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The isolation, structural determination, and total synthesis of terfestatin A, a novel auxin signaling inhibitor from *Streptomyces* sp.

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Abstract—Indole 3-acetic acid, a plant hormone auxin, regulates every aspects of plant growth and development. In the screening for specific inhibitors on auxin signaling using the transgenic Arabidopsis plant harboring the auxin-responsive reporter gene, terfestain A was found to be a novel inhibitor of auxin signaling from the culture of *Streptomyces* sp. F40. The structure of terfestatin A was determined to be *p*-terphenyl β-glucoside on the basis of spectroscopic analyses, chemical degradation, and total synthesis.

Plant growth and development are controlled by plant hormones such as auxin, cytokinin, abscisic acid, ethylene, and gibberellin that are low molecular organic compounds, in contrast to proteinous hormones in mammal. Indole 3-acetic acid, auxin, regulates cell division, elongation, and differentiation in every aspects of plant growth and development.[1] Auxin elicits the rapid and dramatic change of the expression patterns of a number of genes. Therefore, its profound and diverse effects of auxin on plant development would result from regulation of the expression pattern of a number of genes. Recent molecular biological studies have revealed that the expressions of *AUX/IAA* genes are specifically induced by auxin within a minute and play essential roles in early steps of auxin signal transduction.[2,3] AUX/IAA proteins are short-lived transcriptional repressors localized in the nucleus and regulate the expression of late auxin-responsive genes linked to cell division and elongation.[4] However, the molecular mechanism of regulation on the early auxin-responsive gene expression is not fully understood. In addition, auxin receptor has not been identified although many efforts have been made to identify the receptor for several decades. Biochemical and pharmacological approaches with specific inhibitors on signal transduction have greatly contributed to the study of signaling in cellular response. Identification of the specific inhibitors of auxin signal transduction would also provide useful tools for the study of auxin signaling machinery. The plant growth regulatory activity of many natural products has been reported. However, most of this activity has been evaluated with intact plants, and therefore, the site of action have still been unclear. To screen for specific inhibitors on auxin signaling, we used the reporter gene assay for target-oriented screening using the transgenic Arabidopsis plant, which harbors the early auxin-inducible promoter and β-glucuronidase (GUS) reporter gene.[5] This transgenic reporter line expresses GUS protein in response to auxin. The GUS induction in the root is rapid and highly specific to auxin, and can be detected easily by enzyme activity measurement. This reporter system enables us to perform rapid and specific screening for inhibitors on auxin signaling. We have reported yokonolides as an auxin signaling inhibitor using this reporter assay from *Streptomyces* sp.[6,7] In the continuous screening for specific inhibitors of auxin signal transduction from microbial metabolites using the reporter assay, we found that *Streptomyces* sp. F40 produced a new inhibitor, designated terfestatin A (**1**). This report deals with the isolation, structure determination, total synthesis, and some biological activities of **1**.

The terfestatin A-producing organism identified as *Streptomyces* sp. F40 was isolated from soil collected

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at Okayama city, Japan.[8] Two hundred milliliters of seed culture of the producing strain was inoculated into a 10-liter jar-fermenter containing 8 L of the production medium (soluble starch 1%, glucose 0.5%, phamamedia 0.4%, polypeptone 0.1%, meat extract 0.1%, yeast extract 0.1%, and corn steep liquor 0.1%), and cultivated at 28 °C for 96 h under agitation at 400 rpm. The resultant culture was filtrated. The filtrate was adjusted to pH 7.0 and extracted with EtOAc. In each purification step, the fractions were assayed for the inhibitory activity against auxin-induced reporter gene expression with the Arabidopsis transgenic *BA-GUS* reporter line.[5] The auxin-induced β-glucuronidase (GUS) expression was detected by the histochemical staining of GUS enzymatic activity using 5-bromo-4-chloro-3-indyl-β-D-glucuronic acid (X-Glc) as a substrate. In this reporter assay, auxin-induced GUS expression was monitored by the visible blue stain derived from X-Glc in root or by a fluorescent GUS enzyme activity measurement.[5] The EtOAc layer was concentrated in vacuo to give 0.48 g of extract. The EtOAc extract (2.4 g from 40 L of culture) was applied onto a silica gel column and developed with stepwise elution ($CHCl_3$–MeOH). The active fractions were concentrated and further purified by Sephadex LH-20 column chromatography eluting with $CHCl_3$–MeOH = 2:1 solvent. The active fraction was chromatographed by ODS low-pressure column (MeOH:H_2O = 1:1) to give a white powder, as an active compound (**1**: 13.7 mg).[16]

The HR-FABMS of **1** gave a molecular formula of $C_{24}H_{24}O_8$ (*m/z* $[M+Na]^+$ calcd for $C_{24}H_{24}O_8Na$, 463.1368. Found, 463.1360). IR spectrum showed the existence of hydroxy group (3393 cm^{-1}). The ^1H NMR spectrum (Table 1) showed two pairs of monosubstituted benzene protons at δ 7.54 (2H), 7.37 (2H), 7.31 (1H), 7.43 (2H), 7.38 (2H), 7.26 (1H), a singlet benzene proton at δ 6.39 (1H), five oxymethine protons of sugar including an anomeric proton (δ 4.29, 3.34, 3.22, 3.22, and 2.99, each 1H) and oxymethylene protons of a sugar at δ 3.49 and 3.41. The ^{13}C NMR (Table 1) and DEPT spectra indicated 20 signals including 8 signals from two pairs of monosubstituted benzene rings, 6 signals from a pentasubstituted benzene, and 6 signals from a pyranohexose. ^1H–^1H-COSY, HMQC spectral analyses also confirmed the presence of two monosubstituted benzene rings (A and C rings), a pentasubstituted benzene ring (B ring) and pyranohexose. H-2 at the benzene A ring and H-2″ at C ring displayed HMBC correlations to C-4′ and C-1′ in B ring, respectively. These NMR evidences together with the molecular formula suggest that **1** is terphenyl glycoside having two phenolic hydroxy groups at B ring. HMBC cross-peak (H-6′ to C-1″) and NOESY cross-peak (H-6′ to H-2″) indicated that benzene C ring is attached to *o*-position (C-1′) of C-6′. Pyronohexose moiety was elucidated to be β-glucose by NOESY correlations (Fig. 1) and coupling constant (H-1‴, J = 8.0 Hz). We could not obtain any clear structural information from the spectroscopic analyses.

To determine the structure of aglycone, **1** was hydrolyzed by 3 N-HCl in MeOH at reflux for 3 h to give the aglycone **2** and D-glucose (Scheme 1). Compound

Table 1. ^1H and ^{13}C NMR data of **1** (CD_3OD)

No.	^1H (δ)	^{13}C (δ)
1		135.6
2	7.43 (d, 1H, J = 7.6 Hz)	132.2
3	7.38 (m, 1H)	128.9
4	7.26 (m, 1H)	127.7
5	7.38 (m, 1H)	128.9
6	7.43 (d, 1H, J = 7.6 Hz)	132.2
1′		136.4
2′		137.2
3′		149.6
4′		118.1
5′		153.0
6′	6.39 (s, 1H)	108.8
1″		140.2
2″	7.54 (d, 1H, J = 7.3 Hz)	130.8
3″	7.37 (m, 1H)	128.6
4″	7.31 (m, 1H)	128.2
5″	7.37 (m, 1H)	128.6
6″	7.54 (d, 1H, J = 7.3 Hz)	130.8
1‴	4.29 (d, 1H, J = 8.0 Hz)	107.7
2‴	3.34 (t, 1H, J = 8.9, 8.0 Hz)	75.2
3‴	3.22 (m, 1H)	77.6
4‴	3.22 (m, 1H)	71.0
5‴	2.99 (m, 1H)	77.8
6‴	3.49 (dd, 1H, J = 11.7, 2.6 Hz)	62.4
	3.41 (dd, 1H, J = 11.7, 5.1 Hz)	

TMS is used as internal reference.

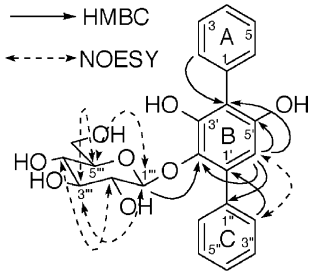
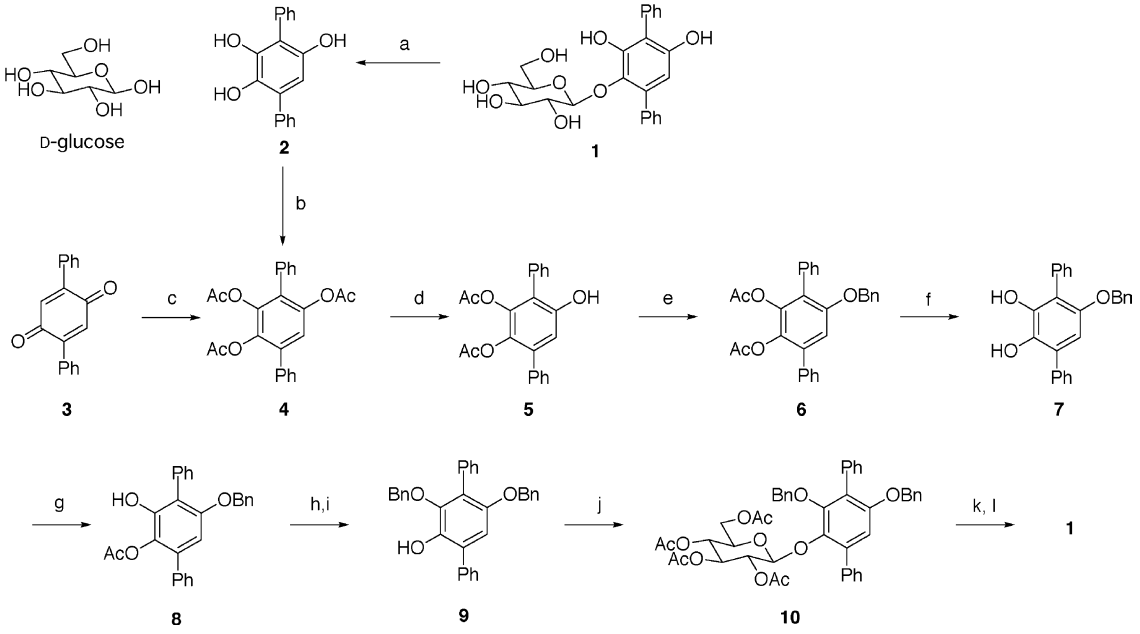


Figure 1. Structure of terfestatin A (**1**), and HMBC and NOESY correlations.

2 was treated with acetic anhydride in pyridine at room temperature for 24 h to give triacetate **4**.[16] We deduced the *p*-terphenyl skeleton to be the most plausible structure by detailed spectral analyses. Therefore, **4** was synthesized by Thiel acetylation from diphenyl benzoquinone **3**.[9] The synthesized **4** was completely identical to triacetylated aglycone from **1**. From these results, the structure of aglycone was determined to be 2′,3′,5′-trihydroxy-*p*-terphenyl.

Benzene proton at H-6′ displayed HMBC correlation with C-2′ (δ137.2) and C-5′ (δ 153.0). The up-field shift of the C-2′ carbon signal was attributed to hydroxyl group at *o*-position. The anomeric proton (H-1‴) showed an HMBC cross-peak with C-2′. These evidences strongly support that glucose binds to C-2′ position at B ring.

To confirm the structure of **1**, we attempted the total synthesis of **1** (Scheme 1). 3′,5′-Dibenzyl protected com-



Scheme 1. Reagents and conditions: (a) 3 N-HCl, MeOH, reflux, 3 h; (b) Ac_2O, pyridine, rt, 24 h; (c) $HClO_4$, Ac_2O, 55 °C to rt, 4 h, 80%; (d) KOH, MeOH, 0 °C, 30 min, 92%; (e) BnBr, NaH, TBAI, THF, 24 h, 87%; (f) 3 N-HCl, MeOH, reflux, 2 h, 84%; (g) Cs_2CO_3, AcCl, CH_3CN, −40 °C, 1 h, 73%; (h) BnBr, NaH, TBAI, THF, rt, 24 h, 92%; (i) KOH, MeOH, 50 °C, 2 h, 90%; (j) Cs_2CO_3, acetobromo-α-D-glucose, rt, 24 h, 97%; (k) H_2, Pd–C, EtOAc, rt, 24 h, 92%; (l) KOH, MeOH, rt, 1 h, 92%.

pound **9** was designed as the key intermediate to introduce β-D-glucoside at C-2′ position in our route and **3** was selected as a starting material. Triacetate **4** was selectively hydrolyzed with KOH in MeOH at 0 °C for 30 min to yield diacetate **5**. This diacetate **5** was treated with benzyl bromide, sodium hydride, and tetra-*n*-butylammonium iodide in THF at room temperature for 24 h to give **6**. Diol **7** was obtained by the hydrolysis of **6** with 3 N-HCl in MeOH at reflux for 2 h. For selective protection on C-2′ position, **7** was mixed with cesium carbonate and acetyl chloride in acetonitrile at −40 °C for an hour to afford monoacetate **8**.[16] The introduction of benzyl group to C-3′ was carried out with the treatment of **8** with benzyl bromide, sodium hydride, and tetra-*n*-butylammonium iodide in THF at room temperature for 24 h. The key dibenzyl protected intermediate **9**[16] was obtained by the hydrolysis of **8** with potassium hydroxide in MeOH. The structure of **9** was confirmed by 2D NMR spectra including NOESY spectrum.

We performed several glycosylation methods of phenolic compounds to introduce a β-glucoside at C-2′ position of **9**. First, **9** was treated with diisopropylethylamine or Lewis acid and acetobromo-α-D-glucose, but no reaction proceeded. We next carried out the glycosylation with acetobromo-α-D-glucose as glycosyl donor and tributyltin methoxide and tin(IV) chloride as catalyst.[10] This reaction afforded **10** in low yield (16%). Therefore, we developed a new glycosylation reaction to the phenolic group using cesium carbonate and acetobromo-α-D-glucose. Phenol **9** was treated with an equimolecular amount of cesium carbonate and threefold excess of acetobromo-α-D-glucose in acetonitrile at room temperature for 24 h to give **10**[16] in very high yield (97%). The benzyl ether **10** was further deprotected by the hydrogenolysis (H_2, Pd–C) and hydrolysis of acetate (KOH in MeOH) to yield **1**. Overall yield of **1** was 27% from starting material **3**. Physico-chemical, spectral, and biological properties of synthetic **1** completely agreed with those of **1** from natural origin.

There are few reports on the isolation of 2′,3′,5′-trisubstituted *p*-terphenyl compounds from natural sources. Terphenyllins[11] and terprenins[12] from *Aspergillus candidus*, terferol[13] from *Streptomyces showdoensis*, and butlerins[14] from *Parmelia butleri* have been reported. These *p*-terphenyl derivatives have *O*-alkyl or acyl groups at benzene B-ring. However, there are no reports on *p*-terphenyl glycoside. Thus, terfestatin A is the first *p*-terphenyl glycoside.

The inhibitory activities of **1** against auxin-induced gene expression were assayed by the transgenic Arabidpsis *BA-GUS* reporter line carrying the GUS gene under the control of auxin-responsive promoter.[5] Compound **1** (20 μM) inhibited auxin-induced reporter gene expression by histochemical staining of GUS enzymatic activity. In addition, the quantitative GUS activity measurement by the fluorometric substrate, 4-methyl-umbelliferyl-β-D-glucuronide, showed that IC_{50} of **1** against auxin-induced GUS gene expression in *BA-GUS* line was 10 μM. On the other hand, GUS enzymatic activity was not affected by **1** at 50 μM. In contrast to auxin-induced gene expression, **1** showed no inhibitory effects at 50 μM on estrogen-induced reporter gene expression in *pER 8::GFP*.[15] Arabidopsis transgenic plant, suggesting **1** is a specific inhibitor of auxin signaling leading to auxin-responsive gene expression, but not an inhibitor of the general translation and transcription process. The detailed biological activities of **1** will be published elsewhere.

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16. Spectral data of **1**, **4**, **8**, **9**, **10**. Compound **1**: IR (KBr): ν 3393, 1625, 1420, 1071, 757, 701 cm^{-1}, UV (MeOH) λ_{max} (nm) ($\log\varepsilon$) 224.0 (5.00), 264.0 (4.83), mp 134.7 °C, $[\alpha]_D$ = (c 0.1, MeOH) −37.2°. Compound **4**: Yellow powder, IR (KBr): ν 3063, 1765, 1628, 1371, 1192, 1045, 916, 757, 702 cm^{-1}, UV (MeOH): λ_{max} (nm) ($\log\varepsilon$) 251.2 (4.57), mp 189.7 °C, ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.47 (d, 2H, J = 5.0 Hz), 7.33–7.42 (m, 6H), 7.31 (d, 2H, J = 5.0 Hz), 7.13 (s, 1H), 2.09 (s, 3H), 1.96 (s, 6H), ^{13}C NMR (500 MHz, $CDCl_3$): δ 168.8, 167.9, 167.8, 146.0, 141.5, 138.3, 136.0, 135.9, 131.8, 129.5 (2C), 129.4, 128.7 (2C), 128.4 (2C), 128.0 (2C), 127.8, 121.9, 100.5, 20.4, 20.3, 20.1, HR-EIMS: m/z $[M]^+$; calcd for $C_{24}H_{20}O_6$, 404.1260. Found, 404.1287. Compound **8**: white powder, IR (KBr): ν 3424, 3059, 1742, 1566, 1205 1063, 745, 697 cm^{-1}, UV (MeOH): λ_{max} (nm) ($\log\varepsilon$) 258.0 (4.64), mp 148.0 °C, ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.34–7.51 (m, 9H), 7.23–7.29 (m, 4H), 7.16–7.19 (m, 2H), 6.64 (s, 1H), 5.21 (s, 1H, OH), 5.02 (s, 2H), 2.12 (s, 3H), ^{13}C NMR (500 MHz, $CDCl_3$): δ 169.4, 153.7, 145.7, 137.5, 136.9, 135.2, 131.8, 130.8 (2C), 129.1, 129.0 (2C), 128.7 (2C), 128.3 (2C), 128.3 (2C), 128.3, 127.7, 127.6, 126.7 (2C), 118.2, 106.3, 70.6, 20.5, HR-EIMS: m/z $[M]^+$; calcd for $C_{27}H_{22}O_4$, 410.1518. Found, 410.1505. Compound **9**: white powder, IR (KBr): ν 3498, 3061, 1600, 1381, 1227, 1098, 754, 696 cm^{-1}, UV (MeOH): λ_{max} (nm) ($\log\varepsilon$) 260.8 (4.61), mp 117.3 °C, ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.60–7.63 (m, 4H), 7.38–7.49 (m, 6H), 7.32–7.35 (m, 1H), 7.19–7.30 (m, 7H), 7.06–7.08 (m, 2H), 6.87 (s, 1H), 5.76 (s, 1H, OH), 4.94 (s, 2H), 4.42 (s, 2H), ^{13}C NMR (500 MHz, $CDCl_3$): δ 149.3, 144.2, 141.2, 137.6, 137.3, 136.3, 133.5, 130.9 (2C), 129.0 (2C), 128.6 (2C), 128.5 (2C), 128.5, 128.3 (2C), 128.3 (2C), 128.0 (2C), 127.5, 127.4, 127.3, 127.0 (2C), 126.8, 124.6, 111.9, 75.4, 71.8, HR-EIMS: m/z $[M]^+$; calcd for $C_{32}H_{26}O_3$, 458.1882. Found, 458.1881. Compound **10**: white powder. IR (KBr): ν 3503, 3288, 3057, 1746, 1609, 1405, 1229, 1043, 755, 698 cm^{-1}, UV(MeOH): λ_{max} (nm) ($\log\varepsilon$) 260.4 (4.63), mp 208.6 °C, ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.51–7.53 (m, 2H), 7.35–7.44 (m, 8H), 7.19–7.29 (m, 8H), 6.94–6.96 (m, 2H), 6.80 (s, 1H), 5.15 (d, 1H, J = 5.0 Hz), 4.97–5.06 (m, 5H), 4.86 (d, 1H, J = 10 Hz), 4.58 (d, 1H, J = 10 Hz), 3.96 (dd, 1H, J = 12.3, 3.8 Hz), 3.75 (dd, 1H, J = 12.3, 2.1 Hz), 3.37–3.40 (m, 1H), 1.95 (s, 6H), 1.93 (s, 3H), 1.85 (s, 3H), ^{13}C NMR (500 MHz, $CDCl_3$): δ 170.5, 170.2, 169.3, 169.1, 152.8, 149.7, 140.3, 138.2, 137.0, 136.8, 136.0, 133.5, 130.8 (2C), 129.5 (2C), 128.3 (2C), 128.1 (2C), 128.1 (2C), 128.0 (2C), 127.8, 127.7 (2C), 127.5, 127.3, 127.1, 126.7 (2C), 126.1, 111.0, 100.2, 75.0, 73.1, 71.8, 71.5, 70.7, 67.9, 61.2, 20.7, 20.6, 20.5, 20.5, HR-FABMS: m/z $[M+Na]^+$; calcd for $C_{46}H_{44}O_{12}Na$, 811.2731. Found, 811.2720.