Electronic Supplementary Material (ESI) for ChemComm.

SUPPORTING INFORMATION

for

Development and Evaluation of a non-peptidic ligand for the molecular imaging of inflammatory processes using S100A9 (MRP14) as novel target

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[b.] *Institute of Immunology, University Hospital of Münster, Röntgenstr. 21, 48149 Münster, Germany.*
[c.] *Department of Nuclear Medicine, University Hospital of Münster, Albert-Schweitzer-Campus 1, 48149 Münster, Germany.*
[d.] *Interdisciplinary Center for Clinical Research (IZKF Münster), Domagkstr. 3, University of Münster, 48149 Münster, Germany.*
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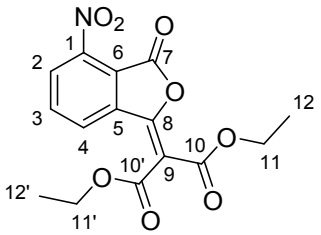
Contents

Chemical synthesis and characterization:

Synthetic procedures were carried out under dry argon atmosphere, unless otherwise specified. All reagents and solvents were purchased at the highest commercial quality available and used without further purification, unless otherwise stated. Column chromatography was carried out on silica gel Merck-60 (230–400 mesh, 60 A°), or on an automated column chromatography system (Reveleris® X2 Flash Chromatography System, GRACE). For TLC aluminum sheets pre-coated with silica gel 60 F254 (E. Merck) were used. NMR spectra were recorded on an ARX 300 and AMX 400 from Bruker Analytische Messtechnik (Karlsruhe, Germany) or on DD2 600 from Agilent spectrometer at a constant temperature of 298 K. 1H NMR and 13C NMR: chemical shifts δ are given relative to TMS (δ = 0) and referenced to the solvent signal. Electrospray ionization (ESI) mass spectra were recorded on a Bruker Daltonics (Bremen, Germany) MicroTof with loop injection. MALDI-TOF MS were recorded with a Bruker Reflex III spectrometer.

Diethyl 2-(4-nitro-3-oxo-3H-isobenzofuran-1-ylidene)malonate



To diethyl malonate (152mL, 160g, 1mol), acetic anhydride (480mL, 519g, 5.1mol) and triethylamine (137mL, 99.2g, 0.98mol) was added at 35°C under slight cooling (temperature should be maintained between 35 and 40°C) 3-nitrophthalic acid anhydride (193.1g, 1mol). After stirring for 2h at 40°C and cooling to room temperature, the mixture was poured onto crushed ice (1.4kg) and hydrochloric acid (32% w/v; 186 mL). After stirring for 30min, the solvent was decanted and the residue suspended with 360mL acetone. The precipitate was isolated by suction, washed with cold acetone and dried in vacuo. Yield: 293.2g (0.87mol; 87%)

mp.: 169 °C (acetone)

^{1}H NMR (600 MHz, chloroform-*d*)

δ [ppm] = 9.01 (dd, $^{3}J_{H,H}$ = 8.1 Hz, $^{4}J_{H,H}$ = 0.8 Hz, 1H, 2-CH), 8.11 (dd, $^{3}J_{H,H}$ = 7.9 Hz, $^{4}J_{H,H}$ = 0.8 Hz, 1H, 4-CH), 7.98 (m, 1H, 3-CH), 4.40, 4.38 (q, $^{3}J_{H,H}$ = 7.1 Hz, 4H, 11-CH, 11‘-CH), 1.37, 1.36 (t, $^{3}J_{H,H}$ 7.1 Hz, 6H, 12-CH, 12‘-CH).

^{13}C NMR (151 MHz, chloroform-*d*)

δ [ppm] = 162.8, 162.3 (s, C-10, C-10‘), 158.5 (s, C-8), 152.5 (s, C-7), 146.9 (s, C-1), 138.2 (s, C-5), 136.5 (d, C-3), 131.7 (d, C-2), 127.6 (d, C-4), 118.5 (s, C-6), 112.0 (s, C-9), 62.7 (t, C-11, C-11’), 14.1, 14.1 (q, C-12, C-12’).

MS (ESI^{+}): m/z =

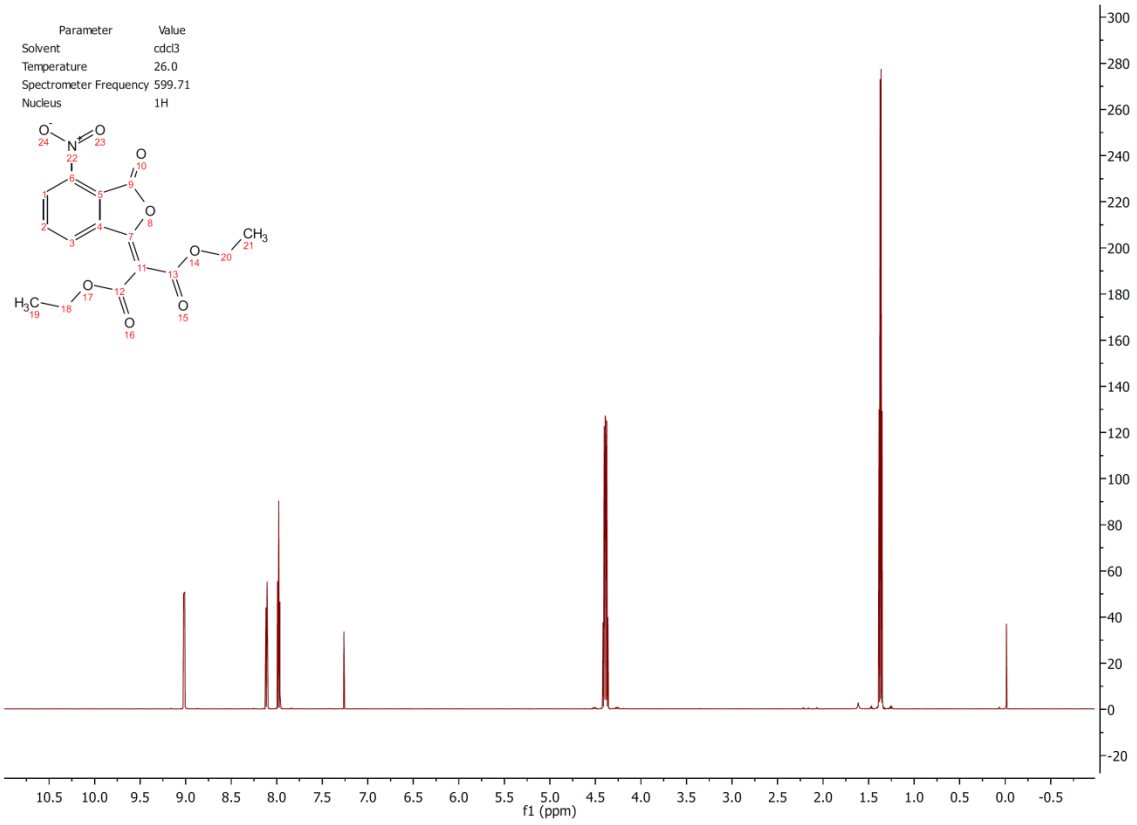
358.0536; calculated $[C_{15}H_{13}NO_8]Na^{+}$ ([M + Na]$^{+}$): 358.0533.

390.0797; calculated $[C_{15}H_{13}NO_8]Na^{+}\cdot MeOH$ ([M + Na + MeOH]$^{+}$): 390.0796.

693.1176; calculated $[C_{30}H_{26}N_2O_{16}]_2Na^{+}$ ([2M + Na]$^{+}$): 693.1175.

725.1435; calculated $[C_{30}H_{26}N_2O_{16}]_2Na^{+}\cdot MeOH$ ([2M + Na + MeOH]$^{+}$): 725.1437.

757.1693; calculated $[C_{30}H_{26}N_2O_{16}]_2Na^{+}\cdot 2MeOH$ ([2M + Na + 2MeOH]$^{+}$): 757.1699.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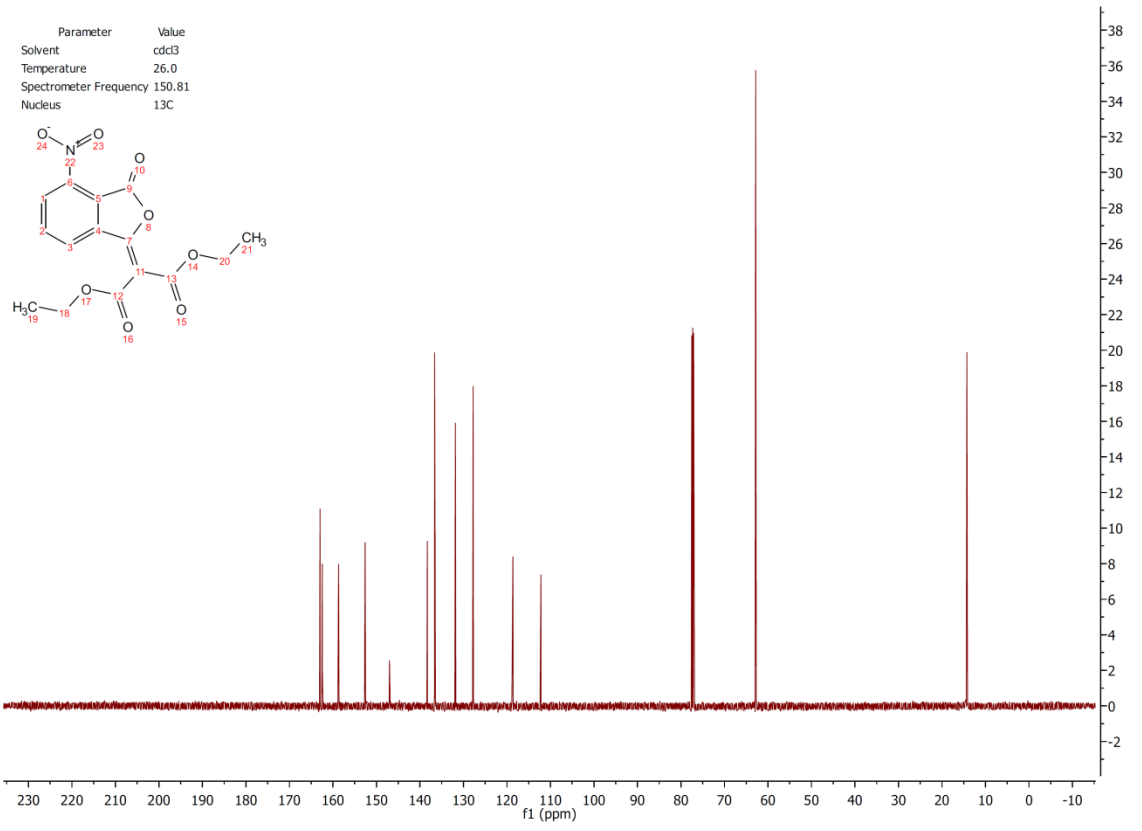
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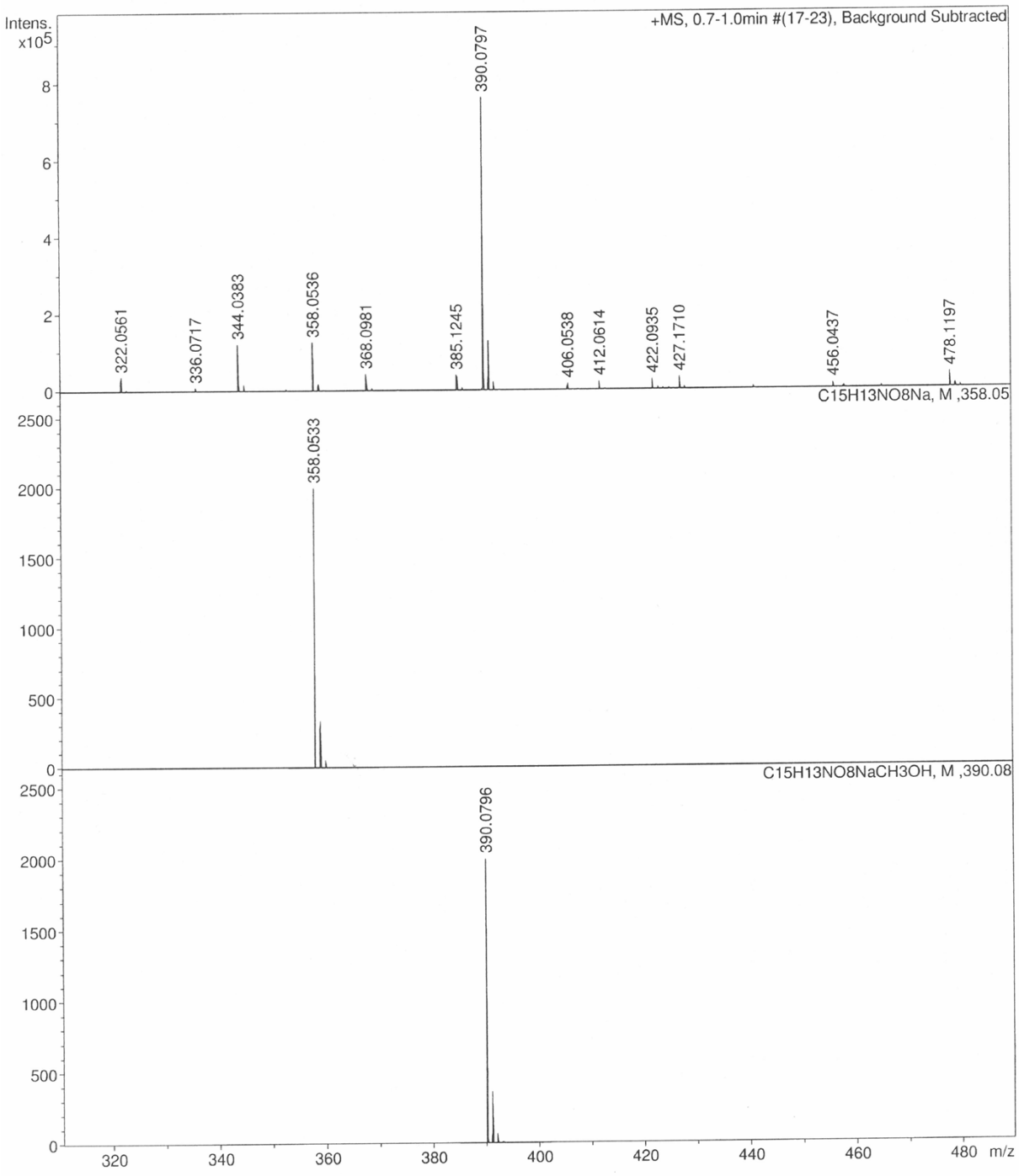
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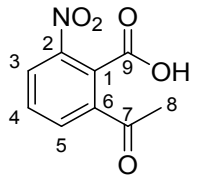
m/z

2-Acetyl-6-nitrobenzoic acid



Hydrochloric acid (32% w/v; 351mL), water (38mL) and toluene (7.5mL) were charged in a round bottom flask and diethyl 2-(4-nitro-3-oxo-3H-isobenzofuran-1-ylidene)malonate (147g, 438mmol) was added. The mixture was stirred and warmed to 95°C over 2h. At 70°C evolution of carbon dioxide started. Stirring was continued for 20h at 95°C, and the mixture then cooled to 10°C. The solid product was filtered off, washed with water and dried at 65°C in vacuo. Yield: 84.5g (404mmol; 92%).

mp.: 169 °C (toluene)

^{1}H NMR (400 MHz, NaOD, 0.1M in deuterium oxide)

δ [ppm] = 8.26 (dd, $^{3}J_{H,H}$ = 8.3, $^{4}J_{H,H}$ = 1.1 Hz, 1H, 3-CH), 8.17 (dd, $^{3}J_{H,H}$ = 7.8, $^{4}J_{H,H}$ = 1.1 Hz, 1H, 5-CH), 7.64 (dd, $^{3}J_{H,H}$ = 8.3, 7.8 Hz, 1H, 4-CH).

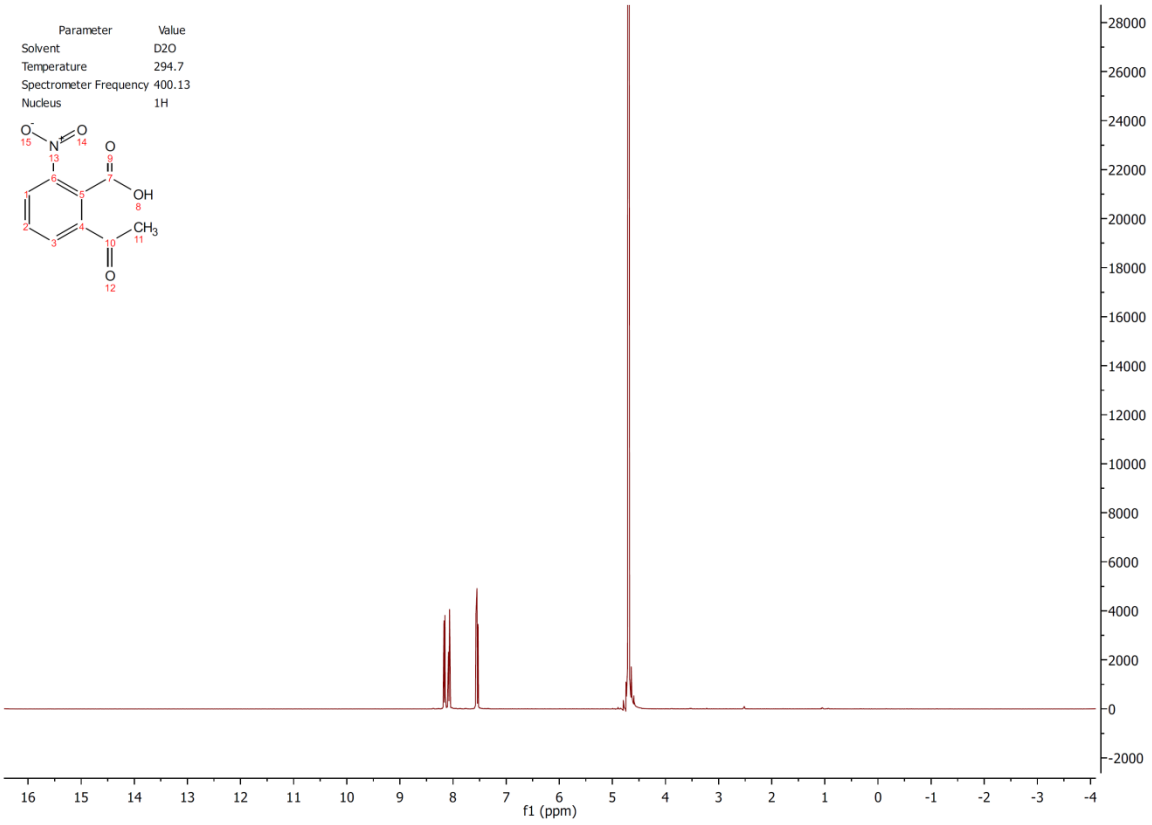
^{13}C NMR (101 MHz, NaOD, 0.1M in deuterium oxide)

δ [ppm] = 202.5 (s, C-7), 173.0 (s, C-9), 145.3 (s, C-2), 135.9 (s, C-6), 135.0 (d, C-5), 134.5 (s, C-1), 128.6 (d, C-4), 127.9 (d, C-3), 27.9 (m, C-8).

MS (ESI^{+}): m/z =

232.0220; calculated $[C_{10}H_9NO_3]Na^{+}$ ($[M + Na]^{+}$): 232.0216.

245.0038; calculated $[C_{10}H_8NNaO_3]2Na^{+}$ ($[M - H + 2Na]^{+}$): 254.0036.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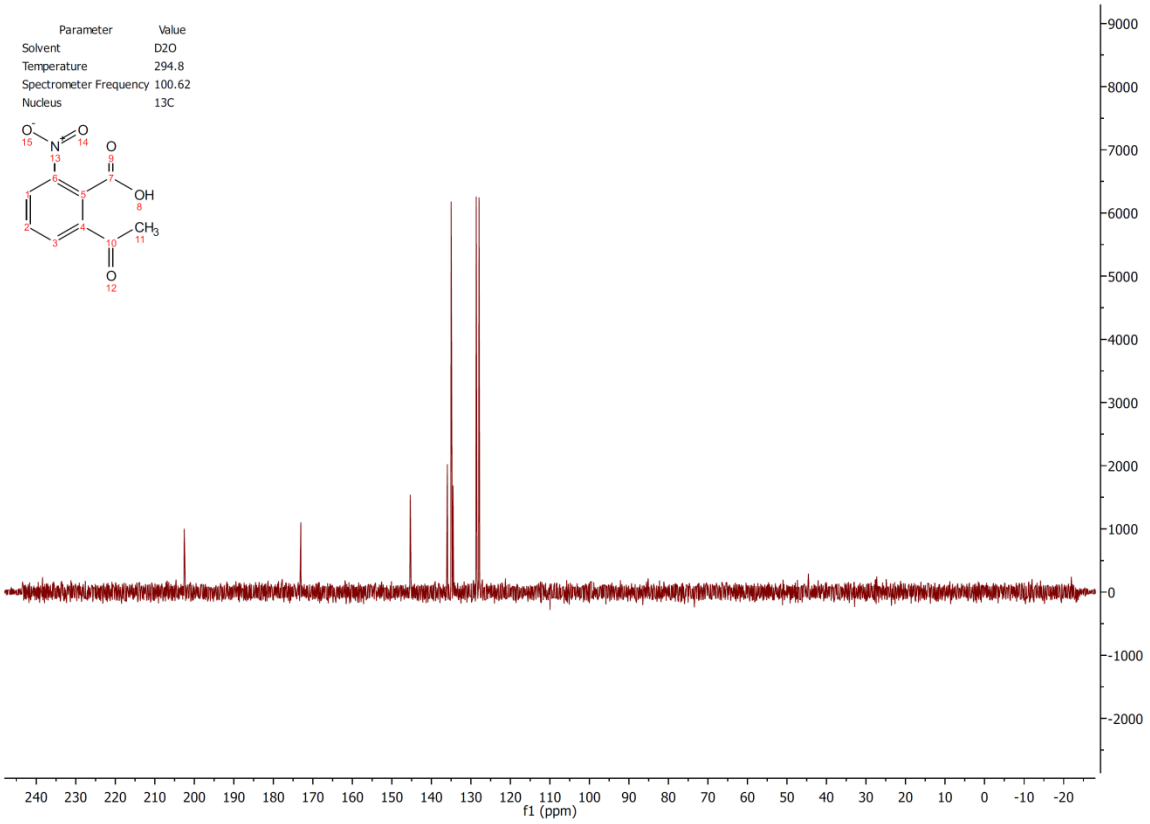
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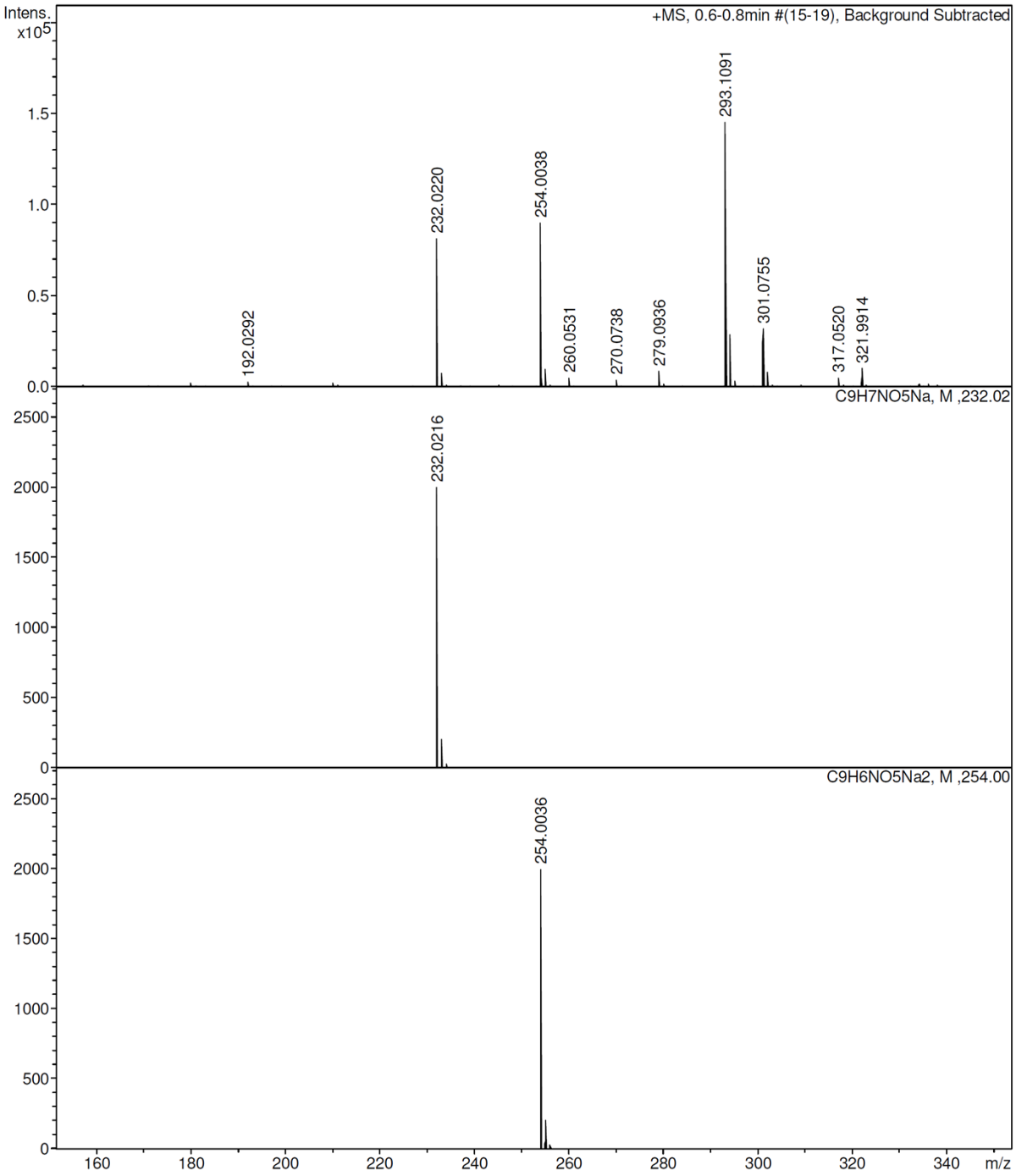
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321.9914
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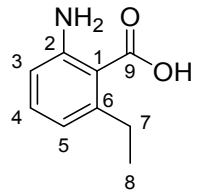
254.0036
m/z

2-amino-6-ethylbenzoic acid



Sodium hydroxide (1M, 50mL) and water (50mL) were charged in a steel tube for high pressure hydrogenations and 2-acetyl-6-nitrobenzoic acid (10g, 48mmol) was added (resulting pH: 12.3). After adding Platinum(IV)oxide (200mg) the reaction vessel was flushed with hydrogen three times and the mixture was stirred at 90°C for 3h at 20bar (H_2). Then Raney-Nickel suspension (3.6g) was added and after further stirring (3h, 110°C, 20bar H_2) the resulting mixture was filtrated through Celite®. The filtrate was adjusted to pH 3.5 with concentrated hydrochloric acid and extracted with ethyl acetate (3 x 200mL). After removing the solvent and drying in vacuo a white solid was obtained. Yield: 6.1g (37mmol; 77%).

mp.: 105 °C (ethyl acetate)

^{1}H NMR (400 MHz, DMSO-d_6)

δ [ppm] = 8.04 (s, 2H, NH_2), 7.02 (dd, $^3J_{H,H}$ = 8.2, 7.5 Hz, 1H, 4-CH), 6.59 (dd, $^3J_{H,H}$ = 8.2, $^4J_{H,H}$ =1.2 Hz, 1H, 3-CH), 6.42 (dd, $^3J_{H,H}$ = 7.5Hz, $^4J_{H,H}$ =1.2 Hz, 1H, 5-CH), 2.71 (q, $^3J_{H,H}$ = 7.6 Hz, 2H, 7-CH), 1.12 (t, $^3J_{H,H}$ = 7.6 Hz, 3H, 8-CH).

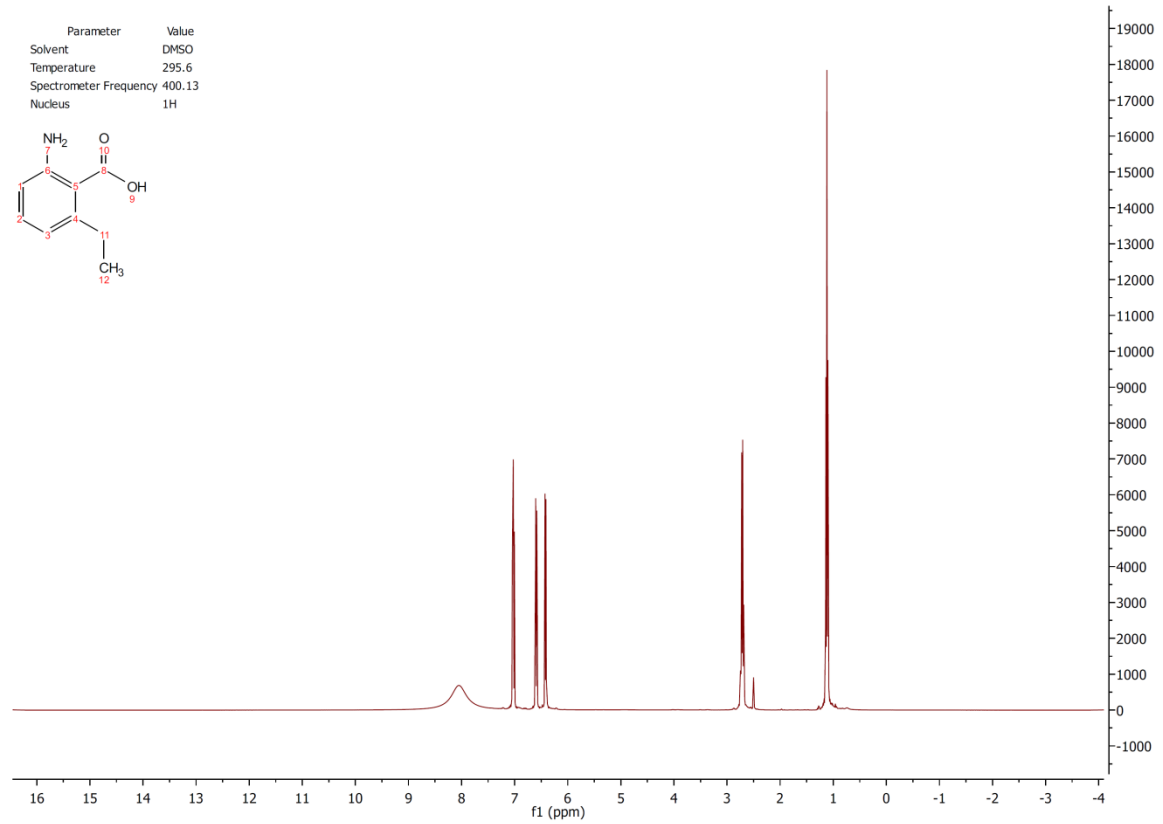
^{13}C NMR (101 MHz, DMSO-d_6)

δ [ppm] = 170.5 (s, C-9), 148.8 (s, C-2), 144.6 (s, C-6), 131.2 (d, C-4), 117.2 (d, C-5), 114.7 (s, C-1), 114.0 (d, C-3), 27.9 (t, C-7), 16.3 (q, C-8).

MS (ESI$^-$): m/z =

166.0864; calculated $[C_9H_{11}NO_2]H^+$ ($[M + H]^+$): 166.0864.

186.0689; calculated $[C_9H_{11}NO_2]Na^+$ ($[M + Na]^+$): 188.0682.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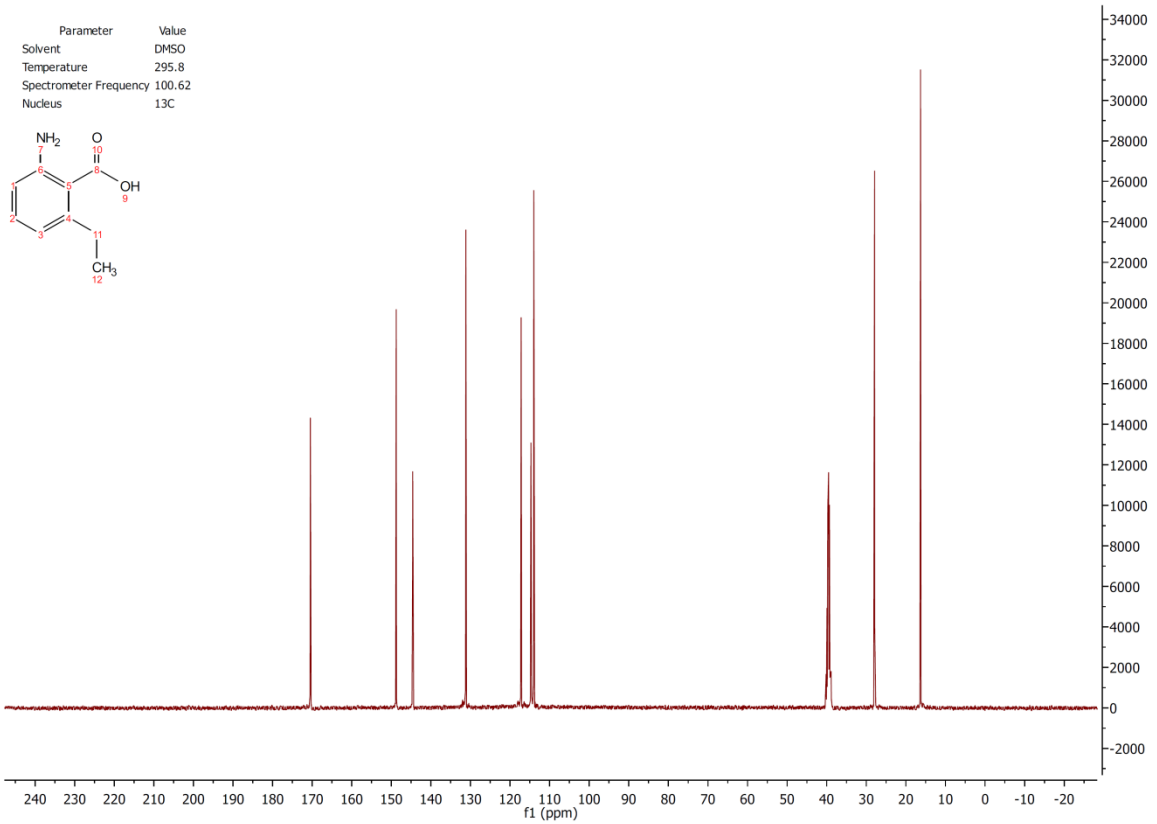
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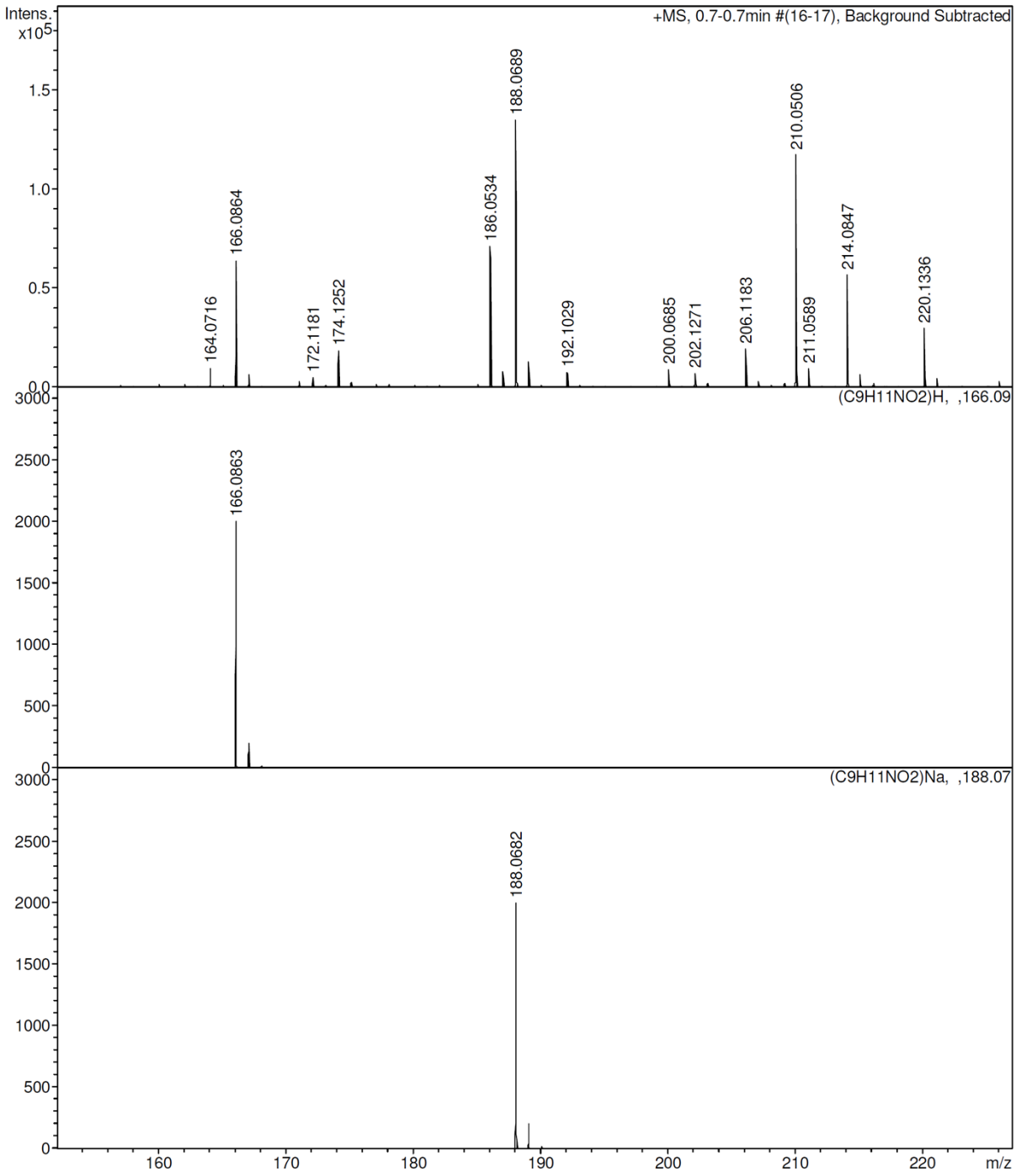
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211.0589
214.0847
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(C9H11NO2)H, ,166.09
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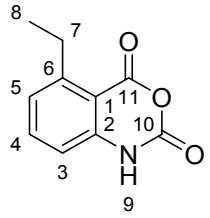
m/z

5-Ethyl-1H-benzo[d][1,3]oxazine-2,4-dione



A solution of phosgene in toluene (20%, 14.7mL, 28mmol) was added dropwise to a slurry of 2-amino-6-ethylbenzoic acid (3.7g, 22.4mmol) in absolute THF (20mL) keeping the temperature below 20 °C (ice cooling). After the mixture was stirred for 1h at room temperature, the reaction mixture was poured onto ice water (110mL) and the resulting precipitate was collected, washed with water, and dried in vacuo to yield the isatoic anhydride: yield 3.73 g (87%).

mp.: 204 °C (H_2O).

^{1}H NMR (400 MHz, DMSO-d_6)

δ [ppm] = 11.59 (s, 1H, 9-NH), 7.54 (dd, $^3J_{H,H}$ = 8.2, 7.6 Hz, 1H, 4-CH), 7.00 (dd, $^3J_{H,H}$ = 7.6 Hz, $^4J_{H,H}$ = 1.1 Hz, 1H, 5-CH), 6.95 (dd, $^3J_{H,H}$ = 8.2, $^4J_{H,H}$ = 1.2 Hz, 1H, 3-CH), 2.97 (q, $^3J_{H,H}$ = 7.4 Hz, 2H, 7-CH_2), 1.11 (t, $^3J_{H,H}$ = 7.4 Hz, 3H, 8-CH_3).

^{13}C NMR (101 MHz, DMSO-d_6)

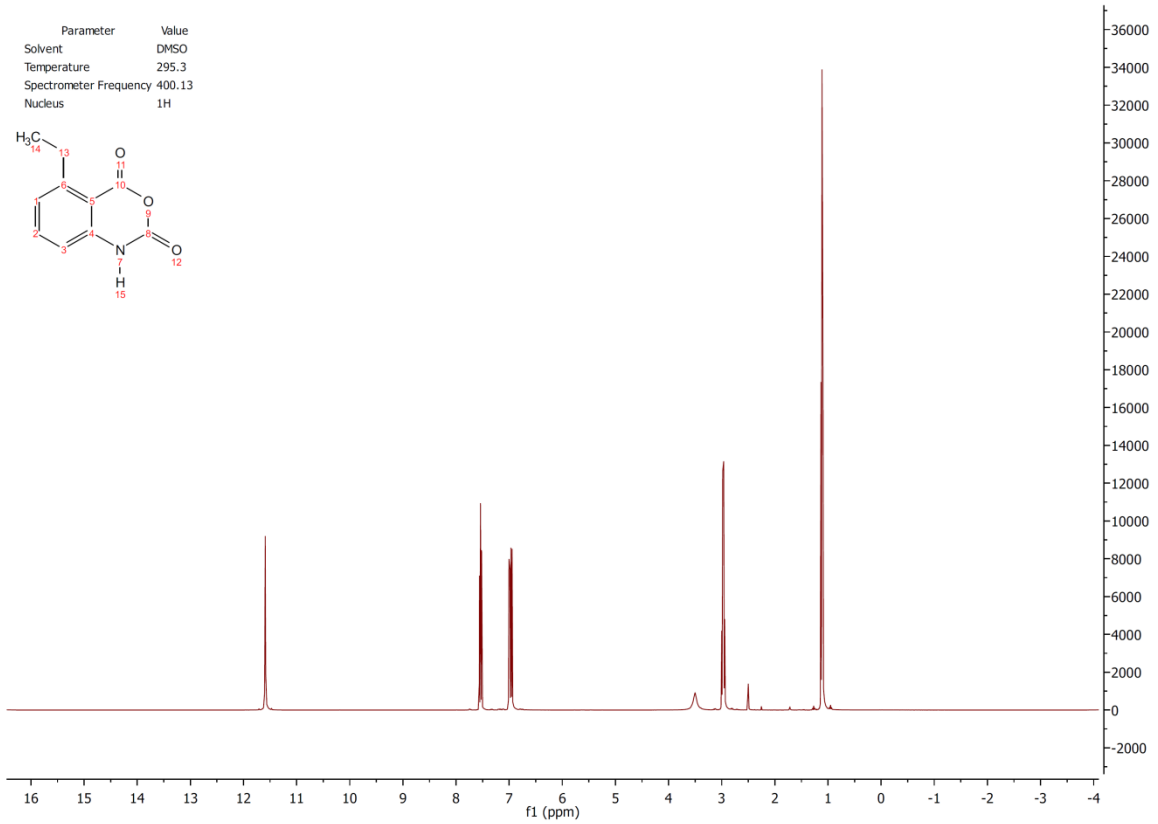
δ [ppm] = 158.5 (s, C-11), 148.5 (s,C-6), 147.1 (s, C-10), 142.7 (s, C-2), 136.1 (d, C-4), 124.5 (d, C-5), 113.5 (d, C-3), 107.8 (s, C-1), 27.1 (t, C-7), 15.0 (q, C-8).

MS (ESI$^+$): m/z =

192.0655; calculated $[C_{10}H_9NO_3]H^+$ ($[M + H]^+$): 192.0655.

214.0475; calculated $[C_{10}H_9NO_3]Na^+$ ($[M + Na]^+$): 214.0475.

405.1056; calculated $[C_{20}H_{18}N_2O_6]_2Na^+$ ($[2M + Na]^+$): 405.1057.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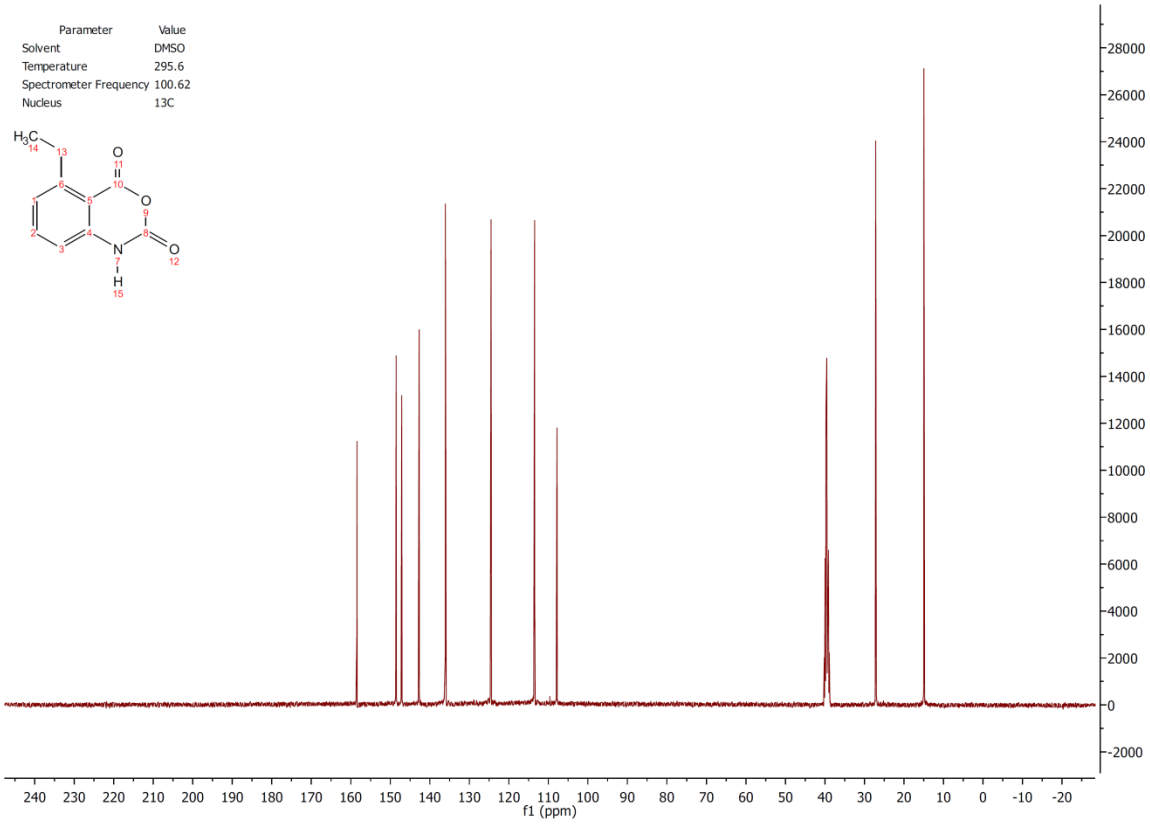
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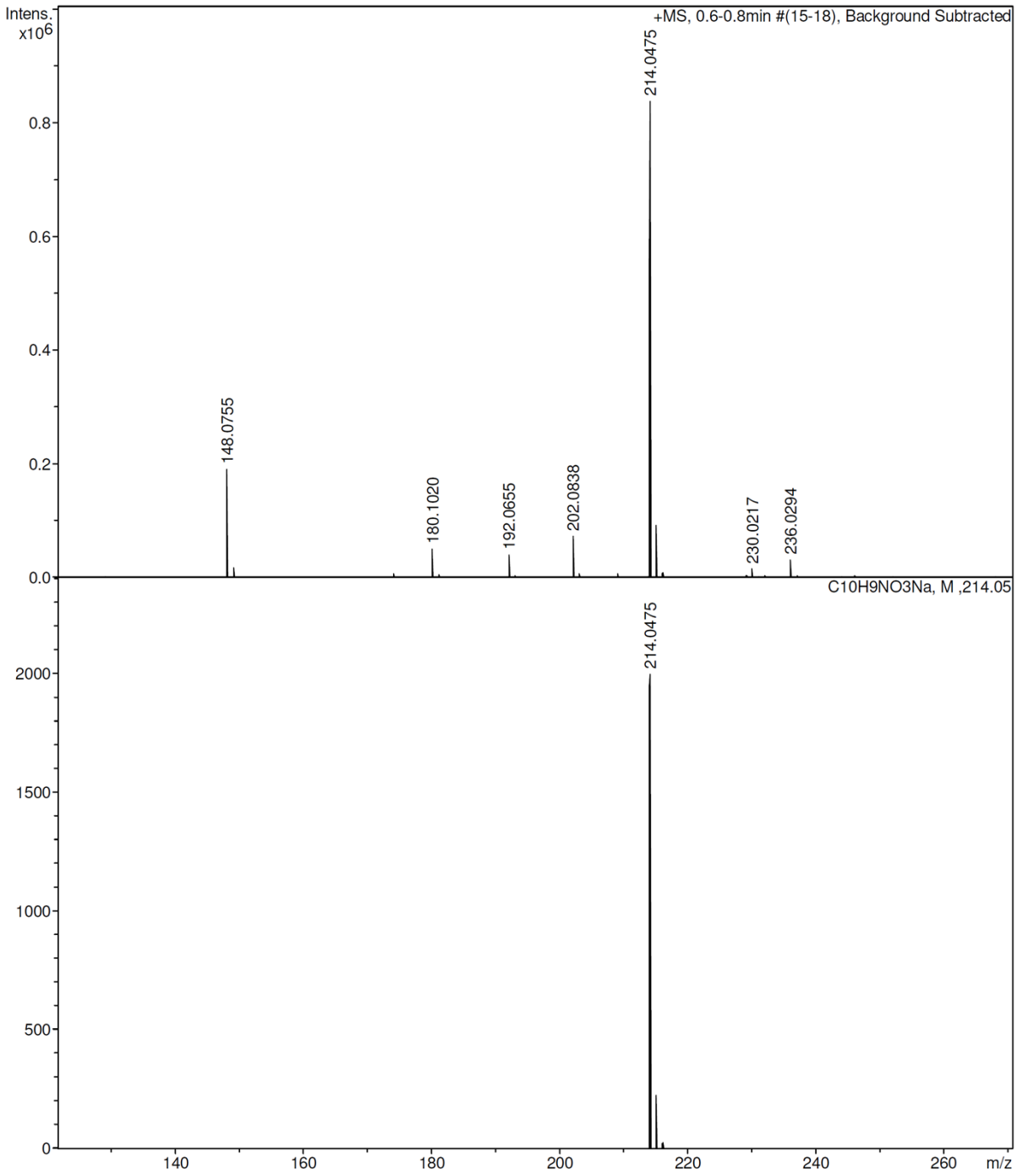
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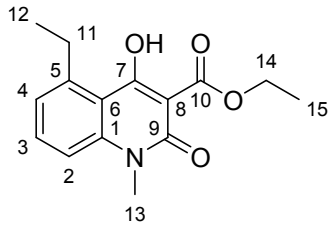
m/z

Ethyl 5-ethyl-4-hydroxy-1-methyl-2-oxo-1,2-dihydroquinoline-3-carboxylate



The isatoic anhydride (10g, 52.3mmol) was dissolved in DMF (100mL) and cooled on an ice bath, and sodium hydride (95%, 1.58g, 62.8mmol) followed by methyl iodide (4.2mL, 9.6g, 68mmol) was added at a rate to keep the temperature below 5 °C. After stirring at room temperature overnight, excess methyl iodide was removed by evacuating for 30 min at approximately 30 mbar. Sodium hydride (95%, 1.58g, 62.8mmol) followed by diethylmalonate (9.5mL, 10.06g, 62.8mmol) was added, and the mixture was heated at 85 °C for 2 h, then cooled, and quenched with water (500 mL). The aqueous solution was acidified with 1 M HCl, and the resulting precipitate was collected by filtration, washed with water, and dried to afford a light brown solid. Yield: 7.54g (27.4mmol; 52%)

mp.: 73 °C (H_2O).

^{1}H NMR (400 MHz, chloroform-*d*)

δ [ppm] = 14.98 (s, 1H, 7-OH), 7.47 (dd, $^3J_{H,H}$ = 8.6, 7.5 Hz, 1H, 3-CH), 7.12 (dd, $^3J_{H,H}$ = 8.6 Hz, $^4J_{H,H}$ = 1.1 Hz, 1H, 2-CH), 6.98 (dd, $^3J_{H,H}$ = 7.5 Hz, $^4J_{H,H}$ = 1.1 Hz, 1H, 4-CH), 4.46 (q, $^3J_{H,H}$ = 7.1 Hz, 2H, 14-CH_2), 3.58 (s, 3H, 13-CH_3), 3.18 (q, $^3J_{H,H}$ = 7.4 Hz, 2H, 11-CH_2), 1.44 (t, $^3J_{H,H}$ = 7.1 Hz, 3H, 15-CH_3), 1.23 (t, $^3J_{H,H}$ = 7.4 Hz, 3H, 12-CH_3).

^{13}C NMR (101 MHz, chloroform-*d*)

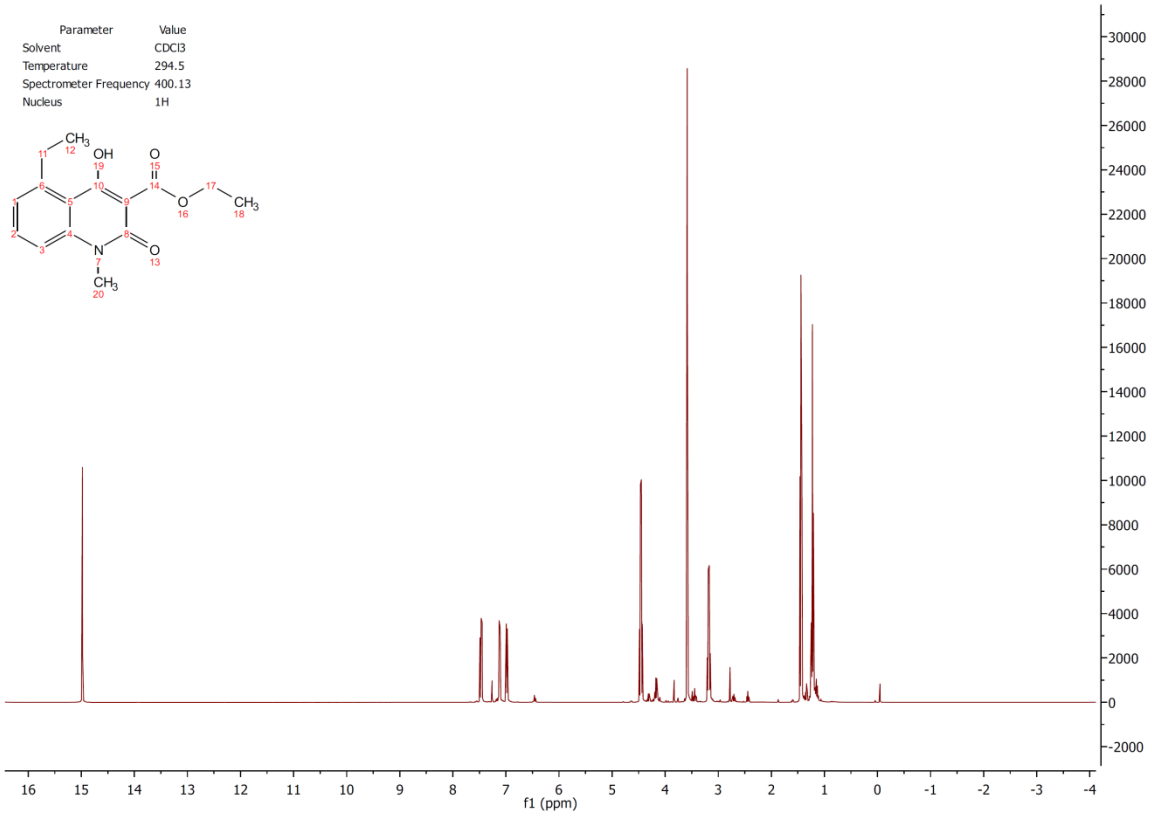
δ [ppm] = 174.5 (s, C-7), 173.5 (s, C-10), 159.2 (s, C-9), 147.0 (s, C-5), 142.9 (s, C-1), 133.6 (d, C-3), 124.7 (d, C-4), 113.3 (s, C-6), 112.6 (d, C-2), 97.5 (s, C-8), 62.3 (t, C-14), 30.3 (t, C-11), 29.9 (q, C-13), 16.4 (q, C-12), 14.2 (q, C-15).

MS (ESI$^+$): m/z =

276.1234; calculated $[C_{15}H_{17}NO_4]H^+$ ($[M + H]^+$): 276.1230.

298.1049; calculated $[C_{15}H_{17}NO_4]Na^+$ ($[M + Na]^+$): 298.1050.

573.2207; calculated $[C_{30}H_{34}N_2O_8]_2Na^+$ ($[2M + Na]^+$): 573.2207.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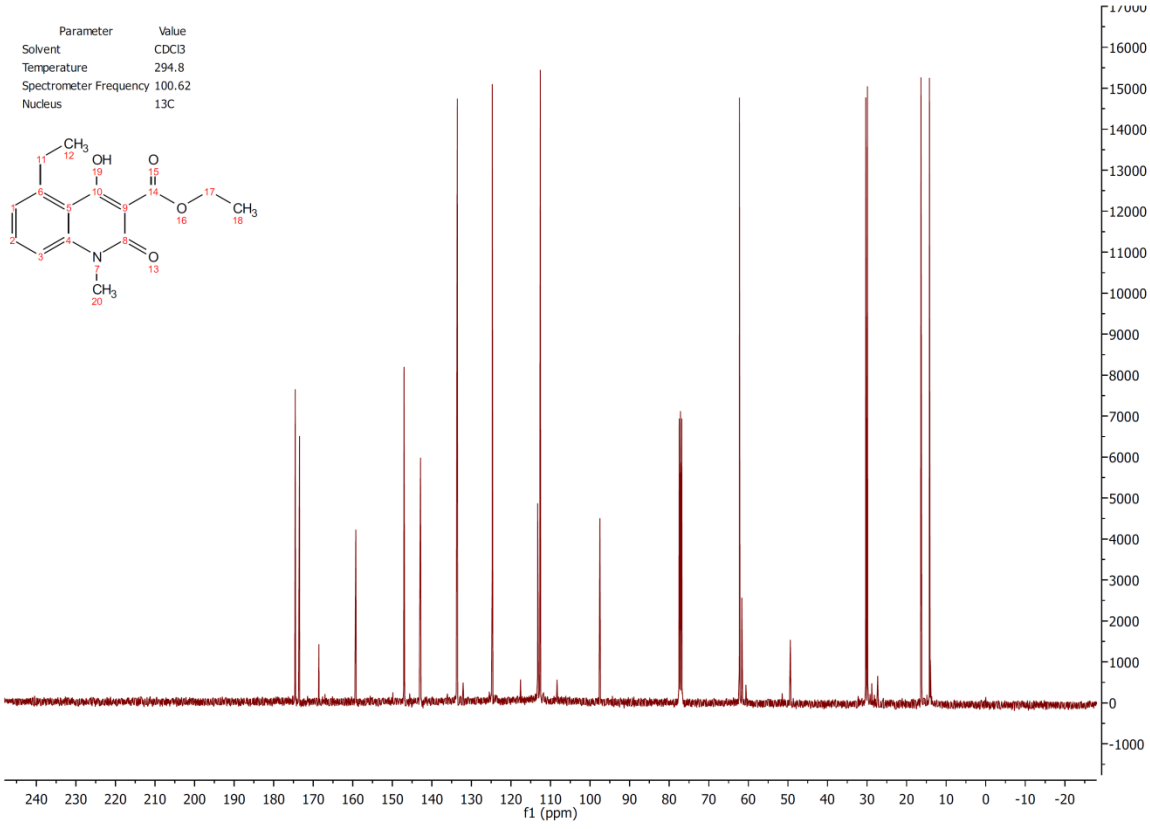
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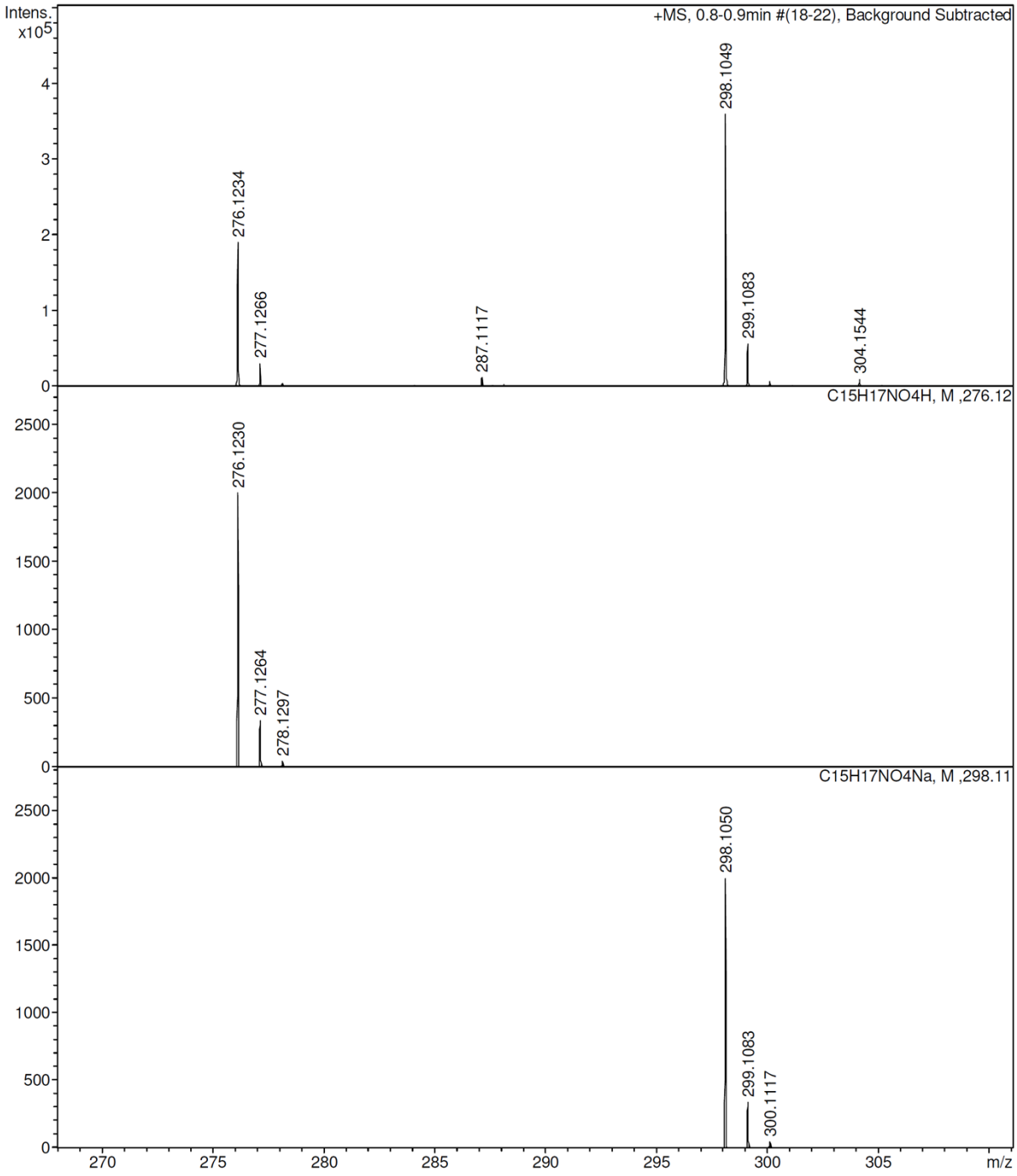
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C15H17NO4Na, M ,298.11
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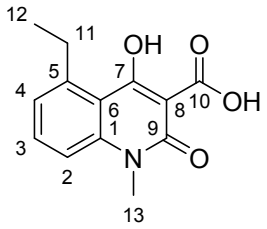
m/z

5-ethyl-4-hydroxy-1-methyl-2-oxo-1,2-dihydroquinoline-3-carboxylic acid



The ethyl ester (7.15g, 26mmol) was heated at 60 °C for six hours in a mixture of hydrochloric acid and acetic acid (80mL, 2.8M HCl in AcOH). Afterwards, the reaction mixture was poured on *iso*-Propanol (200mL). The formed precipitate washed with *iso*-Propanol (10mL) and the product was obtained as a white solid. Yield: 3.18g (12.9mmol; 50%)

mp.: decomposition at 220 °C (*iso*-Propanol).

^{1}H NMR (400 MHz, chloroform-*d*)

δ [ppm] = 16.12 (s, 1H, OH), 15.60 (s, 1H, OH), 7.66 (dd, $^{3}J_{H\text{-}H}$ = 8.6, 7.5 Hz, 1H, 3-CH), 7.33 (dd, $^{3}J_{H\text{-}H}$ = 8.7 Hz, $^{4}J_{H\text{-}H}$ = 1.1 Hz, 1H, 2-CH), 7.20 (dd, $^{3}J_{H\text{-}H}$ = 7.5, $^{4}J_{H\text{-}H}$ = 1.1, 1H, 4-CH), 3.72 (s, 3H, 13-CH_3), 3.24 (q, $^{3}J_{H\text{-}H}$ = 7.4 Hz, 2H, 11- CH_2), 1.26 (t, $^{3}J_{H\text{-}H}$ = 7.4 Hz, 3H, 12-CH_3).

^{13}C NMR (101 MHz, chloroform-*d*)

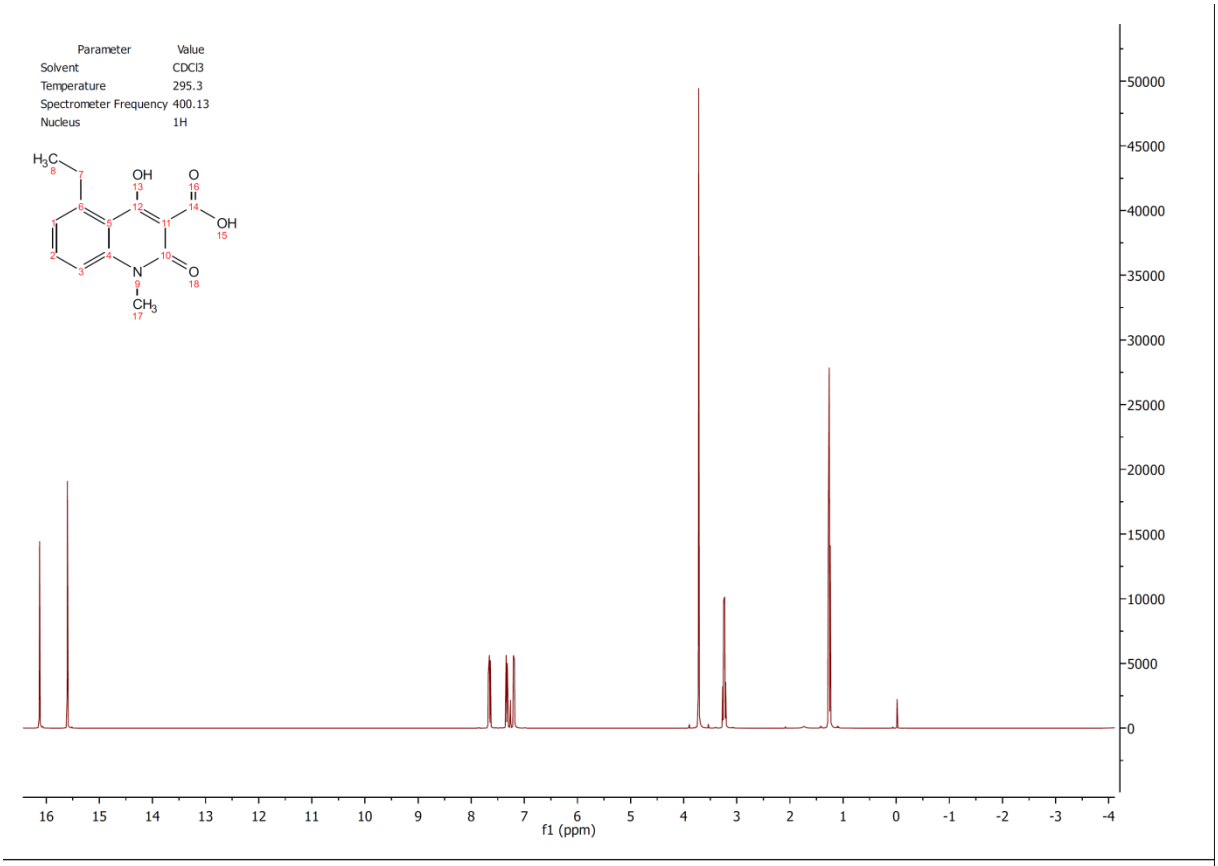
δ [ppm] = 174.6 (s, C-10) , 174.4 (s, C-7), 164.4 (s, C-9), 147.8 (s, C-5), 141.4 (s, C-1), 134,5 (d, C-3), 126.4 (d, C-4), 114.3 (s, C-6), 113.4 (d, C-2), 94,7 (s, C-8), 30.4 (q, C-13), 30.0 (t, C-11), 16.3 (q, C-12).

MS (ESI^{+}): m/z =

248.0917; calculated $[C_{13}H_{13}NO_4]H^{+}$ ([M + H]$^{+}$): 248.0917.

270.0735; calculated $[C_{13}H_{13}NO_4]Na^{+}$ ([M + Na]$^{+}$): 270.0737.

517.1581; calculated $[C_{26}H_{26}N_2O_8]_2Na^{+}$ ([2M + Na]$^{+}$): 517.1490.


Parameter Value
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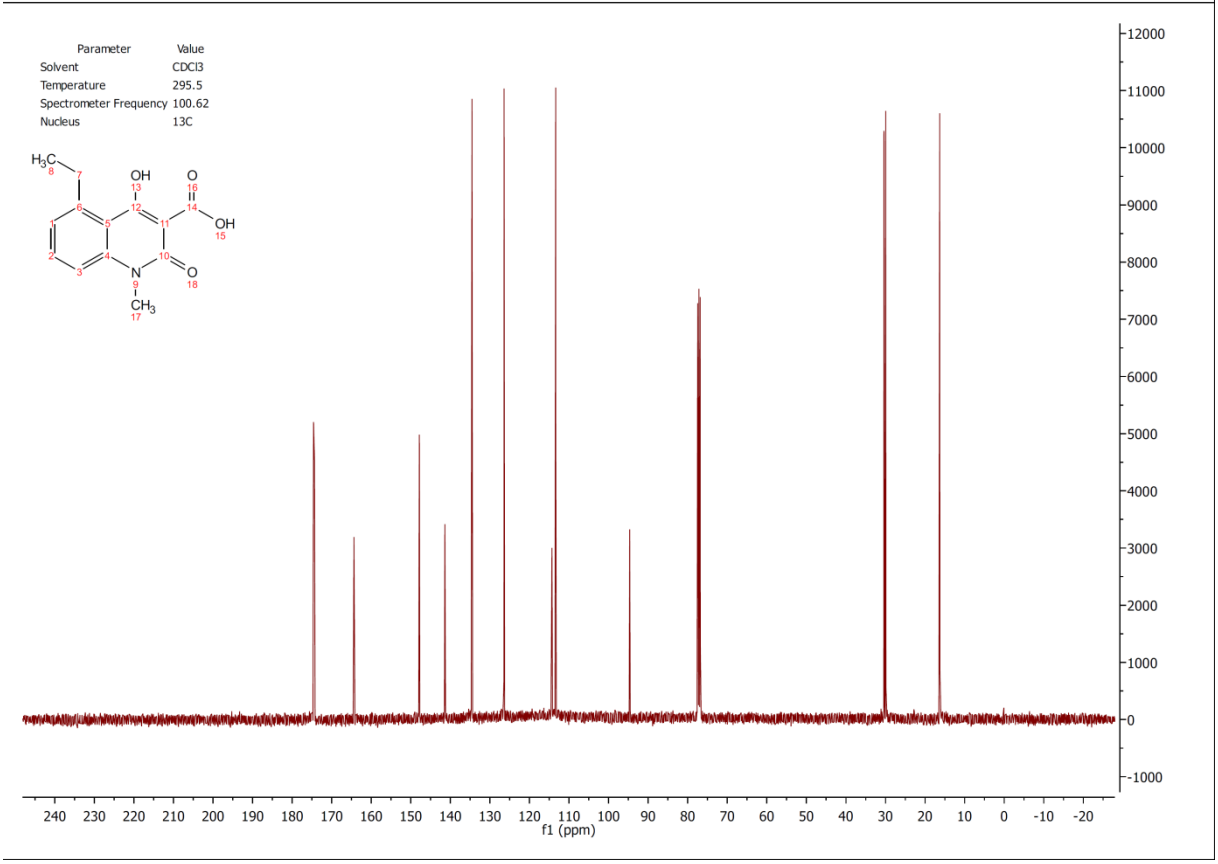
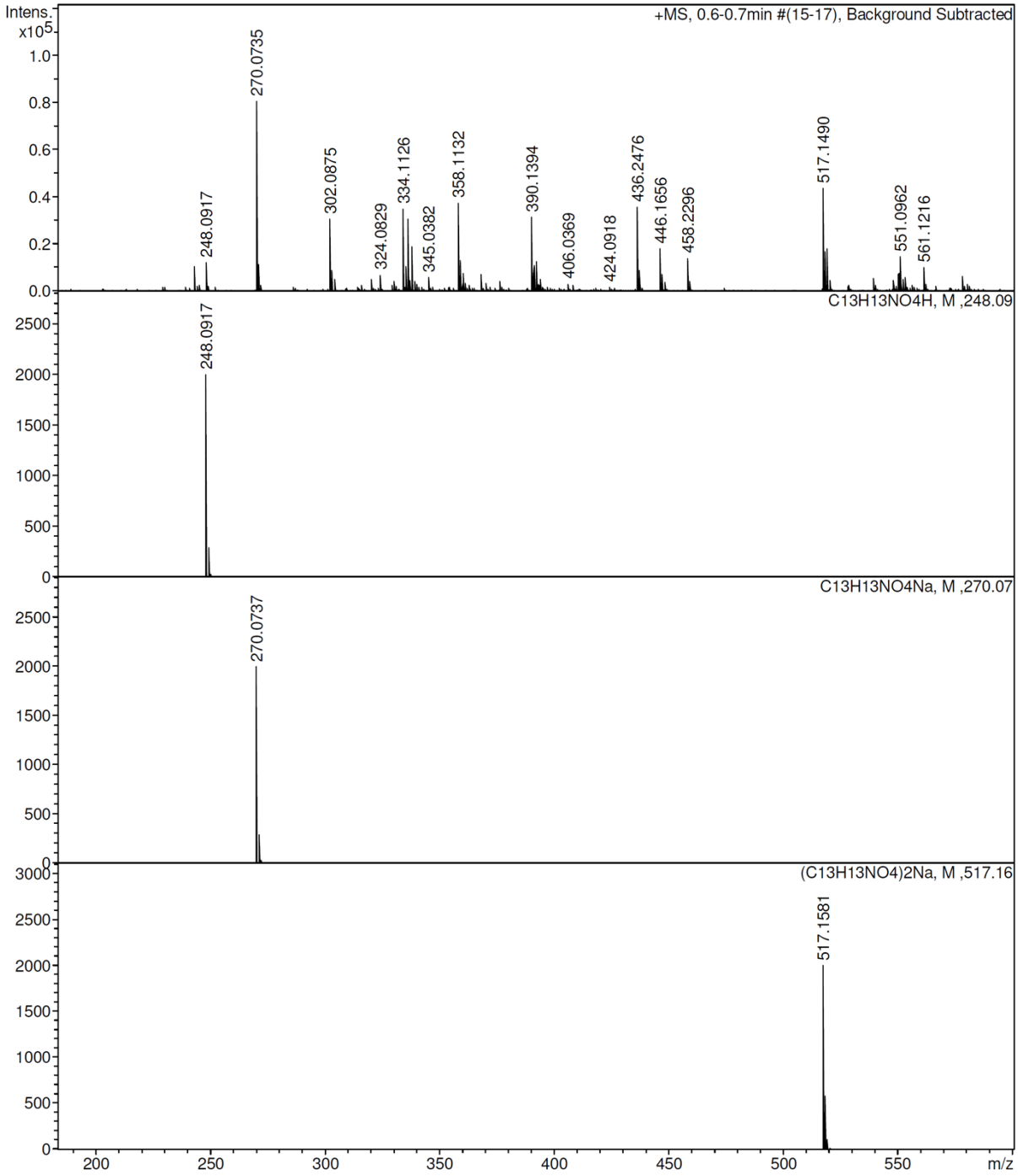
Temperature 295.3
Spectrometer Frequency 400.13
Nucleus 1H
f1 (ppm)


Parameter Value
Solvent CDCl3
Temperature 295.5
Spectrometer Frequency 100.62
Nucleus 13C
f1 (ppm)


Intens.
x10^5
+MS, 0.6-0.7min #(15-17), Background Subtracted
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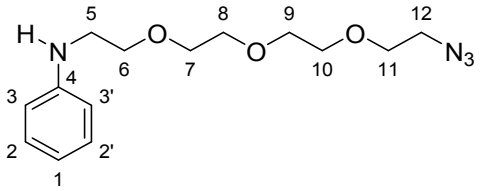
270.0735
302.0875
324.0829
334.1126
345.0382
358.1132
390.1394
406.0369
424.0918
436.2476
446.1656
458.2296
517.1490
551.0962
561.1216
C13H13NO4H, M ,248.09
248.0917
C13H13NO4Na, M ,270.07
270.0737
(C13H13NO4)2Na, M ,517.16
517.1581
m/z

***N*-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethyl)aniline**



Subsequently the azido-PEG-bromide (7.34g, 26mmol) and aniline (4.75mL, 52mmol) were emulsified in water (30mL) and heated to reflux overnight. After cooling to room temperature water (100mL) was added and the aqueous emulsion was extracted with ethyl acetate (100mL) four times. The organic phases were dried over magnesium sulphate and concentrated to dryness. The resulting oil was chromatographed on a silica gel column (cyclohexane/EtOAc 2/1 to 1/1) to give a yellow oil. Yield: 3.41 g (11.6 mmol, 45%).

^{1}H NMR (400 MHz, chloroform-*d*)

δ [ppm] = 7.20-7.14 (m, 2H, 2-CH, 2‘-CH), 6.73-6.68 (m, 1H, 1-CH), 6.64-6.60 (m, 2H, 3-CH, 3‘-CH), 3.71-3.62 (m, 12H, 6-CH_2 bis 11-CH_2), 3.35 (t, $^{3}J_{H,H}$ = 5.1 Hz, 2H, 12-CH_2), 2.29 (t, $^{3}J_{H,H}$ = 5.1 Hz, 2H, 5-CH_2).

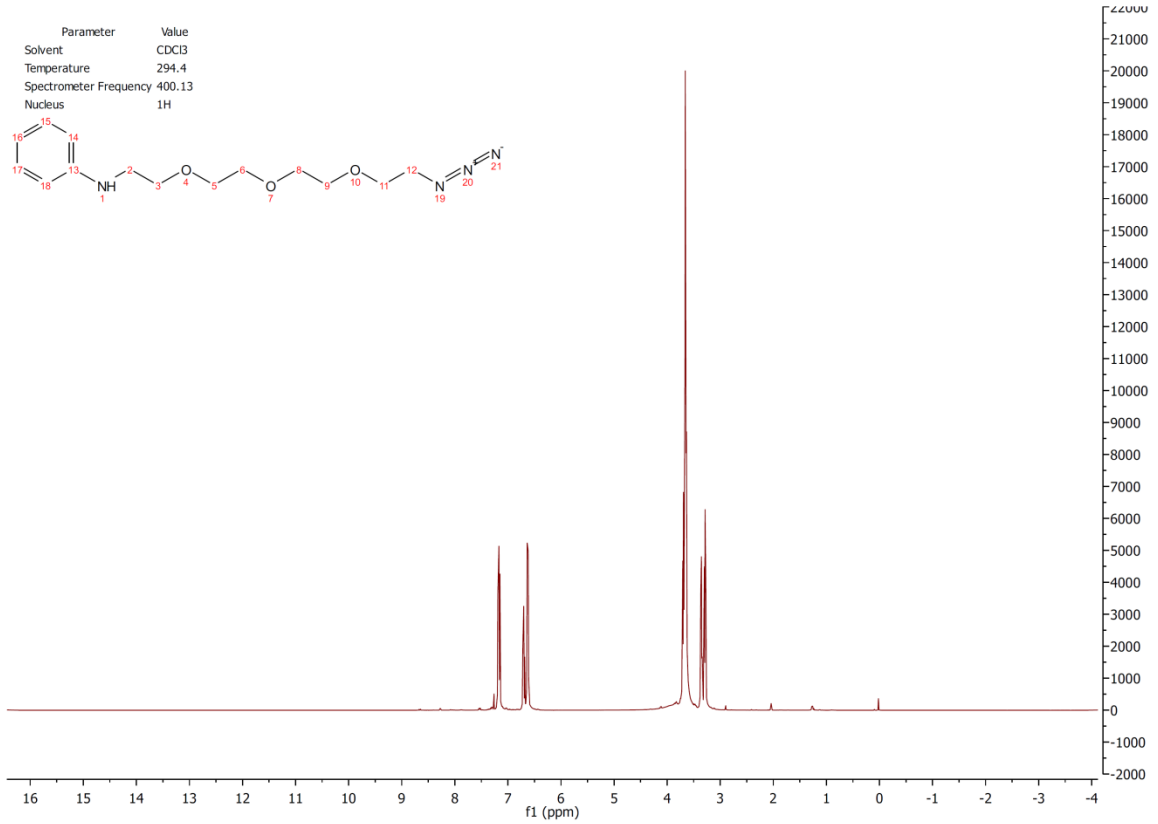
^{13}C NMR (101 MHz, chloroform-*d*)

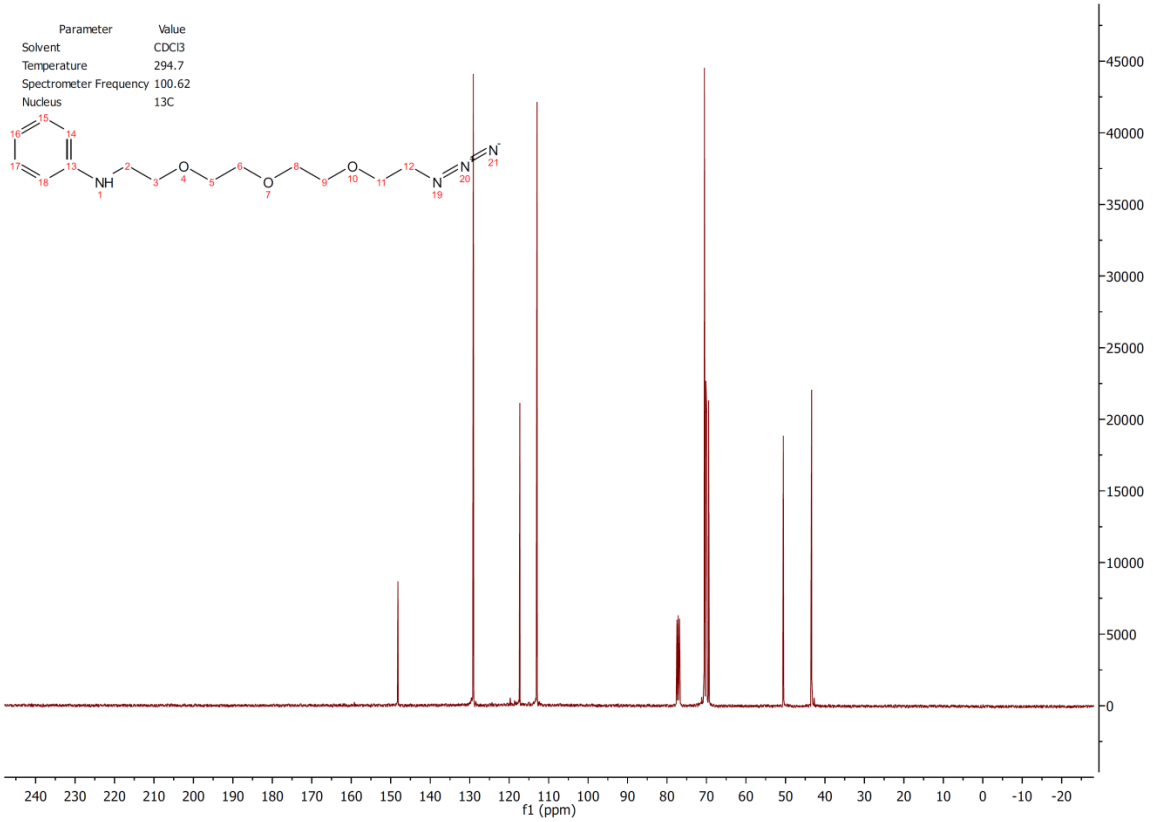
δ [ppm] = 148.2 (s, C-4), 129.1 (d, C-2, C-2’), 117.3 (d, C-1), 112.9 (d, C-3, C-3’), 70.6, 70.5, 70.5, 70.2, 69.9 (t, C-7 bis C-11), 69.5 (t, C-6), 50.5 (t, C-12), 43.4 (t, C-5).

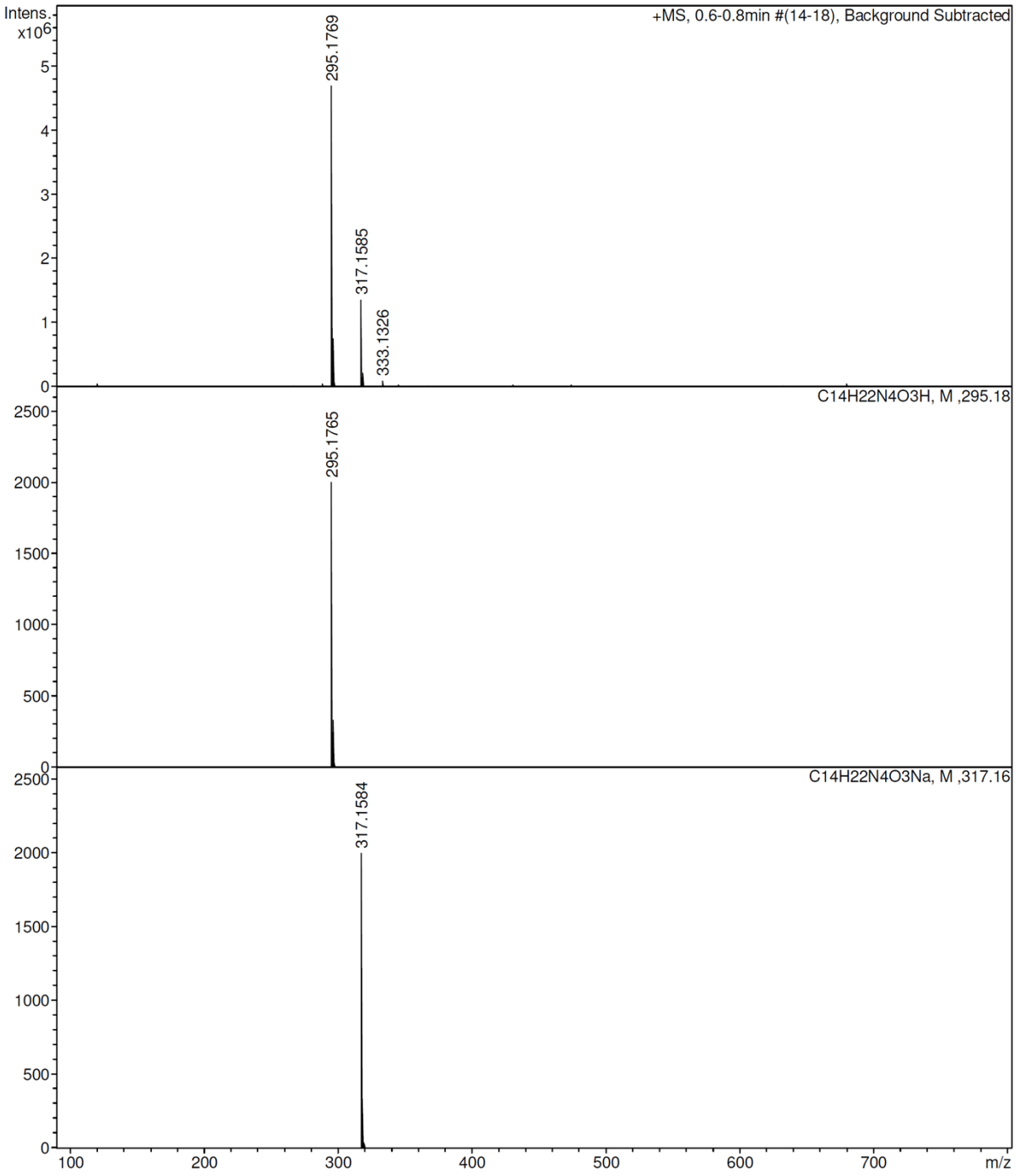
MS (ESI^{+}): m/z =

295.1769; calculated $[C_{14}H_{22}N_4O_3]H^{+}$ ($[M + H]^{+}$): 295.1765.

317.1585; calculated $[C_{14}H_{22}N_4O_3]Na^{+}$ ($[M + Na]^{+}$): 317.1584.


Parameter Value
Solvent CDCl3
Temperature 294.4
Spectrometer Frequency 400.13
Nucleus 1H
f1 (ppm)


Parameter Value
Solvent CDCl3
Temperature 294.7
Spectrometer Frequency 100.62
Nucleus 13C
f1 (ppm)


Intens.
x10^6
+MS, 0.6-0.8min #(14-18), Background Subtracted
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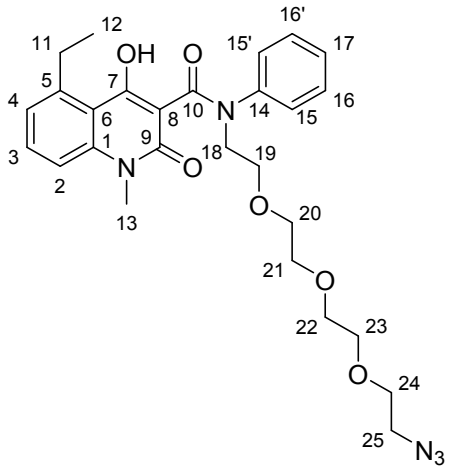
317.1585
333.1326
C14H22N4O3H, M ,295.18
295.1765
C14H22N4O3Na, M ,317.16
317.1584
m/z

***N*-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethyl)-5-ethyl-4-hydroxy-1-methyl-2-oxo-*N*-phenyl-1,2-dihydroquinoline-3-carboxamide**



The 1,2-dihydroquinoline-3-carboxylic acid (1g, 4.05mmol) was dissolved under argon atmosphere in dichloro methane (10mL) and triethylamine (2.1mL, 1.54g, 15.2mmol) and the *N*-PEG-aniline (1.43g, 4.86mmol) were added. At 0°C (ice bath) thionyl chloride (0.38mL, 0.63g, 5.3mmol), dissolved in dichloromethane (0.6mL), was added dropwise within 30 minutes. The reaction mixture was stirred for 4h at 0°C and then overnight at room temperature. After removing the solvent in vacuo the resulting oil was chromatographed on a silica gel column (EtOAc/MeOH 9/1 to 6/1) to give a yellow sticky oil. Yield: 1.73 g (3.3 mmol, 82%).

^{1}H NMR (400 MHz, chloroform-*d*)

δ [ppm] = 7.42-7.37 (m, 1H, 3-CH), 7.23-7.10 (m, 5H, 15-CH bis 17-CH), 7.03-7.00 (m, 1H, 2-CH), 7.00-6.96 (m, 1H, 4-CH), 3.71-3.58 (m, 12H, 19-CH_2 bis 24-CH_2), 3.62 (s, 3H, 13-CH_3), 3.36-3.32 (m, 2H, 25-CH_2), 3.29-3.24 (m, 2H, 18-CH_2), 3.20 (q, $^{3}J_{H,H}$ = 7.4 Hz, 2H, 11-CH_2), 1.25 (t, $^{3}J_{H,H}$ = 7.4 Hz, 3H, 12-CH_3).

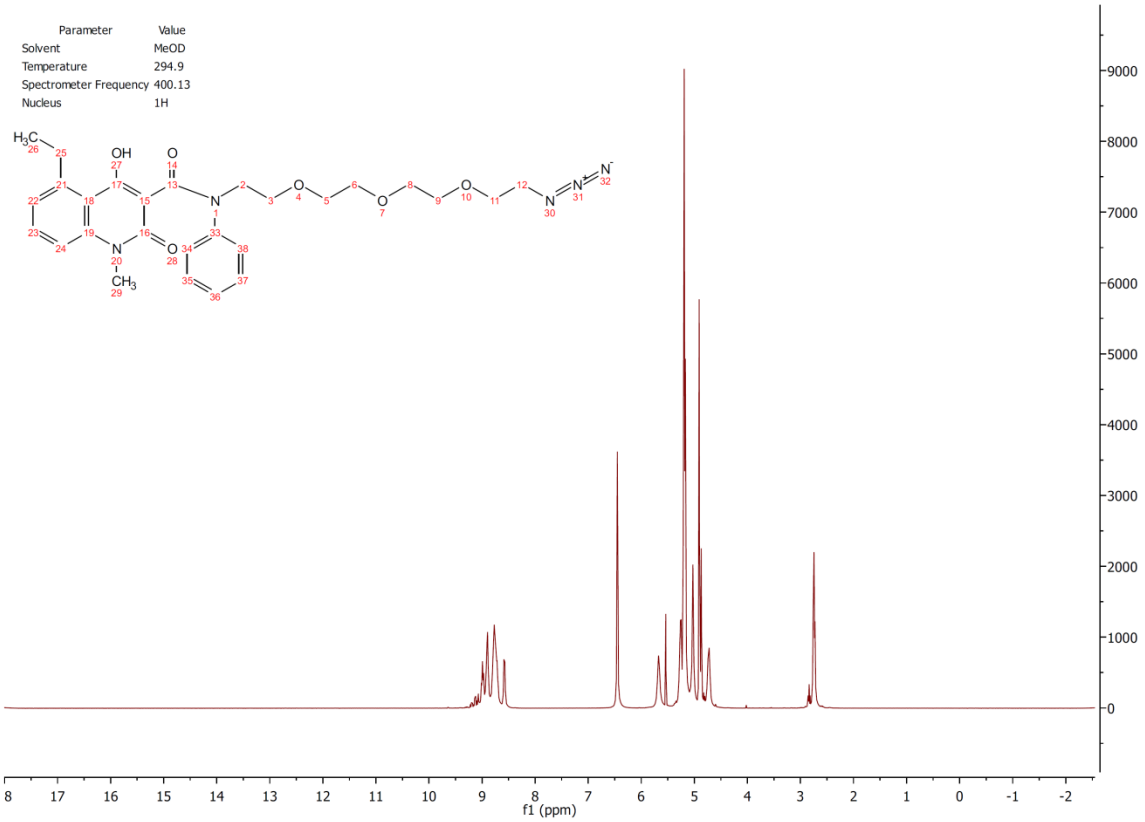
^{13}C NMR (101 MHz, chloroform-*d*)

δ [ppm] = 174.5 (s, n.z.), 170.1 (s, n.z.), 167.7 (s, n.z.), 148.2 (s, C-14), 145.8 (s, C-5), 142.0 (s, C-1), 131.9 (d, C-3), 129.2, 128.5, 126.3 (d, 15-C bis 17-C), 124.4 (d, C-4), 113.6 (s, C-6), 112.4 (d, C-2), 103.9 (s, C-8), 70.6, 70.6, 70.4, 70.0, 69.6, 67.8 (t, 19-C bis 24-C), 50.6 (t, C-25), 43.5 (t, C-18), 30.0 (t, C-11), 29.6 (q, C-13), 16.7 (q, C-12).

MS (ESI^{+}): m/z =

524.2507; calculated $[C_{27}H_{33}N_5O_6]H^{+}$ ($[M + H]^{+}$): 524.2504.

546.2326; calculated $[C_{27}H_{33}N_5O_6]Na^{+}$ ($[M + Na]^{+}$): 546.2323.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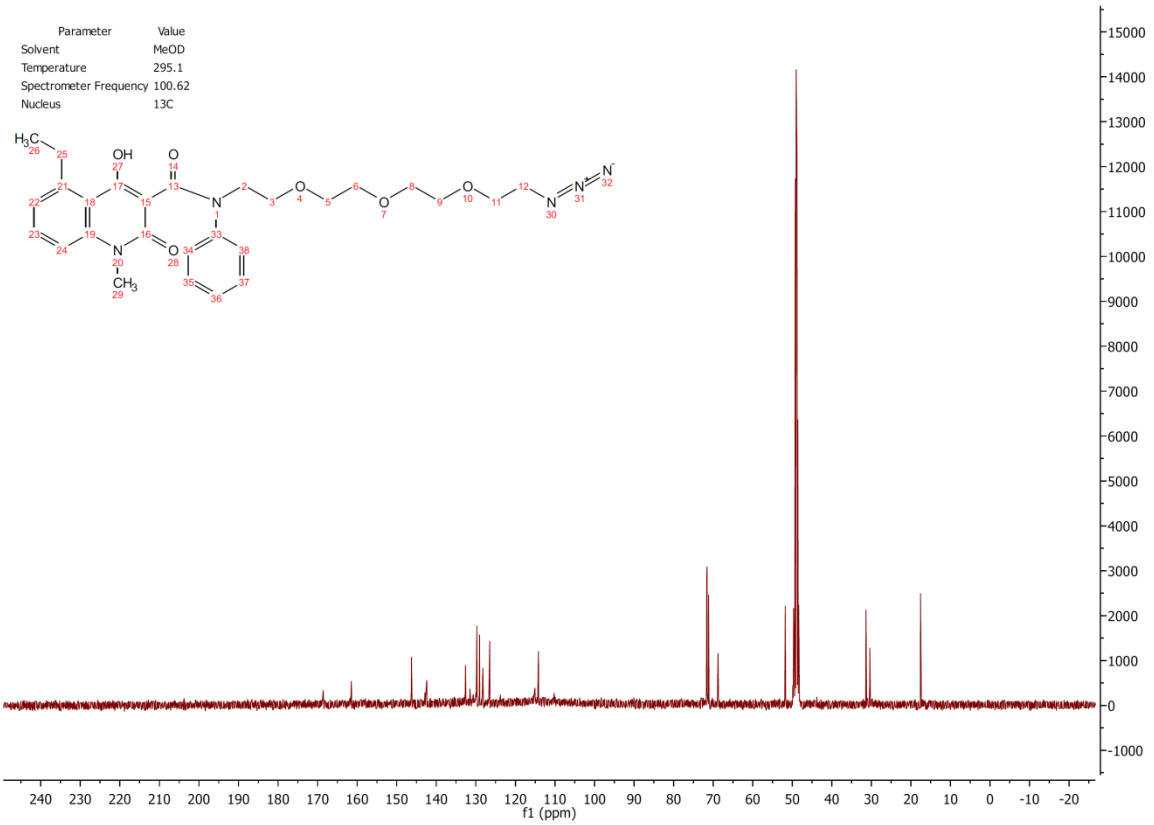
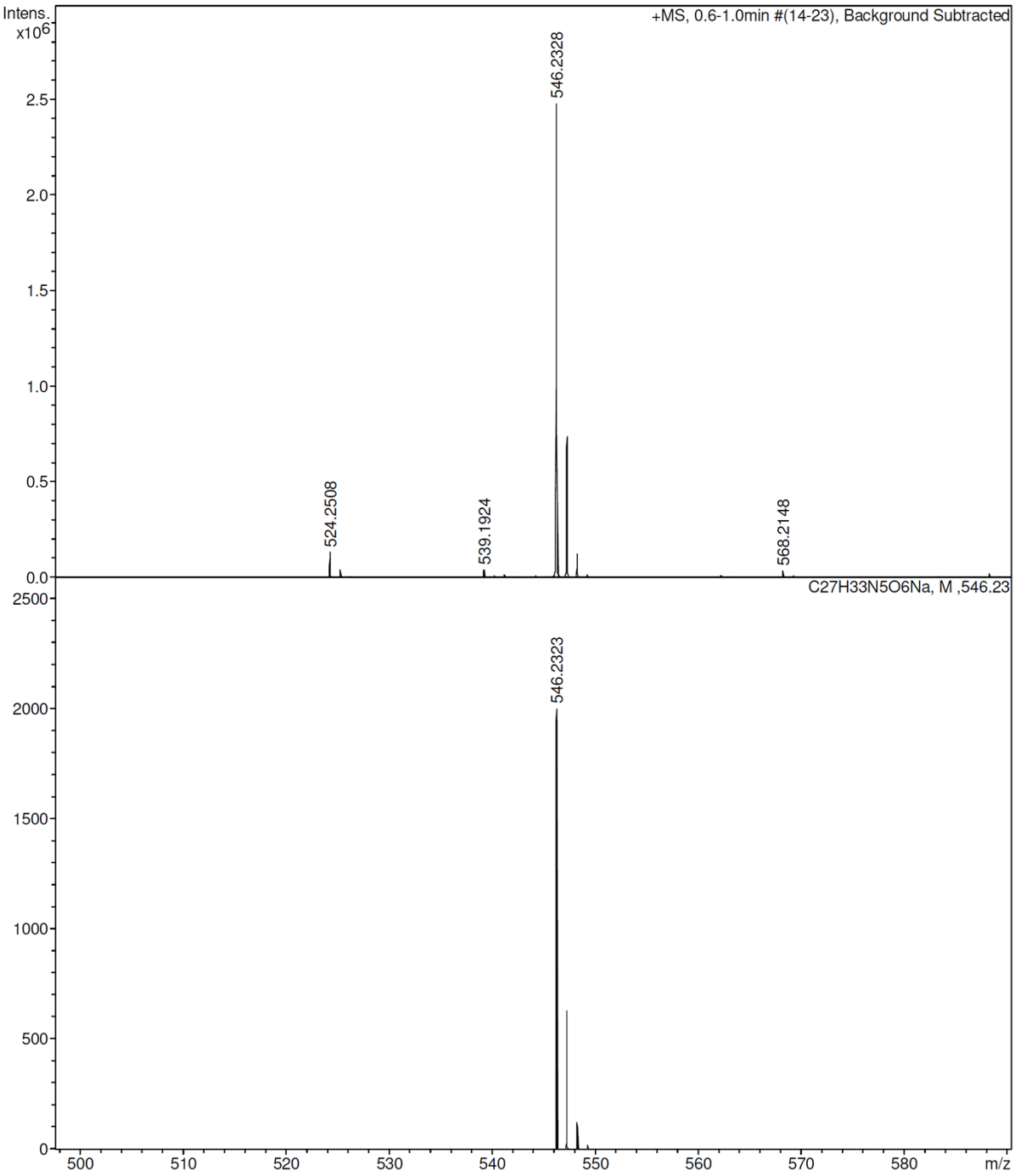
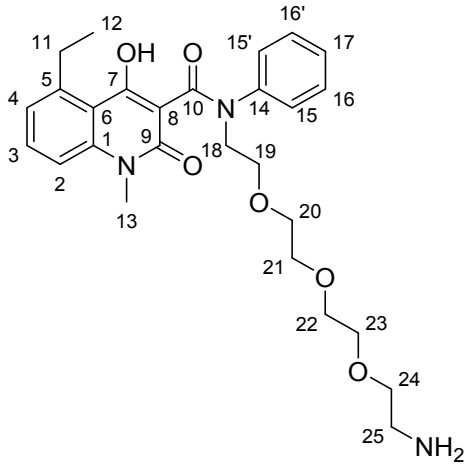
f1 (ppm)


Parameter Value
Solvent MeOD
Temperature 295.1
Spectrometer Frequency 100.62
Nucleus 13C
f1 (ppm)


Intens.
x10^6
+MS, 0.6-1.0min #(14-23), Background Subtracted
546.2328
524.2508
539.1924
568.2148
C27H33N5O6Na, M ,546.23
546.2323
m/z

***N*-(2-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)ethyl)-5-ethyl-4-hydroxy-1-methyl-2-oxo-N-phenyl-1,2-dihydroquinoline-3-carboxamide**



N-(2-{2-[2-(2-Azidoethoxy)ethoxy]ethoxy}ethyl)-5-ethyl-4-hydroxy-1-methyl-2-oxo-*N*-phenyl-1,2-dihydrochinolin-3-carboxamide (1.128 g, 2.15 mmol, 1.0 Äq.) was solved under Ar in tetrahydrofurane (15 mL) and a few mg of Pd/C were added. The Ar-atmosphere was changed to hydrogen (balloon) and the reaction mixture was stirred at room temperature for 16h. After filtration on Celite® and evaporation of the solvent the desired product was obtained as a light brown solid.

yield: 840 mg (1.69 mmol, 79%).

mp.: 53 °C (THF)

^{1}H NMR (400 MHz, methanol-d_4)

δ [ppm] = 7.42-7.39, 7.15-7.10 (m, 5H, 15-CH bis 17-CH), 7.26-7.21 (m, 1H, 3-CH), 7.10-7.05 (m, 1H, 2-CH), 6.82-6.79 (m, 1H, 4-CH), 3.91-3.86, 3.77-3.74, 3.68-3.52 (m, 12H, 19-CH_2 bis 24-CH_2), 3.52-3.46 (m, 2H, 11-CH_2), 3.40 (s, 3H, 13-CH_3), 3.29-3.22 (m, 2H, 18-CH_2), 3.21-3.18 (m, 2H, 25-CH_2), 1.14 (t, $^{3}J_{H,H}$ = 7.4 Hz, 3H, 12-CH_3).

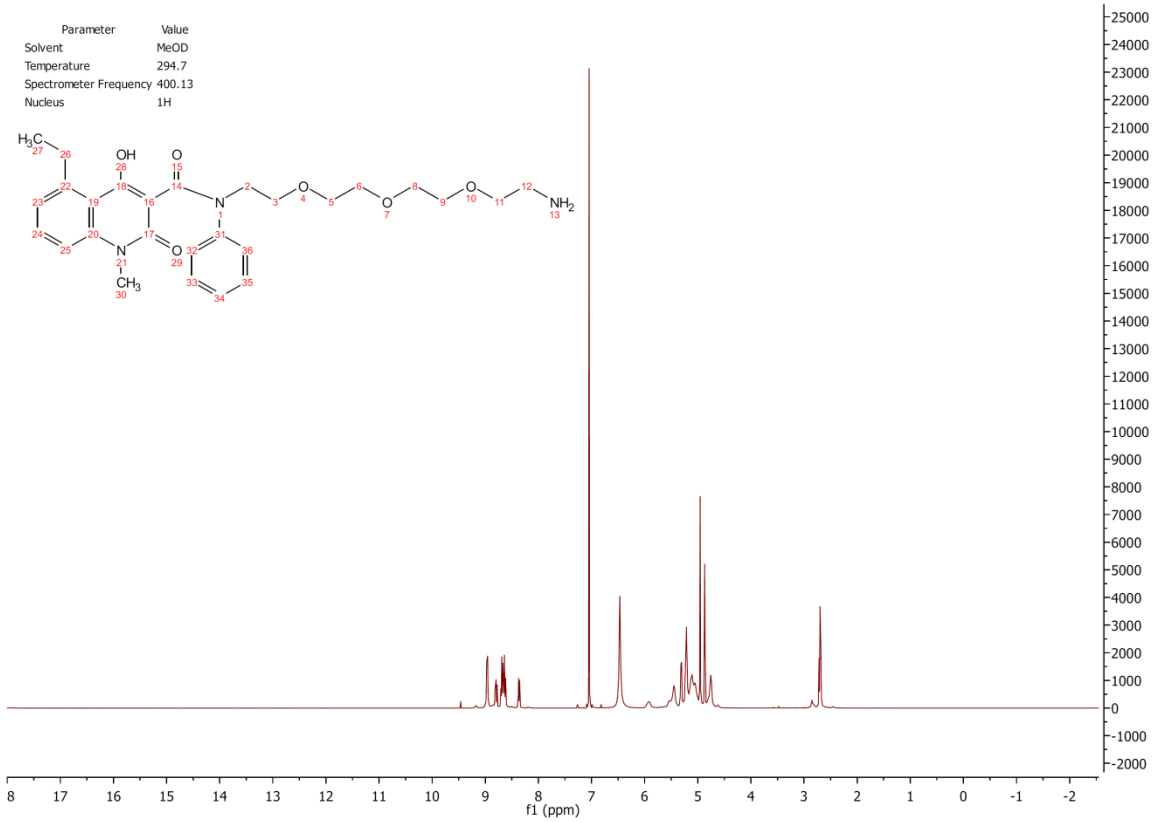
^{13}C NMR (101 MHz, methanol-d_4)

δ [ppm] = 174.5 (s, C-7), 174.2 (s, C-10), 162.8 (s, C-9), 147.2 (s, C-5), 143.0 (s, C-1), 142.8 (s, C-14), 130.4 (d, C-3), 129.2, 128.3, 128.2, (d, C-15 bis C-17), 124.4 (d, C-4), 121.7 (s, C-6), 113.5 (d, C-2), 109.2 (s, C-8), 71.3, 71.0 , 71.0, 70.9, 70.7, 68.8, 68.1 (t, C-19 bis C-24), 44.7 (t, C-18), 40.7 (t, C-25), 30.3 (t, C-11), 29.8 (q, C-13), 17.8 (t, C-12).

MS (ESI$^+$): m/z =

498.2598; calculated $[C_{27}H_{35}N_3O_6]H^+$ ($[M + H]^+$): 498.2599.

520.2420; calculated $[C_{27}H_{35}N_3O_6]Na^+$ ($[M + Na]^+$): 520.2418.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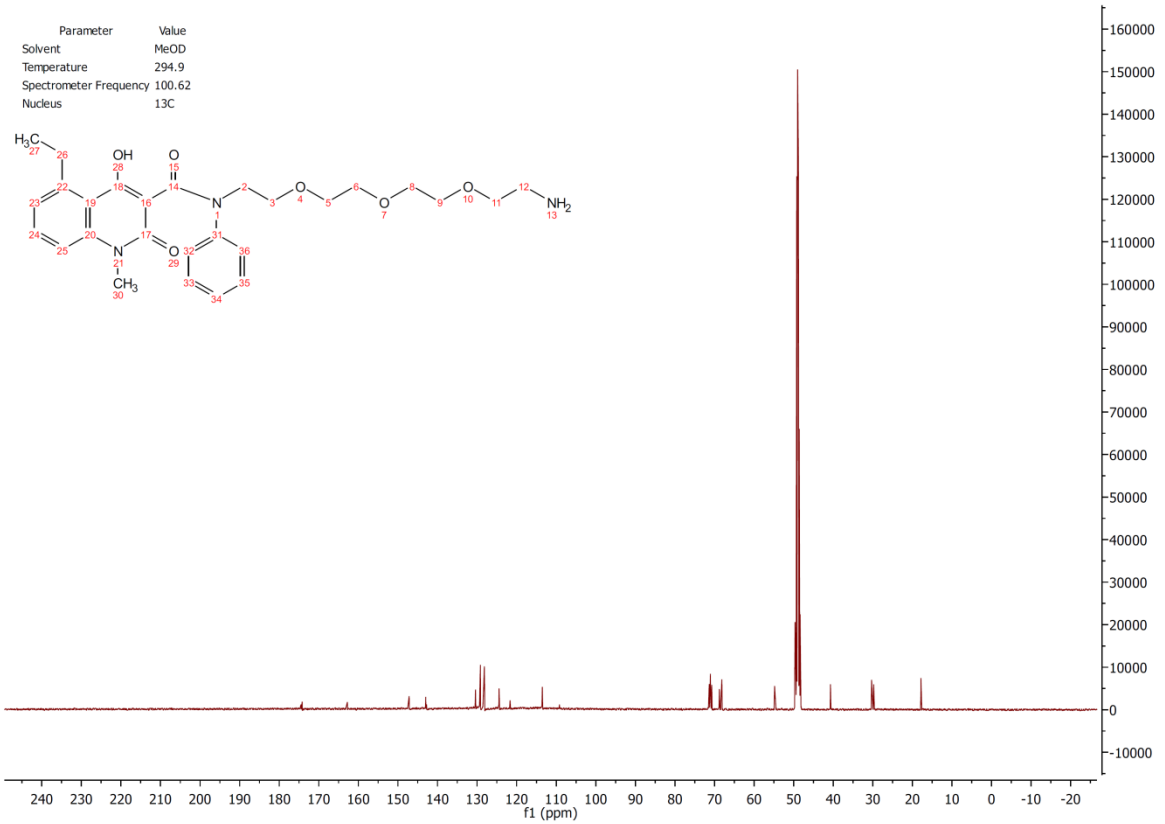
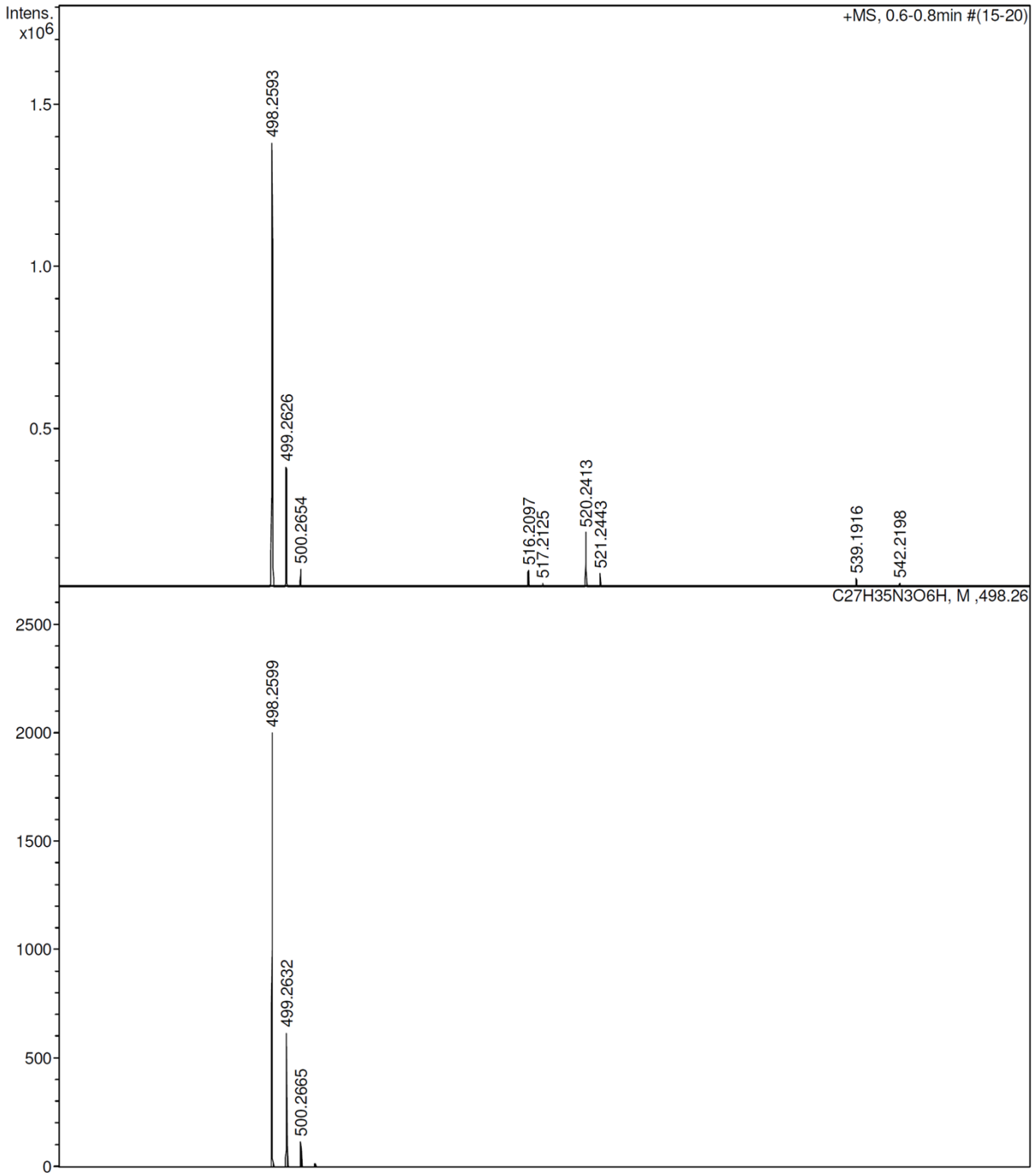
Nucleus 1H
f1 (ppm)


Parameter Value
Solvent MeOD
Temperature 294.9
Spectrometer Frequency 100.62
Nucleus 13C
f1 (ppm)


Intens.
x10^6
+MS, 0.6-0.8min #(15-20)
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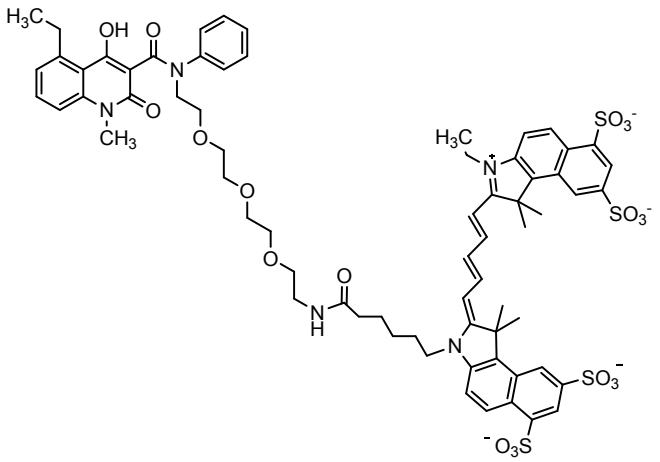
1.0
0.5
498.2593
499.2626
500.2654
516.2097
517.2125
520.2413
521.2443
539.1916
542.2198
C27H35N3O6H, M ,498.26
2500
2000
1500
1000
500
0
498.2599
499.2632
500.2665

3-ethyl-2-((1E,3E,5E)-5-(3-(1-(5-ethyl-4-hydroxy-1-methyl-2-oxo-1,2-dihydroquinolin-3-yl)-1,15-dioxo-2-phenyl-5,8,11-trioxa-2,14-diazanonadecan-19-yl)-1,1-dimethyl-6,8-disulfonato-1H-benzo[e]indol-2(3H)-ylidene)penta-1,3-dien-1-yl)-1,1-dimethyl-1H-benzo[e]indol-3-ium-6,8-disulfonate (Cy5.5-CES271)



The amino-functionalized precursor (3.0 mg, 4.2 µmol) was dissolved in 400 µL dry dimethylformamide provided with 10 µL triethylamine. To this solution, Cy5.5-NHS ester (GE) (1 mg, 0.9 µmol) was added. The reaction mixture was vortexed for 16 h at room temperature in the dark. Purification of Cy5.5-CES271 was performed by gradient-HPLC using a Knauer system with two K-1800 pumps, an S-2500 UV detector and a RP-HPLC Nucleosil 100-5 C18 column (250 mm × 4.6 mm). Eluent A: water (0.1 % TFA). Eluent B: Acetonitrile (0.1 % TFA). Gradient from 95 % A to 40 % A over 19 minutes, holding for 5 minutes and back to 95 % in one minute at a flow rate of 5.5 ml/min, detection at λ = 254 nm. The appropriate fractions (t_R = 16.5 min) were collected, lyophilized, redissolved in 1 mL water and finally stored at - 20 °C. The average content of Cy5.5-CES271 was 0.45 ± 0.02 µmol/ml (≈ 50 %) as determined by fluorometer measurements with λ_{abs} = 678 nm and ε_{678} = 250000 $M^{-1\,cm-1}$.

HRMS (ES^-): m/e = 347.84493, 348.09560, 348.34570, 348.59556, 348.84580 $[M]^{4-}$

calculated : 347.84511, 348.09589, 348.34575, 348.59593, 348.84592 $[M]^{4-}$

Binding studies

For our binding studies all proteins were dissolved in phosphate buffered saline (PBS) containing 100 µM calcium, unless otherwise specified.

ELISA

The binding of **Cy5.5-CES271** to S100A9 was determined by blocking the specific binding of S100A9 to its receptor TLR4/MD2. 50 µl of TLR4/MD2 (3146-TM-050/CF, R&D Systems) was coated to a 96-well plate at a concentration of 1µg/ml at 4°C over night and served as capturing molecule. After a washing step, the unspecific binding sites were blocked with PBS/5% skim milk powder, plates were washed three times. S100A9 protein was added at a concentration of 1 µg/ml in the presence or absence of 100 µM **Cy5.5-CES271** and incubated for two hours at room temperature. Unbound S100 protein was removed by three additional washing steps, followed by the addition of a primary anti-S100A9-antibody (1 µg/ml, polyclonal, rabbit IgG, 1 h at 4°C). The secondary anti-rabbit-IgG-antibody coupled to HRP (1 µg/ml, Cell Signalling) was added for 1 h at room temperature. TMB was used as substrate for HRP for 30 minutes and blocked with sulfuric acid to quantify binding efficiency by absorbance readings at 450 nm in an ELISA reader (Anthos Mikrosysteme). Addition of the non-peptidic S100A9 ligand (**Cy5.5-CES271**) markedly blocked binding of S100A9 to TLR4/MD2, as indicated by a decrease of signal given by the TLR4-S100A9 ELISA (Figure 2A).

Binding constant

The binding constants of **Cy5.5-CES271** to murine and human S100A9 by fluorimetric measurements (Fluoromax II, Spex Instruments). We used an excitation waveleght of 675 nm and an emission wavelenght of 690 nm. 0,3788 µM S100A9 (5 µg/ml of homodimer S100A9) solved in 50 µl phosphate buffered saline (PBS) were coated to the bottom of a 96-well plate and served as capturing molecule over night at 4°C. For each S100A9 coated well a control well was used with 50 µl PBS alone to determine unspecific binding. After three washing steps, unspecific binding sites were blocked by PBS/5% skim milk powder at room temperature for 1h. **Cy5.5-CES271** was added at increasing concentrations into washed wells. After 1h incubation at 4°C, the supernatants were removed and the fluorescence intensity was measured with the fluorimeter (F = concentration of free ligand; B = concentration of bound ligand).

We performed a non-linear regression analysis with a one site saturation modell (equation 1), to calculate the binding constant (K_d) of **Cy5.5-CES271** to either murine or human S100A9 (Figure 2B). K_d is equal to the equilibrium concentration of the ligand at which 50% of the binding sites are occupied. The ammount of coated S100A9 provided the total number of binding sites in the well (B_{max}).

$$B = \frac{B_{max} \times F}{K_d + F}$$ (where B is “bound” and F is “free”) *(1)*

Mice

Balb/c mice (Harlan Laboratories) were used at the age of 10–14 weeks, sex and age matched for each set of experiments and housed under specific pathogen-free conditions. All experiments with mice were performed with the approval of the State Review Board of Nordrhein-Westfalen (Germany) according to the German law for animal welfare (Permit Number: 84-02.04.2012.A058).

Biodistribution

Cy5.5-CES271 was intravenously injected via the tail vein at a dose of 2 nmol per mouse. Either 1 h or 3 h post injection blood and urine samples were taken under 2% isoflurane inhalation anesthesia. Then the mice were sacrificed and perfused with phosphate buffered saline. The organs were removed and fluorescence reflectance imaging was performed with an IVIS Spectrum small-animal imaging system (PerkinElmer, Waltham, MA, USA). Excitation of Cy5.5-CES271 was achieved at 675 ± 15 nm, and emitted light was filtered by a bandpass filter (720 ± 10 nm). The following settings were chosen: binning: 1, f-stop: 2, field of view: B (6.6 cm), exposure time: automatic. Images were analyzed with the software Living Image 4.2.0 (PerkinElmer, Waltham, MA, USA). Grayscale photographic images and fluorescence colour images were superimposed. Regions of interest (ROIs) were drawn over the tissue samples using the photographic images. Fluorescence emission was measured by fluorescence emission radiance per incident excitation irradiance (p/sec/cm^2/sr/µW/cm^2). The tissue samples were weighted and the fluorescence intensity per gram organ weight was calculated (Figure 3).