Supporting Information

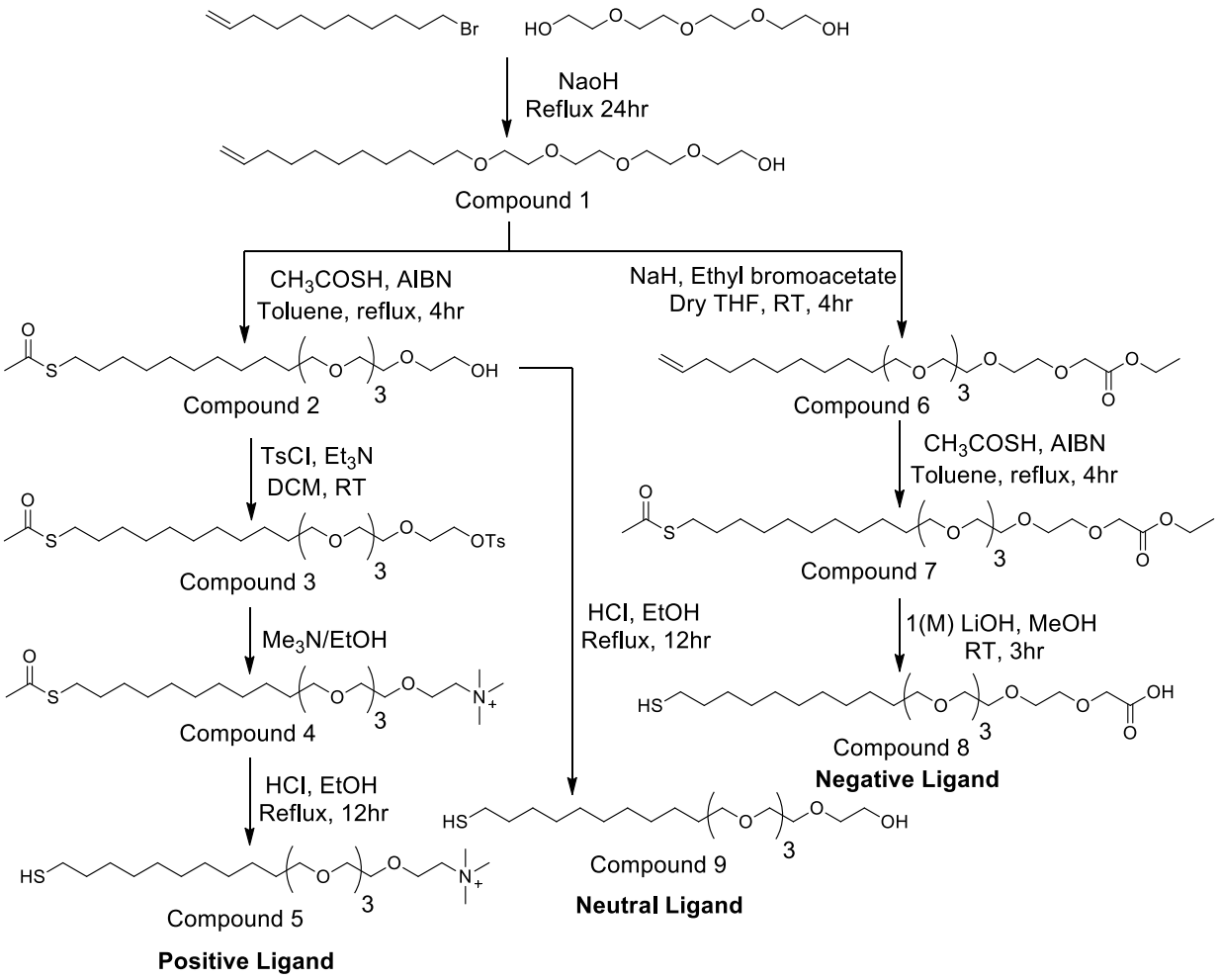
2D-MoS_2 Based β-Lactamase Inhibitor for Combination Therapy against Drug Resistant Bacteria

*Sk Rajab Ali, Subhendu Pandit and Mrinmoy De**

Department of Organic Chemistry, Indian Institute of Science, Bangalore, India

E-mail: md@iisc.ac.in

Synthesis of Ligands. All the ligand molecules were synthesized by following the scheme 1.[1-2] The synthetic procedures for the preparation of various ligand molecules are as follows.



Scheme S1. Synthesis of various thiolated ligands with positive, negative and neutral charged headgroup.

Synthesis of Compound 1. In a double neck round bottom flask, sodium hydroxide (21.4 mmol) was dissolved in minimum quantity of water. Then ethylene glycol (107.2 mmol) was added and stirred for 1 h at 100 ^{0}C under argon atmosphere. Then 11-bromoundec-1-ene (21.4 mmol) was added slowly and the reaction mixture was refluxed for 24 h. After reflux, it was cooled to room

temperature followed by extraction of the substituted products using hexane, repeatedly. The combined hexane extract was concentrated which gives a mixture of mono sustituted and disustituted compounds. The desired mono-substituted compound was collected after purification using column chromatography. The compound was confirmed by ^{1}H-NMR. The yield of the reaction was 76%.

Synthesis of Compound 2. In a round bottom flask, compound 1 (2.88 mmol) was dissolved in 12 mL of toluene. AIBN (0.865) and thioacetic acid (8.66 mmol) were added and the reaction mixture was refluxed for 4 h at 110 ^{0}C under argon atmosphere. After that, the reaction mixture was cooled to room temperature and ethyl acetate was added to it. Then the product was washed with saturated sodium bicarbonate solution for three times. The organic layer was dried over anhydrous sodium sulfate, and concentrated by using rotary evaporator. The crude product was purified using column chromatography. The compound was confirmed by ^{1}H-NMR. The yield of the reaction was 78%.

Synthesis of Compound 3. In a round bottom flask, compound 2 (2.59 mmol) was dissolved in 10 mL of DCM and kept on the ice bath at 0 ^{0}C. Triethylamine (5.18 mmol) was added to it and the mixture was stirred for 15 minutes. Then tosyl chloride (6.48 mmol) in DCM, was added dropwise over a period of several minutes. The resulting reaction mixture was stirred for overnight. At the end of the reaction, the solvent was evaporated from the reaction mixture and redissolved in ethyl acetate. Then washed with HCl (1M) solution followed by washed with saturated sodium bicarbonate solution for three times. The organic layer was dehydrated by using anhydrous sodium sulfate, then the concentrated crude product was subjected to column purification. The product was confirmed by ^{1}H-NMR. The yield of the reaction was 82%.

Synthesis of Compound 4. In a round bottom flask, compound 3 (2.5 mmol) was dissolved in 12 mL of EtOH. Then ethanolic solution of trimethylamine (5 mmol) was added to it and stirred the reaction mixture at room temperature under inert gas atmosphere. The reaction was monitored by TLC. Based on requirement, additional trimethylamine was added after monitoring the reaction at different time interval. After completion of reaction, the product was dissolved in hexane:ether (1:1) solvent mixture and kept in refrigerator for overnight. Then the triturated product was collected and confirmed by ^{1}H-NMR. The yield of the reaction was 88%.

Synthesis of Compound 5 (Cationic Ligand). The compound 4 (1.868 mmol) was dissolved in 10 mL of EtOH. Then 0.5 ml of concentrate HCl was added and reaction mixture was refluxed for 12 h. After reflux, EtOH was evaporated. Then the crude product was dissolved in hexane:ether (1:1) solvent mixture and kept inside the refrigerator for precipitation. The triturated compound was collected and confirmed by ^{1}H-NMR. The yield of the reaction was 90%.

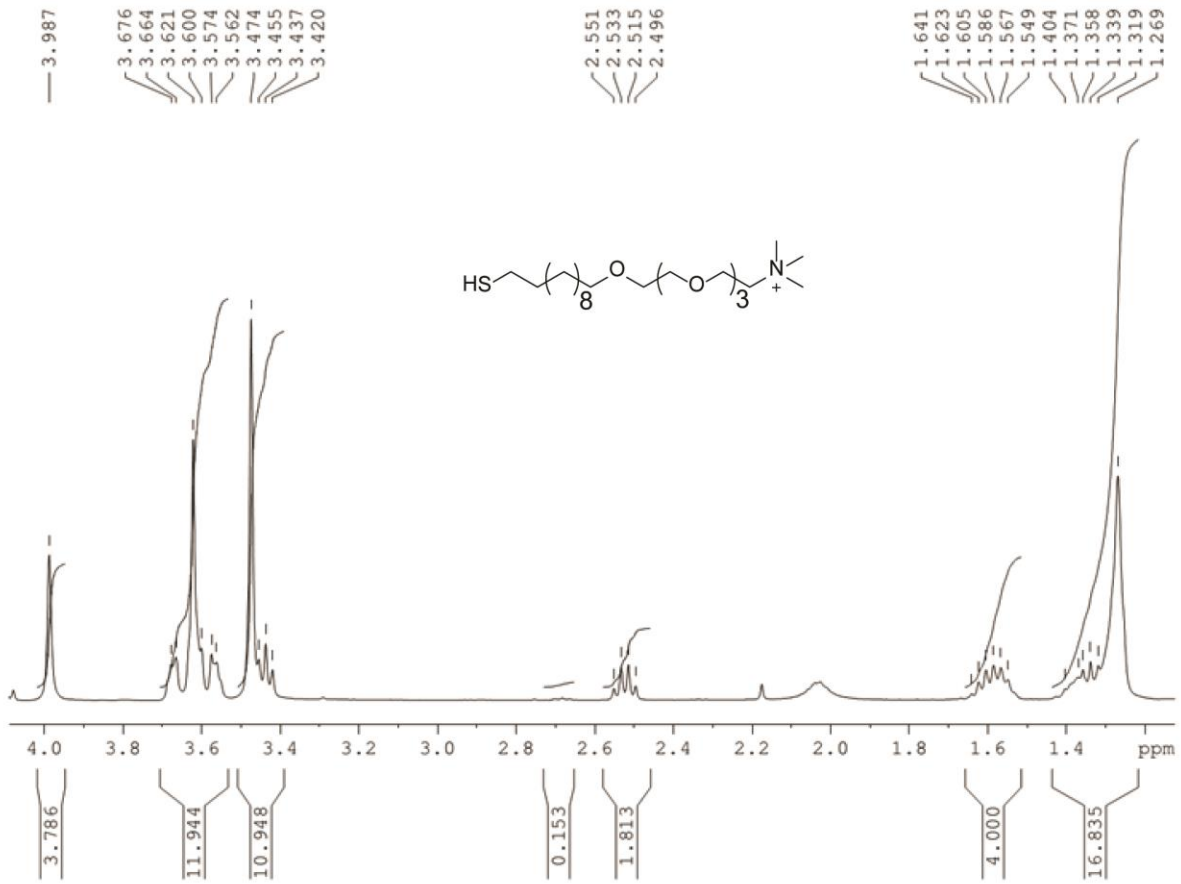


Figure S1: ^{1}H-NMR of compound 5.

^{1}H-NMR (400 MHz, $CDCl_3$): δ 3.987 (s, 2H,-O-CH_2-CO-), 3.676-3.562 (m, 16H, -O-CH_2- CH_2-O-), 3.474 (s, 9H, -N-CH_3), 3.455-3.420 (t, 2H, -CH_2-CH_2-O-), 2.551-2.496 (q, 2H, -CH_2-SH), 1.641-1.549 (m, 4H, -CH_2-), 1.404-1.269 (m, 14H, -CH_2).

Synthesis of Compound 6. In a double neck round bottom flask, sodium hydride (7.21 mmol) was taken and kept on the ice bath under inert gas atmosphere. Then 5 mL of dry THF was added to it. Similarly in an another round bottom flask, compound 1 (2.89 mmol) was dissolved in 5 mL of dry THF under inert atmosphere. Then the latter solution was slowly added to the flask containing sodium hydride solution followed by stirring for 15 min. Ethyl bromoacetate (7.22

mmol) was added to the reaction mixture and the resulting solution was stirred for 4 h under inert gas atmosphere. After completion of the reaction, the reaction mixture was quenched by adding a small amount of water. Then THF was evaporated using rotary evaporator. The obtained crude product was redissolved in ethyl acetate and washed with saturated sodium chloride solution. The organic layer was dehydrated using anhydrous sodium sulfate followed by concentrated by rotary evaporator. The product was purified using column chromatography. The compound was confirmed by ^{1}H-NMR. The yield of the reaction was 75%.

Synthesis of Compound 7. In a double neck round bottom flask, compound 6 (1.99 mmol) was dissolved in 10 mL of toluene. Then AIBN (0.597 mmol) and thioacetic acid (5.97 mmol) were added and the reaction mixture was refluxed for 4 h at 110 ^{0}C under nitrogen atmosphere. After reflux, the reaction mixture was cooled to room temperature. Then ethyl acetate was added and washed with saturated sodium bicarbonate solution for three times. The combined organic layer was dehydrated over anhydrous sodium sulfate and concentrated by using rotary evaporator. The column chromatography is used for the purification of product. The compound was confirmed by ^{1}H-NMR. The yield of the reaction was 76%.

Synthesis of Compound 8 (Negative Ligand). In a round bottom flask, compound 6 (1.96 mmol) was dissolved in 10 mL of MeOH. Then 4 mL of LiOH (1M) solution was added and the reaction mixture was stirred at room temperature for 3 h. After completion of reaction, the reaction mixture was cooled down to 0 ^{0}C and acidified by adding 1M HCl solution to make the pH 2. After that, MeOH was evaporated from the reaction mixture and redissolved in ethyl acetate. Then it was washed with saturated sodium chloride solution and the organic layer was concentrated to get the product. The obtained compound was confirmed by ^{1}H-NMR. The yield of the reaction was quantitative.

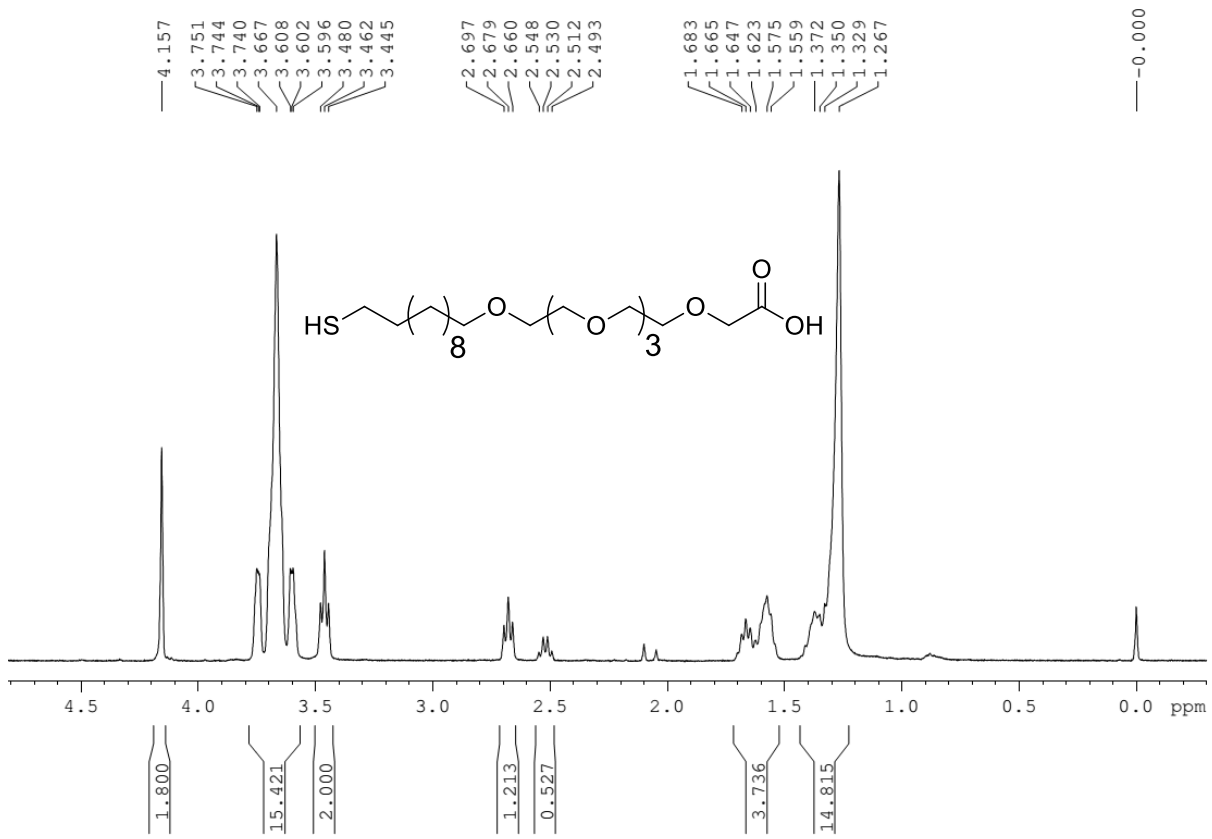


Figure S2: ^{1}H-NMR of compound 8.

^{1}H-NMR (400 MHz, $CDCl_3$): δ 4.157 (s, 2H,-O-CH_2-CO-), 3.751-3.596 (m, 14H, -O-CH_2-CH_2-O-), 3.480-3.445 (t, 2H, -CH_2-CH_2-O-), 2.697-2.660 (t, 2H, -CH_2-S-S-), 2.548-2.493 (q, 2H, -CH_2-SH), 1.683-1.559 (m, 4H, -CH_2-), 1.372-1.267 (m, 14H, -CH_2).

Synthesis of Compound 9 (Neutral Ligand). In a round bottom flask, compound 2 (4.72 mmol) was dissolved in 15 mL of EtOH. Then 2 mL of concentrate HCl was added and the resulting solution was refluxed for overnight. After reflux, the reaction mixture was cooled down to room temperature. Then it was washed with saturated sodium chloride solution and extracted with ethyl acetate for three times. The combined organic layer was concentrated and the product was purified

using column chromatography. The obtained compound was confirmed by ^{1}H-NMR and the yield of the reaction was 95%.

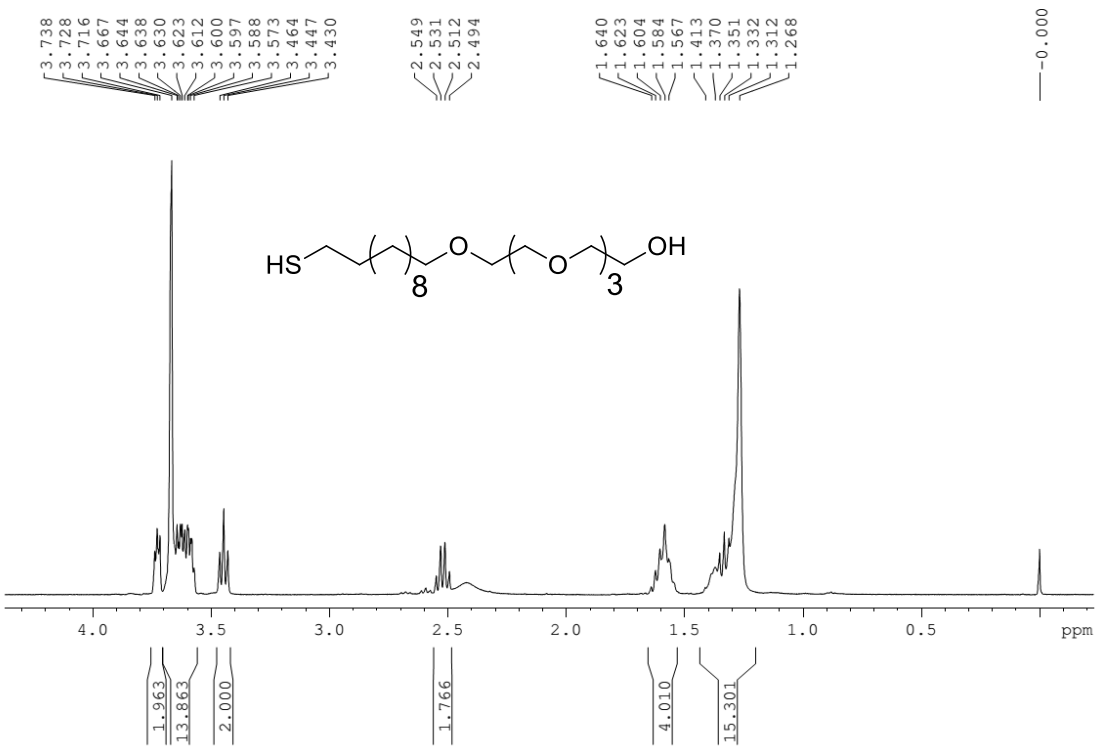


Figure S3: ^{1}H-NMR of compound 9.

^{1}H-NMR (400 MHz, $CDCl_3$): δ 3.738-3.716 (t, 2H, -CH_2-O-), 3.667-3.573 (m, 14H, -O-CH_2-CH_2-O-), 3.464-3.430 (t, 2H, -CH_2-O-), 2.549-2.494 (q, 2H, -CH_2-SH), 1.640-1.567 (m, 4H, -CH_2-),1.413-1.268 (m, 14H, -CH_2-).

Characterization of Exfoliated MoS_2. Exfoliated MoS_2 has been characterized by using various techniques namely atomic force microscopy (AFM), Transmission electron microscopy (TEM) (JOEL JEM-2100F with an accelerating voltage of 200kV), and also x-ray diffraction (using PANalytical Empyrean diffractometer with Cu-Kα irradiation), where revealed the single layer exfoliation of bulk MoS_2.

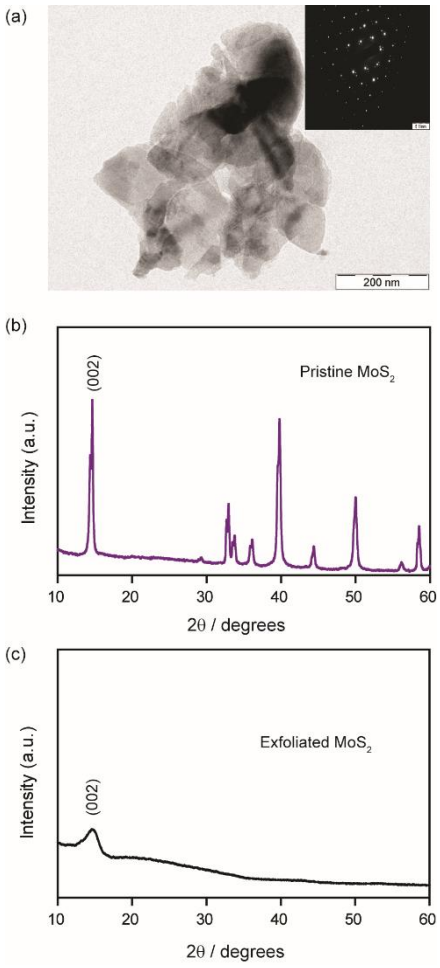


Figure S4: (a) TEM image and the diffraction pattern of exfoliated MoS_2, (b) XRD patterns of pristine MoS_2 and (c) exfoliated MoS_2.

Concentration Measurement of Functionalized MoS_2. Inductively coupled plasma mass spectrometry (ICP-MS) was used to measure the concentration of various MoS_2 samples. In brief, 50 µL of each functionalized MoS_2 solutions were taken and mixed with 100 µL of concentrated HNO_3. The solutions were kept inside the oven at 100 ^{0}C for 4 h for digestion. After digestion, the total volume of the solution was made up to 5 ml by adding DI water. Then, a series of the externals standard solutions were prepared by dissolving required amount of ammonium heptamolybdate $(NH_4)_6Mo_7O_{24}.4H_2O$ in DI-water with maintaining 2% HNO_3 concentrations. The linear regression fitting of the ICP-MS value for external standard solutions gives the accuracy, with

R^2>99.99. After determination of the Mo concentration for sample solutions, the stock solution concentrations were determined by multiplying the dilution factor with the corresponding measured ICP-MS values.

Inhibition Assay. The inhibition assays for β-lactamase were performed in presence of various functionalized MoS_2 following the method mentioned in experimental section. We have monitored the enzymatic activity of β-lactamase over the period of 135 min for native MoS_2 and 240 min for other functionalized MoS_2.

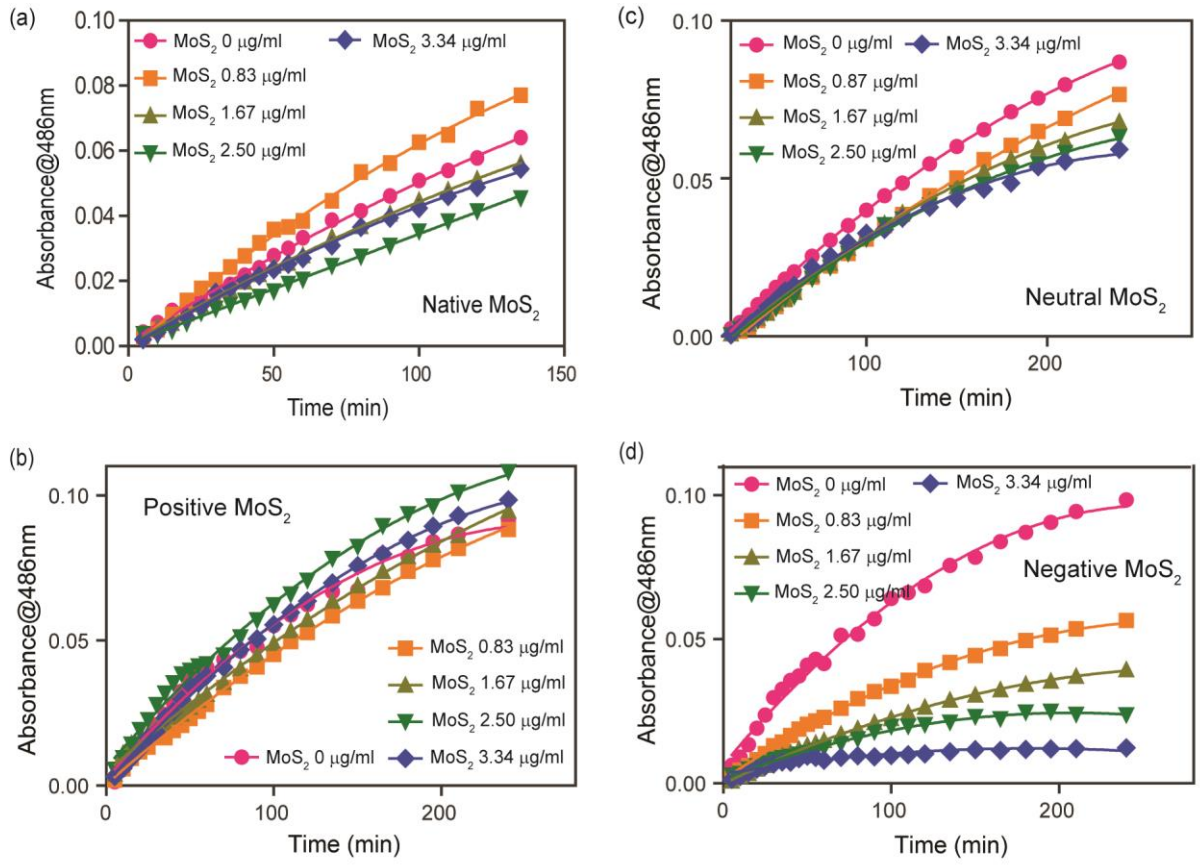


Figure S5: Enzyme activity assay for β-lactamase in presence of (a) native, (b) positive, (c) neutral and (d) negative ligand functionalized MoS_2.

Gel Electrophoresis. Agarose gel electrophoresis was carried out using gel prepared from 1% agarose solution in PBS buffer (pH 7.4). 25 μL of protein solution in PBS buffer of concentration 20 μM and that with MoS_2 solution of 42 μg/mL were incubated 30 min at room temperature. Then 5 μL of 80% of glycerol was mixed with these solutions and finally placed inside the gel cavity. Gel was ran at a constant voltage (100 V) for 1 h. Subsequently the gel was placed in staining solution (0.5% coomassie blue, 40% methanol, 10% acetic acid aqueous solution) for 1 h followed by extensive destaining (40% methanol, 10% acetic acid aqueous solution) until protein bands clearly visualize. At lower concentration of protein (2.5 nM) we have not able to observe the protein band so to visualize the protein band we need to use 20 μM (8000 times) of protein solution. But similarly we are not able to use similar high concentration of functionalized MoS_2 due to the stability issue. So we have used 42 μg/mL (12.5 times) solution of MoS_2. Hence we have observed some bounded and free protein in mixture band.

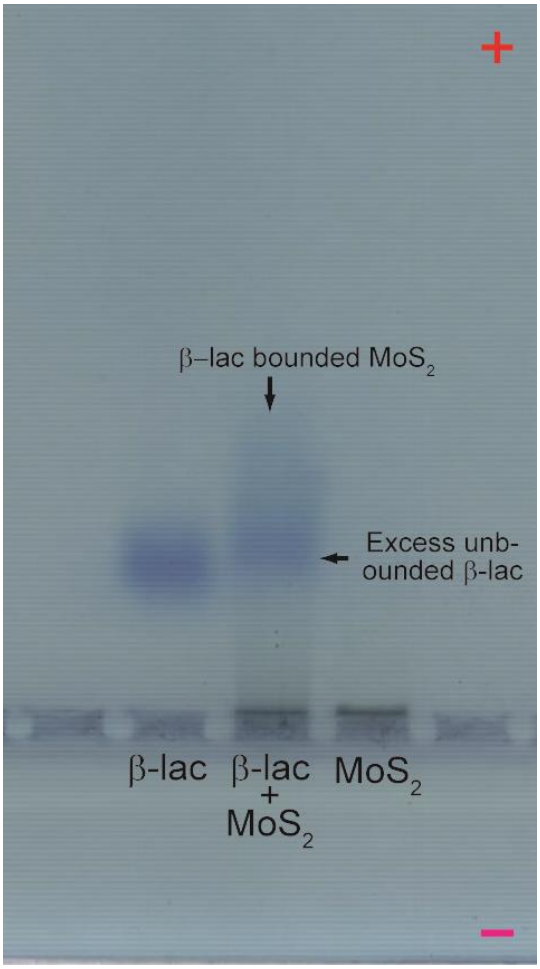


Figure S6: The combination ability of beta lactamase and functionalized MoS_2 analyzed by electrophoresis on 1% agarose gel.

Binding constant estimation from activity assay. The analysis was done by using previously reported method.[3] In our experiment we have assumed that *n* number of clustered MoS_2 is considered as active site and responsible for inhibition of one β-lactamase activity, then the binding between β-lactamase and MoS_2 can expressed as,

$$\text{Active Site + β-lactamase} \underset{}{\overset{K_S}{\rightleftharpoons}} \text{Active Site - β-lactamase} \quad \text{(S-1)}$$

Where K_S is the binding constant. Because the activity of b-lactamase decreases with increasing concentration of MoS_2 which attributes the complex formation is proportional to the activity difference (ΔZ) i.e. $\Delta Z = \alpha \cdot$[Active Site - β-lactamase]. The α is the proportionality coefficient. Then K_S could be defined as:

$$K_S = \frac{[\text{ActiveSite} \cdot \beta\,\text{lactamase}]}{[\text{ActiveSite}][\beta\,\text{lactamase}]} = \frac{\Delta Z/\alpha}{([\text{ActiveSite}]_0 - \Delta Z/\alpha)([\beta\,\text{lactamase}]_0 - \Delta Z/\alpha)} \quad \text{(S-2)}$$

Where $[\text{Active Site}]_0$ and $[\beta\text{-lactamse}]_0$ is the initial concentrations of binding sites and β-lactamase, respectively and the active site concentration can be measured by considering the MoS_2 concentration measured by ICP-MS ($[\text{Active Site}]_0 = 1/n\ [MoS_2]_0$. After a few manipulation, the above equations can be expressed as,

$$\Delta A = \frac{\alpha}{2} \cdot \{([\text{En}]_0 + \frac{1}{n}[MoS_2]_0 + 1/K_S) - \sqrt{([\text{En}]_0 + \frac{1}{n}[MoS_2]_0 + 1/K_S)^2 - \frac{4}{n}[\text{En}]_0[MoS_2]_0}\} \quad \text{(S-3)}$$

$[\text{En}]_0$ is the initial concentration of β-lactamase. Using the above equation, nonlinear curve fitting analysis was done in Origin 8 data analysis software and the analysis data is mentioned in the main text.

References:

1. Chou, S. S.; De, M.; Kim, J.; Byun, S.; Dykstra, C.; Yu, J.; Huang, J. X.; Dravid, V. P. Ligand Conjugation of Chemically Exfoliated MoS_2. *J. Am. Chem. Soc.* **2013**, *135*, 4584-4587.

2. Pandit, S.; Karunakaran, S.; Boda, S. K.; Basu, B.; De, M. High Antibacterial Activity of Functionalized Chemically Exfoliated MoS_2. *ACS Appl. Mater. Interfaces* **2016**, *8*, 31567-31573.
3. You, C. C.; De, M.; Han, G.; Rotello, V. M. Tunable Inhibition and Denaturation of Alpha-Chymotrypsin with Amino Acid-Functionalized Gold Nanoparticles. *J. Am. Chem. Soc.* **2005**, *127*, 12873-12881.