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Syntheses of Conjugated Pyrones for the Enzymatic Assay of Lovastatin Nonaketide Synthase, an Iterative Polyketide Synthase

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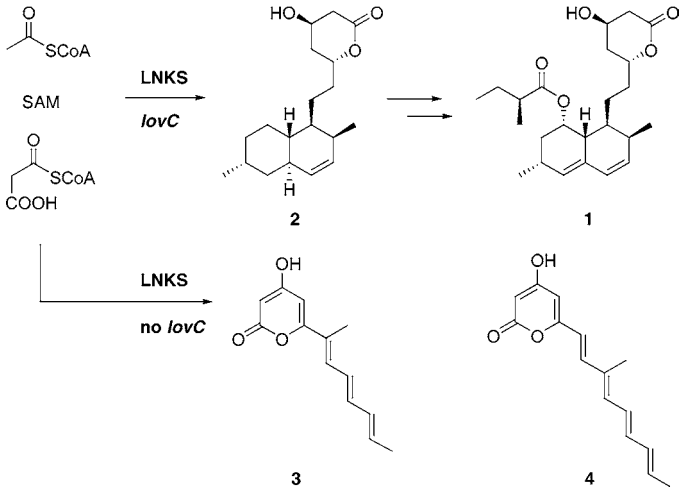
ABSTRACT



Lovastatin nonaketide synthase (LovB, LNKS) is an iterative type I polyketide synthase (PKS) from *Aspergillus terreus* that produces dihydromonacolin L (2), the biosynthetic precursor of lovastatin (1), from acetyl CoA, malonyl CoA, and *S*-adenosylmethionine (SAM) if the accessory protein LovC is present. In the absence of LovC, LNKS forms the conjugated pyrones 3 and 4 as truncated PKS products. Short syntheses of these pyrones provide material for the assay of LNKS activity by HPLC and radioisotope dilution analysis.

Lovastatin (**1**) is a fungal polyketide metabolite produced by *Aspergillus terreus* that has been used as a drug (Mevacor) to lower cholesterol levels,[1,2] but a significant amount of it is now converted by chemical methylation of its side chain to simvastatin (**5**) (Zocor) (Scheme 1). In 2005, Zocor was the fifth best selling pharmaceutical drug with annual worldwide sales of ca. $5.3 billion in a global market of $32.4 billion for cholesterol and triglyceride lowering drugs.[3] Our initial investigations[4] on biosynthesis of **1** eventually led to identification and heterologous expression of genes

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(4) (a) Chan, J. K.; Moore, R. N.; Nakashima, T. T.; Vederas, J. C. *J. Am. Chem. Soc.* **1983**, *105*, 3334−3336. (b) Moore, R. N.; Bigam, G.; Chan, J. K.; Hogg, A. M.; Nakashima, T. T.; Vederas, J. C. *J. Am. Chem. Soc.* **1985**, *107*, 3694−3701. (c) Yoshizawa, Y.; Witter, D. J.; Liu, Y.; Vederas, J. C. *J. Am. Chem. Soc.* **1994**, *116*, 2693−2694.

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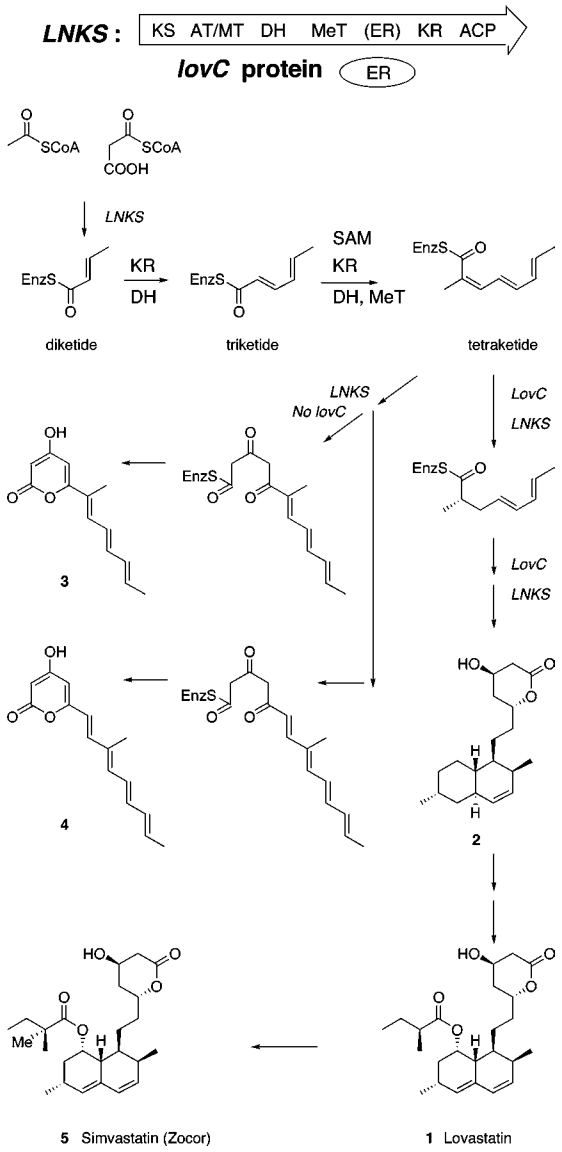
Scheme 1. Biosynthesis of **2** by LNKS with LovC and of **3** and **4** by LNKS without LovC[a]



[a] The functional domains of LNKS are ketosynthase (KS), acyl/malonyl transferase (AT/MT), dehydratase (DH), methyl transferase (MeT), enoyl reductase (ER), keto reductase (KR), and the acyl carrier protein (ACP).

responsible for its production.[2,5] The main skeleton, dihydromonacolin L (**2**), is assembled by an iterative type 1 polyketide synthase (PKS) called lovastatin nonaketide synthase (LNKS, LovB) in conjunction with an accessory protein, LovC. These two enzymes, along with cofactors (NADPH, FAD, and Mg^{++}), are sufficient to make **2** in ca. 35 chemical steps from acetyl-CoA, malonyl-CoA, and *S*-adenosylmethionine (SAM).[5] The process appears to involve an enzyme-catalyzed endo Diels−Alder reaction to make the decalin system of **2**.[6] Subsequently, post-PKS oxidation enzymes (P_{450}) introduce the second double bond in the decalin system and add the diketide side chain (produced by LovF, another iterative type 1 PKS) through esterification by LovD. Interestingly, the accessory protein LovC is necessary for enoyl reductase activity during chain assembly by LNKS. In the absence of a functional LovC, the LNKS protein is still able to assemble part of the skeleton and produces unstable conjugated pyrones **3** and **4** as principal products.[2,5,7] Iterative type 1 PKS enzymes, which are common in fungi, have only a single copy of each functional domain necessary for precursor loading, chain assembly, and reduction.[5,8,9] This is in contrast to the modular PKS enzymes in bacteria wherein multiple copies of the domains act as a sequential assembly line for the metabolite.[10,11] Hence, in type 1 iterative PKS enzymes, the structure of the growing substrate linked as a thioester to the protein must somehow determine which functional domains are accessible and what the next assembly step will be.

As part of our investigation of the in vitro activity of purified LNKS, it proved useful to have ready access to pyrones **3** and **4** for enzyme assays, including the use of isotope dilution analysis in conjuction with transformation of [^{14}C]-malonyl-CoA. Although these compounds have been previously obtained by fermentation of fungal strains that have LNKS but no active LovC, the small amounts produced together with the instability of these pyrones make facile synthetic access desirable.[5] Apparently, in contrast to polypropionate-derived unsaturated pyrones that bear additional methyl groups,[12] these seemingly simple compounds decompose in a few hours at room temperature under acidic or basic aqueous conditions. In the present work, we report chemical syntheses of **3** and **4** and their use in an assay of LNKS.

The hexaketide pyrone **3** can be prepared as shown in Scheme 2. Wittig reaction of (carbethoxyethylidene)triphenylphosphorane with 2,4-hexadienal generates the adduct **6**, which reacts with the dianion of ethyl acetoacetate[13] to afford the diketoester **7**. Hydrolysis of the ester functionality using

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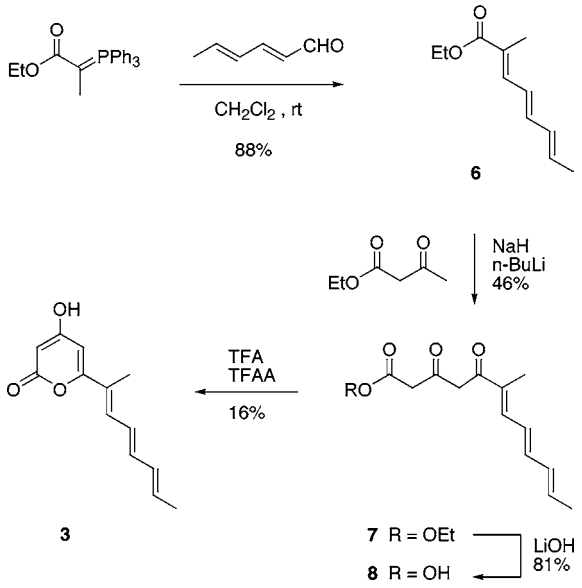
(10) Hill, A. M. *Nat. Prod. Rep.* **2006**, *23*, 256−320.

(11) Type I iterative PKS enzymes have also been found in bacteria. See ref 10 and: (a) Wenzel, S. C.; Kunze, B.; Hoefle, G.; Silakowski, B.; Scharfe, M.; Bloecker, H.; Mueller, R. *Chem. Bio. Chem.* **2005**, *6*, 375−385. (b) Shao, L.; Qu, X.-D.; Jia, X.-Y.; Zhao, Q.-F.; Tian, Z.-H.; Wang, M.; Tang, G.-L.; Liu, W. *Biochem. Biophys. Res. Commun.* **2006**, *345*, 133−139. (c) Yu, F.; Zaleta-Rivera, K.; Zhu, X.; Huffman, J.; Millet, J. C.; Harris, S. D.; Yuen, G.; Li, X.-C.; Du, L. *Antimicrob. Agents Chemother.*, in press; doi:10.1128/AAC.00931−06.

(12) For a recent review of syntheses of propionate-derived unsaturated pyrones, see: Trauner, D.; Miller, A. K. *Synlett* **2006**, 2295−2316.

(13) Huckin, S. N.; Weiler, L. *Tetrahedron Lett.* **1972**, *13*, 2405−2408.

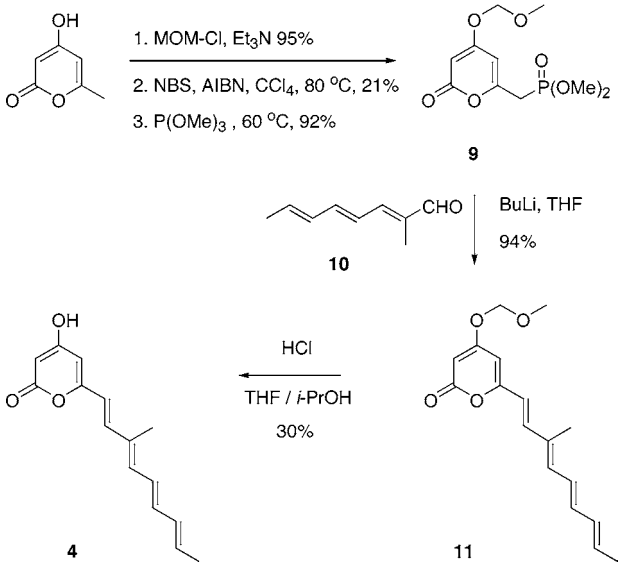
Scheme 2. Synthesis of the Hexaketide Pyrone **3**



lithium hydroxide gives **8**, which cyclizes[14] with trifluoroacetic anhydride and trifluoroacetic acid to the desired product **3**. The yield for the last step is quite low (16%) because of the tendency of the product to decompose rapidly in the presence of acids. Nevertheless, after purification, **3** can be stored at −20 °C for a month with minimal decomposition.

The heptaketide pyrone **4** can be synthesized as depicted in Scheme 3. Commercially available 4-hydroxy-6-methyl-

Scheme 3. Synthesis of the Heptaketide Pyrone **4**



2-pyrone is protected as its methoxymethyl (MOM) ether. Bromination of the methyl group followed by Michaelis−Arbuzov reaction gives the phosphonate **9**. Horner−Emmons−Wadsworth olefination of phosphonate **9** with the triene aldehyde **10**, generated from reduction of triene ester **6**, affords MOM-protected product **11**. The MOM ether can then be removed using HCl to produce the desired product **4**. This pyrone is considerably less stable than **3** and undergoes substantial decomposition when stored pure at −20 °C within a week. The ^{1}H and ^{13}C NMR spectra of both **3** and **4** fully match those previously reported[5] for the corresponding substances produced by fermentation.

To examine the utility of **3** and **4** in a preliminary assay of the activity of LNKS, the heterologous host *A. nidulans* containing the *lovB* gene was fermented, and a cell free extract (CFE) was prepared as previously described.[6] The CFE (15 mL, 2.23 mg of protein per mL) was incubated with acetyl-CoA, unlabeled malonyl-CoA (4.0 mg, 4.7 μmol), 6.0 μCi of [2-^{14}C]-malonyl-CoA (55 mCi/mmol), NADPH, SAM, and FAD at room temperature for 3 h. The synthetic unlabeled pyrones **3** and **4** were added to the mixture after each hour of incubation to help protect any radioactive pyrone that forms from catalytic decomposition (total added: 7.3 mg of **3** and 4.0 mg of **4**). At the end of this period, *N*-acetyl cysteamine (NAC) was added to the mixture to aid in the release of metabolites that may be bound as thioesters with the LNKS protein. The CFE mix was then extracted with ethyl acetate and subjected to HPLC purification. The pyrone peaks were collected, analyzed by scintillation counting, and then reinjected twice onto HPLC followed by collection and scintillation counting to ensure that the pyrones had constant specific activity. Fractions collected prior to and following each of the pyrone peaks were at approximately background radioactivity levels. The isolation of radioactive pyrones of constant specific activity (**3**, 12 ± 3 nCi/mmol; **4**, 22 ± 3 nCi/mmol) indicates that LNKS is active under the assay conditions and produces hexaketide **3** and heptaketide **4** from [2-^{14}C]-malonyl-CoA in vitro, albeit with only 0.013% conversion. The latter figure is based on the total radioactivity recovered in both pyrone **3** and **4**. The apparent low overall conversion may be due to the low inherent activity of the enzyme but may also be caused by other factors such as the tendency of malonyl-CoA to decarboxylate to acetyl-CoA as well as steady decomposition of the product pyrones during the enzyme incubation.

To examine the utility of **3** and **4** for the detection of pyrones in fermentation mixtures, an *A. nidulans* clone containing a heterologously expressed *lovB* gene was fermented as previously described.[5,6] Two days after induction for expression, a total of 250 μCi of [1-^{14}C]-sodium acetate was added every 4 h for a duration of 48 h. The mycelia were removed by vacuum filtration, and the synthetic unlabeled pyrones **3** and **4** were added to the filtrate. The filtrate was then extracted with ethyl acetate. HPLC purification of the extract using an acetonitrile and water gradient elution allowed recovery of ^{14}C-labeled pyrones **3** and **4** (10 900 dpm and 9450 dpm, respectively; 0.0037% total conversion). To ensure the radiochemical purity of the recovered pyrones, the purified fractions were subjected to repeated HPLC separation and scintillation counting two

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more times. No significant change in specific activity was observed. Interestingly, the overall efficiency conversion of sodium [1-^{14}C]-acetate to pyrones **3** and **4** appears very low compared to the high levels of incorporation of the carbon-13-labeled acetate reported earlier.[5,6] However, the amount of the acetate added in the stable isotope study was much larger than that in the current radioactive incorporation experiment. This results in a large pool of [^{13}C]-acetate being continuously available, whereas the small quantities of [^{14}C]-acetate are rapidly consumed in other metabolic pathways. It is probable that the rate of conversion of the substrate by the LNKS (LovB) enzyme is inherently low, at least in the absence of its partner protein, LovC.

In summary, facile syntheses of highly conjugated and unstable pyrones **3** and **4** have been completed and added to the repertoire of recent syntheses of other natural pyrones.[12] This affords access to pure substances for further development of radioisotope dilution assays of the LNKS enzyme that complement standard UV absorption assays. Studies on the mechanism of LNKS and other fungal iterative type 1 PKS enzymes are ongoing.

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Supporting Information Available: Experimental procedures and characterization data for synthetic intermediates to **3** and **4** together with details of incorporation experiments and assay procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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