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Insecticidal activity of a novel fatty acid amide derivative from *Streptomyces* species against *Helicoverpa armigera*

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ABSTRACT

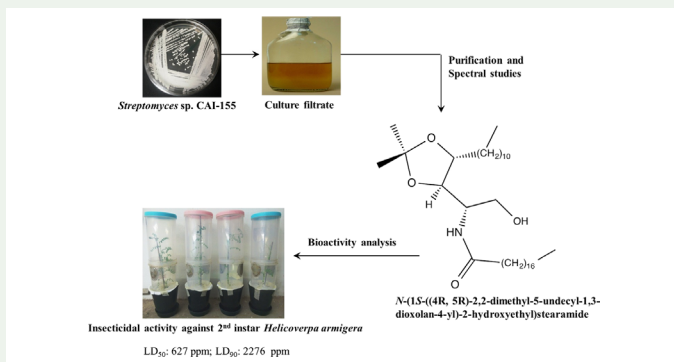
Helicoverpa armigera, an important pest causes serious damage to grain legumes. The main objective of this study was to isolate and identify the metabolite against *H. armigera* from a previously characterised *Streptomyces* sp. CAI-155. The culture filtrate of CAI-155 was extracted using Diaion HP-20 and the active fractions were fractionated on Silica and C18 column chromatography. The C18 active fraction was further fractionated on Silica gel 60 F₂₅₄ thin layer chromatography (TLC). The most active fraction (Rf 0.64) purified from TLC led to the identification of a novel metabolite *N*-(1-(2,2-dimethyl-5-undecyl-1,3-dioxolan-4-yl)-2-hydroxyethyl)stearamide by spectral studies. The purified metabolite showed 70–78% mortality in 2nd instar *H. armigera* by diet impregnation assay, detached leaf assay and greenhouse assay. The LD₅₀ and LD₉₀ values of the purified metabolite were 627 and 2276 ppm, respectively. Hence, this novel metabolite can be exploited for pest management in future.

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1. Introduction

Legume pod borer, *Helicoverpa armigera* (Hübner), is one of the most important lepidopteran pests worldwide. It feeds on more than 300 species of plants including chickpea, pigeonpea, groundnut, cowpea, cotton, maize and sorghum and a range of vegetable and fruit crops, and tree species. The larvae of *H. armigera* feed on leaves and stems but, they prefer buds, inflorescences, fruits and pods, thus causing damage to both vegetative and reproductive plant parts (Moral García 2006). A total of US\$ 500 million worth of soybean and cotton has been lost in Brazil by *H. armigera* where it has been introduced in recent past (Czepak et al. 2013). Synthetic pesticides belonging to pyrethroids, cyclodienes, carbamates and organophosphates have been used efficiently to control *H. armigera*. Because of indiscriminate use of pesticides, *H. armigera* have developed resistance to a range of conventional insecticides (Yang et al. 2013). Over dependence on chemical pesticides has resulted in outbreaks of secondary pests, environmental contamination and a decrease in biodiversity (Lacey & Shapiro-Ilan 2008).

Therefore, there is a need for research on novel metabolites to identify safer alternate products for pest management. Among the various sources of natural products, microorganisms play a key role as they constitute an infinite pool for novel secondary metabolites (Subramani & Aalbersberg 2012). As of 2010, there were around 60–80,000 microbial natural products identified, in which fungi are the major contributors (~30,000) followed by almost equal contribution of actinobacteria and unicellular bacteria (~20,000). In the context of bioactive microbial metabolites also fungus (15,600) contributes higher than actinobacteria (13,700). However, fungal metabolites are the contribution from all groups of fungi whereas, in actinobacteria, the single genus *Streptomyces* (10,400) is the principal contributors (Bérdy 2012). Some of the secondary metabolites produced by the *Streptomyces* sp. with the key role in agricultural sector include, Blastidicin-S from *S. griseochromogenes* (Kimura & Yamaguchi 1996) and kasugamycin from *S. kasugaensis* (Tanaka et al. 1966) which have been used against rice blast, mildiomyacin from *S. rimofaciens* against powdery mildew (Om et al. 1984) and polyoxins from *S. cacaoi* for the control of fungal diseases (Isono & Suzuki 1979). Non-*Streptomyces* actinobacteria also produce glycopeptides and orthosomycins (Nicolaou et al. 2009). Secondary metabolite from actinobacteria has the advantage over the fungal metabolites in terms of lower phytotoxic activity (Bérdy 2005). Previously, we had reported three strains of *Streptomyces* sp. (CAI-155, SAI-25 and BCA-698) isolated from herbal vermicompost for their insecticidal activity against lepidopteran pests including *H. armigera* (Vijayabharathi et al. 2014). The main objective of the present study was to isolate and identify the secondary metabolite(s) responsible for the mortality of *H. armigera* from the *Streptomyces* sp. strain CAI-155.

2. Results and discussion

The present study was designed for purification and characterisation of an insecticidal metabolite from *Streptomyces* sp. CAI-155 against *H. armigera*, originally isolated from vermicompost of *Thevetia peruviana* (yellow oleander) foliage. CAI-155 was demonstrated to have insecticidal activity in extracellular, intracellular and whole cells against *H. armigera*, *Spodoptera litura* and *Chilo partellus* (Vijayabharathi et al. 2014) and plant growth promoting (PGP) activity in rice (Gopalakrishnan et al. 2014) and chickpea (Gopalakrishnan et al. 2015).

Diaion HP-20 fractions of the culture filtrate of *Streptomyces* sp. CAI-155 yielded non-adsorbed fraction (NAF), adsorbed and MilliQ water fractions. Among the three fractions, significant larvicidal activity ($p < 0.05$) of *H. armigera* was recorded only in adsorbed fraction with 85% mortality at 2 days after treatment (DAT) and 100% mortality by 6 DAT. Diaion is a polystyrene divinylbenzene (PS-DVB) copolymer with polymethacrylate structure used for adsorbing hydrophobic compounds, antibiotics and biomolecules. It has been widely used in purification of bioactive compounds from microbes including *Streptomyces* sp. (Liu et al. 2012).

The adsorbed fraction was further fractionated on silica gel column chromatography and eluted with a conclave concentration gradient of chloroform: methanol. When the fractions were evaluated for their insecticidal activity, all the fractions (except F8 and F14) resulted in larval mortality, however, F4 showed 100% mortality. Hence, F4 was selected for purification of the active metabolite. The active F4 fraction on the Silica gel column was further fractionated on C18 column and eluted with gradient of methanol: water. Significant mortality was recorded in 60% methanol [F4(5); 96% mortality], 80% methanol [F4(6); 42% mortality] and 100% methanol [F4(7); 21% mortality] fractions. Therefore, 60% methanol fraction [F4(5)] was selected for further purification of the active metabolite.

The F4 (5) fraction was further fractionated on Silica gel 60 F₂₅₄ thin layer chromatography (TLC) plates using mobile phase of n-butanol/acetic acid/water. When the plates were observed under short wave UV light (254 nm), three bands were recorded, C1 (Rf 0.10), C2 (Rf 0.64) and C3, (Rf 0.91). All the three bands resulted in mortality of 2nd instar *H. armigera*, with the highest mortality of 83% in band C2. Therefore, band C2 was considered as purified compound and subjected to identification by NMR and MS analysis.

Accurate mass determination analysis of the isolated compound lead to the molecular ion [M + H] molecular formula C₃₆H₇₁NO₄. Electron ionisation analysis showed no molecular ion and the presence of a major peak at m/z 283 easily explained via McLafferty rearrangement leading to fragment C₁₈H₃₇NO⁺. This last result pointed towards the asymmetric alkyl chains of 17 and 11 carbons, respectively. Data from the spectral studies confirmed that, the identified compound is a novel metabolite *N*-(1-(2,2-dimethyl-5-undecyl-1,3-dioxolan-4-yl)-2-hydroxyethyl)stearamide, a fatty acid amide derivative (Figure 1).

A similar compound with the same molecular mass was synthesised and reported earlier by Katiyar et al. (1998). Though having the same total number of C-atoms, the lengths of

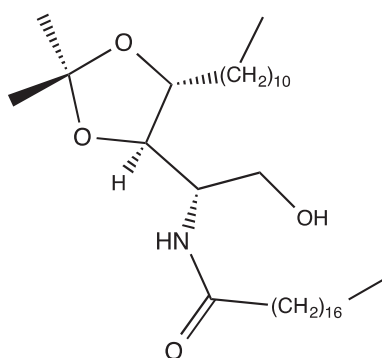


Figure 1. Structure of the *N*-(15-((4R, 5R)-2,2-dimethyl-5-undecyl-1,3-dioxolan-4-yl)-2-hydroxyethyl)stearamide (1).

Table 1. Larvicidal activity of the purified compound, *N*-(1-(2,2-dimethyl-5-undecyl-1,3-dioxolan-4-yl)-2-hydroxyethyl)stearamide, against 2nd instar larvae of *H. armigera*.

Conc [†] (ppm)	Diet impregnation assay	Standard*	Detached leaf assay	Standard*	Greenhouse experiment	Standard*
250	24.6 ± 1.4 ^d	43.5 ± 2.5 ^c	18.5 ± 3.7 ^d	40.0 ± 5.8 ^c	20.0 ± 0.0 ^d	40.0 ± 5.8 ^c
500	40.6 ± 1.4 ^c	82.6 ± 5.0 ^b	37.0 ± 3.7 ^{cd}	76.7 ± 6.7 ^b	36.7 ± 3.3 ^{cd}	73.3 ± 3.3 ^b
1000	78.3 ± 2.5 ^b	100.0 ± 0.0 ^a	74.1 ± 3.7 ^b	100.0 ± 0.0 ^a	70.0 ± 5.8 ^b	96.7 ± 3.3 ^a

Note:

Values are the mean of three replicates ± SE; Mean values followed by lower case letters are not significant ($p < 0.05$);

[†]Concentration;

*Azadirachtin was used as standard.

alifatic chains are different in **1** than in the synthesised compound. We confirmed that by MS experiments with successive fragmentation, which unambiguously showed the length of the chains in **1**. In addition, stereochemistry of the 1,3-dioxolane ring of **1** is also different, according to NOESY data.

The purified metabolite, *N*-(1-(2,2-dimethyl-5-undecyl-1,3-dioxolan-4-yl)-2-hydroxyethyl)stearamide, was evaluated for its insecticidal activity against 2nd instar *H. armigera* at 250, 500 and 1000 ppm. As plant-based metabolites also has the larvicidal properties (Suryawanshi et al. 2015), azadirachtin, a well-known bio-pesticide isolated from *Azadirachta indica* foliage was used as standard (Koul 2012) in the present study. Highest larvicidal activity of 78, 74 and 70% was observed in diet impregnation, detached leaf and greenhouse assays, respectively, at 1000 ppm. This was followed by lower insecticidal activity of 36–40% and 20–24% at 500 and 250 ppm, respectively, which indicates the dose-dependent larvicidal activity of *N*-(1-(2,2-dimethyl-5-undecyl-1,3-dioxolan-4-yl)-2-hydroxyethyl)stearamide. Azadirachtin at 500 ppm exhibited the same level of larvicidal activity of *N*-(1-(2,2-dimethyl-5-undecyl-1,3-dioxolan-4-yl)-2-hydroxyethyl)stearamide (Table 1). The LD₅₀ and LD₉₀ values of the purified metabolite, *N*-(1-(2,2-dimethyl-5-undecyl-1,3-dioxolan-4-yl)-2-hydroxyethyl)stearamide, were 627 and 2276 ppm (χ^2 (df) = 15.7(7); p value = 0.027), respectively, which were twofold higher, i.e. 305 and 744 ppm (χ^2 (df) = 30.2(7); p value = 0.000), respectively, for azadirachtin.

Omura (2011) reported a wide range of secondary metabolites identified from bacteria, fungi and actinomycetes. Reports on bioactive metabolites of *Streptomyces* from forest soil (Balachandran et al. 2015; Velayudam & Murugan 2015), marine (Sánchez López et al. 2003; Rashad et al. 2015) and terrestrial environments (Raju et al. 2013; Duraipandiyar et al. 2014) further supports the potentiality of this genus. Arasu et al. (2013) identified a novel polyketide metabolite with larvicidal, antifeedant and growth inhibitory properties on *H. armigera* and *S. litura* from a marine *Streptomyces* sp. AP-123. Sun et al. (2014) reported a new glutarimide derivative, 3-[2-[2-hydroxy-3-methylphenyl-5-(hydroxymethyl)]-2-oxoethyl] glutarimide, from ethyl acetate fraction of the fermentation broth of a marine sponge-derived *S. anulatus* S71. Sathya et al. (2016) identified a secondary metabolite, diketopiperazine, cyclo(Tre-Phe), from *Streptomyces* sp. SAI-25 with insecticidal activity against *H. armigera*.

Since, the identified compound is a novel fatty acid amide derivative, comparison of its biological activity could be more reasonable with related fatty acids and its amide derivatives. Fatty acid amides and their derivatives exhibited a broad-spectrum antimicrobial activity against human pathogenic bacteria, yeasts and moulds (Novak et al. 1969; Kabara et al. 1972; Omura et al. 1974). Antibacterial activity against bacteria and yeast increased with

the number of carbon atoms in the skeleton and so with methylamide and di-methylamide derivatives of the compound (Kabara et al. 1972; Omura et al. 1974).

Fatty acids are produced by all organisms, where it is catalysed by a type I fatty acid synthase (FAS), while in plants and bacteria it is type II FAS system in which each reaction is catalysed by a discrete protein (Gago et al. 2011). An acyl carrier protein (ACP) involved in the synthesis of FAS produced by *Streptomyces coelicolor* (Revill et al. 1996). Fatty acid biosynthetic pathway is well established in *Escherichia coli* (White et al. 2005) but for other bacteria including actinomycetes such as *Streptomyces* sp, has received little attention. The genes involved in fatty acid biosynthesis in *Streptomyces* genomes (such as *S. coelicolor*) revealed that some of the genes encoding the core enzymes involved in saturated fatty acid biosynthesis lie in a conserved fatty acid biosynthesis (*fab*) cluster that comprises the genes including *fabD*, *fabH*, *acpP* and *fabF* (Gago et al. 2011). The *fabH* gene was demonstrated for encoding one of the FAS proteins in *S. coelicolor* (Revill et al. 1995).

CAI-155 has been reported to produce chitinase, hydrocyanic acid (HCN), indole acetic acid (IAA), siderophore, cellulase, lipase, protease and β -1,3-glucanase. Siderophore gene from CAI-155 is highly up-regulated (25-folds) which provides further evidence of its biocontrol potential (Gopalakrishnan et al. 2014). This strain registered highest enzyme production of all the traits (HCN, siderophore, cellulase, lipase, protease and β -1,3-glucanase except IAA) when compared to other five strains of *Streptomyces* sp. CAI-155 was demonstrated to colonise roots of chickpea (Gopalakrishnan et al. 2015). It is a known fact that bacteria stimulates plant growth directly by solubilising nutrients, fixing nitrogen and producing growth hormones such as IAA and indirectly by antagonising insect pests and plant pathogens through production of siderophores, chitinase, β -1,3-glucanase, antibiotics and secondary metabolites (Patten & Glick 2002). Chitinase, HCN, siderophore, lipase, β -1,3-glucanase and protease-producing bacteria not only play an important role in the nutrient mineralisation, composting and PGP, but also act as biocontrol agents (Vansuyt et al. 2007; Macagnan et al. 2008). Therefore, it is concluded that the *Streptomyces* strain CAI-155 can be a potential tool in the integrated pest and disease management programs.

3. Experimental

3.1. Actinomycete used

Streptomyces sp. CAI-155 (GenBank Acc. No: KF770896) reported previously for insecticidal activity against *H. armigera* (Vijayabharathi et al. 2014) was further used.

3.2. Extraction of the active metabolites

The *Streptomyces* strain CAI-155 was grown on starch casein broth at 28 °C for eight days in a shaker at 120 rpm. At the end of the incubation, the culture broth was centrifuged at 12,000 *g* for 10 min at 4 °C, and the supernatants (cell free extracts) collected. The Diaion HP-20 resin (30 g) was conditioned by solvating with methanol (150 mL for 30 min) and equilibrated with MilliQ water (two times with 150 mL for 30 min). The conditioned resin was incubated with cell free extracts of CAI-155 for 2 h in a shaker at 150 rpm. At the end of the incubation, the culture filtrate soaked resin was passed into a glass column (20 × 2.5 cm) at a steady flow rate of 10–15 mL/min. The discharge hereafter was called NAF. The column was washed

with MilliQ water to remove the residues of non-adsorbed materials. The column was eluted with MeOH and termed as adsorbed fraction. The NAF, adsorbed and MilliQ water fractions were analysed for its efficacy against 2nd instar *H. armigera* by using a diet impregnation assay (section 3.5.2).

3.3. Purification of the active metabolite

The active fraction obtained from Diaion HP-20 resin fractionation was further purified on Silica gel column chromatography (23 × 3.6 cm). The column was packed with Silica gel for one day in advance in order to allow the Silica to settle in the column. The active fraction of the Diaion HP-20 resin was injected into the column and eluted with a conclave concentration gradient of chloroform: methanol (starting with 95% chloroform and ending with 0% chloroform). A total of 20 fractions were collected and evaluated for biological activity against 2nd instar larvae of *H. armigera* by diet impregnation assay. The active fraction was further subjected to C₁₈ column chromatography (23 × 3.6 cm), after conditioning the column, and eluted with gradient of methanol in MilliQ water (5, 10, 20, 40, 60, 80 and 100% MeOH). All the fractions were analysed for their biological activity against 2nd instar larvae of *H. armigera* by diet impregnation assay (section 3.5.2). The most active fraction in C₁₈ column chromatography was subjected to TLC.

Samples of the active fraction from C₁₈ column chromatography were spotted as 1 cm bands alternating with 1 cm non-spotted areas on Silica gel TLC plates (Silica gel 60 F₂₅₄, Merck, Germany). The plates were developed in a mobile phase consisting of n-butanol/ acetic acid/water at 2:1:1 (v/v). When the solvent ran to within 1 cm of the top of the plate, the solvent front was marked and immediately removed from the chromatography tank. After drying, the plates were examined under normal light, and short (254 nm) and long (350 nm) wave ultraviolet light. Conspicuous bands were marked and R_f values were calculated. The bands were scraped separately from the TLC plates, transferred into Eppendorf tubes (1.5 mL) and agitated with methanol (500 µl). Other areas of the plate adjacent to the bands and areas devoid of the sample, as controls, were also scraped from the TLC plate and agitated with methanol. Tests and controls were centrifuged at 6500 g for 5 min. The supernatants were transferred into a vial and the remaining Silica agitated again in methanol (500 µl) and centrifuged. The process was repeated twice and the supernatants were amalgamated. All the fractions were checked for their biological activity against 2nd instar larvae of *H. armigera* by diet impregnation assay (section 3.5.2). The active fraction (purified metabolite) was subjected to NMR and MS for structural elucidation.

3.4. NMR and MS analysis

All NMR data were recorded using a Bruker Avance 600 spectrometer, operating at a proton frequency of 600.18 MHz with a 5 mm triple-resonance cryo probe equipped with a z-gradient. NOESY experiment was run on a Bruker Avance 400 spectrometer, (proton frequency 400.13 MHz), equipped with a 5 mm dual probe for ¹H and ¹³C, equipped with a z-gradient. The samples containing a solution of 5 mg of the substance in CDCl₃ were measured at 300 K, using TMS signal as a reference. Following 1D and 2D pulse sequences from the Bruker user library were used for the NMR experiments: ¹H 1D (600 MHz), ¹³C 1D (150 MHz), HSQC (600/150 MHz), HMBC (600/150 MHz), DQF-COSY (600 MHz) and NOESY (400 MHz).

3.4.1. NMR data of compound 1

^1H NMR (CDCl_3 , 600 MHz) δ : 1.22–1.34 (m, 44H, H4-17 and H10'-17'), 1.38 (m, 4H, H20' and H9'a), 1.42 (s, 3H, H19'), 1.51–1.68 (m, 4H, H3 and H8'), 2.22 (t, 2H, $J = 7.6$ Hz, H2), 2.66 (bs, 1H, OH), 3.66 (m, 1H, H2'a), 3.79 (dd, 1H, $J = 3.9/8.2$ Hz, H3'), 3.88 (td, 1H, $J = 3.5/8.2$ Hz, H7'), 4.00 (m, 1H, H2'b), 4.04 (m, 1H, H1'), 6.20 (d, 1H, NH). ^{13}C NMR (CDCl_3 , 150 MHz) δ : 14.1 (C18 and C18'), 22.7 (C17 and C17'), 25.6 (C3), 26.2 (C9'), 27.0 (C19'), 27.2 (C20'), 29.3–29.7 (C5-C14 and C10'-C14'), 29.4 (C4), 29.7 (C15 and C15'), 31.9 (C16 and C16'), 33.5 (C8'), 36.8 (C2), 50.6 (C1'), 62.1 (C2'), 78.9 (C7'), 82.7 (C3'), 108.9 (C5'), 173.2 (C1).

Low resolution analyses with electron ionisation were performed on an Agilent GC7890-MS5975 (SQ). The sample was introduced with a direct probe and no chromatography separation was used previous to the mass analysis. Accurate mass determination in positive and negative mode was performed on a 'Synapt G2-S' Q-TOF instrument from Waters™. Samples were ionised by the use of ASAP probe (APCI). Calculated exact mass and spectra processing was done by Waters™ Software Masslynxs V4.1 SCN871.

3.5. Characterisation of the purified metabolite

The purified metabolite was evaluated for biological activity at three concentrations (250, 500 and 1000 ppm) against 2nd instar larvae of *H. armigera* by diet impregnation assay, detached leaf assay and under greenhouse assay, as discussed below in sections 3.5.2, 3.5.3 and 3.5.4, respectively, and compared with the standard, azadirachtin. The LD_{50} and LD_{90} values of the purified compound against 2nd instar larvae of *H. armigera* also calculated and compared with standard azadirachtin.

3.5.1. *H. armigera* rearing and maintenance

Rearing of *H. armigera* was done as per Babu et al. (2014).

3.5.2. Diet impregnation assay

Various fractions and the metabolite were bio assayed against 2nd instar *H. armigera* using diet impregnation assay (Narayanamma et al. 2007). Larval mortality was recorded on 2, 4 and 6 DAT.

3.5.3. Detached leaf assay

Various fractions and the metabolite were also bio assayed by using detached leaf assay (Sharma 2005). Larval mortality was recorded on 2, 4 and 6 DAT.

3.5.4. Greenhouse assay

Chickpea plants were grown under greenhouse at 25 ± 2 °C under natural light conditions. When chickpea plants were at 8–10 leaf stage, 10 2nd instar larvae of *H. armigera* were released on the plants after sprayed with test samples. The plants were covered with a plastic jar (with wire mesh at the top) to restrict the movement of larvae. The experiment was repeated two times. The larval mortality was recorded at 2, 4 and 6 DAT.

3.6. Statistical analysis

The data were subjected to analysis of variance, followed by *post hoc* Tukey's test. LD₅₀ and LD₉₀ values were calculated by Probit regression analysis. All of the statistical analysis was conducted using SPSS version 13.0 (SPSS Inc., Chicago, Illinois, USA).

4. Conclusions

A novel metabolite *N*-(1-(2,2-dimethyl-5-undecyl-1,3-dioxolan-4-yl)-2-hydroxyethyl) stearamide was purified from *Streptomyces* sp., CAI-155 with insecticidal activity against *H. armigera*. This is a novel metabolite reported for the first time. Exploration of such novel metabolites fills the void for environment friendly pest management.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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