ISOLATION AND CHARACTERIZATION OF THE MAJOR EQUILIBRIUM PRODUCT OF FK-520

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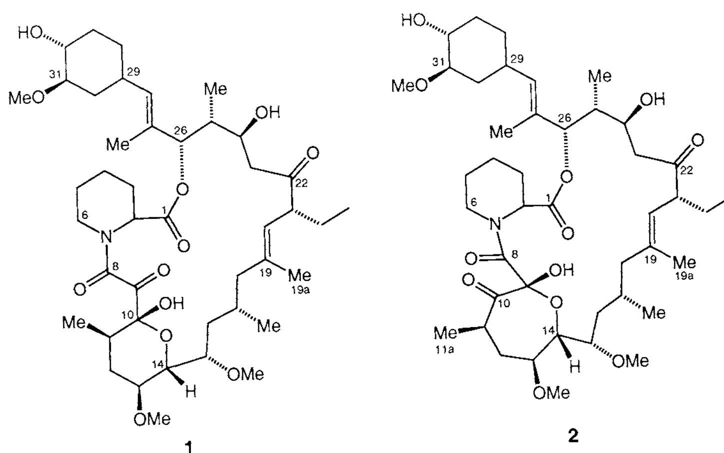
The existence of an isomeric form in equilibrium with the major component of FK-520 in polar solutions has been demonstrated. This minor component has been isolated in high yield and purity by a novel crystallization strategy and preparative HPLC. The equilibrium product was characterized by NMR and MS.

FK-520 (**1**), a macrolide produced by the microorganism *Streptomyces hygroscopicus* var. *ascomyceticus*, is the C-21 ethyl analog of the potent immunosuppressant FK-506[1], which bears an allyl group at C-21. The potency of FK-506 *in vitro*[2] and its use in human organ transplantation[3] have been demonstrated, and interest in FK-506 analogs has arisen from the search for compounds with enhanced efficacy. The structure **1** (Fig. 1) contains a large array of functionalities, most notably the reactive C-8~C-10 "tricarbonyl region" described by FISHER *et al.*[4].

The analysis of FK-520 and related compounds by HPLC provides many challenges in the accurate determination of the purity of the solid. One of the critical challenges was the identification of some minor components, the levels of which were dependent upon the assay used, and which were found to increase with time under certain conditions. It was essential for determination of the final product purity to eliminate the possibility that the unknown components were either degradates derived from FK-520 or artifacts of the assay.

Fig. 1. Structure of FK-520 (**1**), and the major equilibrium product (**2**).



This paper describes the recognition of a significant minor component as the major equilibrium product **2** (Fig. 1), and its isolation by a combination of crystallization and preparative HPLC. Structural characterization of **2** was achieved by NMR and MS.

Experimental

Isolation of the Major Equilibrium Product (**2**)

A methanolic solution of **1** (370 g/liter) was stored overnight at room temperature to form **2**. The solution then contained 24 g/liter of **2** and 330 g/liter of **1** (ratio 1 : 14) by analytical HPLC. From this step forward all operations, including inprocess assays, were expedited to minimize the back-conversion of **2** to **1**. An aliquot (100 ml) was diluted with MeOH (50 ml), and **1** was crystallized by the addition of water (150 ml) over 1 hour. The solids were removed by filtration, and the filtrate (243 ml) was injected within 5 minutes onto a Prochrom axial compression HPLC column. The filtrate contained 3.2 g/liter of **2** and 4.2 g/liter of **1** (ratio 3 : 4), as determined by analytical HPLC. The mobile phase for the preparative column was acetonitrile - aqueous 0.1% phosphoric acid (1 : 1 v/v) at flow rate of 900 ml/minute, and the column (3 kg of Zorbax Pro10, C8 phase, 10 μm particles; 15 × 21 cm) was held at 50 ~ 55°C. The eluate was monitored by UV absorbance at 210 nm. The fraction corresponding to **2** was collected (about 3,600 ml), and was cooled in an ice-water bath. It contained 0.14 g/liter of **2** and 0.013 g/liter of **1** (ratio 10 : 1) when assayed immediately after collection. The fraction was separated into organic and aqueous layers by the addition of dichloromethane (10% by vol). The organic extract was then washed with water to remove acetonitrile, and chilled in a methanol-dry ice bath prior to analytical studies.

Analytical Methods

Analytical HPLC was performed on a Zorbax RX-C8 column (250 × 4.6 mm), with a mobile phase of acetonitrile - aqueous 0.1% phosphoric acid (50 : 50 v/v initially, to 95 : 5 v/v over a 30-minute linear gradient), and a flow rate of 1.0 ml/minute. The column was held at 55°C, and UV detection was at 220 nm. HPLC/MS analysis was achieved using the HPLC conditions described above, except that phosphoric acid was omitted from the mobile phase. The UV detector was coupled to a Finnigan MAT TSQ 70B mass spectrometer *via* a TSP2 thermospray interface held at 72°C, with an ion source temperature of 250°C. Ionization was effected by applying 1,000 volts to the discharge electrode, the repeller was held at −120 volts, and negative ion spectra were acquired over the range *m/z* 750 ~ 850. LC/MS/MS spectra were generated by collision induced dissociation with argon at 1.5 mtorr, at a collision energy of 20 eV. Positive ion EI and methane CI mass spectra were recorded on a Finnigan 4500 mass spectrometer, using a fast-heating direct exposure probe which was ramped at 50 mA/second to about 750°C over 0.75 second; the source temperature was 140°C. For NMR studies, the organic extract was evaporated to dryness under a stream of nitrogen at room temperature, and the residue was dissolved in CD_3CN. ^1H spectra were observed at 400.13 MHz, and were referenced to CHD_2CN at 1.93 ppm. ^{13}C spectra (100.61 MHz) were referenced to CD_3CN at 1.3 ppm. Spectra were recorded on a Bruker AM-400 spectrometer.

Results and Discussion

A typical analytical HPLC/UV profile of FK-520 is shown in Fig. 2A. Analysis by thermospray ionization LC/MS gave *m/z* 777.7 for peak A (the known C-21 methyl analog), while peak B gave *m/z* 805.7, which corresponded to a homolog of FK-520 containing an extra methylene group. Both peak C and the major component of FK-520 gave *m/z* 791.7 as the molecular anion, which established that peak C is due to an isomer of FK-520. Chromatographic behavior and mass spectrometric evidence strongly suggest that the broad peak at Rt 12.5 ~ 13 minutes in Fig. 2A is also an equilibrium product[†] of FK-520.

† Isolation and characterization of this minor equilibrium product is currently underway in this laboratory.

Fig. 2. Analytical HPLC/UV profiles of FK-520 (A), and of isolated equilibrium product (B).

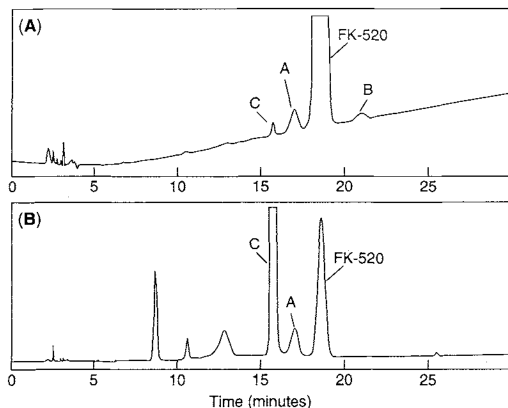


Fig. 4. Equilibration of 2 with FK-520 major component 1.

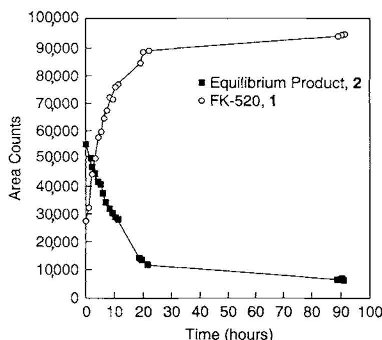
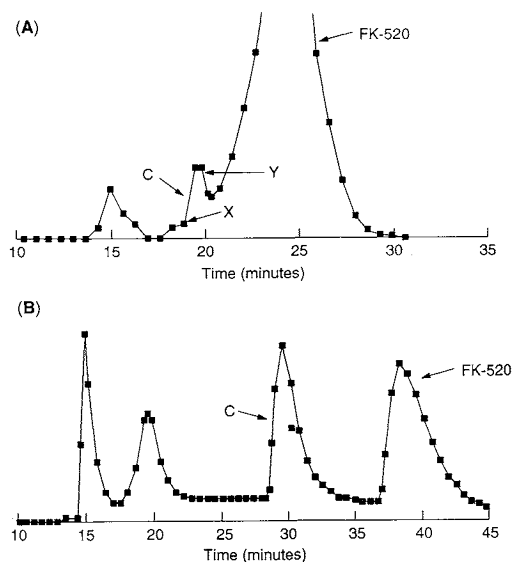


Fig. 3. Preparative HPLC/UV profiles of aged, methanolic solution of FK-520 (A), and of crystallization mother liquors of FK-520 (B).



An indication that peak C might be due to an equilibrium species was a report that FK-506 exists as tautomeric forms when dissolved in polar solvents such as alcohols[5]. This phenomenon is different from the well known *cis* and *trans* forms associated with restricted rotation around the N-7–C-8 amide bond[1,6]. Since the *cis* and *trans* forms of FK-506 are observed by HPLC only at lower temperatures[7], resolution at 55°C was not expected.

Direct proof of an equilibrium product was obtained using preparative chromatography to isolate a fraction enriched in the unknown peak. Collection of the front shoulder peak from point X to point Y in Fig. 3A produced a fraction containing a 6:4 ratio of the unknown component **2** to FK-520.

Conversion of the unknown back to FK-520 was monitored by HPLC, and resulted in the system reaching an equilibrium after approximately 20 hours, as shown in Fig. 4. It was subsequently determined that the level of **2** in solutions of FK-520 could be controlled by using specific solvents and vials in the HPLC analyses (unpublished data).

In order to analyze **2** by MS and NMR, the rate of conversion had to be decreased. Since interconversion was rapid in the presence of an alcohol or water, sufficient water-immiscible solvent (toluene or dichloromethane) to form a two-phase mixture was added to the eluant fraction. Both **2** and **1** partitioned almost completely into the organic layer.

The isolation of higher purity **2** in larger quantities was achieved by a novel approach combining a simple crystallization with a single, preparative-scale HPLC injection. Since slow crystallizations and long aging times allowed **2** to convert back to **1**, a fast crystallization was used to increase the ratio of **2** to **1** in the mother liquors 4-fold, from ~1:10 to 3:4. Comparison of the LC/UV profile of the column effluent

Fig. 5. MS/MS daughter ion spectrum of ion at m/z 791.7 from **2**.

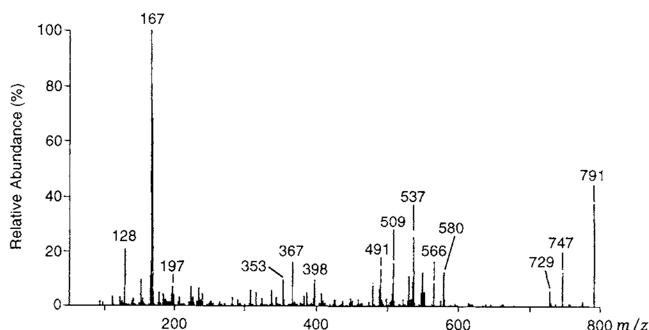
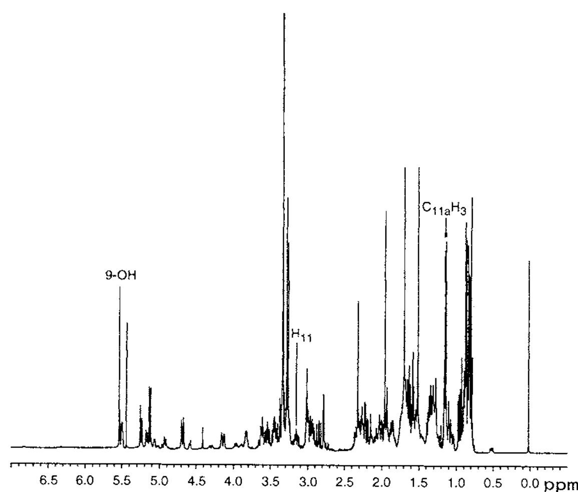


Fig. 6. ^1H NMR spectrum of **2** in CD_3CN.



of the crystallization mother liquors (Fig. 3B) with the profile obtained using an aged solution of FK-520 (Fig. 3A), shows that the injection of crystallization mother liquors, rather than a solution of FK-520, dramatically improved the resolution of the preparative-scale HPLC. This approach allowed the isolation of more than 500 mg of **2** with 90% purity from a single HPLC injection.

The organic fraction obtained from the rapid sequential crystallization and preparative HPLC method was analyzed by HPLC (Fig. 2B), and LC/MS to check that the correct product had been collected, and that the equilibrium product had not degraded. The negative ion mass spectrum of **2** was identical to that of **1**, and showed an intense molecular ion cluster centered at m/z 791.7, as the base peak; a low intensity peak at m/z 773.7 was ascribed to the elimination of water from the molecular anion. This, together with chromatographic retention data, confirmed the integrity of the collected peak. Because of the lack of fragmentation observed under thermospray ionization conditions, MS/MS spectra of the m/z 791.7 ion were generated from both **1** and **2**. The spectra were virtually indistinguishable (only the spectrum obtained from **2** is shown, Fig. 5), which indicated very similar structural characteristics of the two compounds. The EI and CI (CH_4) positive ion mass spectra of **2** were each also indistinguishable from those of **1** (data not shown).

NMR studies were performed on solutions in acetonitrile-d_3, and the ^1H NMR spectrum of 2 in this solvent is shown with some key assignments in Fig. 6.

Various 1D and 2D NMR experiments, including a heteronuclear multiple bond connectivity run[8] (HMBC), were carried out to determine the structure of the equilibrium product. It was clear in the ^{13}C spectrum that C-9 at about 197 ppm was absent, and a signal for a new carbonyl was observed at 211.4 ppm. It should be noted that the data presented (Table 1) are for the major rotamer, since the compound exists as a 3:1 mixture of amide rotamers (as is observed in most compounds related to FK-506). Key HMBC data include a correlation from 11a-H_3 to the new carbonyl at C-10 and correlations from the 9-OH to C-8, C-9, and C-10. Proton-coupled ^{13}C NMR and selective irradiation experiments established the presence of the seven-membered oxepane ring, with $^3J_{H\text{-}14,C\text{-}9}=5.6$ Hz. A magnetization transfer experiment with irradiation of the adventitious water showed the three expected -OH signals, including the 9-OH singlet at 5.52 ppm. NOE difference experiments confirmed the stereochemistry of the hydroxyl group at C-9, since NOE enhancements were observed from 14-H to 9-OH and from 9-OH to 14-H.

Table 1. ^{13}C chemical shifts of **1** and **2** in CD_3CN, $\delta_{CD_3}=1.3$ ppm.

	1	**2**		**1**	**2**
C-22	212.8	211.2	C-23	46.1	49.4
C-9	198.6	99.7	C-25	41.0	41.2
C-1	170.4	171.1	C-6	39.9	39.9
C-8	166.4	168.2	C-30	36.0	35.9
C-19	139.3	139.4	C-29	35.7	35.5
C-27	132.6	135.1	C-11	35.5	39.1
C-28	132.5	123.2	C-16	34.1	37.3
C-20	124.7	125.4	C-12	33.4	39.4
C-10	98.2	211.4	C-33	32.9	32.9
C-31	84.9	84.9	C-34	31.4	31.3
C-26	79.8	83.5	C-3	28.5	28.5
C-15	76.1	77.7	C-17	27.1	26.9
C-13	74.4	78.1	C-21a	25.5	24.9
C-32	74.2	74.1	C-5	25.1	25.5
C-14	73.8	78.9	C-4	21.9	21.5
C-24	70.4	69.8	C-17a	20.3	19.8
C-2	57.5	56.60	C-11a	16.5	16.9
$-OCH_3$	57.5	57.9	C-19a	16.1	16.8
$-OCH_3$	57.0	57.6	C-27a	13.6	12.3
$-OCH_3$	56.7	57.0	C-21b	12.0	11.8
C-21	55.8	56.58	C-25a	10.1	10.6
C-18	49.6	48.5			

Several reports have recently described the isolation and characterization of equilibrium products from macrolides which are structurally very similar to FK-520. An oxepane ring structure, based upon ^{13}C NMR and COSY ^1H NMR data, was also proposed for the isomer in equilibrium with rapamycin[9]. In contrast, two tautomeric forms of FK-506 were isolated and neither was characterized as containing an oxepane ring[5]. In one proposed structure, the tetrahydropyran ring was intact but the stereochemistry at C-10 had been inverted. In the other, the tetrahydropyran ring had opened to give the acyclic form with a free hydroxyl group at C-14, and C-10 as the carbonyl. A molecule of water was proposed to have added to the C-9 carbonyl to produce a geminal diol.

An HPLC assay of the NMR solution after two days at ambient temperature showed minimal conversion of **2** to **1**. After 11 days, the ratio of **2** to **1** was 60:40, and no significant effect on this ratio was observed two days after the addition of water. The addition of a trace amount of trifluoroacetic acid to the NMR solution increased the rate of conversion, with **1** predominating (60:40) after 20 hours. The ratio of **2** to **1** after six days was about 5:95, as determined by both NMR and HPLC analysis. Additional support for an equilibrium between **1** and **2** came from an NMR experiment in which crystalline **1** was dissolved in CD_3CN, and the solution was examined immediately. Initially, **2** was not detected. However, after standing at room temperature for four days the presence of **2** was observed.

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