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Toyocamycin specifically inhibits auxin signaling mediated by SCF^{TIR1} pathway

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ABSTRACT

The auxins, plant hormones, play a crucial role in many aspects of plant development by regulating cell division, elongation and differentiation. Toyocamycin, a nucleoside-type antibiotic, was identified as auxin signaling inhibitor in a screen of microbial extracts for inhibition of the auxin-inducible reporter gene assay. Toyocamycin specifically inhibited auxin-responsive gene expression, but did not affect other hormone-inducible gene expression. Toyocamycin also blocked auxin-enhanced degradation of the Aux/IAA repressor modulated by the SCF(TIR1) ubiquitin–proteasome pathway without inhibiting proteolytic activity of proteasome. Furthermore, toyocamycin inhibited auxin-induced lateral root formation and epinastic growth of cotyledon in the *Arabidopsis thaliana* plant. This evidence suggested that toyocamycin would act on the ubiquitination process regulated by SCF(TIR1) machineries. To address the structural requirements for the specific activity of toyocamycin on auxin signaling, the structure-activity relationships of nine toyocamycin-related compounds, including sangivamycin and tubercidin, were investigated.

1. Introduction

Plant hormones are small molecules known as auxin, cytokinin, gibberellin, ethylene, abscisic acid, brassinolide, and jasmonate, which regulate plant growth and development. Indole 3-acetic acid (IAA, Fig. 1), natural auxin, plays a crucial role in many aspects of plant development, including cell division, elongation and differentiation. At the whole plant level, auxin regulates tropisms, apical dominance and root development, and ultimately controls the architecture of adult plants (Woodward and Bartel, 2005).

Forward and reverse genetic approaches using a model plant *Arabidopsis thaliana* have identified fundamental auxin signaling components, and demonstrated the molecular mechanism of auxin perception and subsequent events (Kepinski and Leyser, 2002; Woodward and Bartel, 2005). In auxin signaling (Fig. 8), ubiquitin–proteasome dependent proteolysis of the Aux/IAA repressors plays a central role in primary auxin-responsive gene expression and subsequent plant developmental processes. Aux/IAAs are nuclear localized transcriptional repressors whose expressions are regulated by auxin. Aux/IAAs heterodimerize with the auxin-responsive factor (ARF) family of transcription regulators, and block the expression of primary auxin-responsive genes by repressing the ARF transactivator on auxin-responsive promoters (Hagen and Guilfoyle, 2002; Liscum and Reed, 2002). As the initial step in auxin signaling, auxin is perceived by TIR1, an F-box protein auxin receptor that interacts with the Skp1 (ASK1) and Cullin (CUL1) proteins to form E3 ubiquitin ligase complexes called SCFs (Kepinski and Leyser, 2004, 2005; Dharmasiri et al., 2005a). If auxin is present, E3 SCF^{TIR1} ubiquitin ligase catalyzes the ubiquitination of Aux/IAAs, indicating that auxin regulates the degradation of Aux/IAA repressors via the SCF^{TIR1}–proteasome pathway. Auxin acts as molecular glue between TIR1 and Aux/IAA to enhance the interaction between these two proteins, leading to the ubiquitination and subsequent degradation of Aux/IAA (Dharmasiri et al., 2005a; Kepinski, 2007).

The physiological and developmental role of auxin is extensively investigated using the *Arabidopsis* plant. However, the physiological role of auxin signaling through the SCF^{TIR1} pathway is not yet fully understood (Kepinski, 2007). In *Arabidopsis*, TIR1 is one of six F-box auxin receptors (TIR1 and AFBs 1–5) and the $SCF^{TIR1/AFB}$ pathway is essential for embryo development (Dharmasiri et al., 2005b). Therefore, a reverse genetic approach to investigating the physiological function of the $SCF^{TIR1/AFB}$ pathway can be hindered by the redundant function of the TIR1/AFB family and the embryo lethality in multiple knock-out mutants of the TIR1/AFB family. Additionally, some evidence suggests the presence of alternative auxin signaling in the pathway (Kepinski, 2007).

The physiological roles of auxin in diverse plant species are also still unclear. Land plants (liverwort, moss, fern, and gymnosperm and angiosperm plants) have diverse morphology and show distinct life cycles in various environments. Auxin regulates the body plan and life cycle of land plants in response to environmental cues (Woodward and Bartel, 2005). However, it is hard to assess the

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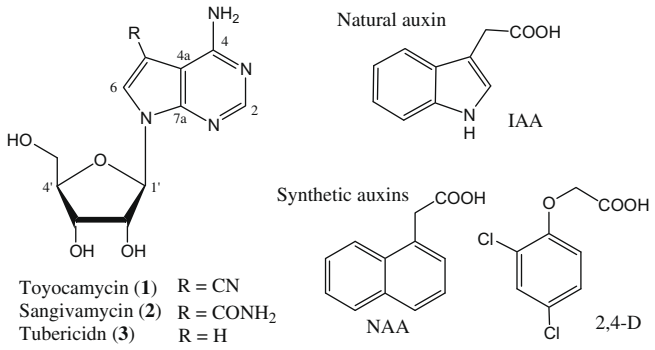

Fig. 1. Structures of toyocamycin (**1**), sangivamycin (**2**), tubercidin (**3**), and auxins (indole 3-acetic acid: IAA, naphthalene 1-acetic acid: NAA and 2,4-dichlorophenoxyacetic acid: 2,4-D).

physiological role of auxin in such diverse land plants using a conventional molecular genetics approach. This is because biological resources, such as genetic markers, genome information, in vitro culture, and established molecular genetic methods are available only in a model plant. To complement the molecular genetic approach to auxin biology, we have investigated specific inhibitor of auxin signaling. Chemical inhibitors can potentially overcome the functional redundancy of cognate target proteins and accomplish the regulation of orthologous protein function in multiple species (Blackwell and Zhao, 2003). Previously, two novel auxin signaling inhibitors, yokonolide B (Hayashi et al., 2003) and terfestain A (Yamazoe et al., 2005), have been reported by our group from a forward screen of microbial extracts using a transgenic auxin-inducible reporter line. Structure-based probe design approach and chemical library screening have identified some synthetic auxin signaling probes (Armstrong et al., 2004; Dai et al., 2005; Sungur et al., 2007; Christian et al., 2008; Hayashi et al., 2008). As a result of our ongoing efforts to identify inhibitors specific for auxin signaling, we have identified the known nucleoside-type antibiotic toyocamycin (Nishimura et al., 1956) as potent inhibitor of auxin signaling.

Herein, we describe the biological activity, mode of action and the structure-activity relationships of toyocamycin on auxin signaling.

2. Results and discussion

*2.1. Identification of toyocamycin **(1)** as an auxin signaling inhibitor using the auxin-responsive reporter assay*

To screen for specific inhibitors of auxin signaling, we used the reporter gene assay with two *Arabidopsis* transgenic *DR5::GUS* and *BA3::GUS* reporter lines (Ulmasov et al., 1997; Oono et al., 1998). Both reporter lines harbor the β-glucuronidase (GUS) reporter gene under the control of a primary auxin-inducible promoter. These transgenic reporter lines express the GUS protein in response to auxin via the SCF^{TIR1} pathway. The GUS induction is very rapid, and highly specific to auxin. The induced GUS enzyme protein in roots was monitored by the visible blue stain resulting from the chromogenic substrate X-Gluc, or by fluorometically quantitative measurements using a fluorogenic substrate (Fig. 2A and B). This reporter system enabled us to perform rapid and specific screening for auxin signaling inhibitors. The transgenic *Arabidopsis pER8::GFP* line (Zuo et al., 2000) was used as a control line to remove false positives, such as unspecific inhibition of transcription and translation processes. This transgenic line constitutively expresses the human estrogen receptor, and the expression of the GFP reporter gene was tightly regulated by estrogen under the control of the estrogen-inducible XVE transactivation system (Zuo et al., 2000).

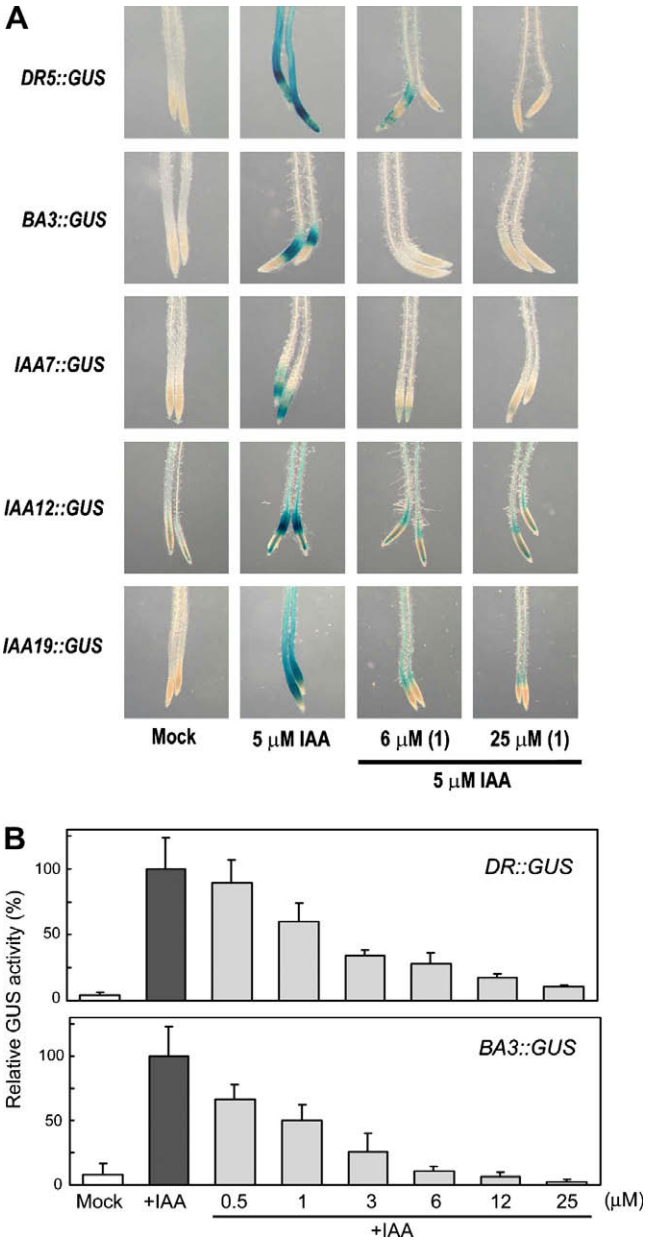

Fig. 2. Effects of toyocamycin (**1**) on primary auxin-responsive reporter gene expression. (A) Effect of toyocamycin (**1**) on the activities of auxin-responsive synthetic promoters (*BA3* and *DR5*) and native promoters (*IAA7*, *IAA12* and *IAA19*). Transgenic *Arabidopsis* seedlings (5-days-old) were treated with 5 μM IAA for 7 h (*BA3::GUS* and *DR5::GUS*) or for 12 h (*IAA7::GUS*, *IAA12::GUS*, and *IAA19::GUS*). After induction, IAA-induced GUS expression was visualized by histochemical staining using X-Gluc. The photographs of the root tips were taken on two representatives. (B) Quantitative measurement of the inhibitory activity of **1**. 5-day-old *Arabidopsis BA3::GUS* and *DR5::GUS* transgenic reporter lines were incubated with 5 μM IAA and various concentrations of **1** for 7 h. IAA-induced GUS expression was determined fluorometrically by the fluorogenic substrate, 4-methyl umbelliferyl β-D-glucuronide. The GUS activity induced by IAA is adjusted to 100% value. Values are the mean +/−SE of three independent experiments.

From the primary screening of 2,500 culture extracts of *Actinomycete* isolated from soil, we found that several active strains selectively inhibited auxin-responsive *DR5* and *BA3::GUS* expression. *Streptomyces* sp. strain G8 showed the most potent inhibitory activity among active strains and was used for further study. The active substance (18 mg) was isolated from the culture broth

(20 L) of strain G8 by activity-guided purification. The active compound was identified as toyocamycin (**1**) by spectroscopic analyses and FAB-MS measurement. Toyocamycin **(1)** was originally isolated as an anti-candida antibiotic from *Streptomyces toyocansis* (Nishimura et al., 1956), and was later found to show cytotoxic activity and antiviral activity (Gupta et al., 1989a). Our finding is the first report on the biological activity of toyocamycin **(1)** on plant development.

2.2. Toyocamycin *(1)* specifically inhibits primary auxin-responsive gene expression

Auxins (Fig. 1) rapidly induce the primary auxin-responsive gene expression via the SCF^{TIR1} pathway. Toyocamycin **(1)** completely blocked 5 μM IAA-induced GUS expression driven by synthetic *BA3* and *DR5* promoters at 25 μM (Fig. 2A). The IC_{50} values were 1.5 μM and 2 μM for *BA3::GUS* and *DR5::GUS* expression, respectively (Fig. 2B). We further investigated the effects on the expression of the native primary auxin-responsive gene using *Arabidopsis IAA7::GUS*, *IAA12::GUS* and *IAA19::GUS* transgenic reporter lines (Tian et al., 2002; Tatematsu et al., 2004; Weijers et al., 2005). *IAA7*, *12* and *19* encode the Aux/IAA family proteins, the expression of which are specifically induced by auxin through the SCF^{TIR1} pathway. Toyocamycin **(1)** also inhibited these three native auxin-responsive promoter activities to the same extent as *DR5* and *BA3* promoters (Fig. 2A). Higher IAA (10–50 μM) could not reverse the inhibition by **1** (5–20 μM), suggesting that **1** would not compete with auxin in the binding site of the TIR1 auxin receptor.

To address the specific inhibition of **1** on auxin signaling, *Arabidopsis* estrogen-inducible *pER8::GFP*, cytokinin-inducible *ARR5::GUS* and tobacco stress-inducible *parB::GUS* transgenic lines were used for the assay. The *pER8::GFP* line has a XVE transactivation system derived from mammal and bacterial transactivation machineries and its activation is tightly regulated by estrogen. The *ARR5::GUS* line expresses GUS in response to cytokinin, a plant hormone perceived via a two-component response regulator system involving protein phosphorylation (D'Agostino et al., 2000). The tobacco *parB* gene, which encodes glutathione S-transferase, was initially identified as an auxin-inducible gene (Takahashi and Nagata, 1992; Takahashi et al., 1995) and also found to be a stress-responsive gene (Ezaki et al., 2000). 2,4-dichlorophenoxyacetic acid (2,4-D) is a potent synthetic auxin and has been used as an auxinic herbicide (Fig. 1). 2,4-D not only activates primary auxin-responsive gene expression, but also strongly induces *parB* gene expression by activating 2,4-D-driven oxidative stress signaling, distinct from the SCF^{TIR1} pathway.

Toyocamycin **(1)** did not inhibit estrogen-induced *pER8::GFP* or cytokinin-induced *ARR5::GUS* expression at 100 μM, and showed no effects on 2,4-D-induced *parB::GUS* expression at 100 μM (Fig. 3). These results clearly demonstrate that **1** is not an inhibitor of general transcription and translation processes, but specifically blocks auxin signaling via the SCF^{TIR1}–proteasome pathway.

2.3. Toyocamycin (*1*) blocks the auxin-dependent degradation of the Aux/IAA repressor via SCF^{TIR1}

Auxin enhances the degradation of Aux/IAA repressor proteins via the SCF^{TIR1}–proteasome pathway and thereby induces primary auxin-responsive gene expression. To investigate the site of action of **1** on auxin signaling, we examined its effect on Aux/IAA protein stability using the *Arabidopsis HS::AXR3NT-GUS* line, in which a translational fusion protein consisting of AXR3 (Aux/IAA) and GUS protein is expressed under the control of a heat shock promoter (Gray et al., 2001). In this line, the AXR3NT-GUS fusion protein is rapidly degraded in the presence of auxin via the SCF^{TIR1}–proteasome pathway. The presence of the fusion protein can be monitored by histochemical staining of the GUS activity. After heat induction of the *HS::AXR3NT-GUS* line, the seedlings were treated with **1** or MG132, a potent proteasome inhibitor, in the presence or absence of 1 μM IAA. After 30 min incubation without IAA, the AXR3NT-GUS fusion protein was observed (Fig. 4A). In contrast, 1 μM IAA accelerated the break-down of fusion proteins, as previously described (Fig. 4A). Toyocamycin **(1)** reduced IAA-enhanced degradation of the fusion protein at 20 μM, and treatment (50 μM) caused the accumulation of the fusion protein to the same extent as treatment with 50 μM MG132 (Fig. 4A).

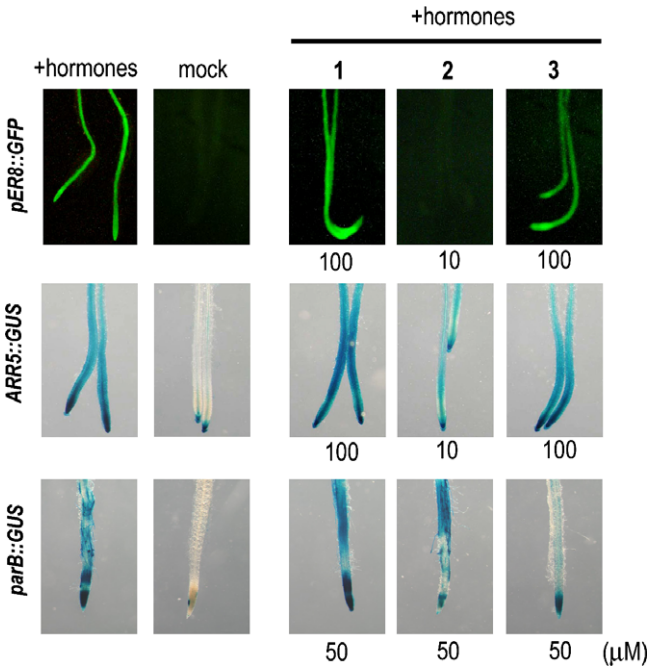

Fig. 3. Effects on toyocamycin (**1**), sangivamycin (**2**) and tubericidin (**3**) on hormones and stress-responsive reporter gene expression. Transgenic *Arabidopsis pER8::GFP* and *ARR5::GUS* seedlings (6-days-old) were treated with 20 μM β-estradiol and 10 μM benzyladenine for 24 and 7 h, respectively, together with the test compound. Seven-day-old transgenic tobacco *parB::GUS* seedlings were incubated with 5 μM 2,4-D and the test compound. After induction, GFP expression in *pER8::GFP* was directly observed with a fluorescent microscopy and GUS expression in *ARR5::GUS* and *parB::GUS* were visualized by histochemical staining. The photographs of the root tips were taken on two representative samples.

In order to exclude the possibility that toyocamycin **(1)** is a general proteasome inhibitor similar to MG132, we examined the effect of **1** on ATP-dependent proteasome activity in *Arabidopsis* roots (Fig. 4B). The proteolytic activity of the 20 S core unit of the 26 S proteasome in root homogenates was directly measured with a fluorogenic peptide substrate (Fujinami et al., 1994). As shown in Fig. 4B, while MG132 completely repressed the proteolytic activity in the 26 S proteasome, **1** had no effect. These results suggest that toyocamycin **(1)** specifically affects the SCF machinery by modulating the degradation of Aux/IAA proteins.

2.4. Toyocamycin *(1)* blocks physiological auxin responses in *Arabidopsis*

Auxin regulates cell elongation and division. The lateral root promotion and epinastic curvature of cotyledons are typical auxin responses resulting from cell division and elongation (Woodward and Bartel, 2005). To assess the effects of **1** on the physiological auxin response, the effects of **1** on the auxin-induced lateral root formation and epinastic curvature of cotyledons was examined. Three-day-old *Arabidopsis* seedlings were cultured for 3 days in

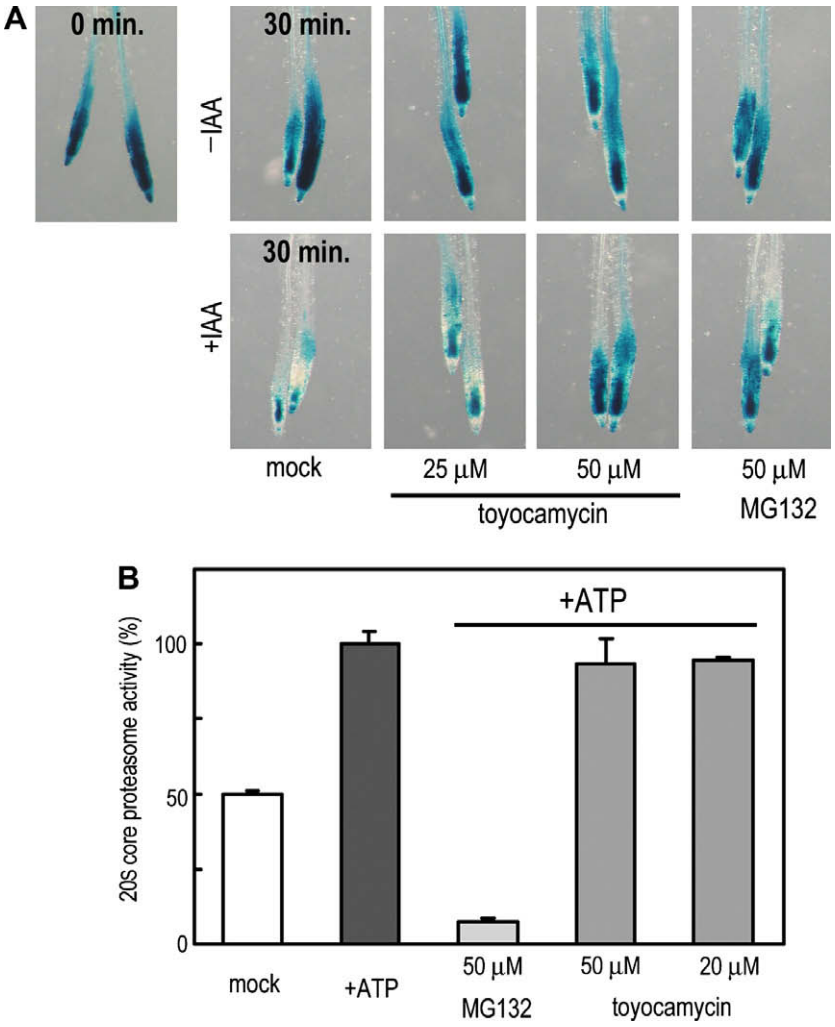

Fig. 4. Effects of toyocamycin (**1**) on the auxin-dependent degradation of the Aux/IAA protein. (A) Effects of **1** and MG132 on AXR3NT-GUS fusion protein degradation. Seven-day-old *HS::AXR3NT-GUS* transgenic seedlings were heat-shocked for 2 h at 37 °C to induce the fusion protein expression. The seedlings were incubated in **1** or MG132 with/without 1 μM IAA for 30 min at 23 °C. The presence of AXR3NT-GUS fusion was visualized by histochemical staining after treatment with cold aqueous acetone. (B) *Arabidopsis* ATP-dependent proteasome inhibition by **1** and MG132 in *Arabidopsis* root. The 20 S core unit activity in the 26 S proteasome was assayed as described in the experimental. The proteasome activity enhanced by endogenous ATP is adjusted to 100% value. Values are the mean +/−SE of two replicates.

the presence of auxin (IAA or 2,4-D) and **1**. Fig. 5 indicates that **1** blocked the 2,4-D-induced epinastic curvature of cotyledons. The cotyledon curvature was observed in 1 μM 2,4-D treatment. Toyocamycin **(1)** blocked 2,4-D-induced cotyledon curvature (Fig. 5A). Fig. 5B displayed that 15 μM toyocamycin **(1)** completely blocked 5 μM IAA-induced lateral root formation (Fig. 5B). Additionally, toyocamycin **(1)** reduced the lateral root number by blocking the endogenous auxin action when seedling was grown in the presence of **1**, and toyocamycin also inhibited lateral root promotion by other synthetic auxins, naphthalene-1-acetic acid, NAA, and 2,4-D (data not shown). These results indicate that toyocamycin **(1)** inhibits the physiological auxin response by blocking the SCF^{TIR1} pathway.

*2.5. The structure-activity relationships of the nucleoside moiety in toyocamycin **(1)***

Sangivamycin (**2**) and tubercidin (**3**) have been reported to be structurally related nucleoside-type compound of toyocamycin (**1**). Sangivamycin **(2)**, which has an amide group in place of the cyano group of **1**, was originally isolated as a cytotoxic compound from *Streptomyces* sp. (Rao, 1968) and found to be a potent inhibitor of protein kinases (Osada et al., 1989; Lee and Jung, 2007). Tubercidin **(3)**, which lacks the cyano group of **1**, has been reported to show cytotoxic activity as well as anti-microbial activity, and acts as an adenosine analog (Anzai et al., 1957; Owen and Smith, 1964; Mooberry et al., 1995). Sangivamycin (**2**) showed inhibition on auxin-induced *DR5::GUS* expression, as did **1** (Fig. 6). In contrast to **1**, sangivamycin (**2**) inhibited *pER8::GFP* and *ARR5::GUS* expression (Figs. 3 and 6). The tobacco *parB::GUS* expression in root tips was also inhibited by **2** (Fig. 3). This evidence suggests that **2** is an unspecific inhibitor of plant gene expression. Surprisingly, tubercidin (**3**) exhibited no inhibition of any reporter gene expression in *Arabidopsis* transgenic lines at 50–100 μM (Figs. 3 and 6), while tobacco *parB::GUS* expression was inhibited by **3** at 100 μM (Fig. 3). All three nucleoside antibiotics were reported to show potent cytotoxic activity towards mammal cell lines. However, in our study, only toyocamycin **(1)** displayed specific effects on auxin-responsive gene expression. This suggests the cyano group in **1** plays a crucial role in toyocamycin's specific action on the SCF^{TIR1} pathway.

*2.6. The structure-activity relationships of the sugar moiety in toyocamycin **(1)***

To address the role of the sugar moiety in **1** in inhibitory activity, we examined seven toyocamycin **(4–10)** derivatives with

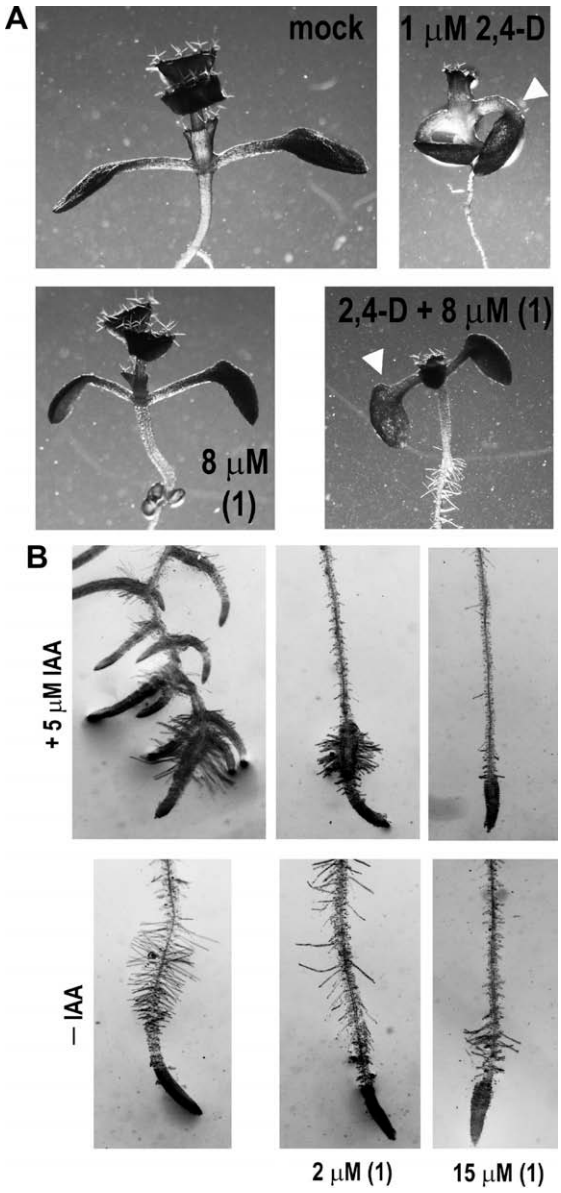

Fig. 5. Effects of toyocamycin (**1**) on auxin responses in the *Arabidopsis* plant. Three-day-old seedlings were transferred into liquid GM medium containing the indicated concentration of toyocamycin (**1**) with or without auxins and then cultured for an additional 3 days under continuous light. (A) Effects of toyocamycin on the epinastic curvature of cotyledon induced by 1 μM 2,4-D. (B) Effects of toyocamycin (**1**) on IAA-induced lateral root formation.

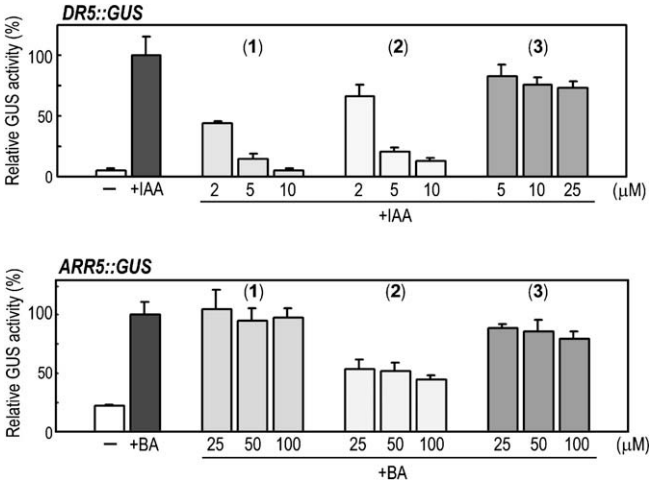

Fig. 6. Effects on toyocamycin (**1**), sangivamycin (**2**) and tubericidin (**3**) on the auxin-responsive *DR5::GUS* and cytokinin-responsive *ARR5::GUS* reporter gene expression. *Arabidopsis DR5::GUS* and *ARR5::GUS* transgenic seedlings (6-days-old) were treated with 5 μM IAA and 10 μM benzyladenine (BA) for 7 h, respectively, together with the test compound. After induction, GUS expression was fluorometrically determined. The induced GUS activity by hormone was adjusted to a 100% value. Values are the mean +/−SE of three independent experiments.

different sugar moieties (Fig. 7). Compounds **4** and **5** have ring-opened sugar analogs mimicking the ribose moiety, while compounds **6**–**10** are the deoxy or dideoxy derivatives of the ribose moiety of **1**. Among the seven derivatives, compounds 2′-deoxytoyocamycin (**9**) and 5′-deoxytoyocamycin (**10**) displayed potent inhibitory activity on auxin-induced *DR5::GUS* expression, with IC_{50} values of 3 μM for **9** and 10 μM for **10** (Table 1). Other derivatives displayed no or slight inhibition on *DR5::GUS* expression, with IC_{50} values of over 50 μM. These results suggest that the 2′-hydroxyl group in ribose is essential for activity, but that the 3′ or 5′-hydroxy group also faintly contributes. Additionally, compounds **9** and **10** had no effects on *ARR5::GUS* and *pER8::GFP* expression at 50 μM (data not shown), suggesting that both **9** and **10** retained specific inhibitory activity towards auxin signaling to the same extent as **1**.

3. Discussion

We have identified toyocamycin (**1**) as a potent inhibitor of auxin signaling from microbial metabolites, using an auxin-responsive reporter assay. Toyocamycin **(1)** specifically inhibited primary auxin-responsive gene expression, but did not block other reporter gene expression in *pER8::GFP*, *ARR5::GUS*, and *parB::GUS* lines. Toyocamycin **(1)** blocked the Aux/IAA–GUS fusion protein degradation regulated by the SCF^{TIR1}–proteasome pathway to a similar extent as MG132, a proteasome inhibitor. In contrast to MG132, **1** did not affect the proteolytic activity of the proteasome. This suggests

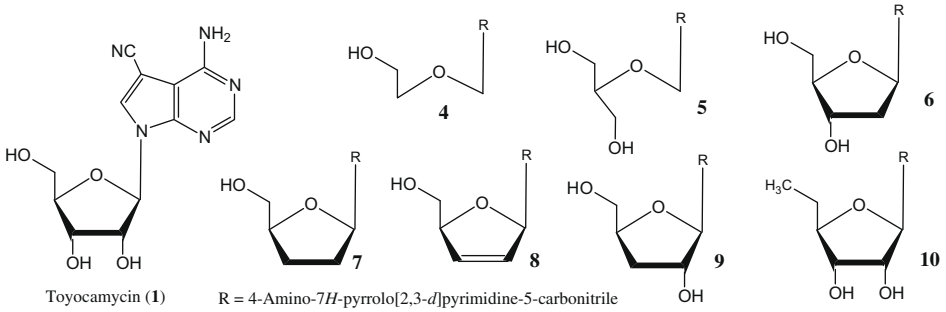

Fig. 7. Structures of toyocamycin derivatives (**4**–**10**).

Table 1
Inhibitory activity of toyocamycin derivatives **4**–**9** on IAA-induced *DR5::GUS* expression.

Compounds	IC_{50}[a]
Toyocamycin (**1**)	2 μM
4	>50 μM (0%)[b]
5	>50 μM (34%)
6	>50 μM (10%)
7	>50 μM (22%)
8	>50 μM (19%)
9	3 μM
10	10 μM

[a] Five-day-old *DR5::GUS* seedlings were incubated with 5 μM IAA and the compound for 7 h. The induced GUS reporter enzyme expression was fluorometrically determined as described in the Experimental section.
[b] The values in parentheses represent the inhibitory activity (%) of the compound at 50 μM on *DR5::GUS* induction.

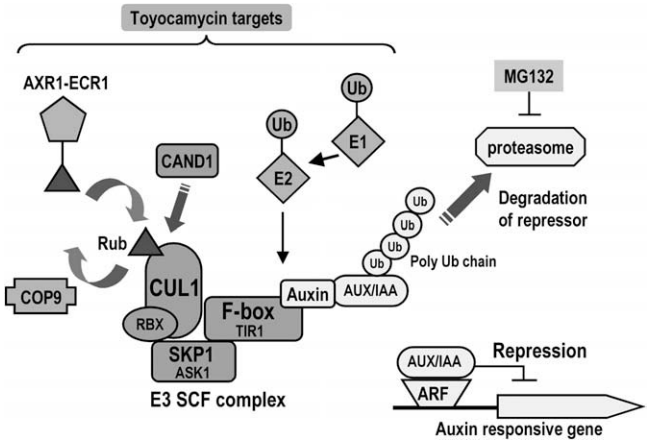

Fig. 8. Regulation of auxin-responsive gene expression by the SCF^{TIR1}–proteasome pathway. Possible toyocamycin target(s) are indicated in dark gray.

that **1** would act on the ubiquitination process of Aux/IAA via the SCF^{TIR1} machinery. In this process, ubiquitin is activated by the E1 ubiquitin-activating enzyme. Activated ubiquitin is then passed to the E2 ubiquitin-conjugating enzyme, as shown in Fig. 8. Finally, the target protein is conjugated with ubiquitin to form a poly-ubiquitin chain by an E3 SCF ubiquitin ligase (ASK1 as SKP subunit, Cul1 as CULLIN subunit and TIR1 as the F-box protein) and an E2 enzyme (Kepinski, 2007). Toyocamycin **(1)** might block a certain step in the ubiquitination process of Aux/IAA involved in E1, E2 and E3 enzymes. Alternatively, toyocamycin **(1)** targets the modification of the cullin subunit regulating SCF activity. The cullin subunit was conjugated with the ubiquitin-like protein RUB/Nedd8 by an E1-like RUB1-activating enzyme, the AXR1-ECR1 complex, and an E2-like RUB1-conjugating enzyme RCE1 (del Pozo et al., 1998; del Pozo and Estelle, 1999; Parry and Estelle, 2004). This RUB-conjugation of cullin plays an essential role in the regulation of the activity of SCF E3 ubiquitin ligases (del Pozo et al., 2002). On the other hand, the COP9 signalosome cleaves RUB from the modified cullin (Schwechheimer et al., 2001). The deconjugation of RUB was also involved in the regulation of E3 SCF ubiquitin ligase. CAND1 (cullin-associated and neddylation-dissociated) selectively binds unmodified cullin (Chuang et al., 2004). Therefore, CAND1 is believed to antagonize the RUB modification of cullin to regulate SCF E3 ubiquitin ligases.

From the structure-activity relationships of toyocamycin-related antibiotics (**1**–**3**), we can deduce that the cyano group in **1** is essential for the specific activity of toyocamycin on the SCF^{TIR1} pathway. Additionally, the 2′-hydroxy group on the riboside was found to be important for the activity of **1**. De Clercq et al. (1987) reported that 2′-deoxytoyocamycin (**6**) and 3′-deoxytoyocamycin (**9**) have both been reported to show the same potent anti-proliferative activity against mammalian Hela and Vero cells. Three nucleoside antibiotics, **1**, **2** and **3**, showed a potent anti-proliferative effect on human cell lines, but tubercidin (**3**) did not inhibit all reporter gene expression in the *Arabidopsis* transgenic line. This evidence supports the idea that toyocamycin **(1)** inhibits auxin signaling by a specific action on SCF machinery, but not by unspecific effects stemming from its cytotoxicity on cells. The ubiquitination by E3 SCF-type ubiquitin ligases plays a central role in many cellular processes such as cell cycle regulation in eukaryotes, and the E3 SCF ubiquitin ligases are involved in several human diseases including certain cancers (Strohmaier et al., 2001; Tanaka et al., 2004). Therefore, part of the anti-proliferative effects of **1** might be due to the inhibition of the SCF pathway. We anticipate that toyocamycin and its derivatives would not only be chemical tools for auxin biology, but also be potential probes to investigate the function of the SCF-ubiquitination pathway in human cell biology.

4. Experimental

4.1. Plant materials

Arabidopsis thaliana ecotype Columbia was used for this study. Transgenic *Arabidopsis* reporter lines, *DR5::GUS* and *ARR5::GUS* lines were provided by Drs. A.M. Jones and J. Kieber (University of North Carolina). *Arabidopsis pER8::GFP* lines were provided from Dr. N.H. Chua (The Rockefeller University). *Arabidopsis IAA3::GUS, IAA7::GUS*, and *IAA19::GUS* lines were gifts from Drs. D. Weijers (ZMBP, Germany), JW Reed (University of North Carolina) and KT. Yamamoto (Hokkaido University, Japan). The *HS::AXR3NT-GUS* lines were provided by Dr. M. Estelle (Indiana University). Tobacco *parB::GUS* line was provided by Dr. Y. Takahashi (Hiroshima University).

4.2. Chemicals

Toyocamycin **(1)** was isolated from culture broth of *Streptomyces* sp. strain G8 by a repeated silica gel ($CHCl_3$-MeOH = 8:2) and Sephadex LH-20 ($CHCl_3$-MeOH = 1:1) column chromatography (cc) and used in this work. Toyocamycin was identified by spectroscopic analyzes and FAB-MS spectrum. [^{1}H NMR (DMSO-d_6) δ: 3.56 (1H, *m*, H-5′), 3.67 (1H, *m*, H-5′), 3.94 (1H, *dd*, *J* = 3.7, 7.3, H-4′), 4.10 (1H, *m*, H-3′), 4.37 (1H, *m*, H-2′) 5.21 (2H, *br s*, 3′-OH and 5′-OH), 5.49 (1H, *d*, *J* = 6.1, 2′-OH), 6.06 (1H, *d*, *J* = 5.5), 6.92 (NH_2, *br s*), 8.23 (1H, *s*, H-6), 8.45 (1H, *s*, H-2); ^{13}C NMR (DMSO-d_6) δ: 61.2 (*d*, C-5′), 70.2 (*d*, C-3′), 74.2 (*d*, C-2′), 83.0 (*s*, C-5), 85.5 (*d*, C-4′), 87.7 (*d*, C-1′), 101.2 (*s*, C-4a), 115.3 (*s*, CN), 132.4 (*d*, C-2), 150.1 (*d*, C-7a), 153.6 (*d*, C-6), 157.0 (*s*, C-4); Positive FABMS *m/z* 292 $[M + H]^+$. Sangivamycin **(2)** and tubericidin **(3)** was obtained from Sigma–Aldrich (Tokyo, Japan). MG132 was purchased from The Peptide Institute (Osaka, Japan). Toyocamycin derivatives **4**–**10** were previously synthesized and used for this study (De Clercq et al., 1987; Gupta et al., 1989a,b; Krawczyk and Townsend, 1989; Gupta et al., 1990; Krawczyk et al., 1995).

4.3. Hormone induction

Arabidopsis seedlings (*n* = 10–15) were grown vertically on germination agar medium (GM, 0.5x Murashige and Skoog salts (Gibco BRL, Gaithersburg, MD), 1% sucrose, 1x B5 vitamins, and 0.2 g/L 2-(4-morpholino)-ethane sulfonic acid (MES), pH 5.8, 1.4% agar) under continuous light and were transferred to 12-well micro-titer plated containing liquid GM medium supplemented with the indi-

cated hormone and/or chemicals. Seedlings were then incubated for the indicated time to induce each responsive gene.

4.4. Histochemical and quantitative measurements of GUS reporter activity

For GUS histochemical analyzes, transgenic seedlings were washed 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 transferred to a staining buffer containing 1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc), the substrate for histochemical staining. The seedlings were then incubated at 37 °C until sufficient staining developed (3–4 h). For quantitative measurements, after reporter gene induction ($n = 20$), the excised roots were homogenized in an extraction buffer as previously described (Oono et al., 2003). After centrifugation, the GUS activity of the supernatant was measured using 1 mM 4-methyl umbelliferyl β-D-glucuronide as a fluorogenic substrate at 37 °C. Protein concentrations were determined by Bradford protein assay (Bio-Rad Japan, Japan). The experiments were repeated at least three times with three replications.

4.5. Aux/IAA protein degradation and proteasome assays

Seven-day-old *HS::AXR3NT-GUS* transgenic seedlings were incubated in liquid GM medium for 2 h at 37 °C. Seedlings were then transferred into liquid GM medium containing 20 μM cycloheximide at 23 °C to block additional protein synthesis. The chemicals were added to the medium after 10 min of incubation at 23 °C. IAA was added after an additional 10 min of incubation with chemicals. After incubation for another 30 min at 23 °C, the seedlings were immersed in 70% cold aqueous acetone and washed with H_2O. GUS activity was histochemically stained until sufficient staining was developed. For the proteasome assay, the ATP-dependant 20 S core unit activity of the 26 S proteasome in 6-day-old *Arabidopsis* root was measured by peptide-hydrolysis activity using succinyl–Leu–Leu–Val–Tyr-4-methyl-coumaryl-7-amide as the substrate with or without ATP and Mg^{2+}, as previously described (Fujinami et al., 1994). Briefly, the excised roots from 6-day-old *Arabidopsis* seedlings ($n = 10$) were homogenized in 100 μL of 50 mM Tris–HCl buffer (pH 8.0, 20 mM 2-mercaptoethanol). This homogenate (50 μL) was added to 150 μL of reaction solution (50 mM Tris–HCl buffer, 20 mM 2-mercaptoethanol, 10 mM substrate with or without 4 mM ATP and 10 mM $MgCl_2$ as final conc.). The reaction mixture was then incubated for 50 min at 37 °C. The reaction was stopped by the addition of 0.2 M Na_2CO_3 (800 μL). The fluorescence from the hydrolyzed substrate was measured by a fluorometer (*Ex* 380 nm, *Em* 440 nm) and the value was normalized by protein concentration. The experiments were repeated three times with three replications.

4.6. Auxin-related phenotype assay in Arabidopsis plants

To study the effects of toyocamycin **(1)** on *Arabidopsis* phenotypes, 3-day-old seedlings were transferred into liquid GM medium containing the indicated concentration of 1 with or without auxins (5 μM IAA and 1 μM 2,4-D). The seedlings were then cultivated under continuous light for an additional 3 days at 24 °C. The representative phenotype of root and cotyledon were recorded by digital camera.

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