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Production of Insect Toxin Beauvericin from Entomopathogenic Fungi *Cordyceps militaris* by Heterologous Expression of Global Regulator

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ABSTRACT

Cordyceps militaris is one of entomopathogenic fungi species that is well known to be a traditional medicine in China for decades. Although the pharmaceutical and/or toxic properties of *C. militaris* has attracted attention as a promising resource for finding bioactive compounds, only a few substances including cordycepin have been reported so far. In the previous report heterologous expression of LaeA, a global regulator for secondary metabolites production in fungi, has been succeeded in *C. militaris*. The *LaeA*-engineered transformants are proved to produce new and/or elevated production of secondary metabolites, as detected by HPLC analysis. In order to further characterize the secondary metabolites that were being significantly produced by *LaeA* transformant, HPLC profiling and structure elucidation by proton NMR were conducted in two target compounds, designated as compound 1 and compound 2. Compound 1 possessed the highly similar characters to insect toxin beauvericin in UV spectrum, molecular weight, and retention time in HPLC analysis. Proton NMR analysis revealed that compound 1 had the same proton signals as beauvericin.

INTRODUCTION

Entomopathogenic fungi (henceforth EF) are group of fungi that parasite insects, and thus are able to kill or seriously disable them. During the infection to insects, these fungi grow either in the inside of insects body or on the exoskeleton surface, causing the death of the host insect, which they differentiate to form fruiting bodies afterward (Lee, Kinoshita, Ihara, Igarashi, & Nihira, 2008). Several reports, however, showed that this group of fungi posses the significant additional roles in nature. The roles includes plant disease antagonists, fungal endophytes, rhizosphere colonizers, and plant growth promoting agents, or known as PGPF (Plant Growth Promoting Fungi). *Beauveria bassiana* (Ascomycota: Hypocreales) and *Lecanicillium* spp. (Ascomycota: Hypocreales) are two well-known EF that can function as dual biological control for both insect pests and plant pathogens (Ownley, Gwinn, & Vega, 2009). The ability of entomopathogenic fungi to function as reservoir for natural product resource starts to receive wide attention to date. These insect fungi now are known to be rich sources of structurally novel biolocally active substances.

During infection and proliferation in insects, entomopathogenic fungi are thought to produce various bioactive compounds, because they need to overcome immune defence system of host insects and produce toxin to kill the hosts. The recent studies on this revealed that these bioactive compounds are not only function for fungi defence system, but also possess a various beneficial effects to human, as it can be functioned as antimicrobial, anti-cancer, and/or anti hypercolesterol agents. The capability of EF to play role as reservoir for natural product resource starts to receive wide attention to date.

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The megagenus *Cordyceps* that is known to be highly host specific genus are the most notable EF and are objects for significant number of researches (Isaka et al., 2005). This genus has been commercially used as traditional Chinese medicine and is most characterized with respect to their bioactive compound production. There are several bioactive compounds have been reported from *Cordyceps*, including cordycepin and other subtances in the form of adenosine derivatives that posses anti-tumour and antibacterial activities (Ng & Wang, 2005), an antimalarial agent isolated from *C.nipponica* designated as cordypyridones A dan B (Isaka, Tanticharoen, Kongsaeree, & Thebtaranonth, 2001).

The fungus *C. militaris* has raised attention recently for the fact that it has notable number of biological and pharmacological activities. This fungi, also known as north cordyceps, is proved to have activities including anticancer, antibacterial, antifungal, and immunoregulative activities. The extract of these fungi may thus be a potential therapy to combat human leukemia (Lee et al., 2006).

Although the pharmaceutical and/or toxic properties of *C. militaris* has attracted attention as a promising resource for finding bioactive compounds, only a few substances including cordycepin have been reported so far. This is possibly due not only to the mutual characters among filamentous fungi, such as slow growth, but the difficulty to reproduce the culture conditions under which EF produce bioactive compounds, because EF is considered to synthesize various bioactive compounds only when it infects or proliferates in the host insects but at quite low level under conditions applied in laboratories. To discover newly emerged bioactive compounds from this fungus efficiently, the method to activate secondary metabolism even under laboratory conditions need to be developed.

Despite the fact that these fungi face difficulties in producing secondary metabolites, the recent reports showed that EF have a notable number of secondary metabolism pathways that encode potential sources for novel and beneficial bioactive compounds. Nevertheless, the biosynthetic pathways of the secondary metabolites are mostly unknown, possibly due to the fact that the productions of the compounds are in trace amounts, making it difficult to detect or reproduce. An important feature that can facilitate genetic analysis of secondary metabolism biosynthesis pathways in fungi is the fact that the biosynthetic pathway are usually exist in adjacent region and form cluster in the genome (Keller, Bok, Chung, Perrin, & Shwab, 2006). These biosynthetic gene clusters are usually silenced or has an extremely low expression resulting in difficulties of production under standard culture condition in laboratory (Chiang, Lee, Sanchez, Keller, & Wang, 2009).

The expressions of some biosynthetic gene clusters known to be regulated by pathway specific regulators. However, recent studies revealed that histone methylase homolog, namely LaeA, play role as a global regulator for the production of secondary metabolite in fungi, and regulates the expression of various gene clusters in several genera of filamentous fungi. LaeA was originally named for loss of aflR expression. This methyltransferase has S-adenosyl methionine (SAM) binding site that is required for several functions (Patananan, Palmer, Garvey, Keller, & Clarke, 2013). This global regulator has raised attention and become model in studying the secondary metabolism in various filamentous fungi.

In the previous report (Rachmawati, Kinoshita, & Nihira, 2013), we succeeded in creating *C. militaris* transformant through heterologous expression by introducing *laeA* gene to discover newly produced bioactive compounds. The transformants successfully produced new and/or enhanced peaks indicating the enhanced production of metabolites. The next step that is reported in this article is the structure elucidation of the compounds. The aim of this research was to search for newly produced or enhanced bioactive compounds production of *C. militaris* using genetic engineering.

MATERIALS AND METHODS

Time and Place of the Research

The research was conducted on October 2009-July 2011 in the Laboratory of Molecular Microbiology, Division of Advanca Biotechnology, Graduate School of Engineering, Osaka University Japan.

Reproducibility Analysis of Secondary Metabolite

In the previous research (Rachmawati, Kinoshita, & Nihira, 2013) metabolites profiling by HPLC analysis were performed in all *LaeA* transformant to determine the effect of *Lae*A. Among all eight *LaeA* transformants, transformants encoded with 3F produced two notable peaks under A3M medium condition supplemented with Sanagiko. To check the reproducibility of the compounds, same culture condition were used to cultivate 3F transformant, under shaking condition (28 ^{o}C, 120 spm). After 14 days, the mycelia of the cultures were extracted with half volume of acetone. The mixtures of broth were centrifuged to separate the solvent layers from the aqueous layers. In vacuo evaporation were conducted with one millilitre solvent layer that were being re-dissolved in 1 mL DMSO and filtered with a 0.2 µm Polytetrafluoroethylene (PTFE) filter (Advantec, Japan).

HPLC coupled with DAD analysis were conducted using 100-µl transformant extract. HPLC system used was an Agilent HP1100 with a photodiode array detector (200-600 nm) using Rainin Microsorb C18 (4.6 x 75 mm) column. A stepwise gradient of CH_3CN-0.15 % KH_2PO_4 (pH 3.5) (15 %-85 % v/v) were used as the mobile phase, with 1.2 mL per minute flow rate. The condition for the gradient were 15 % (0-3 minutes), 15-40 % (3-6 minutes), 40 % (6-12 minutes), 40-55 % (12-19 minutes), 55-85 % (19-22 minutes), 85%(22-29 minutes), and 85-15 % (29-32 minutes).

Purification of Target Compounds

Purification of target peaks was started by making large-scale cultivation involving 16 flasks of 30 ml culture in 100 ml Erlenmeyer flasks (total 480 ml) and 6 flasks of 100 ml culture in 500 ml baffle flasks (total 600 ml). From the 480 ml culture, 2.19 g of crude extract was obtained; and from 600 ml culture, 2.6 g of crude extract was obtained. This crude extract was further purified.

The extraction of the mycelium and fermentation broth was conducted in transformant 3F after 7 days fermentation. Half-volume of *n*-butanol were added to the broth and stirred for one hour. The extract were then being centrifuged to separate the mixture, continued with separation of organic layer from the aqueous layer in an extraction funnel, and being dried with anhydrous Na_2SO_4. The reversed-phase C-18 column chromatography were used to further purify the crude extract, with a step gradient of hexane and ethyl acetate with the ratio of 10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, and 0:10 using Sep-Pak© Vac 35 cc (10 g) C18 cartridge (Waters, USA). Target compound was completely eluted in 90 % and 100 % ethyl acetate (fraction 10 and 11 respectively). Final purification was conducted by subjecting fractions 10-11 to reversed-phase HPLC with 80 % methanol + 0.1 % TFA to yield purified target compounds.

To further purify the target compound, 0.2 g of crude extract were used to be separated by column chromatography using Sep-Pak Silica 35 cc column. The solvent used as mobile phase were a mixture of hexane and ethyl acetate respectively. After checking with HPLC-DAD, it was revealed that the target compound was eluted in 90 % and 100 % ethyl acetate (fraction 10 and 11, respectively). The target compounds in this fraction were collected by HPLC, and yielded 37.31 mg and 20.63 mg for compound 1 and compound 2, respectively. These purified compounds were sent to mass spectrometry to gain the information about the molecular weight.

Structure Elucidation of Target Compounds

Before being subjected to structure elucidation, HPLC analysis on an Agilent HP-1100 system were conducted to yield the target compounds, using a Cosmosil 5C18-AR-II column (Nacalai Tesque Inc., 4.6 x 100 mm), with methanol as the solvent, unless otherwise stated.

The purified compounds obtained from fraction 1:9 and 0:10 of hexane and ethyl acetate were analysed by mass spectrometry (HRFABMS using JEOL JMS-700 spectrometer) and their structures were elucidated by proton Nuclear Magnetic Resonance (NMR) (400 MHZ, using JEOL JNM-ECP 400 spectrometer) in $CDCl_3$.

RESULTS AND DISCUSSION

Reproducibility Analysis of Secondary Metabolite

In the previous research (Rachmawati, Kinoshita, & Nihira, 2013) metabolites profiling by HPLC analysis were performed in all *LaeA* transformants to determine the effect of *Lae*A towards the production of secondary metabolite. Among all eight *LaeA* transformants, transformants encoded with 3F produced two notable peaks, indicating two newly production of compounds. The reproducibility analysis proved that these peaks were reproducible under A3M medium condition supplemented with insect derived materials Sanagiko. The similar peaks were not detected in the wild-type strain (Fig.1). First compound, designated as compound 1, were eluted at 24.9 min with λmax: 210 nm and absorbent for UV Spectrum. Compound 2 were eluted at 25.8 min and has similar UV spectrum.

By comparing the position of eluted compounds in the HPLC condition and the UV spectra with those registered in-house compounds database, compound 1 and compound 2 has strong possibility to be derivatives of beauvericin, a common compound within genus Isaria, the teleomorph form of *Cordyceps* (Luangsa-Ard, Berkaew, Ridkaew, Hywel-Jones, & Isaka, 2009). Since compound 1 and beauvericin overlapped completely in HPLC when co-injected, compound 1 was assumed to be beauvericin itself or closely related compound to beauvericin. To further confirm this assumption, mass spectrometry analysis were performed to both compounds. Subsequently the molecular weight of both compounds were estimated by HRFAB-MS. MS spectra reveals that compound 1 has the same molecular weight as beauvericin (783.4), while compound 2 has molecular weight 797.4, suggesting the presence of additional methylene (CH_2) group in the compound (Fig.2).

To obtain further information of compound 1, both compound 1 and beauvericin standard were subjected to HREI-MS analysis to compare their fragmentation pattern. It was found that compound 1 has the same fragmentation pattern with beauvericin ($C_{45}H_{57}N_3O_9$). Since the pattern of fragmentation of compound 1 is almost identical to that of beauvericin, it is being strongly suggested that compound 1 might be beauvericin (Fig.3).

Beauvericin contains three N-methyl-L-phenylalanyl and D-a-hydroxy-isovaleryl residues in alternating sequence. At least 15 analogs of beauvericin have so far been reported, and each analog of beauvericin possesses molecular weight, ranging from 735-837. The molecular weight of compound 1 and compound 2 were being compared with that of beauvericin and its reported analogs. Among the reported beauvericin and beauvericin analogs, compound 1 has the same molecular weight only with beauvericin itself, while compound 2 possesses the same molecular weight with that of beauvericin A and beauvericin F (Table 1).

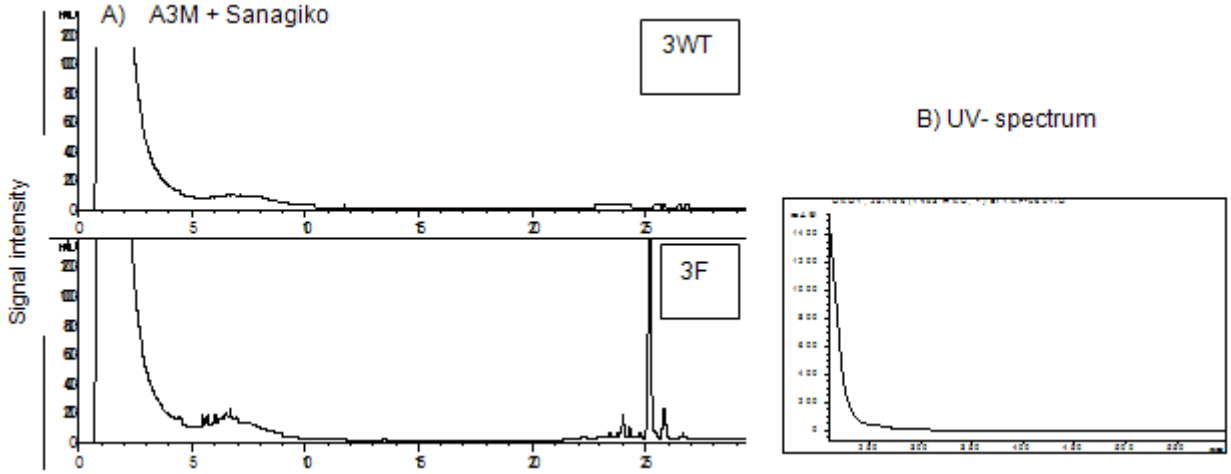

Fig. 1. HPLC profile of newly emerged compounds in LaeA transformant 3F from C. militaris HF 374-1 strain. A) Peaks production of wild type (3WT) strain is shown in upper chart, and of the transformant 3F is shown in below part. B) UV spectrums of each compound produced by transformant 3F.

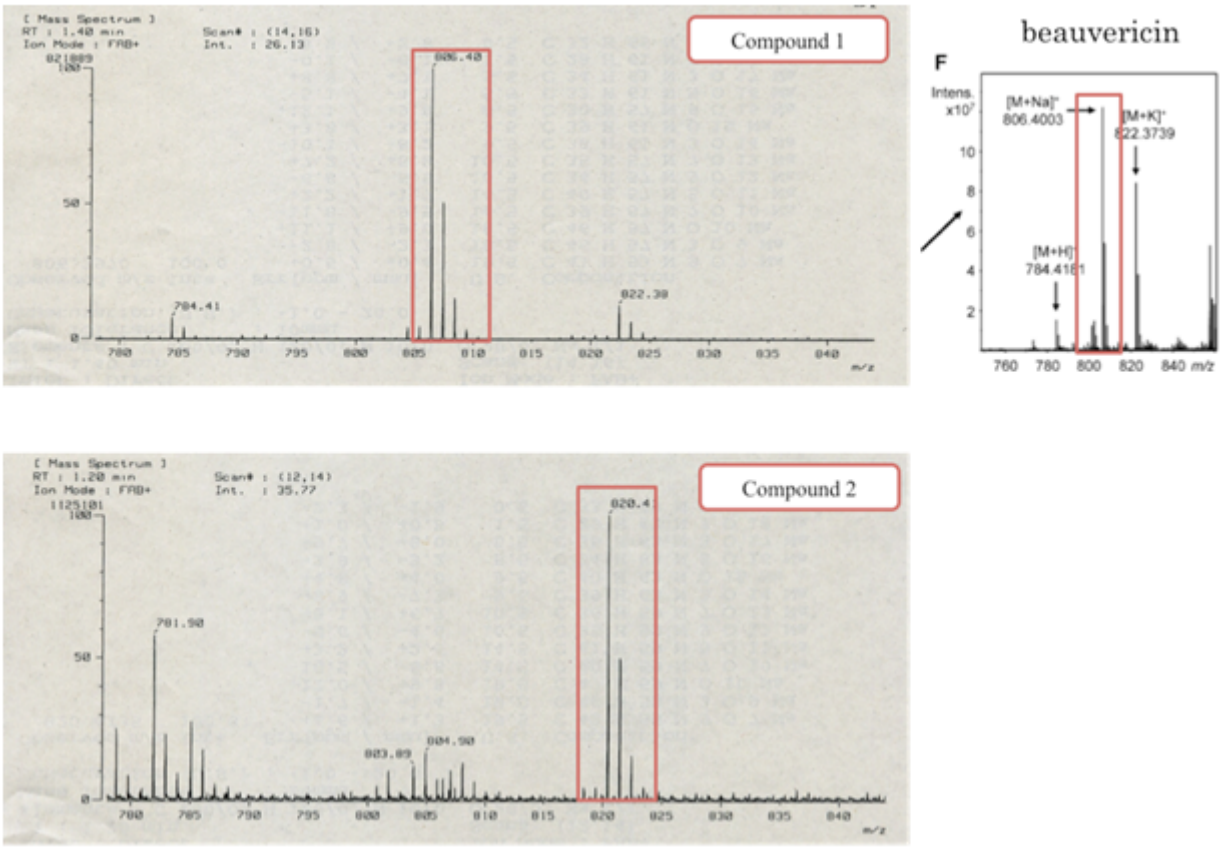

Fig. 2. Molecular weight of compound 1 and compound 2 from laeA transformant and comparison with beauvericin

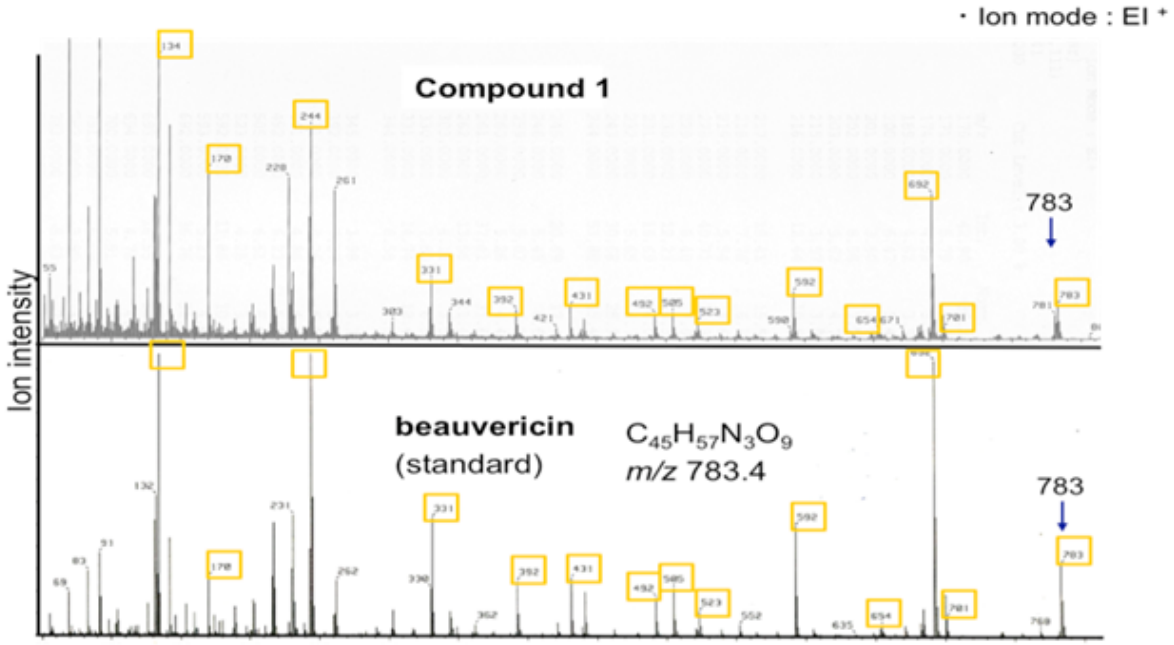

Fig. 3. Comparison of fragmentation patterns of Compound 1 and beauvericin

Table 1. List of molecular weights of beauvericin, beauvericin analogs, compound 1, and compound 2

Name	Molecular Weight	Molecular Formula	Name	Molecular Weight	Molecular Formula
Beauvericin	783	$C_{45}H_{57}N_3O_9$	allobeauvericin C	825	$C_{48}H_{63}N_3O_9$
beauvericin A	797	$C_{46}H_{59}N_3O_9$	beauvericin G1	769	$C_{44}H_{55}N_3O_9$
beauvericin B	811	$C_{47}H_{61}N_3O_9$	beauvericin G2	755	$C_{43}H_{53}N_3O_9$
beauvericin C	825	$C_{48}H_{63}N_3O_9$	beauvericin G3	741	$C_{42}H_{51}N_3O_9$
beauvericin D	769	$C_{44}H_{55}N_3O_9$	beauvericin H1	801	$C_{45}H_{56}FN_3O_9$
beauvericin E	735	$C_{41}H_{57}N_3O_9$	beauvericin H2	819	$C_{45}H_{55}F2N_3O_9$
beauvericin F	797	$C_{46}H_{59}N_3O_9$	beauvericin H3	837	$C_{45}H_{54}F3N_3O_9$
allobeauvericin A	798	$C_{46}H_{59}N_3O_9$	compound 1	783	$C_{45}H_{57}N_3O_9$
allobeauvericin B	811	$C_{47}H_{61}N_3O_9$	compound 2	797	$C_{46}H_{59}N_3O_9$

Table 2. The Proton signal of beauvericin and compound 1

beauvericin (Ref.)	beauvericin (Standard)	Compound 1
0.42, d, CH_3	0.375,d	
0.79, d, CH_3	0.789, d	0.75, d
		0.86, d
2.03, m, β-CH, hiv	1.96, m	1.24, s
		1.67, m
2.98,s, N-CH_3	3.008, s	3.026,s
2.96, m. β-CH(H), phe	2.97, m	2.96,s
3.34, dd, β-CH(H), phe	3.42, m	3.52, dd
4.93, d, α-CH,hiv	4.85, d	
5.42, dd, α-CH,phe	5.56, m	5.05, m
		5.75, dd
7.23, m, aromatic H, phe	7.23, m	7.24, m

In order to confirm whether compound 1 was indeed beauvericin and compound 2 was one of known analogs of beauvericin, both compounds were subjected to Nuclear Magnetic Resonance (NMR) analysis by proton-NMR in 400 MHz in $CDCl_3$. With this partial structure elucidation, the proton signal of both signals could be determined. The NMR spectra revealed that compound 1 missed one methyl signal, but acquired a double-doblet signal, different from beauvericin, but was still indicating that compound 1 might be beauvericin (Table 2).

Since compound 2 has the same molecular weight with beauvericin A and beauvericin F, we decided to compare the proton signal of compound 2 with those two analogs of beauvericin. Interestingly, from the comparison result of proton-NMR spectra among those compounds, it was demonstrated that compound 2 was not identical neither to beauvericin A nor beauvericin F, indicating that compound 2 might be an unknown analog of beauvericin (Table 3). Further structure elucidation by carbon MNR and two-dimensional NMR is needed to reveal the chemical structure of compound 2.

Beauvericin was originally isolated from the well-known EF *Beauveria bassiana*. The fungus *B.bassiana* is the most widely distributed species of the genus and is generally found on infected insects both in temperate and tropical areas throughout the world (Zimmermann, 2007). One of the most important compounds produced by *B. bassiana* is beauvericin which showed insecticidal and antibacterial activity. Beauvericin is a cyclic hexadepsipeptide comprised of three molecules of N-methyl phenylalanine and three molecules of 2-hydroxyisovaleric acid, and has symmetrical structure. The structural feature of beauvericin is attractive because it is capable of accepting several precursors that would lead to the biosynthesis of many ‑unnatural- natural products in a single fermentation (Isaka, Kittakoop, Kirtikara, Hywel-Jones, & Thebtaranonth, 2005).

Unlike the other species of Cordyceps, there is not many reports on the secondary metabolite being produced by *C. militaris*. The most studied compound from this EF is cordycepin, that possesses antifungal, antivirus, and antitumor activities. This compound also is being reported to have potential and interesting biological and pharmacological activities, e.g. anti-cancer, anti-virus, anti-infection, and immunological stimulating activities.

Table 3. The Proton signal of beauvericin A, beauvericin F and compound 2

beauvericin (Ref.)	Compound 2	beauvericin A	beauvericin F
0.42, d, CH_3		0.43 (3H,d)	
		0.68 (3H,m)	
0.79, d, CH_3	0.76, s	0.76 (3H,d)	0.80 (3H, d)
	0.87, m	0.81 (3H,d)	0.86 (3H, d)
	1.1, s		1.28 (1H, m)
	1.2, s		1.64 (1H, m)
		1.78 (1H,m)	1.46 (1H, m)
2.03, m, β-CH, hiv		2.04 (2H,m)	
2.98,s, N-CH_3	2.98, m	2.95 (3H,s)	2.76 (3H, s)
2.96, m. β-CH(H), phe	3.49, m	3.35 (3H,s)	3.12 (1H, dd)
3.34, dd, β-CH(H), phe	3.66, s	4.93 (1H,d)	3.30 (1H, dd)
5.42, dd, α-CH,phe		5.43 (3H,m)	5.20 (1H, dd)
7.23, m, aromatic H, phe	7.22, s 7.29, s, 7.5, s	7.22 (15H,m)	7.18-7.32 (5H, m)

The other bioactive compounds found in this EF are three new-10 memberedmacrolides, cepharosporolides, and pyridine-2,6-dicarboxylic acid (Rukachaisirikul, Pramjit, Pakawatchai, Isaka, & Supothina, 2004). Those huge potential of cordycepin makes *C. militaris* an economically important fungi, and its potential bioactive compounds are believed yet to be fully explored. The finding about newly produced beauvericin in this fungus when is regulated by global regulator LaeA opened up the potential for highly valuable secondary metabolite production.

Other than in *B. bassiana*, production of beauvericin also was reported in soil-borne fungal pathogens *Fusarium oxysporum*. This ascomycete that can attack 100 various crop relies on beauvericin as its virulence factor. López-Berges et al. (2013) found that velvet protein complex regulates the fungal development and production of secondary metabolite including beauvericin in this fungus. Velvet protein is a complex protein consists of VeA, VelB, VelC, and LaeA. VeA and LaeA were also found to be responsible for the full virulence of *F. oxysporum*. This report emphasizes the importance of LaeA in the survivorship of various filamentous fungi.

The previous study proved that the enrichment of LaeA in the genome of *C. militaris* effectively enhance the production of secondary metabolite in this EF. LaeA were proved to regulate the production of both new and enhanced compounds in eight *C. militaris* transformants. In this report we only focused on one transformant (strain HF 374) that produced significant number production of new compounds that is not detected in the wild type. Nevertheless, the other *lae*A transformants that produced reproducible compounds wait to be elucidated. This study confirmed that one of the bioactive compound produced by the transformant is beauveria, the essential virulence factor for controlling insect pest. As for the other compound has potential to be new analog of beauveria.

CONCLUSION

Eights newly emerged compounds have reproducibly been observed in the culture of three *LaeA* transformants, transformants 3F and 3H (from HF 374-1), and 0C (from CM 001-5). Among these 8 compounds, compound 1 and compound 2 were significantly highly and reproducibly produced, and therefore were chosen as primary target compounds to be elucidated their structure.

Compound 1 and compound 2, possesses similar physico-chemical properties with beauvericin or its reported analogs. Based on ^{1}H-NMR analysis, compound 1 proved to be beauvericin, as for compound 2 proved to be different from the reported ones, suggesting that those two compounds might be new analogs of beauvericin.

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