BIOLOGICAL ACTIVITY EVALUATION OF NOVEL N-HETEROCYCLIC CARBENE PRECURSORS

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ABSTRACT: Five novel benzimidazolium salts were synthesized as N-heterocyclic carbene (NHC) precursors (1a-e). They were characterized by different techniques. The antimicrobial activities of these compounds were tested. The compounds showed moderate activity against three Gram (+) and seven Gram (-) bacteria in comparison to tetracycline, which was used as the standard antibiotic, whereas all the compounds (1a-e) showed no activity against the test yeast C. albicans.

INTRODUCTION: In the past few decades, infections caused by multi-drug resistant bacteria have increased at frightening levels all over the world. Microbial infections are a growing problem in contemporary medicine and the use of antibiotics is common worldwide. In particular, infections caused by the Gram-positive bacterium Staphylococcus aureus and species of the genus Enterococcus have become a major worldwide health problem due to their ability to develop resistance to multiple antibiotics. To overcome these emerging resistance problems, there is an urgent need to discover novel chemotherapeutic agents, which have a broad spectrum of activity and, if possible, with new modes of action.

The benzimidazole nucleus is a constituent of many bioactive N-containing heterocyclic compounds. Specifically, this nucleus is a constituent of Vitamin-B12. Benzimidazole and its derivatives have been reported to show various pharmacological activities, such as antiarrhythmic, antifungal, antiviral, antihelmintic and inotropic activities and are used as a treatment for intestinal cystitis.

Additionally, it was reported to possess antitumor, antihistamine, antiulcer, antibacterial, antiproliferative activities and cytotoxicity as well as anti-inflammatory, analgesic, antioxidant, antiallergic, antikinase, anticancer and anti-HIV activities. Its application in the treatment of diverse diseases like diabetes, epilepsy and for antifertility have also been reported. The biological activities showed by compounds containing benzimidazole moiety have prompted chemists to synthesis more and more benzimidazole libraries and screen them for potential activities.
Encouraged by these observations, and as a continuation of our earlier work on biologically important heterocycles, we report herein the synthesis of a novel series of 1-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl) methyl) - 3-alkyl benzimidazolium salts (1a-e) and the in vitro screening results of their antimicrobial activities.

Experimental:
General considerations: Using Schlenk techniques, the necessary reactions for the synthesis of benzimidazolium salts (1a-e) were carried out under argon. All reagents and chemicals were commercially purchased from various firms. For all the new benzimidazolium salts, 1D NMR methods such as 1H NMR and 13C NMR were taken in DMSO-d6 and CDCl3. The 1H and 13C NMR spectra were recorded by using a Bruker AC300P FT spectrometer operating at 300.13 MHz (1H NMR) and 75.47 MHz (13C NMR). Taking into account TMS, chemical shifts (δ) were given in ppm. Coupling constants (J) were generated in hertz (Hz). 1H NMR peaks are labeled as singlet (s), doublet (d), triplet (t), quartet (q), pentet (p), sextet (h) and multiplet (m) signals. The FT-IR spectra of the synthesized benzimidazolium salts were recorded in the 450–4000 cm⁻¹ region with a Shimadzu FT-IR 8400 spectrophotometer and wave numbers are given in cm⁻¹. Melting points (m.p.) were determined in glass capillary tubes by using an Electrothermal-9200 melting point appliance. Elemental analyses were performed by means of a TruSpec MICRO elemental analysis device.

General preparation of 1-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methyl)-3-alkyl benzimidazolium salts, 1a-e: Firstly, the benzimidazole nucleus was synthesized from o-phenylenediamine (20 g), formic acid (11 ml) and water (2 ml). Then, alkyl halides (1 mmol) and potassium hydroxide were added to a solution of benzimidazole (1 mmol) in ethyl alcohol (10 ml). The resulting mixture was refluxed for 12 h. In the third step, 1-bromomethyl-1,4-benzodioxane was added to N-alkyl benzimidazole for the synthesis of different benzimidazolium salts (1a-e) and was stirred at 80 °C for 24 h. The obtained compounds were washed with diethylether (3x15 ml) and dried under vacuum. The product was crystallized from ethyl alcohol-diethyl ether (2:1) mixture at room temperature.

1-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methyl)-3-methylbenzimidazolium bromide, 1a: Yield: 87%; m.p.: 309-310 °C; FT-IR νCN: 1492.8 cm⁻¹. 1H NMR (300 MHz, CDCl3), δ: 4.29 (s, 3 H, CH3); 4.32 and 4.92 [m, 2 H, NCH2CH(CH2)O]; 4.98 [m, 1 H, NCH2CH(CH2)O]; 5.45 and 5.57 [m, 2 H, NCH2CH(CH2)O]; 6.79-6.88 (m, 4 H, Ar-HBenzodioxane); 7.46-7.74 (m, 4 H, Ar-HBenzimidazole); 11.21 (s, 1 H, 2-CH). 13C NMR (75.47 MHz, CDCl3), δ: 32.5 (CH3); 47.5, 64.7 and 72.3 [NCH2CH(CH2)O]; 112.5, 113.9, 117.5, 122.0, 122.1, 125.1, 127.4, 127.5, 131.6, 132.2, 141.4 and 142.8 (Ar-C); 143.4 (2-CH). Anal. Calcd. for C17H17N2O2Br (361.23 g/mol): C 56.52, H 4.74, N 7.70 %.

1-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methyl)-3-ethylbenzimidazolium bromide, 1b: Yield: 79%; FT-IR νCN: 1448.7 cm⁻¹. 1H NMR (300 MHz, DMSO-d6), δ: 1.54 (t, 3 H, J: 6.9 Hz, CH2CH3); 4.52 (q, 2 H, J: 6.9 Hz, CH2CH3); 4.53 and 4.82 [m, 2 H, NCH2CH(CH2)O]; 4.97 (m, 1 H, NCH2CH(CH2)O); 4.12 and 4.81 [m, 2 H, NCH2CH(CH2)O]; 6.83-6.86 (m, 4 H, Ar-HBenzodioxane); 7.45-7.92 (m, 4 H, Ar-HBenzimidazole); 10.10 (s, 1 H, 2-CH). 13C NMR (75.47 MHz, DMSO), δ: 14.5 and 42.7 (CH2CH3); 47.2, 64.9 and 71.2 [NCH2CH(CH2)O]; 114.0, 114.3, 117.6, 117.7, 122.3, 127.2, 131.2, 132.0, 142.1 and 142.5 (Ar-C); 143.0 (2-CH). Anal. Calcd. for C19H19N2O2Br (375.26 g/mol): C 57.61, H 4.85, N 7.70 %.

1-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methyl)-3-butylbenzimidazolium bromide, 1c: Yield: 89%; m.p.: 287-288 °C; FT-IR νCN: 1485.1 cm⁻¹. 1H NMR (300 MHz, CDCl3), δ: 1.01 (t, 3 H, J: 6.9 Hz, CH3CH2CH2CH3); 1.49 (h, 2 H, J: 6.9 Hz, CH2CH2CH2CH3); 2.02 (p, 2 H, J: 6.9 Hz, CH2CH2CH2CH3); 4.88 (t, 2 H, J: 6.9 Hz, CH2CH2CH2CH3); 4.31 and 4.32 [m, 2 H, NCH2CH(CH2)O]; 4.93 [m, 1 H, NCH2CH(CH2)O]; 4.58 and 5.45 [m, 2H, NCH2CH(CH2)O]; 6.66-6.85 (m, 4 H, Ar-HBenzodioxane); 7.56-7.73 (m, 4 H, Ar-HBenzimidazole); 11.35 (s, 1 H, 2-CH). 13C NMR (75.47 MHz, CDCl3), δ: 13.5, 19.9, 31.1 and 47.6 (CH3CH2CH2CH3); 47.7, 64.8 and 72.4 [NCH2CH(CH2)O]; 112.7, 114.2, 117.4, 117.6, 122.0, 122.1, 127.2, 130.8, 132.4 and 141.4 (Ar-C); 142.8 (2-CH). Anal. Calcd. for C20H23N2O2Br

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1-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methyl)-
(2-methoxyethyl)benzimidazolium bromide, 1d:
Yield: 81%; m.p.: 167-168 °C; FT-IR (KBr): 1486.3
cm⁻¹. ¹H NMR (300 MHz, CDCl₃), δ: 3.36 (s, 3 H,
CH₂CH₂OCH₃); 3.95 (t, 2 H, J: 4.5 Hz,
CH₂OCH₂CH₃); 4.01 (t, 2 H, J: 4.5 Hz,
CH₂CH₂OCH₃); 4.29 and 4.58 [m, 2 H,
NCH₂(CH₂)₂O]; 4.90 (m, 1 H, OCH₂CHO); 4.88
and 5.38 [m, 2 H, NCH₂(CH₂)₂O]; 6.67-6.84 (m,
4 H, Ar-H(Benzimidazole)); 7.44-8.02 (m, 4 H, Ar-
H(Benzimidazole)); 10.86 (s, 1 H, 2-CH). Anal. Calcd.
for C₂₁H₂₁N₂O₂Br (403.29 g/mol): C 58.17, H 5.26,
N 6.47 %. Found: C 58.25, H 5.33, N 6.96 %.

1-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methyl)-
3-(2-ethoxyethyl)benzimidazolium bromide, 1e:
Yield: 86%; m.p.: 139-140 °C; FT-IR (KBr): 1496.7
cm⁻¹. ¹H NMR (300 MHz, CDCl₃), δ: 1.14 (t, 3 H,
J: 7.2 Hz, CH₂CH₂OCH₂CH₃); 3.84 (q, 2 H, J: 7.2
Hz, CH₂OCH₂CH₃); 3.90 (t, 2 H, J: 4.8 Hz,
CH₂OCH₂CH₃); 4.72 (t, 2 H, J: 4.8 Hz,
CH₂CH₂OCH₂CH₃); 4.34 and 4.58 [m, 2 H,
NCH₂(CH₂)₂O]; 4.98 (m, 1 H, OCH₂CHO); 4.68
and 5.45 [m, 2 H, NCH₂(CH₂)₂O]; 6.75-6.98 (m,
4 H, Ar-H(Benzimidazole)); 7.32-7.69 (m, 4 H, Ar-
H(Benzimidazole)); 10.95 (s, 1 H, 2-CH). ¹³C NMR
(75.47 MHz, CDCl₃), δ: 15.0, 47.6, 48.3 and 48.3
(CH₂CH₂OCH₂CH₃); 64.8, 66.1 and 72.1
[OCH₂(CH₂)₂O]; 113.2, 113.8, 117.5, 117.6,
122.0, 122.1, 127.0, 127.1, 131.6, 132.1, 141.1
and 142.8 (Ar-CH); 143.1 (Ar-CH). Anal. Calcd.
for C₂₁H₂₁N₂O₂Br (419.31 g/mol): C 57.29, H 5.53,
N 6.68. Found: C 57.38, H 5.48, N 6.72 %.

Evaluation of antimicrobial activity: Eleven
microorganisms, consisting of ten bacteria and one
yeast, were used as test organisms: Aeromonas
hydrophila (ATCC 7965), Escherichia coli (ATCC
25922), Klebsiella pneumoniae (FMC 5), Proteus
mirabilis (BC 3624), Pseudomonas aeruginosa
(ATCC 27853), Salmonella typhimurium (NRRLE
4463), Yersinia enterocolitica (ATCC 1501),
Bacillus cereus (FMC 19), Listeria monocytogenes
(1/2B), Staphylococcus aureus (ATCC 29213) and
Candida albicans (ATCC 1223).

The antimicrobial activities of the five compounds
1a-e were studied in vitro using the agar-well
diffusion method. The stock solutions (10 mg ml⁻¹)
of the test compounds were prepared by dissolving
them in dimethyl sulfoxide (DMSO). All
samples were sterilized through a 0.2 µm
membrane filter. C. albicans and Y. enterocolitica
were grown in malt extract and nutrient broths at
25°C for 18 h, respectively. The other
microorganisms were grown in nutrient broth at
35°C for 18 h and suspensions were adjusted to 0.5
McFarland standard turbidity. Then 250 µl of each
microorganism was added into a flask containing
25 ml sterile Mueller hinton agar or malt extract
agar at 45 °C and poured into Petri dishes (9 cm
diameter). The agars were then allowed to solidify
at 4 °C for 1 h. Holes were made in the agar using
sterile cork borers (Ø = 6 mm). Solutions of the
compounds (50 µl) were applied to the holes using
a pipettor and DMSO was used as a control. Y.
enterocolitica and C. albicans were incubated at
25°C for 14-24 h in the inverted position. The other
microorganisms were incubated at 35 °C for 18-24
h. At the end of the period, the inhibition zones
which formed on the medium were measured in
millimeters (mm). Tetracycline (10 mg ml⁻¹)
(Sigma T3258-56) standard antibiotic was used as
positive control.

RESULTS AND DISCUSSION:
Synthesis of new benzimidazolium salts: Five
novel benzimidazolium salts as NHC precursors
were synthesized by quaternization of N-
alkylbenzimidazole with 2-bromomethyl-1,4-
benzodioxane in dimethylformamide (Scheme 1).
These synthesized salts 1a-e were characterized by
means of FT-IR, 1D NMR methods such as ¹H and
¹³C NMR and elemental analysis. In the ¹H NMR
spectra, the resonances of important protons NCHN
from benzimidazole moiety prove the structures of
the synthesized novel benzimidazolium salts as
NHC precursors. Characteristic peaks (NCHN)
were observed as sharp singlets at 11.21, 10.10,
11.35, 10.86 and 10.95 ppm for 1a-e, respectively.
¹³C NMR chemical shifts corresponded to the
suggested structures of benzimidazolium salts; the
imino carbons are typical singlets in the ¹H-
decoupled mode at 143.4, 143.0, 142.8 and 143.1
ppm for 1a-c and 1e, respectively.
The FT-IR data clearly indicate the presence of \(-C=N-\) with a \(\nu(C=N)\) at 1492.8, 1448.7, 1485.1, 1486.3 and 1496.7 cm\(^{-1}\) for benzimidazolium salts 1a-e, respectively.

**SCHEME 1: SYNTHESIS OF NOVEL BENZIMIDAZOLIUM SALTS (1a-e).**

Evaluation of the antimicrobial activity: Five novel synthesized compounds (1a-e) were assayed *in vitro* for their antimicrobial activity against seven Gram (-) bacteria (*A. hydrophila, E. coli, K. pneumoniae, P. mirabilis, P. aeruginosa, S. typhimurium* and *Y. enterocolitica*), three Gram (+) bacteria (*B. cereus, L. monocytogenes* and *S. aureus*) and one yeast (*C. albicans*). The results of antibacterial activity are shown in *Table 1*. Compounds 1a-e exerted moderate activity against the tested bacterial species. Among the tested compounds only 1c, containing the n-butyl substituent, and 1a, containing the methyl group, were effective against *E. coli*. Also only 1b, containing the ethyl group, and 1e had an inhibitory effect on *S. typhimurium* among the tested compounds. It could be said that 1c had a wide spectrum of antibacterial activity as it was able to inhibit all test bacterial organisms comparable to other tested compounds. This result suggests that n butyl group substituted benzimidazole ring showed higher antibacterial activity in comparison to the other substituted analogues. On the other hand 1a, which only had a slight effect against *E. coli* (7.0 mm), had the least activity among the tested compounds. Compounds 1a-e showed no inhibitory effect on *C. albicans* at 10 mg ml\(^{-1}\) concentration (not shown in the *Table 1*). For evaluating antimicrobial activity tetracycline was used as the standard drug for comparison. As can be seen from *Table 1*, it is evident that all of the tested compounds showed slight activity in comparison to tetracycline.
CONCLUSION: Five novel benzimidazolium salts (1a-e) were synthesized and their structures were completely verified by means of elemental analysis, FT-IR, 1D NMR: $^1$H NMR, $^{13}$C NMR. The biological activities of these salts were examined and were found to show moderate activity. The 1c salt displayed the best activity against A. hydrophila, P. aeruginosa, E. coli, S. aureus and L. monocytogenes as Gram +/- microorganisms. The 1b salt showed significant activity compared to the other salts (1a, 1c-e) against S. typhimurium and Y. enterocolitica. Finally, it could be concluded that compounds 1b-e exerted moderate antibacterial activity while 1a, which contained the methyl group, showed slight activity against the tested bacterial species.

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