

SUPPORTING INFORMATION

Discovery of (ANT3310), a Novel Broad-Spectrum Serine β -Lactamase Inhibitor of the Diazabicyclooctane Class, which Strongly Potentiates Meropenem Activity against Carbapenem Resistant Enterobacterales and *Acinetobacter baumannii*

David T. Davies,^{1*} Simon Leiris,¹ Magdalena Zalacain,¹ Nicolas Sprynski,¹ Jérôme Castandet,¹ Justine Bousquet,¹ Clarisse Lozano,¹ Agustina Llanos,¹ Laethitia Alibaud,¹ Srinivas Vasa,² Ramesh Pattipati,² Ravindar Valige,² Bhaskar Kummari,² Srinivasu Pothukanuri,² Cytia De Piano,³ Ian Morrissey,³ Kirsty Holden,⁴ Peter Warn,⁴ Francesca Marcoccia,⁵ Manuela Benvenuti,⁶ Cecilia Pozzi,⁶ Giusy Tassone,⁶ Stefano Mangani,⁶ Jean-Denis Docquier,⁵ David Pallin,⁷ Richard Elliot,⁷ Marc Lemonnier,¹ Martin Everett¹

¹*Antabio SAS, 436 rue Pierre et Marie Curie, 31670 Labège, France*

²*GVK Biosciences Pvt. Ltd, Survey No. 125 and 126, IDA, Mallapur, Hyderabad-500 076, Telangana, India*

³*International Health Management Associates (IHMA), Rte. De l'Ile-au-Bois 1A, 1870 Monthey, Switzerland*

⁴*Evotec (UK) Ltd., Block 23, Alderley Park, Macclesfield, Cheshire, SK10 4TG, UK*

⁵*Dipartimento di Biotecnologie Mediche, University of Siena, Viale Bracci 16, Siena, 53100, Italy ;*

⁶*Dipartimento di Biotecnologie, Chimica e Farmacia, University of Siena, Via Aldo Moro 2, Siena, 53100, Italy*

⁷*Charles River Laboratories, 8-9 The Spire Green Centre, Harlow, Essex, CM19 5TR, UK*

***Corresponding author : David Davies - Antabio SAS, 436 rue Pierre et Marie Curie, 31670 Labège, France ; E-mail: david.davies@antabio.com**

Contents

S2-S3 NMR studies to distinguish Isomers A and B after fluorination reaction

S4-S9 Characterisation of **21**, ANT3310

S9-S10 Description of enzymology and MIC testing

S11 Description of CYP inhibition assays

S12 Description of plasma protein binding assays and murine thigh infection model

S13-S15 Xray crystallography and references

Isomer Determination by NMR of (2*R*,5*R*)-6-(benzyloxy)-2-fluoro-1,6-diazabicyclo[3.2.1]octan-7-one (A) and (2*S*,5*R*)-6-(benzyloxy)-2-fluoro-1,6-diazabicyclo[3.2.1]octan-7-one (B):

Isomer A

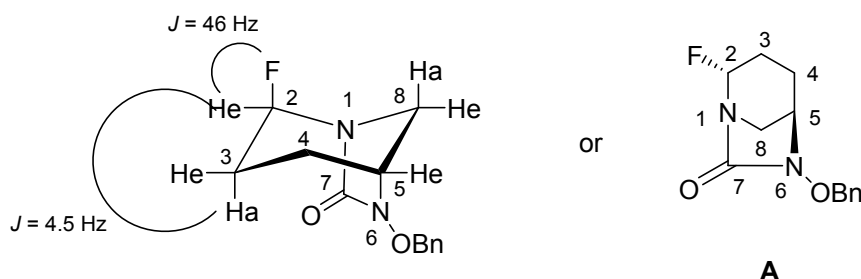


Figure S1. NMR analysis of Isomer A

The coupling constant for H2 proton (5.45 ppm), $^2J_{\text{H2-F}} = 46$ Hz and $^3J_{\text{H2-H3 (axial)}} = 4.5$ Hz, implying that the H2 proton is in the equatorial position. The vicinal coupling constants for the H5 proton (3.36 ppm) are $J = 5.5$ Hz and 2.5 Hz, indicating that it is in the equatorial position. Based on the coupling constants of H2 and H5 protons, it was concluded that this isomer is in *trans* configuration as shown.

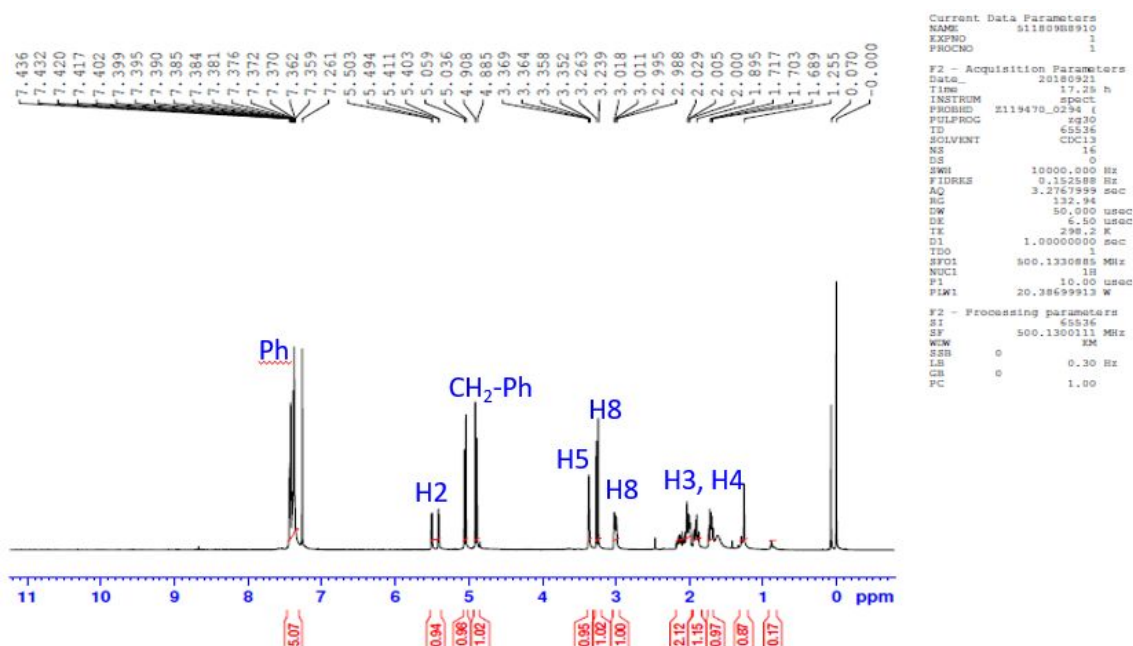


Figure S2. ^1H NMR of Isomer A

Isomer B

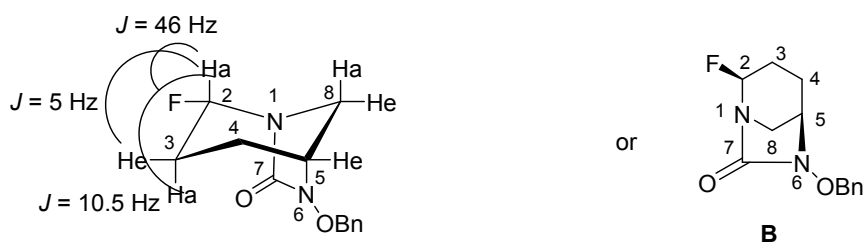


Figure S3. NMR analysis of Isomer B

The coupling constants for H2 proton (5.95 ppm), $^2J_{\text{H2-F}} = 46 \text{ Hz}$, $^3J_{\text{H2-H3 (axial)}} = 10.5 \text{ Hz}$ and $^3J_{\text{H2-H3 (equatorial)}} = 5 \text{ Hz}$, imply that it is in the axial position. The vicinal coupling constant for H2 proton (3.71 ppm), $J = 3 \text{ Hz}$, indicates that it is in the equatorial position. Based on the coupling constants of H2 and H5 protons, it was concluded that this isomer is in the *cis* configuration as shown.

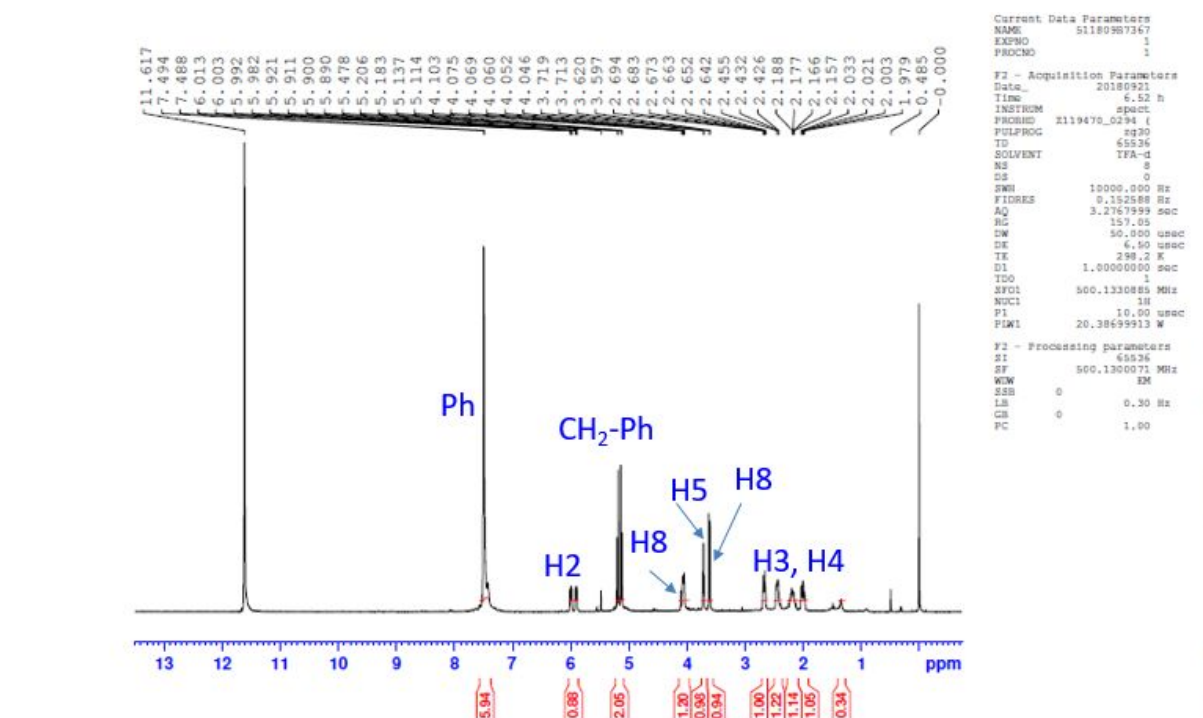


Figure S4. ^1H NMR of Isomer B

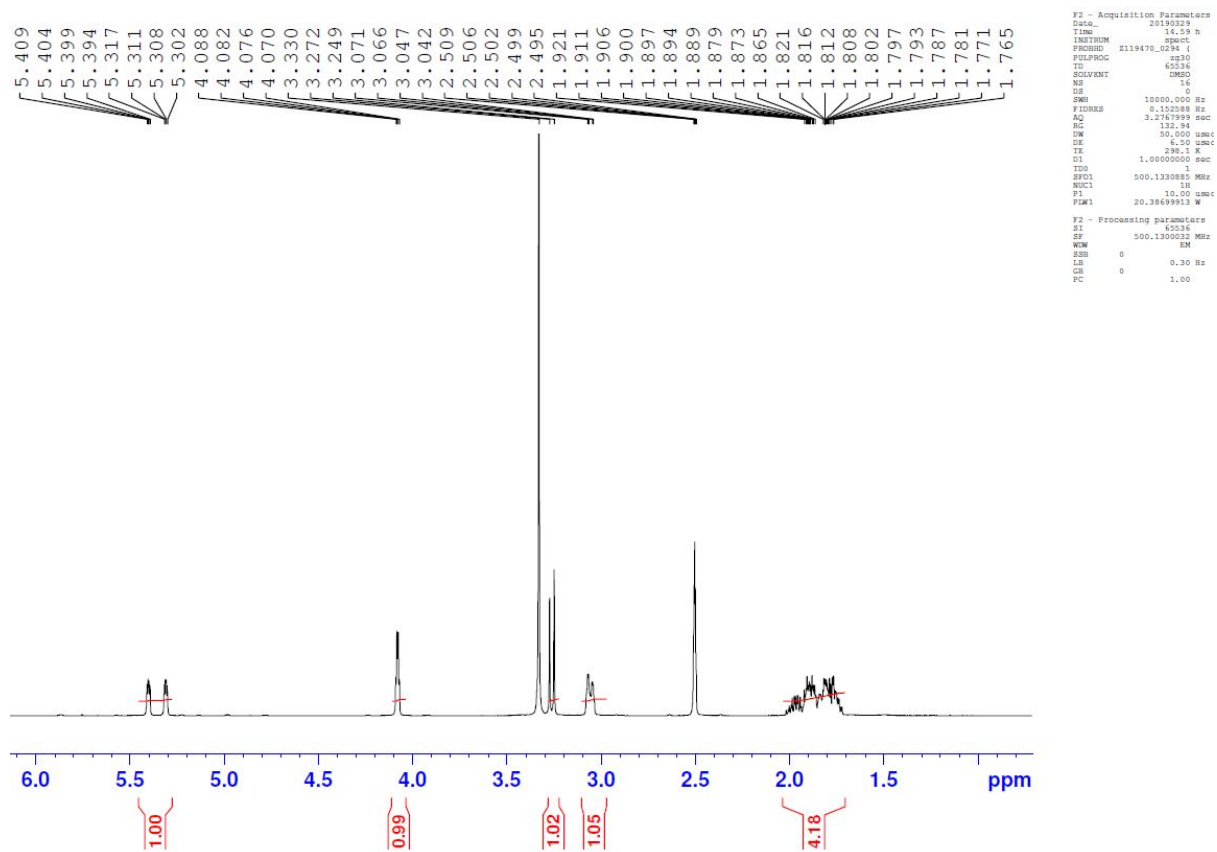


Figure S5. ¹H NMR spectrum of compound **21** (ANT3310)

AV-022-Na-C4282-025-Lot-7

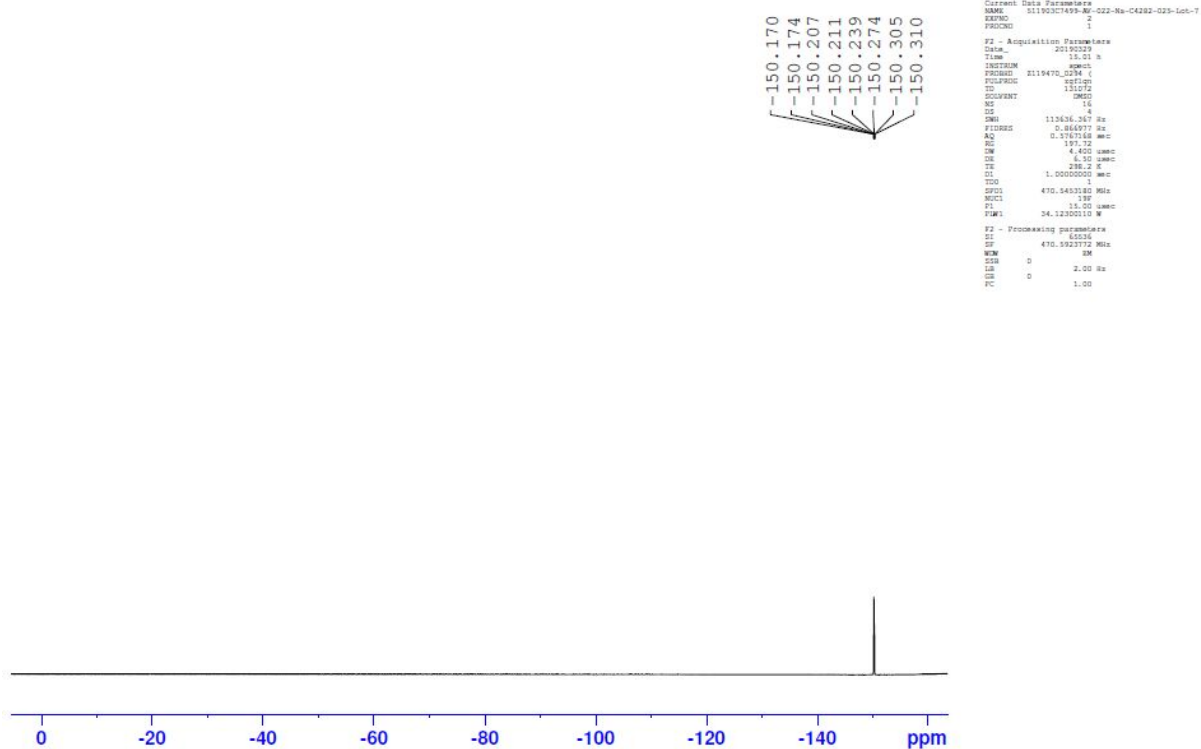


Figure S6. ^{19}F NMR spectrum of compound **21** (ANT3310)

AV-022-Na-C4282-025-Lot-7

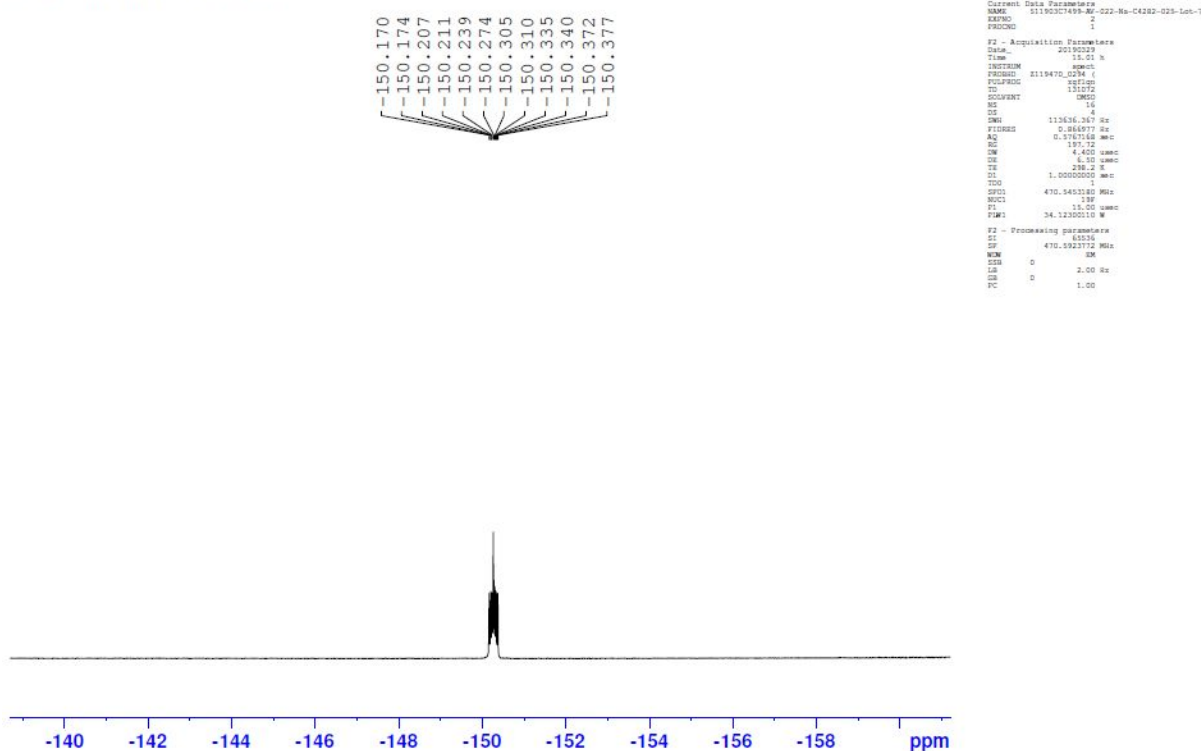


Figure S7. Expanded ^{19}F NMR spectrum of compound **21** (ANT3310)

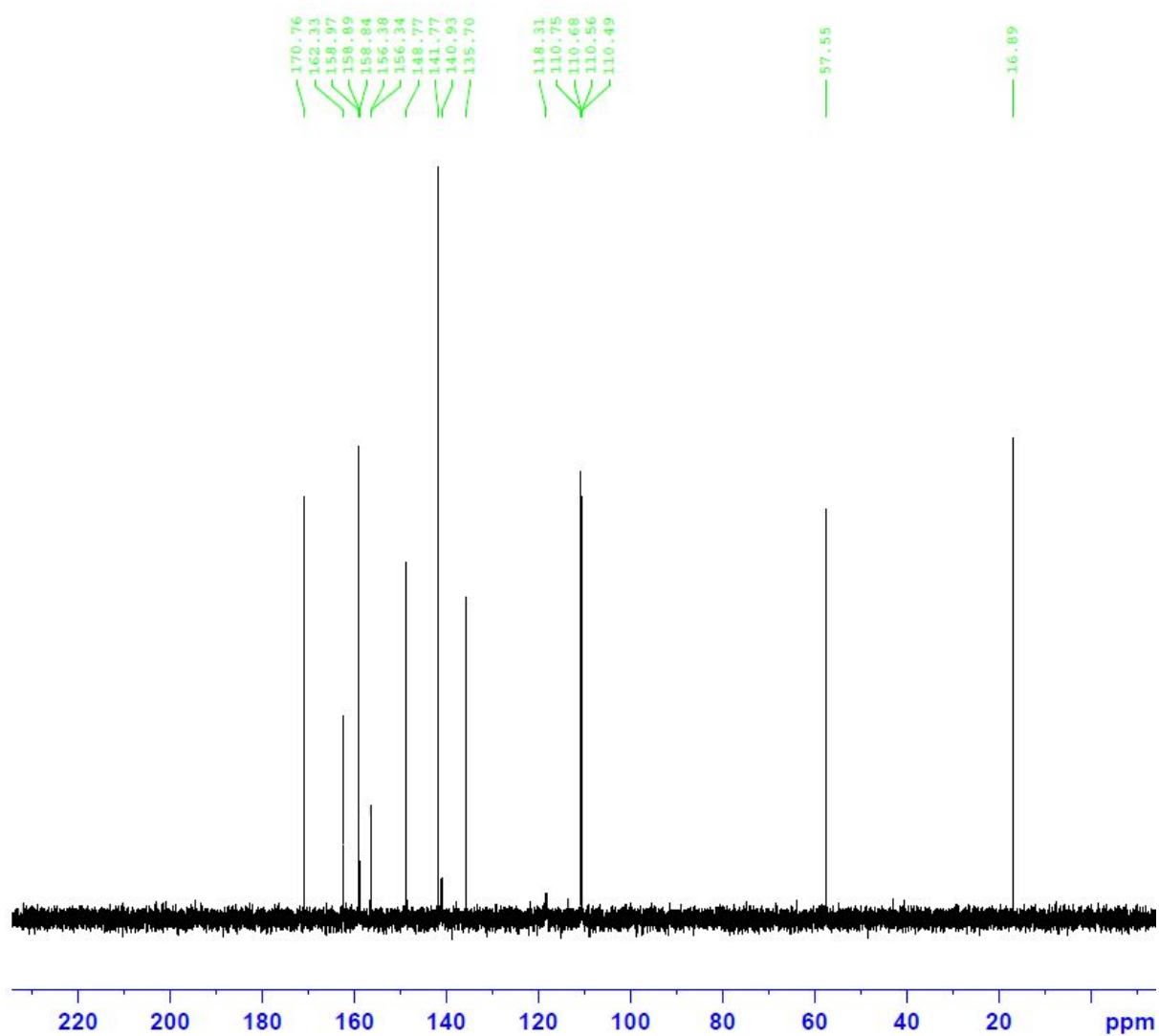
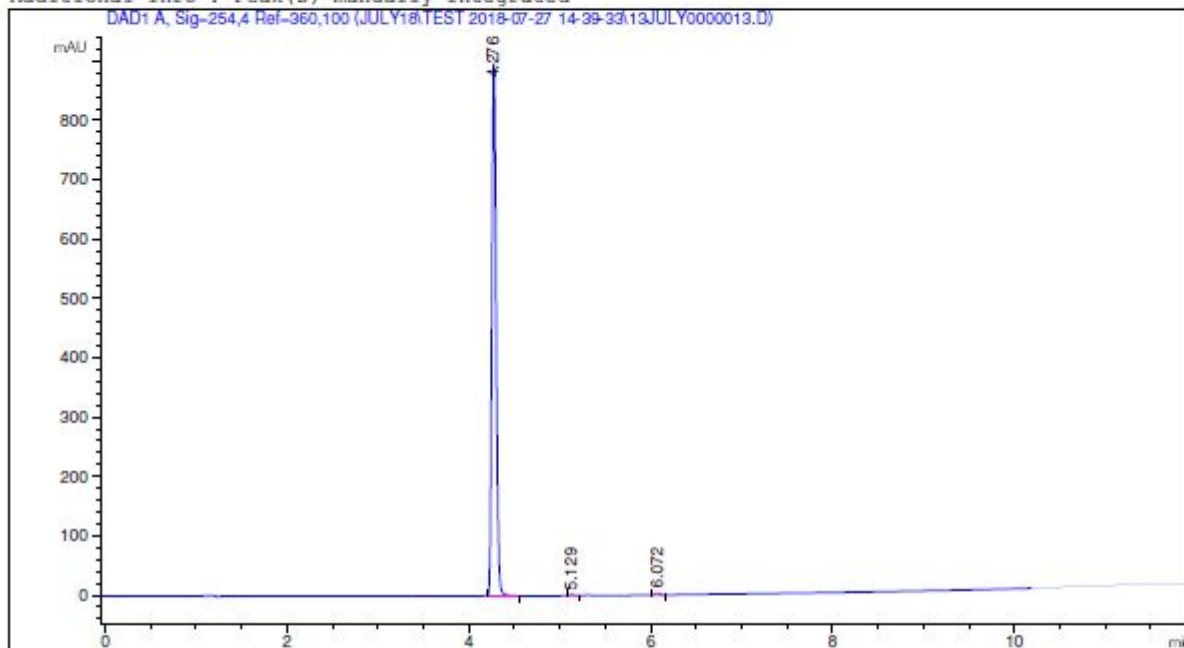


Figure S8. ^{13}C NMR of compounds **21** (ANT3310)

Acq. Operator : SMacB Seq. Line : 13
 Acq. Instrument : Instrument 1 Location : Vial 6
 Injection Date : 27/07/2018 16:59:24 Inj : 1
 Inj Volume : 5.0 µl
 Different Inj Volume from Sequence ! Actual Inj Volume : 2.5 µl
 Acq. Method : C:\CHEM32\1\DATA\JULY18\TEST 2018-07-27 14-39-33\ACID_LONG-ANALYSIS_254_
 POS.M
 Last changed : 13/04/2018 15:24:24 by SMacB
 Analysis Method : C:\CHEM32\1\METHODS\ACID_FAST_POS.M
 Last changed : 13/04/2018 12:41:00 by SMacB
 Additional Info : Peak(s) manually integrated



=====
 Area Percent Report
 =====

Sorted By : Signal
 Multiplier: : 1.0000
 Dilution: : 1.0000
 Use Multiplier & Dilution Factor with ISTDs

Signal 1: DAD1 A, Sig=254,4 Ref=360,100

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	4.276	VB	0.0523	2961.36694	902.61493	99.4826
2	5.129	VB	0.0489	6.08477	1.92516	0.2044
3	6.072	VB	0.0501	9.31824	2.85304	0.3130

Totals : 2976.76995 907.39313

=====
 *** End of Report ***

Figure S9. HPLC analysis of compound **21** (ANT3310)

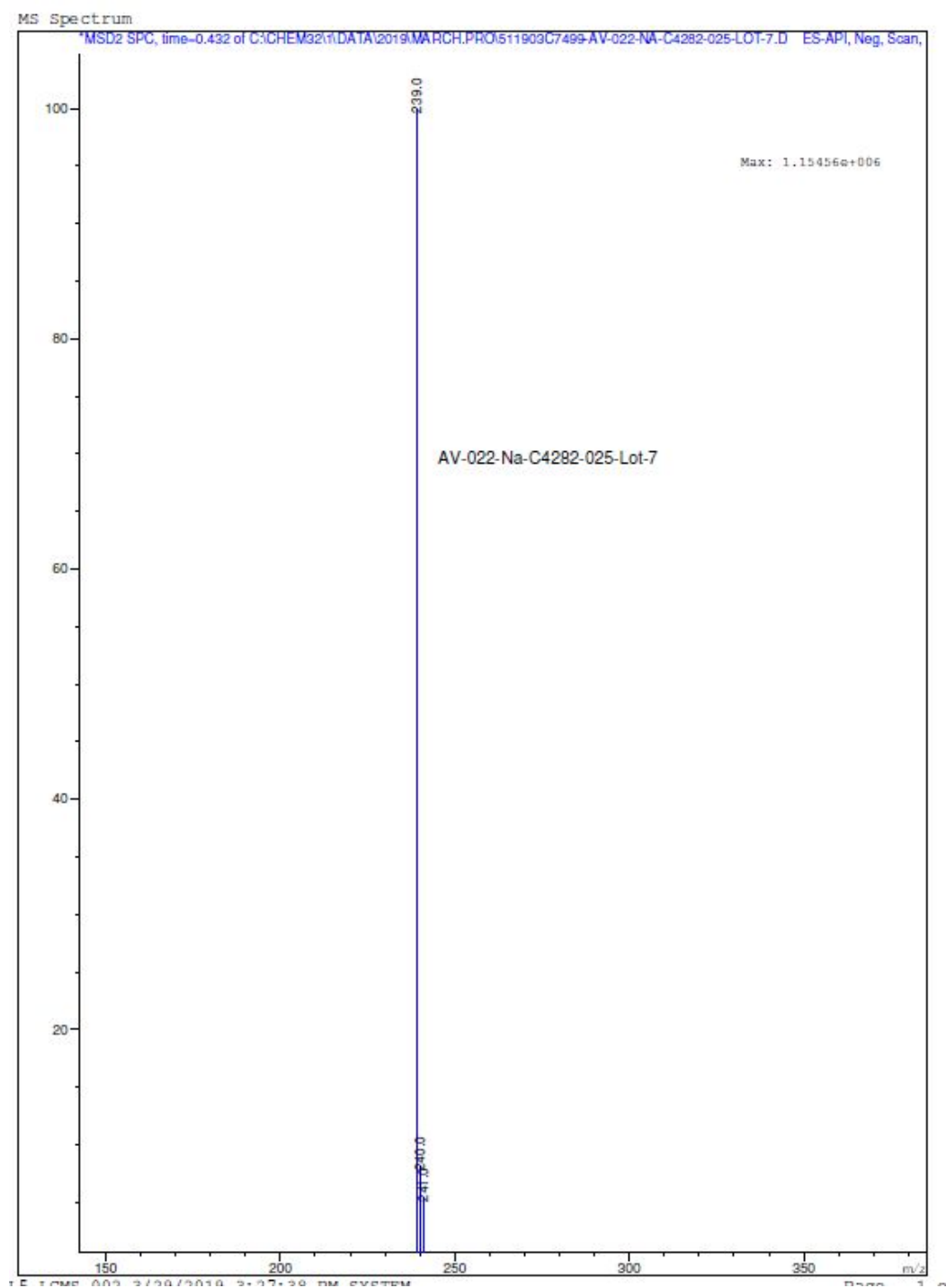


Figure S10. MS spectrum of compound **21** (ANT3310)
S8

Sample ID: ANT3310(AV-022)
Date : 25-02-2020

Instrument ID: ANL-MCL3-LCMS-014

D:\GVL\1\FEB\HRMS\ANT3310(AV-022)-

02/25/20 16:44:57

ANT3310(AV-022)-#184 RT: 1.79 AV: 1 NL: 1.16E10
T: FTMS -c ESI Full ms [100.0000-1500.0000]

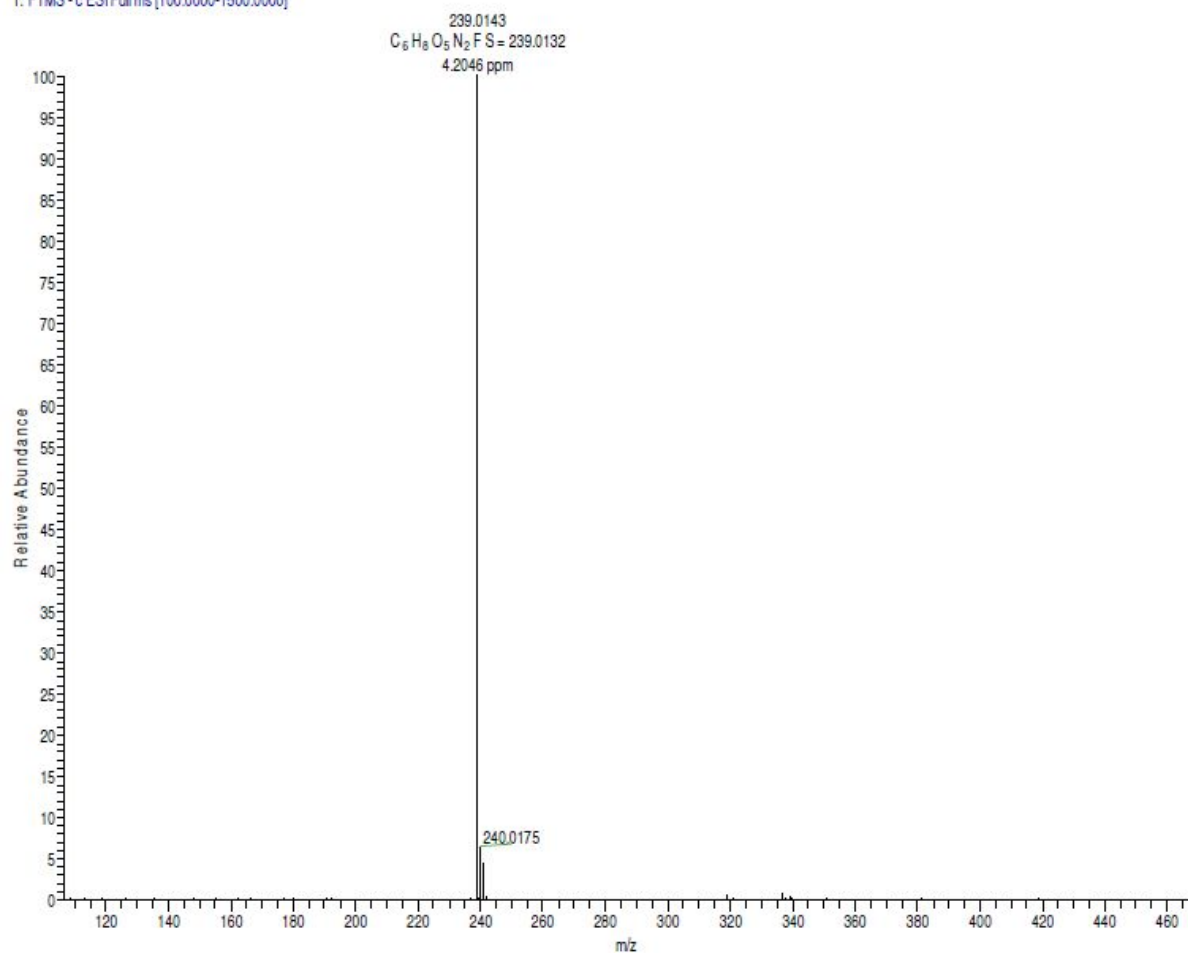


Figure S11. HRMS analysis of compound **21** (ANT3310)

***In vitro* enzyme assays and IC₅₀ determination**

Enzyme inhibition assays were performed using six clinically relevant purified β -lactamase enzymes in a 96-well microplate setup: TEM-1 (0.55 ng/well), KPC-2 (10 ng/well), AmpC (0.42 ng/well), OXA-48 (4.7 ng/well), OXA-23 (1.65 ng/well) and CTX-M-15 (8.8 ng/well) in 10 mM HEPES buffer (pH7.5) (and supplemented with 50 mM NaHCO₃ for OXA-23 assay) in 96-well microtiter plates.

Nitrocefin (Sigma-Aldrich, St Louis, Miss.), a chromogenic cephalosporin (λ , 482 nm; ϵ_M , 15,000 M⁻¹·cm⁻¹), was used as a reporter substrate at 100 μ M for TEM-1, KPC-2, AmpC, OXA-23, OXA-48 and at 50 μ M for CTX-M-15. Enzymes were preincubated in the presence of varying concentrations of inhibitors (0.006 to 3,000 nM, compound were dissolved in DMSO and subsequently diluted in the reaction buffer, the maximum residual DMSO concentration did not exceed 1%) for 10 minutes (to allow the formation of the enzyme-inhibitor covalent complex). Nitrocefin was then added and its hydrolysis by the uninhibited enzyme fraction followed for 10 min at 30 °C using a Envision UV fluorescence plate reader (Perkin Elmer, Waltham, Mass.).

Hydrolysis rate data were used to determine inhibitory activities, expressed in IC₅₀ values (corresponding to the compound concentration (μ M) required to inhibit 50% of enzymatic reaction).

Bacterial strains and antimicrobial susceptibility testing

The following SBL-positive clinical isolates were used to support SAR: *Enterobacter cloacae* KPC-2/TEM-1, *Klebsiella pneumoniae* SHV-11/TEM-1/KPC-3, *K. pneumoniae* SHV-11/OXA-181, *Acinetobacter baumannii* OXA-23 and *K. pneumoniae* TEM-OSBL/CTX-M-14/OXA-48. The first four isolates were supplied by P. Nordmann and the last isolate was purchased from IHMA (International Health Management Associates, Inc). Additional studies were performed against 50 KPC-CRE, 48 OXA-CRE and 50 OXA-CRAB from the 2017 global IHMA collection.

MEM and MEM/ANT3310 minimal inhibitory concentrations (MICs) were determined by broth microdilution according to Clinical and Laboratory Standards Institute (CLSI) guidelines, using cation-adjusted Mueller-Hinton (CAMBH; Becton Dickinson).

Colonies were taken directly from a culture plate and prepared to a suspension equivalent to

the 0.5 McFarland standard using normal saline. Microplates were inoculated within 15 min after adjustment of the inoculum suspension turbidity, and incubated at 37°C for 16 to 20 h. Quality control (QC) testing was performed each day of testing as specified by the CLSI using the following isolates: *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853.

CYP Inhibition Assay Procedure

158 µL of human liver microsomes working stock solution was added to all wells of the reaction plate (0.253 mg/mL, 158 µL/ well) then 2 µL of test compound/positive inhibitor working solutions were added to the reaction plate (2 µL/ well). For the control, 2 µL of acetonitrile containing DMSO (without inhibitor) were added to the reaction plate. Then 20 µL of substrate pool were added to all the wells of the reaction plate. The reaction plate was shaken and pre-incubated at 37°C for 20 min. 20 µL of the NADPH solution (pre-warmed at 37°C for 5 minutes) were added to the reaction plate which was then incubated at 37°C for 10 min. The reaction was terminated by adding cold acetonitrile (200 µL/well) to the reaction mixture. The plate was then centrifuged at 4,000 rpm for 20 minutes. After centrifugation, the supernatant was separated and analyzed by LC-MS/MS.

CYP inhibition (Time dependent) assay procedure

158 µL of human liver microsomes working stock solution was added to all wells of the reaction plate (0.253 mg/mL, 158 µL/ well) then 2 µL of test compound/positive inhibitor working solutions were added to the reaction plate (2 µL/ well). The reaction plate was pre warmed for 10 min at 37 degrees. 25 µL of NADPH solution were added to 'NADPH pre-incubation' wells. 25 µL of substrate solution were added to 'substrate pre-incubation' wells. The plate was mixed and incubated for 5 min at 37°C on a Julabo shaking water bath. After 10 minutes the reaction was terminated by adding ice cold acetonitrile (200µL) containing the internal standard to all the wells. The plate was then centrifuged at 4000 rpm for 30 minutes. The supernatant was diluted 1:1 with water and the corresponding metabolite was assayed by LC-MS/MS analysis.

Generalised Protocol for Plasma Protein Binding

3 μL of 1 mM of test compound in methanol was added to 297 μL of plasma (mouse, rat, dog human). 200 μL of this solution was added to the donor well. 350 μL of PBS buffer pH7.4 was added to the receiver well. The plate was incubated at 37°C in a thermomixer at 400 rpm for 5 hours. The samples were matrix equilibrated with the opposite matrix (ie equal volumes of plasma/buffer samples were matched with blank buffer/plasma). Samples were precipitated with 200 μL of acetonitrile containing the internal standard then vortexed at 1000 rpm for 5 minutes and centrifuged at 4000 rpm for 10 minutes. The supernatant was analysed by LC-MS/MS. The percentage of unbound drug was determined by the equation $\% \text{ Unbound} = 100 \times F_c/T_c$ and the percentage recovery by the equation $\% \text{ Recovery} = 100 \times (F_c + T_c)/T_0$ where T_c = total compound concentration as determined by the calculated concentration on the plasma side of the membrane; F_c = total compound concentration as determined by the calculated concentration on the buffer side of the membrane; T_0 = total compound concentration as determined before dialysis.

Murine Thigh Infection Model

Infection models in the thigh muscle tissue of neutropenic mice have been the bedrock of *in vivo* pre-clinical validation studies and are routinely used to translate *in vitro* data to *in vivo* efficacy of antimicrobial agents. In these studies, the efficacy of test articles in reducing the bacterial burden in the mouse thigh following infection with two different strains of *K. pneumoniae* was determined. All procedures were conducted in accordance with the United Kingdom Animal (Scientific Procedures) Act 1986, approved by institutional ethical review committees (Agenda Resources, Alderley Park Animal Welfare and Ethical Review Board) and conducted under the authority of the Project Licence PA67E0BAA. Specific pathogen free male CD-1 mice (11-15 g on receipt) (Charles River Laboratories, Margate, Kent, UK) were allowed to acclimatize for ~10 days. Mice were kept in individually ventilated cages (IVCs; Tecniplast, Italy) at a temperature of 20°C to 24°C,

humidity of 50% to 60%, 60 air exchanges per hour in the cages, and a 12/12-hour light/dark cycle with the lights on at 6:00 AM. The maximum caging density was five mice per cage. As bedding, aspen chip (Datasand Stockport UK) were provided. Mice were fed a standardized mouse chow and provided drinking water *ad libitum*. All materials, including IVCs, lids, feeders, bottles, bedding, and water were autoclaved before use. Throughout the duration of the experiment, mice were monitored for changes in health as frequently as clinically indicated but at least twice daily pre-infection and every 2h post infection. Following acclimatization mice were rendered neutropenic by immunosuppression with cyclophosphamide by subcutaneous injection at 150 mg/kg 4 days before infection and 100 mg/kg 1 day before infection. The immunosuppression regime leads to neutropenia starting 24 h post administration of the first injection continuing throughout the study. Previously prepared frozen stocks of *K. pneumoniae* NR-48977 (KPC-2, TEM-1, SHV-11, SHV-12) and *K. pneumoniae* ACC00783 (OXA-48) were diluted immediately prior to infection to a concentration of 6.33×10^6 CFU/mL and 2.3×10^7 CFU/mL, respectively. Mice were randomized into groups of 5 mice per group, then infected with 0.05 mL of the corresponding bacterial suspension by intramuscular (IM) injection under brief inhaled anaesthesia (2.5% isoflurane in 87.5% oxygen for 3-4 minutes) into both thighs (3.17×10^5 cfu/thigh and 1.15×10^6 cfu/thigh, respectively). Meropenem/vaborbactam and tigecycline were used as positive controls against KPC-2 *K. pneumoniae* NR-48977 and OXA-48 *K. pneumoniae* ACC00783, respectively. Test articles were administered intravenously (IV) at 10 mL/kg. Meropenem alone or with either ANT3310 or vaborbactam was dosed at 1, 3, 5, and 7 h post-infection and tigecycline was administered once 1 h post-infection. One group of animals was humanely euthanized using pentobarbitone overdose 1-hour post-infection to provide a pre-treatment control group. All animals in the additional groups were euthanized at the end of the study, 9 h post-infection. Thigh samples were homogenized in ice cold sterile phosphate buffered saline; the homogenates were quantitatively cultured onto CLED agar and incubated at 37°C for 18 - 24 h before colonies were counted. The data from the culture burdens were analyzed using appropriate non-parametric statistical models (Kruskal-Wallis using Conover-Inman to make all pairwise comparisons between groups) with StatsDirect software v. 3.1.22, and compared to vehicle control. For all calculations, the thighs from each animal were treated as two separate data points even though they are not completely independent samples.

X-ray crystallography

The OXA-48 carbapenem-hydrolyzing β -lactamase was purified and crystallized using the sitting drop method essentially as previously described¹⁻³. Crystals of the native enzyme were obtained at pH 7.5 in 0.1 M HEPES buffer containing 12% PEG4000 and 3% 1-butanol. Crystals were soaked for 15 minutes by adding 0.7 μ L of a 50 mM solution of compound **21** (**ANT3310**) in the precipitant solution, prior to transferring to the cryoprotectant (20% PEG400 in the precipitant solution) and flash-freezing in liquid nitrogen. X-ray diffraction data were collected at the Diamond Light Source (beamline I04; Didcot, United Kingdom). Data were processed as described elsewhere^{1,2,4} and the structures obtained by molecular replacement using the native structure of the corresponding enzyme (PDB entry, 3HBR), excluding solvent and other ligands. Refinement was performed with REFMAC5⁵ from the CCP4 suite⁶, using an iterative manual rebuilding and modelling of missing atoms in the electron density using COOT⁷. Water molecules were added using the standard procedures implemented in the ARP/wARP suite⁸. Data collection and model refinement statistics are shown in Table S1. The coordinates and structure factors were deposited in the Protein Data Bank under code 6ZXI.

Table S1. Data collection and refinement statistics.

Parameter	OXA-48: 21 (ANT3310)
X-ray source	Diamond beamline I04
Wavelength (\AA)	0.9795
Data collection temp (K)	100
Space group	P2 ₁ 2 ₁
Cell dimensions (\AA)	a = 44.80, b = 105.11, c = 125.43
subunits/asymmetric unit	2
Matthews coefficient ($\text{\AA}^3 \text{Da}^{-1}$)	2.62
Solvent content (%)	53.13
Resolution limits (\AA) ^a	53.85 – 1.85 (1.95 – 1.85)
No. of reflections measured ^a	387,486 (56,588)
No. of unique reflections ^a	51,511 (7,403)

Completeness (%) ^a	100.0 (99.9)
R _{merge} (%) ^{a,b}	6.8 (87.1)
Multiplicity ^a	7.5 (7.6)
I/σ(I) ^a	14.8 (2.2)
Wilson B factor (Å ²)	28.27
R _{cryst} (%) ^{a,b}	17.41 (29.0)
R _{free} (%) ^{a,b}	21.75 (31.4)
Protein atoms	3,981
Ligand atoms	42
Water molecules	428
Avg B factor (Å ²)	33.25
RMSD bond length (Å)	0.010
RMSD bond angle (°)	1.617

^aData in parentheses refer to results for the highest-resolution shell.

$$^bR_{\text{merge}} = \frac{\sum_h \sum_i |I_{i,h} - \bar{I}_h|}{\sum_h \sum_i I_{i,h}} \times 100. \quad R_{\text{cryst}}(R_{\text{free}}) = \frac{\sum_h ||F_{h,\text{obs}}| - |F_{h,\text{calc}}||}{\sum_h |F_{h,\text{obs}}|} \times 100.$$

References

- (1) Docquier, J.-D.; Calderone, V.; De Luca, F.; Benvenuti, M.; Giuliani, F.; Bellucci, L.; Tafi, A.; Nordmann, P.; Botta, M.; Rossolini, G. M.; Mangani, S. Crystal Structure of the OXA-48 Beta-Lactamase Reveals Mechanistic Diversity among Class D Carbapenemases. *Chem. Biol.* 2009, 16 (5), 540–547. <https://doi.org/10.1016/j.chembiol.2009.04.010>.
- (2) Lahiri, S. D.; Mangani, S.; Jahić, H.; Benvenuti, M.; Durand-Reville, T. F.; De Luca, F.; Ehmann, D. E.; Rossolini, G. M.; Alm, R. A.; Docquier, J.-D. Molecular Basis of Selective Inhibition and Slow Reversibility of Avibactam against Class D Carbapenemases: A Structure-Guided Study of OXA-24 and OXA-48. *ACS Chem. Biol.* 2015, 10 (2), 591–600. <https://doi.org/10.1021/cb500703p>.

- (3) De Luca, F.; Benvenuti, M.; Carboni, F.; Pozzi, C.; Rossolini, G. M.; Mangani, S.; Docquier, J.-D. Evolution to Carbapenem-Hydrolyzing Activity in Noncarbapenemase Class D β -Lactamase OXA-10 by Rational Protein Design. *PNAS* **2011**, *108* (45), 18424–18429. <https://doi.org/10.1073/pnas.1110530108>.
- (4) Pozzi, C.; Di Pisa, F.; De Luca, F.; Benvenuti, M.; Docquier, J. D.; Mangani, S. Atomic-Resolution Structure of a Class C β -Lactamase and Its Complex with Avibactam. *ChemMedChem* **2018**, *13* (14), 1437–1446. <https://doi.org/10.1002/cmdc.201800213>.
- (5) Murshudov, G. N.; Skubák, P.; Lebedev, A. A.; Pannu, N. S.; Steiner, R. A.; Nicholls, R. A.; Winn, M. D.; Long, F.; Vagin, A. A. REFMAC5 for the Refinement of Macromolecular Crystal Structures. *Acta Crystallogr. D Biol. Crystallogr.* **2011**, *67* (Pt 4), 355–367. <https://doi.org/10.1107/S0907444911001314>.
- (6) Winn, M. D.; Ballard, C. C.; Cowtan, K. D.; Dodson, E. J.; Emsley, P.; Evans, P. R.; Keegan, R. M.; Krissinel, E. B.; Leslie, A. G. W.; McCoy, A.; McNicholas, S. J.; Murshudov, G. N.; Pannu, N. S.; Potterton, E. A.; Powell, H. R.; Read, R. J.; Vagin, A.; Wilson, K. S. Overview of the CCP4 Suite and Current Developments. *Acta Crystallogr. D Biol. Crystallogr.* **2011**, *67* (Pt 4), 235–242. <https://doi.org/10.1107/S0907444910045749>.
- (7) Emsley, P.; Lohkamp, B.; Scott, W. G.; Cowtan, K. Features and Development of Coot. *Acta Crystallogr. D Biol. Crystallogr.* **2010**, *66* (Pt 4), 486–501. <https://doi.org/10.1107/S0907444910007493>.
- (8) Langer, G.; Cohen, S. X.; Lamzin, V. S.; Perrakis, A. Automated Macromolecular Model Building for X-Ray Crystallography Using ARP/WARP Version 7. *Nat Protoc* **2008**, *3* (7), 1171–1179. <https://doi.org/10.1038/nprot.2008.91>.

