Innovare Academic Sciences

International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 6, Issue 5, 2014

Original Article

4-PYRROLIDINO/MORPHOLINO-1-(4-SUBSTITUTED PHENYLMETHYL/2-ETHOXY-2-OXOETHYL)PYRIDINIUM BROMIDE: SYNTHESIS, ANTIMICROBIAL EVALUATION AND ANTI-MRSA STUDIES

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Received: 28 Mar 2014 Revised and Accepted: 01 May 2014

ABSRACT

Objective: To synthesize a series of 1-(4-substituted phenylmethyl/2-ethoxy-2-oxoethyl)-4-pyrrolidino/morpholinopyridinium bromide (**1-12**), evaluate their *invitro* antimicrobial activities against some Gram positive/Gram negative bacteria and fungi species and perform the anti-MRSA activity of potent compounds (**2, 6 & 8**).

Methods: A series of 1-(4-substituted phenylmethyl/2-ethoxy-2-oxoethyl)-4-pyrrolidino/morpholinopyridinium bromides (**1-12**) were synthesized by stirring 4-substituted phenylmethyl/2-ethoxy-2-oxoethyl bromide and pyrrolidino/morpholinopyridine in dry acetone. The physical and spectral (IR, ^{1}H & ^{13}C NMR and MS) data were collected to confirm their structures. The compounds **1-12** were screened for antimicrobial activity by twofold serial dilution technique. The anti-MRSA studies were performed by agar-well diffusion method and the data were analyzed using One Way Analysis of Variance (ANOVA).

Results: The results of the *invitro* antimicrobial screening studies revealed that compounds **2**, **8** against *Staphylococcus aureus, Klebesilla pneumonia, Escherichia coli, Aspergillus niger* and **6** against *Streptococcus mutants*, *Rhizopus arrhizus* were found better activity than the standard drugs. The compound **8** has exhibited the highest inhibitory effect against methicillin-resistant *Staphylococcus aureus.* The ANOVA of anti-MRSA data showed significant differences at $P<0.01$.

Conclusion: The compound **8** has shown promising antibacterial activity against methicillin-resistant *Staphylococcus aureus.*

Keywords: 4-Pyrrolidinopyridine, 4-Morpholinopyridine, Pyridinium bromides, Antimicrobial, Methicillin-resistant *Staphylococcus aureus* (MRSA).

INTRODUCTION

As a result of the indiscriminate use of antimicrobial drugs in the treatment of infectious diseases, microorganisms have developed resistance to many antibiotics [1]. *Staphylococcus aureus* is one of the most well-known and prevalent human pathogens, causing skin and tissue infections, deep abscess formation, pneumonia, osteomyelitis, endocarditis, toxic shock syndrome and bacteriemia. Methicillin-resistant *Staphylococcus aureus* (MRSA) has been recognized as one of the predominant pathogenic causes of nosocomial infections throughout the world [2,3]. Infections caused by MRSA are a serious problem for intensive care unit patients, leading to resistance of drugs [4]. Solving the problem of drug resistance will depend partially on the development of chemotherapeutic agents that selectively attack new bacterial targets.

The chemistry of pyridinium and their fused heterocyclic derivatives has received considerable attention owing to their synthesis and effective biological importance [5-10]. Due to their ease preparation and rich biological activity, pyridinium framework plays an essential role and represents an interesting template for combinatorial and medicinal chemistry [11,12]. It was proven that the antimicrobial activity of pyridinium salts depends on the adsorptive activities on the surface of bacterial cells as well as their destruction [13]. It is evidenced from the detailed literature survey that the presence of pyridinium nuclei in a molecule plays an important role in enhancing the antimicrobial activity [14,15]. Prompted by these observations and in continuation of authors search for biologically potent molecules [16], the authors report herein the syntheses, antimicrobial evaluation and anti-MRSA studies on 4-pyrrolidino/morpholino-1-(4-substituted phenylmethyl/2-ethoxy-2-oxoethyl) pyridinium bromides.

MATERIALS AND METHODS

Reagents used in the present study were purchased from Aldrich and used without further purification. Reagents and solvents were handled by using standard syringe techniques. Uncorrected melting points were determined on a Veego (India) capillary melting point apparatus. IR spectra (KBr) were recorded on a FT-IR-400 Spectrophotometer (Perkin Elmer). ^{1}H NMR (300 MHz) and ^{13}C NMR (100 MHz) spectra were recorded in DMSO – d_6 on a Bruker AC 300. Chemical shifts were reported as δ values relative to tetramethylsilane (TMS) as internal reference. Mass spectra were obtained on API 3000 Centroid Turbo Spray Analyzer. Elemental analyses were performed using a Perkin-Elmer 2400 C, H, N analyzer.

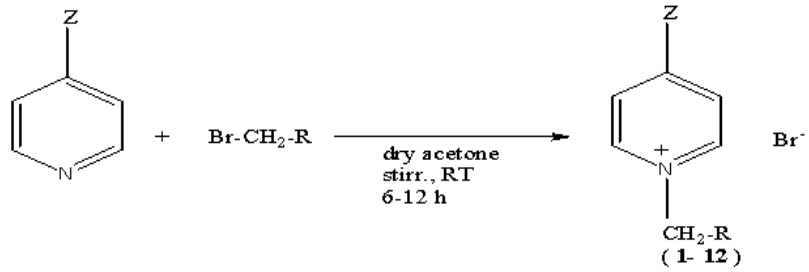
Synthesis

To a solution of 4-pyrrolidino/morpholinopyridine (0.1 mol) in dry acetone (50 mL), equimolar amounts of 4-substituted phenylmethyl bromide/2-ethoxy-2-oxoethyl bromide (s) was added and the reaction mixture was refluxed for 6-12 h. The reactions were monitored by TLC (analytical plates, Merck silica gel 100-200 mesh, India) using chloroform: methanol (8:2 v/v). The solid product obtained upon cooling was separated by filtration, washed with toluene and dried in a vacuum. It was recrystallized from chloroform-acetone (1:1, v/v) to give **1-12** as shown in

4-Pyrrolidino-1-(phenylmethyl)pyridinium bromide **(1):** IR (KBr, cm^{-1}): 3017 (CH str., aromatic), 2868 (C-H str., aliphatic), 2555 (C-H of -CH_2 str.), 1643 (C=C str.), 1559 (C-N str.), 1443 (N^+-C str.), 1355 (N-C str.); ^{1}H NMR (300MHz, DMSO-d_6): δ 8.47-8.46 (d, 2H, C_2- and C_6-H), 7.44-7.36 (m, 5H, -CH_2Ph), 6.93-6.91 (d, 2H, C_3-and C_5-H), 5.45 (s, 2H, N^+CH_2-), 3.50-3.47 (t, 4H, C'$_2$- and C'$_5$-H), 2.51– 2.50 (t, 4H, C'$_3$- and C'$_4$-H) ppm; ^{13}C NMR (100MHz, DMSO-d_6): δ 152, 142.00, 135.91, 128.91, 128.89, 128.62, 127.95, 108.58 (N^+CH_2-), 55.23, 48.34, 24.63 ppm; ESI-MS *m/z*: 239 (Calcd for $C_{16}H_{19}BrN_2$: 239). MS *m/z*: (M^+).

4-Pyrrolidino-1-[(4-bromophenyl)methyl]pyridinium bromide **(2):** IR (KBr, cm^{-1}): 2976 (C-H str., aromatic), 2868 (C-H str., aliphatic), 2070 (C-H of -CH_2 str.), 1644 (C=C str.), 1560 (C-N str.), 1413 (N^+-C str.), 1169 (N-C str.), 802 (C-Br str.); ^{1}HNMR (300MHz, DMSO-d_6): δ

8.43-8.42 (d, 2H, C_2- and C_6-H), 7.63-7.61 (d, 2H, -C'$_3$- and C'$_5$-H), 5.42 (s, 2H, N^+ CH_2-), 6.93-6.91 (d, 2H, C_3-and C_5-H), 7.39-7.37 (d, 2H, C'$_2$- and C'$_6$-H), 3.49-3.47 (t, 4H, C'$_2$- and C'$_5$-H), 1.99–1.98 (t, 4H, C'$_3$- and C'$_4$-H) ppm; ^{13}C NMR (100MHz, DMSO-d_6): δ 153.03, 141.96, 135.23, 131.91, 130.22 (-C_4-Br), 122.00, 108.63 (N^+CH_2-), 58.49, 48.36, 24.63 ppm; ESI-MS *m/z*: 317 (Calcd for $C_{16}H_{18}Br_2N_2$: 316). MS *m/z*: (M+1).



Scheme 1: Synthesis of compounds 1-12

Compd. No	1	2	3	4	5	6
Z						
R	-C_6H_5	-C_6H_4-Br(4)	-C_6H_4-NO_2(4)	-C_6H_4-CH_3(4)	-COC_6H_5	-$OCOCH_2CH_3$
Compd. No	**7**	**8**	**9**	**10**	**11**	**12**
Z						
R	-C_6H_5	-C_6H_4-Br(4)	-C_6H_4-NO_2(4)	-C_6H_4-CH_3(4)	-COC_6H_5	-$OCOCH_2CH_3$

4-Pyrrolidino-1-[(4-nitrophenyl)methyl]pyridinium bromide **(3):** IR (KBr, cm^{-1}): 3046 (C-H str., aromatic), 2873 (C-H str., aliphatic), 2360 (C-H of -CH_2 str.), 1646 (C=C str.), 1523 (C-N str.), 1344 (N^+-C str.), 1176 (N-C str.), 840 (C-NO_2 str.); ^{1}H NMR (300MHz, DMSO-d_6): δ 8.49-8.48 (d, 2H, C_2- and C_6-H), 8.27-8.26 (d, 2H, C''$_5$- and C''$_3$-H), 7.67-7.65 (d, 2H, C''$_2$- and C''$_6$-H), 6.97-6.96 (d, 2H, C_3-and C_5-H), 5.64 (s, 2H, N^+ CH_2-), 3.52-3.49 (t, 4H, C'$_2$- and C'$_5$-H), 2.20 – 1.98 (t, 4H, C'$_3$- and C'$_4$-H) ppm; ^{13}C NMR (100MHz, DMSO-d_6): δ 147.46, 143.17, 142.17, 129.12, 124.04, 108.76 (N^+CH_2-), 48.44 (-C_4-NO_2), 24.63 ppm; ESI-MS *m/z*: 284 (Calcd for $C_{16}H_{18}BrN_3O_2$: 284). MS *m/z*: (M^+).

4-Pyrrolidino-1-[(4-methylphenyl)methyl]pyridinium bromide **(4):** IR (KBr, cm^{-1}): 3017 (C-H str., aromatic), 2014 (C-H str., aliphatic), 2361 (C-H of -CH_2 str.), 1643 (C=C str.), 1559 (C-N str.), 1352 (N^+-C str.), 1172 (N-C str.); ^{1}H NMR (300MHz, DMSO-d_6): δ 8.44-8.43 (d, 2H, C_2- and C_6-H), 7.33-7.31 (d, 2H, -C''$_2$- and C''$_6$-H), 7.22-7.20 (d, 2H, -C''$_3$- and C''$_5$-H), 6.91-6.90 (d, 2H, C_3- and C_5-H), 3.49-3.46 (t, 4H, C'$_2$- and C'$_5$-H), 2.28 (s, 3H-C''$_4$-CH_3), 2.0 –1.97 (t, 4H, C'$_3$- and C'$_4$-H) ppm; ^{13}C NMR (100MHz, DMSO-d_6): δ 152.99, 141.91, 138.09, 132.89, 129.51, 128.22, 128.01, 108.53 (N^+CH_2-), 59.08, 48.32, 24.63, 20.68 (-C_4-CH_3) ppm; ESI-MS *m/z*: 253 (Calcd for $C_{17}H_{21}BrN_2$: 253). MS *m/z*: (M^+).

4-Pyrrolidino-1-(2-oxo-2-phenylethyl)pyridiniumbromide **(5):** IR (KBr, cm^{-1}): 3017 (C-H str., aromatic), 2858 (C-H str., aliphatic), 2394 (C-H of -CH_2 str.), 1717 (C=O str.), 1559 (C=C str.), 1443 (C-N str.), 1355 (N^+-C str.), 1171 (N-C str.); ^{1}H NMR (300MHz, DMSO-d_6): δ 8.47-8.46 (d, 2H, C_2- and C_6-H), 7.44-7.36 (m, 5H, -COPh), 6.93-6.91 (d, 2H, C_3-and C_5-H), 5.45 (s, 2H, N^+ CH_2-), 3.50-3.47 (t, 4H, C'$_2$- and C'$_5$-H), 2.0-1.98 (t, 4H, C'$_3$- and C'$_4$-H) ppm; ^{13}C NMR (100MHz, DMSO-d_6): δ192.15 (C=O), 153.01, 152.01, 142.00, 135.91, 128.99, 128.62, 127.95, 108.58 (N^+CH_2-), 59.23 ppm; ESI-MS *m/z*: 267 (Calcd for $C_{17}H_{19}BrN_2O$: 267). MS *m/z*: (M^+).

4-Pyrrolidino-1-(2-ethoxy-2oxoethyl)pyridinium bromide **(6):** IR (KBr, cm^{-1}): 3017 (C-H str., aromatic), 2858 (C-H str., aliphatic), 2394 (C-H of -CH_2 str.), 1744 and 1654 (C=O str.), 1552 (C=C str.), 1382 (N^+-C str.), 1210 (C-N str.), 1019 (N-C str.); ^{1}H NMR (300MHz, DMSO-d_6): δ 8.27-8.26 (d, 2H, C_2- and C_6-H), 6.94-6.92 (d, 2H, C_3-and C_5-H), 5.19 (s, 2H, N^+ CH_2-), 3.52-3.48 (quadret, 2H,-CH_2- CH_3), 3.90 (t, 4H, C'$_2$- and C'$_5$-H), 2.51-2.50 (t, 3H,-CH_2-CH_3), 2.0 (t, 4H, C'$_3$- and C'$_4$-H); ^{13}C NMR (100MHz, DMSO-d_6): δ 169.43 (carbon of –COO gp), 154.63, 153.85, 143.53, 139.37, 139.00, 108.37, 108.07 (N^+CH_2-), 57.25 (-CH_2-CH_3), 48.89, 48.66 (-CH_2-CH_3) ppm; ESI-MS *m/z*: 235 (Calcd for $C_{13}H_{19}BrN_2O_2$: 235). MS *m/z*: (M^+).

4-Morpholino-1-(phenylmethyl)pyridinium bromide **(7):** IR (KBr, cm^{-1}): 3026 (C-H str., aromatic), 2853 (C-H str., aliphatic), 2372 (C-H of -CH_2 str.), 1643 (C=C str.), 1550 (C-N str.), 1440 (N^+-C str.), 1262 (C-O-C str.), 1170 (N-C str.); ^{1}H NMR (300MHz, DMSO-d_6): δ 8.51-8.49 (d, 2H, C_2- and C_6-H), 7.44-7.37 (m, 5H, -CH_2Ph), 7.29-7.27, (d, 2H, C_3- and C_5-H), 5.44 (s, 2H, N^+CH_2-), 3.73 – 3.71 (t, 4H, C'$_2$- and C'$_5$-H), 3.68-3.66 (t, 4H, C'$_2$- and C'$_6$-H) ppm; ^{13}C NMR (100MHz, DMSO-d_6): δ 155.72, 142.65, 135.64, 129.03, 128.71, 128.04, 108.37 (N^+CH_2-), 65.42 (C-O), 59.41 (C-N) ppm; ESI-MS *m/z*: 255 (Calcd for $C_{16}H_{19}BrN_2O$: 255). MS *m/z*: (M^+).

4-Morpholino-1-[(4-bromophenyl)methyl]pyridinium bromide **(8):** IR (KBr, cm^{-1}): 2992 (CH str., aromatic), 2854 (CH str., aliphatic), 1973 (CH_2 str.), 1642 (C=C str.), 1546 (C-N str.), 1416 (N^+-C str.), 1263 (C-O-C str.), 1177 (N-C str.), 787 (C-Br str.); ^{1}H NMR (300MHz, DMSO-d_6): δ 8.50-8.49 (d, 2H, C_2- and C_6-H), 7.64-7.63 (d, 2H, C''$_3$- and C''$_5$-H), 7.42-7.41 (d, 2H, C''$_2$- and C''$_6$-H), 7.29-7.28 (d, 2H, C_3-and C_5-H), 5.43 (s, 2H, N^+CH_2-), 3.73 – 3.72 (t, 4H, C'$_3$- and C'$_5$-H), 3.69 –3.67 (t, 4H, C'$_2$- and C'$_6$-H) ppm; ^{13}C NMR (100MHz, DMSO-d_6): δ 156.22, 143.13, 135.50, 132.44, 130.85 (C_4-Br), 122.60, 108.90 (N^+CH_2-), 65.93 (C-O), 59.07, 46.63 (C-N) ppm; ESI-MS *m/z*: 383 (Calcd for $C_{16}H_{19}Br_2N_2O$: 383). MS *m/z*: (M^+).

4-Morpholino-1-[(4-nitrophenyl)methyl]pyridinium bromide **(9):** IR (KBr, cm^{-1}): 2858 (C-H str., aromatic), 2453 (C-H str., aliphatic), 2153 (C-H of -CH_2 str.), 1643 (C=C str.), 1535 (C-N str.), 1346 (N^+-C str.), 1263 (C-O-C str.), 1180 (N-C str.), 839 (C-NO_2 str.); ^{1}H NMR (300MHz, DMSO-d_6): δ 8.52-8.50 (d, 2H, C_2- and C_6-H), 8.30-8.29 (d, 2H, -C'$_3$- and C'$_5$-H), 7.68-7.67 (d, 2H, -C''$_2$- and C''$_6$-H), 7.23-7.22 (d, 2H, C_3-and C_5-H), 5.60 (s, 2H, N^+ CH_2-), 3.73 –3.72 (t, 4H, C'$_3$- and C'$_5$-H), 3.69-3.67 (t, 4H, C'$_2$- and C'$_6$-H) ppm; ^{13}C NMR (100MHz, DMSO-d_6): δ 156.25, 148.05, 143.35, 140.30, 131.34, 131.04, 129.71, 129.29, 127.51, 124.57, 124.45, 124.32, 124.14, 108.98, 107.90 (N^+CH_2-), 65.93 (C-O), 58.88, 46.67 (C-N), 46.40 (-C_4-NO_2), 32.59 ppm; ESI-MS *m/z*: 300 (Calcd for $C_{16}H_{19}BrN_3O_3$: 300). MS *m/z*: (M^+).

4-Morpholino-1-[(4-methylphenyl)methyl]pyridinium bromide **(10):** IR (KBr, cm^{-1}): 3013 (C-H str., aromatic), 2855 (C-H str., aliphatic), 1972 (C-H of -CH_2 str.), 1643 (C=C str.), 1544 (C-N str.), 1424 (N^+-C str.), 1262 (C-O-C str.), 1113 (N-C str.); ^{1}H NMR (300MHz, DMSO-d_6): δ 8.48-8.47 (d, 2H, C_2- and C_6-H), 8.26-8.24 (d, 2H, C''$_2$- and C''$_6$-H), 7.34-7.33 (d, 2H, -C''$_3$- and C''$_5$-H), 7.08-7.07 (d, 2H, C_3-and C_5-H), 5.38 (s, 2H, N^+ CH_2-), 3.72-3.70 (t, 4H, C'$_3$- and C'$_5$-H), 3.54 -3.52 (t, 4H, C'$_2$- and C'$_6$-H), 2.29 (s, 3H-C''$_4$-CH_3) ppm; ^{13}C NMR (100MHz, DMSO-d_6): δ 156.20, 143.05, 138.71, 133.14, 130.04, 128.62, 108.83 (N^+CH_2-), 66.05 (C-O), 65.92, 59.73, 46.59, 46.30 (C-N), 21.18 (C'$_4$-

CH_3) ppm; ESI-MS *m/z*: 270 (Calcd for $C_{17}H_{21}BrN_2O$: 270). MS *m/z*: (M^+).

4-Morpholino-1-(2-oxo-2-phenylethyl)pyridinium bromide **(11):** IR (KBr, cm^{-1}): 3026 (C-H str., aromatic), 2853 (C-H str., aliphatic), 1962 (C-H of -CH_2 str.), 1743 (C=O str.), 1550 (C=Cstr.), 1440 (C-N str.), 1308 (N^+-C str.), 1262 (C-O-C str.), 1170 (N-C str.); ^{1}H NMR (300MHz, DMSO-d_6): δ 8.20-8.18 (d, 2H, C_2- and C_6-H), 7.43-7.35 (m, 5H, -COPh), 6.83-6.82 (d, 2H, C_3-and C_5-H), 5.42 (s, 2H, N^+ CH_2-), 3.73-3.71 (t, 4H, C'$_3$- and C'$_5$-H), 3.27–3.25 (t, 4H, C'$_2$- and C'$_6$-H) ppm; ^{13}C NMR (100MHz, DMSO-d_6): δ 194.41 (C=O), 155.31, 150.27, 128.62, 108.70 (N^+CH_2-), 66.19 (C-O), 46.09 (C-N) ppm; ESI-MS *m/z*: 283 (Calcd for $C_{17}H_{19}BrN_2O_2$: 283). MS *m/z*: (M^+).

4-Morpholino-1-(2-ethoxy-2-oxoethyl)pyridinium bromide **(12):** IR (KBr, cm^{-1}): 2954 (C-H str., aromatic), 2834 (C-H str., aliphatic), 2103 (C-H of -CH_2 str.), 1747 and 1655 (C=O str.), 1551 (C=C str.), 1376 (C-N str.), 1242 (C-O-C str.), 1210 (N^+-C str.), 1114 (N-C str.); ^{1}H NMR (300MHz, DMSO-d_6): δ 8.31-8.29 (d, 2H, C_2- and C_6-H), 7.33-7.28 (d, 2H, C_3-and C_5-H), 5.22 (s, 2H, N^+CH_2-), 4.23-4.19 (quadret, 2H,-CH_2- CH_3), 3.75-3.74 (t, 4H, C'$_3$- and C'$_5$-H), 3.73-3.71 (t, 4H, C'$_2$- and C'$_6$-H), 3.65-3.63 (t, 3H,-CH_2-CH_3) ppm; ^{13}C NMR (100MHz, DMSO-d_6): δ 167.97 (carbon of –COO gp), 156.29, 144.24, 144.21, 141.28, 108.16, 108.04, 107.99 (N^+CH_2-), 65.99 (-CH_2-CH_3), 65.95 (C-O), 62.30, 57.09 (-CH_2-CH_3), 46.68 (C-N), 46.60, 46.37, 14.47 ppm; ESI-MS *m/z*: 251 (Calcd for $C_{13}H_{19}BrN_2O_3$: 251). MS *m/z*: (M^+).

Antimicrobial activity

The minimum inhibitory concentration (MIC) assays of the newly synthesized compounds (**1-12**) were conducted against a Gram positive bacteria *Staphylococcus aureus*, *Streptococcus mutants,* Gram negative bacteria *Escherichia coli, Klebesilla pneumonia* and fungi *Rhizopus arrhizus, Aspergillus niger* by the twofold serial dilution technique [17,18]. Initially bacteria/fungi were grown at 37°C/25°C in Nutrient Broth/Sabouraud dextrose broth I.P. This culture was used to inoculate 100 ml of media so that an initial number of 2 x 10^6 CFU/mL could be achieved. Test compound was dissolved in DMSO and was added to 20 mL inoculated media to get a final concentration of 100 µg/mL. Two-fold dilution of this compound was achieved by transferring 10 mL of this 100 µg/mL test compound containing media to another 10 mL inoculated media to get a final concentration 50 µg/mL of the test compound. All other dilutions of the test compounds were prepared in a similar fashion to get the minimal dilution to 0.39 µg/mL. Each 10 mL inoculated media along with the test compound was dispensed equally into 3 screws capped 10 mL glass culture tubes. Controls without the test compound, only media and in the presence of Ciprofloxacin/Fluconazole were set up simultaneously. The samples were incubated at 37°C for 24 h (bacteria), at 25°C for 72 h (*Aspergillus niger*) and at 37°C for 48 h (*Rhizopus arrhizus*) respectively. After incubation, the growth of bacteria / fungi was determined by measuring optical density at 600 nm using SYSTRONICS UV-VISIBLE Spectrophotomer 117. The lowest concentration at which there was more than 90% inhibition of bacteria/fungi as compared to the culture without test compound was taken up as minimal inhibitory concentration (MIC).

Anti-MRSA activity

Sample collection and isolation

In the present study, a total of 205 clinical samples obtained from Government Hospitals in Perambalur and Namakkal, Tamilnadu, India. All the isolates were identified according to colonial and microscopic morphology and standard tests like catalase, tube coagulase, motility, oxidase and biochemical tests [19].

DNA isolation for PCR

For nucleic acid isolation from staphylococcal isolates, the frozen samples were thawed rapidly, and were cultivated in brain-heart infusion broth (Merck, Germany) at 37°C with continuous shaking for overnight. Total DNA was isolated from 5 ml of a broth culture grown for overnight [20].

PCR detection of genes

The mecA/TSST genes were amplified with the respective primers by the method followed by Schmitz *et al* [21]. The PCR was performed with an initial denaturation step of 5 min 94°C, followed by 25 cycles of 20 s 94°C, annealing at 55°C for 20 s and the extension step of 50s 72°C. Agarose gels were prepared with TBE buffer (Tris, Boric acid, EDTA, pH 8) and stained with ethidium bromide (1 µg/15 ml gel). PCR product (5 µl) of each sample was mixed with 5 µl of sample buffer (6X: 0.25% bromophenol, 0.25% xylene cyanol, 15% ficol 400) and loaded on 1.5% agarose and electrophoresed in 75 volts for 60 min. The band of the fragment was observed by ultraviolet (UV) transilluminator and was documented by gel analyzer machine [22].

Antibacterial activity against MRSA

The compounds **2**, **6** and **8** were proven as highly potent antimicrobial agents and selected for anti-MRSA activity. The active compounds **2**, **6** and **8** were tested against selected isolates of MRSA by the agar-well diffusion method [23]. All the tests were performed in triplicate and the anti-MRSA activity produced by the test compounds was expressed as the mean diameter of the inhibition zones (mm).

Statistical analysis

All the experiments were conducted in triplicate and statistical analysis of the data was performed by analysis of variance (ANOVA), using the statistical package for social sciences (SPSS) program, version 17.0. A probability value of difference $p \leq 0.01$ was considered to be statistically significant. All the data were presented as mean values ± standard deviation (SD).

RESULTS AND DISCUSSION

The synthesis of compounds **1-12** were achieved through the versatile and efficient synthetic route as depicted in **Scheme 1**. In the present investigation, group at 4-position of pyridinium moiety makes, the more electron density of the ring nitrogen [24]. The dry acetone is more appropriate solvent for quaternisation reactions [25].

The physical data of **1-12** were collected and are presented in the **Table 1**. The yield of the compound **5** is found to be 50%, whereas, other compounds are more than 70%. This may be due to the solubility of compound **5** may be more in dry acetone than the others.

Spectral analysis

The IR spectra of the compounds **1-12** have been analyzed. The presence of N^+CH_2- has been recognized by the absorption bands in the region at 1443-1344 cm^{-1}. This may be due to the nitrogen cation which makes the bond stronger by electron attraction. The presence of >C=O group in **5** and **12** is revealed by the absorption in the region 1717 and 1747 cm^{-1} respectively. Absorption in the region 840 and 839 cm^{-1} for the compounds **3** and **9** indicates the characteristic bands for C-NO_2. The proton NMR spectra of the compounds **1-12** were recorded and the chemical shift values for methylene (-CH_2-) proton of **1-12** goes to the down field in the range at δ 5.64-5.19 ppm. This may attributed to the attachment of nitrogen cation with a methylene group (N^+CH_2-)**.** In the all compounds for C_2-H and C_6-H protons showed doublet in the down field compared to C_3-H and C_5-H protons of pyridine. This revealed the presence of positive charge on the ring nitrogen. The chemical shift values of other protons and corresponding assignments are presented in the experimental section.

Antimicrobial activity

The minimum inhibitory concentration (MIC) assays for the synthesized compounds (**Figure 1)** revealed that some of the compounds showed a good deal of activity against all the mentioned bacteria and fungi. Pyrrolidino derivative (**2**) with the substitution of a bromine atom at the 4-position of the phenyl ring of pyridinium base displayed strong inhibitory action against both gram positive (*Staphylococcus aureus*) and gram negative (*Klebesilla pneumonia)* bacteria in 3.12 µg/ml of MIC. The carboethoxymethyl derivative (**6**)

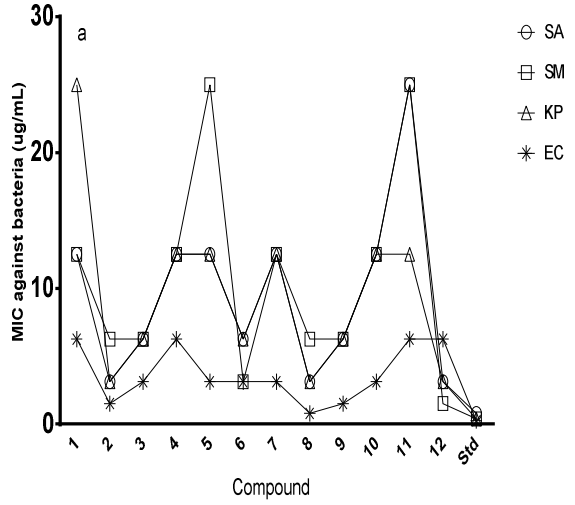
with pyrrolidinopyridnium moiety exhibited good activity against *Streptococcus mutants*. Compound **8** has a morpholinopyridnium moiety with a phenyl ring bearing halogen substitute in the form of bromine atom at the para position displayed promising activity against *Escherichia coli* at the low MIC value of 0.78 µg/ml. Morpholinopyridnium derivatives with phenacyl group appeared with good inhibitory potential toward *Rhizopus arrhizus* at the MIC value of 0.78 µg/mL. Morpholinopyridnium derivatives with a phenyl ring bearing bromine substituent (**8**) at 4-position showed strong inhibition of *Aspergillus niger* at the MIC value of 3.12 µg/mL. The above results suggested that the compounds **2**, **6** and **8** exerted excellent antimicrobial activities compared to the standard drugs ciprofloxacin (antibacterial) / fluconazole (antifungal) respectively. Taking these findings into consideration, we designated the compounds **2**, **6** and **8** as potent compounds and selected for anti-MRSA activity.

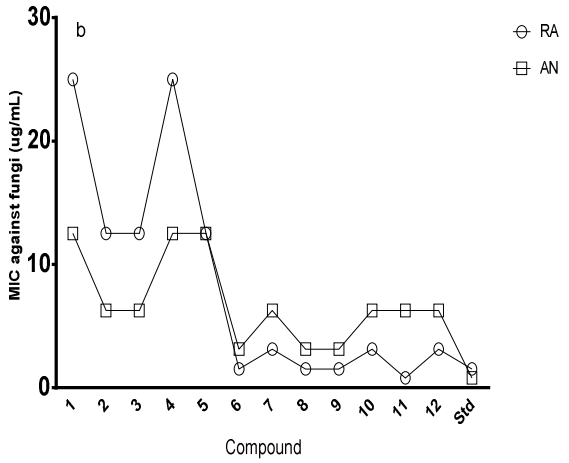
Table 1: Physical data of 1-(4-substituted phenylmethyl/2-ethoxy-2-oxoethyl)-4-pyrrolidino/morpholinopyridinium bromide (1-12)

Compd. No	MF	Colour	Yield (%)	MP (°C)	Analytical data of C, H, N Found (Calculated)
1.	$C_{16}H_{19}BrN_2$	Colourless	78	114-116	80.32(80.83), 7.92 (7.94), 11.70 (11.71)
2.	$C_{16}H_{18}Br_2N_2$	Colourless	80	100-102	60.73 (60.75), 5.68 (5.69), 8.88 (8.89)
3.	$C_{16}H_{18}BrN_3O_2$	Yellow	84	150-152	67.58 (67.60), 6.37 (6.38), 14.77 (14.78)
4.	$C_{17}H_{21}BrN_2$	Pale Yellow	76	178-180	80.62 (80.63), 8.28(8.30), 11.05 (11.06)
5.	$C_{17}H_{19}BrN_2O$	Colourless	50	124-126	76.42(76.20), 7.10 (7.11), 10.46 (10.48)
6.	$C_{13}H_{19}BrN_2O_2$	Colourless	65	165-167	66.36 (66.38), 8.07 (8.08), 11.90 (11.91)
7.	$C_{16}H_{19}BrN_2O$	Colourless	70	288-290	75.27 (75.29), 7.44 (7.45), 10.96, 10.98
8.	$C_{16}H_{19}Br_2N_2O$	Colourless	72	295-297	50.25 (50.26), 4.70 (4.71), 7.30 (7.30)
9.	$C_{16}H_{19}BrN_3O_3$	Yellow	69	240-242	63.98 (64.00), 6.01 (6.00), 13.99 (14.00)
10.	$C_{17}H_{21}BrN_2O$	Pale Yellow	74	282-284	76.10 (76.11), 7.81 (7.83), 10.42 (10.44)
11.	$C_{17}H_{19}BrN_2O_2$	Colourless	72	235-237	72.06 (72.08), 6.70 (6.71), 9.87 (9.89)
12.	$C_{13}H_{19}BrN_2O_3$	Colourless	68	208-210	62.14(62.15), 7.54 (7.56) 11.13 (11.15)

Anti-MRSA activity

The World Health Organization (WHO) has identified antimicrobial resistance as one of the 3 most important problems for human health (WHO 2002) [26]. Over the past decade, there has been an increasing in the rate of infection and disease caused by *Staphylococcus aureus* particularly MRSA throughout the world from 22% in 1995 to 57% in 2001 [27,28]. Therefore, understanding the epidemiology of this organism as well as the potential pathogenicity of the infecting strains is important to design control strategies for the concerned patients. In our study MRSA isolates were in majority of wound discharges (53.4%), followed by burn discharges (46%), blood samples (42.8%), urine (40%) and sputum (30%). The major sites of isolation for nosocomial MRSA infection was from wound discharges which is similar to the study conducted by Montensinos [29]. In our present study, among the 100 tested isolates 46% were resistant to methicillin. Results about resistance to methicillin found in this study were higher than 38% resistance which was reported CLSI 2007, but were in accordance with those in similar studies from New Delhi (51.6%) and Assam (52.9%) in India [30,31].





SA: *Staphylococcus aureus*, SM: *Streptococcus mutans*, KP: *Klebsiella pneumonia*, EC: *Echerichia coli*, Rh: *Rhizopus arrhizus*, AN: *Aspergillusniger*

Fig. 1: Graph showing relation between the synthesized compounds and their MIC against bacteria (a) and fungi (b).

The results of PCR showed that the protection of enterotoxin genes, which play a major role in the pathogenecity of *Staphylococcus aureus*. A recent study indicated that high mobility of mecA gene may be more prevalent than the movement of the enterotoxin genes [19]. One of the most unique manifestations among the various staphylococcal infections is staphylococcal toxic shock syndrome (TSS) associated toxin TSS toxin-1 (TSST-1) and might also be involved in the genesis of some autoimmune diseases [32]. The present study found that over 14% of clinical MRSA isolates carried the TSST gene. This ratio is compatible with recent reports of other countries and increasing annually [33]. These results were confirmed by PCR using the specific primers of resistance genes. All primers produced amplicons of predicted sizes were reported in **Table 2** and the PCR products of mecA and TSST genes were shown in **Figure 2**.

Table 2: The primer sequences and predicted sizes used in the PCR

Gene	Oligonucleotide sequences (5' - 3')	Size of amplified product (bp)
mecA	5' - CACTTGGTATATCTTCACC - 3' 5 - CTCAGGTACTGCTATCCACC - 3'	449 bp
TSST	5'-AGCAGGGCTATAATAAGGACTC-'3 5AAGCCCTTTGTTGCTTGCGAC-3'	250 bp

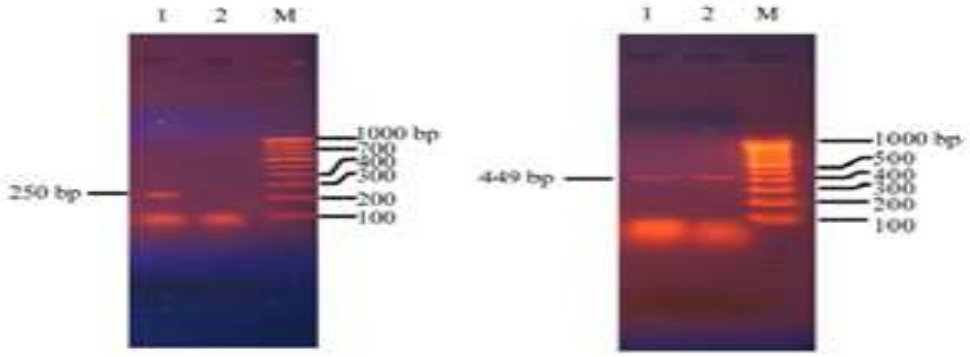


Fig. 2: PCR Product of mecA and TSST genes

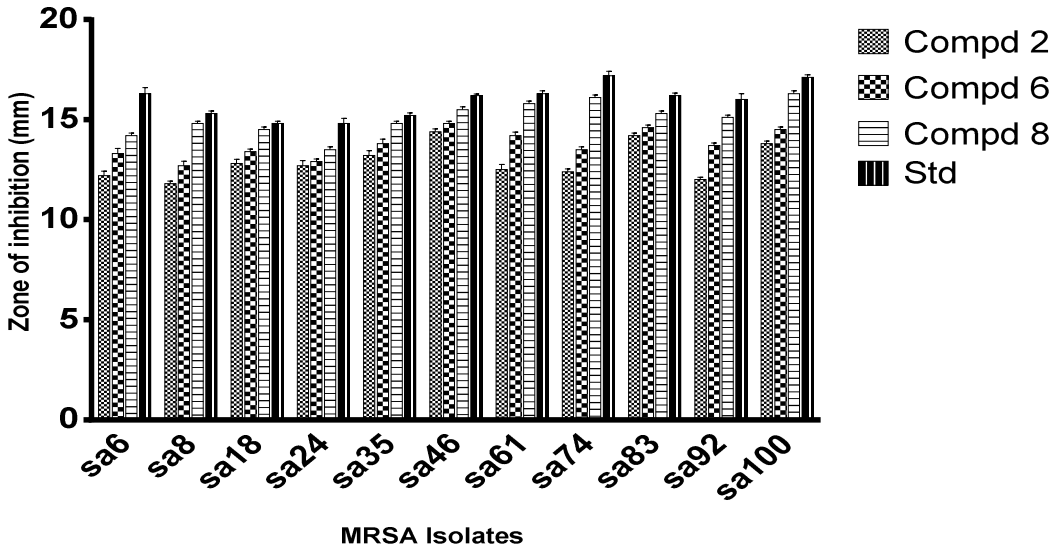


Fig. 3: Anti-MRSA activity of 1-(4-substituted)benzyl/carboethoxymethyl-4-pyrrolidino/morpholinopyridinium bromide

This study indicates that, mecA gene present in 46 isolates and TSST gene in 15 isolates. Both mecA and TSST genes are present in eleven isolates. The distribution of eleven isolates according to the units was as follows: 5 (45%) were from wound discharge, each of one (6.6%) from burn discharge, sputum, blood and 3 (27.2%) were from urine isolates.

Pyrrolidinopyridinium derivatives with 4-bromo substituted phenyl ring (**2)** and 2-ethoxy-2-oxyethyl group **(6)** and morpholino pyridinium derivative with 4-bromo substituted phenyl ring **(8)** were screened against eleven MRSA isolates by agar-well diffusion assay at the concentration of 100 µg/ml. The mean difference of zone of inhibition of compounds **2, 6** and **8** at 95% Confidence interval is shown in **Figure 3**. Based on the obtained values of the zone inhibition, it was revealed that the compound **8** that is a morpholino derivative containing bromine substituted phenyl ring as well as pyridinium nuclei was found to be very effective against MRSA isolates. The factors controlling antimicrobial activity of the substituted pyridinium salts are the hydrophobicity, adsorbability, surface activity, position of the substituents and electron density of the ammonium nitrogen atom [6].

Pyridinium salts have previously been reported to have anti-MRSA activity [34]. Another important pharmacophore group is the morpholino nucleus incorporated in a pyridinium salt, which is important for its antimicrobial properties [35].

CONCLUSION

A series of 4-pyrrolidino/ morpholino-1- (4-substituted phenylmethyl/2-ethoxy-2-oxoethyl) pyridinium bromides were synthesized in good yield and evaluated for *in vitro* antimicrobial activity. Methicillin-resistance (46%) of our study indicated the prevalence of MRSA and the pattern of antibiotic susceptibility to first line antibiotics are changing. The pyridinium bromide bearing a 4-bromo substituted phenyl ring and morpholino nucleus (**8)** has shown promising antibacterial activity against methicillin-resistant

Staphylococcus aureus, which suggests that it could serve as a lead member in a class of pyridinium anti-MRSA agents.

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