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Electronic Supplementary Information

Systematic study on discrepancy of fluorescent properties between in solutions and in cells: Super-bright, environment-insensitive benzocoumarin dyes

Ye Jin Reo,[‡a] Yong Woong Jun,*[‡b] Seo Won Cho,[a] Jinseong Jeon,[a] Hajung Roh,[cd] Subhankar Singha,[a] Mingchong Dai,[a] Sourav Sarkar,[a] Hye Rim Kim,[a] Sojeong Kim,[ac] Yusung Jin,[c] Yun Lim Jung,[a] Yun Jae Yang,[a] Changil Ban,[a] Jinmyoung Joo,*[ce] and Kyo Han Ahn*[a]

[a]Department of Chemistry, Pohang University of Science and Technology (POSTECH), 77 Cheongam-Ro, Nam-Gu, Pohang, Gyungbuk 37673, Republic of Korea

[b]Department of Chemistry, Stanford University, Stanford, CA 94305, USA

[c]Department of Convergence Medicine, Biomedical Engineering Research Center, Asan Institute for Life Science, Asan Medical Center, University of Ulsan College of Medicine, 88 Olympic-Ro 43-gil, Songpa-Gu, Seoul 05505, Republic of Korea

[d]Department of Chemical Engineering, Pohang University of Science and Technology (POSTECH), 77 Cheongam-Ro, Nam-Gu, Pohang, Gyungbuk 37673, Republic of Korea

[e]Department of Biomedical Engineering, School of Life Science, Ulsan National Institute of Science and Technology (UNIST), 50 UNIST-gil, Ulsan 44919, Republic of Korea

[‡]These authors contributed equally.

General information.

The chemical reagents were purchased from Aldrich or TCI. Commercially available reagents were used without further purification. Anhydrous solvents for organic synthesis were prepared by passing through a solvent purification tower. All reactions were performed under argon atmosphere unless otherwise stated. Thin-layer chromatography was performed on pre-coated silica gel 60F-254 glass plates. ^{1}H and ^{13}C NMR spectra were measured with a Bruker AVANCE III 300MHz, AVANCE III 500MHz and AVANCE III 600 MHz FT-NMR spectrometer. Coupling constants (J value) are reported in Hertz. The chemical shifts (δ) are shown in ppm, multiplicities are indicated by s (singlet), d (doublet), t (triplet), dd (doublet of doublets) and m (multiplet). Spectra are referenced to residual chloroform (7.26 ppm, 1H, 77.16 ppm, ^{13}C). UV/Vis absorption spectra were obtained using a HP 8453 UV/Vis spectrophotometer. Fluorescence emission spectra were recorded on a Photon Technical International Fluorescence System using a 1 cm standard quartz cell. High-resolution mass spectra was recorded on a JEOL JMS-700 spectrometer at the Korea Basic Science Center, Kyungpook National University and the values are reported in units of mass to charge (*m/z*).

Synthesis

8-Methylamino-4,4-di-fluro-4-bora-3a, 4a-diaza-s-indacene (BODIPY). This compound was synthesized by following the reported procedure.[1]

5-(Dimethylamino)-N-methylnaphthalene-1-sulfonamide (Dansyl). This compound was synthesized by following the reported procedure.[2]

2-(2-Hydroxyethyl)-6-(methylamino)-1H-benzo[*de*]isoquinoline-1,3(2H)-dione (Naphthalimide). This compound was synthesized by following the reported procedure.[3]

N-Methyl-7-nitrobenzo[*c*][1,2,5]oxadiazol-4-amine (NBD). This compound was synthesized by following the reported procedure.[4]

1-(6-(Dimethylamino)naphthalen-2-yl)ethanone (Acedan). This compound was synthesized by following the reported procedure.[5]

1,3,3-Trimethyl-2-((*E*)-2-((*E*)-2-(methylamino)-3-((*E*)-2-(1,3,3-trimethylindolin-2-ylidene)ethylidene)cyclohex-1-enyl)vinyl)-3H-indolium (Cy7). This compound was synthesized by following the reported procedure.[6]

Methyl 7-(diethylamino)-2-oxo-2H-chromene-3-carboxylate (Coumarin). This compound was synthesized by following the reported procedure.[7]

(*Z*)-3-(1-Hydroxy-3-oxobut-1-enyl)-8-(methylamino)-2H-benzo[*g*]chromen-2-one (BC 1). A solution of 6-methylamino-3-hydroxy-2-naphthaldehyde[8] (100 mg, 0.50 mmol), 4-hydroxy-6-methyl-2-pyrone (75 mg, 0.60 mmol), and benzyltriethylammonium chloride (23 mg, 0.01 mmol) in ethanol (5 mL) was refluxed for 4 h. The reaction mixture was concentrated by rotary-evaporator and the residue was purified by column chromatography (eluent: EtOAc/CH_2Cl_2 = 2/98) to afford **BC 1** as a red solid (110 mg, 72%): ^{1}H NMR (300 MHz, $CDCl_3$, 296 *K*): δ 8.70 (s, 1H), 7.94 (s, 1H), 7.68 (d, J = 9.0 Hz, 1H), 7.42 (s, 1H), 7.03 (s, 1H), 6.84 (dd, J = 9.0, 2.4 Hz, 1H), 6.71 (d, J = 2.1 Hz, 1H), 4.35 (br, 1H), 2.99 (d, J = 5.1 Hz, 3H), and 2.26 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$, 298 *K*): δ 198.72, 173.94, 159.13, 151.78, 150.05, 146.43, 139.30, 131.05, 130.62, 124.87, 118.92, 118.11, 115.16, 109.81, 102.07, 101.33, 30.53, and 27.47. HRMS (EI positive): *m/z*, calculated for $C_{18}H_{15}NO_4$ $[M]^+$ 309.10; found 309.10.

3-Benzoyl-8-(methylamino)-2H-benzo[*g*]chromen-2-one (BC 2). A solution of 6- methylamino-3-hydroxy-2-naphthaldehyde[8] (50 mg, 0.21 mmol) and ethyl benzoylacetate (43 µL, 0.25 mmol) in ethanol (2 mL) was treated with piperidine (1 drop). After being refluxed for 2 h, the reaction mixture wash filtered and washed with ethanol to afford **BC 2** as a red solid (62 mg, 91%): ^{1}H NMR (DMSO, 500 MHz, 298 *K*): δ 8.41 (d, J = 9.5 Hz, 2H), 8.17 (d, J = 9.5 Hz, 2H), 7.89 (t, J = 8.0 Hz ,1H), 7.70 (m, 2H), 7.52 (m, 3H), 7.00 (t, J = 8.0 Hz, 1H), 7.76 (d, J = 4.0 Hz, 1H), and 6.71 (d, J = 7.5 Hz, 1H). ^{13}C NMR (150 MHz, DMSO, 298 *K*): δ 192.39, 159.05, 151.85, 151.04, 147.21, 139.12, 137.34, 133.85, 131.37, 130.38, 129.82 (two carbons), 129.04 (two carbons), 123.99, 123.06, 119.41, 113.92, 109.02, 100.61, and 29.81. HRMS (EI positive): *m/z*, calculated for $C_{21}H_{15}NO_3$ $[M]^+$ 329.11; found 329.11.

3-(Benzo[*d*]thiazol-2-yl)-8-hydroxy-2H-benzo[g]chromen-2-one (BC 3). This compound was synthesized by following the reported procedure.[9]

3-(Benzo[*d*]thiazol-2-yl)-8-(methylamino)-2H-benzo[*g*]chromen-2-one (BC 4). A solution of 6-methylamino-3-hydroxy-2-naphthaldehyde[8] (50 mg, 0.25 mmol) and ethyl 2-(benzo[*d*]thiazol-2-yl)acetate (52 µL, 0.30 mmol) in ethanol (2 mL) was treated with piperidine (1 drop). After being refluxed for 2 h, the reaction mixture wash filtered and washed with ethanol to give **BC 4** as a red solid (80 mg, 90%): ^{1}H NMR (500 MHz, DMSO, 298 *K*): δ 9.19 (s, 1H), 8.36 (s, 1H), 8.16 (d, J = 8.0 Hz , 1H), 8.07 (d, J = 8.0 Hz , 1H), 7.76 (d, J = 9.0 Hz , 1H), 7.56 (m, 2H), 7.45 (m, 1H), 7.02 (dd, J = 1.5, 7.0 Hz, 1H), 6.84 (d, J = 5.0 Hz , 1H), 6.72 (d, J = 1.5 Hz ,1H), and 2.84 (d, J = 5.0 Hz, 3H). ^{13}C NMR (125 MHz, DMSO, 298 *K*): δ 160.88, 160.46, 152.48, 151.08 (two carbons), 143.13, 139.10, 136.20, 131.70, 130.45, 126.95, 125.45, 124.25, 122.67, 122.53, 119.51, 116.10, 114.50, 108.95, 100.65, and 29.76. HRMS (EI positive): *m/z*, calculated for $C_{21}H_{14}N_2O_2S$ $[M]^+$ 358.08; found 358.08.

4-(8-Methylamino-2-oxo-2H-benzo[*g*]chromen-3-yl)phenyl acetate (BC 5). A solution of 6-allylamino-3-hydroxy-2-naphthaldehyde[8] (100 mg, 0.41 mmol) and 2-(4-acetoxyphenyl)acetic acid (120 mg, 0.62 mmol) in dichloromethane (4 mL) was treated with triethylamine (290 µL, 2.07 mmol). After being stirred for 10 min, the solution was further treated with 1-(3- dimethylaminopropyl)-3-ethylcarbodiimide (EDC, 97 mg, 0.62 mmol) and 1-hydroxy-benzotriazole (HOBt, 95 mg, 0.62 mmol). The resulting mixture, after being stirred for 12 h at room temperature, was subjected to extraction with dichloromethane. The organic layer was washed with brine, dried over anhydrous Na_2SO_4, and concentrated. The residue was purified by silica gel column chromatography (eluent: MeOH/CH_2Cl_2 = 3/97) to afford **BC 5** as a yellow solid (120 mg, 76%). ^{1}H NMR (300 MHz, $CDCl_3$, 298 *K*): δ 7.85 (d, J = 5.7 Hz, 2H), 7.75 (dd, J = 2.1, 4.5 Hz, 2H), 7.67 (d, J = 9.0 Hz ,1H), 7.45 (s, 1H), 7.18 (d, J = 8.7 Hz , 1H), 6.89 (d, J = 2.4, 6.6 Hz, 1H), 6.77 (d, J = 2.1 Hz, 1H), 6.02 (m, 1H), 5.36 (dd, J = 1.5, 15.9 Hz, 1H), 5.25 (dd, J = 1.5, 9.0 Hz, 1H), 4.27 (s, 1H), 3.94 (d, J = 3.6 Hz, 2H), and 2.33 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$, 298 *K*): δ 169.60, 161.21, 151.37, 151.03, 147.81, 140.62, 137.38, 134.55, 133.16, 129.99 (two carbons), 128.27, 125.31, 124.82, 121.79 (three carbons), 118.64, 117.16, 116.41, 110.04, 103.23, and 46.35. HRMS (EI positive): *m/z*, calculated for $C_{24}H_{19}NO_4$ $[M]^+$ 385.13; found 385.13.

Methyl 8-(methylamino)-2-oxo-2H-benzo[*g*]chromene-3-carboxylate (BC 6). A solution of 6-methylamino-3-hydroxy-2-naphthaldehyde[8] (20 mg, 0.09 mmol) and diethyl malonate (15 µL, 0.10mmol) in ethanol (1 mL) was treated with piperidine (1 drop). After being refluxed for 4 h, the solvent was concentrated by rotary-evaporator and the residue was subjected to column chromatography (eluent: EtOAc/hexane = 5/95) to afford **BC 6** as orange solid (22 mg, 80%): ^{1}H NMR (500 MHz, $CDCl_3$, 298 *K*): δ 8.63 (s, 1H), 7.93 (s, 1H), 7.77 (d, J = 9.0 Hz, 1H), 7.40 (s, 1H), 7.14 (dd, J = 2.5, 7.0 Hz ,1H), 6.79 (d, J = 2.5 Hz, 1H), 3.96 (s, 3H), and 3.16 (s, 6H).). ^{13}C NMR (125 MHz, $CDCl_3$, 298 *K*): δ 164.60, 157.86, 150.99, 150.12, 138.98, 131.16, 130.63, 123.78 (two carbons), 116.34, 114.76, 114.47, 109.80, 104.18, 52.87, and 40.51 (two carbons).HRMS (EI positive): *m/z*, calculated for $C_{17}H_{15}NO_4$ $[M]^+$ 297.10; found 297.10.

4-(8-(Allyl(methyl)amino)-2-oxo-2H-benzo[*g*]chromen-3-yl)-1-methylpyridinium trifluoromethanesulfonate (BC 7). The compound was synthesized by following the reported procedure.[10]

1-(8-(Pyrrolidin-1-yl)-2H-benzo[*g*]chromen-3-yl)ethanone (BC 8). A solution of 6-(pyrrolidin-1-yl)-3-hydroxy-2-naphthaldehyde[8] (24 mg, 0.10 mmol) and ethyl acetoacetate (25 mL, 0.20 mmol) in ethanol (3 mL) was treated with piperidine (1 drop). After being stirred for 2 h at room temperature, the reaction mixture was diluted with dichloromethane (15 mL) and washed with water (10 mL). The aqueous layer was extracted with dichloromethane (10 mL), and the combined organic layer was concentrated by rotary-evaporator. The residue was subjected to column chromatography (eluent: EtOAc/CH_2Cl_2 = 5/95) to afford **BC 8** (28 mg, 92%) as a red solid: ^{1}H NMR (300 MHz, $CDCl_3$, 298 K): δ 8.54 (s, 1H), 7.91 (s, 1H), 7.71 (d, J = 9.0 Hz, 1H), 7.34 (s, 1H), 6.96 (dd, J = 9.0, 2.1 Hz, 1H), 6.63 (s, 1H), 3.45 (t, J = 6.3 Hz, 4H), 2.72 (s, 3H), and 2.07 (t, J = 6.6 Hz, 4H). ^{13}C NMR (125 MHz, $CDCl_3$, 298 *K*): δ 195.81, 160.47, 152.18, 148.54, 148.38, 139.12, 132.20, 130.84, 123.20, 121.11, 116.64, 114.26, 109.06, 103.40, 47.95 (two carbons), 30.79, and 25.63 (two carbons). HRMS (EI positive): *m/z*, calculated for $C_{19}H_{17}NO_3$ $[M]^+$ 307.12; found 307.12.

4-(8-(Allylamino)-2-oxo-2H-benzo[*g*]chromen-3-yl)-2,2-difluoro-6-methyl-2H-1,3,2-dioxaborinin-1-ium-2-uide (BC 9). To a solution of (*Z*)-3-(1-hydroxy-3-oxobut-1-enyl)-8-(methylamino)-2H-benzo[*g*]chromen-2-one (50 mg, 0.15 mmol) in toluene (5 ml) was added borontrifluoride etherate (92 µL, 0.75 mmol) at 0 °C. The reaction mixture was stirred at room temperature for 30 min, and then it was diluted with dichloromethane (10 mL) and washed with water. The organic layer was concentrated and the residue was subjected to column chromatography (eluent: CH_2Cl_2) to afford **BC 9** (52 mg, 92% yield) as violet colored solid: ^{1}H NMR (500 MHz, DMSO, 298 *K*): δ 9.11 (s, 1H), 8.43 (s, 1H), 7.43 (d, J = 9.0 Hz ,1H), 7.41 (d, J = 12.5 Hz, 3H), 7.07 (d, J = 9.0 Hz , 1H), 6.77 (s, 1H), 5.96 (m, 1H), 5.29 (d, J = 17.5 Hz, 1H), 5.18 (d, J = 10.0 Hz, 1H), 3.9 (s, 1H), and 2.47 (s, 3H). ^{13}C NMR (75 MHz, DMSO, 298 *K*): δ 193.72, 175.67, 158.06, 151.46, 151.16, 140.58, 134.46, 134.37, 131.41, 123.89,

119.29, 116.19 (two carbons), 114.06, 112.30, 107.97, 101.41, 100.32, 44.72, 24.73.HRMS (EI positive): *m/z*, calculated for $C_{20}H_{16}BF_2NO_4$ $[M]^+$ 383.11; found 383.11.

8-(Allylamino)-3-isonicotinoyl-2H-benzo[*g*]chromen-2-one (BC 10). A solution of 6-allylamino-3-hydroxy-2-naphthaldehyde[8] (50 mg, 0.21 mmol) and ethyl 3-oxo-3-(pyridin-4-yl)propanoate (48 mg, 0.25 mmol) in ethanol (2 mL) was treated with piperidine (1 drop). After being refluxed for 4 h, the reaction mixture was filtered and washed with ethanol to afford **BC 10** as a yellow solid (66 mg, 89%): 1H NMR (500 MHz, DMSO, 298 K): δ 8.00 (d, J = 4.5 Hz, 2H), 7.91 (d, J = 8.5 Hz, 2H), 7.84 (d, J = 8.5 Hz ,1H), 7.61 (s, 1H), 7.33 (d, J = 4.5 Hz, 2H), 7.05 (dd, J = 2.0, 7.0 Hz, 1H), 6.94 (s, 1H), 6.16 (m, 1H), 5.52 (d, J = 17.0 Hz, 1H), 5.41 (d, J = 10.5 Hz, 1H), and 4.09 (d, J = 5.0 Hz, 2H). ^{13}C NMR (75 MHz, DMSO, 298 *K*): δ 191.18, 163.63, 152.05, 149.66, 149.02 (two carbons), 148.27, 138.32, 135.43, 135.35, 129.37, 124.51 (two carbons), 122.22, 121.14 (two carbons), 116.86 (two carbons), 115.70, 115.07, 108.68, 101.56, and 44.99. HRMS (EI positive): *m/z*, calculated for $C_{22}H_{16}N_2O_3$ $[M]^+$ 356.12; found 356.12.

3-Acetyl-8-(methylamino)-2H-benzo[*g*]chromen-2-one (BC 11). A solution of 6-methylamino-3-hydroxy-2-naphthaldehydes[8] (20 mg, 0.10 mmol) and ethyl acetoacetate (25 mL, 0.20 mmol) in ethanol (3 mL) was treated with piperidine (1 drop). After being stirred for 2 h at room temperature, the reaction mixture was diluted with dichloromethane (15 mL) and then washed with water (10 mL). The aqueous layer was extracted with dichloromethane (10 mL), and the combined organic layer was concentrated by rotary-evaporator. The residue was subjected to column chromatography (eluent: EtOAc /CH_2Cl_2 = 5/95) to afford **BC 11** (24 mg, 90%) as a red solid: 1H NMR (300 MHz, $CDCl_3$, 298 *K*): δ 8.58 (s, 1H), 7.94 (s, 1H), 7.67 (d, J = 9.0 Hz, 1H), 7.41 (s, 1H), 6.84 (dd, J = 9.0, 2.4 Hz, 1H), 6.69 (d, J = 2.1 Hz, 1H), 4.38 (br, 1H), 2.99 (d, J = 4.8 Hz, 3H), and 2.73 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$, 298 *K*): δ 191.12, 162.14, 153.95, 150.33, 141.24, 133.72, 132.28, 128.14, 126.42, 120.58 (two carbons), 111.30, 103.46, 102.58, 31.92, and 31.54. HRMS (EI positive): *m/z*, calculated for $C_{16}H_{13}NO_3$ $[M]^+$ 267.09; found 267.09.

Benzyl 6-acetylnaphthalen-2-yl(methyl)carbamate (Probe_Acedan). To a solution of 1-(6-(methylamino)naphthalen-2-yl)ethanone (acedan) (10 mg, 0.05 mmol) in CH_2Cl_2 (3 mL), were added benzyl chloroformate (37 µL, 0.25 mmol), pyridine (40 µL, 0.50 mmol), and DMAP(catalytic amount) at room temperature. The reaction mixture was stirred for 4 h at room temperature, and then it was diluted with dichloromethane (10 mL) and washed with water. The organic layer was concentrated, and the crude mixture was subjected to column chromatography (eluent: EtOAc / CH_2Cl_2 = 1/99) to afford the product (15 mg, 90% yield): 1H NMR (300 MHz, $CDCl_3$, 298 *K*): δ 8.44 (s, 1H), 8.03 (dd, J = 1.2, 7.5 Hz, 1H), 7.93 (d, J = 8.7 Hz ,1H), 7.82 (d, J= 8.7 Hz ,1H), 7.70 (s , 1H), 7.54 (d, J = 8.4 Hz, 1H), 7.33 (s, 5H), 5.22 (s, 2H), 3.45 (s, 3H), and 2.72 (s, 3H).

Benzyl 3-(benzo[*d*]thiazol-2-yl)-2-oxo-2H-benzo[g]chromen-8-ylcarbamate (Probe_BC 4). To a solution of 3-(benzo[*d*]thiazol-2-yl)-8-(methylamino)-2H-benzo[*g*]chromen-2-one (**BC 4**) (10 mg, 0.03 mmol) in a mixed solution of CH_2Cl_2 (4 mL) and DMF (0.1 mL) were added benzyl chloroformate (6 µL, 0.04 mmol), pyridine (23 µL, 0.28 mmol), and DMAP (catalytic amount) sequentially at room temperature. The reaction mixture was stirred for 4 hours at room temperature, and then it was diluted with dichloromethane (10 mL) and washed with water. The organic layer was concentrated, and the crude mixture was subjected to column chromatography (eluent: CH_2Cl_2) to afford the product (7.0 mg, 53% yield): ^{1}H NMR (300 MHz, CDCl3, 298 *K*): δ 9.19 (s, 1H), 8.23 (s, 1H), 8.12 (d, J = 8.1 Hz, 1H), 8.01 (d, J = 7.8 Hz ,1H), 7.95 (d, J = 9.0 Hz ,1H), 7.74 (s , 2H), 7.57 (m, 2H), 7.45 (m, 1H), 7.36 (s, 1H), 5.23 (s, 2H), and 3.48 (s, 3H).

Fluorescence assays of dyes

UV-vis absorption spectra were obtained using a HP 8453 UV-vis spectrophotometer. Fluorescence spectra were recorded on a Photon Technology International Fluorimeter with a 1.0 cm standard quartz cell. The excitation and emission wavelength band paths were both set at 2.0 nm. All of the solvents used were of analytical grade. The solution was allowed to cool down to room temperature before carrying out spectrometric measurements. A stock solution of each of the dyes was prepared in dimethyl sulfoxide (DMSO) at a concentration of 1.0 mM. A required amount of the mixed solution was transferred to a cuvette (1.0 mL) for spectroscopic measurement. In the case of an aqueous solution, it was added to the aqueous solvent by keeping the concentration of DMSO within 1% of the total volume. The final titrant volume is the same for all measurements (1.0 mL).

Cell culturing and imaging

HeLa human cervical cancer cells, HEK93T normal human embryonic kidney cells, MCF-7 human breast cancer cells, and A549 human lung cancer cells were obtained from Korean Cell Line Bank. The cells were maintained in DMEM supplemented with 10% (*v/v*) fetal bovine serum (FBS) and 1% (*w/v*) penicillin-streptomycin (PS) at 37 °C in a humidified atmosphere of 5% CO_2 in air. The cells were passaged when they reached approximately 80% confluence. Cells were seeded onto a cell culture dish at a density of 1×10^5 cells and incubated overnight at 37 °C under 5% CO_2. The cells were incubated in DMEM containing 10 μM of commonly used dyes and new dyes for 30 min at 37 °C under 5% CO_2. The cells were washed with PBS to remove the remaining probe and then fixed with 4% formaldehyde solution. The images of the cells were recorded by confocal laser scanning microscopy. Prepared cells were mounted on a tight-fitting holder. The excitation laser power was approximately 4 mW. The imaging field-of-view (FOV) was 97 × 97 μm consisting of 1024 × 1024 pixels. Acquired images were processed by using LAS AF Lite (Leica, Germany). For confocal fluorescence imaging experiments were performed on Leica TCS SP5 II, which Advanced System was equipped with multiple visible laser lines (405, 458, 476, 488, 496, 514, 561, 594, and 633 nm) and fluorescence signals were obtained through Hyd PMT (Hybrid detector Photo Multiplier Tube; Leica).

Tissue preparation and imaging

The experimental procedures regarding mice tissues herein were performed in accordance with protocols approved by The Pohang University of Science and Technology Committee on Animal Research and we followed the guidelines for the use of experimental animals established by The Korean Academy of Medical Science. We made every effort to minimize animal suffering and reduce the number of animals used to prepare samples for imaging. BALB/c type mice (6 weeks) were used for this experiment. Basically, the experiments were conducted under light protected conditions (in a dark-room and using aluminum foil). The mouse was dissected after dislocation of the cervical vertebra. The five organs (brain, liver, kidney and spleen) were removed and washed with PBS buffer. The tissues were incubated in the PBS buffer solution containing 10 μM of each dye for 24 h in an incubator maintained with 5% of CO_2 in the air and at 37 ˚C. The stained sample was washed with PBS buffer three times to remove the remaining dye on the surface and sliced with a vibrating blade microtome (VT1000S, Leica, Germany) in 50 μm thickness. Each of the sliced samples was placed on a slide glass for imaging, and images of the tissue sample were recorded using two-photon microscopy (2PM). 2PM imaging was performed using a Ti-Sapphire laser (Chameleon Vision II, Coherent) at a 140 fs pulse width and 80 MHz pulse repetition rate (TCS SP5 II, Leica, Germany) through a 20× objective lens (obj. HCX PL APO 20×/1.10 W CORR CS, Leica, Germany). The two-photon excitation wavelength for the dyes was tuned to 900 nm. The tissue samples were prepared as above and were mounted on a tight-fitting holder. The excitation laser power was applied in the compensation manner from 5 to 95 mW in all of the experiments. The spectrum was obtained and processed using the LAS AF Lite (Leica, Germany).

Fluorescence correlation spectroscopy (FCS)

HeLa cells were cultured on a glass-bottom dish (Nunc, ThermoFisher Scientific), and mounted on the confocal laser scanning microscopy (LSM 780, Zeiss, Germany), followed by exposure to 10 μM of dye at 37 °C under 5% CO_2. Fluorescence correlation spectroscopy (FCS) measurements were performed in the live cells at the same condition using a ConfoCor2 as described previously.[11,12] Firstly, solution sample containing Rhodamine 6G as a standard dye was measured for verifying correct FCS optical setup and stable confocal detection volume. After that, cells were imaged using confocal scanning setup, and the FCS collects fluorescent signals from confocal detection volume of the live cells to calculate fluorescence correlation function which contains parameters of molecular concentration and mobility. Fluorescence correlation curves in live cells were measured sequentially 10 times with duration of 10 sec to minimize the photobleaching effect.

Acquired fluorescence correlation curve was fitted with a three-dimensional single-component model to determine the average structure parameters. Diffusion times and translational diffusion coefficients of the dyes obtained from the fitting analysis inform the molecular concentration of dyes in the cell accordingly.

Cell lysate (Lysis)

HeLa cells were seeded onto a well plate at a density of 1×10^5 cells and incubated in phenol red-free DMEM overnight at 37 °C under 5% CO_2. In the following day, the culture media was replaced to phenol red-free DMEM containing 10 μM of each dye for 30min at 37 °C under 5% CO_2. The cells were washed three times with PBS to remove the remaining probe in the culture media (not in the cells), and then the ice-cold lysis buffer (ThermoFisher Scientific) was added to release the dye from the cells body according to the manufacturer's protocols. After 5 min of incubation at 4 °C with periodic mixing, the lysate was transferred to microcentrifuge tubes and centrifuged at 15000 rpm for 10 min at 4 °C to remove cell debris. The supernatant was transferred to a new tube for quantification of the dye presented in the cells. Fluorescence characteristics were measured using a spectrofluorometer (FP-8300, Jasco Corporation, Japan). The concentration of dyes released from the cell was determined using a standard curve, which was obtained under same condition in the lysis buffer with serial dilution of each dye with known concentration.

Cellular Intensity Comparison

HeLa cells stained with each dye were imaged with a confocal microscope equipped with different laser lines. The celluar images were obtained with a laser line that provided the brigtest cellular images and by varying the laser power (in the range of ±10% from its optimum inpur laser power). The cellular fluorescence intensity was extracted from the obtained cellular images, which is then divided by the corresponding laser density measured by the internal instrument with LAS-AF software. Cellular fluorescent intensities were collected from at least 10 different cells per condition.

Figures and table

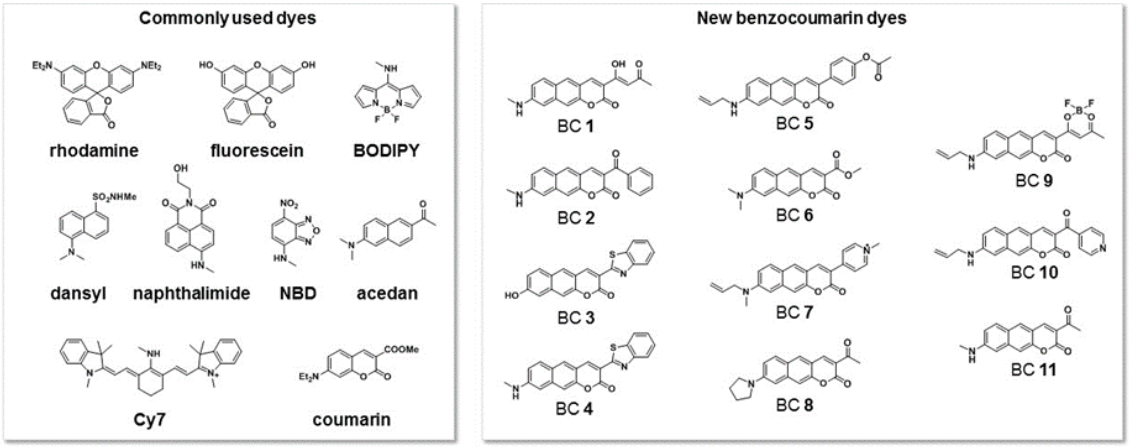


Fig. S1 Structure of commonly used dyes and new benzocoumarin (**BC**) dyes

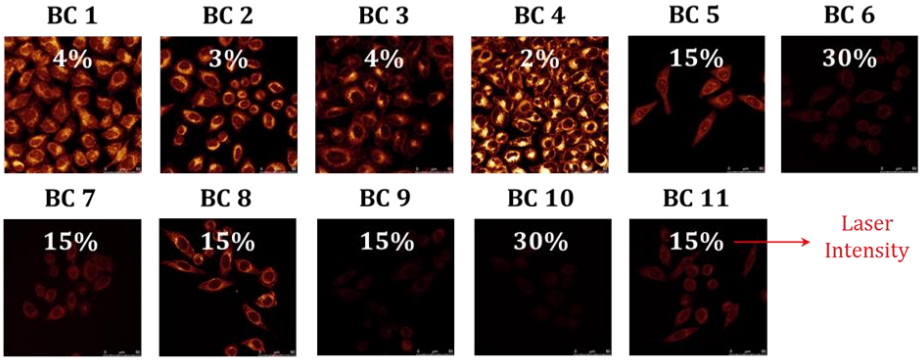


Fig. S2 Confocal microscopic images of Hela cells incubated with different benzocoumarin dyes, **BC 1–11**. Dyes were incubated for 30 min with 10 µM. Percentage indicated in the images.

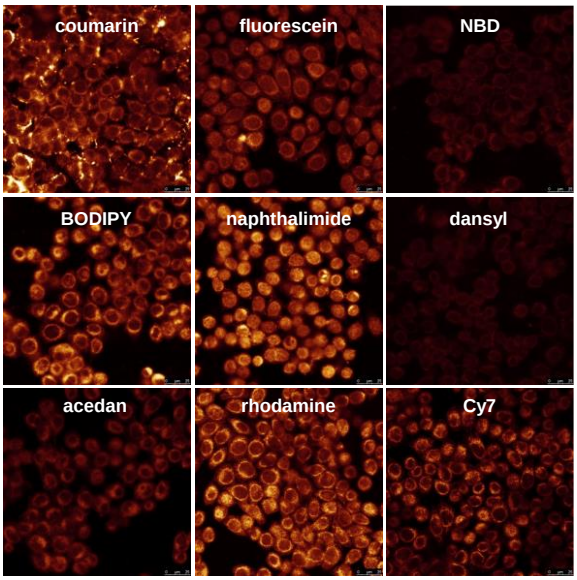


Fig. S3 Confocal microscopic images of HeLa cells incubated with commonly used dyes. Dyes were incubated for 30 min with 10 µM. Percentage indicated in the images.

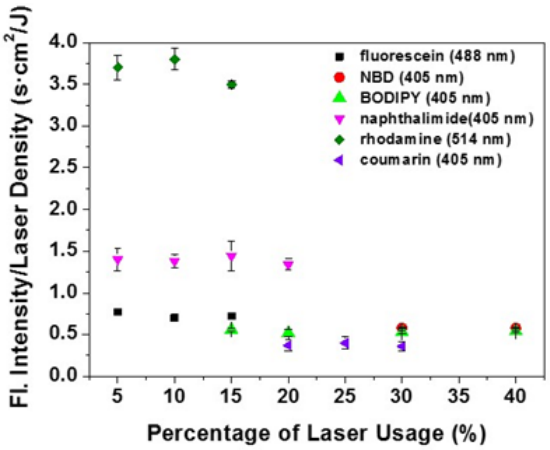


Fig. S4 Comparison of the *in cellulo* brightness of the commonly used dyes depending on the input laser power.

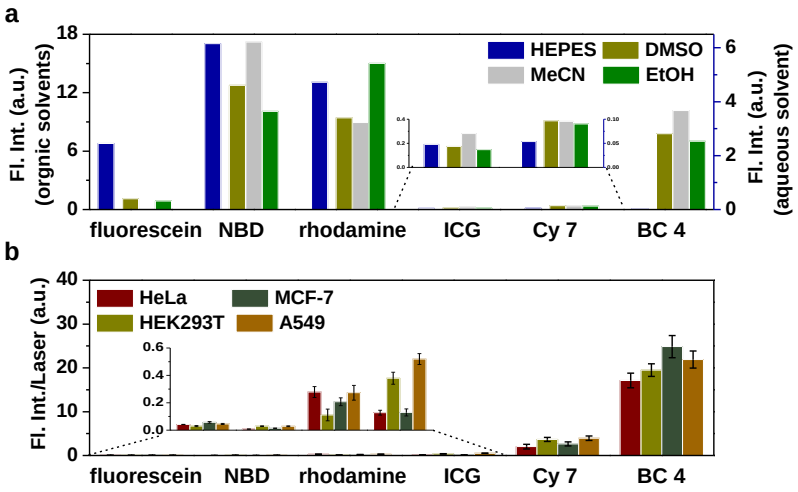


Fig. S5 Comparison of fluorescent intensity of commonly-used fluorophores (fluorescein, NBD, rhodamine, ICG, and Cy7(10 μM)) and **BC 4** (10 μM) in (a) HEPES buffer (10 mM, pH 7.4) on the right y-axis, and organic solvents (DMSO, MeCN, and EtOH) on the left y-axis and in (b) different cell lines (HeLa, HEK293T, MCF-7, A549). The cells were incubated with each of the dyes for 30 min and the cellular intensity was obtained under excitation at each dye's maximum absorption wavelength.

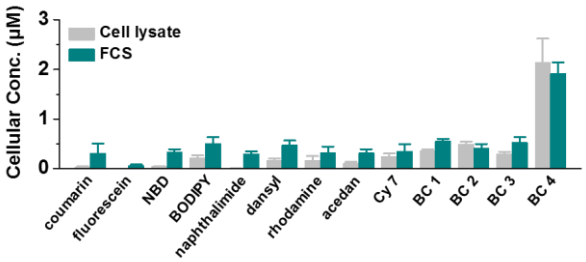


Fig. S6 Comparison of cellular concentration of the fluorophores determined by cell lysate and FCS analyses.

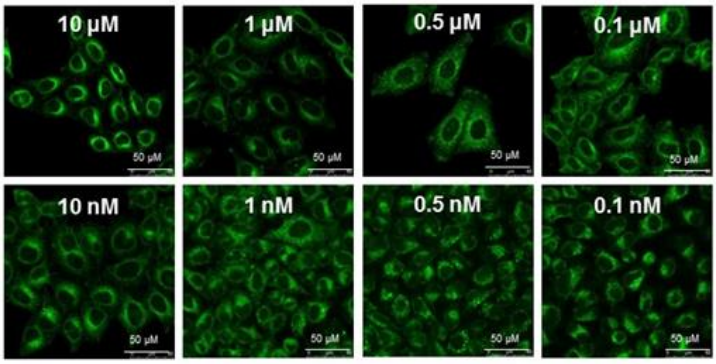


Fig. S7 Cell imaging of **BC 4** at different concentrations. The images of HeLa cells were obtained under excitation at 405 nm and collection of emissions between 450–800 nm.

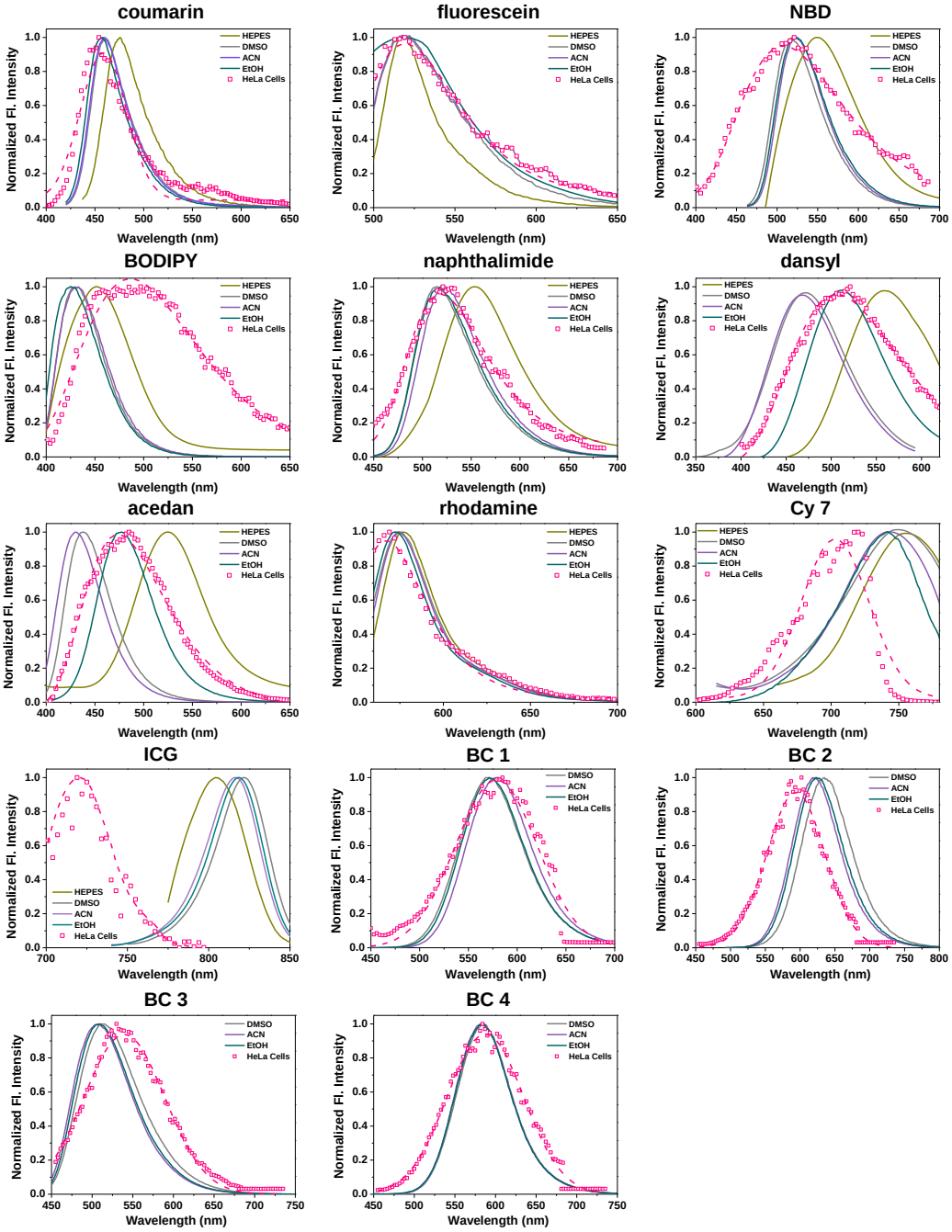


Fig. S8 Normalized fluorescence spectra of the commonly used dyes and new benzocoumarin dyes (**BC 1–4**), measured in different media: an aqueous solution (HEPES, pH 7.4), organic solutions (DMSO, MeCN, and EtOH), and cells (HeLa). The fluorescence emission spectra were measured under excitation at the maximum absorption wavelength of each dye.

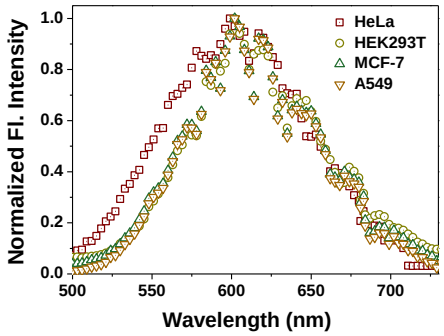


Fig. S9 Normalized fluorescence spectra of **BC 4** measured in different cells (HeLa, HEK293T, MCF-7, and A549).

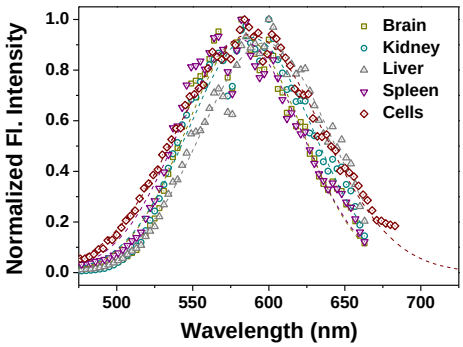


Fig. S10 Normalized fluorescence spectra of **BC 4** measured in different organs of tissue (brain, kidney, liver and spleen) and HeLa cells. All the measurements were taken under two-photon excitation at 900 nm and one-photon excitation at 405 nm, respectively, and the emissions were collected in the range of 450–700 nm.

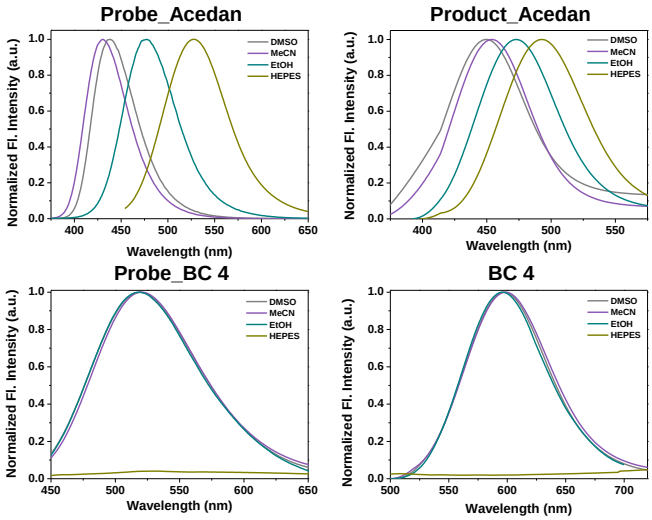


Fig. S11 Normalized emission spectra of the dummy probes and products based on acedan and **BC 4**, measured in different solvents (DMSO, MeCN, EtOH, and HEPES buffer). All the measurements were taken at 1.0 µM concentration of the compounds under excitation at 350 nm (acedan) and 420 nm (**BC 4**), respectively (the isosbestic point).

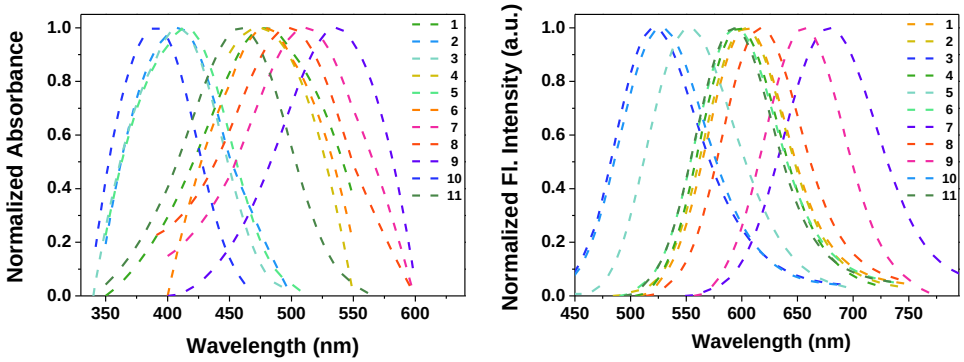


Fig. S12 Normalized absorption and emission spectra of **BC 1–11**. All the measurements were taken in ethanol under excitation at the maximum absorption wavelength of each dye.

Fig. S13 Photographs of **BC 1–11 (**10 µM) in DMSO under the excitation of 365 nm.

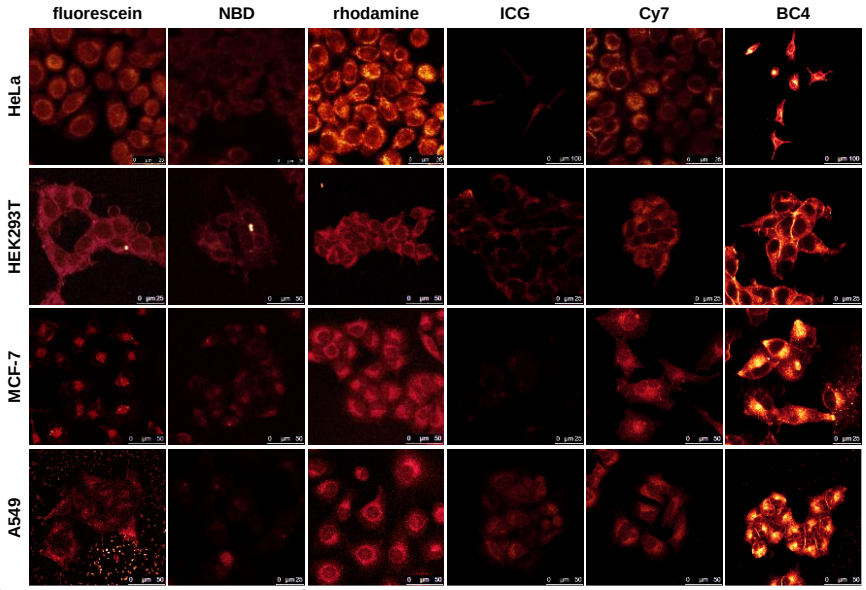


Fig. S14 Confocal microscopic images of HeLa, HEK293T, MCF-7, and A549 cells incubated with 10 µM of commonly used dyes (fluorescein, NBD, rhodamine, ICG, and Cy7) and **BC 4** for 30 min.

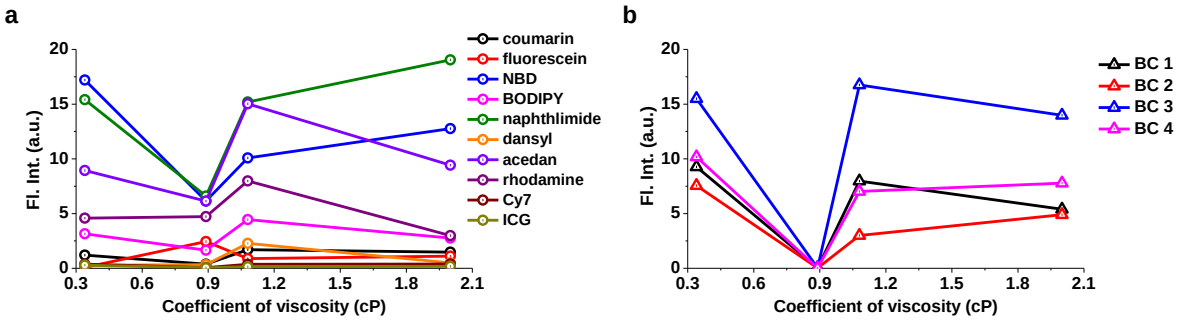


Fig. S15 Fluorescence intensity of commonly used dyes and **BC 1–4** in four different solvents with different coefficient of viscosity (MeCN: 0.34 cP, HEPES buffer: 0.89 cP, EtOH: 1.08 cP, and DMSO: 2.00 cP).[13]

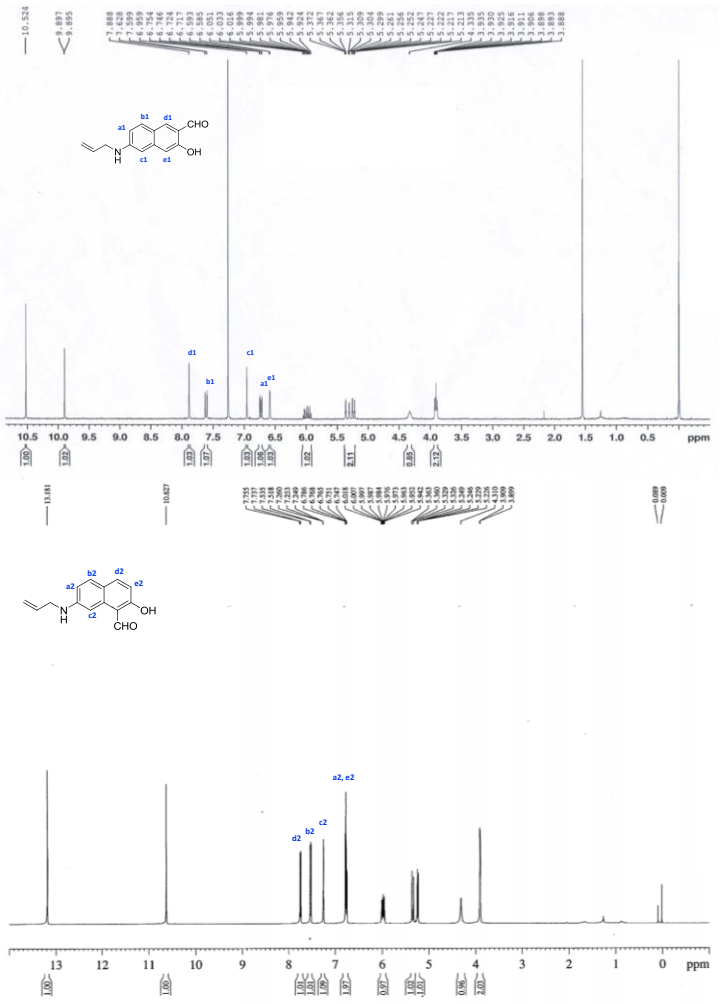


Fig. S16. ^{1}H-NMR of the key intermediates (in $CDCl_3$), one for linear **BC** dyes and the other for bent **BC** dyes.

Table S1. The photophysical properties of the **BC** dyes studied.

	Solvents	λ_{abs} (nm)[a]	λ_{em} (nm)[a]	ε[b]	Φ_F[c,d]
BC 1	DMSO	480	612	19812	0.414[c]
	MeCN	463	595	25211	0.503[c]
	EtOH	475	603	32503	0.487[c]
	HEPES	400	624	5080	0.082[c]
BC 2	DMSO	448	599	8183	0.301[c]
	MeCN	432	579	1519	0.337[c]
	EtOH	448	598	14170	0.134[c]
	HEPES	447	602	17109	0.002[c]
BC 3	DMSO	412	525	15180	0.588[d]
	MeCN	402	508	57979	0.632[d]
	EtOH	410	522	27109	0.667[d]
	HEPES	393	511	18011	0.036[d]
BC 4	DMSO	487	600	82899	0.599[c]
	MeCN	466	595	78891	0.619[c]
	EtOH	480	595	74095	0.576[c]
	HEPES	464	600	37185	0.010[c]

[a]The maximum wavelengths of the absorption (λ_{abs}) and emission (λ_{em}) of the compounds, measured at 1.0 μM. [b]L mol^{-1} cm^{-1}. [c]Fluorescence quantum yields determined using rhodamine 6G as a reference dye (ϕ_F = 0.91 in EtOH). [d]Fluorescence quantum yields determined using coumarin 343 as a reference dye (ϕ_F = 0.68 in EtOH).

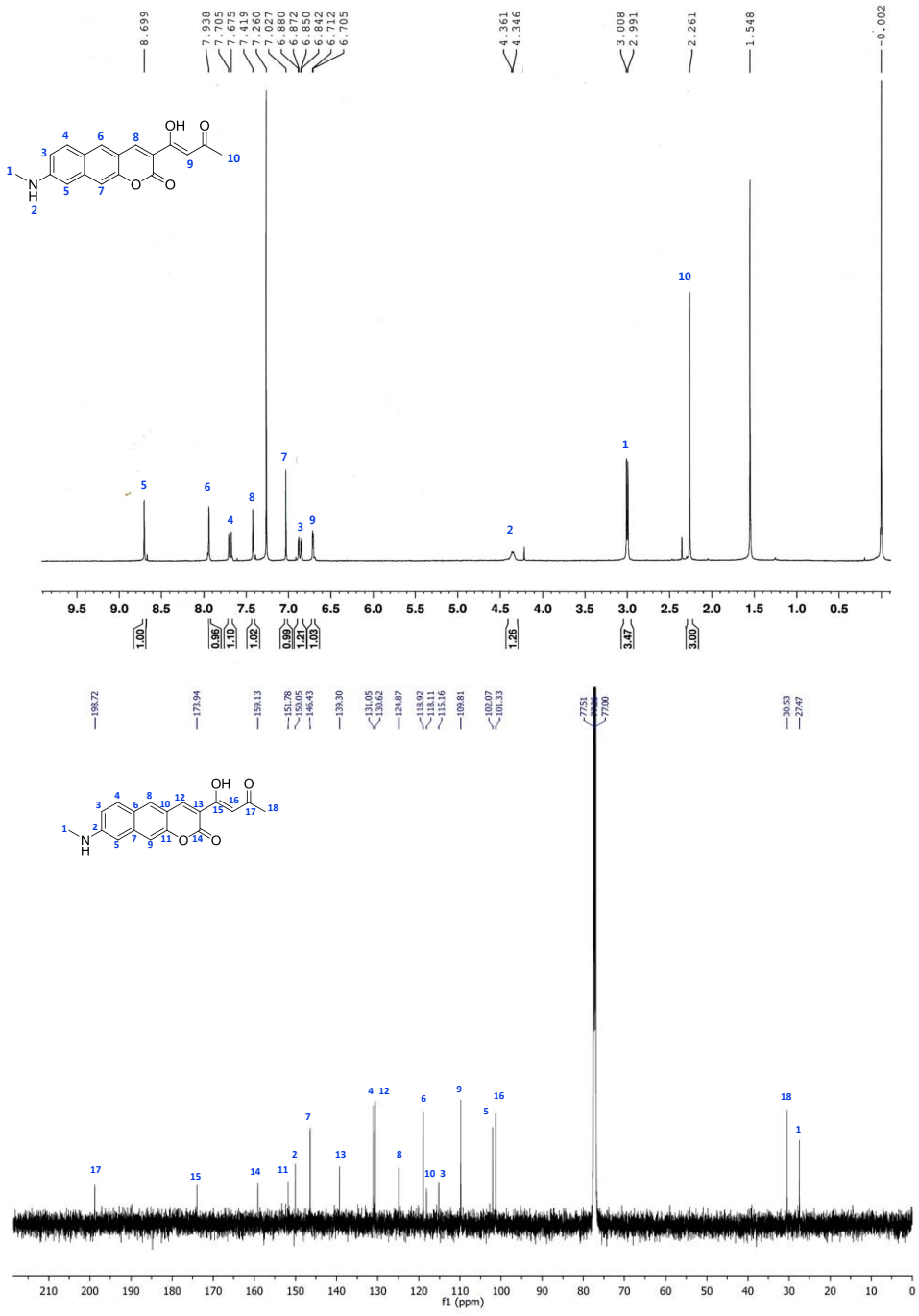
Table S2. Photophysical properties of the **BC 1–11**.

BC	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
λ_{abs} (nm)	475	448	410	480	417	480	515	497	539	392	470
λ_{em} (nm)	603	598	522	595	550	594	679	619	656	527	593
ε ($Lmol^{-1}$ cm^{-1})	32503	14170	27109	74095	42638	3608	16518	6704	38401	41262	31149

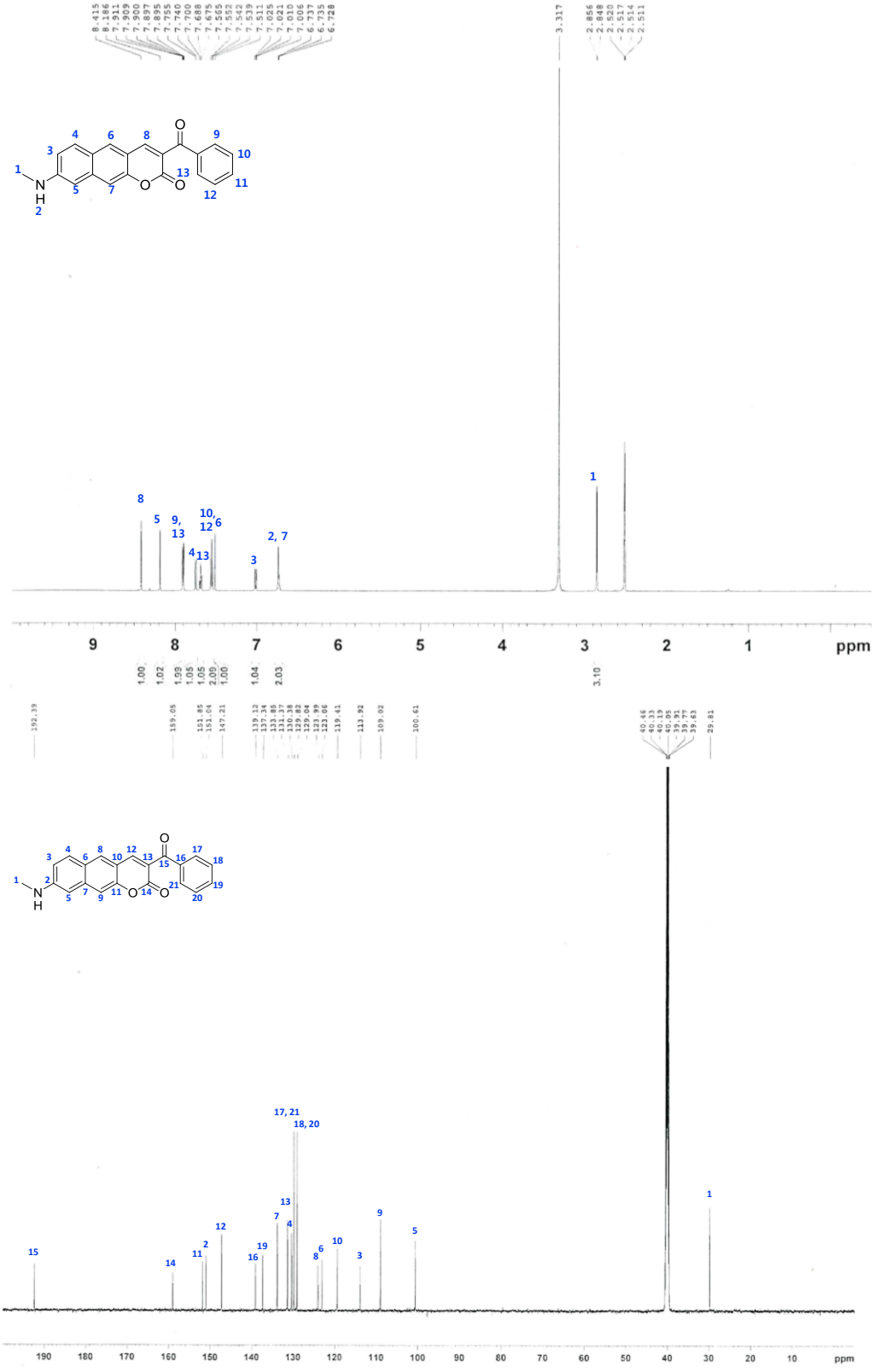
All the measurements were taken in ethanol under excitation at the maximum absorption wavelength of each dye.

^{1}H, ^{13}C, and HRMS spectra of the BC dyes

BC 1



BC 2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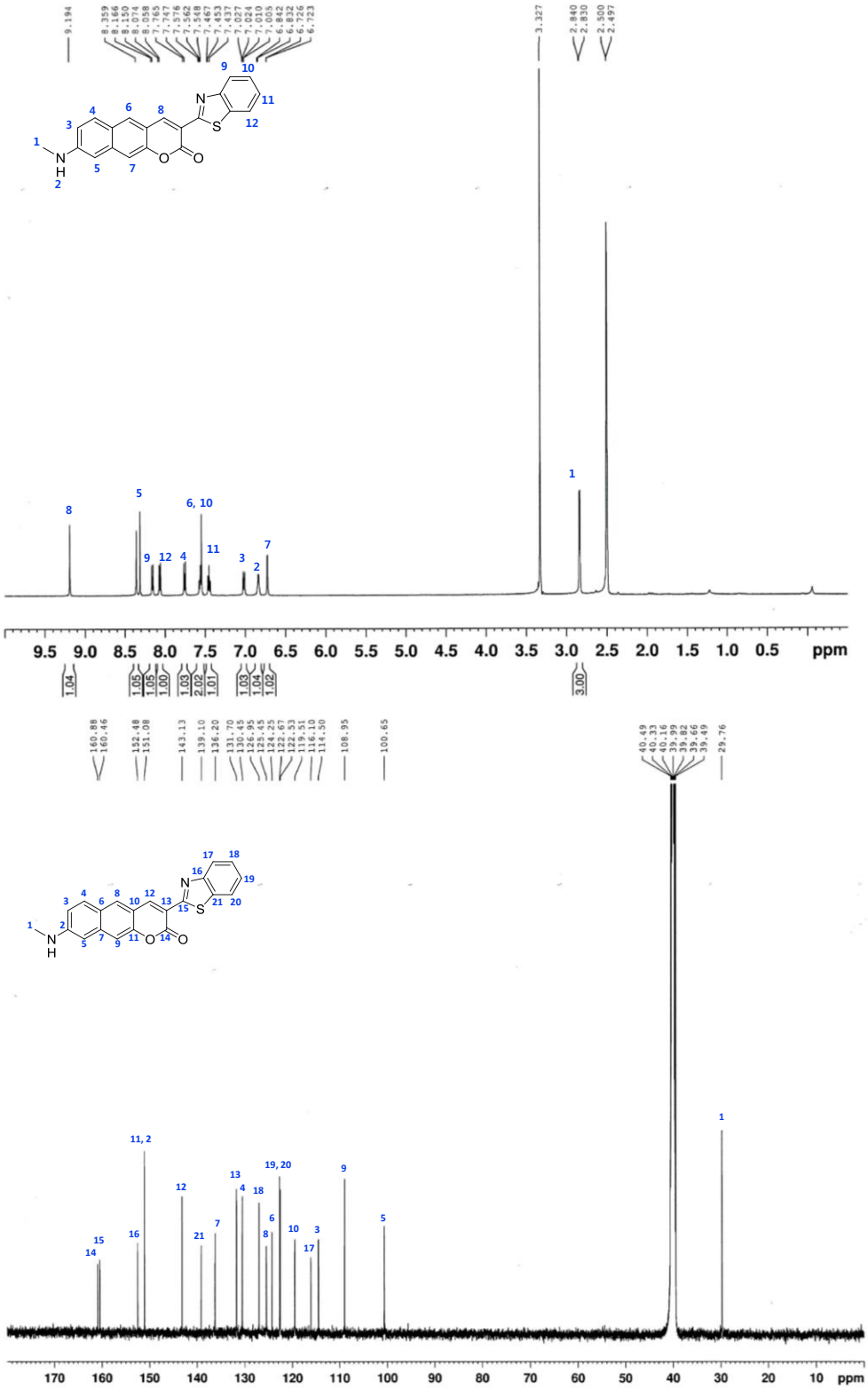
7.911
7.909
7.900
7.897
7.895
7.755
7.740
7.700
7.688
7.675
7.555
7.552
7.542
7.539
7.511
7.025
7.021
7.000
7.006
6.737
6.735
6.728
3.317
2.886
2.868
2.520
2.517
2.514
2.511
1
2
3
4
5
6
7
8
9
10
11
12
13
8
5
9, 13
10, 12
6
4
13
3
2, 7
1
9
8
7
6
5
4
3
2
1
ppm
1.00
1.02
1.99
1.05
1.05
2.09
1.00
1.04
2.03
3.10
192.39
159.05
151.85
151.04
147.21
133.85
131.37
129.84
129.04
123.99
121.06
119.41
113.92
109.02
100.61
40.46
40.33
40.19
40.05
39.91
39.77
39.63
29.81
17, 21
18, 20
15
14
11
2
12
16
19
13
7
4
8
6
10
3
9
5
1
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
ppm

BC 4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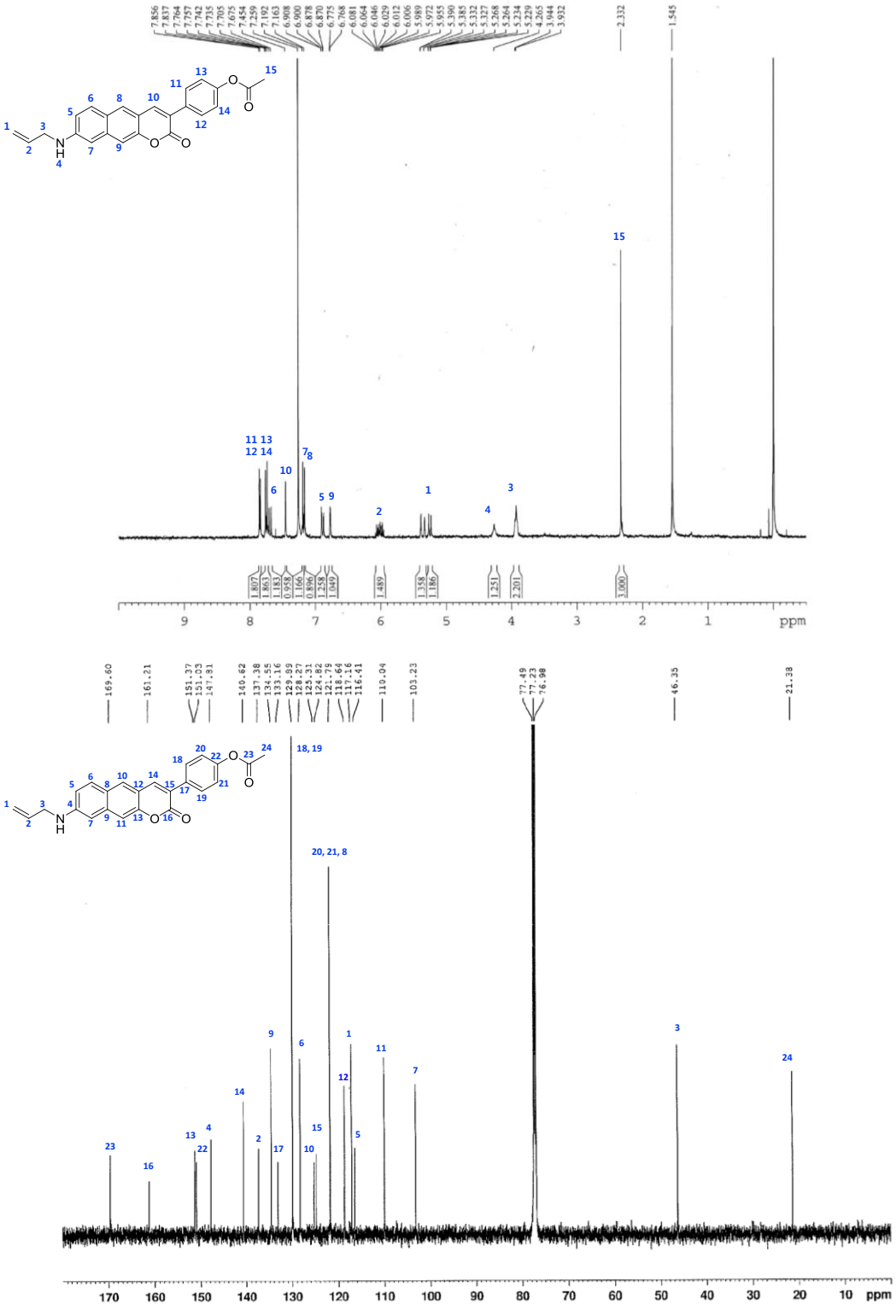
8.166
8.150
8.074
8.058
7.765
7.747
7.576
7.562
7.548
7.467
7.433
7.027
7.024
7.010
7.005
6.832
6.812
6.726
6.723
3.327
2.840
2.830
2.500
2.497
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 ppm
1.04
1.05
1.05
1.00
1.03
2.02
1.01
1.03
1.04
1.02
3.00
160.88
160.46
152.48
151.08
143.13
139.10
136.20
131.70
130.45
126.95
125.45
124.25
122.67
122.53
119.51
116.10
114.50
108.95
100.65
40.49
40.33
40.16
39.99
39.82
39.66
39.49
29.76
170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 ppm

BC 5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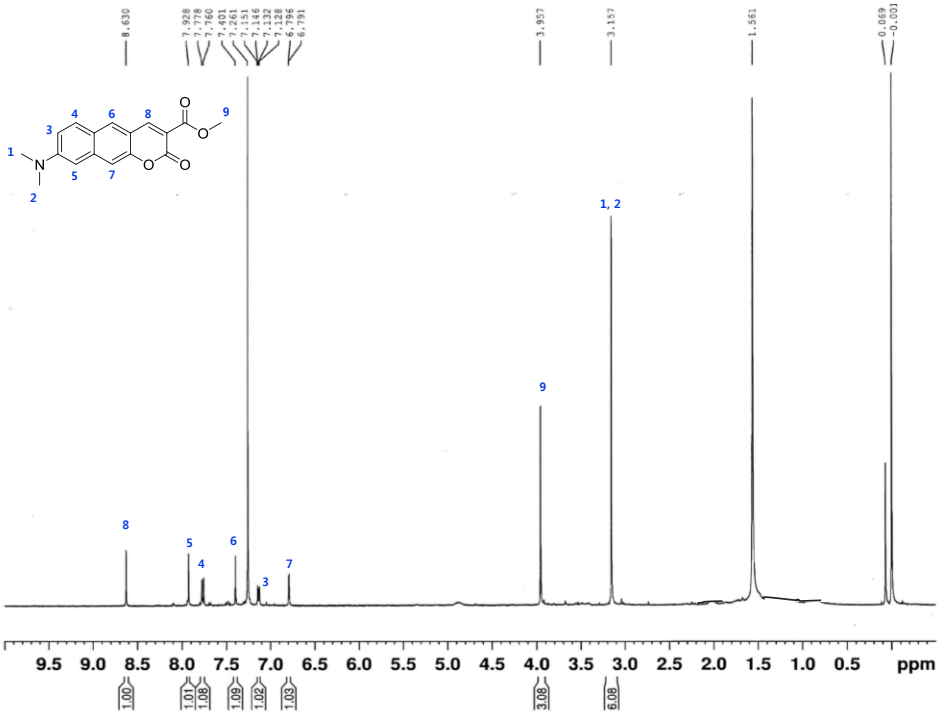
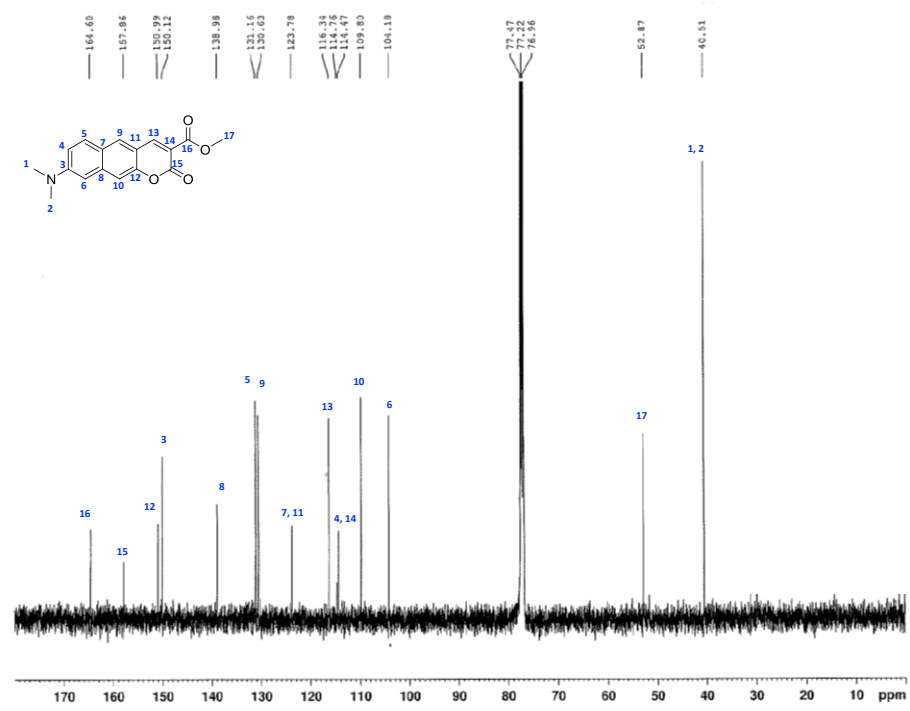
7.837
7.764
7.757
7.742
7.735
7.705
7.675
7.454
7.259
7.192
7.163
6.908
6.900
6.878
6.870
6.775
6.768
6.081
6.064
6.046
6.029
6.012
6.006
5.989
5.972
5.955
5.396
5.385
5.332
5.327
5.268
5.264
5.234
5.229
4.265
3.944
3.932
2.332
1.545
11 13
12 14
10
6
7
8
5 9
2
1
4
3
15
1.807
1.863
1.183
0.958
1.166
0.896
1.248
1.049
1.489
1.358
1.186
1.251
2.201
3.000
9
8
7
6
5
4
3
2
1
ppm
169.60
161.21
151.37
151.03
147.81
140.62
137.38
134.55
133.16
129.09
128.27
125.31
124.82
121.79
118.64
117.16
116.41
110.04
103.23
77.49
77.23
76.98
46.35
21.38
18, 19
20, 21, 8
9
6
1
11
7
12
5
14
2
17
10
15
4
13
22
23
16
3
24
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
ppm

BC 6


8.630
7.928
7.778
7.760
7.401
7.261
7.151
7.146
7.132
7.128
6.796
6.791
3.957
3.157
1.561
0.069
-0.001
1, 2
9
8
5
6
4
3
7
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 ppm
1.00
1.01
1.08
1.09
1.02
1.03
3.08
6.08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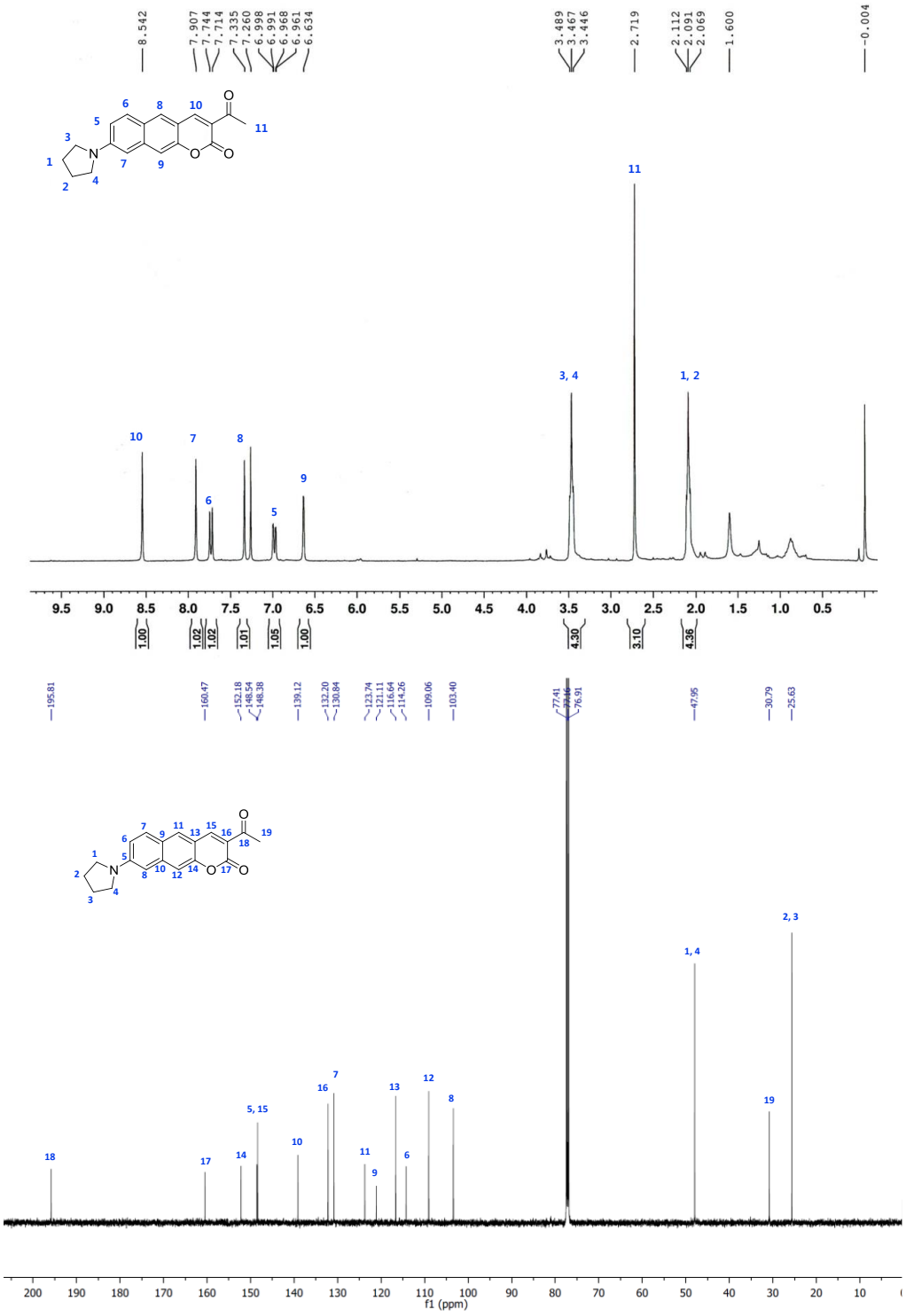
157.86
150.99
150.12
138.98
131.16
130.63
123.78
116.34
114.75
114.47
109.80
104.18
77.47
77.22
76.96
52.87
40.51
1, 2
5
9
13
10
6
17
3
8
16
12
7, 11
4, 14
15
170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 ppm

BC 8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7.335
7.260
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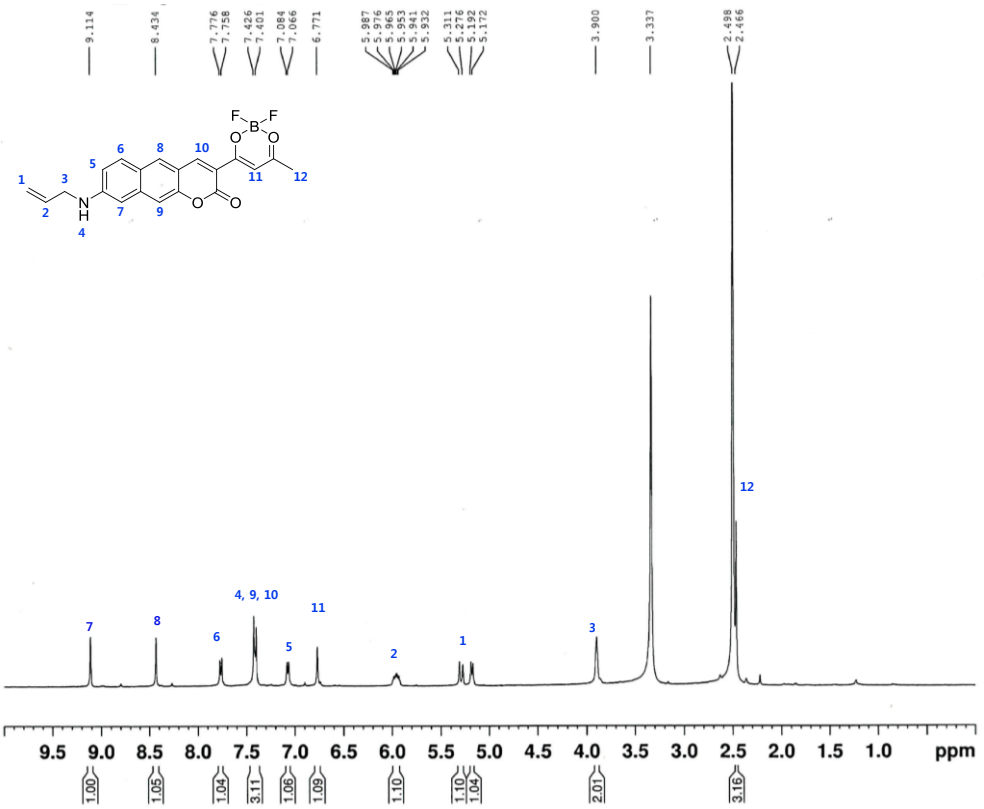
6.991
6.968
6.961
6.634
3.489
3.467
3.446
2.719
2.112
2.091
2.069
1.600
-0.004
11
3, 4
1, 2
10
7
8
6
5
9
1.00
1.02
1.02
1.01
1.05
1.00
4.30
3.10
4.36
195.81
160.47
152.18
148.54
148.38
139.12
132.20
130.84
123.74
121.11
116.64
114.26
109.06
103.40
77.41
77.16
76.91
47.95
30.79
25.63
2, 3
1, 4
19
18
17
14
5, 15
10
16
7
11
9
13
6
12
8
f1 (ppm)

BC 9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7.776
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7.426
7.401
7.084
7.066
6.771
5.987
5.976
5.965
5.953
5.941
5.932
5.311
5.276
5.192
5.172
3.900
3.337
2.498
2.466
12
4, 9, 10
11
7
8
6
5
2
1
3
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 ppm
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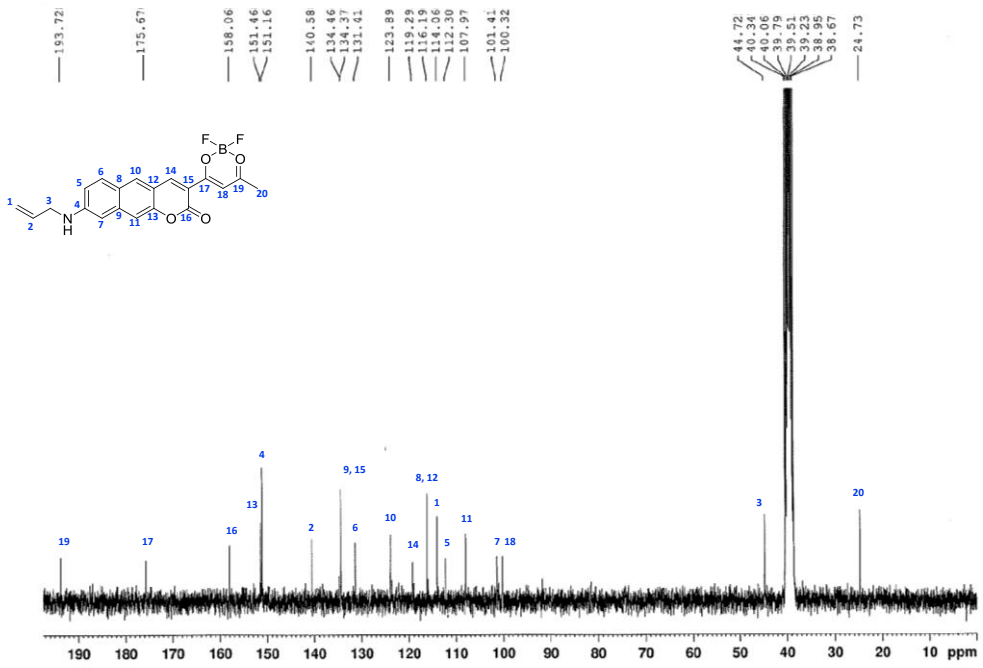
1.09
1.10
1.10
1.04
2.01
3.16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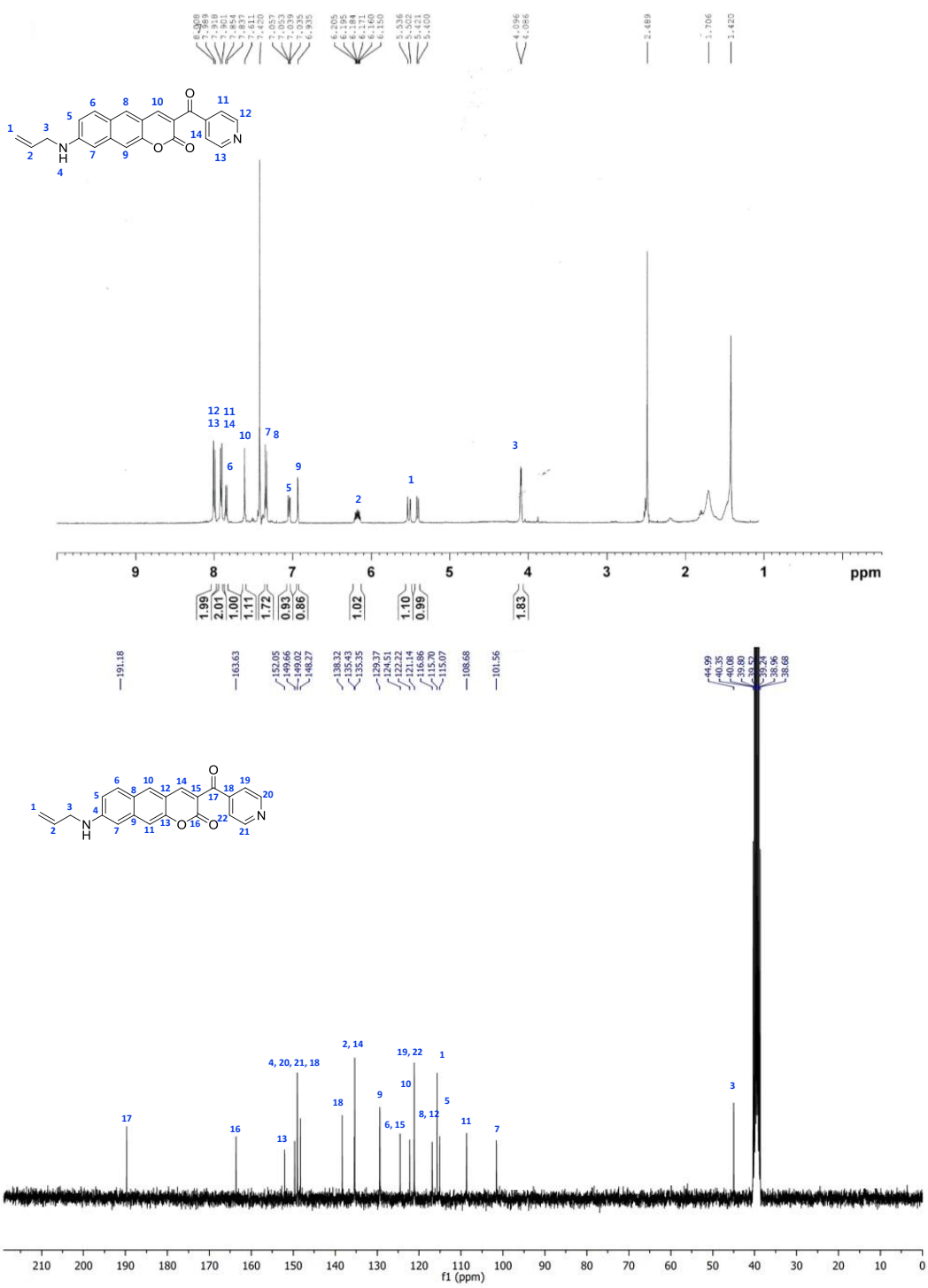
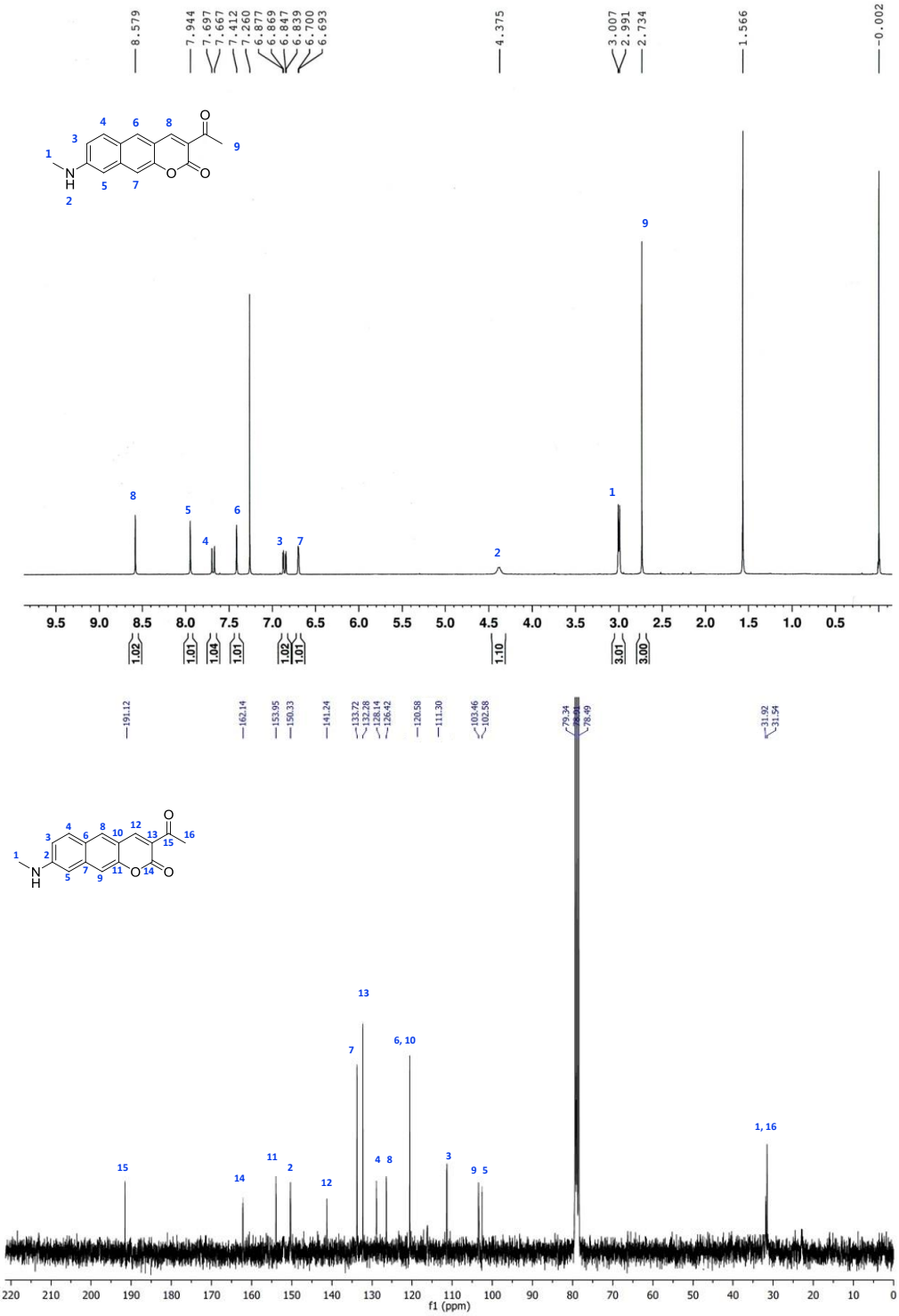
151.16
140.58
134.46
134.37
131.41
123.89
119.29
116.19
114.96
112.30
107.97
101.41
100.32
44.72
40.34
40.06
39.79
39.51
39.23
38.95
38.67
24.73
4
9, 15
8, 12
13
16
2
6
10
1
14
5
11
19
17
7 18
3
20
190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 ppm

BC 10



BC 11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7.667
7.412
7.260
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6.869
6.847
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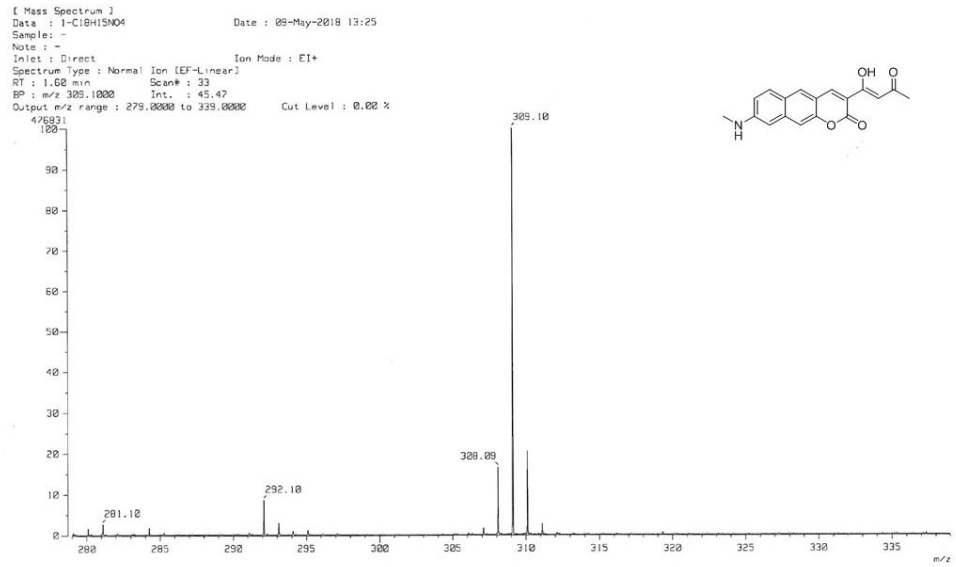
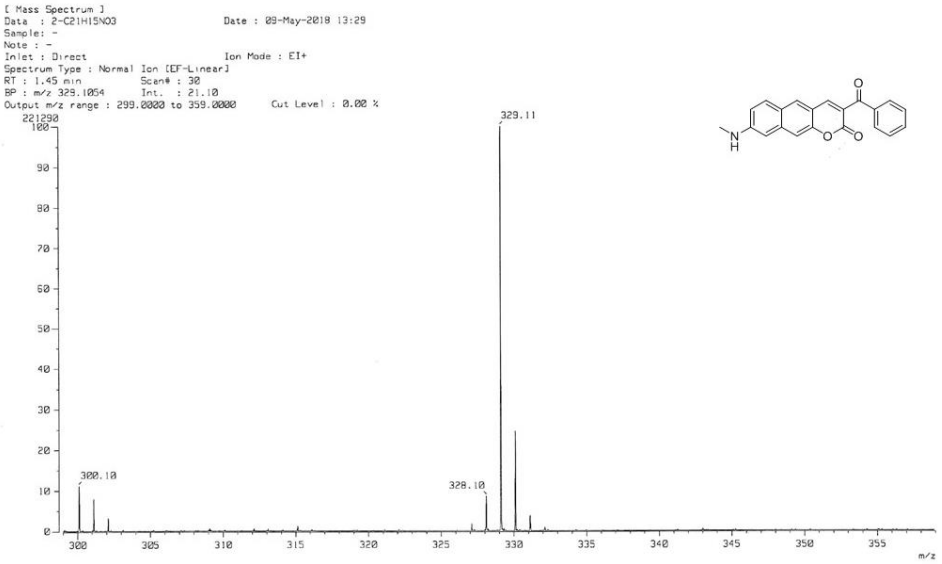
6.700
6.693
4.375
3.007
2.991
2.734
1.566
-0.002
1.02
1.01
1.04
1.01
1.02
1.01
1.10
3.01
3.00
191.12
162.14
153.95
150.33
141.24
133.72
132.28
128.14
126.42
120.58
111.30
103.46
102.58
79.34
78.49
31.92
31.54
f1 (ppm)

HRMS spectra

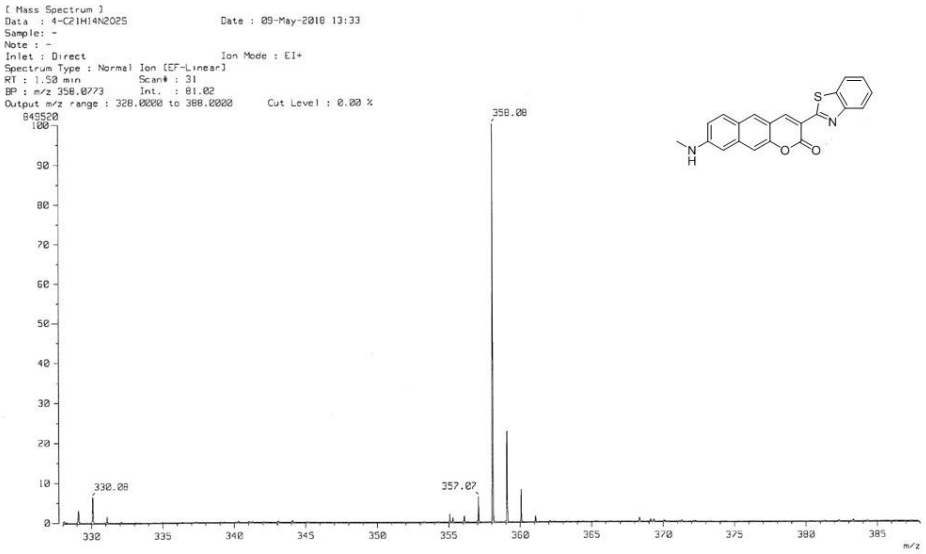
BC 1



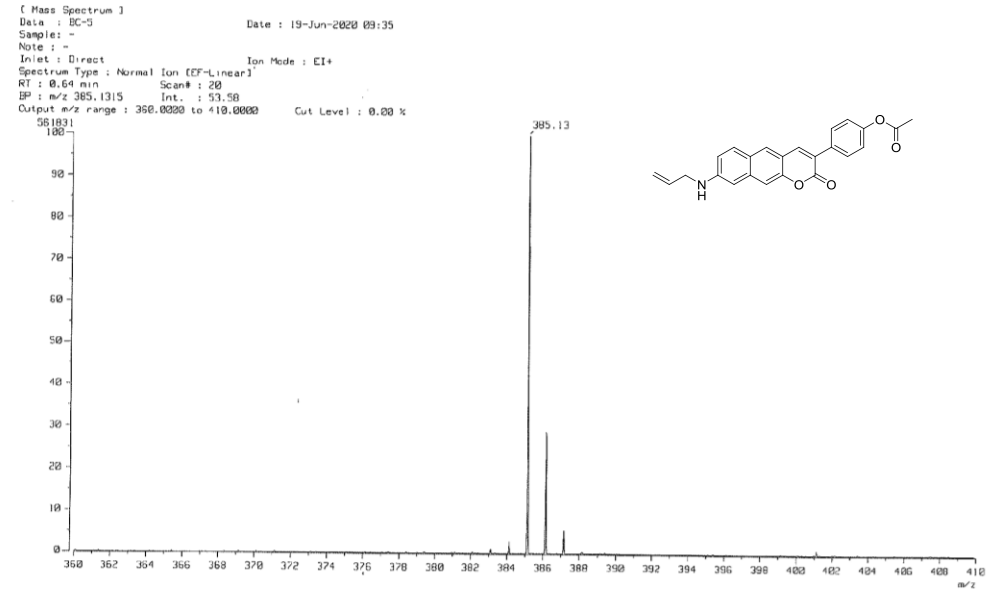
BC 2



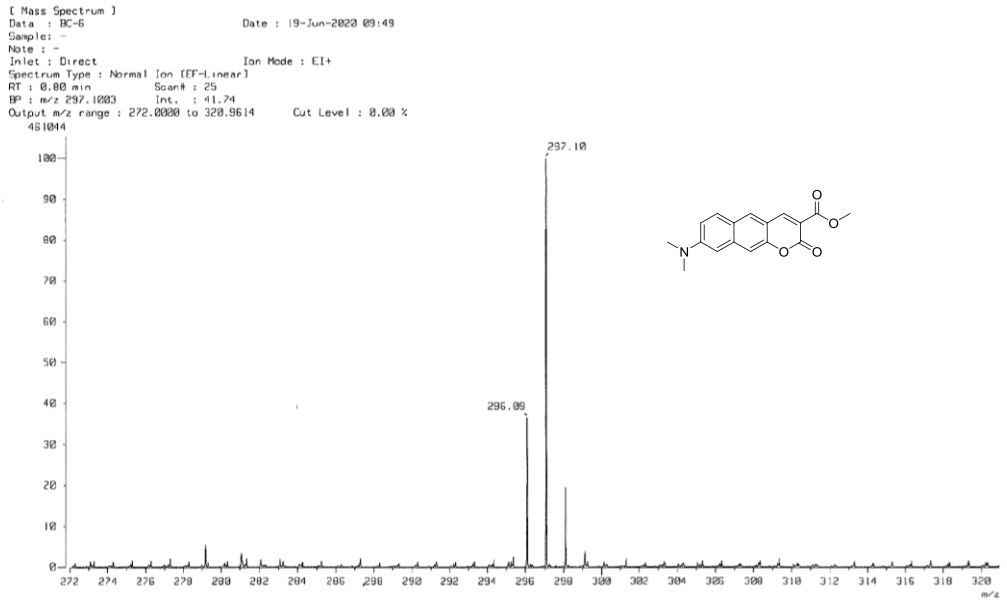
BC 4


[Mass Spectrum]
Data : 4-C21H14N2O2S Date : 09-May-2018 13:33
Sample: -
Note : -
Inlet : Direct Ion Mode : EI+
Spectrum Type : Normal Ion [EF-Linear]
RT : 1.58 min Scan# : 31
BP : m/z 358.0773 Int. : 81.02
Output m/z range : 328.0000 to 388.0000 Cut Level : 0.00 %
849520
358.08
330.08
357.07
m/z

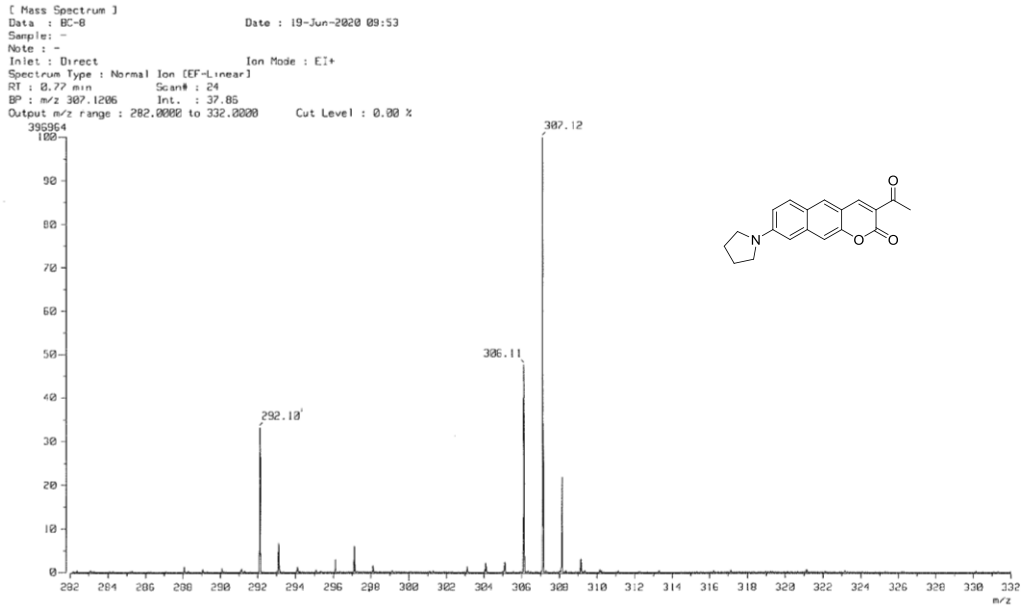
BC 5


[Mass Spectrum]
Data : BC-5 Date : 19-Jun-2020 09:35
Sample: -
Note : -
Inlet : Direct Ion Mode : EI+
Spectrum Type : Normal Ion [EF-Linear]
RT : 0.64 min Scan# : 20
BP : m/z 385.1315 Int. : 53.58
Output m/z range : 360.0000 to 410.0000 Cut Level : 0.00 %
561831
385.13
m/z

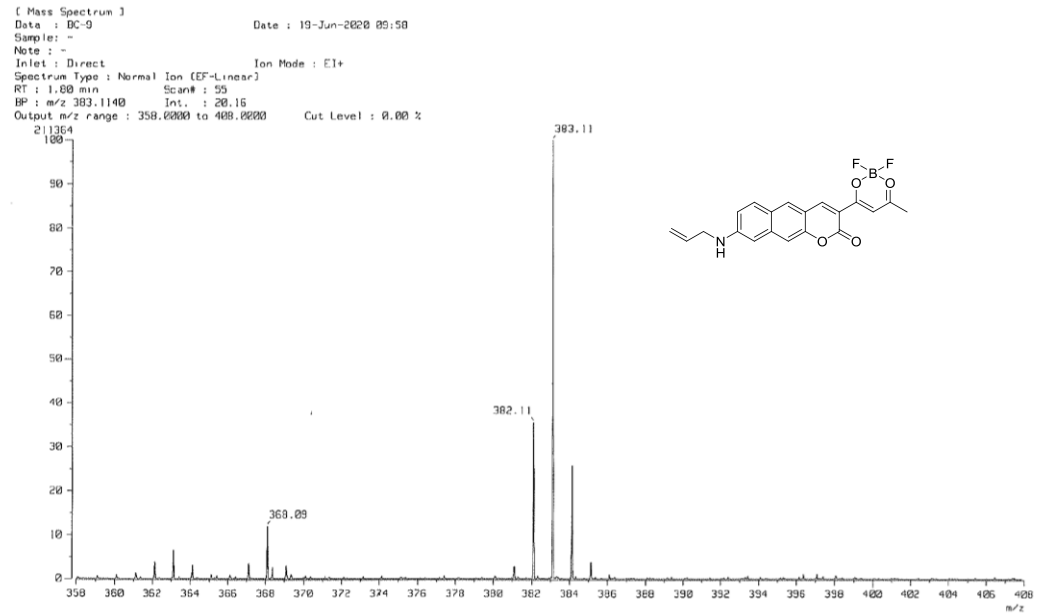
BC 6


[Mass Spectrum]
Data : BC-6
Date : 19-Jun-2020 09:49
Sample: -
Note : -
Inlet : Direct
Ion Mode : EI+
Spectrum Type : Normal Ion (EF-Linear)
RT : 0.80 min
Scan# : 25
BP : m/z 297.1003
Int. : 41.74
Output m/z range : 272.0000 to 320.9614
Cut Level : 0.00 %
461044
297.10
296.09
m/z

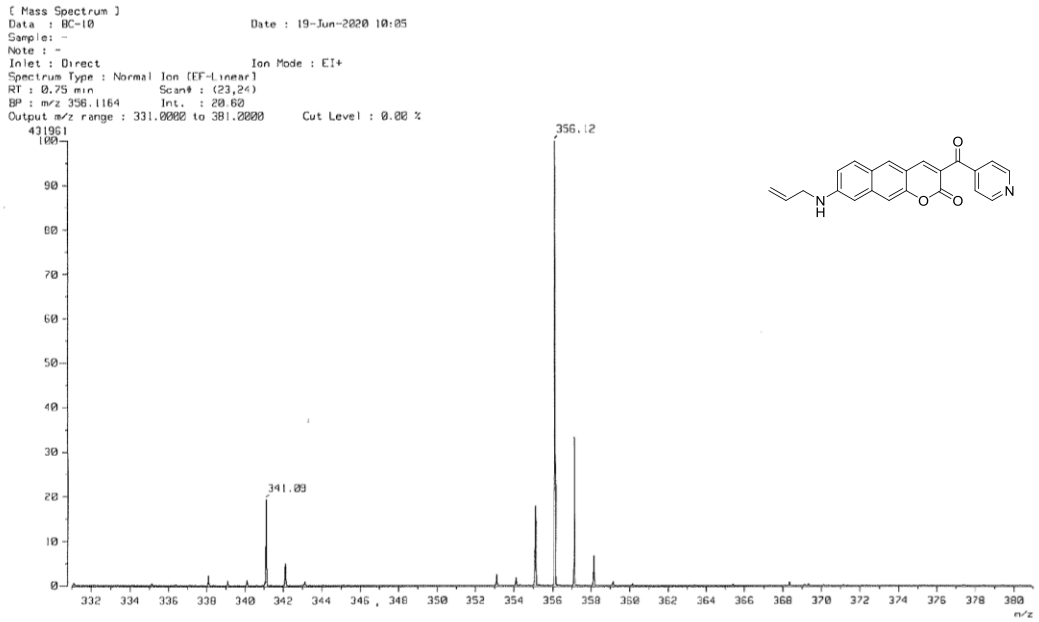
BC 8


[Mass Spectrum]
Data : BC-8
Date : 19-Jun-2020 09:53
Sample: -
Note : -
Inlet : Direct
Ion Mode : EI+
Spectrum Type : Normal Ion (EF-Linear)
RT : 0.77 min
Scan# : 24
BP : m/z 307.1206
Int. : 37.86
Output m/z range : 282.0000 to 332.0000
Cut Level : 0.00 %
395964
307.12
306.11
292.10
m/z

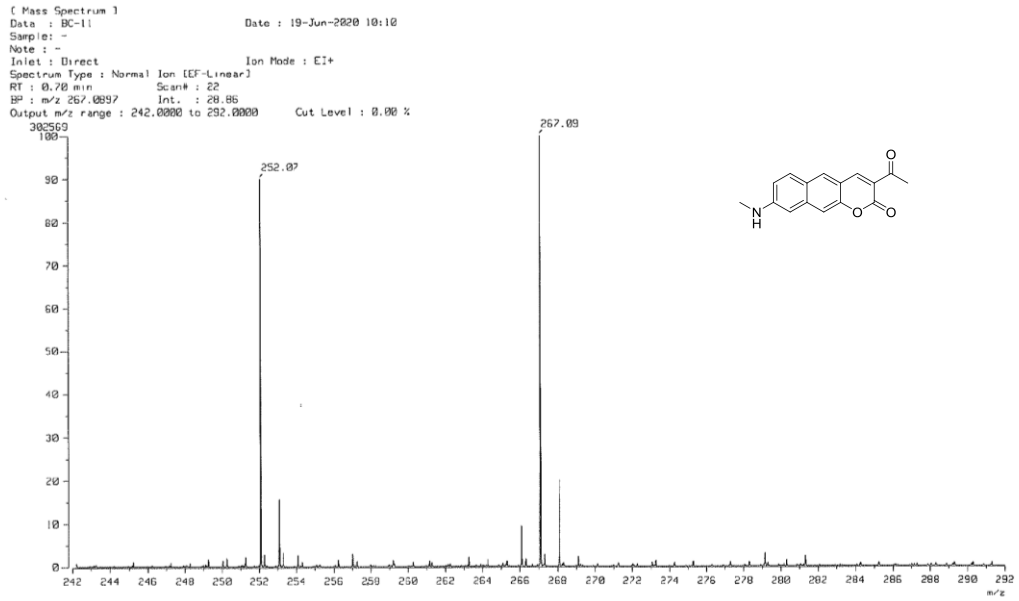
BC 9


[Mass Spectrum]
Data : BC-9 Date : 19-Jun-2020 09:58
Sample: -
Note : -
Inlet : Direct Ion Mode : EI+
Spectrum Type : Normal Ion (EF-Linear)
RT : 1.00 min Scan# : 55
BP : m/z 383.1140 Int. : 20.16
Output m/z range : 358.0000 to 408.0000 Cut Level : 0.00 %
383.11
382.11
368.09
m/z

BC 10


[Mass Spectrum]
Data : BC-10 Date : 19-Jun-2020 10:05
Sample: -
Note : -
Inlet : Direct Ion Mode : EI+
Spectrum Type : Normal Ion (EF-Linear)
RT : 0.75 min Scan# : (23,24)
BP : m/z 356.1164 Int. : 20.60
Output m/z range : 331.0000 to 381.0000 Cut Level : 0.00 %
356.12
341.09
m/z

BC 11



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