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Synthesis and Antibacterial Evaluation of 5-Aminosalicylic Acid Derivatives

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Abstract

The anti-inflammatory scaffold 5-aminosalicylic acid, which is widely used in therapeutic applications, was chosen for the synthesis of *N*-[3-(hydrazinecarbonyl)-4-hydroxyphenyl]acetamide (**1**) to enhance its antibacterial properties. The condensation of hydrazide **1** with aromatic aldehydes provided hydrazone derivatives **2a–f**, whereas cyclocondensation reactions and other related transformations afforded five-membered heterocycles, including pyrrole **3**, pyrazole **4**, pyrrolidinone **7**, oxadiazoles **9**, **10**, thiadiazole **14**, and triazole **15**. Additional modifications yielded acetylhydrazine derivative **11**, which was O-alkylated to analogue **12**. Antibacterial evaluation showed stronger activity against Gram-positive bacteria such as *S. aureus* and MRSA than against Gram-negative strains of *E. coli* and *S.* Enteritidis, consistent with differences in cell membrane permeability. Notably, derivatives containing pyrrolidinone **7**, thiosemicarbazide **13**, and 1,3,4-thiadiazole **14** exhibited potent bactericidal activity against *S. aureus* and MRSA, while hydrazones **2b**, **2c**, **2f**, pyrrole **3**, and pyrrolidinone **7** exhibited activity against *E. coli*. These results provide a practical strategy for the discovery of heterocyclic compounds and emphasise the potential of functionalised 5-aminosalicylic acid derivatives as prime candidates for the development of broad-spectrum antibacterial agents.

Keywords: MRSA; hydrazone; pyrrole; pyrazole; oxadiazoles; triazole; pyrrolidinone; antibacterial; *Escherichia coli*; *Staphylococcus aureus*

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1. Introduction

Antibiotics remain indispensable for the treatment and prevention of bacterial infections, but their widespread use has accelerated the emergence of resistance, creating an urgent need for innovative therapeutic strategies [1]. To restore efficacy, current regimens must be optimised and new biological pathways explored, along with the development of novel molecular structures [2,3]. Infections caused by methicillin-susceptible *Staphylococcus aureus* (MSSA) and methicillin-resistant *Staphylococcus aureus* (MRSA) are widespread [4]. MRSA infections are challenging to manage because new classes of antibiotics have not yet been developed to combat resistant strains [5]. *Salmonella enterica* and certain strains of *Escherichia coli* (*E. coli*) are significant foodborne pathogens, and the increasing

reliance on antibiotics to control infections and associated outbreaks raises concerns about the development of antibiotic resistance [6]. Therefore, identifying diverse bacterial targets is essential to increase the efficacy of current antibiotics and discover new drugs, as many essential processes remain unexplored as potential therapeutic options [7].

The prospect of target selection is important for new strategies for the synthesis of small molecule compounds; for example, studies reported in the literature [8] have shown that targeting virulence factors such as sortase A (SrtA) and caseinolytic peptidase P (ClpP) represents a promising pharmacological approach for the treatment of MRSA infections. In addition to virulence factors, reduced antibiotic efficacy is also mediated by the NorA efflux pump, which expels drugs and biocides. Inhibition of the NorA efflux pump mechanism has the potential to restore antimicrobial activity in resistant strains [9]. A study by F. Guo et al. demonstrated that inhibition of 6-phosphoglucosamine synthetase (GlmS) resulted in strong antibacterial activity against MRSA by disrupting bacterial cell wall biosynthesis, thereby identifying this enzyme as a promising target for small-molecule development [10]. Furthermore, regulation of biofilm formation by SarA inhibitors represents an important strategy for managing chronic infections caused by virulent *Staphylococcus aureus* (*S. aureus*) strains [11].

Studies by F. Liu et al. have shown that the anti-inflammatory drug 5-aminosalicylic acid can modulate bacterial growth in a strain-dependent manner [12], while J. Kaufman et al. have revealed that this effect may be mediated by changes in bacterial gene expression [13]. In addition, newly synthesised compounds containing the 5-aminosalicylic acid moiety have demonstrated antibacterial, anticancer, and anti-inflammatory effects [14]. Studies by F. S. Fu et al. on sulfasalazine have produced strong antibacterial activity (97%) and promoted bone regeneration in MRSA-induced osteomyelitis [15].

In order to expand the synthetic possibilities for the formation of new derivatives, compounds are often functionalised with a hydrazide group, which facilitates their broad application in the preparation of nitrogen-containing derivatives [16–20]. To develop new derivatives with antimicrobial activity, molecules are frequently designed to contain a hydrazone moiety [21–24]. In addition, heterocyclic structures serve as important scaffolds in the design and synthesis of new biologically active compounds, with particular emphasis on classes such as pyrroles [25–28], pyrazoles [29–33], 1,3,4-oxadiazoles [34–36], 1,2,4-triazoles [37–39], and 1,3,4-thiadiazoles [40,41] due to their well-known antibacterial activity.

Treatment of infectious and inflammatory diseases often requires both antibiotics and anti-inflammatory drugs; however, such combinations can cause adverse effects. This underscores the need for dual-action drugs, and anti-inflammatory agents that also exhibit antimicrobial activity are promising candidates [42–44]. In this study, the 5-aminosalicylic acid scaffold was chosen as a well-known anti-inflammatory agent, and its modification was intended to enhance antibacterial activity. A novel library of acetyl-5-aminosalicylic acid derivatives was synthesised, and their antibacterial potential was evaluated using structure–activity relationship (SAR) insights to optimise properties and improve efficacy against resistant bacterial strains.

2. Materials and Methods

The reagents and solvents were used from Sigma-Aldrich (St. Louis, MO, USA), TCI (Europe, Zwijndrecht, Belgium), and Fisher Scientific (Europe, V. A. Graiciuno g., Vilnius). All ^{1}H and ^{13}C NMR data were obtained at 25 °C in DMSO-d_6 solution using Bruker Avance III spectrometers operating at 400 MHz and 101 MHz, respectively (Bruker BioSpin AG, Fällanden, Switzerland). Chemical shifts (δ) are expressed in ppm, with DMSO-d_6 used as an internal standard (2.50 ppm for ^{1}H and 39.43 ppm for ^{13}C). See Supplementary Materials, Figures S1–S40 or on the Zenodo website. To follow the transformation

and study the purity of the synthesised materials, thin-layer chromatography was performed using silica gel F254 plates (Merck KGaA, Darmstadt, Germany). LC-MS measurements were performed using an Agilent G1956B LC/MSD SL single quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) with an electrospray ionization (ESI) source. The instrument was operated in positive and negative ion modes with a mass range of *m*/*z* 50–3000 and unit resolution (≤0.7 Da FWHM). See Supplementary Materials, Figures S41–S48 for representative spectra. The melting points were obtained using a Büchi B-540 instrument and are not corrected. Elemental analysis (C, H, N, S) was performed on an EA3100 series CHNSO analyser (EuroVector S.p.A., Pavia, Italy) using Weaver software CFR 21 art.11, and the results were consistent with the theoretical values with an accuracy of ±0.3%.

General procedure for the synthesis of *N*-[3-(hydrazinecarbonyl)-4-hydroxyphenyl]acetamide (**1**)

N-[3-(Hydrazinecarbonyl)-4-hydroxyphenyl]acetamide (**1**) was obtained following the previously reported procedure [45,46], which involved a three-step synthesis.

Step 1. A mixture of 5-aminosalicylic acid (0.20 mol, 30.63 g), 250 mL of methanol, and sulfuric acid (0.22 mol, 21.56 g) was refluxed for 20 h. After the reaction, the volatiles were removed under reduced pressure. The residue was neutralised with 10% aqueous sodium carbonate solution to pH ~8. The precipitated solid was filtered, washed with water, and purified by recrystallisation from propan-2-ol. The yield of methyl 5-amino-2-hydroxybenzoate was 65% (21.73 g), grey solid, m. p. 98–100 °C (m. p. found in ref. [47], 97–99 °C).

Step 2. Methyl 5-amino-2-hydroxybenzoate (0.13 mol, 24.53 g) was dissolved in 250 mL of glacial acetic acid. Acetic anhydride (0.20 mol, 20.40 g, 18.89 mL) was added by portion wise to the solution at room temperature over 10 min, and the reaction mixture was stirred for 30 min. The reaction mixture was diluted with 500 mL of water containing crushed ice. The precipitate was filtered, washed with water, and purified by recrystallisation from water. The yield of methyl 5-acetamido-2-hydroxybenzoate was 78% (21.21 g), grey solid, m. p. 146–147 °C (m. p. found in ref. [48], 147 °C).

Step 3. A mixture of methyl 5-acetamido-2-hydroxybenzoate (0.10 mol, 20.90 g), hydrazine monohydrate (0.30 mol, 15.00 g, 14.53 mL), and 200 mL of propan-2-ol was refluxed for 6 h, and the reaction progress was monitored by TLC (CH_3OH:CCl_3, 1:8). The reaction mixture was cooled, the solid was filtered, washed with propan-2-ol, and the resulting product **1** was purified by recrystallisation from propan-2-ol containing a small amount of water. The yield of hydrazide **1** was 70% (14.64 g), white solid, m. p. 218–220 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 1.98 (s, 3H, CH_3), 6.01 (br. s, 4H, $NHNH_2$, OH), 6.77 (d, J = 8.8 Hz, 1H, H_{ar}), 7.43 (dd, J = 8.8, 2.7 Hz, 1H, H_{ar}), 7.89 (d, J = 2.7 Hz, 1H, H_{ar}), 9.70 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, 101 MHz): δ (ppm) 23.68, 116.57, 117.51, 120.50, 125.00, 129.02, 156.69, 166.54, 167.66. Anal. calcd for $C_9H_{11}N_3O_3$ (209.205 g/mol) %: C, 51.67; H, 5.30; N, 20.09. Found %: C, 51.61; H, 5.24; N, 19.97. Compound **1** ESI-MS *m*/*z* = 210.198 $[M + H]^+$ (100%), 211.200 $[M + 2]^+$ (8.43%).

A general procedure for the synthesis of compounds **2a–f**

A mixture of *N*-[3-(hydrazinecarbonyl)-4-hydroxyphenyl]acetamide (**1**) (0.209 g, 1 mmol), the corresponding aromatic aldehyde (1.1 mmol), and 20 mL of propan-2-ol was refluxed for 1–4 h. The progress of the reaction was monitored by TLC (CH_3OH:CCl_3, 1:8). The reaction mixture was cooled to room temperature and diluted with 10 mL of water. The precipitate was filtered and washed with propan-2-ol. Compounds **2a–f** were purified by recrystallisation method from the appropriate solvent.

N-{3-[(2*E*)-2-Benzylidenehydrazine-1-carbonyl]-4-hydroxyphenyl}acetamide (**2a**)

White solid, yield 84% (0.25 g), m. p. 274–276 °C (methanol). ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 2.02 (s, 3H, CH_3), 6.94 (d, J = 8.8 Hz, 1H, H_{ar}), 7.30–7.53 (m, 3H, H_{ar}), 7.55–7.64 (m, 1H, H_{ar}), 7.69–7.80 (m, 2H, H_{ar}), 7.99 (s, 1H, H_{ar}), 8.43 (s, 1H, CH=N), 9.88 (s, 1H, NH), 11.22 (s, 1H, OH), 11.73 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, 101 MHz): δ (ppm) 23.71, 116.98, 117.29, 120.61, 125.37, 127.21, 128.85, 130.19, 131.17, 134.24, 148.22, 153.21, 163.33, 167.92. Anal. calcd for $C_{16}H_{15}N_3O_3$ (297.314 g/mol) %: C, 64.64; H, 5.09; N, 14.13. Found %: C, 64.59; H, 4.95; N, 14.04.

N-(4-Hydroxy-3-{(2*E*)-2-[(2-methoxyphenyl)methylidene]hydrazine-1-carbonyl}phenyl)acetamide (**2b**)

White solid, yield 91% (0.30 g), m. p. 148–150 °C (propan-2-ol). ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 2.02 (s, 3H, CH_3), 3.87 (s, 3H, OCH_3), 6.92 (d, J = 8.8 Hz, 1H, H_{ar}), 7.03 (t, J = 7.6 Hz, 1H, H_{ar}), 7.11 (d, J = 8.4 Hz, 1H, H_{ar}), 7.43 (t, J = 7.9 Hz, 1H, H_{ar}), 7.56 (d, J = 8.1 Hz, 1H, H_{ar}), 7.87 (d, J = 7.7 Hz, 1H, H_{ar}), 7.98 (d, J = 2.5 Hz, 1H, H_{ar}), 8.75 (s, 1H, CH=N), 9.86 (s, 1H, NH), 11.31 (s, 1H, OH), 11.80 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, 101 MHz): δ (ppm) 23.67, 55.72, 111.89, 116.81, 117.05, 120.41, 120.78, 122.17, 125.68, 130.90, 131.77, 143.80, 153.95, 157.86, 163.87, 167.93. Anal. calcd for $C_{17}H_{17}N_3O_4$ (327.340 g/mol) %: C, 62.38; H, 5.23; N, 12.84. Found %: C, 62.29; H, 5.16; N, 12.72.

N-(4-Hydroxy-3-{(2*E*)-2-[(3-methoxyphenyl)methylidene]hydrazine-1-carbonyl}phenyl)acetamide (**2c**)

White solid, yield 83% (0.27 g), m. p. 234–236 °C (propan-2-ol). ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 2.02 (s, 3H, CH_3), 3.81 (s, 3H, OCH_3), 6.94 (d, J = 8.8 Hz, 1H, H_{ar}), 7.02 (d, J = 8.1 Hz, 1H, H_{ar}), 7.20–7.33 (m, 2H, H_{ar}), 7.38 (t, J = 8.0 Hz, 1H, H_{ar}), 7.61 (d, J = 8.7 Hz, 1H, H_{ar}), 7.98 (d, J = 2.5 Hz, 1H, H_{ar}), 8.40 (s, 1H, CH=N), 9.88 (s, 1H, NH), 11.20 (s, 1H, OH), 11.72 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, 101 MHz): δ (ppm) 23.70, 55.19, 111.31, 116.36, 116.97, 117.34, 120.13, 120.62, 125.35, 129.96, 131.19, 135.66, 148.10, 153.13, 159.54, 163.28, 167.91. Anal. calcd for $C_{17}H_{17}N_3O_4$ (327.340 g/mol) %: C, 62.38; H, 5.23; N, 12.84. Found %: C, 62.27; H, 5.11; N, 12.77.

N-(4-Hydroxy-3-{(2*E*)-2-[(4-methoxyphenyl)methylidene]hydrazine-1-carbonyl}phenyl)acetamide (**2d**)

White solid, yield 86% (0.28 g), m. p. 242–244 °C (methanol). ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 2.02 (s, 3H, CH_3), 3.81 (s, 3H, OCH_3), 6.93 (d, J = 8.8 Hz, 1H, H_{ar}), 7.03 (d, J = 8.1 Hz, 1H, H_{ar}), 7.20–7.33 (m, 2H, H_{ar}), 7.38 (t, J = 8.0 Hz, 1H, H_{ar}), 7.61 (d, J = 8.7 Hz, 2H, H_{ar}), 7.59 (d, J = 7.9 Hz, 1H, H_{ar}), 7.68 (d, 8.40, J = 8.3 Hz, 2H, H_{ar}), 7.98 (s, 1H, H_{ar}), 8.36 (s, 1H, CH=N), 9.88 (s, 1H, NH), 11.28 (s, 1H, OH), 11.63 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, 101 MHz): δ (ppm) 23.72, 55.34, 114.37, 116.99, 117.23, 120.56, 125.35, 126.77, 128.87, 131.12, 148.18, 153.34, 160.95, 163.22, 167.94. Anal. calcd for $C_{17}H_{17}N_3O_4$ (327.340 g/mol) %: C, 62.38; H, 5.23; N, 12.84. Found %: C, 62.30; H, 5.18; N, 12.76.

N-(4-Hydroxy-3-{(2*E*)-2-[(2,4-dimethoxyphenyl)methylidene]hydrazine-1-carbonyl}phenyl)acetamide (**2e**)

White solid, yield 81% (0.29 g), m. p. 235–237 °C (propan-2-ol). ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 2.02 (s, 3H, CH_3), 3.82 (s, 3H, OCH_3), 3.86 (s, 3H, OCH_3), 6.64 (s, 1H, H_{ar}), 6.91 (d, J = 8.8 Hz, 1H, H_{ar}), 7.55 (d, J = 8.8 Hz, 1H, H_{ar}), 7.81 (d, J = 9.1 Hz, 1H, H_{ar}), 7.98 (s, 1H, CH=N), 9.85 (s, 1H, NH), 11.39 (s, 1H, OH), 11.69 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, 101 MHz): δ (ppm) 23.65, 55.47, 55.80, 98.30, 106.45, 114.95, 116.64, 117.04, 120.34, 125.70, 126.87, 130.83, 143.98, 154.12, 159.29, 162.62, 163.76, 167.92. Anal. calcd for $C_{18}H_{19}N_3O_5$ (357.366 g/mol) %: C, 60.50; H, 5.36; N, 11.76. Found %: C, 60.39; H, 5.28; N, 11.69.

N-(4-Hydroxy-3-{(2*E*)-2-[(3,4-dimethoxyphenyl)methylidene]hydrazine-1-carbonyl}phenyl)acetamide (**2f**)

White solid, yield 80% (0.29 g), m. p. 237–239 °C (methanol). ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 2.02 (s, 3H, CH_3), 3.82 (d, J = 4.6 Hz, 6H, 2OCH_3), 6.93 (d, J = 8.7 Hz, 1H, H_{ar}), 7.03 (d, J = 8.2 Hz, 1H, H_{ar}), 7.22 (d, J = 8.2 Hz, 1H, H_{ar}), 7.35 (s, 1H, H_{ar}), 7.60 (d, J = 9.5 Hz, 1H, H_{ar}), 7.98 (s, 1H, H_{ar}), 8.35 (s, 1H, CH=N), 9.88 (s, 1H, NH), 11.25 (s, 1H, OH), 11.64 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, 101 MHz): δ (ppm) 23.69, 55.45, 55.58, 108.33, 111.48, 116.97, 117.29, 120.57, 122.05, 125.32, 126.89, 131.13, 148.48, 149.06, 150.84, 153.25, 163.16, 167.92. Anal. calcd for $C_{18}H_{19}N_3O_5$ (357.366 g/mol) %: C, 60.50; H, 5.36; N, 11.76. Found %: C, 60.41; H, 5.30; N, 11.67.

Synthesis of 5-acetamido-*N*-(2,5-dimethyl-1*H*-pyrrol-1-yl)-2-hydroxybenzamide (**3**)

A mixture of *N*-[3-(hydrazinecarbonyl)-4-hydroxyphenyl]acetamide (**1**) (2 mmol, 0.42 g), hexane-2,5-dione (2.5 mmol, 0.28 g), and five drops of glacial acetic acid in 30 mL of methanol was refluxed for 8 h. The reaction mixture was cooled down and diluted with 50 mL of water, and the precipitate was filtered and washed with water. The product **3** was purified by recrystallisation from a mixture of propan-2-ol and water (3:1). The yield of compound **3** was 66% (0.38 g), white solid, m. p. 229–231 °C (propan-2-ol). ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 2.01 (s, 3H, CH_3), 2.03 (s, 6H, 2CH_3), 5.69 (s, 2H, CH_{pyrr}), 6.93 (d, J = 8.8 Hz, 1H, H_{ar}), 7.62 (d, J = 8.8 Hz, 1H, H_{ar}), 7.94 (d, J = 2.6 Hz, 1H, H_{ar}), 9.87 (s, 1H, OH), 10.95 (s, 2H, 2NH). ^{13}C NMR (DMSO-d_6, 101 MHz): δ (ppm) 11.55, 24.16, 103.49, 117.36, 120.94, 125.80, 127.40, 131.65, 153.44, 166.79, 168.36. Anal. calcd for: $C_{15}H_{17}N_3O_3$ (287.319 g/mol) %: C, 62.71; H, 5.96; N, 14.63. Found %: C, 62.65; H, 5.92; N, 14.54. Compound **3** ESI-MS m/z = 286.065 $[M - 1]^+$ (100%), 287.049 $[M]^+$ (17.92%), 288.055 (2.12%) $[M + H]^+$.

Synthesis of *N*-[3-(3,5-dimethyl-1*H*-pyrazole-1-carbonyl)-4-hydroxyphenyl]acetamide (**4**)

A mixture of *N*-[3-(hydrazinecarbonyl)-4-hydroxyphenyl]acetamide (**1**) (2 mmol, 0.42 g), pentane-2,4-dione (2.5 mmol, 0.25 g), and five drops of glacial acetic acid in 20 mL of methanol was refluxed for 24 h. The reaction mixture was cooled and diluted with 75 mL of distilled water. The precipitated solid was filtered, washed with water, and purified by column chromatography using acetone:hexane (1:1) as the eluent, R_f = 0.43. The yield of compound **4** was 28% (0.15 g), white solid, m. p. 200–202 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 2.00 (s, 3H, CH_3), 2.08 (s, 3H, CH_3), 2.54 (s, 3H, CH_3), 6.22 (s, 1H, H_{pyr}), 6.82 (d, J = 8.8 Hz, 1H, H_{ar}), 7.46 (d, J = 8.8 Hz, 1H, H_{ar}), 7.53 (s, 1H, H_{ar}), 9.67 (s, 1H, NH), 9.81 (s, 1H, OH). ^{13}C NMR (DMSO-d_6, 101 MHz): δ (ppm) 13.49, 13.89, 23.77, 111.38, 116.12, 119.75, 122.85, 122.94, 130.58, 143.45, 150.62, 151.57, 167.82, 168.12. Anal. calcd for: $C_{14}H_{15}N_3O_3$ (273.292 g/mol) %: C, 61.53; H, 5.53; N, 15.38. Found %: C, 61.47; H, 5.44; N, 15.29. Compound **4** ESI-MS m/z = 274.160 $[M + H]^+$ (100%), 275.091 $[M + 2]^+$ (11.88%).

4-Acetamido-2-(3,5-dimethyl-1*H*-pyrazole-1-carbonyl)phenyl 5-acetamido-2-hydroxybenzoate (**5**)

The yield of compound **5** was 24% (0.21 g), white solid, acetone:hexane (1:1) R_f = 0.30, m. p. 196–198 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 1.91 (s, 3H, CH_3), 2.00 (s, 3H, CH_3), 2.08 (s, 3H,CH_3), 2.46 (s, 3H, CH_3), 5.98 (s, 1H, H_{pyr}), 6.92 (d, J = 9.0 Hz, 1H, H_{ar}), 7.41 (d, J = 8.8 Hz, 1H, H_{ar}), 7.66 (dd, J = 8.9, 2.5 Hz, 1H, H_{ar}), 7.82 (dd, J = 8.9, 2.3 Hz, 1H, H_{ar}), 7.86 (d, J = 2.4 Hz, 1H, H_{ar}), 7.93 (d, J = 2.1 Hz, 1H, H_{ar}), 9.86 (s, 1H, OH), 9.94 (s, 1H, NH), 10.25 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, 101 MHz): δ (ppm) 13.02, 13.41, 23.74, 23.99, 111.39, 111.68, 117.44, 120.02, 122.26, 123.65, 127.70, 127.94, 131.26, 137.01, 142.36, 143.75, 152.58, 156.09, 165.66, 165.90, 167.91, 168.65. Anal. calcd for: $C_{23}H_{22}N_4O_6$ (450.451 g/mol) %: C, 61.33; H, 4.92; N, 12.44. Found %: C, 61.22; H, 4.89; N, 12.40. Compound **5** ESI-MS m/z = 451.169 $[M + H]^+$ (100%), 452.161 $[M + 2]^+$ (28.41%).

Methyl 5-acetamido-2-hydroxybenzoate (**6**)

The yield of compound **6** was 11% as white solid, acetone:hexane (1:1) R_f = 0.73, m. p. 146–147 °C, (ref. [46], 147 °C). ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 2.01 (s, 3H, CH_3), 3.89 (s, 3H, CH_3), 6.93 (d, J = 9.0 Hz, 1H, H_{ar}), 7.63 (d, J = 7.9 Hz, 1H, H_{ar}), 8.10 (s, 1H, H_{ar}), 9.91 (s, 1H, OH), 10.25 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, 101 MHz): δ (ppm) 23.78, 52.50, 112.33, 117.50, 119.85, 127.22, 131.33, 155.84, 168.01, 169.10. Anal. calcd for: $C_{10}H_{11}NO_4$ (209.201 g/mol) %: C, 57.41; H, 5.30; N, 6.70. Found %: C, 57.37; H, 5.29; N, 6.64.

Synthesis of 1-(5-acetamido-2-hydroxybenzamido)-5-oxopyrrolidine-3-carboxylic acid (**7**)

A mixture of *N*-[3-(hydrazinecarbonyl)-4-hydroxyphenyl]acetamide (**1**) (1 mmol, 0.21 g), itaconic acid (1.1 mmol, 1.28 g), and 10 mL of water was refluxed for 5 h. The reaction mixture was cooled down, and the precipitate was filtered and washed with cold water. The product **7** was purified by recrystallisation from methanol. White solid, yield 75% (0.24 g), m. p. 282–284 °C (methanol). ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 2.01 (s, 3H, CH_3), 2.53–2.66 (m, 3H, $COCH_2$), 3.29–3.40 (m, 2H, CH), 3.66–3.81 (m, 3H, NCH_2), 6.92 (d, J = 8.8 Hz, 1H, H_{ar}), 7.60 (d, J = 8.8 Hz, 1H, H_{ar}), 7.98 (s, 1H, H_{ar}), 9.87 (s, 1H, NH), 10.35 (s, 1H, NH), 11.17 (s, 1H, OH), 12.74 (s, 1H, OH). ^{13}C NMR (DMSO-d_6, 101 MHz): δ (ppm) 23.72, 31.25, 34.15, 49.67, 115.76, 117.05, 120.59, 125.82, 131.22, 153.29, 165.31, 167.92, 170.87, 173.97. Anal. calcd for: $C_{14}H_{15}N_3O_6$ (321.289 g/mol) %: C, 52.34; H, 4.71; N, 13.08. Found %: C, 52.30; H, 4.67; N, 13.00.

Synthesis of ethyl (*E*)-*N*-(5-acetamido-2-hydroxybenzoyl)formohydrazonate (**8**)

A suspension of *N*-[3-(hydrazinecarbonyl)-4-hydroxyphenyl]acetamide (**1**) (2 mmol, 0.42 g) in 6 mL of triethyl orthoformate was refluxed for 8 h, and the progress was monitored by TLC (CH_3OH:CCl_3, 1:8). The reaction mixture was cooled down, and the precipitate was filtered and washed with propan-2-ol. The product **8** was purified by recrystallisation from propan-2-ol. White solid, yield 63% (0.33 g), m. p. 198–200 °C (propan-2-ol). ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 1.29 (t, J = 6.9 Hz, 3H, $CH_2C\underline{H}_3$), 2.00 (s, 3H, CH_3), 4.21 (q, J = 7.0 Hz, 2H, $C\underline{H}_2CH_3$), 6.90 (d, J = 8.7 Hz, 1H, H_{ar}), 7.03 (s, 1H, H_{ar}), 7.67 (d, J = 8.7 Hz, 1H, H_{ar}), 8.05 (s, 1H, N=CH), 9.87 (s, 1H, NH), 11.24 (s, 1H, OH), 11.36 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, 101 MHz): δ (ppm) 23.72, 31.25, 34.15, 49.67, 115.76, 117.05, 120.59, 125.82, 131.22, 153.29, 165.31, 167.92, 170.87, 173.97. Anal. calcd for: $C_{12}H_{15}N_3O_4$ (265.269 g/mol) %: C, 54.33; H, 5.70; N, 15.84. Found %: C, 54.25; H, 5.66; N, 15.81.

Synthesis of *N*-[4-hydroxy-3-(1,3,4-oxadiazol-2-yl)phenyl]acetamide (**9**)

A suspension of *N*-[3-(hydrazinecarbonyl)-4-hydroxyphenyl]acetamide (**1**) (2 mmol, 0.42 g), and 4-toluenesulfonic acid monohydrate (3 mmol, 0.57 g) in 6 mL of triethyl orthoformate and the reaction mixture was refluxed for 24 h. The progress of the reaction was monitored by TLC (CH_3OH:CCl_3, 1:8). The mixture was cooled, and the precipitate was filtered and washed with propan-2-ol. The product **9** was purified by recrystallisation from methanol. Yellowish solid, yield 62% (0.27 g), m. p. 232–234 °C (methanol) (m. p. found in ref. [45] 235–236 °C (acetone)). ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 2.03 (s, 3H, CH_3), 7.03 (d, J = 8.8 Hz, 1H, H_{ar}), 7.57 (d, J = 10.7 Hz, 1H, H_{ar}), 8.15 (s, 1H, H_{ar}), 9.34 (s, 1H, CH), 9.96 (s, 1H, OH), 10.05 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, 101 MHz): δ (ppm) 23.83, 108.88, 117.32, 118.74, 124.78, 131.75, 152.01, 153.93, 163.05, 168.06. Anal. calcd for: $C_{10}H_9N_3O_3$ (219.200 g/mol) %: C, 54.79; H, 4.14; N, 19.17. Found %: C, 54.70; H, 4.11; N, 19.12.

Synthesis of *N*-[4-hydroxy-3-(5-sulfanylidene-4,5-dihydro-1,3,4-oxadiazol-2-yl)phenyl]acetamide (**10**)

N-[3-(Hydrazinecarbonyl)-4-hydroxyphenyl]acetamide (**1**) (0.42 g, 2 mmol) was dissolved in pyridine (10 mL). Carbon disulphide (0.76 g, 3 mmol) was added to the solution, and the reaction mixture was heated at 80–90 °C for 30 h. The progress of the reaction was monitored by TLC (CH_3OH:CCl_3, 1:8). The reaction mixture was acidified with 10% hydrochloric acid to pH 4 and diluted with distilled water (50 mL). The precipitate was

filtered, washed sequentially with water and methanol, and dried. The compound **10** was purified by recrystallisation from 1,4-dioxane. Yellowish solid, yield 59% (0.29 g), m. p. 256–258 °C (1,4-dioxane). ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 2.01 (s, 3H, CH_3), 6.98 (d, J = 8.9 Hz, 1H, H_{ar}), 7.55 (d, J = 8.9 Hz, 1H, H_{ar}), 7.99 (s, 1H, H_{ar}), 9.93 (s, 1H, OH), 10.19 (s, 1H, NH), 14.49 (s, 1H, SH or NH). ^{13}C NMR (DMSO-d_6, 101 MHz): δ (ppm) 24.26, 109.22, 117.76, 119.33, 125.16, 131.95, 152.44, 160.06, 168.46, 177.37. Anal. calcd for: $C_{10}H_9N_3O_3S$ (251.260 g/mol) %: C, 47.80; H, 3.61; N, 16.72; S, 12.76. Found %: C, 47.73; H, 3.57; N, 16.64; S, 12.70. Compound **10** ESI-MS m/z = 249.952 $[M - H]^+$ (100%), 251.949 $[M]^+$ (5.48%).

Synthesis of *N*-[3-(2-acetylhydrazine-1-carbonyl)-4-hydroxyphenyl]acetamide (**11**)

Potassium thiocyanate (1.166 g, 12 mmol) was dissolved in 10 mL of glacial acetic acid at room temperature. *N*-[3-(Hydrazinecarbonyl)-4-hydroxyphenyl]acetamide (**1**) (2.092 g, 10 mmol) was added to the solution, and the reaction mixture was gently refluxed for 48 h. Reaction progress was monitored by TLC (CH_3OH:CCl_3, 1:8). The mixture was distilled under reduced pressure on a rotary evaporator, and then the residue was diluted with 20 mL of distilled water. The precipitate was filtered and washed with water. The product **11** was purified by recrystallisation from methanol. Grey solid, yield 57% (0.29 g), m. p. 299–300 °C (methanol). ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 1.93 (s, 3H, CH_3), 2.01 (s, 3H, CH_3), 6.90 (d, J = 8.8 Hz, 1H, H_{ar}), 7.59 (dd, J = 8.8, 2.2 Hz, 1H, H_{ar}), 7.99 (d, J = 2.2 Hz, 1H, H_{ar}), 9.85 (s, 1H, NH), 10.35 (s, 1H, NH), 10.89 (br. s, 2H, NH and OH). ^{13}C NMR (DMSO-d_6, 101 MHz): δ (ppm) 20.46, 23.71, 116.03, 117.01, 120.63, 125.50, 131.10, 153.33, 164.31, 167.15, 167.91. Anal. calcd for: $C_{11}H_{13}N_3O_4$ (251.091 g/mol) %: C, 52.59; H, 5.22; N, 16.73. Found %: C, 52.42; H, 5.09; N, 16.57. Compound **11** ESI-MS m/z = 252.108 $[M]^+$ (96.66%), 253.135 $[M + H]^+$ (5.39%).

Synthesis of *N*-[3-(2-acetylhydrazine-1-carbonyl)-4-(2-oxo-2-phenylethoxy)phenyl]acetamide (**12**)

A mixture of *N*-[3-(2-acetylhydrazine-1-carbonyl)-4-hydroxyphenyl]acetamide (**11**) (0.25 g, 1 mmol), 2-bromoacetophenone (0.20 g, 1 mmol), sodium bicarbonate (3 mmol, 0.25 g), and 10 mL of DMF was heated at 120 °C for 6 h. Reaction progress was monitored by TLC (CH_3OH:CCl_3, 1:8). The reaction mixture was cooled down and poured onto 50 mL of crushed ice, and the precipitate was filtered and washed with water. The product **12** was purified by recrystallisation from propan-2-ol to afford a yellow solid, yield 64% (0.24 g), m. p. 202–204 °C (propan-2-ol). ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 1.95 (s, 3H, CH_3), 2.03 (s, 3H, CH_3), 5.78 (s, 2H, CH_2), 7.23 (d, J = 9.0 Hz, 1H, H_{ar}), 7.59 (t, J = 7.6 Hz, 2H, H_{ar}), 7.72 (t, J = 7.4 Hz, 1H, H_{ar}), 7.83 (dd, J = 9.0, 2.7 Hz, 1H, H_{ar}), 8.00–8.16 (m, 3H, H_{ar}), 10.02 (s, 1H, NH), 10.13 (s, 1H, NH), 10.41 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, 101 MHz): δ (ppm) 20.64, 23.85, 71.78, 114.21, 120.31, 121.84, 123.84, 128.00, 128.95, 133.33, 133.84, 134.19, 151.55, 163.32, 168.03, 168.13, 194.29. Anal. calcd for: $C_{19}H_{19}N_3O_5$ (369.132 g/mol) %: C, 61.78; H, 5.18; N, 11.38. Found %: C, 61.59; H, 5.11; N, 11.22. Compound **12** ESI-MS m/z = 368.079 $[M - H]^+$ (100%), 369.063 $[M]^+$ (22.58%).

Synthesis of *N*-{4-hydroxy-3-[2-(phenylcarbamothioyl)hydrazine-1-carbonyl]phenyl}acetamide (**13**)

N-[3-(Hydrazinecarbonyl)-4-hydroxyphenyl]acetamide (**1**) (1.04 g, 5 mmol) was dissolved in 10 mL of 1,4-dioxane. Phenyl isothiocyanate (5.2 mmol, 0.70 g) was added to the solution, and the reaction mixture was stirred with a magnetic stirrer at room temperature for 4 h. The progress of the reaction was monitored by TLC (CH_3OH:CCl_3, 1:8). The reaction mixture was diluted with water, and the precipitates were filtered and washed with water. The product **13** was purified by recrystallisation from 1,4-dioxane.

White solid, yield: 91% (1.57 g), m. p. 180–182 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 2.01 (s, 3H, CH_3), 6.92 (d, J = 8.7 Hz, 1H, H_{ar}), 7.14 (t, J = 7.1 Hz, 1H, H_{ar}), 7.33 (t, J = 7.4 Hz, 2H, H_{ar}), 7.43–7.69 (m, 3H, H_{ar}), 7.99 (s, 1H, OH), 9.81 (s, 1H, NH), 9.87 (s, 1H, NH), 10.45 (s, 1H, NH), 11.37 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, 101 MHz): δ (ppm) 23.73, 116.63,

116.83, 120.76, 122.76, 124.84, 125.61, 128.25, 131.32, 139.13, 153.39, 167.11, 167.94. Anal. calcd for: $C_{16}H_{16}N_4O_3S$ (344.389 g/mol): C, 55.80; H, 4.68; N, 16.27; S, 9.31. Found: C, 55.70; H, 4.62; N, 16.13; S, 9.22.

Synthesis of *N*-[3-(5-anilino-1,3,4-thiadiazol-2-yl)-4-hydroxyphenyl]acetamide (**14**)

N-{4-Hydroxy-3-[2-(phenylcarbamothioyl)hydrazine-1-carbonyl]phenyl}acetamide (**13**) (0.34 g, 1 mmol) was added in 10 mL of 80% sulphuric acid, and the reaction mixture was stirred at room temperature for 72 h. The progress of the reaction was monitored by TLC (CH_3OH:CCl_3, 1:8). The reaction mixture was poured onto 50 mL crushed ice, and the precipitate was filtered and washed with water. The product **14** was purified by recrystallisation from methanol, yellow solid, yield: 67% (0.22 g), m. p. 246–248 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 2.03 (s, 3H, CH_3), 6.96 (d, J = 8.8 Hz, 1H, H_{ar}), 7.02 (t, J = 7.3 Hz, 1H, H_{ar}), 7.37 (t, J = 7.8 Hz, 2H, H_{ar}), 7.54 (dd, J = 8.8, 2.3 Hz, 1H, H_{ar}), 7.60–7.69 (m, 2H, H_{ar}) 8.28 (d, J = 2.7 Hz, 1H, NH), 9.91 (s, 1H, NH), 10.48 (s, 1H, OH). ^{13}C NMR (DMSO-d_6, 101 MHz): δ (ppm) 23.89, 116.52, 117.40, 117.81, 122.13, 122.18, 122.90, 122.92, 126.71, 129.22, 131.81, 140.59, 149.85, 153.22, 165.32, 167.99. Anal. calcd for: $C_{16}H_{14}N_4O_2S$ (326.374 g/mol): C, 58.88; H, 4.32; N, 17.17; S, 9.82. Found %: C, 58.80; H, 4.24; N, 17.11; S, 9.73. Compound **14** ESI-MS m/z = 325.041 $[M - H]^+$ (100%), 326.029 $[M]^+$ (21.03%), 327.018 $[M + H]^+$ (7.46%).

Synthesis of *N*-[4-hydroxy-3-(4-phenyl-5-sulfanylidene-4,5-dihydro-1*H*-1,2,4-triazol-3-yl)phenyl]acetamide (**15**)

A mixture of *N*-{4-hydroxy-3-[2-(phenylcarbamothioyl)hydrazine-1-carbonyl]phenyl}acetamide (**13**) (0.326 g, 1 mmol), potassium carbonate (1.38 g, 10 mmol), and 30 mL methanol was refluxed for 42 h. The progress of the reaction was monitored by TLC (CH_3OH:CCl_3, 1:8). Then, the volatile fraction was removed under reduced pressure using a rotary evaporator. The residue was dissolved in 10 mL water, and the solution was acidified by acetic acid to pH 6. The precipitates were filtered, washed with water, and dried. The product **15** was purified by recrystallisation from methanol, yielding a white solid with a yield of 71% (0.23 g), m. p. 289–291 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 2.04 (s, 3H, CH_3), 7.02 (d, J = 7.7 Hz, 2H, H_{ar}), 7.37 (t, J = 7.6 Hz, 2H, H_{ar}), 7.44 (d, J = 8.8 Hz, 1H, H_{ar}), 7.63 (d, J = 7.9 Hz, 2H, H_{ar}), 8.19 (s, 1H, H_{ar}), 9.98 (s, 2H, NH and OH), 10.83 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, 101 MHz): δ (ppm) 23.90, 108.85, 117.08, 117.12, 117.15, 121.99, 123.68, 129.12, 131.83, 138.55, 151.61, 157.53, 159.28, 168.03. Anal. calcd for: $C_{16}H_{14}N_4O_2S$ (326.084 g/mol): C, 58.88; H, 4.32; N, 17.17; S, 9.82. Found %: C, 58.80; H, 4.24; N, 17.11; S, 9.73.

The Antibacterial Assays

The bacterial strains used in this study were obtained from the American Type Culture Collection (ATCC) and included the Gram-positive cocci *Staphylococcus aureus* subsp. *aureus* (ATCC 9144) and methicillin-resistant *S. aureus* (MRSA, ATCC 43300), as well as the Gram-negative rods *Escherichia coli* (ATCC 8739) and *Salmonella enterica* subsp. *enterica* serovar Enteritidis (ATCC 13076). Strains were cultured on tryptic soy agar (Liofilchem, Italy) at 37 °C for 24 h. Several well-isolated colonies were suspended in sterile saline to a turbidity of 0.5 McFarland standard prior to antimicrobial testing.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined using the microdilution method described by Balouiri et al. [49]. Two-fold serial dilutions of the test compounds (1:2 to 1:128) were prepared in 96-well microplates. Each well was inoculated with approximately 5×10^4 CFU, obtained by diluting the bacterial suspension 1:150 in Mueller–Hinton broth. Plates were incubated at 37 °C for 24 h. The MIC was defined as the lowest concentration inhibiting visible bacterial growth. To determine the MBC, aliquots from wells without visible growth were subcultured onto Mueller–Hinton agar (Liofilchem, Italy) and incubated at 37 °C for 24 h. MBC

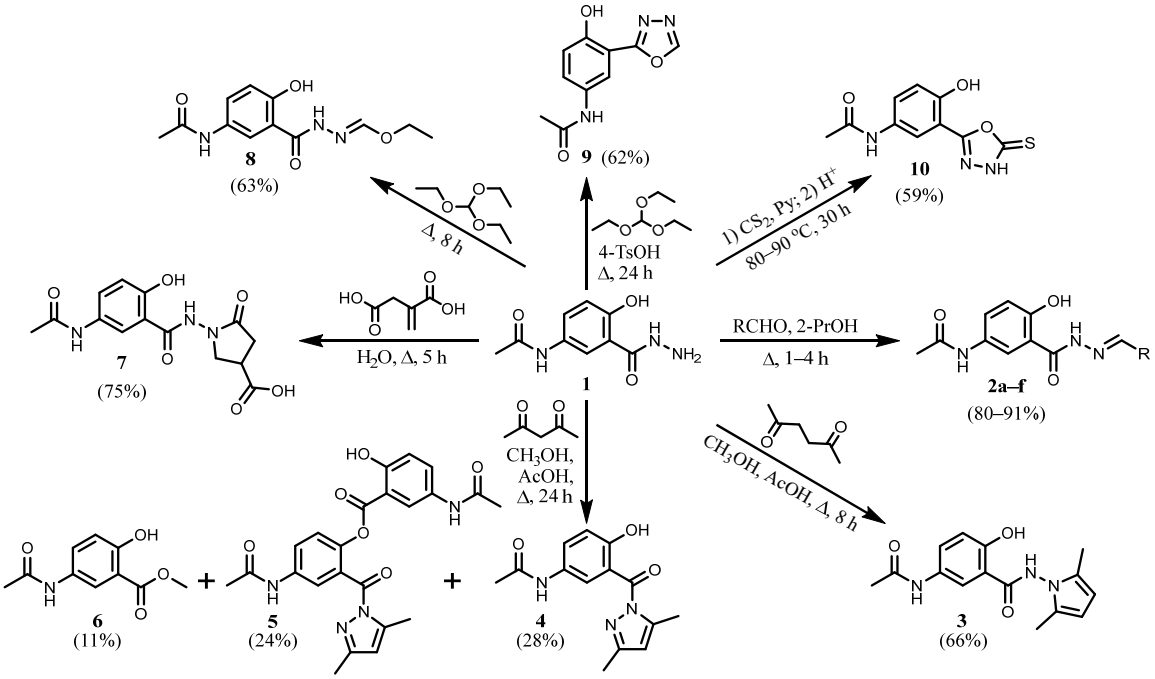
was defined as the lowest concentration resulting in ≥99.9% reduction in viable bacteria relative to the initial inoculum. Ampicillin was used as the positive control. All assays were performed in triplicate, and results are presented as mean ± standard deviation (SD).

3. Results and Discussion

3.1. The Chemistry

The starting material for this study was *N*-[3-(hydrazinecarbonyl)-4-hydroxyphenyl]acetamide (**1**), which was obtained by a three-step synthesis from 5-aminosalicylic acid. The synthesis proceeded via the intermediates methyl 5-amino-2-hydroxybenzoate and methyl 5-acetamido-2-hydroxybenzoate. All related compounds, including the target molecule, have been previously reported in the literature [45,47,48,50]. Methyl 5-amino-2-hydroxybenzoate was obtained by using the classical Fischer esterification method, and a large excess of concentrated sulphuric acid was required to shift the chemical equilibrium towards ester formation. In the subsequent step, methyl 5-acetamido-2-hydroxybenzoate was synthesised in glacial acetic acid by treating the ester with acetic anhydride. Finally, *N*-[3-(hydrazinecarbonyl)-4-hydroxyphenyl]acetamide (**1**) was prepared from the acetamide ester by treatment with hydrazine monohydrate in 1,4-dioxane. The compounds **1–15** synthesised in this study are described in the experimental section, and their NMR and MS spectra are provided in the Supplementary Material (Figures S1–S48).

The reaction pathways of *N*-[3-(hydrazinecarbonyl)-4-hydroxyphenyl]acetamide (**1**) studied in this work are presented in Scheme 1.



R: 2a) C_6H_5 (84%); **R: 2b)** 2-$CH_3OC_6H_4$ (91%); **R: 2c)** 3-$CH_3OC_6H_4$ (83%); **R: 2d)** 4-$CH_3OC_6H_4$ (86%); **R: 2e)** 2,3-$(CH_3O)_2C_6H_3$ (81%); **R: 2f)** 3,4-$(CH_3O)_2C_6H_3$ (80%).

Scheme 1. The reactions of hydrazide derivative **1.**

Condensation of *N*-[3-(hydrazinecarbonyl)-4-hydroxyphenyl]acetamide (**1**) with various aromatic aldehydes gave hydrazone derivatives **2a–f**, as shown in Scheme 1. Structural studies of salicylic acid derivatives reported in the literature indicate that only the *E* isomer is formed [51,52]. In the present study, the ^{1}H NMR spectra of compounds **2a–f** showed the azomethine (-CH=N-) proton as a singlet, with chemical shifts ranging from 7.98 to 8.75 ppm.

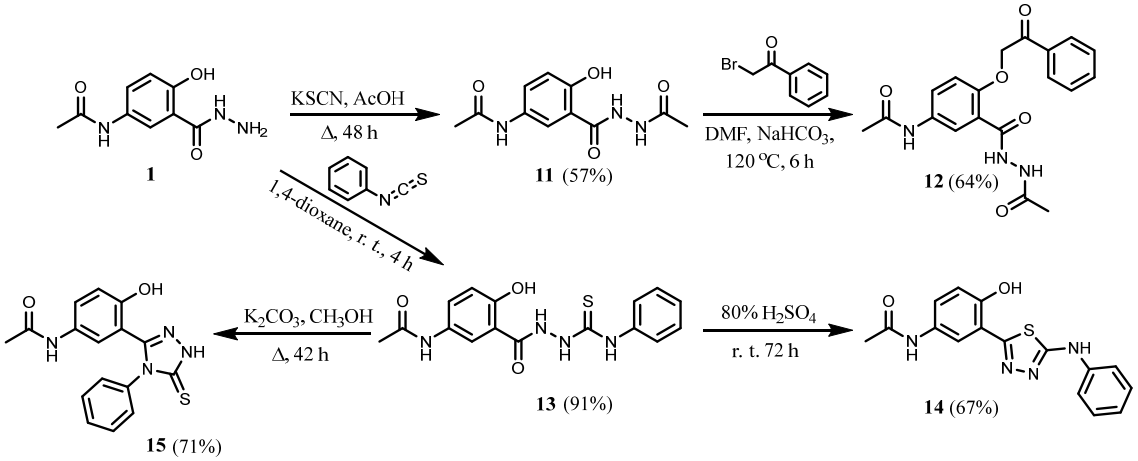
Derivatives containing a hydrazide moiety are often used for the synthesis of pyrazole and pyrrole derivatives in condensation reactions with pentane-2,4-dione and hexane-2,5-dione in the presence of an acid catalyst [53–56]. When hydrazide **1** was treated with hexane-2,5-dione in methanol in the presence of a catalytic amount of glacial acetic acid, the 5-acetamido-*N*-(2,5-dimethyl-1*H*-pyrrol-1-yl)-2-hydroxybenzamide (**3**) was obtained in 66% yield. Moreover, under analogous conditions, the reaction of hydrazide **1** with pentane-2,4-dione afforded a mixture of products **4**–**6** (Scheme 1), which were subsequently separated by column chromatography. Notably, pyrazole **4** was the major product in this reaction, although the amounts of by-products **5** and **6** were also significant. The acidic catalyst likely promoted the cleavage of the hydrazine moiety in derivative **1**, thereby facilitating dimerisation via the phenolic functional group and the formation of the by-product **5**. Furthermore, when methanol was used as the solvent, part of hydrazide **1** underwent reaction under these conditions, resulting in the formation of methyl 5-acetamido-2-hydroxybenzoate (**6**). When cyclocondensation reactions of hydrazide **1** with diketones were carried out in 1,4-dioxane or acetonitrile without acid, these side reactions were suppressed, and the target products **3** and **4** were obtained in higher yields. A comparison of the reactions under boiling conditions of 1,4-dioxane and acetonitrile revealed that the formation of the dimeric compound **5** was more likely in 1,4-dioxane. Considering the boiling points of the solvents, higher temperatures seem to increase the likelihood of by-product formation, while reaction time had little effect, as the cyclocondensation products remained stable after the reaction was complete. Detailed investigations of reactions involving acetic acid with derivatives containing the hydrazide fragment have been reported in the paper [57]. Behalo, M. S. et al. observed acylation of the hydrazide fragment during its reaction with acetylacetone in the presence of acetic acid [58], whereas Baeva L. A. et al. reported the formation of an ester as a side product when $ZnCl_2$ was employed as a catalyst [59].

Typically, the aza-Michael addition reaction between primary amines and itaconic acid is followed by an intermolecular cyclocondensation reaction, which forms pyrrolidinone derivatives [60]. In this study, the treatment of hydrazide **1** with itaconic acid in water resulted in the formation of pyrrolidinone **7** (Scheme 1).

Compounds containing a hydrazide moiety can be reacted with ethyl orthoformate without solvent, usually using a large excess of ester, to form oxadiazole or intermediate hydrazonoformate derivatives [61–63]. A mixture of hydrazide **1** and triethyl orthoformate was refluxed for 8 h to afford ethyl (5-acetamido-2-hydroxybenzoyl)methanehydrazonate (**8**). When the reaction was carried out for 24 h in the presence of a catalytic amount of 4-toluenesulfonic acid, the *N*-[4-hydroxy-3-(1,3,4-oxadiazol-2-yl)phenyl]acetamide (**9**) was obtained (Scheme 1). Notably, during the synthesis of compound **8**, a small amount of 1,3,4-oxadiazole **9** was observed even in the absence of an acid catalyst. An attempt was made to extend the reaction time of hydrazide **1** with triethyl orthoformate to obtain 1,3,4-oxadiazole **9** without acid catalysis. The reaction was prolonged to 72 h, which increased the relative proportion of compound **9** in the crude mixture. However, complete conversion was not achieved. Since derivative **9** had already been reported [45] previously, its synthesis was not further optimised in this study.

It is known that derivatives containing a 1,3,4-oxadiazole-2(3*H*)-thione moiety can be obtained by the reaction of a hydrazide with carbon disulphide in the presence of potassium hydroxide [64]. These conditions were initially investigated in this study, but the reaction mixture darkened, and the desired product **10** could not be isolated. According to a literature report [65], the derivative containing the 1,3,4-oxadiazole-2(3*H*)-thione moiety **10** was synthesised from hydrazide **1** by treatment with carbon disulphide in pyridine at 80–90 °C (Scheme 1). The product **10** was isolated by acidifying the reaction mixture to pH 4 with hydrochloric acid.

Our previous studies [66,67], together with reports by other researchers [68–72], have shown that 1,2,4-triazole-5-thione derivatives can be synthesised by treating hydrazides with potassium thiocyanate. Following this methodology, this study aimed to synthesise a derivative containing a 1,2,4-triazole-5-thione moiety, but the desired product could not be obtained (Scheme 2).



Scheme 2. Synthesis of the compounds **11–15.**

N-[3-(2-Acetylhydrazine-1-carbonyl)-4-hydroxyphenyl]acetamide (**11**) was obtained when hydrazide **1** was refluxed with potassium thiocyanate in acetic acid. The progress of the reaction was monitored by thin-layer chromatography, which showed that only a single product was formed under these conditions. The ^{1}H NMR spectrum of compound **11** displayed two singlets corresponding to the methyl group protons at 1.93 ppm and 2.01 ppm. Similarly, the ^{13}C NMR spectrum showed signals for the two methyl carbon atoms at 20.46 ppm and 23.71 ppm, together with two additional signals at 167.15 ppm and 167.91 ppm, indicating the presence of two acyl groups in molecule **11**. A peak at *m*/*z* 252.108 was observed in the mass spectrum, corresponding to the molecular ion of compound **11**. The signal resembled that expected for a compound containing a 1,2,4-triazole-5-thione moiety. The absence of sulphur in compound **11** was confirmed by elemental analysis, which supports the proposed structure. According to literature data [57,73], it is known that hydrazides can react with acetic acid. In this study, attempts were made to perform the reaction under various conditions, such as refluxing hydrazide **1** in glacial acetic acid and in other solvents in the presence of an equivalent or excess amount of acetic acid. In glacial acetic acid, compound **11** was obtained within 1 h, whereas in acetonitrile and 1,4-dioxane, the reaction did not reach completion after 24 h, although significant formation of derivative **11** was observed.

In this study, the structural properties of compound **11** were investigated by reacting it with 2-bromoacetophenone, following the procedure reported in the literature [74,75]. The reaction was carried out in a DMF solution at 120 °C in the presence of sodium bicarbonate, and *N*-[3-(2-acetylhydrazine-1-carbonyl)-4-(2-oxo-2-phenylethoxy)phenyl]acetamide (**12**) was obtained. In the ^{1}H NMR spectrum of compound **12**, a singlet was observed at 5.78 ppm, assigned to the CH_2 group. In the ^{13}C NMR spectrum, we assigned a signal at 71.78 ppm was attributed to the carbon atom of this group, and an additional signal was also observed at 194.29 ppm, characteristic of the C=O group, indicating alkylation of the phenolic group.

It is known that thiosemicarbazide compounds can be obtained by treating hydrazides with phenyl isothiocyanate, and these compounds can undergo cyclisation through intermolecular cyclocondensation reactions to form five-membered heterocyclic systems

[76–78]. *N*-{4-Hydroxy-3-[2-(phenylcarbamothioyl)hydrazine-1-carbonyl]phenyl}acetamide (**13**) was obtained by reacting hydrazide **1** with phenyl isothiocyanate in 1,4-dioxane at room temperature for 4 h. When the reaction was carried out at a higher temperature, formation of side products was observed. The ^{1}H NMR spectrum of compound **13** displayed broadened NH and OH proton signals, likely due to rapid proton exchange between different nucleophilic centres. Due to the low solubility of the compound in DMSO-d_6, the quality of the ^{13}C NMR spectrum was not optimal. The ^{13}C NMR spectrum of compound **13** showed broadened carbon signals, consistent with dynamic proton exchange.

Based on these literature sources, the cyclocondensation of thiosemicarbazide **13** was investigated using the described procedures [79,80]. *N*-[3-(5-Anilino-1,3,4-thiadiazol-2-yl)-4-hydroxyphenyl]acetamide (**14**) was obtained by treatment of thiosemicarbazide **13** with 80% sulfuric acid at room temperature for 72 h. Similarly, *N*-[4-hydroxy-3-(4-phenyl-5-sulfanylidene-4,5-dihydro-1*H*-1,2,4-triazol-3-yl)phenyl]acetamide (**15**) was synthesised by treatment of thiosemicarbazide **13** with potassium carbonate in methanol under reflux for 42 h. Attempts to perform this reaction using aqueous solutions of sodium hydroxide, potassium carbonate, or sodium bicarbonate were unsuccessful because the reaction mixture turned dark and resinous.

3.2. The Evaluation of Antibacterial Activity

The antibacterial activity of the synthesised compounds **1–15** was evaluated against four bacterial strains: the Gram-positive cocci *Staphylococcus aureus* subsp. *aureus* (ATCC 9144) and methicillin-resistant *S. aureus* (MRSA, ATCC 43300), and the Gram-negative rods *Escherichia coli* (ATCC 8739) and *Salmonella enterica* subsp. *enterica* serovar Enteritidis (ATCC 13076). MIC and MBC values were determined, and the MBC/MIC ratio was calculated to classify each tested compound as bactericidal (MBC/MIC ≤ 4) or bacteriostatic (MBC/MIC > 4). The detailed MIC and MBC values against Gram-positive and Gram-negative bacteria for all tested compounds are presented in Table 1, and the corresponding MBC/MIC ratios are depicted in Figure 1.

Table 1. MIC, MBC, and MBC/MIC ratios of compounds tested against different bacterial strains.

Compound	Concentration (mg/mL)	Bacterial Strains			
		Gram-Positive		Gram-Negative	
		***S. aureus* ATCC 9144**	**MRSA ATCC 43300**	***E. coli* ATCC 8739**	***S.* Enteritidis ATCC 13076**
1	MIC	31.3 ± 0.0	62.5 ± 0.0	62.5 ± 0.0	3.9 ± 0.0
	MBC	500 ± 0.0	500 ± 0.0	250 ± 0.0	62.5 ± 0.0
	MBC/MIC	16	8	4	16
2a	MIC	31.3 ± 0.0	15.6 ± 0.0	62.5 ± 0.0	3.9 ± 0.0
	MBC	500 ± 0.0	125 ± 0.0	250 ± 0.0	62.5 ± 0.0
	MBC/MIC	16	8	4	16
2b	MIC	3.9 ± 0.0	62.5 ± 0.0	15.6 ± 0.0	31.3 ± 0.0
	MBC	62.5 ± 0.0	250 ± 0.0	125 ± 0.0	250 ± 0.0
	MBC/MIC	16	4	8	8
2c	MIC	31.3 ± 0.0	31.3 ± 0.0	31.3 ± 0.0	62.5 ± 0.0
	MBC	125 ± 0.0	125 ± 0.0	125 ± 0.0	250 ± 0.0
	MBC/MIC	4	4	4	4
2d	MIC	3.9 ± 0.0	7.8 ± 0.0	15.6 ± 0.0	15.6 ± 0.0
	MBC	62.5 ± 0.0	62.5 ± 0.0	125 ± 0.0	125 ± 0.0
	MBC/MIC	16	8	8	8

2e	MIC	15.6 ± 0.0	15.6 ± 0.0	15.6 ± 0.0	31.3 ± 0.0
	MBC	125 ± 0.0	125 ± 0.0	62.5 ± 0.0	125 ± 0.0
	MBC/MIC	8	8	4	4
2f	MIC	31.3 ± 0.0	31.3 ± 0.0	62.5 ± 0.0	62.5 ± 0.0
	MBC	125 ± 0.0	250 ± 0.0	250 ± 0.0	250 ± 0.0
	MBC/MIC	4	8	4	4
3	MIC	62.5 ± 0.0	62.5 ± 0.0	62.5 ± 0.0	31.3 ± 0.0
	MBC	250 ± 0.0	500 ± 0.0	250 ± 0.0	125 ± 0.0
	MBC/MIC	4	8	4	4
4	MIC	3.9 ± 0.0	31.3 ± 0.0	15.6 ± 0.0	31.3 ± 0.0
	MBC	62.5 ± 0.0	125 ± 0.0	125 ± 0.0	250 ± 0.0
	MBC/MIC	16	4	8	8
5	MIC	31.3 ± 0.0	7.8 ± 0.0	125 ± 0.0	7.8 ± 0.0
	MBC	500 ± 0.0	500 ± 0.0	250 ± 0.0	125 ± 0.0
	MBC/MIC	16	64	2	16
7	MIC	62.5 ± 0.0	31.3 ± 0.0	62.5 ± 0.0	31.3 ± 0.0
	MBC	125 ± 0.0	250 ± 0.0	250 ± 0.0	125 ± 0.0
	MBC/MIC	2	8	4	4
8	MIC	7.8 ± 0.0	15.6 ± 0.0	62.5 ± 0.0	7.8 ± 0.0
	MBC	250 ± 0.0	500 ± 0.0	250 ± 0.0	125 ± 0.0
	MBC/MIC	32	32	4	16
9	MIC	15.6 ± 0.0	62.5 ± 0.0	125 ± 0.0	3.9 ± 0.0
	MBC	125 ± 0.0	250 ± 0.0	500 ± 0.0	62.5 ± 0.0
	MBC/MIC	8	4	4	16
10	MIC	3.9 ± 0.0	15.6 ± 0.0	15.6 ± 0.0	31.3 ± 0.0
	MBC	62.5 ± 0.0	62.5 ± 0.0	125 ± 0.0	250 ± 0.0
	MBC/MIC	16	4	8	8
11	MIC	7.8 ± 0.0	15.6 ± 0.0	15.6 ± 0.0	31.3 ± 0.0
	MBC	62.5 ± 0.0	125 ± 0.0	125 ± 0.0	125 ± 0.0
	MBC/MIC	8	8	8	4
12	MIC	31.3 ± 0.0	31.3 ± 0.0	15.6 ± 0.0	31.3 ± 0.0
	MBC	250 ± 0.0	250 ± 0.0	125 ± 0.0	125 ± 0.0
	MBC/MIC	8	8	8	4
13	MIC	62.5 ± 0.0	31.3 ± 0.0	31.3 ± 0.0	31.3 ± 0.0
	MBC	250 ± 0.0	250 ± 0.0	250 ± 0.0	125 ± 0.0
	MBC/MIC	4	8	8	4
14	MIC	62.5 ± 0.0	62.5 ± 0.0	31.3 ± 0.0	31.3 ± 0.0
	MBC	250 ± 0.0	250 ± 0.0	250 ± 0.0	125 ± 0.0
	MBC/MIC	4	4	8	4
15	MIC	62.5 ± 0.0	62.5 ± 0.0	31.3 ± 0.0	125 ± 0.0
	MBC	250 ± 0.0	500 ± 0.0	250 ± 0.0	500 ± 0.0
	MBC/MIC	4	8	8	4
Ampicillin	MIC	0.25 ± 0.0	16 ± 0.0	2 ± 0.0	1 ± 0.0
	MBC	0.5 ± 0.0	32 ± 0.0	4 ± 0.0	2 ± 0.0
	MBC/MIC	2	2	2	2

Notes: MIC and MBC values are presented as the mean ± standard deviation (SD) from three independent investigations. In all cases, the calculated SD was equal to 0 because the repeated measurements yielded identical values. MRSA: methicillin-resistant *Staphylococcus aureus*; MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration; ATCC: American Type Culture Collection.

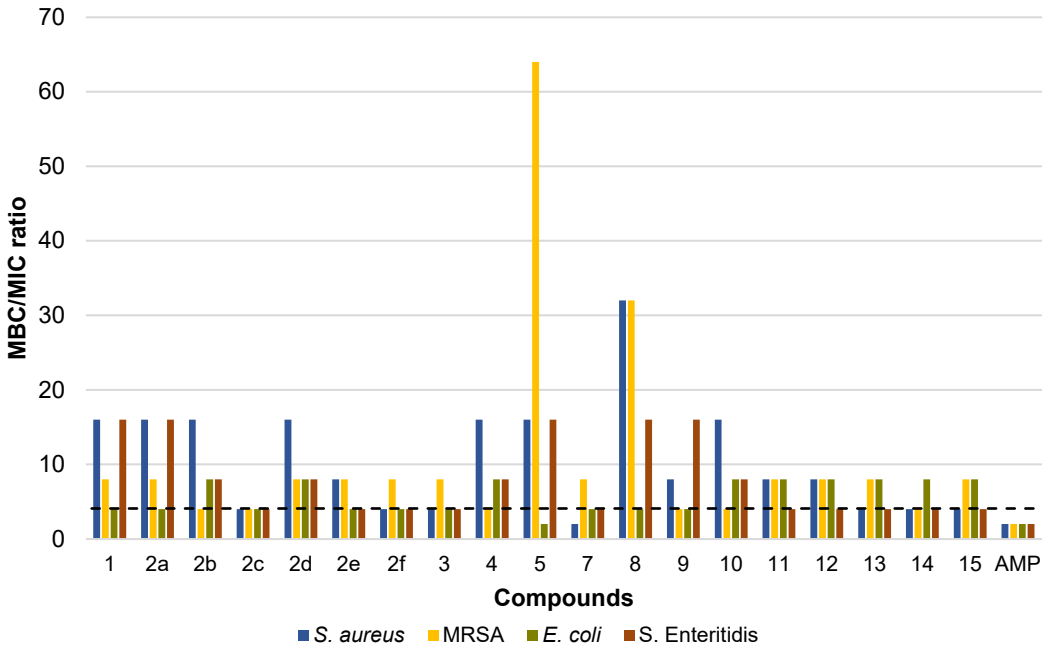


Figure 1. MBC/MIC ratios of tested compounds **1–15** against different bacterial strains. The dashed line represents an MBC/MIC ratio of 4. MBC: minimum bactericidal concentration, MIC: minimum inhibitory concentration, MRSA: Methicillin-resistant *Staphylococcus aureus*, AMP: ampicillin.

Evaluation of antibacterial activity revealed a general trend that the tested compounds **1–15** were more active against Gram-positive bacteria than against Gram-negative strains. Against *S. aureus*, compounds containing the pyrrolidinone **7**, thiosemicarbazide **13**, and 1,3,4-thiadiazole **14** moieties in the molecule exhibited MIC values of 62.5 μg/mL and MBC values of 125–250 μg/mL, resulting in MBC/MIC ratios ≤ 4, which are indicative of bactericidal activity. Other compounds, such as hydrazide **1**, hydrazones **2a** (phenyl)**, 2b** (2-methoxyphenyl)**,** and pyrrole **3**, showed growth inhibition with MBC/MIC ratios > 4, indicating a combination of bactericidal and bacteriostatic effects.

In the case of MRSA, bactericidal activity was observed for derivatives consisting of 2-methoxyphenyl-substituted hydrazone **2b**, pyrrole **3**, and thiosemicarbazide **13**, with MBC/MIC values ranging from 4 to 8. Several compounds exhibited higher MBC/MIC ratios, indicating that complete bacterial killing required higher concentrations. MIC values for MRSA ranged from 7.8 to 62.5 μg/mL, and MBC values ranged from 62.5 to 500 μg/mL, demonstrating variable susceptibility.

The strongest activity against *E. coli* was observed for scaffolds containing hydrazones **2b** (2-methoxyphenyl), **2c** (3-methoxyphenyl), **2f** (3,4-dimethoxyphenyl), pyrrole **3**, and pyrrolidinone **7** derivatives, with MIC values of 15.6–62.5 μg/mL and MBC values of 125–250 μg/mL. Most of these compounds exhibited MBC/MIC ratios of 4, indicating bactericidal effects. Compound **5** exhibited moderate inhibition, with an MBC/MIC ratio of 2, although its MIC value was relatively high at 125 μg/mL.

S. Enteritidis was most sensitive to compounds bearing hydrazide **1**, phenyl substituted hydrazone **2a**, dimerised pyrazole **5**, and hydrazonoformate **8**, with MIC values as low as 3.9–7.8 μg/mL. However, the corresponding MBC values were higher, resulting in MBC/MIC ratios mostly > 4, which indicates a predominantly bacteriostatic effect.

Ampicillin, used as a positive control, demonstrated strong bactericidal activity against all tested strains, as indicated by low MIC and MBC values and an MBC/MIC ratio of 2, confirming the reliability of the assay.

The present study showed that the tested compounds displayed differential antibacterial activity depending on both the bacterial species and the compound structure [7]. In general, *S. aureus* and MRSA were more susceptible to the compounds than *E. coli*, likely due to differences in cell membrane structure [8–11]. The outer membrane of *E. coli* may hinder compound penetration, thereby reducing susceptibility [6].

Derivatives containing pyrrolidinone **7**, thiosemicarbazide **13**, and 1,3,4-thiadiazole **14** moieties were particularly effective against *S. aureus*, whereas 2-methoxyphenyl-substituted hydrazone **2b**, pyrrole **3**, and thiosemicarbazide **13** exhibited notable activity against MRSA. These findings indicate that certain structural characteristics of the compounds may play a role in their differential antibacterial activity across bacterial strains.

In contrast, antibacterial activity was less consistent across certain bacterial strains. Although scaffolds containing hydrazones **2b**, **2c**, **2f**, pyrrole **3**, and pyrrolidinone **7** derivatives demonstrated bactericidal activity against *E. coli*, most compounds exhibited predominantly bacteriostatic effects against *S.* Enteritidis, as reflected by higher MBC/MIC ratios. This difference may be attributed to the stronger permeability barrier and efflux mechanisms in *S.* Enteritidis, which can reduce compound uptake or retention.

Ampicillin, used as a positive control, consistently showed low MIC and MBC values with bactericidal MBC/MIC ratios across all tested strains, confirming the reliability of the assay and the observed compound activities. Taken together, these results emphasise that while several compounds possess promising bactericidal activity against Gram-positive pathogens, additional optimisation may be required to enhance efficacy against Gram-negative bacteria. Differences in bacterial susceptibilities highlight the importance of considering cell envelope properties in the design of new antibacterial agents

4. Conclusions

In this study, the hydrazide functional group of 5-aminosalicylic acid was used for the synthesis of hydrazones **2a–f** and several heterocyclic derivatives, including pyrrole **3**, pyrazole **4**, 1,3,4-oxadiazoles **9**, **10**, 1,3,4-thiadiazole **14**, and 1,2,4-triazole **15**. The results indicate that the reaction course and product selectivity are greatly influenced by the medium, catalysts, and thermal conditions: acidic environments promote oxidative dimerisation, while prolonged heating reduces the purity. This suggests that hydrazide derivatives are sensitive to both acidic and oxidative environments. The synthesis of oxadiazole **10** and 1,2,4-triazole **15** required careful pH control, resulting in the formation of dark by-products. In addition, some intermediates, such as hydrazonoformates **8**, were thermally unstable and readily rearranged to cyclic products **9**. These results emphasise that the development of novel antibacterial heterocyclic derivatives requires not only structure optimisation but also careful control of reaction conditions to avoid undesired transformations and ensure compound stability.

Selected synthesised compounds exhibited significant antibacterial activity against specific bacterial strains. Derivatives containing pyrrolidinone **7**, thiosemicarbazide **13**, and 1,3,4-thiadiazole **14** showed bactericidal activity against *S. aureus*, whereas derivatives of 2-methoxyphenyl hydrazone **2b**, pyrrole **3**, and thiosemicarbazide **13** were active against MRSA. Among the tested compounds, hydrazones **2b**, **2c**, and **2f**, as well as pyrrole **3** and pyrrolidinone **7** derivatives, displayed notable activity against *E. coli*. Most compounds exhibited predominantly bacteriostatic effects against *S. Enteritidis*, as indicated by MBC/MIC ratios > 4.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/app16020703/s1, Figures S1–S40: NMR spectra; Figures S41–S48: MS spectra; or the NMR spectra of 5-aminosalicylic acid derivatives **1–15** are available on the Zenodo website at the following link: https://doi.org/10.5281/zenodo.18090251.

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