Mer-NF5003B, E AND F, NOVEL SESQUITERPENOIDS AS AVIAN MYELOBLASTOSIS VIRUS PROTEASE INHIBITORS PRODUCED BY *Stachybotrys* sp.

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Aspartic retroviral proteases play an important role in the processing of viral polyproteins into mature functional proteins[1]. An inhibitor of avian myeloblastosis virus protease (AMV-protease) could be a possible candidate for antiviral-therapy and could be used, for example, as an anti-HIV agent[2]. In the course of screening for AMV-protease inhibitors, we discovered Mer-NF5003B, E and F, novel sesquiterpenoids (Fig. 1), from the culture broth of a fungal strain *Stachybotrys* sp. Mer-NF5003 (FERM p-12344). In this paper, the fermentation of the producing strain, isolation, physico-chemical properties, structure elucidation and the some biological activities of Mer-NF5003B, E and F are described.

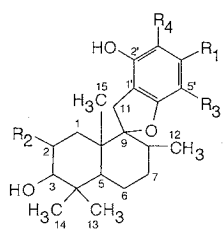
Screening of AMV protease were carried out in the following manner: AMV-protease (Molecular Genetic Resources, 1 μg) was incubated with Thr-Phe-Gln-Ala-Tyr-Pro-Leu-Arg-Glu-Ala (Peptide Institute, Inc. 0.2 mM) and sample broths in a final volume of 60 μl containing 2 M NaCl, 0.1 M sodium citrate (pH 5.5) for 20 minutes at 37°C. Cleavage of the substrate peptide was monitored by HPLC using the following conditions: column; ODS-120T column (6 mm i.d. × 15 cm, Tosoh), solvent; 20~40% acetonitrile (0.1% TFA) linear gradient, detection; UV at 210 nm, flow rate; 1.0 ml/minute. Normally, about 50% of substrate peptide was digested in this assay system. The inhibitory activity was calculated from the remaining substrate as follows: $[1-(a-c)/(a-b)]\times 100$ (%), here 'a' is peak area of a substrate without enzyme; 'b' is peak area of a substrate with enzyme; and 'c' is the peak area of a sample with samples or inhibitors.

The fungal strain Mer-NF5003 was originally isolated from a soil sample collected at Taketomi Island, Okinawa prefecture, Japan, and was identified as *Stachybotrys* sp. using the method previously described[3].

A sample of the slant culture of *Stachybotrys* sp. Mer-NF5003, grown on potato-dextrose agar slant, was inoculated into 500-ml Erlenmeyer flasks containing 50 ml of seed medium (potato starch 2.0%, glucose 1%, soybean meal 2%, KH_2PO_4 0.1%, $MgSO_4\cdot 7H_2O$ 0.05%). The flasks were shaken on a rotary shaker for 3 days at 28°C. One hundred ml of the seed culture was transferred to a 10-liter jar fermentor containing 5 liters of production medium. The production medium contained potato decoction prepared from potato 200 g and glucose 20 g in 1 liter tap-water (pH 7.0). Fermentations were carried out at 28°C with the aeration of 5 liters/minute and agitation at 300 rpm. Active compounds were isolated from the supernatant of culture broth.

The supernatant from the culture broth was applied to a Diaion HP-20 (Mitsubishi-Kasei) column. After washing with distilled water and 30% of MeOH, active fractions were eluted with gradient solution of acetone-water (50~60%). Fractions were collected and concentrated *in vacuo* to remove acetone. The concentrate was extracted twice with the same volume of EtOAc. The organic layer was dried over Na_2SO_4 and filtered and concentrated to dryness. The residue was dissolved in MeOH and applied to a Sephadex LH-20 column and developed

Fig. 1. Structure of Mer-NF5003B, E and F.



	R_1	R_2	R_3	R_4
Mer-NF5003B	CH_2OH	OH	CHO	H
Mer-NF5003E	CH_2OH	H	CHO	H
Mer-NF5003F	CHO	H	CHO	H
K-76	CHO	OH	CHO	H
L-671,776	CH_2OH	H	H	CHO

Table 1. Physico-chemical properties of Mer-NF5003B, E and F.

	Mer-NF5003B	Mer-NF5003E	Mer-NF5003F
Appearance	White prism	Colorless prism	White amorphous
UV in MeOH	328 (4,700), 281 (9,410), 229 (12,200)	328 (5,430), 286 (10,800), 225 (15,100)	352 (4,090), 301 (5,610), 245 (14,900)
FAB-MS (*m/z*)			
NBA-positive	405	389	387
NBA-negative	403	387	385
EI-MS (*m/z*)	nt[a]	387, 370, 368, 354, 207, 189	nt
HREI-MS	nt	370.2166 ($C_{20}H_{30}O_4$; 370.2144)	nt
Formula	$C_{23}H_{32}O_6$	$C_{23}H_{32}O_5$	$C_{23}H_{30}O_5$
TLC Rf value[b]			
EtOAc - hexane (2 : 1)	0.03	0.16	0.46
Toluene - acetone (1 : 1)	0.17	0.41	0.63

[a] Not tested.
[b] Merck $60F_{254}$.

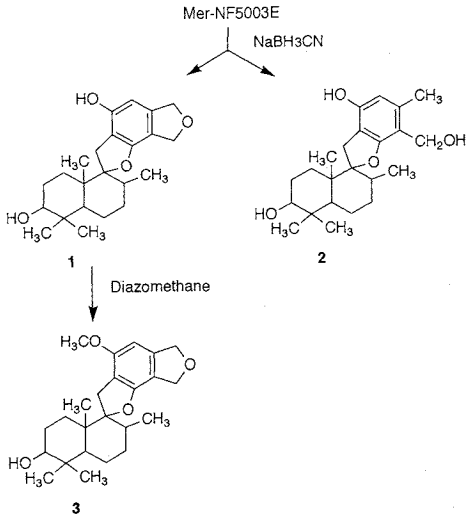
Table 2. ^{13}C and ^{1}H NMR spectra data of Mer-NF5003B, E and F.

Position	Mer-NF5003B		Mer-NF5003E				Mer-NF5003F	
	δ_C	δ_H	δ_C	DEPT	δ_H	HMBC correlation[a]	δ_C	δ_H
1	34.07	1.33 dd (12.0, 4.0), 1.77 t (11.7)	24.98	CH_2	1.05 1.96		24.96	1.05 1.9～2.0
2	67.25	3.97	26.02	CH_2	1.62 1.96		25.99	1.4～1.7 1.9～2.0
3	79.66	3.31	75.33	CH	3.33		75.36	3.34
4	39.52	—	38.33	C	—		38.35	—
5	40.91	2.09	40.87	CH	2.25	4, 6, 13, 15	40.86	2.26
6	21.91		21.71	CH_2	1.63		21.67	1.4～1.7
7	32.39	1.4～1.7	32.10	CH_2	1.62		32.02	1.4～1.7
8	38.08	1.92	37.77	CH	1.89		37.75	1.9～2.0
9	100.64	—	100.42	C	—		101.32	—
10	44.73	—	43.20	C	—		43.23	—
11	31.82	2.80 d (16.4), 3.18 d (16.4)	31.31	CH_2 CH_2	2.79 d (16.9), 3.26 d (16.9)	8, 9, 10, 1′, 2′, 6′, 8, 9, 10, 1′, 2′, 6′	31.47	2.92 d (17.0), 3.25 d (17.0)
12	15.93	0.77 d (6.6)	16.00	CH_3	0.77 d (6.6)	7, 8, 9	15.93	0.79 d (6.6)
13	22.57	0.91 s	22.82	CH_3	0.88 s	3, 4, 5, 14	22.79	0.89 s
14	29.33	1.03 s	29.04	CH_3	0.99 s	3, 4, 5, 13	29.02	1.00 s
15	17.52	1.09 s	16.44	CH_3	1.05 s	1, 5, 9, 10	16.40	1.07 s
1′	113.02	—	112.64	C	—		112.05	—
2′	170.24	—	169.64	C	—		168.29	—
3′	108.83	6.53 s	108.54	CH	6.62 s		109.03	6.86 s
4′	146.78	—	146.80	C	—		139.59	—
5′	110.30	—	110.37	C	—		119.81	—
6′	161.52	—	159.95	C	—		159.18	—
7′	190.16	10.18 s	188.77	CH	10.26 s	4′, 5′	188.40[b]	10.37[b] s
8′	64.17	4.75 s	63.97	CH_2	4.71 s	3′, 4′, 5′	192.74[b]	10.58[b] s

[a] Correlated carbon Number.
[b] Interchangeable.

with MeOH. Active fractions were evaporated and applied to a silica gel column (Merck Silica gel 60, $CHCl_3$ - MeOH, 50 : 1), developed with $CHCl_3$ - MeOH, 50 : 1～10 : 1 and two active fractions were obtained. Further purifications of each active fraction were carried out separately by preparative reverse-phase HPLC (YMC Pack S-343 I-15 ODS). RP-HPLC of fraction 1 and elution with 50～

Fig. 2. Reduction products of Mer-NF5003E.



60% acetonitrile yielded Mer-NF5003F and Mer-NF5003E, while RP-HPLC of fraction 2 and elution with 50~55% acetonitrile yielded Mer-NF5003E and Mer-NF5003B. Finally, each compound was purified by crystallization from CH_2Cl_2-hexane. From 5 liters fermentation broth, 5.6 mg of Mer-NF5003B, 10.3 mg of Mer-NF5003E and 5.1 mg of Mer-NF5003F were obtained.

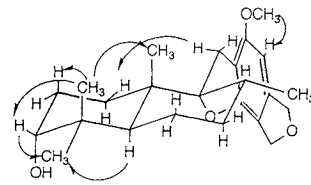
Mer-NF5003B, E and F are soluble in MeOH, $CHCl_3$ and acetone but insoluble in H_2O and hexane. The other physico-chemical properties and the ^{13}C, ^1H and ^{13}C-^1H NMR data of Mer-NF5003s are summarized in Tables 1 and 2, respectively. The results of HMBC spectra of Mer-NF5003E are also included in Table 2.

Elucidation of the structures was accomplished with the help of chemicaly modifying the compounds (Fig. 2). A mixture of 13.1 mg of Mer-NF5003E, 3 mg of $NaBH_3CN$ and 0.5 ml of MeOH was stirred for 2.5 hours at room temperature. The reaction mixture was diluted with 30 ml of water and extracted with EtOAc. The EtOAc layer was washed with water and dried over Na_2SO_4, filtered and evaporated to give 13.3 mg of mixture. The mixture was chromatographed on preparative silica gel TLC with the solvent of EtOAc-hexane, 2:1. Two products, **1** (5.8 mg) and **2** (2.4 mg), were obtained.

1: TLC (silica gel, EtOAc-hexane, 2:1) Rf 0.58, ^1H NMR ($CDCl_3$-CD_3OD, 1:1); δ 0.73 (3H, d, J=5.9 Hz), 0.88 (3H, s), 0.99 (3H, s), 1.02 (3H, s), 2.77 (1H, d, J=16.1 Hz), 3.16 (1H, d, J=16.1 Hz), 3.36 (1H, m), 4.9~5.1 (4H, m), 6.15 (1H, s).

2: TLC (silica gel, EtOAc-hexane, 2:1) Rf 0.42,

Fig. 3. A part of NOE correlation in **3**.



^1H NMR ($CDCl_3$-CD_3OD, 9:1); δ 0.70 (3H, d, J=6.6 Hz), 0.87 (3H, s), 0.99 (3H, s), 1.00 (3H, s), 2.11 (3H, s), 2.79 (1H, d, J=16.1 Hz), 3.16 (1H, d, J=16.9 Hz), 3.37 (1H, m), 4.53 (2H, m), 6.28 (1H, s).

Methylation of **1** was carried out as following: Diazomethane diethyl ether solution was added to the suspension of **1** (5.0 mg) in 1 ml of MeOH at room temperature until the reactant spot on silica gel TLC was disappeared. The reaction mixture was stirred for additional 15 hours at room temperature, and was concentrated to dryness. A single product **3** (4.8 mg) was obtained by chromatography on Sephadex LH-20 column with MeOH.

3: TLC (Silica gel, $CHCl_3$-MeOH, 20:1) Rf 0.63, ^1H NMR ($CDCl_3$); δ 0.72 (3H, d, J=5.9 Hz), 0.87 (3H, s), 0.99 (3H, s), 1.00 (3H, s), 1.10 (1H, ddd, J=13.2, 3.7, 3.7 Hz), 1.92 (1H, ddt, J=2.2, 3.7, 13.9 Hz), 2.06 (1H, dd, J=12.4, 2.2 Hz), 2.75 (1H, d, J=16.1 Hz), 3.13 (1H, d, J=16.1 Hz), 3.48 (1H, m), 3.80 (3H, s), 5.0~5.2 (4H, m), 6.21 (1H, s).

A methoxyl signal was observed in the ^1H NMR spectrum of **3** in $CDCl_3$ at 3.80 ppm. In the differential nuclear Overhauser effect (NOE) spectra of **3** (Fig. 3), the $-OCH_3$ group in **3** was shown to be geometrically closed to the aromatic proton at 6.21 ppm. Therefore, the structures of NF5003E, as well as **1**, **2** and **3** were determined as shown in Figs. 1 and 2.

Some physico-chemical analytical data supported that Mer-NF5003B and Mer-NF5003F were structurally related to Mer-NF5003E. The molecular weight of Mer-NF5003B was found to be 404 by the FAB mass spectrometry. From the ^1H and ^{13}C NMR spectra, there is one additional oxymethine carbon in Mer-NF5003B as compared with Mer-NF5003E. The additional oxygen was determined to be at C-2 position by the NMR spectra. The ^1H and ^{13}C NMR spectra data of Mer-NF5003F, with a molecular weight of 386, showed the presence of two aldehyde moieties. The structures shown in Fig. 1 are rationalized from these spectra.

The IC_{50} values of the compounds were calculated according to the method as described above. Mer-NF5003B, E and F showed inhibitory activity

against AMV-protease at the concentration of 16.5, 12.8 and 7.8 μM, respectively. In our system, the IC_{50} value of pepstatin, a specific inhibitor of aspartic protease, was 10 μM, and Mer-NF5003s are as same active as pepstatin. HIV-1 protease inhibitory activity of the compounds was measured using the method previously described[4]. No inhibitory activities were detected at the concentration of 100 μg/ml.

Mer-NF5003s are novel AMV-protease inhibitors produced by *Stachybotrys* sp. Mer-NF5003. Structure of Mer-NF5003s are related to K-76[5,6], a complement inhibitor produced by *Stachybotrys complementi* nov. sp. K-76, or L-671,776[7], a inositol mono-phosphatase inhibitor from *Memnoniella echinata*. Because of the toxicity against the host cell, anti-HIV activity was not detected in the MT-4 cell/HIV-1 system. Studies on the structure-activity relationships of them, as well as the improving the bio-availability are now under investigation.

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