

Novel 2-mercaptobenzimidazole derivatives: synthesis and evaluation of their antibacterial and antioxidant activities

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Abstract: In the present study, a series of novel 2-mercaptobenzimidazolium were synthesized. These compounds can be prepared by condensation of 2-mercaptobenzimidazole with the various alkylating agents under the conditions of phase transfer catalysis, followed by a quaternization. The newly synthesized compounds were subjected to in vitro biological evaluation. The antibacterial activity was evaluated with diffusion assay and optical density method. The antioxidant activity was carried out using DPPH free radical scavenging assay. The result indicated that some compounds show convincing antibacterial activities against two microorganisms: *Escherichia coli* and *Staphylococcus aureus*. While these molecules have not shown any interesting antioxidant effects.

Keywords: 2-mercaptobenzimidazole, 2-mercaptobenzimidazolium, Antibacterial activity, Antioxidant activity.

Introduction

The benzimidazole ring is an important pharmacophore in modern drug discovery. The compounds bearing benzimidazole moiety are reported to possess a number of interesting biological activities. Its derivatives and particularly 2-mercaptobenzimidazoles were evidenced promising biological efficacies enabling them to perform as new drug. Exceptional structural features of this class of heterocycle and versatile biological applications made it a privileged structural backbone in new drug design and discovery.

The compounds derived from 2-mercaptobenzimidazole have attracted the attention of several researchers for their properties which exhibit significant biological and pharmacological activities such as antibacterial, antioxidant¹⁻³, anticonvulsant⁴, antimicrobial⁵, antihistaminic⁶, nootropic⁷, anti-tubercular⁸ and analgesic⁹ activities. Although, a great interest of the scientific literature concerning 2-mercaptobenzimidazole is in the area of medicinal chemistry, 2-mercaptobenzimidazole is also used in non-biological applications, it serves as plant growth

regulators¹⁰, as natural rubber¹¹ and used as corrosion inhibitor for mild steel¹²⁻¹⁶.

Nowadays, a large number of benzimidazole derivatives used as surfactants have been reported in various researches¹⁷⁻²⁴. Indeed, the benzimidazolium with long-chain constitute a very interesting family of cationic amphiphiles whose structure and nature of against ion can easily be modulated. This makes them of special interest for biological and especially biomedical applications. Consequently, great effort has been made to develop efficient methods for the preparation of new compounds may present interesting surfactant properties, thus it was of value to synthesize some new benzimidazolium derivatives of 2-mercaptobenzimidazole and evaluate their antibacterial and antioxidant activity.

Results and Discussion

Synthesis

We report here our results on the synthesis of 2-mercaptobenzimidazolium using a two-step procedure as shown in (Scheme 1) and (Scheme 2). First, we investigated the reaction conditions for the synthesis of 2-mercapto-benzimidazole N,S-disubstituted. The

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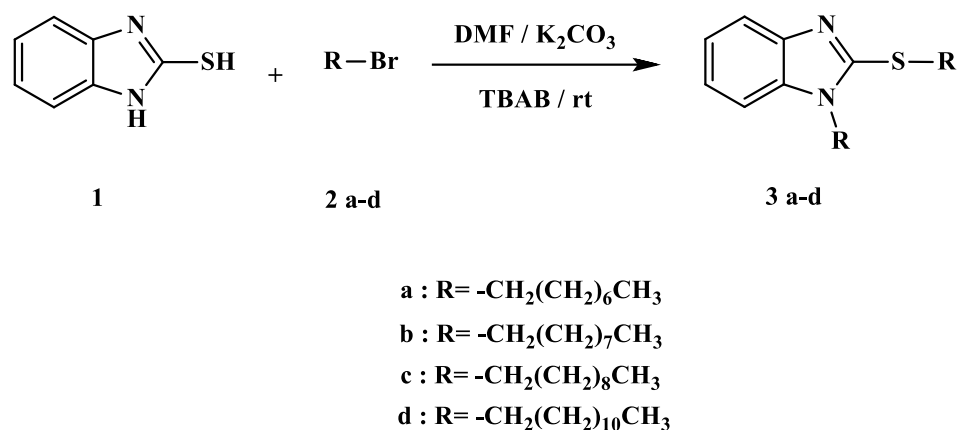
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reaction of 2-mercaptobenzimidazole with n-alkyl bromides reagent using K_2CO_3 as base in N,N-Dimethylformamide as solvent at room temperature for 24 h to provide N,S-disubstituted 2-mercaptobenzimidazoles **3a-d** in good yields (Table 1).

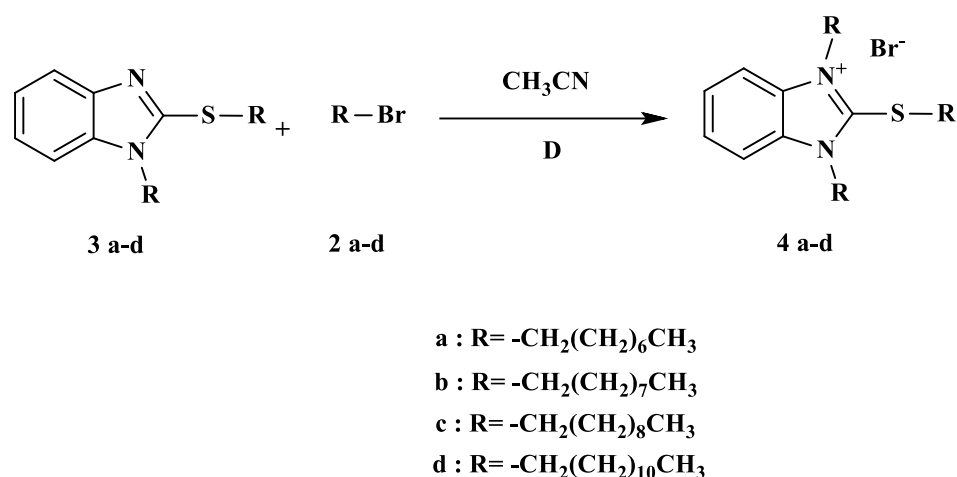
In continuation of our studies, we then focused to convert these compounds corresponding salts in a quaternization type reaction. As expected, reaction of compounds 2-mercapto-benzimidazole N,S-disubstituted **3a-d** with n-alkyl bromides in acetonitrile as solvent under reflux for 72 h furnished the compounds **4a-d** in good yields (Table 2).



Scheme 1 1-alkyl-2-(alkylthio)-1H-benzimidazoles **3a-d**

Table 1. 1-alkyl-2-(alkylthio)-1H-benzimidazoles **3a-d** yields.

Entry	R	Yield %
3a	$-\text{CH}_2(\text{CH}_2)_6\text{CH}_3$	88
3b	$-\text{CH}_2(\text{CH}_2)_7\text{CH}_3$	91
3c	$-\text{CH}_2(\text{CH}_2)_8\text{CH}_3$	93
3d	$-\text{CH}_2(\text{CH}_2)_{10}\text{CH}_3$	93



Scheme 2. Synthesis of 1,3-dialkyl-2-(alkylthio)-1H-benzimidazolium bromides **4a-d**

Table 2. Yields of 1,3-dialkyl-2-(alkylthio)-1*H*-benzimidazolium bromides **4a-d**.

Entry	R	Yield %
4a	-CH ₂ (CH ₂) ₆ CH ₃	88
4b	-CH ₂ (CH ₂) ₇ CH ₃	80
4c	-CH ₂ (CH ₂) ₈ CH ₃	83
4d	-CH ₂ (CH ₂) ₁₀ CH ₃	78

The structure of the obtained compounds **3a-d** and **4a-d** was confirmed by the NMR and MS spectra. The proton spectrum ¹H-NMR of **4a** in CDCl₃ showed a multiplet between 7.18 ppm and 7.26 ppm for the aromatic protons, a triplet at 4.29 ppm for NCH₂ and a triplet at 3.38 ppm for SCH₂, a multiplet between 1.24 ppm and 1.86 ppm corresponding to the protons of methylene groups of the hydrocarbon chain and a triplet at 0.85 ppm for CH₃. On the other hand, the ¹³C-NMR spectrum of **4a** in CDCl₃ shows a signal at 169.0 ppm for the carbon bonded to the sulphur atom and other signals between 32.8 ppm and 22.6 ppm corresponding to the methylene groups then a signal at 14.0 ppm for CH₃. The mass spectra were in accord with the structures of the obtained compounds.

Antibacterial activity

The results of the antimicrobial activity of new of 2-mercaptobenzimidazole salts evaluated against two pathogenic strains (*E. coli* and *S. aureus*) are presented in (Figure 1) (inhibition zones in the Agar-well diffusion assay). The molecules **4b**, **3b** and **3d**

are the most active against the tested strains. In fact, the salt **4b** showed significant activity against *S. aureus* (30±2.12 mm) and *E. coli* (19.66±1.29 mm). The molecule **3b** also shows an important zone of inhibition (15.66±1.93 mm for *E. coli* and 25.33±4.03 mm for *S. aureus*). Compounds **3d**, **4c** and **3a** have some antibacterial activity against *S. Aureus*; while, the expected molecule **3d** proved some inhibition against *S. aureus*, two all other molecules were not active. From our preliminary results, it appears that these compounds have an effect against Gram positive-bacteria more than Gram-negative bacteria^{25, 26}. Generally, the Gram-negative bacteria are the most sensitive to antibacterial agents than the Gram positive-bacteria. This difference is explained by the extra lipopolysaccharides and protein cell wall of Gram-negative bacteria that provides a permeability barrier to the antibacterial agent. On the other hand, the sensitivity of Gram-positive bacteria is related to the single layer of their cell wall, while the double membrane of Gram-negative bacteria should make them less sensitive²⁷.

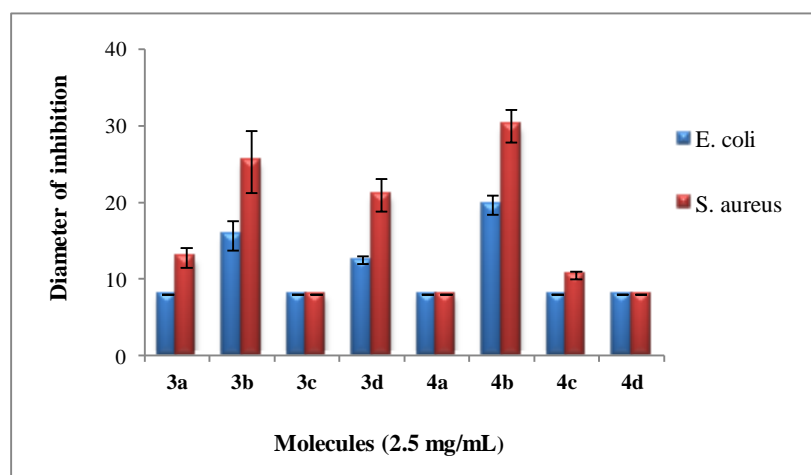


Figure 1. Antibacterial activity of 8 compounds at 2.5 mg/mL against *E. coli* and *S. aureus*. Bacterial density was about 10⁶ CFU/mL, the diameter of well (8 mm) is included and the experiment was carried in triplicate. The results are expressed as diameter around the well in mm ± SD

Determination of the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC)

The minimum inhibitory concentration (MIC) is defined as the minimum level of compound concentration that induces 90% reduction in the

growth of microbial colonies. The MBC is defined as the minimum level of compound concentration that induces at least a 99.9% reduction in the growth of microbial colonies²⁸. The MIC and MBC were determined by optical density and microdilution agar plate methods. Inoculums were prepared by inoculating medium LB with an overnight culture of

S. aureus and *E. coli* incubating for three hours. 1 mL of aliquot of inoculums was added to 9 mL of medium of LB containing 0.15% of agar. Compounds were added to give the following final concentrations: 15, 10, 5, 2.5, 1.25 and 0.75 mg/mL. After 24 h of incubation at 37°C, the optical density of the broths at 615nm was measured with a UV-visible spectrophotometer (blank curve of absorbance at 620 nm).

$$\text{Absorbance} = 61.901 (\text{CFU/mL}) - 7.8769 \text{ for } S. \text{ aureus}$$

$$\text{Absorbance} = 5.9081 e^{2.5973 (\text{CFU/mL})} \text{ for } E. \text{ coli}$$

The optical densities of cultures of the inoculums in LB with different concentrations of compounds were determined and the equation above was used to infer the microbial populations. In that way, profiles of microbial counts vs. compounds concentration were obtained as indicated in the profile of **4b** against *S. aureus*. The microbial populations for molecule-free tests were 1.5×10^6 CFU/mL (Standard value). Therefore, the MIC was established through the microbial concentration of 1.5×10^5 CFU/ml as the same for the MBC one.

The profiles obtained **3b** and **4b** are shown in Figures 2,3 respectively. For the two tested compounds, the antibacterial effect is dose-dependent. Indeed, at low concentrations, small changes in concentration produced large changes in the microbial growth. While, at high concentration, large changes in concentrations were needed to produce only minor changes in microbial growth. The values of MICs and MBCs are indicated in (Table 3). The compound **4b** showed a MIC value at 6.5 mg/mL against *S. aureus* and 13 mg/ml against *E. coli*. While, the molecule **3b** has a MIC = 11.5 mg/mL against *S. aureus* and MIC = 13 mg/mL. On

To evaluate the MIC and MBC, only compounds with inhibition halo with a higher diameter were tested (compounds **4b** and **3b**). The optical density method using a reference curve of absorbance at 620 nm against the microbial concentration as CFU/mL, was constructed and used to test the effect of two molecules on the bacterial populations. The resulting points were fitted with a logarithmic tendency line of the form.

the other hand, MBC was not reached at the highest concentration tested (15 mg/mL). This difference between MIC and MBC reveals that these two compounds at MIC values have a bacteriostatic action on the two bacterial strains. This action could be attributed to some specific interactions with bacterial targeting pathways, which induce a decrease in bacterial growth without bacterial lysis. Indeed, several studies showed numerous targeting pathways such as a disruption of potassium transport, protein coagulation and quorum sensing inhibition²⁹⁻³⁴. These results would be partially in accordance with the results of the Agar-well diffusion method in which the Compound **4b** presented the largest inhibition halo than other molecules including the molecule **3b**. Several studies have shown the antibacterial activity of benzimidazole derivatives^{1,35}. The effects of the latter have demonstrated an inhibition, at low concentration, against the same tested bacteria in our studies (*E. coli* and *S. aureus*). The difference between these results is explained by the difference in functional molecular structure and methods used to reveal antibacterial inhibition.

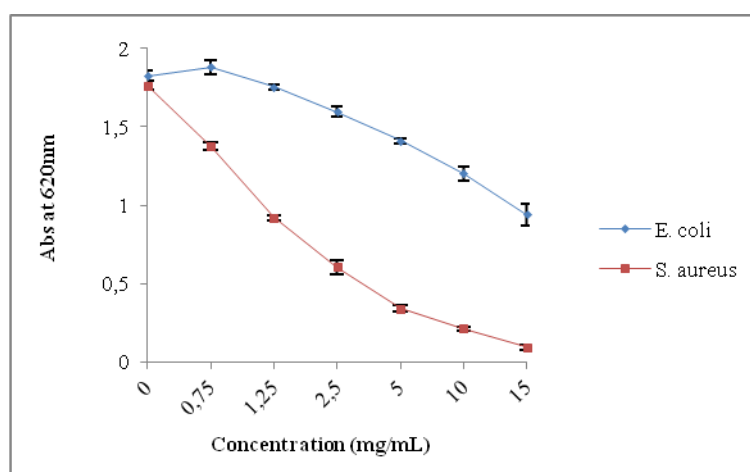


Figure 2. Absorbance at 620 nm of culture media incubated for 24 h with different concentrations of compound **3b** against *E. coli* and *S. Aureus*

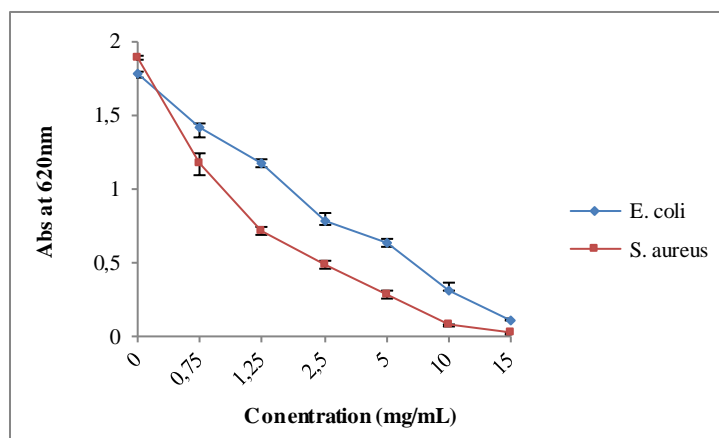


Figure 3. Absorbance at 620 nm of culture media incubated for 24 h with different concentrations of compound **4b** against *E. coli* and *S. aureus*

Table 3. Values of MIC and MBC for 3b and 4b compounds as obtained by the optical density method

Entry	<i>E. coli</i>		<i>S. aureus</i>	
	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)
3b	13	>15	11.5	>15
4b	13	>15	6.5	>15

Kinetics of bacterial growth

This study was conducted to evaluate the effect of compound **4b** (showing the best antibacterial activity) on the kinetics of bacterial growth. Growth curves of bacteria in the presence of different concentrations of this compound against *E. coli* as showed in Figure 4 and against *S. Aureus* (Figure 5).

For *E. coli*, the growth is totally reduced at a concentration 2MIC = 26 mg/mL. While, at the MIC and MIC/2 = 6.5 mg/mL concentrations the growth reduction starts after 4hs of incubation. These results revealed that a sub-inhibitor affects *E. coli* in the initial phase of growth, while at the concentration of MIC and (MIC / 2), this compound affects only the

bacterial growth during stationary stage. For *S. Aureus*, compound **4b** affects the bacterial growth at MIC and 2 MIC (13 mg/mL) just after incubation (action during exponential stage). After 4hs of incubation, *S. aureus* has a normal growth. This result could be explained by a resistance mechanism against *S. aureus* after 4h of incubation (time necessary for *S. aureus* to establish a resistance mechanism). On the other hand, several studies have showed that *S. aureus* can resist against numerous antibacterial agents by several mechanisms³⁶⁻³⁸. However, the finding of our results suggesting that our molecules exert their action on specific targeting against this strain.

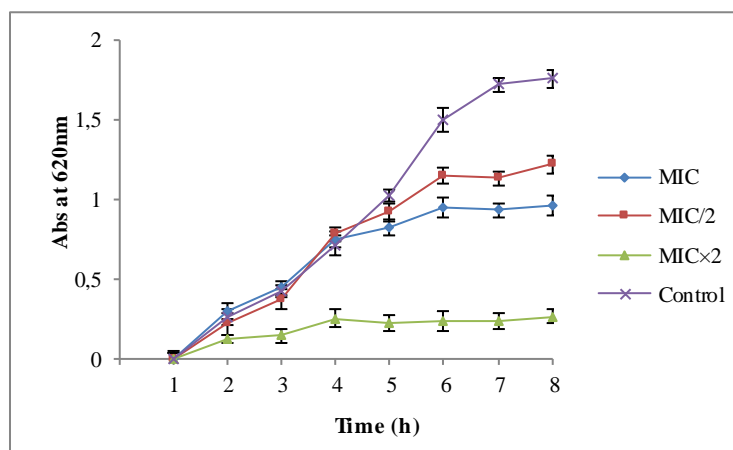


Figure 4. Kinetics of bacterial growth of *E. coli* of different concentrations of compound **4b** (MIC, MIC/2, MICx2) and control without molecule. Experiments were performed in duplicate.

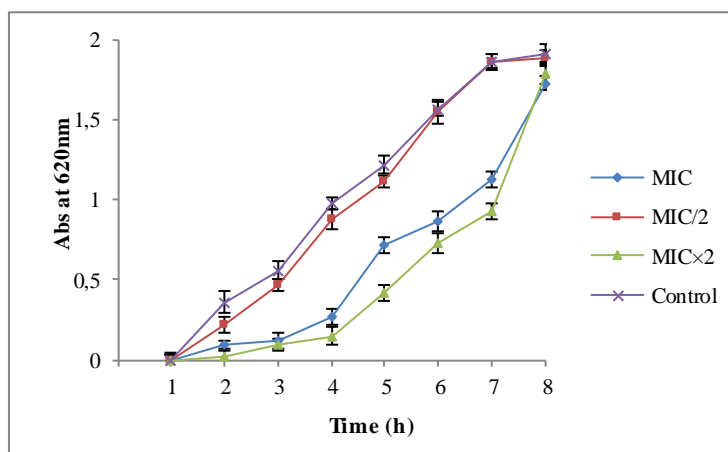


Figure 5. Kinetics of bacterial growth of *S. aureus* of different concentrations of compound **4b** (MIC, MIC/2, MIC \times 2) and control without compound. Experiments were performed in duplicate.

Antioxidant activity

DPPH radical scavenging capacity assay

The results of the antioxidant activity tested by scavenging DPPH radical of cations 2-mercaptobenzimidazolium salts are shown in the

(Figure 6 and Figure 7). They are expressed in scavenging percentage of DPPH radical. All compounds have showed similar antioxidant effect with some non-significant variability.

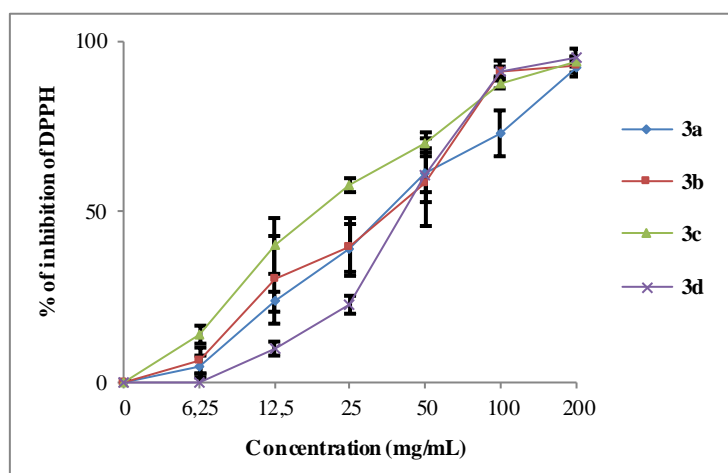


Figure 6. DPPH radical scavenging activities (%) of compounds (**3a**, **3b**, **3c**, **3d**) Values are means \pm standard deviation of three determinations.

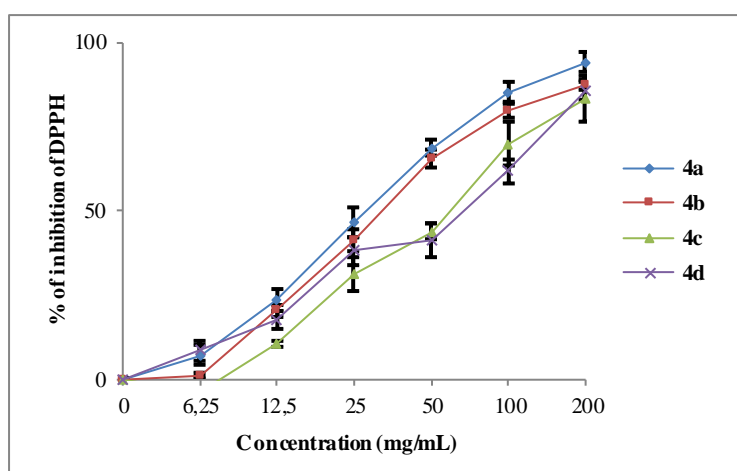


Figure 7. DPPH radical scavenging activities (%) of compounds (**4a**, **4b**, **4c**, **4d**) Values are means \pm standard deviation of three determinations.

Furthermore, for each compound, the ability to reduce the DPPH radical is concentration dependent. Therefore, the calculation of the effective concentration which reduces by 50% the initial concentration of DPPH (IC_{50}) is used to express the antiradical activity of studied molecules (Table 4). This capacity was determined from the graph of inhibition capacity (AA in %) against compounds by using linear extrapolation. The value of the antioxidant capacity 50 is inversely proportional to the antiradical effectiveness of the compounds tested. It is clear that all compounds showed a lower capacity to reduce DPPH radical compared to the standard antioxidant (Trolox and ascorbic acid). The molecule **3c** is the most active ($IC_{50}=19.53$ mg/mL). While, **4d** was the most inactive molecule ($IC_{50}=71.87$ mg/mL). Some studies have revealed the antioxidant activity of benzimidazole derivatives ^{1,3}. The difference between results could be attributed to the molecular structure of synthesized compound and used method to provide antioxidant properties. The DPPH assay is a very common spectrophotometric method to determine the activity of any antioxidant. We have chosen DPPH radical due to the advantage of this method as the antiradical activity is measured at ambient temperature, and thus, the risk of the thermal degradation of the compound tested is eliminated ³⁹

Table 4. IC_{50} (mg/mL) values of **3a**, **3b**, **3c**, **3d**, **4a**, **4b**, **4c**, **4d**

Entry	IC_{50} (mg/mL)
3a	37,5
3b	39,06
3c	19,53
3d	43,75
4a	29,68
4b	34,37
4c	62,5
4d	71,87

Conclusion

In summary, we have developed an easy and efficient procedure for the synthesis of novel benzimidazolium. These compounds were prepared by condensation of 2-mercaptobenzimidazole with the various alkylating agents under the conditions of phase transfer catalysis, followed by a quaternization. There were screened for them in-vitro antibacterial activity against: Escherichia coli as Gram negative and Staphylococcus aureus as Gram positive. Results revealed that, compounds 1,3-Dinonyl-2-(nonylthio)-1H-benzimidazolium bromide **4b** and 1-nonyl-2-(nonylthio)-1H-benzimidazole **3b** showed important antibacterial activity against two microorganisms especially Gram positive-bacteria. The kinetics of bacterial growth showed that compound **4b** has a specific targeting against S. aureus and E. coli. Therefore, this

molecule could be developed as a promising antibacterial agent. However, other studies regarding its mechanisms of action are needed, especially focusing on intracellular targeting pathways that leading to the bacterial cell death.

The radical scavenging ability of compounds **3a-d** and **4a-d** was tested by DPPH assay method. Higher the percentage inhibition for lower concentration is considered to be more antioxidant. It was found that synthesized compounds possess moderate antioxidant activity. These results demonstrated that molecules are not capable to give free electron to reduce the DPPH free radical. However, other studies on antioxidant activities of our molecules need investigation by other antioxidant assays such as Ferric reduction assay, APTS assay and FRAP to compare results.

Acknowledgments

****This work is dedicated to the memory of our Great Professor Rachid ZNIBER who passed away on December 27th 2016.**

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Experimental

Reagents were purchased from commercial sources and used as received without purification. Solvents were dried by standard procedures. ¹H NMR spectra were recorded in CDCl₃ on a Bruker 300 spectrometer (300 MHz) and ¹³C NMR spectra were recorded in CDCl₃ on a Bruker 300 spectrometer (75 MHz). Chemical shift values were quoted in part per million and the coupling constants (J) in Hertz. Chemical shift multiplicities were reported as s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet....

Typical Procedure for Synthesis of **3a-3d**

To a solution of (133.10^{-4} mol) of 2-mercapto-benzimidazole and 80 ml of N,N-dimethylformamide was added (26.6. mmol) of potassium carbonate, (1.33 mmol) of tetra-n-butylammonium bromide and (39.9 mmol) of 1-bromodecane.

The reaction mixture was stirred at room temperature for 24 hours. After filtration, the solvent was removed under reduced pressure and the residue is taken up in dichloromethane, filtered and the solvent was evaporated under reduced pressure.

1-octyl-2-(octylthio)-1H-benzimidazole (**3a**).

Yield: 88%, pale yellow liquid,

¹H NMR spectrum (300 MHz, CDCl₃), δ , ppm (J, Hz): 0.86 (m, 6H, CH₃); 1.25-1.81 (m, 24H, CH₂); 3.44 (t, 2H, SCH₂, J= 14.7); 4.06 (t, 2H, NCH₂, J = 14.7); 7.18-7.73 (4H, m, H Ar).

^{13}C NMR spectrum (75 MHz, CDCl_3), δ , ppm (J , Hz): 14.1 (CH_3); 22.6-31.8 (CH_2); 32.8 (SCH_2); 44.3 (NCH_2); 108.8-142.4 (C Ar); 152.1 (C=N).
Mass spectrum $[\text{MH}]^+ m/z = 375$.

1-nonyl-2-(nonylthio)-1H-benzimidazole (3b).

Yield: 91%, pale yellow liquid,

^1H NMR spectrum (300 MHz, CDCl_3), δ , ppm (J , Hz): 0.97 (6H, m, CH_3); 1.20-1.76 (28H, m, CH_2); 3.42 (t, 2H, SCH_2 , $J = 14.7$); 4.05 (t, 2H, NCH_2 , $J = 14.7$); 7.16-7.70 (4H, m, H Ar).

^{13}C NMR spectrum (75 MHz, CDCl_3), δ , ppm (J , Hz): 14.1 (CH_3); 22.6-31.8 (CH_2); 32.9 (SCH_2); 44.4 (NCH_2); 108.9-141.7 (C Ar); 152.0 (C=N).

Mass spectrum $[\text{MH}]^+ m/z = 403$.

1-decyl-2-(decylthio)-1H-benzimidazole (3c).

Yield: 93%, pale yellow liquid,

^1H NMR spectrum (300 MHz, CDCl_3), δ , ppm (J , Hz): 0.86 (6H, m, CH_3); 1.24-1.84 (32H, m, CH_2); 3.52 (t, 2H, SCH_2 , $J = 14.7$); 4.09 (t, 2H, NCH_2 , $J = 14.7$); 7.24-7.80 (4H, m, H Ar).

^{13}C NMR spectrum (75 MHz, CDCl_3), δ , ppm (J , Hz): 14.1 (CH_3); 22.7-31.9 (CH_2); 33.1 (SCH_2); 44.6 (NCH_2); 109.1-135.1 (C Ar); 151.9 (C=N).

Mass spectrum $[\text{MH}]^+ m/z = 431$.

Anal. Calc for $\text{C}_{27}\text{H}_{46}\text{N}_2\text{S}$ C, 75,34%, H, 10,69%, N, 6,51%, S, 7,44% Found: C, 74,49%, H, 10,64%, N, 6,24%, S, 7,03%.

1-dodecyl-2-(dodecylthio)-1H-benzimidazole (3d).

Yield: 93%, pale yellow liquid,

^1H NMR spectrum (300 MHz, CDCl_3), δ , ppm (J , Hz): 0.84 (6H, m, CH_3); 1.21-1.81 (40H, m, CH_2); 3.50 (t, 2H, SCH_2 , $J = 14.4$); 4.08 (t, 2H, NCH_2 , $J = 14.7$); 7.22-7.77 (4H, m, H Ar).

^{13}C NMR spectrum (75 MHz, CDCl_3), δ , ppm (J , Hz): 13.9 (CH_3); 22.7-34.1 (CH_2); 36.5 (SCH_2); 44.6 (NCH_2); 109.1-135.1 (C Ar); 151.9 (C=N).

Mass spectrum $[\text{MH}]^+ m/z = 487$.

Anal. Calc for $\text{C}_{31}\text{H}_{54}\text{N}_2\text{S}$ C, 76,54%, H, 11,11%, N, 5,76%, S, 6,59% Found: C, 75,19%, H, 10,86%, N, 5,53%, S, 6,39%.

General Procedure for Synthesis of 4a-4d

To a solution of (26.10^{-4} mol) of 1-alkanyl-2-(alkanylthio)-1H-benzimidazole and 40 ml of acetonitrile was added (53.10^{-4} mol) of 1-bromoalkane.

The reaction mixture was refluxed for 72 hours. After evaporating, the solvent was removed under reduced pressure. The oil obtained is chromatographed on silica gel column (eluent: hexane).

1,3-Dioctyl-2-(octylthio)-1H-benzimidazolium bromide (4a).

Yield: 88%, pale yellow liquid,

^1H NMR spectrum (300 MHz, CDCl_3), δ , ppm (J , Hz): 0.85 (6H, m, CH_3); 1.24-1.86 (36H, m, CH_2); 3.38 (t, 2H, SCH_2 , $J = 13.8$); 4.29 (t, 2H, NCH_2 , $J = 15.3$); 7.18-7.26 (4H, m, H Ar).

^{13}C NMR spectrum (75 MHz, CDCl_3), δ , ppm (J , Hz): 14.1 (CH_3); 22.6-32.9 (CH_2); 34.1 (SCH_2); 44.8 (NCH_2); 109.1-132.1 (C Ar); 168.9 (C=N).

Electrospray ionization mass spectrometry (ESI-MS) $[\text{M}]^+ m/z = 487$.

1,3-Dinonyl-2-(nonylthio)-1H-benzimidazolium bromide (4b).

Yield: 80%, pale yellow liquid,

^1H NMR spectrum (300 MHz, CDCl_3), δ , ppm (J , Hz): 0.85 (6H, m, CH_3); 1.24-1.86 (36H, m, CH_2); 3.38 (t, 2H, SCH_2 , $J = 13.8$); 4.29 (t, 2H, NCH_2 , $J = 15.0$); 7.17-7.26 (4H, m, H Ar).

^{13}C NMR spectrum (75 MHz, CDCl_3), δ , ppm (J , Hz): 14.1 (CH_3); 22.6-32.9 (CH_2); 34.0 (SCH_2); 44.8 (NCH_2); 109.0-132.1 (C Ar); 169.0 (C=N).

Electrospray ionization mass spectrometry (ESI-MS) $[\text{M}]^+ m/z = 529$.

1,3-Didecyl-2-(decylthio)-1H-benzimidazolium bromide (4c).

Yield: 83%, pale yellow liquid,

^1H NMR spectrum (300 MHz, CDCl_3), δ , ppm (J , Hz): 0.84 (6H, m, CH_3); 1.22-1.84 (36H, m, CH_2); 3.36 (t, 2H, SCH_2 , $J = 13.8$); 4.27 (t, 2H, NCH_2 , $J = 15.3$); 7.16-7.40 (4H, m, H Ar).

^{13}C NMR spectrum (75 MHz, CDCl_3), δ , ppm (J , Hz): 14.1 (CH_3); 22.7-32.8 (CH_2); 33.9 (SCH_2); 44.8 (NCH_2); 108.9-132.1 (C Ar); 169.1 (C=N).

Electrospray ionization mass spectrometry (ESI-MS) $[\text{M}]^+ m/z = 571$.

1,3-Didodecyl-2-(dodecylthio)-1H-

benzimidazolium bromide (4d). Yield: 78%, pale yellow liquid,

^1H NMR spectrum (300 MHz, CDCl_3), δ , ppm (J , Hz): 0.87 (6H, m, CH_3); 1.25-1.86 (36H, m, CH_2); 3.38 (t, 2H, SCH_2 , $J = 13.8$); 4.29 (t, 2H, NCH_2 , $J = 15.0$); 7.17-7.26 (4H, m, H Ar).

^{13}C NMR spectrum (75 MHz, CDCl_3), δ , ppm (J , Hz): 14.1 (CH_3); 22.7-32.9 (CH_2); 33.8 (SCH_2); 44.9 (NCH_2); 108.9-132.1 (C Ar); 169.1 (C=N).

Electrospray ionization mass spectrometry (ESI-MS) $[\text{M}]^+ m/z = 655$.

Antibacterial activity

In order to evaluate the antibacterial activity of the synthesized compounds, two bacteria strains were used: *Escherichia coli* K12 (Laboratory of Food Microbiology, UCL, Belgium: MBLA) and *Staphylococcus aureus* CECT 976 (Spanish Type Culture Collection: CECT). Strains are maintained on an inclined agar medium at 4°C . Before use, the bacteria were revived by two subcultures in an appropriate culture medium: Luria-Bertoni (LB) broth at 37°C for 18 to 24 hours. For the test, final inoculum concentrations of 10^6 CFU/ml bacteria were used.

A basal layer was prepared by Muller-Hinton agar. The agar plates were then solidified and sterile 8 mm diameter cylinders were deposited.

Six ml of LB medium in superfusion containing 0.8% agar were inoculated by a fresh culture of bacterial strain indicator (a final concentration was 10^6 CFU/mL). After solidification, the wells were filled with 50 μ L of diluted molecules at 2.5 mg/mL²⁵. After incubation at appropriate temperature for 24 h, all plates were examined for any zone of growth inhibition, and the diameter of these zones were measured in millimetres.

Minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC)

The minimum inhibitory concentration (MIC) is defined as the minimum level of compound concentration that induces 90% reduction in the growth of microbial colonies. The MBC is defined as the minimum level of compound concentration that induces at least a 99.9% reduction in the growth of microbial colonies²⁸. The MIC and MBC were determined by optical density and microdilution agar plate methods. Inoculums were prepared by inoculating medium LB with an overnight culture of *S. aureus* and *E. coli* incubating for three hours. 1 mL of aliquot of inoculums was added to 9 mL of medium of LB containing 0.15% of agar. Compounds were added to give the following final concentrations: 15, 10, 5, 2.5, 1.25 and 0.75 mg/mL. After 24 h of incubation at 37°C, the optical density of the broths at 615nm was measured with a UV-visible spectrophotometer (blank curve of absorbance at 620 nm).

Kinetics of bacterial growth

Inoculums were prepared by inoculating medium LB with an overnight culture of *S. aureus* and *E. coli* incubating for three hours. 1 mL of aliquot of inoculums was added to 9 ml of medium of LB containing 0.15% of agar. The compounds **4b** and **3b** were added to each tube to achieve final concentrations of compound of 2 MIC, MIC and MIC/2. The bacterial culture used without compounds was considered as negative control. The tubes were incubated at 37° C. The optical density at a wavelength of 620 nm was measured every hour during the period of growth in order to monitor the bacterial growth. Experiments were performed in triplicate.

Antioxidant activity: DPPH radical scavenging capacity assay

Aliquots (0.2 mL) of various concentrations (6.25–200 mg/mL) of the synthetic compounds ~~samples~~ were added to 1.8 mL of a 0.004% methanolic solution of DPPH. After an incubation period of 30 min in darkness at room temperature, the absorbance was recorded against a blank at 517 nm with a spectrophotometer. Absorption of a blank sample containing the same amount of methanol and DPPH solution used as control. DPPH free radical-

scavenging activity in percentage (%) was calculated using the following formula:

$$\% \text{ Inhibition} = [(A_{\text{blanc}} - A_{\text{sample}}) / A_{\text{blanc}}] \times 100$$

Where A_{blanc} is the absorbance of the control reaction (containing all reagents except the test compound) and A_{sample} is the absorbance of the test compound. Compounds concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotted of inhibition percentage.

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