Folic acid-sulfonamide conjugates as antibacterial agents: Design, synthesis and molecular docking studies

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1. Bacterial strains
The newly synthesized hybrids were investigated for antibacterial activities against gram negative bacteria *P. aeruginosa* (ATCC-27853), *E. coli* (ATCC-25922), and gram positive *S. aureus* (ATCC-25923) and *P. mirabilis* (ATCC 43071). The stock cultures were collected from the Institute of Biochemistry and Biotechnology, University of the Punjab, Lahore. Strains were recultured in the specific culture media i.e. tryptic soy broth. Inoculum was spread with the help of sterile L-shaped glass spreader and then placed in shaking incubator for 24 h at 37 °C. In the present work, 1-3×10⁸ cfu/mL of gram positive (*S. aureus & P. mirabilis*) and gram negative (*E. coli & P. aeruginosa*) were obtained after setting with broth to an optical density at 0.3 to 0.4 and 0.2 to 0.3, respectively at 620 nm wavelength using spectrophotometer.

2. Antibacterial assay
2.1. Zone of Inhibition
A series of the 2 fold dilutions (0.5-0.00097 mg/mL) was prepared from stock solution of synthetic compounds as well as standard reference drug (ampicillin and trimethoprim) were used and concentrations were made in acetone which has no activity against the test microorganisms. Microfiltration of testing solutions was done with 0.2 µM pore size micro filter (Amicon, USA). 5 µL of each testing compounds’ dilution was separately loaded on 6 mm sterilized disks of filter paper. Dimethylsulfoxide was used as a control. Sterilized forceps was used to place disk onto the medium. 100 µL microbial suspension of 0.5 McFarland nephelometry standard (10⁸ cells/mL) was spread over the TSA media (20 mL in each petri plate) with the help of sterilized glass spreader to ensure the even growth of microorganism. The soaked discs were placed aseptically with the help of sterile forceps at equal distances over the inoculated plates. The plates were incubated at 37±1 °C for 24 h and distinct zone was visualized surrounding the discs. The zones of inhibition (mm) were measured using digital vernier caliper (Starrett 799A-6/150, USA), evaluated the antibacterial activities and all studies were performed in triplicates.

2.2. MIC Calculation
Biological activities of the synthesized compounds were also studied by measuring MIC values and comparison was made with standards. Minimum inhibitory concentration is the lowest concentration of compound that prevents visible growth. MIC quantifies the effectiveness of testing compound. Lower the minimum inhibitory concentration value, higher is the efficacy of a testing compound. Minimum inhibitory concentration (MIC) value was calculated by using two fold serial dilution technique and concentration range 2000-1.95 μg/mL was used for MIC determination. 150 μL tryptic soy broth and 50 μL of bacterial suspension was added to each serially diluted concentration in 96-well plate then incubated at 37±1 °C. After 24 h of incubation the optical density of mixture in 96-well plate was recorded at 600 nm using a microplate reader (BioRad, USA). The lowest concentration of the derivative that prevented the development of visible growth (OD₆₀₀ less than 0.05) is considered to be the MIC value.
Table 1S. Antibacterial data as zone of inhibition at three gradient concentrations (n=3) for folic acid-sulfonamide hybrids for Gram (+) bacteria

| Compound | S. aureus | P. mirabilis |
|----------|-----------|--------------|
|          | 3 mg/mL   | 1.5 mg/mL   | 0.75 mg/mL |
|          | 3 mg/mL   | 1.5 mg/mL   | 0.75 mg/mL |
| MS1      | 31.2,31.1,31.1 | 29.9,29.8,29.9 | 27.9,27.8,27.8 | 29.3,29.4,29.3 | 27.9,27.8,27.8 | 22.0,21.8,22.1 |
| MS2      | 29.9,29.8,29.8 | 29.9,28.9,28.7 | 26.9,26.8,26.8 | 27.9,27.8,27.8 | 24.1,23.9,23.9 | 22.1,22.1,22.0 |
| MS3      | 31.9,31.9,31.8 | 27.9,27.9,27.8 | 22.2,22.2,22.0 | 28.2,28.1,28.1 | 24.3,24.3,24.2 | 22.6,22.5,22.5 |
| DS1      | 32.8,32.8,32.7 | 30.9,30.8,30.9 | 28.7,28.8,28.8 | 33.4,33.2,33.2 | 29.9,29.8,29.8 | 28.5,28.5,28.4 |
| DS2      | 36.6,36.6,36.5 | 31.5,31.5,31.6 | 30.5,30.5,30.4 | 35.9,35.7,35.7 | 30.2,30.0,30.0 | 29.5,29.6,29.6 |
| DS3      | 30.7,30.8,30.8 | 27.9,27.8,27.8 | 26.9,26.8,26.8 | 29.9,29.7,29.7 | 23.9,23.8,23.8 | 22.4,22.3,22.3 |
| DS4      | 31.9,31.8,31.8 | 29.2,29.1,29.1 | 26.8,26.7,26.8 | 30.8,30.9,30.8 | 28.9,28.9,28.8 | 26.5,26.3,26.5 |
| DS5      | 34.7,34.8,34.8 | 30.7,30.8,30.8 | 28.6,28.6,28.5 | 33.6,33.5,33.5 | 29.6,29.5,29.5 | 28.5,28.5,28.3 |
| DS6      | 33.9,33.8,33.8 | 31.8,31.7,31.7 | 28.5,28.5,28.3 | 33.3,33.1,33.1 | 29.3,29.2,29.2 | 28.6,28.7,28.7 |
| TS1      | 32.6,32.5,32.5 | 26.6,26.5,26.5 | 23.8,23.8,23.7 | 31.3,31.2,31.3 | 29.3,29.3,29.3 | 24.1,24.1,24.2 |
| TS2      | 31.8,31.7,31.8 | 28.9,28.8,28.8 | 25.8,25.8,25.7 | 30.8,30.7,30.7 | 27.8,27.7,27.7 | 26.1,26.2,26.2 |
| TS3      | 32.5,32.4,32.5 | 26.8,26.7,26.8 | 23.3,23.4,23.4 | 30.9,30.8,30.8 | 29.7,29.7,29.8 | 24.0,24.1,24.1 |
| TS4      | 20.2,20.3,20.3 | 15.8,15.8,15.7 | 10.9,10.8,10.8 | 18.2,18.3,18.3 | 14.3,14.4,14.4 | 10.8,10.8,10.7 |
| TS5      | 34.9,34.8,34.8 | 31.8,31.7,31.7 | 28.5,28.4,28.4 | 33.3,33.2,33.2 | 29.3,29.2,29.2 | 28.6,28.6,28.5 |
| TS6      | 23.2,23.1,23.1 | 15.4,15.3,15.3 | 11.2,11.1,11.1 | 19.1,19.0,19.0 | 14.5,14.4,14.4 | 10.8,10.7,10.7 |
| Folic acid | –         | –            | –            | –            | –            | –            |
| p-toluenesulfonyl chloride | –         | –            | –            | –            | –            | –            |
| benzenesulfonyl chloride | –         | –            | –            | –            | –            | –            |
| 2,4-dibromo benzenesulfonyl chloride | –         | –            | –            | –            | –            | –            |
| *Ampicillin | 29.8,29.8,29.7 | 28.0,28.1,28.1 | 26.7,26.7,26.8 | 30.5,30.6,30.5 | 29.5,29.5,29.4 | 28.2,28.2,28.1 |
| *Trimethoprim | 28.2,28.3,28.3 | 20.2,20.3,20.3 | 18.4,18.2,18.2 | 25.1,24.9,24.9 | 20.2,20.3,20.3 | 14.3,14.3,14.5 |

*aControl drug. bReference DHFR inhibitor, Zone of inhibition was measured in mm ±SD, Gradient concentration of 3 mg/mL, 1.5 mg/mL, and 0.75 mg/mL was used, S. aureus = Staphylococcus aureus, E. coli = Escherichia coli, P. aerugenosa = Pseudomonas aerugenosa, P. mirabilis = Proteus mirabilis
Table 2S. Antibacterial data as zone of inhibition at three gradient concentrations (n=3) for folic acid-sulfonamide hybrids for Gram (-) bacteria

| Compound | Gram (-) bacteria | | | | | |
|----------|------------------|---|---|---|---|---|
|          | E. coli          | P. aeruginosa | | | | |
|          | 3 mg/mL | 1.5 mg/mL | 0.75 mg/mL | 3 mg/mL | 1.5 mg/mL | 0.75 mg/mL |
| MS1      | 31.0,31.2,31.2  | 29.7,29.8,29.8 | 24.0,24.2,24.2 | 30.9,30.9,30.8 | 28.1,28.2,28.2 | 17.8,17.8,17.9 |
| MS2      | 29.9,29.8,29.8  | 29.0,28.9,28.8 | 23.6,23.7,23.7 | 27.8,27.9,27.9 | 25.7,25.8,25.8 | 15.8,15.8,15.9 |
| MS3      | 33.0,33.2,33.2  | 28.3,28.4,28.4 | 24.6,24.7,24.7 | 29.1,29.2,29.2 | 27.9,27.8,27.8 | 20.2,20.3,20.3 |
| DS1      | 34.9,34.9,34.8  | 32.2,32.3,32.3 | 29.3,29.3,29.4 | 32.9,32.8,32.8 | 31.9,31.8,31.9 | 18.4,18.5,18.5 |
| DS2      | 37.8,37.7,37.9  | 33.2,33.3,33.3 | 31.5,31.6,31.6 | 34.7,34.8,34.8 | 32.1,32.2,32.1 | 30.9,30.8,30.9 |
| DS3      | 31.9,31.8,31.8  | 29.2,29.3,29.3 | 23.6,23.7,23.7 | 28.9,28.8,28.8 | 27.7,27.8,27.8 | 17.8,17.9,17.9 |
| DS4      | 34.8,34.9,34.9  | 32.3,32.4,32.5 | 28.6,28.6,28.8 | 30.8,30.8,31.0 | 29.3,29.4,29.4 | 27.3,27.4,27.6 |
| DS5      | 36.4,36.5,36.5  | 32.2,32.3,32.3 | 28.9,28.9,29.0 | 32.7,32.8,32.8 | 31.2,31.3,31.3 | 29.1,29.3,29.3 |
| DS6      | 36.6,36.7,36.7  | 32.3,32.4,32.4 | 29.6,29.8,29.8 | 33.9,34.0,34.0 | 31.2,31.3,31.3 | 29.8,29.9,29.9 |
| TS1      | 34.6,34.7,34.7  | 30.9,30.8,30.8 | 25.9,26.0,26.0 | 29.3,29.4,29.4 | 27.5,27.6,27.7 | 24.1,24.2,24.2 |
| TS2      | 34.7,34.8,34.8  | 31.8,31.9,31.9 | 29.4,29.6,29.6 | 31.1,31.3,31.3 | 29.2,29.4,29.4 | 27.2,27.4,27.4 |
| TS3      | 34.5,34.6,34.6  | 30.0,30.2,30.2 | 24.3,24.5,24.5 | 29.6,29.8,29.8 | 27.8,27.9,27.9 | 24.2,24.6,24.6 |
| TS4      | 19.2,19.4,19.4  | 12.0,12.1,12.2 | 10.7,10.9,10.9 | 20.0,20.2,20.2 | 17.6,17.8,17.8 | 14.2,14.3,14.3 |
| TS5      | 36.6,36.7,36.7  | 32.3,32.4,32.5 | 29.6,29.7,29.8 | 33.9,33.9,34.0 | 31.2,31.4,31.4 | 29.8,30.0,30.0 |
| TS6      | 19.5,19.6,19.6  | 12.3,12.5,12.5 | 10.9,10.8,10.9 | 20.8,20.9,20.9 | 17.9,17.8,17.8 | 14.5,14.6,14.6 |
| Folic acid | –, –, – | –, –, – | –, –, – | –, –, – | –, –, – | –, –, – |
| p-toluensulfonyl chloride | –, –, – | –, –, – | –, –, – | –, –, – | –, –, – | –, –, – |
| benzenesulfonl chloride | –, –, – | –, –, – | –, –, – | –, –, – | –, –, – | –, –, – |
| 2,4-dibromo benzenesulfonyl chloride | –, –, – | –, –, – | –, –, – | –, –, – | –, –, – | –, –, – |
| a Aminopenicillin | 33.5,33.8,33.6 | 30.0,30.2,30.1 | 29.0,29.2,29.1 | 29.1,29.3,29.2 | 27.2,27.3,27.3 | 26.2,26.1,26.3 |
| b Trimethoprim | 31.2,30.9,30.8 | 25.2,25.0,25.0 | 20.1,20.0,20.2 | 14.1,14.2,14.2 | 9.3,9.3,9.4 | 4.1,4.1,4.2 |

a Control drug, b Reference DHFR inhibitor, Zone of inhibition was measured in mm ±SD, Gradient concentration of 3 mg/mL, 1.5 mg/mL and 0.75 mg/mL was used, S. aureus = Staphylococcus aureus, E. coli = Escherichia coli, P. aeruginosa = Pseudomonas aeruginosa, P. mirabilis = Proteus mirabilis
$^{1}$HNMR and $^{13}$CNMR
MS2

\[\text{Diagram of MS2 molecule}\]
DS3
DS5
TS2
TS4
TS6
