Epoxide hydrolases (EHs) have been characterized and engineered as biocatalysts that convert epoxides to valuable chiral vicinal diol precursors of drugs and bioactive compounds. Nonetheless, the regioselectivity control of the epoxide ring opening by EHs remains challenging. Alp1U is an α/β-fold EH that exhibits poor regioselectivity in the epoxide hydrolysis of fluostatin C (compound 1) and produces a pair of stereoisomers. Herein, we established the absolute configuration of the two stereoisomeric products and determined the crystal structure of Alp1U. A Trp-186/Trp-187/Tyr-247 oxirane oxygen hole was identified in Alp1U that replaced the canonical Tyr/Tyr pair in α/β-EHs. Mutation of residues in the atypical oxirane oxygen hole of Alp1U improved the regioselectivity for epoxide hydrolysis on 1. The single site Y247F mutation led to highly regioselective (94%) nucleophilic attack at C-2. Furthermore, single-crystal X-ray structures of the two regioselective Alp1U variants in complex with 1 were determined. These findings allowed insights into the reaction details of Alp1U and provided a new approach for engineering regioselective epoxide hydrolases.

Stereoselective hydrolytic opening of epoxide rings is attractive in asymmetric synthesis, because the resultant vicinal diols are valuable building blocks of chiral drugs and bioactive compounds (1, 2). In biological systems, epoxide hydrolases (EHs) are extensively studied to catalyze the cofactor-independent hydrolysis of epoxides to vicinal diols (3, 4). The enzymatic mechanisms of EHs have been well-characterized by substrate selectivity and crystal structures combined with molecular dynamics simulations and directed evolution approaches (5–9). Most EHs belong to members of a family with an α/β-hydrolase-fold, sharing a catalytic core domain and a cap domain (10). The α/β-EHs usually feature a catalytic triad and an oxirane oxygen hole that enable a two-step mechanism involving an S_n2 reaction and ester hydrolysis (Fig. 1A) (10, 11). The catalytic triad contains two aspartates and one histidine (10). In the first step, the attack of the nucleophilic aspartate ion against the epoxide ring forms a covalently linked enzyme-acyloxoy-substrate adduct (5, 6). In the second step, the His and the other Asp activate a water molecule to hydrolyze the enzyme-acyl-oxo-substrate intermediate and release the products as trans-vicinal diols (12, 13). The oxirane oxygen hole contains a pair of tyrosines, which are proposed to donate hydrogen bonds to the oxirane oxygen, thereby assisting the first step of reaction sequence by activating the epoxide and stabilizing the formed oxoanion (6, 14). Another family of EHs, represented by the limonene-1,2-epoxide hydrolases, catalyzes a one-step mechanism with S_n12 ring opening of the epoxide ring by an enzyme-activated water molecule (Fig. 1B) (15).

These EH-catalyzed epoxide ring-opening mechanisms generally follow the S_n2 type, with backside attack by the nucleophile resulting in inversion of stereoconfiguration at the reactive carbon center (6, 15). However, engineering control of the regioselectivity into the epoxide ring-opening reaction remains challenging for EHs (15, 16). In synthetic epoxide hydrolysis, the regioselectivity depends on the substituents on the epoxide carbons (17) and the strength of the nucleophilic reagent. However, in the EH-catalyzed epoxide ring-opening reactions, the regioselectivity is influenced more by the structure of the enzyme active site (18). These steric constraints can potentially also control the stereochecmistry of the product. This hypothesis is supported by enzyme engineering of EHs by directed evolution strategy or small mutagenic library screening (15, 16, 19–21). Unfortunately, mechanistic details of what causes regioselectivity improvement of engineered EHs are still elusive.

In the biosynthesis of vicinal diols, the chirality of these functional natural products derived from oxiranes plays important roles toward directing of postmodifications of the hydroxy groups, such as acylation and glycosylation. Representative examples include the EH Nasvi-EH1 involved in the biosynthesis of an insect sex attractant (22), the α/β-EHs NcsF2 and SgcF-catalyzed epoxide ring opening in the biosynthesis of endiye antitumor antibiotics neocarzinostatins and C-1027 (23, 24), the Tsrl-mediated endopeptidyl hydrolysis and epoxide ring opening/macrocyclization in thiostrepton biosynthesis (25), and the Alp1U-catalyzed epoxide hydrolysis of epoxykainamycins (Fig. 1C) (26). Interestingly, we have shown that the Alp1U found in Streptomyces ambofaciens also uses fluostatins...
Regioselectivity switch of the $\alpha/\beta$-epoxide hydrolase Alp1U

A two-step epoxide hydrolysis by $\alpha/\beta$-fold EHs

B one-step epoxide hydrolysis by limonene EHs

C epoxide hydrolysis by Alp1U

(FST) C (1) from *Micromonospora rosaria* (27, 28) as a substrate and catalyzes the epoxide hydrolysis to produce a pair of stereoisomers, FST C1 (1a) and FST C2 (1b). The origins of these stereoisomers were proposed to result from nonregioselective hydrolysis by nucleophilic attack at the C-2 and C-3 position of 1 (Fig. 1C) (29).
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Herein, we determined the absolute stereochemistry of the two products FSTs C1 (1a) and C2 (1b) from Alp1U-catalyzed epoxide ring opening of FST C (1) and solved the X-ray crystal structure of Alp1U. An atypical oxirane oxygen hole is identified in Alp1U, consisting of three residues (Trp-186/Trp-187/Tyr-247) that are distinct from the Tyr/Tyr pair in classic α/β-EHs. The regioselectivity was improved for the epoxide ring opening of I in the single mutant Y247F to occur predominantly at C-3 and in the double mutant W187F/Y247F mainly at C-2. Single-crystal X-ray structures of these two Alp1U variants complexed with I were determined to help infer mechanistic insights into their opposite regioselectivities.

Results and discussion

Determination of the stereochemistry of epoxide ring-opening products

We have previously shown that Alp1U converts fluostatin C (1) to two stereoisomers, 1a and 1b, the structures of which have not yet been elucidated (29). Subsequently, compounds 1a and 1b had a molecular formula of C_{18}H_{14}O_{7} (1b) to two stereoisomers, 1a and 1b, the structures of which have not yet been elucidated (29). Subsequently, compounds 1a and 1b had a molecular formula of C_{18}H_{14}O_{7} (1b). The NOESY correlation of OH-1 and H3-12 was indicated by the NOESY correlation of OH-1 and H3-12. The trans 12,3-triol moiety in 1a was established by the NOESY correlation of H-1 and CH3-3 and a second Tyr is putatively replaced by a tryptophan (Trp-186). The regioselectivity was improved for the epoxide carbon and forms a covalent alkyl-enzyme intermediate. In the second step, His-300 activates a water molecule, in accordance with (1a)-EHs reveals that the catalytic triad of classic EHs is conserved overall fold (Z-score 38.2) (28). The trans relative configuration of H-1 and CH3-3 in 1a was indicated by the NOESY correlation of OH-1 and H3-12 (Fig. 2A). To determine the relative configuration of H-1 and H-2, a propylidene acetal derivative of 1a (1a-At) was prepared (Fig. 2A). The cis relative configuration of the 1,2-diol moiety in 1a-At was established by the markedly different proton chemical shifts of the isopropylidene methyl groups (ΔδH 0.36) and the relatively small coupling constant between H-1 and H-2 (ΔJ_{H-1,H-2} = 5.5 Hz) (Fig. 2A, Fig. S3, and Table S2) (30, 31). The NOESY correlation of H-2/OH-3 in 1a-At confirmed the relative trans configuration of OH-2 and OH-3 (Fig. 2A and Fig. S3). Therefore, the relative configuration of the 1,2,3-triol moiety in 1a was determined as (1R,2R*,3S*) in accordance with the configurations of 1a-At. Considering that 1a was a ring-opened product of 2,3-epoxide 1, it must preserve the original (R) absolute configuration at C-1, so the absolute configuration of 1a was assigned as (1R,2R*,3S*). The trans relative configuration of H-1 and H-2 in 1b was indicated by the NOESY correlation of H-1 and OH-2 and the large value of the 3J_{H-1,H-2} coupling constant in methanol-d4 (Table S3 and Fig. S4). The NOESY correlation of H-1 and H-2 was also observed in 1b (Fig. 2B and Fig. S2), suggesting the cis axial orientation of H-1 and CH3-13 and a trans-vcinal diol moiety at C-2/C-3 in 1b (Fig. 2B). In accordance, the three hydroxyl groups adopted equatorial arrangement in the low-energy computed ωB97X/TZVP polarizable continuum model (PCM)/MeCN conformers of (1R,2S,3R)-1b, whereas H-1 and CH3-3 were axial. The absolute configuration of 1b was determined as (1R,2S,3R) based on the good agreement between the experimental and calculated electronic circular dichroism (ECD) spectra of (1R,2S,3R)-1b (Fig. 2B). To further confirm the absolute configuration of 1b, we tried derivatization with p-bromobenzoyl that was reported to enhance crystallization (32). A pyridine derivative of 1b (1b-Py) could be obtained as single crystals from the reaction system, and it was determined to have a (3R) configuration by X-ray diffraction analysis, with a Flack parameter of 0.04 (10) (Fig. 2C, Fig. S5, and Table S4, CCDC 2016594). This supports the (3R) absolute configuration in 1b. Taken together, the structures of 1a and 1b suggest that the nucleophilic attack by Alp1U at C-2 of 1 produces 1a, whereas 1b derives from the attack at C-3.

Crystal structure of Alp1U reveals an atypical oxirane oxanion hole

To learn more details about the catalytic mechanism of the Alp1U reaction, the ligand-free structure of N-terminal His-tagged Alp1U was determined to 2.45 Å, with four chains per asymmetric unit (Table 1 and Fig. S6). The four Alp1U chains form two dimers according to PISA calculation (33). Each chain takes the canonical α/β-hydrolase fold, comprising a catalytic domain (residues 27–166 and 251–319) linked to an α-helical cap (residues 167–250) (Fig. 3A). The 26 residues at the N terminus that share no sequence similarity to reported crystal structures of EHs are not resolved in the structure likely because of the flexibility of this region. A PDB database search of the Alp1U coordinates by the Dali server (34) reveals that Alp1U is most similar to a fluoroacetate dehalogenase (FAcD, PDB entry 3R3U, Z-score 38.5) (35). However, Alp1U shares a conserved overall fold (Z-score 38.2–25) with a batch of α/β-EHs, despite low identities in their amino acid sequences (<30%), including the extensively studied epichlorohydrin epoxide hydrolase (EChA) from Agrobacterium radiobacter AD1 (PDB entry 1EHY) (6), BmEH from Bacillus megaterium ECU1001 (PDB entry 4IIO0) (13), the human soluble epoxide hydrolase (sEH, PDB entry 4HAI) (36), and the bacterial virulence factor EH (cif) from Pseudomonas aeruginosa (PDB entry 5TN2) (12) (Fig. S7). Structural alignment of Alp1U with these α/β-EHs reveals that the catalytic triad of classic EHs is conserved in Alp1U, consisting of residues Asp-137, His-300, and Asp-161 (Fig. 3B). We propose that Alp1U catalyzes a two-step epoxide ring-opening reaction as the classic α/β-EHs (Fig. 1A). In the first step, Asp-137 launches a nucleophilic attack at the epoxide carbon and forms a covalent alkyl-enzyme intermediate. In the second step, His-300 activates a water molecule, in cooperation with Asp-161, hydrolyzing the alkyl-enzyme intermediate to release the vicinal diol product and Asp-137. However, the Alp1U active site is different from those of classic α/β-EHs in the key oxirane oxygen hole (Fig. 3B). The canonical oxirane oxygen hole of α/β-EHs contains two Tyr residues that play critical roles in the first catalytic step, by forming H-bonds with the epoxide oxygen and stabilizing the liberated oxirane oxanion (Figs. 1A and 3B) (8, 14). However, Alp1U has only one of the two conserved tyrosines (Tyr-247), whereas the second Tyr is putatively replaced by a tryptophan (Trp-186).
with its indole side chain being positioned for donating an H-bond to the oxirane oxygen (Fig. 3B). Also, the indole side chain of the adjacent Trp-187 is properly positioned to donate an H-bond to the oxirane oxygen (Fig. 3B). Therefore, the three residues Trp-186, Trp-187, and Tyr-247 putatively form an atypical oxirane oxygen hole in Alp1U to donor H-bonds to the epoxide oxygen.

Substrate regioselectivity by oxirane oxygen hole engineering

To confirm the proposed roles of the catalytic residues, the Alp1U mutants were generated by site-directed mutagenesis (Fig. 3C). As expected, three separate mutants, D137N, H300F, and H300G, completely abolished catalytic activity for 1 (Fig. 3C). The W186F mutant completely lost the activity, whereas the W187F mutant maintained the activity (Fig. 3C). It seems that Trp-186, rather than Trp-187, is required catalytically to donate an H-bond to the oxirane oxygen in 1. Subsequently, the W186Y mutant was made to mimic the classic oxirane oxygen hole in EHs (10). However, W186Y showed no activity (Fig. 3C), likely because the side chain of Tyr-186 could not properly orient to interact with the oxirane oxygen.

It should be noted that a histidine (His-115) forms the oxirane oxygen hole with Tyr-219 in the bacterial virulence factor EH cif (Fig. 3B) (37), and a W186H mutant was also constructed. However, the W186H mutant only showed trace catalytic activity for the epoxide ring opening of 1 (Fig. 3C), likely because the side chain of Tyr-186 could not properly orient to interact with the oxirane oxygen.

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Figure 2. Establishment of stereochemistry of products 1a and 1b. A, key COSY, HMBC, and selected NOESY correlations for 1a; chemical derivatization of 1a to afford 1a-At, and the NMR analysis of the derivative (1a-At). B, key COSY, HMBC, and selected NOESY correlations for 1b, geometries of the overlapped six lowest-energy ωB97X/TZVP PCM/McCN conformers of (1R,2S,3R)-1b, and experimental ECD spectrum of 1b (black line) compared with the B3LYP/TZVP PCM/McCN//ωB97X/TZVP PCM/McCN spectrum of (1R,2S,3R)-1b (red line). The bars represent rotational strength values for the lowest-energy solution conformers. C, a bromobenzoyl derivatization reaction of 1b yields single crystals of the pyridine derivative 1b-Py with (3R) absolute configuration.

Regioselectivity switch of the α/β-epoxide hydrolase Alp1U

Figure 2. Establishment of stereochemistry of products 1a and 1b. A, key COSY, HMBC, and selected NOESY correlations for 1a; chemical derivatization of 1a to afford 1a-At, and the NMR analysis of the derivative (1a-At). B, key COSY, HMBC, and selected NOESY correlations for 1b, geometries of the overlapped six lowest-energy ωB97X/TZVP PCM/McCN conformers of (1R,2S,3R)-1b, and experimental ECD spectrum of 1b (black line) compared with the B3LYP/TZVP PCM/McCN//ωB97X/TZVP PCM/McCN spectrum of (1R,2S,3R)-1b (red line). The bars represent rotational strength values for the lowest-energy solution conformers. C, a bromobenzoyl derivatization reaction of 1b yields single crystals of the pyridine derivative 1b-Py with (3R) absolute configuration.
conserved Tyr-247 in Alp1U is a key H-bond donor to stabilize the oxirane oxygen of 1. To verify this hypothesis, the W187F/ Y247F double mutant was constructed. Unexpectedly, the Alp1U\textsubscript{W187F/Y247F} mutant selectively produced \textit{1a} (94%) as a result of preferentially attacking at C-2. These observations are in sharp contrast to previous reports that classic \textit{α}/\textit{β}-EHSs slightly lost the activity if one of the two catalytic Tyr residues in the oxirane oxygen hole was mutated and became completely inactive if both Tyr residues were mutated (7). To the best of our knowledge, such significant regioselectivity improvement and switch by mutated residues of the oxirane oxygen hole in Alp1U are unprecedented in EHSs that catalyze epoxide ring-opening reactions.

**Structural basis for the switch in regioselectivity**

To investigate how the Alp1U mutants achieved the regioselectivity, the crystal structures of the Alp1U\textsubscript{Y247F} (1.78 Å) and Alp1U\textsubscript{W187F/Y247F} (2.1 Å) mutants were determined in complex with the substrate FST C (1), by soaking with an excess of 1 (Table 1). In both crystal structures, the clear electron density of 1 is observed (Fig. 4B and Fig. S8). Superposition reveals that the two structure complexes are essentially identical to the apo-Alp1U structure (Fig. S9). The catalytic cavity of Alp1U forms a deep groove, with a negatively charged surface, which binds the substrate FST C (1) (Fig. 4A). FST C (1) is bound to Alp1U mainly by direct and water-mediated hydrogen bond networks (Fig. 4B). The OH-1 hydroxy group of 1 orients to the inner side of the active site. Therefore, the O-acetyl or O-methyl derivatization at OH-1 could hamper FSTs from entering the Alp1U active site for a steric crowding effect. This might explain our previously reported phenomenon that Alp1U fails to convert FSTs containing an O-acetyl or O-methyl group at the C1 position (29).

Similar binding patterns of 1 are observed in both structures of Alp1U\textsubscript{Y247F} and Alp1U\textsubscript{W187F/Y247F} in complex with 1 and suggest that Alp1U uses the same epoxide ring–opening mechanism as that of classic \textit{α}/\textit{β}-EHSs (Fig. 4, C–E). In both structures (Fig. 4, C and D), the carboxyl side chain of Asp-137 locates just below the epoxide ring of 1, ready for the nucleophilic attack. His-300 locates adjacent to Asp-137 and forms a hydrogen bond with Asp-161, consistent with its general role to activate one water molecule for hydrolyzing the covalent substrate-enzyme adduct and the subsequent release of Asp-137. The epoxide ring of FST C (1) is accommodated in the putative oxirane oxygen hole defined by the side chains of Trp-186, Trp-187 (3.24 Å) and OH of Tyr-247 (2.81 Å). However, the N-H···O angle (Fig. 4F) to the indole N-H of Trp-187 (3.24 Å) and OH of Tyr-247 (2.81 Å), however, the N-H···O angle and O-H···O angle (Fig. 4F) are inappropriate to donate H-bonds, considering that the ideal X-H···Y angle for a hydrogen bond is generally linear or close to 180° (38). This may explain why the W187F and Y247F mutants retain the catalytic activity.

**Table 1**

Crystal parameters, data collection, and refinement statistics

|                  | Alp1U | Alp1U\textsubscript{Y247F} | Alp1U\textsubscript{W187F/Y247F} |
|------------------|-------|---------------------------|-------------------------------|
| PDB ID           | 6KXR  | 6KXH                      | 7CLZ                         |
| Wavelength (Å)   | 0.97894 | 0.97853                  | 0.97853                      |
| Resolution range (Å) | 70.19–2.45 | 31.98–1.78                  | 44.7–2.1                       |
| Space group      | P 21 21 21 | P 21 21 21                | P 21 21 21                   |
| Unit cell (Å)    | a = 97.09, b = 101.58, c = 117.42, α = β = γ = 90 | a = 97.48, b = 101.56, c = 117.48, α = β = γ = 90 | a = 96.66, b = 100.21, c = 117.45, α = β = γ = 90 |
| Total reflections | 86,069 (8505) | 111,803 (10,937) | 134,284 (13,302) |
| Unique reflections | 43,063 (4255) | 10,016 (9,979)            | 9,125 (9,078)                |
| Multiplicity     | 2.0 (2.0) | 16.18 (2.99)              | 5.85 (2.95)                  |
| Completeness (%) | 99.35 (99.51) | 99.88 (99.97)            | 99.98 (99.97)                |
| Mean I/σ(I)      | 1.1 (1.1) | 26.43                     | 31.22                        |
| Wilson B-factor  | 0.02819 (0.0004) | 0.1120 (0.0756)             | 0.03037 (0.188)              |
| Reactions used for R-free (%) | 4.77 | 4.97                     | 5.20                          |
| R-work           | 0.0168 (0.2150) | 0.1712 (0.2162)            | 0.1932 (0.2190)              |
| R-free           | 0.2344 (0.3197) | 0.2061 (0.2691)            | 0.2468 (0.3006)              |
| No. of nonhydrogen atoms | 9233 | 10,016 (9,979) | 9,125 (9,078) |
| Macromolecules   | 9097 | 9,125                     | 9,125                        |
| Ligands          | 127 | 117                      | 117                          |
| Water            | 127 | 354                      | 354                          |
| Protein residues | 1170 | 1173                     | 1172                         |
| RMSD (bonds) (Å) | 0.008 | 0.007                    | 0.009                        |
| RMSD (angles) (degrees) | 1.11 | 1.14                  | 1.22                         |
| Ramachandran favored (%) | 96 | 97                     | 97                           |
| Ramachandran outliers (%) | 0.34 | 0.34                  | 0.34                         |
| Clashscore       | 10.12 | 5.81                     | 7.51                         |
| Average B-factor | 45.30 | 34.10                    | 32.40                        |
| Macromolecules   | 45.40 | 33.70                    | 32.30                        |
| Ligands          | 49.50 | 36.80                    | 39.30                        |
| Solvent          | 39.30 | 38.50                    | 33.50                        |
Phe-247 and Phe-187 in Alp1UW187F/Y247F. This rotation brings the carboxyl oxygen of Asp-137 significantly closer to C-2 of 1 in the Alp1UW187F/Y247F/1 complex (2.91 Å) than in the Alp1UY247F/1 complex (3.18 Å). This new positioning likely directs the regioselective attack at C-2 of the epoxide ring to selectively produce 1a (94%). This observation is consistent with previous studies describing the distances between the nucleophile and epoxide carbons as essential toward determining the regioselectivity of other EHs (14, 15, 20). However, the Y247F mutant prefers a regioselective attack at C-3 of 1 to produce 1b (98%), despite the similar distances between the nucleophile Asp-137 and C-2 (3.18 Å) and between Asp-137 and the C-3 carbon (3.24 Å) in the Alp1UY247F/1 complex. It is suggested from the Alp1UY247F/1 complex that the hydrophobic effect of the phenyl side chain, introduced by the Y247F mutation, disfavors the carboxyl anion of Asp-137 to approach C-2 of 1 and largely reduces the preference for attack at C-2. Thus, Alp1UY247F led to a regioselective attack at C-3. The C-3 selectivity of the Y247F mutant suggests that the regioselectivity for epoxide hydrolysis may not be solely determined by the distance of the interacting atoms, especially when the distances to both epoxide carbons are similar. Other parameters, such as hydrophobic effects, must be considered to account for the unexpected regioselectivity.

**Conclusion**

In this work, we identified that Alp1U structurally shows a conserved overall fold as classic α/β-EHs, but it features a unique triad (Trp-186/Trp-187/Tyr-247) to assemble an atypical oxirane oxygen hole that is usually defined by two residues of Tyr/Tyr for the canonical α/β-EHs. Mutation of residues in the atypical oxirane oxygen hole of Alp1U led to improved and switched regioselectivity for the epoxide hydrolysis of 1. The
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A

B

C

D

E

F
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single site mutant Y247F regioselectively attacks at C-3 (98%) of FST C (1) to form 1a, the (1R,2R,3S)-isomer, whereas the double mutant W187F/Y247F prefers attacking regioselectively at C-2 (96%) to form the (1R,2S,3R)-1b. Crystal structures of the two mutants with distinctly different regioselectivities in complex with FST C (1) to form 1a, the (1R,2R,3S)-isomer, whereas the double mutant W187F/Y247F prefers attacking regioselectively with an N-terminal His6 fusion tag. Site-directed mutagenesis basically as reported previously (29). Briefly, the Alp1U coding plasmid as template. W187F/Y247F double mutant was constructed using the vectors (Fast Mutagenesis System, TransGen). The cloning, expression, and purification of Alp1U and variants were conducted in methanol followed by semipreparative HPLC using a Phenomenex C18 column (250 mm × 10 mm, 2.5 ml min⁻¹) with an isocratic solvent system: solvent A, 10% acetonitrile in water supplemented with 0.1% formic acid; solvent B, 90% acetonitrile in water; elution profile: 5% B to 80% B (0–20 min), 100% B (21–24 min), 100% B to 5% B (24–25 min), 5% B (25–30 min); flow rate at 1 ml min⁻¹.

Isolation of 1a and 1b

An assay was scaled up and contained 10 μM recombinant Alp1U and 100 μM substrate 1 in 100 mM phosphate buffer (pH 7.0) of total volume 1.5 liters at 30 °C for 2 h. The reaction was extracted using butanone (3 × 1.5 liters), and the butanone was removed under vacuum. The remaining residue was dissolved in methanol followed by semipreparative HPLC using a Phenomenex C18 column (250 mm × 10 mm, 2.5 ml min⁻¹) with an isocratic solvent system: solvent A, 10% acetonitrile in water supplemented with 0.1% formic acid; solvent B, 90% acetonitrile in water; elution profile: 5% B to 80% B (0–20 min), 100% B (21–24 min), 100% B to 5% B (24–25 min), 5% B (25–30 min); flow rate at 1 ml min⁻¹.

Synthesis of the diacetate derivative of 1a

The conversion of triol compound 1a to the isopropylidene derivative was carried out by following the previously reported

Figure 4. Crystal structures of ALP1UY247F and ALP1UW187F/Y247F in complex with 1. A, the surface charge potential representation of ALP1UY247F indicates the negatively charged groove-like active site. B and C, the binding mode of the ALP1UY247F-enzyme-substrate complex in different perspectives. Hydrogen bond interactions are indicated by black dashed lines. In Fig. 3b, the 2F₁ – F₁ maps are shown for FST C (1, blue mesh, contoured at 1.5σ). Catalyric triad and axinane oxynion hole residues forming critical hydrogen bonds with 1 are shown as yellow sticks, whereas other peripheral residues are colored gray. D, binding pattern of ALP1UW187F/Y247F in complex with 1. E, proposed catalytic mechanism for the first nucleophilic attack of ALP1U. F, structural alignment of the WT ALP1U (gray sticks), ALP1UW247F/1 (cyan sticks), and ALP1UW187F/Y247F/1 (pink sticks). Distances are indicated by red dashed lines.
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To define the absolute configuration of 1b, we reacted 1b with p-bromobenzoyl chloride to convert it to its bromobenzoyl analog, improve the conditions for crystallization, and introduce a heavy atom. Following the previously reported method (32), the powdered 1b (0.04 mmol) and p-bromobenzoyl chloride (0.21 mmol) and dimethylaminopyridine (0.12 mmol) are taken together and slowly dissolved with pyridine (4 ml) in a pear-shaped glass flask. The solution was stirred at room temperature for 24 h. The reaction was stopped after diluting with water and extracted with butanone (3 × 5 ml). The combined organic layers were reduced to dryness under vacuum, and light brown crystals appeared. A single crystal of 1b-Py (Fig. 2C, Fig. S5, and Table S4) was diffracted on an XtaLAB AFC12 (RINC): k single diffractometer with Cu Kα radiation (λ = 1.54184 Å). The crystal was kept at 104.5 ± 10 K during data collection. Using Olex2, the structure was solved with the ShelXT structure solution program using intrinsic phasing and refined with the ShelXL refinement package using least squares minimization. Crystallographic data have been deposited in the Cambridge Crystallographic Data Center with the deposition number CCDC 2016594.

Acknowledgments—The Governmental Information-Technology Development Agency (KIFU) is acknowledged for CPU time. We thank the staff of beamlines BL17U1, BL18U1, and BL19U1 of the Shanghai Synchrotron Radiation Facility (China) for access and help with the X-ray data collection. We are grateful to Z. Xiao, C. Li, A. Sun, Y. Zhang, and X. Ma in the analytical facilities of SCSIO for recording spectroscopic data.

Funding and additional information—This work was supported in part by National Natural Science Foundation of China (NSFC) Grants 31820103003, 31700042, and 41676165; Chinese Academy of Sciences Grants QYZDJ-SSW-DQC004 and XDA13020302; Special Project for Introduced Talents Team of Southern Marine Science and Engineering Guangdong Laboratory (Guangzhou) Grant GML2019ZD0406; and Guangdong Province Grants GDME-2018C005 and 2019B030302004. T. K. and A. M. were supported by National Research Development and Innovation Office Grant K120181. B. C. D. was supported by the CAS-TWAS President’s Ph.D. Fellowship.

Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: EH, epoxide hydrolase; FST, fluostatin; PDB, Protein Data Bank; PCM, polarizable continuum model; ECD electronic circular dichroism.

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J. Biol. Chem. (2020) 295(50) 16987–16997