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Synthesis of Bis-Nicotinonitrile Derivatives and Investigations of Antimicrobial and Antibiofilm Activities

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In this study aimed to the synthesis of novel bis-nicotinonitrile derivatives starting from 1,3-bis-chalcones and investigate their antimicrobial activity potentials. Bis-nicotinonitrile derivatives were synthesized from 1,3-bis-chalcone derivatives reacted with malononitrile in ethanol in the presence of potassium carbonate. Characterization of the obtained compounds was carried out by spectroscopic methods. In total, 12 new 1,3-phenylene-bis-nicotinonitrile derivatives were synthesized. Finally, the antimicrobial and antibiofilm activities of synthesized 1,3-phenylene-bis-nicotinonitrile derivatives were tested against 5 microorganisms. As a result, it was observed that the synthesized new nicotinonitrile derivatives were quite active especially in terms of antibiofilm activity.

Introduction

A biofilm is a complex microbial habitat created by microorganisms adhering to any place. Microorganisms are contained in extra-polysaccharides (EPS), an extracellular substance they secrete. Thus, they can be protected from the physical and chemical negative effects of the environment.[1] This situation especially causes the bacteria contained in the biofilm to develop resistance to antibiotics. As a result, the treatment of infectious diseases becomes more difficult.[2] Resistance to antimicrobial substances is one of the most important public health problems of last years. Biofilms are one of the most important causes of antimicrobial resistance.[3,4] Currently, there is no antibiotic that can only destroy biofilms. Therefore, there is a need for new antimicrobials with antibiofilm activities.[5]

Nicotinonitrile derivatives are known as compounds with bioactive potential and they are also called 3-cyanopyridines.[6] It has been reported in the literature that some of these compounds have antimicrobial, enzyme inhibitor and anticancer properties.[7] Nicotinonitrile derivatives are occupy an important place among heterocyclic compounds due to their potential antitumor activity and other important pharmacological benefits.[8] These compounds are known to have a broad spectrum of biological activity such as analgesic, antipyretic, anti-inflammatory,[6] potential anti-HIV inhibitor, anti-parkinsonism,[9] antifungal,[10] anti-hypertensive[11] and antiviral.[12] In addition, nicotinonitrile derivatives show fluorescent properties because they consist a conjugated π-bond system. For this reason, these compounds are among the important materials with application in electrochemical and optical studies.[13]

Synthesis of nicotinonitrile derivatives are among the interesting and current topics in terms of their pharmacological activities and their use as intermediates in the synthesis of new and polyfunctional compounds.[14] There are different methods for the synthesis of nicotinonitrile derivatives in the literature, but one of the most common methods is the reactions of α,β-unsaturated carbonyl compounds with malononitrile. These reactions are usually catalyzed by amines, as well as sodium hydroxide and alkoxides.[14–16] Chalcones are very useful starting compounds that used in the synthesis of many heterocyclic compounds. They are also widely used in the synthesis of cyanopyridines.[17,18] Studies of the synthesis of the cyanopyridines, especially over the chalcone derivatives have a large place in the literature.[19–22] Recently, various studies on the synthesis of heterocyclic compounds from bis-chalcones have been reported,[23–26] but there are rare record of the synthesis of nicotinonitrile derivatives from bis-chalcones.[27]

In our previous work, we reported the investigation of antimicrobial and antibiofilm activities by performing the synthesis and characterization of 1,3-bis-chalcones (**1a–h**, **2a–d**).[28] In this study, which is a continuation of our previous study, we carried out the synthesis and characterization of new bis-nicotinonitrile derivatives (**4a–h**, **5a–d**) from a series of 1,3-bis-chalcone derivatives and the investigation of antimicrobial and antibiofilm properties on five microorganisms.

Results and Discussion

A total of 12 new bis-nicotinonitrile derivatives (**4a–h**, **5a–d**) were synthesized from the reaction of 1,3-bischalcones with malononitrile in the presence of K_2CO_3 in yields varying between 51–75% (Table 1). The synthesized compounds were characterized with spectral methods and investigated of their antibacterial and antibiofilm activities against two Gram-positive, two Gram-negative bacteria and one fungus (*Bacillus subtilis* ATCC 6633, *Streptococcus pyogenes* ATCC 19615, *Pseudo-*

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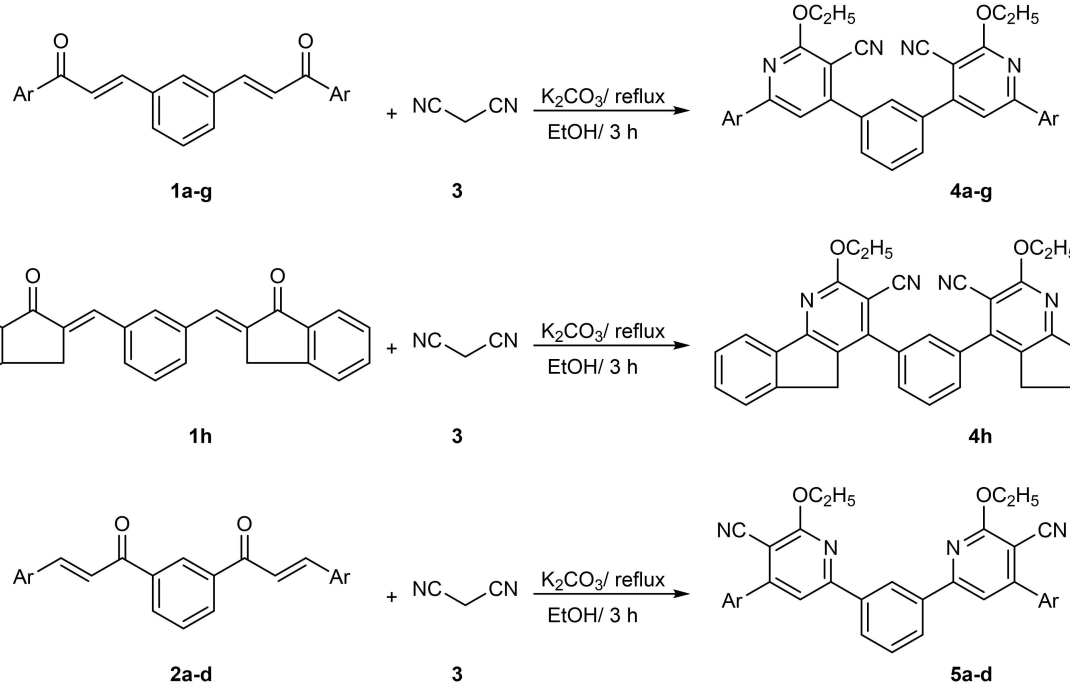
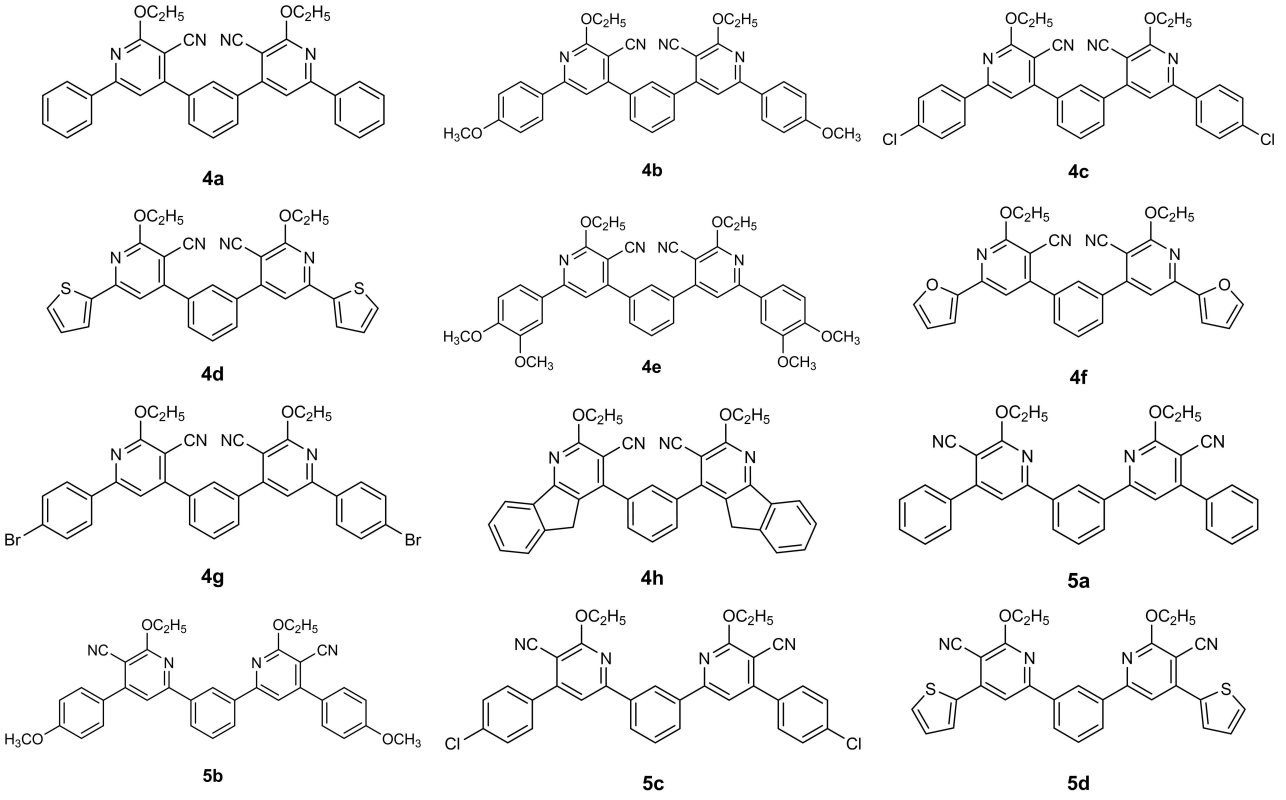
Table 1. Synthesis of bis-nicotinonitrile derivatives (**4a–h**, **5a–d**).



Ar = **a** Ph; **b** 4-OCH_3Ph; **c** 4-ClPh; **d** 2-thiophenyl; **e** 3,4-OCH_3Ph; **f** 2-furyl; **g** 4-BrPh



monas aeruginosa ATCC 27853, *Shigella boydii* ATCC 9905 and *Candida albicans* ATCC 10231)[29–31] by MIC method at concentrations varying between 800 and 12.5 μg/mL of the compounds. In the tests, while DMSO was used as negative control, piperacillin/tazobactam (8:1) and fluconazole were used as positive controls.

Table 2 shows the results of the antimicrobial activity tests of the synthesized bis-nicotinonitrile derivatives (**4a–h**, **5a–d**). As can be seen from the Table 2, **4f**, **4d** and **4h** compounds were the most active compounds against *B. subtilis* microorganism with a MIC value of 100 μg/mL and showed the same level of activity as the positive control. The compounds exhibiting the lowest level of activity are compounds **5b** with a value of 800 μg/mL. Other compounds have an average activity of 200–400 μg/mL. Compounds **4c**, **4e**, **4h**, **5a**, **5c** and **5d** have a very high activity against *S. pyogenes* at the same level (50 μg/mL) as the positive control. Positive control showed activity at a concentration of 50 μg/mL, compound **4d** showed activity at a concentration of 100 μg/mL, and other compounds at a concentration of 200 μg/mL against *P. aeruginosa*. Table 2 shows that bis-nicotinonitrile derivatives have very low activity concentrations when the activities of the positive controls used are taken into account against *S. boydii* and *C. albicans* bacterial strains.

In terms of antimicrobial activity values, when compared the 1,3-biscalcones (**1a–h**, **2a–d**), we synthesized in our previous study,[28] with the bis-nicotinonitrile derivatives (**4a–h**, **5a–d**), we obtained in our current study, it is seen that the results are in harmony with each other. Because the starting compounds **1e**, **1d** and **1h** showed the same activity against the *B. subtilis* as the positive control, while the **2c** and **2d** compounds showed the same activity against the *S. pyogenes* as the positive control. All 1,3-biscalcones (**1a–h**, **2a–d**), like bis-nicotinonitrile derivatives (**4a–h**, **5a–d**), showed an average activity against *P. aeruginosa* and showed low activity levels against *S. boydii* and *C. albicans* bacterial strains.[28]

Table 3 shows the results of the antibiofilm activity tests of the bis-nicotinonitrile derivatives (**4a–h**, **5a–d**). All compounds were found to have antibiofilm activities. The new compounds reduced *B. subtilis* biofilms between 83.7% and 97.5% and similarly reduced *S. pyogenes* biofilms by up to 94.2%. The bis-nicotinonitrile derivatives (**4a–h**, **5a–d**) have an antibiofilm activity between 15.6% and 96.2% against *S. boydii* strains. Moreover, other than **4a**, **4h** and **5a** significantly destroyed the biofilms of *P. aeruginosa*, which are highly resistant bacteria. The compounds **4a**, **4b**, **4h** and **5b** did not effect of *C. albicans* yeast biofilms, while others reduced biofilms by 91.5%.

When the antibiofilm activities of 1,3-bis chalcones (**1a–h**, **2a–d**) are compared with bis-nicotinonitrile derivatives (**4a–h**, **5a–d**), it is seen that 1,3-bis chalcones are generally more active. Although the newly synthesized compounds **4a**, **4h** and **5a** did not have any activity against *P. aeruginosa* and similarly compounds **4a**, **4b**, **4h** and **5b** did not have any activity against *C. albicans*, 1,3-bis chalcones (**1a**, **1b**, **1h**, **2a** and **2b**) had a certain level of antibiofilm activity.[28]

Conclusions

In conclusion, in this study, a series of novel bis-nicotinonitrile derivatives (**4a–h**, **5a–d**) (Table 1) were synthesized in high yields (51–75%) and tested for antimicrobial and antibiofilm activities. It has been observed that the bis-nicotinonitrile derivatives have remarkable effects, especially in terms of antibiofilm activity (Tables 2 and 3). Therefore, these compounds could be useful in developing and synthesizing new antimicrobials. For this purpose, further *in vitro* and *in vivo* studies should be performed.

Table 2. Minimum inhibition concentrations (MIC) of synthesized nicotinonitrile derivatives (μg/mL).

Entry	Compounds	Microorganism				
		B.subtilis	*S.pyogenes*	*P.aeruginosa*	*S.boydii*	*C.albicans*
1	**4a**	400	200	200	200	800
2	**4b**	400	200	200	400	800
3	**4c**	200	50	200	200	100
4	**4d**	100	100	100	800	200
5	**4e**	100	50	200	200	200
6	**4f**	100	100	200	200	400
7	**4g**	200	200	200	200	200
8	**4h**	100	50	200	400	400
9	**5a**	200	50	200	200	100
10	**5b**	800	800	200	400	800
11	**5c**	200	50	200	400	400
12	**5d**	400	50	200	400	400
	P/T[a]	100	50	50	50	–
	Flu[b]	–	–	–	–	12.5

[a] Piperacillin/Tazobactam (8:1)
[b] Fluconazole

Table 3. Reduction in biofilm formation on MIC[a] value of synthesized nicotinonitrile derivatives (%), (± SD).[b]

Entry	Compounds	Microorganism				
		B.subtilis	*S.pyogenes*	*P.aeruginosa*	*S.boydii*	*C.albicans*
1	**4a**	94.3 ± 3.5	94.2 ± 0.2	–	46.6 ± 3.7	–
2	**4b**	95.6 ± 0.1	94.2 ± 0.2	22.0 ± 3.0	49.6 ± 1.5	–
3	**4c**	92.5 ± 0.3	50.3 ± 0.5	63.6 ± 3.2	83.7 ± 0.5	76.8 ± 4.9
4	**4d**	83.7 ± 0.5	31.0 ± 1.7	64.7 ± 1.2	96.2 ± 0.9	89.6 ± 0.2
5	**4e**	94.0 ± 1.7	87.4 ± 0.2	76.6 ± 2.8	90.6 ± 1.2	85.7 ± 0.8
6	**4f**	89.6 ± 0.2	85.3 ± 4.6	37 ± 1.7	88.3 ± 0.7	90.3 ± 0.5
7	**4g**	93.8 ± 0.4	90.6 ± 1.2	61.6 ± 2.8	89.4 ± 0.1	86.0 ± 0.3
8	**4h**	94.3 ± 0.5	89.4 ± 0.1	–	15.6 ± 1.5	–
9	**5a**	93.8 ± 0.4	91.0 ± 0.0	–	75.6 ± 0.4	20.6 ± 0.5
10	**5b**	97.5 ± 0.5	89.0 ± 0.1	20.0 ± 2.0	46.3 ± 3.7	–
11	**5c**	93.6 ± 1.5	89.5 ± 0.5	60.8 ± 0.8	90.0 ± 3.0	91.5 ± 0.5
12	**5d**	94.6 ± 0.5	56.0 ± 1.7	55.1 ± 0.3	93.5 ± 1.5	90.6 ± 1.2

[a] Minimum inhibitory concentration
[b] Data are reported as the mean ± standard deviation of three experiments.

Experimental

General information

Melting points were measured on an Electrothermal 9100 apparatus (Bibby Scientific, Staffordshire, UK). Infrared (IR) spectra (KBr disc) were recorded on a Jasco FT/IR-430 Spectrometer (JASCO, Tokyo, Japan). Elemental analyses were performed using a LECO CHNS 932 elemental analyzer (Michigan, ABD). ^{1}H- and ^{13}C-NMR spectra were recorded on an Agilent 600 MHz Premium COMPACT NMR instrument (Agilent, Santa Clara, CA). The multiplicities of the signals in the ^{1}HNMR spectra are abbreviated by s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), br (broad), and combinations thereof.

General procedure for the synthesis of 4a–h and 5a–d

The synthesis of 1,3-Biscalcone derivatives was given in our previous study.[28] To a solution of 1,3-bischalcone derivative (**1a–h**, **2a–d**) (2 mmol) and malononitrile (**3**) (4 mmol) in EtOH (50 mL) was added K_2CO_3 (8 mmol) and the mixture was refluxed for 3 hours (Table 1). At the end of the reaction, the mixture was taken into a separatory funnel and extracted with chloroform. After drying over Na_2SO_4 and removal of the solvent, the crude product was separated from a silica gel column with a mixture of chloroform/hexane (3:1) was purified and crystallized with ethanol.[22]

Determination of antimicrobial and antibiofilm activity

All biological activity tests were performed as reported in our previous paper.[28]

Antimicrobial activity, determination of MIC values

The broth micro-dilution method which was implemented to assess the antimicrobial activities of the bis-nicotinonitrile derivatives was done in line with the suggestions of the NCCLS.[32]

A micro-well dilution method was taken as a basis for determining the minimum inhibitory concentration (MIC) of the compounds against bacterial strains (*Bacillus subtilis* ATCC 6633, *Streptococcus pyogenes* ATCC 19615, *Pseudomonas aeruginosa* ATCC 27853, *Shigella boydii* ATCC 9905 and *Candida albicans* ATCC 10231). The broth cultures were used to prepare the inoculums of the bacteria, and suspensions were adjusted to standart turbity of 0.5 McFarland. The compounds were dissolved in nutrient broth (NB) containing 10% (v/v) DMSO, and serial two-fold dilutions were prepared in 96-well plates with NB at a concentrations varying between 800–12.5 μg/mL. Wells containing only NB medium with inoculum, and piperacillin/tazobactam (8:1), fluconazole served as positive controls. A microplate reader (Thermo Scientific Microplate Photometer, Multiskan FC, USA) was used to measure the absorbance of the plates at 570 nm after incubation for 24 h at 37 °C. The optical density at the 24th h of the inoculum remains the same or decrease at the MIC which was detected as the lowest concentration of the compounds, when compared to the reading at the beginning.

Determination of antibiofilm activity

In order to determine the anti-biofilm activity of bis-nicotinonitriles against *Bacillus subtilis* ATCC 6633, *Streptococcus pyogenes* ATCC 19615, *Pseudomonas aeruginosa* ATCC 27853, *Shigella boydii* ATCC 9905 and *Candida albicans* ATCC 10231. The microtiter plate method was used. This method was carried out with some modifications as described by Adukwu et al.[33] Overnight cultures of microorganisms in the TSB (Tryptic Soy Broth) medium with the containing of 2% (w/v) glucose were prepared as 10^8 CFU/mL and then dispensed 100 μL into with per each test well. Then 100 μL of different concentrations of compounds (800–12.5 μg/mL) were dispensed into each well. The negative control contained only TSB whereas the positive control contained cell cultures without compound addition. The supernatant were decanted and each well gently rinsed three times with 300 μL of sterile distilled water and discarded after incubation at 37 °C for 48 h. The plates were dried with air during 40 min. They were stained with 0.1% (w/v) crystal violet at room temperature for 30 min, and washed 3 times with sterile distilled water. Afterwards the crystal violet was solubilized in 95% ethanol and absorbance was read in a microplate reader (Thermo Scientific Microplate Photometer, Multiskan FC, USA) at 570 nm. This test was performed in triplicate, and the mean was

taken as an average of three readings. The percentage of biofilm inhibition was calculated according to the following formula:[34]

$$\text{Biofilm eradication } (\%) = \{(\text{OD positive control} - \text{OD sample})/\text{OD positive control}\} \times 100$$

Statistical analysis

All experiments were done in triplicate. Data were expressed in the form of arithmetic mean ± standard deviation (x ± SD). OneWay analysis of variance (ANOVA) and post-hoc Tukey analyses were used to reveal the relationships between groups. The differences were accepted as significant for $p < 0.05$.

Supporting Information Summary

Full spectral data and the NMR spectra of the compounds, are given in the Supporting Information.

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

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