Biosynthesis of the Shikimate-derived Starter Unit of the Immunosuppressant Ascomycin: Stereochemistry of the 1,4-Conjugate Elimination

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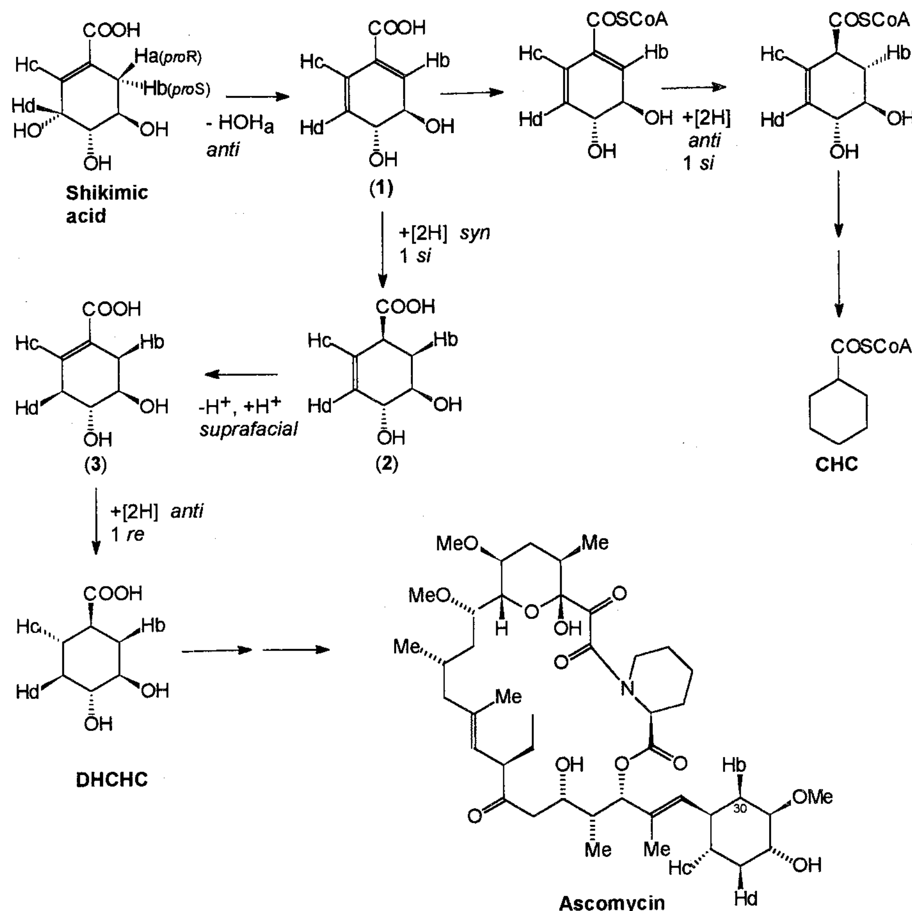
The potent immunosuppressants ascomycin, FK506 and rapamycin are polyketide secondary metabolites that are biosynthesized from an usual shikimate-derived (1*R*,3*R*,4*R*)-3,4,-dihydroxycyclohexanecarboxylic acid (DHCHC) starter unit[1~4]. The biosynthetic pathway to this DHCHC moiety of ascomycin has been investigated in *Streptomyces hygroscopicus* var. *ascomyceticus* by a series of incorporation experiments with isotopically labeled shikimic acid and various putative pathway intermediates[1]. The results obtained were consistent with a process that initiates with either a *syn* or an *anti* 1,4-conjugate elimination of a C6 hydrogen and the C3 hydroxyl group of shikimic acid to produce (3*R*,4*R*)-3,4-dihydroxy-1,5-cyclohexadienecarboxylic acid (**1**) (Fig. 1). A *syn* reduction of the Δ^1-double bond of **1** gives of (1*S*,4*R*,5*R*)-4,5-dihydroxycyclohex-2-enecarboxylic acid (**2**), which is subsequently converted to (4*R*,5*R*)-4,5-dihydroxycyclohex-1-enecarboxylic acid (**3**) by an isomerization of the remaining double bond from the Δ^2 to the Δ^1 position. In the final step of the process **3** is converted to DHCHC by an *anti* addition of hydrogen to the *re* faces of C1 and C2. An alternative pathway in which the first double bond is reduced by an *anti* addition of hydrogen (**1**→**2**), while consistent with the experimental results, seems unlikely as it would require a subsequent unprecedented antarafacial rearrangement to generate **3**[1].

The only stereochemical detail of DHCHC biosynthesis that remains undetermined is the steric course of the initial 1,4-conjugate elimination. Previous incorporation studies with (−)-6(*R*,*S*)-[6-$^2H_1$]shikimic acid had generated ascomycin which exhibited a single ^2H single at 0.9 ppm, consistent with deuterium occupying the C30*R* (axial) position[1]. This signal clearly demonstrated that the pathway to DHCHC involves loss of one hydrogen (no signal was observed for deuterium occupying the C30*S* position), but it does not distinguish which of the heterotropic C6 hydrogens is removed. We have now circumvented this problem by developing a synthetic strategy for preparing stereospecifically deuterated (−)-(6*R*)-[6-$^2H_1$] and (−)-(6*S*)-[6-$^2H_1$]shikimic acid from mannose[5]. In separate experiments each stereospecifically labeled shikimic acid (30 mg) was added to 5×25 ml 24 hours cultures of *S. hygroscopicus* var. *ascomyceticus* to a final concentration of 1.4 mM. After an additional 5 days of fermentation the cells were combined with those obtained from a 2×25 ml six day fermentation, and the ascomycin was isolated, purified and characterized by ^1H and ^2H NMR as described previously[1]. Inspection of the 46.07 MHz ^2H NMR spectra of ascomycin (71 mg) from the feeding experiment with (−)-(6*R*)-[6-$^2H_1$]shikimic acid exhibited no discernible signal even after prolonged accumulation. In contrast, the ^2H NMR spectrum of ascomycin (56 mg) from the (−)-(6*S*)-[6-$^2H_1$]shikimic acid experiment was indistinguishable from that obtained previously using (−)-6(*R*, *S*)-[6-$^2H_1$]shikimic acid, with a single ^2H signal at 0.9 ppm.

These results clearly indicate that the initial 1,4-elimination step proceeds in an *anti* fashion with loss of the *pro*-6*R* hydrogen of shikimate. The same *anti* elimination has been shown to occur in the conversion of shikimate to **1** during the biosynthesis of cyclohexanecarboxylic acid (CHC), a process which occurs in *Streptomyces collinus* and *Alicyclobacillus acidocaldarius* (Fig. 1)[5]. The CHC and DHCHC pathways apparently diverge after this first step. Analysis of the rapamycin polyketide synthase provides strong evidence that this enzyme utilizes DHCHC as a free acid suggesting that the *syn* reduction of the Δ^1-double bond of **1** in this pathway occurs with the free acid[3,6]. Evidence indicates that in the CHC pathway **1** is first converted to a coenzyme A thioester and that the Δ^1-double bond of this compound is then reduced in an *anti* fashion (Fig. 1)[7~9]. It has been proposed that these stereochemical differences reflect differing progenitors for the enoyl reductases in these two pathways[10]. By the same token the dehydratases responsible for converting shikimate to **1** in the same stereochemical fashion in these two pathways may be related. It has previously been noted that chorismate synthase also catalyzes an *anti* 1,4-elimination reaction using 5-enolpyruvylshikimate

Fig. 1. Proposed commonality in the initial step of the pathways to DHCHC and CHC from shikimic acid.



3-phosphate in a later step in the shikimate pathway[5]. The evolutionary relationship of all of these dehydratases, however, will only be determined once the corresponding genes have been cloned. Some progress has been made in this direction; an enoyl CoA reductase involved in the CHC pathway has already been cloned and sequenced[7], and analysis of the surrounding genes has provided evidence of a CHC biosynthetic gene cluster (unpublished data).

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