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Isolation and identification of a FK-506 C_{36}–C_{37} dihydrodiol from erythromycin-induced rabbit liver microsomes

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Abstract: A new metabolite of FK-506 extracted from erythromycin-induced rabbit liver microsomes, isolated by normal- and reversed-phase HPLC chromatography, identified by FAB mass spectrometry and NMR spectroscopy is described.

Keywords: *FK-506; dihydrodiol metabolite; MS; HPLC.*

Introduction

FK-506 is a neutral macrolide of known structure ($C_{44}H_{69}NO_{12}$) isolated from the fermentation broth of a strain of *Streptomyces tsukubaensis* [1]. Structural identification was determined chemically [2, 3] and by X-ray crystallography [4]. This newly developed macrolide immunosuppressive drug includes a hemiketal function and an α–β diketonamide group as a result of the presence of at least two tautomeric forms as illustrated in Fig. 1.

Pharmacological and immunologic studies have shown that FK-506 has strong immunosuppressive properties against mixed lymphocyte reactions [5] and its activity has been reported to be 100 times more potent than that of cyclosporin A (CsA).

Since metabolites of FK-506 may still have immunosuppressive properties which may be less or more pronounced than the parent compound and also be the cause of certain adverse effects such as nephrotoxicity, it is essential that the metabolic profile of the drug is determined [6, 7].

FK-506 is metabolized by the liver and intestinal cytochrome P-450 enzymic system to yield several metabolites including O-demethylated, hydroxylated and O-demethylated hydroxylated compounds [8, 9]. The present communication describes the isolation and identification by FAB mass spectrometry and

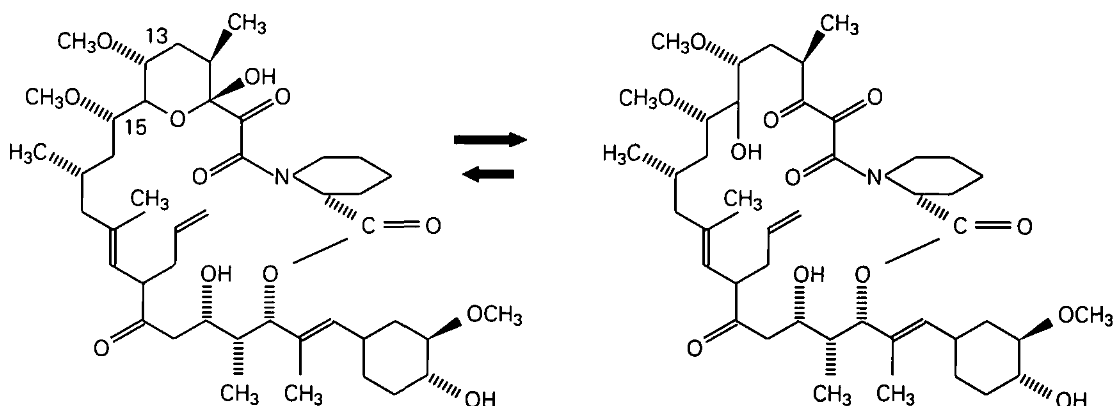

Figure 1
Tautomeric forms of FK-506.

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NMR spectroscopy of a FK-506 dihydrodiol extracted from erythromycin-induced rabbit liver microsomes.

Experimental

Chemicals and reagents

FK-506 was kindly supplied by Fujisawa Pharmaceuticals (Japan). Spectrograde solvents such as methanol, acetonitrile, dichloromethane used in the extraction and analytical procedures were purchased from Labscan Limited Unit T26 (Dublin, Ireland) and hexane from Alltech, Applied Science Labs (Deerfield, IL). NADH, NADP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase (5 mg ml^{-1}) were purchased from Boehringer (Mannheim, Germany). Analytical grade 0.2 M tris(hydroxymethyl)-aminomethane hydrochloride and 1.0 M NaOH used to prepare buffered solutions were purchased from Merck (Darmstadt, Germany). The matrix used in FAB-MS, 3-nitrobenzyl alcohol was obtained from Aldrich-Chemie (Steimheim, Germany) as well as benzo(a)pyrene-7(8H)one. Demineralized and filtered water (Milli-Q water purification system) was used.

Animals

Male New Zealand white rabbits (≈2.4 kg) were maintained in individual cages and were given free access to commercial food pellets (UAR, France) and water. Induced rabbits received 1.5 μmol kg^{-1} day^{-1} erythromycin in drinking water for 5 days. Non-induced rabbits produce lower amounts of metabolites.

Preparation of rabbit liver microsomes

After intramuscular injection of 0.75 ml kg^{-1} Hypnorm, the livers were removed, weighed, washed with ice-cold homogenizing 3 mM imidazole buffer containing 0.5 M sucrose, blotted with filter paper and minced with scissors. The minced liver was treated and fractionated according to the method described by Amar-Costesec *et al.* [10] to produce a microsomal fraction diluted five times (P/5) in the incubation medium, the characteristics are given in Table 1. Protein and cytochrome P-450 concentration were determined [11, 12] according to published standard procedures. 3-Hydroxy-benzo(a)pyrene hydroxylase and ethoxycoumarin deethylase were assayed by high-performance liquid chromatography using fluorimetric detection [13].

FK-506 microsomal incubation medium and extraction of metabolites

The NADPH-generating medium (1 ml) containing 0.88 mg NADPH, 2.54 mg NADP, 0.2 ml $MgCl_2$ (0.5 M), 15 mg glucose-6-phosphate and 0.6 ml Tris pH 7.4 was pre-incubated in a Gallenkamp shaking incubator for 15 min at 37°C in small Erlenmeyer flasks. To this solution was added 2.5 ml of erythromycin-induced rabbit liver microsomes, 6 μl glucose-6-phosphate dehydrogenase and 20 μg FK-506 dissolved in ethanol (20 μl).

This mixture was incubated for 1 h at 37°C. After the elapsed time it was transferred to a centrifuge tube and 7 ml dichloromethane added. After 2 min mixing (vortex mixer), the tube was centrifuged for 10 min at 6000 rpm. The aqueous phase was discarded, the dichloromethane was evaporated to dryness, the residue was dissolved in 1 ml of acetonitrile–water (3:7, v/v) and the resulting solution was subsequently washed with 1.5 ml hexane (2 min vortex mixing) which was discarded after centrifugation for 5 min at 3000 rpm. The acetonitrile–water phase was extracted again with 2 ml of dichloromethane. After 2 min mixing (vortex mixer) and centrifugation for 10 min at 6000 rpm, the water phase was discarded and the dichloromethane layer was evaporated to dryness under a stream of nitrogen. The residue was dissolved in 50 μl isopropanol and the resulting solution analysed by HPLC.

Table 1
Characterization of pooled erythromycin-induced rabbit liver microsomes

Assays	Unit	Erythromycin-induced rabbit liver microsomes
Protein conc.	mg g^{-1} liver	12.4
Cytochrome P-450	nmol mg^{-1} prot.	0.60
Aryl hydrocarbon hydroxylase	nmol min^{-1} mg^{-1} prot.	2.69
Ethoxycoumarin deethylase	nmol min^{-1} mg^{-1} prot.	7.73

High-performance liquid chromatography

The HPLC system consisted of an isocratic pump (Waters model 6000 A), a variable-wavelength Pye Unicam LC–UV detector connected to a HP 3392 A integrator. FK-506 metabolites were first separated on a Biorad Rosil CN column (3 μ particles, 150 mm × 4.6 mm i.d.) using hexane–isopropanol (73:27, v/v) as the mobile phase. The flow rate was adjusted to 3 ml min^{-1} and the UV detector was set at 210 nm. Under those conditions two groups of metabolite peaks were observed at retention times of 18–20 min (group 1) and 20–30 min (group 2) respectively. The column effluent corresponding to group 2 was collected and subsequently rechromatographed on a Macherey Nagel C_8 Nucleosil column (5 μ particles, 250 mm × 4 mm i.d.) maintained at 60°C by a column heater module coupled to a temperature control module (TCM, Waters-Millipore, Milford, MA). The mobile phase was acetonitrile–water (50:50, v/v), the flow rate and UV detector settings were, 0.6 ml min^{-1} and 210 nm, respectively. Under these conditions the effluent corresponding to a metabolite peak observed at 10.5 min was collected and subsequently rechromatographed on the same column at 60°C acetonitrile–water (55:45, v/v) as mobile phase at a flow rate of 0.5 ml min^{-1}.

The effluent corresponding to the metabolite peak with a retention time of 9.8 min was collected.

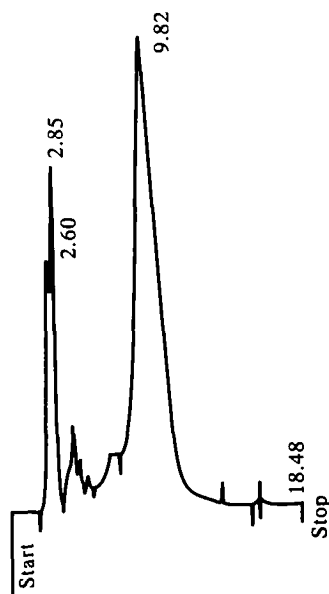

Figure 2
Reversed-phase chromatogram of collected peak RT9.

FAB-MS

FAB mass spectra were obtained by means of a Kratos MS80 RFA instrument. Samples (1–20 μg) in diethyl ether solution were evaporated on a copper probe tip (standard Kratos FAB probe) before adding a standard amount of a 3-nitrobenzyl alcohol matrix. Xenon gas was used in the Kratos FAB source with a primary energy of approximately 7.8 kV.

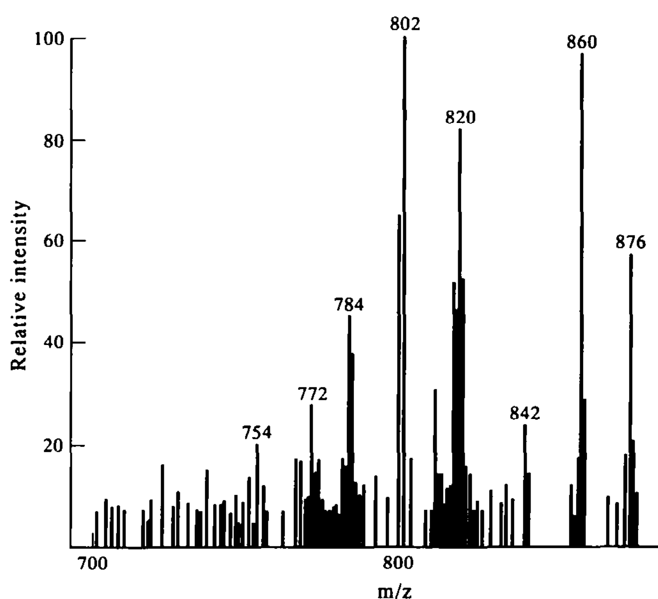

Figure 3
FAB mass spectrum of metabolite RT9.

NMR

^{1}H-NMR spectra were measured on a Bruker AM500 spectrometer. Samples (500–700 μg) were dissolved in 0.4 ml of $CDCl_3$ under nitrogen and spectra measured at 300 K. Each spectrum was acquired as 8 K data points, multiplied by a Gaussian window (LB = −3, GB = 0.3), zero-filled to 32 K, Fourier transformed, phase and baseline corrected with a fourth order polynomial. With a recycle time of 2 s, 4096 scans were accumulated.

Results and Discussion

The reversed-phase chromatogram of the collected peak (RT9) obtained after incubation of FK-506 with erythromycin-induced rabbit liver microsomes (extraction from the microsomal medium and preliminary separations as described earlier) is shown in Fig. 2. The peak collected at a retention time of 9 min was labelled as metabolite RT9.

The FAB mass spectrum (Fig. 3) of metabolite RT9 reveals the presence of quasi-

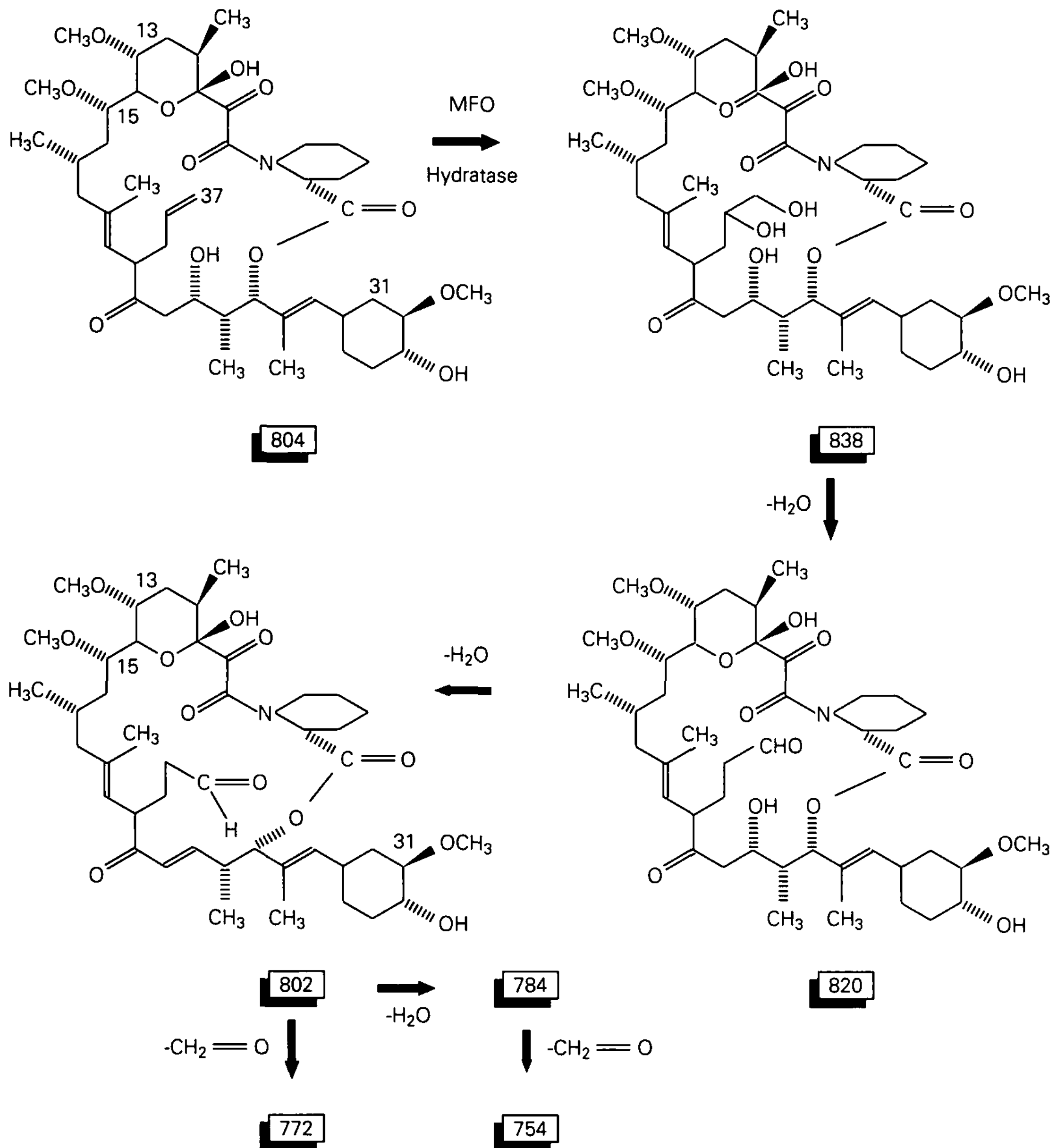

Figure 4
Fragmentation pathway of metabolite RT9.

molecular ions of mass m/z = 876 $(M + K)^+$, 860 $(M + Na)^+$ and 838 $(M + H)^+$, as well as fragmentation ions of mass m/z = 820 $(838\text{-}H_2O)^+$, 842 $(820 + Na)^+$, 802 $(838\text{-}2H_2O)^+$, 784 $(802\text{-}H_2O)^+$, 772 $(802\text{-}CH_2{=}O)^+$, 754 $(784\text{-}CH_2{=}O)^+$. The synthetic C_{36}–C_{37} dihydrodiol [16] chromatographed using acetonitrile–water (50:50, v/v) as eluent was found to have a retention time of 10.8 min and it produced, without rechromatography, the same FAB mass spectrum as metabolite RT9, proving that no coeluting impurity resulted from the extraction of the microsomal medium was present. The existence of tautomeric forms of the FK-506 dihydrodiol will be detailed in a future publication [14] and the presence of certain uneven fragments in the FAB mass spectrum of the FK-506 dihydrodiol resulting

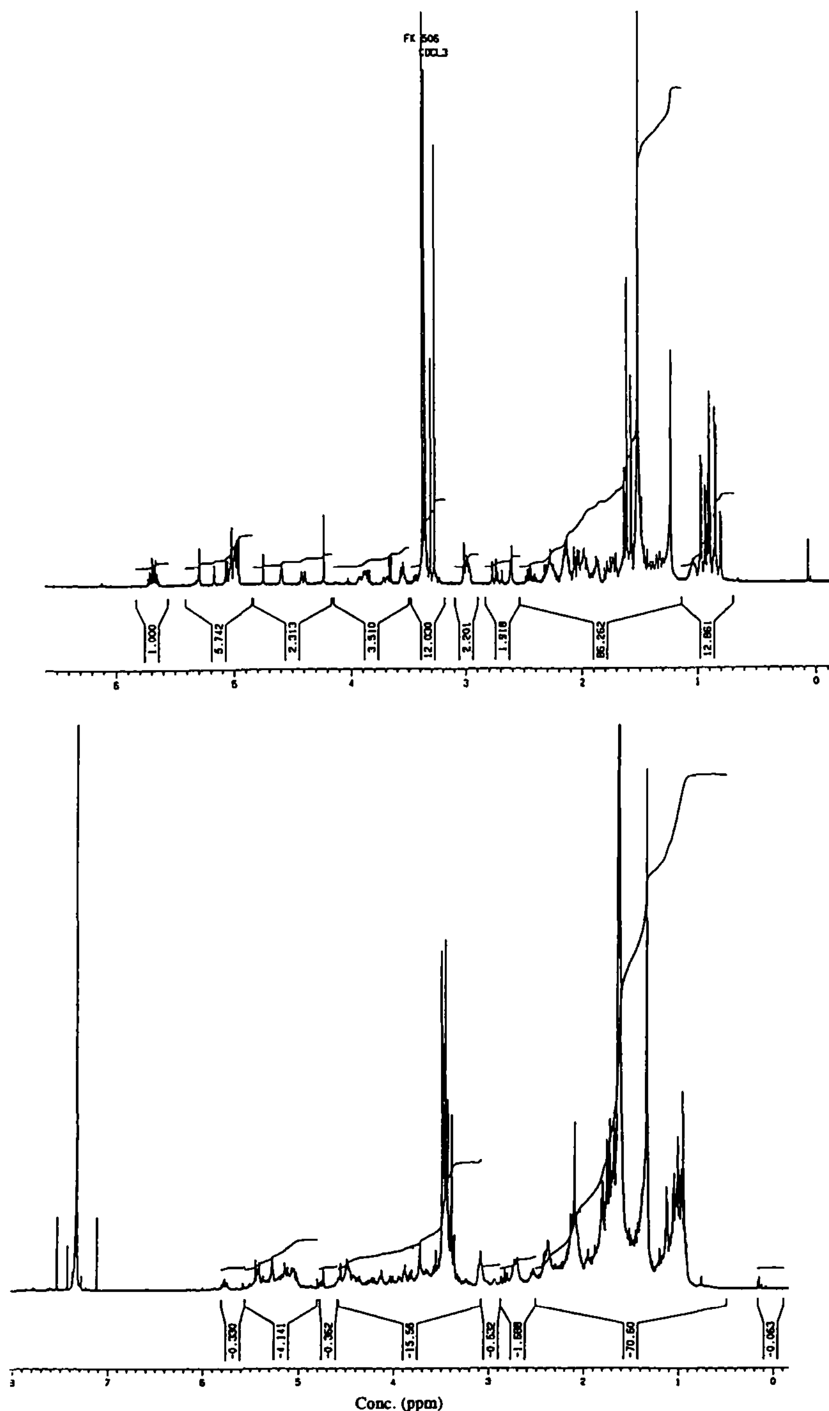

Figure 5
NMR spectrum of FK-506 (top) and metabolite RT9.

from the loss of pipecolic acid (129 amu) [14] is the direct consequence of the existence in the 3-nitrobenzyl alcohol matrix of interactions occurring between the C_{37} hydroxy group and the amide function and producing a carbinolamine intermediate. FAB mass spectrum demonstrates the presence of a dihydrodiol of FK-506 as illustrated in the fragmentation pathway shown in Fig. 4. These results are supported by NMR data. Comparison of the NMR spectra (500 MHz, $CDCl_3$) of FK-506 metabolite RT9 and of FK-506 (Fig. 5) reveals the presence of three unmetabolized methoxy groups as confirmed by the presence of quasi-molecular ions of mass m/z = 876, 860 and 838 (small) in the FAB mass spectrum.

The NMR signals of the six protons belonging to the vinylic double bonds (5H) and to proton H_{26} (1H) and chemically shifts in-between 4.99 and 5.71 ppm for FK-506 as reported by Hane *et al.* [15] are considerably modified for FK-506 metabolite RT9. The number of protons found in this region for metabolite RT9 was reduced to three due to the oxidation of the C_{36}–C_{37} double bond by the mixed function oxygenase enzymic system.

The NMR signal observed at 1.28 ppm for FK-506 and attributed to three OH groups (3H) is more intense for metabolite RT9 (1.32 ppm, 5H) and is the consequence of the addition of two vicinal OH groups. The signal at 1.65 ppm is mainly due to residual water in $CDCl_3$.

These results demonstrated that FK-506 under the influence of cytochrome P-450 dependent monooxygenases and the hydrase enzymic activity is metabolized to a transient epoxide and finally to a dihydrodiol, as illustrated in Fig. 6.

Conclusions

FK-506 metabolite RT9 results from C_{36} double bond epoxidation and subsequent hydration of the epoxide by water and/or by the hydrase enzymic system to produce a C_{36}–

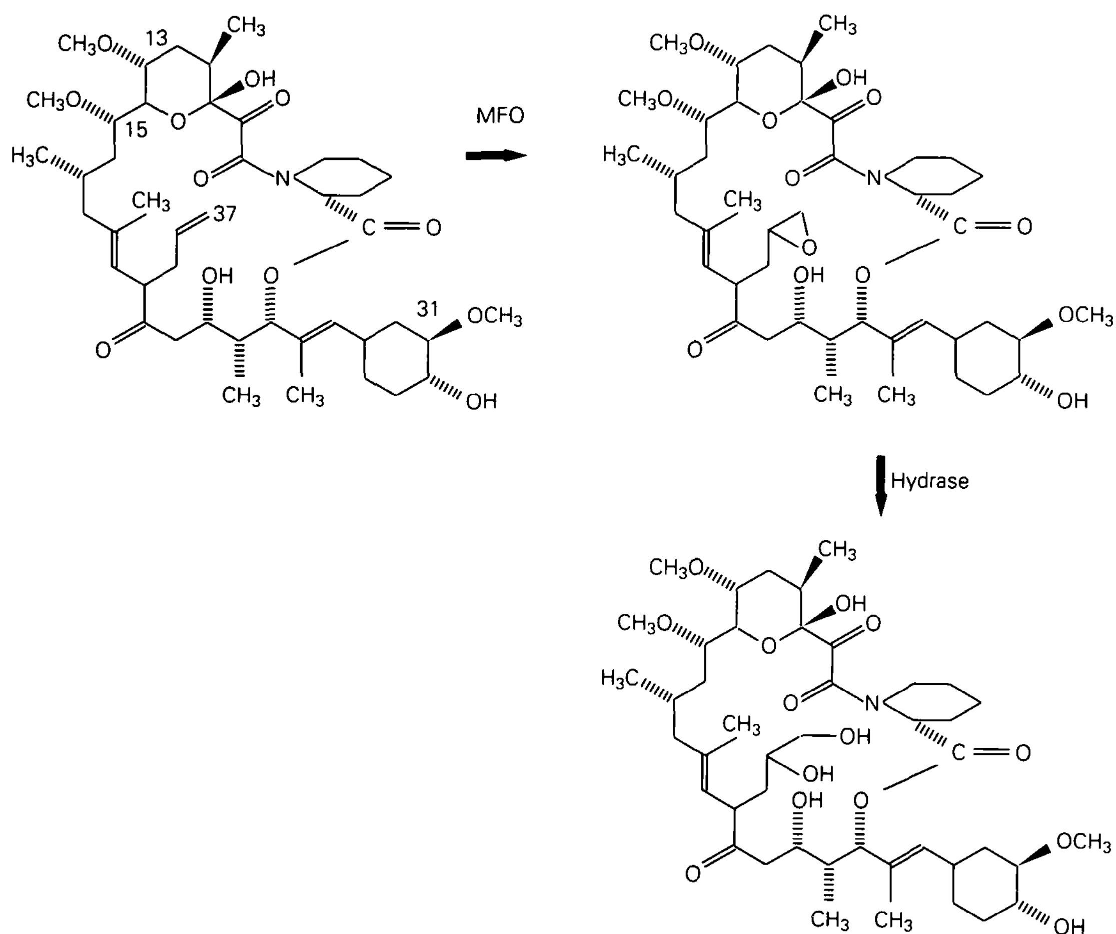

Figure 6
Metabolic pathway of FK-506.

C_{37} dihydrodiol of FK-506. The presence, in the NMR spectrum of the FK-506 dihydrodiol, of a signal (2.1 ppm), not present in the NMR of FK-506, attributed to an OH or NH group is most probably the consequence of the existence for this metabolite as tautomeric forms in $CDCl_3$ resulting from the interaction of the diol with the amide and/or lactone group of FK-506 producing either a carbinolamin or a hemiketal function. Such addition of an OH group to the lactone function of FK-506 may be related to the formation of the so-called iso-FK-506 [16] in the fermentation broth of *Streptomyces tsukubaensis*.

Moreover it may be argued that tautomers of FK-506 and FK-506 metabolites in equilibrium *in vivo* may produce an enhanced or decreased immunosuppressive response depending most probably of the tautomer-receptor specific interactions.

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