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The cyclohexane moiety of rapamycin is derived from shikimic acid in *Streptomyces hygroscopicus*

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SUMMARY

Although the addition of shikimic acid to the medium had no effect on the level of production of rapamycin by *Streptomyces hygroscopicus*, ^{14}C-shikimic acid was incorporated into rapamycin to a very high degree. ^{13}C-Shikimic acid was successfully prepared from 1-[^{13}C]-glucose using a mutant of *Klebsiella pneumoniae*, and used to label rapamycin. It was found that ^{13}C-shikimic acid was incorporated into the cyclohexane moiety of rapamycin, thereby establishing the shikimic acid pathway origin of the seven-carbon starter unit.

INTRODUCTION

Rapamycin was originally isolated from *Streptomyces hygroscopicus* as an antifungal agent [23], but has since been found to have antitumor and potentially useful immunosuppressant activities [2,6,7,8,13,16]. Rapamycin shares many structural features with FK506 [21], a compound also undergoing evaluation as an immunosuppressant, and may share a common mode of action [20]. We have previously demonstrated [18] that much of rapamycin is synthesized from acetate, propionate, and methionine in a fashion similar to many polyketide molecules. Recently, we have shown that the heterocyclic ring is derived from pipecolic acid which, in turn, comes from lysine [19]. The origin of the substituted cyclohexane ring, the apparent 'starter unit' of the biosynthesis, remained to be identified. Our proposed biosynthetic scheme [18] suggested shikimic acid as the precursor for this moiety.

Since ^{13}C-shikimic acid is not commercially available, previous studies on other systems have used various indirect approaches to demonstrate that precursors were from the shikimate pathway. In the case of the ansamycin rifamycin [25], the incorporation pattern of 1-[^{13}C]-glucose and 1-[^{13}C]-glycerate indicated that the C7 starter unit was derived from shikimate. These findings were confirmed by studies with non-producing mutants which were blocked at DAHP synthase (the first enzyme in the shikimate pathway), wherein antibiotic production could be restored by feeding appropriate precursors [10]. At the time our work was begun, no study involving the direct incorporation of ^{13}C-shikimic acid had been published. We chose the approach of using an aromatic amino acid auxotrophic mutant of *Klebsiella pneumoniae* to first 'biosynthesize' ^{13}C-shikimic acid from ^{13}C-glucose, and then determine if shikimate is incorporated into rapamycin. The present study establishes that shikimate is indeed the precursor of the substituted cyclohexane ring.

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MATERIALS AND METHODS

Production and analysis of labeled rapamycin by S. hygroscopicus

The rapamycin-producing strain, the culture conditions, assays and purification procedures for rapamycin, and ^{13}C-NMR parameters were all as described in a previous paper [18].

Preparation of ^{13}C-shikimic acid

The procedure for the preparation of ^{13}C-shikimic acid is a modification of published procedures for the production and purification of ^{14}C-shikimic acid from *Escherichia coli* [12,15]. The medium used was the original Davis A medium less citrate which inhibits the accumulation of shikimic acid and its 5-phosphate by *K. pneumoniae* ATCC 25597 (formerly *Aerobacter aerogenes* mutant A170-40) [24]. The medium contained (per liter): glucose (sterilized separately), 5 g; K_2PO_4, 7.0 g; KH_2PO_4, 3.0 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; $(NH_4)_2SO_4$, 1.0 g; L-tryptophan, 5 mg; L-tyrosine, 10 mg; D,L-phenylalanine, 20 mg; *p*-aminobenzoic acid, 10 μg; *p*-hydroxybenzoic acid, 10 μg. The medium was used at 100 ml per 500-ml flask. The flasks were inoculated with agar-

grown cells of *K. pneumoniae* ATCC 25597. Unlabeled glucose was autoclaved whereas ^{13}C-glucose (Cambridge Isotope Laboratories) was filter-sterilized.

The flasks were incubated at 37 °C on a rotary shaker at 250 rpm (2-in diameter orbit) for 3.5 days. The cultures were centrifuged, supernatants were pooled (400 ml) in screw-cap media bottles, small samples were removed, and the supernatants frozen. The samples were acidified and autoclaved for 2 h as described in the assay procedure. The control supernatant contained at least 300 mg L^{-1} free shikimic acid while the ^{13}C fermentation contained at least 250 mg L^{-1} shikimate. The supernatants were then thawed, acidified to pH 4.9 with acetic acid, and sampled. Fifty mg of acid phosphatase was added to both supernatants, a few drops of toluene were added to the bottles, the bottles were sealed and incubated at 37 °C. After 14 h, the bottles were sampled, 50 mg acid phosphatase was added, additional toluene was added, and the bottles were resealed and returned to 37 °C. This procedure was repeated periodically, adding 40 mg phosphatase at 23, 48, 64, and 110 h. Between 65 and 110 h, there was no significant increase in free shikimic acid in either of the supernatants. The final concentrations were 375 mg L^{-1} (150 mg total) in the ^{13}C fermentation and 500 mg L^{-1} in the control fermentation. Previous trials with unlabeled glucose had also produced approximately 500 mg L^{-1}. It is not known why the yield was lower in the ^{13}C fermentation.

The supernatant from the 1-[^{13}C]-glucose fermentation was loaded onto a column containing 180 ml AG1×8 ion exchange resin (acetate form; Biorad). The column was washed with 300 ml distilled water. Elution with 0.1 M ammonium acetate (pH 6.5) was then started. Shikimic acid was detected after about 1100 ml had passed through the column. Elution was continued until no more shikimic acid was detected in the effluent. The shikimic acid-containing fractions were combined (425 ml total). The assay showed approximately 250 mg L^{-1} (106 mg total).

The combined effluents were loaded onto a second ion-exchange column (100 ml AG1×8 resin, acetate form). The column was washed with 100 ml distilled water. No shikimic acid was detected in either the load or wash effluents. Elution with 0.1 M acetic acid was begun. The shikimic acid started to elute after approximately 600 ml had passed through the column. The fractions containing shikimic acid were pooled (1.2 L). The pool was then freeze-dried. The final product was made up of light brown to off-white crystals. The product was collected by dissolving the crystals in distilled water and pooling the solutions. The total volume was brought to 20 ml and the pool was assayed; the yield was approximately 110 mg. The product had the same UV spectrum as authentic shikimic acid with a maximum absorbance at 205 nm. From the relative absorbances at 205 nm, it was also calculated that the final yield of ^{13}C-shikimic acid was 110 mg.

Chemical assay for shikimic acid

The method used was basically that of Yoshida and Hasegawa [26], except that all reagent volumes were reduced by half. This assay is specific for shikimic acid. Shikimic-5-phosphate must be hydrolyzed by heating in acid or by incubation with acid phosphatase (Sigma). To hydrolyze the samples, they were mixed with an equal volume of 0.2 N HCl in glass tubes, then capped with plastic caps and autoclaved. Approximately 40–50% of the shikimate was released after 1 h of autoclaving, while autoclaving overnight released approximately 70–80% [15].

Preparation of the trimethylsilyl derivative of shikimic acid

We used the method of Cranswick and Zabkiewicz [4]. A Hewlett-Packard GC/MS system with microbore column and electron-impact ionization was used to analyze the samples.

RESULTS

Effect of the addition of shikimic acid

In our hypothetical biosynthetic scheme for rapamycin [18], we proposed an early intermediate of the shikimic acid pathway as the source of the C7 starter unit. None of our previous labeling experiments [18, 19] has contradicted this proposal. Shikimic acid was added to cultures to see if it would stimulate rapamycin production.

When added to cultures at the time of inoculation and after 2.5 days incubation at a concentration of 1.0 g L^{-1}, shikimic acid had no effect on either growth or rapamycin production. Possible explanations include the following: 1) shikimic acid is a precursor, but its formation is not rate-limiting for the biosynthesis of rapamycin; 2) shikimic acid is the precursor of the C7 unit, but is not entering the cells; 3) shikimic acid is not a precursor of rapamycin, the C7 unit being derived from an earlier intermediate in the shikimic acid pathway, or from an unrelated pathway.

Incorporation of ^{14}C-shikimic acid

In order to determine whether or not shikimic acid is entering the cells and if shikimic acid is incorporated into rapamycin, ^{14}C-shikimic acid was added to cultures. Fifty μl of ^{14}C-shikimic acid (1.0 μCi, 19.7 mCi $mmol^{-1}$; Amersham) was added to 4 ml of a filter-sterilized solution of shikimic acid (25 mg ml^{-1}, pH 5.0). One ml of this solution was added to each of three 50-ml cultures after 2.5 days of incubation (final concentration of 0.5 g L^{-1} shikimic acid). After 6 days of incubation, the contents of the flasks were pooled and the rapamycin extracted. A portion of the hexane extract was applied to a TLC plate while another portion was used to determine by HPLC the concentration of rapamycin in the solution. The plate was developed in 50/50 acetone/hexane, and the rapamycin band scraped off and transferred to a scintillation vial. From the specific activity of the recovered rapamycin, it appeared that one out of every three rapamycin molecules had incorporated a shikimic acid molecule. The entire experiment was repeated and an incorporation of one shikimic acid molecule per 4.4

rapamycin molecules was observed. (These calculations assume no incorporation through scrambling and that all incorporation of shikimate is directly into the C7 unit.)

More conclusive and more easily analyzed results could be obtained from ^{13}C-labeling experiments, but ^{13}C-shikimic acid was not commercially available. A small amount of ^{13}C-shikimic acid was therefore biosynthesized and used to label rapamycin.

Preparation of ^{13}C-shikimic acid

Since the *Klebsiella* mutant accumulates predominantly shikimic acid phosphate, it was necessary to treat with acid phosphatase to remove the phosphate group enzymatically before purification of the shikimic acid. All the procedures were optimized using unlabeled glucose prior to work with 1-[^{13}C]-glucose. A full-scale trial with unlabeled glucose produced approximately 125 mg of shikimic acid. ^{1}H-NMR of this material in D_2O was found to be similar to that of commercial shikimic acid.

Determination of the labeling pattern of ^{13}C-shikimic acid

In order to interpret the incorporation of ^{13}C-shikimate into rapamycin, it was necessary to know the sites of ^{13}C incorporation in the shikimic acid. ^{13}C-NMR was used to determine the relative enrichments of the carbons.

Figure 1 shows the ^{13}C-NMR spectrum of the ^{13}C-labeled shikimic acid. The spectrum contained seven peaks, corresponding to the seven carbons. Spectral assignments were made based on the relative chemical shifts of the peaks, except for the C3- and C5-hydroxyl groups which could not be absolutely assigned from the information available. The ^{13}C-NMR spectrum of commercial shikimic acid (Sigma Chemical Co.) was obtained after it was purified by passing it through the same column that had been used to purify the labeled sample. (The unpurified commercial acid had a slight brownish color, and showed additional peaks in the ^{13}C-NMR spectrum, indicating the presence of an impurity or strongly complexing metal contaminant.) The spectrum contained only seven peaks, in one-to-one correspondence with those in the labeled sample.

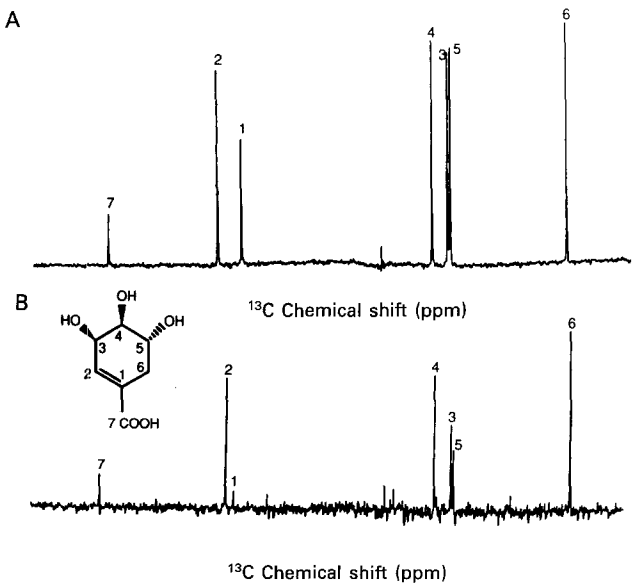

Fig. 1. (A) Natural abundance ^{13}C-NMR spectrum of shikimic acid. (B) ^{13}C-NMR spectrum of ^{13}C-enriched shikimic acid.

Figure 2(A) shows the calculated enrichments for the seven carbons based on the ^{13}C-NMR data. These were estimated by normalizing the peak heights in each spectrum by the C1 peak height, then dividing the normalized peak heights of the ^{13}C-shikimic acid by those of the unlabeled sample. These calculated relative enrichments actually represent the minimum possible enrichments, assuming no enrichment of C1. If there is some incorporation at C1, then the actual enrichments would be higher for each carbon, although the relative enrichments would stay the same. There is also some uncertainty (±10%) in the measurement of the height of the peak for C1, since the peak is small relative to the magnitude of the noise in the spectrum.

It had been predicted that C2 and C6 would be the most intensely labeled carbons, based on known pathways for glucose metabolism. These carbons were enriched (9.2- and 13.2-fold, respectively), but C7 and C4 were also very enriched (9.1- and 8.5-fold), and C3 and C5 were slightly enriched (5.8- and 4.1-fold). It appears that modes of scrambling other than those predicted occur in *K. pneumoniae*.

It had been estimated that 20–30% of the glucose utilized by enteric bacteria is assimilated by the oxidative pentose phosphate cycle. In this pathway, glucose is converted to 6-phosphogluconate, and then the C1 of glucose is lost

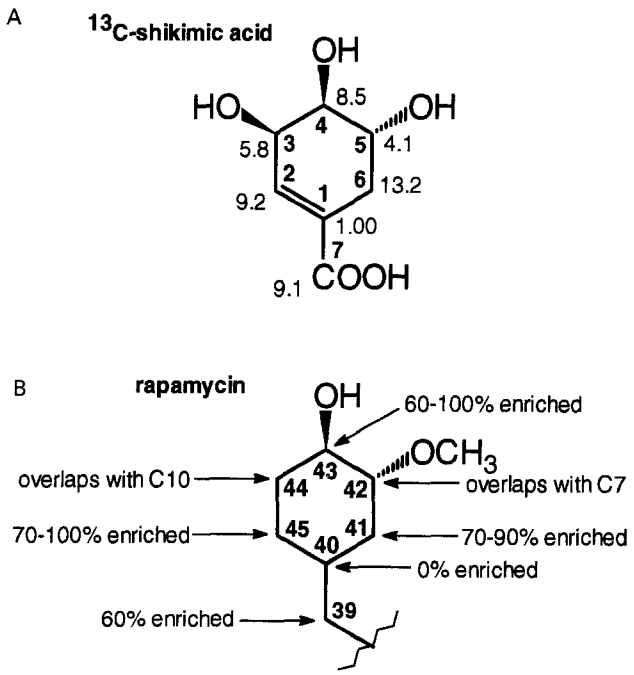

Fig. 2. Comparison of the ^{13}C-enrichment patterns of ^{13}C-shikimic acid and the cyclohexane region of rapamycin. (A) Relative enrichments of ^{13}C-shikimic acid biosynthesized from 1[^{13}C]-glucose. (B) Enrichments of the carbons of the cyclohexane moiety of rapamycin.

as CO_2, yielding ribulose-5-phosphate, which is further metabolized by transketolase and transaldolase reactions [11]. Since 1[^{13}C]-glucose was used to produce the ^{13}C-shikimic acid, some of the label was lost as $^{13}CO_2$. The maximum labeling possible at C6 (methylene carbon) of shikimic acid would be 35–40% (35- to 40-fold enrichment) if the Embden–Meyerhof (EM) pathway was the only other pathway used for glucose utilization. (If processed through the EM pathway, each molecule of ^{13}C-glucose would generate two molecules of phosphoenolpyruvate, only one of which would be labeled). This maximum level might not be reached if the oxidative pentose phosphate cycle is more active than normal, which is quite likely considering the type of *Klebsiella* mutant used. By stimulating the demand for erythrose-4-phosphate for the production of shikimic acid, the normal balance of the cell may have been shifted. The maximum enrichment may not have been reached if yet another route of glucose assimilation was also active, namely the Entner–Doudoroff (ED) pathway. This pathway is not normally used by enterics for glucose accumulation, but is induced by the presence of gluconate. Stimulation of the oxidative pentose cycle might also induce the ED pathway enzymes. The relatively high enrichments of C4 and C7 are evidence that the ED pathway is probably operating. The ED pathway would produce pyruvate labeled in the carboxyl group, which would label C4 and C7 of shikimate, in contrast to the EM pathway which only produces pyruvate labeled in the methyl group.

Gas chromatography/mass spectroscopy (GC/MS) was used to confirm the identity of the biosynthesized compound. Samples of both repurified commercial (unlabeled) and purified ^{13}C-labeled shikimic acid were converted to the tetra-substituted trimethylsilane derivative. The samples were subjected to GC/MS under identical conditions. (These GC profiles were produced using MS as the detector, monitoring the total ions from 50 to 500 amu.) The GC profiles for the two samples were identical, with a major peak at 7.8 min and a slight shoulder peak at 8.0 min.

The mass spectrum of each sample was taken at approximately the maximum of the major peak (retention time 7.7–7.8 min). The overall MS pattern appeared to match that reported in the Eight Peak Index of Mass Spectra:

m/z:	73	204	147	205	75	74	206	45	462
%:	100	88	25	17	10	9	8	7	3.1

where % = relative peak height expressed as the percent of the peak height of the most abundant peak (m/z = 73) and m/z = 462 is the parent peak. While the exact experimental conditions used to obtain these published values may have been different from those used in this study, electron impact ionization was used in both cases.

The above GC/MS data confirms that the ^{13}C-labeled compound is indeed ^{13}C-shikimic acid. Although the absolute enrichment of each of the carbons is not known, the relative enrichments from the NMR data provide sufficient information for the interpretation of the incorporation of ^{13}C-shikimic acid into rapamycin.

Incorporation of ^{13}C-shikimic acid

Flasks containing a total of 950 ml medium were inoculated with *S. hygroscopicus* and incubated 2.5 days. A solution of filter-sterilized ^{13}C-shikimic acid was added to each flask to give a final concentration of 0.075 g L^{-1}. After an additional 24 h, a second dose of ^{13}C-shikimic acid (0.025 g L^{-1}) was added to each flask. After a total of 6.5 days of incubation, the contents of the flasks were pooled and extracted as described earlier [18]. The rapamycin was purified using the procedures described earlier yielding approximately 9–10 mg. The enriched sample was then analyzed by ^{13}C-NMR using a 360-MHz spectrometer. Visual inspection of the spectrum and comparison with spectra from unenriched samples of rapamycin revealed four carbons that were significantly enriched (60–100%) and all were related to the C7 unit, namely carbons 39, 41, 43, and 45. No other carbons showed significant enrichment.

Carbons 39, 41, 43, and 45 correspond to the four most highly enriched carbons in the labeled shikimic acid (see Fig. 2(B)). C40 overlapped with C12 in the rapamycin spectrum and showed no enrichment, as expected, since the corresponding shikimate carbon was relatively unenriched. C42 overlapped with C7 and showed perhaps some slight increase as did the resonance corresponding to C44 and C10. The slight increases in these overlapping resonances could indicate incorporation of the two lesser enriched carbons of the ^{13}C-shikimate into carbons 42 and 44 of rapamycin. These results indicate that ^{13}C-shikimate was successfully incorporated into the cyclohexane moiety of rapamycin, and that this moiety is derived from a shikimate pathway intermediate, perhaps shikimic acid itself.

DISCUSSION

The combined results of our previous work [18,19] and that of McAlpine et al. [14] have identified the origins of the 51 carbons of rapamycin. These results are summarized in Fig. 3 and match our original predicted scheme quite well. ^{13}C-Shikimic acid, prepared from 1-[^{13}C]-glucose was successfully incorporated into the cyclohexane moiety of rapamycin. We conclude that the seven-carbon starter unit of rapamycin arises from an intermediate of the shikimic acid pathway, probably shikimic acid itself. This intermediate could be incorporated directly, or modified before incorporation.

The nearly symmetrical labeling of the ^{13}C-shikimate does not allow an absolute determination of the orientation of this unit when incorporated into rapamycin. However, based on the relative stereochemistry of the hydroxy and methoxy substituents it is most likely that carbons 1, 2, 3, 4, 5, 6, and 7 of shikimic acid correspond to rapamycin carbons 40, 45, 44, 43, 42, 41, and 39, respectively. In ^{13}C-shikimate, the enrichment of C6 is slightly higher than that of C2, and in rapamycin, the enrichment of C41 appears slightly higher than C45, supporting this hypothesis.

Saturated cyclohexane rings are rare among known natural products, especially those linked through carbon–carbon bonds to the rest of a molecule. One example of a terminal

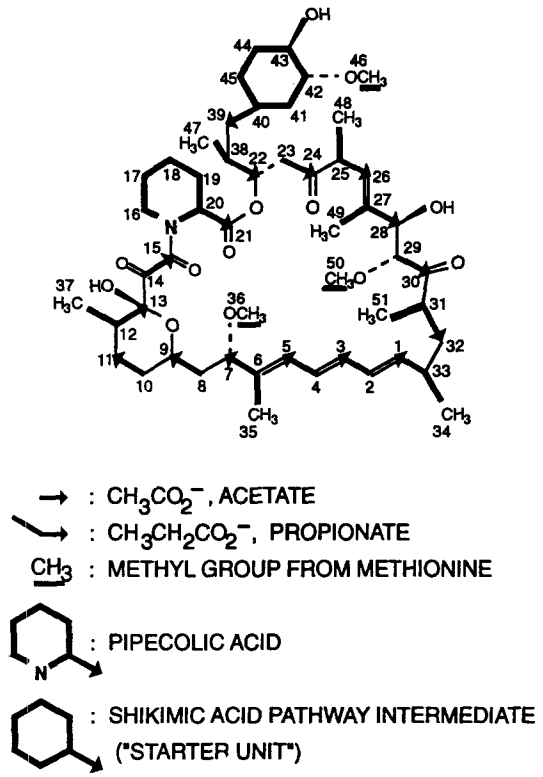

Fig. 3. Origins of the carbon atoms of rapamycin.

cyclohexane ring whose biosynthetic origin is known and which appears to serve as a starter unit is that of the fatty acids of acidophilic–thermophilic bacteria. The fatty acids of one such species, *Bacillus acidocaldarius*, contain as much as 95% omega-cyclohexyl fatty acids (11-cyclohexyl undecanoate and 13-cyclohexyl tridecanoate) [17]. The cyclohexyl ring was found to be derived from shikimate via cyclohexane carboxylic acid [9]. The shikimate origin of the cyclohexane carboxylic acid moiety of asukamycin was established by the incorporation of 2-[^{13}C]-shikimate [9]. The shikimic acid is converted into cyclohexane carboxylic acid, which is then linked to asukamycin through an amide linkage. Casati et al. [3] reported the successful incorporation of chemically synthesized shikimic acid into the cyclohexane carboxylic acid moiety of ansatrienin. In contrast to these three metabolites, the cyclohexane ring of rapamycin retains two oxygen substituents in the same stereochemical relationship as in the original shikimic acid, and thus does not appear to be converted to cyclohexane carboxylic acid before incorporation.

The incorporation of ^{13}C-shikimate into rapamycin is also significant in light of the results recently published for other 'cyclohexane carboxylic acid' type antibiotics. Valienamine is a component of both validamycins and acarbose, and other (hydroxymethyl)cyclitols, including validamine, have been found in related antibiotics. Studies on the biosynthesis of validamycins have shown that both validamine and valienamine are not derived from the shikimate pathway, but instead from a seven-carbon sugar related to the pentose phosphate pathway [22]. Independently, studies on acarbose provided almost identical results for the biosynthesis of valienamine in this secondary metabolite [5]. In both cases, the C7 unit is formed from a three-carbon unit (derived from glycerol or glyceraldehyde-3-phosphate) to which two C2 units are successively added, via transketolase reactions. These results are consistent with earlier observations that ^{14}C-shikimate was not readily incorporated into validamycin.

One benefit of biosynthetic studies is that information gained about one natural product is often applicable to structurally similar compounds. When discovered in 1975, rapamycin represented a unique combination of some rare structural features, including a saturated cyclohexane moiety linked to the molecule through a C–C bond, a diketo group forming a peptide bond to a pipecolic acid molecule, and a triene region. In 1987, a surprisingly similar immunosuppressing antibiotic, FK506, was isolated from *Streptomyces tsukubaensis* [21]. Approximately one half of this molecule is nearly identical to a large portion of rapamycin. The similarities include the substitutions and stereochemistry of their substituted cyclohexane rings, the locations of the L-pipecolic acid and diketo units, and the presence of a hemiketal ring. It is likely that the shared structural features are derived from the same biosynthetic precursors. The recent paper of Byrne et al. [1] on the biosynthesis of immunomycin (FK-520), the ethyl analog of FK-506, is in agreement with our data on rapamycin and agrees with our conclusion on the orientation of the shikimate when incorporated into rapamycin.

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