Electronic Supplementary Information (ESI)

A mitochondria-targeted protonophoric uncoupler derived from fluorescein

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Contents

Abbreviations

mitoFluo, mitofluorescein, 10-[2-(3-hydroxy-6-oxo-xanthen-9-yl)benzoyl]oxydecyl-triphenyl-phosphonium bromide; TPP, triphenylphosphonium; TPP^+, tetraphenylphosphonium; C_8-FL, fluorescein octyl ester; CCCP, carbonyl cyanide m-chloro phenyl hydrazone; TMRE, tetramethylrhodamine ethyl ester; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ANT, adenine nucleotide translocase; BLM, bilayer lipid membrane; DPhyPC, 1,2-diphytanyl-sn-glycero-3-phosphocholine; IMM, inner mitochondrial membrane; RLM, rat liver mitochondria; FCS, fluorescence correlation spectroscopy.

Materials and methods

General comments

^{1}H, ^{13}C and ^{31}P - NMR spectra were obtained on a Bruker AV-500 spectrometer operating at 600, 151 and 162 MHz respectively. ESI-MS measurements were carried out with an Acquity UPLC System (Waters, Milford, MA, USA) and TQD (Waters, Milford, MA, USA). Fluorescence spectra were recorded with a Panorama Fluorat 02 spectrofluorometer (Moscow, Russia).

Materials

Tetramethylrhodamine ethyl ester (TMRE) was from Invitrogen. *E. coli* total lipid extract was from Avanti Polar Lipids (Alabaster, Alabama). 1,10-dibromodecane, triphenylphosphine, rotenone, phloretin and carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) were from Sigma. Fluorescein was from Reakhim.

Planar bilayers

Bilayer lipid membrane (BLM) was formed by the brush technique[1] from a 2% decane solution of 1,2-diphytanyl-sn-glycero-3-phosphocholine (DPhyPC) or *E. coli* polar lipid extract on a 0.6-mm aperture in a Teflon septum separating the experimental chamber into two compartments of equal 3-ml volumes. Electrical parameters were measured with two AgCl electrodes placed into the solutions at both sides of the BLM via agar bridges, using a Keithley 428 amplifier (Cleveland, Ohio, USA).

Isolation of rat liver mitochondria

Rat liver mitochondria were isolated by differential centrifugation[2] in a medium containing 250 mM sucrose, 10 mM MOPS, 1 mM EGTA, pH 7.4. The final washing was performed in the medium additionally containing bovine serum albumin (0.1 mg/ml). Protein concentration was determined using the Biuret method. Handling of animals and experimental procedures were conducted in accordance with the international guidelines for animal care and use and were approved by the Institutional Ethics Committee of A.N. Belozersky Institute of Physico-Chemical Biology at the Moscow State University.

Mitochondria respiration and mitochondrial membrane potential measurements

Respiration of isolated mitochondria was measured using a standard polarographic technique with a Clark-type oxygen electrode ("Strathkelvin Instruments", UK) at 25˚C using 782 system software. The incubation medium contained 250 mM sucrose, 5 mM MOPS, 1 mM EGTA, pH 7.4. Mitochondrial protein concentration was 0.4 mg/ml. Membrane potential was measured with the help of a tetraphenylphosphonium (TPP^+)-sensitive electrode (NIKO-ANALIT, Moscow, Russia) in the medium described above plus 5 mM KH_2PO_4. TPP^+ measurements were accompanied by measurements of respiration by a Clark-type electrode in a home-made two-electrode setup similar to that described in[3].

Binding to mitochondria measured by fluorescence correlation spectrometry (FCS)

FCS experimental setup. The home-made setup was described previously in[4]. Briefly, excitation of fluorescence and detection utilized a Nd:YAG solid state laser with a 532-nm beam attached to an Olympus IMT-2 epifluorescent inverted microscope equipped with a 40x, NA 1.2 water immersion objective (Carl Zeiss, Jena, Germany). The fluorescence light passed through an appropriate dichroic beam splitter and a long-pass filter and was imaged onto a 50-μm core fiber coupled to an avalanche

photodiode (SPCM-AQR-13-FC, PerkinElmer Optoelectronics, Vaudreuil, Quebec, Canada). The signal from an output was sent to a PC using a fast interface card (Flex02-01D/C, Correlator.com, Bridgewater, NJ). The data acquisition time was 30 s. The fluorescence was recorded from the confocal volume located at about 50 μm above the coverslip surface with 50 μl of the buffer solution added. Most of the data were collected under the conditions of stirring a suspension by a paddle-shaped 3-mm plastic bar rotated at 600 rpm. To calibrate the setup, we recorded the autocorrelation function of fluorescence of a solution of Rhodamine 6G which was characterized by the correlation time τ_D estimated from the equation (**1**). Assuming the diffusion coefficient of the dye to be 2.5 x 10^{-6} cm^2/s, the value of the confocal radius ω=0.42 μm was obtained. The correlated fluorescence emission signals were fitted to the three-dimensional autocorrelation function[5;6].

$$G(\tau) = 1 + \frac{1}{N}\left(\frac{1}{1+\frac{\tau}{\tau_D}}\right)\left(\frac{1}{\sqrt{1+\frac{\omega^2\tau}{z_0^2\tau_D}}}\right) \qquad (\mathbf{1})$$

with τ_D being the characteristic correlation time during which a molecule resides in the observation volume of radius ω and length z_0, given by $\tau_D = \omega^2/4D$, where D is the diffusion coefficient, N is the mean number of fluorescent particles in the confocal volume.

Treatment of the fluorescence signal (PIA procedure). Fluorescence traces with the sampling time of 25 μs were analyzed using WinEDR Strathclyde Electrophysiology Software designed by J. Dempster (University of Strathclyde, UK). The software, originally designed for the single-channel analysis of electrophysiological data, enables one to count the number of peaks ($n(F>F_0)$) of the FCS signal having amplitudes higher than the defined value (F_0)[4].

Confocal fluorescence microscopy

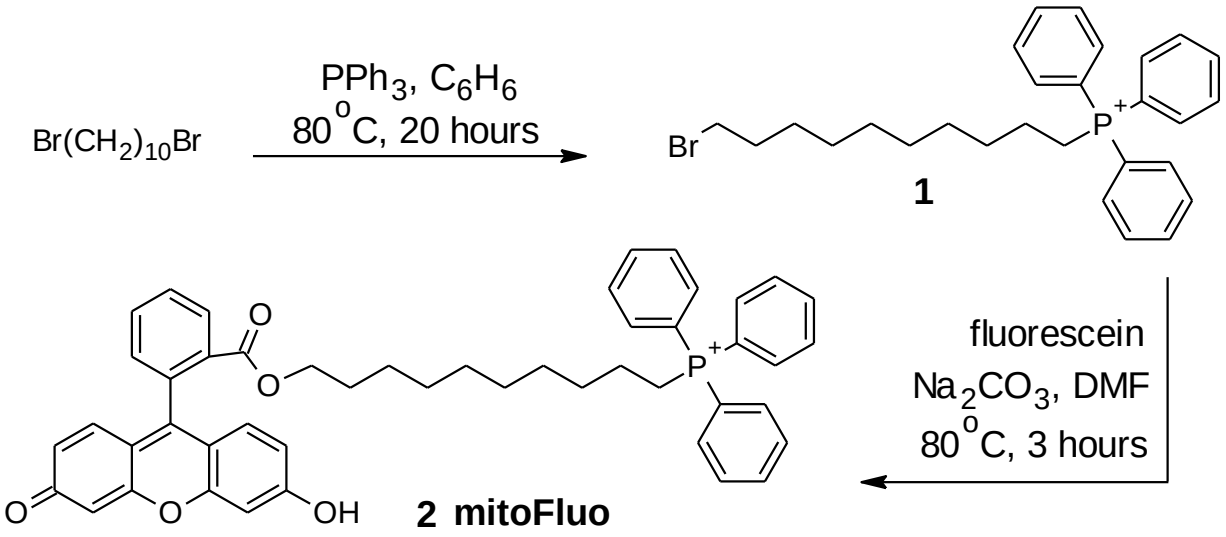
Renal tubular cells cultures were obtained from 3–7 day old rats, as described in[7]. Cells were cultivated in a CO_2 (5%) incubator in glass-bottom dishes for 1–2 days before the experiments. Cells were first incubated with 200 nM TMRE (Invitrogen, USA) for 30 min, followed by washing in DMEM/F-12 medium. Next the mitoFluo solution was added to the cells to a concentration of 1 μM and incubated for 10 min at 37°C in DMEM/F-12 medium containing 10 mM Hepes-NaOH. Then renal tubular cells were imaged with an LSM510 inverted confocal microscope (Carl Zeiss Inc., Jena, Germany). Analysis of localization of fluorophores was performed in glass-bottom dishes (MatTek Corp., USA) with excitation at 488 and 543 nm and emission collected at 510–530 and 560–590 nm, for MitoFluo and TMRE, correspondingly. To minimize contribution of photo-induced mitochondria and/or cell damage to relative fluorescence intensities, image analysis was performed on the average of the first four scans only. Images were processed using ImageJ software (NIH, Bethesda, MD, USA).

MTT assay

Cell viability was evaluated by a widely used methyl thiazol tetrazolium (MTT) test. Micromodification of the method was used for 96-well plates for microcultivation[8]. Briefly, cells were incubated over 24 hours with an appropriate concentration of mitoFluo in DMEM/F12 culture medium without serum. After the incubation, cells were washed twice with the medium, and the MTT solution was added (1 mg/ml in DMEM/F12) for 3 hours at 37˚C. Then the dye solution was removed, 100 μl DMSO per well was added and absorbance at 540 nm was measured. Experiments were performed twice, in which 6 wells per each concentration were analyzed.

Synthesis of mitoFluo

Scheme S1



Synthesis of 10-bromdecyl-triphenyl-phosphonium bromide (1)

A solution of 1,10-dibromdecane (2.17 g, 7.2 mmol.) and triphenylphosphine (1.25 g, 4.7 mmol) in benzene (0.5 ml) was heated at 80°C during 20 hours in a tightly closed flask. After completing the reaction, the reaction mass was cooled at 20°C and transferred to a round-bottom flask with dichloromethane to evaporate to dryness. The residue obtained was dissolved in a minimal volume of dichloromethane, then an excess of diethyl ester was added and the suspension formed was kept at 4°C until the solution became clear. Then the liquid phase was decanted, the residue was dissolved again in dichloromethane and treated with diethyl ether to complete precipitation. This procedure was repeated three times. Finally the residue was dissolved in a minimal volume of the solvent system ethanol-dichloromethane (1:5) and applied to a chromatographic silica gel column (MN Kieselgel 60,240-400 mesh) in the same solvent system as an eluent. Detection was carried out with the help of TLC by UV-absorbance and Dragendorf reaction. Fractions with the same chromatographic mobilities were combined and evaporated in vacuo **(yield 1,43 g, 63 %).**

LCMS: m/z: 483.3 MH^+, requires 483.4

^{1}H-NMR (600 MHz, $CDCl_3$): δ 7.94 – 7.78 (m, 9H), 7.75 – 7.67 (m, 6H), 3.92 – 3.76 (m, 2H), 3.45 – 3.35 (t, J= 6.68 Hz, 2H), 2.10 – 1.95 (m, 2H), 1.87 – 1.77 (m, 2H), 1.72 – 1.57 (m, 4H), 1.44 – 1.25 (m, 2H), 1.30 – 1.15 (m, 4H).

^{13}C-NMR (151 MHz, $CDCl_3$): δ 135.07 (d, J= 2.93 Hz), 133.87 (d, J= 10.3 Hz), 130.06 (d, J= 12.5 Hz), 118.60 (d, J= 85.8 Hz), 34.31, 32.85, 30.52 (d, J= 15.4 Hz), 29.35, 29.31, 29.16, 28.72, 28.16, 22.95 (d, J= 44.8 Hz), 22.77 (d, J= 9.5 Hz).

^{31}P-NMR (162 MHz, $CDCl_3$):): δ 24.32

Synthesis of 10-[2-(3-hydroxy-6-oxo-xanthen-9-yl)benzoyl]oxydecyl-triphenyl-phosphonium bromide (2)

A solution of fluorescein (0.664 g, 2.0 mmol) and sodium carbonate (0.4 g) in DMF (40 ml) was heated to 60^0C. The solution of 1,10-brom decyltriphenylphosphonium bromide (1 g, 2 mmol) in a minimal volume of dichloromethane was added dropwise and the reaction was carried out for 3 hours under stirring at 60^o C. Then the reaction mass was cooled and diluted with 100 ml of dichloromethane. The organic solution was washed twice with 5% hydrochloric acid and was dried over calcium chloride. The organic phase was filtered and evaporated in vacuo. The residue was dissolved in a minimal volume of the solvent mixture ethanol-dichloromethane (1:5) and applied to a silica gel column (MN Kieselgel 60, 240-400 mesh) in the same solvent system used as an eluent for chromatographic purification. Fractions were detected by direct visualization of colored zones and additionally analyzed with the help of thin layer chromatography, those having equal chromatographic mobilities were combined. The solvents were removed and the residues were dried in *vacuo* and analyzed by LC-MS. Compound **2** was eluted as an orange band and after evaporation of solvents was obtained as orange powder **(341 mg, 21%)**.

LC-MS m/z: requires 733.84 (M^+); found 733.52

^{1}H-NMR (600 MHz, $CDCl_3$): δ 8.27 (d, J= 7.8 Hz, 1H), 7.91 – 7.78 (m, 11H), 7.77 – 7.69 (m, 8H), 7.67 (t, J = 7.8 Hz, 1H), 7.29 (d, J= 7.4 Hz, 2H), 6.88 (s, 1H), 6.86 (s, 1H), 6.78 (br.s, 1H, OH), 3.90 (t, J= 6 Hz, 2H), 3.80 – 3.72 (m, 2H), 1.74 – 1.65 (m, 4H), 1.40 – 1.34 (m, 2H), 1.20 – 1.12 (m, 2H), 1.11 -1.01 (m, 4H), 1.00 – 0.94 (m, 2H), 0.678 (quin, J= 7.6 Hz, 2H).

^{13}C-NMR (151 MHz, $CDCl_3$): δ 166.43, 151.84, 135.21 (d, J= 2.9 Hz, 3C), 135.13, 133.87, 133.84, 133.80 (d, J= 10.3 Hz, 6C), 133.78, 132.43, 131.62, 131.05, 130.66, 130.65 (d, J= 12.5 Hz, 6C), 130.58, 130.55, 129.97, 129.59, 118.53 (d, J= 85.8 Hz, 3C), 66.00, 30.33 (d, J= 16.1 Hz), 29.82, 29.41, 29.39, 29.03, 28.96, 28.26, 25.83, 22.94 (d, J= 5.1 Hz), 22.82 (d, J= 48.4 Hz).

^{31}P-NMR (162 MHz, $CDCl_3$):): δ 24.32

Absorption and emission spectra of mitoFluo

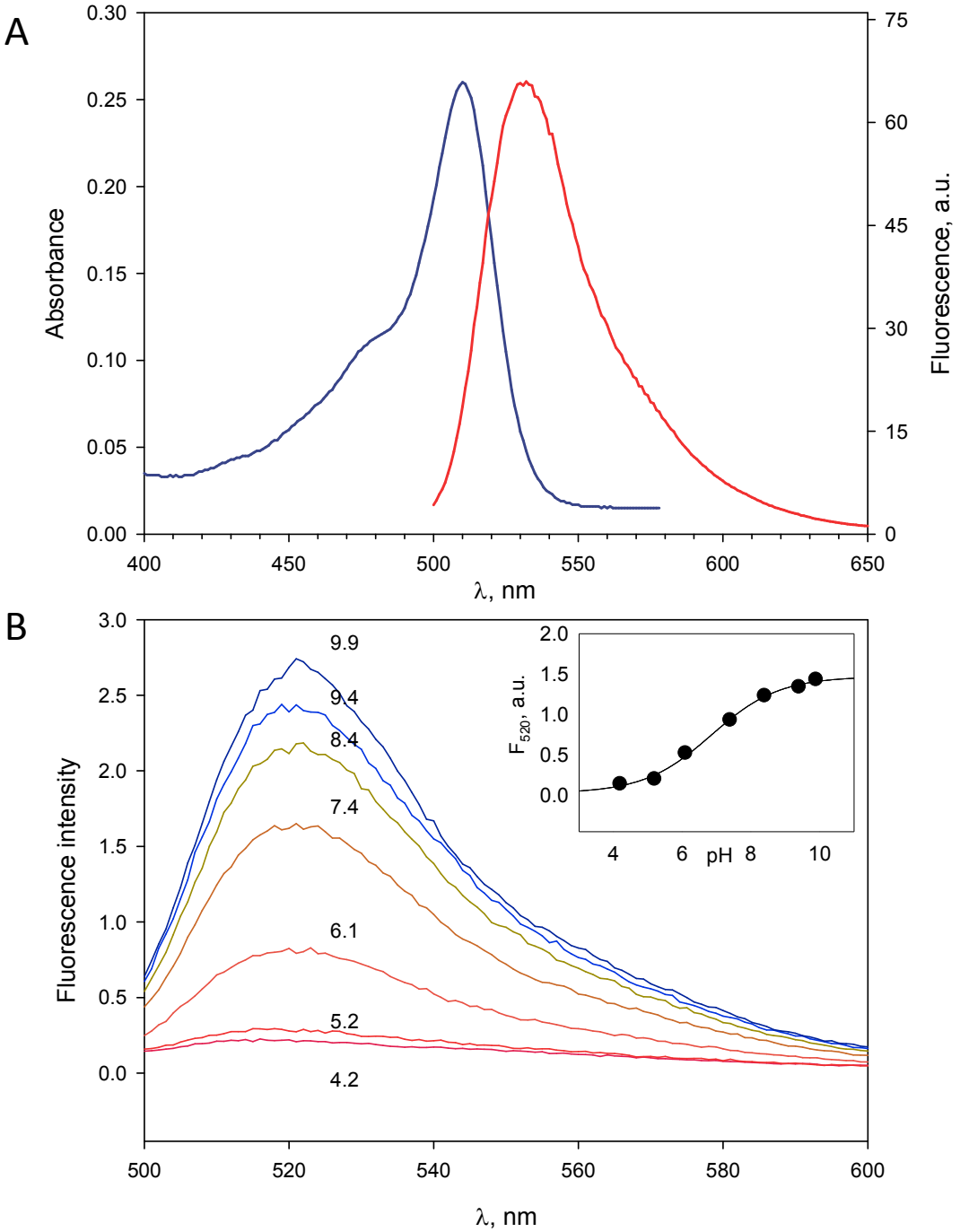


Fig. S1 A – Absorption and emission (λ_{ex} = 490 nm) spectra of 1 μM mitoFluo in ethanol. B – emission spectra of mitoFluo in aqueous buffer solution at different pH. Inset: pH dependence of mitoFluo fluorescence and a fitting curve according to Henderson–Hasselbalch equation with apparent pKa =6.9±0.1. The solution was 100 mM KCl, 10 mM Tris, 10 mM MES, 10 mM β-alanine, 5 nM mitoFluo.

According to Fig. S1A, the absorption spectrum of mitoFluo in ethanol peaks at 508 nm, and the fluorescence emission spectrum – at 530 nm. Figure S1B shows the dependence of mitoFluo emission spectrum on pH of the aqueous buffer solution. As expected for a derivative of fluorescein, fluorescence intensity increased upon shifting pH to alkaline values, while the position of the maximum was pH-independent. Insert to Figure S1B displays a pH dependence of the fluorescence intensity at the maximum of mitoFluo spectra and a fitting curve obtained by the Henderson-Hasselbalch equation with pK_a=6.9±0.1, obviously associated with deprotonation of hydroxyl in the xantene ring. The shift in pK_a with respect to that of unsubstituted fluorescein (6.4)[9] can be related to the effect of local electrostatic potential[10,11] or formation of mitoFluo micelles in aqueous solution.

A scheme of an experimental chamber used to measure electric current through a planar bilayer lipid membrane (BLM)

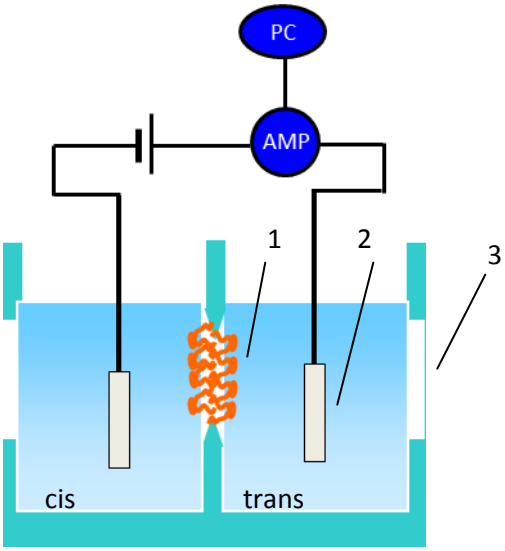


Fig.S2 A scheme of a measurement chamber. 1 – septum; 2 – AgCl/Ag electrodes with agar bridges; 3 – a glass window

Proton selectivity of mitoFluo

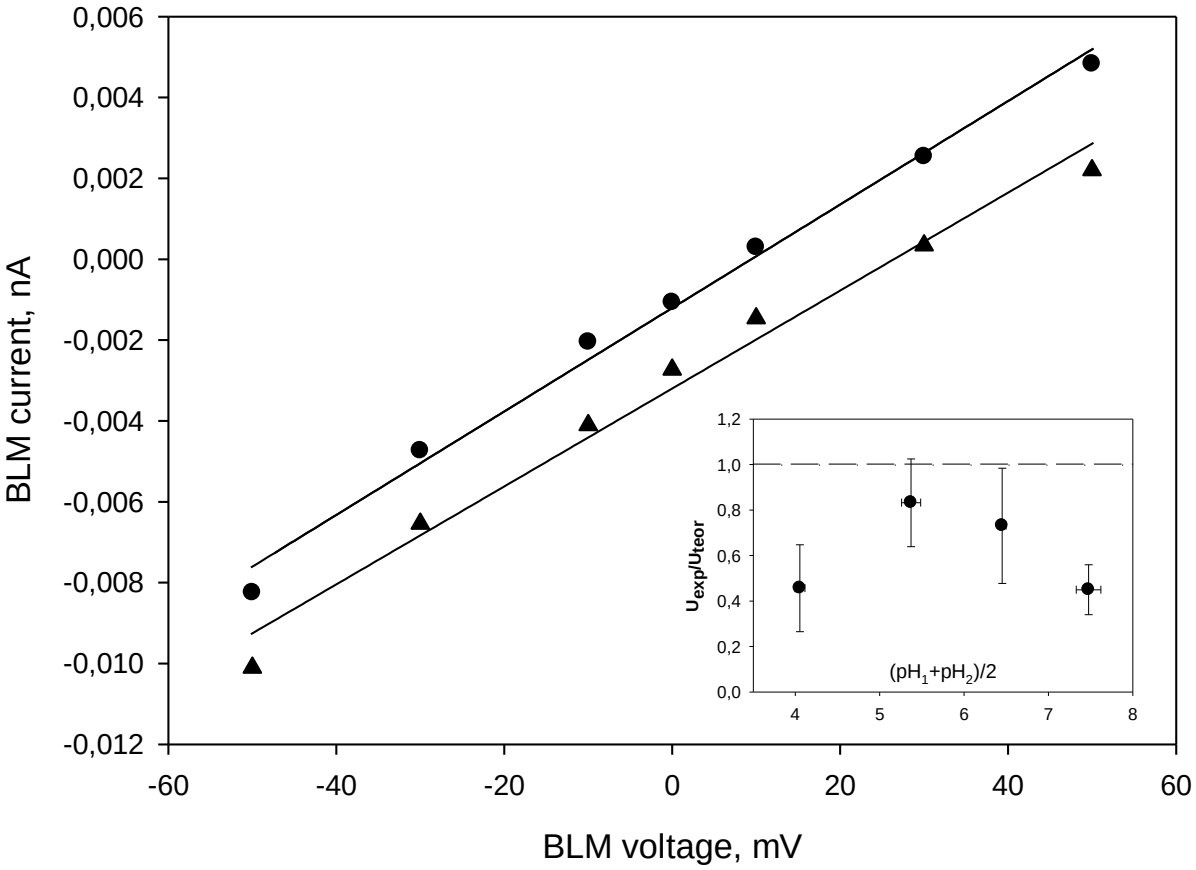
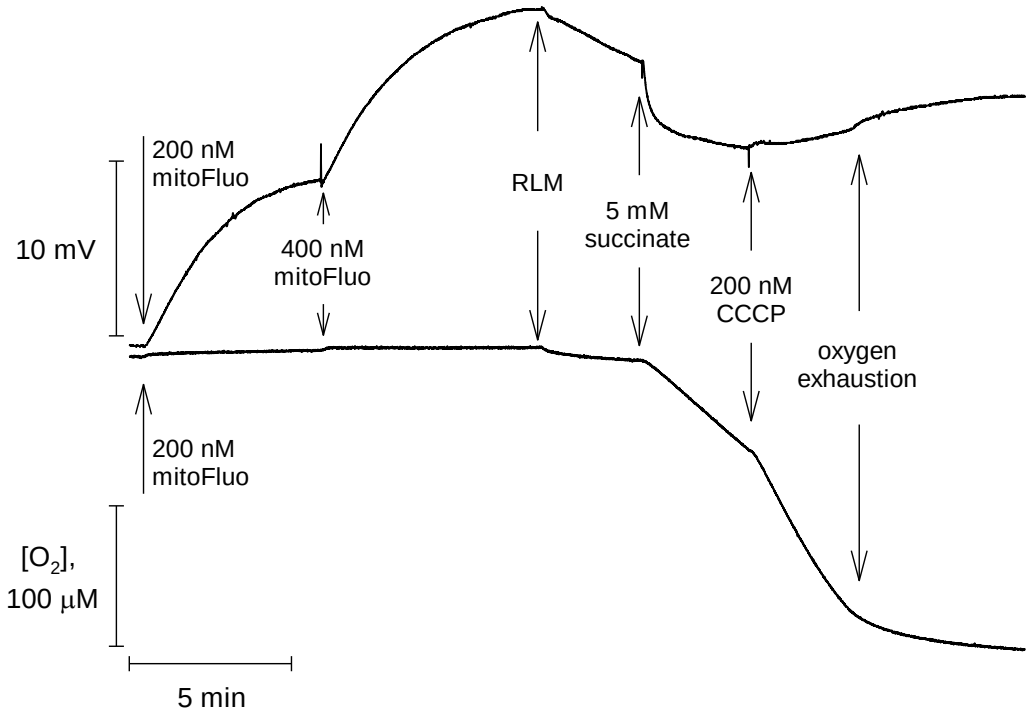


Fig. S3 Current-voltage curve for 1 μM mitoFluo under symmetrical (circles, pH cis and trans were 5.07) and asymmetrical conditions (triangles, pH cis was 5.07, pH trans was 5.42). Inset: pH dependence of the zero current potential. The solution was 100 mM KCl, 10 mM Tris, 10 mM MES, 10 mM β-alanine. BLM was formed from *E. coli* total lipid extract; room temperature.

To test ion selectivity of mitoFluo-mediated permeability of BLM, current-voltage dependences were measured under symmetrical and asymmetrical conditions (Figure S3, circles and triangles, respectively). The experimentally obtained zero-current potential V_{zero} was compared with the value V_{max} calculated for ideal proton selectivity from the Nernst equation[12] $V_{max}= \frac{RT}{F} \cdot \Delta pH$. High proton selectivity which decreased upon shifting the pH in either direction was observed in the pH range from 5.5 to 6.5 (insert to Figure S3).

Uptake of mitoFluo by rat liver mitochondria (RLM) and respiration of RLM as monitored by a TPP^+-selective electrode and a Clark oxygen electrode, respectively



Fi. S4 Effect of respiring rat liver mitochondria (RLM) on the potential of a TPP^+-selective electrode and oxygen concentration in the presence of mitoFluo. Recordings were made with a dual-channel setup containing a TPP^+-selective electrode and a Clark-type electrode immersed in a 1-mL chamber with a constantly stirred buffer solution. The solution was 250 mM sucrose, 20 mM MOPS, 1 mM EGTA, 3 mM $MgCl_2$, 5 mM KH_2PO_4, pH = 7.4, 2 μM rotenone, T = 23°C. Concentration of mitochondrial protein – 1 mg/ml.

Weak colocalization of TMRE and C_8-FL

Mitochondria-targeting properties of mitoFluo (Figs.2C and 2D) result from the presence of a TPP cation in the molecule. In the case of C_8-FL lacking a TPP moiety, the targeting was poor if any. Fig.S5 shows confocal images of cultured renal cells loaded with TMRE (B, red fluorescence) and C_8-FL (A, green fluorescence). Overlay of A and B demonstrated weak colocalization of dyes (C) in contrast to Fig.2D.

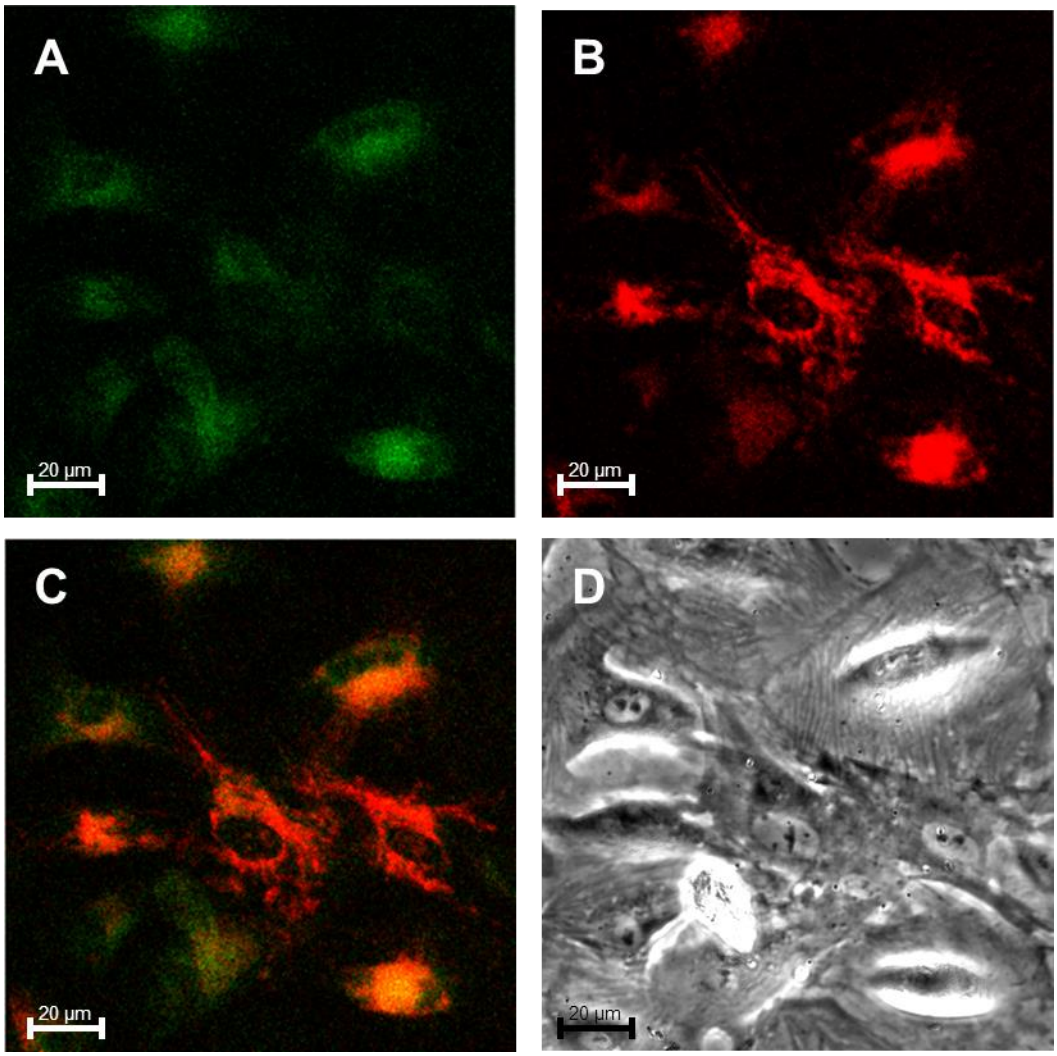


Fig. S5 Confocal images of cultured renal cells loaded with TMRE (B, red fluorescence) and C_8-FL (A, green fluorescence). Overlay of A and B demonstrated weak colocalization of dyes (C). Phase contrast microscopy of the cells is presented in D.

MTT assay

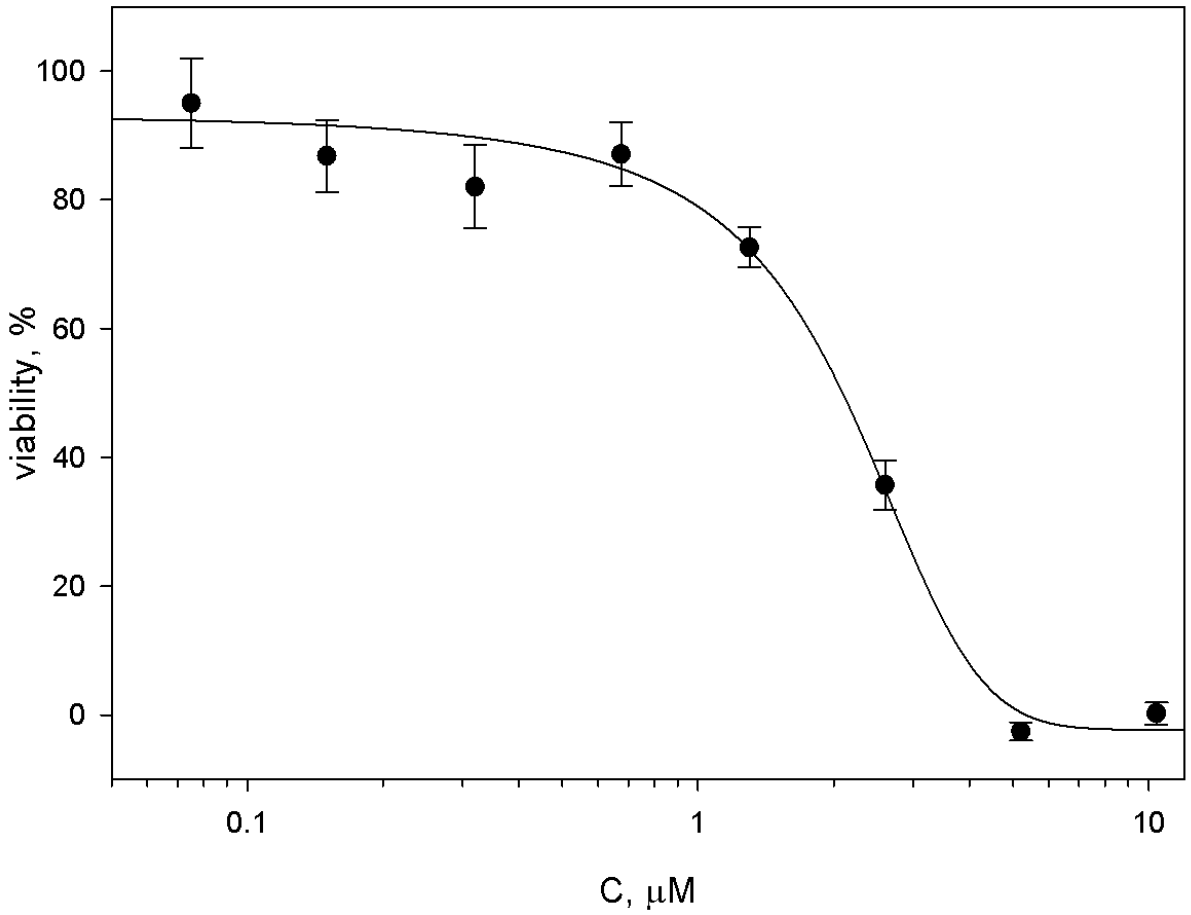


Fig. S6 Viability of L929 fibrosarcoma cells as a function of the mitoFluo concentration.

Electronic supplementary information references

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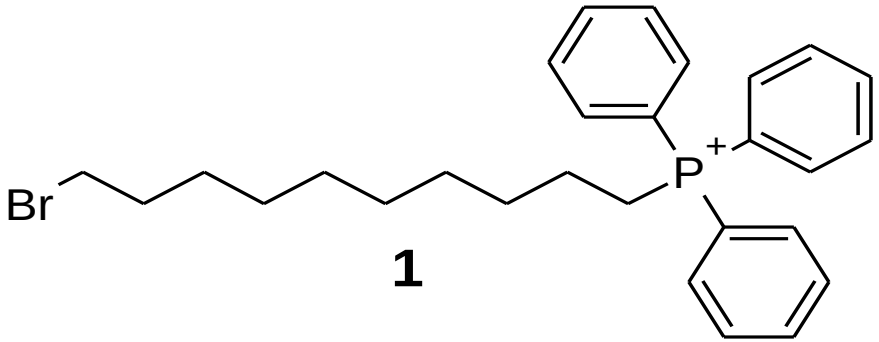
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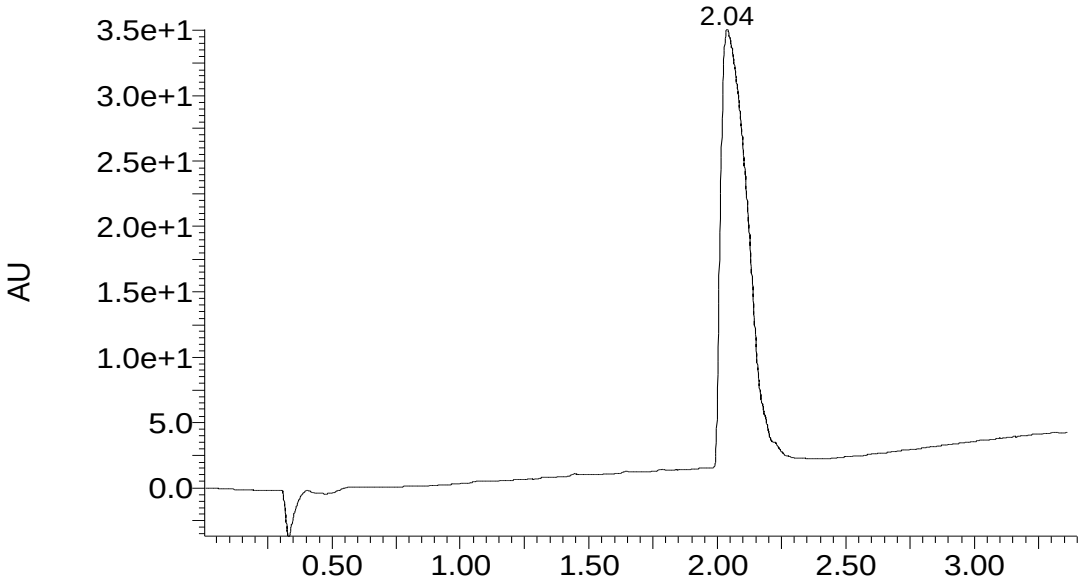
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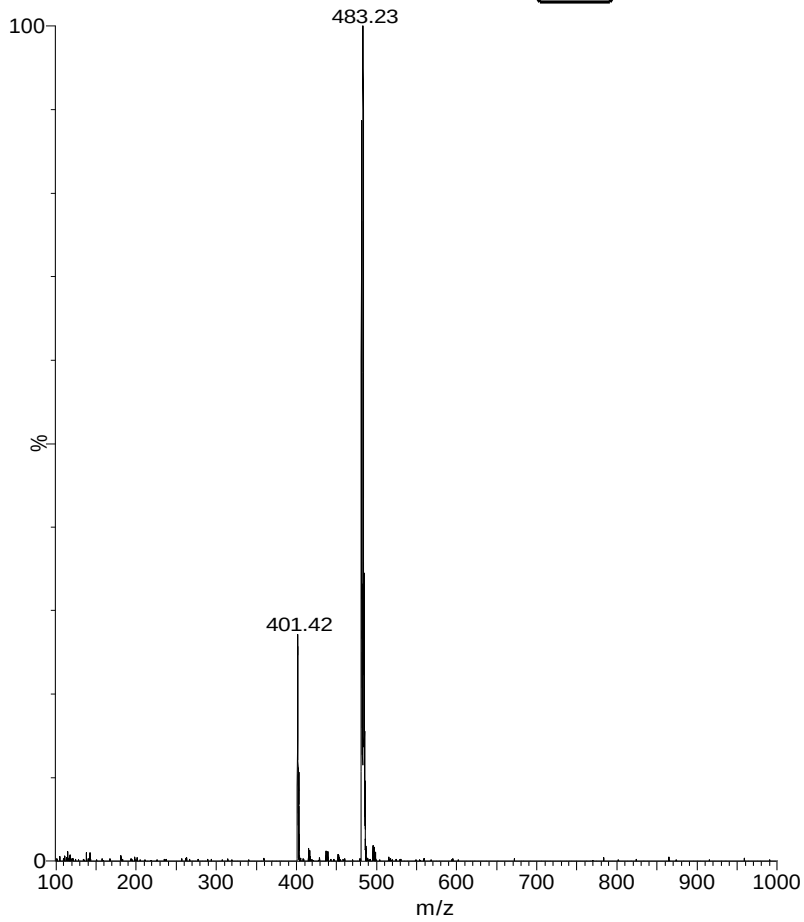
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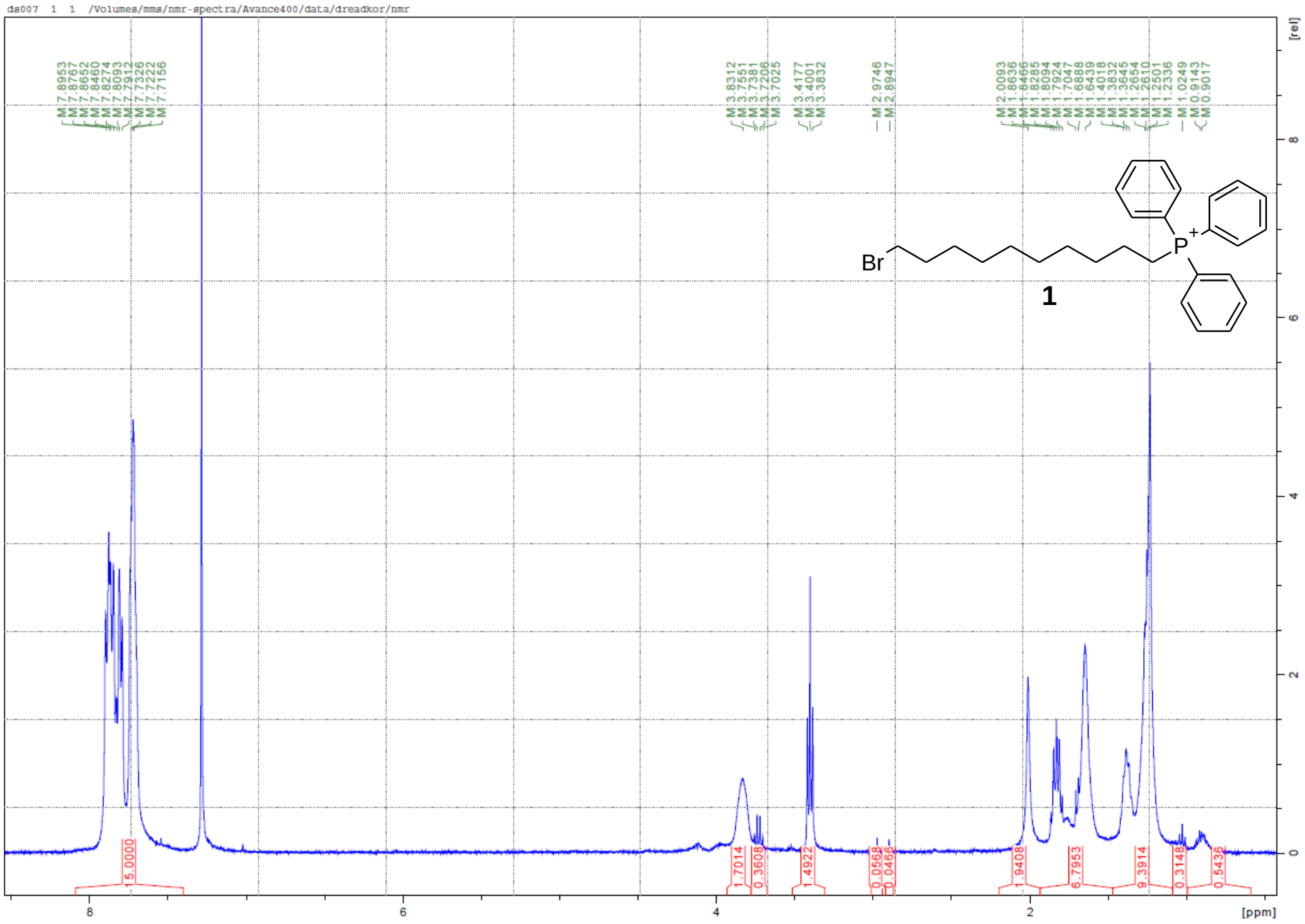
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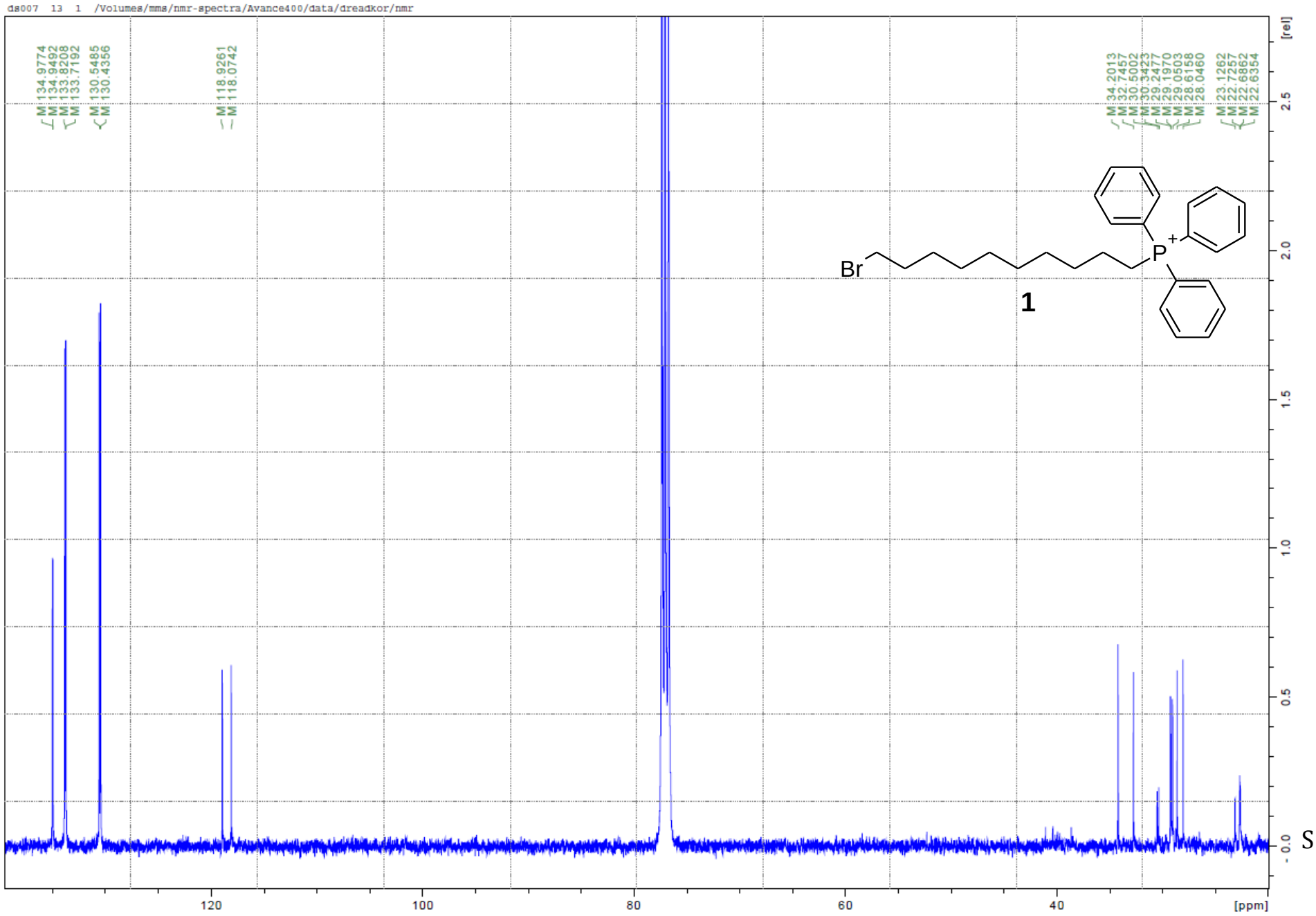
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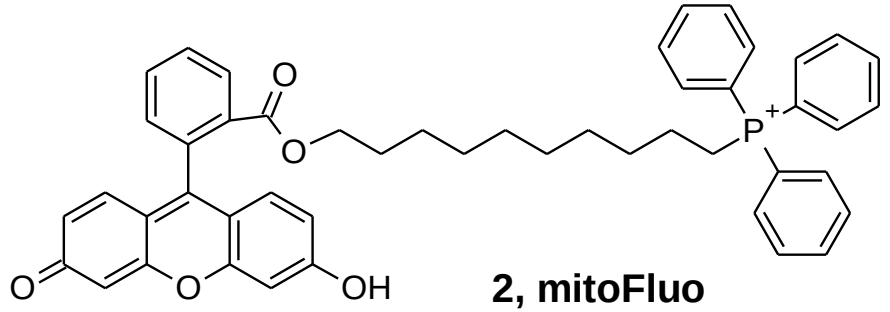
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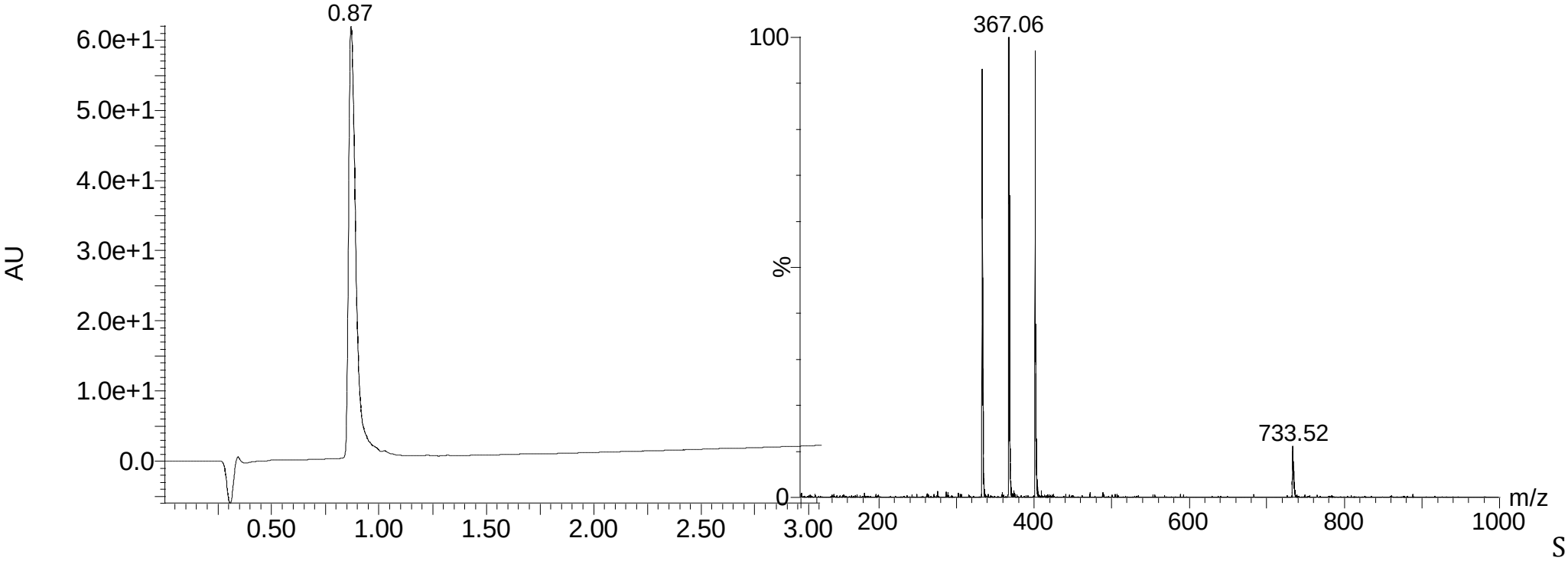
2, mitoFluo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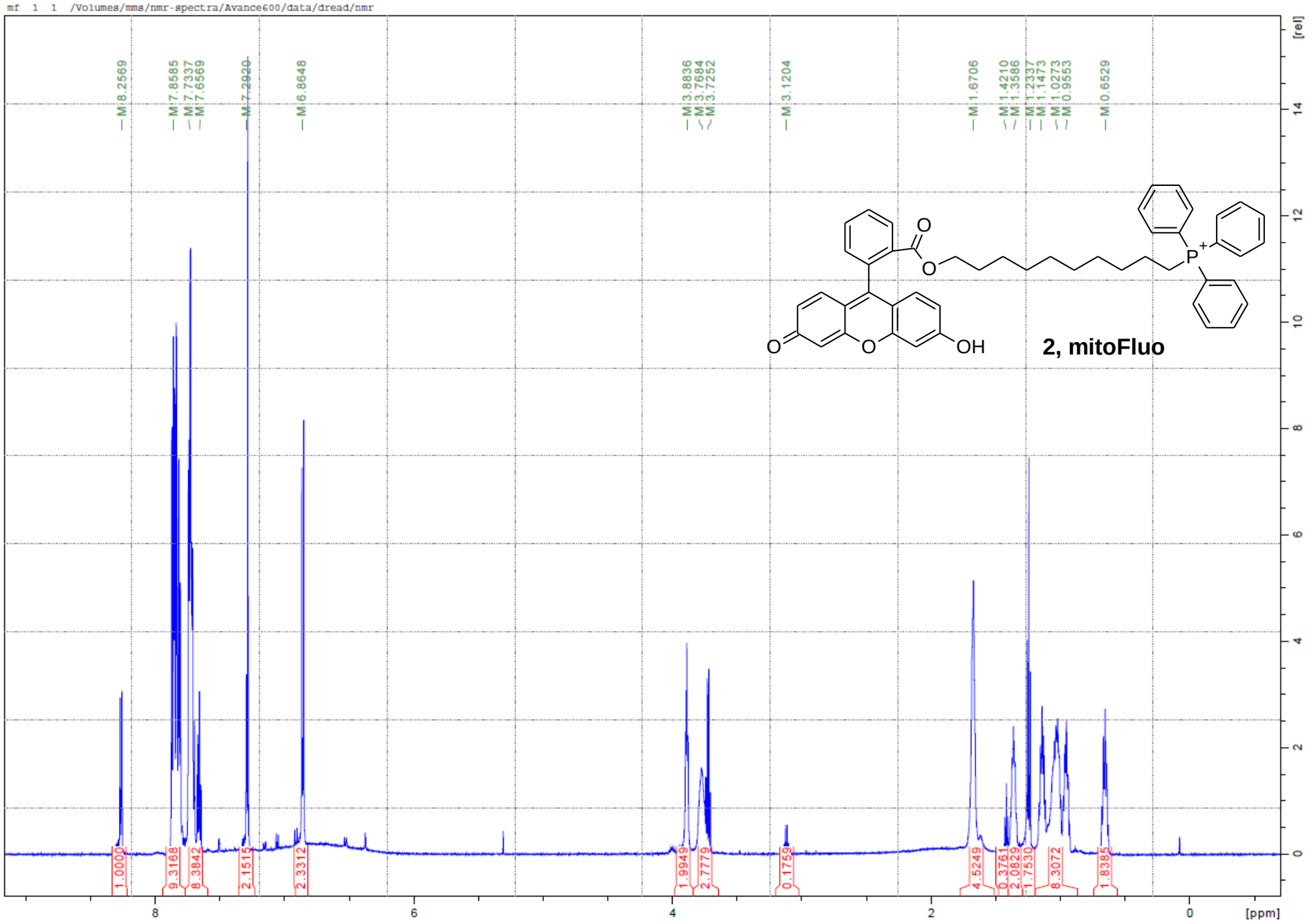
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8.3842
2.1515
2.3312
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0.3761
2.0829
1.7530
8.3072
1.8385
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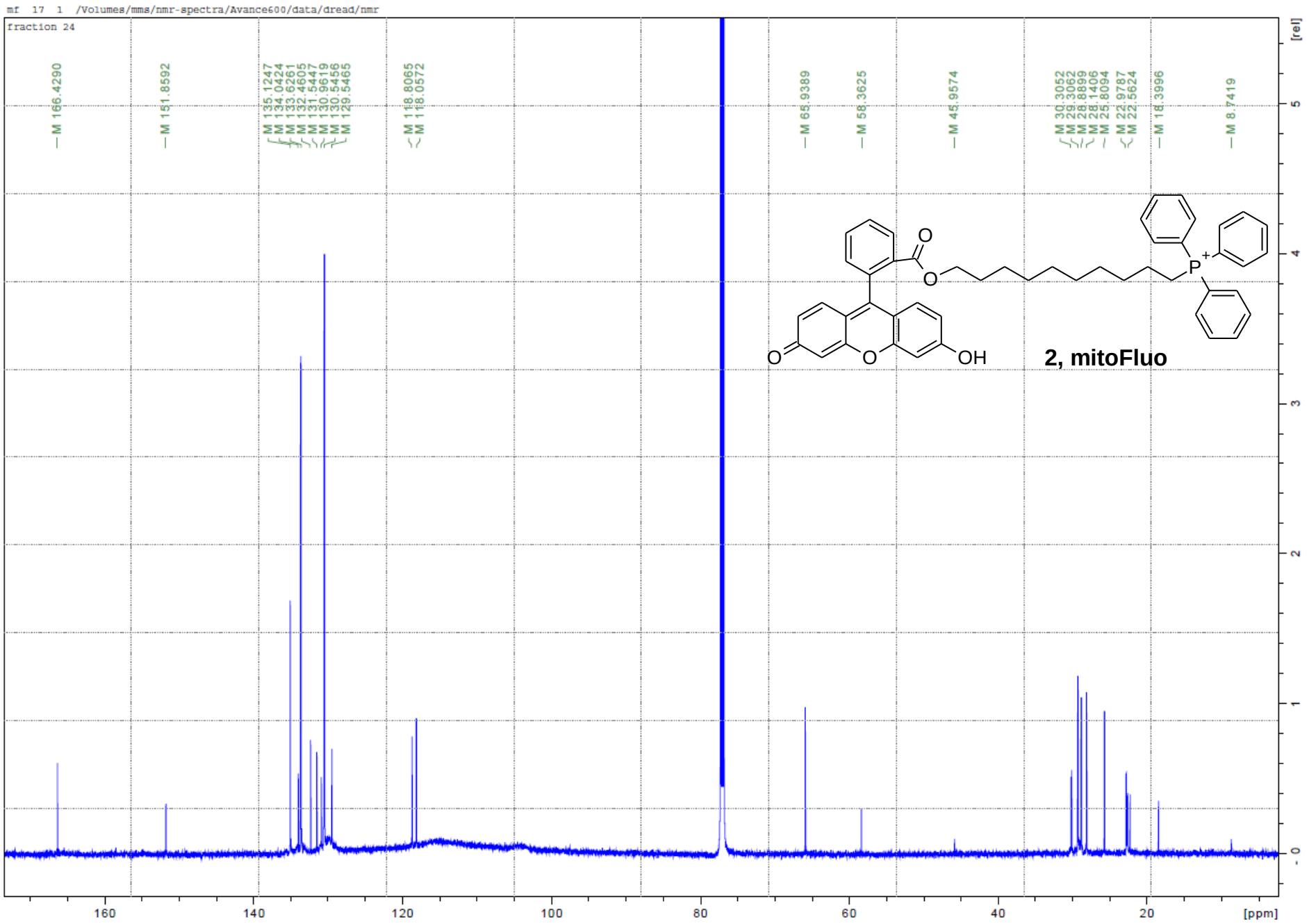
2, mitoFluo


mf 17 1 /Volumes/mms/nmr-spectra/Avance600/data/dread/nmr
fraction 24
M 166.4290
M 151.8592
M 135.1247
M 134.0424
M 133.6261
M 132.4605
M 131.5447
M 130.9619
M 130.5456
M 129.5465
M 118.8065
M 118.0572
M 65.9389
M 58.3625
M 45.9574
M 30.3052
M 29.3062
M 28.8899
M 28.1406
M 25.8094
M 22.9787
M 22.5624
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4
3
2
1
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140
120
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80
60
40
20
[ppm]
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O
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P+
2, mitoFluo