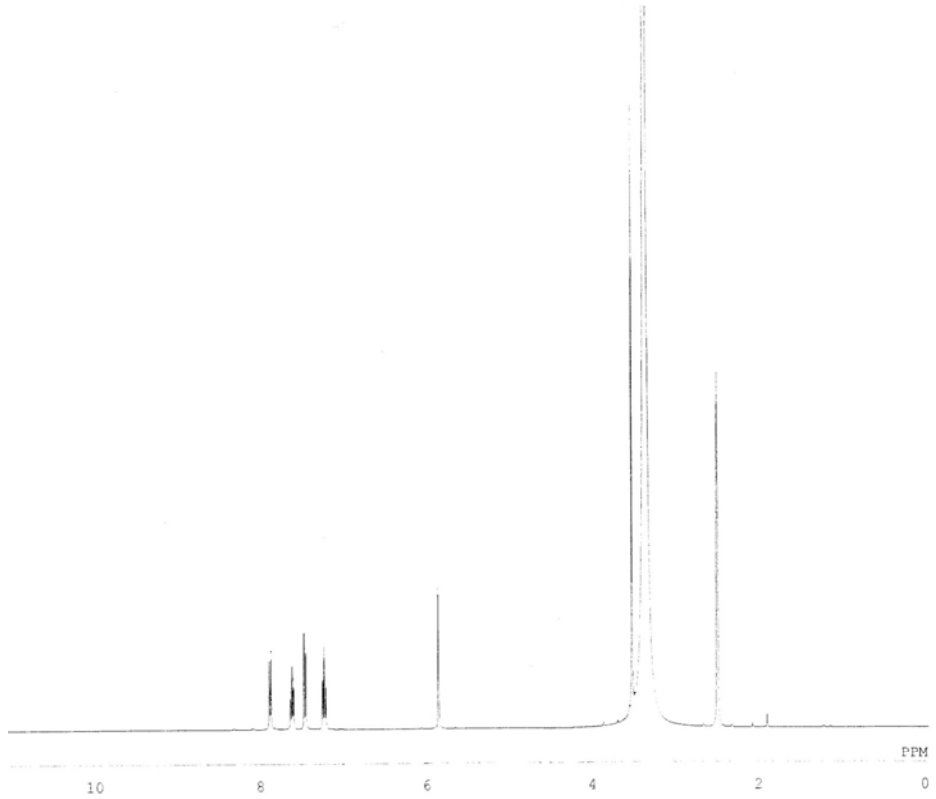
^{1}H NMR (400 MHz, DMSO-d_6) of 4-hydroxy-3-methyl-2(1*H*)-quinolone (**2**).



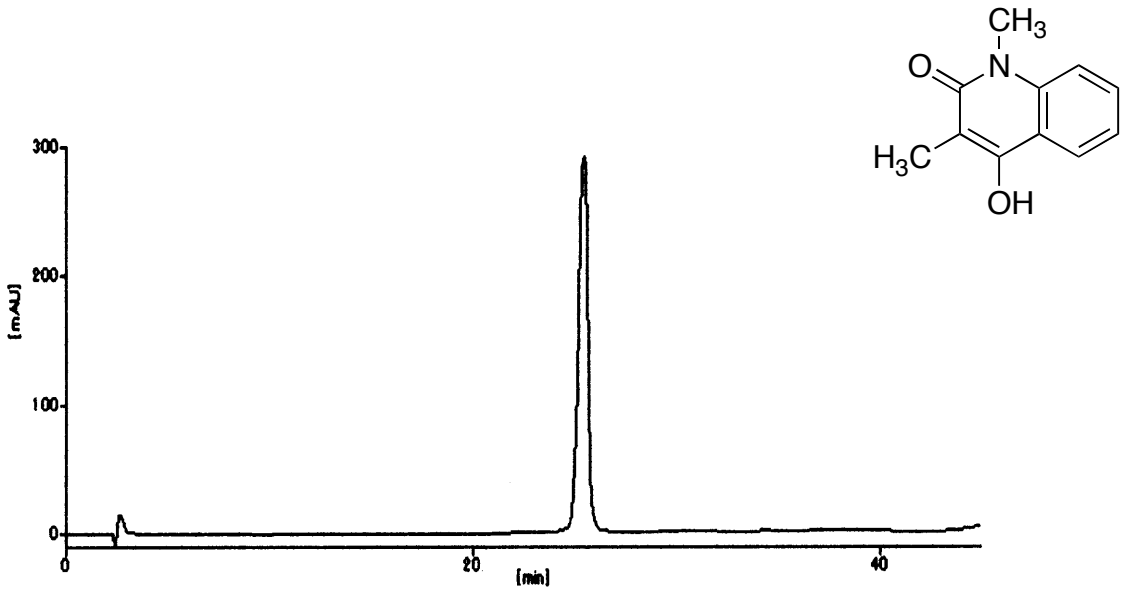
HPLC profile of 4-hydroxy-1-methyl-2(1*H*)-quinolone (**3**)



^{1}H NMR (400 MHz, DMSO-d_6) of 4-hydroxy-1-methyl-2(1*H*)-quinolone (**3**).



HPLC profile of 4-hydroxy-1,3-dimethyl-2(1*H*)-quinolone (**4**).



^{1}H NMR (400 MHz, DMSO-d_6) of 4-hydroxy-1,3-dimethyl-2(1*H*)-quinolone (**4**).