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A NOVOFUMIGATAMIDE, NEW CYCLIC TRIPEPTIDE FROM *ASPERGILLUS NOVOFUMIGATUS*

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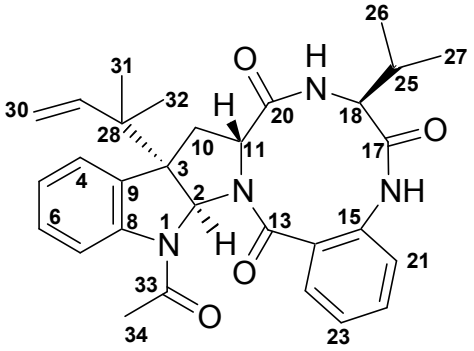
Abstract – A new cyclic tripeptide, novofumigatamide (**1**) has been isolated from *Aspergillus novofumigatus* CBS117520. The relative structure of **1** was established on the basis of spectroscopic and physico-chemical data and chemical investigations. The absolute structure of **1** was determined by using a Marfey's method. Compound **1** showed non-specific antifungal activities against some human pathogenic fungi and did not inhibit cell proliferation for any tumor cells.

The fungus *Aspergillus fumigatus* is known as an important human pathogen, which produces many secondary metabolites. Recently, the fungus *Aspergillus novofumigatus* CBS11520 was re-identified as the new *Aspergillus* sp., closely related to *A. fumigatus* by Hong *et al*.[1] We have previously isolated diketopiperazines, novoamauromine and *ent*-cycloechinulin from the methanolic extract of this fungus cultivated on rice using a thin layer chromatography (TLC) analysis-guided fractionation.[2] Further investigations for this fungal metabolite led to the isolation of a new cyclic tripeptide, novofumigatamide (**1**), from this fungus. This report describes the isolation, structure, and antifungal and cytotoxic activities of **1**.

Solid-substrate fermentation cultures of *A. novofumigatus* CBS117520 grown on rice were extracted with MeOH, and the evaporated extract was suspended in water and extracted with ethyl acetate. The evaporated extract was partitioned with acetonitrile (MeCN) and n-hexane to yield a MeCN fraction. The fraction was extracted sequentially with *n*-hexane, benzene, chloroform, ethyl acetate, and MeOH. The benzene extract was chromatographed using a Sephadex LH-20 column, followed by medium pressure liquid column chromatography (MPLC) on silica gel. A positive color test (blue) with 5%

phosphomolybdic acid - trace ceric acid in 5% H_2SO_4 on TLC led to the isolation of compounds. Further purification of these fractions by HPLC yielded novofumigatamide (**1**), along with novoamauromine, *ent*-cycloechinulin, *epi*-aszonalenins A and C, and helivolic acid, all of which were identified by comparison with the spectral data from reports in the literature.[2,3]



novofumigatamide (**1**)

The molecular formula of novofumigatamide (**1**) was determined as $C_{30}H_{34}N_4O_4$ by HREIMS. The presences of amine NH groups were deduced from the broad absorption at 3447 cm^{-1} in the IR spectrum. Amide groups were inferred from the ^{13}C-NMR spectra (151.1, 160.3, 164.7, and 170.1 ppm) and the broad absorption at 1673 cm^{-1} in the IR spectrum. Two singlet methyl signals at δ 1.03 (s) and δ 1.21 (s), vinyl groups at H_2-30 (δ 5.12 and δ 5.13) and H-29 (δ 5.48), CH_2-CH units at H_2-10 (δ 2.62 and δ 2.99) and H-11 (δ 4.43), CH-CH$(CH_3)_2$ units at H-18 (δ 5.22), H-25 (δ 1.98), H_3-26 (δ 0.86) and H_3-27 (δ 0.94), acetyl group at H_3-34 (δ 2.69) and a carbonyl carbon (170.1 ppm), and two *ortho*-disubstituted benzene rings, were deduced from ^{1}H-, ^{13}C-NMR, ^{1}H-^{1}H COSY and HSQC data. The results of HMBC correlations of H-4 to C-3, and H-10 to C-2 and C-3, showed hexahydropyrrolo[2,3-*b*]indole unit including an *ortho*-disubstituted benzene ring (C4 to C9) and CH_2-CH units at H_2-10 and H-11. The HMBC correlations of a quaternary carbon signal (C-28) at δ 40.4 between the methyl signals at δ 1.03 (s) / δ 1.21(s) and the vinylic resonance at H_2-30, showed the presence of the dimethylpropenyl unit. The HMBC correlations of methyl proton signals of the dimethylpropenyl unit to C-3 indicated that the dimethylpropenyl unit should be attached to the C-3 of hexahydropyrrolo[2,3-b]indole unit.

The HMBC correlations were observed from H-24 at an another *ortho*-disubstituted benzene ring (C-14, C-15, and C-21 to C-24) to C-13 (δ160.3), and from two methyl signals at δ 0.94 (d) and δ 0.86(d) to CH-CH units carbon signals at C-18 (δ 62.3), and C-25 (δ 31.9), and from CH-CH units proton signals H-18 (δ 5.22), and H-25 (δ 1.98) to C-17 (δ 164.7). These results indicated the presence of anthranyl and valinyl groups in **1**. Furthermore, the results of HMBC correlations of H-18 to C-20 and C-17, and H-11 to C-13 and C-20 indicated that three partial structures of hexahydropyrrolo[2,3-*b*]indole unit, anthranyl

and valinyl groups constructed cyclic structure in amide linkage. ROESY correlations were observed with the signal for H-2 to H-31 and H-32, and the signals for H-18 and H-10a (δ 2.99) to two methyl proton signals of the dimethylpropenyl unit, and then the signal for H-10b (δ 2.62) also showed correlation with H-11. Thereby, H-2, H-10a, H-18, and the dimethylpropenyl unit must be on the same face of the ring system, and H-10b and H-11 must be on the opposite side of them. Furthermore, ROESY results also showed the linkage position of acetyl group. The ROESY correlation with H_3-34 (δ 2.69) and H-2 (δ 6.05) showed that the acetyl group was attached to the N-1 position in a hexahydropyrrolo[2,3-*b*]indole unit. Thus, the gross relative structure of **1** was determined to be that of **1** as shown in Figure 1.

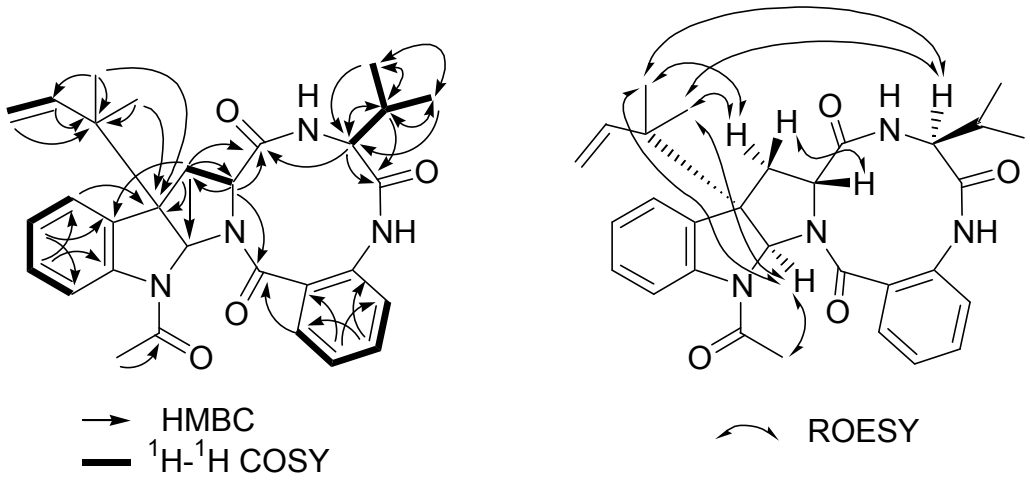


Figure 1. Selected 2D NMR Correlations and Relative Stereochemistry of novofumigatamide (**1**)

The absolute stereochemistry of **1** was determined with using a Marfey's method. The acid hydrolysis of **1**, followed by derivatization using 1-fluoro-2,4-dinitrophenyl-5-L-alanine-amide (Marfey's reagent),[4] and HPLC comparison to the valine standards indicated that valinyl residues obtained as the degradation products had the *L* configuration. From the above results, it was determined that C-2, C-3, C-11, and C-18 positions in **1** have *S*, *S*, *R*, and *S* configuration, respectively, and the absolute configuration of **1** is that of **1** as shown in Figure 1.

Antifungal and cytotoxicity assay of **1** were studied using a previously reported method.[5] Unfortunately, **1** showed non-specific antifungal activities against some human pathogenic fungi at 100 μg per disk and did not inhibit cell proliferation for any tumor cells at 100 μM.

EXPERIMENTAL

EI-MS data were measured using a JMS-MS600W spectrometer (JEOL Co. Ltd., Tokyo, Japan), respectively. UV and IR spectra were recorded on an Ultrospec 2100 pro UV-visible spectrophotometer

(Amersham Biosciences Ltd., Tokyo, Japan) and a JASCO FT/IR-4100 instrument (JASCO Co. Ltd.), respectively. ^{1}H-and ^{13}C-NMR spectra were recorded using a Bruker AVANCE-400 spectrometer (400.13 MHz for ^{1}H, 100.61 MHz for ^{13}C, Bruker Biospin K. K., Kanagawa, Japan). 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 rotations were measured with a JASCO P-1020 photopolarimeter (JASCO Co. Ltd.). TLC spots were visualized by UV light at 254 nm, and 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 (GE Healthcare Bio-Science AB, Uppsala, Sweden). MPLC was performed using a Chemco Low-Prep 81-M-2 pump (Chemco Scientific Co. Ltd., Osaka, Japan) and an ULTRA PACK SI-40B column (300 × 26 mm, Yamazen Corp., Osaka, Japan). HPLC was performed using a Senshu SSC-3160 pump (flow rate 7 mL/min, Senshu Scientific Co. Ltd., Tokyo, Japan) and a YMC-Pack PEGASIL Silica 60-5 column (300 × 10 mm, YMC Co. Ltd., Kyoto, Japan), equipped with a YRD-883 RI detector (Shimamuratech Ltd., Tokyo, Japan). HPLC analytical conditions for Marfey's method were as follows: column, Inertsil ODS-3, 4.6 × 250 mm (GL sciences Inc., Tokyo, Japan); mobile phase, MeCN-0.1% TFA (50:50); flow rate, 1.0 mL/min; column oven temperature at 40 °C; detector, MD-2010 PLUS photodiode array, (JASCO Co. Ltd.).

Isolation of Metabolites from *Aspergillus novofumigatus* CBS117520

Polished rice (Akitakomachi, 24 kg) was soaked in water for 30 min and then sterilized with an autoclave. *A. novofumigatus* CBS117520 was cultivated for 14 days in Roux flasks, each containing 140 g of moisted rice. The cultivated rice was extracted with MeOH and the extract was concentrated in *vacuo*. The resulting extract was suspended in water and extracted with EtOAc. The EtOAc extract (52.3 g) was partitioned between hexane and MeCN to yield a MeCN fraction. The MeCN fraction (29.4 g) was extracted sequentially with n-hexane (100 mL), benzene (100 mL), $CHCl_3$ (100 mL), EtOAc, and MeOH (100 mL). The benzene extract (18 g) was chromatographed using a Sephadex LH-20 column [solvent system: *n*-hexane/$CHCl_3$ (1:4) (180 mL), $CHCl_3$/acetone (3:2) (220 mL), (1:4) (200 mL), acetone (200 mL), and MeOH (500 mL)] to yield five fractions. Fraction 2 [$CHCl_3$/acetone (3:2) eluate] was rechromatographed using MPLC with a silica gel [*n*-hexane/acetone (2:1) to acetone] to give novofumigatamide (**1**: 3 mg), along with novoamauromine (2 mg), *ent*-cycloechinulin (5 mg), *epi*-aszonalenin A (217 mg), and C (85 mg), and helivolic acid (162 mg). The spectral data of these compounds were identical to those from reports in the literature.[2,3]

Novofumigatamide (1): Colorless amorphous solid; $[\alpha]_D^{20}$ -31° (c 0.15, MeOH); HREIMS obsd 514.2574, calcd for $C_{30}H_{34}N_4O_4$ (M^+) 514.2580; UV λ_{max}^{MeOH} nm (log ε) 208 (4.5), 227 (4.4), 267 (3.9), 304 (3.5) and 317 (3.5); IR ν_{max}^{KBr} cm^{-1} 3447, 1684, 1607 and 1572; CD (c 6.52 × 10^{-5}, MeOH) Δε (nm) -27.8

(201), 17.5 (209), -7.0 (218), -2.5 (226), -3.5 (232), 8.4 (250) and -1.3 (307). ^{1}H NMR (400 MHz, $CDCl_3$): 8.27 (1H, d, J = 7.8 Hz, H-24), 8.02 (1H,d, J = 7.7 Hz, H-7), 7.78 (1H, t, J = 7.8 Hz, H-22), 7.66 (1H, d, J = 7.8 Hz, H-21), 7.50 (1H, t, J = 7.8 Hz, H-23), 7.39 (1H, d, J = 7.7 Hz, H-4), 7.35 (1H, t, J = 7.7 Hz, H-6), 7.18 (1H, t, J = 7.7 Hz, H-5), 6.04 (1H, s, H-2), 5.84 (1H, dd, J = 17.4, 10.8 Hz, H-29), 5.22 (1H, d, J = 7.5 Hz, H-18), 5.13 (1H, brs, J = 17.4 Hz, H-30), 5.12 (1H, brs, J = 10.9 Hz, H-30), 4.43 (1H, dd, J = 10.7, 5.5 Hz, H-11), 2.99 (1H, dd, J = 12.4, 5.5 Hz, H-10), 2.69 (3H, s, H_3-34), 2.62 (1H, dd, J = 12.4, 10.7 Hz, H-10), 1.98 (1H, m, H-25), 1.21 (3H, s, H_3-32), 1.03 (3H, s, H_3-31), 0.94 (3H, d, J = 6.8 Hz, H_3-26), and 0.86 (3H, d, J = 6.8 Hz, H_3-27). ; ^{13}C NMR (100MHz, $CDCl_3$): 170.1 (C-33), 164.7 (C-17), 160.3 (C-13), 151.1 (C-20), 147.0 (C-15), 143.1 (C-29), 143.0 (C-8), 134.8 (C-22), 132.4 (C-9), 130.1 (C-7), 129.3 (C-6), 127.3 (C-23), 127.2 (C-24), 127.1 (C-21), 124.6 (C-5), 124.5 (C-4), 120.4 (C-14), 114.6 (C-30), 79.2 (C-2), 62.3 (C-18), 61.0 (C-3), 59.0 (C-11), 40.4 (C-28), 37.5 (C-10), 31.9 (C-25), 23.5 (C-34), 23.2 (C-31), 22.5 (C-32), 19.8 (C-26), and 18.3 (C-27).

Amino acid analysis of novofumigatamide (1): Novofumigatamide (**1** : 1.0 mg) was dissolved in 100 μL of 6 M HCl and heated at 110 °C for 16 h. The resulting hydrolyzate was allowed to cool and then neutralized with $NaHCO_3$. Then, 200 μL of Marfey's reagent (PIERCE, IL, USA) and 40 μL of 1M $NaHCO_3$ were added to this mixture, and the mixture was heated at 40°C for 1 h. HCl (2 M, 20 μL) was added upon cooling to room temperature. The solution was then analyzed by reversed-phase HPLC as previously described. Coinjection with L- and D-valine standards (tR = 10.4 and 14.9 min, respectively) indicated that the valinyl residue (tR = 10.4 min) in **1** has L- configuration.

Antifungal assay using the paper disk method: Antifungal assay was performed using a previously reported method, the paper disk method, against *Aspergillus niger* IFM 41398, *A. fumigatus* IFM 41362, *Candida albicans* IFM 40009 and *Cryptococcus neoformans* ATCC 90112 as test organisms.[5] Novofumigatamide (**1**) and was applied to the paper disk (diameter: 8 mm) at 100 μg per disk and the disks were 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, the zones of inhibition (the diameter measured in millimeters) were recorded.

Cytotoxicity assay: Cytotoxicity assay was performed by a modified method of in the previous paper.[5] Cell were seeded into 96-well microplates at 4000 cells per well, and allowed to attach for 4-6 h, A549 human lung cancer cells and Hela human cervical cancer cells were then incubated in Dulbecco's modified Eagle's medium (Invitrogen Co. Ltd, Carlsbad, CA, USA), and LNCap human prostate adenocarcinoma cells in RPMI-1640 medium (Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 10% fetal bovine serum, penicillin G (100 U/mL), streptomycin (100 μg/mL) and amphotericin B (0.25 μg/mL) until 80% confluency. Media were supplemented with the indicated

concentrations of isolated compounds for 48–72 h. Cell proliferation was measured using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) to count living cells by combining WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium) and 1-methoxy-PMS (1-methoxy-5-methylphenazinium methylsulfate). Briefly, 10 μL of Cell Counting Kit8 solution was added to each well after the medium was removed, and the plates were incubated for 4 h. Cell number was determined by scanning with a Bio-Rad Model Q4 550 microplate reader at 450 nm.

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