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FOUR CYCLODIPEPTIDES, ASNOVOLENINS A-B AND ASNOVOZINES A-B, FROM *ASPERGILLUS NOVOFUMIGATUS*

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Abstract – Four new cyclodipeptides, asnovolenins A (**1**) and B (**2**), and asnovozines A (**3**) and B (**4**), were isolated from the fungus *Aspergillus novofumigatus* CBS 117520. The structures of **1-4** were determined by the detailed analysis of mainly 1D- and 2D-NMR and MS data. Compounds **1** and **2** are composed of *epi*-aszonalenin (**5**) and dihydroterrein (**6**), and they are 2'-epimers of each other. Compounds **3** and **4** consist of D-alanine and tryptophan attached to a 3-methyl-1-butene group. The stereochemistry of **1** and **2** was determined from ROESY spectra and the exciton chirality method from CD spectra, and that of **3** and **4** was determined from NOE or NOESY spectra using the modified Marfey's method.

INTRODUCTION

The genus *Aspergillus* includes an extremely diverse array of filamentous ascomycetous fungi found ubiquitously around the world. One member in particular, *A. fumigatus*, is an important bioresource that produces diverse secondary metabolites exhibiting a variety of pharmacologic activities.[1] In a previous study, we reported the isolation of secondary metabolites from methanol extracts of *A. novofumigatus* CBS 117520 cultivated on rice medium. These metabolites included novobezomalvins A-C[2] and asnovolins A-E[3] as fibronectin expression regulators. The cyclodipeptides novoamauromine and *ent*-cycloechinulin were also isolated from same extract of *A. novofumigatus*.[4] We therefore hypothesized that further investigation of methanol extracts of *A. novofumigatus* would lead to the isolation of other novel compounds. In this report, we describe the isolation and structure elucidation of the novel

cyclodipeptides asnovolenins A (**1**) and B (**2**) and asnovozines A (**3**) and B (**4**) using detailed analysis of 1D- and 2D-NMR, MS, and CD spectral data and Marfey's method.[5]

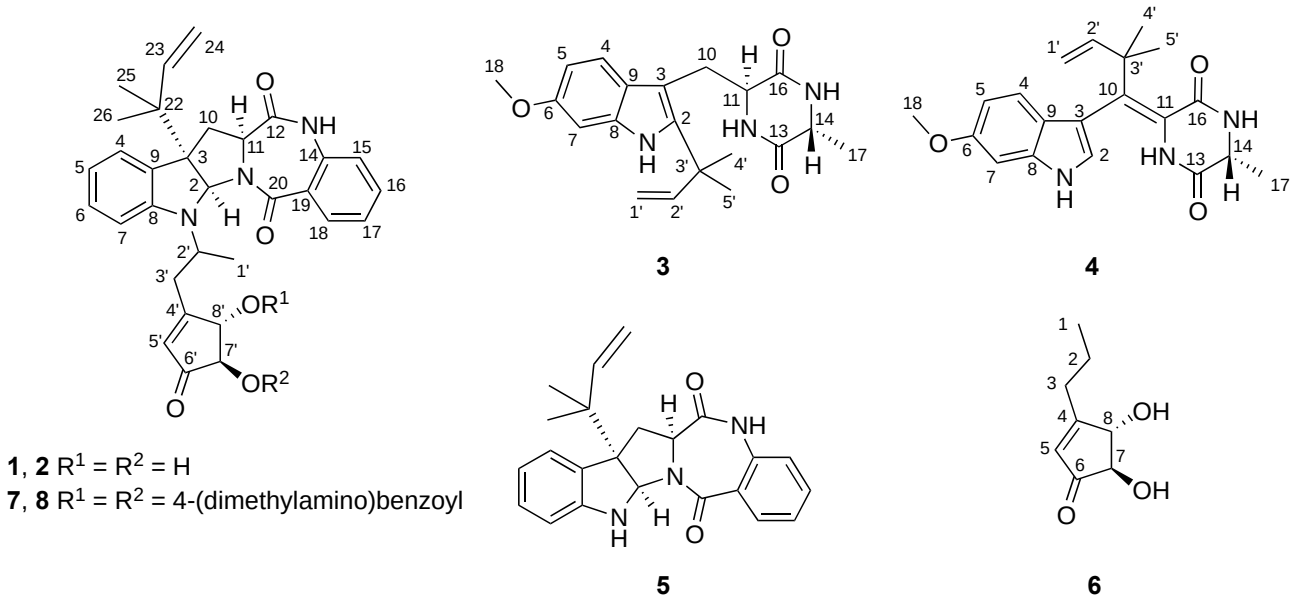


Figure 1. Chemical structures of **1**-**6** isolated from *A. novofumigatus*, and the benzoate derivatives **(7** and **8)** of asnovolenins **A** and **B**

RESULTS AND DISCUSSION

Methanol extracts of *Aspergillus novofumigatus* CBS 117520 cultured on rice medium were separated and purified by HPLC to obtain asnovolenins A (**1**) and B (**2**) and asnovozines A (**3**) and B (**4**), along with *epi*-aszonalenin C (**5**)[6] and dihydroterrein (**6**).[7]

Compound **1** was obtained as a colorless amorphous solid, and the molecular formula was determined as $C_{31}H_{33}N_3O_5$ (17 degrees of unsaturation) by high-resolution chemical ionization mass spectrometry (HRCIMS). The IR bands (1699 and 1636 cm^{-1}) indicated the presence of amide groups.

The ^{1}H-NMR spectrum of **1** exhibited signals for three methyl protons (δ_H 1.38, 1.15, and 1.02), two methylene proton signals (δ_H 3.08 and 2.74, δ_H 2.79 and 2.54), two oxygenated methine protons (δ_H 4.43 and 3.99), three nitrogenous methine protons (δ_H 5.96, 4.39, and 4.05), two sp^2 methine protons (δ_H 5.91 and 5.81), one exomethylene group (δ_H 5.09 and 5.06), and eight aromatic protons constituting two 1,2-disubstituted benzene moieties. The ^{13}C-NMR spectrum of **1** showed the presence of two amide carbonyl carbons (δ_C 170.6 and 167.8) and an α,β-unsaturated ketone carbon (δ_C 203.1), 16 sp^2 carbons including 12 aromatic sp^2 carbons, 3 methyl carbons (δ_C 24.0, 22.9, and 15.7), 5 sp^3 methine carbons (δ_C 85.7, 80.0, 77.4, 58.2, and 52.6) at the adjacent heteroatom positions, 2 sp^3 methylene carbons (δ_C 35.6 and 33.8), and 2 sp^3 quaternary carbons (δ_C 61.0 and 41.9) (Table 1). ^{1}H-^{1}H COSY and HMBC

Table 1. NMR spectroscopic data for asnovolenins A (**1**) and B (**2**)

	asnovolenin A (**1**)				asnovolenin B (**2**)			
Position	δ_C[a]	δ_H[b] (*J* in Hz)			δ_C[a]	δ_H[b] (*J* in Hz)		
1-N								
2	85.7	5.96	s		81.7	6.10	s	
3	61.0				61.9			
4	124.8	7.27	m		124.3	7.27	m	
5	118.4	6.71	m		118.3	6.67	dd	(7.5, 7.3)
6	128.1	7.08	dd	(7.5, 7.5)	128.5	7.09	dd	(7.7, 7.3)
7	109.9	6.69	d	(8.3)	108.2	6.55	d	(7.7)
8	146.7				148.2			
9	133.5				132.7			
10	33.8	2.79	m		34.2	2.74	dd	(14.3, 6.4)
		2.54	dd	(13.6, 8.5)		2.56	m	
11	58.2	4.05	dd	(8.5, 6.4)	58.4	4.00	dd	(8.5, 6.4)
12	170.6				170.9			
13-NH		9.19	brs			9.23	brs	
14	136.5				136.8			
15	122.0	6.96	d	(6.8)	122.0	7.00	d	(6.7)
16	133.4	7.33	dd	(6.8, 6.6)	133.5	7.33	dd	(6.7, 6.7)
17	125.1	7.19	dd	(7.6, 6.6)	124.8	7.17	dd	(7.5, 6.7)
18	130.9	7.93	d	(7.6)	131.0	7.97	d	(7.5)
19	125.7				125.5			
20	167.8				167.5			
21-N								
22	41.9				41.7			
23	144.3	5.91	dd	(17.1, 10.6)	144.5	5.94	dd	(17.5, 11.1)
24	114.4	5.09	d	(17.1)	114.2	5.08	d	(17.5)
		5.06	d	(10.6)		5.04	d	(11.1)
25	22.9	1.15	s		23.2	1.16	s	
26	24.0	1.02	s		24.3	1.03	s	
1'	15.7	1.38	d	(6.3)	16.9	1.36	d	(6.4)
2'	52.6	4.39	m		49.9	4.08	m	
3'	35.6	3.08	dd	(14.0, 10.3)	34.3	2.92	dd	(17.3, 9.0)
		2.74	m			2.60	m	
4'	175.8				176.4			
5'	128.4	5.81	s		127.4	5.79	s	
6'	203.1				203.6			
7'	80.0	3.99	brs		81.2	4.08	brs	
8'	77.4	4.43	brs		77.9	4.30	brs	

[a]Recorded at 100 MHz in $CDCl_3$. [b]Recorded at 400 MHz in $CDCl_3$.

correlations of **1** are shown in Figure 2. ^{1}H-^{1}H COSY correlations indicated six sequences (H-4 to H-7, H_2-10 to H-11, H-15 to H-18, H-23 to H_2-24, H_3-1' to H_2-3', and H-7' to H-8'), as shown by bold lines in Figure 1. HMBC correlations of H-4 (δ_H 7.27) with C-8 (δ_C 146.7), H-7 (δ_H 6.69) with C-9 (δ_C 133.5), H-17 (δ_H 7.19) with C-19 (δ_C 125.7), and H-18 (δ_H 7.93) with C-14 (δ_C 136.5) indicated the existence of two benzene rings. The presence of a 3-methyl-1-butene group was revealed from HMBC correlations of H_3-25 (δ_H 1.15) and H_3-26 (δ_H 1.02) with C-23 (δ_C 144.3) and C-22 (δ_C 41.9). HMBC correlations of H-2 (δ_H 5.96) with C-3 (δ_C 61.0) and C-10 (δ_C 33.8) indicated the existence of a hexahydropyrrolo[2,3-*b*]indole unit. Moreover, it was revealed that the 3-methyl-1-butene group was attached at the C-3 position of a hexahydropyrrolo[2,3-*b*]indole unit, based on HMBC correlation of H_3-25 with C-3. HMBC correlations of H-10 (δ_H 2.54) with C-12 (δ_C 170.6) and H-18 with C-20 (δ_C 167.8) indicated that two amide bonds were located between a hexahydropyrrolo[2,3-*b*]indole unit and a benzene ring. The linkage of N-1 between the sequence of C-1' to C-3' was revealed from the HMBC correlation of H-2 with C-2'. HMBC correlations of H-5' (δ_H 5.81) with C-6' (δ_C 203.1), C-7' (δ_C 80.0), and C-8' (δ_C 77.4) indicated the presence of a 4,5-dihydroxy-2-cyclopenten-1-one group. The planar structure of **1** was deduced from the combination of C-3' (δ_C 35.6) with C-4' (δ_C 175.8) in which the dihydroterrein[8] residue is attached to the N-1 of the aszonalenin[7] residue.

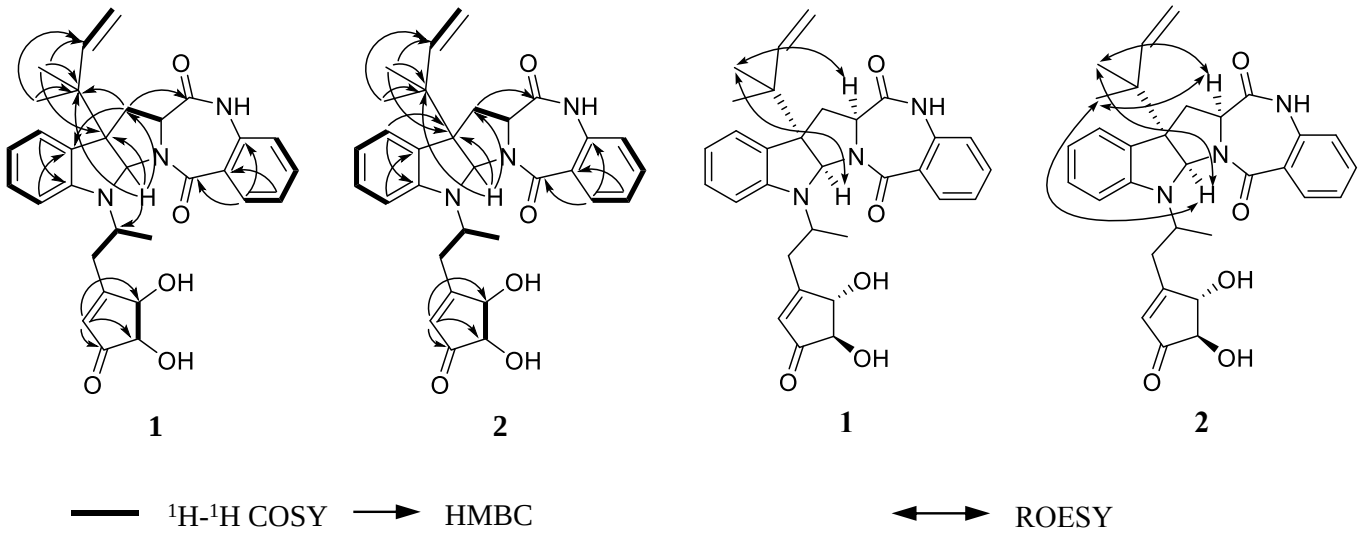


Figure 2. Key 2D-NMR spectra correlations for **1** and **2**

Compound **2** was isolated as a colorless amorphous solid, and the molecular formula was determined as $C_{31}H_{33}N_3O_5$ (17 degrees of unsaturation), the same as **1**, by high-resolution electrospray ionization mass spectrometry (HRESIMS). Furthermore, ^{1}H- and ^{13}C-NMR spectra were very similar to those of **1**, except for chemical shifts at the C-2' position (**2**: δ_H 4.08 and δ_C 49.9; **1**: δ_H 4.39 and δ_C 52.6). Therefore, the planar structure of **2** was determined to be the same as that of **1**. However, the difference in retention time

on preparative HPLC indicated that **1** (t_R = 8.2 min) and **2** (t_R = 8.7 min) are diastereomers at the C-2' position. The relative structure of the aszonalenin residue in **1** and **2** was established from analysis of rotating-frame Overhauser enhancement and exchange spectroscopy (ROESY) spectra (Figure 2). ROESY correlations of H_3-25/H-2 and H-11 in **1** and H_3-25/H_3-26 and H-2/H-11 in **2** indicated the relative configuration of the aszonalenin residue in **1** and **2** was same as that of *epi*-aszonalenins C (**5**).[6] A previous study[8] indicated that the stereochemistry of C-2, C-3, and C-11 depends on the cotton effect at 250 nm, and the same positive cotton effect at 250 nm was observed in each CD spectrum of **1**, **2**, and **5**. On the other hand, **6** showed no positive cotton effect at 250 nm on the CD spectrum (Figure 3).

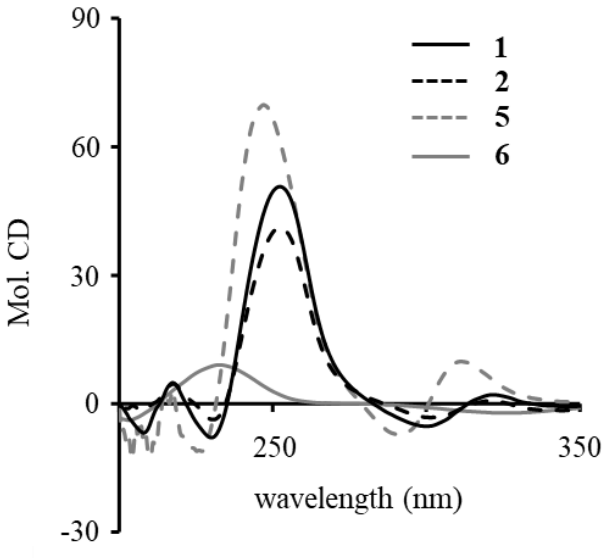
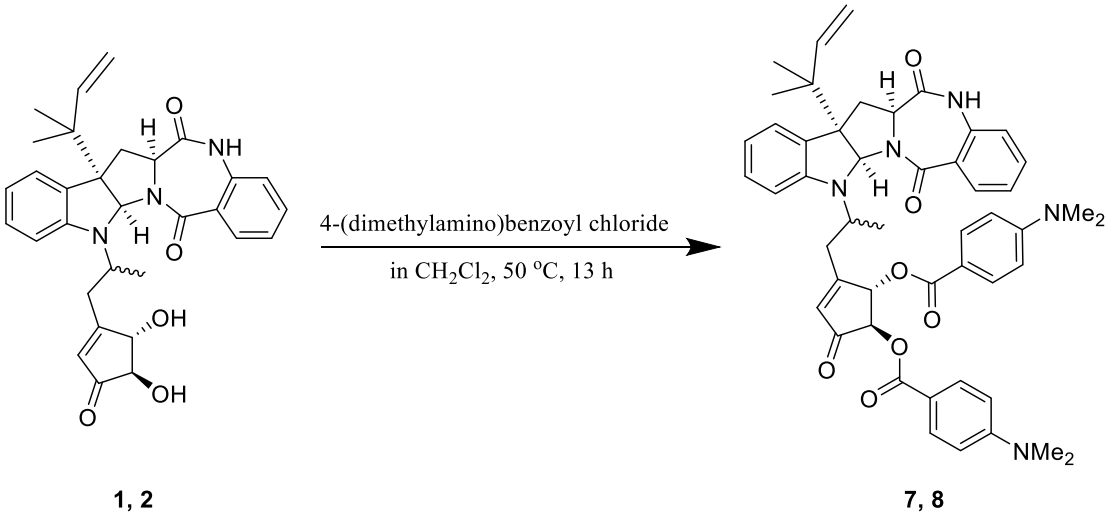


Figure 3. CD spectra of **1**, **2**, **5** and **6**

Therefore, the absolute configuration of the aszonalenin residues in **1** and **2** was determined to be 2*R*, 3*R*, and 11*S* (Figure 1).



Scheme 1. Esterification of **1** and **2** to **7** and **8**

On the other hand, the small coupling constants at H-7' and H-8' in the ^{1}H-NMR spectrum indicated that the relative stereochemistry of the hydroxy groups at C-7' and C-8' in **1** and **2** are *trans*. In order to determine the absolute configuration of C-7' and C-8' of the dihydroterrein residue, **1** and **2** were esterified to **7** and **8** using 4-(dimethylamino)bezoyl (DMAB) chloride in dichloromethane (Scheme 1). Positive cotton effects (325 nm) and negative cotton effects (300 nm) in CD spectra of the DMAB derivatives of **7** and **8** (Figure 4) indicated the absolute configurations of C-7' and C-8' as 7'*R* and 8'*S* based on exciton chirality analysis.[8] From these results, the absolute configurations of **1** and **2**, except for C-2', were determined, as shown in Figure 1.

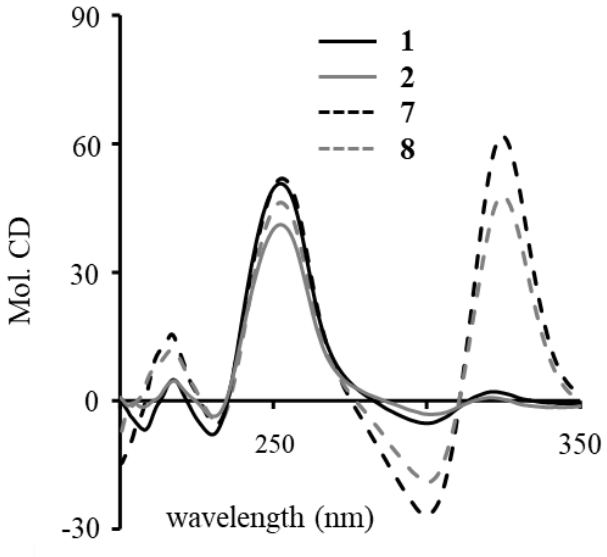


Figure 4. CD spectra of **1**, **2**, **7** and **8**

Compound **3** was isolated as a colorless amorphous solid. The molecular formula of **3** was determined as $C_{20}H_{25}N_3O_3$ based on high-resolution electron ionization mass spectrometry (HREIMS). IR bands (3382, 1676, and 1631 cm^{-1}) and ^{13}C-NMR signals (δ_C 168.9 and 168.6) revealed the presence of two amide bonds. Moreover, ^{1}H-NMR spectra of **3** exhibited signals for three methyl groups (δ_H 1.45 × 2 and 1.19), a methoxy group (δ_H 3.73), three methine protons (δ_H 6.14, 3.82 and 3.17), methylene and exomethylene protons (δ_H 3.20 and 3.08; 5.06 and 5.02), and three aromatic protons constituting a 1,2,4-trisubstituted benzene moiety (Table 2). In addition, ^{1}H-^{1}H COSY spectra indicated four substructures of **3**: the sequence of H-4 to H-5, H_2-10 to H-11, H-14 to H_3-17, and H-1'to H-2', as shown by bold lines in Figure 5. HMBC correlations from H-2' to C-3', C-4' and C-5', and between H_3-4' and H_3-5' to C-2' established the presence of a 3-methyl-1-butene group (Figure 1).

Table 2. NMR spectroscopic data for asnovozines A (**3**) and B (**4**)

	asnovozine A (**3**)				asnovozine B (**4**)			
Position	δ_C[a]	δ_H[b] (*J* in Hz)			δ_C[a]	δ_H[b] (*J* in Hz)		
1-NH		10.40	brs			10.87	brs	
2	139.8				110.4	6.88	s	
3	104.4				103.2			
4	118.6	7.27	d	(8.6)	119.6	7.08	d	(8.6)
5	108.1	6.59	dd	(8.6, 1.6)	109.2	6.68	dd	(8.6, 2.4)
6	155.1				155.3			
7	94.1	6.82	d	(1.6)	95.2	6.93	d	(2.4)
8	135.4				135.9			
9	123.3				120.3			
10	29.4	3.20	m		142.8			
		3.08	dd	(14.5, 8.6)				
11	48.6	3.17	m		124.6			
12-NH		7.79	brs			8.56	s	
13	168.9				156.0			
14	56.8	3.82	m		50.6	4.15	q	(7.0)
15-NH		8.13	brs			8.30	brs	
16	168.6				166.5			
17	17.1	1.19	d	(7.0)	19.7	1.37	d	(7.0)
18	55.1	3.73	s		55.3	3.76	s	
1'	110.8	5.06	dd	(16.9, 9.6)	111.6	5.03	dd	(10.6, 1.2)
		5.02	dd	(10.5, 9.6)		5.01	dd	(17.6, 1.2)
2'	146.6	6.14	dd	(16.9, 10.5)	145.3	6.05	dd	(17.6, 10.6)
3'	39.1				39.0			
4'	27.9	1.45	s		27.6	1.45	s	
5'	27.9	1.45	s		27.6	1.45	s	

[a]Recorded at 100 MHz in DMSO-d_6. [b]Recorded at 400 MHz in DMSO-d_6.

Furthermore, HMBC correlations from H-4 to C-6, C-8, and C-9; H-5 to C-7 and C-9; H-7 to C-5, C-6, C-8, and C-9; H_2-10 (δ_H 3.20 and 3.08) to C-2, C-3, C-9, and C-16; H-11 to C-16, and H_3-18 to C-6 revealed the presence of a 6-methoxytryptophan group. In addition, HMBC correlations from H-2', H_3-4', and H_3-5' of a 3-methyl-1-butene group to the C-2 position of the 6-methoxytryptophan group indicated the linkage of a 3-methyl-1-butene group and a 6-methoxytryptophan group. Moreover, an alanine residue was revealed by HMBC correlations from H-14 and H_3-17 to C-13. HMBC correlations from H-11 to C-13, C-16 and H-14 to C-13, C-16 indicated the presence of a diketopiperazine ring consisting of 6-methoxytryptophan and alanine residues. The relative structure of **3** was determined by NOE spectra

and the modified Marfey's method. The key NOE correlation of H-14 to H-10 (δ_H 3.08) was observed; therefore, the stereochemistry of the diketopiperazine ring was *anti* (Figure 5). Moreover, acid hydrolysis of **3** followed by derivatization using 1-fluoro-2,4-dinitrophenyl-L-alanine-amide (Marfey's reagent) and comparison of HPLC retention time with those of D/L-alanine derivative standards revealed that the alanine residue of **3** was the D- form. Thus, the stereochemistry of C-11 and C-14 were determined as *S* and *R* configuration, respectively, and the absolute configuration of **3** was established.

Compound **4** was isolated as a colorless amorphous solid, and the molecular formula was determined as $C_{20}H_{23}N_3O_3$ based on HRESIMS. The molecular formula of **4** had two fewer hydrogen atoms compared with that of **3**. Moreover, ^{1}H- and ^{13}C-NMR spectra of **4** were very similar to those of **3**, except for the absence of H_2-10 and H-11 signals and the appearance of an H-2 signal (δ_H 6.88). Therefore, it was thought that the planar structure of **4** was formed by dehydration of C-10/11 and attachment to the 3-methyl-1-butene group at the C-10 position of **3**. HMBC correlations from H-2 of the 6-methoxyindole ring and H-2', H_3-4', and H_3-5' of the 3-methyl-1-butene group to the C-10 position indicated that the 3-methyl-1-butene group was attached to the C-10 position (Figure 1). Detailed analysis of NOESY spectrum indicated that key NOESY correlations of NH-12 (δ_H 8.56 [s])/H-2 (δ_H 6.88 [s]) and H-4 revealed that C10/11 was in the *Z* form (Figure 5). The stereochemistry of the C-14 position was determined as 14*R* using Marfey's method, similar to **3**.

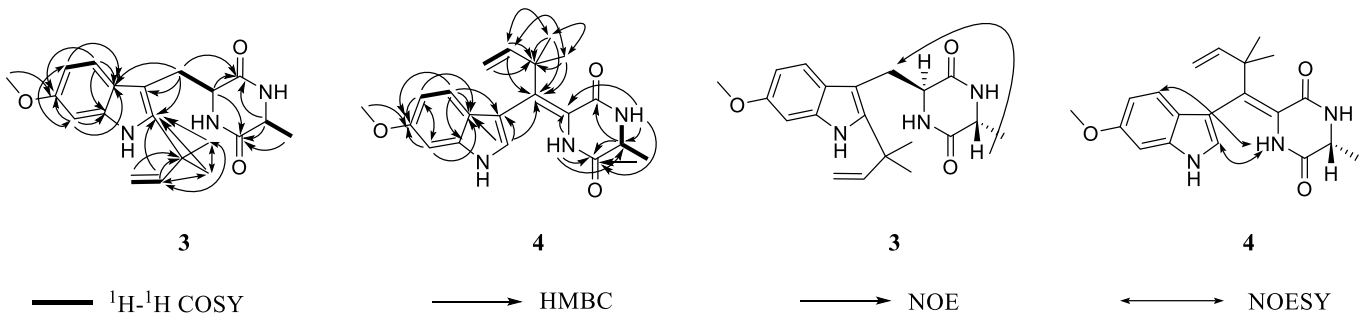


Figure 5. Key 2D-NMR and NOE correlations for **3** and **4**

Antifungal and cytotoxic activity of **1** and **2** was studied using the paper disk method, as described previously.[9] Compounds **1** and **2** showed non-specific antifungal activity against *A. fumigatus*, *A. niger*, *Candida albicans*, and *Cryptococcus neoformans* at 100 μg per disk. Neither **1** nor **2** exhibited cytotoxic activity against A549 human lung cancer cells, HeLa human cervical cancer cells, and LNCap human prostate adenocarcinoma cells.

In a previous study,[2] we reported several novel compounds along with known compounds such as helvolic acid and terrein. In this study, we isolated four novel cyclodipeptides and determined their

structures using a spectroscopic analysis and chemical approach. Asnovolenins A (**1**) and B (**2**) were found to consist of *epi*-aszonalein C (**5**) and dihydroterrein residues. The *epi*-aszonalein C (**5**) was found to be a cyclodipeptide consisting of two amino acids, L-tryptophan and anthranilic acid, and was isolated only from *A. novofumigatus*. By contrast, aszonalenins typically consist of D-tryptophan and anthranilic acid, and there are numerous reports of their isolation from a variety of fungi, such as *Aspergillus* sp. and *Neosartorya fischeri*. Asnovozines A (**3**) and B (**4**) were found to consist of two amino acids, D-alanine and L-tryptophan, and our results suggested that **3** is an intermediate of *ent*-cycloechinulin. Asnovozine B (**4**) had a 3-methyl-1-butene group attached at the C-10 position. To our knowledge, there are no reported examples of a 3-methyl-1-butene group attached at the C-10 position. Interestingly, a novoamauromine and an *ent*-cycloechinulin isolated only from *A. novofumigatus* were also epimers.[4] These results suggest that *A. novofumigatus* could be characterized based on differences in the stereochemistry of compounds isolated from the fungus.

None of the compounds isolated in this study exhibited antifungal activity. Previous studies reported that aszonalenins act as substance P inhibitors for the human neurolinin-1 receptor[10] and that terrein is a melanogenesis inhibitor.[11] These results suggest that **1** and **2**, which include *epi*-aszonalenin C and dihydroterrein residues, would exhibit similar bioactivity.

EXPERIMENTAL

EI and CIMS data were collected using a JMS-MS600W spectrometer (JEOL Co., Ltd., Tokyo, Japan), and ESIMS data were collected using a JMS-T100LP spectrometer (JEOL Co. Ltd.). UV and IR spectra were recorded using an Ultrospec 2100 pro UV-visible spectrophotometer (Amersham Biosciences Ltd., Buckinghamshire, UK) and FT/IR-4100 instrument (JASCO Co. Ltd., Tokyo, Japan), respectively. ^{1}H- and ^{13}C-NMR spectra were recorded using an AVANCE-400 spectrometer (400.13 MHz for ^{1}H, 100.61 MHz for ^{13}C, Bruker Biospin, Billerica, MA). Chemical shifts (δ) were measured in ppm using tetramethylsilane as an internal standard. CD curves were determined on a J-820 spectropolarimeter (JASCO Co., Ltd.). Optical rotation was measured using a P-1020 photopolarimeter (JASCO Co., Ltd.). TLC plates were visualized under UV light at 254 nm and/or by spraying with phosphomolybdic acid (5%)-ceric acid (trace) in 5% H_2SO_4 and then heating. Column chromatography was performed using a Sephadex LH-20 column (GE Healthcare Bio-Science AB, Uppsala, Sweden). Middle-pressure liquid chromatography (MPLC) was performed using a Chemco Low-Prep 81-M-2 pump (Chemco Scientific Co., Ltd., Osaka, Japan) and ULTRA PACK SI-40B column (300 × 26 mm, Yamazen Corp., Osaka, Japan). Preparative HPLC was performed using a Senshu SSC-3160 pump (flow rate 4 mL/min, Senshu Scientific Co., Ltd., Tokyo, Japan) and Inertsil ODS-P column (250 × 10 mm, GL Sciences Inc., Tokyo, Japan) on a system equipped with a YRD-883 RI detector (Shimamuratech Ltd., Tokyo, Japan). Samples

were examined by Marfey's method using PDA-HPLC with PU-980 and PU-1580 pumps (flow rate 1 mL/min; JASCO Co., Ltd.) and Inertsil ODS-3 column (5 μm, 4.6 mm × 250 mm; GL Science Inc., Tokyo, Japan) maintained at a temperature of 40 °C using a CO-2065 Plus column oven (JASCO Co.) and equipped with a MD-2010 Plus photodiode array detector (JASCO Co.).

Fermentation and Isolation: Polished rice (Akitakomachi, 24 kg) was soaked in water for 30 min and then sterilized in an autoclave. *Aspergillus novofumigatus* CBS 117520 was cultivated on sterilized rice (140 g) at 30 °C for 21 days in Roux flasks. Cultivated rice was then extracted with MeOH, and the MeOH extract was evaporated at 40 °C under reduced pressure. The resulting residue was suspended in water and extracted with EtOAc. The concentrated EtOAc extract (52 g) was partitioned between MeCN and *n*-hexane to yield an MeCN extract. The MeCN extract (29.4 g) was sequentially extracted by a solid-liquid separation method using *n*-hexane, benzene, $CHCl_3$, EtOAc, and MeOH. Then, *n*-hexane extract, benzene extract, $CHCl_3$ extract, EtOAc extract, and MeOH extract was obtained by removing organic solvent, respectively. The benzene extract was chromatographed on a Sephadex LH-20 column (solvent system: *n*-hexane/$CHCl_3$ [1:4], 200 mL; $CHCl_3$/acetone [3:2], 200 mL; $CHCl_3$/acetone [1:4], 200 mL; acetone, 200 mL; and then MeOH, 500 mL). Fraction 3 ($CHCl_3$/acetone [1:4] elute) was chromatographed by MPLC on a silica gel column ($CHCl_3$/MeOH [10:1]) followed by HPLC on a silica gel column ($CHCl_3$/MeOH [20:1]) to isolate asnovolenins A (**1**: 44.9 mg) and B (**2**: 72.0 mg) and asnovozine A (**3**: 1.0 mg). The other known compounds, *epi*-aszonalenin A (217 mg) and C (**5**: 85 mg), terrein, and dihydroterrein were isolated from the benzene extract, and identified by comparison with spectral data in the literature.[7,8] The $CHCl_3$ extract was chromatographed on a Sephadex LH-20 column (solvent system: *n*-hexane/ $CHCl_3$ [1:4], 200 mL; $CHCl_3$/acetone [3:2], 200 mL; $CHCl_3$/acetone [1:4], 200 mL; acetone, 200 mL; and then MeOH, 500 mL). Fraction 3 ($CHCl_3$/acetone [1:4] elute) was chromatographed by MPLC on a silica gel column ($CHCl_3$/MeOH [15:1]) followed by HPLC on a silica gel column ($CHCl_3$/MeOH [35:1] to [6:1]) to isolate asnovozine B (**4**: 3.1 mg).

Asnovolenin A (1): Colorless amorphous solid; $[\alpha]_D^{18}$ +211.7 (c 0.29, MeOH); UV (MeOH) λ_{max} (logε) 216 (4.9), 252 (4.5), 300 (4.0) nm; IR (KBr)ν_{max} 3447, 1699, 1636 cm^{-1}; CD (c 3.80×10^{-5}, MeOH) Δε (λ_{max}) −6.8 (208), 5.5 (217), −7.9 (230), 50.7 (252), −5.3 (300), 2.1 (322), −0.7 (345) nm. HRCIMS obsd. 528.2519 $[M+H]^+$ (calcd. 528.2498 for $C_{31}H_{34}N_3O_5$); The ^{1}H- and ^{13}C-NMR signal assignments are summarized in Table 1.

Asnovolenin B (2): Colorless amorphous solid; $[\alpha]_D^{20}$ +218.8 (c 0.25, MeOH); UV (MeOH) λ_{max} (logε) 215 (4.5), 249 (4.1), 300 (3.6) nm; IR (KBr) ν_{max} 3430, 1700, 1636 cm^{-1}; CD (c 3.80×10^{-5}, MeOH) Δε (λ_{max}) −3.3 (204), 6.7 (216), −5.6 (231), 6.7 (216), 50.7 (252), −4.8 (302), 1.0 (321), −2.5 (343) nm; HRCIMS obsd. 528.2512 $[M+H]^+$ (calcd. 528.2498 for $C_{31}H_{34}N_3O_5$); The ^{1}H- and ^{13}C-NMR signal assignments are summarized in Table 1.

Asnovolenin A di-4-(dimethylamino)benzoate (7): Compound **1** (6.2 mg, 0.012 mmol), 4-(dimethylamino)benzyl chloride (32.9 mg, 0.18 mmol) and DCM (1 mL) were mixed and stirred at 50 °C for 13 h. The reaction mixture was extracted with $CHCl_3$ and water. The extract was evaporated and residue purified by HPLC (benzene/acetone [10:1]) to afford 3.0 mg of **7**.

Colorless amorphous solid; UV (MeOH) λ_{max} (logε) 208 (4.8), 215 (4.8), 251 (4.4), 317 (4.8) nm; CD (c 2.44×10^{-5}, MeOH) $\Delta\varepsilon$ (λ_{max}) 12.2 (214), 15.5 (217), −5.5 (231), 51.9 (253), −26.8 (300), 61.8 (325) nm; ESIMS obsd. 844.0 $[M+Na]^+$; ^{1}H-NMR ($CDCl_3$) δ : 1.03 (3H, s, H-26), 1.15 (3H, s, H-25), 1.33 (3H, d, *J*=6.9 Hz, H_3-1'), 2.60 (2H, m, H-3'), 2.93 (1H, dd, *J*=14.4, 4.8 Hz, H-10), 3.03 (6H, s, N-$(CH_3)_2$ × 2), 3.04 (6H, s, N-$(CH_3)_2$ × 2), 3.16 (1H, dd, *J*=14.4, 9.5 Hz, H-10), 4.14 (1H, dd, *J*=9.5, 4.8 Hz, H-11), 4.53 (1H, m, H-2'), 5.09 (1H, dd, *J*=10.9, 1.0 Hz, H-24), 5.10 (1H, *J*=17.2, 1.0 Hz, H-24), 5.56 (1H, d, *J*=3.0 Hz, H-7'), 5.94 (1H, dd, *J*=17.2, 10.9 Hz, H-23), 5.97 (1H, s, H-2), 6.30 (1H, dd, *J*=3.0, 0.8 Hz, H-8'), 6.51 (1H, d, *J*=8.2 Hz, H-4), 6.57 (2H, d, *J*=9.2 Hz), 6.58 (2H, d, *J*=9.2 Hz), 6.65 (1H, dd, *J*=7.6, 7.4 Hz, H-6), 6.86-6.92 (2H, m, H-7, H-15), 7.22-7.28 (2H, m, H-5, H-17), 7.42 (1H, ddd, *J*=7.8, 7.7, 1.5 Hz, H-16), 7.76 (2H, d, *J*=9.2 Hz), 7.80 (1H, s, NH), 7.85 (2H, d, *J*=9.2 Hz), 8.03 (1H, dd, *J*=7.9, 1.5 Hz, H-18).

Asnovolenin B di-4-(dimethylamino)benzoate (8): Compound **2** (7.1 mg, 0.013 mmol), 4-(dimethylamino)benzoyl chloride (32.9 mg, 0.18 mmol) and DCM (1 mL) were mixed and stirred at 50 °C for 13 h. The reaction mixture was extracted with $CHCl_3$ and water. The extract was evaporated and residue purified by HPLC (benzene/acetone [9:1]) to afford 1.0 mg of **8**.

Colorless amorphous solid; UV (MeOH) λ_{max} (logε): 207 (4.7), 215 (4.7), 251 (4.2), 317 (4.7) nm; CD (c 2.44×10^{-5}, MeOH) $\Delta\varepsilon$ (λ_{max}) 8.1 (212), 12.0 (217), −5.0 (232), 46.3 (252), −19.2 (300), 47.8 (325) nm; ESIMS obsd. 844.0 $[M+Na]^+$; ^{1}H-NMR ($CDCl_3$) δ : 1.03 (3H, s, H-26), 1.15 (3H, s, H-25), 1.33 (3H, d, *J*=6.9 Hz, H_3-1'), 2.59 (1H, dd, *J*=13.9, 9.7 Hz, H-10), 2.66 (1H, dd, *J*=17.7, 11.3 Hz, H-3'), 2.87 (1H, dd, *J*=17.7, 8.1 Hz, H-3'), 2.90 (1H, dd, *J*=13.9, 4.4 Hz, H-10), 3.04 (6H, s, N-$(CH_3)_2$ × 2), 3.04 (6H, s, N-$(CH_3)_2$ × 2), 4.14 (1H, dd, *J*=9.7, 4.4 Hz, H-11), 4.28 (1H, m, H-2'), 5.09 (1H, dd, *J*=17.6, 1.1 Hz, H-24), 5.10 (1H, dd, *J*=10.5, 1.1 Hz, H-24), 5.26 (1H, d, *J*=3.0 Hz, H-7'), 5.99 (1H, dd, *J*=17.6, 10.5 Hz, H-23), 6.08-6.13 (2H, m, H-2, H-8'), 6.31 (1H, d, *J*=0.7 Hz, H-5'), 6.49 (1H, d, *J*=8.0 Hz, H-4), 6.61 (4H, m), 6.71 (1H, dd, *J*=7.5, 7.3 Hz, H-6), 6.88 (1H, d, *J*=8.1 Hz, H-15), 7.02 (1H, t, *J*=7.5 Hz, H-17), 7.20-7.32 (2H, m, H-5, H-7), 7.44 (1H, ddd, *J*=8.1, 7.5, 1.5 Hz, H-16), 7.87 (2H, d, *J*=9.0 Hz), 7.87 (2H, d, *J*=9.0 Hz), 7.90 (1H, s, NH), 8.05 (1H, dd, *J*=7.5, 1.5 Hz, H-18).

Asnovozine A (3): Colorless amorphous solid; $[\alpha]_D^{26}$ −17.6 (c 0.05, MeOH); UV (MeOH) λ_{max} (logε) 212 (4.3), 226 (4.4), 298 (3.6) nm; IR (KBr) ν_{max} 3382, 1676, 1631 cm^{-1}; CD (c 5.63×10^{-5}, MeOH) $\Delta\varepsilon$ (λ_{max}) −7.4 (219), 1.9 (239) nm; HREIMS obsd. 355.1880 $[M]^+$ (calcd. for $C_{20}H_{25}N_3O_3$ 355.1896); The ^{1}H- and ^{13}C-NMR signal assignments are summarized in Table 2.

Asnovozine B (4): Colorless amorphous solid; $[\alpha]_D^{27}$ +51.8 (c 0.16, MeOH); UV (MeOH) λ_{max} (logε): 223 (4.4), 301 (3.9), 344 (3.9) nm; IR (KBr) ν_{max} 3216, 1673, 1629 cm^{-1}; CD (c 8.50×10-5, MeOH) $\Delta\varepsilon$ (λ_{max}) 1.7 (211), 2.4 (221), −1.4 (241), −0.8 (262), −0.9 (300), 0.6 (346) nm; HRESIMS obsd. 376.16304 $[M+Na]^+$ (calcd. 376.16371 for $C_{20}H_{23}N_3O_3Na$); The ^1H- and ^{13}C-NMR signal assignments are summarized in Table 2.

Amino acid analysis of 3 and 4: Compounds **3** and **4** (each 1.0 mg) were dissolved in 100 μL of 6 M HCl and heated at 110 °C for 12 h. The resulting hydrolysates were allowed to cool and then neutralized with $NaHCO_3$. Then, 200 μL of Marfey's reagent (PIERCE, IL, USA) and 40 μL of 1 M $NaHCO_3$ were added, and the mixture were heated at 40 °C for 1 h. Upon cooling to room temperature, 20 μL of 2 M HCl was added, respectively. The solution was then analyzed by reversed-phase PDA-HPLC (Analysis condition; Flow rate was 1 mL/min, and the column oven temperature was maintained at 30 °C. Mobile phase was 60% MeCN, and compounds were detected at a UV wavelength of 340 nm.), as previously described.[10] Comparison with t_R value for D- and L-alanine standards indicated that the alanyl residue in **3** and **4** has a D-configuration, respectively.

Antifungal assay using the paper disk method: Antifungal assay was performed according to the paper disk method using *A. niger* IFM 41398, *A. fumigatus* IFM 41362, *C. albicans* IFM 40009, and *C. neoformans* ATCC 90112 as test organisms. Asnovolenins A and B (**1** and **2**) were applied to the paper disk (diameter: 8 mm) at 100 μg per disk, and the disks were then placed on the assay plates. The test organisms were cultivated in potato dextrose agar (Nissui Pharmaceutical Co., Ltd, Tokyo, Japan) at 25 °C. After 48-72 h of incubation, zones of inhibition (diameter measured in millimeters) were recorded.

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