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The shikimic acid pathway and polyketide biosynthesis

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The shikimic acid pathway, ubiquitous in microorganisms and plants, provides precursors for the biosynthesis of primary metabolites such as the aromatic amino acids and folic acid. Several branchpoints from the primary metabolic pathway also provide aromatic and, in some unusual cases, nonaromatic precursors for the biosynthesis of secondary metabolites. We report herein recent progress in the analysis of two unusual branches of the shikimic acid pathway in streptomycetes; the formation of the cyclohexanecarboxylic acid (CHC)-derived moiety of the antifungal agent ansatrienin and the dihydroxycyclohexanecarboxylic acid (DHCHC) starter unit for the biosynthesis of the immunosuppressant ascomycin. A gene for 1-cyclohexenylcarbonyl-CoA reductase, *chcA*, which plays a role in catalyzing three of the reductive steps leading from shikimic acid to CHC has been characterized from *Streptomyces collinus*. A cluster of six open reading frames (ORFs) has been identified by sequencing in both directions from *chcA* and the putative role of these in CHC biosynthesis is discussed. The individual steps involved in the biosynthesis of DHCHC from shikimic acid in *Streptomyces hygroscopicus* var *ascomyceticus* has been delineated and shown to be stereochemically and enzymatically distinct from the CHC pathway. A dehydroquinate dehydratase gene (*dhq*) likely involved in providing shikimic acid for both DHCHC biosynthesis and primary metabolism has been cloned, sequenced and characterized.

Keywords: shikimic acid; streptomycetes; immunosuppressant; polyketide

Introduction

The classical shikimate pathway of plants and bacteria represents a reaction sequence that proceeds from two precursors of carbohydrate biosynthesis, d-erythrose 4-phosphate and phospho*enol*pyruvate, through shikimate to aromatic compounds [3] (Figure 1). Products of the shikimate pathway include not only the aromatic amino acids but also other metabolites such as folate, *p*-aminobenzoate and enterobactin [10].

The shikimate pathway and variations of this pathway also play a critical role in providing aromatic precursors for the biosynthesis of natural products of medicinal importance [32], particularly in the bacterial genus Streptomyces. For instance, shikimate-derived *p*-aminobenzoic acid is used as a precursor for the biosynthesis of the polyene antibiotic candicidin [19], and tryptophan, derived from the shikimic acid pathway, is used to provide aromatic precursors for both actinomycin and streptonigrin biosynthesis [13,14]. Pathways closely related to the shikimate pathway also provide aromatic precursors for natural product biosynthesis. 3-Amino-5-hydroxybenzoic acid (AHBA), also derived from erythrose 4-phosphate and phosphenolpyruvate, is used in the biosynthesis of a wide variety of antibiotics such as rifamycin, geldanamycin and mitomycin C [15].

In some unusual circumstances shikimic acid is converted to a nonaromatic precursor for natural product biosynthesis. Cyclohexanecarboxylic (CHC) acid derived from shikimic acid is used in the biosynthesis of ansatrienin in *Streptomyces collinus* (Figure 2) [33,34]. Cyclohexyl rings, which are likely derived from shikimic acid, are also observed in other antibiotics such as asukamycin and phoslactomycin [22]. (1*R*,3*R*,4*R*)-3,4 Dihydroxycyclohexanecarboxylic (DHCHC) acid, derived from shikimic acid, is used in the biosynthesis of the immunosuppressant ascomycin (Figure 3) by *Streptomyces hygroscopicus* var *ascomyceticus* [23]. DHCHC is also used in the biosynthesis of FK506 (structurally related to ascomycin) and rapamycin [6,8].

In this paper we report recent developments concerning the unusual pathways that lead from shikimic acid to CHC and DHCHC.

The cyclohexanecarboxylic (CHC) acid pathway

The side-chain of the antibiotic ansatrienin contains a shikimic acid-derived cyclohexyl moiety. A comprehensive series of incorporation studies using both labeled shikimic acid and pathway intermediates has delineated the individual steps in the conversion of shikimic acid to CHC (Figure 2). It has been suggested that most of the intermediates in this pathway are activated as coenzyme A thioesters [21,22,32].

The CHC pathway involves three separate steps that involve reduction of α, β-double bonds of either an enoyl or dienoyl CoA substrate: 1-cyclohexenylcarbonyl CoA (**A**), 5-hydroxy-1-cyclohexenylcarbonyl CoA (**B**), and 4,5-dihydroxy-1,5-cyclohexadienylcarbonyl CoA (**C**). *In vitro* studies have shown that these reductions proceed with the same stereochemistry, *anti* fashion with addition of solvent hydrogen to the *si* face of the α-carbon (Figure 2) [20,22]. Based on this observation, it has been suggested that one

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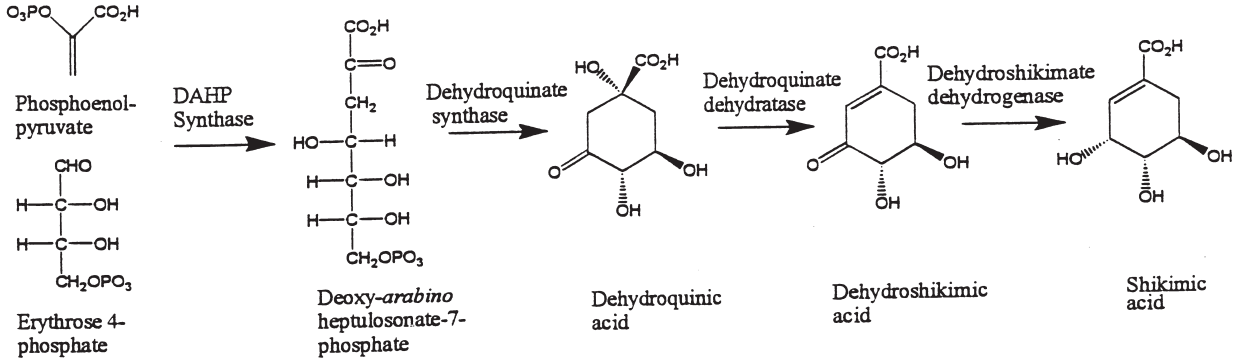

Figure 1 The first four steps of the shikimic acid pathway.

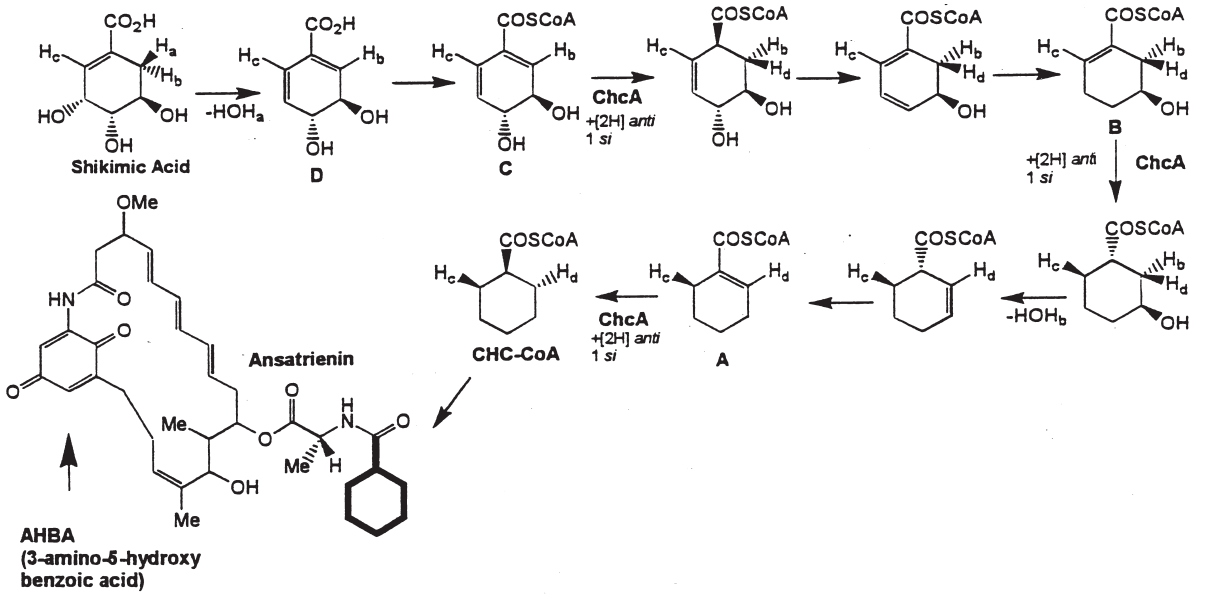

Figure 2 Biosynthesis of the cyclohexanecarboxylic acid (CHC)-derived moiety of ansatrienin from shikimic acid.

enoyl CoA reductase may be responsible for catalyzing all three reductions [20,22,33]. Consistent with these observations has been the isolation and characterization from *Streptomyces collinus* of an enzyme, 1-cyclohexenylcarbonyl CoA reductase (ChcA), capable of catalyzing all three reductions *in vitro* [32]. The corresponding *chcA* gene has been cloned and sequenced [33]. The predicted amino acid sequence of ChcA has statistically significant matches within the short-chain alcohol dehydrogenase protein superfamily, but no significant sequence similarity with enoyl thioester reductases from either fatty acid synthases or polyketide synthases. Supporting evidence for the role of ChcA in the CHC pathway has been provided by analysis of a recombinant ChcA which demonstrates properties indistinguishable from those observed with the native protein, and by the generation and analysis of a *chcA* mutant [33]. The *chcA*-deficient *S. collinus* mutant lost the ability to produce ansatrienin or ω-cyclohexylfatty acids (fatty acids generated from a CHC starter unit), clearly demonstrating that *chcA* is involved in CHC biosynthesis. Further analysis demonstrated that cell-free extracts of this mutant were unable to reduce the α,β-double bond of either **A** or **B**, activities present in cell-free extracts of *S. collinus* under identical conditions.

Genes which code for enzymes involved in the biosynthesis of a given metabolite in Streptomyces are often clustered [4,7,9,18,28]. This phenomenon raises the question of whether *chcA* is clustered with other genes involved in CHC biosynthesis. We have recently sequenced 9 kb of DNA surrounding *chcA* and discovered six open reading frames transcribed in the same direction as *chcA* (Figure 4). ORF1 encodes a protein with strong similarity to plant 3-deoxy-*arabino*-heptulosonate-7-phosphates (approximately 45% sequence similarity to various plant DAHP synthases). DAHP synthase catalyzes the first committed step in the shikimic acid pathway (Figure 1) and it is possible that ORF1 encodes an isozyme of DAHP synthase that ensures a supply of shikimic acid for CHC biosynthesis. Ansatrienin also contains a structural moiety derived from AHBA (Figure 2) and ORF1 may encode a modified DAHP synthase that catalyzes the formation of aminoDAHP (the first intermediate in the AHBA biosynthetic pathway) [2,15].

ORF2 has similarity to 3-enolpyruvylshikimate-5-phosphate synthase (EPSPS), an enzyme involved in the conver-

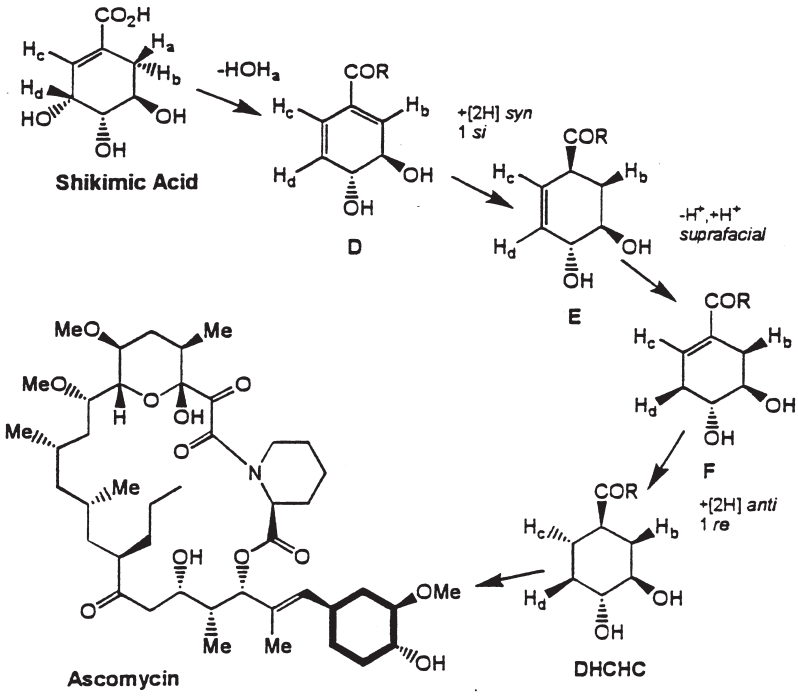

Figure 3 Biosynthesis of the dihydroxycyclohexanecarboxylic acid (DHCHC)-derived moiety of ascomycin from shikimic acid.

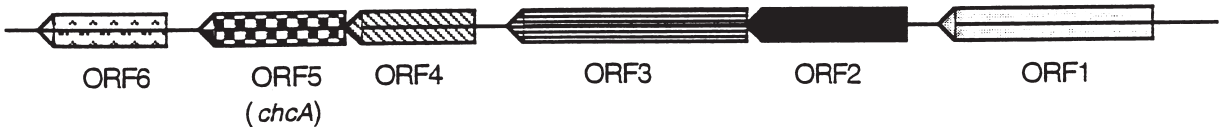

Figure 4 Physical map of the *S. collinus chcA* region.

sion of shikimic acid to chorismic acid. Chorismic acid is not used in CHC biosynthesis [21] and the sequence similarity to EPSPS is relatively weak (less than 25% sequence similarity). ORF2 *may* be involved in the first step of the CHC pathway catalyzing the conversion of either shikimic acid or shikimate 3-phosphate to 4,5-dihydroxycyclohexa-1,5-dienecarboxylic acid (**D**) and not act as a true EPSPS. It is not yet clear whether shikimic acid or shikimate-3-phosphate is the substrate for the first step in CHC biosynthesis [21]. Thus the substrate for formation of both chorismate and **D** is likely the same. This similarity and the fact that both reactions involve dehydrations led to our speculation. Evidence for this speculation however requires additional experimentation. ORF3 has strong amino acid sequence similarity to a number of 4-coumarate CoA ligases (29% identity with the *Mycobacterium leprae* enzyme, GeneBank Accession No. U15181) and dihydroxybenzoyl AMP ligases (44.5% identity to the 2,3-dihydroxybenzoate AMP ligase of *Escherichia coli*) [29]. It is hypothesized that the gene product of ORF3 catalyzes the conversion of **D** to the corresponding CoA thioester (**C**) (Figure 2).

ORF4 has approximately 30% amino acid sequence similarity to acyl CoA dehydrogenases involved in fatty acid degradation. ORF6 (downstream of *chcA*/ORF5) has some amino acid sequence similarity to NADPH oxidoreductases (41% to the yeast NADPH-dependent flavin oxidoreductase). The physiological role of NADPH oxidoreductases is elusive although recent studies suggest they may be involved in a novel dismutation reaction [30] and that NADPH is its physiological reductant (the *in vivo* electron acceptor is unknown). An NADPH-FAD oxidoreductase gene, *vlmR*, has recently been found clustered with the valinomycin biosynthetic genes in *Streptomyces viridifaciens* [24]. The assignment of putative roles for ORFs 4 and 6 in CHC biosynthesis based on sequence similarity is less obvious than ORFs 1–3, but it does not preclude them from playing a role. The sequence similarity of ChcA (encoded by ORF5) to alcohol dehydrogenases but not to enoyl CoA reductases demonstrates that sequence similarity used to assign function to a gene product can be misleading.

The roles of ORFs 1–6 in CHC biosynthesis, with the exception of ORF5 (*chcA*), remain to be determined. Nonetheless, our analysis of these ORFs suggests a putative role for some of them in CHC biosynthesis. We are also interested in determining whether *chcA* is clustered with other genes involved in ansatrienin biosynthesis, particularly the polyketide synthase that is involved in the assembly of the ansatrienin carbon backbone. Analysis of 20 kb of DNA downstream of *chcA* has shown no evidence of polyketide synthase genes. However recent work carried out at the University of Bonn (E Leistner and D Bloomberg, unpublished data) has identified a polyketide synthase gene cluster upstream of *chcA*. These data suggest that clustering of CHC and ansatrienin biosynthetic genes occurs in *S. collinus*.

The dihydroxycyclohexanecarboxylic acid (DHCHC) pathway

The potent immunosuppressants, ascomycin (Figure 3), rapamycin, and FK506 are structurally similar polyketides assembled from a shikimate-derived starter unit [6,8,27]. Recent evidence has shown that this starter unit is (1*R*,3*R*,4*R*)-3,4-dihydroxycyclohexanecarboxylic acid (DHCHC) [17,32]. We have used a series of biosynthetic studies, with both isotopically labeled shikimate and putative pathway intermediates, to elucidate the pathway to DHCHC in *S. hygroscopicus* var *ascomyceticus* [26,32] (Figure 3).

The DHCHC pathway initiates in the same way as the CHC pathway with an *anti* 1,4-elimination of water from either shikimate or shikimate 3-phosphate to provide 4,5-dihydroxycyclohexa-1,5-dienecarboxylic acid (**D**) [26]. The pathway to DHCHC then diverges from the CHC pathway with a *syn* fashion reduction of the α,β-double bond of **D** to give (4*R*,5*R*)-4,5-dihydroxycyclohex-2-enecarboxylic acid (**E**). A suprafacial 1,3-allylic rearrangement of **E** then provides (4*R*,5*R*)-4,5-dihydroxycyclohex-1-enecarboxylic acid (**F**). This compound is converted to DHCHC by an *anti* reduction (addition of hydrogen to the 1 *re* face) of the α,β-double bond. The α,β-double bond reduction steps in this pathway are stereochemically distinct both from each other and the reduction steps catalyzed by ChcA in the CHC biosynthetic pathway. We have proposed that this stereochemical diversity indicates that the enzymes involved in the CHC and DHCHC biosynthetic processes are unrelated [16,25,26,32].

To date neither the genes nor enzymes involved in the DHCHC pathway have been identified. It seems reasonable to propose that the genes involved in DHCHC biosynthesis may be clustered within the immunosuppressant biosynthetic gene cluster (as appears to be the case for the CHC and ansatrienin biosynthetic genes). The entire rapamycin polyketide synthase gene cluster has been sequenced [1,17] and shown to contain numerous ORFs for which no clear functions can be attributed. Nonetheless it has not yet been determined if any of these ORFs are involved in DHCHC biosynthesis.

Sequence analysis of the genes responsible for initiating the biosynthesis of the rapamycin carbon backbone has revealed a CoA ligase domain, suggesting that DHCHC is loaded onto the polyketide synthase in the form of a free carboxylic acid [1,17]. Therefore, the DHCHC pathway, unlike the CHC pathway appears to occur with most or all of the intermediates as free carboxylic acids. This difference together with the stereochemical differences clearly suggests that despite the obvious similarities, enzymes catalyzing analogous reactions in the CHC and DHCHC pathways have evolved separately.

The shikimic acid pathway—dehydroquinate dehydratase

In addition to studying pathways that diverge from shikimic acid in streptomycetes we have undertaken a study of the shikimic acid pathway itself. Despite the clear role of the shikimic acid pathway in providing precursors for natural product biosynthesis, the enzymes involved in the pathway have not been extensively studied [31].

We have cloned the *Streptomyces hygroscopicus* var *ascomyceticus dhq* gene. This gene encodes dehydroquinate dehydratase (DHQase) which catalyzes the conversion of dehydroquinic acid to dehydroshikimic acid, the third step in the shikimic acid pathway (Figure 1) and the second step in the quinic acid catabolic pathway [11]. Type II DHQases are small heat-stable enzymes (subunit M_r 16 000–18 000) that typically oligomerize into dodecameric structures. The type I enzymes by contrast are heat-labile. There is no clear pattern regarding the role of type I and type II DHQases in the shikimic acid biosynthetic pathway and quinic acid catabolic pathway [11,12].

Type I and type II DHQases show no clear amino acid sequence similarity while clear similarity is observed within each class. This similarity allowed us to design PCR primers based on a multiple alignment of type II DHQase peptide sequences and use these to amplify a 0.35-kb fragment of the *S. hygroscopicus dhq*. This PCR product, in turn, was used to isolate the entire *dhq* gene from a λ-library of *S. hygroscopicus* genomic DNA. The predicted amino acid sequence of DHQase encoded by cloned *S. hygroscopicus dhq* exhibited strong similarity to the bacterial and fungal type II DHQases.

High level expression of *S. hygroscopicus* DHQase was achieved in *E. coli* and the recombinant DHQase was shown to have properties typical of type II DHQases: relatively high K_m for dehydroquinate (650 mM), subunit M_r of 19 000 (SDS-PAGE), and high thermal stability. The enzyme appears to be heptameric, differing from most type II DHQases which form dodecamers [5]. Correlation by gel-chromatography of the M_r of purified, native protein with the M_r of DHQase activity in a crude cell-free extract of *S. hygroscopicus*, and the absence of any additional DHQase activity (particularly a type I heat-labile protein) are consistent with a role for this enzyme in providing shikimic acid for both primary metabolic processes and ascomycin biosynthesis. The clustering of DHQase genes with either shikimate or quinate pathway genes is often used to provide supporting evidence for a physiological role. For instance, *aroQ* encoding the DHQase of *Mycobacterium tuberculosis* is clustered with a gene encoding dehydroquinate synthase [12]. No such supporting evidence was found for a biosynthetic role of *S. hygroscopicus dhq*. Analysis of the flanking DNA regions failed to show any ORFs with sequence homology to either shikimate or quinate pathway enzymes. The definitive assignment of a physiological role for this enzyme, therefore, must await the generation and analysis of DHQase-deficient mutants.

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