



Green mechanochemical synthesis of bis-imine derivatives: Multi-target docking, MD simulations, and in silico evaluation as potential antibacterial agents

HemanthKumar Dharmapuri^a, György Marcell Tokaji^{b,c}, R. Udaya Rajesh^d, Béla Fiser^{b,*}, A. Singhal^{a,*}

^a Department of Chemistry, School of Chemical Sciences, St Joseph's University, Bangalore, India

^b Institute of Chemistry, Faculty of Materials and Chemical Engineering, University of Miskolc, Miskolc, Egtetenváros 3515, Hungary

^c Wanhua-BorsodChem Zrt, Bolyai tér 1, Kazincbarcika, Hungary

^d Materials Research Centre, Department of Chemistry, IISc Bangalore, 560012, India

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ABSTRACT

This study reports a green, solvent-free mechanochemical synthesis of novel bis-imine derivatives, offering a rapid and sustainable alternative to conventional thermal methods. The target compounds were obtained in 10 to 15 min via grinding, compared to 60 to 90 min under reflux. Although mechanochemical method yields (48–64%) were moderately lower than conventional yields (53–83%), the approach significantly reduces reaction time and eliminates the use of organic solvents. All the synthesized compounds were structurally characterized using ¹H NMR, ¹³C NMR, HR-MS, FT-IR, and CHN analysis. Molecular docking against four bacterial targets (DNA Gyrase, Topoisomerase IV, Dihydropteroate Synthetase, and tRNA Synthetase) identified tRNA Synthetase and DNA Gyrase as the most promising ones based on docking scores. To further validate the stability of the protein-ligand interactions, 100 ns molecular dynamics (MD) simulations were performed on all the ligands in complexed with tRNA Synthetase and DNA Gyrase using GROMACS. Additional ADMET predictions have been done to describe toxicity and pharmacological properties of the synthesized bis-imines. This work combines synthetic and computational approaches to highlight the potential of mechanochemically synthesized bis-imines to identify promising candidates that may be useful for developing new antibacterial agents against resistant bacteria.

1. Introduction

Imines, also known as Schiff bases, are a highly sought-after class of organic molecules characterized by the presence of carbon-nitrogen double bond (C=N). This versatile functional group serves as a key structural motif for the synthesis of diverse N-containing heterocyclic compounds and emphasizes their broad utility in medicinal chemistry [1]. They exhibit a wide spectrum of pharmacological activities including antimalarial [1,2], antimicrobial [3,4], antitubercular [5], anti-fungal [6], anti-alzheimer [7], and anti-cancer [8], making them attractive scaffolds for drug discovery. Further derivatization of imine yields various heterocycles with a wide range of therapeutic potential such as quinolines, oxazines, imidazo[2,1-b]thiazoles, pyridines, triazoles, and thiadiazoles to name a few. For instance, imidazo[2,1-b]

thiazole-based thiadiazole derivatives have demonstrated promising anti-proliferative effects against hepatic cancer, supported by docking studies revealing strong binding to Glypican-3 protein [9]. Similarly, pyridine-thiazole hybrids have shown potent antiviral properties and were identified as potential SARS-CoV-2 inhibitors via molecular docking and dynamics studies [10]. While novel bis-imine curcuminoids and bis-isatin Schiff bases have demonstrated potent and selective anticancer activity against MCF-7 and HeLa cell lines [8,11,12].

Given their broad applicability and role as key intermediates, the development of efficient synthetic routes to imines and derived heterocycles remains highly relevant. The classical method of synthesis of imines via the condensation of primary amines with aldehydes or ketones [13], has been extensively optimized under various green-approaches including solvent-free [2], microwave-assisted [14–16]

* Corresponding authors

E-mail addresses: bela.fiser@uni-miskolc.hu (B. Fiser), anchal.singhal@sju.edu.in (A. Singhal).

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and ultrasonic techniques [17]. While compounds **5a** and **5c** were previously synthesized under catalytic reflux conditions with piperidine [18], the present work introduces a solvent-free mechanochemical grinding protocol for the synthesis of four novel bis-imine derivatives (**5a**, **6a-c**). This approach aligns with green chemistry principles by eliminating organic solvents and reducing energy consumption. Recent studies have explored related heterocyclic scaffolds and their biological potential [19–22].

The relentless rise of multidrug resistance (MDR) bacteria has become a worldwide health issue, necessitating the discovery of new antibiotic agents with novel mechanisms of action [23,24]. Bis-imines, with their structural flexibility and established bioactivity including antimicrobial [25] and anticancer [26,27] properties represent a promising scaffold in this pursuit. Their versatility extends to catalysis [25] and coordination chemistry [26], further highlighting their utility.

Integrating sustainable synthesis with rational design is crucial for modern drug discovery. Molecular docking provides valuable insights such as key interactions and binding energy in identification of potential pharmacophores. In this study, we report the solvent-free mechanochemical synthesis of novel bis-imines and their computational evaluation via molecular docking and molecular dynamics simulations against key bacterial protein targets. These simulations provide critical insights into binding interactions and affinities, and complex stability helping to identify the most promising candidates for future development.

2. Materials and methods

4-hydroxybenzaldehyde, 4-chloroaniline and 1,4-dibromobutane were purchased from LobaChemie. 4-formylbenzoic acid was purchased from Spectrochem. Aniline was purchased from Merck. *p*-Anisidine (LR) was purchased from High Purity Lab Chemicals (HPLC). Glacial acetic acid was purchased from EMPLURA, Merck. Potassium hydroxide and dimethylformamide were purchased from EMPARTA, Merck. Ethyl acetate (AR) and hexane (AR) were purchased from Molychem. Ethanol was purchased from Laboratory Solutions India. FT-IR spectral data were recorded using BRUKER FTIR. ¹H NMR and ¹³C NMR were analyzed using BRUKER using CDCl₃ and DMSO-*d*₆ as solvents. Mass spectrometry was analyzed using High Resolution Accurate Mass (HRAMS) orbitrap analyzer coupled with quadrupole precursor ion selection (Thermo Q Exactive HF Benchtop LC-MS/MS system). Elemental analysis was conducted utilizing a CHNS elemental analyzer (Perkin Elmer 2400 CHNS/O series). Melting points of the synthesized compounds were recorded using the Duralab Digital Melting/ Boiling Point Apparatus. Molecular docking studies were carried out using the following software: Auto Dock Tools 1.5.7, Avogadro, and PyMOL. LigPlot was used to visualize 2D protein-ligand interactions. MD simulations were performed using the GROMACS software package.

2.1. Experimental procedure

2.1.1. Synthesis of ether-linked dialdehyde [4,4'-(butane-1,4-diylbis(oxy)) dibenzaldehyde] (**3a**)

10 mmol (1.2212 g) of 4-hydroxybenzaldehyde (**1a**) was dissolved in 10 mL of *N,N*-dimethylformamide (DMF) in a round-bottom flask. 10 mmol (0.561 g) of KOH was dissolved in a minimum amount of water and was slowly added to the solution of 4-hydroxybenzaldehyde in DMF while stirring continuously. The reaction mixture turned slightly turbid and was stirred at room temperature for 10–15 min. After stirring, 5 mmol (0.597 mL) of 1,4-dibromobutane (**2**) was added and refluxed for about 20–25 min in an oil bath. The progress of the reaction was monitored by TLC at regular intervals using hexane and ethyl acetate (7:3) as mobile phase. After completion of the reaction, ice-cold water was gradually added to the reaction mixture with continuous stirring. The precipitate obtained was filtered to yield a light brown solid with an 87% yield.

2.1.2. Synthesis of ester-linked dialdehyde [Butane-1,4-bis (4-formyl benzoate)] (**3b**)

10 mmol (1.5 g) of 4-formylbenzoic acid (**1b**) was dissolved in DMF in a round-bottom flask. 10 mmol (0.561 g) of KOH was dissolved in a minimum amount of water and was slowly added to the solution of 4-formylbenzoic acid in DMF while stirring continuously. The reaction mixture turned slightly turbid and was stirred at room temperature for 10–15 min. After stirring, 5 mmol (0.597 mL) of 1,4-dibromobutane (**2**) was added and refluxed for about 20–25 min in an oil bath. The progress of the reaction was monitored by TLC at regular intervals using hexane and ethyl acetate (7:3) as mobile phase. After completion of the reaction, ice-cold water was gradually added to the reaction mixture with continuous stirring. The obtained precipitate was filtered to get a colorless solid with an 85% yield.

2.1.3. General procedure for synthesis of bis-imines

Bis-imines were synthesized using conventional reflux (Method A) and mechanochemical grinding (Method B).

2.1.3.1. Method a. A solution of 3 mmol of respective amine (**4a-c**) in 5 mL ethanol was taken in a round bottom flask, and 1–2 drops of glacial acetic acid were added. 1.5 mmol of corresponding dialdehyde (**3a**, **3b**) was added, and the reaction mixture was heated to reflux for 60–90 min. The completion of the reaction was monitored by TLC using hexane and ethyl acetate (7:3) as mobile phase. The crude solid obtained was recrystallized using DMF (**5a** and **5b**, due to low solubility) and by using acetone (**6a**, **6b**, **6c**, **5c**) affording bis-imines in yields ranging from 53% to 83% respectively.

2.1.3.2. Method B. 1.5 mmol of corresponding dialdehyde (**3a**, **3b**) and 3 mmol of respective amine (**4a-c**) were taken together in a mortar and a drop of glacial acetic acid was added. The reaction mixture was ground for 10–15 min using a pestle. The progress of the reaction was monitored by TLC using hexane and ethyl acetate (7:3) as mobile phase. The crude solid obtained was recrystallized in the same way as mentioned above yielding the desired bis-imines in yields ranging from 48% to 64% respectively.

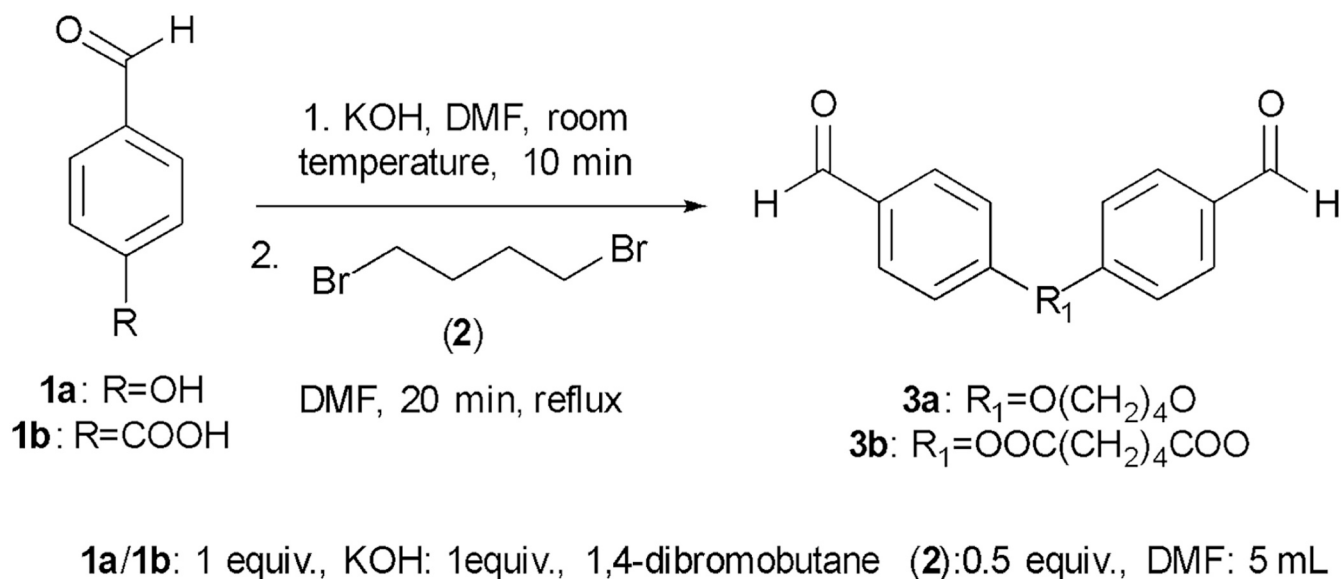
3. Results and discussion

The synthesis of dialdehydes **3a** and **3b** (Scheme 1) was achieved by reacting commercially available *p*-hydroxybenzaldehyde (**1a**) or *p*-carboxybenzaldehyde (**1b**) with 1,4-dibromobutane (**2**), setting the step for bis-imine formation (Scheme 2). The target bis-imines (**5a-c**, **6a-c**) were synthesized via two distinct routes, conventional reflux (Method A, 60–90 min) and solvent-free mechanochemical grinding (Method B, 10–15 min), enabling a comparison of yield and efficiency between the two approaches. While Method A generally gave higher yields, Method B provided a greener, solvent-free alternative at room temperature with acceptable yields (48–64%). Extending the grinding time to 25 min did not improve the yield of the reaction. The yields and melting points of all synthesized dialdehydes and bis-imines are given in Tables 1 and 2, respectively.

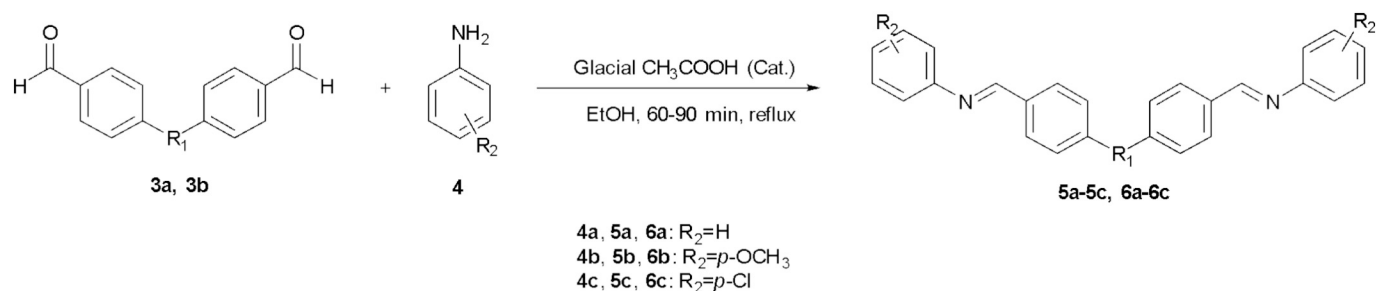
Yields were influenced by the electronic nature of amine substituents. The amine bearing electron-donating group (EDG) exhibited slightly higher reactivity, leading to enhanced yields while the electron withdrawing group (EWG) resulted in marginally lower yield. This trend was observed across both synthetic methods.

The structures of all synthesized bis-imines (**5a-c** and **6a-c**) were confirmed using a combination of ¹H NMR, ¹³C NMR, FT-IR, HR-MS, and CHN elemental analysis.

In the ¹H NMR spectrum of representative compound **6a** (recorded in CDCl₃ at 400 MHz), a characteristic signal appeared as a sharp singlet at δ 8.713 ppm (2H) corresponding to the two equivalent imine (-CH=N-) protons. The aromatic protons resonated as two doublets at δ



Scheme 1. Synthesis of dialdehydes



3a/3b: 1 equiv., 4: 2 equiv., EtOH: 5-10 mL

Scheme 2. Synthesis of bis-imines

Table 1
Yield and melting points of the synthesized dialdehydes.

Sl.no	Aldehyde	Dialdehyde	Yield (%)	Melting point range found (°C)
1	1a	3a	87	102–105
2	1b	3b	85	106–110 [28]

Table 2
Yield and Melting points of the synthesized bis-imines.

Sl. no	Dialdehyde	Amine	Product	Method A Yield (%)	Method B Yield (%)	Melting point range (°C)
1	3a	4a	5a	53	48	183–186
2	3a	4b	5b	83	58	220–225
3	3a	4c	5c	68	48	202–204
4	3b	4a	6a	70–72	57	131–133
5	3b	4b	6b	75–80	64	185–190
6	3b	4c	6c	73	60	203–205

7.966–7.987 ppm (4H) and δ 8.135–8.156 ppm (4H) with the phenyl protons resonating between δ 7.232–7.433 ppm with δ 7.26 ppm being the solvent peak the butyl protons are assigned to the peaks at δ 4.452 ppm (broad singlet, -O-CH₂, 4H) and δ 2.000 ppm (broad singlet, -CH₂-, 4H). Although these methylene protons are expected to show triplet,

they appeared as broad singlets. This is commonly observed due to signal averaging in very similar chemical environments of the methylene groups leading to unresolved triplet. Similar NMR spectrum where the protons in the butyl chain is seen as singlet rather than a multiplet was reported in the literature [29].

The ¹³C NMR spectrum of 6a displayed imine carbon at δ 159.10 ppm confirming successful condensation. The ester carbonyl appeared at δ 166.09 ppm, while the aromatic carbons resonated between δ 120.93–151.92 ppm. The butyl linker carbons appeared at δ 64.77 ppm (-OCH₂-) and δ 25.57 (-CH₂-), consistent with the proposed structure.

FT-IR spectroscopy provided further evidence of imine formation. Compound 6a exhibited a strong absorption band at 1621.72 cm⁻¹ corresponding to the -C=N- stretching vibration. The absence of characteristic aldehyde (-CHO-) and primary amine (-NH₂) stretching bands confirmed complete conversion of the starting materials.

In HR-MS (ESI positive ion mode), 6a showed a prominent [M + H]⁺ peak at *m/z* 505.21262, which matched the calculated value.

The dichloro derivatives 5c and 6c displayed the characteristic isotopic cluster with an intensity ratio of approximately 9:6:1 for [M + H]⁺: [M + H + 2]⁺: [M + H + 4]⁺, confirming the presence of two chlorine atoms in the molecule.

Elemental (CHN) analysis of 6a showed observed (%): C, 75.024; H, 5.331; N, 5.679, closely matching the expected values of C, 76.17; H, 5.59; N, 5.55, confirming its purity. Similar spectral and analytical patterns were observed for all other bis-imine derivatives, with minor

expected variations based on substituents.

Compounds **5a** and **5b** exhibited poor solubility in common deuterated solvents, preventing acquisition of solution state ^1H NMR spectra. However, their structures were confirmed by solid-state ^{13}C NMR showing characteristic imine peaks, HR-MS peaks matching with the calculated $[\text{M} + \text{H}]^+$ values (**5a**: $[\text{M} + \text{H}]^+$ calculated for $\text{C}_{30}\text{H}_{28}\text{N}_2\text{O}_2$ 449.22235, found, 449.22257, **5b**: $[\text{M} + \text{H}]^+$ calculated for $\text{C}_{32}\text{H}_{32}\text{N}_2\text{O}_4$ 509.24348, found, 509.24418.), FT-IR and CHN elemental analysis.

3.1. Spectral data

3.1.1. Butane-1,4-diylbis(oxy))bis(4,1-phenylene)bis(N-phenylmethanimine) [5a]

^{13}C NMR (solid state, 100 MHz) δ (ppm) 162.33 ($\text{Ar}^{13}\text{C}=\text{O}$), 160.80 ($^{13}\text{C}=\text{N}$), 154.52 ($\text{Ar}^{13}\text{C}-\text{N}$), 134.31, 131.87, 130.22, 128.39, 124.97, 118.56, 112.09, 68.741, 25.089; IR (cm^{-1}) ν 2949.84, 2932.34, 2919.38, 1633.93, 1620.61 ($\text{C}=\text{N}$ stretch), 1604.54, 1586.95, 1572.10, 1507.82, 1482.04, 1464.44, 1445.37, 1422.10, 1237.96 (ether $\text{C}-\text{O}$ stretch), 1162.99, 1106.21, 1043.95, 1023.54, 969.73, 906.40, 876.54, 837.59, 817.71, 757.53, 691.04, 643.69, 624.92, 612.20; Mass (m/z) $[\text{M} + \text{H}]^+$: calculated for $\text{C}_{30}\text{H}_{28}\text{N}_2\text{O}_2$ 449.22235, found, 449.22257. Elemental analysis: calculated for $\text{C}_{30}\text{H}_{28}\text{N}_2\text{O}_2$, C, 80.33; H, 6.29; N, 6.25; found, C, 79.824; H, 6.012; N, 6.073.

3.1.2. Butane-1,4-diyl bis(4-((phenylimino)methyl)benzoate) [6a]

IR (cm^{-1}) ν 1715.63 (ester $\text{C}=\text{O}$ stretch), 1621.72 ($\text{C}=\text{N}$ stretch), 1588.05, 1578.08, 1484.1, 1412.75, 1395.65, 1360.44, 1288.18 (ester $\text{C}-\text{O}$ stretch), 1251.93, 190.96, 1177.83, 1167.30, 1123.22, 1018.99, 974.07, 958.78, 909.38, 841.82, 767.71, 693.79; ^1H NMR (400 MHz, CDCl_3) δ (ppm) 8.52 (s, 2H, $2 \times \text{CH} = \text{N}$), 8.16–8.14 (d, 4H, $\text{Ar}-\text{H}$), 7.99–7.97 (d, 4H, $\text{Ar}-\text{H}$), 7.43–7.41 (d, 4H, $\text{Ar}-\text{H}$), 7.28–7.23 (m, 6H, $\text{Ar}-\text{H}$), 4.45 (br s, 4H, $2 \times \text{CH}_2$), 2.00 (br s, 4H, $2 \times \text{CH}_2$); ^{13}C NMR (100 MHz, CDCl_3) δ (ppm) 166.09 ($^{13}\text{C}=\text{O}$), 159.10 ($^{13}\text{C}=\text{N}$), 151.52 ($\text{Ar}^{13}\text{C}-\text{N}$), 140.06, 132.42, 129.98, 129.26, 128.68, 126.54, 120.93, 64.77 ($^{13}\text{CH}_2-\text{O}$), 25.57 ($^{13}\text{CH}_2-\text{C}$); Mass (m/z) $[\text{M} + \text{H}]^+$: calculated for $\text{C}_{32}\text{H}_{28}\text{N}_2\text{O}_4$ 505.21218, found, 505.21262. Elemental analysis: calculated for $\text{C}_{32}\text{H}_{28}\text{N}_2\text{O}_4$, C, 76.17; H, 5.59; N, 5.55; found, C, 75.024; H, 5.331; N, 5.679.

3.1.3. Butane-1,4-diylbis(oxy))bis(4,1-phenylene)bis(N-(4-methoxyphenyl)methanimine) [5b]

^{13}C NMR (solid state, 100 MHz) δ (ppm) 160.69 ($\text{Ar}^{13}\text{C}-\text{O}$), 158.61 ($^{13}\text{C}=\text{N}$), 156.90 ($\text{Ar}^{13}\text{C}-\text{OMe}$), 143.71 ($\text{Ar}^{13}\text{C}-\text{N}$), 136.63, 130.59, 128.75, 117.89, 110.68, 68.13, 55.07, 25.94; IR (cm^{-1}) ν 1620.04 ($\text{C}=\text{N}$ stretch), 1603.64, 1572.83, 1507.66, 1464.56, 1439.06, 1418.58, 1396.36, 1304.85, 1289.14, 1239.73 (ether $\text{C}-\text{O}$ stretch), 1192.34, 1167.41, 1110.46, 1028.68, 1010.98, 956.59, 943.34, 840.33, 816.04, 796.59, 753.98; Mass (m/z) $[\text{M} + \text{H}]^+$: calculated for $\text{C}_{32}\text{H}_{32}\text{N}_2\text{O}_4$ 509.24348, found, 509.24418. Elemental analysis: calculated for $\text{C}_{32}\text{H}_{32}\text{N}_2\text{O}_4$, C, 75.57; H, 6.34; N, 5.51; found, C, 75.53; H, 6.71; N, 5.34.

3.1.4. Butane-1,4-diyl bis(4-(((4-methoxyphenyl)imino)methyl)benzoate) [6b]

IR (cm^{-1}) ν 1714.09 ($\text{C}=\text{O}$ stretch), 1622.66 ($\text{C}=\text{N}$ stretch), 1592.33, 1504.52, 1475.10, 1442.30, 1411.44, 1273.73 (ester $\text{C}-\text{O}$ stretch), 1246.34, 1161.45, 1114.54, 1027.29, 833.19, 768.47; ^1H NMR (400 MHz, CDCl_3) δ (ppm) 8.47 (s, 2H, $2 \times \text{CH} = \text{N}$), 8.14–8.12 (d, 4H, $\text{Ar}-\text{H}$), 7.89–7.87 (d, 4H, $\text{Ar}-\text{H}$), 7.20–7.19 (d, 4H, $\text{Ar}-\text{H}$), 6.89–6.86 (d, 4H, $\text{Ar}-\text{H}$), 4.37 (br s, 4H, $2 \times \text{CH}_2$), 3.77 (s, 6H, $2 \times \text{OCH}_3$), 1.92 (br s, 4H, $2 \times \text{CH}_2$); ^{13}C NMR (100 MHz, CDCl_3) δ (ppm) 166.14 ($^{13}\text{C}=\text{O}$), 158.79 ($^{13}\text{C}=\text{N}$), 156.78 ($\text{Ar}^{13}\text{C}-\text{O}$), 144.27 ($\text{Ar}^{13}\text{C}-\text{N}$), 140.38, 132.06, 129.95, 128.41, 122.42, 114.47, 64.72 ($^{13}\text{CH}_2-\text{O}$), 55.52 ($^{13}\text{CH}_3-\text{O}$), 25.58 ($^{13}\text{CH}_2-\text{C}$); Mass (m/z) $[\text{M} + \text{H}]^+$: calculated for $\text{C}_{34}\text{H}_{32}\text{N}_2\text{O}_6$, 564.23331, found, 565.23404. Elemental analysis: calculated for $\text{C}_{34}\text{H}_{32}\text{N}_2\text{O}_6$, C, 72.32; H, 5.71; N, 4.96; found, C, 72.024; H, 5.301; N, 5.029.

3.1.5. Butane-1,4-diylbis(oxy))bis(4,1-phenylene)bis(N-(4-chlorophenyl)methanimine) [5c]

IR (cm^{-1}) ν 2946.27, 2875.88, 1619.46 ($\text{C}=\text{N}$ stretch), 1603.13, 1571.95, 1505.24, 1454.75, 1396.88, 1305.34, 1243.01 (ether $\text{C}-\text{O}$ stretch), 1189.06, 1165.83, 1092.54, 1007.97, 960.9, 879.80, 843.76, 826.96, 826.96, 813.00, 752.89, 725.32, 688.48, 664.37; ^1H NMR (400 MHz, CDCl_3) δ (ppm) 8.29 (s, 2H, $2 \times \text{CH} = \text{N}$), 7.28–7.25 (d, 4H, $\text{Ar}-\text{H}$), 7.08–7.06 (d, 4H, $\text{Ar}-\text{H}$), 6.94–6.90 (m, 8H, $\text{Ar}-\text{H}$), 4.06 (br s, 4H, $2 \times \text{CH}_2$), 1.97 (br s, 4H, $2 \times \text{CH}_2$); ^{13}C NMR (100 MHz, CDCl_3) δ (ppm) 162.07 ($\text{Ar}^{13}\text{CH}_2-\text{O}$), 160.23 ($^{13}\text{C}=\text{N}$), 151.07 ($\text{Ar}^{13}\text{C}-\text{N}$), 131.28 ($\text{Ar}^{13}\text{C}-\text{Cl}$), 130.88, 129.43, 129.24, 122.44, 114.97, 67.86 ($^{13}\text{CH}_2-\text{O}$), 26.15 ($^{13}\text{CH}_2-\text{C}$); Mass (m/z) $[\text{M} + \text{H}]^+$: calculated for $\text{C}_{30}\text{H}_{26}\text{Cl}_2\text{N}_2\text{O}_2$, 517.14441, found, 517.14488. Elemental analysis: calculated for $\text{C}_{30}\text{H}_{26}\text{Cl}_2\text{N}_2\text{O}_2$, C, 69.64; H, 5.06; N, 5.41; found, C, 69.024; H, 5.012; N, 6.003.

3.1.6. Butane-1,4-diyl bis(4-(((4-chlorophenyl)imino)methyl)benzoate) [6c]

IR (cm^{-1}) ν 1706.98 (ester $\text{C}=\text{O}$ stretch), 1681.50, 1621.04 ($\text{C}=\text{N}$ stretch), 1581.92, 1566.49, 1484.11, 1470.29, 1456.16, 1410.53, 1388.18, 1362.46, 1271.24, 1245.20, 1186.06, 1167.70, 1103.29, 1088.96, 1053.68, 1007.31, 955.24, 843.06, 808.48, 764.53, 702.15, 688.97; ^1H NMR (400 MHz, CDCl_3) δ (ppm) 8.49 (s, 2H, $2 \times \text{CH} = \text{N}$), 8.15–8.13 (d, 4H, $\text{Ar}-\text{H}$), 7.97–7.95 (d, 4H, $\text{Ar}-\text{H}$), 7.39–7.38 (d, 4H, $\text{Ar}-\text{H}$), 7.19–7.17 (d, 4H, $\text{Ar}-\text{H}$), 4.45 (br s, 4H, $2 \times \text{CH}_2$), 2.00 (br s, 4H, $2 \times \text{CH}_2$); ^{13}C NMR (100 MHz, CDCl_3) δ (ppm) 166.01 ($^{13}\text{C}=\text{O}$), 159.37 ($^{13}\text{C}=\text{N}$), 149.92 ($\text{Ar}^{13}\text{C}-\text{N}$), 139.77, 132.62 ($\text{Ar}^{13}\text{C}-\text{Cl}$), 132.14, 130.01, 129.36, 128.73, 122.27, 64.80 ($^{13}\text{CH}_2-\text{O}$), 25.56 ($^{13}\text{CH}_2-\text{C}$); Mass (m/z) $[\text{M} + \text{H}]^+$: calculated for $\text{C}_{32}\text{H}_{26}\text{Cl}_2\text{N}_2\text{O}_4$ 573.13424, found, 573.13497. Elemental analysis: calculated for $\text{C}_{32}\text{H}_{26}\text{Cl}_2\text{N}_2\text{O}_4$, C, 67.02; H, 4.57; N, 4.88; found, C, 69.71; H, 4.18; N, 5.19.

4. Protein-ligand docking studies

Molecular docking studies were performed on four bacterial targets: DNA Gyrase (PDB ID: 5CPH), Topoisomerase IV (PDB ID: 2INR), Dihydropteroate Synthetase (PDB ID: 1AD1), and tRNA Synthetase (PDB ID: 1FFY) to evaluate the binding interactions and affinity of the synthesized bis-imine derivatives. These specific targets were selected due to their critical roles in bacterial DNA replication and protein synthesis. DNA Gyrase, a type II topoisomerase, is essential for relieving the torsional strain generated ahead of the replication fork during DNA replication. It introduces negative supercoils into DNA, which is vital for maintaining the proper topology of the DNA molecule. Inhibitors of DNA Gyrase can effectively halt bacterial growth by disrupting DNA replication processes [30]. Topoisomerase IV, another type II topoisomerase, is involved in the decatenation of intertwined DNA molecules following replication. It plays a crucial role in ensuring that daughter DNA strands are separated, allowing for proper cell division. Targeting Topoisomerase IV with bis-imine derivatives could provide a mechanism to interfere with bacterial cell division, thereby exerting antibacterial effects [31]. Dihydropteroate Synthetase is an enzyme in the folate biosynthesis pathway, which is essential for the synthesis of nucleic acids and amino acids in bacteria. By inhibiting this enzyme, bis-imine derivatives could disrupt the production of folate, leading to impaired bacterial growth and survival [32]. tRNA Synthetase is responsible for the charging of tRNA molecules with their corresponding amino acids, a critical step in protein synthesis. Inhibition of tRNA Synthetase can lead to a decrease in protein production, further contributing to the antibacterial activity of the bis-imine derivatives [33]. The selection of these targets reflects a strategic approach to identify compounds that can simultaneously disrupt multiple essential bacterial processes. The molecular docking studies aimed to elucidate the binding affinities and interactions of the bis-imine derivatives with these targets, providing insights into their potential as effective antibacterial agents. By understanding the molecular interactions at play, the design of these compounds can be

optimized to enhance their efficacy and selectivity against bacterial pathogens.

4.1. Preparation of receptors and ligands

Molecular docking was conducted using Autodock 4.2 [34]. The three-dimensional structures of the bis-imine derivatives were constructed and geometrically optimized using the Avogadro software [35] prior to conversion into PDBQT format. Protein structures obtained from Protein Data Bank (PDB) were prepared by removing water molecules and metal ions, adding polar hydrogens, assigning Kollman charges and converting to PDBQT format.

The grid box for each protein was defined with $40 \times 40 \times 40$ points. The protocol was validated by redocking the co-crystallized ligand into the active sites of 1FFY and 5CPH. For 1FFY, the top-ranked pose of MRC reproduced the crystallographic binding mode with a RMSD of 1.346 Å. For 5CPH, the corresponding RMSD was 0.156 Å. In both cases, the respective grid box differed. RMSD values ≤ 2.0 Å were taken as confirmation that the protocol reliably recovers experimental poses. For 2INR and 1 AD1, potential binding sites were identified using the PrankWeb: Ligand Binding Site Prediction server, and the grid box was positioned accordingly.

The docking study employed Lamarckian Genetic Algorithm (LGA) for conformational search, with 100 independent docking runs to ensure convergence to the best binding pose. The docking poses were analyzed using LigPlot and PyMOL to visualize interactions with key active site residues.

Table 3
Binding energies and interactions of bis-imines with 1FFY.

Target Protein	Bis-imine	Binding energy (kcal/mol)	H-bond interactions	Hydrophobic interacting residues
tRNA synthetase (1FFY)	5a	−9.41	Val588, Gly555	Lys595, Met589, Met596, Gly66, His64, His67, Asp557, Asn70, Glu554, Gly586, His585, Phe587, Asp630
	6a	−10.52	Lys74, Lys71, Gly555, Gly558	Asp95, Pro57, His585, Gly55, Glu554, Asn70, His64, Asp557, Gly66, Met596, Val588, Phe587, His67, Trp528, Pro56
	5b	−9.76	Gly555, Pro56	Asp95, Tyr58, Trp528, Val527, Glu554, Pro57, Gly55, His585, Asn70, Gly66, Gly586, Met596, His6, Phe587, Val588
	6b	−10.09	Lys71	Val603, Met596, Ser597, His64, Gly586, Gly66, Asn70, His67, His585, Gly55, Gly555, Pro57, Trp528, Val527, Asp95, Pro56, Glu554, Phe587, Val588
	5c	−10.05	Met596, His585, Glu554, Ser531, Val588	Ser597, Phe587, His64, Gly66, Gly586, Asp557, His67, Trp562, Gln558, Pro56, Gly555
	6c	−10.63	His585, Lys71	Met596, Phe587, Gly66, Asn70, Gly555, His67, Pro57, Gly55, Glu554, Pro56, Asp95, Trp528, Asp557, His64

4.2. Docking results

The docking results, including binding energy values and key interactions are summarized in Table 3, 4, 5 and 6.

The analysis indicates that all six derivatives demonstrate strong binding affinity toward tRNA Synthetase, identifying it as a potential primary bacterial target. Notably, compound **6c** exhibited the highest binding energy (−10.63 kcal/mol), followed by **6a** (−10.52 kcal/mol) and **6b** (−10.09 kcal/mol), suggesting their pronounced ability to inhibit this crucial enzyme in protein translation. The derivatives also demonstrated better binding to 5CPH ranging from −7.31 to −8.72 kcal/mol. It can be noted that **5a** demonstrated higher binding affinity for Topoisomerase IV suggesting their potential as dual inhibitors.

Overall, compounds **6a** and **6c** stood out as potent inhibitors of tRNA synthetase, while **5a** demonstrated the broadest versatility across all four targets. The high binding affinities are attributed to multiple stabilizing hydrogen bonds and hydrophobic interactions within respective active sites.

The poor aqueous and polar organic solubility of **5a** and **5b** suggests strong hydrophobic character which could negatively impact their drug-like properties and bioavailability. To address these limitations, future work could explore strategies such as prodrug derivatization, salt formation or advanced formulation techniques.

5. Structure-activity relationship (SAR) analysis

The docking results for the six synthesized bis-imine ligands across four bacterial targets: tRNA synthetase (1FFY), Topoisomerase IV (2INR), dihydropteroate synthetase (1 AD1), and DNA gyrase subunit B (5CPH), revealed consistent trends that allowed clear structure-activity relationships to be drawn. These patterns demonstrated that none of the ligands uniformly dominated every protein pocket; instead, performance shifted depending on the nature of each binding site.

5.1. Effect of linker type

Analysis revealed that binding affinity and interaction profile were greatly governed by the linker's chemical structure. The incorporation of additional carbonyl functionalities in the di-ester derivatives (**6a-c**) increased molecular polarity and provided hydrogen-bond acceptor sites. In pockets containing basic or polar residues: such as Lys71, His585, and Glu554 in 1FFY, the di-ester ligands engaged stronger anchoring interactions and achieved more favorable docking scores, with **6c** reaching −10.63 kcal/mol (See Figs. 1 and 2). In contrast, the hydrophobic tunnel of 2INR was dominated by Leu and Ile residues, and the less polar, more flexible di-ether linker of **5a** enabled superior

Table 4
Binding energies and interactions of bis-imines with 2INR.

Topoisomerase IV (2INR)	5a	−8.67	Leu425	Tyr426, Arg427, Gln427, Leu423, Ile420, Ala382, Leu383, Leu428, Ile433, Leu386, Ile390, Val389, Ile393, Val421
	6a	−7.24	NA	Ala382, Leu386, Leu383, Val389, Leu423, Ile420, Leu428, Val421, Ile390, Leu425, Ile393, Arg394
	5b	−6.83	NA	Leu383, Leu423, Val389, Val421, Ile393, Arg394, Leu425, Ile390, Leu428, Ile420, Leu386
	6b	−6.23	Ile420	Leu428, Ile390, Leu386, Val389, Leu423, Ala382, Leu383, Leu425, Lys399, Ile393, Val421
	5c	−6.99	NA	Leu386, Leu423, Leu428, Leu425, Ile390, Ile393, Arg394, Val421, Ile420, Val389
	6c	−6.44	NA	Leu428, Ile390, Leu386, Val389, Leu423, Leu425, Arg394, Ile420, Val421, Ile393

Table 5

Binding energies and interactions of bis-imines with 1 AD1.

Dihydropteroate Synthetase (1 AD1)	5a	−6.65	His241, Arg219, Arg239	Arg66, Asp15, His55, Thr13, Gly48, Glu56, Asn11, Val49, Ser50, Pro216, Arg52, Arg202, Ile9, Lys203
	6a	−6.72	His241, Arg239	Gly48, Ser50, Gln56, His55, Val49, Arg66, Thr13, Ser16, Ser201, Asp15, Ile9, Asn11, Arg52, Arg204, Lys203, Arg219, Arg202, Lys207
	5b	−6.20	Arg239, His55	Arg202, Lys207, Arg219, Lys203, Arg204, Ile9, His241, Asp84, Asn11, Glu105, Arg52, Thr13, Val49, Ser50, Asp15, Phe17, Ser18, Ser16, His241, Ile9, Lys203, Arg52, His55, Glu56, Val49, Ser50, Ile58, Arg66, Gly48, Thr13, Asp15, Ser16, Thr13, Ser50, Val49, His55, Arg52, Ser201, His241, Lys203, Ile9
	6b	−6.00	Asn11, Arg239, Asp15	Asp15, Arg202, Ser16, Lys203, Phe172, His241, Pro216, Ser201
	5c	−6.83	Asn11	
	6c	−6.69	Arg52, Arg219, Arg239, His55	

Table 6

Binding energies and interactions of bis-imines with 5CPH.

DNA Gyrase subunit B (5CPH)	5a	−8.35	NA	Ser128, Ser129, Glu50, Asn54, Asp81, Ile86, Gly83, Gly85, Ile102, Leu103, Thr173, Glu58, Arg84, Ala61, Asp57
	6a	−7.94	Asn54	Ser55, Gly85, Glu58, Asp81, Ile86, Thr173, Ile51, Leu103, Ser129, Ile175, Ile102, Ser128, Glu50, Asp57
	5b	−7.53	NA	Glu58, Ser55, Gly85, Ile51, Pro87, Ile86, Asn54, Arg84, Asp81, Ile175, Val79, Ile102, Ser128, Ser129, Val130
	6b	−7.13	Thr173, Asn54	Thr80, Asp81, Val79, Ile86, Ser55, Gly83, Ile51, Ile175, Arg84, Gly85, Glu58, Asp57, Glu50, Ser129, Ser128, Leu103
	5c	−7.94	NA	Pro87, Gly85, Glu58, Ile102, Ile86, Ser55, Thr173, Asp81, Asn54, Leu103, Ser129, Glu50, Ser128
	6c	−8.72	Asn54	Arg84, Pro87, Glu58, Gly85, Arg144, Asp57, Ile86, Asp81, Ile102, Ser128, Ser129, Leu103, Ile51

hydrophobic packing (−8.67 kcal/mol). For 5CPH and 1 AD1, the di-ester and di-ether scaffolds performed more comparably, indicating that a balance of polarity and hydrophobicity governed their interactions.

5.2. Effect of aryl substituents

The binding was further influenced by the electronic properties of the aniline derivatives. The *p*-chloro derivatives (**5c** and **6c**) exhibited increased lipophilicity and polarizability, thereby enhancing van der Waals interactions in mixed or hydrophobic areas like 1FFY and 5CPH. The *p*-methoxy analogues (**5b** and **6b**) increase the electron density through resonance and introduce a weak hydrogen-bond acceptor, which sometimes strengthens interactions with basic residues (Arg239

and His241 in 1 AD1) but could incur penalties in hydrophobic tunnels like 2INR. The unsubstituted phenyls (**5a** and **6a**) behaved as electronic baselines, adapting flexibility across all sites.

5.3. Key residue interactions

Residue analysis supported these trends. In 1FFY, the strongest binders (**6c** and **6a**) formed hydrogen bonds with Lys71, His585, Glu554, and Gly555, while packing against Phe587, Met596, and Trp528. In 2INR, high-scoring **5a** buried deeply within Leu386, Leu423, Val389, Ile420, and Leu428, demonstrating that extensive hydrophobic burial was sufficient for strong binding. At 1 AD1, scores clustered between −6.00 and −6.83 kcal/mol, and successful ligands oriented their carbonyl or methoxy groups toward Arg239, His241, or Arg219 without introducing steric strain. In 5CPH, **6c** achieved the most favorable score (−8.72 kcal/mol) by simultaneously engaging polar residues such as Asn54 and Glu58 and burying aromatic rings among Ile86, Ile102, and Leu103.

Collectively, these results demonstrate that linker polarity governs the primary binding mode, while electronic effects of aniline substituents fine-tune the interactions with specific residues. Di-ester linkers offered polar anchoring that benefitted pockets rich in hydrogen-bond donors or polar residues, whereas di-ether linkers excelled in predominantly hydrophobic environments. Analysis of substituents revealed distinct roles; the *p*-chloro group conferred superior hydrophobic packing and polarizability in mixed sites, the *p*-methoxy group promoted favorable polar interactions within charged grooves, and the unsubstituted phenyls served as versatile and adaptable motifs. These results underscore a key design principle for the bis-imine derivatives that optimal binding requires alignment of linker polarity with overall character of the binding pocket, complemented by matching the electronic properties of the aryl substituent to local residue environments.

6. ADMET studies

The pharmacokinetic and toxicity profiles of the synthesized compounds (**5a-c**, **6a-c**) were predicted using the ADMETLab 3.0 online server. The canonical SMILES of each compound were taken from ChemDraw tool and submitted individually to the server, and predicted all the required parameters, including molecular weight, cLogP, Topological polar surface area (TPSA), Lipinski's rule of five, human intestinal absorption (HIA), Caco-2, BBB permeability, P-glycoprotein (P-gp) interaction, cytochrome P450 inhibition, AMES mutagenicity, hERG inhibition, hepatotoxicity, and acute oral toxicity (LD50), were recorded and analyzed.

The predicted ADMET properties of compounds **5a-c**, **6a-c** are summarized in Table 7. all compounds exhibited molecular weights ranging between 448.22 and 572.13 g/mol and cLogP values between 4.35 and 6.66, indicating moderate to high lipophilicity. The TPSA values ranged from 43.18 to 95.78 Å², suggesting acceptable polarity for membrane permeation. Few compounds satisfied Lipinski's rule of five, whereas compounds **5c**, **6a**, and **6c** showed one rule violation, primarily due to increased molecular weight or lipophilicity. The predicted pharmacokinetic profile indicated low HIA and relatively low Caco-2 permeability for all compounds. BBB permeability was predicated only for compounds **5b** and **5c**. all compounds predicated P-glycoprotein inhibitors but not as P-gp substrates, suggesting possible modulation of drug efflux mechanisms. **6a** and **6c** inhibited CYP2D6, whereas **6a** inhibited CYP3A4, indicating a comparatively greater potential for cytochrome P450-mediated drug-drug interactions. Toxicity assessment predicted that all compounds were AMES positive and exhibited a high probability of hERG inhibition, suggesting potential mutagenic and cardiotoxic liabilities. Compounds **5a-c** showed high predicted hepatotoxicity, whereas compounds **6a-c** demonstrated comparatively moderate hepatotoxicity. Overall, compound **5b** showed the most favorable drug-

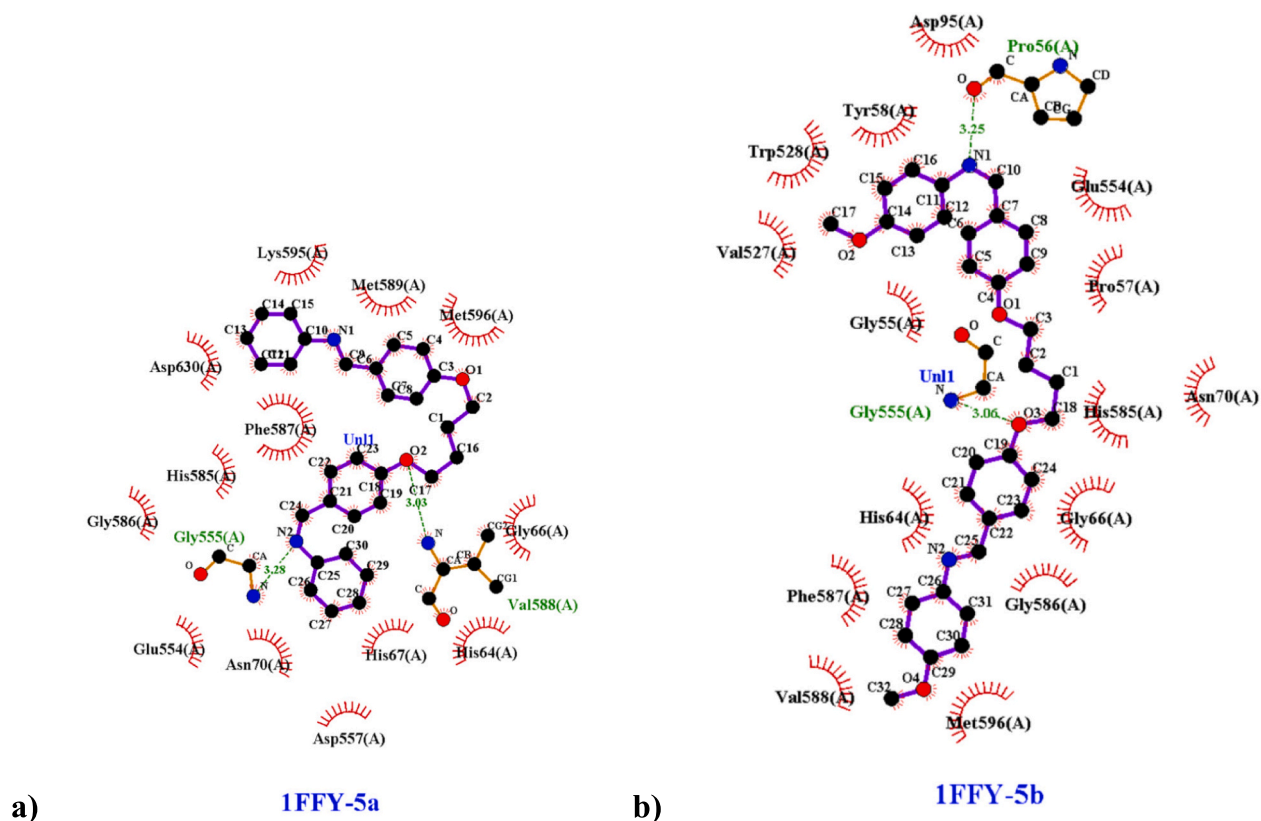


Fig. 1. (a-f). 2D representation of tRNA Synthetase (1FFY) with a) 5a b) 5b c) 5c d) 6a e) 6b f) 6c.

likeness profile owing to absence of Lipinski's rule violations and acceptable physicochemical properties, although the predicted toxicity liabilities suggest that further structural optimization is necessary.

7. Molecular dynamics (md) simulations

To evaluate the stability of the most promising protein-ligand complexes obtained from docking studies, 100 ns molecular dynamics simulations were performed using GROMACS simulation package on all the complexes formed with 1FFY and 5CPH. These two targets were selected because they exhibited the strongest docking scores with several bis-imine derivatives. All the figures related to molecular dynamics are included in the supplementary information (Fig. S36-S42).

7.1. Methodology

All molecular dynamics simulations were performed using the GROMACS simulation package. Periodic boundary conditions were applied in all three spatial dimensions using a cubic simulation box with an initial size of $15 \times 15 \times 15 \text{ nm}^3$. Each system contained one protein (PDB ID: 1FFY and 5CPH) and one ligand (5a, 5b, 5c, 6a, 6b, and 6c). To model the proteins the AMBER99SB-ILDN force field, while for ligands the General Amber Force Field (GAFF) was employed. Explicit solvation was achieved by using the TIP3P water model.

The protein-ligand complexes were solvated in water, and the system was neutralized by the addition of sodium counterions. Depending on the simulated system, the number of water molecules ranged from 104,067 to 107,690, and either 37 or 11 Na^+ ions were added. The composition of the individual simulation systems is summarized in Table 8.

Prior to production runs, energy minimization and subsequent equilibration were carried out in two stages for each system. An initial

500 ps equilibration was performed in the canonical (NVT) ensemble at 298.15 K using the velocity-rescaling (V-rescale) thermostat. This was followed by a 500 ps equilibration in the isothermal-isobaric (NPT) ensemble at 298.15 K and 1 bar pressure. Pressure coupling during NPT equilibration and production simulations was achieved using the Parrinello-Rahman barostat.

Following equilibration, 100 ns production MD simulations were carried out under NPT conditions at 298.15 K and 1 bar. Short-range van der Waals and electrostatic interactions were calculated using the Verlet cutoff scheme with a cutoff distance of 1.2 nm. Long-range electrostatic interactions were treated using the Particle Mesh Ewald (PME) method. Covalent bonds involving hydrogen atoms were constrained using the LINCS algorithm, while all other bonds remained unconstrained.

The resulting trajectories from the 100 ns production simulations were used for subsequent structural and dynamical analyses of the protein-ligand complexes.

7.2. Simulation results

It was found that the 6c ligand has the most stable interaction with 1FFY enzyme, while the binding is the least "favorable" for 5a ligand with the 1FFY enzyme (Fig. S36).

For the 5CPH enzyme, 5b, 5c, and 6b display very similar binding free energies (-37.96 , -37.93 , and -38.35 kcal/mol , respectively), indicating comparable binding affinities. The remaining ligands exhibit less favorable interactions with 5CPH, as reflected by their less negative ΔG_{MD} values (Fig. S37).

There is no significant positive correlation between the docking and MD derived energy values, which is expected, because docking does not mimic the real behaviour of the system well enough, it only finds the most optimal environment for the ligands to dock in.

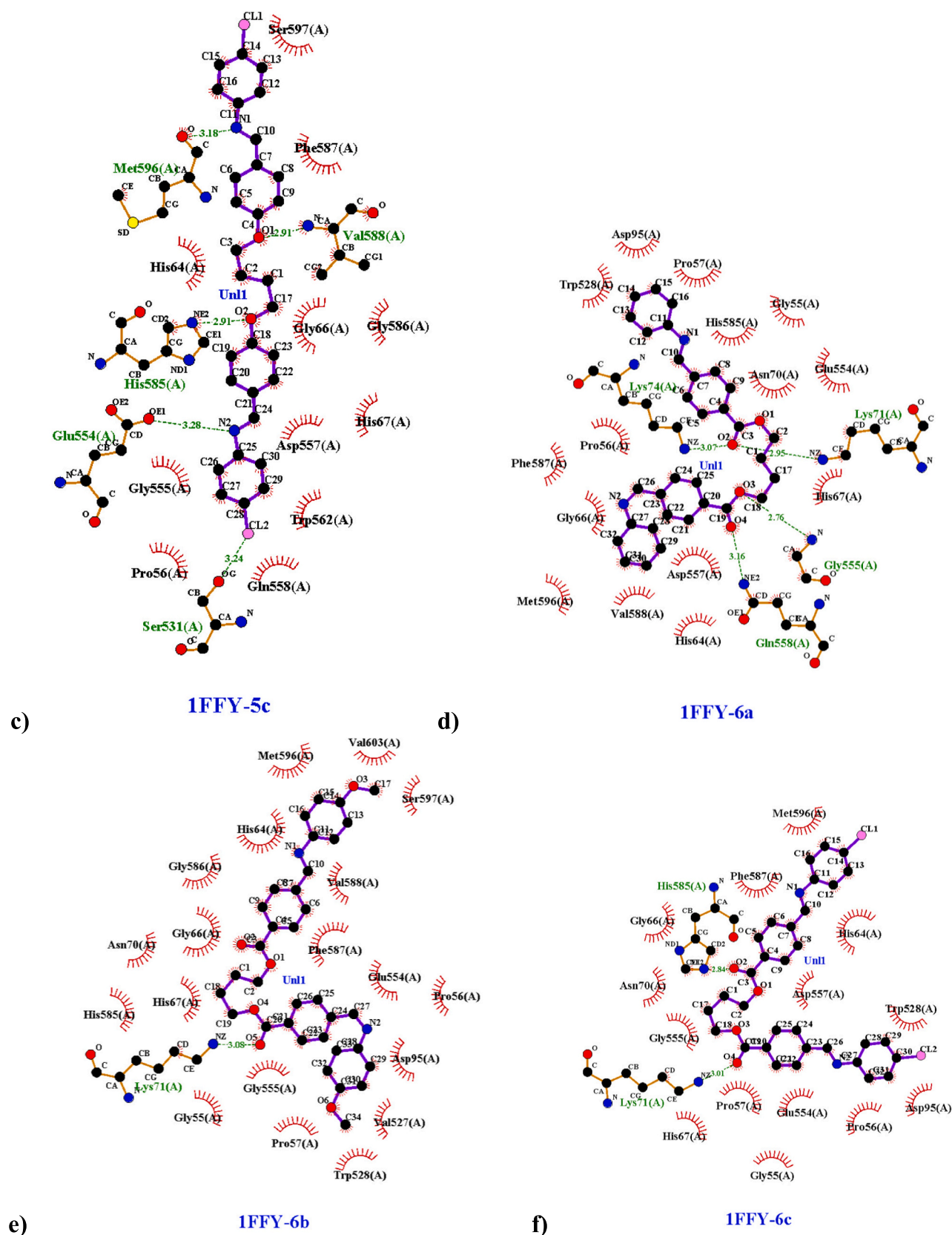


Fig. 1. (continued).

7.3. Protein backbone RMSD analysis

First, the 1FFY protein's backbone RMSD was analyzed to describe

the structural stability of the system (Fig. S38).

Based on the backbone RMSD profiles it can be concluded that the 1FFY-ligand complexes reached equilibrium within the first few

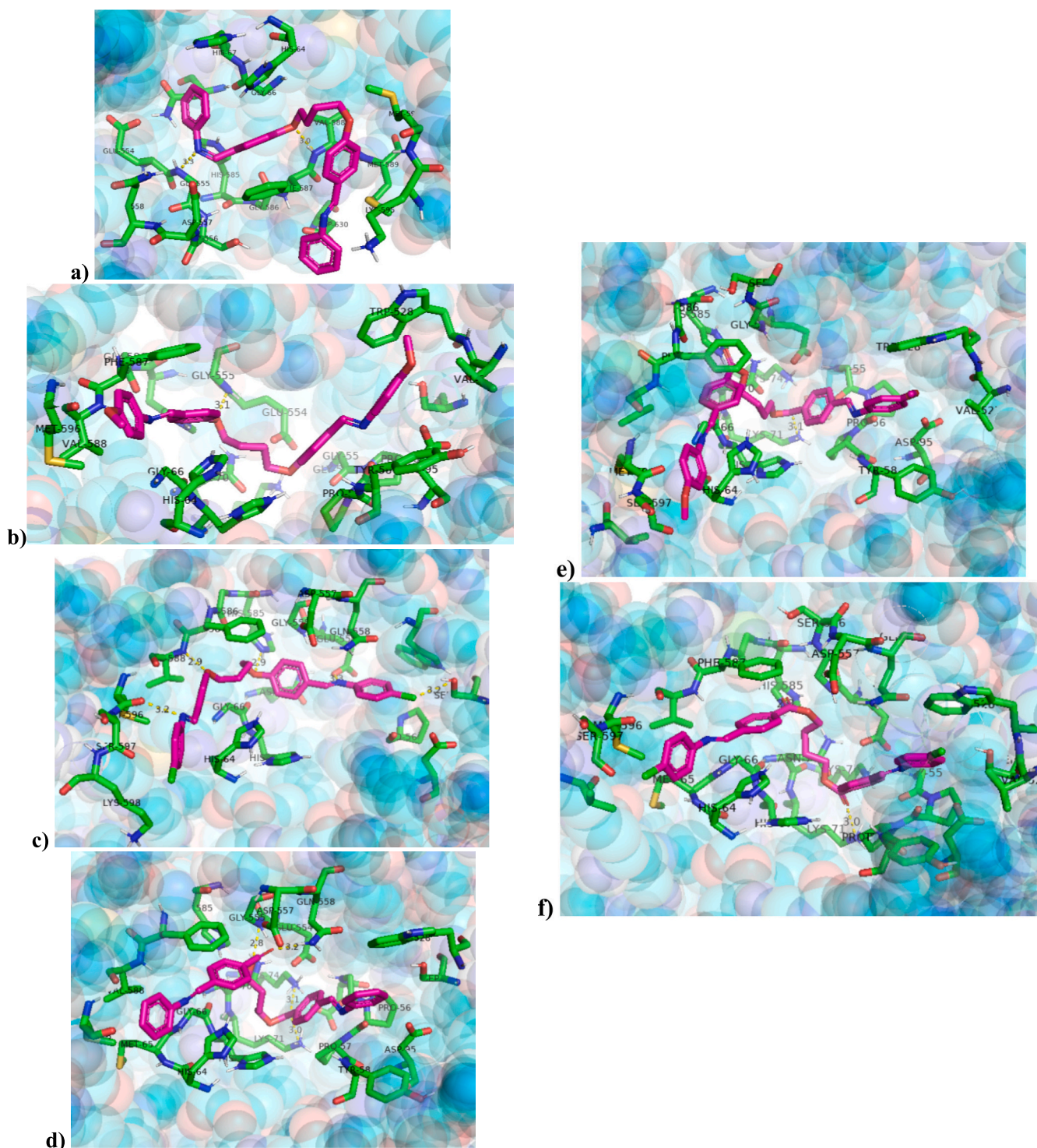


Fig. 2. (a-f). 3D representation of tRNA Synthetase (1FFY) protein with a) 5a b) 5b c) 5c d) 6a e) 6b f) 6c.

nanoseconds of the simulations and remained structurally stable during the 100 ns trajectory. Nevertheless, the bound ligands influenced the magnitude of the backbone fluctuations. Complexes containing ligands **5b** and **6b** exhibited the lowest RMSD values (~ 0.25 – 0.35 nm), whereas **6a** and particularly **6c** produced larger deviations, reaching approximately 0.6 – 0.7 nm. Ligands **5a** and **5c** showed intermediate behaviour. Despite these differences, none of the trajectories displayed a continuous increase in RMSD, indicating that the overall structure remained stable throughout the simulations.

The protein backbone RMSD in case of the 5CPH-ligand systems was

also analyzed (**Fig. S39**).

The 5CPH complexes also demonstrated structural stability throughout the simulations. Most ligand-bound systems maintained RMSD values between 0.10 and 0.15 nm, indicating only minor deviations from the initial structure. The only notable exception was the **5CPH-6a** complex, which underwent a conformational transition after approximately **30 ns**, stabilizing at a higher RMSD (~ 0.20 – 0.30 nm). However, this transition was followed by a stable plateau rather than a continuous increase, suggesting the formation of an alternative stable conformation of the protein instead of structural destabilization.

Table 7
ADMET Profiling.

Product	Lipinski violations	Caco-2 ^a	BBB ^b	CYP3A4 ^c inhibitor	CYP2D6 ^d inhibitor	AMES ^e toxicity	Hepatotoxicity
5a	0	−4.916	No	No	No	Positive (0.981)	High
5b	0	−4.858	Yes	No	No	Positive (0.981)	High
5c	1	−4.589	Yes	No	Yes	Positive (0.937)	High
6a	1	−5.129	No	Yes	Yes	Positive (0.901)	Moderate
6b	0	−5.357	No	No	No	Positive (0.905)	Moderate
6c	1	−4.796	No	No	Yes	Positive (0.735)	Moderate

^a Cancer coli-2 permeability, ^bBlood-Brain Barrier permeability, ^cCytochrome P450 3A4, ^dCytochrome P450 2D6, ^eBacterial Reverse Mutation Assay.

Table 8
Number of water molecules and sodium counterions in the simulation boxes.

	Number of Solvated H ₂ O molecules	Number of Na ⁺ counter ions
5a_1FFY	104,085	37
5a_5CPH	107,686	11
5b_1FFY	104,067	37
5b_5CPH	107,671	11
5c_1FFY	104,083	37
5c_5CPH	107,690	11
6a_1FFY	104,120	37
6a_5CPH	107,678	11
6b_1FFY	104,124	37
6b_5CPH	107,660	11
6c_1FFY	104,088	37
6c_5CPH	107,677	11

Table 9
Top 3 residues of 1FFY and 5CPH involved in the binding of the studied ligands.

	Residue	GLN558	ASP557	SER531
5a_1FFY	Probability	93.7	93.1	92.6
5a_5CPH	Residue	ILE102	ILE86	ASN54
	Probability	100.0	99.3	98.7
5b_1FFY	Residue	GLN558	PRO56	TRP562
	Probability	100.0	99.8	98.3
5b_5CPH	Residue	ILE102	ASN54	ILE86
	Probability	99.9	99.5	97.9
5c_1FFY	Residue	HIS64	HIS67	GLU554
	Probability	99.8	98.4	97.8
5c_5CPH	Residue	ILE102	ASN54	ILE86
	Probability	100.0	100.0	100.0
6a_1FFY	Residue	HIS67	GLN558	HIS64
	Probability	99.9	99.9	99.9
6a_5CPH	Residue	ILE102	SER105	ASN54
	Probability	99.9	99.9	99.3
6b_1FFY	Residue	TRP528	PRO56	HIS67
	Probability	100.0	100.0	100.0
6b_5CPH	Residue	ILE102	ILE86	ASN54
	Probability	100.0	100.0	99.8
6c_1FFY	Residue	HIS64	PHE587	GLN558
	Probability	100.0	99.9	99.5
6c_5CPH	Residue	ILE102	ASN54	ILE86
	Probability	100.0	99.8	99.9

By comparing the two proteins it can be seen that they have clear differences in their structural flexibility. Overall, 1FFY exhibited considerably higher backbone RMSD values and stronger ligand-dependent conformational changes than 5CPH, indicating greater structural flexibility (Fig. S38 and S39). In contrast, 5CPH remained highly rigid for most ligand-bound systems, with only the 6a complex showing a noticeable conformational rearrangement. These results suggest that ligand binding induces larger global conformational adaptations in 1FFY, whereas the backbone of 5CPH remains largely

Table 10
Number of intramolecular hydrogen bonds established within the proteins in complexed with the ligands.

Protein	Ligands	Mean	Std.
1FFY	5a	741	15
	5b	744	15
	5c	743	13
	6a	748	14
	6b	743	14
	6c	742	13
5CPH	5a	166	6
	5b	167	6
	5c	165	6
	6a	167	6
	6b	164	6
	6c	165	6

Table 11
Number of intermolecular hydrogen bonds established between the proteins and studied ligands.

Protein	Ligands	Mean	Std.
1FFY	5a	0	1
	5b	1	1
	5c	1	1
	6a	1	1
	6b	1	1
	6c	2	1
5CPH	5a	0	0
	5b	0	0
	5c	0	0
	6a	0	0
	6b	1	1
	6c	0	0

unaffected by the different ligands, reflecting its more rigid structural nature.

7.4. Ligand flexibility during binding

After investigating the proteins' stability, and behaviour, the next step was to determine the ligands' flexibility, or conformational changes by analysing the RMSD of the heavy atoms of the ligands (excluded the H-atoms, because they are more flexible so prone to distort the RMSD graphs). The heavy atom RMSD profiles indicate that all ligands remained associated with the 1FFY protein throughout the simulations, although their conformational stability differed considerably (Fig. S40). Ligands 6a and 6b exhibited the lowest RMSD values (approximately 0.3–0.5 nm), suggesting relatively stable binding poses.

Ligands 5b, 5c, and 6c showed intermediate RMSD values with moderate fluctuations, indicating conformational rearrangements

within the binding pocket (Fig. S40). In contrast, 5a displayed the largest deviations, reaching approximately 1.5 nm, suggesting substantial changes in ligand orientation or conformation during the simulation. Nevertheless, all trajectories reached stable plateaus without abrupt increases indicative of complete ligand dissociation (Fig. S40).

To get a broader view on the effect of the different proteins on the ligands' structural stability the effect of 5CPH on the ligand conformations was also investigated (Fig. S41).

By looking at these RMSD graphs it can be seen that the RMSD analysis of the 5CPH complexes revealed more pronounced ligand-dependent differences than those observed for the protein backbone. Ligands 5a, 5c, and 6b maintained relatively low RMSD values (approximately 0.2–0.5 nm), indicating stable binding conformations (Fig. S41). In contrast, ligands 5b, 6a, and 6c underwent distinct conformational transitions during the simulation, resulting in higher RMSD values of approximately 0.8–1.5 nm. Despite these rearrangements, the RMSD profiles remained stable after the transitions, suggesting that the ligands adopted alternative binding orientations (Fig. S41).

Comparing the two proteins with each other it can be said that ligand mobility depends strongly on the protein environment. In the 1FFY complexes, only ligand 5a exhibited markedly larger conformational changes, whereas the remaining ligands fluctuated within a relatively narrow RMSD range (Fig. S40 and S41). In contrast, the 5CPH complexes displayed greater ligand-dependent variability, with ligands 5b, 6a, and 6c undergoing substantial conformational rearrangements while 5a, 5c, and 6b remained comparatively stable. These results suggest that the binding pocket of each protein imposes different constraints on ligand dynamics, leading to distinct binding behaviour despite the structural similarity of the ligand series (Fig. S40 and S41).

7.5. Binding site composition

The composition of the binding site was determined by analysing the trajectories using the MDAnalysis tool. The top 3 residues which had the largest probability of finding within 4.5 Å (reasonable binding distance) from the ligands have been investigated and selected as binding site components (Table 9 and Fig. S42).

The selected residues have been further analyzed by plotting the corresponding RMSDs (Fig. S43 and S44).

The binding site residues of 1FFY remained structurally stable throughout the simulations, although the extent of local flexibility depended on the bound ligand (Fig. S43). Ligands 5b and 6b produced the lowest binding-site RMSD values (approximately 0.10–0.18 nm), indicating minimal perturbation of the binding pocket (Fig. S43). Ligands 5a, 5c, and 6a induced moderate fluctuations, while 6c exhibited the highest RMSD values (up to approximately 0.50 nm), suggesting the largest local conformational changes within the binding site. Nevertheless, all trajectories fluctuated around relatively stable average values without evidence of continuous structural drift. Moreover, the binding site of 5CPH was also investigated in contact with the studied ligands (Fig. S44).

In the case of the 5CPH complexes, the RMSD profiles of the ligand-binding residues indicate that the binding pocket remained highly stable during the 100 ns simulations (Fig. S44). All complexes showed low binding-site RMSD values, mostly fluctuating between approximately 0.08 and 0.20 nm, suggesting only minor structural deviations within the ligand-binding region. Ligands 5a, 5b, 5c, and 6c generally maintained RMSD values close to 0.08–0.14 nm, indicating a particularly stable binding-site environment. Ligand 6b showed slightly higher values during the simulation, while the 6a complex exhibited the largest binding-site fluctuations, especially after approximately 50 ns, reaching around 0.20–0.22 nm. However, these changes remained limited and no continuous increase or major conformational rearrangement was observed.

Overall, the binding-site RMSD analysis suggests that the 5CPH

binding pocket is structurally rigid and only weakly affected by the investigated ligands. Compared with the 1FFY complexes, where larger ligand-dependent binding-site fluctuations were observed, the 5CPH systems maintained consistently lower RMSD values. This indicates that the ligand-binding residues of 5CPH are less susceptible to ligand-induced conformational changes than those of 1FFY.

7.6. Hydrogen bond analysis

Hydrogen bonding analysis of the systems was also carried out. Number of intramolecular hydrogen bonds in case of both proteins have been determined which shows that 1FFY has almost 5 times more contact (>740) than 5CPH (>160) in each ligand bounded cases (Fig. S45 and Table 10.).

The protein-ligand intermolecular hydrogen bonds were also analyzed (Fig. S46 and Table 10.). It was found that hydrogen bonds were established in case of 1FFY and 5 of the studied ligands (5b, 5c, 6a-c), while in case of the 5CPH only with the 6b ligand (Table 11, Fig. S46).

In the supplementary information, the protein-solvent and ligand-solvent hydrogen bond analysis can also be seen (see Table S1). Regarding this analysis, it can be concluded that the protein-solvent hydrogen bond profiles show relatively stable hydration of both proteins throughout the 100 ns simulations, with no systematic loss or continuous increase in the number of established hydrogen bond. The absolute number of protein-solvent H-bonds is much higher for 1FFY (~2021–2034) than for 5CPH (~448–454), which most likely reflects differences in protein size/surface area rather than stronger hydration by itself (Fig. S47, Table S1). In both proteins, the different ligands caused only moderate fluctuations in protein-solvent hydrogen bonding, indicating that ligand binding did not strongly disturb the overall protein hydration shell. For the ligand-solvent hydrogen bonds, clearer ligand-dependent differences can be observed. In the 1FFY complexes, ligand 6b and later 6a showed relatively higher ligand-solvent H-bond numbers, while 5c and 5b generally exhibited fewer solvent interactions during parts of the simulation (Fig. S48, Table S2). In the 5CPH complexes, ligands 6a and 6b formed the most ligand-solvent hydrogen bonds, whereas 5a and 5c remained lower, usually around 2–3H-bonds. Overall, the ligand-solvent results suggest that some ligands, especially 6a and 6b, are more exposed to solvent or form more stable interactions with water during the simulations.

8. Future aspects

While molecular docking and MD simulations provide valuable insights into binding affinities, key interactions and complex stability, it is important to recognize some limitations. To validate the computational findings and assess the true therapeutic potential of the synthesized compounds, *in vitro* antimicrobial susceptibility testing, including minimum inhibitory concentration (MIC) assays against Gram-positive and Gram-negative bacterial strains, including multidrug-resistant isolates and cytotoxicity studies on mammalian cell lines will be performed to better understand the potential of these compounds. These experimental studies will help us in correlating the computational and biological aspects of the study.

9. Conclusion

This study presents a sustainable synthetic route to a series of novel ether- and ester-linked bis-imine derivatives through both conventional reflux and solvent-free mechanochemical grinding methods. The mechanochemical approach offered comparable yields in significantly shorter reaction time (10–15 min) at room temperature without the use of organic solvents, highlighting its efficiency and environmental benefits. All the synthesized compounds were fully characterized by ¹H NMR, ¹³C NMR, HRMS, FT-IR and CHN analysis. Molecular docking and

MD simulations identified tRNA synthetase 1FFY and DNA Gyrase 5CPH as the most promising bacterial targets. Compounds **6c** (−10.63 kcal/mol) and **6a** (−10.52 kcal/mol) exhibited strongest binding affinities and stable interactions with 1FFY. MD simulations were performed on two of the protein 1FFY and 5CPH due to their complexes with ligands giving better binding energy than the other two protein targets.

The structure-activity relationship demonstrated that ester linkers facilitated key polar interactions including hydrogen-bonding whereas ether linkers promoted hydrophobic packing. Among the aryl substituents, the *p*-chloro group enhanced hydrophobic packing, while the *p*-methoxy group provided tunable polarity and the unsubstituted phenyl ring offered good versatility across all targets, as evidenced by strong broad-spectrum binding of **5a** and **6a**.

In silico AMDT profiling indicated that most compounds possess accepted drug-like properties, with compound **5b** showing the most balanced profile. However, potential liabilities such as CYP inhibition, mutagenicity, and hepatotoxicity were predicted, suggesting that structural optimization will be necessary.

In summary, the present work emphasizes the significance of mechanochemical synthesis as a sustainable and efficient synthetic strategy for the synthesis of bis-imine scaffolds along with their multi-level in silico evaluation of therapeutic potential as novel bacterial enzyme inhibitors. The influence of linker polarity and aryl substituents on binding affinity provides a rational framework for further optimization. These findings position the bis-imine scaffold, particularly compounds **6c** and **6a** as potential leads for the development of new antibacterial agents targeting tRNA Synthetase and DNA Gyrase.

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CRediT authorship contribution statement

Hemant Kumar Dharmapuri: Writing – review & editing, Writing – original draft, Validation, Software, Methodology, Investigation, Formal analysis. **György Marcell Tokaji:** Writing – review & editing, Validation, Data curation. **R. Udaya Rajesh:** Writing – review & editing, Methodology. **Béla Fiser:** Supervision, Formal analysis. **A. Singhal:** Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Conceptualization.

Ethics declaration

The author(s) declare(s) that the study does not involve humans nor animal subjects.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.rechem.2026.103762>.

Data availability

Complete spectral data and molecular docking details of all the synthesized compounds are available in the Electronic Supplementary Information (ESI).

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