Supplementary Information

for

Enzymatic Synthesis of D-Sorbose and D-Psicose with Aldolase RhaD: Effect of Acceptor Configuration on Enzyme Stereoselectivity

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Bacterial strains, plasmids, and materials.

Oligonucleotide primers were synthesized at Invitrogen (CA, USA). Pfx DNA polymerase was purchased from Invitrogen. Restriction enzymes and T4 ligase were purchased from Fermentas (MBI, Canada). *Escherichia coli* DH5α [*lacZΔM15 hsdR recA*] was purchased from Gibco-BRL (Gaithersburg, MD). *Escherichia coli* MG1655 (rph-1, fnr) was from lab stock. *E. coli* BL21 (DE3) [F^- *ompT hsdSB*(r_B^- m_B^-) *gal dcm* (DE3)] and plasmid pET-28a were purchased from Novagen (Carlsbad, CA). L-glycerol 3-phosphate bis(cyclohexylammonium) salt, D-glyceraldehyde, glycerol phosphate oxidase, catalase, acid phosphatase from sweet potato, D-psicose, D-sorbose, isopropyl-1-thio-β-D-galactopyranoside (IPTG), imidazole, kanamycin and Ca^{2+} exchange resin were purchased from Sigma-Aldrich (St. Louis, MO). Ni^{2+}-NTA column was purchased from Qiagen (Hilden, Deutschland). L-glyceraldehyde was purchased from Capot Chemical Co (Hangzhou, China). Amicon Ultra centrifugal filter (10 K) was purchased from Millipore (Billerica, MA). Bio gel P-2 gel, Aminex HPX-87H column (300 × 7.8 mm) was purchased from Bio-Rad Laboratories, Inc. (Hercules, CA). XK column (100 × 2.6 cm) was purchased from GE Healthcare (Piscataway, NJ). Pierce BCA Protein Assay Kit was purchased from Thermo Scientific (IL, USA).

Cloning, expression and purification of RhaD

Construction of pET28a-rhaD plasmid

The gene encoding L-rhamnulose-1-phosphate aldolase (RhaD) was amplified by PCR from *E.coli* MG1655 (gene information is available at http://www.genome.jp/kegg). The primers were: forward: 5'-GCGTGGATCCATGCAAAACATTACTCAGT-3' (BamHI); reverse: 5'-TATA AAGCTTTTACAGCGCCAGCGCACT-3' (HindIII). The rhaD gene amplified was digested with BamHI and HindIII then ligated into the pET-28a plasmid with the same enzymes digested. The recombinant plasmid pET28a-rhaD was transformed into DH5α strain for amplifying and sequencing.

Expression and purification of RhaD

The pET28a-rhaD plasmid was transformed into *E. coli* BL21 (DE3) to express RhaD. 20 mL overnight culture of *E.coli* BL21 (DE3) with the recombinant plasmid was inoculated to 1 L fresh LB medium containing 50 μg/mL kanamycin and cultivated at 37 ^{o}C, 200 rpm until the OD_{600} reached 0.8-1.0. Then the temperature was switched to 16 ^{o}C and IPTG was added at a final concentration of 0.1 mM to induce the expression of RhaD for 20 h. Cells were harvested by centrifugation at 4,000 × g for 20 min at 4 ^{o}C. The cell pellet was resuspended in 50 mM Tris-HCl (pH 7.5) buffer and sonicated on ice. The cell lysate was clarified by centrifugation at 15,000 × g for 30 min at 4 ^{o}C. The supernatant was loaded onto Ni^{2+}-NTA column equilibrated with 50 mM Tris-HCl (pH 7.5), then the column was washed with 50 mL wash buffer (50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM imidazole). Finally, RhaD protein was eluted with elution buffer (50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 500 mM imidazole). The eluant was concentrated and desalted with Amicon Ultra (10 K) by centrifugation at 4,000 × g at 4 ^{o}C with

50 mM Tris-HCl buffer (pH 7.5) containing 10% glycerol to give the final volume of 2.5 mL which was stored at -20 ^{o}C. The purity of the target protein was examined by 12% (v/v) SDS-PAGE and the gels were stained by Coomassie brilliant blue. The protein concentrations were determined using Pierce BCA Protein Assay Kit according to the instructions.

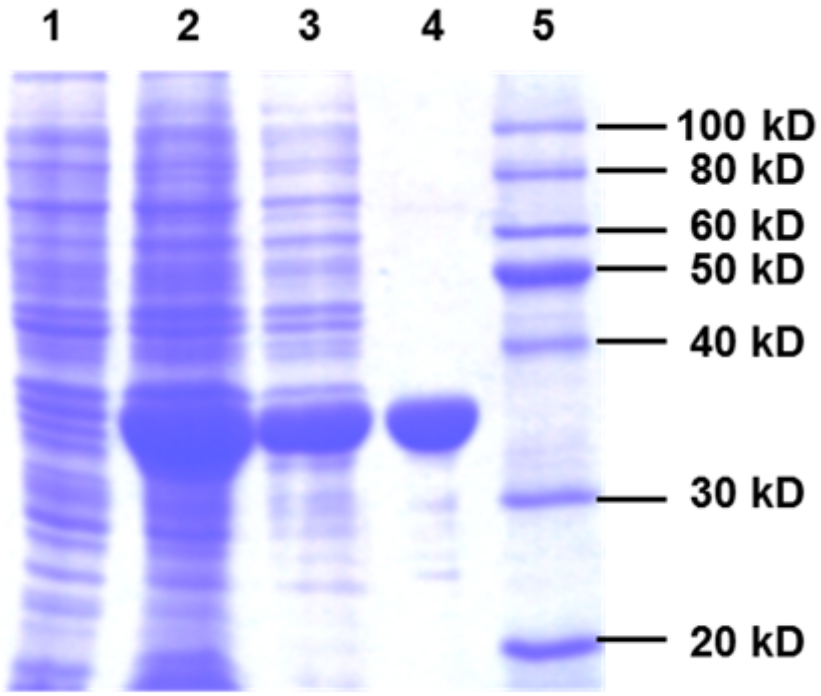


Figure S1. SDS-PAGE analysis of RhaD expression and purification. Lanes: 1, whole cells not induced; 2, whole cells induced for 20 h; 3, supernatant of cell lysate; 4, purified RhaD; 5, protein marker.

Synthesis of D-sorbose/D-psicose with RhaD and AP. DHAP (0.2 M, 874 μL) solution and D-glyceraldehyde (0.5 M, 435 μL) were combined and the resulting solution was brought to a total volume of 2335 μL by adding ddH_2O. RhaD (18.4 mg/mL, 65 μL in pH 7.5 Tris buffer, final concentration 0.5 mg/mL) was added and the reaction mixture was shaken at rt. for 16-22 h and the reaction was monitored by TLC (developed by nBuOH/AcOH/H_2O 2/1/1 (v/v/v) and stained with anisaldehyde sugar stain). The pH was adjusted to pH ~5 with 6 N HCl and 2 μL acid phosphatase (3 U) was added and the mixture was shaken at 37 ^oC for another 24 h. The ratio of D-sorbose and D-psicose in the final mixture was determined by HPLC. (Aminex HPX-87H, 300×7.8 mm, column temperature 60 ^oC, 5 mM sulfuric acid as mobile phase and detected with Refractive Index Detector). After cooling to rt., the pH was adjusted to 7 with 1 N NaOH and the mixture was diluted with methanol. The solution was filtered through Celite and washed with methanol. The filtrate was concentrated under reduced pressure and the residue was purified by silica gel column chromatography (EtOAc/iPrOH/H_2O 9/3/1 (v/v/v)) to afford a pale yellow syrup which was further purified by Bio gel P-2 column to afford D-sorbose and D-psicose mixture (23 mg, 73% totally). The mixture containing D-sorbose and D-psicose only could be isolated as described below. NMR data were consistent with authentic samples. HRMS (ESI, D-sorbose) calcd for $C_6H_{12}O_6Na$ $(M+Na)^+$ 203.0526, found 203.0530 *m*/*z*. HRMS (ESI, D-psicose) calcd for $C_6H_{12}O_6Na$ $(M+Na)^+$ 203.0526, found 203.0524 *m*/*z*.

One-pot four enzymes synthesis of D-sorbose and D-psicose. To a solution of L-glycerol 3-phosphate bis(cyclohexylammonium) salt (370 mg, 1.0 mmol) in 7.56 mL ddH_2O was added D-glyceraldehyde (2.44 mL, 0.5 M, 1.22 mmol) at pH 7, glycerol phosphate oxidase (70 U, 2 mg), catalase (1000 U, 1.18 μL) and RhaD (272 μL, final concentration 0.5 mg/mL). The mixture was

shaken at rt. for 22 h and the reaction was monitored by TLC (developed by nBuOH/AcOH/H_2O 2/1/1 (v/v/v) and stained with anisaldehyde sugar stain). The pH was then adjusted to pH ~5 with 6 N HCl and 11 μL acid phosphatase (18 U) added and the mixture was shaken at 37 ^oC for another 24 h. After cooling to rt., the pH was adjusted to 7 with 1 N NaOH and the mixture was diluted with methanol. The solution was filtered through Celite and washed with methanol. The filtrate was concentrated under reduced pressure and the residue was purified by silica gel column chromatography (EtOAc/iPrOH/H_2O 9/3/1 (v/v/v)) to afford a pale yellow syrup which was further purified by Bio gel P-2 column to afford D-sorbose and D-psicose mixture (87 mg, 48% totally). The mixture containing D-sorbose and D-psicose only could be isolated as described below. Synthesis of L-fructose was performed with the same procedure using L-glyceraldehyede (66% yield). NMR data were consistent with ref 11 and authentic sample. HRMS (ESI, L-fructose) calcd for $C_6H_{12}O_6Na$ $(M+Na)^+$ 203.0526, found 203.0522 *m*/*z*.

Typical procedure for isolation of D-sorbose and D-psicose using cation exchange resin (Ca^{2+} form). D-Sorbose and D-psicose mixture (150 mg from one-pot reactions) was dissolved in 3 mL ddH_2O and applied to a cation exchange resin (Ca^{2+} form, 26×100 mm) which was pre-heated to 65^oC with a thermostatic jacket. The column was eluted with ddH_2O (flow rate ~1.5 mL/min) and the whole isolation process was performed at 65-70 ^oC. Fractions were collected with an automatic fraction collector and identified by HPLC. Fractions containing pure D-sorbose or D-psicose were pooled and lyophilized to give D-sorbose (62 mg) and D-psicose (50 mg). A mixture of both sugars (~ 30 mg) was also collected which can be further separated if necessary.

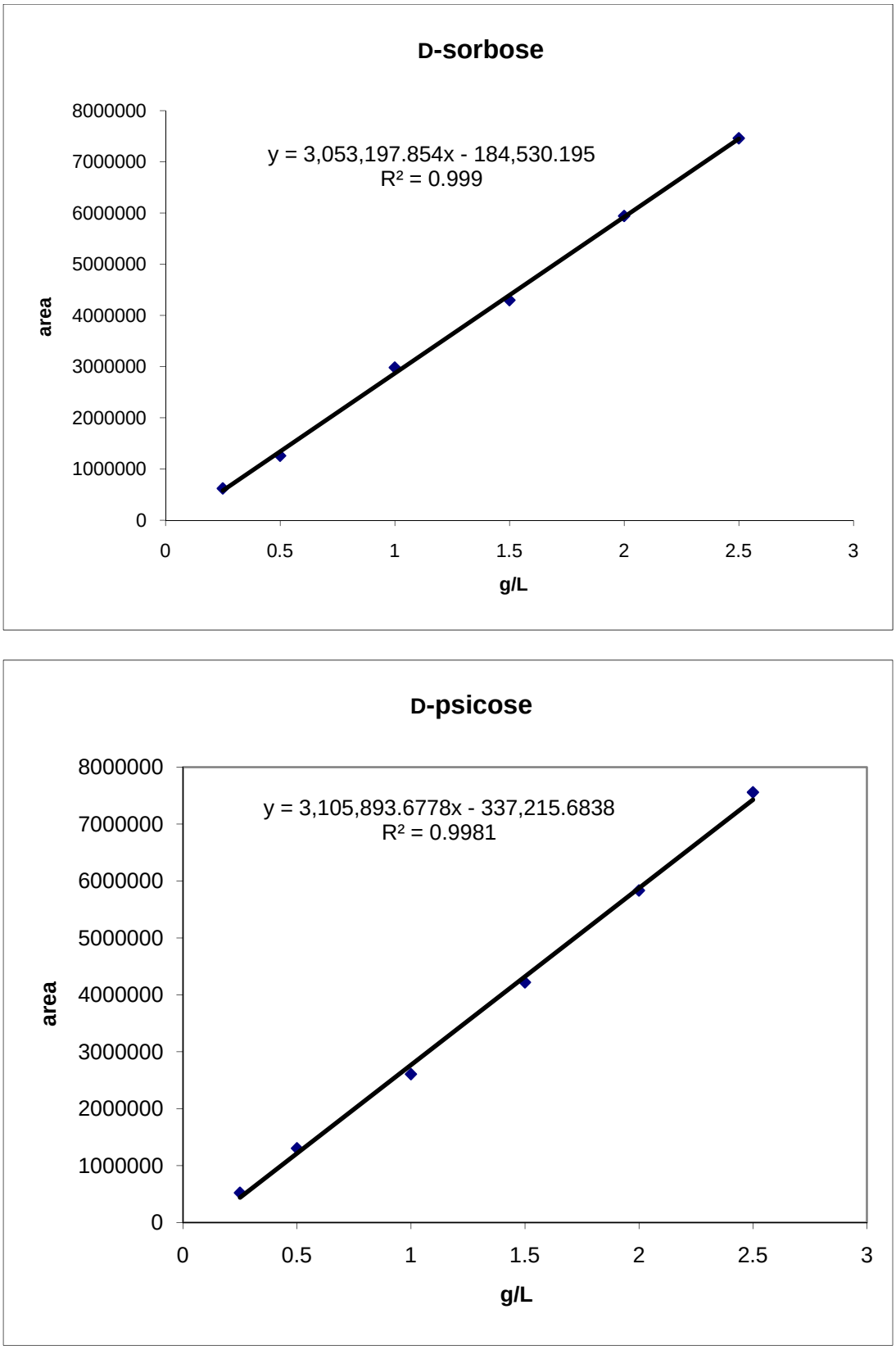
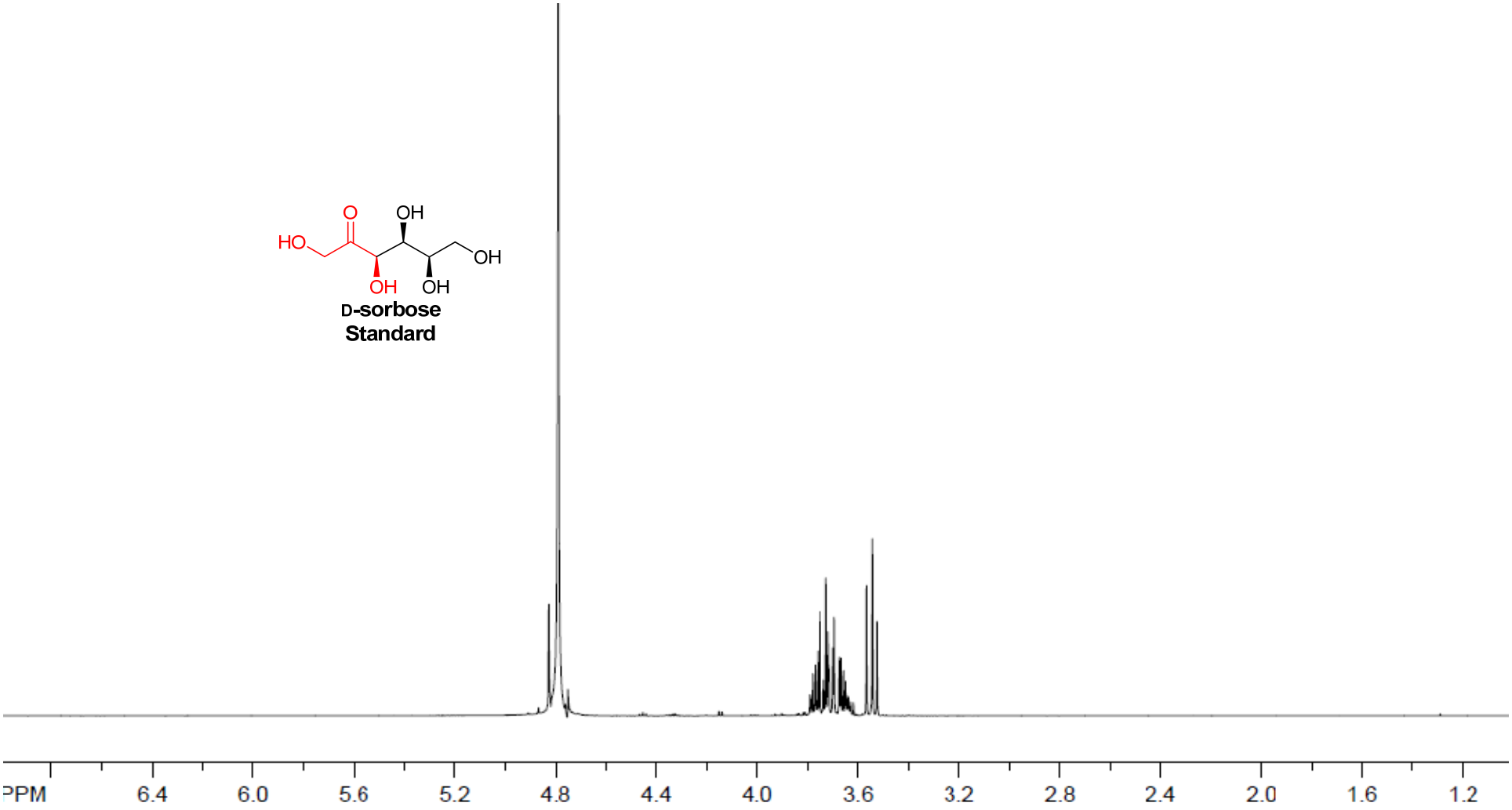


Figure S2. HPLC Standard curve for D-sorbose and D-psicose quantification (Area/C)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HO
OH
OH
OH
D-sorbose
Standard
PPM
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6.0
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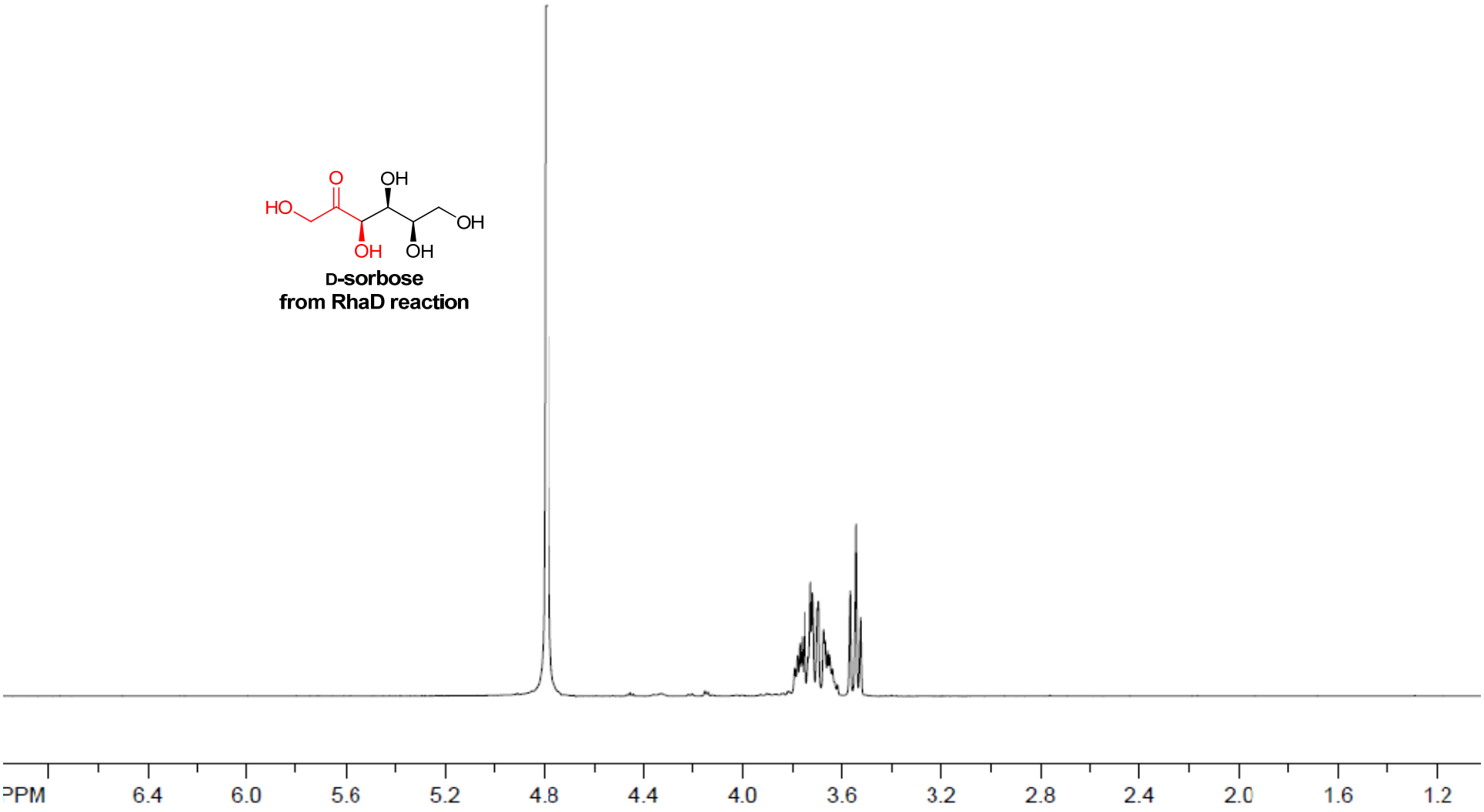
3.2
2.8
2.4
2.0
1.6
1.2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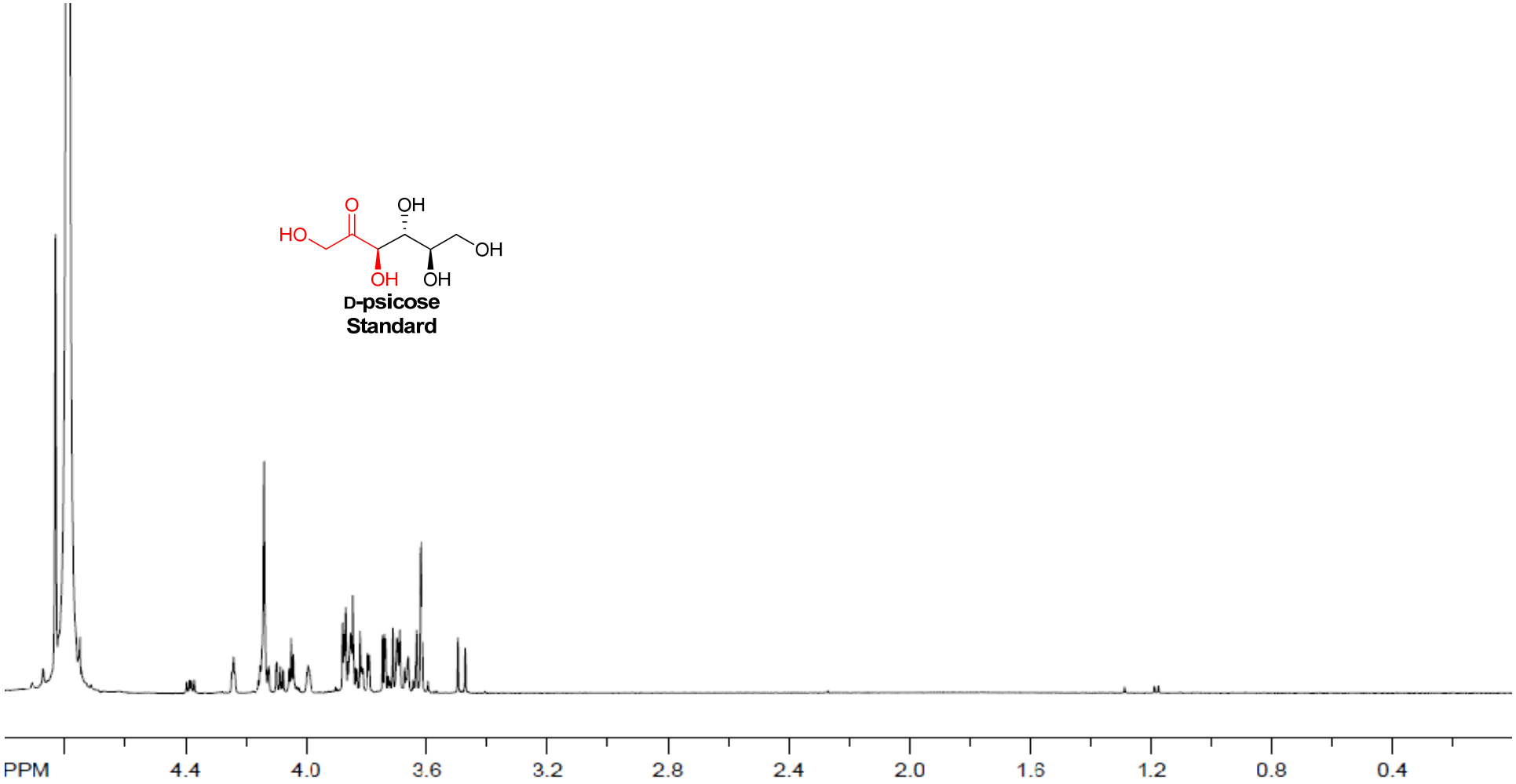
OH
HO
OH
OH
OH
D-sorbose
from RhaD reaction
PPM
6.4
6.0
5.6
5.2
4.8
4.4
4.0
3.6
3.2
2.8
2.4
2.0
1.6
1.2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HO
OH
OH
OH
D-psicose
Standard
PPM
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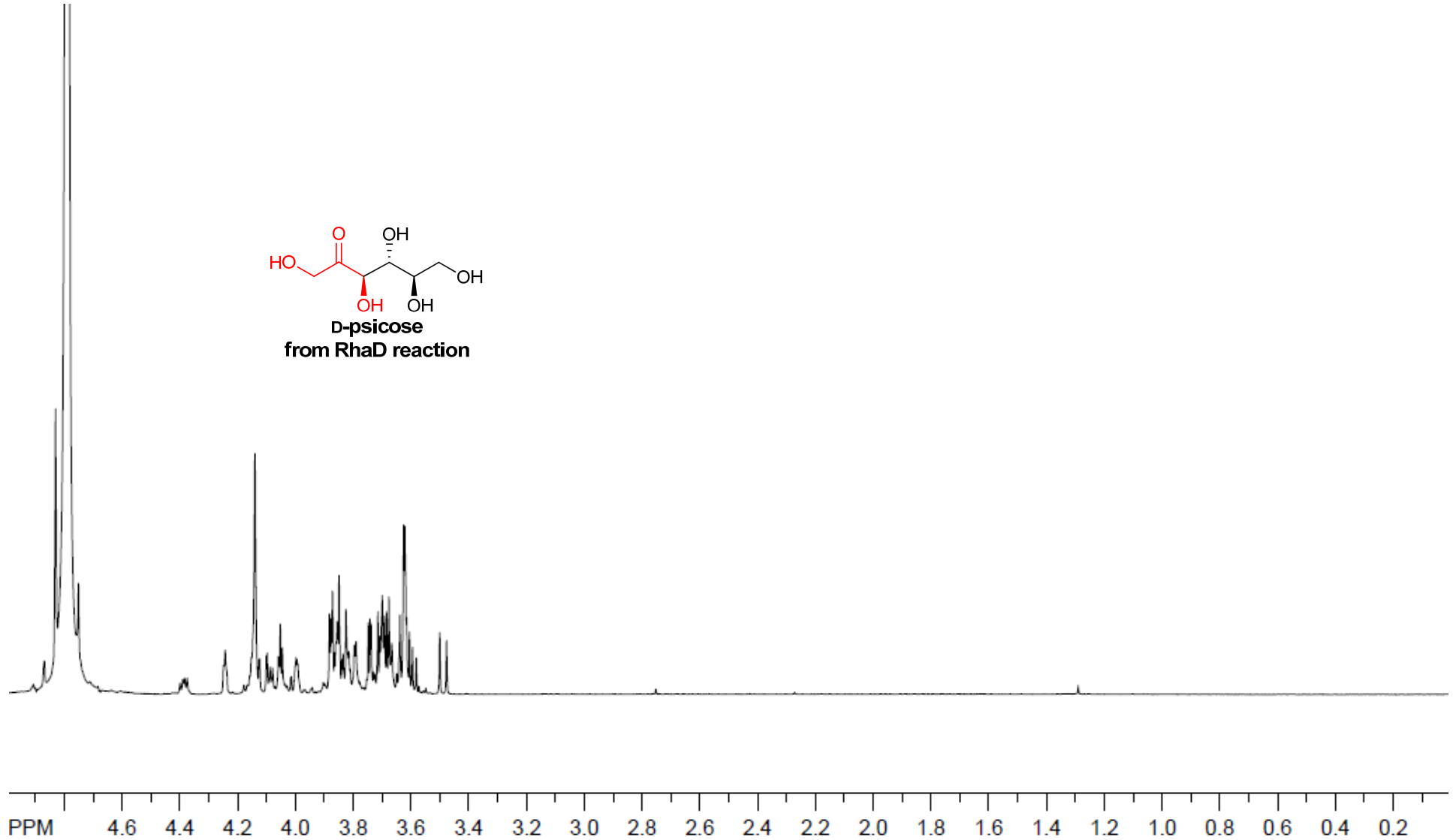
1.6
1.2
0.8
0.4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D-psicose
from RhaD reaction
PPM
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3.2
3.0
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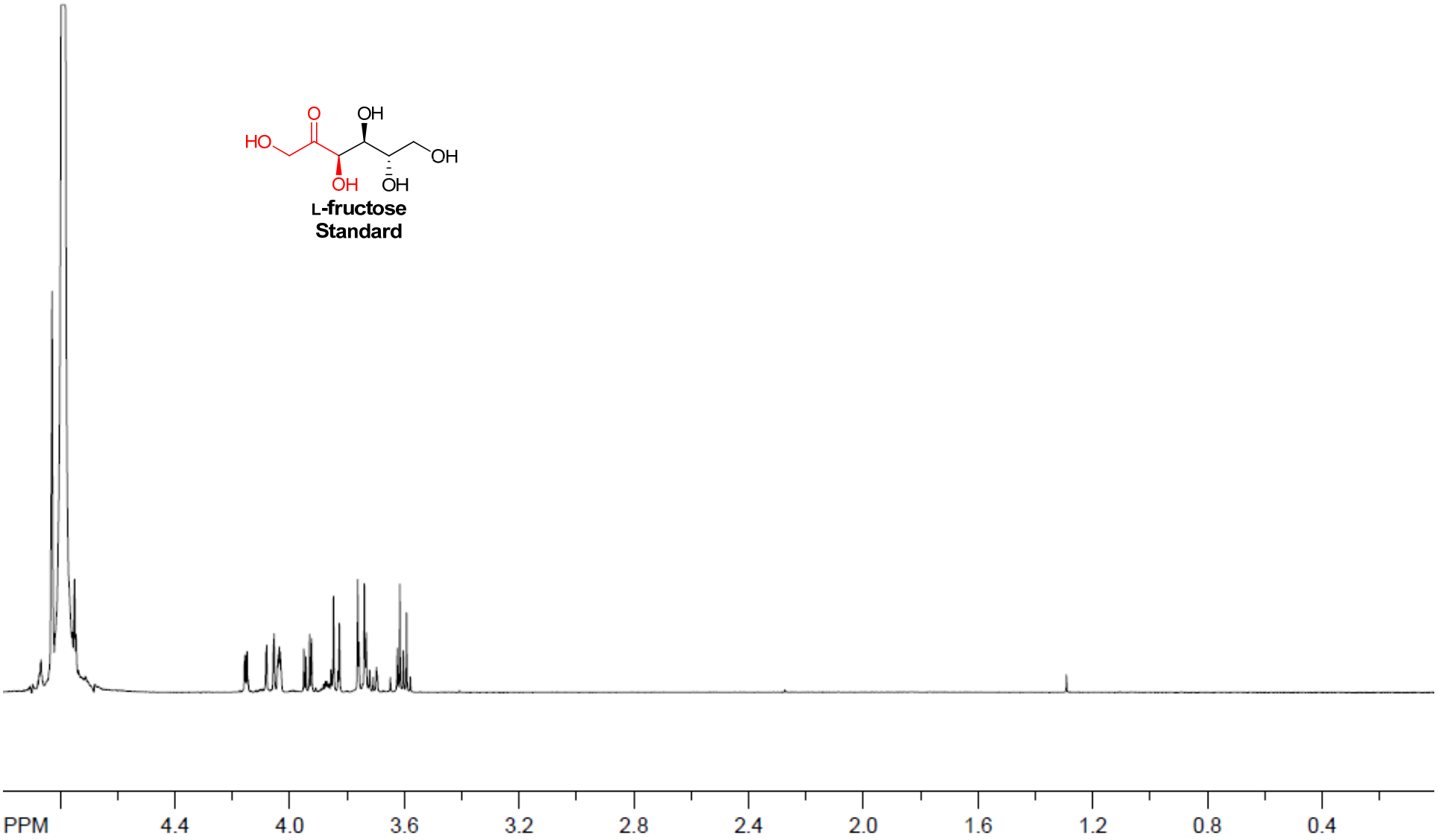
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HO
OH
OH
OH
L-fructose
Standard
PPM
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3.2
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2.4
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1.6
1.2
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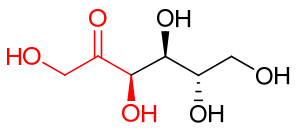
0.4



L-fructose
from RhaD reaction