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New Antibiotics Miyakamides Produced by a Fungus

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New antibiotics, miyakamides A_1, A_2, B_1, and B_2, were isolated from the cultured broth of *Aspergillus flavus* Link var. *columnaris* FKI-0739 together with known compounds, parasiticolide A, hydroxyaspergillic acid, and kojic acid. The structure of miyakamide A_1 is *N*-acetyl-L-phenylalanyl-*N*-methyl-L-phenylalanyl-(αZ)-α,β-didehydrotryptamine, and miyakamide A_2 is *E* isomer of A_1 at didehydrotryptamine. The structure of miyakamide B_1 is *N*-acetyl-L-tyrosyl-*N*-methyl-L-phenylalanyl-(αZ)-α,β-didehydrotryptamine, and B_2 is *E* isomer of B_1. Both miyakamides A_1 and B_1 existed as equilibrium isomers in solvents, and this isomerism was associated with *cis-trans* rotation of the amide bond between two amino acids. Conformational isomerism between two amino acids of miyakamides A_2 and B_2 is *cis*-form. Miyakamides showed growth inhibitory activity against brine shrimp, *Artemia salina*.

In the course of screening for insecticidal and nematocidal antibiotics, we have isolated some new antibiotics from microbial metabolites.[1~5] Our continuous screening efforts to find antibiotics were rewarded by the finding of new antibiotics, miyakamides A_1, A_2, B_1, and B_2 (**1**~**4**, Fig. 1), which were isolated from a cultured broth of *Aspergillus* sp. FKI-0739. In this report, we describe taxonomy of the producing strains and fermentation, isolation, structure elucidation, and biological properties of miyakamides.

Results and Discussion

Taxonomy of Producing Strain FKI-0739

Strain FKI-0739 was originally isolated from a fallen leaf collected at Miyakojima Island, Japan. For the taxonomic studies of the fungus, Czapek yeast extract agar (CYA), malt extract agar (MEA), and Czapek yeast extract agar with 20% sucrose (CY20S) were used. Colonies on CYA were 55~60 mm in diameter after 7 days at 25°C, floccose to farinaceous, and light olive (1 1/2 ie) to golden olive (1 1/2 lg) in color with abundant sporulation; the reverse was dusty yellow (1 1/2 gc). Colonies on MEA were 57~63 mm in diameter after 7 days at 25°C, floccose to farinaceous, and butter yellow (1 1/2 ga) in color with abundant sporulation; the reverse was white to cream (1 1/2 ca). Colonies on CY20S were 53~55 mm in diameter after 7 days at 25°C, floccose to farinaceous, and olive gray (1 ig) in color with abundant sporulation; the reverse was white to light yellow (1 1/2 ea). Morphological properties were observed after 7 days on CYA under a microscope and a scanning electron microscope (Fig. 2). Conidial heads were loosely columnar up to 500 μm. Conidiophores were born from the substratum and aerial hyphae and 400~1,000×3.5~5.0 μm in diameter. They were almost rough-walled and hyaline to slightly brown. Vesicles were clavate to subglobose, 30~60 μm in diameter, and fertile over the upper one-half of their surface. Phialides were consistently in a single series, ampulliform, closely packed, 6~11×3~5 μm in size, and producing conidia in long chain. Conidia were globose to subglobose, 3.5~5.5 μm in diameter, conspicuously echinulate, and olive-brown in mass.

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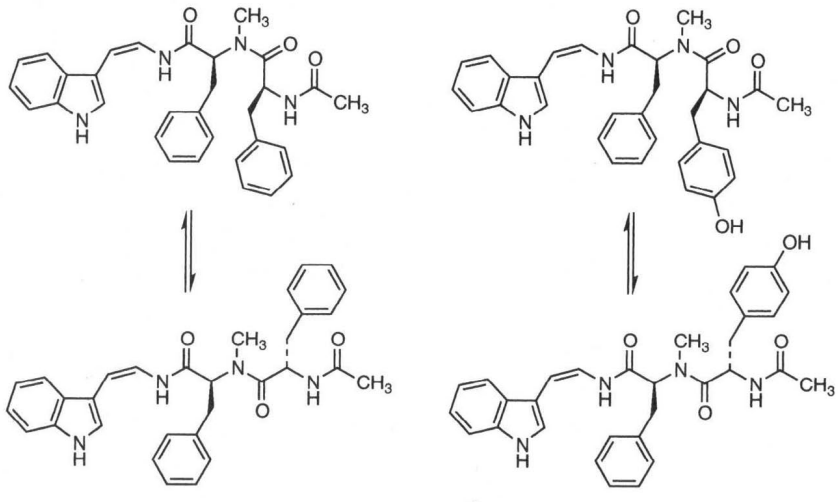
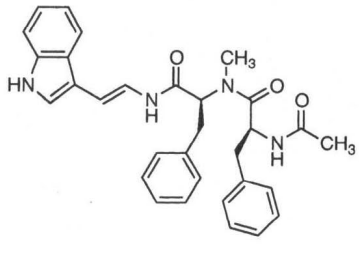
Fig. 1. Structures of miyakamides A_1 (**1**), A_2 (**2**), B_1 (**3**), and B_2 (**4**).

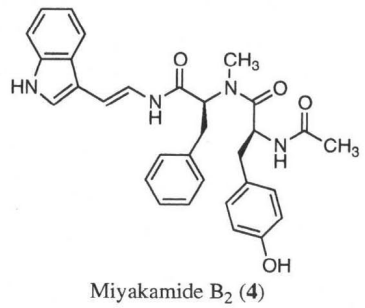


Miyakamide A_1 (**1**)

Miyakamide B_1 (**3**)



Miyakamide A_2 (**2**)



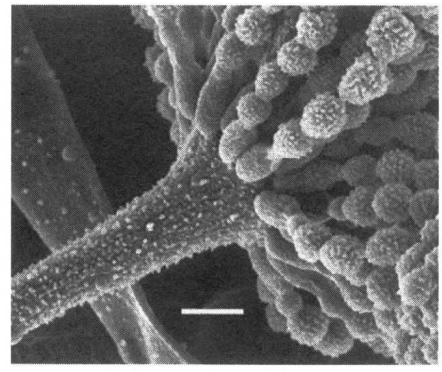
Miyakamide B_2 (**4**)

From the above characteristics, strain FKI-0739 was considered to belong to genus *Aspergillus*,[6,7] and above characteristics corresponded to the species description of *A. flavus* Link var. *columnaris* by Key to species.[7] Thus, we identified the strain FKI-0739 as *A. flavus* Link var. *columnaris* and named it *Aspergillus flavus* Link var. *columnaris* FKI-0739. The strain has been deposited at the National Institute of Advanced Industrial Science and Technology, International Patent Organism Depositary, Japan, as FERM BP-7786.

Fermentation of Miyakamides

A stock culture of strain FKI-0739 grown on potato dextrose agar was inoculated into a large test tube containing 10 ml of a seed medium and incubated on a reciprocal shaker at 27°C for 3 days. One milliliter of the seed culture was transferred into each of five 500-ml Erlenmeyer flasks containing 100 ml of a production

Fig. 2. Scanning electron micrograph of strain FKI-0739.



Bar represents 5 μm.

medium. The fermentation was carried out on a rotary shaker (210 rpm) at 27°C for 6 days.

Isolation

The cultured broth (500 ml) was centrifuged and the supernatant was extracted with ethyl acetate. The mycelia were extracted with methanol, which was then removed from the extract by evaporation. The aqueous extract was partitioned with ethyl acetate, and the organic layer was combined with the supernatant extract and concentrated to dryness *in vacuo* to afford a crude material (388 mg). It was chromatographed over a silica gel column. The eluate of $CHCl_3$ was concentrated to yield 7.6 mg of yellow powder, which was identified as parasiticolide A (astellolide A, **5**).[8,9] Active fractions eluted with $CHCl_3$-methanol (100:1) and $CHCl_3$-methanol (50:1) were concentrated to yield crude miyakamides A (258 mg) and B (18.8 mg), respectively. The $CHCl_3$-methanol eluate (10:1) was concentrated (32.8 mg) and further purified by Sephadex LH-20 chromatography to yield an orange powder (18.4 mg) of hydroxyaspergillic acid[10] (**6**) and a white powder (2.3 mg) of kojic acid[11] (**7**).

The crude miyakamide A was purified by HPLC under the following conditions: column, Senshu pak Pegasil ODS (i.d. 20×250 mm, Senshu Scientific Co.); mobile phase, 60% CH_3CN; flow rate, 7 ml/minute; detection, multi-wavelength detector MD-910 (JASCO). Miyakamides A_1 (**1**) and A_2 (**2**) were eluted at 28.0 and 22.7 minutes, respectively, under the above conditions. The HPLC eluates were concentrated to remove CH_3CN, extracted with ethyl acetate, and concentrated to dryness to give pale yellow powders of **1** (220 mg) and **2** (10.2 mg). The crude miyakamide B was purified by HPLC using the same condition as miyakamide A except that the mobile phase was 40% CH_3CN with 0.05% phosphate buffered saline, pH 7.0. Pale yellow powders of miyakamides B_1 (**3**, 2.0 mg) and B_2 (**4**, 2.9 mg) were obtained from the eluate at 79.7 and 63.1 minutes, respectively.

Physico-chemical Properties

Physico-chemical properties of miyakamides are summarized in Table 1. They showed similar UV spectra. The common IR absorbances observed at 1630~1650 cm^{-1} suggested the presence of amide carbonyl groups in their structures. The molecular formulae of **1** and **2** were established as $C_{31}H_{32}N_4O_3$ and the molecular formulae of **3** and **4** were established as $C_{31}H_{32}N_4O_4$ by HR-FAB-MS. Chemical shifts in the ^1H and ^{13}C NMR of **1**~**4** are shown in Tables 2 and 3. Miyakamides showed positive color reaction by Rydon-Smith reagent. As ^{13}C NMR assignment of **6** has not been reported, its NMR data are shown in the experimental section.

Table 1. Physico-chemical properties of miyakamides A~D (**1**~**4**).

		Miyakamide A_1 (**1**)	Miyakamide A_2 (**2**)	Miyakamide B_1 (**3**)	Miyakamide B_2 (**4**)
Appearance		Pale yellow powder	Pale yellow powder	Pale yellow powder	Pale yellow powder
Melting point		92–94° (decomp.)	98–100° (decomp.)	103–106° (decomp.)	107–111° (decomp.)
$[\alpha]_D^{25}$		–34.9° (*c* 0.230, MeOH)	–27.1° (*c* 0.244, MeOH)	–24.2° (*c* 0.153, MeOH)	–34.1° (*c* 0.291, MeOH)
Molecular formula		$C_{31}H_{32}N_4O_3$	$C_{31}H_{32}N_4O_3$	$C_{31}H_{32}N_4O_4$	$C_{31}H_{32}N_4O_4$
Molecular weight		508.6	508.6	524.6	524.6
FAB-MS (*m/z*)	Positive:	508 $[M]^+$, 531 $[M+Na]^+$	508 $[M]^+$, 531 $[M+Na]^+$	524 $[M]^+$, 547 $[M+Na]^+$	524 $[M]^+$, 547 $[M+Na]^+$
	Negative:	507 $[M-H]^-$	507 $[M-H]^-$	523 $[M-H]^-$	523 $[M-H]^-$
HR-FAB-MS (*m/z*)		$C_{31}H_{31}N_4O_3$ $[M-H]^-$	$C_{31}H_{32}N_4O_3Na$ $[M+Na]^+$	$C_{31}H_{31}N_4O_4$ $[M-H]^-$	$C_{31}H_{31}N_4O_4$ $[M-H]^-$
	Calcd:	507.2396	531.2372	523.2345	523.2345
	Found:	507.2373	531.2363	523.2337	523.2347
UV λ_{max}^{MeOH} nm (ε)		212 (sh, 8,600), 228 (13,600), 284 (sh, 8,200), 288(sh, 9,000), 300 (10,400)	204 (25,900), 258 (5,800), 280 (6,100), 306 (6,400)	204 (37,700), 225 (29,300), 288 (15,200), 301 (sh, 14,150)	202 (26,700), 225 (18,860), 280 (sh, 10,500), 307 (13,100)
IR ν_{max} (KBr) cm^{-1}		3307, 2927, 1651, 1633, 1538, 1496, 1456, 1230, 1103, 748, 700	3419, 2962, 2926, 2854, 1628, 1549, 1454, 1261, 1097, 1029, 800, 743, 700	3417, 2925, 1653, 1633, 1537, 1497, 1456, 1230, 1103, 744, 700	3415, 2923, 1651, 1633, 1552, 1516, 1456, 1230, 1101, 947, 743, 702
Solubility	Soluble:	Acetone, MeOH, EtOAc, Acetonitrile, $CHCl_3$	Acetone, MeOH, EtOAc, Acetonitrile, $CHCl_3$	Acetone, MeOH, EtOAc, Acetonitrile, $CHCl_3$	Acetone, MeOH, EtOAc, Acetonitrile, $CHCl_3$
	Insoluble:	H_2O, *n*-Hexane	H_2O, *n*-Hexane	H_2O, *n*-Hexane	H_2O, *n*-Hexane
Color reaction	Positive:	5% H_2SO_4, Rydon-Smith	5% H_2SO_4, Rydon-Smith	5% H_2SO_4, Rydon-Smith	5% H_2SO_4, Rydon-Smith

Table 2. ^{1}H and ^{13}C NMR data of miyakamides A_1 (**1**) and A_2 (**2**) (in acetone-d_6).

	Miyakamide A_1 (**1**)				Miyakamide A_2 (**2**)	
	cis-amide		*trans*-amide			
	^{13}C (ppm)	^{1}H (ppm)[a]	^{13}C (ppm)	^{1}H (ppm)[a]	^{13}C (ppm)	^{1}H (ppm)[a]
AcPhe Ac (Me)	21.7 q	1.49 s	22.6 q	1.79 s	22.5 q	1.92 s
AcPhe Ac (C=O)	171.8 s		169.3 s		172.7 s	
AcPhe NH		7.65 m		7.16 m		7.81 d (6.6)
AcPhe C=O	172.8 s		174.0 s		172.3 s	
AcPhe α	51.0 d	4.74 ddd (9.1, 6.7, 6.1)	51.4 d	5.06 ddd (7.8, 7.8, 7.8)	50.8 d	4.90 ddd (10.2, 6.6, 4.7)
AcPhe β	37.6 t	2.14 dd (13.8, 6.1)	39.1 t	2.80 m,	37.3 t	1.78 dd (13.9, 4.7),
		2.70 dd (13.8, 9.1)		2.88 m		2.52 dd (13.9, 10.2)
AcPhe 1	138.2 s		138.0 s		138.1 s	
AcPhe 2,6	130.14 dx2	7.11 m	130.12 dx2	7.11 m	130.1 dx2	7.19 m
AcPhe 3,5	129.5 dx2	7.25 m	129.1 dx2	7.15 m	129.1 dx2	7.28 m
AcPhe 4	127.34 d	7.17 m	127.29 d	7.10 m	127.37 d	7.18 m
MePhe NMe	29.7 q	2.94 s	31.9 q	2.82 s	29.3 q	2.87 s
MePhe C=O	167.8 s		167.7 s		166.6 s	
MePhe α	62.4 d	5.48 dd (8.7, 6.6)	59.5 d	5.31 dd (8.7, 6.9)	63.2 d	5.21 dd (9.4, 5.2)
MePhe β	35.7 t	2.55 dd (13.9, 8.7)	34.0 t	3.04 dd (14.6, 8.7)	34.9 t	2.74 dd (14.0, 9.4),
		3.24 dd (13.9, 6.6)		3.31 dd (14.6, 6.9)		3.26 dd (14.0, 5.2)
MePhe 1	138.9 s		138.5 s		139.3 s	
MePhe 2,6	130.5 dx2	7.31 m	129.9 dx2	7.23 m	130.5 dx2	7.37 d (7.1)
MePhe 3,5	129.0 dx2	7.26 m	129.2 dx2	7.25 m	129.5 dx2	7.24 m
MePhe 4	127.4 d	7.13 m	127.25 d	7.19 m	127.41 d	7.14 m
Tra NH[b]		9.18 d (10.2)		8.47 d (10.8)		10.11 d (10.0)
dhTra α	119.5 d	6.79 dd (10.2, 9.6)	119.5 d	6.84 dd (10.8, 9.4)	121.0 d	7.46 dd (14.9, 10.0)
dhTra β	104.5 d	5.98 d (9.6)	103.1 d	6.05 d (9.4)	107.7 d	6.56 d (14.9)
dhTra 1		10.4 br.s		10.5 br.s		10.24 s
dhTra 2	124.9 d	7.66 d (2.5)	123.7 d	7.58 d (2.5)	123.8 d	7.36 d (2.2)
dhTra 3	111.1 s		111.5 s d		113.5 s	
dhTra 3a	128.0 s		127.9 s		126.4 s	
dhTra 4	119.3 d	7.56 d (8.0)	119.3 d	7.64 d (7.7)	120.2 d	7.73 d (7.9)
dhTra 5	120.2 d	7.06 m	120.3 d	7.09 m	120.3 d	7.09 dd (7.9, 7.4)
dhTra 6	122.6 d	7.11 m	123.0 d	7.16 m	122.6 d	7.12 dd (8.3, 7.4)
dhTra 7	112.1 d	7.38 d (7.2)	112.3 d	7.44 d (8.3)	112.5 d	7.40 d (8.3)
dhTra 7a	137.0 s		137.1 s		138.2 s	

a: The coupling constants are in parentheses. *b*: dhTra is α,β-didehydrotryptamine.

Structure Elucidation

The structure of **2** was elucidated by NMR study. Analysis of the ^{1}H NMR, ^{13}C NMR, DEPT, and HMQC spectra revealed the presence of eight quaternary, nineteen methine, two methylene, and two methyl carbons. Aromatic protons were assigned as two monosubstituted benzenes and one 1,2-disubstituted benzene, and they were deduced to be two phenylalanines and one indole, respectively, by ^{1}H-^{1}H-COSY and HMBC (Fig. 3). ^{1}H-^{13}C long-range couplings by HMBC indicated that the amino residue of one phenylalanine is acetylated (AcPhe) and the other is methylated (MePhe). A partial structure –CH=CH–NH– is connected to C-3 of the indole by ^{1}H-^{13}C long-range couplings, which forms α,β-didehydrotryptamine (dhTra). ^{1}H-^{13}C long-range couplings between MePhe α (δ_H 5.20) and AcPhe C=O (δ_C 172.3) and between MePhe NMe (δ_H 2.87) and AcPhe C=O proved the connection of AcPhe-MePhe. ^{1}H-^{13}C long-range couplings between dhTra α (δ_H 7.46) and MePhe C=O (δ_C 166.6) and between dhTra NH (δ_H 10.10) and MePhe C=O indicated the connection of MePhe-dhTra. Thus the planar structure of **2** was elucidated as AcPhe-MePhe-dhTra. NOESY experiment indicated that the conformational isomerism of the amide bond between two phenylalanines is *cis* by NOE between AcPhe α-H (δ_H 4.90) and MePhe α-H (Fig. 3). The geometrical isomerism between α and β methines of dhTra was elucidated as *E* by the vicinal coupling constants of the methine protons (J=14.9 Hz) and NOEs between dhTra β (δ_H 6.56)/dhTra NH, dhTra β/dhTra 2 (δ_H 7.36), and dhTra α/dhTra 4 (δ_H 7.73). The chirality of each amino acid was elucidated by chiral HPLC of acid hydrolysate of **2**, which revealed the presence of 1 mol of L-phenylalanine and *N*-methyl-L-phenylalanine. Consequently, the structure of **2** was elucidated as *N*-acetyl-L-phenylalanyl-*N*-methyl-L-phenylalanyl-(α*E*)-α,β-didehydrotryptamine in which the amide bond between *N*-acetylphenylalanine and *N*-methylphenylalanine is *cis*.

The molecular formula of **1** was the same as that of **2**. More than 90% of **1** was converted to **2** in methanol at

Table 3. ^{1}H and ^{13}C NMR data of miyakamides B_1 (**3**) and B_2 (**4**) (in acetone-d_6).

	Miyakamide B_1 (**3**)				Miyakamide B_2 (**4**)	
	cis-amide		*trans*-amide			
	^{13}C (ppm)	^{1}H (ppm)[a]	^{13}C (ppm)	^{1}H (ppm)[a]	^{13}C (ppm)	^{1}H (ppm)[a]
AcTyr Ac (Me)	21.8 q	1.52 s	22.6 q	1.80 s	22.6 q	1.94 s
AcTyr Ac (C=O)	171.8 s		169.3 s		172.2 s	
AcTyr NH		7.60 d (7.3)		7.12 m		7.77 d (7.7)
AcTyr C=O	172.9 s		174.3 s		172.9 s	
AcTyr α	51.5 d	4.67 ddd (8.8, 7.3, 6.3)	51.7 d	4.98 ddd (7.7, 7.7, 7.7)	51.2 d	4.84 ddd (9.9, 6.1, 5.3)
AcTyr β	37.0 t	2.15 dd (13.8, 6.3)	38.5 t	2.71 m,	36.6 t	1.80 dd (13.9, 5.3),
		2.61 dd (13.8, 8.8)		2.80 m		2.46 dd (13.9, 9.9)
AcTyr 1	128.7 s		128.5 s		128.7 s	
AcTyr 2,6	131.2 dx2	6.95 d (8.5)	131.1 dx2	6.93 d (8.5)	131.2 dx2	7.03 d (8.5)
AcTyr 3,5	115.9 dx2	6.72 d (8.5)	116.1 dx2	6.67 d (8.5)	115.9 dx2	6.74 d (8.5)
AcTyr 4	157.05 s		156.98 s		157.0 s	
AcTyr OH		8.19 s		8.15 s		8.20 s
MePhe NMe	29.6 q	2.90 s	31.9 q	2.79 s	29.3 q	2.88 s
MePhe C=O	167.8 s		167.7 s		166.6 s	
MePhe α	62.4 d	5.46 dd (8.0, 7.2)	59.6 d	5.29 dd (8.5, 7.2)	63.2 d	5.19 dd (9.1, 5.8)
MePhe β	35.7 t	2.50 dd (13.8, 8.0)	33.9 t	3.03 dd (14.6, 8.5)	34.9 t	2.71 m,
		3.24 dd (13.8, 7.2)		3.21 dd (14.6, 7.2)		3.18 dd (14.0, 5.8)
MePhe 1	139.0 s		138.6 s		139.3 s	
MePhe 2,6	130.5 dx2	7.31 d (8.0)	129.9 dx2	7.21 d (7.6)	130.6 dx2	7.38 d (8.0)
MePhe 3,5	129.4 dx2	7.28 m	129.2 dx2	7.27 m	129.5 dx2	7.26 dd (8.0, 8.0)
MePhe 4	127.4 d	7.16 m	127.2 d	7.16 m	127.4 d	7.16 m
dhTra NH[b]		9.19 d (9.8)		8.52 d (10.8)		10.14 d (10.0)
dhTra α	119.5 d	6.77 dd (9.8, 9.8)	119.5 d	6.83 dd (10.8, 9.4)	121.0 d	7.47 dd (14.9, 10.0)
dhTra β	104.4 d	5.97 d (9.8)	103.0 d	6.03 d (9.4)	107.7 d	6.57 d (14.9)
dhTra 1		10.39 br.s		10.49 br.s		10.24 br.s
dhTra 2	125.0 d	7.68 d (2.5)	123.7 d	7.61 d (1.9)	123.7 d	7.36 d (2.5)
dhTra 3	111.1 s		111.5 s d		113.6 s	
dhTra 3a	128.0 s		127.9 s		126.4 s	
dhTra 4	119.35 d	7.57 d (8.0)	119.33 d	7.64 d (8.0)	120.2 d	7.74 d (7.7)
dhTra 5	120.2 d	7.04 ddd (8.0, 8.0, 5.2)	120.3 d	7.07 dd (8.4, 8.0)	120.3 d	7.07 dd (7.7, 7.7)
dhTra 6	122.6 d	7.11 m	122.9 d	7.16 m	122.6 d	7.14 dd (7.7, 7.7)
dhTra 7	112.1 d	7.38 m	112.3 d	7.44 d (8.0)	112.5 d	7.41 d (7.7)
dhTra 7a	137.0 s		137.1 s		138.2 s	

a: The coupling constants are in parentheses. *b*: dhTra is α,β-didehydrotryptamine.

room temperature under room light for one week. Therefore, **1** was suggested to be an isomer of **2**. The ^{13}C NMR spectrum of **1** showed the double number of carbon signals comparing with that of **2**, and the signals of **1** can be separated to two groups in the signal intensity ratio of 5 : 4 by further NMR study (Table 2). ^{1}H-^{1}H-COSY and HMBC study deduced the planar structure of the major group of **1** (having larger signals) to be AcPhe-MePhe-dhTra, which was the same as the structure of **2** (Fig. 4). However, it has *Z* isomer between α and β methines of dhTra, which was revealed by the vicinal coupling constants ($J_{\alpha,\beta}$=9.6 Hz) and NOEs between dhTra NH (δ_H 9.18)/dhTra 2 (δ_H 7.66) and dhTra β (δ_H 5.98)/dhTra 4 (δ_H 7.56). The amide bond between two phenylalanines was revealed to be *cis* conformer by NOESY experiment, and the chirality of two phenylalanines was both L-form. The planar structure of the minor group of **1** (having smaller signals) was also elucidated as AcPhe-MePhe-dhTra by NMR study, and *Z* isomer between α and β methines of dhTra was revealed by the vicinal coupling constants ($J_{\alpha,\beta}$=9.4 Hz) and NOEs between dhTra NH (δ_H 8.47)/dhTra 2 (δ_H 7.58) and dhTra β (δ_H 6.05)/dhTra 4 (δ_H 7.64). The amide bond between two phenylalanines was revealed to be *trans* conformer by NOE between AcPhe α (δ_H 5.06) and MePhe NMe (δ_H 2.82). Consequently, the structure of **1** was elucidated as *N*-acetyl-L-phenylalanyl-*N*-methyl-L-phenylalanyl-(αZ)-α,β-didehydrotryptamine in which the amide bond between *N*-acetylphenylalanine and *N*-methylphenylalanine is a mixture of *cis* and *trans* conformers.

The molecular formula of **4** has one more oxygen than that of **2**. NMR analysis of **4** comparing **2** indicated that *N*-acetylphenylalanine (AcTyr) of **2** is replaced with *N*-acetyltyrosine in **4** (Fig. 5). Isomerisms of **4** were shown to be *cis*-amide and (αE)-dhTra. The amino acids were revealed as L-tyrosine and *N*-methyl-L-phenylalanine by chiral HPLC of acid hydrolysate of **4**. Thus, the structure of **4** was elucidated as *N*-acetyl-L-tyrosyl-*N*-methyl-L-phenyl-

Fig. 3. Selected ^{1}H-^{1}H COSY, HMBC, and NOESY correlations of **2**.

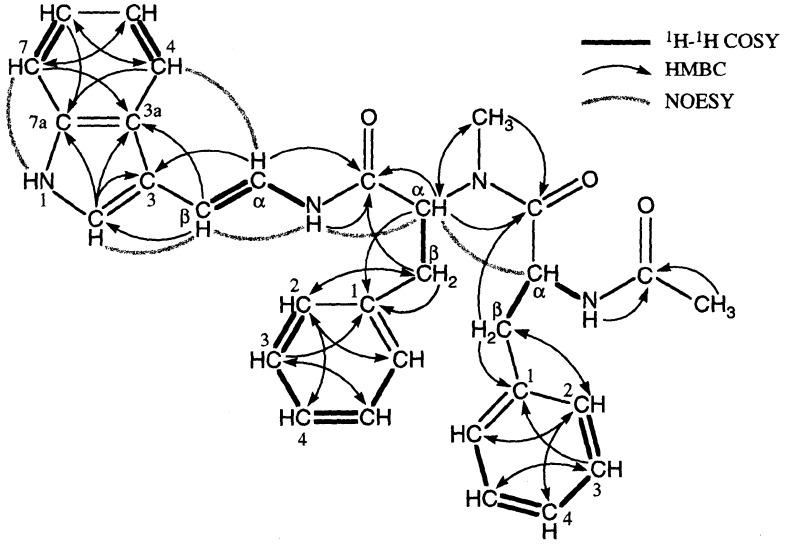


Fig. 4. Selected ^{1}H-^{1}H COSY, HMBC, and NOESY correlations of **1**.

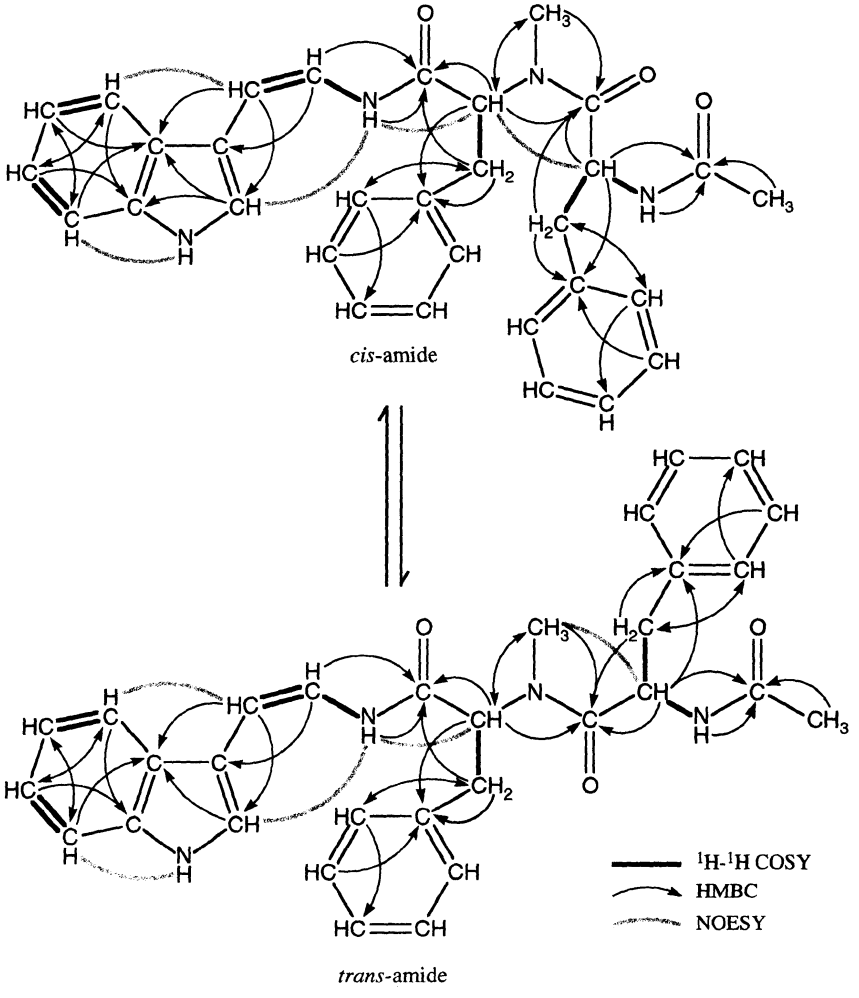
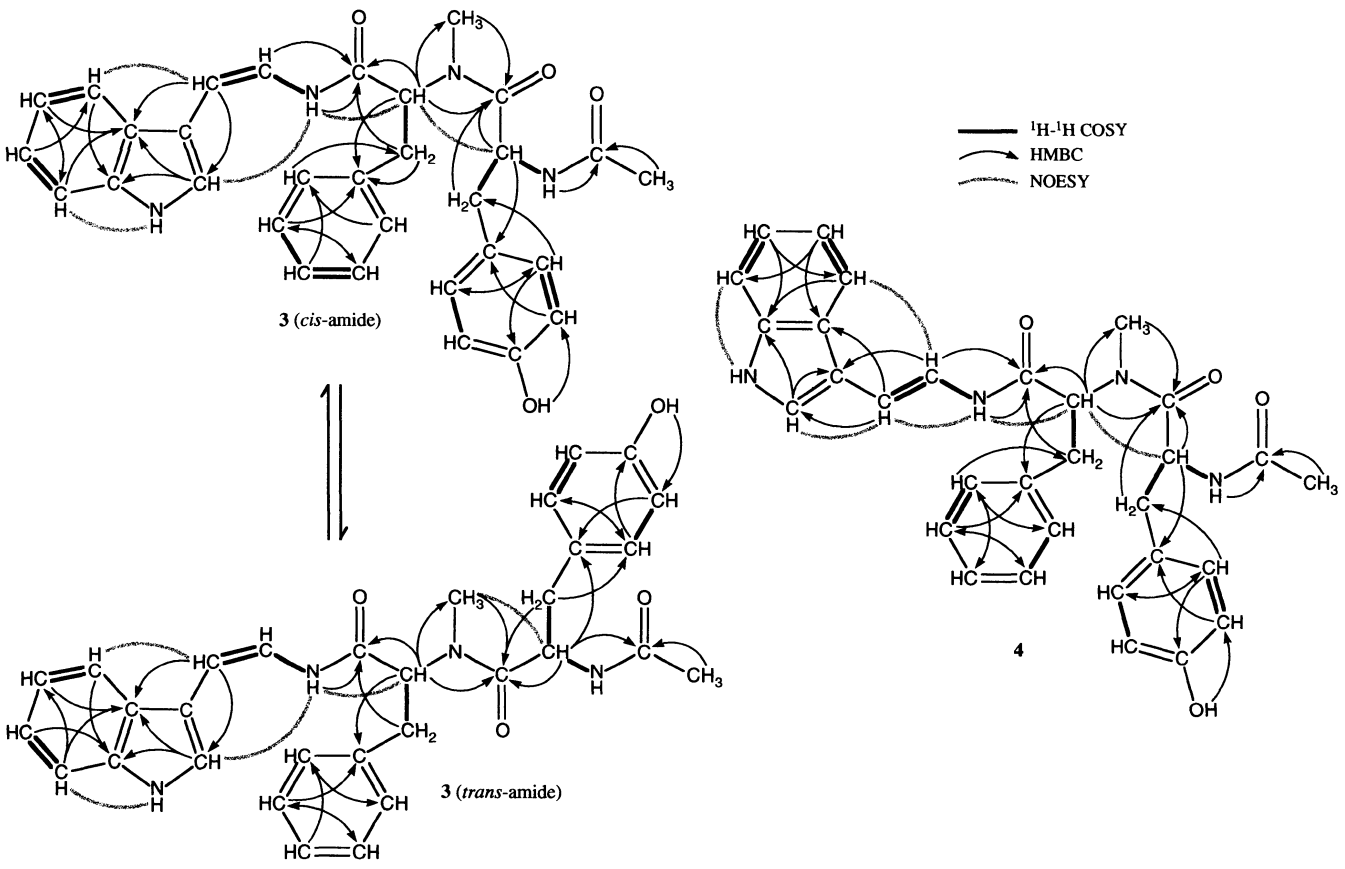


Fig. 5. Selected ^{1}H-^{1}H COSY, HMBC, and NOESY correlations of **3** and **4**.



alanyl-(αE)-α,β-didehydrotryptamine in which the amide bond between *N*-acetyltyrosine and *N*-methylphenylalanine is *cis*.

The molecular formula of **3** was the same as that of **4**, and more than 90% of **3** was converted to **4** in methanol for one week (room temperature under room light). The ^{13}C NMR spectrum of **3** showed a double number of carbon signals comparing with that of **4**. NMR signals of **3** can be also separated to two groups in the signal intensity ratio of 5 : 4. Therefore, **3** was suggested to be (αZ)-dhTra isomer of **4** by analogy with the conversion from **1** to **2**. Further NMR analysis (Fig. 5) revealed that **3** is *N*-acetyl-L-tyrosyl-*N*-methyl-L-phenylalanyl-(αZ)-α,β-didehydrotryptamine in which the amide bond between *N*-acetyltyrosine and *N*-methylphenylalanine is a mixture of *cis* and *trans* conformers.

Miyakamides have a common structure of dipeptidyl-α,β-didehydrotryptamine. *l*-Phenylalaninamide, terpeptin, and aspergillamides A and B belong to the same group, and they are all produced by *Aspergillus* spp.[12~14] Similar compounds have been also isolated from, *Penicillium* sp. (penidiamide), red algae (fragilamide, chondriamides A~C), and invertebrates (halocyamines A and B, coscinamides A~C).[15~20] Among them, aspergillamides A and B are closely related to miyakamides. Their planar structure is AcLeu-MePhe-dhTra, and isomerisms of aspergillamides A and B are (αZ)-dhTra with *cis*- and *trans*-amide mixture and (αE)-dhTra with *cis*-amide, respectively. It is interesting that *l*-phenylalaninamide, terpeptin, and miyakamides were all isolated from *Aspergillus* spp. collected from soil samples in Ryukyu Islands, Japan. Aspergillamides producing strain was collected in Acklins Island, the Bahamas, and most other α,β-didehydrotryptamine containing compounds were isolated from marine algae or marine invertebrates. Therefore, it is suggested that the producing organisms of α,β-didehydrotryptamine containing compounds are related to the marine environment.

Table 4. Antimicrobial spectra of **1**~**4**.

Test microorganism	MIC (μg/ml)			
	1	**2**	**3**	**4**
Staphylococcus aureus ATCC6538p	>100	>100	>50	>50
Bacillus subtilis ATCC6633	>100	>100	>50	>50
Micrococcus luteus ATCC9341	>100	>100	—	—
Mycobacterium smegmatis ATCC607	>100	>100	—	—
Escherichia coli NIHJ	>100	>100	>50	>50
Pseudomonas aeruginosa IFO3080	>100	>100	>50	>50
Xanthomonas campestris pv. *oryzae* KB88	100	100	—	—
Candida albicans KF1	>100	>100	>50	>50
Saccharomyces cerevisiae KF26	>100	>100	>50	>50
Aspergillus niger ATCC6275	>50	50	—	—
Mucor racemosus IFO4581	>100	>50	>50	>50

—, not tested

Biological Activities

Insecticidal and nematocidal activities of miyakamides were studied by a microplate assay using brine shrimp *Artemia salina* and free-living nematode *Caenorhabditis elegans*. The assay method was reported previously.[1,5] Minimum growth inhibitory concentrations of **1** and **2** against *A. salina* were 5 μg/ml, and those of **3** and **4** were 20 μg/ml. However, all miyakamides did not affect *C. elegans* at 100 μg/ml. Antimicrobial activities of miyakamides are shown in Table 4. Compounds **1** and **2** showed weak activity against *Xanthomonas campestris*, but **3** and **4** did not show any antimicrobial activities. The IC_{50} values of **1**, **2**, **3**, and **4** against P388 cells were 10.5, 12.2, 8.8, and 7.6 μg/ml, respectively. Though anti-*A. salina* activities of **1** and **2** were more potent than those of **3** and **4**, their cytotoxicities against P388 cells were similar.

Experimental

General

Morphological observations of the miyakamides producing strain were carried out using an Olympus Vanox-S AH-2 microscope and a JEOL JSM-5600 scanning electron microscope.

NMR spectra were recorded on a Varian Inova 600 spectrometer ($^{2\text{-}3}J_{CH}$=8 Hz in HMBC). Chemical shifts are shown in δ values (ppm) relative to acetone-d_6 at 2.06 ppm for ^{1}H NMR and at 29.8 ppm for ^{13}C NMR. Mass spectrometry was conducted on a JEOL JMS-AX505 HA spectrometer. UV and IR spectra were measured with a Shimadzu UV-240 spectrophotometer and a Horiba FT-210 Fourier transform infrared spectrometer, respectively. Optical rotations were recorded on a JASCO model DIP-181 polarimeter. Melting points were measured with a Yanaco micro melting point apparatus MP-S3.

Taxonomic Studies of the Producing Organism

The strain FKI-0739 was isolated from a fallen leaf collected at Miyakojima Island, Japan. Taxonomic studies and identification were conducted according to the procedure described by KLICK and PITT,[6] and RAPER and FENNEL.[7] Morphological observations were done under a microscope (Olympus Vanox-S AH-2) and a scanning electron microscope (JEOL JSM-5600). Color hues were described according to Color Harmony Manual, 4th Ed.[21]

Media

The seed medium was composed of 2.4% glucose, 0.5% Polypepton (Nihon Pharmaceutical Co.), 0.5% yeast extract (Oriental Yeast Co.), 0.4% $MgSO_4 \cdot 7H_2O$, 0.1% KH_2PO_4, and 0.1% agar (pH 5.6 prior to sterilization). The production medium was composed of 2.0% glycerol, 1.0% sucrose, 0.5% ammonium acetate, 0.2% Cultivator #100 (fish extract, Yaizu Suisankagaku Industry Co.), 0.1% agar, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.05% KCl (pH 6.0 prior to sterilization).

Hydroxyaspergillic Acid (**6**)

FAB-MS (*m/z*) 503 $(2M+Na)^+$, 765 $(3M+2Na-H)^+$; HR-FAB-MS (*m/z*) found 503.2759 $(2M+Na)^+$, calcd 503.2845 (for $C_{24}H_{40}N_4O_6Na$); ^1H NMR (600 MHz, acetone-d_6) δ 7.50 s (1H, 6-H), 6.73 br.s (1H, 4-OH), 2.56 m (2H, $CH_2CH(CH_3)_2$), 2.12 m (1H, $CH(CH_3)_2$), 1.94 m (2H, CH_2CH_3), 1.50 s (3H, $C(CH_3)OH$), 0.85 d (6H, J=6.3 Hz, $CH(CH_3)_2$), 0.81 t (3H, J=7.3 Hz, CH_2CH_3); ^{13}C NMR (125 MHz, acetone-d_6) δ 158.3 s (C-3), 150.5 s (C-2), 144.2 s (C-5), 124.4 d (C-6), 75.0 s ($C(CH_3)OH$), 42.6 t ($CH_2CH(CH_3)_2$), 33.2 t (CH_2CH_3), 28.6 d ($CH(CH_3)_2$), 24.8 q ($C(CH_3)OH$), 22.9 q ×2 ($CH(CH_3)_2$), 8.8 q (CH_2CH_3).

Acid Hydrolysis of Miyakamides

Each miyakamide (100 μg) was hydrolyzed with 6 N HCl (990 μl) - 1% phenol (10 μl) vapor at 150°C for 5 hours by Pico-Tag Workstation (Waters). The reaction mixture was concentrated to dryness, dissolved in a small amount of water, and charged on a chiral HPLC: column, Sumichiral OA-5000 (Sumika Chemical Analysis Service, i.d. 4.6× 150 mm); mobile phase, 2 mM $CuSO_4$ - MeOH (85 : 15); flow rate 1.0 ml/minute; detection, UV 254 nm. The amino acids were identified by comparison with each authentic optically pure sample. Authentic L-phenylalanine, D-phenylalanine, *N*-methyl-L-phenylalanine, *N*-methyl-D-phenylalanine, L-tyrosine, and D-tyrosine were eluted at 45.1, 62.0, 39.8, 54.1, 16.3, and 22.3 minutes, respectively.

Conversion of **1** to **2** and **3** to **4**

Compound **1** or **3** (1 mg) was dissolved in 1 ml of MeOH in a glass test tube and kept at room temperature under room light for one week. Then the solution was analyzed by HPLC.

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