Chlamydocin Analogues and Their Plant Growth-Retardant Activity

Chlamydocin 類縁体とイネ矮化活性

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Contents

List of abbreviations used

aao	2-amino-9-acetoxy-8-oxodecanoic acid
ABA	abscisic acid
aba	2-aminobutyric acid
ada	2-aminodecanoic acid
aeo	2-amino-9, 10-epoxy-8-oxodecanoic acid
aho	2-amino-9-hydroxy-8-oxodecanoic acid
aib	2-aminoisobutyric acid
aoa	2-aminooctanoic acid
aod	2-amino-8-oxodecanoic acid
Boc	*tert*-butyloxycarbonyl
CIMS	chemical ionization mass spectrometry
DCC	*N, N'*-dicyclohexylcarbodiimide
DMF	dimethyl formamide
EEDQ	*N*-(ethoxycarbonyl)-2-ethoxy-1, 2-hydroquinoline
EIMS	electron ionization mass spectrometry
ELISA	enzyme-linked immunosorbent assay
EtOAc	ethyl acetate
FABMS	fast atom bombardment mass spectrometry
GA	gibberellin
GC–MS	gas chromatography mass spectrometry
^{1}H–^{1}H COSY	^{1}H–^{1}H correlation spectroscopy
HDAC	histone deacetylase
HMBC	heteronuclear multiple bond connectivity
HMQC	heteronuclear single quantumn coherence
HPLC	high-performance liquid chromatography
HOBt	1-hydroxybenzotriazole monohydrate
HOSu	*N*-hydroxysuccinimide
HREIMS	high resolution electron ionization mass spectrometry
IR	infrared
LC–MS	liquid chromatography mass spectrometry
MeOH	methanol

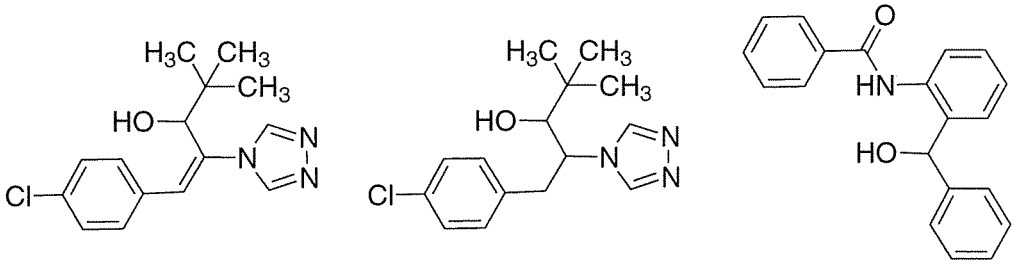
MRM	multiple reaction monitoring
$NaBH_4$	sodium borohydride
$NaIO_4$	sodium periodate
NaOAc	sodium acetate
nle	norleucine
NMR	nuclear magnetic resonance
NOE	nuclear overhauser effect
SIM	selected ion monitoring
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin-layer chromatography
UV	ultraviolet

Chapter 1
General introduction

Agricultural chemicals include not only agents that protect agricultural products from pathogens, insects and weeds, but also agents that regulate plant growth. They have contributed to a revolution in food production. However, they were produced by chemical industries and used by farmers without forethought of their substantial potential to harm the environment. Along with the increasing use of chemicals in agriculture, chemical contamination of food and of the environment has become a vast and enormously important worldwide problem. At the same time, however, agricultural chemicals are indispensable for the stable supply and quality control of agricultural products.

As a category of agricultural chemicals, plant growth regulators are widely used in plant production to control plant size, leaf shedding, growth cessation and other characteristics (Geissbuehler et al., 1987). They are an effective way to create a uniform appearance for marketing purposes as well as for creating other characteristics that improve handling and shipping. In rice cultivation, the use of plant growth regulators to control plant height could reduce lodging, permit higher nitrogen application rates, and increase yields. Most nitrogen recommendations are based on an application rate that will produce the maximum yield. Lodging generally results in lower yield and grain quality. A plant shortener or stalk stiffener would be valuable for the rice industry (Eastin, 1983). Uniconazole (Izumi et al., 1984), paclobutrazol (Pulley and Davis, 1986) and inabenfide (Okatake, 1986) (Fig. 1-1) are highly effective at preventing lodging. However, they are chemically synthesized and perhaps persistent in the environment.

A number of researches have been conducted to find new agricultural chemicals with low toxicity, low persistence, and high activity. Natural substances are believed to have the

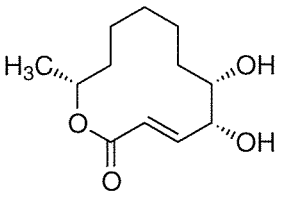


Uniconazole Paclobutrazol Inabenfide

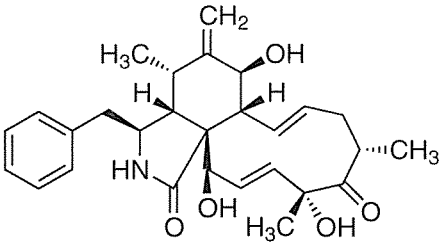
Fig. 1-1 Structures of retardants

advantage of low persistency in the environment; if they have high activity, they can be used in place of synthetic agricultural chemicals. In screening programs for agricultural chemicals, the quality and structural diversity of the compounds tested are also quite important. The exceptional biosynthetic capabilities of microorganisms suggest that the search for novel microbial metabolites possessing bioactivity against plant disease, insects, and weeds would be particularly valuable (Duke and Lydon, 1987, Cutler, 1984). Fungi produce structurally diverse secondary metabolites, most of which remain to be identified and tested for their bioactivities. These metabolites can be used for directly to control the height of the rice plant, and may also lead to the development of new plant growth retardants. Therefore, I chose fungi as a source of natural substances for this study.

I tested the retardant activity of fungal metabolites with the microdrop bioassay. One known method to detect GAs was developed using dwarf rice (cv. Tanginbozu or cv. Waito C). Instead of dwarf rice, I used normal rice (cv. Koshihikari) to test the retardant activity of the fungal metabolites. The Koshihikari is a cultivar whose elongate stem gives it a tendency toward lodging. By this assay I can detect the retardation activity sensitively. In the course of screening in my laboratory, I found that cladospolide B and zygosporin D (Fig. 1-2) are plant growth retardants from *Cladosporium tenuissimum* and *Metarrhizium anisopliae*, respectively (Fujii et al., 1995, 2000).



Cladospolide



Zygosporin D

Fig. 1-2 Structures of retardants reported previously in this laboratory

The objective of this research is to find new natural plant growth retardants. For this purpose, I searched more than 300 soil samples collected in Tottori Prefecture, Japan, for fungi whose culture filtrates displayed plant growth retardant activity. I found a fungus that had this property, and from it I isolated three chlamydocin analogues as active metabolites. Chlamydocin is a bioactive cyclic tetrapeptide and is reported to inhibit HDAC activity (Brosch et al., 1995).

My thesis is composed of five chapters. Chapter 2 deals with the identification of the fungus isolated from the soil in Tottori Prefecture, and the isolation and structural

determination of the active metabolites (**1-3**). In chapter 3, the plant growth retardant activities of compounds **1-3** are described. To investigate the structure-activity relationship in greater detail, I prepared and assayed six derivatives and two synthetic analogues. In chapter 4 the results are discussed. In chapter 5, the mechanisms of retardation are discussed. To elucidate such mechanisms, the endogenous ABA and GA_1 levels in the rice plants treated with those retardants are determined. In addition, I investigated the inhibition of HDACs by those retardants to study the relationship between the retardation and inhibition of HDACs.

References

Brosch, G., Ransom, R., Lechner, T., Walton, J.D., Loidl, P., 1995. Inhibition of maize histone deacetylases by HC toxin, the host-selective toxin of *Cochliobolus carbonum*. Plant Cell 7, 1941-1950.

Cutler, H. G., 1984. Biologically active natural compounds from fungi: templates for tomorrow's pesticide. Am. Chem. Soc. Symp. Ser. 257, 153.

Duke, S. O., Lydon, J., 1987. Herbicides from natural compounds. Weed Technol. 1, 122-128.

Eastin, E.F., 1983. Plant growth regulators in rice. In: Nickell, L.G. (Ed.), Plant Growth Regulating Chemicals, Vol. II. CRC Press, Boca Raton, FL, pp. 150-160.

Fujii, Y., Fukuda, A., Hamasaki, T., Ichimoto, I., Nakajima, H., 1995. Twelve-membered lactones produced by *Cladosporium tenuissimum* and the plant growth retardant activity of cladospolide B. Phytochemistry 40, 1443-1446.

Fujii, Y., Tani, H., Ichinoe, M., Nakajima, H., 2000. Zygosporin D and two new cytochalasins produced by the fungus *Metarrhizium anisopliae*. J. Nat. Prod. 63, 132-135.

Geissbuehler, H. Kerber, E., Mueller, U., 1987. Prospects for chemical plant growth regulation: An industrial viewpoint: An industrial viewpoint, In: Pinfield, J.R. (Ed.), In Plant Growth Regulators for Agricultural and Amenity Use. BCPC Monograph 36, 11-17.

Izumi, K., Yamaguchi, I., Wada, A., Oshio, H., Takahashi, N., 1984. Effects of a new plant growth retardant (*E*)-1-(4-chlorophenyl)-4, 4-dimethyl-2-(1, 2, 4-triazol-1-yl)-1-penten-3-ol (*S*-3307) on the growth and gibberellin content of rice plants. Plant Cell Physiol. 25, 611-617.

Okatake, S., 1986. Prevention of lodging in rice plant with chemical control. Nogyogijyutsu 41, 55-59.

Pulley, G.E., Davis, T.D., 1986. Effect of paclobutrazol on growth and interior performance of several foliage plant species. HortScience 21, 702.

Chapter 2
Isolation and structure determination of compounds 1-3 from a soil fungus, *Peniophora nuda*

2.1 Introduction

Plant growth retardants play important roles in agriculture. In the cultivation of grains, they can reduce lodging by shortening the stem length and thus increase yield. During a screening-based search for natural plant growth retardants in more than the 300 collections of soil fungi, I found a fungus whose culture filtrate could reduce the height of rice seedlings without causing either blotch or wilting. I describe here the identification of a soil fungus known as *Peniophora nuda*, and the isolation and structural elucidation of three active metabolites (**1**, **2** and **3**) (Fig. 2-1).

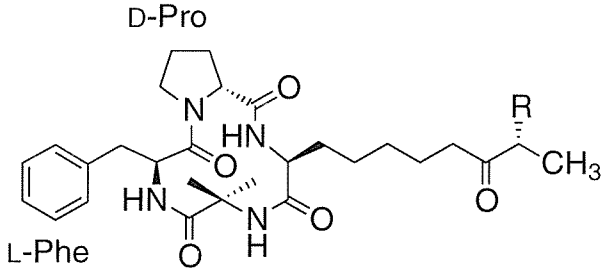


Fig. 2-1 Structures of compounds **1**, **2** and **3**

2.2 Results and discussion

2.2.1 Identification of a soil fungus

A fungus (Fig. 2-2), whose culture filtrate had the retardant activity, did not form spores in many trials, making identification of the fungus difficult. Eventually, it was determined to be *Peniophora* sp. on the basis of electron microscopy observations and its 18S rRNA gene sequence. Electron microscopy showed that this fungus has a Dolipore-parenthesome septum in its hyphae (Fig. 2-3), indicative that it belongs to the holobasidiomycetes (Khan and Kimbrough, 1982). The sequence of its 18S rRNA gene (1737 bases) was determined (data not shown) and a BLAST similarity search of the sequence showed that *Peniophora nuda* is the most closely related fungus (97% similarity).

Fig. 2-2 *Peniophora nuda* grown on potato-dextrose-agar medium 24°C for 10 days

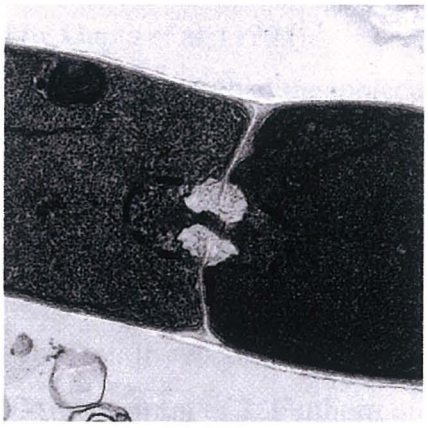


Fig. 2-3 Dolipore-parenthesome septum

2.2.2 Isolation of active metabolites

The *P. nuda* was grown on malt extract medium without shaking at 24°C for 14 days in the dark. Extraction of metabolites from the culture filtrate and purification by chromatography gave compounds **1**, **2** and **3** in respective yields of 3.3, 0.42 and 0.07 mg/l of medium.

Compound **1** had the molecular formula $C_{28}H_{40}N_4O_6$ from its HREIMS, with a molecular ion peak at *m/z* 528.2955, and NMR spectroscopic data. The IR spectrum of **1** showed absorption bands at 3454 cm^{-1} for hydroxyl and/or amide groups, at 1697 cm^{-1} for a ketone carbonyl group, and at 1667cm^{-1} for amide carbonyl groups. A ketone carbonyl carbon resonance appeared at δ 212.4 in its ^{13}C NMR spectrum, and four amide carbonyl carbon resonances at δ 171.9, 172.8, 174.3 and 175.6. Acetylation of **1** gave a monoacetate, suggesting it has one hydroxyl group. Structural clarifications were based on ^{1}H-^{1}H COSY,

HMQC and HMBC data. Analysis of the NMR spectral data (Table 2-1) for compound **1** suggested that it was the same as chlamydocin analogue isolated from *Verticillium coccosporum* by Gupta et al. (1994). The reported spectral data, however, were insufficient for unambiguous identification because the NMR data were recorded in C_6D_6 and the optical rotation was not described. I therefore collected NMR spectral data in C_6D_6 and found them identical to the reported data, indicative that compound **1** has the same structure and same relative stereochemistry as the reported analogue. To confirm the absolute stereochemistry of **1**, acid hydrolysis of the compound was carried out. HPLC analysis after derivatizing the hydrolyzate with (+)-1-(9-fluorenyl) ethyl chloroformate (Hayashi and Sasagawa, 1993) showed that the phenylalanine derivative in the hydrolyzate had the same retention time as the L-phenylalanine derivative, evidence that the stereochemistry of the phenylalanine in **1** is L, the same as the reported configuration. Thus **1** was definitively identified as the same as the chlamydocin analogue reported by Gupta et al. (1994).

Compound **2** was characterized as follows: it had the molecular formula $C_{28}H_{40}N_4O_5$ on the basis of its HREIMS data, which showed a molecular ion peak at *m/z* 512.3001. Its NMR spectral data were similar to those of **1**, indicating that **2** is also a chlamydocin analogue. Its structure was clarified by comparing its NMR spectral data (Table 2-2) with those for **1**. The carbon resonance (δ 72.6) produced by an oxygenated methine carbon C-9 of aod, present in the ^{13}C NMR spectrum of **1**, was absent. Instead, there was a carbon resonance (δ 35.9) due to a methylene carbon in the ^{13}C NMR spectrum of **2**. Furthermore, its ^{1}H NMR spectrum showed a two-proton quartet signal at δ 2.41 and a three-proton triplet signal at δ 1.05, whose signals were coupled. These findings indicate that compound **2** has a structure in which the hydroxyl at the C-9 of aod in compound **1** has been lost. Loss of chirality at the C-9 of aod changed the shape of the proton signal (H-7 of aod) from the AB type triplet in compound **1** to a simple triplet in compound **2**. The amino acid sequence of compound **2** was confirmed from the HMBC correlations between the NH of aod and C-1 of Pro, between the NH of aib and C-1 of aod, and between the NH of Phe and C-1 of aib. NOE data (Fig. 2-4) support this sequence. The fact that the NOE data for compound **2** are almost the same as those reported for compound **1** suggests that the compounds have the same relative stereochemistry. Compound **2** has been obtained from chlamydocin by chemical conversion (Closse and Huguenin, 1974), but there were insufficient spectroscopic data for unequivocal characterization. Ours is the first report of compound **2** as a natural product and of its full spectroscopic data.

Table 2-1 NMR data (^{1}H, 500 MHz and ^{13}C, 125 MHz) for compound **1** in $CDCl_3$

Unit	position	**1**	
		δ_C	δ_H
aho	1	174.3	
	2	54.3	4.18 (1H, *ddd*, 10.2, 7.8, 7.6)
	3	28.74	1.64 (1H, *m*)
			1.79 (1H, *m*)
	4	25.2	1.33 (2H, *m*)
	5	28.69	1.29 (2H, *m*)
	6	23.2	1.63 (2H, *m*)
	7	37.2	2.41 (1H, *ddd*, 17.3, 7.3, 7.3)
			2.50 (1H, *ddd*, 17.3, 7.3, 7.3)
	8	212.4	
	9	72.6	4.23 (1H, *q*, 7.1)
	10	19.8	1.37 (3H, *d*, 7.1)
	NH		7.13 (1H, *d*, 10.2)
aib	1	175.6	
	2	58.8	
	3	23.5	1.77 (3H, *s*)
	4	26.4	1.34 (3H, *s*)
	NH		6.00 (1H, *s*)
Phe	1	172.8	
	2	53.4	5.15 (1H, *ddd*, 10.2, 10.2, 5.8)
	3	35.8	2.95 (1H, *dd*, 13.6, 5.8)
			3.25 (1H, *dd*, 13.6, 10.2)
	4	137.0	
	5, 9	129.0	7.22 (2H, *m*)
	6, 8	128.6	7.29 (2H, *m*)
	7	126.7	7.20 (1H, *m*)
	NH		7.51 (1H, *d*, 10.2)
Pro	1	171.9	
	2	57.7	4.65 (1H, *dd*, 7.8, 2.2)
	3	24.7	1.74 (1H, *m*)
			2.31 (1H, *m*)
	4	25.0	1.75 (1H, *m*)
			2.16 (1H, *m*)
	5	46.9	3.21 (1H, *ddd*, 10.2, 7.3, 7.3)
			3.86 (1H, *ddd*, 10.2, 9.0, 4.9)

^{1}H NMR data represent chemical shift, multiplicity (*J* in Hz).

Table 2-2 NMR data (^{1}H, 500 MHz and ^{13}C, 125 MHz) for compound **2** in $CDCl_3$

Unit	position	**2**	
		δ_C	δ_H
aod	1	174.4	
	2	54.3	4.18 (1H, *ddd*, 10.3, 7.6, 7.6)
	3	28.8	1.56 (1H, *m*)
			1.78 (1H, *m*)
	4	25.3	1.28 (2H, *m*)
	5	28.69	1.28 (2H, *m*)
	6	23.6	1.56 (2H, *m*)
	7	42.1	2.40 (1H, *t*, 7.6)
	8	211.7	
	9	35.9	2.41 (2H, *q*, 7.3)
	10	7.8	1.05 (3H, *d*, 7.3)
	NH		7.09 (1H, *d*, 10.3)
aib	1	175.6	
	2	58.8	
	3	23.5	1.77 (3H, *s*)
	4	26.4	1.34 (3H, *s*)
	NH		6.00 (1H, *s*)
Phe	1	172.8	
	2	53.4	5.15 (1H, *ddd*, 10.2, 10.2, 5.8)
	3	35.8	2.95 (1H, *dd*, 13.6, 5.8)
			3.26 (1H, *dd*, 13.6, 10.2)
	4	137.0	
	5, 9	129.0	7.22 (2H, *m*)
	6, 8	128.6	7.28 (2H, *m*)
	7	126.7	7.19 (1H, *m*)
	NH		7.51 (1H, *d*, 10.2)
Pro	1	171.8	
	2	57.8	4.66 (1H, *dd*, 7.8, 2.2)
	3	24.7	1.78 (1H, *m*)
			2.31 (1H, *m*)
	4	25.0	1.78 (1H, *m*)
			2.17 (1H, *m*)
	5	47.0	3.22 (1H, *ddd*, 10.2, 7.3, 7.3)
			3.86 (1H, *ddd*, 10.2, 8.5, 5.1)

^{1}H NMR data represent chemical shift, multiplicity (*J* in Hz).

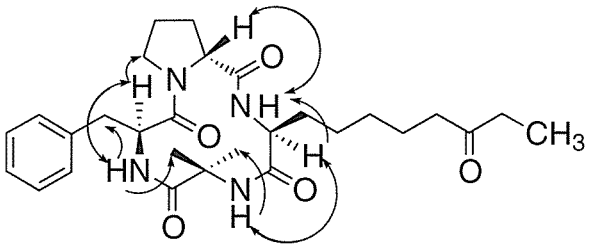


Fig. 2-4 Key NOE correlations in compound **2**

The structure of compound **3** was determined as follows: on the basis of its HREIMS, which showed a molecular ion peak at *m/z* 570.3035, and its NMR spectral data, this compound has the molecular formula $C_{30}H_{42}N_4O_7$. The NMR spectrum is similar to that of **1**, evidence that **3** also is a chlamydocin analogue. Its structure was established by comparing its NMR spectral data (Table 2-3) with those for **1** and by chemical conversion. Two new additional carbon resonances (δ 170.4 and 20.7), not observed in the ^{13}C NMR spectrum of **1**, were present in the ^{13}C NMR spectrum of **3**, and an additional new three proton singlet signals appeared at δ 2.15 in its ^{1}H NMR spectrum. These findings indicate there is an additional acetyl group in **3**. Furthermore, the hydroxyl methane proton signal produced by the H-9 of aao was shifted down field (δ 4.23 in the ^{1}H NMR spectrum of **1** to δ 5.08 in that of **3**), indicative that the acetoxyl group attached to the C-9 of aao. To verify this, compound **1** was acetylated by the usual method. The resulting product was identical in all respects to **3**. Compound **3**, therefore, is a new compound, never before reported.

Table 2-3 NMR data (^{1}H, 500 MHz and ^{13}C, 125 MHz) for compound **3** in $CDCl_3$

Unit	position	**3**	
		δ_C	δ_H
aao	1	174.4	
	2	54.3	4.18 (1H, *ddd*, 10.2, 7.6, 7.6)
	3	28.74	1.64 (1H, *m*)
			1.79 (1H, *m*)
	4	25.2	1.29 (2H, *m*)
	5	28.62	1.29 (2H, *m*)
	6	22.9	1.56 (2H, *m*)
	7	37.9	2.41 (1H, *ddd*, 17.6, 7.3, 7.3)
			2.51 (1H, *ddd*, 17.6, 7.3, 7.3)
	8	207.6	
	9	74.6	5.08 (1H, *q*, 7.1)
	10	16.1	1.39 (3H, *d*, 7.1)
	NH		7.08 (1H, *d*, 10.2)
	CH_3CO	170.4	
	CH_3CO	20.7	2.15 (3H, *s*)
aib	1	175.6	
	2	58.8	
	3	23.5	1.77 (3H, *s*)
	4	26.4	1.34 (3H, *s*)
	NH		6.00 (1H, *s*)
Phe	1	172.8	
	2	53.4	5.15 (1H, *ddd*, 10.2, 10.2, 5.8)
	3	35.8	2.95 (1H, *dd*, 13.6, 5.8)
			3.26 (1H, *dd*, 13.6, 10.0)
	4	137.0	
	5, 9	129.0	7.22 (2H, *m*)
	6, 8	128.6	7.29 (2H, *m*)
	7	126.7	7.20 (1H, *m*)
	NH		7.51 (1H, *d*, 10.0)
Pro	1	171.9	
	2	57.7	4.66 (1H, *dd*, 8.0, 2.7)
	3	24.7	1.74 (1H, *m*)
			2.31 (1H, *m*)
	4	25.0	1.75 (1H, *m*)
			2.16 (1H, *m*)
	5.9		3.22 (1H, *ddd*, 10.2, 7.6, 7.6)
			3.86 (1H, *ddd*, 10.2, 8.3, 4.9)

^{1}H NMR data represent chemical shift, multiplicity (J in Hz).

2.3 Experimental

2.3.1 General experimental procedures

Optical rotation was measured with a Horiba SEPA-200 polarimeter. UV spectra were recorded with a Hitachi U-2001 spectrophotometer, and IR spectra with a JASCO FT/IR 7000 spectrometer. NMR spectra were measured with a JEOL Lambda 400 NMR spectrometer. Chemical shifts were referenced to $CDCl_3$ (δ_H 7.26, δ_C 77.0). Mass spectra were obtained with a JEOL AX505HA spectrometer (direct probe, 70 eV). HPLC was carried out with a Cosmosil $5C_{18}$-AR column (Nacalai Tesque, 10 × 250 mm), a flow rate of 1.0 ml/min, and detection at 220 nm. Daisogel IR-60 was used for silica gel column chromatography.

2.3.2 Fungal material

A fungal isolate, obtained from soil sample collected in Tottori prefecture, Japan was found to be very close to *Peniophora nuda* based on electron microscopy observations and the sequence of its 18S rRNA gene: for the electron microscopy study the fungus was grown on PDA medium at 25°C for 7 days. Sections prepared according to the procedure reported (Suh et al., 1993) were observed with a JEOL JEM-1010 transmission electron micromascopic at 100 kV. For analysis of the 18S rRNA sequence, the isolate was grown in YM medium (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1% glucose; pH 5.6) at 25°C for three days with shaking. Mycelia were collected by centrifugation, freeze-dried, and disrupted in liq. N_2. Genomic DNA was obtained with a Dneasy Plant Mini Kit (QIAGEN GmbH, Hilden, Germany). The PCR used genomic DNA as the template and NS1 and NS8 (White et al., 1990) as the PCR primers. Amplification conditions were 94°C for 1 min, followed by 30 cycles of 94°C for 1 min, 52°C for 30 s, 72°C for 2 min, and lastly 72°C for 2 min. PCR products were purified with a QIA quick PCR purification kit (QIAGEN GmbH, Hilden, Germany) and directly sequenced. NS1–8 were the sequencing primers (White et al., 1990). All the sequencing reactions were done with a Perkin-Elmer BigDye Terminator Kit, and the sequence determination with a Perkin-Elmer ABI PRISM 377 sequencer.

2.3.3 Fermentation and extraction

The fungus was grown without shaking at 24°C for 14 days in the dark in 500 ml conical flasks (100) containing liquid medium (200 ml/flask) composed of glucose (30 g/l), peptone (3 g/l), the extract from 50 g/l of malt, and H_2O. Metabolites were extracted from the culture filtrate with EtOAc (3 × 10 l) after adjusting the pH to 2.0 with 6 M HCl. The EtOAc solution

was washed with 1M $NaHCO_3$ (2 × 0.5 volume), dehydrated over Na_2SO_4 and concentrated to dryness for a residue (1.6 g).

2.3.4 Purification

The residue (1.6 g) was subjected to silica gel column chromatography (Daiso gel IR-60, 16 × 230 mm), with 200 ml (40 ml × 5) each of 30, 40 and 60% acetone in *n*-hexane as eluant. The first fraction (143 mg), eluted with 40% acetone in *n*-hexane, was further purified by Sephadex LH-20 column chromatography (20 × 370 mm, MeOH). Five milliliter portions of the eluate were collected. Fraction 18 was dried by evaporation, giving **1** as a colorless oil (66 mg). The third fraction (48 mg), eluted with 30% acetone in *n*-hexane and purified by HPLC, gave **2** as a colorless oil (8.4 mg, R_t 30.7 min). The forth fraction (39 mg), eluted with 30% acetone in *n*-hexane and purified by HPLC gave **3** as a colorless oil (1.5 mg, R_t 26.9 min).

2.3.5 Compound 1

$[\alpha]^{25}_D$ –51° (*c* 0.10; EtOH). UV λ_{max} nm (log ε): 237 (3.58), 205 (4.26). IR ν_{max} (film) cm^{-1}: 3454, 3288, 1697, 1667, 1622, 1528. ^1H NMR and ^{13}C NMR data: see Table 2-1. ^1H-^{13}C HMBC correlations: aho H-2/aho C-1, aho C-3; aho H-3/aho C-4; aho H-4/aho C-3; aho H-5/aho C-3; aho H-6/aho C-4, aho C-7; aho H-7/aho C-5, aho C-6, aho C-8; aho H-9/aho C-8, aho C-10; aho H-10/aho C-8, aho C-9; aib H-3/aib C-1, aib C-2, aib C-4; aib H-4/ aib C-1, aib C-2, aib C-3; aib NH/ aib C-2, aib C-3, aho C-1; Phe H-2/Phe C-1; Phe H-3/Phe C-1, Phe C-2, Phe C-4, Phe C-5, 9; Phe H-5,9/ Phe C-3; Pro H-2/Pro C-3, Pro C-4, Pro C-5; Pro H-3/Pro C-1, Pro C-5; Pro H-5/Pro C-3, Pro C-4. EIMS *m/z* (rel. int.): 528 $[M]^+$ (26), 498 (8), 485 (20), 471 (27), 428 (50), 426 (15), 409 (28), 343 (7), 330 (23), 314 (15), 269 (14), 229 (26), 219 (10), 172 (14), 120 (37), 70 (100), 58 (66). HREIMS *m/z:* 528.2955 (calc. for $C_{28}H_{40}N_4O_6$, 528.2948).

2.3.6 Compound 2

$[\alpha]^{25}_D$ –55.6° (*c* 0.36; EtOH). UV λ_{max} nm (log ε): 237 (3.56), 205 (4.02). IR ν_{max} (film) cm^{-1}: 3453, 3310, 1692, 1667, 1628, 1530. ^1H NMR and ^{13}C NMR data: see Table 2-2. ^1H-^{13}C HMBC correlations: aod H-2/aod C-1, aod C-3, Pro C-1; aod H-7/aod C-5; aod H-9/aod C-8; aod H-10/aod C-8; aod NH/Pro C-1; aib H-3/aib C-1, aib C-2; aib H-4/ aib C-1, aib C-2; aib NH/ aib C-3, aib C-4, aod C-1; Phe H-2/Phe C-1, aib C-1; Phe H-3/Phe C-1, Phe C-2; Phe NH/aib C-1; Pro H-2/Pro C-1, Pro C-4; Pro H-5/Pro C-4. EIMS *m/z* (rel. int.): 512 $[M]^+$ (16),

456 (14), 455 (16), 413 (13), 412 (44), 400 (11), 393 (22), 315 (18), 314 (51), 253 (22), 252 (11), 229 (26), 201 (10), 156 (21), 131 (12), 120 (37), 91 (10), 70 (100), 58 (59), 57(24). HREIMS *m/z*: 512.3001 (calc. for $C_{28}H_{40}N_4O_5$, 512.2999).

2.3.7 Compound 3

$[\alpha]^{25}_D$–45.7° (*c* 0.21; EtOH). UV λ_{max} nm (log ε): 237 (3.52), 211 (4.00). IR ν_{max} (film) cm^{-1}: 3456, 1705, 1690, 1456, 1032. ^1H NMR and ^{13}C NMR data: see Table 2-3. ^1H-^{13}C HMBC correlations: aao H-2/aao C-1, aao C-3; aao H-4/aao C-3, aao C-4; aao H-5/aao C-4; aao H-6/aao C-4; aao H-7/aao C-5, aao C-6, aao C-8; aao H-9/aao C-8, aao C-10, aao CH_3CO; aao H-10/ aao C-8, aao C-9; aao NH/Pro C-1; aao CH_3CO/aao CH_3CO; aib H-3/ aib C-1, aib C-2, aib C-4; aib H-4/ aib C-1, aib C-2, aib C-3; aib NH/ aib C-2, aib C-3, aib C-4, aao C-1; Phe H-2/Phe C-1, Phe C-3; Phe H-3/Phe C-1, Phe C-2, Phe C-4, Phe C-5,9; Phe H-5,9/ Phe C-3, Phe C-6,8, Phe C-7; Phe H-6,8/Phe C-4; Phe H-7/Phe C-5,9, Phe C-6,8; Phe NH/aib C-1; Pro H-2/Pro C-3, Pro C-4, Pro C-5; Pro H-3/Pro C-1; Pro H-5/Pro C-3, Pro C-4. EIMS *m/z* (rel. int.): 570 $[M]^+$ (24), 514 (20), 513 (29), 512 (13), 470 (47), 451 (26), 412 (18), 372 (43), 314 (31), 311 (19), 310 (12), 229 (28), 214 (20), 120 (34), 70 (100), 58 (55). HREIMS *m/z*: 570.3035 (calc. for $C_{30}H_{42}N_4O_7$, 570.3054).

2.3.8 Acetylation of compound 1

Compound **1** (9.5 mg) was treated overnight with 1 ml of acetic anhydride and 0.5 ml of pyridine. The product was purified by HPLC, giving compound **3** (8.8 mg).

2.3.9 Amino acid analysis

Compound **1** (0.6 mg) was hydrolysed in 0.12 ml of 6 M HCl at 110°C for 20 h. The reaction mixture was dried by evaporation and dissolved in 50 µl of 0.1 M borate buffer (pH 9.0). Ten microliters of CH_3CN and 2 µl of (+)-1-(9-fluorenyl) ethyl chloroformate (Aldrich, 18 mM in acetone) were added to 10 µl of the hydrolysate solution, after which the mixture was kept at 50°C for 15 min. The reaction was terminated by adding 25 µl of 100 mM cysteic acid in 0.1 M borate buffer (pH 9.0). Before HPLC analysis, the reaction mixture was diluted with 153 µl of 0.1 M NaOAc buffer (pH 4.0) (Hayasaki and Sasagawa, 1993). Analytical HPLC employed a Shim pack CLC-C8 (Shimazu, 6.0 × 150 mm) column. Delivertives were detected with a Shimazu RF 550 fluorescence detector (excitation: 260 nm; emission; 315 nm). Two solvents were used; solvent A consisting of THF-NaOAc buffer (pH 4.0) (25:75,

v/v) and solvent B consisting of THF-NaOAc buffer (pH 5.1) (50:50, v/v). Gradient elution conditions were 0-25 min, 0-60% B; 25-80 min, 60% B; 80-90 min, 60-100% B; 90-95 min, 100% B; 95-100 min, 100-0% B. The flow rate was 0.8 ml/min. Under these conditions the R_f of D-phenylalanine derivative was 81.4 min and that of the L-form 85.6 min.

2.4 References

Closse, A., and Huguenin, R. 1974. Isolierung und strukturaufklärung von chlamydocin. Helv. Chim. Acta 57, 533-545.

Gupta, S., Peiser, G., Nakajima, T., Hwang, Y., 1994. Characterization of a phytotoxic cyclotetrapeptide, a novel chlamydocin analogue, from *Verticillium coccosporum*. Tetrahedron Lett. 35, 6009-6012.

Hayashi, T., Sasagawa, T., 1993. A method for identifying the carboxy terminal amino acid of a protein. Analytic. Biochem. 209, 163-168.

Khan, S.R., Kimbrough, J.W., 1982. A reevaluation of the basidiomycetes based upon septal and basidial structures. Mycotaxon 15, 103-120.

Suh, S.-O., Hirata, A., Sugiyama, J., Komagata, K., 1993. Septal ultrastructure of basidiomyceteous yeasts and their taxonomic implications with observations on the ultrastructure of *Erythrobasidium hasegawaianium* and *Sympodiomycopsis paphopedili*. Mycologia 85, 30-37.

White, T.J., Bruns, T., Lee, S., Taylor, J.W., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. (Eds.), PCR Protocols, a Guide to Methods and Applications. Academic Press, New York, pp. 315-322.

Chapter 3
Plant growth-retardant activity of compounds 1-3

3.1 Introduction

In previous chapter the structures of compounds **1-3** were shown. These metabolites have same framework as chlamydocin, but differ on the side chain. I describe here the plant growth-retardant activity of compounds **1-3**, and the important parts of their structure.

To test the retardant activity I used the microdrop bioassay (Murakami, 1968). The method was developed for the detection of GAs using dwarf rice (cv. Tanginbozu and cv. Waito C). I used this to test the retardant activity of fungal metabolites using normal rice (cv. Koshihikari). The Koshihikari is a cultivar susceptible to lodging due to its elongated stem. By this assay I can detect the retardation activity sensitively.

3.2 Results and discussion

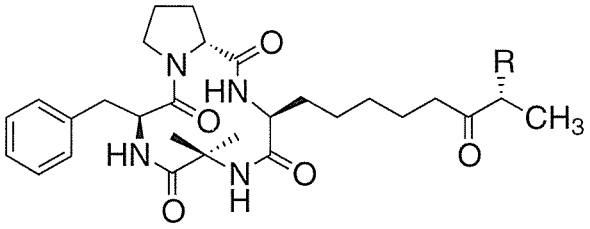
Plant growth-retardant activities for compounds **1-3** in a microdrop bioassay of rice seedlings are shown in Table 3-1 and Fig. 3-1. Administration of 10 nmol/plant of **1** or **2** reduced the second leaf sheath of the treated plants, respectively to 73 and 71% of the value for the water control. The same amount of **3** produced 83% growth of the second leaf sheath in the treated as compared to control plants. Interestingly, compound **2** was more active than **3** as active as **1**. This suggests that the hydroxyl group at the C-9 of aho in **1** is not important for plant growth retardation and that the acetoxyl group at the C-9 of aao in **3** reduced activity. These data indicated that the hydroxyl at the C-9 of aho is not essential for the retardant activity, and thus the ketone function at the C-8 of aho is important.

Cyclic tetrapeptides, which have both proline and an adjacent novel amino acid, aeo, include Cyl-2 from *Cylindrocladium scoparium* (Takayama et al., 1984), chlamydocin from *Diheterospora chlamydosporia* (Closse and Huguenin, 1974), and HC toxin from *Helminthosporium carbonum* (Walton et al., 1982), all of which are reported to have phytotoxic activity. A chlamydocin analogue that has aho instead of aeo has phytotoxic activity (Gupta et al., 1994). This means that the phtyotoxic activity is not produced by epoxide function of aeo. My present findings show that the 9-hydroxyl of aho is not essential for the retardant activity, and thus the 9-hydroxyl of aho may also not be essential for phytotoxicity.

Table 3-1 Effects of compounds **1**-**3** on second leaf sheath length of rice (*Oryza sativa* L., cv. Koshihikari) seedlings

Amount (nmol/plant)	Second leaf sheath length		
	1 R=OH	**2** R=H	**3** R=OAc
0	100 (49.2 mm)	100 (49.2 mm)	100 (46.8 mm)
0.3	94.5 ± 2.1	95.3 ± 2.9	100.0 ± 4.9
1	81.6 ± 1.3	84.8 ± 3.2	100.4 ± 6.3
3	78.3 ± 1.4	79.2 ± 3.5	94.0 ± 1.8
10	73.0 ± 1.4	71.0 ± 3.2	82.9 ± 3.1
30	57.6 ± 3.0	59.6 ± 5.2	79.1 ± 3.1

Values are presented as percentage of the control. Data are mean ± standard error (n = 5)



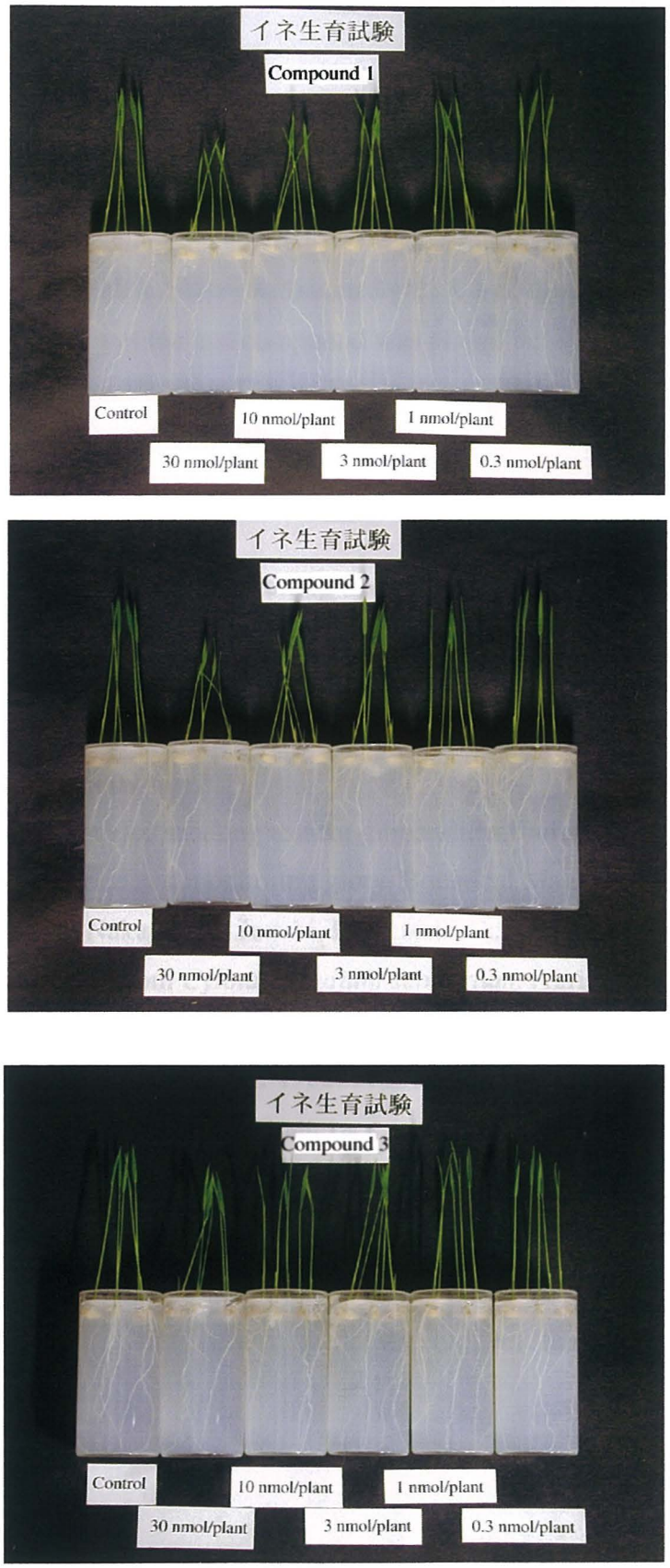


Fig. 3-1 Effects of compounds **1**-**3** on rice (*Oryza sativa* L., cv. Koshihikari) seedlings

3.3 Experimental

3.3.1 Biological assays

Compounds **1-3** were evaluated for their ability to retard the growth of rice plants. Rice seeds (*Oryza sativa* L., cv. Koshihikari) were soaked in H_2O for 48 h at 28°C under fluorescent light. Sets of six germinated seeds were planted on 1% agar medium in 30 ml beakers then incubated for 48 h under continuous light. A 1 μl drop of 50% aq. MeOH containing a certain amount of the test compound was placed between the shoot and first leaf of each seedling. After incuvation for 72 h under continuous light, the length of the second leaf sheath of the seedling was measured.

3.4 References

Closse, A., and Huguenin, R., 1974. Isolierung und strukturaufklärung von chlamydocin. Helv. Chim. Acta 57, 533-545.

Gupta, S., Peiser, G., Nakajima, T., Hwang, Y., 1994. Characterization of a phytotoxic cyclotetrapeptide, a novel chlamydocin analogue, from *Verticillium coccosporum*. Tetrahedron Lett. 35, 6009-6012

Murakami, Y., 1968. A new rice seedling test for gibberellins, 'microdrop metod' and its use for testing extracts of rice and monitoring glory. Botanical Magazine Tokyo 81, 33-43.

Takayama, S., Isogai, A., Nakata, M., Suzuki, H., Suzuki, A., 1984. Structure of Cyl-1, a novel cyclotetrapeptide from *Cylindrocladium scoparium*. Agric. Biol. Chem. 48, 839-842.

Walton, J.D., Earle, E.D., Gibson, B.W., 1982. Purification and structure of the host–specific toxin from *Helminthosporium carbonum* race 1. Biochem. Biophys. Res. Commun. 107, 785-794.

Chapter 4
Synthesis of test compounds and their growth-retardant activities

4.1 Introduction

In previous chapter, the structure-activity relationship of **1** indicated that the hydroxyl at the C-9 of aho is not important, but the ketone function at the C-8 of aho is important for the retardant activity. I therefore investigated the structure-activity relationship of **1** in more detail. I describe here the preparation of six derivatives (**4–9**) and two synthetic analogues (**10** and **11**) (Fig. 4-1), and their plant growth-retardant activities.

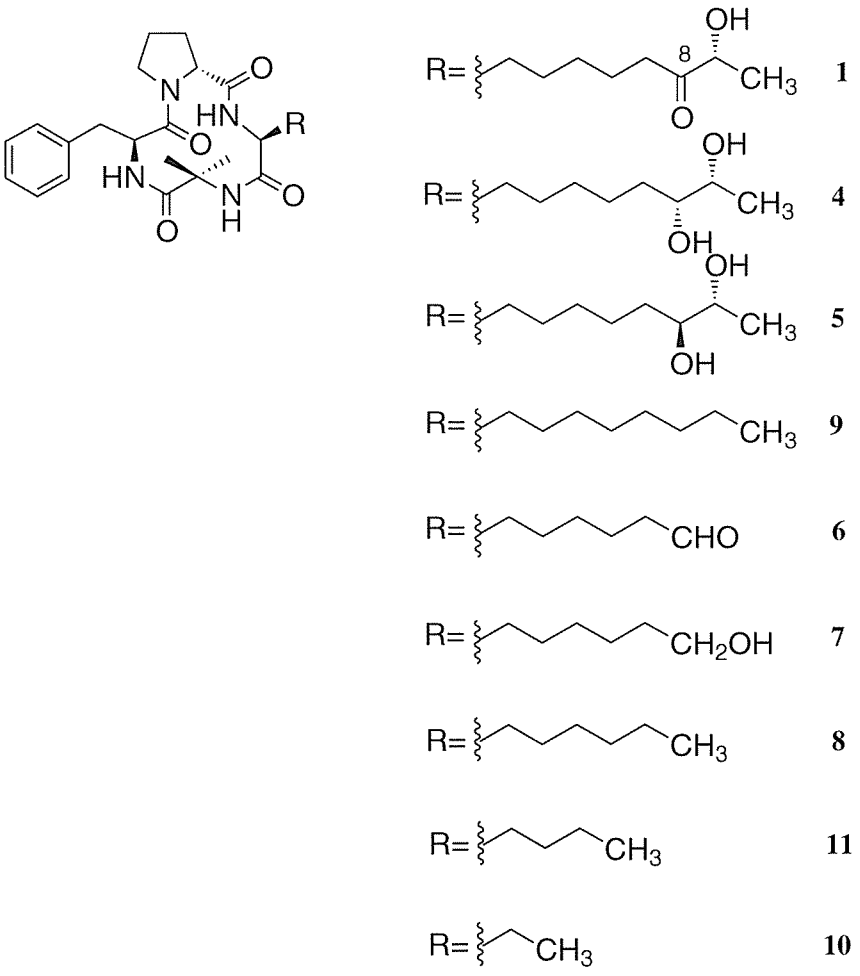


Fig. 4-1 Structures of compounds **1** and **4–11**

4.2 Results and discussion

4.2.1 Preparation of test compounds

Compounds **4–8** were prepared from **1**, the major metabolite of the fungus, as shown in Fig.

4-2. The stereochemistry of the 8-hydroxyl of **4** and **5** was determined as follows. Compounds **4** and **5** were treated with 2, 2-dimethoxypropane and *p*-toluenesulfonic acid to afford the corresponding acetonides, **4a** and **5a**. In the NOE experiments, irradiation of the H-8 of **4a** enhanced the H-9 methine signal, while irradiation of the H-8 of **5a** enhanced the H-10 methyl signal. These results revealed that the configuration of the 8-hydroxyl of **4** is *R* and that of **5** is *S*. Compound **9** was prepared from **2** by chemical conversion as shown in Fig. 4-2. Compounds **10** and **11** were synthesized according to the procedure reported by Pastuszak et al. (1982). The linear sequence was synthesized by a two-plus-two strategy as shown in Fig. 4-3. These prepared compounds were checked by EIMS, and ^{1}H and ^{13}C NMR.

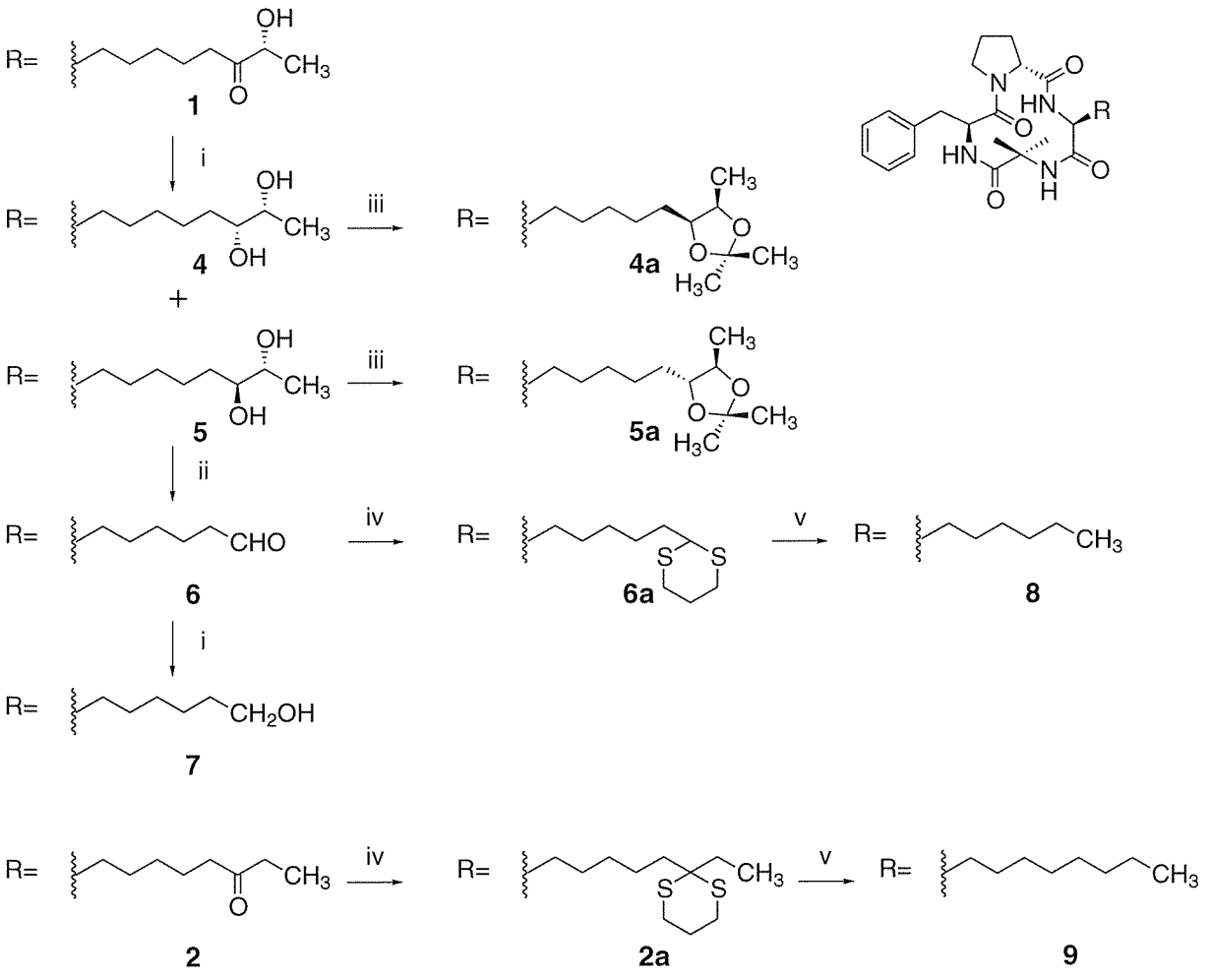


Fig. 4-2 Preparation of compounds **4–9**, Reagents and conditions: i, $NaBH_4$, MeOH; ii, $NaIO_4$, aq. MeOH; iii, 2, 2-dimethoxypropane, *p*-toluenesulfonic acid; iv, 1, 3-propanedithiol, boron trifluoride-diethyl ether complex, CH_2Cl_2; v, Raney Ni (W-2), Dioxane, reflux.

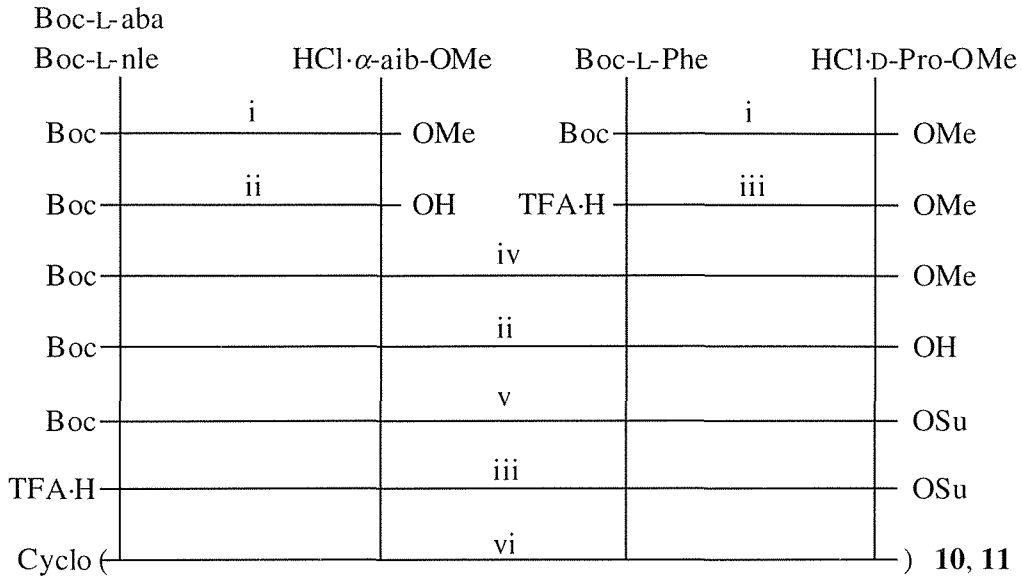


Fig. 4-3 Synthesis of compounds **10** and **11**. Reagents: i, EEDQ; ii, NaOH; iii, TFA; iv, triethylamine, HOBt, DCC; v, HOSu, DCC; vi, pyridine.

4.2.2 Plant growth-retardant activities

Plant growth-retardant activities for compounds **1** and **4–11** were determined with a microdrop bioassay using rice (cv. Koshihikari) seedlings (Table 4-1). The test compounds were applied to the rice seedlings at the dose of 30 nmol/plant. Compounds **1**, **4** and **5** reduced the length of the second leaf sheath to be 58, 78 and 69% of the control, respectively. In the rice seedlings treated with **6** and **7**, the lengths of the second leaf sheaths were 51 and 67% of the control, respectively. These results indicated that the 8-oxo in aho and aoa moieties is important, but not essential for the retardant activity. Compound **5** was a little more active than **4**, indicating that a small structural change, such as a change of the stereochemistry of only one carbon atom in the molecule, influences the retardant activity. Compound **9** completely lost its retardant activity. This result suggested that all of the compounds that have a chlamydocin framework need the oxygen atom at the C-8 position of the ada moiety to exhibit their retardant activity. Surprisingly, compound **8** retained its retardant activity despite the absence of the oxygen atom at the C-8 position. Actually, compound **8** reduced the length of the second leaf sheath to be 70% of the control. Compounds **10** and **11** almost lost their retardant activity. The retention of retardant activity in compound **8** and the loss of the activity in compounds **9**, **10** and **11** revealed that the length of the alkyl side chain in the compounds with no functionalities in the side chain is specific for the retardant activity. Compound **6** was

the most active among the compounds tested, presumably due to the additive effect of these two types of retardation. It is noteworthy that in the rice plant treated with **8** the shoots were remarkably curved (Fig. 4-4) compared with the control and with that treated with compound **1**. These phenomena suggested that the retardation mechanism of **8** is different from that of **1**.

Table 4-1 Effects of compounds **1** and **4–11** (30 nmol/plant) on second leaf sheath length of rice (*Oryza sativa* L., cv. Koshihikari) seedlings

Compound	R	Second leaf sheath length
1	$(CH_2)_5COCH(R\text{-}OH)CH_3$	57.6 ± 3.0
4	$(CH_2)_5CH(R\text{-}OH)CH(R\text{-}OH)CH_3$	78.1 ± 2.0
5	$(CH_2)_5CH(S\text{-}OH)CH(R\text{-}OH)CH_3$	68.8 ± 1.5
6	$(CH_2)_5CHO$	51.1 ± 1.7
7	$(CH_2)_5CH_2OH$	67.5 ± 1.9
8	$(CH_2)_5CH_3$	69.5 ± 3.3
9	$(CH_2)_7CH_3$	97.3 ± 1.9
10	CH_2CH_3	91.6 ± 2.7
11	$(CH_2)_3CH_3$	90.6 ± 3.8

Values are presented as percentage of the control. Data are mean ± standard error (n=5)

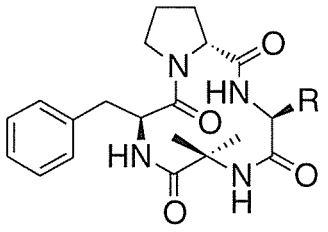


Fig. 4-4 Effect of compound **8** on rice (*Oryza sativa* L., cv. Koshihikari) seedlings

4.3 Experimental

4.3.1 General experimental procedures

Optical rotation was measured with a Horiba SEPA-200 polarimeter. UV spectra were recorded with a Hitachi U-2001 spectrophotometer. NMR spectra were measured with a JEOL JNM-ECP 500 NMR spectrometer. Chemical shifts were referenced to $CDCl_3$ (δ_H 7.26, δ_C 77.0). Mass spectra were obtained with a JEOL AX505HA spectrometer (direct probe). Glycerol was the matrix used for FABMS. In EIMS the ionization voltage was 70 eV. In CIMS the reaction gas was *iso*-butane. HPLC was carried out with a Cosmosil $5C_{18}$-AR column (Nacalai Tesque, 10 × 250 mm), a flow rate of 1.0 ml/min, and detection at 220 nm. Daisogel IR-60 was used for silica gel column chromatography and Merck Kieselgel 60 F_{254} for TLC.

4.3.2 Compounds 4 and 5

A soln of **1** (25.6 mg, 0.048 mmol) and $NaBH_4$ (3.0 mg, 0.072 mmol) in dry MeOH (1.0 ml) was stirred for 15 min at room temp. The reaction mixture was diluted with 15 ml of brine (pH 2.0 with 6 M HCl), and the products were extracted with EtOAc (15 ml × 3). The EtOAc extract was dried over Na_2SO_4 and evaporated *in vacuo*. The residue was purified by HPLC (70% MeOH) to give **4** (8.0 mg, 0.015 mmol, 31%, R_t 19.2 min) and **5** (11.5 mg, 0.022 mmol,

46%, R_t 20.5 min) as colorless oils. **4**: $[\alpha]^{25}_D$–49° (*c* 0.10; EtOH). UV λ_{max} nm (log ε): 236 (3.54), 213 (3.91). ^{1}H NMR (500 MHz, $CDCl_3$): ada δ 1.15 (3H, *d*, *J* = 6.5 Hz, H-10), 1.25-1.84 (10H, *m*, H-3-7), 3.60 (1H, *m*, H-8), 3.78 (1H, *dq*, *J* = 3.5, 6.5, H-9), 4.19 (1H, *ddd*, *J* = 7.6, 7.8, 10.1 Hz, H-2), 7.11 (1H, *d*, *J* = 10.1 Hz, NH), aib δ 1.34 (3H, *s*, H-4), 1.77 (3H, *s*, H-3), 5.97 (1H, *s*, NH), Phe δ 2.95 (1H, *dd*, *J* = 5.8, 13.5 Hz, H-3), 3.26 (1H, *dd*, *J* = 10.1, 13.5 Hz, H-3), 5.16 (1H, *ddd*, *J* = 5.8, 10.1, 10.1 Hz, H-2), 7.19-7.29 (5H, *m*, H-5-9), 7.51 (1H, *d*, *J* = 10.1 Hz, NH), Pro δ 1.25-1.84 (2H, *m*, H-3 and 4), 2.14-2.34 (2H, *m*, H-3 and 4), 3.22 (1H, *ddd*, *J* = 7.6, 7.6, 10.1 Hz, H-5), 3.86 (1H, *ddd*, *J* = 4.4, 8.3, 10.1 Hz, H-5), 4.66 (1H, *dd*, *J* = 2.3, 8.1 Hz, H-2). ^{13}C NMR (125 MHz, $CDCl_3$): δ 16.7, 23.6, 24.7, 25.0, 25.3, 25.6, 26.5, 28.8, 29.1, 31.5, 35.8, 47.0, 53.4, 54.4, 57.8, 58.8, 70.4, 74.8, 126.7, 128.6, 129.0, 137.0, 171.9, 172.8, 174.4, 175.6. EIMS *m/z* (rel. int.): 530 $[M]^+$ (11), 485 (17), 412 (14), 411 (13), 332 (12), 314 (13), 230 (11), 229 (26), 120 (32), 70 (100), 69 (11), 58 (66). **5**: $[\alpha]^{25}_D$–48° (*c* 0.15; EtOH). UV λ_{max} nm (log ε): 237 (3.34), 214 (3.85). ^{1}H NMR (500 MHz, $CDCl_3$): ada δ 1.19 (3H, *d*, *J* = 6.2 Hz, H-10), 1.24-1.90 (10H, *m*, H-3-7), 3.32 (1H, *m*, H-8), 3.59 (1H, *dq*, *J* = 6.4, 6.2 Hz, H-9), 4.19 (1H, *ddd*, *J* = 7.6, 7.6, 10.1 Hz, H-2), 7.12 (1H, *d*, *J* = 10.1 Hz, NH), aib δ 1.34 (3H, *s*, H-4), 1.77 (3H, *s*, H-3), 6.02 (1H, *s*, NH), Phe δ 2.95 (1H, *dd*, *J* = 5.8, 13.5 Hz, H-3), 3.26 (1H, *dd*, *J* = 10.1, 13.5 Hz, H-3), 5.16 (1H, *J* = *ddd*, 5.8, 10.1, 10.1 Hz, H-2), 7.19-7.29 (5H, *m*, H-5-9), 7.51 (1H, *d*, *J* = 10.1 Hz, NH), Pro δ 1.24-1.90 (2H, *m*, H-3 and 4), 2.12-2.35 (2H, *m*, H-3 and 4), 3.21 (1H, *ddd*, *J* = 7.6, 7.6, 10.1 Hz, H-5), 3.86 (1H, *ddd*, *J* = 4.1, 8.3, 10.1 Hz, H-5), 4.66 (1H, *dd*, *J* = 2.3, 8.0 Hz, H-2). ^{13}C NMR (125 MHz, $CDCl_3$): δ 19.5, 23.6, 24.7, 25.0, 25.2, 25.3, 26.5, 28.8, 29.1, 33.1, 35.8, 47.0, 53.4, 54.4, 57.8, 58.8, 70.9, 76.1, 126.7, 128.6, 129.0, 137.0, 171.9, 172.8, 174.4, 175.6. EIMS *m/z* (rel. int.): 530 $[M]^+$ (11), 486 (12), 485 (20), 430 (24), 412 (11), 411 (15), 332 (20), 314 (11), 229 (25), 120 (35), 70 (100), 69 (12), 58 (64), 57 (11), 55 (15).

4.3.3 Compound 4a

A soln of **4** (3.2 mg, 0.0060 mmol) was dissolved in 2, 2-dimethoxypropane (1.0 ml) and a catalytic amount of *p*-toluenesulfonic acid was added and stirred for 30 min at room temp., and the solvent was then evaporated *in vacuo*. The residue was purified by silica gel column chromatography to give **4a** (2.4 mg, 0.0042 mmol, 70%) as a colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$): ada δ 1.13 (3H, *d*, *J* = 6.2 Hz, H-10), 1.23-1.85 (10H, *m*, H-3-7), 1.33 (3H, *s*, CH_3C), 1.44 (3H, *s*, CH_3C), 4.01 (1H, *m*, H-8), 4.22 (1H, *dq*, *J* = 7.3, 6.2 Hz, H-9), 4.18 (1H, *ddd*, *J* = 7.6, 7.6, 10.1 Hz, H-2), 7.08 (1H, *d*, *J* = 10.1 Hz, NH), aib δ 1.34 (3H, *s*, H-4), 1.77

(3H, *s*, H-3), 5.89 (1H, *s*, NH), Phe δ 2.95 (1H, *dd*, *J* = 5.7, 13.8 Hz, H-3), 3.26 (1H, *dd*, *J* = 10.1, 13.8 Hz, H-3), 5.16 (1H, *ddd*, *J* = 5.7, 10.1, 10.1 Hz, H-2), 7.19-7.29 (5H, *m*, H-5-9), 7.52 (1H, *d*, *J* =10.1 Hz, NH), Pro δ 1.23-1.85 (2H, *m*, H-3 and 4), 2.12-2.35 (2H, *m*, H-3 and 4), 3.22 (1H, *ddd*, *J* = 7.6, 7.6, 10.1 Hz, H-5), 3.86 (1H, *ddd*, *J* = 4.2, 8.3, 9.6 Hz, H-5), 4.66 (1H, *dd*, *J* = 2.2, 7.6 Hz, H-2). EIMS *m/z* (rel. int.): 570 $[M]^+$ (36), 556 (23), 555 (49), 513 (20), 485 (26), 470 (26), 455 (23), 451 (21), 412 (35), 314 (21), 229 (26), 120 (34), 70 (100), 58 (70).

4.3.4 Compound 5a

Compound **5** (8.0 mg, 0.015 mmol) was treated with 2, 2-dimethoxypropane and *p*-toluenesulfonic acid under the same conditions as **4** to give **5a** (5.0 mg, 0.0088 mmol, 59%) as a colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$): ada δ 1.24 (3H, *d*, *J* = 6.0 Hz, H-10), 1.26-1.86 (10H, *m*, H-3-7), 1.38 (3H, *s*, CH_3C), 1.39 (3H, *s*, CH_3C), 3.49 (1H, *m*, H-8), 3.69 (1H, *dq*, *J* = 8.0, 6.0 Hz, H-9), 4.18 (1H, *ddd*, *J* = 7.8, 7.8, 10.1 Hz, H-2), 7.08 (1H, *d*, *J* = 10.1 Hz, NH), aib δ 1.34 (3H, *s*, H-4), 1.77 (3H, *s*, H-3), 5.87 (1H, *s*, NH), Phe δ 2.95 (1H, *dd*, *J* = 5.7, 13.5 Hz, H-3), 3.26 (1H, *dd*, *J* = 10.1, 13.5 Hz, H-3), 5.16 (1H, *ddd*, *J* = 5.7, 10.1, 10.1 Hz, H-2), 7.19-7.30 (5H, *m*, H-5-9), 7.50 (1H, *d*, *J* = 10.1 Hz, NH), Pro δ 1.26-1.90 (2H, *m*, H-3 and 4), 2.13-2.36 (2H, *m*, H-3 and 4), 3.22 (1H, *ddd*, *J* = 7.4, 7.5, 10.0 Hz, H-5), 3.86 (1H, *ddd*, *J* = 4.6, 8.5, 10.1 Hz, H-5), 4.66 (1H, *dd*, *J* = 2.3, 7.8 Hz, H-2). EIMS *m/z* (rel. int.): 570 $[M]^+$ (16), 556 (9), 555 (29), 513 (11), 485 (14), 470 (14), 455 (13), 451 (13), 412 (19), 314 (14), 229 (18), 120 (27), 70 (100), 58 (75).

4.3.5 Compound 6

A mixture of **4** and **5** (39.6 mg, 0.075 mmol) in MeOH (2.0 ml) was added to a soln of $NaIO_4$ (19.3 mg) in H_2O (2.0 ml) at 0°C. After being stirred at room temp. for 1 hour, the reaction mixture was diluted with 20 ml of H_2O. The products were extracted with EtOAc (18 ml × 3). The EtOAc soln was dried over Na_2SO_4 and evaporated *in vacuo*. The residue was purified by HPLC (70% MeOH) to give **6** (33.9 mg, 0.070 mmol, 93%, R_t 21.5 min) as a colorless oil. $[\alpha]^{25}_D$ –51° (*c* 0.13; EtOH). UV λ_{max} nm (log ε): 236 (3.40), 211 (3.85). ^{1}H NMR (500 MHz, $CDCl_3$): aoa δ 1.21-1.85 (8H, *m*, H-3-6), 2.43 (2H, *dt*, 1.4, 7.4 Hz, H-7), 4.18 (1H, *ddd*, *J* = 7.6, 7.8, 10.3 Hz, H-2), 7.07 (1H, *d*, *J* = 10.3 Hz, NH), 9.76 (1H, *t*, *J* = 1.4 Hz, H-8), aib δ 1.34 (3H, *s*, H-4), 1.77 (3H, *s*, H-3), 5.93 (1H, *s*, NH), Phe δ 2.95 (1H, *dd*, *J* = 5.8, 13.5 Hz, H-3), 3.26 (1H, *dd*, *J* = 10.3, 13.5 Hz, H-3), 5.16 (1H, *ddd*, *J* = 5.8, 10.3, 10.3 Hz, H-2),

7.19-7.29 (5H, *m*, H-5-9), 7.50 (1H, *d*, *J* = 10.3 Hz, NH), Pro δ 1.21-1.85 (2H, *m*, H-3 and 4), 2.10-2.36 (2H, *m*, H-3 and 4), 3.22 (1H, *ddd*, *J* = 7.6, 7.6, 10.1 Hz, H-5), 3.86 (1H, *ddd*, *J* = 4.1, 8.7, 10.1 Hz, H-5), 4.66 (1H, *dd*, *J* = 2.4, 7.8 Hz, H-2). ^{13}C NMR (125 MHz, $CDCl_3$): δ 21.8, 23.5, 24.7, 25.0, 25.3, 26.5, 28.72, 28.73, 35.8, 43.7, 47.0, 53.4, 54.3, 57.8, 58.8, 126.7, 128.6, 129.0, 137.0, 171.8, 172.8, 174.3, 175.6, 202.5. EIMS *m/z* (rel. int.): 484 $[M]^+$ (17), 428 (14), 427 (14), 399 (13), 384 (33), 365 (24), 356 (30), 286 (26), 229 (27), 128 (19), 120 (38), 70 (100), 58 (71).

4.3.6 Compound 7

A soln of **6** (11.2 mg, 0.023 mmol) and $NaBH_4$ (1.2 mg) in dry MeOH (1.0 ml) was stirred for 15 min at room temp. The reaction mixture was diluted with 10 ml of brine (pH 2.0 with HCl), after which the products were extracted with EtOAc (10 ml × 3). The EtOAc extract was dried over Na_2SO_4 and evaporated *in vacuo*. The residue was purified by HPLC (70% MeOH) to give **7** (9.3 mg, 0.019 mmol, 83%, R_t 22.8 min) as a colorless oil. $[\alpha]^{25}_D$ –54° (*c* 0.16; EtOH). UV λ_{max} nm (log ε): 236 (3.45), 214 (3.78). ^{1}H NMR (500 MHz, $CDCl_3$): aoa δ 1.25-1.86 (10H, *m*, H-3-7), 3.63 (2H, *t*, *J* = 6.5 Hz, H-8), 4.19 (1H, *ddd*, *J* = 7.6, 7.8, 10.1 Hz, H-2), 7.10 (1H, *d*, *J* = 10.1 Hz, NH), aib δ1.34 (3H, *s*, H-4), 1.77 (3H, *s*, H-3), 5.98 (1H, *s*, NH), Phe δ 2.95 (1H, *dd*, *J* = 5.8, 13.6 Hz, H-3), 3.26 (1H, *dd*, *J* = 10.1, 13.6 Hz, H-3), 5.16 (1H, *J* = *ddd*, 5.8, 10.1, 10.1 Hz, H-2), 7.17-7.29 (5H, *m*, H-5-9), 7.52 (1H, *d*, *J* = 10.1 Hz, NH), Pro δ 1.25-1.86 (2H, *m*, H-3 and 4), 2.12-2.36 (2H, *m*, H-3 and 4), 3.22 (1H, *ddd*, *J* = 7.6, 7.6, 10.3 Hz, H-5), 3.86 (1H, *ddd*, *J* = 4.1, 8.7, 10.3 Hz, H-5), 4.66 (1H, *dd*, *J* = 1.6, 7.6 Hz, H-2). ^{13}C NMR (125 MHz, $CDCl_3$): δ 23.6, 24.7, 25.0, 25.4, 25.5, 26.5, 28.9, 29.0, 32.5, 35.8, 47.0, 53.4, 54.4, 57.8, 58.8, 62.9, 126.7, 128.6, 129.0, 137.0, 171.8, 172.8, 174.4, 175.6. EIMS *m/z* (rel. int.): 486 $[M]^+$ (12), 430 (16), 429 (18), 387 (19), 386 (69), 367 (30), 314 (14), 288 (29), 229 (51), 227 (18), 217 (13), 201 (13), 131 (10), 130 (24), 120 (48), 70 (100), 58 (96).

4.3.7 Compound 8

To a soln of **6** (33.9 mg, 0.070 mmol) in CH_2Cl_2 (2.5 ml) was added 1, 3-propanedithiol (0.0098 ml, 15.0 mg, 0.14 mmol) and boron trifluoride-diethyl ether complex (0.026 ml, 29.8 mg, 0.098 mmol) at 0°C. After being stirred at room temp. for 30 min, the reaction mixture was diluted with EtOAc (10 ml). The EtOAc extract was washed with saturated $NaHCO_3$ and brine, dried over Na_2SO_4 and evaporated *in vacuo* to give thioacetal **6a** (21.9 mg, 0.038 mmol,

54%) as a colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$): aoa δ 1.25–1.92 (12H, *m*, H-3-7 and SCH_2CH_2), 2.77-2.89 (4H, *m*, SCH_2), 4.02 (1H, *dd*, *J* = 6.9, 7.0 Hz, H-8), 4.17 (1H, *ddd*, *J* = 7.6, 7.6, 10.0 Hz, H-2), 7.09 (1H, *d*, *J* = 10.0 Hz, NH), aib δ 1.33 (3H, *s*, H-4), 1.76 (3H, *s*, H-3), 6.00 (1H, *s*, NH), Phe δ 2.94 (1H, *dd*, *J* = 5.7, 13.5 Hz, H-3), 3.25 (1H, *dd*, *J* = 10.3, 13.5 Hz, H-3), 5.15 (1H, *ddd*, *J* = 5.7, 10.3, 10.3 Hz, H-2), 7.18-7.30 (5H, *m*, H-5-9), 7.52 (1H, *d*, *J* = 10.3 Hz, NH), Pro δ1.25–1.92 (2H, *m*, H-3 and 4), 2.07-2.34 (2H, *m*, H-3 and 4), 3.21 (1H, *ddd*, *J* = 7.6, 7.6, 10.3 Hz, H-5), 3.84 (1H, *ddd*, *J* = 5.0, 8.6, 10.3 Hz, H-5), 4.65 (1H, *dd*, *J* = 2.7, 8.4 Hz, H-2). EIMS *m/z* (rel. int.): 574 $[M]^+$ (27), 330 (53), 245 (15), 244 (76), 153 (49), 142 (33), 125 (80), 120 (21), 119 (56), 108 (21), 107 (12), 106 (100), 91 (41), 78 (20), 73 (15), 70 (70), 64 (30), 60 (15), 58 (37).

A soln of **6a** (21.9 mg, 0.038 mmol) in dioxane (2.0 ml) was refluxed for 20 min with Raney Ni (700 mg) freshly prepared from alloy. The soln was filtered, the metal washed thoroughly with methanol, and the filtrate and washings evaporated *in vacuo*. The residue was purified by prep. TLC [EtOAc-*n*-hexane, 4:6, R_f 0.51] and then HPLC (70% MeOH) to give **8** (10.5 mg, 0.022 mmol, 58%, R_t 26.8 min) as a colorless oil. $[\alpha]^{25}_D$ –63° (*c* 0.24; EtOH). UV λ_{max} nm (log ε): 236(3.52), 212 (3.92). ^{1}H NMR (500 MHz, $CDCl_3$): aoa δ 0.88 (3H, *t*, *J* = 7.1 Hz, H-8), 1.20-1.90 (10H, *m*, H-3-7), 4.18 (1H, *ddd*, *J* = 7.3, 7.6, 10.2 Hz, H-2), 7.07 (1H, *d*, *J* = 10.2 Hz, NH), aib δ 1.34 (3H, *s*, H-4), 1.77 (3H, *s*, H-3), 5.91 (1H, *s*, NH), Phe δ 2.95 (1H, *dd*, *J* = 5.7, 13.5 Hz, H-3), 3.27 (1H, *dd*, *J* = 10.1, 13.5 Hz, H-3), 5.16 (1H, *ddd*, *J* = 5.7, 10.1, 10.1 Hz, H-2), 7.19-7.29 (5H, *m*, H-5-9), 7.53 (1H, *d*, *J* = 10.1 Hz, NH), Pro δ1.20-1.90 (2H, *m*, H-3 and 4), 2.14-2.36 (2H, *m*, H-3 and 4), 3.23 (1H, *ddd*, *J*= 7.3, 7.3, 10.1 Hz, H-5), 3.86 (1H, *ddd*, *J* = 5.5, 8.6, 10.1 Hz, H-5), 4.66 (1H, *dd*, *J* = 2.1, 8.0 Hz, H-2). ^{13}C NMR (125 MHz, $CDCl_3$): 14.0, 22.5, 23.6, 24.7, 25.0, 25.5, 26.5, 28.9 × 2, 31.6, 35.8, 47.0, 53.4, 54.5, 57.8, 58.8, 126.7, 128.6, 129.0, 137.1, 171.8, 172.8, 174.5, 175.7. EIMS *m/z* (rel. int.): 470 $[M]^+$ (34), 370 (60), 351 (31), 229 (34), 120 (37), 70 (100), 58 (69).

4.3.8 Compound 9

Compound **2** (5.9 mg, 0.012 mmol) in CH_2Cl_2 (0.5 ml) was treated with 1, 3-propanedithiol (0.017 ml, 2.6 mg, 0.024 mmol) and boron trifluoride-diethyl ether complex (0.046 ml, 5.2 mg, 0.017 mmol) under the same conditions used in the preparation of **6a** to give thioketal **2a** (5.1 mg, 0.0085 mmol, 71%) as a colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$): ada δ 0.97 (3H, *t*, *J* = 7.4 Hz, H-10), 1.24–1.97 (14H, *m*, H-3-7, 9 and SCH_2CH_2), 2.75-2.84 (4H, *m*, SCH_2), 4.17 (1H, *ddd*, *J* = 7.6, 7.6, 10.1 Hz, H-2), 7.08 (1H, *d*, *J* = 10.1 Hz, NH), aib δ 1.34 (3H, *s*,

H-4), 1.77 (3H, *s*, H-3), 5.92 (1H, *s*, NH), Phe δ 2.95 (1H, *dd*, *J* = 5.7, 13.6 Hz, H-3), 3.26 (1H, *dd*, *J* = 10.1, 13.6 Hz, H-3), 5.16 (1H, *ddd*, *J* = 5.7, 10.1, 10.3 Hz, H-2), 7.18-7.29 (5H, *m*, H-5-9), 7.52 (1H, *d*, *J* = 10.3 Hz, NH), Pro δ 1.24–1.97 (2H, *m*, H-3 and 4), 2.13-2.36 (2H, *m*, H-3 and 4), 3.22 (1H, *ddd*, *J* = 7.3, 7.3, 10.1 Hz, H-5), 3.86 (1H, *ddd*, *J* = 4.4, 8.3, 10.1 Hz, H-5), 4.65 (1H, *dd*, *J* = 2.5, 8.0 Hz, H-2). EIMS *m/z* (rel. int.): 602 $[M]^+$ (52), 502 (19), 496 (24), 229 (24), 147 (45), 120 (40), 73 (19), 70 (100), 69 (22), 60 (22), 58 (68), 57 (24), 55 (25).

Compound **2a** (5.1 mg, 0.0085 mmol) was treated with Raney Ni (100 mg) under the same conditions used in the preparation of **8**. The product was purified by silica gel column chromatography followed by HPLC (90% MeOH) to give **9** (1.5 mg, 0.0030 mmol, 35%, R_t 26.8 min) as a colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$): ada δ 0.88 (3H, *t*, *J* = 6.9 Hz, H-10), 1.24-1.86 (14H, *m*, H-3-9), 4.18 (1H, *ddd*, *J* = 7.6, 7.6, 10.1 Hz, H-2), 7.07 (1H, *d*, *J* = 10.1 Hz, NH), aib δ 1.34 (3H, *s*, H-4), 1.77 (3H, *s*, H-3), 5.89 (1H, *s*, NH), Phe δ 2.95 (1H, *dd*, *J* = 5.7, 13.5 Hz, H-3), 3.26 (1H, *dd*, *J* = 10.1, 13.5 Hz, H-3), 5.16 (1H, *ddd*, *J* = 5.7, 10.1, 10.1 Hz, H-2), 7.19-7.29 (5H, *m*, H-5-9), 7.53 (1H, *d*, *J* = 10.1 Hz, NH), Pro δ 1.24-1.86 (2H, *m*, H-3 and 4), 2.14–2.36 (2H, *m*, H-3 and 4), 3.22 (1H, *ddd*, *J* = 7.3, 7.3, 10.1 Hz, H-5), 3.86 (1H, *ddd*, *J* = 4.4, 8.3, 10.1 Hz, H-5), 4.66 (1H, *dd*, *J* = 2.1, 7.6, H-2). EIMS *m/z* (rel. int.): 498 $[M]^+$ (34), 399 (22), 398 (63), 386 (18), 379 (30), 229 (34), 142 (15), 120 (36), 70 (100), 58 (66).

4.3.9 Compound 10

_N_-Boc-L-2-aminobutyryl-2-aminoisobutyric acid methyl ester

L-Aba (103.0 mg, 1 mmol) in dioxane (1.0 ml) and H_2O (1.0 ml) was coupled with *N*-Boc azide (290 mg, 1.6 mmol) under alkali conditions to give *N*-Boc-L-aba (183.4 mg, 0.90 mmol, 90%). ^{1}H NMR (500 MHz, $CDCl_3$): aba δ 0.99 (3H, *t*, *J* = 7.4 Hz, H-4), 1.45 (9H, *s*, $(CH_3)_3C$), 1.73 (1H, *m*, H-3), 1.92 (1H, *m*, H-3), 4.11 (1H, *m*, H-2), 4.27 (1H, *m*, NH), 4.99 (1H, *m*, NH). FABMS *m/z*: 204 $[M+H]^+$.

Aib methyl ester hydrochloride (10.5 mg, 0.070 mmol) that was prepared by the method reported by Pastuszak et al. (1982) in EtOAc (0.5 ml) was neutralized with triethylamine (0.0098 ml, 7.17 mg, 0.070 mmol) and coupled with the *N*-Boc-L-aba (15.2 mg, 0.075 mmol) by use of EEDQ (18.9 mg, 0.075 mmol) in EtOAc (0.1 ml) to give the title compound (16.5 mg, 0.055 mmol, 79%). ^{1}H NMR (500 MHz, $CDCl_3$): aba δ 0.94 (3H, *t*, *J* = 7.4 Hz, H-4), 1.45 (9H, *s*, $(CH_3)_3C$), 1.62 (1H, *m*, H-3), 1.84 (1H, *m*, H-3), 3.96 (1H, *m*, H-2), 4.99 (1H, *m*,

NH), aib δ 1.54 (3H, *s*, H-3 or 4), 1.55 (3H, *s*, H-3 or 4), 3.73 (3H, *s*, CH_3O), 6.67 (1H, *s*, NH). FABMS *m/z* (rel. int.): 325 $[M+Na]^+$, 303 $[M+H]^+$.

4.3.10 *N*-Boc-L-2-aminobutyryl-2-aminoisobutyryl-L-phenylalanyl-D-proline methyl ester

N-Boc-L-aba-aib methyl ester (16.5 mg, 0.055 mmol) in MeOH (0.11 ml) was saponified with 1 M NaOH (0.083 ml) to give the acid (14.2 mg, 0.049 mmol, 89%). ^{1}H NMR (500 MHz, $CDCl_3$): aba δ 0.93 (3H, *t*, *J* = 7.6 Hz, H-4), 1.43 (9H, *s*, $(CH_3)_3C$), 1.61 (1H, *m*, H-3), 1.82 (1H, *m*, H-3), 4.01 (1H, *m*, H-2), 5.05 (1H, *m*, NH), aib δ 1.52 (3H, *s*, H-3 or 4), 1.53 (3H, *s*, H-3 or 4), 6.65 (1H, *s*, NH). CIMS *m/z* (rel. int.): 289 $[M+H]^+$ (18), 233 (100).

L-Phe-D-Pro methyl ester trifluoroacetate (82.5 mg, 0.23 mmol), which was prepared by the method reported by Pastuszak et al. (1982), in $CHCl_3$ (2.0 ml) was neutralized with triethylamine (0.032 ml, 23.4 mg, 0.23 mmol) and coupled with the *N*-protected dipeptide acid (95 mg, 0.33 mmol) in DMF (0.8 ml) by use of HOBt (40.2 mg, 0.26 mmol) in DMF (0.5 ml) and DCC (56.5 mg, 0.26 mmol) in $CHCl_3$ (0.5 ml) to give the title compound (99.7 mg, 0.18 mmol, 78%). ^{1}H NMR (500 MHz, $CDCl_3$): aba δ 0.95 (3H, *t*, *J* = 7.3 Hz, H-4), 1.58-1.89 (2H, *m*, H-3), 1.45 (9H, *s*, $(CH_3)_3C$), 3.97 (1H, *m*, H-2), 5.11 (1H, *d*, *J* = 5.8 Hz, NH), aib δ 1.53 (3H, *s*, H-3 or 4), 1.55 (3H, *s*, H-3 or 4), 6.72 (1H, *s*, NH), Phe δ 2.95 (1H, *dd*, *J* = 9.2, 13.1 Hz, H-3), 3.05 (1H, *dd*, *J* = 6.0, 13.1 Hz, H-3), 4.91 (1H, *ddd*, *J* = 6.0, 9.2, 9.2 Hz, H-2), 6.96 (1H, *d*, *J* = 9.2 Hz, NH), 7.16-7.27 (5H, *m*, H-5-9), Pro δ 1.58-1.89 (4H, *m*, H-3 and 4), 2.71 (1H, *ddd*, *J* = 2.7, 6.9, 7.1 Hz, H-5), 3.54 (1H, *m*, H-5), 3.69 (3H, *s*, CH_3O), 4.30 (1H, *dd*, *J* = 3.7, 8.3 Hz, H-2). EIMS *m/z* (rel. int.): 546 $[M]^+$ (16), 271 (27), 259 (11), 243 (14), 215 (30), 187 (19), 130 (58), 120 (43), 70 (25), 58 (100), 57 (20).

4.3.11 Cyclo (L-2-aminobutyryl-2-aminoisobutyryl-L-phenylalanyl-D-prolyl) (10)

The *N*-protected tetrapeptide methyl ester (18.2 mg, 0.033 mmol) in MeOH (0.66 ml) was saponified with 1 M NaOH (0.50 ml) to give the acid (14.9 mg, 0.028 mmol, 85%). ^{1}H NMR (500 MHz, $CDCl_3$): aba δ 0.93 (3H, *t*, *J* = 7.4 Hz, H-4), 1.55-1.88 (2H, *m*, H-3), 1.44 (9H, *s*, $(CH_3)_3C$), 4.00 (1H, *m*, H-2), 5.43 (1H, *d*, *J* = 6.9 Hz, NH), aib δ 1.51 (6H, *s*, H-3 and 4), 7.02 (1H, *s*, NH), Phe δ 2.95 (1H, *m*, H-3), 3.05 (1H, *m*, H-3), 4.92 (1H, *ddd*, *J* = 6.9, 8.6, 8.6 Hz, H-2), 7.17-7.28 (5H, *m*, H-5-9), 7.41 (1H, *d*, *J* = 6.9 Hz, NH), Pro δ 1.55-1.88 (4H, *m*, H-3 and 4), 2.68 (1H, *m*, H-5), 3.65 (1H, *m*, H-5), 4.31 (1H, *dd*, *J* = 3.7, 8.0 Hz, H-2). EIMS *m/z* (rel. int.): 532 $[M]^+$ (5), 488 (7), 332 (7), 271 (14), 243 (15), 217 (12), 215 (18), 201 (13), 187

(14), 186 (6), 130 (6), 120 (37), 116 (32), 102 (9), 91 (5), 70 (26), 59 (6), 58 (100), 57 (20).

The *N*-protected tetrapeptide acid (20.5 mg, 0.038 mmol) in CH_2Cl_2 (0.5 ml) was coupled with HOSu (8.9 mg, 0.076 mmol) in DMF (200 µl) by use of DCC (9.1 mg, 0.042 mmol) in CH_2Cl_2 (200 µl). The succinimide ester was *N*-deprotected using TFA (0.38 ml). The TFA salt was dissolved in DMF (2.0 ml) and then cyclized by being slowly added to a large volume of pyridine (60 ml). The pyridine was evaporated *in vacuo*. The residue was purified by silica gel column chromatography followed by HPLC (85% MeOH) to give the cyclic peptide **10** (4.1 mg, 0.01 mmol, 26%, R_t 16.2 min) as a colorless powder. $[\alpha]^{25}_D$ −71° (*c* 0.19; EtOH). UV λ_{max} nm (log ε): 236 (3.09), 209 (3.67). ^{1}H NMR (500 MHz, $CDCl_3$): aba δ 0.91 (3H, *t*, *J* = 7.3 Hz, H-4), 1.59-1.91 (2H, *m*, H-3), 4.11 (1H, *ddd*, *J* = 7.6, 7.6, 10.3 Hz, H-2), 7.09 (1H, *d*, *J* = 10.3 Hz, NH), aib δ 1.34 (3H, *s*, H-4), 1.77 (3H, *s*, H-3), 5.92 (1H, *s*, NH), Phe δ 2.95 (1H, *dd*, *J* = 5.7, 13.5 Hz, H-3), 3.26 (1H, *dd*, *J* = 10.1, 13.5 Hz, H-3), 5.16 (1H, *ddd*, *J* = 5.7, 10.1, 10.1 Hz, H-2), 7.19-7.29 (5H, *m*, H-5-9), 7.52 (1H, *d*, *J* = 10.1 Hz, NH), Pro δ 1.59-1.91 (2H, *m*, H-3 and 4), 2.13-2.36 (2H, *m*, H-3 and 4), 3.22 (1H, *ddd*, *J* = 7.4, 7.6, 10.3 Hz, H-5), 3.86 (1H, *ddd*, *J* = 4.8, 8.7, 10.3 Hz, H-5), 4.66 (*dd*, *J* = 2.5, 7.8 Hz, H-2). ^{13}C NMR (125 MHz, $CDCl_3$): 10.1, 22.3, 23.6, 24.7, 25.0, 26.5, 35.8, 47.0, 53.4, 55.9, 57.8, 58.8, 126.7, 128.6, 129.0, 137.0, 171.9, 172.8, 174.4, 175.7. EIMS *m/z* (rel. int.): 414 $[M]^+$ (29), 371 (12), 314 (43), 302 (14), 295 (19), 229 (27), 120 (44), 70 (100), 58 (91).

4.3.12 Compound 11

_N_-Boc-L-norleucyl-2-aminoisobutyric acid methyl ester

L-Nle (131.2 mg, 1 mmol) in dioxane (2.0 ml) and H_2O (1.0 ml) was coupled with *N*-Boc azide to give *N*-Boc-L-nle (197 mg, 0.85 mmol, 85%). ^{1}H NMR (500 MHz, $CDCl_3$) nle δ 0.91 (3H, *t*, *J* = 7.1 Hz, H-6), 1.30-1.90 (6H, *m*, H-3-5), 1.45 (9H, *s*, $(CH_3)_3C$), 4.12 (1H, *m*, H-2), 4.29 (1H, *dd*, *J* = 7.6, 13.1 Hz, NH), 4.96 (1H, *d*, *J* = 7.6 Hz, NH). FABMS *m/z*: 254 $[M+Na]^+$, 232 $[M+H]^+$.

Aib methyl ester hydrochloride (13.7 mg, 0.091 mmol) was neutralized with triethylamine and coupled with *N*-Boc-L-nle (23.2 mg, 0.10 mmol) by use of EEDQ to give the title compound (21.1 mg, 0.064 mmol, 70%). ^{1}H NMR (500 MHz, $CDCl_3$): nle δ 0.89 (3H, *t*, *J* = 7.1, H-6), 1.30-1.80 (6H, *m*, H-3-5), 1.45 (9H, *s*, $(CH_3)_3C$), 4.00 (1H, *m*, H-2), 4.95 (1H, *m*, NH), aib δ 1.53 (3H, *s*, H-3 or 4), 1.55 (3H, *s*, H-3 or 4), 3.73 (3H, *s*, OCH_3), 6.59 (1H, *s*, NH). CIMS *m/z* (rel. int.): 331 $[M+H]^+$ (58), 275 (100).

4.3.13 *N*-Boc-L-norleucyl-2-aminoisobutyryl-L-phenylalanyl-D-proline methyl ester

The *N*-protected dipeptide methyl ester (48.5 mg, 0.15 mmol) was saponified with 1 M NaOH to give the acid (41.9 mg, 0.13 mmol, 87%). ^{1}H NMR (500 MHz, $CDCl_3$): nle δ 0.90 (3H, *t*, *J* = 6.7 Hz, H-6), 1.44 (9H, *s*, $(CH_3)_3C$), 1.30-1.85 (6H, *m*, H-3-5), 4.14 (1H, *m*, H-2), 5.18 (1H, *m*, NH). aib δ 1.57 (3H, *s*, H-3 or 4), 1.59 (3H, *s*, H-3 or 4), 6.99 (1H, *s*, NH). CIMS *m/z* (rel. int.): 317 $[M+H]^+$ (52), 261 (100).

L-Phe-D-Pro methyl ester trifluoroacetate (189.8 mg, 0.53 mmol) was neutralized with triethylamine and coupled with the *N*-Boc-L-nle (253 mg, 0.80 mmol) by use of HOBt and DCC to give the title compound (195.2 mg, 0.34 mmol, 64%). ^{1}H NMR (500 MHz, $CDCl_3$): nle δ 0.90 (3H, *t*, *J* = 6.9 Hz, H-6), 1.30-2.00 (6H, *m*, H-3-5), 1.45 (9H, *s*, $(CH_3)_3C$), 4.01 (1H, *m*, H-2), 4.78 (1H, *m*, NH), aib δ 1.50 (3H, *s*, H-3 or 4), 1.55 (3H, *s*, H-3 or 4), 5.54 (1H, br. *s*, NH), Phe δ 2.95 (1H, *dd*, *J* = 9.4, 12.9 Hz, H-3), 3.03 (1H, *dd*, *J* = 4.9, 12.9 Hz, H-3), 4.91 (1H, *ddd*, *J* = 4.9, 8.9, 9.4 Hz, H-2), 7.15-7.31 (6H, *m*, H-5-9), 7.46 (1H, br. *s*, NH), 4.95 (1H, *m*, NH), Pro δ 1.30-2.00 (4H, *m*, H-3 and 4), 2.72 (1H, *ddd*, *J* = 2.8, 6.9, 7.1 Hz, H-5), 3.54 (1H, *m*, H-5), 3.71 (3H, *s*, CH_3O), 4.30 (1H, *dd*, *J* = 3.7, 8.3 Hz, H-2). EIMS *m/z* (rel. int.): 574 $[M]^+$ (15), 346 (13), 299 (30), 271 (12), 261 (14), 259 (14), 243 (31), 215 (16), 185 (11), 131 (12), 130 (78), 128 (12), 120 (50), 86 (24), 70 (35), 58 (100), 57 (26), 56 (12).

4.3.14 Cyclo (L-norleucyl-2-aminoisobutyryl- L-phenylalanyl-D-prolyl) (11)

The *N*-protected tetrapeptide methyl ester (46.0 mg, 0.084 mmol) was saponified with 1 M NaOH to give the acid (40.7 mg, 0.073 mmol, 87%). ^{1}H NMR (500 MHz, $CDCl_3$): nle δ 0.88 (3H, *d*, *J* = 6.9 Hz, H-6), 1.24-2.00 (6H, *m*, H-3-5), 1.43 (9H, *s*, $(CH_3)_3C$), 4.09 (1H, *m*, H-2), 5.54 (1H, *m*, NH), aib δ 1.43 (3H, *s*, H-3 or 4), 1.50 (3H, *s*, H-3 or 4), 7.47 (1H, *m*, NH), Phe δ 2.90 (1H, *dd*, *J* = 6.0, 12.8 Hz, H-3), 2.98 (1H, *dd*, *J* = 9.4, 12.8 Hz, H-3), 4.78 (1H, *m*, H-2), 7.18-7.26 (5H, *m*, H-5-9), 5.54 (1H, *m*, NH), Pro δ 1.24-2.00 (4H, *m*, H-3 and 4), 2.67 (1H, *m*, H-5), 3.53 (1H, *m*, H-5), 4.19 (1H, *m*, H-2). EIMS *m/z* (rel. int.): 560 $[M]^+$ (8), 299 (17), 271 (16), 243 (21), 217 (14), 215 (14), 202 (20), 130 (23), 126 (13), 120 (52), 116 (37), 91 (11), 86 (33), 70 (41), 58 (100), 56 (11).

The *N*-protected tetrapeptide acid (7.0 mg, 0.013 mmol) was converted to the corresponding *N*-hydroxysuccinimide ester. The succinimide ester was *N*-deprotected by use of TFA and then cyclized in pyridine. The crude product was purified by silica gel column chromatography followed by HPLC (85% MeOH) to give the cyclic peptide **11** (1.3 mg, 0.0030 mmol, 23%, R_t 20.3 min) as a colorless powder. $[\alpha]^{25}_D$ –44° (*c* 0.10; EtOH). UV λ_{max}

nm (log ε): 237 (3.36), 213 (3.76). ^{1}H NMR (500 MHz, $CDCl_3$): nle δ 0.90 (3H, *t*, *J* = 7.1 Hz, H-6), 1.14-1.85 (6H, *m*, H-3-5), 4.18 (1H, *ddd*, *J* = 7.6, 7.8, 10.3 Hz, H-2), 7.10 (1H, *d*, *J* = 10.3 Hz, NH), aib δ 1.34 (3H, *s*, H-4), 1.77 (3H, *s*, H-3), 5.97 (1H, *s*, NH), Phe δ 2.95 (1H, *dd*, *J* = 5.7, 13.6 Hz, H-3), 3.26 (1H, *dd*, *J* = 10.1, 13.6 Hz, H-3), 5.16 (1H, *ddd*, *J* = 5.7, 10.1, 10.3 Hz, H-2), 7.19-7.29 (5H, *m*, H-5-9), 7.54 (1H, *d*, *J* = 10.3 Hz, NH), Pro δ 1.14-1.81 (2H, *m*, H-3 and 4), 2.12-2.35 (2H, *m*, H-3 and 4), 3.22 (1H, *ddd*, *J* = 7.3, 7.4, 10.3 Hz, H-5), 3.86 (1H, *ddd*, *J* = 4.4, 8.3, 9.9 Hz, H-5), 4.66 (*dd*, *J* = 2.1, 7.8 Hz, H-2). ^{13}C NMR (125 MHz, $CDCl_3$): 13.9, 22.4, 23.6, 24.7, 25.0, 26.5, 27.7, 28.6, 35.8, 47.0, 53.4, 54.4, 57.8, 58.8, 126.7, 128.6, 129.0, 137.0, 171.8, 172.8, 174.5, 175.7. EIMS *m/z* (rel. int.): 442 $[M]^+$ (34), 399 (10), 343 (12), 342 (51), 330 (15), 323 (25), 314 (12), 229 (35), 217 (10), 183 (12), 120 (44), 86 (19), 70 (100), 58 (80).

4.3.15 Biological assays

The retardant activity of compounds **1** and **4-11** was determined with the same procedures as described Chapter 3.3.1.

4.4 References

Pastuszak, J., Gardner, J.H., Singh, J., Rich, D.H., 1982. Improved synthesis of [4-alanine] chlamydocin: cyclization studies of tetrapeptides containing five α-substituents. J. Org. Chem. 47, 2982-2987.

Chapter 5
Mechanisms of retardation

5.1 Introduction

In the previous chapter, I showed that there are two types of retardant in chlamydocin analogues. One, for example in compound **1**, requires the oxygen atom at C-8 of aod moiety to show the retardant activity. The other, for example in compound **8**, requires no oxygen atom at the C-8 position but requires a specific length of alkyl group to show the activity. The shoots of rice seedlings treated with **8** were markedly curved, suggesting that the dwarfism induced by **8** was apparently different from that induced by **1**. To understand the mechanism of their action, I focused on the contents of endogenous GAs and endogenous ABA. GAs are known to stimulate shoot elongation in higher plants. GA_1 is the active GA in the rice plant, and promotes shoot elongation (Graebe, 1987). ABA is involved in many physiological responses in plant development and sometimes shows inhibitory activity, although the role of this hormone is not clearly defined (Zeevaart and Creelman, 1988).

Chlamydocin and HC-toxin (Fig. 5-1) have been reported to inhibit HDAC activity (Brosch et al., 1995). HDACs are enzymes that regulate the gene expression (Wolffe, 1996), and their inhibition causes cell differentiation and cell cycle arrest (Yoshida et al., 1995). It had been proposed that epoxyketone unit of aeo is essential for the HDAC inhibitory activity (Furumai, et al. 2001). However apicidins and their analogues have been reported to have the inhibitory activity without the epoxide of aeo (Singh et al., 2002). Therefore, I also tested the HDAC inhibitory activity of compounds **1** and **8**.

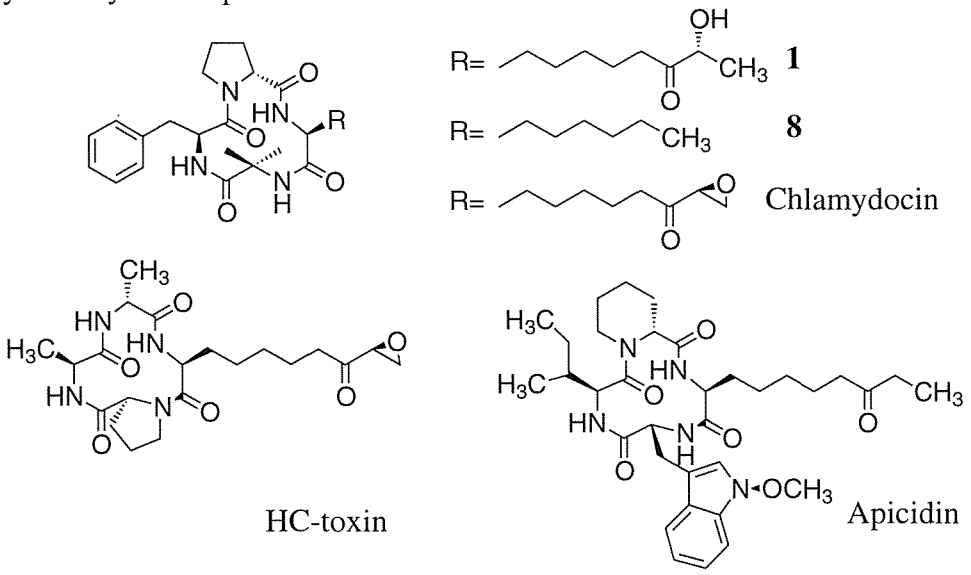


Fig. 5-1 Structures of compounds **1** and **8**, chlamydocin, HC-toxin and apicidin

I describe here the levels of endogenous ABA and GA_1 in the rice seedlings whose dwarfism was induced by **1** or **8**. In addition, HDAC inhibitory activity of compounds **4**–**8** is described in comparison with trichostatin A (Fig. 5-2), which is a potent inhibitor of HDACs.

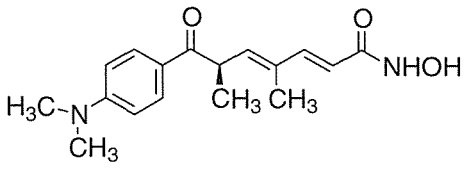


Fig. 5-2 Structure of trichostatin A

5.2 Results and discussion

To determine the mechanisms of the dwarfism induced by these two types of retardant, the changes of the endogenous GA_1 and ABA levels caused by compounds **1** and **8** were investigated. Ten nmol/plant of **1** or 30 nmol/plant of **8** was applied to the seedlings as in the bioassay to equalize the retardation effect. The aerial parts of the treated rice seedlings were homogenized in 80% acetone and filtrated. The filtrates were concentrated *in vacuo* and the d_6 ABA was added to the aqueous concentrate. This solution was subjected to a solvent fractionation to give the acidic ethyl acetate (AE) fraction, which was purified by HPLC after passing through a Sep-Pak C18 Cartridge. The ABA equivalent fractions from HPLC were combined and analyzed by LC–MS. On the other hand, the GA_1 equivalent fractions from HPLC were combined and analyzed by ELISA after methylation with CH_2N_2. GC–SIM analysis after methylation and trimethylsilylation of the HPLC fractions confirmed the occurrence of GA_1 in these fractions. The hormonal levels determined in the treated plants are shown in Table 5-2. In the rice plants treated with **1**, the ABA level was four times higher than that of the control, and the GA_1 level was 20% of that of the control. In the rice plant treated with **8**, the ABA level was almost the same as that of the control, and the GA_1 level was 5% of that of the control. These results indicated that the increase in the ABA level and the decrease

Table 5-2 The endogenous levels of ABA and GA_1 in the aerial parts of the rice seedlings treated with compound **1** (10 nmol/plant) or **8** (30 nmol/plant)

	ABA (ng/g)	GA_1 (pg/g)
Control	2.2	315.9
1	9.1	60.3
8	2.3	15.9

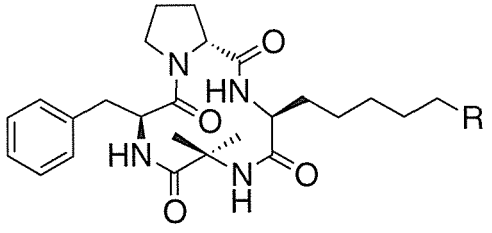
in the GA_1 level caused growth retardation in the case of **1**, and the dramatic reduction of the GA_1 level caused it in the case of **8**. The exogenously applied ABA reduced the height of the rice seedlings, and thus it is reasonable that the accumulation of endogenous ABA causes such effects (data not shown). Compounds **1** and **8** both reduced endogenous GA_1 levels in the treated rice seedlings. There is an important and interesting question, which step in GA biosynthesis they inhibit, still remains to be solved.

I also investigated the HDAC inhibitory activity of compounds **1** and **8** (Table 5-3). Compound **1** was as active as trichostatin A, which is a potent inhibitor of HDACs. On the other hand, compound **8** did not have the inhibitory activity at 10000 nM. In the case of **1**, an increase in ABA level and a decrease in GA_1 level retarded plant growth. It is likely that HDAC inhibition stimulate the ABA synthesis as an adaptation to stress and an increase in ABA level may produce plant growth retardation. I tested the plant growth retardant activity of trichostatin A, and found that it did not have the retardant activity at the dose of 30 nmol/plant. This revealed that HDAC inhibitory activity was not related to retardant activity of both compounds **1** and **8**, confirming that the alteration of endogenous hormonal levels was not related to HDAC inhibition.

Table 5-3 HDAC (nuclear extract from HeLa cell) inhibitory activity of compounds **1** and **4–8**

Compound	R	10000 nM	1000 nM	100 nM	10 nM
Trichostatin A		100	100	100	39
1	COCH(*R*-OH)CH_3	100	100	100	53
4	CH(*R*-OH)CH(*R*-OH)CH_3	40	17		
5	CH(*S*-OH)CH(*R*-OH)CH_3	100	100	75	0
6	CHO	70	32	0	0
7	CH_2OH	0	0	0	0
8	CH_3	0	0	0	0

Values are inhibition activity presented as percentage of the control.



Compounds **4**-**6** also inhibited the HDAC activity (Table 5-3). Compounds **4** and **5** were less active than **1**. This indicated that the 8-oxo in aho is important for the inhibition like the retardation. Compound **6** was 100 fold less active than **1**. This indicated that the 9-hydroxyl and/or alkyl group chain length of **1** was important in the inhibition not like in the retardation. Singh et al. reported that the 9-hydroxyl in aho slightly enhanced the inhibitory activity (2000). This indicated that the alkyl group chain length of **1** was more important than 9-hydroxyl for the inhibitory activity. Surprisingly, compound **5** was 100 hold more active than **4**, indicating that the stereochemistry of 8-hydroxyl is important. Singh et al. also reported the probability that the slight difference of the backbone conformation affect the inhibitory activity. Further studies on the alkyl group chain length and the backbone conformation may lead to the development of specific HDAC inhibitors.

5.3 Experimental

5.3.1 Extraction and purification of plant hormones

Compound **1** (10 nmol/plant) or **8** (30 nmol/plant) in aq. MeOH was applied to the rice seedlings as in the bioassay described chapter 3.3.1. As a control only aq. MeOH was applied. After incubation for 72 h under continuous light, the aerial parts of the rice seedlings were collected. The weights of the plant materials collected were 8.2 (treated with **1**), 8.4 (treated with **8**) and 10.2 g (the control). Each plant material was homogenized in 60 ml of 80% aq. acetone. The homogenate was filtered and the debris was further extracted twice with 80% aq. acetone. The extracts were combined and concentrated *in vacuo*. The aq. residue equivalent to 7.2 g of plant materials was diluted up to 100 ml by adding H_2O and d_6-ABA was added to it. The amounts of d_6-ABA added were 144 (treated with **1**), 100 (treated with **8**) and 93 ng (the control). The aq. residue was adjusted to pH 2.5 with 6 M HCl, and extracted with EtOAc (80 ml × 3). The EtOAc solution was extracted with saturated $NaHCO_3$ (50 ml × 3). The $NaHCO_3$ solution was adjusted to pH 2.5 with 6 M HCl, and extracted with EtOAc (180 ml × 3). The EtOAc solution was dried over Na_2SO_4 and evaporated *in vacuo*. The residue was dissolved in a small amount of 80% aq. MeOH, and loaded onto a Sep-Pak C18 Cartridge (Millipore). The eluate eluted from the cartridge with 7.5 ml of 80% aq. MeOH was evaporated. The residue was subjected to HPLC with a column of Capcell Pak C18 (Shiseido, 6.0 × 150 mm) and eluted with 0.5% AcOH in 10% aq. CH_3CN (solvent A) and 0.5% AcOH in 80% aq. CH_3CN (solvent B) at 30°C as follows: 0-10 min, elution with solvent A; 10-50 min, linear gradient of 0 to 100% solvent B. The flow rate of the solvent was 1.0 ml/min. Eluate was collected every

1 min as one fraction. The retention times of ABA and GA_1 were 31 and 19 min, respectively. The authentic GA_1 was a gift of Prof. Takeshi Sassa (Yamagata University, Yamagata, Japan) and (+)-ABA was purchased from Sigma. The d_6-ABA was prepared according to the procedure of Milborrow et al. (1971), and its purity and labeling pattern were checked with ^1H NMR and EIMS.

5.3.2 LC–MS analysis for ABA

Negative electrospray mass spectra were obtained using a Quattro micro mass spectrometer (Micromass, electrospray voltage: 3.5 kV; desolvation temp.: 400°C; sheath gas: N_2) coupled with a JASCO PU-980 Gulliver LC system equipped with a PR-18 GP column (Kanto Chemical, 250 × 2.0 mm, 5 μm). For LC, a gradient system was used that started from 0.25% AcOH in 10% aq. CH_3CN (solvent C) to 0.25% AcOH in 80% aq. CH_3CN (solvent D) at 40°C as follows: 0-10 min, elution with solvent C; 10-50 min, linear gradient of 0 to 100% solvent D. The flow rate was 0.2 ml/min (injection volume: 10 μl). The MRM was obtained during LC analysis under the following conditions: cone voltage, 30 V; collision energy, 13 eV; collision pressure, 1.7 mT; collision gas, Ar. The MRM transitions from *m/z* 269 to 159 of the internal d_6-ABA and from *m/z* 263 to 153 of the natural ABA were monitored, and these product ions of 159 and 153 were used for quantitation.

5.3.3 GC–MS analysis for methyl trimethylsilyl GA_1

A portion of GA_1 equivalent fraction from ODS-HPLC was subjected to GC/SIM analysis after methylation with CH_2N_2 and trimethylsilylation with *N*-methyl-*N*-trimethylsilyl trifluoroacetamide. A Trace MS (Thermo) equipped with a Trace GC 2000 (Thermo) and a capillary column SPB™-1 (Supelco, 30 m × 0.25 mm) was used. The ionization voltage was 70 eV. The GC conditions were as follows: injection temperature, 250°C; initial temperature, 60°C (1.5 min); liner gradient up to 200°C at 60°C/min, to 280°C at 5°C/min, and maintained at 280°C (10 min). In the GC/SIM analysis, characteristic ions, *m/z* 506 (M^+) and 448 were monitored.

5.3.4 ELISA

ELISA was performed according to the procedure of Yamaguchi et al. (1990).

5.3.5 HDAC inhibitory assays

HDAC inhibition activity assays were conducted by the use of HDAC Colorimetric Activity Assay/Drug Discovery Kit-AK-501 instruction (Funakoshi), and according to the instruction.

5.4 References

Brosch, G., Ransom, R., Lechner, T., Walton, J.D., Loidl, P., 1995. Inhibition of maize histone deacetylases by HC toxin, the host-selective toxin of *Cochliobolus carbonum*. Plant Cell 7, 1941-1950.

Furumai, R., Komatsu, Y., Nishino, N., Khochbin, S., Yoshida, M., Horiuchi, S., 2001. Potent histone deacetylase inhibitors built from trichostatin A and cyclic tetrapeptide antibiotics including trapoxin. Proc. Natl. Acad. Sci. USA 98, 87-92.

Graebe, J.E., 1987. Gibberellin biosynthesis and control. Ann. Rev. Plant Physiol. 38, 419-465.

Marks, P.A., Richon, V.M., Rifkind, R.A., 2000. Histone deacetylase inhibitors: Inducers of differentiation or apoptosis of transformed cells. J. Natl. Cancer Inst. 92, 1210-1216.

Milborrow, B.V., 1971. Abscisic acid. In: Goodwin T.W. (Ed), Aspects of Terpenoid Chemistry and Biochemistry. Academic Press, London, pp. 137-151.

Singh, S.B., Zink, D.L., Liesch, J.M., Mosley, R.T., Dombrowski, A.W., Bills, G.F., Darkin-Rattray, S.J., Schmatz, D.M., Goetz, M.A., 2002. Structure and chemistry of apicidins, a class of novel cyclic tetrapeptides without a terminal α-keto epoxide as inhibitors of histone deacetylase with potent antiprotozoal activities. J. Org. Chem. 67, 815-825.

Wolffe, A.P., 1996. Histone deacetylase: A regulator of transcription. Science 272, 371-372.

Yamaguchi, I., Nakazawa, R., Nakagawa, R., Suzuki, Y., Kurogochi, S., Murofushi, N., Takahashi, N., Weiler, E.W., 1990. Identification and semi-quantification of gibberellins from the pollen and anther of *Zea mays* by immunoassay and GC/MS. Plant Cell Physiol. 31, 1063-1069.

Yoshida, M., Horiuchi, S., Beppu, T., 1995. Trichostatin A and trapoxin: novel chemical probes for the role of histone acetylation in chromatin structure and function. BioEssays 17, 423-430.

Zeevaart, J.A.D. and Creelman, R.A., 1988. Metabolism and physiology of abscisic acid. Ann. Rev. Plant Physiol. Plant Mol. Biol. 39, 439-473.

Chapter 6
Conclusion

During a screening-based search for natural plant growth retardants, I found a fungus whose culture filtrate acted as a plant growth retardant. The fungus was identified as *Peniophora nuda* on the basis of electron microscopy observations and its 18S rRNA gene sequence. Three metabolites (**1**-**3**) with rice plant growth retardant activity were isolated from the culture filtrate. Compound **1** was definitively identified as being identical to the chlamydocin analogue reported by Gupta et al. (1994). Compounds **2** and **3** were also chlamydocin analogues, and were first reported as natural products. The bioassay data indicated that the hydroxyl at the C-9 of aho is not essential for the retardant activity, but the ketone function at the C-8 of aho is important. I attempted to confirm the importance of the ketone function at the C-8 of aho in **1** by preparing some derivatives and determining their activities. The results indicated that all of the compounds that have a chlamydocin framework need the oxygen atom at the C-8 position to exhibit their retardant activity. In the course of my research on the structure-activity relationship, I discovered a novel compound **8** that requires no oxygen atom at the C-8 position but requires a specific alkyl group chain length to show the activity. The shoots of rice seedlings treated with **8** were markedly curved, suggesting that the dwarfism induced by **8** was apparently different from that induced by **1**.

This phenomenon prompted me to clarify the mode of action of **1** and **8**. For this purpose I investigated the levels of endogenous ABA and GA_1 in rice seedlings whose dwarfism was induced by **1** or **8**. In the case of **1**, the results revealed that an increase in the ABA level and a decrease in the GA_1 level retarded growth. In the case of **8**, the dramatic reduction of the GA_1 level retarded growth. I also determined the HDAC inhibitory activity of these compounds, because chlamydocin is known to inhibit HDAC activity. The results showed that the alteration of endogenous hormonal levels was not caused by HDAC inhibition, although **1** and **4**-**6** inhibited HDAC activity. There is an important and interesting question, which step in GA biosynthesis both **1** and **8** inhibit, that still remains to be solved.

Acknowledgement

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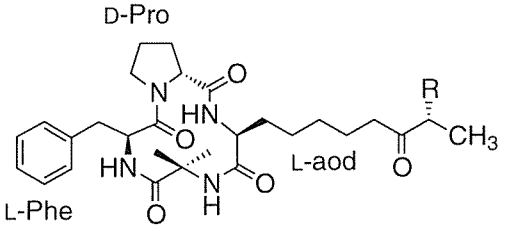
Summary

In rice cultivation, the use of plant growth retardant to control plant height is very important to reduce lodging. During a screening of more than 300 soil fungi collected from Tottori Prefecture, Japan, for natural plant growth retardant, I found a fungus, whose culture filtrate could reduce the height of rice seedlings without causing either blotch or wilting. I identified the fungus as *Peniophora nuda* on the basis of electron microscopy observations and its 18S rRNA gene sequence. And from the culture filtrate, three active metabolites (**1**–**3**) were isolated. The structures of compounds **1**–**3** were characterized by spectral data and chemical conversion. Compound **1** was definitively identified as being identical to the chlamydocin analogue reported by Gupta et al. (1994). Compounds **2** and **3** were also chlamydocin analogues and differ on the C9 of aod. They were the first isolation as natural products. I tested their plant growth retardant activities in a microdrop bioassay of rice (cv. Koshihikari) seedlings. Administration of 10 nmol/plant of **1** or **2** reduced the second leaf sheath length of the treated plants, respectively to 73 and 71% of the value for the water control. The same amount of **3** reduced growth of the second leaf sheath to 83% in the treated as compared to control plants. This suggested that the hydroxyl at the C-9 of aho is not essential for the retardant activity, but the ketone function at the C-8 of aho is important. Six derivatives from **1** and **2**, and two synthetic analogues were prepared to investigate the structure-activity relationship of **1** more. These data revealed two types of retardant in chlamydocin analogues. One, for example in compound **1**, requires the oxygen atom at C-8 of aod to exhibit the retardant activity. The other, for example in compound **8**, requires no oxygen atom at the C-8 position but requires a specific alkyl group chain length to exhibit the activity.

In order to understand the difference in the mode of action of these two types of retardant, the endogenous ABA and GA_1 in the rice seedlings treated with them were analyzed by LC–MS and ELISA, respectively. Treatment with **1** (10 nmol/plant) increased the ABA level to 4 times higher than that of the control and decreased the GA_1 level to 20% of that of the control. Treatment with **8** (30 nmol/plant) did not affect the ABA level but decreased GA_1 content to 5% of that of the control. These results indicated that the increase in the ABA level and the decrease in the GA_1 level caused growth retardation in the case of **1**, and the dramatic reduction of the GA_1 level caused it in the case of **8**. I also determined the HDAC inhibition by their retardants by using HDACs from HeLa cells, because chlamydocin is known to

inhibit HDAC. The inhibitory activity of **1** was almost same as trichostatin A at 10 nM. On the other hand, even a 10000 nM of **8** did not inhibit the HDAC. To understand the relationship between the plant growth retardation of **1** and HDAC inhibition, I tested the plant growth retardant activity of trichostatin A, and found that it did not have the retardant activity at the dose of 30 nmol/plant. These results showed that the inhibitory activity of HDAC was not linked to the plant growth retardant activity.



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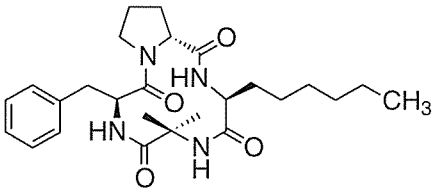
2 R=H
3 R=OAc



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摘要

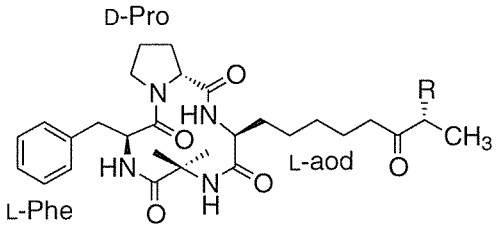
矮化剤はイネなどの倒伏軽減による増収を目的として用いられる。鳥取県内の土壌から分離した糸状菌約 300 種類の代謝産物中にイネ矮化物質を検索した。結果に基づいて 1 種類の糸状菌を選抜し、電子顕微鏡による観察と 18S rRNA 配列の相同性から、担子菌類の *Peniophora nuda* と同定した。*P. nuda* を培養し、その培養濾液より活性物質 **1–3** を得た。各種機器分析ならびに化学的誘導の結果、化合物 **1** は 1994 年に Gupta らが報告した chlamydocin の類縁化合物であることがわかった。化合物 **2** と **3** も chlamydocin の類縁化合物であり、2-アミノ-8-オキソデカン酸の C9 位の置換基だけが異なっていた。化合物 **2** と **3** は新規化合物であった。点滴法を用いたイネ生育試験において **1** と **2** は 10 nmol/plant の投与量で対照の 73%と 71%までイネを矮化させた。化合物 **3** は同じ投与量で対照の 83%までイネを矮化させた。化合物 **1** と **2** で活性が変わらないことから、2-アミノ-9-ヒドロキシ-8-オキソデカン酸の C9 位の水酸基は矮化活性に関与しないことがわかった。また C9 位の置換基が嵩高くなることで活性が弱まったことから、2-アミノ-9-ヒドロキシ-8-オキソデカン酸の C8 位のケトン基の重要性が示唆された。

この C8 位のケトン基の重要性を調べるため、いくつかの化合物を調製または合成し、イネ生育試験を行った。この結果、矮化には、2-アミノ-8-オキソデカン酸部分の C8 位の酸素原子もしくは特有の炭素鎖の長さが関わることがわかった。C8 位に酸素原子を持つ化合物として **1** を、特有の炭素鎖の長さを持つ化合物として **8** を用い、これらの作用機作を検討した。

化合物 **1** もしくは **8** をイネ幼鞘に与えた際の、地上部に含まれる ABA と GA_1 量を調べた。ABA については抽出時にd_6 ABA を加え、LC–MS により同定、定量した。GA_1 については GC–MS により同定し、ELISA で定量を行った。化合物 **1** を 10 nmol/plant ずつ処理したイネの ABA 含量は対照の 4 倍に増加していた。また GA_1 含量は対照の 20% に減少していた。これらのことから、化合物 **1** による矮化現象は ABA の増加と GA_1 の減少により引き起こされていることがわかった。一方、化合物 **8** を 30 nmol/plant ずつ処理したイネの ABA 含量は対照と同じであった。また GA_1 含量は対照の 5% に減少しており、化合物 **8** による矮化現象は GA_1 の著しい減少により引き起こされていることがわかった。

Chlamydocin は HDAC を阻害することが知られている。そこで、得られた矮化物質が HDAC を阻害するかどうかを調べた。HeLa 細胞由来の HDAC を用い、HDAC 阻害剤として知られる trichostatin A と阻害活性の強さを比較した。化合物 **1** は 10 nM

で trichostatin A と同程度の阻害活性を示した。一方、化合物 **8** は 10000 nM でも阻害活性を示さなかった。HDAC 阻害と **1** による矮化との関係を調べる為、trichostatin A の矮化活性を調べたところ、30 nmol/ plant の投与量でも矮化作用を示さなかった。従って HDAC 阻害は矮化の原因ではないことがわかった。



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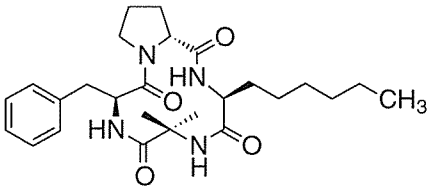

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List of Publications

1. Tani, H., Fujii, Y., Nakajima, H., 2001. Chlamydocin analogues from the soil fungus *Peniophora* sp.: structures and plant growth-retardant activity. Phytochemistry 58, 305-310 (Chapters 2 and 3).

2. Tani, H., Honma, T., Fujii, Y., Yoneyama, K., Nakajima, H., 2003. A plant growth retardant related to chlamydocin and its proposed mechanism of action. in press (Chapters 4 and 5).