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

Asfia Qureshi, Jacob B. Mauger, Raúl J. Cano, Jorge L. Galazzo and May D. Lee

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 actinopyrane A (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), 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% CaCO₃, 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.
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 NH₄OHAc, 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 C₁₈ silica gel (Amicon) that had been pre-equilibrated with 75% methanol in 0.1 M NH₄OHAc (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 C₁₈, 4.6 mm × 100 mm, 1.7 ml; 75% methanol in 0.1 M NH₄OHAc, 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 C₁₈ column (MODco®, 2.12 cm × 25 cm, 90 ml) eluted with 75% methanol in 0.1 M NH₄OHAc 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 C₉₀H₁₇₂O₂, which requires ten degrees of unsaturation, was established by HRFABMS [m/z 295.1688 (M+H)+] and by interpretation of the ¹H and ¹³C 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 sp² 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

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<td>[M+H]+</td>
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<td>3390, 1710</td>
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Table 2. $^1$H NMR (400 MHz, 25°C, CDCl$_3$) and $^{13}$C NMR (100 MHz) data of MF-EA-705α (2).

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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).

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<th>δ$_c$ (ppm)</th>
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* 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 C-15-C-16 double bond. The signals at δ$_c$ 137.6 and 177.2 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.

Acknowledgements

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References
