VOL. 54 NO. 7, JULY 2001 THE JOURNAL OF ANTIBIOTICS pp. 573 — 581

Yokonolide A, a New Inhibitor of Auxin Signal Transduction, from *Streptomyces diastatochromogenes* B59

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(Received for publication February 19, 2001)

Bio-probes that inhibit the action of auxin are useful tools for the study of auxin signaling. To screen for specific inhibitors of auxin signaling, we used an Arabidopsis transgenic line harboring the auxin-inducible promoter derived from *PS-IAA4/5* and the reporter gene, GUS (β-glucuronidase). In this transgenic plant, the exogenous auxin specifically enhanced the expression of the GUS reporter gene. A novel 22-membered spiroketal-macrolide, yokonolide A (**1**), and related previously known compound, A82548A (**2**), were isolated from *Streptomyces diastatochromogenes* B59 as inhibitors of auxin inducible gene expression. The absolute structure of **1** was determined by detailed spectral analyses and chemical derivatization. **1** and **2** completely inhibited the auxin-induced transcription of the reporter gene at 5 and 1 μM, respectively. In contrast, **1** and **2** did not affect the translation of GUS reporter transcripts. In addition, **1** and **2** did not inhibit the gibberellin-induced α-amylase expression at 100 μM in barley aleurone cells. These results suggest that **1** and **2** specifically inhibit auxin signaling leading to auxin-mediated gene expression.

The plant hormone auxin regulates various aspects of plant growth and development by controlling cell division, cell elongation, and cell differentiation[1]. Auxin causes dramatic changes in the expression pattern of a number of genes[2]. Recent evidence from molecular and genetic studies has indicated that *AUX/IAA* genes, one of the auxin-regulated gene families, play essential roles in auxin signaling[3]. The expression of most *AUX/IAAs* are specifically enhanced by exogenously applied auxin within minutes, without requiring *de novo* protein synthesis, suggesting that *AUX/IAAs* are primary auxin-responsive genes[4,5]. *AUX/IAAs* encode short-lived nuclear proteins and possibly regulate the expression of late auxin genes as transcriptional factors[6,7]. However, the molecular mechanism of how auxin activates the expression of early auxin-regulated genes is not fully understood. In addition, the lack of specific inhibitors of auxin signaling has prevented the biochemical and pharmacological approaches to the study for the early steps of auxin perception responsible for gene activation. The search for specific inhibitors of auxin signal transduction from microbial metabolites may provide useful biochemical tools for understanding the mechanism of auxin signal transduction.

The transgenic Arabidopsis plant (BA3) harboring the early auxin-inducible promoter gene derived from *PS-IAA 4/5* and β-glucuronidase (GUS) reporter gene showed GUS activity in the root tip in response to exogenous auxin[8]. The induced GUS activity in the root tip is highly specific to auxin and can be detected easily by histochemical staining or fluorometry. This system is useful in monitoring early auxin gene expression and enables us to perform rapid and specific screening for bio-probes affecting the auxin

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signaling system.

In the screening for specific inhibitors of auxin signal transduction from microbial metabolites using the BA3 reporter system, *S. diastatochromogenes* B59 was found to produce an active new compound, yokonolide A (**1**), and an active known compound, A82548A (**2**). The isolation and structural determination of **2** has already been reported[9]. However, no biological activity of **2** has yet been reported. This report deals with the taxonomy, fermentation, isolation, structure determination and biological activities of **1** and **2**.

Material and Methods

Taxonomic Studies

The B59 strain was isolated from a soil sample collected in Okayama City, Yokoikami Town, Japan. The methods and media described by the International Streptomyces Project (ISP) were used to determine the cultural and physiological characteristics[10]. The morphological observations were made with a scanning electron microscope (JEOL JXA-8900). The isomer of diaminopimelic acid in the cell wall was determined by the method of BECKER *et al.*[11]. The utilization of carbon sources was determined by the method of PRIDHAM and GOTTILIEB[12].

Fermentation Studies

A well-grown agar slant of the B59 strain was inoculated into a 500-ml flask containing 100 ml of a culture medium consisting of soluble starch 1%, glucose 0.5%, soy bean meal 0.4%, polypeptone 0.1%, meat extract 0.1%, yeast extract 0.1% and corn steep liquor 0.1% (pH 7.2 before sterilization). The flask was shaken on a reciprocal shaker at 28°C for 48 hours, after which 200 ml of this culture was transferred to a 10-liter jar-fermenter containing 8 liters of the same medium described above. The fermentation was carried out at 28°C for 72 hours under agitation at 400 rpm with an aeration rate of 8 liters per minute.

Plant Growth Condition and Reporter Gene Assay

The transgenic Arabidopsis BA3 line carries the GUS gene regulated *via* the auxin-responsive domains A and B and the core promoter from *PS-IAA 4/5*[13]. The biological properties of this line have been reported previously[8]. The transgenic tobacco line containing the *PS-IAA 4/5* promoter (Δ-2309)-GUS fusion gene has been described previously[14]. The surface-sterilized BA3 seeds were placed on a germination medium (GM medium) containing 1.6% agar[15]. The plates were kept at 4°C for 2 days and then grown vertically at 25°C for 5 days under continuous light. For the histochemical staining, 4 to 5 seedlings were transferred into a 24-well microtiter plate containing 500 μl of the GM medium. The seedlings were incubated with a test sample for 10 minutes and then IAA (auxin, indole 3-acetic acid) was added to the medium. The induction of the GUS reporter gene was carried out for 6 hours at 25°C under continuous light. The seedlings were washed 3 times with a staining buffer (100 mM sodium phosphate, pH 7.0, 10 mM EDTA, 0.5 mM $K_4Fe[CN]_6$, 0.5 mM $K_3Fe[CN]_6$ and 0.1% triton X-100) and then incubated in a staining buffer containing 1 mM X-gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronide, the substrate for histochemical staining). For the quantitative fluorometric GUS assay, the roots excised from 5-day-old seedlings (20 individual) were incubated in the GM medium containing 20 μM IAA at 25°C. After induction, the roots were homogenized in an extraction buffer as described by JEFFERSON[16]. The GUS activity was determined with a fluorophotometer using 4-methyl umbelliferyl β-D-glucuronide as the substrate.

α-Amylase Enzyme Assays

The aleurone layers were prepared from deembryonated barley seeds (*Hordeum vulgare* L. cv. Kinuyutaka) that had been sterilized and imbibed in sterile water at room temperature for 48 hours. The isolated aleurone layers of the barley seeds were incubated in 5 mM $CaCl_2$ solution containing 0 or 5 μM GA (gibberellin A_3) at 25°C with reciprocal shaking (100 rpm). The incubation period was 24 hours for the barley aleurone layers.

The α-amylase activity was measured using the colorimetric RBB-starch method of RINDERKNECHT *et al.*[17]. The aleurone layers were homogenized in 50 mM acetate buffer (pH 5.4, 20 mM $CaCl_2$) after the incubation. The homogenates and the medium used for the incubation were combined and centrifuged. After centrifugation, the α-amylase activity of the supernatant was measured with 2% Starch Azure, RBB-starch (Sigma) as the substrate at 36°C. The amount of protein in the supernatant was determined by the method of BRADFORD[18] and BSA was used as the standard.

Reduction of **2** to **1**

2 (15 mg) was dissolved in MeOH, and then 2 equivalents of $NaBH_4$ dissolved in MeOH were added, and the solution was left for 1 hour at room temperature. The MeOH solution was added to water and extracted with EtOAc at pH 9.0. The organic layer was concentrated and purified with silica gel column chromatography to give a white powder of **1** (12 mg).

Table 1. Cultural characteristics of the B59 strain.

Yeast extract-malt extract agar (ISP-2)	G:	Good
	AM:	Moderate. Light gray.
	R:	Pale yellow
	S:	None
Oatmeal agar (ISP-3)	G:	Good
	AM:	Abundant. Light gray.
	R:	Pale yellow
	S:	None
Inorganic salts-starch agar (ISP-4)	G:	Good
	AM:	Abundant. Light gray.
	R:	Pale yellow
	S:	None
Glycerol-asparagine agar (ISP-5)	G:	Good
	AM:	Abundant. Light gray.
	R:	Pale yellow
	S:	None
Peptone-yeast extract iron agar (ISP-6)	G:	Good
	AM:	None
	R:	Pale brown
	S:	Dark brown
Tyrosine agar (ISP-7)	G:	Good
	AM:	Abundant.Gray.
	R:	Brown
	S:	Brown

G: growth, AM: aerial myceria and color, R: reverse side color, S: soluble pigments.

Results and Discussion

Taxonomic Studies

The substrate and aerial hyphae of the cultured strain were well branched and the spore chains consisted of 10 to 50 spores on each were flexuous. The spore was cylindrical and 0.5~0.6×1.0~1.2 μm in size with a smooth surface. Whorls, sclerotic granules, sporangia, and flagellated spores were not observed. The strain produced melanoid pigments in ISP-6 and 7 media, utilized all the carbon sources tested (L-arabinose, D-xylose, D-glucose, D-fructose, L-rhamnose, D-galactose, sucrose, inositol, raffinose and D-mannitol), hydrolyzed starch and liquefied gelatin. The coagulation and peptonization of milk were not observed. The cultural characteristics of strain B59 are shown in Table 1. The cell wall of this strain contained LL-diaminopimelic acid. These taxonomic data suggest that strain B59 belongs to the genus *Streptomyces* and is very similar to *S. diastatochromogenes* which was reported to be the producing strain of **2**[9]. Therefore, strain B59 was identified as *Streptomyces diastatochromogenes*.

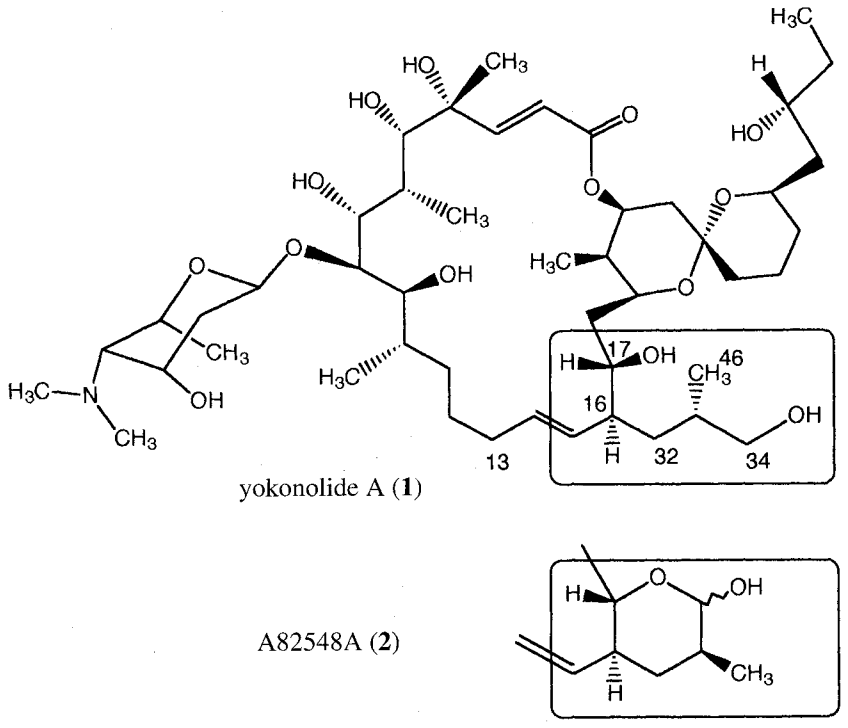
Fermentation

The typical time course of the B59 strain in a 500-ml flask is shown in Fig. 2. The growth of the strain increased until 3 days after cultivation. The inhibitory activity of the culture extract on the auxin-induced GUS expression reached a maximum at 3 days after cultivation (Fig. 2).

Isolation

The culture broth (14 liters) was centrifuged to separate the supernatants and cells. The supernatant was extracted with EtOAc at pH 10. The EtOAc layer was concentrated *in vacuo* to yield a brown reside (441 mg). This residue was applied onto a silica gel column (Silica gel 60, Merk, 230~400 mesh, column 3 i.d.×40 cm) with $CHCl_3$-MeOH as the eluent with increasing a content of MeOH content. The fractions from the eluent of $CHCl_3$: MeOH=9 : 1 showed two active peaks on auxin-induced GUS reporter assay.

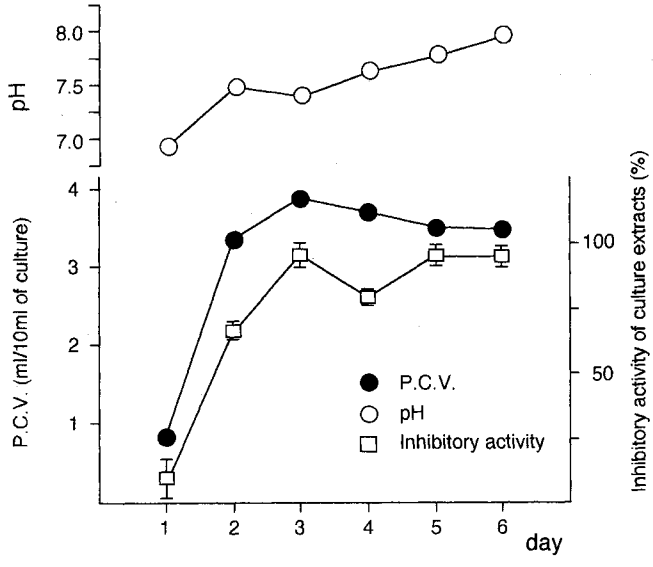
Fig. 1. Structure of yokonolide A (**1**) and A82548A (**2**).



Each active peak was evaporated to give a brown residue (135 mg) and a pale yellow residue (37.5 mg), respectively. The brown residue (135 mg) from the first active peak was further purified by ODS low-pressure column chromatography (ODS DM 2035T, Fuji silycia, MeOH : H_2O=95 : 5) to give a white powder, as an active compound (**2**: 28 mg; A82548A, a known spiroketal-macrolide). The pale yellow residue (37.5 mg) from the second active peak was further purified with a silica gel column (Silica gel 60, Merk, 230~400 mesh, benzene - MeOH=85 : 15) to give a white powder as a new active compound (**1**: 9.5 mg; designated as yokonolide A). During the isolation process, the inhibitory activity of each fractions on auxin-induced GUS expression in the BA3 line was monitored using histochemical staining.

The physico-chemical properties of **1** are as follow: (MP: 117°C; $[\alpha]_D$ (c=0.54, $CHCl_3$)=−45°; UV (ε_{max}, MeOH) End absorption; Molecular formula $C_{47}H_{83}NO_{14}$; FAB-MS (m/z) 887 $(M+H)^+$; HRFAB-MS (m/z) found 886.5912 $(M+H)^+$, calcd. For $C_{47}H_{84}NO_{14}$ 886.5891; IR (KBr) 3404, 2926, 1711, 1387, 1260 cm^{-1}.

Fig. 2. Time course of the fermentation of the B59 strain.



Structure Determination of **1** and **2**

The NMR spectra of **2** showed the characteristics of a

macrolide compound containing an amino sugar moiety. The molecular formula was determined as $C_{47}H_{81}NO_{14}$ by FAB-MS and NMR spectra. From these data, **2** was identified as the 22-membered spiroketal-macrolide, A-82548A. The spectral and physico-chemical data of **2**, including specific rotation, agreed with the reported data of A-82548A[9]. The structure of the active compound **1** was determined by detailed NMR spectra analyses and chemical modification of **2**. The NMR spectra of **1** were very similar to those of **2**. This suggests that **1** is a compound related to **2**. The molecular formula of **1** was determined as $C_{47}H_{83}NO_{14}$ by HRFAB-MS. The ^{1}H-NMR spectrum showed the presence of 4 olefinic proton signals and 8 methyl signals, including 2 nitrogen-attached methyl signals. The ^{13}C-NMR and DEPT spectra indicated 47 carbon signals including 23 methine, 12 methylene, 9 methyl, 2 quaternary and an ester carbonyl group. The molecular formula of **1** is two protons larger than that of **2**, and a hydroxymethylene signal was observed in the NMR spectra of **1**. In addition, NMR spectra of **1** did not show the hemiacetal signal attributed to C-34 observed in **2**. This evidence suggests that **1** has hydroxymethine at C-17 and hydroxymethylene at C-34, instead of ether methine at C-17 and hemiacetal methine at C-34 as in **2**. This was further confirmed by the detailed 2D NMR analyses of the HMQC, HMBC, ^{1}H-^{1}H COSY and TOCSY spectra. The long range ^{13}C-^{1}H couplings from H-46 to C-32, C-33 and C-34 were observed in the HMBC spectra. The connections from H-13 to H-17 and from H-16 to H-34 were established by ^{1}H-^{1}H COSY and TOCSY experiments. The correlations between the signals found in the HMBC and TOCSY spectra of **1** are shown in Fig 3. The assignment of NMR signals was conducted on the basis of 2D NMR spectra and is indicated in Table 2.

The absolute stereochemistry of **2** has been determined by X-ray crystallography and chemical degradation[9]. Therefore, **2** was reduced with $NaBH_4$ to determine the stereostructure of **1**. The spectral and physico-chemical data of reduced product of **2** were completely identical to **1**. Thus, the absolute stereostructure of **1** was determined as shown in Fig. 1.

Biological Activities of **1** and **2**

In the transgenic Arabidopsis BA3 line, the IAA (auxin, indole-3-acetic acid)-induced GUS activity in 5-day-old seedlings was seen in the elongation zone of the root tips. In the fluorescent GUS reporter assay, **1** and **2** completely inhibited 20 μM IAA-induced GUS expression in the root tips at 5 and 1 μM, respectively (Fig. 4). The inhibitory

Table 2. The assignment of NMR signals of **1**.

Position	^{13}C	^{1}H
1	165.4	
2	119.5	6.20
3	149.5	6.86
4	74.6	
5	79.4	3.83
6	35.7	1.64
7	76.6	3.97
8	84.0	3.60
9	74.6	2.86
10	37.5	1.47
11	31.3	1.67/0.91
12	27.7	1.56/1.26
13	33.0	2.38/1.97
14	132.2	5.19
15	132.6	5.10
16	49.4	1.96
17	70.8	3.58
18	40.3	1.93/1.00
19	64.5	3.99
20	35.1	2.07
21	70.3	5.32
22	35.5	1.81/1.73
23	97.2	
24	35.2	1.88/1.45
25	19.2	1.88/1.59
26	31.4	1.52/1.24
27	65.5	3.81
28	43.2	1.57/1.38
29	68.6	3.92
30	30.5	1.48
31	9.7	0.95
32	35.5	1.93/1.02
33	32.8	1.66
34	66.1	3.55/3.45
35	96.1	4.96
36	36.3	2.12/1.75
37	64.5	4.24
38	64.8	2.34
39	71.2	4.41
40	15.6	1.49
41/42	43.7	2.43
43	28.4	1.36
44	5.2	0.88
45	15.9	0.83
46	18.7	0.93
47	6.6	0.74

NMR spectra (500 MHz for ^{1}H, 125 MHz for ^{13}C) were recorded in $CDCl_3$. TMS was used for internal standard.

Fig. 3. HMBC and ^{1}H-^{1}H correlations of yokonolide A (**1**)

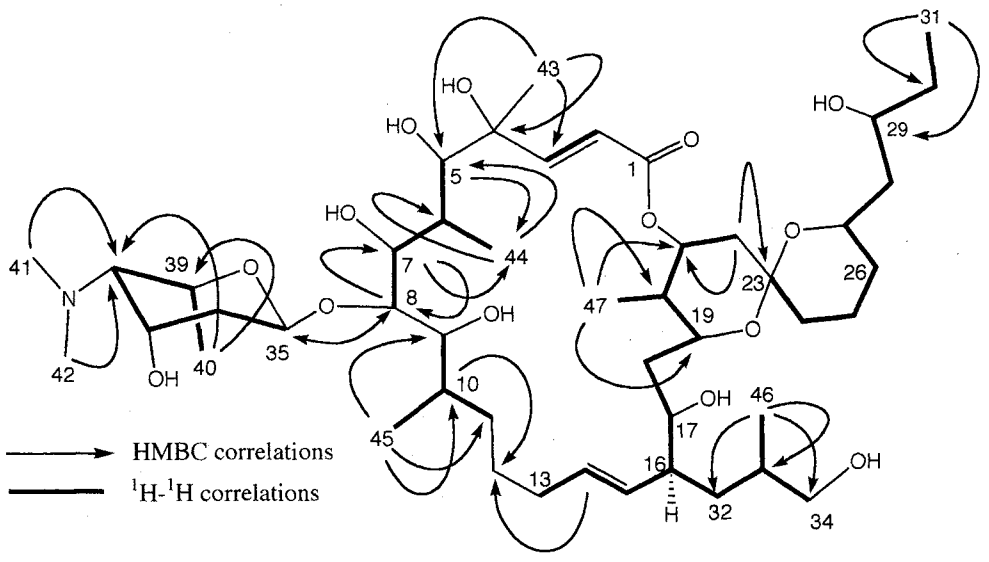
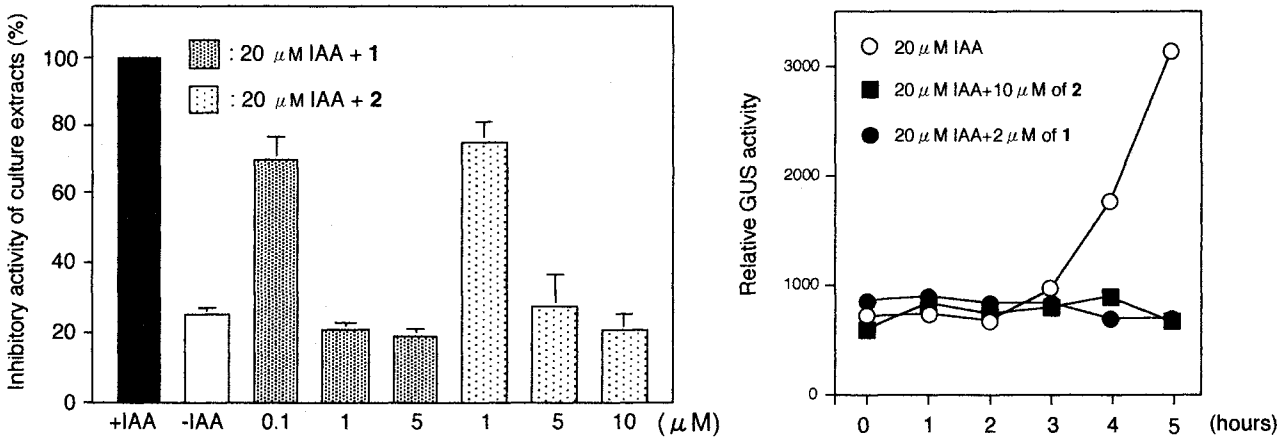


Fig. 4. The inhibitory activity of **1** and **2** on the expression of IAA-induced GUS reporter activity in the transgenic Arabidopsis BA3 line.



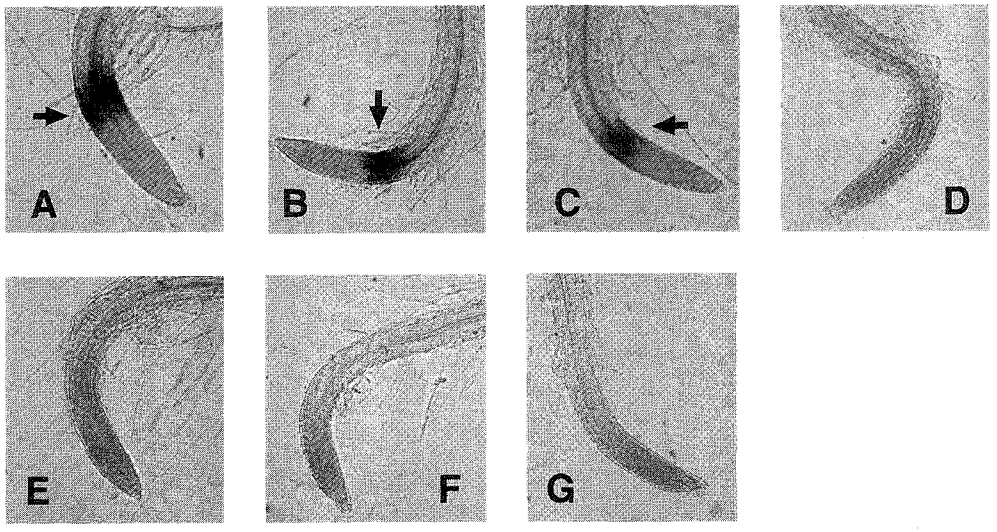
BA3 seedlings treated with IAA for 6 hours.

Time course of GUS induction with IAA.

activity of **1** and **2** was also observed by histochemical staining of GUS activity in the root tips (Fig. 5-A, E, F and G). In the case of the transgenic tobacco harboring *PS-IAA 4/5* promoter-GUS fusion gene (*Δ*-2309), **1** and **2** also inhibited the IAA-induced GUS expression in the transgenic tobacco at the same concentration as in the BA3 line (data not shown).

To elucidate whether **1** and **2** have an inhibitory effect on the translation of GUS mRNA, the following experimental system was designed; the BA3 seedlings were pre-treated with IAA for 2 hours and then extensively washed with an IAA-free medium. After washing, the seedlings were incubated in an IAA-free medium with or without **1**, **2** or cycloheximide (protein synthesis inhibitor) for an additional 3 hours. The histochemical staining of the seedlings was carried out in the presence of cycloheximide. It is known that the transcription of *AUX/IAA* mRNA does not require *de novo* protein synthesis and starts within minutes

Fig. 5. The photographs of the root tip of the BA3 line stained with X-gluc.



The roots of the BA3 line were stained with staining buffer containing 1mM X-gluc and 50μM cycloheximide after the incubation. The arrows indicate the location of GUS activity.

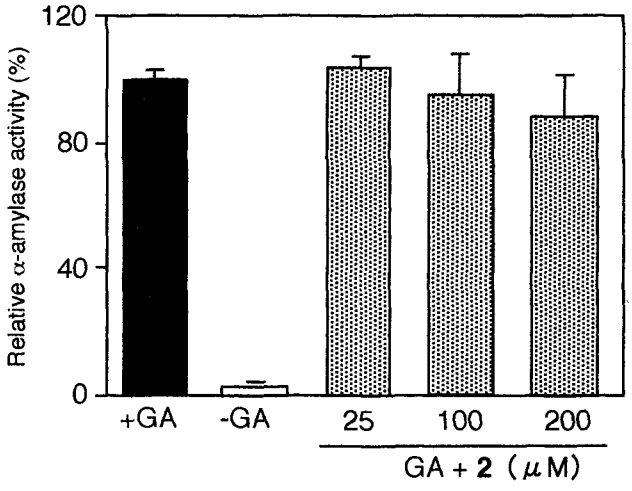
A: Incubated without IAA for 3 hours after a 2-hour-induction with 10 μM IAA.
B: Incubated with 5 μM of **2** for 3 hours aftera 2-hour-induction with 10 μM IAA.
C: Incubated with 10 μM of **1** for 3 hours a 2-hour-induction with 10 μM IAA.
D: Incubated with 100 μM of cycloheximide for 3 hours after a 2-hour-induction with 10 μM IAA.
E: Incubated with 10 μM IAA for 2 hours.
F: Incubated with 5 μM of **2** in the presence of 10 μM IAA for 5 hours.
G: Incubated with 10 μM of **1** in the presence of 10 μM IAA for 5 hours.

after the application of IAA[5]. In contrast, the detection of IAA-induced GUS activity in the BA3 line requires at least a 3-hour incubation in the presence of IAA (Fig. 4). This suggests that there is a time lag between the accumulation and translation of the GUS mRNA. Therefore, the GUS activity resulting from the translation of the GUS mRNA accumulated for 2 hours by the IAA incubation could be detected if **1** and **2** do not inhibit the translation of the GUS mRNA, but could not be detected if **1** and **2** do inhibit it.

Both **1** and **2** did not inhibit the expression of GUS activity at 10 and 5 μM, respectively, when the BA3 line seedlings were pre-treated with IAA for 2 hours, while both **1** and **2** inhibited the expression of GUS activity at same concentrations when the seedlings were continuously treated with **1** or **2** together with IAA for 5 hours. In contrast, cycloheximide (protein synthesis inhibitor) inhibited the expression of GUS activity, even when the seedlings were pre-treated with IAA for 2 hours. The photographs of histochemical staining in the root tip in the BA3 line are shown in Fig. 5. From these results, the inhibition of IAA-induced GUS expression by **1** and **2** are not a result of non-specific inhibition of the translation or later processes.

To determine whether **1** and **2** inhibit other plant hormone signaling, the inhibitory effect of **1** and **2** on gibberellin-induced α-amylase expression was examined in the deembryonated barley aleurone layers. The cereal aleurone cells in the cereal seeds produce α-amylase for germination in response to the plant hormone, gibberellin. This regulation system in cereal aleurone cells has been investigated for studying the molecular mechanisms of gibberellin signaling[19]. The results showed that **1** and **2** have no inhibitory activity on the α-amylase expression induced by gibberellin at 200 μM (Fig. 6, data not shown for **1**), indicating that the inhibitory effects of **1** and **2** on GUS expression are specific to auxin signaling. The antimicrobial activity of **2** was tested against Gram-positive and negative bacteria and yeast fungi using agar dilution methods. **2** did not show any antimicrobial activity at the concentration of 50 μg/ml. The antimicrobial activity of **1**

Fig. 6. The effect of **2** on gibberellin-induced α-amylase expression in the barley aleurone layers.



The isolated barley aleurone layers were incubated for 24 hours with or without 5 μM gibberellin A3.

was not determined because of the limited sample amount. The data described above indicate that **1** and **2** specifically inhibit one of the steps involved in auxin signaling linked to auxin-inducible gene expression.

In the course of the screening of inhibitors on auxin signal transduction, **1** and **2** were isolated as active compounds. These inhibitors were the 22-membered spiroketal-macrolides containing an amino sugar moiety. Several spiroketal-macrolides exhibiting various biological activities have been isolated.[20~24] However, the biological activities on plant hormone signaling have not yet been presented. The detailed biological activities of **1** and **2** on auxin action will be published elsewhere.

Acknowledgement

We are very grateful to Dr. A. THEOLOGIS (Plant Gene Expression Center) for providing the transgenic tobacco seeds.

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