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. ¹H- and ¹³C-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-[6,6-²H₂], (6*S*)-D-[6-²H₁], and (6*R*)-D-[6-²H₁]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 NH₄OH 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% CH₃OH 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-[6,6-²H₂], (6*S*)-D-[6-²H₁], and (6*R*)-D-[6-²H₁]glucose, respectively. NMR spectra were recorded on a Bruker DRX500 spectrometer. ¹H-NMR spectra were analyzed in D₂O (99.8 atom% enriched, Kanto Chemical) and ²H-

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-[6⁻²H₁] and (6*R*)-D-[6⁻²H₁]glucose 6-phosphate, 2 mM of (6*S*)-D-[6⁻²H₁] or (6*R*)-D-[6⁻²H₁]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 MgCl₂ 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 (HCO₂⁻ 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 H₂O:CH₃OH:CHCl₃ = 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-[6⁻²H₁]G6P (25.6 mg, 82.1%) and (6*R*)-D-[6⁻²H₁]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-[6-²H₁]G6P or (6*R*)-D-[6-²H₁]G6P, 0.1 mM NAD⁺, 1 mM NH₄Cl, 1 mM MgCl₂, 1 U of phosphoglucose isomerase (Sigma) and 25 μ M 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 μ M of 2-deoxy-*scyllo*-inosose (2DOI) synthase, BtrC³ 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 (HCO₂⁻ 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 H₂O:CH₃OH:CHCl₃ = 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 program⁶ with the MIPS structure (PDB code: 1GR0)⁷ as a search model. The structural model of Ari2 was manually constructed with Coot.⁸ Refmac⁹ 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).

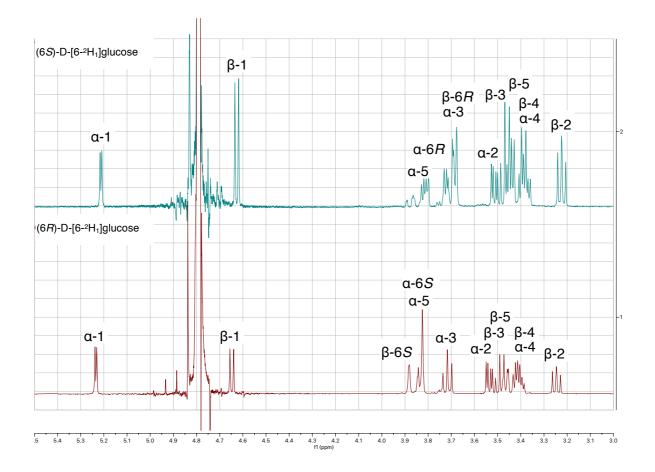


Figure S1. ¹H-NMR of (6*S*)-D-[$6^{-2}H_{1}$]glucose and (6*R*)-D-[$6^{-2}H_{1}$]glucose (500 MHz, in D₂O). These data are identical to the ¹H-NMR data of (6*S*)-D-[$6^{-2}H_{1}$]glucose and (6*R*)-D-[$6^{-2}H_{1}$]glucose in literature.¹

(6S)-D-[6-2H1]glucose

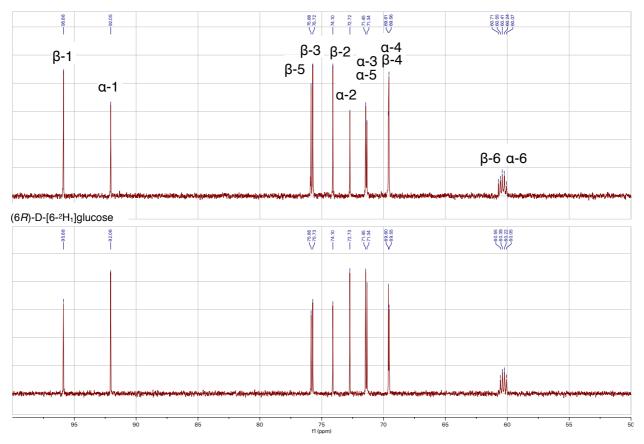


Figure S2. ¹³C-NMR of (6*S*)-D-[6-²H₁]glucose and (6*R*)-D-[6-²H₁]glucose (125 MHz, in D₂O).

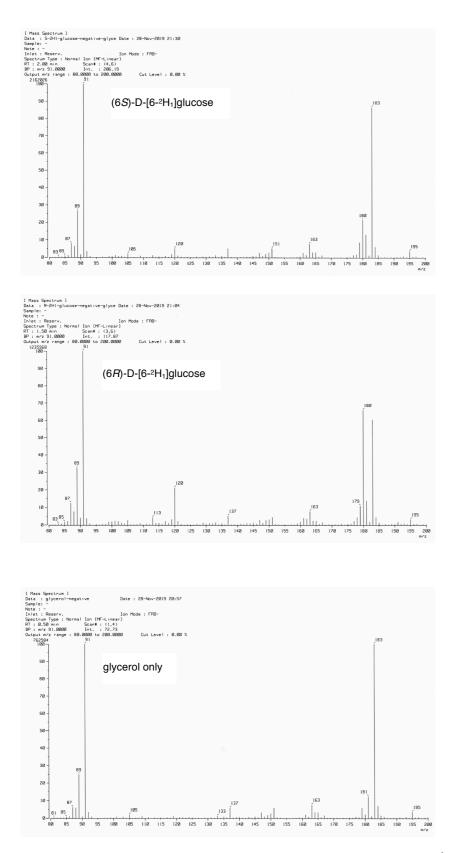


Figure S3. FAB-MS spectra (glycerol matrix, negative mode) of (6S)-D- $[6^{-2}H_1]$ glucose, (6R)-D- $[6^{-2}H_1]$ glucose and glycerol only.

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-[6-²H₁]glucose and (6*R*)-D-[6-²H₁]glucose were estimated to be approximately 80% and 90%, respectively.

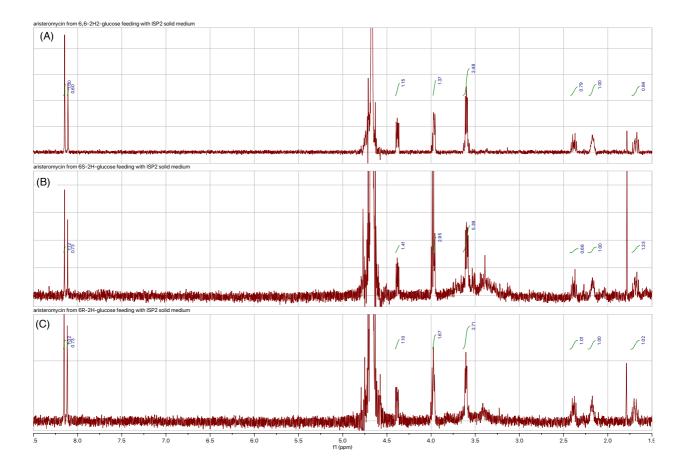


Figure S4. ¹H-NMR of aristeromycins isolated from the solid cultures with D-[$6,6-^{2}H_{2}$]glucose (A), (6S)-D-[$6-^{2}H_{1}$]glucose (B) and (6R)-D-[$6-^{2}H_{1}$]glucose (C) (500 MHz, in D₂O).

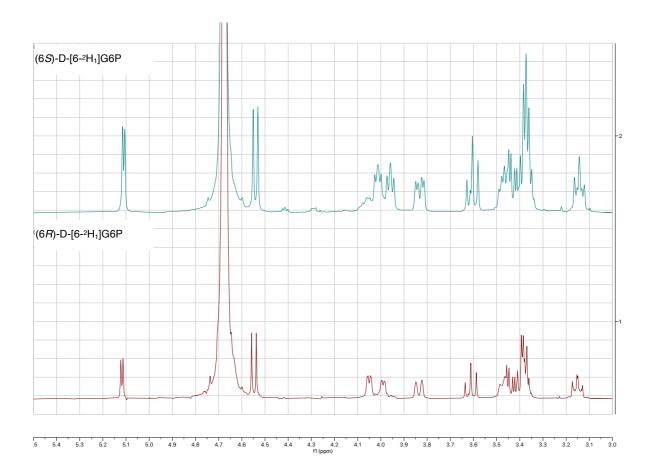


Figure S5. ¹H-NMR of (6*S*)-D-[6-²H₁]G6P and (6*R*)-D-[6-²H₁]G6P (400 MHz, in D₂O).

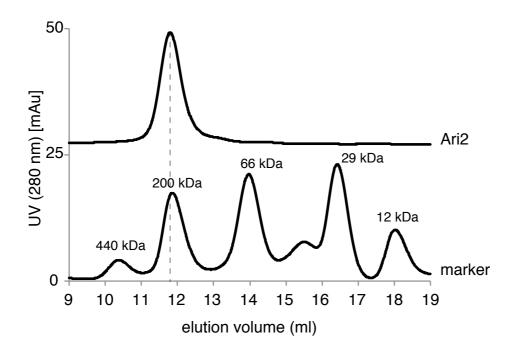


Figure S6. Gel-filtration analysis of Ari2 (38.8 kDa)

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).

Consensus	1 MTEDNIAPITSVK		³⁰ LTKYSYENA	VVTKTASGRF	50 D V T P T X X X X X	SXXAXXMAESXRVA
1. Ari2 2. WP_091283573 3. WP_066928793.1 4. SCO_3899 5. SGR_3681 6. 1GR0					MSEHQ	MAESIRLA MAEDIRLA MGSVRVA MGSVRVA MGSVRVA SLPAPEASTEVRVA FKLDLKKPEKLGTM MKVW MRTGIW
0. FGR0 7. 1RM0 8. 3QVT 9. SCO_3243 10. SCO_6573 11. SGR_1112	M T E D N I A P I T S V K	V V T D K C T Y K D N E I	L T K Y S Y E N A	<mark>V V</mark> T K T A S <mark>G R F</mark>	M S A	S P S A S S R G S R T G V W
Consensus 1. Ari2 2. WP_091283573 3. WP_066928793.1	70 VGV-GNNASTV VAGV-GNNISALF VAGV-GNNISALF VAGV-GNNISALF	OGAELYRKMSAEC OGAELYRKMYRDC OGAELYRKLSAEC		K R <mark>P R I G G</mark>	120	130
4. SCO_3899 5. SGR_3681 6. 1GR0 7. 1RM0 8. 3QVT 9. SCO_3243	VAGV-GNNISALF VAGV-GNNISALF VAGV-GNNISALF VGV-GNNISALF VGV-GNCASLV VGV-GNCASSLV LYGV-GNCASSLV LYGVGVGIVSTAM LYGARGSVATTTV LIGLGGNNGTTVI LIGARGSVATTTVI IGARGSVATTTVI 100	Q G V E Y Y K D A Q G V E Y Y K D A Q G V E Y Y K D A A S V L AN K N K V G A R A I E R C V G A R A I E R C	ADPAGKVPGL ADPAGKVPGL ADDTSTVPGL IVEFQTKEGV IAPKIGLVS	M H V Q F G D M H V Q F G D M H V R F G P K Q P N Y F G S M T K Q P H F E G I A L E A F D G V	Q C S T L K L G I D	A E G N D V Y A P F N S L L
10. SCO_6573 11. SGR_1112 Consensus		– – – – F D V X X X K V (δΕΧΙΑDΑΧVΑ	E X N N X X X L – A		200 XAGVX
1. Ari2 2. WP_091283573 3. WP_066928793.1 4. SCO_3899 5. SGR_3681	I G V S D L T F V A A I A V S D L A F V A A I S V S D L S F V A A Y H V S D I E F V A A Y H V S D I E F V A A		RSETEAVIA	FPNNYPRDV	EL P EL P EL P DV P DV P DV P	E V G F T
6. 1GR0 7. 1RM0 8. 3QVT 9. SCO_3243 10. SCO_6573	YHYRDVKFVAA PMVSPNDFVVSG- EKYAPFSFEFGGH GLPALPALVFGGH		F D L S D A I F A E A M Q R S Q V L E H W E L N R H F E Q L A E A G V V E A L A A G G V L	S E NNT I K I – A E Y D L Q Q R L K A D R E I L E A V K S P R G L P G V L T A P H G L P S A V H A	D V A A K M S L V K P L P S D L E G I V A E L A E L A	
11. SGR_1112 Consensus 1. Ari2		D C P L A K R A 230 - T - X T E E S X A A F D P S S P A F	VADVQXLXE RRIVERLRE	SXADVIVVVI	PVGS	270 AIADAE
2. WP_091283573 3. WP_066928793.1 4. SCO_3899 5. SGR_3681 6. 1GR0 7. 1RM0	V Q P G L A R E E H V Q R G H T H D G L G K Y	- T - X T E E S X A A F D P S S P A F A E P G S A A F A E P G S A A F A E P G S A A F Y R - Q T E S A A A A Y R - Q T E S A D A F Y R - Q T E S A D A F Y A - D T E S A D		KQVDVLVCYL	P T G L P T G L P V G S P V G S P V G S P V G S	
7. TRNU 8. 3QVT 9. SCO_3243 10. SCO_6573 11. SGR_1112	LNCGSGIKELGGD IRPAPPGTEEGGD IRPGGPLPGD IRPGGPLPGD 280	Y A - Q T E S A D A F Y A - D T E S D A F T T - R G K W T H L Q R I K T L E G E G L S L A E N G T - P D Q A S A G A - T - R D E E L I A A F - T - R D Q E L I A A F - T - R T D Q E L I A A F - 300	ADLTGFRE ADLTGFRE ADLTDFAR ADLTDFAR ADLTDFAH	S F A D D E T V V R L G L D R V V V R S G V D R T V V R H D L A R T V V 320	NVASTEPLPN NVSSTOPPAV NVASTEPAPA NVASTEPAPA NVASTEPAPA NVASTEPAP3 330	V S P G V N D T M E N L L O Y S E E Y H G S L E G F E R G
Consensus 1. Ari2 2. WP_091283573 3. WP_066928793.1		- LYAXAALXAGVA - AYARAALEAKVA - GYARAALEANVA	A F <mark>V N</mark> F T P S X G A F <mark>V N</mark> C T P E L V A F V N C T P E V V	ARTPXLXXXF ARTPELLEEF ARSPEMLAAF	EEAGVPIXGD	DXKS GXTLVHRV
4. SCO_3899 5. SGR_3681 6. 1GR0 7. 1RM0 8. 3QVT	S I K N D H E E I A P S T	- FYAQCAIDAKVA - FYAQCAIDAGVA - IFAAASILEGV	X F W N A L P V F I X F W N A L P V F I X F W N A L P V F I Y I N G S P V F I Y A N F T P S P G X F W D F T P S T G		EKAGWPLVGD ODAKWPLVGD TEAGWPLVGD TEAGWPLVGD TEAGWPLVGD TEAGWPLVGD TEAGWPLVGD TEAGWPLVGD EKKGUPLAGS REGULPHAGS ASGULPHAGS ASGULPHAGS ASGULPHAGS	DLASHEGTSVYRA DDIKSVVGATITHRV DIKSVVGATITHRV DLKSVVGATITHRV DLKSVVGATITHRV DLKST-GQTKV
9. SCO_3243 10. SCO_6573 11. SGR_1112 Consensus	G A L P A S S G D P R - L P A S S 350		SYANFTPSTG 2221 380	ARLPALDELA LHHPALSAPA LRTPALTDTV * K232 * K2360 × K232 * K2360 × K236	A S G L P Y A G S A S G L P Y A G R A A S G L P H A G R 40	
1. Ari2 2. WP_091283573 3. WP_066928793.1 4. SCO_3899 5. SGR_3681					A Q E G A Q E G L A Q E G V T S Q I P D R D - V T S Q I P D R D -	VD TSNVEVI VD TSNVEVI VD TSNVEVI VD TSNVEVI GADNVHIG
6. 1GR0 7. 1RM0 8. 3QVT 9. SCO_3243			L NM L E RERL YNLSAPKOF K VLSARDNK A TLADPERV			
10. SCO_6573 11. SGR_1112 Consensus 1. Ari2						
2. WP_091283573 3. WP_066928793.1 4. SCO_3899 5. SGR_3681 6. 1GR0	PSAGFVPHLKDNK PSAGYVPHLRDHK PS-DYVAWLDDRK PS-DYVAWLDDRK		T P V S L D L K L T P V S L D L K L O V P L N L E Y K L O V P L N L E Y K L	K V Q D S S N A A G K V Q D S S N A A G E V W D S P N S A G E V W D S P N S A G		A S S R L G I S G A S S R L N R G G I A K D R G I G G I A K D R G I G G
7. 1RN0 8. 3QVT 9. SCO_3243 10. SCO_6573 11. SGR_1112	VI-KYMKPVGDSK EI-QYFPSLVDNK HI-HHVPDLGEWK HI-DDVPVLGDWK	VANDEYYSELMU TAFDFVHFKGFLC TAWDHVTFEGFLC TAWDHUTAFDGFLC	GHNRISIHN KLMKFYFIW ARMTLQFTW ARMFLQTTW			A S S R L G T S G
Consensus 1. Ari2 2. WP_091283573	490 	500 * K327 E - L X X F X K S P A - A V K V L K S P A - A V R I L K S P	510 PGGHPX AGGHP AGGHTSYTS		EXFXXGLXEX DAVTEAMAEK DRLESEDIVP	AXXAXXRXXXXXX AASAR
3. WP_066928793.1 4. SCO_3899 5. SGR_3681 6. 1GR0 7. 1RM0		A – A V R V L K S P – – – S A S S Y F MK S P – – – S A S S Y F MK S P – – – P A S A Y L MK S P – – – T F S Y W I K A P I T F	AGGHPKYTA PVQYFD PVQYFD PEQLPD PEQLPD	E D V E E G F R K L D E A R A N V D I A R A Q L N G N K O R T A	D G P G A A P Q D G E K F I A G E V E R E K F I N G E T E E E F I I G E N F I R I I G I	V L E A S S R P S O N F I R F F F F I I
8. 3QVT 9. SCO_3243 10. SCO_6573 11. SGR_1112		E - M A F F F K S P E - L G F F F K D P E - L G F F F K D P G F F F K D P F - L G F Y F K D P 570	MDTNVİ VGSAEH VGDGPS DGGTSA	- NTHEOFVVL - DLAAQYASL - ALAEQYGEL - ALAEQYAAL	K E W Y S N L K A A W A R S V G A P K R F A G R L R E D L T F A E R L R E R	A X X A X X R X X X X X X X X X X X X X
Consensus 1. Ari2 2. WP_091283573 3. WP_066928793.1	ANGSAGQ ŠGDAVA					
4. SCO_3899 5. SGR_3681 6. 1GR0 7. 1RM0 8. 3QVT						
9. SCO_3243 10. SCO_6573 11. SGR_1112	A N G S A G Q S G D A <mark>V</mark> A	<mark>V</mark> R <mark>P G G R G V E G G A C</mark>	R			

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.

Data collection statistics				
Beamline	PF-AR NE-3A			
Wavelength (Å)	1.00000			
Space group	<i>P</i> 6 ₅ 22			
Unit-cell parameters				
<i>a</i> (Å)	101.18			
<i>b</i> (Å)	101.18			
<i>c</i> (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)			
$R_{\rm merge}$ (%)	13.8 (40.7)			
Mean	12.4 (4.6)			
$CC_{1/2}$	0.990 (0.867)			
Refinement statistics				
$R_{ m work}$ (%)	22.6			
$R_{\rm free}$ (%)	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			

Table S1. Data collection and refinement statistics

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