SYNTHESIS OF VERNOLAMIDES CONTAINING TERTIARY AMINO GROUPS FROM *VERNONIA GALAMENSIS* OIL AND THEIR BIOLOGICAL ACTIVITIES

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ABSTRACT. The vernonia oil obtained by soxhlet and cold extractions from *Vernonia galamensis* seeds was reacted with 1-(2-aminoethyl)pyrrolidine, 4-(3-aminopropyl)-morpholine and 1-(3-aminopropyl)-2-pyrrolidinone (molar ratio 1:3) at 50°C to obtain: N-2-(1-pyrrolidino)ethylvernolamide, N-3-(4-morpholino)propylvernolamide and N-3-[1-(2-oxopyrrol-idino)]propylvernolamide, respectively. The reactions were complete after 16-18 hours. The antimicrobial activities of the vernolamides were investigated against *Staphylococcus aureus* (gram-positive), *Escherichia coli* (gram-negative), *Bacillus subtilis*, *Trichophyton mentagrophyte*, *Microsporum gypseumCandida albicans* and *Saccharomyces cerevisiae*. The vernolamides exhibited both antibacterial and antifungal activities.

INTRODUCTION

Vernonia oil extracted from *Vernonia galamensis* seeds is a viable starting material for synthesis of new chemical derivatives with higher added value. The oil, besides being a good source of dibasic acids [1, 2], can also be used as a reactive diluent in paints and in the synthesis of interpenetrating polymer networks.

The oil is extracted from the seeds of *Vernonia galamensis* with petroleum ether or hexane by soxhlet and cold extraction after seeds are lipase-deactivated and coarse ground, where yields of up to 42% have been reported [3, 4]. One unique characteristic of vernonia oil is that about 80% of the oil is the triglyceride of vernolic acid (Figure 1).

$$CH_3-(CH_2)_4-\overset{O}{\overset{\diagdown}{CH}}-CH-CH_2-CH=CH-(CH_2)_7-COOH$$

(A)

$$CH_2-O-\overset{O}{\overset{\|}{C}}-(CH_2)_7-CH=CH-CH_2-\overset{O}{\overset{\diagdown}{CH}}-CH-(CH_2)_4-CH_3$$
$$CH-O-\overset{O}{\overset{\|}{C}}-(CH_2)_7-CH=CH-CH_2-\overset{O}{\overset{\diagdown}{CH}}-CH-(CH_2)_4-CH_3$$
$$CH_2-O-\overset{O}{\overset{\|}{C}}-(CH_2)_7-CH=CH-CH_2-\overset{O}{\overset{\diagdown}{CH}}-CH-(CH_2)_4-CH_3$$
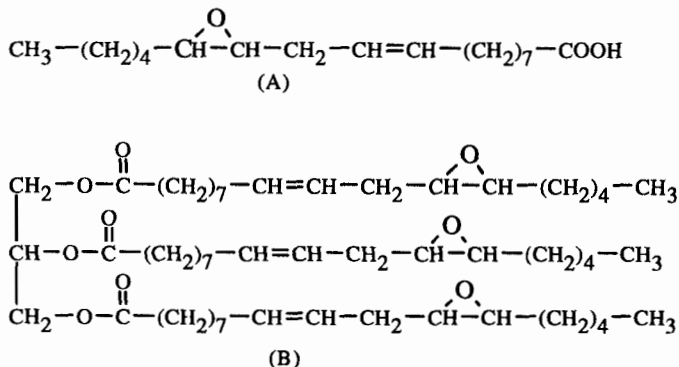
(B)

Figure 1. Vernolic acid (A) and trivernolin (B).

Fatty amides, which are important chemical intermediates, have been synthesised by several methods under different conditions. The fatty amides prepared so far are from alkylamines and hydroxyamines. Primary aminoamides have also been synthesised. Fatty alkanolamides, fatty diamides and fatty arylamides were synthesised directly from triglycerides (vernonia oil, tallow and tripalmitin) and primary amines at low temperatures [5].

Vernonia oil has been reacted with aliphatic diamines to obtain bisamides [6, 7] with the general formula R-CO-NH-$(CH_2)_n$NH-CO-R

$$R= CH_3(CH_2)_4CH-CH-CH_2-CH=CH-(CH_2)_7-$$

The reaction of diamines and acid chlorides results in the formation of N,N'-diacylalkyldiamines which have antimicrobial activities [8].

Although the reactions of vernonia oil with the amines are all nucleophilic in nature, they do not always result in epoxy ring opening implying that the epoxy group which is located on carbons 6 and 7 from the end of the alkyl chain is substantially hindered. It is, therefore relatively stable under alkaline conditions. However, it is prone to ring opening under acidic conditions. This paper reports the synthesis of vernolamides containing tertiary amino groups and tests on their behaviour as antimicrobial agents.

MATERIALS AND METHODS

Thin layer chromatographic analyses (TLC) were performed on commercial precoated plates (0.25 mm silica gel 60, MN). Developing solvents were hexane:diethyl ether (70:30 or 60:40 v/v) and chloroform:methanol (90:10). Visualisation with iodine was carried out by placing the developed, dried layer in a jar containing crystalline iodine. Column chromatography was run on silica gel 60 (0.063-0.100 mm, E. Merck) packed in a 2.0 x 50 cm glass column. Reaction mixtures were separated on silica gel by elution with chloroform. Infrared (IR) spectra of the solids in nujol mull were recorded on Pye unicam SP3-300 IR spectrophotometer. Electron impact ionisation (EI) mass spectral data were obtained by a TSQ 70 (FINNIGAN MAT) spectrometer. ^{1}H- and ^{13}C-NMR spectra were obtained with a Bruker (AC-250) spectrometer in $CDCl_3$ solution (TMS). ^{1}H-NMR was recorded at 250 MHz while ^{13}C-NMR spectra were recorded at 50 MHz. The melting points of the derivatives were determined using the Gallenkamp melting point apparatus.

Extraction and purification of vernonia oil. Dry and powdered seeds of *Vernonia galamensis* (705 g) were extracted in a soxhlet with commercial hexane (2.5 L). Hexane was removed by a rotary evaporator to obtain the slightly dark coloured crude oil. The crude oil was degummed by stirring the oil with distilled water in the ratio of 21:1 at 50 °C for 1 h on a magnetic stirrer, followed by centrifugation for 3 h. The degummed oil was then decolourised with activated charcoal (5% by weight) by stirring the mixture at 60 °C for 1 h on a magnetic stirrer followed by filtration.

The degummed and charcoal-treated oil was bleached with fuller's earth (1%) by mixing on a rotary evaporator at 60 °C for 30 min and filtered. Then finally, alkali refining was done by adding 2 N NaOH in the ratio 34:1 with stirring for 30 min and

dried on a rotary evaporator at 60 °C. The yield was 27%. The infrared (IR) in nujol mull gave major peaks at 1760 cm^{-1} (ester group) and 850, 840 cm^{-1} (epoxy group).

Synthesis of N-2-(1-pyrrolidino)ethylvernolamide. Vernonia oil (2.70 g, 2.92 mmol) based on a molecular weight of 926 and 1-(2-aminoethyl)pyrrolidine (1 g, 8.76 mmol) were added to a round-bottomed flask (25 mL) equipped with a reflux condenser and a magnetic stirrer. The reaction was allowed to proceed at 50 °C for 15 h and the formation of the product monitored by TLC. After cooling, the reaction mixture was dissolved in 10 mL of chloroform and transferred into a 100 mL separatory funnel, to which 10 mL of water was added. Then it was shaken and allowed to partition. After removing and discarding the aqueous layer, the organic portion was washed five times with 10 mL portions of water to remove any unreacted amine. The organic portion was transferred into a 100 mL round-bottomed flask and the chloroform evaporated under vacuum. Hexane was added and placed in ice to precipitate the crude yellow epoxy amide, which was filtered and washed several times with cold hexane to remove any unreacted oil.

The product was dried in air to afford an amorphous yellow compound with a melting point of 42-44 °C in 45% yield (1.55 g). Infrared (IR) (cm^{-1}) in nujol: 3300 (NH), 1640 (C=O), 1550, 845 and 825 (epoxy group). Mass spectral data for the isolated N-2-(1-pyrrolidino)ethylvernolamide exhibited major peaks at *m/z* 84 (100), 156 (3.1), 169 (7.6), 225 (21), 279 (22.3), 321 (46.6), 363 (2.3) and 393.4 (9.2). ^{1}H-NMR (CDCl$_3$) δ ppm: 6.30 (s, 1H, NH), 5.42-5.65 (m, 2H, CH=CH), 3.39-3.48 (m, 2H, CH$_2$N), 2.97-3.02 (t, 2H, CHOCH), 2.6 (m, 4H, N(CH$_2$)$_2$), 2.4 (m, 4H, CH$_2$C=C), 2.1 (m, 2H, CH$_2$C=O), 1.32-1.89 (m, (CH$_2$)$_n$) and 0.9 (t, 3H, CH$_3$); ^{13}C-NMR (CDCl$_3$) δ ppm: 173.25 (C=O), 123.86-132.57 (CH=CH) and 56.54-57.21 (epoxy carbons).

Synthesis of N-3-(4-morpholino)propylvernolamide. The above procedure for the synthesis of N-2-(1-pyrrolidino)ethylvernolamide was repeated where vernonia oil (2.14 g, 2.31 mmol) and 4-(3-aminopropyl)morpholine (1 g, 6.93 mmol) were used. A white, shiny, soft and low density amorphous compound was obtained with a melting point of 50-52 °C after drying in air. The yield was 20% (0.59 g). Infrared (IR) (cm^{-1}) in nujol: 3300 (NH), 1640 (C=O), 1550, 840 and 825 (epoxy group). Mass spectral data for the isolated amide exhibited major peaks at *m/z* 43 (18.7), 57 (8.9), 86 (16.7), 100 (100), 114 (12.4), 128 (8.9), 143 (3.7), 167 (6.0), 171 (3.1) 186 (5.1), 199 (14.6), 213 (2.7), 236 (7.8), 279 (7.8), 295 (2.4), 309 (4.1), 336 (10.6), 351 (27.2), 365 (0.6), 379 (2.6), 423 (9.3) and 424 (10.0). ^{1}H-NMR (CDCl$_3$) δ ppm: 6.85 (s, 1H, NH), 5.40-5.60 (m, 2H, CH=CH), 3.80 (m, 4H, CH$_2$O), 3.39-3.48 (m, 2H, CH$_2$N), 2.90-3.0 (t, 2H, CHOCH), 2.45 (m, 4H, N(CH$_2$)$_2$), 2.20-2.45 (m, 4H, CH$_2$C=C), 2.05 (m, 2H, CH$_2$C=O), 1.30-1.80 (m, (CH$_2$)$_n$) and 0.9 (t, 3H, CH$_3$); ^{13}C-NMR (CDCl$_3$) δ ppm: 172.89 (C=O), 123.92-132.53 (CH=CH) and 56.53-57.87 (epoxy carbons).

Synthesis of N-3-[1-(2-oxopyrrolidino)]propylvernolamide. A mixture of 2.70 g (2.92 mmol) of vernonia oil and 1.25 g (8.79 mmol) of 1-(3-aminopropyl)-2-pyrrolidinone was stirred with a magnet at 50 °C for 17 h. The reaction mixture was monitored by TLC. Then the reaction mixture was pre-adsorbed on silica gel and poured as a powder on a short column packed with silica gel to trap excess amine. It was eluted with chloroform and TLC analysis of the effluent collected confirmed the absence of the amine. The chloroform was stripped off by a rotavapor under vacuum and the mixture dissolved in hexane. Then allowed to cool for crytallisation to occur. A white compound crystallised

out, which was filtered and washed several times with hexane to remove unreacted oil. The product, which was white amorphous, thin, shiny sheets, 0.74 g (20% yield) had a melting point of 52 °C. Infrared (IR) data (cm^{-1}) in nujol: 3300, 1680, 1645, 1550, 840 and 825. The mass spectrum of the isolated epoxy amide exhibited major peaks at *m/z* 98 (46.4), 112 (56.4), 126 (100), 143 (26.5), 169 (4.1), 184 (7.2), 197 (9.0), 211 (0.7), 253 (1.4), 279 (1.0), 307 (8.8), 349 (2.9) and 420 (0.4).^{1}H-NMR (CDCl$_3$) δ ppm: 6.71 (s, 1H, NH), 5.30-5.58 (m, 2H, CH=CH), 3.16-3.43 (m, 2H, CH$_2$N), 2.90-2.92 (t, 2H, CHOCH), 2.35 (m, 4H, CH$_2$C=C), 2.1 (m, 2H, CH$_2$C=O), 1.30-1.70 (m, (CH$_2$)$_n$) and 0.91(t, 3H, CH$_3$); ^{13}C-NMR (CDCl$_3$) δ ppm: 123.89-132.68 (CH=CH) and 56.60-57.25 (epoxy carbons).

Antimicrobial tests. The epoxy amides were tested for antimicrobial activities. The amines: 1-(2-aminoethyl)-   pyrrolidine,   4-(3-aminopropyl)morpholine,   1-(3-aminopropyl)-2-pyrrolidinone and vernonia oil were also tested.

Antibacterial properties. Nutrient agar (composition: 31.4 g/L) was sterilised at 121 °C for 15 min. Then cooled before pouring on petri dishes which had been sterilised in an autoclave. Portions of *Staphylococcus aureus, Bacillus subtilis* and *Escherichia coli* were spread on the petri dishes of nutrient agar. After drying the petri dishes in a sterile hood (Lamina Air flow, gelaine HF 72, flow laboratories from Italy) for 15 to 30 min, blank sterile filter paper discs (5 mm diameter) were placed with sterile forceps. A solution of the sample (4 µL, 2 µL, and 1 µL of 25 mg/mL chloroform solution equivalent to 100 µg, 50 µg, and 25 µg of the respective samples) were spotted at the centre of the paper discs. The petri dishes were then placed in the incubator overnight at 37 °C. The diameter of the inhibition zone was obtained from the average of two determinations in each case.

Antifungal properties. The media was prepared by weighing 39 g of potato dextrose agar (composition: potato extract 4.0 g/L, dextrose 20.0 g/L and agar 15.0 g/L) and dispersed in 1 litre of de-ionised water. The agar mixture was left standing for 10 min and swirled to mix and sterilised at 121 °C for 15 min. It was cooled to 47 °C and mixed well before pouring on sterilised petri dishes. Portions of *Trichophyton mentagrophyte, Microsporum gypseum, Candida albicans* and *Saccharomyces cerevisiae* were spread on the petri dishes of the potato dextrose agar. After drying the petri dishes in a sterile hood for 15 to 30 min, sterile filter paper discs (5·mm diameter) were placed on the petri dishes with sterile forceps. The samples were spotted in solution form (4 µl, 2 µl and 1 µl of 25 mg/mL chloroform solution) on the paper discs. In addition, a blank disc with only chloroform was used as a control. The plates were placed in the incubator at 37 °C for four days. The diameter of the inhibition zone was obtained from the average of three determinations in each case.

RESULTS AND DISCUSSION

Reaction of vernonia oil with the primary amines . The reactions did not go to completion at room temperature due to the formation of semi-solid reaction mixtures. Therefore, the reactants were maintained at 50 °C for complete solubility. The amines were used in excess in molar ratios of 1:3 (vernonia oil : amine). The amines acted as

reactants and solvents in the reactions. The reaction of vernonia oil with 1-(2-aminoethyl)pyrrolidine, 4-(3-aminopropyl)- morpholine and 1-(3-aminopropyl)-2-pyrrolidinone resulted in the formation of vernolamides. The reactions were complete after 16 -18 hours. The general reaction is according to the following scheme:
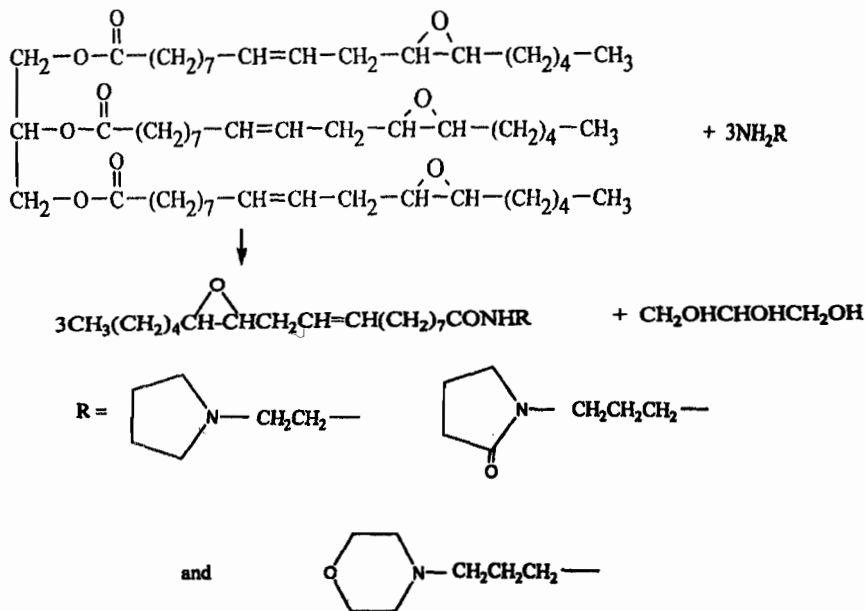
$$CH_2-O-\overset{\overset{O}{\|}}{C}-(CH_2)_7-CH=CH-CH_2-\overset{O}{\overset{/\backslash}{CH-CH}}-(CH_2)_4-CH_3$$
$$CH-O-\overset{\overset{O}{\|}}{C}-(CH_2)_7-CH=CH-CH_2-\overset{O}{\overset{/\backslash}{CH-CH}}-(CH_2)_4-CH_3 \qquad + \ 3NH_2R$$
$$CH_2-O-\overset{\overset{O}{\|}}{C}-(CH_2)_7-CH=CH-CH_2-\overset{O}{\overset{/\backslash}{CH-CH}}-(CH_2)_4-CH_3$$

$$\downarrow$$

$$3CH_3(CH_2)_4\overset{O}{\overset{/\backslash}{CH-CH}}CH_2CH=CH(CH_2)_7CONHR \qquad + \ CH_2OHCHOHCH_2OH$$

R =   [pyrrolidine ring] N— CH_2CH_2—   [2-oxopyrrolidine ring] N— $CH_2CH_2CH_2$—

and   [morpholine ring] N— $CH_2CH_2CH_2$—

Table 1. Experimental results from synthesis of epoxy amides.

Epoxy amides	Reaction time (h)	R_f value	Yield (%)	m.p. (°C)
N-2-(1-pyrrolidino)ethylvernolamide	16	0.4	45	42- 44
N-3-(4-morpholino)propylvernolamide	18	0.63	20	50-52
N-3-[1-(2-oxopyrrolidino)] propylvernolamide	17	0.7	20	52

Purity of the amides was confirmed by obtaining a single spot in each case on thin layer chromatography plates, while the amines did not move but remained at the origin when using chloroform:methanol (90:10) as the developing system. It was observed that the reactivity of 1-(2-aminoethyl)pyrrolidine with vernonia oil was faster, resulting in a higher yield (45%) of N-2-(1-pyrrolidino)ethylvernolamide, as compared to the other two amides. The yields are slightly lower than expected probably due to the formation of diglycerides and monoglycerides as the reactions proceed and also because of steric hindrance.

The infrared spectra for N-2-(1-pyrrolidino)ethylvernolamide showed sharp absorptions at 3300 cm^{-1} typical of secondary amides. Absorptions at 1640 cm^{-1} suggested the carbonyl of the amide group, indicating that the amminolysis reaction had taken place at the ester bond, giving an amide as a product. The epoxy absorption was still present at 825 cm^{-1} and 845 cm^{-1}.

The M+1 peak was observed at m/z 393.4 in the EI MS while the base peak at m/z 84.1 was due the α-cleavage to the nitrogen. The McLafferty rearrangement gave a fragment ion at m/z 156.1 and β-cleavage to the carbonyl group gave a peak at m/z 169. Cleavage between C7 and C8 (allylic cleavage) resulted in a fragment at m/z 225 while cleavage at alpha to the epoxy group gave rise to a fragment ion at m/z 279. This could also represent the acyl group of vernolic acid. The fragment at m/z 321 was due to the loss of -$CH_2CH_2CH_2CH_2CH_3$ group while that at 363 was due to the loss of -CH_2CH_3 group.

Chemical ionization (CI) spectrometry gave rise to a similar fragmentation pattern where the CH_4 induced CI spectrum shows the quasimolecular ion (MH$^+$, m/z 393.306) as the base peak (100%). The peak at m/z 421.346 was due the $MC_2H_5^+$ ion. Both the ^{1}H-NMR and ^{13}C-NMR spectra were consistent with the structure.

Reaction of vernonia oil with 4-(3-aminopropyl)morpholine. The reaction resulted in the formation of an amide that still contained the epoxy group in the molecule as confirmed by IR with absorptions at 825 cm^{-1} and 840 cm^{-1}. Sharp absorptions at 1640 cm^{-1} and 1550 cm^{-1} corresponding to amide I (C=O) and amide II (NH) bands, respectively, were also observed. A very sharp peak at 3300 cm^{-1} due to NH stretching indicated that it was a secondary amide.

The peak at m/z 424 in the EI MS represents the M+2 ion while the base peak at m/z 100 is due to the cleavage of the C-C bonds next to the nitrogen in the ring. Cleavage between C-11 and C-12 (allylic cleavage) gave a fragment ion at m/z 309. The ion at m/z 351 was attributed to cleavage alpha to the epoxy group (C13-C14) while a McLafferty rearrangement gave the ion at m/z 186. Alpha cleavage of the group attached to the amide nitrogen gave rise to a fragment ion at m/z 114 and β-cleavage of C-C bond to the carbonyl group gave rise to a fragment ion at m/z 199.

The CH_4 induced CI spectrum shows the quasimolecular ion (MH$^+$, m/z 423.416) as the base peak (100%) and a peak at m/z 451.457 was due to $MC_2H_5^+$ ion. Other major peaks were observed at m/z 100.088 (15%), 279.272 (6%) and 351.319 (10%).

The NMR spectra was consistent with the structure. Protons on the terminal methyl groups gave a peak at 0.9 ppm, methylene group protons gave signals between 1.30 and 1.80 ppm except for the allylic methylenes (2.20 ppm and 2.45 ppm), the methylene alpha to the carbonyl functionality (2.05 ppm) and the methylene adjacent to the NH group (3.35 ppm). The epoxy hydrogens produced signals at 2.9-3.0 ppm, while the olefinic hydrogens gave complex signals at 5.4 and 5.6 ppm. The broad singlet at 6.85 ppm was due to the NH proton. The -CH_2O protons in the morpholine ring showed an absorption at 3.8 ppm, while the methylenes adjacent the nitrogen in the ring absorbed at 2.45 ppm.

^{13}C-NMR of the amide also confirmed the presence of the epoxy carbons at 56.53 and 59.87 ppm. Olefinic carbons gave signals at 123.91 and 132.53 ppm, while the carbonyl carbon had a peak at 172.89 ppm. The peak at 67.05 ppm was attributed to the methylnene carbons adjacent to the oxygen in the morpholine ring, while the methylenic carbons adjacent to the nitrogen in the ring absorbed at 53.72 ppm.

Antimicrobial activity. The screening of activity was carried out using the amines and their derivatives in chloroform. The activity of the amine derivatives was greater against the gram-negative bacteria than the gram-positive bacteria as shown by the inhibition zones. The gram-positive bacteria were inhibited to a small extent by the vernolamides.

The absence of activity of the amines highlighted the enhanced effect of the vernolamides.

The activity of N-2-(1-pyrrolidino)ethylvernolamide was more pronounced against *Trichophyton mentagrophyte* than against the other fungi. All the vernolamide derivatives showed inhibition against *Saccharomyces cerevisiae*, *Trichophyton mentagrophyte* and *Microsporum gypseum*. The presence of additional bonds (pyrrolidine and morpholine rings) in the epoxy acid resulted in enhanced inhibition as compared to vernonia oil.

The amines, (1-(2-aminoethyl)pyrrolidine, 1-(3-aminopropyl)- 2-pyrrolidinone and vernonia oil did not show any activity against the microorganisms tested (both bacteria and fungi) except 4-(3-aminopropyl)morpholine which was active against *Eschericha coli* at a concentration of 500 µg.

CONCLUSION

The study has shown that under appropriate conditions, vernonia oil reacts with 1-(2-aminoethyl)pyrrolidine, 1-(3-aminoprpropyl)-2-pyrrolidinone, and 4-(3-aminopropyl)-morpholine to form their respective vernolamides. The synthesized aminoamides contain tertiary amino groups.

The chemical form of the amine moiety has an effect on amidation as reflected in the yields of the products. The pyrrolidine, morpholine, and pyrrolidinone rings do not affect the reactivity of the amines to a great extent because they are not attached directly to the -NH$_2$ group. However, solubility of the reactants influences the rate of a reaction.

The synthesised amide derivatives have the secondary and tertiary nitrogens separated either by two or three CH$_2$ groups. These derivatives have lone pairs of electrons on the nitrogens, therefore the compounds can function as precursors in the making of polymeric resins for removing hydrogen ions in solution by forming quaternary ammonium ions.

The vernolamides were found to be bio-active against some micro-organisms (fungi and bacteria). Thus the vernolamides could be used as bactericides and fungicides.

ACKNOWLEDGEMENTS

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