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# THE PREPARATION OF $^{14}$C-LABELED FK-506

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## SUMMARY

Utilizing sodium [1-$^{14}$C]propionate as a precursor, [$^{14}$C]FK-506, labeled at carbon atoms 10, 16, 18, 21a, 24, and 26, was produced by fermentative biosynthesis. Extractive isolation followed by chromatographic purification provided material of purity suitable for metabolism studies.

Key Words: [$^{14}$C]FK-506, immunosuppressant, sodium [1-$^{14}$C]propionate, fermentative biosynthesis.

## INTRODUCTION

The immunosuppressant compound FK-506, first described in 1987[1], is a 21-membered macrolide similar in effect to cyclosporin A, but with one hundred times the *in vitro* potency.[2] Both inhibit the production of interleukin-2 and interleukin-3 as well as γ-interferon.[3] While cyclosporin A is currently the leading drug for use in immune system suppression to prevent rejection of transplanted organs, the potency of FK-506 makes it a promising alternative form of treatment. FK-506 has been shown to be effective in liver, kidney, and pancreas transplant patients with minor side effects.[4] Metabolism studies to evaluate the *in vivo* disposition of FK-506 necessitated the availability of the $^{14}$C-labeled material.[5]

The first total synthesis of FK-506 was accomplished at Merck[6], and two $^{13}$C-labeling schemes by chemical synthesis have been achieved.[7] However, the $^{14}$C-labeling of compounds as large and as structurally diverse as FK-506 is usually a process too lengthy for chemical synthesis. Also, the prospects of chemically synthesizing sufficient quantities of a multiple-labeled compound such as that achievable by fermentation and favored for metabolism studies within a reasonable time frame appeared small. Thus, isotope incorporation by fermentation was undertaken. The positions of label derived from sodium [1-$^{13}$C]propionate (carbon atoms 10, 16, 18, 21a, 24, and 26) have recently been reported[8] and are the same as those indicated as C-14 labeled in Figure 1.

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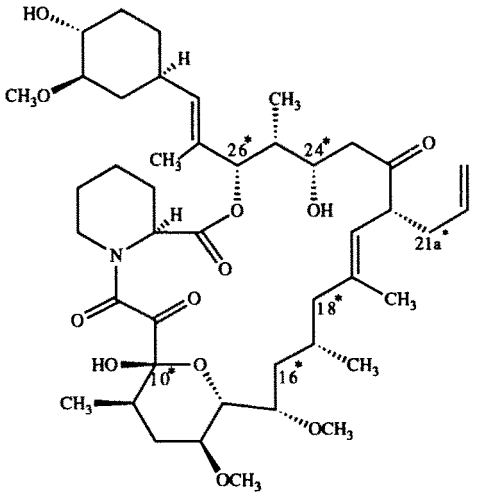
## DISCUSSION

The addition of aliquots of sodium [1-$^{14}$C]propionate (614 mCi, 57.7 mCi/mmol) in water (~8 mg/0.5 ml, pH 6.9) to 50 ml of broth in each of thirty-two 250 ml Erlenmeyer flasks was carried out at 48, 72, 80, and 96 hours after inoculation of the flasks with the FK-506 producing culture Streptomyces tsukubaenis No. 9993. The flasks were incubated in a rotary shaker at 29°C and 220 rpm. At 120 hours, the contents of the flasks were pooled and filtered through celite, and the filter cake washed with water (600 ml). The filter cake was extracted first with $CH_3OH$ (1000 ml) and then with $CH_3OH$:$H_2O$; 80:20 (1000 ml). The extracts were combined, concentrated in vacuo to 400 ml, and extracted with ethyl acetate (2 x 100 ml). The combined ethyl acetate extracts contained 7.3 mCi of radioactivity which by TLC analysis (silica; $CH_3OH$:$CHCl_3$; 1:9) was 73% product and by HPLC (C-18; 50°C; THF:$H_2O$; 45:55) was 70% product. Preparative reverse phase HPLC (Zorbax C-8; THF:$H_2O$; 35:65) followed by preparative normal phase HPLC (Whatman M-9 silica; EtOH:$CHCl_3$; 5:95) provided 3.6 mCi (0.59% from sodium [1-$^{14}$C]propionate) of product existing as a 9:1 equilibrium mixture[9] by HPLC (as above) with a combined radiochemical purity of 98% .




FK-506

(* indicates C-14)

Figure 1

## EXPERIMENTAL

A New Brunswick Series 25 incubator shaker was used. Aqueous sodium [1-$^{14}$C]propionate was sterilized by filtration through a Nalgene 115 ml sterile filter

(45 μ). Media and glassware sterilizations were performed with a Getinge PACS 50 autoclave. The following materials were used in the fermentation : Pharmamedia™ (Traders Protein Co., Memphis, Tenn.), soluble starch (Difco Laboratories Co., Detroit, Mich.), corn steep liquor (Grain Processing Corp., Muscatine, Iowa), dried yeast (Champlain Industries Corp., Clifton, N. J.), and antifoam P-2000 (Dow Chemical Co., Midland, Mich.). Radioactivity determinations were carried out with a Packard Tri-Carb Model 3320 liquid scintillation counter using 0.42% Omnifluor™ in toluene:ethanol (7:3) as scintillation medium. Analytical TLC was performed using silica gel 60 F-254 (E. Merck glass plates, 5 x 20 cm) with radioactivity measurements made with a Berthold Model LB2760 scanner. The HPLC system used for analysis consisted of a 4.6 mm x 25 cm E. Merck RP-18 or Whatman Partisil C-8 column, a Fiatron Model CH-30 column heater, two Beckman Model 100A pumps, a Beckman Model 421 controller, and an LDC Spectromonitor II UV detector. HPLC radioactivity measurements were done using a Berthold LB504 Radioactivity Monitor with Hewlett Packard HP3388A computer integration. Preparative HPLC was accomplished using a Whatman M9 (9.4 mm x 50 cm) Partisil column and a DuPont Zorbax C-8 column (21.2mm x 25 cm). All chemicals, unless specified otherwise, were obtained from Aldrich Chemical Co. (Milwaukee, Wis.).

Medium and Culture Preparation

Seed medium contained the following : glycerol (10 g/liter), glucose (5 g/liter), corn starch (10 g/liter), Pharmamedia™ (10 g/liter), corn steep liquor (5 g/liter), dried yeast (5 g/liter), and calcium carbonate (2 g/liter). The pH was adjusted to 6.5. Complete seed medium was dispensed at 50 ml per nonbaffled 250 ml Erlenmeyer flask and sterilized at 121$^0$C, 15 psi, for 30 minutes. Complete production medium consisted of soluble starch (45 g/liter), corn steep liquor (10 g/liter), dried yeast (10 g/liter), calcium carbonate (1 g/liter), and antifoam P-2000 (1 g/liter); the pH was adjusted to 6.8. Complete production media (50 ml) was dispensed into each of thirty-two nonbaffled 250 ml Erlenmeyer flasks and sterilized at 121$^0$C, 15 psi, for 30 minutes. Culture development was begun by inoculating 1.0 ml from frozen vegetative mycelia of Streptomyces tsukubaensis No. 9993 into 50 ml of seed medium and incubating at 29$^0$C, 220 rpm, 85% humidity for 72 hours. The resultant culture was aseptically transferred (2% inoculum) to each of the thirty two production flasks. Fermentation was then begun in a nonhumidified shaker incubator at 29$^0$C / 220 rpm.

Sodium [1-$^{14}$C]Propionate Addition

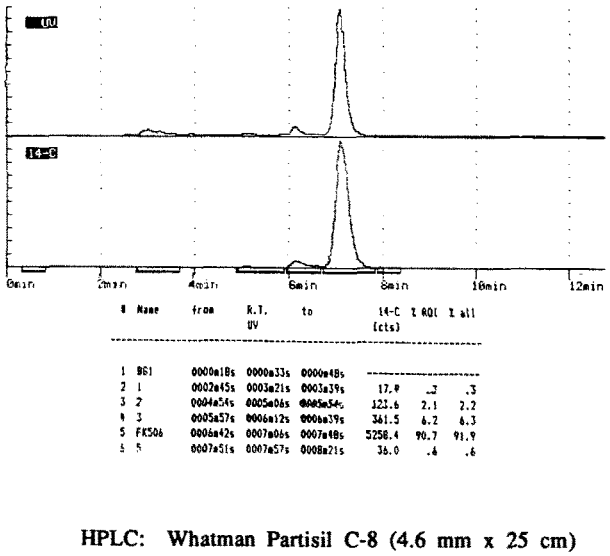
A solution of sodium [1-$^{14}$C]propionate (614 mCi, 57.7 mCi/mmol, 1042 mg) in water (15 ml) was prepared. The pH was adjusted from 8.0 to 6.9 with 0.1 N HCl. This solution was sterilized by filtration and diluted with aqueous rinse to 65 ml (0.5 ml = 8 mg). At 48, 72, 80, and 96 hours after inoculation, 0.5 ml aliquots of this solution were aseptically added to each flask.

[$^{14}$C]FK-506 Isolation

After 120 hours incubation, the contents of all thirty-two flasks were filtered through a ~1/2 inch pad of celite on an 8 inch (i.d.) Buchner funnel. The filter cake was washed with 600 ml of water. The solids and celite on the funnel were then suspended in 1000 ml of methanol and mechanically stirred for one hour. The solids were filtered and reextracted in the same manner with 1000 ml of methanol:water; 80:20, combining both filtrates to provide 21.8 mCi of radioactivity. Analysis by silica TLC (methanol:chloroform; 1:9) indicated 29% of the radioactivity to be associated with FK-506 (Rf 0.55). In vacuo concentration to 400 ml followed by extraction with ethyl acetate (100 ml) provided in the organic layer 7.3 mCi (73 % product by TLC). Assay of this material by HPLC (C-18; 50°C; THF:$H_2O$; 45:55) indicated 70% of [$^{14}$C]FK-506 ($t_R$ = ~10 mins.). This solution was dried over sodium sulfate and then concentrated in vacuo to give 1.3 g as a brown oil.

[$^{14}$C]FK-506 Purification

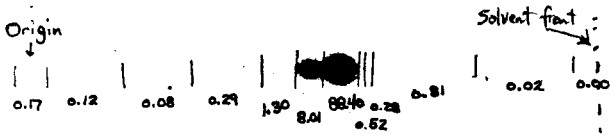
The sample from above was dissolved in 1.5 ml THF and purified by preparative HPLC (Zorbax C-8, 21.2 mm x 25 cm; THF:$H_2O$; 35:65, room temperature) in three runs. This provided 3.6 mCi of product. Analysis by both TLC and HPLC indicated ~90% radiochemical purity with ~10% of the radioactivity in a slightly more polar species (Rf 0.50 and $t_R$ = ~9 mins. using the above TLC and HPLC




HPLC: Whatman Partisil C-8 (4.6 mm x 25 cm)
Acetonitrile:water:phosphoric acid: 72:28:0.1
1.0 ml/min, 50°C, 205 nm.

Figure 2

conditions, respectively). Repeated attempts to remove the minor component by both normal and reverse phase chromatography were unsuccessful. Collecting separately the 9 and 10 minute peaks from an analytical HPLC injection, followed by concentration with nitrogen and reinjection gave the same ~1:9 ratio for both. Also, two dimensional TLC (E. Merck 20 x 20 cm; methanol:chloroform; 1:9) showed a reequilibration with the second development. These observations lead us to conclude that the two components are in equilibrium with one another. A number of plausible species can be suggested such as the tri-keto compound resulting from opening of the cyclic hemiketal, the C-10 epimer, and the seven-membered cyclic hemiketal from ring closure at C-9 instead of C-10.[9] However, the rapid nature of this equilibrium has prevented isolation of the minor component and thus precluded its identification. Although it is known that FK-506 can exist as two amide rotamers,[10] HPLC analysis at 50°C would coalesce rotamers into a single peak and not give the two resolved peaks found here. Further purification of this material by preparative normal phase chromatography (Whatman M9 Partisil, 9.4 mm x 50 cm: methanol:chloroform; 5:95: 4 runs) gave quantitative recovery of radioactivty with a specific activity of 66 μCi/mg. Analysis using the slightly modified HPLC conditions shown in Figure 2 gave the same results as the above system. The TLC profile with percent radioactivity indicated is also included (Figure 3); this was obtained by exposing the plate to x-ray film, then scraping and counting in the standard manner. In conclusion, analysis by HPLC showed the radiochemical purity to be ~98% while that with TLC was ~96%.




TLC: Methanol:chloroform; 1:9

Figure 3

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9) a. There have been two published reports supporting a six-membered/seven-membered cyclic hemiketal phenomenon in similar systems, but the correlation with FK-506 is tenuous at this point. Jones et. al. (ref. 6 above) report in their total synthesis of FK-506 that a tri-keto ester intermediate cyclized to a 1-4:1 mixture of six- and seven-membered cyclic hemiketals. In addition, Schreiber et. al. [9b] mentions the isolation of a 2:1 mixture of six- and seven-membered cyclic hemiketals in the synthesis of FK-506 BD (binding domain), an FK-506 analog.

b. Schreiber, S.L., Bierer, B. E., Somers, P. K., Wandless, T. J. and Burakoff, S. J. - Science 250: 556 (Oct. 26, 1990).

10) a. The existence of rotamers due to hindered rotation about the amide bond in FK-506 has been observed by others (see ref. 1b). The antifungal antibiotic rapamycin contains the same masked tri-keto amide grouping as in FK-506 and is also reported to exist as a mixture of rotamers.[10b]

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