Structural insights into inhibition of lipid I production in bacterial cell wall synthesis

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Antibiotic-resistant bacterial infection is a serious threat to public health. Peptidoglycan biosynthesis is a well-established target for antibiotic development. MraY (phospho-MurNAc-pentapeptide translocase) catalyses the first and an essential membrane step of peptidoglycan biosynthesis. It is considered a very promising target for the development of new antibiotics, as many naturally occurring nucleoside inhibitors with antibacterial activity target this enzyme1-3. However, antibiotics targeting MraY have not been developed for clinical use, mainly owing to a lack of structural insight into inhibition of this enzyme. Here we present the crystal structure of MraY from Aquifex aeolicus (MraYAA) in complex with its naturally occurring inhibitor, muraymycin D2 (MD2). We show that after binding MD2, MraYAA undergoes remarkably large conformational rearrangements near the active site, which lead to the formation of a nucleoside-binding pocket and a peptide-binding site. MD2 binds the nucleoside-binding pocket like a two-pronged plug inserting into a socket. Further interactions it makes in the adjacent peptide-binding site anchor MD2 to and enhance its affinity for MraYAA. Surprisingly, MD2 does not interact with three acidic residues or the Mg2+ cofactor required for catalysis, suggesting that MD2 binds to MraYAA in a manner that overlaps with, but is distinct from, its natural substrate, UDP-MurNAc-pentapeptide. We have determined the principles of MD2 binding to MraYAA, including how it avoids the need for pyrophosphate and sugar moieties, which are essential features for substrate binding. The conformational plasticity of MraY could be the reason that it is the target of many structurally distinct inhibitors. These findings can inform the design of new inhibitors targeting MraY as well as its paralogues, WecA and TarO.

MraY is a member of the polyprenylphosphate N-acetyl hexosamine 1-phosphate transferase (PNPT) superfamily. The PNPT superfamily includes bacterial and eukaryotic integral membrane enzyme families such as MraY, WecA, TarO, WbcO, WbpL, RgpG and GPT, which are involved in cell envelope polymer synthesis and protein N-linked glycosylation1. WecA and TarO are also targets for antibiotic development. MraY catalyses the transfer of phospho-MurNAc-pentapeptide from UDP-MurNAc-pentapeptide (UM5A) to the lipid carrier undecaprenyl phosphate (C55-P), yielding undecaprenyl-phosphoryl-MurNAc-pentapeptide, known as lipid I (Extended Data Fig. 1a). This step is essential, rate limiting, and Mg2+-dependent1. It is blocked by five classes of natural nucleoside antibiotics (for example, muraymycin and tunicamycin), and bacteriolytic protein E from bacteriophage φX174, with various modes of inhibition2-8 (Extended Data Fig. 1a). MraY-targeted natural products have gained attention because of their in vivo efficacy against pathogenic bacteria including Mycobacterium tuberculosis; methicillin-resistant Staphylococcus aureus (MRSA), and vancomycin-resistant Enterococcus (VRE)9-12.

Despite their promise, no antibacterial natural products that target MraY have been developed for clinical use, in part owing to a lack of structural information on MraY catalysis and inhibition. We carried out structural studies of MraY in complex with a naturally occurring inhibitor of MraY, muraymycin, which shows antibacterial effects against MRSA, VRE and Pseudomonas aeruginosa, and S. aureus11-13. We used MD2 for our structural, enzymatic and biophysical studies17 (Extended Data Fig. 1b). The muraymycins are known to be competitive inhibitors for the natural substrate UM5A (ref. 14). Unlike UM5A, the muraymycins do not have pyrophosphate and sugar moieties and have a 5-aminoaribosyl group (Extended Data Fig. 1b). Using a radiochemical transfer assay19, we determined the Michaelis constant (Km) for UM5A with purified MraYAA to be ~190μM (Extended Data Fig. 1c), which is within the range of measured cellular UM5A concentrations20. MraYAA activity is markedly reduced after addition of 0.3μM MD2 (Fig 1a). Using isothermal titration calorimetry (ITC), we measured the dissociation constant (Kd) of MD2 for MraYAA to be ~20 nM (Fig. 1b). We generated crystals of MraYAA in the presence of MD2, which diffracted to 2.95 Å. Phasing was obtained by molecular replacement using the apoMraYAA structure (PDB code 4J72) with all

Figure 1 | The natural product MD2 binds to and inhibits MraYAA.

a. Specific activity measurements of wild-type MraYAA in the presence and absence of MD2 using radiolabelled substrate, [14C]UM5A. The radiolabelled product, [14C]lipid I, was quantified using a liquid scintillation counting method (disintegration per min, d.p.m.). Three technical replicates are shown with the mean value indicated by a line. b. Representative ITC raw data (top) and binding isotherm (bottom) for MD2 interacting with wild-type MraYAA in the presence of 10 mM MgCl2; Kd = 17.2 nM, ΔH° = −10.1 kcal mol⁻¹. This ITC experiment was performed in triplicate (technical replicates) and mean thermodynamic parameters are shown in Extended Data Table 2.
the cytoplasmic loops and TM9b removed as a search model. The structure was refined to good statistics ($R/R_{\text{free}} = 0.247/0.261$) (Extended Data Table 1).

MraYAA in complex with MD2 crystallizes as a dimer, as does apoMraYAA$^{21}$. Each protomer contains ten transmembrane helices (TM1–TM10) and five cytoplasmic loops (loops A–E) (Fig. 2a). TM9 breaks into two helical fragments (TM9a and TM9b), and TM9b bends outward towards the membrane (Fig. 2b). We previously outlined the active site as a cleft formed by the inner-leaflet membrane regions of TM3, TM4, TM5, TM8 and TM9b and cytoplasmic loops B, C, D and E. Many absolutely conserved polar/charged amino acid residues are localized in this cleft, including three catalytically critical acidic residues: Asp117, Asp118 and Asp265 (Fig. 2a, c), which are conserved in the PNPT superfamily$^{1,19,21–23}$. Asp265 interacts with Mg$^{2+}$ in the apoMraYAA structure, and Asp117 has been proposed to bind to the phosphate moiety of C55-P (refs 21, 22). Situated in the active site cleft, the nucleoside portion of MD2 is inserted between loop C and D and the peptide portion interacts with TM9b and loop E (Fig. 2c). High sequence conservation is observed around the MD2-binding region in the active site (Fig. 2d). Notably, MD2 does not interact with any of the three catalytically critical acidic residues (Fig. 2c and Extended Data Fig. 1d).

MraY undergoes remarkable conformational rearrangements near the active site upon binding MD2 (Fig. 3). The amphipathic TM9b rotates away from the active site while loop E rearranges, packs against the hydrophilic part of TM9b (Extended Data Fig. 2 and Supplementary Video 1), and a helical segment of the conserved HHH motif (PHXHHXEXGX) extends$^{21}$. This TM9b–loop E rearrangement widens and reshapes the active site, allowing the peptidic moiety of MD2 to bind to the side of TM9b and loop E (Extended Data Fig. 3). The carboxy-terminal portion of TM5 and loop C unwinds (cyan, Fig. 3a, b) and loop D rearranges (magenta, Fig. 3a, b), creating a pocket where the 5-aminoribosyl moiety and the uracil base of uridine interact (Fig. 3d and Extended Data Fig. 4a–c). The concerted motions of TM5 and loops C and D lead to the rearrangement of loop A and part of TM1 (green, Fig. 3a, b), although these regions do not appear to interact with MD2 directly. It is noteworthy that the amino acid residues interacting with the uracil base move large distances (5–17 Å) while the residues interacting with the 5-aminoribosyl moiety move shorter distances (Extended Data Fig. 4a–c and Supplementary Video 1). The active-site structural rearrangement leads to substantial changes in its electrostatic potential, including enlargement of the acidic milieu around the nucleoside-binding pocket, which may play a role in MD2 binding (Fig. 3c, d).

Developing nucleotide-sugar mimicking inhibitors for glycosyltransferases has been challenging largely owing to the difficulty of developing prophophosphate mimics capable of cellular entry with high affinity for the target enzyme$^{24–27}$. MraY is a phosphoglycosyltransferase that shares a common nucleotide sugar substrate with glycosyltransferases. Nucleoside antibiotics targeting MraY have garnered additional interest because they can enter the cell with high affinity for MraY$^{24,25,28}$. MD2 does not contain a prophophosphate or sugar moiety and has a 5-aminoribosyl group, which was thought to mimic the prophophosphate in the natural substrate UM5A (refs 23, 29) (Extended Data Fig. 1b). To understand the principle of MD2 inhibition of MraYAA, we performed mutagenesis and measured the mutational effects on MD2 binding using ITC. Amino acid residues involved in binding MD2 were grouped based on the substructures with which they interact: (1) uracil (Lys70, Gly194, Asp196, Asn255 and Phe262); (2) 5-amino ribose (Thr75, Asn190, Asp193 and Gly264), and (3) peptidic side chain (Gln305, Ala321 and His325). We performed site-directed mutagenesis on the residues that form side-chain interactions with MD2 (Lys70, Thr75, Asn190, Asp193, Asp196, Asn255, Phe262, Gln305 and His325) (Extended Data Fig. 4). The MraYAA mutants that showed substantial enzymatic activity (Extended Data Table 2) were used for ITC experiments with MD2 (Extended Data Fig. 5). We used ITC to explore the MD2-MraYAA complex (Extended Data Fig. 6).

The affinity of MD2 for MraYAA was most perturbed with Asp193Asn and Phe262Ala, mutations that disrupt interactions with the 5-aminoribose and uracil moieties of MD2, respectively (Extended Data Table 2 and Extended Data Fig. 6). Phe262 interacts

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**Figure 2** | MD2 binds to a conserved site in MraYAA. a, The MD2-bound MraYAA dimer viewed from the membrane. One protomer is shown as surface representation and the other as a cartoon. MD2 (green sticks) resides in the pocket formed by TM3–TM5 and TM8–TM9b and cytoplasmic loops B–E. Conserved catalytic aspartic acid residues (from the pocket formed by TM9b (yellow) and loop E (salmon). b, View from the membrane rotated 90° about a horizontal axis relative to a. One protomer is shown for clarity. c, Cytosolic view of the MraYAA–MD2 complex, rotated 90° about a vertical axis relative to a. d, Conservation mapping of MraYAA from high (magenta) to low (cyan) sequence identity, based on the alignment of 28 MraY homologues$^{21}$.

**Figure 3** | Conformational rearrangement of MraYAA upon MD2 binding. a, apoMraYAA (PDB code 4J72) viewed from the cytoplasm, as in Fig. 2c. Residues involved in interactions with MD2 are shown as sticks. b, MD2-bound MraYAA with MD2 omitted. Part of TM1 (light green) is transparent owing to its absence in the apoMraYAA structure. c, Electrostatic surface representation of apoMraYAA, viewed from the cytoplasm as in a and b. d, Electrostatic surface representation of MraYAA in complex with MD2. MD2 is green and shown in ball-and-stick representation.
elucidates the chemical logic of MraY AA inhibition. a, Composite simulated annealing 2F_{o} – F_{c} omit electron density of MD2 in the crystal structure of MD2-bound MraY AA at 1.7 \sigma. The transmembrane helices are coloured as in Fig. 3a, b. The residues forming side-chain interactions with MD2 are labelled. b, A two-dimensional representation of the interactions between MD2 and MraY AA. Hydrogen bonds (3.2 Å cutoff) are indicated with black dashed lines and \pi–\pi contacts are indicated with red dashes. Mutation of residues with red coloured labels resulted in a larger than fivefold increase in the \( K_d \) values of MD2, and those with blue residue labels are nearly inactive. c, Representative ITC raw data and binding isotherm for MD2 titrated into MraY AA in the absence of added Mg\(^{2+}\); \( K_d = 14.8 \text{ nM}, \Delta H^\circ = -8.3 \text{ kcal mol}^{-1}\). A similar \( K_d \) value is observed for MD2 titrated into MraY AA with added Mg\(^{2+}\). d, Representative ITC raw data and binding isotherm for 5-aminoribosyl-3-deoxy uridine titrated into wild-type MraY AA; \( K_d = 283 \text{ nM}, \Delta H^\circ = -16.4 \text{ kcal mol}^{-1}\). Each ITC experiment was performed in triplicate (technical replicates) and mean thermodynamic parameters are shown in Extended Data Table 2.

with the uracil base via a \pi–\pi interaction (Fig. 4 and Extended Data Fig. 4d). When Phe262 is mutated to another aromatic amino acid, such as tryptophan, there is a smaller effect on the \( K_d \) value relative to the alanine mutation, indicating the importance of this \pi–\pi interaction. Residue Asp193 makes side-chain interactions with the 5-aminoribose moiety of MD2 (Extended Data Fig. 4e). Because the Asp193Ala mutant is nearly inactive (Extended Data Fig. 5b), we used functionally competent Asp193Asn for ITC with MD2 (Extended Data Fig. 5a). However, the heat associated with binding was too low to measure, suggesting that the Asp193Asn mutation greatly reduces the affinity of MD2 for MraY AA (Extended Data Fig. 6). This observation is consistent with previous studies indicating the antibacterial activity of MraY inhibitors with a 5-aminoribose is dependent on the amino group of that moiety.\(^{29,30}\). The Gln305Ala mutant exhibits a larger than fivefold increase in \( K_d \) (Fig. 4 and Extended Data Table 2), indicating that the interactions formed by the peptidic moiety of MD2 contribute to the binding affinity. Asp193, Phe262 and Gln305 are absolutely conserved in MraY orthologues.\(^{31}\). The results from the equilibrium binding experiments are consistent with the enzymatic inhibition experiments because the Phe262Ala mutation results in partial inhibition and the Asp193Asn mutant is not inhibited in the presence of 1 \( \mu \text{M} \) MD2 (Extended Data Fig. 5a).

We infer that MD2 and the natural substrate, UMS5A, use different strategies for binding MraY. First, the three catalytically critical acidic residues, including the Mg\(^{2+}\)-binding Asp265, do not participate in direct interactions with MD2 (Extended Data Fig. 1d). Second, the Asp193Asn mutant remains functionally active, although it disrupts an interaction MraY makes with the 5-aminoribosyl group and affects the binding affinity of MD2 markedly (Extended Data Table 2 and Extended Data Fig. 6). This suggests the 5-aminoribosyl group does not function as a pyrophosphate mimic and instead forms interactions that are not present in or important for UMS5A binding. If MD2 lacks a pyrophosphate mimic, it is unlikely that Mg\(^{2+}\) has an important role in MD2 binding. To test this idea, we performed ITC in the absence of Mg\(^{2+}\) and found that MD2 does not require Mg\(^{2+}\) for MraY binding (Fig. 4c). It is possible that the amino group of the 5-aminoribose mimics Mg\(^{2+}\) and Asp193 interacts with Mg\(^{2+}\), as previously suggested.\(^{14}\). However, in such a case, we would expect Asp193Asn to be inactive. Furthermore, the amino group of the 5-aminoribose is not near the Asp265, which coordinates Mg\(^{2+}\) in the apoMraY structure.\(^{21}\).

In summary, the 5-aminoribosyl and uracil moieties of MD2 bind to the nucleoside-binding pocket like a two-pronged electrical plug inserts into a socket, and these interactions are the most critical for binding. The peptidic moiety also contributes to the binding energy by anchoring MD2 to MraY, probably contributing to its specificity for MraY. With the 5-aminoribosyl moiety of MD2 positioned as a second prong alongside uridine in the nucleoside binding site, MD2 binds to MraY with increased affinity, making the pyrophosphate and sugar moieties unnecessary for binding. To confirm the importance of the 5-aminoribosyl and uracil moieties of MD2, we synthesized 5-aminoribosyl-3-deoxy uridine\(^{29}\) and found it retains substantial binding affinity for MraY AA (Fig. 4d). Our structural and biochemical studies demonstrate the principles of MD2 inhibition of MraY and explain why MD2 does not require pyrophosphate and sugar moieties for binding, unlike the natural substrate, UMS5A. This illustrates an example of nature circumventing a long-standing problem in chemical biology: developing a nucleotide-sugar-like inhibitor for glycosyltransferases.\(^{24–27}\). Finally, the large conformational arrangement observed in MraY indicates...
conformational plasticity, which could be the reason why MraY accommodates so many structurally different nucleoside inhibitors, as well as protein E, with distinct modes of action7.

Online Content Methods, along with any additional Extended Data display items and online Content Methods, along with any additional Extended Data display items and protein E, with distinct modes of action7.

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Supplementary Information is available in the online version of the paper.

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Author Contributions B.C.C. solved the structure and performed some of ITC experiments and E.H.M. carried out the enzymatic assays and performed most of ITC experiments, both under the guidance of S.-Y.L. T.T. carried out chemical synthesis of MD2 under the guidance of S.I. and A.M. M.K. synthesized 5-amino-2-borono-3-deoxyuridine under the guidance of J.H. S.-Y.L., E.H.M. and B.C.C. wrote the paper. All authors discussed the results and commented on the manuscript.

Author Information Atomic coordinates and structure factors for the reported crystal structure are deposited in the Protein Data Bank under accession code 5CKR. Reprints and permissions information is available at www.nature.com/ reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to S.-Y.L. (seok-yong.lee@duke.edu).
METHODS

No statistical methods were used to predetermine sample size.

Crystalization. Wild-type MraYAA and mutants were expressed and purified as described21. All MraYAA bacterial expression plasmids were 10 × histidine (His6) maltose binding protein (MBP) fusion constructs expressed in C41 (DE3) cells. The cells were lysed by microfluidizer and the protein was extracted from the crude lysate using 40 mM dodecyl-maltoside. The lysates were centrifuged to remove the insoluble fraction and the supernatant was applied to a Co2+ -affinity column for purification. The His6-MBP tag was cleaved overnight by PreScission Protease and MraYAA was isolated by gel filtration using a Superdex 200 10/300 GL column in the presence of 5 mM n-decyl-β-D-maltopyranoside (DM), 150 mM NaCl, 20 mM Tris-HCl, pH 8.0, and 2 mM diethiothreitol (DTT). All purification steps were performed at 4°C. After gel filtration, the protein was concentrated to 10 mg ml−1 (~250 μM) and MD2 was added to a final concentration of 400–500 μM before crystallization. Crystals were grown using sitting-drop vapour diffusion in the presence of 50 mM MgCl₂, 40% PEG400, and 100 mM sodium cacodylate, pH 5.6. Crystals were harvested after 10–14 days and flash frozen in liquid nitrogen.

Data collection and structure determination. X-ray data were collected at beamlines 22-ID-D and 24-ID-C at the Advanced Photon Source in Argonne National Laboratory at a wavelength of 1.0 Å and processed using iMosflm. The data were ellipsoidally truncated at 3.0 Å on the c axis and anisotropically scaled using the UCLA anisotropy diffraction server (http://services.mbi.ucla.edu/anisoyscale/). Phases of the MD2 complex structures were solved by molecular replacement using PHASER31 with a partial apoMraYAA structure (PDB code 4J72, TM9b and the corresponding to MD2 were prominent from the beginning of the refinement. For wild-type MraYAA, 145–240 μM MD2 was titrated into 7–10.5 μM substrate, [14C]UM5A, with butanol extraction (200 μl) of 6 M pyridinium acetate, pH 3.0. The radiolabelled product, [14C]lipid I, was isolated from the hydrophilic substrate, [14C]UM5A, with butanol extraction (200 μl) after vortexing (30 s) and centrifugation at 3,000 g (5 min), the upper butanol phase was removed, added to 5 ml scintillation fluid (Fisher Chemical), and analysed using a liquid scintillation counting method (d.p.m.) for 14C detection (Packard 2500 TR Liquid Scintillation Analyzer). Control reactions lacking enzyme and inhibitor were incubated, extracted and analysed following the same protocol described above and were used for background subtraction. Each reaction rate was calculated by converting the d.p.m. measured (with background subtraction) to moles of [14C]lipid I formed using the specific radioactivity and dividing by the reaction time. All experiments were performed in triplicate (technical replicates).

Chemical synthesis. MD2 was synthesized as published17. The MD2 analogue, 5-aminoborosyl 3-deoxyuridine, was synthesized as published20, with a minor modification as follows. In the coupling of the hexose with the ribose, we replaced 5-azido-5-deoxy-β-ribose with 5-azido-5-deoxy-β-D-ribose since the ribofuranosyl fluoride was previously prepared in our laboratory26. Except for this minor modification, our synthesis of the MD2 analogue was identical to the original synthesis35.

ITC. Wild-type MraYAA and mutants (Lys70Ala, Thr75Ala, Asp193Asn, Asn255Ala, Phe262Ala, Phe262Trp, Gln305Ala and His325Ala) were purified as previously described21 in a buffer containing 150 mM NaCl, 20 mM Tris-HCl, pH 8.0, 4 mM DM, 2 mM DTT and 10 mM MgCl₂. This same buffer was used to dilute the ligand, MD2. One triplicate set of titrations with wild-type MraYAA and MD2 did not include MgCl₂. For wild-type MraYAA, 145–240 μM MD2 or 118–130 μM 5-aminoborosyl 3-deoxy uridine was titrated into 6.6–35 μM enzyme. For MraYAA mutants Lys70Ala, Thr75Ala, Asp193Asn, Asn255Ala, Phe262Trp and His325Ala, 210 μM MD2 was titrated into 30 μM enzyme. For MraYAA, Gln305Ala, 315–430 μM MD2 was titrated into 25–27 μM enzyme. For MraYAA, 80–110 μM MD2 was titrated into 7–105 μM enzyme. All titrations were performed in triplicate (technical replicates) at 37°C using either a MicroCal iTC200 or VP-ITC system (GE Healthcare). The total heat exchanged during each injection was fit to a single-binding isotherm with Kd and ΔH‡ as independent parameters. Data were analysed and figures were generated using Origin software (OriginLab Corp).

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Extended Data Figure 1 | MraY catalyses the formation of lipid I and binds MD2. a, Scheme of the reaction catalysed by MraY. The U-labelled blue hexagon represents uridine and the M-labelled orange hexagon represents MurNAc. The phosphates associated with the lipid carrier C_{55}-P are shown as red circles, and the phosphates from the substrate, UM5A, are shown as yellow circles. b, Chemical structures of the substrate, UM5A (top) and the inhibitor MD2 (bottom). c, Michaelis–Menten kinetic characterization of MraYAA translocase activity. The reaction monitored is the MraYAA-catalysed transfer of [^{14}C]phospho-MurNAc-pentapeptide from [^{14}C]UM5A to C_{55}-P, forming [^{14}C]lipid I. The enzymatic parameters measured are as follows: $K_m = 190 \pm 60 \mu M$, $k_{cat} = 20 \pm 2 \text{ min}^{-1}$, $k_{cat}/K_m = 0.11 \pm 0.03 \text{ min}^{-1} \mu M^{-1}$. Data are mean and s.e.m. of three technical replicates. d, MD2 (green) in complex with MraYAA. The distances between MD2 and the three catalytic acidic residues Asp117, Asp118 and Asp265 (magenta) are all greater than 4.5 Å.
Extended Data Figure 2 | Conformational changes of the TM9b and loop E region of MraY_{AA} upon MD2 binding. a, Zoomed-in view of TM9b (yellow) and loop E (salmon) of apoMraY_{AA} viewed from within the membrane. b, TM9b and loop E of MD2-bound MraY_{AA} viewed from within the membrane. MD2 is omitted to illustrate the conformational change of TM9b and loop E associated with MD2 binding. c, 45° rotated view of a about a horizontal axis. d, 45° rotated view of b about a horizontal axis, including the model of MD2 (green). The rotation of TM9b and rearrangement of loop E, including the HHH motif, allows for MD2 binding, especially its peptidic moiety.
Extended Data Figure 3 | Quality of electron density map surrounding MD2. a, Stereo view of $2F_o - F_c$ electron density map at 1σ for TM9b and loop E. b, Stereo view of $2F_o - F_c$ electron density map at 1σ for the MD2 binding pocket. The electron density peaks corresponding to MD2 are carved for clarity and all transmembrane helices are coloured as in Fig. 3.
Extended Data Figure 4 | Conformational changes in MraY_A that create binding pockets for the uridine and 5-aminoribosyl groups of MD2. a, A close-up view of apoMraY_A with key residues that participate in conformational changes upon MD2 binding shown as sticks in various colours. b, A close-up view of the nucleoside-binding pocket in the MraY_A–MD2 complex with MD2 omitted. Key residues are coloured as in a. c, A close-up view of the interactions MD2 (green) makes with the nucleoside-binding pocket of MraY_A. Interactions between MraY_A and MD2 are shown as dotted lines. It is noteworthy that residues interacting with the uridine moiety of MD2 move large distances (5–17 Å for residues Lys70, Asp196, Asn255 and Phe262), while the residues binding the 5-aminoribosyl group of MD2 (Thr75, Asn190 and Asp193) do not make large side-chain movements after MD2 binding. The uridine and 5-aminoribosyl groups of MD2 are circled. d, Interactions between the uracil base of MD2 (green) and the nucleoside-binding pocket of MraY_A. The uracil base forms H-bonds with side chains of Asn255, Asp196 and Lys70 and forms a π–π interaction with Phe262. e, The 5-aminoribosyl group of MD2 forms H-bond interactions with side chains of Thr75, Asn190 and Asp193, and the backbone amide of Gly264.
Extended Data Figure 5 | Specific activity of wild-type and mutant MraYAA in the presence and absence of MD2. a, Normalized specific activity of wild-type (WT) MraYAA and enzymatically active mutants with and without MD2 treatment. Wild-type MraYAA or mutant MraYAA was added to the reaction mixture to a final concentration that enabled product detection within the enzymatic linear range: 50 nM (WT), 500 nM (Lys70Ala), 400 nM (Thr75Ala), 350 nM (Asp193Asn), 250 nM (Asn255Ala), 200 nM (Phe262Ala), 50 nM (Phe262Trp), 400 nM (Gln305Ala), and 500 nM (His325Ala). Each reaction was carried out in the presence of either 0 μM, 0.3 μM or 1 μM MD2. Data are shown for three technical replicates ± s.e.m. Specific activity measurements for each mutant were normalized relative to that without added MD2. b, Specific activity of wild-type MraYAA and enzymatically inactive mutants. MraYAA Asn190Ala, Asp193Ala, Asp196Ala and Asp196Asn were each added to a final concentration of 500 nM, while wild-type MraYAA was present at 50 nM. All enzymatic reactions were conducted with a radiochemical assay monitoring the transfer of [14C]phospho-MurNAc-pentapeptide from [14C]UM5A to C55-P, forming [14C]lipid I. The radiolabelled product, [14C]lipid I, was quantified using a liquid scintillation counting method (d.p.m.). Specific activity was calculated by determining moles of [14C]lipid I formed, divided by the reaction time and the quantity of enzyme added. Three technical replicates are shown with the mean value indicated by a line.
Extended Data Figure 6 | Representative ITC raw data and binding isotherms for MD2 interacting with mutant MraYAA. All titrations were performed in triplicate (technical replicates); see source data for all titrations. Representative data are shown. For MraYAA mutants Lys70Ala, Thr75Ala, Asp193Asn, Asn255Ala, Phe262Trp and His325Ala, 210 \( \mu \)M MD2 was titrated into 30 \( \mu \)M enzyme. For MraYAA Gln305Ala, 315–430 \( \mu \)M MD2 was titrated into 25–27 \( \mu \)M enzyme. For MraYAA Phe262Ala, 80–110 \( \mu \)M MD2 was titrated into 7–10.5 \( \mu \)M enzyme. Mean thermodynamic parameters for triplicate titrations are shown in the Extended Data Table 2. Mean \( K_d \) values for each triplicate are as follows: 63.9 \( \pm \) 4.7 nM for Lys70Ala; 27.4 \( \pm \) 1.5 nM for Thr75Ala; \( K_d \) not determined for Asp193Asn; 29.7 \( \pm \) 0.8 nM for Asn255Ala; 228 \( \pm \) 4 nM for Phe262Ala; 68.4 \( \pm \) 0.9 nM for Phe262Trp; 117 \( \pm \) 10 nM for Gln305Ala; 24.4 \( \pm \) 0.4 nM for His325Ala.
Extended Data Table 1 | Data collection and refinement statistics

MraY<sub>aa</sub>-Muraymycin D2

### Data collection

| Parameter                      | Value(s)                  |
|--------------------------------|----------------------------|
| Space group                    | C 2 2 2<sub>i</sub>       |
| Cell dimensions                | a, b, c (Å)                |
|                               | 94.48, 102.05, 135.8       |
| α, β, γ (°)                    | 90, 90, 90                |
| Resolution (Å)                 | 2.95 (3.06 – 2.95)*       |
| R<sub>merge</sub> (%)          | 27.7 (>100)               |
| R<sub>free</sub> (%)           | 11.6 (64.5)               |
| I / σI                         | 4.6 (1.2)                 |
| CC<sub>1/2</sub> (%)           | 87.4 (50.2)               |
| Completeness (%)               | 100 (100)                 |
| Redundancy                     | 7.0 (6.3)                 |

### Refinement

| Parameter                      | Value(s)                  |
|--------------------------------|----------------------------|
| Resolution (Å)                 | 2.95 (3.18 – 2.95) †       |
| No. reflections                | 14053                      |
| Completeness (%)               | 99.40 (93.90)              |
| R<sub>merge</sub> / R<sub>free</sub> (%) | 24.7/26.1                 |
| No. atoms                      |                            |
| Protein                        | 2576                       |
| Ligand/ion                     | 64                         |
| Water                          | 9                          |
| B-factors                      |                            |
| Protein                        | 63.50                      |
| Ligand/ion                     | 60.50                      |
| Water                          | 50.00                      |
| R.m.s. deviations              |                            |
| Bond lengths (Å)               | 0.008                      |
| Bond angles (°)                | 1.14                       |
| Molprobity Overall             | 1.75                       |
| Ramachandran (%)               |                            |
| Favored                        | 99.1                       |
| Allowed                        | 0.9                        |
| Outlier                        | 0                          |

*Values in parentheses are for highest-resolution shell.
†Anisotropic truncation at 3.0 Å on c axis by the UCLA Diffraction Anisotropy Server (http://services.mbi.ucla.edu/anisoscale/)
Extended Data Table 2 | Equilibrium dissociation constants and binding parameters demonstrating the effect of mutation in MraY<sub>AA</sub> on MD2 binding

|               | K<sub>D</sub> (nM) | N (sites) | ΔH (kcal/mol) | ΔS (cal/mol/deg) |
|---------------|-------------------|-----------|---------------|-----------------|
| WT +Mg<sup>2+</sup> | 20.4 ± 1.9        | 0.62 ± 0.02 | -15.0 ± 3.5   | -11.5 ± 9.8     |
| WT -Mg<sup>2+</sup> | 15.1 ± 0.2        | 0.65 ± 0.05 | -9.1 ± 0.6    | 6.5 ± 2.0       |
| K70A          | 63.9 ± 4.7        | 0.72 ± 0.05 | -7.2 ± 0.2    | 9.8 ± 0.7       |
| T75A          | 27.4 ± 1.5        | 0.71 ± 0.01 | -11.7 ± 0.6   | -3.3 ± 1.7      |
| D193N         | -                 | -          | -             | -               |
| N255A         | 29.7 ± 0.8        | 0.68 ± 0.06 | -7.6 ± 0.3    | 10.1 ± 1.1      |
| F262A         | 228 ± 4           | 0.77 ± 0.07 | -4.9 ± 0.8    | 14.7 ± 2.5      |
| F262W         | 68.4 ± 0.9        | 0.81 ± 0.03 | -10.4 ± 0.7   | -0.7 ± 2.2      |
| Q305A         | 117 ± 10          | 0.73 ± 0.08 | -9.8 ± 0.3    | 0.1 ± 1.0       |
| H325A         | 24.4 ± 0.4        | 0.56 ± 0.07 | -11.3 ± 0.7   | -1.7 ± 2.2      |
| WT + 5-aminoribosyl-3-deoxy uridine | 283 ± 3 | 0.44 ± 0.02 | -15.0 ± 1.5 | -19.9 ± 4.8 |

Data are shown as mean and s.e.m. of three technical replicates.
| Web summary | The crystal structure of the MraY enzyme from *Aquifex aeolicus* in complex with the naturally occurring nucleoside inhibitor muraymycin D2 (MD2) reveals that MraY undergoes a large conformational rearrangement near the active site after the binding of MD2, leading to the generation of a nucleoside-binding pocket and a peptide-binding site. |