Supporting Information

Molecular Mechanism of Avibactam Mediated β-Lactamase Inhibition

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METHODS

Dynamic light scattering.

Dynamic light scattering was performed using a Zetasizer NanoS (Malvern Instruments). All measurements were taken using a 12 µL quartz cell (ZEN2112) at 25°C. Size distribution of the samples was calculated based on the correlation function provided by the Zetasizer Nano S software.

LC-MS analysis of avibactam-CTX-M-15 mutants.

LC-ESI-MS data were obtained by using an Agilent 1100 Series LC system (Agilent Technologies Canada, Inc.) and a QTRAP LC/MS/MS System (Applied Biosystems). The reverse phase HPLC was performed using C_{18} column (SunFire C18 5 µm, 4.6x50 mm, Waters) with Agilent 1100 LC binary pump at a flow rate of 1 mL/min, under the following conditions: isocratic 5% solvent B (0.05% formic acid in acetonitrile) and 95% solvent A (0.05% formic acid in water) for 1 min, followed by a linear gradient to 97% B over 10 min. CTX-M-15 WT, K73A, N104A, S130A, N132A, E166Q, K234A; and KPC-2 (7 µM) were incubated with 14 µM avibactam in buffer containing 30 mM HEPES pH 7.5, 300 mM NaCl, and 20 % v/v glycerol and analyzed at both 0 h and 24 h.

Protein expression and purification for crystallographic studies.

The *P. aeruginosa* OXA-10 protein (UniProt ID: P14489) corresponding to the mature sequence (20-266) was cloned, overexpressed and purified as previously described [1].

The *E. coli* CTX-M-15 and *Klebsiella pneumoniae* OXA-48 expression vectors were constructed as described above. The expression vectors were then transformed into *E. coli* BL21 DE3 cells. The cells were grown in Lauria Bertani (LB) broth at 37°C until an OD_{600} of 0.7 was reached at which point the culture was cooled to room temperature. Protein expression was induced by addition of 1mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and the cultures were grown at 22°C for 12-16 hours. The cells (~20g) were then harvested and resuspended in 50mL lysis buffer (50mM Tris, pH 7.5, 350mM NaCl, and one complete, EDTA-free protease inhibitor tablet from Roche). The cells were lysed by two passes on a French Press at ~12,000 p.s.i., and the lysate was centrifuged (45,000 rpm in a Beckman 70 Ti rotor) for 35 minutes. The supernatant was then filtered using a 0.22 µM syringe filter and passed through a 1mL Hi-Trap HP His column, which was pre-equilibrated in lysis buffer. Elution buffer (50 mM Tris, pH 7.5, 350mM NaCl, 1M imidazole) was used to elute the His-tagged proteins from the column with a gradient of imidazole from 0 to 500mM in 50 minutes. Fractions enriched in the protein of interest were pooled and 1U/mL of bovine α-thrombin (Roche) was added and the samples were incubated overnight at 4°C. Samples were then exchanged via a 10 kDa cut-off Amicon centrifugation concentrator into crystallization buffer (20mM Tris, pH 7.5, 100mM NaCl). Samples were passed over a Superdex 200 column using crystallization buffer, as running buffer and pooled fractions were concentrated to 30 mg/mL for CTX-M-15, 50 mg/mL for OXA-48 and 10mg/mL for OXA-10.

TABLES

Table S1. Data collection and refinement statistics.

	CTX-M-15-AVI	OXA-10-AVI	OXA-48-AVI8.5	OXA-48-AVI7.5	OXA-48-AVI6.5	OXA-48-Native
Data collection						
Space group	$P2_1$	$P2_12_12_1$	$P3_2$	$P3_2$	$P2_12_12_1$	$P22_12_1$
Cell dimensions						
a, *b*, *c* (Å)	62.0, 60.6, 71.5	48.6, 96.5, 125.7	142.0, 142.0, 52.4	142.8, 142.8, 52.4	64.1, 108.1, 162.8	43.4, 102.9, 124.7
□□□□□□□□□□□□□□ (°)	90, 104, 90	90, 90, 90	90, 90, 120	90, 90, 120	90, 90, 90	90, 90, 90
Resolution (Å)	34.7-1.6 (1.69-1.60)	52.66-1.70 (1.73-1.70)	46.64-2.00 (2.11-2.00)	52.42-2.10 (2.21-2.10)	65.03-2.54 (2.65-2.54)	41.6-1.70 (1.73-1.70)
R_{merge}	0.052(0.296)	0.040(0.290)	0.090(0.295)	0.096(0.457)	0.065(0.150)	0.055(0.424)
I / □*I*	13.7(3.5)	24.5(5.2)	6.1(2.9)	8.5(2.9)	12.7(6.1)	12.1(2.3)
Completeness (%)	96.7(95.2)	98.0(99.9)	99.2(99.4)	99.8(100.0)	91.2(91.4)	99.7(99.9)
Redundancy	3.9(3.9)	4.8(4.9)	2.5(2.5)	3.4(3.4)	4.0(3.8)	5.0(4.9)
Refinement						
Resolution (Å)	34.7-1.60	52.66-1.70	46.64-2.00	52.42-2.10	65.03-2.54	41.6-1.70
No. reflections	65645(9368)	64493(3478)	76256(11167)	69650(10200)	34471(4158)	62226(3297)
R_{work} / R_{free}	0.165/0.198	0.185/0.230	0.171%/0.205	0.172/0.205	0.184/0.222	0.192/0.226
Avibactam occupancy chainA, chainB, etc.	1.00, 1.00	1.00, 1.00	0.70, 0.70, 1.00, 1.00	1.00, 1.00, 1.00, 1.00	1.00, 1.00, 1.00, 1.00	N/A, N/A
No. atoms						
Protein	3930	3957	8000	8043	7972	3963
Ligand/ion	34	38	68	68	68	N/A
Water	549	468	362	480	203	372
B-factors						
Protein	17.8	22.6	40.2	30.3	34.1	27.2
Ligand/ion	17.8	17.5	36.7	22.4	35.4	N/A
Water	27.4	28.7	37.6	32.3	29.2	35.4
R.m.s. deviations						
Bond lengths (Å)	0.012	0.012	0.014	0.012	0.011	0.014
Bond angles (°)	1.62	1.70	1.72	1.68	1.49	1.53
Favored/allowed/ disallowed (%)[+]	98.2, 1.4, 0.4	97.8, 2.0, 0.2	97.6, 2.4, 0.0	97.2, 2.8, 0.0	97.9, 2.1, 0.0	97.7, 2.3, 0.0

*All datasets correspond to diffraction data collected from a single crystal.
*Values in parentheses are for highest-resolution shell.
*Avibactam (AVI)
[+]phenix.ramalyze; "allowed" is the percentage remaining after "favored" and "outlier" residues are subtracted.

Table S2. Primers used in this study. Underline shows restriction sites.

Primer	Sequence (5'-3')
CTX-M-15 F	AATATCATATGCAAACGGCGGACGTACAGCA
CTX-M-15 R	TATTAGAATTCTTACCGTCGGTGACGATTTTAGCC
OXA-48 F	GCTTCATATGGAATGGCAAGAAAACAAAAGTTGGAATGCT
OXA-48 R	CGTACTCGAGCTAGGGAATAATTTTTTCCTGTTTGAGCAC
K73A F	GCGATGTGCAGCACCAGTGCGGTGATGG
K73A R	CGCTACACGTCGTGGTCACGCCACTACC
N104A F	CGAGTTGAGATCAAAAAATCTGACCTTGTTGCGTATAATCCGATTGC
N104A R	GCTCAACTCTAGTTTTTTAGACTGGAACAACGCATATTAGGCTAACG
S130A F	CGCTACAGTACGCGGATAACGTGGCGATGAATAAGC
S130A R	GCGATGTCATGCGCCTATTGCACCGCTACTTATTCG
N132A F	GCTACAGTACAGCGATGCGGTGGCGATGAATAAGC
N132A R	CGATGTCATGTCGCTACGCCACCGCTACTTATTCG
E166Q F	GCTGGGAGACGAAACGTTCCGTCTCGACC
E166Q R	CGACCCTCTGCTTTGCAAGGCAGAGCTGG
K234A F	GGTTGTGGGGGATGCGACCGGCAGC
K234A R	CCAACACCCCCTACGCTGGCCGTCG
T7 terminator	GCTAGTTATTGCTCAGCGG

Table S3. Kinetic values for the hydrolysis of nitrocefin by CTX-M-15 mutants.

Parameter	***WT***	***K73A***	***N104A***	***S130A***	***N132A***	***E166Q***	***K234A***
K_m (μM)	9.9	13	11	33	6.2	8.3	2.6
k_{cat} (s^{-1})	57	4.1	85	270	55	0.06	4.0
k_{cat}/K_m (M^{-1} s^{-1})	5.8×10^6	3.1×10^5	8.2×10^6	8.2×10^6	8.8×10^6	7.9×10^3	1.6×10^6

FIGURES

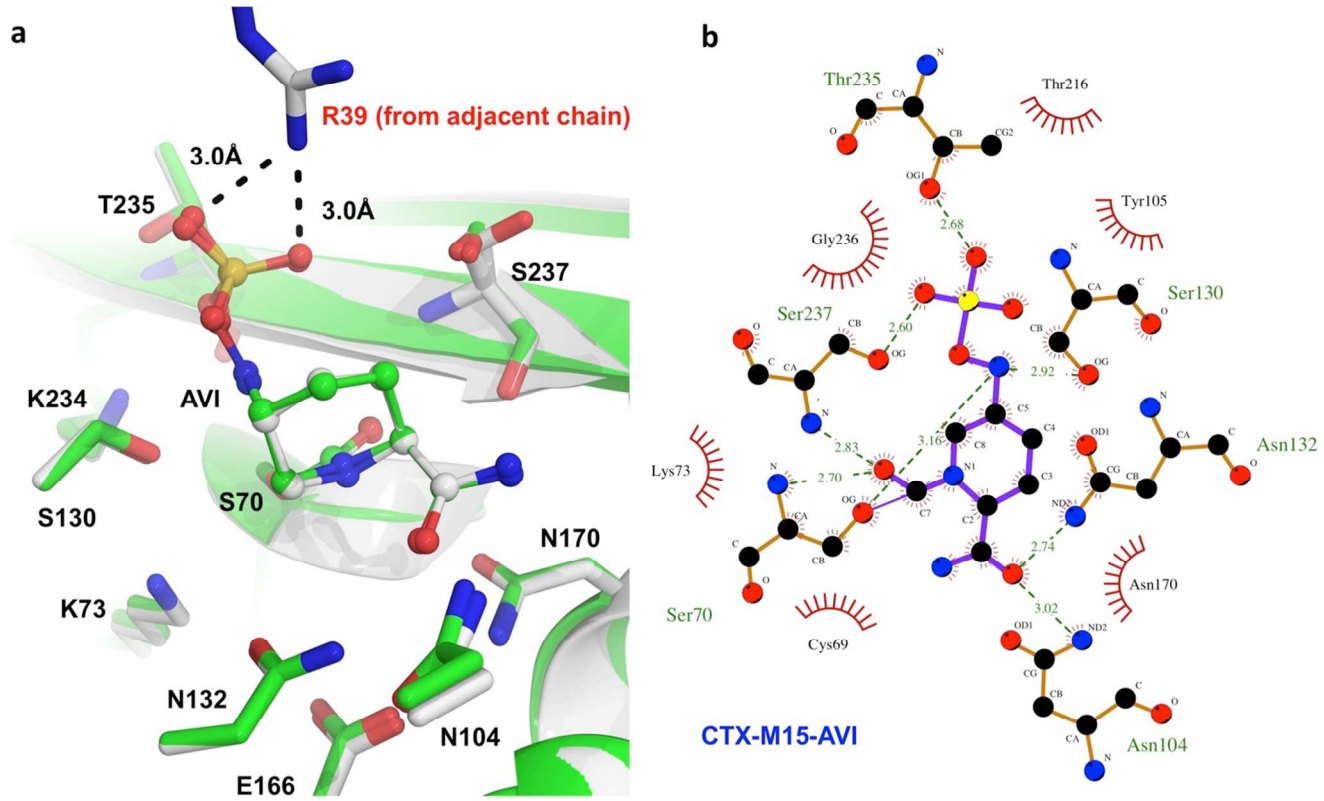


Figure S1. Carbamyl-avibactam bound CTX-M-15 active site details. (a) Active site overlay of carbamyl-avibactam CTX-M-15 complexes in spacegroups $P2_12_12_1$ (PDB ID: 4HBU) [2], and $P2_1$ (PDB ID: XXXX). Carbon atoms for the 4HBU and XXXX active site residues and avibactam are displayed in grey and green, with all other non-carbon atoms colored by type (N, blue; O, red; S, yellow). The 4HBU and XXXX CTX-M-15 protein backbones are displayed as grey and green cartoons. (b) Protein-ligand interactions between CTX-M-15 and avibactam depicted in monomer A using LigPlot+ [3]. Avibactam and CTX-M-15 are displayed as purple and orange sticks with atoms colored by type. Hydrogen bonding and electrostatic interactions are shown as green dashes. Ligand-protein hydrophobic contacts are shown as curved red combs.

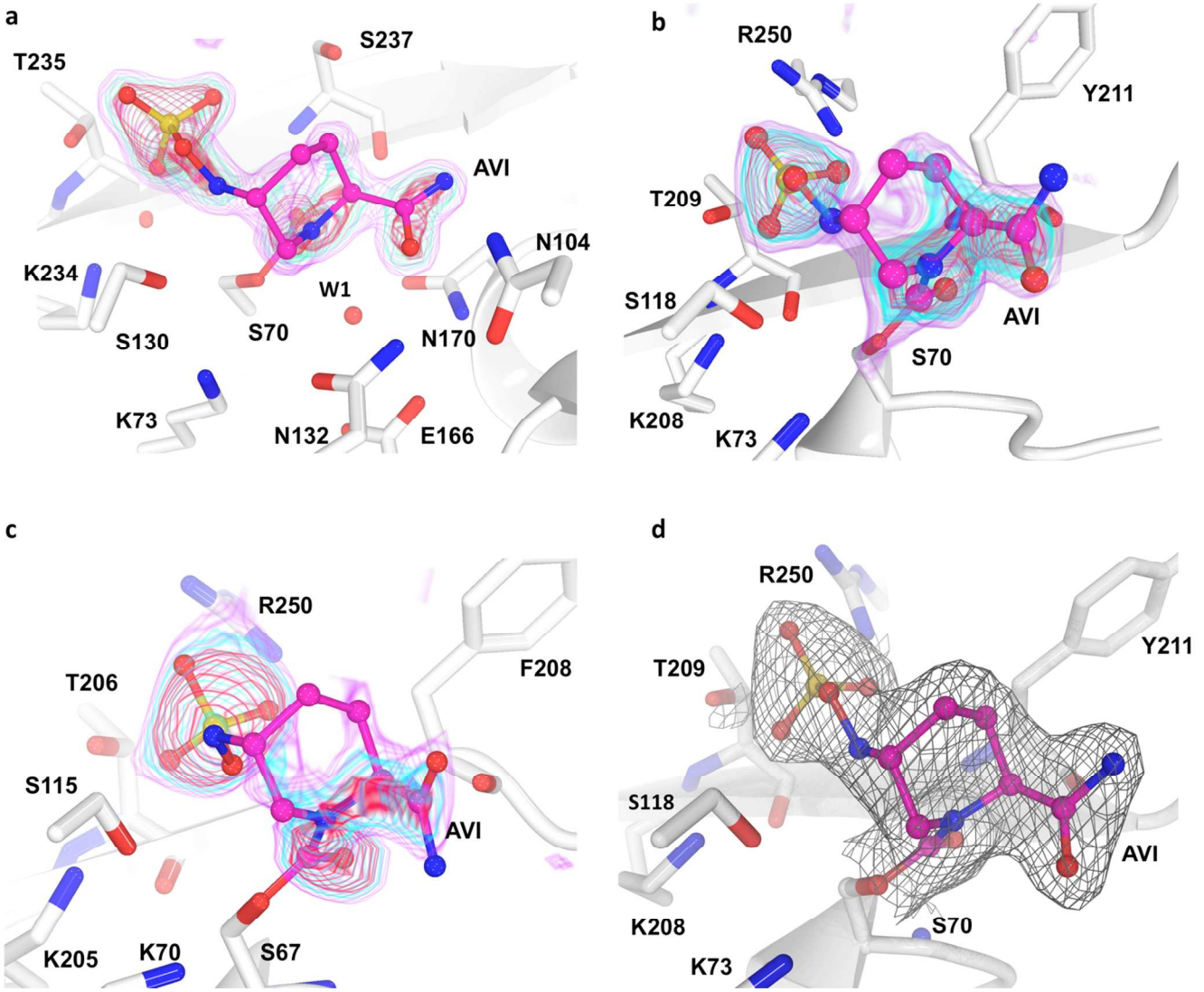


Figure S2. Avibactam electron density for carbamylated CTX-M-15, OXA-48 and OXA-10 crystal structures. In a-c, the F_o-F_c ligand omit maps are contoured at 3.0, 4.0 and 5.0 σ and are shown as pink, cyan and red transparent surfaces. (a) Carbamyl-avibactam CTX-M-15 ligand omit F_o-F_c electron density. The CTX-M-15 cartoon is shown in white with selected active site residues displayed in stick representation and non-carbon atoms are colored by type. (b) and (c), Carbamyl-avibactam OXA-48-AVI7.5 and OXA-10 ligand omit F_o-F_c electron density. In B and C, the OXA-48-AVI7.5 and OXA-10 protein backbones are shown in white cartoon representation with selected active site residues displayed as white sticks with non-carbon atoms colored by type. In all panels, the carbamyl-avibactam is represented as pink sticks with atoms colored by type. (d) Carbamyl-avibactam OXA-48-AVI7.5 final refined $2F_o$-F_c electron density. The OXA-48-AVI7.5 protein and bound avibactam are displayed as in b. The $2F_o$-F_c electron density map is contoured at 1.0σ and is displayed as a grey mesh.

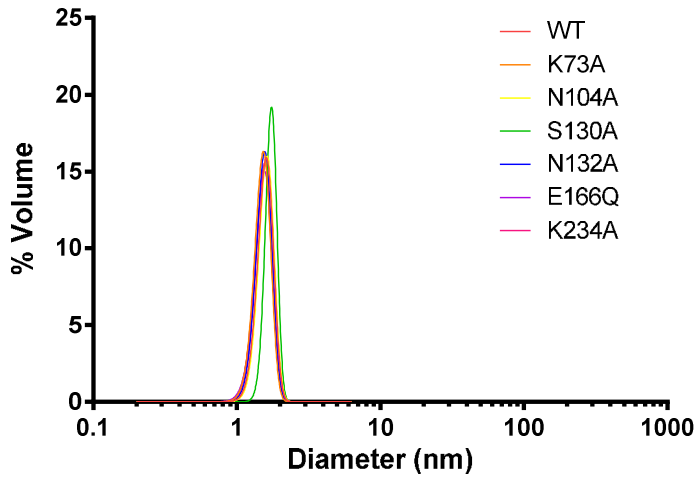


Figure S3. CTX-M-15 variants are stable in solution. Characterization of the particle size distribution for CTX-M-15 variants using dynamic light scattering.

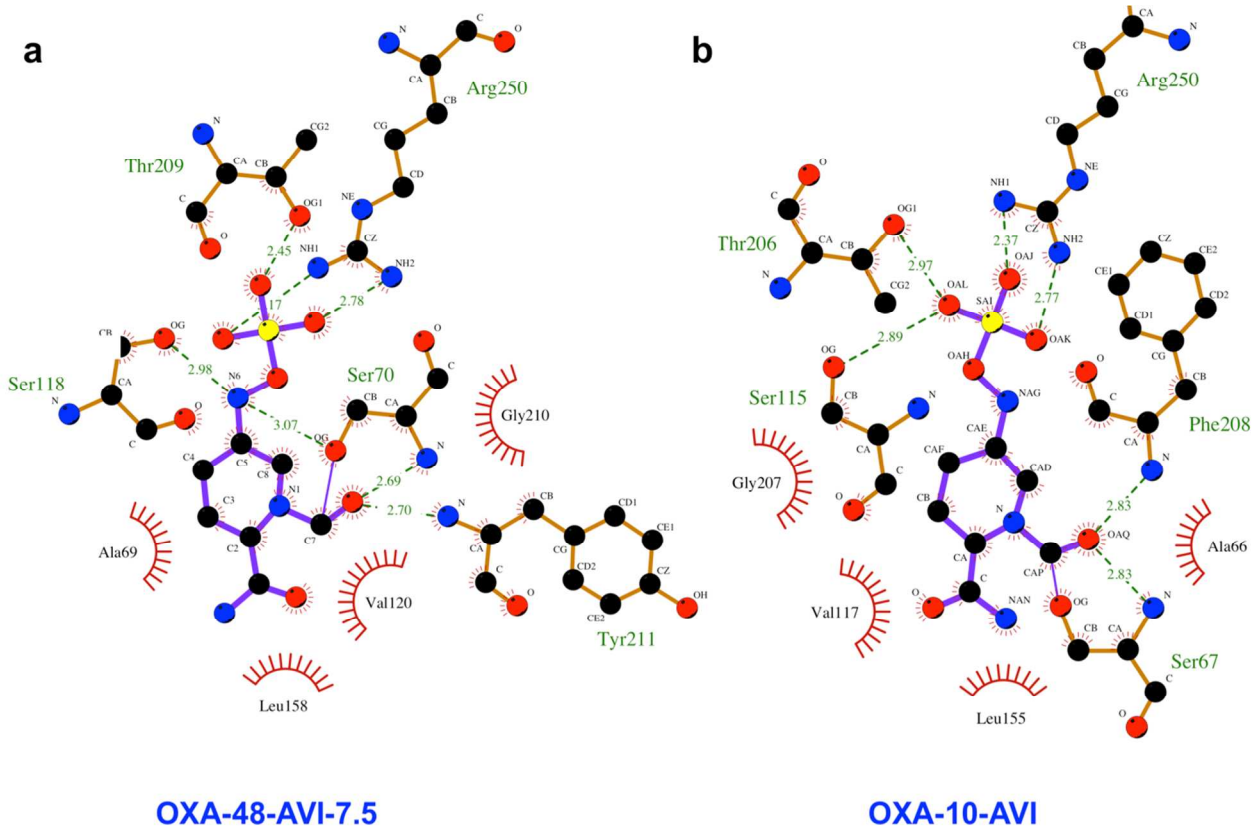


Figure S4. Interactions between avibactam and active site residues in OXA-48 and OXA-10. (a) and (b), Chain A-avibactam interactions in OXA-48-AVI-7.5 and OXA-10-AVI crystal complexes designed using LigPlot+ [3]. In all panels, the carbamyl-avibactam and active site residues are displayed as purple and orange sticks with atoms colored by type. Hydrogen bonding and electrostatic interactions are shown as green dashes. Ligand-protein hydrophobic contacts are displayed as curved red combs.

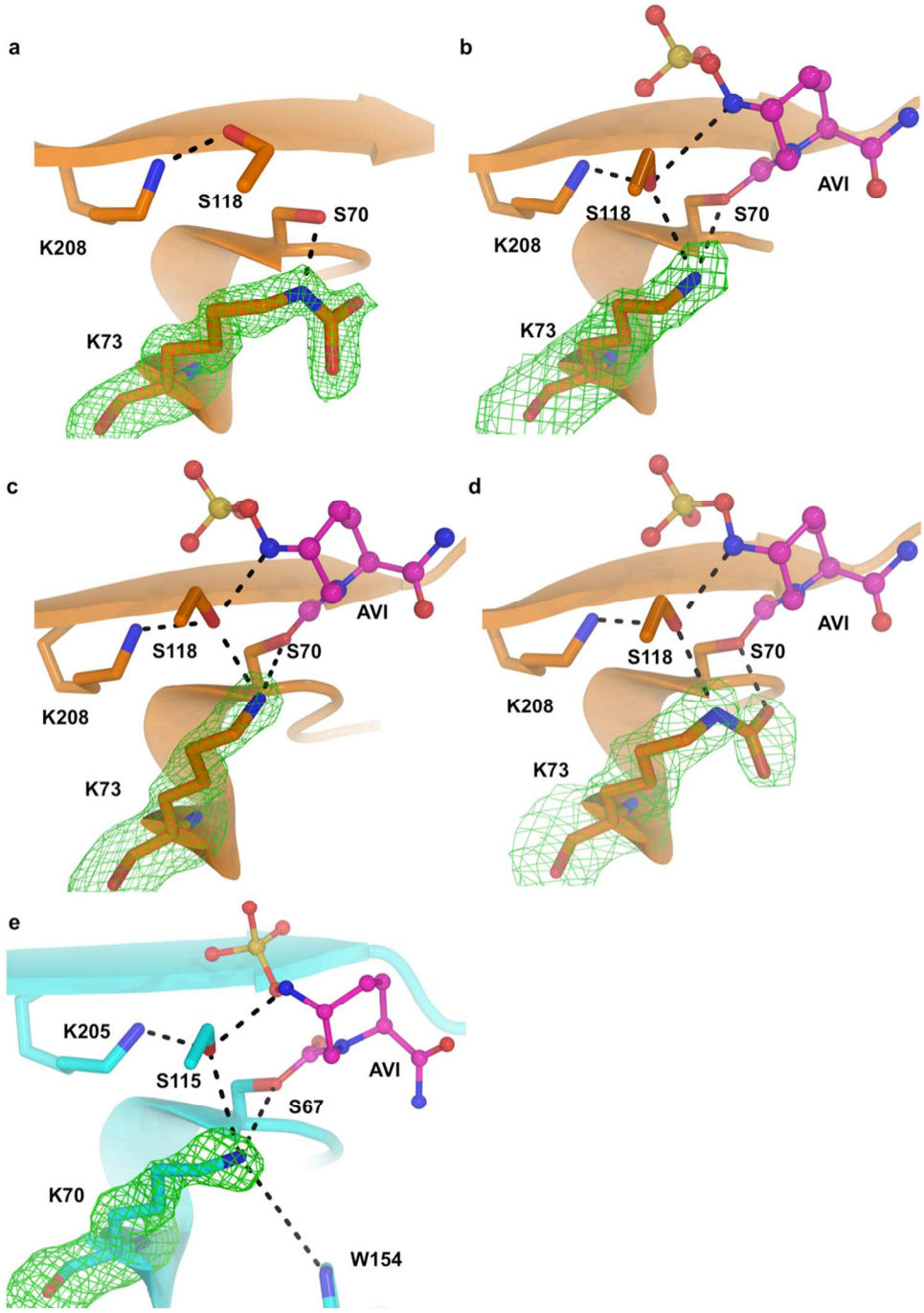


Figure S5. Carboxylation state of the SXXK lysine in OXA-48 and OXA-10. (a) Native OXA-48 (pH 7.5), chain A K73 omit F_o-F_c electron density. The OXA-48 protein backbone is displayed as an orange cartoon with selected active site residues shown as sticks with all non-carbon atoms colored by type. The F_o-F_c K73 omit electron density map is contoured at 3.0σ and is shown as a green mesh. (b), (c) and (d) OXA-48-AVI6.5 (pH 6.5), OXA-48-AVI7.5 (pH 7.5) and OXA-48-AVI8.5 (pH 8.5), chain A K73 omit F_o-F_c electron density. The OXA-48 protein backbone, active site residues and F_o-F_c K73 omit electron density maps are shown as in A. The carbamyl-avibactam is represented as pink sticks with all non-carbon atoms colored by type. (e) OXA-10-AVI (pH 6.5) chain A K70 omit F_o-F_c electron density map. The OXA-10 protein backbone is displayed in cyan cartoon representation with selected active site residues shown as sticks with all non-carbon atoms colored by type. The F_o-F_c K70 omit electron density map is represented as in a. The carbamyl-avibactam is represented as in b.

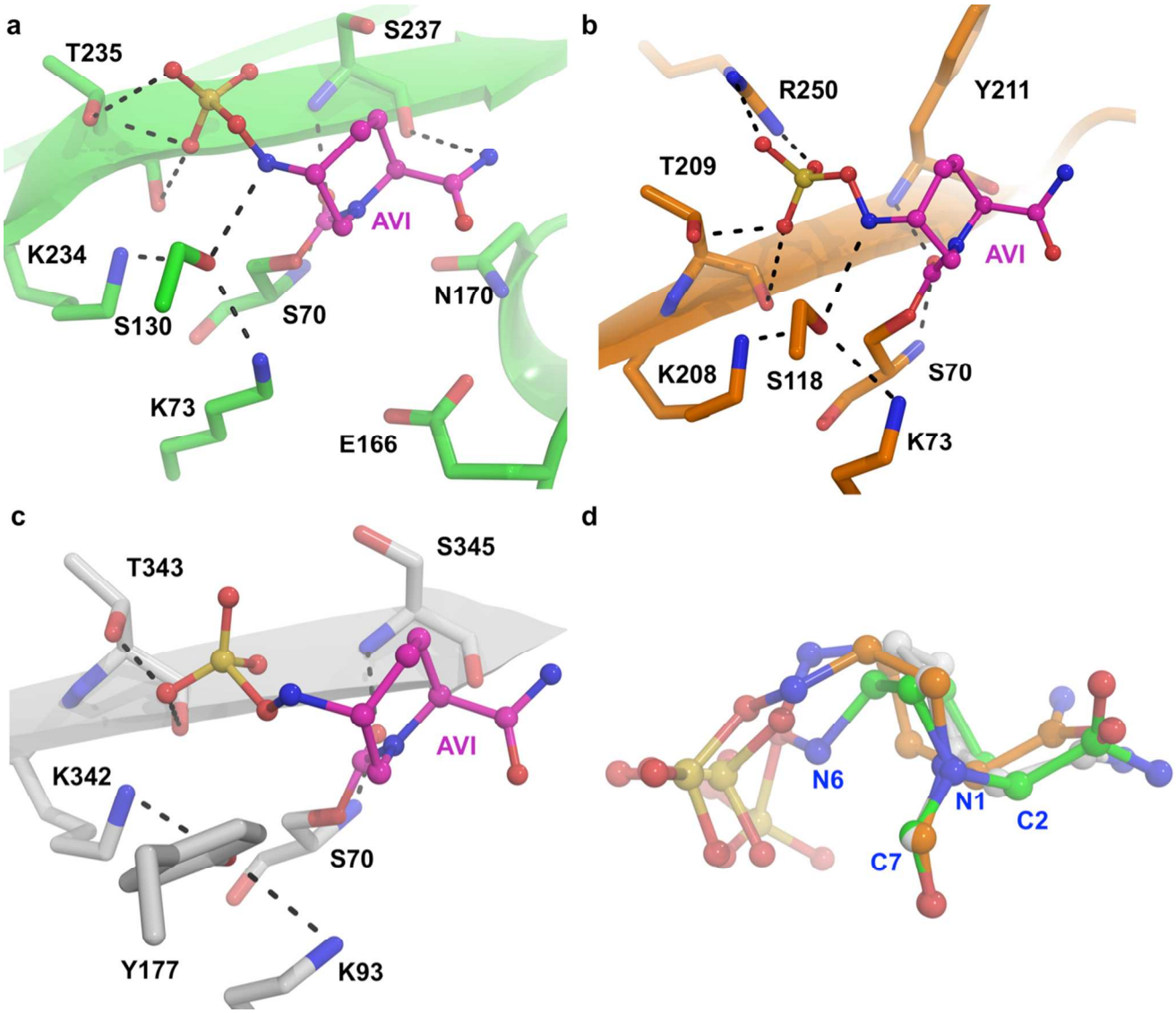
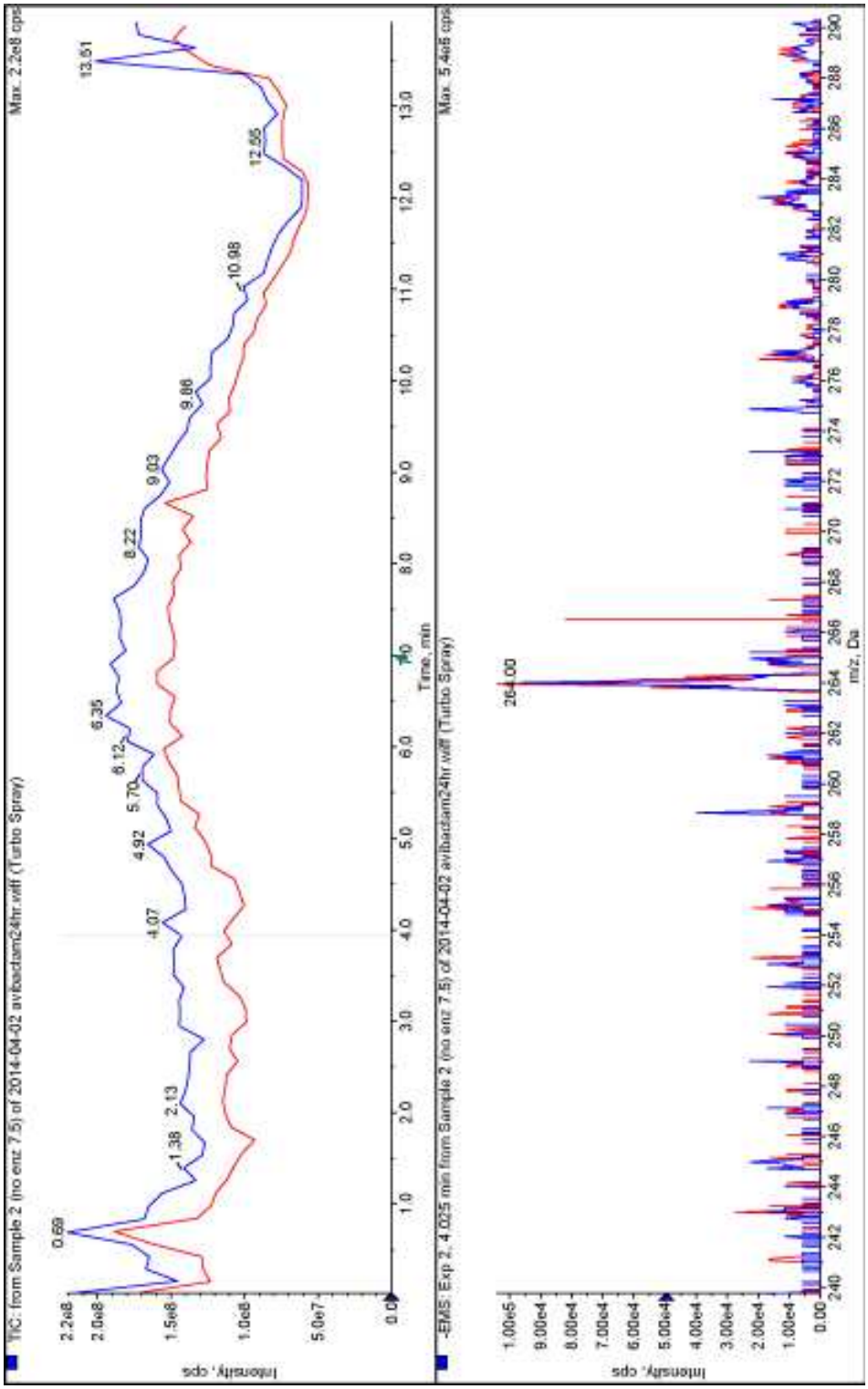


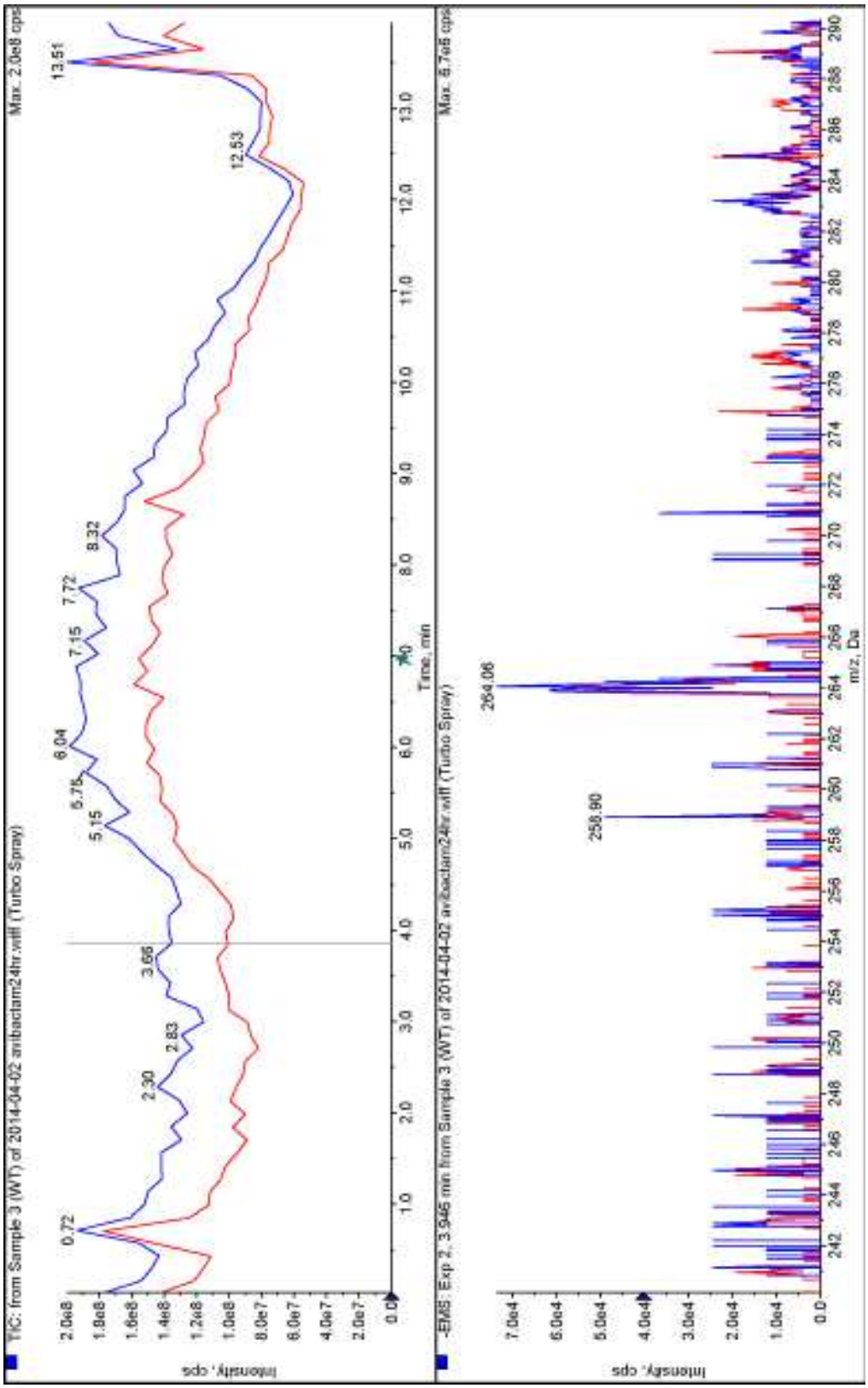
Figure S6. Comparison of carbamyl-avibactam CTX-M-15, OXA-48 and AmpC co-crystal structures. (a) Active site close-up of carbamyl-avibactam CTX-M-15. The carbon atoms of avibactam are pink with non-carbon atoms colored by atom type. The avibactam bound CTX-M-15 protein chain is represented as a green cartoon, with key active site residues shown as sticks with atoms colored by type. (b) and (c) Active site overlay of carbamyl-avibactam OXA-48, and AmpC (PDB ID: 4HEF) [2]. In b and c, the bound avibactam is represented as in a. The OXA-48 and AmpC protein chains are illustrated as orange and grey cartoons, and active site residues are depicted as sticks with non-carbon atoms colored by type. In a-c, hydrogen bonding and electrostatic interactions are shown as black dashes. (d) Overlay of carbamyl-avibactam from the CTX-M-15, OXA-48 and AmpC co-crystal structures (PDB ID's: XXXX, XXXX, 4HEF) [2]. Carbamyl-avibactam from the CTX-M-15, OXA-48 and AmpC structures are displayed as green, orange and white sticks with all non-carbon atoms colored by type. The carbamyl-avibactam C7 carbon, carbonyl oxygen and N1 atoms were fixed in the exact same positions.

Figures S7-S16. ESI-LC-MS trace overlays of avibactam incubated with β-lactamase as noted at pH 7.5 (Figs S7-S15). Samples were analyzed at 0 hours (red trace) and 24 hours (blue trace). Avibactam remains intact in all samples with the exception of KPC-2 and no enzyme pH 8.5

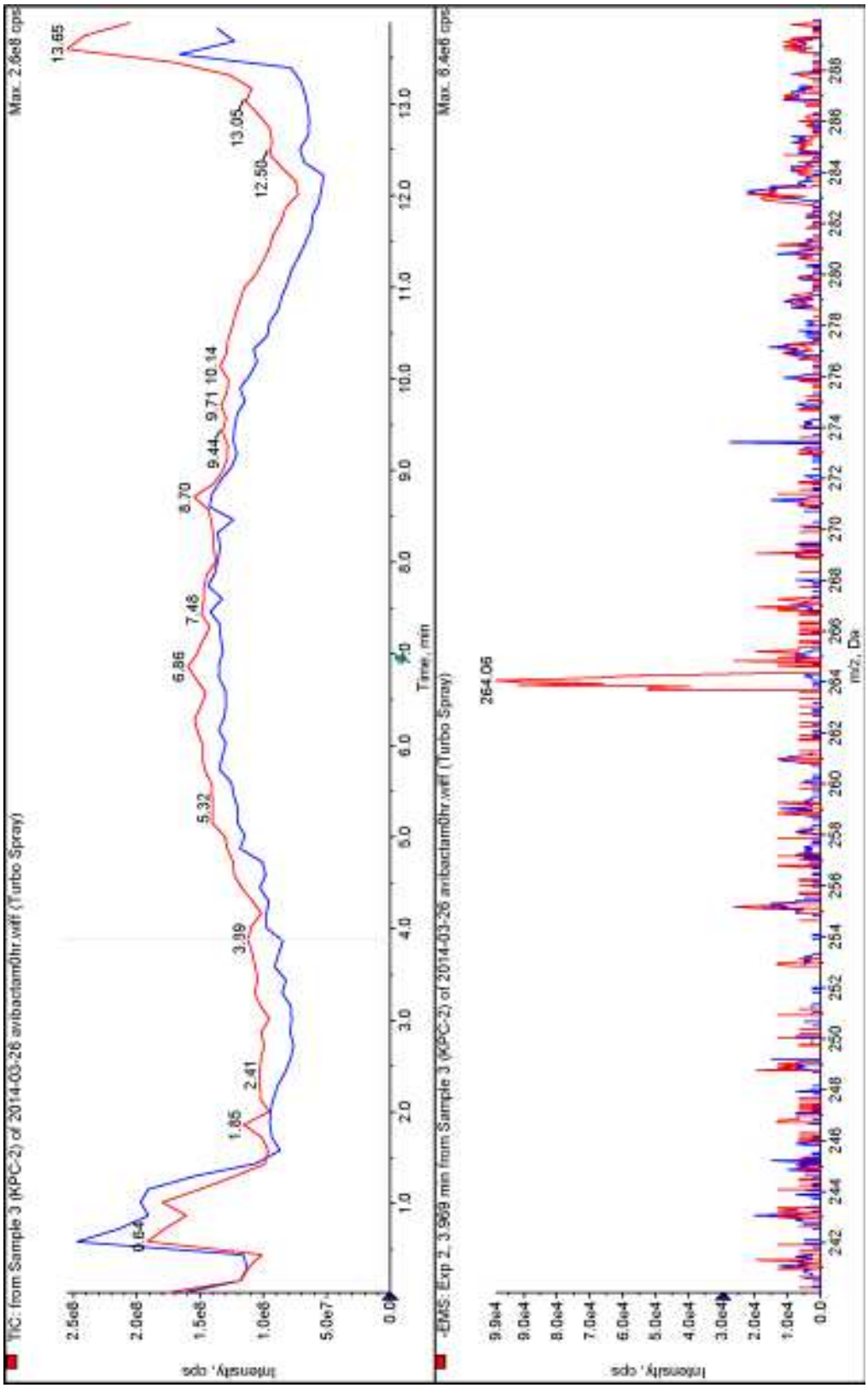
No enzyme



CTX-M-15


TIC: from Sample 3 (WT) of 2014-04-02 aribactam24hr.wiff (Turbo Spray)
Max: 2.0e8 cps
Intensity, cps
Time, min
0.72
2.30
2.83
3.66
5.15
5.76
6.04
7.15
7.72
8.32
12.53
13.51
-EMS: Exp 2, 3.946 min from Sample 3 (WT) of 2014-04-02 aribactam24hr.wiff (Turbo Spray)
Max: 8.7e5 cps
Intensity, cps
m/z, Da
258.90
264.06

KPC-2


TIC: from Sample 3 (KPC-2) of 2014-03-26 avibactam0hr.wiff (Turbo Spray)
Max. 2.6e8 cps
Intensity, cps
Time, min
0.64
1.85
2.41
3.99
5.32
6.86
7.46
8.70
9.44
9.71
10.14
12.50
13.05
13.65
-EMS: Exp 2, 3.969 min from Sample 3 (KPC-2) of 2014-03-26 avibactam0hr.wiff (Turbo Spray)
Max. 8.4e6 cps
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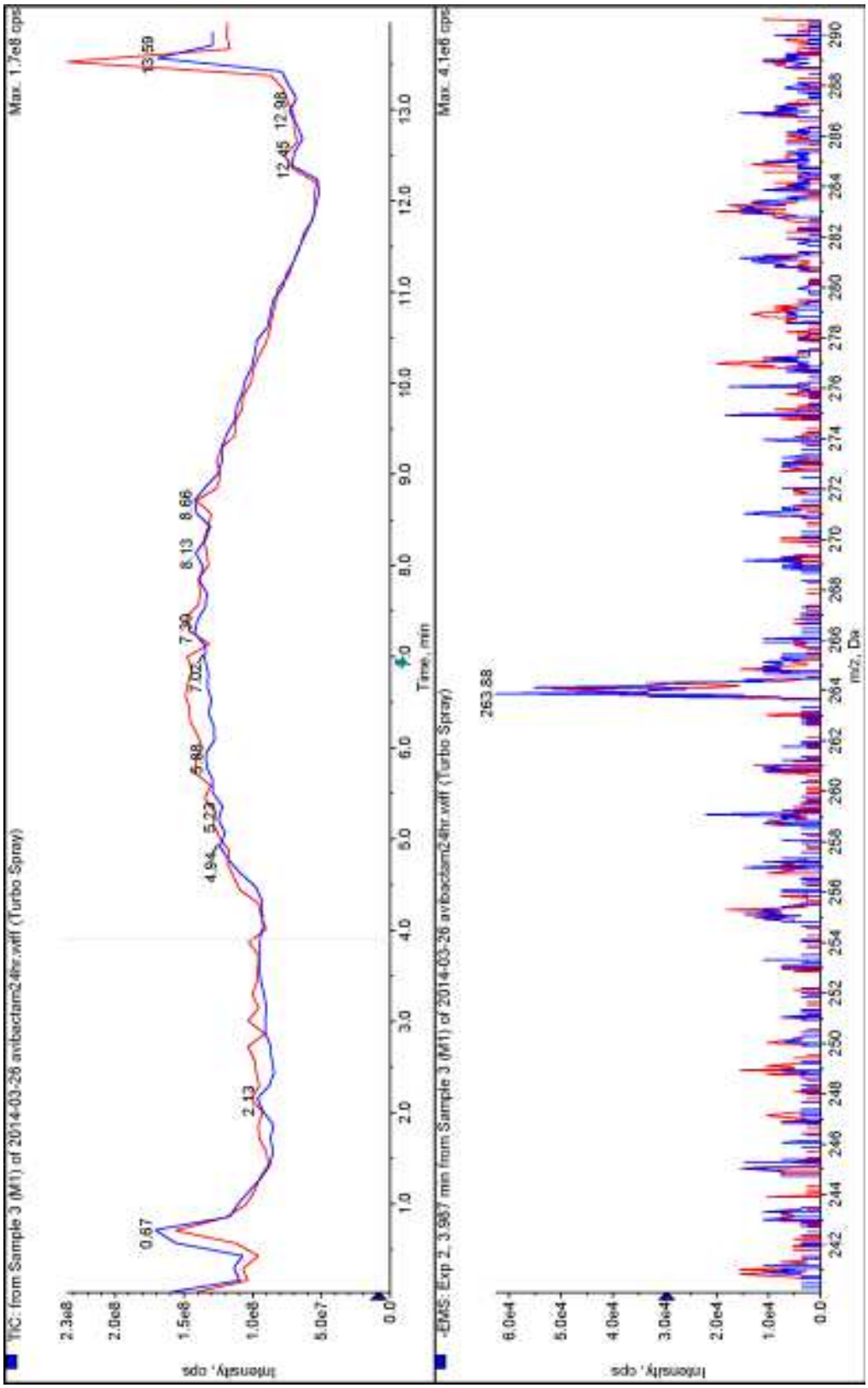
m/z, Da

CTX-M-15 K73A


TIC: from Sample 3 (M1) of 2014-03-26 avibactam24hr.wiff (Turbo Spray)
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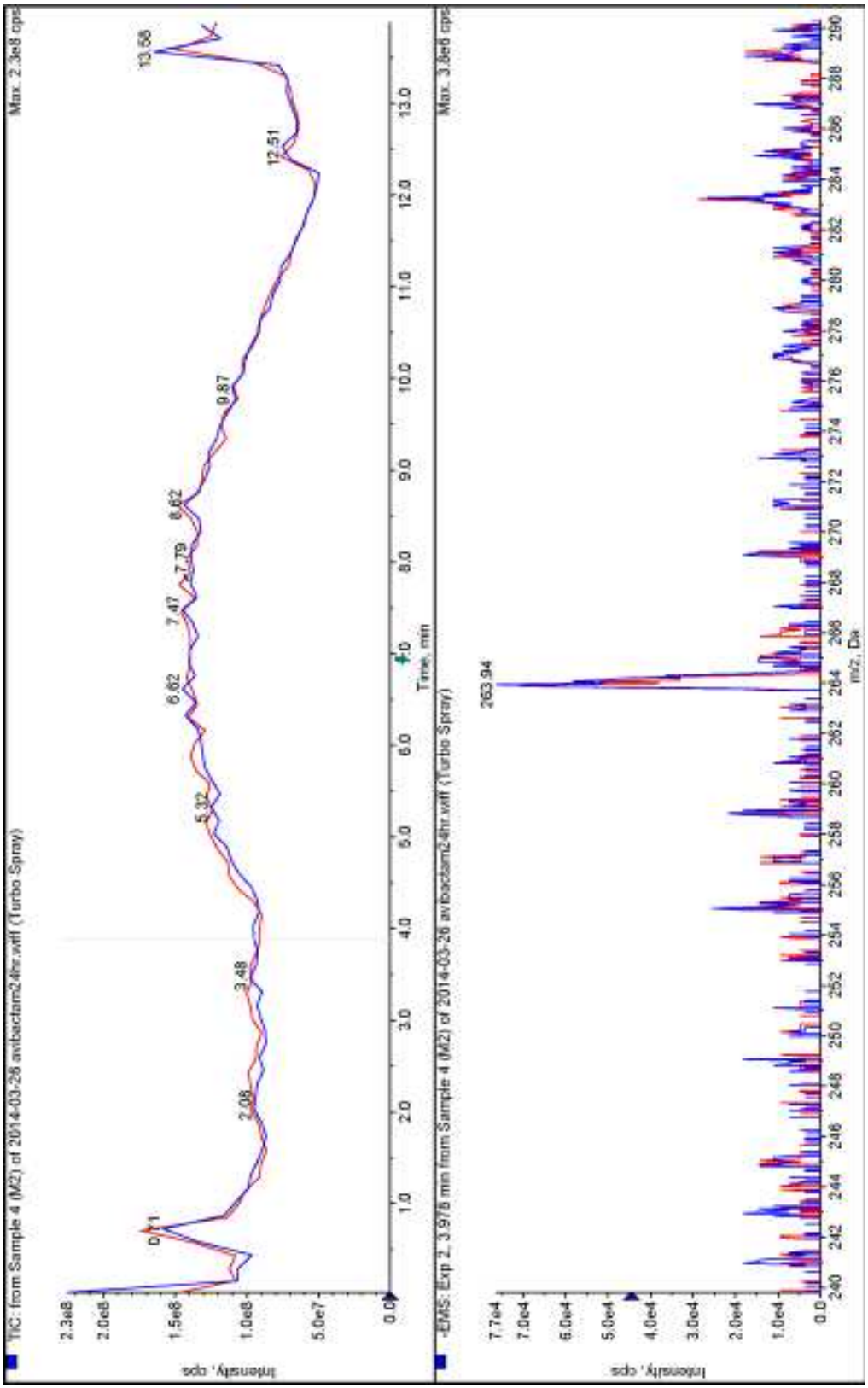
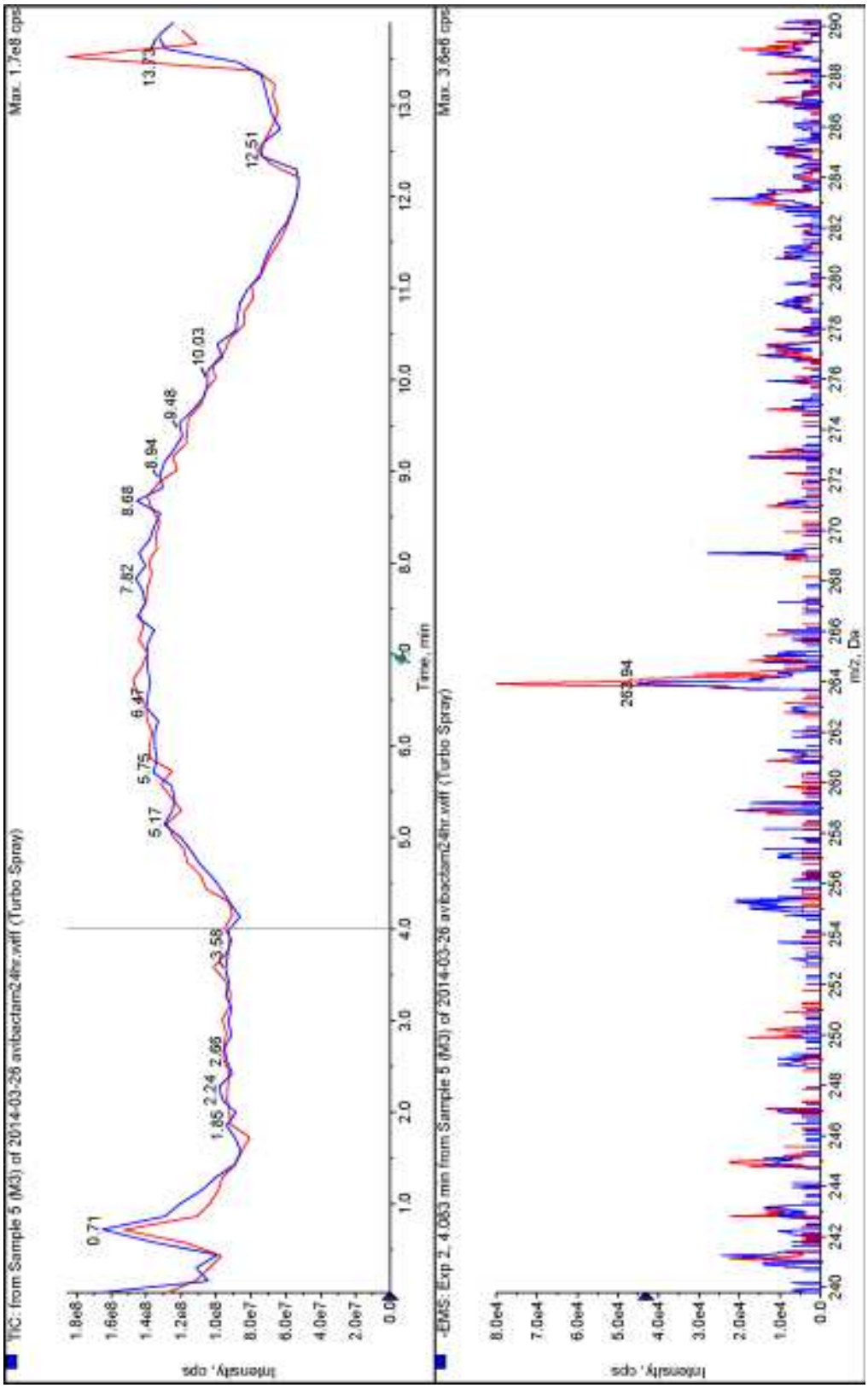
Intensity, cps
Time, min
0.67
2.13
4.94
5.22
5.88
7.02
7.30
8.13
8.66
12.45
12.98
13.59
-EMS: Exp 2, 3.987 min from Sample 3 (M1) of 2014-03-26 avibactam24hr.wiff (Turbo Spray)
Max. 4.1e6 cps
Intensity, cps
m/z, Da
263.88

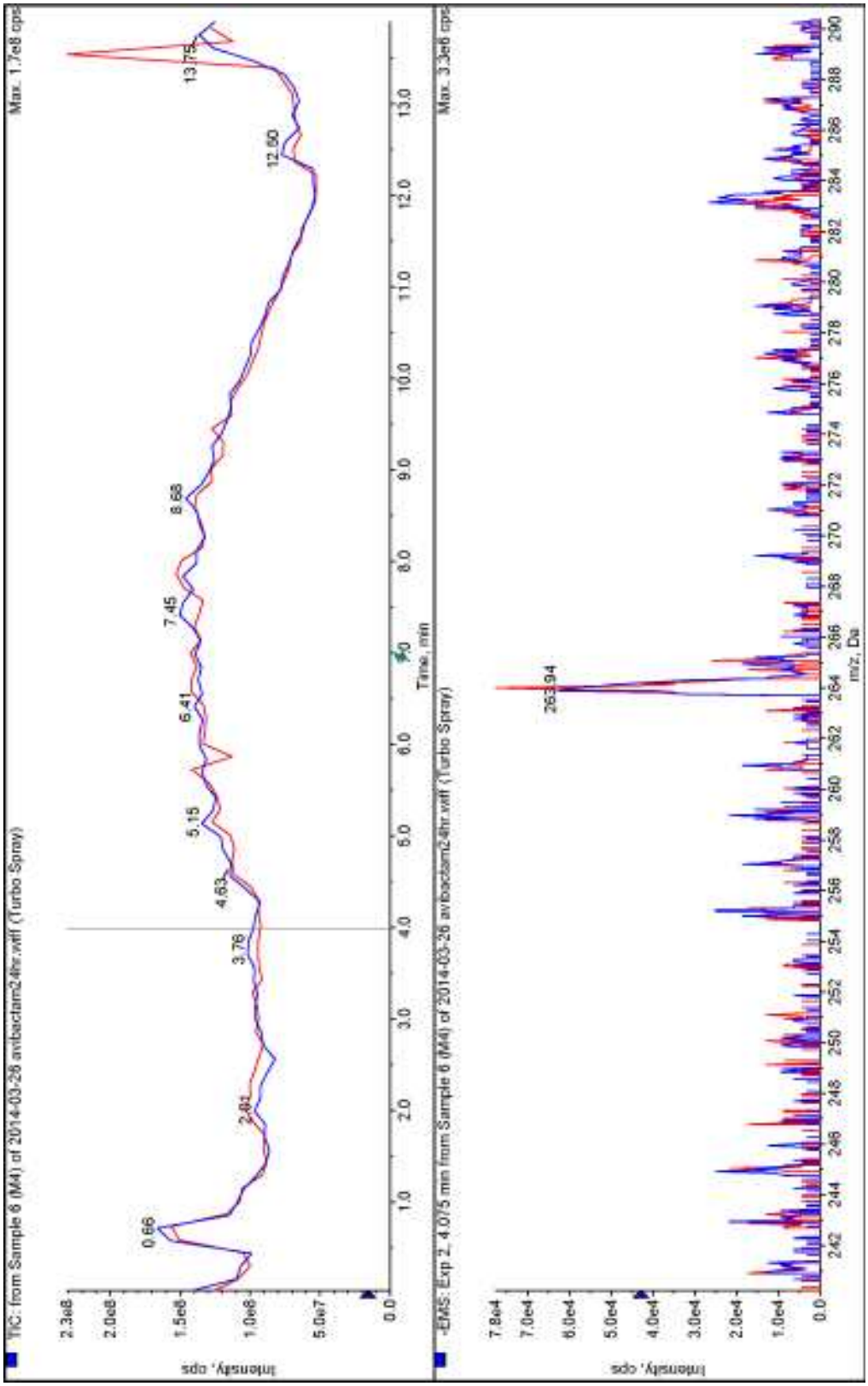
CTX-M-15 N104A



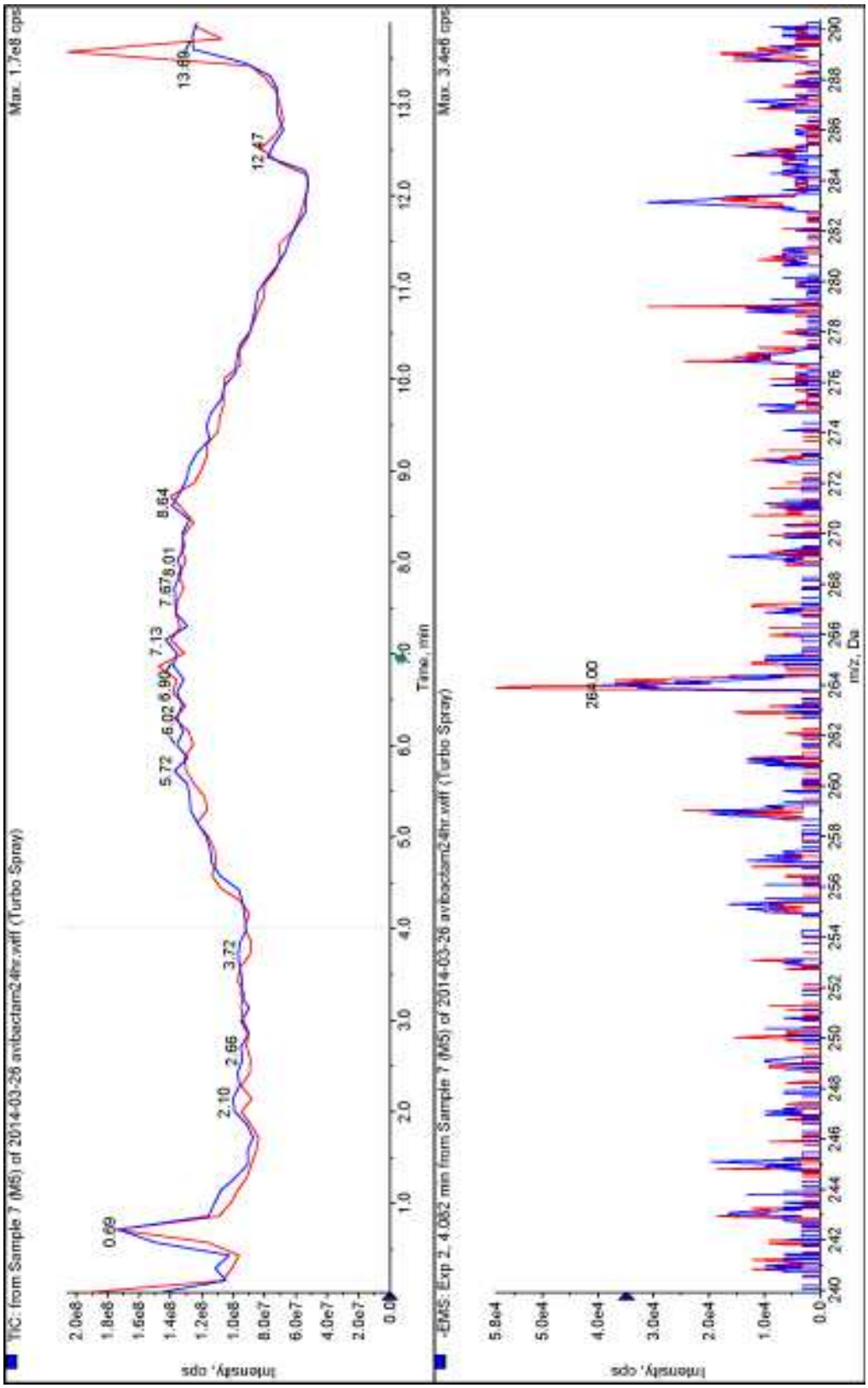
CTX-M-15 S130A



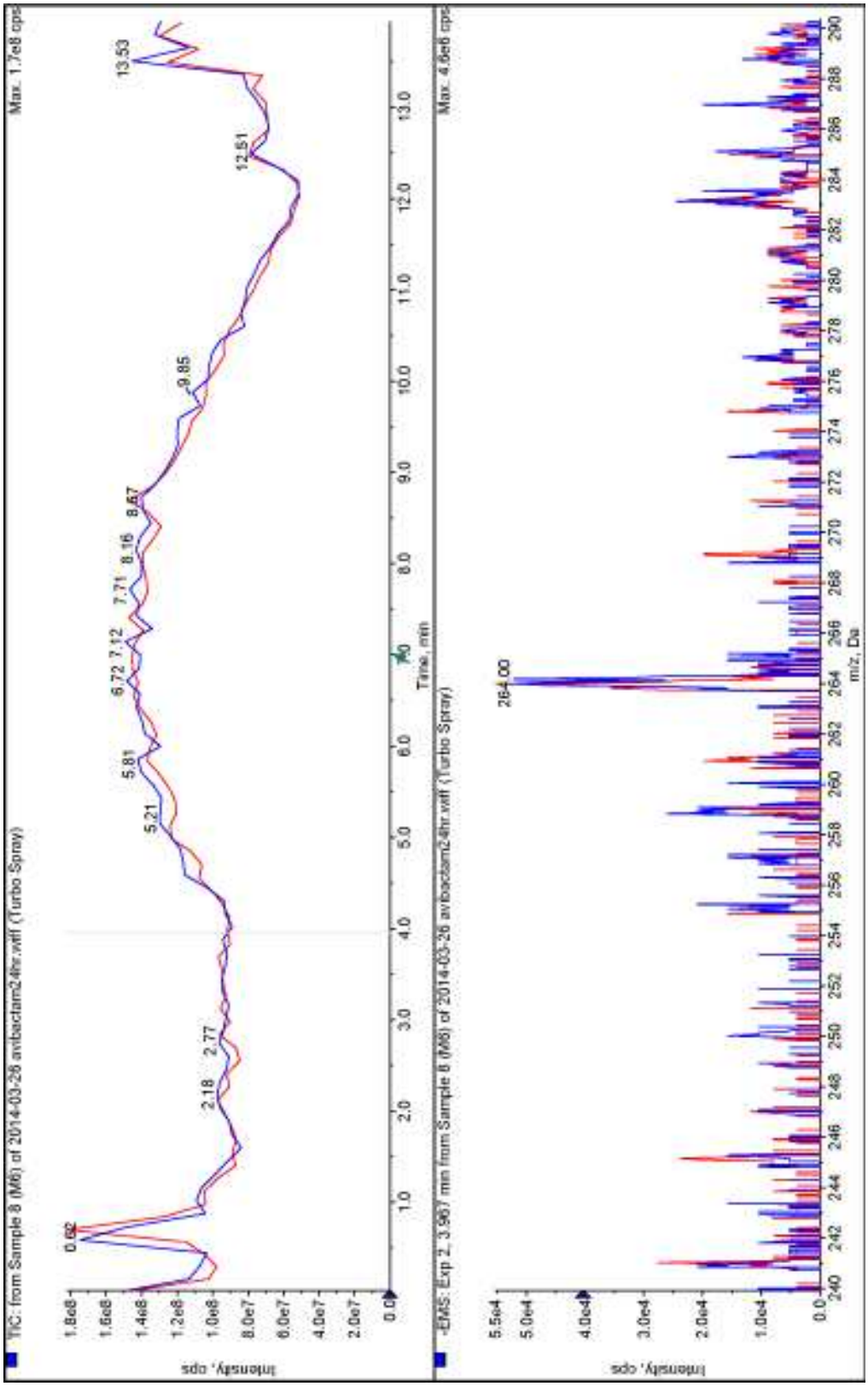
CTX-M-15 N132A



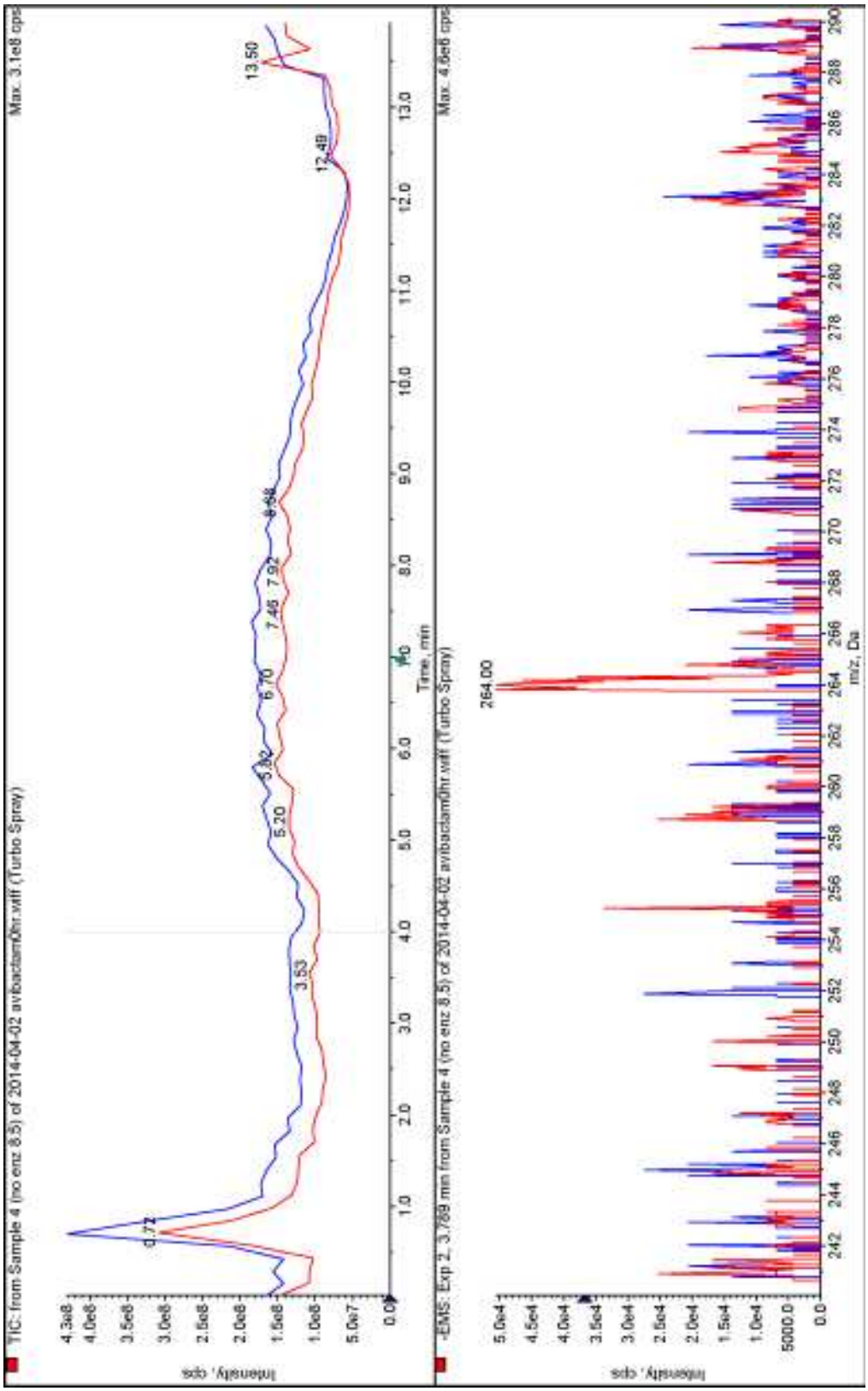
CTX-M-15 E166Q



CTX-M-15 K234A



No enzyme (pH 8.5)



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