Introduction

A good number of phenothiazine derivatives have been known for their biological activities, yet considerable attention is still paid to them as a result of their varied uses in medicine which have prompted greater focus on phenothiazine moieties especially the aza analogues [1-3]. For instance, prothipendyl, 1, the 1-azaphenothiazine analog of promazine is found to be a superior drug to chlorpromazine, 2, in the treatment of mental disorders particularly in acute psychosis complicated with latent epilepsy [3] (Figure 1). This and other reports led to redirection of attention once more to azaphenothiazine ring systems as they aroused greater interest in the aza analogues of phenothiazine [4]. Phenothiazine derivatives have been found to possess potent wide spectrum CNS activities viz: anticonvulsant, antipsychotic, antidepressant and anti-neuroleptics [5-9]. Besides, they also possess potent anti-tubercular, antitumor, anti-histaminic and antibacterial activities among others [10-13]. Most reports on the pharmacology activities of phenothiazines were centred on the linear derivatives, only few reports have appeared in the literature on the angular type especially the non-linear aza analogues [14-16]. In this direction, we report the bactericide activity of newly synthesized angular aza phenothiazine derivatives against E.coli, Staphylococcus pseudointermedius, Staphylococcus scuri, Bacillus spp and Pseudomonas aeruginosa.

Results and Discussion

Two key functional intermediates were used in this work namely, 2-amin-5-bromopyrazine-3-[4H]-thione, 3, and 6,7-dibromo-5,8-quinolinequinone, 4 (Figure 2). Intermediate 3 was obtained in good yield according to literaturea colorless needles by treating 2-amino-s compound with bromine in glacial acetic acid. The reaction mixture was heated at 100°C for 15 min. The product was isolated as a yellow solid by vacuum distillation. The product was recrystallized from methanol to obtain colorless needles. The melting point of the product was recorded at 200°C. The product was characterized by IR, UV-VIS, NMR and mass spectrometry. The IR spectrum showed the presence of the characteristic bands of the amine, thione and quinone moieties. The UV-VIS spectrum showed the characteristic absorption bands of the aza and quinone moieties. The NMR spectrum showed the presence of the characteristic protons of the aza and quinone moieties. The mass spectrum showed the molecular ion peak at m/z 347. The structure of the product was confirmed by these spectral data. The product was screened for its antibacterial activity against E. coli, Staphylococcus pseudointermedius, Staphylococcus scuri, Bacillus spp and Pseudomonas aeruginosa at varying concentrations using Agar-well diffusion method and the results showed that these phenothiazine moieties had significant biological activity and inhibited the growth of these bacterial isolates even at low concentrations. Structures were assigned based on spectroscopic data and elemental analysis.

Keywords: Angular phenothiazines; Coupling; Biological activity; Halogeno derivatives
acetic acid at 0 to -5 °C followed by thiation using sodium hydrosulphide in methanol and recrystallization using DMF (Norit) (Scheme 1) [17,18].

Intermediate 4 was prepared by converting 8-hydroxyquinoline, 6, to the nitroso moiety, 7 which was subsequently reduced under nitrogen atmosphere with sodium dithionite to 5-amino-8-hydroxyquinoline sulphate, 8 [19-21]. This reduced product was treated with 24% hydrobromic acid and aqueous sodium bromate to furnish the intermediate of interest, 4 (Scheme 2).

Base mediated condensation (Scheme 3) using equimolar mixture of compound 3 and 6,7-dibromo-5, 8-quinonquinone, 4 furnished two isomers, 6,9-dibromo-1, 8, 11-triaza-5H-benzo[a]phenothiazin-5-one, 9 and 6, 9-dibromo-4, 8, 11-triaza-5H-benzo[a]phenothiazin-5-one, 10. The two isomers were separated by column chromatography and were identified on the basis of UV-Vis, IR and NMR spectra. The elemental analysis of the found and the calculated also agreed. This formation of angular polycyclic phenothiazine heterocycles by base mediated reaction of ortho amino heterocyclic thiones and halogenated quinolinequinones is consistent with previous reports in the literature [8-14].

The possible mechanism for this reaction is rationalized as follows (Scheme 4): the mercaptide ion, 11, mounts a nucleophilic attack at the 7-bromo position of compound 7 leading to the formation of the diaryl sulphide, 12. The electron-rich amino group mounts another nucleophilic attack at the carbonyl carbon of the quinolinequinone moiety which leads to elimination of water molecule (condensation) resulting to the formation of compounds 9 and 10.

Due to considerable biological and pharmaceutical activities of quinonemines, the antibacterial activity of these novel non-linear phenothiazine derivatives was evaluated.
This was largely as a result of the fact that the precursors, 8-hydroxyquinolinequinone, 6, and 6, 7-dibromo-8-quinolinequinone, 4, are known to have fungistatic and bacteriostatic activities [19-22]. An effective approach of antimicrobial therapy of an infection is based on the isolation and identification of the infected organism and determining its sensitivity to antimicrobial drugs. The microorganisms tested were E.coli (strains used were coded Eco 3, Eco 4 and Eco 12), Staphylococcus pseudointermedius and Staphylococcus scuiri (G101 and G84 respectively), Bacillus spp. and Pseudomonas aeruginosa. The assay was conducted using agar-well diffusion method in which a 20 mg/ml concentration of each compound was constituted by dissolving 0.04g of each compound in 2 ml of dimethyl sulfoxide (DMSO) [23]. A single colony of each test isolate was suspended in 2 ml of sterile nutrients broth. Inoculation was done followed by incubation at 37°C for 24 hr after which the plates were observed for inhibition zones around the wells. The diameters of the zones were measured with metre rule to the nearest whole millimetre. The result of the preliminary screening of the novel compounds tested against some Gram negative and Gram positive bacteria are shown in Table 1.

Table 1: Results of Antibacterial susceptibility test (tested with 20 mg/ml of each compound).

<table>
<thead>
<tr>
<th>Cpd</th>
<th>G101</th>
<th>G84</th>
<th>Eco 3</th>
<th>Eco 4</th>
<th>Eco 12</th>
<th>Bacillus Spp.</th>
<th>Pseudomonas Aeruginosa</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>18</td>
<td>19</td>
<td>11</td>
<td>11</td>
<td>12</td>
<td>17</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>21</td>
<td>17</td>
<td>11</td>
<td>0</td>
<td>0</td>
<td>16</td>
<td>0</td>
</tr>
</tbody>
</table>

Cpd = compound.

The figures in Table 1 show the inhibition zone diameter produced by each compound against each bacterial organism at 20 mg/ml concentration. The IZD ranged between 0 and 21 mm in diameter; the higher the IZD, the higher the sensitivity. The compounds showed significant activity against the test organisms except Pseudomonas aeruginosa which was resistant to both isomers; Eco 4 and Eco 12 were resistant to compound 10. Minimum inhibitory concentration of the compounds was also determined by the agar-well diffusion method with various concentrations ranging between 20 mg/ml and 0.15625 mg/ml and was compared with those of some standard drugs. Minimum inhibitory concentration is the minimal concentration of drug that inhibits visible growth after overnight incubation [23-25]. The lowest concentration of each compound that produced no zone was regarded as MIC. Hence, the essence of MIC is to determine the least concentration of drug that can inhibit the growth of the micro-organism.

Table 2: Result of Minimum Inhibitory Concentration (MIC) (mg/ml) of the Synthesized Compounds and Some Standard Drugs.

<table>
<thead>
<tr>
<th>Compound/Drug</th>
<th>Eco3</th>
<th>G84</th>
<th>Bacillus ssp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>20</td>
<td>5</td>
<td>1.25</td>
</tr>
<tr>
<td>10</td>
<td>20</td>
<td>5</td>
<td>1.25</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>100</td>
<td>2.5</td>
<td>1.25</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>6.25</td>
<td>2.5</td>
<td>0.15625</td>
</tr>
</tbody>
</table>

The antimicrobial activity of these compounds was compared with those of some standard drugs. Table 2 below gives the result of Minimum Inhibitory Concentration (MIC) of the synthesized compounds, Ampicillin and Gentamycin (standard drugs) against Eco 3, G84 and Bacillus spp. The MIC for the compounds against Eco 3, G84 and Bacillus spp. ranged from 1.25 mg/ml to 20 mg/ml. For instance, the MIC for Eco 3 for compounds 9 and 10 is 20 mg/ml because there was no inhibition at the next concentration which is 10 mg/ml. It became necessary to compare the MICs of the synthesized compounds with those of some standard antibiotics against the microbes tested. Ampicillin and Gentamycin were used because they are standard drugs used for treating such strains of microorganisms; plain agar (i.e, without any of the test compounds) was also spot-inoculated to serve as the control because it had no effect against the isolates as it showed no zone of inhibition. The MIC of the standard drugs ranged between 100 mg/ml and 0.15625 mg/ml. From Table 2 is synthesized phenothiazine derivatives were active against Eco 3 at lower concentrations than Ampicillin and compared favourably well in their resistance against G84 and Bacillus spp. showing that the synthesized phenothiazine compounds are highly biologically active; hence, they may be of pharmaceutical interest.

Conclusion

We have shown that these angular (non-linear) azaphenothiazines are pharmaco-actively effective against some strains of bacteria thereby increasing the number of biologically active phenothiazine derivatives. More so, some of the isolates are methicillin resistant and they are imagined problems because they are difficult to treat and they infect humans and animals. However, we are glad that these synthesized compounds resisted them effectively even at very low concentrations. However, the possible industrial values of these heterocycles need investigation as they are deeply coloured and phenothiazines are known to be extensively used as dyes and pigments.
Experimental

General Information

Melting points were taken using Fisher-Johns melting point apparatus and they remain uncorrected. Compounds were characterized by HNMR, CNMR, IR spectroscopy, UV/Visible spectroscopy and elemental analysis. UV-Visible spectra were recorded on a JENWAY 6405 UV/VIS Spectrophotometer using matched 1cm quartz cells, Department of Pure and Industrial Chemistry, University of Nigeria Nsukka; IR spectra on a SHIMADZU FTIR-8400S FOURIER TRANSFORM INFRARED SPECTROPHOTOMETER (KBr Pellets), (NARICT, ZARIA). Nuclear magnetic resonance (HNMR and CNMR) spectra were determined using Varian NMR Mercury-200BB (Central Science Lab. Obafemi Awolowo University Ife). Chemical shifts are recorded on the δ-scale (neat). The elemental analyses were done at the Central Science laboratory, University of Cairo, Egypt on a CE440 Elemental Analyzer. The antimicrobial screening was done at the Faculty of Veterinary Medicine, University of Nigeria, Nsukka.

8-Hydroxy-5-nitrosoquinoline hydrochloride, 10

This compound was prepared following the procedure of Pratt and Drake. To a solution of 8-hydroxyquinoline (58g, 0.4 mol) in 200 ml of water, 75 ml of concentrated HCl and 200g of ice was added a solution of 30g of sodium nitrite in 100 ml of water in portions with vigorous shaking over 1hr at 0 to 4°C. The mixture was allowed to stand overnight at 0°C before the product was filtered off and washed with cold water. The product was air-dried giving a yield of 76g (87%) of the hydrochloride of 8-hydroxy-5-nitrosoquinoline (m.p, 181-183°C, dec) as fine yellow compound. The compound loses its brightness in time and darkens due to auto-oxidation.

5-Amino-8-hydroxyquinoline sulphate, 14

20 g (0.11 mol) of the freshly prepared hydrochloride of 8-hydroxy-5-nitrosoquinoline was dissolved in a mixture of water (80 ml) and 5M NaOH (130 ml) placed in a three-necked flask (500 ml) equipped with a long magnetic bar. The reaction mixture was heated to 50°C. A solution of sodium bromated (38.5 g, 0.225 mol) in water (150 ml) was added at such a rate that the temperature does not exceed 50-60°C. After the addition, the reaction mixture was heated at that temperature with gentle stirring for 0.5h (30 min) and then cooled. The cooled mixture was poured onto ice (500 g) and further chilled in an ice-salt mixture. The resulting precipitate was filtered and recrystallized from ethanol to give yellow precipitate of 6,7-dibromo-5,8-quinolinequinone (71.3 g, 66%); m.p 242-243°C (Lit. 243-245°C).

UV-Vis (MeOH): λmax 265 (3.69), 290 (3.92), 345 (3.45) and 505 (2.20) nm.

IR (KBr): ʋmax 3093, 1686, 1541, 1289, 664, 662 cm⁻¹

2-Amino-3,5-dibromopyrazine

This compound was prepared following Okafor and Okoro’s procedure. 2-Aminopyrazine (9.5 g, 100 mmol) was placed in a reaction flask containing glacial acetic acid (70 ml) and warmed on a steam bath until it dissolved. Sodium acetate trihydrate (33 g, 243 mmol) was added with constant swirling. The slurry was stirred in an ice-salt bath maintained at -5°C and bromine (16 ml) was added dropwise over a 4h period (if the bromine addition was speeded up the reaction became turbulent and potentially hazardous). The mixture was stirred in the ice bath for 2h and then at room temperature for 24h. It was then poured into ice (50 g) and neutralized with concentrated ammonia (pH 8). The crude product were collected and recrystallized from methanol (Norit) to give colourless needles of 2-amino-3,5-dibromopyrazine (16.8 g, 66%), m.p 113-114°C (Lit. 114-115°C) (Figure 3).
2-Amino-5-bromopyrazine-3[4H]-thione, 5

A mixture of 2-amino-3,5-dibromopyrazine (7.69 g, 30 mmol) and sodium hydrosulphide (13.33 g, 238 mmol) was added to methanol (60 ml) and the mixture was refluxed for 4.5 h. Methanol was then removed by distillation and the residual dark moist solid was dissolved in water (150 ml), treated with activated charcoal, boiled and filtered. The filtrate was cooled and the product filtered. Recrystallization from DMF (Norit) yielded pure 2-amino-5-bromopyrazine-3[4H]-thione (3.75 g, 61%) as a yellow solid, m.p 209-211°C (dec); (lit. 208-210°C).

6,9-Dibromo-1,8,11-triaza-5H-benzo[a]phenothiazin-5-one, 7 and 6,9-dibromo-4,8,11-triaza-5H-benzo[a]phenothiazin-5-one, 8

A mixture of benzene (40 ml) and dimethylformamide (5 ml) was added to 2-amino-5-bromopyrazine-3[4H]-thione (1.03 g, 5 mmol) mixed with anhydrous sodium carbonate (1.06 g, 10 mmol) in a 100 ml three-necked flask equipped with a magnetic stirring bar. The entire mixture was refluxed on a water-bath at 70-75°C for 45 min. while stirring. Then, 6,7-dibromo-5-8-quinolinequinone (1.59 g, 5 mmol) was added and the stirring continued for 6 h while the refluxing temperature was maintained at 70-75°C. At the end of the reflux period, the solvent was evaporated and the reddish-brown crude product was column chromatographed using methanol-acetone (2:1) as eluting solvent. From the first fraction, a dark red solid was obtained, compound 7. It weighed 0.68 g (32.1%) and melted at 148-150°C (dec.). The second fraction gave compound 8 as a brown solid which weighed 1.23 g (58.0%) and melted at 291-293°C (dec).

UV-Vis (MeOH) for Compound 7: λmax 222.2(4.57), 247.4(4.82), 341.8(4.50) nm.

IR (KBr): ʋmax 3085, 1638, 1553, 1262, 740 cm⁻¹

HNMR (DMSO): δ 186 (C=O), 153 (C-2), 143 (C-10), 123 (C-3), 120 (C-6), 38.6-41.1 (DMSO)

Anal. Calcd for C13H4N4Br2SO: C, 36.80; H, 0.94; N, 13.21; Br, 37.74; S, 7.55

Found:     C13H4N4Br2SO: C, 36.68; H, 0.91; N, 13.25; Br, 37.89; S, 7.59

Evaluation of the Synthesized Angular Phenothiazinones for Antibacterial Activity

The assay was conducted using agar-well diffusion method [23]. An 20 mg/ml concentration of each compound was constituted by dissolving 0.04 g of each in 2 ml dimethyl sulfoxide. A single colony of each test isolate was suspended in 2 ml of sterile nutrient broth. The suspension of each isolate was standardized by adjusting to correspond to 0.5 McFarland turbidity standards corresponding to approximately 108 cfu/ml and used to inoculate the surface of the iso-sensithest nutrient agar and the excess fluid drained into discard pot containing disinfectant. The inoculated agar surface was allowed to dry and the plates appropriately labelled. Using a cork borer of 6 mm in diameter, wells were bored in the inoculated iso-sensitest nutrient agar. With a micropipette, 50 µl of each test compound solution was delivered into each well. The plates were left on the bench for 30 minutes to allow the compound to diffuse into the agar. Thereafter, the plates were incubated at 37°C for 24 h. After incubation, the plates were observed for inhibition zones around the wells. The diameters of the zones were measured with metre rule to the nearest whole millimetre.

Determination of Minimum Inhibitory Concentration (MIC) of the Synthesized Angular Phenothiazinones

This was carried out using agar dilution following the procedure outlined by Clinical Laboratory Standards Institute [25]. Sterile test tubes were arranged on a test tube rack and 1 ml of DMSO was dispensed into each of them. From the stock compound solutions, 1 ml was transferred into the first test tube and two-fold serial dilution of each compound solution was carried out and the resultant concentration in the test tubes were 10, 5, 2.5, 1.25, 0.625, 0.3125, and 0.15625 (mg/ml) (i.e., graded concentrations of the compounds) [26]. A single colony of each test isolate was suspended in 2 ml of sterile nutrient broth. The suspension of each isolate was standardized and used to inoculate the surface of the nutrient agar and the excess fluid drained into discard pot containing disinfectant.

The inoculated agar surface was allowed to dry and the plates appropriately labelled. Using a cork borer of 6 mm in diameter, wells were bored in the inoculated nutrient agar. With a micropipette, 50 µl of each test compound solution was delivered into each well. The plates were left on the bench for 30 minutes to allow the compound to diffuse into the agar. Thereafter, the plates were incubated at 37°C for 24 h. After incubation, the plates were observed for inhibition zones around the wells. The diameters of the zones were measured with meter rule to the nearest whole millimetre. MIC was taken as the lowest concentration of the compounds that resulted in no visible growth on the surface of the agar.
References


