Supporting Information

Efficient Cryopreservation of Human Midbrain Organoids Using Conventional DMSO Protocols with Ice Recrystallization Inhibitors

Ellyssa M. Walsh [a], Cecilia Rocha [b], Paula Lépine [b], Julien Sirois [b], Thomas M. Durcan [b], Robert N. Ben[a]*

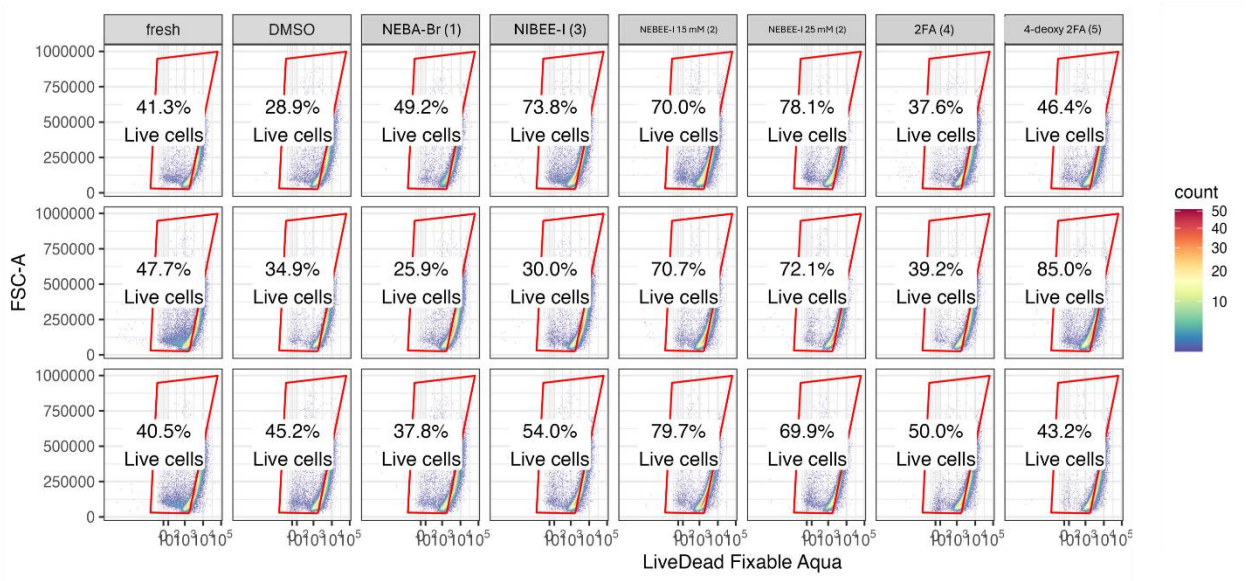
[a] Department of Chemistry and Biomolecular Sciences, University of Ottawa, Ottawa, Ontario, Canada, K1N 6N5

[b] The Neuro's Early Drug Discovery Unit (EDDU), McGill University, 3801 University, Montreal, QC H3A 2B4

*Corresponding author: Robert N. Ben robert.ben@uottawa.ca

Flow Cytometry Gating Information

Stained



Unstained

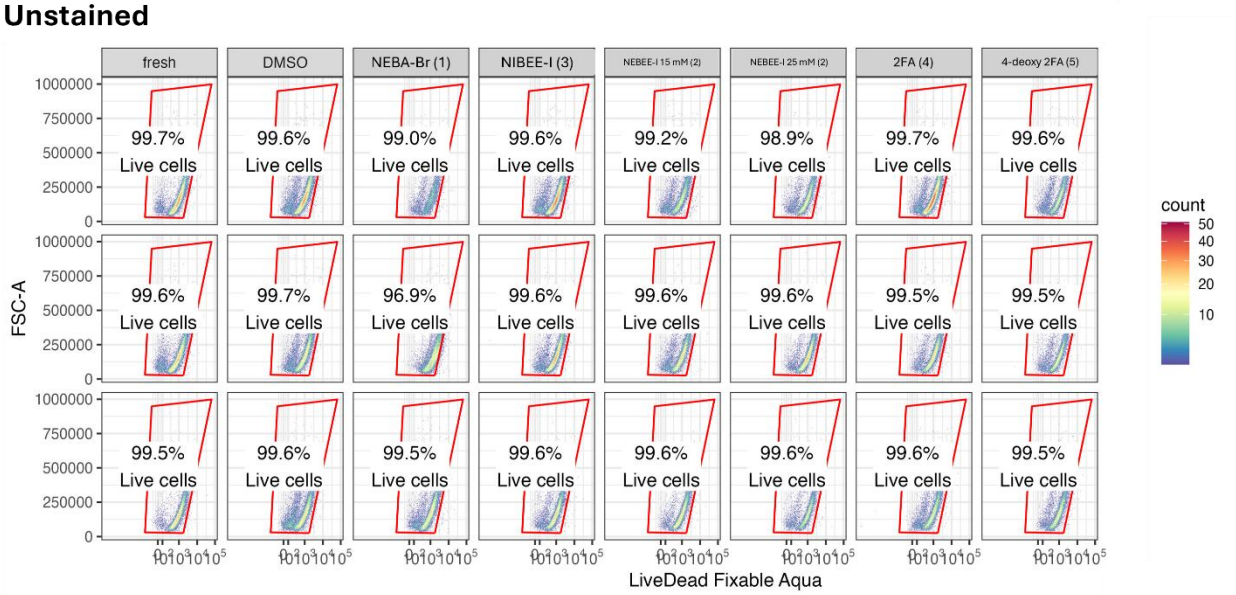


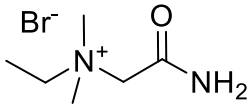
Figure S1. Representative flow cytometry gating strategy used for viability analysis with Fixable Viability Dye (FVD) Aqua. *Cell populations were sequentially gated to exclude debris based on forward and side scatter, remove doublets using forward scatter area versus height, and identify live (FVD Aqua-negative) and dead (FVD Aqua-positive) cells. The percentage of live cells was used for all viability analyses. Each biological replicate represents a single dissociated organoid analyzed independently by flow cytometry (n = 3), and the reported values in the main text are presented as the mean ± SD.*

General Experimental

All anhydrous reactions were performed in flame-dried glassware under a positive pressure of argon with distilled anhydrous solvents (LC Technology Solutions – SPBT 1). Air or moisture-sensitive reagents and anhydrous solvents were transferred with oven-dried syringes. All flash chromatography was performed with SiliFlash® P60 silica gel. Reaction progress was monitored using thin layer chromatography (TLC) with 0.2 mm pre-coated silica gel aluminum plates and components were visualized by illumination with a short-wavelength (254 nm) ultraviolet light and staining (orcinol, p-anisaldehyde). ^{1}H (400 or 600 MHz) and ^{13}C (100 or 150 MHz) NMR spectra were recorded at room temperature on a Bruker Avance II 400 spectrometer or Bruker Avance III HD 600 spectrometer with deuterated dimethyl sulfoxide ($(CD_3)_2SO$) or deuterium oxide (D_2O) as the solvent. Chemical shifts are reported in ppm using the residual solvent peak as an internal standard. Splitting patterns are designated as follows: s, singlet; d, doublet; t, triplet; q, quartet; quint, quintet; sext, sextet; m, multiplet and br, broad. High resolution mass spectrometry (HRMS) was performed by the John L. Holmes Mass Spectrometry Facility at the University of Ottawa.

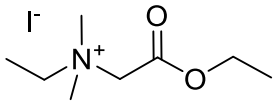
Synthesis and Characterization

***N*-ethyl betaine amide bromide (*NEBA-Br, (2-amino-2-oxoethyl)-ethyl-dimethylazanium bromide*) [1]**



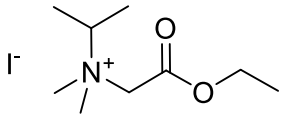
N-ethyl-*N,N*-dimethylamine (0.75 mL, 7.25 mmol) and 2-bromoacetamide (1.0 g, 7.25 mmol) were dissolved in acetonitrile (5.0 mL) in a flame-dried flask under an argon atmosphere. The reaction mixture was heated to reflux (85 °C) with a water condenser and stirred overnight. The solvent was removed under reduced pressure, and the residue was recrystallized from methanol and diethyl ether to afford the desired product as a white crystalline solid (1.49 g, 98%). **^{1}H NMR (400 MHz, D_2O):** δ 4.07 (s, 2H), 3.60 (*q*, *J* = 7.2 Hz, 2H), 3.22 (s, 6H), 1.36 (t, *J* = 7.2 Hz, 3H). **^{13}C NMR (100 MHz, D_2O):** δ 166.8, 61.6, 61.4, 51.2, 7.6. **HRMS (ESI):** *m/z* calcd. for $C_7H_{18}NO^+$ $[M]^+$131.1179; found 131.1183.

***N*-ethyl betaine ethyl ester iodide *(NEBEE-I, (2-ethoxy-2-oxoethyl)-ethyl-dimethylazanium iodide)* [2]**



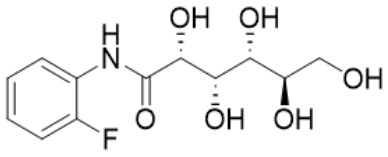
N,N-dimethylglycine ethyl ester (0.25 mL, 3.2 mmol) and iodoethane (0.76 mL, 3.2 mmol) were dissolved in acetonitrile (7.0 mL) in a flame-dried flask under an argon atmosphere. The reaction mixture was heated to reflux (85 °C) with a water condenser and stirred overnight. The solvent was removed under reduced pressure, and the residue was recrystallized from methanol and diethyl ether to afford the desired product as an orange crystalline solid (0.67 g, 71%). **^{1}H NMR (400 MHz, D_2O):** δ 4.31 (q, *J* = 7.2 Hz, 2H), 4.25 (s, 2H), 3.64 (q, *J* = 7.3 Hz, 2H), 3.23 (s, 6H), 1.37 (t, *J* = 7.3 Hz, 3H), 1.29 (t, *J* = 7.2 Hz, 3H). **^{13}C NMR (100 MHz, D_2O):** δ 165.2, 63.4, 61.6, 60.9, 51.0, 13.1, 7.6. **HRMS (ESI):** *m/z* calcd. for $C_8H_{18}NO_2^+$ $[M]^+$ 160.1333; found 160.1320.

_N_-isopropyl betaine ethyl ester iodide *(NIBEE-I, (2-ethoxy-2-oxoethyl)-dimethyl-propan-2-ylazanium iodide)* [3]



N,N-dimethylglycine ethyl ester (0.3 mL, 2.12 mmol) and 2-iodopropane (0.2 mL, 2.0 mmol) were dissolved in acetonitrile (5.0 mL) in a flame-dried flask under an argon atmosphere. The reaction mixture was heated to reflux (85 °C) with a water condenser and stirred overnight. The solvent was removed under reduced pressure, and the residue was recrystallized from methanol and hexanes to afford the desired product as an orange crystalline solid (0.13 g, 22%).**^{1}H NMR (400 MHz, D_2O):** δ 4.30 (q, *J* = 7.2 Hz, 2H), 4.24 (s, 2H), 4.09 (sext., *J* = 6.6 Hz, 1H), 3.18 (s, 6H), 1.39 (d, *J* = 6.6 Hz, 6H), 1.28 (t, *J* = 7.2 Hz, 3H).**^{13}C NMR (100 MHz, D_2O):** δ 165.4, 67.6, 63.4, 60.3, 48.2, 15.6, 13.1. **HRMS (ESI):** *m/z* calcd. for $C_9H_{20}NO_2^+$ $[M]^+$ 174.1489; found 174.1518.

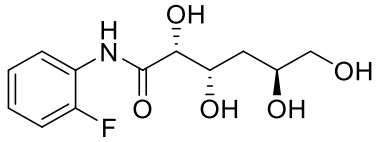
_N_-(2-fluorophenyl)-D-gluconamide (*2FA*) [4]



N-(2-fluorophenyl)-D-gluconamide was synthesized as described previously [32]. The product was obtained as a white powder (3.86 g, 47%). All spectroscopic data (^{1}H NMR, ^{13}C NMR, and HRMS) were consistent with those previously reported [32]. **^{1}H NMR (400 MHz, $(CD_3)_2SO$):** δ 9.17 (br, 1H), 8.09 (dt, *J* = 8.0, 1.9, 1H), 7.28 –7.20 (m, 1H), 7.17 – 7.06 (m, 2H),

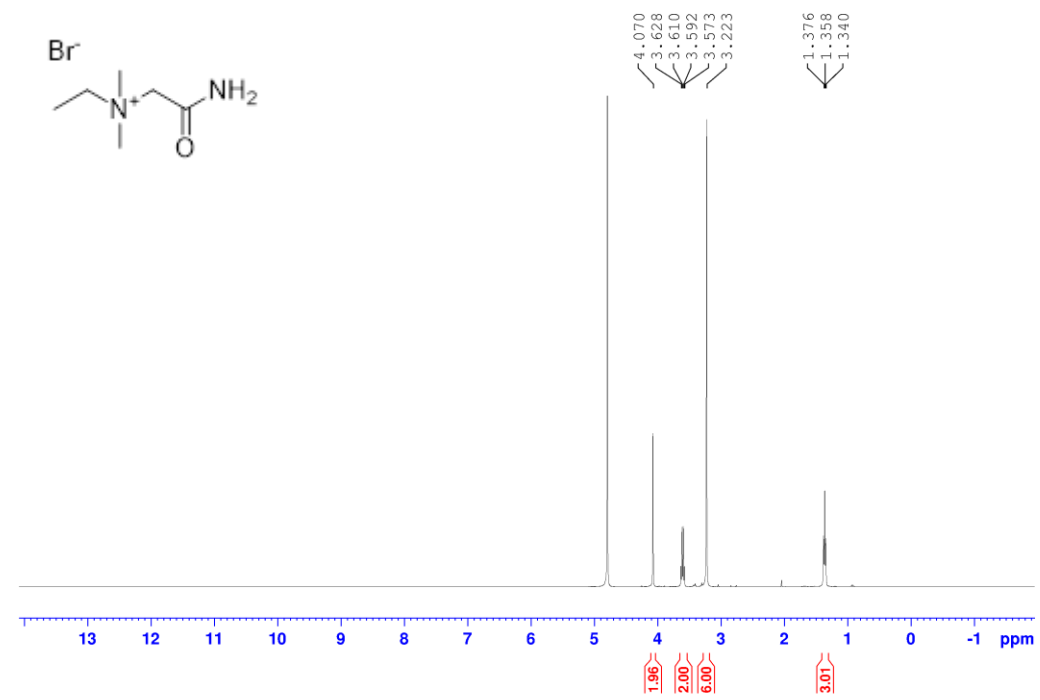
5.93 (d, *J* = 4.9 Hz, 1H), 4.65 (d, *J* = 7.3 Hz, 1H), 4.60 (dd, *J* = 9.1, 5.6 Hz, 2H), 4.37 (t, *J* = 5.6 Hz, 1H), 4.21 (dd, *J* = 4.7, 1.4 Hz, 1H), 3.99 (dt, *J* = 7.2, 3.0 Hz, 1H), 3.60 – 3.44 (m, 3H), 3.40 – 3.36 (m, 1H). **^{13}C NMR (100 MHz, $(CD_3)_2SO$):** *δ* 172.0, 154.0, 152.4, 126.4, 125.2, 122.5, 115.7, 74.5, 72.7, 72.1, 70.7, 63.8. **HRMS (ESI):** *m/z* calcd. for $C_{12}H_{16}FNO_6$ $[M + Na]^+$ 312.0859; found 312.0859.

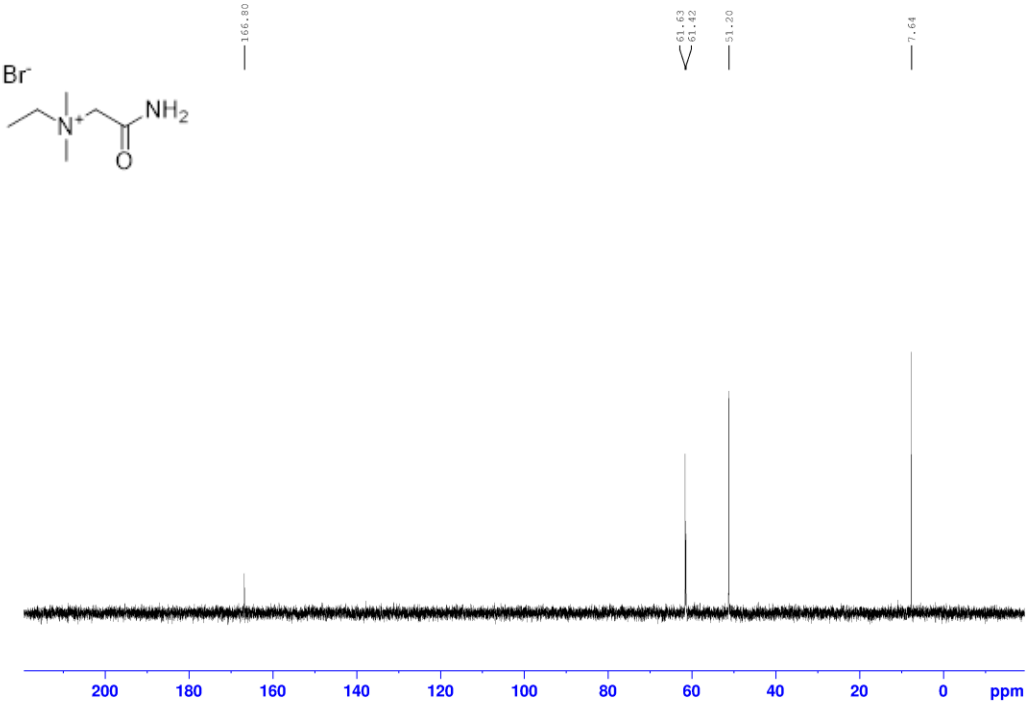
4-deoxy-*N*-(2-fluorophenyl)-D-gluconamide (*4-deoxy 2FA*) [5]

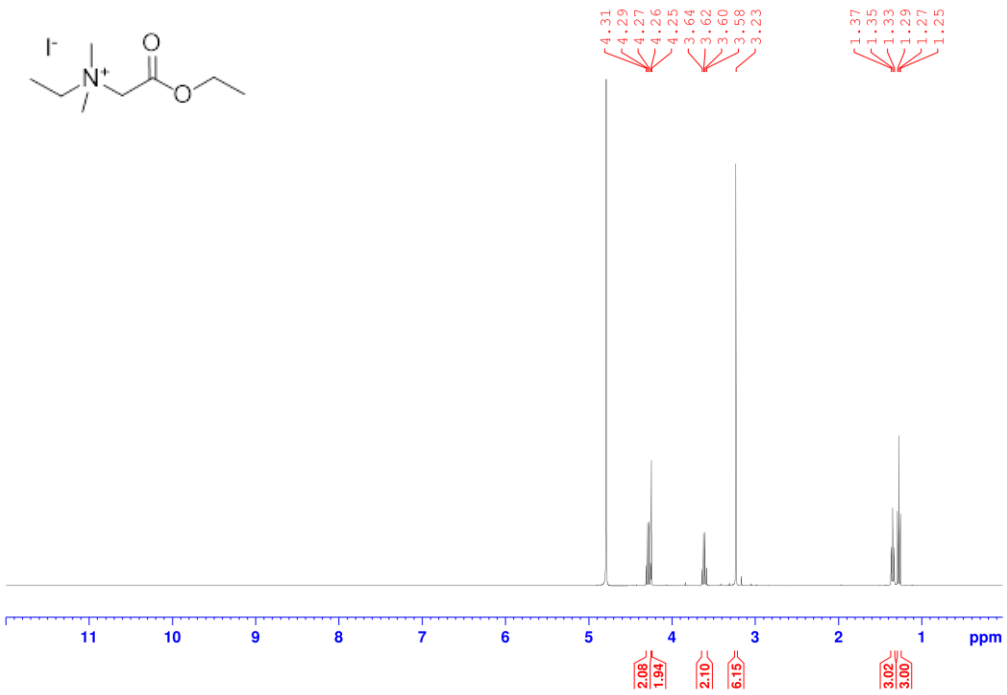


4-deoxy-*N*-(2-fluorophenyl)-D-gluconamide was synthesized according to the previously reported procedure [39]. The product was obtained as a white powder (72 mg, 1% total yield over 9 steps). All spectroscopic data (^{1}H NMR, ^{13}C NMR, and HRMS) were consistent with those previously reported [39]. **^{1}H NMR (600 MHz, D_2O):** δ 7.56 (t, J = 7.3 Hz, 1H), 7.29 (t, J = 7.1 Hz, 1H), 7.22 (q, J = 8.3 Hz, 2H), 4.24 (q, J = 4.2 Hz, 2H), 3.88 (q, J = 6.6 Hz, 1H), 3.60 (ddd, J = 11.7, 4.0, 1.7 Hz, 1H), 3.55 – 3.43 (m, 1H), 1.85 – 1.73 (m, 1H), 1.60 (ddd, J = 14.4, 9.8, 2.4 Hz, 1H). **^{13}C NMR (150 MHz, D_2O):** δ 174.2, 156.4, 154.8, 128.2, 128.2, 126.2, 124.7, 124.6, 123.3, 123.3, 116.1, 115.9, 74.9, 68.6, 68.4, 65.9, 36.0. **HRMS (ESI):** *m/z* calcd. for $C_{12}H_{16}FNO_5$ $[M + Na]^+$ 296.0907; found 296.1018.

Spectral Data



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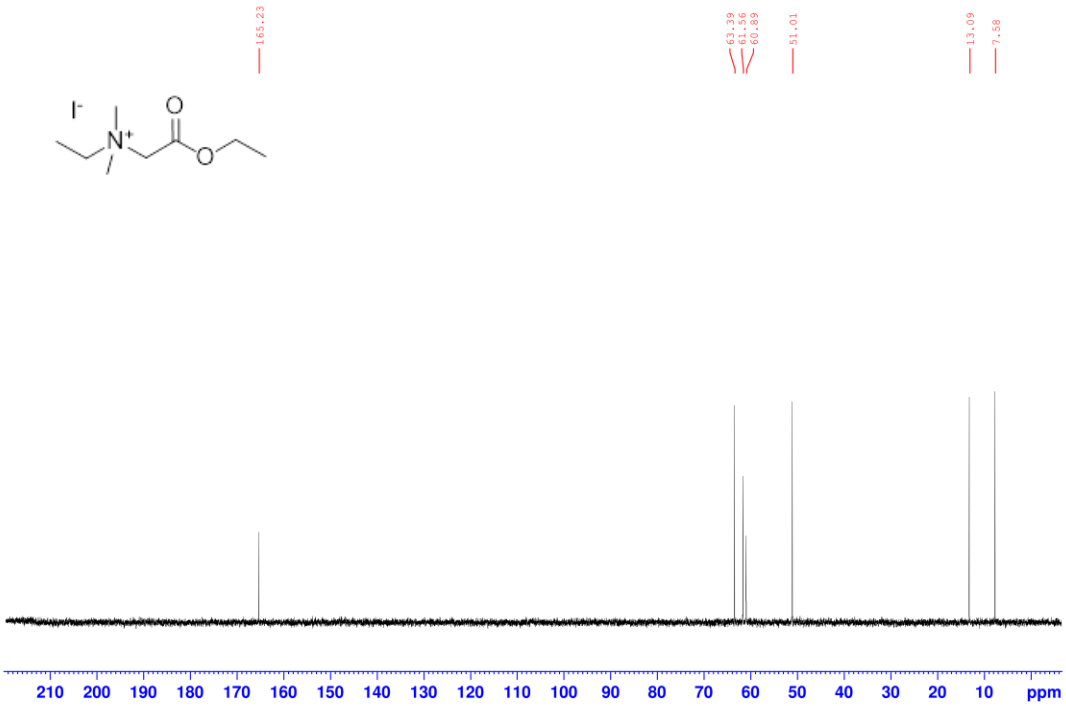
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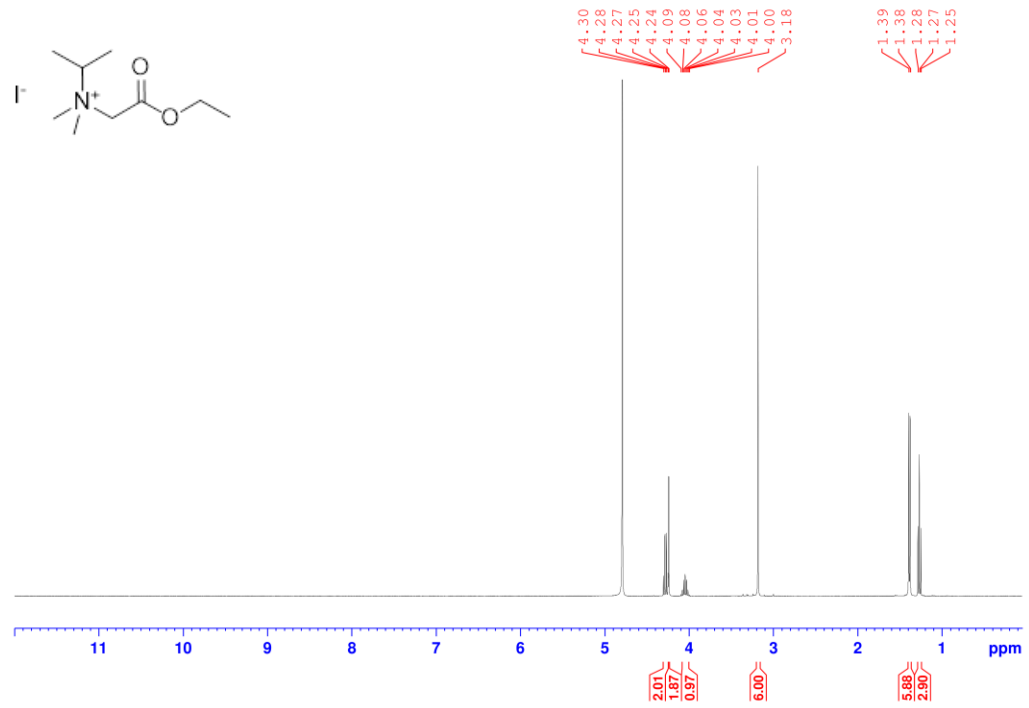
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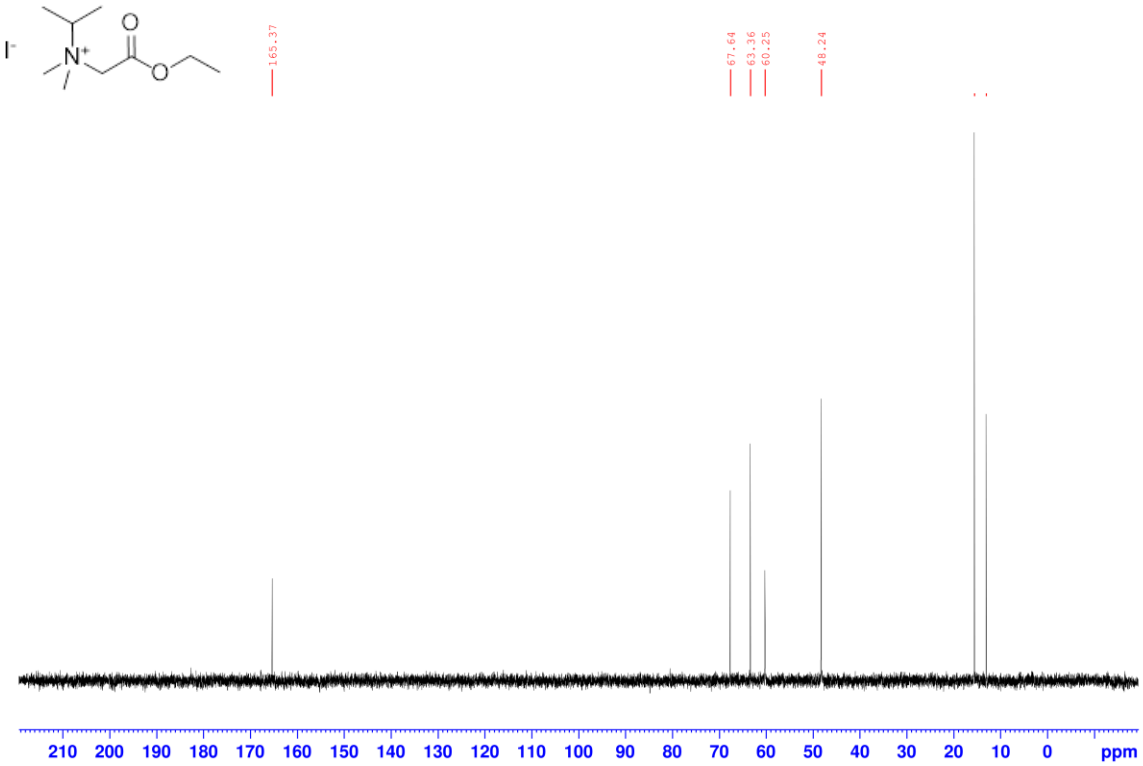
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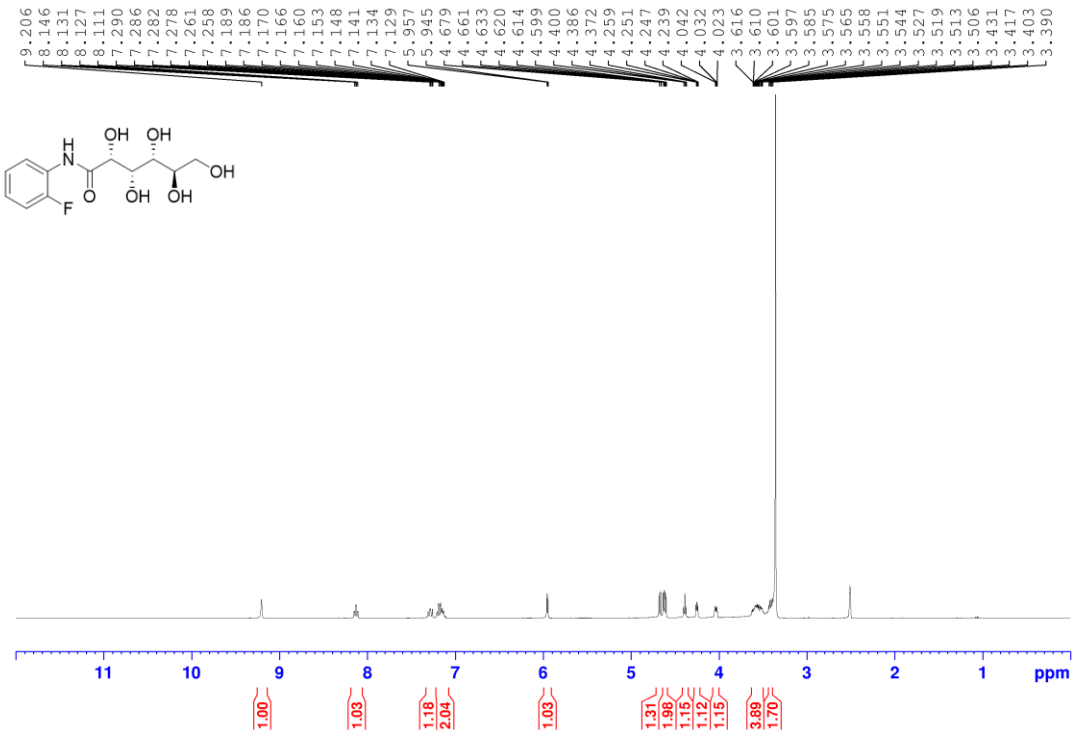
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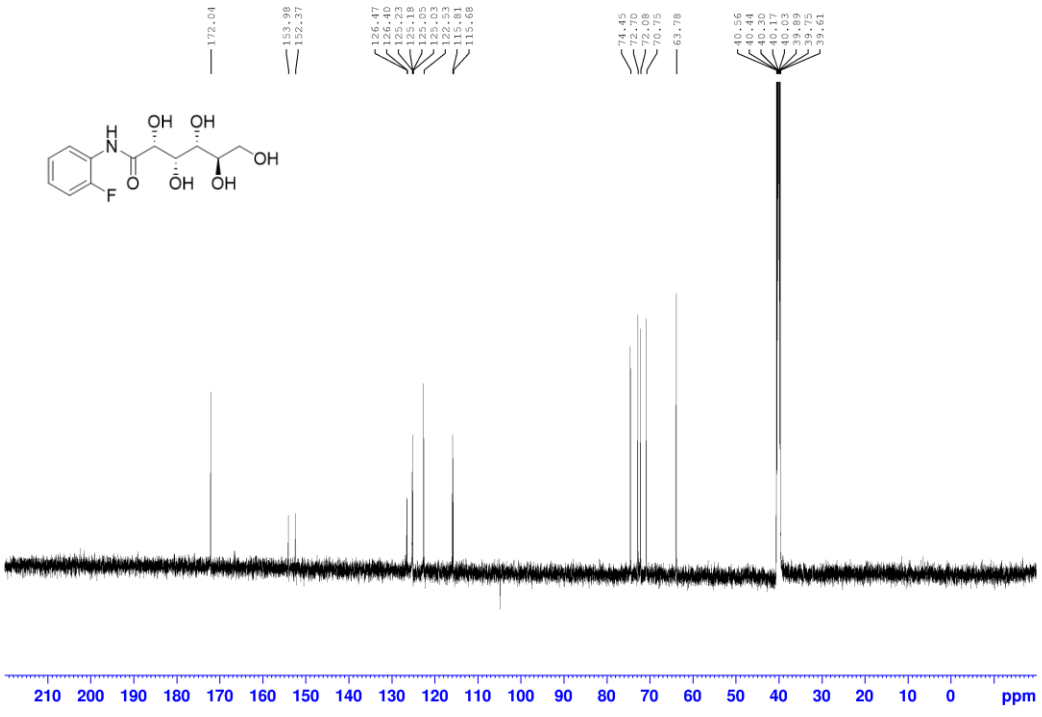
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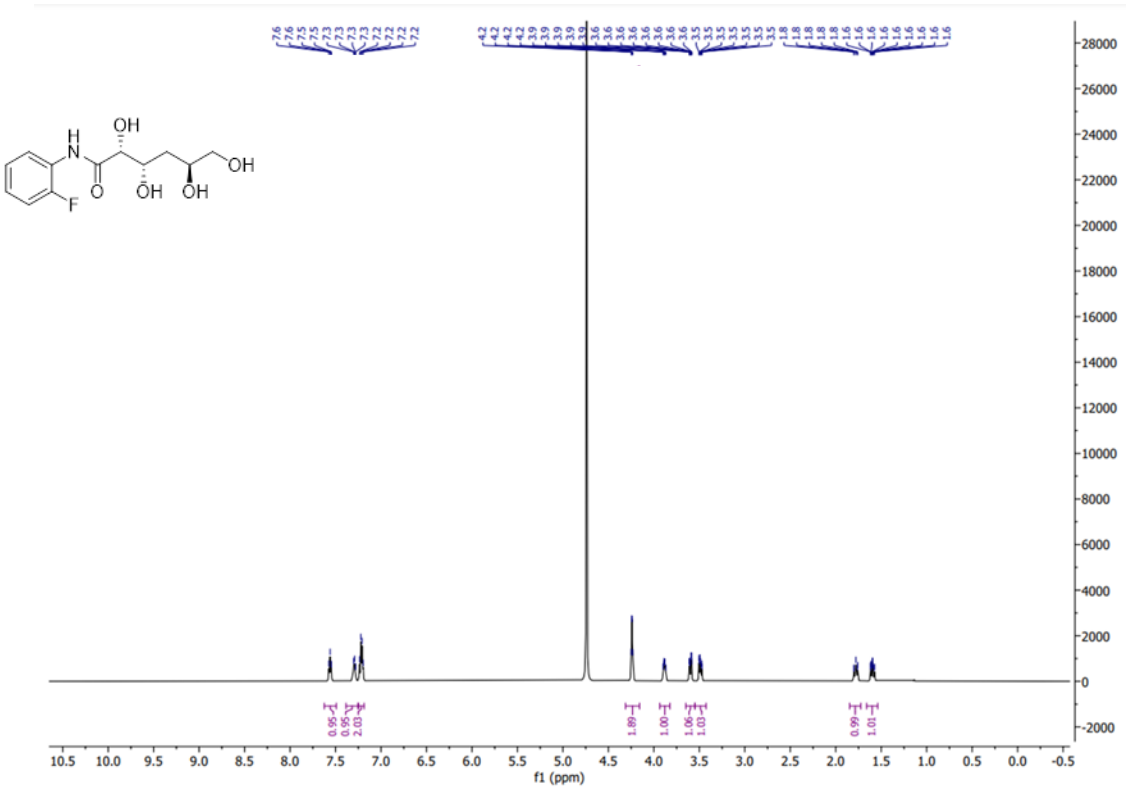
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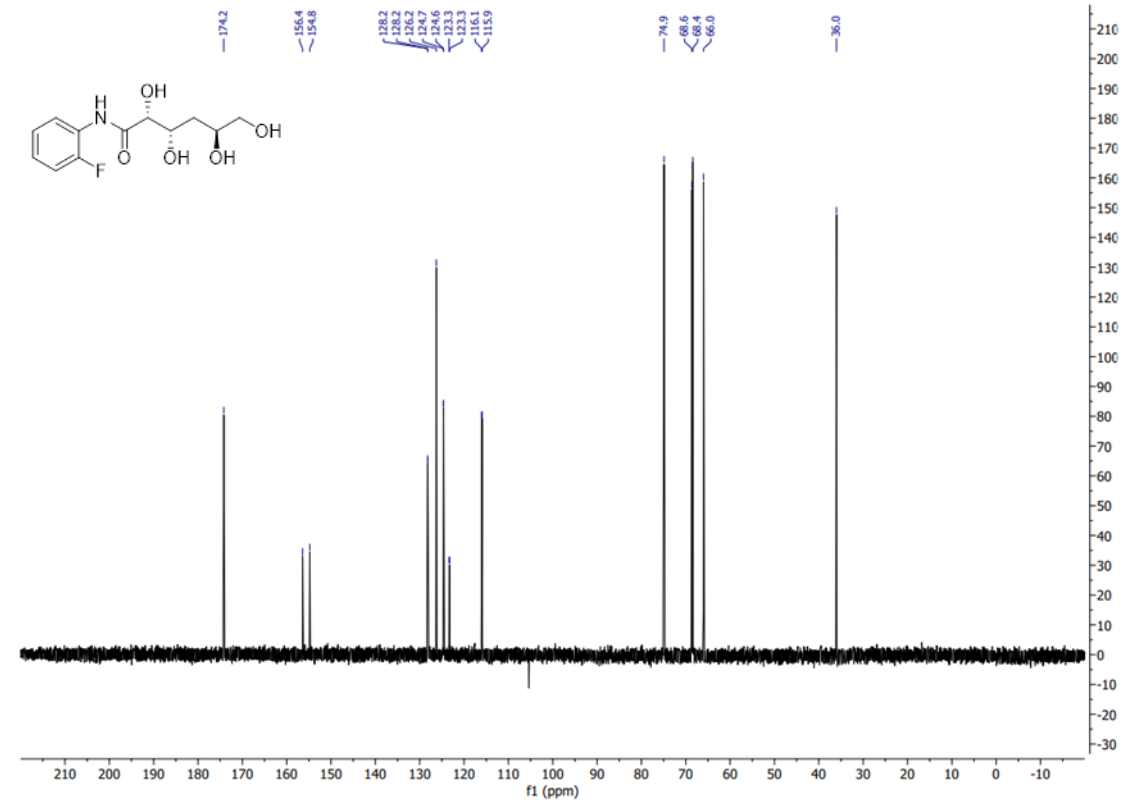
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