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Antibacterial Properties of Metallocenyl-7-ADCA Derivatives and Structure in Complex with CTX-M β -Lactamase

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Abstract

A series of six novel metallocenyl-7-ADCA (metallocenyl = ferrocenyl or ruthenocenyl; 7-ADCA = 7-aminodesacetoxycephalosporanic acid) conjugates were synthesized and their antibacterial properties evaluated by biochemical and microbiological assays. The ruthenocene derivatives showed a higher level of inhibition of DD-carboxypeptidase 64-575, a Penicillin Binding Protein (PBP), than the ferrocene derivatives and the reference compound penicillin G. Protein X-ray crystallographic analysis revealed a covalent acyl-enzyme complex of a ruthenocenyl compound with CTX-M β -lactamase E166A mutant, corresponding to a similar complex with PBPs responsible for the bactericidal activities of these compounds. Most interestingly, an intact compound was captured at the crystal-packing interface, elucidating for the first time the structure of a metallocenyl β -lactam compound that previously eluded small molecule crystallography. We propose that protein crystals, even from biologically unrelated molecules, can be utilized to determine structures of small molecules.

Graphical abstract

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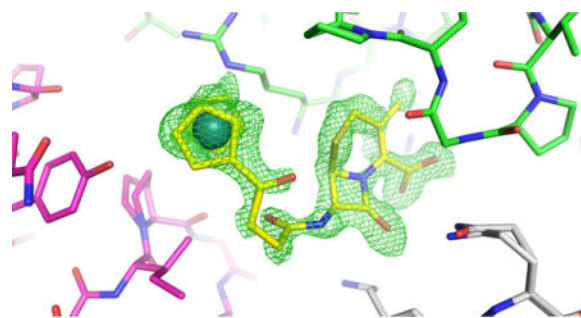
#E.L. and Ł.S. contributed to this work equally.

Supporting Information

Compound synthesis, conformational analysis, computational data, microbiological data, computed Cartesian coordinates of all of the molecules reported in this study, and x-ray crystallographic statistics.

Notes

The coordinates and structure factors have been deposited in the Protein Data Bank (PDB), www.rcsb.org, with the accession code PDB ID: 5UJO. The authors declare no competing financial interest.



First used clinically in the 1940's, the β -lactam antibiotics treat bacterial infections by inhibiting Penicillin Binding Proteins (PBPs), which are enzymes that catalyze the final steps of bacterial cell wall biosynthesis.^{1, 2} The β -lactams inhibit the PBPs by forming a covalently linked acyl-enzyme adduct with a catalytic serine in the active site.³⁻⁵ One of the main bacterial resistance mechanisms against β -lactam antibiotics, particularly in Gram-negative bacteria, is the production of β -lactamases.⁶⁻¹¹ Among the four classes of β -lactamases, Classes A, C, and D all share a common active site serine residue, like the PBPs, whereas Class B are Zn^{2+} -based metallo enzymes.^{12, 13} It is hypothesized that serine β -lactamases originated from PBPs by evolving the ability to hydrolyze the covalent acyl-enzyme linkage.⁹

Organometallic compounds have been explored within medicinal chemistry mainly as anticancer¹⁴⁻¹⁷ and antimalarial agents.¹⁸ However, they have recently attracted increased attention for their antibacterial properties.¹⁹⁻²² Antibacterial mechanisms of organometallic compounds are diversified, and in some cases they overcome antibiotic resistance in pathogenic bacteria.^{20, 21} The idea of metallocene to β -lactam antibiotic conjugation is not new and fits into the more general concept of organometallic drug derivatization often used in bioorganometallic chemistry.¹⁵ The first ferrocenyl (Fc) derivatives of 6-aminopenicillanic acid (6-APA) and 7-aminocephalosporanic acid (7-ACA) were reported in 1970s.²³ Recently, novel ferrocenyl-penems with antibacterial activity against Gram-positive and Gram-negative bacteria have been reported, further substantiating the importance of metallocenyl conjugates of β -lactams as antibacterial agents.²⁴

In continuing our program studying the biologically active organometallic compounds,²⁵ we became interested in metallocenyl derivatives of 6-APA.^{26, 27} Our preceding results show noticeable activity of ferrocenyl and ruthenocenyl (Rc) 6-APA conjugates against Gram-positive bacteria, including clinically isolated *S. aureus* strains.^{26, 27} We have also determined a 1.18 Å resolution X-ray crystal structure of a hydrolyzed Rc-6-APA derivative in a complex with CTX-M-14 E166A β -lactamase.²⁷ However, in all previous studies including our own, no experimental structure has been determined for any of the intact metallocenyl β -lactam compounds, mainly due to the difficulty in obtaining suitable small molecule crystals.

Here we report the synthesis, biological, and structural properties of ferrocenyl and ruthenocenyl conjugates of 7-aminodesacetoksycephalosporanic acid (7-ADCA; **1**) (Scheme 1). The desired metallocenyl- β -lactams have been obtained by an active ester

methodology.²⁶ Compounds were characterized by ¹H NMR, ¹³C NMR, FTIR, MS and elemental analysis, confirming the proposed structures. ¹H-NMR spectra of **2–7** are shown in the SI.

Inhibitory activity of compounds **2–7** was evaluated against several bacterial enzymes: a D-alanyl-D-alanine carboxypeptidase/transpeptidase from *Saccharopolyspora erythraea* 64-575 (DD-carboxypeptidase 64-575), CTX-M-14 Class A β-lactamase,^{28–31} and *Bacillus cereus* 569/H9 β-lactamase (Table 1).^{32–34} DD-carboxypeptidase 64-575 is an exocellular serine peptidase. Similar to high molecular mass (HMM) PBPs, DD-carboxypeptidase 64-575 shows affinity to a plethora of common β-lactam antibiotics, including cephalosporin C, cefamandole, and cefotaxime. These antibiotics cause 50% inhibition of the DD-carboxypeptidase enzyme in the low concentration range from 5.8×10⁻⁹ to 7.5×10⁻⁶ M.³⁵ Therefore, DD-carboxypeptidase 64-575 can be utilized as a model enzyme in the study of β-lactam-PBP interactions and inhibition. CTX-M-14 β-lactamase belongs to the class A β-lactamase family and its active site shares many key catalytic features with that of the PBP's.^{9, 36, 37} Moreover, high-resolution X-ray crystal structures of CTX-M β-lactamases in their apo, as well as substrate-bound, states were solved, providing detailed insights into the mode of action of these enzymes.^{27, 30, 31, 38, 39} The third enzyme of interest, a *B. cereus* 569/H9 β-lactamase, belongs to the class B metallo-β-lactamases (MBLs).⁴⁰ In our biochemical assay, compounds **2–7** were treated as 'competitive inhibitors' to compare their interactions with the enzyme active site, although they function as covalent ligands for the DD-carboxypeptidase and substrates for β-lactamases. Compared with the 7-ADCA starting scaffold and the benzyl side chain of penicillin G, the ferrocenyl and ruthenocenyl moieties have enhanced the interactions with the protein and consequently improved the inhibitory activity as demonstrated by the IC₅₀ values (Table 1). In particular, for the DD-carboxypeptidase, the ruthenocenyl group appears to enhance binding more than the ferrocenyl group.

The antibacterial activity of compounds **2–7** was tested against a panel of Gram-positive strains, namely: methicillin-sensitive *Staphylococcus aureus* (MSSA), methicillin-resistant *S. aureus* (MRSA), vancomycin-intermediate *S. aureus* (VISA), and *S. epidermidis* (Table S3). In contrast to the biochemical results, the compounds displayed overall less activity in the cell-based assays compared with the reference compounds penicillin G and ampicillin. This suggests the metallocenyl moieties may have affected the compounds' ability to reach the PBPs or may have varied effects on PBPs from different bacterial species.

To investigate the molecular interactions between these compounds and the target protein, we used CTX-M-14 E166A mutant to prepare complex crystals with compound **3**. Class A β-lactamases such as CTX-M are hypothesized to have evolved from PBPs, acquiring Glu166 to activate a water molecule that breaks the acyl-enzyme linkage with the β-lactam compound.^{9, 36, 37} CTX-M-14 E166A mutant behaves like a PBP, forming a stable acyl-enzyme intermediate with β-lactam antibiotics. This is indeed what we observed in a 1.35 Å resolution crystal structure of CTX-M-14 E166A complexed with **3** (Fig. 1). The electron density maps clearly identified the binding pose of the compound in the protein active site (Fig. 1A), as well as a covalent bond between the β-lactam ring carbon and Ser700γ (Fig. 1B). The β-lactam ring carbonyl oxygen is nestled in the oxyanion hole formed by the

backbone –NH groups of Ser70 and Ser237. The structure also illustrates other favorable interactions between the compound and the protein, including hydrogen bonds (HBs) between the C4 carboxylate group and Thr235/Ser130, and between the ligand amide group and Asn104/Asn132/Ser237. Interestingly, the ruthenocetyl group appears to display two conformations: conformation 1 with the base ring of the cyclopentadienyl group stacked against the protein backbone of Gly238-Asp240 (note: there is a gap in the residue numbering due to class A β -lactamase convention); conformation 2 has the base ring rotated away from the β -strand and the distal ring establishing more contact with Thr171. Together with our previous structure, these observations demonstrate the versatility of metallocenyl groups in interacting with protein.

The CTX-M crystal also allowed us to capture an intact compound **3** at the crystal-packing interface. This is particularly worth noting because none of the metallocenyl β -lactam antibiotics have been structurally characterized by small molecule X-ray crystallography due to the challenges in obtaining suitable single crystals. We initially resorted to computational modeling using the B3LYP hybrid functional as described in the SI. The conformation of the compound is identified unambiguously in the unbiased $F_o - F_c$ electron density map (Fig. 2A). The intact compound resides outside the active site and establishes multiple interactions with three monomers at the crystal-packing interface (Fig. 2B). The six-membered ring is sandwiched between Arg65 and Gly175 of one monomer (monomer 1), forming extensive non-polar and stacking interactions with these residues. The C4-carboxylate group forms a HB with the Gly175 backbone, while establishing favorable electrostatic interactions with nearby Arg43 and Lys88 from two different monomers (monomers 1 and 2 respectively). The β -lactam ring contacts both monomer 1 and 2, forming a HB with Gln89. The ruthenocetyl moiety and the linker also interact with two monomers (1 and 3), including non-polar interactions with Pro177 (monomer 1) and Ile173/Pro174 from monomer 3, as well as stacking interactions with the Arg65 side chain. There is also close proximity (4.5 Å) between the ruthenium atom and the hydroxyl group of Tyr241, suggesting a possible HB. This contact highlights a unique feature of Ru in interacting with –OH,^{41, 42} particularly in the context of protein-ligand interactions.²¹

The observation of an intact compound **3** at the crystal-packing interface, unrelated to CTX-M's biological activity, highlights the value of protein X-ray crystallography in determining novel structures for small molecules. Although this is certainly not an entirely new concept because protein-small molecule complex structures are determined routinely, including those in the intended binding pockets, as well as some accidental discoveries at the crystal packing interface. But in most cases, the structure of the ligand has already been characterized by small molecule crystallography, or the ligand is bound by a biologically related macromolecule. However, based on previous studies, we propose that protein crystals can be utilized as 'crystalline sponges' in a fashion similar to those formed by certain framework metallo compounds as previously described.⁴³ First, protein crystals provide an anisotropic binding environment allowing the capture of specific and ordered ligand conformations. Second, although the vast majority of protein crystals are filled with water, it has been shown that crosslinking can stabilize protein crystals in organic solvent in multiple-solvent crystal structure (MSCS) experiments, and such crosslinking does not block the access of

small molecules to the protein surface.^{44–47} Lastly, there is a wide range of protein crystals described in the Protein Data Bank, including many capable of diffracting to sub-Angstrom resolution, like those of CTX-M. These crystals can provide a diverse set of interfaces to capture different small molecules and the high diffracting quality of some of these crystals can permit accurate structure determination even for ligands with low occupancies. It is therefore conceivable that a series of high quality and diverse protein crystals can be analyzed and prepared to screen and capture small molecule ligands for structure determination, independent of the biological relationship between the proteins and the small molecules.

In conclusion, our synthesis and characterization of metallocenyl-7-ADCA conjugates has demonstrated how metallocenyl groups can be utilized to enhance the interactions between β -lactam compounds and the target proteins. These results provide valuable information for designing future metallocenyl compounds with biological activities, and offer insights into how protein crystals can facilitate the structural analysis of novel small molecules.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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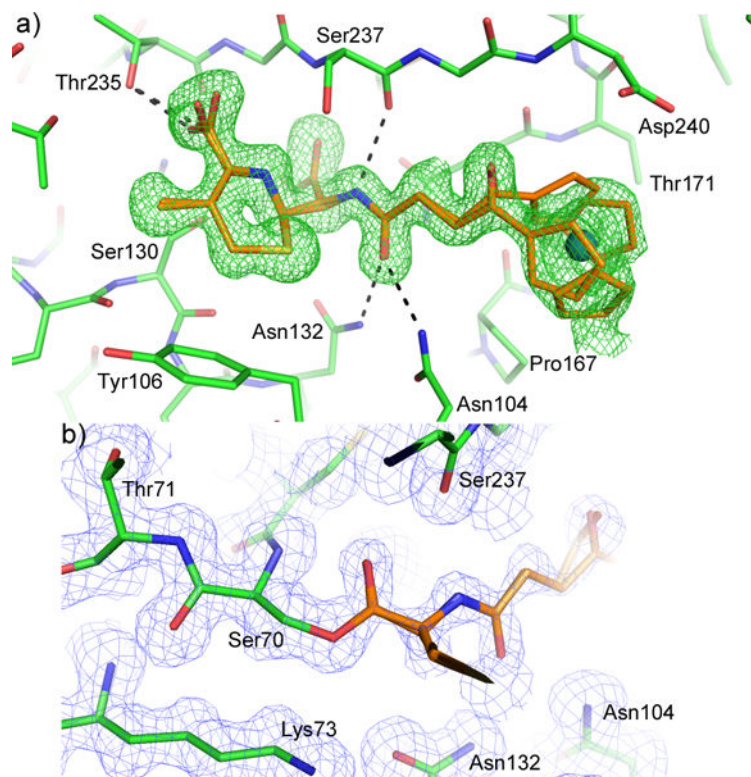


Figure 1.

Acyl-enzyme complex of CTX-M-14 E166A mutant with compound **3**. The protein and compound are shown in green and orange respectively. (A) The unbiased F_o-F_c density map is shown in green at 2σ . (B) $2F_o-F_c$ map (0.5σ , blue) shows the covalent acyl-enzyme linkage between Ser70 and compound **3**.

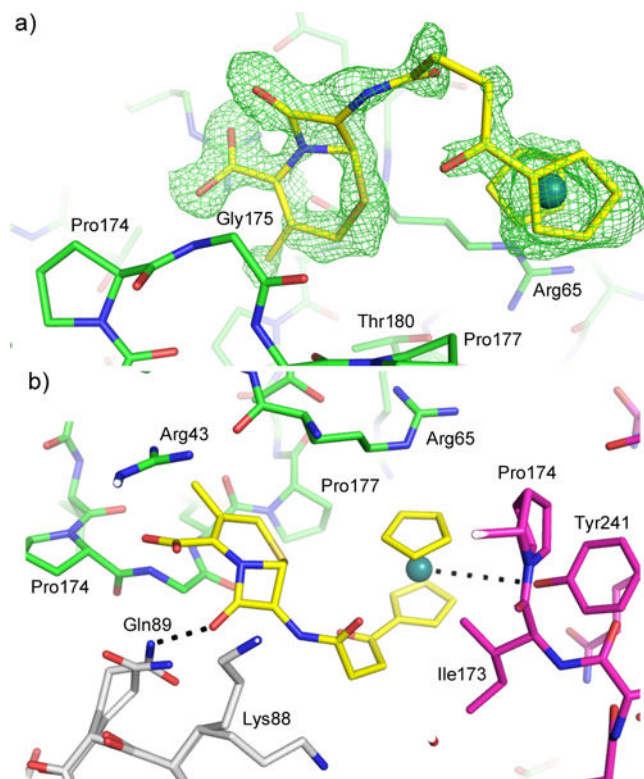
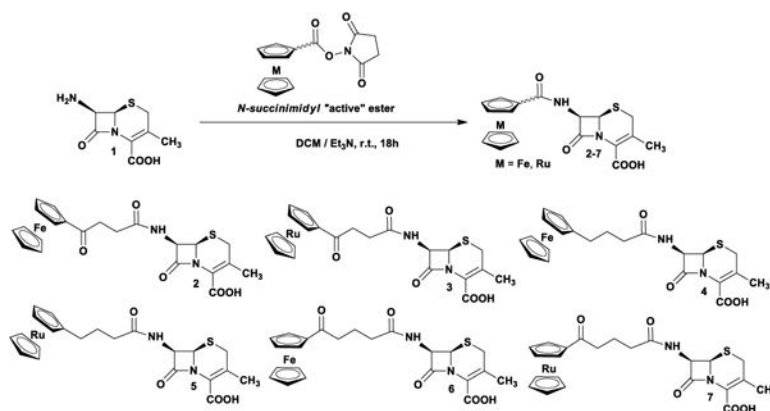


Figure 2. Intact compound **3** (yellow) observed at the crystal-packing interface. (A) The unbiased $F_0 - F_c$ density map is shown in green at 3σ . (B) Interactions between compound **3** and three protein monomers. Monomers 1, 2, and 3 are colored in green, white, and magenta respectively. Potential hydrogen bonds are shown as black dashed lines.



Scheme 1.
Synthesis of metallo-7-ADCA compounds 2-7.

Table 1

Inhibition of compounds 2–7 on PBP and β -lactamases.

Enzyme	IC ₅₀ μ M							penicillin G
	1	2	3	4	5	6	7	
DD-carboxypeptidase 64-575	176 \pm 2.38	0.158 \pm 0.002	0.052 \pm 0.001	0.133 \pm 0.002	0.027 \pm 0.000	0.083 \pm 0.003	0.081 \pm 0.003 \pm	0.153 \pm 0.002
CTX-M-14	1620 \pm 144	31 \pm 5	44 \pm 4	716 \pm 108	7 \pm 1	36 \pm 4	363 \pm 49	nd*
<i>B. cereus</i> 569/H9	4661 \pm 422	142 \pm 28	200 \pm 25	82 \pm 12	117 \pm 10 \pm	126 \pm 27	65 \pm 5	nd

* nd, not determined.