Electronic Supplementary Material (ESI) for Chemical Communications

A Near-Infrared Fluorescent Calcium Probe: a New Tool for Intracellular Multicolour Ca^{2+} Imaging

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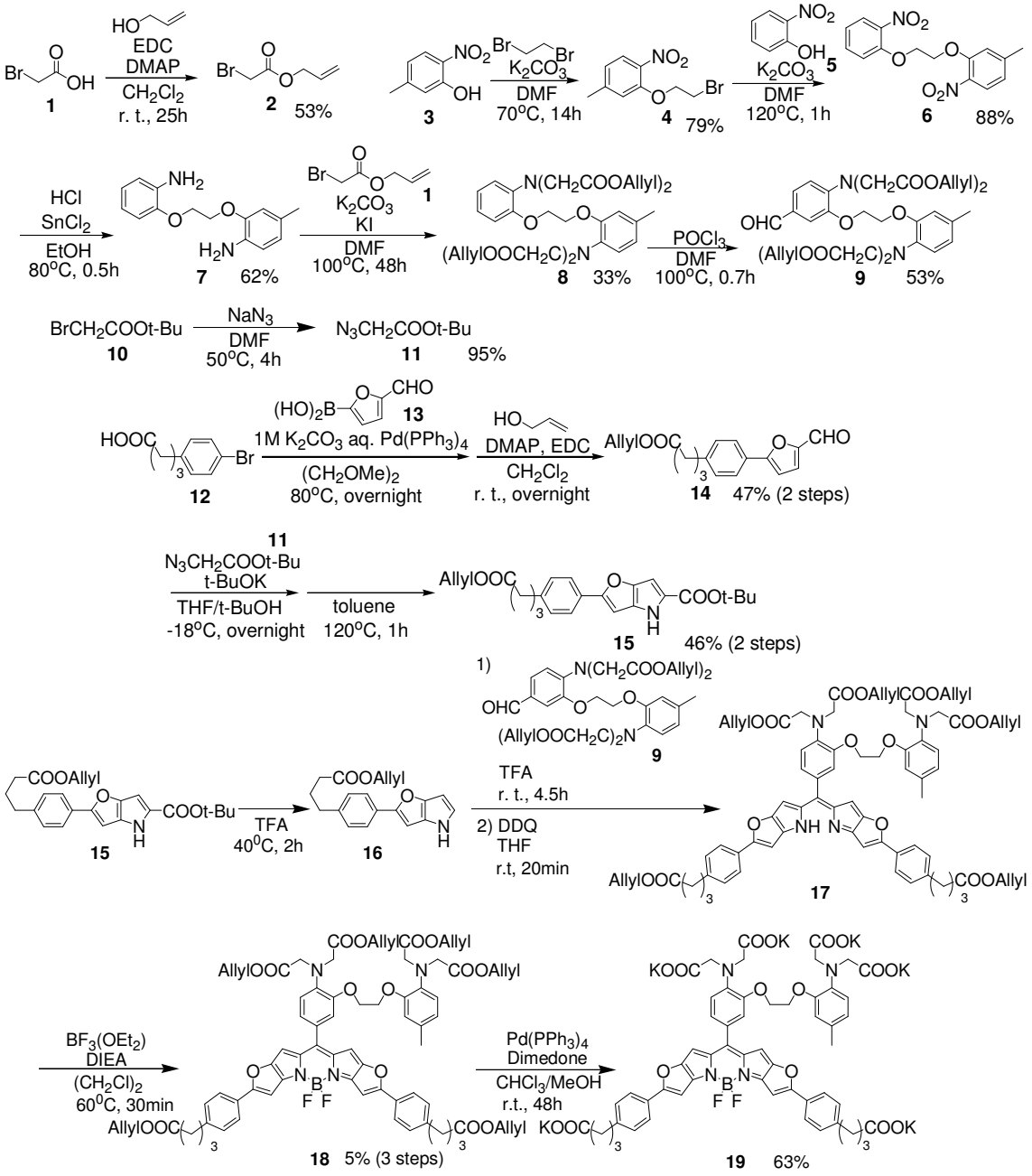
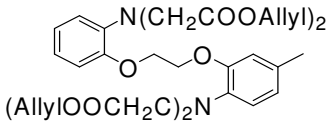
Synthesis

All chemical reagents and solvents for synthesis were purchased from commercial suppliers (Wako Pure Chemical, Tokyo Kasei Industry and Aldrich Chemical) and were used without further purification. All solvents for HPLC were purchased from Wako Pure Chemical and Kanto Chemical. All moisture-sensitive reactions were carried out under an atmosphere of argon. The composition of mixed solvents is given by volume ratio (v/v). ^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian MVX-300 (Varian Inc.) or an ECA-500 (JEOL Ltd.) spectrometer at room temperature. The measurements for ^{1}H NMR and ^{13}C NMR were performed at 500 MHz (ECA-500), and 125 MHz (ECA-500), respectively. All chemical shifts are relative to an internal standard of tetramethylsilane (δ = 0.0 ppm) or solvent residual peaks ($CDCl_3$: δ = 7.26 ppm, DMSO-d_6: δ = 2.50 ppm for ^{1}H, $CDCl_3$: δ = 77.16 ppm, DMSO-d_6: δ = 39.52 ppm for ^{13}C), and coupling constants are given in Hz. Flash chromatography separation was undertaken using a YFLC-Al-560 chromatograph (Yamazen Co. Ltd.). HPLC purification was performed on a reversed-phase column, Inertsil ODS-3 (30 × 50 mm) (GL Sciences Inc.), fitted on an LC-918 recycling preparative HPLC system (Japan Analytical Industry Co. Ltd.). High-resolution MS spectra (HR-MS) were recorded on a JEOL JMS-T100LCS (JEOL Ltd.) with MeOH as the eluent. MALDI-TOF (matrix-assisted laser desorption ionization—time-of-flight) mass spectra were recorded on an Ultraflex TOF/TOF spectrometer (Bruker) with sinapinic acid (SA) as matrix. The following abbreviations are used in this manuscript: DMAP: *N,N*-dimethyl-4-aminopyridine, EDC: 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; DIEA: *N,N*-diisopropylethylamine DDQ: 2,3-dichloro-5,6-dicyano-*p*-benzoquinone, DMF: *N,N*-dimethylformamide, THF: tetrahydrofuran, TFA: trifluoroacetic acid. Compounds **2**,[1] **7**,[2] and **11**[3] were synthesised according to previously reported literature.



Scheme 1. Synthetic scheme for KFCA

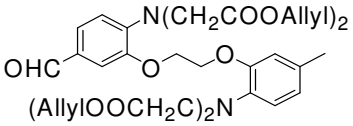
[Allyloxycarbonylmethyl-(2-{2-[2-(bis-allyloxycarbonylmethylamino)-5-methyl-phenoxy]-ethoxy}-phenyl)-amino]-acetic acid allyl ester 8



2-[2-(2-Amino-phenoxy)-ethoxy]-4-methyl-phenylamine **7** (1.50 g, 5.81 mmol, 1eq.), potassium iodide (4.82 g, 29.1 mmol, 5 eq.), potassium carbonate (4.02 g, 29.1 mmol, 5 eq.) and bromoacetic acid allyl ester **2** (4.80 g, 26.8 mmol, 4.6 eq.) were dissolved in DMF (50 ml) and stirred for 2 days at 90 °C. After cooling to room temperature, water was added into the reaction mixture, followed be extraction with ethyl acetate. The combined organic phase was washed with water and brine, dried over Na_2SO_4 and evaporated. The resulting residue was purified by flash chromatography (silica gel, eluent: *n*-hexane/ethyl acetate = 77/23) to obtain [allyloxycarbonylmethyl-(2-{2-[2-(bis-allyloxycarbonylmethylamino)-5-methyl-phenoxy]-ethoxy}-phenyl)-amino]-acetic acid allyl ester **8** (1.25 g, 33.1 %).

^{1}H-NMR (500 MHz, $CDCl_3$): δ (ppm) = 2.26 (s, 3H), 4.15 (s, 4H), 4.18 (s, 4H), 4.27 (s, 4H), 4.48–4.51 (m, 8H), 5.16–5.24 (m, 8H), 5.77–5.85 (m, 4H), 6.67–6.68 (m, 2H), 6.76 (d, 1H, *J* = 8.6 Hz), 6.83–6.93 (m, 4H). ^{13}C-NMR (125 MHz, $CDCl_3$): δ (ppm) = 21.0, 53.6, 53.7, 65.35, 65.42, 67.2, 67.3, 113.7, 114.5, 118.6, 118.7, 119.4, 119.5, 121.7, 121.9, 122.4, 131.96, 132.00, 132.3, 136.9, 139.4, 150.47, 150.53, 171.27, 171.30. HR-MS: *m/z* calcd. for $C_{35}H_{42}N_2O_{10}$: 651.2918, found: 651.2893 $[M+H]^+$.

[Allyloxycarbonylmethyl-(2-2-[2-(bis-allyloxycarbonylmethyl-amino)-5-methyl-phenoxy]-ethoxy}-4-formyl-phenyl)-amino]-acetic acid allyl ester 9

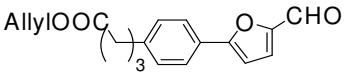


$POCl_3$ (0.35 ml, 3.84 mmol, 5 eq.) was added dropwise to DMF (2 ml) at 0 °C and stirred for 10 min. A solution of [allyloxycarbonylmethyl-(2-{2-[2-(bis-allyloxycarbonylmethylamino)-5-methyl-phenoxy]-ethoxy}-phenyl)-amino]-acetic acid allyl ester **8** (498 mg, 0.768 mmol, 1 eq.) in DMF (2 ml) was then added to the mixture and heated at 100 °C for 40 min. After cooling to 0 °C, water was added to the reaction mixture, followed by treatment with aqueous $NaHCO_3$ solution until a neutral pH was obtained. After that, the solution was extracted with ethyl acetate, and the combined organic phase was washed with water and brine, dried over Na_2SO_4 and evaporated. The resulting residue was purified by flash chromatography (silica gel, eluent: n-hexane/ethyl

acetate = 70/30) to obtain [allyloxycarbonylmethyl-(2-2-[2-(bis-allyloxycarbonylmethyl-amino)-5-methyl-phenoxy]-ethoxy)-4-formyl-phenyl]-amino]-acetic acid allyl ester **9** (278 mg, 53.4 %).

^{1}H-NMR (500 MHz, $CDCl_3$): δ (ppm) = 2.26 (s, 3H), 4.15 (s, 4H), 4.26–4.28 (m, 6H), 4.32 (d, 2H, *J* = 4.9 Hz), 4.49–4.52 (m, 8H), 5.16–5.26 (m, 8H), 5.77–5.85 (m, 4H), 6.67–6.70 (m, 2H), 6.77 (s, 1H), 6.78 (s, 1H), 7.38–7.40 (m, 2H), 9.80 (s, 1H). ^{13}C-NMR (125 MHz, $CDCl_3$): δ (ppm) = 20.9, 53.6, 53.7, 65.3, 65.7, 66.8, 67.4, 111.1, 114.6, 116.8, 118.5, 119.0, 119.6, 122.1, 126.6, 130.0, 131.6, 131.8, 132.3, 136.9, 145.0, 149.7, 150.2, 170.4, 171.1, 190.5. HR-MS: *m/z* calcd. for $C_{36}H_{42}N_2O_{11}$: 679.2867, found: 679.2838 $[M+H]^+$.

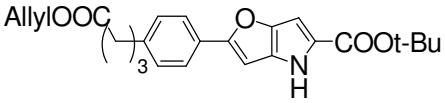
4-[4-(5-Formyl-furan-2-yl)-phenyl]-butyric acid allyl ester 14



4-(4-Bromo-phenyl)-butyric acid **12** (3.95 g, 16.2 mmol, 1.05 eq.) and 5-formyl-2-furanboronic acid **13** (2.17 g, 15.5 mmol, 1 eq.) were dissolved in 1,2-dimethoxyethane (150 ml) and 1 M K_2CO_3 aqueous solution (40 ml), and degassed *in vacuo*. A catalytic amount of tetrakis(triphenylphosphino)palladium(0) was added into the solution, and the mixture was refluxed overnight. After cooling to room temperature, the solution was filtered through a Celite pad and evaporated to remove most of the solvent. Concentrated aqueous HCl solution was added to the residue at 0 °C in order to acidify the solution (pH < 2), and the resulting precipitates were filtered, washed with water and dried *in vacuo*. The resulting compound (4-[4-(5-formyl-furan-2-yl)-phenyl]-butyric acid) was dissolved in CH_2Cl_2, and allyl alcohol (1.05 ml, 15.5 mmol, 1 eq.), DMAP (283 mg, 2.32 mmol, 0.15 eq.) and EDC (2.96 g, 15.5 mmol, 1 eq.) were added to the solution at 0 °C and stirred for 2 days at room temperature. Water was added to the reaction mixture, followed by extraction with CH_2Cl_2. The combined organic phase was washed with water and brine, dried over Na_2SO_4 and evaporated. The resulting residue was purified by flash chromatography (silica gel, eluent: *n*-hexane/ethyl acetate = 80/20 to 70/30) to obtain 4-[4-(5-formyl-furan-2-yl)-phenyl]-butyric acid allyl ester **14** (2.15 g, 46.5 %).

^{1}H-NMR (500 MHz, $CDCl_3$): *δ* (ppm) = 1.99 (tt, 2H, *J* = 7.2 Hz, 7.5 Hz), 2.37 (t, 2H, *J* = 7.5 Hz), 2.70 (t, 2H, *J* = 7.2 Hz), 4.58 (d, 2H, *J* = 5.7 Hz), 5.24 (d, 1H, *J* = 10.2 Hz), 5.31 (d, 1H, *J* = 17.4 Hz), 5.92 (ddd, 1H, *J* = 5.7 Hz, 10.2 Hz, 17.4 Hz), 6.80 (d, 1H, *J* = 3.6 Hz), 7.26 (d, 2H, *J* = 8.4 Hz), 7.31 (d, 1H, *J* = 3.6 Hz), 7.74 (d, 2H, *J* = 8.4 Hz), 9.63 (s, 1H). ^{13}C-NMR (125 MHz, $CDCl_3$): δ (ppm) = 26.3, 33.5, 35.1, 65.2, 107.4, 118.4, 123.8, 125.5, 127.0, 129.2, 132.3, 143.5, 152.0, 159.7, 173.0, 177.2. HR-MS: *m/z* calcd. for $C_{18}H_{18}O_4$: 299.1283, found: 299.1276 $[M+H]^+$.

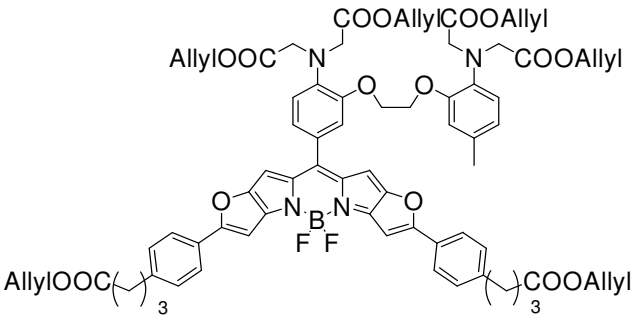
2-[4-(3-Allyloxycarbonyl-propyl)-phenyl]-4H-furo[3,2-b]pyrrole-5-carboxylic acid tert-butyl ester 15



4-[4-(5-Formyl-furan-2-yl)-phenyl]-butyric acid allyl ester **14** (2.00 g, 6.70 mmol, 1 eq.) and azidoacetic acid *tert*-butyl ester **11** (3.51 g, 22.3 mmol, 3.3 eq.) were dissolved in THF (150 ml) and stirred at -18 °C. A solution of *tert*-butanol (12 ml) containing potassium *tert*-butoxide (1.51 g, 13.4 mmol, 2 eq.) was added dropwise into the mixture, and stirred for 5.5 h. Excess saturated aqueous NH_4Cl solution was added to the reaction mixture, THF was removed by evaporation, and the remaining aqueous phase was extracted with ethyl acetate. The combined organic phase was washed with water and brine, dried over Na_2SO_4 and evaporated. The resulting residue was purified by flash chromatography (silica gel, eluent: *n*-hexane/ethyl acetate = 90/10 to 80/20) to obtain a crude solid. This solid was dissolved in toluene (80 ml) and heated to reflux for 1 h. After cooling, the solvent was evaporated. The residue was purified by flash chromatography (silica gel, eluent: *n*-hexane/ethyl acetate = 90/10 to 80/20) to obtain 2-[4-(3-allyloxycarbonyl-propyl)-phenyl]-4*H*-furo[3,2-*b*]pyrrole-5-carboxylic acid *tert*-butyl ester **15** (1.27 g, 46.0%).

^{1}H-NMR (500 MHz, $CDCl_3$): δ (ppm) = 1.60 (s, 9H), 1.98 (tt, 2H, J = 7.5 Hz, 7.5 Hz), 2.37 (t, 2H, J = 7.5 Hz), 2.67 (t, 2H, J = 7.5 Hz), 4.58 (d, 2H, J = 5.7 Hz), 5.23 (d, 1H, J = 10.5 Hz), 5.31 (d, 1H, J = 17.1 Hz), 5.92 (ddd, 1H, J = 5.7 Hz, 10.5 Hz, 17.1 Hz), 6.64 (s, 1H), 6.73 (s, 1H), 7.21 (d, 2H, J = 8.1 Hz), 7.64 (d, 2H, J = 8.1 Hz), 8.93 (s, 1H). ^{13}C-NMR (125 MHz, $CDCl_3$): δ (ppm) = 26.4, 28.5, 33.6, 35.0, 65.2, 81.1, 93.2, 96.5, 118.4, 124.2, 125.4, 129.0, 129.2, 130.0, 132.3, 141.4, 147.8, 159.8, 161.6, 173.2. HR-MS: *m/z* calcd. for $C_{24}H_{27}NO_5$: 354.1341, found: 354.1326 $[M–C_4H_9+H]^+$ (TFA in the solution for HR-MS cleaved the *tert*-butyl group *in situ*).

KFCA Allyl ester 18

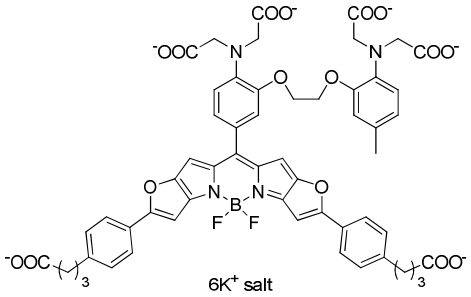


2-[4-(3-Allyloxycarbonyl-propyl)-phenyl]-4*H*-furo[3,2-*b*]pyrrole-5-carboxylic acid *tert*-butyl ester **15** (300 mg, 0.733 mmol, 2 eq.) was dissolved in TFA (3 ml) and stirred at 40 °C for 2 h. (α-decarboxylated furopyrrole **16** was obtained, and used for the next reaction without further purification.)

[Allyloxycarbonylmethyl-(2-2-[2-(bis-allyloxycarbonylmethyl-amino)-5-methyl-phenoxy]-ethoxy)]-4-formyl-phenyl]-amino]-acetic acid allyl ester **9** (249 mg, 0.366 mmol, 1 eq.) was added into the reaction mixture and stirred for further 4.5 h at room temperature. After compound **9** was completely reacted, the solvent was removed *in vacuo* and THF (5 ml) was added. DDQ (166 mg, 0.733 mmol, 2 eq.) in THF (1 ml) solution was slowly added to the reaction solution and stirred for 20 min. The reaction mixture was extracted with CH_2Cl_2, washed with water and brine, dried over Na_2SO_4 and evaporated. The crude compound **17** and DIEA (0.5 ml) was dissolved in 1,2-dichloroethane and stirred at 60 ℃ for 10 min. BF_3 Et_2O complex (0.5 ml) was added to the reaction mixture and stirring was continued for further 30 min. The reaction mixture was diluted with toluene, washed with water and brine, dried over Na_2SO_4 and evaporated. The resulting residue was purified by chromatography (silica gel, eluent: *n*-hexane/ethyl acetate = 67/33) and preparative thin layer chromatography (silica gel, eluent: toluene/ethyl acetate = 80/20) to obtain KFCA allyl ester (23.4 mg, 4.8 %).

^{1}H-NMR (500 MHz, DMSO-d_6): δ (ppm) = 1.89 (tt, 4H, *J* = 7.4 Hz, 7.8 Hz), 2.21 (s, 3H), 2.38 (t, 4H, *J* = 7.4 Hz), 2.68 (t, 4H, *J* = 7.8 Hz), 4.09 (s, 4H), 4.21 (s, 2H), 4.30 (s, 4H), 4.34 (s, 2H), 4.43 (d, 4H, *J* = 5.5 Hz), 4.52–4.56 (m, 8H), 5.09–5.32 (m, 12H), 5.75–5.96 (m, 6H) 6.64 (s, 2H), 6.66 (s, 2H), 6.78 (s, 1H), 6.85 (d, 1H, *J* = 8.3 Hz), 7.22 (d, 1H, *J* = 8.3 Hz), 7.29 (s, 1H), 7.36 (d, 4H, *J* = 8.3 Hz), 7.40 (s, 2H), 7.93 (d, 4H, *J* = 8.3 Hz). ^{13}C-NMR (125 MHz, DMSO-d_6): δ (ppm) = 20.5, 25.9, 32.8, 34.2, 53.2, 53.4, 64.3, 64.5, 64.8, 67.0, 67.5, 95.4, 104.0, 114.5, 116.0, 116.9, 117.7, 117.8, 118.0, 118.4, 121.3, 124.5, 125.56, 125.62, 127.0, 129.3, 130.8, 132.4, 132.5, 132.8, 136.3, 138.3, 140.9, 142.6, 144.4, 148.2, 148.7, 149.7, 152.8, 167.2, 170.3, 170.5, 172.3. HR-MS: *m/z* calcd. for $C_{74}H_{75}BF_2N_2O_{16}$: 1325.5317, found: 1325.5294 $[M+H]^+$.

KFCA 19



KFCA allyl ester 18 (22.3 mg, 16.3 μmol, 1 eq.) and dimedone (47.2 mg, 336.6 μmol, 20 eq.) was dissolved in degassed MeOH/CH_2Cl_2 mixture (1.6 ml, 50/50, v/v) and stirred at room temperature. A catalytic amount of tetrakis(triphenylphosphino)palladium(0) was added into the solution, and the mixture was stirred for 2 days. An excess amount of saturated aqueous $KHCO_3$ solution was added to the reaction mixture and the organic solvents were evaporated. For desalting of potassium ions, the residue was adsorbed on an ODS-18 column (waters Sep-Pack), the column was washed with water (to remove $KHCO_3$), KFCA was eluted from the column with MeOH, and the solvent was evaporated. The crude compound was purified by semipreparative reversed-phase HPLC (eluent: MeCN/H_2O = 75/25 with 0.1% formic acid), and the collected fraction was treated with an excess amount of saturated aqueous $KHCO_3$ solution to quench formic acid (pH ~ 8), and desalting was performed according to the above described protocol to obtain KFCA 19 (14.0 mg, 63.3%)

^{1}H-NMR (500 MHz, CD_3OD): δ (ppm) = 1.94 (tt, 4H, *J* = 7.5 Hz, *J* = 7.8 Hz), 2.22 (t, 4H, *J* = 7.8 Hz), 2.30 (s, 3H), 2.71 (t, 4H, *J* = 7.5 Hz), 3.54 (s, 4H), 3.69 (s, 4H), 4.43 (s, 2H), 4.51 (s, 2H), 6.64 (s, 2H), 6.70 (d, 1H, *J* = 8.1 Hz), 6.85 (s, 1H), 6.93 (d, 1H, *J* = 8.1 Hz), 7.08 (s, 2H), 7.21–7.25 (m, 2H), 7.32 (s, 1H), 7.36 (d, 4H, *J* = 8.3 Hz), 7.83 (d, 4H, *J* = 8.3 Hz). ^{13}C-NMR (125 MHz, CD_3OD): δ (ppm) = 21.2, 29.5, 36.9, 38.8, 59.1, 59.3, 67.2, 67.8, 95.7, 104.9, 113.6, 115.5, 119.3, 119.5, 122.4, 125.5, 126.6, 128.8, 129.8, 130.5, 134.0, 139.3, 140.5, 144.2, 144.5, 147.0, 150.6, 151.7, 151.9, 155.0, 169.5, 178.0, 178.5, 182.4. HR-MS: *m/z* calcd. for $C_{56}H_{45}BF_2K_6N_4O_{16}$, H^+ form): 1065.3377 $[M–F]^+$, Found: 1065.3376 $[M–F]^+$. MALDI-TOF-MS: *m/z* calcd for: 1084.3 $[M]^+$, 1065.3 $[M–F]^+$; found: 1084.4 $[M]^+$, 1065.4 $[M–F]^+$.

Measurements:

All solvents for spectrometry were purchased from Kanto Chemical. Absorption spectra were recorded on a Hitachi U-2001 double beam spectrophotometer (Hitachi, Tokyo, Japan). Measurements of the extinction coefficients were performed according to the following protocol: Around 1 mg of dye was weighed using a digital scale (Δw = 0.01 mg) and dissolved into 100 ml of MeOH. A number of further diluted solutions with different dye concentrations (C: 10^{-7} to 10^{-6} M) were prepared from this stock solution. The absorption spectra of these diluted solutions were measured, and the absorbance (A) and the concentration (C) were plotted on a graph of A versus C to determine the extinction coefficients (from the gradient). Fluorescence emission spectra and quantum yields were recorded on a SREX Fluorolog-3 (Model FL-3–11, Horiba Jobin Yvon, Kyoto, Japan) at 25 °C. The instrument was equipped with a R2658P photomultiplier tube (Hamamatsu Photonics, Shizuoka, Japan) as a fluorescence detector. Measurement of fluorescence emission spectra of KFCA depending on different Ca^{2+} concentration were performed by following the method recommended in the Calcium Calibration Buffer Kit of Molecular Probes.[4] Each KFCA buffer solution (0.4 μM) was prepared using Calcium Calibration Buffer Kit #1, which contains 30 mM MOPS/KOH, pH 7.2, 100 mM KCl and 10 mM EGTA or 10 mM CaEGTA. The excitation wavelength was set to 635 nm, and the excitation and emission bandwidths were set to 6 nm. The apparent dissociation constant (K_d) for Ca^{2+} (mol/l) was calculated from the fluorescence spectra using plots of log $[(F-F_{min})/(F_{max}-F)]$ against log$[Ca^{2+}]_{free}$, following a previously described method[6] (F: fluorescence intensity, F_{max}: maximum fluorescence intensity at saturating $[Ca^{2+}]$, F_{min}: minimum fluorescence intensity at zero $[Ca^{2+}]$). Measurements of quantum yields were performed by following the method recommended by Horiba Jobin Yvon (see: http://www.jp.jobinyvon.horiba.com/product_j/spex/quantum_yield/img/quantum_yields.pdf). A number of diluted solutions of different dye concentrations (A < 0.10, to prevent reabsorption) were prepared, and the absorbance (A) and the integrated fluorescence intensity (F) at each concentration were recorded. Then a graph of F versus A was plotted to determine the gradient ($Grad$). Quantum yields ϕ were calculated by using Equation (1):

$$\Phi_{sample} = \Phi_{ref} \cdot \left(\frac{Grad_{sample}}{Grad_{ref}} \right) \cdot \left(\frac{n_{sample}}{n_{ref}} \right)^2 \quad (1)$$

The subscripts *ref* and *sample* denote the reference dye and the sample, respectively, and n is the refractive index of the solvent. 3,3'-diethyl-thiadicarbocyanine (ϕ = 0.35 in ethanol) was used as a reference dye.[5]

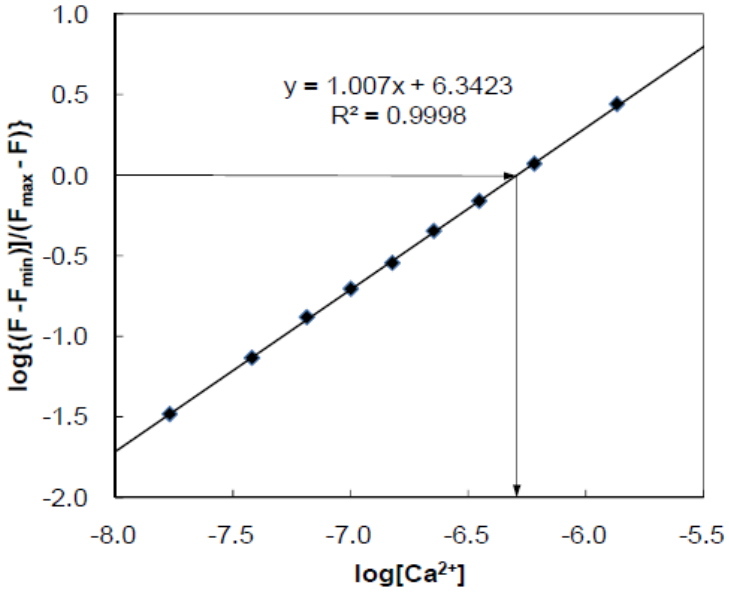


Fig.S1 Plot of log $[(F–F_{min})/(F_{max}–F)]$ against $\log[Ca^{2+}]_{free}$ for determination of K_d.

Cell culture

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen Corp.), supplemented with 10% (v/v) fetal bovine serum (Invitrogen Corp.), penicillin (100 units/mL) and streptomycin (100 μg/mL) at 37 °C in a 5% CO_2/95% air incubator in a humidified atmosphere. For fluorescence imaging, the Hela cells were seeded on a Glass BASE DISH φ = 12 mm (IWAKI) and cultured overnight in DMEM. The cells were loaded with KFCA and/or Fluo-4 using bead-loading according to a published procedure.[7] Briefly, the medium was removed from the dish, 20 μl of 100 μM KFCA and/or Fluo-4 solution in DMEM containing 10% FBS was added to the middle of the dish. Glass beads (radius 100 μm Sigma), which were neutralized by 2N NaOH aq., washed and dried beforehand, were added to the dish. The dish was then pulsated a few times in order to load KFCA into the cells. Afterward, the cells were washed several times with Hanks' balanced salt solutions (HBSS) for removing the beads, and the cells were incubated for 15 min at 37 °C. (HBSS includes the following components: 137 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$, 0.3 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 5.6 mM D-glucose, 5 mM HEPES, and NaOH for adjusting the pH to 7.4.)

To stimulate cells to cause a calcium signal, 100 ul of 2 mM of adenosine 5'-triphosphate (ATP) solution or 100 μl of 100 μM of ionomycin solution in HBSS(+) was added to the dish (final concentration; 100 μM and 5 μM, respectively)

The time delay in the stimulation of the cells after ATP addition resulted from the diffusion of

ATP in the bathing solution.

Protein expression of DsRed2

To construct DsRed2-Actin, human-β-actin was inserted into the *XhoI*/*BamHI* site of the pDsRed2-C1 vector (BD Bioscience, Franklin Lakes, NJ, USA). This plasmid vector was transfected to HeLa cells by lipofection using Lipofectamine LTX (Invitrogen).

Fluorescence microscopy

Fluorescence imaging experiments were performed using a confocal laser scanning microscope system (FluoView FV1000; Olympus, Tokyo, Japan) mounted on an inverted microscope (IX81; Olympus) with a 40× oil immersion objective lens. A semiconductor laser (635 nm), an Ar laser (488 nm), and a semiconductor laser (559 nm) were used as excitation sources for KFCA, Fluo-4, and DsRed2, respectively.

For simultaneous fluorescence imaging of HeLa cells stained by KFCA and Fluo-4, KFCA and Fluo-4 were simultaneously excited by the above-mentioned lasers, and the resulting signals were separated using a 560 nm dichroic mirror. Signals from KFCA and Fluo-4 were observed at 650-750 nm and 500-545 nm, respectively. For fluorescence imaging of HeLa cells stained with KFCA and expressing DsRed2, KFCA and DsRed2 were sequentially excited by the above-mentioned lasers, and the resulting signals were observed at 655-755 nm and 575-620 nm, respectively. Fluorescence images were acquired and analyzed with the FluoView software package (Olympus). Fluorescence differences were calculated as the mean intensity over a defined region of interest (ROI) containing the cell body of each cell.

References

1. R. J. Mart, K. P. Liem, X. Wang, S. J. Webb, *J. Am. Chem. Soc.* **2006**, *128*, 14462–14463.
2. G. Grynkiewicz, M. Poenie, R. Y. Tsien, *J. Biol. Chem.* **1985**, *260*, 3440–3450.
3. A. Vollmar, M. S. Dunn, *J. Org. Chem.* **1960**, *25*, 387–390.
4. http://probes.invitrogen.com/media/pis/mp03008.pdf.
5. D. N. Dempster, T. Morro, R. Rankin, G. F. Thompson, *J. Chem. Soc. Faraday Trans. 2* **1972**, *68*, 1479–1496.
6. A. Minta, J.P.Y. Kao, R.Y. Tsien, *J. Biol. Chem.* **1989**, *264*, 8171–8178.
7. P. L. McNeil, E. Warder, *J. Cell Sci.* **1987**, *88*, 669–678.

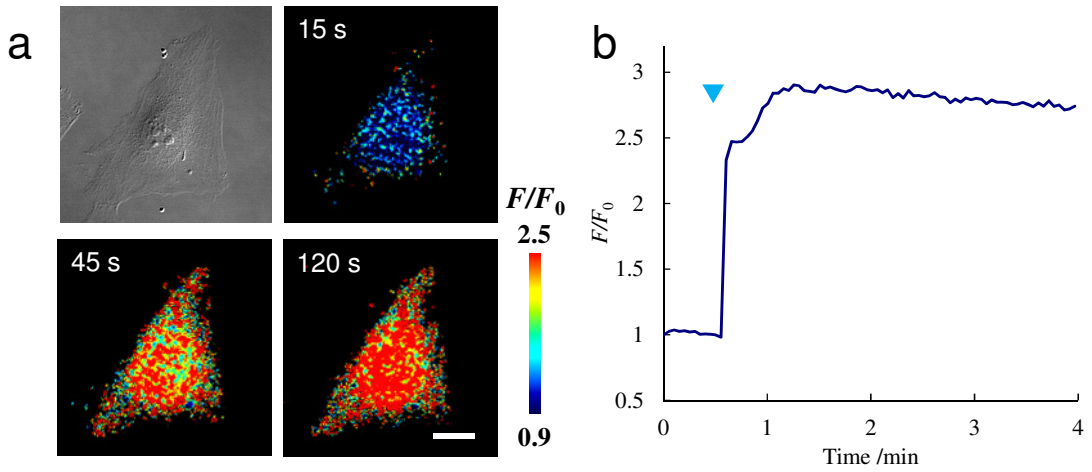


Fig. S2 (a) Bright-field and pseudocoloured images of KFCA-loaded HeLa cells with ionomycin stimulation (5 μM) at 30 s. Images were captured at 15 s, 45 s, and 120 s; Scale bar, 20 μm. (b) Time course of fluorescence change of KFCA. The arrowhead indicates the timing of ionomycin addition.

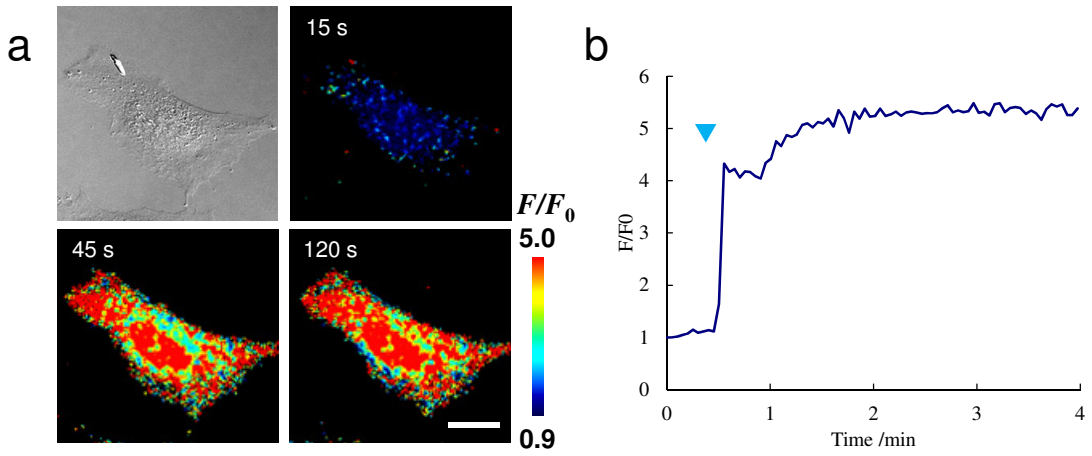


Fig. S3 (a) Bright-field and pseudocoloured images of Fluo-4-loaded HeLa cells with ionomycin stimulation (5 μM) at 30 s. Images were captured at 15 s, 45 s, and 120 s; Scale bar, 20 μm. (b) Time course of fluorescence change of KFCA. The arrowhead indicates the timing of ionomycin addition.

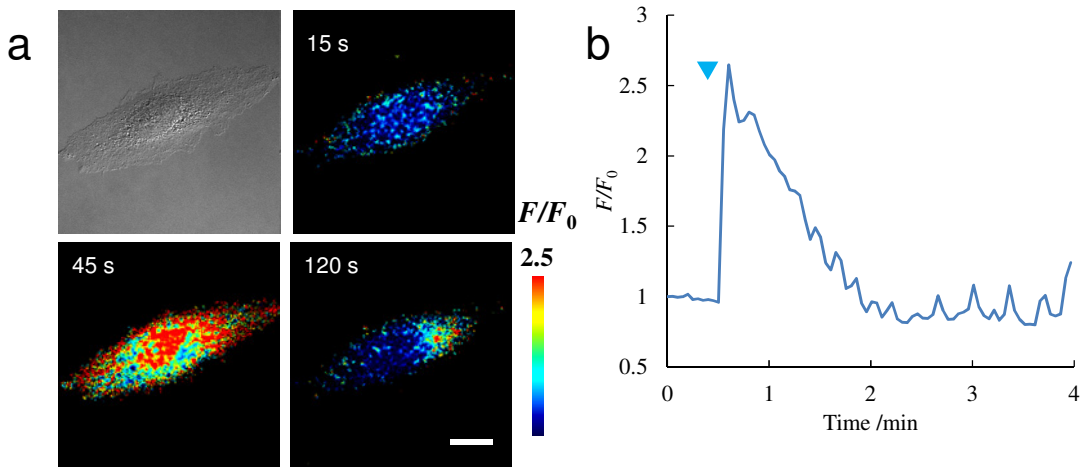
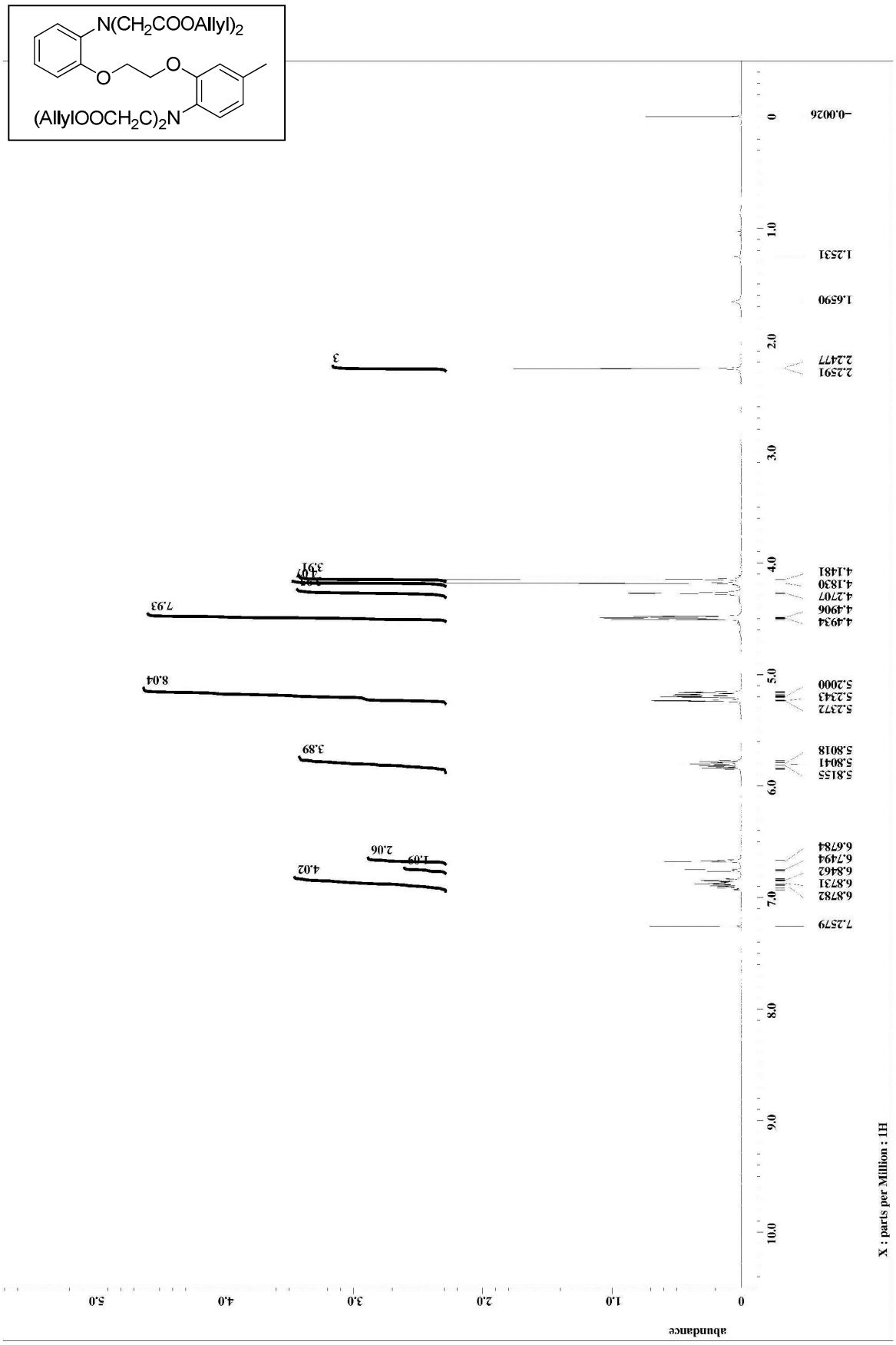
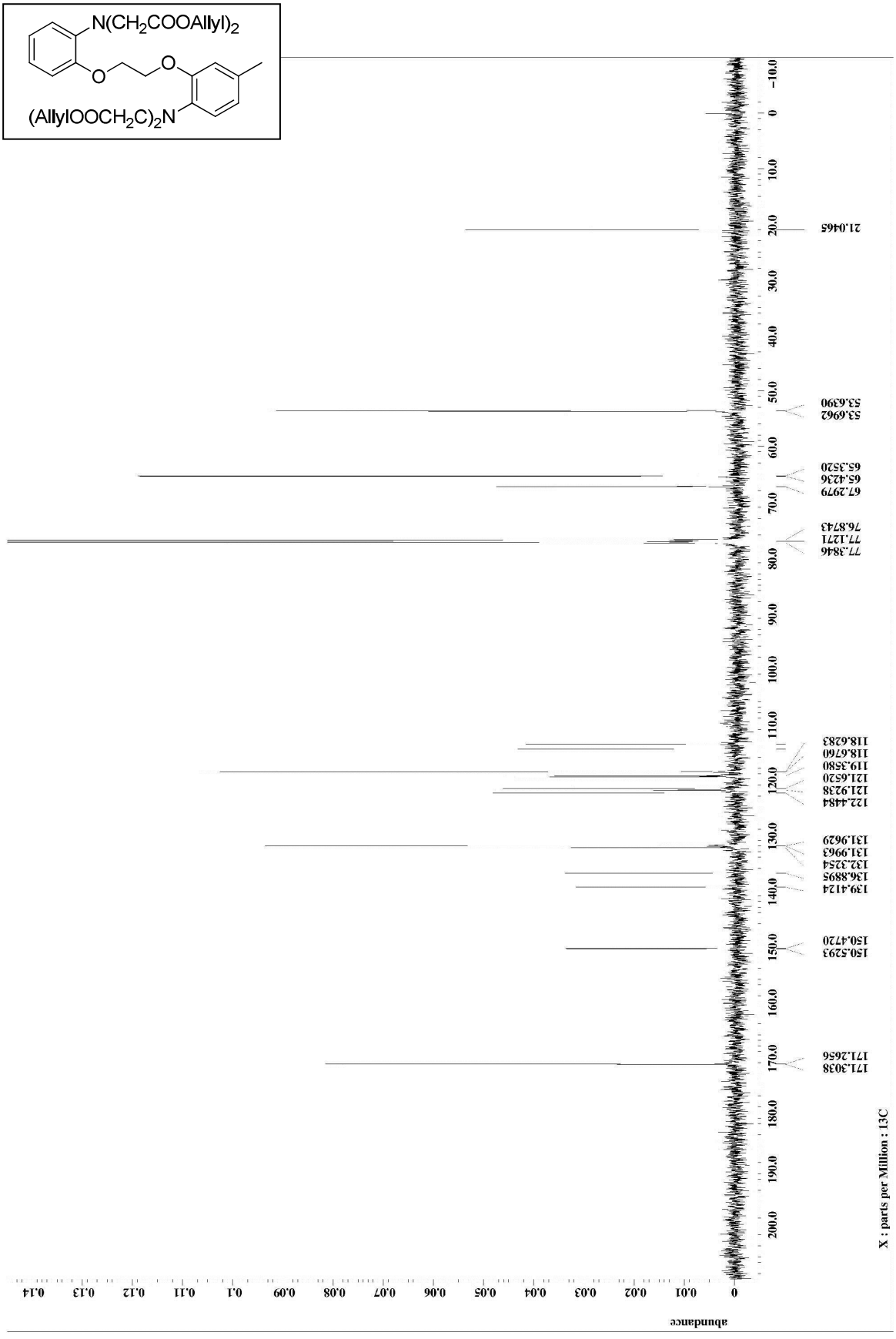
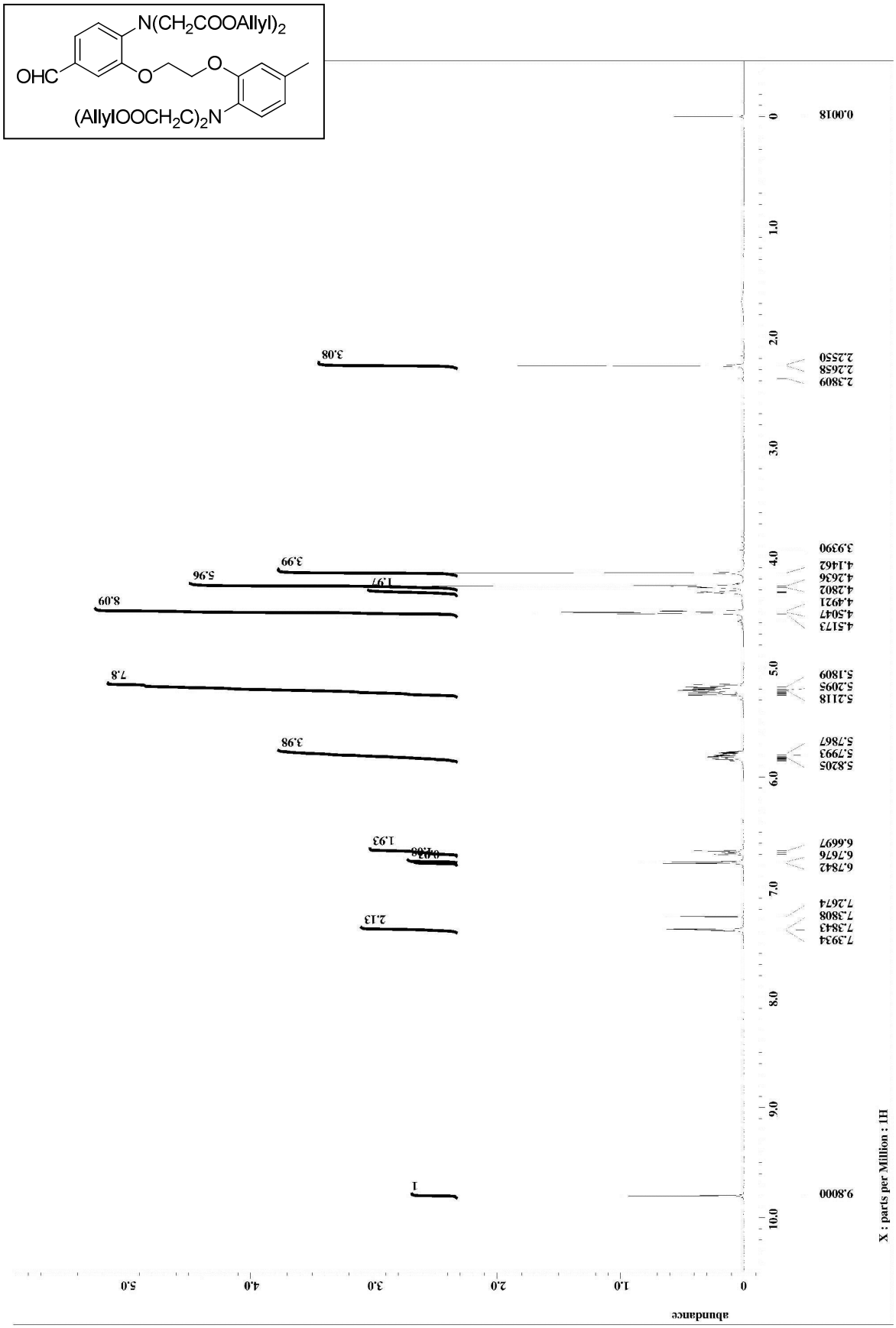
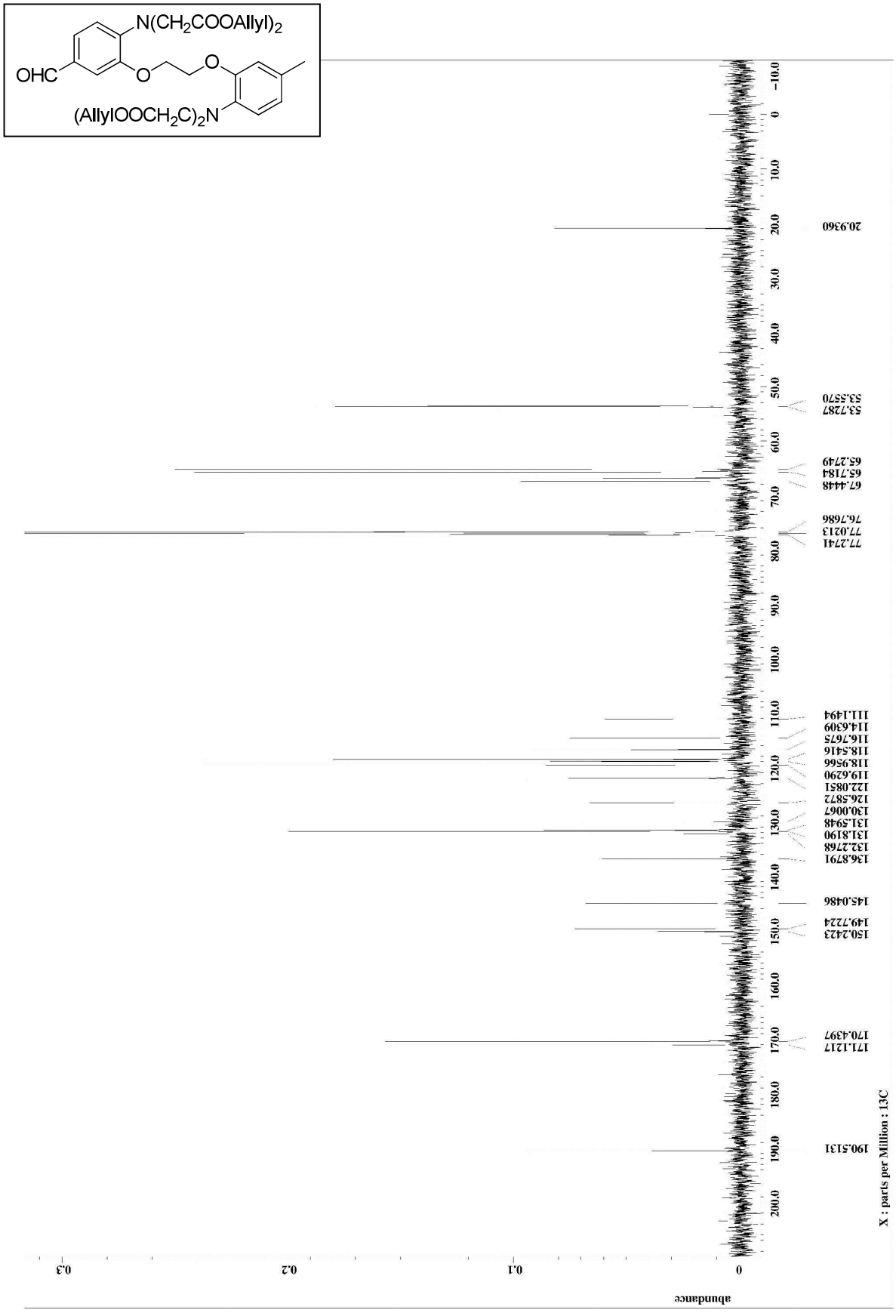


Fig. S4 (a) Bright-field and pseudocoloured images of Fluo-4-loaded HeLa cells with ATP stimulation (100 μM) at 30 s. Images were captured at 15 s, 45 s, and 120 s; Scale bar, 20 μm. (b) Time course of fluorescence change of KFCA. The arrowhead indicates the timing of ATP addition.





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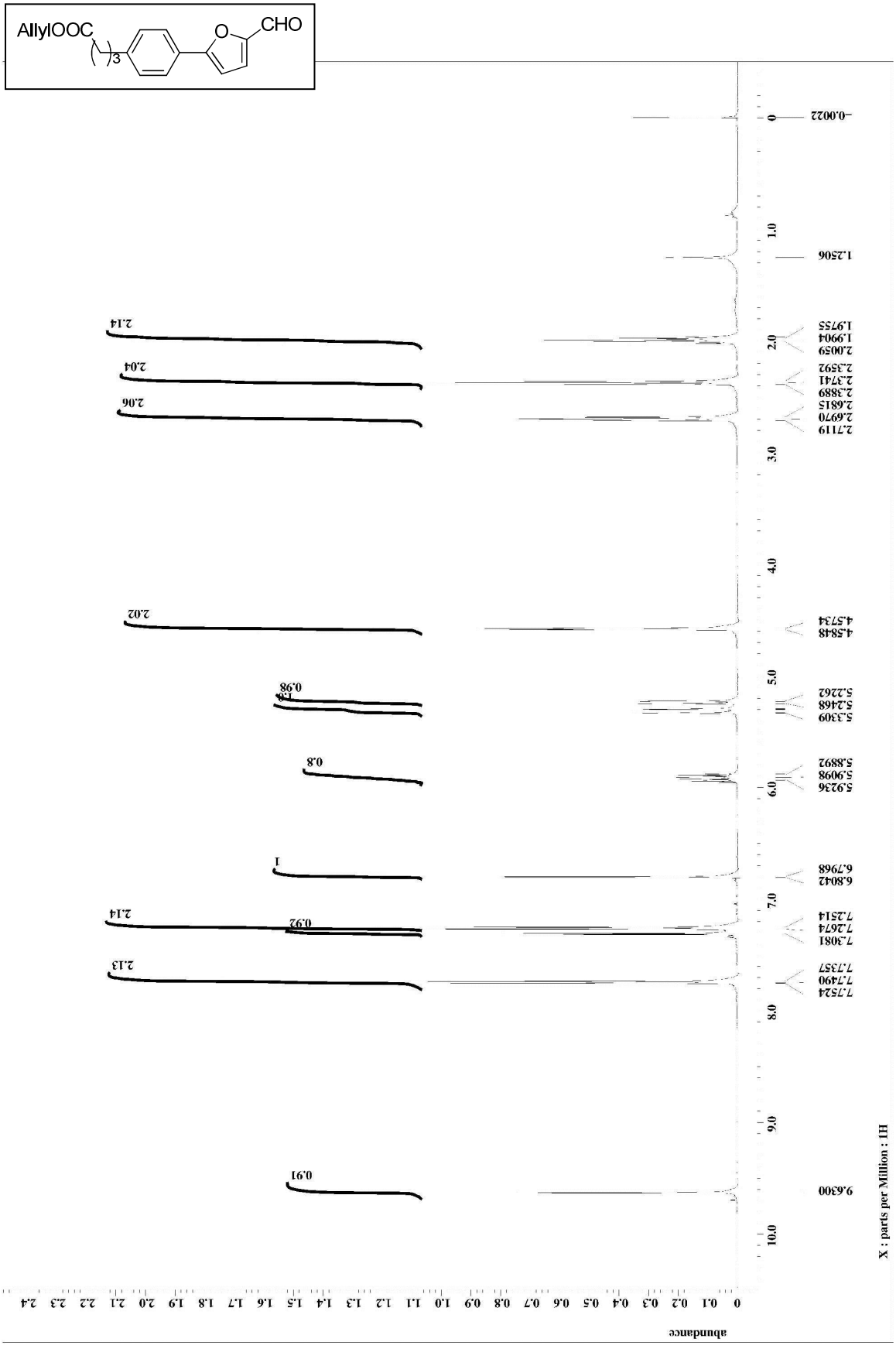
OHC
O
O
(AllylOOCH2C)2N
20.9360
53.7287
53.5570
65.2749
65.7184
67.4448
76.7686
77.0213
77.2741
111.1494
114.6309
116.7675
118.5416
118.9566
119.6290
122.0851
126.5872
130.0067
131.5948
131.8190
132.2768
136.8791
145.0486
149.7224
150.2423
170.4397
171.1217
190.5131
X : parts per Million : 13C
abundance


AllylOOC
3
O
CHO
abundance
X : parts per Million : 1H
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7.7524
7.7490
7.7357
7.3081
7.2674
7.2514
6.8042
6.7968
5.9236
5.9098
5.8892
5.3309
5.2468
5.2262
4.5848
4.5734
2.7119
2.6970
2.6815
2.3889
2.3741
2.3592
2.0059
1.9904
1.9755
1.2506
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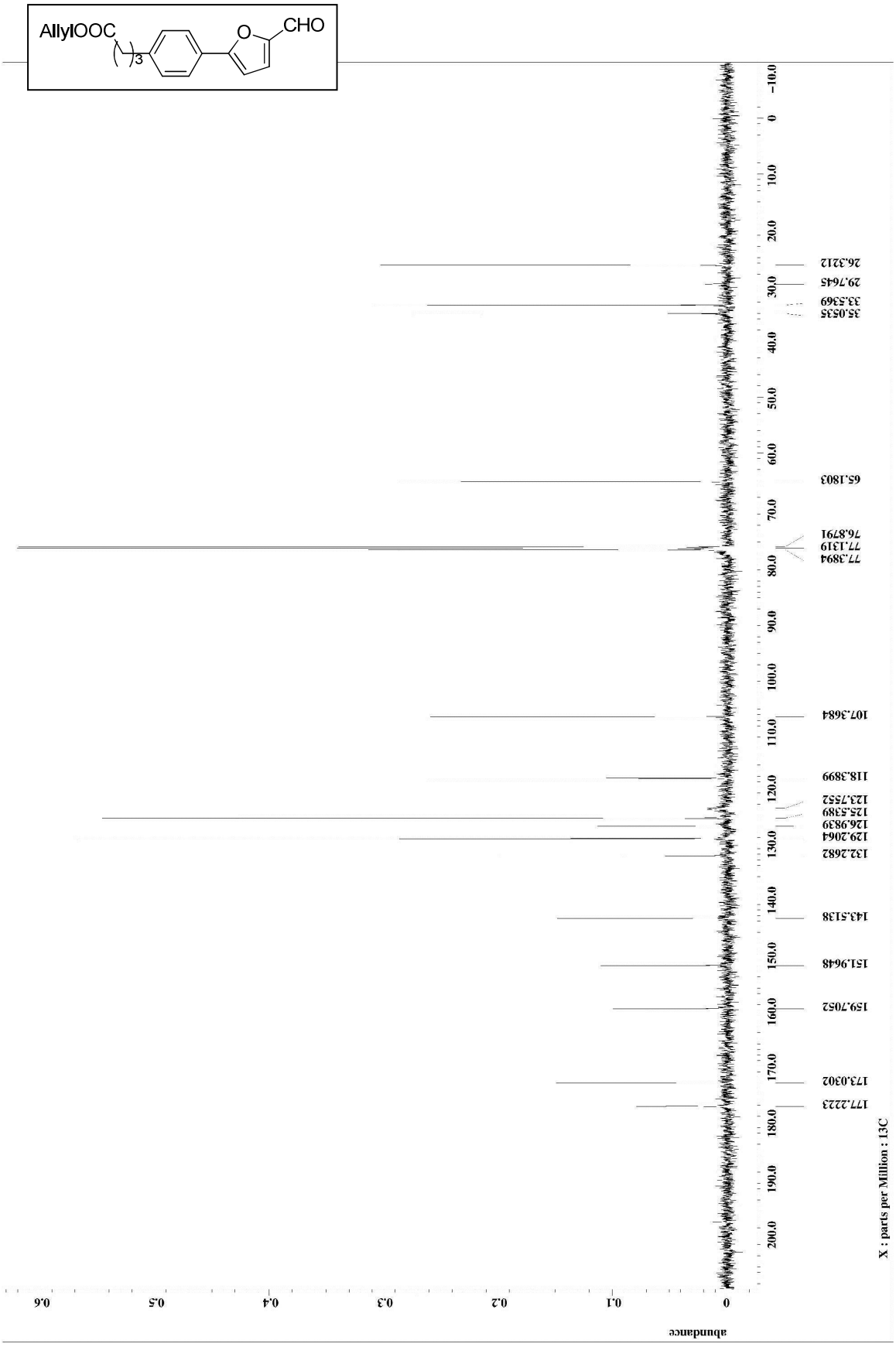
2.13
0.92
2.14
1
0.8
0.98
2.02
2.06
2.04
2.14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O
CHO
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77.3894
107.3684
118.3899
123.7552
125.5389
126.9839
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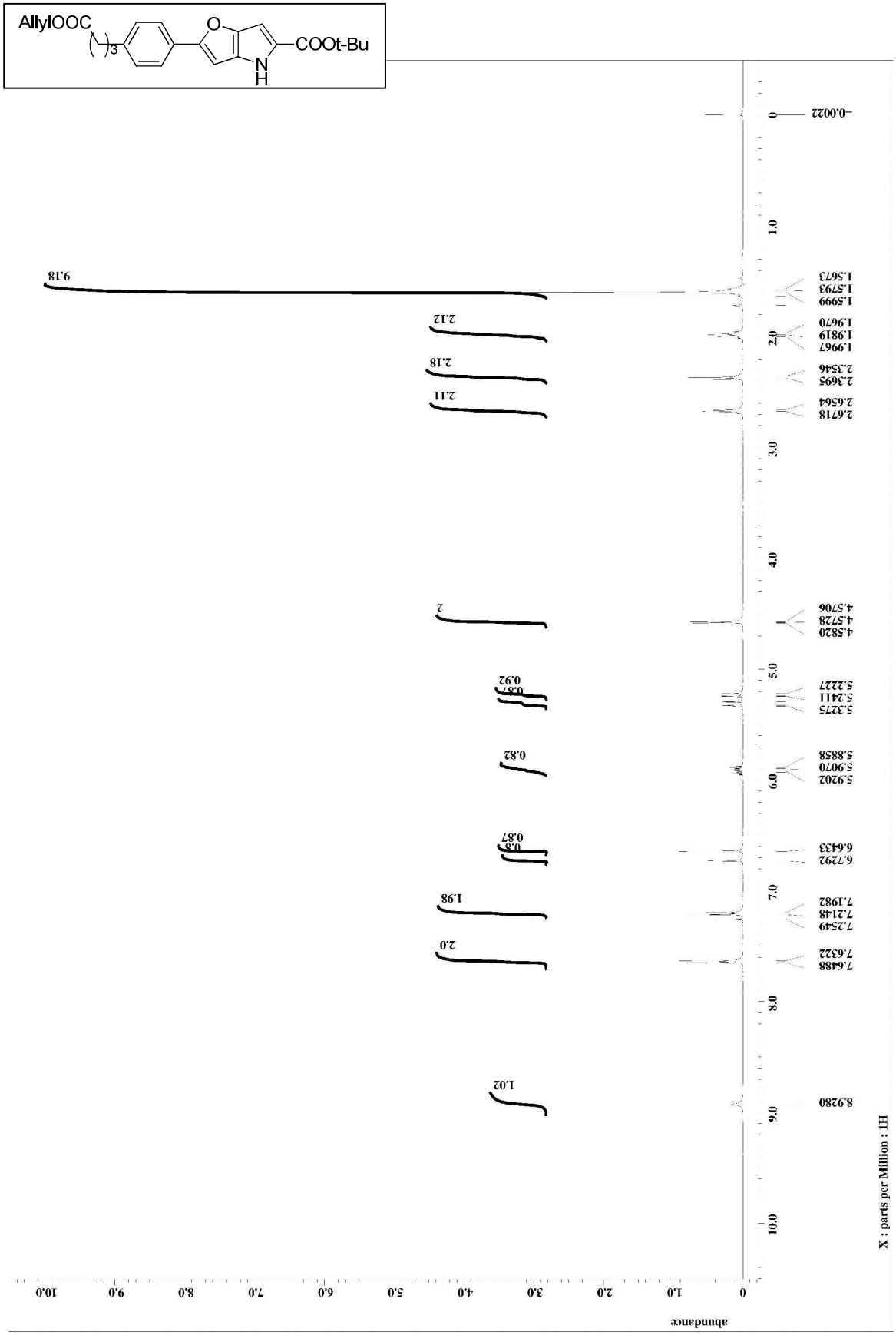
177.2223
X : parts per Million : 13C
abundance


AllylOOC
3
O
N
H
COOt-Bu
X : parts per Million : 1H
abundance
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2.12
2.18
2.11
2
0.92
0.87
0.82
0.87
1.98
2.0
1.02
8.9280
7.6488
7.6322
7.2549
7.2148
7.1982
6.7292
6.6433
5.9202
5.9070
5.8858
5.3275
5.2411
5.2227
4.5820
4.5728
4.5706
2.6718
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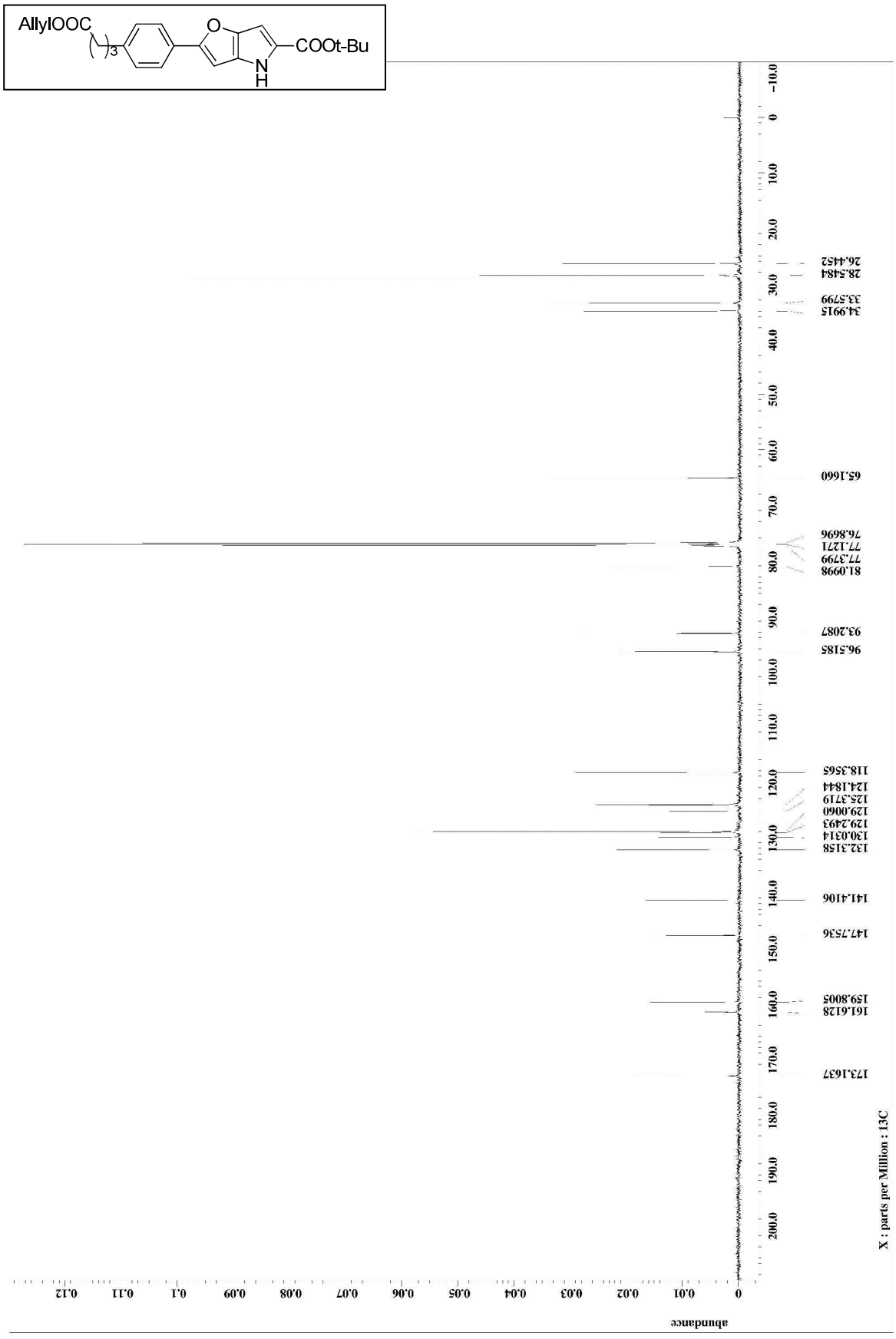
1.9967
1.9819
1.9670
1.5999
1.5793
1.5673
-0.0022


AllylOOC
3
O
N
H
COOt-Bu
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34.9915
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77.3799
81.0998
93.2087
96.5185
118.3565
124.1844
125.3719
129.0060
129.2493
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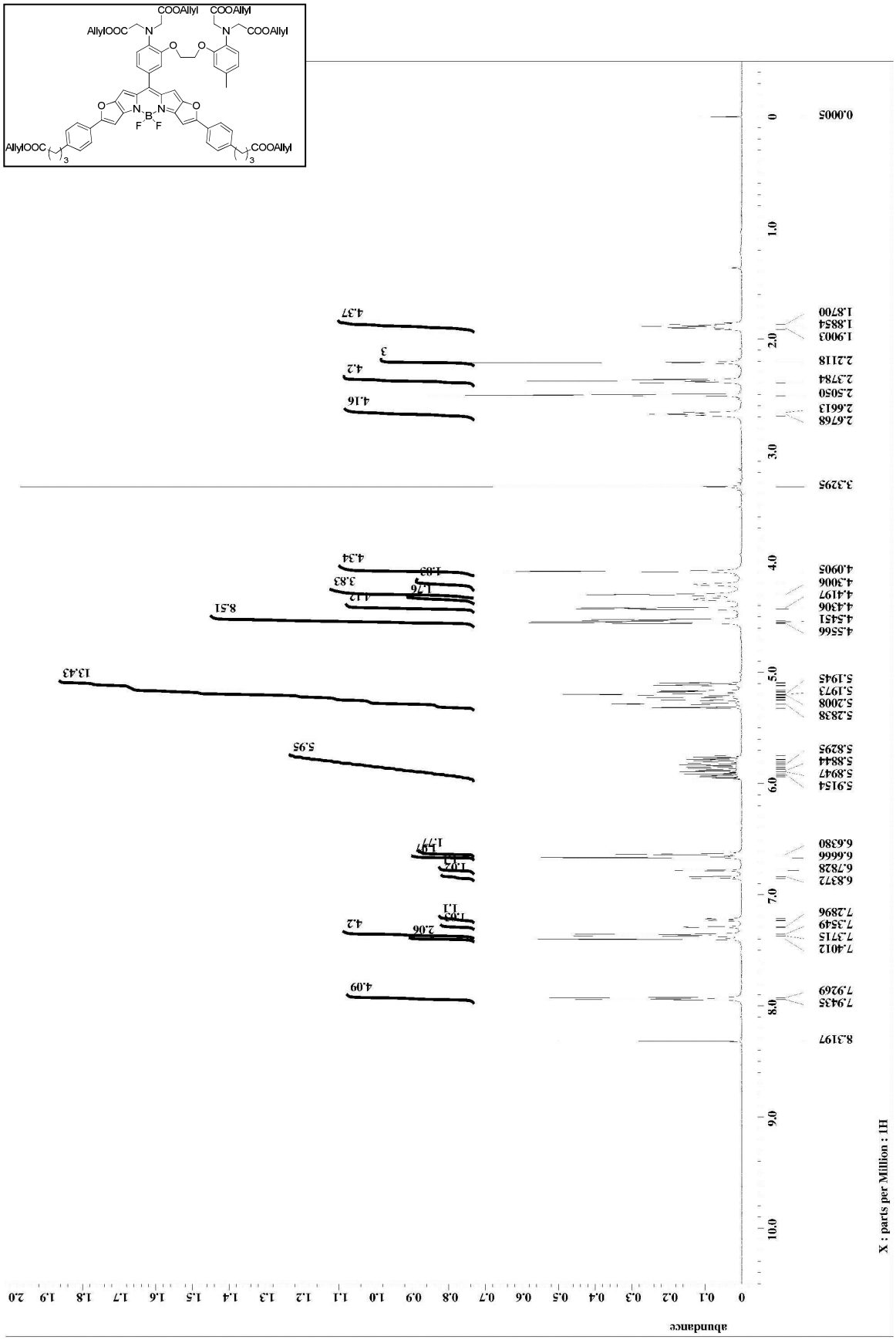
141.4106
147.7536
159.8005
161.6128
173.1637
X : parts per Million : 13C
abundance


COOAllyl
COOAllyl
AllylOOC
N
N
COOAllyl
O
O
O
N
B
F
F
N
O
AllylOOC
3
COOAllyl
3
X : parts per Million : 1H
abundance
0.0005
8.3197
7.9435
7.9269
7.4012
7.3715
7.3549
7.2896
6.8372
6.7828
6.6666
6.6380
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5.8947
5.8844
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5.1973
5.1945
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4.5451
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4.4197
4.3006
4.0905
3.3295
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2.6613
2.5050
2.3784
2.2118
1.9003
1.8854
1.8700
4.37
4.34
4.16
4.2
4.09
13.43
8.51
5.95
2.06
3
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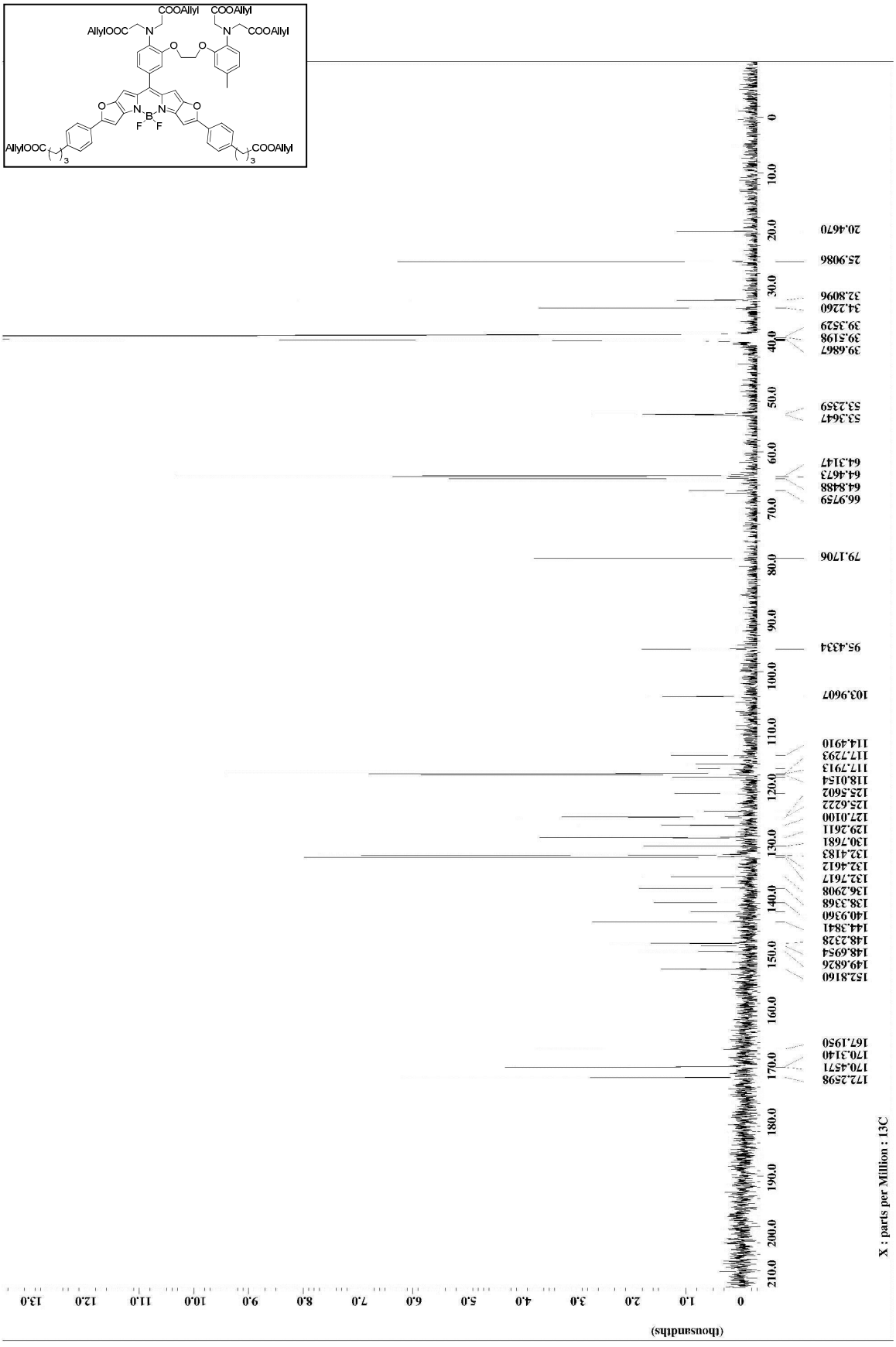
1.03
1.02
1.00
0.97
1.77
1.1


COOAllyl
COOAllyl
AllylOOC
N
N
COOAllyl
O
O
O
N
B
N
O
F
F
AllylOOC
3
3
COOAllyl
20.4670
25.9086
32.8096
34.2260
39.3529
39.5198
39.6867
53.2359
53.3647
64.3147
64.4673
64.8488
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95.4334
103.9607
114.4910
117.7293
117.7913
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125.5602
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127.0100
129.2611
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132.4183
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136.2908
138.3368
140.9360
144.3841
148.2328
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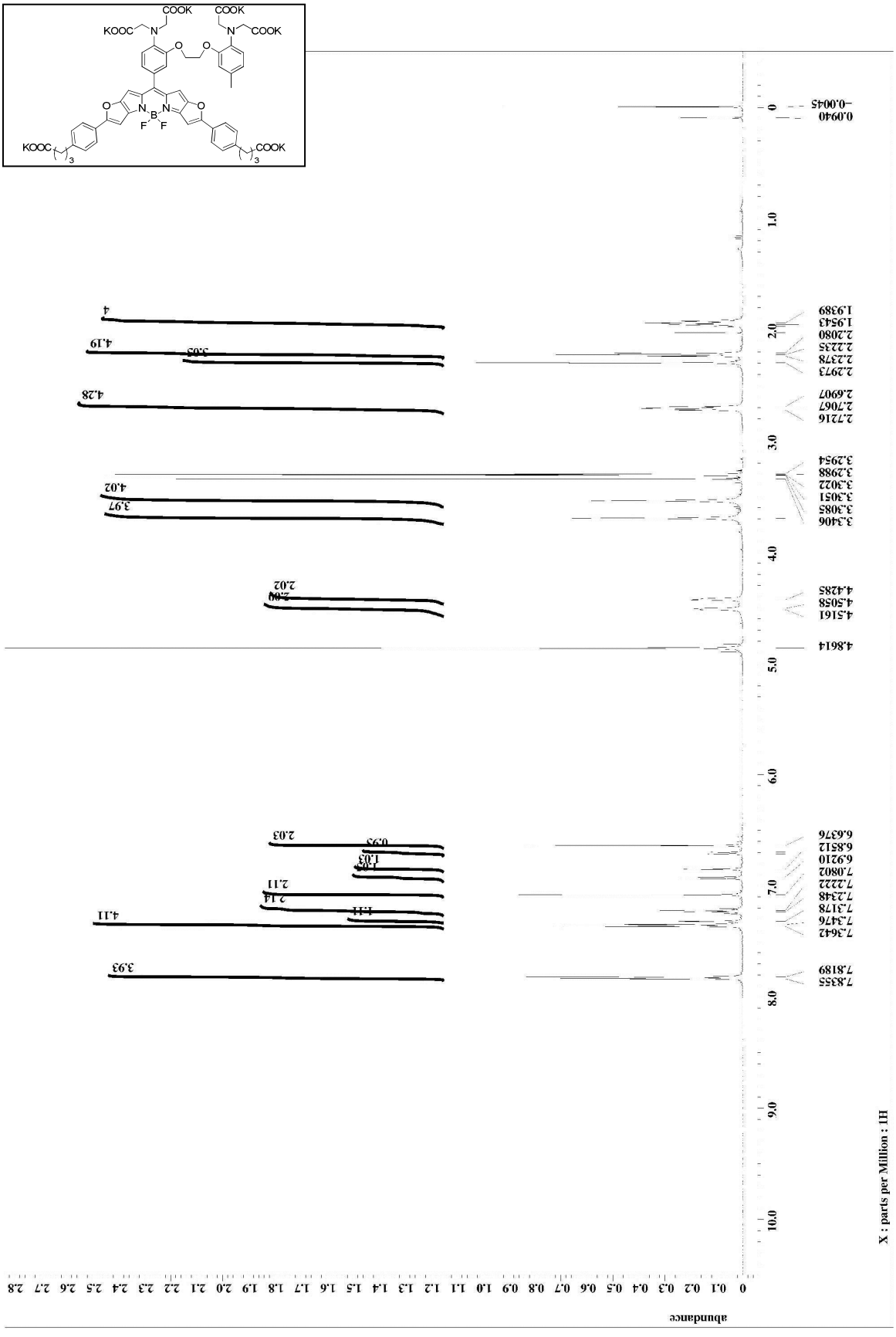
172.2598
X : parts per Million : 13C
(thousandths)


COOK
COOK
KOOC
N
N
COOK
O
O
N
B
N
F
F
O
O
KOOC
3
COOK
3
X : parts per Million : 1H
abundance
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1.9543
2.2080
2.2235
2.2378
2.2973
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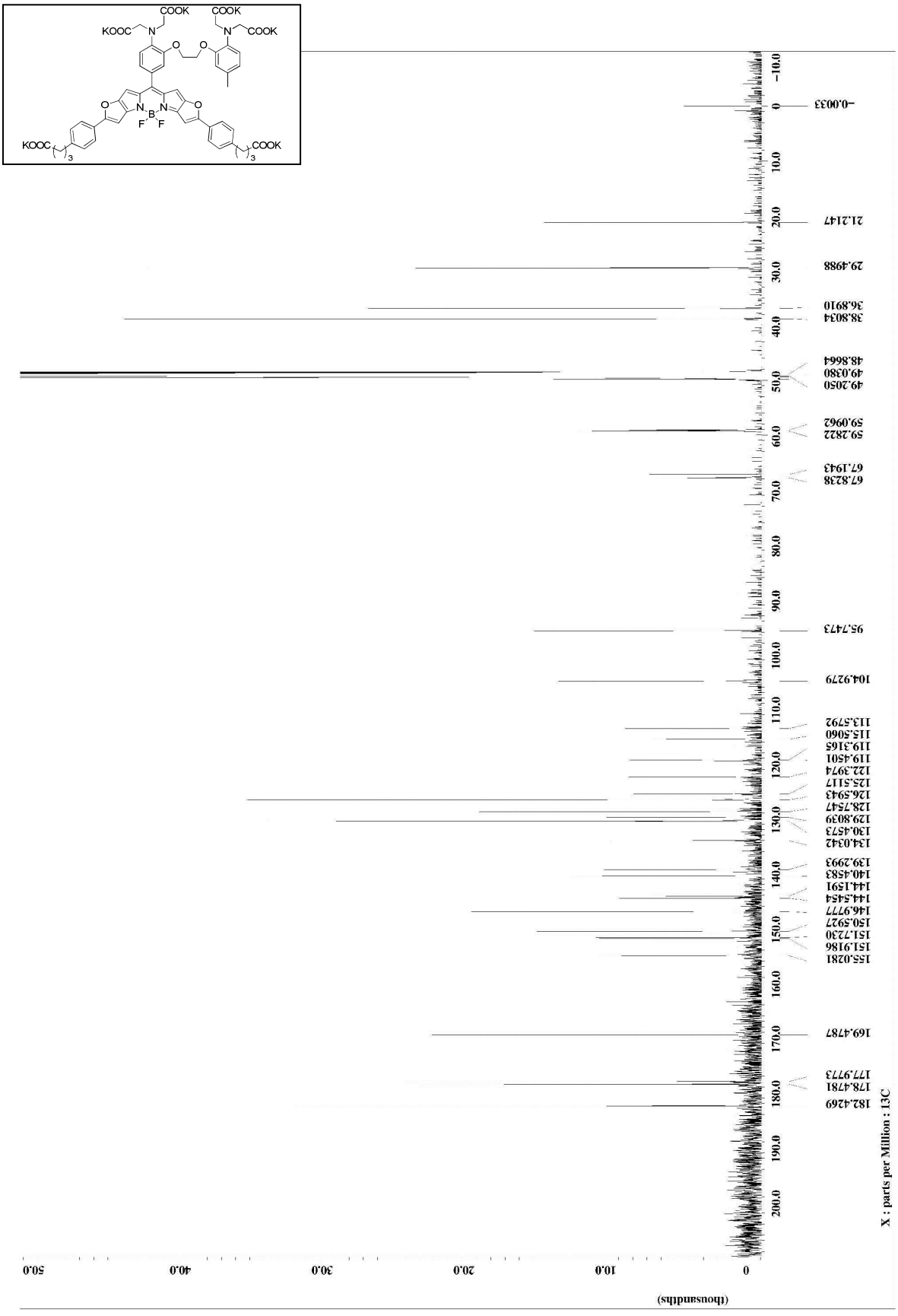
COOK
COOK
KOOC
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N
COOK
O
O
O
N
B
N
O
F
F
KOOC
3
COOK
3
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178.4781
182.4269
(thousandths)
X : parts per Million : 13C