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Research paper

Identification of novel scaffolds for potential anti-*Helicobacter pylori* agents based on the crystal structure of *H. pylori* 3-deoxy-D-manno-octulosonate 8-phosphate synthase (*Hp*KDO8PS)





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ABSTRACT

The crystal structure of 3-deoxy-D-manno-octulosonate-8-phosphate synthase (KDO8PS) from Helicobacter pylori (HpKDO8PS) was determined alone and within various complexes, revealing an extra helix (HE) that is absent in the structures of KDO8PS from other organisms. In contrast to the metal coordination of the KDO8PS enzyme from Aquifex aeolicus, HpKDO8PS is specifically coordinated with Cd²⁺ or Zn²⁺ ions, and isothermal titration calorimetry (ITC) and differential scanning fluorimetry (DSF) revealed that Cd^{2+} thermally stabilizes the protein structure more efficiently than Zn^{2+} . In the substratebound structure, water molecules play a key role in fixing residues in the proper configuration to achieve a compact structure. Using the structures of HpKDO8PS and API [arabinose 5-phosphate (A5P) and phosphoenolpyruvate (PEP) bisubstrate inhibitor], we generated 21 compounds showing potential HpKDO8PS-binding properties via in silico virtual screening. The capacity of three, avicularin, hyperin, and MC181, to bind to HpKDO8PS was confirmed through saturation transfer difference (STD) experiments, and we identified their specific ligand binding modes by combining competition experiments and docking simulation analysis. Hyperin was confirmed to bind to the A5P binding site, primarily via hydrophilic interaction, whereas MC181 bound to both the PEP and A5P binding sites through hydrophilic and hydrophobic interactions. These results were consistent with the epitope mapping by STD. Our results are expected to provide clues for the development of *Hp*KDO8PS inhibitors.

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1. Introduction

Helicobacter pylori (H. pylori) is a gram-negative, microaerophilic

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bacterium that colonizes the stomach. Marshall and Warren first identified this microbe from patients suffering from chronic gastritis and peptic ulcerations [1]. Currently, more than half of the general population is infected with *H. pylori*, which is linked to gastritis, duodenal ulcer, gastric cancer, gastric mucosa-associated lymphoid tissue lymphoma (MALT), and sudden infant death syndrome (SIDS) [2–7].

Triple therapy involving a proton pump inhibitor (omeprazole) and antibiotics (amoxicillin and clarithromycin) was initially recommended for treating *H. pylori* infection [8]; however, this conventional multi-therapy is no longer effective due to the prevalence of antibiotic resistance [9,10]. Moreover, these antibiotics commonly disrupt the normal gastrointestinal flora, causing diarrhea as a side effect [11]. Therefore, it is necessary to develop new anti-*H. pylori* agents based on the structure of novel cellular targets.

Abbreviations: KDO8PS, 3-deoxy-D-manno-octulosonate-8-phosphate synthase; PEP, phosphoenolpyruvate; A5P, D-arabinose-5-phosphate; *Hp*KDO8PS, KDO8PS from *Helicobacter pylori*; *Ec*KDO8PS, KDO8PS from *Escherichia coli*; AdKDO8PS, KDO8PS from *Aquifex aeolicus*; DAH7PS, 3-deoxy D-arabino-heptulosonate-7phosphate synthase; apoHpKDO8PSwt, the metal- and substrate-free form of *Hp*KDO8PS; *Hp*KDO8PS_H2O4A, a mutant of *Hp*KDO8PS (His2O4 to Ala2O4); *Hp*KDO8PS_C18A, a mutant of *Hp*KDO8PS (Cys18 to Ala18); *Hp*KDO8PS-Cd, the cadmium-bound form of *Hp*KDO8PS; *Hp*KDO8PS-PEP-Zn, the PEP and zinc-bound form of *Hp*KDO8PS; API, an A5P and PEP bisubstrate inhibitor.

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3-Deoxy-p-manno-octulosonate 8-phosphate synthase (KDO8PS)¹ is the enzyme that catalyzes the condensation reaction between arabinose 5-phosphate (A5P)² and phosphoenolpyruvate $(PEP)^3$ to synthesize KDO8P, the precursor of the 8-carbon sugar 3deoxy-p-manno-octulosonate (KDO). KDO is the essential component of gram-negative bacterial lipopolysaccharides (LPS) [12]. functioning as the linkage between lipid A and O-antigen [13]. As the enzymes related to KDO synthesis and its incorporation within the LPS structure play an important role in the survival and growth of gram-negative bacteria, inhibition of KDO synthesis results in a loss of the LPS endotoxin component and reduced pathogenicity [14–16]. Because the enzymes that participate in bacterial LPS synthesis reactions are absent in mammalian systems, they are considered to be potential targets for the development of antibiotics with fewer potentially undesirable cross-reactions [17]. Members of the KDO8PS family have been grouped into two classes according to their transition metal requirements [18]. Escherichia coli (E. coli) KDO8PS is metal independent (class I), whereas the Aquifex aeolicus (A. aeolicus) and H. pylori enzymes (HpKDO8PS)⁴ require a transition metal (class II) [18,19]. KDO8PS crystal structures, both alone and in complex with various ligands, have been previously reported (e.g., KDO8PSs from E. coli, A. aeolicus, Neisseria meningitidis, Burkholderia pseudomallei and Pseudomonas aeruginosa) [20–24]. The KDO8PSs from E. coli $(EcKDO8PS)^5$ and A. aeolicus (AaKDO8PS)⁶ are homotetramers in which each monomer forms a $(\beta/\alpha)_8$ barrel fold structure [20,21]. In addition, similarities in the enzymatic reaction and active site structure between KDO8PS and 3-deoxy D-arabino-heptulosonate-7-phosphate synthase $(DAH7PS)^7$ suggest that they share a common ancestor [21]. The active site cavity in the PEP- and A5P-bound forms is closed, whereas it is open in the substrate-free form [20]. It is possible that its binding site allows PEP to form its distorted C-2 geometry by adopting sp^3 instead of sp^2 hybridization, thereby facilitating the intermediate form prior to the condensation with A5P [20]. Based on the crystal structures of binary complexes of EcKDO8PS with PEP and through the use of a mechanism-based inhibitor ($K_d = 0.4 \,\mu\text{M}$), the condensation reaction between the substrates was shown to be mediated by an intermediate form containing a transient oxocarbenium ion formed at the C-2 position in PEP [25]. Additionally, the structures of AaKDO8PS in complex with R5P and PEP, along with the bisubstrate inhibitor API [arabinose 5-phosphate (A5P) and phosphoenolpyruvate (PEP) bisubstrate inhibitor],⁸ suggest that the water molecule coordinated to the metal ion on the si side of PEP is necessary to trigger the reaction [26]. These structures also support the hypothesis that a proton of the PEP-phosphate group is transferred to the A5P-aldehyde group to first form the hydroxyl group and that condensation is completed by a syn addition of water and A5P to the *si* side of PEP [26].

*Aa*KDO8PS Cys11, His185, Glu222, and Asp233 are the residues that interact with the aforementioned transitional metal ions. Among them, His185 in particular directs PEP to its active site and places a water molecule on the *si* side of PEP [27]. The Fe²⁺ or Zn²⁺ ions bound to the *Aa*KDO8PS active site are replaced by other divalent metal ions, such as Cd²⁺ and Cu²⁺ [27–29], and these metal ion substitutions affect enzymatic activity by altering the environment of the active site [29]. For example, Cd²⁺-bound *Aa*KDO8PS exhibits maximal enzymatic activity over the other metal-bound proteins [27].

Based on the catalytic mechanism and *Aa*KDO8PS structures, Gatti and colleagues proposed novel inhibitors that mimic the intermediate form of the condensation reaction [26,30]. In this study, we determined the crystal structures of *Hp*KDO8PS alone (apo*Hp*KDO8PSwt),⁹ in complex with PEP and zinc (*Hp*KDO8PS-PEP-Zn)¹⁰, and in complex with cadmium (*Hp*KD08PS-Cd)¹¹ in an effort to develop anti-*H. pylori* agents. The structure of an *Hp*KDO8PS mutant (*Hp*KDO8PS_H204A)¹² was also determined.

Chemical compounds binding to the *Hp*KDO8PS active site were evaluated by *in silico* virtual screening using the determined *Hp*KDO8PS structure. Among the 21 compounds initially selected, three were validated for binding to *Hp*KDO8PS via STD NMR spectroscopy and waterLOGSY experiments. We also performed docking simulations for *Hp*KDO8PS in complex with the three compounds. These compounds could serve as novel scaffolds for the development of antibiotics that inhibit the function of *Hp*KDO8PS.

2. Materials and methods

2.1. Cloning, expression, and protein purification

The gene encoding HP0003 from H. pylori 26695 was amplified by PCR using H. pylori genomic DNA (strain ATCC 700392/26695) as a template. The PCR primers used to clone the HpKDO8PS expression plasmid, in which the restriction enzyme sites are underlined, were as follows: 5'-GGGAATTCCATATGAAAACTTCTAAAACAAAAA and 5'-CCGCTCGAGTTAAAATAAATTTTGGATTTTTAACA CCCC-3'TGTCGG-3'. The PCR product and pCold I vector (Takara, Japan) were digested with Ndel and Xhol (NEB, UK) and ligated together. After confirming the sequence, the recombinant plasmid was overexpressed in chaperone-expressing E. coli cells pTf16/BL21 (Takara, Japan) grown in LB broth. When the culture media reached an O.D. of 0.8, 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) was added to induce protein expression; cells were transferred to 15 °C and grown for an additional 20 h. The cells were collected by centrifugation at $4293 \times$ g for 10 min, and the pellet was resuspended in lysis buffer (20 mM Tris, 500 mM NaCl, 5 mM imidazole, 0.5 mM TCEP, pH 7.8) and then sonicated at 4 °C using a 30% duty cycle setting for 20 min (Cole Parmer Inc., USA). The lysate was centrifuged at $6708 \times g$ for 60 min at 4 °C, and the supernatant was loaded into an open Ni-NTA column (Qiagen, USA) pre-equilibrated with lysis buffer. The column was washed with a 30-fold excess volume of buffer containing 150 mM imidazole; the protein was eluted when the buffer reached 400 mM imidazole using an imidazole gradient (from 150 to 500 mM). After concentrating the protein, the buffer was changed to 20 mM MES, 200 mM NaCl, and 0.5 mM TCEP at pH 6 by dialysis using an Amicon Ultra-15 Centrifugal Filter Unit with a 10 K molecular weight cutoff (MWCO) (Millipore, USA). SDS-PAGE revealed purity up to 95%. The enzyme was concentrated to 0.12 mM for crystallization.

The *Hp*KDO8PS_metal-free form used for circular dichroism (CD) and ITC experiments was prepared by dialyzing purified *Hp*KDO8PSwt against a buffer containing 20 mM MES, 200 mM NaCl, 2 mM 1, 10-phenanthroline, and 2 mM EDTA at pH 6. Subsequently, dialysis with a buffer containing 20 mM MES, 200 mM

¹ 3-Deoxy-D-manno-octulosonate-8-phosphate synthase.

² Arabinose 5-phosphate.

³ Phosphoenolpyruvate.

⁴ KDO8PS from Helicobacter pylori.

⁵ KDO8PS from *Escherichia coli*.

⁶ KDO8PS from Aquifex aeolicus.

⁷ 3-Deoxy _D -arabino-heptulosonate-7-phosphate synthase.

⁸ A5P and PEP bisubstrate inhibitor.

⁹ Metal- and substrate-free form of *Hp*KDO8PS.

¹⁰ PEP and zinc-bound form of *Hp*KDO8PS.

¹¹ Cadmium-bound form of *Hp*KDO8PS.

¹² Mutant of HpKDO8PS (His204 to Ala204).

NaCl, and 0.5 mM TCEP at pH 6 was conducted to reach a 1000-fold dilution of the metal ion chelators. The resulting *Hp*KDO8PS_metal-free form was concentrated to 0.03 mM for CD spectroscopy and 0.1 mM for ITC.

Mutagenesis primers (Table 1) targeting the four metal-binding residues were designed, and EZchangeTM Site-Directed Mutagenesis Kit (Enzynomics, Korea) was used to generate point mutations according to the manufacturer's instructions. Briefly, the PCR conditions consisted of 25 repeated cycles including a 30-s melting step at 94 °C, a 1-min annealing step at 55 °C and a 5-min elongation step at 72 °C. Template removal and ligation of the PCR products were performed using EZ-MIX buffer (Enzynomics, Korea). After transformation of the mutant plasmids into DH5 α -competent cells, the sequence of the plasmid DNA sequence was confirmed. BL21/pTF16 was used for overexpression of the mutant proteins, and the successfully expressed mutants (*Hp*KDO8PS_C18A and *Hp*KDO8PS_H204A) were purified.

2.2. Crystallization and structure determination

The most efficient crystallization conditions for each form of HpKD08PS were selected using the Crystal ScreenTM and IndexTM crystal screening kits (Hampton Research, USA) with the hanging drop vapor diffusion method. The protein solution and reservoir solution containing 18% polyethylene glycol (PEG) 3350, 0.1 M HEPES, pH 7.5, and 0.25 M magnesium chloride hexahydrate were mixed together in a 1:1 drop ratio. Crystals were obtained through drop equilibration with the reservoir solution at 20 °C for 14 days. Prior to data collection, the crystals were flash-cooled with liquid nitrogen with protection by the addition of 25% glycerol to the cryosolution. An identical procedure was applied for HpKD08PS_H204A crystal preparation.

To obtain better diffraction-quality crystals of HpKDO8PS-Cd and HpKDO8PS-PEP-Zn, we performed streak seeding using pulverized apoHpKDO8PSwt crystals as microseeds for nucleation. The protein solution contained the protein-binding partners (Cd²⁺, Zn^{2+} and PEP) at a 2-fold excess concentration over the protein molecules (*i.e.*, protein at 0.12 mM and protein binding partners at 0.24 mM). For the crystallization drop, 1 µL of prepared protein solution was added to the same volume of reservoir solution. The seed stock was prepared after washing an apoHpKDO8PSwt crystal; the stock was continuously transferred to the original crystallization solution for stabilization and crushed using a glass homogenizer. This concentrated seed stock was serially diluted with the stabilizing buffer by a factor of 1000. Each drop was streaked with the diluted solution using a clean whisker-like fiber. Crystals appeared in 7–14 days at 20 $^\circ\text{C}$ after the start of the incubation and were additionally soaked in a cryo-solution containing 1 mM of Zn^{2+} , PEP or Cd²⁺ for 12 h prior to the flash-freezing step.

The diffraction dataset for the apo*Hp*KDO8PSwt crystal was collected at 100 K at a resolution of 2.0 Å using an MAR225HE CCD detector at beamline BL44XU in the SPring-8 radiation facility

(Hyogo, Japan). The dataset was processed and scaled with the HKL-2000 program package [31]. The apo*Hp*KDO8PSwt crystal was identified from the *C*2 space group with the following unit cell parameters: a = 137.83 Å, b = 50.66 Å, c = 78.67 Å, and $\beta = 110.5^{\circ}$. There were two monomers in each asymmetric unit. The calculated crystal volume per protein weight (V_M) was 2.12 Å³ Da⁻¹, and the solvent content was 42.0% [32]. The structure of apo*Hp*KDO8PSwt was determined by molecular replacement using the PHENIX Phaser-MR program [33] based on the *Aa*KDO8PS structure as a search model (PDB: 1FX6) [20]. The initial structures were refined by alternately using the Refmac [34] and Phenix.refine [33] programs. The solvent molecules were inserted with Coot [35].

The X-ray diffraction data from a single *Hp*KD08PS_H204A crystal were collected at PAL (Pohang, Korea) using an ADSC quantum 315r CCD detector on beamline 5C-SBII at 100 K and a resolution of 2.4 Å. The raw data were processed using the HKL-2000 program [31]. The *Hp*KD08PS_H204A crystal belongs to the space group *C*2, with two monomers in each asymmetric unit (unit cell parameters: a = 139.86 Å, b = 50.87 Å, c = 78.30 Å, and β = 104.73°). Its V_M was 2.22 Å³ Da⁻¹, and the solvent content was 44.6% [32]. The structure of the *Hp*KD08PS_H204A crystal was determined by molecular replacement using the PHENIX Phaser-MR program [33] based on the determined model of ap0*Hp*K-D08PSwt. The refinement step for *Hp*KD08PS_H204A was conducted in a manner identical to that for ap0*Hp*KD08PSwt.

Raw X-ray diffraction data for the *Hp*KD08PS-Cd crystals were collected at a resolution of 1.93 Å at 100 K using the same detector as for the *Hp*KD08PS_H204A crystal at PAL (Pohang, Korea). The data were processed and scaled using the HKL-2000 program [31]. The *Hp*KD08PS-Cd crystal was identified from the *C*2 space group, with unit cell parameters of a = 140.35 Å, b = 51.02 Å, c = 78.67 Å, and β = 104.7°. Two monomers of *Hp*KD08PS-Cd were found in each asymmetric unit, with a V_M = 2.24 Å³ Da⁻¹ and a solvent content of 45.2% [32]. The structure was determined by molecular replacement using apo*Hp*KD08PSwt as a model with the PHENIX Phaser-MR program [33]. Refinement of the *Hp*KD08PS-Cd model was performed by alternately using the Refmac [34] and Phenix.refine [33] programs, as mentioned above.

The *Hp*KDO8PS-PEP-Zn diffraction dataset was collected at a resolution of 1.68 Å at 100 K using a Saturn A200 mosaic CCD detector at beamline 26B1 in SPring-8 (Hyogo, Japan). The dataset was processed and scaled with the HKL-2000 program [31]. The *Hp*KDO8PS-PEP-Zn crystal was identified from the C2 space group, with unit cell parameters of a = 140.3 Å, b = 51.01 Å, c = 78.55 Å, and β = 104.37°. There were two monomers in each asymmetric unit. The V_M was 2.24 Å³ Da⁻¹, and the solvent content was 45.2% [32]. The structure of *Hp*KDO8PS-PEP-Zn was determined by molecular replacement using the PHENIX Phaser-MR program [33] with the apo*Hp*KDO8PSwt structure as a search model. The initial structures were refined by alternately using the Refmac [34] and Phenix.refine [33] programs.

Table 1

Primers for site-directed mutagenesis targeting the residues involved in metal binding within the active site of *Hp*KD08PS. In primer IDs, "F" represents forward, and "R" represents reverse. Mutated nucleotides are underlined in the primer sequences used for site-directed mutagenesis.

| Primer ID | Primer sequence | Mutated nucleotide |
|-----------|--|--------------------|
| C18p-F | GTTTTAATCGCTGGGCCA <u>GCT</u> GTCATTGAGAGCTTAG | C18A |
| C18p-R | CTAAGCTCTCAATGACAGCTGGCCCAGCGATTAAAAC | C18A |
| H204p-F | GATTTTTGACGCTACCGCTAGCGTGCAAATGCCAG | H204A |
| H204p-R | CTGGCATTTGCACGCTAGCGTAGCGTCAAAAATC | H204A |
| E241p-F | ATTGATGGGTTGTTTGCTGCTACGCATGTTGATCCTAAA | E241A |
| E241p-R | TTTAGGATCAACATGCGT <u>AGC</u> AGCAAACAACCCATCAAT | E241A |
| D252p-F | CTAAAAACGCCCTAAGCGCTGGAGCAAACATGCTAAAAC | D252A |
| D252p-R | GTTTTAGCATGTTTGCTCCAGCGCTTAGGGCGTTTTTAG | D252A |

2.3. Circular dichroism spectroscopy

For CD experiments, the *Hp*KDO8PS forms (*Hp*KDO8PS_metalbound, *Hp*KDO8PS_metal-free, *Hp*KDO8PS_C18A, and *Hp*KDO8P-S_H204A) were used at a concentration of 0.03 mM in a buffer containing 20 mM MES, 200 mM NaCl, and 0.5 mM TCEP, pH 6. *Hp*KDO8PS_metal-bound represents the sample used in the crystallization of apo*Hp*KDO8PSwt. CD spectra were collected using a Chirascan[™]-plus CD spectrometer (Applied Photophysics, UK) with the following settings: scan rate of 120 nm min⁻¹, response time of 0.5 s, step size of 1 nm, and bandwidth of 1 nm. The path length was 0.2 mm using a quartz cell (106-0.20-40) with detachable windows (Hellma, Germany). All scans were conducted in the far-UV range (190–260 nm). The data were processed by baseline subtraction and smoothing using the Pro-Data Viewer program (Applied Photophysics, UK).

2.4. Isothermal titration calorimetry

A 0.1 mM solution of HpKDO8PS_metal-free was prepared as described above. Zinc chloride and cadmium chloride were dissolved to a concentration of 1.5 mM in the same buffer as the protein. The protein and metal ion solutions were degassed in a vacuum before measurement. All ITC experiments were performed using a MicroCal iTC200 microcalorimeter (GE healthcare, UK) at 25 °C. The protein was added to the sample cell, and a metal solution (zinc or cadmium chloride) was charged in the injection syringe. During the titration, 20 aliquots of metal solution were injected into the sample chamber. The titrations began with a 60-s delay time and 0.4-µL injection volume, followed by a 2-µL injection-volume with 5-s delay times. Intervals of 150 s between injections were included at the end of each titration. The stirring speed in the sample chamber at 25 ± 0.1 °C was 1000 rpm. Heat generation during the titration experiments was measured using the integrated data from the ITC calorimeter with the Origin 7.0 software package supplied by MicroCal, subtracting the heat generated by the buffer. K_a (binding constant), ΔH (enthalpy change) and N (stoichiometry) values were calculated by applying the one-site fitting model.

2.5. Differential scanning fluorimetry

*Hp*KD08PS_metal-free was diluted to 5 μ M in a white 96-well plate using the identical buffer as mentioned above. A 5000X solution of the dye SYPRO Orange (Sigma Aldrich, USA) was added to each well to achieve a final concentration of 5X. Measurements were performed in 100 μ L in the presence and absence of 75 μ M metal ion (zinc or cadmium chloride). The temperature was ramped from 25 to 95 °C at a rate of 1 °C/min using an Applied Biosystems 7500 Fast Real-Time PCR Instrument System (ThermoFisher Scientific, USA). The raw fluorescence data were plotted as a function of temperature, which generated a sigmoidal curve [Fig. 5(A)]. The inflection point of the transition curve (T_m , midpoint melting temperature) was calculated using Prism 5 (GraphPad software, USA) by applying the Boltzmann sigmoidal fitting [Fig. 5(B)].

2.6. Preparation of compounds for docking-based virtual screening

A total of 415 compounds from our in-house database were processed for docking with the Sybyl-X suite v. 1.3 (Certara, USA) [36]. The compounds were prepared by adding hydrogen atoms and rectifying wrong valences and then energy-minimized using the conjugated gradient method in the Tripos force field with the Gasteiger-Hückel charge method until a convergence value of

0.001 kcal $Å^{-1}$ mol⁻¹ was reached.

2.7. Preparation of protein structures

The crystal structure of *Aa*KDO8PS in complex with API (PDB: 1JCX) [26] was selected to gain insight into the ligand binding site. Superimposition of apo*Hp*KDO8PSwt and *Aa*KDO8PS in complex with API revealed that the C-terminal active sites of the two enzymes are highly conserved (motifs C18/11, K47/41, N54/48, R55/49, S56/50, A108/102, K130/124, H207/185, and E241/222). Therefore, the API molecule from the *Aa*KDO8PS-API structure was extracted and merged into our structure. The resulting API:*Hp*KD08PS complex was energy-minimized. Both proteins were prepared with the Protein Preparation module of the Sybyl-X suite v. 1.3 [36] using the default parameters.

2.8. Docking-based virtual screening

Receptor-based virtual screening was performed to obtain new compounds harboring the desired activity profiles. The chemical database containing natural products and synthetic compounds was docked into the validated *Hp*KD08PS binding site using Surflex-Dock [36]. First, a protomol was generated to define the active site, with a threshold of 0.4 and bloat set of 1 around the embedded ligand. Core interactions (Lys47, Asn54, Arg55, Ser56, Ala108, and Lys130) were also assigned in the protomol. The number of docking runs was set to 50, and other parameters were set as the Surflex-Dock Geom default settings. The final hit compounds were evaluated for binding by combining the consensus scoring function Cscore3 (Cscore >3) and Surflex-Dock total score ($-\log K_d$). Visual inspection considering the important interactions was necessary to evaluate binding.

2.9. Synthesis and preparation of ligands

Hyperin was prepared as reported by Lee and colleagues [37], and avicularin was purchased (Jinan Boss Chemical Industry, China). MC181 was synthesized using the following 2-step method. (see Scheme 1).

2.9.1. Step 1) ethyl 1-(3-phenylpropyl)piperidine-4-carboxylate (compound 1)

A mixture of ethyl-4-piperidinecarboxylate (500 mg, 3.18 mmol), 3-phenylpropylbromide (576.2 μ L, 3.82 mmol), and potassium carbonate (in excess) prepared in DMF (1 mL) was stirred at 70 °C for 2 h. The reaction mixture was cooled to room temperature and then diluted with ethyl acetate, washed with water and brine, dried on anhydrous MgSO₄, and concentrated *in vacuo*. The residue was purified by flash chromatography on silica gel (MeOH:CH₂Cl₂ = 1:20) to generate ethyl 1-(3-phenylpropyl) piperidine-4-carboxylate (863.9 mg, 98.6%), referred to as Compound 1.

¹H NMR (600 MHz, CD₃OD): δ 7.26–7.23 (m, 2H), 7.19–7.17 (m, 2H), 7.16–7.13 (m, 1H), 4.13–4.09 (m, 2H), 2.89 (d, J = 11.46 Hz, 2H), 2.61 (t, J = 15.24 Hz, 2H), 2.38–2.36 (m, 2H), 2.35–2.30 (m, 1H), 2.07 (t, J = 22.02 Hz, 2H), 1.91–1.87 (m, 2H), 1.84–1.81 (m, 2H), 1.75–1.68 (m, 2H), 1.23(t, J = 14.28, 3H); ¹³C NMR (150 MHz, CDCl₃): δ 175.07, 142.10, 128.37, 128.27, 125.72, 60.25, 58.14, 52.94, 41.16, 33.70, 28.57, 28.20, 14.21, 14.20; LC/MS (ESI⁺) m/z: 276.3 [M+H]⁺.

2.9.2. Step 2) N-(3-(furan-2-yl)phenyl)-1-(3-phenylpropyl) piperidine-4-carboxamide (MC181)

A mixture of Compound 1 (79.6 mg, 0.289 mmol) and 3N-HCl (2 mL) was subjected to microwave synthesis (Monowave 300) at 150 °C for 20 min. The reaction mixture was concentrated *in vacuo*



Fig. 1. ApoHpKDO8PSwt structure (PDB: 4Z1A). (A) The crystal structure of apoHpKDO8PSwt exhibited a (β/α)₈ topology. The secondary structures are drawn in a cartoon diagram with distinctive structures in different colors (α -helix, *green*, β -strand, *cyan*, and loop, *orange*). (B) ApoHpKDO8PSwt is superimposed onto *Ec*KDO8PS (PDB: 1D9E) and *Aa*KDO8PS (PDB: 1FX6) for comparison. Both structures are drawn in a cartoon diagram (*Ec*KDO8PS, *pink*, *Aa*KDO8PS, *brown*, and *Hp*KDO8PS, *green*). A hairpin in *Ec*KDO8PS is highlighted in *hot pink*. An extra helix (HE) in *Hp*KDO8PS is highlighted in *lime green*. (C) Topological models of *Hp*KDO8PS, *brown*, and *Aa*KDO8PS. Cylinders represent α -helices (*Ec*KDO8PS, *pink*, *Aa*KDO8PS, *orange*, and *Hp*KDO8PS, *green*), and *cyan* arrows represent β -strands. The differences between models are highlighted in *red* boxes. (D) Interactions of *HE (raspberry)*. HE interacts with neighboring residues in H5 and S6 as well as loops through both hydrophobic interaction (left) and hydrogen bonding (right). The interactions are shown in the surface diagram by *black* dashed lines. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

to produce the corresponding acid, and the resulting residue was used for the next reaction without further purification. A mixture of the acid (20 mg, 0.070 mmol), 3-(2-furyl)aniline hydrochloride (12.9 mg, 0.081 mmol), and triethylamine (113 μ L, 0.81 mmol) in CH₂Cl₂ (10 mL) was added to 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (77.7 mg, 0.405 mmol) in a dropwise manner and

stirred at room temperature for 1.75 h. The mixture was added to 4dimethylaminopyridine (3.5 mg, 0.03 mmol) and stirred at room temperature for 75 min. The reaction mixture was diluted with CH₂Cl₂, washed with water and brine, dried on anhydrous MgSO₄, and concentrated *in vacuo*. The crude product was purified by flash chromatography on silica gel (MeOH:CH₂Cl₂ = 1:20) to generate



Fig. 2. Interactions within the active sites in four *Hp*KD08PS X-ray crystal structures. The residues related to the interactions are shown as sticks; water molecules (numerically ordered) are drawn as *gray* balls; hydrogen bonds are drawn as *black* dashed lines. (A) Interactions between apo*Hp*KD08PSwt (*green*) and water molecules. (B) Interactions between *Hp*KD08PS_H204A (*cyan*) and water molecules (see also Fig. S1). (C) Interactions between *Hp*KD08PS-Cd (*orange*) and water molecules. The cadmium ion (Cd²⁺) is shown as a *green* ball. (D) Interactions between *Hp*KD08PS-PEP-Zn (*light blue*) and water molecules. The zinc ion (Zn^{2+}) is shown as a *stick* (carbon, *green*; oxygen, *red*; phosphorus, *olive*). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

MC181 (10 mg, 37%).

¹H NMR (600 MHz, CD₃OD): δ 7.94 (t, J = 4.2 Hz, 1H), 7.54 (dd, J = 1.8, 0.66 Hz, 1H), 7.43–7.41 (m, 1H), 7.31 (t, J = 15.8 Hz, 1H), 7.28–7.25 (m, 2H), 7.21–7.20 (m, 2H), 7.18–7.15 (m, 1H), 6.73 (dd, J = 3.36, 0.66 Hz, 1H), 6.50 (dd, J = 3.39, 1.92 Hz, 1H), 3.22 (d, J = 12.1 Hz, 2H), 2.66 (t, J = 11.94 Hz, 2H), 2.67–2.62 (m, 2H), 2.53–2.48 (m, 1H), 2.40 (t, J = 20.7 Hz, 2H), 1.96–1.89 (m, 6H); ¹³C NMR (150 MHz, CD₃OD): δ 174.08, 153.51, 142.11, 142.09, 141.15, 138.90, 131.45, 128.06, 127.99, 125.7, 119.08, 118.64, 114.94, 114.93, 111.32, 111.29, 104.99, 104.97, 57.37, 52.26, 42.16, 32.90, 27.35, 27.12; LC/MS (ESI⁺) m/z: 389.3 [M+H]⁺.

2.10. NMR spectroscopy

NMR experiments [38,39] were performed at 298 K using a Bruker Avance DRX 600 MHz spectrometer equipped with a 5-mm TXI (1 H/ 13 C/ 15 N) probe. The NMR sample was prepared as a mixture of 5 μ M *Hp*KD08PS and 0.2 mM ligand, which were dissolved in a solution containing 98% D₂O and 2% DMSO. On- and off-resonance

irradiations were applied at chemical shifts of 0 and -30 ppm, respectively. *Hp*KDO8PS was saturated using a train of Gaussian-shaped 50-ms-long pulses. The total length of the saturation train was set to 2 s.

Prior to acquisition, a 15-ms spin-lock pulse with a 1-W strength (T2 filter) was applied to remove protein signals from the STD spectrum. The ¹H NMR spectrum was acquired with 32 K real points and 6320 scans.

Single pseudo-2D data from two serial free-induction decays were divided into two separate 1D data (on- and off-resonance). To increase the signal-to-noise ratio, the 1D raw data were processed with a 0.5-Hz line broadening and exponential window function prior to Fourier transformation. For comparison, the STD effects of individual peaks were quantified by the simple equation $(I_{off} - I_{on})/I_{off}$, where I_{on} and I_{off} represent the absolute intensities of the on- and off-resonance spectra peaks, respectively.

The off-resonance spectrum is identical to a conventional ${}^{1}\text{H}$ NMR spectrum. The largest STD value was set to 100%, and the other STD values were normalized to the largest value [40,41].



Fig. 3. Metal and PEP binding sites in *Hp*KD08PS. Omit difference maps (*Fo* – *Fc*) contoured at 3.0 σ (gray) are shown around the metal, water and PEP. (A) Cd²⁺ (green ball) has a distorted octahedral coordination in *Hp*KD08PS, in contrast with the geometry in gas phase or *Aa*KD08PS, which exhibit a tetrahedral or square pyramidal coordination, respectively. (B) Zn^{2+} (*pink* ball) shows a distorted square pyramidal geometry. The metal geometry of the Cd²⁺ ion and Zn²⁺ ion *Hp*KD08PS is remarkably opposite to that in *Aa*KD08PS. (C) Zn²⁺ (*pink* ball) and PEP binding sites in *Hp*KD08PS. PEP (carbon, *green*; oxygen, *red*; phosphorus, *olive*) interacts directly with Ser49, Lys52, and Lys130. Gln133 also participates in PEP binding via water molecules (*gray* balls) (see also Table S1). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

WaterLOGSY NMR experiments [42] were conducted to confirm the results of the STD experiments. All of the WaterLOGSY spectra were recorded at 298 K using a Jeol ECA 600 MHz spectrometer equipped with a 5-mm triple resonance inverse probe. The ligand (0.2 mM) was dissolved in the presence and absence of 5 μ M *Hp*KDO8PS in a solution containing 10% D₂O and 90% H₂O in a total volume of 300 μ L. To selectively excite water, Gaussian-shaped 20ms pulses were irradiated at approximately 4.7 ppm. The mixing time for the magnetization transfer was set at 2 s. The NMR spectra were acquired with 256 transients and 8 K data points. WATERGATE pulse sequences were interleaved in the WaterLOGSY sequences to suppress water signals. To increase the signal-to-noise ratio, freeinduction decays were processed with a 0.5-Hz line broadening and exponential window function prior to Fourier transformation.

For STD-NMR competition experiments, PEP or A5P was added to the NMR samples, which contained either *Hp*KDO8PS and hyperin or *Hp*KDO8PS and MC181. The same acquisition and processing parameters mentioned above were used to obtain the STD NMR spectra.



Fig. 4. ITC data for Cd^{2+} titration onto metal-free *Hp*KDO8PS (*Hp*KDO8PS_metal-free). Cd^{2+} injection induces an exothermal reaction and stabilizes the enzyme. The data are well fitted in a single-site binding isotherm model.

The peaks of the 1D ¹H NMR spectra were assigned using a server (www.Acdlabs.com/resources/ilab) for the assignment of peaks and analysis of STD and WaterLOGSY data. All STD and WaterLOGSY spectra were analyzed in a spectral range of approximately 6 ppm–8 ppm, where aromatic protons appear.

3. Results

3.1. Protein expression and structure determination

Several *Hp*KDO8PS constructs were prepared using the pCold I vector (Takara, Japan). Five types of different chaperone-expressing BL21 competent cells (pG-KJE8, pGro7, pKJE7, pG-Tf2, and pTf16, Takara, Japan) were tested for the most efficient production of soluble protein extracts. Among them, the pTf16 chaperone-expressing BL21 competent cells provided the best results, and soluble protein was prepared.

We determined four crystal structures, namely, apoHpK-DO8PSwt, HpKDO8PS_H204A, HpKDO8PS-Cd, and HpKDO8PS-PEP-Zn. The data and refinement statistics are presented in the Supplementary material Table S1. The electron density map of apoHpKDO8PSwt showed some poorly resolved regions. Several residues between residues 212–218 in chain A and 211–217 in chain B exhibited poor electron density. In the N-terminal region, loop-forming residues 1–9 were also invisible on the electron density map, possibly due to their level of structural disorder.



Fig. 5. Thermal shift assay of *Hp*KDO8PS_metal-free by differential scanning fluorimetry measured in the presence and absence of metal (Cd^{2+} or Zn^{2+}). (A) Protein thermal denaturation is depicted by a fluorescence versus temperature plot in the presence of SYPRO Orange. (B) Mid-point melting temperatures (T_m) calculated from the melting curve inflections shown in Fig. 5(A).

substrate-free forms of wild-type *Hp*KDO8PS), as well as *Ec*KDO8PS and *Aa*KDO8PS [20], are similar to each other, with r.m.s. deviations of 1.38 Å and 1.08 Å, respectively, with 463 and 478 equivalent $C_{\alpha S}$ and harboring sequence identity levels of 46.9% and 51.5%, respectively. An extra α -helix (HE, residues 160–166)¹³ is found between the H5 α -helix and S6 β -strand in *Hp*KDO8PS (Fig. 1); a 3₁₀-helix between S8 and H8 (residues 246–249) was also found, as in *Aa*KDO8PS. However, due to poor electron density, the 3₁₀-helix was not observed in the structure of *Ec*KDO8PS (not shown). No hairpins were observed at the N-terminus of *Hp*KDO8PS and *Ac*KDO8PS [Fig. 1(C)], in contrast with *Ec*KDO8PS.

The crystal structure of the H204A mutant (HpKD08PS_H204A) was similar to that of apoHpKDO8PSwt. However, several residues located primarily at the active site (Lys52, Asn54, Arg55, Gln133, Asp252, and Asn255) were oriented in different directions in HpKDO8PS_H204A compared with the corresponding residues in apoHpKDO8PSwt [Fig. 2(A) and (B)]. These residues are charged amino acids or have similar polar groups that can form hydrogen bonds. Asn255 in HpKDO8PS_H204A is flipped into the active site, forming a hydrogen bond with a water molecule (water-7) [Fig. 2(B)]. In addition, water-7 and -8 are linked together by a hydrogen bond. In apoHpKDO8PSwt, Glu241, Asp252, and Asn255 are moved toward the space previously filled with His204 [Fig. 2(B)]. The distance between the residues in HpKDO8PS_H204A is shorter compared with apoHpKDO8PSwt, with the Glu241-Asp252 distance changing from 9.0 Å to 8.2 Å, the Glu241-Asn255 distance changing from 7.2 Å to 5.3 Å, and the Asp252-Asn255 distance from 7.6 Å to 5.0 Å. In the *Hp*KDO8PS H204A structure, a hvdrogen bond between Arg55 and Ser251 is absent: therefore. Arg55 moves toward the outside of the active site [Fig. 2(A) and (B)]. Lys52, which hydrogen bonds with water-5 in apoHpK-DO8PSwt, interacts with water-12 and Asn54 through multiple hydrogen bonds, moving closer to Asn54 by a distance of 5.0 Å compared with the Lys52-N-Asn54-O distance of 9.3 Å observed in apoHpKDO8PSwt [Fig. 2(A) and (B)].

To analyze the metal geometry in the active site, crystals of the Cd^{2+} -bound form (*Hp*KD08PS-Cd) were obtained. A Cd^{2+} ion is bound to the metal binding residues (Cys18, His204, Glu241, and



Scheme 1. (a) 3-Phenylpropylbromide, potassium carbonate, DMF, 70 °C, 2 h; (b) i. 3N-HCl, microwave 150 °C, 20 min; ii. 3-(2-furyl)aniline hydrochloride, Et₃N, EDC, DMAP, room temperature, 3.5 h.

In the refined model of *Hp*KD08PS_H204A, *Hp*KD08PS-Cd, and *Hp*KD08PS-PEP-Zn, two monomers were observed in each asymmetric unit, and they were well superimposed with the apo*Hp*K-D08PSwt model, with root mean square (r.m.s.) deviation values of 0.50 Å, 0.36 Å, and 0.36 Å, respectively, for the 513, 514, and 514 C_α atom pairs. In these structures, two loop regions involving residues 1 to 9 and 210 to 220, which correspond to the similar sites in apo*Hp*KD08PSwt, were also invisible.

3.2. HpKDO8PS crystal structures

*Hp*KD08PS is a 30.3-kDa enzyme with two monomers in each asymmetric unit. The monomers adopt the $(\beta/\alpha)_8$ barrel topology [Fig. 1(A)], similar to their previously reported homologs. The secondary structures were assigned using the STRIDE server [43]. Both apo*Hp*KD08PSwt monomeric structures (*i.e.*, the metal- and

Asp252) of each protomer, forming a distorted octahedral geometry with a water molecule (*i.e.*, water-**19**) [Figs. 2(C) and 3(A)]. In the active site, several residues and water molecules are linked to each other by hydrogen bonds making space for substrate binding [20]. Compared with the apo*Hp*KDO8PSwt structure, the *Hp*KDO8PS-Cd Asp252 residue moves toward Asn255 when it is bound to a Cd²⁺ ion [Fig. 2(A) and (C)]; the distance between the two residues then changes from 7.6 Å to 4.8 Å by linking a water molecule (*i.e.*, water-**17**).

Instead of forming a hydrogen bond with Ser251, the Arg55-NH residue is bound to water-**24**, and the Arg55-N ε residue is linked to Thr56 via water-**23**, favoring a rigid conformation [Fig. 2(A) and (C)]. The link between Ser49-water-**5**-Lys52 [Fig. 2(A)] is complex

¹³ An extra helix in the HpKDO8PS structure.

in *Hp*KD08PS-Cd due to water-**18** and -**20**, which mediate the interaction with Asn54 via two additional water molecules (*i.e.*, water-**21** and water-**22**) [Fig. 2(C)]. The overall structure of the active site could become compact through polar interactions. Consequently, the distances between Ser49, Lys52, and Asn54 become shorter. More precisely, between apo*Hp*KD08PSwt and *Hp*KD08PS-Cd, the Ser49-Lys52 distance changes from 4.0 Å to 3.3 Å, the Ser49-Asn54 from 12.0 Å to 9.7 Å, and the Lys52-Asn54 from 9.3 Å to 7.0 Å.

We attempted to crystallize HpKDO8PS in complex with substrates to determine which residues participate in the binding of specific substrates, and HpKDO8PS-PEP-Zn crystals were obtained using various substrates and metal ion combinations. In *Hp*KDO8PS-PEP-Zn, a Zn^{2+} ion is bound to a site identical to the one bound by the Cd^{2+} ion in *Hp*KDO8PS-Cd. This binding forms a distorted square pyramidal geometry involving neighboring residues and a water molecule (*i.e.*, water-**34**) [Figs. 2(D) and 3(B)]. The overall folding conformation in *Hp*KDO8PS-PEP-Zn crystals appears to be similar to that of the model apoHpKDO8PSwt, though some residues generate differences in the active site responsible for substrate binding. Indeed, several residues interact directly with PEP. Notably, Ser49 and Lys52 are linked to PEP O2', whereas Lys130 captures PEP O2P, O2, and O1 [Fig. 2(C)]. More water molecules are observed in the HpKDO8PS-PEP-Zn active site, as they form hydrogen bonds with PEP and amino acids. Among them, water-27 and water-31 form a direct link with PEP O1P, whereas water-28 and water-30 bind to PEP O3P [Fig. 2(C)]. In particular, as in EcK-DO8PS and AaKDO8PS, water-**31** is consistently found on the PEP si side, which is expected to trigger the condensation reaction [25,26]. As the structure of HpKDO8PS-PEP-Zn was obtained at higher resolution compared to apoHpKDO8PSwt, it shows a more detailed water network together with Ser49, Lys52, Asn54, Arg55, Gln133, Ser251, Asp252, and Zn²⁺ [Fig. 2(D)].

In addition, structural comparison between *Hp*KDO8PS-Cd and *Hp*KDO8PS-PEP-Zn shows that the water molecules are located in similar positions in both crystals. Water-**18** in *Hp*KDO8PS-Cd is substituted to PEP O2' in linking Ser49 and Lys52 in *Hp*KDO8PS-PEP-Zn. Additionally, the connections found in *Hp*KDO8PS-Cd are consistently observed in *Hp*KDO8PS-PEP-Zn, starting at the trigonal link between Ser49, Lys52, and water-**20** (or water-**42** in *Hp*KDO8PS-PEP-Zn) and extending to Asn54 via water-**21** and water-**22** (or water-**40** and water-**43**) [Fig. 2(C) and (D)].

3.3. Thermal scanning for metal and metal-free HpKDO8PS interaction

The thermostabilizing effects of Cd^{2+} and Zn^{2+} on HpKDO8PS were measured by ITC and DSF experiments. The ITC data with Cd^{2+} injection were well fitted to the single-site binding in the isotherm model (Fig. 4). However, Zn^{2+} ion titration with the enzyme caused an exothermic reaction up to the 6th injection and an endothermic reaction in the following injections (from the 7th to the 20th injections), generating data that could not be fitted to the single-site binding isotherm model. Indeed, HpKDO8PS contains a single site for a Cd^{2+} ion, and the binding affinity (K_d) is 460.5 ± 75.5 nM, with a heat change (ΔH) of -5741 ± 61.83 cal/mol.

The DSF experiments were conducted as described in the Materials and Methods section. The fluorescent dye (SYPRO Orange) binds to the hydrophobic residues of the protein and fluoresces. During the thermal unfolding process, hydrophobic regions of the protein are exposed, and fluorescence intensity increases with enhanced dye binding [44]. Thus, thermostability can be evaluated from the shift in T_m . In the absence of metal, T_m was up to 48 °C, suggesting destabilization. In contrast, T_m was observed to shift to 63 °C in the presence of Cd²⁺ and was 59 °C in the presence of Zn²⁺

(Fig. 5). This result supported the hypothesis that Cd^{2+} contributes more to the thermostability of *Hp*KDO8PS than Zn^{2+} .

3.4. Validation of the virtual docking method

Before virtually screening our in-house chemical database with the Surflex-Dock program [36], the docking protocol was evaluated for its ability to reproduce the binding modes of known KDO8PS inhibitors [API and 2,8-bis(phosphonooxy)-octanoic acid] (Supplementary material Fig. S2). API [26], which mimics the intermediate form of the KDO8PS substrate condensation reaction, was used as a reference molecule by re-docking it into the active site of apo*Hp*KDO8PSwt. The API phosphate and phosphonate groups formed a hydrogen bond network with Ala108, Lys130 and Asn54, which was highly consistent with the reported co-crystal structure (not shown) [26], thereby validating our docking protocol.

3.5. HpKDO8PS-targeted virtual screening

A step-wise strategy for virtual screening was employed in our study to identify novel HpKDO8PS inhibitors, and various criteria were applied in combination to select hit compounds, as outlined in Fig. 6. First, 65 top-ranked compounds (out of 415 docked) were selected based on their Surflex-Dock energy score and Cscore (Cscore > 3) [36]. Then, their interactions with the receptor active site were then analyzed to select compounds with the required hydrogen bond interactions. Compounds that made contact with residues that have the desired hydrogen bonds (residues Asn54/ Arg55 and Lys130/Ala108) were considered to be real hits. The third filter was a visual inspection of the binding pose considering the diversity of ligand scaffolds, such as the number of hetero atoms, hydrogen bond donor/acceptor, different types of aromatic, and non-aromatic ring and alkyl groups. Finally, 21 compounds, all of which have drug-like profiles obeying Lipinski's rule-of-five [45], were selected for further bioactivity evaluation. For comparison, the 21 compounds were docked into HpKDO8PS-Cd, EcKDO8PS, and AaKDO8PS; the docking scores and ranks of the 21 compounds are listed in Supplementary material Table S2. In addition, virtual screening using those structures was conducted (Supplementary



Fig. 6. Scheme of structure-based virtual screening (see also Fig. S2 and Table S2).

material Table S3). The ranking of Surflex-Dock scores is slightly worse for the *Hp*KD08PS-Cd docking results and falls significantly when the compounds were docked into KD08PS from other species (*E. coli* and *A. aeolicus*).

3.6. NMR analysis of interactions between hit compounds and HpKDO8PS

To validate the results from our *in silico* virtual screening, we applied saturation transfer difference (STD) NMR spectroscopy and water-ligand observed via gradient spectroscopy (waterLOGSY) to evaluate whether the individual hit compounds bind to *Hp*KDO8PS [38,39,42,46].

STD NMR spectroscopy is a useful approach to detect a reduction in a ligand's NMR signal, which is caused by the nuclear Overhauser effect (NOE) between the protein and its ligand [39]. The onresonance spectrum records a 1D ¹H NMR signal for each compound, which is reduced by the magnetization transfer from *Hp*KDO8PS, whereas the off-resonance spectrum serves as a reference [Figs. 7 and 8(B), and Supplementary material Fig. S3(B)] that is identical to a standard 1D ¹H NMR spectrum for ligands [47]. The STD spectrum represents the difference between the on- and offresonance signals [Figs. 7 and 8(D), and Supplementary material Fig. S3(D)], and the signals on the STD spectrum reflect the interaction between the protein and its ligand. Figs. 7 and 8(C) and Supplementary material Fig. S3(C) show representative STD spectra for each compound in the absence of *Hp*KDO8PS.

Among the 21 potential ligands derived from our *in silico* virtual screening, three ligands, avicularin (quercetin-3-O- α -L-arabinofuranoside) (Supplementary material Fig. S3), hyperin (quercetin-3-O- β -D-galactopyranoside) (Fig. 7), and MC181 (*N*-(3-(furan-2-yl) phenyl)-1-(3-phenylpropyl)piperidine-4-carboxamide) (Fig. 8), produced STD spectra that indicated interactions with *Hp*KD08PS. Because hyperin and avicularin [Fig. 7(A) and Supplementary material Fig. S3(A)] have identical aromatic moieties involved in *Hp*KD08PS binding and exhibited only a minor difference in their auxiliary sugar moieties (β -D-galactopyranose for hyperin and α -L-arabinofuranose for avicularin), the following analysis was performed only for hyperin.

All of the five protons of hyperin exhibited STD signals, among which proton-**2** showed the largest reduction in signal ratio between the on- and off-resonance spectra [Fig. 7(D)]. For comparison, the levels of STD signals of the other protons were normalized to that of proton-**2**, as shown in Fig. 7(A). Proton-**1** exhibited the second-largest STD signal, which represented 75 percent of that of proton-**2**. The protons from the sugar moieties could not be observed because of buffer signals, though the buffer demonstrated no STD signals as an internal reference.

For MC181, proton-**6** exhibited the largest STD effect, and the STD effects of protons-**1**, -**3**, -**5**, -**8**, and -**10** were quantified and normalized to that of proton-**6**, demonstrating relatively low STD effects [Fig. 8(A)]. To corroborate the STD data, waterLOGSY experiments were performed for hyperin and MC181. This technique uses magnetization transfer from water to the free ligand and the ligand-bound protein via intermolecular NOE [48,49].

We compared the waterLOGSY spectra of the ligand in the absence [Figs. 7 and 8(G)] and presence [Figs. 7 and 8(H)] of *Hp*KDO8PS, showing the peaks in the opposite signs. The protons corresponding to these peaks are believed to interact with *Hp*KDO8PS via water molecules within the active site, and they were observed only at 6–7.5 ppm (hyperin) and 6.5–8 ppm (MC181). These results were highly consistent with those from the STD experiments.

To identify the *Hp*KDO8PS ligand-binding site, STD competition experiments were conducted using the natural substrates of



Fig. 7. ¹H STD and WaterLOGSY NMR spectra in the aromatic region and docking conformation of hyperin. (A) Chemical structure of hyperin and epitope mapping (each proton is numbered in red, and the values of the normalized STD effect are presented as percentages). (B) Reference ¹H NMR spectrum of hyperin. (C) STD spectrum of hyperin in the absence of HpKDO8PS. (D) STD spectrum of hyperin in the presence of HpKDO8PS. (E) STD spectrum of hyperin in the presence of HpKDO8PS and PEP. (F) STD spectrum of hyperin in the presence of HpKDO8PS and A5P. (G) WaterLOGSY spectrum of hyperin in the absence of HpKDO8PS. (H) WaterLOGSY spectrum of hyperin in the presence of HpKDO8PS. (I) Docking conformation of hyperin. The distances between residues (carbon atoms, orange), shown as sticks, and the compound are less than 3.5 Å. Hyperin (carbon atoms, green) forms hydrogen bonds (black dashed lines) with Asn54, Arg55, Asp87, His89, Lys130, Arg173, and Ser251. (J) Distances between the protons of hyperin and residues. The protons exhibiting an STD effect are numbered as in Fig. 7(A). Although proton-2 exhibited the largest STD effect, it is not consistent with the distances between the proton and residues (dashed arrows and indicated in *blue*) (see also Figs. S3 and S4). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

KDO8PS, PEP [Figs. 7 and 8(E)] and A5P [Fig. 7 and 8(F)], as competitors. These substrates are well known to bind to different parts of the same active site in KDO8PS [20,50].

The hyperin competition STD data showed that the STD peak intensities or hyperin in the presence of A5P were significantly



Fig. 8. ¹H STD and WaterLOGSY NMR spectra in the aromatic region and docking conformation of MC181. (A) Chemical structure of MC181 and epitope mapping (each proton is numbered in red, and the values of the normalized STD effect are presented as percentages). (B) Reference ¹H NMR spectrum of MC181. (C) STD spectrum of MC181 in the absence of HpKDO8PS. (D) STD spectrum of MC181 in the presence of HpKDO8PS. (E) STD spectrum of MC181 in the presence of HpKDO8PS and PEP. (F) STD spectrum of MC181 in the presence of HpKDO8PS and A5P. (G) WaterLOGSY spectrum of MC181 in the absence of HpKDO8PS. (H) WaterLOGSY spectrum of MC181 in the presence of HpKDO8PS. (I) Docking conformation of MC181. The distances between residues (carbon atoms, orange), shown as sticks, and the compound are less than 3.5 Å. MC181 (carbon atoms, green) forms hydrogen bonds (black dashed lines) with Ala53, Asn54, Arg55, and Asp87. (J) Distances between the protons of MC181 and residues. The protons exhibiting an STD effect are numbered as in Fig. 8(A). The epitope mapping result is consistent with the distances between the compound and enzyme. except for proton-12 (dashed arrows and indicated in blue) (see also Figs. S3 and S4). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

decreased but that PEP was less affected in 6-7.5 ppm of the hyperin STD spectrum compared with A5P [Fig. 7(E) and (F)]. These results indicate that hyperin competes with A5P for *Hp*KDO8PS binding, suggesting that hyperin binds to the A5P binding site (A5P-subsite) of *Hp*KDO8PS.

For MC181, the overall intensities of the STD spectra were significantly decreased in the presence of PEP or A5P [Fig. 8(E) and (F)], indicating that MC181 binds to a broad range of PEP and A5P binding sites within the HpKDO8PS active site. This result is in agreement with the fact that MC181 is a long molecule.

3.7. Docking analyses of HpKDO8PS ligands

The binding modes of representative hit compounds were predicted by Surflex-Dock docking [36]. As shown in Fig. 7 and 8(I), these results revealed that the hit compounds fit well in the area covering the combined binding sites for PEP and A5P, the natural KDO8PS substrates. The compounds were docked into the *Hp*KDO8PS-Cd structure, which is presumably more similar to the Zn²⁺-bound *Hp*KDO8PS structure than its apo-form. The binding modes of hyperin and MC181 were compared with that of the known inhibitor API [25] (Supplementary material Fig. S4), which was used as the reference compound for our docking analysis.

According to the hyperin-bound models, the hydroxyl groups of the sugar moiety are docked into the PEP binding site (PEP-subsite) [20,25] with hydrogen bonds to Lys130 and Arg173, which interact with nearby His204 [Fig. 7(I)]. However, hyperin exhibits no interaction with the metal because it is placed farther from the bound Cd²⁺ ion compared with the API binding site. In addition, the hydrogen bonds with Lys47, Lys52, and Ala108 in the API-bound model disappear in the hyperin-docked conformation [Fig. 7(I) and Supplementary material Fig. S4]. The hyperin benzopyran ring occupies the site where A5P binds (A5P-subsite) in the *Aa*KDO8PS structure [20,25] [Fig. 7(I)], forming multiple hydrogen bonds with Asn54, Arg55 and Ser251, which are not observed in the API-bound model. The hyperin dihydroxyphenyl ring is placed near Asp87, His89, and Pro107 via hydrogen bonds and hydrophobic interactions [Fig. 7(I)].

In the MC181 binding mode analysis [Fig. 8(I)], the PEP-subsite [20] is occupied by the MC181 furan ring, which interacts with Gln105, Pro107, and Lys130 at a short distance. Although the furan ring of MC181 is farther away from the Cd²⁺ ion than the PEP moiety in the API-bound model, the phenyl group of MC181 is well matched to API [Fig. 8(I) and Supplementary material Fig. S4]. Indeed, it binds to Leu182 via a hydrophobic interaction and also interacts with His204, Gln207, and Asp252. MC181 covers the A5P-subsite with its piperidine ring, and the peptide oxygen forms hydrogen bonds with Ala53, Asn54, and Arg55 [Fig. 8(I)]. Asn54 also forms a hydrogen bond with the peptide's nitrogen atom.

MC181 was also docked into *Ec*KDO8PS and *Aa*KDO8PS, and the results showed that the key residues for MC181 binding are different from those in *Hp*KDO8PS. MC181 binds to Asn62, Arg63, Ser64, Ala116, Lys138, His202, and Gln205 in *Ec*KDO8PS, or Asn48, Arg49, Ser50, Ala102, Lys124, His185, and Gln188 in *Aa*KDO8PS. Considering the protein sequence differences between *Hp*KDO8PS, *Ec*KDO8PS, and *Aa*KDO8PS, the binding key residues are 45% (*Ec*KDO8PS) or 52% (*Aa*KDO8PS) identical compared with those of *Hp*KDO8PS.

Analysis of the three catalytic pockets (*Hp*KDO8PS, *Ec*KDO8PS and *Aa*KDO8PS) revealed that although the active sites are highly conserved, the dimensions of the catalytic channel rim differ greatly [Fig. 9(A)]. Fig. 9(A) shows four regions, A–D, that represent the boundaries of the catalytic channel rim. Region A corresponds to Asn54 in *Hp*KDO8PS, Ser232 in *Aa*KDO8PS and Asn26 in *Ec*K-DO8PS, region B to Arg55 in *Hp*KDO8PS and Arg49, Ser50 in



Fig. 9. Top–down view of the active site of AaKDO8PS (top), *Hp*KD08PS (middle) and EcKD08PS (bottom). (A) The distances between the boundaries of the catalytic channel rim are shown with red arrows. Letters A–D denote four boundary areas surrounding the channel rim. (B) The binding poses of MC181 in the active sites of AaKD08PS (top), *Hp*KD08PS (middle) and EcKD08PS (bottom). (A) The distances between the boundaries of the catalytic channel rim are shown with red arrows. Letters A–D denote four boundary areas surrounding the channel rim. (B) The binding poses of MC181 in the active sites of AaKD08PS (top), *Hp*KD08PS (middle) and EcKD08PS (bottom). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

AakDO8PS, region C to Gln207 in *Hp*KDO8PS, Phe103 in *Aa*KDO8PS and Phe117 in *Ec*KDO8PS, and region D to Phe134 in *Hp*KDO8PS and Pro190 in *Aa*KDO8PS. Variation at the A, B and C regions produces a significantly wider channel rim in the crystal structure of *Hp*KDO8PS compared to the other two KDO8PSs. The structural manifestation of this variation is that the measured B–D distance is 6.21 Å in *Aa*KDO8PS compared to 11.7 Å in *Hp*KDO8PS, whereas the measured A–C distance is 10.06 Å in *Ec*KDO8PS compared to 20.6 Å in *Hp*KDO8PS.

The binding poses of MC181 in the active site of *Aa*KDO8PS or *Ec*KDO8PS revealed that the catalytic channel is too narrow to accommodate the entire MC181 molecule, with the furan ring and phenyl group of MC181 protruding out of the binding pocket [Fig. 9(B)]. Nonetheless, MC181 can bind deeply at the bottom of the *Hp*KDO8PS catalytic channel, which occupies both the PEP- and A5P-subsites.

4. Discussion

*H*pKDO8PS possesses an extra helix (HE) (Fig. 1) that is not observed in the structures of KDO8PS from *E. coli, A. aeolicus, B. cenocepacia* [23], *P. aeruginosa* [24], and *N. meningitides* [22]. This HE interacts with its neighboring H5 α-helix, S6 β-strand, and loops (H6–S7 and HE–S6) through hydrogen bonds and hydrophobic interactions [Fig. 1(D)]. Arg151 interacts with Gly167 at the HE–S6 loop [Fig. 1(D), right panel], an interaction that affects the PEP-binding residues Lys130 and Gln133, which are located at the N-

terminus of the loop. Moreover, Leu164, which is located in this same loop, forms a hydrophobic interaction with Pro197 in the H6–S7 loop [Fig. 1(D), left panel], which in turn affects the S7–H7 loop, where Gln207 and His204 form an A5P and a metal ligand-binding site, respectively. Based on these observations, the additional HE may play a role in indirectly stabilizing the conformation of the active site. However, further studies are needed to evaluate the possible role of the HE.

*Hp*KD08PS is a metal-dependent enzyme that has four metalcoordinating residues, namely, Cys18, His204, Glu241, and Asp252, in the active site, similar to *Aa*KD08PS [20]. These residues were mutated to determine whether they are related to protein stability and active site coordination. Of the four mutants (C18A, H204A, E241A, and D252A), the crystal structure of the H204A (*Hp*KD08PS_H204A) was determined. As shown in our CD spectroscopy studies, *Hp*KD08PS_H204A demonstrated a better stability in its secondary structure than the other mutants, which presumably led to crystallization success (Supplementary material Fig. S1). The two other mutants, E241A and D252A, were unavailable due to low protein stability during purification.

The structure of *Hp*KD08PS_H204A [Fig. 2(B)] showed that the residues adjacent to His204 in the active site became closer to each other. Moreover, the conformations of Asn54, Lys52, and Asn255 were significantly altered with respect to our other wild-type structures. Because the residues adjacent to His204 are related to substrate and metal binding, His204 is believed to not only serve as a metal-binding residue but also as a contributor to active site

formation. These results are consistent with a previous report showing that the His185 residue in the active site of *Aa*KDO8PS is responsible for forming the substrate binding space [27].

The structures of *Hp*KDO8PS in complex with Cd^{2+} or PEP/Zn²⁺ (*Hp*KDO8PS-Cd and *Hp*KDO8PS-PEP-Zn) [Fig. 2(C) and (D)] were determined, and different hydrogen bond networks with water molecules were observed within the active site. In particular, the *Hp*KDO8PS-PEP-Zn active site appeared rigid and therefore stabilized via its complicated hydrogen bond network [Fig. 2(D)]. By facilitating the condensation process, the water molecules in the structures appear to be involved in the formation of a stable complex between the protein and ligands. The idea of a protein-ligand interaction mediated by water was supported by waterLOGSY-NMR experiments [51].

Interestingly, in these structures, *Hp*KDO8PS binds to Zn^{2+} and Cd^{2+} ions in distorted square pyramidal and octahedral geometries [Fig. 3(A) and (B)], respectively, in contrast to the AaKDO8PS enzyme structure [29]. Moreover, in contrast with Cd²⁺, the ITC data (Fig. 4) for Zn^{2+} , the naturally occurring metal ion binding to HpKDO8PS, did not fit to the single binding site isothermal model, possibly for the following reasons: (i) multiple binding modes could exist during the equilibration process between Zn^{2+} and *Hp*KDO8PS; (ii) inevitable interactions could occur with the buffer; or (iii) metal ion precipitation could generate heat [52] (data not shown). The DSF data supported the idea that Cd^{2+} enhances *Hp*KDO8PS thermostability to a greater degree than Zn^{2+} (Fig. 5). According to Krosky and colleagues, the K_{cat} value increases by approximately 2-fold, and the A5P K_m value decreases by approximately 6.5-fold upon replacement of the Zn^{2+} ion by Cd^{2+} in Zn^{2+} bound *Hp*KDO8PS [19]. Consistently, our metal coordination results indicated that the metal geometries of Cd²⁺ and Zn²⁺ could affect enzyme activity. Indeed, the properties of metal ions as Lewis acids play a particularly important role in biology: metal ions can activate coordinated ligands for reactivity by affecting either bond length, bond angles, or coordination site number [53]. In *Hp*KDO8PS, Cd²⁺ may favor a more ideal coordination than Zn²⁺ for the enzymatic reaction.

To date, only a few inhibitors of KDO8PS have been reported (Supplementary material Fig. S2), all of which were designed to mimic the intermediate form of the condensation reaction between A5P and PEP, and the lack of known ligands has limited the use of conventional ligand-based screening methods to identify novel KDO8PS inhibitors. Instead, structural information for the crystal structure of *Hp*KDO8PS determined for the first time in our present study was utilized to identify novel scaffolds that specifically bind to *Hp*KDO8PS using structure-based virtual screening.

This step-wise virtual screening approach successfully identified three novel chemotypes (avicularin, hyperin, and MC181) as HpKDO8PS inhibitors. To the best of our knowledge, this is the first in silico study on the identification of novel KDO8PS inhibitors. For comparison, an API-based 3D similarity search against the same inhouse database was conducted using the SurflexSim program [36]. Notably, three active compounds that directly bind to HpKDO8PS were highly ranked in the output of Surflex-Dock scoring (docking ranks ranged from 16 to 21), and the ranking was significantly lower when they were docked into EcKDO8PS and AaKDO8PS, suggesting that the compounds may specifically bind to HpKDO8PS. However, a simple 3D similarity search failed to select the most active compounds (similarity ranks ranged from 77 to 146), as shown in the Supplementary material Table S2. Taken together, the results demonstrated that the virtual screening approach was successful for the discovery of novel KDO8PS inhibitors.

The *Hp*KDO8PS binding modes of hyperin and MC181 were investigated using STD-NMR experiments and docking simulations. According to the docking results, the A5P-subsite is spatially

occupied by the benzopyran ring of hyperin, which is consistent with the epitope mapping results from STD experiments [Fig. 7(A)], even though the STD values could not be easily converted to the distance between the protein and ligand [Fig. 7(J)]. This result from the docking experiments was also confirmed by competition data, which indicated that hyperin competes with A5P for the ligand binding site [Fig. 7(F)]. Although the sugar moiety of hyperin binds to the PEP-subsite, according to the docking simulation results, it was difficult to confirm these data by NMR experiments because the protons of the sugar moiety could not be detected on the NMR spectra.

According to docking simulations, MC181 and API share similar binding HpKDO8PS modes, despite their different chemical structures (Fig. 8 and Supplementary material Fig. S4). MC181, through hydrogen bonds and hydrophobic interactions, binds to and broadly covers the PEP- and the A5P-subsites in HpKDO8PS. These results were highly consistent with the results obtained from STD competition experiments against PEP or A5P [Fig. 8(E) and (F)], which showed a decline in the STD spectral intensities. The epitope mapping results [Fig. 8(A)] were also consistent with the observed distances between the compounds and enzyme, as shown in Fig. 8(J). MC181 was also docked into EcKDO8PS and AaKDO8PS to investigate whether it specifically binds to HpKDO8PS (Fig. 9). The active sites are highly conserved, yet the catalytic channel rims of the enzymes differ significantly [Fig. 9(A)], with HpKDO8PS having a wider channel rim, which is consistent with the MC181 docking results showing that MC181 binds well to the HpKDO8PS PEP- and A5P-subsites. Converselv. due to their narrow channel rims. MC181 iuts out from the active sites of EcKDO8PS and AaKDO8PS [Fig. 9(B)]. Based on the results, MC181 can be considered a promising scaffold for developing new antibiotics that specifically act against H. pylori.

The interactions observed for MC181 involved both hydrophobic and hydrophilic binding modes, whereas hyperin primarily interacted with *Hp*KDO8PS via hydrophilic interactions. Furthermore, the compound docking results provided clues for the modification of these inhibitors. Hyperin and avicularin are derivatives of quercetin, representing a typical subclass of flavonoids [54], which have been reported to exert various biological effects, including antimicrobial, antihypertensive, neuroprotective, and chemoprotective effects [54–56]. Among those biological activities, the antimicrobial effect is supported by the interactions between *Hp*KDO8PS and these compounds (hyperin [57,58] and avicularin [59]).

5. Conclusions

Due to increased resistance to antibiotics and the gastrointestinal side effects of conventional multiple therapy, the development of new anti-*H. pylori* drugs is urgently needed. The LPS synthesis pathway is considered to be a target for developing antibiotics against *H. pylori*, and *Hp*KDO8PS is an essential enzyme that catalyzes the condensation of A5P and PEP to generate KDO8P, the precursor of LPS biosynthesis. The current study provides information of *Hp*KDO8PS crystal structures, and among 21 possible ligands generated via *in silico* virtual screening, the capacity of 3 compounds to bind to *Hp*KDO8PS was demonstrated through STD and waterLOGSY experiments. It is expected that this study will provide a basis for the design of new and selective *Hp*KDO8PS inhibitors.

Author contributions

B.L. conceived this project and designed the experiments, S.C. contributed to the protein purification, site-directed mutagenesis, CD spectroscopy, ITC experiments. S.C., H.I., and H.Y. performed the

protein crystallization and structure determination. S.C. and K.L. carried out the STD and waterLOGSY NMR experiments. H.P. and J.C. conducted the docking simulation, virtual screening, and compound preparation. K.H.M and H.J.K. synthesized MC181. S.C., H.I., K.L., and B.L. wrote the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2015.11.036.

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