Brevicompanines A and B: new plant growth regulators produced by the fungus, *Penicillium brevicompactum*

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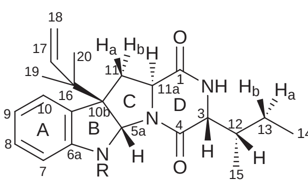
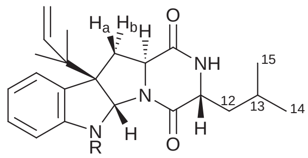
New plant growth regulators, named brevicompanines A 1 and B 2, have been isolated from *Penicillium brevicompactum*, and their structures have been established by spectroscopic methods including 2D NMR. The biological activities of 1 and 2 have been examined using bioassay methods with lettuce and rice seedlings.

Introduction

So far, many compounds have been isolated as plant growth regulators, such as dihydroampullicin,[1] BSF-A,[2] penienone and penihydrone.[3] In the course of our screening search for new plant growth regulators, using bioassay methods with lettuce and rice seedlings, suitable for developing new herbicides and for new lead compounds, we found the presence of plant growth regulators in the cultural metabolite of *Penicillium brevicompactum*. Bioassay-guided fractionation led to isolation of the compounds named brevicompanines A **1** and B **2**. In this report, we describe the isolation, structural determination and biological activities of **1** and **2**.



1 R = H
3 R = Ac



2 R = H
4 R = Ac

Results and discussion

The fungus was stationarily cultured in a Czapek–Dox medium (19 l) at 24 °C for 21 days. The culture filtrate was adjusted to pH 2.0 and then extracted twice with ethyl acetate. The ethyl acetate-soluble acidic (11.1 g) and neutral fractions (10.5 g) were then obtained according to standard methods (see Experimental section). The latter fraction was purified with a silica gel column, and final purification using reversed-phase high-performance liquid chromatography (HPLC) afforded brevicompanines A **1** and B **2**.

Brevicompanine A **1** was obtained as a white amorphous solid. The molecular formula of **1** was established as $C_{22}H_{29}$-N_3O_2 by high-resolution electron impact mass spectrometry (HREIMS), requiring ten degrees of unsaturation. The compound was positive to Ehrlich and Dragendorff reagents. The UV spectrum showed absorption maxima at λ_{max} 210, 245 and 303 nm, which was similar to those found for aszonalenin.[4] The IR spectrum showed characteristic absorption bands at ν_{max} 3336 and 1680 cm^{-1}, indicating that the compound possesses a diketopiperazine unit.[5] Two signals at δ_C 169.38 and 165.95 in the ^{13}C NMR spectrum also supported this result. ^{13}C NMR, distortionless enhancement by polarization transfer (DEPT) and ^1H NMR spectral data (Table 1) indicated the presence of a total of 22 carbons, including two aromatic and two aliphatic quaternary carbons, two amides, one vinyl group, four aromatic and four aliphatic methine carbons, two aliphatic methylene carbons and four methyl carbons, and revealed the presence of two D_2O exchangeable protons. The assignments of ^1H and ^{13}C NMR shown in Table 1 were confirmed by analyses of pulsed field gradient double quantum filter correlation spectroscopy (PFG-DQF COSY)[6] and ^1H–^{13}C pulsed field gradient heteronuclear multiple quantum coherence spectral (^1H–^{13}C PFG-HMQC)[7] data. In particular, ^1H–^{13}C pulsed field gradient heteronuclear multiple-bond correlation (^1H–^{13}C PFG-HMBC) experiments[8] (Fig. 1) established the connectivities of partial structures and assignments of all other quaternary carbons and two amido carbons at C-1 and C-4. ^1H–^{15}N PFG-HMQC[9] spectral data were very useful for distinction of a secondary amino proton at δ_H 4.92 (6-NH) and a secondary amido proton at δ_H 6.69 (2-NH). In the spectrum, the amido proton at δ_H 6.69 was correlated to a nitrogen at δ_N 87.3 (2-NH) and the other amido proton at δ_H 4.92 was correlated to a nitrogen at δ_N 61.6 (6-NH). These chemical shifts for δ_N were with respect to an external reference of $^{15}NH_4NO_3$ at 0 ppm in DMSO solution. A remaining nitrogen (5-N) was observed at δ_N 124.9 by ^1H–^{15}N PFG-HMBC experiments[10–12] (Fig. 2) through correlations

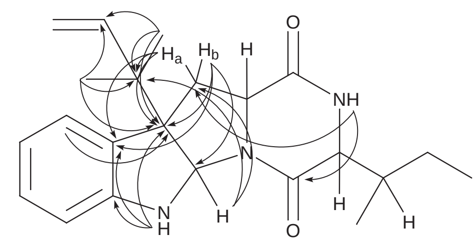


Fig. 1 Key ^1H–^{13}C PFG HMBC experimental data for **1**

Table 1 ^1H (600 MHz) and ^{13}C (150 MHz) NMR data for **1** and **2** in $CDCl_3$

	1		**2**	
Position	$^{13}C^a$	$^1H^b$	^{13}C	^1H
1	169.15 (s)		169.04 (s)	
2		6.18 (br, s)		6.18 (d, 2.5)
3	61.10 (d)	3.93 (dd, 3.4, 3.4)	56.00 (d)	3.90 (ddd, 10.3, 4.9, 2.5)
4	165.93 (s)		166.57 (s)	
5a	77.66 (d)	5.59 (s)	77.67 (d)	5.56 (s)
6		4.90 (s)		4.90 (s)
6a	150.05 (s)		149.94 (s)	
7	108.77 (d)	6.59 (d, 7.8)	109.04 (d)	6.59 (d, 7.8)
8	128.97 (d)	7.10 (dd, 7.8, 7.3)	128.92 (d)	7.11 (dd, 7.8, 7.3)
9	118.81 (d)	6.78 (dd, 7.8, 7.3)	118.28 (d)	6.76 (dd, 7.3, 7.3)
10	125.12 (d)	7.15 (d, 7.8)	125.08 (d)	7.16 (d, 7.3)
10a	128.67 (s)		128.80 (s)	
10b	61.17 (s)		61.17 (s)	
11	37.06 (t)	2.38 (dd, 12.2, 11.7), Ha 2.54 (dd, 12.2, 5.9), Hb	36.66 (t)	2.43 (dd, 12.7, 11.7), Ha 2.56 (dd, 12.7, 6.4), Hb
11a	57.91 (d)	3.92 (dd, 11.7, 5.9)	57.76 (d)	3.90 (dd, 11.7, 6.4)
12	39.85 (d)	2.02 (dddq, 8.3, 6.8, 6.4, 3.4)	42.82 (t)	1.52 (ddd, 13.7, 10.3, 5.4) 1.58 (ddd, 13.7, 9.3, 4.9)
13	25.78 (t)	1.26 (ddq, 13.7, 8.3, 7.3), Hb 1.36 (ddq, 13.7, 7.3, 6.4), Ha	24.27 (d)	1.69 (ddqq, 9.3, 5.4, 6.8, 6.8)
14	11.51 (q)	0.92 (d, 7.3)	23.02 (q)	0.92 (d, 6.8)
15	13.83 (q)	0.77 (d, 6.8)	21.21 (q)	0.91 (d, 6.8)
16	40.90 (s)		40.90 (s)	
17	143.46 (d)	5.97 (dd, 19.6, 10.8)	143.43 (d)	5.97 (dd, 17.6, 10.8)
18	114.55 (t)	5.08 (d, 17.6), 5.12 (d, 10.8)	114.57 (t)	5.08 (d, 17.6), 5.12 (d, 10.8)
19	22.41 (q)	1.12 (s)	22.40 (q)	1.12 (s)
20	22.91 (q)	1.01 (q)	22.85 (q)	1.01 (s)

^{a} The s, d, t and q in the ^{13}C NMR spectral data show multiplicities determined by DEPT experiments. ^{b} The s, d and q, and the numbers in parentheses in the ^1H NMR spectral data show multiplicities and coupling constants in Hz.

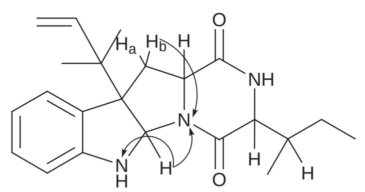


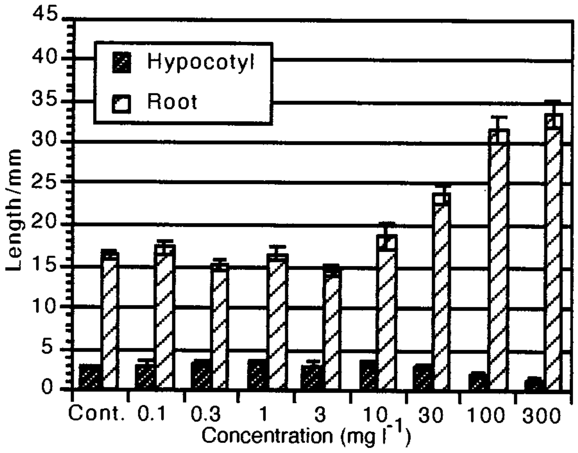
Fig. 2 ^1H–^{15}N PFG HMBC experimental data for **1**

from 5a-H and 11-Hb. In this spectrum, two-bond correlation from 5a-H to 6-N was observed, and direct coupling constant values ($^1J_{NH}$) of 91 and 86 Hz for the amide 2-NH and secondary amine 6-NH were also observed, respectively. Thus, the planar structure of **1** was established by consideration of those data. The structure was also supported strongly by the MS fragmentation pattern.[13] Namely, a prominent peak at *m*/*z* 298 (base peak) was assigned to be the fragmentation of **1** by the loss of a C_5H_9 radical. The fragment peaks at *m*/*z* 194 and 130 were reasonable as the peaks originated from the cleavage of the diketopiperazine unit, and the peaks at *m*/*z* 185 and 157 originating from the cleavage of diketopiperazine unit were also observed.

The relative stereochemistry of **1** was determined by differential nuclear Overhauser enhancement (NOE) and one-dimensional selective rotating frame nuclear Overhauser enhancement spectroscopy (1D selective ROESY)[14] experiments. In the differential NOE spectra, NOEs from 17-H to 5a-H and from 20-H_3 and 5a-H indicated that the B and C rings were connected with a *cis* junction. The coupling constant between 11a-H and 11-Ha (J = 11.7 Hz) as well as the NOE enhancement from 15-H_3 to 11a-H suggested that the vinyl allyl group on C-10b, methine protons of C-3, C-5a and C-11a were of β, β, β and α orientation, respectively. The ROESY correlation from 13-Hb to 2-NH along with the J-value between 3-H and 12-H (J = 3.4 Hz) indicated that the relative configurations of C-3 and C-12 were 3*R** and 12*S** of the *allo*-isoleucine unit, respectively.

Brevicompanine B **2** was also obtained as a white amorphous solid with a molecular formula of $C_{22}H_{29}N_3O_2$ by HREIMS, which was identical with that of **1**. The UV, IR and mass spectra of **2** were similar to those of **1**, indicating the presence of a 2,3-substituted indole moiety and diketopiperazine unit in the molecule of **2** like **1**. The ^{13}C and ^1H NMR spectra of **2** (Table 1) indicated the presence of two methyl signals and one methylene signal, and the remaining signals were similar to those found for **1**. These data suggested that the *allo*-isoleucine unit in **1** is replaced with a leucine moiety in **2**. Analyses of the differential NOE and 1D selective ROESY data provided the relative stereochemistry of **2**.

The absolute configurations of compounds **1** and **2** were established as follows. Hydrolysis of the mixture of **1** and **2** (7 : 3, 10 μg) with 6 M HCl followed by amino acid analysis confirmed the presence of *allo*-isoleucine, leucine and tryptophan. The absolute configurations of the *allo*-isoleucine, leucine and tryptophan were determined by using FLEC methods.[15] The resulting derivatives were analyzed by reversed-phase HPLC. From these results, the absolute configurations of *allo*-isoleucine, leucine and tryptophan were determined to be D-*allo*-isoleucine, D-leucine and L-tryptophan, respectively. Hence, the absolute configurations of **1** and **2** were established as 3*R*, 11a*S*. Furthermore, by comparison of CD data for a mixture of monoacetamides **3** and **4** (see Experimental section) [+25 700 (248 nm)] with those of related compounds, dihydrofructigenines A **5** [+27 400 (247 nm)] and B **6** [+25 100 (248 nm)][5] possessing the same ring systems, **1** and **2** should have the same type of absolute configurations as those of **5** and **6** at the B and C ring junction. The presence of a D-amino acid in these molecules of **1** and **2** has not been reported among alkaloids with similar skeletons. The biological activities of **1** and **2** were examined using bioassay with lettuce[16] and rice seedlings.[17] With lettuce seedlings, both **1** and **2** showed inhibitory activity toward the hypocotyl elongation of the seedlings at a concentration of 100 mg l^{-1} as shown in Fig. 3. However, **1** accelerated the root growth of the seedlings in proportion to its concentration from 10 mg l^{-1} to 300 mg l^{-1}, and **2** promoted it weaker than **1** in proportion to its concentration from 10 mg l^{-1} to 300



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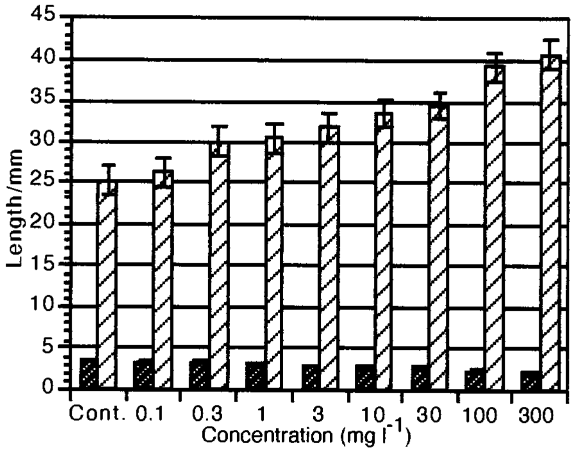

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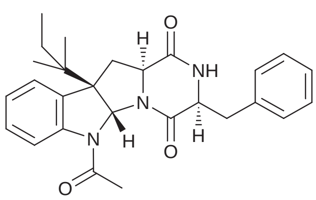
Fig. 3 Effects of **1** and **2** on the hypocotyl and root length of lettuce seedlings (cv. Kingcisco)



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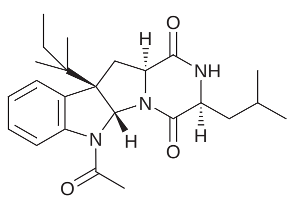

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mg l^{-1}. Both **1** and **2** showed no inhibitory effect on the root and stem elongation of rice seedlings at a concentration of 300 mg l^{-1}. These results may suggest that each active site or the receptor of **1** and **2** on lettuce seedlings should be different from that on rice seedlings.

Experimental

General

Melting point (mp) data were determined with a YANACO MP-S3 instrument. Optical rotation values were recorded with a HORIBA SEPA-200 instrument. The IR and UV spectra were recorded with JASCO FT/IR 700 and SHIMADZU UV 2200 instruments, respectively. The NMR spectra were recorded with a JEOL-JNM-A600 spectrometer (at 600 MHz for ^{1}H and 150 MHz for ^{13}C). *J* Values are given in Hz. The mass spectra were recorded with a HITACHI M-80B apparatus. The CD spectra were measured with a JASCO J-720 spectropolarimeter. Reversed-phase HPLC purifications were performed on Wakosil 5C18 column (7.5 × 250 mm) using a SHIMADZU LC-3A pump with a flow rate of 2.0 ml min^{-1}. Column chromatography was performed on silica gel of 200 mesh. HPLC analysis of amino acid derivatives employed a SHIMADZU LC-9A system controller using PEGASIL ODS-3 column (4.6 × 250 mm). Analysis of the amino acids in hydrolysates was carried out using an HITACHI L-8500 A amino acid analyzer. Analytical TLC and preparative TLC were performed on Merck pre-coated silica gel 60 F_{254} and Merck Kiselgel 60 GF_{254} (10 g silica gel spread on 20 × 20 cm glass plates), respectively.

Extraction and isolation

Seventy-six Erlenmeyer flasks (500 ml), each flask containing 250 ml of Czapek–Dox medium supplemented with 3% polypeptone were inoculated with spores of *P. brevicompactum* previously grown on solid potato dextrose agar. The fungus was stationarily grown at 24 °C for 21 days. The culture broth (19 l) was filtered, and the filtrate was adjusted to pH 2.0 with a 2 M HCl solution. The filtrate was then extracted with ethyl acetate and evaporated to 1 l. The remaining solvent was washed with water saturated with $NaHCO_3$. The remaining ethyl acetate extract was concentrated *in vacuo* and the residue (10.5 g) was fractionated by a Wacogel C-200 column with *n*-hexane–ethyl acetate mixture. The fraction eluted with 40% ethyl acetate (6.99 g) was further chromatographed on a Wacogel C-200 column with *n*-hexane–acetone mixture, giving a fraction (3.72 g, eluting with 15% acetone) containing a mixture of brevicompanines A **1** and B **2** (7 : 3, 8.4%). A part of the mixture was fractionated *via* C_{18} reversed-phase HPLC. Preparative HPLC using a column with 60:40 H_2O–acetonitrile as eluent gave **1** (10.5 mg, 33 min) and **2** (4.3 mg, 38 min).

Brevicompanine A 1. A white amorphous solid; mp 61–65 °C; $[\alpha]_D^{20}$ −237.5 (*c* 0.73, EtOH); λ_{max}(EtOH)/nm 210, 245 and 303; ν_{max}(KBr)/cm^{-1} 3336, 1680, 1647, 1446, 1319, 745; *m*/*z* (EI) 367 (M^+, 25%) (HREIMS found M^+, 367.2236. Calc. for $C_{22}H_{29}N_3O_2$, 367.2214); ^{1}H and ^{13}C NMR data are listed in Table 1; δ_N($CDCl_3$) 61.6 ($^{1}J_{NH}$ 86, N-6), 87.3 ($^{1}J_{NH}$ 91, N-2), 124.9 (N-5).

Brevicompanine B 2. A white amorphous solid; mp 79–82 °C; $[\alpha]_D^{20}$ −228.3 (*c* 0.46, EtOH); λ_{max}(EtOH)/nm 208, 245 and 300; ν_{max}(KBr)/cm^{-1} 3340, 1680, 1607, 1446, 1319, 746; *m*/*z* (EI) 367 (M^+, 20%) (HREIMS found M^+, 367.2246. Calc. for $C_{22}H_{29}N_3O_2$, 367.2235); ^{1}H and ^{13}C NMR data are listed in Table 1; δ_N($CDCl_3$) 61.7 ($^{1}J_{NH}$ 86, N-6), 92.2 ($^{1}J_{NH}$ 93, N-2), 122.0 (N-5).

Hydrolysis of a mixture of 1 and 2

A mixture of **1** and **2** (7 : 3, 10 µg) was hydrolyzed in 1 µl of 6 M HCl at 110 °C for 5 h in a sealed tube. The cooled reaction mixture was evaporated to dryness and then analyzed using a HITACHI L-8500 A amino acid analyzer with standard solution of amino acids.

FLEC Derivatization of amino acids and HPLC analysis

Derivatization of hydrolysate residue of **1** and **2** was carried out according to the reported procedure.[15] The derivatives were analyzed by reversed-phase HPLC with fluorescence detection for DL-*allo*-isoleucine and DL-leucine and with UV detection at 280 nm for DL-tryptophan. The mobile phase containing of acetonitrile, tetrahydrofuran and an acetic acid buffer (1.8 ml of glacial acetic acid was dissolved in 1 l of water; pH was adjusted to 4.35 with NaOH).

Acetylation of a mixture of 1 and 2

A mixture of **1** and **2** (20 mg) was acetylated with pyridine–acetic anhydride (0.75 ml, 2:1) and the solution was kept at 24 °C for 24 h. Purification by preparative TLC in *n*-hexane–ethyl acetate (45:55 v/v) gave a mixture of monoacetamides **3** and **4** as colorless amorphous solids.

Bioassay for the growth of lettuce seedlings

Lettuce seeds were sown in a Petri dish (150 × 25 mm) lined with a filter paper containing deionized water. After 1 day under light at 24 °C, seedlings were selected for uniformity (radicles; 2 mm) and transferred into a mini-Petri dish (35 × 15 mm) lined with filter paper containing 1 ml of deionized water and a defined amount of the test compound. The Petri dishes were kept at 24 °C for 4 days under continuous light. The length of the hypocotyls and roots treated with the compounds **1** and **2** were measured and the mean value of the length was compared with an untreated control.

Bioassay for the growth of rice seedlings

The rice seeds (*Oriza sativa* L., cv. Yamabiko) were sterilized with 75% ethanol for 30 s, rinsed with sodium hypochlorite solution (antihormin) for 2 h and placed in a Petri dish (150 × 25 mm) containing deionized water. After 3 days at 30 °C under light, seven seedlings were selected for uniformity (radicles; 2–3 mm) and transferred into a test tube lined with filter paper containing 1 ml of deionized water and a defined amount of the test compound. The test tubes (23 × 140 mm) sealed with a sheet of polyethylene film were incubated at 30 °C for 7 days under continuous light. The length of total, second leaf sheath and primary root after treated with the compounds **1** and **2** were measured and the mean value of the length was compared with an untreated control.

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