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Microwave-Assisted Synthesis of Bioactive Pyridine-Functionalized *N*-Alkyl-Substituted (Benz)Imidazolium Salts

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Imidazolium salts have always been well recognized as the precursors to ionic liquids. Various studies have been extended to their biological properties, in which many have discovered interesting results. In this study, a series of pyridine-functionalized (benz)imidazolium salts were synthesized using the conventional synthetic method, and microwave irradiation. The products were tested biologically against a list of microorganisms via broth microdilution assay and cancer cell lines using a standard MTT assay. Lead compound **4e** exhibited great antimicrobial properties with lowest MIC value of 3.91 μg/mL against *Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus* and *Enterococcus faecalis*. Remarkable anticancer properties of **3f** were shown with IC_{50} value as low as 0.71 μM against human colon cancer (HCT116). From our study, the MIC of **4e** and IC_{50} values of **3f** surpassed the potency of positive controls such as chloramphenicol (8.08–16.2 μg/mL) and cisplatin (10.78 μM) respectively. Additionally, the use of microwave irradiation has significantly reduced the reaction time from 5 days in a conventional setup, to a 3-hour synthetic reaction in a microwave synthesizer.

Introduction

Heterocycles are ubiquitous in nature and have an important role biologically and industrially. Imidazole is a five-membered azole heterocycle with two nitrogen atoms in position 1 and 3. Imidazolium salts can be easily derived from imidazole by performing the conventional *N*-alkylation reaction at each of the nitrogen atom.[1] Imidazolium salts are often utilized in various applications such as anion receptors[2] and ionic liquids.[3] Its medicinal applications have also been studied extensively. Over the last few decades, various promising antimicrobial[4] and anticancer[5] activities possessed by imidazolium salts have been highlighted in many review articles.

N-alkylation using conventional laboratory setup often requires a longer reaction duration and generally generates more by-products, resulting in lower yield production.[6] The issues can be easily overcome with the use of microwave irradiation in *N*-alkylation reactions. Microwave irradiation has gained attention over the past decades due to its drastic reduction in reaction time compared to conventional heating and the ease of workup after the reaction with enhanced selectivity and yield.[7]

Several studies have established the structure-activity relationship of imidazolium salts. Microbial inhibitions are heavily influenced by the alkyl chain length in position 1 and 3 of the imidazolium ring. In general, an increase in alkyl group chain length and the number of alkyl groups substituted on the imidazolium cation ring shows correlation with an increase in potency.[4c,8] Salts containing more than 10 carbon atoms show very strong antimicrobial activities.[4a] It was also established that the higher hydrophobicity of the compound, the lower the minimum inhibition concentration (MIC).[9] In these investigations, no observable trend was established between the antimicrobial activities and different counter anions. Imidazolium salts with long alkyl chains in position 4 and 5 are also reported to possess remarkable antibacterial activities.[10]

Analogues of imidazolium salts with other functionalities such as peptide,[11] pyridine,[12] binapthol,[13] cyclodextrin[14] and polymer[15] are also reported for its profound antimicrobial activities. Pyridine-functionalized isoniazid, prescribed in combination with other drugs such as ethambutol, rifampin, streptomycin or pyrazinamide, is often used to treat latent tuberculosis.[16] In our previous studies, we have reported the synthesis and characterization of a series of bioactive pyridine-functionalized alkyl-substituted imidazolium salts bearing methyl, *tert*-butyl and phenyl at the *N*-wingtip which possess weak antimicrobial properties with MIC values as low as 1.25 mM.[17] This is not comparable to other imidazolium salts which were reported to have MIC values in the μM range.[8] The synthesized salts were also found not cytotoxic to the tested human carcinoma cell lines (H103, HCT116, and MCF7) at the highest tested concentration (40 μM).

In our continued endeavor to discover more potent antimicrobial and cytotoxic agents, we herein present a chemi-

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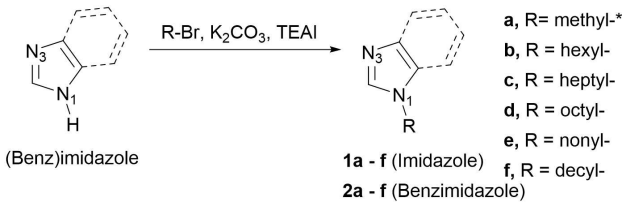
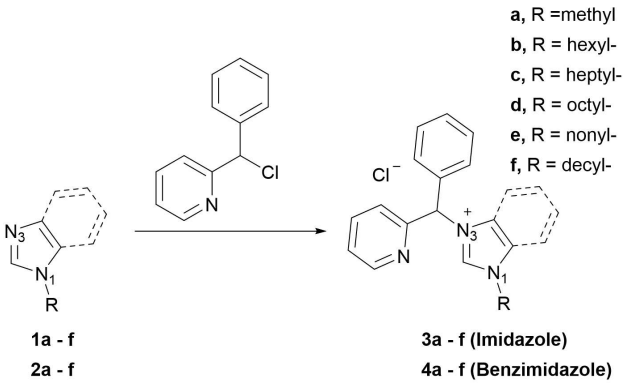
cally robust and efficient synthesis route for commercially available alkyl-(benz)imidazole derivatives (hexyl-, heptyl-, octyl-, nonyl-, and decyl-), and their respective novel pyridine-functionalized alkyl-(benz)imidazolium salts. Furthermore, the influence of alkyl chain length on the biological properties and the potency between imidazole and benzimidazole are discussed.



Scheme 1. Synthesis pathway for alkylation of (benz)imidazole into **1 b**–**f** and **2 b**–**f**. *__1 a__ and **2 a** are commercially available.



Scheme 2. Synthesis pathway for subsequent alkylation of **1 a**–**f** and **2 a**–**f** to **3 a**–**f** and **4 a**–**f**.

Results and Discussion

Synthesis of (benz)imidazolium salts

The pyridinyl ligand was synthesized as per literature method.[18] The (benz)imidazole derivatives (**1 b**–**f**, imidazole; **2 b**–**f**, benzimidazole) on the other hand were synthesized in an analogous fashion with their respective alkyl bromide (C_6 to C_{10}). All imidazole derivatives **1**–**2** showed similar trend, with the only difference of two additional protons in the upfield region (approximately δ 1.00–1.50 ppm) for each increment in the methylene protons of the alkyl chain. Compounds **1 b**–**f** and **2 b**–**f** was confirmed with the presence of methyl peak on ^{1}H-NMR at approximately δ 0.87 ppm, a quintet ($CH_2CH_2CH_2$) and a triplet (CH_2CH_2N) at approximately δ 1.75 and 3.90 ppm respectively, and the remaining $-CH_2$ found within the δ 1.00–1.50 ppm range. The characteristics (benz)imidazolium proton peak (NC*H*N) can also be observed at approximately δ 7.43 ppm. The four additional protons from the extended π-conjugated benzene ring in benzimidazole, **2** can be observed at the aromatic region, approximately δ 7.20–7.90 ppm. Besides, the number of carbons in the ^{13}C-NMR spectrum corresponded to the respective (benz)imidazole derivatives, further confirming the chemical structure of the synthesized compounds.

Subsequent *N*-alkylation of **1 a**–**f** and **2 b**–**f** was performed, yielding **3 a**–**f** and **4 a**–**f** respectively. The products were confirmed with the presence of characteristic imidazolium proton peak (NC*H*N) at approximately δ 10.30 ppm. The downfield shifting of the characteristic imidazolium proton peak was due to the hydrogen bonding with the anion and the inductive effect of its proximity to the imidazolium ring.[19] The proton at the chiral center of the compound has a downfield shift that appeared in the aromatic region on the ^{1}H-NMR spectrum and was indistinguishable from other aromatic protons. This finding is consistent with those reported in the literatures.[17–18] Like their parent (benz)imidazole derivatives, the respective alkyl chain can be observed at the aliphatic region, where the number of carbon and hydrogen agrees to their respective chemical structure.

Microwave-assisted synthesis

Like conventional heating method, the ratio among the reactants remained unchanged. The parameters and conditions needed were optimized on the synthesis of **1 b**, and the optimized parameters and conditions were applied on to the corresponding derivatives (**1 c**–**f**, **2 b**–**f**, **3 a**–**f** and **4 a**–**f**) as outlined in Scheme 1 and 2. Before the parameters and conditions were decided for the synthesis, optimization of reaction conditions was performed using imidazole and 1-bromohexane to identify the best reaction parameter combinations.

The reaction was found to be unsuccessful when tetraethylammonium iodide (TEAI) was absent (Table 1, Entry 1). Despite having reaction carried forward with the use of triethylamine, the use of potassium carbonate is more favored as the reaction only produces water and carbon dioxide as by-products. Thus, Entry 2 was selected to screen for the reaction duration, ranging from 30 minutes to 210 minutes (Table 2). The reaction was found to be completed at 180 minutes, with the yield of 86.3 % (Table 2, Entry 13), as there was no significant increment in percentage yield at 210 minutes (Table 2, Entry 14). It was found that the duration of first *N*-alkylation reaction yielding

Table 1. Screening of reaction conditions for alkylation of imidazole with 1-bromohexane.

Entry	Base	Catalyst	Solvent	Yield [%][a]
1	K_2CO_3	–	CH_3CN	–
2	K_2CO_3	TEAI	CH_3CN	31.1
3	K_2CO_3	TEAI	CH_2Cl_3	–
4	K_2CO_3	TEAI	$CHCl_3$	–
5	K_2CO_3	TEAI	H_2O	–
6	NaOH	TEAI	CH_3CN	–
7	KOH	TEAI	CH_3CN	–
8	Triethylamine	TEAI	CH_3CN	25.6

[a] Yield (%) refer to isolated yield.

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Table 2. Screening of reaction duration for alkylation of imidazole with 1-bromohexane.

Entry	Time [min]	Temperature [°C]	Yield [%][a]
9	30	150	31.1
10	45	150	44.4
11	60	150	62.7
12	120	150	71.9
13	180	150	86.3
14	210	150	85.5

[a] Yield (%) refer to isolated yield.

1b–f and **2b–f** can be shortened from 5–7 days to 3 hours (Table 3).

The conventional laboratory setup produces heat through series of conduction and convection, from the heat source to the reaction mixture. This forms a temperature gradient, which often lead to overheating and result in formation of by-products. With the use of microwave dielectric heating, the heat is generated from the reaction mixture itself, which prevented the formation of temperature gradient.[6] Microwave irradiation also allowed a more uniform heating and the reaction proceeded much faster than the conventional refluxing.[7b] Hence, the product yield can be improved in a shorter reaction duration with the use of microwave irradiation.

Similarly, the reaction parameters for synthesis of (benz)imidazolium salts, **3** and **4** derivatives using microwave irradiation were first optimized using **1a** and 2-(chloro(phenyl)methyl)pyridine to identify the best reaction duration (Scheme 2). The reaction was found to be completed at 90 minutes, with a yield of 79.9% (Table 4, Entry 4). The duration of subsequent *N*-alkylation yielding **3a–f** and **4a–f** can be shortened from 2 days to 1 hour, with approximately 20% increment in yield production (Table 5). All the synthesized (benz)imidazole, **1**–**2** and (benz)imidazolium salts, **3**–**4** were confirmed with ^{1}H-NMR.

Table 3. Comparison of reaction yields for (benz)imidazole (**1b–f** and **2b–f**) under microwave irradiation (3 hours) and conventional heating (120 hours).

Compound	Yield [%] Conventional heating	Microwave irradiation
1b	67.7	86.3
1c	56.4	88.0
1d	62.3	82.1
1e	47.7	83.1
1f	68.0	84.8
2b	54.2	76.3
2c	60.5	81.4
2d	50.2	60.9
2e	54.8	77.1
2f	56.8	68.8

Table 4. Screening of reaction conditions for subsequent alkylation of **1a**.

Entry	Time [min]	Temperature [°C]	Yield [%][a]
1	15	150	-
2	30	150	10.5
3	60	150	79.1
4	90	150	79.9

[a] Yield (%) refer to isolated yield.

Antimicrobial property

Previously, we reported the antimicrobial properties of **1a** and **3a**, and other derivative bearing phenyl and *tert*-butyl R-group.[17] In this study, we tested the synthesized compounds **1b–f**, **2b–f**, **3b–f**, and **4b–f** against a panel of 15 micro-organism strains, which included eight Gram-positive, four Gram-negative bacteria, and three yeast strains. Based on the results, all synthesized compounds showed low to moderate antimicrobial properties with MIC values reported as low as 3.91 μg/mL, which was comparable to reported literature.[4] Interestingly, it was found that the antimicrobial properties increased as the carbon chain length is increased, up to C_9. Microbial inhibition properties were found to increase with chain length up to a point exhibiting a cut-off effect at chain lengths of 16 or 18 for the imidazolium cation and the Cl^- anion.[4b] The retardation in antimicrobial properties observed in C_{10} is most probably due to the antimicrobial mode of action dependent on the surface activity of the molecule suggesting that surface activity may contribute to the cut-off effect in the biological activity of imidazolium salts.[4b] Hence, it can be deduced that the "cut-off" point for **1** and **2** are nine carbons.

Imidazole bearing hexyl group (**1e**) was 160 times more effective against the tested microorganisms as compared to imidazole bearing methyl group (**1a**).[17] However, the benzimidazole derivatives (**2b–f**) in comparison to the imidazole derivatives (**1b–f**) were more bacteriostatic (lower MIC value), but possessed weaker or no bactericidal property (higher MBC value).

Upon subsequent alkylation, yielding compound **3b–f** and **4b–f**, similar trend of antimicrobial properties was seen, where increasing carbon chain length enhanced the antimicrobial

Table 5. Comparison of reaction yields for (benz)imidazolium salts (**3a–f** and **4b–f**) under microwave irradiation (1 hour) and conventional heating (48 hours).

Compound	Yield [%] Conventional heating	Microwave irradiation
3a	49.1	79.1
3b	53.6	71.1
3c	49.9	79.4
3d	50.2	83.7
3e	36.6	80.8
3f	46.1	82.4
4b	46.5	86.4
4c	34.1	88.7
4d	40.1	89.6
4e	63.4	91.3
4f	52.0	87.6

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activity. Based on Table 6–7, most of the synthesized compounds display moderate antimicrobial activities against the chosen bacterial strains. Among all the imidazolium salts, **3e** and **4e** possessed the most potent antimicrobial activity, with MIC values as low as 7.81 and 3.91 μg/mL respectively. As expected, as the number of carbons in the alkyl chain increased (up to $C_n = 9$), the antimicrobial properties increase as observed for **3e** and **4e**.

A retardation in the antimicrobial property of the compound when the chain length is increased to 10 carbons was observed in both imidazole-derived and benzimidazole-derived salts from 7.81 to 31.25 μg/mL and 3.91 to 7.81 μg/mL, respectively, as demonstrated by **3f** and **4f** against *S. aureus*, which the trend is like their parent imidazole and benzimidazole derivatives.

Compound **4** was found to have better bacteriostatic properties of the compounds, but the bactericidal properties of the benzimidazole-derived compounds remained weaker than their imidazolium counter parts. For examples, **4** showed MIC values as low as 3.91 μg/mL against the tested microorganisms, whereas **3** showed lowest MIC values of 7.81 μg/mL. However, **4** were almost non-bactericidal at highest tested concentration (MBC values > 1000 μg/mL) while **3** showed MBC values as low as 31.25 μg/mL against the tested microorganisms.

Comparing to our previously reported pyridine-functionalised imidazolium salts,[17] the salts bearing a longer alkyl chain showed better antimicrobial activities, with the increment of at least 160-fold. Generally, the pyridine functionality showed superiority in antimicrobial properties than the reported ester- and amide-functionalized imidazolium salts with MIC values as low as 75 and 9.375 μg/mL respectively,[20] and alcohol-functionalized imidazolium salts with MIC value as low as 8 μg/mL.[8] However, the antimicrobial profiles of this class of pyridine-functionalized imidazolium salts were inferior to a class of dialkyl-substituted imidazolium salts, where the MIC values were found to be as low as 0.013 μg/mL.[21]

Analogues of **4b–f** were found to be active against all three yeast strains with MIC values as low as 3.91 μg/mL, in which their imidazolium counter parts **3b–f** did not display any activity at the highest tested concentration. This is not surprising as benzimidazole is a well-known scaffold present in many bioactive molecules with antifungal properties.[22]

Table 6. Minimum inhibitory concentration (MIC) in μg/mL of synthesized compounds against the chosen microorganism strains (CL – chloramphenicol, CX – cycloheximide).

Microorganisms tested	Minimum inhibitory concentration, MIC [μg/mL]																			
	1b	**1c**	**1d**	**1e**	**1f**	**2b**	**2c**	**2d**	**2e**	**2f**	**3b**	**3c**	**3d**	**3e**	**3f**	**4b**	**4c**	**4d**	**4e**	**4f**	CL[a]	CX[a]
Bacillus cereus ATCC 14579	1000	62.5	62.5	7.81	125	250	250	125	3.91	3.91	250	62.5	31.3	15.6	125	62.5	62.5	62.5	3.91	3.91	1.94	> 14.1
Bacillus subtilis ATCC 8188	1000	62.5	31.3	7.81	250	250	250	250	3.91	3.91	250	62.5	62.5	7.81	125	62.5	62.5	62.5	3.91	3.91	1.94	> 14.1
Staphylococcus aureus ATCC 29213	1000	250	62.5	15.6	250	500	500	250	3.91	7.81	250	62.5	31.3	15.6	31.3	125	125	62.5	3.91	7.81	8.08	> 14.1
Staphylococcus aureus ATCC 6538P	1000	125	62.5	7.81	250	500	500	125	3.91	3.91	250	31.3	31.3	7.81	31.3	125	62.5	62.5	3.91	7.81	8.08	> 14.1
Staphylococcus aureus ATCC 43300	1000	250	62.5	7.81	250	500	500	250	7.81	7.81	500	62.5	31.3	15.6	15.6	125	125	62.5	3.91	7.81	8.08	> 14.1
Staphylococcus aureus ATCC 33591	1000	62.5	31.3	7.81	500	500	500	250	3.91	7.81	500	62.5	31.3	31.3	62.5	62.5	62.5	62.5	3.91	7.81	8.08	> 14.1
Enterococcus faecalis ATCC 29212	> 1000	250	125	15.6	> 1000	1000	1000	500	7.81	7.81	500	250	62.5	7.81	250	500	500	125	3.91	7.81	16.2	> 14.1
Enterococcus faecalis ATCC 700802	> 1000	250	125	31.3	> 1000	500	500	125	7.81	15.6	500	500	125	31.3	250	500	500	250	7.81	7.81	16.2	> 14.1
Escherichia coli ATCC 25922	500	62.5	31.3	31.3	500	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	62.5	62.5	62.5	1000	1000	1000	500	15.6	31.3	8.08	> 14.1
Klebsiella. pneumoniae ATCC 10031	500	125	62.5	15.6	500	500	500	250	7.81	125	500	250	125	125	250	125	125	31.3	7.81	31.3	0.97	> 14.1
Pseudomonas aeruginosa ATCC 10145	1000	500	500	500	1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	250	250	> 16.2	> 14.1
Shigella flexneri ATCC 12022	250	62.5	62.5	31.3	500	> 1000	> 1000	> 1000	> 1000	> 1000	500	250	125	125	1000	500	500	250	31.3	31.3	8.08	> 14.1
Candida albicans IMR	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	250	250	125	15.6	500	> 16.2	14.1
Candida albicans Clinical isolates	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	250	250	31.3	3.91	250	> 16.2	14.1
Candida tropicalise Clinical isolates	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	125	125	125	7.81	500	> 16.2	14.1

[a] Results are expressed in ng/mL.

Bacterial growth curve

The lead compound, **3e**, was selected to study their inhibition kinetics. *B. cereus* ATCC 14579 and *E. coli* ATCC 25922 were chosen and cultured overnight in the presence of the compounds at various concentrations, ranging from 3.91 μg/mL to 1000 μg/mL. As the bacterial cultures were growing, the absorbance readings were measured spectrophotometrically every 1-hour interval at 620 nm. The growth curves for each tested compound against each bacterial strain were plotted as shown in Figure 1–2.

Based on Figure 1–2, it was observed that the tested compounds acted immediately upon exposing to the bacterial cultures. Despite **3e** was found to have MIC values against *E.*

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Table 7. Minimum bactericidal concentration (MBC) in μg/mL of synthesized compounds against the chosen microorganism strains (CL – chloramphenicol, CX – cycloheximide).

Microorganisms tested	Minimum bactericidal concentration, MBC [μg/mL]																				
	1b	**1c**	**1d**	**1e**	**1f**	**2b**	**2c**	**2d**	**2e**	**2f**	**3b**	**3c**	**3d**	**3e**	**3f**	**4b**	**4c**	**4d**	**4e**	**4f**	CL[a]	CX[a]
Bacillus cereus ATCC 14579	>1000	125	125	31.3	250	500	500	500	>1000	>1000	500	125	125	62.5	1000	>1000	>1000	>1000	>1000	>1000	>16.2	>14.1
Bacillus subtilis ATCC 8188	>1000	125	62.5	15.6	250	500	500	500	>1000	>1000	500	250	125	31.3	500	>1000	>1000	>1000	>1000	>1000	>16.2	>14.1
Staphylococcus aureus ATCC 29213	>1000	500	500	62.5	>1000	1000	500	500	>1000	>1000	1000	125	125	31.3	250	>1000	>1000	>1000	>1000	>1000	>16.2	>14.1
Staphylococcus aureus ATCC 6538P	>1000	500	500	62.5	1000	1000	500	500	>1000	>1000	1000	125	125	31.3	125	>1000	>1000	>1000	>1000	>1000	>16.2	>14.1
Staphylococcus aureus ATCC 43300	>1000	1000	500	62.5	>1000	1000	500	500	>1000	>1000	1000	250	125	31.3	125	>1000	>1000	>1000	>1000	>1000	>16.2	>14.1
Staphylococcus aureus ATCC 33591	>1000	500	250	62.5	>1000	1000	500	500	>1000	>1000	1000	250	125	62.5	500	>1000	>1000	>1000	>1000	>1000	>16.2	>14.1
Enterococcus faecalis ATCC 29212	>1000	500	250	62.5	>1000	>1000	1000	1000	62.5	500	1000	500	250	125	1000	1000	1000	500	125	250	>16.2	>14.1
Enterococcus faecalis ATCC 700802	>1000	500	250	62.5	>1000	1000	500	500	>1000	>1000	1000	1000	500	125	>1000	1000	1000	500	15.6	31.3	>16.2	>14.1
Escherichia coli ATCC 25922	>1000	250	125	62.5	>1000	>1000	>1000	>1000	>1000	>1000	>1000	250	125	125	>1000	>1000	>1000	1000	250	1000	>16.2	>14.1
Klebsiella. pneumoniae ATCC 10031	>1000	250	125	31.3	>1000	1000	1000	500	>1000	>1000	1000	500	250	125	250	>1000	>1000	>1000	>1000	>1000	0.97	>14.1
Pseudomonas aeruginosa ATCC 10145	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	1000	>1000	>16.2	>14.1
Shigella flexneri ATCC 12022	500	250	125	62.5	500	>1000	>1000	>1000	>1000	>1000	1000	500	250	125	1000	>1000	>1000	1000	500	>1000	16.2	>14.1
Candida albicans IMR	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>16.2	14.1
Candida albicans Clinical isolates	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>16.2	14.l
Candida tropicalise Clinical isolates	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>16.2	14.1

[a] Results are expressed in ng/mL.

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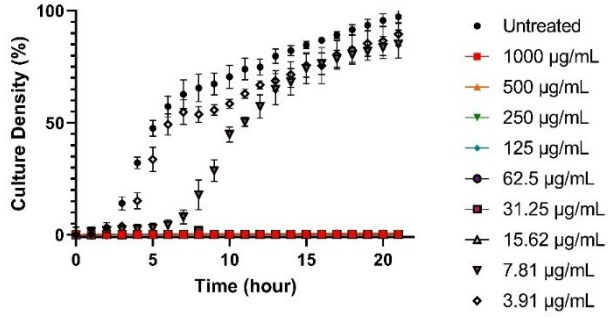

Figure 1. Growth curve of *B. cereus* ATCC 14579 over 21 hours cultured in MHB containing compound **3e**.

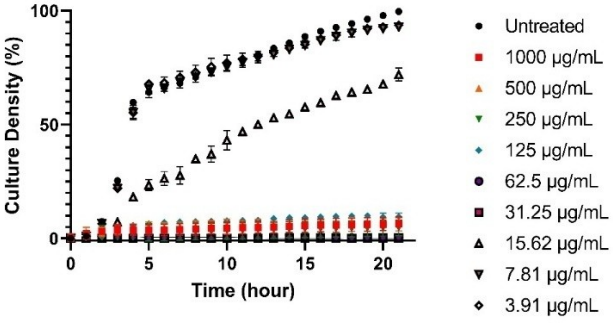

Figure 2. Growth curve of *E. coli* ATCC 25922 over 21 hours cultured in MHB containing compound **3e**.

coli at 62.5 µg/mL as shown in broth microdilution assay, the sub-MIC (concentration that is half of the MIC = 31.25 µg/mL) of **3e** against *E. coli* were found to retard the growth of the bacterial culture (Figure 2). As shown in Figure 1 and Figure 2, the log phase of *B. cereus* and *E. coli* was delayed by 6 and 4 hour(s) respectively, upon exposing to **3e** at sub-MIC and $^1/_4$ MIC. The delay in growth of the bacteria culture indicated that compound **3e** were able to suppress the bacterial growth despite at sub-MIC values.

Anticancer property

MTT cell viability assay was performed to determine the cell cytotoxicity profiles of the synthesized (benz)imidazolium salts. The absorbance values based on the dissolved formazan were converted to their respective cell viability at different concentration to generate cell viability-concentration plots. The cytotoxicity profiles of the synthesized (benz)imidazolium salts were expressed in terms of IC_{50} or half maximal inhibitory concentration, where 50% of the cell growth were inhibited by the compounds. The IC_{50} values were summarized and tabulated in Table 8.

In this study, four selected human carcinoma cell lines, including H103 (oral squamous carcinoma), HCT-116 (colorectal epithelial carcinoma), MCF-7 (breast epithelial carcinoma), and HT1080 (skin fibrosarcoma), were treated to various concentration of synthesized (benz)imidazolium salts, **3b–f** and **4b–f**,

Table 8. Cell viability (half inhibitory concentration) IC_{50} values (in µM) of screened (benz)imidazolium salts and cisplatin against human skin HT1080 tumour cells, human colon HCT116, human oral H103, human breast MCF-7.

Complex	HT-1080	HCT116	H103	MCF-7
3b	>4.00	3.30 ± 0.18	3.49 ± 0.04	3.70 ± 0.20
3c	>4.00	3.06 ± 0.12	3.08 ± 0.10	3.65 ± 0.03
3d	3.80 ± 0.41	2.17 ± 0.09	2.53 ± 0.03	2.39 ± 0.07
3e	1.68 ± 0.15	1.40 ± 0.06	1.99 ± 0.02	2.38 ± 0.07
3f	1.07 ± 0.07	0.71 ± 0.03	1.80 ± 0.14	2.00 ± 0.08
4b	>4.00	3.85 ± 0.25	3.70 ± 0.11	>4.00
4c	>4.00	3.36 ± 0.44	3.28 ± 0.31	>4.00
4d	3.15 ± 0.15	3.13 ± 0.11	2.53 ± 0.04	3.07 ± 0.07
4e	2.86 ± 0.15	2.43 ± 0.20	2.20 ± 0.07	2.62 ± 0.10
4f	1.92 ± 0.20	1.23 ± 0.04	1.99 ± 0.12	1.55 ± 0.07
cisplatin	Not determined	10.78 ± 0.21	6.16 ± 0.04	19.78 ± 0.21

ranging from 1.0 µM to 4.0 µM. All the synthesized imidazolium salts and benzimidazolium salts were cytotoxic to the selected human carcinoma cell lines ranging from 0.71 to 3.80 µM, except for **3b–c** against HT1080 and **4b–c** against HT1080 and MCF-7 where no cytotoxicity was observed at highest tested concentration (4.00 µM). The activity is comparable to other reported imidazolium salts with IC_{50} values ranging from 3–30 µM.[23]

In addition, all benzimidazolium salts, **4** demonstrated slightly weaker cytotoxicity when compared to their imidazolium salt, **3** analogues as shown in Table 8, where imidazolium salt analogues were about two-fold more cytotoxic than benzimidazolium salt analogues against HT1080 and HCT-116 with IC_{50} value of 1.07 and 0.71 µM respectively.

The structural-activity relationship was like those of antimicrobial property, except that the cut-off point was not seen in C_{10}, where C_{10} was 2-fold more cytotoxic to HCT-116 than C_9 in both the imidazolium and benzimidazolium series. The relationship between alkyl chain residues and anticancer property does not always present a clear-cut trend. For example, a series of 5-alkyl-tetrazolatobridged dinuclear Pt(II) complexes with different alkyl chain lengths were investigated for its anticancer activity.[24] The complexes that possessed an alkyl chain of intermediate length were less potent than were those with shorter or longer alkyl chains. Another recent study showed that anticancer efficacy of cyclometalated iridium complexes against the human breast cancer are most biologically active with short alkyl chains.[25] In contrast, a series of *p*-alkylaminophenols bearing a longer alkyl side chain were found to exhibit better anticancer property.[26]

Selectivity index study

Despite that the cytotoxic profiles of tested (benz)imidazolium salts against the selected cell lines were remarkable, low toxicity to non-cancerous cells was required to ensure the compounds only selectively targeting the cancer cells. Hence, a selectivity index study was conducted to compare the cytotoxicity profiles of these compounds against both cancer cells and non-cancerous cells. In this selectivity index study, two non-

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cencerous cell lines were selected, namely HaCaT (human skin keratinocytes) and BEAS2B (human lung epithelial cells). The cytotoxicity profiles of synthesized (benz)imidazolium salts against HaCaT and BEAS2B were recorded and tabulated in Table 9.

The selectivity index (SI) is defined as the cytotoxic selectivity of the tested compound against cancer cells from non-cancerous cells. It was calculated from the ratio of the IC_{50} value obtained from the cell viability test on normal cells versus the IC_{50} value obtained for the cancer cells. Compound with SI value higher than 3 is deduced as compound with high selectivity towards the targeted cells instead of the non-targeted cells.[27] An SI value less than 2 indicates the toxicity of the pure compounds.[28] In this study, the SI values were calculated based on the IC_{50} values of four cancer cell lines and a non-cancerous cell line at a time. The SI values of the tested compounds towards HaCaT and BEAS2B were tabulated in Table 10 and 11, respectively.

Although the salts were cytotoxic towards the selected cell lines, all tested compounds did not have a distinct selectivity towards neither of the human non-cancerous cell lines namely BEAS-2B (human lung epithelial) nor HaCaT (human skin keratinocytes). The compounds inhibit both cancer cells and non-cancerous cells equally as the SI values for most compounds were close to 1 (Table 10 and 11). Notably, **3f** was the only candidate that portrayed selectivity index of close to 3 against HCT-116 and approximately 2 against HT1080, towards BEAS2B and HaCaT cell lines. On the other hand, **3e** also demonstrated moderate SI value of 2 against HCT-116 towards Beas2B and HaCaT cell lines.

Conclusions

In this study, a series of (benz)imidazolium salts were successfully synthesized using the conventional heating method and microwave irradiation method, and characterized with NMR and MS. Compound **3e** and **4e** were most potent in antimicrobial profiles, with MIC values as low as 7.81 and 3.93 μg/mL respectively against the tested microorganisms, with a cut-off point at C_9. Meanwhile, the anticancer profiles were led by **3f** and **4f**, with IC_{50} values as low as 0.71 and 1.23 μM respectively. The biological properties were found to be greatly associated with the alkyl chain length attached at the *N*-wingtip, where increasing the alkyl chain length enhances the biological properties.

Table 9. Cell viability (half inhibitory concentration) IC_{50} values (in μM) of screened imidazolium salts and cisplatin against normal human cells including human skin keratinocytes (HaCAT) and human lung epithelial cells (BEAS-2B).

Complex	BEAS-2B	HaCAT
3b	>4.00	3.58±0.09
3c	>4.00	3.11±0.15
3d	3.20±0.17	2.82±0.06
3e	2.73±0.06	2.38±0.03
3f	2.15±0.02	2.07±0.06
4b	>4.00	3.85±0.04
4c	>4.00	3.19±0.10
4d	3.41±0.04	2.84±0.04
4e	3.12±0.04	2.60±0.06
4f	2.39±0.05	1.20±0.01

Table 10. Selectivity index (SI) of selected compounds for lung epithelial cells (BEAS2B) against skin cancer (HT1080), colon cancer (HCT-116), oral cancer (H103) and breast cancer (MCF-7).

Compounds tested	Selectivity Index (SI) towards BEAS2B[a]			
	HT1080	HCT-116	H103	MCF-7
3b	ND	ND	ND	ND
3c	ND	ND	ND	ND
3d	0.84	1.47	1.26	1.34
3e	1.63	1.95	1.37	1.15
3f	2.01	3.03	1.19	1.08
4b	ND	ND	ND	ND
4c	ND	ND	ND	ND
4d	1.08	1.09	1.35	1.11
4e	1.09	1.28	1.42	1.19
4f	1.23	1.94	1.20	1.54

[a] Selectivity index (SI) was determined from the ratio of the IC_{50} obtained from the test against normal cells versus the IC_{50} for cancer cells. Abbreviation: ND, not determined.

Experimental Section

General procedures

All the commercially available chemicals and solvents were used without prior drying or purification. Synthesis of 2 – (chloro-(phenyl)methyl)pyridine were conducted as per literature method with slight modifications.[18] The synthesis of alkyl-substituted (benz)imidazoles was performed in analogous fashion with the respective alkyl bromide (hexyl, heptyl, octyl, nonyl, decyl). The synthesised alkyl-substituted (benz)imidazoles were then reacted with 2 – (chloro(phenyl)methyl)pyridine to yield the respective alkyl-substituted (benz)imidazolium salts. The solvent system used for all TLC was similar to the solvent system used in the respective column chromatography. Mass spectra were recorded on Waters

Table 11. Selectivity index (SI) of selected compounds for skin keratinocytes (HaCaT) against skin cancer (HT1080), colon cancer (HCT-116), oral cancer (H103) and breast cancer (MCF-7).

Compounds tested	Selectivity Index (SI) towards HaCaT[a]			
	HT1080	HCT-116	H103	MCF-7
3b	ND	1.08	1.03	0.97
3c	ND	1.02	1.01	0.85
3d	0.74	1.30	1.11	1.18
3e	1.42	1.70	1.20	1.00
3f	1.93	2.92	1.15	1.04
4b	ND	1.00	1.04	ND
4c	ND	0.95	0.97	ND
4d	0.90	0.91	1.12	0.93
4e	0.91	1.07	1.18	0.99
4f	0.63	0.98	0.60	0.50
3b	ND	1.08	1.03	0.97

[a] Selectivity index (SI) was determined from the ratio of the IC_{50} obtained from the test against normal cells versus the IC_{50} for cancer cells. Abbreviation: ND, not determined

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Xevo TQ-XS Triple Quadrupole Mass Spectrometer. ^{1}H- and ^{13}C-NMR spectroscopy were performed on a Bruker Advance 300 NMR Spectrometer. The number of protons (n) for a given resonance was indicated by n H. Coupling constants were reported as a *J* value in Hz. ^{1}H-NMR spectra were reported as δ in units of parts per million (ppm) downfield from SiMe4 (δ = 0.00). ^{13}C-NMR spectra were reported as δ in units of parts per million (ppm) relative to the signal of chloroform-d (δ = 77.16 ppm, triplet) and DMSO-d6 (δ = 39.52 ppm, septet). All chemical shifts reported were referenced to the chemical shifts of their respective residual solvent resonances. Unless stated otherwise, all NMR experiments were carried out at 300 K. The microwave-assisted *N*-alkylation was performed using Anton Paar Monowave 400 (monowave, maximum power 850 W, temperature control via IR-sensor) employing a 10 mL Pyrex vial in a closed vessel mode. Like conventional heating method, the ratio between the reactants remained unchanged. A mixture of imidazole in acetonitrile (10 mL), potassium carbonate, tetraethylammonium iodide and alkyl bromide in a closed vial was irradiated in a microwave reactor at 150 °C for the appropriate time. The work-up was similar to that described for the conventional *N*-alkylation.

Broth microdilution assay (BMD)

Broth microdilution assay was conducted using 96-well microtiter plates as per shown in the protocol provided by Clinical & Laboratory Standards Institute (CLSI, 2012) with slight modifications.[29] The synthesised compounds were prepared in 10% DMSO in PBS solution. Positive controls used in this study were chloramphenicol (0.25 mg/mL) for bacterial strains and cycloheximide (1.00 mg/mL) for yeast species. Three negative controls were used, namely blank control (MHB broth only), solvent control (MHB and 2.5% DMSO only), and sterility control (MHB and compound only). The microorganisms were freshly cultured on MHA and inoculated into MHB. The overnight cultures in MHB were adjusted to match 0.5 McFarland Standard and 100-fold diluted with MHB. The 96-well microtiter plates were charged with synthesised compounds per the protocol provided by CLSI with final working concentration ranging from 1.00 mg/mL to 3.91 μg/mL. Then, the diluted microorganism suspensions were added into each well and the 96-well microtiter plates were allowed to incubate at 37 °C for 24 hours. The well with the lowest concentration where no growth was observed (non-turbid) was determined as the minimum inhibitory concentration (MIC). The clear wells were restreaked onto fresh MHA and incubated overnight. The lowest concentration where the microorganisms did not grow on MHA was determined as the minimum bactericidal concentration (MBC). All the experiments were conducted in triplicates.

Bacterial growth curve

Two selected microorganisms namely *Bacillus cereus* ATCC 14579 and *Escherichia coli* ATCC 25922 were used for the study of bacterial growth curve. Overnight bacteria cultures in MHB were adjusted to match 0.5 McFarland standard. Bacteria were incubated with different concentrations of 3e in Tecan Infinite® M200 PRO where the absorbance of bacterial suspensions were recorded spectrophotometrically at 625 nm at one-hour interval for 24 hours. The growth curves were plotted. The experiment was performed in triplicates.

MTT cell viability assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell viability assay was performed to evaluate the cell cytotoxicity profiles of the synthesised compounds.[30] The compounds tested were prepared in PBS solution containing 2% DMSO. The cells were seeded in 96-well microtiter plate at a density of 5,000 to 10,000 cells/well in 100 μL cell culture medium and incubated at 37 °C (5% CO2). After 24 hours of seeding, the medium was removed and the cells were further incubated with freshly prepared medium containing compound of interest with various concentration ranging from 1.0, 2.0, 2.5, 3.0, 3.5 and 4.0 μM at 37 °C for 24 hours. After incubation, 10 μL of MTT dye (5 mg/mL in PBS) was added into each well. The plates were incubated again for 4 hours in the CO_2 incubator at 37 °C. After that, the MTT-containing medium was removed, and the purple formazan crystals formed were dissolved in 100 μL of dimethyl sulfoxide. The plates were then measured spectrophotometrically at 570 nm, with reference wavelength at 620 nm. The cell viability was determined by using the formula: Cell viability % = (optical density of sample/optical density of control) × 100 (solvent controls set to 100% viable cells). IC_{50} value was defined as the concentration where 50% inhibition of proliferation on the tested cell lines. The experiment was carried out in three technical replicates and two biological replicates. Positive control results were adopted from our previous finding as reference in this study.[17]

Supporting Information Summary

Characterization data of compounds are reported in the Supporting Information.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: Anticancer · Antimicrobial · Benzimidazolium salts · Imidazolium salts · Long alkyl chain

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