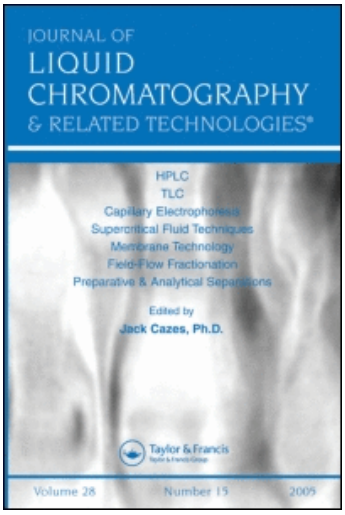

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ISOLATION, SPECTROSCOPIC CHARACTERIZATION, AND IMMUNOSUPPRESSIVE ACTIVITIES OF TWO RAPAMYCIN DEGRADATION PRODUCTS*

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ABSTRACT

A high performance liquid chromatographic method has been developed for the isolation of two degradation products of rapamycin which is currently under development as an immunosuppressive agent. Prior to isolation, the drug was incubated at 37°C in rat bile or ammonium acetate (pH 8.0). The isolation was achieved by a Supelco, PLC-18 21.2 x 250 mm, 18 μm column using methanol/ammonium acetate gradient mobile phase. After evaporation of methanol, the remaining eluates were lyophilized. The isolated degradation products were characterized by negative ion fast atom bombardment mass spectrometry (FAB MS) and proton nuclear magnetic resonance spectroscopy (^{1}H NMR). Degradation product A was found to be a macrolide ring-opened hydrolysis product of rapamycin where the C25 ester bond had been hydrolyzed. Degradation product B was determined to be a ring-opened isomer of rapamycin. B had less than 4% of the potency of rapamycin in a thymocyte proliferation assay, while A had minimal activity at concentrations tested.

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INTRODUCTION

Rapamycin, [3S-[3R*[S*(1R*,3S*,4S*)],6S*,7E,9S*,10S*,12S*,14R*,15E,17E, 19E,21R*,23R*,26S*,27S*,34aR*]]-9,10,12,13,14,21,22,23,24,25,26,27,32,33,34a-Hexadecahydro-9,27-dihydroxy-3-[2-(4-hydroxy-3-methoxycyclohexyl)-1-methylethyl]-10,21-dimethoxy-6,8,12,14,20,26-hexamethyl-23,27-epoxy-3H-pyrido[2,1-c] [1,4]oxaazacyclohentriacontine-1,5,11,28,29 (4H,6H,31H)-pentone, (Figure 1), an antitumor and antifungal agent isolated from the fungus *Streptomyces hygroscopicus*(1,2), has been found to have potent immunosuppressive activity while exhibiting little toxicity in primates(3,4). The drug is currently under clinical trials as an immunosuppressive agent.

Rapamycin is chemically unstable; under basic or acidic conditions, it degrades via β-elimination to form a ring-opened isomer of rapamycin. The drug is also unstable in biological fluids; extensive degradation was observed in rat serum samples even after storage at -20°C for 18 days. Therefore, prior to pursuing the isolation of biliary metabolites of rapamycin in rats, the drug was incubated at 37°C in rat bile. Two major rapamycin degradation products, designated A and B, were observed. To further verify that the degradation process is not induced or catalyzed by endogenous materials and to facilitate the isolation of large quantities of the products, we have also incubated rapamycin in an ammonium acetate solution of a similar pH to bile (pH 8.0).

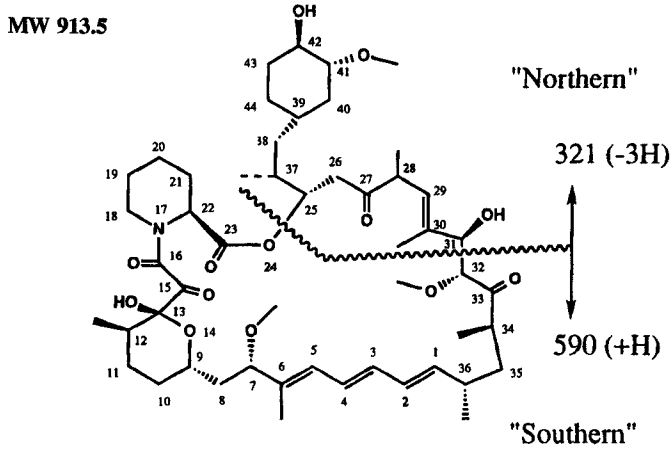

FIGURE 1 Chemical Structure of Rapamycin and its Major Fragmentation Pathway

We hereby report the isolation, structural characterization and immunosuppressive activity of the major degradation products of rapamycin after incubation of rapamycin at 37°C in rat bile or ammonium acetate (pH 8.0).

EXPERIMENTAL

Materials

Rapamycin was obtained from Wyeth-Ayerst Research, Rouses Point, NY. Ammonium acetate (HPLC grade) and ammonium hydroxide were obtained from J.T. Baker (Phillipsburg, NJ). All solvents used in the study were HPLC grade. Control rat bile was obtained in-house (Drug Metabolism Division, Wyeth-Ayerst Research, Princeton, NJ).

Instrumentation

Two HPLC systems were used in the isolation. One consists of a LDC Analytical, Model CM 4000 pump, a Waters WISP Model 710B autosampler (Waters Associate, Milford, MA), and a LDC Analytical SpectroMonitor 5000, photodiode array detector. The second system is composed of a Waters 600E system controller and pump, a Waters 490E programmable multiwavelength detector, a Waters U-6K manual injector and a Hewlett-Packard 3390A integrator. Isolation of degradation products was achieved using Supelcosil LC-18, 5 μm, 4.6 x 250 mm column (Supelco, Bellefonte, PA) and Supelcosil PLC-18, 18 μm, 21.2 x 250 mm column. The nuclear magnetic resonance spectrometers used were Bruker AM-400 (Billerica, MA) and Varian VXR-400 (Palo Alto, CA). A MS 50 mass spectrometer equipped with a FAB ion source operated in the negative ion mode (Kratos Analytical, Ramsey, NJ) was used.

Preliminary isolation of compounds A and B from *in vitro* incubation of rapamycin at 37°C in rat bile

Rapamycin (50 μg in 20 μl methanol) was added to control rat bile per 1.0 ml bile or 500 μg in 40 μl methanol per 1.25 ml bile. The samples were vortexed and incubated at 37°C in a shaking water bath for up to 15 hr.

The samples were then injected directly onto a Supelco LC-18, 25 cm x 4.6 mm, 5 μm column to evaluate degradation. Separation was achieved with a linear gradient from 65% to

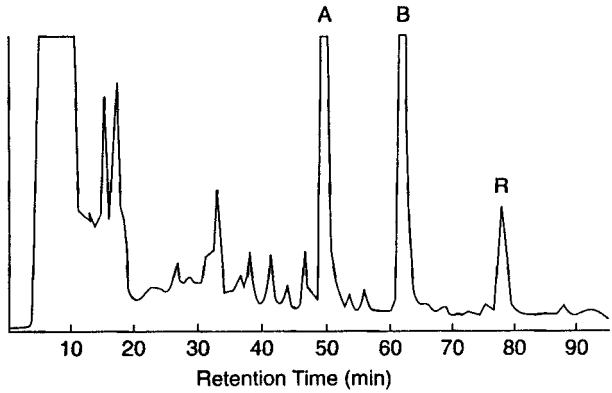

FIGURE 2 HPLC (Supelco LC-18, 25 cm x 4.6 mm, 5 μm) Chromatogram of Rapamycin Incubated with Rat Bile for 15 hr (A: Peak A, B: Peak B, R: Rapamycin)

86% methanol in 0.05M ammonium acetate over 80 min at a flow rate of 0.5 ml/min. Detection was by monitoring UV absorbance at 276 nm. Two major degradation peaks were observed in the chromatogram (Figure 2). The peaks were designated as compound A (retention time 49.2 min.) and compound B (retention time 61.5 min.). The eluates containing compounds A and B were collected separately from multiple injections. The collections were dried in a Savant AS-160 concentrator at room temperature in the manual mode.

Isolation of compounds A and B after incubation of rapamycin at 37°C in rat bile

To isolate sufficient quantities of compounds A and B, 30 tubes that each contained 0.9 mg rapamycin in 50 μl ethanol and 2.0 ml control rat bile were prepared. The tubes were incubated in a 37°C water bath for 13.5 hr and then stored at -80°C until analysis.

Isolation of compounds A and B was performed with a preparative Supelco PLC-18, 25 cm x 21.2 mm, 18 μm column. The mobile phase gradient profile is described in Table 1. Detection was by UV absorbance at 276 nm. The injection volume was 2 ml of the biliary sample. As shown in Figure 3, the retention times of compounds A and B and rapamycin were 31.1, 39.4, and 60.6 min., respectively. The HPLC eluates containing compounds A and B were collected into 20 ml scintillation vials, and the purity of the collected eluates was analyzed by the analytical HPLC system described above. Methanol was then removed from the eluates with the Savant AS-160 at room temperature in the manual mode. The remaining aqueous residues were pooled and then lyophilized. The material was redissolved and purified again as described in this section.

TABLE 1

Gradient System used in the Supelco PLC-18, 250 x 21.2 mm, 18 µm Column for the Isolation of Rapamycin Degradation Products[a]

Time (min.)	Flow rate (ml/min.)	Percent methanol	Percent 0.05M ammonium acetate
0.0	4.0	77	23
45	4.0	86	14
50	4.0	95	5
60	4.0	95	5
65	4.0	77	23

[a]Linear gradient was used between each time point.

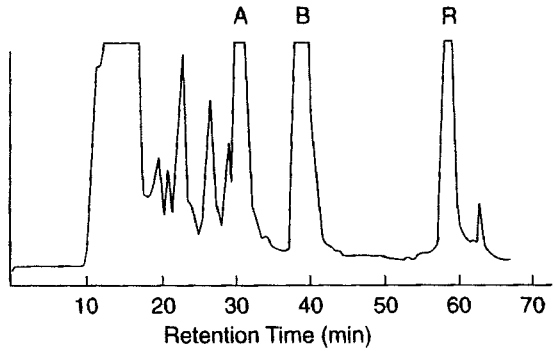

FIGURE 3 HPLC (Supelco PLC-18, 25 cm x 21.2 mm, 18 µm) Chromatogram of Rapamycin Incubated with Rat Bile for 13.5 hr (A: Peak A, B: Peak B, R: Rapamycin)

Isolation of compounds A and B after incubation of rapamycin in 0.1M ammonium acetate (pH 8.0)

Rapamycin (150 mg) was dissolved in 15 ml ethanol, which was added to 300 ml of 0.1M ammonium acetate (pH 8.0 with ammonium hydroxide). The mixture was incubated in a 37°C water bath for 24 hr. The suspension was filtered while it was still warm, and the filtrate was lyophilized to dryness. The dried white powder was redissolved in methanol/water (65/35). Isolation of compounds A and B was performed on the preparative system as described above (Figure 4). After removal of methanol using the Savant concentrator, the aqueous solution was pooled and lyophilized.

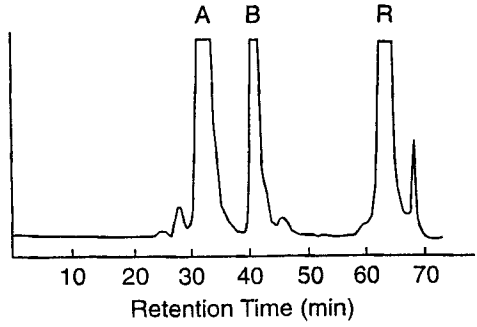

FIGURE 4 HPLC (Supelco PLC-18, 25 cm x 21.2 mm, 18 μm) Chromatogram of Rapamycin Incubated with Ammonium Acetate Solution (pH 8.0) (A: Peak A, B: Peak B, R: Rapamycin)

The thymocyte proliferation assay

The isolated rapamycin degradation products A and B were subjected to an *in vitro* immunosuppressive activity test. This thymocyte proliferation assay was conducted in-house at Wyeth-Ayerst Research, Princeton, NJ.

Spectroscopic characterization of compounds A and B

All FAB mass spectra were obtained in the negative ion mode. The primary beam was 1 mA of 7 kV xenon atoms. Resolution of the instrument was adjusted to 2000 (10% valley). Triethanolamine was employed as the matrix for the samples. Each sample was dissolved in methylene chloride, and an aliquot of the solution was mixed with the matrix on a copper probe tip before insertion into the mass spectrometer for analysis. High resolution measurements of the molecular ion species were carried out by peak matching with reference ions from cesium iodide at a resolution of 10,000. All ^{1}H-NMR spectra were obtained from samples dissolved in alumina neutralized, deuterated chloroform.

RESULTS

Immunosuppressive activity of A and B by thymocyte proliferation assay

When compared to the IC50 of rapamycin, the immunosuppressive activity of B is less than 4% of that of rapamycin. Product A appears to be inactive.

Structural elucidation of compound A

The mass spectra of A isolated from *in vitro* incubation of rapamycin in rat bile or 0.1M NH_4OAc are essentially the same. The exact mass of the molecular ion species at m/z 930 was found to be 930.5595 by high resolution MS. This mass is consistent with an elemental composition of $C_{51}H_{80}NO_{14}$ (theoretical mass = 930.5578) and suggests that the ion at m/z 930 is the $[M-H]^-$ of a product in which one mole of H_2O has been added to rapamycin. The observation of the $[M-H]^-$ suggests that the macrolide ring is opened and the fragment ions at m/z 339 and 590 show that hydrolysis has occurred in the northern portion of rapamycin (Figure 1). Therefore, formation of A is likely due to hydrolysis of the C_{25} ester bond (Figure 5).

In the ^{1}H-NMR spectra of A, the H-25 resonance, which occurs at 5.15 ppm for rapamycin, is observed at 3.85 ppm. This chemical shift is similar to those observed for H-25 in spectra of hydrolyzed rapamycin analogs containing C25-OH. Upon dissolution of A in $CDCl_3$, no evidence of a C_{25}=C_{26} alkene group is observed. A has been nomenclatured as 1-{[2R-Hydroxy-6S-[22-(4R-hydroxy-3R-methoxy-1S-cyclohexyl)-14R,20-dihydroxy-2S,13R-dimethoxy-3, 9S,11R,15,17R,21R-hexamethyl-12,18-dioxo-docosa-3E,5E,7E,15E-tetraenyl]-3R-methyl-tetrahydro-pyran-2-yl]-oxo-acetyl}-piperidin-2S-carboxylic acid.

Structural elucidation of compound B

The negative ion FAB mass spectra of B isolated after *in vitro* incubation of rapamycin in rat bile or ammonium acetate solution were essentially identical. The mass spectrum of B is very similar to that of rapamycin with the exception of the molecular ion species being detected at m/z 912 instead of 913. The exact mass of this ion determined by high resolution MS was 912.5513, which is consistent with an elemental composition of $C_{51}H_{78}NO_{13}$ (i.e. one hydrogen less than rapamycin, theoretical mass = 912.5473). These data suggest that B is an isomer of rapamycin in its macrolide ring opened form (Figure 6) because the free carboxylic acid in ring opened rapamycin can favor the formation of $[M-H]^-$ and generate the carboxylate anion more easily than rapamycin.

Similarly, the ^{1}H-NMR spectra of B obtained from rat bile and 0.1M NH_4OAc solution incubations match, indicating that B isolated from the two sources is the same. The ^{1}H-NMR spectra of all B materials examined by NMR contain the H-25 double-doublet resonance at 6.75 ppm which is diagnostic for the presence of a C_{26}=C_{25} alkene functionality and characteristic of this ring opened form of rapamycin. The nomenclature of B is 23,25-Deepoxy-25,26-didehydro-23-hydroxyrapamycin.

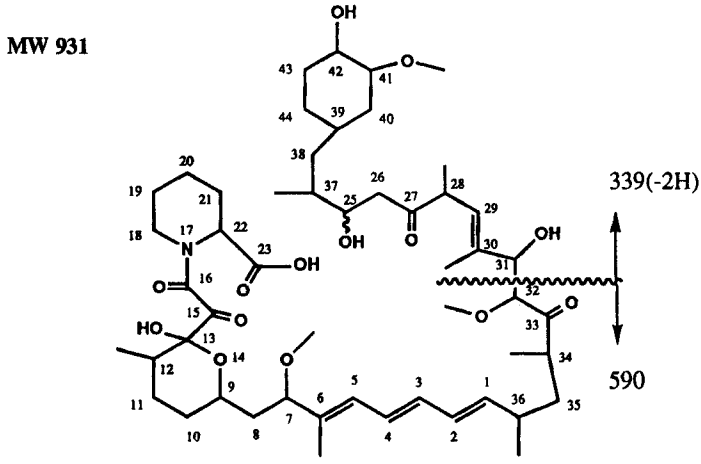

FIGURE 5 Chemical Structure of Degradation Product A and its Major Fragmentation Pathway

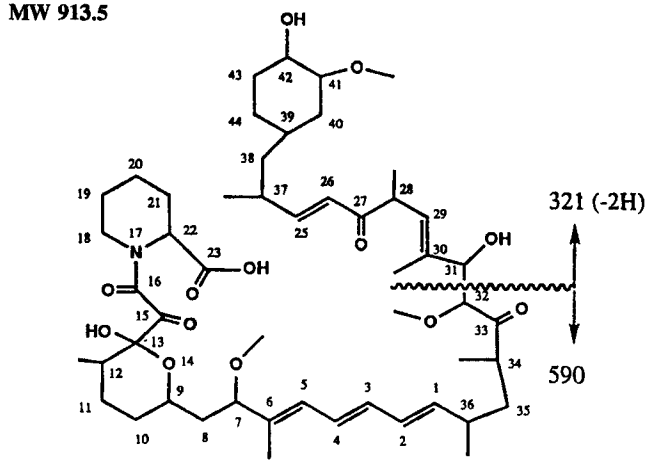

FIGURE 6 Chemical Structure of Degradation Product B and its Major Fragmentation Pathway

DISCUSSION

An analytical and a preparative HPLC procedures for the isolation of rapamycin degradation products A and B have been developed. Using ammonium acetate in the mobile phase is a critical factor to achieve satisfactory resolution and isolation of the degradation products. Since rapamycin degradation products are unstable, the sample handling procedure described earlier is critical for the successful isolation of the degradation products. A and B are generated by *in vitro* incubation of rapamycin with rat bile at 37°C for 13 hr. This degradation process is not necessarily catalyzed by endogenous materials because it also occurs in 0.1M NH_4OAc to form the same products. However, the endogenous compounds appear to affect the degradation of rapamycin since there is a difference in the relative peak areas of A and B when rapamycin is incubated in rat bile and 0.1M NH_4OAc (pH 8.0) (Figures 3 - 4).

The results described in this report show that the degradation products formed in either matrix are the same; they have been identified by HPLC, ^{1}H-NMR, and MS. Compound B was determined by MS and NMR to be a macrolide ring-opened isomer of rapamycin (Figure 6). Compound A has been characterized by MS and NMR to be a ring-opened hydrolysis product of rapamycin in which the C_{25} ester bond has been hydrolyzed (Figure 5). Both A and B are unstable to air, acid and base so that samples are best stored under nitrogen and kept at -80°C.

The immunosuppressive activities of A and B have been measured by the thymocyte proliferation assay; the potency of the degradation products was found to be less than 4% of that of rapamycin. Therefore, unless they are present *in vivo* at much higher concentrations than is rapamycin, they do not contribute significantly to the immunosuppressive action of rapamycin.

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