Supplementary material

to

Phosphate promotes the recovery of *Mycobacterium tuberculosis* β-lactamase from clavulanic acid inhibition.

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Supplementary Tables

Table S1. M9 medium constituents per liter, in MilliQ water.

KH_2PO_4	13.0 g
K_2HPO_4	10.0 g
Na_2HPO_4	9.0 g
Na_2SO_4	2.4 g
$^{15}NH_4Cl$	0.3 g
Glucose	4.0 g
thiamine	30 mg
$MgCl_2$	2 g
Biotin	1 mg
Choline chloride	1 mg
Folic acid	1 mg
Niacinamide	1 mg
D-pantothenate	1 mg
Pyridoxal	1 mg
Riboflavin	0.1 mg
$FeSO_4$ (7 H_2O)	10 mg
$CaCl_2$ (2 H_2O)	60 mg
$MnCl_2$ (4 H_2O)	12 mg
$CoCl_2$ (6 H_2O)	8 mg
$ZnSO_4$ (7 H_2O)	7 mg
$CuCl_2$ (2 H_2O)	3 mg
H_3BO_3	0.2 mg
EDTA	50 mg

Table S2. Data collection and refinement statistics of structures 5NJ2 and 5OYO.

Parameter	Value for 5NJ2	Value for 5OYO
Space group	P1	P1
Unit-cell parameters a, b, c, α, β, γ	39.59, 41.68, 76.84, 101.28, 90.11, 90.43	39.59, 41.42, 76.52, 104.29, 90.07, 90.67
Number of observations	310711	73383
Number of unique reflections	96841	33346
Completeness (%)	62.7 (97.8 inner, 4.5 outer)	94.2 (96.4 inner, 81.4 outer)
R_{pim} (%), CC(1/2) highest resolution bin	0.053, 0.998	0.074, 0.991
$\langle I/\sigma(I)\rangle$	1.10	6.8
Multiplicity	3.2 (3.2 inner, 2.0 outer)	3.2 (3.2 inner, 2.0 outer)
Resolution range (Å)	40.88 – 1.19	40.13 – 2.10
R factor (%)	16.7	21.6
R free	17.6	25.3
R.m.s. deviations		
Bond lengths (Å)	1.04	0.75
Bond angles (°)	1.21	0.93
Number of atoms	4416	4237
Protein (Chains A, B)	2076, 1992	2038, 2005
Phosphate ions	15	0
Acetate ions	16	28
Polyethylene glycol	29	25
Glycerol	0	6
Water	288	135
Ramachandran plot (%)		
Preferred regions	98	98
Allowed regions	2	2
Outliers	0	0

Table S3. Nitrocefin hydrolysis by BlaC with and without His-tag. [a]

BlaC	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\cdot 10^5$ $M^{-1}s^{-1}$)
His-tagged	81 ± 7	73 ± 3	11.1 ± 0.4
Not tagged	77 ± 1	80 ± 1	9.5 ± 0.1

[a] Buffer was 100 mM MES, pH 6.4. Errors represent the standard deviation over duplicate measurements.

Table S4. Simulation parameters of BlaC inhibition by clavulanic acid. [a]

	K_D [b] (μM)	k_a ($\mu M^{-1}s^{-1}$)	k_b (s^{-1})	k_c (s^{-1})	K_I (μM)	k_1 ($\mu M^{-1}s^{-1}$)	k_2 (s^{-1})	k_3 (10^{-4} s^{-1})
MES 100 mM, pH 6.4	170	1.05	3000	148	20	1	0.045	0.25
NaP_i, 100 mM, pH 6.4	220	1.05	2500	103	20	1	0.045	18

[a] The parameters used for the simulations shown in Figure S6 are listed. The model is described in equations 3 and 4. Note that some parameters are correlated and the values should be considered as indicative. The large difference in k_3 under different buffer conditions is, however, very significant. The concentrations of BlaC and nitrocefin were 2 nM and 125 μM, respectively.

[b] $K_D = k_{-a}/k_a$

Supplementary figures

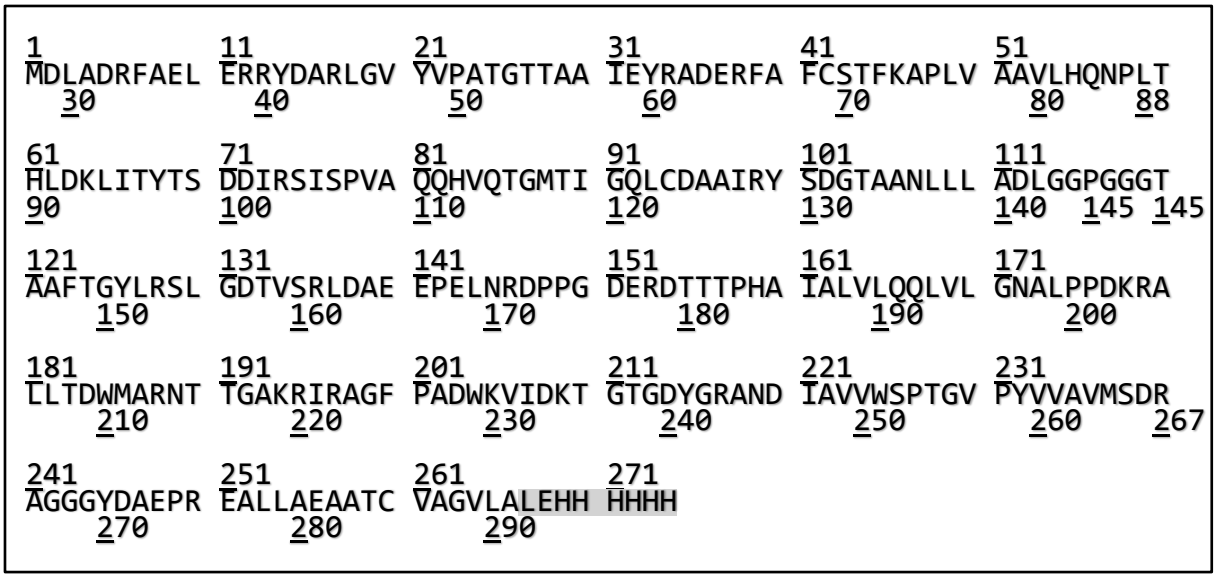

Figure S1. Amino acid sequence of the BlaC protein used in this study. The upper numbering corresponds to the actual sequence, the lower to the Ambler notation.[S1] Residues 2-266 (upper numbering) are residues 43-307 of BlaC Uniprot sequence P9WKD3-1. The His-tag residues are highlighted in grey.

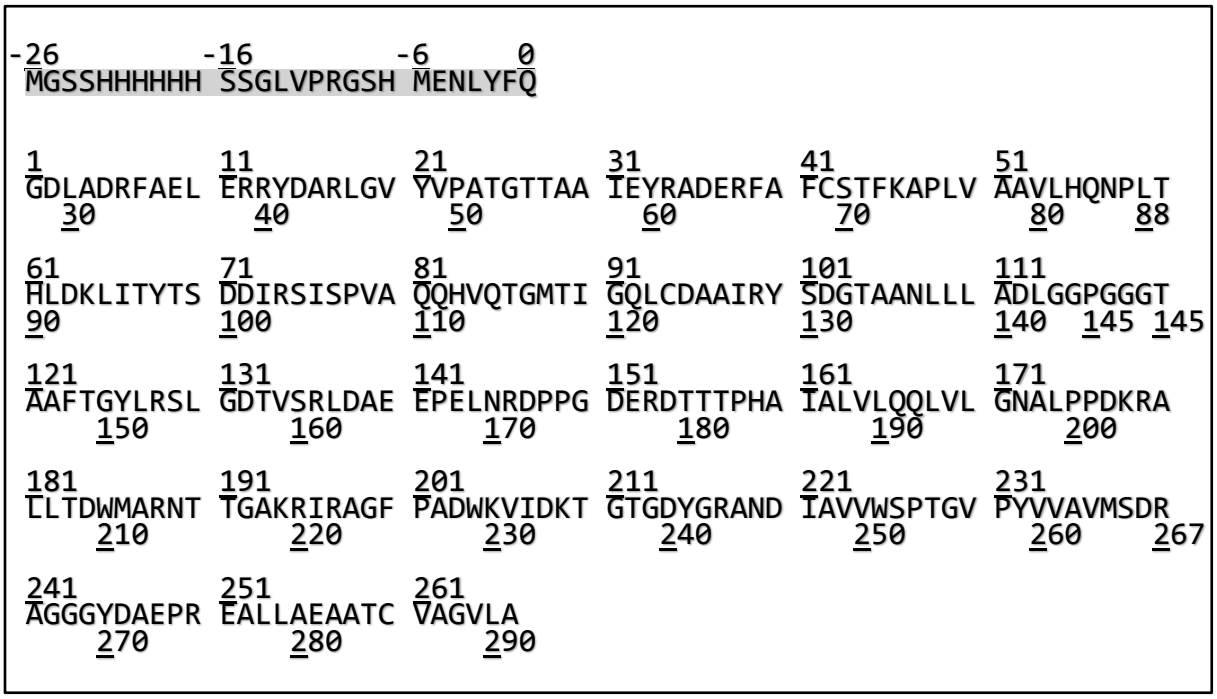

Figure S2. Amino acid sequence of the BlaC protein with cleavable His-tag. The upper numbering corresponds to the sequence of the protein after cleavage, the lower to the Ambler notation.[S1] Residues 2-266 (upper numbering) are residues 43-307 of BlaC Uniprot sequence P9WKD3-1. The residues of the TEV-cleavable His-tag are highlighted in grey. The sequence after cleavage with TEV protease (residues 1-266, no highlight) is the same as that shown in Figure S1, except that the N-terminal Met in that sequence is replaced by Gly and the eight residues constituting the C-terminal His-tag are lacking.

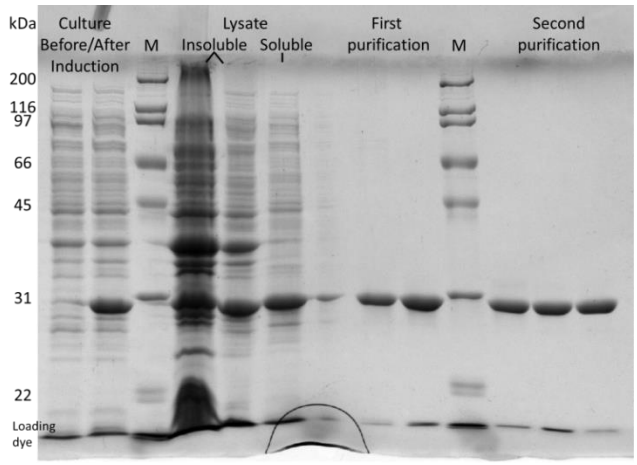

Figure S3. SDS-PAGE illustrating the production and purification process of BlaC (29 kDa). Minor impurities present after the first (Nickel column) purification are no longer visible after the second (gel filtration column) purification. This gel shows a representative protein batch. 'M' indicates a protein ladder, with band sizes shown on the left.

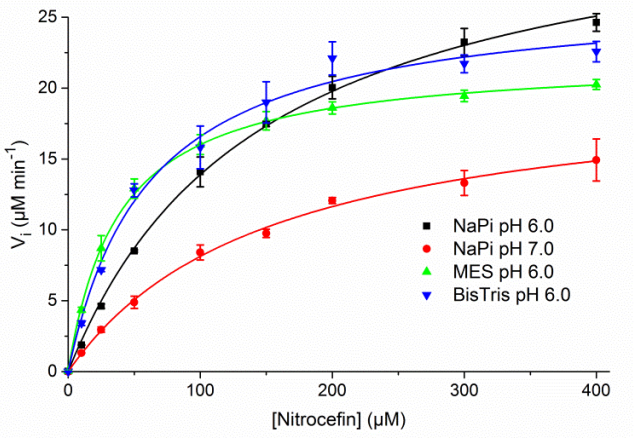

Figure S4. Michaelis-Menten curves for the hydrolysis of nitrocefin by 5.4 nM BlaC in various buffers. Error bars represent standard deviation of triplicate measurements.

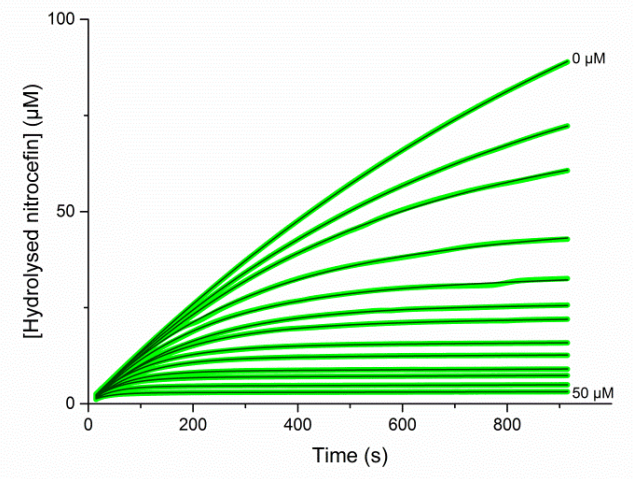

Figure S5. Inhibition curves of BlaC nitrocefin hydrolysis with increasing concentrations of clavulanic acid in 100 mM MES buffer pH6.4. Green lines represent experimental data, black lines are fits using equation 1.

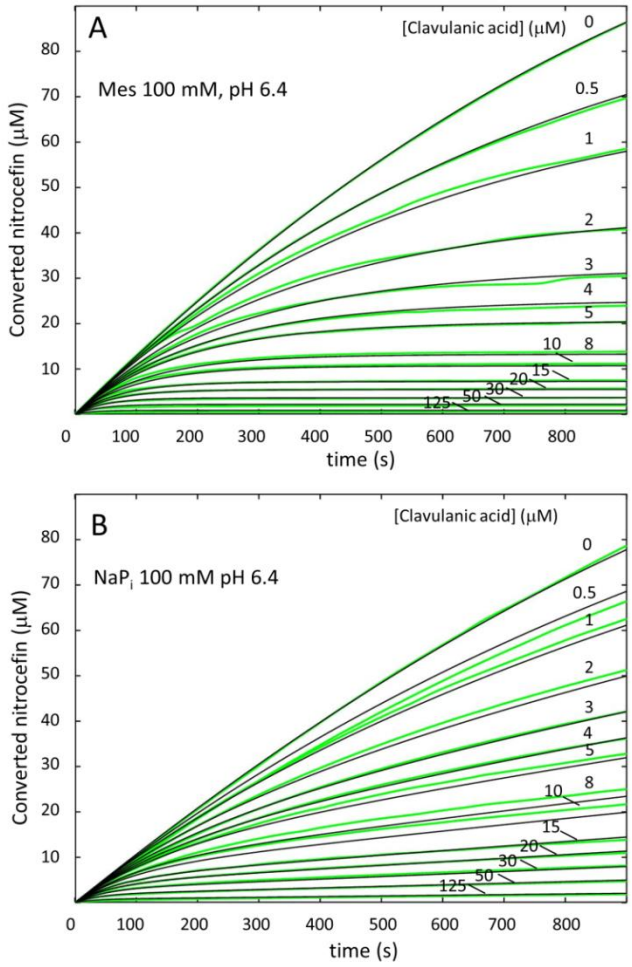

Figure S6. Inhibition of BlaC nitrocefin hydrolysis by clavulanic acid, in MES buffer (**A**) and phosphate buffer (**B**). The experimental curves (green) were simulated using GNU Octave software (black) and the models described in equations (3) and (4). [Nitrocefin], 125 µM; [BlaC], 2 nM; temperature, 298 K. The concentrations of clavulanic acid and the buffer conditions are indicated. Note the difference in the slopes of the curves at the end of the experiments. The larger slopes in NaP_i buffer reflect the larger hydrolysis of BlaC-clavulanic acid intermediate (rate constant k_3).

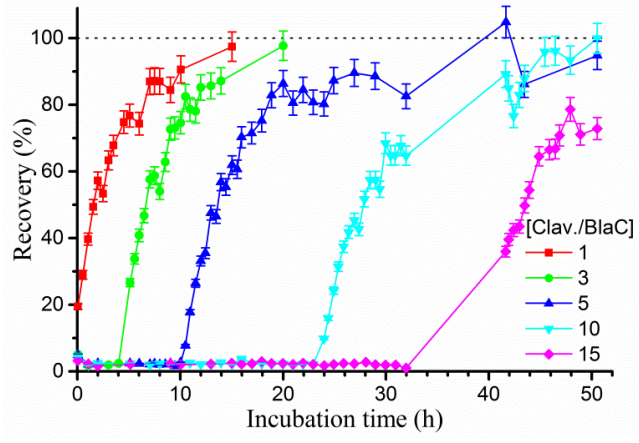

Figure S7. BlaC recovery from clavulanic acid inhibition with varying inhibitor / enzyme ratios. BlaC (100 µM) was incubated with 0.1, 0.3, 0.5, 1 and 1.5 mM clavulanic acid, respectively. Samples were taken at various time points, diluted to 2.0 nM BlaC and tested for hydrolase activity using 100 µM nitrocefin. Between 32 and 41.5 h, no measurements were performed due to a technical failure. The error bars are relative to the activity and represent the standard deviation in the 100% activity control measurements.

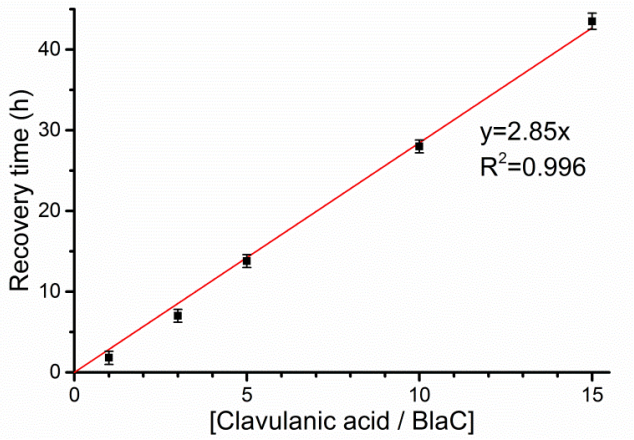

Figure S8. Relation between recovery time and molar ratio of enzyme and inhibitor. Recovery times (black squares) are the incubation times required to reach 50% activity, as estimated from Figure S7. Error bars represent the estimated error in reading recovery times from single curves. The text inset describes the linear fit through the origin, consistent with a first order reaction (red line). The slope represents the turn-over rate in h^{-1}.

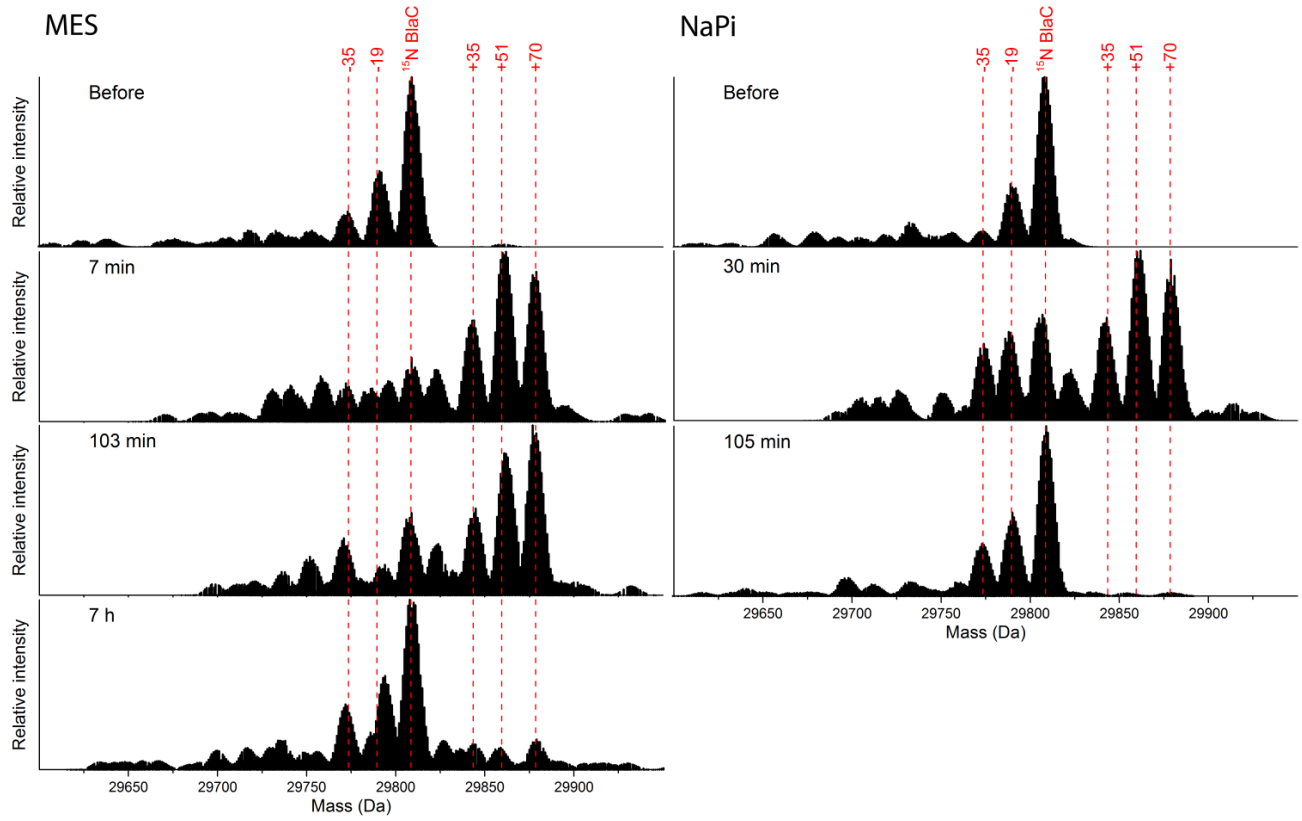

Figure S9. Charge-deconvoluted ThermoScientific Orbitrap mass spectra of BlaC before and during incubation with 1.5 mM clavulanic acid (protein: clavulanic acid ratio 1:5) in 100 mM MES (left) or NaPi (right) buffer, pH 6.4. Upon inhibition with clavulanic acid, the main species contain covalently bound adducts. After prolonged incubation, the enzyme returns to its free form in either buffer, but with different rates. For this experiment, ^{15}N labelled BlaC was used. This sample was also used in the NMR experiment. Each spectrum was normalised to the highest signal intensity.

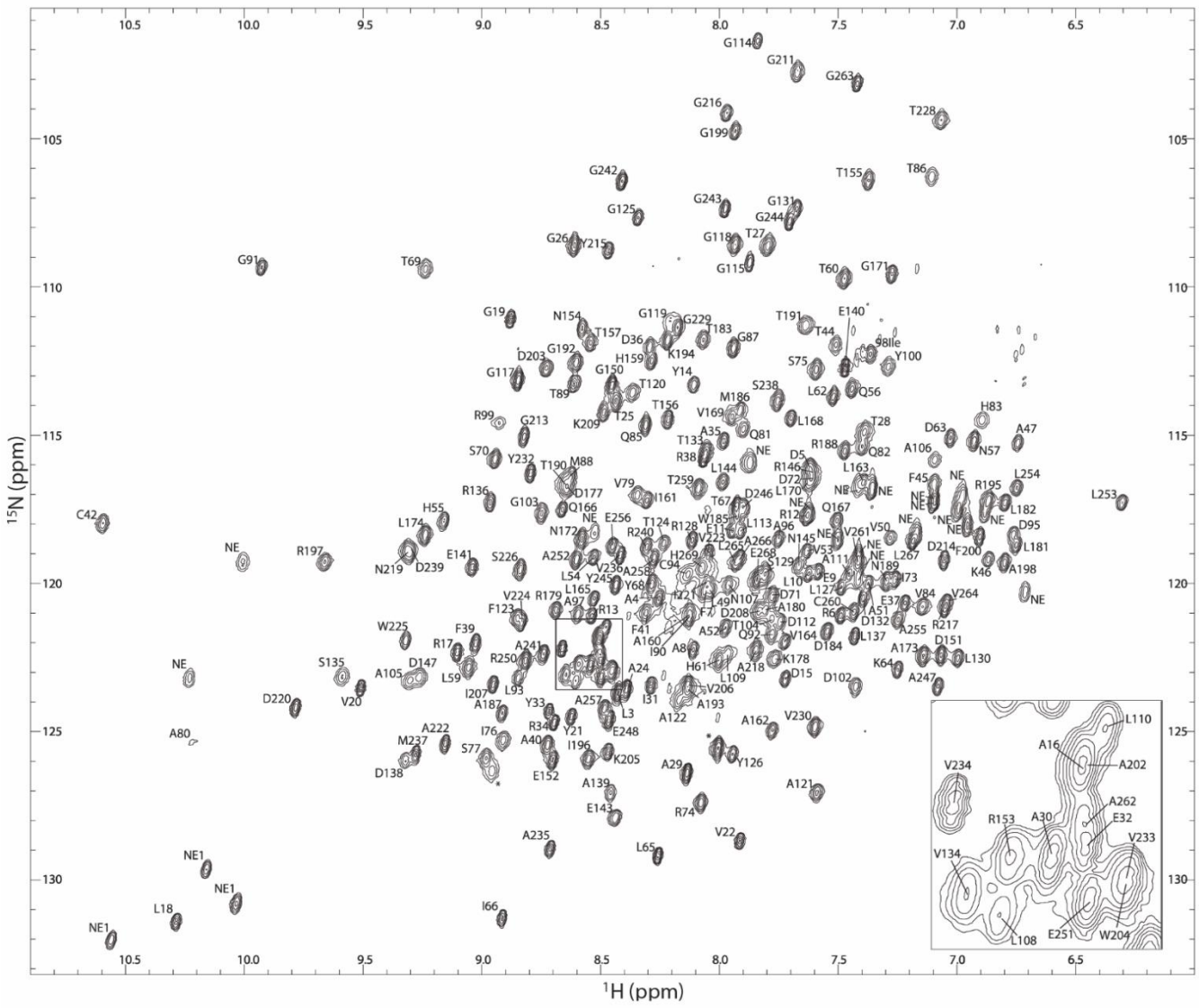

Figure S10. TROSY-HSQC spectrum of BlaC with residue assignments. 'NE' labels indicate arginine amide ε signals, 'NE1' labels indicate tryptophan amide ε1 signals. Numbering refers to the protein as it was measured, conversion to the Ambler numbering scheme can be found in Figure S1. The assignment can be accessed under Biological Magnetic Resonance Bank ID 27067.

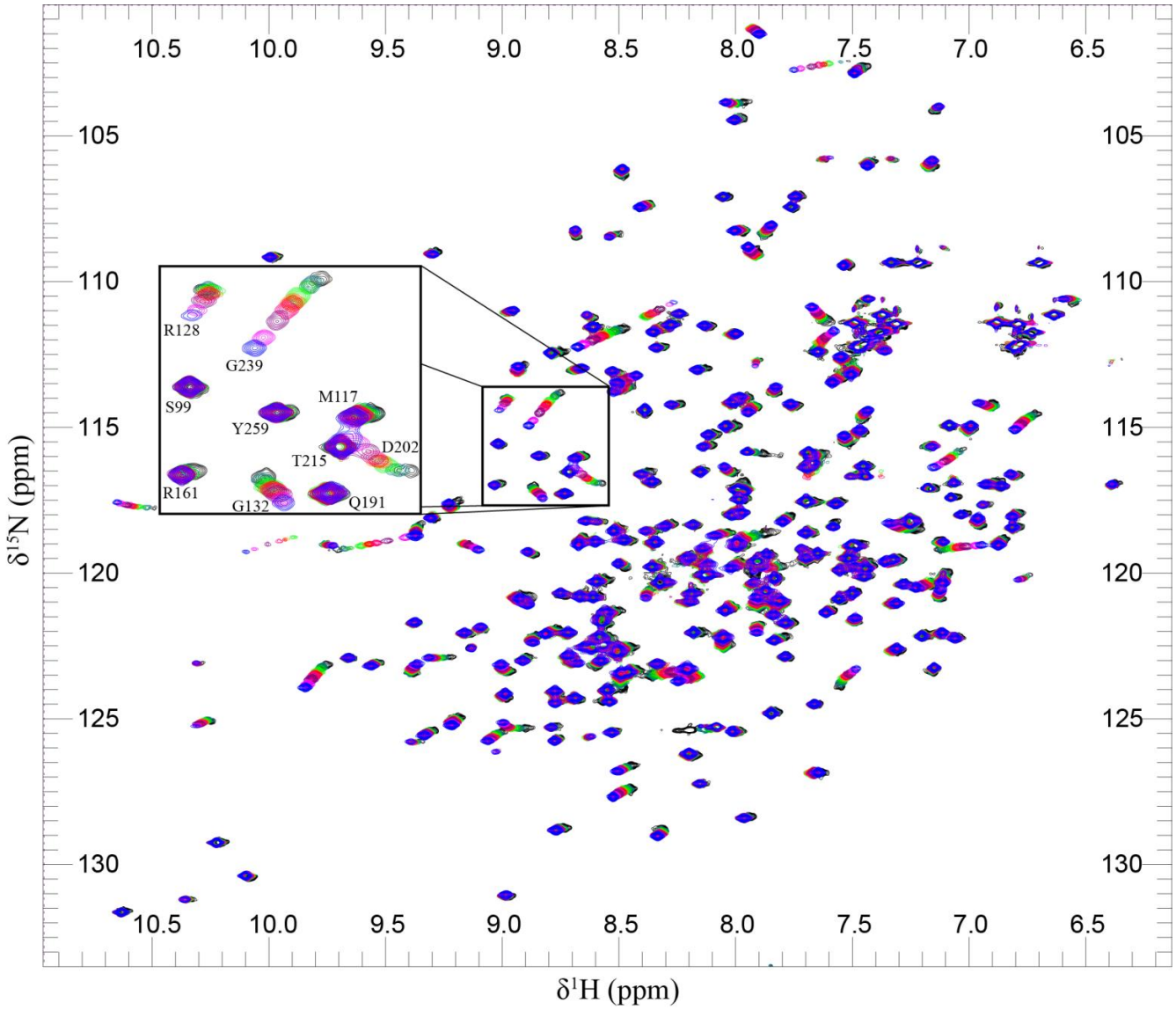

Figure S11. Overlay of ^{1}H-^{15}N HSQC spectra of BlaC upon titration with phosphate. The sample contained 350 µM BlaC, 20 mM MES buffer, pH 6.2, 1 mM DTT, 6% D_2O, without (blue peaks) and with 5 mM (magenta), 10 mM (purple), 15 mM (pink), 20 mM (red), 30 mM (orange), 50 mM (lime), 100 mM (green), 150 mM (teal) or 250 mM (black) of sodium phosphate, respectively. Selected residue numbers are shown.

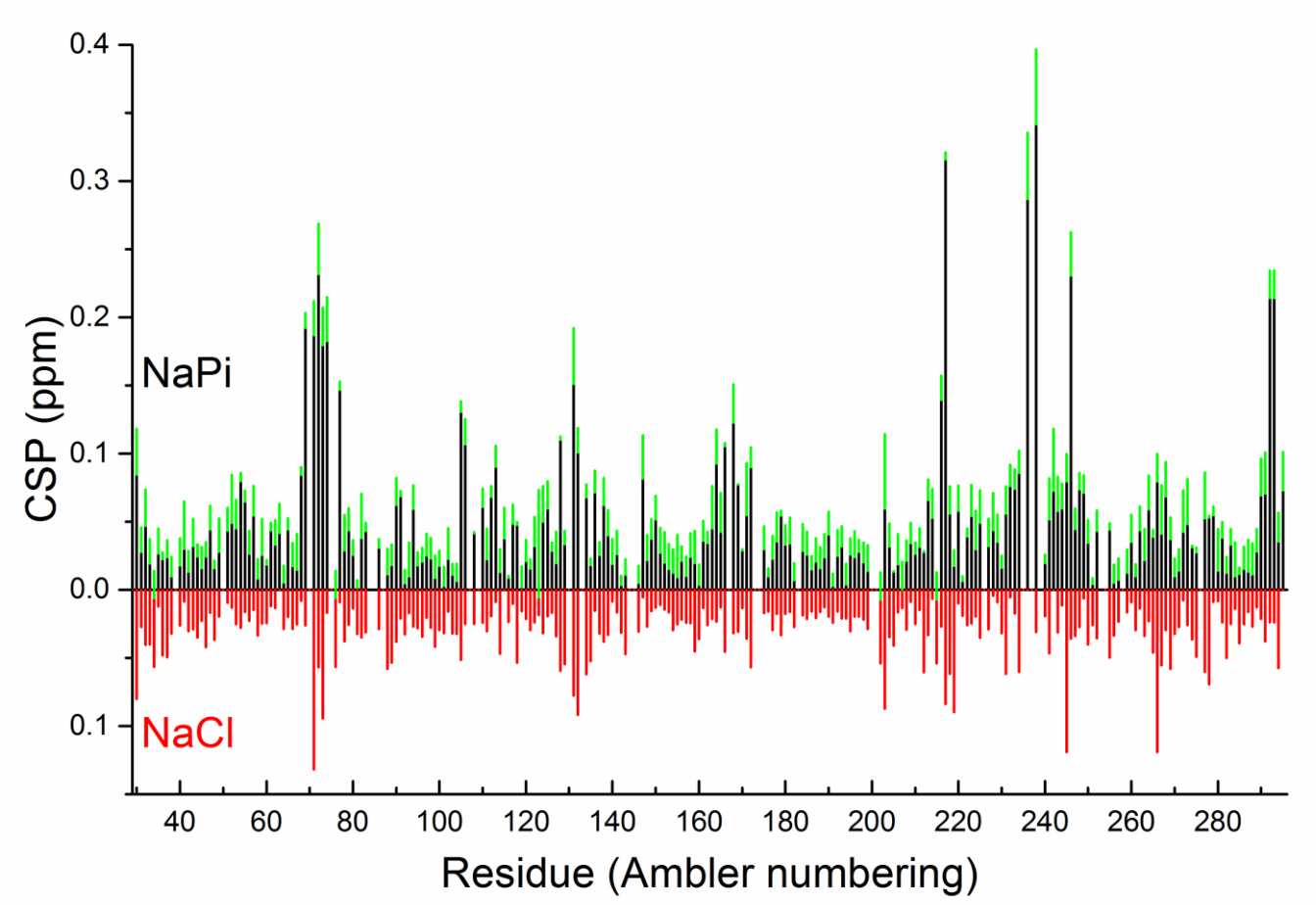

Figure S12. Absolute amide CSP upon titration from 0 – 250 mM with sodium phosphate (NaPi) or chloride (NaCl). Green error bars represent standard deviation over duplicate measurements.

Octave script for simulation of inhibition curves

```
#! /usr/bin/octave -qf
# S(1) + E(2) k2<Ki>k1 SE(3) >k3 PSE(4) >k4 PS(5) + E(2)
# I(6) + E(2) k6<K2i>k5 IE(7) >k7 PIE(8) >k8 PI(9) + E(2)

# Load data and adjust
delay = 15;  #deadtime
ext = 0.0173;#extinction coeff of product in uM-1cm-1
data = load ("WE_BlaC_CLA_Phosphate_inhibition_data.txt");
time = data(:,1)-delay;
#abs = data(:,2:end)/ext; #convert abs to conc in uM
abs = data(:,2:end); #data already in uM
conc = load ("WE_BlaC_CLA_Phos_CLAconcen.txt"); #conc of clavulanate in each exp
m1 = rows(abs);
m2 = abs(1,:);

for i = 1:m1
 abs(i,:) = abs(i,:)-m2;
endfor

num1 = numel(conc);
num2 = numel(time);
res = zeros(num2,num1);
sq = zeros(num1,2);
sq(:,1)=conc';

for i = 1:num1
#for i = 1:1

con = conc(i);

# Define rates and differential equations
function xdot = f (x,t)
  Ki = 220;
  k1 = 1.05;
  k2 = Ki*k1;
  k3 = 2500;
  k4 = 103;
  K2i = 20;
  k5 = 1;
  k6 = K2i*k5;
  k7 = 0.045;
  k8 = 0.0018;
  xdot = zeros (9,1);
  xdot(1) = -k1*x(1)*x(2)+k2*x(3);
  xdot(2) = -k1*x(1)*x(2)+k2*x(3)+k4*x(4)-k5*x(6)*x(2)+k6*x(7)+ k8*x(8);
  xdot(3) = k1*x(1)*x(2)-(k2+k3)*x(3);
  xdot(4) = k3*x(3)-k4*x(4);
  xdot(5) = k4*x(4);
  xdot(6) = -k5*x(6)*x(2)+k6*x(7);
  xdot(7) = k5*x(6)*x(2)-(k6+k7)*x(7);
  xdot(8) = k7*x(7)-k8*x(8);
  xdot(9) = k8*x(8);

endfunction;
x0 = [125; 0.002; 0; 0; 0; con; 0; 0; 0];
#t = logspace(-3,8,10000);       #adjust to see entire range
y = lsode ("f", x0, time);
res(:,i) = y(:,5);
sq(i,2) = sum((abs(:,i)-res(:,i)).^2);

endfor

sqsum = sum(sq(:,2));
sqsum

# Plot the results
```

```
## Log plot of all concentrations
#plot (log10(t),log10(y));
#limits = axis([-3,8,-4,2]);
#xlabel ("log(10) time (s)");
#ylabel ("log(10) concentration (uM)");
#legend ("S", "E", "SE", "PSE", "PS", "I", "IE", "PIE", "PI", "location", "west");

#pause();

# Normal plot of data and predicted product curve
figure('Position',[100,350,1000,850]);
plot (time, abs, "-k", time, res,"-r");
limits = axis([1, 900, 0.1, 90]);
xlabel ("time (s)");
ylabel ("concentration (uM)");

print -djpg WE_BlaC_CLA_Phosphate_inhibition_data.jpg;
#save simul02.txt sqsum sq
pause();
```

Supplementary references

(S1) Ambler, R. P., and Coulson, A. F. W. (1991) A standard numbering scheme for the Class A beta-lactamases. *Biochem. J 276*, 269–272.