Mer-NF8054A AND X, NOVEL ANTIFUNGAL STEROIDS, ISOLATED FROM *Aspergillus* sp.

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In the course of screening for new antifungal agents from microbial metabolites, we found novel steroids Mer-NF8054A and X in a culture of *Aspergillus* sp. There were some steroids that were reported to have antifungal activity, ergokonin A[1] and A25822A[2], *etc.* Mer-NF8054A and X were different from these steroids on their structures and specific activities to *Aspergillus fumigatus*. In this paper we describe the fermentation, isolation, structure determination and biological activities of Mer-NF8054A (**1**) and X (**2**).

The producing microorganism, strain NF8054, was isolated from a soil sample collected in Chichi-jima, Ogasawara islands, Tokyo, Japan, and was identified as *Aspergillus ustus* on the basis of its cultural properties[3].

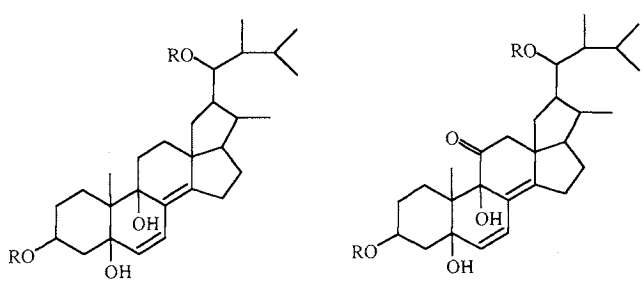
Further search resulted in the finding of Mer-NF8054A and X producing microorganisms; *Aspergillus versicolor* NF8054b, *Aspergillus versicolor* PF1003, and *Aspergillus fischeri* var *glaber* LF1019. Therefore, Mer-NF8054 like compounds are produced widely by various species of *Aspergillus*.

A slant culture of the strain *Aspergillus ustus* NF8054 was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of the medium consisting of glycerol 4.0%, potato starch 2.0%, glucose 1.0%, soy bean meal (Ajinomoto Co., Inc.) 2.0%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05% (pH not adjusted) and incubated on a rotary shaker at 28°C for 3 days. The seed culture was transferred into 500-ml Erlenmeyer flask containing 100 ml of the same medium as described above. The fermentation was carried out for 90 hours at 28°C and 200 rpm on a rotary shaker.

Antifungal activity of the resulting broth was assayed by the paper-disk agar diffusion method using *Aspergillus fumigatus* IFM4942 as the test organism.

The antifungal activity were found both in mycelial extract and culture filtrate, and the isolation of the active compounds was carried out from both fractions. After filtration, mycelial cake was extracted with methanol. The methanol extract was concentrated *in vacuo* to an aqueous solution, and was diluted with water and then extracted twice with equal volume of ethyl acetate. The culture filtrate was extracted twice with equal volume of ethyl acetate. Both organic extracts were combined, dried over anhydrous sodium sulfate, and concentrated *in vacuo* to an oily residue. The residue was dissolved in 50 ml of chloroform and applied to a silica gel column (Merck, Art7734, 330 ml). The active compounds were eluted with chloroform - methanol (20 : 1). The active fractions were combined, concentrated *in vacuo* to dryness, and dissolved in 5 ml of toluene - acetone (2 : 1), following the chromatography on a column of silica gel (300 ml) with toluene - acetone (2 : 1). The active fractions were combined, concentrated *in vacuo*, and dissolved in 2 ml of methanol. The methanol solution was purified by chromatography on a column of Sephadex LH-20 (Pharmacia, 130 ml). Final separation of **1** and **2** was achieved by preparative HPLC (column, YMC-Pack S343 I-15 ODS; mobile phase,

Fig. 1. Structures of Mer-NF8054A, X and their acetylated derivatives.



Mer-NF8054A (**1**) R = H
3 R = Ac

Mer-NF8054X (**2**) R = H
4 R = Ac

Table 1. Physico-chemical properties of Mer-NF8054A and X.

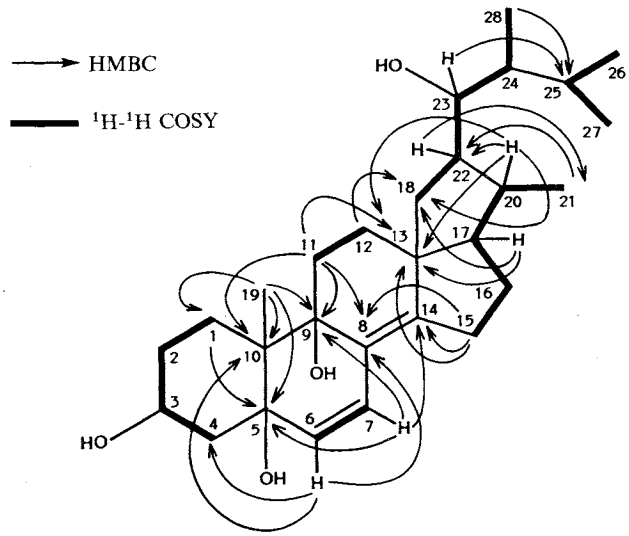
	Mer-NF8054A (1)	Mer-NF8054X (2)
MP	105~107°C	109~111°C
Molecular formula	$C_{28}H_{44}O_4$	$C_{28}H_{42}O_5$
MW	444	458
FAB-MS (*m/z*) Positive:	427 $(M+H-H_2O)^+$	441 $(M+H-H_2O)^+$
Negative:	443 $(M-H)^-$	457 $(M-H)^-$
HRFAB-MS (*m/z*)		
Obsd:	409.3110 $(M+H-2H_2O)^+$	441.2995 $(M+H-H_2O)^+$
Calcd:	409.3107 ($C_{28}H_{41}O_2$)	441.3005 ($C_{28}H_{41}O_4$)
UV λ (nm) (ε)	247.2 (21,700)	241.2 (16,900)
IR KBr cm^{-1}	3385 (br), 2959, 1655, 1462, 1383, 1073, 997	3425 (br), 2955, 1707, 1661, 1470, 1385, 1078, 1009
Rf value		
Toluene - EtOAc (1 : 4)	0.29	0.27
Toluene - Acetone (1 : 1)	0.46	0.45
$CHCl_3$ - MeOH (10 : 1)	0.38	0.41

Table 2. NMR spectral data of Mer-NF8054A and X in CD_3OD.

Position	Mer-NF8054A (1)		Mer-NF8054X (2)	
	δ_C	δ_H	δ_C	δ_H
1	32.10	1.15 (m), 1.39 (dd, J=10.3, 3.7 Hz)	33.49	1.28 (m), 1.50 (td, J=12.8, 3.3 Hz)
2	31.25	1.34 (td, J=3.7, 11.7 Hz), 1.67 (m)	31.15	1.38 (td, J=12.8, 3.7 Hz), 1.72 (t, J=11.7 Hz)
3	68.53	3.40 (m)	67.79	3.42 (m)
4	45.99	1.72 (m)	45.33	1.71 (m), 1.79 (ddd, J=12.5, 4.8, 1.8 Hz)
5	74.49		74.50	
6	131.35	5.44 (d, J=10.3 Hz)	133.32	5.58 (d, J=10.3 Hz)
7	126.55	6.28 (d, J=10.3 Hz)	125.12	6.31 (d, J=10.3 Hz)
8	127.61		127.99	
9	76.70		81.04	
10	44.75		43.88	
11	32.74	1.75 (m), 2.39 (m)	212.80	
12	36.54	1.20 (dd, J=12.5, 4.4 Hz), 1.78 (m)	54.58	2.61 (ABq, J=12.1 Hz)
13	52.72		55.95	
14	153.99		153.26	
15	30.71	2.29 (m), 2.55 (ddd, J=15.4, 7.3, 2.9 Hz)	30.37	2.35 (m), 2.63 (ddd, J=16.1, 7.0, 3.3 Hz)
16	25.91	1.47 (td, J=10.3, 3.7 Hz), 1.78 (m)	26.29	1.67 (m), 1.88 (m)
17	56.53	2.14 (td, J=8.8, 3.7 Hz)	55.72	2.38 (m)
18	36.47	1.10 (m), 1.72 (m)	38.54	1.25 (m)
19	14.76	1.26 (s)	15.07	1.18 (s)
20	37.84	2.46 (q, J=7.1 Hz)	38.30	2.48 (q, J=7.3 Hz)
21	10.81	1.01 (d, J=7.3 Hz)	10.47	1.01 (d, J=7.3 Hz)
22	47.82	2.18 (m)	47.92	2.17 (m)
23	73.08	3.78 (dd, J=10.3, 1.5 Hz)	72.71	3.72 (dd, J=10.3, 1.5 Hz)
24	44.31	1.11 (m)	44.35	0.97 (q, J=6.2 Hz)
25	31.69	1.60 (oct, J=7.3 Hz)	31.65	1.58 (qd, J=6.6, 1.5 Hz)
26	21.53	0.93 (d, J=6.6 Hz)	21.45	0.91 (d, J=7.0 Hz)
27	21.71	0.94 (d, J=6.6 Hz)	21.66	0.92 (d, J=6.6 Hz)
28	10.19	0.86 (d, J=6.6 Hz)	10.05	0.84 (d, J=6.6 Hz)

Assignments were established based on DEPT, ^1H-^1H COSY, HMQC and HMBC experiments. ^1H and ^{13}C chemical shifts were given in δ downfield of TMS at 400 and 100 MHz, respectively.

Fig. 2. Structure of Mer-NF8054A from HMBC.



acetonitrile-water (1:1); flow rate, 7 ml/minute; detection, UV at 210 nm). The retention times of **1** and **2** were 25.9 and 15.8 minutes, respectively. The peak fractions of **1** and **2** were collected, respectively and concentrated *in vacuo* to dryness to give 5.6 mg of **1** and 7.4 mg of **2** from 100 liters culture broth.

The physico-chemical properties of Mer-NF8054A (**1**) and X (**2**) are summarized in Table 1. Both **1** and **2** were white powder, and have very similar properties. They were soluble in methanol, ethyl acetate, acetone, and chloroform, but insoluble in water and hexane. The compounds showed positive responses to phosphomolybdic acid and Liebermann-Burchard reagents, but were negative to Ninhydrin and Rydon-Smith reagents.

The ^{1}H and ^{13}C NMR spectra data for **1** and **2** are shown in Table 2. The molecular formula of **1** and **2** were determined as $C_{28}H_{44}O_4$ and $C_{28}H_{42}O_5$, respectively, on the basis of HRFAB-MS and ^{13}C, ^{1}H NMR spectra. All C–H connectivities were elucidated by DEPT and HMQC analysis. Comparison of ^{1}H NMR spectra in different solvents and elucidation of spectral data of acetylated derivatives (**3** and **4**) proved that **1** and **2** had four hydroxy groups and two double bond connectivities. Partial structures of **1** were determined by ^{1}H-^{1}H COSY spectrum. The long range ^{1}H-^{13}C correlations were derived from HMBC spectrum, following the whole structure of **1** was contrived. ^{1}H-^{1}H COSY and ^{1}H-^{13}C long range coupling data necessary to determine the structure were shown in Fig. 2. Though the ^{1}H and ^{13}C NMR spectra of **2** were almost coincident with those of **1**, ^{13}C NMR spectrum of **2** showed a carbonyl carbon signal at δ_C 212.80 (C-11) ppm corresponding to the methylene carbon signal at δ_C 32.74 ppm of **1**. Any other remarkable differences were not seen in ^{13}C NMR spectrum between **1** and **2**. IR signal at 1707 cm^{-1} ascertained that **2** had carbonyl carbon. Therefore, the structure of **2** was established (shown in Fig. 1). Both compounds possessed unique structures, 18,22-cyclosterol skeletons. The structures of **1**, **2**, **3**, and **4** were confirmed in comparison with the chemical shifts of ^{1}H, ^{13}C NMR spectra. Studies of the stereochemistry are now in progress, and details will be reported elsewhere.

Table 3. *In vitro* antifungal activity of Mer-NF8054A.

Test organism	MIC (μg/ml)
Aspergillus fumigatus IFM4942	0.16
A. fumigatus IFM41088	0.63
A. fumigatus TIMM0063	>50
A. fumigatus A44	>50
A. fumigatus IAM2612	>50
A. terreus F822	>50
A. ochraceus F90	>50
A. nidulans F101	>50
Candida albicans IFM40009	>50

Antimicrobial activity of Mer-NF8054A was examined by the serial micro-broth dilution method[4], using Yeast Nitrogen Base (Difco) supplemented with glucose at 35°C for 40 hours incubation. As shown in Table 3, Mer-NF8054A was active only against several strains of *Aspergillus fumigatus*, but not against the other species of

Aspergillus and *Candida albicans*. Further biological studies including the mode of action are now in progress.

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References

1) AUGUSTINIAK, H.; E. FORCHE, H. REICHENBACH, V. WRAY, U. GRAEFE & G. HOEFLE: Isolation and structure elucidation of ergokonin A and B. Liebigs Ann. Chem. 4: 361~366, 1991
2) BOECK, L. D.; M. M. HOEHEN, J. E. WESTHEAD, R. K. WOLTER & D. N. THOMAS: New azasteroidal antifungal antibiotics from *Geotrichum fravo-brunneum*. J. Antibiotics 28: 95~117, 1975
3) RAPER, K. B. & D. I. FANNEL (*Eds*): The Genus *Aspergillus*. The Williams & Wilkins Co., Baltimore, 1965
4) FUKAZAWA, Y.; T. NISHIKAWA, K. UCHIDA, T. SHINODA, K. NISHIMURA & T. HAMAMOTO: A proposal for the standardization in the field of medical mycology. Jpn. J. Med. Mycol. 32: 239~260, 1991