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Synthesis and Biological Activity of Azine Heterocycle Functionalized Quaternary Phosphonium salts

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Abstract. Various azine heterocycles (pyrazine, quinoxaline and quinoline) possessing phosphonium salts (**3a-3c**) were prepared as cationic biocides. The structural characterization of the phosphonium compounds was confirmed by FTIR, NMR and HR-Mass spectroscopy. These compounds has shown excellent bactericidal activity against two Gram-positive bacteria (*Staphylococcus aureus, Bacillus subtilis*) and two Gram-negative bacteria (*Escherichia coli, Klebsiella pneumoniae*). Quinoline functionalized phosphonium has shown more antibacterial activity than pyrazine and quinoxaline.

1. Introduction

Recently, more attention towards to positively charged compounds such as quaternary ammonium and phosphonium salts for the application as disinfectants in agriculture, the food processing industry and clinics etc. Because of owing high antibacterial and a broad spectrum of antimicrobial activity, these organic cations are particularly important to act as disinfectants. Quaternary ammonium compounds are actively prevented the growth bacterial growth against both Gram-positive and Gram-negative bacteria, as well as against other pathogenic species of fungi and protozoa [1]. It has been found that quarternary phosphonium salts also showed beneficial antibacterial activity [2,3].

Microbial protection is mainly dependent on long alkyl chains which are attached with the phosphonium salts. These can be enhanced strongly by the molecular structure, correlation between antimicrobial activity and molecular structure [4]. It is significant that the double decyl groups of phosphonium biocide exhibited the broadest spectrum of microbial activity and showed the greatest bacteriostatic activity against MRSA infection. It was determined that the phosphonium salts showed better bactericidal activity and killing rate than corresponding ammonium salts [5]. On the other hand, higher cytotoxicity and complimentary selectivity of few cancer cells was detected towards for the derivative of mono and di phosphonium salt [6]. Shtyrlin et al synthesized the pyridine and pyridoxine analogue of quaternary phosphonium salts with excellent antibacterial and cytotoxic activities **[7]**.

Based on these observations, azine heterocycles of pyrazine, quinoxaline, and quinoline was bromo methylated and converted to quaternary phosphonium salts. These compounds was well

characterized and tested its biocidal performance against against gram-positive and gram-negative bacteria using disc diffusion method.

2. Experimental

2.1. Materials

2,3 dimethyl pyrazine, 2,3 dimethyl quinoxaline, triphenylphosphine, N-bromo succinimide, were purchased from Sigma Aldrich, Mumbai, India. 2,4, dichloro 6,8 dimethyl quinoline was prepared according to the literature data [8]. Benzoyl peroxide, chloroform, acetonitrile, methanol, acetone, carbon tetra chloride were purchased from SD fine chemicals, India and the solvents were purified by standard procedure.

2.2 Characterization methods

All NMR spectra were acquired using a Bruker AVANCE III 500 MHz spectrometer. The chemical shifts are reported in part per million (ppm) with tetramethylsilane (TMS) as a reference. The exact molecular weight of the intermediates was measured from JEOL GCMATE II GC-MS spectrometer with high resolution data system. Maximum resolution is 6000 and calibrated mass is 1500 daltons. Elementar vario El III, Carlo Erba 1108 system has been used for elemental analysis with detection limit of 0.1 to 1 μg.

2.3. Biological studies

2.3.1. Source of Microorganism

Staphylococcus aureus (S.aureus) (ATCC 700699), Escherichia coli (E.coli) (ATCC 10412), Bacillus subtilis (B.subtilis) (ATCC 11778), Klebsiella pneumoniae (K.pneumoniae) (MTCC 139) were used as micro organisms for the present investigation.

2.3.2. Preparation of inoculum

The inoculum was prepared by inoculating a loop of each test organism for 24 hours culture into a sterile nutrient broth and incubated at 37 ^{o}C for 3 hours, till an optical density value of 0.3 was reached in polarimeter.

2.3.3. Disc diffusion method

The medium was sterilized by autoclaving at 121 ^{o}C for 15 min, cooled to 45 ^{o}C and then poured in 20 ml quantity of petri dishes. A loopful of overnight broth culture was spread evenly over whole plate with sterile cotton wool swab. The culture plates were dried in an incubator with the lid until its surface was free from visible moisture. Subsequently 5mm diameter sterile discs (made from whatmann filter paper sterilized in UV lamp) are dipped in solutions of modified polymers; standard (chloramphenicol) and control (DMSO) were placed on the surface of agar plates.

The plates were left for one hour at room temperature as a period of pre-incubation diffusion to minimize the effects of variation in time between the applications of different solutions of modified polymers. The plates were incubated at 37 ^{o}C for 24 hours and observed for antibacterial activity. The diameter of the zones of inhibition was observed and measured. The average area of zone of inhibition was compared with that of standard.

2.3.4. Determination of relative percentage inhibition

The relative percentage inhibition of the phosphonium salt (**3a-3c**) with respect to standard was calculated by using the following formula

$$\textbf{Relative percentage inhibition} = 100 \times (x-y) / (z-y)$$

Where,

X = total area of inhibition of the phosphonium salt
Y = total area of inhibition of the solvent
Z = total are of inhibition of the standard drug
The total area of the inhibition was calculated by using area = πr^2;
where, r-radius of zone of inhibition.

2.4. General procedure for the bromination of dimethyl N-heterocyclic compound

The corresponding N-heterocyclic compound and N-bromosuccinimide (NBS) was taken in 1:2 molar ratios which was dissolved in 150 ml of carbon tetrachloride. A small amount of benzoyl peroxide was added as an initiator. The reaction mixture was refluxed for 3 hour under a nitrogen atmosphere. The completion of the reaction was indicated by the appearance of succinimide salt. This salt was filtered out, and a crude solution was concentrated under reduced pressure. The white solid product was obtained by precipitation in n-hexane.

2,3 (bis bromo methyl) pyrazine (2a):

General procedure has been followed to synthesize the compound **2a**. 2,3 dimethyl pyrazine (1.08 g, 10 mmol) and N- bromo succinimide (3.64 g, 20 mmol) were reacted to give reddish brown liquid. Yield: (0.8 g, 74.5 %). FTIR (KBr, cm^{-1}): 3052 (aromatic CH), 1628 (aromatic C=N), 1435 (aromatic C=C), 543 (CH_2Br). ^1H NMR ($CDCl_3$, ppm): 8.0 (2H, aromatic), 4.5 (4H, C<u>H</u>$_2$Br), ^{13}C NMR ($CDCl_3$, ppm): 154, 145, 142 (pyrazine carbons), 28.3 (<u>C</u>H$_2$ Br). Anal.Calcd for $C_6H_6Br_2N_2$ (%): C, 27.10 ; H, 2.27; N, 10.53; Found; C, 27.07; H, 2,05; N,10.99.

2, 3-Bis (bromo methyl) quinoxaline (2b):

The general procedure was applied for the reaction between 2,3 dimethyl quinoxaline (l.58 g, 10 mmol) and N-bromosuccinimide (3.64 g, 20 mmol) to furnish light red colored solid. Yield (2.04 g, 52 %, mp 58 ^oC). %). FTIR (KBr, cm^{-1}): 2907 (aromatic-CH), 1627 (aromatic C=N), 1435 (aromatic C=C), 541 (CH_2Br). ^1H NMR ($CDCl_3$, ppm): 7.8, 8.1(4H, aromatic), 4.8 (4H, C<u>H</u>$_2$Br), ^{13}C NMR ($CDCl_3$, ppm): 158, 144, 128 (quinoxaline carbons), 27.3 (<u>C</u>H$_2$ Br). Anal.Calcd for $C_{10}H_8Br_2N_2$ (%): C, 38.01; H, 2.55; N, 8.87; Found; C, 37.87; H, 2,43; N, 8.12.

2,4 dichloro 6,8-bis (bromomethyl) quinoline (2c):

Using the general procedure the resulting white solid of 2.4 dichloro 6, 8 dimethyl quinoline (2.25 g, 10 mmol) and N-bromo succinmide (3.64 g, 20 mmol) were reacted to form light red colored solid. Yield (1.5 g, 66.7 %, mp 55 ^oC). FTIR (KBr, cm^{-1}): 3034 (aromatic-CH), 1694 (aromatic C=N), 1450 (aromatic C=C), 628 (CH_2Br). ^1H NMR ($CDCl_3$, ppm): 7.4, 7.6, 8.1 (3H, aromatic), 4.6 (4H, C<u>H</u>$_2$Br). ^{13}C NMR ($CDCl_3$, ppm): 152, 148, 147, 135, 132, 128, 123, 122, 122 (quinoline carbons), 33.6, 32, 2 (<u>C</u>H$_2$ Br). Anal.Calcd for $C_{11}H_7Br_2Cl_2N$ (%): C, 34.42; H, 1.84; N, 3.65; Found; C, 33.17; H, 1,50; N,3.55.

2.5. General procedure for the Synthesis of N-heterocyclic phosphonium ylides

In a 250 ml three necked RB flask, 1 mole ratio of bromo methylated compound **2(a-c)** and 2 molar ratio of triphenyl phosphine were dissolved with 20 ml of acetonitrile. Under nitrogen atmosphere, the reaction mixture was stirred overnight at 40 ^oC. The final colorless precipitate was recrystallized from 2:1 ratio of methonol-toluene mixture.

2,3(triphenylphosphonium dibromo methyl) pyrazine (3a)

According to the general procedure, 2,3 dibromo methyl pyrazine **(2a)** (0.45 g, 1.69 mmol) and triphenylphosphine (0.89 g, 3.4 mmol) was reacted to furnish sticky brown solid with the yield (3.62 g, 85 %, mp. 125 ^{o}C). FTIR (KBr, cm^{-1}): 3250 (aromatic-CH), 1627 (aromatic C=N), 1435 (aromatic C=C), 790 (C-P), 695 (C-Br), 484 (P-Br). ^{1}H NMR ($CDCl_3$, ppm): 7.41-7.65 (30H, $\underline{Ph}_3P$), 7.8-8.3 (2H, pyrazine), 3.2 ($C\underline{H}_2P$). ^{13}C NMR ($CDCl_3$, ppm):158.2, 145.8, 140.1, 138.0 (pyrazine carbons), 132.3, 132.2, 132.0 128.7, 128.5, 128.4, 128.2, 128.1 (phenyl carbons), 58.3 ($\underline{C}H_2PPh_3$). Anal.Calcd for $C_{37}H_{34}Br_2N_2P_2$: C, 61.01; H, 4.71; N, 3.85; Found; C, 61.09; H, 4,31; N,3.21. HRMS calculated for $C_{37}H_{34}Br_2N_2P_2$ m/z = 788.0172, found, 787.8137.

2,3(triphenylphosphonium dibromo methyl) quinoxaline (3b)

According to the general procedure, dibromo methyl quinoxaline **(2b)** (0.634 g, 2.0 mmol) and triphenylphosphine (1.05 g, 4.0 mmol) was reacted to result sticky brown solid with the yield (3.4 g, 76 %, mp 130 ^{o}C). FTIR (KBr, cm^{-1}): 2973 (aromatic-CH), 1687 (aromatic C=N), 1485 (aromatic C=C), 766 (C-P), 670 (C-Br), 533 (P-Br). ^{1}H NMR ($CDCl_3$, ppm): 7.5 (30H, $\underline{Ph}_3P$) 7.6-7.7 (4H, quinoxaline), 2.7 ($C\underline{H}_2$), ^{13}C-NMR ($CDCl_3$, ppm): 149.5, 141.9, 142.2, 140.1 (quinoxaline carbons), 133.5, 133.8, 133.2, 127.9, 127.6, 127.3, 127.1, 126.4 (phenyl carbon), 52.2 ($\underline{C}H_2PPh_3$). Anal.Calcd. for $C_{46}H_{38}Br_2N_2P_2$: C, 65.73; H, 4.56; N, 3.33; Found; C, 64.98; H, 4,30; N, 2.82. HRMS calculated for $C_{46}H_{38}Br_2N_2P_2$ m/z =838.0877, found, 837.6734.

2,4 dichloro 6,8(triphenylphosphonium dibromo methyl) quinoline (3c)

According to the general procedure, dibromo methyl quinoline **2c** (0.5 g, 2.2 mmol) and triphenylphosphine (1.2 g, 4.6 mmol) was reacted to provide sticky brown solid with the yield (3.4 g, 76 %, mp 120 ^{o}C).). FTIR (KBr, cm^{-1}): 2923 (aromatic-CH), 1628 (aromatic C=N), 1450 (aromatic C=C), 781 (C-P), 690 (C-Br), 476 (P-Br). ^{1}H NMR ($CDCl_3$, ppm): 7.4-7.6 (30H, $\underline{Ph}_3P$) 7.7 (3H, quinoline), 2.1 ($C\underline{H}_2$), ^{13}C-NMR ($CDCl_3$, ppm): 158.8, 157.3 156.9, 146.9, 143.9, 142.4, 139.5, 139.2, 138.4 (quinoline carbons), 133.6, 132.5, 132.4, 132.3, 132.2, 128.8, 128.6, 128.5, 128.1, 128.0 (phenyl carbons), 49.6 ($\underline{C}H_2PPh_3$). Anal.Calcd for $C_{47}H_{37}Br_2Cl_2NP_2$: C, 62.14; H, 4.11; N, 1.54; Found; C, 61.07; H, 4,50; N,1.21. HRMS calculated for $C_{47}H_{37}Br_2Cl_2NP_2$ (m/z) = 905.0145, found, 906.5511.

3. Results and discussion

Three various nature of imine nitrogen comprising heterocyclic compound were selected for the present synthesis.

1. Diazine heterocycle such as., pyrazine
2. Fused diazine heterocycle such as., quinoxaline
3. Fused azine heterocycle such as., quinoline respectively

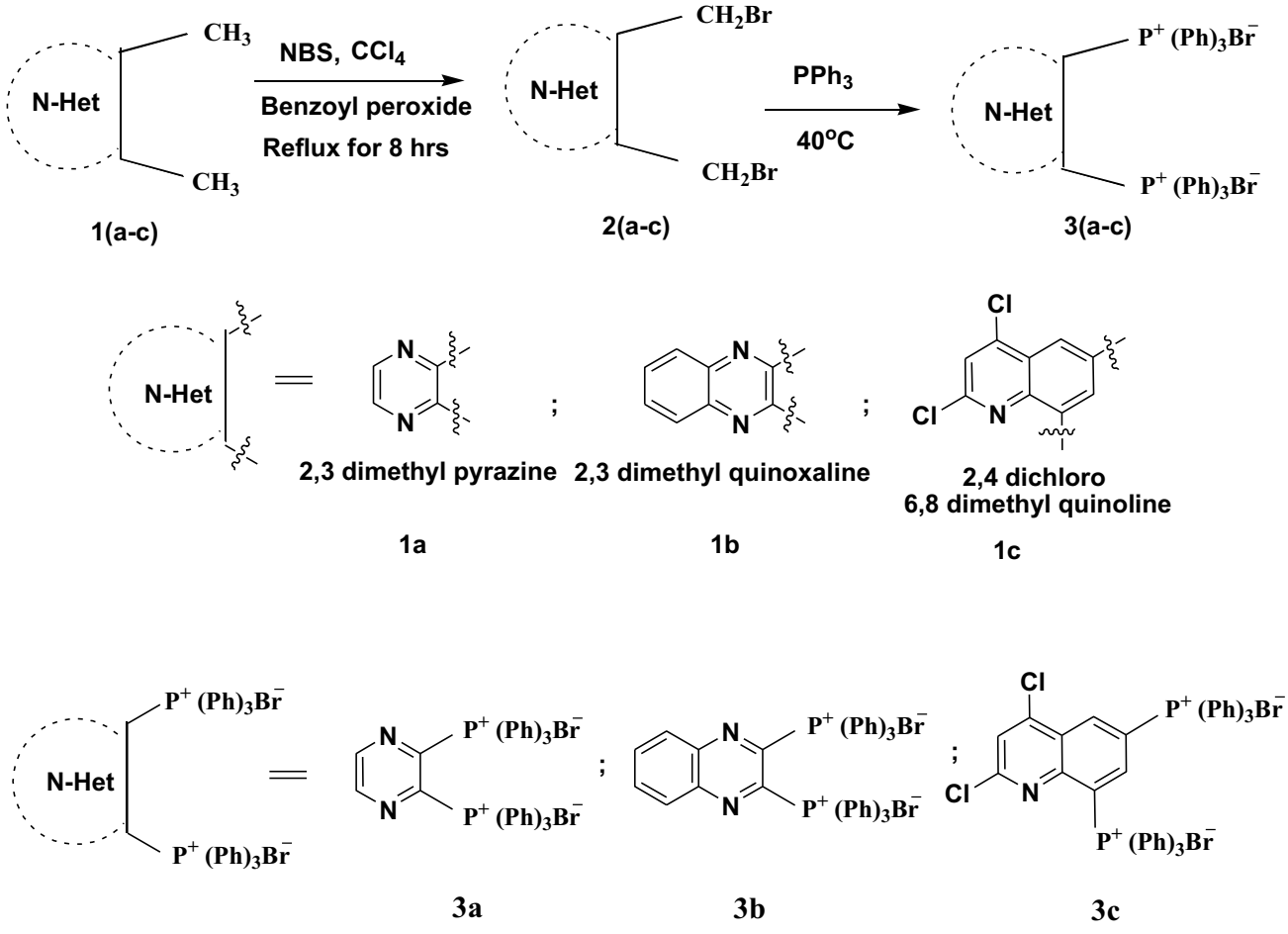
These heterocyclic groups generally assist or induce the solubility, thermal stability and increase the antimicrobial activity. **Scheme 1** shows the synthetic route to produce the nitrogen heterocyclic bearing phosphonium groups. In the first key step of the synthesis, phosphonium salt **3(a-c)** was prepared from bromomethyl groups using NBS (Bromination technique). High purified monomer was obtained by successive crystallizations which was verified by chromatographic and elemental analysis.

3.1 Characterization of Bromo methylated N-heterocyclic compound 2(a-c)

N-bromo succinimide (NBS) is used as source for bromine in radical reactions and act as brominating and oxidizing agent which is applied for various electrophilic reactions. The bromination of the N-heterocyclic compounds has been done by using benzoyl peroxide as an initiator and CCl_4 as a solvent [9].

In the first step, catalytically active species of Br_2 was generated from NBS, benzoyl peroxide acts as a radical initiator which is needed for the hemolytic bond cleavage of Br_2 to form HBr. Thus, these reagents (Br_2 and HBr) are generated insitu from NBS. It is very important to keep the lowest concentration of the reagent to prevent side reactions.

The brominated N-heterocyclic compounds **2(a-c)** were confirmed by FTIR and NMR spectra and elemental analysis respectively. Presence of bands at around 541-628 cm^{-1}, 2907-3052 cm^{-1} in the spectrum clearly indicated for the functional groups of C-Br and phenyl nucleus respectively. This fact was further confirmed by appearance of signals at 4.5-4.7 cm^{-1} (for $C\underline{H}_2$ protons attached to N-heterocycle and bromo group) in ^1H NMR spectra and singlet formed at 122-158 and 27.3-32.2 (for heterocyclic carbon and $\underline{C}H_2$ –Br moiety) in ^{13}C NMR spectra, which was listed in the **Table 1**. The results of the elemental analysis were well agreed for the calculated values for the corresponding structure of the brominated compounds, which was mentioned in the **Scheme 1**.



Scheme 1. Synthetic route of N-heterocyclic phosphonium salts

3.2 Characterization of N-heterocyclic phosphonium groups 3(a-c)

Bromomethylated N-heterocyclic compound **2 (a-c)** was obtained as a syrup and directly converted into 2,5-bis (triphenyl phosphoniomethyl)-N-heterocyclic dibromide **3 (a-c)** (phosphonium ylide or salt) with triphenylphosphine in an overall yield of 82-83%**.** The ylide formation was favored by the relatively high acidity of the methylenic protons of the phosphonium salt which caused by the electron withdrawing strength of pyrazine, quinoline and quinoxaline groups. The spectral values were shown in **Table 1**. The structure of the phosphonium ylides were confirmed by appearance of strong bands at 760-790 and 484-690 cm^{-1} (for C-P and P-Br stretching frequency) in IR spectra, multiplet formed at 7.41-7.65 ppm for aromatic hydrogens of phosphonium salt (zwitter ion) in ^{1}H NMR spectra. ^{1}H NMR spectra is clearly depicted in the **Figure 1**. Methylene signal of 4.5-4.7 ppm shifted to 2.7-3.2 ppm (^{1}H NMR) and 28.3 - 33.6 ppm shifted to 49.6-58.3 ppm (^{13}C NMR) indicates methylene group attached with phosphonium salt was confirmed. Presence of phosphorus atom in phosphonium ylide was confirmed from ^{31}P NMR spectra and it is shown in **Figure 2**. Only one signal was observed at 28.05 ppm for all phosphonium salt. The percentage of carbon, hydrogen and nitrogen was found from the elemental analysis, which was agreed with the theoretical values of the empirical structure of the Phosphonium ylide **(3a-3c)**.

Intermediates	**FTIR**			**^{1}H-NMR**	
	C-Br	**C-P**	**Aromatic C-H**	**Aromatic**	**CH_2**
2a	543	-	3052	7.9	4.5
2b	541	-	2907	7.5-8.2	4.7
2c	628	-	3034	7.5-7.8	4.7
3a	695	790	3250	7.2-7.8	2.6
3b	670	766	2973	7.5-8.0	2.8
3c	690	781	2923	7.3-7.7	2.9

Table 1. Characterization of N-heterocyclic bromo and ylide intermediates

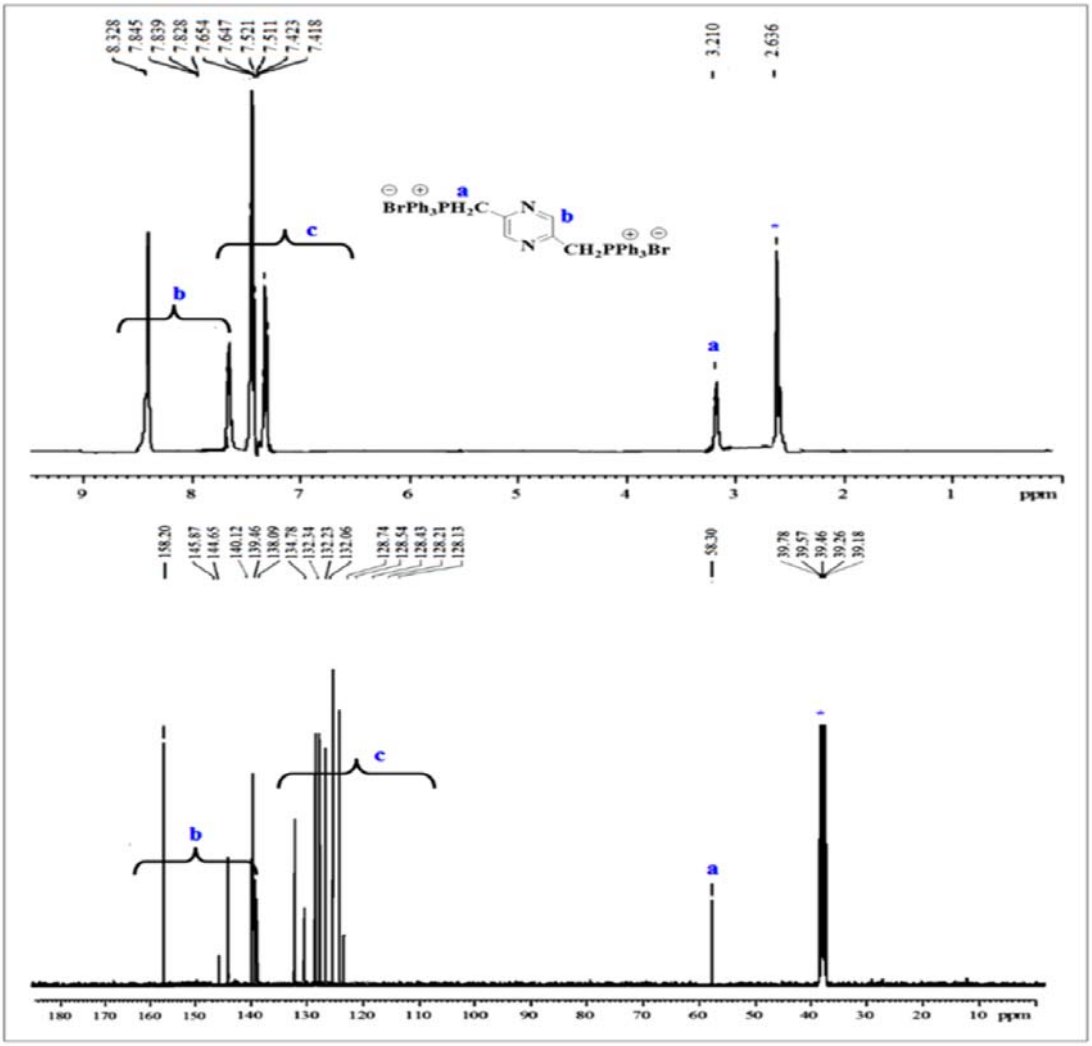


Figure. 1. ^{1}H NMR and ^{13}C NMR spectra of pyrazine substituted phosphonium salt (3a)

HRMS spectrum of the phoshonium ylides were presented in the **Figure 3**. The molecular weight of the phosphonium intermediates has been found to have 787.8237 for **3a**, 837.6734 for **3b** and 905.0145 g/mole for **3c** respectively. This value also agreed well with the theoretical value of the corresponding structure, which was represented in the **Scheme 1**.

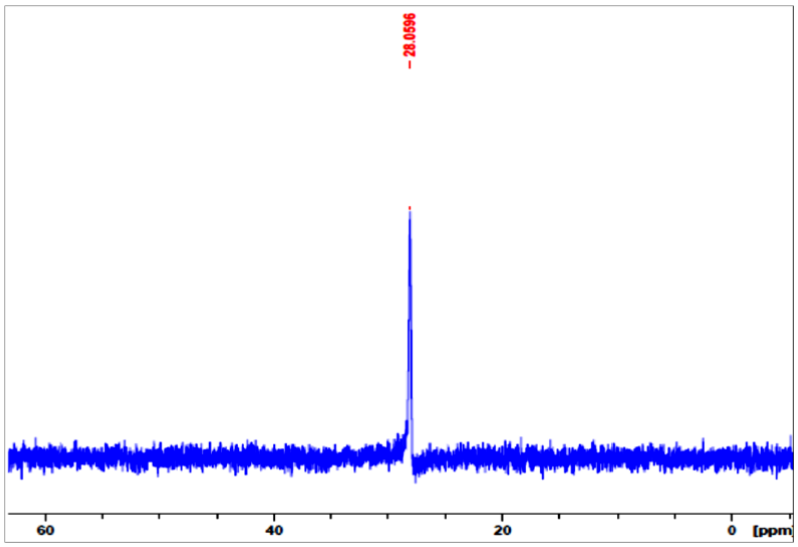


Figure. 2. ^{31}P NMR spectra of Phosphonium ylide (3a)

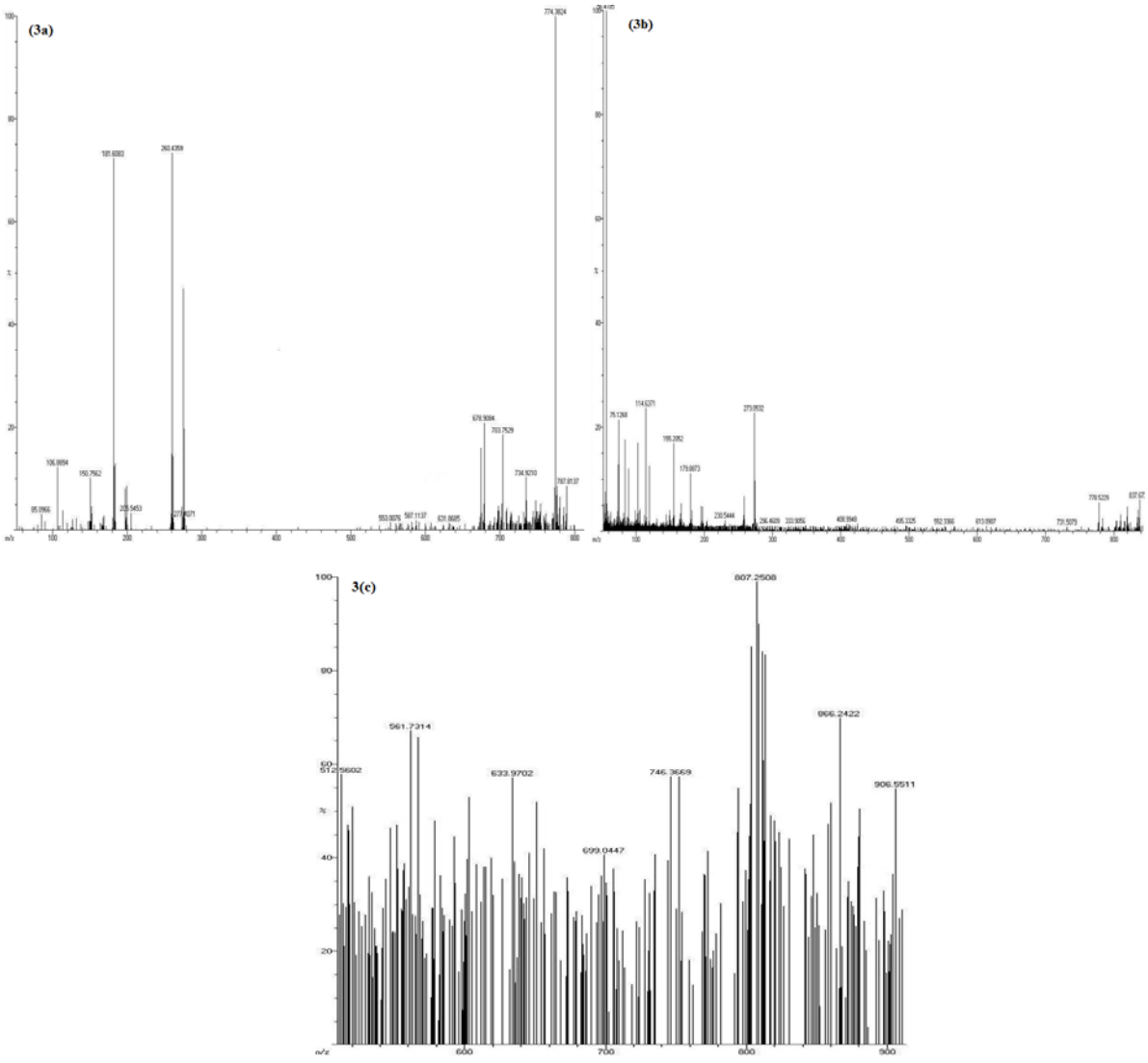


Figure 3. HRMS spectra of N-heterocyclic containing phosphonium ylides

3.3 Antibacterial Activity

Organic cations such as phosphonium and ammonium salts are famous antimicrobial agent in biological field [9]. Extensive research report was observed on the antimicrobial activity of cationic biocides. The target sites of cationic biocides are the cytoplasmic membranes of microbes, which kill microbial cells thus exhibiting bactericidal action. From the cationic biocides, phosphonium and ammonium cation was played a major role. Active phosphorus atom in phosphonium salts, may be expected to show a high antibacterial activity by strong interaction and high affinity with bacteria than ammonium salts [10]. Anitimicrobial analysis of phosphonium salts **3(a-c)** were carried out using four different bacteria

(*S.aureus,* B.*subtilis, E.coli, K.pneumonia).* The diameters of inhibition zones ranged between 8.0– 12 mm after incubation and its relative percentage inhibition was presented in **Figure 4**. Order of decreasing antibacterial activity for synthesized compounds were arranged as

3c > 3a > 3b

As expected, the triphenylphosphonium salt of the *N*-heterocyclic compounds **3(a-c**) has a large effect on bacterial species. These results suggested that the intermediate of phosphonium ylides can be used as effective antibacterial agents due to their strong ability in killing bacteria. Moreover, phosphonium salt was possessed structurally as ylide with positive and negative charges can be expected to show strong interaction (due to ease of charge transfer) and high affinity towards bacteria.

From the examination of the biological response of phosphonium salts, quinoline functionalized phosphonium salt showed higher activity than pyrazine and quinoxaline **(Table 2)**. The increased activity might be due to the presence of two chlorine atoms attached in quinoline moiety and also considered as broad biological activity [11]. The growth inhibitory effect of the intermediate **(3c)** was differed among the bacteria species and the order is

B. Subtilis > K.pneumoniae > S.aureus > E. coli.

From this observation, gram positive showed larger effect when compared to gram negative bacteria. The reason may be gram-positive cells have a simple cell wall structure contrast to gram-negative cells; the outside cytoplasmic membrane has only a rigid peptidoglycan layer [12]. This layer relatively thick, which allows foreign molecules come into the cell without difficulty [13].

Test bacteria	Zone of inhibition (mm)			
	3a	3b	3c	Chlromphenicol (Standard)
S.Aureus	10	10	11	24
B.Subtilis	11	9	8	22
E.coli	8	8	7	19
K.pneumoniae	12	7	0	22

Table 2. Zone of inhibition for phosphonium salt 3(a-c)

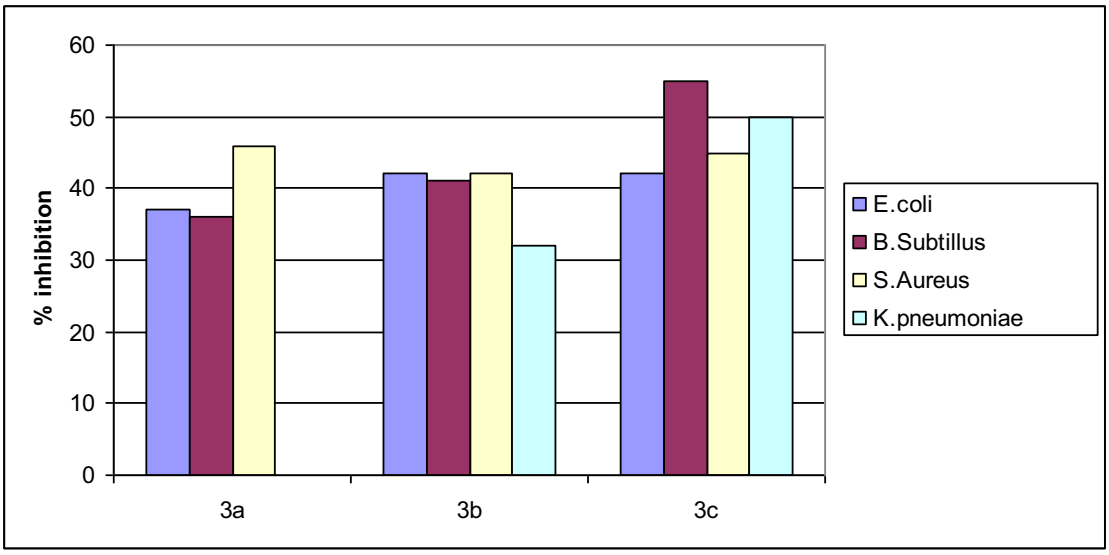


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Figure 4. Antimicrobial analysis for the intermediates and the polymers

Conclusion

Nitrogen heterocycles such as pyrazine, quinoxaline, quinoline substituted phosphonium salts **3(a-c)** was synthesized. The resulting compounds were characterized by FTIR, NMR, HRMS and elemental analysis. From the biological studies, quinoline attached phosphonium salt (**3c**) had more antibacterial activity than pyrazine and quinoxaline groups (**3a-b**). These groups possessing structurally positive and negative charges can be expected to show strong interaction and high affinity towards bacteria.

Acknowledgements

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