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Maclafungin, a New Antifungal Macrocyclic Lactone from *Actinomycete* sp.Y-8521050

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Abstract :

A new antifungal macrocyclic lactone maclafungin, belonging to the oligomycin class has been isolated from an *actinomycete sp.* Y-8521050. The antibiotic, having a molecular formula of $C_{46}H_{80}O_{12}$, is active against several fungal species. Its structure was elucidated by analysis of 2D NMR experiments.

Keywords : Antifungal; Macrocyclic lactone; Stucture elucidation; Microorganism; *Actinomycete*

During the course of screening for new antifungal metabolites active against either human or plant pathogens, we isolated a new macrocyclic lactone named maclafungin (**1**) [1] from the fermentation broth of an unidentified *Actinomycete* species Y-8520050. Herein, we report the fermentation, isolation, structure elucidation and biological activities of maclafungin (**1**).

Results and Discussion

Maclafungin (**1**) was found to be present both in the culture filtrate and the mycelium. The crude compound was obtained from the culture filtrate by $CHCl_3$ extraction and from the mycelium by MeOH extraction. The combined crude material was purified by repeated

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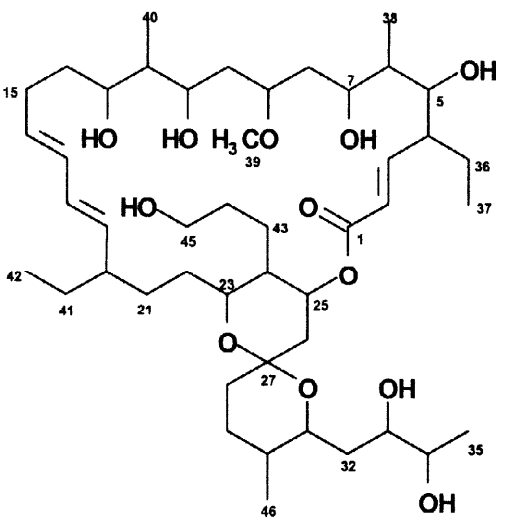
flash silica gel chromatography using $CHCl_3$-MeOH mixtures for elution to get maclafungin as a white amorphous powder. The purification was monitored both by HPLC and bioactivity against *Fusarium culmorum*.

The IR spectrum of maclafungin showed bands at 3450 and 1720 cm^{-1} indicating the presence of hydroxyls and a carbonyl respectively. The HRDCI-MS of maclafungin gave a molecular ion at *m/z* 825.578 $(M + H)^+$ (calculated for $C_{46}H_{81}0_{12}$ *m/z* = 825.573) corresponding to the molecular formula $C_{46}H_{80}0_{12}$ which is in accordance with the elemental analysis and NMR spectra. The molecular formula of **1** suggested the presence of seven degrees of unsaturation out of which four were accountable (three double bonds and one carbonyl) indicating that maclafungin was a tricyclic compound.



(1)

The ^1H and ^{13}C NMR spectral data of maclafungin (**1**) are summarized in Table 1. The ^{13}C NMR and DEPT-135 spectra of **1** revealed the presence of seven methyl groups [6 x CH_3 and 1 x OCH_3], fifteen methylene groups [14 x CH_2 and 1 x OCH_2], twenty two methine groups [6 x CH, 10 x OCH and 6 x =CH] and two quaternary carbons [1 x OCO and 1 x C=O]. The protonated carbons were identified by the analysis of the HMQC spectrum [2,3].

The ^1H NMR spectrum (400 MHz) of **1** could unambiguously resolve only 53 % of all proton resonances due to overlapping signals and hence the ^1H-^1H COSY spectrum [4] could only partially establish ^1H-^1H correlations. In addition, the analysis of the HMQC-TOCSY spectrum of **1** [5] utilizing the well resolved ^{13}C chemical shift scale allowed the unravelling of overlapping of proton resonances. The interpretation of ^1H-^1H connectivities thus resulted in four spin systems, covering carbon atoms C.2 to C.26 (**A**), C.28 to C.35 (**B**), C.44 to C.45 (**C**) and C.39 (**D**).

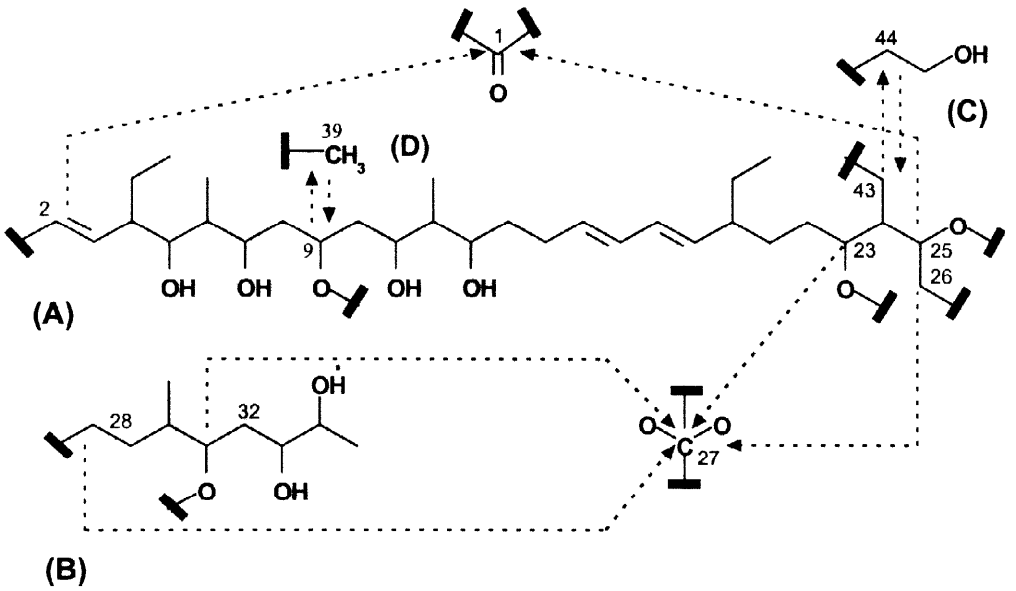


Figure 1. HMBC correlations observed establishing the connectivities of the sub-units (- - - -► $^{1}H—^{13}C$)

^{1}H-^{13}C long range couplings, detected in a HMBC spectrum [5,6] of maclafungin (**1**) optimized for long range coupling constants of 6.25 Hz (80 ms mixing time), were utilized in order to link these three spin-systems together.

The chemical shift of the quaternary carbon (δ_C 97.44) indicated that it should be attached to two oxygen substituents and two aliphatic carbons. In the HMBC spectrum of **1**, this carbon showed $^{2}J_{CH}$ correlations with C.26-H_2 (δ_H 1.740 and 1.615) and C.28-H_2 (δ_H 1.585 and 1.380) suggesting its connectivity to these aliphatic methylene carbons. Further, $^{3}J_{CH}$ correlations were observed for this quaternary carbon with C.23-H (δ_H 3.735) and C.31-H (δ_H 3.980) which could be attributed to an ether linkage of C.23 and C.31 to this quaternary carbon, leading to a spiro ketal system in maclafungin (**1**) which is known for other oligomycins [7,8].

Long range ^{1}H-^{13}C correlations also localized the lactone betweeen C.1 (δ_C 164.75) and C.25-H (δ_H 5.250); correlations to C.2-H (δ_H 5.695) and C.3-H (δ_H 6.395) could be observed from the carbonyl resonance.

Although the ^{1}H-^{1}H COSY spectrum did not allow the identification of any correlations between C.43-H_2 (δ_H 1.390 and 1.330) and C.44-H_2 (δ_H 1.450 and 1.280), long range ^{1}H-^{13}C correlations were observed between C.43-H_2 and C.44 (δ_C 32.93) and between C.44-H_2 and C.43 (δ_C 17.65). Similarly, the position of the -OCH_3 group was established by the long range ^{1}H-^{13}C correlations observed between C.39-H (δ_H 3.295) and C.9 (δ_C 81.01) and between C.9-H (δ_H 3.715) and C.39 (δ_C 55.87).

Table 1.
^{1}H and ^{13}C NMR spectral data of **Maclafungin (1)** in $CDCl_3$

Position*	δ_C	δ_H (m)	Coupling partner** (J in Hz)		
1	164.75 (s)	-			
2	123.45 (d)	5.695 (d)	3-H (15.7)		
3	148.42 (d)	6.395 (dd)	4-H (10.6)		
4	49.16 (d)	2.110 (dq)	36-H (2.6)	36-H' (10.4)	5-H (10.0)
5	78.63 (d)	3.670 (dd)	6-H (0.7)		
6	40.68 (d)	1.250 (m)	7-H (2.0)	38-CH_3 (6.0)	
7	78.75 (d)	4.020 (dt)	8-H (10.0)	8-H' (1.2)	
8	38.01 (t)	1.515 (ddd)	8-H' (14.5)	9-H (1.8)	
		1.450 (ddd)	8-H (14.5)	9-H (10.1)	
9	81.01 (d)	3.715 (dt)	10-H (2.9)	10-H' (10.1)	
10	38.03 (t)	1.800	10-H' (13.4)	11-H (11.6)	
		1.350	10-H (13.4)	11-H (3.2)	
11	73.79 (d)	3.365 (ddd)	12-H (8.9)		
12	45.28 (d)	1.440 (tq)	40-CH_3 (6.8)	13-H (8.8)	
13	75.24 (d)	3.470 (ddd)	14-H(11.2)	14-H' (2.3)	
14	32.88 (t)	1.670 (ddt)	14-H' (14.0)	15-H (4.6)	15-H' (10.7)
		1.420 (ddt)	14-H (14.0)	15-H (2.0)	15-H' (4.6)
15	28.44 (t)	2.225 (ddt)	15-H' (14.8)	16-H (4.6)	
		2.220 (ddt)	15-H (14.8)	16-H (9.3)	
16	130.97 (d)	5.385 (ddd)	17-H (15.0)		
17	132.05 (d)	6.030 (dd)	18-H (10.6)		
18	130.04 (d)	5.885 (dd)	19-H (15.2)		
19	137.87 (d)	5.305 (dd)	20-H (9.5)		
20	45.41(d)	1.880 (m)	21-H (6.7)	41-H (5.6)	41-H' (7.8)
21	30.75 (t)	1.450 (dt)	22-H (4.9)	22-H' (4.9)	
22	29.43 (t)	1.580 (ddd)	22-H' (16.20)	23-H (10.4)	
		1.105 (dt)	22-H (16.2)	23-H (5.4)	
23	70.33(d)	3.735 (ddd)	24-H (1.4)		
24	39.99 (d)	1.825 (dq)	25-H (4.8)	43-H (5.1)	43-H' (5.1)
25	70.90 (d)	5.250 (dt)	26-H (4.8)	26-H' (12.2)	
26	36.45 (t)	1.740 (dd)	26-H' (13.2)		
		1.615 (dd)	26-H (13.2)		
27	97.45 (s)	-			
28	29.86	1.585 (ddd)	28-H' (14.0)	29-H (11.4)	29-H' (4.0)
		1.380 (ddd)	28-H (14.0)	29-H (4.1)	29-H' (1.4)
29	26.52 (t)	2.060 (ddt)	29-H' (15.6)	30-H (4.1)	
		1.330 (ddt)	29-H (15.6)	30-H (1.5)	
30	30.82 (d)	1.535 (dtq)	31-H (2.0)	46-H (7.0)	
31	67.30 (d)	3.980 (dt)	32-H (10.8)	32-H' (2.0)	
32	37.17 (t)	1.565 (ddd)	32-H' (14.2)	33-H (2.2)	
		1.235 (ddd)	32-H (14.2)	33-H (9.5)	
33	72.85(d)	3.495 (ddd)	34-H (6.2)		
34	71.61(d)	3.520 (quint)	35-H (6.0)		
35	19.81 (q)	1.170 (d)	34-H (6.0)		
36	23.83 (t)	1.980 (ddq)	36-H'	37-H (7.6)	4-H (2.6)
		1.130 (ddq)	36-H	37-H (7.6),	4-H (10.4)

Position*	δ_C	δ_H (m)	Coupling partner** (J in Hz)			
37	11.48 (q)	0.735 (t)	36-H (7.6)	36-H' (7.6)		
38	4.06 (q)	0.800 (d)	6-H (6.0)			
39	55.87 (q)	3.295 (s)	-			
40	12.89 (q)	0.690 (d)	12-H (6.8)			
41	27.73 (t)	1.325 (ddq)	41-H' (13.6)	20-H (5.6)	42-H (7.5)	
		1.230 (dquint)	41-H (13.6)	20-H (7.8)	42-H (7.5)	
42	12.16 (q)	0.740 (t)	41-H (7.5)	41-H' (7.5)		
43	17.65 (t)	1.390 (m)	43-H'	44-H (5.1)	44-H' (5.1)	24-H (5.1)
		1.330 (m)	43-H	44-H (5.1)	44-H' (5.1)	24-H (5.1)
44	32.93 (t)	1.450 (dtt)	44-H' (11.9)	45-H (5.9)	45-H' (5.9)	
		1.280 (dtt)	44-H (11.9)	45-H (5.9)	45-H' (5.9)	
45	62.59 (t)	3.370 (dt)				
		3.350 (dt)				
46	11.27 (q)	0.855 (d)	30-H (7.0)			
5-OH	-	4.220 (br s)				
7-OH	-	4.310 (br)				
33-OH	-	2.220 (br)				

* The hydroxyl protons at C.11, C.13, C.34 and C.45 could not be detected.

** Only those coupling partners (and their J values) which are relevant to establish connectivities are listed.

The large coupling constants (J= ~15-16 Hz) of the olefinic protons suggested that all the three double bonds had *E*-configuration. The conformation of the C.17-C.18 bond is assumed to be same as that reported for rutamycin[9,10] based on the comparison of the coupling constants.

The structure of maclafungin was thus established to be as represented in **1**. The stereochemistry of the chiral centres was not established.

The *in vitro* activity (MIC) of maclafungin (**1**) is shown in Table 2. Maclafungin is more active against filamentous fungi (human and phytopathogenic) than against yeast like *Candida*

Table 2.
In vitro activity of maclafungin (**1**)

Test organism	MIC (μg/ml)	Test organism	MIC (μg/ml)
Candida albicans	12.50	*Trichophyton mentagrophytes*	< 0.10
Penicillium digitatum 135	0.80	*Botrytis cinerea* 57	0.10
Aspergillus niger	1.50	*Botrytis cinerea* 213	0.10
Microsporum canis	0.20	*Botrytis cinerea* 214	0.20
Microsporum gypseum	0.20	*Pyricularia oryzae* 154	0.10
Fusarium culmorum 100	0.10	*Cercospora beticola* 71	0.20
Cladosporium resinae	0.40	*Alternaria solani* 5	0.10

albicans. The compounds belonging to oligomycin class are known to exihibit mainly antifungal properties [7,11]; antibacterial activity is generally poor[12]. The compounds are known to be toxic and LD_{50} values have been reported[7,11]. In addition cytotoxicities and anti -tumor activities have also been studied[7,12].

Although maclafungin belongs to the oligomycin class it has several distinct features which are absent in all the other members of the group. The specific features observed were substitution patterns at C.7 and C.11 (CHOH instead of CO), C.9 (OCH_3 instead of OH), C.10 (no CH_3), C.24 (CH_2CH_2OH instead of CH_3) and at C.33 ($CHOHCH_3$ instead of CH_3). Maclafungin is therefore a unique member of this class of compounds.

Experimental

General experimental procedures

Melting points are uncorrected. The UV spectrum was recorded on a UVIKON 810 double beam spectrophotometer. The IR spectrum was obtained on a Perkin-Elmer 157 spectrophotometer. The optical rotation was measured on a Perkin-Elmer 141 polarimeter. HRDCI-MS and FAB-MS spectra were recorded on a VG-ZAB SEQ spectrometer. NMR spectra were recorded on a Bruker AM-400 spectrometer.

Fermentation

Strain Y-8520050 was isolated from a soil sample collected at Billimora, Gujarat, India. The strain was characterized as belonging to the order *Actinomycetales*. A loopful of mature slant culture of Y-8520050 was innoculated into 500 mL Erlenmeyer flasks containing 60 mL each of seed medium consisting of glucose 1.5%, soybean meal 1.5%, cornsteep liquor 0.5%, NaCl 0.5%, and $CaC0_3$ 0.2% (pH adjusted to 6.8 before autoclaving). The flasks were shaken on a rotary shaker at 220 rpm for 96 hours at 28^0C. The resultant seed culture (1 L) was innoculated into a 15 L fermenter containing 10 L of the above seed medium. The aeration and agitation of the fermentation were maintained at 8 $Lmin^{-1}$ and 180 rpm respectively and the temperature at 28^0C.

The fermentation was carried out for 42 hours and the resultant seed culture (8.5 L) was innoculated into a 100 L fermenter containing 85 L of production medium consisting of glucose 2.5%, soybean meal 1.0%, peptone 0.5%, $CaCO_3$ 0.02%, $CoC1_2.6H_2O$ 0.0001% (pH 6.6 before autoclaving). The aeration and agitation were maintained at 70 $Lmin^{-1}$ and 95 rpm respectively and the temperature at 28^0C. The fermentation was carried out for 110 hours. The production of the antibiotic was monitored by activity against *Fusarium culmorum*.

Isolation

The culture broth (86 L, pH 6.5) was harvested and centrifuged to separate the mycelium. The culture filtrate (80 L) was extracted with chloroform (40 L) using a counter current extractor (Westfalia). The extract was concentrated under reduced pressure to get 28 g of crude material. This material was flash chromatographed on silica gel (200-400 mesh, 400 g) at a flow rate of 40 $mLmin^{-1}$ using increasing amounts (1%, 1.5%, 3% and 4 %) of methanol in chloroform. The active fractions (3% methanol) were pooled and concentrated and then precipitated with petroleum ether (60-80) to get 1.2 g of enriched maclafungin.

The mycelium (5 kg) was extracted with methanol (40 L) and concentrated under reduced pressure. The concentrate was diluted to 12 L using water and extracted with chloroform (20 L). The extract was concentrated under reduced pressure to get 27 g of crude material. This material was flash chromatographed using the same conditions as above. The active fractions were pooled and concentrated and then triturated with petroleum ether (60-80) to get 1.6 g of enriched maclafungin.

The enriched materials, thus obtained, from mycelium and culture filtrate were combined and chromatographed on silica gel (100-200 mesh, 250 g) at a flow rate of 10 $mLmin^{-1}$ using increasing amounts (1%, 1.5%, 3% and 5 %) of methanol in chloroform. The active eluates (3% methanol) were combined and concentrated to get 1.4 g of semi-pure material. This was then chromatographed on reverse phase silica gel (RP-18, 50μ, 100 g) at a flow rate of 10 $mLmin^{-1}$ with increasing percentage of acetonitrile (30%, 35%, 40%, 50% and 100%) in water. All the active samples were analyzed by HPLC. Pure fractions (40% acetonitrile) were combined and concentrated under reduced pressure and then lyophilized to obtain 0.6 g of maclafungin.

Maclafungin (1) : White powder; m.p. 195^{0}C; soluble in benzene, chloroform, acetone, methanol and DMSO; $[\alpha]_D$ +32.8^0 (*c* 0.41, chloroform); UV (MeOH) 224, 230(sh); TLC Rf : 0.5 [silica gel / MeOH:$CHCl_3$ (1:9)]; HPLC Rt : 3.6 mins [(10 + 3) cm x 0.4 cm ODS-Hypersil, 10μ column/ methanol : water (8:2)/ flow rate (ml/min): 0.7 / detection : 220 nm]. IR (KBr) cm^{-1} : 3450, 2980, 2940, 2900, 1720, 1650, 1470, 1400, 1285, 1200, 1090, 1000; Elemental analysis : Calculated for $C_{46}H_{80}O_{12}.0.5H_2O$ C, 66.27; H, 9.72; Found C, 66.14, H, 9.70; FABMS m/z 825 $(M+H)^+$; HRDCIMS : Observed m/z 825.578 $(M+H)^+$; Calculated for $C_{46}H_{81}O_{12}$ 825.573 $(M+H)^+$.

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