Biosynthetic Studies of Ascomycin (FK520): Formation of the (1*R*,3*R*,4*R*)-3,4-Dihydroxycyclohexanecarboxylic Acid-Derived Moiety

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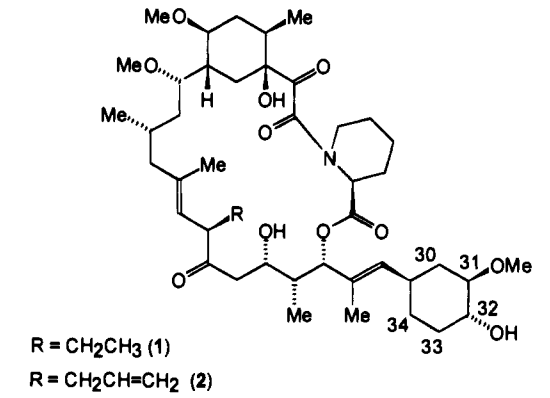
The potent immunosuppressants ascomycin (FK520) (immunomycin) (**1**, produced by *Streptomyces hygroscopicus* subsp. *yakushimaensis*),[1] FK506 (**2**, produced by *Streptomyces tsukabaensis*),[2] and rapamycin (**3**, produced by *S. hygroscopicus* subsp. *hygroscopicus*)[3] are secondary metabolites that contain a structural moiety likely derived from (1*R*,3*R*,4*R*)-3,4-dihydroxycyclohexanecarboxylic acid (**4**, DHCHC) (Figure 1). In the case of FK520 and rapamycin, incorporation experiments have indicated that this moiety is derived from shikimic acid.[4,5] In FK520 the methylation of the C-4 hydroxyl group of the DHCHC moiety occurs after assembly of the macrolide structure.[4] The pathway from shikimic acid to DHCHC has not been delineated in any of these systems. We report here results of incorporation studies[6] in *S. hygroscopicus* (the FK520 producer) using specifically-labeled shikimic acids and putative pathway intermediates[7] (Figure 2) that are consistent with a proposed pathway (Figure 3).[8]

Evidence that DHCHC (**4**) is formed from shikimic acid was obtained from an incorporation study with [2-^{13}C]shikimic acid (**5**). The ^{13}C NMR spectrum of the FK520 produced in this study exhibited a single enhancement (13-fold above natural abundance) for the C-34 signal.[9]

The hydrogens at C-2, C-4, and C-5 of shikimic acid were shown to be retained during the conversion to DHCHC by two



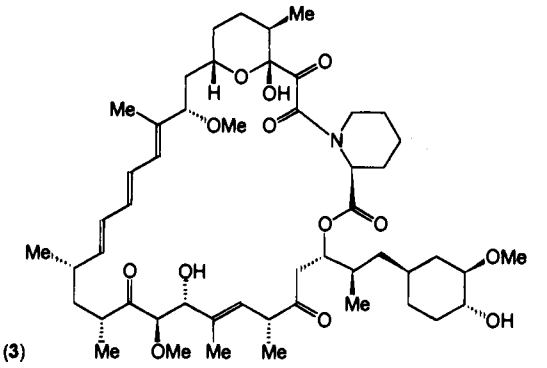


Figure 1. Structures of the novel immunosuppressants FK520 (1), FK506 (2), and rapamycin (3).

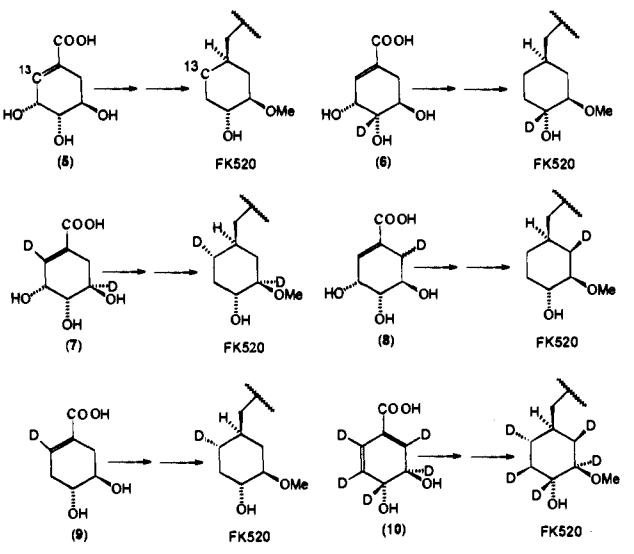


Figure 2. Interpretation of the results of incorporation of [2-^{13}C]-shikimic acid (**5**), (−)-[4-^{2}H]shikimic acid (**6**), (±)-[2,5-^{2}H$_2$]shikimic acid (**7**), (−)-(6*R*,*S*)-[6-^{2}H$_1$]shikimic acid (**8**), *trans*-4,5-dihydroxy[2-^{2}H]cyclohex-1-enecarboxylic acid (**9**), and *trans*-3,4-dihydroxy[2,3,4,5,6-^{2}H$_5$]cyclohexa-1,5-dienecarboxylic acid (**10**) into FK520 by *S. hygroscopicus*.

incorporation studies: FK520 produced in the presence of (-)-[4-^{2}H]shikimic acid (**6**) exhibited a single ^{2}H NMR resonance at 3.37 ppm, consistent with deuterium occupying the C-32 position;[10] FK520 produced in the presence of (±)-[2,5-^{2}H$_2$]-shikimic acid (**7**) exhibited two ^{2}H NMR resonances (1.63 and 3.00 ppm), consistent with deuterium occupying the C-34*S* (equatorial) and C-31 positions, respectively. The pathway to DHCHC was shown to involve loss of one hydrogen from C-6 of shikimic acid by an incorporation experiment with (−)-(6*R*,*S*)-

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(6) Incorporation experiments were conducted by addition of labeled compounds, to a final concentration of between 1.0 and 1.4 mM, to separate 24 h fermentations of *S. hygroscopicus*. After an additional 5 days the cells were harvested and the FK520 was isolated, purified, and characterized by ^{1}H, ^{2}H, and ^{13}C NMR spectroscopy.

(7) All labeled compounds except compound **10** were kindly provided by Professor Heinz Floss at the University of Washington. Compound **10** was prepared according to literature procedures.[13]

(8) It is possible that all or most of the transformations from shikimic acid to DHCHC occur with the carboxylic acid group of the intermediates activated as either coenzyme A or acyl carrier protein (ACP) thioesters.

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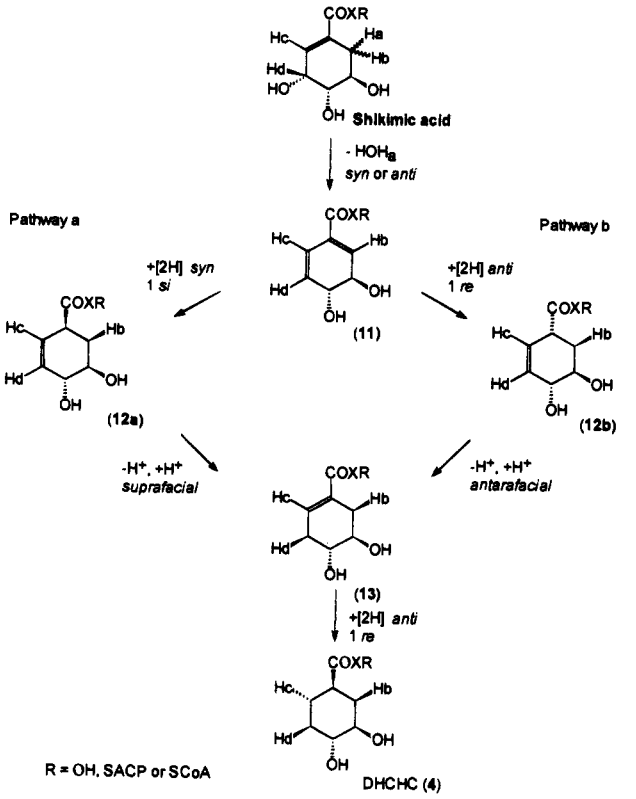

Figure 3. Proposed pathway to DHCHC (**4**) from shikimic acid in *S. hygroscopicus*.

[6-$^2H_1$]shikimic acid (**8**). The resultant FK520 exhibited only a single ^2H NMR resonance at 0.9 ppm, consistent with deuterium occupying only the C-30*R* (axial) position.

Two putative intermediates in the pathway to DHCHC from shikimic acid were successfully incorporated into FK520: *trans*-4,5-dihydroxy[2-^2H]cyclohex-1-enecarboxylic acid (**9**) produced FK520 which exhibited a single deuterium resonance at 1.63 ppm, consistent with a deuterium occupying the C-34*S* (equatorial) position of FK520; *trans*-3,4-dihydroxy[2,3,4,5,6-$^2H_5$]-cyclohexa-1,5-dienecarboxylic acid (**10**)[7] produced FK520 which exhibited five separate deuterium resonances (3.37, 3.0, 1.95, 1.63, and 0.9 ppm), consistent with deuterium occupying the C-32, C-31, C-33*S* (equatorial), C-34*S* (equatorial), and C-30*R* (axial) positions, respectively.

These results are consistent with the proposed pathway which commences with either *syn* or *anti* 1,4-conjugate elimination of the C-3 hydroxyl group and a C-6 hydrogen of shikimic acid to produce (3*R*,4*R*)-3,4-dihydroxycyclohexa-1,5-dienecarboxylic acid (**11**). A reduction of the Δ^1 double bond of **11** produces (4*R*,5*R*)-4,5-dihydroxycyclohex-2-enecarboxylic acid (**12a** or **12b**), which is subsequently converted to (4*R*,5*R*)-4,5-dihydroxycyclohex-1-enecarboxylic acid (**13**) by an isomerization of the remaining double bond from the Δ^2 to the Δ^1 position. The incorporation of a deuterium label from **8** into the C-30*R* position of FK520 indicates that the reduction of **11** proceeds with hydrogen addition to the *si* face of C-2. The stereochemical course of this double-bond reduction at C-1 and of the subsequent isomerization step cannot be determined from these incorporation results. However, the incorporation of a deuterium label from C-5 of **10** in the C-33*S* (equatorial) position of FK520 can only have arisen from two possible pathways: a double-bond reduction of **11** with hydrogen addition to the *si* face of C-1 (*syn* addition) to produce **12a**, and a subsequent suprafacial 1,3-allylic rearrangement of **12a** to produce **13** (Figure 3, pathway a); a double-bond reduction of **11** with hydrogen addition to the *re* face of C-1 (*anti* addition) to produce **12b**, and a subsequent antarafacial 1,3-allylic rearrangement of **12b** to produce **13** (Figure 3, pathway b). All previously studied rearrangements of this type,[11,12] including the interconversion of 2-cyclohexenylcarbonyl-CoA and 1-cyclohexenylcarbonyl-CoA,[13-16] have been shown to be suprafacial. In the final step of the proposed pathway, **13** is reduced to DHCHC (**4**) by an *anti* addition of hydrogen to the *re* faces of C-1 and C-2.[17]

The two Δ^1 double bond reduction steps in the pathway from shikimic acid to DHCHC show some notable stereochemical differences from the three Δ^1 double bond reduction steps involved in the conversion of shikimic acid to fully reduced cyclohexanecarboxylic acid (CHC) that has been studied in *Streptomyces collinus* and *Alicyclobacillus acidocaldarius*.[13,14,18] In the CHC pathway all three Δ^1 double bond reductions (including the reduction of **11**) proceed in an *anti* fashion with addition of hydrogen to the *si* face of C-1. The proposed Δ^1 double bond reductions of **13** to DHCHC and **11** to **12b** (pathway b) in the DHCHC pathway also proceed in an *anti* fashion, but with opposite absolute stereochemistry (addition of hydrogen to the *re* face of C-1). In the alternative pathway for DHCHC formation (pathway a) the reductive conversion of **11** to **12a** proceeds with *syn* addition of hydrogen. The biosyntheses of DHCHC and CHC apparently diverge at or before the first intermediate, *trans*-3,4-dihydroxycyclohexa-1,5-dienecarboxylic acid (**11**).

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Supplementary Material Available: The ^{13}C and deuterium NMR spectra of FK520 samples from the incorporation studies (5 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

(10) The deuterium/proton resonances for the (29*R*,31*R*,32*R*)-31-methoxy-32-hydroxycyclohexane moiety of FK520 are based on the published ^{13}C and ^1H NMR assignments.[9] These assignments are consistent with the cyclohexane ring adopting a locked chair conformation with all three substituents in the equatorial positions. In this conformation the C-34, C-33, and C-30 equatorial hydrogens are deshielded between 0.5 and 1.0 ppm with respect to the axial hydrogens (Bovey, F. A. *Nuclear Magentic Resonance Spectroscopy*, 2nd ed.; Academic Press: San Diego, 1988; Chapter 3).

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(17) An alternative pathway in which **12a** is directly reduced to DHCHC by a *syn* addition to the Δ^2 double bond and that therefore does not involve **13** as an intermediate cannot be ruled out from these experiments. Such a pathway, however, would require that the exogenous **9** added to the fermentations was converted to DHCHC by a process unrelated to the normal pathway. The simplest interpretion of all of the incorporation results is that shown in Figure 3.
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