

# Antibacterial Properties and Atomic Resolution X-ray Complex Crystal Structure of a Ruthenocene Conjugated $\beta$ -Lactam Antibiotic

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## Supporting Information

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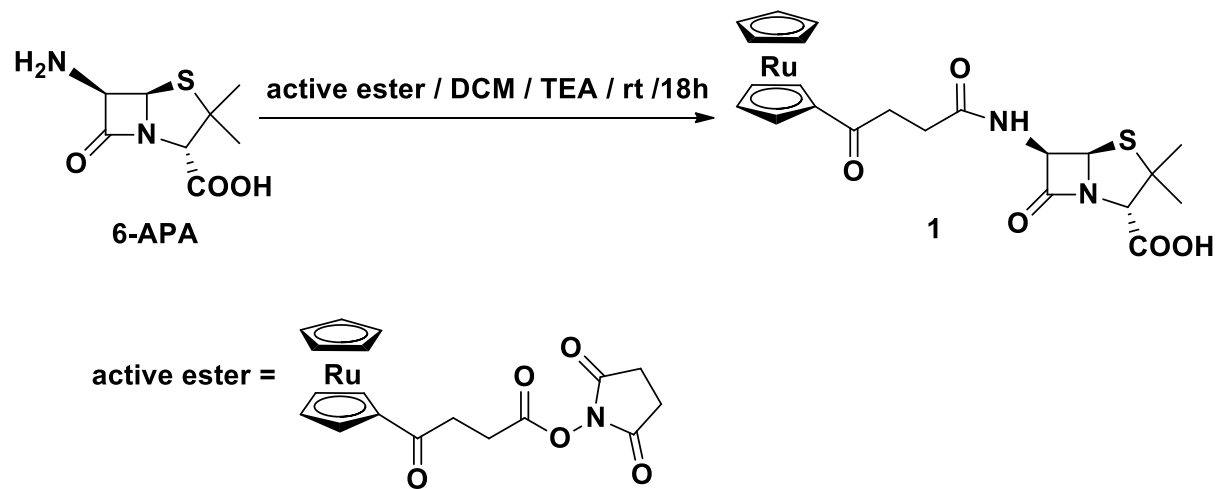
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## 1.1 General Comments.

Synthesis of complex **1** was carried out using standard Schlenk techniques. Chromatographic separation was carried out using silica gel 60 (Merck, 230-400 mesh ASTM). Dichloromethane and triethylamine were distilled and deoxygenated prior to use. Other solvents were of reagent grade and were used without prior purification. All other chemicals were purchased from the Aldrich Chemical Co. The NMR spectra were recorded on a Bruker AV600 Kryo (600 MHz) spectrometer. Chemical shifts are reported in  $\delta$  (ppm) using residual  $\text{CHCl}_3$  ( $^1\text{H}$   $\delta$  7.26 ppm) signal as the reference. Mass spectrum was recorded using positive FAB method on a Voyager Spec mass spectrometer (cyano-4-hydroxycinnaminic acid – saturated in 0.1% TFA  $\text{H}_2\text{O}$ /acetonitrile 1:1 as a matrix). IR spectra were recorded on a FTIR Nexus Nicolet apparatus.

## 1.2 Synthesis of compound 1

The synthetic approach for the preparation of compound **1** is shown in Scheme 1.



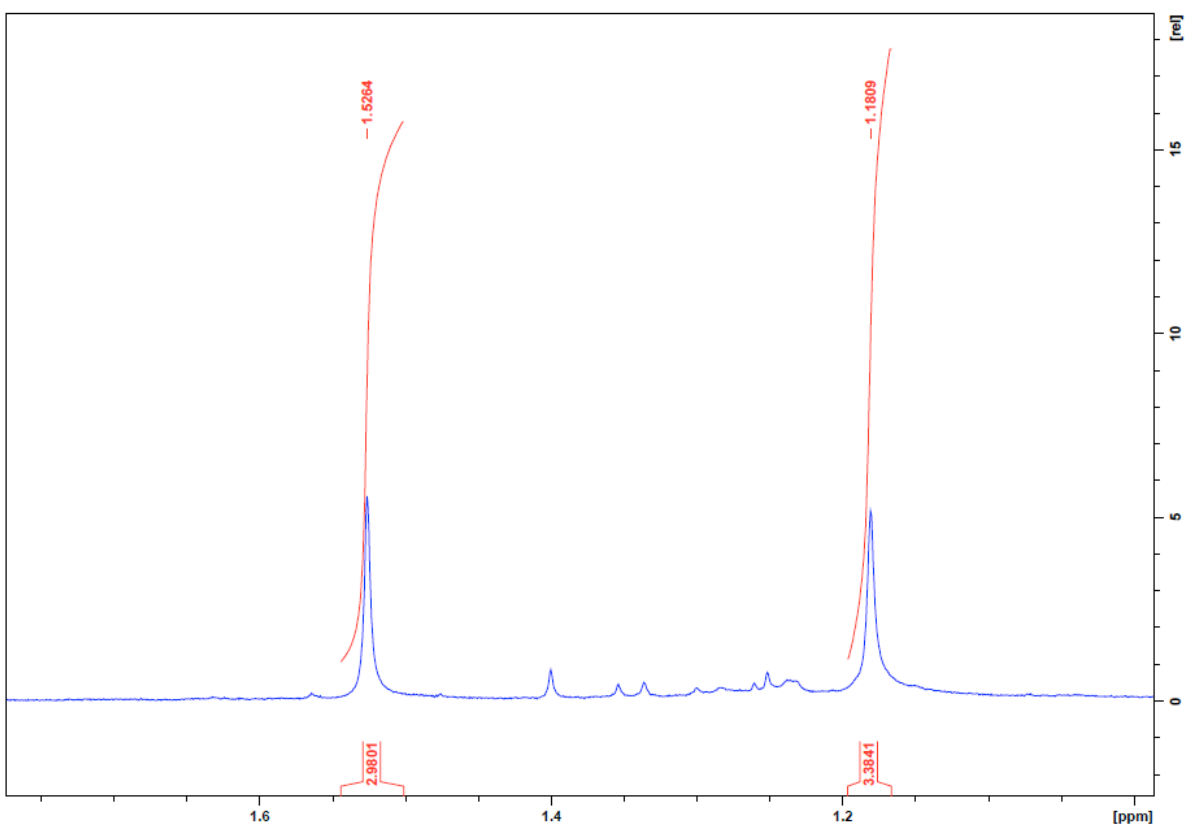
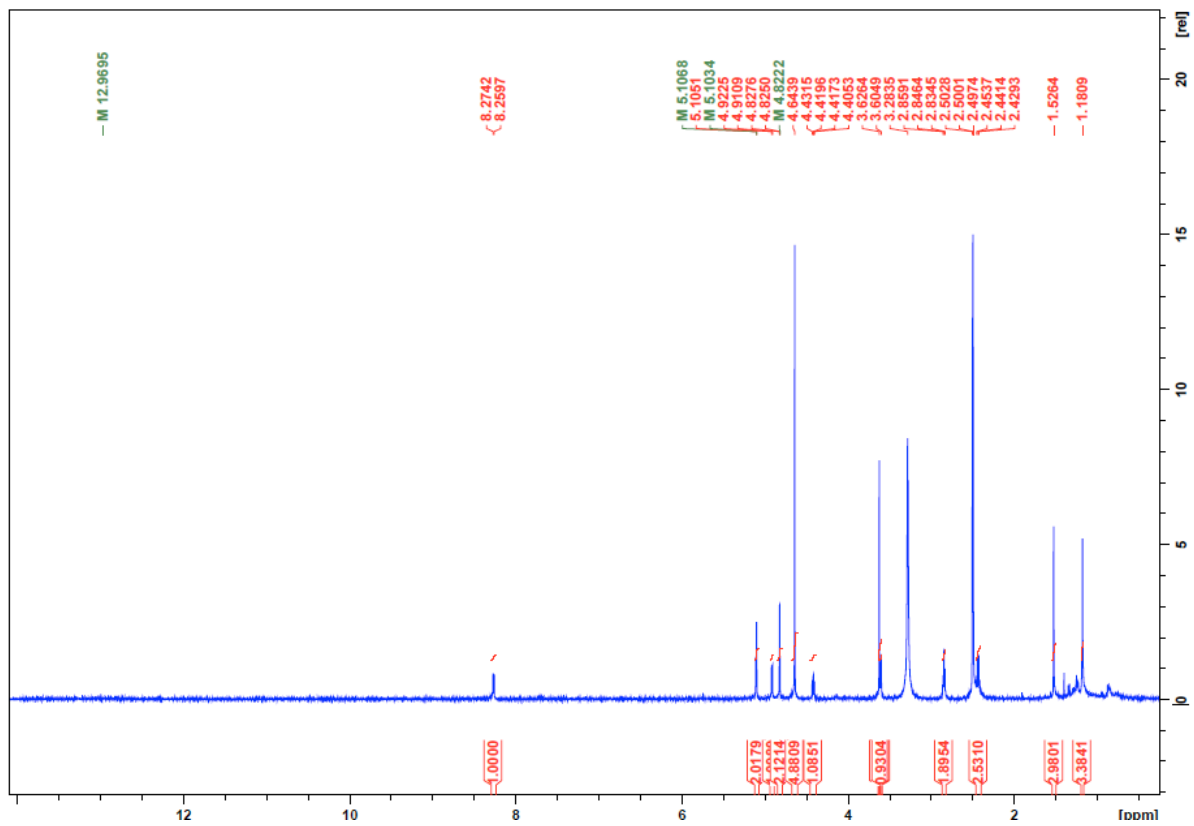
Scheme 1

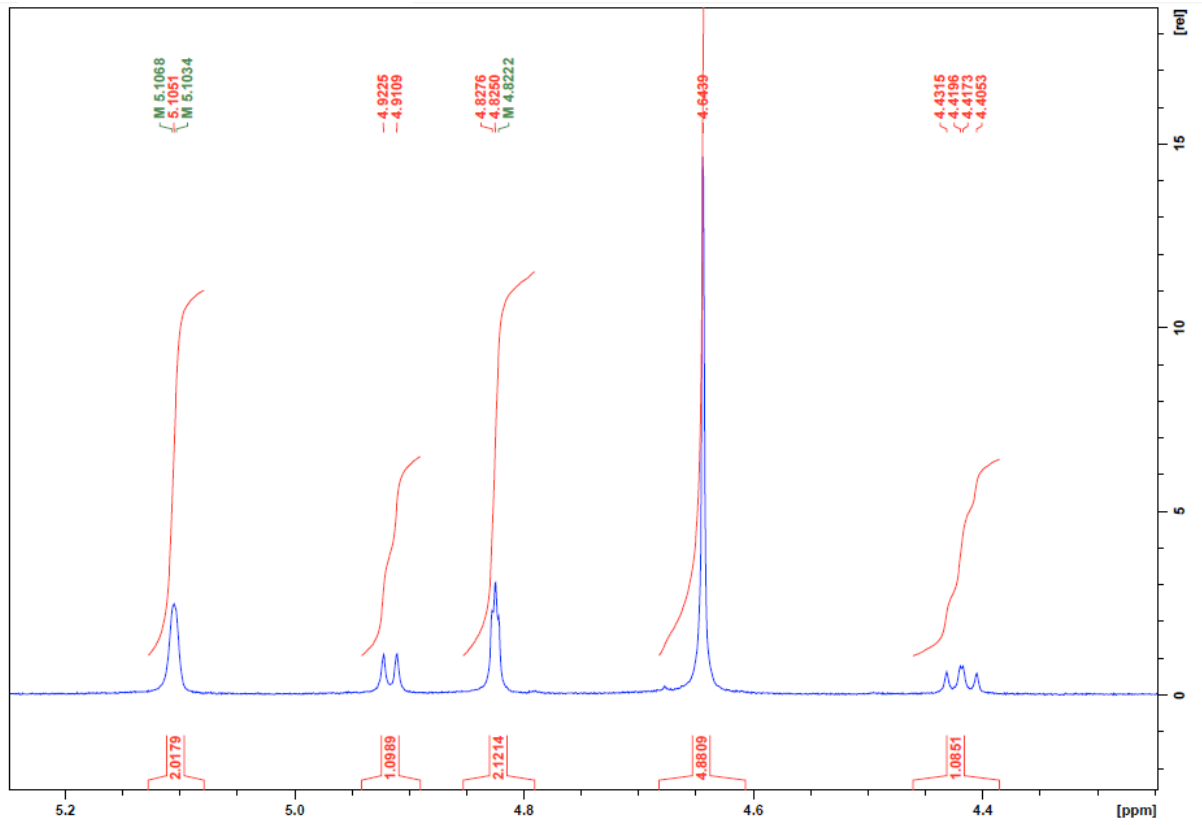
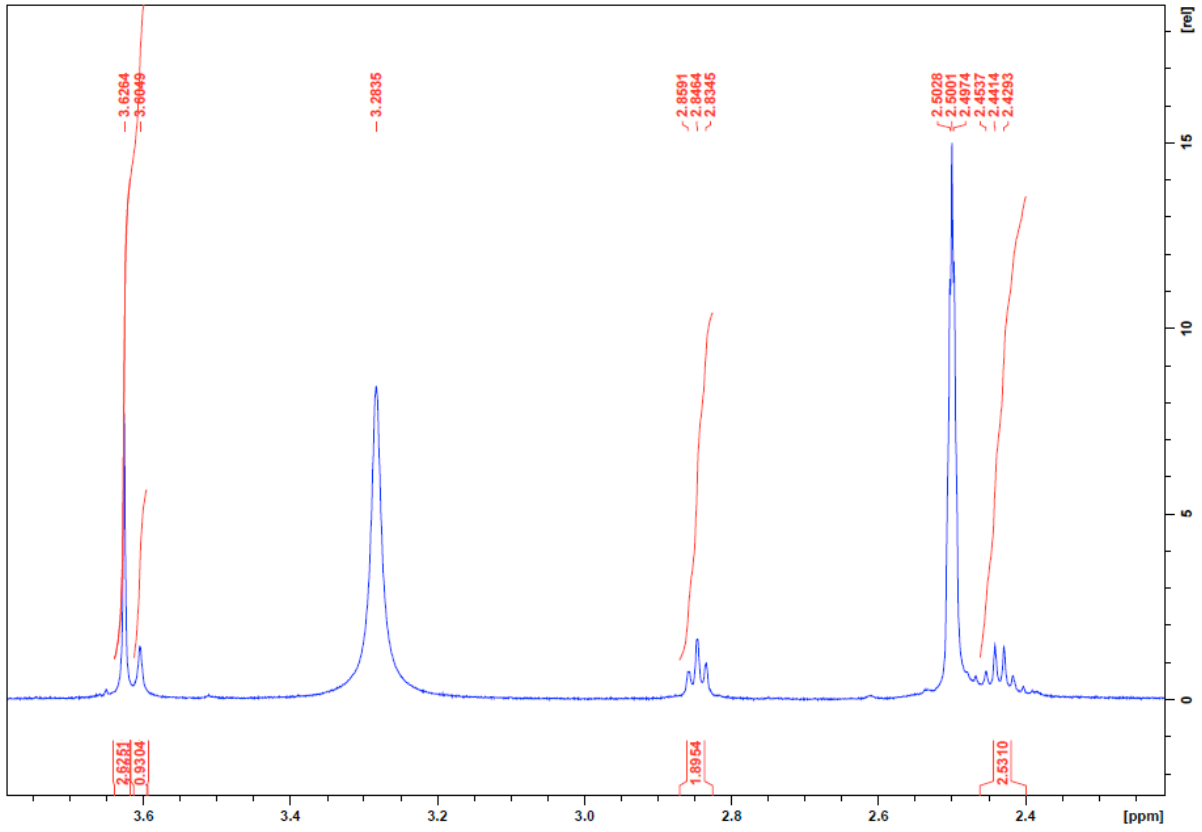
It involves reaction of 6-APA with 4-oxo-4-(ruthenocenyl) butanoic *N*-succinimidyl ester (active ester). Analytically pure sample of complex **1** has been obtained after workup and chromatography with 73% yield. Complex **1** is thermally and air-stable yellow crystalline solid. Active ester has been prepared according to the literature [1].

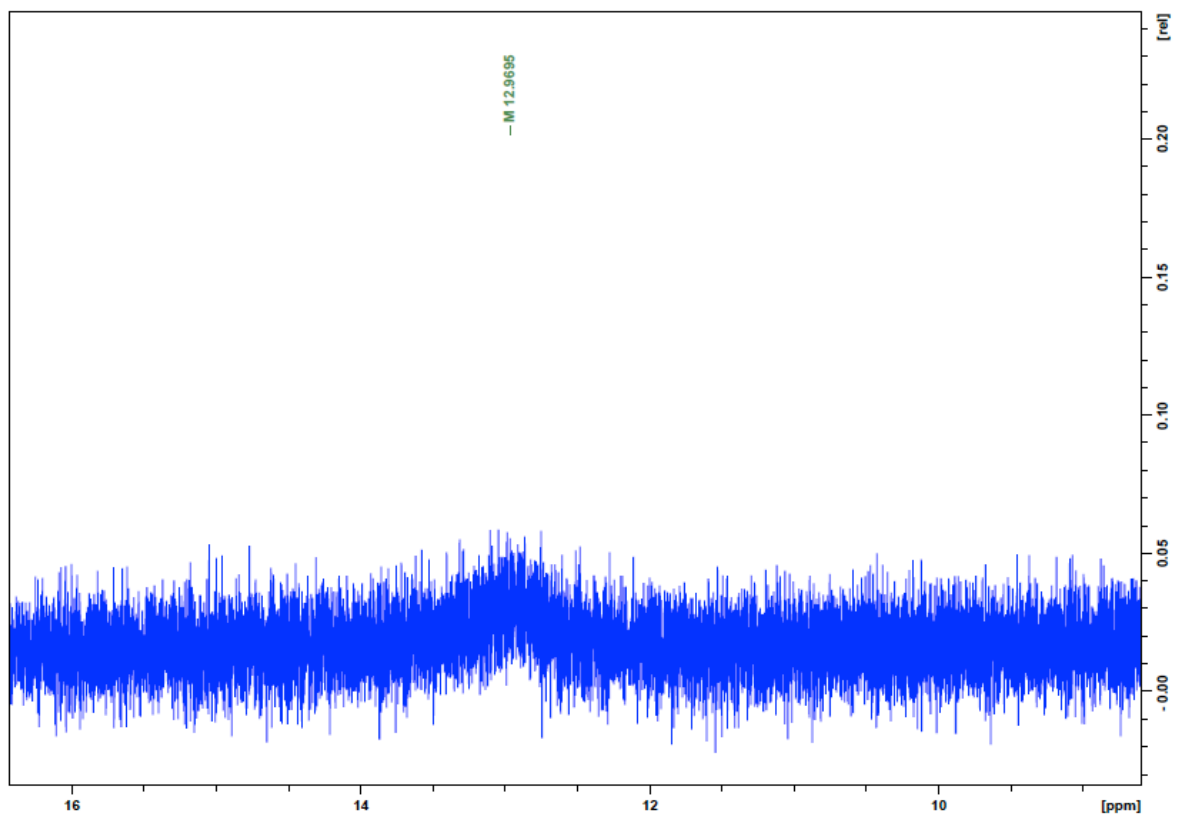
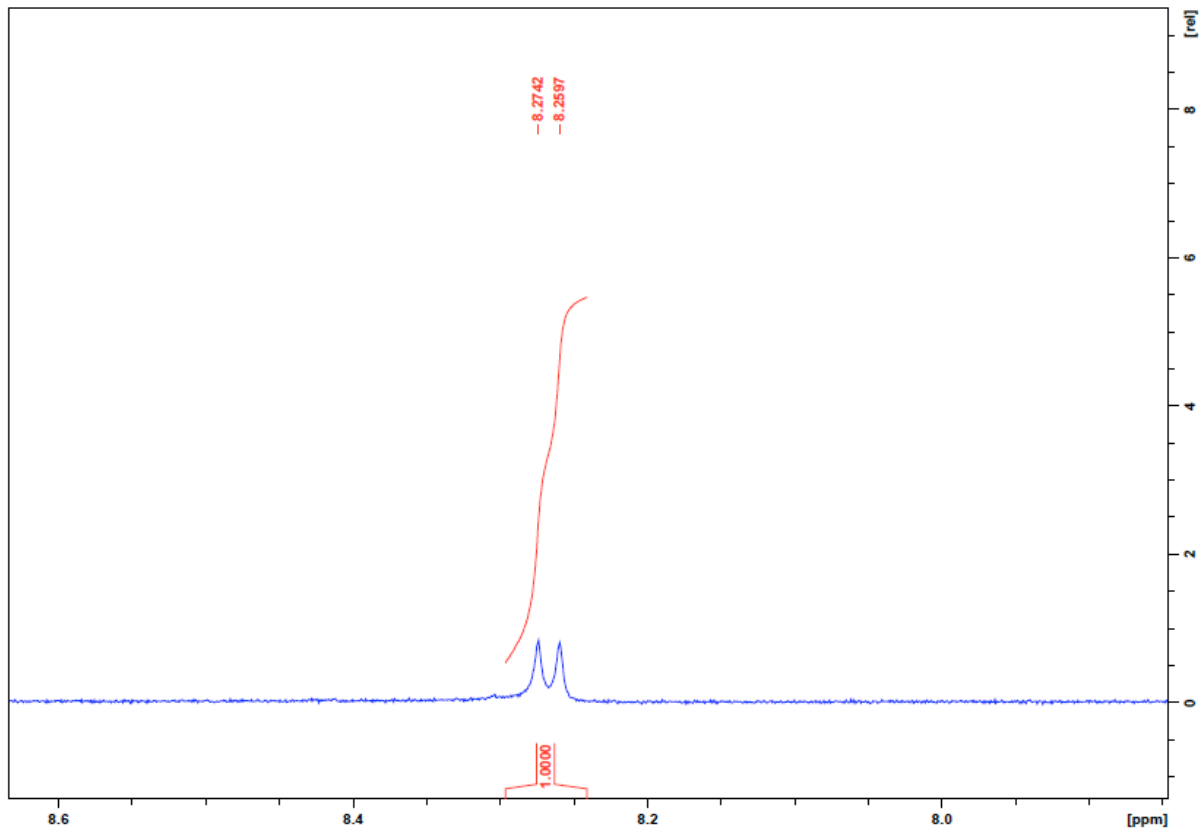
### 1.3 Procedure for the synthesis of compound 1

Solution of 6-APA (108 mg, 0.50 mmol) in dichloromethane/triethylamine (10 ml:800  $\mu$ l) was added to an argon-saturated dichloromethane (10 ml) solution of 4-oxo-4-(ruthenocenyloxy)butanoic *N*-succinimidyl ester (194 mg, 0.45 mmol). The reaction mixture was stirred for 18 h at room temperature. Then solvents were evaporated and the residue was subjected to column chromatography on SiO<sub>2</sub> (CHCl<sub>3</sub>/MeOH, 50:5). Chromatographically purified complex **1** was dissolved in chloroform and washed with 3% HCl aq. Organic layer was separated, dried over anhydrous MgSO<sub>4</sub> and evaporated. Crystallization from chloroform/*n*-hexane mixture afforded **1** as yellow solid in 73% yield (175 mg).

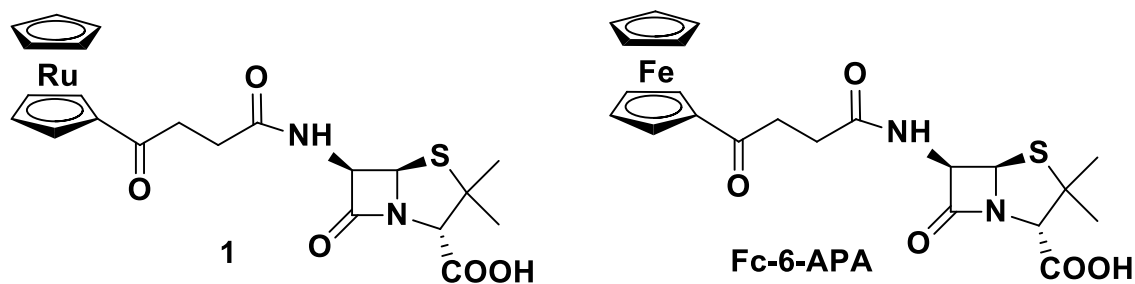
<sup>1</sup>H NMR (600 MHz, DMSO-d<sub>6</sub>):  $\delta$  = ~13 (bs, H, COOH), 8.26 (d, J<sub>HH</sub> = 8.7 Hz, 1H, NH), 5.10 (bs, 2H, Rc), 4.91 (d, J<sub>HH</sub> = 7 Hz, 1H, H  $\beta$ -lactam), 4.82 (pt, J<sub>HH</sub> = 1.6 Hz, 2H, Rc), 4.64 (s, 5H, Rc), 4.41 (dd, J<sub>HH</sub> = 8.2 Hz, 7.1 Hz, 1H, H  $\beta$ -lactam), 3.60 (s, 1H, H  $\beta$ -lactam), 2.84 (t, J<sub>HH</sub> = 7.6 Hz, 2H, CH<sub>2</sub>), 2.43 (m, 2H, CH<sub>2</sub>), 1.52 (s, 3H, CH<sub>3</sub>), 1.18 (s, 3H, CH<sub>3</sub>) ppm. FAB-MS (positive ions):  $m/z$  = 531 (M+H<sup>+</sup>). FTIR (KBr v [cm<sup>-1</sup>]): 3686-2142 (NH+OH), 1782 (CO), 1740 (CO), 1663 (2CO).











**Table S1** *In vitro* antibacterial activity of **1** and Fc-6-APA compounds (MIC  $\mu\text{g}/\text{mL}$ )<sup>a</sup>.

Microorganism	MIC ( $\mu\text{g}/\text{mL}$ )			
	Fc-6-APA	1	Ampicillin	6-APA
<i>Staphylococcus aureus</i> subsp. <i>aureus</i> ATCC® 29213™ (MSSA)	10 <sup>b</sup>	2	0.5	200
<i>Staphylococcus aureus</i> subsp. <i>aureus</i> ATCC® 43300 (MRSA)	160 <sup>b</sup>	256	10	1600
<i>Staphylococcus epidermidis</i> ATCC® 12228™	10 <sup>b</sup>	4	5	400
<i>Enterococcus faecalis</i> ATCC® 29212	-	16	10	n.d.
Clinical strains: methicillin sensitive <i>Staphylococcus aureus</i>				
30/11	-	4		
26/11	-	2		
1255/11	-	8		
1637(1)/11	-	0.5		
1730/11	-	4		
1826/11	-	2		
1863/11	-	2		
1933/11	-	4		
2187/11 (37)	-	2		
2271/11 (41)	-	2		
57/12 (47)	-	4		
12/10	-	0.5		

<sup>a</sup>MICs values of the reference compound, penicillin G against *S. aureus* ATCC® 29213 – 2.0  $\mu\text{g ml}^{-1}$ , against *S. aureus* ATCC 43300® > 4.0  $\mu\text{g ml}^{-1}$ . <sup>b</sup> Values from Eur. J. Med. Chem. 57 (2012) 234-239.

## 2.1 Antibacterial activity of compound 1. Liquid microdilution method

The antimicrobial spectrum of complex **1** was 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 [2]. A panel of Gram-positive bacterial strains, *Staphylococcus aureus* ATCC<sup>®</sup> 29213 (sensitive to methicillin, (MSSA)), *Staphylococcus aureus* ATCC<sup>®</sup> 43300 (resistant to methicillin, (MRSA)), *Staphylococcus epidermidis* ATCC<sup>®</sup> 12228<sup>™</sup>, *Enterococcus faecalis* ATCC<sup>®</sup> 29212 were used. Twelve clinical isolates of methicillin sensitive *Staphylococcus aureus* (MSSA) obtained from the Department of Pharmaceutical Microbiology of Medical University of Warsaw, Poland were also used. The clinical isolates were collected from various patients hospitalized in several clinics. Bacterial strains were cultivated on tryptic soy agar (TSA) according to ATCC recommendation. All strains were incubated for 24 hrs at 37 °C.

Reference method (broth microdilution susceptibility test) was as follows: The bioconjugate **1** was dissolved in DMSO. A series of two-fold **1** dilutions were diluted with cation-adjusted Mueller–Hinton broth (CAMHB). 95 µl aliquots were dispensed into microdilution sterile plates (Mar-Four). Then, 5 µl of bacteria inoculum, containing  $5 \times 10^4$  CFU ml<sup>-1</sup>, was added. The final concentration of **1** ranged from 256 to 0.5 µg ml<sup>-1</sup> all in two-fold dilution steps. Penicillin G was used as a control (from 8 – 0,15 µg ml<sup>-1</sup>). The plates were incubated at 35 °C for 24 hours. Results were obtained with the use of Spectrostar Omega (BMG Labtech), Absorbance was measured at  $\lambda = 540$  nm and  $\lambda = 595$  nm. The experiments for each sample were conducted in triplicate. Penicillin G was used as a control (from 8 – 0,15 µg ml<sup>-1</sup>). MIC was defined as the lowest drug concentration that reduced growth by 100 %.

## 2.2 Hemolysis Testing

The hemolysis test was evaluated following the literature method [3]. Red blood cells were obtained from a healthy donor. The erythrocytes were separated from blood plasma and leukocytes by centrifugation (2500 g, 5 min) at 4 °C and washed three times with the phosphate buffered saline (PBS, pH 7.4). Prepared suspension of 1% hematocrit was incubated with serial concentrations (0.001, 0.03, 0.15, 0.4 mM, respectively) of complex **1** for 30 min at 23 °C. After centrifugation (1,000 rpm, 5 min) the absorbance of the supernatant was measured at 540 nm (Jasco V-630). A value of 100% hemolysis was determined by incubation of erythrocytes with double-distilled water (30 min at 23°C). No hemolytic activity of compound **1** was observed on human erythrocytes.

### **3.1 Crystallization, Data Collection, and Refinement**

CTX-M-14 E166A  $\beta$ -lactamase crystals were grown from seeds over the course of 3-4 days at 20°C in 1M potassium phosphate buffer at pH 7.9. 10mM **1** dissolved in the crystallization buffer was then added to the crystal drop and allowed to soak into the crystal for four hours, 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 [4]. CCP4 and Coot were used to complete the model building and refinement [5, 6]. PyMOL (<http://www.pymol.org>) was used to generate all images for figures.

**Table S2. X-ray Data Collection and Refinement Statistics****Data Collection**

Space Group	P2 <sub>1</sub>
Cell Dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	44.9, 106.8, 47.9
$\alpha$ , $\beta$ , $\gamma$ (°)	90.0, 101.9, 90.0
Resolution (Å)	50.00 - 1.18 (1.20-1.18)
No. Reflections	141750 (6158)
<i>R</i> <sub>merge</sub> (%)	5.4 (28.9)
<i>I</i> / $\sigma$ <i>I</i>	16.54 (3.15)
Completeness (%)	98.3 (85.9)
Redundancy	3.5

**Refinement**

resolution (Å)	46.92-1.18
<i>R</i> <sub>work</sub> / <i>R</i> <sub>free</sub> (%)	12.5/15.3
no. heavy atoms	
protein/ligand/water	4474/146/601
<i>B</i> -factors (Å <sup>2</sup> )	
protein/ligand/water	8.76/10.73/23.28
rms deviations	
bond lengths (Å)	0.008
bond angles (°)	1.38
ramachandran plot	
most favored region(%)	91.3
additionally allowed (%)	7.8
generously allowed (%)	0.9

\* Values in parentheses represent highest resolution shells

## References

- [1] K. Kowalski, R. F. Winter, A. Makal, A. Pazio, K. Woźniak, *Eur. J. Inorg. Chem.* (2009) 4069-4077.
- [2] CLSI. *Method for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard-Eighth Edition. M07-A8* [ISBN 1-56238-689-1]. CLSI, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA, 2009].
- [3] A. Knopik-Skrocka, J. Bielawski, *Biological Lett.* 42 (2005) 49-60
- [4] Otwinowski, Z.; Minor, W. *Processing of X-ray diffraction data collected in oscillation mode Methods Enzymol.* 1997, 276, 307– 326
- [5] Collaborative Computational Project, N. *The CCP4 suite: programs for protein crystallography. Acta Crystallogr., Sect. D: Biol. Crystallogr.* 1994, 50, 760– 763
- [6] Emsley, P.; Cowtan, K. *Coot: model-building tools for molecular graphics Acta Crystallogr., Sect. D: Biol. Crystallogr.* 2004, 60, 2126– 2132