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Article

New bis(thieno[2,3-*b*]pyridine) hybrids linked to arene units as potential bacterial biofilm and MRSA inhibitors

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Abstract

Using sonication and mediated by piperazine, new arene-linked bis(thieno[2,3-*b*]pyridine) hybrids were efficiently prepared in this study. The target hybrids were prepared by reacting bis(α-haloketone) with two equivalents of the appropriate pyridine-2(1H)-thiones in the presence of 1.4 equivalents of piperazine. The reaction mixture was subjected to sonication at 60 °C for 30–40 min to produce the desired products in 88%–95% yields. When tested against six different ATCC bacterial strains, the new products demonstrated a wide range of antibacterial activity. The 4-(4-methoxyphenyl)-linked hybrids **1i** and **1j**, attached to 6-(4-methoxyphenyl) and 6-(*p*-tolyl) units, respectively, had the best efficacy against *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, and *Pseudomonas aeruginosa* strains. Both hybrids showed more effective potency than the reference ciprofloxacin with MIC and MBC values in the ranges from 2.0 to 2.1, and 4.1 to 4.2 μM, respectively. Additionally, hybrids **1i** and **1j** demonstrated stronger efficacy than linezolid with MIC values ranging from 2.0 to 4.2 μM, and MBC values ranging from 8.2 to 8.5 μM, respectively, against the MRSA ATCC:33 591 and ATCC:43 300 strains. Furthermore, hybrids **1i** and **1j** showed inhibitory antibacterial biofilm activity comparable to the standard ciprofloxacin. They had IC_{50} values ranging from 3.8 to 4.6 μM against *S. aureus*, *E. faecalis*, *E. coli*, and *P. aeruginosa* strains. The cytotoxicity of hybrids **1i** and **1j** against the human breast epithelial cell line MCF-10A lends credence to both hybrids' potential as safe antibacterial agents.

Key words: bacterial biofilm inhibitors, MRSA inhibitors, piperazine-mediated reactions, thieno[2,3-*b*]pyridine, sonication-mediated reactions

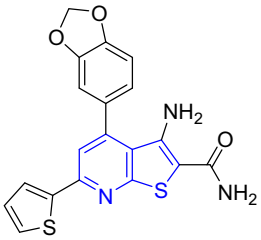
1. Introduction

Infectious diseases caused by bacteria and fungi have recently risen.[1] Despite many significant advances in antimicrobial therapy, widespread use and misuse of antibiotics has resulted in the emergence of antibiotic resistance, posing a serious threat to public health.[2] The emergence of multi-drug resistant gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA), has become a significant issue in the treatment of bacterial diseases.[3,4] As a result, one of the most important areas of antibacterial research today is the development of new compounds to combat resistant bacteria.[5,6] In the same context, biofilms are surface-attached microbial communities that differ from free swimming, and planktonic counterparts in architecture, phenotypic, and biochemical properties.[7,8] In ordinary or hospital community settings, the biofilm causes severe human infections (up to 60%). One of the most well-known of these biofilm-specific properties is the development of antibiotic resistance, which can be 1000-fold greater than in planktonic cells.[9–11]

Thieno[2,3-*b*]pyridine hybrids have fascinating antimicrobial activity, including good inhibitory activity against bacterial biofilm and MRSA strains.[12–15] Incorporating aryl units to the thieno[2,3-*b*]pyridine skeleton at C4 and C6 results in the formation of arene-linked hybrids with promising antimicrobial activity, as demonstrated in numerous publications (see Fig. 1).[14,16,17] Additionally, the previous hybrids demonstrated interesting biological applications, including antiviral,[18,19] anti-inflammatory,[20,21] and antidiabetic activity.[22] They also act as effective inhibitors of DNA gyrase,[23] COX-2,[24] and AChE enzymes.[25–27] The previous hybrids are usually prepared starting from the corresponding 2-thioxo-1,2-dihydropyridine-3-carbonitriles and α-haloketones. Inorganic bases such as sodium methoxide[28,29] and ethoxide,[30] as well as potassium hydroxide,[31,32] and carbonate[33] were used to mediate the previous synthesis.

Several publications have documented piperazine's ability to mediate various chemical transformations,[34–36] including Thrope–Ziegler reaction.[37] Our research group reported the synthesis of promising bacterial biofilm and MRSA inhibitors, particularly thieno[2,3-*b*]pyridine hybrids.[38–44] In this context, we investigated in this study to prepare efficiently new arene-linked bis(thieno[2,3-*b*]pyridines). The new hybrids were investigated as potential bacterial biofilm and MRSA inhibitors.

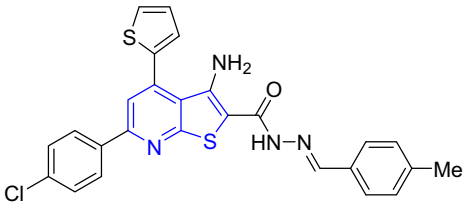
Fig. 1. Structure of some thieno[2,3-*b*]pyridines I–IV with promising antimicrobial activity.



I

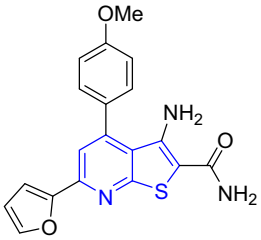
MIC values up to 4.8 μM against *S. aureus, S. mutans* and *E. coli* strains

Anti-biofilm activity against *S. aureus*, *S. mutans,* and *E. coli* strains with IC_{50} values up to 10.0 μM



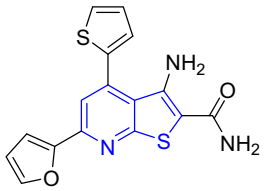
II

MIC values up to 12.3 μM against *S. aureus, S. mutans* and *E. coli* strains



III

MIC values up to 42.8 μM against *S. aureus, P. aeruginosa* and *E. coli* strains



IV

MIC values up to 45.8 μM against *S. aureus, P. aeruginosa* and *E. coli* strains

2. Experimental

2.1. Materials

All solvents were acquired from commercial sources and used as received unless otherwise stated. All other chemicals were acquired from Merck or Aldrich and used without further purification. The melting points were measured on a Stuart melting point apparatus and are uncorrected. IR spectra were recorded on a Smart iTR, which is an ultra-high performance, versatile Attenuated Total Reflectance sampling accessory on the Nicolet iS10 FT-IR spectrometer. NMR spectra were recorded on Bruker Avance III 400 MHz spectrophotometer (400 MHz for ^{1}H-NMR and 100 MHz for ^{13}C-NMR) using TMS as an internal standard and DMSO-d_6 as solvent and chemical shifts were expressed as δ ppm units. Elemental analyses were carried out on a EuroVector instrument C, H, N, S analyzer EA3000 Series. For all characterization data and general procedures, see Electronic supplementary file.

2.2. General procedure for the synthesis of bis(thieno[2,3-*b*]pyridine) hybrids 1

2.2.1. Conventional procedure for the synthesis of 1a

A mixture of pyridine-2(1H)-thione **2a** (10 mmol) and bis(α-haloketone) **3** (5 mmol) in ethanol (15 mL) in the presence of piperazine (7 mmol) was stirred at 80 °C for 150 min. The reaction was continued until the starting materials **2a** or **3** were no longer detectable by TLC. The product was collected by filtration, washed with water then with ethanol, dried and then recrystallized from dioxane/ethanol mixture in 64% yield.

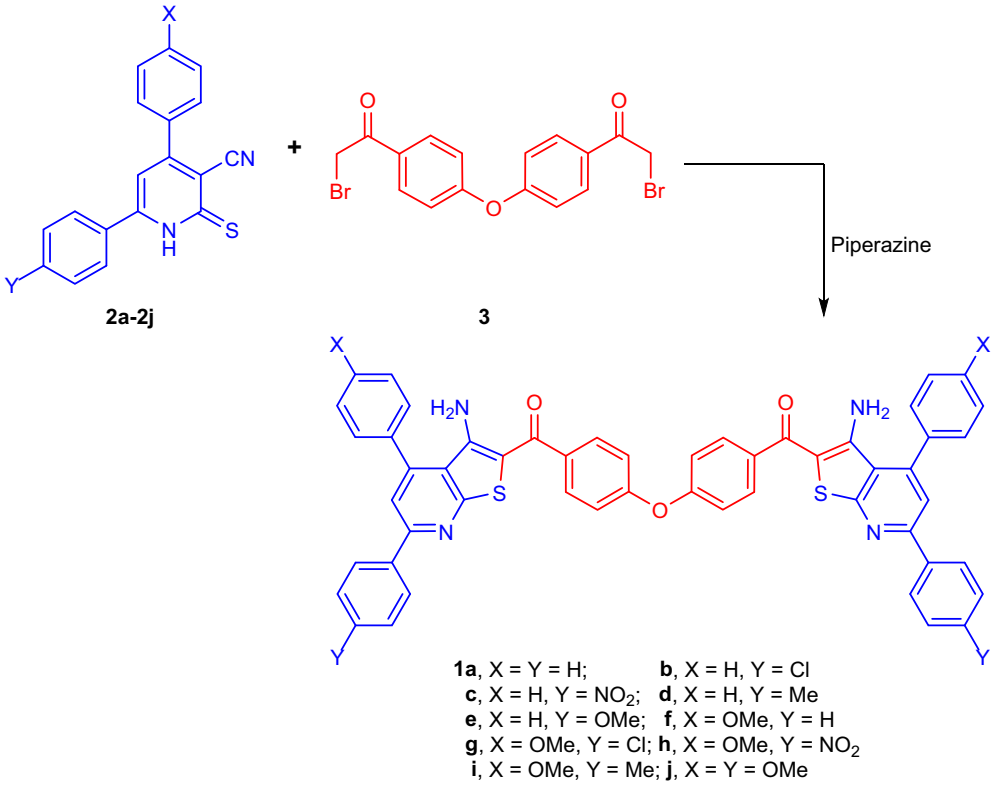
2.2.2. Sonication-mediated procedure

A mixture of pyridine-2(1H)-thiones **2a–2j** (10 mmol) and bis(α-haloketone) **3** (5 mmol) in ethanol (15 mL) in the presence of piperazine (7 mmol) was subjected to sonication at 60 °C for 30–40 min. The flask of the reaction mixture was put in the middle of sonicator bath to achieve effective cavitation. The sonochemical reaction was continued until the starting materials **2** or **3** were no longer detectable by TLC. The product was collected by filtration, washed with water then with ethanol, dried and then recrystallized from the appropriate solvent.

2.2.3. (Oxybis(4,1-phenylene))bis((3-amino-4,6-diphenylthieno[2,3-*b*]pyridin-2-yl)methanone) (1a)

Yellow solid (dioxane/ethanol mixture); m.p. 270-273 °C; IR (υ cm^{-1}): 3421, 3244 (NH_2); ^{1}H-NMR (DMSO-d_6): δ 7.09 (d, J = 8.8 Hz, 4 H, ArH), 7.25 (br s, 4 H, 2 NH_2), 7.46 (t, J = 7.6 Hz, 2 H, ArH), 7.50–7.55 (m, 6 H, ArH), 7.59 (t, J = 7.6 Hz, 4 H, ArH), 7.81 (s, 2 H, 2 pyridine-H), 7.88 (d, J = 8.8 Hz, 4 H, ArH), 7.94 (d, J = 7.6 Hz, 4 H, ArH), 8.24 (d, J = 7.6 Hz, 4 H, ArH); ^{13}C-NMR (DMSO-d_6): δ 116.2, 118.7, 119.4, 120.6, 127.7, 128.1, 128.6, 129.2, 129.6, 129.8, 130.0, 134.2, 136.2, 136.5, 149.4, 154.0,

Scheme 1. General synthesis of the target arene-linked bis(thieno[2,3-*b*]pyridine) hybrids 1.



158.5, 159.4, 161.8, 189.4; Anal. for $C_{52}H_{34}N_4O_3S_2$ (826.9) Calcd: C, 75.52%; H, 4.14%; N, 6.77%; found: C, 75.35%; H, 3.98%; N, 6.86%.

2.3. In vitro antibacterial screening

2.3.1. Determination of MIC values

MIC values were determined against all tested ATCC strains according to the previously published procedure by Sanad et al.[45] (for a detailed assay, see Electronic supplementary file). The inhibitory activity was determined using the microbroth serial dilution method and ciprofloxacin (100 g susceptibility disc) or Linezolid (30 μg susceptibility disc) as reference drugs.[46] The concentrations of the tested hybrids and ciprofloxacin used in the study ranged from 250 to 0.9 μg/mL. The assay was carried out in triplicate to ensure consistency, in accordance with CLSI guidelines (2012).[47]

2.3.2. Determination of MBC values

MBC values[48] were determined against all tested ATCC strains according to the previously published procedure by Sanad et al.[39] (for a detailed assay, see Electronic supplementary file). The assay was carried out with concentrations of the tested derivatives or the reference ciprofloxacin ranging from 250 to 0.9 μg/mL. All of the results were obtained in duplicate, and the average values were calculated.

2.3.3. Biofilm inhibition assay

The bacterial biofilm inhibitory activity[49] were determined against four different ATCC strains according to the previously published procedure by Sanad et al.[39] (for a detailed assay, see Electronic supplementary file). The assay was carried out with concentrations of the tested derivatives or the reference ciprofloxacin ranging from 250 to 0 μg/mL. All of the results were obtained in triplicates, and the IC_{50} values were indicated as mean ± SD.

2.4. Neutral red uptake assay

The cytotoxicity of hybrids **1i**, and **1j** as well as the reference doxorubicin was screened using the neutral red uptake assay.[50] The cytotoxicity was determined against the human breast epithelial cell line MCF-10 A according to the previously published procedure by Sanad et al.[16] (for a detailed assay, see Electronic supplementary file). The assay was carried out with eight concentrations (2.5, 5, 10, 15, 25, 50, 75 and 100 μg/mL) of the tested hybrids or doxorubicin.

3. Results and discussion

3.1. Chemistry

The goal of this study was to efficiently produce new arene-linked bis(thieno[2,3-*b*]pyridine) hybrids **1**. Pyridine-2(1H)-thiones **2**[51,52] were prepared for this purpose and used as key synthons to efficiently prepare the target hybrids **1**. The general strategy was to react two equivalents of the

Scheme 2. Reaction of pyridine-2(1H)-thione 2a and bis(α-haloketone) 3.

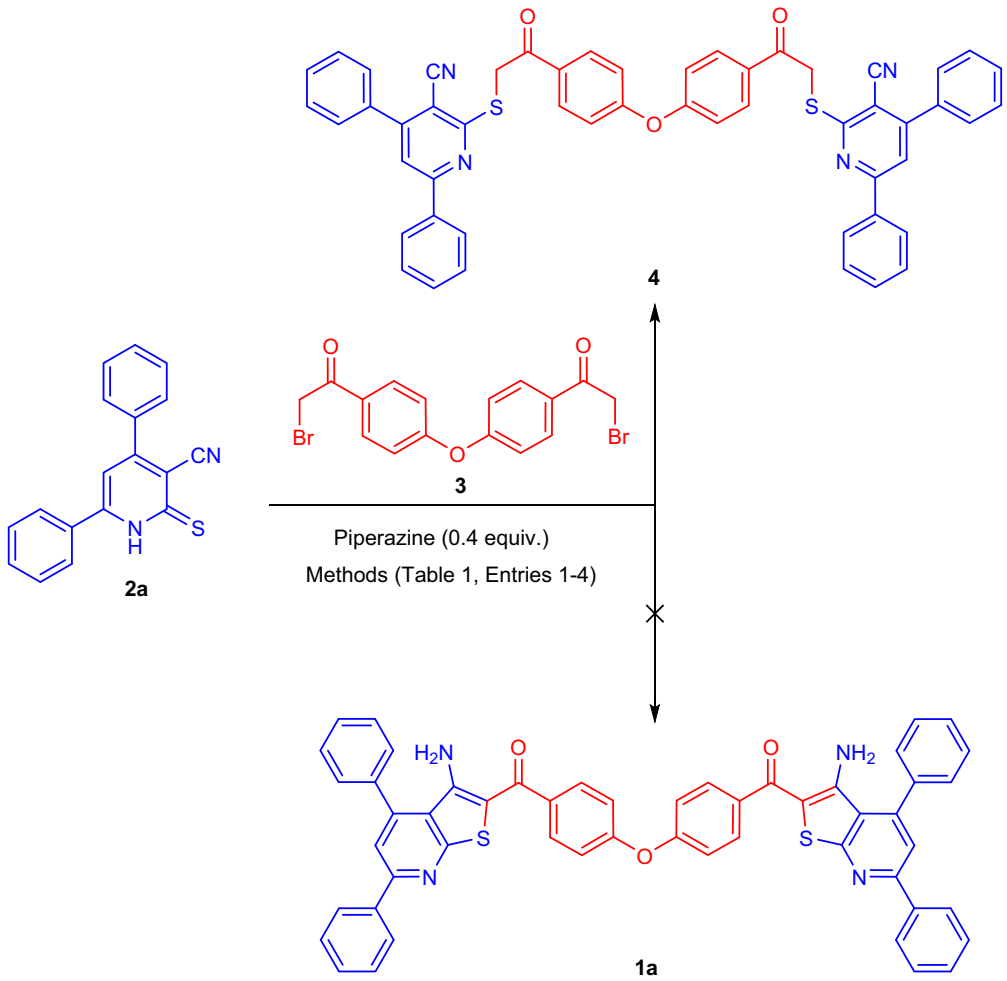


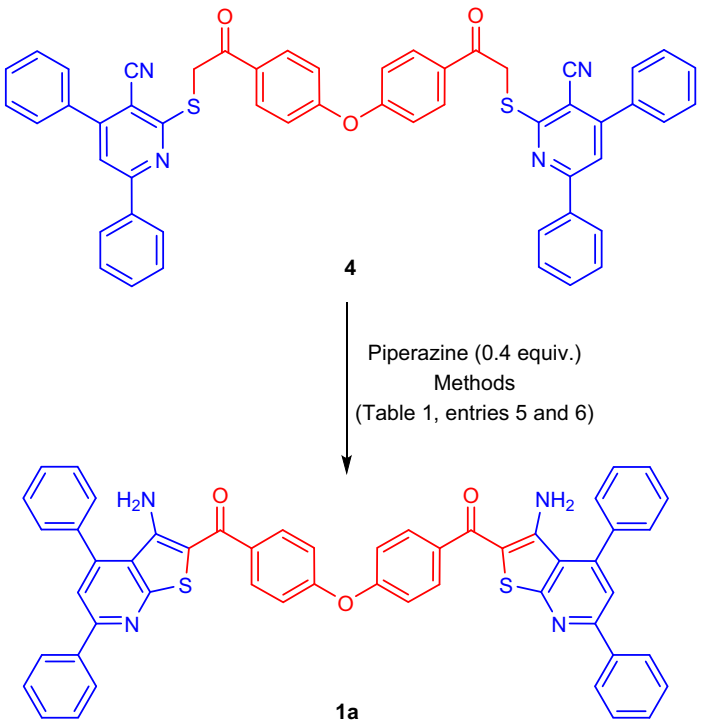
Table 1. The reaction conditions for the synthesis of 1a/4 in ethanol containing 0.4 equivalent of piperazine, either under conventional stirring or sonication-mediated procedures.

Entry	Reactants	Procedure	Temp. (°C)	Time (min)	Yield (%)	
					1a	4
1	2a + 3	Conventional stirring	rt	240	None	36
2	2a + 3	Conventional stirring	80	240	None	39
3	2a + 3	Sonication	rt	60	None	45
4	2a + 3	Sonication	60	60	None	50
5	4	Conventional stirring	80	120	76	—
6	4	Sonication	60	5	86	—

respective pyridine-2(1H)-thiones **2** with bis(α-haloketone) **3**[53] (see Scheme 1). Piperazine, a low-hazard organic base, was used to mediate the synthesis of the desired bis(thieno[2,3-*b*]pyridine) hybrids. To achieve the highest yield of the target products, both conventional heating and sonication were examined.

The synthesis of **1a** was taken as an example to optimize the reaction conditions. To begin, two equivalents of pyridine-2(1H)-thione **2a** and one equivalent of bis(α-haloketone) **3** were separately reacted in ethanol. The reaction was mediated using 0.4 equivalent of piperazine at different reaction temperatures with conventional stirring or sonication (see Scheme 2).[54] All reactions were monitored using TLC analyses Interestingly, bis(nicotinonitrile) **4** was obtained in 36%–50% instead of bis(thieno[2,3-*b*]pyridine) **1a** (see Table 1, entries 1–4). The elemental analysis and spectral data of **4** were used to confirm its structure. The IR spectrum of **4** revealed the absorption bands at 2216 and 1690 cm^{-1} owing to nitrile and carbonyl functions. The ^{1}H-NMR spectrum of **4** showed the presence of a singlet signal at δ 4.97 due to SCH_2 protons (see the Experimental section).

Scheme 3. Conversion of bis(nicotinonitrile) 4 into bis(thieno[2,3-*b*]pyridine) 1a.



Furthermore, the isolated bis(nicotinonitrile) **4** was reacted with 0.4 equivalent piperazine in ethanol using either conventional stirring at 80 °C or sonication at 60 °C (see Scheme 3). The reaction produced **1a** in 76% and 86% yields under both conditions (see Table 1, entries 5 and 6).[43] The IR spectrum of **1a** showed the absorption bands at 3421, and 3244 cm^{-1} corresponding to the amino functions. Additionally, the ^{1}H-NMR spectrum of **1a** revealed a broad singlet signal at δ 7.25 due to the amino protons (see the Experimental section).

The previous findings prompted us to investigate further the reaction conditions for the synthesis of bis(thieno[2,3-*b*]pyridine) **1a** and bis(nicotinonitrile) **4**. Therefore, the reaction of two equivalents of pyridine-2(1H)-thione **2a** and one equivalent of bis(α-haloketone) **3** in the presence of 0.6 equivalent of piperazine was repeated to determine the optimal conditions. The model reaction was investigated in a variety of solvents, including toluene, ethanol, and dioxane separately, with either conventional stirring or sonication at room temperature (rt) (see Table 2, Entries 1–6). It was found that instead of bis(thienopyridine) **1a**, bis(nicotinonitrile) **4** was formed as a sole product in 16%–56% yields, and that using sonication results in a higher yield with a faster reaction. It was also found that ethanol is the best solvent for this reaction when subjected to sonication. The model reaction was tested with various amounts of piperazine in ethanol. The results show that in the absence of piperazine, neither conventional stirring nor sonication produces any product (see Table 2, Entries 7 and 11). Under conventional stirring or sonication, increasing the amount of piperazine to 1.0 equivalent results in yields of 84% and 93%, respectively (see Table 2, Entries 9 and 13). More piperazine (1.2 equivalents) had no discernible effect on the reaction yield (see Table 2, Entries 10 and 14).

The effect of temperature was also studied in the abovementioned model reaction in ethanol with varying amounts of piperazine. Under standard heating or sonication conditions, 1.0 equivalent piperazine produced the sole product bis(nicotinonitrile) **4** (see Table 3, entries 1, 2, and 7). In all cases, 1.2 equivalents of piperazine were found to catalyze the Thrope–Ziegler reaction, resulting in the desired bis(thieno[2,3-*b*]pyridine) **1a** accompanied by **4** (see Table 3, entries 3, 4, and 8). Furthermore, the use of 1.4 equivalents of piperazine resulted in the sole product being target **1a** (see Table 3, entries 5, 6, 9, and 10). The best conditions for preparing **1a** were found to be conventional stirring in ethanol containing 1.4 equivalents of piperazine at 80 °C for 150 min or sonication at 60 °C for 30 min. The reaction yields 64% and 92% under conventional stirring and sonication, respectively (see Table 3, entries 6 and 9).

Motivated by the aforementioned findings, the optimal sonication-mediated procedure was used to prepare a new series of arene-linked bis(thieno[2,3-*b*]pyridines) **1b-1j**. As a result, in ethanol containing 1.4 equivalents of piperazine, a mixture of two equivalents of pyridine-2(1H)-thiones **2b-2j** was reacted with one equivalent of bis(α-haloketone) **3**. For 30-40 min, the mixture was stirred at 60 °C while being subjected to sonication. The desired products were obtained in yields ranging from 88%–95% (see Scheme 4 and Experimental section).

3.2. Biology

3.2.1. In vitro antibacterial screening

3.2.1.1. Evaluation of MIC and MBC values against standard susceptible ATCC bacterial strains

The new arene-linked bis(thieno[2,3-*b*]pyridines) **1** were screened in vitro against each of *S. aureus* (ATCC:6538), *Streptococcus mutans* (ATCC:25 175), *Enterococcus faecalis* (ATCC:29 212), *Escherichia coli* (ATCC:9637), *Pseudomonas aeruginosa* (ATCC:27 953), and *Klebsiella pneumonia* (ATCC:10 031) bacterial strains. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values against the selected strains were assessed using ciprofloxacin as a standard drug (MIC/MBC values of 2.9/5.9 μM) (Table 4).[55–48] The hybrids **1i** and **1j** showed the best antibacterial activity against all strains tested. They had more effective efficacy than the reference ciprofloxacin against the *S. aureus*, *E. faecalis*, *E. coli*, and *P. aeruginosa* strains with MIC or MBC values in the range from 2.0 to 4.2 μM. Also, they had lower potency against the *S. mutans*, and *K. pneumonia* strains with MIC values ranging from 8.2–8.5 μM, and MBC values ranging from 16.4 to 17.0 μM. Furthermore, hybrids **1d**, **1e**, and **1f** showed lower antibacterial activity with MIC or MBC values in the range from 4.4 to 17.6 μM against the *S. aureus*, *E. faecalis*, *E. faecalis*, and *P. aeruginosa* strains. Additionally, the previous hybrids had MIC values in the range from 17.6 to 35.2 μM, and MBC values in the range

Table 2. Optimization of the reaction conditions for 1a/4 using different solvents and amounts of piperazine at room temperature, either under conventional stirring or sonication-mediated procedures.

					Yield (%)	
Entry	Procedure	Solvent	Piperazine (equiv.)	Time (min)	1a	4
1	Conventional stirring	Toluene	0.6	240	None	16
2	Conventional stirring	Ethanol	0.6	240	None	39
3	Conventional stirring	Dioxane	0.6	240	None	20
4	Sonication	Toluene	0.6	30	None	17
5	Sonication	Ethanol	0.6	30	None	56
6	Sonication	Dioxane	0.6	30	None	40
7	Conventional stirring	Ethanol	None	420	None	None
8	Conventional stirring	Ethanol	0.8	60	None	68
9	Conventional stirring	Ethanol	1.0	60	None	84
10	Conventional stirring	Ethanol	1.2	60	None	81
11	Sonication	Ethanol	None	30	None	None
12	Sonication	Ethanol	0.8	5	None	71
13	Sonication	Ethanol	1.0	3	None	93
14	Sonication	Ethanol	1.2	6	None	87

Table 3. Optimization of the reaction conditions for 1a/4 using ethanol as a solvent, either under conventional stirring or sonication-mediated procedures.

					Yield (%)	
Entry	Procedure	Temp. (°C)	Piperazine (equiv.)	Time (min)	1a	4
1	Conventional stirring	60	1.0	60	None	84
2	Conventional stirring	80	1.0	60	None	87
3	Conventional stirring	60	1.2	150	32	38
4	Conventional stirring	80	1.2	150	35	33
5	Conventional stirring	60	1.4	200	59	Traces
6	Conventional stirring	80	1.4	150	64	None
7	Sonication	60	1.0	40	None	93
8	Sonication	60	1.2	40	51	21
9	Sonication	60	1.4	30	92	None
10	Sonication	70	1.4	30	90	None

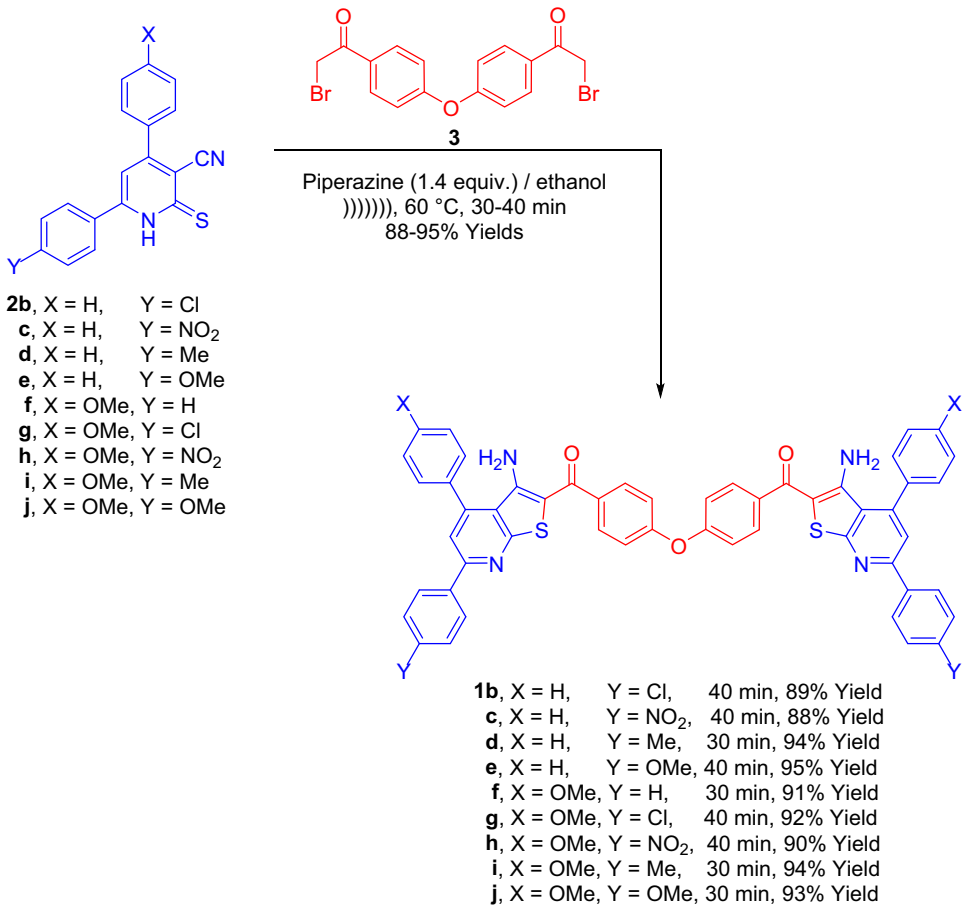
from 35.2 to 36.5 μM against the *S. mutans* and *K. pneumonia* strains. Other hybrids tested had weak potency against all strains tested with MIC/MBC values ranging from 16.3 to 139.4 μM.

3.2.1.2. Structure-activity relationship

The new bis(thieno[2,3-*b*]pyridines) **1** series is linked to two aryl units at C4 and C6. The series exhibited a wide range of antibacterial efficacy, which could be correlated to the electronic properties of the attached arene units. Using the *S. aureus* strain as an example, we discovered that hybrid **1a** with 4,6-diphenyl units had moderate activity with MIC/MBC values of 18.8/37.7 μM. Incorporating electron-releasing units such as *p*-Me or *p*-OMe attached to 6-aryl units, as in hybrids **1d** and **1e**, resulted in higher activity than in hybrid **1a**, with MIC and MBC values of 4.4–4.5 μM. In the same context, incorporation of electron withdrawal units such as *p*-Cl or *p*-NO_2 attached to 6-aryl units, as seen in hybrids **1b** and **1c**, resulted in lower activity than in hybrid **1a**, with MIC and MBC values ranging from 34.0 to 69.7 μM.

The same pattern of activity was seen in hybrids **1f–1j**, where hybrids **1i** and **1j**, attached to 6-(*p*-tolyl) and 6-(4-methoxyphenyl), respectively, demonstrated superior activity with MIC/MBC values of 2.0–2.1 μM compared to hybrid **1f**, which had MIC/MBC values of 8.8 μM. Additionally, hybrids **1g** and **1 h**, attached to 6-(4-chlorophenyl) and 6-(4-nitrophenyl), respectively, demonstrated lower activity with MIC/MBC values of 16.3–32.6 μM compared to hybrid **1f**. Incorporating electron-releasing *p*-OMe attached to 4-aryl units in hybrids **1f-1j** resulted in superior antibacterial activity compared to hybrids **1a–1e** attached to 4-phenyl units, as shown in Table 5. However, incorporating electron-releasing units attached to 6-aryl units has a greater influence on the antibacterial activity of the tested hybrids than incorporating electron-releasing *p*-OMe attached to 4-aryl units. This finding is supported by the superior antibacterial activity of hybrid **1e** over **1f**. The hybrid **1e**, attached to 4-phenyl and 6-(4-methoxyphenyl) units, had MIC/MBC values of 4.4 μM, while hybrid **1f**, attached to 4-(4-methoxyphenyl) and 6-phenyl units, had MIC/MBC values of 8.8 μM.

Scheme 4. Synthesis of the target arene-linked bis(thieno[2,3-*b*]pyridine) hybrids **1b-1j**.



3.2.1.3. Evaluation of MIC and MBC values against MRSA strains

Encouraged by the findings of antibacterial activity against the *S. aureus* strain, some of new hybrids were examined as potential inhibitors of MRSA strains. In this regard, the inhibitory activity of hybrids **1d–1f**, **1i**, and **1j** were examined against two different MRSA ATCC:33 591 and ATCC:43 300 strains using the reference linezolid with MIC/MBC values of 5.2/31.1 and 2.6/31.1 μM, respectively (Table 5). The hybrids **1i** and **1j** demonstrated stronger efficacy than linezolid with MIC values of 2.0–8.5 μM, and MBC values of 4.1–8.5 μM against the MRSA ATCC:33 591, and ATCC:43 300 strains. Furthermore, hybrids **1d**, and **1e** demonstrated lower efficacy with MIC values of 8.8–9.1 μM, and MBC values of 17.6–18.2 μM against the previous strains. Finally, hybrid **1f** had moderate efficacy with MIC/MBC values of 17.6–35.2 μM.

3.2.1.4. Evaluation of anti-biofilm activity

The bis(thieno[2,3-*b*]pyridines **1i** and **1j** were chosen for further investigation of their antibacterial biofilm activity.[49] The results were obtained by using ciprofloxacin, which has IC50 values of 4.0, 4.2, and 3.7 μM against *S. aureus* (ATCC:6538), *E. faecalis* (ATCC:29 212), *E. coli* (ATCC:9637), and *P. aeruginosa* (ATCC:27 953) strains, respectively (Table 6). Both hybrids tested had inhibitory activity comparable to the standard ciprofloxacin. Hybrid **1i** had IC_{50} values of 3.9, 4.6, 4.0, and 4.1 μM against the strains tested, while hybrid **1j** had IC_{50} values of 3.8, 4.4, 4.2, and 4.0 μM.

3.2.2. Cytotoxicity against eukaryotic cells

Because it will come into contact with infected tissues and their neighboring eukaryotic cells, an antibacterial agent must be cytocompatible rather than cytotoxic. The potential antibacterial agents **1i**, and **1j** were tested for cytotoxicity in the human breast epithelial cell line MCF-10 A. Both compounds were tested at eight different concentrations in comparison to the reference drug doxorubicin using the neutral red uptake assay.[16,50] Doxorubicin has been shown to be cytotoxic in a dose-dependent manner. When MCF-10 A cells were treated with 22.9 μM (12.5 μg/mL) doxorubicin, their viability was less than 70% (lowest value established by the ISO 10993–5[56] to consider a material as non-cytotoxic).

According to the findings, hybrid **1i** is more cytotoxic than hybrid **1j**, but both hybrids tested are less toxic to eukaryotic cells than doxorubicin. A dose of hybrid **1i** of 27.3 μM (25 μg/mL) is sufficient to reduce MCF-10 A cell viability below 70%, whereas a dose of 52.7 μM (50 μg/mL) or higher is required to consider hybrid **1j** toxic to MCF-10 A cells (see Fig. S3).

The previously mentioned concentrations are significantly higher than their MIC/MBC values against *S. aureus*, *E. faecalis*,

Table 4. MIC/MBC values in μM of new arene-linked bis(thieno[2,3-*b*]pyridines) 1.

Compound	MIC (MBC) in μM					
	Staphylococcus aureus	*Streptococcus mutans*	*Enterococcus faecalis*	*Escherichia coli*	*Pseudomonas aeruginosa*	*Klebsiella pneumonia*
1a	18.8	37.7	18.8	37.7	18.8	75.5
	(37.7)	(75.5)	(18.8)	(37.7)	(37.7)	(75.5)
1b	34.8	139.4	34.8	34.8	69.7	139.4
	(69.7)	(139.4)	(34.8)	(69.7)	(69.7)	(139.4)
1c	34.0	136.3	34.0	68.1	68.1	136.3
	(68.1)	(136.3)	(68.1)	(68.1)	(68.1)	(136.3)
1d	4.5	18.2	9.1	4.5	9.1	18.2
	(4.5)	(36.5)	(9.1)	(9.1)	(9.1)	(36.5)
1e	4.4	17.6	4.4	4.4	8.8	17.6
	(4.4)	(35.2)	(8.8)	(4.4)	(8.8)	(35.2)
1f	8.8	35.2	8.8	8.8	17.6	35.2
	(8.8)	(35.2)	(17.6)	(8.8)	(17.6)	(35.2)
1g	16.3	65.3	16.3	16.3	32.6	65.3
	(32.6)	(130.7)	(32.6)	(32.6)	(32.6)	(130.7)
1h	31.9	63.9	15.9	31.9	31.9	63.9
	(31.9)	(127.9)	(31.9)	(31.9)	(31.9)	(127.9)
1i	2.1	8.5	4.2	2.1	4.2	8.5
	(2.1)	(17.0)	(4.2)	(4.2)	(4.2)	(17.0)
1j	2.0	8.2	2.0	2.1	4.1	8.2
	(2.0)	(16.4)	(4.1)	(4.1)	(4.1)	(16.4)
Ciprofloxacin	2.9	2.9	2.9	2.9	2.9	2.9
	(5.9)	(5.9)	(5.9)	(5.9)	(5.9)	(5.9)

Note: MBC: minimum bactericidal concentration; MIC: minimum inhibitory concentration.

Table 5. MIC and MBC values in μM of some bis(thieno[2,3-*b*]pyridines) 1 against MRSA strains.

Compound	MRSA ATCC:33 591		MRSA ATCC:43 300	
	MIC (μM)	MBC (μM)	MIC (μM)	MBC (μM)
1d	9.1	18.2	9.1	18.2
1e	8.8	17.6	8.8	17.6
1f	17.6	17.6	17.6	35.2
1i	4.2	8.5	2.1	8.5
1j	4.1	8.2	2.0	8.2
Linezolid	5.2	31.1	2.6	31.1

Note: MBC: minimum bactericidal concentration; MIC: minimum inhibitory concentration; MRSA: methicillin-resistant *Staphylococcus aureus*.

Table 6. Bacterial biofilm inhibitory activity (IC_{50} in μM ± SD) of arene-linked bis(thieno[2,3-*b*]pyridines **1i** and **1j**.

Compound	IC_{50} in μM ± SD			
	Staphylococcus aureus	*Enterococcus faecalis*	*Escherichia coli*	*Pseudomonas aeruginosa*
1i	3.9 ± 0.10	4.6 ± 0.15	4.0 ± 0.11	4.1 ± 0.13
1j	3.8 ± 0.11	4.4 ± 0.12	4.2 ± 0.11	4.0 ± 0.11
Ciprofloxacin	4.0 ± 0.13	4.5 ± 0.14	4.2 ± 0.12	3.7 ± 0.09

E. coli, *P. aeruginosa*, and MRSA strains, which ranged from 2.0 to 8.5 μM. Additionally, the previously mentioned concentrations are significantly higher than the concentrations required for their antibacterial biofilm activity, which ranged from 3.8 to 4.6 μM. This lends credence to the potential of both hybrids as safe antibacterial agents.

4. Conclusion

A three-component tandem protocol involving the reactions of pyridine-2(1H)-thiones, bis(α-haloketone), and piperazine yielded a new series of arene-linked bis(thieno[2,3-*b*]pyridines). The target hybrids were formed by an initial bis(nicotinonitrile) formation, followed by sonication and piperazine-mediated Thrope–Ziegler reaction. The new products demonstrated a wide range of antibacterial activity. The 4-(4-methoxyphenyl)-linked hybrids attached to 6-(4-methoxyphenyl) or 6-(*p*-tolyl) units had more effective potency than the references ciprofloxacin or linezolid against different bacterial strains, including MRSA strains. Furthermore, they showed inhibitory antibacterial biofilm activity comparable to the standard ciprofloxacin. The cytotoxicity of the previous hybrids lends credence to both hybrids' potential as safe antibacterial agents.

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Supplementary material

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