

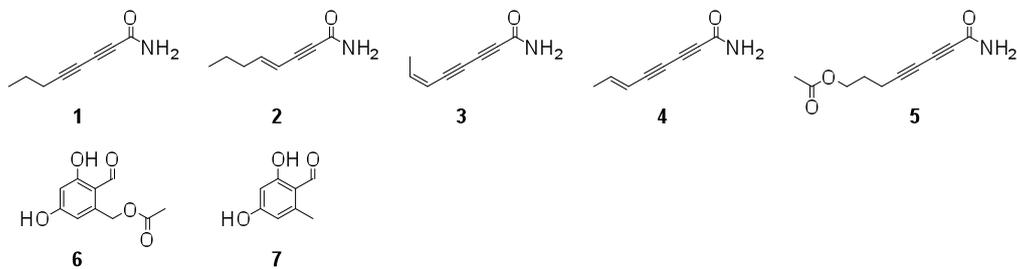
Agrocybynes A-E from the culture broth of *Agrocybe praecox*

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Graphical abstract

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Title

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Abstract

Five novel compounds (**1-3**, **5** and **6**), and two known ones (**4** and **7**) were isolated from the culture broth of *Agrocybe praecox*. Their structures were determined by the interpretation of spectroscopic data. Compounds **1** to **4** inhibited hypocotyl growth of lettuce, and **1**, **3** and **4** inhibited the root growth.

1. Introduction

In 2007, abnormal enlargement of strawberry fruits was observed and a kind of mushroom grew near the stimulated fruits in a greenhouse in Niigata prefecture, Japan. It was identified as *Agrocybe praecox* (English name, Spring Fieldcap; Japanese name, Fumizukitake) . On the other hand, it has been reported that an *Agrocybe* sp. caused

cringing of strawberry fruits.¹ These widely varying phenomena related to growth stimulation and suppression, suggest that *Agrocybe* genus produces plant growth regulator(s). *A. praecox* is edible and widespread in the northern temperate zone throughout the world. The mushroom is litter degrading fungus that is able to grow in the forest soil and produces non-specific extracellular enzymes, which decompose soil detritus (including polymers such as cellulose and hemicellulose) into monomers and oligomers, which in turn are made available to microbes and plants.² The fungus has been in focus for various application due to the hydrolytic and ligninolytic enzymatic activities for bioremediation, however it has not yet been reported as environmental adaptation by coexisting with plants.³⁻¹¹

In this article we focused on plant growth regulators from the fungus *A. praecox*. Here, we describe the isolation, structural determination, and biological activity of five novel compounds (**1-3**, **5** and **6**), and two known ones (**4** and **7**) from the culture broth of the fungus.

2. Results

Culture broth of *A. praecox* was partitioned between EtOAc and water, and then *n*-BuOH and water. Each fraction was tested to plant growth regulatory activity

using lettuce and the EtOAc-soluble part showed significant inhibitory activity. The EtOAc-soluble part was fractionated by repeated chromatography, guided by the bioassay results leading to discovery of five novel compounds (**1-3**, **5** and **6**), and two known ones (**4** and **7**) were purified.

Agrocybyne A (**1**) was purified as colorless needle shaped. Its molecular formula was determined as C_8H_9NO by HRESIMS m/z 158.05586 $[M+Na]^+$ (calcd for C_8H_9NNaO , 158.05818), indicating the presence of five degrees of unsaturation in the molecule. Structure of **1** was elucidated by interpretation of NMR spectra including DEPT, COSY, HMQC, and HMBC (Figure 1). The DEPT experiment indicated the presence of a methyl, two methylenes, and five quaternary carbons. The molecular formula, the unsaturation degrees, and the DEPT data suggested the presence of two substituted triple bonds and a carboxamide in the molecule. The complete assignment of the protons and carbons of NMR was accomplished as shown in Table 1. The structure of propyl moiety (C6 to C8) was elucidated by the COSY correlations (H7/H6, H8) and the HMBC correlations (H6/C7, C8; H7/C6, C8; H8/C6, C7). The presence and position of butadiyne moiety (C2 to C5) was confirmed by the HMBC correlations (H6/C2 to C5; H7/C5) and the characteristic chemical shifts (δ_C 63.7, 67.0, 71.7, 87.3). As a result, structure of **1** was supposed to be octa-2,4-diyneamide and was confirmed by

X-ray crystallography (Figure 2).

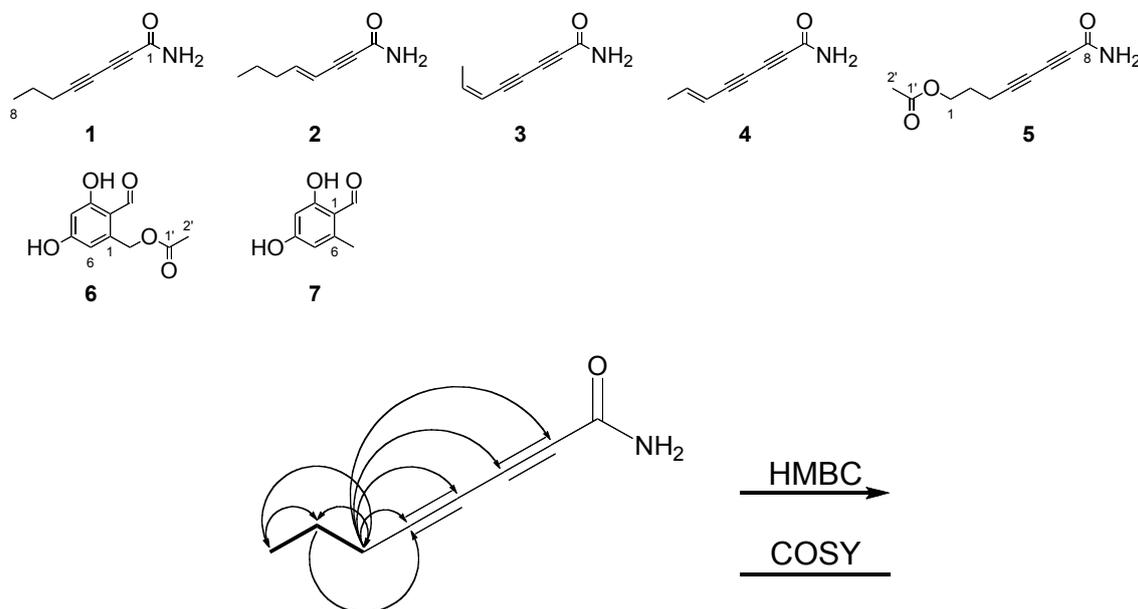


Figure 1. COSY and HMBC correlations in **1**.

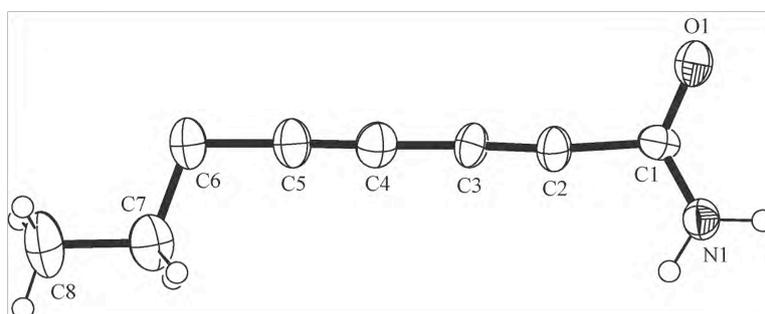


Figure 2. ORTEP drawing of **1**.

Agrocybyne B (**2**) was purified as colorless needle shaped. Its molecular formula was determined as $C_8H_{11}NO$ by HRESIMS m/z 160.07311 $[M+Na]^+$ (calcd for $C_8H_{11}NNaO$, 160.07383), indicating that **2** has two more hydrogens than **1**. The NMR data of **2** were similar to those of **1** (Table 1). The DEPT experiment indicated the presence of a methyl, two methylenes, two methines and three quaternary carbons.

Table 1 ^1H and ^{13}C NMR data for **1-5** (**1**, **2**, and **5** in CDCl_3 ; **3** and **4** in CD_3OD)

Position	1		2		3		4		5	
	^1H δ (multiplicity, J in Hz)	^{13}C δ								
1		153.5		154.8		156.3		156.4	4.13 (dd, 6.4,6.1)	62.7
2		67.0 ^a		81.0		75.5 ^a		70.9 ^a	1.88 (m)	27.0
3		71.7 ^a		85.5		76.8 ^a		74.2 ^a	2.43 (dd, 7.3,6.7)	16.4
4		63.7 ^a	5.52 (d, 16.2)	107.3		70.5 ^a		70.8 ^a		85.4 ^a
5		87.3	6.42 (ddd, 16.2,7.3,7.2)	151.2		80.9 ^a		83.1		64.3 ^a
6	2.30 (t, 7.0)	21.3 ^b	2.13 (m)	35.4	5.62 (d, dd, 11.0,1.5)	108.8	5.67 (dd, 15.8,1.8)	109.6		71.2 ^a
7	1.57 (m)	21.4 ^b	1.43 (m)	21.5	6.37 (dq, 11.0,7.0)	147.3	6.50 (dq, 15.8,7.0)	148.4		67.5
8	0.99 (t, 7.3)	13.4	0.90 (t, 7.3)	13.5	1.93 (dd, 7.0,1.5)	16.7	1.85 (dd, 7.0,1.8)	19.1		153.4
1'										170.9
2'									2.04 (s)	20.8

^{a, b} interchangeable between the same letter.

These results show that **2** has a double bond instead of one of triple bonds in **1**. The COSY correlations (H5/H4, H6; H7/H6, H8), the HMBC correlations (H4/C2, C6; H5/C3, C6, C7; H6/C4, C5, C7, C8; H7/C5, C6, C8; H8/C6, C7), and the coupling constant ($J = 16.2$ Hz) between H-4 and H-5 indicate that **2** is (*E*)-oct-4-en-2-ynamide.

Agrocybyne C (**3**) was purified as a white amorphous. Its molecular formula was determined as C_8H_7NO by HRESIMS m/z 156.04148 $[M+Na]^+$ (calcd for C_8H_7NNaO , 156.04253). The formula was smaller by two mass unit than that of **1** and the NMR data of **3** were similar to those of **1** (Table 1). The DEPT experiment indicated the presence of a methyl, two methines and five quaternary carbons. These results show that **3** possesses an olefin instead of one of two methylenes in **1**. The COSY correlations (H7/H6, H8), the HMBC correlations (H8/C4, C5, C6, C7) and the coupling constant ($J = 11.0$ Hz) between the olefinic protons indicate that **3** is (*Z*)-octa-6-en-2,4-dynamide.

Agrocybyne D (**4**) was obtained as white amorphous. Its molecular formula was determined as C_8H_7NO by HRESIMS m/z 156.04110 $[M+Na]^+$ (calcd for C_8H_7NNaO , 156.04253). The formula was the same as that of **3** and the NMR data of **4** were very similar to those of **3** (Table 1). The COSY correlations (H7/H6, H8), the HMBC correlations (H6/C4, C7, C8; H7/C5, C8; H8/C4, C5, C6, C7) and the coupling constant of chemical shift at δ_H 5.67 (H6, dd, $J = 15.8$ Hz) suggest that **4** is the *cis*

isomer of **3**. As a result, the structure of **4** was identified as (*E*)-octa-6-en-2,4-diyamide.

Although this compound has been synthesized, this is its first reported isolation from a natural source.^{12, 13}

Agrocybyne E (**5**) was purified as white amorphous. Its molecular formula was determined as C₁₀H₁₁NO₃ by HRESIMS *m/z* 216.06076 [M+Na]⁺ (calcd for C₁₀H₁₁NNaO₃, 216.06366). The NMR data of **5** were similar to those of **1** (Table 1). The chemical shift at δ_C 170.9 in ¹³C-NMR and the molecular formula suggest that **5** has additional two carbons including a carboxy group compared with **1**. The DEPT experiment indicated the presence of a methyl, three methylenes, and six quaternary carbons. The methyl proton was correlated with the carboxy (δ_C 170.9) in HMBC experiment. The results and other data (COSY correlations, H2/H1, H3; HMBC correlations, H1/C2, C3, C1'; H2/C1, C3, C4; H3/C1, C2, C4 to C7'); the characteristic chemical shifts of a hydroxy methyl at δ_H 4.13 and δ_C 62.7) indicate that **5** is 8-amino-8-oxoocta-4,6-diynyl acetate.

Compound **6** was purified as colorless oil. Its molecular formula was determined as C₁₀H₁₀O₅ by HRESIMS *m/z* 233.04095 [M+Na]⁺ (calcd for C₁₀H₁₀NaO₅, 233.04259). The complete assignment of the protons and carbons of NMR was accomplished by interpretation of NMR spectra including DEPT, COSY, HMQC, and

HMBC (Experimental). As a result, the structure of **6** was determined as

2-formyl-3,5-dihydroxybenzyl acetate.

Compound **7** was identified as *o*-orsellinaldehyde. It has been isolated from different mutant strains of *Aspergillus rugulosus* as a cytotoxic compound towards Hep 3B human hepatoma cells through apoptosis.¹⁴⁻¹⁶

Since bioassay using strawberry needs large amounts of the compounds, and we do not have sufficient amounts of the compounds, effects of the compounds on plant growth were tested towards lettuce. Compounds **5** to **7** did not exhibit any noteworthy activity. Compounds **1** to **4** inhibited the hypocotyl growth (rate of growth length compared with control \pm standard deviation, **1**, $29.6 \pm 4.23\%$; **2**, $70.4 \pm 4.41\%$; **3** $38.4 \pm 13.5\%$; **4**, $12.0 \pm 3.86\%$), and **1**, **3** and **4** inhibited the root (**1**, $20.7 \pm 3.67\%$; **3** $17.3 \pm 5.85\%$; **4**, $6.67 \pm 2.49\%$) at 1 μmol .

We are now trying to synthesize these compounds and planning cultivating experiments of strawberry fruits using them.

3. Experimental

3.1. General

¹H NMR spectra (one- and two-dimensional) were recorded on a JEOL

lambda-500 spectrometer at 500 MHz, while ^{13}C NMR spectra were recorded on the same instrument at 125 MHz. The HRESIMS spectra were measured on a JMS-T100LC mass spectrometer. A JASCO grating infrared spectrophotometer was used to record the IR spectra. HPLC separations were performed with a JASCO Gulliver system using normal-phase HPLC column (Senshu PAK AQ, Senshu scientific Co., Ltd, Japan) and reverse-phase HPLC columns (COSMOSIL μ NAP Waters, nacalai tesque, Japan; COSMOSIL 5PYE Waters, nacalai tesque, Japan). Silica gel plate (Merck F254) and silica gel 60N (Merck 100–200 mesh) were used for analytical TLC and for flash column chromatography, respectively.

3.2. Fungus and plant materials

The strain of *Agrocybe praecox* F450 used in this study was isolated from the fruiting body appeared in the greenhouse in Niigata prefecture, Japan, and identified by one of the authors (N. M). Lettuce seeds (*Lactuca sativa* L. cv. Great Lakes 366; Takii Co., Ltd., Japan) were used in this study.

3.3. Incubation

The culture medium (24 g/L) was prepared from dextrose broth (Difco)

containing 0.1% actcol (Takeda Chemical Industries Ltd., Japan). The medium was poured into a jar fermenter (480 g/30 L jar; Biott Co., Ltd., Japan) and sterilized. The pre-incubated mycelia of *A. praecox* were inoculated to the jar fermenter and incubated under the condition (25°C, shaking with 100 rpm, bubbling with 1 L/min) for 3 weeks.

3.4. Extraction and isolation

The culture broth of *A. praecox* (175 L) was filtrated and then concentrated under reduced pressure. The filtrate was successively partitioned between EtOAc and water (four times), and then *n*-BuOH and water (four times). The EtOAc-soluble part (19.8 g) was fractionated by silica gel flash column chromatography (CH₂Cl₂; CH₂Cl₂/EtOAc 90:10, 80:20, 70:30, 60:40, 50:50; EtOAc; EtOAc/MeOH 50:50; and MeOH) to obtain 12 fractions.

Fraction 3 (54.1 mg) was fractionated by normal-phase HPLC (Senshu PAK AQ, hexane/CHCl₃ 20:80) to obtain 7 fractions. Fraction 3-6 (8.0 mg) was further separated by reverse-phase HPLC (COSMOSIL πNAP Waters, 40% MeOH) to afford compound **7** (2.3 mg).

Fraction 4 (348.6 mg) was fractionated by normal-phase HPLC (Senshu PAK AQ, hexane/CHCl₃ 20:80) to obtain 9 fractions. Fraction 4-5 (20.6 mg) was further

separated by reverse-phase HPLC (COSMOSIL π NAP Waters, 40% MeOH) to afford compounds **1** (5.6 mg), **2** (6.7 mg), **3** (1.1 mg), **4** (2.2 mg), and **6** (0.6 mg).

Fraction 5 (606.1 mg) was separated by normal-phase HPLC (Senshu PAK AQ, hexane/CHCl₃ 20:80) to obtain 8 fractions. Each fraction 5-5 (52.9 mg) and 5-6 (29.0 mg) was further separated by reverse-phase HPLC (COSMOSIL π NAP Waters, 40% MeOH) to obtain 7 fractions, respectively. Compound **2** (0.6 mg) was obtained from fraction 5-5-6 (1.3 mg) by reverse-phase HPLC (COSMOSIL 5PYE Waters, 40% MeOH). Fraction 5-6-6 (3.9 mg) was further separated by reverse-phase HPLC (COSMOSIL 5PYE Waters, 40% MeOH) to afford compound **5** (3.5 mg).

3.4.1. Compound 1. mp 101-103°C; IR (neat): 1652, 2244, 3136 cm⁻¹; ¹H and ¹³C NMR, see Table 1; ESIMS *m/z* 158 [M+Na]⁺; HRESIMS *m/z* 158.05586 [M+Na]⁺ (calcd for C₈H₉NNaO, 158.05818).

3.4.2. Compound 2. mp 114-116°C; IR (neat): 1652, 2197, 3136 cm⁻¹; ¹H and ¹³C NMR, see Table 1; ESIMS *m/z* 160 [M+Na]⁺; HRESIMS *m/z* 160.07311 [M+Na]⁺ (calcd for C₈H₁₁NNaO, 160.07383).

3.4.3. Compound 3. mp 82-84°C; IR (neat): 1611, 2216, 3160 cm⁻¹; ¹H and ¹³C NMR, see Table 1; ESIMS *m/z* 156 [M+Na]⁺; HRESIMS *m/z* 156.04148 [M+Na]⁺ (calcd for C₈H₇NNaO, 156.04253).

3.4.4. Compound 4. mp 86-88°C; IR (neat): 1650, 2222, 3158 cm⁻¹; ¹H and ¹³C NMR, see Table 1; ESIMS *m/z* 156 [M+Na]⁺; HRESIMS *m/z* 156.04110 [M+Na]⁺ (calcd for C₈H₇NNaO, 156.04253).

3.4.5. Compound 5. mp 72-74°C; IR (neat): 1613, 2243, 3178 cm⁻¹; ¹H and ¹³C NMR, see Table 1; ESIMS *m/z* 216 [M+Na]⁺; HRESIMS *m/z* 216.06076 [M+Na]⁺ (calcd for C₁₀H₁₁NNaO₃, 216.06366).

3.4.6. Compound 6. IR (neat): 1627, 3287 cm⁻¹; ¹H NMR (CDCl₃): δ 2.11 (H2', s), 5.29 (1-CH₂, s), 6.35 (H4, d, 2.0), 6.45 (H6, d, 2.0), 10.1 (2-CHO, s), 12.3 (C3-OH, s); ¹³C NMR (CDCl₃): δ 20.9 (C2'), 62.5 (1-CH₂), 103.7 (C4), 110.5 (C6), 112.8 (C2), 141.3 (C1), 162.8 (C5), 166.4 (C3), 170.3 (C1'), 192.5 (C2-CHO); ESIMS *m/z* 233 [M+Na]⁺; HRESIMS *m/z* 233.04095 [M+Na]⁺ (calcd for C₁₀H₁₀NaO₅, 233.04259).

3.4.7. Compound 7. IR (neat): 1626, 3245 cm^{-1} ; ^1H NMR (CDCl_3): δ 2.51 (C6-CH_3 , s), 6.19 (H3 and H5, s), 10.1 (1-CHO, s), 12.4 (C2-OH, s); ^{13}C NMR (CDCl_3): δ 18.2 (C6-CH_3), 101.3 (C3), 110.5 (C5), 113.6 (C1), 144.9 (C6), 163.2 (C4), 166.3 (C2), 192.9 (C1-CHO); ESIMS m/z 153 $[\text{M}+\text{H}]^+$.

3.5. X-ray crystallography analysis of agrocybyne A (1)

Crystal data: $\text{C}_8\text{H}_7\text{ON}$, $M_r = 133.15$, triclinic $P-1$, $a = 8.8879(17)$, $b = 9.2843(19)$, $c = 10.594(2)$ Å, $\alpha = 115.980(10)$, $\beta = 95.452(8)$, $\gamma = 92.299(6)^\circ$, $V = 779.1(3)$ Å³; $D_x = 1.135$ Mg m^{-3} ; $Z = 4$; $\mu(\text{Mo } K\alpha) = 0.076$ mm^{-1} , $T = 90$ K. The final R values for 1199 unique reflections ($2\theta_{\text{max}} = 50^\circ$) with $I > 2\sigma(I)$ are 0.1147 and 0.2624 for $R(F)$ and $wR(F^2)$, respectively.

Single crystals of **1** were obtained from a hexane solution as colorless needles. Diffraction data were collected using a RIGAKU AFC-8 diffractometer equipped with a Saturn70 CCD detector with Mo $K\alpha$ radiation by an ω -scan method with 0.5° oscillation for each frame at 90 K. X-rays were monochromated and focused by a confocal mirror. Bragg spots were integrated using the HKL2000 program package.¹⁷ No absorption corrections were applied. The structure was solved by a direct method using the program of SIR2004,¹⁸ and refined by a full-matrix least squares method using

the program SHELXL-97.¹⁹ Anisotropic temperature factors were applied to all non-hydrogen atoms. The hydrogen atoms were put at calculated positions, and refined applying riding models.

Crystallographic data have been deposited with Cambridge Crystallographic Data Centre: Deposition code CCDC 851177. Copy of the data can be obtained free of charge via <http://www.ccdc.cam.ac.uk/products/csd/request>.

3.6. Bioassay

Plant growth activity against lettuce was examined as follows. Lettuce seeds were put on filter paper (Advantec No. 2, ϕ 55 mm; Toyo Roshi Kaisha, Ltd., Japan), soaked in distilled water in a petri dish (ϕ 60 \times 20 mm) and incubated in a growth chamber under dark at 25°C for one day. The pre-incubated lettuces (n = 7 in each petri dish) were transferred onto filter paper soaked in 1 mL of distilled water (control) or each sample solution (1 μ mol/mL) in a petri dish and incubated in a growth chamber under dark at 25°C for 3 days. The lengths of the hypocotyl and the root were measured using a ruler.

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Figure Legends

Figure 1. COSY and HMBC correlations in **1**.

Figure 2. ORTEP drawing of **1**.