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Larvicidal activity of (oxiran-2-yl)methylpentanoate extracted from mushroom *Cyptotrama asprata* against mosquito *Aedes aegypti*

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ABSTRACT

In our continuing search for mosquito larvicidal compounds from defined laboratory cultures of higher fungi (ascomycete and basidiomycetes), one basidiomycete *Cyptotrama asprata* showed strong larvicidal activity against mosquito *Aedes aegyptii*. From submerged cultures of *C. asprata*, a secondary metabolite (oxiran-2-yl)methylpentanoate was isolated due to its mosquito larvicidal activity against *Ae. aegyptii*. The compound is a new secondary metabolite, whose structure was determined by NMR spectroscopy and comparison with closely related structures from literature. The compound, (oxiran-2-yl)methylpentanoate, showed strong larvicidal activity against the *Ae. aegypti* larvae with LC_{50} of 1.50 ppm and an LC_{90} of 1.90 ppm after 24 hours. These were found to be much lower concentrations than those reported for some natural compounds recently. These results are of value since search for new natural larvicidal compounds from ascomycetes and basidiomycetes is novel.

Keywords: basidiomycete, submerged cultures, mosquito larvicidal, purification and structure elucidation.

INTRODUCTION

Sub-Saharan Africa is faced with an unprecedented magnitude of disease burden in the 21st century due to the global climate change (UNEP, 2006). The prevailing warm moist climates tend to increase the rate of pathogen and vector reproduction, thus intensifying transmission of diseases (Campbell-Lendrum, 2009). There has been increased frequency of episodic departures from normal climatic conditions associated with epidemics of mosquito-borne diseases like malaria, yellow fever and Rift Valley Fever (Onyango et al., 2004; Ndiaye et al., 2006). In the last decade, there have been consistent reports from Kenya that malaria outbreaks occur in areas where the disease was previously non-existent, such as highlands around Mount Kenya (Boko et al., 2007). Furthermore, the rapidly changing land use pattern, increase in urbanization with weak infrastructure support, aggressive socio-economic activities and the inevitable development projects, modify climatic pattern and create appropriate microhabitats for proliferation of *Anopheles gambiae* and *Ae. aegyptii* (Service, 1989; Kuno, 1997; Carlson et al., 2004). An obvious method for the control of mosquito-borne diseases is the use of insecticides, and many synthetic agents have been developed and employed in the field with considerable success. However, one

major drawback with the use of chemical insecticides is that they are non-selective and could be harmful to other organisms in the environment (Wattal et al., 1981). The toxicity problem, together with the growing incidence of insect resistance, has called attention to the need for novel insecticides (Macedo et al., 1997), and for more detailed studies of naturally-occurring insecticides (Ansari et al., 2000). Pyrethrin, extracted from *Chrysanthemum cinnerariaefolium* (Asteraceae), is one of the best known of the natural insecticides and has provided a lead for the synthesis of various pyrethroid derivatives. Unfortunately, there are reported cases of insect resistance to the pyrethroids (Fanello, 1999; Enayati et al., 2003). Numerous studies have been conducted concerning the larvicidal properties of plants in general (Green et al., 1991; Chantraine et al., 1998; Loset et al., 2000; Ciccia et al., 2000; Vahitha et al., 2002; Cavalcanti et al., 2004; Ndung'u et al., 2004; de Omena et al., 2007) but not much is reported beyond the laboratory findings (Shaalan et al., 2005). Apart from plants, fungi are known as prolific producers of novel biologically active molecules with some notable applications as pharmaceuticals and agrochemicals (McCartney et al., 2007). Besides their potential as prolific producers of biologically active compounds, fungi have been under-researched for larvicides as agents in the control of mosquito proliferation. Interestingly, sub-Saharan Africa is documented as the reservoir of most fungal biodiversity on planet earth (Hawksworth, 2001) and these can be investigated for beneficial eco-dynamics information (Ffrench-Constant, 2005). It is in this regard that we report from our continuing search for mosquito larvicidal compounds from nutrient sub-merged cultures of Kenyan higher fungi, that a basidiomycete *Cyptotrama asprata* was found to produce larvicidal compounds against the larvae of *Ae. aegypti*. *C. asprata* is a small agaric mushroom with striking bright orange-yellow and spiny looking but soft surface. It is found mostly on decaying wood in moist forests; lowland rain forests and moist lower mountain forests (Harkonen et al., 2003). The fruiting body of *C. asprata* was collected from an undisturbed habitat in Londiani Forest in Rift Valley in Kenya. In this paper, we report the cultivation of *C. asprata*, purification and structure elucidation of a larvicidal compound named (oxiran-2-yl)methylpentanoate.

MATERIALS AND METHODS

General

The basidiomycete was cultivated on 250 ml scale in 500 ml Erlenmeyer flasks as starter cultures and on 1 L scale in 2 L Erlenmeyer flasks in 60 replicates. The media and flasks were initially heat sterilized using an autoclave for 15 minutes at a temperature of 115 °C and pressure of 1.5 bars. The inoculation and monitoring of growth parameters were done under lamina flow hood backed with a hot flame. Analytical TLC was performed with Macherey–Nagel pre-coated silica gel 60 F_{254} plates (ALUGRAM ® SIL G/UV_{254} 0.25 mm). Column chromatography was packed with silica gel 60 (0.063 – 0.2 mm/70-230 mesh). The developed TLC plate was viewed under dual fixed wavelength UV lamp (λ = 254 nm and 365 nm) and the spots visualised by spraying with freshly prepared *p*-anisaldehyde solution, heated to 115 ^{o}C. The larvicidal experiments were set up in glass beakers. The crude extract and the purified compound were kept under 4 °C except when undergoing analysis. The purified compound was dissolved in deuterated chloroform $CDCl_3$). ^{1}H NMR and ^{13}C NMR spectra were recorded with NMR on Bruker AV 300 MHz spectrometer using TMS as internal standard. COSY, HMBC, HSQC and NOESY were acquired using the standard Bruker software.

Producing organism

A freshly sprouted fruiting body of the basidiomycete, *C. asprata* was collected from undisturbed habitat under a tight canopy in Londiani Forest in Kenya in July 2005. The spores were immediately brought into pure culture and the strain of the *C. asprata* as well as the corresponding herbarium material is deposited in the culture collection in

Integrated Biotechnology Research Laboratory (IBRL), Egerton University. The culture is grown and kept on potato dextrose agar (PDA) and the corresponding herbarium material, were both serialized JO5444 and preserved.

Cultivation of *C. asprata*

C. asprata was cultured in a constituted medium comprising 1% industrial refined molasses, 0.5% glucose and 0.4% of yeast extract for 21 days using a procedure adapted from Thines and Anke (1998). Sixty culture media were prepared as 1 litre sterile replicates in 2 litre Erlenmeyer flasks. The cultures were allowed to grow as still cultures with regular mechanical agitations.

Preparation of crude extracts from cultures of *C. asprata*

Immediately growth was stopped, mycelium was separated from culture filtrate by filtration and the culture filtrate discarded as it had no larvicidal activity. The mycelium was directly soaked in acetone with constant agitation using magnetic stirrer for 4 hours. The acetone filtrate was then concentrated to an aqueous solution. The aqueous solution was extracted thrice with equal volumes of ethyl acetate and the combined volume of ethyl acetate solution was dried with anhydrous sodium sulphate. The dried ethyl acetate was concentrated to dryness using a rotary evaporator under reduced pressure. This gave 3.3 g of oily crude extract.

Purification of the compound

Chromatography on silica gel 60 (0.063–0.2 mm, column size 230 x 25 mm) in cyclohexane-ethyl acetate yielded intermediate products 1~4 eluting successively with cyclohexane-ethyl acetate ratios 4:1, 3:2, 1:1, and 2:3, respectively. From the intermediate products the pure compound was obtained from fraction 2 by isocratic chromatography using 1:1 cyclohexane-ethyl acetate solvent ratio (column size 230 x 10 mm). For further details, see Figure 1.

Mosquito larvicidal activity tests

Eggs of the *Ae. aegypti* mosquitoes were hatched by submerging them in de-chlorinated tap water at a temperature in the range 25–27 °C, as described by the standard WHO protocol (1973). The larvicidal experiments were set-up following procedure described by WHO (2005). In brief, the crude extracts were tested at 1.00, 2.50, 5.00 and 10.00 ppm while the pure compound was tested at the concentrations 1.00 ppm, 1.25 ppm, 1.50 ppm, 1.75 ppm and 2.00 ppm as solutions in 20 ml. To each of the concentrations, batches of 25 late third and early fourth instars larvae were transferred. Four replicates were set for each concentration and an equal number of controls set up simultaneously with tap water to which 1 ml of methanol was added. Each test was done three times on different days. The test containers were held at room temperatures and photoperiod of 12 hours light followed by 12 hours dark (12 L:12 D). Larvae mortality was recorded after 24 hours exposure. Larvae which pupated during the test period were treated as having negated the test. To get the percentage mortality, Abbott's formula was used;

$$Mortality(\%) = \frac{X - Y}{X} x100$$

where X = percentage survival in the untreated control and, Y= percentage survival in the treated sample.

The data obtained from the mosquito larvicidal bioactivity was analysed by linear regression that was in-built in Microsoft Excel 2003 software. The LC_{50} and LC_{90} were calculated only for the purified compound with a reliability interval of 95% adapted from McLaughlin et al. (1993).

RESULTS AND DISCUSSION

Taxonomy of the producing organism

Pure cultures of *C. asprata* were prepared from the fruiting body of a freshly

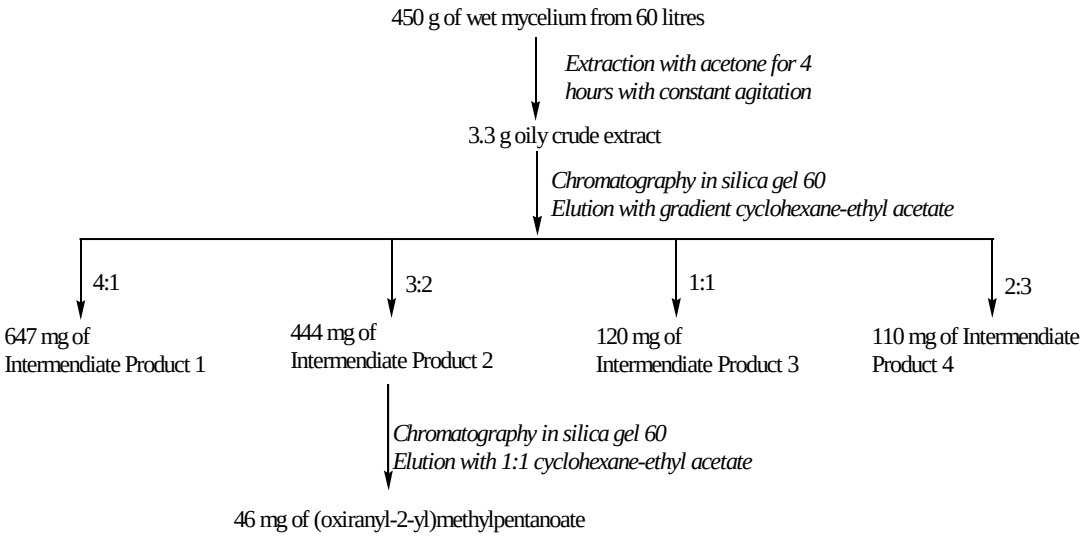


Figure 1: Isolation scheme for the metabolites produced by *C. asprata*

sprouted single mushroom. At the time of collection, the mushroom was bright-orange in colour, with a 3 cm-wide cap which was densely covered with erect tufty scales. The gills were white in colour. The stipe was 4 x 1 cm, hollow, fibrous, slightly swollen towards the base, concolorous with upper surface, with floccose tufts especially at the base; no veil but with an indistinct ring-like collar close to the apex. The spore print was white and each spore being elliptical in shape with 8.3 x 5.4 µm size. Once the spores were brought into axenic cultures, the colonies emerged as smooth hyphae which quickly developed into intense brick-red pigmentation on PDA. Microscopically the hyphal strands had distinct clamp connections, typical characteristic of a basidiomycete. All these observations fitted the description for *Cyptotrama asprata* (Harkonen et al., 2003).

Cultivation and purification of the compound

During the cultivation of the *C. asprata* in liquid medium, it was noted that it produces secondary metabolites with mosquito larvicidal activities against larvae of *Ae. aegyptii*. The cultures were harvested after 21 days, when glucose in the medium was used up and the larvicidal activity had reached a plateau. It was noted that only intra-cellular secondary metabolites from mycelium were showing the activity, whereas the extra-cellular metabolites from the culture filtrate never showed larvicidal activity, hence was discarded. The mycelium crude extract when tested at different concentrations in the range 1.00-20 ppm, it was observed that from 2.50 ppm all the larvae of *Ae. aegypti* were killed, that is 100% mortality, after 30 minutes of the experiment.

Fractionation of the crude extract by column chromatography afforded 4 enriched intermediate products (Figure 1). Intermediate product 1 was the least polar while intermediate product 4 was the most polar. All the four intermediate products were subjected to mosquito larvicidal activity at two concentrations 1.25 ppm and 2.50 ppm (Table 1), with the latter found to be least lethal for the crude extract. The former concentration of 1.25 ppm was chosen to help in zeroing on the actual larvicidal compound(s). Intermediate product 1 showed strongest larvicidal activity of 100% mortality at 1.25 ppm after 2 hours of the experiment (Table 1). Close analysis of the components of this enriched fraction on TLC indicated that it comprised mostly of

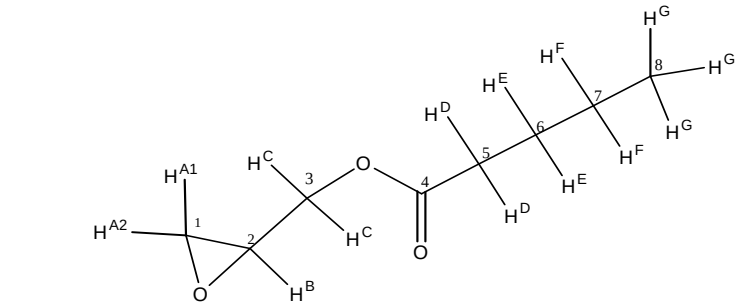
fatty acids. Fatty acids are known to show non-specific biological activities (Stadler et al., 1993) hence, were of no interest for this research. Intermediate product 2 showed larvicidal activity of 100% mortality at 1.25 ppm after 8 hours. On TLC, this enriched fraction had a compound in company of fatty acids, which were definitely residually retained as intermediate product 1 was eluted. The compound was purified and is named (oxiran-2-yl)methylpentanoate. The purified compound, (oxiran-2-yl)methylpentanoate, showed high potency against the *Ae aegypti* larvae with LC_{50} of 1.50 ppm and an LC_{90} of 1.90 ppm after 24 hours from regressions analysis (Table 2). These concentrations are much lower than those reported for some other natural compounds like limonoids, vilasininoid isolated from *Turraea wakefieldii*, and havanensinoids (Ndung'u et al., 2004). Intermediate product 3 showed weaker larvicidal activity than those of intermediate products 1 and 2, with 80% mortality at the same concentration after 24 hours. Further examination on TLC revealed many spots and intermediate product 3 was not purified. Intermediate product 4 did not show any larvicidal activity even after 24 hours, hence was of no interest for this study. It is clear that the crude extract showed a much stronger activity than the purified compound. This is not unusual given that presence of fatty acids was observed and the activity could have resulted from synergistic effects of the compounds present in the crude extract.

Structure determination of (oxiran-2-yl)methylpentanoate (1)

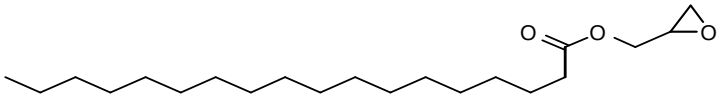
The purified compound was obtained from intermediate product 2 that yielded 46.2 mg (Figure 1). On TLC plate, the pure compound (oxiran-2-yl)methylpentanoate (Figure 2) gave an R_f of 0.54. The compound (oxiran-2-yl)methylpentanoate was yello-wish–oily liquid. The ^{13}C NMR spectrum and DEPT spectrum revealed presence of one ester carbonyl group (δ = 174.6 ppm), two oxygenated methylene carbons (δ = 65.3 ppm and 63.6 ppm) and one oxygenated methine carbon (δ = 70.5 ppm). In addition, three methylene carbons and one methyl carbons were identified. These observations have indicated presence of cyclic functional group (glycidyl) and existence of an alkyl was suggested according to signals in the ^1H NMR spectra. From ^{13}C–NMR spectrum, there is a carbon having a chemical shift at 70.5 ppm; this is a characteristic region for sp^3-hybridised carbon atoms attached to oxygen atom. In the proposed chemical structure, this carbon is labeled number 2 i.e. C_2 in Figure 2. From DEPT spectrum this carbon (at chemical shift=70.5 ppm) is clearly seen to be a methine carbon and from $^1H/^{13}C$ HSQC-DEPT spectrum, this carbon has a proton with a chemical shift at 3.93 ppm - a characteristic region for protons attached to a carbon which is in turn attached to an electronegative group like oxygen (H^B). From COSY $^1H/^1H$-NMR spectrum, H^B couples with protons at 4.14 ppm.

These protons (at δ = 4.14 ppm), from $^1H/^{13}C$ HSQC-DEPT spectrum were assigned to the carbon at 65.3 ppm which is a methylene group based on $^1H/^{13}C$ HSQC-DEPT experiment (H^c) while the carbon is labeled C_3. From the COSY $^1H/^1H$-NMR spectrum, Proton H^B is also coupled to two other protons; at 3.59 ppm and the other at 3.70 ppm. On the hand, from the $^1H/^{13}C$ HSQC-DEPT spectrum, the protons at 3.59 ppm and 3.70 ppm are attached to same carbon at 63.6 ppm which also happens to be methylene but having non equivalent protons. Two non equivalent protons on same carbon can only be found in cyclic structures. These protons have been labelled H^{A1} and H^{A2} while the carbon is labelled C_1 in the proposed chemical structure. Furthermore, the protons (H^{A1} and H^{A2}) do not couple to any other proton except for H^B.

From COSY $^1H/^1H$ spectrum, the protons at 4.14 ppm (H^C) do not couple with any other proton except H^B which therefore means that C_3 is attached to only one carbon atom that is C_2. The chemical shift of C_3 and



Name proposed: (oxiran-2-yl)methyl pentanoate **(1)**



Glycidyl octadecanoate **(2)**

Figure 2: The proposed chemical structure of (oxiran-2-yl)methylpentanoate **(1**) and Glycidyl octadecanoate (**2**).

its protons is in a region characteristic of carbon atoms attached to an oxygen atom. Table 3 gives a summary of NMR spectra and its correlations.

Compounds with an epoxide/glycidyl and ester functional groups' have been reported both from synthetic and natural sources. However, the purified compound **(1)** (Figure 2) has not been reported from both natural and synthetic sources as evidenced by search in the electronic databases for chemical compounds. Comparison of ^{13}C NMR data (Table 4) of compound **(1)** (Figure 2) with those of (R)-glycidyl octadecanoate **(2)** (Figure 2) isolated from basidiomycete *Cortinarius umidicola* (Hu et al., 2003) indicates that the two compounds have similar structures except for the length of the alkyl chain. In addition, compound **(1)** has ester functional group, a property found in many compounds isolated from basidiomycetes, e.g. pulvinic acids isolated from mycelia cultures of *Suillus bovines* (Besl et al., 2008) which has an ester carbonyl atom with chemical shift 172.7 ppm, lovastitin isolated from *Pleurotus ostreatus* (Alarcon et al., 2003) which has two ester carbonyl signals at δ = 170.9 and δ = 176.9), cyclopinol, cyclocalopin A and acetylcyclocalopin A isolated from *Boletus calopuis* (Liu et al., 2008) which also have ester carbonyl functional groups. Purpuracolide isolated from *Gomphus purpuraceus* (Jiang et al., 2008) has an ester carbonyl group at δ = 175.0 ppm. The stereochemistry at C-2 for compound 1 has not been established. The presence of the ester and the epoxide functional groups are most likely responsible for the observed mosquito larvicidal activity. The results presented in this paper indicate of presence of compounds from fungal cultures with mosquito larvicidal activities that may not only rival but supersedes those of the synthetic chemicals.

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Table 1: Results for the mosquito larvae bioactivity of the enriched fraction.

Intermediate Products	Percentage Mortality (%)							
	1.25 ppm				2.50 ppm			
	2 hrs	4 hrs	8 hrs	24 hrs	2 hrs	4 hrs	8 hrs	24 hrs
1	100	-	-	-	100	-	-	-
2	0	60	100	-	0	100	-	-
3	0	0	0	80	100	-	-	-
4	0	0	0	0	100	-	-	-

Table 2: The LC_{50} and LC_{90} values (ppm) for (oxiran-2-yl)methylpentanoate (**1**)

	LC_{50}	**LC_{90}**
Concentration (ppm)	1.50	1.90

Table 3: NMR spectroscopic data (300 MHz) for (oxiran-2-yl)methylpentanoate **(1)**

Entry	^{13}C	^{1}H	COSY	HMBC (C→H)	NOESY
1	63.6	3.59 m, 3.70 m	2	1, 2, 4	1, 2
2	70.5	3.92 m	1, 3	1, 3	1
3	65.3	4.14 m	2	2, 3	2
4	174.6			5, 6	
5	34.3	2.31 m	6	4, 6, 7	8, 6
6	25.1	1.62 m	5, 7	4, 5, 7, 8	5, 7
7	22.9	1.30 m	6, 8,	5, 6, 8	5, 6
8	14.3	0.84 m	7	6, 7	5

Table 4: The ^{1}H and ^{13}C NMR data for compound 1 and 2 (δ in ppm**)**

	Compound 1		Compound 2	
Entry	^{13}C	^{1}H	^{13}C	^{1}H
1	63.6	3.59 m 3.70 m	66.8	4.68
2	70.5	3.92 m*	71.0	4.45*
3	65.3	4.14 m	64.3	4.12
4	174.6		173.8	
5	34.3	2.31 m	34.4	2.35
6	25.1	1.62 m	25.3	1.63
7	22.9	1.30 m	23.0	1.25
8	14.3	0.84 m	14.3	0.85

* Comparison may suggest different stereochemistry at this position for both.

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