Supporting Information

Stereochemistry in the Reaction of the *myo*-Inositol Phosphate Synthase Ortholog Ari2 during Aristeromycin Biosynthesis

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Experimental details

General procedure

All commercial reagents derived from TCI, Kanto Chemical and Sigma Aldrich were used as provided unless otherwise indicated. ^{1}H- and ^{13}C-NMR spectra were recorded with a Bruker DRX500 spectrometer or JEOL ECX400 spectrometer. FAB-MS spectra were recorded with a JEOL JMS700 MStation.

Feeding experiments

Streptomyces citricolor NBRC 13005 was maintained on a plate of ISP-2 agar (0.4% yeast extract, 1% malt extract, glucose 0.4%, agar 2.0%, pH was adjusted to 7.3 with NaOH) at 28 °C. D-[6,6-$^{2}H_2$], (6*S*)-D-[6-$^{2}H_1$], and (6*R*)-D-[6-$^{2}H_1$]Glucose were prepared according to the reported method.[1] All of glucose in the ISP-2 medium was replaced with the deuterium labeled glucose for the feeding experiments. Two plates containing 15 mL agar medium (total 30 mL) were used for each feeding experiment. After incubation for 7 days at 28 °C, the solid culture was grinded in mortar with pestle. The grinded culture was then suspended in water and stirred overnight. After centrifugation, the obtained supernatant was loaded on Dowex 50W X-8 (H^+ form) and washed with water. The bound cationic compounds were eluted with 4 M of NH_4OH and fractionated. The UV-positive fractions were combined and the solvent was evaporated. The residue was dissolved in water and further purified with HPLC system with a Chromaster 5110 Pump (Hitachi), a 996 Photodiode Array Detector (Waters), and a column oven L-7300 (Hitachi) at 40 °C. Senshu Pak ODS SP100, 20ø x 150 mm (Senshu, Japan) with 14% CH_3OH at a flow rate of 4.0 mL/min was used for the first separation. Further, YMC-Triart PFP, 10ø x 150 mm (YMC, Japan) with 14% CH_3OH at a flow rate of 3.0 mL/min was used for the second separation to yield 0.6 mg, 0.5 mg, and 0.8 mg of aristeromycin from D-[6,6-$^{2}H_2$], (6*S*)-D-[6-$^{2}H_1$], and (6*R*)-D-[6-$^{2}H_1$]glucose, respectively. NMR spectra were recorded on a Bruker DRX500 spectrometer. ^{1}H-NMR spectra were analyzed in D_2O (99.8 atom% enriched, Kanto Chemical) and ^{2}H-

NMR spectra were analyzed in deuterium-depleted water (≤ 1 ppm, ISOTEC). Chemical shifts are reported in ppm relative to the solvent peaks (4.7 ppm for water).

Ari2 reaction analysis

To prepare (6*S*)-D-[6-$^2H_1$] and (6*R*)-D-[6-$^2H_1$]glucose 6-phosphate, 2 mM of (6*S*)-D-[6-$^2H_1$] or (6*R*)-D-[6-$^2H_1$]glucose, 2.5 mM of ATP, 5 U of hexokinase (Sigma) in the 50 mM of Tris buffer (pH 8.5) containing 100 mM of KCl and 6.5 mM of $MgCl_2$ were reacted at 28°C overnight. The reaction volume was 60 ml that was divided to 12 tubes (5.0 ml each tube). The resultant solution was transferred to the centrifugal filter (Amicon Ultra, 10K) and proteins were removed at 5,000 *g* for 20 min. The obtained solution was loaded on a DOWEX AG1-X8 column (HCO_2^- form, 25 mL), and the column was washed with water (100 mL) and the enzyme reaction product was eluted with 1.5 M of formic acid and fractionated in 10 mL each fraction. After TLC analysis of each fraction with a developing solution H_2O:CH_3OH:$CHCl_3$ = 1:5:5, several positive fractions with *p*-anisaldehyde stain reagent (Rf = 0.2) were combined and the solvent was removed by rotary evaporator to obtain (6*S*)-D-[6-$^2H_1$]G6P (25.6 mg, 82.1%) and (6*R*)-D-[6-$^2H_1$]G6P (20.8 mg, 66.7%).

Ari2 was prepared according to our previous report.[2] The Ari2 reactions were conducted under the following conditions: 0.5 mM of (6*S*)-D-[6-$^2H_1$]G6P or (6*R*)-D-[6-$^2H_1$]G6P, 0.1 mM NAD^+, 1 mM NH_4Cl, 1 mM $MgCl_2$, 1 U of phosphoglucose isomerase (Sigma) and 25 μM Ari2 at 28 °C overnight. The reaction volume was 70 ml that was divided to 70 tubes (1.0 mL each tube). After incubation, final 5 μM of 2-deoxy-*scyllo*-inosose (2DOI) synthase, BtrC[3] was added and incubated further at 28 °C overnight to consume G6P. The resultant solution was transferred to the centrifugal filter (Amicon Ultra, 10K) and proteins were removed at 5,000 *g* for 20 min. The obtained solution was loaded on a DOWEX AG1-X8 column (HCO_2^- form), and the column was washed with water and 0.75 M of formic acid to remove NAD^+. The enzyme reaction product was then eluted with 1 M of formic acid and fractionated. After TLC analysis of each fraction with a developing solution H_2O:CH_3OH:$CHCl_3$ = 1:5:5, the positive fractions with *p*-anisaldehyde stain reagent (Rf = 0.2) were combined and the solvent was removed by rotary evaporator to obtain the Ari2 reaction products.

Crystallization, Data Collection and Structural Determination. Ari2 crystals were grown from a 1:1 mixture of a protein solution (10 mg mL^{-1} in 10 mM Tris-HCl (pH 8.0), 10% glycerol and 1 mM NAD^+) and a reservoir solution containing 0.4 M NaCl, 0.2 M Tris-HCl (pH 8.0) and 1.0 M sodium citrate using the sitting-drop vapor diffusion method at 5 °C. Prior to collection of the X-ray data, the crystals were soaked in reservoir solution containing 25% (v/v) glycerol as a cryoprotectant and flash-frozen in a stream of liquid nitrogen. The X-ray diffraction data were collected on a beamline AR-NE3A at the Photon Factory (Tsukuba, Japan) and were subsequently indexed, integrated, and scaled using the iMosflm program.[4,5] The initial phase was determined by molecular replacement using the Molrep program[6] with the MIPS structure (PDB code: 1GR0)[7] as a search model. The structural model of Ari2 was manually constructed with Coot.[8] Refmac[9] was used for refinement of the structures. The non-crystallographic symmetry was applied as restraints in the refinement. The structural representations were prepared with PyMOL (DeLano Scientific, Palo Alto, CA, USA). The geometries of the final

structure were evaluated using the program MolProbity.[10] The type of metal ion was assigned as Na^+ based on Metal Binding Site Validation Server.[11,12] The resulting coordinates and structure factors have been deposited in the Protein Data Bank (PDB code: 6K96).

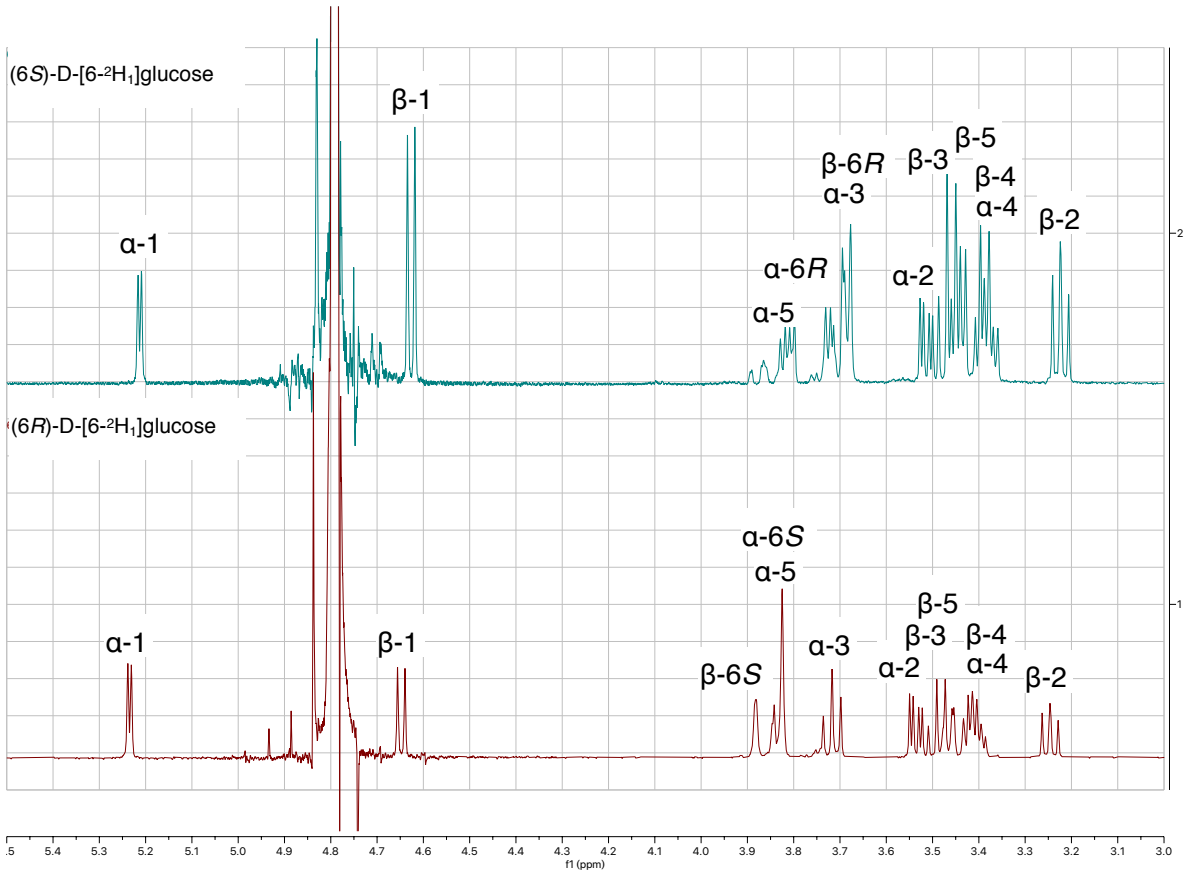


Figure S1. ^{1}H-NMR of (6*S*)-D-[6-$^2H_1$]glucose and (6*R*)-D-[6-$^2H_1$]glucose (500 MHz, in D_2O).

These data are identical to the ^{1}H-NMR data of (6*S*)-D-[6-$^2H_1$]glucose and (6*R*)-D-[6-$^2H_1$]glucose in literature.[1]

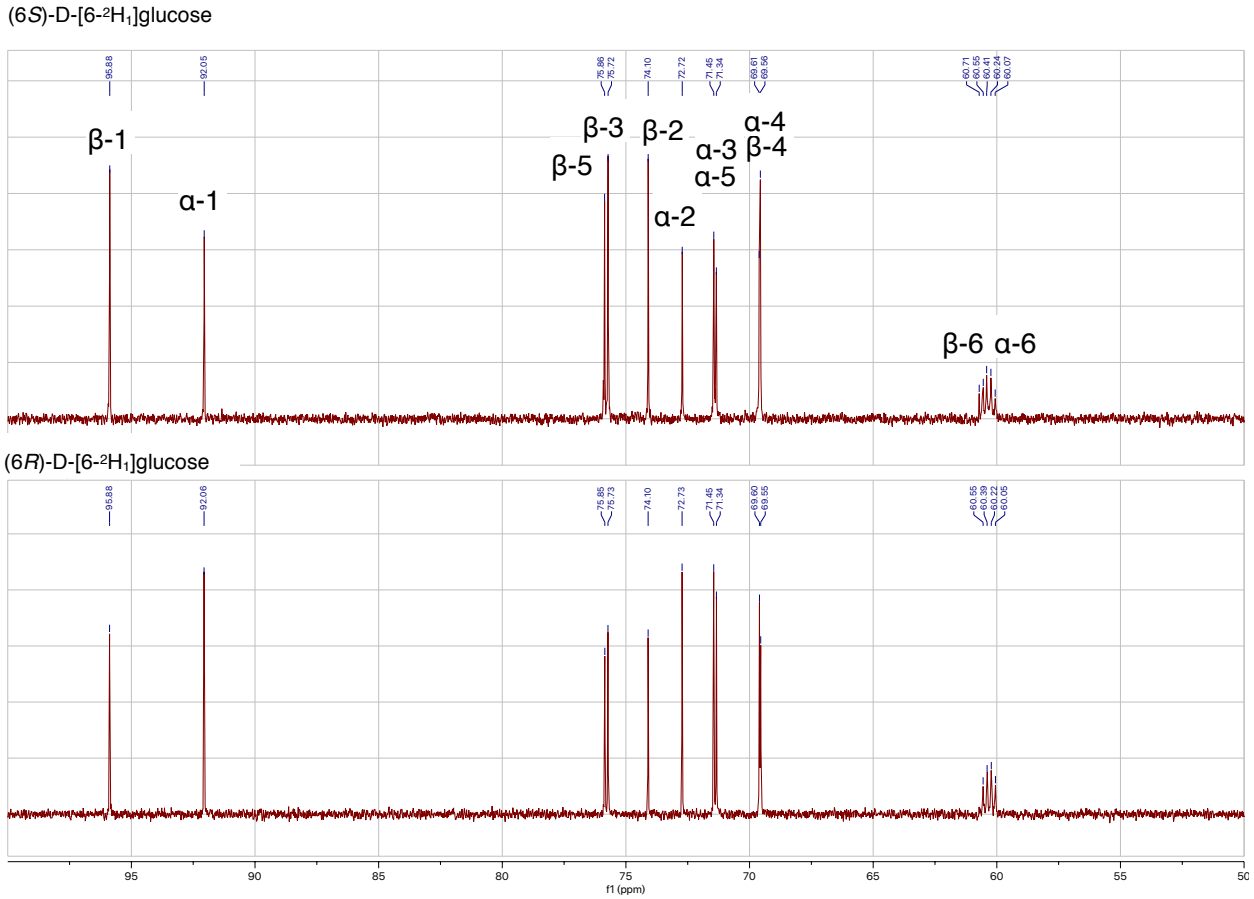
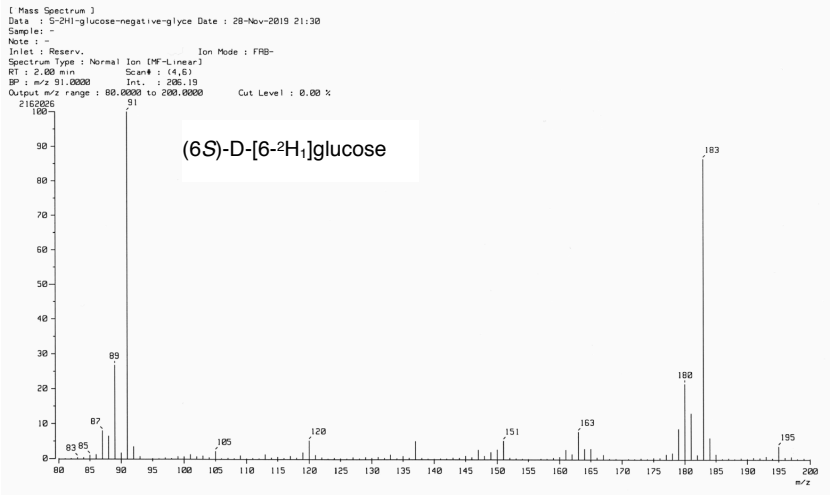
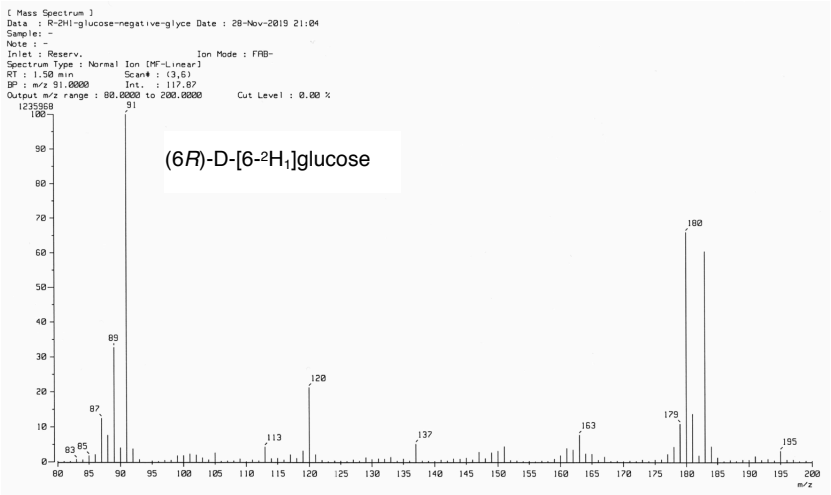


Figure S2. ^{13}C-NMR of (6*S*)-D-[6-$^{2}H_1$]glucose and (6*R*)-D-[6-$^{2}H_1$]glucose (125 MHz, in D_2O).





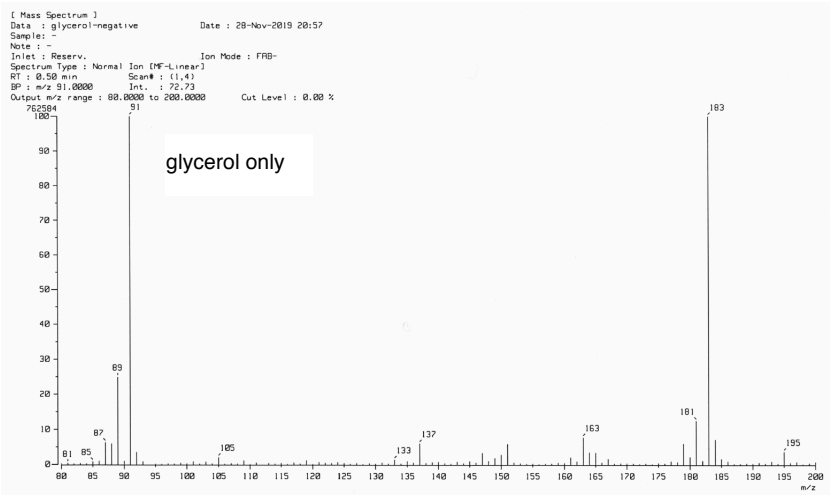


Figure S3. FAB-MS spectra (glycerol matrix, negative mode) of (6*S*)-D-[6-$^2H_1$]glucose, (6*R*)-D-[6-$^2H_1$]glucose and glycerol only.

Based on the intensities of *m*/*z* 179 for non-labeled glucose and *m*/*z* 180 for mono-deuterium-labeled glucoses, the deuterium enrichment in (6*S*)-D-[6-$^2H_1$]glucose and (6*R*)-D-[6-$^2H_1$]glucose were estimated to be approximately 80% and 90%, respectively.

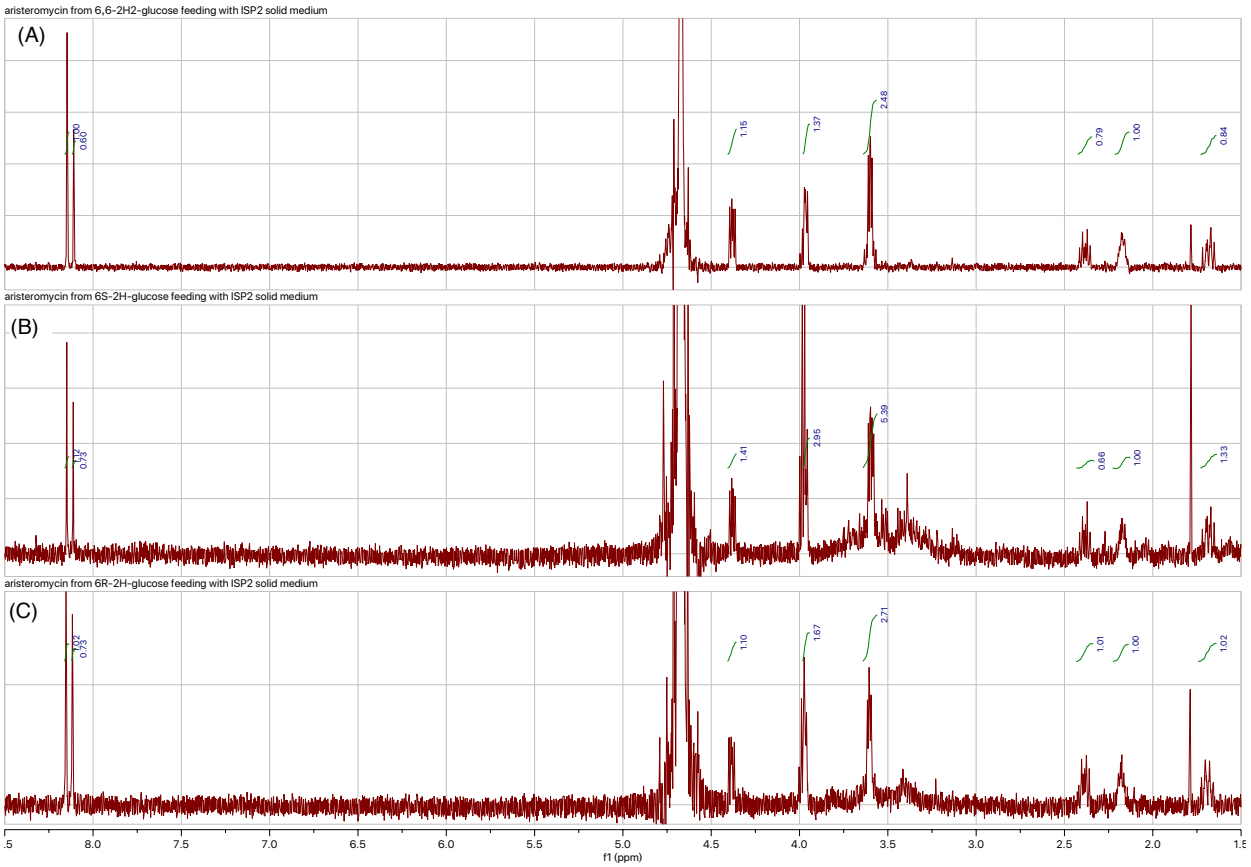


Figure S4. ^{1}H-NMR of aristeromycins isolated from the solid cultures with D-[6,6-$^{2}H_2$]glucose (A), (6*S*)-D-[6-$^{2}H_1$]glucose (B) and (6*R*)-D-[6-$^{2}H_1$]glucose (C) (500 MHz, in D_2O).

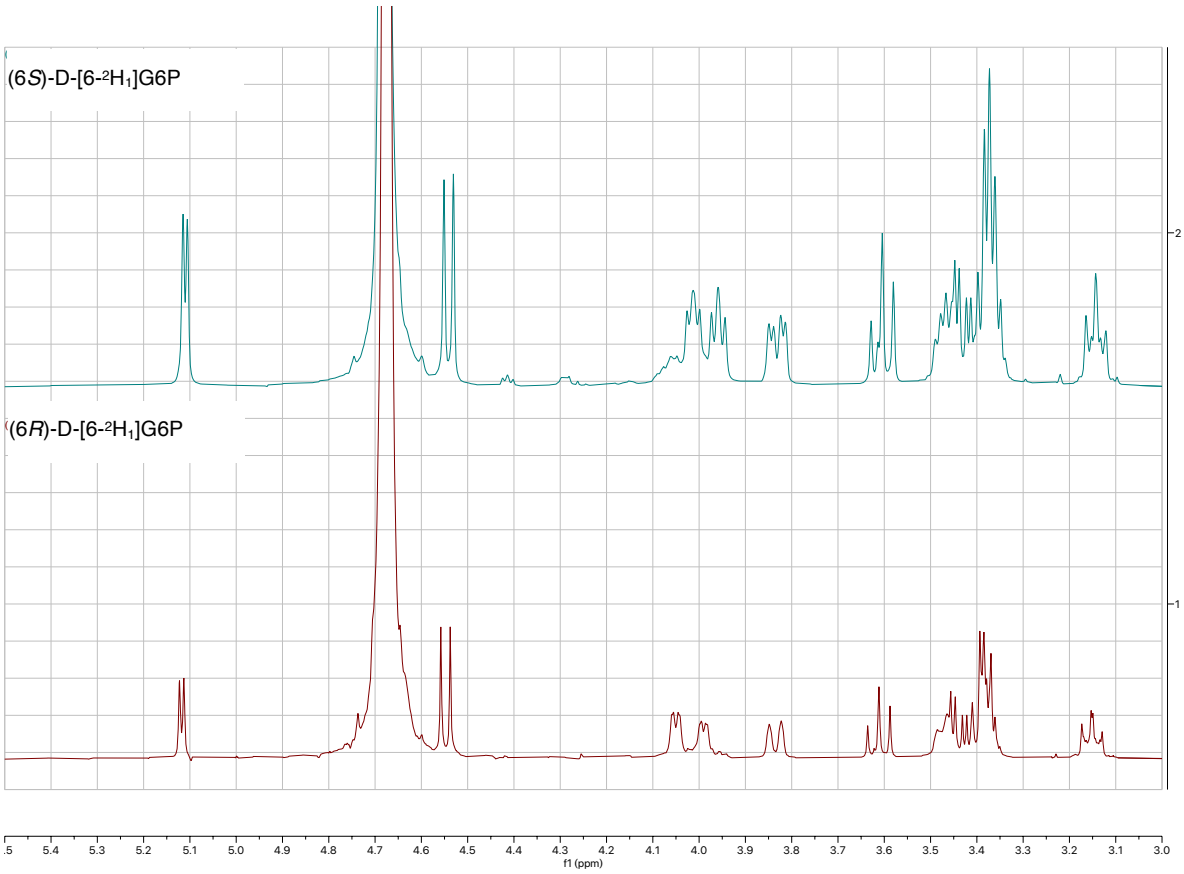


Figure S5. ^{1}H-NMR of (6*S*)-D-[6-$^{2}H_1$]G6P and (6*R*)-D-[6-$^{2}H_1$]G6P (400 MHz, in D_2O).

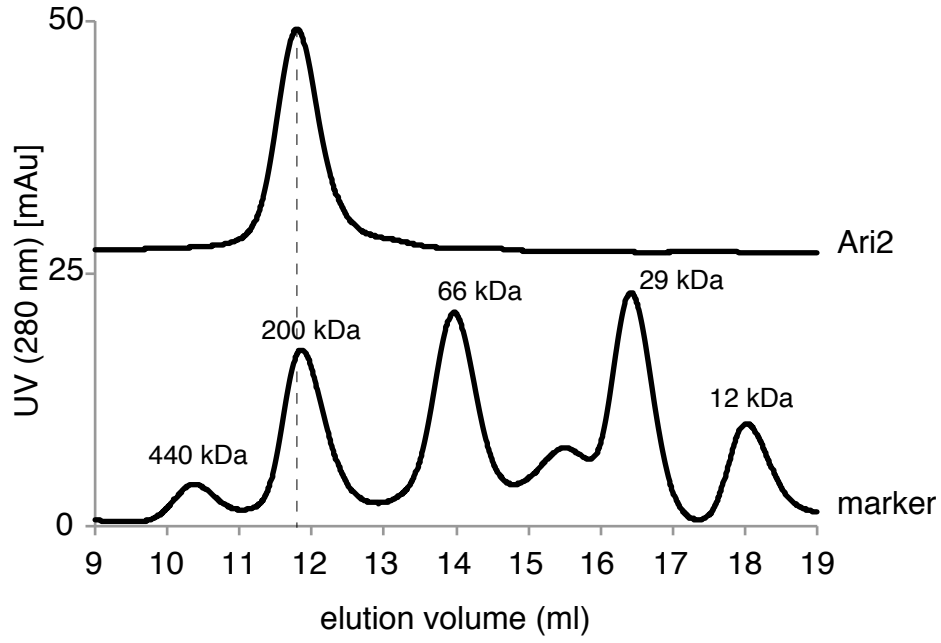


Figure S6. Gel-filtration analysis of Ari2 (38.8 kDa)

Ari2 protein was loaded onto the Superdex 200 10/300 column (GE Healthcare) equilibrated in buffer containing 20 mM HEPES-Na (pH 7.5), 150 mM NaCl and 10% glycerol. Eluted peaks were observed by monitoring the absorbance at 280 nm. Top line: Ari2, Bottom line: molecular weight marker; apoferritin (440 kDa), β-amylase (200 kDa), albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome c (12 kDa).

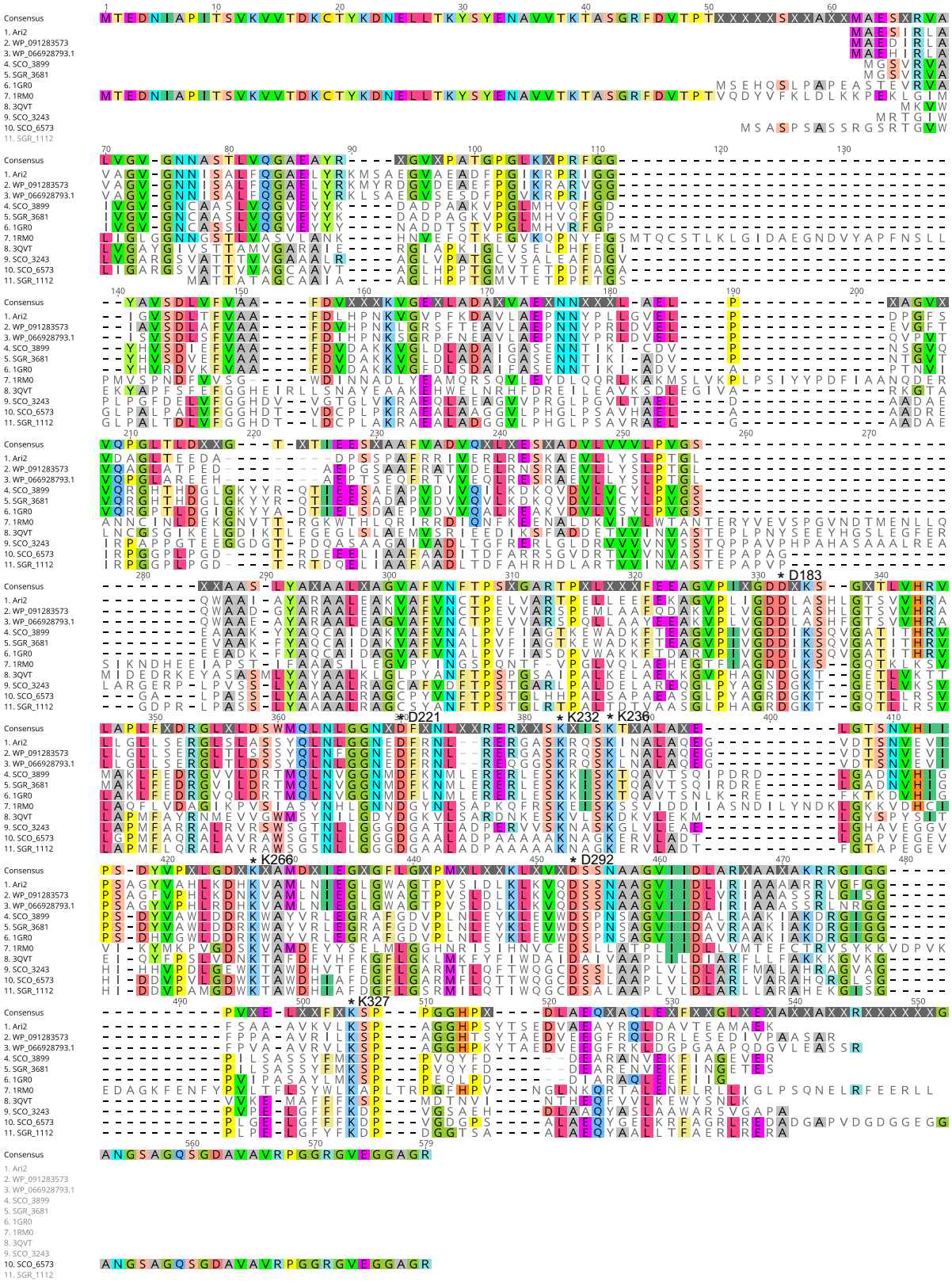


Figure S7. Multiple-sequence alignment of Ari2 homologs and some MIPSs using Geneious software (Biomatters) Ari2; five-membered cyclitol phosphate synthase in aristeromycin biosynthesis,[2] WP_091283573; Ari2 homolog in *Micromonospora haikouensis* DSM 45626,[13] WP_066928793.1; Ari2 homolog in *Streptomyces* sp. NBRC 110611,[14] SCO_3899, SCO_6573, SCO_3243, MIPS orthologs in *Streptomyces coelicolor* A3(2),[15] SGR_3681, SGR_1112 in *Streptomyces griseus* IFO 13350,[16] 1GR0; Mtb_MIPS,[7] 1RM0; MIPS in *Saccharomyces cerevisiae,*[17] 3QVT; MIPS in *Archaeoglobus fulgidus*.[18] Conserved catalytically important residues are marked with asterisk.

Table S1. Data collection and refinement statistics

Data collection statistics	
Beamline	PF-AR NE-3A
Wavelength (Å)	1.00000
Space group	$P6_522$
Unit-cell parameters	
a (Å)	101.18
b (Å)	101.18
c (Å)	389.66
Resolution (Å) (outer shell)	50.00–2.50 (2.64–2.50)
Unique reflections	40,934 (5,751)
Redundancy	8.4 (8.7)
Completeness (%)	97.4 (96.3)
R_{merge} (%)	13.8 (40.7)
Mean <I/σ (I)>	12.4 (4.6)
$CC_{1/2}$	0.990 (0.867)
Refinement statistics	
R_{work} (%)	22.6
R_{free} (%)	28.0
R.m.s. deviations	
Bond lengths (Å)	0.013
Bond angles (°)	1.928
No. of chains in the asymmetric unit	2
No. of non-hydrogen atoms	
Protein	5,048
NAD^+	88
Na^+	2
Solvent	227
Average B-factors (Å^2)	
Protein	43.1
NAD^+	29.7
Na^+	26.5
Solvent	44.1
Ramachandran plot	
Favored region (%)	94.9
Allowed region (%)	5.1
Outlier region (%)	0.0

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