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CONVERSION OF *VERNONIA GALAMENSIS* OIL TO PYRIDINYL-VERNOLAMIDES AND THEIR ANTIMICROBIAL ACTIVITIES

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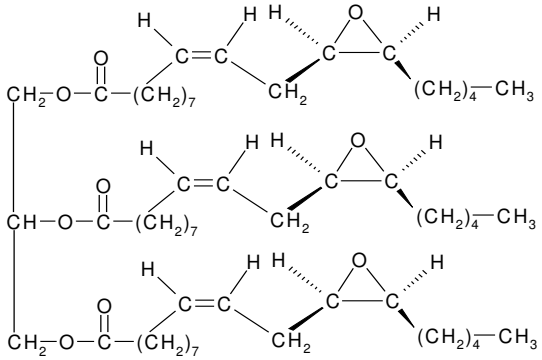
ABSTRACT. Vernonia oil obtained by Soxhlet extraction from the seeds of *Vernonia galamensis* ssp. *nairobensis* was reacted with 2-aminopyridine, 2-(aminomethyl)pyridine and 2-(aminoethyl)pyridine using a 1:3 mole ratio of vernonia oil to amine at varied temperatures to give the corresponding vernolamides. The expected vernolamide from the reaction of 2-aminopyridine and the oil was not formed. N-(2-pyridinylmethyl)vernolamide and N-(2-pyridinylethyl)vernolamide exhibited antibacterial activity that was shown to be higher against gram-positive (*Bacillus subtilis*) than gram-negative (*Escherichia coli*) bacteria. The vernolamides did not show any antifungal activity.

KEY WORDS: *Vernonia galamensis*, 2-Aminopyridine, N-(2-pyridinylmethyl)vernolamide, N-(2-pyridinylethyl)vernolamide, Antimicrobial activity

INTRODUCTION

Vernonia galamensis oil contains about 80 % trivernolin (**1**), a triglyceride of vernolic acid (*cis*-12,13-epoxy-cis-9-octadecenoic acid).



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Vernolic acid (**2**) is an unsaturated fatty acid containing one epoxy and one double bond. The high content of trivernolin, which has three epoxy rings and three double bonds, makes it a prime candidate for application in the coating and plastic industries [1, 2]. The oil has excellent solubility in many organic solvents and could possibly be used as a reactive diluent for high solid coating formulations [3, 4]. Incorporation of vernonia oil in the production of high solid coatings would lead to reduction in the use of non-reactive diluents in coating formulations. The non-reactivity of

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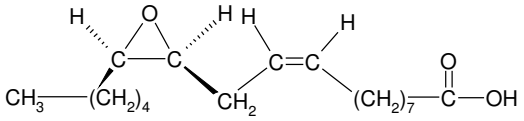
vernonia oil`s epoxy group under alkaline conditions has been reported [5]. Thus under appropriate conditions it is possible to derivatize vernonia oil and obtain a wide range of products.



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Applications includes its transformation to fatty amides, which are important chemical intermediates for commerce, with applications ranging from paper coatings and printing ink additives to slip and anti-block additives for polyethylene films. Basic diamides have a wide variety of applications such as detergent additives, fungicides, rust inhibitors, antistatic agents and water repellants [6]. Industrially, secondary amides are produced from purified fatty acids and primary amines at high temperatures under moderate pressure. They are also produced by reacting fatty acyl chlorides with amines or, alternatively, by reacting fatty esters with amines while using sodium methoxide as a catalyst [5].

Mambo *et al.* [7] reported the synthesis of tertiary vernolamides, which exhibited both antifungal and antibacterial activities. Other studies on direct amidation using vernonia oil, showed that synthesis of N,N′-ethylenebis(vernolamide), N-(aminoalkyl)vernolamide and N,N′-polymethylene(vernolamide) in the absence of a catalyst could be successful [8, 9]. Alkanoamides, diamides and arylakylamides have been prepared using tallow oil, a triglyceride, at low temperatures of 50-60 °C. These reaction temperatures are approximately 100 °C lower than the present conventional practice employed for the synthesis of fatty amides [10]. In another study, where N-substituted amides were screened for anti-microbial activity against bacteria, yeast and molds, it was reported that amides containing an epoxy group exhibits a broad spectrum of antimicrobial activity which is further enhanced by unsaturation [11]. This paper describes the synthetic transformation of vernonia oil to novel epoxy containing basic fatty amides by reacting the oil with primary amines containing a pyridinyl group. The anti-microbial properties of these products are also reported.

EXPERIMENTAL

The amines were purchased from Aldrich Chemical Co. (USA). Solvents were obtained from Kobian Kenya Limited (Kenya). Chromatography was performed using MN-Silica gel (70-230 mesh). All fractions were concentrated in *vacuo* using a rotary evaporator. Precoated analytical MN-Silica gel TLC plates were used. IR were carried out in Nujol on a Perkin-Elmer 435 V-04 spectrometer; GC-MS analysis were performed on a Hewlett Packard 589 DA instrument; ^{1}H NMR spectra were obtained with a Bruker WP 200 SY Spectrometer operating at 500 MHz; the melting points were measured by a Gallenkamp melting point apparatus and are uncorrected.

The bioassays were done using Sabouraud dextrose agar and Muller Hinton agar obtained from Biochemistry Department, University of Nairobi (Kenya). The fungal tests were done using *Saccharomyces cerevisiae* (S.C.), *Microsporum gypseum* (M.G.) and *Trichophyton metagrophyte* (T.M.), while the antibacterial tests were done on *Escherichia coli* (E.C.), *Baccilus subtilis* (B.S.) and *Staphylococcus aureus* (S.A.).

Extraction and purification of the oil. Dry seeds were tempered for lipase deactivation by placing them in an oven and heating for 1 h at 90 °C. Seed moisture was maintained at approximately 15

%. The ground seeds were heated together with *n*-hexane (2.5 L) at 55-60 °C for about 5 h in a Soxhlet extractor. The crude extract was concentrated in *vacuo* at 40 °C., decolorized by mixing it with 5 % w/w of activated charcoal at 60 °C for 1 h. The decolorized oil was then obtained by filtration. The oil was then degummed by stirring with distilled water in the ratio of 21:1 at 50 °C for 1 h followed by centrifuging for 3 h. The gum and oil were separated and the oil dried at 60 °C under reduced pressure for 1 h. The degummed oil was then alkali refined by adding 2 M NaOH (1 mL) and stirring the mixture at 40 °C for 30 min. The refined oil was centrifuged, washed with saturated NaCl solution (10 mL) and bleached with Fuller's earth (1 %).

Reaction of vernonia oil with 2-aminopyridine at 70 °C. Vernonia oil (1 g, 1.08 mmol), and 2-aminopyridine (0.33 g, 3.24 mmol) were heated in a 50 mL flask with stirring at 70 °C for 12 h. The residue was dissolved in chloroform (40 mL) and washed with water (3 x 20 mL). The organic layer was dried over anhydrous magnesium sulfate and solvent removed in a rotary evaporator. The product was shown by IR spectroscopy to contain a strong ester carbonyl stretch at 1730 cm^{-1} and the absence of the amide NH stretch at 3300 cm^{-1}, an indication that the reaction had not taken place. When the above reaction was repeated at 150 °C, red sticky product, believed to be a polymer, was formed via epoxide ring opening.

Synthesis of N-(2-pyridinylmethyl)vernolamide. A mixture of vernonia oil (1.01 g, 1.08 mmol) and of 2-(aminomethyl)pyridine (0.35 g, 3.24 mmol) was heated together at 70 °C for 12 h. The crude product was then washed with water (3 x 20 mL). The organic layer was separated and dried over anhydrous magnesium sulfate and solvent removed in a rotary evaporator. The residue was taken up in warm n-hexane (20 mL) and upon cooling gave N-(2-pyridinylmethyl)vernolamide (0.79 g, 63 %). M.p. 40-42 °C. EIMS: *m/z* (%), 386 (M^+, 8), 273 (3), 135 (15), 163 (26), 150 (34), 92 (47), 109 (100), 107 (86). Analysis found: C, 74.50; H, 9.78; N, 7.32 %; calc. for $C_{24}H_{38}N_20_2$ (386.58): C, 74.59; H, 9.91; N, 7.25 %). V_{max} (Nujol, cm^{-1}); 820, 830 (epoxy); 3260 (N-H); 3060 (Ar-H); 1640-1660 (CON). ^{1}H NMR ($CDCl_3$ 500 MHz) δ: 0.44 (3H, t, J = 7.0 Hz, CH_3), 0.85-1.82 (22H, m, -CH_2), 2.46 (2H, t, J = 7.4 Hz, -CH_2-CO), 2.93 (2H, m, epoxy protons), 4.1 (2H, s, N-CH_2), 4.98-5.1 (2H, dt, J = 11 Hz, J = 12.75 Hz, olefinic H), 6.30 (1H, m, -CONH-), 6.8 (1H, d, J = 7.7 Hz, Ar-H-3), 6.75 (1H, br s, Ar-H-5), 7.2 (1H, d, J = 5.5, Ar-H-4) and 8.1 (1H, s, Ar-H-6).

Synthesis of N-(2-pyridinylmethyl)vernolamide at 80 and 25 °C. Vernonia oil (1 g, 1.08 mM) and 2-(aminomethyl)pyridine (0.35 g, 3.24 mM) were reacted at 80 °C. The crude product was then purified by column chromatography on 30 g silica gel. The column was eluted with hexane and then with a gradient of hexane and increasing amounts of ethyl acetate. Two compounds were obtained, one recrystallized from hexane in 16 % yield and was identified through m.p. 40-42 °C as N-(2-pyridinylmethyl)vernolamide. The other was a dark sticky material that was probably polymeric. The procedure was again repeated but on half the scale at 25 °C for a period of 12 h. The crude product was chromatographed to give the crystalline N-(2-pyridinylmethyl)vernolamide (0.09 g, 45 %). M.p. 40-42 °C.

Synthesis of N-(2-pyridinylethyl)vernolamide at 70 °C. A mixture of vernonia oil (1.01 g, 1.08 mmol) and 2-(2-aminoethyl)pyridine (0.4 g, 3.27 mmol) was refluxed for 12 h at 70 °C. The crude product was then taken up into warm n-hexane and allowed to cool. This gave N-(2-pyridinylethyl)vernolamide as white crystals (0.77 g, 59 %). M.p. 38-40 °C. EIMS, *m/z* (%), 400 (M^+, 8) 273 (3), 135 (15), 163 (26), 150 (34), 92 (47), 109 (100), 107 (86). Analysis found: C, 74.92; H, 10.09; N, 7.01 %. Calc. for $C_{25}H_{40}N_2O_2$ (400.61): C, 74.95; H, 10.06; N, 6.99 %. V_{max} (Nujol, cm^{-1}); 820, 830 (epoxy), 3260 (N-H), 3060 (Ar-C-H), 1640-1660 (-CONH-). ^{1}H NMR ($CDCl_3$ 500 MHz) δ: 0.43 (3H, t, J = 7.2 Hz, CH_3), 0.78-1.73 (22H, m, -CH_2), 2.51 (2H, t, J = 7.7 Hz, -CH_2-CO), 2.7 (2H, t, J = 8.11, N-CH_2), 2.99 (2H, m, epoxy protons), 3.22 (2H, t, 9.2 Hz, CH_2-

pyridinyl), 4.78-5.03 (2H, dt, J = 10.80 Hz, J = 13.11 Hz, olefinic H), 6.30 (1H, m, -CONH-), 6.9 (1H, d, J = 7.5 Hz, Ar-H-3), 6.73 (1H, br s, Ar-H-5), 7.3 (1H, d, J = 5.3, Ar-H-4) and 8.3 (1H, s, Ar-H-6).

Synthesis of N-(2-pyridinylethyl)vernolamide at 25 and 80 ºC. The procedure for the reaction above was repeated twice using oil (0.5 g, 0.55 mmol) and 2-(2-aminoethyl)pyridine (0.18 g, 1.62 mmol) at 25 and 80 °C for a period of 12 h. The products were chromatographed to give the amide N-(2-pyridinylethyl)vernolamide (0.09 g, 32 %), m.p. 38-40 °C and (0.04 g, 16.3 %), m.p. 42-44 °C, respectively. The results are shown in Table 1.

Table 1. Results of the reactions at different temperatures.

Amide	Temperature (ºC)	Yield (%)	M.p. (°C)
N-(2-Pyridinylmethyl)vernolamide	25	45	40-42
	70	63	40-42
	80	16	40-42
N-(2-Pyridinylethyl)vernolamide	25	32	38-40
	70	59	38-40
	80	16	42-44

Biological activity of the epoxy amides

The antimicrobial activities of the amines, N-(2-pyridinylmethyl)vernolamide, N-(2-pyridinylethyl)vernolamide and vernonia oil were tested. About 500 µg each of the above were dissolved in 1 mL of chloroform and serially diluted to 100, 50 and 25 µg/mL solutions. Blank disks (0.5 cm diameter) were soaked in these solutions until saturation and allowed to dry. The discs were used to challenge the growth of *Saccharomyces cerevisiae*, *Microsporum gypseum* and *Trichophyton metagrophyte (T.M.)* fungi, while *Escherichia coli* (gram –ve), *Bacillus subtilis* (gram +ve), *Candida albicans* and *staphylococcus aureus (S.A.)* were used for antibacterial tests.

Media preparation. For the antifugal tests, 62 g of Sabouraud dextrose agar (SDA) was dispersed in 1 L of deionised water and soaked for 10 min. Mixture was swirled and sterilized at 121 ºC for 15 min., cooled to 47 ºC and transferred into petri dishes (10 mL each) and left to cool in a laminar flow cabinet before use. The same procedure was repeated for the antibacterial tests using Mueller Hinton agar 38 g/L.

Inoculation. The appropriate medium was inoculated with the test microorganisms on radial axis starting from the centre of the medium. The discs containing the extracts were placed on the surface of the medium and then incubated. The incubation of the fungi was done at 26 ºC and fungal growth monitored for three days. The incubation temperature for the bacteria was 30 ºC and growth monitored for 24 h. The results are presented in Table 2.

Table 2. Results of antimicrobial activities of the synthesized vernolamides.

Compound	Conc. (μg/mL)	Bacteria/inhibition (mm)			Fungi/inhibition (mm)		
		B.S.	S.C.	E.C.	T.M.	M.G.	C.A.
N-(2-Pyridinylmethyl)-vernolamide	100	23	-	17	-	-	-
	50	20	-	15	-	-	-
	25	15	-	-	-	-	-
N-(2-Pyridinylethyl)-vernolamide	100	20	-	15	-	-	-
	50	15	-	15	-	-	-
	25	10	-	9	-	-	-
Vernonia oil	100	17	-	13	-	-	-
	50	13	-	11	-	-	-
	25	7	-	7	-	-	-

Where, B.S. E.C., S.C., T.M., M.G. and C.A. are *Bacillus subtilis*, *Escherichia coli*, *Saccharomyces cerevisiae*, *Trichophyton metagrophyte*, *Microsporum gypseum* and *Candida albicans*, respectively.

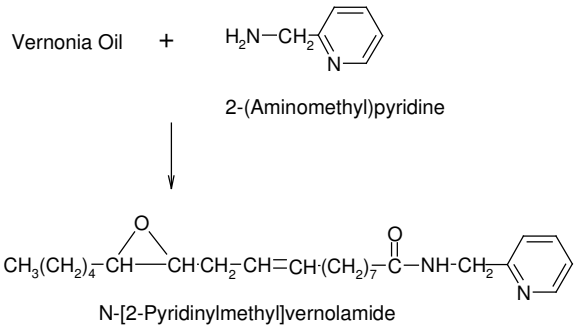
RESULTS AND DISCUSSION

Reaction of vernonia oil with 2-aminopyridine at 70 ºC. When the reaction of vernonia oil and 2-aminopyridine (1:1) mole ratio at 70 °C was carried out for 12 h it was evident that no reaction had taken place. This was confirmed by the presence of strong absorption bands at 1730 cm^{-1} characteristic of the ester group and the absence of amide NH stretching bands at 3300 $cm.^{-1}$ Extending the reaction time to 18 h and decreasing the molar ratio of oil to amine to 1:3 gave the same result, which indicated that the reaction had not taken place. The reason 2-aminopyridine did not react with the vernonia oil to give the desired vernolamide, was probably due to the poor nucleophilicity of 2-aminopyridine. The lone pairs of electrons on the primary amino nitrogen are withdrawn into the pyridine ring via resonance, making the amino group less nucleophilic.

Reaction of vernonia oil with 2-aminopyridine at 150 ºC. When the reaction was repeated by increasing the temperature to 150 ºC for 12 h at molar ratio oil to amine 1:3 a viscous product was obtained. In this case, the absorption bands characteristic of epoxy group at 825 and 840 cm^{-1} disappeared, indicating that the reaction of the amine with the epoxy group had occurred.

Reaction of vernonia oil with 2-(aminomethyl)pyridine at 70 ºC. The reaction of vernonia oil with 2-(aminomethyl)pyridine yielded N-(2-pyridinylmethyl)vernolamide as shown in Scheme I. The disappearance of the triglycerides (vernonia oil) and formation of the product was confirmed by TLC, IR, EIMS and NMR spectroscopy.

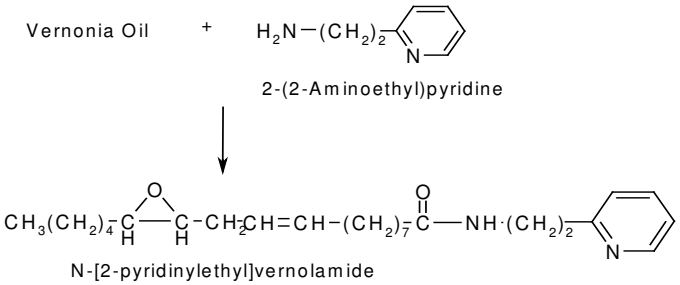
The infrared spectrum for the epoxy fatty amide showed sharp absorption at 3260 cm^{-1} typical of secondary amides. Absorptions at 1660, 1640 and 1560 cm^{-1} were taken as an indication of the carbonyl group of the amide function. Aminolysis had taken place at the ester bond, giving amide as the product. The epoxy absorption was still present at 820 and 830 cm^{-1}. The mass spectral data further confirmed the structure for the isolated N-(2-pyridinylmethyl)vernolamide. The peak at *m/z* 386 represents the molecular ion peak M^+ that is in agreement with the molecular mass of N-(2-pyridinylmethyl)vernolamide. Cleavage at C-11 to C-12 bond gave a fragment at *m/z* 273. The ions at *m/z* 135 and 163 arose from simple cleavages alpha and gamma to the carbonyl group, respectively while a McLafferty rearrangement gave the ion at *m/z* 150. Alpha cleavage of the alkyl group attached to the amide nitrogen gave rise to the fragment ion at *m/z* 92. The peak at *m/z* 107 was due to simple cleavage of C and N bond of the amide group.



Scheme I. Reaction of vernonia oil with 2-(aminomethyl)pyridine.

The NMR spectrum was consistent with the structure. Protons on the terminal methyl groups gave a triplet at δ 0.44; methylene group protons gave multiplets between δ 0.85 and δ 1.82, except for the methylene attached to the carbonyl, which gave a triplet at δ 2.46 and pyridinyl methylene at δ 4.1. Epoxy protons produced a multiplet at δ 2.93, while aromatic protons gave a doublet at δ 6.8, a broad singlet at δ 6.75, doublet at δ 7.2 and a singlet at δ 8.1.

Formation of N-(2-pyridinylethyl)vernolamide. Reaction of vernonia oil with 2-(2-aminoethyl)pyridine resulted in the formation of N-[2-pyridinylethyl]vernolamide (Scheme II), which was then identified by IR, EIMS, and NMR spectroscopy.



Scheme II. Reaction of vernonia oil with 2-(2-Aminoethyl)pyridine.

Infrared spectroscopy absorptions at 820-830 cm^{-1} indicated that the epoxy group was still stable. Absorption at 3260 cm^{-1} was due to NH of amide and at 3060 cm^{-1} due to the C-H stretch of the benzene. Absorption at 1640 cm^{-1} was due to amide I band and at 1560 cm^{-1} was due to the amide II band. Peak at 1660 cm^{-1} was caused by carbonyl stretching of the amide bond. Mass spectrometric data indicated fragment ions that were similar to those previously described for N-(2-pyridinylmethyl)vernolamide. The molecular ion M^+ was observed at *m/z* 400 and the base peak at *m/z* 106. Cleavage at C-11 to C-12 bond gave a fragment at *m/z* 287. The ions at *m/z* 149 and 177 arose from simple cleavages alpha and gamma to the carbonyl group respectively, while a McLafferty rearrangement gave the ion at *m/z* 164. Alpha cleavage of the alkyl group attached to the nitrogen gave rise to the fragment ion at *m/z* 106 and was the base peak, while the signal at *m/z* 123 could be due to a quaternary ammonium ion. The peak at *m/z* 121 was due to simple cleavage of the C-N bond of the amide group. From the ^1H NMR spectrum, terminal methyl

group gave a triplet at δ 0.43; methylene group protons gave a multiplet between δ 0.78 and δ 1.73 except the methylene attached to the carbonyl, which gave a triplet at δ 2.51, and another triplet at δ 2.7 (pyridinyl methylene). Epoxy protons gave a multiplet at δ 2.99, while aromatic protons a doublet at δ 6.9, a broad singlet at δ 6.73, a doublet at δ 7.3 and a singlet at δ 8.3.

In terms of biological activity, from the inhibitions zones it is evident that the activity of the amine derivatives was greater against the gram-positive bacteria than the gram-negative bacteria. The gram-negative bacteria were inhibited to a smaller extent by the amides. The additional bonds in the epoxy acid resulted to enhanced inhibition as compared to vernonia oil. The synthesized amides N-(2-pyridinylmethyl)vernolamide, N-(2-pyridinylethyl)vernolamide and the oil showed activity against both *Bacillus subtilis* and *Escherichia coli* bacteria. There was no antifungal activity shown.

CONCLUSIONS

The reactions of vernonia oil with 2-(aminomethyl)pyridine and 2-(2-aminoethyl)pyridine resulted in the formation of their respective vernolamides in high yields, while 2-aminopyridine did not react. When the effect of temperature was examined, it was evident that a temperature of 70 °C gave the highest yields of the corresponding vernolamides while reactions done at 80 °C gave the lowest yields. Reactions at 25 °C interestingly gave high yields a pointer that aminolysis can be carried out even at room temperature. Reaction temperatures above 80 °C led to opening of the epoxy ring giving polymeric material, hence ring opening seems to be temperature dependent.

The vernolamides, N-((2-pyridinylmethyl)vernolamide and N-(2-pyridinylethyl)vernolamide, were found to be bioactive against *Bacillus subtilis* (gram +ve) and *Escherichia coli* (gram -ve). Thus they are potential chemicals that are likely to be used as bactericides, though more research work needs to be done on other strains for comparison purposes.

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