Supplementary Material (ESI) for New Journal of Chemistry

Efficient immobilization of fructose-6-phosphate aldolase in layered double hydroxide: improved stereoselective synthesis of sugar analogues

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General procedures

Nuclear magnetic resonance (**NMR**) spectra were measured in deutered solvents ($CDCl_3$, D_2O) on a Bruker AC-400 spectrometer, operating at 400 MHz for ^1H and 100 MHz for ^{13}C J-modulation. Residual solvent signals were used as internal reference. Chemical shifts (δ) are reported in ppm, coupling constant values (*J*) are given in hertz. Multiplicity abbreviations: s = singlet, d = doublet, t = triplet, q = quadruplet, qu = quintuplet, dd : double doublet, dq = double quadruplet, bs = broad singlet, m = multiplet. The NMR spectra of all main products are totally interpreted. Optical rotations were measured on a Jasco DIP-370 polarimeter, using a 10 cm quartz cell. Values for $[\alpha]^T_D$ were obtained with the D-line of sodium at the indicated temperature T, using solutions of concentration (*c*) in units of g/100 mL. Electrospray ionization mass spectra (**ESI-MS**) were recorded on a micro q-tof Micromass (3000 V) and high resolution mass spectra (**HR-MS**) were recorded on the same instrument with an internal lock mass (H_3PO_4) and an external lock mass (Leu-enkephalin). **TLC** were carried out on silica gel (60 F254, on alumina; Merck), visualised by UV flurescence at 254 mn and revealed with vanillin for sugars and carbonyl compounds (3% in ethanol). Powder X-ray diffraction (PXRD) patterns were obtained with a X'Pert Pro Philips X-ray diffractometer equipped with a PW301120 proportional detector, fitted with a graphite back-end monochromator and using Cu K_α radiation (λ = 1.5415 Å). The samples were scanned from 2° to 70° (2θ) using steps of 0.08° and a counting time of 4 s per step. D-fructose-6-phosphate aldolase (FSA) was prepared and purified as previously described[1]. **Unit (U) definition** : one U will cleave 1 μmol of D-fructose-6-phosphate per minute, at pH 8.5 (glycyl-glycine 50mM buffer) and 25°C to afford D-glyceraldehyde-3-phosphate (D-G3P) and dihydroxyacetone (DHA). Phosphoglucose isomerase, glucose-6-phosphate dehydrogenase, triose-phosphate isomerase and glycerol-3-phosphate dehydrogenase from *Cellulomonas sp*. were purchased from Sigma-Aldrich. $Mg(NO_3)_2.6H_2O$, $Al(NO_3)_3.9H_2O$, NaOH, glycylglycine were of analytical grade and purchased from Aldrich chemicals. All aqueous salt solutions were prepared using MilliQ water. Some liquid reagents were purified by distillation under argon, as follows: propionaldehyde, butyraldehyde, hydroxyacetone.

Preparation and characterisation of FSA@LDH

Preparation of LDH materials:

Mg_2Al-NO_3 LDH was prepared by the coprecipitation method[2] under nitrogen atmosphere (in order to minimise the contamination with atmospheric CO_2) and vigorous magnetic stirring. 11 mL of an aqueous solution of Mg and Al nitrate with a Mg/Al molar ratio equal to 2 and total metal ion concentration of 0.1 M was added with a constant flow (0.050 mL/min) to a flask containing 100 mL of deionised water. A solution of sodium hydroxide (0.1 M) was simultaneously added to fix the pH of coprecipitation at the constant value of 9.0 ± 0.1 using an automated pH titrator. The addition of the salt solution was completed in 5 h and the suspension was aged for 12h under stirring. The precipitate was washed by three dispersion and centrifugation cycles in deionised water, and finally air-dried. Mg_2Al-GlyGly was prepared using the same procedure except that coprecipitation was performed in a glycylglycine solution with an excess of glycylglycine (glycylglycine/Al^{3+} = 2).

FSA immobilization:

Immobilization of FSA was realized by the coprecipitation method. FSA was directly encapsulated in Mg_2Al LDH during the coprecipitation process. The method was adapted from the procedure described above for the preparation of small amounts of biohybrid materials (< 100 mg). Typically, 10.89 ml of a $Mg(NO_3)_2.6H_2O$ and $Al(NO_3)_3.9H_2O$ mixed aqueous solution ([Mg^{2+}+Al^{3+}] = 0.1M) was introduced at a rate of 0.050 mL/min into a reactor containing 188.68 mg of FSA (53 w%)/Glygly dissolved in 100 mL water. The coprecipitation was performed for a FSA/LDH mass ratio (Q) equal to 1.0, at a pH = 9.0 and room temperature. Addition was completed in 3.6 hours. The biohybrid materials were collected and stored, as previously described, in dried and wet forms. Elemental analysis of Mg^{2+} and Al^{3+} was performed by ICP-ES (Perkin-Elmer Optima 3000XL atomic emission spectrometer) (Mg^{2+} 6.85 ppm, Al^{3+} 3.86 ppm for 50mg of FSA@LDH solubilized in 1L of acidified water). The experimental ratio Mg^{2+}/Al^{3+} = 1.97 was found to be very close to the expected one for a full coprecipitation of a LDH phase with an initial ratio equal to 2.0.

FSA@LDH kinetic measurements:

Steady-state kinetic measurements of FSA@LDH were determined as previously described[1].

To a solution of D-fructose-6-phosphate (50 mM) was added FSA@LDH (20 μL, 0.02 U) in 50 mM glycyl-glycine buffer pH 8.5 at 25°C. NADH (0.5 mM), auxiliary enzymes triose-phosphate isomerase and glycerol-3-phosphate dehydrogenase were added in order to isomerise and reduce D-glyceraldehyde-3-phosphate. The final volume was 1 mL. The reaction was monitored by spectrophotometry at 340 nm following the consumption of NADH. One mmol of NADH oxidized was equivalent to 1 mmol of D-fructose-6-phosphate cleaved.

Km of dihydroxyacetone (DHA): A solution of D,L-G3P was obtained by deprotection of the commercially available D,L-glyceraldehyde-3-phosphate diethylacetal following the manufacturer protocol. To the solution of D,L-G3P (6 mM) was added 50 mM of glycyl-glycine buffer (pH 8.5, 25°C) and FSA@LDH (0.02 U). DHA (in a concentration varying from 20 to 100 mM, from 1.8 mg to 9 mg respectively) was added to the solution. The formation of D-fructose-6-phosphate was monitored by spectrophotometry at 340 nm and 25 °C, using the combined enzymes phosphoglucose isomerase (18 U) and glucose-6-phosphate dehydrogenase (5 U) in the presence of NADP (0.5 mM). The final volume was 1 mL. One mmol of NADP reduced was equivalent to 1 mmol of D-fructose-6-phosphate formed.

Reaction rates of hydroxyacetone (HA) and formaldehyde with FSA@LDH **over several cycles**: reactions were carried out at analytic level and reaction rates were evaluated over 30 min. Rates were determined at t = 0 and t = 30 min. In order to find the concentration of HA for each time, solutions of 0.1, 0.25, 0.5, 1, 2.5 and 5 mM were prepared to generate a calibration curve.

A solution of HA (500 mM) and a solution of formaldehyde (500 mM) in water were freshly prepared. In two Amicon® microcon YM-50 for t = 0 and t = 30 min, were blended a solution of HA (20 µL), formaldehyde (20 µL), 80 µL H_2O and finally FSA@LDH (80 µL; 0.14 U) (final volume 200 µL). Reactions were shaken at 25°C. Each Amicon® was centrifuged and 100 µL of supernatant was diluted with 900 µL of HPLC-grade water and injected directly in GC-MS to quantify disappearance of HA. Concentration of HA was found after triplicate measurements. Each FSA@LDH biomaterial was washed 5 times with 300 µL of water to completely remove HA and formaldehyde from FSA@LDH. The washed material was reused in another cycle with the same procedure.

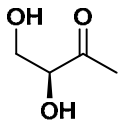
GC-MS method: The GC-MS analysis for HA was performed on Agilent 6890 gas chromatography instrument coupled with an Agilent MS-5973 mass detector and an Agilent autosampler 7683-B injector (Agilent Technologies, Little Fall, NY, USA). A capillary column supelcowax 10 (30 m x 0.25 mm i.d x 0.25 lm film thickness (Supelco 595 North Harrison Road, Bellefonte, USA)) was used for the separation. The initial temperature of 50 °C was maintained for 1 min raised to 200 °C at the rate of 15 °C/min, and kept at 200 °C for 5 min. Helium was used as a carrier gas with the flow rate of 0.9 ml/min, 1 µL splitless injections were made, and the purge valve was turned on after 1.0 min with a 50 mL/min split vent flow. Injector and detector temperatures were 250 and 230 °C, respectively. The mass spectrometer was operated in the electron impact (EI) mode at 70 eV. GC-MS quantification was performed by selected-ion monitoring of the HA at m/z 74 Th.

Sugar analogues synthesis and characterization

General procedure for enzymatic aldol reactions

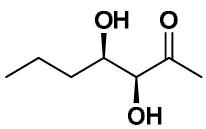
To a solution of FSA@LDH (40 U) in water (15 mL) into a Falcon®, was added respectively hydroxyacetone and formaldehyde (37% in water) and the reaction mixture was stirred overnight at RT on a shaking plate. Then the solution was centrifuged (8000 rpm, 15', 5°C) and the supernatant was transferred in a round flask. FSA@LDH was washed two times with water and the supernatants were poured in the round flask. Water was evaporated under reduced pressure to give the desired aldol without any further purification.

(3*S*)-3,4-dihydroxybutan-2-one **1**



1 Aldol **1** was obtained using the general procedure with hydroxyacetone (0.600 g, 8.10 mmol) and formaldehyde (37% in water) (0.740 g, 9.12 mmol). The aqueous solution was evaporated under reduced pressure to give the desired aldol **1** as a clear oil (0.85 g, 7.68 mmol, 95 %). (α_D: +66.0 (c 0.98, H_2O); **reference enantiomer 3*R*** - 70.1[3] (c 5.8, $CHCl_3$)] R_f = 0.53 (CH_2Cl_2/MeOH, 9:1); ^{1}H NMR (D_2O) δ (ppm): 2.25 (s, 3H), 3.87 (dd, *J* = 12 x 3 Hz, 1H), 3.93 (dd, *J* = 12 x 4 Hz, 1H), 4.40 (m, 1H); ^{13}C NMR (D_2O) δ (ppm): 25.8, 62.5, 77.9, 212.7.

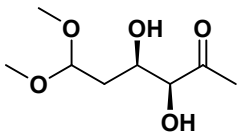
(3*S*,4*R*)-3,4-dihydroxyheptan-2-one **2**



2 Aldol **2** was obtained using the general procedure with hydroxyacetone (0.150 g, 2.03 mmol) and butyraldehyde freshly distilled (0.120 g, 1.66 mmol). The aqueous solution was evaporated under reduced pressure to give the desired aldol **2** as a clear oil (0.22 g, 1.49

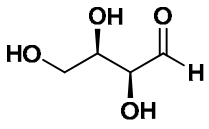
mmol, 90 %). [α_D: +102.1 (c 0.95, $CHCl_3$); **reference enantiomer 3*R*,4*S***[4]: - 103.1 (c 0.26, $CHCl_3$)]; R_f = 0.47 (CH_2Cl_2/MeOH, 95:5); ^{1}H NMR ($CDCl_3$) δ (ppm): 0.97 (t, *J* = 7.1 Hz, 3H), 1.37-1.54 (m, 2H), 1.63 (m, 2H), 1.86 (d, *J* = 8.5 Hz, 1H), 2.27 (s, 3H, H-1), 3.72 (d, *J* = 3.5 Hz, 1H); 4.00 (d, *J* = 5.7 Hz, 1H) 4.07 (m, 1H); ^{13}C NMR ($CDCl_3$) δ (ppm): 14.0, 19.2, 25.4, 36.5, 71.7, 79.4, 208.4.

(3*S*,4*R*)-3,4-dihydroxy-6,6-diethoxyhexan-2-one **3**



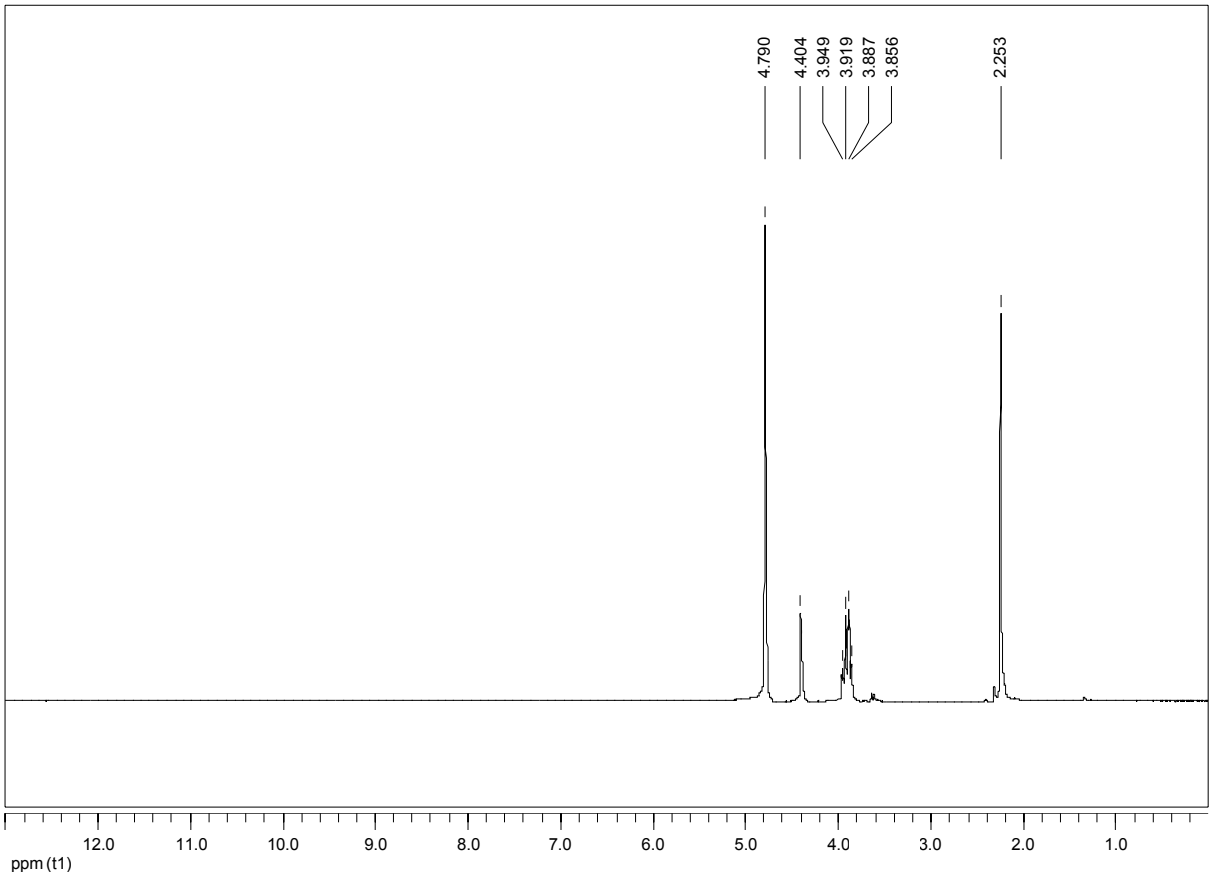
3 Aldol **3** was obtained using the general procedure with hydroxyacetone (0.100 g, 1.28 mmol) and 3,3-dimethoxypropional (0.100 g, 0.85 mmol). The aqueous solution was evaporated under reduced pressure to give the desired aldol **3** as a clear oil (0.16 g, 0.83 mmol, 98 %). (α_D: +72.1 (c 1.0, H_2O); R_f = ketone : 0.50 + hydrate form : 0.66 (CH_2Cl_2/MeOH, 95:5); ^{1}H NMR (D_2O) δ (ppm): 1.95 (m, 2H), 2.28 (s, 3H), 3.41, 3.44 (2s, 6H), 4.26 (m, 1H), 4.29 (m, 1H), 4.69 (m, 1H); ^{13}C NMR (D_2O) δ (ppm): 25.8, 36.1, 53.4, 53.9, 67.8, 79.6, 102.8, 212.7.

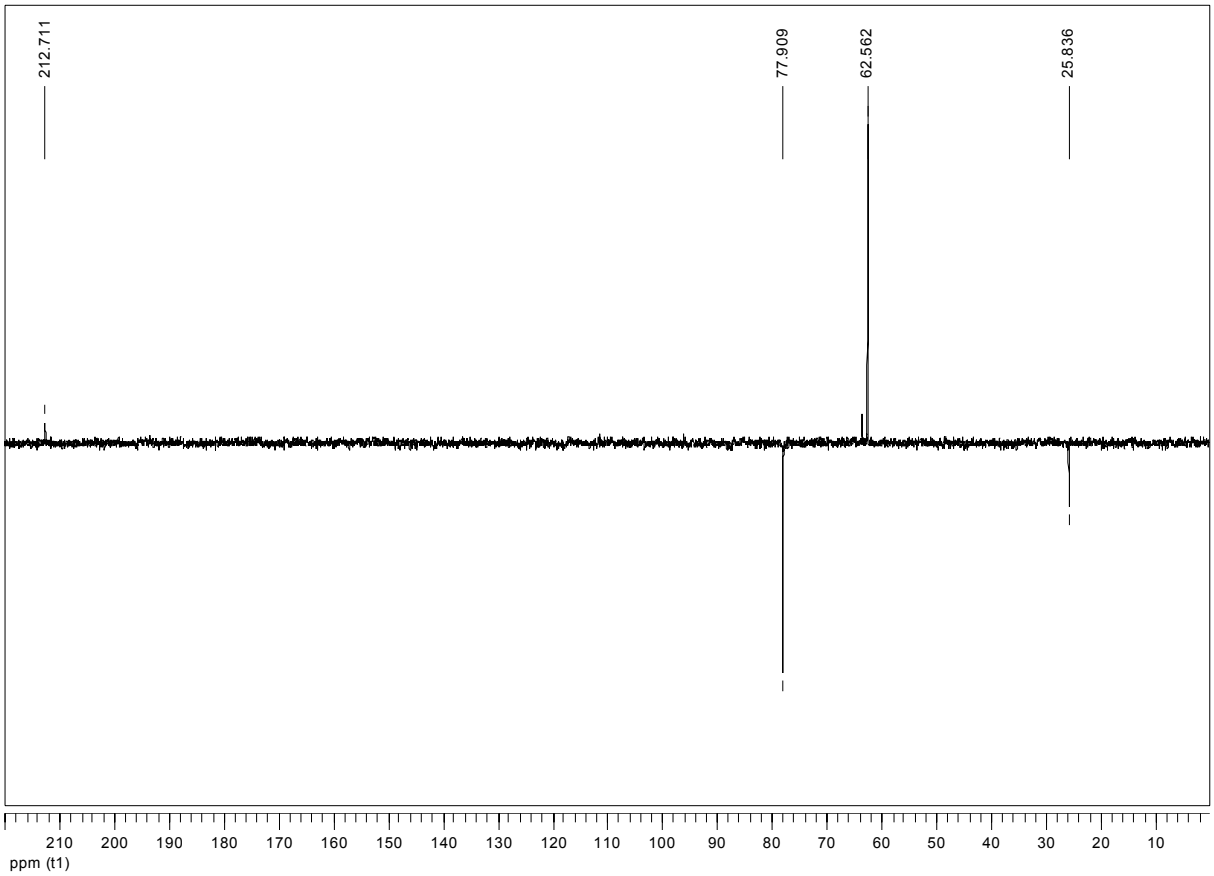
D-threose **4**

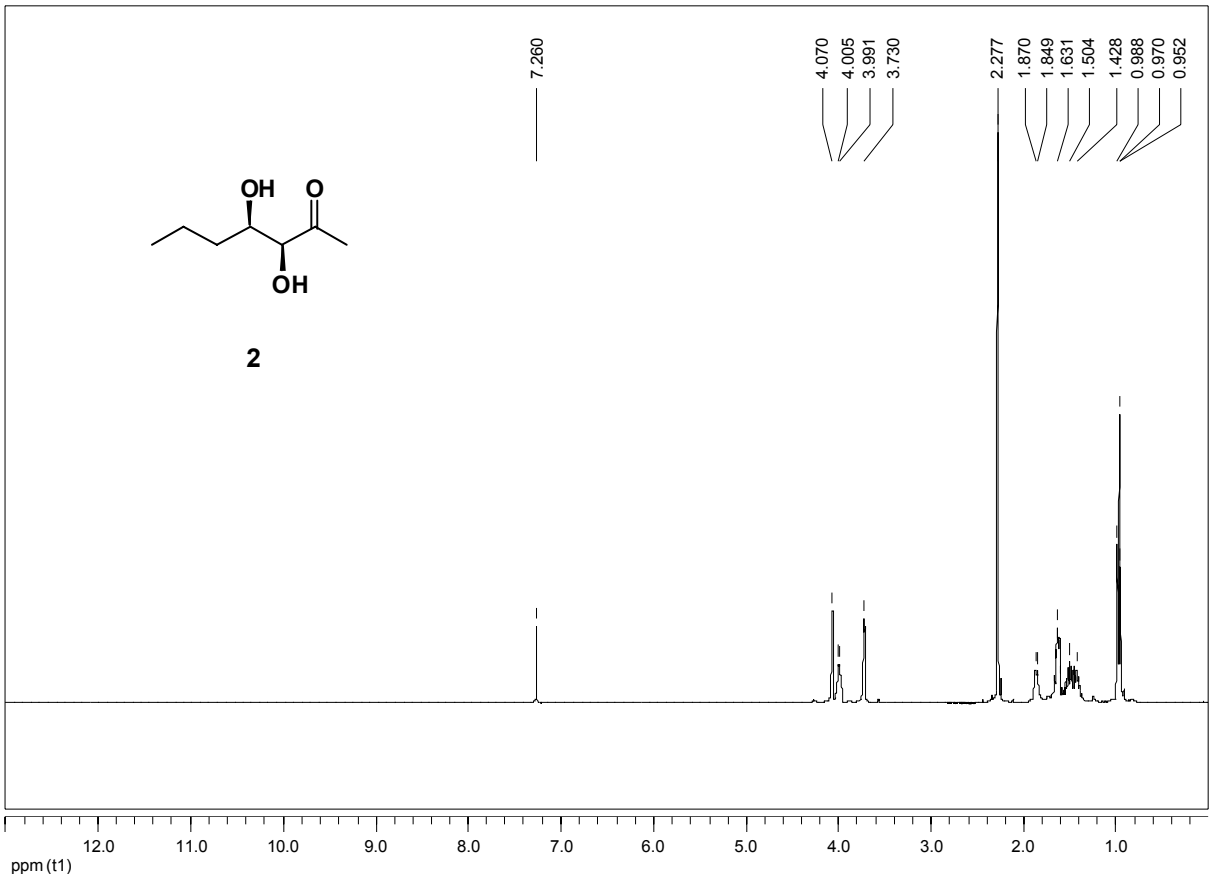


4 Aldol **4** was obtained using the general procedure with glycolaldehyde (0.054 g, 0.45 mmol). The aqueous solution was evaporated under reduced pressure to give the desired aldol **4** as a clear oil (0.054 g, purity 60 %, 0.27 mmol, 60 %). (α_D: –11.0 (c 0.6, H_2O); The data matched those reported previously[5].

^{1}H and ^{13}C NMR spectra







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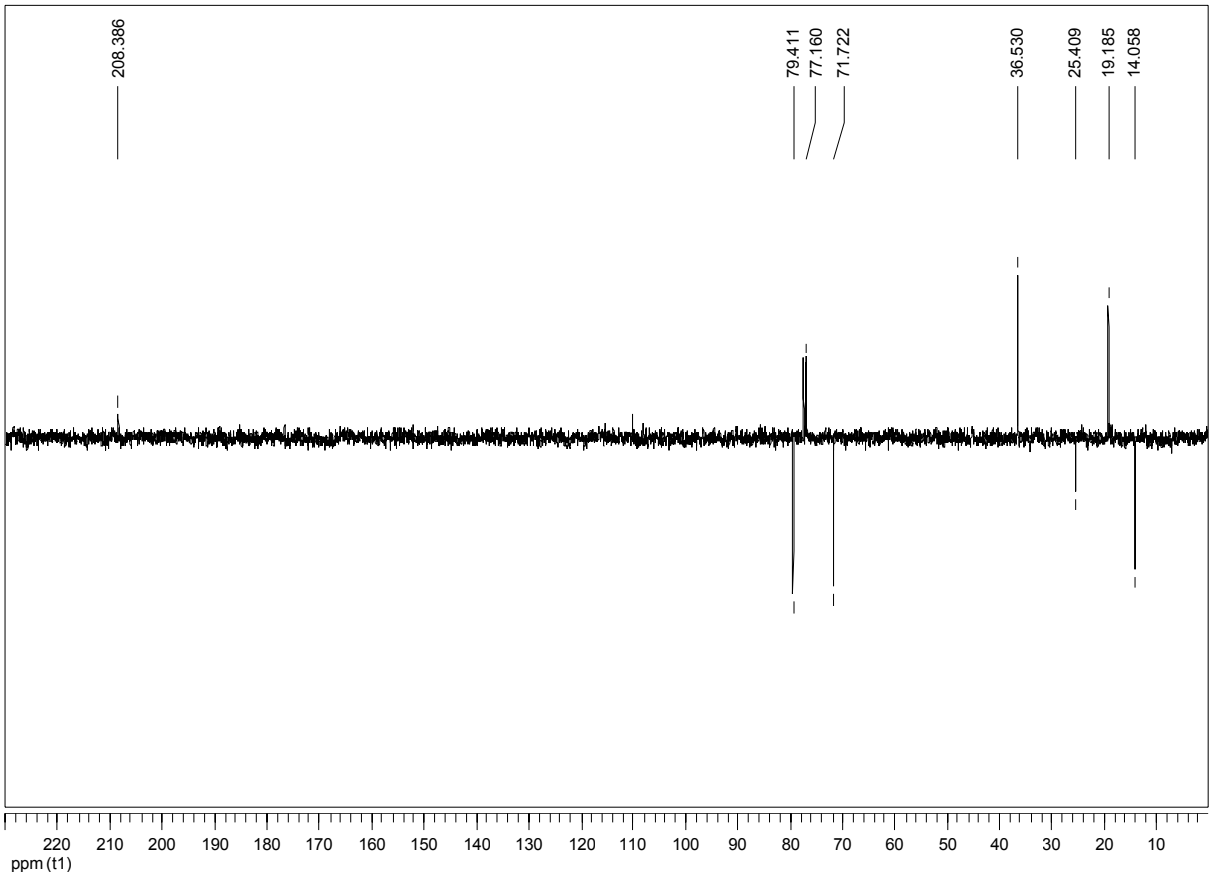

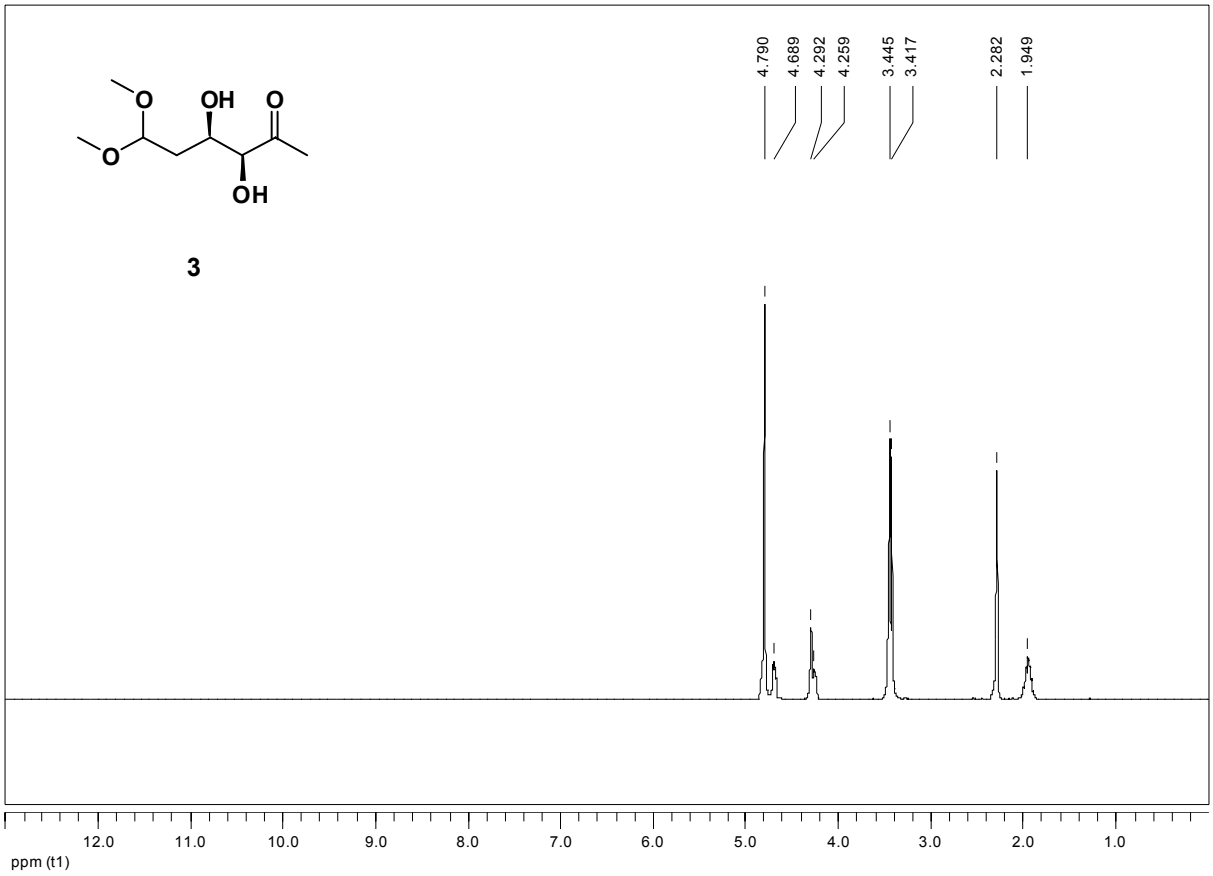

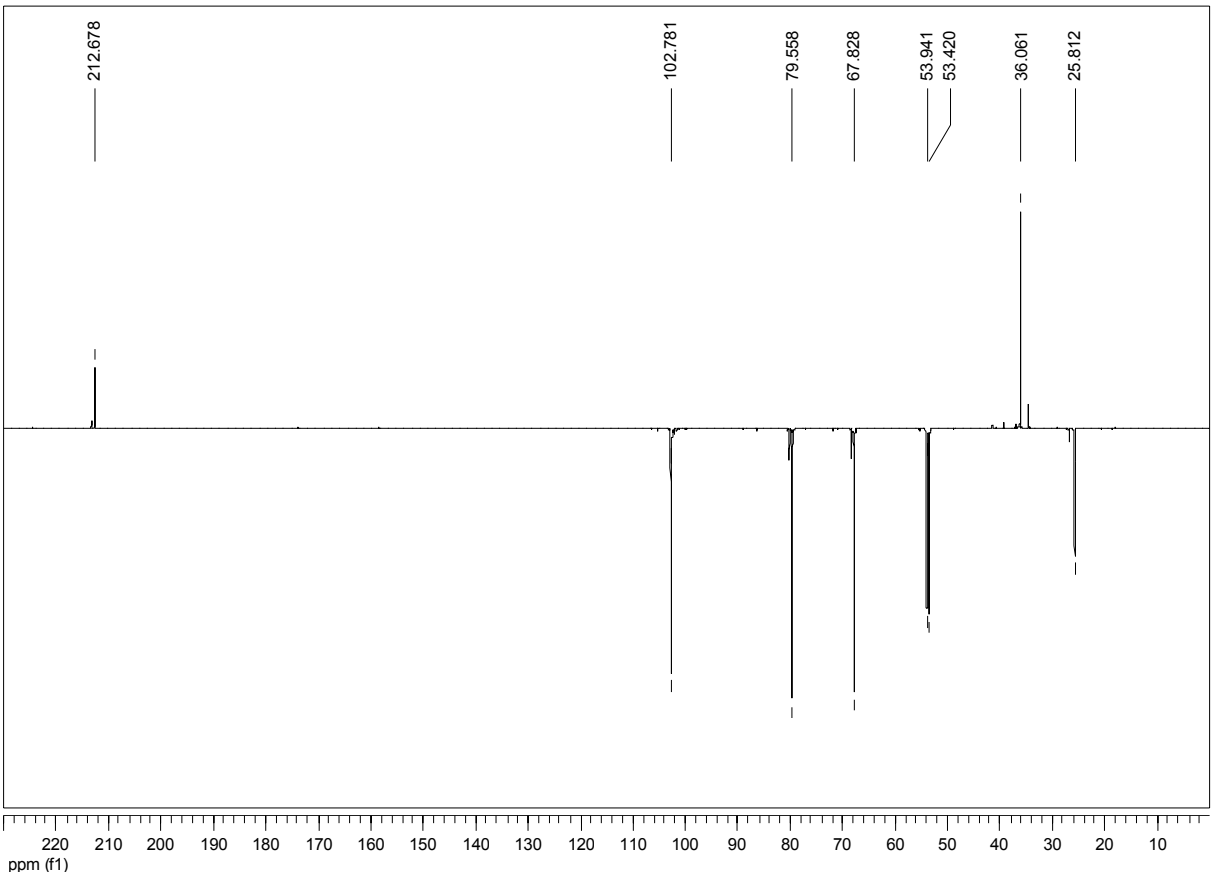

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