Synthesis, Biological Evaluation and in Silico Studies of Several Substituted Benzene Sulfonamides as Potential Antibacterial Agents

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Abstract. Four substituted benzene sulfonamides were synthesized to obtain antibacterial agents. The antibacterial activities were evaluated by the cup plate method. Compound 2d showed the most obvious inhibitory effect on seven kinds of strains with MIC ranging from 16 to 64µg/mL. The inhibitory effect of compound 2d on A. niger, C. albicans, B. subtilis, and S. aureus was better than that of the control drug SAN. Moreover, the cytotoxicity was evaluated by MTT assay. Finally, the binding patterns of the compounds with dihydrofolate synthetase and the prediction of oral bioavailability were preliminarily studied by computer simulation. The molecular docking result revealed that compound 2d had a stronger interaction with dihydrofolate synthetase than SAN. The oral bioavailability prediction by Molinspiration online program proved that these compounds exhibited drug-like properties. In conclusion, compound 2d is promising as a new antibacterial agent.

1. Introduction

Sulfonamide derivatives have antibacterial, antifungal, anticancer, antiviral, carbonic anhydrase inhibitor, anti-inflammatory activities, and can promote insulin secretion. Dihydrofolate synthetase is a target enzyme for the antimicrobial activities of sulfonamides [1]. In 1940, Woods [2] and Fildes [3] successively reported that the addition of excessive p-aminobenzoic acid (PABA) in the culture environment of microorganisms inhibited by sulfonamides could effectively antagonize the antibacterial effects of sulfonamides. Based on this result, they suggest that sulfonamides can inhibit bacterial growth by competitively inhibiting the synthesis and metabolism of PABA utilized by bacteria [4]. The antibacterial effects of many N1-substituted sulfonamides on human and animal pathogens have been widely tested since the antibacterial activity of sulfonamide (SAN) in vivo and in vitro was discovered [5].

PABA is an indispensable substance for bacteria to synthesize dihydrofolate synthase. Lack of folic acid can cause bacteria to fail to reproduce and survive normally. Therefore, blocking the anabolism of folic acid can inhibit or kill bacteria. In many pathogens, microorganisms and plants, folic acid must be synthesized by itself. However, the human can obtain folic acid directly from food, so this kind of folic acid anabolic inhibitor is less toxic to the human body [4]. The process of synthesis of folic acid in bacteria consists of a series of reactions. The key step is that 6-hydroxymethyl-7,8-dihydropterinpyrophosphate and PABA are condensed under the catalysis of dihydrofolate synthetase to form 6-hydroxymethyl-7,8-dihydropteroate. Then, 6-hydroxymethyl-7,8-dihydropteroateis condensed with glutamic acid under the catalysis of
folypolyglutamate synthetase (FPGS) to produce 7,8-dihydrofolic acid [6]. The structural unit of SAN is very close to PABA in both molecular scale and charge distribution, so it has a similar effect to PABA [7]. Woods and Fildes suggest that SAN may act as an analog of PABA to competitively bind to dihydrofolate synthetase that catalyzes the further transformation of PABA, thereby inhibiting the participation of PABA in metabolic reactions.

SAN and PABA can competitively bind in the active site of dihydrofolate synthetase. Therefore, sulphonamides are one of the most useful antibacterial drugs found in the clinic [8]. Sulfonamides with good antibacterial effect can be obtained by chemical modification of aromatic and aliphatic sulphonamides. In many reports, alkyl, acyl, heterocyclic, and aromatic substituents have been used to study a class of important N₁-substituents in the sulfonamide group (-SO₂NH₁-), which enhance antibacterial properties and clinical applications.

In recent years, the study of antibacterial effect has been focused on the p-amino group, or the amino nitrogen atom [9], but the study of substituted benzene sulphonamides without p-amino group has been rarely reported. In this paper, four substituted benzensulphonamide compounds without the p-amino group were synthesized. The effect of compounds 2a-2d on the viability of HepG2 and RAW264.7 cell lines was evaluated by the MTT method. Antibacterial activity of compound 2a-2d was measured using the cup plate method. Besides, the oral bioavailability of compounds was predicted by the Molinspiration online program based on Lipinski's rule of five. Finally, the binding patterns of compounds with dihydrofolate synthetase were studied by molecular docking.

2. Experimental Method

2.1. Material and Chemistry

Tryptic soy broth (TSB), tryptic soy agar medium (TSA), and sabouraud dextrose agar medium (SDA) were purchased from Qingdao Hope Bio-Technology Co.Ltd (China). Norfloxacin (NFX), fluconazole (FLUCZ), sulphonamide (SAN), and dimethyl sulfoxide (DMSO) were purchased from Aladdin. Escherichia coli (E. coli) CMCC44102, Staphylococcus aureus (S. aureus) CMCC 26003, Salmonella typhi (S. typhi) CMCC 50094, Pseudomonas aeruginosa (P. aeruginosa) CMCC 10104, Bacillus subtilis (B. subtilis) CMCC 63501, Aspergillus niger (A. niger) CMCC 98003, and Candida albicans (C. albicans) CMCC 98001 were purchased from Shandong Food and Drug Research Institute (China). The HepG2 and RAW264.7 cell lines were provided from Prof. HoSub Lee (WonKwang University, Korea). Dulbecco's modified eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco BRL Co. (Grand Island, NY, USA). Thiazolyl blue tetrazolium bromide (MTT) was obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Biological Evaluation

2.2.1. Preparation of the compound solution and the bacterial suspension. NFX was used as a positive control for bacteria, FLUCZ was a positive control for fungus, and SAN was a positive control for the seven kinds of strains. Four compounds and positive control used deionized water and DMSO to prepare solutions of the following concentrations: 512, 256, 128, 64, 32, 16, 8, 4, 2, 1, and 0.5 μg/mL. 100% DMSO and 50% DMSO were used as negative controls. The tested strains were inoculated into TSB sterilized at 115°C for 30 minutes and cultured in a constant temperature incubator. Five kinds of bacteria cultured at 30-37°C for 18-24 hours, A. niger was cultured at 23-28°C for 2-3 days, and C. albicans was incubated at 37°C for 2-3 days. After the culture was completed, the bacterial suspension was diluted into 1×10⁶ CFU/mL.

2.2.2. Evaluation of antibacterial activity. Different concentrations of compounds 2a, 2b, 2c, 2d, NFX, FLUCZ, and SAN solutions were added to small steel tubes and marked, respectively. The Petri dishes were cultured in constant temperature incubator under the corresponding culture conditions of each strain. The culture conditions of the strains were the same as the cultivation of the bacterial suspension.
2.2.3. Cytotoxicity assay. The HepG2 and RAW264.7 cell lines were cultured in DMEM medium containing 10% FBS and 1% penicillin/streptomycin, and the medium was placed in an environment with 95°C humid air and 5% CO₂ at 37°C cultured. The effect of synthetic compounds on the viability of cell culture in vitro was evaluated by MTT assay. The cells were inoculated in a 96-well plate at a density of 1.0×10⁶ cells/mL and were allowed to grow until fusion for 24 hours. The cells were cultured for 24 hours. Added 5mg/mL MTT solution to each well and incubated under the same conditions for 4 hours. After the medium was sucked out, DMSO dissolved the purple precipitate formed in the living cells. The absorbance of each well was measured by a microplate reader at 490nm. The absorbance of the control group was considered to be 100% viability.

2.3. In Silico Studies

2.3.1. Physicochemical property prediction. This study estimated the pharmacokinetic parameters by using an online program called Molinspiration based on Lipinski’s rule of five. The percentage of absorption % ABS = 109 - [0.345 × TPSA] [10]. Compounds conforming to this rule have better pharmacokinetic properties and higher oral bioavailability in the metabolic process in vivo, so they are more likely to become oral drugs.

2.3.2. Molecular docking. With the development of computer-aided drug design, molecular docking technology is widely used to explore the interaction between compound molecules and protein receptors. Autodock4.2 software [11] was used for molecular docking to explore the interactions between compounds and dihydrofolate synthetase. Autodock4.2 used the Lamarckian Genetic Algorithm to find the optimal binding site for the receptor and ligand, and used a semi-empirical free energy calculation to evaluate the match between the receptor and the ligand.

The structure of dihydrofolate synthetase [12] (PDB: 1AJ0) was downloaded from the RCSB protein database. The ligand SAN of the enzyme active center was extracted. Besides, the water molecules, hydrogen bonds, and charges are optimized. Other parameters are default. The MM2 force field was used to minimize the energy of the compounds. The compounds that minimized energy were induced to fit the molecular docking at the active site of dihydrofolate synthetase to obtain .dlg file.

The optimal docking conformations of the compounds with dihydrofolate synthetase were analyzed by binding energy, inhibition constant, hydrogen bonds, and other related parameters. Furthermore, the validation of this docking protocol was confirmed by redocking the co-crystalized ligand SAN into the active site of dihydrofolate synthetase with root mean square deviation (RMSD) ≤ 2.

3. Results and Discussion

3.1. Chemistry

Scheme 1 is the synthetic routes of compounds 2a, 2b, 2c, and 2d. Put compounds 1a, 1b and 1c (0.5mol) into a three-necked flask, respectively. Separately, 100mL of dichloromethane was added to the flask and cooled to 0°C, then 30% ammonia water was slowly dropped into the flask, the pH was adjusted to 9, and stirred at 0°C. During the reaction, ammonia water was added to adjust the pH to 9 until the reaction was completed. Then the white solid was formed, the solution was spun dry, and washed with water for 3-5 times. The crude product was recrystallized from acetone to obtain compounds 2a, 2b and 2c. Compound 2d was synthesized using acetonitrile as the solvent, and other reaction conditions were the same as above. We recrystallized crude product to get refined product 2d.

![Scheme 1. Synthetic routes of compounds 2a-2d.](image)
FT-IR, $^1$H-NMR, and ESI-MS methods were used to characterize the structure of the compounds. In FT-IR, characteristic along with N-H stretch of -SO$_2$NH$_2$ group in the absorption range 3240-3420 cm$^{-1}$. Due to the -SO$_2$NH$_2$ group, two distinct bands of SO$_2$ stretch were observed in the absorption ranges 1304-1350 cm$^{-1}$ and 1150-1160 cm$^{-1}$, respectively. In $^1$H NMR, an exchangeable singlet due to two protons displayed in the range at δ 7.23-8.09 was attributed to -SO$_2$NH$_2$ group (Table 1).

### Table 1. The FT-IR and $^1$H-NMR of compounds 2a-2d.

| Compounds | Structure | FT-IR result (cm$^{-1}$) | $^1$H-NMR (DMSO) |
|-----------|-----------|-------------------------|-----------------|
| 2a        | ![Structure](image) | ν$_{N-H}$.3326.25, 3241.66 | 7.68 (d, 2H) |
|           |           | ν$_f$-H:3124.96          | 7.38 (d, 2H) |
|           |           | ν$_C=H$:2923.51         | 7.23 (s, 2H) |
|           |           | ν$_C\equiv C$:1596.64,1495.86 | 2.43 (s, 3H) |
|           |           | ν$_S=O$:1325.26, 1151.22 |               |
| 2b        | ![Structure](image) | ν$_{N-H}$.3337.11, 3242.81 | 7.65 (d, 1H) |
|           |           | ν$_f$-H:3110.97          | 7.58 (m, 1H) |
|           |           | ν$_C=H$:2980.23, 2951    | 7.32 (m, 1H) |
|           |           | ν$_C\equiv C$:1598.62, 1487.92 | 7.23 (s, 2H) |
|           |           | ν$_S=O$:1328.73, 1158.12 | 2.34 (s, 6H) |
| 2c        | ![Structure](image) | ν$_{N-H}$.3373.72, 3266.29 | 8.17 (d, 1H) |
|           |           | ν$_f$-H:3113.51          | 7.94 (d, 1H) |
|           |           | ν$_C=F$:1343.66-1270.43 | 7.89 (m, 1H) |
|           |           | ν$_S=O$:1304.45, 1159.76 | 7.72 (s, 2H) |
| 2d        | ![Structure](image) | ν$_{N-H}$.3419.5, 3315.47 | 8.95 (s, 1H) |
|           |           | ν$_f$-H:3108.08          | 8.77 (d, 1H) |
|           |           | ν$_N=O$:1543.82, 1376.99 | 8.32 (d, 1H) |
|           |           | ν$_S=O$:1607.78         | 8.09 (s, 2H) |

### 3.2. In Vitro Antibacterial Activity

This study determined the MIC and MBC of four compounds and three positive control drugs (Table 2). The MIC values of compound 2d against the seven tested strains were less than 64 μg/mL. Among the four compounds, compound 2d had the smallest MIC value, indicating that it had a higher antibacterial effect on both bacteria and fungus than other compounds. Compared with SAN, the MIC values of compound 2d against *S. aureus*, *B. subtilis*, *C. albicans*, and *A. niger* were all less than that of SAN. This phenomenon suggested that the inhibitory effect of compound 2d against the four kinds of strains was superior to that of SAN.

### Table 2. The MIC and MBC of compounds 2a-2d and positive controls.

| Strains               | Antimicrobial susceptibility |
|-----------------------|-----------------------------|
|                       | 2a                          | 2b | 2c | 2d | NFX | FLUCZ | SAN  |
|                       | MIC(μg/mL)                  |    |    |    |     |       |      |
| *E. coli* CMCC 44102  | 128/256                     | 128/256 | 64/128 | 64/128 | 1/1 | -/-  | 64/16 |
| *S. aureus* CMCC 26003| 128/512                     | 128/256 | 128/512 | 64/64 | 1/1 | -/-  | 64/64 |
| *P. aeruginosa* CMCC 10104| 128/256                    | 128/256 | 128/256 | 64/128 | 1/1 | -/-  | 32/32 |
| *S. typhi* CMCC 50094 | 256/256                     | 256/256 | 128/256 | 64/64 | 1/1 | -/-  | 32/32 |
| *B. subtilis* CMCC 63501| 256/512                    | 256/512 | 256/512 | 16/32 | 1/1 | -/-  | 32/32 |
| *C. albicans* CMCC 98001| 128/256                    | 128/128 | 128/256 | 32/128 | -/- | 4/16 | 64/128|
| *A. niger* CMCC 98003 | 64/64                       | 128/256 | 128/256 | 16/32 | -/- | 4/4  | 32/64 |

This study has determined the MIC and MBC of compounds 2a-2d (Table 2). The tested compounds have bactericidal activity when MBC=MIC, but the tested compounds have antibacterial activity when MBC>MIC. From Table 2, we could see that almost all the compounds were bacteriostatic.

The diameters of the inhibitory zone of compound 2d, SAN, NFX, and FLUCZ were determined (Figure 1). As shown in Figure 1(a), when the concentration of compound 2d exceeded 32μg/mL, the diameter of the inhibitory zone of compound 2d against *C. albicans* was the largest, which indicated
that the inhibitory effect of compound 2d against *C. albicans* was better than other tested strains. As shown in Figure 1(a) and Figure 1(b), the antibacterial effect of compound 2d and SAN were equivalent when the concentration was 512μg/mL. And with the decrease of the concentration, the inhibitory effect of compound 2d on *C. albicans* was greater than that of SAN. This suggested that the inhibitory activity of compound 2d against *C. albicans* was higher than that of SAN when the concentration exceeded 32μg/mL. Besides, the diameter of the inhibitory zone of compound 2d against *A. niger* was second to that against *C. albicans*, and larger than that of SAN. The results showed that the inhibitory effect of compound 2d on fungus was stronger than that on bacteria when the concentration of compound 2d exceeded 32μg/mL. Figure 1(c) showed the diameters of the inhibitory zone of NFX and FLUCZ against seven kinds of strains. NFX was a positive control for five types of bacteria, and FLUCZ was a positive control for *A. niger* and *C. albicans*. It could be seen from Figure 1(c), the diameters of the inhibitory zone gradually increased with the increasing concentrations of NFX and FLUCZ. The results indicated that the antibacterial effects of NFX and FLUCZ were by the concentration dependence, indicating that this experiment was operated correctly with small error and high reliability. And they could be used as positive controls to evaluate the antibacterial activities of other compounds.

![Figure 1. The diameter of inhibitory zone. (a) compound 2d, (b) SAN, (c) NFX and FLUCZ.](image)

3.3. Cytotoxicity Assay

![Figure 2. Cell viability results of SAN and compound 2d against HepG2 (a) and RAW264.7 (b). Each represented values were mean ± SD(n = 3), * p < 0.05, ** p < 0.01 compared to SAN.](image)
shown in Figure 2(b). The result showed that the viability of HepG2 and RAW264.7 cell lines gradually decreased with increasing the concentration of compound 2d.

3.4. Physicochemical Property Prediction
Physicochemical parameters of synthesized compounds were calculated by Molinspiration online program Lipinski’s rule of five that is considered to predict oral bioavailability. MW<500 predicting their easy movement, diffusion and absorption. The number of hydrogen bond donors and the number of hydrogen bond acceptors in all the compounds were less than 5 and 10, respectively. The LogP values of all synthesized compounds were all less than 5, indicating that they were likely to have good membrane permeability. The TPSA values of most compounds were less than 140 Å², except compound 2d, indicating that they might have good oral bioavailability. Besides, all tested compounds displayed reasonable %ABS in the range of 56.63 to 88.24%, suggesting that they probably had excellent oral bioavailability.

3.5. Molecular Docking
To better understand the mechanism of antibacterial activity, molecular docking was performed to fit compounds 2a-2d into the active site of dihydrofolate synthetase using AutoDock4.2 software (Table 3). The result showed that compound 2c, compound 2d, PABA, and SAN had similar hydrogen bonds and hydrophobic interactions with residues at the active site of dihydrofolate synthetase. It could be seen from Table 3 that compound 2d had the largest binding energy and the smallest inhibition constant with dihydrofolate synthetase compared with PABA and the control inhibitor SAN. Binding energy and hydrogen bonds can increase the stability of ligand and receptor. And the inhibition constant is an index to indicate the effect of the inhibitor. It is the concentration required to produce half-maximum inhibition. Therefore, we speculated that the antibacterial effect of compound 2d was because it occupied the binding site of PABA and dihydrofolate synthetase, which resulted in a greater inhibitory effect on dihydrofolate synthetase.

Table 3. Docking results of the compounds.

| Compound code | Free binding energy ΔG (kcal/mol) | Inhibition constant Ki (μM) | Hydrogen bonds |
|---------------|-----------------------------------|----------------------------|----------------|
|               |                                   |                           | Atom of ligand | Amino acids | Distance(A°) |
| PABA          | -4.19                             | 853.78                    | O             | SER222      | 2.063        |
|               |                                   |                            | H             | THR62       | 1.873        |
| SAN           | -4.25                             | 763.09                    | O             | SER222      | 2.215        |
|               |                                   |                            | H             | THR62       | 2.021        |
| 2c            | -4.12                             | 961.01                    | O             | SER222      | 2.233        |
| 2d            | -5.6                              | 78.02                     | O             | SER222      | 2.058        |
|               |                                   |                            | O             | ARG235      | 2.246        |

Figure 3 shows the 3D docking diagrams of all the compounds with dihydrofolate synthetase at the active site. PABA formed two hydrogen bonds with dihydrofolate synthetase. -OH oxygen formed a hydrogen bond with SER222 amino acid residue, the hydrogen of -NH₂ formed a hydrogen bond with the amino acid residue of THR62. The control inhibitor SAN formed two hydrogen bonds at the active site of dihydrofolate synthetase. The oxygen of -SO₂ formed a hydrogen bond with the amino acid residue of SER222, and the hydrogen of -NH₂ formed another hydrogen bond with the amino acid residue of THR62. Compound 2c only formed a hydrogen bond with dihydrofolate synthetase, which is formed by the oxygen in -SO₂ and the amino acid residue of SER222. Compound 2d formed two hydrogen bonds with dihydrofolate synthetase. The ortho-oxygen of -NO₂ formed a hydrogen bond with the amino acid residue of SER222, and the para-oxygen of -NO₂ formed another hydrogen bond with the amino acid residue of ARG235. We could see that compound 2d, PABA and SAN had the same number of hydrogen bonds and similar binding sites, indicating that compound 2d had the potential to compete with PABA to bind dihydrofolate synthetase. And we speculated that the inhibitory effect of compound 2d on bacteria and fungus might be related to the inhibition of dihydrofolate synthetase activity.
Figure 3. The 3D docking diagrams of PABA, SAN, compound 2c and compound 2d with dihydrofolate synthetase. (a) PABA, (b) SAN, (c) 2c, (d) 2d. The dotted line represents a hydrogen bond; red represents an oxygen atom; blue represents a nitrogen atom; white represents a hydrogen atom; and green represents a fluorine atom.

4. Conclusion
In this study, four new substituted benzene sulphonamides were synthesized. We found that compound 2d had an obvious inhibitory effect on bacteria and fungi. In particular, compound 2d exhibited good potencies in inhibiting the growth of *S. aureus*, *B. subtilis*, *C. albicans*, and *A. niger*. Moreover, compound 2d could kill cancer cells at a certain concentration and had certain toxicity to normal cells. Molecular docking predicted the possible binding mode of compound 2d with dihydrofolate synthetase and provided a reference for the study of the antibacterial mechanism of compound 2d. In summary, compound 2d was promising as a candidate for antibacterial agents.

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References
[1] Griffith, E. C., Wallace, M. J., Yinan, W., Gyanendra, K., Stefan, G., and Pamela, J. 2018. The structural and functional basis for recurring sulfa drug resistance mutations in staphylococcus aureus dihydropteroate synthase. *Frontiers in Microbiology*. 9: 1369.
[2] Woods, D. D. 1940. The relation of p-aminobenzoic acid to the mechanism of the action of sulphanilamide. *British journal of experimental pathology*. 21(2): 74-90.
[3] Fildes, and Paul. 1940. A rational approach to research in chemotherapy. *Lancet*. 235(6091): 955-957.
[4] Brown, G. M. 1962. The biosynthesis of folic acid. ii. inhibition by sulphonamides. *journal of biological chemistry*. 237(1): 536.
[5] Premasis Sukul, and Michael Spiteller. 2006. Sulphonamides in the environment as veterinary drugs. *reviews of environmental contamination and toxicology*. 187(187): 67.
[6] Baca, A. M., Sirawaraporn, R., Turley, S., Sirawaraporn, W., and Hol, W. G. 2000. Crystal structure of Mycobacterium tuberculosis 6-hydroxymethyl-7,8-dihydropteroate synthase in
complex with pterin monophosphate: new insight into the enzymatic mechanism and sulfa-drug action. *Journal of Molecular Biology*, 302(5): 1193–1212.

[7] Bermingham, A., and Derrick, J. P. 2002. The folic acid biosynthesis pathway in bacteria: evaluation of potential for antibacterial drug discovery. *BioEssays*. 24(7): 637-648.

[8] Bell, P. H., and Roblin, R. O. 1942. Studies in chemotherapy. vii. a theory of the relation of structure to activity of sulfanilamide type compounds1. *Journal of the American Chemical Society*. 64(12): 2905 - 2917.

[9] Achari, A., Somers, D. O., Champness, J. N., Bryant, P. K., Rosemond, J., and Stammers, D. K. 1997. Crystal structure of the anti-bacterial sulfonamide drug target dihydropteroate synthase. *Nature Structural Biology*. 4(6): 490-497.

[10] Shankar, S., Pangeni, R., Park, J. W., and Rhim, J. W. 2018. Preparation of sulfur nanoparticles and their antibacterial activity and cytotoxic effect. *Materials Science and Engineering: C*. 92: 508–517.

[11] Husain, A., Ahmad, A., Khan, S. A., Asif, M., Bhatuni, R., and Al-Abbasi, F. A. 2016. Synthesis, molecular properties, toxicity and biological evaluation of some new substituted imidazolidine derivatives in search of potent anti-inflammatory agents. *Saudi Pharmaceutical Journal*. 24(1): 104–114.

[12] Idrees, D., Hadianawala, M., Mahapatra, A. D., Datta, B., Roy, S., Ahamad, S., and Imtaiyaz Hassan, M. 2018. Implication of sulfonyleurea derivatives as prospective inhibitors of human carbonic anhydrase II. *International Journal of Biological Macromolecules*. 115: 961–969.