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DESIGN, SYNTHESIS AND MOLECULAR DOCKING OF NEW SPIRO HETEROCYCLIC COUMARIN AS ANTIBACTERIAL AGENTS

Abdallah FATHI AL-BURGUS, Omar THANOON-ALI and Omar YOUNIS AL-ABBASY

University of Mosul, College of Education for Pure Science, Department of Chemistry, Mosul, Ninava, Iraq

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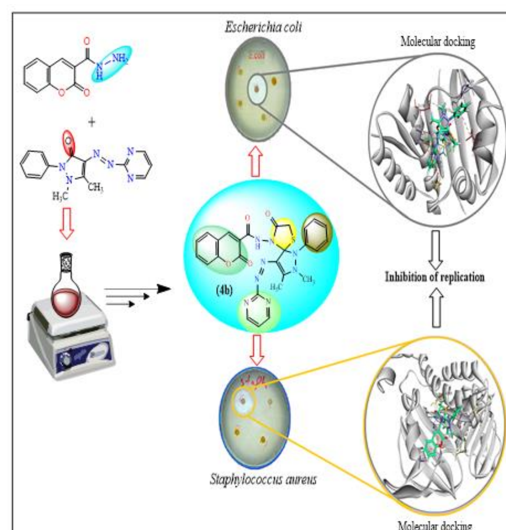
In this study, coumarin derivatives were synthesized from coumarin 3-carbohydrazide (1) and 4-(pyrimidin-2-yl diazenyl)-antipyrine (2), leading to the intermediate compound (3). The final compounds were synthesized through the cyclocondensation of compound (3) with mercaptoacetic acid, acetic anhydride, sodium azide, 2-aminobenzoic acid, and maleic anhydride. This process resulted in the formation of five spiro heterocyclic coumarins (4a-e) respectively. The novel compounds were purified by column chromatography, and were identified by FT-IR,^{1}H, and ^{13}C-NMR**.** The antibacterial activity of the prepared compounds was evaluated *in vitro* using the disk diffusion method against *Escherichia coli* and *Staphylococcus aureus* bacteria. Compound (4b) showed significant antibacterial activity, among others. Furthermore, the docking study of (4b) with DNA gyrase for both bacterial strains was investigated using (Auto Dock Vina), and (Discovery Studio software), which revealed vital interactions and binding conformations.



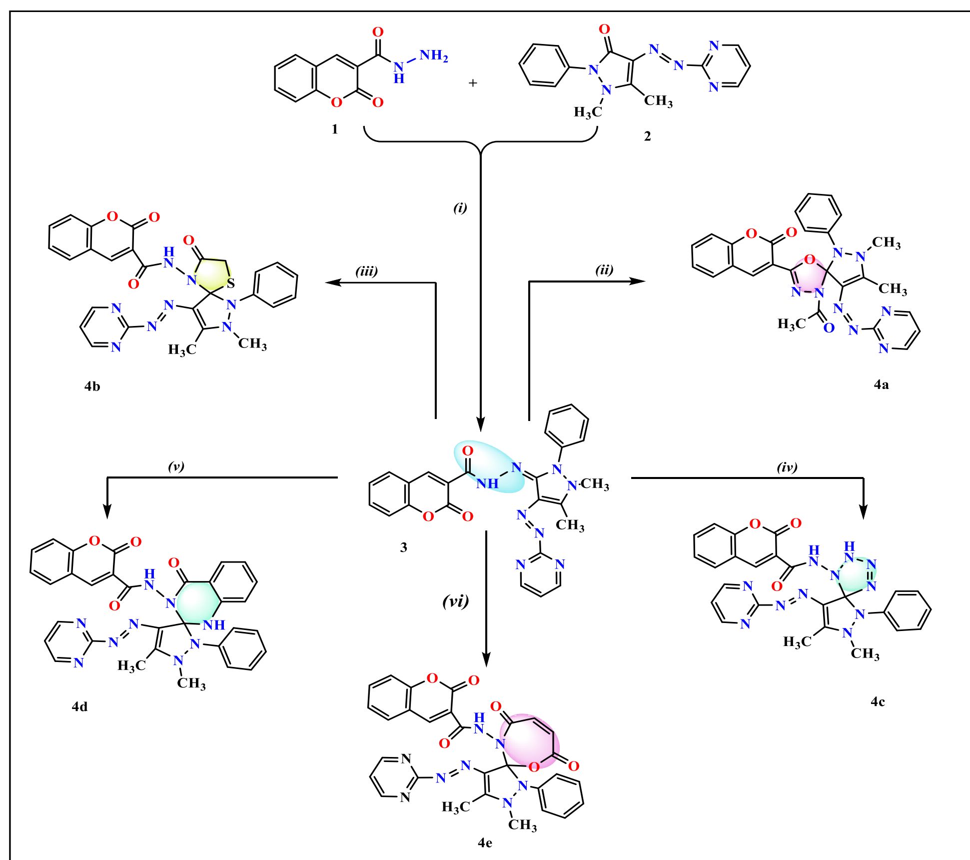
INTRODUCTION

Coumarins (α-benzopyrones, 2*H*-chromen-2-ones) are a large family of a natural origin compounds that exhibit numerous medicinal properties and biological activities[1,2] such as anti-oxidant, anti-cancer, anti-coagulant, anti-diabetic, and anti-inflammatory properties.[3] Coumarin compounds and their derivatives are used also as additives in food, cosmetics, dyes, and pharmacological industry.[4] Illness via bacterial infections is widely spreading and developing globally.[5,6] It is expected that ten million people will die annually from infectious diseases around the world in the absence of enhanced medical treatments.[7] The reason behind that expected scenario is the dramatic increase of multidrug resistance of microorganisms such as *E. coli* and *S. aureus* to known antibiotics.[8] Several studies focus on attempting to change the molecular structure and function of bacterial protein, gyrase, that is involved in DNA replication and transcription. DNA gyrase (topoisomerases II) is an enzyme that mainly responsible for bacterial chromosome

* Corresponding author: abdallah.21esp11@student.oumosul.edu.iq

replication. It can introduce a double-stranded break in DNA and is believed to have a central role in reducing topological strain in an ATP-dependent manner.[9] DNA gyrase inhibitors can prevent bacterial growth through two different mechanisms: directly inhibiting DNA gyrase, called "gyrase poisoning" or indirectly by inhibiting gyrase ATPase activity (*e.g.*, ciprofloxacin), which blocks the negative supercoils into DNA such as aminocoumarin.[10]

Many literatures have revealed that derivatives of coumarin as bacterial DNA gyrase inhibitors were utilized.[11] As observed, inhibiting of DNA gyrase by using antibiotics such as novobiocin and chlorobiocin (containing a coumarin skeleton) can cause bacterial death. Due to the drug resistance issue and developing of bacterial properties, new and effective medicine is needed. Therefore, some heterocyclic coumarin derivatives (Scheme 1) were designed and synthesized. Spectroscopic techniques such as FT-IR, ^{1}H-NMR and ^{13}C-NMR were used to characterize the structure of these compounds. In addition, the *in vitro* assessment of their antibacterial activities was evaluated. Molecular docking simulation of compound (4b) with DNA gyrase in *E. coli*. and *S. aureus* was also studied.



Scheme 1 – Synthesis of spiro heterocyclic coumarin derivatives (**4a-e**).

Reagents and conditions: (i) abs. EtOH, Gla.CH_3CO_2H, reflux for10 h. (ii) Acetic anhydride, reflux for 4 h. (iii) 1,4-dioxane, $HSCH_2COOH$, $ZnCl_2$, reflux for10 h, $NaHCO_3$. (iv) NaN_3, dissolved in 5 mL of water, 1,4-dioxane, reflux for 12 h. (v) 2-Amino benzoic acid, 1,4-dioxane, reflux for 12 h, $NaHCO_3$. (vi) Maleic anhydride, 1,4-dioxane, reflux for10 h.

RESULTS AND DISCUSSION

Synthesis

Starting material **(1 and 2)** were prepared and obtained in 80 % yield, all data were identical to literatures.[12,13] FT-IR, ^{1}H-NMR and ^{13}C-NMR, spectra of the new synthesized compounds (3 and 4a-e) were given in the supplementary data.

***N'-(1,5-dimethyl-2-phenyl-4-(pyrimidin-2-yldiazenyl)-1,2-dihydro-3H-pyrazol-3-ylidene)-2-oxo-2H-chromene-3-carbohydrazide* (3).** Red solid; m.p = 220°C; yield = 77 %; Rf = 0.83 (Hex: EtOAc 7:3); IR (ATR, cm^{-1}): 3341(N-H), 3065(CH $_{aromatic}$), 2981 (CH $_{aliphatic}$), 1736(C=O$_{lactone}$), 1672(C=O$_{amide}$), 1554(N=N$_{azo}$); ^{1}H-NMR(400 MHz, DMSO-d_6, δ/ppm): 10.34 (s,1H,-NH), 8.56 – 8.45 (d, 2H, J = 8.0 Hz, Arom-H),

8.32 (s, 1H, coumarin, H_4), 8.04–7.96 (m, 2H, Arom-H), 7.84–7.72 (m, 2H, Arom-H), 7.66–7.52 (m, 1H, Arom-H), 7.53–7.34 (m, 1H, Arom-H), 7.32–7.23 (m,1H, Arom-H), 3.39 (s, 3H, $-CH_3$), 2.43 (s, 3H, $-CH_3$); ^{13}C-NMR (DMSO-d_6, 100 MHz, δ/ppm): 168.0 (C=O, amide), 158.3 (C=O, lactone), 144.4 (C=N imine), 151.4, 149.6, 141.48, 137.6, 133.5, 131.5, 128.7, 127.7, 127.0, 126.0, 125.2, 124.8, 121.0 (Arom-C), 158.2, 118.2, 115.1, 112.0. (olefinic- C), 37.5, 7.7 (antipy-CH_3).

3-(1-acetyl-7,8-dimethyl-6-phenyl-9-(pyrimidin-2-yldiazenyl)-4-oxa-1,2,6,7-tetraazaspiro [4.4]nona -2,8-dien-3-yl)-2H-chromen-2-one **(4a).** Red solid; m.p = 236°C; yield = 69 %; Rf = 0.75 (Hex: EtOAc 7:3); IR (ATR, cm^{-1}): 3081(CH $_{aromatic}$), 2980 (CH $_{aliphatic}$) , 1729(C=O $_{lactone}$), 1596(N=N $_{azo}$); ^1H-NMR (400 MHz, DMSO-d_6, δ/ppm): 8.49 (d,2H, J = 7.0 Hz, Arom-H), 8.34 (s, 1H, coumarin, H_4), 8.07–7.98 (m, 2H, Arom-H), 7.81–7.74 (m,1H, Arom-H), 7.52–7.45 (m,1H, Arom-H), 7.39–7.29 (m, 2H, Arom-H), 7.03–6.94 (m, 1H, Arom-H), 3.18 (s, 3H, $-CH_3$), 2.40 (s, 3H, $-CH_3$), 2.00 (s, 3H, N-acetyl CH_3); ^{13}C-NMR (DMSO-d_6, 100 MHz, δ/ppm): 167.2 (C=O, N-acetyl), 159.2 (C=N), 153.3 (C=O, lactone), 150.9, 150.3, 143.5, 143.0, 132.6, 128.2, 125.1, 124.8, 119.1, 117.4, 116.0, 112.9, 110.4 (Arom-C), 125.3 (spiro-C), 145.4, 131.1, 135.8, 124.2 (olefinic- C) , 27.8 (acetyl-CH_3), 40.3, 8.4 (antipy-CH_3).

N-(2,3-dimethyl-8-oxo-1-phenyl-4-(pyrimidin-2-yldiazenyl)-6-thia-1,2,9-triazaspiro [4.4]non-3-en-9-yl)-2-oxo-2H-chromene-3-carboxamide **(4b).** Brown solid; m.p = 254°C; yield = 64 %; Rf = 0.72 (Hex: EtOAc 7:3); IR (ATR, cm^{-1}): 3460(N-H), 3063 (CH $_{aromatic}$), 2980(CH $_{aliphatic}$), 1710(C=O $_{lactone}$), 1566(N=N $_{azo}$); ^1H-NMR(400 MHz, DMSO-d_6, δ/ppm): 10.31 (s,1H,-NH), 8.60 (d, 2H, J=8.0 Hz, Arom-H), 8.39-8.31 (m, 1H, Arom-H), 8.27 (s, 1H, coumarin, H_4), 8.06–7.99 (m, 2H, Arom-H), 7.66–7.59 (m, 1H, Arom-H), 7.47–7.36 (m, 1H, Arom-H), 7.32–7.24 (m, 1H, Arom-H), 7.21–7.09 (m,1H, Arom-H), 3.95 (d, 1H, J = 7.4 Hz, S-CH_2), 3.74 (d, 1H ,J = 9.3 Hz, S-CH_2), 3.43 (s, 3H, -CH_3), 2.15 (s, 3H, $-CH_3$); ^{13}C-NMR (DMSO-d_6, 100 MHz, δ/ppm): 174.5, 169.7 (C=O, amide), 159.6 (C=O lactone), 164.5, 161.2, 151.3, 150.7, 145.8, 143.9, 143.5, 143.4, 136.2, 131.6, 125.5, 125.2, 119.6 (Arom-C), 153.7, 117.8, 116.4, 113.4 (olefinic- C), 104.5 (spiro-C), 28.2 (S-CH_2), 40.7, 8.8 (antipy-CH_3).

N-(7,8-dimethyl-6-phenyl-9-(pyrimidin-2-yldiazenyl)-1,2,3,4,6,7-hexaazaspiro [4.4] nona-3,8-dien-1-yl)-2-oxo-2H-chromene-3-carboxamide **(4c).** Brownish solid; m.p = 243°C; yield = 67 %; Rf = 0.79 (Hex: EtOAc 7:3); IR (ATR, cm^{-1}): 3300(N-H), 3053(CH $_{aromatic}$), 2980(CH $_{aliphatic}$), 1714(C=O $_{lactone}$), 1589(N=N $_{azo}$); ^1H-NMR(400 MHz, DMSO-d_6, δ/ppm): 10.46 (s, 1H, -NH), 8.86 (d, 2H, J = 7.0 Hz), 8.55 (s, 1H, coumarin, H_4), 8.32–8.20 (m, 2H, Arom-H), 8.09–8.02 (m, 1H, Arom-H), 7.97–7.88 (m, 2H, Arom-H), 7.82–7.77 (m, 2H, Arom-H), 4.12 (s, 1H, -NH), 3.10 (s, 3H, -CH_3), 2.37 (s, 3H, $-CH_3$); ^{13}C-NMR(DMSO-d_6, 100 MHz, δ/ppm): 167.2 (C=O, amide), 159.2 (C=O, lactone), 153.3,150.9, 150.3, 143.4, 143.0, 132.6, 131.1, 128.2, 125.3, 119.1, 117.4, 116.0, 112.9 (Arom-C), 135.81 (spiro-C), 145.4, 125.1, 124.2, 124.8 (olefinic- C), 40.3, 8.4 (antipy-CH_3).

N-(1,5-dimethyl-4'-oxo-2-phenyl-4-(pyrimidin-2-yldiazenyl)-1,1',2,4'-tetrahydro-3'H-spiro[pyrazole-3,2'-quinazolin]-3'-yl)-2-oxo-2H-chromene-3-carboxamide **(4d).** Brown, solid; m.p = 280°C; yield=65%; Rf = 0.68 (Hex: EtOAc 7:3); IR(ATR, cm^{-1}): 3229(N-H), 3053(CH $_{aromatic}$), 2980 (CH $_{aliphatic}$), 1711(C=O $_{lactone}$), 1565(N=N $_{azo}$); ^1H-NMR(400 MHz, DMSO-d_6, δ/ppm): 10.02(s,1H,-NH), 8.56–8.45(d, 2H, J= 7.0 Hz, Arom-H), 8.39–8.29(m, 2H, Arom-H), 8.23(s, 1H, coumarin, H_4), 8.06–7.92 (m, 2H, Arom-H), 7.87 (s, 1H, -NH), 7.84–7.72 (m, 2H, Arom-H), 7.67–7.38 (m, 3H, Arom-H), 7.30–7.20(m, 2H, Arom-H), 3.22 (s, 3H, $-CH_3$), 2.43(s, 3H, $-CH_3$); ^{13}C-NMR(DMSO-d_6, 100 MHz, δ/ppm): 166.9, 160.4 (C=O, amide), 158.8 (C=O, lactone), 152.9, 150.5, 149.9, 143.1, 142.6, 135.4, 134.4, 132.3, 130.8, 125.0, 124.8, 124.7, 124.6, 124.5, 118.8, 117.5, 117.0, 112.6, 110.1 (Arom-C), 128.8 (spiro-C), 145.1, 127.8, 123.8, 115.7 (olefinic-C), 43.2, 8.1 (antipy-CH_3).

N-(2,3-dimethyl-7,10-dioxo-1-phenyl-4-(pyrimidin-2-yldiazenyl)-6-oxa-1,2,11-triazaspiro [4.6] undeca-3,8-dien-11-yl)-2-oxo-2H-chromene -3-carboxamide **(4e).** Red solid; m.p = 240°C; yield=66%; Rf = 0.77 (Hex: EtOAc 7:3); IR (ATR,cm^{-1}): 3424(N-H), 3070(CH $_{aromatic}$), 2948 (CH $_{aliphatic}$), 1737(C=O $_{lactone}$), 1586(N=N $_{azo}$); ^1H-NMR (400 MHz, DMSO-d_6, δ/ppm): 10.60(s, 1H, NH), 8.65(d, 2H,J=6.5 Hz, Arom-H), 8.55 (s,1H, coumarin, H_4), 8.44–8.32 (m, 2H, Arom-H), 8.31–8.24 (m,1H, Arom-H), 8.17–8.04 (m, 1H, Arom-H), 7.99–7.89(m, 1H, Arom-H), 7.86–7.79(m, 1H, Arom-H), 7.64–7.59(m, 1H, Arom-H), 7.00 (d, 1H, J = 9.9 Hz, olefinic-H), 6.92(d,1H, J=9.7 H, olefinic H), 3.42(s, 3H, $-CH_3$), 2.35(s, 3H, $-CH_3$); ^{13}C-NMR (DMSO-d_6, 100 MHz, δ/ppm): 168.2, 161.8, 160.1 (C=O, amide), 154.3 (C=O, lactone), 151.8, 151.2, 143.9, 130.1, 126.3, 126.2, 126.0, 125.9, 125.8, 118.8, 118.4, 117.0, 111.4 (Arom-C), 135.8 (spiro-C), 144.4, 124.2, 120.1, 113.9 (olefinic-C), 132.1, 129.1 (oxazp-C), 44.5, 9.4 (antipy-CH_3).

Antibacterial Activity

The new synthesized heterocyclic coumarin derivatives were evaluated against *E. coli* (ATCC 25922) and *S. aureus* (ATCC 5923), using dimethyl sulfoxide (DMSO) and Ceftriaxone (30 μg/mL), a broad-spectrum antibiotic, as negative and positive controls, respectively. Figure 1 showed significant antibacterial activity for some synthesized compounds, specifically, compound (4b) which showed a better activity against both *E. coli* and *S. aureus* pathogenic bacteria.

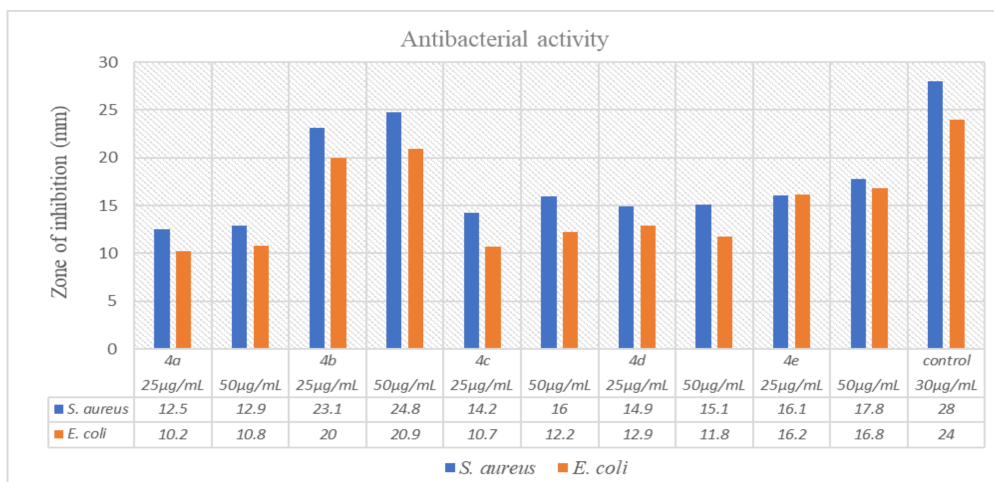


Fig. 1 – Antibactrial activity of heterocyclic coumarin derivatives.

Molecular docking analysis

Molecular docking results revealed that compound (4b) shows binding modes with DNA gyrase that closely resemble clorobiocin binding mode. Figure 2 (1A–1C) illustrates the formation of (4b) ligand-protein complex in the active site of *E. coli* DNA gyrase (ID: 1KZN), with $\Delta G_{\text{binding energy}}$ value of –9.2 kcal/mol. The formed complex was stabilized by hydrogen bonds (shown by green dotted lines) with two amino acids, ASP35 and ASN32. Along different types of bonding interactions (like pi-Anion, pi-alkyl, and pi-Sigma etc.) are shown by different color dotted lines. As compared with the known antibiotic clorobiocin, the ligand-protein complex inside the active site of the *E. coli* DNA gyrase has $\Delta G_{\text{binding energy}}$ value of –7.3 kcal/mol (2A–2C).

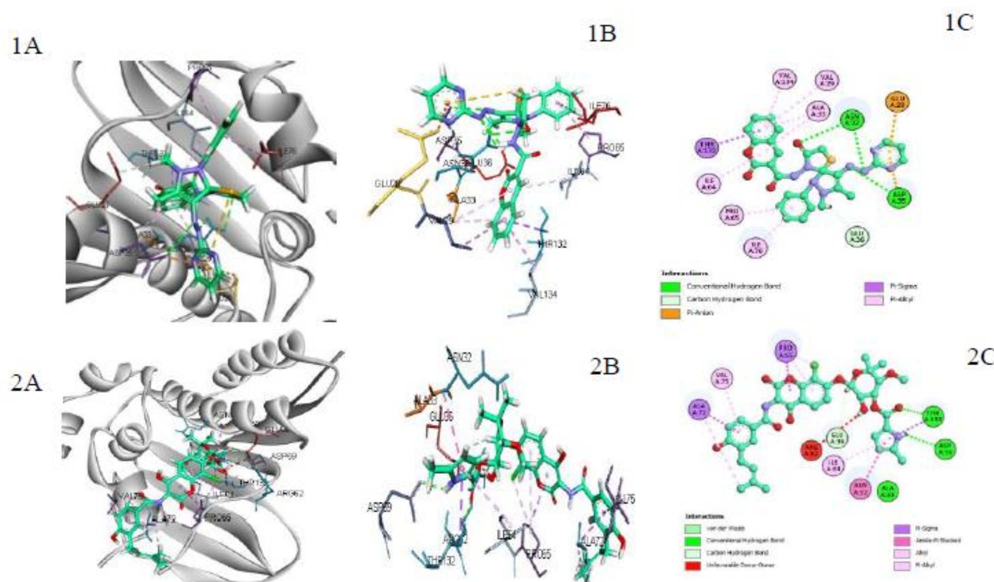


Fig. 2 – Illustrates the formed interactions between the compound (4b) **(1A)**, the clorobiocin **(2A)**, with the enzyme of *E. coli* (ID: 1KZN) in a 3D ribbon. Stick model for compound (4b) **(1B)**, the clorobiocin **(2B)** show 3D interactions with specific amino acids residues of *E. coli* (ID: 1KZN) enzyme. Compound (4b) **(1C)**, with clorobiocin **(2C)**: show 2D interactions with specific amino acids residues of *E. coli* (ID: 1KZN) enzyme.

On the other hand, Fig. 3 (3A–3C) showed the formation of compound (4b) ligand-protein complex in the active site of *S. aureus* DNA gyrase (ID: 3g75) with ΔG $_{\text{binding energy}}$ value of –9.0 kcal/mol. The ligand-protein complex was stabilized by pi-alkyl with ALA: 75, ALA: 38, and IELA: 129, IELA. 28. hydrophobic interactions and different types of bonding interactions (like van der Waal's, pi-cation, and pi-Sigma etc.). As compared with the known antibiotic clorobiocin, the ligand-protein complex inside the active site of the *S. aureus*. DNA gyrase has ΔG $_{\text{binding energy}}$ of –7.0 kcal/mol (4A–4C).

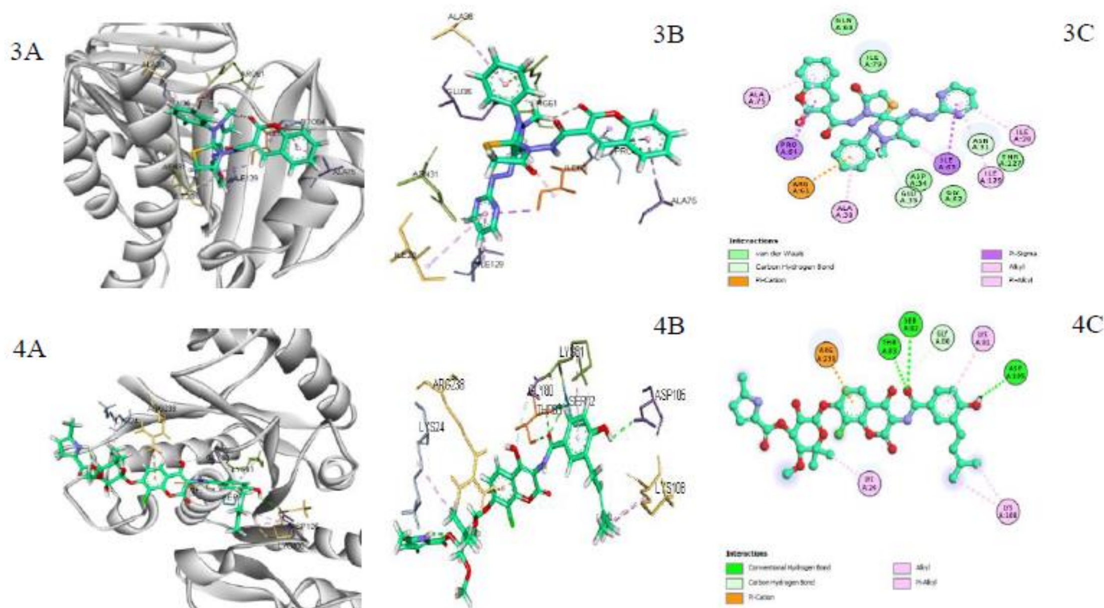


Fig. 3 – Illustrates the formed interactions between the compound (4b) **(3A)**, the clorobiocin **(4A)**, with the enzyme of *S. aureus* (ID: 3g75) in a 3D ribbon. Stick model for compound (4b) **(3B)**, the clorobiocin **(4B)** show 3D interactions with specific amino acids residues of *S. aureus* (ID: 3g75) enzyme. Compound (4b) **(3C)**, with clorobiocin **(4C)**: show 2D interactions with specific amino acids residues of *S. aureus* (ID: 3g75) enzyme.

Compound (4b) showed both main hydrogen bond and interactions with different groups such as pyridine, azo group, and thiazolidin-4-one. Also, possible antibacterial effects may be attributed to further interactions with amino acid residues. According to the findings reached, the selected compound (4b) had a better docking score of ΔG $_{\text{binding energy}}$ with bacterial strains compared to clorobiocin. The results provide a sufficient explanation and a convenient correlation between *in vitro* antibacterial assay and scores of docking study.

EXPERIMENTAL

Materials and instrumentation

In this work, the chemicals used were of high purity, purchased from Sigma Aldrich and BDH Companies. Melting points were determined in open capillary tubes on a Stuart melting point apparatus. The reactions were monitored by thin-layer chromatography (TLC) using silica gel $60F_{254}$ Merck, Darmstadt and compounds were visualized by exposure to UV. FT-IR spectra were recorded on ATR-Bruker in the range (400–4000 cm^{-1}). ^{1}H-NMR spectra were recorded on Bruker (400 MHz, Bio Spin GmbH) using DMSO-d_6 as a solvent with (TMS) Tetramethylsilane as an internal reference and ^{13}C-NMR spectra were recorded on (Messtechnik Gmb Hwere,100MHz), using DMSO-d_6 as a solvent. The selected NMR samples were measured in the department of chemistry, university of Sharif, Tehran, Iran. All used bacterial cultures were received from the microbial culture collection from the college of sciences, department of biology, university of Mosul, Iraq.

Synthesis of spiro heterocyclic coumarin derivatives

The synthesized coumarin 3-carbohydrazide (1) was reacted with 4-(pyrimidin-2-yl-diazenyl)-antipyrine (2) through a Schiff base condensation, resulting in the formation of an intermediate molecule (3). A series of chemical steps involving the reaction of a Schiff base (3) with various cyclization reagents, to synthesized five new compounds (4a–e) have been conducted. The final products were obtained in 64–69% yield. Various reagents, including thioglycolic acid,[14,15] sodium azide,[16] anthranilic acid,[16] maleic anhydride,[17] and acetic anhydride[15] were used. The conditions for each reaction were showed in Scheme1.

Evaluation of antibacterial activity

The antibacterial activity of the newly prepared compounds was evaluated *in vitro* against both *E. coli* (ATCC 25922), and *S. aureus* (ATCC 5923) clinical antibiotic-resistant isolates. The agar-disc diffusion approach was used to assess the antibacterial activities according to Özer H. *et al.* method.[18]

Molecular docking studies

Molecular docking investigations were implemented by utilizing the (AutoDock Vina 1.1.2) software was used in molecular docking simulation investigations.[19] The 3D structure of DNA gyrase proteins were imported from the RCSB Protein Data Bank (https://www.rcsb.org/ref). *E. coli* (PDB ID: 1KZN) and *S. aureus* (PDB ID: 3g75).[20,21] All the water molecules and the ligand were removed before the docking calculations for the compound studied, and the 3D structures of ligand and conformations were created by the ChemAxon Marvin Sketch 5.3.735 program and saved in the mol2 format. The Gaussian 09 software was used to optimize and minimize the energy of ligand structure. Auto Dock Tools (ADT) 1.5.6 was utilized to prepare both the ligand and protein.[21,22] The binding affinities of compound (4b) towards gyrase DNA protein of *E. coli* and *S. aureus* were simulated by mimicking the interactions between the protein and compound. Discovery Studio Visualizer (BIOVIA, Discovery Studio, v4.0.100.13345) was employed to examine the interactions between the ligand and targeted proteins.

CONCLUSION

Mortality statistics shows that, about 10 million deaths worldwide were recorded from infectious diseases. This may increase due to the development of bacteria and their resistance to known antibiotics. One of the most important strategies to reduce antimicrobial resistance is preparing new drugs or developing the pharmacological properties of the existent ones. Inhibition of bacterial topoisomerase, which is involved in DNA replication, is one of these strategies. Because of coumarin compounds have antibacterial properties, herein new hybrid coumarin derivatives were synthesized and screened *in vitro* against *E. coli* and *S. aureus* bacteria. Compound (4b) was observed to possess best antibacterial activity among others. Furthermore, these results have been supported by molecular docking study to illustrate how (4b) compound can bind with bacterial DNA gyrase. It can be concluded that the selected compound is a potential antibacterial agent in comparison with the antibiotic clorobiocin throughout its binding energy.

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