Research Article

# *b*-Lactam Based *a*-Amino and *a*-Hydroxy Phosphonate Ester Molecular Hybrids: Synthesis, Docking Studies and Evaluation of Anti-microbial Activity Against Various Gram-positive and Gram-negative Species

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**ABSTRACT**

Novel β-lactam containing α-amino phosphonate esters and α-hydroxy phosphonates esters were synthesized. Both the lactams were evaluated for antimicrobial activities. It was observed that two of α-amino phosphonates and one α-hydroxy phosphonate exhibited relatively good antimicrobial activities.

**Keywords:** β-Lactams, α-Aminophosphonate esters, β-Hydroxy phosphonates esters and Antimicrobial activities.

## INTRODUCTION

α-Amino phosphonates are intriguing class of bioactive compounds that share structural similarity with α-amino acids and mimic transition state of active peptides. Prominence of α-amino phosphonate derivatives is particularly due to their proven potential as enzyme inhibitors[1], antibiotics[2], peptide mimics [3], herbicides [4], and other pharmacological agents [5]. α-Amino phosphonate derivatives are in general synthesized through various modifications of the original Kabachnik-Fields reaction [6], or Pudovik reaction conditions [7]. In quest of novel compounds with increased pharmacological activity [8, 9] and some of the synthetic efforts dealt with the preparation of hybrid α-amino phosphonate derivatives bearing skeletons such as indoles, thiazoles, pyrazoles, etc. However, to our knowledge there are no reports of α-amino phosphonate derivatives containing β-lactam skeleton. Several β-lactam derivatives such as Cephalosporins, Carbapenems, Nocardicins and Monobactams are known for their anti-microbial activity and pharmacokinetic performance [10]. Additionally, molecules having β-lactam moiety are known for other interesting biological properties, such as Cholesterol absorption inhibitors [11], Human cytomegalovirus protease inhibitors [12], Thrombin inhibitors [13], Antitumour [14], Anti-HIV [15], and Serine-dependent enzyme inhibitors [16, 17]. In the current article, we wish to report the synthesis and biological activities of novel hybrids of α-amino and hydroxy phosphonate analogues bearing substituted β-lactam skeleton.

## RESULTS AND DISCUSSION

The β-lactam skeleton is a four-membered cyclic amide framework and is a core of many pharmacologically active compounds. It was envisaged that the novel molecules designed with structural integrity of α-amino and α-hydroxy phosphonates and β-lactam functionalities would impart synergistic effect towards anti-microbial properties (Figure 1). Hence, the syntheses of the starting materials 4-formyl-2-azetedinones **(3a-c)** were carried out by following the protocol reported by Alcaide and co-workers [18], wherein the substituted acetic acid chlorides were dehydrochlorinated by a base, and in-situ reacted with glyoxal di-imines in toluene underwent cycloaddition, and resulted into the corresponding imine intermediates. Further, acidic work up of those imine intermediates gave rise to the desired racemic *cis* aldehydes exclusively with good to excellent yields. The *cis* stereochemical outcome of racemic products was assigned based on the $H_3$-$H_4$ coupling constants of 4-formyl-2-azetedinones **3a-c** and NOE experiment [18]. The relative stereochemistry of the vicinal protons presents within the β-lactam ring with those present outside the ring were determined, by isolating a crystal of one of the synthesized analogues and analyzed by XRD [19]. With an objective of evaluating the biological profile of the designed molecular hybrids, we synthesized β-lactam based *α*-amino and *α*-hydroxy phosphonates, using mild acid catalysts tartaric and fumaric acid.

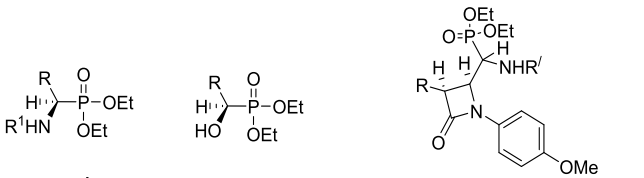


**Figure 1:** General structures of *α*-amino phosphonates (**1a**), *α*-hydroxy phosphonates (**1b**) and β-lactam containing α-amino phosphonate esters (**2a-2l**)

### Chemistry

### Synthesis of *α*-Amino phosphonates

The original syntheses of α-amino phosphonate esters **(1a)** involved the usage of aldehyde/ketone, amine and dialkyl/trialkyl phosphites in the presence of an acid or a base under harsh conditions and those methods were


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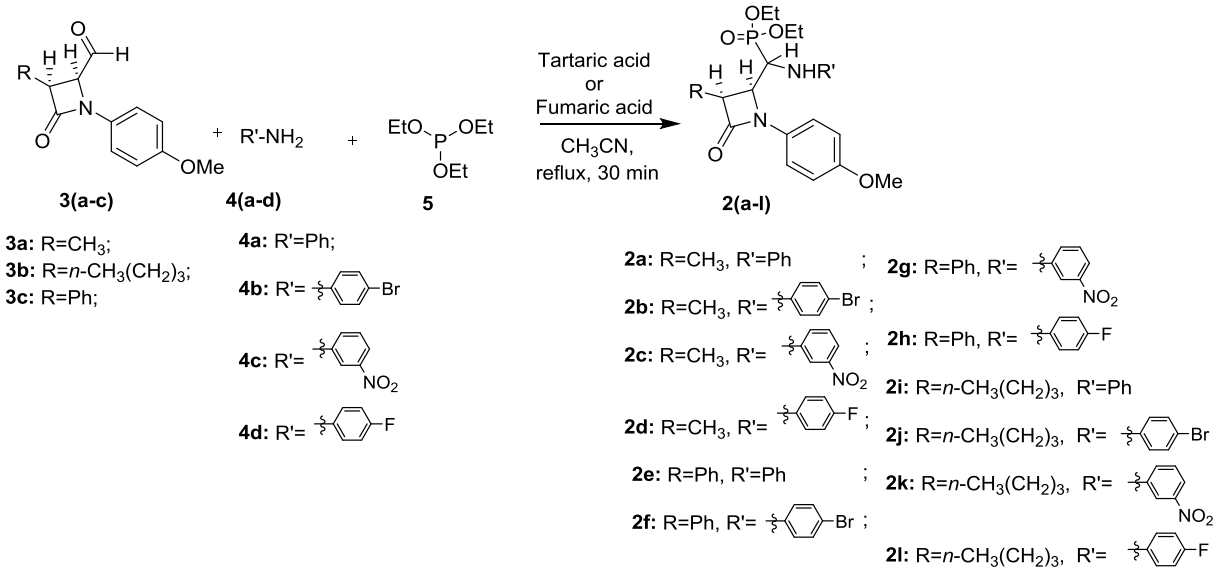
incapable for the synthesis in presence of sensitive functional groups such as β-lactam. Hence, finding the milder reaction conditions is crucial to obtain novel β-lactam containing α-amino phosphonate esters. Cognizant of the sensitivity of β-lactam functionality present in one of the reactants and subsequently in the final products, milder acid conditions were explored by following Kabachnik-Fields type reaction[20] conditions using mild acid catalyst. During our trials probing various mild acidic catalysts with pKa ranging from 2-5, not so advantage was gained from lactic acid and tartaric acid, since the yields of the reactions varied between 10% and 30%; which led us to explore other mild catalysts. However, when fumaric acid was used under identical reaction conditions, product yields improved moderately >50%. Encouraged by these results, a diverse set of analogs **2b-2l**, (Scheme 1) were synthesized by employing fumaric acid as a catalyst in three component synthesis involving *N*-(4-methoxyphenyl) -2-(substituted) propiolactam-3-carbaldehyde (**3a-3c)**, triethylphosphite and aromatic amines **4a-4c** (Scheme 1). The target compounds (**2a-2l**) were isolated as their racemic diastereomeric mixtures after subjecting their crude compounds to purification and recrystallization in di-isopropyl ether produced the products with yields ranging from 45-70%. Overall, the products **2a, 2b, 2e, 2f, 2g** and **2k** were isolated as their racemic single diastereomers, confirmed by interpretation of their respective $^{1}$H and $^{13}$C NMR spectra, while others **2c, 2d, 2h, 2i, 2j** and **2l** were isolated as mixture of diastereomers (Table **1**). All the compounds (**2a-2l**) were characterized by IR, $^{1}$H NMR, $^{13}$C NMR spectroscopy, and mass spectrometry [21].




**Scheme 1:** Synthesis of α-amino phosphonate ester derivatives of N-4-methoxyphenyl-3-substituted-β-propiolactam (**2a-2l**)

### Synthesis of α-Hydroxy phosphonates

Several reports describe the synthesis of α-hydroxy phosphonate esters (**1b**) by reacting aldehydes or ketones with dialkyl phosphonates in the presence of an acid or a base. Acid catalysts like $BF_3.Et_2O$ and $AlCl_3$ or HCl, TFA or TfOH and $Ti(OPri)_4$; and the use of various bases such as, sodium alkoxide, triethylamine, ethyl magnesium bromide, potassium or cesium fluoride, LDA, MgO, and DBU have been reported [22, 23, 24] to synthesize α-hydroxy phosphonate esters (**1b**). However, the reported conditions are not suitable for preparing compounds containing sensitive functionalities such as β-lactam ring since it is susceptible to hydrolysis under basic and acidic reaction conditions. Hence the developed methodology was followed to obtain novel β-lactam containing α-hydroxy phosphonate esters (**7a-7h**) by reacting *N*-(4-methoxyphenyl)-3-(substituted) propiolactam-2-carbaldehyde (**3a-3d**) with trialkyl phosphite (**6a** and **6b**) using a catalytic amount of tartaric acid or fumaric acid (10 mol %) in acetonitrile at 80°C in 30 min produced **7a-7h** (Scheme 2). The products were found to be a mixture of diasteromers and were isolated as their single diastereomers by crystalizing with di-isopropyl ether as a solvent and the yields were ranging from 41-69%. The structure of one of the compounds **7f** was confirmed unequivocally by x-ray diffraction studies [19]. All the compounds (**7a-7h**) were characterized by IR, $^{1}$H NMR, $^{13}$C NMR spectroscopy, and mass spectrometry. The prepared analogues were evaluated against a series of Gram-positive and Gram-negative bacteria for their anti-bacterial activities by comparing with a standard drug.

### Biology

#### *Biological activity α-Amino phosphonates*

#### Molecular docking studies: α-Amino phosphonates

Actinomadura R39 DD-peptidase, a class C Penicillin binding protein (PDB id: 2XLN) was used in docking studies for antimicrobial activity. Co-crystallized ligands and water molecules are removed from target protein using Argus lab. Ligands are prepared using hemoffice (Cambridge). Energy minimization was done using molecular mechanics. The minimized was executed until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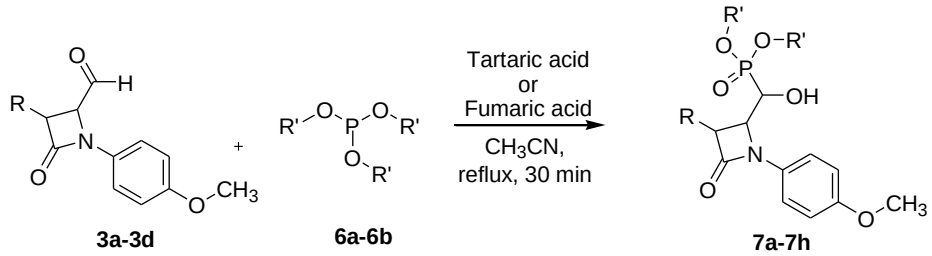
root mean square value reached smaller than 0.001 kcal/mol. Such energy minimized ligands and receptor used for docking studies using GEMDOCK (Generic Evolutionary Method for Molecular Docking) is a generic evolutionary method with an empirical scoring function for the protein–ligand docking, which is a problem of paramount importance in structure-based drug design, combines both continuous and discrete search mechanisms. A population size of 300 with 70 generations and three solutions were used in docking accuracy setting. Docking studies results are summarized in Table (1).




**3a-3d** **6a-6b** **7a-7h**

**3a:** R=$CH_3$;
**3b:** R=*n*-$CH_3(CH_2)_3$;
**3c:** R=Ph;
**3d:** R=PhO;

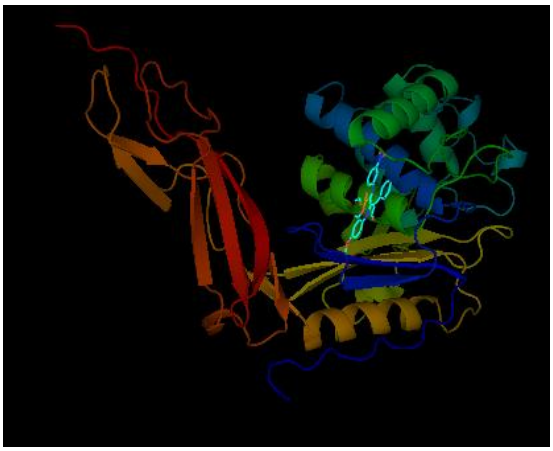
**6a:** R'=$CH_3$;
**6b:** R'=$CH_3CH_2$;

**7a:** R=$CH_3$, R'=$CH_3$;
**7b:** R=$CH_3$, R'=$CH_3CH_2$;
**7c:** R=*n*-$CH_3(CH_2)_3$, R'=$CH_3$;
**7d:** R=*n*-$CH_3(CH_2)_3$, R'=$CH_3CH_2$;
**7e:** R=Ph, R'=$CH_3$;
**7f:** R=Ph, R'=$CH_3CH_2$;
**7g:** R=PhO, R'=$CH_3$;
**7h:** R=PhO, R'=$CH_3CH_2$.

**Scheme 2:** Synthesis of β-lactam containing α-hydroxy phosphonates **7a-7h**

**Table 1:** Results of the molecular docking studies of compounds **2a**-**2l**

| Compounds | Penicillin binding protein |
|---|---|
| | Dock score (-Kcal) |
| **2a** | -119.44 |
| **2b** | -114.51 |
| **2c** | -112.28 |
| **2d** | -110.73 |
| **2e** | -120.98 |
| **2f** | -111.33 |
| **2g** | -132.72 |
| **2h** | -129.45 |
| **2i** | -121.21 |
| **2j** | -123.45 |
| **2k** | -114.5 |
| **2l** | -132.44 |
| **Penicillin** | -121.34 |



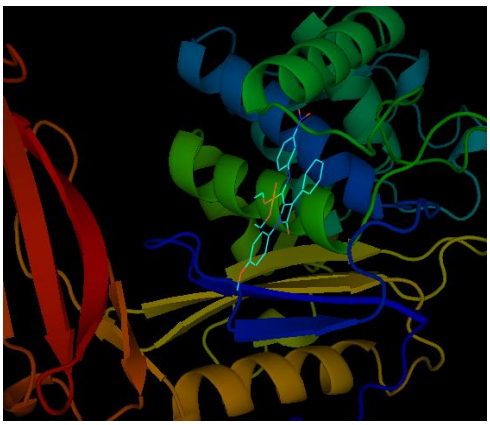


**Figure 2:** Binding orientation of **2g** on penicillin binding protein

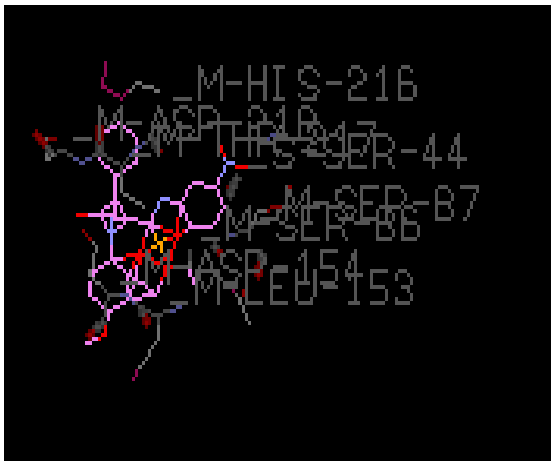



**Figure 3:** Compound **2g** interacts with active site amino acids**.** Molecular docking studies of **2g** on penicillin binding protein (PDB ID 2XLN). (A) Binding orientation of **2g** on penicillin binding protein. (B) **2g** interacts with active site amino acids SER-44, SER-86, SER-87, LEU-153, ASP-154, HIS-216, THR-217, ASP-218 of penicillin binding protein.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***In vitro* MIC studies: α-Amino phosphonates**

Antibacterial activity studies were performed by agar well method. The bacterial strains were grown in Muller-Hinton media (HiMedia Pvt. Ltd., Mumbai, India) at 37 °C and maintained on nutrient agar slants at 4 °C and stored at -20 °C. Inoculum of test organisms were prepared by growing pure isolate in nutrient broth at 37 °C for overnight. The overnight broth cultures were sub-cultured in fresh nutrient broth and grown for 3 h to obtain log phase cultures. The agar plates were prepared by pour plate method using 20 ml M-H medium. The sterile M-H agar medium is cooled to 45 °C and mixed thoroughly with 1 ml of growth culture of concerned test organism (1 x $10^8$ cells) and then poured into the sterile petri dishes and allowed to solidify. Wells of 6 mm size were made with sterile cork borer and compounds were added. The agar plates were incubated at 37 °C for 24 h. The diameter of zones of inhibition was measured in mm using HiMedia zone reader. Results are summarized in Table **2**.

**Table 2:** Antibacterial activity of newly synthesized compounds **2a**-**2l** on various Gram -positive and Gram-negative bacteria

| Compounds | *Staphylococcus aureus* (G+ve) | *Klebsiella pneumoniae* (G-ve) | *Bacillus subtilis* (G+ve) | *Escherichia coli* (G-ve) |
|---|---|---|---|---|
| **2a** | 2 | 3 | 2 | 2 |
| **2b** | 1 | 2 | 2 | 1 |
| **2c** | 4 | 6 | 5 | 4 |
| **2d** | 6 | 5 | 5 | 4 |
| **2e** | 5 | 4 | 4 | 6 |
| **2f** | 6 | 5 | 4 | 3 |
| **2g** | 8 | 4 | 8 | 7 |
| **2h** | 10 | 11 | 8 | 10 |
| **2i** | 4 | 5 | 5 | 5 |
| **2j** | 5 | 4 | 3 | 4 |
| **2k** | 6 | 6 | 6 | 4 |
| **2l** | 9 | 11 | 9 | 10 |
| **Penicillin** | 9 | 8 | 9 | 8 |
| **Ciprofloxacin** | 11 | 10 | 10 | 10 |

Overall, based on the *in vitro* studies, the newly synthesized compounds **2a**-**2l** showed substantial antibacterial activity against Gram-positive and Gram-negative bacteria. Based on results in Table **(2)**, compounds **2h** and **2l** among the tested compounds **2a**-**2l** exhibited antibacterial activities better than Penicillin against all four bacterial strains used in the study. In addition, molecular docking studies demonstrated that compounds **2h** and **2l** among tested compounds showed better binding affinity on penicillin binding protein (Table **1**). These results suggested that wet lab experimental results and *in silico* studies were well correlated. On the other hand, **2d**, **2e**, **2f, 2g** and **2k** showed moderate antibacterial activity and these two compounds, **2a** and **2b** did not show any antibacterial activity.

**Biological evaluation of α-hydroxy phosphonates**

**Molecular docking studies: α-hydroxy phosphonates**

Actinomadura R39 DD-peptidase, a class C Penicillin binding protein (PDB id: 2XLN) was used in docking studies for antimicrobial activity. Co-crystallized ligands and water molecules are removed from target protein using Argus lab. Ligands are prepared using hemoffice (Cambridge). Energy minimization was done using molecular mechanics. The minimized was executed until root mean square value reached smaller than 0.001 kcal/mol. Such energy minimized ligands and receptor used for docking studies using GEMDOCK (Generic Evolutionary Method for Molecular Docking) is a generic evolutionary method with an empirical scoring function for the protein–ligand docking, which is a problem of paramount importance in structure-based drug design, combines both continuous and discrete search mechanisms. A population size of 300 with 70 generations and three solutions were used in docking accuracy setting. Docking studies results are summarized in Table (3).


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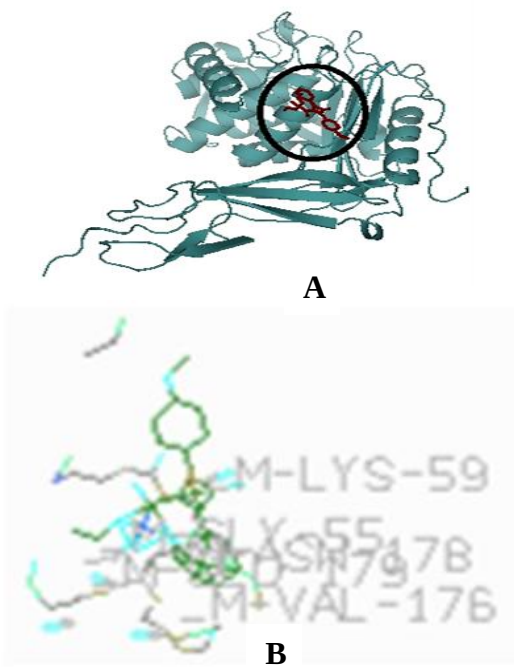


**Figure 4:** (A): Binding orientation of **7f** on actinomadura R39 DD-peptidase crystal protein. (B): Binding interactions of **7f** with active site amino acid residues of actinomadura R39 DD-peptidase protein. Molecular docking studies of 7f on actinomadura R39 DD-peptidase, a class C Penicillin binding protein (PDB id: 2XLN).

***In vitro* MIC studies: α-Hydroxy phosphonates**

Antibacterial activity studies were performed by agar well method. The bacterial strains were grown in Muller-Hinton media (Hi Media Pvt. Ltd., Mumbai, India) at 37 °C and maintained on nutrient agar slants at 4 °C and stored at -20 °C. Inoculum of test organisms were prepared by growing pure isolate in nutrient broth at 37 °C for overnight.

**Table 3:** Results of the molecular docking studies of compounds **7a**-**7h**

| Compounds | Dock score (-Kcal) |
|---|---|
| **7a** | -122.46 |
| **7b** | -110.47 |
| **7c** | -120.58 |
| **7d** | -140.67 |
| **7e** | -131.59 |
| **7f** | -143.22 |
| **7g** | -124.47 |
| **7h** | -129.23 |
| **Penicillin** | -121.34 |

The overnight broth cultures were sub-cultured in fresh nutrient broth and grown for 3 h to obtain log phase cultures. The agar plates were prepared by pour plate method using 20 ml M-H medium. The sterile M-H agar medium is cooled to 45 °C and mixed thoroughly with 1 ml of growth culture of concerned test organism (1 x 108 cells) and then poured into the sterile petri dishes and allowed to solidify. Wells of 6 mm size were made with sterile cork borer and compounds were added. The agar plates were incubated at 37 °C for 24 h. The diameter of zones of inhibition was measured in mm using HiMedia zone reader. Results are summarized in Table 4.

**Table 4:** Antibacterial activity of newly synthesized compounds **7a**-**7h** on various Gram-positive and Gram-negative bacteria

| Compound | *Staphylococcus aureus* (G+ve) | *Klebsiella pneumoniae* (G-ve) | *Bacillus subtilis* (G+ve) | *Escherichia coli* (G-ve) |
|---|---|---|---|---|
| **7a** | 2 | 2 | 3 | 2 |
| **7b** | 0 | 1 | 1 | 1 |
| **7c** | 1 | 1 | 1 | 1 |
| **7d** | 6 | 5 | 5 | 5 |
| **7e** | 3 | 2 | 3 | 3 |
| **7f** | 10 | 9 | 10 | 10 |
| **7g** | 2 | 1 | 1 | 1 |
| **7h** | 3 | 2 | 2 | 2 |
| **Penicillin** | 9 | 8 | 9 | 8 |
| **Ciprofloxacin** | 11 | 10 | 10 | 10 |

**Determination of minimum inhibitory concentration:**

The minimum inhibitory concentration (MIC) of compound was determined using broth dilution assay. The medium containing different concentrations of compound namely 1, 10, 100, 1000 µg/ml prepared by serial dilution. After inoculation with bacterial culture, the tubes were incubated for 24 h at 37 °C. The MIC of each sample was determined by measuring the optical density in the spectrophotometer at 620 nm and compared with the optical density (O.D) value of control in which contain the non-inoculated broth contain same concentration of compound. The MIC values are summarized in Table (5).


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**Table 5:** Minimum inhibitory concentration of β-lactam analogs **7a**-**7h** against Gram-positive and Gram-negative bacteria

| Compound | MIC (µM) | | | |
|---|---|---|---|---|
| | ***Staphylococcus aureus*** **(G+ve)** | ***Klebsiella pneumoniae*** **(G-ve)** | ***Bacillus subtilis*** **(G+ve)** | ***Escherichia coli*** **(G-ve)** |
| **7a** | 100 | 100 | 100 | 100 |
| **7b** | 1000 | 1000 | 1000 | 1000 |
| **7c** | 1000 | 1000 | 1000 | 1000 |
| **7d** | 1 | 1 | 1 | 1 |
| **7e** | 100 | 100 | 100 | 100 |
| **7f** | 10 | 10 | 1 | 10 |
| **7g** | 100 | 100 | 1000 | 100 |
| **7h** | 100 | 100 | 100 | 100 |
| **Penicillin** | 1 | 10 | 10 | 10 |

Overall, based on the in vitro studies, the newly synthesized compounds **7a-7h** showed substantial antibacterial activity against Gram-positive and Gram-negative bacteria. Based on results in Table **4**, compounds **7d** and **7f** among the tested compounds exhibited antibacterial activities better than Penicillin against all four bacterial strains used in the study. These results correlated well with MIC values for compounds **7d** and **7f**, which are comparable to Penicillin, suggesting the potency of these compounds (Table 5). In addition, molecular docking studies demonstrated that compounds **7d** and **7f** among tested compounds showed better binding affinity on penicillin binding protein (Table 3). These results suggested that wet lab experimental results and *in silico* studies were well correlated. On the other hand, **7a**, **7g**, **7e** and **7h** showed moderate antibacterial activity and these two compounds, **7b** and **7c** did not show any antibacterial activity.

## CONCLUSION

In conclusion, fumaric acid and tartaric acids were found to be the suitable catalysts for synthesis of β-lactam-containing α-amino and hydroxy phosphonates. We believe that the synthetic protocol described in this paper present a practical alternative to the existing procedures for the synthesis of these novel analogues possessing functionalities that are unstable in acidic and basic conditions. Most of the synthesized novel -lactam based -amino and hydroxy phosphonate hybrids were tested against several Gram-positive and Gram-negative bacterial strains which displayed interesting anti-bacterial activities better than Penicillin and Ciprofloxacin. The results indicate that, studying further on these candidates may lead to potential hit compounds for making leads for developing NCEs against several multi drug resistant bacterial strains.

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