MF-EA-705α & MF-EA-705β, New Metabolites from Microbial Fermentation of a *Streptomyces* sp.

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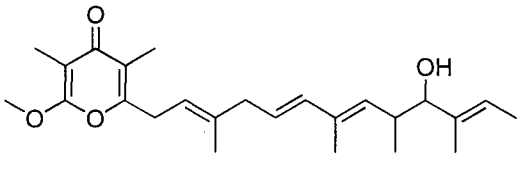
As part of an antifungal discovery program, the crude extract of a *Streptomyces* sp. from Ecuador, MF-EA-705, was found to display broad-spectrum antifungal activity against *Candida* spp. and *Aspergillus* spp. The extract was found to contain the known weakly-antibacterial compound actinopyrone A (**1**),[1] previously isolated from *Streptomyces pactum*. We report herein the production, isolation, structure elucidation and biological activities of two new compounds MF-EA-705α (**2**) and MF-EA-705β (**3**). A structurally similar compound has been reported in the microbial natural product literature, NFAT-133 (**4**),[2] known to be an immunosuppressive agent *in vitro*.

An 8-cm sporulation agar plate was inoculated with a 100 μl portion of frozen MF-EA-705 stock, and incubated at 28°C until growth (medium, beige-white) was observed (16 days). Sterile phosphate-buffered saline (6 ml) was then added. The MF-EA-705 mycelia and spores were gently scraped with a sterile loop, and a 3-ml portion of the resultant suspension was pipetted into a 500-ml flask containing sterile ATCC-172 media (150 ml, 2% soluble starch, 1% dextrose, 0.5% NZ amine type A, 0.3% Difco beef extract, 0.5% Difco bacto peptone, 0.5% yeast extract, 0.1% $CaCO_3$, presterile pH 7.0). The seed culture was incubated for 24 hours at 28°C, 200 rpm, and 85% humidity. Each of five production flasks (2800-ml Fernbach) containing 500 ml of production media (5% glycerol, 2.5% corn meal, 0.5% Hyeast 444, presterile pH 7.0) was inoculated with 20 ml of seed culture prepared

Fig. 1. Structures of compounds.



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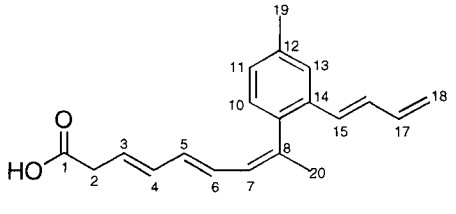

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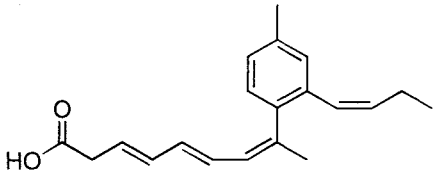

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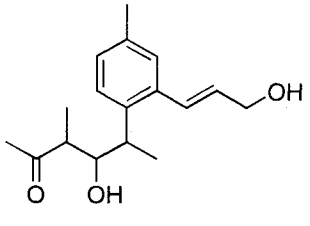

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above and incubated at 28°C, 160 rpm, and 85% humidity for 192 hours (8 days). The fermentation was harvested by centrifugation (4,500 rpm, 15 minutes, 4°C) and the supernatant (2200 ml) was decanted and stored at 4°C until used. The pellet was extracted with 1000 ml of 90% aqueous acetone for 2 hours and the extract was separated from the cell debris by centrifugation (4,500 rpm, 15 minutes, 4°C) and concentrated *in vacuo* (30 mbar, 30°C). The remaining aqueous suspension (200 ml) was also stored at 4°C until used.

The production of the bioactive components and their purification was monitored by inhibitory activity against *Candida albicans* in a cut-well agar diffusion assay. The fermentation supernatant and the concentrated pellet extract were recombined and adsorbed onto a column (2.5 cm i.d.× 22 cm L, 110 ml) packed with Diaion® HP20 (Mitsubishi Kasei) that had been equilibrated in water (1000 ml). The column was sequentially eluted at 5 ml/minute with water (600 ml), a gradient of 0 to 20% acetone (350 ml), 20% acetone (350 ml), a gradient of 20 to 100% acetone (550 ml) and 100% acetone (550 ml), collecting fractions at 5-minute intervals. The active eluates (60~80% acetone) were combined and concentrated *in vacuo* (<30°C), and the residual aqueous solution was lyophilized to yield 1.8 g of crude material. The sample was dissolved in a mixture of 1.25 ml of buffer A (0.1 M NH_4OAc, pH 4.9) and 3.75 ml of methanol, and was top-loaded on a column (2.2 cm×35 cm, 135 ml) packed with C_{18} silica gel (Amicon) that had been pre-equilibrated with 75% methanol in 0.1 M NH_4OAc (pH 4.9). The column was eluted at 5 ml/minute with the same solvent mixture. The active fractions were each analyzed by HPLC (Amicon C_{18}, 4.6 mm×100 mm, 1.7 ml; 75% methanol in 0.1 M NH_4OAc, pH 4.9) and the fractions corresponding to separate peaks (as detected by UV) combined accordingly. These pooled active fractions were further purified by reverse-phase HPLC on a Matrex C_{18} column (MODcol®, 2.12 cm×25 cm, 90 ml) eluted with 75% methanol in 0.1 M NH_4OAc pH 4.9, to obtain MF-EA-705α (**2**, 65 mg), MF-EA-705β (**3**, 20 mg) and actinopyrone A (**1**, 12 mg). The physico-chemical properties of compounds **2** and **3** are summarized in Table 1.

MF-EA-705α (**2**) was isolated as an optically-inactive colorless oil. The molecular formula $C_{20}H_{22}O_2$, which requires ten degrees of unsaturation, was established by HRFABMS [*m/z* 295.1688 $(M+H)^+$] and by interpretation of the ^1H and ^{13}C NMR data. The IR spectrum contained typical carboxylic acid bands at 3420 and 1710 cm^{-1}, and the UV absorption at 280 nm was consistent with a chromophore arising due to conjugation. The aliphatic region of the ^1H NMR spectrum (Table 2) contained two methyl singlets at δ 2.34 (Me-19) and 2.02 (Me-20) and a single methylene signal at δ 3.05 (H-2). Two one-proton coupled signals at 5.10 and 5.27 (H-18a and H-18b) were correlated (HMQC) to a single carbon at δ 117.2, confirming the presence of a terminal methylene group. From ^1H NMR, DEPT and HMQC experiments, the presence of eleven additional methine carbons and five sp^2 quaternary carbons, one of which was an acid carbonyl (δ 177.0), was strongly inferred. In addition to the data presented above, the COSY experiment allowed the

Table 1. Physico-chemical properties of MF-EA-705α and β.

	MF-EA-705α	MF-EA-705β
Appearance	clear oil	clear oil
Molecular Formula	$C_{20}H_{22}O_2$	$C_{20}H_{24}O_2$
HRFABMS	$[M+H]^+$	$[M+H]^+$
Calc.	295.1698	297.1855
Found	295.1688	297.1856
UV λ_{max} (MeOH) nm, ε	280 (1.8×10^6)	240 (35630) 280 (51330)
IR ν_{max} (neat) cm^{-1}	3420, 1710	3390, 1710
Solubility	MeOH, $CHCl_3$, ethyl acetate	MeOH, $CHCl_3$, ethyl acetate

Table 2. ^{1}H NMR (400 MHz, 25°C, $CDCl_3$) and ^{13}C NMR (100 MHz) data of MF-EA-705α (**2**).

C no.	δ_C	δ_H	mult, J (Hz)	HMBC
1	177.0			
2	37.7	3.05	d, 2H, 7.1	177.0, 123.4, 134.6
3	123.4	5.64	dt, 1H, 15, 7.3	177.0, 37.7, 130.3
4	134.6	5.97	dd, 1H, 15, 11	37.7, 130.6
5	130.3	6.09	dd, 1H, 15.6, 11.3	123.4, 134.6, 128.3
6	130.6	5.77	dd, 1H, 14.8, 11	134.6
7	128.3	6.15	d, 1H, 11.2	130.6, 138.1, 26.2
8	139.5			
9	138.1			
10	128.9	6.91	d, 1H, 7.7	139.5, 134.2, 136.7
11	128.4	7.02	dd, 1H, 7.7, 1.1	138.1, 125.5, 21.2
12	136.7			
13	125.5	7.40	br s, 1H	138.1, 128.4, 130.8, 21.2
14	134.2			
15	130.8	6.50	d, 1H, 15.7	125.5, 130.1, 137.6
16	130.1	6.71	dd, 1H, 15.7, 10.4	134.2, 130.8, 137.6
17	137.6	6.42	dt, 1H, 16.8, 10	
18a	117.2	5.10	dd, 1H, 10.1, 0.8	130.1
18b		5.27	dd, 1H, 16.8, 0.8	130.1, 137.6
19	21.2	2.34	s, 3H	125.5, 136.7, 128.4
20	26.2	2.02	s, 3H	128.3, 139.5, 138.1
1-OH		5.60	br s, 1H	

construction of several partial structures that could then be interconnected using data obtained from the HMBC experiment. The 1,2,4-trisubstituted benzene moiety was assigned on the basis of the coupling constants of the H-10, H-11 and H-13 signals and COSY correlations between H-10 and H-11. Key long-range HMBC correlations (Figure 2a) are as follows: H-13 to C-9, C-11, C-15 and C-19; H-11 to C-9 and H-10 to C-8, C-14 and C-12. These results are consistent with a trisubstituted aromatic ring.

The remainder of the molecule was established as follows. The ^{1}H NMR signals at δ 5.64 (H-3), 5.97 (H-4), 6.09 (H-5), 5.77 (H-6) and 6.15 (H-7), each integrating to one proton, were assigned on the basis of COSY to the hydrogens on a set of three conjugated olefins. The geometries of the C3–C4 and C5–C6 olefins were assigned as *trans* on the basis of the ^{1}H coupling constants (Table 2) and the NOESY data (Figure 2b). The configuration of the C7–C8 olefin was assigned as shown based on a NOESY correlation between H-7 and H-20. The placement of the C-20 methyl group at C-8 was assigned on the basis of COSY and HMBC data. Further HMBC correlations from H-3 to C-1 and C-5, and from H-2 to C-4 established the terminal carboxylic acid portion of the chain. The remaining portion of the molecule, C-15 to C-18, was determined similarly.

Fig. 2. (a) Selected HMBC and (b) NOESY correlations for EA-705α (**2**).

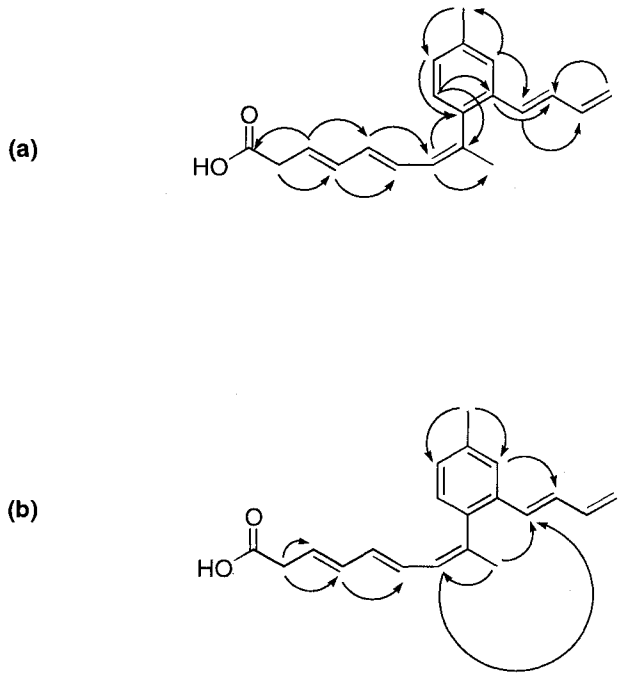

Table 3. ^{1}H NMR (400 MHz, 25°C, $CDCl_3$) and ^{13}C NMR (100 MHz) data of MF-EA-705α (**3**).

C no.	δ_C	δ_H	mult, J (Hz)	HMBC
1	177.2			
2	37.7	3.06	d, 2H, 7	177.2, 123.0, 134.8
3	123.0	5.65	dt, 1H, 15, 7	177.2, 37.7, 129.8
4	134.8	5.99	dd, 1H, 15.2, 11	37.7
5	129.8	6.09	m, 1H	
6	131.0	5.81	dd, 1H, 15, 11	134.8
7	127.6	6.09	m, 1H	138.3, 25.6
8	140.4			
9	138.3			
10	128.5	6.94	d, 1H, 7.4	140.4, 135.4
11	127.4	7.02	br d, 1H, 8.2	138.3, 130.0, 21.2
12	136.0			
13	130.0	7.06	br s, 1H	138.3, 127.3, 21.2
14	135.4			
15	127.3	6.20	d, 1H, 11.2	130.0, 21.8
16	134.4	5.56	dt, 1H, 11, 7	135.4, 21.8, 14.4
17	21.8	2.19	q, 2H, 7.5	127.3, 134.4, 14.4
18	14.4	0.97	t, 3 H, 7.5	134.4, 21.8
19	21.2	2.33	s, 3H	130.0, 136.0
20	25.6	1.98	s, 3H	127.6, 140.4, 138.3
1-OH*				

* not observed

The C-19 methyl group was placed at C-12 on the basis of HMBC correlations between CH_3-19 to C-11 and C-13.

MF-EA-705β (**3**) was isolated as an optically-inactive colorless oil. Its molecular formula, $C_{20}H_{24}O_2$, was 2 amu heavier than that of MF-EA-705α (**2**). Analysis of the NMR data (Table 3) revealed that the only structural changes were saturation of the C-17 to C-18 double bond of **2**, and a *cis* geometry of the C15–C16 double bond. The signals at δ_C 137.6 and 117.2 and δ_H 6.42, 5.10 and 5.27 in the NMR spectra of **2** were replaced by a methylene carbon signal at 21.8 and an additional methyl signal at δ_C 14.4 and δ_H 0.97 (t, 3H, J 7.5, 7.5 Hz). The new CH_3 signal showed HMBC correlations to C-16, and H-17 correlated to C-15. Further HMBC correlations indicated the chain was still attached to C-14 of the benzene ring, although the effect of the change was noted in the chemical shift of H-13 (δ 7.40 to 7.06), H-15 (6.50 to 6.20) and H-16 (6.71 to 5.56). The remaining spectral data suggested the rest of the molecule was identical to **2**.

The purified compounds MF-EA-705α (**2**) and MF-EA-705β (**3**) were inactive at a concentration of 128 μg/ml against *Candida albicans*, *C. glabrata*, *C. krusei*, *Cryptococcus neoformans*, and *Aspergillus fumigatus*. The minimum inhibitory concentration (MIC) of (**2**) against *C. albicans* was determined to be 1 mg/ml, suggesting the unsuitability of this compound as a drug candidate. The activity observed and followed during bioassay-guided fractionation was thus due to high concentrations of the compound being assayed in the cut-well agar plates. Nevertheless, these compounds are interesting novel metabolites, with a similar compound having been isolated only once previously in the microbial natural product literature.[2)]

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