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Supporting Information

DOI: 10.1002/cbic.200900214

Supporting Information

for

Development of Ratiometric Fluorescent Probes for Phosphatases by Using a pK_a Switching Mechanism

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General Information

In general, reactions were carried out in a flame-dried glassware under an argon atmosphere using standard inert atmosphere techniques. Solvents and reagents were obtained from commercial sources unless otherwise noted. PTP1B and CD45 were purchased from BIOMOL Research Laboratories Inc. Acid phosphatase (ACP), alkaline phosphatase (ALP), and PP1 were purchased from Sigma-Aldrich. PP2A1 was purchased from CALBIOCHEM. Synthesized probes were dissolved in DMSO (biochemical grade) before fluorescence measurement.

NMR spectra were recorded at 270 or 400 MHz for NMR and at 67.5 or 100 MHz for ^{13}C NMR, using tetramethylsilane as an internal standard. Excitation and emission spectra of the synthesized compounds were measured at 1 μM in 100 mM HEPES buffer (pH 7.4) at 25 ºC. Each sample contained less than 0.1% DMSO as a cosolvent. Fluorescence quantum yields of the synthesized compounds were estimated by a relative method with reference to a fluorescence standard, quinine sulfate (Φ = 0.55 in 0.05 M H_2SO_4 aq.).[S1] The pH profiles of the synthesized compounds were evaluated by measuring the fluorescence intensities (λ_{ex} = 380 nm, λ_{em} = 470 nm) in 100 mM sodium phosphate buffer at each pH at 25 ºC.

All enzymatic reactions were performed in a quartz cuvette at 30 ºC. Synthesized probes were dissolved at 10 □M in a reaction buffer (50 mM HEPES (pH 7.4), 1 mM EDTA, 1 mM DTT, 0.05% NP-40 for CD45 and PTP1B; 25 mM imidazole (pH 7.4), 0.1 mg/mL BSA, 1mM DTT, 50 mM NaCl for PP1; 100 mM HEPES (pH7.4), 1 mM EDTA, 1 mM DTT for PP2A1, ALP, and ACP). The excitation spectra (λ_{ex} = 380 nm) were measured before and after the addition of the enzyme solution.

Kinetic parameters such as K_m and V_{max} were determined by plotting the initial reaction velocity against the probe concentration and fitting the plot to the Michaelis-Menten equation. The initial velocities were evaluated from measurements of the absorbance at 380 nm or the fluorescence intensity (λ_{ex} = 380 nm, λ_{em} = 470 nm) in 100 mM HEPES (pH 7.4) containing 1.0 mM DTT and EDTA.

Probe Synthesis

*7-Hydroxy-8-methyl-2-oxo-2H-1-benzopyran (**I**)*: 2-Methylresorcinol (1.0 g, 8.06 mmol) and conc. H_2SO_4 (one drop) were added to propiolic acid (1.13 g, 16.1 mmol). The mixture was stirred at 120 ºC for 15 min and solids appeared. The solid was washed with saturated $NaHCO_3$ aq. and water. This compound was recrystallized from MeOH to afford **I** (700 mg, y. 50%). ^{1}H NMR (400 MHz, [D_6]DMSO) δ 2.15 (s, 3H), 6.19 (d, 1H, *J* = 9.6 Hz), 6.84 (d, 1H, *J* = 8.8 Hz),7.37 (d, 1H, *J* = 8.8 Hz),7.92 (d, 1H, *J* = 9.6 Hz), 10.5 (s, 1H); ^{13}C NMR (100 MHz, [D_6]DMSO) δ 7.8, 110.6, 110.7, 111.2, 111.9, 126.2, 144.7, 153.3, 158.9, 160.5; HRMS (ESI$^+$) *m*/*z*: 177.0579 (calcd for M^+ ($C_{10}H_8O_3$): 177.0552).

*7-Methoxymethoxy-8-methyl-2-oxo-2H-1-benzopyran (**II**)*: 7-Hydroxy-8-methyl-2-oxo-2*H*-1-benzopyran **I** (1.0 g, 5.68 mmol) was dissolved in dry DMF (10 mL), then diiso-propylethylamine (1.49 g, 11.5 mmol) and methoxymethyl chloride (925 mg, 11.5 mmol) were added. The mixture was stirred at 0 ºC for 3 h under Ar. The reaction mixture was diluted with diethylether and washed with sat. citric acid aq., 2 M NaOH aq., water, and brine. After evaporation, the residue was purified with silica gel chromatography, eluted with CH_2Cl_2 to afford **II** (720 mg, y. 58%). ^{1}H NMR (270 MHz, $CDCl_3$) δ 2.33 (s, 3H), 3.50 (s, 3H), 5.28 (s, 2H), 6.26 (d, 1H, *J* = 9.4 Hz),7.03 (d, 1H, *J* = 8.6 Hz),7.25 (d, 1H, *J* = 8.6 Hz),7.63 (d, 1H, *J* = 9.4 Hz); ^{13}C NMR (67.5 MHz, $CDCl_3$) δ 8.3, 56.3, 94.4, 110.3, 113.3, 113.4, 115.1, 125.7, 143.6, 153.1, 158.0, 161.3; HRMS (EI$^+$) *m*/*z*: 220.0730 (calcd for M^+ ($C_{12}H_{12}O_4$): 220.0736).

*7-Methoxymethoxy-8-bromomethyl-2-oxo-2H-1-benzopyran (**III**)*: 7-Methoxymethoxy-8-methyl-2-oxo-2*H*-1-benzopyran **II** (590 mg, 1.97 mmol) and *N*-Bromosuccinimide (590 mg, 2.34 mmol) was suspended in CCl_4 (25 mL). Then catalytic amount of AIBN (10 mg) was added and refluxed for 3 h. The organic layer was washed with water, and dried over brine and sodium sulfate. After evaporation, the residue was purified with silica gel chromatography, eluted with CH_2Cl_2 to afford **III** (400 mg, y. 68%). ^{1}H NMR (270 MHz, $CDCl_3$) δ 3.51 (s, 3H), 4.79 (s, 2H), 5.34 (s, 2H), 6.30 (d, 1H, *J* = 9.6 Hz),7.06 (d, 1H, *J* = 8.9 Hz),7.39 (d, 1H, *J* = 8.9 Hz),7.63 (d, 1H, *J* = 9.6 Hz) ; ^{13}C NMR (67.5 MHz, $CDCl_3$) δ 20.4, 56.7, 94.3, 110.4, 113.4, 113.9, 114.9, 128.8, 143.2, 152.7, 157.7, 160.2; HRMS (CI$^+$) *m*/*z*: 298.9922 (calcd for [M+H]$^+$ ($C_{12}H_{12}BrO_4$): 298.9919).

*7-Hydroxy- 8-phosphonomethyl-2-oxo-2H-1-benzopyran (**1**)*: 7-Methoxymethoxy-8-bromomethyl-2-oxo-2*H*-1-benzopyran **III** (300 mg, 1.00 mmol) was dissolved in trimethylphosphite (5 mL), and stirred at 90 ºC for 2 h. After evaporation, the crude 7-Methoxymethoxy-8-(dimethoxyphosphorylmethyl)-2-oxo-2*H*-1-benzopyran (**IV**) (300 mg, 0.91 mmol) was dissolved in dry CH_2Cl_2 (10 mL), and TMSBr (1.40 g, 9.10 mmol) was added dropwise at 0 ºC under Ar. After removing the solvent, the resulting crude silyl ester was dissolved in MeOH (10 mL), and the solution was stirred for 1 h at RT. After evaporation, the residue was purified with RP-HPLC, eluted with 100 mM triethylamine-acetic acid (TEAA) buffer (pH = 6.5) to yield **1** as a triethylammonium salt (80 mg, y. 22% (2 steps)). ^{1}H NMR (270 MHz, D_2O) δ 1.13 (t, 9H, *J* = 7.2 Hz),3.05 (q, 6H, *J* = 7.2 Hz), 3.10 (d, 2H, *J* = 21.0 Hz),6.15 (d, 1H, *J* = 9.4 Hz),6.80 (d, 1H, *J* = 8.6 Hz), 7.30 (d, 1H, *J* = 8.6 Hz), 7.81 (d, 1H, *J* = 9.4 Hz); ^{13}C NMR (67.5 MHz, CD_3OD) δ 25.6 (d, $J_{C\text{-}P}$ = 83.9 Hz) , 47.5, 111.9, 112.6 (two carbon), 113.7 (two carbon), 116.3, 116.4, 146.4, 154.4, 154.5, 161.9 (two carbon), 163.6; MS (CI^+) *m*/*z*: 358.1 $[M+H]^+$; E. A.: C, 53.41; H, 6.73; N, 3.94 (calcd for $C_{17}H_{26}NO_7P$: C, 53.78; H, 6.77; N, 3.92).

*7-Allyloxy-2-oxo-2H-1-benzopyran (**6**)*: 7-Hydroxycoumarin (1.0 g, 6.2 mmol) and K_2CO_3 (1.2 g, 8.6 mmol) was suspended in acetone (30 mL). The mixture was refluxed for 2 h under Ar. After cooled, inorganic salt was filtrated off. After evaporation of the filtrate, the product was purified with silica gel chromatography, eluted with CH_2Cl_2 to afford **6** (1.20 g, y. 96%). ^{1}H NMR (270 MHz, $CDCl_3$) δ 4.61 (d, 2H, *J* = 5.1 Hz), 5.34 (dd, 1H, *J* = 10.5 Hz , 1.5 Hz), 5.44 (dd, 1H, *J* = 17.5 Hz, 1.5 Hz),6.01 (m, 1H),6.25 (d, 1H, *J* = 9.7 Hz),6.83 (s, 1H),6.85(d, 1H, *J* = 8.6 Hz),7.36 (d, 1H, *J* = 8.6 Hz),7.63(d, 1H, *J* = 9.7 Hz); ^{13}C NMR (67.5 MHz, $CDCl_3$) δ 69.3, 101.7, 112.6, 113.0, 113.1, 118.5, 128.6, 132.0, 143.2, 155.6, 161.0, 161.6; MS (CI^+) *m*/*z*: 203 $[M+H]^+$; E. A.: C, 71.17; H, 4.98 (calcd for $C_{12}H_{10}O_3$: C, 71.28; H, 4.98).

*7-Hydroxy- 8-allyl-2-oxo-2H-1-benzopyran (**7**)*: 7-Allyloxy-2-oxo-2*H*-1-benzopyran **6** (1.0 g, 27.2 mmol) was dissolved in *N*,*N*-diethyl aniline (60 mL) and refluxed for 3 h at 220 ºC under Ar. After cooling to 0 ºC, *n*-hexane (50 mL) was added for precipitating the product. The precipitate was filtered, washed with *n*-hexane, and recrystallized from ethyl acetate to yield light yellow crystals of **7** (2.7 g, y. 50%). ^{1}H NMR (270 MHz, $CDCl_3$) δ 3.67 (d, 2H, *J* = 5.1 Hz); 5.14-5.24 (m, 2H,), 5.89 (s, 1H), 6.01 (m, 1H), 6.25 (d, 1H, *J* = 9.5 Hz),6.81(d, 1H, *J* = 8.4 Hz),7.26 (d, 1H, *J* = 8.4 Hz),7.63 (d, 1H, *J*

= 9.5 Hz); ^{13}C NMR (67.5 MHz, [D_6]DMSO) δ 26.2, 110.7, 111.1, 112.0, 113.0, 114.8, 126.9, 135.0, 144.6, 152.9, 158.6, 160.1; MS (CI^+) *m*/*z*: 203 [*M*+H]$^+$; E. A.: C, 71.14; H, 5.03 (calcd for $C_{12}H_{10}O_3$: C, 71.28; H, 4.98).

*7-Methoxymethoxy- 8-allyl-2-oxo-2H-1-benzopyran (**9**)*: 7-Hydroxy-8-allyl-2-oxo-2*H*-1-benzopyran **7** (300 mg, 1.48 mmol) was dissolved in dry DMF (10 mL), then diisopropylethylamine (239 mg, 2.97 mmol) and methoxymethyl chloride (384 mg, 2.97 mmol) were added. The mixture was stirred at 0 ºC for 3 h under Ar. The reaction mixture was diluted with diethyl ether and washed with sat. citric acid aq., 2 M NaOH aq., water, and brine. After evaporation, the residue was purified with silica gel chromatography, eluted with CH_2Cl_2 to afford **9** (340 mg, y. 93%). ^{1}H NMR (270 MHz, $CDCl_3$) δ 3.49 (s, 3H), 3.63 (d, 2H, *J* = 5.9 Hz), 4.98-5.01 (m, 2H), 5.28 (s, 2H), 5.97 (m, 1H),6.26 (d, 1H, *J* = 9.2 Hz),7.06 (d, 1H, *J* = 8.4 Hz),7.29 (d, 1H, *J* = 8.4 Hz),7.63 (d, 1H, *J* = 9.2 Hz); ^{13}C NMR (67.5 MHz, $CDCl_3$) δ 27.1, 56.4, 94.2, 110.4, 113.5 (two cabons), 115.3, 116.8, 126.5, 134.9, 143.6, 152.9, 157.7; MS (CI^+) *m*/*z*: 247 [*M*+H]$^+$; E. A.: C, 68.22; H, 5.39 (calcd for $C_{14}H_{14}O_4$: C, 68.28; H, 5.73).

7-Methoxymethoxy-8-(2-oxoethyl)-2-oxo-2*H*-1-benzopyran (10): 7-Methoxymethoxy-8-allyl-2-oxo-2*H*-1-benzopyran **9** (1.0 g, 4.07 mmol) was dissolved in THF (50 mL), then OsO_4 in water (3 mL of 1.5% (*w*/*w*) in water, 0.4 mmol) was added to the mixture and stirred for 1 h at RT under Ar. Then $NaIO_4$ (5.5 g, 24 mmol) was added in 3 portions and stirred at RT for 12 h. THF was removed and diluted with water and extracted with CH_2Cl_2. The extracts were dried over brine and sodium sulfate. After evaporation, the residue was chromatographed on silica gel, eluted with CH_2Cl_2/ethyl acetate (9:1) to afford **10** (650 mg, y. 65%). ^{1}H NMR (270 MHz, $CDCl_3$) δ 3.45 (s, 3H), 4.02 (s, 2H), 5.26 (s, 2H), 6.28 (d, 1H, *J* = 9.2 Hz), 7.11 (d, 1H, *J* = 8.4 Hz), 7.40 (d, 1H, *J* = 8.4 Hz), 7.66 (d, 1H, *J* = 9.2 Hz), 9.78 (s, 1H); ^{13}C NMR (67.5 MHz, $CDCl_3$) δ 38.1, 56.5, 94.4, 110.0, 110.3, 113.5, 113.7, 127.9, 143.5, 153.1, 158.2, 160.4, 197.5; MS (CI^+): 249 [*M*+H]$^+$; E. A.: C, 62.80; H, 4.73 (calcd for $C_{13}H_{12}O_5$: C, 68.90; H, 4.87).

*7-Methoxymethoxy-8-(2-hydroxyethyl)-2-oxo-2H-1-benzopyran (**11**)*: $NaBH_4$ (20 mg, 0.48 mmol) was suspended in dry THF (20 mL) at 0 ºC under Ar. Then, 7-methoxymethoxy-8-(2-oxoethyl)-2-oxo-2*H*-1-benzopyran **10** (100 mg, 0.41 mmol) solution in THF (5 mL) was added dropwise. The mixture was stirred for 1 h, and quenched with sat. citric acid aq. (5 mL). THF was removed and the residue was diluted with water,

then extracted with CH_2Cl_2. After evaporation, the residue was purified with silica gel chromatography, eluted with CH_2Cl_2/ethyl acetate (1:1) to afford **11** (76 mg, y. 75%). ^{1}H NMR (270 MHz, $CDCl_3$) δ 3.19 (t, 2H, *J* = 6.8 Hz), 3.50 (s, 3H),3.88 (t, 2H, *J* = 6.8 Hz), 5.30 (s, 2H),6.28 (d, 1H, *J* = 9.2 Hz),7.08 (d, 1H, *J* = 8.4 Hz),7.32 (d, 1H, *J* = 8.4 Hz),7.64 (d, 1H, *J* = 9.2 Hz); ^{13}C NMR (67.5 MHz, $CDCl_3$) δ 26.5, 56.4, 61.8, 94.4, 110.5, 113.4, 113.6, 115.6, 126.9, 143.9, 153.4, 158.3, 161.3; HRMS (EI^+) *m*/*z*: 250.0845 (calcd for M^+ ($C_{13}H_{14}O_5$): 250.0841).

*7-Hydroxy-8-(2-phosphoethyl)-2-oxo-2H-1-benzopyran (**3a**, PEHC)*: $POCl_3$ (307 mg, 2.0 mmol) and triethylamine (405 mg, 4.0 mmol) were dissolved in dry THF (10 mL). 7-Methoxymethoxy-8-(2-hydroxyethyl)-2-oxo-2*H*-1-benzopyran **11** (100 mg, 0.41 mmol) in THF (5 mL) was added dropwise at 0 ºC under Ar. The mixture was stirred for 2 h, and quenched with 1 M $NaHCO_3$ aq. (10 mL). The reaction mixture was stirred for 1 h at RT, then $NaHCO_3$ was filtered off and washed with THF. After evaporation of the filtrate, MeOH (10 mL) was added to the residue oil and stirred for 2 h at 40 ºC. After evaporation, the residue was purified with RP-HPLC, eluted with 100 mM triethylamine-acetic acid (TEAA) buffer (pH = 6.5) to yield **3a** as a triethyl-ammonium salt (30 mg, y. 20%). ^{1}H NMR (270 MHz, D_2O) δ 1.12 (t, 9H, *J* = 7.6 Hz),2.98 (t, 2H, *J* = 6.8 Hz),3.06 (q, 6H, *J* = 7.3 Hz),3.91 (q, 2H, *J* = 7.0 Hz),6.05 (d, 1H, *J* = 9.2 Hz),6.77 (d, 1H, *J* = 8.4 Hz),7.26 (d, 1H, *J* = 8.4 Hz), 7.77 (d, 1H, *J* = 9.2 Hz); ^{13}C NMR (67.5 MHz, CD_3OD) δ 9.2, 25.6 (d, *J* = 7.5 Hz), 47.4, 64.5 (d, *J* = 5.4 Hz), 111.8, 113.1 (two carbons), 113.9, 128.3, 146.3, 155.2, 161.3, 163.5; MS (FAB^+) *m*/*z*: 388.2 [*M*+H]$^+$; E. A.: C, 52.43; H, 6.77; N, 3.65 (calcd for $C_{17}H_{26}NO_7P$: C, 52.71; H, 6.77; N, 3.62).

*7-Hydroxy-8-(2-hydroxyethyl)-2-oxo-2H-1-benzopyran (**3b**, HEHC)*: 7-Methoxymeth-oxy-8-(2-hydroxyethyl)-2-oxo-2*H*-1-benzopyran **11** (50 mg, 0.2 mmol) was dissolved in CH_2Cl_2 (10 mL) at 0 ºC under Ar. TMSBr (122 mg, 0.8 mmol) was added drop-wise. The mixture was stirred for 4 h. After evaporation of the CH_2Cl_2, the residue was purified with RP-HPLC, eluted with 100 mM TEAA buffer (pH = 6.5) to afford **3b** (10 mg, yield 24%). ^{1}H NMR (270 MHz, CD_3OD) δ3.09 (t, 2H, *J* = 7.3 Hz),3.74 (t, 2H, *J* = 6.8 Hz),6.17 (d, 1H, *J* = 9.2 Hz),6.81 (d, 1H, *J* = 8.4 Hz),7.32 (d, 1H, *J* = 8.4 Hz), 7.83 (d, 1H, *J* = 9.2 Hz); ^{13}C NMR (67.5 MHz, CD_3OD) δ 27.3, 61.7, 111.8, 113.2, 113.6, 113.7, 128.2, 146.4, 155.1, 161.1, 163.7; HRMS (EI^+) *m*/*z*: 206.0576 (calcd for M^+ ($C_{11}H_{10}O_4$): 206.0579).

*7-Methoxymethoxy- 6-allyl-2-oxo-2H-1-benzopyran (**12**)*: 7-Allyloxy-2-oxo-2*H*-1-benzopyran **6** (5.5 g, 27.2 mmol) was dissolved in *N*,*N*-diethylaniline (60 mL) and refluxed for 3 h at 220 ºC under Ar. After cooling to 0 ºC, *n*-hexane (50 mL) was added, then the precipitate was filtered, washed with hexane, and evaporated to give crude product **8**. The crude **8** (2.00 g, 9.90 mmol) was dissolved in DMF (10 mL), then diisopropylethylamine (1.56 g, 19.8 mmol) and methoxymethyl chloride (2.56 g, 19.8 mmol) were added. The mixture was stirred for 3 h at 0 ºC under Ar. The reaction mixture was diluted with diethyl ether and washed with sat. citric acid aq., 2 M NaOH, water and brine. After evaporation, the residue was purified with silica gel chromatography, eluted with CH_2Cl_2, then CH_2Cl_2: ethyl acetate (1:1) to afford **12** (600 mg, y. 9% (2 steps)). ^{1}H NMR ($CDCl_3$, 270 MHz) δ 3.41 (d, 2H, J=6.5 Hz), 3.48 (s, 3H), 5.03-5.11 (m, 2H), 5.26 (s, 2H), 5.97 (m, 1H), 6.26 (d, 1H, *J* = 9.5 Hz), 7.05 (s, 1H), 7.23 (s, 1H), 7.61 (d, 1H, *J* = 9.5Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 33.7, 56.3, 94.3, 101.7, 112.8, 113.4, 116.2, 126.3, 128.2, 135.9, 143.3, 154.2, 157.8, 161.2; HRMS (EI$^+$) *m*/*z*: 246.0892 (calcd for M^+ ($C_{14}H_{14}O_4$): 246.0886).

*7-Methoxymethoxy-6-(2-oxoethyl)-2-oxo-2H-1-benzopyran (**13**)*: 7-Methoxymethoxy-6-allyl-2-oxo-2*H*-1-benzopyran **12** (400 mg, 1.63 mmol) was dissolved in THF (50 mL), then OsO_4 (3 mL of 1.5% (*w*/*w*) in water, 0.16 mmol) was added and stirred for 1 h at RT under Ar. Then, $NaIO_4$ (2.2 g, 9.6 mmol) was added in 3 portions and stirred at RT for 12 h. After evaporation of THF, the residue was diluted with water and extracted with CH_2Cl_2. After evaporation of the CH_2Cl_2, the residue was purified with silica gel chromatography, eluted with CH_2Cl_2/ethyl acetate (9:1), to afford **13** (186 mg, y. 46%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.45 (s, 3H),3.75 (s, 2H),5.25 (s, 2H),6.27 (d, 1H, *J* = 9.5 Hz),7.10 (s, 1H),7.28 (s, 1H),7.62 (d, 1H, *J* = 9.5 Hz), 9.75 (s, 1H); ^{13}C NMR (100 MHz, CD_3CN) δ 45.2, 56.8, 95.2, 102.4, 113.9, 114.5, 120.8, 131.4, 144.4, 155.8, 158.8, 161.2, 200.2; HRMS (EI$^+$) *m*/*z*: 248.0685 (calcd for M^+ ($C_{13}H_{12}O_5$), 248.0680).

*7-Methoxymethoxy-6-(2-hydroxyethyl)-2-oxo-2H-1-benzopyran (**14**)*: $NaBH_4$ (28 mg, 0.67 mmol) was suspended in THF (20 mL) at 0 ºC under Ar. Then, 7-methoxymethoxy-8-(2-oxoethyl)-2-oxo-2*H*-1-benzopyran **13** (150 mg, 0.61 mmol) dissolved in THF (5 mL) was added dropwise. The mixture was stirred for 40 min, and quenched with *sat.* citric acid aq. (5 mL). After evaporation of THF, the residue was diluted with water and extracted with CH_2Cl_2. After evaporation, the residue was purified with silica

gel chromatography, eluted with CH_2Cl_2/ethyl acetate (1:1) to afford **14** (56 mg, y. 30%). The crude product was used without further purification.

*7-Hydroxy-8-(2-phosphoethyl)-2-oxo-2H-1-benzopyran (**4a**, 6-PEHC)*: $POCl_3$ (46 mg, 0.31 mmol) and triethylamine (60 mg, 0.62 mmol) were dissolved in THF (10 mL), then crude 7-methoxymethoxy-8-(2-hydroxyethyl)-2-oxo-2*H*-1-benzopyran **14** (15 mg, 0.06 mmol) dissolved in THF (5 mL) was added dropwise at 0 ºC under Ar. The mixture was stirred for 1 h, and added 1 N $NaHCO_3$ aq. (10 mL) and stirred. After 1 h, the reaction mixture was filtrated and evaporated, then MeOH (10 mL) was added to the residue oil and stirred for 2 h at 40 ºC. After evaporation, the residue was purified with silica gel with RP-HPLC, eluted with TEAA buffer (pH = 6.5) to yield **4a** as a triethylammonium salt (8 mg, y. 34%). ^{1}H NMR (400 MHz, CD_3OD) δ 1.29 (t, 9H, *J* = 7.6 Hz), 2.98 (t, 2H, *J* = 6.8 Hz),3.15 (q, 6H, *J* = 7.6 Hz),4.09 (q, 2H, *J* = 6.8 Hz),6.16 (d, 1H, *J* = 9.5 Hz),6.70 (s, 1H),7.46 (s, 1H),7.84 (d, 1H, *J* = 9.5 Hz); ^{13}C NMR (100 MHz, CD_3OD) δ 9.1, 32.2 (d, *J* = 8.0 Hz), 47.5, 65.2 (d, *J* = 5.0 Hz), 102.8, 112.1, 112.9, 125.0, 131.4, 146.3, 155.8, 161.3, 163.9; HRMS (ESI$^-$) *m*/*z*: 285.0163 (calcd for $[M\text{-H}]^-$ ($C_{11}H_{10}O_7P$): 285.0164).

*7-Hydroxy-6-(2-hydroxyethyl)-2-oxo-2H-1-benzopyran (**4b**, 6-HEHC)*: Crude 7-Methoxymethoxy-6-(2-hydroxypropyl)-2-oxo-2*H*-1-benzopyran **14** (15 mg, 0.06 mmol) was dissolved in CH_2Cl_2 (10 mL), and TMSBr (35 mg, 0.24 mmol) was added dropwise at 0 ºC under Ar. The mixture was stirred for 1.5 h, then evaporated. The residue was purified with silica gel chromatography, eluted with 7% MeOH/CH_2Cl_2 to afford **4b** (5 mg, y. 35%). ^{1}H NMR (400 MHz, CD_3OD) δ 2.86 (t, 2H, *J* = 6.8 Hz),3.76 (t, 2H, *J* = 6.8 Hz),6.15(d, 1H, *J* = 9.5 Hz),6.71(s, 1H),7.36 (s, 1H),7.82 (d, 1H, *J* = 9.5 Hz); ^{13}C NMR (67.5 MHz, $CDCl_3$) δ 34.3, 62.4, 102.9, 112.2, 112.9, 125.4, 131.2, 146.2, 155.8, 161.4, 163.9; HRMS (ESI$^+$) *m*/*z*: 207.0661 (calcd for M^+ ($C_{11}H_{11}O_4$): 207.0657).

*7-Methoxymethoxy-8-(2-hydroxypropyl)-2-oxo-2H-1-benzopyran (**15**)*: 7-Methoxymethoxy-8-allyl-2-oxo-2*H*-1-benzopyran **9** (1.0 g, 4.06 mmol) was dissolved in THF (10 mL). Four mL of 1 M BH_3·THF (4.00 mmol) was added at 0 ºC under Ar and stirred for 1 h at RT, then 2 M NaOH aq (2 mL) was added and stirred for 30 min at 0 ºC. Then, 30% H_2O_2 aq. (1.7 mL) was added and stirred for 2 h at 0 ºC. After evaporation of the THF, the residue was extracted with diethyl ether, washed with water and dried with brine and sodium sulfate. After evaporation, the residue was purified with silica gel chromatography, eluted with CH_2Cl_2/ethyl acetate (1:1) to af-

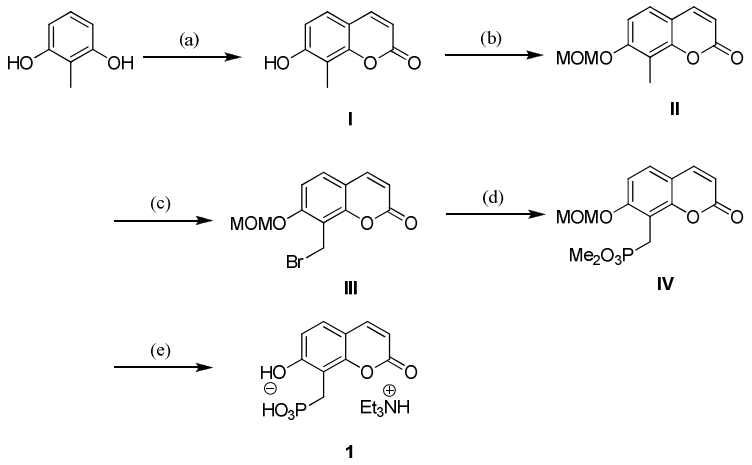
ford **15** (426 mg, y. 42%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.89 (q, 2H, *J* = 6.8 Hz),2.99 (t, 2H, *J* = 7.1 Hz),3.50 (s, 3H),3.65 (t, 2H, *J* = 6.8 Hz),5.30 (s, 2H),6.30 (d, 1H, *J* = 9.4 Hz),7.09 (d, 1H, *J* = 8.8 Hz),7.31 (d, 1H, *J* = 8.8 Hz),7.66 (d, 1H, *J* = 9.4 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 10.8, 31.8, 56.4, 62.0, 94.5, 114.5, 110.5, 113.4, 113.6, 118.5, 126.4, 143.8, 153.1, 158.0, 161.2; HRMS (CI^+) *m*/*z*: 265.1074 (calcd for $[M+H]^+$ ($C_{14}H_{17}O_5$): 265.1076).

*7-Hydroxy-8-(2-phosphopropyl)-2-oxo-2H-1-benzopyran (**5a**, PPHC)*: $POCl_3$ (150 mg, 1.0 mmol) and triethylamine (200 mg, 2.0 mmol) were dissolved in THF (10 mL). 7-Methoxymethoxy-8-(2-hydroxyethyl)-2-oxo-2*H*-1-benzopyran **15** (50 mg, 0.20 mmol) dissolved in THF (5 mL) was added dropwise at 0 ºC under Ar. The mixture was stirred for 1 h, and 1 M $NaHCO_3$ aq (10 mL) was added. The reaction mixture was stirred for 1 h at RT, then filtrated. After evaporation of the filtrate, MeOH (10 mL) was added to the residue oil and stirred for 2 h at 40 ºC. After evaporation, the residue was purified with RP-HPLC, eluted with TEAA buffer (pH = 6.5) to yield **5a** as a triethylammonium salt (30 mg, yield 20%). ^{1}H NMR (400 MHz, CD_3OD) δ 1.31 (t, 9H, *J* = 7.6 Hz),1.93 (m, 2H),2.92 (t, 2H, *J* = 7.8 Hz),3.20 (q, 6H, *J* = 7.3 Hz),3.96 (q, 2H, *J* = 6.6 Hz), 6.17 (d, 1H, *J* = 9.6 Hz),6.80 (d, 1H, *J* = 8.6 Hz),7.31 (d, 1H, *J* = 8.6 Hz),7.83 (d, 1H, *J* = 9.6 Hz); ^{13}C NMR (100 MHz, CD_3OD) δ 9.1, 20.4, 31.3 (d, *J* = 8.0 Hz), 47.5, 66.9 (d, *J* = 6.0 Hz), 111.9, 113.3, 113.7, 117.0, 128.0, 146.6, 155.0, 161.0, 163.9; HRMS (ESI^-) *m*/*z*: 299.0317 (calcd for $[M-H]^-$ ($C_{12}H_{12}O_7P$): 299.0321); E. A.: C, 53.68; H, 6.91: N, 3.60 (calcd for $C_{18}H_{28}NO_7P$: C, 53.86; H, 7.03; N, 3.49).

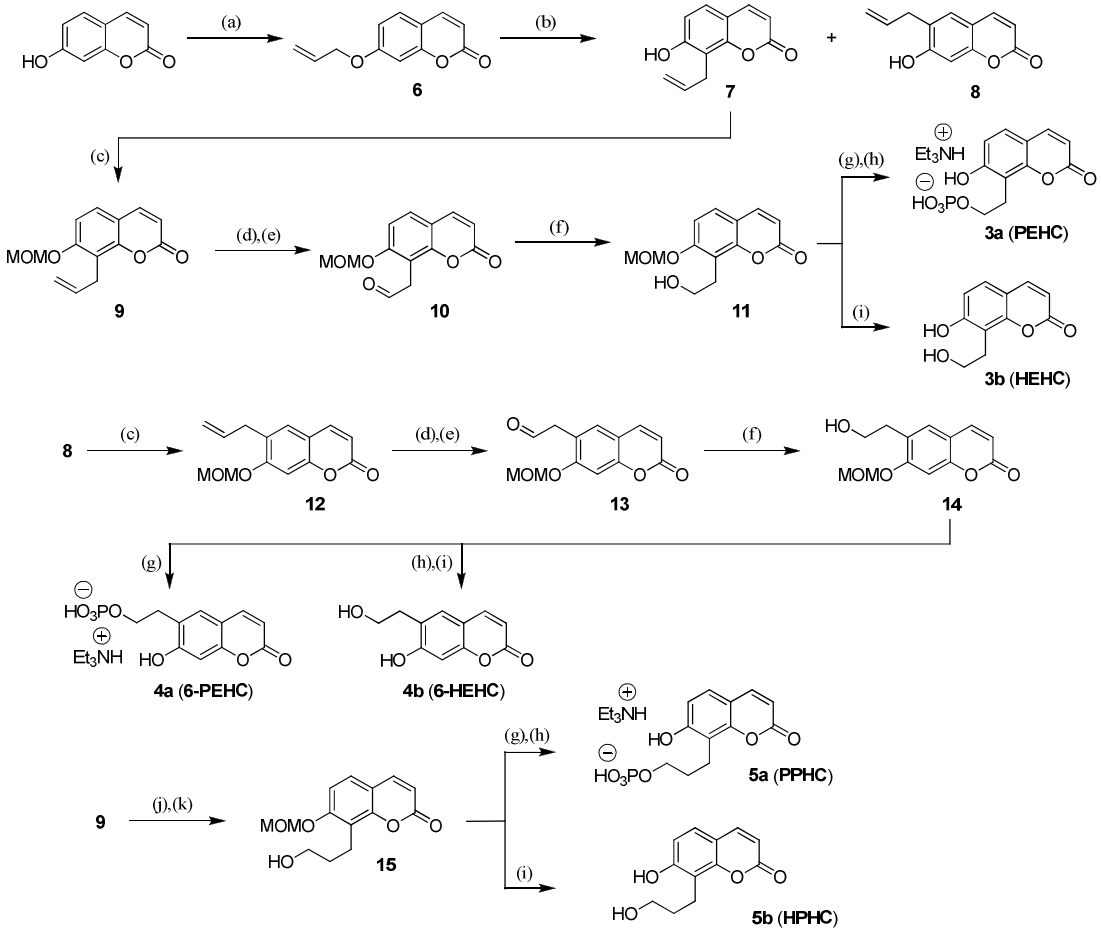
*7-Hydroxy-8-(2-hydroxypropyl)-2-oxo-2H-1-benzopyran (**5b**, HPHC)*: 7-Methoxymethoxy-8-(2-hydroxypropyl)-2-oxo-2*H*-1-benzopyran **15** (50 mg, 0.2 mmol) was dissolved in CH_2Cl_2 (15 mL). TMSBr (122 mg, 0.8 mmol) was added dropwise at 0 ºC under Ar, then the mixture was stirred for 3 h. After evaporation, the residue was purified with silica gel chromatography, eluted with 7% MeOH/CH_2Cl_2 to afford **5b** (36 mg, y. 86%). ^{1}H NMR (400 MHz, CD_3OD) δ 1.83 (q, 2H, *J* = 6.8 Hz), 2.88 (t, 2H, *J* = 7.8 Hz), 3.61 (t, 2H, *J* = 6.8 Hz),6.17 (d, 1H, *J* = 9.6 Hz),6.81 (d, 1H, *J* = 8.6 Hz),7.31 (d, 1H, *J* = 8.6 Hz),7.84 (d, 1H, *J* = 9.6 Hz); ^{13}C NMR (100 MHz, CD_3OD) δ 20.0, 32.8, 62.9, 111.8, 113.3, 113.6, 117.0, 127.9, 146.6, 154.9, 160.8, 164.0; HRMS (ESI^+) *m*/*z*: 221.0828 (calcd for $[M+H]^+$ ($C_{12}H_{13}O_4$): 221.0814).

Reference

[S1] J. N. Demas, G. A. Crosby, *J. Phys. Chem.* **1971**, *75*, 991–1024.

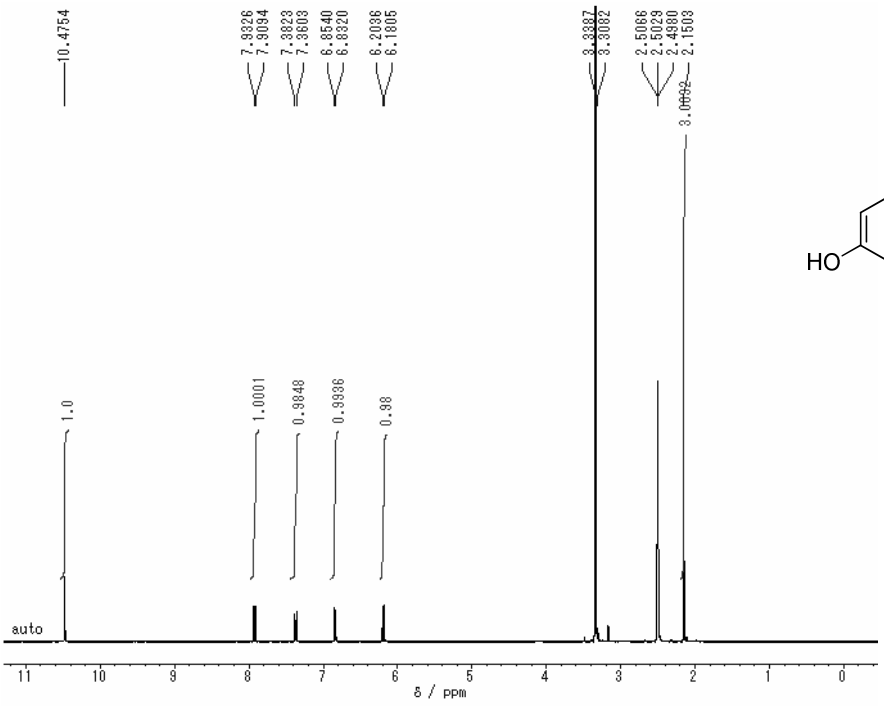


Scheme 1. Synthetic schemes of compound **1**. a) 2-methylresorcinol, propiolic acid, H_2SO_4, 100 ºC. b) MOMCl, diisopropylethylamine, DMF, 0 ºC. c) NBS, AIBN, CCl_4, reflux. d) trimethylphosphite, 90 ºC. e) TMSBr, CH_2Cl_2, 0 ºC.



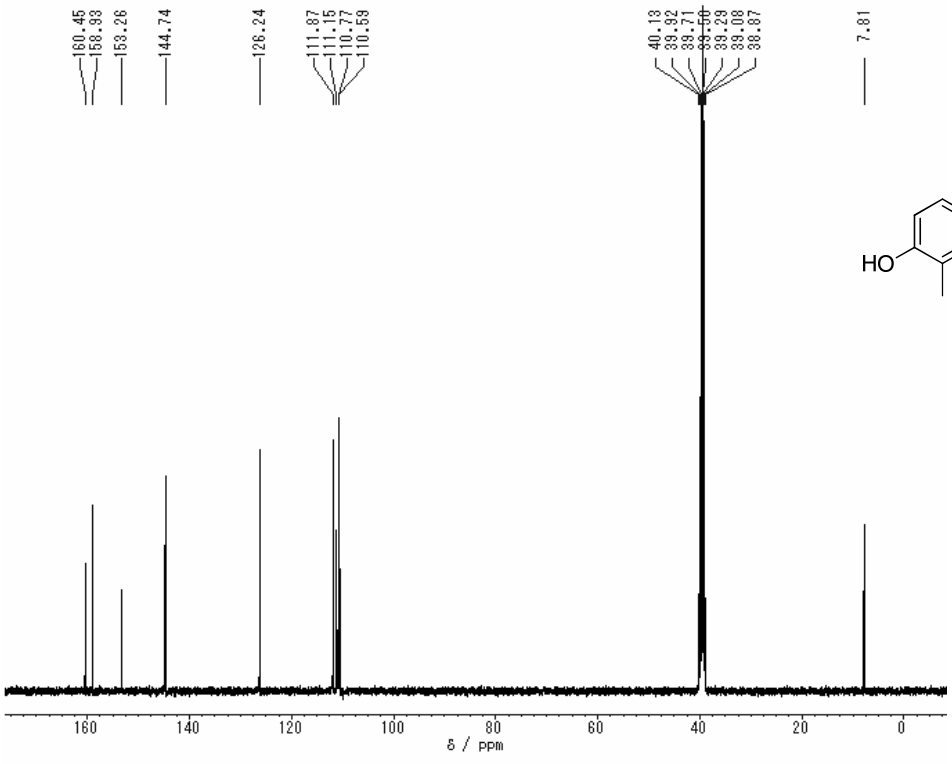
Scheme 2. Synthetic schemes of compound **3a**–**5b**. a) allyl bromide, K_2CO_3, acetone, reflux. b) *N*,*N*-diethylaniline, 220 ºC. c) MOMCl, DIEA, DMF, 0 ºC. d) OsO_4, THF/H_2O, RT. e) $NaIO_4$, RT. f) $NaBH_4$, THF, 0 ºC. g) $POCl_3$, TEA, THF. h) MeOH, 40 ºC. i) TMSBr, CH_2Cl_2, 0 ºC. j) BH_3·THF, THF, 0 ºC. k) 2 M NaOH aq., H_2O_2, RT.

^{1}H NMR spectrum of **I** in [D_6]DMSO (400 MHz)



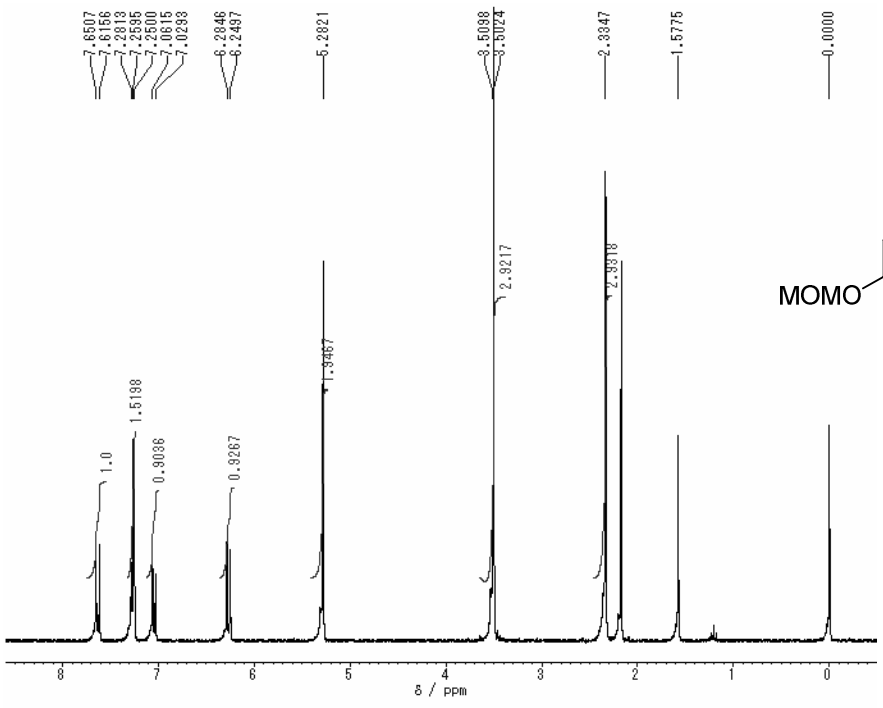
I

^{13}C NMR spectrum of **I** in [D_6]DMSO (100 MHz)



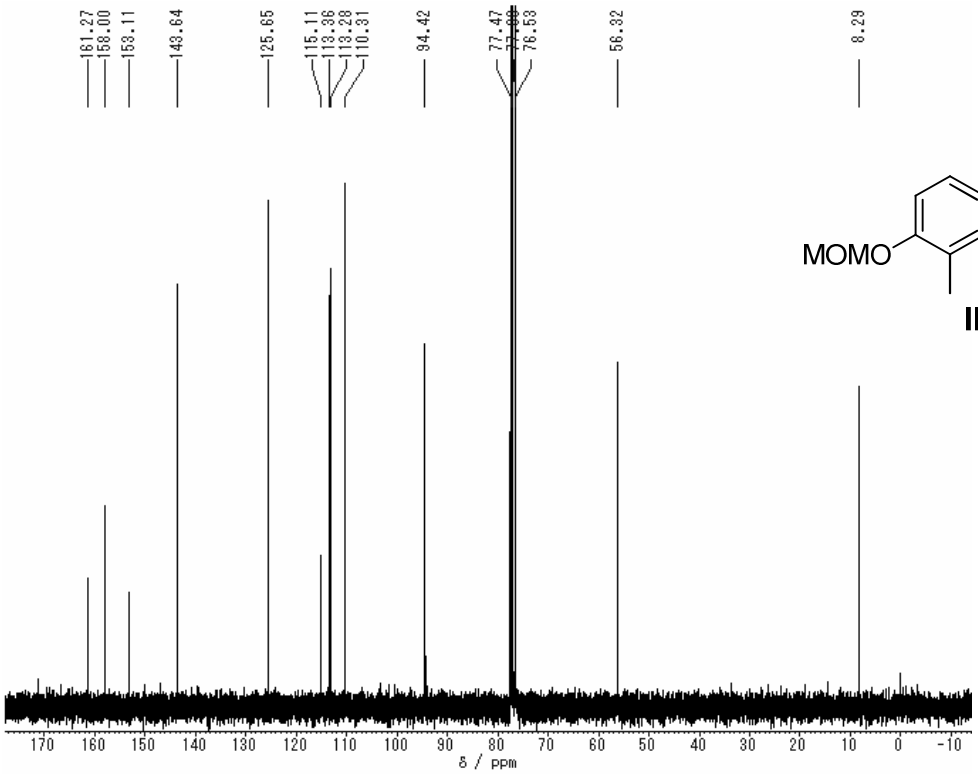
I

^{1}H NMR spectrum of II in $CDCl_3$ (270 MHz)



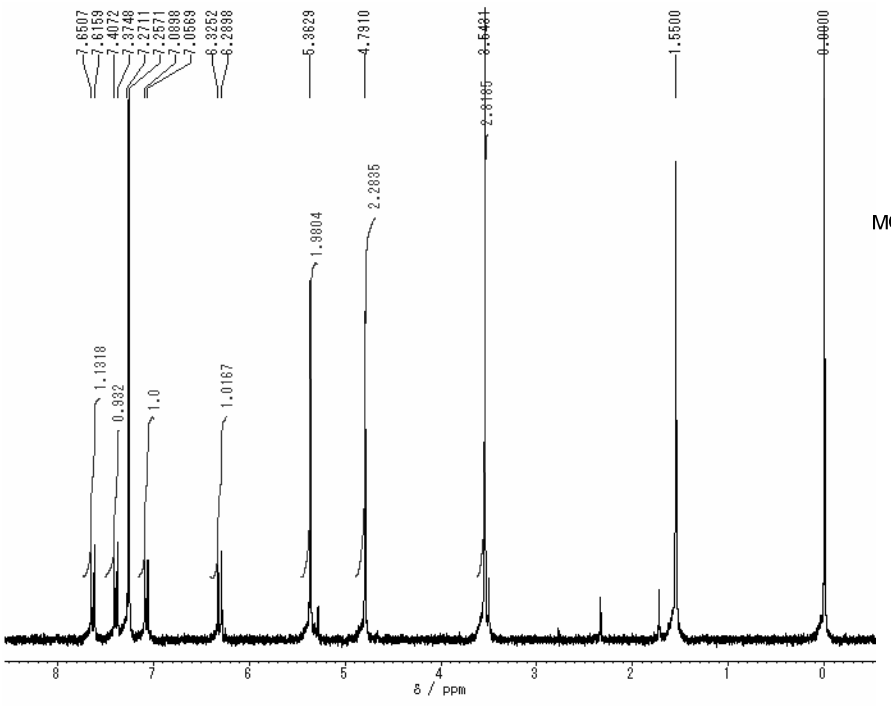
II

^{13}C NMR spectrum of II in $CDCl_3$ (67.5 MHz)

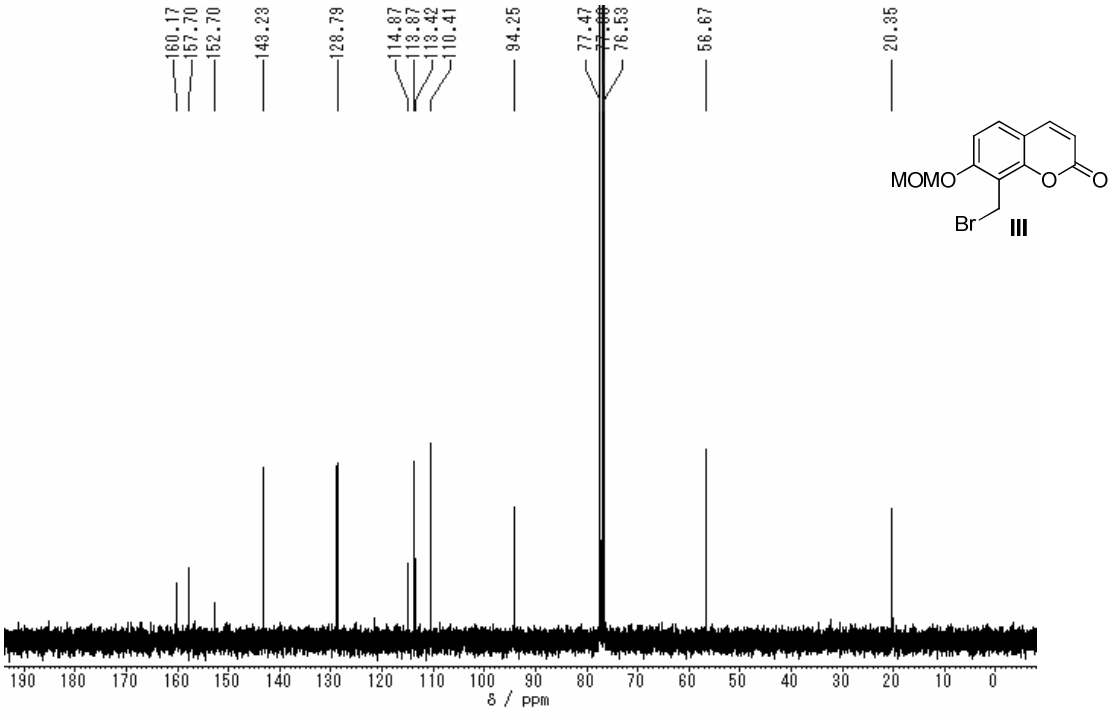


II

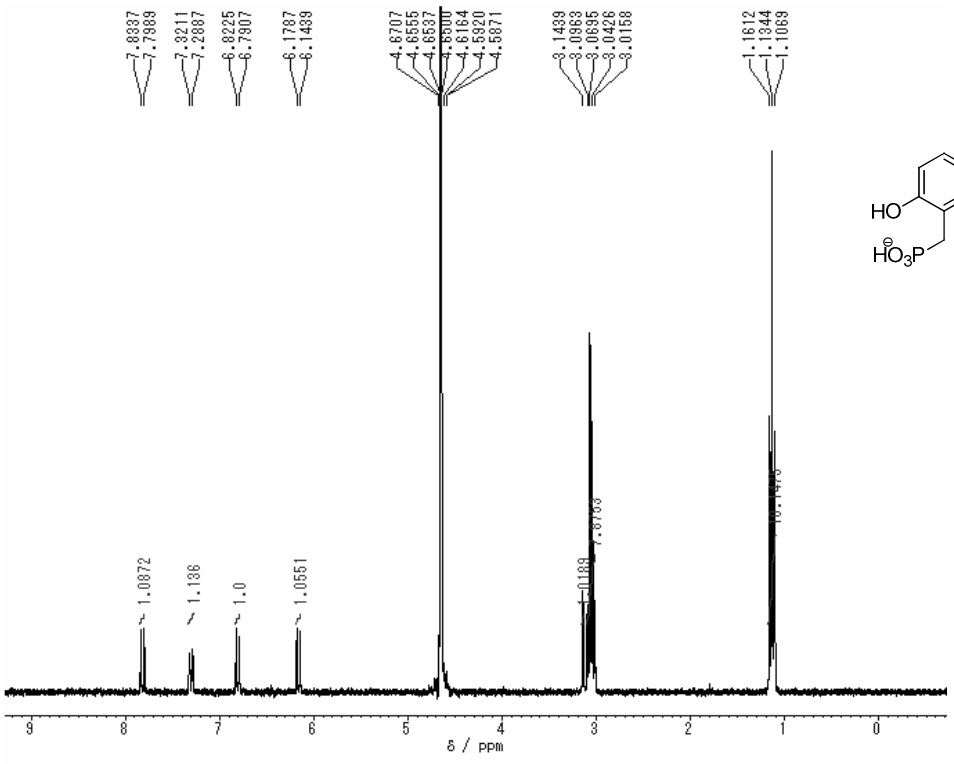
^{1}H NMR spectrum of III in $CDCl_3$ (270 MHz)



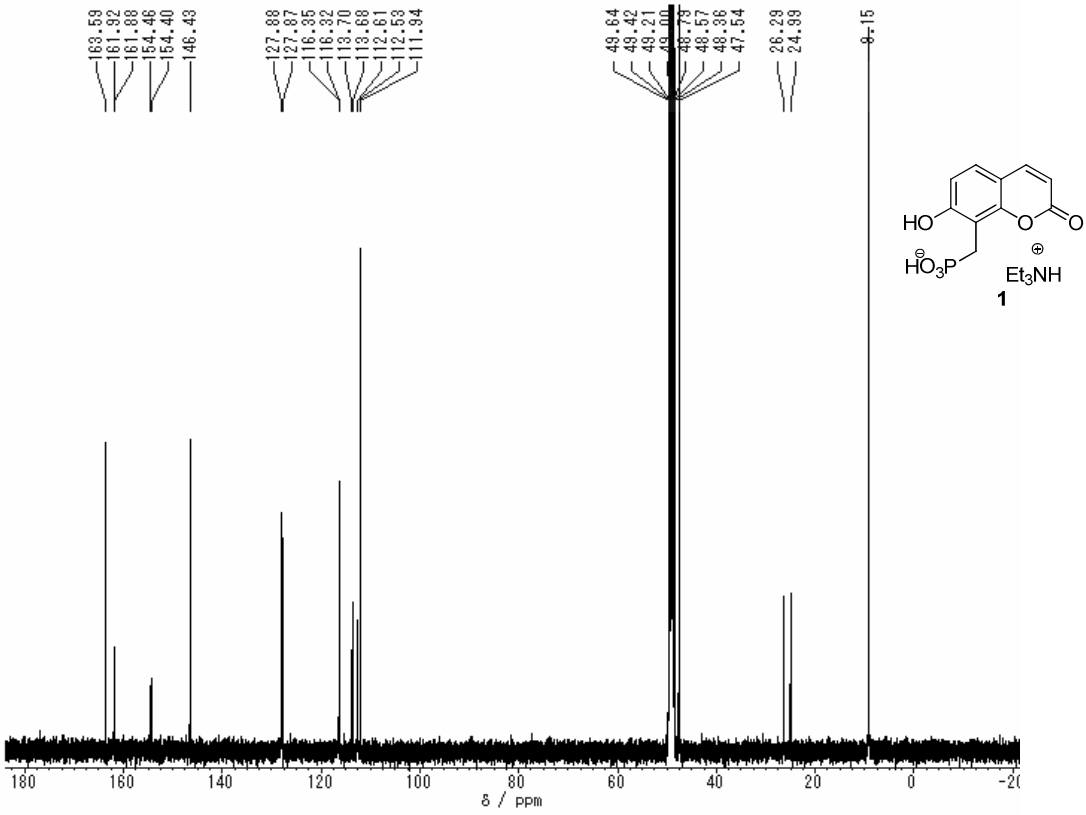
^{13}C NMR spectrum of III in $CDCl_3$ (67.5 MHz)



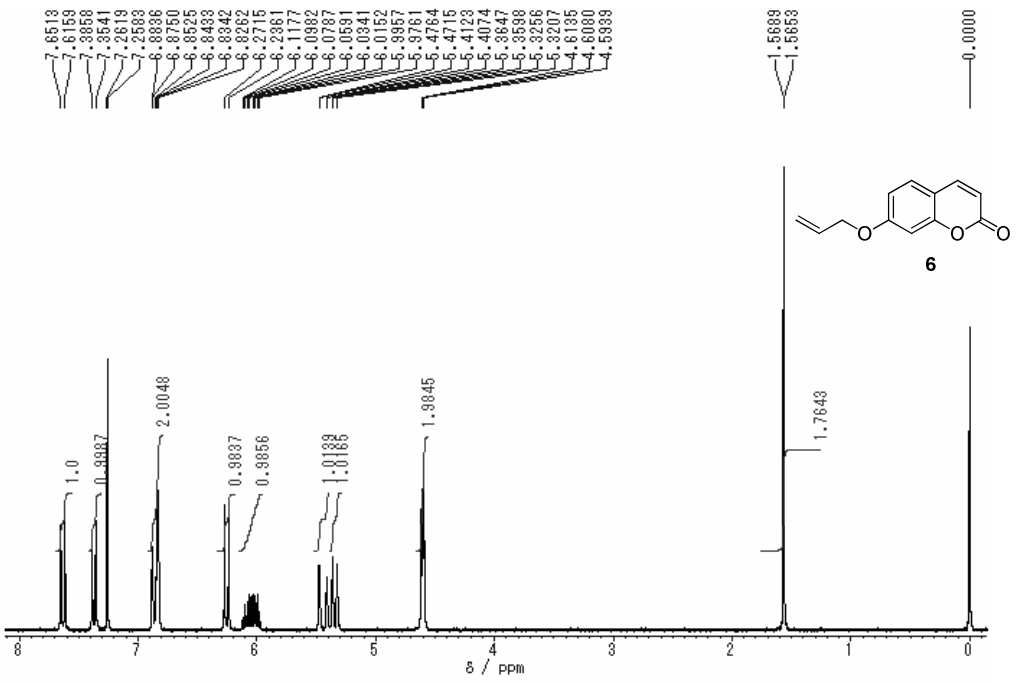
^{1}H NMR spectrum of 1 in CD_3OD (270 MHz)



^{13}C NMR spectrum of 1 in CD_3OD (67.5 MHz)

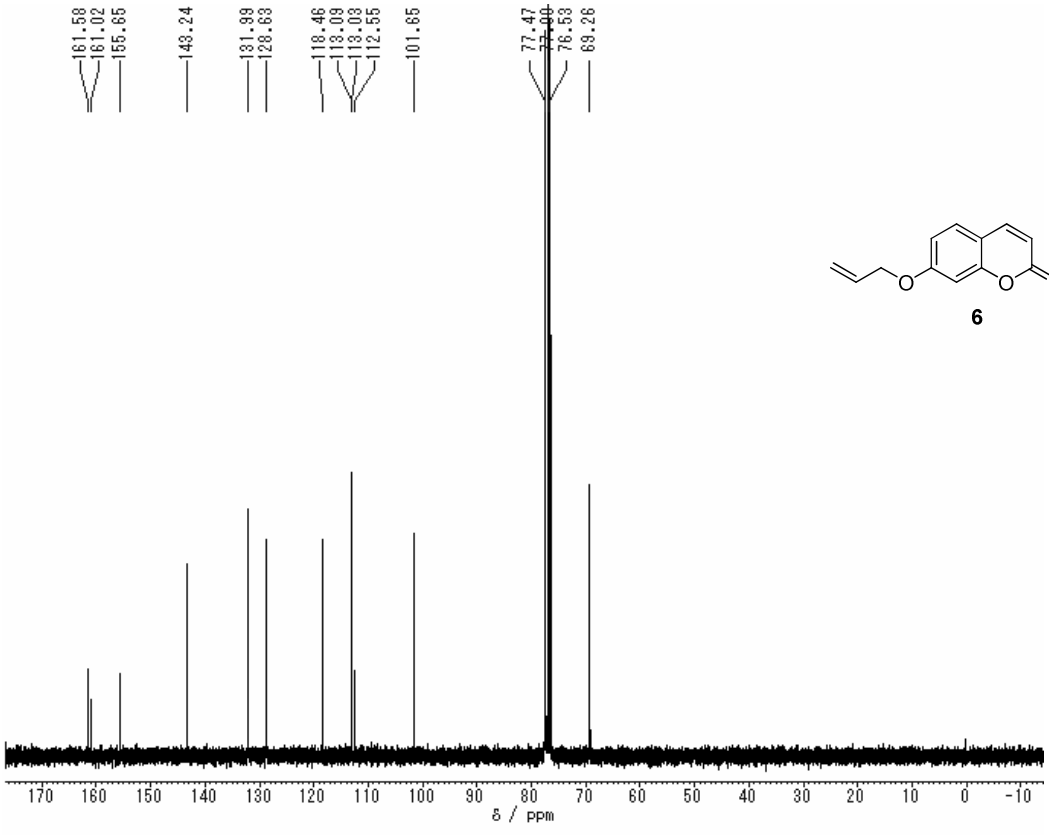


^{1}H NMR spectrum of 6 in $CDCl_3$ (270 MHz)



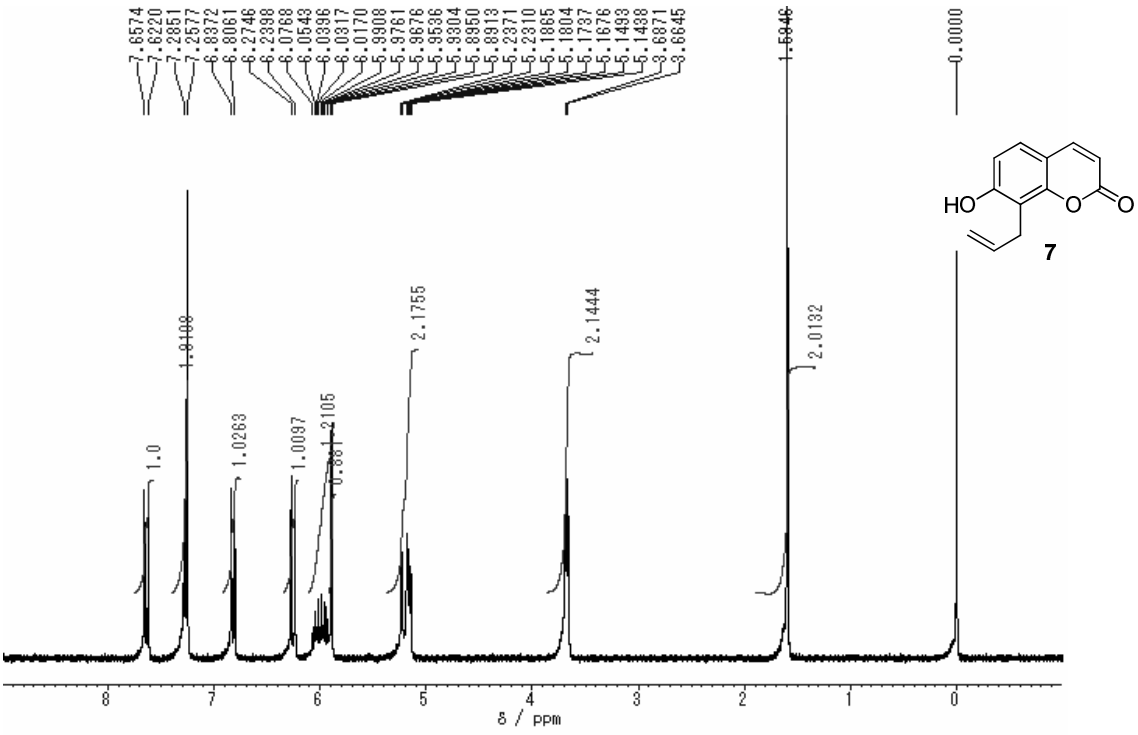
6

^{13}C NMR spectrum of 6 in $CDCl_3$ (67.5 MHz)

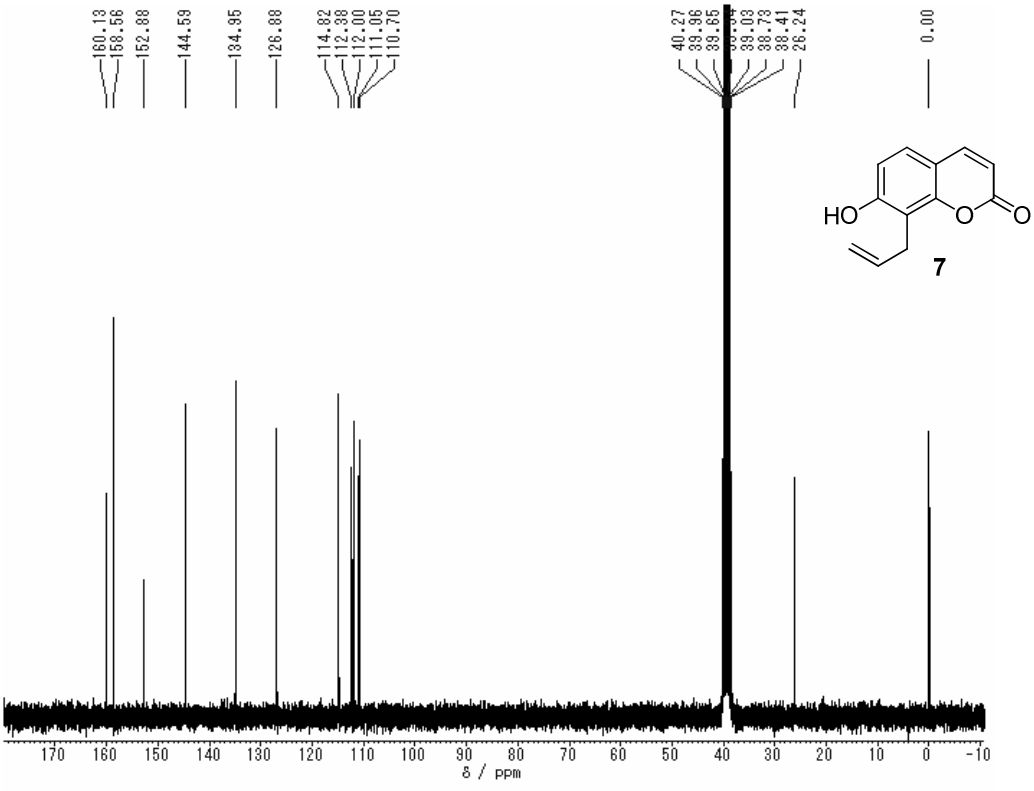


6

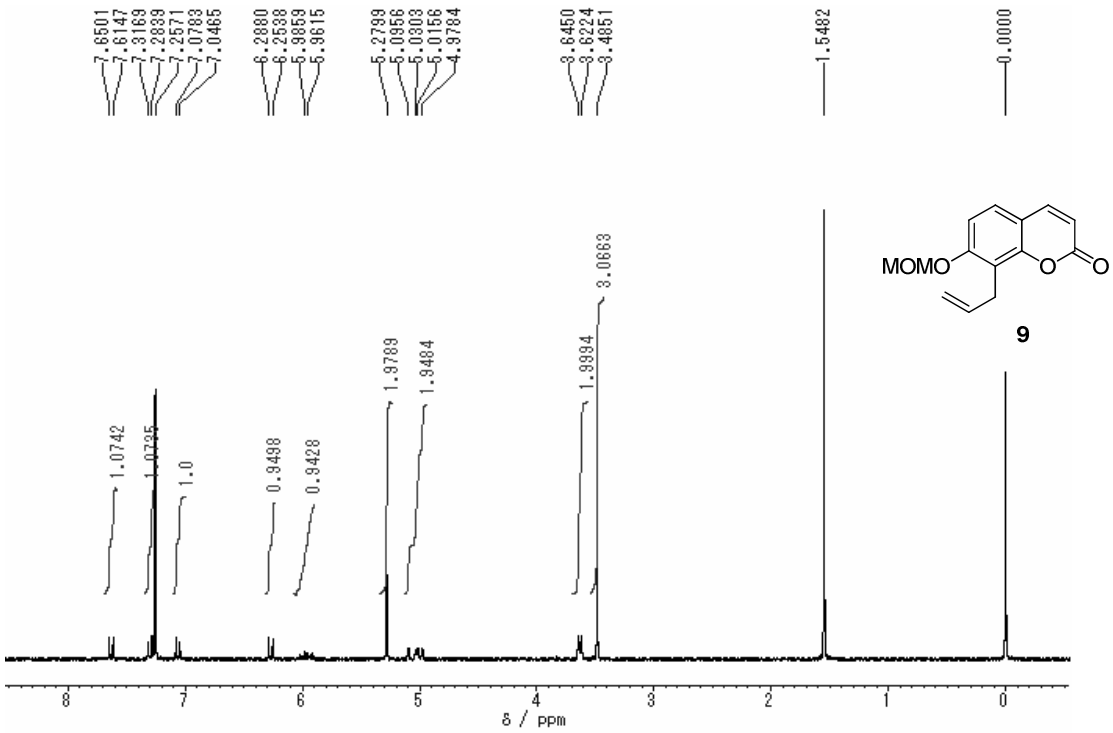
^{1}H NMR spectrum of 7 in $CDCl_3$ (270 MHz)



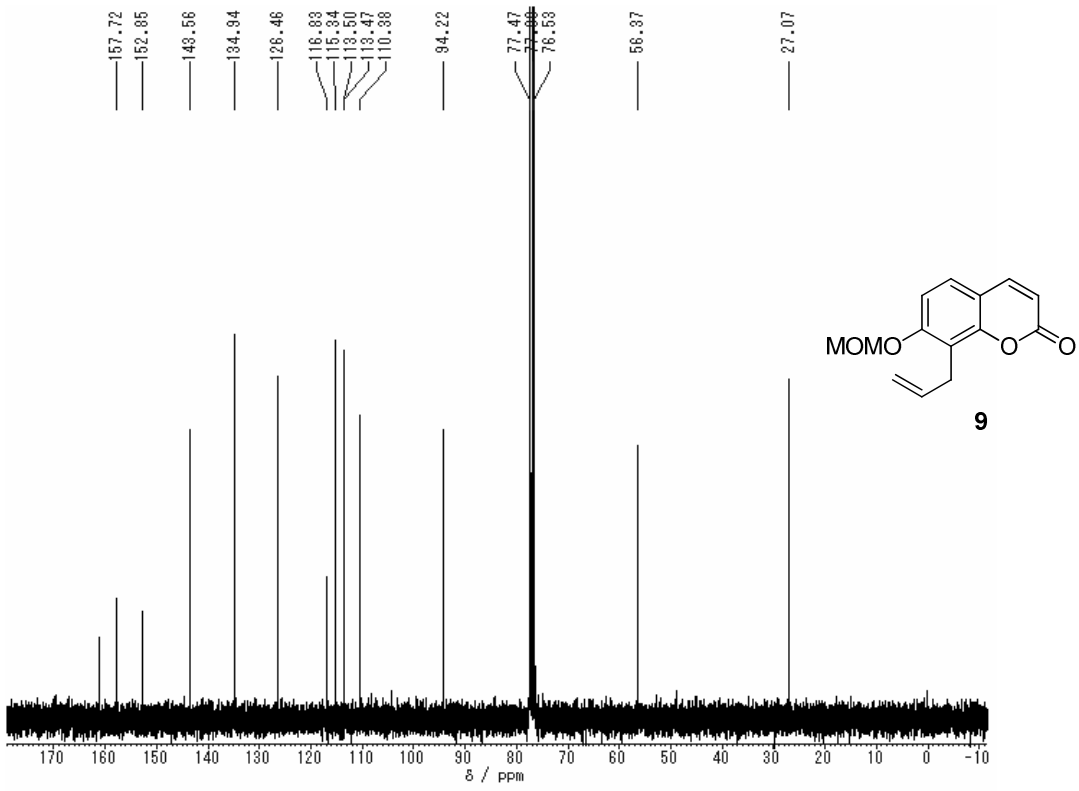
^{13}C NMR spectrum of 7 in DMSO-d_6 (67.5 MHz)



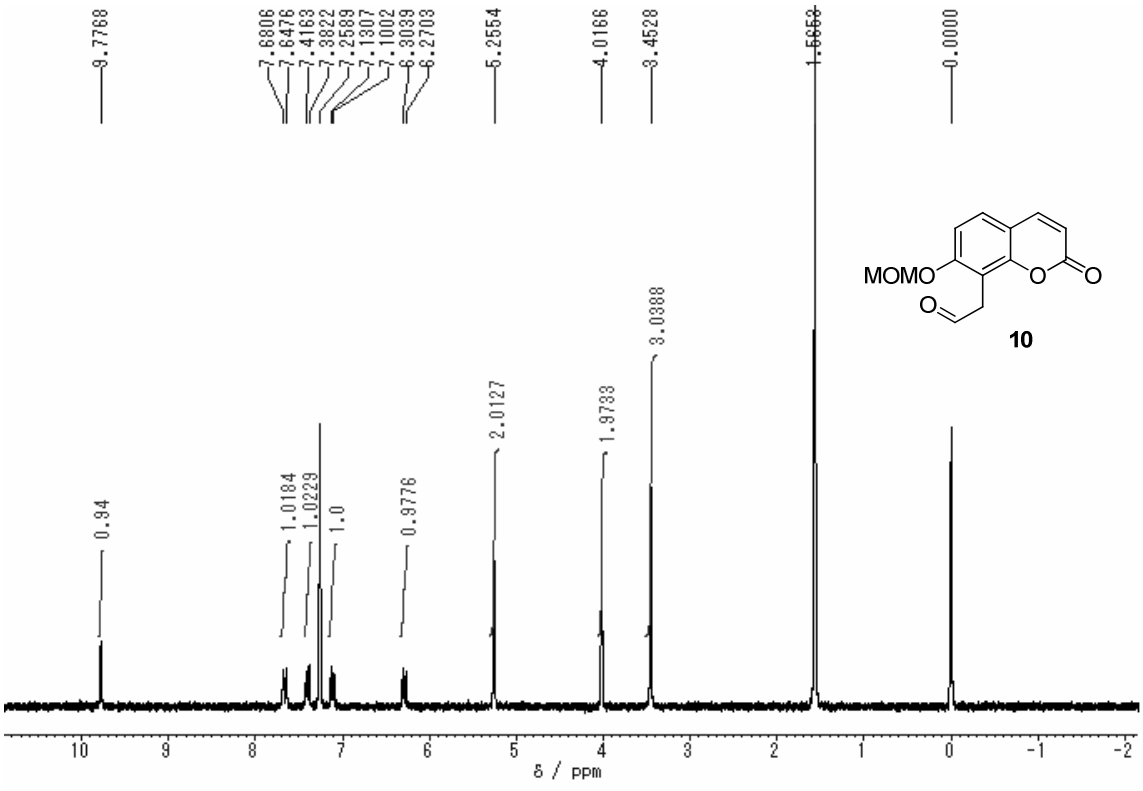
^{1}H NMR spectrum of 9 in $CDCl_3$ (270 MHz)



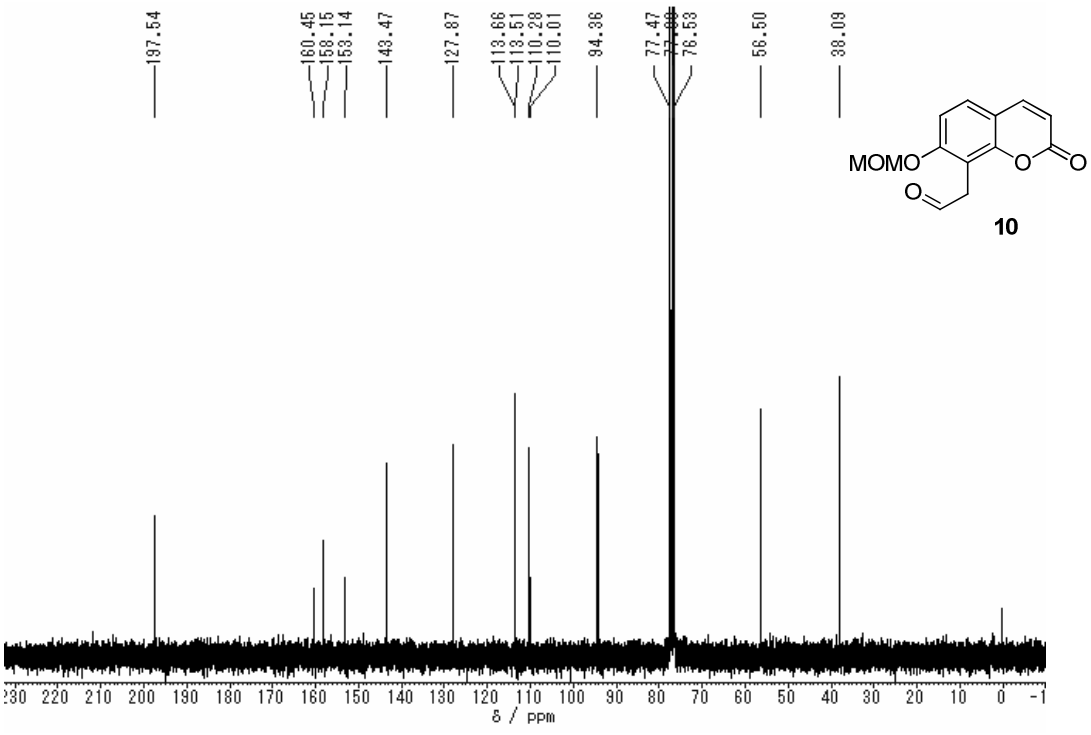
^{13}C NMR spectrum of 9 in $CDCl_3$ (67.5 MHz)



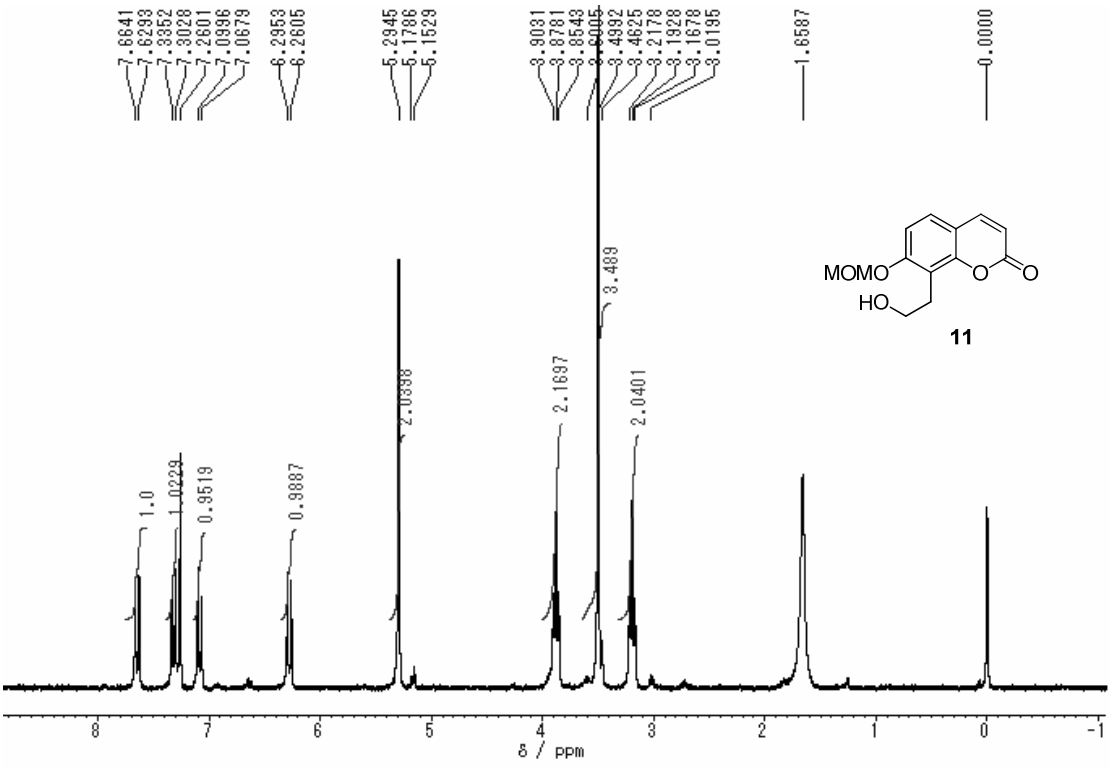
^{1}H NMR spectrum of 10 in $CDCl_3$ (270 MHz)



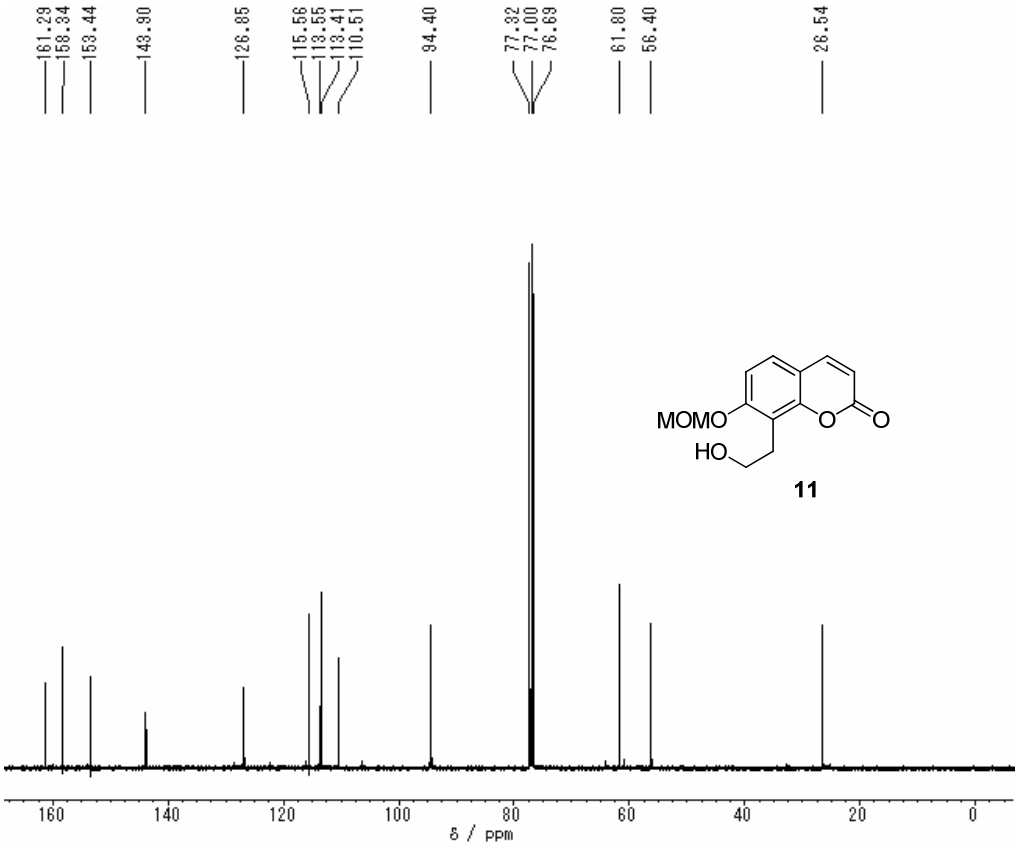
^{13}C NMR spectrum of 10 in $CDCl_3$ (67.5 MHz)



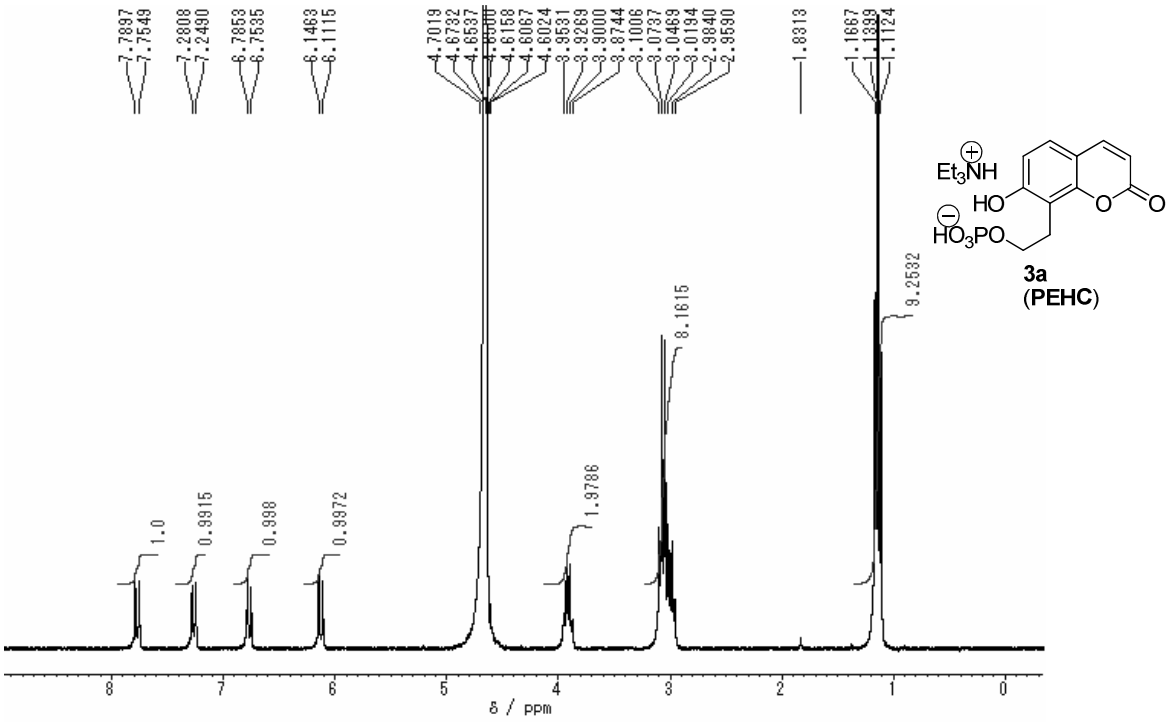
^{1}H NMR spectrum of 11 in $CDCl_3$ (270 MHz)



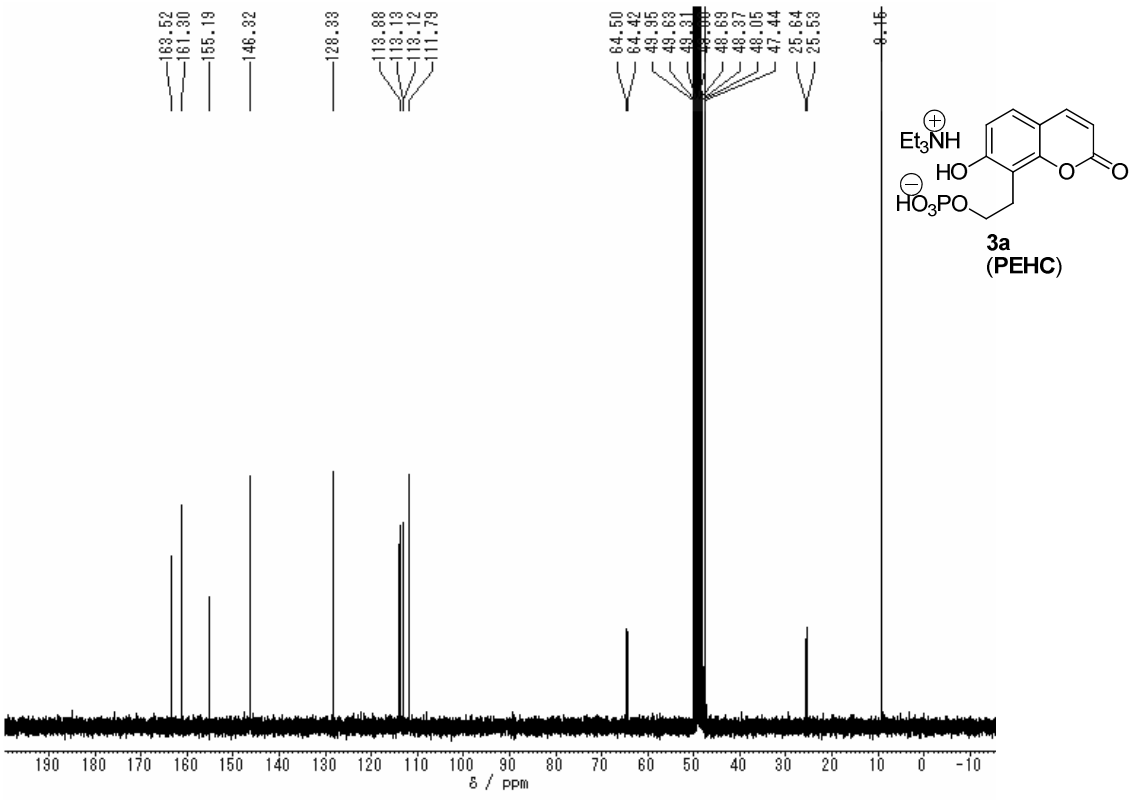
^{13}C NMR spectrum of 11 in $CDCl_3$ (67.5 MHz)



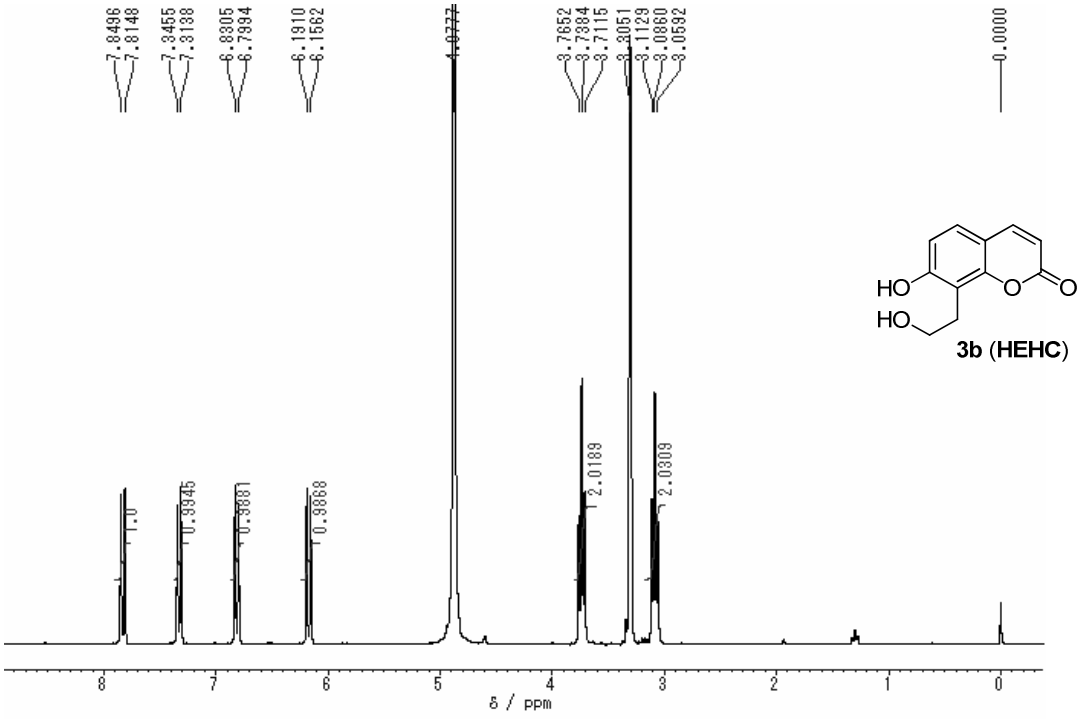
^{1}H NMR spectrum of 3a in D_2O (270 MHz)



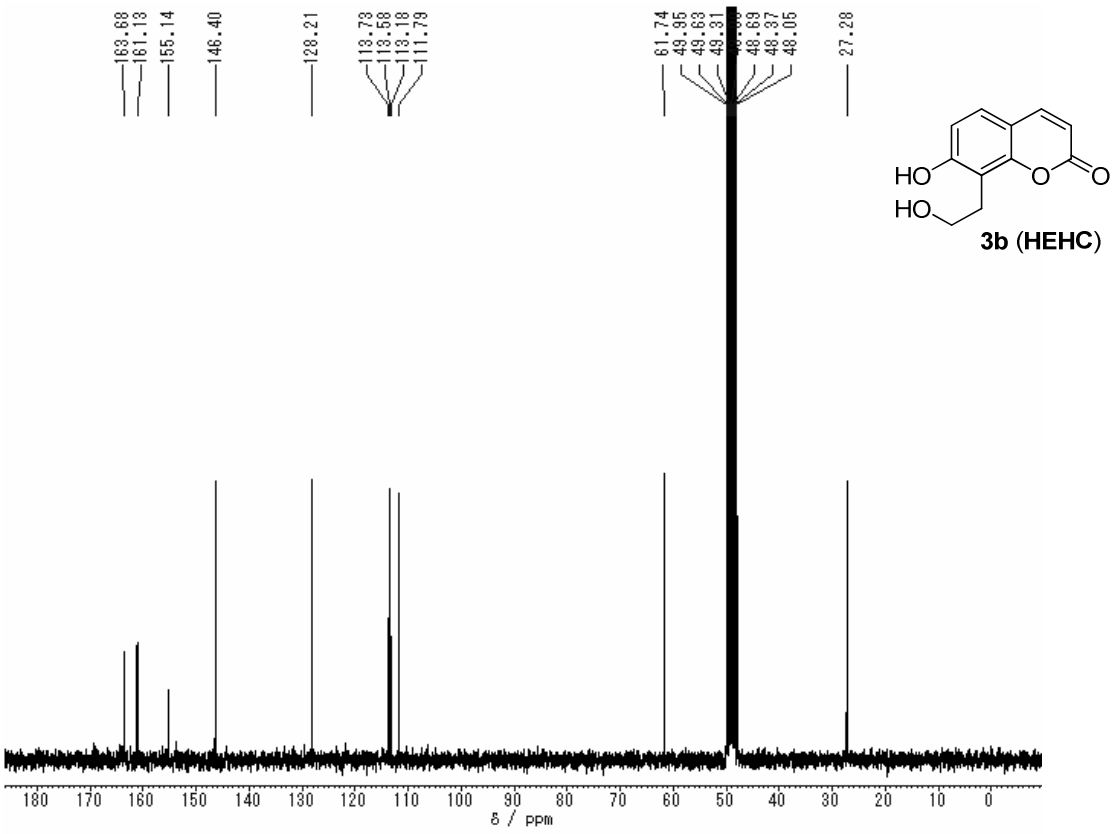
^{13}C NMR spectrum of 3a in CD_3OD (67.5 MHz)



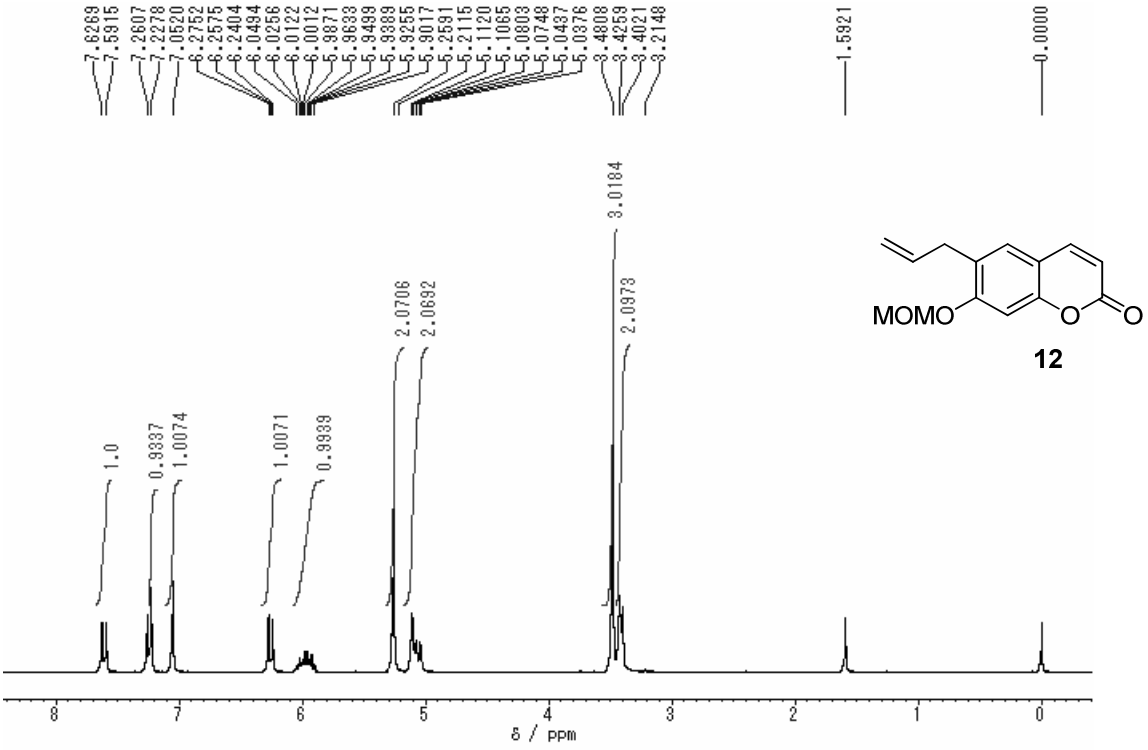
^{1}H NMR spectrum of 3b in CD_3OD (270 MHz)



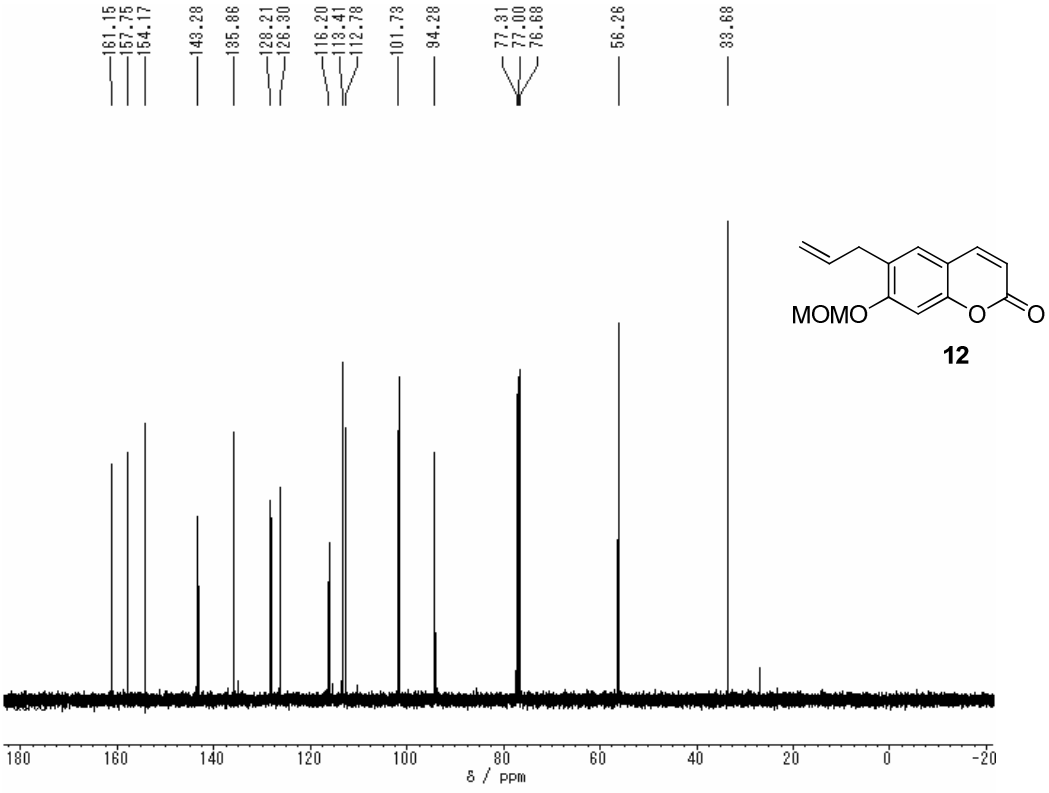
^{13}C NMR spectrum of 3b in CD_3OD (67.5 MHz)



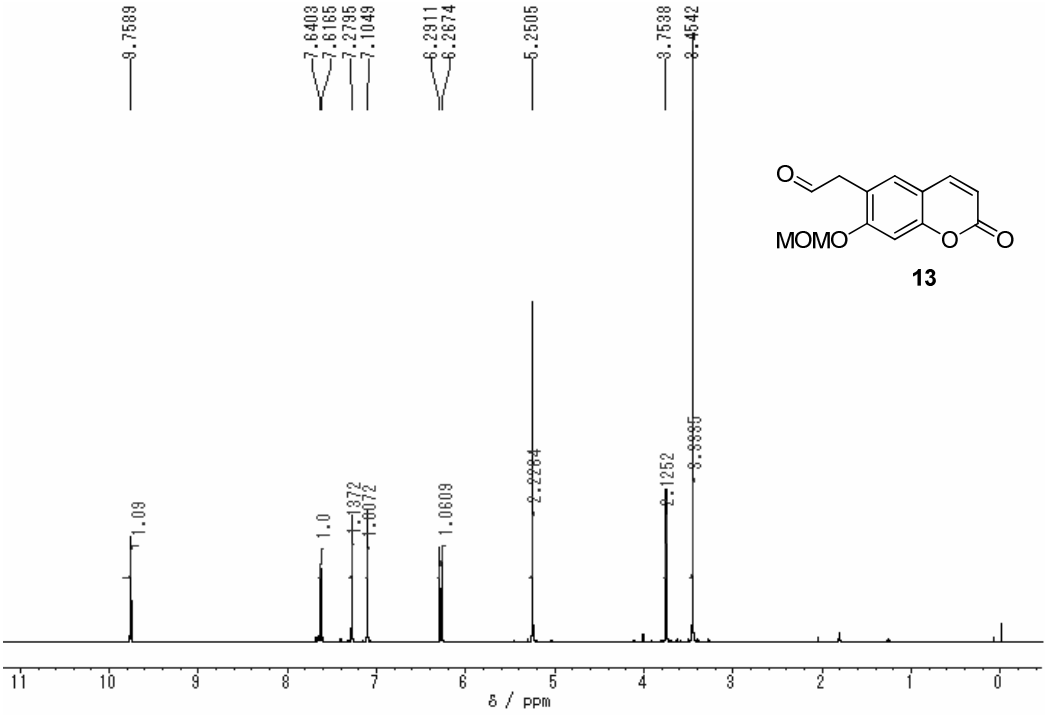
^{1}H NMR spectrum of 12 in $CDCl_3$ (270 MHz)



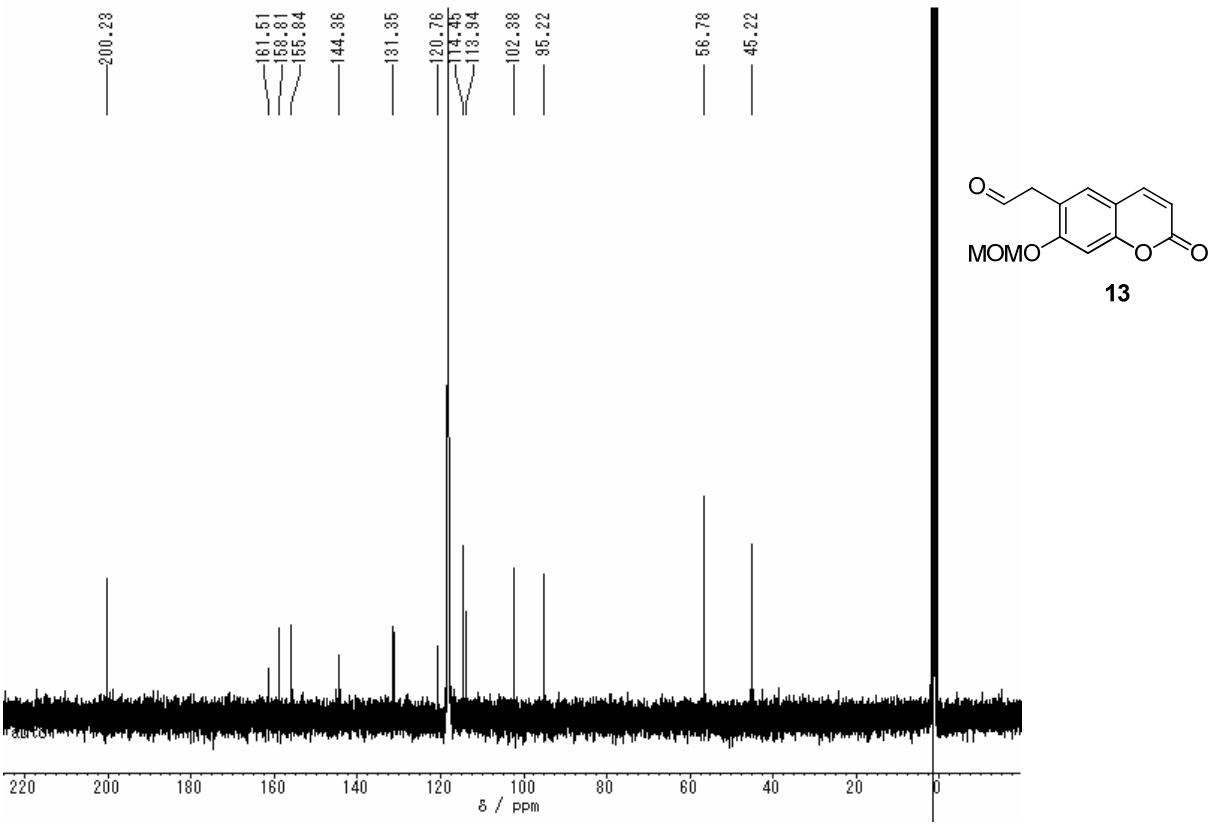
^{13}C NMR spectrum of 12 in $CDCl_3$ (67.5 MHz)



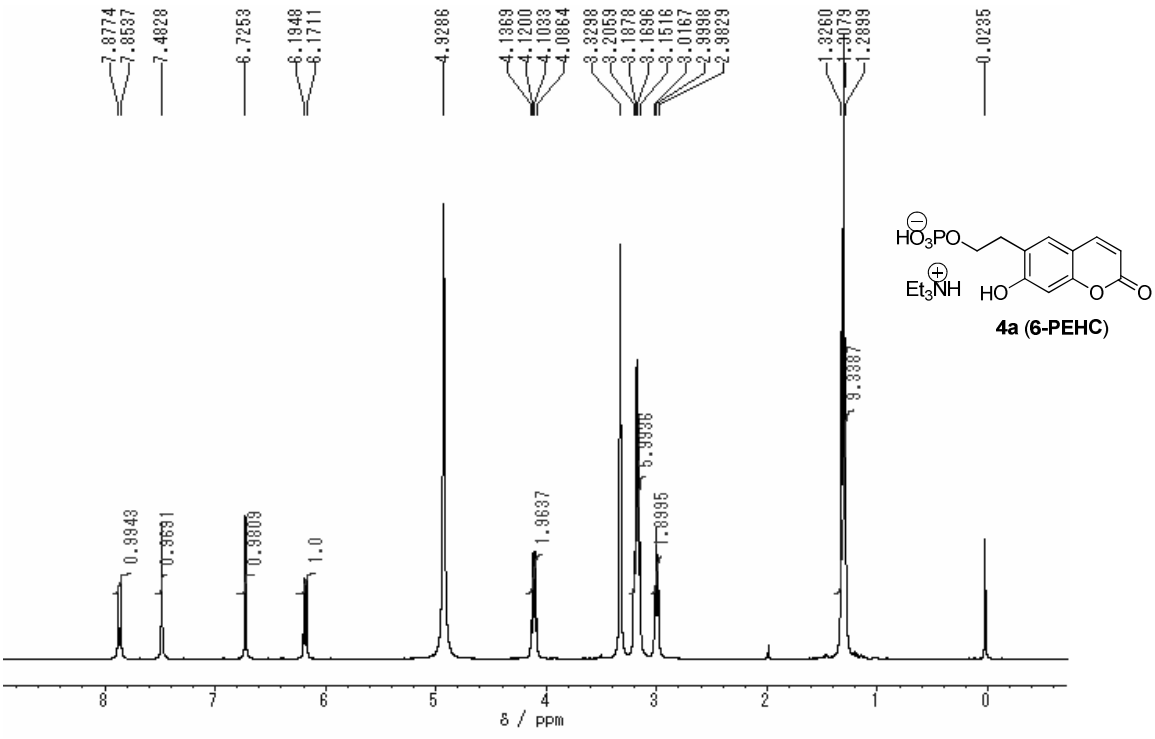
^{1}H NMR spectrum of 13 in $CDCl_3$ (270 MHz)



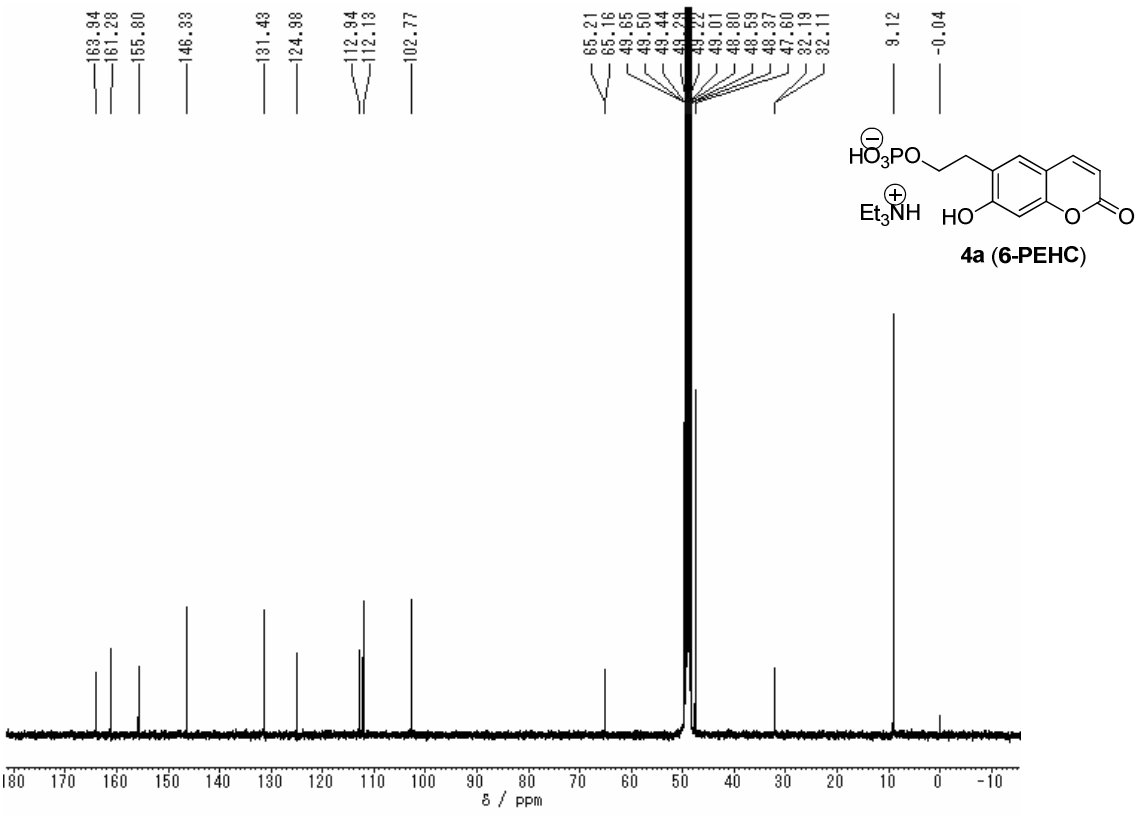
^{13}C NMR spectrum of 13 in CD_3CN (67.5 MHz)



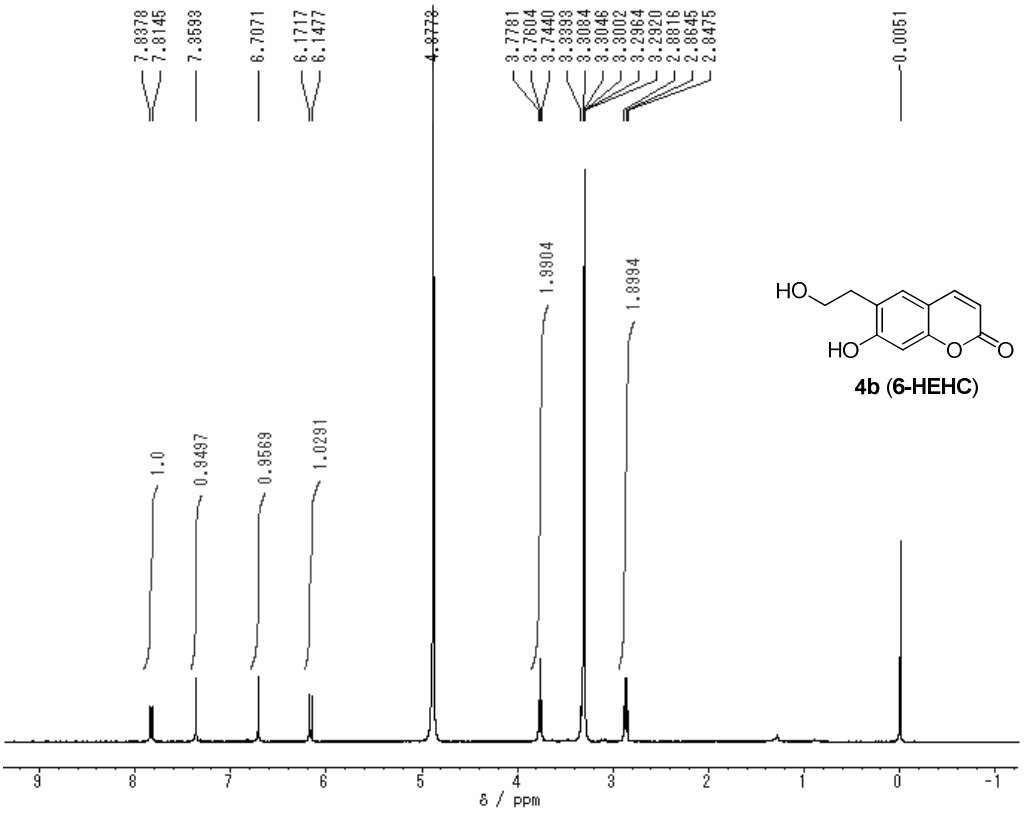
^{1}H NMR spectrum of 4a in CD_3OD (270 MHz)



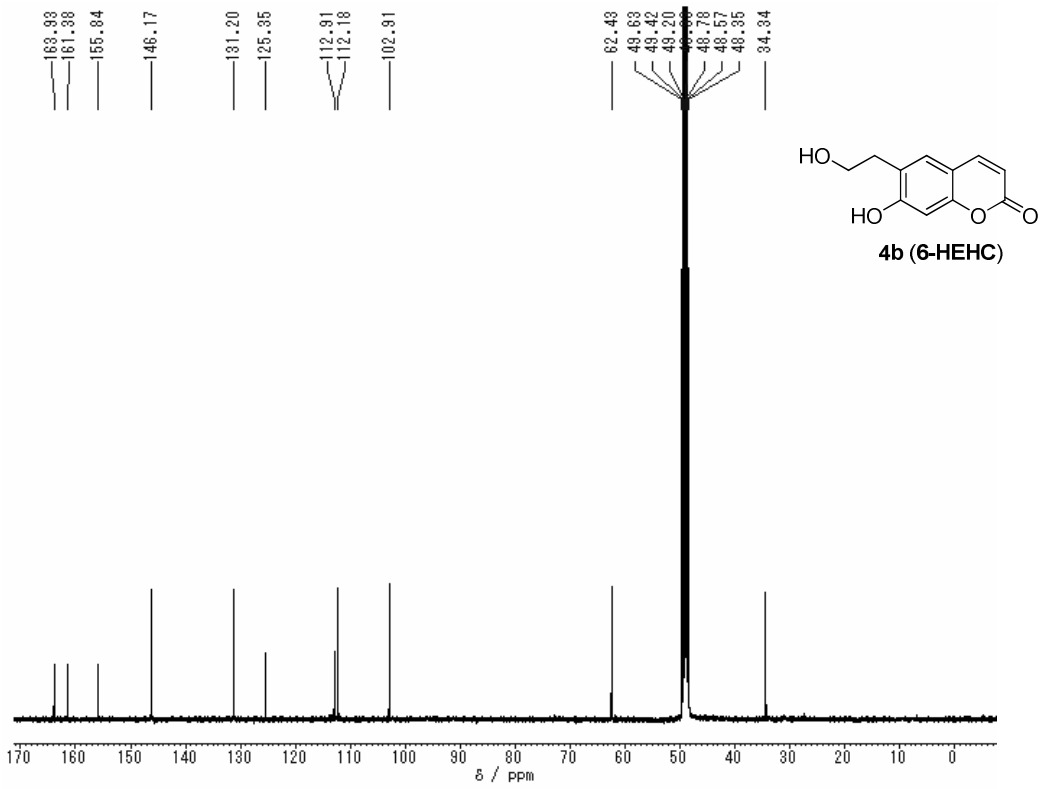
^{13}C NMR spectrum of 4a in CD_3OD (67.5 MHz)



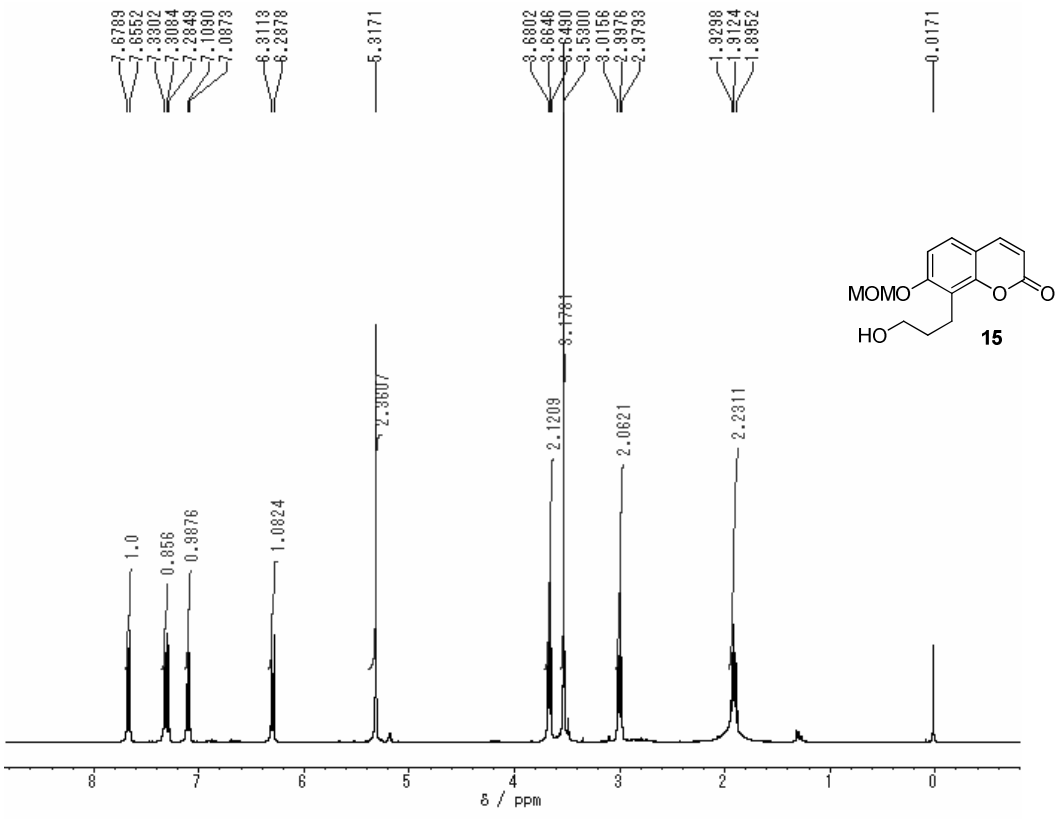
^{1}H NMR spectrum of 4b in CD_3OD (400 MHz)



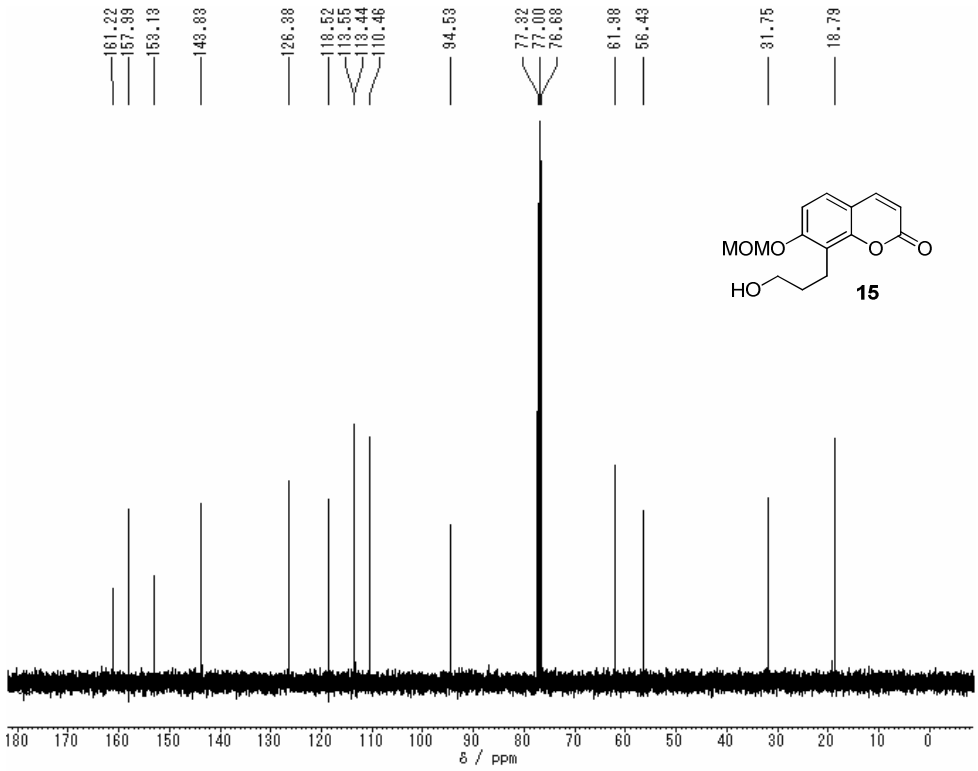
^{13}C NMR spectrum of 4b in CD_3OD (100 MHz)



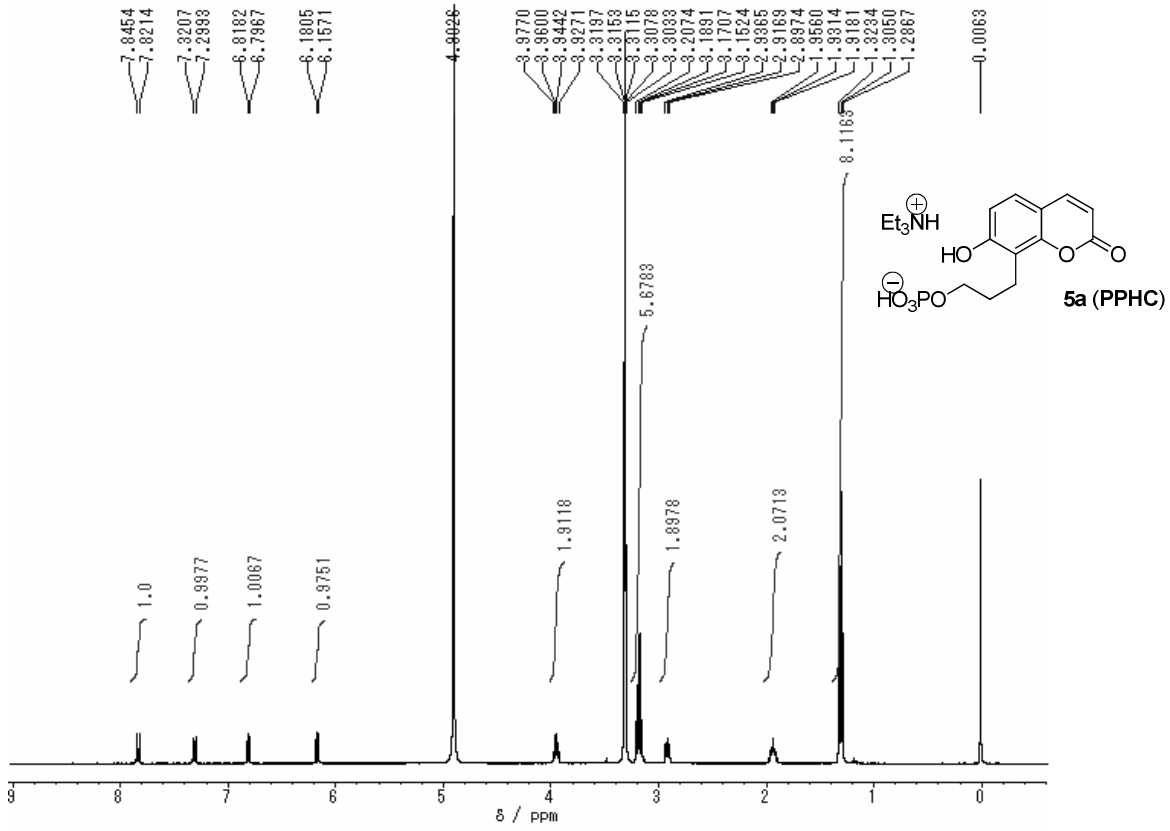
^{1}H NMR spectrum of 15 in $CDCl_3$ (400 MHz)



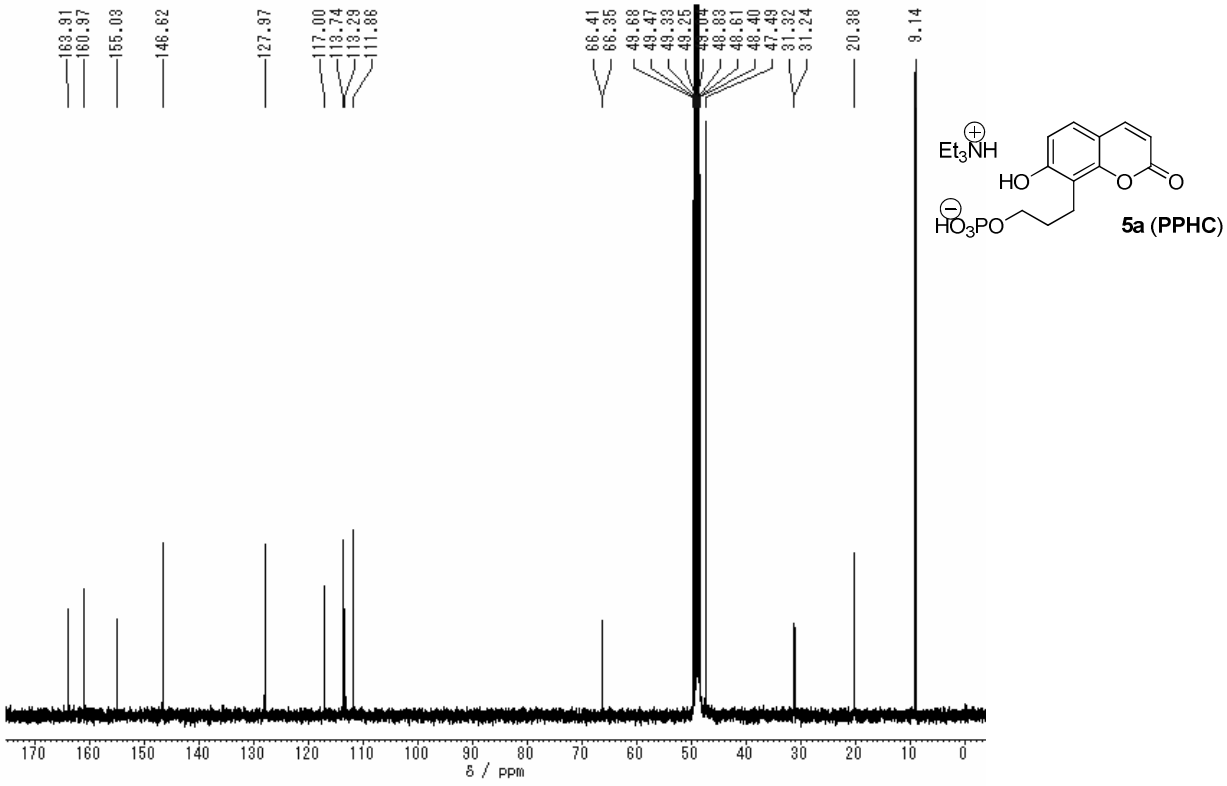
^{13}C NMR spectrum of 15 in $CDCl_3$ (100 MHz)



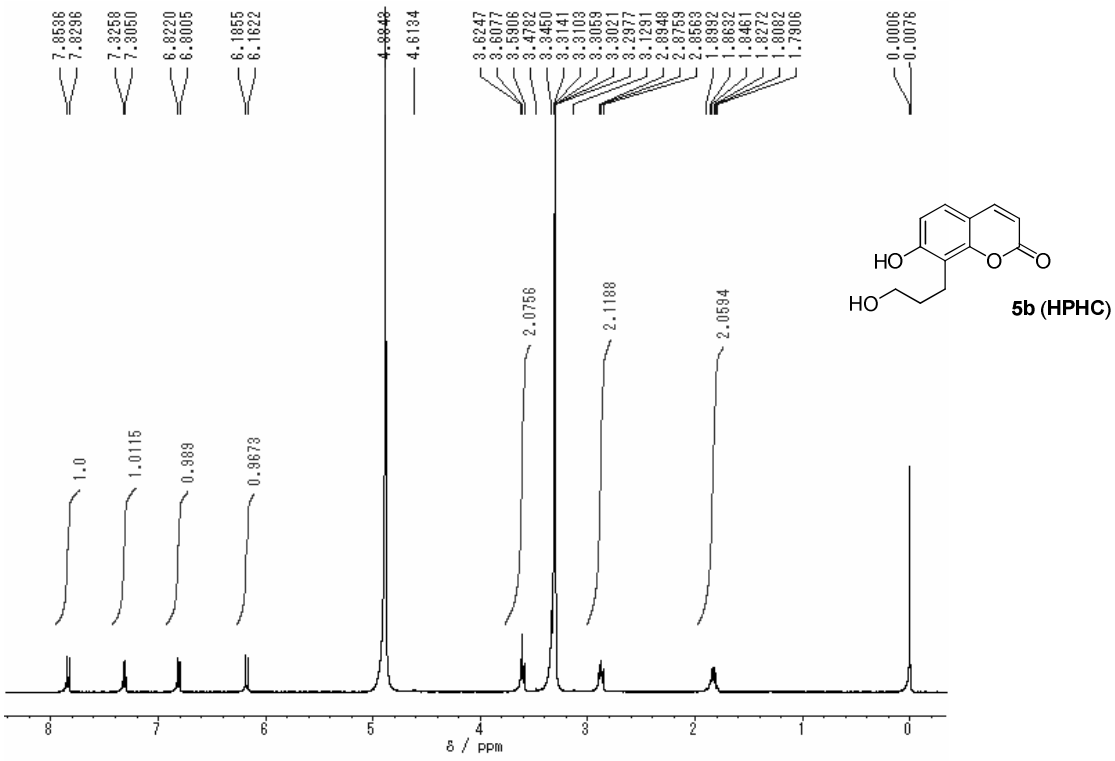
^{1}H NMR spectrum of 5a in CD_3OD (400 MHz)



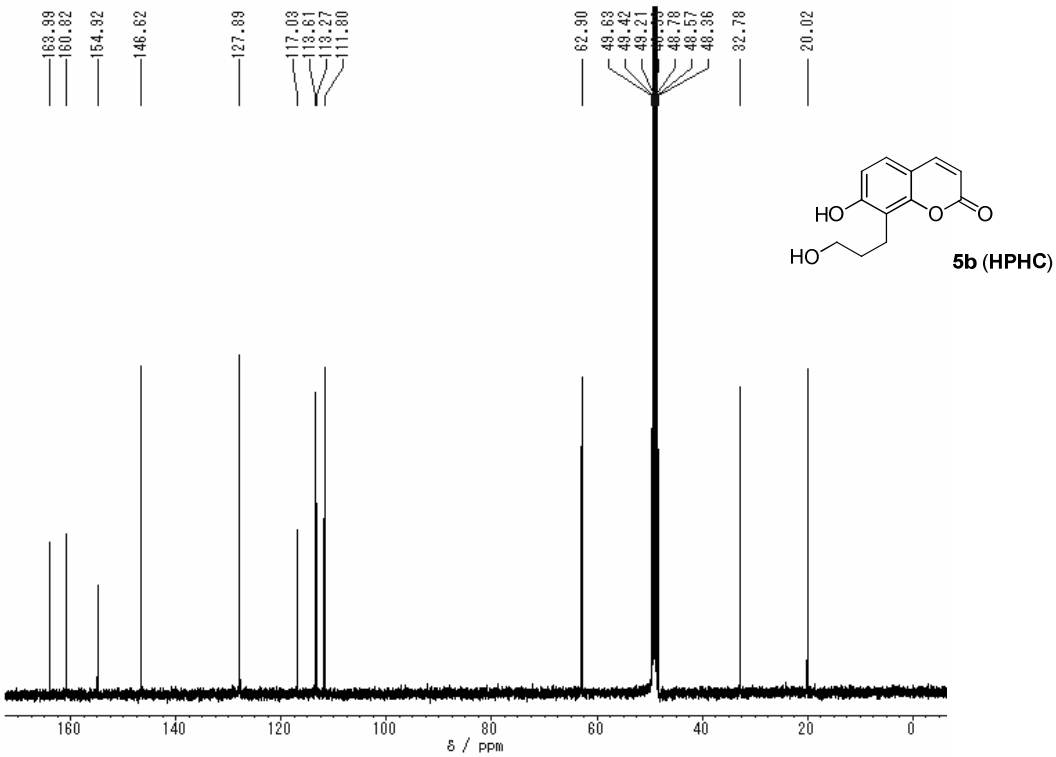
^{13}C NMR spectrum of 5a in CD_3OD (100 MHz)



^{1}H NMR spectrum of 5b in CD_3OD (400 MHz)



^{13}C NMR spectrum of 5b in CD_3OD (100 MHz)



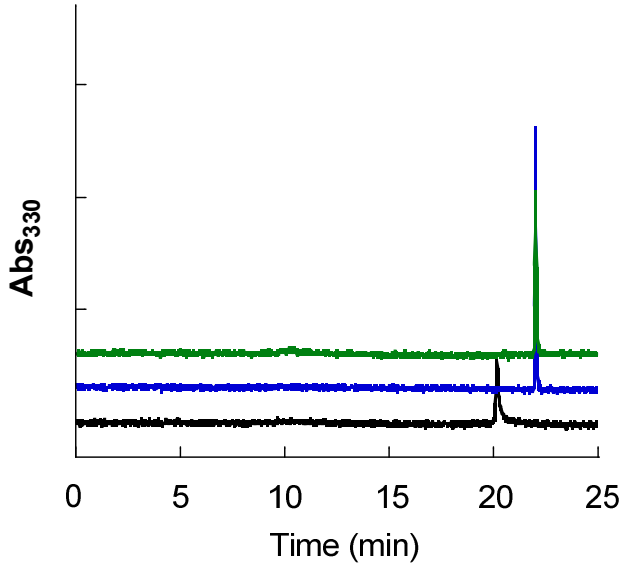


Figure S1. Reversed-phase HPLC with absorbance detection of **3a** (lower), **3a** with ACP (middle), and **3b** (upper). Eluent: 100 mM triethylamine-acetic acid buffer (pH 6.5) / acetonitrile (gradient mode).

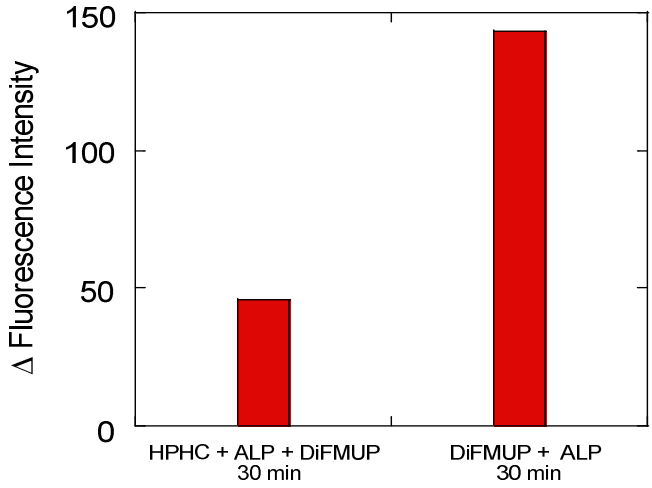


Figure S2. Inhibition assay of ALP with **3b**. Fluorescence increases of 10 µM DiFMUP after incubation (30 min) with ALP (left) and with ALP preincubated with 1 µM **3b**.