MICROBIAL TRANSFORMATION OF IMMUNOSUPPRESSIVE COMPOUNDS

I. DESMETHYLATION OF FK506 AND IMMUNOMYCIN (FR 900520) BY *Actinoplanes* sp. ATCC 53771

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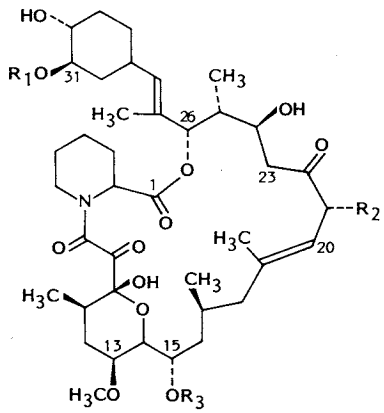
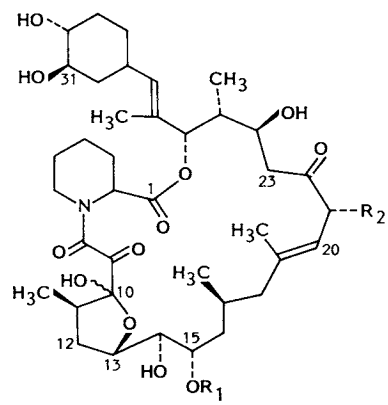
The immunosuppressants FK506 and FR 900520 were desmethylated by *Actinoplanes* sp. ATCC 53771 to yield various *O*-desmethylated products. The products were isolated and purified by solvent extraction and HPLC chromatography, and identified by NMR and MS spectroscopy.

In 1987, Fujisawa Pharmaceutical Company reported the identification of novel natural product immunosuppressants, FK506 and FR 900520[1~5]. It has been established that FR 900520 (immunomycin) was actually ascomycin[6], an old antifungal compound discovered by Bristol-Meyers & Co. in 1960. FK506 and FR 900520 are members of a new class of 23-membered polymethoxylated macrolides. They have attracted considerable interest as immunosuppressive agents due to their potential therapeutic usefulness[3]. They inhibit T cell activation both *in vivo* and *in vitro* and are reputed to be 50~100 times more effective than cyclosporin[1]. Microbial cutlures were examined for their ability to modify the compounds. Our goal was to better understand which structural and conformational aspects of the parent structures are critical for biological activity. The present communication deals with the microbial desmethylation of FK506/FR 900520 by *Actinoplanes* sp. ATCC 53771.

Fig. 1. The structure of FK506/FR 900520 and desmethyl derivatives by *Actinoplanes* sp. ATCC 53771.





FK506	$R_1=CH_3$	$R_2=CH_2CH=CH_2$	$R_3=CH_3$
FR 900520	$R_1=CH_3$	$R_2=CH_2CH_3$	$R_3=CH_3$
L-682,993	$R_1=H$	$R_2=CH_2CH=CH_2$	$R_3=CH_3$
L-683,742	$R_1=H$	$R_2=CH_2CH_3$	$R_3=CH_3$
L-686,292	$R_1=H$	$R_2=CH_2CH_3$	$R_3=H$

L-692,992	$R_1=CH_3$	$R_2=CH_2CH=CH_2$
L-683,756	$R_1=CH_3$	$R_2=CH_2CH_3$
L-687,795	$R_1=H$	$R_2=CH_2CH_3$

Materials and Methods

^1H NMR spectra were recorded in $CDCl_3$ on a Varian XL 400 NMR spectrometer at ambient temperature at 400 MHz using the solvent peak at 7.26 ppm as internal references downfield of TMS at 0 ppm. MS measurements were obtained on a MAT 731 instrument.

Microbiological

More than one thousand microorganisms were screened for their ability to achieve useful biotransformation reactions to modify FK506 and FR 900520. A two stage fermentation procedure was used. Frozen vegetative mycelium was used to inoculate a 250-ml baffled flask containing 50 ml seed medium. The seed flasks were incubated on a rotary shaker (220 rpm) at 27°C for 1~2 days. A 1 ml aliquot of the seed medium was used to inoculate a 50 ml non-baffled shake flask containing 10 ml of transformation medium. FK506/FR 900520 was dissolved in DMSO and added to the fermentation at 0 hour. The shake flasks were subsequently incubated for 1~4 days. Following incubation, 0.5 ml acetonitrile was added to 0.5 ml whole broth and vortexed for 1 minute. The resulting solution was centrifuged and subjected to HPLC analysis for biotransformation products as described below.

Production of Metabolites

Culture *Actinoplanes* sp. ATCC 53771 was stored and maintained on skim milk in the culture collection of Merck & Co., Inc. as MA 6559. The lyophilized culture was used to inoculate a 250-ml baffled shake flask containing 50 ml of an autoclaved seed medium consisting of (in g/liter) dextrin 10.0, glucose 1.0, beef extract 3.0, Ardamine PH (Yeast Products, Inc.) 5.0, N-Z Amine type E 5.0, $MgSO_4 \cdot 7H_2O$ 0.05, KH_2PO_4 0.37, and $CaCO_3$ 0.5. The pH of the seed medium was adjusted to 7.1 before autoclaving. The lyophilized seed was incubated in the seed medium at 27°C for 48 hours on a rotary shaker operating at 220 rpm. Alternatively, when frozen vegetative mycelia or a slant source is used, the culture is incubated in the seed medium at 27°C for 24 hours at 220 rpm. A 2.5 ml aliquot of the resulting seed medium was used to inoculate a 250-ml non-baffled Erlenmeyer shake flask containing 50 ml of production medium. Product medium consisted of (in g/liter) glucose 10.0; Hy-case SF 2.0; beef extract 1.0; corn steep liquor 3.0. pH was adjusted to 7.0 before autoclaving. Substrate (FK506 or FR 900520) was added as a solution in DMSO to achieve a final concentration of 0.1 mg/ml concentration. The shake flask contents were subsequently incubated for 24 hours at 27°C on a rotary shaker operating at 220 rpm.

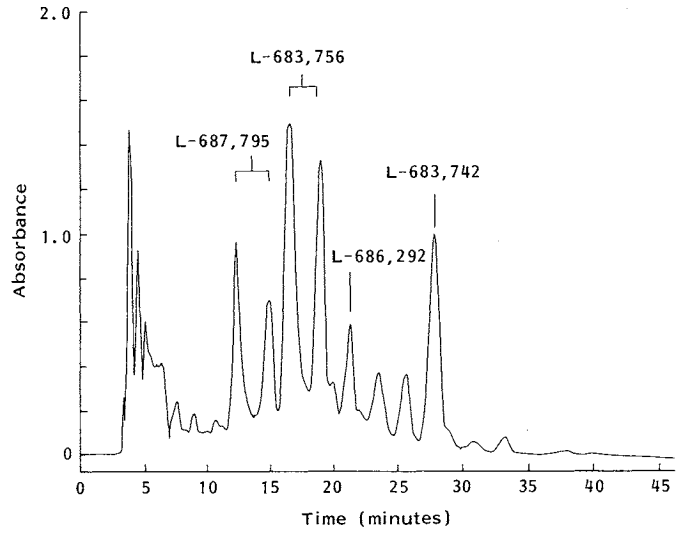
HPLC Analysis

The microbial transformation metabolites can be detected easily in whole broth using HPLC analysis. The T-cell proliferation assay provides a sensitive indicator of biological activity. For assay the whole broth was mixed with an equal volume of methanol. The mixture was centrifuged and the clear supernatant was analyzed by HPLC. Samples (50 μl) were loaded onto a Whatman Partisil 10 ODS-3, 4.6 mm × 25 cm column maintained at 55°C and eluted with a gradient at 1 ml/minute. The gradient was obtained by mixing a 0.1% aqueous phosphoric acid solution and acetonitrile. The initial ratio 55:45 was increased to 20:80 in 30 minutes. Products and substrates (FK506 or FR 900520) were detected by measuring the absorbance at 205 nm.

Assessment of Immunosuppressive Activity

The immunosuppressive activities of the isolated desmethyl derivatives were determined in an *in vitro* T cell proliferation assay described previously[7]. Briefly, nylon wool purified splenic T cells were prepared from C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME). Microcultures were performed in flat-bottom 96-well plates containing 5×10^4 T cells per well. Proliferation was induced by the addition of 250 ng/ml ionomycin and 10 ng/ml phorbol PMA (phorbol 12-myristate 13-acetate). Cell division was assessed by the addition of 2 μCi/well of tritiated thymidine for the last 4 hours of a 48 culture period. Compounds were added at the initiation of culture. The IC_{50} for inhibition by each compound was calculated using a 4-parameter algorithm.

Fig. 2. HPLC of the methylene chloride extracts of the whole broth 24 hours after addition of FR 900520.



Isolation and Purification of Desmethylated FR 900520

The whole broth (100 ml) was extracted three times with methylene chloride (3 × 100 ml). Methylene chloride extracts were combined, dried over sodium sulfate, and concentrated under vacuum to an oily residue. The residue was dissolved in acetonitrile and subjected to HPLC. The preparative HPLC was carried out on Whatman Partisil 10 ODS-3, 9.4 mm × 25 cm at 60°C and monitored at 205 nm and 225 nm. The column was developed at 3 ml/minute with linear gradient from 0.1% aqueous H_3PO_4-CH_3CN (55:45) to 0.1% aqueous H_3PO_4-CH_3CN (20:80) in 40 minutes. The compounds were collected during repeated injections of the above described extract. The fractions (Fig. 2) at Rt, 12.3 (L-687,795), 16.6 (L-683,756), 21.4 (L-686,292), 28.0 (L-683,742) minutes, were pooled, respectively, adjusted to pH 6.5 and evaporated to remove acetonitrile. The compounds were further purified using a C-18 Sep-Pak (Waters Associates) and acetonitrile-water elution solvent to yield 4 mg of 31-desmethyl, 6 mg of 13,31-bisdesmethyl, 3.6 mg of 13,15,31-tridesmethyl, and 1 mg of 15,31-bisdesmethyl FR 900520.

Isolation and Purification of Desmethylated FK506

The whole broth obtained from 2 flasks was processed as described above yielding 2.5 mg of 31-desmethyl and 4 mg 13,31-bisdesmethyl FK506.

Results and Discussion

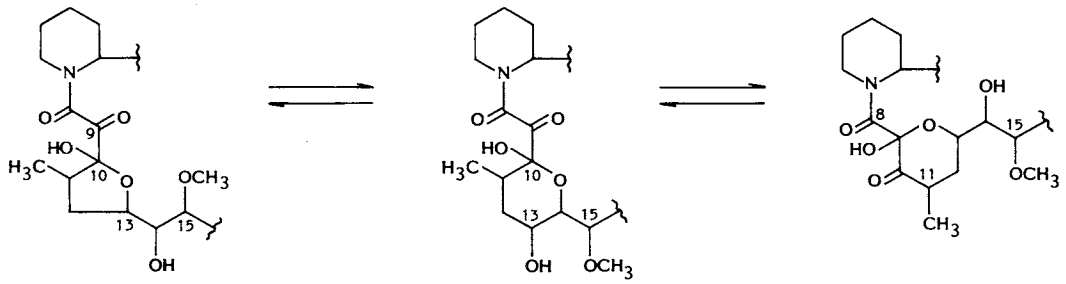
Biotransformation

Of the active cultures obtained from our biotransformation screen *Actinoplanes* sp. ATCC 53771 was selected for subsequent experiments. FR 900520 added was converted to 31-desmethyl, 13,31-bisdesmethyl, 15,31-bisdesmethyl, and 13,15,31-tridesmethyl derivatives in yields of 20, 30, 5 and 18%, respectively, after 24 hours of incubation. FK506 was transformed to 13,15,31-tridesmethyl and 15,31-bisdesmethyl derivatives in yields $<2\%$.

Structure Determination

The structure determination of the desmethyl derivatives was based on virtually complete analysis of 400 MHz ^1H NMR. The proton resonances were assigned by using a combination of NMR techniques including COSY, NOESY, decoupling difference, NOE difference, proton-proton decoupling, solvent

Fig. 3. The possible isomers after C-13 methoxyl group is desmethylated.



effects, and temperature effects.

31-Desmethyl FK506 (L-682,993) and 31-Desmethyl FR 900520 (L-683,742)

The key ^1H NMR observation was the loss of a methoxy signal coupled with the absence of the 31-H resonance at its characteristic chemical shift near 3.1 ppm. Since a CH with an attached OH is generally 0.1~0.3 ppm downfield relative to a CH-OCH_3 it was reasonable to conclude that the 31 methyl was lost and that 31-H was displaced into the 3.3~3.5 ppm multiple containing 13-H, 21-H, 33-H and the remaining methoxyls. Further support for assigning the missing methoxyl to C-31 rather than to C-13 or C-15 was the fact that the chemical shifts of 14-H and 15-H were identical to those in the parent molecule. It was also reasonable to infer that the C-31 desmethylation was the sole structural change since no new resonances were seen and all recognizable proton signals from 2-H to 28-H were virtually superimposed with those in parent compounds. The FAB mass spectra were consistent with the proposed structure in revealing an M + Li of 796 and 784 which correspond to a loss of 14 mass units from FK506 and FR 900520, respectively.

13,31-Bisdesmethyl FK506 (L-692,992) and 13,31-Bisdesmethyl FR 900520 (L-683,756)

The desmethylation of C-13 methoxyl group can result in isomers that differ in the point of formation of hemiketal as shown in Fig. 3. The HPLC chromatographic study shows that there are two constituents existing in equilibrium as shown in Fig. 2. The FAB mass spectra gave an M + Li of 782 and 770 which correspond to a loss of 28 mass units from FK506 and FR 900520, respectively, indicating that they are bisdesmethylated derivatives. The NMR spectra show many major differences from parent compounds. Select proton chemical shifts in FK506 and L-692,992 are shown in Table 1. Appreciable downfield displacement of 2-H, 11-H, 12-H and 13-H and upfield displacement of 15-H can be attributed to the formation of the new ring which causes the perturbed protons to be closer to or farther from a nearby nucleus. The five membered ring structure is strongly favored as the major component primarily because of the large vicinal coupling constants between 11-H and the 12 methylene protons (10.8 and 8.7 Hz). Two large vicinal coupling constants are most unusual, do not occur to our knowledge in six membered rings or in flexible open chain sequences but are seen in appropriately substituted five membered ring systems containing oxygen.

13,15,31-Tridesmethyl FR 900520 (L-687,795)

A tridesmethylated FR 900520 was clearly indicated by the absence of a methoxy signal and the five membered ring rearranged structure was inferred from the close similarity with the distinctive features in 13,31-bisdesmethyl derivatives. These include the downfield displacement of 2-H, 11-H, 12-H and 13-H relative to FR 900520 and the increase in the 23-H geminal coupling constant from 16 Hz in FR

Table 1. Select proton chemical shifts in FK506 and L-683,756.

H	FK506 (ppm)	L-683,756 (ppm)	$\Delta\delta$ (ppm)
2	4.62	5.18	0.56
6	4.43	4.48	0.05
11-CH_3	1.00	1.04	0.04
11	2.08	2.92	0.91
12	1.75,	2.45	0.70,
	1.4~1.5	1.80	0.3~0.4
13	3.40	4.42	1.02
14	3.69	3.82	0.13
15	3.58	3.23	−0.35

Table 2. Select chemical shift comparison; L-687,795 and L-683,756.

H	L-687,795 (ppm)	L-683,756 (ppm)
2	5.20	5.20
11	2.95	2.95
12	1.80, 2.35	1.85, 2.45
13	4.40	4.40
23	2.65, 2.58	2.70, 2.63

Table 3. Inhibition of T-cell proliferation by desmethylated FK506 and FK520.

Desmethylated compounds	IC_{50} (nM)
FK506	0.4
FK520	0.8
31-Desmethyl FK506 (L-682,993)	0.9
31-Desmethyl FK520 (L-683,742)	1.7
13,31-Bisdesmethyl FK506 (L-692,992)	37.0
13,31-Bisdesmethyl FK520 (L-683,756)	50.0
15,31-Bisdesmethyl FK520 (L-686,292)	>1,000
13,15,31-Tridesmethyl FK520 (L-687,795)	>1,000

900520 to 18 Hz. A comparison of the pertinent chemical shifts in L-683,756 and L-687,795 is given in Table 2. A conformational point of interest: a change in the dihedral angle between 26-H and 25-H is indicated by an increase in the coupling constant from 2.5 Hz in FR 900520 to 7 Hz in L-687,795. It is unlikely that this change is consequent to chemistry occurring in the "upper" region of the molecule since 23-H, 24-H, 28-H and 29-H are all within 0.1 ppm of their chemical shifts in FR 900520. The FAB mass spectrum of the sample exhibits a M + Li of 756 corresponding to 42 mass units loss from FR 900520.

15,31-Bisdesmethyl FR 900520 (L-686,292)

A bisdesmethylated structure was immediately evident from the ^1H NMR spectrum which showed only one isomeric pair of methoxy peaks in about a 4/3 ratio. Unlike several previous compounds, no rearrangement of the tertrahydropyran to the tetrahydrofuran has occurred since the spectrum showed none of the features that characterize the five membered ring structures. Desmethylation at C-15 rather than C-13 is strongly preferred since 15-H is displaced 0.37 ppm downfield relative to its chemical shift in FR 900520 while 13-H is unchanged. The EI spectrum of the sample gave a molecular ion (*m/z* 763) 28 mass units less than that of FR 900520. The fragment representative of a cleavage at O-C_1 and C_{25}-C_{24} (*m/z* 550) was 14 mass units lower, indicating a loss of CH_3 at C_{31} and eithr C_{13} or C_{15}.

Biological Activity

The T-cell inhibition data of our desmethylated FK506 and FR 900529 are summarized in Table 3. 13,31-Bisdesmethyl analogs retained modest activity, however, tridesmethyl and 15,31-bisdesmethyl compounds were inactive up to 10 μM. In contrast, the activity of 31-desmethyl analogs did not decrease significantly.

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