2-Demethylazalomycins F_{4a} and F_{5a}, Two New Antifungal Metabolites from *Actinomycete* sp. HIL Y-9120362

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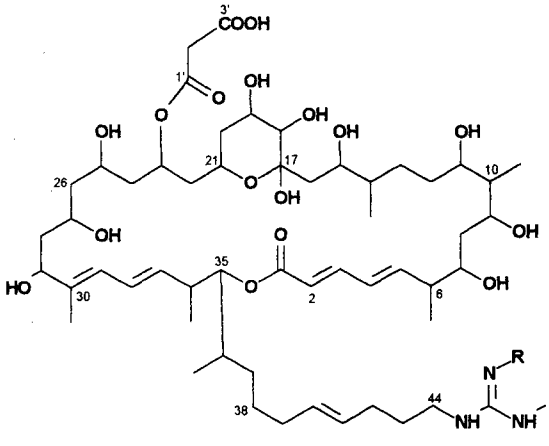
During the course of our screening for novel and potent antifungal agents active against phytopathogenic fungi, we isolated two new members of azalomycin class, 2-demethylazalomycin F_{4a} (**1**) and 2-demethylazalomycin F_{5a} (**2**) from the fermented broth of an *Actinomycete* sp. HIL Y-9120362. Herein, we report the production, isolation, structure elucidation and biological properties of **1** and **2**.

Strain HIL Y-9120362 was isolated from a soil sample collected from Madhya Pradesh, India. A loopful of mature slant culture of Y-9120362 was inoculated into Erlenmeyer flasks (1 liter capacity) containing 150 ml of seed medium consisting of glucose 1.5%, soyabean meal 1.5%, corn steep liquor 0.5%, NaCl 0.5%, $CaCO_3$ 0.2%, pH 7.0 before autoclaving. The flasks were cultivated on a rotary shaker at 220 rpm for 72 hours. The seed culture (3 liters) was inoculated into a 150-liter fermentor containing 90 liters of the production medium consisting of soluble starch 2.5%, glucose 1.0%, soyabean meal 2.0%, yeast extract 0.5%, NaCl 0.5%, $CaCO_3$ 0.3% and $MgSO_4 \cdot 7H_2O$ 0.1%, pH 7.0 before autoclaving. The aeration and agitation of the fermentation were maintained at 60 liters per minute and 100 rpm, respectively, and the temperature at 28°C. Desmophen was added as the antifoam during the fermentation cycle of 44 hours. The production of the antibiotic as well as its purification were monitored by activity against *Fusarium culmorium* 100.



2-Demethylazalomycin F_{4a} (**1**): R = H
2-Demethylazalomycin F_{5a} (**2**): R = CH_3

The culture broth was harvested and centrifuged to separate the mycelium (6 kg). The culture filtrate (75 liters) was passed through a column of Diaion HP-20 (4 liters). The column was washed with demineralized water and eluted with MeOH. The active eluates were concentrated under reduced pressure to give crude antibiotic (41 g) as brown powder. The mycelium was extracted with MeOH (2 × 10 liters), concentrated, diluted with water, passed through HP-20 (1 liter) and processed further as above to get additional crude antibiotic (27 g). Both the crude materials were combined and subjected to MPLC on RP-18 (50~70 μ) using a step gradient of 0~70% (in increments of 5%) CH_3CN-MeOH (1:1) in 0.02 M phosphate buffer, pH 5.8. The active fractions, which eluted out in 40~50% CH_3CN-MeOH (1:1) in 0.02 M phosphate buffer, were pooled, concentrated and desalted on HP-20 (eluant-MeOH). The concentrated active fractions were subjected to silica gel (55~75 μ) chromatography using a step gradient of 0~2% water (in increments of 0.5%) in EtOAc-*n*-PrOH (5:3). All the fractions were monitored by their bioactivity and also by HPLC (Column: 4 mm × (30+100) mm ODS-Hypersil (10 μ); Eluant: CH_3CN-MeOH-0.1% $NH_4H_2PO_4$ (14:11:10); Flow rate: 1.5 ml/minute; Detection: 240 nm). The semi-pure **1** (6.2 g) and **2** (5 g), thus obtained, were purified separately by preparative HPLC (Column: 16 mm × (30+120) mm ODS-Hypersil (10 μ); Eluant: CH_3CN-MeOH-0.1% $NH_4H_2PO_4$ (14:11:10); Flow rate: 9 ml/minute; Detection: 240 nm) to obtain pure **1** (2.2 g) and **2** (3.8 g). Both **1** and **2** were obtained as white powders and found to be soluble in MeOH and DMSO.

The physico-chemical properties of **1** and **2** are given in Table 1. The UV absorption maxima of both the compounds **1** and **2** were indicative of a conjugated diene and α, β, γ, δ-unsaturated conjugated carbonyl moiety. IR bands at 3380, 1700, 1645 and 975 cm^{-1} in case of **1** and correspondingly at 3400, 1710, 1645 and 975 cm^{-1} in case of **2** were suggestive of the presence of hydroxyls, carbonyls and *trans*-disubstituted double bonds. The ^1H NMR spectrum of **1** is given in Fig. 1. The ^1H NMR of **2** was practically identical to that of **1** except that the N-methyl signal (δ 2.88) was twice as intense.

The ^{13}C NMR and DEPT-135 spectra of both the compounds revealed the presence of 6 × CH_3, 16 × CH_2, 5 × CH, 12 × OCH, 9 × =CH, 3 × C and 3 × C=O, in addition to one N–CH_3 in case of **1** and two N–CH_3 in

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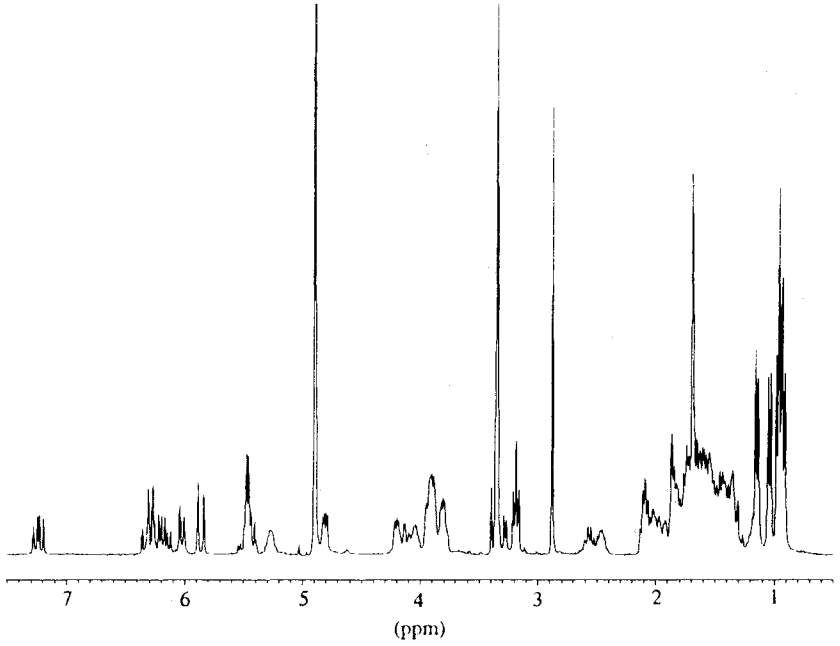
Table 1. Physico-chemical properties of 2-demethylazalomycin F_{4a} (**1**) and 2-demethylazalomycin F_{5a} (**2**).

	1	**2**
MP	136~138°C	134~136°C
$[\alpha]_D$	+39.8° (*c* 0.206, MeOH)	+37.0° (*c* 0.2, MeOH)
FAB-MS $(M+H)^+$	1068	1082
Molecular formula	$C_{55}H_{93}N_3O_{17}$	$C_{56}H_{95}N_3O_{17}$
Elemental analysis:		
Calcd for:	$C_{55}H_{93}N_3O_{17}\cdot 6H_2O$	$C_{56}H_{95}N_3O_{17}\cdot 6H_2O$
	C 57.17, H 8.93, N 3.57	C 56.51, H 8.49, N 3.53
Found:	C 56.64, H 8.13, N 3.16	C 56.99, H 8.27, N 3.32
UV (MeOH) nm	242, 262	242, 264
IR (KBr) cm^{-1}	3380, 2940, 1700, 1645, 1460, 1385, 1365, 1300, 1280, 1145, 1095, 1075, 1000 and 970	3400, 2930, 1710, 1645, 1460, 1380, 1300, 1150, 1070, 1010 and 975
^{13}C NMR (75 MHz, CD_3OD, δ)	11.2, 14.1, 14.8, 15.6, 17.8, 18.3, 28.7, 29.1, 30.6, 31.4, 34.4, 35.3, 35.7, 40.1, 41.5, 41.7, 41.9, 42.4, 42.5, 42.7, 44.7, 44.9, 45.2, 45.3, 47.2 (×2), 66.2 (×2), 66.8, 70.4, 71.4, 72.9, 73.1, 74.9, 75.4, 76.3, 77.8, 81.0, 100.5, 121.4, 125.9, 129.4, 130.9, 131.1, 133.3, 137.0, 140.7, 147.5, 148.2, 158.9, 169.8, 172.3 and 174.9	11.2, 14.1, 14.8, 15.6, 17.8, 18.3, 28.7, 29.1, 30.5, 31.4, 34.4, 35.2, 35.7, 40.2, 41.5, 41.7, 41.9, 42.4, 42.5, 42.8, 44.9 (×2), 45.2, 45.3, 47.3 (×2), 66.2, 66.3, 66.8, 70.5, 71.5, 73.0, 73.1, 75.0, 75.5, 76.3, 77.9, 81.1, 100.5, 121.4, 125.9, 129.4, 131.0, 131.1, 133.3, 136.9, 140.8, 147.5, 148.2, 158.1, 169.8, 172.3 and 174.7

Fig. 1. 300 MHz ^1H NMR spectrum of 2-demethylazalomycin F_{4a} (**1**) in CD_3OD.



case of **2**. The molecular formulae of **1** and **2** required eleven degrees of unsaturation, out of which only nine were accountable in the form of six double bonds and three carbonyls, indicating that both the compounds contained two rings.

The ^1H and ^{13}C NMR data of **1** and **2** indicated that they were macrocyclic lactones like azalomycins F_{4a}[1] and F_{5a}[2]. Whereas azalomycins F_{4a} and F_{5a} had seven C-methyl groups, ^1H and ^{13}C NMR of **1** and **2** accounted only for six C-methyls. Thus, C-2 methyl, which appeared at δ_C 12.8 and δ_H 1.98 in azalomycins F_{4a} and F_{5a}, was absent in **1** and **2**. Instead, ^1H NMR spectra of **1** and **2** indicated the presence of an additional olefinic proton at δ 5.86 (d, J=15.6 Hz) having a correlation with 3-H (δ 7.23, dd, J=15.6, 11 Hz) in its DQF shift-correlated HH COSY spectrum. Further, an additional olefinic methine doublet at δ 121.4 in ^{13}C NMR spectrum of **1** and **2** coupled with the absence of an olefinic quaternary carbon singlet, which is observed at δ 126.7 in that of azalomycin F_{4a} and F_{5a} suggested that both **1** and **2** were 2-demethylazalomycins.

The position of the two N–CH_3 groups (δ_H 2.89, 6H) in **2** as a part of either N,N-dimethylguanidine or N,N'-dimethylguanidine was determined[2] by alkaline hydrolysis (3 N NaOH, reflux, 6 hours, N_2/HCl) of **2**, followed by treating the amine hydrochloride with benzoyl chloride. The product, thus obtained, was identified as N-methylbenzamide, indicating the presence

Table 2. "*In vitro*"* and "*in vivo*"** (in parentheses) antifungal activity of 2-demethylazalomycin F_{4a} (**1**) and F_{5a} (**2**).

Test organism	1	2
Candida albicans	11	Slight
Penicillium digitatum	15	14
Fusarium culmorum 100	17	16
Alternaria mali P37	20	18
Pyricularia oryzae K02	16	20
Leptosphaeria nodorum J02	23	27
Pellicularia sasakii J03	28	25
Pseudomonas herpotrichoides 008	19	16
Neurospora crassa SGF-18	11	12
Botrytis cinerea E02	22	20
Botrytis cinerea A06	13 (97)	12 (97)
Botrytis cinerea D01	17 (97)	13 (97)
Phytophthora infestans J08	13 (75)	15 (40)
Plasmopara viticola	NT (95)	NT (65)

*Zone size in mm at a concentration of 5 μg/disc; **Protection in % at 150 ppm under green house conditions; NT: not tested.

of N,N'-dimethylguanidine moiety in **2**.

The stereochemistries of the double bonds were established on the basis of coupling constants and by the analysis of phase-sensitive 2D NOESY spectrum. In the ^{1}H NMR spectra of **1** and **2**, the large coupling constants between 2-H (δ 5.86) and 3-H (δ 7.23) (J=15.6 Hz), 4-H (δ 6.32) and 5-H (δ 6.16) (J=15.8 Hz) and 32-H (δ 6.26) and 33-H (δ 5.46) (J=14.8 Hz) indicated that these double bonds were oriented in *E*-configuration. The stereochemistry of C-40=C-41 double bond could not be established due to overlapping signals; however, considering the identity of the spectra with those reported in literature[1,2], it is assumed to be *E*.

Based on these data, it can be concluded that **1** and **2** are 2-demethylazalomycin F_{4a} and 2-demethylazalomycin F_{5a} respectively. **1** was isolated by us earlier from another culture Y-8521242[3]. During the course of this work, we came across a report[4] on MDL 63766 complex belonging to the azalomycin class. One of the components was reported to have the same molecular weight of 1081 as in the case of **2**. However, the structure of this compound has not been reported. Subsequent to the preparation of this manuscript, two compounds RS-22B and RS-22C, which are identical to **1** and **2**, have been reported[5].

Biological Properties

Both the compounds **1** and **2** exhibited "*in vitro*" and "*in vivo*" activity against a wide range of fungal strains (Table 2). They did not exhibit any antibacterial activity.

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