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Research Article

(E)-4-benzamido-N'-(2,4-dinitrophenyl) benzohydrazonic acid: A Novel Molecule with Remarkable Bactericidal Activity against Both Gram-positive and Gram-negative Microbes

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A B S T R A C T

After knowing the multifarious potentials of benzamide compounds, a multi-aromatic compound was designed for overcoming the challenges imposed by the pathogens. The present investigation aimed at exploring the anti-bacterial perspectives of a rationally developed therapeutically privileged molecule 4-benzamido-N'-(2,4-dinitrophenyl)-benzohydrazonic acid, produced through two-step chemical synthesis, against gram-negative microbial species (*Escherichia coli*) and gram-positive microbial species (*Staphylococcus aureus*). The novel benzamide-based Schiff's base compound expressed a fair anti-bacterial activity against anti-E. coli activity (ZOI = 17.4 mm, MIC = 500 µg/mL), the Gram-negative strain and anti-S. aureus activity (ZOI = 19.6 mm, MIC = 500 µg/mL), the Gram-positive strain as compared to ciprofloxacin (ZOI = 31.5 mm, MIC = 6.25 µg/mL), the standard drug. However, the study opened new opportunities towards anti-microbial drug development by deeply focusing and exclusively highlighting the unexplored class of hybrid-benzamides or benzohydrazonic acids. More pre-clinical studies and investigations at various levels are essentially required as well as the establishment of structure-activity-relationship (SAR) of the whole series is needed which will open avenues for better pharmacotherapy against a large number of resistant pathogenic strains.

Keywords: Benzamide, Benzohydrazonic acid, *Escherichia coli*, *Staphylococcus aureus*, Anti-microbial, Anti-bacterial

Introduction

Microbes of all forms have imposed a serious threat to the progress of human civilization.[1] From ancient times, humans relied majority on precautions and preventions, and later depended mostly on nature for some hidden medical

treasures.[2] With the discovery of penicillin in the early 20th Century, the scenario changed rapidly.[3] The survival rate among communities gets drastically improved as compared to previous decades which eventually motivated the researchers and the population becomes use-too with the upcoming challenges.[4] Meanwhile, the creation of a number of its derivatives, exploration of new classes, development of hybrid molecules, and improvement in drug discovery elements have flooded the market with anti-microbial products with varied advantages.[5] For a limited period of time, everything remained fine and easy-going but in due course of time, resistance against these products was frequently observed that again inflicted challenge to the humans.[6]

Benzamides are a class of compounds with an amide-bond backbone, have a wide range of biological activities when coupled with aliphatic, aromatic or heteroaromatic systems. They are characterized by a general formula R-CO-NH_2. Among the natural and synthetic substituted amide derivative, there are a number of reported therapeutically active compounds possessing activity such as anti-proliferative, anti-viral, anti-malarial, general anesthetics, anti-inflammatory, anti-bacterial, anti-fungal, anti-convulsant, analgesic, anti-depressants, and anti-Alzheimer's disease.[8] It is versatile in organic compounds since all the three atoms in the O-C-N chain are potentially reactive due to their ability to form hydrogen bonding quite readily.[9]

After knowing the multifarious potentials of benzamide compounds, a multi-aromatic compound was designed for overcoming the challenges imposed by the pathogens. The present investigation aimed at exploring the anti-bacterial perspectives of a rationally developed therapeutically privileged molecule 4-benzamido-N'-(2,4-dinitrophenyl)-benzohydrazonic acid, produced through two-step chemical synthesis, against gram-negative microbial species (*Escherichia coli*) and gram-positive microbial species (*Staphylococcus aureus*).

Materials and Methods

Chemicals and Instrumentation

The starting material, reactants, and the solvents were of analytical grade and exclusively purchased from Sigma Aldrich, Germany through a local vendor at Nagpur. Double distilled water (Borosil®, India) was employed in the study. The reaction progress was monitored through Merck® pre-coated Silica gel-G TLC plates. Elemental analysis (PerkinElmer 2400 model), Fourier-transformed infrared spectroscopy (Shimadzu® IR-Affinity-1 model), Mass Spectroscopy (MICROMASS® Q-TOF model), and Proton (^{1}H)-Nuclear Magnetic Resonance (NMR) Spectroscopy (Bruker® Avance-II model) were done for the characterization.

Synthesis of Target Compounds

The chemical synthesis involved the reaction of benzamide (1) and 4-chlorobenzoic acid (2) where a hydrochloride (HCl) moiety, Cl from (2) and hydrogen from (1) gets eliminated during the reaction. In the successive step, Schiff's base was formed by reacting the active carbonyl group, present in 4-benzamidobenzoic acid (3) with an amino group, present in 2,4-dinitrophenyl hydrazine (4) to form azomethine component, 4-benzamido-N'-(2,4-dinitrophenyl)-benzohydrazonic acid (5) (Figure 1). The mechanism involved an electrophilic attack of the carbonyl carbon (3) with the nucleophilic amine (4) of the reactant.[10]

Synthesis of 4-benzamidobenzoic acid (3)

An equimolar solution (0.01 M) of benzamide (1) and 4-chlorobenzoic acid (2) in anhydrous sodium carbonate (1 mol) was made to stir at room temperature for the duration of 6 hr. The solvent was evaporated under vacuum and the collected precipitate was washed with distilled water and further recrystallized from ethanol to get the desired product.

White powder; 83% yield; R_f: 0.92 (ethanol: chloroform: n-hexane 8:1:1); m.p.: 150-152°C; FTIR (KBr) υ (cm^{-1}):

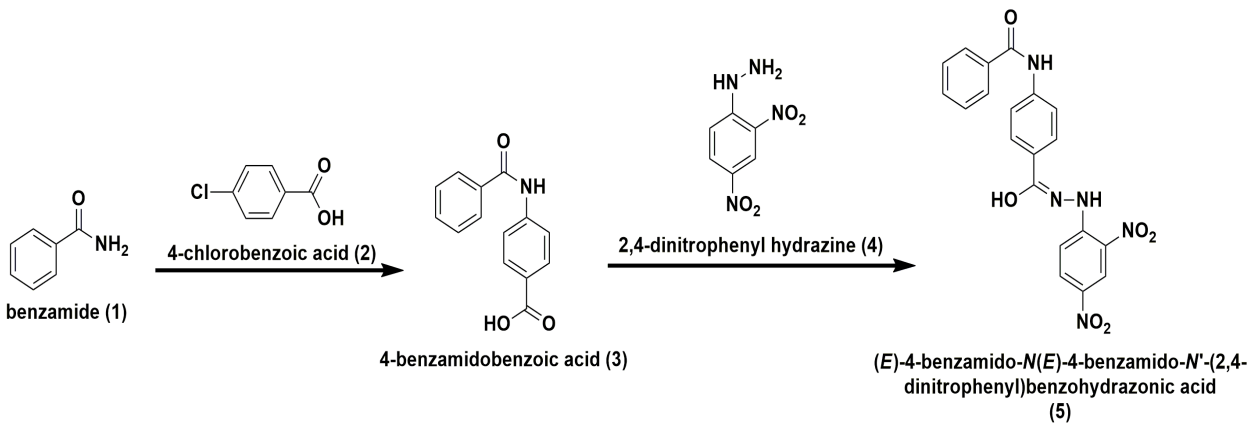

Figure 1.Synthesis of a Benzohydrazonic acid Derivative

3367 (-OH, stretching), 3181 (-NH, stretching), 3106 (C-H, aromatic), 1723 (C=O, stretching), 1622 (C=C, aromatic), 1568 (-NH, bending), 1287 (C-N, stretching), 1204 (C-O); ^{1}H-NMR (δ, ppm, $CDCl_3$): 11.23 (Hydroxyl, 17, 1H), 9.34 (Amide, 9, 1H), 7.6-8.2 (Aromatic, 9H). MS: M^+ 241. Anal. Calcd. for $C_{14}H_{11}NO_3$: C, 69.70; H, 4.60; N, 5.81. Found: C, 69.12; H, 4.34; N, 5.27.

Synthesis of 4-benzamido-N'-(2,4-dinitrophenyl)-benzohydrazonic acid (5)

In a round bottom flask, equimolar quantity (0.01 M) of ethanolic solution of 4-benzamidobenzoic acid (3) and 2,4-dinitrophenyl hydrazine (4) were made to refluxed for a period of 6 hr in the presence of 7-8 drops of glacial acetic acid. The reaction content was cooled in crushed ice to precipitate the final product. The obtained product was washed with cold water, suitably dried, and recrystallized by using absolute ethanol.

Reddish powder; 61% yield; R_f: 0.85 (acetone: chloroform 1:1); m.p.: 263-265°C; FTIR (KBr) υ (cm^{-1}): 3319 (-OH, stretching), 3192 (-NH, stretching), 3127 (C-H, aromatic), 1712 (C=O, stretching), 1675 (C=N, stretching), 1619 (C=C, aromatic), 1578 (-NH, bending), 1543 (-NO_2, stretching), 1302 (C-N, stretching), 1133 (C-O); ^{1}H-NMR (δ, ppm, $CDCl_3$): 9.19 (Amide, 9, 1H), 7.6-8.8 (Aromatic, 12H), 3.59 (Amide, 19, 1H), 2.17 (Hydroxyl, 17, 1H). MS: M^+ 421. Anal. Calcd. for $C_{20}H_{15}N_5O_6$: C, 57.01; H, 3.59; N, 16.62. Found: C, 69.12; H, 4.34; N, 5.27.

Anti-microbial Screening

The synthesized compound was screened for anti-microbial activity against bacterial species; *Escherichia coli (E. coli*, MTCC 2961) and *Staphylococcus aureus (S. aureus,* MTCC 3160) by using disc diffusion method. By using the Muller Hinton Agar medium, the bacterial species were cultured in the nutrient broth media under laminar air-flow condition. The cultured species were selectively transferred into the agar plates. The molecule was dissolved in dimethyl sulfoxide, soaked over a Whatman filter paper (no. 1), placed cautiously over the microbial agar plates, and finally incubated under conditions of 37±1ºC for 24 hrs.[11] By employing the agar streak dilution method, the minimum inhibitory concentration (MIC) was determined. By serial dilution with DMSO, the microbial suspension of concentration (10^6 CFU/mL) was prepared and transferred into the Petri dish up to 5 mm of depth at a temperature of 40-50ºC. The average value of MIC was calculated in triplicate manner. Ciprofloxacin served as the positive control and DMSO act as negative control.[12]

Results and Discussion

Chemistry

The structure of the novel benzamide-based compound was established correctly by employing sophisticated instruments such as FT-IR spectroscopy,^{1}H-NMR spectroscopy, and Mass spectroscopy. The FT-IR analysis of the compound presented some well-known features of the molecule such as amide (-NH) stretching at 3192 cm^{-1}, amide (-NH) bending at 1578 cm^{-1}, carbonyl (C=O) stretching at 1712 cm^{-1}, hydroxyl (-OH) stretching at 3319 cm^{-1}, and azomethine (C=N) stretching at 1675 cm^{-1} suggested the formation of Schiff's base, that ascertained the formation of the proposed compound. In addition to it, the miscellaneous features characteristics such as C-N stretching at 1302 cm^{-1} suggesting the presence of benzamide linkage, C-O stretching at 1133 cm^{-1} suggested the presence of hydroxyl linkage, C-H aromatic stretching at 3127 cm^{-1} and C=C aromatic stretching at 1619 cm^{-1} suggested the presence of aromatic ring, and -NO_2 stretching at 1543 cm^{-1} suggested the two nitro moieties on the aromatic ring were principally observed which helped in partial confirmation of the chemical structure.

At the same time, by using the ^{1}H-NMR technique, the proposed structure of the benzamide-based compound was entirely ascertained. The signals from the 12 protons of the three bulky aromatic rings appeared in the range of 7.6-8.8 ppm. Additionally, the essential features of the chemical structure like hydroxyl proton and amide proton were seen predominately at 9.19 ppm and 2.17 ppm, respectively. Another signal response of proton at 17-position was also located in the spectra at 4.63 ppm. From this technique, the whole structure was understood in a much better way.

The utilization of the final system, mass spectroscopy, demonstrated the appearance of base peak (M^+) of the compound at m/z 346 sharply which match up with the compound's molecular mass along with the appearance of multiple fragmented peaks. The structure of the proposed novel compound was entirely established by the sophisticated analytical technique.

The experimentally obtained elemental analysis data almost matched up with the theoretically calculated values which directed towards the creation of the proposed compound.

Anti-microbial Screening

The novel benzamide-based Schiff's base compound 4-benzamido-N'-(2,4-dinitrophenyl)-benzohydrazonic acid (5) expressed a fair anti-bacterial activity against anti-*E. coli* (ZOI = 17.4 mm, MIC = 500 µg/mL), the Gram-negative strain (Figure 2A) and also expressed fair anti-bacterial activity against anti-*S. aureus* (ZOI = 19.6 mm, MIC = 500 µg/mL), the Gram-positive strain (Figure 2B) as compared to ciprofloxacin (ZOI = 31.5 mm, MIC = 6.25 µg/mL), the standard drug. The novel Schiff's base molecule expressed somehow promising activity against both the Gram-positive and Gram-negative strains as compared to the marketed product.

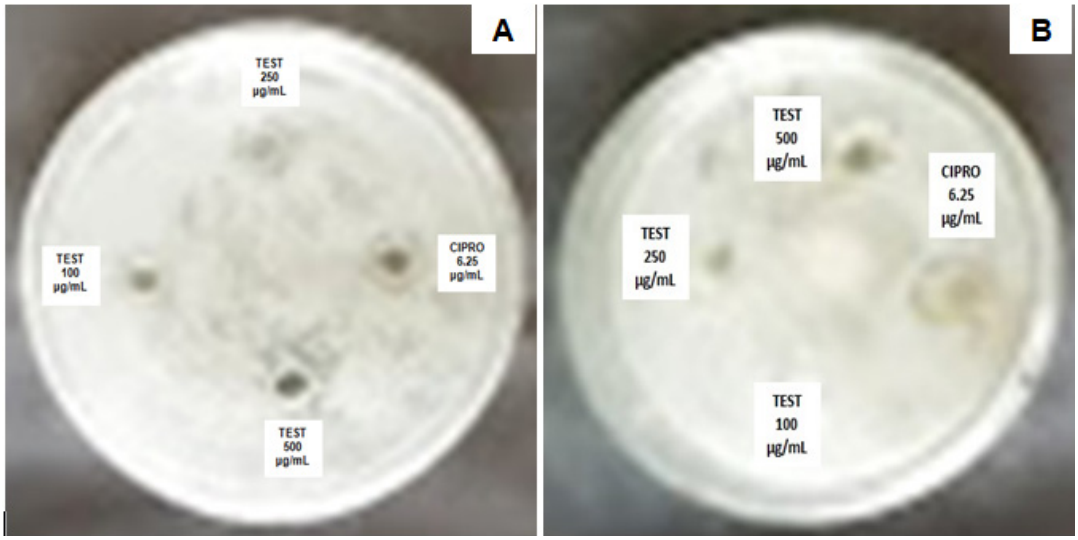

Figure 2. Anti-bacterial Activity of the testbenzamidebased compound against*(A)E.coli(B)S.aureus*

Conclusion

The present investigation involves an exploration of the therapeutically active compound against both Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) strains with an objective to find new analogs that could potentially be a privilege in terms of resistance. The structure of the proposed molecule was established by utilizing sophisticated analytical tools such as FT-IR, ^{1}H-NMR, Mass spectroscopy, and CHN Analyzer. The molecule displayed a fair activity against both the strains in *in-vitro* anti-microbial screening model, but with a very low potency. However, the study opened new opportunities towards anti-microbial drug development by deeply focusing and exclusively highlighting the unexplored class of hybrid-benzamides or benzohydrazonic acids. More pre-clinical studies and investigations at various levels are essentially required as well as the establishment of Structure-Activity-Relationship (SAR) of the whole series is needed which will open avenues for better pharmacotherapy against a large number of resistant pathogenic strains.

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