TAUTOMERIC PHENOMENON OF A NOVEL POTENT IMMUNOSUPPRESSANT (FK506) IN SOLUTION

I. ISOLATION AND STRUCTURE DETERMINATION OF TAUTOMERIC COMPOUNDS

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Tautomeric phenomenon was observed in the HPLC chromatogram obtained from a novel potent immunosuppressant, FK506, in ethanol solution. Two tautomeric compounds derived from FK506 were isolated and purified by HPLC. Their structures were elucidated by spectral analyses. The mechanisms for this tautomerization were also established based on the results of structure analysis.

Cis (major component[1]) and *trans* conformational isomers of FK506 resulting from the restricted rotation of the amide bond in the pipecolic acid moiety (Fig. 1) have been known to exist in chloroform as the equilibrium mixture[1,2] which could be detected by HPLC under low temperature column condition[3]. However, this equilibrium is not detectable on the HPLC chromatogram obtained by increasing the column temperature in order to improve a peak shape, while FK506 in ethanol solution provided three peaks which suggest the existence of another kind of equilibrium phenomenon (Fig. 2). This means that the origin of the equilibrium in ethanol is different from the equilibrium associated with the rotation around the amide bond. We report the conclusion that a new equilibrium is explained by tautomerism of FK506; the resulting mixture consists of three species, FK506, Tautomeric compounds I and II in ethanol solution[4]. Herein we report structures of two tautomeric compounds of FK506 in comparison with spectral data of FK506[4] and postulate a mechanism of interconversion between these three compounds.

Materials and Methods

Isolation of Tautomeric Compounds

FK506 ($C_{44}H_{69}NO_{12}\cdot H_2O$, 1 g), obtained from Fujisawa Pharmaceutical Co., Ltd., was dissolved in 100 ml of ethanol, and then 100 ml of

Fig. 1. Structure of FK506.

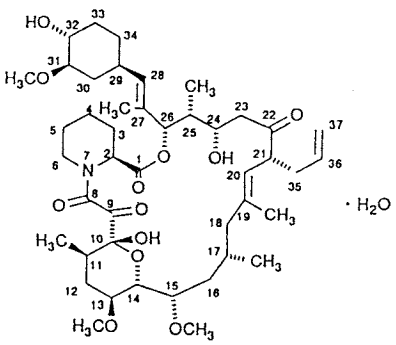
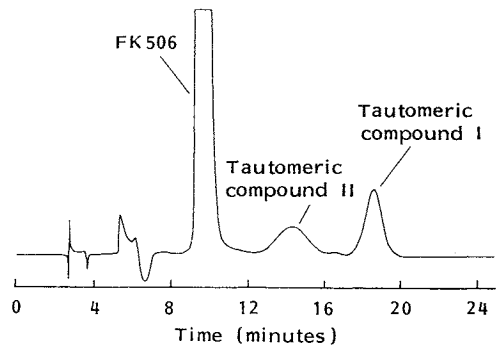


Fig. 2. HPLC chromatogram of FK506 ethanol solution under the following conditions; detector: UV at 220 nm, column: TSKgel OH-120 (TOSOH, 4.6 × 250 mm), column temperature: ambient, mobile phase: a mixture of *n*-hexane - ethylene dichloride - acetonitrile (6 : 3 : 1).



water was added. The suspended solution was kept for one hour at room temperature, and then lyophilized after adding appropariate water and 2-propanol. Lyophilized sample was dissolved in a mixture of hexane and THF (2 : 1), and then submitted to the preparative HPLC, Hitachi 638 system consisting of a column of TSKgel OH-120 (TOSOH, 4.6 × 250 mm, 5 μm), a mobile phase of a mixture of hexane and THF (2 : 1) set at 1 ml/minute, and a detector set at 230 nm. The peak eluted at about 16 minutes was fractionated, and then the solvent was evaporated *in vacuo* with a calcium chloride tube to avoid the decomposition. The residue dissolved in the mixture was again submitted to the same HPLC, and treated in the same manner to obtain Tautomeric compound I as a white powder. On the other hand, lyophilized sample was suspended in a mixture of hexane, ethylene dichloride and acetonitrile (6 : 3 : 1), and a small quantity of chloroform was added to dissolve. This solution was injected into the preparative HPLC at ambient temperature with a mobile phase, the mixture of hexane, ethylene dichloride and acetonitrile (6 : 3 : 1), flow rate at 1 ml/minute. The peak eluted at about 13 minutes was fractionated, and then the solvent was evaporated *in vacuo*. The residue dissolved in the mixture was submitted to the same HPLC and treated in the same manner to obtain Tautomeric compound II as a white powder.

Preparation of Anhydrous FK506

In order to confirm that water molecules attack C-9 position, the water of crystallization in FK506 was removed to produce anhydrous FK506 according to the following procedures. FK506 (0.01 g) was dissolved in 0.2 ml of a mixture of hexane, ethylene dichloride and acetonitrile (6 : 3 : 1), and then submitted to preparative HPLC. The peak eluted at about 10 minutes was fractionated, and then the solvent was evaporated *in vacuo* to obtain anhydrous FK506 as an oily compound.

Spectral Measurements

NMR spectra, *i.e.* ^{1}H, ^{13}C, double quantum filtered-correlated spectroscopy (DQF-COSY), hetero nuclear multiple quantum coherence (HMQC), correlation spectroscopy for long-range couplings (COLOC), hetero nuclear multiple bond connectivity (HMBC), of isolated and purified Tautomeric compounds I and II, and FK506 itself, were recorded on a Bruker AMX500 spectrometer operated at 500.13 MHz for protons and 125.77 MHz for carbons in THF-d_8 and $CDCl_3$ containing TMS as an internal reference at 20°C.

FAB-MS spectra of Tautomeric compounds I and II were measured on a Finnigan MAT TSQ-70 spectrometer in a matrix of thioglycerol containing sodium iodide. Additionally, daughter ion spectra of $(FK506+Na)^+$ obtained from the reactant of anhydrous FK506 dissolved in a mixture of $H_2{}^{18}O$ and dehydrated alcohol (1 : 1) were measured on the freshly prepared sample, and after 30 hours.

IR spectra in mineral oil mull were recorded on a Shimadzu IR-420 spectrophotometer.

Equilibration in a Mixture of Water and Ethanol

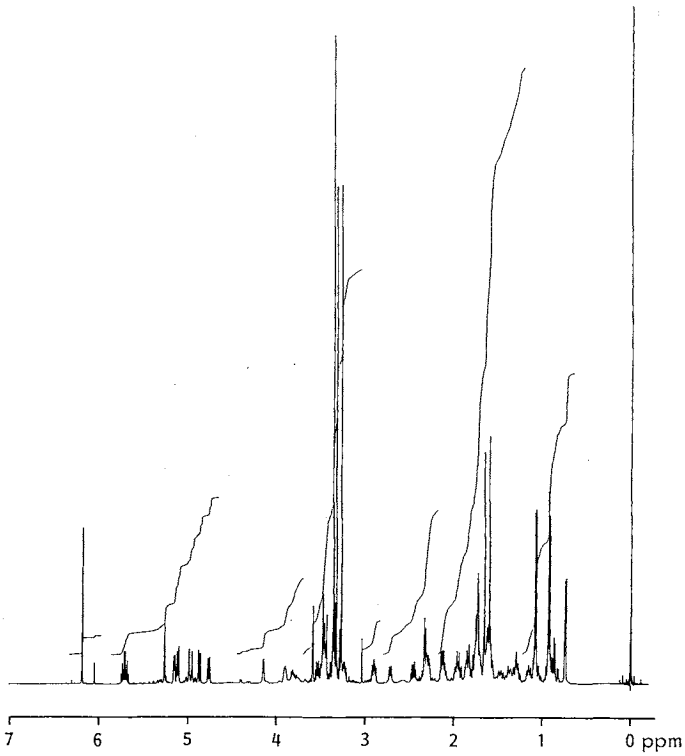
FK506, anhydrous FK506, Tautomeric compound I and Tautomeric compound II were dissolved in the mixture of water and dehydrated alcohol (1 : 1) at the concentration of 2 mg/ml, respectively. At appropriate intervals, the area percentages of FK506, Tautomeric compound I and Tautomeric compound II in these solutions being kept at 25°C were measured by the analytical HPLC, a Shimadzu LC-6A system with a column of TSKgel ODS-80T_M (TOSOH, 4.6 × 150 mm, 5 μm). This system consisted of a Shimadzu LC-6A pump set at 1 ml/minute with a mixture of acetonitrile and water (3 : 2), SPD-6A detector (wavelength: 220 nm), SIL-6A with a system controller, SCL-6A, and CTO-6A column oven kept at about 50°C.

Results and Discussion

Structure Determination

No characteristic IR spectrum of Tautomeric compound I was observed compared with that of FK506: 3400 (stretching vibration of OH), 1743, 1700 and 1695 (stretching vibraton of C=O), 1655 (stretching vibration of C=O and C=C), 1195 and 1165 (stretching vibration of C–O and C–O–C), and 1090 cm^{-1}

Fig. 3. ^{1}H NMR spectrum of Tautomeric compound I (500.13 MHz, THF-d_8).



(stretching vibration of C–O–C). The ^{1}H NMR spectrum, however, provided a new signal located at 6.18 ppm, which was assigned to OH group as shown in Fig. 3. In addition, carbons at the 9 and the 10 positions were found to be significantly changed by using a combination of H-H COSY, HMQC and HMBC techniques, although other carbon signals were quite similar to those of FK506 (see Table 1) and the minor signal associated with the *cis-trans* isomerization was not observed. The quaternary carbon C-9 observed at 198 ppm in FK506 shifted to 98 ppm due to the bonding of two oxygen atoms such as acetal, ketal or gem-diol.

Hemiketal C-10 carbon at 98 ppm changed into carbonyl carbon observed at 199 ppm to form triketone compound as an intermediate. The FAB-MS spectrum of Tautomeric compound I exhibited a $(M+Na-H_2O)^+$, $m/z=826$, instead of $(M+Na)^+$, $m/z=844$ due to the interconversion to FK506.

In order to clarify the position of water molecule added to triketone compound finally, mass spectral analysis was carried out. Daughter ion analysis of anhydrous FK506 in the mixture of $H_2{}^{18}O$ and ethanol revealed the ^{18}O labeling at C-9 which was a central carbonyl group known to be hydrated much easier[5]. Consequently, Tautomeric compound I was elucidated to have the structure generating from the cleavage at hemiketal C-10 position and the addition of water to C-9 carbonyl group in FK506, as presented in Fig. 4.

The ^{1}H NMR and IR spectra of Tautomeric compound II did not significantly differ from that of FK506. IR: 3400 (stretching vibration of OH), 1738, 1720 and 1700 (stretching vibration of C=O), 1638 (stretching vibration of C=O and C=C), 1195 and 1170 (stretching vibration of C–O and C–O–C), and 1090 cm^{-1} (stretching vibration of C–O–C). On the other hand, the result from FAB-MS, $m/z=826$,

Table 1. ^{13}C NMR assignments for FK506 and Tautomeric compound I (δ: ppm, internal reference: TMS).

Carbon No.	FK506[a]	Tautomeric compound I	Carbon No.	FK506[a]	Tautomeric compound I
1	169.40, C	169.51, C	25	41.36, CH	42.69, CH
2	57.04, CH	51.46, CH	26	80.08, CH	76.80, CH
3	28.60, CH_2	27.36, CH_2	27	132.91, C	134.19, C
4	21.89, CH_2	22.35, CH_2	28	132.83, CH	129.72, CH
5	25.29, CH_2	25.85, CH_2	29	35.92, CH	36.03, CH
6	39.34, CH_2	44.22, CH_2	30	36.44, CH_2	36.64, CH_2
		(or 44.50)	31	85.28, CH	85.45, CH
8	165.88, C	166.02, C	32	74.20, CH	74.27, CH
9	197.53, C	98.20, C	33	33.08, CH_2	33.18, CH_2
10	98.12, C	199.09, C	34	31.64, CH_2	31.89, CH_2
11	35.70, CH	40.41, CH	35	36.42, CH_2	35.46, CH_2
12	33.37, CH_2	34.21, CH_2	36	137.18, CH	137.63, CH
13	74.48, CH	74.95, CH	37	116.06, CH_2	115.60, CH_2
14	73.38, CH	76.44, CH	—	—	—
15	76.28, CH	77.25, CH	13-OMe	56.13	56.33
16	34.73, CH_2	36.03, CH_2	15-OMe	57.32	57.00
17	26.71, CH	27.00, CH	31-OMe	57.09	57.15
18	49.95, CH_2	48.64, CH_2	—	—	—
19	139.00, C	141.39, C	11-Me	16.61	15.94
20	124.54, CH	122.45, CH			(or 16.01)
21	53.78, CH	54.15, CH	17-Me	20.06	18.66
22	210.47, C	210.46, C	19-Me	16.61	16.01
23	47.19, CH_2	44.50, CH_2			(or 15.94)
		(or 44.22)	25-Me	10.40	9.96
24	70.00, CH	71.95, CH	27-Me	13.32	14.44

[a] Chemical shift of major isomer.

Fig. 4. Structure of Tautomeric compound I.

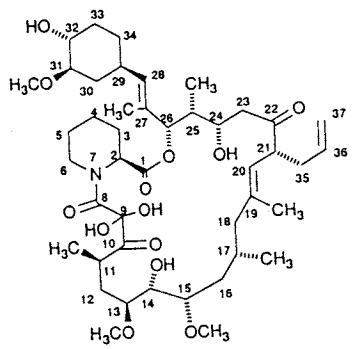
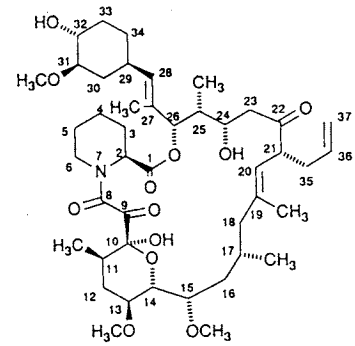


Fig. 5. Structure of Tautomeric compound II.



showed the same mass units as FK506. The NMR spectra with a combination of DEPT, H-H COSY, C-H COSY and COLOC, however, showed the same *cis-trans* isomerism as FK506 and the signal of C-9 carbonyl was found to shift remarkably to downfield along with the change of signals at C-11, 12, 13 and 14 (Table 2). As a result, Tautomeric compound II was identified as an epimer of FK506 at C-10 (Fig. 5).

Equilibrium Phenomenon

As shown in Fig. 6, it was found that Tautomeric compounds I and II reached the same equilibrium state to form FK506 within approximately 2 hours and 20 hours, respectively. Similar phenomenon was also observed in the equilibrium experiment employing FK506 and anhydrous FK506. FK 506 (crystallized

Table 2. ^{13}C NMR assignments for FK506 and Tautomeric compound II (δ: ppm, Internal reference: TMS).

Carbon No.	FK506[a]	Tautomeric compound II[a]	Carbon No.	FK506[a]	Tautomeric compound II[a]
1	169.03, C	170.06, C	25	39.87, CH	40.51, CH
2	56.59, CH	55.88, CH	26	77.50, CH	81.09, CH
3	27.66, CH_2	27.49, CH_2	27	132.28, C	131.59, C
4	21.09, CH_2	20.76, CH_2	28	130.03, CH	132.59, CH
5	24.47, CH_2	24.41, CH_2	29	34.87, CH	34.92, CH
6	39.23, CH_2	39.32, CH_2	30	34.91, CH_2	34.57, CH_2
8	164.75, C	166.92, C	31	84.18, CH	84.12, CH
9	196.31, C	210.80, C	32	73.45, CH	73.50, CH
10	97.10, C	98.25, C	33	31.36, CH_2	31.21, CH_2
11	34.62, CH	38.15, CH	34	30.63, CH_2	30.46, CH_2
12	32.65, CH_2	38.86, CH_2	35	35.23, CH_2	35.08, CH_2
13	73.64, CH	77.62, CH	36	135.60, CH	135.45, CH
14	72.79, CH	78.26, CH	37	116.60, CH_2	116.62, CH_2
15	75.20, CH	76.53, CH	—	—	—
16	33.06, CH_2	36.04, CH_2	13-OMe	56.30	57.43
17	26.18, CH	26.43, CH	15-OMe	56.98	57.73
18	48.62, CH_2	47.73, CH_2	31-OMe	56.59	56.50
19	138.91, C	138.68, C	—	—	—
20	122.51, CH	123.61, CH	11-Me	16.21	16.68
21	52.83, CH	53.85, CH	17-Me	20.36	19.71
22	212.46, C	210.36, C	19-Me	15.93	16.60
23	43.63, CH_2	47.09, CH_2	25-Me	9.52	9.71
24	69.88, CH	69.64, CH	27-Me	13.97	12.79

[a] Chemical shift of major isomer.

Fig. 6. Equilibration of FK506, anhydrous FK506, Tautomeric compounds I and II in the mixture of water-dehydrated alcohol (1 : 1).

○ FK506, □ Tautomeric compound I, △ Tautomeric compound II, ● anhydrous FK506.

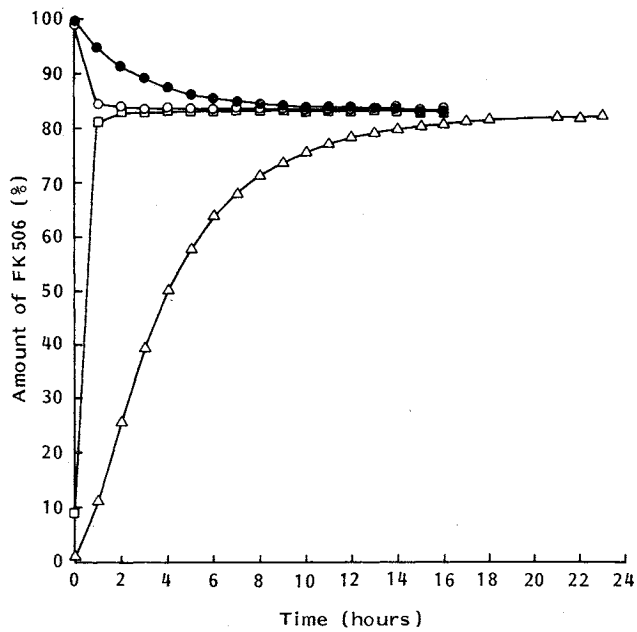
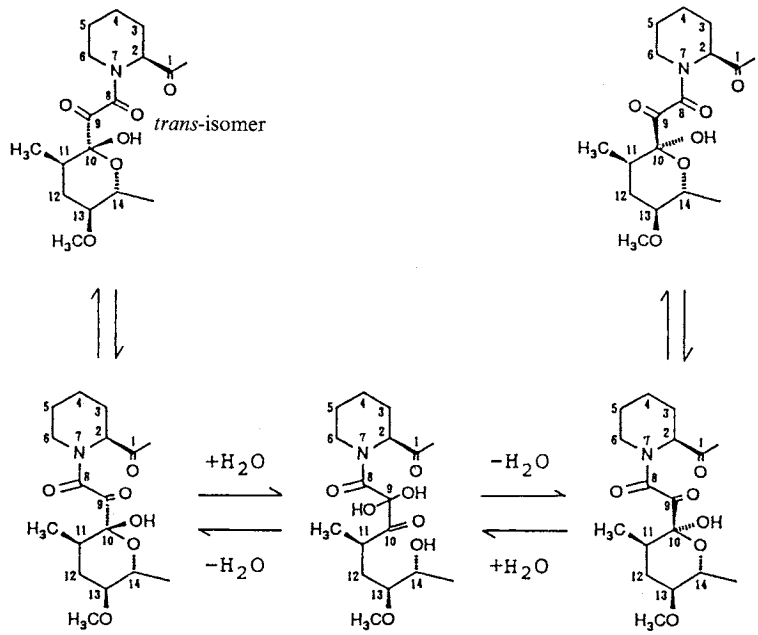


Fig. 7. Equilibrium of FK506 in solution.



as monohydrate) was equilibrated faster remarkably than anhydrous FK506. These findings suggest that crystalline water in FK506 plays an important role to facilitate the reaction at C-10. This assumption is supported by the evidence[6] revealed by the single crystal X-ray analysis in which water molecule contained in FK506 is situated near carbonyl oxygens at C-8, C-9 and hydroxyl group at C-10 and forming hydrogen bonding with them.

Formation Mechanisms of Tautomeric Compound I and Tautomeric Compound II

In addition to the phenomenon observed in ethanol solution of FK506, the structure determinations of Tautomeric compounds I and II lead us to a plausible formation scheme (Fig. 7) for Tautomeric compound I and Tautomeric compound II. FK506 might be firstly cleaved at C-10 hemiketal, and then addition of water might occur at C-9 ketone to form Tautomeric compound I. Similar conversion from Tautomeric compound II to Tautomeric compound I must occur and furthermore provide *cis* and *trans* conformational isomers like FK506 itself; the conversion of FK506 and Tautomeric compound II into Tautomeric compound I might happen by the reversed reaction mechanism.

It is of quite interest that Rapamycin[7], structurally related to FK506 and known to inhibit distinct signaling pathways in T-lymphocytes[8,9], does not show this phenomenon remarkably. This fact implies the possibility that the different biological effect in these two compounds may be caused by this mode of action in tautomeric phenomenon. The result obtained will be presented elsewhere.

Acknowledgments

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