AB5046A AND B, NOVEL CHLOROSIS-INDUCING SUBSTANCES FROM *Nodulisporium* sp.

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Novel chlorosis-inducing substances, AB5046A and B, were isolated from the culture broth of a fungal strain. The producing organism, designated AB5046, was identified as a member of *Nodulisporium*.

AB5046A and B were purified by extraction with EtOAc and silica gel chromatography. The structure of AB5046A and B were determined to be 2-butyryl-3,5-dihydroxy-cyclohex-2-ene-1-one and 2-acetyl-3,5-dihydroxy-cyclohex-2-ene-1-one, respectively, by spectroscopic analyses.

AB5046A and B induced chlorosis against Japanese barnyard millet *in vitro*. The chlorosis activity of these compounds was stronger against monocotyledons than dicotyledons.

Inhibitions of chloroplast formation, photosynthesis and essential amino acid's biosynthesis are considered as the important and attractive targets in the development of new herbicides with low toxicity to mammals. Some herbicides such as pyrazolate[1], methoxy-phenone[2], fluridone[3] are practically used as the inhibitors of chloroplast formation (chlorosis-inducing chemicals). Although it is known that some natural products (virdomic acids[4], alternaric acid[5], rhizobitoxin[6], 1-amino-2-nitrocyclopentanecarboxylic acid[7], tentoxin[8], *etc.*) have chlorosis-inducing activity, they are not utilized for control of weeds.

In the course of screening for new herbicides, we found that a soil fungus produced new herbicidal substances, named AB5046A (**1**) and B (**2**), which induced chlorosis against Japanese barnyard millet *in vitro*. The color of generating foliage of the barnyard millet became completely white, as if they were bleached, when the seeds were treated with these compounds.

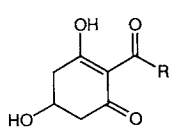
In this paper, we describe the identification of the producing organism together with the isolation, fermentation, structure determination and biological activities of **1** and **2**.

Materials and Methods

Taxonomy

The AB5046A and B producing organism, strain AB5046, was isolated from a soil sample collected at Seta-gun in Gumma prefecture. Morphological observations of the strain grown on malt extract agar were carried out with a light microscope (Olympus Model BHS) and a scanning electron microscope (Hitachi S-430). The characteristics of the culture on potato-dextrose agar and malt extract agar were determined after 2 weeks of incubation

Fig. 1. Structures of AB5046A (**1**) and B (**2**).



1 R = $CH_2CH_2CH_3$
2 R = CH_3

at 25°C.

Fermentation

A loop-full of spores from a slant culture of strain AB5046 on malt extract agar medium was inoculated into a 500-ml Erlenmeyer flask containing 110 ml of a medium consisting of glucose 10 g, dextrin 10 g, Polypeptone 5 g, yeast extract 2 g, malt extract (Difco) 5 g, V-8 juice (Cambell Soup Co.) 80 ml and $CaCO_3$ 3 g in 1 liter of deionized water (pH 6.5 before autoclaving). The culture was fermented at 25°C for 5 days on a rotary shaker (180 rpm).

Physico-chemical Characteristics

Optical rotation was measured with a Perkin-Elmer model 241 polarimeter. IR and UV spectra were recorded with a Hitachi 285 spectrophotometer and a Hitachi 557 spectrophotometer, respectively. The ^{1}H and ^{13}C NMR spectra were measured with JEOL GX400 and EX270 spectrometers. The mass spectrum was recorded with a JEOL JMS-HX110 mass spectometer.

Biological Activity

The biological studies were performed using the sodium salts of compounds **1** and **2** which were prepared by neutralizing with equimolar of NaOH. Sea sand (12.5 g, Kanto Chem. Co.) in test tube (4 cm × 12.5 cm) was fully moistened with a sample solution (4 ml) diluted with distilled water. Then, seeds of some weeds and crops were placed on the sea sand and incubated in a growth cabinet at 25°C under 16 hours in illumination of 2,000 lux and 8 hours in darkness. After 7- to 10-days incubation, the chlorosis activity was observed. The plants used to test chlorosis activity of **1** and **2** were as follows; Japanse barnyard millet (*Echinochola utilis*), large crabgrass (*Digitaria sanguinalis*), green foxtail (*Setaria viridis*), rice flatsedge (*Cyperus iria*), green gram (*Phaseolus aureus*), Chinese radish (*Rhaphanus sativus*), hairy beggarticks (*Bidens pilosa*) and livid amaranth (*Amaranthus viridis*).

Influence of **1** on chlorophyll formation in Japanese barnyard millet was investigated by the following procedures: Isolated foliage, dissected from 7-days old seedlings of barnyard millet, were extracted with absolute MeOH at 60°C. The MeOH solution was concentrated *in vacuo* and then the concentrate containing chlorophyll was dissolved in acetone. Optical density of the acetone solution was measured at 661.6 and 644.6 nm by using a Hitachi 557 spectrophotometer. From the result, chlorophyll content of the extract was calculated with the method of WATANABE *et al.*[9].

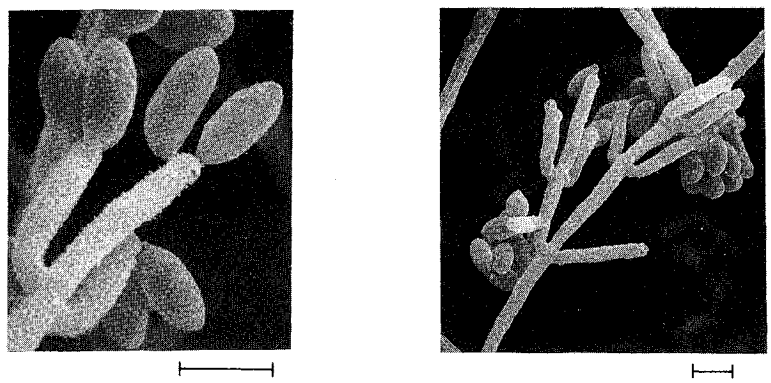
Results

Taxonomic Features of Strain AB5046

Morphological observations with scanning electron microscope of strain AB5046 are shown in Photo. 1. Conidiophores were erected or suberected, 100~180 μm in length and 2.5~3.5 μm in diameter, and

Photo. 1. Scanning electron micrographs of strain AB5046.

Bars represents 5 μm.



had septa. Main axis of the conidiophores were simple or branched, forming 1~5 branches. Sometimes, verticillous branching were observed in the terminal branches. The surface and color of the conidiophores were smooth to minutely verruculose and hyaline to pale olive-brown, respectively.

Conidiogeneous cells which bore singly or plurally as terminal branches, were cylindrical or silghtly clavate in shape. After conidial dehiscence, minute denticles remained on the conidiogeneous cells.

Conidia were produced sympodually. The conidia were dry, hyaline and obovoid or ellipsoid, and had smooth to slightly truncate surface, 6.5~9.0×3.5~4.5 μm in size.

Strain AB5046 showed good growth on malt extract agar and potato-dextrose agar. On malt extract agar, this strain rapidly grew and the size of the colony attained 65~69 mm in diameter after incubation for 5 days. The color of the colony was light yellow, becoming pale gray to greenish gray with conidial development. The reverse color of the colony was light gray to greenish gray.

The strain showed good growth at 10~30°C on malt extract agar and potato-dextrose agar. The optimum temperature for growth was 20~30°C.

Based on these morphological and cultural characteristics, strain AB5046 was identified as a member of the genus *Nodulisporium*[10]. The culture has been deposited in the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Tsukuba, Japan, with an accession No. of FERM P-13066.

Isolation

The active substances were monitored by the chlorosis-inducing activity toward Japanese barnyard millet seedlings and by ethanolic $FeCl_3$ color reaction on a TLC plate (Kieselgel 60 F_{254}, art 5715, Merck) during the purification process. The culture broth (10 liters) was filtered by using Hyflo Super-Cel (John-Manville Co., U.S.A.). The culture filtrate was adjusted to pH 2.0 with 6 M HCl and extracted with EtOAc. The EtOAc extract was concentrated *in vacuo*.

The concentrate was charged on a column of silica gel (42 × 340 mm, Kieselgel 60, art 7734, Merck) and developed with $CHCl_3$-MeOH-AcOH (98:2:1; 20 g fractions). The active fractions (Nos. 68~82) were collected and concentrated *in vacuo* to give a brownish oil (2.65 g). The crude oil was further chromatographed on a silica gel column (38 × 300 mm) developing with toluene-acetone-AcOH (40:10:1; 11 g fractions). The active fractions (Nos. 21~34) were collected and concentrated yielding pure compound **1** (1.45 g as colorless oil) and the other fractions (Nos. 38~46) were collected and concentrated to give crude oil containing **2** (1.45 g).

Compound **2** was further purified by silica gel PTLC (Kieselgel 60 F_{254}, art 5715, Merck) with $CHCl_3$-MeOH-AcOH (95:5:1) as development solvent (**2**, Rf 0.42). Active band was collected and extracted with $CHCl_3$-MeOH (1:1). The extract was concentrated *in vacuo* yielding pure compound **2** (oil, 0.20 g).

Physico-chemical Properties

AB5046A (**1**) was obtained as an oil, $[\alpha]_D^{23}$ 0° (*c* 1.0, $CHCl_3$). It was soluble in MeOH, acetone, EtOAc and $CHCl_3$, and slightly soluble in water. The UV and IR spectral data of **1** are as follows: UV λ_{max}^{MeOH} nm (ε) 272 (8,200), 232 (8,800); $\lambda_{max}^{0.01\,M\,HCl\text{-}MeOH}$ nm (ε) 273 (8,600), 232 (8,700); $\lambda_{max}^{0.01\,M\,NaOH\text{-}MeOH}$ nm (ε) 268 (15,000): IR ν_{max} (film) cm^{-1} 3400, 2960, 1660, 1565, 1450, 1410, 1065. Compound **1** was positive to ethanolic $FeCl_3$ reaction. The molecular formula of **1** was established as $C_{10}H_{14}O_4$ (MW 198) by ^{13}C NMR spectrum and HRFAB-MS; $(M+H)^+$ *m/z* 199.0972 (calcd for $C_{10}H_{15}O_4$ 199.0971).

AB5046B (**2**) was also obtained as an oil, $[\alpha]_D^{23}$ 0° (*c* 1.0, $CHCl_3$). Solubility and color reaction of **2** were same as **1**. The UV and IR spectral data of **2** are as follows: UV λ_{max}^{MeOH} nm (ε) 272 (7,700), 230 (8,000); UV $\lambda_{max}^{0.01\ M\ HCl\text{-}MeOH}$ nm (ε) 272 (7,100), 230 (8,500); $\lambda_{max}^{0.01\ M\ NaOH\text{-}MeOH}$ nm (ε) 264 (14,700); IR ν_{max} (KBr) cm^{-1} 3400, 3330, 2930, 1645, 1550, 1450, 1090, 1065, 1040. The molecular formula of **2** was established as $C_8H_{10}O_4$ (MW 170) by ^{13}C NMR spectrum and FAB-MS; $(M+H)^+$ *m/z* 171.

The Rf values of **1** and **2** on silica gel TLC (Kieselgel 60 F_{254}, art 5715, Merck) with $CHCl_3$-MeOH-AcOH (95:5:1) as development solvents were 0.49 and 0.42, respectively.

Structure Determination

The UV spectra mentioned above and characteristic IR absorption (**1**; 1660, 1560 cm^{-1}, **2**; 1645, 1550 cm^{-1}) of **1** and **2** suggested the presence of a conjugated β-diketone moiety in their structures. The positive color reaction (orange colored spot) for ethanolic $FeCl_3$ of these compounds indicated that the conformation of the β-diketone moiety assumed *cis*-enol form in ethanol solution.

The ^1H and ^{13}C NMR spectral data of **1** and **2** are shown in Tables 1 and 2, respectively. The multiplicity of carbon signals was determined by DEPT experiment. Correlations between protons were obtained by ^1H-^1H shift COSY and correlations between proton and carbon were obtained by the ^1H-^{13}C heteronuclear shift COSY experiment. The ^1H-^{13}C long range connectivities were determined by ^1H-detected heteronuclear multiple-bond ^1H-^{13}C corrleation spectroscopy (HMBC) experiments.

These NMR studies of **1** showed the presence of the following two carbon chains; $-C(=O)-CH_2-CH(OH)-CH_2-C(=O)-$ and $-C(=O)-CH_2-CH_2-CH_3$, and a quaternary carbon (δ 112.9). Although the quaternary carbon was not connected to the two carbon chains in the HMBC spectrum, it is clear that the carbon is located at α-position in the β-diketone moiety from physico-chemical property of **1** described above. From these results, the structure of **1** was determined to be 2-butyryl-3,5-dihydroxy-cyclohex-2-ene-1-one (Fig. 1). The NOE and ^1H-^1H spin decoupling experiments were performed to clarify conformation of the cyclohexenone moiety of **1**. As shown in Fig. 2, the coupling constants ($J_{4a,5}$=6.7 Hz, $J_{4b,5}$=4.1 Hz, $J_{6a,5}$=5.8 Hz, $J_{6b,5}$=4.1 Hz), NOE between 4-Hb and 6-Hb and long range coupling between 4-Ha and 6-Ha ($J_{4a,6a}$=1.3 Hz) suggested that the hydroxy group attached at C-5 existed in *quasi-axial* position and also suggested that the conformation of the cyclohexenone moiety was in the

Table 1. ^1H NMR assignments of **1** and **2**.

Position	δ value in ppm	
	1[a]	**2**[a]
3-OH	18.4 (s)[b]	18.4 (s)
4-Ha	2.64 (ddd, *J*=17.1, 6.7, 1.3)	2.65 (dd, *J*=16.4, 6.0)
4-Hb	2.76 (dd, *J*=17.1, 4.1)	2.76 (dd, *J*=16.4, 1.0)
5-H	4.40 (m)	4.41 (m)
6-Ha	2.80 (ddd, *J*=17.6, 5.8, 1.3)	2.80 (dd, *J*=18.4, 5.4)
6-Hb	2.95 (dd, *J*=17.6, 4.1)	2.90 (dd, *J*=18.4, 4.2)
8-H	3.00 (t, *J*=7.2)	2.60 (s)
9-H	1.66 (m)	
10-H	0.98 (t, *J*=7.2)	

[a] 400 MHz ($CDCl_3$, ref TMS).
[b] Multiplicity, coupling constant (Hz).

Table 2. ^{13}C NMR assignments of **1** and **2**.

Position	δ value in ppm	
	1[a]	**2**[b]
1	196.3 (s)[c]	196.5 (s)
2	112.9 (s)	113.3 (s)
3	193.4 (s)	193.7 (s)
4	47.1 (t)	41.5 (t)
5	63.1 (d)	63.3 (d)
6	41.4 (t)	46.9 (t)
7	205.4 (s)	202.4 (s)
8	42.1 (t)	28.5 (q)
9	17.9 (t)	
10	13.8 (q)	

[a] 100 MHz ($CDCl_3$, ref TMS).
[b] 67.5 MHz ($CDCl_3$, ref TMS).
[c] Multiplicity, coupling constants (Hz).

Fig. 2. NOE and ^1H-^1H spin decoupling experiments of **1**.

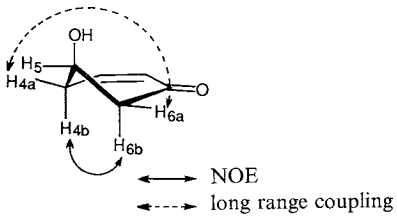


Fig. 3. Summary of ^1H-^1H COSY and HMBC experiments of **1** and **2**.

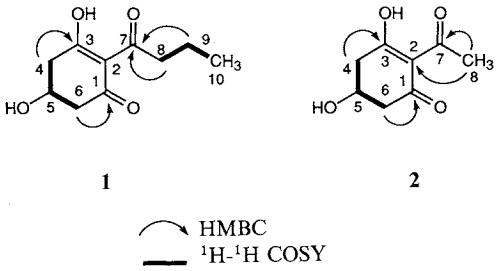


Table 3. Chlorosis activity of **1** and **2** against Japanese barnyard millet.

Conc. (ppm)	Chlorosis activity	
	1	**2**
100	2	2
50	2	2
25	2	1
12.5	1	0

0: No effect, 1: moderate chlorosis, 2: complete chlorosis.

Table 4. Chlorosis activity of **1** against mono- and dicotyledonous plants.

Plants	Chlorosis activity		
	Concentration of **1** (ppm)		
	100	25	6.25
Large crabgrass	2	2	1
Green foxtail	2	1	0
Rice flatsedge	2	2	1
Green gram	0	0	0
Chinese radish	0	0	0
Hairy beggarticks	0	0	0
Livid amaranth	2	1	0

0: No effect, 1: moderate chlorosis, 2: complete chlorosis.

half chair form.

As shown in Table 1, the ^1H NMR spectrum of **2** was very similar to that of **1**. The NMR experiments of **2** showed the presence of the following two carbon chains, $-C(=O)-CH_2-CH(OH)-CH_2-C(=O)-$ and $-C(=O)CH_3$. Furthermore, the HMBC spectrum showed the long range connectivity between acetyl methyl protons (δ 2.60) and a quaternary carbon (δ 113.3). Thus, from all these results, the structure of **2** was determined to be 2-acetyl-3,5-dihydroxy-cyclohex-2-ene-1-one (Fig. 1).

The results of all NMR experiments are shown in Fig. 3.

Biological Properties

Compounds **1** and **2** caused chlorosis against Japanese barnyard millet whose foliage turned completely white without inhibition of germination. This activity of **1** was about twice as strong as **2** (Table 3). On the other hand, the chlorosis activity of these compounds did not show against Chinese radish even at the concentration of 100 ppm (Table 4). As the result suggested that these compounds might have selective chlorosis activity toward monocotyledons, the chlorosis effect of **1** was examined on the following mono- and dicotyledonous plants: monocotyledons; Japanese barnyard millet, large crabgrass, green foxtail and rice flatsedge, and dicotyledons; green gram, Chinese radish, hairy beggarticks and livid amaranth. As shown in Table 4, all of monocotyledons used in this test induced chlorosis at 6.25 ppm or 12.5 ppm with compound **1**, while three dicotyledonous plants except livid amaranth did not show chlorosis.

The formation of chlorophyll by Japanese barnyard millet was completely inhibited at the concentration of 0.25 mM of **1**. The 50% inhibition concentration of **1** against chlorophylls formation of the plant was about 0.094 mM.

AB5046A and B showed no toxicity in mice by single oral administration of 300 mg/kg.

Discussion

2-Acyl-1,3-cyclohexanedione, which is the common skeleton of **1** and **2**, is rare in natural products and was not found in microoraganisms. A few compounds having this skeleton such as 3,5-dihydroxy-2-dodecanoyl-cyclohex-2-ene-1-one[11] and 3,6-dihydroxy-2-[1-oxo-10(*E*)-tetradecenyl]cyclohex-2-ene-1-one[12] were known in insects. However, their herbicidal activities including chlorosis were not reported.

Compounds **1** and **2** induced chlorosis against Japanese barnyard millet whose foliage color turned completely white without inhibition of germination. Production of chlorophyll in this plant was completely inhibited at 0.25 mM. Inhibition of chloroplast formation (chlorosis-phenomenon as the result) is known to be induced by either inhibition of chlorophyll biosynthesis[13,14], inhibition of carotenoid biosynthesis[15] or protein biosynthesis in chloroplast[4]. Mode of action of AB5046A and B is under investigation.

It is important to develop and discover chemicals with highly selective activity among plants, for example selectivity between crops and weeds, and between monocotyledons and dicotyledons. From this point of view, it is of interest that monocotyledons such as Japanese barnyard millet, large crabgrass, rice flatsedge and green foxtail were more susceptible than dicotyledons such as green gram, Chinese radish and hairy beggarticks to AB5046.

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