Electronic Supplementary Information

Mapping of a Lipoglycopeptide Antibiotic Binding Site on *Staphylococcus aureus* Penicillin-Binding Protein 2 Using a Vancomycin Photoaffinity Analogue

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1. General

Reagents and solvents were used as provided from commercial suppliers, and all reactions were carried out at room temperature unless otherwise noted. LC-ESI-TOF mass spectra (MS) were recorded on a Bruker Daltonics micrOTOF focus spectrometer. The LC part was performed on an Agilent 1100 Series, by using an Imtakt UNISON UK $3C_8$ column (150 x 2 mm) under the following conditions: flow rate of 0.2 mL/min, 65% to 95% acetonitrile/water containing 0.1% formic acid, duration 5 min, temperature 40 °C, and UV 214, 254, 280, and 190-400 nm. A monoisotopic mass was used to calculate the exact mass (C= 12.0000, H= 1.0078, O= 15.9949, N= 14.0031, Cl= 35.9689, P= 30.9738, Na= 22.9898). The analysis of mass fragmentation to obtain further information about the structures of the new vancomycin derivatives was carried out using LC-ESI-TOF MS under the conditions described above.

Nuclear magnetic resonance (NMR) spectra were recorded at room temperature on a JEOL ECA600 at 600 MHz. Chemical shifts (δ) are reported in parts per million (ppm) in ^{1}H NMR spectra relative to the residual solvent peak of $CHCl_3$ (7.26 ppm), CHD_2OD (3.31 ppm), $(CHD_2)(CD_3)SO$ (2.5 ppm), and water (3.33 ppm in DMSO-d_6); in ^{13}C NMR spectra relative to the residual solvent peak of $CHCl_3$ (77.16 ppm). Coupling constants are given in Hz. Multiplicities are reported as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), comp. (overlapping signals of chemical nonequivalent protons), br. (broad), or combinations thereof.

Thin-layer chromatography (TLC) was performed using Merck silica gel 60 F254 precoated plates (0.25 mm). Flash chromatography was carried out using 60–230 mesh silica gel (silica gel 60N, Kanto Chemical). Analytical reversed-phase HPLC was performed as follows. Column: Nacalai Tesque, Cosmosil $5C_{18}$-AR-II (150 x 4.6 mm); conditions: flow rate of 1.0 mL/min, 15% to 100% acetonitrile/water containing 0.1% TFA, duration of 10 min, temperature of 30 °C, and UV detected at 280 nm.

Protein quantification was performed with a BCA Protein Assay Kit (Thermo Scientific) using bovine serum albumin as a standard. Readings were obtained on a microplate reader at 550 nm (model 550, Bio-Rad Laboratories). Western blotting analysis was performed with a semi-dry blotting method (TransBlot SD Cell, Bio-Rad Laboratories), followed by chemiluminescence detection (Immun-Star HRP Chemiluminescent Kit, BioRad Laboratories) using LAS-1000plus (Fujifilm). Ultracentrifugation was performed on a model L-011 (Beckman) using a Type 70.1 Ti rotor (Beckman) and ultraclear centrifuge tubes (76 x 16 mm, No. 355603, Beckman).

Centrifugation was performed in a Himac CF15D (Hitachi) and in a Himac CR15 (Hitachi).
LC-MS/MS analysis was performed in TOF/TOF 5800 (ABSciex), and micro-HPLC was performed in a DiNa-AM (Kya Tech).

2. Materials and Methods

Preparation of the membrane fraction of *Staphylococcus aureus*.[1] After incubation in trypticase soy broth (TSB, two duplicates of 5 mL) at 37 °C for 24 h, the culture solution of *Staphylococcus aureus* (8 mL) was inoculated to TBS medium (400 mL). Static culture at 37 °C for 18 h followed by centrifugation at 6,500 x g at 4 °C for 10 min resulted in the formation of a bacterial pellet. This pellet was suspended in 9 mL Tris buffer (50 mM Tris, 0.1 mM $MgCl_2$, pH 8.0) and added lysostaphin (1.0 mg, L-4402, Sigma). After incubation at 30 °C for 30 min, the suspension was sonicated at 0 °C (ice bath) with a Sonifier 450 (sonic 1 min and interval 30 sec duty cycle 50%, power 3, Branson, Danbury CT, USA). These digestion and sonication protocols were repeated four times. A supernatant fluid, obtained from centrifugation at 6,500 x g at 4 °C for 10 min three times and at 8,000 x g at 4 °C for 10 min twice, was ultracentrifuged at 100,000 x g at 4 °C for 35 min. The resulting precipitate was suspended in 200 μL of Tris buffer (50 mM Tris, 0.1 mM $MgCl_2$, pH 8.0). This suspension was used as a membrane fraction of *S. aureus*.

Photoaffinity labelling. After preincubation of a suspension (10 μL) taken from a mixture containing 5 μL of the membrane fraction and 200 μL of Tris buffer (60 mM Tris, 0.1 mM $MgCl_2$, 0.1 mM 2-mercaptoethanol (2-ME), pH 8.0) with probe **1** in DMSO (final concentration, 50 nM) at 30 °C for 20 min, UV light was irradiated at 365 nm from a US lamp (UVGL-25, 4 W, Funakoshi) at a distance of 10 cm on ice for 20 min.

SDS-PAGE and Western blotting by avidin-HRP conjugate. The photolabeled proteins were separated by SDS-PAGE in 7.5% polyacrylamide gel and transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was incubated with a blocking solution in 50 mL of Tris buffer (20 mM Tris, 500 mM NaCl, 0.1% Tween 20, 5% (w/v) skim milk, pH 7.5) and washed with 30 mL of Tris buffer (20 mM Tris, 500

mM NaCl, 0.1% Tween 20, pH 7.5) three times. The membrane was incubated with a solution of blotting-grade avidin-horseradish peroxidase conjugate (1 μL, Bio-Rad Laboratories) in 30 mL of Tris buffer (20 mM Tris, 500 mM NaCl, 0.1% Tween 20, pH 7.5) at room temperature for 1 h three times, and washed with 30 mL of Tris buffer (20 mM Tris, 500 mM NaCl, 0.1% Tween 20, pH 7.5) five times. This membrane was treated with Immun-Star HRP substrate (1.5 mL, Bio-Rad Laboratories) and the emission was detected by use of an LAS-1000plus.

Competitive assay. The membrane fraction (5 μL) was added 195 μL of Tris buffer (50 mM Tris, 0.1 mM $MgCl_2$, pH 8.0) and mixed vigorously. The solution was then dispensed into four vials (15 μL each), to which Van-M-02[1] was added for final concentrations of 0 (none), 1, 5, and 20 μM. After incubation at 30 °C for 5 min, to each vial was added photoaffinity probe **1** for the final concentration of 1 μM. After incubation at 30 °C for 5 min, UV irradiation was performed at 365 nm at a distance of 10 cm on ice for 20 min by use of a UV lamp (model UVGL-25, 4 W, Funakoshi). Then each sample was added buffer, incubated at 80 °C for 5 min, and subjected to SDS-PAGE (7.5% polyacrylamide, 0.02 A, 1 h). The gel was transferred to PVDF membrane and treated with blocking solution (20 mM Tris, 500 mM NaCl, 0.1% Tween 20, 5% (w/v) skim milk, pH 7.5) and washed with Tris buffer (20 mM Tris, 500 mM NaCl, 0.1% Tween 20, pH 7.5) three times (10 min each). The membrane was incubated with a solution of blotting-grade avidin-horseradish peroxidase conjugate (1 μL, Bio-Rad Laboratories) in 2 mL of Tris buffer (20 mM Tris, 500 mM NaCl, 0.1% Tween 20, pH 7.5) at room temperature for 1 h, and washed with 30 mL of Tris buffer (20 mM Tris, 500 mM NaCl, 0.1% Tween 20, pH 7.5) three times (10 min each). This membrane was treated with Immun-Star HRP substrate (1.5 mL, Bio-Rad Laboratories), and the emission was detected by use of an LAS-1000plus.

Pull-down assay and Western blotting analysis by anti-PBP2 antibody. A suspension (10 μL) taken from a mixture of the membrane fraction (5 μL) and 200 μL of Tris buffer (60 mM Tris, 0.1 mM $MgCl_2$, 0.1 mM 2-mercaptoethanol (2-ME), pH 8.0) was added compound **1** for a final concentration of 1 μM, followed by incubation at 30 °C for 1 h and then UV irradiation at 365 nm on ice for 20 min. After photoirradiation, the solution was added cold acetone (200 μL, -30 °C) and cooled at -30 °C for 2 h. The supernatant obtained by centrifugation at 10,000 x g at 4 °C for 20 min was removed. The precipitate was added to cold methanol (200 μL, -30 °C) and

then suspended in methanol by ultrasonic treatment (model UT-205, Sharp, Osaka, Japan). The suspension was centrifuged at 10,000 x g at 4 °C for 20 min, and the resulting precipitate was washed with methanol twice and, after the removal of methanol, dissolved in Tris buffer (20 mM Tris, 500 mM NaCl, 0.2% SDS, pH 7.5). This suspension was added to 20 μL of a streptavidin magnetic bead suspension (Dynabeads MyOne streptavidin C1, Veritas, Tokyo, Japan) followed by mixing at room temperature for 30 min (model TM-282, As One) and then washing with Tris buffer (20 mM Tris, 500 mM NaCl, 0.2% SDS, pH 7.5) three times. After the supernatant was removed, the resin was incubated at 95 °C for 3 min in Laemmli sample buffer (Bio-Rad Laboratories). The sample was subjected to SDS-PAGE and then transferred to PVDF membrane. The membrane was blocked with Tris buffer containing skim milk (20 mM Tris, 500 mM NaCl, 0.1% Tween 20, 5% (w/v) skim milk, pH 7.5), washed with 30 mL of Tris buffer (20 mM Tris, 500 mM NaCl, 0.1% Tween 20, pH 7.5), added to a solution of anti-PBP2 antibody[2] (1 μL, provided by Shionogi Research Laboratories) in Can Get Signal Solution (3 mL, Toyobo), and finally incubated at room temperature for 1 h. After washing with 30 mL of Tris buffer (20 mM Tris, 500 mM NaCl, 0.2% SDS, pH 7.5) five times, the membrane was added to a solution of goat anti-rat IgG-HRP (1 μL, Cosmobio) in Can Get Signal Solution 2 (2 mL), followed by incubation at room temperature for 1 h. This membrane was washed with 30 mL Tris buffer (20 mM Tris, 500 mM NaCl, 0.1% Tween 20, pH 7.5) five times and treated with Immun-Star HRP substrate (1.5 mL, Bio-Rad Laboratories). The emission was detected by use of an LAS-1000plus.

Recombinant PBP2 protein expression and purification. The *pbp2* gene was amplified from *S. aureus* (RN4220) by using PCR with the primer pair previously reported.[3] The gene encoding PBP2 was cloned into the bacterial expression vector pET21b, and the protein was overexpressed in *Escherichia coli* as a C-terminal His_6 fusion protein after induction of a log-phase culture with 1 mM isoprenyl β-D-thiogalactopyranoside (IPTG) at 37 °C for 4 h as previously described.[2] The cells were lysed with a French pressure cell at 16,000 lb/in^2 in buffer (10 mM Tris-HCl, 150 mM NaCl, pH 7.4) supplemented with 360 kU of lysozyme. The resulting supernatant was removed, and the pellet was resuspended in buffer containing 0.5% sarkosyl. The solubilized fusion protein was loaded onto a Ni^{2+} column, followed by washing with an imidazole gradient. The eluted fractions were dialyzed and finally used as a recombinant PBP2 solution composed of PBP2-His_6 fusion protein (0.888 mg/mL),

Tris buffer (50 mM, pH 8.0), KH_2PO_4 (200 mM, pH 8.0), dithiothreitol (DTT, 1 mM), 50% glycerol, and protease inhibitor cocktail (Complete, EDTA-free, Roche).

LC-MALDI MS/MS analysis. A mixture of the solution of recombinant PBP2 (1 μL) and 10 μL of Tris buffer (60 mM Tris, 0.1 mM $MgCl_2$, 0.1 mM 2-ME, pH 8.0) was added to a solution of probe **1** in DMSO for a final concentration of 2 μM. After incubation at 4 °C for 2 h, UV light was irradiated at 365 nm at a distance of 10 cm on ice for 20 min by use of UV lamp, followed by the addition of cold acetone (200 μL, -20 °C) and cooling at -20 °C for 2 h. The supernatant obtained by centrifugation at 10,000 x g at 4 °C for 20 min was removed. The precipitate was added to cold methanol (200 μL, -30 °C) and then suspended in the methanol by ultrasonic treatment (model UT-205, Sharp). This washing process with cold methanol was repeated twice. The precipitate was dried and then added to aqueous solution of 25 mM ammonium bicarbonate (30 μL) followed by the addition of a solution of trypsin (100 ng/μL, 5 μL, Wako Pure Chemical) to digest the proteins. The resulting suspension was added to 30 μL of Tris buffer (20 mM Tris, 500 mM NaCl, 0.1% SDS, 8 M urea, 2 M thiourea, 4% CHAPS, pH 7.5) to dissolve the contents. After the addition of a suspension of magnetic beads (Dynabeads MyOne streptavidin C1), the suspension was mixed for 2 h, and then the beads were washed with 100 μL Tris buffer (20 mM Tris, 500 mM NaCl, 0.1% SDS, 8 M urea, 2 M thiourea, 4% CHAPS, pH 7.5) three times. At that point, the beads were transferred into a new vial. This washing process was repeated twice. The beads were washed with 0.1% water solution of SDS (100 μL) and 0.1 M HCl_{aq} (50 μL). The supernatant was added 0.1 M HCl_{aq} (20 μL). This suspension was incubated at 100 °C for 20 min. The resulting supernatant was transferred to a new vial and added to 2% acetonitrile/water containing 0.1% TFA (20 μL) and then desalted on a ZipTip (C_{18}, size P10, ZTC18S096, Millipore). After concentration by vacuum centrifugation and the addition of 2% acetonitrile/water containing 0.1% TFA (10 μL), the filtrate was fractionated into 117 spots by micro-HPLC under the following conditions: matrix of α-cyano-4-hydroxycinnamic acid (α-CHCA), HiQ sil C18W3 (a guard column, 1.0 x 0.5 mm, Kya Tech), HiQ sil C18W3 (50 x 0.15 mm, Kya Tech), flow rate 150 nL/min, acetonitrile/water/TFA= 2:98:0.1 to 85:15:0.1, duration 73 min, room temperature.

Molecular docking studies. The crystal structure of a macromolecule, PBP2, was obtained from the Protein Data Bank (PDB ID: 2olu),[4] and 12 selenomethionine in this file was changed to methionine, after which its structure was minimized in the OPLS_2005 force field without solvent using Macromodel 9.1 (Schrödinger). The initial 3D structure of ligand **1´** (Fig. S1A), which was used instead of compound **1**, was drawn based on the crystal structure of vancomycin (PDB ID, 1fvm),[5] and the stable conformations of this compound were computed by a conformational search in Macromodel using the mixed torsional/low-mode sampling method in the force field of OPLS_2005 with water solvation. The resulting conformers were clustered based on the direction of the sugar moiety and sorted into three groups, labeled A to D, with 47%, 36%, 12%, and 5% of the occurring ratios of these groups, respectively. The most stable conformer in each group was displayed and designated conf A to D in Figure S1B, and the potential energies of the conformers in the force field of OPLS_2005 were -768.4 kcal/mol, -768.8 kcal/mol, -763.2 kcal/mol, and -764.3 kcal/mol, respectively. Docking experiments between PBP2 and these conformers were performed with AutoDock 4.2 (The Scripps Research Institute) using conf A to D as the initial structure of the ligand and the default Lamarckian genetic algorithm, which generated 10 complex structures in a run. Finally, structures of the four complexes were refined by minimization with Macromodel in the force field of OPLS_2005 with water solvation. During this calculation, **1´** was set to be flexible and the amino acid residues within 5 Å from **1´** were allowed to move, and this computation was limited by the gradient criterion of 0.05, resulting in the complex state shown in Figure 8 (complex A) and Figure S2 (complex B to D). These four results are bound and depicted in Figure S3.

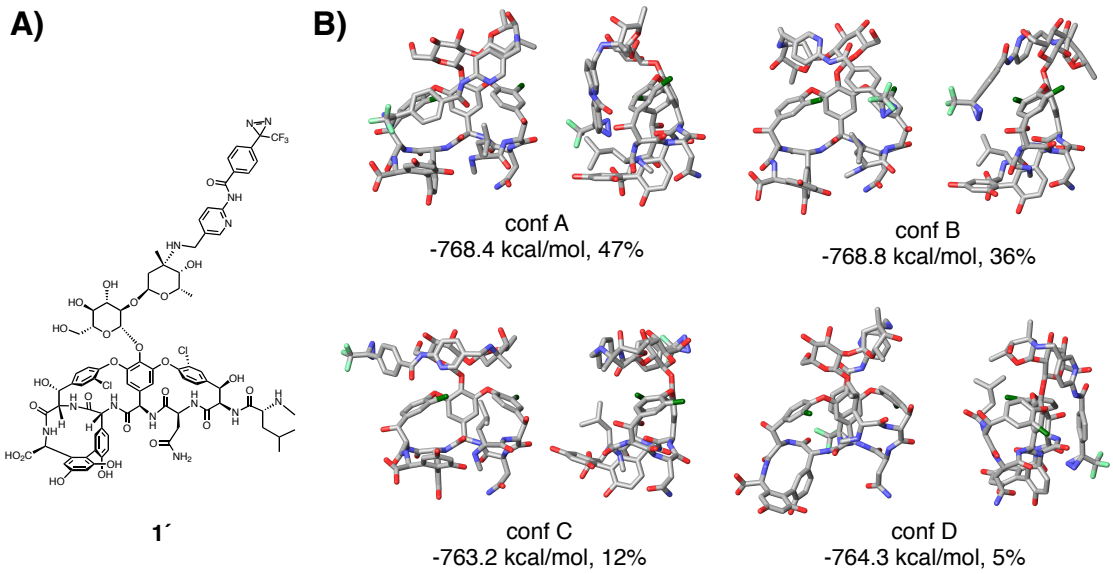


Fig. S1 Structure of compound **1´** (A) and its stable conformers: A to D (B). The potential energy and ratios of occurrence in the force field of OPLS_2005 are shown under the structure.

Table S1 Results and analysis of the molecular modeling using PBP2[4] and compound **1´**.

			complex A	complex B	complex C	complex D
ligand[a]	name		conf A	conf B	conf C	conf D
	potential energy after conformational search (kcal/mol)[b]		-768.4	-768.8	-763.2	-764.3
	occurring ratio (%)		47	36	12	5
results	potential energy	**after minimization (kcal/mol)[b]**	**-1215.1**	**-1204.2**	**-891.2**	**-717.2**
	distance between 19-Cl and β-oxygen atom of Ser398 (Å)		17.7	17.4	37.2	34.9

[a]See Figure S1 for the structure and conformations of the ligand; [b]Calculated in the force field of OPLS_2005 with water solvation.

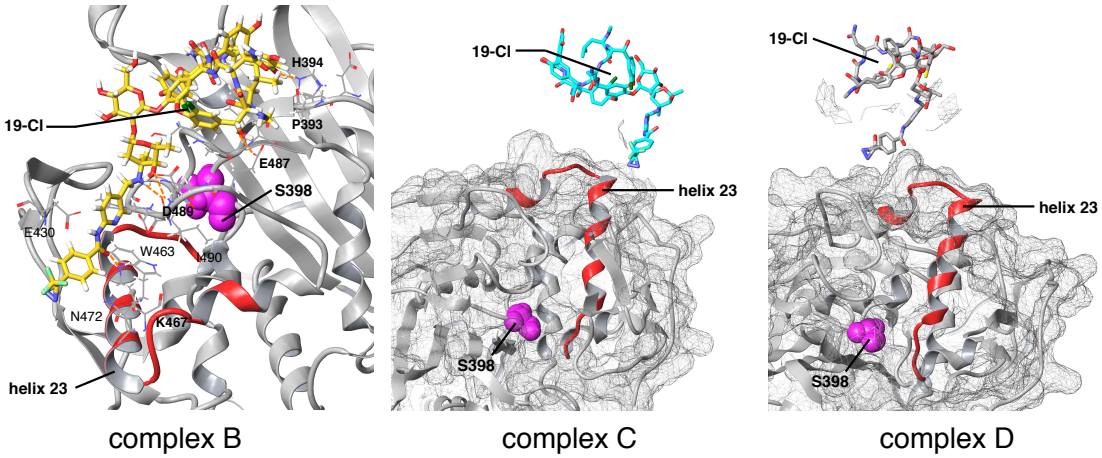


Fig. S2 Minimized structures of the complexes B to D. PBP2 is represented with ribbons. The probe-binding region (Q453-K477) characterized in this work and TP catalytic S398 residue are highlighted in red and magenta, respectively. In the model of complex B, residues within 4 angstroms are shown using a line representation. In the model of both complexes C and D, there is no amino acid residue within 4 angstroms from the probe **1´**. The distances between an oxygen atom of the hydroxyl group in Ser398 at the active site, and the 19-Cl of probe **1´** in the complexes B to D were 17.4, 37.2, and 34.9 angstroms, respectively.

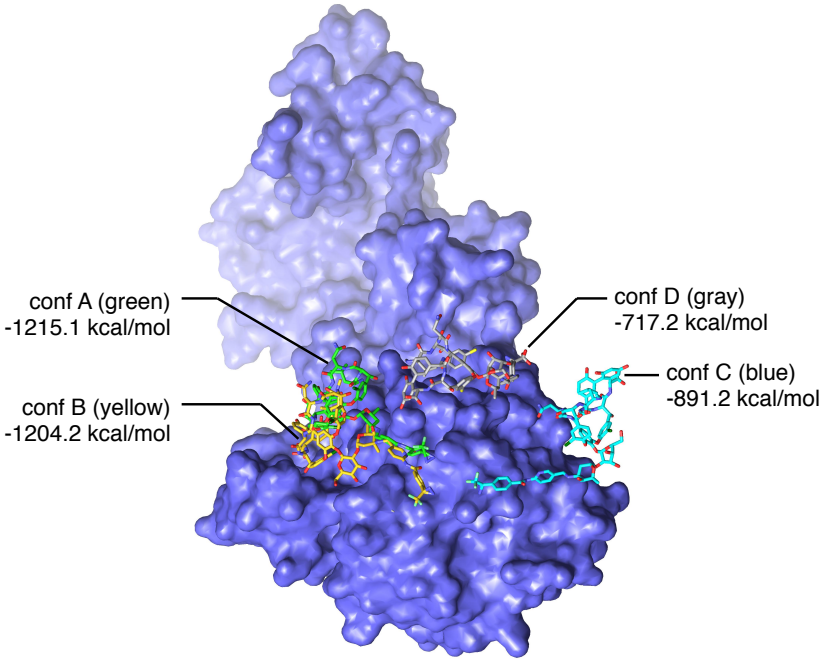
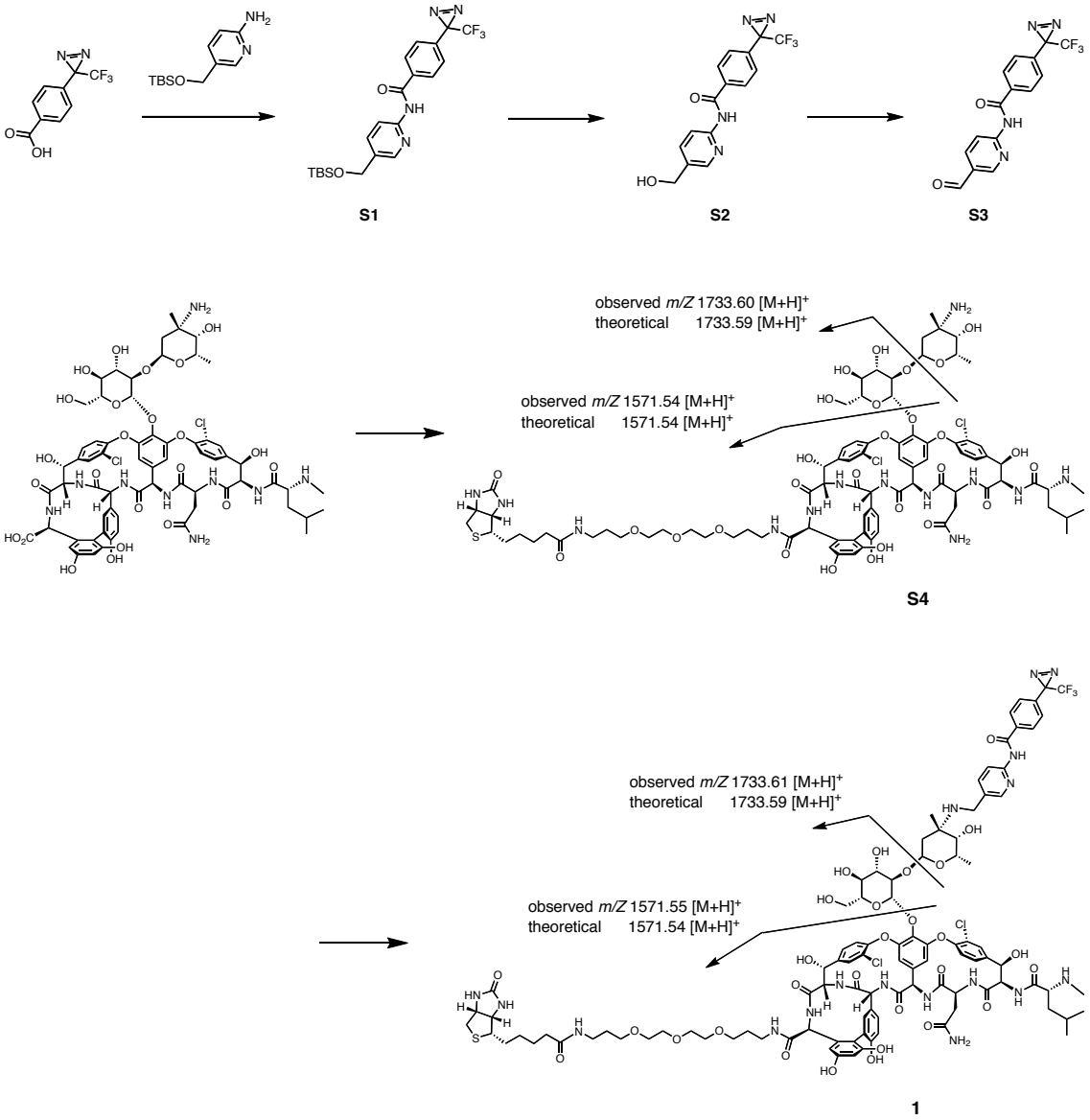


Fig. S3 Three-dimensional structure of PBP2 interacting with the four conformers generated from the docking simulation in this study.

3. Preparation of Photoaffinity Probe 1

(1) Experimental procedures of the compounds.



Scheme S1 Synthetic route of compound **1**.

Compound S1. 4-[3-(Trifluoromethyl)-3*H*-diazirin-3-yl]benzoic acid (46 mg, 0.20 mmol) and 5-[[[(1,1-dimethylethyl)dimethylsilyl]oxy]methyl]-2-pyridinamine were dissolved in dry DMF (1 mL) and cooled to 0 °C (ice-water bath). The mixture was treated with 4-(dimethylamino)pyridine (DMAP, 49 mg, 0.40 mmol) and *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC, 77 mg, 0.40

mmol) and then warmed to room temperature and stirred in the dark for 23 h. The reaction was quenched by the addition of 1 M HCl_{aq} (1 mL), and the mixture was partitioned between water (2 mL) and ethyl acetate (1 x 10 mL and 3 x 5 mL). The organic layers were combined, washed with brine, and dried over anhydrous magnesium sulfate. Filtration and evaporation of the solvent gave a yellow solid (0.12 g), which was purified by column chromatography on silica gel (ethyl acetate/hexanes= 1:6) to afford amide **S1** as a colorless solid (67 mg, 47% yield): R_f 0.63 (ethyl acetate/hexanes= 2:1); ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.72 (s, 1H), 8.34 (d, *J*= 8.2 Hz, 1H), 8.22 (s, 1H), 7.96 (d, *J*= 8.3 Hz, 2H), 7.74 (dd, *J*= 8.6, 2.1, 1H), 7.13 (d, *J*= 8.3, 2H), 4.72 (s, 2H), 0.93 (s, 9H), 0.11 (s, 6H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 164.5, 150.4, 146.1, 137, 135.5, 133.5, 133.2, 127.8, 127, 122.9 (q, $^1J_{C–F}$= 273 Hz), 113.9, 62.6, 29.4 (q, $^2J_{C–F}$= 51.7 Hz), 26, 18.5, -5.1; HRMS (LC-ESI-TOF) calcd. for $C_{21}H_{26}F_3N_4O_2Si$: 451.1777 $[M+H]^+$, found 451.1797 $[M+H]^+$.

Compound S2. A solution of compound **S1** (0.18 g, 0.40 mmol) in THF (10 mL) was added conc. HCl_{aq} (1 mL) and stirred at room temperature in the dark for 35 h. This reaction mixture was then diluted with ethyl acetate (50 mL) and added to saturated sodium hydrogen carbonate solution (20 mL). This mixture was partitioned between a water layer and ethyl acetate (2 x 50 mL). The organic layers were combined, washed with brine, and dried over anhydrous magnesium sulfate. Filtration and evaporation of the solvent gave compound **S2** as a colorless solid (0.13 g), which was used for the next reaction without further purification: R_f 0.05 (ethyl acetate/hexanes= 2:1).

Compound S3. A solution of compound **S2** (0.13 g) in DMSO (10 mL) was added to 2-iodoxybenzoic acid (IBX, 0.22 g, 0.79 mmol), and the solution was stirred at room temperature in the dark for 3 h. This reaction mixture was partitioned between water (5 mL) and diethyl ether (1 x 30 mL and 3 x 5 mL). The combined organic layer was washed with saturated sodium carbonate solution, water, and brine, and dried over anhydrous magnesium sulfate. Filtration and evaporation of the solvent gave a yellow solid (0.12 g), which was purified by column chromatography on silica gel (ethyl acetate/hexanes= 1:3) to afford aldehyde **S3** (0.13 g, 95% yield in two steps from **S1**) as a colorless solid: R_f 0.28 (ethyl acetate/hexanes= 1:2); ^{1}H NMR (600 MHz, $CDCl_3$) δ 10.03 (s, 1H), 8.82 (s, 1H), 8.78 (d, *J*= 1.7 Hz, 1H), 8.55 (d, *J*= 8.6 Hz, 1H), 8.23 (dd, *J*= 8.6, 2.4, 1H), 7.98 (m, 2H), 7.35 (d, *J*= 8.2 Hz, 2H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 190.4, 164.6, 155.2, 151.5, 139.2, 134.7, 134, 128.9, 127.9, 127.1, 123.8 (q, $^1J_{C–F}$= 239

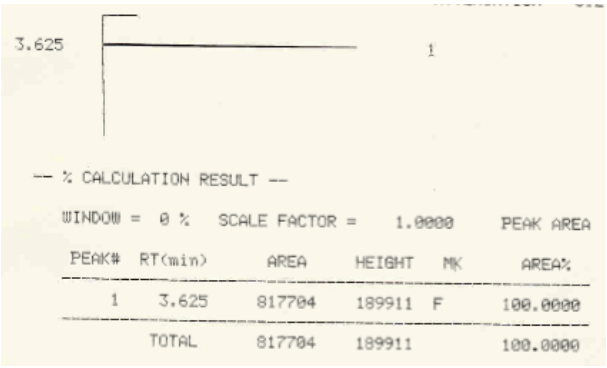
Hz), 114, 29.5 (q, $^2J_{C-F}$= 54.1 Hz); HRMS (LC-ESI-TOF) calcd. for $C_{15}H_{10}F_3N_4O_2$: 335.0756 $[M+H]^+$, found 335.0758 $[M+H]^+$.

Compound S4. Vancomycin hydrochloride (0.50 g, 0.34 mmol) was dissolved in dry DMSO/dry DMF= 1:1 mixture (10 mL), followed by the addition of 1-hydroxybenzotriazole (HOBt, 45 mg, 0.33 mmol), (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP, 0.52 g, 1 mmol), and *N*,*N*-diisopropylethylamine (DIPEA, 0.18 mL, 1 mmol). The mixture was stirred at room temperature for 30 min and then added *N*-(13-amino-4,7,10-trioxatridecanyl)-D-biotinamide. The reaction mixture was stirred for an additional 4 h, and then poured into ethyl acetate (0.7 L) with stirring. The resulting white precipitate was collected by use of a membrane filter (Millipore, pore size 1.0 μm, JAWP04700) and dried by an air stream to give a crude solid of compound **S4** (0.75 g). This crude sample was dissolved in methanol/water containing 0.1% TFA and purified by reversed-phase column chromatography (Yamazen, Ultra Pack ODS-S-50B, 300 mm x 26 mm, 50 μm particle size, methanol/water= 20:80 to 40:60 containing 0.1% TFA). The combined fractions were concentrated and the residue was poured into ethyl acetate with stirring. The resulting white precipitate was collected by use of a membrane filter (Millipore, pore size 1.0 μm, JAWP04700) and dried by an air stream to yield compound **S4** (0.27 g, 42% yield, 96% purity). Further purification of this sample (12 mg) by HPLC (Nacalai Tesque, Cosmosil 5C_{18}-AR-II water, 250 x 20 mm, 15% to 65% acetonitrile/water containing 0.1% TFA, duration of 30 min, flow rate of 5 mL/min) gave a white solid (10 mg, >99% purity): R_t 3.6 min; ^{1}H NMR (600 MHz, DMSO-d_6 and one drop of D_2O, 21 °C, water) δ 8.52 (s, 1H), 8.26 (s, 1H), 7.66 (s, 1H), 7.37 (d, *J*= 8.2 Hz, 1H), 7.33 (s, 1H), 7.28 (d, *J*= 8.6 Hz, 1H), 7.14 (t, *J*= 6.9 Hz, 1H), 7.01 (d, *J*= 7.6 Hz, 2H), 6.59 (d, *J*= 8.9 Hz, 2H), 6.53 (d, *J*= 8.2 Hz, 1H), 6.18 (s, 1H), 6.05 (br., 1H), 5.56 (br., 1H), 5.4 (s, 1H), 5.08-5.06 (comp., 4H), 4.99 (br., 2H), 4.73 (s, 1H), 4.49 (br., 1H), 4.26 (s, 1H), 4.18 (s, 1H), 4.13 (t, *J*= 4.8 Hz, 1H), 4.02 (s, 1H), 3.96 (q, *J*= 4 Hz, 1H), 3.77 (s, 1H), 3.49-3.36 (comp., 8H), 3.23 (m, 3H), 3.2 (m, 3H), 3.08 (m, 2H), 2.99 (comp., 4H), 2.93 (m, 1H), 2.88 (t, *J*= 6.9 Hz), 2.65 (dd, *J*= 12.7, 5.1 Hz, 1H), 2.44 (br., 3H), 2.41 (s, 1H), 2.39 (s, 1H), 2.32 (s, 3H), 1.96 (br., 1H), 1.86 (t, *J*= 7.2 Hz, 3H), 1.73 (d, *J*= 10 Hz, 1H), 1.58-1.26 (comp., 16H), 1.11 (br., 6H), 0.87 (br., 3H), 0.72 (br., 3H), 0.67 (br., 3H); HRMS (LC-ESI-TOF) calcd. for $C_{86}H_{113}Cl_2N_{13}O_{28}S$: 938.8458 $[M+2H]^{2+}$, found 938.854 $[M+2H]^{2+}$.

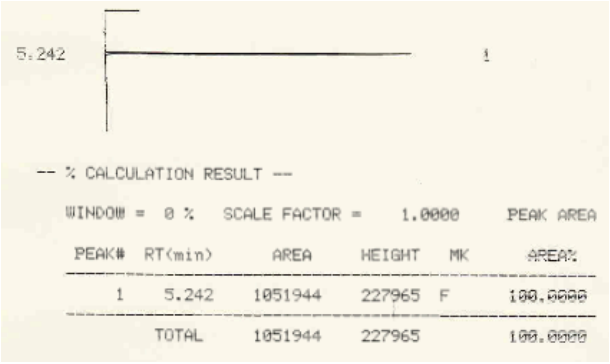
Compound 1. A solution of compound **S4** (0.20 mg, 0.10 mmol) in dry methanol/dry DMF= 1:1 (5 mL) was added DIPEA (0.20 mL, 1.2 mmol) and a solution of compound **S3** (34 mg, 0.10 mmol) in dry methanol/dry DMF= 1:1 (1 mL), and the mixture was stirred at 60 °C in the dark for 6 h. The reaction mixture was cooled to room temperature, followed by an addition of sodium cyanoborohydride (45 mg, 0.71 mmol). The reaction was stirred at 60 °C in the dark for 12 h. After being cooled to room temperature, the reaction solution was pored into ethyl acetate with stirring at 0 °C (ice-water bath). The resulting white precipitate was collected with membrane filter (Millipore, pore size 1.0 μm, JAWP04700), and dried by passing air stream to give a crude solid of compound **1** (0.20 g). This sample was dissolved in methanol/water containing 0.1% TFA and purified by reverse-phase column chromatography (Yamazen, ULTRA PACK ODS-S-50B, 300 mm x 26 mm, 50 μm particle size; methanol/5 mM HCl_{aq}= 40:60 to 50:50). The combined fractions were concentrated, and the residue was pored into ethyl acetate (0.8 L) with stirring. The resulting white precipitate was collecting by use of membrane filter (Millipore, pore size 1.0 μm, JAWP04700) and dried by passing air stream to yield compound **1** (0.11 g, 48% yield, 99% purity). Further purification of this sample (12 mg) by HPLC (Nacalai Tesque, Cosmosil 5C_{18}-AR-II waters, 250 x 20 mm, 15% to 65% acetonitrile/water containing 0.1% TFA, duration of 30 min, flow rate of 5 mL/min) gave a white solid (4.9 mg, >99% purity): R_t 5.2 min; ^{1}H NMR (600 MHz, DMSO-d_6 and one drop of D_2O, 21 °C, water) δ 8.53 (s, 1H), 8.27 (br., 2H), 8.02 (m, 1H), 7.92 (br., 2H), 7.74 (m, 1H), 7.67 (s, 1H), 7.37 (d, *J*= 7.6 Hz, 1H), 7.29 (d, *J*= 7.9 Hz, 1H), 7.24 (d, *J*= 7.6 Hz, 2H), 7.14 (m, 1H), 7.04 (s, 1H), 7.02 (br., 1H), 6.59 (d, *J*= 8.2 Hz, 1H), 6.53 (d, *J*= 7.2 Hz, 1H), 6.18 (s, 1H), 6.05 (br., 1H), 5.59 (br., 1H), 5.44 (br., 1H), 5.17 (m, 1H), 5.11 (s, 1H), 5.06 (s, 1H), 4.99 (s, 1H), 4.98 (s, 1H), 4.73 (s, 1H), 4.49 (s, 1H), 4.27 (s, 1H), 4.18 (br., 1H), 4.12 (t, *J*= 5.5 Hz, 1H), 4.03 (br., 1H), 3.94 (m, 1H), 3.85 (s, 1H), 3.77 (s, 1H), 3.5-3.39 (comp., 8H), 3.23 (br., 1H), 3.19 (br., 1H), 3.1-3.08 (comp., 2H), 2.99 (br., 1H), 2.9-2.87 (comp., 4H), 2.63 (m, 1H), 2.44 (s, 3H), 2.4 (s, 1H), 2.38 (s, 1H), 1.96 (br., 1H), 1.85 (t, *J*= 6.5 Hz, 3H), 1.67 (d, *J*= 12 Hz, 1H), 1.52 (comp., 2H), 1.42 (comp., 3H), 1.31 (br., 3H), 1.1 (br., 2H), 0.94 (br., 3H), 0.72 (br., 3H), 0.68 (br., 3H); HRMS (LC-ESI-TOF) calcd. for $C_{101}H_{122}Cl_2F_3N_{17}O_{29}S$: 1097.8822 $[M+2H]^{2+}$, found 1097.8833 $[M+2H]^{2+}$.

(2) HPLC chart of the compounds. (R_t and compound purity were assessed under the following conditions: Cosmosil 5C_{18}-AR-II waters (Nacalai Tesque), 150 x 4.6 mm, 15% to 100% acetonitrile/water containing 0.1% TFA, duration 10 min, flow rate 1 mL/min, detection at 280 nm).

Compound S4

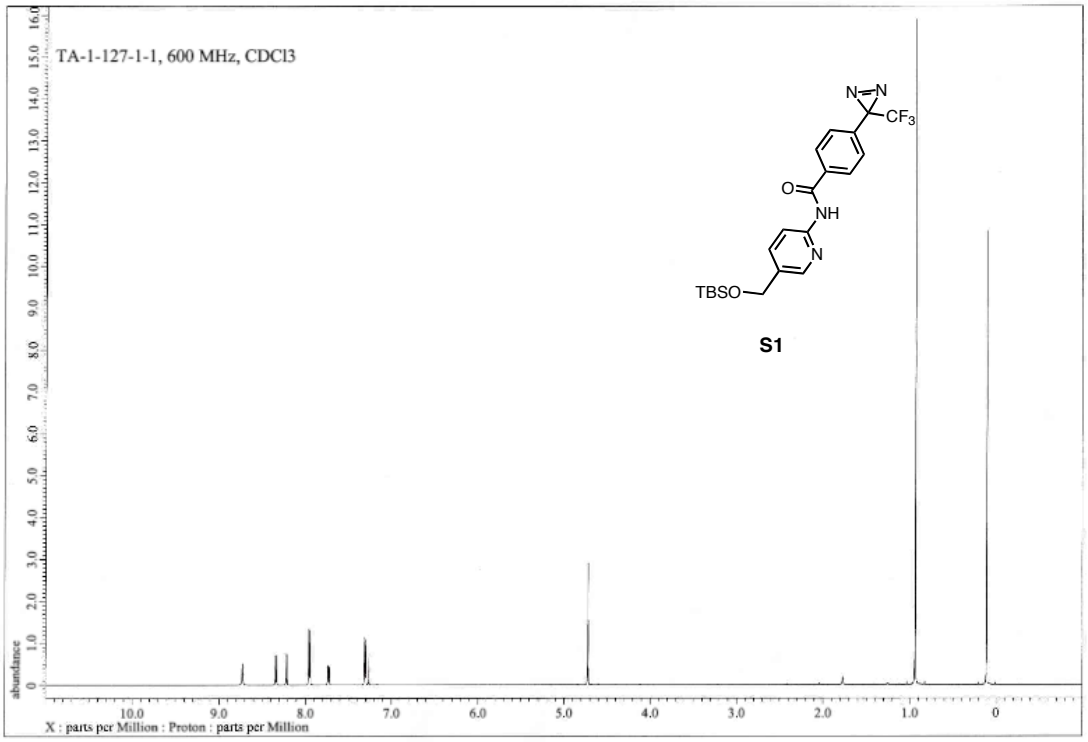


Compound 1

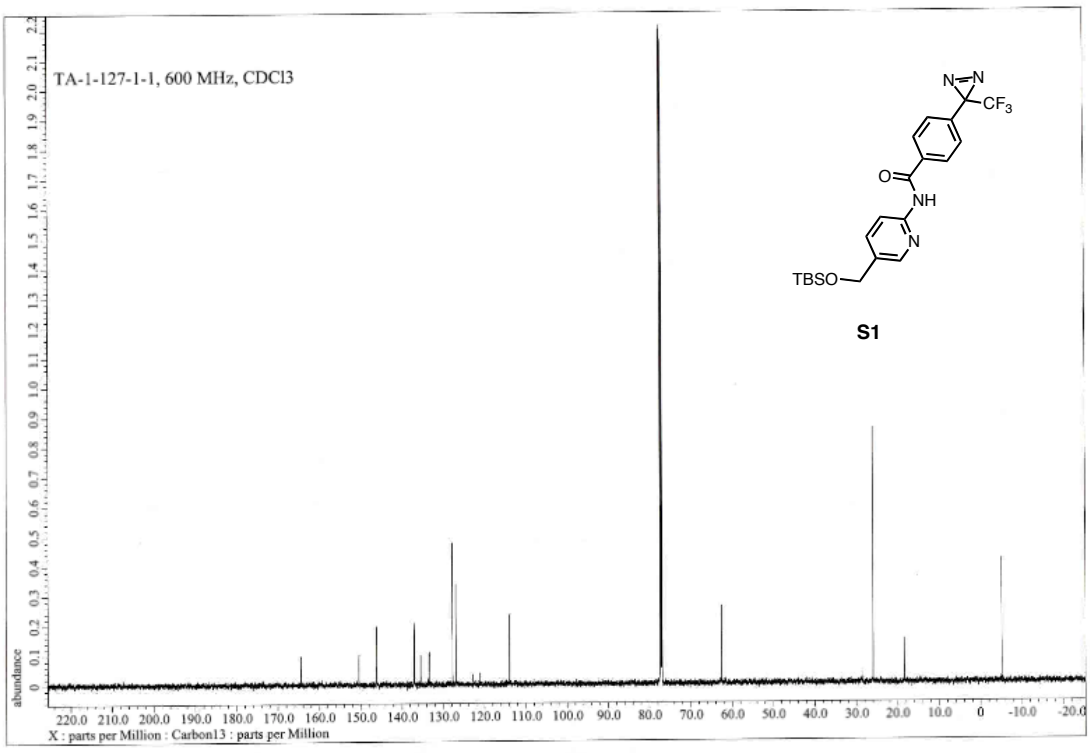


(3) ^{1}H and ^{13}C NMR spectra of the compounds

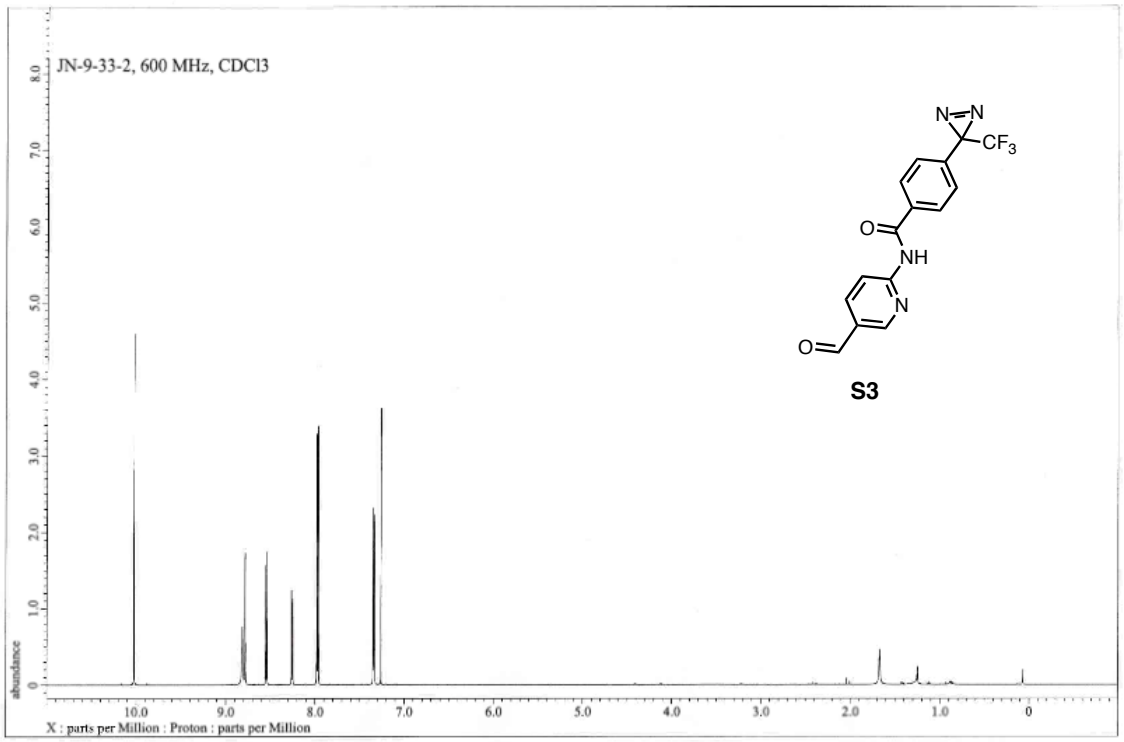
^{1}H NMR of compound S1 (600 MHz, $CDCl_3$)



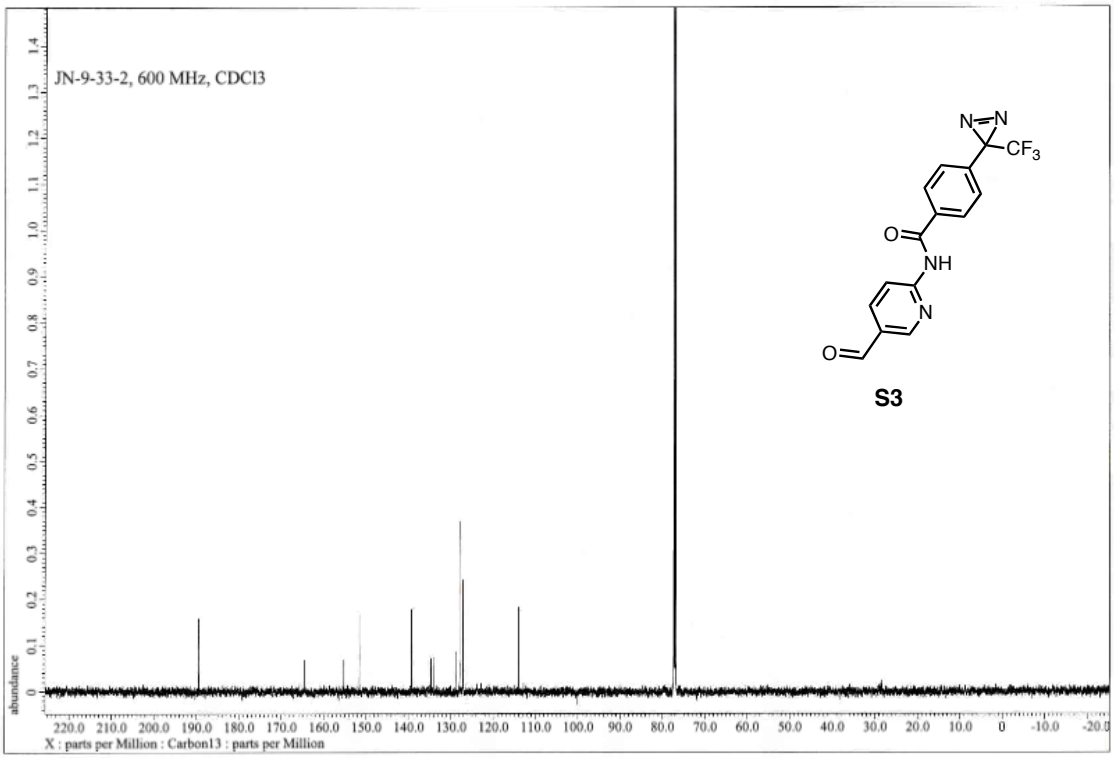
^{13}C NMR of compound S1 (150 MHz, $CDCl_3$)



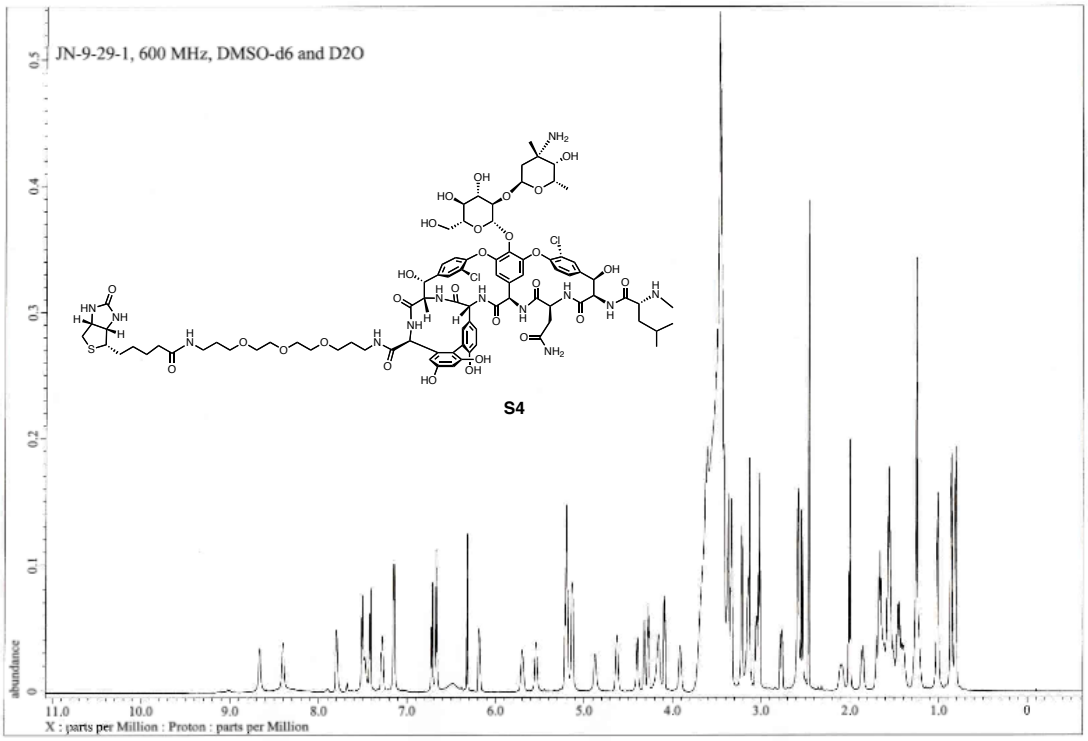
^{1}H NMR of compound S3 (600 MHz, $CDCl_3$)



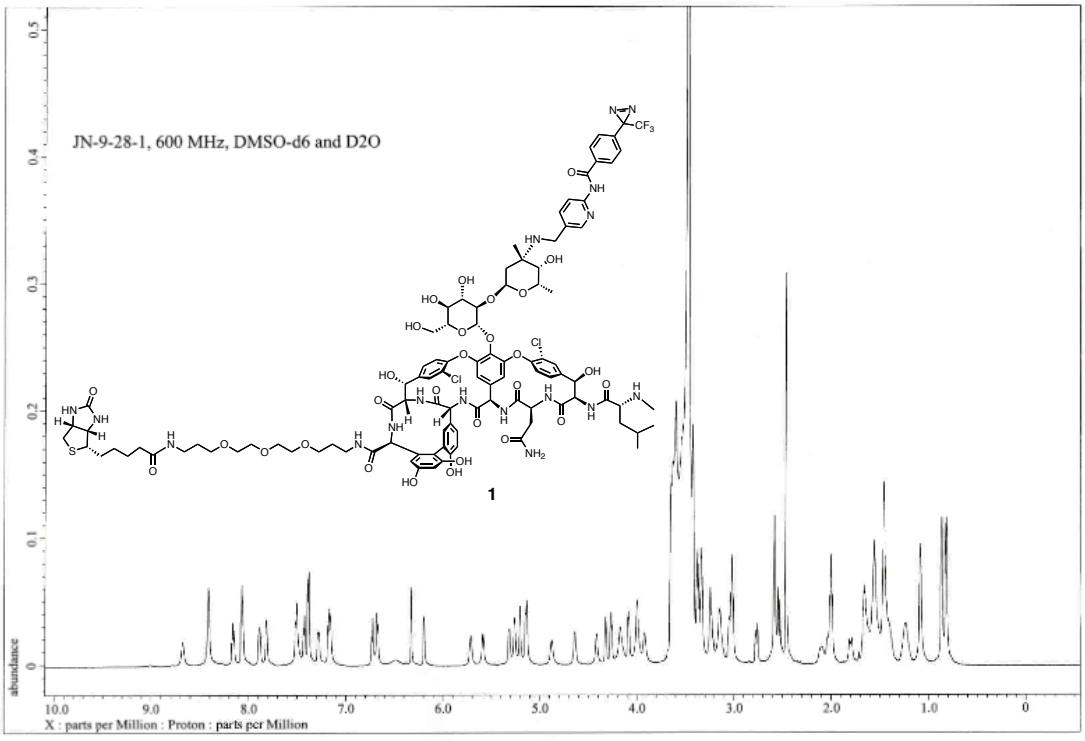
^{13}C NMR of compound S3 (150 MHz, $CDCl_3$)



^{1}H NMR of compound S4 (600 MHz, DMSO-d_6 and one drop of D_2O)



^{1}H NMR of compound 1 (600 MHz, DMSO-d_6 and one drop of D_2O)



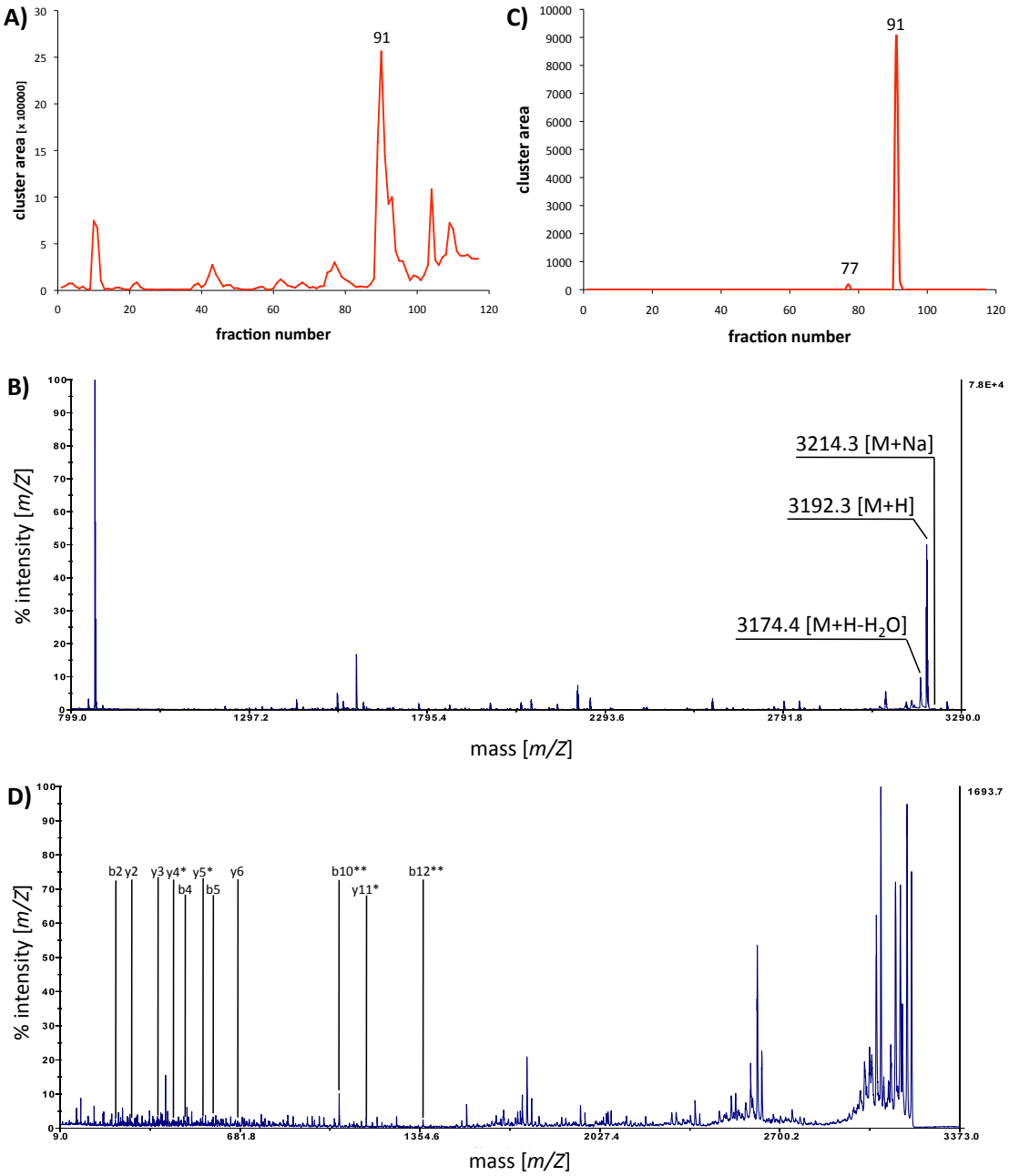
4. Supplemental Table and Figures

Table S2 Antibacterial activity of compound **1**, Van-M-02, and vancomycin.

	MIC (μg mL^{-1})[a]				
	S. aureus[b]	*E. faecium*[c]	*S. aureus*[d]	*E. faecium*[e]	*E. faecium*[f]
	susceptible	susceptible	resistant	resistant	resistant
compounds			(VanA)	(VanA)	(VanB)
1	1	0.5	2	2	0.5
Van-M-02[1]	0.125	0.25	0.5	1	0.25
vancomycin	1	1	>64	>64	>64

[a]Minimum inhibitory concentration. [b]RN4220. [c]SR16972. [d]VRS-2. [e]SR7940. [f]SR23598.

Fig. S4 Spectra of LC-MS/MS analysis. (A) Total ion chromatography of the RP-HPLC part. The strongest peak was detected in fraction No. 91; (B) Mass spectrum of fraction No. 91. A positive ion *m/Z* 3192.3 $[M+H]^+$ was observed mainly with a dehydration peak $[M+H-H_2O]^+$ and a sodium salt peak $[M+Na]^+$; (C) XIC of the candidate precursor ion (3192.3 ± 0.25 mass unit). This ion was extracted in fraction No. 91 in maximum intensity; D) MS/MS spectrometry of candidate precursor ions (*m/Z* 3192.3). The ions labeled with *asterisks* and *double asterisks* are $-NH_3$ ions and H_2O ions, respectively.



5. References

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