Notes

FK-506 RELATED COMPOUNDS PRODUCED BY *STREPTOMYCES TSUKUBAENSIS* No. 9993

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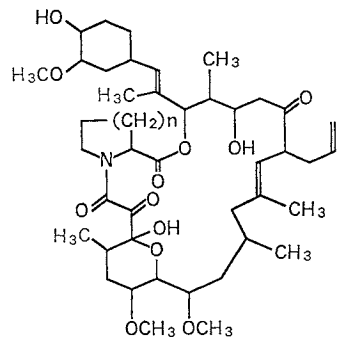
In a previous paper[1], we reported that *Streptomyces tsukubaensis* No. 9993 produced a novel and potent immunosuppressant, FK-506. In the course of FK-506 production, two minor components were isolated from a culture broth of *S. tsukubaensis* No. 9993 (deposit No. FERM BP-927[2]). Both suppressed immune response *in vitro* and are structually related to FK-506. One of them was purified[2] from a FK-506 crude product by preparative HPLC (YMC column, ODS, 5 μm, 20×250 mm, CH_3CN - BuOH - H_2O - H_3PO_4 (28 : 10 : 62 : 0.075) containing 3.75 mM sodium dodecyl sulfate, 12 ml/minute, UV at 210 nm, retention times 85~90 minutes). Based on the spectroscopic analyses, the component was identified as FR-900520[3] produced by *Streptomyces hygroscopicus* subsp. *yakushimaensis* No. 7238[4]. The other was designated FR-900525. The structure of FR-900525 (**1**) differs from that of FK-506 (**2**) by replacement of the piperidine-2-carboxylic acid moiety with proline as shown in Fig. 1. This paper describes the fermentation of *S. tsukubaensis*, the isolation, characterization and biological properties of **1**.

A loopful from a slant culture of the producing strain was transferred to a 500-ml Erlenmeyer flask containing 160 ml of a sterile seed medium and incubated on a rotary shaker at 30°C for 4 days. The seed medium (adjusted to pH 6.5) was composed of glycerol 1%, corn starch 1%, glucose 0.5%, cotton seed meal 1%, dried yeast 0.5%, corn steep liquor 0.5% and $CaCO_3$ 0.2%.

Fig. 1. Structures of FR-900525 and FK-506.



FR-900525 (**1**) n=1
FK-506 (**2**) n=2

The incubated seed culture (1.6 liters) were transferred to the production medium (150 liters, pH 6.8) containing soluble starch 5%, peanut powder 0.5%, dried yeast 0.5%, gluten meal 0.5%, $CaCO_3$ 0.1% and Adekanol (defoaming agent, Asahi Denka Co., Ltd.) 0.1%, in a 200-liter jar fermentor. Fermentation was carried out at 30°C for 4 days under agitation at 250 rpm and aeration (150 liters/minute).

The broth filtrate (135 liters) and mycelium acetone extract (50 liters) were combined, and then passed through a column of Diaion HP-20 (Mitsubishi Chemical Industries Ltd.) (10 liters). After washing with 50% aq acetone, the column was eluted with 75% aq acetone. The eluate was concentrated under reduced pressure and extracted with EtOAc. The extract was concentrated under reduced pressure to an oily residue and applied to a column of acidic silica gel (800 ml, Fuji Devison Co., Ltd., grade 12) which was packed with hexane. Elution with a mixture of hexane and EtOAc gave active fractions which were collected and concentrated under reduced pressure. The oily residue was dissolved in hexane - EtOAc (1 : 1) and chromatographed on silica gel (500 ml). **2** was eluted with hexane - EtOAc (1 : 1 to 1 : 2) as a crude, yellowish powder (3 g). Slower running fraction containing **1**, following rechromatography on silica gel, were subjected to column chromatography of acidic silica gel (100 ml, grade 12) packed and developed with hexane. Active

fractions were concentrated under reduced pressure to yield a pale yellowish powder (380 mg). This powder was dissolved in hexane - EtOAc (1 : 2) and applied to acidic silica gel (100 ml, grade 922) with the same solvent. Elution with EtOAc gave **1** (230 mg) as a white powder. **1** was analyzed by reversed-phase HPLC (YMC column, ODS, 5 μm, 4.6×150 mm, MeOH - CH_3CN - 0.5% aq TFA (4 : 4 : 2), UV at 210 nm, 1 ml/minute, retention time 9.3 minutes). **1** displayed a sharp peak as opposed to the broad ones of **2** and FR-900520.

The physico-chemical properties of **1** are as follows: $C_{43}H_{67}NO_{12}$, white powder, secondary ion (SI)-MS *m/z* 790 (M+1); mp 85~89°C; $[\alpha]_D^{23}$ −88° (*c* 1.0, $CHCl_3$); *Anal* calcd for $C_{43}H_{67}NO_{12}$: C 65.37, H 8.55, N 1.77, found: C 65.17, H 8.53, N 1.76; UV end absorption; IR $\nu_{max}^{CHCl_3}$ cm^{-1} 3475, 3340, 1755, 1705, 1635, 1093. **1** gives positive color reactions for cerium sulfate, sulfuric acid, iodine vapor, Ehrlich and Dragendorff, and negative for ferric chloride, ninhydrin and Molisch. **1** is soluble in MeOH, EtOH, $(CH_3)_2CO$, EtOAc, $CHCl_3$, diethyl ether and benzene, sparingly soluble in hexane and petroleum ether, and insoluble in water. Its physico-chemical properties were similar to those of **2**. ^1H NMR ($CDCl_3$, 200 MHz) and ^{13}C NMR ($CDCl_3$, 50 MHz) spectra of **1** are shown in Figs. 2 and 3, respectively. Similar to **2**[5], the ^{13}C NMR spectrum of **1** indicates an equilibrium mixture, presumably due to rotamers associated with the amide bond. The molecular formula was determined to be $C_{43}H_{67}NO_{12}$ by SI-MS and elemental analysis. We previously reported the molecular formula[1,5], $C_{44}H_{69}NO_{12}$ for **2**. The 14 mass unit difference in the molecular ion between **2** (M+1, *m/z* 804) and **1** (M+1, *m/z* 790) in SI-MS suggests the absence of one methylene group in **1**. In addition, the ^{13}C NMR spectrum of **1** indicated three methoxy groups as observed in **2**. Acid hydrolysis of **1** and **2** (6 N HCl, 110°C, 16 hours) gave proline and pipecolic acid, which were identified by comparison with authentic samples on TLC and amino acid analyses (835 Hitachi amino acid analyzer), respectively. The Rf values for both amino acids on silica gel TLC were 0.17 and 0.22 (authentic samples, proline 0.17, pipecolic acid 0.22) in BuOH - AcOH - H_2O (4 : 1 : 2), and 0.47 and 0.53 in $CHCl_3$ - MeOH - 17% NH_4OH (2 : 2 : 1). They also gave brownish yellow and violet spots by ninhydrin detection on TLC, respectively. The signals due to the α-carbon of proline (δ 60.0, 56.0) were observed in the ^{13}C NMR spectrum of **1**, whereas the corresponding signals of pipecolic acid (δ 56.5, 52.6) in **2** were observed. These results indicated that the pipecolic acid moiety in **2** should be replaced by proline in **1**. Further structural information of **1** will be published in a seperate paper.

A mouse mixed lymphocyte reaction for **1**

Fig. 2. ^1H NMR spectrum of FR-900525 ($CDCl_3$, 200 MHz).

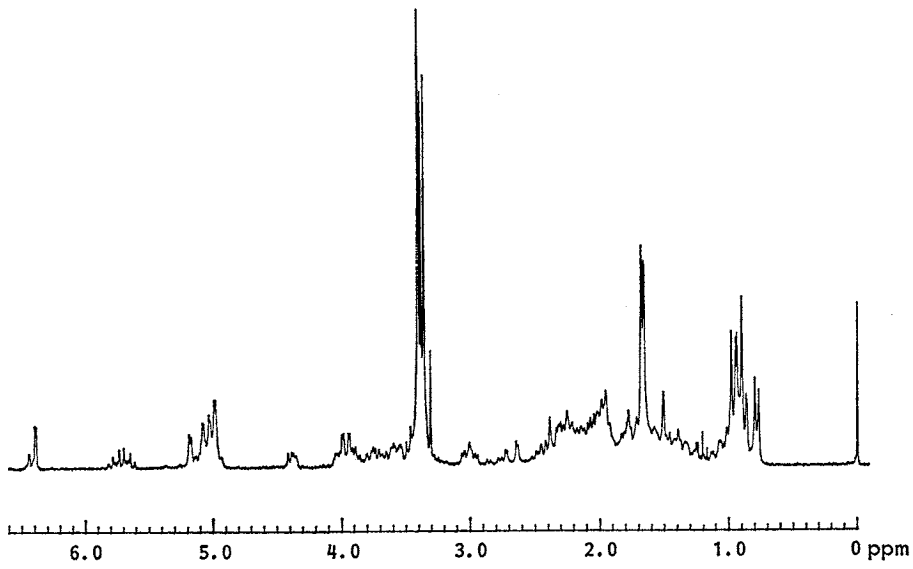
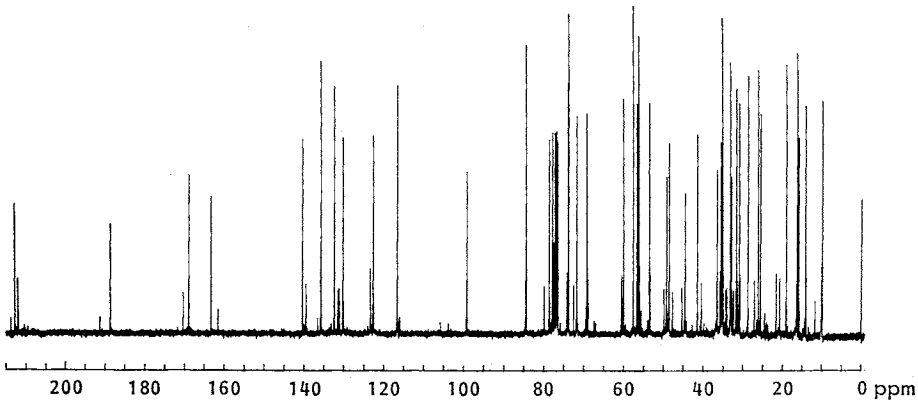


Fig. 3. ^{13}C NMR spectrum of FR-900525 ($CDCl_3$, 50 MHz).



was examined according to the method described in a previous paper[6]. In multiple experiments, the IC_{50} value was 1.9 nM. The cytotoxicity for EL-4 lymphoma, IL-2 independent T cell line, was not observed at concentrations less than 3,200 nM. The percent inhibition was 17% at 3,200 nM. **1** showed antifungal activity, *e.g. Aspergillus fumigatus* IFO 5840 by a conventional agar dilution method. The MIC value was 0.5 μg/ml. **1**, dissolved in olive oil, showed no adverse effect when administered intraperitoneally to *dd*Y mice at 100 mg/kg.

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