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Research Article

Synthesis and Biological Evaluation of Imidazole-2-thione Fused Furazan as a New Biheterocyclic Scaffold

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ABSTRACT

A new imidazole-2-thione fused furazan biheterocyclic group was synthesized and its biological activity was investigated. Nitrogen rich heterocycles, imidazole-2-thione and 1,2,5-oxadiazole (furazan) have been conveni ently fused. To accomplish this, we have improved a new strategy that silica gel supported solvent free micro wave dehydration of vicinal dioximes. The target furazan "*N,N'*-diphenyl-4H-imidazo[4,5-c]furazan-5(6H) –thio ne" [II] (DFTFrz) was obtained from (4E,5Z)-4,5-bis(hydroxyimino)-*N,N'*-diphenylimidazolidine-2-thione [I] (DFTD) with acceptable yield and characterized by LC-MS/MS, FT-IR and NMR spectroscopy. The biological evaluation of the obtained thiourea furazan and its precursor dioxime were investigated by antibacterial effect studies using broth microdilution and disc diffusion methods. It was observed that thioure furazan was more effective on the tested pathogenic bacteria than its precursor dioxime. The compounds did not show any effect on gram negative bacteria, while they exhibited a moderate effect on gram positive bacteria, especially *Staphylococcus* spp.

Keywords: *Thiourea, Furazan, Imidazole-2-thione, Biheterocyclic, Antibacterial*

Yeni İki Hetero halkalı Bir Yapı Olarak İmidazol-2-tiyon ile Kaynaşmış Furazan Sentezi ve Biyolojik Değerlendirmesi

ÖZ

İmidazole-2-tiyon grubunun furazan halkasıyla birleşmesinden oluşan iki heterohalkalı yeni bir bileşik sentez lenmiş ve biyolojik aktivitesi araştırılmıştır. Vic-dioksimlerinsilikajel destekli mikrodalga dehidrasyonunu içeren yeni bir stratejiyle, azot bakımından zengin heterohalkalar olan imidazol-2-tiyon ve 1,2,5-oksadiazol (furazan) uygun bir şekilde kaynaştırılmıştır. Hedeflenen furazan bileşiği olan "*N,N'*-difenil-4H-imidazo[4,5-c]furazan-5(6H)-tiyon" [II] (DFTFrz), kabul edilebilir bir verimle "(4E,5Z)-4,5-bis(hidroksimino)-*N,N'*-difenil imidazoli din-2-tiyon" [I] (DFTD) bileşiğinden sentezlendi ve LC-MS/MS, FT-IR ve NMR spektroskopisi metotlarıyla karakterize edildi. Elde edilen tiyoürefurazan ve çıkış maddesi dioksimin biyolojik değerlendirmesi, broth mikro dilüsyon ve disk diffüzyon yöntemlerini içeren antibakteriyel etki çalışmalarıyla araştırıldı. Tiyoüre furazanın test edilen patojenik bakteriler üzerinde öncülü olan dioksime göre daha etkili olduğu bulunmuştur. Bileşikler gram negatif bakteriler üzerinde herhangi bir etki göstermezken gram pozitif bakteriler özellikle de stafilakok türleri üzerinde orta derecede etki göstermiştir.

Anahtar kelimeler: *Tiyoüre, Furazan, Imidazol-2-tiyon, İkili heterohalka, Antibakteriyel*

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I. INTRODUCTION

The compounds containing furazan ring (1,2,5-oxadiazole) have found utility in many different areas with interesting properties. Furazans are heterocyclic compounds generally obtained by dehydration of glyoximes [1],[2]. The aromaticity of the furazan ring strengthens the bonds and yielding very stable compounds. At the same time, the planar formation of the ring has led to smooth and tight crystal stacking, allowing the synthesis of high-density compounds. Since the oxygen atom in the ring is not bound to any carbon and hydrogen, it combines with carbons in high temperature decomposition and exhibits advanced oxidation and redox properties. Such causes have included furazans among the materials used to improve the properties of rocket fuels and explosives [3],[4]. Furazans also attract attention with their biological activity properties. For example; combretastatin A-4 (CA-4) is a potent cytotoxic agent isolated from a kind of African willow tree with an anti-tubulin effect. The furazan analogue of CA-4, combretafurazan, was synthesized and found to be more effective than combretastatin [5]. Furazabol, a steroid used in the clinic with its androgenic effect [6], is an example of biological active furazans (Figure 1). However, a wide variety of bioactive furazan derivatives have been synthesized. Some of those; COX-1 and COX-2 inhibitor [7], antimicrobial and antibiofilm activity [8],[9], anticancer activity [10],[11], antimitotic activity [12]. Thioureas are of great importance due to their wide application in chemical and pharmaceutical industry. These compounds exhibit a diverse range of bioactivities associated with antimicrobial, antitumor, antiviral, antithyroidal and have been fully reviewed [13]-[15]. Imidazole-2-thiones; cyclic analogs of thiourea, which are of importance for application in pharmaceutical and synthetic chemistry as well as other fields [16], [17]. Some of their applications comprise drugs treating hyperthyroidism like Carbimazol or Methimazol [18],[19] (Figure 1). L-(+)- Ergothioneine, a rare natural amino acid, is an imidazole-2-thione derivative discovered in the fungus *claviceps purpurea* and proven to act as an antioxidant in vivo [20]. The imidazoline-2-thione moiety fused with cyclic coumponds were also used as a two-photon fluorescent probe for the selective detection of mitochondrial OCl^- [21],[22]. Although imidazole-2-thiones are widely used, their fused cyclic derivatives are very rare. To our knowledge, there is no derivative of imidazole-2-thione fused with furazan ring. Keeping this in mind, a new compound starting from dioxime of N,N′-Diphenylimidazole-2-thione have been synthesized and subjected to antibacterial tests.

Nitrogen, oxygen, sulfur alone or together are the most common hetero atoms in a cyclic structure. The electronic configuration of the heteroatom and the ring tension of the heterocyclic perform an important role in physicochemical and biological properties. Fused cyclic systems, of which at least one heterocycle is a furazan ring, deserve close attention both from the theoretical view point and due to the possible application of these compounds as intermediates in synthesis and have been considered in several reviews [23]-[26].

In this paper; a new nitrogen-rich, sulfur and oxygen containing ring was synthesized by fusing the imidazole-2-thione ring with furazan ring. To accomplish this, we have improved a new strategy that silica gel supported solvent free microwave dehydration of vicinal dioximes. The synthesis of the new biheterocycle group is shown in scheme 1. The biological activities of the obtained thiourea furazan and its precursor dioxime were investigated by in vitro antibacterial effect studies against some nasocomial pathogens, using broth microdilution and disc diffusion methods.

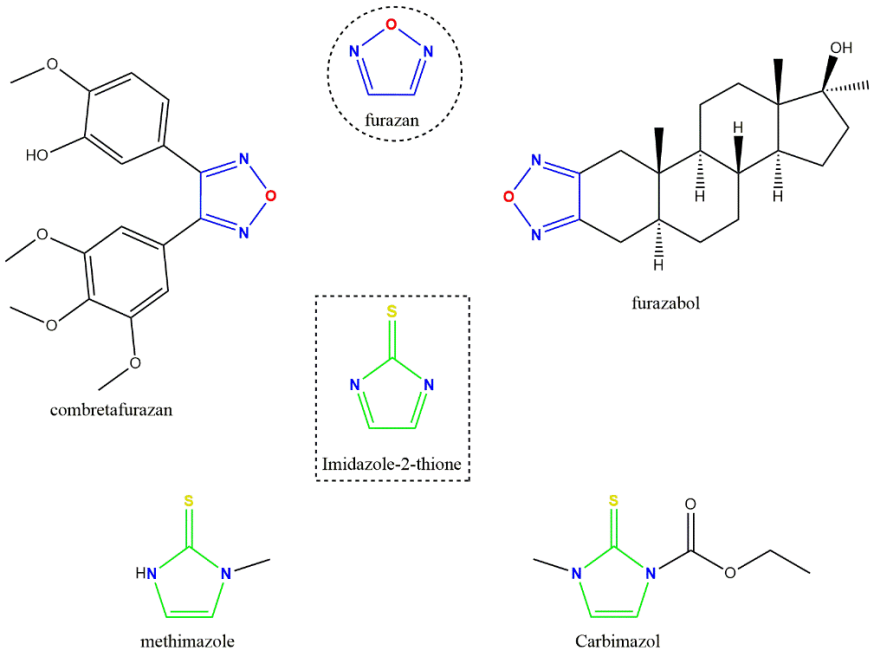


Figure 1. *The Structure of furazan, Imidazole-2-thione and related pharmaceuticals.*

II. EXPERIMENTAL SECTION

A. EXPERIMENTAL

A.1.Materials and Physical Measurements

All chemical reagents were supplied from Sigma and Merck and were used without further purification. Melting points were measured on Stuart Melting Point SMP10. ^{1}H-NMR (400Mhz) spectra were recorded with a Varian 400-MR. FT-IR spectrum were recorded in Bruker Vertex 70FT-IR Spectrometer. The LC-MS-MS analysis was performed using an Ulti Mate 3000 HPLC (Thermo Fisher Scientific) system coupled to TSQ Quantum Access Max mass spectrometer. Dichloroglyoxime [27] and cyanogen-di-N-oxide [28] were prepared according to the referenced procedures.

A.2.Synthesis

A.2.1.Synthesis of (4Z,5E)-4,5-bis(hydroxyimino)-*N,N*'-diphenylimidazoline-2-thione [I] (DFTD)

The amphi isomer of 4,5-bis(hydroxyimino)-*N,N*'-diphenylimidazoline-2-thione was prepared following protocol [29]. 1.14 g (5 mmol) of *N,N'*-Diphenylthiourea was dissolved in 50 ml dichloro methane at -10 ^{0}C. The cyanogen-di-N-oxide solution obtained by treatment with sodium carbonate and (1.18 g 7.5 mmol) dichloroglyoxime in 50 ml dichloromethane was quickly added to the solution. The color of the solution immediately turned yellow. The solution was stirred at -10^{0}C for 10 hours and stirred at room temperature for 3 hours. The yellow precipitates were filtered. Washed with cold dichloromethane and diethyl ether. The crude material was dissolved in 10 ml ethanol and refluxed on a water bath for 5 hours. Then crystalline *amphi* isomer of DFTD filtered, washed with cold ethanol and dried. Yield; 0.75 g, (48%), m.p. 205 ^{0}C (dec.) ^{1}H-NMR: (400 MHz, DMSO-d_6) (ppm): 13.07 (s, 1H, N-OH), 11.26 (s, 1H, N-OH), 7.29-7.50 (m, 7H, Ar-H), 7.09 (t, 1H, Ar-H), 6.87 (t, 2H, Ar-H). FT-IR: 3503, 3249, 3058, 2250-1712, 1590, 1300, 999, 967 LC-MS: (M+1, %100) 313.

A.2.2. Synthesis of *N,N'*-diphenyl-4H-imidazo[4,5-c]furazan-5(6H)-thione [II] (DFTFrz)

0.15 g (0.5 mmol) of *amphi* DFTD compound is mixed with 1.5 g of dry powder SiO_2 and thoroughly mixed in mortar. The mixture, which was taken to the glass vial, was heated with 800 W microwave rays (Samsung) at 4-minute intervals. The maximum temperature reached 185 ^{0}C at the end of the irradiations. From the results of TLC (n-hexane/Ethyl acetate, 2/1) of the irradiated material for 20 minutes, the starting material appears to be present in the medium, but new transformation products are also observed. The mixture extracted from the medium with chloroform and solvent is evaporated to dryness. The crude matter was recrystallized with ethanol/water (9:4). Yield; 0.033 g (23.6%), m.p. 123-125 ^{0}C (dec.) ^{1}H-NMR: (400 MHz, $CDCl_3$): 7.80 (d, 2H, Ar-H), 7.56 (t, 2H, Ar-H),7.22-7.42 (m, 4H, Ar-H), 7.01 (t, 2H, Ar-H). FT-IR: 3058, 3049, 2920, 2850, 2250-1712, 1585, 1276. LC-MS: (M+1, %100) 295,09.

A.3.Biological Activity Research

A.3.1.Bacterial Strains and Growth Media

For determining the antibacterial potential of the synthesized compounds, disc diffusion and broth microdilution assay was applied. The bacterial strains used were six Gram positive strains (*Staphylococcusaureus* ATCC 29213, *Staphylococcus aureus* ATCC 25923, Methicillin-resistant *Staphylococcusaureus* ATCC 43300, *Staphylococcus epidermidis* ATCC 35984, *Bacillus cereus* ATCC 11778 and *Enterococcus faecalis* ATCC 29212) and four Gram negative strains (*Escherichia coli* ATCC 25922, *Escherichia coli* JM109, *Pseudomonas aeruginosa* ATCC 27853 and *Klebsiella pneumoniae* ATCC 700603). Overnight cultures were grown routinely in Tryptic Soy Broth (TSB, Sigma-Aldrich) medium with shaking at 37°C. Mueller-Hinton Agar (MHA, Merck) and Mueller Hinton Broth (MHB, Merck) was used for the Disc Diffusion and broth microdilution assay respectively.

A.3.2.Determination of Antibacterial Activity by Disc Diffusion Method

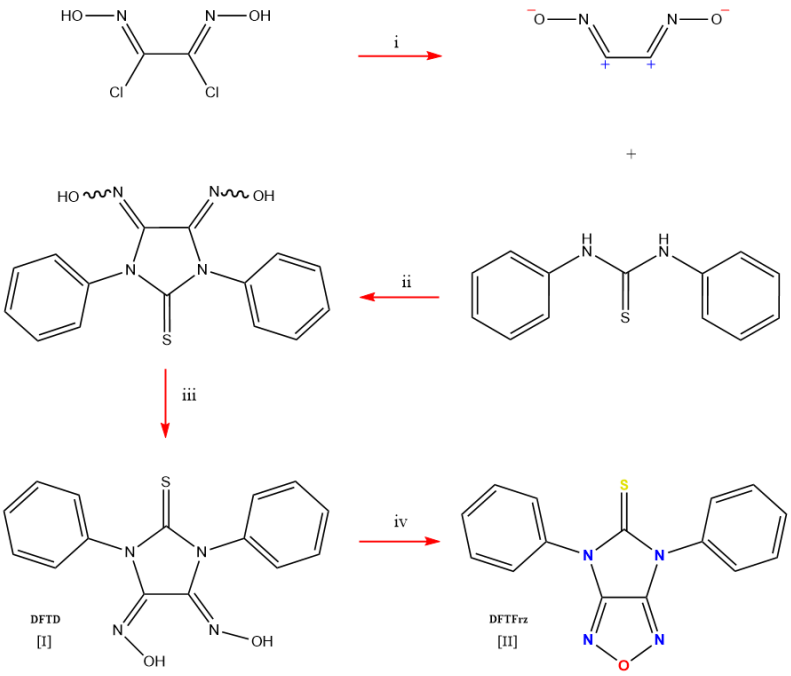
The modified Kirby-Bauer disc diffusion method was used to determine the antibacterial effects of the synthesized compounds [30]. Stock solutions of the synthesized compounds were prepared with DMSO at a concentration of 10 mg mL^{-1}. After the MHA was prepared and sterilized, it was poured into sterile petri dishes in a thickness of 4 mm. Then turbidity of bacterial suspension was adjusted to 0.5 McFarland ($\approx 10^8$ cfumL^{-1}), and swabbed homogeneously on the plates using sterile cotton swabs. 15 µL solution of each compound was added to antibiotic-free paper discs. Clarithromycin (CLR, 15 mcg, Bioanalyse) was used as a positive control antibiotic, DMSO impregnated paper discs were used as negative control. The petries were incubated at 37^{0}C overnight and the zone diameters were determined carefully. The values are reported as the average of three measurements at Table 1.

A.3.3.Determinationof Antibacterial Activity by Broth Microdilution Method

The antibacterial effects of the synthesized compounds were evaluated by finding the minimum inhibitory concentration (MIC). MIC values were determined spectrophotometrically at 600 nm [31]. Briefly, bacterial cells were grown overnight to obtain single colonies and resuspended in isotonic solution to give a turbidity equivalent to 0.5 Mc Farland. The cells were then diluted to 100-fold in MHB and were added to 96-well microtiter plates (100 µl/well) containing same volumes of 1/2 serial dilutions of synthesized compounds were prepared in MHB with stock solution of compounds (4,096 mgmL^{-1} DMSO). As a result final concentration of compounds range from 1024,512, 256, ... to 2 µgmL^{-1} and $5x10^5$cfumL^{-1} bacteria in each well (last wells are MHB control well). The same procedures were applied to Clarithromycin (Klacid, Abbott) which was used as the control standard (control standard adjusted to 128 µgmL^{-1} H_2O in the first well). Then, the prepared plates were incubated at 37^{0}C for overnight and the growths were measured at 600 nm by using a microplate reader (µQuant, BioTek). The MIC values were determined as the minumum concentration of

compounds whose Optical Density (OD) values were comparable to the negative control wells. The experiments are three replicates and the results are given in Table 2.

III. RESULTS AND DISCUSSION



Scheme 1. *Synthesis of compounds. Reagent and conditions: (i) Na_2CO_3+CH_2Cl_2, -10^0C; (ii) CH_2Cl_2, -10^0C 10 hours, room temp. 3 hours; (iii) EtOH, reflux, 5 hours; (iv) SiO_2, microwave, 20 minutes.*

The designed thiourea group condensed with furazan (1,2,5-oxadiazole) ring system was prepared as illustrated in scheme 1. The key intermediate highly reactive cyanogen-di-N-oxide was obtained by treatment dichloroglyoxime with sodium carbonate [28],[32]. 1,3-Diphenyl-2-thioxo-4,5-bis (hydroxy imino)-Imidazoline was synthesized from the reaction of cyanogen-di-N-oxide with N,N′-Diphenyl thiourea as *anti*- and *amphi*- isomer mixtures and converted into pure *amphi*- isomer [26]. The intra molecular hydrogen bond in the *amphi*-isomer fixes both oxime groups on the same side of the C—C bond, which is favorable for cyclization of dioxime to target furazan [33]-[34]. The structure of *amphi*- isomer (DFTD) was confirmed in ^{1}H-NMR by presence of characteristic two singlets (δ = 11.26 ppm and δ = 13.07 ppm) of oxime peaks. The phenyl and hydroxyl protons are at the expected values in accordance with the literature [29], as can be seen in the ^{1}H-NMR spectrum given in Figure 2. In the IR spectrum of DFTD (Figure 3), the stretching vibrations at 3249, 1647 and 967 cm^{-1} are assigned to the oxime O–H, C=N and N–O groups, respectively. The (O-H) deformation vibration has shifted to 1300 cm^{-1}. These values are consistent with previously reported dioxime [29]. This was also supported by the presence of the characteristic molecular ion peak *m/z* = 313.03 $[M+1]^+$ at mass spectrum, supporting the proposed formulation (Figure 4).

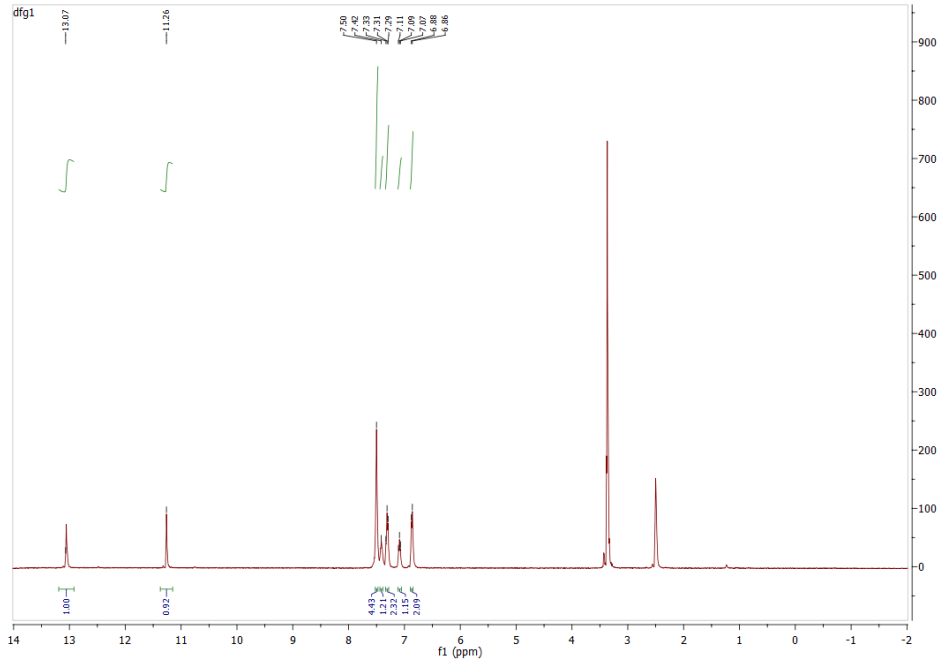
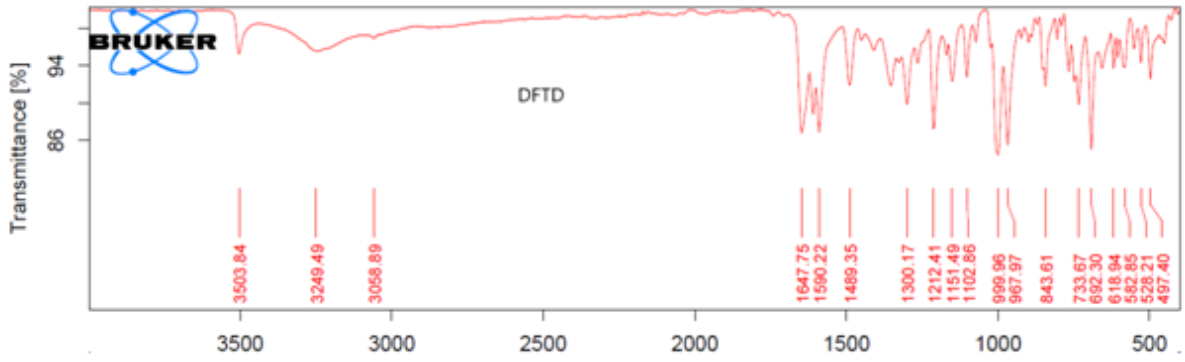
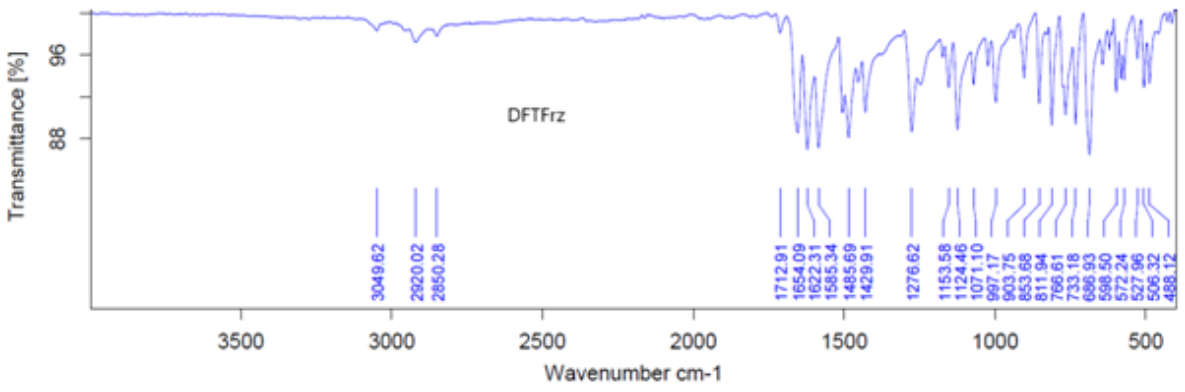


Figure 2. *^{1}H-NMR Spectrum of DFTD in DMSO-d_6.*



(a)



(b)

Figure 3. *Comparison of FT-IR spectrum of (a) DFTD and (b) DFTFrz.*

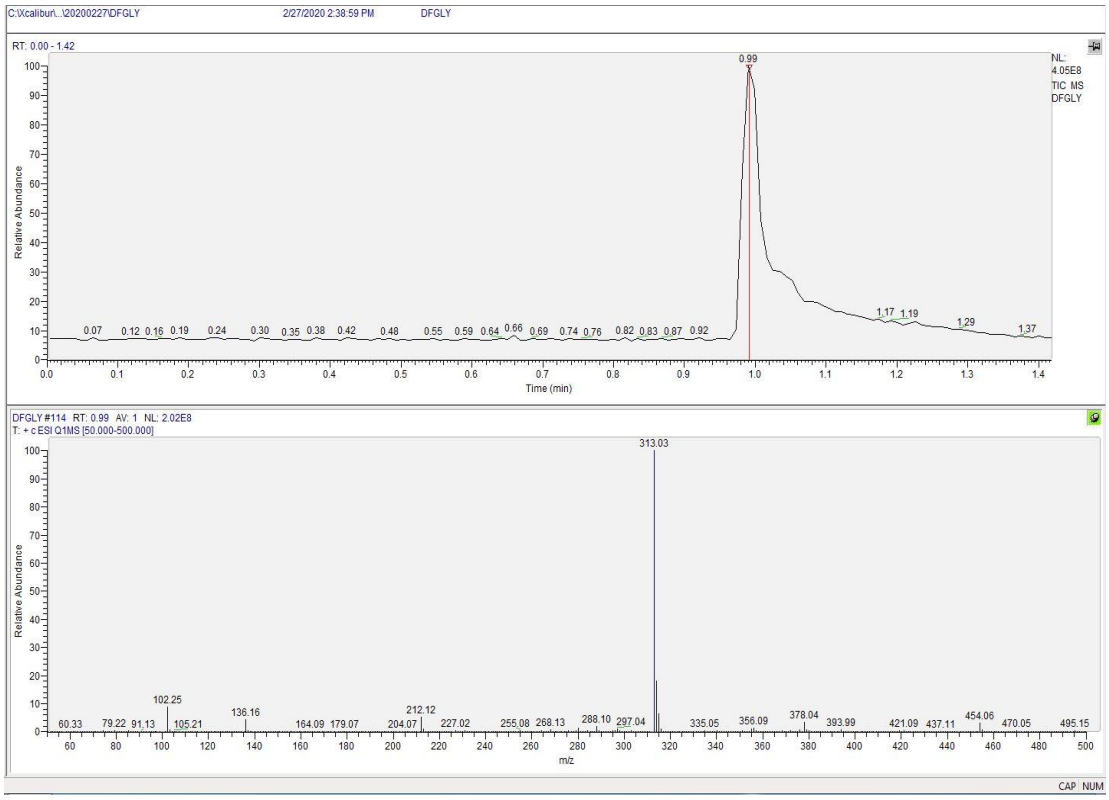


Figure 4. *LC-MS results of the DFTD compound.*

The reactions of commercial thioureas containing electron donating side groups such as N,N′-Dibutyl thiourea, N,N′-Diethylthiourea, N,N′-Diisopropylthiourea with cyanogen-di-N-oxide have been tried, but the targeted dioximes could not be obtained. We then attempted to close the synthesized *amphi*-dioxime to furazan for construction a new biheterocyclic scaffold. A more generalized route for the construction of furazan ring that have been improved in the literature contain in the dehydration of vicinal dioximes in acidic or basic medium [1],[5],[24],[35]. We tried these methods, which require high temperatures and long reaction times in basic media, resulted in mixtures containing degradation products. Although these methods have been the classical in furazan synthesis, it possesses hard reaction conditions and is applied only on unfunctionalized or poorly functionalized dioximes [5]. Perhaps the acidic and basic conditions and long reaction times required preclude the formation of furazan from the presence highly strained five membered 1,3-Diphenyl-2-thioxo Imidazoline in the molecule. As a result of this, microwave synthesis techniques were used, which have been success fully applied for difficult reactions that require high temperatures and short reaction times for a long time. Firstly, microwave basic dehydration method was tried in closed vessel at high temperature but no results were obtained. Then, the synthesis of silica gel-supported furazan method made by Kamitori [36] was examined and adapted to the microwave conditions. In this method, silica gel is used both as a solid support and as a catalyst, however, the use of microwave irradiation has also attracted attention as an effective method for providing the high temperature and short time required for this conversion reaction. After all, the target compound *N,N'*-diphenyl-4H-imidazo[4,5-c]furazan-5(6H)-thione has been synthesized with satisfactory yield via silica gel supported new microwave method.

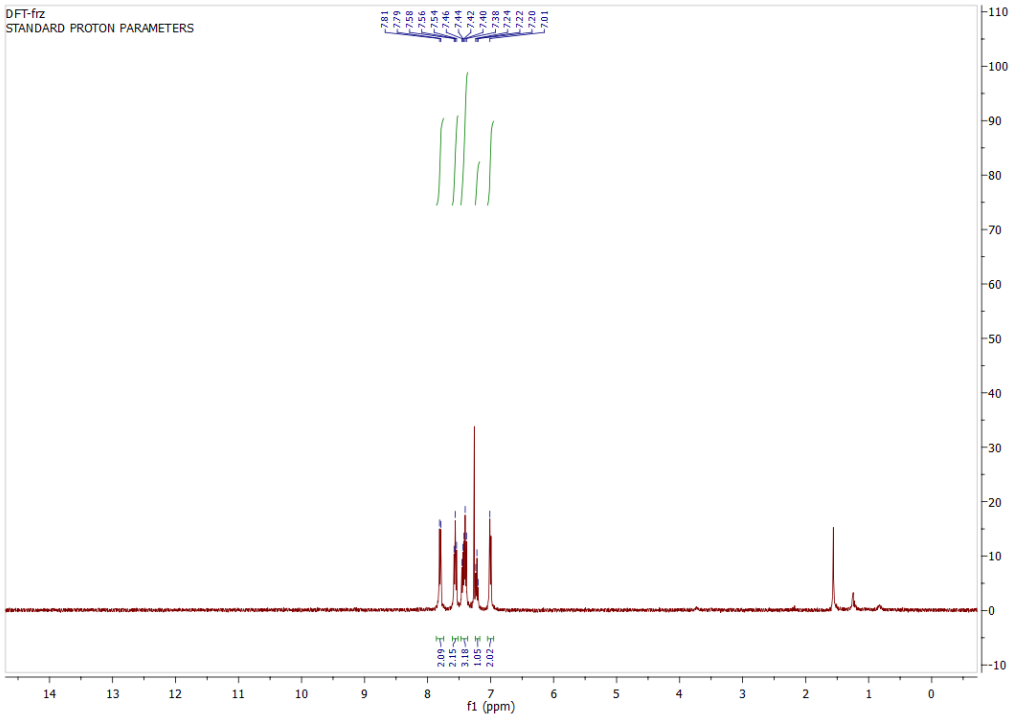


Figure 5. *^{1}H-NMR spectrum of DFTFrz in $CDCl_3$.*

^{1}H-NMR results of DFTD and DFTFrz are given in Figures 2 and 5 respectively. The structure of the new furazan was confirmed in ^{1}H-NMR by loss of oxime protons signals and presence of only phenyl protons at (7.01-7.81) ppm. The furazan ring system is an aromatic heterocycle. The field of this aromatic ring creates a deshielding effect for the protons on the phenyl skeletons. Comparing the chemical shifts of new established furazan ring with dioxime it is notable that all chemical shifts are shifted downfield. This also confirms that an aromatic furazan ring constructed to the structure. When the FT-IR spectra (Figure 3) of the compounds are examined; ν(O-H) vibration bands (3249-3503 cm^{-1}) of the dioxime disappeared with the formation of furazan. In addition, the strong (N-O) stretching vibration seen in the dioxime (967 cm^{-1}) disappeared with the formation of furazan. This was also supported by the presence of the characteristic molecular ion peak m/z = 295.09 $[M+1]^{+}$ at mass spectrum (Figure 6), supporting the proposed furazan formulation.

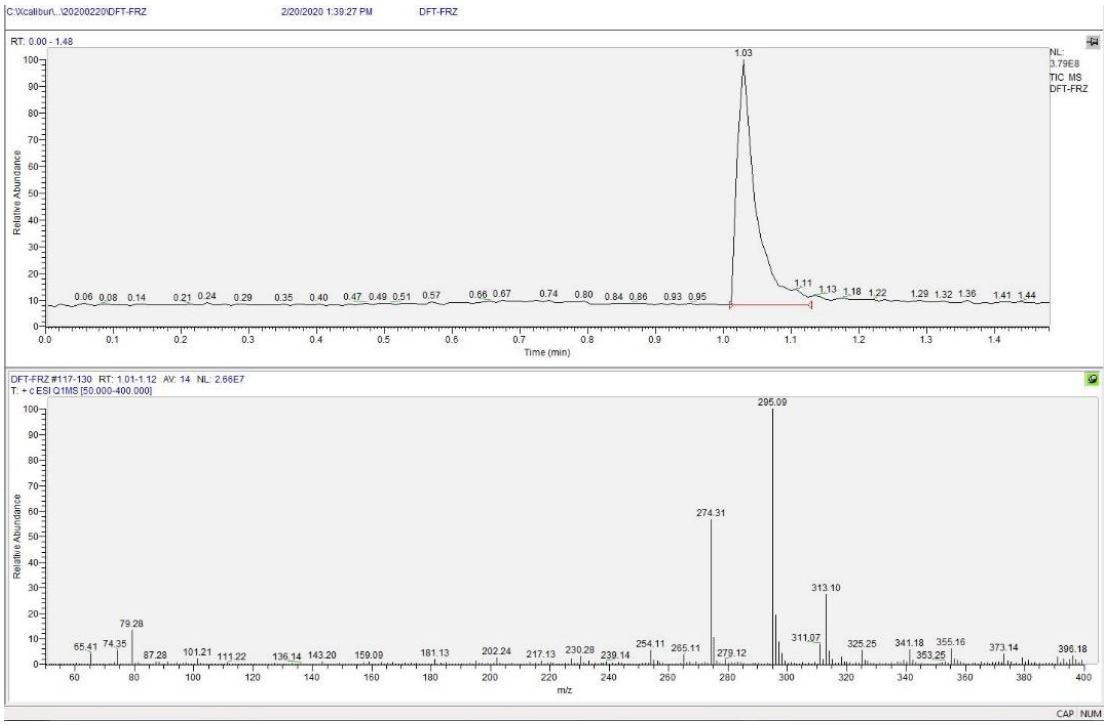


Figure 6. *LC-MS results of the DFTFrz compound.*

The biological activity of the obtained thiourea-furazan compound was demonstrated by antibacterial effect studies using disc diffusion method and broth microdilution method. For this purpose, six Gram positive strains and four Gram negative strains, total of ten microorganisms were studied.

The results obtained are given in Table 1 for disc diffusion assay and Table 2 for micro dilution assay. When the results are examined, it is seen that the substances are sensitive to gram positive bacteria especially *Staphylococcus aureus* than gram negative bacteria. The effect of DFTFrz on staphylococcal bacteria is remarkable with its 12-13 mm zone diameter and 32-64 μgmL^{-1} inhibitor concentration.We extended the study on *Staphylococcus aureus* ATCC 43300 and *Staphylococcus epidermidis* ATCC 35984, which are known to be resistant to methicillin and responsible of nosocomial infections [37],[38]. DFTFrz also showed noteworthy effect on these resistant bacteria with zone diameters of 8-13 mm and 128 μgmL^{-1} MIC values. DFTFrz has no effect on other gram-positive bacteria *Enterococcus faecalis* and *Bacillus cereus*. Furazan compound had slightly more effect on bacteria than its precursor dioxime (DFTD). We found that compounds did not show any antibacterial effect against the Gram-negative strains evaluated in Table 1-2. Many antibacterial drugs inhibit gram-negative bacteria less effectively than gram-positive bacteria, since the permeability barrier of the outer membrane allows only slow drug entry [39]. The cell wall composition of Gram-positive and Gram-negative differs considerably, as Gram-negative bacteria have two membranes, and also the lipopolysaccharide-coated outer membrane is very forcing for small molecules to cross [40].

Table 1. *Antibacterial activities of compounds according to disc diffusion method*[†]

Compounds	*S.a*	*S.a**	MRSA	*S.e*	*B.c*	*E.f*	*E.c*	*E.c**	*P.a*	*K.p*
DFTD	11	10	12	8	8	-	-	-	-	-
DFTFrz	13	12	13	8	-	-	-	-	-	-
CLR	27	23	-	-	25	23	11	7	14	21
DMSO	-	-	-	-	-	-	-	-	-	-

Table 2. *Antibacterial activities of the compounds by Microdilution BrothMethod (MIC, μg /mL)*

Compounds	*S. a*	*S.a**	MRSA	*S.e*	*B.c*	*E.f*	*E.c*	*E.c**	*P.a*	*K.p*
DFTD	256	256	256	128	512	512	512	512	512	512
DFTFrz	64	128	128	128	256	512	512	512	512	512
CLR	0,5	0,5	>128	>128	<0,25	1	32	64	64	64

S.a:*Staphylococcus aureus* ATCC 25923, ***S.a*:****Staphylococcus aureus* ATCC 29213, **MRSA**: Methicillin-*resistant Staphylococcus aureus* ATCC 43300, ***S.e:****Staphylococcus epidermidis* ATCC 35984, ***B.c:****Bacillus cereus* ATCC 11778, ***E.f:****Enterococcus faecalis* ATCC 29212, ***E.c:****Escherichia coli* ATCC 25922, ***E.c*:****Escherichia coli* JM109, ***P.a:****Pseudomonas aeruginosa* ATCC 27853, ***K.p:*** *Klebsiella pneumoniae* ATCC 700603,**CLR**:Clarithromycin, **DMSO**:Dimethyl sulfoxide.
- : No inhibition
†: The values indicate the diameters (mm) of the inhibition zones.

IV. CONCLUSION

In conclusion, we designed and synthesized new 1,2,5-oxadiazole (furazan) fused imidazole-2-thione heterocyclic group for the first time; this process involved the reaction of highly reactive cyanogen-di-N-oxide intermediate with N,N′-Diphenylthiourea. Formed dioxime; (4Z,5E)-4,5-bis(hydroxyimino)-*N,N*'-diphenylimidazoline-2-thione was converted to new furazan; "*N,N'*-diphenyl-4H-imidazo[4],[5-c]furazan-5(6H)-thione", via silica gel supported solvent-free microwave dehydration method. The structures were characterized and biological activity studies were performed. Furazan compound had slightly more effect on bacteria than its precursor dioxime. The effect of compounds on staphylococcal bacteria including methicillin resistant counterpart are remarkable. However, the compounds did not show any antibacterial effects on gram negative bacteria.

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