Supporting Information

Affinity Capture and Elution/Electrospray Ionization Mass Spectrometry (ACESIMS) Assay of Phosphomannomutase and Phosphomannose Isomerase for the Multiplex Analysis of Congenital Disorders of Glycosylation Types Ia and Ib

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Synthetic Procedures

N-(N'-Methyl-N'-biotinylglycyl)-2-aminoethyl dihydrogenphosphate (**1**). N-Methyl-N-biotinylglycine (*S1*) (200 mg, 0.63 mmol) was mixed with N-hydroxyl succinimide (0.75 mmol), 1,3-dicyclohexylcarbodiimide (0.75 mmol) and 4 mL of N,N-dimethylformamide. The reaction was stirred at room temperature under argon for 9 hours, and solvent was removed under reduced pressure. 2-Aminoethyldihydrogenphosphate (1.25 mmol, Aldrich) was converted to the Na+ form by dissolution in water, addition of two equivalents of NaOH, and removal of water under reduced pressure. The di-sodium 2-aminoethyl phosphate was added to the reaction residue followed by the addition of 12 mL of formamide (dried over 4A molecular sieves). The mixture

was stirred at room temperature for 3 hours under argon, and the product was purified by flash chromatography on silica gel (2.5 x 25 cm, column washed with 400 mLof $CHCl_3/CH_3OH$ (1/1), then the product was eluted with 1 L of $CHCl_3/CH_3OH$/concentrated NH_4OH (1/1/0.25)). The product was further purified by HPLC (C18, Vydac 218TP1022) using a gradient of solvent A (0.05% trifluoroacetic acid in water) and solvent B (methanol) as follows: 0-30 min, 0% B; 30-90 min, 0-40% B. The product eluted at 63.0 min. To convert to the acid form, product-containing fractions were pooled, solvent was removed by rotary evaporation, and the residue was dissolved in water and passed through a column of cation exchange resin (Bio-Rad, AG50W-X2, H^+) to yield 168 mg (61%) of **1** as white solid. ^{1}H-NMR (499 MHz, CD_3OD): δ 4.60-4.64 (m, 1H), 4.42-4.46 (m, 1H), 4.14 and 4.29 (s + s, 2H; rotamers), 3.95-4.08 (m, 2H), 3.42-3.56 (m, 2H), 3.28-3.39 (m, 1H), 3.15 and 2.95 (s + s, 3H; rotamers), 2.95-3.05 (dd, 1H), 2.78-2.82 (d, 1H), 2.50 and 2.33 (t + t, 2H; rotamers), 1.40-1.82 (m, 6H). ESI-MS, *m/z* 437.3 $(M - H)^-$.

N-(N'-Methyl-N'-biotinylglycyl)-3-aminopropyl dihydrogenphosphate (**2**). Compound **2** was prepared as for **1** except that 3-aminopropyl phosphate was used. The latter was made by method of Touet et al. (*S2*) except that the purification was completed by passing an aqueous solution of the product through a cation exchange resin (Amberlite IR 120, H^+ form) instead of by crystallization. N-Methyl-N-biotinylglycine (200 mg) was used to yield 186 mg of **2** (62%). ^{1}H-NMR (499 MHz, CD_3OD): δ 4.60-4.64 (m, 1H), 4.42-4.46 (m, 1H), 4.14 and 4.29 (s + s, 2H; rotamers), 3.95-4.08 (m, 2H), 3.32-3.42 (m, 3H), 3.15 and 2.95 (s + s, 3H; rotamers), 2.95-3.05 (dd, 1H), 2.78-2.82 (d, 1H), 2.50 and 2.33 (t + t, 2H; rotamers) 1.40-1.92 (m, 8H). ESI-MS, *m/z* 451.3 $(M - H)^-$.

N-(N'-Methyl-N'-biotinylglycyl) 2-aminoethyl phosphoromorpholidate (**3**). The morpholidate derived from compound **1** was prepared by a modification of the published procedure (*S3*). Dicyclohexylcarbodiimide (1.61 mmol) was dissolved in *t*-butyl alcohol (8 mL) and added in small portions by syringe over 4 h to a refluxing solution of **1** (160 mg, 0.365 mmol) in a mixture of water (4 mL), *t*-butyl alcohol (4 mL) and morpholine (1.61 mmol). The mixture was refluxed for an additional several hours (typically 9-12 h) until no starting material was detected by thin layer chromatography (silica, isopropyl alcohol/H_2O/concentrated NH_4OH, 3/1/0.5, R_f ~0.35 for starting material). The mixture was cooled to room temperature, and most of *t*-butyl alcohol was removed under reduced pressure. The residue was dissolved in 6 mL of water, and the solvent was reduced to 4 mL under reduced pressure. After filtration through a glass wool-plugged Pasteur pipet, the filtrate was extracted with ethyl ether (4 × 2 mL), and the aqueous layer was concentrated in vacuo to give a glassy residue. The residue was dissolved in a minimum amount of CH_3OH (~0.2 mL). After addition of dry ether (5 mL), a gummy solid precipitated, which changed to a white powder upon trituration with additional dry ethyl ether. The product was dried in vacuo at room temperature to yield 250 mg of **3** (white powder) as the 4-morpholine N, N'-dicyclohexyl-carboxamidine (MDCC) salt in a mixture with additional MDCC (based on ^{1}H-NMR), yield ~65%. ESI-MS, *m/z* 506.4 $(M - MDCC)^-$.

N-(N'-Methyl-N'-biotinylglycyl) 3-aminopropyl phosphoromorpholidate (**4**). Starting from compound **2**, **4** was made as for **3** (yield ~65%). ESI-MS, *m/z* 520.3 $(M - MDCC)^-$.

Substrate conjugate S_{PMI}. Compound **3** was reacted with ribose-5-phosphate by a modification of the published procedure (*S4*). An aqueous solution of disodium D-ribose-5-phosphate (0.196

mmol, Sigma) was passed through a cation-exchange resin (AG50W-X2, pyridinium form, Bio-Rad) to give the pyridinium salt (complete elution of material required washing the column with about four column volumes of water). The eluate was co-evaporated with methanol to concentrate to about 2 mL at room temperature. Tri-*n*-octylamine (0.305 mmol, Aldrich) and D-ribose (1.60 mmol, Sigma) were added to the D-ribose-5-phosphate solution, and the solvent was removed under reduced pressure. D-ribose was added to scavenge the morpholine released during coupling of 3 with ribose-5-phosphate to prevent it from reacting with the hemiacetal group of S_{PMI}. To ensure complete dryness of the mixture, the sample was dissolved in anhydrous pyridine and dried in vacuo; this was repeated four times. Finally, the residue was dried in vacuo over P_2O_5 for two days. It was re-dissolved in anhydrous pyridine (4 mL) and added to dry **3** (125 mg, 0.16 mmol, dried by two-times addition of anhydrous pyridine and solvent removal in vacuo). The mixture was again dissolved in anhydrous pyridine (4 mL) and evaporated it in vacuo; this was quickly repeated three times. Finally, to the mixture was added 4 mL of anhydrous pyridine, and the reaction was stirred at room temperature under argon for 24 h. The solvent was removed in vacuo, and to the residue was added 8 mL of water/ether (1/1) containing NaCl (2 equivalents based on the sum of tri-*n*-octylamine and MDCC). The water layer was washed with ether (4 × 1.5 mL), the combined ether washes were back extracted with 0.5 mL water, and the water layers were combined and concentrated in vacuo to ~2.0 mL. The product was purified by HPLC (C18) using solvent C (0.05 M aqueous NH_4HCO_3) and solvent D (methanol): 0-5 min 0% D; 5-80 min, 0-15% D. The product eluted at 60.5 min. Product fractions were pooled and lyophilized, water was added and the lyophilization was repeated two times to remove NH_4HCO_3. This gave 31 mg of light yellow solid (30%). ^{1}H-NMR (499 MHz CD_3OD): δ 5.15 and 5.35 (m + m, 1H; anomers), 4.60-4.64 (m, 1H), 4.42-4.46 (m, 1H), 3.80-

4.30 (m, 9H), 3.42-3.56 (m, 2H), 3.28-3.39 (m, 1H), 3.15 and 2.95 (s + s, 3H; rotamers), 2.95-3.05 (dd, 1H), 2.78-2.82 (d, 1H), 2.50 and 2.33 (t + t, 2H; rotamers) 1.40-1.82 (m, 6H). ESI-MS, *m/z* 649.3 $(M - H)^-$.

Substrate conjugate **S_{PMM}**. This was prepared as for **S_{PMI}**. The yield was 35% and the product eluted from the HPLC column at 63.7 min. IH-NMR (499 MHz, CD_3OD): δ 5.15 and 5.35 (m + m, 1H; anomers), 4.60-4.64 (m, 1H), 4.42-4.46 (m, 1H), 3.80-4.30 (m, 9H), 3.28-3.41 (m, 3H), 3.15 and 2.95 (s + s, 3H; rotamers), 2.95-3.05 (dd, 1H), 2.78-2.82 (d, IH), 2.50 and 2.33 (t + t, 2H; rotamers), 1.40-1.92 (m, 8H). ESI-MS, *m/z* 663.3 $(M - H)^-$.

Internal standard conjugate **IS_{PMI}**. Isotopically labeled **S_{PMI}** was prepared from compound **1** containing an N-(methyl-d_3)glycyl-l-^{13}C moiety (prepared as described by Zhou et al.(*S1*)), as described above **S_{PMI}**. **IS_{PMI}** was prepared enzymatically from labeled **S_{PMI}** as follows. Labeled **S_{PMI}** (5 mg) was added to a mixture (10 mL) containing $MgCl_2$ (3 mM), TPP (2 mM), L-erythrulose (3 mM) and NADH (3 mM) in 50 mM Tris-HCl buffer, pH 7.5. TK (3 U) and alcohol dehydrogenase (3 U, Sigma Cat. A3263) were added to the mixture. The solution was stirred at 25°C for 10 h. The product was purified by HPLC using the same gradient conditions as for **S_{PMI}** and eluted at 58.3 min. Product fractions were pooled and lyophilized, water was added and the lyophilization was repeated 2 times to remove NH_4HCO_3. This gave 3.5 mg of light yellow solid (65%). ^{1}H-NMR (499 MHz, CD_3OD): δ 4.60-4.64 (m, 1H), 4.42-4.46 (m, 1H), 3.60-4.35 (m, 10H), 3.42-3.54 (m, 4H), 3.28-3.39 (m, 1H), 2.95-3.05 (dd, 1H), 2.78-2.82 (d, 1H), 2.50 and 2.33 (t + t, 2H; rotamers), 1.40-1.82 (m, 6H). ESI-MS, *m/z* 713 $(M - H)^-$.

Internal standard conjugate $\mathbf{IS_{PMM}}$. It was prepared as for $\mathbf{IS_{PMI}}$. The yield was 62% and the product eluted from the HPLC column at 61.4 min. ^{1}H-NMR (499 MHz, CD_3OD): δ 4.60-4.64 (m, 1H), 4.42-4.46 (m, 1H), 3.55-4.30 (m, 10H), 3.50-3.56 (m, 2H), 3.28-3.41 (m, 3H), 2.95-3.05 (dd, 1H), 2.78-2.82 (d, 1H), 2.50 and 2.33 (t + t, 2H; rotamers), 1.40-1.92 (m, 8H). ESI-MS, *m/z* 727.3 $(M - H)^-$.

References

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Figure Captions

Figure S1. Elution profile of $\mathbf{S_{PMI}}$ measured as an extracted ion chromatogram for the $(M - H)^-$ ion at *m/z* 649 from an assay of a cellular extract from a healthy donor.

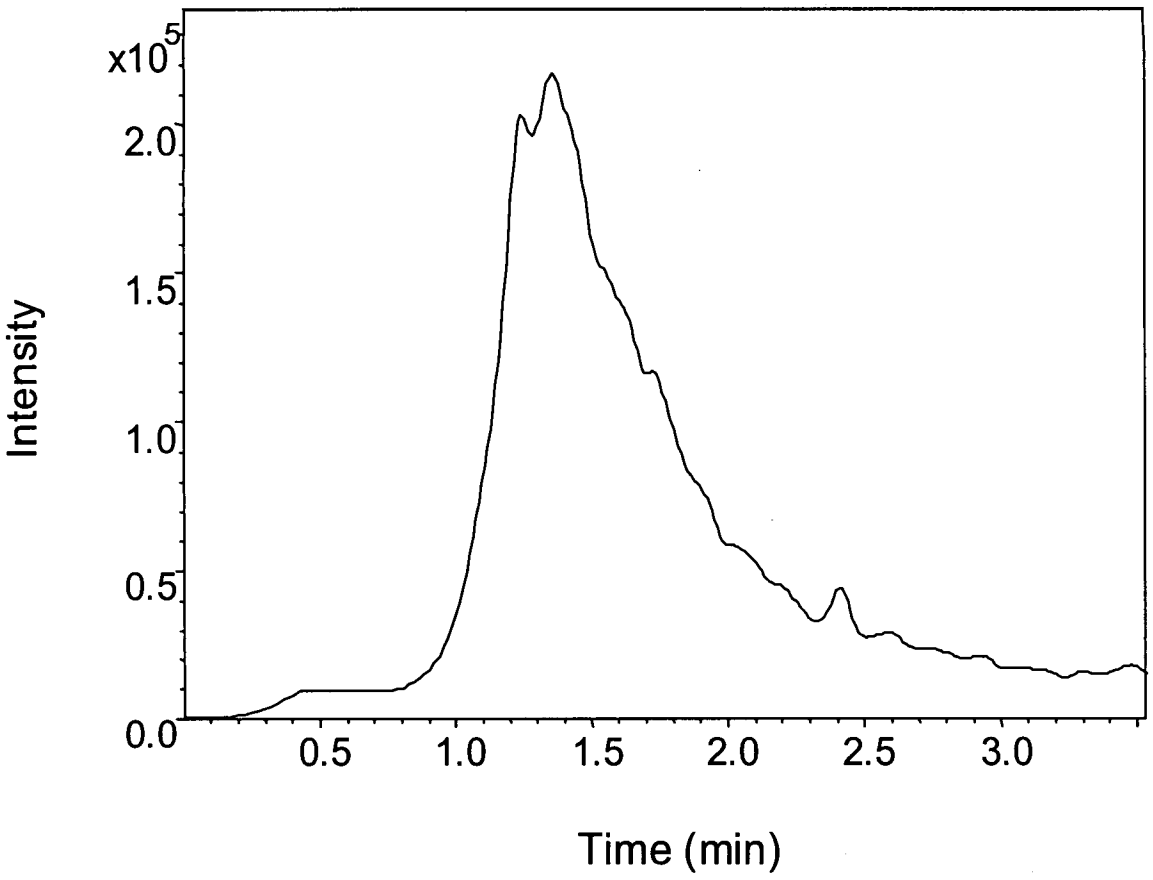

Figure S1.