Cellular Entry and Nuclear Targeting By a Highly Anionic Molecular Umbrella

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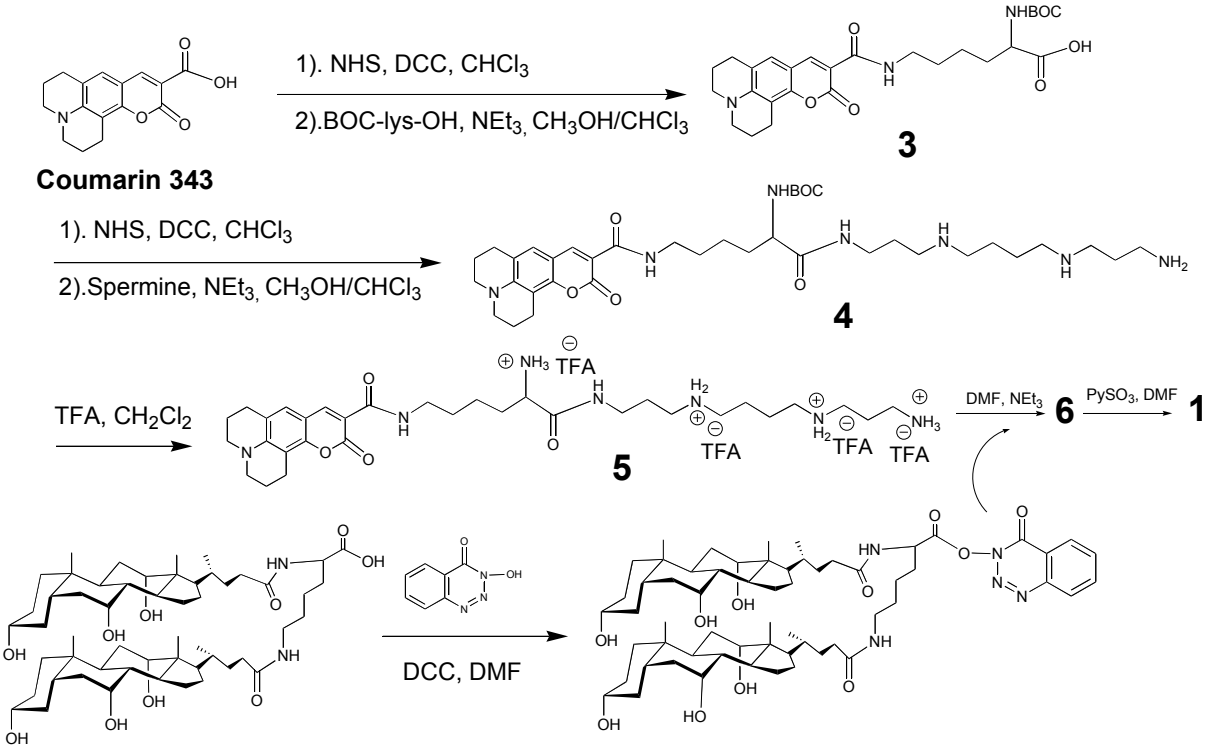
Supporting Information

Materials. Coumarin 343 (Sigma-Aldrich) and PRO 2000 (Indevus Pharmaceuticals, Lexington, MA) were used as obtained. BOC-lys-OH was obtained from Fluka.



Coumarin 343-BOC–lysine (3). To a solution that was made from 300 mg (1.05 mmol) of Coumarin 343, 145 mg (1.26 mmol) of N-hydroxyl succinimide and 20 mL of chloroform was added 260 mg (1.26 mmol) of DCC. After stirring for 3 h at room temperature, the insoluble urea was removed by filtration, and the filtrate was concentrated to ca. 10 mL. The resulting chloroform solution, which contained the NHS-ester of Coumarin, was then added to a solution that was made from 271 mg (1.1mmol) of BOC-lys-OH and 460 μL (3.3 mmol) of triethylamine in 4 mL of methanol. The reaction mixture was stirred at room temperature overnight. Removal of solvent under reduced pressure and purification of the crude product by silica gel column chromatography using chloroform/methanol (10:1, v/v, R_f=0.24) as the eluant afforded 274 mg (51%) of **5** as a yellow solid and ^{1}HNMR ($CDCl_3$): 1.40-2.0(m, 19H), 2.73(t, 2H), 2.83(t, 2H), 3.30-3.50(m, 6H), 4.29(br, 1H), 5.34(s, 1H, -OCON**H**-), 6.99(s, 1H), 8.66(s, 1H). MALDI-TOF MS: *m/z* **514** ($[M+H]^+$).

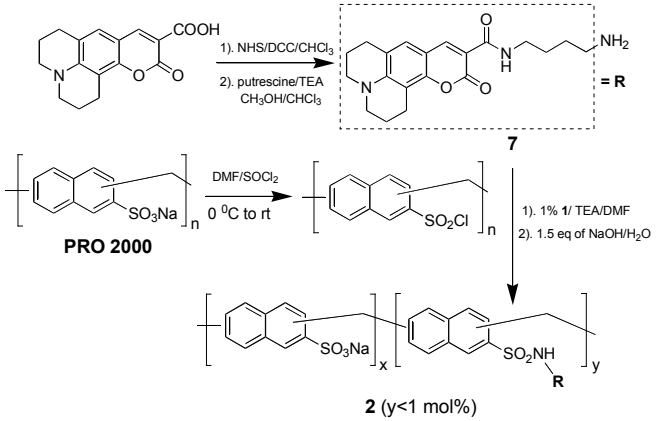
Coumarin 343-BOC-lysine-spermine (4). An activated form of **3** was prepared by dissolving 205 mg (0.4 mmol) of the conjugate in 20 mL of chloroform along with 51 mg (0.44 mmol) of N-hydroxysuccinimide and 91 mg (0.44 mmol) of DCC. After stirring at room temperature for 3 h, the insoluble urea was removed by filtration and the filtrate then added, dropwise, to a stirred solution that was made from 405 mg (2 mmol) of spermine, 167 μL (1.2 mmol) of triethylamine and 50 mL of methanol. The mixture was then stirred for 1 h at room temperature, and the solvent removed under reduced pressure. The resulting residue was purified by silica gel column chromatography using chloroform/methanol/ammonium hydroxide (30%) (60/40/10, v/v/v) as the eluant, affording 187 mg (67%) of **4** having a R_f of 0.28 and ^{1}HNMR (CD_3OD): 1.40-1.80(m, 23H), 1.96(m, 4H), 2.55-2.86(m, 14H), 3.18-3.29(m, 2H), 3.33-3.45(m, 6H), 3.95(s, 1H), 7.06(s, 1H), 8.45(s, 1H). MALDI-TOF MS: *m/z* **698** ($[M+H]^+$).

Coumarin 343-lysine-spermine-TFA salt (5). A solution was prepared by dissolving 151 mg (0.216 mmol) of **4** in 2 mL of a 1/1 mixture of trifluoroacetic acid/chloroform (v/v) at 0°C, and the mixture then stirred at room temperature for 5 h. Removal of solvent under reduced pressure and subsequent

freeze drying overnight afforded 230 mg (91%) of **5** having ^{1}HNMR (CD_3OD): 1.35-2.10(m, 18H), 2.72-2.78(m, 4H), 3.00-3.15(m, 10H), 3.30-3.50(m, 8H), 3.84(t, 1H), 7.03(s, 1H), 8.41(s, 1H). MALDI-TOF MS: *m/z* **598** ($[M+H]^+$).

Conjugate 6. A solution was prepared by dissolving 300 mg (0.323 mmol) of lysine dicholamide, 56 mg (0.344 mmol) of 3-hydroxy-1,2,3-benzotriazin-4(3H)-one, and 72 mg (0.349 mmol) of DCC in 1.5 mL of DMF.[1] The mixture was stirred at room temperature for 3 h, followed by the addition of 71 mg (0.0608 mmol) of **5** and 94 μL (0.674 mmol) of triethylamine. The resulting solution was stirred at room temperature overnight and then poured into 10 mL of an aqueous saturated sodium bicarbonate solution. The precipitate was collected by filtration and purified by silica gel column using chloroform/methanol/ammonium hydroxide (30%) (40/10/1, v/v/v) as the eluant to give 134 mg (52%) of **6**, which was further purified by preparative TLC (silica gel), using the same eluant as that which was used in the column chromatography. Conjugate **6** had an R_f=0.31 and ^{1}HNMR (CD_3OD): 0.69(s, 24H), 0.90-2.40(m, 282H), 2.79(t, 2H), 2.84(t, 2H), 3.16(m, 12H), 3.32-3.50(m, 22H), 3.8(s, 8H), 3.93(s, 8H), 4.16-4.40(br, 3H), 4.76(br, 2H), 7.13(s, 1H), 8.51(s, 1H). MALDI-TOF MS: *m/z* **4258** ($[M+Na]^+$).

Conjugate 1. To 1 mL of DMF was added 67 mg (0.018 mmol) of **6** and 182 mg (1.143 mmol) of pyridine sulfur trioxide at 0 ^{0}C. The reaction mixture was stirred at room temperature overnight and neutralized by addition of cold saturated sodium bicarbonate having a pH of ca. 10. After removal of solvent under reduced pressure, the residue was purified by silica gel column using chloroform/methanol/water (50/50/10, v/v/v) as the eluant to give 90 mg (85%) of **1** having R_f=0.42 ($CHCl_3/CH_3OH/H_2O$, 4/4/1, v/v/v) and ^{1}HNMR (CD_3OD): 0.75-2.40(m, 306H), 2.80(t, 2H), 2.86(t, 2H), 3.14(m, 12H), 3.33-3.50(m, 14H), 4.13(s, 8H), 4.26(br, 3H), 4.44(s, 8H), 4.65(s, 8H), 4.76(br, 2H), 7.15(s, 1H), 8.55(s, 1H).



1, 4'-Aminobutylcoumarin 343 amide (7). To a solution that was prepared by dissolving 200 mg (0.7 mmol) of Coumarin 343 in 10 mL of chloroform was added 100 mg (0.87 mmol) of N-hydroxylsuccinimide and 180 mg (0.87 mmol) of DCC. After stirring for 5 h at room temperature, the insoluble urea was removed by filtration. The filtrate was then diluted with 50 mL of chloroform and the resulting solution added in a dropwise manner (within 1 h) to a stirred solution made from 176 mg (1.4 mmol) of putrescine and 293 μL (2.1 mmol) of triethylamine in 100 mL of methanol. After stirring for 3 h at room temperature, the solvent was removed under reduced pressure and the residue purified by silica gel column chromatography using chloroform/methanol/ammonium hydroxide (30%) (50/10/1, v/v/v, R_f=0.7) affording 133 mg 53% of **7** having ^{1}HNMR (500 MHz, CD_3OD, rt): 1.78(m, 4H), 2.05(m, 4H), 2.81(t, 2H), 2.88(t, 2H), 3.03(t, 2H), 3.42(m, 4H), 3.50(m, 2H), 7.17(s, 1H), 8.57(s, 1H). MALDI-TOF MS: *m/z* **356** ($[M+H]^+$).

Fluorescent Labeling of PRO 2000 (2). The procedure that was used for activating PRO 2000 was similar to that used for forming chlorosulfonate derivatives of aromatic compounds.[2,3] Thus, a suspension that was prepared from 200 mg of PRO 2000 in 1 mL of DMF was cooled to 0°C, followed by addition of 200 μL (2.75 mmol) of thionyl chloride. After stirring for 10 min, the mixture was allowed to reach room temperature and stirred for an additional 5 h. The product mixture was poured into 25 mL of ice water, vortex mixed for 5 min, and centrifuged. The aqueous solution was then removed and an additional 25 mL of ice water added to the precipitate. Further centrifugation and

removal of the aqueous phase, followed by freeze drying overnight to give 180 mg of a chlorosulfonated form of the polymer as a pale powder.

To a solution that was prepared by dissolving 80 mg of this activated form of PRO 2000 in 1 mL of DMF was added 0.5 mL of a DMF solution containing 1.2 mg (0.00337 mmol) of **7**, (corresponding to less than 1% mol of the pendant sulfonate groups present in solution) plus 100 μL (0.717 mmol) of triethylamine. This solution was stirred at room temperature for 4 h, followed by addition of an alkaline solution that was prepared by dissolving 25 mg (0.628 mmol) of NaOH in 1 mL of water. After stirring the solution for an additional 2 h, the solvent was removed under reduced pressure. The residue was dissolved in 1 mL of water and passed through a C-18 reverse-phase column using pure water as eluant. The first fraction (yellow) that was eluted from the column was concentrated under reduced pressure. Additional purification of this residue was carried out by preparative thin layer chromatography (silica gel, two chromatographs) using chloroform/methanol/water (40/40/10, v/v/v) as the eluant. The polymer was then dialyzed against pure water for 24 h, using dialysis tubing having a 500 MW cutoff. Concentration of the dialyzed product under reduced pressure afforded 53 mg of **2** as a yellow powder.

Binding to Liposomal Membranes. Binding of **1** to liposomal membranes was measured via an equilibrium dialysis cell having 2.0 mL compartments (Fisher Scientific, Bel-Art Scienceware), using a Nuclepore membrane (100 nm diameter pores) to separate the two compartments. To the "source" side was added 0.5 mL of a 500 μM solution of **1** in PBS plus 0.5 mL of a liposomal dispersion made from 10 mg of POPC/POPG (95/5, mol/mol, 200 nm diameter, extrusion) in PBS. To the "receiving side" was added 1.0 mL of PBS. The UV absorption (460 nm) in the "receiving" side of the cell was then monitored as a function of time at room temperature with gentle rocking of the cell until equilibrium was reached (24 h). Under these conditions, the amount of the molecular umbrella that was bound to the liposomes corresponded to 0.002 mole of **1** per mole of phospholipid.

Cell Entry Experiments. HeLa (human epithelial cervical carcinoma) cells were cultivated in Minimum Essential Medium (MEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS) at

37°C in a humidified atmosphere containing 5% CO_2. For all experiments, exponentially growing cells were detached from the culture flasks using a trypsin-0.125% EDTA solution, counted using a hemocytometer and the trypan blue dye. The cell suspension was seeded at a concentration of 20×10^4 cells/cm^2 onto a 42 mm glass round coverslip (Hemogenix). Experiments were carried out 24 h later, at a confluence level of approximately 60%-70%. To ensure consistency in uptake, cells were passaged biweekly and discarded after 20 passages.

Compound **1** or **2** was dissolved in PBS Ca^{2+} and Mg^{2+} free (Invitrogen) at a concentration of 150 μM. Before all experiments, compound **1** or **2** stock solutions were diluted to a concentration of 4 μM in 2 mL of DMEM (Invitrogen) lacking both phenol red and fetal bovine serum.

In a typical experiment, the coverslip with the monolayer of HeLa cells was mounted in a POC chamber (PeCon GmbH, Erbach, Germany) and washed three times with 2 mL PBS of Ca^{2+} and Mg^{2+} free to remove nonadherent cells. The attached cells were then labeled with 1 μM Styo-59 (Molecular Probes) in 2 mL of DMEM at 37 °C in CO_2 atmosphere for 20 min. Cells were imaged to measure their autofluorescence prior a cellular entry experiment. The medium was then discarded and the cells were incubated with 2 mL of 4 μM of compound **1** or **2** in DMEM at 37 °C for the indicated time prior image acquisition.

Confocal laser scanning microscopy was performed with an Olympus Fluoview 1000 confocal microscope (Olympus FV 1000, Tokyo, Japan) using a 60X/1.4 NA plan-apochromatic objective. Coumarin 343 was excited by both the 458 nm and 488 nm lines from an argon ion laser. Syto-59 was excited using a 543 nm HeNe laser. The microscope settings were maintained identical for both compounds. Images, usually 1024 x 1024 or 512 x 512 pixels, were acquired using 2-line mean averaging. Optical sections have a depth of 0.75 μm. The background from a region lacking cells was subtracted from each image. The fluorescence intensity (measured using Image J software) within each cell was measured from single optical sections located at the center of the cell (Z dimension). The autofluorescence of the cells was subtracted from each measurement. For presentation, image stacks

were converted to maximum intensity projections using the Olympus Fluoview ver.1.6 viewer software. The Fluoview software was also used to rotate 3-D projections to visualize the X – Z dimension. Images were exported as TIFF files and printed using Photoshop 7.0 (Adobe).

Similar procedures were used for experiments in which cells were depleted of ATP.[4-6] In this case, the cells were incubated in saline containing 5 mM NaN_3 and 1 mM 2-deoxyglucose for 4-5 minutes.[6] The efficiency of inhibition of energy-dependent endocytosis was determined by incubation in saline containing 10 mM Alexa Fluor 488-labeled transferrin (5 mM stock solution in 1x PBS; Molecular Probes) for 3 min, fixation in 2% *p*-formaldehyde, and microscopic examination of either ATP-depleted or untreated cells.

(1) Chen, W. H.; Shao, X.B.; Regen, S. L. *J. Am. Chem. Soc.*, **2005**, *127*, 12717-12735.

(2) Bosshard, H. H.; Mory, R.; Schmid, M.; Zollinger, Hch. *Helv. Chim. Acta*, **1959**, *42*, 1653-1658.

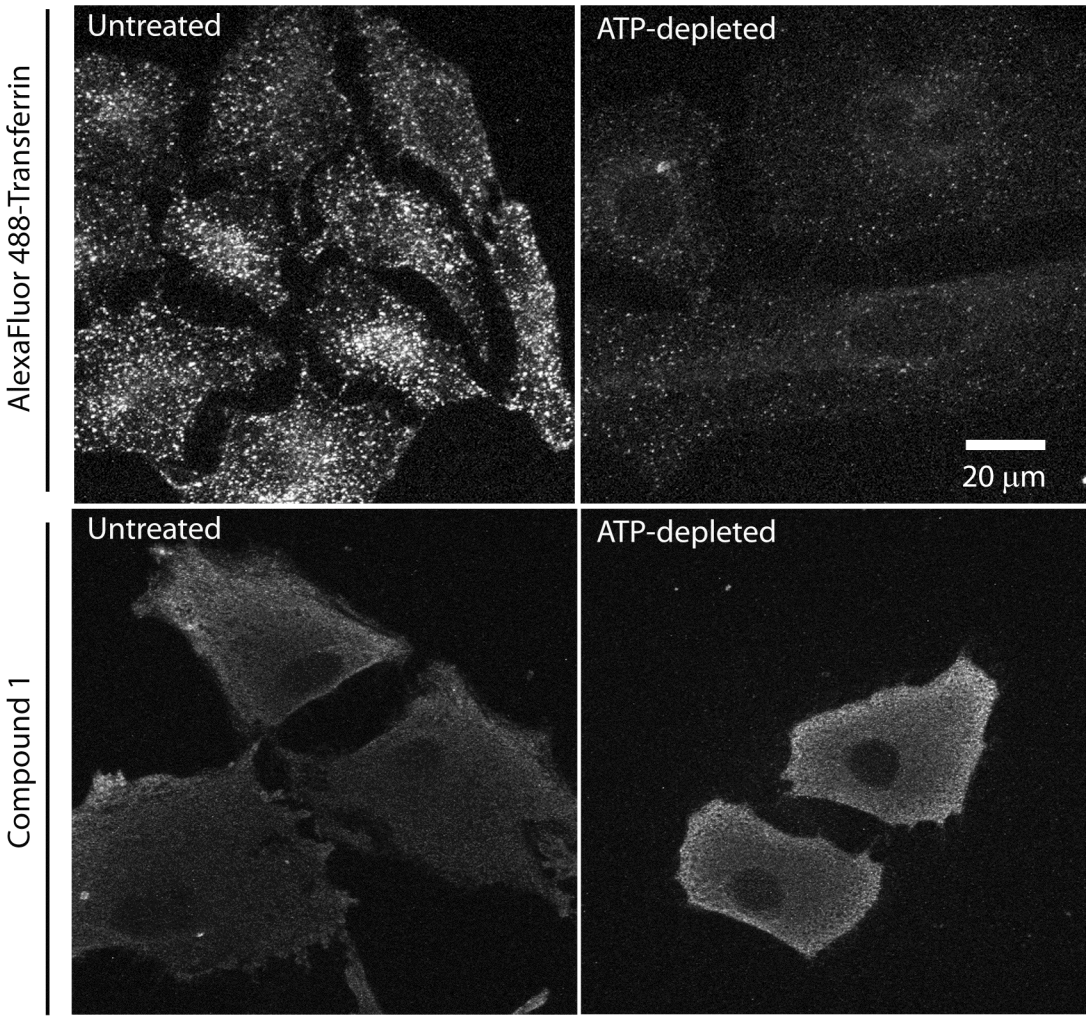
(3) Loewenthal, H. J. E.; Gottlieb, L. *J. Org. Chem.*, **1992**, *57*, 2631-2641.

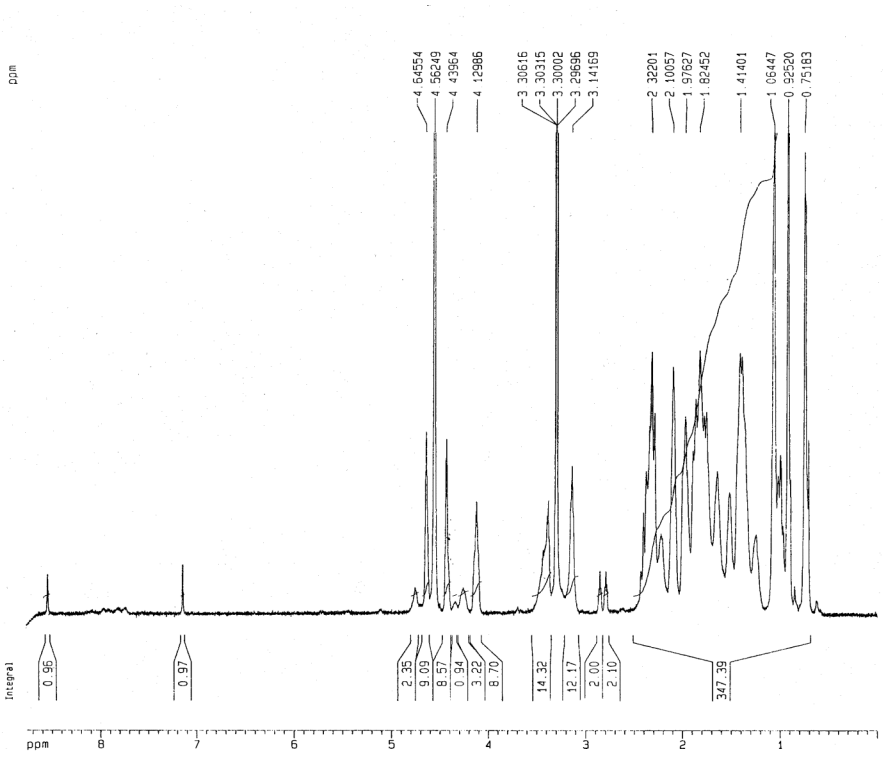
(4) Cassimeris, L.; Morabito, J. *Mol. Biol. Cell.*, **2004**, *15*, 1580-1590.

(5) Piehl, M.; Cassimeris, L. *Mol. Biol. Cell.*, **2003**, *14*, 916-925.

(6) Howell, B.; Hoffman, D. B.; Fang, G.; Murray, A. W.; Salmon, E.D. *J. Cell Biol.*,

Effectiveness of ATP depletion on cellular uptake of AlexaFluor 488-Transferrin:





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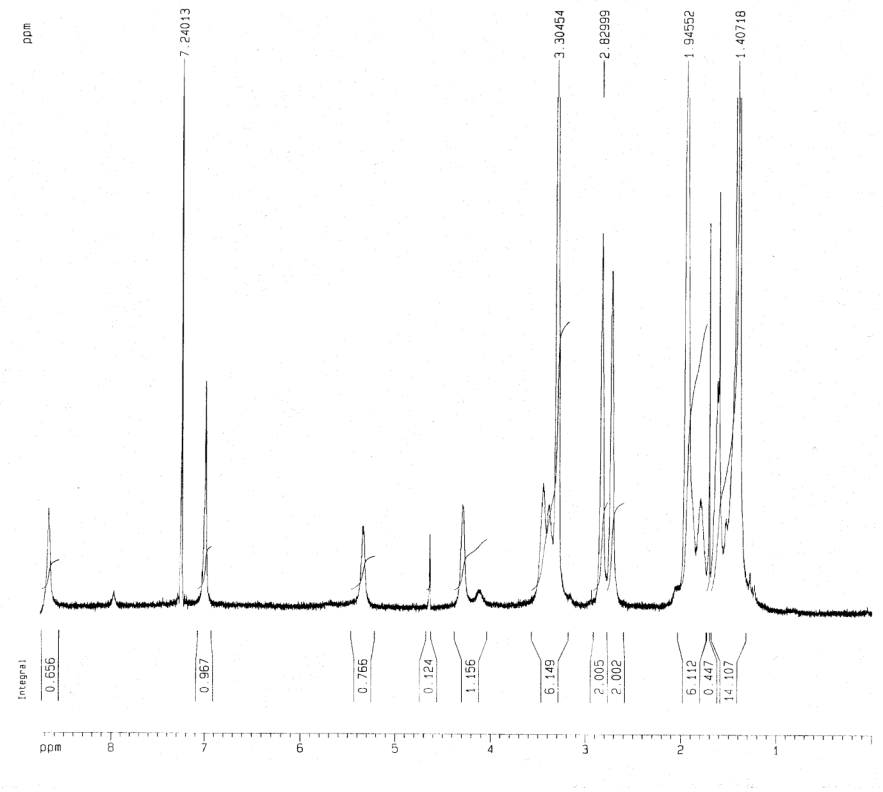

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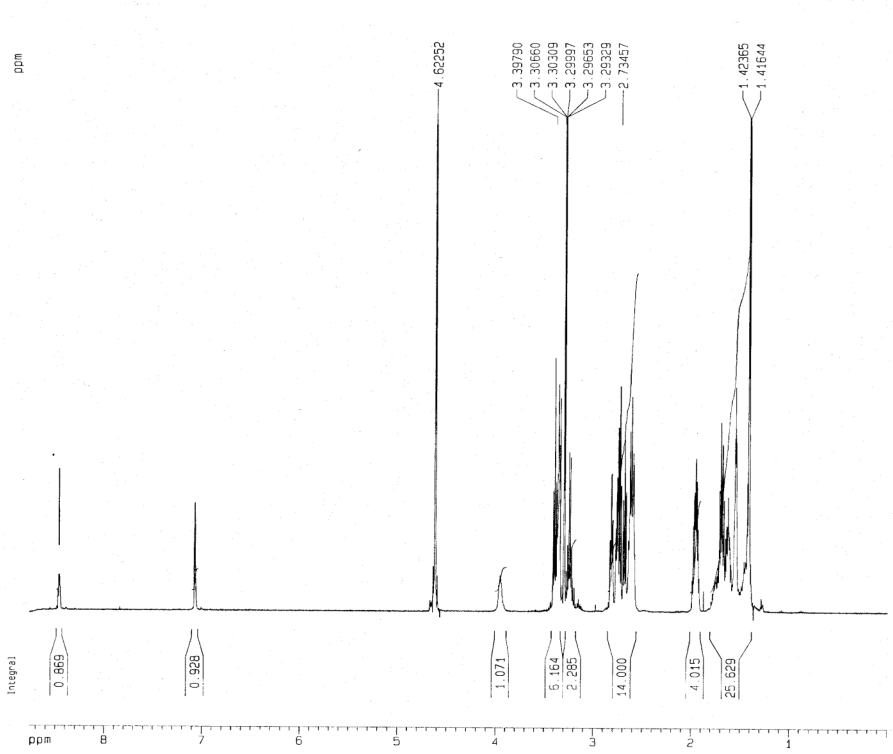


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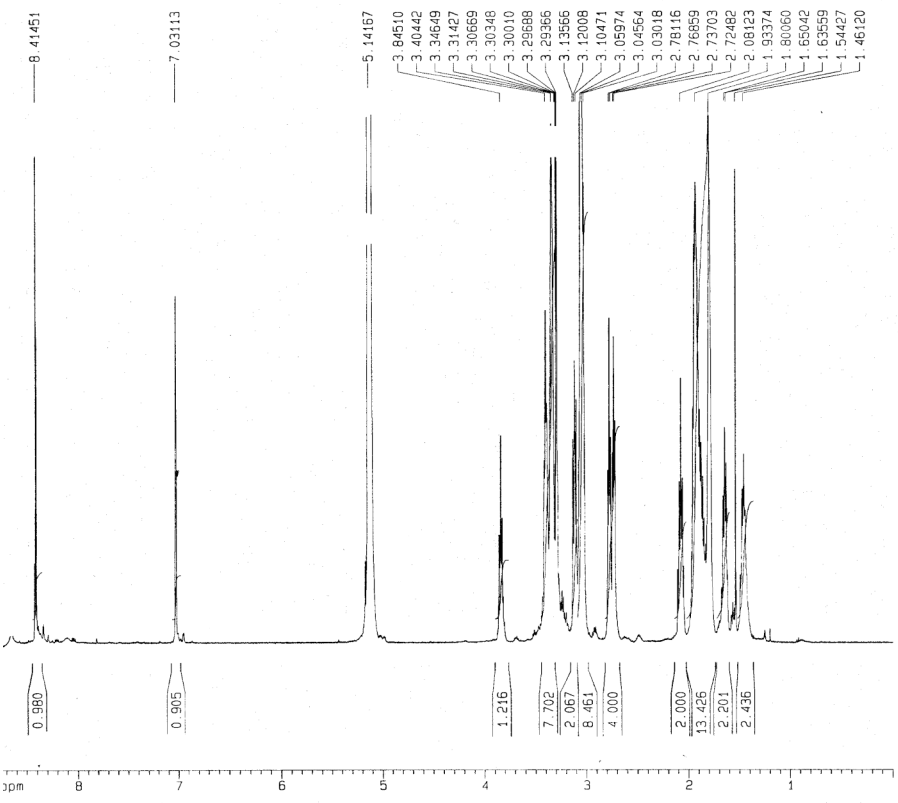


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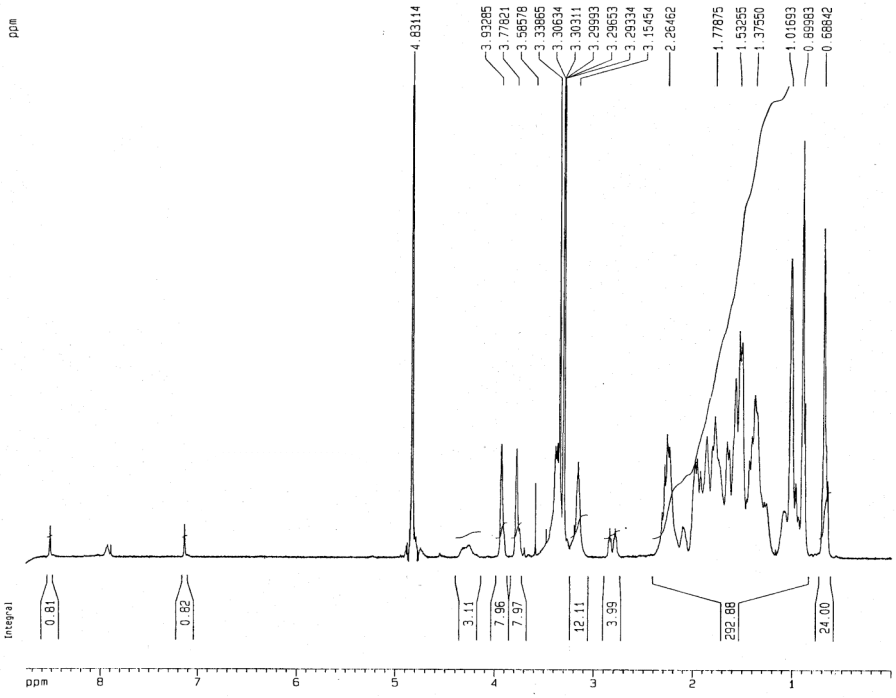


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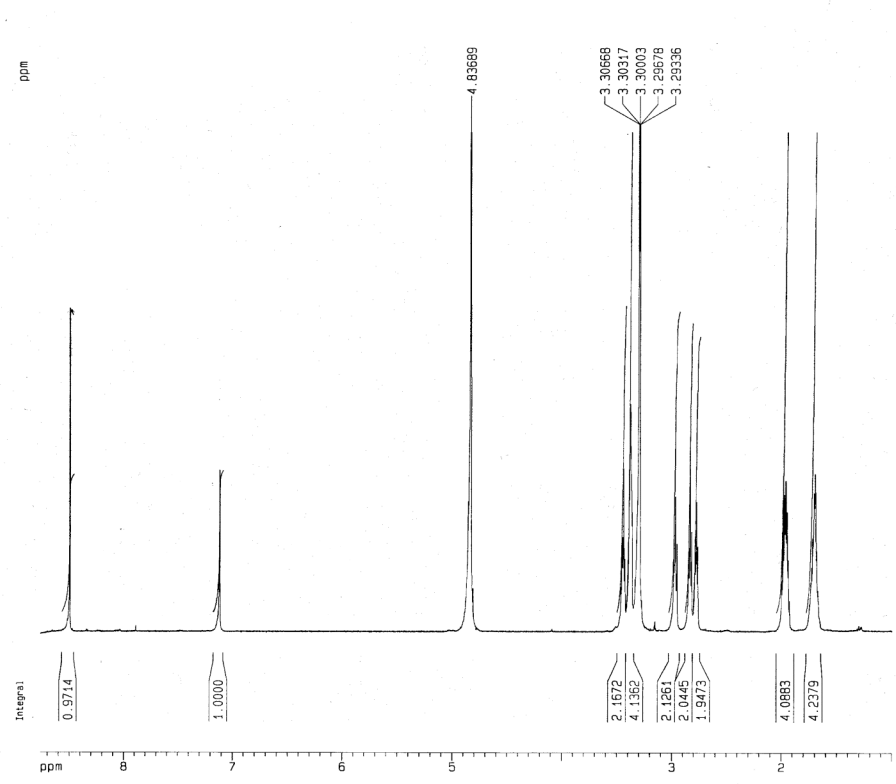


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7

Low Res. Mass Spectrum for Compound 1