Advances in Microbiology, 2022, 12, 254-269
https://www.scirp.org/journal/aim
ISSN Online: 2165-3410
ISSN Print: 2165-3402

Production of Ergot Alkaloids by the Japanese Isolate *Claviceps purpurea* var. *agropyri* on Rice Medium

Yuji Doi[1], Daigo Wakana[1], Hisashi Takeda[1], Eiji Tanaka[2], Tomoo Hosoe[1*]

[1]Faculty of Pharmaceutical Sciences, Hoshi University, Shinagawa, Japan
[2]Department of Environmental Science, Ishikawa Prefectural University, Nonoichi, Japan
Email: *hosoe@hoshi.ac.jp

How to cite this paper: Doi, Y., Wakana, D., Takeda, H., Tanaka, E. and Hosoe, T. (2022) Production of Ergot Alkaloids by the Japanese Isolate *Claviceps purpurea* var. *agropyri* on Rice Medium. *Advances in Microbiology*, **12**, 254-269.
https://doi.org/10.4236/aim.2022.124019

Received: March 29, 2022
Accepted: April 24, 2022
Published: April 27, 2022

Abstract

Ergot alkaloids (EAs) are secondary metabolites produced by ergot fungi (e.g., *Claviceps purpurea*), which are parasites of Gramineae grasses. EAs and their analogs are used to treat migraine, postpartum uterine bleeding, and Parkinson's syndrome. Recent studies have reported additional new bioactive activities of EAs and their analogs, making them essential compounds for drug development, drug repositioning, and clinical applications. EAs are produced industrially by field cultivation of ergot or liquid fermentation in the mycelial phase, but there are few published studies of the production of EAs by cereal culture and thus this approach is poorly understood. This study searched for *Claviceps* strains that produce EAs cultured artificially in the mycelial phase, then the selected strains were cultured on cereal media (white rice, brown rice, and rye) to examine their ability to produce EAs on each medium. *C. purpurea* var. *agropyri* produced the Clavine-type EAs pyroclavine (**1**), festuclavine (**2**), and agroclavine (**3**) in the mycelial phase. When cultured with white rice, brown rice, or rye, *C. purpurea* var. *agropyri* produced **1** - **3** on all cereal media. The total amount of **1** - **3** in each cereal medium (150 g of cereal per Roux flask) was 2220.5 ± 564.1 μg for white rice, 920.0 ± 463.6 μg for brown rice, and 595.4 ± 52.1 μg for rye. The white rice medium supported the highest production of **1** - **3**, with the total amount of EAs (150 g of white rice per Roux flask) being about 34 times higher than that in the T25 liquid medium (190 mL per 1 L Erlenmeyer flask) (equivalent amount per flask).

Keywords

Claviceps, Ergot Alkaloid, Rice Culture, Pyroclavine, Festuclavine, Agroclavine

1. Introduction

Ergot alkaloids (EAs) are secondary metabolites produced by a wide range of fungi, predominantly *Claviceps* spp. (e.g., *C. purpurea*), a parasite of wheat, rye, and other Gramineae grasses. EAs are synthesized within the sclerotia (ergot) that form in the ears of host rice plants. EAs were discovered as the causative compound of wheatgrass poisoning, the main symptom of which is cramps and miscarriages [1] [2].

EAs and their analogs are classified into three types: Clavine-type, peptide-type, and simple amides of the lysergic acid-type, and more than 70 compounds are currently known [3]. EAs and their analogs are compounds with many pharmacological activities; for example, ergotamine is used to treat migraine, ergometrine is used to prevent postpartum hemorrhage after childbirth, and bromocriptine and pergolide are used to treat Parkinson's syndrome [4]. In particular, the market size of ergot-derived dopamine agonists in Japan was approximately 1.5 billion yen in FY2017 and a stable supply is required [5]. EAs and their analogs are thus essential compounds for the development of new drugs and clinical applications, including antibiotics, and treating cancer, psychiatric disorders, and coronavirus infection [6] [7] [8] [9].

EAs have a sterically complex chemical structure and industrial chemical synthesis has not been achieved due to economic disadvantages [10]. The industrial method for producing EAs is to extract them from ergot on rye artificially cultivated with *C. purpurea* in the field, or by the liquid culture of the mycelial phase of *Claviceps* spp. [11] [12]. The field production is greatly influenced by weather conditions, but liquid culture production covered these shortcomings. In addition, solid media changed the EAs composition ratio and increased the amount of EAs production [13]. The production of EAs by cultivating *Claviceps* spp. on solid media has been reported using wheat, rye, or sugarcane dregs but few studies on cereal culture have been published and the process is poorly understood [13] [14]. Furthermore, the ability to produce EAs in the mycelial phase varies significantly between species and strains of *Claviceps* [15].

This study searched for *Claviceps* strains cultured artificially in the mycelial phase and the produced EAs were identified. We cultured selected strains on cereal media (white rice, brown rice, and rye) to examine their ability to produce EAs on each medium.

Twenty-two strains of *Claviceps* spp. isolated in Japan were examined to produce EAs in the mycelial phase of liquid media. The results showed that *C. purpurea* var. *agropyri* produced the Clavine-type EAs pyroclavine (**1**), festuclavine (**2**), and agroclavine (**3**) in the mycelial phase. We report the types and total amounts of EAs produced by the mycelial phase of *C. purpurea* var. *agropyri* cultured on cereal media made from white rice, brown rice, or rye.

2. Materials and Methods

2.1. Experimental Instruments

Analytical TLC was performed on silica gel 60 F_{254} (Merck Ltd., Darmstadt, Germany). HPLC analysis was performed using an 1100 series HPLC system (binary pump: G1312A, autosampler: A1329A, column compartment: G1316A, UV detector: G1314A, Agilent Technologies, Inc., CA, USA) equipped with an Inertsil ODS-3 column (3 µm, 2.1 × 150 mm) (GL Science Inc., Tokyo, Japan). Open column chromatography for compound isolation was performed using 100 g of Sephadex LH-20 (GE Healthcare, IL, USA) in a 3.5 × 80 cm glass column. Preparative HPLC was performed on a Shimadzu LC-20AT and SPD-10AV instrument with a GL Science InertSustain C18 (5 µm, 10 × 250 mm) column. NMR spectra were recorded on an ECA-600II (^{1}H: 600.17 MHz; ^{13}C: 150.91 MHz) (JEOL, Tokyo, Japan). Chemical shifts for ^{1}H and ^{13}C NMR are given in parts per million (δ) relative to residual solvent signals ((δ_H 7.26)/(δ_C 77.0) for $CDCl_3$, (δ_H 3.31)/(δ_C 49.0) for CD_3OD as internal standards). Mass spectra were measured on a JMS-T100LP (JEOL, Tokyo, Japan).

2.2. Materials

Twenty-two strains of the genus *Claviceps* were isolated from parasites of 18 grass hosts from 1990 to 2019 in 12 prefectures in Japan (**Table 1**). Ergotamine tartrate standard was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan).

2.3. Fermentation and Extraction

2.3.1. Fermentation and Extraction of *Claviceps* Spp. in T25 Culture

The culture conditions using a liquid medium were based on the method of Amici *et al.* [16]. *Claviceps* species were pre-cultured on T2 agar (sucrose 100 g, l-asparagine 10 g, yeast extract 0.1 g, $Ca(NO_3)_2$ 1 g, KH_2PO_4 0.25 g, $MgSO_4$-$7H_2O$ 0.25 g, KCl 0.12 g, $FeSO_4$-$7H_2O$ 0.02 g, $ZnSO_4$-$7H_2O$ 0.015 g, agar 20 g, purified water to 1 L, 25% NH_3 aq. pH 5.2). Fermentation was conducted using seed medium (sucrose 100 g, citric acid 10 g, yeast extract 0.1 g, $Ca(NO_3)_2$ 1 g, KH_2PO_4 0.5 g, $MgSO_4$-$7H_2O$ 0.25 g, KCl 0.12 g, $FeSO_4$-$7H_2O$ 0.007 g, $ZnSO_4$-$7H_2O$ 0.006 g, purified water to 1 L, 25% NH_3 aq. pH 5.2) and fermentation T25 medium (sucrose 300 g, citric acid 15 g, yeast extract 0.1 g, $Ca(NO_3)_2$ 1 g, KH_2PO_4 0.5 g, $MgSO_4$-$7H_2O$ 0.25 g, KCl 0.12 g, $FeSO_4$-$7H_2O$ 0.007 g, $ZnSO_4$-$7H_2O$ 0.006 g, purified water to 1 L, 25% NH_3 aq. pH 5.2).

First, the mycelia were cultured on T2 agar for 21 days at 25˚C, then two pieces of cultured agar (1 × 2 cm^2) were transferred into 500 mL Erlenmeyer flasks containing 100 mL of seed culture medium and grown at 25˚C and 150 rpm for six days. Next, 20 mL of the seed culture medium was transferred into 1000 mL Erlenmeyer flasks containing 170 mL of fermentation medium and grown at 25˚C and 150 rpm for 12 days.

T25 culture filtrate was adjusted to pH 8.5 with saturated aqueous Na_2CO_3 and ergot alkaloids were extracted twice using liquid/liquid extraction with an equal volume of chloroform. The chloroform extract was evaporated to dryness in a vacuum evaporator.

Table 1. Sample overview of *Claviceps* spp. and the yield of extract from T25 culture used in the study.

Fungal name	Place	Date	Host plant	Extract yield (mg)
C. purpurea **MAFF 247543**	Ishikawa	6/28/2017	*Lolium arundinaceum*	4.1
Claviceps **sp1 ex** ***Alopecurus*** **MAFF 247299**	Ishikawa	5/23/2016	*Alopecurus aequalis* var. *amurensis*	3.7
Claviceps **sp2 ex** ***Dactylis*** **MAFF 247304**	Chiba	7/4/2016	*Dactylis glomerata*	3.4
Claviceps **sp3 ex** ***Phalaris*** **MAFF 247310**	Chiba	6/23/2017	*Phalaris arundinacea*	3.8
Claviceps **sp4 ex** ***Phalaris*** **MAFF 247311**	Iwate	7/8/2017	*Phalaris arundinacea*	23.0
C. purpurea **var.** ***sasae*** **MAFF 247545**	Tochigi	8/12/2015	*Sasa yahikoensis*	-
Claviceps **sp5 ex** ***Phragmites*** **MAFF 247522**	Ishikawa	10/25/2016	*Phragmites australis*	9.6
C. purpurea **var.** ***agropyri*** **MAFF 247547**	Ishikawa	6/19/2016	*Elymus tsukusiensis* var. *transiensis*	17.9
C. litoralis **MAFF 247555**	Hokkaido	5/31/2014	*Leymus mollis*	6.2
Claviceps **sp6 ex** ***Phragmites*** **MAFF 247549**	Aomori	11/18/2016	*Phragmites australis*	57.1
C. paspali **MAFF 247573**	Fukui	11/30/2016	*Paspalum dilatatum*	13.1
C. queenslandica **MAFF 306124**	Tokyo	1990	*Paspalum scrobiculatum*	1.1
C. queenslandica **MAFF 247574**	Kagoshima	11/22/2018	*Paspalum scrobiculatum*	6.0
C. panicoidearum **MAFF 247571**	Aomori	11/4/2016	*Isachne globosa*	21.2
C. microspore **MAFF 247562**	Ishikawa	11/15/2017	*Arundinella hirta*	-
C. microspore **var.** ***kawatanii*** **MAFF 247557**	Kanagawa	10/10/2015	*Spodiopogon sibiricus*	15.2
C. sorghicola **MAFF 247566**	Miyazaki	11/19/2020	*Sorghum bicolor*	11.9
C. sorghicola **MAFF 306571**	Tochigi	n.d.	*Sorghum bicolor*	12.7
Claviceps **sp7 ex** ***Miscanthus*** **MAFF 247559**	Fukui	11/30/2016	*Miscanthus sinensis*	9.7
C. Africana **MAFF 247564**	Nagasaki	11/2019	*Sorghum bicolor*	11.2
C. bothriochloae **MAFF 247569**	Kagoshima	11/22/2018	*Capillipedium parviflorum*	20.7
C. yanagawaensis **MAFF 247556**	Iwate	8/7/2019	*Zoysia japonica*	11.0

-: No growth; n.d.: No data.

2.3.2. Fermentation and Extraction of *C. purpurea* var. *agropyri* on Cereal Culture

C. purpurea var. *agropyri* was pre-cultured on potato dextrose agar (PDA, Nissui Pharmaceutical Co., Ltd., Tokyo, Japan). Fermentation was conducted using M102 medium (sucrose 30 g, malt extract 20 g, peptone 2 g, yeast extract 1 g, $MgSO_4$-$7H_2O$ 0.5 g, KCl 0.5 g, KH_2PO_4 1.0 g, purified water 1 L, NaOH pH 6.0) and cereal medium (dry cereal (rye, brown rice): 150 g, tap water: 90 mL; white rice: 30, 60, 90, 120, 150 mL) [17].

First, *C. purpurea* var. *agropyri* was pre-cultured on PDA and grown for 21 days at 25˚C, then two pieces of cultured agar ($1 \times 2\ cm^2$) were transferred into 500 mL Erlenmeyer flasks containing 200 mL of M102 medium and grown at 25˚C and 150 rpm for seven days. Next, 10 mL of cultured M102 was transferred into 500 mL Roux flasks containing cereal medium and grown at 25˚C with shaking for 28 days.

Ergot alkaloids were extracted from the cereal cultures with 300 mL of chloroform: 25% NH_3 aq. (500:1), then the chloroform extract was evaporated to dryness in a vacuum evaporator. The amount of extract obtained from each culture was 547.8 ± 296.5 mg for white rice, 829.6 ± 144.3 mg for brown rice, and 257.6 ± 9.4 mg for rye.

2.4. Isolation of Pyroclavine (1), Festuclavine (2) and Agroclavine (3)

C. purpurea var. *agropyri* was incubated using 10 Roux flasks containing rice medium for 28 days at 25˚C, then the cultures were extracted with chloroform. The crude chloroform extract (7.6 g) was obtained by evaporation under vacuum, suspended in 100 mL of 4% tartaric acid aqueous solution, and partitioned twice with an equal volume of *n*-hexane. The pH of the remaining water layer was adjusted to 8.5 with saturated Na_2CO_3 aqueous solution, and alkaloids were extracted twice with an equal volume of chloroform. The solvent was removed under vacuum, and the chloroform extract (273.3 mg) was separated into ten fractions by open column chromatography using Sephadex LH-20. The mobile phases were *n*-hexane:chloroform = 1:4, chloroform:acetone = 3:2, chloroform:acetone = 1:4, acetone, and MeOH, in turn. The volume of each solvent used was 200 mL, and 100 mL fractions were collected. Fractions 4 and 5 (total 92.3 mg) were purified by reverse-phase (RP)-HPLC (solvent: 30% acetonitrile containing 0.05% TFA, room temperature, flow rate: 2 mL/min) to obtain pyroclavine (**1**: 5.4 mg), festuclavine (**2**: 1.8 mg) and agroclavine (**3**: 10.1 mg).

2.5. TLC Analysis

Chloroform extracts of T25 culture were dissolved in chloroform at 50 mg/mL and ergotamine tartrate was dissolved at 1 mg/mL, then 2 μL aliquots were spotted on a TLC plate and developed with chloroform/methanol/acetic acid (90/15/0.1). Chromatographed ergot alkaloids were observed by spraying the plates with van Urk's reagent.

2.6. HPLC Analysis

Compounds **1** - **3** were dissolved in 5% acetonitrile at 100 μg/mL to provide a standard solution, which was diluted stepwise to provide a dilution series of 12.5, 2.5, 0.5, and 0.1 μg/mL. The chloroform extracts were dissolved in 5% acetonitrile to 1 mg/mL, centrifuged at 7200 g for 5 min, and 10 μL of the supernatant was used as the analysis sample. The HPLC mobile phases were 0.1% formic acid solution (A) and acetonitrile containing 0.1% formic acid (B). The gradient was 0 - 1 min 5% B, 1 - 10 min 5% - 20% B, 10 - 28 min 20% - 30% B, 28 min 20% - 30% B, 28 - 34 min 95% B, 34 - 43 min 5% B. An ODS column was used at 40˚C and a flow rate of 0.2 mL/min. A calibration curve was constructed by linear approximation using the obtained area values. The approximations were compound **1**:$y = 0.000001817x + 0.02460$ with an R^2 value of 0.9997, compound **2**:$y = 0.000004338x + 0.002143$ with an R^2 value of 1.0000, and compound **3**:$y = 0.000001982x + 0.05990$ with an R^2 value of 0.9998.

3. Results

3.1. Culture of *Claviceps* Spp. in T25 Liquid Medium

Twenty-two strains were cultured in T25, which has already been reported to produce EAs during culture. All strains except *C. purpurea* var. *sasae* and *C. microspora* grew in T25 (Table 1).

3.2. Detection of EA-Producing Strains by TLC Analysis and Identification of Compounds

The EAs detected in chloroform extracts of the T25 culture media were confirmed by TLC analysis with van Urk's color reagent, using ergotamine tartrate as a color control. *C. purpurea* var. *agropyri* produced compounds showing blue-violet spots **1** (Rf 0.07), **2** (Rf 0.11), and **3** (Rf 0.24) with the same color as ergotamine tartrate (Rf 0.54) (Figure 1(A)).

The molecular formulae of these alkaloids are $C_{16}H_{20}N_2$ (**1**), $C_{16}H_{20}N_2$ (**2**) and $C_{16}H_{18}N_2$ (**3**) as determined by ESI-MS analysis. Compounds 1 - 3 were identified as pyroclavine (**1**) [18], festuclavine (**2**) [19], and agroclavine (**3**) [20] by ^{1}H-, ^{13}C-NMR spectroscopy after isolation by preparative HPLC (Figure 1(B), Tables 2-4, Figures S1-S6).

3.3. Production of 1 - 3 on Cereal Medium by *C. purpurea* var. *agropyri*

C. purpurea var. *agropyri* produced EAs (**1** - **3**) in the mycelial phase cultured in a T25 medium. The production of **1** - **3** on cereal medium (white rice, brown rice, rye) was calculated using the absolute calibration curve method (Figure 2(A)). *C. purpurea* var. *agropyri* produced **1** - **3** on all cereal media, with agroclavine (**3**) as the major EA (Figure 2(B) and Figure 2(C)). The total amount of **1** - **3** in each cereal medium (150 g of cereal per Roux flask) was 2220.5 ± 564.1 μg for white rice, 920.0 ± 463.6 μg for brown rice, and 595.4 ± 52.1 μg for rye. *C. purpurea* var. *agropyri* produced the highest amount of EAs in white rice culture. The total amount of **1** - **3** in white rice medium (150 g of white rice per Roux flask) was about 34 times higher than that in T25 liquid medium (190 mL per 1 L Erlenmeyer flask) (equivalent amount per flask).

3.4. Effects of Moisture Content of White Rice Medium on the Production of 1 - 3 by *C. purpurea* var. *agropyri*

C. purpurea var. *agropyri* efficiently produced **1** - **3** in white rice medium culture. Osmotic pressure (water activity) of the liquid medium affects the production of EAs in the mycelial phase of *Claviceps* spp. [21] [22]. Therefore, we examined the relationship between the water content of white rice medium and the

Table 2. ^{1}H- and ^{13}C-NMR data of **1** and pyroclavine [18].

atom	**1***		pyroclavine [18]*	
	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
2	120.28	7.04 (d, 1.4)	120.29	7.03 (d, 1.5)
3	108.12		108.10	
4a	25.52	3.00 (ddd, 13.8, 11.7, 1.4)	25.55	2.99 (ddd, 14.0, 11.6, 1.7)
4b		3.70 (dd, 14.4, 4.8)		3.69 (dd, 14.2, 4.5)
5b	69.83	3.28 (td, 11.0, 4.1)	69.84	3.30 (td, 11.0, 4.5)
7a	62.83	3.51 (dt, 13.1, 2.1)	62.86	3.51 (dt, 12.8, 1.5)
7b		3.43 (dd, 12.4, 4.1)		3.41 (dd, 12.8, 3.9)
8b	28.12	2.50 m	28.12	2.49 m
9a	32.71	2.63 (dddd, 13.8, 3.4, 2.8, 1.4)	32.72	2.63 (ddt, 13.7, 3.6, 2.1)
9b		1.91 (td, 13.8, 4.8)		1.91 (td, 13.1, 4.9)
10a	35.54	3.41 (td, 11.7, 4.1)	35.56	3.41 (dt, 4.2, 11.6)
11	130.43		130.42	
12	113.95	6.91 (d, 7.6)	113.95	6.90 (dd, 7.2, 0.8)
13	123.88	7.12 (dd, 8.3, 7.6)	123.89	7.11 (dd, 8.2, 7.2)
14	110.64	7.21 (d, 8.3)	110.65	7.20 (d, 8.2)
15	135.09		135.07	
16	126.81		126.81	
17	17.33	1.38 (d, 7.6)	17.29	1.37 (d, 7.5)
18	42.46	3.06 s	42.46	3.07 s

*NMR data were measured using CD_3OD.

Table 3. ^{1}H- and ^{13}C-NMR data of **2** and festuclavine [19].

atom	**2***		festuclavine [19]*	
	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
2	120.30	7.03 s	120.15	7.02 (d, 1.4)
3	108.24		108.22	
4a	25.82	2.93 (dd, 11.7, 10.3)	25.62	2.93 (ddd, 13.9, 11.7, 1.6)
4b		3.69 (d, 12.4)		3.64 (dd, 12.7, 5.0)
5b	68.71	3.23 m	68.35	3.20 (dt, 4.1, 11.3)
7a	63.37	3.58 (d, 11.0)	63.13	3.55 (ddd, 12.1, 3.8, 1.7)
7b		2.88 (dd, 12.4, 11.7)		2.86 (t, 12.4)
8b	30.61	2.22 m	30.01	2.25 m
9a	35.56	2.81 (dd, 13.8, 1.7)	35.54	2.79 (ddt, 13.3, 1.9, 3.6)
9b		1.35 (dt, 13.4, 12.0)		1.33 (dt, 12.2, 12.7)

Continued

10a	40.76	3.23 m	40.13	3.29 (dt, 3.2, 11.4)
11	130.28		130.23	
12	113.96	6.92 (d, 6.9)	113.77	6.90 (d, 7.6)
13	123.88	7.12 (dd, 8.3, 6.9)	123.63	7.11 (t, 7.7)
14	110.67	7.21 (d, 8.3)	110.45	7.21 (d, 8.1)
15	135.10		134.99	
16	126.70		126.71	
17	18.70	1.14 (d, 6.9)	18.56	1.13 (d, 6.6)
18	41.80	3.07 s	41.31	3.06 s

*NMR data were measured using CD_3OD.

Table 4. ^{1}H- and ^{13}C-NMR data of **3** and agroclavine [20].

atom	**3***		agroclavine [18]*	
	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
1		8.10 s		7.98 s
2	117.83	6.87 s	117.77	6.89 s
3	112.31		112.47	
4a	26.70	2.80 (ddd, 13.8, 12.0, 1.7)	26.73	2.80 (t, 13.0)
4b		3.33(dd, 14.4, 4.1)		3.34(dd, 10.1, 4.6)
5	63.86	2.55 m	63.92	2.55 m
7a	60.68	3.26 (d, 16.15)	60.71	3.26 (d, 16.1)
7b		2.96 (d, 16.15)		2.95 (d, 16.0)
8	132.32		132.32	
9	119.38	6.20 s	119.34	6.19 s
10	40.99	3.76 (d, 6.87)	41.01	3.76 (d, 6.7)
11	132.51		132.58	
12	112.70	7.01 m	112.83	7.01 m
13	122.92	7.16 m	122.97	7.17 m
14	108.51	7.16 m	108.37	7.17 m
15	133.54		133.58	
16	126.35		126.42	
17	20.86	1.80 s	20.78	1.79 s
18	40.93	2.51 s	40.91	2.51 s

*NMR data were measured using $CDCl_3$.

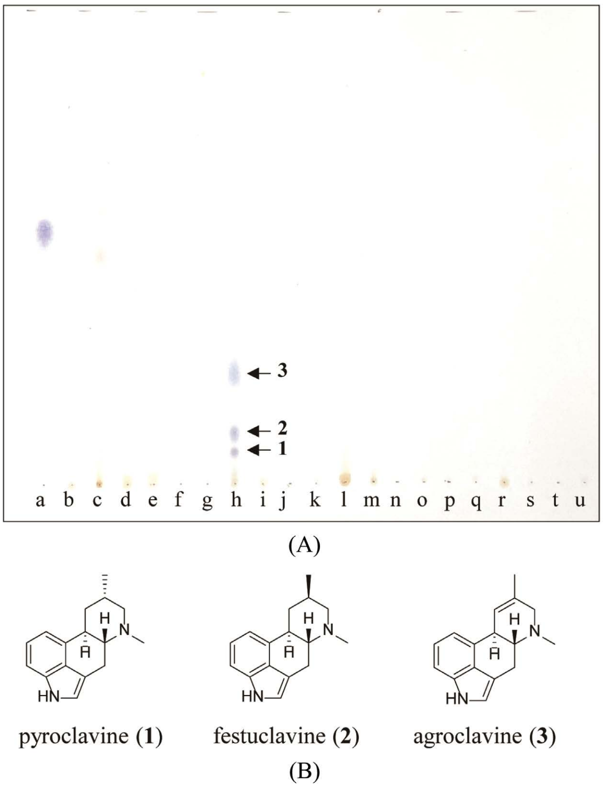


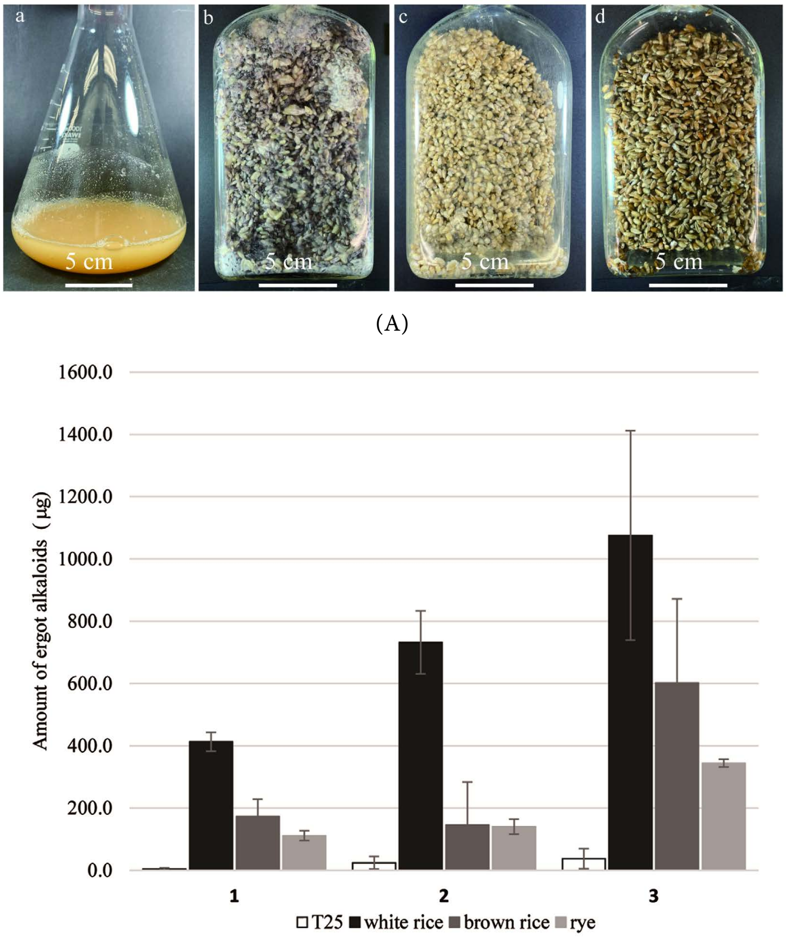
Figure 1. The TLC analysis of chloroform extracts of T25 culture media and the structures of compounds **1** - **3**. (A): TLC analysis of chloroform extracts of T25 culture media. Solvent system: $CHCl_3/MeOH/CH_3COOH$ (90/15/0.1), ergotamine tartrate: 2 μg/spot, chloroform extract: 100 μg/spot, reagent: van Urk's reagent, a: ergotamine tartrate, b: *C. purpurea*, c: *Claviceps* sp1 ex *Alopeculus*, d: *Claviceps* sp2 ex *Dactylis*, e: *Claviceps* sp3 ex *Phalaris*, f: *Claviceps* sp4 ex *Phalaris*, g: *Claviceps* sp5 ex *Phragmites*, h: *C. purpurea* var. *agropyri*, i: *C. litoralis*, j: *Claviceps* sp6 ex *Phragmites*, k: *C. paspali*, l: *C. queenslandica* MAFF 306124, m: *C. queenslandica* MAFF 247574, n: *C. panicoidearum*, o: *C. microspora* var. *kawatanii*, p: *C. sorghicola* MAFF 247566, q: *C. sorghicola* MAFF 306571, r: *Claviceps* sp7 ex *Miscanthus*, s: *C. africana*, t: *C. bothriochloae*, u: *C. yanagawaensis*, (B): The structures of **1** - **3**.

production of **1** - **3** by *C. purpurea* var. *agropyri*. The production of EAs **1** - **3** was maximal at 90 mL of water per 150 g of white rice (**Figure 3**).

4. Discussion

The purpose of our study was to identify *Claviceps* sp. that can be cultured in the mycelial phase, determine their ability to produce EAs, and cultivate the strains on cereal media (white rice, brown rice, and rye), and examine their ability to produce EAs on each medium.

Twenty-two strains of *Claviceps* spp. isolated in Japan were examined for their production of EAs in the mycelial phase using a liquid medium. The results showed that *C. purpurea* var. *agropyri*, isolated from *Elymus tsukusiensis* var. *transiensis* as a host plant, produced the Clavine-type EAs pyroclavine (**1**), festuclavine (**2**), and agroclavine (**3**) in the mycelial phase.



(A)

(B)

	1	2	3	Total (1 + 2 + 3)
T25	4.4 ± 3.3	24.3 ± 20.3	37.2 ± 32.2	65.9 ± 55.7
white rice	412.6 ± 30.3	732.0 ± 100.9	1075.9 ± 336.1	2220.5 ± 564.1
brown rice	173.0 ± 55.4	145.4 ± 138.4	601.7 ± 270.4	920.0 ± 463.6
rye	111.4 ± 15.8	139.9 ± 24.2	344.1 ± 12.6	595.4 ± 52.1

(C)

Figure 2. Production of **1** - **3** by *C. purpurea* var. *agropyri* fermentation. (A) Culture conditions for *C. purpurea* var. *agropyri*, a: T25 culture, b: white rice culture, c: brown rice culture, d: rye culture. [(B), (C)] Amount (μg) of **1** - **3** produced in T25 culture, white rice, brown rice, and rye cultures by *C. purpurea* var. *agropyri.* Data are shown as mean ± standard deviation (SD) of the mean (N = 3).

Clavine-type EAs **1** - **3** were first isolated by Abe *et al.* from *Claviceps* sp. parasitic on *Elymus tsukusiensis* var. *transiensis* [23] [24] [25]. Previous studies reported various physiological activities of agroclavine (**3**), including partial agonist or antagonist effects on adrenergic receptors, dopamine receptors, and serotonin receptors [26]. Furthermore, the carcinocidal effect of agroclavine (**3**) was enhanced by derivatization [7].

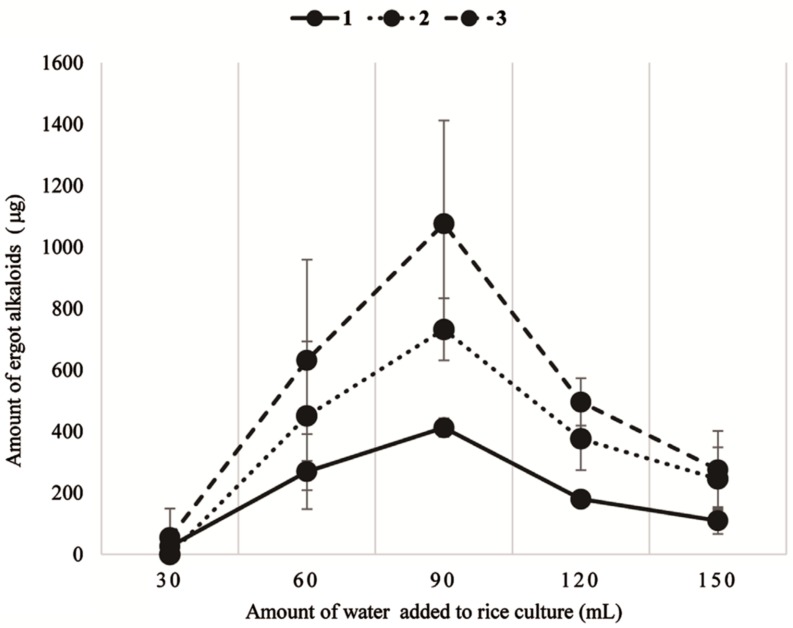


Figure 3. The change in the total amount of **1** - **3** in 150 g white rice with 30, 60, 90, 120, 150 mL water. Data are shown as mean ± SD of the mean.

However, **1** - **3** are difficult to obtain and there are no commercially available standards. *C. fusiformis*, isolated in South Asia and Africa, is a representative Clavine-type EA-producing fungus whose host plant is *Pennisetum typhoideum* Rich [27] [28] [29]. *C. fusiformis* belongs to section Pusillae within the genus *Claviceps*, and *C. purpurea* belongs to section Purpurea in the taxonomic study by Píchová *et al.* [30]. In that study, Clavine-type alkaloids were isolated from a phylogenetically distinct species of fungi from which Clavine-type alkaloids had previously been isolated, suggesting that *C. purpurea* var. *agropyri* could be a useful new Clavine-type EA-producing fungus distinct from *C. fusiformis*.

When cultured with white rice, brown rice, or rye, *C. purpurea* var. *agropyri* produced **1** - **3** on all cereal media. The total amount of **1** - **3** in each cereal medium (150 g of cereal per Roux flask) was 2220.5 ± 564.1 μg for white rice, 920.0 ± 463.6 μg for brown rice, and 595.4 ± 52.1 μg for rye. The highest production of **1** - **3** was found in white rice culture. This is the first report on the production of EAs in white rice medium. The total amount of **1** - **3** in white rice medium (150 g of white rice per Roux flask) was about 34 times higher than that in T25 liquid medium (190 mL per 1 L Erlenmeyer flask) (equivalent amount per flask), indicating the usefulness of white rice medium.

Osmotic pressure (water activity) of the liquid medium affects the production of EAs in the mycelial phase of *Claviceps* spp. [21] [22]. The production of EAs by *C. purpurea* var. *agropyri* was affected by the moisture content of the white rice medium. *C. purpurea* var. *agropyri* produced the highest amount of **1** - **3** when 90 mL of water was added to 150 g of white rice. These data suggest that water content is a major factor affecting the production of EAs in white rice medium. An osmosensor of filamentous fungi is the group III histidine kinase upstream of a mitogen-activated protein kinase (MAPK) cascade [31]. The production of aurofusarin of *Fusarium graminearum* is regulated by the group III histidine kinase FgOs1 [32]. On the other hand, the type III histidine kinase CpHK1 in *Claviceps purpurea* does not directly regulate EAs production, and the relationship between osmotic pressure and EAs production remains unclear [22].

5. Conclusion

This study showed that white rice medium is effective for the production of the Clavine-type EAs pyroclavine (**1**), festuclavine (**2**), and agroclavine (**3**) by *C. purpurea* var. *agropyri*. Further improvement of cultural conditions, such as water content, may increase the production of EAs in white rice medium.

Acknowledgements

This work was supported by JSPS KAKENHI Grant Number 16K07238.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Supporting Information

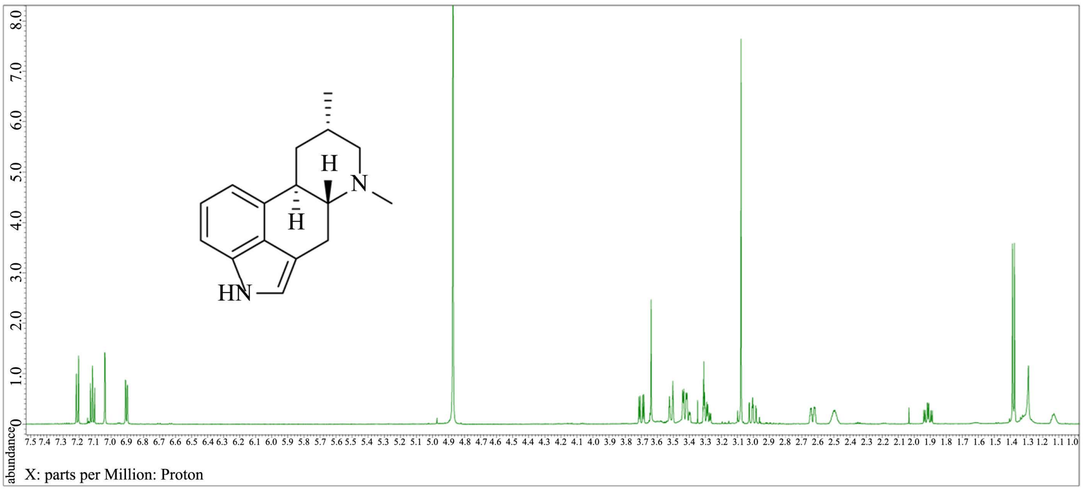

Figure S1. ^{1}H-NMR spectrum of 1 (600.17 MHz) in CD_3OD.

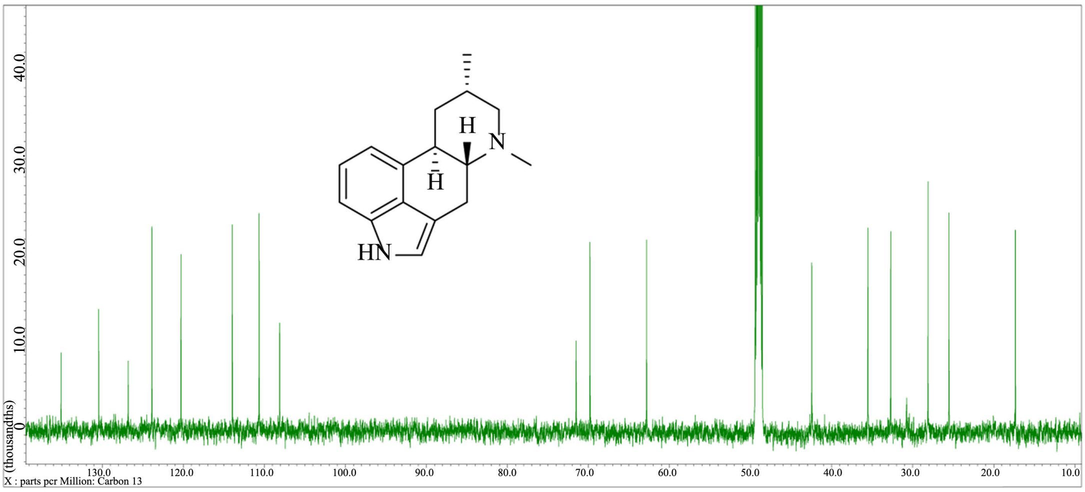

Figure S2. ^{13}C-NMR spectrum of 1 (150.91 MHz) in CD_3OD.

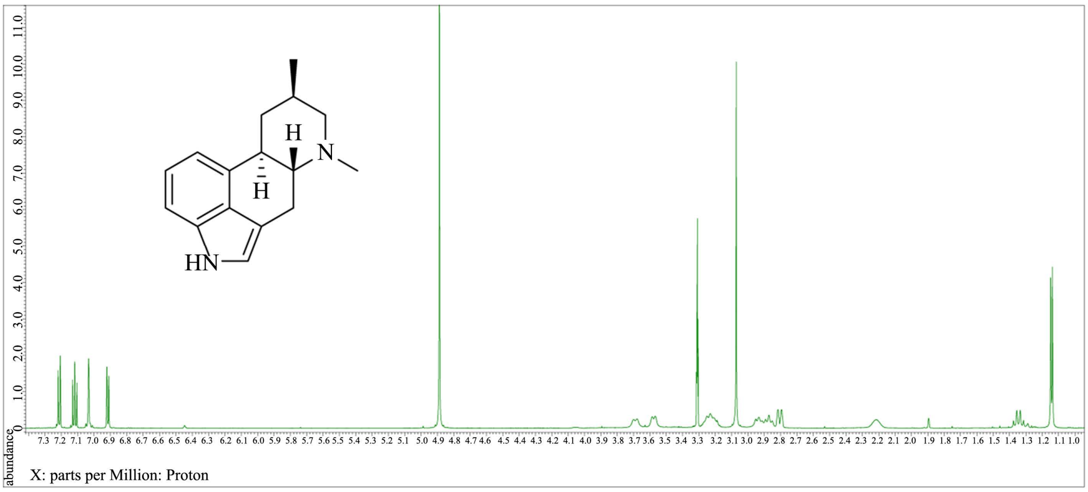

Figure S3. ^{1}H-NMR spectrum of 2 (600.17 MHz) in CD_3OD.

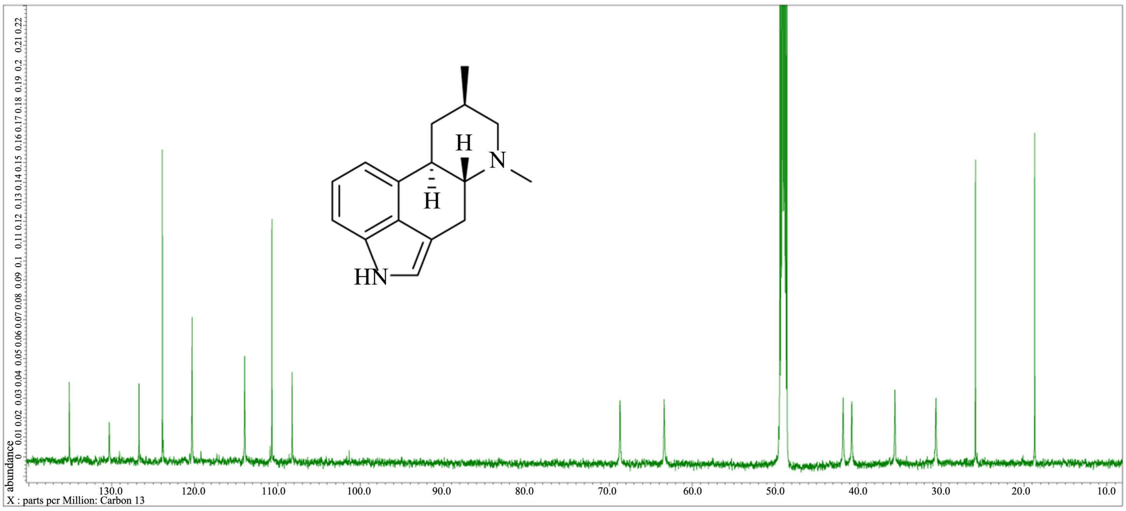

Figure S4. ^{13}C-NMR spectrum of 2 (150.91 MHz) in CD_3OD.

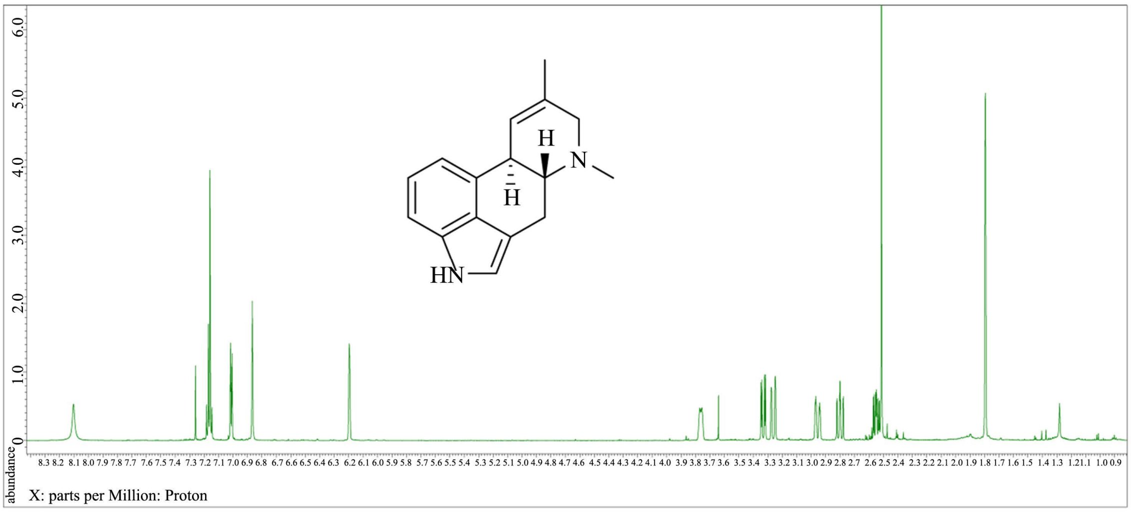

Figure S5. ^{1}H-NMR spectrum of 3 (600.17 MHz) in $CDCl_3$.

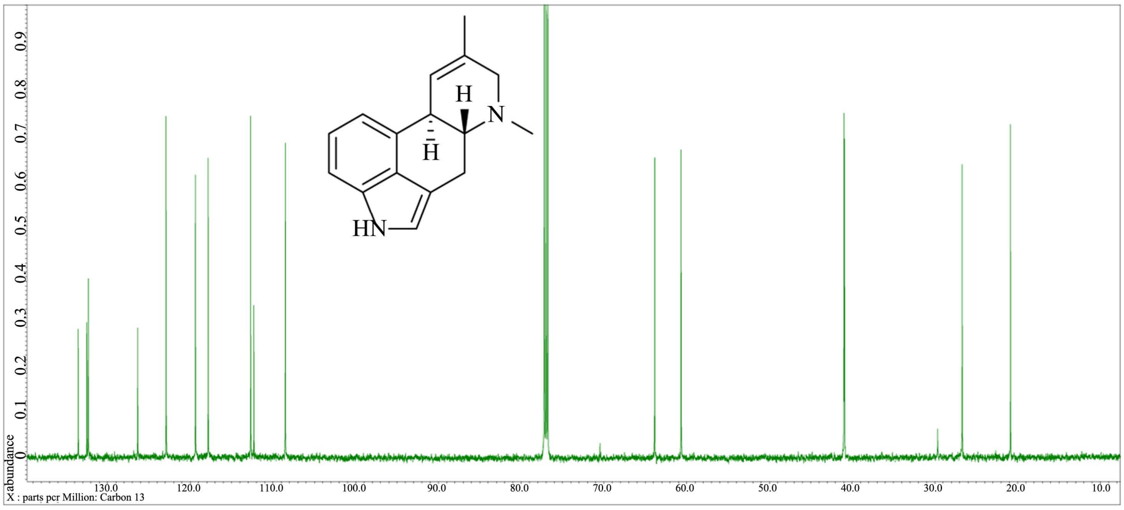

Figure S6. ^{13}C-NMR spectrum of 3 (150.91 MHz) in $CDCl_3$.