Four New Alkaloids, Brevianamides O – R, from the Fungus *Aspergillus versicolor*

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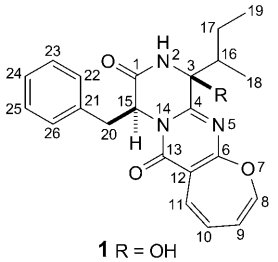
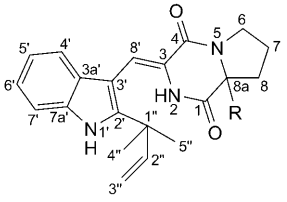
Four new alkaloids, brevianamides O – R (**1** – **4**, resp.), were isolated from the AcOEt extract of the solid-state fermented rice culture of the fungus *Aspergillus versicolor*. Their structures were elucidated on the basis of spectroscopic analyses.

Introduction. – *Aspergillus versicolor*, a harmful fungus, is widely found in human and animal foodstuffs. Its secondary metabolites such as aflatoxins and related sterigmatocystins are carcinogenic [1]. Therefore, a variety of secondary metabolites of *A. versicolor* were extensively characterized, including xanthones such as sterigmatin [2] and sterigmatocystin [3], anthraquinones such as averantin [4], averufin [5], and versicolorone [6], a terpenoid, versiol [7], lactones [8], and chromones such as aspergillitine or aspergiones A – F [9]. Our previous study on the alkaloidal metabolites of the fungus *A. versicolor* led to the identification of five new diketopiperazine alkaloids [10]. The present study disclosed four new diketopiperazine alkaloids, brevianamides O – R (**1** – **4**, resp.), from the AcOEt extract of the solid-state-fermented rice culture of this fungus. Here, the isolation and structure elucidation of compounds **1** – **4** are described.



1 R = OH
2 R = H



3 R = OH
4 R = MeO

Results and Discussion. – 1. *Structure Elucidation.* Compound **1** was isolated as yellow amorphous powder. The molecular formula ($C_{22}H_{23}N_3O_4$) was determined from the *quasi*-molecular-ion peak at *m*/*z* 416.1561 ($[M+Na]^+$) in the HR-ESI-MS. The IR absorption band at $\tilde{\nu}_{max}$ 3331 cm^{-1} suggested the presence of a OH group. The presence of amides could be deduced from the IR absorption bands at 3167, 1693, and 1658 cm^{-1},

and the ^{13}C-NMR signals at δ(C) 166.1 and 163.2 (*Table 1*). The NMR data and the UV maxima at λ_{max} 209 (4.10), 248 (3.78), and 339 (3.53) nm suggested that compound **1** was an analog of brevianamide L [10]. The comparison of the NMR data of **1** with those of brevianamide L suggested that one O-bearing C-atom resonates at δ(C) 84.5 in **1** instead of δ(C) 70.4 in brevianamide L. The OH group must be located at C(3) in view of the key HMBCs of H–C(17) and H–C(18) with δ(C) 84.5 (C(3)). The HMBC of H–C(16) with C(4), of H–C(10) with C(12), and of H–C(8) and H–C(11) with C(6) suggested the presence of a C=N and a C=C bond between C(4) and N(5), and C(6) and C(12), respectively. The structure of compound **1** was finally elucidated by the HSQC and HMBC experiments.

Table 1. *NMR Data of Compounds* **1** *and* **2**. δ in ppm, J in Hz[a])

	1		**2**	
	δ(H)	δ(C)	δ(H)	δ(C)
C(1)		166.1		167.5
H–N(2)	6.45 (*s*)		6.50 (*s*)	
C(3) or H–C(3)		84.5	2.66 (br. *s*)	58.4
C(4)		156.0		156.1
C(6)		160.6		163.0
H–C(8)	6.06 (*d*, *J* = 5.5)	143.5	6.05 (*d*, *J* = 5.6)	143.2
H–C(9)	5.64 (*t*, *J* = 5.5)	117.2	5.64 (*t*, *J* = 5.6)	117.2
H–C(10)	6.22 (*dd*, *J* = 11.2, 5.5)	128.7	6.18 (*dd*, *J* = 11.0, 5.6)	128.0
H–C(11)	6.80 (*d*, *J* = 11.2)	125.2	6.80 (*d*, *J* = 11.0)	125.5
C(12)		111.3		110.0
C(13)		163.2		160.9
H–C(15)	5.45 (*dd*, *J* = 5.6, 3.4)	57.2	5.47 (*t*, *J* = 4.2)	57.0
H–C(16)	2.36–2.40 (*m*)	44.2	2.27–2.30 (*m*)	36.6
CH_2(17)	0.83–0.86 (*m*)	24.6	1.01–1.05 (*m*)	23.1
Me(18)	0.93 (*d*, *J* = 7.2)	10.7	0.84 (*d*, *J* = 7.2)	15.7
Me(19)	0.79 (*t*, *J* = 7.6)	11.6	0.76 (*t*, *J* = 7.6)	11.9
CH_2(20)	3.45 (*dd*, *J* = 14.9, 5.6), 3.55 (*dd*, *J* = 14.9, 3.4)	37.0	3.38–3.41 (*m*)	37.1
C(21)		134.4		134.3
H–C(22,26)	6.98 (*d*, *J* = 7.6)	130.0	6.94 (*d*, *J* = 7.2)	129.8
H–C(23,25)	7.32 (*t*, *J* = 7.6)	129.3	7.24 (*t*, *J* = 7.2)	128.9
H–C(24)	7.33 (*t*, *J* = 7.6)	128.4	7.30 (*t*, *J* = 7.2)	128.1

[a]) Recorded in $CDCl_3$; ^{1}H: 600 MHz, ^{13}C: 150 MHz; assignments based on HSQC and HMBC.

In the NOESY experiment, the cross-signal between HO–C(3) at δ(H) 6.84 and H–C(22) or H–C(26) was observed (*Fig. 1*). The hydrolysis of compound **1** in 6N HCl (aq.) for 12 h at 100° afforded L-phenylalanine ($[\alpha]_D^{20} = -34.0$ (c = 0.1, H_2O)), which was determined by comparing with an authentic sample. Therefore, the absolute configurations of C(3) and C(15) were determined as (*R*) and (*S*), respectively. The configuration at C(16) could not be determined so far.

Compound **2** was obtained as yellow amorphous powder. The molecular formula ($C_{22}H_{23}N_3O_3$) was inferred from the *quasi*-molecular-ion peak at *m*/*z* 400.1635 ($[M + Na]^+$) in the HR-ESI-MS. The NMR (*Table 1*) and UV data of compound **2** were

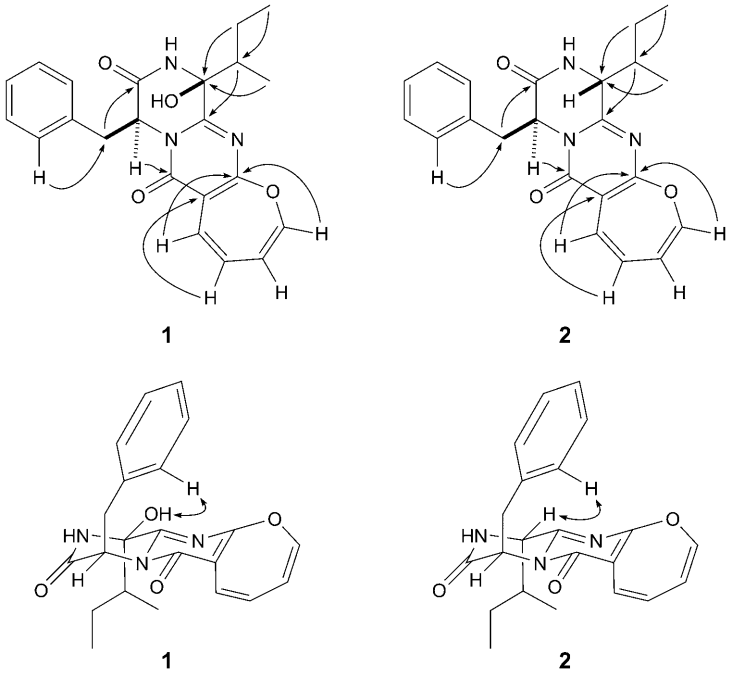


Fig. 1. *Key HMBC* (H → C) *and NOESY* (↔) *correlations of* **1** *and* **2**

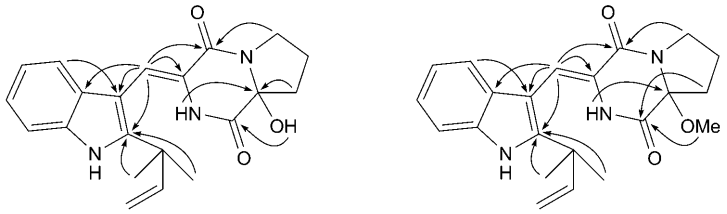
very similar to those of **1**. The molecular formula and IR spectrum suggested that, in compound **2**, there is no HO−C(3) as in compound **1**, which was confirmed by the HMBC of CH_2(17) and Me(18) with δ(C) 58.4 (C(3)) (*Fig. 1*). The structure of compound **2** was confirmed by the HSQC and HMBC experiments. The absolute configurations of both C(3) and C(15) were determined as (*S*) on the basis of the NOESY cross-peak between H−C(3) and H−C(22), and of L-phenylalanine obtained from the hydrolysis of compound **2** in the same way as in the case of **1**. Again, the configuration at C(16) could not be determined.

Compound **3** was obtained as a colorless racemic mixture with $[a]_D^{20} = 0.0$ ($c = 0.10$, acetone), and a *Cotton* effect in the CD spectrum was absent. The *quasi*-molecular-ion peak at *m*/*z* 388.1637 ($[M+Na]^+$) in the HR-ESI-MS gave the molecular formula $C_{21}H_{23}N_3O_3$. Signals for 21 H-atoms in ^{1}H-NMR and 21 signals in ^{13}C-NMR spectra were observed (*Table 2*). The ^{1}H-NMR signals at δ(H) 5.09 (*dd*, $J = 11.9$, 1.1, H−C(3″)), 5.12 (*d*, $J = 10.6$, 1.1, H−C(3″)), 6.14 (*dd*, $J = 11.9$, 10.6, H−C(2″)), and the HMBC of δ(H) 1.56 (*s*, Me(4″)) and 1.57 (*s*, Me(5″)) with δ(C) 39.2 (C(1″)) and 145.1 (C(2″)) indicated the presence of $-Me_2CCH{=}CH_2$ moiety. An *ortho*-substituted benzene ring was evident from the ^{1}H-NMR signals at δ(H) 7.43 and 7.37 (*d*, $J = 8.1$, H−C(4′), H−C(7′)), and 7.08 and 7.01 (*t*, $J = 8.1$, H−C(6′), H−C(5′)). The IR absorptions (1711, 1692 cm^{-1}) and ^{13}C-NMR signals (δ(C) 164.7, 158.8) indicated two amido C=O groups. The above evidence revealed compound **3** as an analog of brevianamide K [10]. The key HMBC of H−N(2) and H−C(7) with δ(C) 87.2 (C(8a))

Table 2. *NMR Data of Compounds* **3** *and* **4**. δ in ppm, J in Hz[a])

	3[b])		**4**[c])	
	δ(H)	δ(C)	δ(H)	δ(C)
C(1)		164.7		162.5
H–N(2)	7.99 (*s*)		7.46 (*s*)	
C(3)		126.7		125.8
C(4)		158.8		158.7
CH_2(6)	3.65–3.69 (*m*), 3.75–3.77 (*m*)	44.7	3.72–3.76 (*m*), 3.89–3.94 (*m*)	45.3
CH_2(7)	2.02–2.04 (*m*), 2.21–2.27 (*m*)[d])	19.4	2.00–2.04 (*m*), 2.13–2.18 (*m*)	19.2
CH_2(8)	2.21–2.27 (*m*)[d]), 2.21–2.27 (*m*)[d])	36.0	2.07–2.12 (*m*), 2.47–2.50 (*m*)	34.6
C(8a)		87.2		91.7
H–N(1′)	10.24 (*s*)		8.32 (*s*)	
C(2′)		144.2		143.9
C(3′)		104.0		103.2
C(3a’)		126.7		126.2
H–C(4′)	7.43 (*d*, J = 8.1)	119.5	7.26 (*d*, J = 7.2)	118.8
H–C(5′)	7.01 (*t*, J = 8.1)	119.7	7.14 (*t*, J = 7.2)	121.1
H–C(6′)	7.08 (*t*, J = 8.1)	121.2	7.19 (*t*, J = 7.2)	122.4
H–C(7′)	7.37 (*d*, J = 8.1)	111.3	7.36 (*t*, J = 7.2)	111.3
C(7a’)		135.3		134.4
H–C(8′)	7.13 (*s*)	111.1	7.29 (*s*)	113.0
C(1″)		39.2		39.2
H–C(2″)	6.14 (*dd*, J = 11.9, 10.6)	145.1	6.06 (*dd*, J = 17.3, 10.5)	144.2
CH_2(3″)	5.09 (*dd*, J = 11.9, 1.1), 5.12 (*dd*, J = 10.6, 1.1)	115.5	5.17 (*dd*, J = 17.3, 0.8), 5.21 (*dd*, J = 10.5, 0.8)	113.4
Me(4″)	1.56 (*s*)	27.0	1.52 (*s*)	27.4
Me(5″)	1.57 (*s*)	27.2	1.52 (*s*)	27.3
MeO			3.33 (*s*)	51.5

[a]) Assignments based on HSQC and HMBC; ^{1}H: 600 MHz, ^{13}C: 150 MHz. [b]) Recorded in $CDCl_3$. [c]) Recorded in (D_6)acetone. [d]) Signals overlapped.



3 **4**

Fig. 2. *Key HMBC* (H → C) *correlations of* **3** *and* **4**

indicated the presence of a OH group at C(8a). The structure of compound **3** was finally elucidated by detailed analysis of the HSQC and HMQC spectra (*Fig. 2*).

Compound **4** was also obtained as a colorless racemic mixture. The molecular formula $C_{22}H_{25}N_3O_3$ was deduced from the HR-ESI-MS. The IR, UV, and NMR spectra of **4** were similar to those of **3**, but compound **4** contains one more MeO group (δ(H)

3.33 (*s*, Me), δ(C) 51.5), which is located at C(8a) in view of the HMBC of δ(H) 3.33 with δ(C) 91.7 (C(8a)). The structure of compound **9** was finally determined on the basis of HSQC and HMQC experiments (*Fig. 2*).

2. *Biological Studies.* Compounds **1**–**4** were evaluated for their cytotoxic properties against human breast cancer (Bre04), human lung (Lu04), and human neuroma (N04) cell lines. They exhibited no cytotoxicity against these cell lines ($GI_{50} > 10$ μg/ml).

Experimental Part

General. All solvents including petroleum ether (PE; 60–90°) were distilled prior to use. Column chromatography (CC): silica gel (SiO_2, 200–300 mesh; *Qingdao Marine Chemical Factory*, Qingdao, P. R. China); *Lichroprep C_{18}* reversed-phase (*RP-18*) SiO_2 (40–63 μm, *Phenomenex Co.*). TLC: SiO_2 *GF_{254}* (10–40 μm; *Qingdao Marine Chemical Factory*, Qingdao, P. R. China). M.p.: *X-6* precise melting-point apparatus (*Beijing Fukai Science and Technology Development Limited Company*). Optical rotations: *Perkin-Elmer 341* polarimeter. UV Spectra: *Perkin-Elmer Lambda 35* UV/VIS spectrometer; λ_{max} (log ε) in nm. IR Spectra: *Perkin-Elmer Spectrum One* FT-IR spectrometer, KBr pellets; in cm^{-1}. 1D- and 2D-NMR Spectra: *Bruker AM-600* spectrometer; δ in ppm rel. to Me_4Si (= 0 ppm), *J* in Hz. ESI- and HR-ESI-MS: *BioTOF-Q* mass spectrometer; in *m*/*z* (rel. %).

Microorganism and Fermentation. A. versicolor (*A.* 3.3968) was obtained from Institute of Microbiology of the Chinese Academy of Sciences (CAS). It was maintained on potato dextrose agar slant (PDA) at 4° and was stocked in Chengdu Institute of Biology, Chinese Academy of Sciences. The seed culture medium was comprised of dextrose (20 g/l), yeast extract (1 g/l), KH_2PO_4 (3 g/l), $MgSO_4 \cdot 7\,H_2O$ (1.5 g/l), and potato extract prepared by extracting 200 g of potato with 1 l of boiling H_2O for 20 min. The pH of the medium was adjusted to 6.0 with 1M NaOH (aq.). The solid culture medium was comprised of rice and 0.3% peptone. The sterilization was carried out at 121° under 15 psi for 30 min.

The fresh mycelium grown on PDA slant at 28° for 3 d was inoculated into 500-ml flasks containing 100 ml of sterilized seed medium. Flasks with inoculated medium were placed in a rotary shaker at 28° and incubated at 180 rpm for 2 d. The seed culture was inoculated into the sterilized solid medium for further fermentation at 28° for 25 d.

Extraction and Isolation. The fermented solid medium (4 kg) was soaked with AcOEt (8 l × 1, 3 d) at r.t. The solvent was evaporated *in vacuo* to afford a residue (42.0 g). This residue was separated over a SiO_2 column (600 g, ∅ 90 mm × 330 mm) eluted with PE/acetone (15:1, 10:1, 5:1, 3:1, 1:1, 0:1; each 3000 ml) to yield five fractions: *A* (18.6 g), *B* (5.1 g), *C* (4.3 g), *D* (6.0 g), and *E* (5.0 g). *Fr. D* was subjected to CC (SiO_2 (110 g, ∅ 50 mm × 110 mm); $CHCl_3$/MeOH 30:1, 20:1, 10:1; each 1000 ml) to afford *Frs. DA* (0.80 g), *DB* (2.55 g), and *DC* (1.50 g). *Fr. DA* was subjected to CC (*RP-18* silica gel (22 mm × 260 mm); MeOH/H_2O 7:3; 1000 ml) to afford compound **2** (18 mg). *Fr. DB* was separated by CC (SiO_2 (145 g, ∅ 44 mm × 185 mm); PE/AcOEt 2:1, 1:1; each 1000 ml) to give **3** (52 mg), **4** (45 mg), and **1** (12 mg) successively.

Brevianamide O (= *(8*S*,11*R*)-8-Benzyl-11-(butan-2-yl)-10,11-dihydro-11-hydroxy-6*H*-oxepino[2,3-*d*]pyrazino[1,2-*a*]pyrimidine-6,9(8*H*)-dione*; **1**): Yellow amorphous powder. $[\alpha]_D^{20} = -125.5$ ($c = 0.10$, acetone). UV (MeOH): 209 (4.10), 248 (3.78), 339 (3.53). IR (KBr): 3331, 3167, 3059, 2963, 2925, 1693, 1658, 1624, 1595, 1427, 1396, 1040, 743. ^{1}H- and ^{13}C-NMR: *Table 1.* HR-ESI-MS (pos.): 416.1561 ($[M + Na]^+$, $C_{22}H_{23}N_3NaO_4^+$; calc. 416.1581).

Brevianamide P (= *(8*S*,11*S*)-8-Benzyl-11-(butan-2-yl)-10,11-dihydro-6*H*-oxepino[2,3-*d*]pyrazino[1,2-*a*]pyrimidine-6,9(8*H*)-dione*; **2**): Yellow amorphous powder. $[\alpha]_D^{20} = -272.3$ ($c = 0.13$, acetone). UV (MeOH): 208 (4.04), 245 (3.68), 336 (3.49). IR (KBr): 3204, 2925, 2854, 1687, 1577, 1535, 1435, 1101, 747. ^{1}H- and ^{13}C-NMR: *Table 1.* HR-ESI-MS (pos.): 400.1635 ($[M + Na]^+$, $C_{22}H_{23}N_3NaO_3^+$; calc. 400.1632).

Brevianamide Q (= *(3*Z*)-8a-Hydroxy-3-{[2-(2-methylbut-3-en-2-yl)-1*H*-indol-3-yl]methylidene}hexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione*; **3**): Colorless crystals. M.p. 184–186°. $[\alpha]_D^{20} = 0.0$ ($c = 0.10$, acetone). UV (MeOH): 204 (4.42), 224 (4.57), 347 (4.20). IR (KBr): 3345, 3268, 2970, 2927, 1711, 1692,

1658, 1576, 1434, 1384, 764. ^{1}H- and ^{13}C-NMR: *Table 2*. HR-ESI-MS (pos.): 388.1637 ($[M+Na]^+$, $C_{21}H_{23}N_3NaO_3^+$; calc. 388.1632).

Brevianamide R (*=(3Z)-8a-Methoxy-3-{[2-(2-methylbut-3-en-2-yl)-1*H*-indol-3-yl]methylidene}hexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione*; **4**): Colorless crystals. M.p. 206–208°. $[\alpha]_D^{20} = 0.0$ ($c = 0.10$, acetone). UV (MeOH): 203 (4.30), 224 (4.45), 350 (4.04). IR (KBr): 3328, 3166, 3058, 2964, 1693, 1658, 1624, 1600, 1427, 1396, 1040, 743. ^{1}H- and ^{13}C-NMR: *Table 2*. HR-ESI-MS (pos.): 402.1802 ($[M+Na]^+$, $C_{22}H_{25}N_3NaO_3^+$; calc. 402.1788).

Cytotoxicity Assay. Cancer cell lines Bre04 (MDA-MB-231), Lu04 (NCI-H460), and N04 (SF-268) were obtained from the *American Type Culture Collection* (ATCC) and cultured according to the supplier's instruction. The cells were seeded into 96-well plates, incubated for 16 h at 37°, and treated with compounds **1**–**4** at different concentrations for 48 h. Taxol was used as positive control. The cytotoxic activities were examined by means of colorimetric chemosensitivity assay with SRB (sulforhodamine B). The GI_{50} value (the drug concentration required to inhibit the cell growth by 50%) was used as a parameter for cytotoxicity [11][12].

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