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Synthesis of Disulfide-Bridged N-Phenyl-N'-(Alkyl/Aryl/Heteroaryl)Urea Derivatives and Evaluation of Their Antimicrobial Activities

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The recognition of new antimicrobial agents is extremely needed to overcome multidrug-resistant bacterial and tuberculosis infections. In the present study, eight novel substituted urea derivatives containing disulfide bond (**10a-h**) were designed, synthesized and screened for their *in vitro* antimicrobial activities on standard strains of Gram-positive and Gram-negative bacteria as well as on *Mycobacterium tuberculosis*. According to the obtained results, antibacterial effects of the compounds were found to be considerably better than their antimycobacterial activities along with their weak cytotoxic effects. Molecular docking studies were performed to gain insights into the antibacterial activity mechanism of the synthesized compounds. The interactions and the orientation of compound **10a** were found to be highly similar to the original ligand within the binding pocket *E. faecalis* β-ketoacyl acyl carrier protein synthase III (FabH). Finally, a theoretical study was established to predict the physicochemical properties of the compounds.

Keywords: Drug design, synthesis; dimerization; antibacterial; urea

Introduction

Bacterial infections, which are caused by mycobacterial and Gram-positive/Gram-negative pathogens, still remain one of the most severe health-threating problems worldwide.[1] Infectious diseases take place among five leading causes of life lost together with cardiovascular diseases and cancer.[2] Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, is ranked in the top ten causes of death and the leading cause of an infectious

disease. The World Health Organization (WHO) estimated that ten million people developed TB disease in 2017.[3] The rising antimicrobial resistance to the current drugs makes the treatment of infectious diseases complicated. Therefore, continuous efforts focus on developing new molecules with diverse structures to control different types of infections successfully.[4]

Urea functionality is one of the most important pharmacophores integrated into chemistry and life sciences. Thus, a remarkable amount of studies focus on alkyl/aryl substituted urea moieties for the rational design of new drug candidates.[5] Various urea-containing molecules have been reported with diverse biological applications as antibacterial,[6] antitubercular,[7] antifungal,[8] anti-inflammatory,[9] antiviral,[10] antidepressant,[11] and anticancer agents.[12,13] Among them, various aryl/alkyl-substituted urea derivatives were reported with their antimicrobial activities.[14–19] (Figure 1)

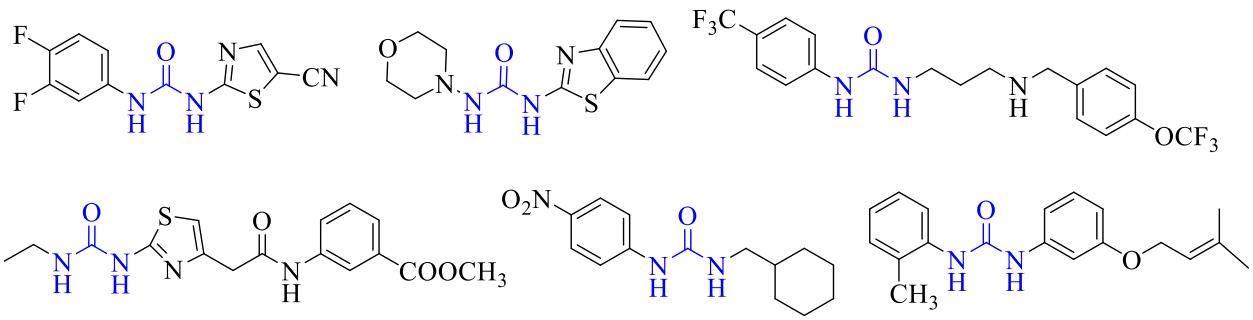


Figure 1. Some examples of substituted urea derivatives possessing antimicrobial activities

Numerous disulfide bonds are present in antimicrobial peptides isolated from both plants and animals.[20,21] These peptides have been shown to possess protective effects against antibacterial infections in the living hosts.[22] Disulfide bond containing analogs of vancomycin (Figure 2) and teixobactin,[23,24] well-known antibacterial compounds, have been reported with improved antibacterial activities. Consequently, combining the pharmacophores in the same molecule by using a disulfide bond is a rational approach to obtain novel antimicrobial agents.

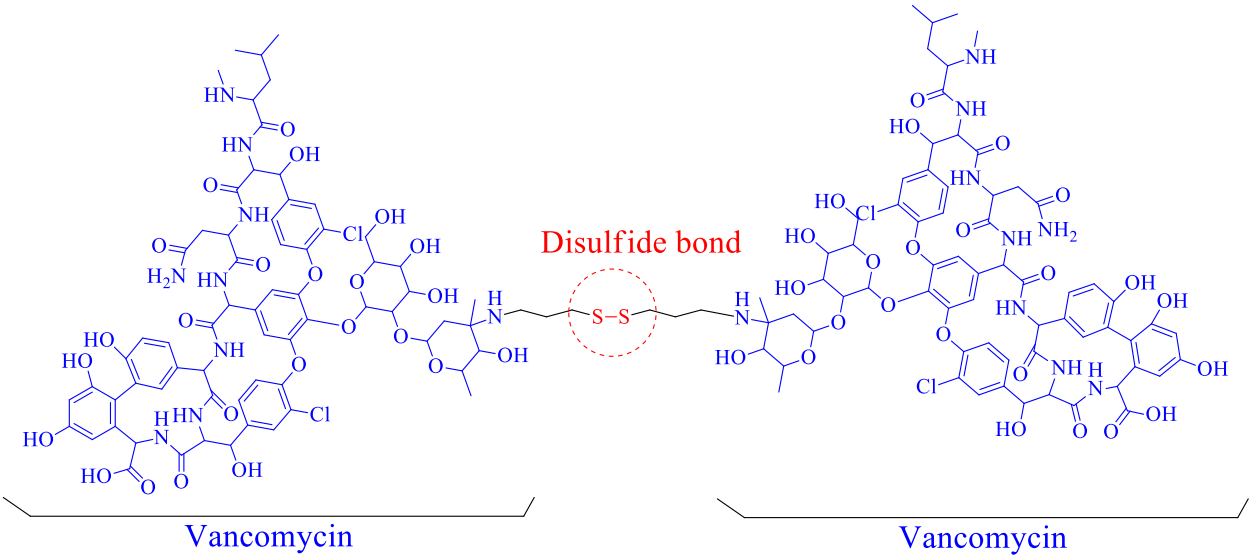


Figure 2. Vancomycin disulfide dimer possessing antibacterial activity

In light of these considerations, we aimed to synthesize disulfide-bridged N-phenyl-N'-(alkyl/aryl/heteroaryl)urea derivatives (Figure 3) and evaluate their antimicrobial activities on different Gram-positive and Gram-negative bacteria as well as on *Mycobacterium tuberculosis*.

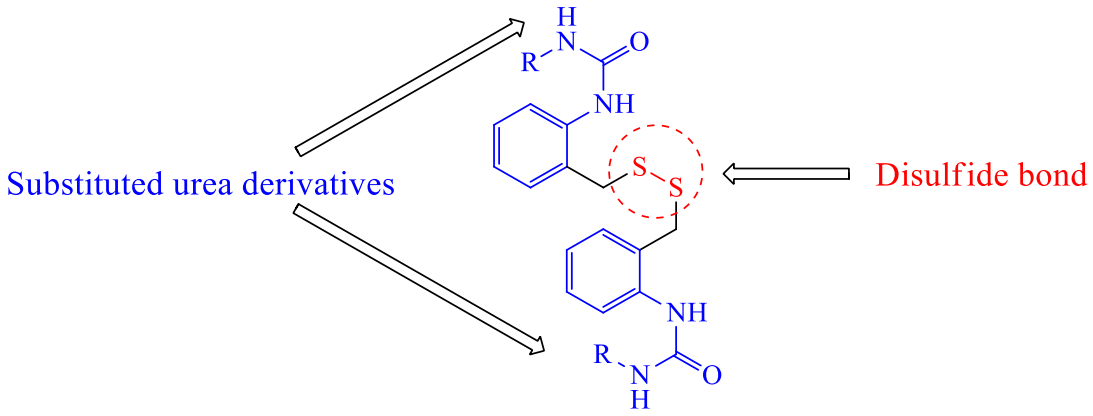
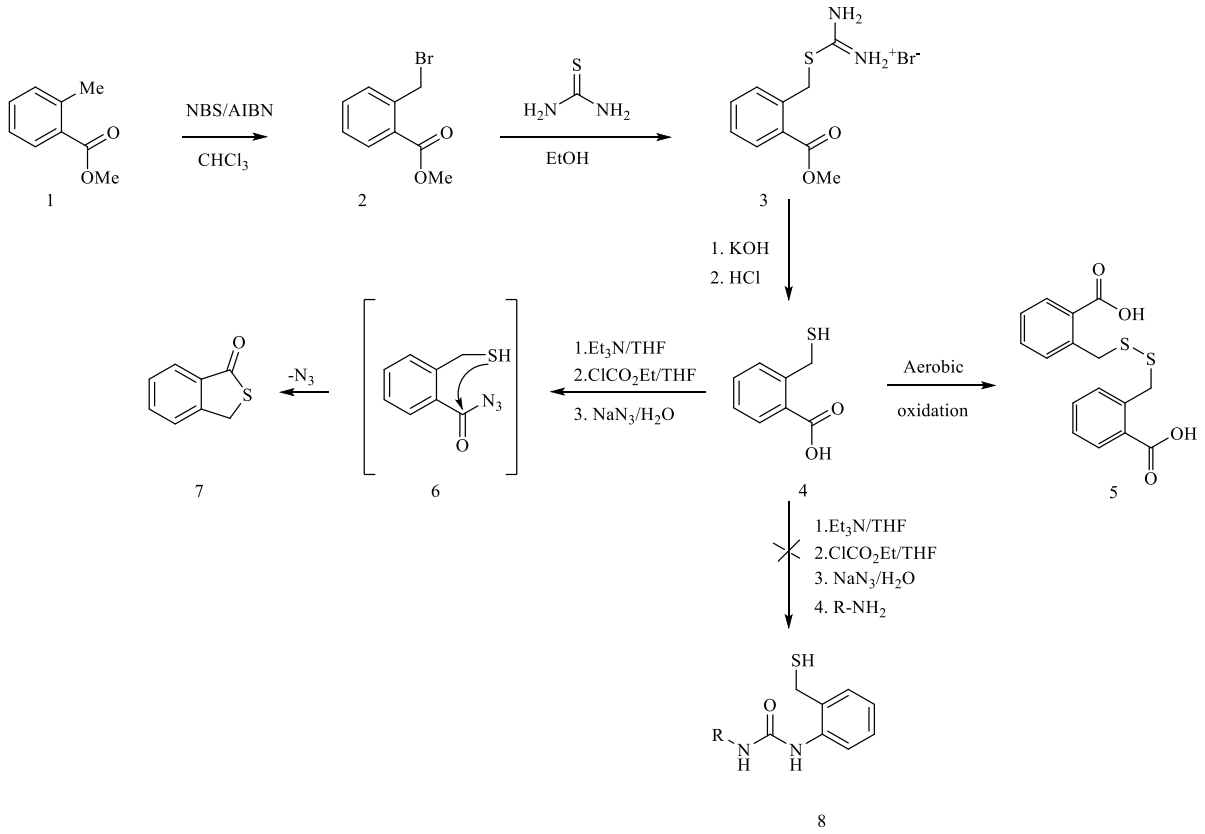


Figure 3. The design strategy of **10a-h**

Results and Discussion

Chemistry

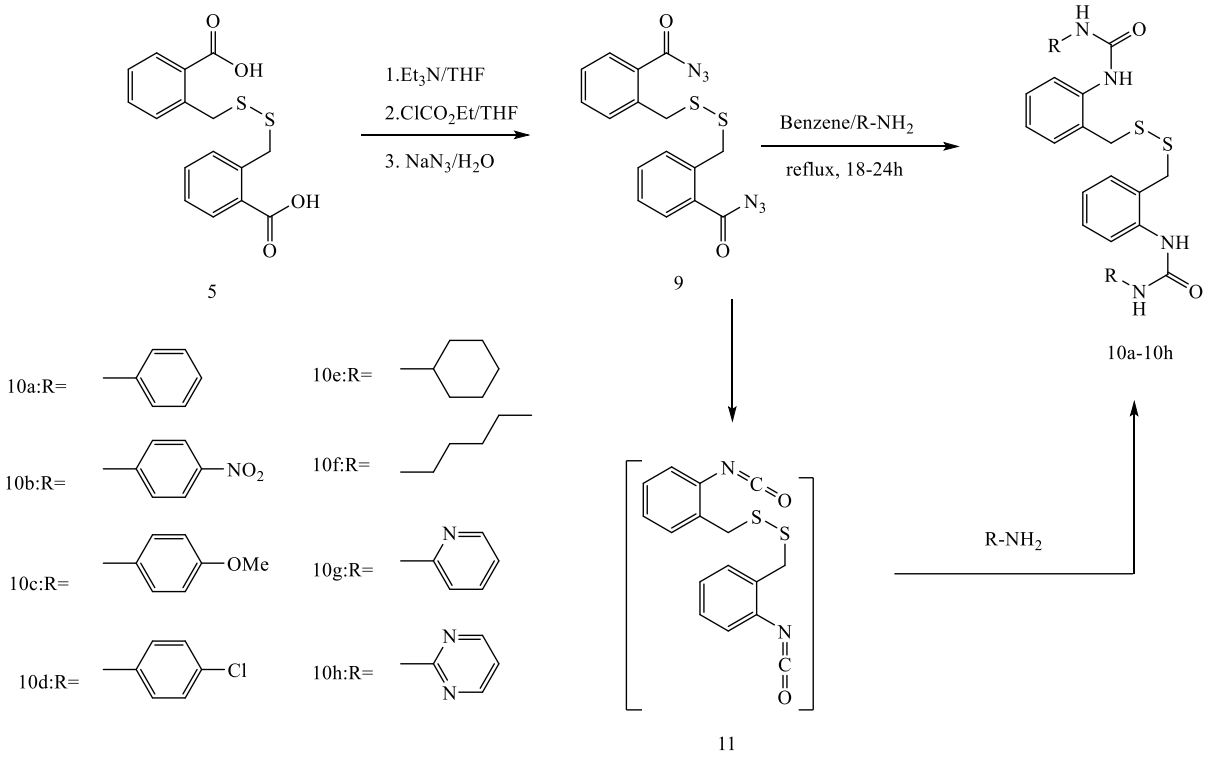
To obtain the target novel disulfide-bridged *N*-phenyl-*N′*-(alkyl/aryl/heteroaryl) urea derivatives, **10a-h**, 2,2'-(disulfanediylbis(methylene))dibenzoic acid (**5)** was employed as the key compound. Commercially available methyl 2-methylbenzoate (**1**) was brominated in the presence of NBS/AIBN at reflux temperature to give the product **2**. 2-Bromomethyl substituted ester **2** treated with thiourea resulted in the formation of thiouronium salt **3**. Basic hydrolysis of thiouronium salt **3** gave carboxylic acid derivative **4** with high yield. The proposed structure of the obtained product **4** was evaluated by NMR spectral data. After the synthesis of acid **4**, the attempts to synthesize urea compound **8** were failed. Thiophthalide (**7**) was formed with the azidination reaction of **4** with ethyl chloroformate in the presence of triethylamine following by the addition of a solution of NaN_3 in water, unexpectedly (Scheme 1). In order to synthesize bisulfide bond containing urea derivative **10a-h**, thiol group oxidative coupling reaction takes place before the azidination reaction. Oxidative coupling reaction of thiol group in **4** took place with air within one or two weeks in open vessel, without any heavy metal ion or a base, giving the disulfide containing compound **5** (Scheme 1). The high-resolution mass spectrum data obviously indicated the formation of product **5**, obtained from two moles of **4**. The experimental value of compound **5** $[M-H]^-$= 333.0245 ($C_{16}H_{13}O_4S_2$) was in an excellent agreement with the theoretical value, $[M-H]^-$=333.0275.



Scheme 1. Synthetic scheme for the preparation of the key compound, 2,2'-(disulfanediylbis(methylene))dibenzoic acid (**5**) .

In order to synthesize target urea derivatives **10a-h**, azidination reaction was performed on the 2,2'-(disulfanediylbis(methylene))dibenzoic acid (**5)**. In the azidination reaction, [25,26] acid **5** was initially reacted with ethyl chloroformate in presence of triethylamine, then a solution of NaN_3 in water was added to synthesize azide **9** in 81% yield (Scheme 2). The azide function was convenient to generate the corresponding isocyanate via Curtius rearrangement.[27,28] Hence, acyl azide **9** was refluxed in benzene and this gave rise to the formation of the isocyanate **11.** It was not isolated and reacted with various amines to achieve the target urea derivatives **10a-h** (Scheme 2).

The structures of the title compounds **10a-h** were evaluated by ^{1}H- and ^{13}C-NMR, IR, HRMS. In the ^{1}H-NMR spectra, the singlet signal for the CH_2 group of **10a-h** was observed at 3.5-4.0 ppm. The signals for -NH groups were observed at 8.0-12.0 ppm in all synthesized urea derivatives **10a-h**.



Scheme 2. General synthesis of urea derivatives **10a-h**.

Antibacterial activity evaluation

The *in vitro* antibacterial activities of the title compounds were evaluated against three Gram-positive and two Gram-negative bacteria by measuring their minimum inhibitory concentration (MIC) values. Gentamicin and piperacillin-tazobactam were used as positive controls during the experiments. Table 1 reports the antibacterial activities of the designed compounds against tested bacteria.

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Table 1. The minimum inhibitory concentration (μg/mL) of **10a-h** against tested bacteria

	Gram-positive bacteria			**Gram-negative bacteria**	
Compound	*S. aureus* ATCC 29213	*E. faecalis* ATCC 29212	*MRSA* ATCC 43300	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853
10a	16	2	32	16	256
10b	256	16	256	256	128
10c	128	8	128	256	256
10d	16	2	32	32	128
10e	64	8	64	128	128
10f	32	4	32	128	128
10g	64	4	64	128	128
10h	128	4	64	128	128
Gentamicin	1	16	32	1	1
Piperacillin-tazobactam	0.5	1	NA	0.5	0.5

NA: Not applicable.

The title compounds possessed antimicrobial activity against all tested microorganisms with MIC values ranging between 2 and 256 μg/mL. According to obtained results, the tested compounds have high antimicrobial activity against *E. faecalis* which is a Gram-positive bacterium present in the gastrointestinal tract and may cause enteric infections (MIC values ranging between 2 and 16 μg/mL). All tested compounds have higher or equal MIC values when compared to reference compound gentamicin against *E. faecalis*. The obtained results clearly revealed that the test compounds (**10a-h)** had higher antimicrobial activity against Gram-positive bacteria. Furthermore, three of the compounds (**10a**, **10d**, and **10f**) had an activity on methicillin-resistant *S. aureus* (MRSA) with a MIC of 32 μg/mL as determined for gentamicin.

When the obtained results are figured out in terms of chemical structure, the substitution of the phenylurea moiety with either aryl or alkyl group did not mediate a significant change in the antibacterial activity of the compounds. Introducing a substituent into the 4- position of the phenyl ring in **10a** did not enhance the activity, nevertheless, compound **10d** carrying chlorine atom possessed equipotent activity to **10a**. Changing one of the phenyl rings in **10a**

with other aromatic rings (pyridine and pyrimidine) did not cause an improvement in the preferential activity, suggesting that this modification is not critical.

Antitubercular activity evaluation

The synthesized compounds were evaluated for their *in vitro* antitubercular activity against *M. tuberculosis* H37Rv using the Microplate Alamar Blue Assay (MABA) method. Isoniazid, rifampin, and ethambutol were employed as reference compounds. The antimycobacterial activities of the compounds are provided as MIC values in Table 2.

Table 2. The antitubercular activity of compound **10a-h**

Compound	MIC (μg/mL)
10a	>25
10b	>25
10c	25
10d	>25
10e	>25
10f	>25
10g	>25
10h	>25
Isoniazid	0.05
Rifampicin	0.1
Ethambutol	1.56

Among the tested compounds, only **10c** exhibited moderate activity with MIC value of 25 μg/mL. The rest of the compounds were found to have MIC values higher than 25 μg/mL. These data indicated that the compounds were not effective inhibitors of *M. tuberculosis.*

In vitro cytotoxicity screening

As the discovery of new antimicrobial agents with low cytotoxicity is of utmost importance, the compounds were also screened for their cytotoxic effects on RAW 264.7 cells at 50 μg/mL concentration using MTT assay. The percentages of inhibition data for all compounds are summarized in Table 3.

Table 3. Cytotoxicity of the synthesized compounds

Compound	% Inhibition at 50 (µg/mL) against RAW cells
10a	16.25
10b	9.86
10c	21.74
10d	22.08
10e	13.90
10f	24.61
10g	22.81
10h	19.42

According to the obtained values, all compounds were found to be non-toxic with <50% inhibition.

Molecular Docking

In order to investigate the antibacterial activity mechanism of the compounds, compound **10a** was selected for molecular docking studies. **10a** was found to be more active on Gram-positive bacteria, especially on *E. faecalis* with MIC value of 2 µg/mL. *E. faecalis* is one of the major causes of hospital-acquired infections, thus the development of new antibacterials against this bacteria is of utmost importance due to the increased antimicrobial drug resistance.[29] The bacterial fatty acid biosynthesis (fab) pathway is essential for cell growth and viability, thus stands as an attractive target to obtain antibacterial activity. β-Ketoacyl acyl carrier protein synthase III (KAS III) encoded by the fabH gene initiates fatty acid synthesis in bacteria and is one of the key enzymes to overcome the antibiotic resistance problem.[30] Particularly, molecules containing disulfide bonds were reported to inhibit FabH,[31] therefore this situation prompted us to dock compound **10a** into the active site of *E. faecalis* FabH with the PDB code 3IL5.

Common interactions of superimposed co-crystallized ligand and the top-ranked docking pose of **10a** within the binding pocket of the enzyme are provided in Figure 4. It is clear that the original ligand and **10a** have a similar orientation in the active site and we surmise that compound **10a** is likely to show its antibacterial effect by inhibiting the mentioned enzyme considering the high number of common interactions with the enzyme.

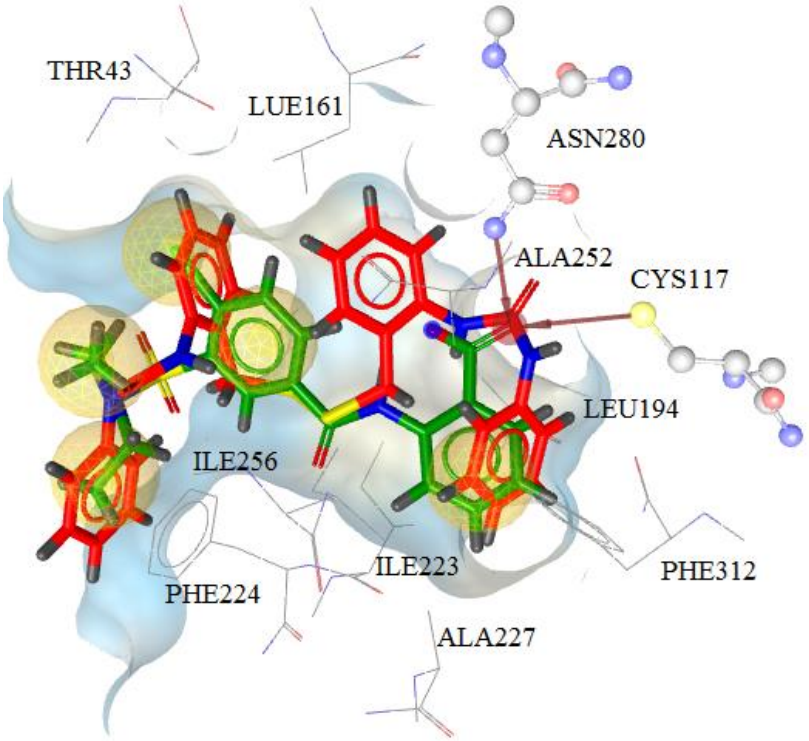

Figure 4. Common interactions of the co-crystallized ligand (green) with compound **10a (red)** in the binding pocket of *E. faecalis* FabH. Color-coded pharmacophore features: hydrogen bond acceptor (red vector), hydrophobic interaction (yellow sphere)

More closely focusing on the interactions of **10a**; the binding conformation of **10a** in the active pocket of FabH, the pharmacophore features in 3D and 2D representations are provided in Figure 5. β-Ketoacyl acyl carrier protein synthase III (KAS III) catalyzes the condensation reaction between acyl-CoA and malonyl-ACP during fatty acid biosynthesis.[30] The active site of FabH consists of the catalytic triad formed by the residues Cys117, His250 and Asn280 in which Cys117 is involved in acetyl transfer, while His250 and Asn280 play an active role in decarboxylation.[32] Thus, it is important to note that **10a** interacts with this catalytic triad via forming three hydrogen bonds. The proposed binding mode of **10a** involves several hydrophobic contacts as well.

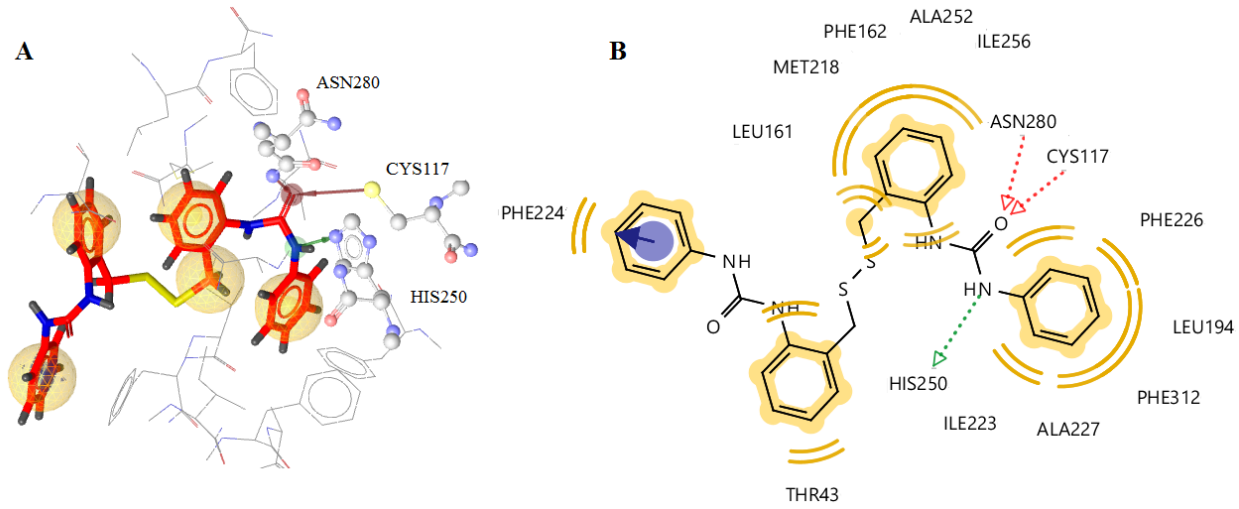

Figure 5. Predicted binding mode of **10a** in the active pocket of 3IL5. 3D- (A) and 2D- (B) representations of the pharmacophores. Hydrophobic and aromatic interactions are represented as yellow spheres and blue circle. Hydrogen bond acceptor and donor interactions are represented as red and green arrows, respectively. The residues forming the catalytic triad are represented as ball and stick models.

In silico prediction of physicochemical properties of 10a-h

Molecular property prediction is considered to be a useful tool to identify chemical leads in the drug discovery process.[33] Here, we carried out some computational calculations, using Molinspiration online software,[34] to obtain the physicochemical properties **10a-h**. (Table 4).

Table 4. Calculated physicochemical properties data of **10a-h**

Compound	LogP[a]	M.W.[b]	HBA[c]	HBD[d]	TPSA[e]	nROTB[f]
10a	6.84	514.68	6	4	82.25	9
10b	6.76	604.67	12	4	173.19	11
10c	6.96	574.73	8	4	100.72	11
10d	8.18	583.57	6	4	82.25	9
10e	7.26	526.77	6	4	82.25	9
10f	7.33	502.75	6	4	82.25	15
10g	5.04	516.65	8	4	108.03	9
10h	4.04	518.63	10	4	133.82	9

[a]LogP: logarithm of *n*-octanol-water partition coefficient
[b]M.W: molecular weight
[c]HBA: number of hydrogen bond acceptors
[d]HBD: number of hydrogen bond donors
[e]TPSA: topological polar surface area
[f]NROTB: number of rotatable bonds

Lipinski's rule of five describes the main molecular descriptors which are used to predict the cell permeability and oral bioavailability of the new drug candidate molecules.[35] Any drug candidate must adhere to at least three of the following rules: M.W≤500 Da, logP≤5, HBA≤10, HBD≤5. In addition to these parameters, we additionally calculated NROTB and TPSA which are also considered to be important descriptors of oral bioavailability of drugs.[36] The TPSA values for the most known drug molecules are below 140–150 $Å^2$.[37] According to the obtained data, most of the compounds showed a slight violation regarding their molecular weights or logP values. Only **10b** has additional violation due to its number of hydrogen bond acceptors. In the present study, we combined repeating pharmacophores with disulfide bonds, so it is expected that the compounds slightly exceed the accepted values of molecular weight. Additionally, only 34.4 % of marketed antibacterial drugs have logP value in 0-5 range.[38] Altogether, it can be concluded that compound **10a-h** exhibit reasonable physicochemical properties as antibacterial drug candidates.

3. Conclusion

In this study, we presented the synthesis of novel compounds containing disulfide bridged repeating urea pharmacophores. The obtained compounds were screened for their antimicrobial activities on various Gram-positive/Gram-negative bacteria and on *Mycobacterium tuberculosis*. Some compounds turned out to be promising antibacterial agents with a good safety profile in this series. The docking results identified FabH as the possible target enzyme for the compounds to possess their antibacterial activities. Altogether, our data show that combining two same pharmacophores with a disulfide bond may serve as a good approach to achieve the antibacterial activity.

Experimental Section

Material and methods

All chemicals were purchased from commercial sources and used as received. The reaction mixtures were monitored by thin layer chromatography (TLC) using glass-backed TLC plates. Melting points were determined using open glass capillaries and were uncorrected. Infrared (IR) spectra were obtained via ATR diamond in the range 4000–600 cm^{-1}. ^1H (400 MHz) and ^{13}C NMR spectra (100 MHz) were obtained on a Bruker AM 400 spectrometer in DMSO-d_6

or $CDCl_3$ solution. Coupling constants, *J*, are reported in hertz. MS spectra were carried out on an LC/MS High-Resolution Time of Flight (TOF) Agilent 1200/6530 instrument at the Atatürk University-East Anatolian High Technology Research and Application Center (DAYTAM).

Chemistry

Synthesis of the methyl-2-(bromomethyl)benzoate (2)[39]

Methyl 2-methylbenzoate (**1**) (4 g, 26.4 mmol), N-bromosuccinimide (NBS) (4.74 g, 26.40 mmol) and azaisobutryonitrile (AIBN) (100 mg, 0.60 mmol) were refluxed in 80 mL chloroform for 24 h. After the reaction was completed, the solid succinimide was filtered, and the organic phase was evaporated to achieve product **2**. This product was used directly for the next step without further purification. Yellow oil. (Yield 95%).

Synthesis of tiouronium salt 3[40]

Thiourea was added (0,11 mol, 8.4 g) was added to the solution of methyl 2-(bromomethyl)benzoate (**2**) (8.7 mmol, 2.0 g) in acetone (30 mL). The resulting mixture was refluxed for 10 h., then cooled and filtered to remove the solid. The filtrate was evaporated to half volume. The desired solid tiouronium salt was filtered. The obtained product was used directly for the next step without further purification. White solid. Yield: 45%. Mp: 171-173 °C. ^{1}H NMR (400 MHz, DMSO) δ 9.12 (s,4H), 7.96 (d, *J* = 7.8 Hz, 1H), 7.67-7.56 (m, 2H), 7.51 (t, *J* = 7.5 Hz, 1H), 4.74 (s, 2H), 3.86 (s,3H). ^{13}C NMR (100 MHz, DMSO) δ 169.7, 167.1, 136.9, 133.5, 131.7, 131.6, 129.2, 129.1, 52.9, 33.7.

Synthesis of 2,2'-(disulfanediylbis(methylene))dibenzoic acid (5) [40]

A solution of 2M KOH (20 mL) was added to a solution of **3** (4.73g, 0,01 mol) in 20 mL of MeOH and the reaction was refluxed for 1h. After the reaction was completed, MeOH was removed using a rotary evaporator, and the residue was acidified with 1N HCl. The obtained mixture was extracted with Et_2O (3X25 mL), organic layers were collected, dried over $MgSO_4$ and evaporated. White solid. Yield 72%. Mp: 117-119 °C. ^{1}H NMR (400 MHz, DMSO) δ 13.06 (s, 1H), 7.84 (d, *J* = 7.7 Hz, 1H), 7.50 (t, *J* = 7.4 Hz, 1H), 7.43 (d, *J* = 7.6 Hz, 1H), 7.34 (t, *J* = 7.5 Hz, 1H), 4.03 (d, *J* = 8.3 Hz, 2H), 2.75 (t, *J* = 8.2 Hz, 1H). ^{13}C NMR (100 MHz, DMSO) δ 168.7, 139.3, 132.2, 132.2, 131.4, 130.1, 128.1, 41.0. After one or two weeks on an open vessel, the colour of synthesized compound **5** became yellow. Mp: 158-160 °C. HRMS (EI): $[M-H]^-$, found 333.0275. $C_{28}H_{26}N_4NaO_2S_2$ requires 333.0255.

General procedure for the synthesis of 10a-h

Compounds **10a-h** were synthesized according to the literature procedure.[41] Briefly, triethylamine in THF was added to the solution of acid **5.** After stirring for 30 min., the cold ethyl chloroformate solution in THF was added slowly. Lastly, sodium azide solution in water was included. The final mixture was extracted with ethyl acetate, washed with saturated sodium bicarbonate and water, and dried. The obtained acyl azide **9**, obtained after removing ethyl acetate, was reacted with the corresponding amines to give final product **10a-h**. The experimental procedure is provided in detail as supplementary data.

1,1'-((Disulfanediylbis(methylene))bis(2,1-phenylene))bis(3-phenylurea) (10a)

White solid, yield: 32%. mp 198-200 °C; IR (ATR) 3250, 2971, 1699, 1634, 1550, 1149. ^{1}H NMR (400 MHz, DMSO) δ 9.02 (s, 1H), 7.97 (s, 1H), 7.87 (d, *J* = 8.0 Hz, 1H), 7.45 (d, *J* = 7.9 Hz, 2H), 7.32-7.20 (m, 3H), 7.14 (d, *J* = 7.6 Hz, 1H), 6.96 (t, *J* = 7.4 Hz, 2H), 3.72 (s, 2H). ^{13}C NMR (100 MHz, DMSO) δ 153.1, 140.2, 137.5, 131.7, 129.3, 123.0, 122.6, 122.3, 118.5, 38.5. HRMS (EI): $[M+Na]^-$, found 537.1327. $C_{28}H_{26}N_4NaO_2S_2$ requires 537.6518.

1,1'-((Disulfanediylbis(methylene))bis(2,1-phenylene))bis(3-(4-nitrophenyl)urea) (10b)

Yellow solid, yield: 24%. mp 215-217 °C; R_f (EtOAc): 0.62; . IR (ATR) 3285, 3031, 1648, 1551, 1477, 1328. ^{1}H NMR (400 MHz, DMSO) δ 9.73 (s, 1H), 8.36 – 8.04 (m, 3H), 7.79 (d, *J* = 8.0 Hz, 1H), 7.69 (d, *J* = 9.0 Hz, 2H), 7.27 (t, *J* = 7.3 Hz, 1H), 7.17 (d, *J* = 7.3 Hz, 1H), 7.03 (t, *J* = 7.3 Hz, 1H), 3.73 (s, 2H).^{13}C NMR (100 MHz, DMSO) δ 152.6, 146.8, 141.4, 136.7, 131.6, 128.5, 128.2, 125.6, 123.9, 122.6, 117.8, 38.4. HRMS (EI): $[M+KH]^-$, found 643.0733. $C_{28}H_{24}KN_6O_6S_2$ requires 643.0839.

1,1'-((Disulfanediylbis(methylene))bis(2,1-phenylene))bis(3-(4-methoxyphenyl)urea) (10c)

Purple solid, yield: 50%. mp 231-233 °C; R_f (EtOAc:Hexane=1:1): 0.27; IR (ATR) 3247, 2902, 1635, 1543, 1443, 1225. ^{1}H NMR (400 MHz, DMSO) δ 8.84 (s, 1H), 7.88 (d, *J* = 2.3 Hz, 1H), 7.87 (s, 1H), 7.36 (d, *J* = 9.0 Hz, 2H), 7.23 (t, *J* = 7.7 Hz, 1H), 7.13 (d, *J* = 7.5 Hz, 1H), 6.95 (t, *J* = 7.7 Hz, 1H), 6.87 (d, *J* = 9.0 Hz, 2H), 3.71 (s, 7H). ^{13}C NMR (100 MHz, DMSO) δ 154.8, 153.2, 137.7, 133.2, 131.7, 128.5, 126.8, 122.7, 122.3, 120.2, 114.4, 55.5, 38.3. HRMS (EI): $[M+H]^+$, found 575.1759. $C_{30}H_{31}N_4O_4S_2$ requires 575.1789.

1,1'-((Disulfanediylbis(methylene))bis(2,1-phenylene))bis(3-(4-chlorophenyl)urea) (10d)

White solid, yield: 50%. mp 239-241 °C; R_f (EtOAc:Hexane=1:1):0.43; IR (ATR) 3271, 2916, 1633, 1584, 1545, 1237. ^{1}H NMR (400 MHz, DMSO) δ 9.15 (s, 1H), 7.99 (s, 1H), 7.82 (d, *J* = 8.1 Hz, 1H), 7.51 – 7.45 (m, 2H), 7.35 – 7.29 (m, 2H), 7.28 – 7.21 (m, 1H), 7.14 (dd, *J* = 7.6, 1.4 Hz, 1H), 6.98 (td, *J* = 7.5, 1.1 Hz, 1H), 3.71 (s, 3H). ^{13}C NMR (100 MHz, DMSO) δ 152.9, 139.1, 137.3, 131.7, 129.1, 128.5, 127.4, 125.7, 123.2, 122.8, 120.0, 38.4. HRMS (EI): $[M+K]^-$, found 621.0355. $C_{28}H_{24}Cl_2KN_4O_2S_2$ requires 621.0259.

1,1'-((Disulfanediylbis(methylene))bis(2,1-phenylene))bis(3-cyclohexylurea) (10e)

White solid, yield: 10%. mp 214-216 °C; R_f (EtOAc:Hexane=1:1):0.42; IR (ATR) 3303, 2917, 1630, 1556, 1451, 1246. ^{1}H NMR (400 MHz, DMSO) δ 7.92 (d, *J* = 8.2 Hz, 1H), 7.59 (s, 1H), 7.18 (t, *J* = 7.8 Hz, 1H), 7.06 (d, *J* = 7.3 Hz, 1H), 6.88 (t, *J* = 7.4 Hz, 1H), 6.53 (d, *J* = 7.6 Hz, 1H), 3.64 (s, 2H), 3.53 – 3.39 (m, 1H), 1.81 (d, *J* = 11.8 Hz, 2H), 1.66 (dd, *J* = 9.0, 4.0 Hz, 2H), 1.53 (dd, *J* = 8.3, 3.9 Hz, 1H), 1.37 – 1.23 (m, 2H), 1.23 – 1.03 (m, 3H). ^{13}C NMR (100 MHz, DMSO) δ 154.9, 138.5, 131.6, 128.3, 125.6, 121.7, 121.2, 48.2, 38.3, 33.4, 25.7, 24.8. HRMS (EI): $[M+K]^-$, found 565.1999. $C_{28}H_{28}KN_4O_2S_2$ requires 564.2073.

1,1'-((Disulfanediylbis(methylene))bis(2,1-phenylene))bis(3-pentylurea) (10f)

White solid, yield: 11%. mp 171-173 °C; R_f (EtOAc:Hexane=1:1): 0.5; IR (ATR) 3276, 2924, 1630, 1531, 1450, 1230.^{1}H NMR (400 MHz, DMSO) δ 7.88 (d, *J* = 8.3 Hz, 1H), 7.66 (s, 1H), 7.19 (t, *J* = 7.7 Hz, 1H), 7.07 (d, *J* = 7.2 Hz, 1H), 6.90 (t, *J* = 7.1 Hz, 1H), 6.56 (t, *J* = 5.2 Hz, 1H), 3.66 (s, 2H), 3.13 – 3.01 (m, 2H), 1.51 – 1.36 (m, 2H), 1.36 – 1.18 (m, 4H), 0.87 (t, *J* = 6.0 Hz, 3H). ^{13}C NMR (100 MHz, DMSO) δ 155.7, 138.5, 131.5, 128.3, 125.9, 121.9, 121.5, 39.5, 38.4, 29.8, 29.0, 22.3, 14.4. HRMS (EI): $[M+K]^-$, found 541.2010. $C_{26}H_{38}KN_4O_2S_2$ requires 541.2073.

1,1'-((Disulfanediylbis(methylene))bis(2,1-phenylene))bis(3-(pyridin-2-yl)urea) (10g)

White solid, yield: 18%. mp 261-263 °C; R_f (EtOAc:Hexane=1:1): 0.71. IR (ATR) 3196, 2978, 1694, 1579, 1552, 1540. ^{1}H NMR (400 MHz, DMSO) δ 11.19 (s, 1H), 9.85 (s, 1H), 8.20 (d, *J* = 4.7 Hz, 1H), 8.05 (d, *J* = 8.2 Hz, 1H), 7.80 – 7.67 (m, 1H), 7.32 – 7.25 (m, 1H), 7.22 (d, *J* = 8.1 Hz, 1H), 7.15 (d, *J* = 7.6 Hz, 1H), 7.01 (td, *J* = 7.5, 1.0 Hz, 1H), 6.95 (dd, *J* = 7.1, 5.2 Hz, 1H), 3.77 (s, 2H). ^{13}C NMR (100 MHz, DMSO) δ 153.5, 152.8, 146.5, 139.3, 137.5, 131.6, 128.7, 126.5, 123.1, 122.1, 117.6, 112.4, 39.1. HRMS (EI): $[M+H]^+$, found 517.1454. $C_{26}H_{25}N_6O_2S_2$ requires 517.1480.

1,1'-((Disulfanediylbis(methylene))bis(2,1-phenylene))bis(3-(pyrimidin-2-yl)urea) (10h)

White solid, yield: 14%. mp 262-264 °C; R_f (EtOAc:Hexane=1:1): 0.46; IR (ATR) 3138, 2975, 1691, 1578, 1446, 1285. ^{1}H NMR (400 MHz, DMSO) δ 11.46 (s, 1H), 10.32 (s, 1H), 8.59 (d, J = 4.8 Hz, 2H), 8.00 (d, J = 8.0 Hz, 1H), 7.30 (t, J = 7.9 Hz, 1H), 7.15 (d, J = 7.5 Hz, 1H), 7.11 – 6.99 (m, 2H), 3.78 (s, 2H). ^{13}C NMR (100 MHz, DMSO) δ 158.5, 158.26, 152.0, 137.2, 131.6, 128.7, 127.0, 123.6, 122.6, 115.4 (-CH_2 signal overlapped with the solvent). HRMS (EI): $[M+K]^-$ found 557.0867. $C_{24}H_{22}KN_8O_2S_2$ requires 557.0944).

Antibacterial activity evaluation

The title compounds were tested against five bacteria including three Gram-positive (*Staphylococcus aureus, Enterococcus faecalis* and methicillin-resistant *Staphylococcus aureus*) and two Gram-negative microorganisms (*Escherichia coli* and *Pseudomonas aeruginosa*) to evaluate their antibacterial activities. Gentamicin and piperacillin-tazobactam were used as positive control antibiotics.

Broth microdilution assay reported by the Clinical and Laboratory Standards Institute (CLSI) was used to determine MIC values. MIC values were identified as the lowest concentration of the test compounds with no bacterial growth.[42]

Antitubercular activity evaluation

Antitubercular activities of the designed compounds **10a-h** were investigated *in vitro* using MABA method. The obtained results are reported as MIC values against *M. tuberculosis* H37Rv.[43]

In vitro cytotoxicity screening

The *in vitro* cytotoxicity of the synthesized compounds was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay against growth inhibition of RAW 264.7 cells at 50 μg/mL concentration.[44,45] The values were calculated for percentage inhibition that enables us to achieve the cytotoxicity data of the test compounds.

The detailed experimental procedures to determine antimicrobial activities and the cytotoxicity of the compounds are provided as supplementary data.

Molecular docking

The chemical formula of compound **10a** was built using ChemDraw Ultra 12.0 and saved as Simplified Molecule Input Entry System (SMILES) file. The file was transferred to LigandScout 4.2,[46] the structure was geometrically optimized and energy minimized to the

3D structure using the MMFF94x force field. The crystal structure of *Enterococcus faecalis* FabH with the inhibitor 2-({4-bromo-3-[(diethylamino)sulfonyl]benzoyl}amino)benzoic acid was obtained from Protein Data Bank with the PDB code 3IL5.[47] **10a** was docked into the active site of the enzyme using AutoDock Vina 1.1, integrated into LigandScout 4.2, with default parameters. 3D pharmacophores from the cocrystallized ligand of the PDB structure 3IL5 were generated using LigandScout. This pharmacophore model was employed to rank nine obtained docking poses of **10a**. The selection of the most plausible one was based on its ability to provide the highest amount of chemical interactions defined in the 3D pharmacophore. Docking results were analyzed using LigandScout 4.2 and Discovery Studio Visuliazer.[48]

In silico prediction of physicochemical properties of 10a-h

The chemical formulas of compounds **10a-h** were built in ChemDraw Ultra 12.0 and saved as Simplified Molecule Input Entry System (SMILES) file. The calculation of physicochemical properties was carried out using Molinspiration (https://www.molinspiration.com).

Supplementary Material

The detailed experimental procedures to synthesize the compounds and determine their antimicrobial activities and the cytotoxicity data as well as their ^{1}H-NMR and ^{13}C-NMR spectra are provided as supplementary material.

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Author Contribution Statement

ŞDD and SB synthesized the compounds and elucidated their structures. CÖ carried out antibacterial activity evaluation. VSK and DS performed *in vitro* antitubercular activity and

cytotoxicity experiments. MGG was responsible for computational studies. ŞDD and MGG wrote the manuscript.

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