Supplementary Information:

Stereoselective oxidation of protected inositol derivatives catalyzed by inositol dehydrogenase from *Bacillus subtilis*.

Richard Daniellou, Christopher P. Phenix, Pui Hang Tam, Michael C. Laliberte, and David R.J. Palmer*

Department of Chemistry, University of Saskatchewan

palmer@sask.usask.ca

EXPERIMENTAL

Chemical reagents, including buffers, salts, *myo*-inositol, NAD^+, and NADH, were obtained from Aldrich, Sigma, or VWR CanLab, and were categorized as Molecular Biology Grade or were the highest grade available. Isomaltose was a gift of Dr. Nicholas Lowe, Department of Applied Microbiology and Food Science, University of Saskatchewan. DNA-manipulating biochemicals, including restriction enzymes, T4 DNA ligase, and associated reagents were obtained from New England Biolabs. Oligonucleotides were obtained from Integrated DNA Technologies, INC. (Coralville, IA). *Pfu* DNA polymerase was obtained from Stratagene. *Bacillus subtilis* 168 (trpC2) was a generous gift of Dr. Luc Masson, NRC-Institute for Biological Sciences.

Centrifugation was performed using a Beckman J2-HS refrigerated centrifuge with a JLA-10.5 or JA-25.5 rotor. Protein chromatography was performed using an AP Bioscience AKTAfplc in a refrigerated chamber. UV-visible absorbance was measured using a Beckman DU-640 spectrophotometer with a circulating-bath-controlled temperature block. PCR amplifications were performed using an Eppendorf gradient thermocycler.

NMR spectra were recorded on a Bruker 500 MHz spectrometer. Chemical shifts are reported in ppm downfield from tetramethylsilane. Optical rotation values were determined with a Digipol DP781 polarimeter. Mass spectra were recorded with VG 70SE mass spectrometer. Reverse-phase HPLC was performed using a Zorbax SB-C18 250 × 4.6 mm (5 μm) analytical column (eluent: water/MeOH 80/20, temperature 25°C, flow rate 0.75 mL/min). Semi-preparative HPLC used a Zorbax SB-C18 250 × 9.4 mm (5 μm) column (same conditions, flow rate 2.75 ml/min). Chiral HPLC used a Chiradex column (eluent: 100% water, temperature 30°C, flow rate 0.8mL/min or 0.5 ml/min). Peaks were detected by UV absorbance at 254 nm. Thin-layer chromatography was performed on aluminium-backed plates of Silica Gel $60F_{254}$ (EM Science, Gibbstown, NJ) using phosphomolybdic acid/ethanol reagent, or a 10% solution of sulfuric acid in ethanol, and/or UV at 254 nm to visualize spots. Silica Gel 60 (40-63 μm) was used for flash chromatography.

Molecular cloning and heterologous gene expression. Standard molecular biology techniques were followed. The gene *iolG*, encoding inositol dehydrogenase, was PCR-amplified from *B. subtilis* 168 genomic DNA using oligonucleotide primers

5′-GGAGTGGCTGCATATGAGTTTACG-3′ and

5′-CGATACATGGCCGGATCCAAT-G-3′,

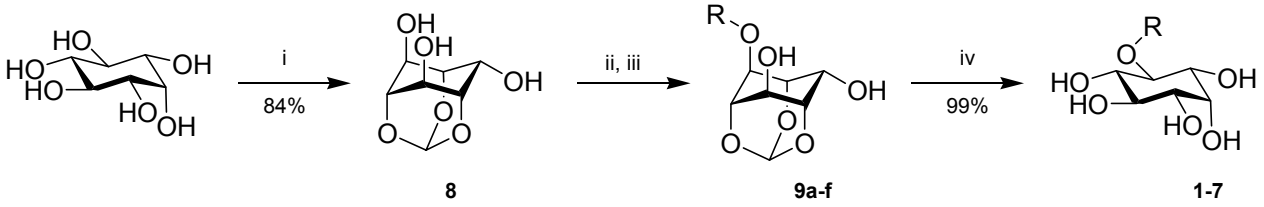
which incorporated *Nde* I and *Bam*H I restriction sites (underlined) at the *5′*- and *3′*-terminus respectively. The *iolG* gene was amplified with *Pfu* DNA polymerase using 30 cycles of the following protocol: 1 minute denaturation at 95 °C, 1 minute annealing at 58 °C, 2 minute extension at 72 °C. The PCR reaction (50 μl) contained 100 μM deoxynucleotide triphosphates, 2 mM $MgSO_4$, 50 ng *B. subtilis* genomic DNA, 50 ng of each oligonucleotide, and 2.5 U of polymerase. The 1082 base pair PCR product was isolated following agarose gel electrophoresis with the QIAquick Gel Extraction Kit as described by the manufacturer. The PCR product was ligated into pCR4blunt-TOPO with the Zero-Blunt TOPO PCR Cloning Kit for sequencing as described by the manufacturer. The resulting construct was used to transform electro-competent TOP10 *E. coli* and transformants selected using 50 μg/ml kanamycin. The plasmid was isolated from positive transformants and digested with *Nde* I and *Bam*H I. The DNA fragment containing *iolG* was isolated following agarose gel electrophoresis with the QIAquick Gel Extraction Kit as described by the manufacturer and was ligated to the 5369 bp *Nde* I/*Bam*H I fragment of the histidine tag prokaryotic protein expression vector pET-28b (Novagen). The resulting construct was designated pET-28b-IDH. Electro-competent XL1-Blue *E. coli* transformed with pET-28b-IDH were selected on LB agar supplemented with 50 μg/ml kanamycin. pET-28b-IDH was isolated from positive transformants and was digested with *Hin*d III to verify the *iolG* insertion. The *iolG* coding sequence of pET-28b-IDH was completely sequenced using an Applied Biosystems PRISM 3700 capillary electrophoresis DNA analyzer at the National Research Council Plant Biotechnology Institute DNA Technologies Unit.

Expression of iolG and Purification of IDH. E. coli BL21 (DE3) was transformed with the plasmid pET-28b-IDH. Transformants were grown at 37 °C in LB medium supplemented with 50 μg/ml kanamycin until the culture reached an OD_{595} of 1.1. Expression of *iolG* was induced by adding isopropyl β-D-thiogalactoside (IPTG) to a final concentration of 1

mM. Incubation was continued for a further 3 hours. The protein expression cell culture was pelleted, and the resulting cell pellet was lysed by sonication on ice in a volume of chilled buffer equivalent to 0.04 × the culture volume. The lysis buffer contained 20 mM sodium phosphate buffer, 10 mM imidazole, pH 7.4. The cells were disrupted by sonication, and the cellular debris was pelleted at 15,000 rpm for ten minutes using a JA-25.5 rotor. The supernatant was filtered, then passed directly onto a column of Chelating Sepharose FF (AP Biosciences) which had been charged with 4 column volumes 50 mM $NiSO_4$, followed by 3 column volumes of lysis buffer. The column was washed with 6 column volumes lysis buffer to remove endogenous protein, and the His tagged IDH was eluted with a 10-volume gradient of increasing imidazole concentration up to 500 mM. Fractions that were homogeneous by SDS-PAGE analysis were pooled and dialyzed overnight against 50 mM potassium phosphate buffer, pH 6.5, containing 0.1 mM 2-mercaptoethanol, previously reported as the conditions under which this enzyme is most stable. Protein concentration was determined spectrophotometrically using the Bradford method. Protein monomer molecular weight was measured by mass spectrometry.

Enzymatic Reactions and Kinetics – Assays were performed in 100 mM Tris-HCl pH 9.0. Activity was routinely assayed in the presence of 0.5 mM NAD^+ at 25 °C. The production of NADH was monitored at 340 nm (ε = 6.2 mM^{-1} cm^{-1}). Kinetic constants were determined using the program *Leonora* by fitting the data to the Michaelis-Menten equation, $v = V^{App}$ [S] / (K^{App} + [S]), where S = the varied substrate (e.g. inositol), V^{App} = the maximal velocity at the concentration of the fixed substrate (NAD^+), and K^{App} = the substrate concentration necessary for half-maximal velocity under these conditions.

The enzymatic reaction coupled to L-lactate dehydrogenase (LDH) was carried out in the following solution: **3c** (100 mM, 0.25 mL), NAD^+ (500 mM, 0.02 mL), pyruvate (11 mg), IDH (0.8 mg / mL, 0.22 mL), LDH (1U / mL, 0.02 mL) and Tris-HCl buffer (100 mM, pH 9.0, 0.69 mL). The enzymatic reaction was allowed to stay in the dark and at room temperature for 3 days. The enzyme was removed by ultrafiltration and the reaction purified by semi-preparative HPLC to give the 1.5 mg of product as a white solid after lyophylisation. NMR matched that of **3c** prepared by chemical synthesis. $[\alpha]_D$ = + 6 (c = 0.15, MeOH, 25 °C).



i. triethylorthoformate, toluene sulfonic acid, DMF, 100 °C, 16 h. ii. NaH, DMF, room temperature, 15 min. iii. alkyl halide or alkanesulfonyl chloride, room temperature, 16 h. iv. 80% Trifluoroacetic acid, room temperature, 5 h.

Synthesis of myo-*Inositol 1,3,5-monoorthoformate (**8**)*. Compound **8** was synthesized by the published method.[1] Spectroscopic characteristics matched those reported previously. In our hands, product could be isolated in 84% yield.

*General method for monoalkylation or monoalkanesulfonylation of **8***. Orthoformate **8** was stirred in dry DMF (20 mL / gram **1**) under Ar atmosphere, and NaH (60% oil dispersion, 1.1 equivalents) was added in one portion. The mixture was stirred at room temperature for 15 minutes, and then alkyl halide (1 equivalent) dissolved in minimal dry DMF was added slowly, and the solution stirred 16 hours at room temperature. The reaction was quenched with MeOH, and the reaction mixture was diluted with $CHCl_3$ (6 mL / mL reaction mixture), washed once with 0.1M HCl and once with saturated brine. The organic phase was dried over $MgSO_4$ and concentrated. The crude product was purified by flash chromatography.

4/6-O-*Methyl*-myo-*inositol 1,3,5-monoorthoformate (**9a**)*. To facilitate purification, **9a** was isolated by acetylation of the crude product after quenching of the reaction. After addition of MeOH to the reaction described above, using a 200 mg scale, the solvents were removed, and the yellow residue was dissolved in 1:1 pyridine/acetic acid (4 mL). After 2 hours at room temperature, the solvents were removed *in vacuo* and the residue was dissolved in ethyl acetate (5 mL), washed with water (2 × 2 mL), dried over $MgSO_4$ and concentrated. The peracetylated compound was diluted in 5 mL of methanol. After adding 100 mg of sodium (4.3 mmol), the mixture was stirred 5 hours at RT and neutralized with Dowex 50x8 (H^+ form) resin. The solution was filtered and concentrated to give 193 mg of **9a** (91% yield). ^{1}H NMR (D_2O) δ 3.47 (3H, s), 4.05 (1H, m), 4.10 (1H, m), 4.18 (1H, m) 4.28 (1H, m), 4.37 (1H, m), 4.45 (1H, m), 5.51(1H, s). ^{13}C NMR (D_2O) δ 59.9, 62.3, 69.2, 70.8, 74.2, 76.4, 77.9, 104.9.

4/6-O-*Allyl*-myo-*inositol 1,3,5-monoorthoformate (**9b**)*. This compound was prepared as described in the general procedure, on a 1 g scale, using allyl bromide as electrophile, isolated in 77% yield. NMR spectra matched those reported previously.[2]

4/6-O-*Benzyl*-myo-*inositol 1,3,5-monoorthoformate (**9c**)*. This compound was prepared as described in the general procedure on a 1 g scale, using benzyl bromide as electrophile, isolated in 88% yield. NMR spectra matched those reported previously.[2]

4/6-O-*para-(Methyloxycarbonyl)benzyl*-myo-*inositol 1,3,5-monoorthoformate* ***(9d)***. This compound was prepared as described in the general procedure on a 0.5 g scale, using methyl 4-(bromomethyl)benzoate as electrophile, isolated in 73% yield. ^{1}H NMR (500 MHz, $CDCl_3$) : δ 3.87 (s, 3H, Me), 4.02 (m, 1H), 4.15 (m, 1H), 4.22 (m, 1H), 4.25 (m, 1H), 4.36 (m, 1H), 4.42 (m, 1H), 4.65 (s, 2H, CH_2 Bn), 5.38 (s, 1H, H orthoformate), 7.31 (d, *J* = 8.2Hz, 2H, H aromatic), 7.99 (d, *J* = 8.2Hz, 2H, H aromatic). ^{13}C NMR (125 MHz, $CDCl_3$) : δ 52.6 (Me), 61.0, 67.6, 68.2, 72.5, 72.7, 75.0 (CH_2 Bn), 75.1, 102.8(C orthoformate), 128.1, 130.6, 131.0, 141.1, 169.7.

4/6-O-*[(1*S*)-10-Camphorsulfonyl]*-myo-*inositol 1,3,5-monoorthoformate* ***(9e)***. This compound was prepared as described above on a 380 mg scale, isolated in 40% yield. ^{1}H ($CDCl_3$) δ: 0.81 (s, 6H, camphor), 0.99 (s, 3H, camphor), 1.0 (s, 3H, camphor), 1.42 (m, 2H, camphor), 1.66 (m, 2H, camphor), 1.89 (d, *J* = 2.8Hz, 1H, camphor), 1.93 (d, *J* = 2.8Hz, 1H, camphor), 2.00 (m, 2H, camphor), 2.09 (m, 2H, camphor), 2.3 (m, 4H, camphor), 3.00 (d, *J* = 15.1Hz, 1H, CH_2 camphor), 3.02 (d, *J* = 15.1Hz, 1H, CH_2 camphor), 3.55 (d, *J* = 15.1Hz, 1H, CH_2 camphor), 3.58 (d, *J* = 15.1Hz, 1H, CH_2 camphor), 4.02 (m, 1H), 4.10 (m, 1H), 4.18 (m, 2H), 4.27 (m, 1H), 4.31 (m, 1H), 4.47 (m, 2H), 4.57 (m, 2H), 5.43 (m, 4H). ^{13}C NMR ($CDCl_3$): δ 19.9, 19.95, 20.09, 20.11, 25.52, 25.58, 27.3, 43.0, 43.1, 49.0, 49.1, 49.2, 49.3, 58.6, 58.7, 60.65, 60.70, 67.5, 67.6, 68.9, 69.1, 72.3, 72.4, 72.7, 72.9, 74.2, 74.4, 103.22, 103.24, 128.6, 129.4, 215.4, 216.0.

myo-*inositol 1,3,5-monoorthoformate 4/6-phosphate, dibenzyl ester* ***(9f)***. This compound was prepared following the published procedure.[2] NMR spectra matched those reported previously.

General method for hydrolysis of the orthoformate group. The monoorthoformate was stirred in 80% trifluoroacetic acid (30 mL / g monoorthoformate) for 5 hours at room temperature. The solvent was removed by rotary evaporation at 45 °C. The residue was extracted into water, then lyophilized to obtain a white solid, in quantitative yield.

4/6-O-*Methyl*-myo-*inositol* ***(1)***. ^{1}H (D_2O) δ 3.27 (m, 2H), 3.40 (dd, *J* = 10.0 Hz, *J* = 2.7 Hz, 1H, H-1), 3.50 (s, 3H), 3.52 (m, 2H), 3.95 (t, 1H, H-2). ^{13}C NMR (D_2O) δ 60.2, 71.0, 71.4, 72.6, 72.9, 74.1, 82.9.

4/6-O-*Allyl*-myo-*inositol* ***(2)***. ^{1}H NMR (D_2O) δ 3.37 (t, *J* = 9.3 Hz, 1H, H-6), 3.45 (m, 4H), 3.91 (t, *J* = 2.7 Hz, 1H, H-2), 4.18 (d, *J* = 12 Hz, 2H, CH_2 allyl), 5.12 (m, 1H), 5.22 (m, 1H), 5.87 (m, 1H). ^{13}C NMR (D_2O) : δ 71.2, 71.4, 72.7, 72.8, 74.3 (d), 81.05, 118.8, 134.7.

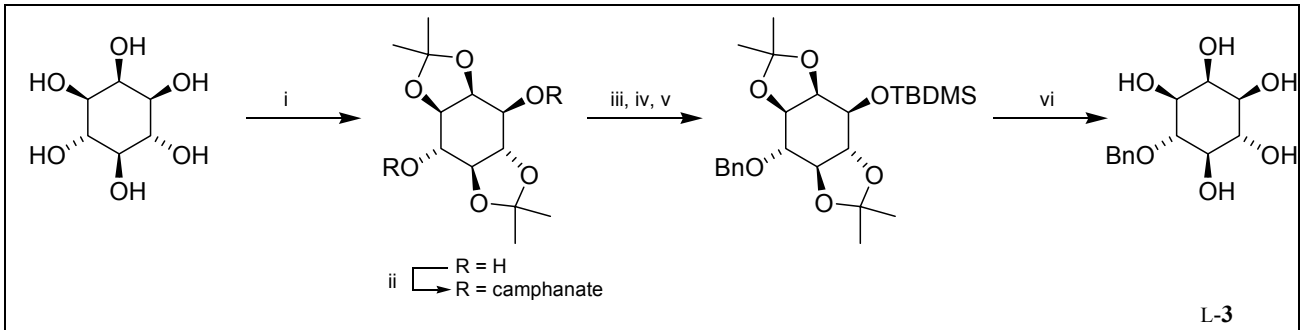
4/6-O-*Benzyl*-myo-*inositol* ***(3)***. ^{1}H NMR (D_2O) δ 3.37 (t, *J* = 9.0 Hz, 1H, H-6), 3.49 (dd, *J* = 9.0 Hz, *J* = 3.9 Hz, 1H, H-1), 3.63 (m, 3H), 4.04 (t, *J* = 2.7 Hz, 1H, H-2), 4.83 (s, 2H, CH_2 benzyl), 7.36-7.51 (m, 5H, Ar). ^{13}C NMR (D_2O) δ 71.3, 71.4, 72.8, 72.9, 74.4, 75.4, 81.6, 128.7, 129.0, 129.2, 138.0.

4/6-O-*para-(Methyloxycarbonyl)benzyl*-myo-*inositol* ***(4)***. ^{1}H NMR (500 MHz, D_2O) : δ 3.30 (t, *J* = 9.0Hz, 1H, H-6), 3.41 (dd, *J* = 9.0Hz, *J* = 3.9Hz, 1H, H-1), 3.55 (m, 3H), 3.82 (s, 3H, Me), 3.95 (m, 1H, H-2), 4.81 (s, 2H, CH_2 Bn), 7.47 (d, *J* = 8.2Hz, 2H, H aromatic), 7.90 (d, *J* = 8.2Hz, 2H, H aromatic). ^{13}C NMR (125 MHz, D_2O) : δ 53.0 (Me), 71.3, 71.4, 72.8, 72.9, 74.4, 74.6 (CH_2 Bn), 82.0, 128.7, 129.4, 130.0, 143.9, 169.7.

4/6-O-*para-carboxybenzyl*-myo-*inositol* ***(5)***. Methyl ester **4** (600 mg, 1.77 mmol) was suspended in 1N NaOH (20 mL) and the mixture was refluxed for 1hour. After cooling to room temperature, the solution was acidified using Dowex H^+ resin and stirred overnight. The resin was filtered off and the filtrate was concentrated to afford the title compound **5** (550 mg, 99%) as a white solid. ^{1}H NMR (500 MHz, D_2O) : δ 3.30 (t, *J* = 9.0Hz, 1H, H-6), 3.41 (dd, *J* = 9.0Hz, *J* = 3.9Hz, 1H, H-1), 3.55 (m, 3H), 3.95 (m, 1H, H-2), 4.81 (s, 2H, CH_2 Bn), 7.47 (d, *J* = 8.2Hz, 2H, H aromatic), 7.90 (d, *J* = 8.2Hz, 2H, H aromatic). ^{13}C NMR (125 MHz, D_2O) : δ 71.3, 71.4, 72.8, 72.9, 74.4, 74.6 (CH_2 Bn), 82.0, 128.7, 129.9, 130.2, 143.8, 171.1.

4/6-O-*[(1*S*)-10-Camphorsulfonyl]*-myo-*inositol* ***(6)***. ^{1}H NMR (500 MHz, D_2O) : δ 0.89 (s, 3H, camphor), 0.90 (s, 3H, camphor), 1.07 (s, 3H, camphor), 1.08 (s, 3H, camphor), 1.52 (m, 2H, camphor), 1.76 (m, 2H, camphor), 2.04 (s, 1H, camphor), 2.08 (s, 1H, camphor), 2.11 (m, 2H, camphor), 2.24 (t, 2H, camphor), 2.36 (m, 2H, camphor), 2.50 (m, 2H, camphor), 3.54-3.66 (m, 6H), 3.72 (t, *J* = 9.7Hz, 2H), 3.79-3.87 (m, 4H), 4.12 (m, 2H), 4.73 (t, *J* = 9.5Hz, 2H, H-4). ^{13}C NMR (125 MHz, D_2O) : δ 18.9(d), 19.2(d), 25.6, 25.7, 26.5(d), 42.8(t), 42.9, 48.9, 49.0, 49.8, 49.7, 58.8, 58.85, 69.3, 69.4, 71.1(d), 72.4, 72.3, 72.6, 72.7(t), 85.7, 85.8. ESI-MS : $[M+Na]^+$= 417.1.

myo-*inositol 4/6-phosphate* ***(7)***. After following the general procedure, the benzyl groups were removed by hydrogenolysis following the published procedure.[2]



L-**3**

i. 2,2-dimethoxypropane, tolenesufonic acid, DMF. ii. (-)-camphanic chloride, pyridine; diastereomers separated by flash chromatography. iii. NaOMe, MeOH. iv. TBDMS-Cl, imidazole, DMF. v. NaH, BnBr, DMF. vi. 80% trifluoroacetic acid.

1L-4-O-*benzyl*- myo-*inositol (L-**3**)*. myo-Inositol was converted (using a 25 g scale) to racemic 1,2:5,6-di-*O*-isopropylidene-*myo*-inositol by the method of Khersonsky and Chang,[3] and 1D-1,2:5,6-di-*O*-isopropylidene-*myo*-inositol was isolated by resolution of the biscamphanic ester following the published procedure.[4] Silylation to form the 3-*O*-*tert*-butyldimethylsilyl derivative was performed as described by Chung and Ryu, and the resulting compound treated with benzyl bromide,[5] and finally deprotected to give **L-3c** in an overall yield of 2% from *myo*-inositol. ^{1}H and ^{13}C spectra matched those reported above, and reported previously.[6]

---

[1] D.C. Billington et al., *J. Chem. Soc. Perkin Trans. 1*, 1989, 1423-1429.

[2] D.C. Billington, UK patent application GB 2 206 581 A, (1989) 21 pp.

[3] S.M. Kheronsky, Y.-T. Chang, *Carbohydr. Res.* 2002, **337**, 75-78.

[4] S.-K. Chung, B.-G. Shin, Y.-T. Chang, B.-C. Suh, K.-T. Kim, *Bioorg. Med. Chem. Lett.* 1998, **8**, 659-662.

[5] S.-K. Chung, Y. Ryu, *Carbohydr. Res.* 1994, **258**, 145-167.

[6] P.J. Garegg, B. Lindberg, I. Kvarnström, S.C.T. Svensson, *Carbohydr. Res.* 1985, **139**, 217-223.