Supplementary Information for

Rational Design of a Highly Sensitive and Selective Fluorogenic Probe for Detecting Nitric Oxide

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Materials and Methods

Solvents and reagents were purchased from commercial suppliers and used without further purification unless otherwise indicated. spectroscopic grade DMSO were used for spectroscopic studies.

The ^{1}HNMR and ^{13}CNMR spectra were recorded on Varian Brüker 400 MHz spectrometer. Mass spectra were obtained from a Quattro microtriple quadrupole mass spectrometer (Waters, Milford, MA, USA). UV-Vis spectra were recorded on a Cary-50 UV-Vis spectrophotometer. Fluorescence spectra were recorded on a Cary Eclipse Fluorescence spectrophotometer (V = 600 volts). The adjustable slit was set at 5 nm. Fluorescence quantum yield of samples were recorded on a Fluormax-4 spectrophotometer at room temperature with an integrating sphere system, and the machine was revaluated using standard sample before measurement. Melting points were taken on a X-5B precise micro melting point apparatus. Single-crystal X-ray diffraction measurements were carried out on Rigaku MicroMax 002 CCD diffractometer at 298k using *CuKα* radiation. All structures were solved by direct methods and refined by full-matrix least squares on F^2 using the Shelxs-97 computer program package.

The nitric oxide (NO) stock solution in de-ionized water was prepared according to the literature.[1] Peroxynitrite was generated from amyl nitrite and H_2O_2.[2]

Quartz cuvettes with 10 mm path lengths and four faces polished were used. Stock solutions of probe **1** (5 mM) were prepared in phosphate buffer (50 mM, pH 7.4) with 20% DMSO.

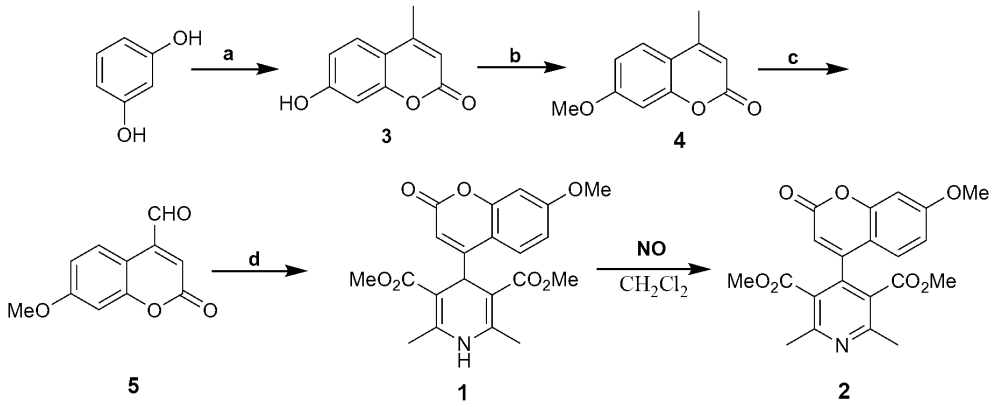
Cell Culture and Imaging Materials and Methods. HeLa cells and Raw 264.7 cells were obtained from Cell Resource Center (IBMS, CAMS/PUMC) and cultured in Dulbecco' s Modified Eagle Medium (DMEM; Thermo Scientific) supplemented with 10% fetal bovine serum (FBS; GIBCO; Invitrogen), and 1% penicillin/streptomycin (Beijing Solarbio Scientific & Technology Co, Ltd). For imaging studies, cells were plated in Class Bottom Cell Culture Dish (Nest) containing 1 mL of complete DMEM and incubated at 37 ℃ under 5% CO_2 for one day. To induce NO production, Raw 264.7 cells were stimulated with 0.5 μg mL^{-1} Lipopolysaccharide (LPS, Sigma-Aldrich) for 4 h. To induce NO production, Raw 264.7 cells were stimulated with 2

mM sodium nitroprusside (SNP) for 30-150 minitues. Bright filed and fluorescence images were taken with a Zeiss Abserver A1 inverted fluorescence microscope equipped with an EM-CCD camera (Hamamatsu) and an X-Cite 120 metal halide lamp (EXFP). Bright field image and fluorescence images were obtained using an 40× objective lens.

1 K. J. Huang, H. Wang, M. Ma, X. Zhang, and H. S. Zhang, *Nitric Oxide*. 2007, **16**, 36-43.

2 R. M. Uppu and W. A. Pryor, *Anal. Biochem*. 1996, **236**, 242–249.

Experiments and characterizations



Scheme S1. Synthetic scheme of **1** and **2**. (a) Ethyl acetoacetate, H_3PO_4, rt 12h; (b) Acetone, K_2CO_3, CH_3I, reflux, 6 h,; (c) SeO_2 , xylene, reflux, 10 h; (d) Methy acetoacetate, $NH_3 \cdot H_2O$, EtOH, reflux, 4 h.

4-Methyl-7-hydroxylcoumarin (3). 3-hydroxylphenol (11 g, 100 mmol) and ethyl acetoacetate (13 mL, 100 mmol) were added into concentrated phosphoric acid (52 mL, 85%). After stirring at room temperature for 12 hours, the reaction mixture was poured into 150 mL water. The crude product was collected by filtration and purified by recrystallization in ethanol to afford **3** as white crystal (23.7 g, yield 98%). Mp. 160～162℃, ^{1}H NMR (400 MHz DMSO-d_6) δ 10.50 (s, 1H), 7.58 (t, 1H, J = 5.3 Hz), 6.82 (t, 1H, J = 7.1 Hz), 6.72 (d, 1H, J = 1.5 Hz), 6.13 (s, 1H), 2.37 (d, 3H, J = 0.8 Hz). ^{13}C NMR (400 MHz DMSO-d_6) δ 161.04, 160.17, 154.71, 153.20, 126.26, 112.68, 111.86, 110.14, 102.06, 17.93; ESI MS calculated 176.1, found 177.1 ($M+H^+$).

4-Methyl-7-methoxylcoumarin (4). To a solution 30 mL acetone solution of **3** (3.5 g, 22 mmol), anhydrous potassium carbonate (6.0 g, 44 mmol) was added. The reaction mixture was refluxed for 10 min, and then methyl iodide (3.1 g, 22 mmol) was added dropwise. The resulting mixture was refluxed for 6 more hours and filtered without cooling. The filtrate was evaporated *in vacuo* to give the crude product which was purified by recrystallization in ethanol to give **4** (3.6 g, 95%) as white crystal. Mp. 163～165 ℃, ^{1}H NMR (400 MHz $CDCl_3$) δ 7.50 (d, 1H, J = 8.7 Hz), 6.86 (m, 1H, J = 2.5 Hz), 6.81 (d, 1H, J = 2.4 Hz), 6.13 (d, 1H, J = 0.9 Hz), 3.87 (s, 3H), 2.40 (d, 3H, J = 1.0 Hz). ^{13}C NMR (400 MHz $CDCl_3$) δ 161.05, 153.53, 152.44, 142.88, 125.34, 124.26, 117.52, 117.10, 113.92, 21.55, 18.56; ESI MS calculated 189.1, found 190.1 ($M+H^+$).

4-Formyl-7-methoxylcoumarin (5). 4 (0.95 g, 5 mmol) was dissolved in 30 mL xylene and heated to reflux. After addition of selenium dioxide (1.1 g. 10 mmol), the reaction mixture was refluxed for 10 hours. The resulting suspension was filtrated without cooling. While filtrate was cooled to room temperature, crude product precipitated out of solution and was collected by filtration. Recrystallization in acetonitrile afforded **5** (0.85 g, 84%) as yellow needle-like crystal. Mp. 126～127℃, ^{1}H NMR (400 MHz $CDCl_3$) δ 10.07 (s, 1H), 8.49 (d, 1H, *J* = 5.0 Hz), 6.93 (m, 1H, *J* = 2.5 Hz), 6.86 (d, 1H, *J* = 2.4 Hz), 6.71 (s, 1H), 3.89 (s, 3H). ^{13}C NMR (400 MHz DMSO-d_6) δ 193.70, 162.55, 160.37, 155.87, 143.50, 126.87, 121.28, 112.84, 108.16, 101.06, 55.93; ESI MS calculated 203.1, found 204.2 ($M+H^+$).

Dimethyl 4-(7-methoxy-2-oxo-2*H*-chromen-4-yl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate (**1**)**. 5** (0.3 g, 1.47 mmol), methyl acetoacetate (1.1 g, 9.2 mmol) and ammomia solution (0.2 g, 9.2 mmol) were dissovled in 20 mL ethanol and refluxed for 4 hours. After cooling to room temperature, the reaction mixture was concentrated in vacuo to give crude product which was purified by recrystallization in ethanol to afford **1** (0.9 g, 42%) as yellowish crystal. Mp. 249～251℃, ^{1}H NMR (400 MHz $CDCl_3$) δ 8.12 (d, 1H, *J* = 9.1 Hz), 6.93 (d, 1H, *J* = 2.5 Hz), 6.91(d, 1H, *J* = 2.5 Hz), 6.79(d, 1H, *J* = 2.4 Hz), 6.24 (s, 1H), 5.35 (s, 1H), 3.88 (s, 3H), 3.51 (s, 6H), 2.35 (s, 6H). ^{13}C NMR (400 MHz DMF-d_6) δ 167.20,165.45, 155.38, 146.83, 127.83, 112.04, 11.98, 111.52, 101.67, 100.23, 55.68, 50.31, 17.90; ESI MS calculated 399.1, found 400.1 ($M+H^+$).

Dimethyl 4-(7-methoxy-2-oxo-2*H*-chromen-4-yl)-2,6-dimethylpyridine-3,5-dicarboxylate (2). NO gas was Bubbled through a CH_2Cl_2 solution containing **1** (0.2 g, 0.5 mmol) at room temperature and the reaction was monitored by TLC. The reaction mixture was sequentially washed with saturated $NaHCO_3$ and NaCl solutions, and dried over anhydrous $MgSO_4$ and evaporated in vacuo. The resulting residue as crude product was purified by flash chromatography with a mixture ethyl acetate and

petroleum ether (1:1) as eluent to afford **2** as yellowish crystalline (0.19 g, yield 95%). Mp. 193～195℃, ^{1}H NMR (400 MHz $CDCl_3$) δ 6.96 (d, 1H, J = 8.8 Hz), 6.85 (d, 1H, J = 2.3 Hz), 6.78 (m, 1H, J = 2.4 Hz), 6.08 (s, 1H), 3.87 (s, 3H), 3.57 (s, 6H), 2.68 (s, 6H).. ^{13}C NMR (400 MHz DMSO-d_6) δ 166.25, 162.72, 159.12, 156.93,154.60, 151.35, 140.23, 127.47, 124.70, 112.59, 111.15, 111.10, 100.87, 56.00, 52.47, 23.18; ESI MS calculated 397.1, found 398.3 ($M+H^+$).

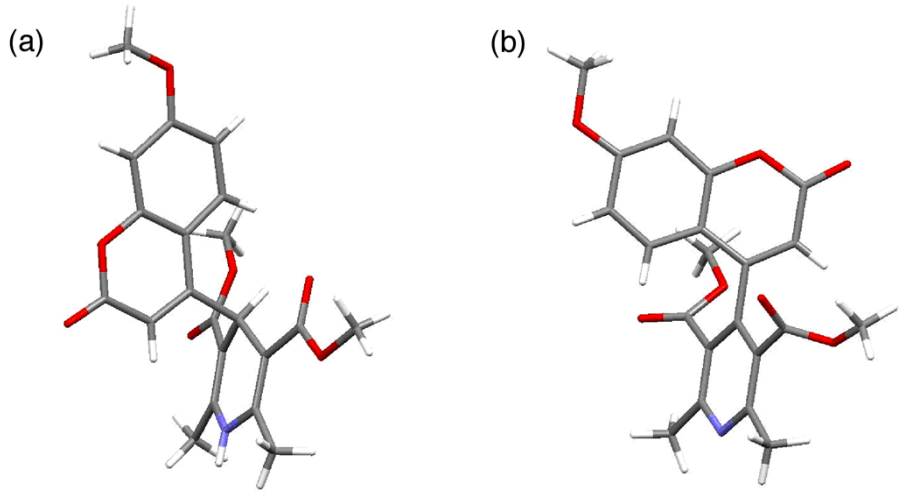


Figure S1. Crystal structures of (a) **1** and (b) **2**.

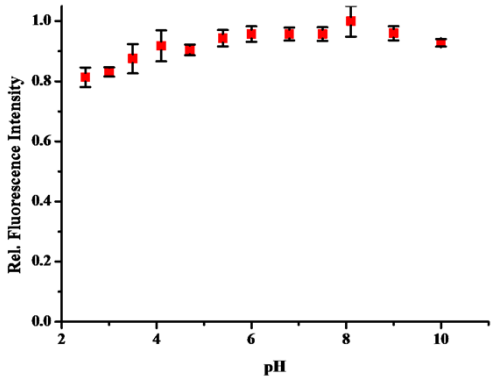


Figure S2. The pH dependence of the fluorescence intensity of compound **2** (10 μM) in phosphate buffer solution with 20% DMSO. λ_{ex} = 334 nm, λ_{em} = 450 nm.

Calculation of the detection limit

The detection limit was calculated on the basis of literature previously reported.[3] The fluorescence emission spectrum of probe **1** was determined by three times and the standard of blank measurement was achieved.

The detection limit was calculated by following formula:

$$\text{Detection limit} = 3s/k$$

Where s is the standard deviation of blank measurement, k is the slope between the fluorescence intensity ratio versus NO concentration.[4]

3 B. Zhu, C. Gao, Y. Zhao, C. Liu, Y. Li, Q. Wei, Z. Ma, B. Du and X. Zhang, *Chem. Commun.* 2011, **47**, 8656-8658.

4 H. Yu, Y. Xiao, L. Jin, *J. Am. Chem. Soc.* 2012, **134**, 17486-17489.

Calculation methods

In this work, the quantum chemical calculations were carried out using the Gaussian 09 program package.[5] The possible ground state structures of probe **1** and compound **2** have been optimized with density functional theory (DFT) at B3LYP/6-31+G (d) level,[6] in which the effects of solvent were considered using polarized continuum model (PCM)[7] with 50 mM phosphate buffer (pH 7.4, 20% DMSO) as solvent. On the basis of optimized configuration for the ground state, TD-DFT[8] calculations were performed using the B3LYP functional (TD-B3LYP-SCRF) within the adiabatic approximation to predict the excitation energies. In order to account for fluorescence properties, TD-B3LYP-SCRF calculations have also been performed to locate the excited state structures of probe **1** and compound **2**.

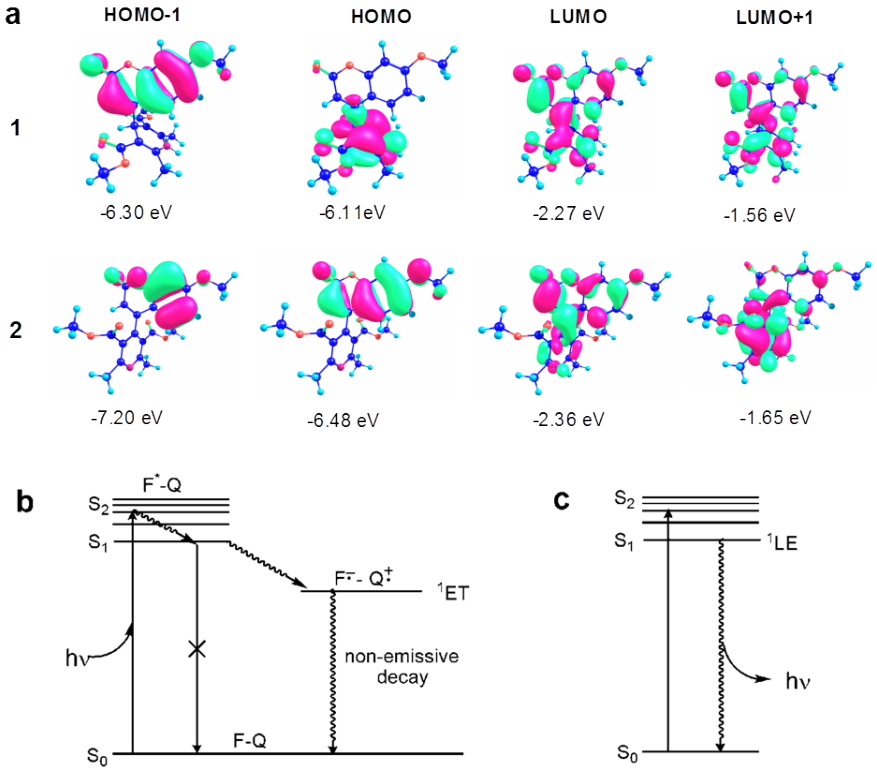


Figure S3. (**a**). Molecular orbital plots for the HOMO-1, HOMO, LUMO and LUMO+1 of **1** and **2**; (**b**). Jablonski diagram for PET pathways of **1;** (c) Jablonski diagram for a local excited state (^{1}LE) emission of **2**. ^{1}ET, electron transfer state.

Table S1. The energy level of selected molecular orbital of **4**, **1** and **2**

Compound	HOMO-1 (eV)	HOMO (eV)	LUMO (eV)	LUMO+1 (eV)
4		-6.38	-2.20	
1	-6.30	-6.11	-2.27	-1.56
2	-7.20	-6.48	-2.36	-1.65

5 M. J. Frisch, G. W. Trucks, H. B. Schlegel, G. E. Scuseria, M. A. Robb, J. R. Cheeseman, G. Scalmani, V. Barone, B. Mennucci, G. A. Petersson, H. Nakatsuji, M. Caricato, X. Li, H. P. Hratchian, A. F. Izmaylov, J. Bloino, G. Zheng, J. L. Sonnenberg, M. Hada, M. Ehara, K. Toyota, R. Fukuda, J. Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H. Nakai, T. Vreven, J. A. Montgomery, Jr., J. E. Peralta, F. Ogliaro, M. Bearpark, J. J. Heyd, E. Brothers, K. N. Kudin, V. N. Staroverov, T. Keith, R. Kobayashi, J. Normand, K. Raghavachari, A. Rendell, J. C. Burant, S. S. Iyengar, J. Tomasi, M. Cossi, N. Rega, J. M. Millam, M. Klene, J. E. Knox, J. B. Cross, V. Bakken, C. Adamo, J. Jaramillo, R. Gomperts, R. E. Stratmann, O. Yazyev, A. J. Austin, R. Cammi, C. Pomelli, J. W. Ochterski, R. L. Martin, K. Morokuma, V. G. Zakrzewski, G. A. Voth, P. Salvador, J. J. Dannenberg, S. Dapprich, A. D. Daniels, O. Farkas, J. B. Foresman, J. V. Ortiz, J. Cioslowski, D. J. Fox, *Gaussian 09, Gaussian, Inc.*, Wallingford CT, 2010.

6 A. D. Becke, *J. Chem. Phys.* 1993, **98**, 5648-5652.

7 (a) M. Cossi, V. Barone, R. Cammi, J. Tomasi, *Chem. Phys. Lett.* 1996, **255**, 327–335; (b) G. Scalmani, M. J. Frisch, *J. Chem. Phys.* 2010, **132**, 114110-114124.

8 (a) F. Furche and R. Ahlrichs, *J. Chem. Phys.*, 2002, **117**, 7433-7447; (b) G. Scalmani, M. J. Frisch, *J. Chem. Phys.* 2006, **124**, 094107:1-15.

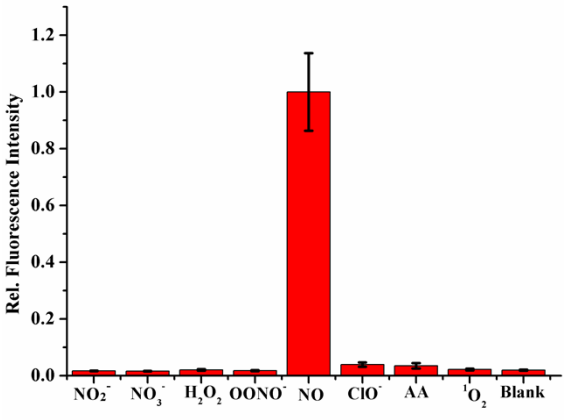


Figure S4. Fluorescence intensity of probe **1** (10 μM, in phosphate buffer 50 mM, pH 7.4) at 450 nm upon the addition of 100 equivalents of various oxidative species: NO, ascorbic acid (AA), H_2O_2, $^1O_2$, KNO_3, $NaNO_2$, $OONO^-$, ClO^-. λ_{ex} = 334 nm.

Cytotoxicity of probe 1 and compound 2

HeLa cells were grown in 96-well plates at an initial density 5×10^3 cells per well for 24 h. Subsequently, the 10 μM, 40 μM and 80 μM of probe **1** and compound **2** were incubated for 12 h, respectively. Cell viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazoilum bromide (MTT) reduction assay. After incubation, MTT (20 μL, 5 mg mL^{-1}) assay was added to each well for 4 h. DMSO (100 μL) was added to each well after removing media. Absorption at 490 nm was measured on a plate reader.

Raw 264.7 cells were grown in 96-well plates at an initial density 5×10^3 cells per well for 24 h. Subsequently, the 10 μM, 40 μM and 80 μM of probe **1** and compound **2** were incubated for 12 h, respectively. Cell viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazoilum bromide (MTT) reduction assay. After incubation, MTT (20 μL, 5 mg mL^{-1}) assay was added to each well for 4 h of. DMSO (100 μL) was added to each well after removing media. Absorption at 490 nm was measured on a plate reader.

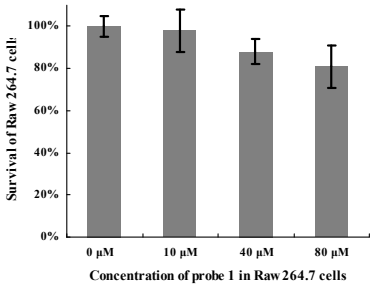


Figure S5. MTT assay of Raw 264.7 cells treated with probe **1**

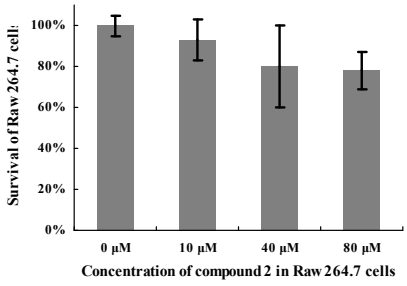


Figure S6. MTT assay of Raw 264.7 cells treated with compound **2**

NO imaging in HeLa cells

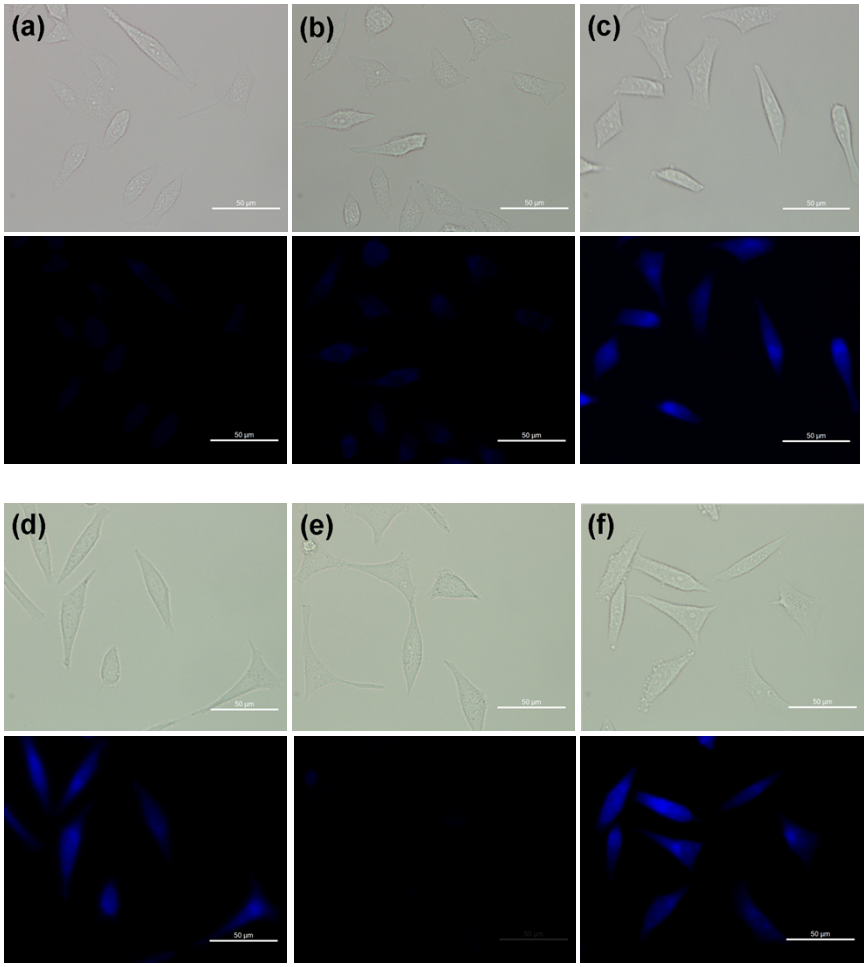


Figure S7. Fluorescence response of probe **1** in HeLa cells. The fluorescence response were monitored for cells after 150 min of incubation with probe **1** (40 μM) **(a)** and for cells co-treated with SNP (2 mM) and probe **1** (40 μM) for 30 min **(b)** , 60 min **(c)**, 90 min **(d)**, 120 min **(e)**, 150 min **(f)**. Images were taken with a Zeiss Abserver A1 inverted fluorescence microscope after removing DMEM and washing the cells three times with PBS.

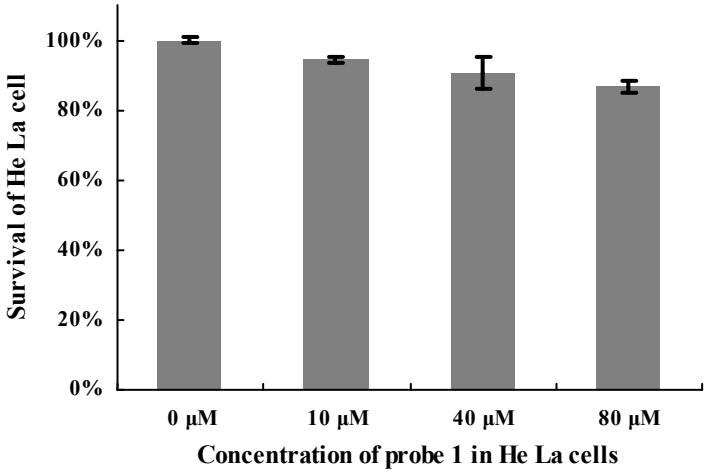


Figure S8. MTT assay of HeLa cells treated with probe **1**

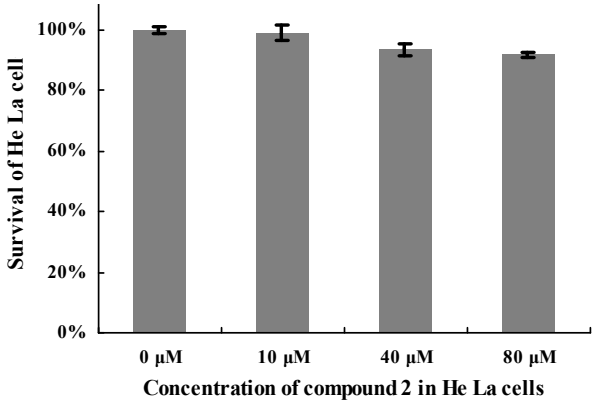
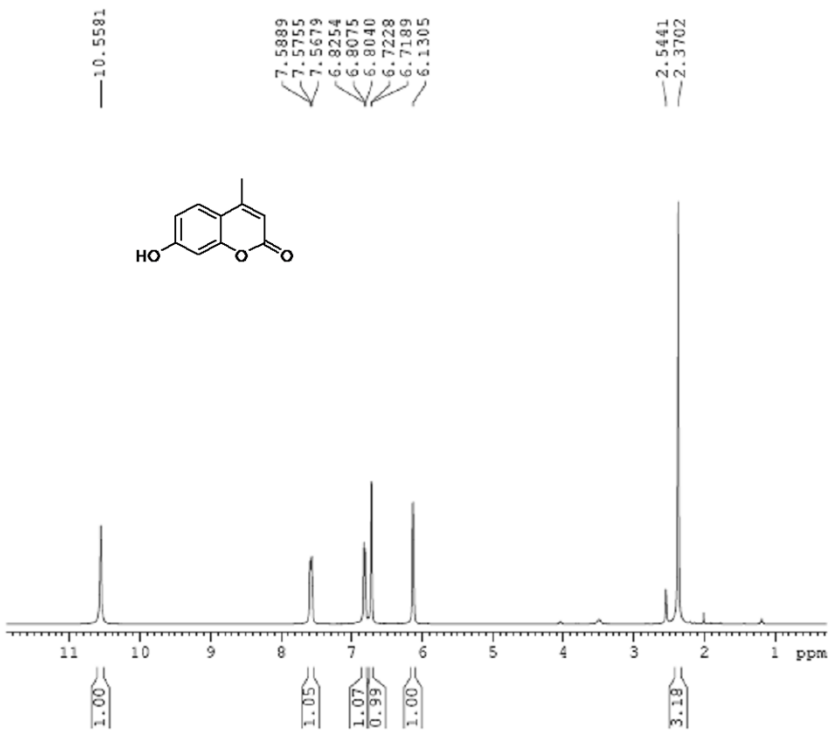


Figure S9. MTT assay of HeLa cells treated with compound **2**

Appendix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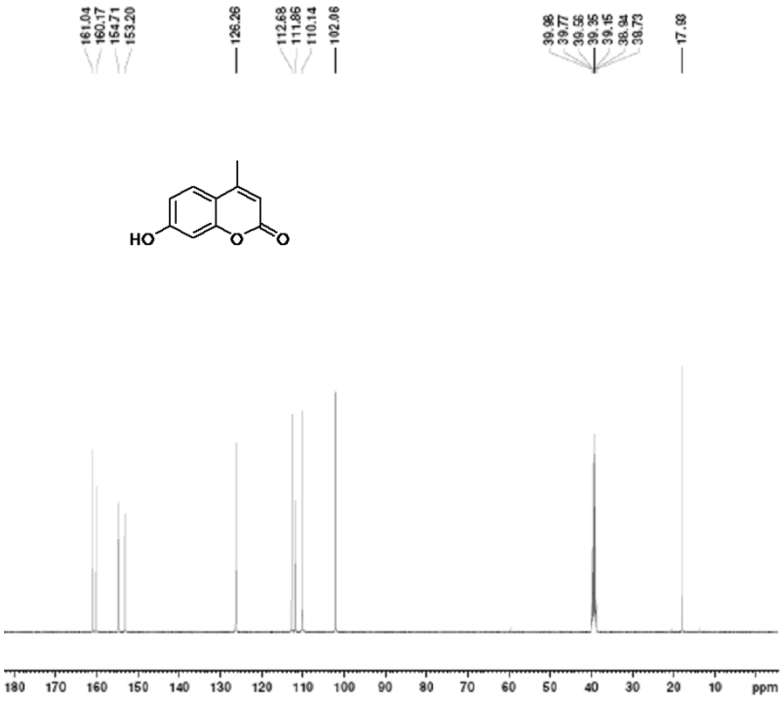
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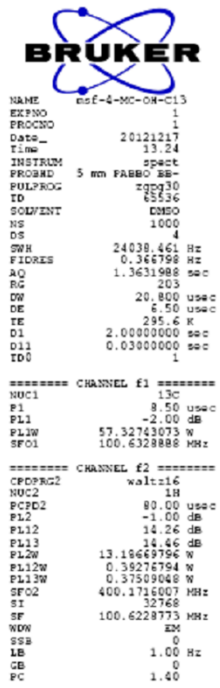
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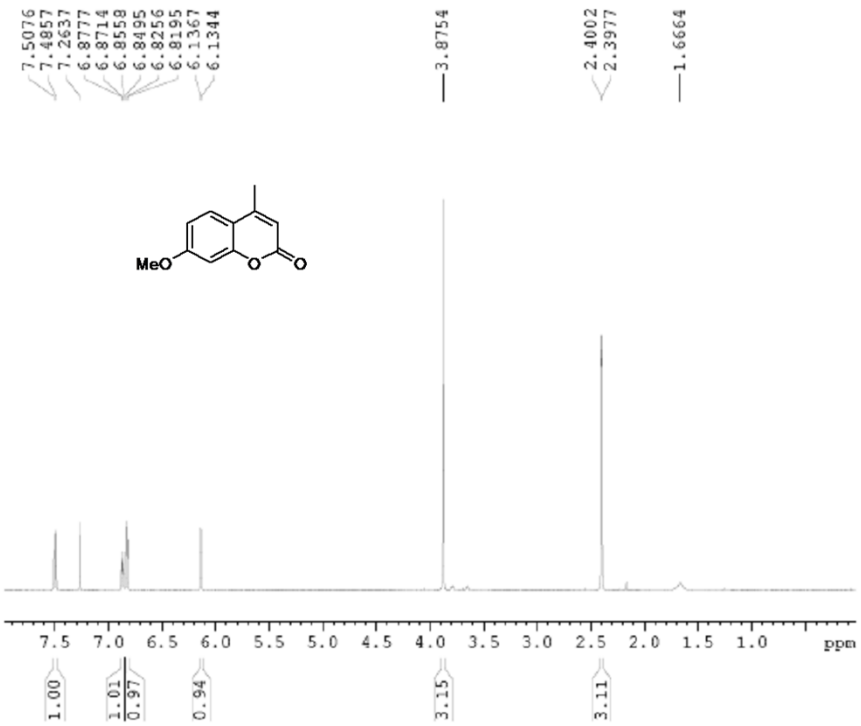

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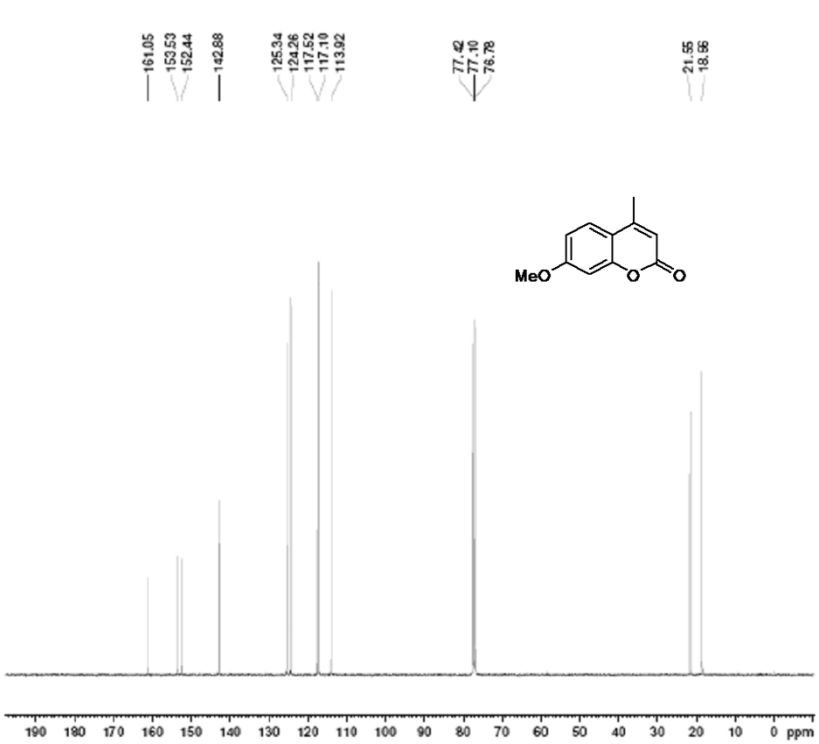

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PC                    1.40
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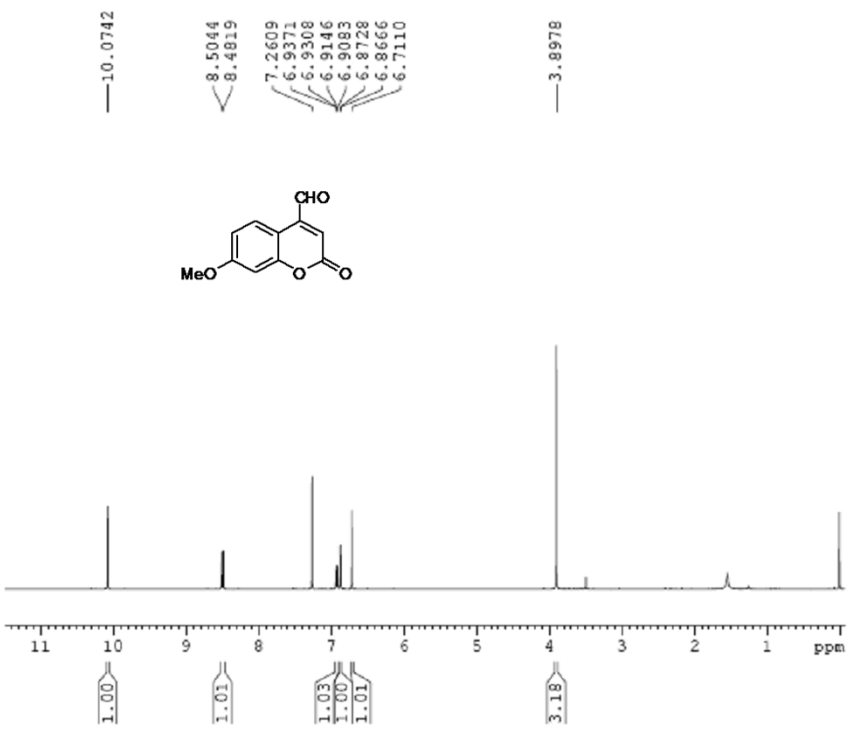
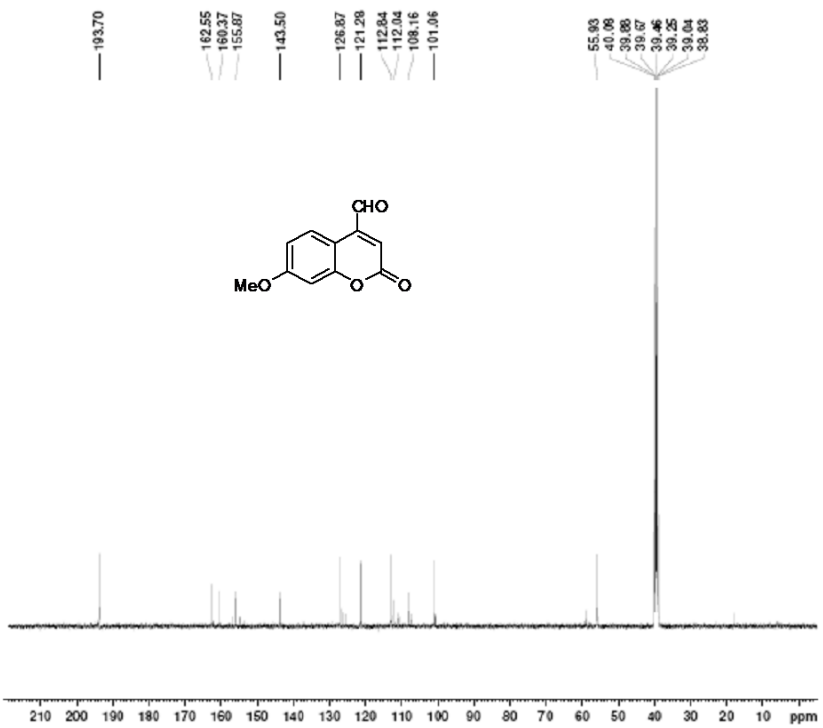
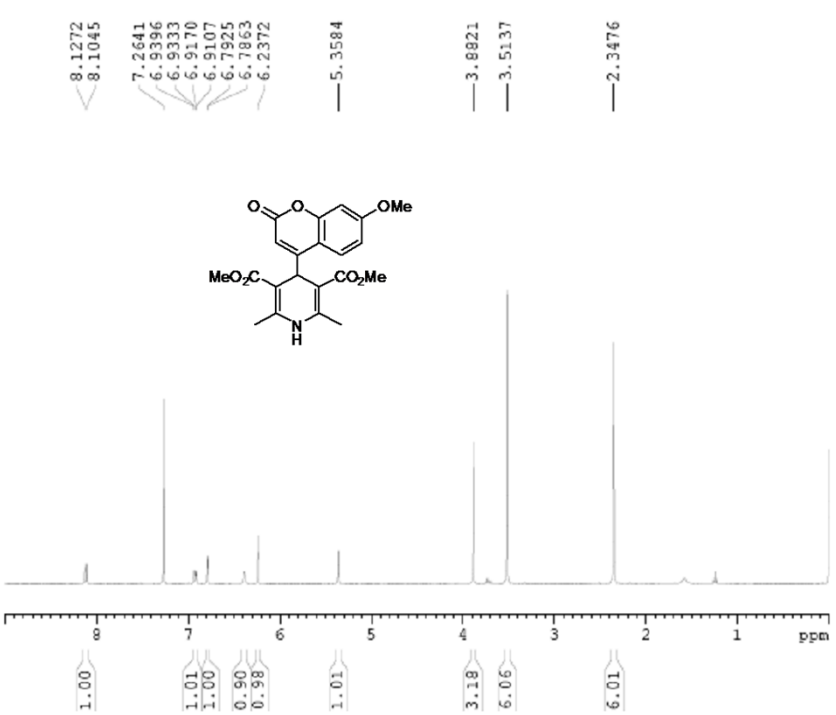


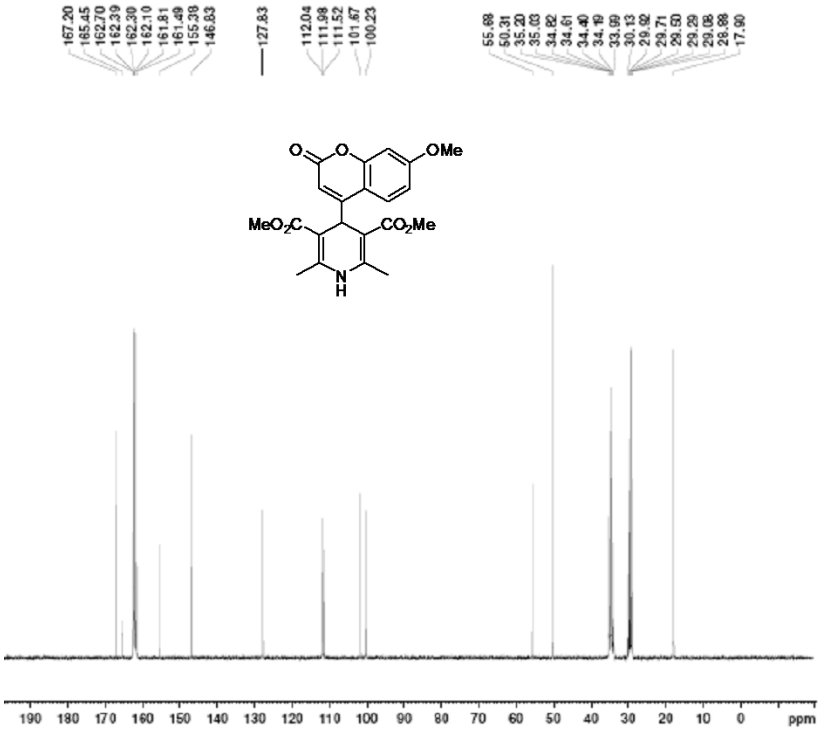
```
NAME                zgq-xhc-2
EXPNO                       1
PROCNO                      1
Date_                20120727
Time                    14.37
INSTRUM                 spect
PROBHD   5 mm PABBO BB-
PULPROG                 zg30
TD                     65536
SOLVENT                CDCl3
NS                        16
DS                         2
SWH                 8223.685 Hz
FIDRES              0.125493 Hz
AQ                  3.9846387 sec
RG                       203
DW                  60.800 usec
DE                   6.50 usec
TE                   300.0 K
D1                1.00000000 sec
TD0                         1

======== CHANNEL f1 ========
NUC1                       1H
P1                      13.80 usec
PL1                     -1.00 dB
PL1W              13.18669796 W
SFO1              400.1724712 MHz
SI                       32768
SF                400.1700032 MHz
WDW                        EM
SSB                         0
LB                       0.30 Hz
GB                          0
PC                       1.00
```



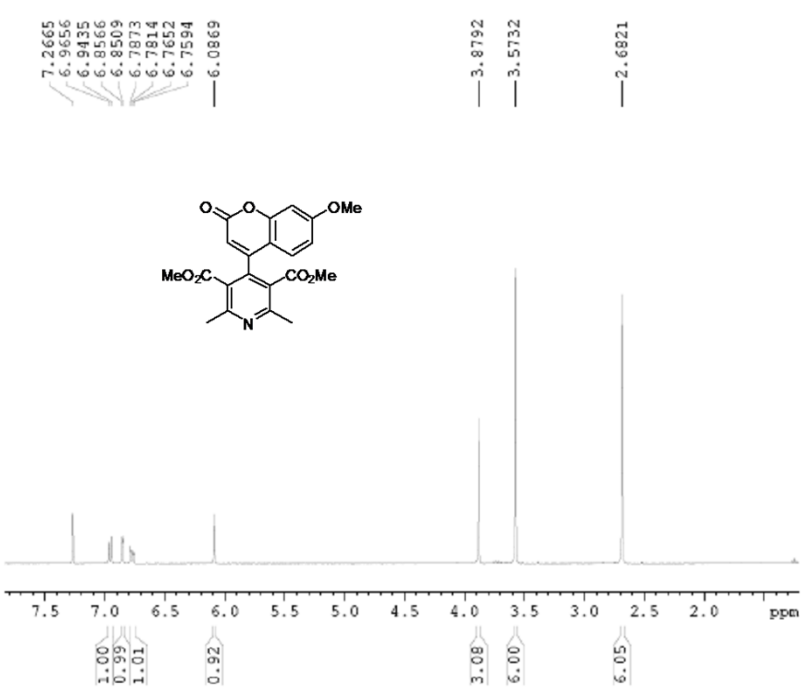
```
NAME          mzf-4-MC-CHO-C13
EXPNO                       1
PROCNO                      1
Date_                20121217
Time                    11.32
INSTRUM                 spect
PROBHD   5 mm PABBO BB-
PULPROG                 zgpg30
TD                     65536
SOLVENT                 DMSO
NS                       1000
DS                          4
SWH                24038.461 Hz
FIDRES              0.366798 Hz
AQ                  1.3631988 sec
RG                       203
DW                  20.800 usec
DE                   6.50 usec
TE                   293.5 K
D1                2.00000000 sec
D11               0.03000000 sec
TD0                         1

======== CHANNEL f1 ========
NUC1                       13C
P1                       9.50 usec
PL1                     -2.00 dB
PL1W              57.32743073 W
SFO1              100.6328888 MHz

======== CHANNEL f2 ========
CPDPRG2                waltz16
NUC2                       1H
PCPD2                   80.00 usec
PL2                     -1.00 dB
PL12                    14.26 dB
PL13                    14.46 dB
PL2W              13.18669796 W
PL12W              0.38276794 W
PL13W              0.37509048 W
SFO2              400.1716007 MHz
SI                       32768
SF                100.6228773 MHz
WDW                        EM
SSB                         0
LB                       1.00 Hz
GB                          0
PC                       1.40
```




```
NAME          msf-12914-c4dhp
EXPNO                       1
PROCNO                      1
Date_              20120914
Time                   11.06
INSTRUM                spect
PROBHD   5 mm PABBO BB-
PULPROG                zg30
TD                    65536
SOLVENT               CDCl3
NS                       16
DS                        2
SWH               8223.685 Hz
FIDRES            0.125493 Hz
AQ                3.9846387 sec
RG                      203
DW                 60.800 usec
DE                  6.50 usec
TE                 298.1 K
D1                1.00000000 sec
TD0                       1

======== CHANNEL f1 ========
NUC1                    1H
P1                  13.90 usec
PL1                 -1.00 dB
PL1W          13.18669796 W
SFO1          400.1732712 MHz
SI                    32768
SF            400.1700019 MHz
WDW                     EM
SSB                      0
LB                  0.30 Hz
GB                       0
PC                  1.00
```

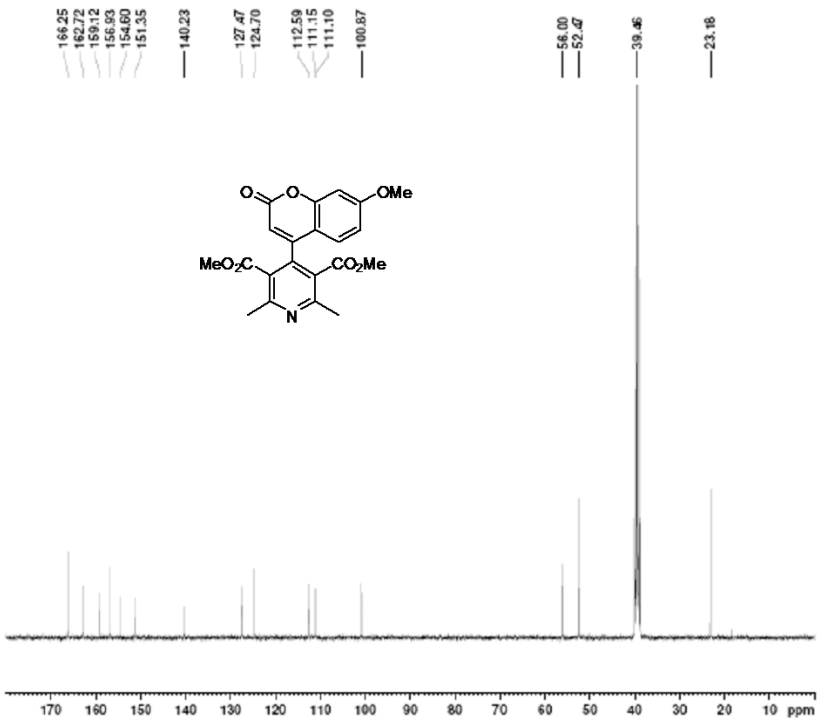



```
NAME          msf-CMeDHP-1-C13
EXPNO                       1
PROCNO                      1
Date_              20121122
Time                    6.28
INSTRUM                spect
PROBHD   5 mm PABBO BB-
PULPROG             zgpg30
TD                    65536
SOLVENT                 DMF
NS                       1024
DS                          4
SWH              24038.461 Hz
FIDRES            0.366798 Hz
AQ                1.3631988 sec
RG                      203
DW                 20.800 usec
DE                  6.50 usec
TE                 300.0 K
D1                2.00000000 sec
D11               0.03000000 sec
TD0                       1

======== CHANNEL f1 ========
NUC1                    13C
P1                   8.50 usec
PL1                 -2.00 dB
PL1W          57.32743073 W
SFO1          100.6328888 MHz

======== CHANNEL f2 ========
CPDPRG2             waltz16
NUC2                    1H
PCPD2               80.00 usec
PL2                 -1.00 dB
PL12                14.26 dB
PL13                14.46 dB
PL2W          13.18669796 W
PL12W          0.39276794 W
PL13W          0.37509049 W
SFO2          400.1716007 MHz
SI                    32768
SF            100.6228270 MHz
WDW                     EM
SSB                      0
LB                  1.00 Hz
GB                       0
PC                  1.40
```



BRUKER

```
NAME                msf-116
EXPNO                     1
PROCNO                    1
Date_              20121106
Time                   9.07
INSTRUM                spect
PROBHD   5 mm PABBO BB-
PULPROG                zg30
TD                    65536
SOLVENT               CDCl3
NS                       16
DS                        2
SWH               8223.685 Hz
FIDRES            0.125493 Hz
AQ                3.9846397 sec
RG                      203
DW                 60.800 usec
DE                  6.50 usec
TE                 293.9 K
D1              1.00000000 sec
TD0                       1

======== CHANNEL f1 ========
NUC1                    1H
P1                 13.90 usec
PL1                -1.00 dB
PL1W          13.18669796 W
SFO1          400.1724712 MHz
SI                    32768
SF            400.1700009 MHz
WDW                     EM
SSB                      0
LB                  0.30 Hz
GB                       0
PC                  1.00
```



BRUKER

```
NAME              msf-7-MC-Py-C13
EXPNO                     1
PROCNO                    1
Date_              20121221
Time                  11.44
INSTRUM                spect
PROBHD   5 mm PABBO BB-
PULPROG             zgpg30
TD                    65536
SOLVENT                DMSO
NS                     1000
DS                        4
SWH               24038.461 Hz
FIDRES            0.366798 Hz
AQ                1.3631988 sec
RG                      203
DW                 20.800 usec
DE                  6.50 usec
TE                 293.7 K
D1              2.00000000 sec
D11             0.03000000 sec
TD0                       1

======== CHANNEL f1 ========
NUC1                   13C
P1                  8.50 usec
PL1                -2.00 dB
PL1W          57.32743073 W
SFO1          100.6328888 MHz

======== CHANNEL f2 ========
CPDPRG2             waltz16
NUC2                    1H
PCPD2              80.00 usec
PL2                -1.00 dB
PL12               14.26 dB
PL13               14.46 dB
PL2W          13.18669796 W
PL12W          0.38276794 W
PL13W          0.37509048 W
SFO2          400.1716007 MHz
SI                    32768
SF            100.6228773 MHz
WDW                     EM
SSB                      0
LB                  1.00 Hz
GB                       0
PC                  1.40
```