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L 970843 and L 970844, Two New Antifungal Metabolites from an Unidentified Fungal Species HIL Y-903146†

E. K. S. VIJAYAKUMAR,*,†† KIRITY ROY, C. P. HIREMATH, S. K. DESHMUKH, TRIPTIKUMAR MUKHOPADHYAY and H. KOGLER†††

Research Centre, Hoechst Marion Roussel, Mulund (W), Mumbai-400 080, India
††† Biophysics, Core Research Functions, Hoechst Marion Roussel, D-65926, Frankfurt am Main, Germany

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During the course of our screening for antifungal metabolites, we have isolated two new compounds L 970843 (**1**) and its stereo isomer L 970844 (**2**) from an unidentified fungal species HIL Y-903146.[1] Herein, we report the production, isolation, structure elucidation and biological properties of **1** and **2**. Literature survey revealed that **1** and **2** are the first pentahydro analogs of xanthocillin class.[2]

The fungal strain, culture number HIL Y-903146, was isolated from a soil sample collected in Chandigarh, India. A loopful of mature slant culture of Y-903146 was inoculated into 500 ml wide mouth Erlenmeyer flasks containing 100 ml of the seed medium[3] and the flasks were incubated on a rotary shaker for 48 hours to get seed culture. The seed culture was then inoculated (1%) into 1 liter Erlenmeyer flasks containing 200 ml each of production medium consisting of glucose 4%, peptone 1%, NaH_2PO_4 0.1%, $ZnSO_4$ 0.022%, $CaCl_2$ 0.000055%, $MnCl_2 \cdot 4H_2O$ 0.00005%, $FeSO_4 \cdot 7H_2O$ 0.00005%, $CuSO_4 \cdot 5H_2O$ 0.000016% in demineralised water, adjusted to pH 6.5 before sterilization. The flasks were cultivated on a rotary shaker at 200 rpm for fermentation at 26°C (±1°C) for 64 hours. The progress of the fermentation was monitored by bioactivity testing against *Staphylococcus aureus* 209P, *Candida albicans*, *Aspergillus niger* and *Pseudomonas aerugenosa*.

The culture broth (40 liters) was harvested and centrifuged to separate the mycelium (1.2 kg). The activity was found both in the culture filtrate and the mycelium. The culture filtrate (33 liters) was passed through a column of Diaion HP-20 (3.5 liters). The column was washed with water (10 liters) and was then eluted with methanol (5 liters). The active methanol eluates were combined, concentrated under reduced pressure and lyophilized to get the crude material. This material was treated with methanol (500 ml) and filtered. The active methanol soluble portion was concentrated under reduced pressure to get crude material (8 g). The mycelium (1.2 kg) was extracted with 2×5 liters of acetone and the combined acetone extracts were concentrated under reduced pressure and lyophilized to get the crude material, which on treatment with methanol (500 ml) gave the crude material. Both the crude materials were pooled and subjected to flash chromatography on silica gel (200~300 mesh). The column was eluted sequentially with 500 ml each of 10%, 20%, 30%, 40% and 50% ethyl acetate in petroleum ether at a flow rate of 5 ml/minute with fractions size being 20 ml. All the fractions were monitored by silica gel TLC (Article No. 5554, E. Merck) using petroleum ether - ethyl acetate (60 : 40) and also by activity against *Candida albicans* and *Aspergillus niger*. L 970843 eluted in 30% ethyl acetate in petroleum ether, while L 970844 eluted in 40% ethyl acetate in petroleum ether. The fractions containing **1** and **2** were pooled separately and concentrated to dryness under reduced pressure. Both **1** and **2** were finally purified by dissolving in diethyl ether and reprecipitating with *n*-pentane.

L 970843 (**1**) was obtained as a pale yellow solid, m.p. 118~119°C, $[\alpha]_D$ −20° (*c* 0.02, MeOH), soluble in $CHCl_3$, EtOAc, MeOH and DMSO, UV (MeOH): 224, 288 and 300 nm. HRFABMS of **1** gave a molecular ion peak at *m/z* 397.2102 $(M+H)^+$ [calcd. for $C_{23}H_{29}N_2O_4$: 397.2127 $(M+H)^+$] corresponding to a molecular formula of $C_{23}H_{28}N_2O_4$ for **1**. The IR bands at 3400, 2120, 1725 and

* Corresponding author: evskumar@rediffmail.com

† Dedicated to Late Dr. P. V. DIVEKAR, Former Head of Microbiology, Research Centre, Hoechst Marion Roussel, Mulund (W), Mumbai-400 080, India.

†† Present address : Merck Development Centre (I) Pvt. Ltd., Plot No. 1A/2, M.I.D.C. Industrial Estate, Taloja, Panvel, Raidad Dist., Maharashtra-410 208, India.

Table 1. ^{1}H and ^{13}C NMR data of L 970843 (**1**) and L 970844 (**2**) in $CDCl_3$.

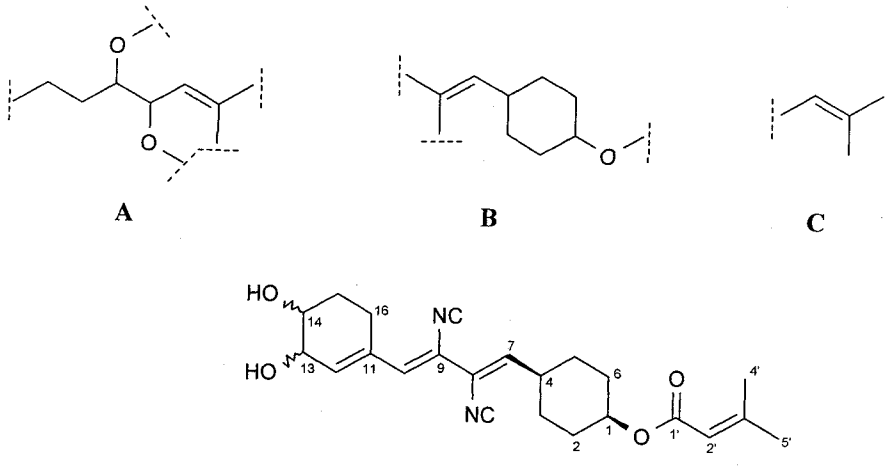
Carbon	L 970843 (**1**)[a]		L 970844 (**2**)[b,c]	
	δ_C	δ_H	δ_C	δ_H
1	67.29 (d)	5.07 (dd, 4.1, 2.3 Hz)	67.27 (d)	4.94 (m)
2, 6	28.83 (2 x t)	1.95 (ddd, 12.7, 3, 2.3 Hz)	28.24 (2 x t)	1.78 (m)
		1.65 (dd, 12.7, 4.1 Hz)		1.68 (m)
3, 5	25.96 (2 x t)	1.63 (ddd, 12.7, 4.4, 3 Hz)	25.51 (2 x t)	1.56 (m)
		1.60 (dd, 12.7, 10 Hz)		1.53 (m)
4	37.29 (d)	2.70 (ddd, 10, 9.5, 4.4 Hz)	36.67 (d)	2.62 (m)
7	138.34 (d)	6.22 (d, 9.5 Hz)	137.73 (d)	6.27 (d, 9.7 Hz)
8	120.34 (s)	-	119.93 (s)	-
9	116.56 (s)	-	114.78 (s)	-
10	130.03 (d)	6.50 (s)	130.72 (d)	6.58 (bs)
11	135.24 (s)	-	132.47 (s)	-
12	136.54 (d)	6.11 (d, 3.6 Hz)	140.96 (d)	6.15 (bs)
13	67.06 (d)	4.33 (dd, 4, 3.6 Hz)	70.09 (d)	3.46 (m)
14	67.26 (d)	3.98 (ddd, 7.8, 4, 2.3 Hz)	71.01 (d)	3.90 (m)
15	26.13 (t)	2.00 (dddd, 13.4, 7.8, 6.1, 5.7 Hz)	27.77 (t)	1.82 (m)
		1.82 (dddd, 13.4, 7.6, 6.1, 2.3 Hz)		1.58 (m)
16	23.72 (t)	2.76 (ddd, 17.8, 7.6, 5.7 Hz)	24.50 (t)	2.56 (m)
		2.58 (ddd, 17.8, 6.1, 6.1 Hz)		2.47 (m)
1'	166.07 (s)	-	165.09 (s)	-
2'	116.34 (d)	5.72 (bs)	115.99 (d)	5.73 (bs)
3'	156.69 (s)	-	156.39 (s)	-
4'	20.23 (q)	2.19 (s)	19.85 (q)	2.12 (s)
5'	27.44 (q)	1.93 (s)	26.79 (q)	1.89 (s)
6"	170.37 (s)	-	170.28 (s)	-
7"	173.05 (s)	-	172.60 (s)	-

[a] : ^{1}H NMR at 600 MHz and ^{13}C NMR at 150 MHz; [b] : ^{1}H NMR at 300 MHz and ^{13}C NMR at 75 MHz; [c] : The assignments were based on comparison with those of **1**.

1700 cm^{-1} indicated the presence of hydroxyls, isonitriles and carbonyls respectively.

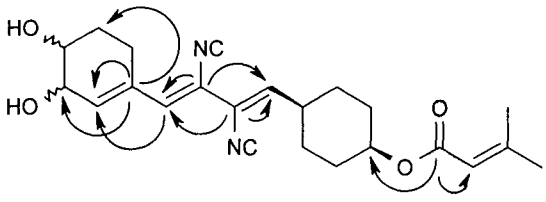
The ^{1}H NMR and ^{13}C NMR spectral data of L 970843 (**1**) in $CDCl_3$ were summarized in Table 1. The analysis of ^{1}H NMR, ^{13}C NMR and DEPT-135 spectra of **1** indicated the presence of seven quaternary carbons [4×=C, 2×≡C and 1×CO], eight methines [4×=CH, 3×OCH and 1×CH], six methylenes, two methyls and two hydroxyls. The molecular formula of **1** required 11 degrees of unsaturation, out of which 9 were accountable in the form of four double bonds, one carbonyl and two isonitriles, indicating that L 970843 had two cyclic units. DQF HH COSY spectral analysis of **1** in $CDCl_3$ gave three isolated spin systems **A**~**C**.

The connectivities between the sub-units **A**~**C**, two isonitriles groups, one carbonyl and the remaining double bonds were unequivocally established by the analysis of HMBC spctrum optimized for 7 Hz. Figure 1 describes the $^{3}J_{CH}$ and $^{2}J_{CH}$ correlations observed in the HMBC spectrum of **1**.



1 and 2

Fig. 1. ^{1}H-^{13}C long range correlations in the HMBC spectrum of **1** ($\rightarrow^{13}C$-^{1}H).



The presence of a cyclohexene diol was established by $^{3}J_{CH}$ couplings observed for C.11 (δ_C 135.24) with C.13-H (δ_H 4.33) and C.15-H_2 (δ_H 1.82 and 2.00). Further, C.12-H (δ_H 6.11) showed $^{3}J_{CH}$ correlation with C.10 (δ_C 130.03) and $^{2}J_{CH}$ correlation with C.11 (δ_C 135.24), indicating the conjugation of C.9–C.10 double bond to C.11–C.12 double bond. Besides, $^{3}J_{CH}$ couplings were observed for C.10-H (δ_H 6.50) and C.7-H (δ_H 6.22) with C.8 (δ_C 120.34) and C.9 (δ_C 116.56) respectively and $^{2}J_{CH}$ correlations with C.9 (δ_C 116.56) and C.8 (δ_C 120.34) respectively, thus establishing not only the presence of a conjugated triene system but also the connectivity of fragment **B**. The connectivity of fragment **C** to C.1 through an ester linkage was envisaged by the fact that the carbonyl at δ_C 166.07 showed $^{3}J_{CH}$ correlation with C.1-H (δ_H 5.07) and $^{2}J_{CH}$ coupling with C.2′-H (δ_H 5.72).

^{1}H-^{13}C long range coupling constants were well documented for unsaturated systems. In principle, $^{2}J_{CH}$ coupling constants are of the order of 0 to 5 Hz, while $^{3}J_{CH}$ coupling constants for the cisoid configuration of proton and carbon substituted at the double bond tend to be smaller (4 to 9 Hz) than for the transoid configuration (8 to 15 Hz) depending upon the electronegativity of the substituents. In **1**, the $^{2}J_{CH}$ coupling constants were in the range of 1 to 2.5 Hz, while $^{3}J_{CH}$ coupling constants were in the range of 6 to 8.4 Hz, suggesting *Z*-configuration. In addition, the size of the coupling constants observed support the substitution pattern of both the isonitrile groups at C.8 and C.9.

The relative stereochemistry of the chiral centres at C.1 and C.4 could be infered from the homonuclear coupling constants. Thus, C.1-H at δ_H 5.07 showed small constants of 2.3 and 4.1 Hz with C.2-H_2 and C.6-H_2, indicating an equatorial orientation, whereas C.4-H showed large coupling constant of 10 Hz with the axial protons of C.3-H_2 and C.5-H_2, indicating an axial orientation. In a classical chair conformation of the cyclohexane moiety, both these protons are on the same side *i.e.* 1,4-*cis*.

The NOESY and ROESY experiments on **1** gave conflicting results which might be due to a dynamic flip of the envelope conformation of the cyclohexene diol moiety. As a result, the stereochemistry of the chiral centres at C.13 and C.14 could not be established.

L 970844 was obtained as a pale yellow shiny flakes, m.p. 116~117°C. $[\alpha]_D$ +20° (*c* 0.02, MeOH), soluble in $CHCl_3$, EtOAc, MeOH and DMSO, ESIMS *m/z* $(M+H)^+$ 397, *Anal* Found: C 69.82, H 7.01, N 6.96; calcd for $C_{23}H_{28}N_2O_4$: C 69.76, H 7.13, N 7.07. UV (MeOH): 224, 288 and 300 nm, IR (KBr): 3400, 2120,1715, 1700, 1650,

Table 2. *In vitro* activities of L 970843 (**1**) and L 970844 (**2**).

Test Organism	Zone size (mm)	
	L 970843	L 970844
Penicillum digitatum	24	25
Fusarium culmorum 100	18	19
Alternaria mali P37	23	27
Botrytis cinera A06	23	27
B. cinera D01	17	18
B. cinera E02	20	23
Pellicularia sasakii	23	27
Leptospheria nodorum JO2	28	27
Pyricularia oryzae 154	23	30
Pseudo herpotrichoides 008	28	30
Phytophthora infestans J08	20	21
Neurospora crassa SGF 18	27	27
Candida albicans	21/23h	18/27h
Staphylococcus aureus 209P	23/26h*	26
Escherichia coli 9632	17	17
Pseudomonas aeruginosa	17	22

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1450, 1240 and 1160 cm^{-1}.

The molecular formula of **2** was determined to be $C_{23}H_{28}N_2O_4$ by the analysis of ^1H NMR, ^{13}C NMR and DEPT-135 spectra in combination with mass and elemental analysis. The NMR data of **2** was found to be almost identical to that of **1** with minor differences in the chemical shifts for C.13 and C.14, suggesting that **2** was a stereo isomer of **1**.

Both **1** and **2** showed good activity against fungal and bacterial strains. The *in vitro* activity at a concentration of 1 mg/ml in MeOH applied on paper discs is given in Table 2. Under green-house conditions at 2500 ppm, **1** showed fungicidal efficiency of 90, 40 and 100% against *Phytophthora infestans* of LILY, *Erysiphie gram. forma hordei* of HOVU and *Sclerotinia fuckeliana* St.E02 of CUSA respectively, while **2** exhibited fungicidal efficiency of 75/2, 65 and 42% against *Phytophthora infestans* of LILY, *Piricularia oryzae* of ORSA and *Sclerotinia fuckeliana* St.E02 of CUSA respectively.

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