Rotihibins, Novel Plant Growth Regulators from *Streptomyces graminofaciens*

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In the course of screening search for plant growth regulators, a culture filtrate of *Streptomyces graminofaciens* 3C02 was found to inhibit the growth of lettuce seedlings. The active substances, named rotihibin A (**1**) and B (**2**), were revealed to be lipo-peptidal compounds. Rotihibins inhibit growth of various plants at below 1 μg/ml, but do not show lethal activity even at higher doses.

In our screening search for plant growth regulators from bacterial origin, we found a strain of actinomycete, identified as *Streptomyces graminofaciens*, that yielded strong plant growth regulators. Based on spectroscopic data, the active substances were revealed to be lipo-peptidal compounds, named rotihibin A (**1**) and B (**2**) and we reported their structures preliminarily[1,2]. This paper describes the taxonomy of producing strain, isolation, structural elucidation and biological activity of rotihibins in detail.

Taxonomy of the Producing Strain

The strain 3C02 was isolated from a soil sample collected in Bunkyo-ku, Tokyo, Japan. Both substrate and aerial mycelia were formed abundantly on International Streptomyces Project (ISP) media No. 2 (yeast extract-malt extract agar), No. 3 (oatmeal agar) and No. 4 (inorganic salt-starch agar). Morphological observation of cultures grown on the above three media was made by electron microscopy (Fig. 1). Spore chains were monopodial and showed spiral to open-spiral style with 10 to 50 spores in each chain. Mature spores were cylindrical and $1 \times 2\,\mu$m in size. Spore surfaces were warty. Fragmentation of substrate, sporangia, motile spores were not observed.

Strain 3C02 grew well on most test media shown in Table 1. No diffusible pigment was formed in any tested medium. White to yellowish-gray aerial mycelium was formed on most test media except for sucrose-nitrate

Fig. 1. Scanning electron microphotography of aerial mycelia of strain 3C02.

Table 1. Cultural characteristics of strain 3C02.

Medium	Growth	Aerial mycelium	Substrate mycerium
Sucrose-nitrate agar	Very good	None	Yellowish grey
Glucose-asparagine agar	Good	Modrate; white	Yellowish grey
Glycerol-asparagine agar (ISP No. 5)	Poor	Scant; white to grey	Colorless
Inorganic salt-Starch agar (ISP No. 4)	Good	Abundant; white to light grey	Colorless to yellow
Tyrosine agar (ISP No. 7)	Poor	Moderate; white	Colorless to yellowish grey
Peptone-yeast extract iron agar (ISP No. 6)	Moderate	None	Colorless to yellowish grey
Yeast extract-malt extract agar (IPS No. 2)	Good	Abundant; white	Colorless to pale yellowish brown
Oatmeal agar (ISP No. 3)	Good	Abundant; whilte to light grey	Yellowish grey

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agar and peptone-yeast extract-iron agar (ISP No. 6) media. The substrate mycelium was colorless to yellowish-gray.

Cell wall analysis of the strain 3C02 as described by YAMAGUCHI[3] showed that the isomer of diaminopimelic acid contained in the hydrolysate of whole-cell was LL-form.

According to Bergey's Manual of Determinative Bacteriology[4], the physiological characteristics of the strain 3C02, as shown in Table 2, placed it in *Streptomyces graminofaciens*.

Isolation and Purification

In the course of purification, activity was monitored by growth inhibition assay with lettuce (*Lactuca sativa*) seedlings. The strain 3C02 was cultured in Bennett's medium[5] with 5 liter volume Erlenmeyer flasks (2 liters in each flask) for 4 days at 26°C . The cultured broth was filtered with celite and the filtrate was adsorbed on Amberlite XAD-7 column and was eluted with 50% methanol. After the purification of this 50% methanolic eluate by SP-Sephadex C-25 ion exchange column, the active substances were further purified by reverse-phase HPLC with an octadodecyl silica gel column, SSC-ODS-742 (10×250 mm) eluted by 0.1% trifluoroacetic acid (TFA) with acetonitrile gradient. The active substances named rotihibin A (**1**) (52.9 mg) and B (**2**) (small amount) were then obtained as an amorphous white powder.

Table 2. Physiological characteristics of strain 3C02.

Liquefaction of gelatin	−
Hydrolysis of starch	+
Milk coagulatin	±
Milk peptonization	−
Melanoid production	−
Temperature	
Growth range	10~40°C
Optimal growth	20~40°C
Utilization of:	
L-Arabinose	+
D-Xylose	+
D-Glucose	+
D-Fructose	+
Sucrose	+
Inositole	+
L-Rhamnose	+
Raffinose	+
D-Mannitol	+

Table 3. Physicochemical properties of rotihibin A (**1**).

Molecular formula	$C_{35}H_{63}N_{11}O_{13}$
High-resolution FAB-MS	846.4596
Calcd for $(M+H)^+$	846.4685
IR μ_{max} (KBr) cm^{-1}	3300, 2940, 1650, 1540, 1200, 1135
Color reaction	Positive: ninhydrine (orange)
Rf value[a]	0.23 BuOH-AcOH-H_2O (4:1:1)
UV spectrum	End absorption

[a] Silicagel TLC (Merck Art. No. 5717).

Structural Elucidation

Physicochemical properties of rotihibin A (**1**) were as shown in Table 3. The typical absorption at 1650 and

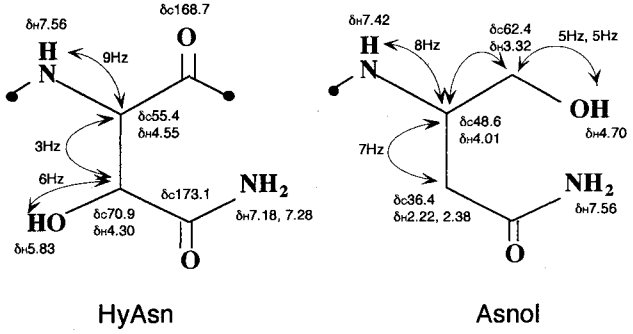
Table 4. ^{13}C and ^{1}H NMR of rotihibin A (**1**).

	Position	δ_H (multiplicity, J (Hz))	δ_C
2-Decenoic acid	1		165.7
	2	5.88 (d, 11)	122.5
	3	5.94 (m)	144.6
	4	2.52 (m), 2.65 (m)	29.6
	5	1.33 (m)	28.6
	6	1.25 (m)	28.9
	7	1.25 (m)	28.5
	8	1.25 (m)	31.1
	9	1.25 (m)	22.0
	10	0.85 (t, 7)	13.8
Cit	1		172.0
	2	4.36 (m)	52.0
	3	1.49 (m), 1.60 (m)	27.8
	4	1.33 (m)	26.5
	5	2.94 (m)	26.5
	2-NH	8.08 (d, 8)	
	5-NH	5.96 (m)	
	CO		158.7
	NH_2	5.38 (s)	
Ser	1		170.8
	2	4.36 (m)	54.6
	3	3.57 (m)	61.6
	2-NH	8.10 (d, 8)	
	3-OH	5.13 (t, 5)	
Dab	1		170.8
	2	4.26 (dd, 7, 13)	51.3
	3	1.73 (m)	30.9
	4	3.18 (m)	35.8
	2-NH	8.34 (d, 7)	
	4-NH_2	8.40 (t, 5)	
HyAsn	1		168.7
	2	4.55 (dd, 3, 9)	55.4
	3	4.30 (dd, 3, 6)	70.9
	4		173.1
	2-NH	7.56 (d, 9)	
	3-OH	5.83 (d, 6)	
	4-NH_2	7.18 (d, 2), 7.28 (d, 2)	
Asnol	1		172.7
	2	2.22 (dd, 7, 15), 2.38 (m)	36.4
	3	4.01 (m)	48.6
	4	3.32 (m)	62.4
	1-NH_2	6.88 (d, 1), 7.37 (d, 1)	
	3-NH	7.42 (d, 8)	
	4-OH	4.70 (t, 5)	
aloThr	1		166.4
	2	3.69 (d, 5)	57.6
	3	4.04 (m)	64.8
	4	1.05 (d, 7)	17.7
	2-NH_2	7.97 (br.)	
	3-OH	5.51 (d, 4)	

1540 cm^{-1} in the IR spectrum and the signals derived from amide –CONH– in the NMR spectra in DMSO-d_6 of **1** suggested its peptidal structure. Amino acid analysis of the hydrolysate of **1** by 6 N HCl (110°C, 20 hours) indicated the presence of serine (Ser), allothreonine (alloThr), 2,4-diaminobutanoic acid (Dab), ornithine (Orn) and some unknown amino acids. Edman's degradation of **1** yielded phenylthiohydantoin (PTH)-alloThr in the first cycle, but no PTH derivatives were given after the second cycle. High-resolution FAB mass spectra of rotihibin A (**1**) and B (**2**) gave $(M+H)^+$ ions at 846.4596 (calcd. for $C_{35}H_{64}N_{11}O_{13}$, 846.4685) and 847.4593 (calcd. for $C_{35}H_{63}N_{10}O_{14}$, 847.4529), respectively.

^1H- and ^{13}C-NMR spectra (Table 4) of **1** indicated that **1** consisted of serine, 2,4-diaminobutanoic acid, allothreonine, 2-decenoic acid and three more residues. ^1H COSY, ^{13}C-^1H COSY and HMBC[6] spectra of **1** revealed that ornithinyl residue detected in amino acid analysis existed as citllurinyl (Cit) residue in **1**, and the other two residues were β-hydroxyasparagine (HyAsn) and 3-amino-4-hydroxybutanamide (asparaginol, Asnol) as shown in Fig. 2. The distinction between hydroxy –OH protons and all amide protons including urea –N*H*$CONH_2$ proton in Cit residue and $-CONH_2$ protons in HyAsn and Asnol residues was confirmed by

Fig. 2. Assignment of the protons and the carbons of hydroxyasparagine (HyAsn) and asparaginol (Asnol) residues in the ^1H and ^{13}C NMR spectra of **1**.



Arrows represent the ^1H-^1H coupling constants.

^{15}H-^1H HMQC[7] spectrum of **1**. The sequence of these seven residues in **1** was clarified by NOESY and HMBC spectra as shown in Fig. 3, satisfying the molecular formula observed in high-resolution FAB mass spectrum. Furthermore, this structure was consistent with the result in Edman's degradation mentioned above.

To confirm the structure of **1** and to elucidate the structure of **2**, FAB mass and mass/mass spectra of **1** and **2** were analyzed. In the mass/mass spectrum of **1**, typical sequential fragment ions[8] were observed as shown in Fig. 4. The fragment ion pattern of **2** was similar to that of **1**, but the mass number of C-terminal ions, Y2, Y3 and Y4[8] were larger than those of **1** by one mass unit besides Y1 ions of both molecule were equal. This indicated that $-CONH_2$ group of HyAsn residue in **1** was exchanged for –COOH group in **2**.

Determination of the stereochemistry of amino acid residues was performed by method of MARFEY[9]. The hydrolysate of **1** by 6 N HCl (110°C, 18 hours) was reacted with an optically active fluorobenzene derivative, *N*-(3-fluoro-4,6-dinitrophenyl)-L-alaninamide (FDAA), and the reaction mixture was analyzed by HPLC with reverse-phase column. The retention times of each amino acids were compared with the standard samples from L- and DL-amino acids. L- and DL-Aspol (3-amino-4-hydroxybutanoic acid) were prepared by reduction of t-butyloxycarbonylasparagine (t-Boc Asn). As the results, Ser, Dab, alloThr and Asnol residues were proved to be L-form and Cit residue to be D-form. HyAsp, which was yielded from HyAsn residue was determined to be L-threo form by the same analysis. The configuration of decenoic acid in **1** was proved to be Z form by the coupling constant between C-2 and C-3 protons (11 ppm).

Biological Activity of Rotihibin A

Rotihibin A (**1**) did not show antimicrobial activity against any bacterial or fungal strain tested as shown in Table 5, and did not show cytotoxic activity to K562, FM3A and HSG cells at 200 μg/ml.

The inhibitory activity of **1** against lettuce seedlings

Fig. 3. Structure of **1** with observed NOEs (arrows) and ^{13}C-^1H long-range couplings (dashed arrows).

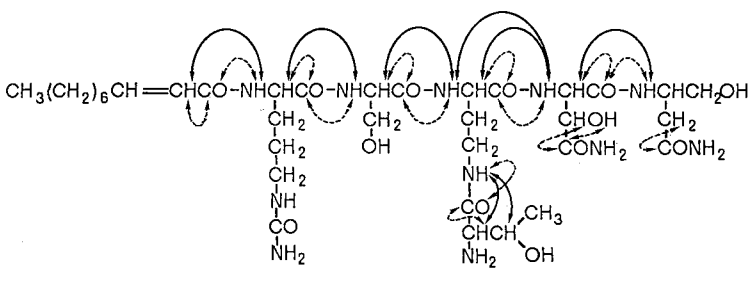
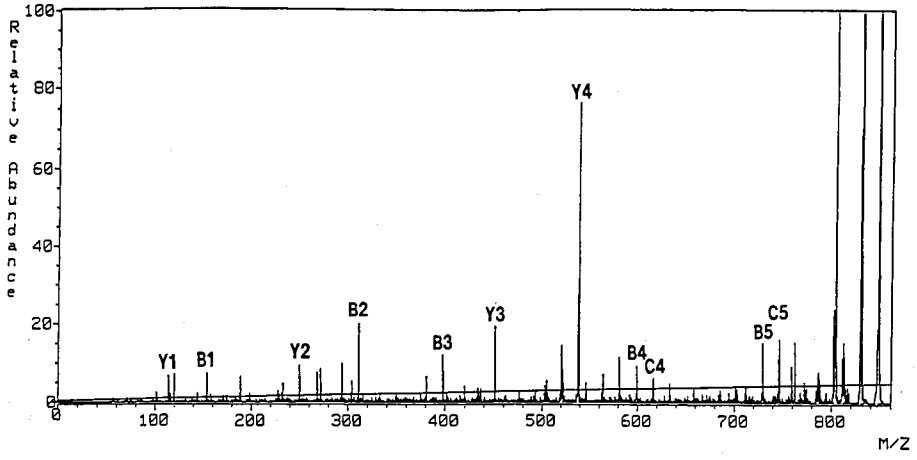


Fig. 4.

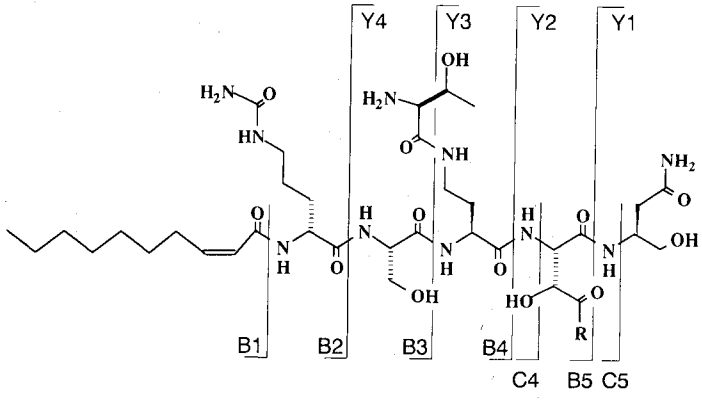
(A) Collision activated desorption (CAD) spectrum of $(M+H)^+$ ion of **1**.

Table shows the comparison between the CAD ions of **1** and **2**.



		Rotihibin A (1)	B (2)
B1	m/z	153	←
B2		310	←
B3		397	←
B4		598	←
C4		615	←
B5		728	729
C5		745	746
Y1		119	←
Y2		249	250
Y3		450	451
Y4		537	538

(B) Fragment ion patterns of **1** and **2**.



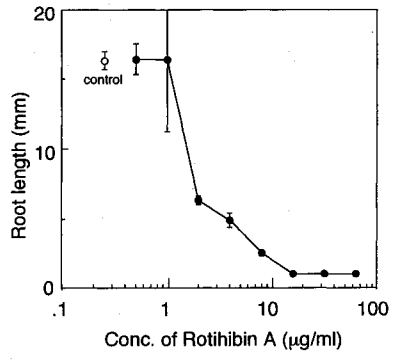
Rotihibin A (**1**) R = NH_2
B (**2**) R = OH

Table 5. Antibiotic activity of rotihibin A (**1**).

The organism	Medium	MIC (μg/ml)
Pyricuraia oryzae	C	>100
Botrytis cinerea	C	>100
Fusarium oxysporum	C	>100
Aspergillus oryzae	C	>100
Phytophthora capsici	C	>100
Candida albicans	P	>100
Bacillus subtilis	N	>100
Staphylococcus aureus	N	>100
Xantomonas oryzae	X	>100
Pseudomonas glumae	N	>100

Media C: Czapeck YE agar, P: PDA agar, N: Nutrient agar, X: Medium for Xantomonas.

Fig. 5. Inhibitory activity of **1** to the lettuce seedlings.



was observed above 1 μg/ml (Fig. 5), but was not lethal at 100 μg/ml, and furthermore, the absence of lethality of **1** to the plant was shown by the fact that lettuce seedlings which were treated by **1** for two days recovered their normal growth after removal of **1** (Fig. 6).

The activity of **1** against *Nicotiana rustica* (tobacco)

Fig. 6. Growth recovery of lettuce seedlings after removal of **1**.

● Treatment of **1** during shaded period (2~4 days); ○ Treatment of **1** throughout the experimental period; □ No treatment.

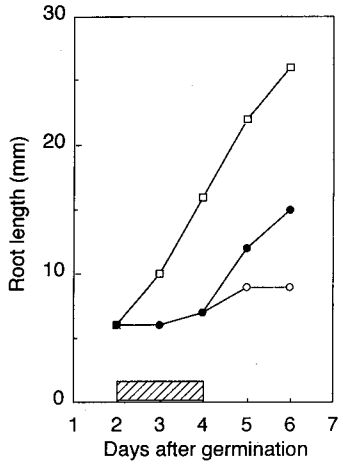
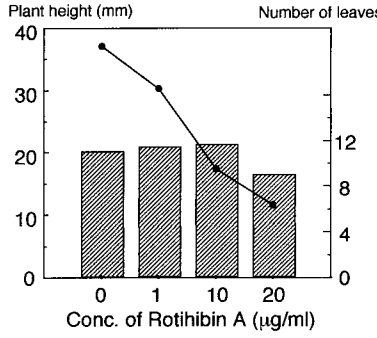


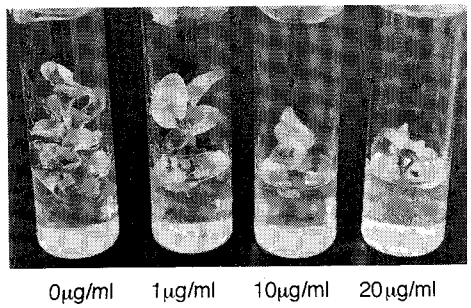
Fig. 7.

(A) Effect of **1** to *N. rustica*.

Bars represent the number of leaves; circles represent the height of plants.



(B) Growth feature of *N. rustica* treated by **1**.



was measured by using shoots with three leaves. The height of *N. rustica* grown on the medium with **1** at above 10 μg/ml was obviously shortened after 30 days' treatment, but there is no differences statistically in the number of leaves (Fig. 7).

Discussion

In the screening search for plant growth regulators, new plant growth regulators, rotihibins, were discovered. Structural studies revealed that rotihibins were novel lipo-peptidal compounds, and biological studies clarified their specific activity against plants. Rotihibin A (**1**) not only inhibited the growth of seedlings, but also dwarfed the plants, for example *N. rustica*, at low concentration. It may be possible to utilize **1** as a growth retardant.

Although many lipo-peptides found from microbial origin show antitumor, antibacterial and antifungal activity, rotihibin A (**1**) does not show any effect on mammalian cells, bacteria or fungi, but only against plants. It is interesting that **1** seems to act as a plant hormone, although it is not a plant hormone mimetic.

Experimental

The UV spectrum was measured with a SHIMADZU UV-160 spectrometer and the IR spectrum was measured with a JASCO A-202. The NMR spectra were measured with a JEOL-JNM GX-400 and a Bruker AM-600 spectrometer. High resolution FAB mass spectra were measured with a JEOL JMS-SX102 and mass/mass spectra were performed on a JEOL JMS-SX102/SX102 spectrometer.

Isolation of Rotihibin A (**1**) and B (**2**)

S. graminofaciens 3C02 was cultured in Bennett's medium[5], which was consisted of 1% glucose, 0.2% polypeptone, 0.1% meat extract and 0.1% yeast extract (pH 7.2) at 26°C for 4 days. The culture filtrate was adsorbed on Amberlite XAD-7 column (4.0 × 40 cm), washed with distilled water (1.5 liter) and was eluted with 50% methanol (2 liter). The methanolic eluate, after being concentrated to 200 ml volume and adjusted to pH 3.5 with acetic acid, was applied to SP-Sephadex C-25 cation ion-exchange column and was eluted with each 280 ml volume of 50 mM ammonium acetate buffer pH 4.0, 6.0 and 8.0, successively, and was fractionated. The activity was recovered in the last 60 ml fractions of the pH 6.0 eluate and the first 90 ml of pH 8.0 fractions. After lyophilization, the residue was dissolved in water and charged on the HPLC with ODS column (SSC-ODS-742, 10 × 250 mm), eluted by 0.1% TFA with acetonitrile/water linear gradient system (0% to 30% in 15 minutes) yielding rotihibin A (**1**) (52.9 mg) and B (**2**) (trace amount) as an amorphous powder.

Amino Acid Analysis of **1**

Rotihibin A (**1**) (100 mg) was hydrolyzed in PICO-TAG workstation (Waters Co.) by constant boiling point 6 N HCl (Pierce Chemical Co.) for 22 hours at 110°C. After removal of HCl from the hydrolysate under reduced pressure, the residue was dissolved in 20 mM HCl (200 nmol/ml) and applied on amino acid analyzer (HITACHI model-835).

Edman's Degradation of **1**

Rotihibin A (**1**) (500 mg), evaporated in a glass micro tube, was dissolved in 50% pyridine (100 μl) and added by phenylisothiocyanate (PITC) (10 μl), purged by N_2 and reacted at 57°C for 15 minutes. After evaporation, the dried sample was added by TFA (20 μl) and reacted at 57°C for 15 minutes, and yielded anilynothianolyzin-(ATZ-) derivatives. After removal of TFA, ATZ-derivatives were extracted in ethylacetate (80 μl) from water phase (50 μl) and dried ATZ-derivatives was reacted with 25% TFA (30 μl) at 55°C for 10 minutes yielding PTH-amino acids. Water phase was applied to next Edman's degradation cycle. A part of PTH-amino acids was analyzed by HPLC with ODS column (SSC-ODS-1251-K, 4.6×250 mm) eluted by 0.1% TFA with acetonitrile/water (15% to 50% in 30 minutes). The retention time of the PTH-amino acid in the first cycle coincided not to PTH-threonine but to PTH-allo-threonine. FD mass spectrum of PTH-derivative in the first cycle gave M^+ ion at m/z 236.

Determination of Stereochemistry

Configurational determination of the amino acids was performed by Marfey's method. Hydrolysate of **1** was obtained as mentioned above. A 50 mM amino acid mixture (10 μl) with 6% triethylamine (4 μl) and 1% *N*-(3-fluoro-4,6-dinitrophenyl)-L-alaninamide (FDAA) in acetone (20 μl) were reacted at 40°C for 1 hour in polypropylene microtube. The reactant was diluted into water (1 ml), and aliquot of the solution (200 pmol for each amino acid) was applied to HPLC analysis with ODS column (SSC-ODS-1151-N, 4.6×150 mm). For separation of basic amino acid derivatives, 10 mM triethylamine-phosphate buffer (pH 3.0) with acetonitrile/water gradient (16% to 30% in 18 minutes and 30% to 50% in 12 minutes) was used as solvent system, and for other amino acids, 10 mM acetic acid-sodium acetate buffer (pH 6.5) with acetonitrile/water gradient (0% to 10% in 10 minutes, 10% to 20% in 15 minutes, 20% to 31.5% in 7 minutes and 31.5% to 50% in 2 minutes). FDAA derivatives of amino acids were detected by their absorbance at 340 nm.

Synthesis of L-3-Amino-4-hydroxy-1-butanoic acid (L-Aspol; **3**) and DL-Aspol

Methanolic solution (15 ml) of t-butyloxycarbonylasparagine (t-Boc-Asn) (500 mg) was added dropwisely by diazomethane in ether (20ml) and evaporated. Residual solid was dissolved in hexane - ethylacetate (3 : 7 v/v) and was purified by a silica gel column (Wacogel C-100, 100 ml), then t-Boc-Asn methyl ester (**4**) was obtained in hexane - ethylacetate (1 : 9 v/v) (160 ml) and ethylacetate eluate (160 ml) as a white solid (390 mg). Anhydrous THF solution (20 ml) of **4** (390 mg) was added by $LiBH_4$ and was stirred at room temperature for 20 hours. After degradation of $LiBH_4$ by water, the reactant was applied to silica gel column (Wacogel C-100, 50 ml) and was eluted by ethylacetate and ethylacetate - methanol (95 : 15 v/v). In the later fraction, L-*N*-t-butyloxycarbonyl-4-amino-3-hydroxybutanamide (t-Boc-Asnol, **5**) was recovered (54 mg). Hydrolysis of **5** by constant boiling point 6 N HCl (110°C, 22 hours) yielded L-Aspol (**3**): ^{13}C NMR (75 MHz, CD_3OD) δ 28.7 ($3\times CH_3$), 38.3 (CH_2), 51.3 (CH), 64.7 (CH_2), 80.2 (C), 157.8 (CO) and 176.4 (CO); ^1H-NMR (300 MHz, CD_3OD) δ 1.49 (9H, s), 2.40 (1H, m), 2.52 (1H, m), 3.58 (2H, m), 3.97 (1H, m).

DL-Aspol was prepared in a similar way from DL-asparagine.

Biological Activity of Rotihibin A (**1**)

Cytotoxity of **1** to K562 cell was measured in RPMI640 medium with 10% fetal calf serum (FCS), and to FM3A and HSG cells was measured in Dulbeco MEM medium with 10% FCS. To the media with each cells, 50% methanol solution of **1** was added. Each cells were seeded to the media at 1×10^5 cells/ml in 96 well plate. After the culture at 37°C under humidified atmosphere in CO_2 incubator for 4 days, the cell number was counted.

Antimicrobial activity of **1** was measured by observing inhibition circles on the agar plates seeded by the test bacteria and fungi and put by the paper disks with sample solution.

Lettuce seedling test was performed by incubating ten lettuce seeds in 5 cm diameter petri dishes containing filter papers and Murashige-Skoog's medium with sample. The petri dishes were incubated under 3000 lux white light at 30°C for 4 days and the length of roots was measured.

Inhibitory activity on *Nicotiana rustica* was measured as below. The shoot with three leaves of *N. rustica* grown to 6~7 leaves height was cut off and cultured on Murashige-Skoog's medium (Nihon Seiyaku Co.) with 0.2% gelan gum in a glass tube. The medium was added by filtered solution of **1** after autoclave. After 30 days of culture at 25°C under 3000 lux of white continuous light, the length of shoot and the number of leaves were counted.

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References

1) FUKUCHI, N.; K. FURIHATA, S. TAKAYAMA, A. ISOGAI & A. SUZUKI: Rotihibin A, a novel plant regulator, from *Streptomyces* sp. Biosci. Biotech. Biochem. 56: 840~841, 1992

2) FUKUCHI, N.; J. NAKAYAMA, S. TAKAYAMA, A. ISOGAI & A. SUZUKI: Structural elucidation of rotihibin B by tandem mass spectrometry. Biosci. Biotech. Biochem. 56:

1152～1152, 1992

3) YAMAGUCHI, T.: Comparison of the cell-wall composition of morphologically distinct actinomycetes. J. Bacteriol. 89: 444～453, 1965
4) PRIDHAM T. G. & H. D. TRESNER: Genus I. Streptomyces Waksman and Henrici, 1943, 339. *In* BERGEY's Manual of Determinative Bacteriology. 8th Ed. *Eds.*, R. E. BUCHAMAN & N. E. GIBBSON, pp. 748～829, Williams & Wilkins Co., 1974
5) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species, Int. J. Syst. Bacteriol. 16: 313～340, 1966
6) BAX, A. & M. F. SUMMER: ^{1}H and ^{13}C assignments from sensitivity-enhanced detection of heteronuclaer multiple-bond connectivity by 2D multiple quantum NMR. J. Am. Chem. Soc. 108: 2093～2094, 1986
7) BAX, A. & S. SUBRAMANIAN: Sensitivity-enhanced two-dimensional heteronuclaer shift correlation NMR spectroscopy. J. Magn. Reson. 67: 565～569, 1986
8) BIEMANN, K.: Contributions of mass spectrometry to peptide and protein structure. Biomed. Environ. Mass Spectro. 16: 99～111, 1988
9) MARFEY, P.: Determination of D-amino acids. II. Use of a bifunctional reagent, 1,5-difluoro-2,4-dinitrobenzene. Carlsberg Res. Commun. 49: 591～596, 1984