Antimicrobial resistance conferred by OXA-48 β-lactamases:

towards a detailed mechanistic understanding

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ABSTRACT. OXA-48-type β-lactamases are now routinely encountered in bacterial infections caused by carbapenem-resistant *Enterobacterales.* These enzymes are of high and growing clinical significance due to the importance of carbapenems in treatment of healthcare-associated infections by Gram-negative bacteria, the wide and increasing dissemination of OXA-48 enzymes on plasmids, and the challenges posed by their detection. OXA-48 confers resistance to penicillin (which is efficiently hydrolyzed) and carbapenem antibiotics (more slowly broken down). In addition to the parent enzyme, a growing array of variants of OXA-48 is now emerging. The spectrum of activity of these variants varies, with some hydrolyzing

expanded-spectrum oxyimino-cephalosporins. The growth in importance and diversity of the OXA-48 group has motivated increasing numbers of studies that aim to elucidate the relationship between structure and specificity and establish the mechanistic basis for β-lactam turnover in this enzyme family. In this review we collate recently published structural, kinetic, and mechanistic information on the interactions between clinically relevant β-lactam antibiotics and inhibitors with OXA-48 β-lactamases. Collectively, these studies are starting to form a detailed picture of the underlying bases for the differences in β-lactam specificity between OXA-48 variants, and the consequent differences in resistance phenotype. We focus specifically on aspects of carbapenemase and cephalosporinase activities of OXA-48 β-lactamases and discuss β-lactamase inhibitor development in this context. Throughout the review, we also outline key open research questions for future investigation.

Introduction

Antimicrobial resistance has been recognized globally as one of the most serious threats to modern medicine. According to the 2014 UK Review on Antimicrobial Resistance, antibiotic resistant bacterial infections are predicted to result in 10 million deaths annually by 2050 if no preventative measures are taken.[1] β-lactams are the single most prescribed antibiotic class, accounting for over half of all antibiotic prescriptions in human patients,[2] thus the consequences of widespread resistance to these agents are especially severe. Resistance against carbapenems is a particular concern, as these drugs are the most recently introduced and potent class of β-lactams; they are for example favored treatments for opportunistic infections of secondary care patients by Gram-negative bacteria resistant to other agents.[3] Furthermore, the continuing weakness of the antibacterial pipeline means that alternative treatments are limited.[4] This existing threat is highlighted by the Centers for Disease Control and Prevention, who

classified carbapenem-resistant *Enterobacterales* as an urgent antibiotic resistance threat in the United States in 2019.[5]

Since the initial introduction of β-lactam antibiotics in the 1940s, bacteria have developed many different mechanisms to bypass their effect; these include changes in expression levels of porins and efflux pumps, target modification through gene acquisition or mutation, and enzymatic drug modification.[6] In Gram-negative bacteria, β-lactamase enzymes are the main resistance mechanism against β-lactam antibiotics. β-Lactamases modify the antibiotic by hydrolytic cleavage of the β-lactam amide bond;[7] as β-lactams work by binding to penicillin-binding proteins (PBPs) and disrupting bacterial cell wall biosynthesis,[8, 9] degrading the β-lactam pharmacophore renders these antibiotics inactive. Over 4500 β-lactamases have now been identified (see www.bldb.eu for details),[10] with the continuing explosion of genomic data driving further discovery of new enzymes from both environmental and clinical sources.

According to the Ambler classification, β-lactamases are divided into four groups: classes A, C, and D are serine β-lactamases (SBLs), which utilize an active site serine nucleophile to hydrolyze β-lactams via a covalent acylenzyme intermediate, while class B metallo-β-lactamases (MBLs) utilize zinc cofactors to activate a water molecule to undertake antibiotic inactivation.[7] Within SBLs, class D β-lactamases form a structurally diverse group of enzymes, which were first identified as having enhanced hydrolytic activity towards semisynthetic penicillins such as oxacillin, and reduced activity towards penicillin (rates compared against class A β-lactamases).[11, 12] Subsequently, they were named as oxacillinases, or OXAs for short. OXA β-lactamases include five recognized subgroups of carbapenem-hydrolyzing enzymes: four of these, namely OXA-23-like, OXA-24/40-like, OXA-51-like, and OXA-58-like β-lactamases, are largely restricted to *Acinetobacter baumannii*, while OXA-48-like β-lactamases are most commonly encountered in the *Enterobacterales.*[13-21] Additionally, some OXA enzymes (OXA-2, OXA-10) classified as narrow-spectrum β-lactamases have demonstrated comparable rates of carbapenem hydrolysis to recognized carbapenem-

hydrolyzing OXAs, which could imply that most OXAs can (to some extent) be considered carbapenemases.[22] OXA-48 β-lactamases are now among the most common carbapenemases[23] and are often co-produced with other β-lactamases (MBLs or ESBLs).[24] For an in-depth overview of the global epidemiology of β-lactamase, specifically OXA-48-type, producing pathogens we refer the reader to recent reviews by Bush and Bradford[25] and Pitout *et al.*[26]

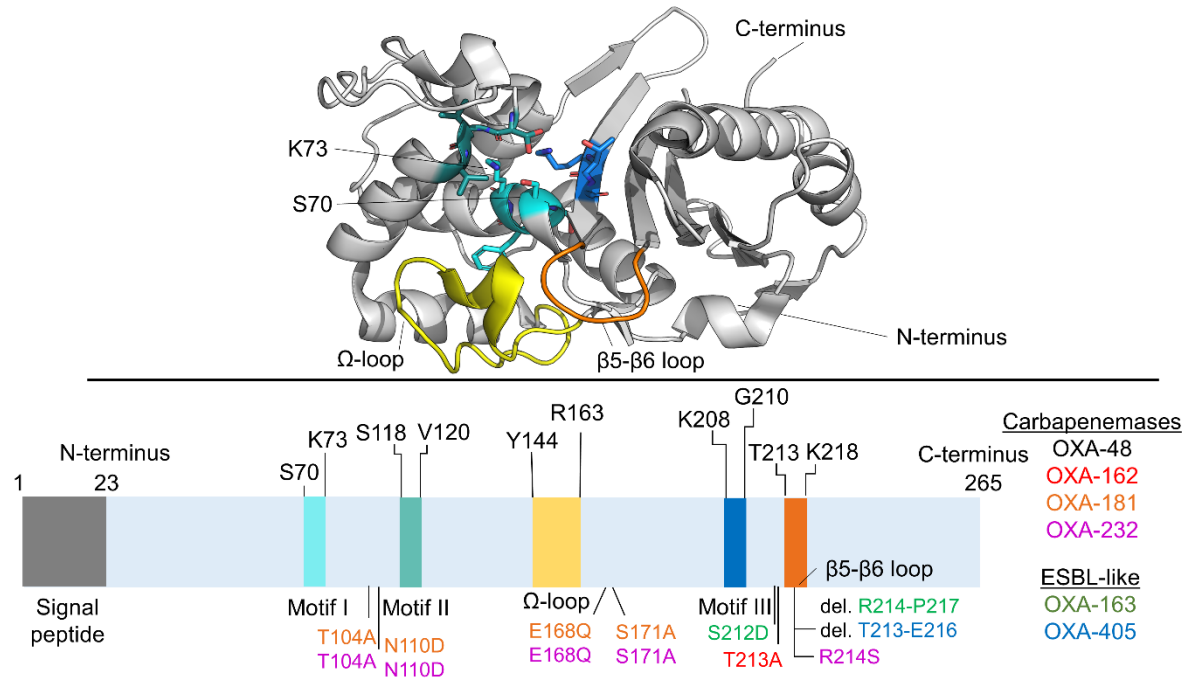


Figure 1. Structure of OXA-48. *Cartoon shows unliganded OXA-48 (PDB ID 6P96)[27] with selected elements of the structure highlighted. The three conserved motifs within class D β-lactamases are shown in blue shades, the Ω-loop in yellow, and the β5-β6-loop in orange. Selected OXA-48 variants are listed according to their primary hydrolysis phenotype, and their amino acid substitutions or deletions highlighted in the corresponding colour in the amino acid sequence. Carbapenemase = efficient imipenem hydrolysis, some activity towards other carbapenem substrates. ESBL-like = only weak activity against all carbapenems, activity against expanded-spectrum oxyimino cephalosporins.*

Even though OXA-48 β-lactamases are not closely related in sequence to other class D β-lactamases (less than 50 % amino acid identity), their sequences include three active site motifs

that are broadly conserved within class D enzymes.[28] Motif I (SxxK) includes the nucleophilic Ser70 and the catalytically important Lys73, which needs to be carboxylated for efficient hydrolysis to take place (Figure 1).[28, 29] Motifs II and III are in the vicinity of these key catalytic residues and include residues Ser118-Val119-Val120 and Lys208-Thr209-Gly210, respectively, in OXA-48-like enzymes. Additionally, the Ω-loop (residues 143-165) and β5-β6-loop (residues 213-218) bordering the active site seem to be important determinants of OXA-48 activity, as discussed below. According to the β-lactamase database, at least 15 plasmid-encoded OXA-48 β-lactamases have been identified and validated (with further variants chromosomally encoded mainly in different *Shewanella* species[30, 31]). These variants differ from wild-type OXA-48 by certain amino acid substitutions or deletions. Selected key family members along with their hydrolytic profiles are listed in Figure 1.

As mentioned above, OXA-48 enzymes degrade a variety of β-lactam antibiotics, including ampicillin and oxacillin (more efficiently than e.g. temocillin),[28, 32] and perhaps most notably the "last-resort antibiotics" carbapenems (Figure 2).[33] However, there are large phenotypic variations within the enzyme family. Compared to the parent OXA-48 enzyme, some variants have enhanced carbapenemase activity (like OXA-162[34] and OXA-181[35]), while others have expanded their hydrolysis profile to better accommodate expanded-spectrum oxyimino cephalosporins (such as OXA-163[36] and OXA-405[37]). OXA-48 carbapenemases tend to favour imipenem over other carbapenems and display only low-level meropenem and ertapenem hydrolysis (Table 1). Weak carbapenem hydrolysis can complicate diagnosis and treatment of bacterial infections involving OXA-48 producers, as their activity can be below the detection limit of clinical tests but still sufficient to confer resistance, especially in strains with reduced antibiotic permeability.[38] OXA-48 itself shows varying activity against cephalosporins; e.g. cefalotin and cefotaxime are inactivated readily, whereas minimal (or no)

activity is measured against ceftazidime and cefepime. Further enzyme kinetic data for OXA-48 and key variants are collated in the Supporting Information (spreadsheet).

Table 1. Kinetic parameters for OXA-48, OXA-163, OXA-181, and OXA-232 with different β-lactam antibiotics. *Presented values taken from ref. [32], and more comprehensive data of enzyme kinetics is provided as part of the SI.*

	OXA-48[a]		***OXA-163***		***OXA-181***		***OXA-232***	
	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})	K_m (μM)
Imipenem	5	13	0.03	520	7.5	13	0.2	9
Meropenem	0.07	10	>0.1	>2000	0.1	70	0.03	100
Ertapenem	0.13	100	0.05	130	0.2	100	0.04	110
Doripenem	-[b]	-[b]	NH	NH	0.04	55	0.005	10
Ceftazidime	NH	NH	8	>1000	-	-	>0.6	>1000
Cefotaxime	>9	>900	10	45	>62	>1000	>6.5	>1000
Cefalotin	44	195	3	10	13	250	13	125
Benzylpenicillin	-[c]	-[c]	23	13	444	90	125	60
Ampicillin	955	400	23	315	218	170	132	220
Temocillin	0.3	45	NH	NH	0.3	60	0.03	60
Oxacillin	130	95	34	90	90	80	156	130

[a] Values for OXA-48 in ref. [32] are from ref. [28].

[b] Data from ref. [34] do not show doripenem hydrolysis by OXA-48, but kinetic data from ref. [22] indicate weak doripenem hydrolysis.

[c] Kinetic data from ref. [33] indicate that benzylpenicillin is hydrolysed by OXA-48.

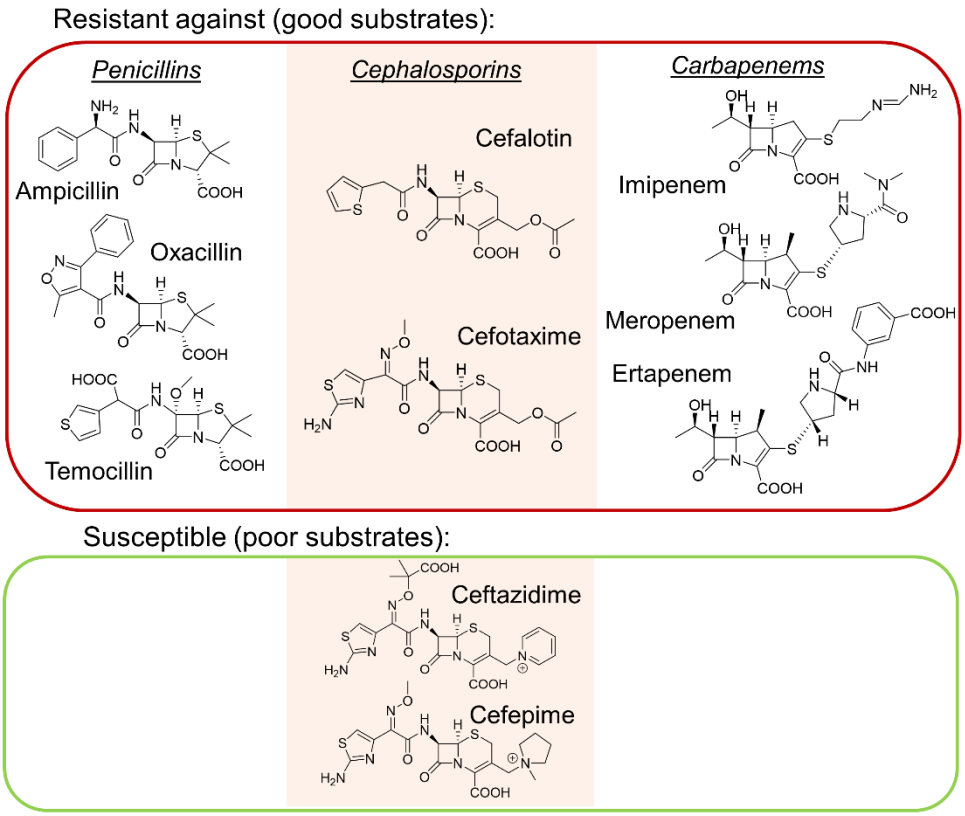
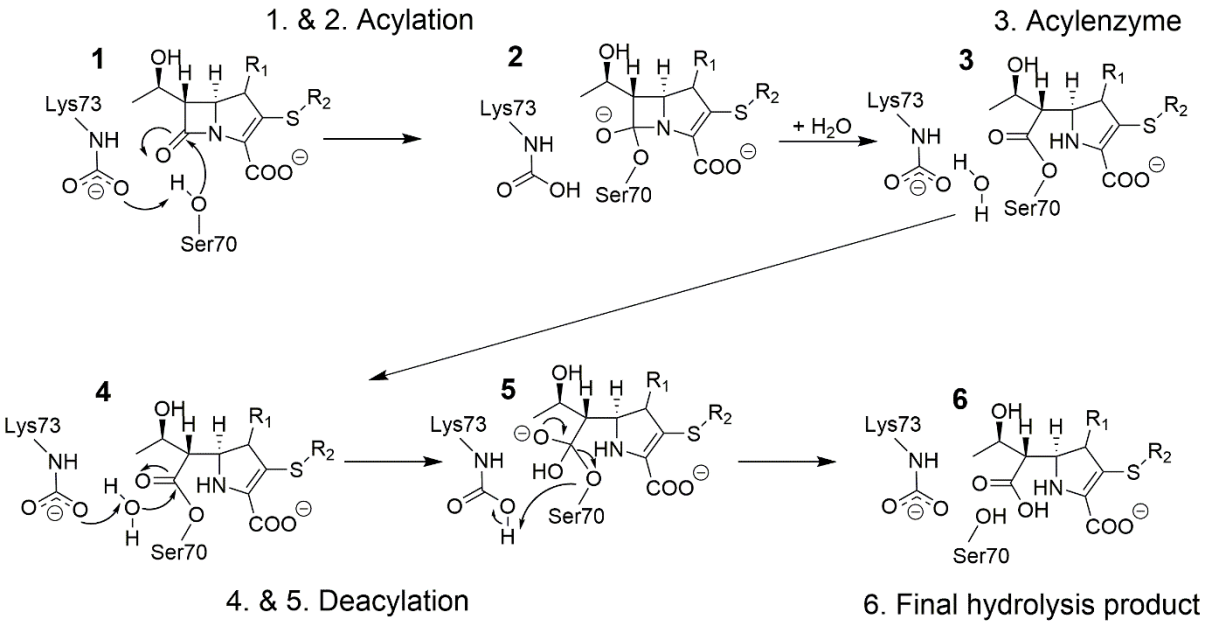


Figure 2. β-lactam antibiotics as substrates for OXA-48. *Examples of penicillin, cephalosporin, and carbapenem antibiotics (left, middle, and right respectively), which are generally ineffective against OXA-48 producers (red box), and which can be used to treat OXA-48-producing infections (green box). Notably, activity profiles vary within the OXA-48 family, as e.g. the ESBL-like OXA-163 has acquired activity against expanded-spectrum oxyimino cephalosporins (ceftazidime).*

General hydrolysis mechanism

In SBLs, the overall hydrolysis reaction consists of two parts, acylation followed by deacylation (Scheme 1).[7] After initial formation of the non-covalent Michaelis complex, the β-lactamase is acylated by the antibiotic resulting in covalent bond formation between Ser70 and the carbonyl carbon of the β-lactam ring. This covalent acylenzyme structure is hydrolyzed in the deacylation step, where an active site water molecule (the so-called deacylating water) acts as the nucleophile to attack the acylenzyme carbonyl. Both acylation and deacylation involve

formation of short-lived tetrahedral intermediate (TI) structures. For OXA-48-like β-lactamases, deacylation was shown to be rate-limiting for carbapenem breakdown.[39]



Scheme 1. Hydrolysis Mechanism of OXA-48 β-lactamases. *Starting from the formation of a Michaelis complex for a general carbapenem substrate (**1**), the substrate is acylated (tetrahedral intermediate formation in **1** → **2**), which yields a covalent acylenzyme structure (**3**). The bound antibiotic is subsequently deacylated (**4** & tetrahedral deacylation intermediate **5**) resulting in the final hydrolysis product (**6**).*

As depicted in Scheme 1, both acylation and deacylation involve a negatively charged general base. For class A β-lactamases, this residue is largely accepted to be Glu166,[40, 41] but for OXA enzymes, the general base is a carboxylated lysine (Lys73 in OXA-48 numbering).[28, 42] This post-translational carboxylation is needed for efficient hydrolysis to take place, as mutating Lys73 results in enzymes incapable of substrate turnover.[29] The degree of carboxylation increases with pH, and preparation of catalytically competent enzymes can be ensured by adding a suitable CO_2 source for carboxylation (bicarbonate), even though atmospheric CO_2 may also be enough.[43] This carboxylation is reversible, and it has been

monitored with ^{19}F NMR spectroscopy in the presence of different inhibitors to understand how (de)carboxylation contributes to enzyme inhibition.[44] The results indicate that Lys73 is carboxylated to a lesser extent with some covalently-bound inhibitors (like avibactam), which may contribute to more efficient inhibition.

Carbapenemase activity

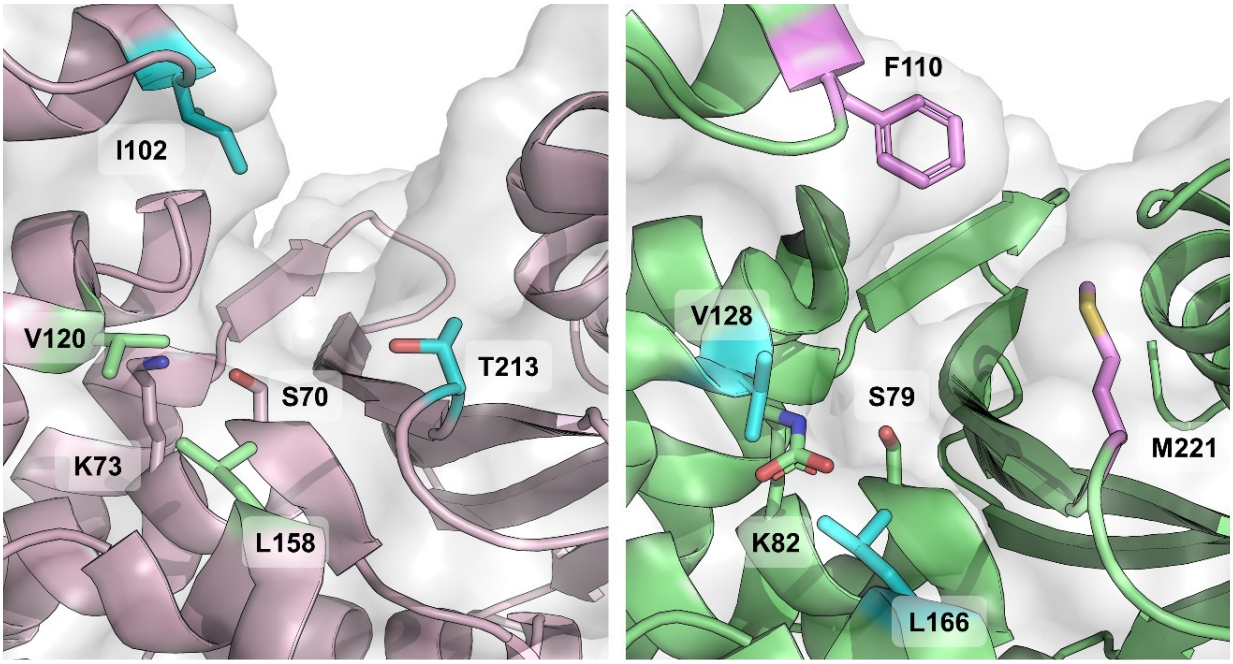


Figure 3. Divergent Active Sites of Carbapenem-Hydrolyzing OXA Enzymes. *Active sites of OXA-48 (PDB ID 6P96, left)[27] and OXA-23 (PDB ID 4K0X, right)[45] highlight the missing hydrophobic bridge in OXA-48 with respect to other class D carbapenemases. In OXA-23, the hydrophobic bridge is formed by residues Phe110 and Met221, while the corresponding residues in OXA-48 are Ile102 and Thr213, which leave the active site more open. Additionally, residues forming the so-called "deacylating water channel" are also highlighted in sticks (V120 and L158 for OXA-48, V128 and L166 for OXA-23).*

OXA-48 enzymes are carbapenemases or, more specifically, imipenemases with weak turnover rates for other carbapenems such as meropenem and ertapenem (Table 1, SI and references therein). Based on the structural information originally derived from other carbapenem-hydrolyzing OXAs,[46] carbapenemase activity in class D β-lactamases was hypothesized to originate from a hydrophobic bridge spanning the active site (Phe110 and Met221 for OXA-23, Tyr112 and Met223 for OXA-24). However, structural comparisons between OXA-48 and other OXA carbapenemases show OXA-48 to be lacking this hydrophobic bridge,[28] which implies that the OXA-48 group has evolutionally diverged from other class D β-lactamases and acquired carbapenemase activity by other means (Figure 3). Fortunately, within the last years a plethora of new crystal structures of OXA-48s complexed with carbapenems have been released, and new mechanistic knowledge has been derived from them (currently available structures of OXA-48 enzymes in the Protein Data Bank (www.rcsb.org/pdb) are listed in the SI).

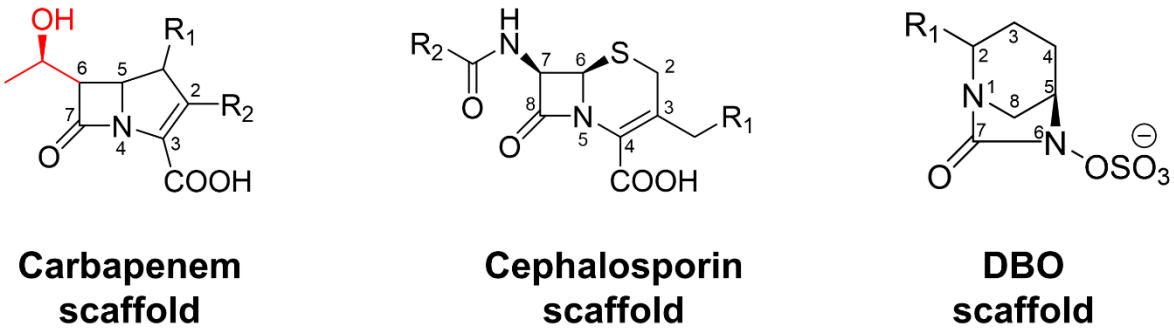
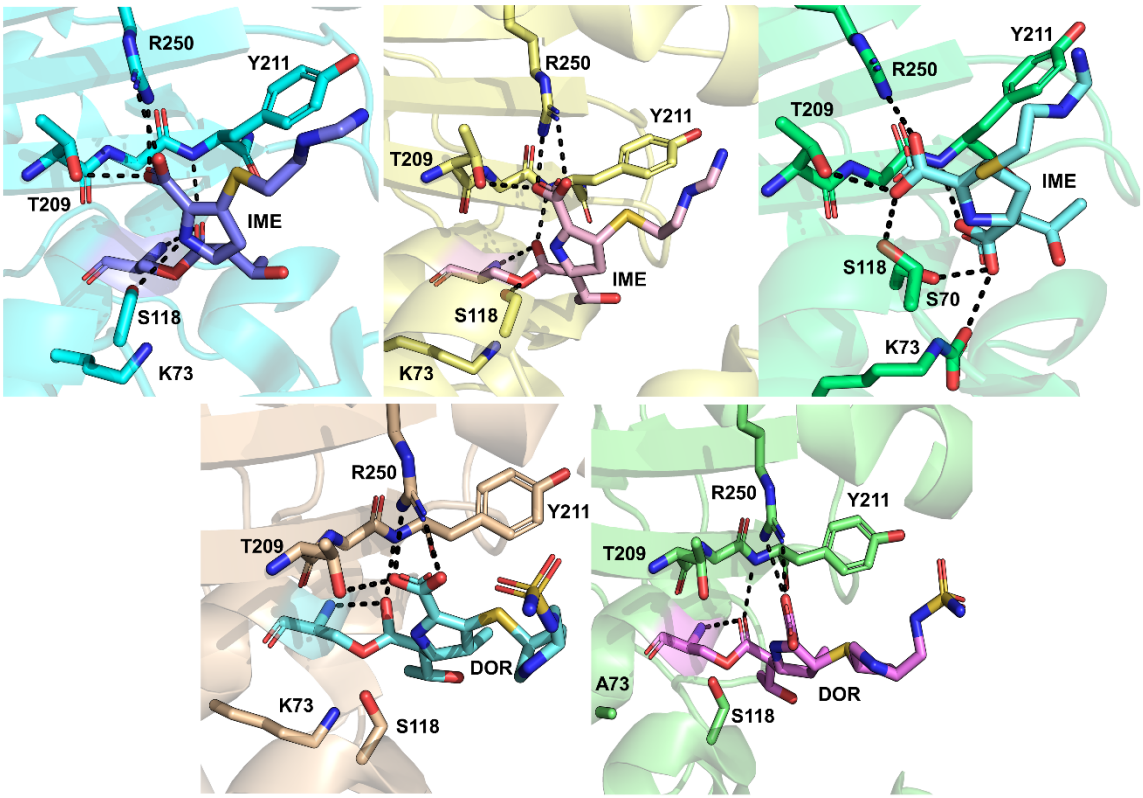


Figure 4. Carbapenem, Cephalosporin, and Diazabicyclooctanone (DBO) Scaffolds with Atom Numbering. *The 6α-hydroxyethyl group (C6 substituent) in the carbapenem scaffold is shown in red.*

The first carbapenem acylenzyme structure of OXA-48 (with imipenem) was released in 2018 (PDB ID 5QB4) alongside multiple structures with small inhibitor fragments.[47] From 2019 onward, further acylenzyme structures have been deposited with imipenem (PDB IDs

6P97, 6PTU, and 7KH9),[27, 39, 48] meropenem (PDB IDs 6P98, 6PT1, and 7KHQ),[27, 39, 48] doripenem (PDB IDs 6P9C and 6PXX),[27, 49] ertapenem (PDB ID 6P99),[27] and faropenem (PDB ID 6PSG)[48]. Additionally, two acylenzyme structures of inactivated OXA-163 (K73A) with imipenem and meropenem are available (PDB IDs 7KHZ and 7KHY, respectively)[39]. Common features in these structures include a covalent bond between Ser70 and the substrate and hydrogen bonds between Thr209/Arg250 and the carbapenem C3 carboxylate (Figure 4). The carbonyl oxygen of the cleaved β-lactam ring is positioned in the oxyanion hole formed by the backbone amides of Ser70 and Tyr211, active site interactions in selected crystallized carbapenem acylenzyme complexes are presented in Figures 5 and 6. Carbapenem "tail" groups (C2 substituents) are not anchored by any strong interactions, which implies that they are dynamic and do not need to adopt any one specific orientation. This likely disorder was also inspected by Papp-Wallace *et al.*, who further refined previously deposited imipenem and doripenem complexes (PDB IDs 5QB4 and 6P9C, respectively).[49] Their analysis of the re-refined structures supports the presence of a covalent bond between Ser70 and the antibiotic, but observation of weak or absent density for the pyrroline ring and C2 tail groups indicates disorder (i.e. multiple conformations) for these regions. In addition to previously mentioned covalent complexes, a structure of OXA-48 with hydrolyzed imipenem has also been published (PDB ID 6PK0).[48] Non-covalently bonded hydrolyzed imipenem forms similar interactions with Thr209 and Arg250 to those observed in the acylenzyme, and the newly formed C7 carboxylate group is hydrogen bonded to Ser70, Lys73, and Tyr211 (Figure 5). Although the deacylating water is not present in any acylenzyme structure, the orientation of hydrolyzed imipenem (specifically coordination of the C7 carboxylate to Ser70 and Lys73) indicates the possible position of the deacylating water molecule prior to deacylation.



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214 ***Figure 5. Carbapenem Complexes of OXA-48***. *Top left: Imipenem acylenzyme (PDB ID 6P97)[27],*
215 *interactions with active site residues highlighted. Imipenem pyrroline ring modelled as the* Δ^2
216 *tautomer. Top middle: Imipenem acylenzyme (PDB ID 6PTU)[48], with the pyrroline ring as the (R)-*Δ^1
217 *tautomer. Top right: Hydrolyzed imipenem (PDB ID 6PK0)[48], with the pyrroline ring as the (S)-*Δ^1
218 *tautomer. Bottom left: Doripenem acylenzyme (PDB ID 6P9C)[27], with the pyrroline ring as the* Δ^2
219 *tautomer. Bottom right: Doripenem acylenzyme (PDB ID 6PXX)[49], with the pyrroline ring as the (R)-*
220 Δ^1 *tautomer.*

221

222 In OXA-48 enzymes the basis for carbapenemase activity has been attributed to the
223 presence of the β5-β6 loop bordering the active site, as for example engineering this loop from
224 OXA-48 into the non-carbapenemase OXA-10 changes its phenotype to hydrolyze imipenem
225 at higher rates than native OXA-48.[50] The specific role of Arg214 (in the β5-β6 loop) was
226 studied by comparing hydrolysis kinetics and crystal structures of OXA-181 and OXA-232,

the difference between these two variants being residue 214 (Arg in OXA-181, Ser in OXA-232).[51] OXA-181 is a slightly better carbapenemase than OXA-48,[35] whilst OXA-232 has decreased carbapenem hydrolysis rates but has also acquired weak activity against ceftazidime (Table 1).[52] The authors suggest that the presence of Arg214 is crucial for carbapenem hydrolysis by OXA-48, as it aids in the formation of a productive binding pose for imipenem. Replacing this arginine with a negatively charged residue (Glu) results in poor affinity, which was reasoned to be due to unproductive binding pose of imipenem (both hypotheses based on molecular docking). Similar results were found by Dabos *et al.*, who substituted the β5-β6 loop of OXA-18 into OXA-48.[53] Steady-state kinetics of the OXA-48loop18 variant showed decreased ampicillin and imipenem hydrolysis and elevated ceftazidime hydrolysis. The importance of the β5-β6 loop for the hydrolysis profile indicated by these studies is further emphasized by the decrease in imipenem hydrolysis and increase in ceftazidime hydrolysis in OXA-163 (Table 1),[32, 36] in which the loop is partially deleted (Figure 1). Pre-steady state kinetics indicate that the loss of efficient imipenemase activity in OXA-163 is due to decreased deacylation rates.[39] However, even though the β5-β6 loop is evidently important for carbapenem hydrolysis, the specific origin of imipenemase activity in OXA-48 enzymes (e.g. over meropenem hydrolysis) remains to be investigated. The presence of the 1β-methyl group e.g. in meropenem and doripenem (instead of the 1β-proton in imipenem) has been suggested to impair hydrolysis, as this methyl group might prevent deacylation by disfavoring rotation of the carbapenem 6α-hydroxyethyl moiety (attached to C6, Figure 3), which would in turn prohibit the nucleophilic attack.[48] In all OXA-48/carbapenem crystal structures (excluding 5QB4), the 6α-hydroxyethyl sidechain adopts a similar orientation where its methyl group points towards Leu158 and Arg214 and values for the C7-C6-C-O dihedral angle are between 147°-192° (Figure 6). However, for hydrolyzed imipenem this orientation has changed, and the methyl group points out of the active site towards bulk solvent (with the same dihedral angle being between 275°-292° depending on the protein chain). As the 6α-hydroxyethyl group is

likely able to rotate in the acylenzyme, verifying the extent of its influence on e.g. positioning and movement of the deacylating water remains as an important aspect for future mechanistic studies.

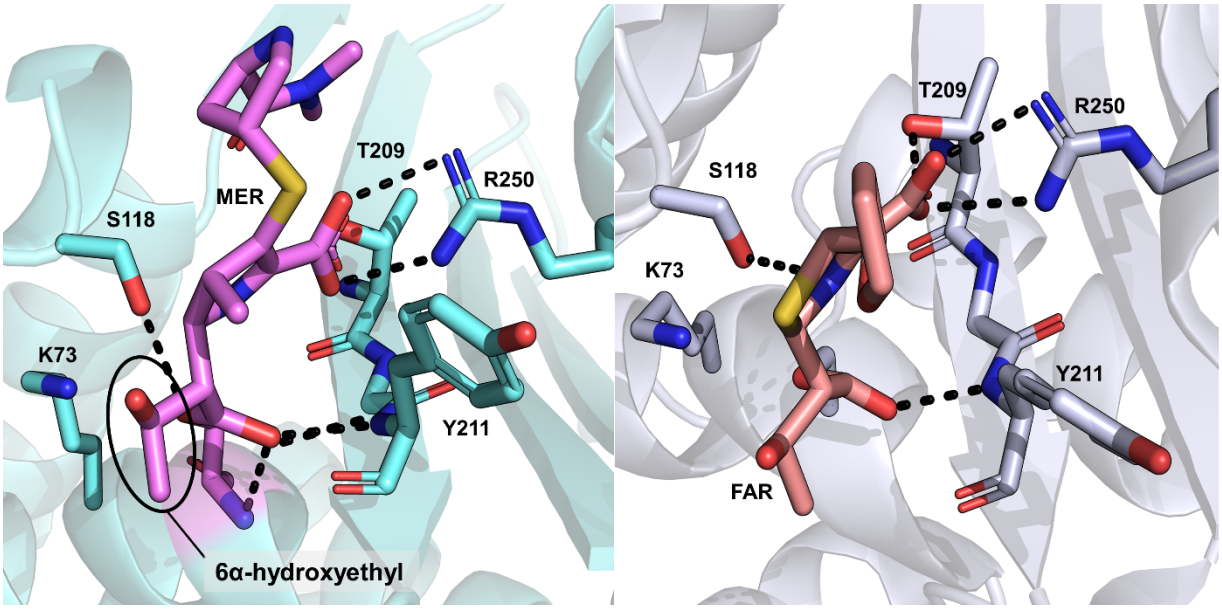
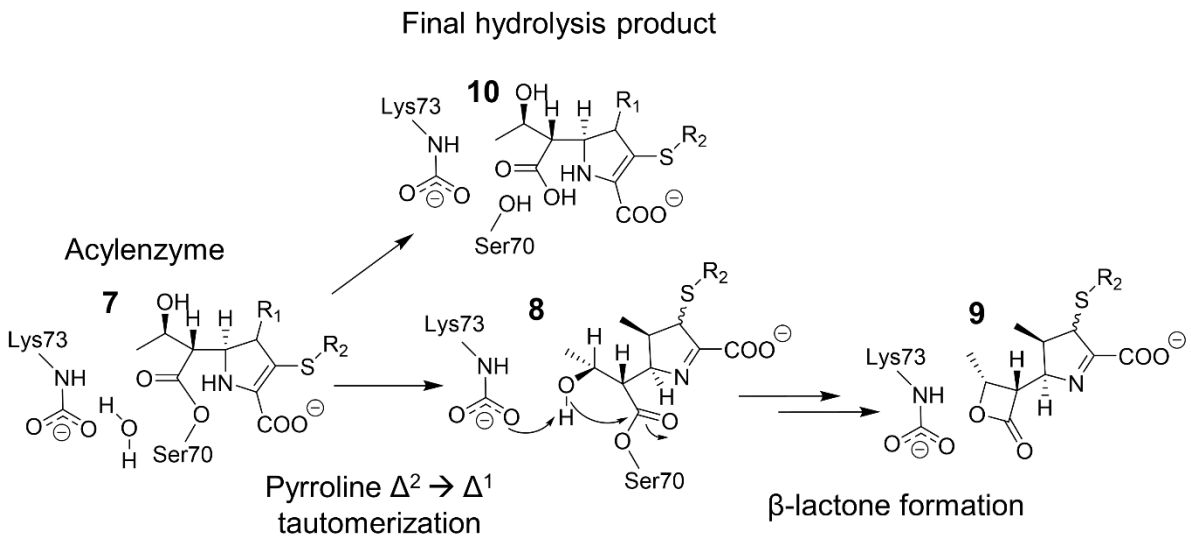


Figure 6. Further Carbapenem Complexes of OXA-48. *Acylenzyme structures with meropenem (left, PDB ID 6P98)[27] and faropenem (right, PDB ID 6PSG)[48]. The pyrroline ring is present as the Δ^2 tautomer in both structures.*

The pyrroline ring of carbapenem acylenzymes can exist as two different tautomers: Δ^2 or Δ^1, the latter of which also has two stereoisomers (*R*)-Δ^1 and (*S*)-Δ^1 (Scheme 2). For class A β-lactamases, the Δ^2 tautomer has been proposed to be the catalytically competent form,[54, 55] and the Δ^1 to not deacylate efficiently (potentially due to displacement of the deacylating water from the active site[56] or loss of stabilizing interactions with the oxyanion hole[57]). The same has been suggested for class D enzymes when comparing the doripenem complex of carbapenem-hydrolyzing OXA-24 against carbapenem-inhibited OXA-1.[58] The tautomeric form can be identified in crystal structures with sufficiently strong electron density for the ligand, as the pyrroline ring C2 – sulphur bond present in all carbapenems is planar (sp^2 hybridized) in the

case of the Δ^2, and sp^3 hybridized for the Δ^1 forms. For previous class D β-lactamases complexed with carbapenems, all three tautomers have been observed.[58, 59] In the case of OXA-48, the Δ^2 form was assigned in the first deposited imipenem complex,[47] and the same tautomer was subsequently observed for the meropenem, imipenem, doripenem, and ertapenem acylenzymes published by C. A. Smith *et al.* (structures prepared by soaking crystals of apo-OXA-48 with 50 mM carbapenem solution over time scales between 30 seconds and 10 minutes).[27] The same authors inspected the possibility of accommodating ligands in the active site in the Δ^1 form by superimposition of their structures onto OXA-23 with (*R*)-Δ^1 and (*S*)-Δ^1 ligands. They suggest that the formation of the (*S*)-Δ^1 tautomer of meropenem is feasible, while the (*R*)-Δ^1 conformer would clash sterically with Tyr211. Shortly after the publication of these carbapenem acylenzymes, a new structure of deacylation-deficient OXA-48 (Lys73Ala) in complex with doripenem was released (also prepared using crystal soaking).[49] The doripenem acylenzyme was observed as both (*R*)-Δ^1 and (*S*)-Δ^1 tautomers (Figure 5), and only a partial salt bridge with Arg250 is formed, which most likely prevents any severe steric clashes between doripenem and Tyr211 for either tautomer. In further structures deposited by Akhtar *et al.*, different carbapenems have different tautomers present: the meropenem acylenzyme is in the Δ^2 form (as depicted for another crystal structure in Figure 6), imipenem and faropenem are found as (*R*)-Δ^1 (Figures 5 and 6), and the imipenem hydrolysis product as the (*S*)-Δ^1 tautomer (Figure 5, structures prepared by soaking OXA-48 crystal with a solution containing the ligand for 30 minutes, or for the OXA-48 imipenem product complex for 2 hours).[48] Characterization of the enzyme-hydrolyzed products by NMR spectroscopy implies that for OXA-48 (as well as all other tested SBLs and MBLs) the preferred hydrolysis product would be either in the Δ^2 or (*R*)-Δ^1 form, but deducing the exact enzyme-catalyzed reaction product was not feasible due to the ability of released products to undergo tautomerization in solution.[60]



Scheme 2. Mechanism for Carbapenem Side Product Formation by OXA-48. *The pyrroline ring in carbapenem substrates can undergo $\Delta^2 \rightarrow \Delta^1$ tautomerization (**7** → **8**) post-acylation. In addition to the general hydrolysis mechanism (**7** → **10**), 1β-methyl carbapenems such as meropenem can form a 1β-lactone product (**8** → **9**), which has been suggested to be mainly in the Δ^1 form.*[61]

In addition to the generic hydrolysis mechanism of serine β-lactamases, OXA-48 enzymes were shown to possess an additional mechanism for carbapenem breakdown that involves the formation of a β-lactone product, as illustrated in Scheme 2.[61, 62] Starting from the acylenzyme, the β-lactone is suggested to form by intramolecular cyclization, where the hydroxyl group of the carbapenem 6α-hydroxyethyl sidechain donates a proton to the carboxylated lysine (Lys73) and attacks the same electrophilic C7 carbon as in deacylation. This results in formation of a four-membered lactone ring, which is structurally close to the original β-lactam ring and capable of reacting further to give (unidentified) reaction products. Interestingly, β-lactone formation by OXA-48 appears carbapenem-dependent, as it was observed only for 1β-methyl carbapenems (such as meropenem, doripenem, and ertapenem), but not for carbapenems with a 1β-hydrogen (imipenem and biapenem). The reason for this dependence on the presence of the 1β-substituent was studied by simulating the dynamics of

OXA-1 (one 100ns simulation), and suggested to be due to more favorable conformational sampling of the 6α-hydroxyethyl sidechain: with a 1β-methyl group, bound carbapenems formed closer interactions with the carboxylated lysine, which would aid in proton transfer from the hydroxyl group to the lysine carboxylate oxygen.[61] More recently, however, lactone formation was shown to also depend upon the structure of the active site: OXA-519 (Val120Leu variant of OXA-48) demonstrated both an increase in the proportion of the lactone product as well as generated lactones from both of 1β-proton and 1β-methyl carbapenems.[62]

Cephalosporinase activity

While OXA-48 is considered of particular importance as a result of its carbapenemase activity, there are variations in hydrolytic phenotypes between different OXA-48 variants. OXA-48 itself does hydrolyze some cephalosporin antibiotics, such as cefalotin and cefotaxime, but shows no significant hydrolysis of the expanded-spectrum oxyimino cephalosporin ceftazidime or the fourth-generation cephalosporin cefepime.[32] However, variants such as OXA-163 and OXA-405 (that contain partial deletions in the β5-β6 loop) are capable of hydrolyzing ceftazidime, at the expense of efficient imipenem breakdown (Figure 1, Table 1).[36, 37] Interestingly, their hydrolysis rates for other carbapenems (such as meropenem) seem to be on the same low level as for OXA-48 (further kinetic information is collated in the SI).

In 2019, the structure of an OXA-48 (Pro68Ala) ceftazidime acylenzyme was deposited (PDB ID 6Q5F, Figure 7);[63] this single point mutant was obtained by passage of a laboratory OXA-48 producer strain against increasing concentrations of ceftazidime. Comparison of this OXA-48 structure with previously deposited OXA/ceftazidime complexes (OXA-225 and

OXA-160, PDB IDs 4X55 and 4X56, respectively)[64] shows that ceftazidime exhibits a different binding pose in OXA-48 than observed in the OXA-23 or OXA-24/40 variants, the difference being in the orientation of the C7 substituents (carboxypropyl oxyimino and thiazole groups, Figure 3). Another distinct feature in the OXA-48/ceftazidime structure was the lack of interpretable electron density for the Ω-loop (including residues Leu158 and Asp159, Figure 1). The authors suggested ceftazidime binding to displace Arg214, which in turn results in a distorted (and thus flexible) Ω-loop; the Pro68Ala mutation might then contribute to Ω-loop distortion by increasing flexibility of the active site. Molecular dynamics simulations and QM/MM reaction modelling of ceftazidime deacylation by OXA-48, OXA-163, and OXA-181 suggest that in addition to the β5-β6 loop and Arg214, Leu158 could also play an important role in determining the efficiency of ceftazidime turnover.[65] The orientation of Leu158 was observed to correlate with active site hydration, and an increase in water molecules in the active site was observed to impair deacylation efficiency in OXA-48. Additionally, the study proposed that distorting the Ω-loop, as is implied by the absence of electron density for this region in the OXA-48 ceftazidime crystal structure, would fully open the active site to bulk water and diminish deacylation rates. Although some consideration has been given to the routes by which the water molecule necessary for deacylation may enter the active site,[27, 59] the importance of active site hydration to the activity of OXA-48 β-lactamases (or of SBLs in general) has to date not been extensively discussed in the literature.

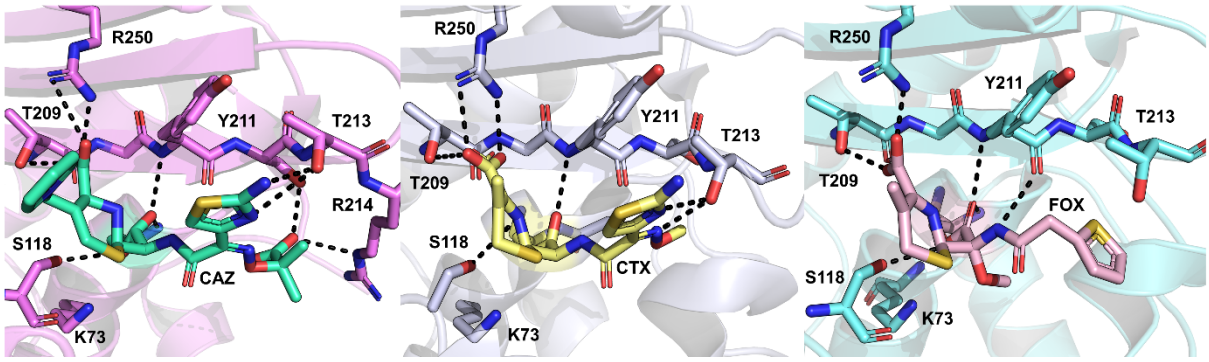


Figure 7. Cephalosporin Acylenzyme Complexes of OXA-48. *Hydrogen bonds between the substrate and active site residues highlighted with dashed lines. Left: ceftazidime (CAZ, PDB ID 6Q5F)[63], middle: cefotaxime (CTX, PDB ID 6PQI)[48], right: cefoxitin (FOX, PDB ID 6PT5)[48].*

In addition to ceftazidime, structures of OXA-48 acylenzyme complexes with cefotaxime and cefoxitin have also been determined (PDB IDs 6PT5 and 6PQI for cefoxitin and cefotaxime, respectively).[48] Cefotaxime has a similar binding pose to ceftazidime, where the thiazole ring orients to make stacking interactions with Tyr211 and the oxyimino group occupies a pocket between residues Leu158, Thr213, and Arg214 (Figure 7). Unlike the ceftazidime complexes, the Ω-loop remains ordered, as found in the apoenzyme, and the salt bridge between Asp159 and Arg214 is preserved. This is most likely due to the smaller size of the cefotaxime C7 methoxyimino group, compared to the equivalent carboxypropyl oxyimino group of ceftazidime. In the case of cefoxitin, the thiophene ring is rotated towards Leu158, breaking the Asp159-Arg214 salt bridge. Low cefotaxime hydrolysis rates are hypothesized to be due to limited access of potential deacylating water molecules to the active site, while cefoxitin hydrolysis ($k_{cat} > 0.05$ s^{-1} and $K_m > 200$ μM, SI) is essentially hindered by the presence of its 7-α-methoxy group, which would sterically clash with any active site water molecules.[48] Additionally, carboxylation of Lys73 could lead to further steric clashes with the 7-α-methoxy group, which could increase preference for lysine decarboxylation in the presence of cefoxitin (Lys73 is decarboxylated in the crystal structure).

OXA-48 Inhibitors

A common strategy for treating challenging, β-lactam resistant bacterial infections is to prescribe a β-lactam antibiotic together with a β-lactamase inhibitor.[66, 67] FDA approved antibiotic/inhibitor combinations include e.g. amoxicillin/clavulanate, piperacillin/tazobactam, ceftazidime/avibactam, and meropenem/vaborbactam.[66-69] In general, OXA-48 β-lactamases are not susceptible to traditional β-lactamase inhibitors like sulbactam, tazobactam, and clavulanate (except for some exceptions like OXA-163).[70] Of the new generation β-lactamase inhibitors, avibactam[71, 72] shows efficacy against OXA-48.[73, 74] Avibactam belongs to the diazabicyclooctanone (DBO) class and exhibits broad-spectrum inhibition of SBLs. The ceftazidime/avibactam combination specifically shows promise as an effective therapy against OXA-48 producers in both *in vitro* testing and clinical practice.[24, 70, 75-77] When compared with other OXAs, it appears that DBOs such as avibactam inhibit OXA-48 better than enzymes with more hydrophobic active site residues.[78] Several crystal structures of OXA-48 with covalently-bound avibactam all show a very similar binding pose for the acylenzyme (PDB IDs 6Q5B[63], 4WMC,[79] 4S2J[43], 4S2K[43], and 4S2N[43]), with the carbamate carbonyl positioned in the oxyanion hole (analogous to the position of the ester carbonyl carbon in β-lactam antibiotics), and the sulfonate group positioned towards motif II and Arg250 (Figure 8). The amide group of avibactam is positioned towards Leu158 on the Ω-loop. Based on the published OXA-48/avibactam structures, the presence of avibactam seems to favour Lys73 decarboxylation: for structures crystallized at pH 6.5 or 7.5 (PDB IDs 4S2J and 4S2K), no carboxylation was observed, and at pH 8.5 only partial occupancy of the carboxylate was seen in two out of four monomers in the asymmetric unit (PDB ID 4S2N).[43] Partial carboxylation of Lys73 was also

observed in another study, where only two out of eight monomers displayed electron density for carboxylated Lys73 (PDB ID 4WMC).[79]

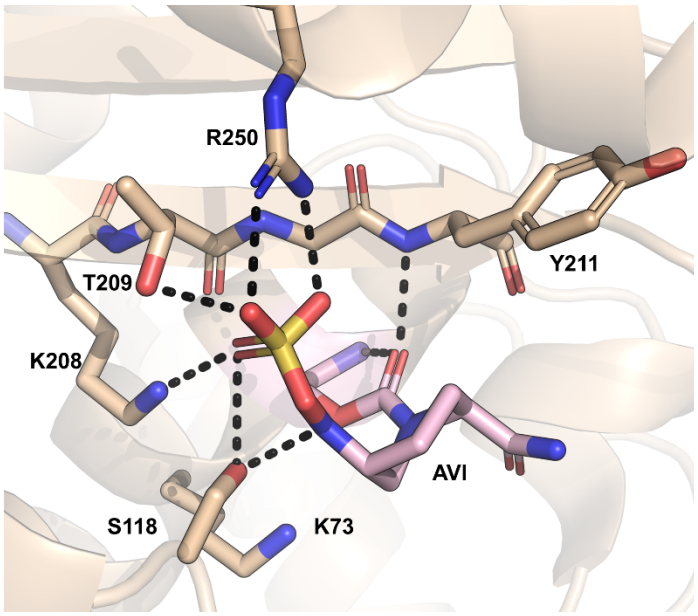
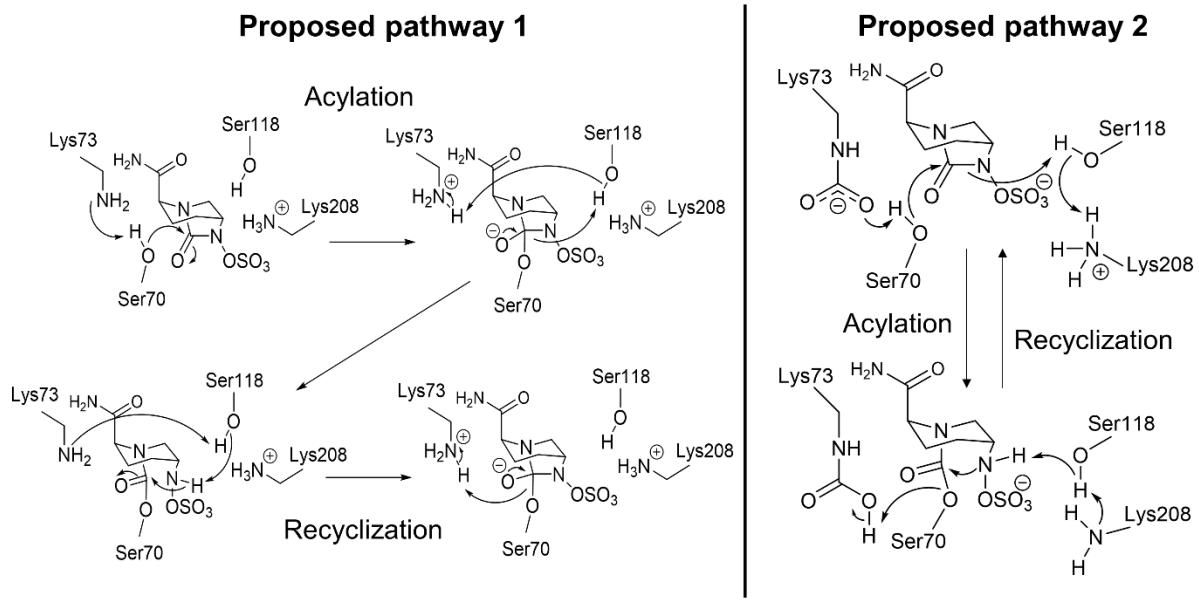


Figure 8. Crystal Structure of the Avibactam-OXA-48 Acylenzyme at pH 7.5 (PDB ID 4S2K)[43].

Inhibition kinetics indicate that avibactam readily acylates OXA-48, but that its recyclization to release intact avibactam happens very slowly (whilst no analogue for the 'standard' β-lactam ring-opened hydrolysis product is observed).[79] In acylation, the C7-N6 bond in the five-membered ring structure is broken (as opposed to the C7-N1 bond, Figure 3), likely due to the N6-sulphate moiety being a better leaving group than N1-R group.[43] At least two different reaction mechanisms for avibactam with OXA-48 have been proposed in the literature (Scheme 3). King *et al.* proposed a general mechanism for all SBLs, which involves a decarboxylated, neutral Lys73 acting as a general base in acylation; Lys73 would then subsequently protonate the N6 ring nitrogen via Ser118.[43] Recyclization occurs as the reverse reaction (Scheme 3, Pathway 1). This mechanism was based on the preference for Lys73 to be decarboxylated in the presence of avibactam. Additionally, mutational studies of the class A ESBL CTXM-15 identified Lys73 to be the most likely general base in avibactam acylation.[43]

Since decarboxylated Lys73 was observed to form a hydrogen bond with Ser118 (Figure 8), it is possible it has a similar role in class D and class A SBLs.

The second proposed mechanism for avibactam inhibition in Scheme 3 (Pathway 2) was suggested by Lahiri *et al.*; in this case, Lys73 is indicated to be carboxylated for the whole reaction cycle.[79] Carboxylated Lys73 acts as the general base in acylation, and Lys208 protonates N6 via Ser118. Recyclization takes place similarly but in reverse, where N6 first donates a proton back to Lys208 via Ser118, and Lys73 acts as a general acid protonating Ser70. As the authors also observed decarboxylation of Lys73 in the presence of avibactam, they attribute the slow avibactam recyclization rates to Lys73 decarboxylation, which hinders reactivity. In addition to these crystal structures, decarboxylation of Lys73 in the presence of covalently bound avibactam has also been measured using NMR spectroscopy.[44] The authors observed that Lys73 favors the decarboxylated form when OXA-48 is complexed with avibactam (or the related DBO inhibitors relebactam and zidebactam). The extent of Lys73 decarboxylation in reactions of OXA-48 with DBOs and its exact mechanistic role remain unclear.



Scheme 3. Two proposed reaction pathways for the avibactam inhibition mechanism with OXA-48. *Left: Pathway 1, based on a proposed "universal" avibactam reaction scheme for SBLs.[43] Neutral Lys73 is suggested to act as a general base in acylation and recyclization, whilst Ser118 (de)protonates the ring nitrogen. Right: Pathway 2, where carboxylated Lys73 is proposed to act as the general base in acylation, and as the general acid in recyclization.[79] Ser118 has the same role as in pathway 1, except it donates a proton to Lys208 instead of Lys73 during recyclization.*

To study the possible emergence of resistance to avibactam, OXA-48 producers were passaged against a combination of ceftazidime and avibactam.[63] Resistance was observed to develop as a result of two amino acid substitutions: Pro68Ala (as discussed above in the section "Cephalosporinase activity"), and Tyr211Ser. The catalytic efficiency of ceftazidime turnover increased >10-fold and >20-fold for the single and double substituted variants, respectively. Inhibitory activity of avibactam stayed on the same level as for OXA-48 for the Pro68Ala variant, but for Pro68Ala/Tyr211Ser the activity of avibactam decreased >5-fold. Tyr211 is known to be a key residue in stabilizing tetrahedral intermediates in β-lactam hydrolysis through the formation of an oxyanion hole (together with the backbone amide of Ser70).

Additionally, Tyr211 was suggested to possibly aid in the formation of a Michaelis complex. Notably, however, the observed evolutionary trajectory towards ceftazidime/avibactam resistance comes at a fitness cost, as the enzyme thermostability is reduced and the primary hydrolysis phenotype (carbapenemase/penicillinase) is compromised.[63]

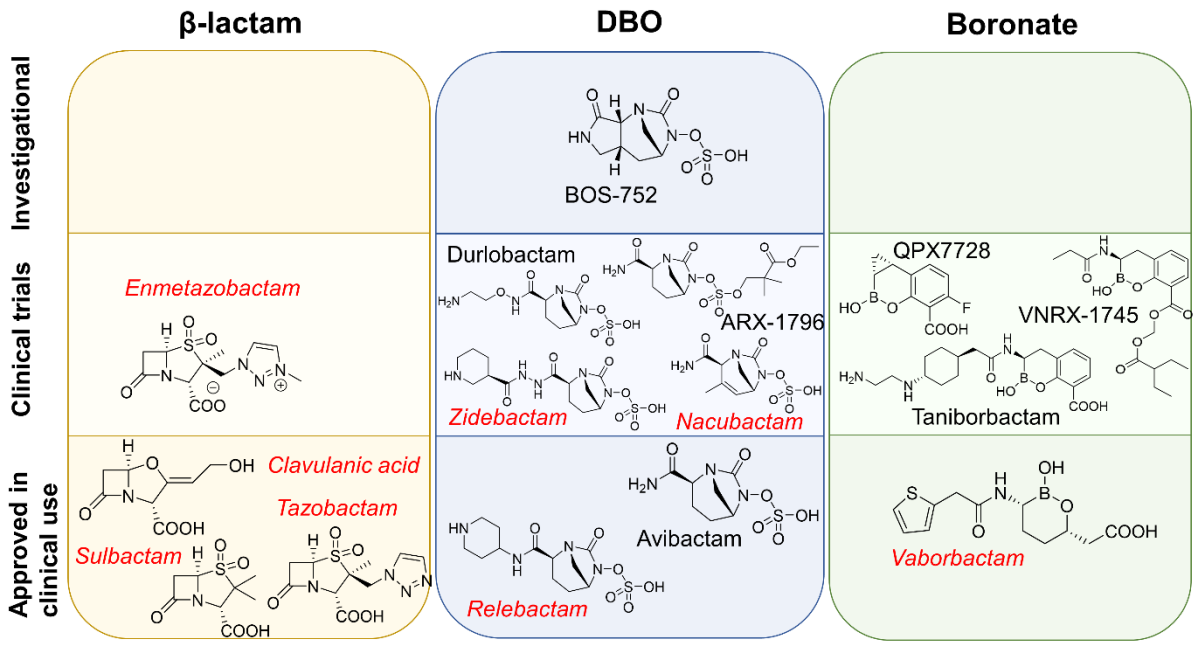


Figure 9. Examples of β-lactamase Inhibitors in Different Inhibitor Classes*: β-lactam ring-based inhibitors, DBO = diazabicyclooctanones, and boronates, each block divided into investigational compounds (top), compounds in clinical trials (middle), and inhibitors approved in clinical use (bottom). Inhibitors in red and cursive do not effectively inhibit OXA-48, inhibitors in black show inhibitory activity.*

Other β-lactamase inhibitors with a DBO scaffold include relebactam, nacubactam, zidebactam, durlobactam (previously ETX2514), ARX-1796 and the investigational compound BOS-752 (Figure 9). The relebactam/imipenem combination has been approved for clinical use, but this inhibitor does not effectively inhibit OXA-48; measured MICs for carbapenems do not change (or change only slightly) in the presence of relebactam.[80-82] Based on MICs,

zidebactam combined with cefepime shows inhibitory activity against OXA-48.[83] This is due to OXA-48 inhibition by cefepime, as *in vitro* kinetics indicate that zidebactam on its own does not inhibit OXA-48.[84] Similarly, nacubactam inhibits class A and C SBLs, but *in vitro* data on its activity against OXA-48 are sparse. In MIC tests, bacterial isolates expressing OXA-48 were susceptible to aztreonam/nacubactam and cefepime/nacubactam, but the potentiation of antibiotic activity by nacubactam was concluded to be mainly due to inhibition of co-expressed ESBLs and AmpC β-lactamases.[85] Durlobactam was originally developed to combat infections involving OXA enzymes in *Acinetobacter baumannii,*[78] and this compound inhibits OXA-48 effectively irreversibly (as well as class A and C SBLs): in MIC tests, durlobactam restored imipenem potency against OXA-48 better than avibactam.[86] Durlobactam is currently in Phase III clinical trials in combination with sulbactam.[87] New β-lactamase inhibitors utilizing the DBO scaffold have been synthesized by substituting the avibactam C2 carboxamide (Figure 3) with new functional groups.[84, 88] The size of the C2 substituent appears to correlate with β-lactamase inhibitory activity: new DBO compounds with larger C2 groups (with respect to avibactam) have approximately an order of magnitude slower on-rates and faster off-rates for OXA-48.[88] However, the studied derivatives with larger C2 substituents inhibit PBPs in bacterial cells. OXA-48 complexes with avibactam derivatives (PDB IDs 5FAQ, 5FAS, and 5FAT)[88] show essentially the same binding pose as observed for avibactam, the main differences being in the respective C2 substituents. Avibactam itself has poor oral bioavailability, but the avibactam prodrug ARX-1796 can be administered orally and subsequently metabolized in the body to produce avibactam.[89] ARX-1796 differs from avibactam through the addition of a neopentyl ester protecting group on the N6 sulfate moiety. Recent data show DBO inhibitory activity towards OXA-48 to be dependent upon the N6 substituent, as replacing the durlobactam N6 sulfate with fluoroacetate reduces potency but can form the basis for an orally available therapy.[90] Another investigational β-lactamase inhibitor in the DBO group is BOS-752, which has a third ring fused to the DBO scaffold (making it a

dioxotriazatricyclohendecane).[91] BOS-752 does not possess antibacterial activity on its own, but combined with piperacillin it lowered measured MICs against SBLs including OXA-48.[91]

In addition to DBOs, other β-lactamase inhibitors currently in clinical development include mechanism-based β-lactam inhibitors and boronic acid compounds (Figure 9). An example of a β-lactam inhibitor is enmetazobactam, which is a penicillanic acid sulfone currently developed in combination with cefepime.[92, 93] This combination was found to be effective against OXA-48 producers, but the efficacy is most likely again attributed to the activity of cefepime and not to efficient inhibition by enmetazobactam, which is active primarily against ESBLs.[93, 94] On the other hand, boronates show promise as broad-spectrum β-lactamase inhibitors. In particular, cyclic boronates can act as analogues of the tetrahedral acylation transition state of SBLs,[95] and have potential for at least moderate activity against MBLs.[96, 97] The first boronic acid inhibitor approved in clinical use was vaborbactam (originally RPX7009),[98] which is currently administered in combination with meropenem.[99, 100] Vaborbactam is a monocyclic boronic acid compound showing inhibition mainly against class A and C SBLs, and it is not able to effectively inhibit OXA-48 based on both biochemical data and MIC measurements (potency of meropenem not restored).[80, 101] Further development of boronic acid derivatives as β-lactamase inhibitors includes taniborbactam (VNRX-5133), which is a bicyclic boronate.[102] Based on both *in vitro* and whole cell assay data, taniborbactam exhibits pan-β-lactamase inhibition (i.e. is able to inhibit all four Ambler classes) including moderate inhibition of OXA-48 (with an IC_{50} value of approximately 0.54 μM).[103] Taniborbactam is currently in clinical development in combination with cefepime.[104] Another potent bicyclic boronate with ultrabroad-spectrum β-lactamase inhibition is the compound QPX7728, which can efficiently inhibit carbapenem-resistant *Enterobacterales* and restore the potency of meropenem against OXA-48.[105, 106] QPX7728 entered Phase I clinical trials in December 2020.[107, 108] VNRX-7145, which is orally bioavailable, also demonstrates OXA-48

inhibition and has entered Phase I clinical trials in 2020 combined with ceftibuten.[109-111] In addition to boronates, other cyclic compounds mimicking the tetrahedral intermediate (such as phosphonates, sulfonates, and sulfonamides), may also provide a source of future inhibitors, but these are yet to be explored in detail.[95] Growing appreciation of the clinical importance of OXA-48 has also motivated exploration of other routes to inhibitors, such as the use of DNA-encoded libraries, but these too remain at an early stage.[112]

Conclusions

Carbapenem-hydrolyzing *Enterobacterales* are classified as an urgent global threat to modern medicine, while OXA-48 β-lactamases are endemic in some regions (especially Turkey and the Mediterranean) and continue to disseminate. In general, OXA-48 enzymes convey penicillin and low-level carbapenem resistance; their weak carbapenem hydrolysis often complicates diagnosis and subsequent treatment of infections involving OXA-48 producers. Most variants within the OXA-48 family are imipenemases with slow turnover rates for other carbapenems, and resist established mechanism-based β-lactam inhibitors. However, certain variants (such as OXA-163 and OXA-405) have acquired a more ESBL-like hydrolysis profile with activity against expanded spectrum oxyimino-cephalosporins (such as ceftazidime) and significantly decreased imipenemase activity. The extent to which further evolution of the OXA-48 scaffold towards genuinely broad-spectrum activity is possible remains to be established.

Recent crystallographic efforts have yielded structures of acylenzyme complexes of OXA-48 not only with clinically relevant carbapenem and cephalosporin substrates, but also with new generation DBO inhibitors (avibactam). These supply much new information

regarding the interactions of substrates and inhibitors with the OXA-48 active site, including the importance of active site structure (specifically the W-loop), hydration and, with respect to carbapenems, rearrangements such as tautomerization and lactone formation that occur after β-lactam cleavage. The origin of preferential activity towards imipenem over other carbapenems, however, remains to be verified. Importantly, structural data for other OXA-48-like enzymes has started to emerge too, which is important to increase understanding of how substitutions affect specificity across the enzyme group. Combining knowledge from biochemical characterization, X-ray crystallography as well as atomistic computational modelling will likely lead to a detailed picture of the origin of activity and specificity in OXA-48 enzymes, ultimately benefitting design of inhibitors effective against this widespread and variable β-lactamase family.

Supporting Information

Table of published crystal structures & Excel spreadsheet with published kinetics values.

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Abbreviations

PBP, penicillin-binding protein; SBL, serine β-lactamase; MBL, metallo-β-lactamase; ESBL, extended-spectrum β-lactamase; DBO, diazabicyclooctanone

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