Lovastatin Nonaketide Synthase Catalyzes an Intramolecular Diels−Alder Reaction of a Substrate Analogue

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The fungal metabolite lovastatin (**1**)[1] and its derivatives are cholesterol-lowering drugs that act as potent inhibitors of (*3S*)-hydroxy-3-methylglutaryl-coenzyme A reductase.[2] Although **1** and compactin[3] have attracted attention from synthetic chemists,[4] these drugs and some analogues (e.g., simvastatin, pravastatin) which are used in humans are manufactured by fermentation, either directly or with subsequent chemical or microbial modification. Studies on the biosynthesis of **1** in *Aspergillus terreus* indicate that it is formed by a polyketide pathway.[5−7] Of special interest is the proposal of an enzyme-catalyzed Diels−Alder cyclization of the intermediate hexaketide triene to generate the decalin system (Figure 1).[7,8] This idea is supported by the formation of dihydromonacolin L (**2**)[9] by a heterologous host, *A. nidulans* containing the *lovB* and *lovC* genes from *A. terreus*.[10,11] Corresponding heterologous expression of the *lovB* protein (lovastatin nonaketide synthase, LNKS) without *lovC* leads to truncated pyrones **3** and **4**, formed due to failure of enoyl reduction at the tetraketide stage.[10]

There are proposals that enzyme-catalyzed Diels Alder reactions may occur during biosynthesis of many secondary metabolites,[12] but the demonstrated ability of pure biological macromolecules to promote this process has been limited to catalytic antibodies generated from synthetic haptens[13] and to synthetic RNA fragments that bind metals.[14] There is also a report of a crude cell-free preparation from the fungus *Alternaria solani* that oxidizes an achiral allylic alcohol, prosolanopyrone II, to a conjugated triene aldehyde, thereby triggering intramolecular Diels−Alder cyclization to an optically active product, solanopyrone A.[15] We now report that purified LNKS catalyzes intramolecular Diels−Alder *endo* closure of a substrate analogue, (*E*,*E*,*E*)-(*R*)-6-methyldodecatri-2,8,10-enoic acid *N*-acetylcysteamine (NAC) thioester (**5**), to a bicyclic system with the same ring stereochemistry as **2**, which is different from that obtained in nonenzymatic cyclization.

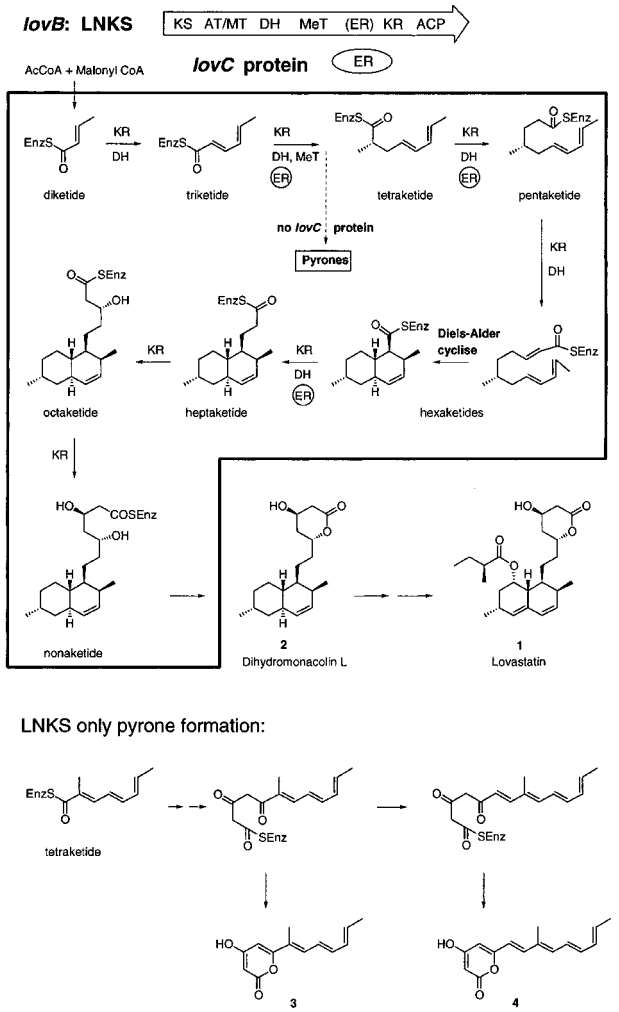

Figure 1. Proposed pathway to dihydromonacolin L (**2**) and lovastatin (**1**). The domains for LNKS and *lovC* protein are assigned functions based on sequence homology to other PKS proteins: KS, *β*-ketoacyl synthase; AT/MT, acetyl/malonyl transferase; DH, dehydratase; MeT, methyltransferase; ER, enoyl reductase; ACP, acyl carrier protein. The boxed region shows reactions catalyzed by LNKS with *lovC* protein to produce **2**. LNKS alone shunts at the tetraketide stage to pyrone formation.

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To ascertain the function of LNKS, this 335 kD enzyme was purified to homogeneity (SDS-PAGE) from the *A. nidulans* clone.[16] Addition of malonyl CoA, FAD, *S*-adenosylmethionine, and NADPH in buffer initiated formation of two pyrones, 4-hydroxy-6-[(1*E*,3*E*,5*E*)-1-methylhepta-1,3,5-trien-1-yl]-2-pyrone (**3**) and 4-hydroxy-6-[(1*E*,3*E*,5*E*,7*E*)-3-methylnona-1,3,5,7-tetraen-1-yl]-2-pyrone (**4**).[10] Acetyl CoA was usually added but was not essential for pyrone production, possibly because of in situ decarboxylation of malonyl CoA. In the absence of malonyl CoA or AdoMet or NADPH, no pyrone formation could be detected by UV spectroscopy. The apparent K_m for malonyl CoA is 0.5 mM and the V_{max} is 1×10^{-7} M·min^{-1} at an enzyme concentration of 0.07 nM with the acetyl CoA concentration fixed at 0.60 mM in Tris-HCl buffer (pH 7.8). The specific activity of the enzyme is 10.9 μmol (of pyrone generated)min^{-1} mg^{-1}.

Although *lovC* protein (363 amino acids) has not yet been purified, preliminary experiments indicate that it complexes strongly to LNKS based on gel shift assays and retention on gel permeation columns (data not shown). Cell-free protein extracts from *A. nidulans* containing *lovC* protein and LNKS produce dihydromonacolin L in the lactone-opened form using the conditions described for the pure enzyme. Since some fungal PKS systems in intact cells can utilize advanced tetraketide intermediates as their *N*-acetylcysteamine (NAC) thioesters,[17] the previously synthesized ^{13}C-labeled hexaketide triene NAC ester (**5**)[8] was added to the cell-free preparation of *lovC* protein and LNKS along with the usual cofactors and substrates. However, no labeling of dihydromonacolin L (**2**) was observed, probably because the cell-free system still contains enzymes that rapidly hydrolyze the NAC thioester to the corresponding acid, which cannot be loaded into the PKS system.

NMR studies show that in the absence of enzyme the hexaketide triene NAC ester (**5**) cyclizes spontaneously in aqueous media at 20 °C to give a 1:1 mixture of *exo* and *endo* adducts **7** and **8** (Figure 2)[8] with a half-life of 60 h (20 mM TrisHCl, pH 7.8) to 72 h (3:1 D_2O:THF). As expected,[18] the cyclization to the same products is considerably slower in nonaqueous media (e.g., $t_{1/2}$ 8 days at 20 °C in $CDCl_3$). The *endo* product **9**, whose stereochemistry corresponds to that of **2**, was not detected in enzyme-free experiments. In contrast, addition of **5** to purified LNKS (TrisHCl buffer, pH 7.7) in the absence of added cofactors or substrates causes cyclization to *endo* Diels Alder product **9** ($k_{cat} = 0.073 \pm 0.001$ min^{-1}), which is obtained along with the previously observed nonenzymatic products **7** and **8** (**7**:**8**:**9** ratio 15:15:1). Possible *exo* product **6** is not detected in any experiments. Since separation of the cyclized thioesters is difficult, they were converted with sodium ethoxide to the known[8] ethyl esters **10**, **11**, and **12**, respectively, to assist characterization.[16] Control experiments demonstrate that no epimerization occurs during this process.[16] To show that active LNKS is essential for Diels−Alder cyclization to **9**, the enzyme was inactivated (e.g., heating), and **5** was added as before. In this case **7** and **8** were formed but no **9** was detected; conversion to the ethyl esters confirmed the result. Exposure of the hexaketide triene ethyl ester **13** to LNKS does not produce detectable amounts of **12** (analogous stereochemistry to **9** and **2**); only nonenzymatic products **10** and **11** are observed. This may be due to the inherent lower reactivity of the oxygen ester as compared to the thioester.

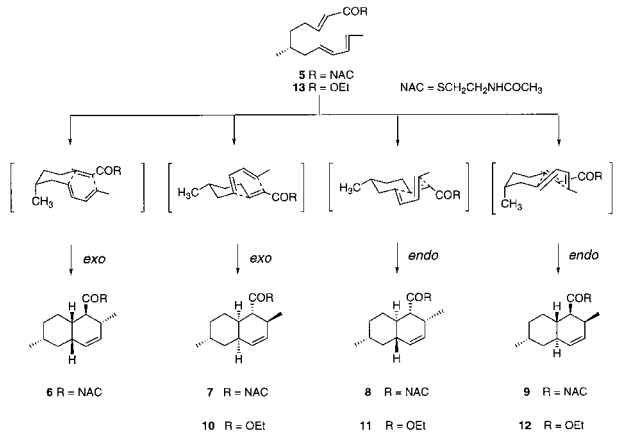

Figure 2. Cyclization of **5** and **13** via proposed transition states (bracketed structures) to *exo* and *endo* Diels−Alder products. Product **9** is observed only in the presence of LNKS.

Both **7** and **8** result from cyclization via transition states wherein the C-6 methyl group occupies a sterically favored pseudoequatorial orientation.[8] Product **9** requires a transition state having the methyl in a crowded pseudoaxial arrangement. Apparently, a key function of LNKS is to bind the substrate in a conformation that resembles the *endo* transition state leading to **9**. Presumably van der Waals interactions in the hydrophobic active site of LNKS confine the substrate so as to bring the reacting atoms into close proximity, analogous to what is seen in catalytic antibodies.[13d,e] The protein is also likely to assist closure through hydrogen bonding of the carbonyl oxygen,[13e] thereby making the dienophile more electron-deficient; essentially this resembles Lewis acid catalysis of Diels−Alder cyclization.[19] In normal PKS function,[5,6] growing polyketide chains remain covalently linked to the protein as thioesters throughout the assembly process. Hence, the route to dihydromonacolin L (**2**) would involve cyclization of the hexaketide while it is thioesterified to LNKS. However, our results suggest that LNKS can catalyze the Diels Alder process without trans-thioesterification to the protein, because if the NAC moiety were to be released as a free thiol into bulk solution, its low concentration would make reformation of the NAC ester unlikely. Since denatured LNKS cannot catalyze the Diels−Alder process, it is clear that nonspecific binding to a chiral protein[20] is insufficient to account for the cyclization. Hence, LNKS represents the first naturally occurring Diels Alderase enzyme to be purified and one of the few fungal PKS enzymes to be isolated and characterized.

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Supporting Information Available: Experimental details and characterization of compounds (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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