Supporting Information

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

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Contents

1.1 Conformational Analysis of Compounds 2, 3, and 5

Any attempts to obtain X-ray quality crystals of **2**-**7** failed. Thus, we decided to investigate the structures of selected products with a computational approach. To obtain more insight into the possible conformational space of metallocenyl-β-lactams **2**, **3**, and **5**, a modeling study was performed using the B3LYP hybrid functional. DFT calculations on **2** and **3,** with varying starting geometries, resulted in an ensemble of 10 different conformations in each case, which span a range of ca. 50 kJ/mol. The two low-energy conformers **2A** and **2B** have been located on the energy landscape of **2** (Fig. S1), with the former being predominant (x = 90%).

The most stable conformer displays an intramolecular hydrogen bond between CO_{Fc} and amidic hydrogen, while the interaction between lactam CO and CH_{Fc} additionally stabilizes its structure. In the conformer **2B,** the lactam moiety resides in an exo-position relative to ferrocene, and therefore only one hydrogen bond is possible. In the case of ruthenocenyl-β-lactam **3**, exactly the same structural motifs have been established in the two lowest-energy conformers **3A** and **3B**, with the latter being 8.3 kJ/mol less stable (Table S1).

In contrast to **2** and **3**, the ruthenocenyl-β-lactam **5** is much more flexible and can adopt a number of different conformations. More than 20 conformer minima have been located for **5** in the energy range < 40 kJ/mol (Table S1). The five lowest-energy conformers are within 5 kJ/mol of each other (Table S1 and Fig. S2).

The particular conformations of **5A**, **5B**, and **5C** are well suited for a possible stabilization by CH_{Fc}…OC intramolecular interactions. Both amidic and lactam carbonyl groups are involved in this bonding, which is, however, absent in conformers **5D** and **5E**. The latters are therefore less stable structures. The conformer **5D** has a fully extended geometry, while the conformer **5E** is characterized by the exo-position of lactam moiety in relation to ruthenocene part. A variety of structural features reveal that the ruthenocenyl-β-lactam **5** possesses a less rigid geometry as compared to **2** and **3**. To conclude, the structural properties of **2** and **3** are governed by intramolecular hydrogen bonds resulting in only one representative conformer of type **A** in either case. In the case of **5**, an array of relevant conformations have been detected, in which only weak (all interaction distances are > 2.5 Å) or no hydrogen bonds exist.

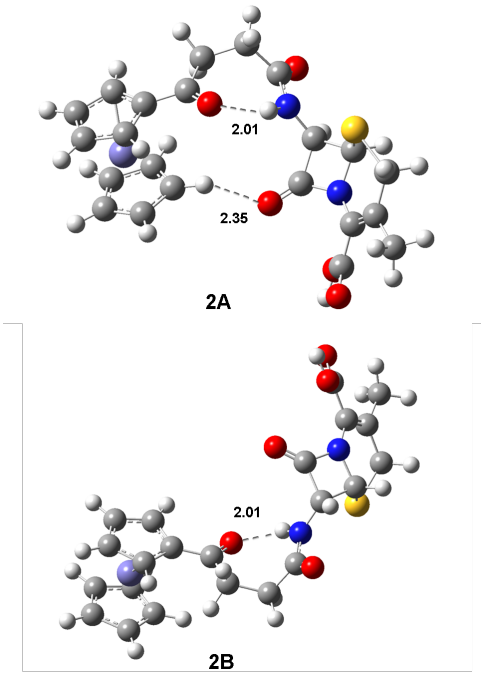

Figure S1. The two lowest-energy conformers, **A** and **B**, of ferrocenyl-β-lactam **2** calculated using the B3LYP hybrid functional. The very similar geometries have been optimized for ruthenocenyl-β-lactam analogs **3A** and **3B** (the central metal Fe replaced by Ru). Distances are in Å.

Table S1. Relative Gibbs free energies (ΔG_{298}), calculated at the B3LYP level, for conformers of metallocenyl-β-lactams **2**, **3**, and **5**.

compound	confor-mer	ΔG_{298} (kJ/mol)	conformer population (x, %)[a]
2	**2A**	0.0	90.0
	2B	5.5	9.9
3	**3A**	0.0	96.6
	3B	8.3	3.3
5	**5A**	0.0	42.8
	5B	1.2	26.8
	5C	2.9	13.5
	5D	3.3	11.5
	5E	5.2	5.2

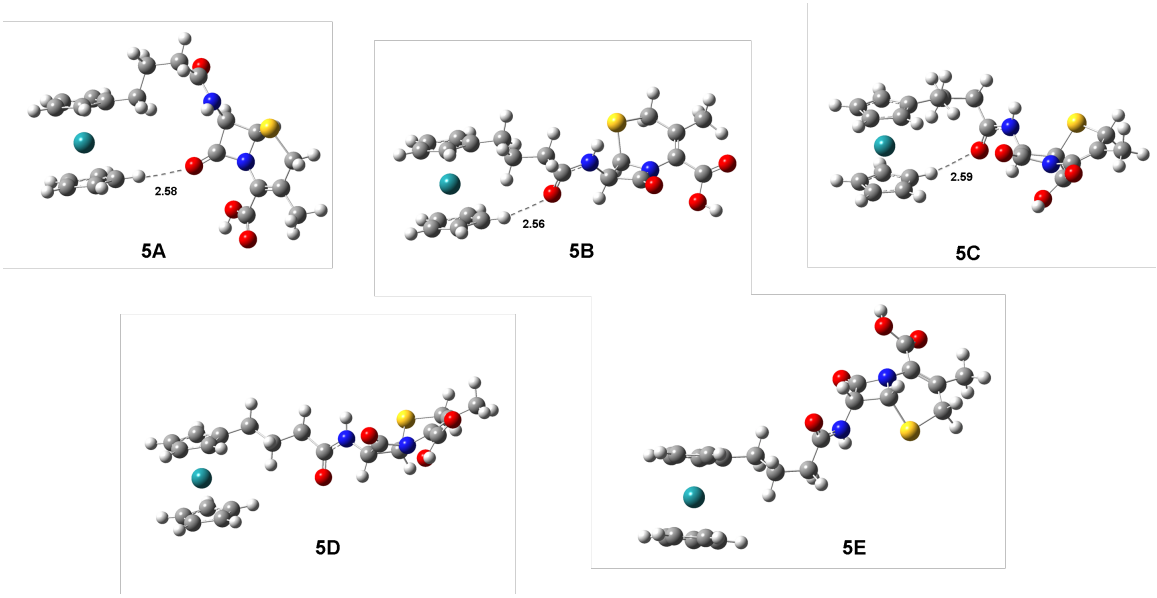

Figure S2. The five lowest-energy conformers of ruthenocenyl-β-lactam **5** calculated using the B3LYP hybrid functional. Distances are in Å.

Table S2. X-ray Data Collection and Refinement Statistics

Data Collection	
Space Group	$P2_1$
Cell Dimensions	
a, *b*, *c* (Å)	44.961, 107.351, 47.956
α, β, γ (°)	90.00, 101.36, 90.00
Resolution (Å)	50.00 - 1.35
No. Reflections	96803 (4747)
R_{merge} (%)	3.7 (23.8)
I / ó*I*	27.07 (5.875)
Completeness (%)	99.3 (97.1)
Redundancy	3.7
Refinement	
resolution (Å)	53.68-1.35
R_{work}/R_{free} (%)	12.5/15.0
no. heavy atoms	
protein/ligand/water	4340/169/521
B-factors (Å^2)	
protein/ligand/water	14.67/11.48/29.58
rms deviations	
bond lengths (Å)	0.003
bond angles (°)	0.813
ramanchandran plot	
most favored region(%)	91.3
additionally allowed (%)	7.8
generously allowed (%)	0.9

* Values in parentheses represent highest resolution shells

Table S3. Antibacterial Activity of Complexes 2-7, Ampicillin, and Penicillin G

Microorganism	MIC (µg/mL)								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**Ampicillin**	**Penicillin G**
S. aureus ATCC® 29213 (MSSA)	>256	128	16	8	16	128	16	0.5	0.25
S. aureus ATCC® 43300 (MRSA)	>256	>256	>256	256	>256	>256	>256	10	8
S. aureus ATCC® 700787™ (VISA)	>256	>256	>256	>256	128	>256	>256	20	>8
S. epidermidis ATCC® 12228™	>256	64	8	8	4	128	4	5	0.5

2.1 General Comments for Synthesis

All reactions were carried out under an atmosphere of argon using standard Schlenk techniques. Chromatographic separations were carried out using silica gel 60 (Merck, 230 - 400 mesh ASTM). Dichloromethane was purified by distillation from CaH_2 prior to use. N-Hydroxysuccinimide and N,N'-diisopropylcarbodiimide were purchased from commercial suppliers and were used without further purification. 4-Oxo-4-(ferrocenyl) butanoic acid,[1] 4-oxo-4-(ruthenocenyl) butanoic acid,[2] 5-oxo-5-(ruthenocenyl) pentanoic acid,[2] 4-(ferrocenyl) butanoic acid,[3,4] 5-(oxo)-5-(ferrocenyl) pentanoic acid,[5] and 4-(ruthenocenyl) butanoic acid,[6] are known compounds and were prepared according to the literature.[1-5] Corresponding N-succinimidyl esters were obtained according to the literature[6,8] and used for the reaction directly after flash chromatography purification.

^{1}H NMR (600 MHz) and $^{13}C\{H\}$ NMR (150 MHz) spectra were recorded with a Bruker Avance III 600 spectrometer operating at 298 K in the Fourier transform mode. Chemical shifts are reported in δ units (ppm) using as residual DMSO-d_6 (^{1}H δ 2.50 ppm, ^{13}C δ 39.70 ppm) as the reference. Infrared spectra were recorded with a FTIR Nexus Nicolet apparatus. Mass spectra were recorded with a Varian 500-MS iT, mass spectrometer (ESI) or with a Voyager Elite mass spectrometer (MALDI). Microanalyses were determined by Analytical Services of the Polish Academy of the Sciences, Łódź. UV-Vis absorption spectra were recorded with a Spectrostar Omega (BMG Labtech) spectrometer. Purification of compounds **2-7** was achieved by normal phase HPLC (Shimadzu Prominence with LC-20AP pumps) with Luna 5u Silica (2) 100A, AXIA Packed 150 X 21.1 mm preparative column.

3.1 Procedure for Synthesis of Compounds 2-7

A mixture of appropriate metallocene *N*-succinimidyl ester (1.3 equiv.) and 7-ADCA **1** in dichloromethane-triethylamine (ratio 40/1.5 (v/v)) was vigorously stirred for 18 hrs at room temperature. Afterwards, the reaction mixture was evaporated to dryness and the obtained solid was subjected to column chromatography on SiO_2 (chloroform- methanol, ratio 50/2 (v/v)). Obtained product was dissolved in chloroform and washed with aqueous HCl (3%). The chloroform layer was separated, dried, and evaporated to dryness. Obtained solid was subjected toward preparative HPLC purification on Luna 5u Silica (2) 100A, AXIA Packed 150 X 21.1 mm preparative column. The HPLC conditions for compounds **2-5** and **7** were a mixture of dichloromethane (98%) and methanol (2%); low-pressure gradient with total flow pump 10 ml/min. The conditions for compound **6** were a mixture of dichloromethane (99%) and methanol (1%); low pressure gradient with total flow pump 10 ml/min. Finally, crystallization from chloroform-*n*-hexane gave analytically pure samples.

Purification of compounds **2-7** was achieved by normal phase HPLC with a Luna 5u Silica (2) 100A, AXIA Packed 150 X 21.1 mm preparative column. The conditions for compounds **2, 3, 4, 5,** and **7** were a mixture of dichloromethane (98%) and methanol (2%); low pressure gradient with total flow pump 10 ml/min. The conditions for compound **6** were a mixture of dichloromethane (99%) and methanol (1%); low pressure gradient with total flow pump 10 ml/min. HPLC retention time for **2:** 16.26 min. HPLC retention time for **3:** 13.14 min. HPLC retention time for **4:** 18.88 min. HPLC retention time for **5:** 8.55 min. HPLC retention time for **6:** 9.92 min. HPLC retention time for **7:** 9.62 min.

3.2 Compound 2

Orange solid, HPLC retention time 16.26 min., 15% yield (139 mg).

^{1}H NMR (600 MHz, DMSO-d_6): δ =13.16 (bs, 1H, COOH), 8.82 (d, $J_{H,H}$ = 8.3 Hz, 1H, N-H), 5.61 (dd, $J_{H,H}$ = 8.3 Hz, $J_{H,H}$ = 4.6 Hz, 1H, C-H), 5.03 (d, $J_{H,H}$ = 4.6 Hz, 1H, C-H), 4.80 (m, 2H, Fc), 4.55 (pt, $J_{H,H}$ = 1.8 Hz, 2H, Fc), 4.27 (s, 5H, Fc), 3.56 (d, $J_{H,H}$ = 18.0 Hz, 1H, CH_2), 3.36 (d, $J_{H,H}$ = 18.0 Hz, 1H, CH_2), 3.01 (m, 2H, CH_2), 2.56 (m, 2H, CH_2), 2.02 (s, 3H, CH_3). ^{13}C NMR (150 MHz, DMSO-d_6): δ = 201.8, 172.5, 164.7, 163.6, 129.8, 123.0, 78.8, 72.01, 69.7, 69.1, 69.0, 58.9, 57.3, 55.0, 34.0, 29.1, 28.6, 26.4, 19.5. ESI-MS: m/z = 482 (M^+). FTIR (KBr): 3432 (OH), 3096 (CH), 2924 (CH), 2854 (CH), 1778 (C=O), 1720 (C=O), 1662 (C=O) , 1535 cm^{-1}. Anal. Calcd. for $C_{22}H_{22}N_2O_5SFe$: C, 54.78 ; H, 4.60. Found: C, 54.83 ; H, 4.68.

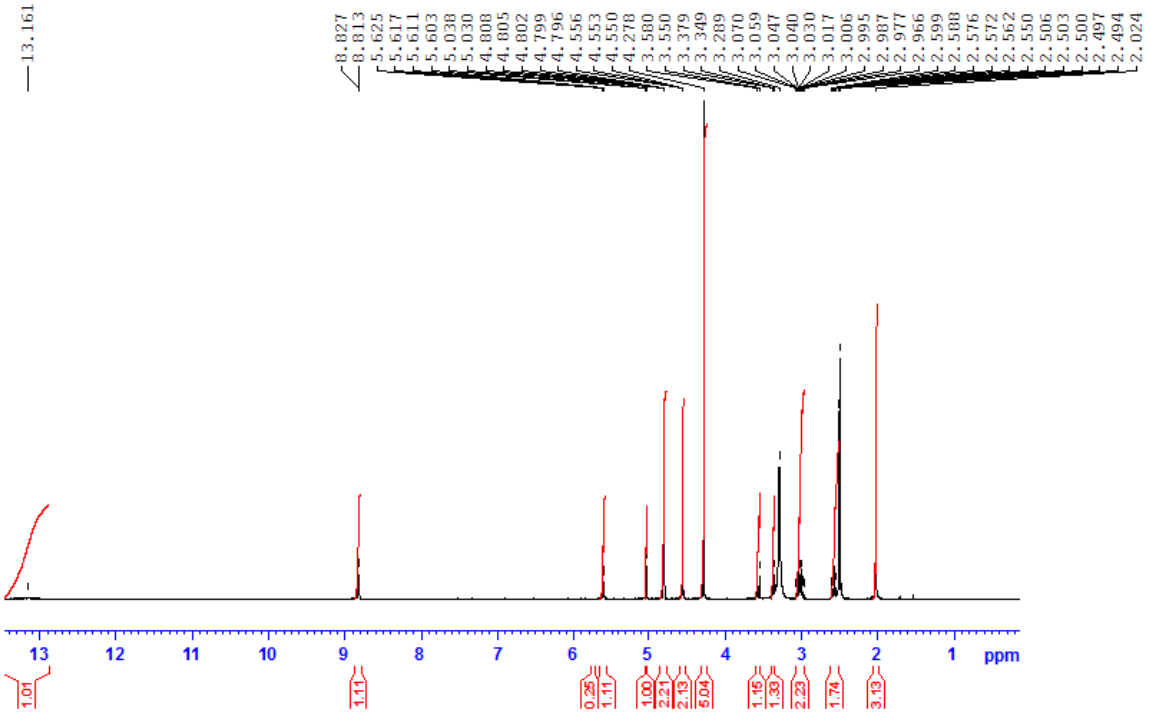

Figure S3. ^{1}H-NMR of Compound 2.

3.3 Compound 3

Yellow solid, HPLC retention time 13.14 min., 16% yield (160 mg).

^{1}H NMR (600 MHz, DMSO-d_6): δ =13.16 (bs, 1H, COOH), 8.77 (d, $J_{H,H}$ = 8.3 Hz, 1H, N-H), 5.586 (dd, $J_{H,H}$ = 8.3 Hz, $J_{H,H}$ = 4.6 Hz, 1H, C-H), 5.11(m, 2H, Rc), 5.02 (d, $J_{H,H}$ = 4.6 Hz, 1H, C-H), 4.82 (pt, $J_{H,H}$ = 1.6 Hz, 2H, Rc), 4.65 (s, 5H, Rc), 3.55 (d, $J_{H,H}$ = 18.7 Hz, 1H, CH_2), 3.35 (d, $J_{H,H}$ = 18.7 Hz, 1H, CH_2), 2.88 (m,2H, CH_2), 2.42 (m, 2H, CH_2), 2.02 (s, 3H, CH_3). ^{13}C NMR (150 MHz, DMSO-d_6): δ = 200.0, 172.3, 164.6, 163.6, 129.8, 123.0, 83.5, 73.5, 72.0, 70.5, 70.5, 58.9, 57.3, 33.3, 29.1, 28.9, 26.4, 19.4. ESI-MS: m/z = 529 (MH^+). FTIR (KBr): 3432 (OH), 3103 (CH), 2924 (CH), 2854 (CH), 1774 (C=O), 1719 (C=O), 1663 (C=O), 1535 cm^{-1}. Anal. Calcd. for $C_{22}H_{22}N_2O_5SRu$ + 2 *n*-hexane : C, 58.35 ; H, 7.20. Found: C, 58.35; H, 7.13.

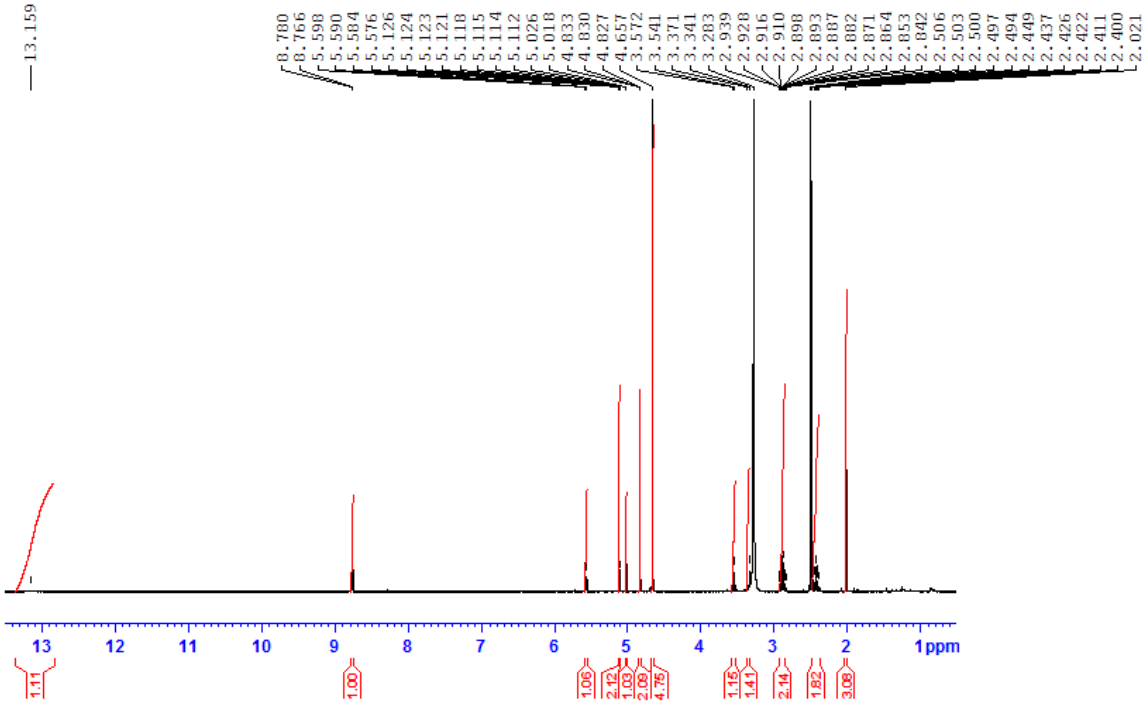

Figure S4. ^{1}H-NMR of Compound 3.

3.4 Compound 4

Orange solid, HPLC retention time 18.88 min., 17% yield (164 mg).

^{1}H NMR (600 MHz, DMSO-d_6): δ =13.13 (bs, 1H, COOH), 8.74 (d, $J_{H,H}$ = 8.4 Hz, 1H, N-H), 5.57 (dd, $J_{H,H}$ = 8.4 Hz, $J_{H,H}$ = 4.8 Hz, 1H, C-H), 5.03 (d, $J_{H,H}$ = 4.8 Hz, 1H, C-H), 4.11 (s, 5H, Fc), 4.099 (dd, $J_{H,H}$ = 3.3 Hz, $J_{H,H}$ = 1.8 Hz, 1H, Fc), 4.082 (dd, $J_{H,H}$ = 3.3 Hz, $J_{H,H}$ = 1.8 Hz, 1H, Fc), 4.03 (pt, $J_{H,H}$ = 1.8 Hz, 2H, Fc), 3.56 (d, $J_{H,H}$ = 18.0 Hz, 1H, CH_2), 3.35 (d, $J_{H,H}$ = 18.0 Hz, 1H, CH_2), 2.25 (m, 4H, CH_2), 2.02 (s, 3H, CH_3), 1.71 (m, 2H, CH_2). ^{13}C NMR (150 MHz, DMSO-d_6): 173.2, 164.6, 163.8, 129.6, 123.4, 88.6, 68.5, 68.0, 67.9, 67.0, 59.1, 57.5, 34.8, 29.2, 28.6, 26.7, 19.6. MALDI-MS: m/z = 468(M). FTIR (KBr): 3427 (OH), 3276 (CH), 3091 (CH), 2929 (CH), 2872 (CH), 1779 (C=O), 1728 (C=O), 1655(C=O), 1624(C=O), 1541 cm^{-1}. Anal. Calcd. for $C_{22}H_{24}N_2O_4SFe$: C,56.42; H, 5.17; Found: C, 56.42 ; H, 5.11.

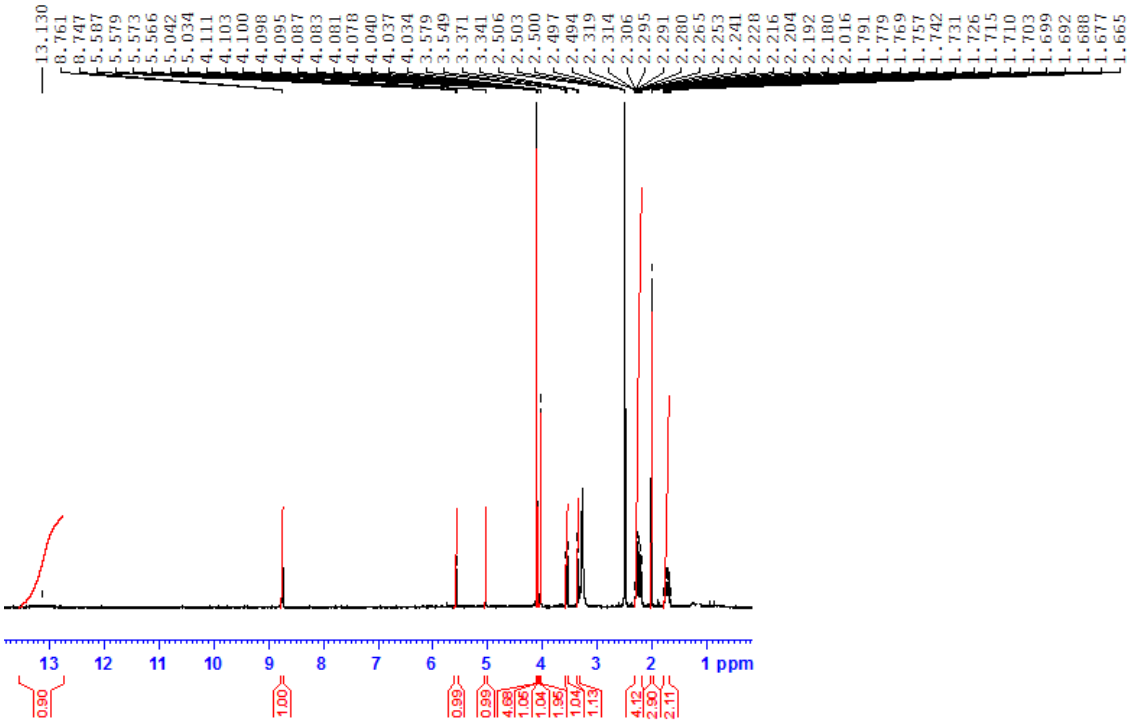

Figure S5. ^{1}H-NMR of Compound 4.

3.5 Compound 5

Yellow solid, HPLC retention time 8.55 min., 20% yield (181 mg).

^{1}H NMR (600 MHz, DMSO-d_6): δ =13.16 (bs, 1H, COOH), 8.74 (d, $J_{H,H}$ = 8.4 Hz, 1H, N-H), 5.57 (dd, $J_{H,H}$ = 8.4 Hz, $J_{H,H}$ = 4.8 Hz, 1H, C-H), 5.03 (d, $J_{H,H}$ = 4.8 Hz, 1H, C-H), 4.51 (bs, 7H, Rc), 4.41 (pt, $J_{H,H}$ = 1.8 Hz, 2H, Rc), 3.56 (d, $J_{H,H}$ = 18.0 Hz, 1H, CH_2), 3.35 (d, $J_{H,H}$ = 18.0 Hz, 1H, CH_2), 2.22 (m, 2H, CH_2), 2.12 (t, $J_{H,H}$ = 6.6 Hz, 2H, CH_2), 2.02 (s, 3H, CH_3), 1.65 (m, 2H, CH_2). ^{13}C NMR (150 MHz, DMSO-d_6): 173.0, 164.5, 163.6, 129.7, 123.1, 92.3, 70.7, 70.6, 70.4, 69.3, 58.9, 57.3, 34.7, 29.1, 28.0, 27.5, 19.4. MALDI-MS: m/z = 514(M). FTIR (KBr): 3436 (OH), 3277 (CH), 3086 (CH), 2925 (CH), 2854 (CH), 1791 (C=O), 1730 (C=O), 1621 (C=O) , 1547 cm^{-1}. Anal. Calcd. for $C_{22}H_{24}N_2O_4SRu$: C, 51.45; H, 4.71. Found: C, 51.42 ; H, 4.93.

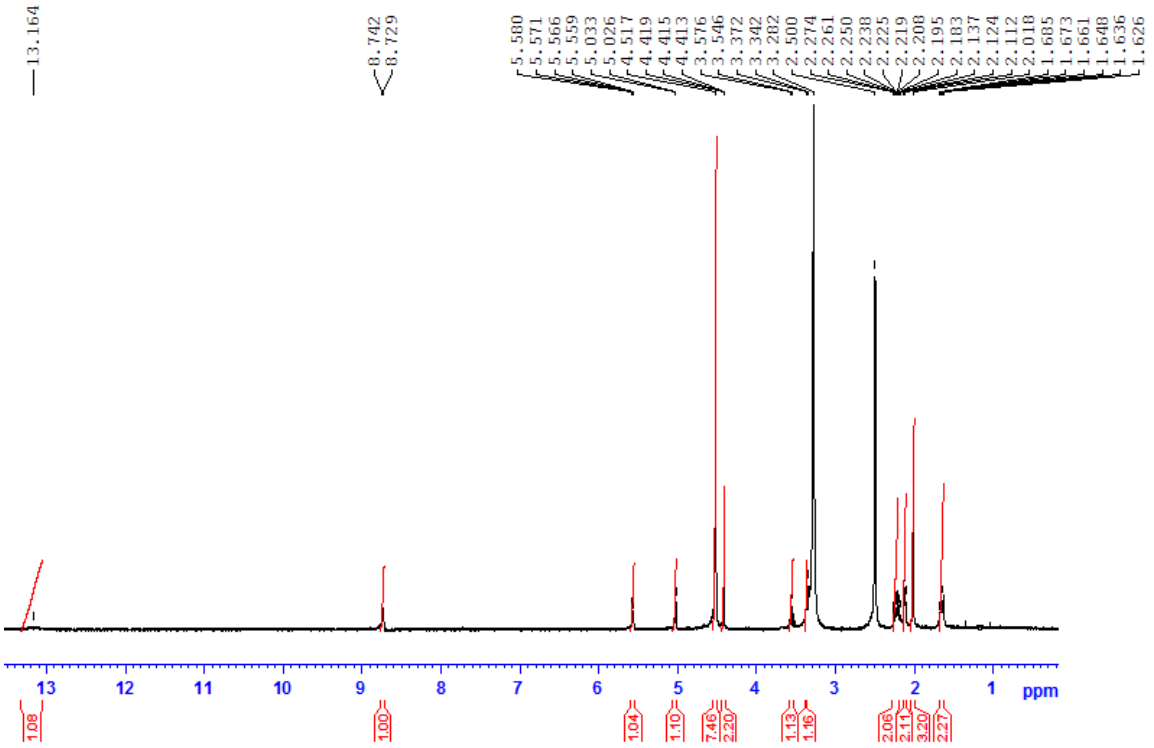

Figure S6. ^{1}H-NMR of Compound 5.

3.6 Compound 6

Orange solid, HPLC retention time for **7**: 9.92 min. 16% yield (161 mg).

^{1}H NMR (600 MHz, DMSO-d_6): δ =13.14 (bs, 1H, COOH), 8.79 (d, $J_{H,H}$ = 7.8 Hz, 1H, N-H), 5.59 (bs, 1H, C-H), 5.05 (d, $J_{H,H}$ = 3.6 Hz, 1H, C-H), 4.78 (bs, 2H, Fc), 4.56 (bs, 2H, Fc), 4.23 (s, 5H, Fc), 3.57 (d, $J_{H,H}$ = 18.0 Hz, 1H, CH_2), 3.36 (d, $J_{H,H}$ = 18.0 Hz, 1H, CH_2), 2.75 (m, 2H, CH_2), 2.29 (t, $J_{H,H}$ = 6.6 Hz, 2H, CH_2), 2.02 (s, 3H, CH_3), 1.83 (t, $J_{H,H}$ = 7.2Hz, 2H, CH_2). ^{13}C NMR (150 MHz, DMSO-d_6): 203.0, 172.9, 164.5, 163.6, 129.9, 123.0, 79.1, 72.1, 69.6, 69.1, 59.0, 57.3, 38.2, 34.1, 29.1, 20.1, 19.4. MALDI-MS: m/z = 496(M), FTIR (KBr): 3432 (OH), 3091 (CH), 2927 (CH), 1774 (C=O), 1724 (C=O), 1655 (C=O), 1545, 1456, 1380, 1245 cm^{-1}. Anal. Calcd. for $C_{23}H_{24}N_2O_5SFe$: C, 55.66; H, 4.87; Found: C, 55.59 ; H, 4.97.

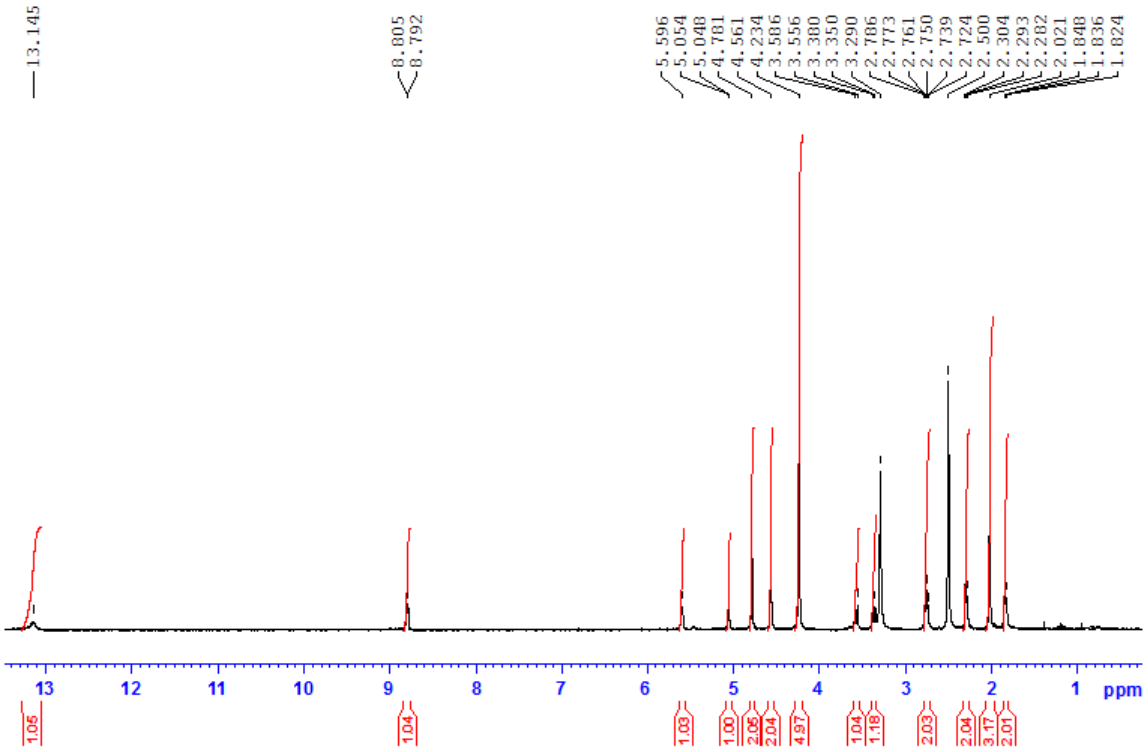

Figure S7. ^{1}H-NMR of Compound 6.

3.7 Compound 7

Yellow solid, HPLC retention time 9.62 min., 14% yield (142 mg).

^{1}H NMR (600 MHz, DMSO-d_6): δ =13.13 (bs, 1H, COOH), 8.77 (d, $J_{H,H}$ = 7.8 Hz, 1H, N-H), 5.58 (dd, $J_{H,H}$ = 8.4 Hz, $J_{H,H}$ = 4.8 Hz, 1H, C-H), 5.09 (s, 2H, Rc), 5.04 (d, $J_{H,H}$ = 4.2 Hz, 1H, C-H), 4.83 (s, 2H, Rc), 4.63 (s, 5H, Rc), 3.56 (d, $J_{H,H}$ = 18.0 Hz, 1H, CH_2), 3.36 (d, $J_{H,H}$ = 18.0 Hz, 1H, CH_2), 2.61 (m, 2H, CH_2), 2.22 (t, $J_{H,H}$ = 6.6 Hz, 2H, CH_2), 2.02 (s, 3H, CH_3), 1.77 (t, $J_{H,H}$ = 6.6 Hz, 2H, CH_2). ^{13}C NMR (150 MHz, DMSO-d_6): 201.1, 172.9, 164.5, 163.6, 130.0, 123.0, 83.9, 73.5, 71.9, 70.6, 59.0, 57.3, 39.2, 37.5, 34.0, 29.1, 20.6, 19.4. MALDI-MS: m/z = 542(M), 541(M-H$^+$). FTIR (KBr): 3432 (OH), 2933 (CH), 1774 (C=O), 1717 (C=O), 1662 (C=O), 1540, 1456, 1378, 1244 cm^{-1}. Anal. Calcd. for $C_{23}H_{24}N_2O_5SRu$: C, 51.01; H, 4.47; Found: C, 50.89 ; H, 4.61.

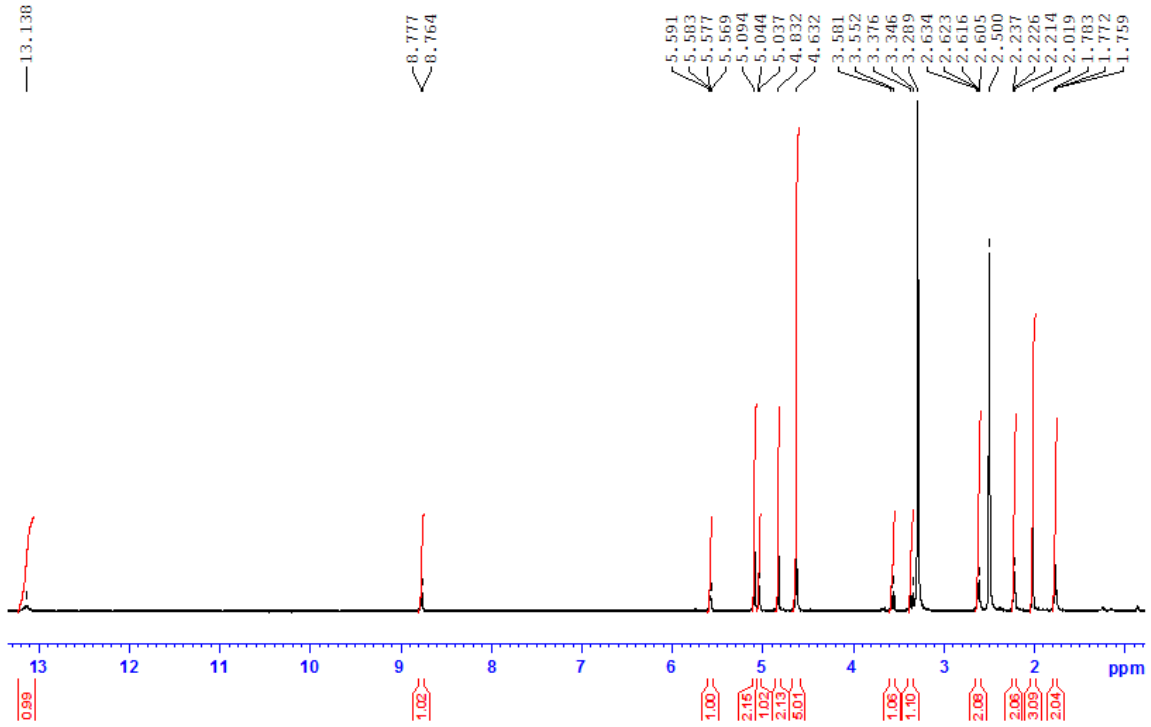

Figure S8. ^{1}H-NMR of Compound 7.

4.1 DFT Calculations

The quantum chemical calculations were performed using the Gaussian09 suite of programs.[9] All conformers were fully optimized with the B3LYP functional,[10] using the augmented Wachters' basis on Fe (8s7p4d),[11] and 6-31G(d) basis on C, H, N, O, and S. For Ru, the Stuttgart-Dresden relativistic effective core potential and the corresponding [6s5p3d] valence basis were employed.[12] Frequency calculations were performed at the corresponding level to characterize the optimized structures as minima (NImag=0). All energies are reported at 298.15 K. Thermal corrections to Gibbs free energies were calculated using the rigid rotor/harmonic oscillator model. A Boltzmann distribution was used to calculate the percentages of each conformer in the equilibrated sample. First, a reference structure *A* (e. g. a global minimum) was selected for a given organometallic species (**3**, **4**, or **6**). Next, for every structure *B*, other than the reference structure *A*, the equilibrium constant K_B was determined $K_B = [B] / [A]$ from the difference in Gibbs free energies for *B* and *A* calculated according to: $\Delta G = R\ T\ \ln K$ where R is the molar gas constant, and T is the temperature (in K). The fraction of *B* in the equilibrated sample is given by: $x_B = K_B / (1 + K_C + K_D + K_E + ...)$ where the sum in the denominator goes through all conformer structures for a given species. The fraction of *A* in the sample is: $x_A = 1 / (1 + K_B + K_C + K_D + ...)$

5.1 Microbiology and DD-carboxypeptidase, MBL, and CTX-M Inhibition Assay

Antibacterial activity of compounds **2-7** and the references ampicillin and penicillin G were tested by the liquid microdilution method. The antimicrobial spectrum of **2-7**, penicillin G, and ampicillin were evaluated by the minimal inhibitory concentrations (MIC) method using the serial two-fold dilution method under standard conditions as described in the Clinical and Laboratory Standards Institute (CLSI) reference method M07-A8.[13] Gram-positive bacterial strains, *Staphylococcus aureus* ATCC® 29213 (sensitive to methicillin, (MSSA)), *Staphylococcus aureus* ATCC® 43300 (resistant to methicillin, (MRSA)), *Staphylococcus aureus* subsp. ATCC 700787™ (intermediate to vancomycin, (VISA)), and *Staphylococcus epidermidis* ATCC® 12228™ were used. All strains were incubated for 24 hrs at 37 ^{o}C. Reference method (broth microdilution susceptibility test) was as follows: assayed compounds (**2-7**) were dissolved in DMSO. A series of two-fold dilutions were made for each of **2-7** with cation–adjusted Mueller–Hinton broth (CAMHB). 95 μL aliquots were dispensed into sterile microdilution plates (Mar-Four). Then, 5 μL of bacteria inoculum, containing 5 x 10^4 CFU mL^{-1}, was added. For each compound, the final concentrations in the two-fold dilution series ranged from 256 to 0.5 μg mL^{-1}. The experiments for each sample were conducted in triplicate. Penicillin G was used as a control (from 8 – 0.15 μg ml^{-1}). The plates were incubated at 37 ^{o}C for 18 to 24 hrs depending on bacterial strain. Results were obtained with the use of Spectrostar Omega (BMG Labtech), and absorbance was measured at λ=540 nm and λ=595 nm. MIC was defined as the lowest drug concentration that reduced growth by 100%.

DD-Carboxypeptidase Inhibition Assay

Inhibition of DD-carboxypeptidase 64-575[14] was based on the determination of the D-Ala cleaved from the substrate (Ac_2-L-Lys-D-Ala-D-Ala) according to the previously described method[7] with modification.[15] The method was adapted to microtitre plates. 15 μL of 0.2 M phosphate buffer pH 8.0, 5 μL of inhibitor solution in DMSO, 20 μL of 6 mM Ac_2-L-Lys-D-Ala-D-Ala in 0.2 M phosphate buffer pH 8.0, and 10 μL of DD-carboxypeptidase 64-575 in 0.2 M phosphate buffer pH 8.0 were mixed and incubated at 37 °C for 30 min. After incubation, 200 μL of coupling reagent (stock solution: 20 mg of

phthaldialdehyde in ethanol (1 mL), β-mercaptoethanol (50 μL), and sodium borate buffer pH 9.5 (50 mL)) was added to the well, mixed, and the fluorescence was read in BMG LABTECH FLUOstar Omega microplate reader (ex. 330 nm, em. 450 nm).

The positive control of the enzyme activity consisted of DMSO (5 μL) instead of inhibitor solution. The analogous blank contained only enzyme in 0.2 M phosphate buffer pH 8.0 (50 μL). Sample, control and related blanks were triplicated and carried out under the same conditions. Different solutions of inhibitor were examined: 2.5 nM – 0.25 mM. For each inhibitor concentration, percentage of inhibition was calculated by using formula:

$$DD - carboxypeptidase\ inhibition\ [\%] = (1 - \frac{F_i}{F_c}) \times 100$$

The fluorescence for the control is F_c and the fluorescence in the presence of the compounds or other inhibitor is F_i. The amount of tested compound needed to inhibit the enzyme by 50%, IC_{50} (inhibitory concentration) was calculated by the linear regression between inhibitor concentration and percentage of inhibition and expressed as μM.

MBL and CTX-M Inhibition Assay

Inhibition of β-lactamase CTX-M-14 from *Escherichia coli* and β-lactamase class B from *Bacillus cereus,* was based on the determination of the nitrocefin (Merck Millipore, Germany) distinctive color change when hydrolyzed by the enzyme. The previously described method[16] was adapted to microtitre plates (a total volume of 100 μL per well). The reaction mixtures (for each enzyme) consisted of 85 μL of 0.01M phosphate buffer pH 7.0, 5 μL of 2.67 μg mL^{-1} β-lactamase CTX-M-14 solution in 0.01M phosphate buffer pH 7.0, 5 μL of inhibitor solution in DMSO, and 5 μL of 1 mg mL^{-1} nitrocefin solution for β-lactamase CTX-M-14; 85 μL of 0.1M phosphate buffer pH 7.0, 5 μL of 12.25 U mL^{-1} β-lactamase *B. cereus* solution in 0.1M phosphate buffer pH 7.0, 5 μL of inhibitor solution in DMSO, and 5 μL of 1 mg mL^{-1} nitrocefin solution for β-lactamase from *Bacillus cereus*. Each reaction mixture was incubated at 37 °C for 20 min and the absorbance was read every minute at λ = 482 nm (FLUOstar Omega microplate reader (BMG LABTECH, Germany)). The positive control of the enzyme activity consisted of DMSO (5 μL) instead of inhibitor solution. Samples and control were

triplicated and carried out under the same conditions. Different solutions of inhibitor were examined (final concentration: 0.0625 μM – 1.25 mM). For each inhibitor concentration, percentage of inhibition was calculated by using formula:

$$\beta - lactamase\ inhibition\ [\%] = \left(1 - \frac{\Delta A_i}{\Delta A_c}\right) \times 100$$

The change of absorbance for the control is A_c and the change of absorbance in the presence of the inhibitor is A_i. The amount of tested compound needed to inhibit the enzyme by 50%, IC_{50} (inhibitory concentration), was calculated by the linear regression between inhibitor concentration and percentage of inhibition, and expressed at μM.

6.1 Crystallization, Data Collection, and Refinement

CTX-M-14 E166A β-lactamase crystals were grown from seeds over the course of 3-4 days at 20 °C in 1 M potassium phosphate buffer at pH 7.9. 10 mM **3** dissolved in the crystallization buffer was then added to the crystal drop and allowed to soak into the crystal for three days, at which point the crystals were collected and cryo-cooled. Data was collected using the 22-ID beamline of SER-CAT at the Advanced Photon Source (APS), Argonne, Illinois. Data were processed using HKL2000.[17] CCP4 and Coot were used to complete the refinement and model building.[18, 19] PyMOL (http://www.pymol.org) was used to generate all images for figures.

R**eferences**

1. Patwa, A.N.; Gupta, S.; Gonnade, R. G.; Kumar, V. A.; Bhadbhade, M. M.; Ganesh, K. N. *J. Org. Chem.* **2008**, *73*, 1508-1515.

2. Siddiqi, M. A.; Siddiqui, R. A.; Atakan, B.; Roth, N.; Lang, H. *Materials* **2010**, *3*, 1172-1185.

3. Rinehart, K. L.; Curby, R. J.; Sokol, P. E. *J. Am. Chem. Soc.* **1957**, *79*, 3420-3424.

4. Blom, N. F.; Neuse, E. W.; Thomas, H. G. *Trans. Met. Chem.* **1987**, *12*, 301-306.

5. Apreutesei, D.; Lisa, G.; Akutsu, H.; Hurduc, N.; Nakatsuji, S.; Scutaru, D. *Appl. Organometal. Chem.* **2005**, *19*, 1022-1037.

6. Hofer, O.; Schlögl, K. *J. Organomet. Chem.* **1968**, *13*, 443-456.

7. Skiba, J.; Rajnisz, A.; de Oliveira, K.N.; Ott, I.; Solecka, J.; Kowalski, K. *Eur. J. Med. Chem.* **2012**, *57*, 234-239.

8. Kowalski, K.; Winter, R. F.; Makal, A.; Pazio, A.; Woźniak, K. *Eur. J. Inorg. Chem.* **2009**, *27*, 4069-4077.

9. Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Scalmani, G.; Barone, V.; Mennucci, B.; Petersson, G. A.; Nakatsuji, H.; Caricato, M.; Li, X.; Hratchian, H. P.; Izmaylov, A. F.; Bloino, J.; Zheng, G.; Sonnenberg, J. L.; Hada, M.; Ehara, M.; Toyota, K.; Fukuda, R.; Hasegawa, J.; Ishida, M.; Nakajima, T.; Honda, Y.; Kitao, O.; Nakai, H.; Vreven, T.; Montgomery, J. A. Jr.; Peralta, J. E.; Ogliaro, F.; Bearpark, M.; Heyd, J. J.; Brothers, E.; Kudin, K. N.; Staroverov, V. N.; Kobayashi, R.; Normand, J.; Raghavachari, K.; Rendell, A.; Burant, J. C.; Iyengar, S. S.; Tomasi, J.; Cossi, M.; Rega, N.; Millam, J. M.; Klene, M.; Knox, J. E.; Cross, J. B.; Bakken, V.; Adamo, C.; Jaramillo, J.; Gomperts, R.; Stratmann, R. E.; Yazyev, O.; Austin, A. J.; Cammi, R.; Pomelli, C.; Ochterski, J. W.; Martin, R. L.; Morokuma, K.; Zakrzewski, V. G.; Voth, G. A.; Salvador, P.; Dannenberg, J. J.; Dapprich, S.; Daniels, A. D.; Farkas, Ö.; Foresman, J. B.; Ortiz, J. V.; Cioslowski, J.; Fox, D. J. *Gaussian 09*, Revision D.01, Gaussian, Inc.: Wallingford, CT, 2009.

10. Becke, A. D. *J. Chem. Phys*. **1993**, *98*, 5648-5652.

11. Wachters, A. J. H. *J. Chem. Phys.* **1970**, *52*, 1033-1036.

12. Andrae, D.; Häussermann, U.; Dolg, M.; Stoll, H.; Preuss, H. *Theor. Chim. Acta*. **1990**, *77***,** 123-141.

13. Approved Standard-Eighth Edition. M07-A8 Method for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; CLSI: 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898, USA, 2009, ISBN 1-56238-689-1.

14. Kurzątkowski, W.; Solecka, J.; Filipek, J.; Kurzątkowski, J. D.; Kuryłowicz, W. *Appl. Microbiol. Biotechnol.* **1990,** *33*, 452-454.

15. Georgopapadakou, N. H.; Liu, F. Y.; Ryono, D. E.; Neubeck, R.; Gordon, E. M.; Pluscec, J. *Anal. Biochem*. **1984**, *137*, 125–128.

16. O'Callaghan, C. H.; Morris, A.; Kirby, S. M.; Shingler, A. H. *Antimicrob. Agents Chemother*. **1972**, *1*, 283–288.

17. Otwinowski, Z.; Minor, W. *Methods Enzymol*. **1997**, *276*, 307– 326.

18. Collaborative Computational Project, N. *Acta Crystallogr., Sect. D: Biol. Crystallogr*. **1994**, *50*, 760– 763.

19. Emsley, P.; Cowtan, K. *Acta Crystallogr., Sect. D: Biol. Crystallogr*. **2004**, *60*, 2126–2132.