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Short communication

SYNTHESIS, ANTIMICROBIAL ACTIVITY AND INTERACTION WITH DNA OF SOME 2-SUBSTITUTED BENZIMIDAZOLE DERIVATIVES

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Abstract

In this study, synthesis and structural illumination of eight compounds that are expected to display antifungal and antibacterial activity in addition to interaction with DNA has been conducted. The syntheses were performed by heating o-phenylendiamine and derivatives with succinic acid and malonic acid in 4N HCl. It has been observed that, the reactions with succinic acid formed bisbenzimidazole derivatives. On the other hand, the reactions with the malonic acid under the same conditions resulted in formation of benzimidazole derivatives instead of bisbenzimidazole derivatives. The structures of all synthesized compounds were analysed with spectroscopic methods (UV, IR, ^{1}H NMR, MS). Among the synthesized compounds, ***1*** *and* ***5*** *which are nonsubstitue compounds, were selected so as to dispose the roles of electron acceptor or donor atoms in the activity and their interaction with DNA has been examined with respect to concentration and time. When these two compounds were compared, it's seen that* ***1*** *requires higher concentrations and longer time to be able to interact with DNA. In addition, all compounds have been examined for antibacterial activities against Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Enterococcus faecalis, and for antifungal activities against Candida albicans. It was found that* ***7*** *was the most effective compound against Escherichia coli, Pseudomonas aeruginosa and Staphylococcus aureus,* ***5*** *was the most effective compound against Enterococcus faecalis,* ***7*** *and* ***8*** *were effective equivalently against Candida albicans.*

Key Words: *Bisbenzimidazole, Benzimidazole, Antimicrobial activity, Electrochemical detection, The interaction with DNA*

Bazı 2-Sübstitüe Benzimidazol Türevlerinin Sentezi, Antimikrobiyal Aktivitesi ve DNA ile Etkileşimi

Bu çalışmada, DNA ile etkileşimin yanı sıra, antifungal ve antibakteriyel aktivite göstermeleri beklenen sekiz bileşiğin sentezi ve yapı aydınlatma çalışmaları yapılmıştır. Sentezler 1,2-fenilendiamin ve türevlerinin süksinik ve malonik asit ile 4N HCl'li ortamda ısıtılmasıyla gerçekleştirilmiştir. Süksünik asit üzerinden yürüyen reaksiyonların bisbenzimidazol türevlerini oluşturdukları görülmüştür. Ancak, aynı şartlarda malonik asit ile bisbenzimidazol türevlerinin yerine benzimidazol türevleri oluşmuştur. Sentezlenen bileşiklerin yapıları spektroskopik yöntemlerle (UV, IR, ^{1}H NMR, Kütle) aydınlatılmıştır. Sentezi gerçekleştirilen bileşiklerden, ***1*** *ve* ***5*** *benzimidazol çekirdeğinde elektron çeken ya da elektron veren atomların etkideki rolünün berteraf edilmesi amacı ile non sübstitüe bileşikler olarak seçilmiş ve DNA ile etkileşimleri konsantrasyon ve zamana karşı araştırılmıştır. Bu iki bileşik karşılaştırıldığında,* ***1****'in DNA ile etkileşebilmesi için daha yüksek konsantrasyonlara ve daha uzun süreye ihtiyacı olduğu görülmüştür. Ayrıca tüm bileşiklerin Esherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Enterococcus faecalis'e karşı antibakteriyel aktiviteleri ve Candida albicans'a karşı antifungal aktiviteleri araştırılmıştır. 7'nin Esherichia coli, Pseudomonas aeruginosa ve Staphylococcus aureus üzerine,* ***5****'in Enterococcus faecalis üzerine en etkili bileşikler olduğu,* ***7*** *ve* ***8****'in ise Candida albicans üzerine eşit oranda etkili oldukları bulunmuştur.*

Anahtar Kelimeler: *Bisbenzimidazol, Benzimidazol, Antimikrobiyal aktivite, Elektrokimyasal tarama, DNA ile etkileşim*

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INTRODUCTION

Benzimidazoles are the key component of many bio-active compounds including natural and synthetic source. The diverse substitutions of compounds which contain benzimidazole ring, cause different pharmacologic activities such as antihelmintic *(1, 2)*, antifungal *(3-5)*, antitumor *(6)*, mammalian DNA topoizomeraz I inhibitor *(7)*, antiviral *(3, 4)*, H_2 receptor blocker and proton pump inhibitor *(8)*, HIV-1 integrase inhibitor *(9)*, angiotensin II receptor antagonist *(10)* activities. Besides, studies about activities upon rhinovirus of the compounds which contain bisbenzimidazole structure have drawn attention *(2)*.

The study of the interactions of some anticancer agents with DNA has been employed by a variety of techniques *(11, 12)* and there is a growing interest in the electrochemical methods *(13-17)*. Electrochemical DNA hybridization biosensors are mostly employed for determining early and precise diagnoses of infectious agents in various environments *(18)* and these devices can be used for monitoring sequence-specific hybridization events directly *(19)* or by DNA intercalators (metal coordination complexes, organic dyes, etc.) *(20-22)*. These studies showed that electrochemical techniques offer a very attractive route for converting the hybridization event into an electrochemical signal.

Phillips' method for the preparation of simple benzimidazoles is well known *(23)*. The same procedure can be used to prepare bisbenzimidazoles by refluxing two moles of diamine with one mole of a dibasic acid in 4 N hydrochloric acid. The aim of our study is to synthesize monobenzimidazoles which carry 1*H*-benzimidazole nucleus and bisbenzimidazole compounds and clarifying their structures and examining their antimicrobial activities together with the interaction with DNA.

EXPERIMENTAL

Chemistry

Melting points were determined with a Buchi 510 capillary melting point apparatus and not corrected. UV spectra were taken on Shimadzu UV-160 spectrometer in methanol solution. The IR spectra of compounds were monitored in potassium bromide pellets on a Jasco FT/IR-430 spectrometer. The NMR spectra were recorded on a Varian AS 400 Mercury Plus. NMR spectra were recorded in the CD_3OD and DMSO-d_6. All chemical shifts were recorded as δ (ppm). *J* values, given in Hz. Mass spectra (LC/MS) were performed on a Waters 2695 Alliance Micromass ZQ LC/MS at Ankara University Faculty of Pharmacy, Ankara, Turkey. Analytical thin-layer chromatography (TLC) was run on Merck silica gel plates (Kieselgel $60F_{254}$) with detection by UV light. All starting materials and reagents were highgrade commercial products purchased from Aldrich, Fluka and Merck.

Synthesis of Bis-1H-benzimidazole Compounds ***(1- 3)***

0.1 mole of 4 and/or 5-substituted-1,2-phenylenediamines and 0,05 mole of succinic acid were heated in 4N 120 ml HCl at 135 ^{0}C oil bath for 4 hours. The product was recrystallized from ethanol.

Because of all derivatives elucidated by preparative TLC (solvent system; C_6H_6: CH_3OH: 25 % NH_4OH 9:1:0.01) (except **1** and **3**), their yields could not shown in this text.

2,2'-(1,2-Ethanediyl)bis[1H-benzimidazole] ***(1)***

Recrystallized from ethanol (yield 69 %, m.p. 300 °C decomp.), [315 °C decomp.] *(24)*. ^{1}H NMR (CD_3OD), *δ* 3.93 (s, 4H, -CH_2CH_2-); 7.62 (dd, 4H, J_m= 3.2, J_o= 6.4 Hz, H-5, H-5', H-6, H-6'); 7.81 (dd, 4H, J_m= 3.2, J_o=6.4 Hz, H-4, H-4', H-7, H-7')ppm. UV λ_{maks} (log ε) 278 (3.13), 258

(2.78), 244 (2.94), 228 (2.73) nm. FT-IR (KBr), cm^{-1}: 3440 (N-H), 1623-1569 (C=N, C=C). ES+ MS: *(m/z)* 132 [Benzimidazole-CH_2+H]$^+$, 263 [M+H]$^+$.

2,2'-(1,2-Ethanediyl)bis[5-chloro-1H-benzimidazole] ***(2)***

Recrystallized from ethanol (m.p. 281 °C), [280 °C] *(25)*. ^{1}H NMR ($CDCl_3$) δ 3.62 (s, 4H, -CH_2CH_2-); 7.38 (dd, 2H, J_m = 2.0, J_o= 9.2 Hz, H-6, 6'); 7.59 (d, 2H, J_o = 8.8, H-7, H-7'); 7.65(d, 2H, J_O = 2.0, H-4, H-4')ppm. UV λ_{maks} (log ε) 290 (3.03), 264 (2.47), 248 (2.90), 232 (2.70), 210 (3.82) nm. FT-IR (KBr), cm^{-1}: 3444 (N-H), 1627-1573 (C=N, C=C). ES+ MS: *(m/z)* 168 [Cl-Benzimidazole-CH_2+H]$^+$, 333 [M+H]$^+$.

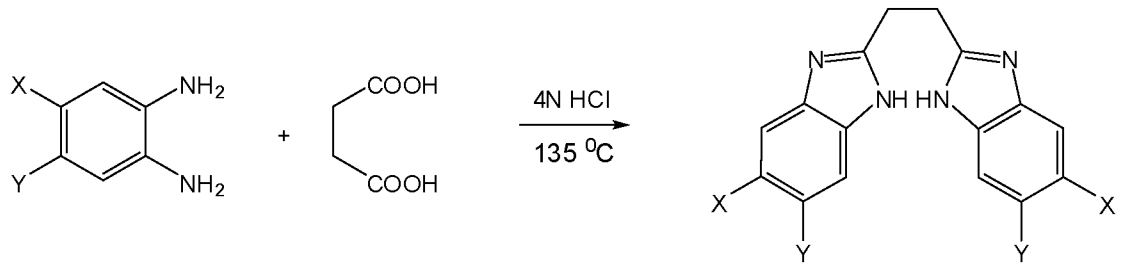
2,2'-(1,2-Ethanediyl)bis [5,6-dimethyl-1H-benzimidazole] ***(3)***

Recrystallized from ethanol (yield 19 %, m.p. 221 °C), [245 °C] *(25)*. ^{1}H NMR ($CDCl_3$) δ 2.43 (s, 12H, H-5 CH_3, H-6 -CH_3, H-5'-CH_3, H-6'-CH_3); 3.85 (s, 4H, -CH_2CH_2-); 7.54 (s, 4H, H-4, H4', H-7, H-7'). UV λ_{maks} (log ε) 288 (3.36), 260 (3.15) nm. FT-IR (KBr), cm^{-1}: 3363 (N-H), 1627-1565 (C=N, C=C). ES+ MS: *(m/z)* 160 [$(CH_3)_2$-Benzimidazole-CH_2+H]$^+$, 319 [M+H]$^+$.

2,2'-(1,2-Ethanediyl)bis[5-nitro-1H-benzimidazole] ***(4)***

0.1 mole of 4-nitrobenzene-1,2-diamine and 0.05 mole of succinic acid were heated in 50 ml of 4 N HCl at 100 ^{0}C oil bath for 48 hours. The crude product was recrystallized from ethanol.

Recrystallized from ethanol (m.p. 288 °C), [286-8 °C] *(26)*. ^{1}H NMR ($CDCl_3$) δ 3.62 (s, 4H, -CH_2CH_2-); 7.67 (s, 2H, H-6, H-6'); 8.07 (d, 2H, J_o =7.6 Hz, H-7, H-7'); 8.39(s, 2H, H-4, H-4'). UV (Because of the solubility problem UV spectrum could not taken.) FT-IR (KBr), cm^{-1}: 3583 (N-H), 1627-1515 (C=N, C=C), 1465 (-NO_2). ES+ MS: *(m/z)*177 [NO_2-Benzimidazole-CH_2+H]$^+$, 353 [M+H]$^+$.



Comp.	1	2	3	4
X	H	-Cl	-CH_3	-NO_2
Y	H	H	-CH_3	H

Scheme 1. Synthetic route of compounds **(1-4)**

Synthesis of 2-methylsubstituted-1H-Benzimidazole Compounds ***(5-8)***

0.1 mole of 1,2-phenylenediamine and 0.05 mole of malonic acid were stirred in 120 ml of 4 N HCl at 135 ^{0}C oil bath for 4 hours. The *pH* of the mixture was maintained as 5.7 with 4 N KOH. The crude product was recrystallized from ethylacetate/water. The structural confirmation of 2-methyl-1*H*-benzimidazole was achieved by interpretation of spectral data. Then, by using

substituted-1,2-phenylenediamines; 5-nitro, 5-chloro and 5,6-dimethyl derivatives of 2-methyl-1*H*-benzimidazole were synthesized.

2-Methyl-1H-benzoimidazole ***(5)***

Recrystallized from ethylacetate/water (m.p. 166 °C), [168-70 °C] *(27)*. ^{1}H NMR (CD_3OD) δ 2.54 (s, 3H, 2-CH_3); 7.16 (dd, 2H, J_m = 3.2, J_o =6.2 Hz, H-5, H-6); 7.45 (dd, 2H, J_m =3.2, J_o =6.2 Hz, H-4, H-7). UV λ_{maks} (log ε) 274 (2.75), 260 (2.5), 244 (2.76), 220 (2.42) nm. FT-IR (KBr), cm^{-1}: 3405 (N-H), 1623-1550 (C=N, C=C). ES+ MS: *(m/z)* 133 $[M+H]^+$.

2-Methyl-5-nitro-1H-benzoimidazole ***(6)***

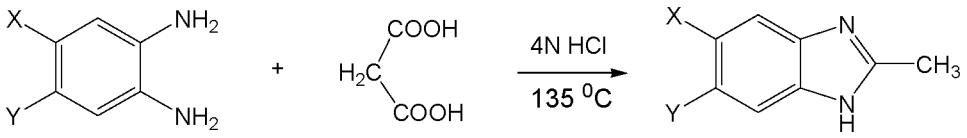
Recrystallized from ethylacetate/water (m.p. 215 °C), [221 °C] *(28)*. ^{1}H NMR (CD_3OD) δ 2.61 (s, 3H, 2-CH_3); 7.56 (d, 1H, J_o =8.4 Hz, H-6); 8.11 (dd, 1H, J_p =0.8, J_o =8.6 Hz, H-7); 8.36 (d, 1H, J_m =1.6 Hz, H-4). UV λ_{maks} (log ε) 308 (2.98), 262 (2.56), 236 (3.27), 212 (2.75) nm. FT-IR (KBr), cm^{-1}: 3563 (N-H), 1627-1519 (C=N, C=C), 1465 (-NO_2). ES+ MS: *(m/z)* 178 $[M+H]^+$.

5-Chloro-2-methyl-1H-benzoimidazole ***(7)***

Recrystallized from ethylacetate/water (m.p. 200 °C), [203 °C] *(29)*. ^{1}H NMR (CD_3OD) δ 2.54 (s, 3H, 2-CH_3); 7.41 (d, 1H, J_o =8.4 Hz, H-7); 7.15 (dd, 1H, J_m =2.0, J_o =8.6 Hz, H-6); 7.45 (1H, d, J_m =2.0 Hz H-4). UV λ_{maks} (log ε) 282 (2.77), 264 (1.92), 248 (2.67), 226 (1.52), 208 (3.66) nm. FT-IR (KBr), cm^{-1}: 1619-1542 (C=N, C=C). ES+ MS: *(m/z)* 167 $[M+H]^+$.

2,5,6-Trimethyl-1H-benzoimidazole ***(8)***

Recrystallized from ethylacetate/water (m.p. 221 °C,), [229-31 °C] *(30)*. ^{1}H NMR (CD_3OD) δ 2.31 (s, 6H, 5-CH_3, 6-CH_3); 2.49 (s, 3H, 2-CH_3); 7.21 (s, 2H, H-4, H-7). UV λ_{maks} (log ε) 290 (2.49), 264 (2.04), 208 (3.39) nm, FT-IR (KBr), cm^{-1}: 3359 (N-H), 1627-1519 (C=N, C=C). ES+ MS: *(m/z)* 161 $[M+H]^+$.



Comp.	5	6	7	8
X	H	-NO_2	-Cl	-CH_3
Y	H	H	H	-CH_3

Scheme 2. Synthetic route of compounds **(5-8)**

Antimicrobial activity

The substrate Fish-sperm double-stranded DNA which is used in biological activity studies is provided from Sigma firm, Mueller-Hinton Broth medium and ethanol, methanol and DMSO which are used as solvent are provided from Merck firm, and streptomycine which is used for the control of antimicrobial activity is provided from Ibrahim Ethem firm.

In order to determine the antimicrobial activities of the compounds, the National Committee for Clinical Laboratory Standard (NCCLS) microdilution method was used *(31)*. The minimum inhibitor concentrations (MIC) of compounds on the reference microorganisms were designated. In the experiment as a growth medium "Mueller-Hilton Broth" for bacteria's and Sabouraud dextrose broth for *Candida albicans* were used. 20 mg of compound was weighed and got decomposed in 250 µl methanol and completed to 1 ml with water to prepare stock solutions. Microdilution plates with 96 wells were used for the test. 50µl broth medium were dispensed to sterile micro plates. 50 µl compound solutions were added to first wells and then by the two folded serial dilution method of the compounds, their solutions were prepared in the concentrations which vary from 5000 µg/ml to 10 µg/ml. The last well (12) in the micro plate was defined sterility control, and the second last well (11) was defined as growth control. The bacteria suspensions were prepared as to allow the final concentrations of the micro-organisms to be 5 x 10^5 colony/ml according to NCCLS criteria. 50 µl bacteria suspension was inoculated wells to all wells except 12th well. Plates were incubated at 35 ^{0}C for 24 hours. At the end of this time the lowest concentration that inhibits growth of organism was accepted as MIC. The accuracy of the method was tested with streptomycin *(32)*. Streptomycin concentrations varied from 1000 µg/ml to 2 µg/ml by two-folded serial dilution method.

Interaction with DNA studies

The measurements in interaction with DNA study were made by using potansiostat AUTOLAB 30 (Eco-Chemie, Holland) and GPES 4.8 software package. Acetate buffer which has 4.80 *pH* is used as buffer solution. Sterilized water and ultra-pure water was used in all experiments. The experiments were made at room temperature (25 °C).

After the substance-DNA interaction, electro-chemical assignation can be effectuated by moving from the obtained substance signal or the signal of a base in the DNA.

For the purpose of determining the interaction with DNA, a pencil graphite electrode (PGE) was used in the concept of our study *(33)*. The interaction of the compounds which were synthesized in this part of the study, with DNA was examined by using the differential pulse voltammeter technique *(15, 16)*. By taking advantage of guanine oxidation signal in the potential of +1.0V, measurements were taken according to the signal changes which come out in the DNA interact with the compounds.

In this study the triple electrode system including reference electrode (Ag^+/AgCl), working electrode PGE and auxiliary electrode (Pt wire) was used. Interactions were inspected by two different methods as interaction at the electrode surface and in the solution phase. For the interactions at the electrode surface; firstly surface of PGE was activated by applying +1.40V for 60 seconds in buffer solution. Afterwards, activeted PGE plunged into 10 µg/ml dsDNA solution that prepared in the 0.50 M acetat buffer. DNA was attached at electrode surface by applying +0.50V for 300 seconds in mixing place. The electrode was washed with acetate buffer. Then, the electrode which has a DNA attached surface is interacted with the substance in a specific concentration for 300 seconds in mixing place without applying voltage. It was re-washed with acetate buffer. In measurement stage, the electrode which was taken into the buffer solution again was scanned in a specific potential range according to the procedure of inverter system. The interaction was sighted according to the alternating signals.

For the interaction in the solution phase; PGE was activated by the same method that used in interaction at the electrode surface. Afterwards dsDNA and substance solutions that prepared in 0.50 M (*pH*= 4.80) acetat buffer in specific concentrations interacted for 300 seconds in mixing place without applying voltage. Interacted dsDNA-substance solution attached to activeted PGE by applying +0.50 V for 300 seconds and measurment were taken by the same method.

After the DNA substance interaction, optimum substance concentration assignment and optimum time assignment was effectuated.

RESULTS AND DISCUSSION

The bisbenzimidazole derivatives were synthesized by the reaction of appropriate 4 and/or 5-substituted-1,2-phenylenediamine derivatives with succinic acid in the presence of HCl. Analytical data for structure elucidation were given in Experimental Part. The preparation of resulting bisbenzimidazole derivatives **(1-4)** were outlined in Scheme 1. Additionally, the benzimidazole derivatives were synthesized by the reaction of appropriate substituted-1,2-phenylenediamine derivatives with malonic acid in the presence of HCl **(5-8)** (Scheme 2). Although dicarboxilyc acid derivative was used in the synthesis of 2-methylsubstituted-1*H*-benzimidazole compounds, result of the instrumental analysis showed that 2-alkyl derivatives formed instead of expected bis-derivatives *(34)*. This showed that 2-alkyl substituted derivatives can also be synthesized by an other method *(5)*.

In this study, antibacterial activities of all compounds against *Escherichia coli*, *Pseudomonas Aeruginosa*, *Staphylococcus Aureus*, *E. feacalis* and antifungal activities against *C. albicans* together with the interaction study of **1** and **5** with DNA are tested.

In the interaction of **1** and **5** with DNA, it's controlled whether DNA gives the signal in the same place with guanine base by supervising the oxidation signals of the compounds primarily. It was found that our compounds did not give any signal in same place with guanine and designated that the compounds were appropriate for the study. Afterwards, the optimum medicine concentration was determined by studying compounds with two methods (1. Interaction in solution phase, 2. Interaction at DNA modified electrode). Also optimum substance interaction was defined for interaction at DNA modified electrode method.

In the first of these methods, the optimum concentration was found 10 μg/ml for **1** and 5 μg/ml for **5** (Figure 1, 4).

In the second method, the interaction was applied on the surface of the electrode. It was seen that the optimum concentration was 50 μg/ml for **1** and 25 μg/ml for **5** (Figure 2, 5).

In case of DNA molecules and substance molecules take place in the solution, substance and DNA molecules can be interacted easier because the interaction will be formed in a wider area. But in the interaction at the electrode surface; because of the interaction formed only on the surface of the electrode, the electrode surface formed a narrower area and substance-DNA interaction was formed more difficultly. As a result, more substances were needed for the interaction. This case displays the optimum concentration discrepancies between the two methods.

In interaction at modified electrode method, a study for determining the optimum interaction time between DNA and substance was also made. An interaction at electrode surface was effectuated in this study. The optimum time was found 5 minutes for **1** and 3 minutes for **5** with this method (Figure 3, 6).

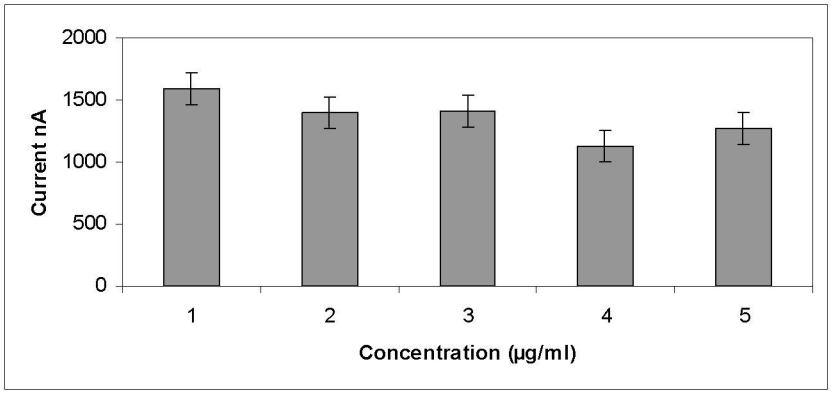


Figure 1. Histomograms for the magnitude of guanine oxidation signals before interaction with **1** (1); after interaction with ethanol (2); after interaction with **1**: 5 ppm (3); 10 ppm (4); 15 ppm (5) at solution phase.

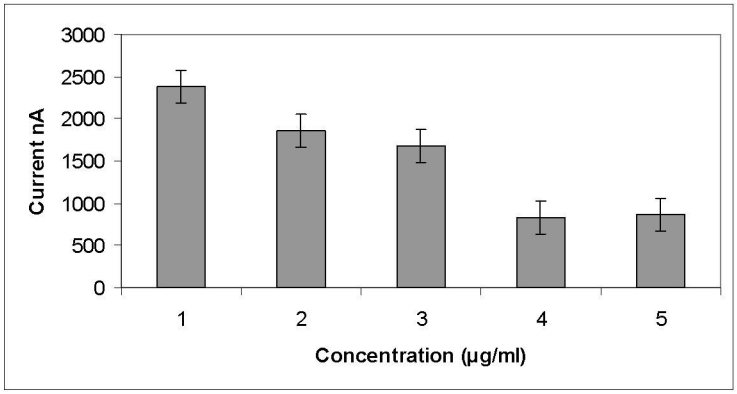


Figure 2. Histomograms for the magnitude of guanine oxidation signals before interaction with **1** (1); after interaction with ethanol (2); after interaction with **1**: 5 ppm (3); 10 ppm (4); 15 ppm (5) at surface of dsDNA modified PGE.

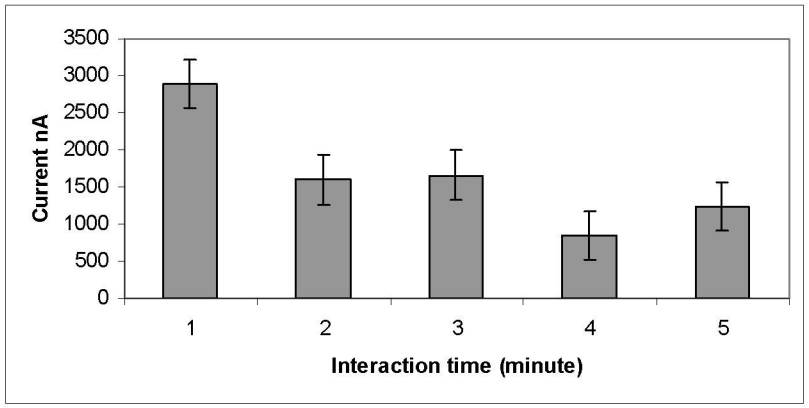


Figure 3. The effect of different interaction times, such as 0 min. (1); 1 min. (2); 3 min. (3); 5 min. (4); 7 min. (5) for interaction of **1** with DNA.

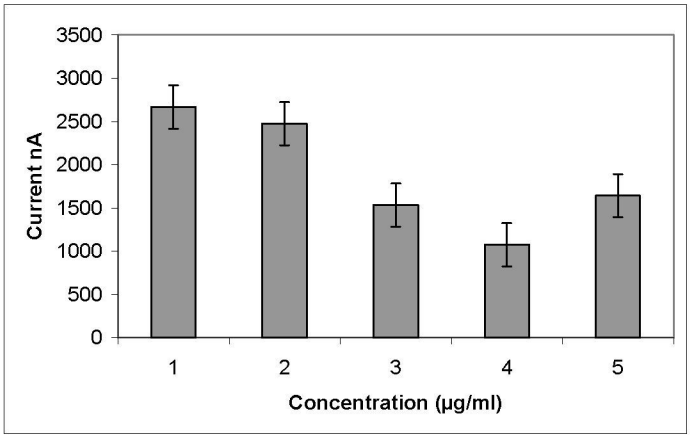


Figure 4. Histomograms for the magnitude of guanine oxidation signals before interaction with **5** (1); after interaction with ethanol (2); after interaction with **5**: 5 ppm (3); 10 ppm (4); 15 ppm (5) at solution phase.

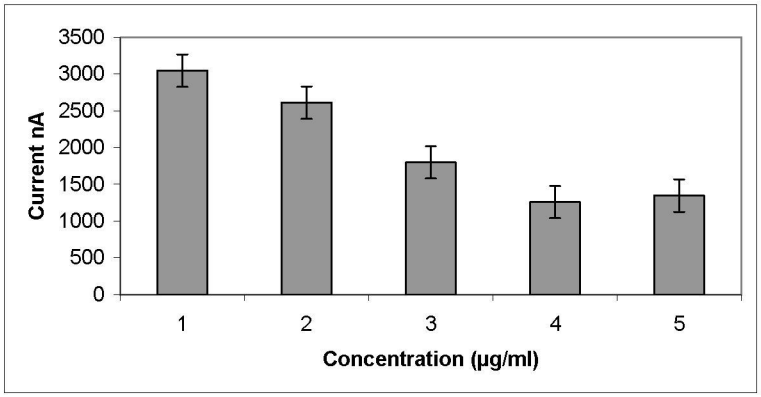


Figure 5. Histomograms for the magnitude of guanine oxidation signals before interaction with **5** (1); after interaction with ethanol (2); after interaction with **5**: 5 ppm (3); 10 ppm (4); 15 ppm (5) at surface of dsDNA modified PGE.

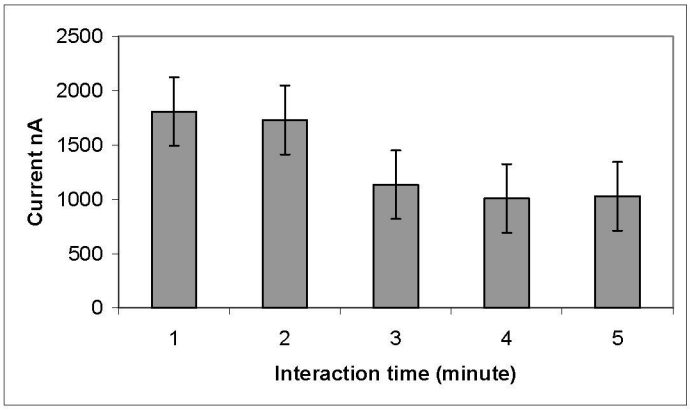


Figure 6. The effect of different interaction times, such as 0 min. (1); 1 min. (2); 3 min. (3); 5 min. (4); 7 min. (5) for interaction of **5** with DNA.

When **1** and **5** were compared, it was found that **1** requires higher concentrations and longer time to be able to interact with DNA. This result leads us to think that it's difficult and less for **1** to hang to and interact with DNA because of being a compound with a bigger volume.

The antimicrobial activities of all synthesized compounds were studied and minimum inhibitor concentrations (MIC) were determined. Streptomycin was used as control drug and tested under the same conditions with the microorganisms in the experiment. The MIC values of the compounds are given in Table 1.

Comparison of antibacterial activities of the compounds showed that 2-methylsubstituted-1*H*-benzimidazole series are more active than bis-1*H*-benzimidazole series. According to the MIC values of the compounds, **7** was the most effective compound against *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, **5** was the most effective compound against *Enterococcus faecalis* and **7** and **8** were effective equally against *Candida albicans*. Executing antimicrobial activity studies on the 2-methylsubstituted-1*H*-benzimidazole and bis-1*H*-benzimidazole derivatives showed that, electron donor or acceptor groups on the aromatic ring increased biological effect intensity.

To determine the antimicrobial activity of the compounds antibacterial and antifungal studies were executed. The microorganisms ATCC (The American Type Culture Collection) used for antimicrobial activity determination were reference origins and their interactions with standard compounds are known *(31)*. In this literature, MIC values change between 0.002-128 μg/ml according to type of strain and antibiotic. Also, MIC values of antifungal activity are between 0.25-2.0 μg/ml *(35)*.

In conclusion, MIC values for antibacterial activity of the synthesized compounds are between 312->5000 μg/ml and MIC values for antifungal activity are between 39.1->5000 μg/ml (Table 1). However, these values don't match with the values given in the literature.

Table 1. Minimum Inhibitor Concentrations of The Compounds (μg/ml)

Compound	1	2	3	4	5	6	7	8	Streptomicin
E. coli	5000	>5000	>5000	2500	2500	2500	312.5	1250	≤ 2
P.aeruginosa	5000	>5000	>5000	5000	2500	5000	625	5000	≤ 2
S. aureus	5000	>5000	>5000	1250	625	1250	312.5	1250	≤ 2
E. feacalis	5000	>5000	>5000	2500	312.5	2500	625	1250	≤ 2
C. albicans	5000	>5000	>5000	312.5	312.5	312.5	39.1	39.1	-

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