Chemical and Biological Characterization of Two FK506 Analogs Produced by Targeted Gene Disruption in *Streptomyces* sp. MA6548

ALI SHAFIEE[a,*], HAIDEH MOTAMEDI[a,*], FRANCIS J. DUMONT[b], BYRON H. ARISON[c] and RANDALL R. MILLER[c]

[a]Departments of Natural Products Drug Discovery, [b]Immunology Research and [c]Drug Metabolism, Merck Research Laboratories, Rahway, New Jersey, 07065, U.S.A.

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Two genetically engineered mutant strains of *Streptomyces* sp. MA6548 produced two FK506 analogs, 9-deoxo-31-*O*-demethylFK506 and 31-*O*-demethylFK506. The structures were determined by a combination of NMR and mass spectrometry. These compounds exhibited immunosuppressive and antifungal activities, albeit reduced, compared to FK506. Both compounds contain a free hydroxyl group at C-31 for the synthesis of novel FK506 derivatives.

Despite the superior potency of FK506 relative to cyclosporine A[1] as a drug in the prevention of graft rejection and in the treatment of autoimmune diseases, neuro- and nephrotoxicities are equivalent in patients treated with either drug[2,3]. As a result, there is an urgent need for novel FK506 derivatives with reduced toxicity and improved therapeutic indices.

A new approach to generate novel structural variation of known natural products is by altering of the genes involved in their biosynthesis[4,5]. In this approach a detailed understanding of the biochemistry and genetics involved in the formation of the desired compound is of critical importance.

We have recently started a comprehensive study of FK506 biosynthesis[6,7] and have reported characterization of three co-transcribed genes from the FK506 gene cluster of *Streptomyces* sp. MA6548[8,9]. Targeted disruption of two of these genes, *fkbD* and *fkbM*, which code for 31-*O*-demethylFK506 methyltransferase (FKMT) and a cytochrome P-450-dependent 9-deoxo-31-demethylFK506 hydroxylase/oxidase (9-deoxo-hydroxylase), resulted in the accumulation of two new biologically active metabolites by the recombinant mutants. Here we report the isolation and the chemical and biological characterization of these two compounds.

Experimental

Mutant Construction

Construction of two genetically engineered mutants of *Streptomyces* sp. MA6548, designated strains M23 and M38, has recently been described[8]. Briefly, M23 was developed by disruption of *fkbM*, coding for FKMT. The second mutant, M38, was constructed by disruption of *fkbD*, the gene for FK-9-oxidase.

Fermentation

Vegetative cells (1 ml) of each mutant strain (M23 or M38) were inoculated in a 250-ml flask containing KE seed medium (50 ml) consisting of glucose 1%, dextrin 10%, beef extract 3%, ardamin PH 5%, NZ-amine E 4%, $MgSO_4 \cdot 7H_2O$ 0.5%, 1 M phosphate buffer (pH 7) 2 ml, $CaCo_3$ 0.5 g (pH 7) and cultivated for 40 hours at 29°C on a rotary shaker (220 rpm). Aliquots (2 ml) of the resulting seed culture were then transferred to 250-ml flasks containing RSPB production medium (50 ml) consisting of dextrin 20%, distillers soluble 1.4%, yeast extract 0.25% and cultivated for 5 days at 29°C and agitation of 220 rpm.

Metabolite Isolation

A 5-day-old culture of each mutant strain (1.5 liters) was centrifuged and the supernatant was recovered. The pellet (collected mycelia) was extracted with 70% methanol (1 liter) and the extract was combined with the original supernatant. The resulting mixture was extracted three times with an equal volume of methylene chloride and the pooled extract was dried over Na_2SO_4 and stripped of solvent using rotary evaporator under reduced pressure. The residue obtained was dissolved in 25 ml of 25% acetonitrile and applied to an activated C-18 reverse-phase column (Spe-ed, Applied Bioscience). The desired metabolites were eluted with 50 ml each of 50% and 75% aqueous acetonitrile which were then combined. After evaporation of the acetonitrile, the remaining solution was extracted with methylene chloride and dried; the

resulting residue was dissolved in 1 ml of acetonitrile and filtered. A small aliquot of the filtrate was fractionated *via* HPLC using a semi-preparative C-18 reverse-phase column (Phenomenex 9.2 mm × 250 mm). The column was eluted with an isocratic solvent system containing 0.1% aqueous phosphoric acid and acetonitrile (30 : 70) at a flow rate of 2 ml/minute and thirty fractions (2 ml/fraction) were collected. The acetonitrile portion of each collected fraction was evaporated under a stream of nitrogen gas and the aqueous portion was extracted with an equal volume of ethyl acetate. An aliquot (1 ml) of the ethyl acetate extract was then dried under nitrogen as above and the residue was dissolved in 50 μl of ethanol for use in antifungal activity evaluation (see below). Antifungal active fractions were pooled and extracted with methylene chloride. The methylene chloride extract was concentrated and a portion was chromatographed on a silica TLC plate (E. M. Merck) using a dichloromethane : methanol (9 : 1) solvent system. UV-absorbing band with Rf value of approximately 0.19 was scraped off from the TLC plate and eluted with ethyl acetate. The ethyl acetate extract was washed with water twice, dried over sodium sulfate and concentrated to dryness. The purified residue was then used for antifungal activity evaluation and structural elucidation.

MS and NMR Analysis

The purified samples were analyzed by LC/MS/MS on a SCIEX API III mass spectrometer using the ionspray interface. Samples were examined by direct injection in a mobile phase that consisted of 50% acetonitrile in 10 mM aqueous ammonium acetate and 0.1% aqueous trifluoroacetic acid. Positive ion detection was used during analysis.

NMR spectra were recorded on a Varian Unity 400 NMR spectrometer.

Estimation of the Metabolites Concentration

HPLC was used to determine the titer of each metabolite in the fermentation broths and to determine the quantity of metabolite in samples submitted for antifungal activity evaluation. For the estimation of the titer, 1 ml of the fermentation broth (pH 6.8) was mixed with an equal volume of methanol, vortexed and centrifuged. An aliquot of the resulting supernatant (50 μl) was quantified by an HPLC system equipped with a Partisil 10 ODS-3 analytical column (Whatman) at 55°C. The column was developed with a linear-gradient solvent system in which the concentration of acetonitrile in 0.1% aqueous phosphoric acid was raised from 55 to 80% during a 30 minutes run. The flow (1 ml/minute) was monitored at 205 nm. The areas of the peaks at retention times corresponding to those for the metabolites were integrated and the results were used for quantitation from a standard curve that had been established for authentic 31-*O*-demethylFK506.

Bioassay for Immunosuppressive Activity

Splenic T cells from C57Bl/6 mice were isolated by nylon wool separation as previously described[10] and resuspended in supplemented RPMI 1640 culture medium (GIBCO, Grand Island, NY) containing 10% heat-inactivated fetal calf serum. Microcultures were set up in flat bottom 96-well plates (Costar, Cambridge, MA) with each well receiving 200 ul of T cell suspension at 0.5×10^6 cells/ml. Various concentrations of compounds were distributed in triplicate wells and the stimulating combination of ionomycin (250 ng/ml) + PMA (10 ng/ml) was added[10]. One set of replicate plates received human recombinant IL-2 (50 U/ml, Biosource International) in addition to ionomycin + PMA. The plates were incubated for 48 hours at 37°C in a humidified atmosphere of 5% CO_2 - 95% air and pulsed with ^{3}H-TdR (2 μCi/well) for the last 5 hours. Cells were harvested on fiber glass filters and incorporated radioactivity was measured in a Betaplate liquid scintillation spectrometer (Pharmacia-LKB, Piscataway, NJ). The results were expressed as percent of the control (no compound) response.

Bioassay for Antifungal Activity

Spores (10^5) of the FK506 sensitive strain *Aspergillus niger* ATCC6275 were added to 250 ml YPAD agar medium containing per liter: YPD broth (Difco), 50 g; adenine, 0.8 g and agar, 20 g and poured into 9 × 9 inch Nunc plates. An aliquot (0.5 ml) of each fraction obtained from HPLC, as described above, was extracted with ethyl acetate (0.5 ml) and the extract was dried under nitrogen and the residue was dissolved in ethanol (50 μl). Filter paper disks were soaked with 10 μl of the ethanol extract, air dried and then placed on top of the indicator plates. The plates were then incubated overnight at 30°C. FK506 and its active analogs were evidenced by the appearance of a zone of growth inhibition around the disks. *Aspergillus niger* MF5661, an FK506 resistant strain was used in the same bioassy to confirm that the antifungal activities of the isolated metabolites are related to FK506 derivatives.

Results and Discussion

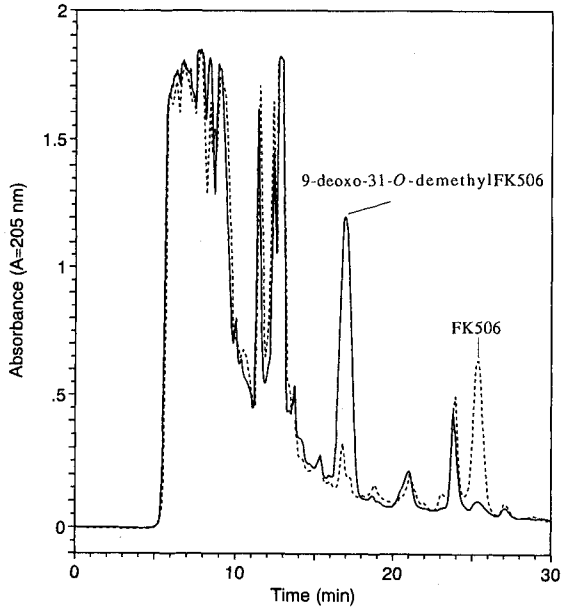
Production of 9-Deoxo-31-*O*-demethylFK506

HPLC trace (205 nm) for the extract from wild type *Streptomyces* sp. MA6548 (broken line) superimposed with that of the mutant strain M38 (solid line) is shown in Fig. 1. The peak eluting at 25.36 minutes, representing FK506, is missing from M38 and instead a distinct peak at 16.76 minutes has emerged. In M38, disruption of *fkbD* and its apparent polarity on the downstream gene, *fkbM*[8], resulted in the generation of a biologically active material represented by the latter peak.

Structural Elucidation of 9-Deoxo-31-*O*-demethylFK506

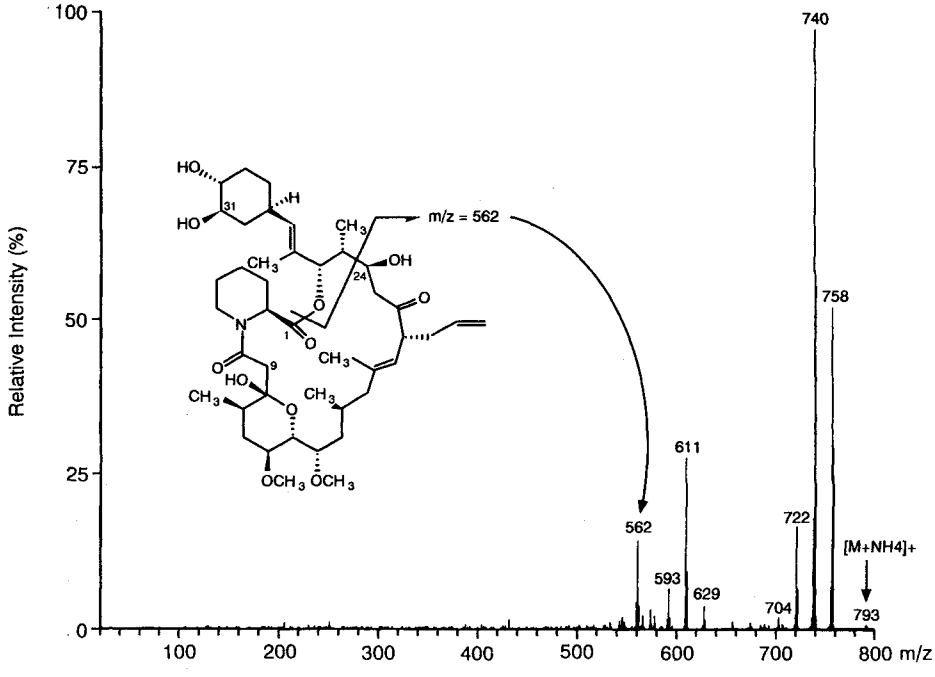
The structure responsible for the peak at 16.76 minutes was elucidated by a combination of MS and NMR spectral analysis. In the mass spectrum of this sample, an $[M+NH_4]^+$ ion at *m/z* 793 is present. This indicated a molecular weight of 775 Da which was 28 Da less than the standard FK506 (803 Da). In the product ion spectrum of this sample (Fig. 2), four distinct signals are observed at *m/z* 758, 740, 722 and 704, indicating the loss of up to four water molecules from the molecular ion at *m/z* 776. Similarly, in the product ion spectrum of the standard 31-*O*-demethylFK506 $[M+NH_4]^+$ (Fig. 3) four distinct signals at *m/z* 772, 754, 736 and 718 indicate the same loss of up to four water molecules; each of these is 14 Da higher than the corresponding fragments of the compound under investigation. Collectively, these results indicated a loss of 28 Da from FK506, of which 14 can thus be assigned to the methyl group at the C31 position. This interpretation is in accord with the disruption of one of the two genes, namely, the gene

Fig. 1. HPLC trace for the extract from wild type *Streptomyces* sp. MA65488 (broken line) superimposed with that of the mutant strain M38 (solid line).



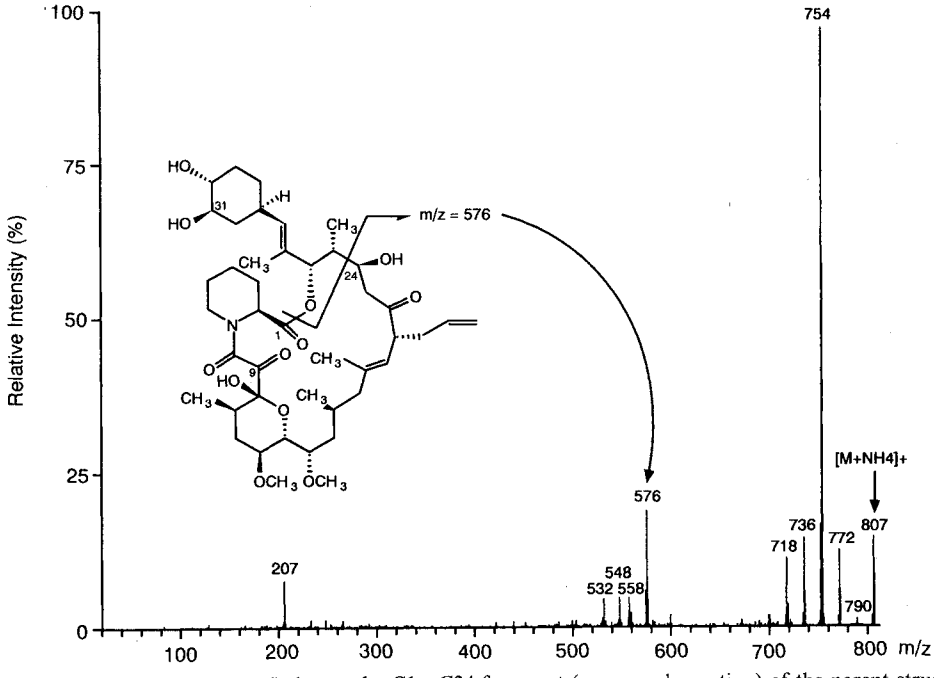
The elution positions of FK506 and 9-deoxo-31-demethylFK506 are shown in this figure.

Fig. 2. Product ion mass spectrum of 9-deoxo-31-*O*-demethylFK506.



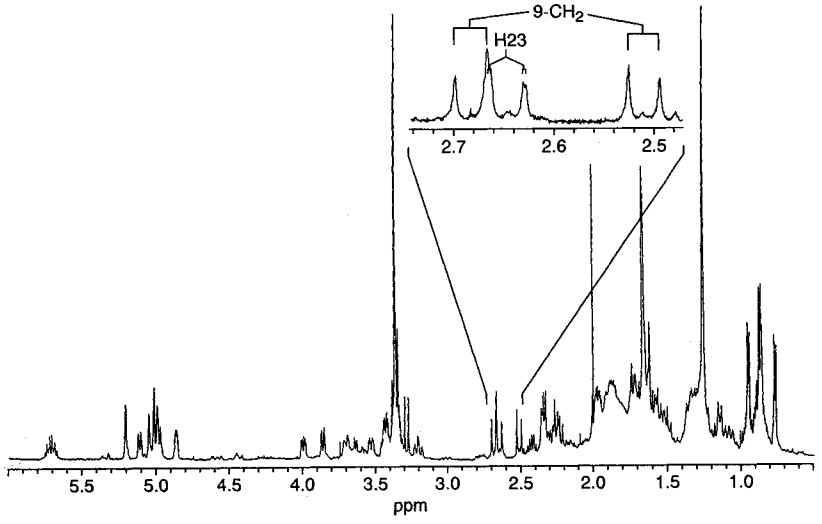
Arrow shows the signal (*m/z* = 562) due to the C1 ~ C24 fragment (macrocycle portion) of the parent structure.

Fig. 3. Product ion spectrum of 31-*O*-demethylFK506.



Arrow shows the signal ($m/z = 576$) due to the C1 ~ C24 fragment (macrocycle portion) of the parent structure.

Fig. 4. NMR spectrum of 9-deoxo-31-*O*-demethylFK506 in which the presence of two non-equivalent protons at C-9 is demonstrated in an expanded scale.



that codes for the methylation of the hydroxyl group at the C-31 position in the producing organism. In order to account for the loss of the remaining 14 Da, a close examination of the product spectra of both 31-*O*-demethylFK506 and FK506 (spectrum not shown) was required. In these two structures, a major fragment ion at m/z 576 encompasses C1 through C24. Similarly, in the product ion spectrum of the unknown compound (Fig. 2), there is a fragment ion at m/z 562 which is 14 Da less than the signals at m/z 576, observed for both FK506 and 31-*O*-demethylFK506. Thus, the difference of the remaining 14 Da can be attributed to a loss from the C1 ~ C24 portion of the macrolactone structure. In order to determine the specific position of the lost element(s), a highly purified sample was subjected to proton NMR analysis. The result, shown in Fig. 4, demonstrates the presence of a novel pair of non-equivalent methylene protons with chemical shifts at 2.51 and 2.68 ppm (d,

15.7 Hz). The chemical shifts are well within the range expected for a methylene group neighboring a carbonyl group. However, since there are methylene groups at C9 and C23 in the macrolactone portion of the structure, both having a neighboring carbonyl group (C8 and C22), an NOE difference experiment was performed. In this study, the methylene proton at 2.51 ppm was irradiated, resulting in the emergence of an NOE signal from the C11 methyl group thus providing evidence that the new methylene is located at C9 position. These results collectively and unequivocally establish 9-deoxo-31-*O*-demethylFK506 as the structure for the isolated compound from the genetically engineered mutant designated M38. This result is in accordance with the genetic analysis of FK506 gene cluster in *Streptomyces* sp. MA6548[8].

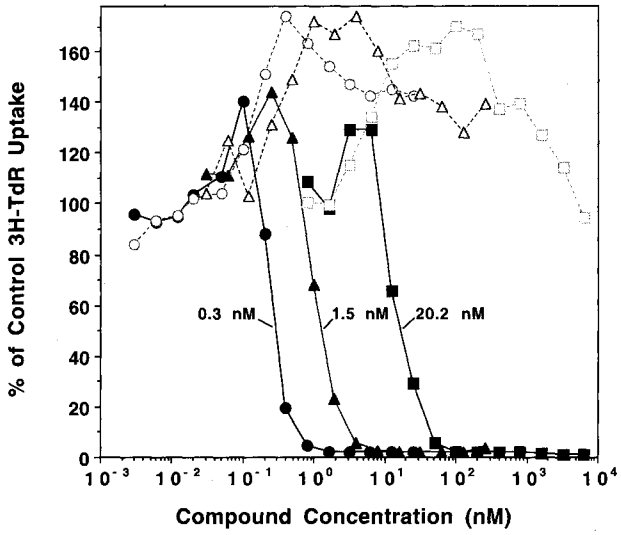
Production and Structural Elucidation of 31-*O*-DemethylFK506

No peak representing the presence of FK506 was detected in the HPLC analysis of the extract from the recombinant strain M23, which had been generated by the disruption of *fkbM*, the gene coding for 31-*O*-demethylFK506 methyltransferase[8]. However, there appeared a peak with a retention time identical to the retention time of standard 31-*O*-demethylFK506 (data not shown). The NMR and MS spectra obtained for the material eluting with this peak were superimposable with the standard 31-*O*-demethylFK506. This confirmed that *fkbM* is the gene encoding FKMT.

Immunosuppressive Activity

The isolated compounds were tested for inhibition of the proliferative response of mouse T cells induced by ionomycin + PMA. As shown in Fig. 5, 9-deoxo-31-*O*-demethylFK506 abrogated the response with an IC_{50} = 20.2 nM while 31-*O*-demethylFK506 and FK506 gave IC_{50}s of 1.5 nM and 0.3 nM, respectively. Importantly, the inhibitory activity of all three compounds was fully reversed by addition of exogenous IL-2 to the cultures, demonstrating that the inhibition is not due to non-specific cytotoxicity of the compounds but rather to a suppression of endogenous IL-2 production. Therefore, the isolated 9-deoxo-31-*O*-demethylFK506 exerts immunosuppressive activity *in vitro*, presumably through the same mechanism as FK506 but with a 70-fold reduced potency. Further studies will be needed to determine whether this reduction in biological activity reflects a lower affinity of the compound for the FK506-binding protein or for calcineurin[3], or both.

Fig. 5. Effects of 9-deoxo-31-*O*-demethylFK506 (squares), 31-*O*-demethylFK506 (triangle) and FK506 (circle) on the *in vitro* proliferation of mouse T cells stimulated with ionomycin (250 ng/ml) + PMA (10 ng/ml) in the absence (closed symbols) or in the presence of exogenous IL-2 (50 U/ml) (open symbols).



The proliferation was measured by ^{3}H-TdR uptake after 2 days of culture and expressed as percent of the response of control wells treated with the stimulating agents only. The IC_{50} values for inhibition of proliferation are indicated on the figure.

Antifungal Activity

The 31-*O*-demethylFK506 isolated from the recombinant strain M23 showed antifungal activity against *Aspergillus niger* strain ATCC6275 identical to that of authentic 31-*O*-demethylFK506 with a MIC of 1 ng. The level of activity of 9-deoxo-31-*O*-demethylFK506 was about one-fifth of FK506. Both isolated compounds were inactive against an FK506 resistant *Aspergillus niger* MF5961.

Conclusions

The targeted gene disruption technology as described here, which is amenable to large scale production, allowed for the production of FK506 analogs that would be difficult to obtain through classical medicinal chemistry. These analogs, 31-*O*-demethylFK506 and 9-*O*-deoxo-31-*O*-demethylFK506, exhibit reduced but significant immunosuppressive activity. Additionally, because both compounds contain a free hydroxyl group at C-31 position, they may also serve as starting materials for further chemical modifications aimed at increasing the immunosuppressive potency and perhaps imparting an improved therapeutic index compared to the parent

FK506 molecule.

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