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Article

Synthesis, In Vitro Antimicrobial Activity, and Molecular Docking Study of Novel Benzylidene Ketamine Analogues as Potential Elongation Factor Tu Inhibitors

Abraham S. Eboh*[,1], Azibanasamesa D. C. Owaba[2], Darlington D. Eboh[3], Okpenkuwo G. Francis[2], Charis Tiemo[2], Laura O. Edward[1]

[1]Department of Biochemistry, Faculty of Basic Medical Science, Niger Delta University, Wilberforce Island, Bayelsa State, Nigeria.
[2]Department of Pharmaceutical and Medicinal Chemistry, Faculty of Pharmacy, Niger Delta University, Wilberforce Island, Bayelsa State, Nigeria.
[3]Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmacy, Niger Delta University, Wilberforce Island, Bayelsa State, Nigeria.

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***Corresponding author:**
eboh.abraham@ndu.edu.ng

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ABSTRACT

The rapid emergence of antimicrobial resistance (AMR) has become a critical global health challenge, leading to prolonged hospitalization, increased healthcare costs, and higher mortality rates. The declining efficacy of conventional antibiotics highlights the urgent need for novel antimicrobial agents with alternative mechanisms of action. This study evaluated the antimicrobial activity of newly synthesized benzylidene ketamine derivatives and explored their potential interactions with bacterial elongation factor Tu (EF-Tu), an essential protein involved in bacterial protein synthesis. Antibacterial and antifungal activities of ketamine and its derivatives (D11-D15) were determined using the broth microdilution method at concentrations ranging from 1 to 512 µg/mL. Ciprofloxacin and fluconazole served as reference antibacterial and antifungal agents, respectively. Molecular docking against EF-Tu (PDB ID: 1DG1) was performed using AutoDock Vina to predict ligand–protein binding affinity. Among the tested compounds, D14 and D15 exhibited the strongest antibacterial activities, with minimum inhibitory concentrations (MICs) of 32 µg/mL against Bacillus subtilis and 64 µg/mL against Staphylococcus aureus, respectively, whereas ketamine showed no detectable antibacterial activity. None of the synthesized compounds inhibited the growth of Candida albicans, while fluconazole displayed an MIC of 1 µg/mL. Docking analysis indicated favorable binding interactions between the active derivatives and the EF-Tu binding site. These findings suggest that benzylidene modification of ketamine enhances antibacterial activity and identifies D14 and D15 as promising lead compounds for the development of EF-Tu-targeted antibacterial agents.

1. Introduction

Antimicrobial resistance (AMR) has emerged as one of the most serious global public health threats of the twenty-first century. The widespread and often inappropriate use of antibiotics has accelerated the evolution of resistant microorganisms, diminishing the clinical efficacy of many first-line antimicrobial agents [1], [2]. Consequently, infections caused by multidrug-resistant pathogens are increasingly associated with prolonged hospitalization, higher treatment costs, therapeutic failure, and elevated mortality rates [3]. Despite the continuous introduction of new antimicrobial agents, the pace of antibiotic discovery has not kept up with the rapid evolution of bacterial resistance, highlighting the urgent need for innovative therapeutic strategies and structurally novel antimicrobial compounds [4], [5].

One of the most effective approaches in modern medicinal chemistry is the structural modification of biologically active molecules to generate new chemical entities with improved pharmacological properties [6]. Rather than developing completely new molecular scaffolds, lead optimization through rational structural modification offers a more efficient strategy for enhancing biological activity, improving selectivity, and overcoming existing mechanisms of drug resistance [7]. Continuous biological evaluation of newly synthesized compounds also provides opportunities to uncover previously unrecognized therapeutic applications and establish new structure activity relationships (SAR).

Ketamine is a well-established dissociative anesthetic widely used in clinical practice because of its rapid onset of action and favorable safety profile. Pharmacologically, ketamine acts primarily as a noncompetitive antagonist of the N-methyl-D-aspartate (NMDA) receptor and has also demonstrated analgesic, antidepressant, anti-inflammatory, and neuroprotective properties [8], [9]. Beyond its established clinical applications, the cyclohexanone framework of ketamine has attracted increasing attention as a versatile pharmacophore for medicinal chemistry because its molecular structure can be readily modified to generate derivatives with diverse biological activities [10].

Among these modifications, the incorporation of a benzylidene moiety into cyclic ketone scaffolds has become an attractive strategy for drug development. Benzylidene-containing compounds share important structural characteristics with chalcones, a class of α,β-unsaturated ketones that has been extensively investigated for their broad spectrum of biological activities, including antibacterial, antifungal, anticancer, anti-inflammatory, antioxidant, and antiviral effects. The presence of the conjugated carbonyl system enhances molecular planarity, lipophilicity, and electrophilicity, facilitating interactions with various biological targets involved in microbial survival [11], [12]. Previous studies have demonstrated that substitution patterns on the aromatic ring of chalcone analogues strongly influence antimicrobial potency, suggesting that similar structural modifications may improve the biological activity of ketamine-derived compounds [13].

In addition to structural optimization, the identification of novel bacterial targets has become increasingly important in antimicrobial drug discovery [5]. Elongation factor Tu (EF-Tu) is an essential GTP-binding protein that mediates the delivery of aminoacyl-tRNA to the ribosome during bacterial protein synthesis [14]. Because EF-Tu is indispensable for bacterial viability and is absent in mammalian cytoplasmic translation, it represents an attractive target for the development of selective antibacterial agents. Several naturally occurring antibiotics exert their antibacterial activity through EF-Tu inhibition, demonstrating the therapeutic relevance of this protein and supporting its use in structure-based drug discovery [15].

Although ketamine has been extensively investigated for its anesthetic and neurological applications, studies exploring the antimicrobial properties of benzylidene-modified ketamine derivatives remain extremely limited. Furthermore, to the best of our knowledge, no previous study has systematically combined in vitro antimicrobial evaluation with in silico molecular docking against bacterial EF-Tu to elucidate the possible antibacterial mechanism of these derivatives. This knowledge gap limits our understanding of how benzylidene substitution influences the antimicrobial activity of ketamine analogues and whether these compounds could serve as promising lead structures for antibacterial drug development [5], [7], [13].

Therefore, the present study aimed to evaluate the antibacterial and antifungal activities of newly synthesized benzylidene ketamine derivatives against representative Gram-positive, Gram-negative, and fungal pathogens using the broth microdilution method. In addition, molecular docking analysis against bacterial elongation factor Tu (PDB ID: 1DG1) was performed to investigate the potential binding interactions and provide preliminary insights into the possible mechanism underlying the

observed antimicrobial activity. By integrating experimental and computational approaches, this study seeks to establish a foundation for the future development of ketamine-derived antibacterial agents with novel structural features and potential activity against drug-resistant bacterial pathogens.

2. Research and Methodology

2.1. Materials

Mueller-Hinton broth (MHB; TM, Manufacturer, Country) and Sabouraud dextrose broth (SDB; TM, Manufacturer, Country) were used as culture media for antibacterial and antifungal susceptibility testing, respectively. Ciprofloxacin injection (200 mg; Fidson Pharmaceuticals, Nigeria) and fluconazole capsules (200 mg; Manufacturer, Country) served as the positive controls for antibacterial and antifungal assays, respectively. Dimethyl sulfoxide (DMSO; JHD, China) was used as the solvent for preparing stock solutions of all test compounds.

Five ketamine-derived benzylidene analogues previously synthesized in our laboratory were investigated in this study: D11, 6 - (4-dimethylaminobenzylidene) -2- (2-chlorophenyl) -2-(methylamino) cyclohexan-1-one; D12, (6E)-6-(4-methoxybenzylidene)-2-(2-chlorophenyl) -2-(methylamino) cyclohexan-1-one; D13, (6E)-6-(4-ethylidene)-2-(2-chlorophenyl)-2-(methylamino)cyclohexan-1-one; D14, (6E)-6-benzylidene-2-(2-chlorophenyl)-2-(methylamino)cyclohexan-1-one; and D15, (6E)-6-(1,3-benzodioxol-5-yl)methylidene-2-(2-chlorophenyl)-2-(methylamino)cyclohexan-1-one. Ketamine hydrochloride (Jawa Pharmaceutical, Indonesia) was included as the reference compound for biological comparison.

2.2. Microorganisms

Antimicrobial activity was evaluated against four clinically relevant bacterial strains, namely Staphylococcus aureus, Bacillus subtilis, Escherichia coli, and Klebsiella pneumoniae, together with the fungal strain Candida albicans. Wherever possible, the corresponding ATCC strain numbers should be reported (e.g., S. aureus ATCC 25923). All microorganisms were maintained on appropriate culture media and subcultured prior to each experiment to ensure cell viability. Fresh bacterial inocula were prepared in Mueller–Hinton broth, whereas C. albicans was cultured in Sabouraud dextrose broth before susceptibility testing..

2.3. Instruments

Routine microbiological procedures were performed using an analytical balance (Ohaus, USA), laboratory autoclave, incubator, and thermostatically controlled water bath. Structural characterization of the synthesized compounds was carried out using ^1H and ^13C nuclear magnetic resonance (NMR) spectroscopy on Bruker Avance III spectrometers operating at 400 and 600 MHz (Bruker BioSpin GmbH, Germany). High-resolution mass spectra (HRMS) were recorded using an Agilent 1290 Infinity UHPLC system coupled to a 6545 quadrupole time-of-flight (QTOF) mass spectrometer (Agilent Technologies, USA) [16].

2.4. Antimicrobial Assay

The antimicrobial activities of ketamine and its benzylidene derivatives (D11–D15) were evaluated using the broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (M07 for bacteria and M27 for yeasts). Serial two-fold dilutions of each compound were prepared in sterile Mueller–Hinton broth to obtain final concentrations ranging from 1 to 512 µg/mL.

Bacterial inocula were adjusted to the turbidity of a 0.5 McFarland standard and diluted to achieve a final inoculum density of approximately 5 × 10^5 CFU/mL. Aliquots of bacterial suspension were dispensed into sterile 96-well microplates containing equal volumes of the test compounds. Ciprofloxacin served as the positive control, while DMSO at the corresponding concentration served as the negative control. Sterility and growth controls were included in every experiment. The plates were incubated at 37°C for 18–24 h, after which the minimum inhibitory concentration (MIC) was recorded as the lowest concentration showing complete inhibition of visible microbial growth. All experiments were performed in triplicate.

Antifungal susceptibility was evaluated against Candida albicans using Sabouraud dextrose broth under the same concentration range (1–512 µg/mL). Fluconazole was employed as the positive control. MIC values were determined after incubation according to CLSI M27 recommendations.

2.5. In Silico Molecular Docking Study

Molecular docking was performed to investigate the binding interactions between ketamine derivatives and bacterial elongation factor Tu (EF-Tu), a highly conserved protein essential for bacterial protein synthesis. The crystal structure of EF-Tu (PDB ID: 1DG1) was retrieved from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB) [14], [15].

Protein preparation was carried out using AutoDock toos, which involved the removal of crystallographic water molecules and co-crystallized ligands, addition of polar hydrogen atoms, assignment of Kollman charges, and conversion of the protein structure into PDBQT format. Three-dimensional structures of ketamine and compounds D11–D15 were obtained from PubChem or generated using ChemDraw and optimized before conversion into PDBQT format with Gasteiger charge assignment.

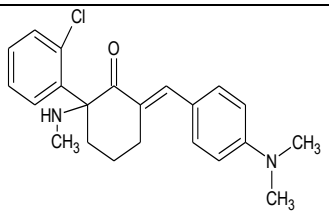
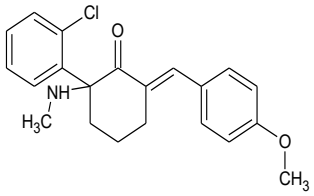
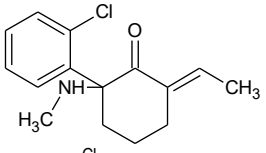
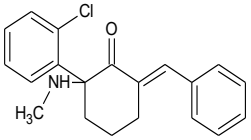
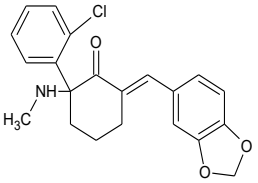
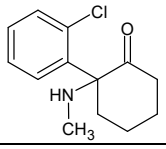
Docking calculations were performed using AutoDock Vina. A grid box centered on the active site of EF-Tu was defined using the coordinates of the native ligand. The grid dimensions, center coordinates, exhaustiveness value, and number of docking modes should be reported here because they are required for reproducibility. Binding poses were ranked according to their predicted binding free energies (kcal/mol), and the conformation with the lowest binding energy was selected for further analysis. Protein–ligand interactions, including hydrogen bonds, hydrophobic contacts, π–π interactions, and van der Waals interactions, were visualized using PyMOL and Discovery Studio Visualizer [17], [18].

3. Results and Discussion

3.1. In Vitro Antimicrobial Activity of Ketamine Benzylidene Derivatives

Microbiological assessment of the compounds (Table 1) showed barely significant antimicrobial activity in a few compounds (D14 and D15) compared to the control (Ciprofloxacin) as shown in Table 3 and 5 and fungi. D14, a benzylidene derivative of ketamine, had an MIC of 32µg/mL against strains of Bacillus sp., a gram positive bacteria. D15, a benzodioxyl methylidene group attached to C-2 position, had an MIC of 64µg/mL against strains of S. aureus, a gram positive bacteria14. This implies that modification enhanced inhibitory effect against gram positive organisms. The antifungal effect of the samples clearly illustrate that the samples (Table 6) were biologically inactive against Candida albican when compared to fluconazole [5], [19].

Table 1. Chemical structures of the samples.

Sample	Chemical name	Chemical Structures
D_{11}	(6*E*)-6-(4-dimethylaminobenzylidene)-2-(2-chlorophenyl)-2-(methylamino)cyclohexan-1-one	
D_{12}	(6*E*)-6-(4-methoxybenzylidene)-2-(2-chlorophenyl)-2-(methylamino)cyclohexan-1-one	
D_{13}	(6*E*)-6-(4-ethylidene)-2-(2-chlorophenyl)-2-(methylamino)cyclohexan-1-one	
D_{14}	(6*E*)-6-benzylidene-2-(2-chlorophenyl)-2-(methylamino)cyclohexan-1-one	
D_{15}	6*E*)-6- benzodioxylmethylidene -2-(2-chlorophenyl)-2-(methylamino)cyclohexan-1-one	
Ketamine	2(2-chlorophenyl)-2-(methylamino)cyclohexan-1-one	

3.2. Antibacterial Activity and Structure-Activity Relationship of Ketamine Benzylidene Derivatives

Figure 1 summarizes the minimum inhibitory concentrations (MICs) of ketamine and its benzylidene derivatives (D11–D15) against the tested bacterial and fungal strains. Overall, the synthesized compounds exhibited selective antibacterial activity rather than broad-spectrum antimicrobial effects. Most derivatives showed MIC values greater than 512 µg/mL against Escherichia coli, Klebsiella pneumoniae, and Candida albicans, indicating that the introduction of the benzylidene moiety alone was insufficient to confer activity against Gram-negative bacteria or fungal pathogens. Similarly, the parent compound ketamine did not exhibit antimicrobial activity against any of the tested microorganisms, suggesting that structural modification is essential for enhancing its biological properties.

Among the synthesized derivatives, D14 demonstrated the highest antibacterial potency against Bacillus subtilis, with an MIC of 32 µg/mL, whereas D15 showed moderate activity against Staphylococcus aureus, exhibiting an MIC of 64 µg/mL. The remaining compounds (D11–D13) were inactive against all tested microorganisms. These findings indicate that subtle structural modifications on the benzylidene aromatic ring significantly influence antibacterial activity. The superior activity of D14 suggests that the unsubstituted benzylidene group may provide a more favorable balance between

steric effects and hydrophobic interactions, facilitating interaction with bacterial targets. Conversely, the benzodioxole substituent present in D15 appears to retain moderate antibacterial activity, particularly against S. aureus, possibly due to enhanced lipophilicity and improved interactions with essential bacterial proteins.

The lack of activity against E. coli and K. pneumoniae can be attributed to the structural characteristics of Gram-negative bacteria, which possess an outer lipopolysaccharide membrane that restricts the penetration of many hydrophobic compounds. In addition, multidrug efflux pumps may further reduce intracellular drug accumulation, thereby limiting antibacterial efficacy. In contrast, Gram-positive bacteria lack this outer membrane, allowing greater accessibility of small lipophilic molecules to intracellular targets. This difference in cell envelope architecture may explain the selective activity observed for D14 and D15 against B. subtilis and S. aureus, respectively.

These results are consistent with previous studies reporting that benzylidene- and chalcone-based derivatives generally exhibit greater antibacterial activity against Gram-positive bacteria than Gram-negative species because of differences in membrane permeability and target accessibility. Collectively, the present findings suggest that benzylidene modification enhances the antibacterial potential of ketamine in a structure-dependent manner, although further structural optimization will be required to improve potency and broaden the antimicrobial spectrum [5], [16], [17], [20].

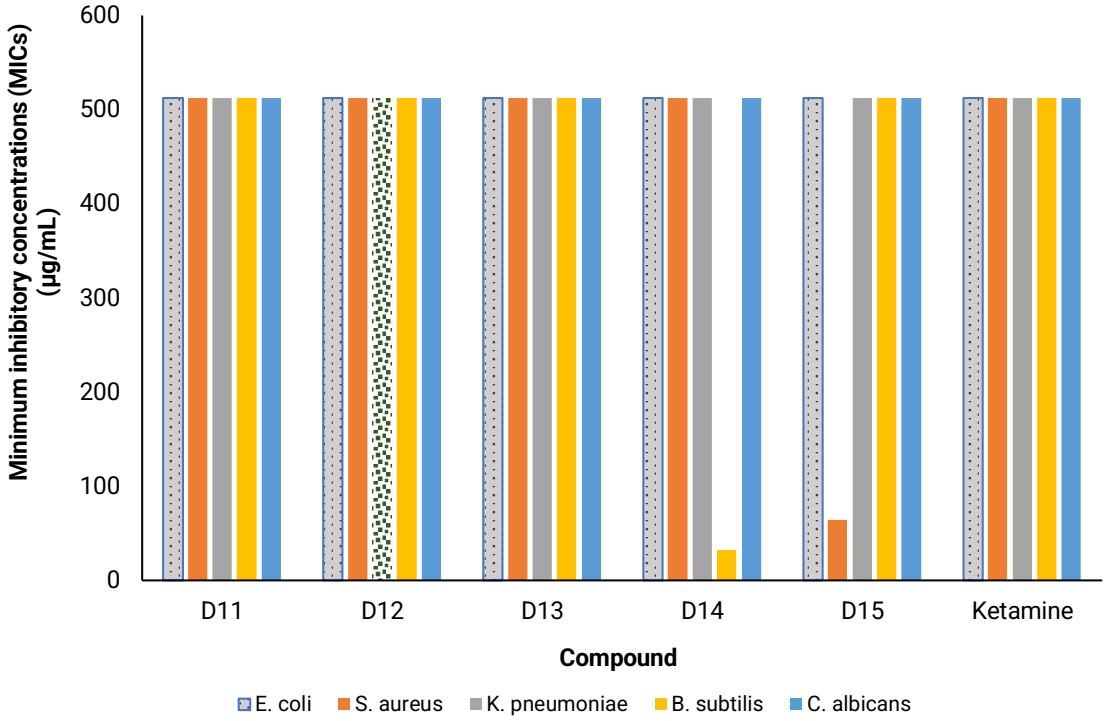


Figure 1. Minimum inhibitory concentrations (MICs) of ketamine and its benzylidene derivatives against tested microorganisms

To gain mechanistic insight into the antibacterial activity of the synthesized compounds, molecular docking simulations were performed against bacterial elongation factor Tu (EF-Tu; PDB ID: 1DG1), an essential GTP-binding protein involved in bacterial protein synthesis. The predicted binding energies of ketamine, its benzylidene derivatives (D11-D15), and the reference drug ciprofloxacin are presented in Table 2. Overall, the compounds exhibited binding energies ranging from -3.612 to -4.985 kcal/mol, indicating moderate binding affinity toward the EF-Tu active site. Among the investigated compounds, ketamine displayed the most favorable docking score (-4.985 kcal/mol), followed by D13 (-4.584 kcal/mol), whereas D14 and D15 exhibited binding energies of -3.639 and -3.948 kcal/mol, respectively. Interestingly, this computational trend was not consistent with the in vitro antimicrobial results, where ketamine showed no detectable antibacterial activity against any of the tested microorganisms, while D14 and D15 demonstrated selective antibacterial activity against Bacillus subtilis (MIC = 32 µg/mL)

and Staphylococcus aureus (MIC = 64 µg/mL), respectively. This apparent discrepancy indicates that binding affinity alone is insufficient to predict antimicrobial potency.

Although molecular docking estimates the thermodynamic favorability of ligand-protein interactions, the biological activity of antimicrobial agents is also governed by several additional factors, including membrane permeability, molecular lipophilicity, aqueous solubility, intracellular accumulation, metabolic stability, and susceptibility to bacterial efflux pumps [21]. Furthermore, the crystal structure employed in this study represents EF-Tu as an isolated protein and does not account for the complex physiological environment of bacterial cells or the influence of cell envelope architecture, particularly the permeability barriers present in Gram-negative bacteria. Consequently, compounds with relatively favorable docking scores may fail to exhibit biological activity if they cannot effectively reach their intracellular targets. Similar inconsistencies between molecular docking predictions and experimental antimicrobial activity have frequently been reported in medicinal chemistry studies, emphasizing that molecular docking should be regarded as a complementary tool for elucidating potential binding modes rather than as a definitive predictor of antimicrobial potency [22]. Therefore, the selective antibacterial activities observed for D14 and D15 are likely attributable not only to their interactions with EF-Tu but also to favorable physicochemical properties that facilitate cellular uptake and target accessibility in Gram-positive bacteria. Collectively, these findings highlight the importance of integrating computational predictions with experimental validation to provide a more comprehensive evaluation of the antibacterial potential of newly synthesized ketamine-derived benzylidene analogues [8], [23].

Table 2. Docking Scores for 1DG1

S/N	Code	Docking Scores (kcal/mol)
1.	D11	-4.099
2.	D12	-4.019
3.	D13	-4.584
4.	D14	-3.639
5.	D15	-3.948
6.	Ketamine	-4.985
7.	Ciprofloxacin	-3.612

3.4. Protein-Ligand Interaction Analysis

To further elucidate the molecular basis underlying the antibacterial activity of the synthesized compounds, the protein–ligand interaction profiles of ketamine, its benzylidene derivatives (D11–D15), and ciprofloxacin within the active site of bacterial elongation factor Tu (EF-Tu; PDB ID: 1DG1) were analyzed and are summarized in Table 8. Overall, all ligands occupied a similar binding pocket and established multiple non-covalent interactions, including hydrophobic contacts, hydrogen bonds, polar interactions, π-cation interactions, salt bridges, and π–π stacking. Several amino acid residues, particularly Val20, Phe46, Pro82, Thr25, Thr26, Asp50, Lys136, and Leu175, were consistently involved in ligand recognition, suggesting that these residues constitute key interaction hotspots within the EF-Tu binding cavity. The recurrence of these residues across different ligand complexes indicates that the synthesized derivatives share a common binding mode despite differences in their chemical substituents.

Among the synthesized compounds, D11 exhibited the most extensive interaction network by simultaneously forming hydrophobic interactions, hydrogen bonds, a salt bridge with Asp50, and a π-cation interaction with Lys136, features generally associated with enhanced complex stabilization. D12 retained a similar interaction pattern but lacked π-cation interactions, whereas D13 was stabilized predominantly through hydrophobic, hydrogen-bonding, and polar interactions without electrostatic contributions such as salt bridges or π-cation contacts. In contrast, the biologically active compounds D14 and D15 displayed a distinct interaction profile characterized by π-cation interactions with Lys136, accompanied by extensive hydrophobic and polar contacts. Notably, D15 interacted with additional residues, including Ser173 and Asn135, which may contribute to a more favorable ligand orientation within the binding pocket. These observations suggest that the spatial arrangement and quality of

specific interactions, rather than the total number of contacts, may play a more important role in ligand recognition and stabilization.

Interestingly, the interaction patterns observed in the docking study did not completely correlate with the experimental antimicrobial results. Although ketamine exhibited the most favorable binding energy and formed a π–π stacking interaction with Phe46, it was biologically inactive against all tested microorganisms. Likewise, D11 and D12 established several favorable interactions, including salt bridges and hydrogen bonds, yet failed to demonstrate measurable antibacterial activity. Conversely, D14 and D15 exhibited selective antibacterial activity against *Bacillus subtilis* and *Staphylococcus aureus*, respectively, despite possessing comparatively weaker docking scores. These findings indicate that predicted binding affinity and interaction profiles alone are insufficient to explain antimicrobial efficacy. Instead, biological activity is likely influenced by additional physicochemical and pharmacokinetic factors, including membrane permeability, lipophilicity, intracellular accumulation, metabolic stability, and bacterial efflux mechanisms, which collectively determine whether a compound can reach and effectively inhibit its intracellular target [24].

Comparison with the reference compound further supports this interpretation. Ciprofloxacin formed a diverse interaction network involving hydrogen bonds, π-cation interactions, hydrophobic contacts, and π–π stacking despite exhibiting a less favorable docking score than ketamine. Nevertheless, ciprofloxacin displayed the strongest antibacterial activity in the biological assay, demonstrating that the overall biological response depends not only on calculated binding energy but also on productive molecular recognition, efficient cellular uptake, and favorable pharmacodynamic properties. Similar discrepancies between molecular docking predictions and experimental antimicrobial activities have been reported in previous medicinal chemistry studies, emphasizing that docking should be considered a hypothesis-generating tool for identifying potential binding modes rather than a quantitative predictor of antimicrobial potency [16], [23], [25]. Collectively, the present findings suggest that benzylidene modification of ketamine enhances target recognition while simultaneously modulating physicochemical characteristics that contribute to the selective antibacterial activities observed for D14 and D15, thereby supporting their potential as promising lead compounds for further antibacterial optimization [26].

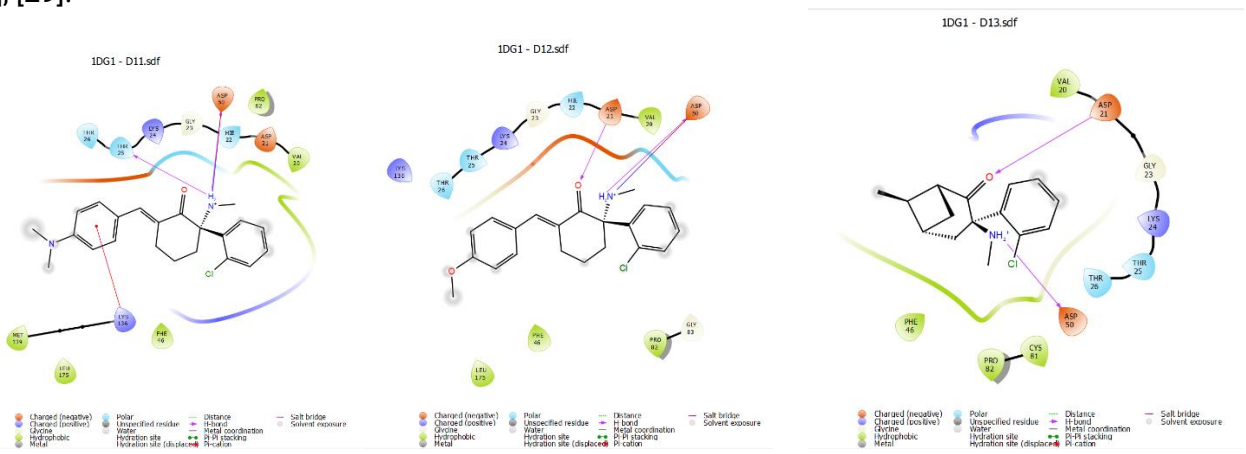
Table 8. Major Protein-ligand interactions for 1DG1

S/N	Code	Type of Interaction and Amino Acid Residues					
		Hydrophobic	**Hydrogen**	**Polar**	**Pi-Cation**	**Salt bridge**	**Pi-pi stacking**
1.	D11	VAL-20, PRO-82, PHE-46, LEU-175, MET-139	THR-25, ASP-50	THR-26, THR-25, HIE-22	LYS-136	ASP-50	-
2.	D12	VAL-20, PRO-82, PHE-46, LEU-175	ASP-50, ASP-21	THR-26, THR-25, HIE-22	-	ASP-50	-
3.	D13	VAL-20, PHE-46, PRO-82, CYS-81	ASP-50, ASP-21	THR-26, THR-25	-	-	-
4.	D14	PHE-46, PRO-82, MET-139, LEU-175, ALA-174	-	THR-26, THR-25, SER-173, ASN-135	LYS-136	-	-
5.	D15	VAL-20, PRO-82, PHE-46, ALA-174, LEU-175, MET-139	GLY-23	HIE-22, THR-26, THR-25, SER-173, ASN-135	LYS-136	-	-
6.	Ketamine	PHE-46, VAL-20, CYS-81, PRO-82	ASP-21, ASP-50, THR-25	THR-25, THR-26	-	-	PHE-46
7.	Ciprofloxacin	PHE-46, VAL-20, CYS-81, PRO-82, LEU-175, LEU-178	ASP-21, GLY-23, THR-25	HIE-22, THR-25, THR-26	PHE-46	-	PHE-46

3.5. Protein-Ligand Interaction Analysis

he two-dimensional protein–ligand interaction maps (Figure 2) provide detailed insights into the binding patterns of ketamine, its benzylidene derivatives (D11–D15), and ciprofloxacin within the active site of bacterial elongation factor Tu (EF-Tu). Overall, all ligands occupied a similar binding pocket and established multiple non-covalent interactions, including hydrogen bonds, hydrophobic contacts, electrostatic interactions, π-cation interactions, salt bridges, and π–π stacking. Several amino acid residues, particularly Asp21, Asp50, Thr25, Thr26, Phe46, Val20, Pro82, Lys136, Leu175, and Met139, were consistently involved in ligand recognition, indicating that these residues constitute the principal binding region of EF-Tu. Among the synthesized compounds, D11 exhibited the most extensive interaction network by forming hydrogen bonds with Thr25 and Asp50, a salt bridge with Asp50, and a π-cation interaction with Lys136, whereas D12 and D13 displayed comparatively simpler interaction patterns. In contrast, D14 and D15, the only compounds exhibiting measurable antibacterial activity, shared a common π-cation interaction with Lys136, while D15 additionally established polar interactions with Ser173 and Asn135, suggesting improved stabilization within the binding cavity.

Despite ketamine exhibiting the most favorable docking score, its interaction profile did not translate into detectable antibacterial activity, whereas D14 and D15 demonstrated selective inhibition against Gram-positive bacteria. This finding indicates that the biological performance of the synthesized compounds cannot be explained solely by predicted binding affinity or the number of molecular interactions. Instead, antibacterial efficacy is likely governed by the combined influence of binding orientation, interaction with key catalytic residues, membrane permeability, intracellular accumulation, and other physicochemical properties [27]. The interaction patterns observed in Figure 4 therefore complement the docking energy analysis by demonstrating that residues such as Lys136, Asp50, Thr25, Thr26, and Phe46 play important roles in ligand recognition and stabilization within EF-Tu. Collectively, these results support the hypothesis that benzylidene substitution modifies the molecular recognition of ketamine derivatives toward EF-Tu and may contribute to the selective antibacterial activity observed experimentally, highlighting these residues as potential targets for future structure-based optimization [28], [29].



(a) **(b)** **(c)**

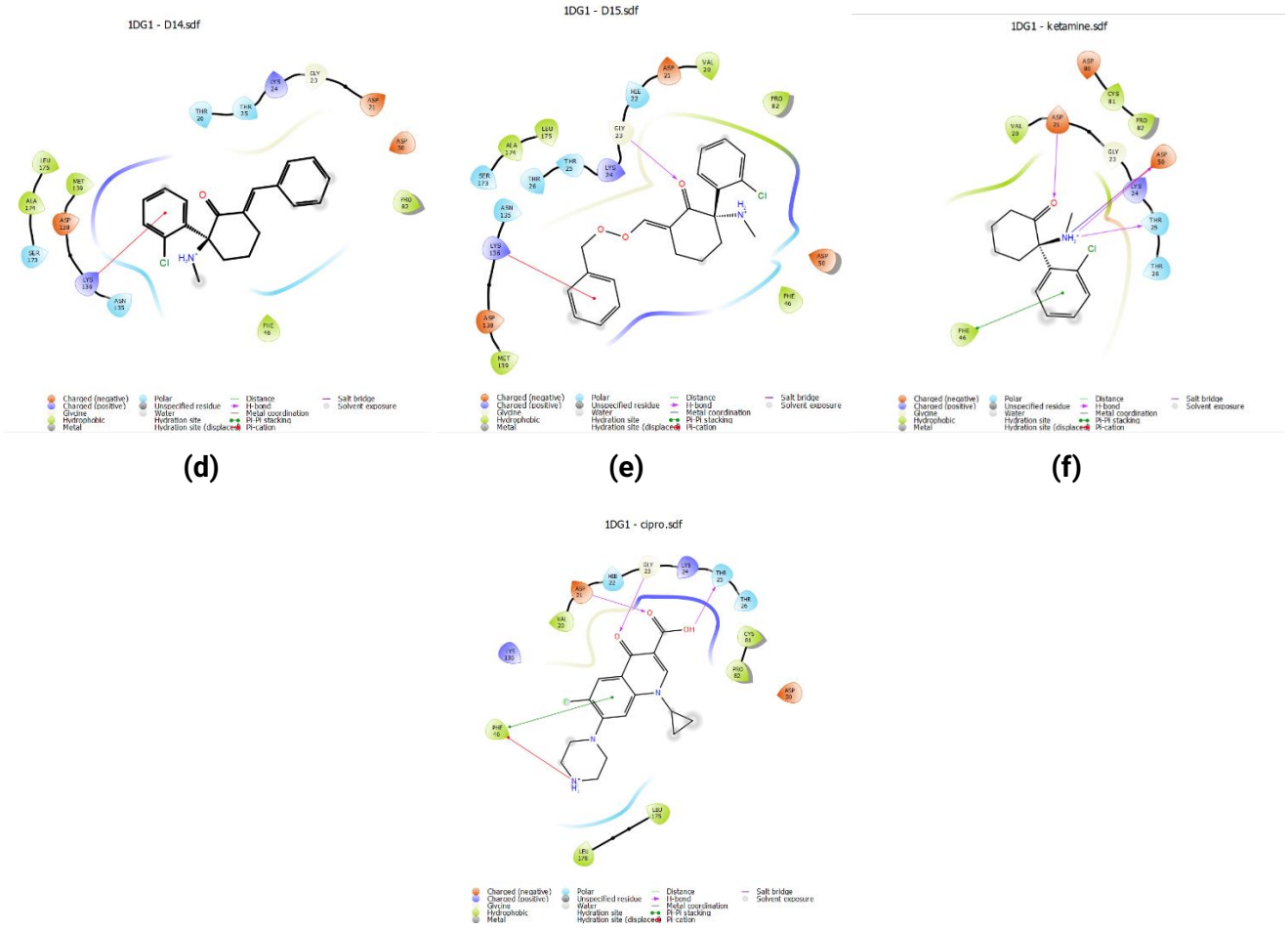


Figure 2. 2D interaction of **(a)** D11, **(b)** D12, **(c)** D13, **(d)** D14, **(e)** D15, **(f)** Ketamin and **(g)** Ciprofloxacin with **1DG1**

To further investigate the molecular recognition patterns responsible for the predicted binding affinities, the three-dimensional binding poses of ketamine, its benzylidene derivatives (D11 –D15), and ciprofloxacin within the active site of bacterial elongation factor Tu (EF-Tu; PDB ID: 1DG1) were analyzed (Figure 3). All compounds occupied a similar binding cavity, suggesting that the synthesized derivatives retained the ability to recognize the EF-Tu active site despite structural modifications on the benzylidene moiety.

The interaction patterns revealed that the synthesized compounds established multiple non-covalent interactions with key amino acid residues lining the binding pocket. Hydrogen bonding predominantly involved Asp21, Asp50, Thr25, and Gly23, whereas hydrophobic contacts were mainly observed with Phe46, Val20, Pro82, Leu175, and Met139. In addition, compounds D11, D14, and D15 formed favorable electrostatic interactions with Lys136, which may contribute to stabilization of the ligand within the active site. These recurring interactions indicate that Asp50, Thr25, Lys136, and Phe46 constitute important recognition residues for ligand binding to EF-Tu.

Interestingly, although ketamine displayed the most favorable docking score, its interaction profile did not translate into measurable antibacterial activity. Conversely, D14 and D15, which demonstrated selective antibacterial activity against Bacillus subtilis and Staphylococcus aureus, respectively, adopted well-oriented binding conformations that enabled productive interactions with key residues within the binding cavity. These observations further support the notion that the spatial arrangement of ligand–protein interactions, rather than binding energy alone, may contribute to antibacterial activity. Similar findings have been reported in medicinal chemistry studies, where effective biological activity

is determined not only by receptor affinity but also by physicochemical properties governing membrane penetration and intracellular target accessibility.

Overall, the three-dimensional interaction analysis complements the docking energy data by illustrating the binding orientations adopted by the synthesized derivatives within EF-Tu. The combined computational and experimental findings suggest that benzylidene substitution modulates ligand orientation and interaction patterns, which may partially explain the selective antibacterial activities observed for D14 and D15 [28], [29].

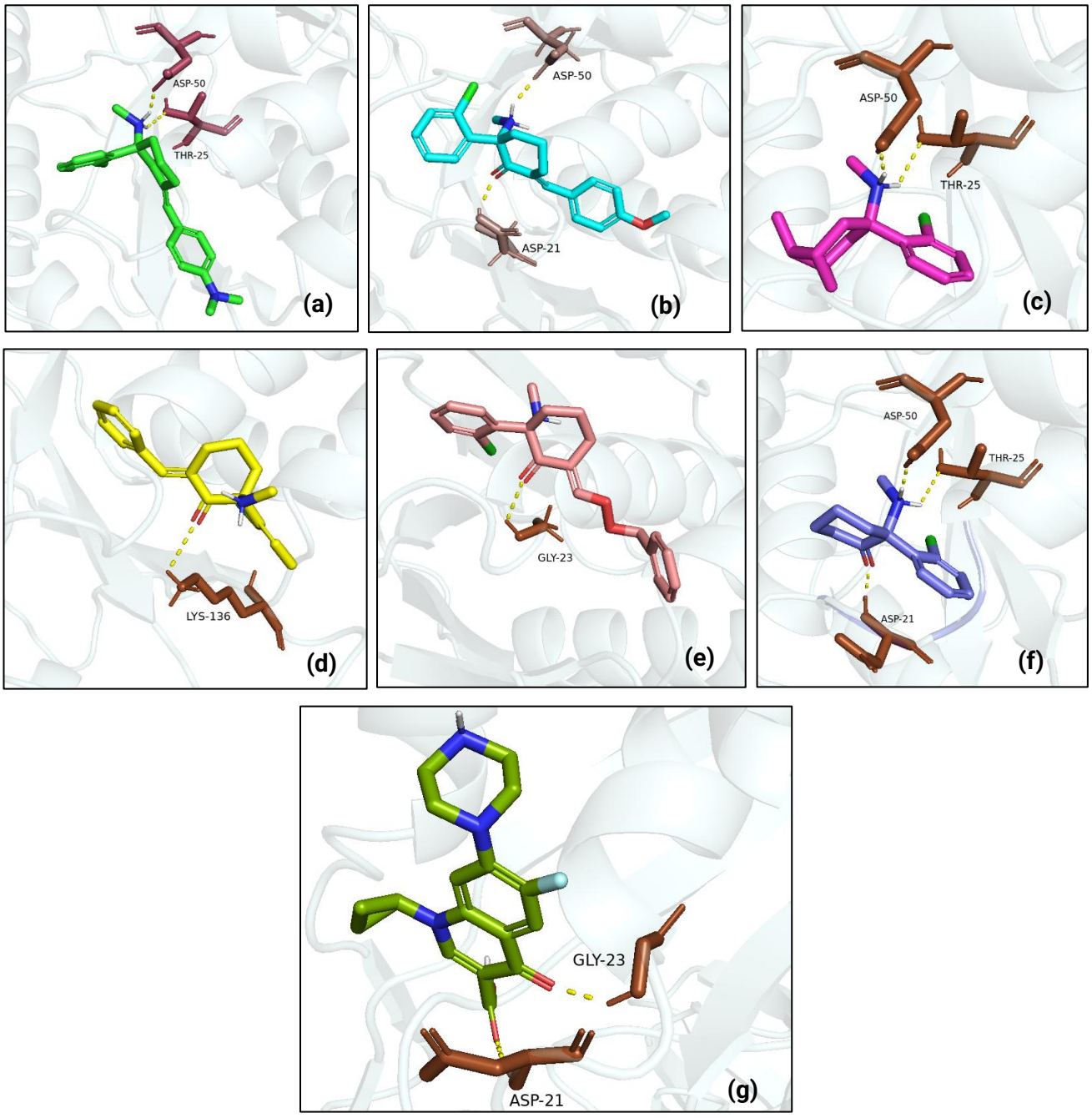


Figure 3. Three-dimensional binding poses of (a) D11, (b) D12, (c) D13, (d) D14, (e) D15, (f) ketamine, and (g) ciprofloxacin within the active site of bacterial elongation factor Tu (EF-Tu; PDB ID: 1DG1). Hydrogen bonds are represented by yellow dashed lines.

3.6. Molecular Docking and Protein-Ligand Interaction Analysis

To further elucidate the potential molecular basis of the observed antibacterial activity, the binding interactions of ketamine and its benzylidene derivatives (D11–D15) with bacterial elongation factor Tu (EF-Tu; PDB ID: 1DG1) were examined using molecular docking. As summarized in Table 2, all compounds exhibited moderate binding affinities toward the EF-Tu active site, with docking scores ranging from −3.612 to −4.985 kcal/mol. Ketamine showed the most favorable predicted binding energy (−4.985 kcal/mol), followed by D13 (-4.584 kcal/mol), whereas D11, D12, D14, and D15 displayed docking scores of -4.099, -4.019, -3.639, and -3.948 kcal/mol, respectively. However, these computational results did not directly correlate with the in vitro antimicrobial findings, as ketamine and compounds D11–D13 were biologically inactive, whereas D14 and D15 exhibited selective antibacterial activity against Bacillus subtilis and Staphylococcus aureus, respectively. These observations indicate that docking scores alone are insufficient to predict antibacterial efficacy and should be interpreted together with experimental data [11], [13], [27].

Detailed interaction analysis revealed that the binding of the synthesized derivatives was stabilized through a combination of hydrogen bonding, hydrophobic contacts, electrostatic interactions, and aromatic interactions within the EF-Tu binding pocket [29]. Compound D11 formed a π-cation interaction with Lys136 and a salt bridge with Asp50, together with several hydrophobic and hydrogen-bond interactions that contributed to its favorable docking pose. Similarly, D12 established a salt bridge with Asp50 in addition to hydrogen-bonding and hydrophobic interactions, whereas D13 was predominantly stabilized by hydrophobic and polar contacts without forming π-cation or salt bridge interactions. Compounds D14 and D15 showed favorable interactions involving Lys136, while ketamine formed a π–π stacking interaction with Phe46. Despite these predicted interactions, only D14 and D15 demonstrated measurable antibacterial activity, suggesting that productive target binding alone is not sufficient for biological efficacy. The ability of a compound to penetrate the bacterial cell envelope, evade efflux systems, and achieve adequate intracellular concentrations likely plays an equally important role in determining antimicrobial activity [30], [31]. Therefore, the docking results should be regarded as supporting evidence for potential target recognition rather than definitive proof of the antibacterial mechanism. Collectively, the computational and experimental findings suggest that the benzylidene modification of ketamine may improve target recognition while simultaneously altering physicochemical properties that contribute to the selective antibacterial activities observed for D14 and D15.

4. Conclusion

The various inferences made from the experimental results suggest that the benzylidene derivatives synthesized from ketamine, particularly (6E)-6-benzylidene-2-(2-chlorophenyl)-2-(methylamino)cyclohexan-1-one, and (6E)-6-[(2H-1,3-benzodioxol-4-yl)methylidene]-2-(2-chlorophenyl)-2-(methylamino)cyclohexan-1-one, have good prospects in developing novel antibacterial agents. Although the MICs of these compounds were relatively high, further modifications and studies may prove their effectiveness in this field.

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