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JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

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Double Oxidation of the Cyclic Nonaketide Dihydromonacolin L to Monacolin J by a Single Cytochrome P450 Monooxygenase, LovA

Jorge Barriuso,[†,#] Don T. Nguyen,[†,#] Jesse W.-H Li,[§] Joseph N. Roberts,[†] Gillian MacNevin,[†] Jennifer L. Chaytor,[§] Sandra L. Marcus,[§] John C. Vederas,[*,§] and Dae-Kyun Ro[*,†]

[†]Department of Biological Sciences, University of Calgary, 2500 University Drive NW, Calgary T2N 1N4, Canada

[§]Department of Chemistry, University of Alberta, Edmonton T6G 2G2, Canada

Supporting Information

ABSTRACT: Lovastatin, a cyclic nonaketide from *Aspergillus terreus*, is a hypercholesterolemic agent and a precursor to simvastatin, a semi-synthetic cholesterol-lowering drug. The biosynthesis of the lovastatin backbone (dihydromonacolin L) and the final 2-methylbutyryl decoration have been fully characterized. However, it remains unclear how two central reactions are catalyzed, namely, introduction of the 4a,5-double bond and hydroxylation at C-8. A cytochrome P450 gene, *lovA*, clustered with polyketide synthase *lovB*, has been a prime candidate for these reactions, but inability to obtain LovA recombinant enzyme has impeded detailed biochemical analyses. The synthetic codon optimization and/or N-terminal peptide replacement of *lovA* allowed the *lovA* expression in yeast (*Saccharomyces cerevisiae*). Both *in vivo* feeding and *in vitro* enzyme assays showed that LovA catalyzed the conversion of dihydromonacolin L acid to monacolin L acid and monacolin J acid, two proposed pathway intermediates in the biosynthesis of lovastatin. LovA was demonstrated to catalyze the regio- and stereospecific hydroxylation of monacolin L acid to yield monacolin J acid. These results demonstrate that LovA is the single enzyme that performs both of the two elusive oxidative reactions in the lovastatin biosynthesis.

Lovastatin (open form acid **5a**, lactone **5b**) is a natural polyketide product produced from the filamentous fungus, *Aspergillus terreus*. Lovastatin, its natural analogues such as compactin, and their semi-synthetic derivatives simvastatin (Zocor) and pravastatin (Pravachol) are potent and widely prescribed cholesterol-lowering drugs.[1] These compounds are effective competitive inhibitors of (3*S*)-hydroxy-3-methylglutaryl-coenzyme A reductase, a rate-limiting enzyme in the cholesterol biosynthetic pathway. The gene cluster for lovastatin biosynthesis was identified from *A. terreus* (Figure 1A), and subsequent biochemical studies revealed that the iterative type I polyketide synthase (PKS) encoded in *lovB* catalyzes the synthesis of dihydromonacolin L acid (**1a**) in concert with an accessory enzyme, LovC (Figure 1B).[2] Recently, it has been shown that *in vitro* reconstitution of purified LovB and LovC recombinant enzymes could synthesize the enzyme-conjugated **1a**.[3] Although this PKS enzyme−product complex was not able to off-load the synthesized product, the addition of a fungal thioesterase (TE) enzyme *in trans* facilitated the release of **1a** from the LovB and LovC enzyme complex. Therefore, coordinated reactions of purified LovB, LovC, and *trans*-TE can synthesize a key lovastatin intermediate, **1a**, *in vitro*. The final C-8 side-chain modification is also

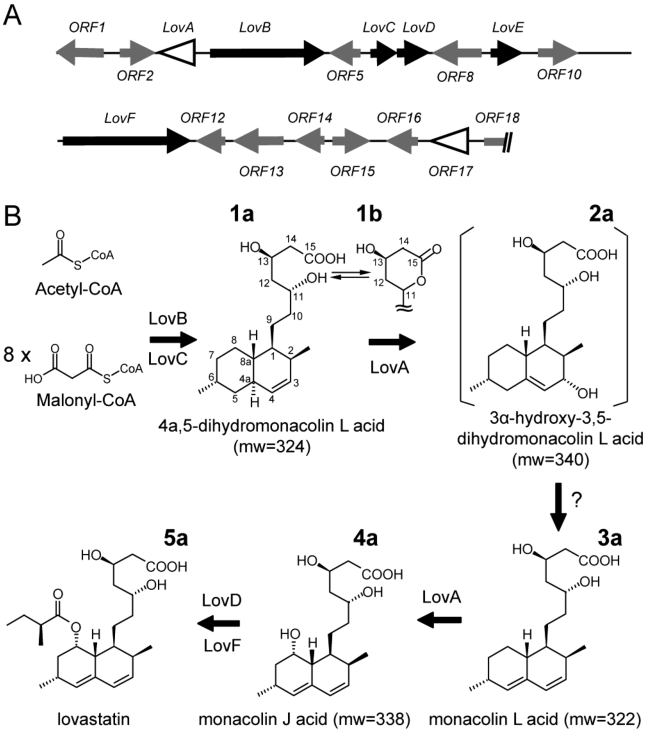

Figure 1. (A) Lovastatin gene cluster. Two cytochrome P450 genes are shown as open triangles. Black arrows are the genes involved in lovastatin biosynthesis, and the gray arrows are the genes of unknown functions. (B) Proposed biosynthetic pathway for lovastatin. Compounds are shown as their hydroxy acids **a**, but corresponding lactone forms **b** (e.g., **1b**) are frequently isolated. The hydroxy acid forms (**1a**−**5a**) will exist as salts in *in vivo* and *in vitro* conditions.

well studied.[2,4] The 2-methylbutyryl side chain is synthesized by the second PKS, LovF, as an enzyme-bound thioester and is transferred directly from this enzyme to the C-8 hydroxyl group of monacolin J acid (**4a**) by the acyl transferase, LovD.

Significant progress has been made in elucidating lovastatin biosynthesis at the entry point (LovB and LovC) and at the last step of side-chain decoration (LovD and LovF). However, the central oxidative transformation of **1a** to **4a** has yet to be fully understood. During the lovastatin purification from *A. terreus*, 3α-hydroxy-3,

Received: February 5, 2011
Published: April 15, 2011

dx.doi.org/10.1021/ja201138v | *J. Am. Chem. Soc.* 2011, 133, 8078–8081

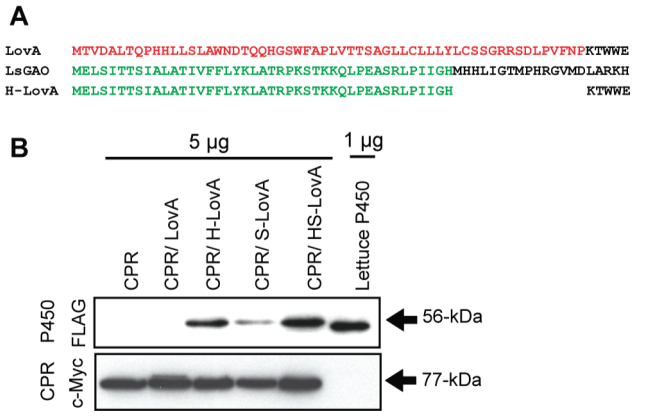

Figure 2. Illustration of the N-terminal modification of LovA (A) and the immunoblot analysis of LovA derivatives and CPR (B). Lettuce P450 (GenBank number GU198171) was used as a control to assess the expression levels of LovA derivatives, and commercial antibodies (anti-FLAG and anti-cMyc) were used to detect the tagged recombinant enzymes.

5-dihydromonacolin L (**2a**) was also isolated, and subsequent chemical analysis demonstrated that this unstable compound undergoes dehydration to yield monacolin L acid (**3a**).[5] In addition, microsomes isolated from *A. terreus* could catalyze the C-8 hydroxylation of **3a** to produce **4a** *in vitro*.[6] This C-8 hydroxylation reaction was blocked by carbon monoxide, implying that the C-8 hydroxylation of **3a** is catalyzed by cytochrome P450 monooxygenase (P450). The gene cluster for lovastatin biosynthesis indeed encodes two P450 genes, *lovA* and *ORF17* (Figure 1A).[2] The *lovA* gene shares its bi-directional promoter with *lovB* with only a 482-bp distance, and therefore it was logical to postulate that *lovA* is involved in oxidative modification of **1a**. In agreement with this idea, genetic disruption of *lovA* in *A. terreus* resulted in the complete absence of downstream intermediates after **1a**.[7] This genetic evidence demonstrated the critical role of *lovA* in oxidative transformation of **1a** and also suggested that the first oxidation is catalyzed by P450 enzyme LovA. Collectively, these results suggested that two consecutive hydroxylations, both catalyzed by P450(s), are responsible for the conversion of **1a** to **4a**. *LovA* is the prime candidate for at least one of these oxidative reactions.

Expression of *lovA* in *Escherichia coli* was not successful, and yeast (*Saccharomyces cerevisiae*) was pursued as an alternative host. A P450 enzyme of this type requires a redox partner, such as cytochrome P450 oxidoreductase (CPR), to receive reducing equivalents from NADPH.[8] It was reported that native CPR activity is limiting when heterologous P450 is expressed in yeast.[9] Therefore, we first isolated and characterized *A. terreus* CPR. Primary sequences of CPR are highly conserved in eukaryotes, and the BLAST search identified *A. terreus cpr* gene from the sequence database. In order to verify CPR activity, *cpr* cDNA was isolated from *A. terreus*, cloned into the high-copy yeast vector *pESC-Leu2d*, and expressed in yeast with a C-terminal cMyc tag.[10] Immunoblot analysis using anti-cMyc antibodies clearly detected the recombinant CPR in the yeast microsomes (Figure 2B). Using the isolated microsomes, the catalytic activity of recombinant CPR was measured by monitoring the reduction of cytochrome *c* (Cyt*c*). In these assays, microsomes from *cpr*-expressing yeast showed 7.3-fold higher Cyt*c* reduction activity (4430 ± 260 nmol of Cyt*c* min^{-1} mg^{-1}) than those from the vector control (610 ± 220 nmol of Cyt*c* min^{-1} mg^{-1}). The basal CPR activity from the control was due to endogenous yeast activities, as previously reported.[9] This result substantiated that the *A. terreus cpr* expressed in yeast is biochemically active.

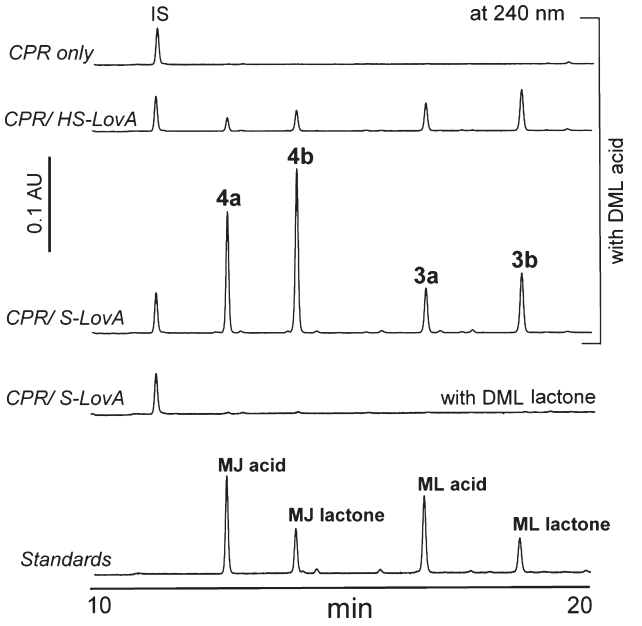

Figure 3. Metabolite profiles of *in vivo* substrate feeding assays using an HPLC-UV detector. Compounds **4a**, **4b**, **3a**, and **3b** were confirmed as monacolin J acid, monacolin J lactone, monacolin L acid, and monacolin L lactone, respectively, by comparison to the authentic standards. Dihydromonacolin L (DML, **1b**) and its corresponding acid **1a** are not detected at this wavelength (240 nm). IS = internal standard (cinnamic acid, 20 μM).

The *lovA* gene in C-terminal FLAG-tagged form was cloned in the same vector coding *cpr*, and both *lovA* and *cpr* were co-expressed in yeast, using a Gal1 (for *cpr*) and Gal10 (for *lovA*) bi-directional promoter. However, LovA was not detected in the immunoblot analysis using FLAG antibodies (Figure 2B), although its mRNAs could be detected by reverse transcriptase PCR analysis. Hence, inefficient translation and/or sub-cellular targeting were suspected, and three LovA transcript and protein variants were created to address this problem. First, *lovA* cDNA was entirely synthesized with optimized yeast codons (synthetic *lovA*, *S-lovA*) by replacing 430 nucleotides in native *lovA* (Supporting Information). Second, to ensure its endoplasmic reticulum localization, the N-terminal 58 amino acids of LovA (putative hydrophobic ER-targeting domain) were replaced with the N-terminal 43 amino acids from lettuce P450 (*LsGAO*), which previously showed a high level of expression in yeast (Figure 2A).[11] This fusion protein is referred to as hybrid LovA (*H-lovA*). Finally, synthetic *lovA* with lettuce N-terminus was generated and referred to as hybrid synthetic *lovA* (*HS-lovA*). The expressions of these three *lovA* variants were examined in comparison to the native *lovA* by immunoblot (Figure 2B). The immunoblot analysis clearly showed that both codon optimization and N-terminal engineering of LovA markedly increased the abundance of LovA protein in the microsomes. The additive effect of the two modifications was found in *HS-lovA*. We focused on the characterization of S-LovA and HS-LovA recombinant enzymes, but H-LovA was not further studied because it encodes the same amino acids as HS-LovA.

The catalytic activities of S-LovA and HS-LovA were first evaluated by feeding the substrate **1a** (Li salt form) to the culture medium at 100 μM. In the HPLC diode array detector (DAD) analysis of culture extract after 8 h of cultivation, four new peaks were detected at 240 nm in both *S-lovA*- and *HS-lovA*-expressing yeast strains (Figure 3). The retention times and characteristic UV spectra (unique triple max peaks at ~240 nm) of these novel

dx.doi.org/10.1021/ja201138v | *J. Am. Chem. Soc.* 2011, 133, 8078–8081

products were identical to those of the authentic standards for **3a**/**b** and **4a**/**b** (Figures 3 and S1). However, the catalytic efficiency of S-LovA for the synthesis of **4a**/**b** was at least 110-fold higher than that of HS-LovA, taking into consideration that HS-LovA showed 11.9-fold higher expression than S-LovA. LC-MS analyses of the four compounds confirmed that the masses of these compounds were consistent with those for **3a**/**b** and **4a**/**b**. In the negative-ion (−)LC-MS, **3a** and **4a** showed $[M-H]^-$ ions of m/z 321 and 337, respectively. In the positive ion (+)LC-MS, the $[M+H]^+$ ions of **3a** and **4a** were labile and underwent dehydration (−18 Da) to form $[M+H-H_2O]^+$ ions whose m/z values corresponded to the predicted values of positive ions for **3a** (m/z = 305) and **4a** (m/z = 321). In addition, $[M+H]^+$ ions of the lactone compounds (**3b** and **4b**) were consistent with the predicted masses (m/z = 305 for **3b** and m/z = 321 for **4b**).

It is potentially feasible that both free acid and lactone forms (**1a** and **1b**) could be used as LovA substrates. In order to test if **1b** can be used as LovA substrate, 100 μM **1b** was fed to the yeast expressing *S-lovA* and *cpr*, and the culture was incubated for 8 h. However, no conversion of **1b** was detected by HPLC-DAD (Figure 3). When the same sample was analyzed by a highly sensitive LC-MS, the four compounds (**3a**/**b** and **4a**/**b**) could be detected, but their abundance was about 250-fold lower than those converted from **1a**. Therefore, it appears that **3a** and **4a** were synthesized from **1a** and then converted to the corresponding lactones in an acidic yeast culture medium. In order to verify this, the same feeding experiments were performed in extended incubation times (24 h) with varying final pH (3.0−6.8) in the medium using different buffer strengths. In acidic conditions (pH 3), almost all of the monacolin L and monacolin J were present as their lactone forms (**3b** and **4b**), whereas their free acid forms (**3a** and **4a**) were dominant in the medium with final pH 6.8 (Figure S2). This result together with the data from the **1b**-feeding assay suggested that **1a** is the LovA substrate. The **3b** and **4b** apparently resulted from non-enzymatic lactonization in acidic yeast medium.

Using the pH-optimized conditions, yeast feeding assays were scaled up (1 L), and **3a** and **4a** were purified by HPLC. The structure of the final product **4a** was verified by spectral comparison to authentic standard, and standard NMR analyses were used to confirm the structure of **3a** (Supporting Information). By using FT-ICR-MS, the exact m/z of the $[M-H]^-$ for **3a** was determined to be 321.20700 and for **4a** to be 337.20212. These values were less than 0.4 ppm deviations from the theoretical masses. To ensure the reactions were catalyzed by LovA, *in vitro* enzyme assays were done using microsomes prepared from yeast expressing *S-lovA* and *cpr*. When **1a** was incubated with the microsomes, **3a** and **4a** were produced as shown by LC-MS analysis (Figure 4A). Two additional $[M-H]^-$ ions displaying m/z 339 were detected. One of these compounds is likely to be 3α-hydroxy-3,5-dihydromonacolin L acid (**2a**), a reported intermediate in the lovastatin biosynthesis, and we propose that the other compound is its isomer, 4aα-hydroxy-4a,5-dihydromonacolin L acid (Figure S3, Supporting Information). No conversion was detected when **1b** was incubated with the microsomes, consistent with the *in vivo* feeding experiment. As many P450 enzymes catalyze epoxidations, it has been proposed that 3,4-epoxy-dihydromonacolin L could be a LovA reaction intermediate.[7] To examine this possibility, the pure α and β isomers of 3,4-epoxy-dihydromonacolin L (open forms **a**, m/z 339) were chemically

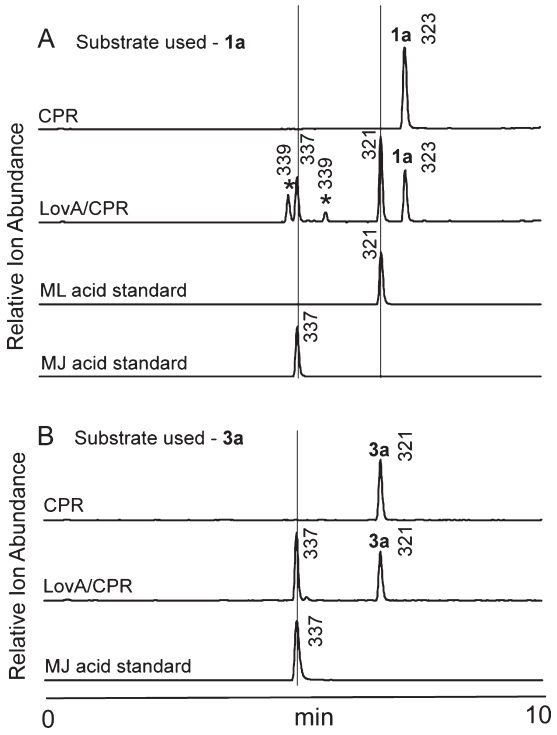

Figure 4. *In vitro* LovA enzyme assays. Total negative ion scans were performed by LC-MS. Selective ions of m/z 323, 339, 321, and 337 were used to detect the metabolites shown in Figure 1B. Beside the peaks, m/z values of $[M-H]^-$ ions are given. (A) **1a**, 4a,5-dihydromonacolin L acid (substrate), was incubated with the microsomes from yeast expressing either *cpr* only or *cpr* and *S-lovA*. Asterisks indicated compounds displaying negative ions of m/z 339, proposed to be **2a** and its isomer 4aα-hydroxy-4a,5-dihydromonacolin L acid (Supporting Information). (B) **3a**, monacolin L acid, was used as substrate in the same experimental conditions as described for panel A.

synthesized from **1b** and also independently incubated with the microsomes, but these were not transformed further (data not shown). In addition, MS/MS analysis suggested that the two m/z 339 compounds from the *in vitro* assays are not 3,4-epoxy-dihydromonacolin L (Figure S4, Supporting Information). On the basis of these results, we propose that **2a** is synthesized by a hydrogen (**1a** C-4a hydrogen) abstraction and subsequent oxygen re-bound (i.e., C-3 hydroxyl group) onto the allylic radical (Figure S3). Using scaled-up yeast cultures (1 L), we attempted to purify the two compounds with m/z 339 after feeding **1a**, but the low abundance of these two compounds did not allow us to acquire sufficient amounts for NMR analyses.

In the assays described thus far using **1a**, it cannot be excluded that the second reaction (the conversion of **3a** to **4a**) is catalyzed by an unknown yeast enzyme. Also, **3a** could, in principle, be a reaction shunt product that is released from LovA but cannot be re-introduced into the LovA biosynthetic pathway. To address these questions, the purified **3a** was used as a substrate for *in vitro* LovA assays. In these assays, clear conversion of **3a** to **4a** was observed, with no trace of catalytic conversion in the control microsomes (Figure 4B). The K_m value of LovA for **3a** was determined to be 6.2 ± 1.1 μM, and the microsomes showed V_{max} = 9.1 ± 0.5 pmol min^{-1} mg^{-1}. The sufficiently low K_m value supported the physiological relevance of LovA activity in *A. terreus*. These results demonstrate that **3a** is a true intermediate in the lovastatin biosynthetic pathway.

In summary, detailed *in vivo* and *in vitro* characterizations of LovA recombinant enzyme demonstrated that LovA is the missing link in the lovastatin biosynthesis, catalyzing the two central oxidative reactions from **1a** to **4a**. Recently, other double oxidations of bacterial polyketides by single P450 tailoring enzymes during biosynthesis have been reported.[12] These LovA studies have completed the molecular characterizations of an entire set of genes required for the lovastatin biosynthesis and hence provide an opportunity to synthesize lovastatin by means of metabolic engineering.

■ ASSOCIATED CONTENT

Supporting Information. Experimental details, synthetic gene sequences, and NMR and HPLC data. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author
daekyun.ro@ucalgary.ca; john.vederas@ualberta.ca

Author Contributions
#These authors contributed equally.

■ ACKNOWLEDGMENT

We thank Dr. Jürgen Schmidt (Leibniz-Institute of Plant Biochemistry) for the FT-ICR-MS analysis of **3a** and **4a**, Dr. Randy Whittal (University of Alberta) for MS/MS analysis of compounds with *m*/*z* 339, and Zhizeng Gao (University of Alberta) for assistance with NMR analysis. This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC), the Canada Research Chair (CRC) program, and the Canada Foundation for Innovation (CFI). We also acknowledge the José Castillejo Fellowship from the Spanish Ministry of Education and Science awarded to J.B. and the Markin Undergraduate Student Research Program (USRP) in Health and Wellness at University of Calgary awarded to J.N.R.

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