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Biosynthesis of Lovastatin Analogs with a Broadly Specific Acyltransferase

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Summary

The natural product lovastatin and its semisynthetic, more effective derivative, simvastatin, are important drugs for the treatment of hypercholesterolemia. Here, we report the biochemical characterization of a dedicated acyltransferase, LovD, encoded in the lovastatin biosynthetic pathway. We demonstrate that LovD has broad substrate specificity towards the acyl carrier, the acyl substrate, and the decalin acyl acceptor. LovD can efficiently catalyze the acyl transfer from coenzyme A thioesters or *N*-acetylcysteamine (SNAC) thioesters to monacolin J. When α-dimethylbutyryl-SNAC was used as the acyl donor, LovD was able to convert monacolin J and 6-hydroxyl-6-desmethylmonacolin J into simvastatin and huvastatin, respectively. Using the *Escherichia coli* LovD overexpression strain as a whole-cell biocatalyst, preparative amounts of simvastatin were synthesized in a single fermentation step. Our results demonstrate LovD is an attractive enzyme for engineered biosynthesis of pharmaceutically important cholesterol-lowering drugs.

Introduction

Lovastatin (Mevacor; **1**; Figure 1) is a fungal polyketide produced by *Aspergillus terreus* [1–4]. It is a pharmaceutically important compound because of its potent inhibitory activity toward hydroxymethylglutaryl coenzyme A (CoA) reductase, the rate-limiting step of cholesterol biosynthesis. Simvastatin (Zocor, **2**) is a semisynthetic analog of **1** and is more effective in treating hypercholesterolemia [5, 6]. Substitution of the α-methylbutyrate side chain with α-dimethylbutyrate significantly increases the inhibitory properties of **2**, while lowering undesirable side effects. In 2005, simvastatin was the second-best-selling drug in the United States, with annual sales exceeding $4.5 billion. Other important natural product-derived statins include pravastatin (**5**) [7] and huvastatin (**6**) [8].

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Because of the importance of simvastatin, various multistep syntheses of **2** starting from **1** have been described in the patent literature [9–11]. For example, a widely used process starts with the hydrolysis of the C8 ester in **1** to yield the triol monacolin J (**3**), followed by selective silylation of the C13 alcohol, esterification of C8 alcohol with dimethylbutyryl chloride, and deprotection of C13 alcohol to yield **2** [12]. Enzymatic transformations using lipases and esterases have been investigated as alternatives to chemical derivation [13, 14]. However, the requirement of regioselective esterification of the C8 alcohol invariably involves protection of other reactive alcohol groups in **3**, and generally leads to lowered overall yield. Therefore, a specific reagent that is able to selectively acylate C8 of **3** is important for the efficient synthesis of **2** and additional statin analogs.

Pioneering works by Reeves [15] and Hutchinson and Vederas [16] have identified the gene cluster for lovastatin biosynthesis in *A. terreus*. The biosynthesis of **1** is coordinated by two iterative type I polyketide synthases and numerous accessory enzymes. Nonaketide **3**, the immediate biosynthetic precursor of **1**, is assembled by the upstream megasynthase LovB [15] (also known as lovastatin nonaketide synthase), enoylreductase LovC, and CYP450 oxygenases. The five carbon unit side chain is synthesized by LovF (also known as lovastatin diketide synthase) through a single condensation between an acetyl-CoA and a malonyl-CoA. The condensed diketide undergoes methylation and reductive tailoring by the individual LovF catalytic domains to yield an α-*S*-methylbutyryl thioester covalently attached to the phosphopantetheine arm on the acyl carrier protein (ACP) domain of LovF [16]. Encoded in the gene cluster is a 46 kDa protein, LovD, which was initially identified as an esterase homolog. LovD was suggested to catalyze the last step of lovastatin biosynthesis that regioselectively transacylates the acyl group from LovF to the C8 hydroxyl group of **3** to yield **1** (Figure 2). Inactivation of either LovD or LovF in *A. terreus* led to accumulation of the precursor **3** [16].

Homologous enzymes to LovD can be found in other fungal polyketide gene clusters. MlcH in the compactin biosynthetic pathway is implicated in the catalysis of the transacylation reaction between 6-desmethylmonacolin J and an identical acyl substrate [17]. An acyltransferase in the recently discovered squalestatin pathway in *Phoma* sp. C2932 likely catalyzes a similar reaction between an ACP bound tetraketide thioester and an aglycone to yield squalestatin [18]. The amino acid sequence of LovD resembles type C β-lactamase enzymes, which catalyze the hydrolytic inactivation of the β-lactam class of antibiotics [19, 20]. Alignment of LovD with the well-characterized *Enterobacter cloacae* P99 lactamase [21] shows moderate sequence homology, including the potentially conserved nucleophilic active site residues Ser76 and other catalytic amino acids, such as Lys79, Tyr188, and Lys315 [21].

We reason that LovD would be an attractive candidate for the chemoenzymatic synthesis of lovastatin analogs if it, indeed, regioselectively acylates the C8 hydroxyl

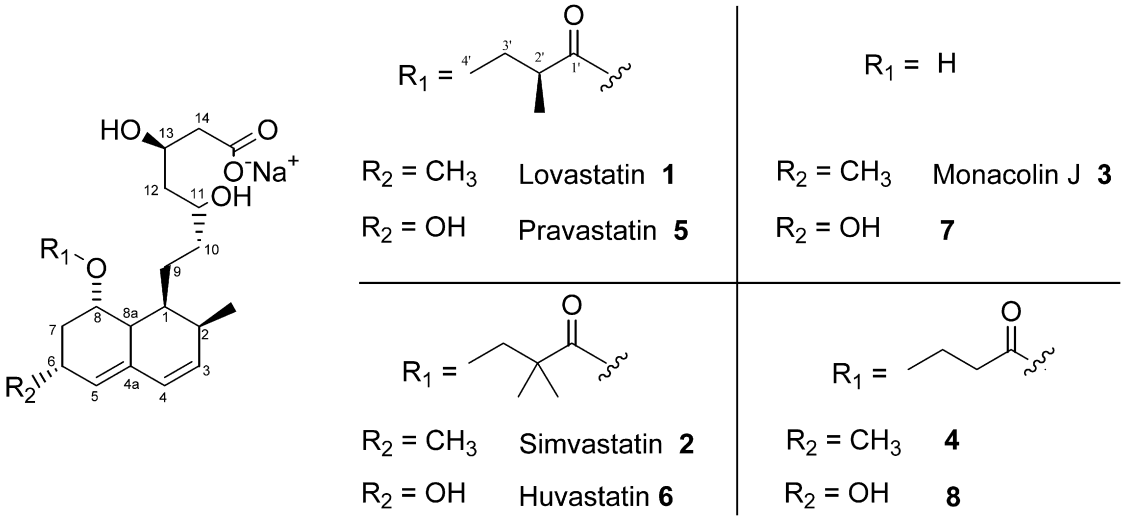


Figure 1. Lovastatin (1), Simvastatin (2), Monacolin J (3), Pravastatin (5), Huvastatin (6), and 6-Hydroxyl-6-Desmethylmonacolin J (7)

position in **3**. Three biochemical properties of LovD remain unexplored. First, can the transacylation activity of LovD be confirmed in vitro and be reconstituted in a heterologous host? Second, does LovD require protein-protein interactions with specific LovF domains for catalysis? Previous work by Kennedy and coworkers suggested LovD-LovF interaction is necessary, although no in vitro characterization was performed [16]. Substitution of acyl-*S*-LovF with synthetic thioesters may significantly increase the chemical diversity of the acyl moieties. Third, what is the substrate specificity of LovD toward the acyl moiety, the thioester carrier, and the decalin core? Can LovD directly acylate **3** with α-dimethybutyrate to yield the pharmaceutically important **2**? In this report, we examine these properties of LovD and demonstrate that LovD displays broad substrate specificity. We also show that LovD can be a useful biocatalyst for the synthesis of **2** and other statin analogs.

Results and Discussion

LovD Regioselectively Acylates Monacolin J

The *lovD* gene was amplified from *A. terreus* genomic DNA by splice-by-overlap extension PCR to delete the two introns, and the amplified DNA was inserted into the pET28a expression vector. Overexpression of soluble, *N*-terminal 6× His fusion LovD was performed using *Escherichia coli* strain BL21(DE3)/pAW31 at 18°C. Elevated expression temperature resulted in significant aggregation of LovD into inclusion bodies. Following overnight expression, soluble LovD was purified using a single step Nickel-NTA affinity column to near homogeneity (Figure 3). The final yield of pure LovD is ~40 mg/l.

Due to lack of the cognate substrate α-methylbutyryl-*S*-LovF, we directly examined whether an acyl-CoA can be used as the acyl donor in the transacylation reaction. The substrate **3** (1 mM) was prepared through the LiOH hydrolysis of **1** and was added to a reaction mixture containing pure LovD (10 μM) and butyryl-CoA (4 mM), which is a commercially available acyl-CoA that best mimics the natural α-methylbutyrate side chain. The reaction mixture was incubated at room temperature, extracted with ethyl acetate, and analyzed by HPLC and LC-MS (Figure 4A).

A single, more hydrophobic compound, with an identical UV absorbance to that of **1**, was formed in the reaction mixture in conjunction with the disappearance of **3**

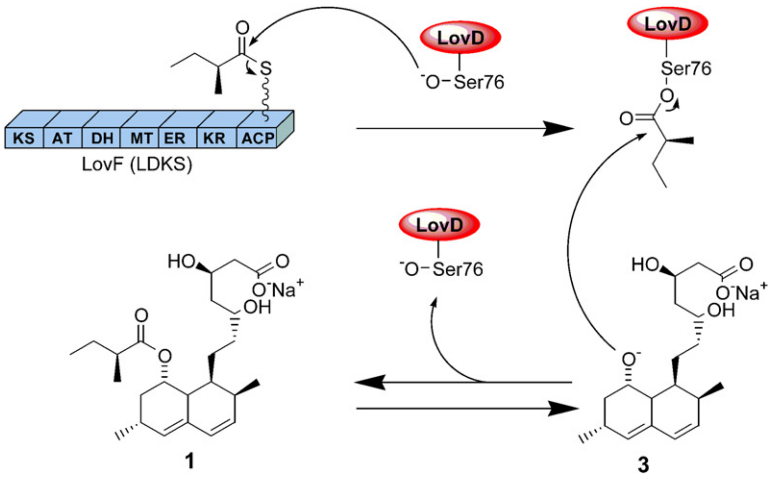


Figure 2. Proposed Acyl Transfer Reaction in *A. terreus*

The reaction likely follows a ping-pong bi-bi mechanism. LovD first captures the α-methylbutyryl acyl group synthesized by the megasynthase LovF, followed by the transacylation of the C8-hydroxyl moiety of monacolin J to yield the natural product lovastatin. Ser76 is the putative active site nucleophile.

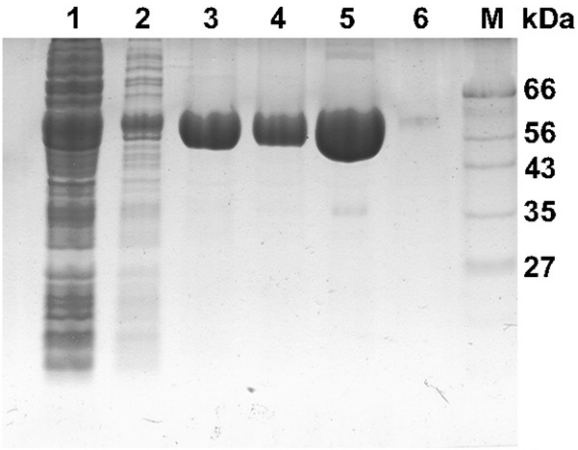


Figure 3. Expression and Purification of LovD from *E. coli* BL21(DE3)/pAW31

Lane 1, Flow through of whole cell lysate soluble protein over the Ni-NTA column; lane 2, 10 mM imidazole wash; lane 3, 20 mM imidazole wash; lane 4, 50 mM imidazole wash; lane 5, 250 mM imidazole elution 1; lane 6, 250 mM imidazole elution 2; and lane M, broad range molecular weight marker. The size of the *N*-6× His-LovD is 48 kDa. Only the protein in lane 5 was concentrated, buffer exchanged, and used in assays described here.

(Figure 4A, trace a). The mass of the new compound was found to be 391 $(M + H)^+$, in accordance with addition of a butyryl group to **3**. The selective esterification of the C8 hydroxyl group of **3** to yield **4** was confirmed by proton NMR spectroscopy ($CDCl_3$, 500 MHz). The proton NMR spectrum of **4** (lactonized form) is nearly identical to that of **1** (see Experimental Procedures), except for the aliphatic signals of the linear acyl side chain. The diagnostic H8 multiplet (assigned using ^1H-^1H COSY, ^1H-^{13}C HMQC, and ^1H-^{13}C HMBC) in **4** is shifted downfield to δ 5.39, compared with δ 4.23 observed for the same proton in **3**. This is consistent with the deshielding effect of the acyloxy substitution at C8. Protons H11 and H13 of carbons bearing other hydroxyl groups were shifted from δ 4.71 and δ 4.38 in **3** to δ 4.61 and δ 4.37 in **4**, respectively.

As shown in Figure 4A (trace a), 87% conversion of **3** to **4** was observed after 10 hr using butyryl-CoA as an acyl donor. A time-course study revealed an initial turnover rate (k_i; V/E_o) of 0.18 min^{-1} (Figure 4B). The observed 87% conversion is likely approaching equilibrium, as evident in Figure 4B. To examine the biochemical nature of the equilibrium conversion, we assayed whether LovD also catalyzes the reverse, hydrolysis reaction. Indeed, when **1** was used as the only substrate in the presence of LovD, formation of **3** was readily detected with k_{cat} and K_m values of 0.21 ± 0.01 min^{-1} and 0.56 ± 0.05 mM, respectively (Figure 4C). An excess of butyryl-CoA was, therefore, necessary for achieving maximum conversion of the forward reaction.

To verify the catalytic role of LovD, we constructed a mutant form of *lovD* that contained a Ser76-to-Ala76 substitution. Ser76 was identified through sequence alignment with type C β-lactamase to be the putative, active site nucleophile. When the LovD S76A mutant was used in place of the wild-type enzyme, neither formation of **4** (Figure 4A, trace b) nor the hydrolysis of **1** could be detected. The enzymatic synthesis of **4** confirms that LovD indeed catalyzes the acyl transfer reaction shown in Figure 2. Furthermore, this result suggests that direct association between domains of LovF and LovD is not an absolute requirement for catalytic turnover, in contrast to the previously suggested mode of LovD catalysis [16]. Acyl-*S*-CoA can substitute for

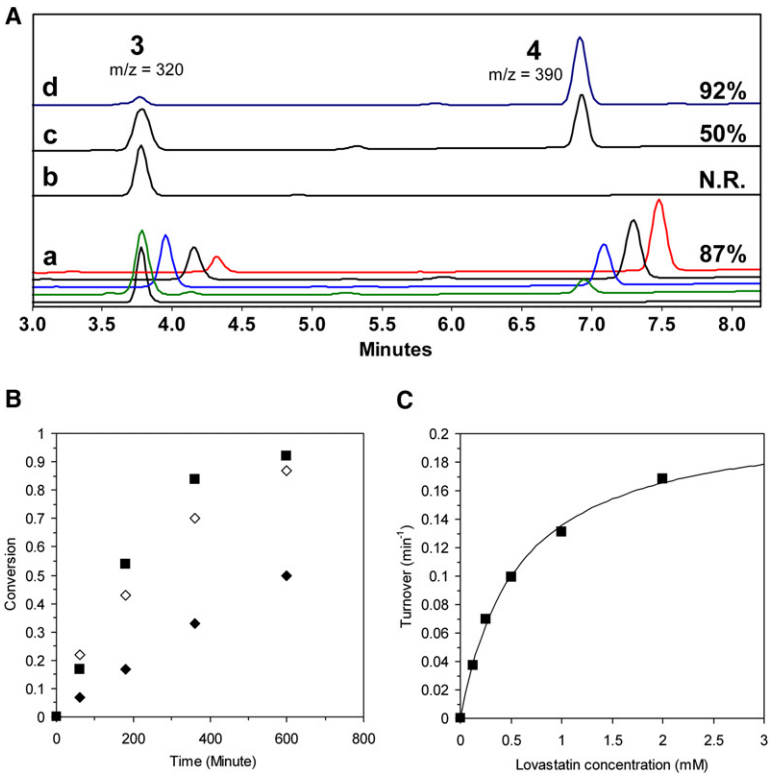


Figure 4. Verification of LovD Activity

(A) HPLC (238 nm) trace showing formation of **4** by LovD-mediated acyl transfer. Gradient: 60% B–95% B, 5 min; 95% B, 15 min; (**a**) LovD + **3** + butyryl-CoA time course (0, 1, 3, 6, and 10 hr); (**b**) LovD S76A + **3** + butyryl-CoA, 10 hr; (**c**) LovD + butyryl-SNAC, 10 hr; (**d**) LovD + butyryl-SMTG, 10 hr. Assay conditions: 1 mM **3**, 4 mM butyryl-CoA, 10 μM LovD, 50 mM HEPES, pH 7.9, 25°C.

(B) Conversion as a function of time for butyryl-CoA (open diamonds), butyryl-SNAC (closed diamonds), and butyryl-SMTG (closed squares). The apparent k_{cat} values reported throughout the text are initial turnover rates in the linear range.

(C) LovD catalyzed hydrolysis of **1** to **3**. The reaction progress is monitored by HPLC. k_{cat}: 0.21 ± 0.01 min^{-1}; K_m: 0.56 ± 0.05 mM.

acyl-*S*-LovF as an acyl donor, albeit likely with a significantly higher K_m due to the loss of potential protein-protein interactions.

Monacolin J Is a Competitive Inhibitor of LovD

With the HPLC assay shown in Figure 4A established, we measured the steady-state kinetic parameters of LovD toward butyryl-CoA and **3** by varying the concentrations of both substrates. Each data point shown is the result of three repeated measurements. The acylation reaction likely proceeds through a ping-pong mechanism, as shown in Figure 2, in which butyryl-CoA first binds to the free LovD, followed by dissociation of free CoA-SH and binding of **3** to effect acyl transfer. The concentrations of butyryl-CoA in the assays varied between 1 and 10 mM, while the sensitivity of the HPLC assay limited the lowest concentration of **3** to 50 μM. The enzyme concentrations ranged between 0.5 and 5 μM, depending upon the concentration of **3**.

Figure 5A shows the V/E_o of the acylation reaction as a function of increasing butyryl-CoA concentration. Interestingly, within the examined concentration range of **3** (50 μM–2 mM), we observed a marked decrease of product turnover rate at higher concentrations of **3**. This is indicative of excess substrate inhibition occurring due to binding of **3** to free LovD, forming a LovD-**3** complex and blocking entrance of butyryl-CoA. In contrast, no substrate inhibition by butyryl-CoA at high concentrations was observed (Figure 5A).

Figure 5B shows the Lineweaver-Burk plot of the data in Figure 5A, and reveals that **3** is a competitive inhibitor of butyryl-CoA, as expected. From the intercept of the plot, the k_{cat} of the reaction is estimated to be 0.53 ± 0.08 min^{-1}. The apparent K_m values of butyryl-CoA at fixed concentrations of **3** were calculated using nonlinear least-squares fitting and are plotted in the inset in Figure 5B. Linear regression of the plot indicates that the K_m for butyryl-CoA and K_I for **3** are 1.59 ± 0.15 mM and 330 ± 36 μM, respectively.

LovD Has Broad Substrate Specificity toward the Acyl Substrate

We assayed the tolerance of LovD toward different acyl substituents by performing the transacylation assay with various commercially available acyl-CoAs (Table 1). All assays were performed with 1 mM **3** to facilitate detection of products, 4 mM acyl-CoA, and 10 μM LovD. The reaction mixtures were kept at room temperature for 10 hr and were then extracted and analyzed by HPLC and LC-MS. With the exceptions of malonyl- and palmitoyl-CoA, a single, new compound matching the expected increase in mass as a result of transacylation was detected in each of the reactions (Table 1). Furthermore, in each case, no product formation could be detected in the absence of LovD.

Our results clearly indicate that LovD displays preference toward medium chain length (C3–C6) acyl groups, with butyryl-CoA being the optimal alkylacyl-CoA substrate. Both acetyl- and octanoyl-CoA were poor substrates of LovD, with <10% acylation of **3**. Surprisingly, the bulkier benzoyl-CoA was one of the best acyl substrates examined, with nearly 70% conversion of **3** to the corresponding C8-benzoyl-monacolin J analog (k_i = 0.16 min^{-1}). Introducing α-β unsaturation significantly

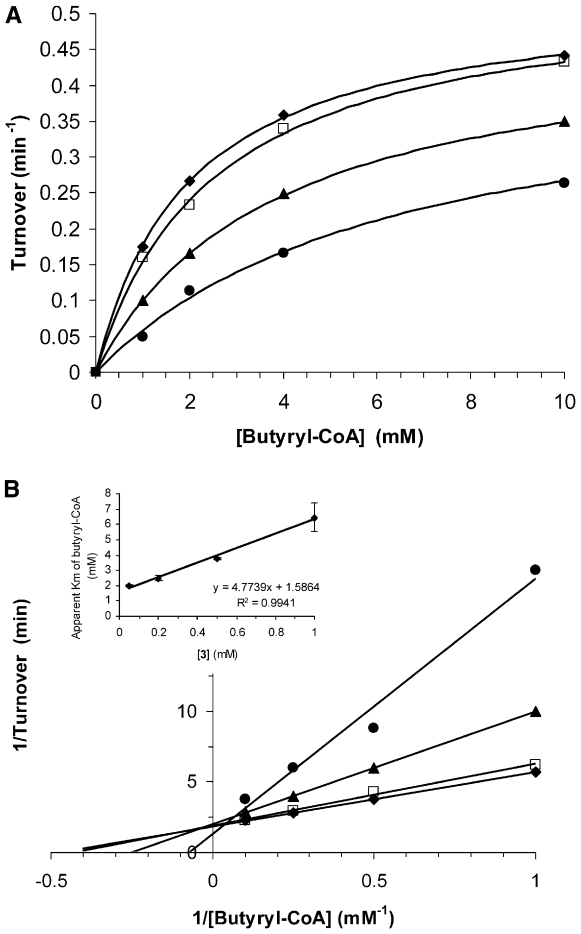


Figure 5. Kinetic Properties of LovD using Butyryl-CoA as an Acyl Substrate

(A) Kinetic analysis of LovD catalyzed acylation of **3** in the presence of butyryl-CoA. Initial turnover rates (V/E_o) as a function of butyryl-CoA concentration with different concentrations of **3**. The lines are fitted with nonlinear least squares regression to Michaelis-Menten kinetics. The concentrations of **3** used in these assays were 0.05 mM (closed diamonds), 0.2 mM (open squares), 0.5 mM (closed triangles), and 1 mM (closed circles).

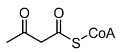
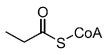
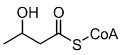
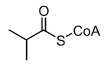
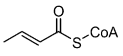
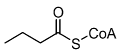
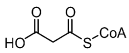
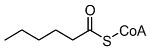
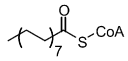
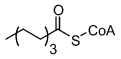
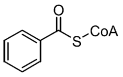
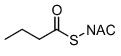
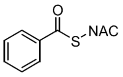
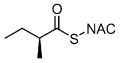
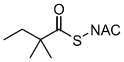
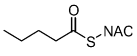
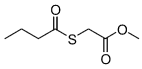
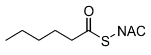
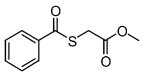
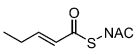
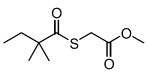
(B) Lineweaver-Burk plot of data shown in Figure 5A reveals that **3** is a competitive inhibitor of the acylation reaction; the k_{cat} is estimated to be ~0.53 min^{-1} from the intercept of the lines. Inset: the apparent K_m values of butyryl-CoA increase as the concentration of **3** is increased. The actual K_m of butyryl-CoA and K_I of **3** are determined to be 1.59 ± 0.15 mM and 330 ± 36 μM.

decreased the reaction rate, as seen in the 6% acylation of **3** in the presence of crotonyl-CoA. Acetoacetyl-CoA and β-hydroxylbutyryl-CoA were both excellent substrates of LovD, in good agreement with the isolation of monacolin X [22] and monacolin M [23] from the natural host, respectively. The side chains of these shunt products are likely formed due to the derailment of the LovF reductive tailoring steps, and were subsequently transferred by the broadly specific LovD.

LovD Accepts Membrane-Permeable Thioesters

To further examine the substrate specificities of LovD toward alternative acyl carriers, especially those that are simpler to prepare synthetically, and can penetrate

Table 1. Acyl-Thioesters as Substrates of LovD[a]

Acyl Thioester Substrate	Conversion (%)[b]/RT (min)[c]	Acyl Thioester Substrate	Conversion (%)/RT (min)
	7/5.1		89/4.6
	35/6.0		35/4.2
	52/6.8		6/6.5
	87/6.8		NR
	32/8.7		NR
	7/10.6		69/7.6
	50/6.8		58/7.6
	22/7.6		10/8.5
	52/7.8		92/6.8
	33/8.7		70/7.6
	2/7.5		17/8.5

NR, no reaction; RT, retention time.

[a] The products of the reactions were verified by LC-MS. Reaction conditions: 10 μM LovD, 1 mM **3**, 4 mM acyl-thioester, 50 mM HEPES, pH 7.9, 25°C, 10 hr.

[b] Conversion is measured by the percent **3** converted to the corresponding lovastatin analog using HPLC (238 nm).

[c] HPLC (C18 reverse phase) retention time of the free acid form of product. The HPLC gradient is the same as described in Figure 4.

cell membrane under in vivo conditions, we assayed two variants of butyryl-thioesters as substrates of LovD (Figure 4A and Table 1). *N*-acetylcysteamine thioesters (SNAC) have been used extensively as probes and precursors in studying natural product biochemistry [24, 25]. Methyl-thioglycolate (SMTG) was recently shown to be a cost-effective substitute for SNAC in the precursor-directed biosynthesis of erythromycin [26]. Figure 4 (traces c and d) shows the conversion of **3** to **4** when these butyryl-thioesters were used as acyl donors. Both SNAC and SMTG thioesters substituted for butyryl-CoA efficiently, with k_i values of 0.09 min^{-1} and 0.23 min^{-1} (Figure 4B), respectively, further highlighting that protein-protein interactions between LovD and LovF, as well as the interaction between LovD and the phosphopantetheine arm, are not required for acyl transfer. Butyryl-thioethane and butyryl-thioethanol, however, were not competent substrates of LovD, and supported only 4% and 1% conversion of **3** to **4**, respectively (data not shown). Similarly, benzoyl-SNAC and benzoyl-SMTG substituted for benzoyl-CoA efficiently, with k_i values of 0.12 min^{-1} and 0.15 min^{-1}, respectively (Table 1).

We then synthesized α-*S*-methylbutyryl-SNAC and α-dimethylbutyryl-SNAC and assayed for the in vitro chemoenzymatic synthesis of **1** and **2**, respectively. The results are shown in Figure 6A and Table 1. Authentic samples of **1** and **2** were used as references for HPLC detection (Figure 6A, trace a). The cognate, α-*S*-methylbutyrate side chain was, surprisingly, a poorer substrate compared with butyryl-, pentanoyl-, and hexanoyl-SNAC. The initial turnover rate ($k_i \approx 0.04$ min^{-1}) of **1** synthesis was more than 50% slower than that of LovD toward butyryl-SNAC under identical conditions. This suggested that the wild-type LovD has not been optimized for transferring the branched substrate. Addition of a second methyl substitutent at the α-position further attenuated the rate of acylation, likely attributed to the increased steric hindrance of the dimethyl moiety. Approximately 10% of **3** was converted to **2** when α-dimethylbutyryl-SNAC was used as a substrate ($k_i = 0.02$ min^{-1}). We were able to reach equilibrium conversions >70% when 100 μM LovD and a 10-fold excess of α-methyl-SNAC or α-dimethyl-SNAC were added to the in vitro reaction mixture (Figure 6A, traces b and c). The hydrolysis rate of **2** ($k_{cat}/K_m = 0.30$ min^{-1} mM^{-1}) by LovD was comparable to that of **1**.

LovD Can Accept Alternative Decalin Cores

To test the substrate specificity of LovD toward monacolin J variants, we assayed the conversion of tetra-ol **7** to **8**, pravastatin (**5**), and huvastatin (**6**) (Figure 6B). Vederas and coworkers have previously shown that, when 6-desmethyl-monacolin J is fed to an *A. terreus* mutant blocked in biosynthesis of **3**, compactin can be readily isolated [27]. This hints that LovD may be tolerant of substitutions at the C6 position of the decalin core. The novel compound **6** has recently been shown to have excellent pharmacological properties as a cholesterol-lowering compound [8]. Its synthesis from pravastatin is, however, further complicated by the presence of an additional reactive alcohol at C6. A LovD-mediated biotransformation that regioselectively acylates **7** to yield **6** could, therefore, be an attractive route.

To prepare the substrate **7**, we utilized the hydrolytic properties of LovD. When **5** is incubated with LovD under standard assay conditions, **7** can be readily formed. Using a 10 kDa MWCO spin column to remove LovD after complete hydrolysis, we were able to recover **7** in quantitative yield. The comparable rate of hydrolysis of **5** ($k_{cat}/K_m = 0.21$ min^{-1} mM^{-1}) by LovD to that of **1** hints that the C6 modified decalin core will not attenuate the rate of the acylation reaction significantly. Indeed, LovD displays relaxed specificity toward the hydroxyl substitution at C6 and catalyzed the acylation of **7** with higher efficiencies. The initial turnover rates for the synthesis of **8**, **5**, or **6** using the corresponding acyl-thioesters were 0.18, 0.11, and 0.03 min^{-1}, respectively. The retention time of authentic **5** was identical to the enzymatically synthesized compound. The mass of the newly formed compounds were verified by LC-MS. We did not detect any di-acylated products in the reaction mixture, further highlighting the exceptional regioselectivity of LovD toward the C8 hydroxyl group.

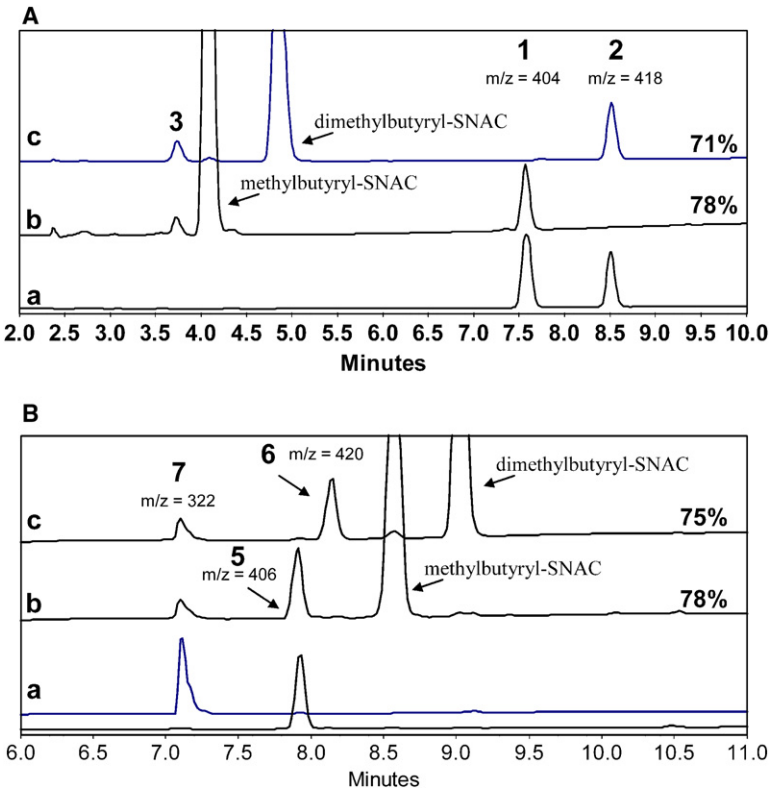


Figure 6. Activity of LovD towards Acyl-SNAC substrates

(A) HPLC (238 nm) trace showing formation of **1** and **2** by LovD-mediated acyl transfer. The gradient is the same as that described in Figure 4. (**a**) authentic standards of **1** and **2**; (**b**) **3** + α-*S*-methylbutyryl-SNAC; (**c**) **3** + α-dimethylbutyryl-SNAC. Assay conditions: 1 mM **3**, 10 mM acyl-SNAC, 100 μM LovD, 50 mM HEPES, pH 7.9, 25°C, 6 hr.

(B) HPLC (238 nm) traces showing formation of **5** and **6** by LovD-mediated acylation of the tetra-ol **7**. Gradient: 5% B–95% B, 5 min; 95% B, 15 min; (**a**) authentic standards of **5** and **7**; (**b**) **7** + α-*S*-methylbutyryl-SNAC; (**c**) **7** + α-dimethylbutyryl-SNAC. Assay conditions: 1 mM **3**, 10 mM acyl-SNAC, 100 μM LovD, 50 mM HEPES, pH 7.9, 25°C, 6 hr.

Whole-Cell Biocatalysis with LovD

To demonstrate that LovD can be used for preparative biosynthesis of lovastatin analogs, we first attempted to perform the benzylation reaction in vivo using *E. coli* as a heterologous host. The BL21(DE3)/pAW31 overexpression strain was grown in a shake flask (200 ml) to an OD_{600} of 1.0, at which time 1 mM IPTG, 0.8 mM **3**, and 4 mM of either benzoyl-SNAC or benzoyl-SMTG were added to the culture. Expression of LovD and bioconversion was performed at 18°C. The culture was extracted and analyzed for the formation of 8-benzoyl-monacolin J. When supplemented with benzoyl-SNAC, 84% conversion of **3** was detected within 20 hr postinduction. The product was lactonized and purified by a single silica gel chromatography step. The NMR spectra confirmed the regioselective benzoylation of the C8 hydroxyl group, as the diagnostic H8 multiplet was shifted downfield to δ 5.61 (see Experimental Procedures). In contrast, only 40% benzoylation was observed for the culture that was supplemented with benzoyl-SMTG, despite the faster in vitro acylation rate to that of benzoyl-SNAC. We discovered that the low conversion was due to the rapid degradation of benzoyl-SMTG by *E. coli*, and no trace of the precursor could be detected in the culture medium after 12 hr.

Encouraged by the high yield of conversion using *E. coli* as a whole-cell biocatalyst, we performed low cell density fermentation with α-dimethylbutyryl-SNAC as a precursor to yield **2** from **3** in a single biosynthetic step. After 2 days of culturing, we observed ~35% conversion of **3** to **2**. The product was purified, and the proton and carbon NMR spectra were identical to those of the commercial standard. The lower yield of **2** is consistent with the slower turnover rate observed in vitro, which may be further decreased at lower intracellular concentration of the SNAC precursor due to diffusion limitations. Other factors, such as the reversible hydrolysis of simvastatin and inactivation of LovD after prolonged fermentation, may lead to the observed conversion. We anticipate that the yield can be significantly improved by several means, such as: (1) using high cell density fermentation to increase the effective concentration of LovD and optimize fermentation conditions; and (2) increasing LovD catalytic efficiencies toward the unnatural precursor by protein engineering.

In conclusion, we have confirmed that LovD catalyzes the final acyl transfer step during lovastatin biosynthesis and regiospecifically acylates the C8 hydroxyl group in **3**. LovD displays broad substrate specificity toward the decalin aglycone, the acyl carrier, and the acyl group. When supplemented with the unnatural substrate α-dimethylbutyryl-SNAC, LovD produced the pharmaceutically important simvastatin both in vitro and in vivo. With optimization of the whole-cell biocatalyst, the one-step biological synthesis of simvastatin from either **1** or **3** will represent a significant upgrade over the conventional five-step chemical synthesis. In addition, we anticipate that, when an α-dimethylbutyryl-thioester precursor is supplied to a *lovF*-deficient strain of *A. terreus*, the lovastatin biosynthetic pathway can be redirected to afford **2** directly. We are currently attempting to obtain the X-ray crystal structure of LovD and use it as a starting point for enhancing LovD catalytic efficiencies.

Significance

Lovastatin is a natural fungal product that inhibits the rate-limiting step of cholesterol biosynthesis.

Understanding the biosynthetic steps of lovastatin from *Aspergillus terreus* is, therefore, important for the engineered biosynthesis of pharmaceutically relevant statin compounds. In this work, we have characterized an acyltransferase (LovD) that catalyzes the last step of lovastatin biosynthesis. First, we demonstrate that LovD is an efficient enzyme that transfers a 2-methylbutyryl acyl group regioselectively to the C8 hydroxyl of monacolin J, the immediate biosynthetic precursor of lovastatin. The reaction proceeds via a ping-pong mechanism, and is inhibited by monacolin J at moderate substrate concentrations. Second, we show that LovD has broad substrate specificity toward the acyl carrier, the acyl substrate, and the decalin core of the acyl acceptor. In particular, LovD does not require protein-protein interactions with the megasynthase LovF for the acyl transfer activity, and can accept a variety of acyl groups tethered to membrane-permeable thioesters. Building upon these biochemical insights, we demonstrate that LovD can be utilized as an effective biocatalyst for the single-step synthesis of simvastatin, a commercially important lovastatin analog, both in vitro and in vivo. Our work, therefore, provides important insights into several novel features of fungal polyketides biosynthesis, and can have significant utility in an improved synthesis of simvastatin.

Experimental Procedures

General Procedures

E. coli XL-1 Blue (Stratagene) was used for the manipulation of plasmid DNA. *E. coli* BL21(DE3) (Novagen) was used for protein expression. *A. terreus* (ATCC 20542) was cultured for extraction of genomic DNA. NMR spectra were obtained on Bruker spectrometers (^{1}H, 500 MHz; ^{13}C, 125 MHz; $CDCl_3$) at the NMR facility in the Department of Chemistry and Biochemistry at UCLA. LC–MS was conducted with a Finnigan LCQ Deca XP quadrupole ion trap mass spectrometer by positive electrospray ionization and a Waters 2.1 × 100 mm C18 reverse-phase column. Samples were separated on a linear gradient of 5%–95% CH3CN (v/v) over 30 min and 95% CH_3CN (v/v) for an additional 30 min in H_2O supplemented with 0.05% (v/v) formic acid at a flow rate of 0.125 ml/min at room temperature.

LovD Cloning, Expression, and Purification

The three exons of *lovD* were individually amplified from the genomic DNA of *A. terreus* and fused to yield a continuous open reading frame by splice-by-overlap extension PCR. The restriction sites *Nde*I and *Hind*III were introduced on the 5′ and 3′ outside primers, respectively. The gene cassette was ligated into pET28 (Novagen) to yield the expression construct pAW31. The *E. coli* BL21(DE3) strain transformed with pAW31 was grown in LB medium at 37°C to an OD_{600} of 0.5, at which time 1 mM IPTG was added to the culture and expression performed at 18°C for 24 hr. Cells were collected by centrifugation, resuspended in Buffer A (50 mM Tris-HCl, pH 8.0, 2 mM DTT, 2 mM EDTA), and lysed by sonication. Cell debris and insoluble proteins were removed by centrifugation (17,000 × g, 4°C, 1 hr). To the cleared lysate, 2 ml of Ni-NTA resin (Qiagen) was added. LovD was purified using a step gradient of buffer A with increasing concentration of imidazole. Pure (>95%) LovD proteins were eluted in buffer A containing 250 mM imidazole, buffer exchanged into buffer A without imidazole, concentrated, aliquoted, and flash frozen. The frozen LovD aliquots were for single use only. We observed significant decreases in enzyme activity after a single freeze-thaw cycle.

Construction of LovD S76A Mutant

The mutant *lovD* (Ser76 → Ala76) construct was made by oligo-directed mutagenesis using pAW31 as template and oligonucleotides 5′-GACACCCCCTGCCGGCTAGCCGCTGCGACCAAGCTGCT-3′ and 5′-AGCAGCTTGGTCGCAGCGGCTAGCCGGCAGGGGGTGTC-3′. The introduced mutation was verified by DNA sequencing. Expression and purification of the S76A mutant was performed in identical fashion as the wild-type enzyme (yield 30 mg/l).

Preparation of 3

Commercially available, lactonized **1** (500 mg) was refluxed in 50 ml 1 M LiOH for 24 hr. After cooling to room temperature, the suspension was acidified to pH 2.0 and extracted three times with 100 ml ethyl acetate. The extract was dried, evaporated, and redissolved in 100 ml toluene. A soxhlet apparatus was used to yield the lactonized form of **3**, which was subsequently purified with silica column chromatography (final yield, 253 mg, 64%). The sodium salt of **3** was obtained by stirring **3** in 100 mM NaOH in methanol, followed by evaporation and redissolving in dH_2O.

Preparation of Acyl Substrates

The acyl thioesters were prepared either by (1) combining the acyl chloride directly with the free thiol in diethyl ether/triethylamine, or (2) adding the free acid, diphenylphosphoryl azide, and the free thiol in DMF/triethylamine. The following is an example of (1): benzoyl chloride (370 μl, 3.2 mmol) was added slowly to a 20 ml solution of HSNAC (288 μl, 3 mmol) and triethylamine (835 μl, 6 mmol) in diethyl ether at 0°C. The reaction was quenched with aqueous NH_4Cl and extracted twice with 80 ml ethyl acetate. The organic layer was dried and evaporated to give a white solid (620 mg). The residue was purified with silica gel chromatography (EA:hexane, 20:80) to yield pure benzoyl-SNAC (602 mg, 90% yield). ^{1}H NMR: δ 7.93 (d, 2H, 7.5 Hz), 7.57 (t, 1H, 7.5 Hz), 7.43 (m, 2H), 6.24 (br s, 1H), 3.50 (q, 2H, 6.3 Hz), 3.21 (t, 2H, 6.3 Hz), and 1.92 (s, 3H). ^{13}C NMR: δ 192.22, 170.51, 136.68, 133.71, 128.71, 128.58, 127.42, 127.29, 39.63, 28.56, and 23.22. The following is an example of (2): α-dimethylbutyric acid (626 μl, 5 mmol) was dissolved in 25 ml DMF at 0°C and treated with diphenylphosphoryl azide (1.6 ml, 7.5 mmol) and triethylamine (1.4 ml, 10 mmol) for 2 hr. HSNAC (535 μl, 5 mmol) was added, and the solution was stirred for an additional 2 hr. The reaction was quenched with the addition of 50 ml H_2O and extracted twice with ethyl acetate. The organic layer was dried, evaporated, and α-dimethylbutyryl-SNAC was purified with silica gel chromatography to give 750 mg of a colorless liquid (69% yield). ^{1}H NMR: δ 6.01 (br s, 1H), 3.38 (q, 2H, 6.3 Hz), 2.97 (t, 2H, 6.3 Hz), 1.93 (s, 3H), 1.58 (q, 2H, 7.5 Hz), 1.17 (s, 6H), and 0.81 (t, 3H, 7.5 Hz). ^{13}C NMR: δ 207.17, 170.20, 50.13, 39.79, 33.65, 27.88, 24.72, 23.13, and 8.9.

In Vitro Assay of LovD Activity

The assays were performed at room temperature in 50 mM HEPES (pH 7.9), 50 mM NaCl, and 10 mM $MgCl_2$. The acyl substrates and **3** were added, and the reaction was initiated with the addition of LovD. To facilitate solubilization of hydrophobic acyl-SNAC and acyl-SMTG substrates, DMSO was added to a final concentration of 10%. Performing the in vitro assay at a slightly elevated temperature (30°C) resulted in aggregation and precipitation of LovD. At desired time points, 20 μl aliquots of the reaction mixture were extracted with ethyl acetate/1% acetic acid. The organic phase was dried in a speedvac, redissolved in 20 μl acetonitrile, and analyzed by HPLC. Similar procedures were followed to monitor the hydrolysis of lovastatin in the presence of LovD.

Preparation of 7

Commercially available sodium salt of **5** (4 mM) was added to a buffered solution containing 50 mM HEPES (pH 7.9), 10 mM NaCl, and 10 mM $MgCl_2$. LovD was added to a final concentration of 10 μM, and the solution was incubated at room temperature for 10 hr. Complete hydrolysis of **5** to **7** was determined by HPLC. After the level of **5** became undetectable, the reaction mixture was applied to a 10 kDa MWCO spin column to remove the enzyme. The filtrate was collected and used directly in subsequent assays.

Fermentation Conditions

Large-scale biosynthesis of **2** with BL21(DE3)/pAW31 was performed in 500 ml LB media with 30 mg/l kanamycin. At an OD_{600} of 1.0, cells were concentrated to a final OD_{600} of 5.0 and induced with 1 mM IPTG. Substrates **3** and acyl thioester were added to a final concentration of 1 mM and 4 mM, respectively. At different time

points, culture samples were collected, centrifuged, filtered, and injected on to HPLC (20 μl). When maximum conversion was reached, the broth was acidified to pH 2.0, extracted with ethyl acetate, dried, and redissolved in toluene. The lactone forms of **3** and acylated products were obtained by refluxing with a soxhlet apparatus. Pure, acylated products were obtained after silica gel column chromatography.

NMR Characterization of Lovastatin Analogs

Substrate **4** (0.5 mg) was purified directly from a scaled-up in vitro reaction. C8-benzoyl-monacolin J and **2** were purified from fermentation broths. All data reported are for the lactonized compounds. The diagnostic H8 and C8 signals in the following characterizations are underlined.

4: ^{1}H NMR: δ 5.98 (d, 1H, 9.7 Hz, H-4), 5.78 (dd, 1H, 6.1 Hz, 9.5 Hz, H-3), 5.53 (m, 1H, H-5), 5.39 (m, 1H, H-8), 4.64 (m, 1H, H-11), 4.37 (m, 1H, H-13), 2.80–2.61 (m, 2H, H-14), 2.45–2.40 (m, 1H, H-6), 2.40–2.30 (m, 1H, H-2), 2.31–2.26 (m, 2H, H-2′), 2.30–2.20 (m, 1H, H-8a), 2.00–1.91 (m, 1H, H-1), 1.96–1.93 (m, 2H, H-7), 1.90–1.80 (m, 1H, H-10), 1.70–1.60 (m, 2H, H-12), 1.70–1.60 (m, 2H, H-3′), 1.49–1.32 (m, 2H, H-9), 1.40–1.30 (m, 1H, C-10), 1.06 (d, 3H, 7.4 Hz, CH_3-6), 0.88 (d, 3H, 7.1 Hz, CH_3-2), and 0.83 (t, 3H, 7.4 Hz, H-4′). ^{13}C NMR: δ 174.32 (C-1′), 171.45 (C-15), 133.21 (C-3), 131.62 (C-4a), 130.07 (C-5), 128.76 (C-4), 76.71 (C-11), 68.19 (C-8), 63.03 (C-13), 38.96 (C-14), 37.85 (C-8a), 36.96 (C-12), 36.53 (C-1), 35.52 (C-2′), 33.21 (C-7), 32.61 (C-10), 30.91 (C-2), 27.63 (C-6), 24.67 (C-9), 23.36 (CH_3-6), 18.11 (C-3′), 14.20 (CH_3-2), and 11.67 (C-4′).

C8-benzoyl-monacolin J: ^{1}H NMR: δ 8.03 (d, 2H, 7.5 Hz, C$_6H_5$-2), 7.56 (t, 1H, 7.5 Hz, C$_6H_5$-4), 7.44 (m, 2H, C$_6H_5$-3), 6.05 (d, 1H, 9.7 Hz, H-4), 5.82 (dd, 1H, 6.1 Hz, 9.6 Hz, H-3), 5.61 (br, 1H, H-5), 5.61 (m, 1H, H-8), 4.53 (m,1H, H-11), 4.21(m, 1H, H-13), 2.70–2.51 (m, 2H, H-14), 2.40–2.35 (m, 1H, H-6), 2.40–2.30 (m, 1H, H-2), 2.25–2.16 (m, 1H, H-8a), 2.03–1.97 (m, 1H, H-1), 1.99–1.93 (m, 2H, H-7), 1.90–1.80 (m, 1H, H-10), 1.72–1.62 (m, 2H, H-12), 1.50–1.36 (m, 2H, H-9), 1.35–1.20 (m, 1H, C-10), 1.01 (d, 3H, 7.4 Hz, CH_3-6), and 0.92 (d, 3H, 7.1 Hz, CH_3-2). ^{13}C NMR: δ 170.18 (C-15), 166.27 (C-1′), 133.26 (C-3), 133.08 (C$_6$H$_5$-4), 131.79 (C-4a), 130.50 (C$_6$H$_5$-1), 129.86 (C-5), 129.76 (C$_6$H$_5$-2), 128.59 (C$_6$H$_5$-3), 128.46 (C-4), 76.15 (C-11), 68.95 (C-8), 62.72 (C-13), 38.52 (C-14), 37.59 (C-8a), 37.12 (C-12), 36.05 (C-1), 32.83 (C-7), 32.63 (C-10), 30.95 (C-2), 27.56 (C-6), 24.17 (C-9), 22.73 (CH_3-6), and 14.07 (CH_3-2).

2: ^{1}H NMR: δ 5.98 (d, 1H, 9.6 Hz, H-4), 5.77 (dd, 1H, 6.1 Hz, 9.6 Hz, H-3), 5.50 (m, 1H, H-5), 5.36 (m, 1H, H-8), 4.61 (m, 1H, H-11), 4.37 (m, 1H, H-13), 2.80–2.60 (m, 2H, H-14), 2.45–2.40 (m, 1H, H-6), 2.40–2.30 (m, 1H, H-2), 2.30–2.20 (m, 1H, H-8a), 2.00–1.91 (m, 1H, H-1), 1.96–1.93 (m, 2H, H-7), 1.90–1.80 (m, 1H, H-10), 1.70–1.60 (m, 2H, H-12), 1.60–1.50 (q, 2H, 7.4 Hz, H-3′), 1.49–1.32 (m, 2H, H-9), 1.40–1.30 (m, 1H, C-10), 1.12 (d, 6H, 3.0 Hz, CH_3-2′), 1.08 (d, 3H, 7.4 Hz, CH_3-6), 0.88 (d, 3H, 7.1 Hz, CH_3-2), and 0.83 (t, 3H, 7.4 Hz, H-4′). ^{13}C NMR: δ 178.29 (C-1′), 170.66 (C-15), 133.22 (C-3), 131.84 (C-4a), 130.05 (C-5), 128.72 (C-4), 76.69 (C-11), 68.36 (C-8), 63.00 (C-13), 43.36 (C-2′), 38.94 (C-14), 37.86 (C-8a), 36.96 (C-12), 36.53 (C-1), 33.34 (C-3′), 33.24 (C-7), 32.60 (C-10), 30.98 (C-2), 27.61 (C-6), 25.13 (CH_3-2′), 25.10 (CH_3-2′), 24.65 (C-9), 23.39 (CH_3-6), 14.22 (CH_3-2), and 9.67 (C-4′).

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