Two New Dioxopiperazine Derivatives, Arestrictins A and B, Isolated from *Aspergillus restrictus* and *Aspergillus penicilloides*

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In the course of searching for bioactive substances, two new dioxopiperazine derivatives, arestrictins A (1) and B (2), were isolated along with the known dioxopiperazine, cristatin A (3), and the known peptide, asperglucide (4), from the organic extract of the xerophilic fungi, *Aspergillus restrictus* and *Aspergillus penicilloides*. The absolute structures of 1 and 2, except for the configuration of the secondary alcohol in 1, were established by spectroscopic and chemical investigation. The absolute configuration of cristatin A (3) was also determined.

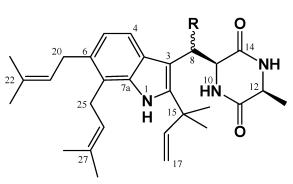
Key words *Aspergillus restrictus*; *Aspergillus penicilloides*; dioxopiperazine; arestrictin A; arestrictin B; cristatin A

Recently large amounts of xerophilic fungi have been found to be widely distributed in our inhabited environment. The typical xerophilic fungi, *Aspergillus restrictus* SMITH and *Wallemia sebi* (FR.) v. ARX., have been very frequently detected in housedust by low water activity (A_w) media such as dichloran 18% glycerol agar (DG-18) medium.[1] From the mycofloristic analyses of housedust samples from asthmatic patients' dwellings collected in Japan,[2] the total fungal counts cultivated on potato-dextrose agar (PDA) medium, which had high A_w value, were 1.3×10^5 CFU (colony formatting units) per 1 g of sample, in which *Penicillium* sp. (3×10^4 CFU) was detected as main fungal flora along with a small amount of xerophilic fungi; *A. restrictus* (10^2 CFU), while the total fungal counts cultivated on DG-18 medium, which had low A_w value, were 1.6×10^6 CFU per 1 g of sample, in which *A. restrictus* (6×10^5 CFU), *Cladosporium sphaerospermum* PENZIG (2×10^5 CFU), *W. sebi* (10^5 CFU), and *Penicillium* sp. (10^5 CFU) were detected as main fungal flora. Therefore, the xerophilic fungi might be considered as the main fungi causing various allergenic diseases such as asthma, but the allergenic activity and the allergens for xerophilic fungus have not been yet investigated, except for reports on the allergenic activity of *W. sebi*[3] and *A. restrictus*.[4] In our studies on the allergenic relationship of xerophilic fungi, we had intended to isolate the metabolites of *A. restrictus* strain A-17, from housedust collected in dwellings in Hyogo prefecture, and *Aspergillus penicilloides* SPEG. strain SUM3319, isolated from the surface of tuna jarky collected in Yokohama city.

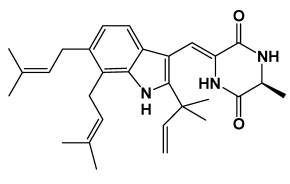
Two new dioxopiperazine derivatives designated as arestrictins A (**1**) and B (**2**) were isolated along with the known dioxopiperazine, cristatin A (**3**), and the known peptide, asperglucide (**4**), from the mycelial CH_2Cl_2–MeOH (1 : 1) extract of *A. restrictus* and *A. penicilloides*, cultivated on M40Y medium. Cristatin A (**3**) had originally been isolated as an immunosuppressive substance from the whole plant of *Lepidagathis cristata* WILLD. (Acanthaceae),[5] but no report of its isolation from the fungal sources had been made, whereas asperglaucide (**4**), identified as *N*-benzoyl-L-phenylalanyl-L-phenylalaninol acetate, had been isolated from *Aspergillus glaucus* LINK: FR.[6] The structural elucidation of **1** and **2** and the absolute configuration of **3** are described in this paper.

The molecular formula of arestrictin B (**2**), a colorless crystalline powder, mp 228 °C (from MeOH), was confirmed as $C_{29}H_{39}N_3O_2$ by high resolution EI-MS. The ^{1}H- and ^{13}C-NMR spectrum of **2** was similar to that of cristatin A (**3**), (Table 1) except for the appearance of aliphatic methylene protons observed at δ 3.17 (dd) and 3.65 (dd) and an aliphatic methine proton at δ 4.38 (ddd) attached to the carbons at δ 29.4 and 54.8, respectively, in **2** instead of an olefinic proton observed at δ 7.20 (d) coupled with two carbons at δ 112.3 and 124.1 in **3**. This fact and the molecular formula indicated that **2** would be a dihydro derivative of **3** at C-8 and C-9. The detailed analysis of the HMBC spectrum of **2** (Fig. 2) determined the planar structure of arestrictins B (**2**).

The molecular formula of arestrictin A (**1**), a pale yellow crystalline powder, mp 209 °C (from MeOH), was confirmed as $C_{29}H_{39}N_3O_3$, from the quasi-dehydration peak in high resolution ESI-TOF-MS [Observed: 460.2967, Calcd for $C_{29}H_{38}N_3O_2$: 460.2959]. The ^{1}H- and ^{13}C-NMR spectrum of **1** was closely similar to that of **2** (Table 1) except for the appearance of an aliphatic methine proton [δ 5.45 (dd)], which was coupled with a hydroxyl group [δ 4.95 (d)], attached to the carbon (δ 69.4) bearing oxygen function in **1** instead of an aliphatic methylene proton observed at δ 3.17 and 3.65 at-



1 : R = OH
2 : R = H



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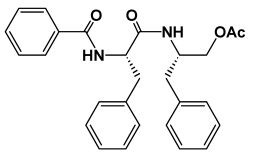

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Table 1. ^{1}H- and ^{13}C-NMR Chemical Shifts of Diketopiperazines (**1**—**3**) in $CDCl_3$

Carbon No.	**1**		**2**		**3**[5]	
	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
1 (NH)		8.21 br s		8.12 br s		8.73 s
2	142.8		140.8		142.9	
3	106.3		104.2		102.9	
3a	125.0		127.2		124.5	
4	117.9	7.54 d (8.2)	115.9	7.28 d (8.2)	116.8	7.06 d (8.1)
5	122.7	6.91 d (8.2)	122.3	6.95 d (8.2)	123.1	6.98 d (8.1)
6	133.2		132.4		133.4	
7	121.5		121.1		121.5	
7a	135.0		134.6		134.6	
8	69.4	5.45 dd (9.2,1.0)	29.4	3.17 dd (14.8. 11.5) 3.65 dd (14.8. 3.5)	112.3	7.20 s
8-OH		4.95 d (1.0)				
9	56.1	4.64 dd (9.2,1.2)	54.8	4.38 ddd (11.5, 3.5, 1.6)	124.1	
10 (NH)		6.73 br s		6.52 br s		6.57 br s
11	167.2		167.9		159.9	
12	50.6	4.08 qd (6.7, 1.2)	50.8	4.08 qd (7.0, 1.6)	51.7	4.30 qd (6.9, 1.8)
12-Me	19.1	1.49 d (6.7)	19.8	1.53 d (7.0)	20.9	1.60 d (6.9)
13 (NH)		5.34 br s		5.72 br s		7.48 br s
14	170.4		168.5		159.9	
15	38.7		39.0		39.2	
16	145.7	6.12 dd (17.6, 10.5)	145.8	6.10 dd (17.4, 10.4)	144.5	6.04 dd (17.3, 10.6)
17	112.5	5.15 dd (10.5, 0.8) 5.21 dd (17.6, 0.8)	112.3	5.15 dd (10.4, 0.9) 5.16 dd (17.4, 0.9)	113.0	5.16 dd (10.6, 1.0) 5.21 dd (17.3, 1.0)
18	28.2	1.51 s	27.8	1.51 s	27.3	1.49 s
19	28.3	1.52 s	28.0	1.51 s	27.4	1.49 s
20	32.0	3.43 d (6.7)	31.9	3.44 d (7.0)	31.9	3.44 d (6.9)
21	124.2	5.21 m	124.2	5.23 m	124.0	5.24 m
22	131.2		131.2		131.4	
23	17.9	1.75 br s	18.0	1.75 d (0.6)	17.9	1.75 br s
24	25.7	1.71 d (1.2)	25.7	1.71 d (1.2)	25.7	1.71 d (1.0)
25	27.2	3.56 d (7.0)	28.2	3.56 d (7.0)	27.2	3.57 d (7.1)
26	123.3	5.32 m	123.2	5.31 m	123.3	5.32 m
27	132.5		133.1		132.7	
28	18.0	1.90 br s	18.0	1.90 d (0.6)	18.0	1.90 br s
29	25.7	1.80 d (0.9)	25.7	1.78 d (0.9)	25.7	1.79 d (1.0)

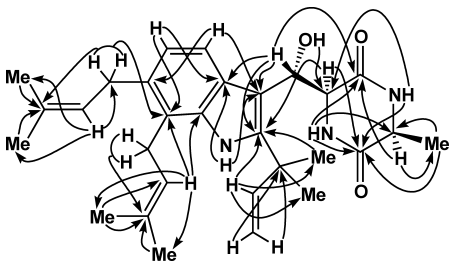

Fig. 1. HMBC Correlations in Arestrictin A (**1**)

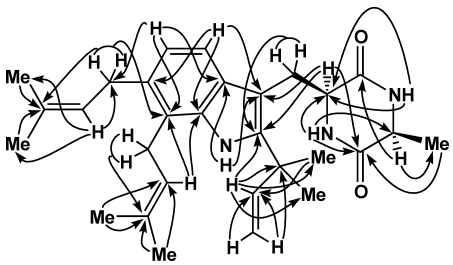

Fig. 2. HMBC Correlations in Arestrictin B (**2**)

tached to the carbon at δ 29.4 in **2**. This result, in consideration of the molecular formula, indicated that **1** would be a hydroxy derivative of **1** at C-8. From the detailed analysis of the HMBC spectrum of **1** (Fig. 1), the planar structure of arestrictins A was confirmed as shown in **2**.

The stereochemistry of the alanine residue in cristatin A (**3**) had not been determined yet.[5] The absolute configuration of **3** ($[\alpha]_D$ $-26.3°$ ($CHCl_3$)), isolated from *A. restrictus* at this time, was determined by the advanced Marfey method.[7,8] For this analysis, **3** was hydrolyzed with HCl to give alanine. Then this alanine was reacted with 1-fluoro-2,4-dinitrophenyl-5-L-alaninamide (L-FDAA) and the product was analyzed with two diastereometric alanine derivatives by reversed-phase HPLC. The alanine residue of cristatin A (**3**) had *S*-configuration (L-alanine residue) and the absolute structure of cristatin A (**3**), isolated from *A. restrictus*, was therefore confirmed. On the other hand, cristatin A (**3**), isolated from the whole plant of *Lepidagathis cristata*, showed a low and opposite optical rotation ($[\alpha]_D$ $+1.2°$ ($CHCl_3$)).[5] Therefore, the alanine residue of cristatin A (**3**) isolated from *L. cristata*, might be *R*-configuration or racemate.

The stereochemistry of the alanine residue in arestrictin A (**1**) was also determined as *S*-configuraton (L-alanine residue) the same as that in cristatin A (**3**), by applying the advanced Marfey method of the acid hydrolysate.[7,8] The absolute configuration of the tryptophan moiety in **1** was determined as *S*-configuration, since the 2.5% of NOE was observed at H-9 [4.64 (dd)] in the difference NOE spectrum when 12-H [4.08 (qd)] was irradiated. The absolute structure of arestrictin A, except for the stereochemistry of the secondary alcohol (8-OH), was consequently confirmed as depicted in **1**. The stereochemistry of the secondary alcohol could not be deter-

mined, since the optical rotation of the obtained 8-*O*-(2-phenylbutyryl)arestrictin A ($[\alpha]_D$ −1.3° (c=0.95, $CHCl_3$)) was very low by the 'partial resolution' method of Horeau[9,10] and the MTPA ester could not be obtained by the advanced Mosher method.[11]

In order to determine the stereochemistry of arestrictin B (**2**), **2** was ozonolyzed in formic acid-water followed by decomposition of the ozonide by warming with hydrogen peroxide and then hydrolysis with 6 M HCl by boiling to detect alanine and aspartic acid in the hydolysate.[12] This hydrolysate was applied to the advanced Marfey method.[7,8] The absolute structure of obtained alanine and aspartic acid was also determined as *S*-configuraton (L-alanine and L-aspartic acid). The absolute structure of arestrictin B was consequently determined as depicted in **2**.

This is the first example that a dioxopiperazine derivative, cristatin A (**3**), was isolated along with new dioxopiperazine derivatives, arestrictins A (**1**) and B (**2**) from fungal sources, the xerophilic fungi, *A. restrictus*, which has quite frequently been detected in housedust. The biological activity of arestrictins A (**1**) and B (**2**) has not been tested yet, but such activity is expected, since the similar dioxopiperazine derivative, cristatin A (**3**), has been reported as an immunosuppressive substance.

Experimental

Melting points were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. EI-MS and ESI-TOF-MS were taken with a JEOL JMS-MS600W spectrometer and a Bruker microTOF spectrometer, respectively. UV and IR spectra were recorded on a Hitachi U-3210 spectrophotometer and a JASCO IR-810 spectrophotometer, respectively. ^{1}H- and ^{13}C-NMR spectra were recorded on a JEOL Lambda-500 (^{1}H, 500.00 MHz; ^{13}C, 125.43 MHz) spectrometer, using tetramethylsilane as an internal standard. CD curves were determined on a JASCO J-600 spectropholarimeter. Column chromatography was performed using Kieselgel 60 (Art. 7734, Merck). Low-pressure liquid chromatography (LPLC) was performed with a FMI LAB RP-SY pump, using a YAMAZEN SI-40B column (ϕ 26×300 mm). HPLC was performed with a JASCO PU-980 pump (flow rate, 7 ml/min), using a SENSHU PEGASIL Silica 60-5 column (ϕ 10×300 mm) equipped with a JASCO RI-930 detector. TLC was conducted on pre-coated Kieselgel 60 F_{254} plates (Art. 5715; Merck). Spots on TLC were detected by UV light on 254 nm, and/or by spraying with phosphomolybdate reagent and then heating.

Isolation of Arestrictins A (1) and B (2) from *Aspergillus restrictus* *A. restrictus* strain A-17 was cultured at 25 °C for 21 d in 100 Roux flasks containing 250 ml of M40Y medium in each flask. The mycelium was extracted with CH_2Cl_2–MeOH (1 : 1) and the organic layer was evaporated *in vacuo*. The residual extract (39.6 g) was extracted in sequence with hexane, CH_2Cl_2, and MeOH. The evaporated CH_2Cl_2 fraction (6.6 g) was chromatographed on silica gel with CH_2Cl_2–MeOH (100 : 1), CH_2Cl_2–MeOH (50 : 1), and then EtOH. The eluate from CH_2Cl_2–MeOH (100 : 1) was purified with LPLC using the solvent system of hexane–acetone (5 : 1) followed by recrystallization from MeOH to give cristatin A (**3**) (166 mg) and asperglucide (**4**) (113 mg). The eluate from CH_2Cl_2–MeOH (50 : 1) was purified with HPLC using CH_2Cl_2–MeOH (50 : 1) followed by recrystallization from MeOH to give arestrictins A (**1**) (23 mg). The EtOH eluate was purified by recrystallization from MeOH to give arestrictins B (**2**) (510 mg).

Arestrictins A (**1**): Pale yellow crystalline powder, ESI-TOF-MS m/z: 460.2967 [$(M-H_2O+H)^+$, 460.2959 for $C_{29}H_{38}N_3O_2$]. UV λ_{max}^{MeOH} nm (log ε): 228 (4.62), 274 (3.95), 283 (3.92), 291 (3.82). IR ν_{max}^{KBr} cm^{-1}: 3480 (OH), 3240 (NH), 1675, 1660 (CONH). CD (MeOH) $\Delta\varepsilon$ (nm): −17.3 (229), +7.2 (272), +5.8 (283), +4.0 (291). The ^{1}H- and ^{13}C-NMR signal assignments are summarized in Table 1.

Arestrictins B (**2**): Colorless crystalline powder, mp 228 °C (from MeOH). $[\alpha]_D^{25}$ −36.8° (c=0.51, $CHCl_3$). EI-MS m/z (%): 461.3044 (M^+, 461.3042 for $C_{29}H_{39}N_3O_2$, 1.5), 334 (M-127, 100). UV λ_{max}^{MeOH} nm (log ε): 230 (4.62), 278 (3.96), 283 (3.96), 294 (3.86). IR ν_{max}^{KBr} cm^{-1}: 3240 (NH,), 1670, 1660 (CONH). CD (MeOH) $\Delta\varepsilon$ (nm): −12.8 (230), +2.0 (277), +1.6 (289), +1.5 (299). The ^{1}H- and ^{13}C-NMR signal assignments are summarized in Table 1.

Isolation of Metabolites from *Aspergillus penicilloides* *A. penicilloides* strain SUM3319 was cultured at 25 °C for 21 d in 100 Roux flasks containing 250 ml of M40Y medium in each flask. The mycelium was extracted with CH_2Cl_2–MeOH (1 : 1) and the organic layer was evaporated *in vacuo*. The residual extract (14.4 g) was suspended in H_2O and extracted in sequence with hexane, CH_2Cl_2, and MeOH. The evaporated CH_2Cl_2 fraction (5.0 g) was chromatographed on silica gel with CH_2Cl_2–MeOH (100 : 1 and 50 : 1) followed by purification with LPLC using the solvent system of CH_2Cl_2–MeOH (30 : 1) to give asperglaucide (**4**) (33 mg), cristatin A (**3**) (24 mg), and arestrictin B (**2**) (12 mg).

Determination of Absolute Configuration of Alanine Residue in 1 and 3 by Hydrolysis by Marfey Method Compound (**1** or **3**) (100 μg) was subjected to acid hydrolysis with 6 M HCl (200 μl) at 110 °C for 12 h. After removal of the solvent, 1% L-FDAA in acetone (100 μl) and 1 M Na_2CO_3 (50 μl) was added to this amino acid solution in H_2O (50 μl). The solution was stirred at 37 °C for 1 h. After the reaction was quenched by the addition of 1 M HCl (20 μl), the reaction mixture was diluted with MeOH (810 μl) and 6 μl of this solution was compared with that similarly prepared from standard amino acids by HPLC [Inartsil ODS-3 (ϕ 4.6×250 mm), flow rate 1 ml/min, column temperature 40 °C, UV detection at 340 nm] using an isocratic elution of MeCN–0.1% TFA (1 : 1). The peaks of L-alanine and D-alanine were observed at 4.60 and 5.04 min, respectively, whereas the peaks derived from **1** and **3** were shown at 4.64 and 4.63 min, respectively.

Determination of Absolute Configuration of Alanine and Aspartic acid Residues in Arestrictin (2), by Ozonolysis Followed by Acid Hydrolysis, using Marfey Method Ozone was introduced in the solution of arestrictin B (**2**) (10 mg) in HCO_2H–H_2O (1 ml) at r.t. The obtained ozonide was decomposed with 3% H_2O_2 (1 ml) in a boiling bath for 30 min, and then hydrolyzed with 6 M HCl (1 ml) by boiling for 2 h. After removal of the solvent, 1% L-FDAA in acetone (100 μl) and 1 M Na_2CO_3 (50 μl) was added to this amino acid solution in H_2O (50 μl). The solution was stirred at 37 °C for 1 h. After the reaction was quenched by the addition of 1 M HCl (20 μl), the reaction mixture was diluted with MeOH (810 μl) and 6 μl of this solution was compared with that similarly prepared from standard amino acids by HPLC [Inartsil ODS-3 (ϕ 4.6×250 mm), flow rate 1 ml/min, column temperature 40 °C, UV detection at 340 nm] using an isocratic elution of MeCN–0.1% TFA (3 : 7). The peaks of L-alanine and D-alanine, and L-aspartic acid and D-aspartic acid were observed at 12.97 and 17.33 min, and 8.09 and 8.53 min, respectively, whereas the peaks derived from **2** were shown at 12.96 and 8.15 min.

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