New Convulsive Compounds, Brasiliamides A and B, from Penicillium brasiliatinum Batista JV-379

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Received February 14, 2002; Accepted May 1, 2002

New convulsive compounds, brasiliamides A (1) and B (2), were isolated by activity-guided fractionation from okara fermented with a soil isolate of Penicillium brasiliatinum Batista JV-379. Their structures were elucidated on the basis of spectral and chemical evidence and by X-ray crystallography of the hydrogenated product of 2. In the 1H- and 13C-NMR spectra of 2, the signals were complicated, all being doubled or broadened in several deuterated solvents at room temperature. The conformational change of 2 was clarified as the rotational isomerization of amide bonds in solution by NMR measurements at various temperatures. Four rotamers of 2 at two amide bonds were presented at −60°C in CDCl3, whereas only two isomers were apparent at room temperature, owing to rapid rotation of one of the amide bonds. Brasiliamides A and B respectively showed convulsive activity against silkworms with ED50 values of 300 and 50 μg/g diet.

Key words: Penicillium brasiliatinum Batista; brasiliamide; rotational isomerization; convulsive activity; okara

Our studies on bioactive fungal metabolites, using a bioassay with the silkworm (Bombyx mori), have yielded several isolates from soil samples collected in and around Sakai. Penicillium simplicissimum ATCC 90288 produced the insecticidal indole alkaloids, okaramines A, B, D-G, J-R, and related compounds,1,2) and Aspergillus japonicus ATCC 204480 produced the paralytic alkaloids, asperpara- lines A-C.3) In the course of screening fungi for bioactive compounds, isolate No. JV-379 causing convulsions in silkworms was obtained. The strain was identified as Penicillium brasiliatinum Batista from its morphological and cultural characteristics. We isolated two new substances named brasiliamides A (1) and B (2) from the strain cultured with okara (the water-insoluble residue of whole soybean). In this paper, we report the isolation and structural elucidation of these compounds and their convulsive activity in the silkworm. In addition, we describe the rotational isomerization of 2 in solution due to rotational restriction of two amide bonds.

Materials and Methods

Melting point (mp) data are uncorrected. Optical rotation was measured in a Horiba model SEPA-300 polarimeter. IR and UV spectra were recorded with a Perkin-Elmer 1760X FT-IR spectrophotometer and a Hitachi UV-3210 spectrophotometer, respectively. Mass spectra were measured with a JEOL JMS-600 instrument. 1H- and 13C-NMR spectra were obtained with a JEOL JNM A-500 spectrometer. Chemical shifts are given on a δ (ppm) scale, and the pulse delay time between scans was 5.0 sec for 13C-NMR measurement. Analytical TLC was carried out with Kieselgel 60F254 (No. 105554) and RP-18 F254S (No. 105559) plates (Merck). Column chromatography was performed with Wakogel C-200 (Wako Pure Chemical Industries), Kieselgel 60 (Merck) and Chromatorex ODS (Fuji Silysia Chemicals). trans-2, 5-Dimethylpiperazine was purchased from Aldrich Chemical Co. Okara used as a medium in this experiment was kindly supplied by Kitagawa Tofu (bean-curd) shop (Sakai, Osaka, Japan).

Bioassay. Larvae of Bombyx mori were used for the bioassay, having been cultured on an artificial diet purchased from Nippon Nosan Kogyo Co. One hundred microlitres of a methanolic extract or a certain amount of the sample to be tested were added to one gram of the diet in a Petri dish. After removing the solvent, ten larvae at the third instar stage were introduced into the Petri dish. Thirty larvae (three Petri dishes) were treated at each dosage, and the behavior of the silkworms was observed 5 h and 24 h after initiating the administration. MeOH was used as a control treatment. The convulsive activities of tested compounds at each dose were examined in duplicated experiments. The average convulsion rate from each dose after 24 h was estimated and used for determining the ED50 values.

Fermentation. Strain JV-379 was isolated from a
soil sample collected around Sakai (Osaka, Japan) in the usual manner, and identified as *Penicillium brasiliannum* Batista at Centraalbureau voor Schimmelcultures (The Netherlands). A loopful of spores from a slant culture of the *P. brasiliannum* strain was inoculated into 30 g of okara in a Petri dish (9 cm in diameter) and cultivated at 25°C for 14 days.

**Extraction and preliminary separation.** The okara (7.0 kg) that had been fermented with strain JV-379 was soaked in MeOH. After removing the solvent from the MeOH extract, an aqueous concentrate was successively extracted with n-hexane and EtOAc. The EtOAc extract was dried over anhydrous sodium sulfate, filtered, and evaporated to dryness. The residue (4.0 g) was chromatographed on Wakogel C-200 (40 g) by eluting with n-hexane and an increasing ratio of EtOAc, and then by EtOAc and an increasing ratio of MeOH to afford a 70% EtOAc eluate (0.90 g), and 100% EtOAc and 5% MeOH eluates (1.16 g).

**Isolation of 1.** The 100% EtOAc and 5% MeOH eluates (1.16 g) were chromatographed on Kieselgel 60 (18 g) by eluting with benzene and an increasing volume of acetone. The 20% acetone eluate (285 mg) was flash-chromatographed on Kieselgel 60 (10 g) by eluting with benzene-acetone (8:1) to afford an active fraction (123 mg). This active fraction was further flash-chromatographed on Chromatorex ODS (5 g) by eluting with H2O-MeOH (4:6) to afford basiliamide A (1, 58 mg).

**Brasiliamide A (1):** colorless prisms; mp 133–134°C; EIMS m/z: 438 (M+), 396, 379, 336, 305, 277, 247, 218, 165, 111, 91. HR-EIMS m/z (M+): Calcd. for C23H26N2O6: 438.1791, Found: 438.1793. UV \( \lambda_{max} \) (MeOH) nm (ε): 253 (42,000), 235 (21,000), 253 (16,000). IR \( \nu_{max} \) (KBr) cm⁻¹: 3434, 3289, 1701, 1673, 1509, 1373, 1198, 1132, 1091, 1043. ¹H- and ¹³C-NMR (CDCl₃): see Table 1.

**Isolation of 2.** The 70% EtOAc eluate (0.90 g) was chromatographed on Kieselgel 60 (18 g) by eluting with benzene and an increasing volume of acetone. The 10% and 20% acetone eluates (652 mg) were chromatographed on Kieselgel 60 (10 g) by eluting with n-hexane and an increasing ratio of EtOAc in 5% steps. The 45% and 50% EtOAc eluates (125 mg) were flash-chromatographed on Kieselgel 60 (6 g) by eluting with n-hexane and EtOAc (65:35) to afford an active fraction (32 mg). This active fraction was further flash-chromatographed on Chromatorex ODS (5 g) by eluting with H2O-MeOH (4:6) to afford basiliamide B (2, 30 mg).

**Brasiliamide B (2):** amorphous powder; mp 59–61°C; [α]D⁺ + 195° (c 0.62, MeOH); EIMS m/z: 422 (M⁺), 380, 337, 289, 247, 165. HR-EIMS m/z (M⁺): Calcd. for C19H22N2O5: 422.1841. Found: 422.1842. UV \( \lambda_{max} \) (MeOH) nm (ε): 208 (46,000), 270 (21,000). IR \( \nu_{max} \) (KBr) cm⁻¹: 3488, 1646, 1508, 1407, 1194, 1124, 1090, 1043. ¹H- and ¹³C-NMR (CDCl₃) at room temperature: see Table 2. ¹H- and ¹³C-NMR (CDCl₃) at 20°C and −60°C (Figs. 3 and 4): see Table 2. ¹H-NMR at 95°C (DMSO-d₆) \( \delta_{H} \): 1.90 (3H, s, H-2’), 2.13* (H-3’), 2.4–2.6* (overlapped with solvent, H-12a), 2.73 (1H, dd, J = 5.5, 13.7 Hz, H-12b), 3.39* (approximately 1H, H-10b), 4.09 (1H, d, J = 15.3 Hz, H-7b), 4.73* (H-11), 5.89 (2H, s, H-20), 6.38 (1H, s, H-6), 6.39 (1H, s, H-2), 6.51* (H-1’), 7.18–7.30 (5H, m, H-14–18). The signals marked * were broadened and their integrated values were insufficient for the corresponding number because of equilibrium in the rotational isomerization on the amide bonds. ¹³C-NMR at 95°C (DMSO-d₆) \( \delta_{C} \): 21.1* (q, C-2’), 22.7 (q, C-4’), 35.8 (t, C-12), 37.4 (t, C-7), 45.0* (t, C-10), 51.6* (d, C-11), 56.7 (q, C-19), 100.9 (t, C-20), 102.3 (d, C-6), 108.8 (d, C-2), 114.2* (d, C-1’), 119.8 (scarcey appeared, s, C-8), 126.5 (d, C-16), 128.4 (2xC, d, C-15, 17), 129.1 (2xC, d, C-14, 18), 133.5 (s, C-4), 134.0 (s, C-1), 137.5* (s, C-13), 143.1 (s, C-3), 148.5 (s, C-5), 166.3 (s, C-1’), 168.6 (s, C-3’). The signals marked * were broadened and of small intensity.

**Hydrogenation of 2.** Into an EtOH solution (2 ml) of 2 (6.0 mg), 5% Pd/C (5 mg, E106NN/W, Degusa Chemicals Co.) was added, and the mixture stirred under hydrogen gas at room temperature for 24 h. After removing the catalyst, the filtrate was evaporated and subjected to Chromatorex ODS column chromatography (H2O-MeOH, 5:5). A hydrogenated product was obtained as a colorless powder (3, 3.8 mg). RF value by TLC (RP-18F254S): 0.42 (H2O-MeOH, 3:7). EIMS m/z: 424 (M⁺), 333, 291, 259, 217. ¹H- and ¹³C-NMR in CDCl₃ at room temperature: see Table 3.

**X-ray analysis of 3.** Dihydrobrasiliamide B (3) was crystallized from MeOH to give rhombic prisms, mp 187–188°C. These crystals, 0.10×0.15×0.20 mm in size, were monoclinic (P2₁) with lattice parameters \( a = 11.492(2), b = 7.927(2), c = 12.879(2) \text{ Å}, V = 1188.7 (5) \text{Å}^3, Z = 2, \) and \( D_\text{c} = 1.260 \text{ g/cm}^3. \) All reflections with \( 2\theta_{\text{max}} < 55.0° \) were collected at 23°C in the \( \omega-2\theta \) scanning mode with a Rigaku AFC5R diffractometer (λ (MoKα)=0.71069Å). Of the 2892 reflections collected, 2758 were judged after correcting for the Lorentz and polarization effects. The structure was solved by the direct method and by using the Fourier technique with the teXsan crystallographic software package (Molecular Structure Corporation). Full-matrix least-squares refinement, with
Acetylation of trans-2,5-dimethylpiperazine. **trans-1,4-Diacetyl-2,5-dimethylpiperazine** was prepared from trans-2,5-dimethylpiperazine by using acetic anhydride in pyridine in the usual way. The reaction mixture was purified by flash-chromatography (Kieselgel 60), using benzene-acetone (4:1) as the eluent to afford a colorless powder. 

1H- and 13C-NMR for E1 form (CDCl3) δH: 1.18 (d, J = 7.3 Hz, 2-Me and 5-Me), 2.08 (s, H-8 and 10), 3.37 (dt, J = 13.7, 1.2 Hz, H-3a and 6a), 3.47 (dd, J = 13.7, 4.0 Hz, H-3b and 6b), 4.89 (m, H-2 and 5); δC: 14.5 (q, 2-Me and 5-Me), 21.6 (q, C-8 and 10), 43.4 (d, C-2 and 5), 45.0 (t, C-3 and 6), 169.6 (s, C-7 and 9). Z1 (ZZ2) form δH: 1.12 (d, J = 7.3 Hz, 2-Me), 1.28 (d, J = 7.3 Hz, 5-Me), 2.08 (s, H-8), 2.16 (s, H-10), 2.95 (dd, J = 13.7, 4.0 Hz, H-3a), 3.42 (dt, J = 13.7, 1.2 Hz, H-3b), 4.91 (m, H-2); δC: 14.7 (q, 2-Me), 15.6 (q, 5-Me), 21.2 (q, C-10), 21.6 (q, C-8), 39.5 (t, C-3), 43.3 (d, C-2), 45.3 (t, C-6), 49.0 (d, C-5), 169.3 (s, C-7), 169.5 (s, C-9). E2 form δH: 1.22 (d, J = 7.3 Hz, 2-Me and 5-Me), 2.17 (s, H-8 and 10), 3.02 (dd, J = 13.7, 4.0 Hz, H-3a and 6a), 4.06 (m, H-2 and 5), 4.35 (dt, J = 13.7, 1.2 Hz, H-3b and 6b); δC: 15.6 (q, 2-Me and 5-Me), 21.1 (q, C-8 and 10), 39.7 (t, C-3 and 6), 49.0 (d, C-2 and 5), 169.7 (s, C-7 and 9).

**Results and Discussion**

**Fermentation and isolation of the active compounds**

A strain of *P. brasiliannum* Batista JV-379 was cultured with okara at 25°C for two weeks. The cultivated okara was soaked in MeOH, and the MeOH extract was concentrated in vacuo. An aqueous residue was successively extracted with n-hexane and EtOAc. The active EtOAc extract was chromatographed on Wakogel with a mixed solvent system (n-hexane-EtOAc and EtOAc-MeOH). The 100% EtOAc and 5% MeOH in EtOAc eluates were combined and purified by column chromatography to afford brasiliamide A (I). The 70% EtOAc eluate was chromatographed in Kieselgel and reversed-phase ODS columns to afford brasiliamide B (2).

**Structural determination of brasiliamide A (I)**

Brasiliamide A (I) was obtained as colorless prisms; mp 133–134°C. The molecular formula was established to be C24H26N2O6 from the HR-EIMS data [m/z 438.1793 (M+); 438.1791 calcd. for C24H26N2O6] together with the NMR data (Table 1), being indicative of thirteen degrees of unsaturation.

The UV and IR spectra revealed the presence of aromatic rings [λmax 211 (ε 42,000), 235sh (ε 21,000), and 233sh (ε 16,000) nm; νmax 1509 cm⁻¹]. In addition, the IR spectrum exhibited the presence of a ketone carbonyl (1701 cm⁻¹) and an amide group (1673 cm⁻¹). The 1H-NMR, 13C-NMR and DEPT spectra revealed the presence of two aromatic rings, a monosubstituted benzene ring [δC 127.4 (d), 128.8 (2xC, d), δC 129.5 (2xC, d), 132.8 (s); δH 7.15–7.35, totally 5H] and a 1,2,3,5-tetrasubstituted benzene ring having three oxygenated carbons [δC 103.1 (d), 108.2 (d), 131.4 (s), 134.3 (s), 143.8 (s), 149.1 (s); δH 6.23 (d, J = 1.5 Hz) and δH 6.28 (d, J = 1.5 Hz)], and a trisubstituted double bond [δH 122.2 (d) and 122.5 (s); δH 6.92 (d, J = 10.7 Hz)]. The 13C-NMR spectra also indicated the presence of three carbonyls, a ketone carbonyl (δC 206.6 ppm) and two amide carbonyls (δC 168.3 and 170.8). These NMR data accounted for twelve (i.e., three carbonyls, two benzene rings, and one double bond functional groups) out of thirteen degrees of unsaturation, indicative of the presence of one more ring system. There were four isolated sp³ methylenes and three singlet methyls [one methoxyl (δC 56.7; δH 3.84) and two acetyl methyls (δC 20.1 and 23.2; δH 1.87 and 2.11)]. One sp² methylene observed at δH 101.5 (δH 5.95 and 5.96) revealed the presence of a methylenedioxy ring on the 1,2,3,5-tetrasubstituted benzene ring. The methoxyl group was also attached to this tetrasubstituted benzene ring, suggesting the presence of a 3-methoxy-4,5-methylenedioxophenyl group on the basis of the chemical shifts of the aromatic carbons and meta-coupled aromatic protons. An olefinic proton (δH 6.92, d, J = 10.7 Hz) and an amide proton (δH 9.62, d, J = 10.7 Hz) were mutually coupled, indicating the linkage between a trisubstituted double bond and NH in the acetamide group. The ketone carbonyl should form CH2COCH2 comprising two pairs of isolated methylenes (δH 3.02/4.53 and 3.75/3.81). These partial structures were found to be connected through 1H-13C long-range correlations by COLOC experiments as shown in Fig. 1. The remaining methylene protons (δH 3.19 and 3.29) were correlated to three non-oxygenated carbons at δC 131.4, 103.0, and 108.2 in the 3-methoxy-4,5-methylenedioxophenyl group, suggesting that I had a 3-methoxy-4,5-methylenedioxobenzyl moiety. The methylene protons were also correlated to olefinic carbons at δC 122.2 and 122.5, indicative of the linkage between the 3-methoxy-4,5-methylenedioxobenzyl and a quaternary carbon in the >C = CH-NH-COCH3 moiety. The signals at δH 3.75 and 3.81 for an isolated methylen (H-12) showed cross peaks with carbons in the monosubstituted benzene ring together with a ketone carbonyl, indicating the presence of a ß-CH2COCH2 group. Protons of another methylene (H-10) at δH 3.02 and 4.53 showed cross peaks with the ketone carbonyl and an amide carbonyl at δC 170.8 (C-1δ).
suggesting that the \( \phi \text{-CH}_2\text{COCH}_2 \) group was linked to N-COCH\(_3\). Thus, \( \text{I} \) had an N-acetyl-N-2-oxo-3-phenylpropyl moiety. The N-acetyl-N-2-oxo-3-phenylpropyl group should be linked at N-9 to the quaternary carbon at C-8 in \( \text{II} \) along with the 3-methoxy-4,5-methylenedioxybenzyl group. The geometry of the double bond (C-8-C-1') was confirmed as Z-conformation by the NOE correlation between an olefinic proton (H-1') and aromatic protons (H-2 and 6). Consequently, the structure of \( \text{I} \) was elucidated to be \( N^1, N^2 \)-diacetyl-N\(^2\)-(2-oxo-3-phenylpropyl)-3-(3-methoxy-4,5-methylenedioxyphenyl) - 1,2 - (Z)-propenediamine (Fig. 2).

**Structural determination of brasiliamide B (2)**

Brasiliamide B (2) was obtained as an amorphous powder, mp 59–61°C, \([\alpha]_D^0 +198^\circ\) (c 0.62, MeOH). The molecular formula was established to be C\(_{24}\)H\(_{26}\)N\(_2\)O\(_5\) by the HR-EIMS and NMR data (Table 2), indicating thirteen degrees of unsaturation like \( \text{I} \). The UV and IR spectra exhibited the presence of aromatic rings \( \lambda_{\text{max}} 208 (e 46,000) \) and 270 (e 21,000) nm; \( \nu_{\text{max}} 1508 \text{ cm}^{-1} \). The IR spectrum of \( \text{II} \) also revealed the presence of an amide group \( \lambda_{\text{max}} 1646 \text{ cm}^{-1} \) and the absence of a ketone carbonyl that was observed in \( \text{I} \), indicative of the presence of one more ring system instead of a carbonyl group in \( \text{I} \). In

The spectra were measured at 500 MHz for \(^1\text{H}\) and at 125 MHz for \(^{13}\text{C}\).
on the other hand, sharpened all signals, and some signals derived from minor conformers were observed (Fig. 3b-f). The alteration of an acetyl methyl region was particularly remarkable, and four pairs of acetyl methyl signals were observed in the spectrum at −60°C (Fig. 5). The ratio of these signals was approximately 76:15:7:2. This result suggested that 2 existed in equilibrium with four conformational isomers in solution. We therefore tried to elucidate the structure of 2 with a major conformer in CDCl₃.

In the ¹³C-NMR spectrum measured at −60°C (Fig. 4b), twenty-four carbons were observed, together with three other sets of signals due to minor conformers. The ¹H- and ¹³C-NMR and ¹H-¹H COSY spectra revealed the presence of the same partial structures as those in 1, a monosubstituted benzene ring [δc 126.9 (d), 128.7 (2xC, d), 129.0 (2xC, d), 136.5 (s); δh 7.30–7.40, totally 5H], a 3-methoxy-4,5-methylenedioxyphenyl group [δc 56.0 (q), 101.4 (t), 101.7 (d), 106.2 (d), 132.8 (s), 132.8 (s), 142.7 (s), 147.9 (s); δh 3.89 (3H, s), 6.00 (1H, s), 6.01 (1H, s), 6.38 (2H, s)], and a trisubstituted double bond [δc 112.5 (d) and 120.6 (s); δh 6.23 (s)]. The spectra also indicated the presence of two acetamides [δc 166.6 (s), 21.9 (q); δh 2.34 (3H, s) and δc 169.2 (s), 22.9 (q); δh 1.77 (3H, s)]. Two sets of methylenes (δh 2.98/3.55 and 2.53/3.04) and one methine (δh 4.98) were mutually coupled, substantiating the presence of a CH₃-CHCH₂ moiety. HSQC, HMBC, and COLOC experiments at 20°C were carried out to ascertain the connectivity of these partial structures, because 2D NMR measurements could not be done at −60°C. In the COLOC spectrum in CDCl₃, signals at δh 2.50 and 2.98 (H₂-12) due to one of the methylenes in CH₂CHCH₂ were correlated to aromatic carbon signals at δc 127.0 (C-13) and 129.2 (C-14, 18) in the monosubstituted benzene ring, indicating the presence of a ϕ-CH₂CHCH₂ moiety. On the other hand, the HMBC spectrum in C₆D₆ provided useful and complementary information about the ¹H-¹³C long-range correlations as shown in Fig. 6 and Table 2. One of the benzyl protons at δh 3.23 (H-7) was correlated to olefinic carbons at δc 112.6 (C-1’) and 121.0 (C-8) together with aromatic carbons (C-1, 2 and 6), indicating the linkage of a 3-methoxy-4,5-methylenedioxybenzyl and a quaternary olefinic carbon in the >C=CH- group. As the remaining parts were only two acetamides, the two partial structures might be connected by these acetamides to form a tetrahydropyrazine ring. The correlation of an olefinic proton at δh 6.16 (H-1’) between a methine carbon at δc 51.6 (C-11) and an amide carbonyl (δc 165.5) supports this structure. The whole structure of 2 was thus determined to be 1,4-diacetyl-2-benzyl-5-(3-methoxy-4,5-methylenedioxybenzyl)-1,2,3,4-tetrahydropyrazine (Fig. 2).

Hydrogenation of 2 with 5% Pd/C afforded reductive products. The major product was purified by reversed-phase ODS column chromatography to give a dihydro-derivative (3). The molecular formula of 3 was established to be C₂₄H₂₈N₂O₅, based on EIMS and NMR spectral data. In the ¹H- and ¹³C-NMR spectra of 3, four sets of signals were observed at room temperature due to the conformational change, as had been observed in the spectra of 2 at −60°C. Signals assigned to a methine and a methylene were newly detected in the sp³ region, instead of the two
Fig. 6. 1H-13C Long-range Correlations of 2 from the COLOC and HMBC Spectra.

Table 2. 1H- and 13C-NMR Spectral Data for the Major Rotamer (2a) of 2

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<th>Position</th>
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<th>1H-NMR δh (integral, mult., J Hz)</th>
<th>COLOC (H to C)</th>
<th>13C-NMR δc (mult.)</th>
<th>1H-NMR δh (integral, mult., J Hz)</th>
<th>13C-NMR δc (mult.)</th>
<th>1H-NMR δh (integral, mult., J Hz)</th>
<th>HMBC (H to C)</th>
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The spectra were measured at 500 MHz for 1H and at 125 MHz for 13C. *These signals appeared broadened and small.

Fig. 7. ORTEP Drawing of 3.

olefinic carbon signals of 2, in the 13C-NMR and DEPT spectra, supporting the assertion that 3 was a dihydro-derivative of 2. Crystallization of 3 from methanol yielded rhombic prisms that were suitable for an X-ray crystallographic analysis. A single-crystal X-ray analysis established the complete structure of 3 as trans-1,4-diacetyl-2-benzyl-5-(3-methoxy-4,5-methylenedioxybenzyl)piperazine as shown in Fig. 7. This result indicates that a 2,5-trans-isomer was preferentially produced by hydrogenation. The selectivity of the hydrogen addition was probably influenced by the axial substituent at C-11, a benzyl group, because the catalyst was favorably located on the opposite side of the benzyl group. Consequently, the structure of 2 was absolutely determined to be 1,4-diacetyl-2-benzyl-5-(3-methoxy-4,5-methylenedioxybenzyl)-1,2,3,4-tetrahydropyrazine. The determination of the absolute configuration at C-11 of 2 is now in progress.

Conformational analysis of 2 and 3 in solution Brasiliamide B (2) and its dihydro-derivative (3) showed conformational change in solution, and four sets of isomers appeared in the NMR spectra in CDCl3. To clarify their conformational properties, we first attempted to examine the conformational change by using the model compound, trans-1,4-diacetyl-2,5-dimethylpiperazine. The restricted rotation of an amide bond is well known as the syn- and anti-
isomerization of a C-N bond. The rotational change of monoamide compounds has already been studied by a dynamic NMR technique. However, few reports have dealt with the isomerization of bisamide compounds by using an NMR analysis and an ab initio calculation. G. Montaudo and P. Finocchiaro have investigated the conformational properties of asymmetric bisamides by using the shift reagent, Eu(fod). The conformational change of bisamide compounds was shown to be rotational isomerization of the amide bonds by means of the 1H-NMR spectrum induced by a lanthanide shift. Thus, four conformational isomers corresponding to the rotational change of two C-N bonds were present as rotamers in equilibrium in solution. For example, trans-1,4-diacetyl-2,5-dimethylpiperazine existed as a nearly equipopulated mixture of the four possible rotamers in which the ring methyl groups were biased in the diaxial position. In addition, the ratio of the rotamers (E1, Z1 and Z2 which had an enantiomeric relationship, and E2 as shown in Fig. 8) was respectively estimated to be 3:4:3 in the presence of Eu(fod). The symbol “E” represents a trans-isomer on two carbonyl groups of acetamide, while the symbol “Z” represents a cis-isomer.

In order to evaluate the conformational properties of trans-1,4-diacetyl-2,5-dimethylpiperazine without a shift reagent, the assignment of all signals in the 1H- and 13C-NMR spectra was carried out by the 2D NMR technique (see the Experimental section). Three sets of signals due to each rotamer were completely assigned, and the direction of the acetamide group on each rotamer was determined from differential NOE measurements. In these experiments, the chemical shifts of the ring protons were markedly changed due to the direction of the carbonyl group in acetamide. When the carbonyl group in acetamide (N-1) is directed to the C-2 side, the neighboring methine proton (H-2) is deshielded. When the carbonyl group is opposite the C-6 side, the equatorial proton of methylene (H2-6) is deshielded. The ratio of three rotamers, E1, Z1 (Z2) and E2, was evaluated to be approximately 42:35:23, respectively, on the basis of integral values of the ring methyls.

Four sets of signals due to the rotamers of 3 were distinguishable in the 1H- and 13C-NMR spectra in CDCl3 at room temperature. The ratio of these rotamers was evaluated by the integral values of the acetyl methyls to be approximately 35:33:18:14. Most of the signals, except for the aromatic protons, could be assigned based on their relative intensity, in support of 2D NMR spectral data, and by applying the chemical shifts of the piperazine ring protons in each rotamer (Table 3). The major rotamer was determined to be of E1 form (3a), as both methine protons ([δH 4.99 (H-8) and 5.05 (H-11)]) were deshielded owing to neighboring carbonyl groups. The direction of the acetyl methyls was also confirmed by NOE correlations between an acetyl methyl at δH 1.98 and an equatorial proton of methylene at δH 3.46 (H-1’), and between an acetyl methyl at δH 1.97 and an equatorial proton at δH 3.43 (H-10). The second rotamer was determined to be of E2 form (3d) from the NOE correlations between an acetyl methyl at δH 1.97 and a methine proton at δH 3.97 (H-8), and between an acetyl methyl at δH 1.79 and a methine proton at δH 4.01 (H-11). Even in this case, both methine protons at H-8 and H-11 were shielded, and both equatorial protons [δH 4.67 (H-10) and 4.70 (H-1’)] were deshielded due to the neighboring carbonyl groups. Two minor conformers, the third and fourth ones, were respectively assigned the Z1 form (3b) and Z2 form (3c) on the basis of the chemical shifts of the piperazine ring protons and differential NOE measurements. The structures of each rotamer (3a-d) and their ratios are summarized in Fig. 8. The E-conformers (3a and 3d) were more prominent than the Z-conformers (3b and 3c).

To clarify the conformational properties of 2, the chemical shifts of the tetrahydropyrazine ring protons were compared with those of 3. A methine proton at δH 4.98 (H-11) for the major rotamer was deshielded, indicating that the carbonyl group in N-2’ acetamide was directed to the C-11 side. The direction of the acetyl methyl in N-2’ acetamide was also confirmed by NOE correlation between an acetyl methyl signal at δH 2.34 (H-4’) and an olefinic proton at δH 6.23 (H-1’). On the other hand, an equatorial proton [δH 3.55 (H-10)] was shielded, suggesting that the acetyl methyl in N-9 acetamide was directed to the C-10 side. The direction of the acetyl
the fact that neither the acetyl methyl (H3-2) were presumed to be of Z2 form and the C-8 substituent, a 3-methoxy-4,5-methylenedioxybenzyl group, because they existed at the same planar level. Therefore, the proportions of the rotamers of 2 were quite different from those of 3.

At room temperature, only two conformers were apparent in the NMR spectra of 2 in various solvents. The behavior of the acetyl methyl rotation became apparent by NMR measurements at lower temperatures. The major conformer existed as a mixture of 2a and 2c, and the minor one existed as a mixture of 2b and 2d. This result suggests that an amide bond at N-9 rotated rapidly without any rotational restraint at room temperature, and that another amide bond at N-2’ had rotational restriction. Therefore, only two rotamers corresponding to the syn- and anti-isomers on the amide bond at N-2’ were observed, and some signals due to an acetyl methyl at N-9 and the neighboring ring protons appeared to broaden. The rotational constraint of the C-N bond at N-9 seemed to have been lower than that at N-2.

The convulsive activity of 1-3
The convulsive activity of brasiliamides A (1) and
Convulsive Brasiliamides from *Penicillium brasiliannum*

B (2) was examined with the third instar larvae of silkworm. The activity of 1 and 2 was evaluated as ED₅₀ values of 300 and 50 µg/g of diet, respectively, upon oral administration. The functional moiety for the activity of the brasiliamides was expected to be a 3-methoxy-4,5-methylenedioxybenzyl moiety, namely a piperonyl group, since such natural compounds having a piperonyl group as myristicin and dillapiole have been reported as insecticides. However, the activity of dihydrobrasiliamide B (3) was 400 µg/g of diet, weaker than that of 1, suggesting that a double bond in the tetrahydropyrazine ring was an essential moiety in these structures.

A large number of tremorgens have already been reported. Such tremorgenic mycotoxins as fumitremorgins, verruculogen and penitrems, which showed similar activity against silkworm, have an indole moiety in common. Since brasiliamides do not contain any indole moiety, they could be classified to a new tremorgen group. 1,4-Diacetyl-1,2,3,4-tetrahydropyrazine compounds such as brasiliamide B have not previously been isolated as natural products. Therefore, the biosynthetic pathway of brasiliamides is of interest, and further biological studies on brasiliamides are in progress.

**Acknowledgments**

The authors express their gratitude to Dr. Toshiji Tada of the Research Institute for Advanced Science and Technology at Osaka Prefecture University for the X-ray analysis. They are also grateful to Dr. Kazuhiro Irie of the division of Applied Life Sciences in the Graduate School of Agriculture at Kyoto University for the MS measurements. This research was supported by grant-aid for scientific research (No. 13660111 (C)) from the Ministry of Education, Culture, Sports, Science, and Technology.

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