

## Supporting Information

### Rationale design of cell active C2-modified DGJ analogues for the inhibition of human $\alpha$ -galactosidase A (GALA)

#### Authors:

**Roger A. Ashmus,<sup>a#</sup> Yang Wang,<sup>a,e#</sup> Manuel González-Cuesta,<sup>c</sup> Dustin T. King,<sup>b</sup> Ben Tiet,<sup>a</sup> Pierre-André Gilormini,<sup>a</sup> José M. García Fernandez,<sup>d</sup> Carmen Ortiz Mellet,<sup>c</sup> Robert Britton,<sup>a\*</sup> David J. Vocadlo<sup>a,b\*</sup>**

#### Affiliations:

<sup>a</sup> Department of Chemistry, Simon Fraser University, Burnaby, British Columbia, Canada

<sup>b</sup> Department of Molecular Biology and Biochemistry, Simon Fraser University, Burnaby, British Columbia, Canada

<sup>c</sup> Departamento de Química Orgánica, Facultad de Química, Universidad de Sevilla, Sevilla 41012, Spain

<sup>d</sup> Instituto de Investigaciones Químicas (IIQ), CSIC-Universidad de Sevilla, Sevilla 41092, Spain

<sup>e</sup> *Current address:* Key Laboratory of Marine Drugs, Chinese Ministry of Education, School of Medicine and Pharmacy, Ocean University of China, Qingdao 266003, China and Laboratory for Marine Drugs and Bioproducts, Qingdao National Laboratory for Marine Science and Technology (QNLN), Qingdao 266237, China

<sup>#</sup> *these authors contributed equally to this work.*

#### \*Corresponding authors:

**Robert Britton, robert\_britton@sfu.ca**

**David J. Vocadlo, dvocadlo@sfu.ca**

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**Docking and structure analysis methods.** The protein monomers used for docking consisted of chain A from the 1.51Å and 2.1Å resolution crystallographic structures of DGJ (Galafold®) bound human alpha-N-acetylgalactosaminidase and human alpha-galactosidase (PDB ID: 4DO5, and 3S5Y). The protein monomers were prepared by removing chain B as well as all ligands and water from the coordinate files. Ligands were prepared by modifying the ligand coordinates for DGJ from the PDB in Coot,<sup>40</sup> followed by energy minimization using ProDrg.<sup>41</sup> Docking was carried out using the AutoVina 1.1.2<sup>42</sup> plugin in Chimera<sup>43</sup> using default parameters unless otherwise stated. Polar hydrogen atoms and Kollman charges were added to the protein and ligand using the AM1-BCC method in ANTECHAMBER<sup>44</sup>. The energy range, exhaustiveness, and number of modes to search for were set to 3, 8 and 5. The grid box used to define the docking site was strategically defined around the DGJNac binding site and was defined with X, Y, and Z centers of (GALA: 40.053, 16.917, 30.753, NAGAL; 56.473, -6.716, 17.154). The grid box had x, y, and z dimensions of 20.0, 20.0, and 20.0 Å. Using this method, Autodock Vina generated docking results for the top 5 poses in the pdbqt format and the data were analyzed in Chimera using the ViewDock feature. Omit electron density maps were generated by deleting the (DGJ C2-OH + W1 + S188) from chain A of the 4DO5 coordinate file and subsequently running three rounds of omit-refinement using the original deposited mtz in phenix.refine.<sup>43</sup> All structure figures were generated using PyMOL.

**General chemical methods.** All reagents and starting materials were purchased from Sigma Aldrich, Alfa Aesar, TCI America or Acros and were used without further purification. All solvents were purchased from Sigma Aldrich, EMD, Anachemia, Caledon, Fisher or ACP and used without further purification unless otherwise specified. CH<sub>2</sub>Cl<sub>2</sub> was freshly distilled over CaH<sub>2</sub>; Tetrahydrofuran (THF) was freshly distilled over Na metal/benzophenone. Cold temperatures were maintained by use of the following conditions: 0 °C, ice-water bath; -78 °C, acetone-dry ice bath; temperatures between -78 °C and 0 °C required for longer reaction times were maintained with a Neslab Cryocool Immersion Cooler (CC-100 II) in a 2-propanol bath. All anhydrous reactions described were performed under an atmosphere of nitrogen using flame dried glassware. Normal phase column chromatography was carried out with 230–400 mesh silica gel (Silicycle, SiliaFlash® P60). Concentration and removal of trace solvents was done with a Büchi rotary evaporator using a dry ice/acetone condenser and vacuum applied from a Büchi V-500 pump. Nuclear magnetic resonance (NMR) spectra were recorded using CDCl<sub>3</sub> or CD<sub>3</sub>OD. Signal positions ( $\delta$ ) are given in parts per million from tetramethylsilane ( $\delta$  0) and were measured relative to the signal of the solvent (<sup>1</sup>H NMR: CDCl<sub>3</sub>:  $\delta$  7.26, CD<sub>3</sub>OD:  $\delta$  3.31, D<sub>2</sub>O:  $\delta$  4.79; <sup>13</sup>C NMR: CDCl<sub>3</sub>:  $\delta$  77.16, CD<sub>3</sub>OD:  $\delta$  49.00). Coupling constants (J values) are given in Hertz (Hz) and are reported to the nearest 0.1 Hz. <sup>1</sup>H NMR spectral data are tabulated in the order: multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br., broad), coupling constants, number of protons. High-resolution mass spectra were performed on an Agilent 6210 TOF LC/MS using ESI-MS technique. Optical rotation was measured on a Perkin Elmer 341 Polarimeter at 589 nm. The purity of all inhibitors tested were > 95% as assessed by HPLC.

**Preparation of 1-deoxy-2-O-alkyl-galactonojirimicin derivatives.** *Method A:* A solution of epoxide 10 (0.2 mmol) in the corresponding alcohol (3.4 mL) and 1,4-dioxane (5.2 mL) with concentrated H<sub>2</sub>SO<sub>4</sub> (0.34 mL) was heated to 100 °C and stirred for 5 h. The reaction was then cooled to room temperature, treated with Dowex monosphere 550A (hydroxide form) and filtered. The filtrate was evaporated *in vacuo*. The mixture was redissolved in CH<sub>3</sub>OH:H<sub>2</sub>O (3:2, 0.073 M). NaHCO<sub>3</sub> (5.0 equiv) and benzyl chloroformate (1.2

equiv) were added at 0 °C. The reaction mixture was stirred at room temperature for 2 h. The solvents were removed *in vacuo* and the residue was purified by column chromatography on silica gel to give a mixture of regioisomers of corresponding intermediate 11. The mixture was purified by preparative HPLC eluting with solvent (solvent A: 0.1 % TFA in H<sub>2</sub>O, solvent B: 0.1 % TFA in ACN, flow: 15 mL/min) using a gradient of 20–100% solvent B over 25 min to give corresponding intermediate 11. This intermediate (1.0 equiv) and 10% Pd/C (0.1 equiv) in CH<sub>3</sub>OH (0.1 M) was stirred at rt under H<sub>2</sub> (2 atm). After 1 h, the reaction mixture was filtered through a bed of Celite and the filtrate was evaporated *in vacuo*. The residue was purified by reversed-phase chromatography on a SiliaPrep SPE Cartridges C18 column to yield 7. *Method B*: To a solution of epoxide 10 (0.07 mmol) in the corresponding alcohol (0.5 M) was added BF<sub>3</sub>·OEt<sub>2</sub> (1.7 eq). The mixture was stirred at 80 °C for 18 h. The reaction mixture was concentrated and the resulting residue was purified by column chromatography using DCM:CH<sub>3</sub>OH:H<sub>2</sub>O 100:10:1–30:10:1 to yield 7.

*1-deoxy-2-O-methyl-galactonojirimycin (7a)*. Method A. Colorless oil, isolated 19 mg (55%);  $[\alpha]_D^{20} = 35.7$  ( $c = 0.75$ , CH<sub>3</sub>OH); <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>OD):  $\delta$  4.02 (dd, 1H,  $J = 2.9, 1.3$  Hz, H-4), 3.80 (d, 2H,  $J = 6.4$  Hz, H-6, H-6'), 3.65 (ddd, 1H,  $J = 10.6, 9.3, 5.1$  Hz, H-2), 3.56 (dd, 1H,  $J = 9.3, 3.1$  Hz, H-3), 3.53 (s, 3H, CH<sub>3</sub>O), 3.50 (dd, 1H,  $J = 12.5, 5.2$  Hz, H-1), 3.19 (td, 1H,  $J = 6.6, 1.3$  Hz, H-5), 2.66 (dd, 1H,  $J = 12.4, 10.7$  Hz, H-1'). <sup>13</sup>C NMR (125.7 MHz, CD<sub>3</sub>OD):  $\delta$  75.1, 72.7, 67.2, 59.8, 59.2, 57.2, 44.0; HRMS (ESI):  $m/z$  [M + H]<sup>+</sup> calcd. for C<sub>7</sub>H<sub>16</sub>NO<sub>4</sub>: 178.1074; found: 178.1078.

*1-deoxy-2-O-ethyl-galactonojirimycin (7b)*. Method A. Colorless oil, isolated 9 mg (25%);  $[\alpha]_D^{20} = 34.4$  ( $c = 0.86$ , CH<sub>3</sub>OH); <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>OD):  $\delta$  3.96 (dd, 1H,  $J = 3.1, 1.2$  Hz, H-4), 3.69 (m, 4H, CH<sub>2</sub>O, H-6, H-6'), 3.57 (ddd, 1H,  $J = 10.6, 9.6, 5.2$  Hz, H-2), 3.43 (dd, 1H,  $J = 9.3, 3.1$  Hz, H-3), 3.31 (dd, 1H,  $J = 12.4, 5.2$  Hz, H-1), 2.75 (td, 1H,  $J = 6.5, 1.3$  Hz, H-5), 2.39 (dd, 1H,  $J = 12.5, 10.7$  Hz, H-1'), 1.23 (t, 3H,  $J = 7.0$  Hz, CH<sub>2</sub>CH<sub>3</sub>). <sup>13</sup>C NMR (125.7 MHz, CD<sub>3</sub>OD):  $\delta$  76.1, 74.6, 69.1, 65.7, 61.3, 59.9, 47.1, 14.5; HRMS (ESI):  $m/z$  [M + H]<sup>+</sup> calcd. for C<sub>8</sub>H<sub>18</sub>NO<sub>4</sub>: 192.1230; found: 192.1237.

*1-deoxy-2-O-propyl-galactonojirimycin (DGJ-C2Pr, 7c)*. Colorless oil. Method A: isolated 9 mg (23%); Method B: isolated 9 mg (65%);  $R_f = 0.2$  (50:10:1 DCM: CH<sub>3</sub>OOH:H<sub>2</sub>O);  $[\alpha]_D^{20} = 25.5$  ( $c = 0.32$ , CH<sub>3</sub>OH); <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>OD):  $\delta$  3.98 (dd, 1H,  $J = 3.1, 1.3$  Hz, H-4), 3.73 (d, 2H,  $J = 6.5$  Hz, H-6, H-6'), 3.63 (m, 3H, CH<sub>2</sub>O, H-2), 3.49 (dd, 1H,  $J = 9.4, 3.1$  Hz, H-3), 3.36 (dd, 1H,  $J = 12.5, 5.2$  Hz, H-1), 2.93 (td, 1H,  $J = 6.5, 1.3$  Hz, H-5), 2.51 (dd, 1H,  $J = 12.4, 10.6$  Hz, H-1'), 1.62 (m, 2H, CH<sub>2</sub>CH<sub>3</sub>), 0.97 (t, 3H,  $J = 7.0$  Hz, CH<sub>2</sub>CH<sub>3</sub>). <sup>13</sup>C NMR (125.7 MHz, CD<sub>3</sub>OD):  $\delta$  74.9, 73.6, 71.9, 68.1, 60.2, 59.6, 45.8, 22.4, 8.93; HRMS (ESI):  $m/z$  [M + H]<sup>+</sup> calcd. for C<sub>9</sub>H<sub>20</sub>NO<sub>4</sub>: 206.1387; found: 206.1391.

*1-deoxy-2-O-isopropyl-galactonojirimycin (7d)*. Method A. Colorless oil, isolated 6 mg (15%);  $[\alpha]_D^{20} = 29.1$  ( $c = 0.2$ , CH<sub>3</sub>O); <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>OD):  $\delta$  3.97 (dd, 1H,  $J_{4,5} = 1.2$  Hz,  $J_{3,4} = 3.1$  Hz, H-4), 3.87 (m, 1H, CHCH<sub>3</sub>), 3.63 (m, 3H, H-2, H-6a, H6b), 3.39 (dd, 1H,  $J_{2,3} = 9.4$  Hz, H-3), 3.22 (dd, 1H,  $J_{1a,1b} = 12.5$  Hz, H-1a), 2.76 (td, 1H,  $J_{5,6a} = J_{5,6b} = 6.5$  Hz, H-5), 2.39 (dd, 1H, H-1b), 1.22, 1.19 (d, 6H, CHCH<sub>3</sub>). <sup>13</sup>C NMR (125.7 MHz, CD<sub>3</sub>OD):  $\delta$  74.6 (C-2), 74.0 (CHCH<sub>3</sub>), 71.6 (C3), 69.1 (C-4), 61.3 (C-6), 59.9 (C-5), 47.1 (C-1), 22.0, 21.3 (CHCH<sub>3</sub>); HRMS (ESI):  $m/z$  [M + H]<sup>+</sup> calcd. for C<sub>9</sub>H<sub>20</sub>NO<sub>4</sub>: 206.1387; found: 206.1392.

*1-deoxy-2-O-butyl-galactonojirimycin (7e)*. Method B. Colorless oil, isolated 8 mg (57%).  $R_f = 0.2$  (50:10:1 DCM: CH<sub>3</sub>OH:H<sub>2</sub>O); <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>OD):  $\delta$  3.93 (dd, 1H,  $J = 3.1, 1.2$  Hz, H-4), 3.63 (m, 4H, CH<sub>2</sub>O, H-6, H-6'), 3.53 (ddd, 1H,  $J = 10.6, 9.3, 5.1$  Hz, H-2), 3.40 (dd, 1H,  $J = 9.3, 3.2$  Hz, H-3), 3.27 (dd, 1H,  $J = 12.5, 5.1$  Hz, H-1), 2.70 (app. t, 1H,  $J = 6.4$  Hz, H-5), 2.35 (dd, 1H,  $J = 12.5, 9.5$  Hz, H-1'), 1.58 (m, 2H, CH<sub>2</sub>), 1.40 (m, 2H, CH<sub>2</sub>), 0.95 (t, 3H,  $J = 7.4$  Hz, CH<sub>2</sub>CH<sub>3</sub>); <sup>13</sup>C NMR (125.7 MHz, CD<sub>3</sub>OD):  $\delta$  76.4, 74.7, 70.2, 69.3, 61.5, 59.9, 47.0, 31.9, 18.8, 12.8; ESI:  $m/z$  202.16 [M + H]<sup>+</sup>. HRMS (EI<sup>+</sup>) calcd for [C<sub>10</sub>H<sub>22</sub>NO<sub>4</sub>]<sup>+</sup> 220.1543; found 220.1569.

*1-deoxy-2-O-isobutyl-galactonojirimycin (7f)*. Method B. Colorless oil, isolated 8 mg (52%).  $R_f = 0.2$  (50:10:1 DCM:CH<sub>3</sub>OH:H<sub>2</sub>O). <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>OD):  $\delta$  3.93 (dd, 1 H,  $J = 3.2, 1.2$  Hz, H-4), 3.65 (m, 2H, OCH<sub>2</sub>), 3.52 (ddd, 1H,  $J = 10.6, 9.3, 5.1$  Hz, H-2), 3.49-3.41 (m, 2H, H-6, H-6'), 3.35 (dd, 1H,  $J = 9.3, 3.2$  Hz, H-3), 3.27 (dd, 1H,  $J = 12.5, 5.1$  Hz, H-1), 2.71 (app. t, 1H,  $J = 6.5$  Hz, H-5), 2.35 (dd, 1H,  $J = 12.5, 9.5$  Hz, H-1'), 1.85 (m, 1H, CH), 0.93 (d, 6H, 2  $\times$  CH<sub>3</sub>); <sup>13</sup>C NMR (125.7 MHz, CD<sub>3</sub>OD):  $\delta$  77.5, 75.9, 74.3, 68.9, 61.0, 60.0, 46.5, 29.2, 18.3, 18.1; ESI:  $m/z$  220.16 [M + H]<sup>+</sup>; HRMS (EI<sup>+</sup>) calcd for [C<sub>10</sub>H<sub>22</sub>NO<sub>4</sub>]<sup>+</sup> 220.1543; found 220.1570.

*1-deoxy-2-O-pentyl-galactonojirimycin (7g)*. Method B. Colorless oil, isolated 11 mg (67%).  $R_f = 0.2$  (50:10:1 DCM: CH<sub>3</sub>OH:H<sub>2</sub>O). <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>OD):  $\delta$  3.95 (dd, 1H,  $J = 3.1, 1.2$  Hz, H-4), 3.65 (m, 2H, CH<sub>2</sub>O, H-6, H-6'), 3.56 (ddd, 1H,  $J = 10.6, 9.3, 5.1$  Hz, H-2), 3.43 (dd, 1H,  $J = 9.3, 3.3$  Hz, H-3), 3.31 (dd, 1H,  $J = 12.5, 5.2$  Hz, H-1), 2.81 (app. t, 1H,  $J = 6.4$  Hz, H-5), 2.42 (dd, 1H,  $J = 12.5, 9.4$  Hz, H-1'), 1.60 (m, 2H, CH<sub>2</sub>), 1.36 (m, 4H, 2  $\times$  CH<sub>2</sub>), 0.93 (t, 3H,  $J = 7.3$  Hz, CH<sub>3</sub>); <sup>13</sup>C NMR (125.7 MHz, CD<sub>3</sub>OD):  $\delta$  75.9, 74.4, 70.6, 69.0, 61.1, 59.9, 46.8, 29.5, 27.9, 22.1, 12.9; ESI:  $m/z$  234.15 [M + H]<sup>+</sup>; HRMS (EI<sup>+</sup>) calcd for [C<sub>11</sub>H<sub>24</sub>NO<sub>4</sub>]<sup>+</sup> 234.1700; found 234.1727.

*1-deoxy-2-O-isopentyl-galactonojirimycin (7h)*. Method B. Colorless oil, isolated 10 mg (62%).  $R_f = 0.2$  (50:10:1 DCM:CH<sub>3</sub>OH:H<sub>2</sub>O). <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>OD):  $\delta$  3.94 (dd, 1H,  $J = 3.1, 1.2$  Hz, H-4), 3.67 (m, 4H, CH<sub>2</sub>O, H-6, H-6'), 3.56 (ddd, 1 H,  $J = 10.6, 9.4, 5.1$  Hz, H-2), 3.42 (dd, 1H,  $J = 9.3, 3.2$  Hz, H-3), 3.30 (dd, 1H,  $J = 12.5, 5.1$  Hz, H-1), 2.78 (app. t, 1H,  $J = 6.4$  Hz, H-5), 2.40 (dd, 1H,  $J = 12.5, 9.3$  Hz, H-1'), 1.73 (m, 2H, CH<sub>2</sub>), 1.43 (t, 1H, CH), 0.92 (d, 6H, 2  $\times$  CH<sub>3</sub>); <sup>13</sup>C NMR (125.7 MHz, CD<sub>3</sub>OD):  $\delta$  76.4, 74.7, 70.5, 69.2, 61.4, 59.9, 48.4, 31.4, 29.8, 22.2, 12.9; ESI:  $m/z$  233.61 [M + H]<sup>+</sup>; HRMS (EI<sup>+</sup>) calcd for [C<sub>11</sub>H<sub>24</sub>NO<sub>4</sub>]<sup>+</sup> 234.1700; found 234.1724.

*1-deoxy-2-O-hexyl-galactonojirimycin (7i)*. Method B. Colorless oil, isolated 8 mg (47%).  $R_f = 0.2$  (50:10:1 DCM:CH<sub>3</sub>OH:H<sub>2</sub>O). <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>OD):  $\delta$  3.94 (dd, 1 H,  $J = 3.1, 1.2$  Hz, H-4), 3.63 (m, 4H, CH<sub>2</sub>O, H-6, H-6'), 3.53 (ddd, 1 H,  $J = 10.6, 9.3, 5.1$  Hz, H-2), 3.41 (dd, 1H,  $J = 9.3, 3.1$  Hz, H-3), 3.28 (dd, 1H,  $J = 12.5, 5.2$  Hz, H-1), 2.71 (app. t, 1H,  $J = 6.4$  Hz, H-5), 2.35 (dd, 1H,  $J = 12.5, 9.4$  Hz, H-1'), 1.59 (m 2H, CH<sub>2</sub>), 1.35 (m, 6H, 3  $\times$  CH<sub>2</sub>), 0.92 (t, 3H,  $J = 7.4$  Hz, CH<sub>2</sub>CH<sub>3</sub>); <sup>13</sup>C NMR (125.7 MHz, CD<sub>3</sub>OD):  $\delta$  76.1, 74.5, 69.1, 68.9, 61.2, 59.9, 48.4, 38.7, 24.6, 21.5; ESI:  $m/z$  248.19 [M + H]<sup>+</sup>; HRMS (EI<sup>+</sup>) calcd for [C<sub>12</sub>H<sub>26</sub>NO<sub>4</sub>]<sup>+</sup> 248.1856; found 248.1882.

**Determination of inhibitory activity against human GALA, NAGAL, BGAL, and GALC.** Glycosidases were obtained from R&D Systems. IC<sub>50</sub> values in Table 1 were determined using fluorescence-based stopped assay with appropriate substrate. In brief, glycosidases were preincubated with a dilution series of inhibitors **7a-i** in a 96-well mixing plate in total volume of 20  $\mu$ L for 10 min at RT in appropriate reaction buffer. Reactions were started by addition of appropriate substrate (10  $\mu$ L) in reaction buffer into enzyme/inhibitor mixture. Following a 15–20 min incubation at 37 °C, reactions were terminated by addition of 90  $\mu$ L stop buffer (300 mM glycine + 200 mM NaOH, pH 10). All endpoint measurements were carried out in triplicates in 384-well plates (Nunc 262260) using a BioTek Neo2 microplate reader at Ex/Em 365/445 and background fluorescence signal (no enzyme) subtracted. Nonlinear regression analysis was employed to calculate IC<sub>50</sub> values using Excel and GraphPad Prism. Ten-point IC<sub>50</sub> curves were generated using three-fold inhibitor dilution series.

#### In vitro NAGAL IC<sub>50</sub> assay

The activity was determined with 1 nM recombinant NAGAL enzyme (RD Systems) and 1 mM 4MU- $\alpha$ GalNAc (CarboSynth) in the presence of varying inhibitor concentrations (600  $\mu$ M, 3-fold dilution x 9 for **1**, **7d**, and **7f-i**; 3 mM, 3-fold dilution x 9 for **7a-c** and **7e**) in 100 mM sodium citrate + 200 mM NaCl buffer (pH 4.5) supplemented with 1.8% DMSO.

#### In vitro GALA IC<sub>50</sub> assay

The activity was determined with 1 nM recombinant GALA enzyme (RD Systems) and 1 mM 4MU- $\alpha$ Gal (CarboSynth, recrystallized in MeOH) in the presence of varying inhibitor concentrations (600  $\mu$ M, 3-fold dilution x 9 for **7a-i**; 10  $\mu$ M, 3-fold dilution x 9 for DGJ) in 50 mM sodium citrate + 50 mM NaCl buffer (pH 4.5) supplemented with 1% DMSO.

#### In vitro $\beta$ -Gal IC<sub>50</sub> assay

The activity was determined with 2 nM recombinant  $\beta$ -Gal enzyme (RD Systems) and 160  $\mu$ M 4MU- $\beta$ Gal (CarboSynth) in the presence of varying inhibitor concentrations (600  $\mu$ M, 3-fold dilution x 9) in 50 mM sodium citrate buffer (pH 4.0) supplemented with 3% DMSO.

#### In vitro GALC IC<sub>50</sub> assay

The activity was determined with 0.5 nM recombinant GALC enzyme (RD Systems) and 500  $\mu$ M 4MU- $\beta$ Gal (CarboSynth) in the presence of varying inhibitor concentrations (600  $\mu$ M, 3-fold dilution x 9) in 50 mM sodium citrate + 125 mM NaCl buffer (pH 4.5) supplemented with 0.5% Triton X100 and 5% DMSO.

#### In vitro GALA K<sub>i</sub> assay

The  $K_i$  was determined by variation of iminosugar concentrations. The inhibitor **7c** (60, 45, 15, 3, 1.5, and 0  $\mu$ M) was pre-incubated with and without recombinant GALA (3 nM, RD Systems) for 15 min and then the assays were started by adding 4MU- $\alpha$ Gal substrate (15 mM, 2-fold dilution x 7, CarboSynth, recrystallized in MeOH). Incubation temperature was 37 °C for 3 and 15 min. The reaction was stopped by addition of 300 mM glycine + 500 mM NaOH buffer (pH 10). Endpoint fluorescence measurements were carried out in 384-well plates (Nunc 262260) using a BioTek Neo2 microplate reader at Ex/Em 365/445 nm. The fluorescence measurements of background (without enzyme) was subtracted from the fluorescence measurements with enzyme for each time point to account for spontaneous hydrolysis of 4MU- $\alpha$ Gal. Nonlinear regression analysis was employed using Excel and GraphPad Prism.

**Cell lines and cell culture.** Patient fibroblasts were obtained from the Coriell Institute. Fibroblasts were received fresh in T25 flasks at passage P7 for WT (GM02770) and P13 for R301G (GM00882). Fibroblasts were acclimatized overnight, sub-cultured, and frozen according to Coriell's recommendations. All cells were cultured at 37 °C in a humidified incubator with 5% CO<sub>2</sub> using standard procedures. Briefly, fibroblasts were cultured in EMEM (M5650, Sigma) supplemented with 15% FBS (Gibco), Pen/Strep antibiotics (BioShop), and GlutaMAX™ (LifeTech). Cells were expanded in T175 flasks and media was changed every 3–4 days until cells reached 80-90 % confluency. For each passage, cells were washed with warm PBS, treated with the minimal volume of 0.5% Trypsin/EDTA (LifeTech) in PBS, incubated for ~5 minutes until cells were fully detached, and resuspended in warm culture media. Cells were counted using BioRad Automated Cell Counter TC20 with Trypan blue (Gibco), and either split into a new flask for sub-culturing or plated in 96-well or 6-well plates for treatments and imaging.

**Cell plating and treatment.** After counting, the concentration of cells was adjusted using the corresponding volume of growing media. The cell suspension was plated in 96-well plates (Corning 4680) for treatments and imaging and in 6-well culture plates for immunoblots. Cell seeding (95 to 100 µL) was performed using a multichannel pipette. Depending on the experiment, between 1000 and 4000 cells were dispensed per well. Plates were centrifuged (100 x g, 1 min) and incubated (37 °C, 5 % CO<sub>2</sub>) overnight. For WT and R301G comparison, the cells were grown in the 96 well-plate for 2 days before PFA fixation. For chaperone experiments, cells were treated with 0–50 µM of **7c** or DGJ, centrifuged (100 x g, 1 min) and incubated at 37 °C for 4 days (medium with inhibitor exchanged at 2 days). Inhibitor-containing medium was then replaced with fresh medium (no inhibitor) and cells were incubated for an additional 24 h before PFA fixation. All experiments with patient fibroblasts were carried out with cells having a passage number between P15 and P19 with at least three biological replicates per treatment.

**Immunofluorescence staining.** Cells were washed with cold PBS (100 µL, 2 times) and fixed with 4% paraformaldehyde (EM Sciences) in PBS (60 µL) for 20 min at RT. Cells were subsequently washed with PBS (100 µL, 3 times), permeabilized with 0.1% Triton X-100 (Acros Organics) in PBS (60 µL) for 20 min at RT followed by blocking with 5% BSA in PBS (60 µL) for 1 h at RT. The cells were incubated with mouse monoclonal antibody against Gb3-antigen (A2506 TCI Chemical, 10 µg/mL) in PBS (50 µL) containing 5% BSA overnight (16 h) at 4 °C, washed with PBS (100 µL, 5 times), and then incubated with AlexaFluor568-conjugated goat anti-mouse antibody (2 µg/mL, A11004, Eugene) in PBS (50 µL) containing 5% BSA for 1 h at RT in the dark. The cells were washed with PBS (100 µL, 5 times) and then Hoechst (1 µg/mL, LifeTech) in PBS (100 µL) was added. After 30 min incubation at RT in the dark, the stained cells were examined under microscope.

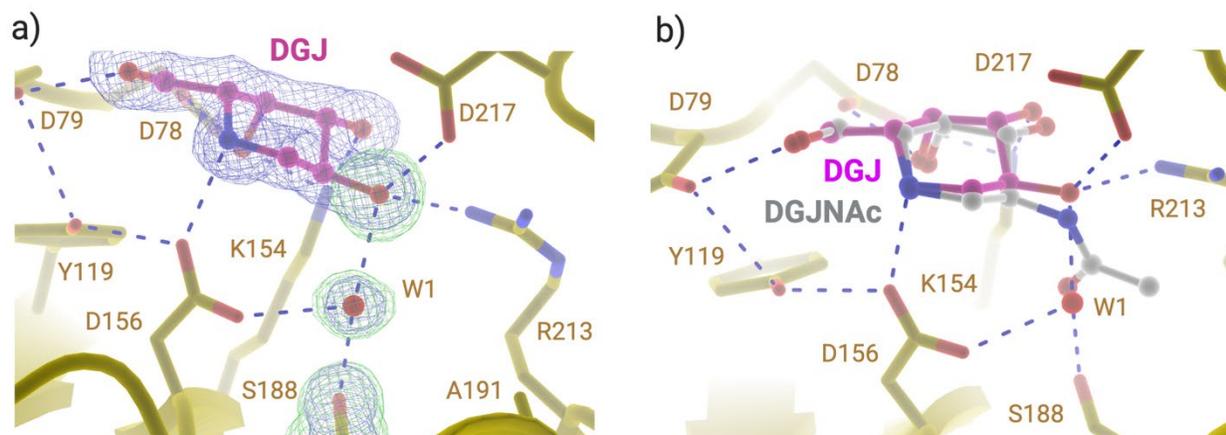
**Image acquisition and analysis.** Imaging was performed using an ImageXpress Micro XLS High-Content Imager (Molecular Devices). Image acquisition was carried out using a 40X objective. For each well, 4 to 6 sites (ROI) were imaged using the DAPI (Ex/Em 377/447) and the Texas Red (Ex/Em 562/624) channels. Before acquisition, the focus was adjusted for both the DAPI and Texas Red channel and set to adjust on plate and well bottom. Exposure times were set at 50 ms for the DAPI and 300 ms for the Texas Red channel. Following acquisition, images were analyzed using the MetaXpress software suite. Data was analyzed in batches using the Multi-wavelength Cell Scoring module. Nuclei were detected and counted using the DAPI channel and analyzed using a 5/50 µm constraint (min/max width) and a 300 gray-level minimal intensity above background. AF594 fluorescence was analyzed from the Texas Red channel image using a 0.1/1 µm constraint (min/max width) and a 750 to 1000 gray-level minimal intensity above background. For each ROI in each well, this analysis module returned the mean integrated intensity for

the Texas Red channel and the number of cells. After combining sites, we generated the integrated intensity normalized by the number of cells for each well and transformed these data to percentage of relative Gb3 immunofluorescence levels using positive control (R301G, no treatment). Data was then analyzed and plotted using Microsoft Excel and GraphPad Prism. Images included are scaled (16-bit) according to the following gray-level values: Figure S16 (DAPI: 200/2000, TR: 1700/5000) and Figure 3c (DAPI: 200/2500, TR: 300/750).

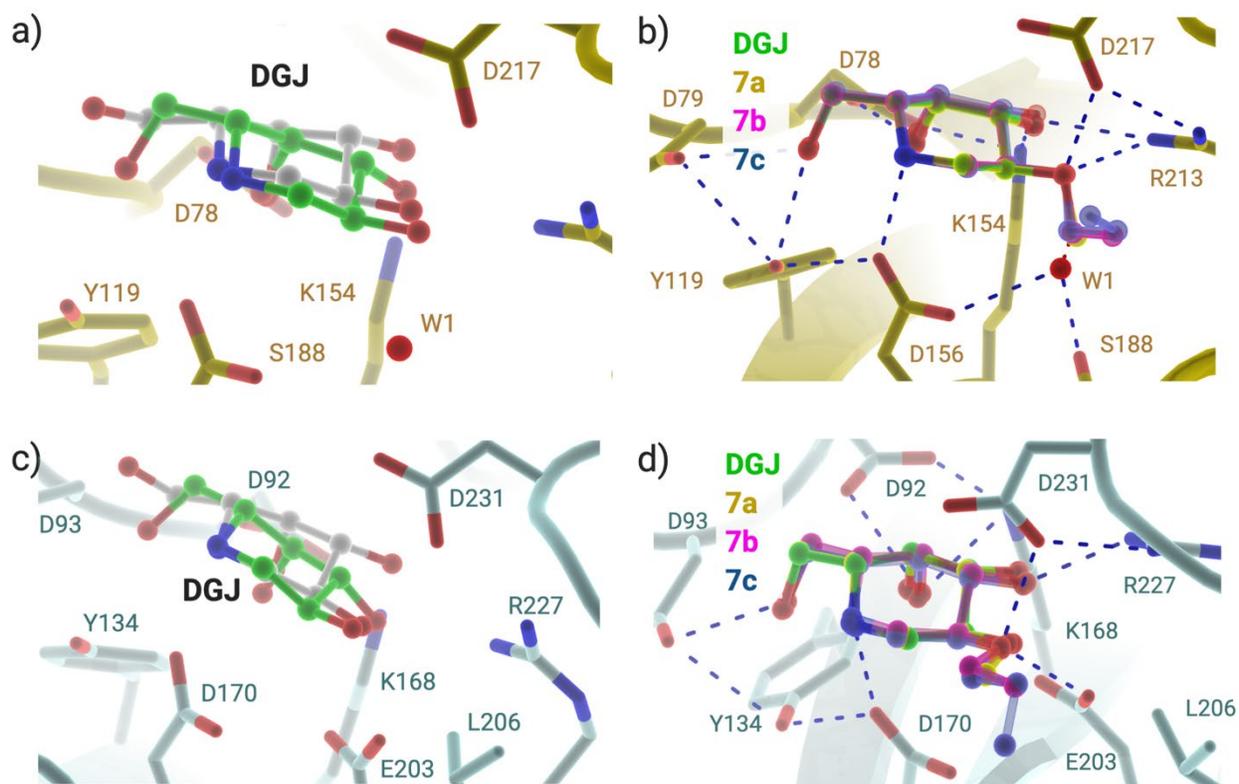
**Determination of GALA activity in cell lysate.** The cells were washed with PBS, detached using Trypsin/EDTA then mixed with equal parts medium, and centrifuged (250 x g, 5 min). The supernatant discarded and the pellets were stored at  $-80^{\circ}\text{C}$ . For GALA activity analysis, each cell pellet was resuspended in 0.5% Triton X100 in assay buffer (200 mM  $\text{Na}_2\text{HPO}_4$ , 100 mM citric acid, pH 4.6 + protease inhibitors) at  $4^{\circ}\text{C}$  for 1 h and then centrifuged (15,000 x g, 15 min,  $4^{\circ}\text{C}$ ). GALA activity was measured in a 96-well plate in triplicates by mixing lysate (20  $\mu\text{L}$ ), 250 mM GalNAc in assay buffer (40  $\mu\text{L}$ ), and 12.5 mM 4-methylumbelliferyl  $\alpha$ -galactopyranoside (4MU- $\alpha$ -Gal) in assay buffer (40  $\mu\text{L}$ ) containing 5% DMSO. The plate was incubated at  $37^{\circ}\text{C}$  for 1 h and then the reaction was quenched by addition of stop solution (300 mM glycine + 200 mM NaOH, pH 10; 300  $\mu\text{L}$ ). The fluorescence was measured following details outlined in enzyme kinetics. The fluorescent signal was normalized to total protein concentrations of each lysate determined by the Bradford Assay.

**Cell viability.** The viability of the cells was examined 72 h after chaperone treatment using ethidium homodimer-1 (Invitrogen), Calcein AM (Invitrogen), and Hoechst (LifeTech). Cells were incubated with ethidium homodimer-1 (2  $\mu\text{M}$ ), Calcein AM (4  $\mu\text{M}$ ), and Hoechst (1  $\mu\text{g}/\text{mL}$ , LifeTech) for 20 min ( $37^{\circ}\text{C}$ ) and then examined under fluorescence microscope. Percentage of live cells was calculated by counting the difference of cells stained with Hoescht (total cell count) and cells stained with ethidium homodimer-1 (dead cell count) divided by the difference of the two stains for the cells treated with vehicle multiplied by 100. Calcein AM was used as a secondary measure for cell viability.

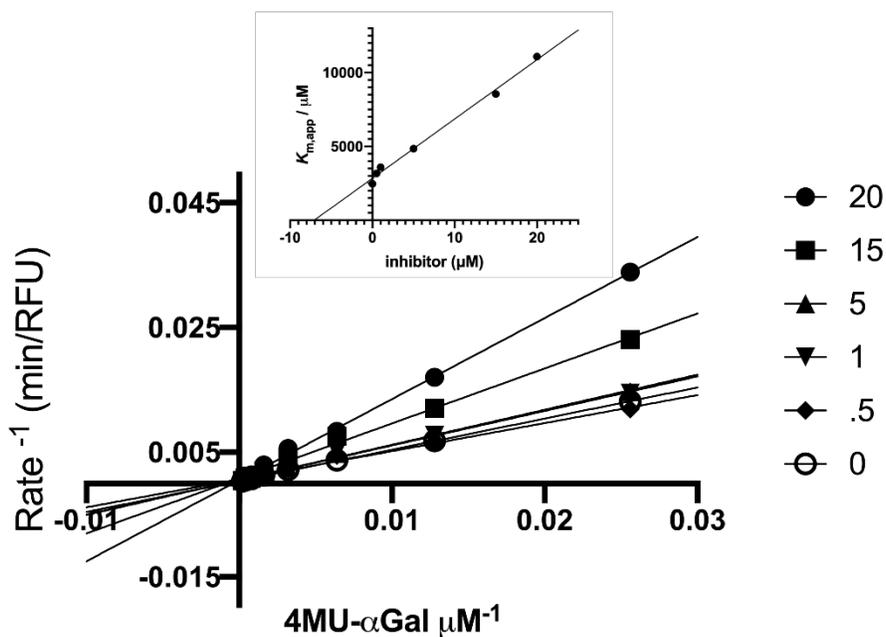
## Supplementary Figures and Tables



**Figure S1. Structural analysis NAGAL.** a) Electron density in support of DGJ C2-OH-W1-S188 O-gamma interaction. The final refined  $2f_o-f_c$  and (DGJ C2-OH + W1 + S188) omit  $f_o-f_c$  difference density maps are contoured at 2.0 sigma and 4.0 sigma and displayed in blue and green mesh representation. b) Overlaid crystal structures of DGJ-NAGAL (PDB ID: 4DO5) and DGJNAc-NAGAL (PDB ID: 4DO4) complexes. The bound DGJ and DGJNAc are depicted as magenta and white sticks with atoms colored by type. In all panels, the NAGAL protein backbone and selected active site residues are depicted as gold cartoons and sticks with atoms colored by type. Hydrogen bonds and electrostatic interactions are illustrated as blue dashes throughout.



**Figure S2. Docking of DGJ analogues bound to NAGAL and GALA** a) Overlay of crystallographic DGJ (white sticks) and docked DGJ (green sticks) to NAGAL. b) Overlay of docked DGJ (green sticks), **7a** (yellow), **7b** (magenta), and **7c** (dark blue) bound to NAGAL. c) Overlay of crystallographic DGJ (white sticks) and docked DGJ (green sticks) bound to GALA. d) Overlay of docked DGJ (green sticks), **7a** (yellow), **7b** (magenta), and **7c** (dark blue) bound GALA. In panels a) and b), the NAGAL protein backbone and selected active site residues are depicted as gold cartoons. In panel b), W1 was omitted in docking experiments, but is overlaid onto the docked structure to show steric clash with **7a-c** ( $<1.3\text{\AA}$ ). In panels c) and d), the GALA protein backbone and selected active site residues are depicted as light blue cartoons. In all panels, sticks with atoms colored by type while hydrogen bonds and electrostatic interactions are illustrated as blue dashes throughout. The **7a-c** docking poses have the lowest theoretical binding energies are shown.



**Figure S3.** Line-Weaver Burk Plot. Inhibition of human GALA catalyzed hydrolysis of 4MU- $\alpha$ Gal by **7c** shows a pattern of competitive inhibition. The concentrations of **7c** ( $\mu\text{M}$ ) used were 20, 15, 5, 1, 0.5, and 0.0. Inset: graphical analysis of  $K_i$  from plotting  $K_m$  apparent against concentration of **7c**.  $K_{m,app}$  is the apparent  $K_m$  value obtained at each inhibitor concentration and the X-intercept represents  $-K_i$  ( $K_i = 7.1 \pm 1.9 \mu\text{M}$ ).

**Table S1.** IC<sub>50</sub> values ( $\mu\text{M}$ ) for the inhibition of human glycosidases  $\beta$ -GAL and GALC by C2-modified DGJ analogues.

Inhibitor	$\beta$ -Gal	GALC
<b>7b</b>	> 600	> 600
<b>7c</b>	> 600	n.i.
<b>7e</b>	> 600	n.i.

n.i. = No inhibition observed up to 600  $\mu\text{M}$ .

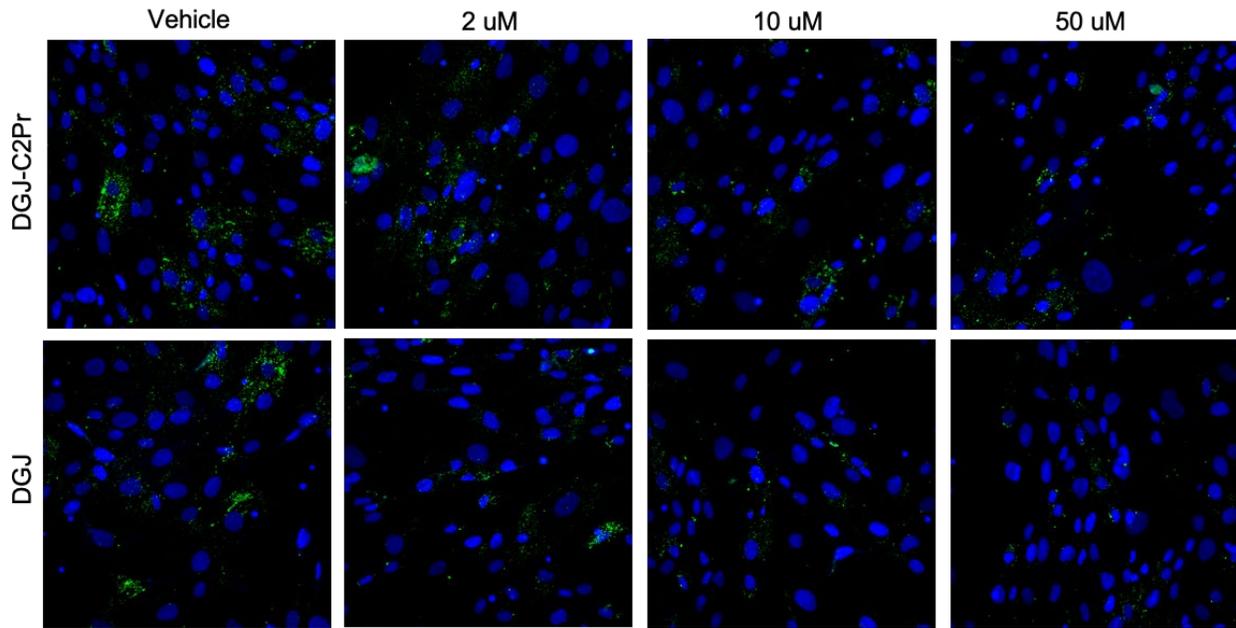


Figure S4. Representative immunofluorescent images in reference to quantitative comparison of relative Gb3 levels of R301G FD patient fibroblasts treated with DGJ-C2Pr and DGJ.

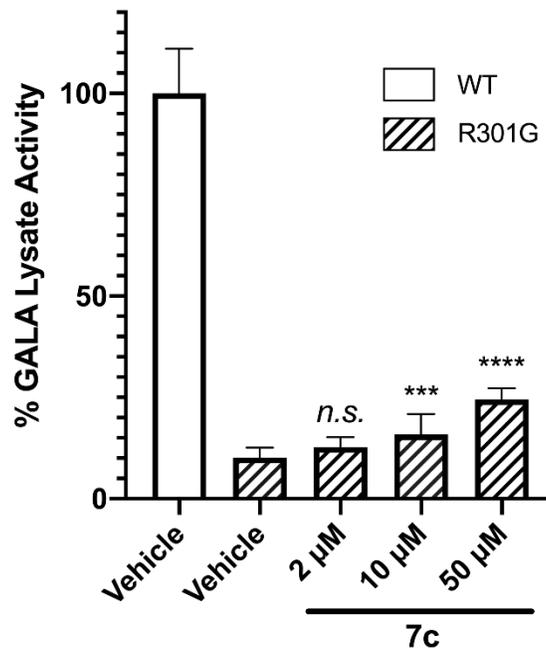


Figure S5. GALA activity in lysates of cells treated with 7c. Statistical significance was tested using one-way ANOVA and Dunnett's post hoc test. *n.s.* not significant, \*\*\* $P$ <0.001, \*\*\*\* $P$ <0.0001.

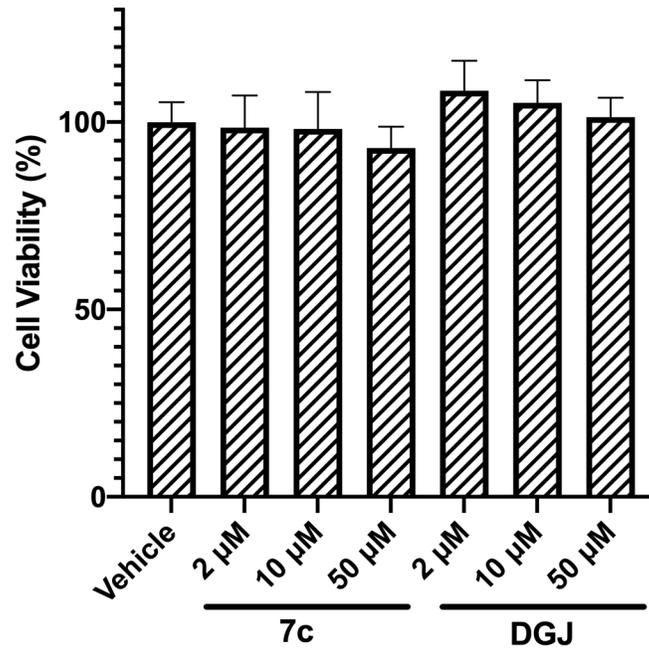
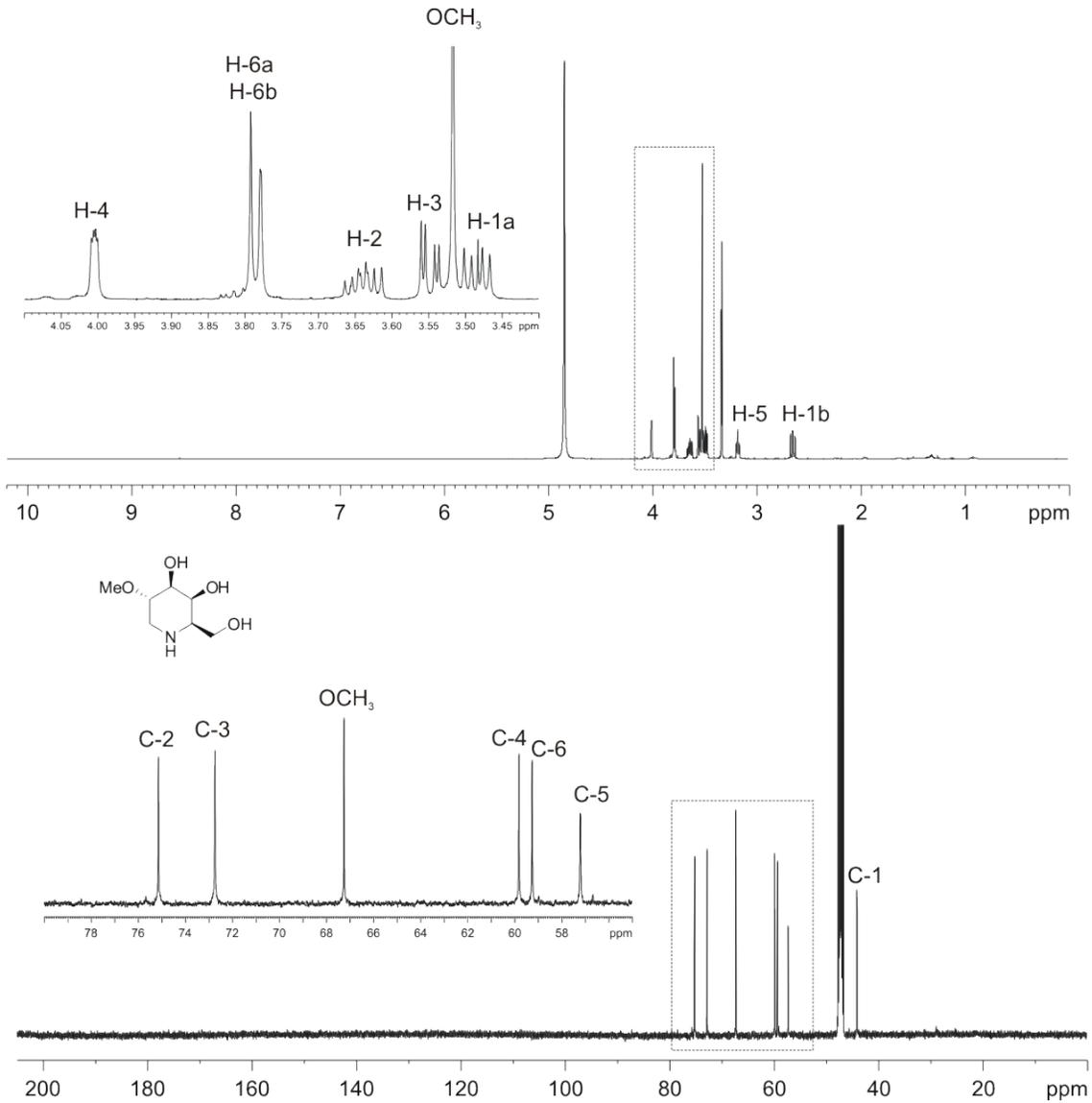


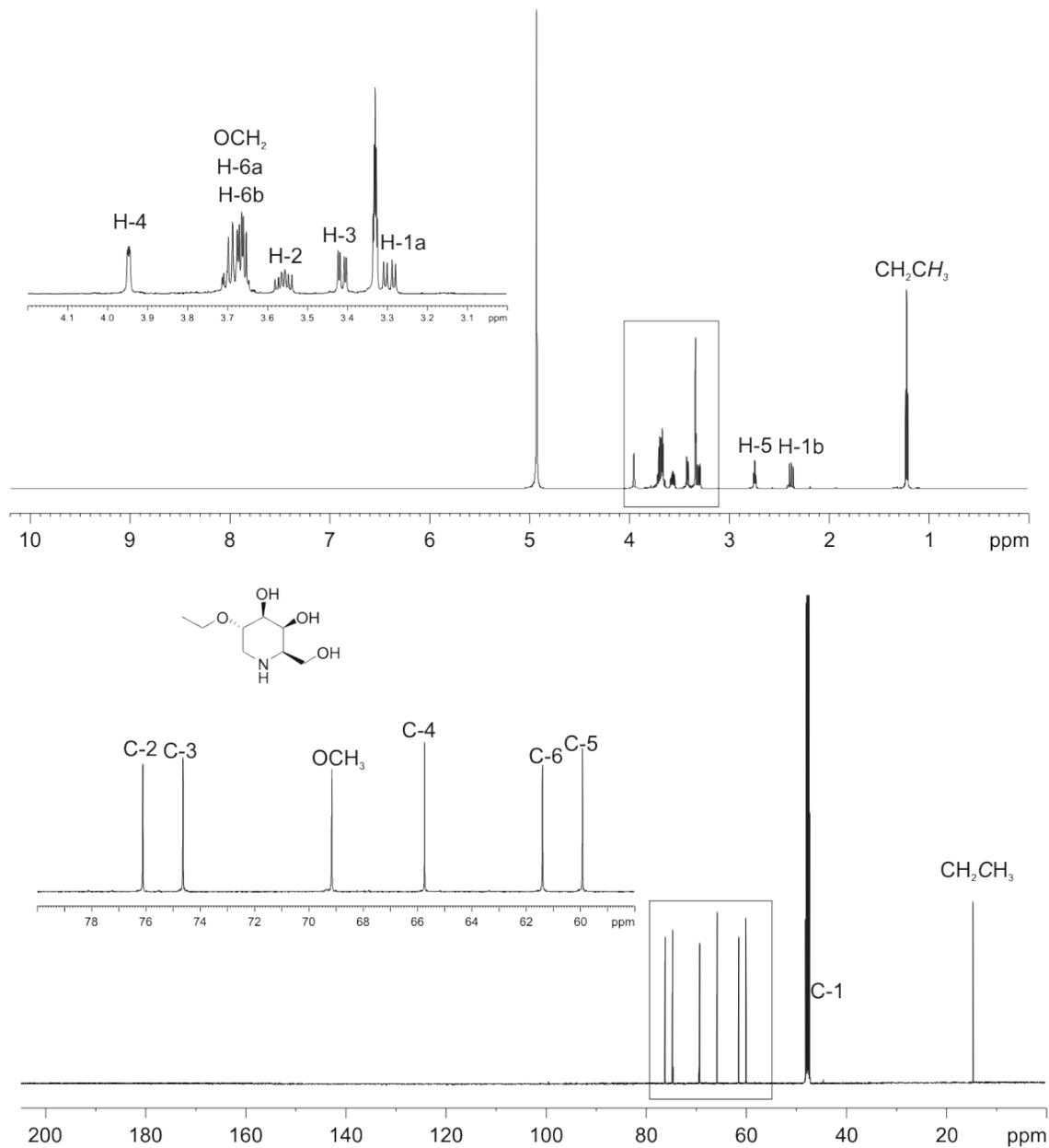
Figure S6. Assessment of cell viability for R301G fibroblasts treated with 7c and DGJ at 2, 10, and 50 μM.

### Compound NMR spectra

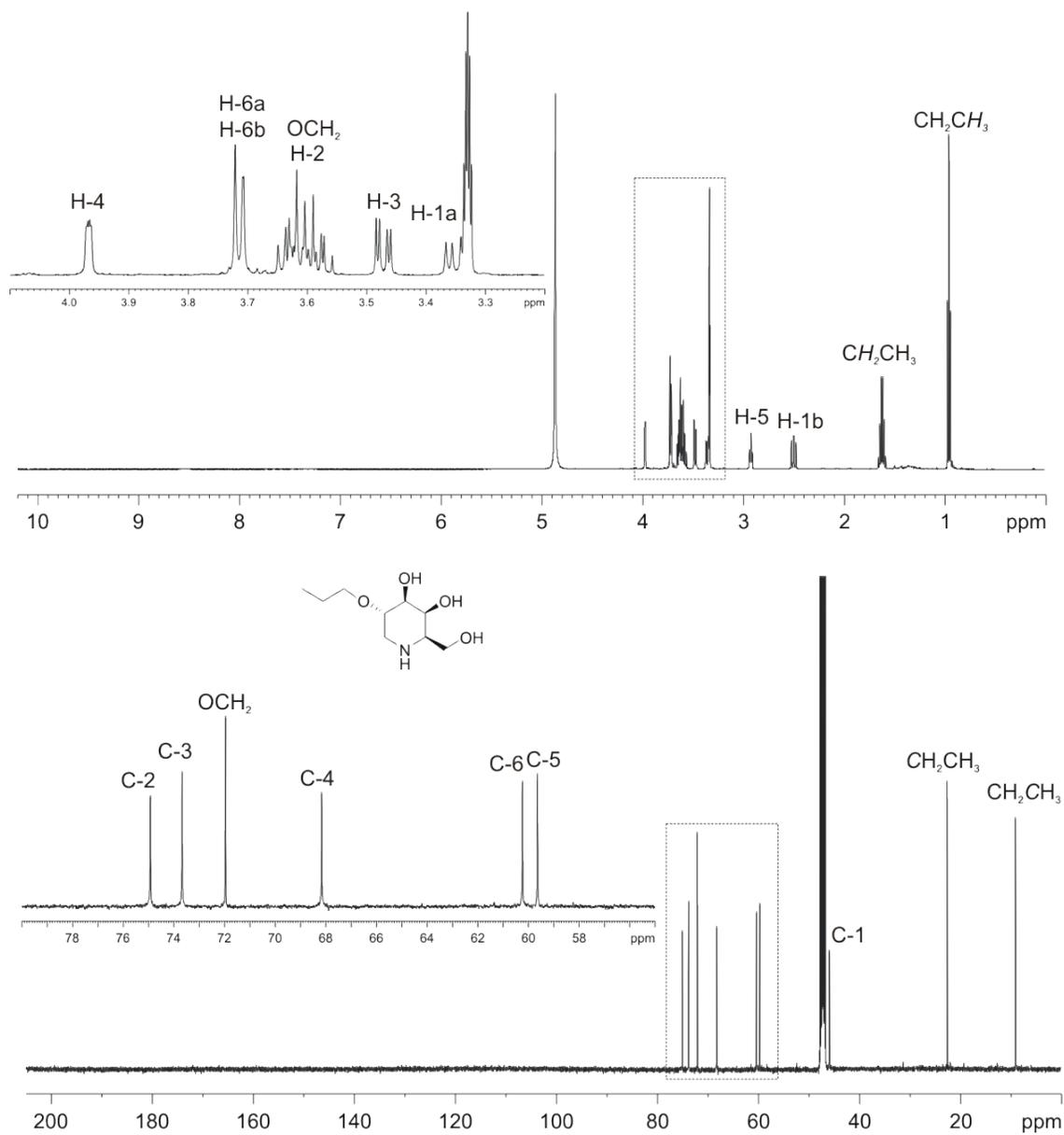
$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra (600 MHz, 151 MHz,  $\text{CD}_3\text{OD}$ ) of **7a**.



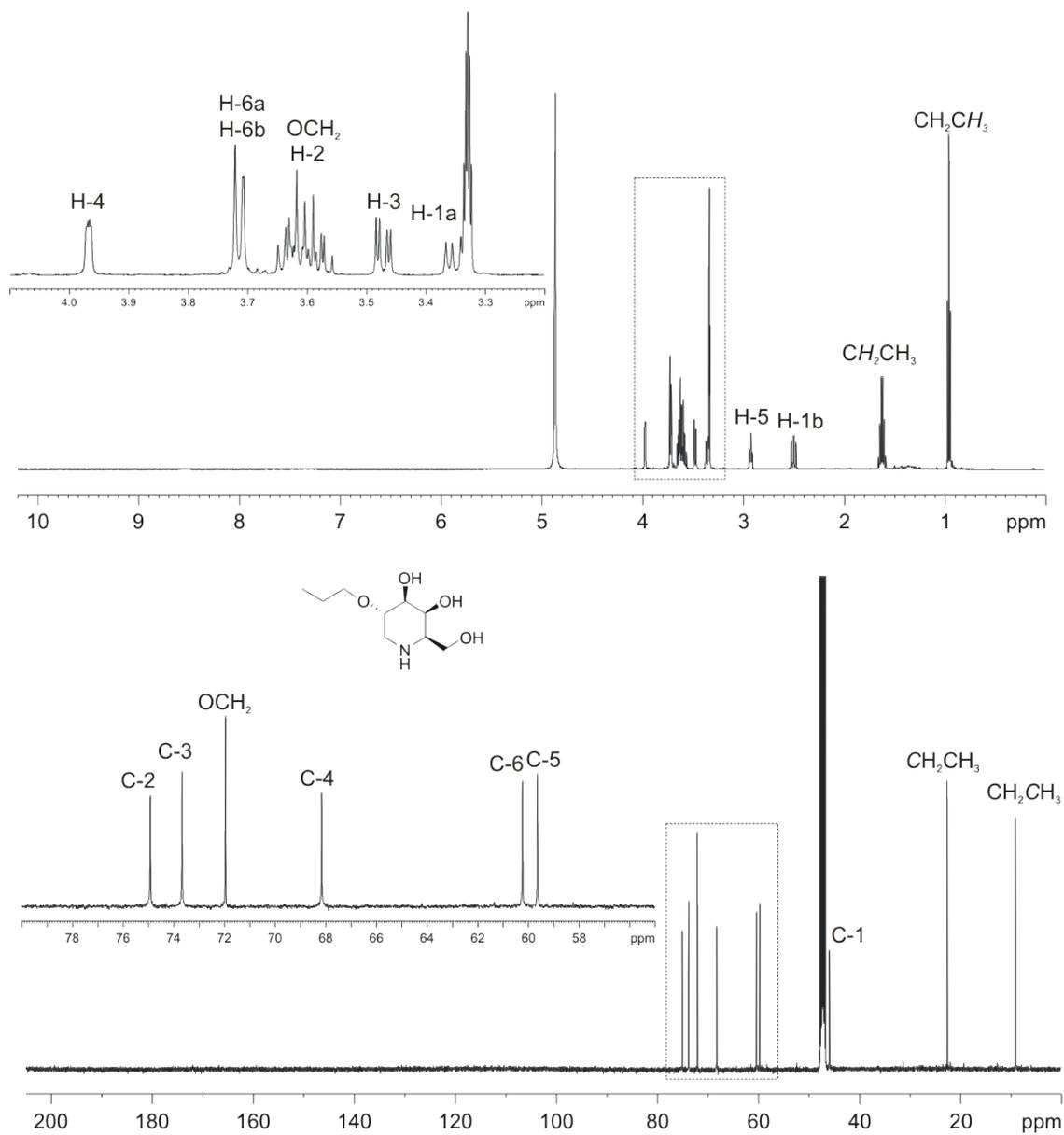
$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra (600 MHz, 151 MHz,  $\text{CD}_3\text{OD}$ ) of **7b**.



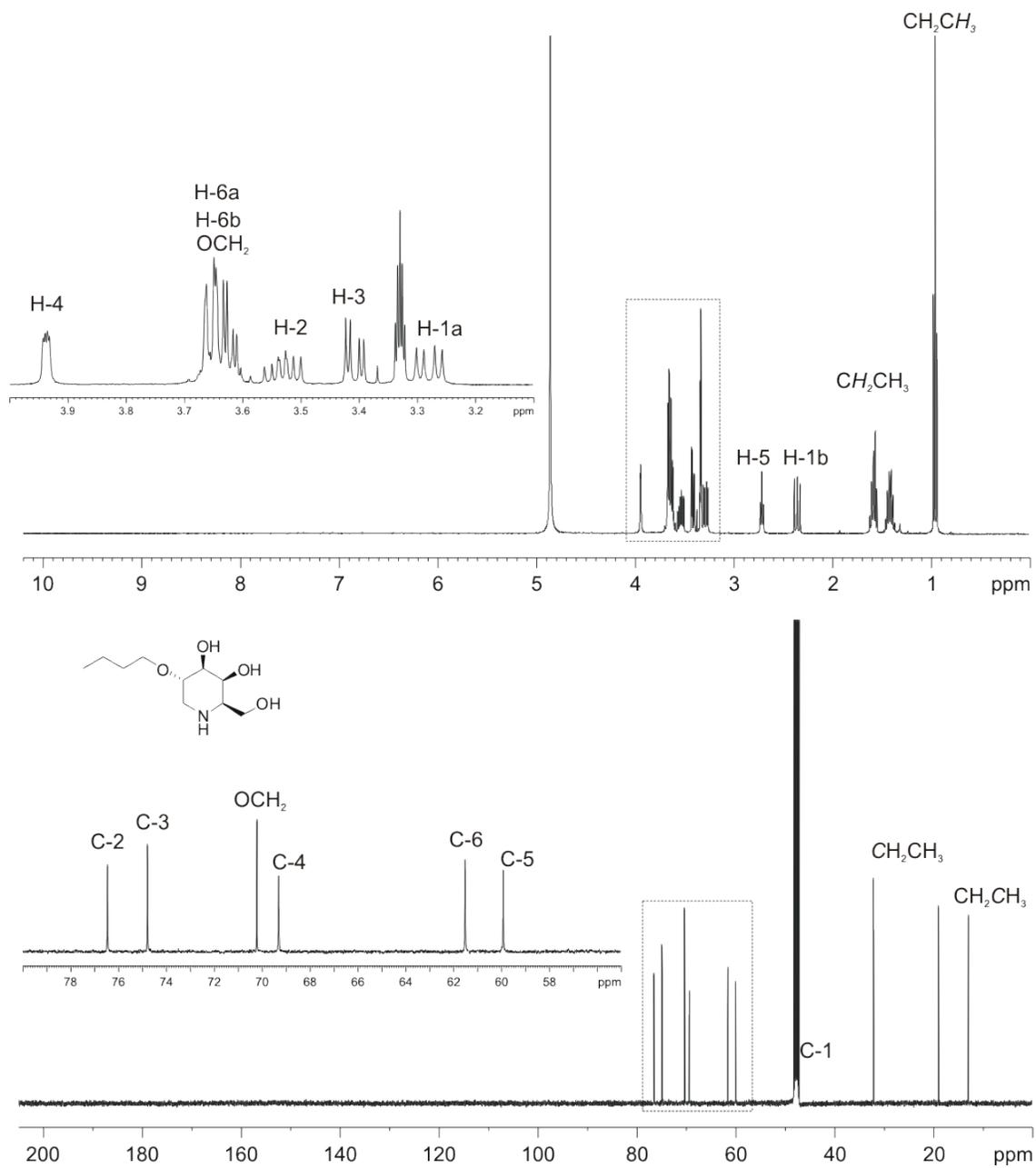
$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra (600 MHz, 151 MHz,  $\text{CD}_3\text{OD}$ ) of **7c**.



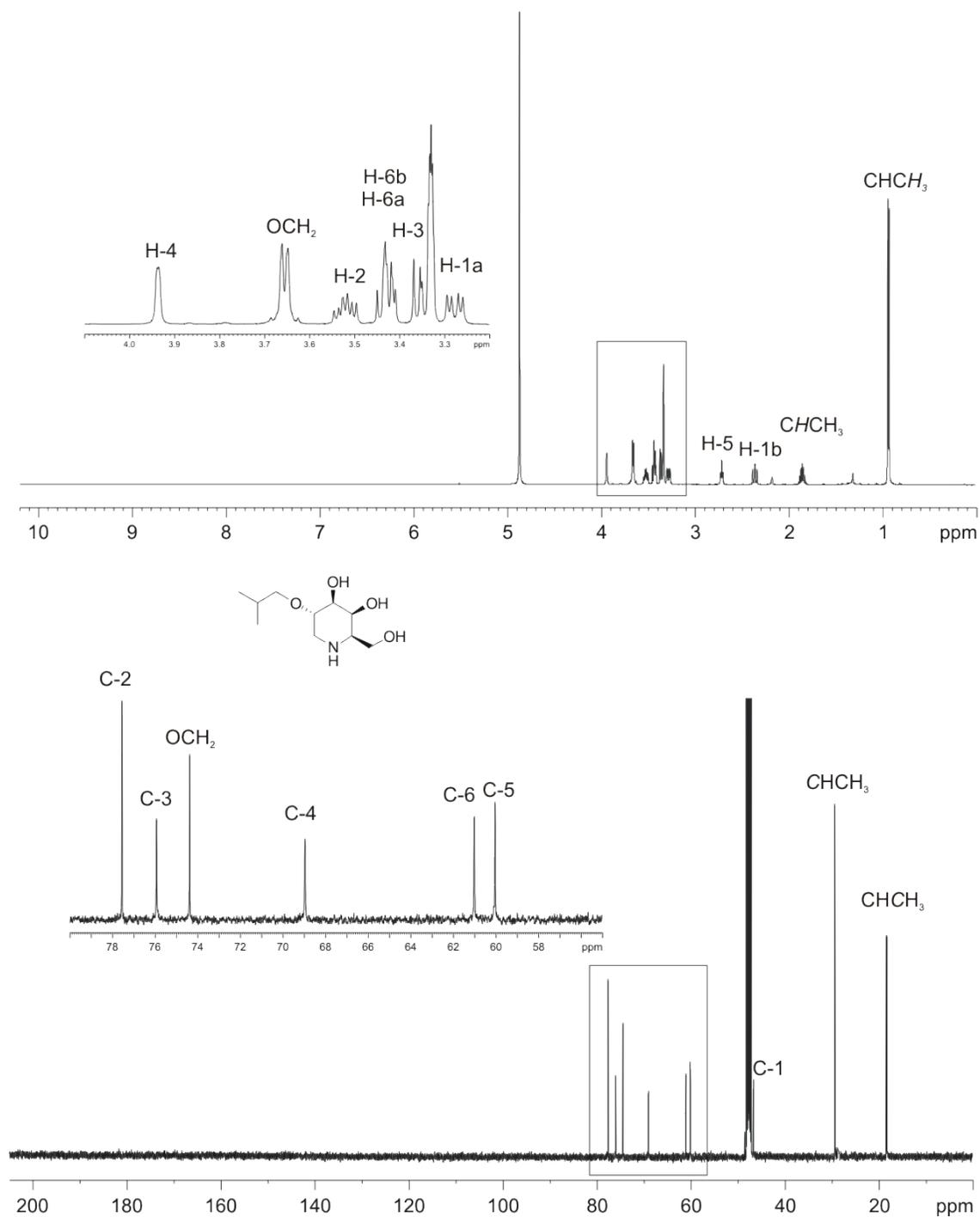
$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra (600 MHz, 151 MHz,  $\text{CD}_3\text{OD}$ ) of **7d**.



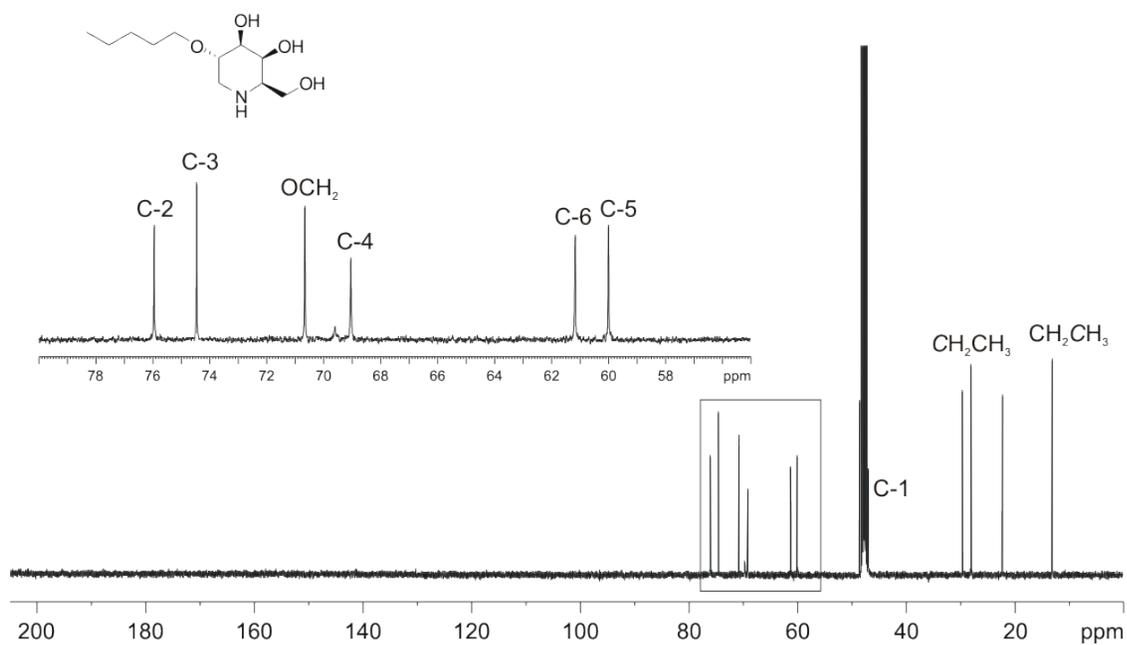
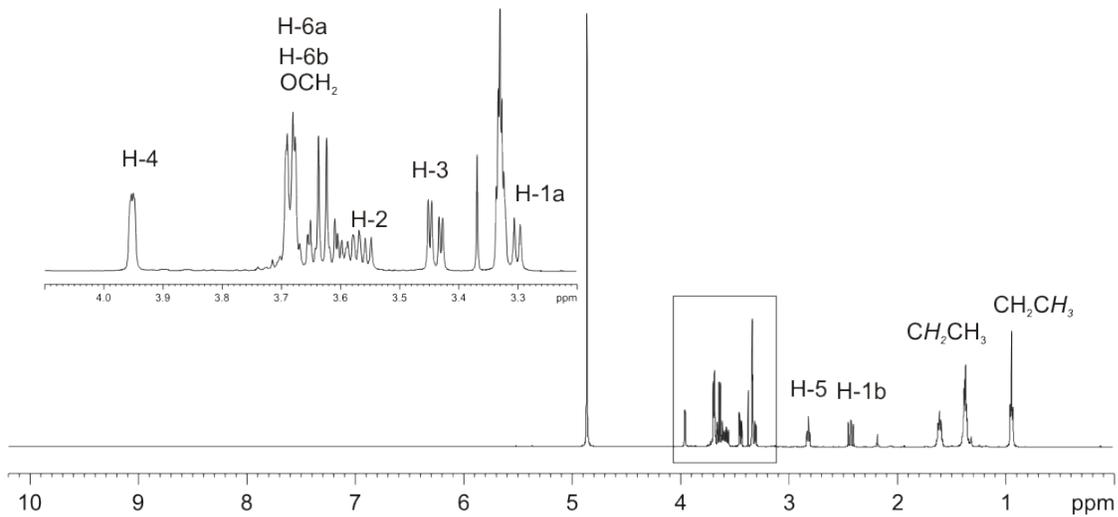
$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra (600 MHz, 151 MHz,  $\text{CD}_3\text{OD}$ ) of **7e**.



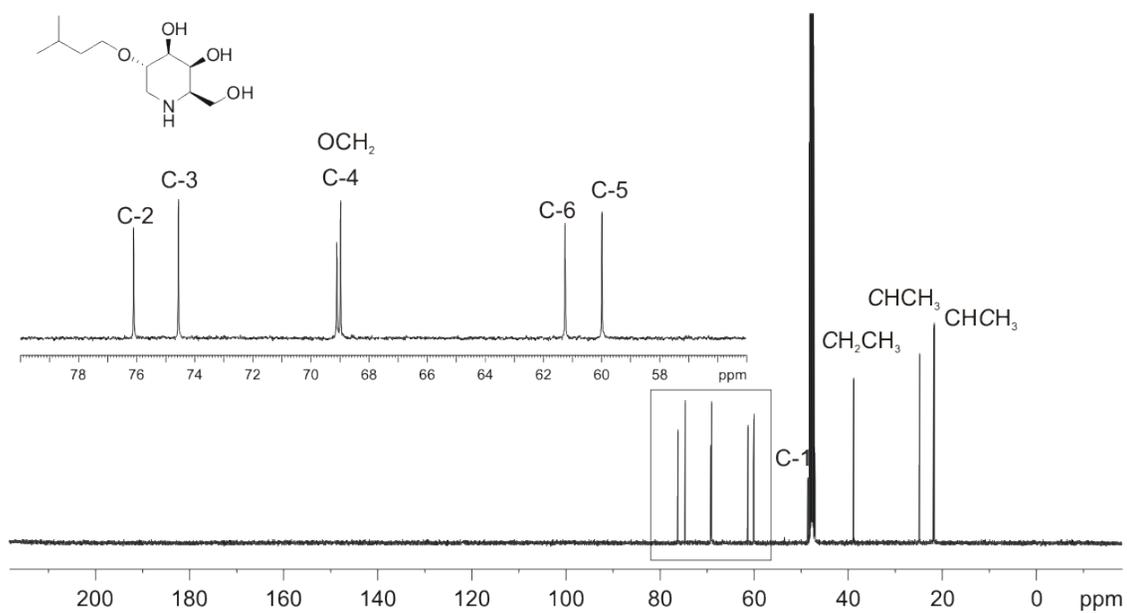
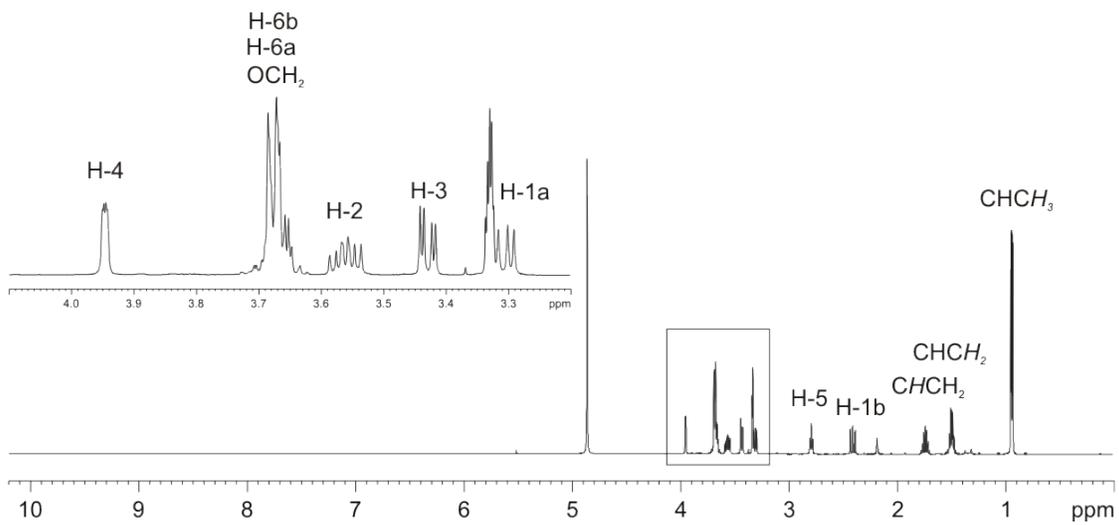
$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra (600 MHz, 151 MHz,  $\text{CD}_3\text{OD}$ ) of **7f**.



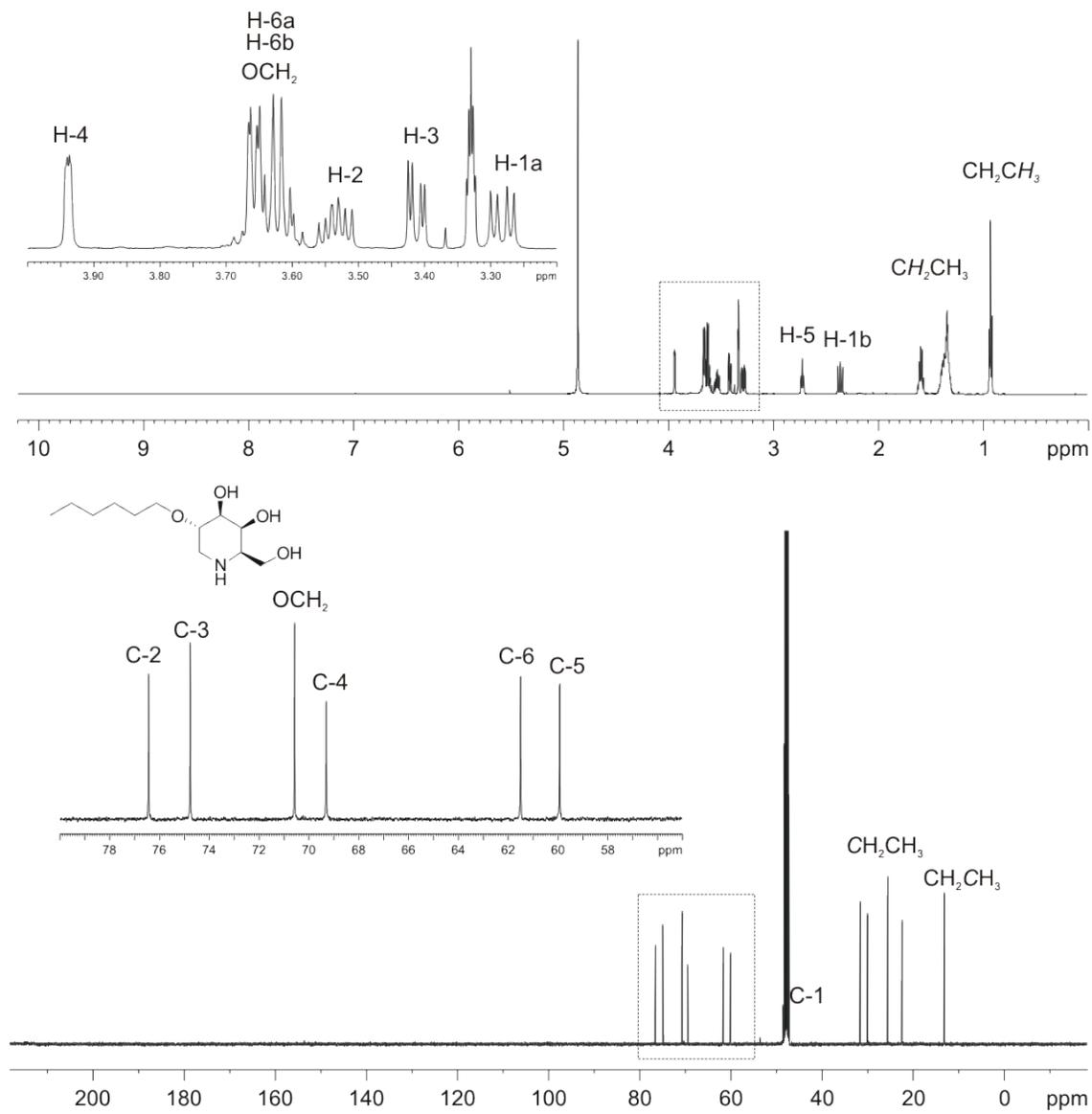
$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra (600 MHz, 151 MHz,  $\text{CD}_3\text{OD}$ ) of **7g**.



$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra (600 MHz, 151 MHz,  $\text{CD}_3\text{OD}$ ) of **7h**.



$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra (600 MHz, 151 MHz,  $\text{CD}_3\text{OD}$ ) of **7i**.



## Compound HPLC details and traces

### Mass spectrometer

Bruker maXis Impact Ultra-High Resolution tandem TOF (UHR-Qq-TOF) mass spectrometer.  
Software: Compass 1.5

### LC-MS Conditions

Mass Spectrometer Ionization Mode: Positive Electrospray Ionization (+ESI)

Gas Temp (°C): 200

Gas Flow (l/min): 8

Nebulizer (bar): 2

Capillary Voltage (V): 4500

Mass Range (Da): 50 to 1500

Calibrant: Sodium Formate

### HPLC

Agilent 1200 HPLC

HPLC Column: Zorbax RX-SiL particle size 5 micron, 150 (length) x 2.1 (diameter) mm from Agilent Technologies

Column Temp (°C): 30

HPLC Gradient Table:

Solvent A: Water with 0.1% formic acid

Solvent B: Acetonitrile with 0.1 % formic acid

Time (min)	% Solvent B	Flow rate (mL/min)
0	90	0.3
1	90	0.3
6	60	0.3
10	60	0.3
12	90	0.3
15 Stop	90	0.3

