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Conversion of Cyclic Nonaketides to Lovastatin and Compactin by a *lovC* Deficient Mutant of *Aspergillus terreus*

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Abstract—Investigation of the post-PKS biosynthetic steps to the cholesterol-lowering agent lovastatin (**1**) using an *Aspergillus terreus* strain with a disrupted *lovC* gene, which is essential for formation of 4a,5-dihydromonacolin L (**3**), shows that **7** and **3** are precursors to **1**, and demonstrates that lovastatin diketide synthase (*lovF* protein) does not require *lovC*. © 2001 Elsevier Science Ltd. All rights reserved.

Lovastatin (**1**) (also known as mevinolin, monacolin K, or Mevacor^{TM}) is a potent cholesterol-lowering drug[1] isolated from *Monascus ruber* by Endo and co-workers[2] and from *Aspergillus terreus* by Merck researchers.[3] This fungal metabolite and its analogue compactin (**2**),[4] also designated ML-236B or mevastatin (Fig. 1), have antifungal activity, but their pharmacological and commercial importance stems from the competitive inhibition of (3*S*)-hydroxy-3-methylglutaryl-coenzyme A reductase, the rate limiting enzyme in the biosynthesis of cholesterol.[1c,5] Biosynthetic studies with fungi (e.g., *A. terreus*) show that lovastatin (**1**) is a polyketide (Fig. 2) derived from 11 acetate units, molecular oxygen, and two methyl groups from *S*-adenosyl-L-methionine (SAM).[6,7] Additional incorporation studies with the compactin (**2**) producer *Penicilium aurantiogriseum* indicated that each atom of **2** has the same biosynthetic origin as its homologue in lovastatin (**1**), suggesting a similar assembly of both metabolites.[7f]

The polyketide synthase (PKS) steps of the biosynthesis of lovastatin (**1**) and compactin (**2**) involve the head-to-tail assembly of all the acetate molecules as well as a cyclization to afford 4a,5-dihydromonacolin L (**3**) and its 6-desmethyl analogue **4**, respectively. Compounds **3** and **4** are further transformed into **1** and **2**, respectively, through post-PKS modification steps that may proceed as proposed by Endo and co-workers (Scheme 1).[8] Using cell-free extracts of *M. ruber*, they demonstrated the conversion of **3** into 3α-hydroxy-3,5-dihydromonacolin L (**5**),[8a] which is suggested to spontaneously dehydrate to give **6**. Hydroxylation at C-8 of monacolin L (**6**) by molecular oxygen present in the cell-free system of Endo and co-workers provides monacolin J (**7**),[8e] but researchers at Merck concluded that **6** is formed from **5** only as an artifact of isolation rather than as a discrete product of biosynthesis since no monacolin L (**6**) can be detected in freshly harvested *A. terreus* cultures, whereas an increasing amount appears during the isolation of lovastatin (**1**).[9] The nonaketide **7** is finally converted to lovastatin (**1**) in the cell-free extract by esterification with the diketide (2*R*)-methylbutyric acid (**8**).[8b] Study of the lovastatin gene cluster of *A. terreus* located 18 putative open reading frames (Fig. 3) for which functions were tentatively assigned based on sequence alignment and on the preparation and characterization of genetically engineered fungal

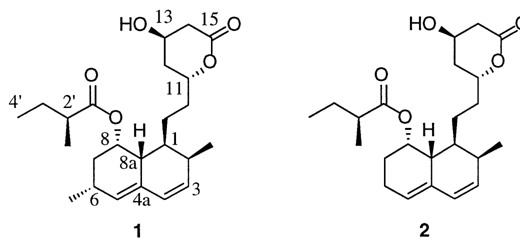

Figure 1. Lovastatin (**1**) and compactin (**2**).

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strains.[10] We now report the use of a strain termed *A. terreus lovC*, in which the *lovC* gene has been disrupted by insertional mutation, to examine the post-PKS steps involved in the biosynthesis of **1**.

The *lovC* protein is an accessory enzyme that complexes to *lovB* protein (also known as lovastatin nonaketide synthase or LNKS) and imparts enoyl reductase activity necessary for successful assembly of the normal PKS product, dihydromonacolin L (**3**).[10] Lacking a functional form of this accessory protein, the *A. terreus lovC* disruptant produces polyketide pyrones **11** and **12** (as does a heterologous host *A. nidulans* having only the *lovB* gene) (Fig. 4), but it does not produce lovastatin (**1**) nor any of the post-PKS intermediates **5**, **6**, or **7**.[10] Nevertheless, this strain is still expected to contain all the post-PKS activities required to assemble lovastatin (**1**) from **3**, with the possible exception of generation of the (*2R*)-methylbutyrate side chain by lovastatin diketide synthase (LDKS, *lovF* protein), which could also

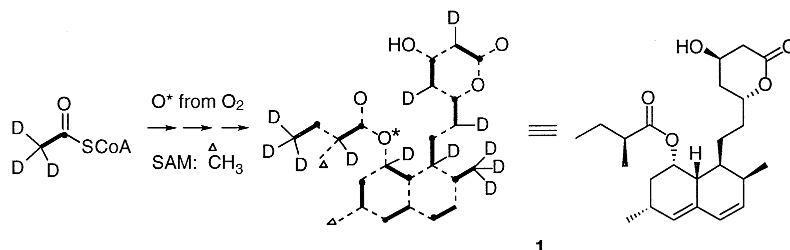
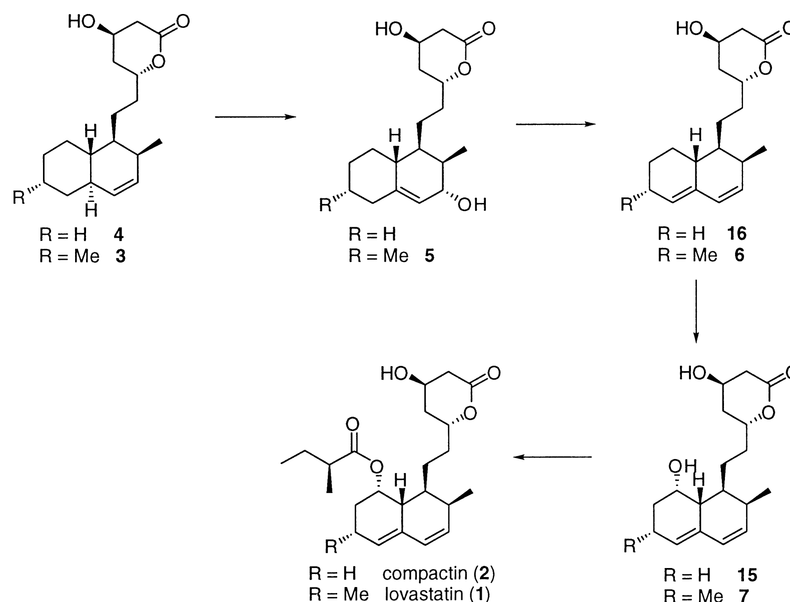


Figure 2. Origin of the atoms in lovastatin (**1**).



Scheme 1. Post-PKS transformations leading to lovastatin (**1**) and compactin (**2**).

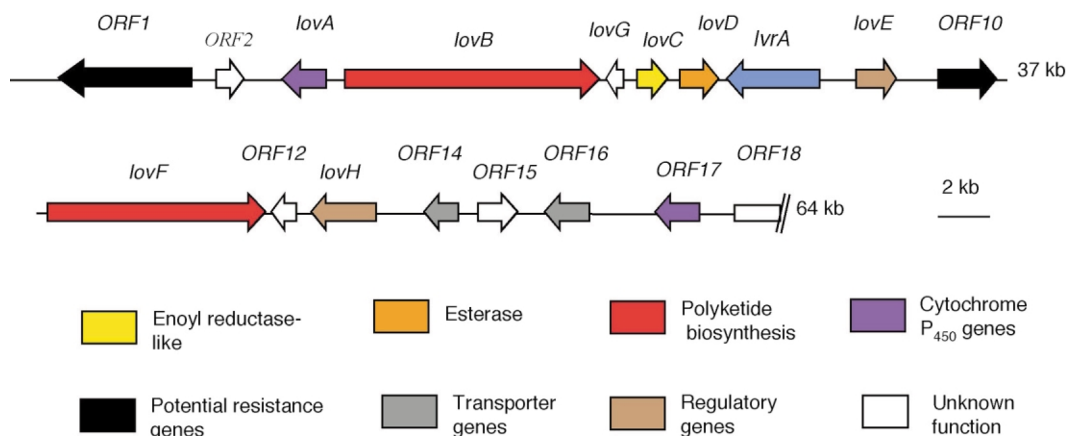


Figure 3. Lovastatin gene cluster.

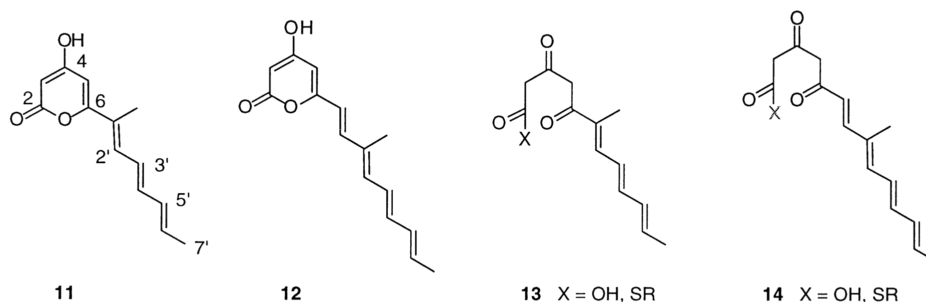


Figure 4. The yellow pigments **11** and **12**, and their homologue compounds **13** and **14**.

require *lovC* protein for enoyl reductase activity. Consequently, the *A. terreus lovC* disruptant represents an ideal strain to verify the post-PKS biosynthetic reactions leading to lovastatin (**1**) and to examine the possible requirement of *lovC* protein for proper formation of the diketide side chain by *lovF* protein.

Extensive analysis of the *A. terreus lovC* disruptant cultures did not reveal the presence of any of the three likely side-chain diketides [i.e., (*2R*)-methylbutyric acid **8**, tiglic acid (**9**), or crotonic acid (**10**)].[11,12] This suggests either that LDKS requires *lovC* for activity, or that **8** is released directly to the esterase (LovD protein) without the release of the free acid. To examine this hypothesis, monacolin J (**7**), prepared by hydrolysis of lovastatin (**1**),[13] was administered to *A. terreus lovC* disruptant cultures.[14] The yield of **1**, which is normally not produced by this strain, accounted for about 70% of the monacolin J (**7**) injected. This experiment demonstrates that, unlike the lovastatin nonaketide synthase (*lovB* protein), LDKS does not require the *lovC* protein for polyketide assembly and that all of its functional domains are active, including its enoyl reductase. Failure to detect free (*2S*)-methylbutyric acid **8** supports our earlier suggestion that *lovD* protein (homologous to esterases) promotes direct esterification of the LDKS enzyme-bound diketide with monacolin J (**7**), and that free methylbutyrate or its CoA thiolester are not involved in this process.[10] This is also in accord with sequence analysis and databases comparisons that indicate the absence of a thioesterase domain on LDKS, which would release **8** into the medium.

In order to examine whether the *lovD* protein of *A. terreus* can esterify an analogue of **7**, 6-desmethylmonacolin J (**15**) was added to the *A. terreus lovC* disruptant cultures. No compactin (**2**) is normally produced by *A. terreus*. The required precursor **15** is readily obtained in 75% yield by basic hydrolysis of compactin (**2**) (Scheme 1).[15] The *A. terreus lovC* disruptant generates compactin (**2**) in 45% yield from **15**, thereby demonstrating the ability of *lovD* protein to accommodate the absence of the methyl group at C-6. Interestingly, ML-236C (**16**), the 6-desmethyl analogue of monacolin L (**6**) (Scheme 1) and a proposed post-PKS precursor of **2** in *P. aurantiogriseum*, was not converted by *A. terreus lovC* disruptant cultures to detectable amounts of compactin (**2**). This suggests that either the enzyme for hydroxylation at C-8 is very specific and requires a methyl at C-6, or that neither ML-236C nor monacolin L (**6**) are intermediates en route to compactin (**2**) or lovastatin (**1**), respectively. As mentioned above, the intermediacy of **6** in the *A. terreus* pathway to **1** has been disputed.[9]

To verify that all of the biosynthetic enzymes required to oxidize C-8 and add the extra double bond into the decalin system are present in the *A. terreus lovC* disruptant, 4a,5-dihydromonacolin L (**3**) (isolated from an engineered *A. nidulans* strain expressing *lovB* and *lovC* proteins[10]) was added to cultures of this organism. This strain, which normally produces the pigments **11** and **12**,[10] converted **3** to lovastatin (**1**) in 40% yield. This confirms that all of the necessary biosynthetic machinery for post-PKS transformation of **3** to **1** is active and indicates that *lovC* protein is not involved in these late stage processes.

In summary, the results demonstrate that the critical role of *lovC* protein is to ensure correct assembly with reductive processing of the nonaketide chain of **1** by *lovB* protein (LNKS), but that it is not necessary for construction of the methylbutyrate side chain by the *lovF* protein, which is also an iterative type I PKS. This work also shows that *lovC* protein has no detectable function in post-PKS processing of dihydromonacolin L (**3**), and indicates that the *lovC* disruptant may prove useful in transformation of a variety of modified derivatives of **3** to new analogues of lovastatin (**1**). Additional studies on such conversions and on the biosynthetic pathway to **1** are in progress.

Acknowledgements

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12. For fermentation of *A. terreus lovC* disruptant, the following media were prepared. A 10×AMM salts solution was made that contained: 60 g of $NaNO_3$, 5.2 g of KCl, and 15.2 g of KH_2PO_4, diluted to 1 L at pH 6.5. The trace elements solution required for production medium had: 1 g of $FeSO_4{\cdot}7H_2O$, 8.8 g of $ZnSO_4{\cdot}7H_2O$, 0.4 g of $CuSO_4{\cdot}5H_2O$, 0.15 g of $MnSO_4{\cdot}4H_2O$, 0.1 g of $Na_2B_4O_7{\cdot}10H_2O$, and 0.05 g of $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, diluted to 1 L with 0.5 mL HCl added. Agar complete medium (ACM) contained: 20 g of malt extract, 1 g of bacto-peptone, 20 g of glucose, and 20 g of agar, in 1 L of water. The ACM slants were inoculated with *A. terreus lovC* and incubated for 5 days at 30 °C. Growth medium (YEPD) contained: 20 g of yeast extract, 1 g of bacto-peptone, and 20 g of glucose, diluted to 1 L. This was inoculated from one slant of *A. terreus lovC* disruptant and then fermented (1 L in a 2 L Erlenmeyer) at 200 rpm and 30 °C for 1 day. Miracloth (Calbiochem, La Jolla, CA, USA) was used to filter the mycelia, which were washed with production medium (2 L) and then transferred into fresh production medium (1 L in a 2 L Erlenmeyer). The production medium (AMM) consisted of: 1 mL of trace elements solution, and 100 mL of 10×AMM salts solution, diluted to 1 L, then autoclaved, and finally treated with 2.5 mL of sterile 20% $MgSO_4{\cdot}7H_2O$ and 25 mL of sterile 40% lactose solution. The AMM culture was incubated at 30 °C and 200 rpm. In order to detect the possible presence of **8**, **9**, or **10**, the mycelia and supernatant were analysed by HPLC using authentic standards on a Novapak radial compression reverse-phase C_{18} column, 8×100 mm, 4 µm (Millipore Waters, Mississauga, ON, Canada). The flow rate was kept at 2 mL/min, and the gradient was raised from 0 to 10% acetonitrile in water and 0.1% TFA over 10 min, raised from 10 to 52% over 15 min, raised from 52 to 100% over 5 min, and kept at 100% for 5 min. The UV detector was set at 220 nm. Retention time was 15.6 min for (2*R*)-methylbutyric acid (**8**), 17.0 min for tiglic acid (**9**), and 38.9 min for crotonic acid (**10**). None of these diketides was present in the cultures.

13. A monacolin J (**7**) standard was prepared by hydrolysis of lovastatin (**1**) as described for desmethylmonacolin J below. It was also purified by HPLC with a µBondapak radial compression reverse-phase column C_{18}, 25×100 mm, 10 µm, 125 Å (Millipore Waters, Mississauga, ON). The flow rate was 15 mL/min. The gradient started at 40% acetonitrile and 0.1% TFA for 10 min, raised from 40 to 49% over 11 min, kept at 49% for 8 min, raised from 49 to 56% over 8 min, from 56 to 100% over 5 min, and kept at 100% for 5 min. The UV detector was set at 240 nm. Monacolin J (**7**) was collected as a white solid with a retention time of 10 min. $[\alpha]_D^{25}$ +17.30 (*c* 0.14, CH_3OH); IR (microscope) 3236 (br s), 2927 (s), 2879 (s), 1707 (m), 1645 (m), 1451 (s), 1318 (s), 1093 (s), 1075 (s), 1060 (s), 1049 (s), 1026 (s), 973 (s), 858 (s) cm^{-1}; ^1H NMR (500 MHz, CD_3OD) δ 5.93 (d, 1H, *J* = 10.0 Hz, H-4), 5.75 (dd, 1H, *J* = 10.0, 6.2 Hz, H-3), 5.45 (br s, 1H, H-5), 4.23 (br dd, 1H, *J* = 6.5, 2.8 Hz, H-8), 3.92 (m, 1H, H-11), 3.79 (m, 1H, H-13), 3.68 (br t, 2H, *J* = 6.5 Hz, H-14), 2.38 (m, 2H, H-2, H-6), 2.13 (br dd, 1H, *J* = 12.1, 2.8 Hz, H-8a), 1.92–1.68 (m, 4H, H-7, H_a-9, H_a-12), 1.67–1.50 (m, 3H, H-1, H_a-10, H_b-12), 1.41 (m, 1H, H_b-10), 1.31 (m, 1H, H-9), 1.18 (d, 3H, *J* = 7.4 Hz, 6-Me), 0.89 (d, 3H, *J* = 6.9 Hz, 2-Me); ^{13}C NMR (125 MHz, CD_3OD) δ 189.96 (C-15), 134.11 (C-4), 133.16 (C-4a), 130.60 (C-3), 130.02 (C-5), 71.73 (C-8), 69.21 (C-11), 65.89 (C-13), 60.08 (C-14), 45.41 (C-7), 40.98 (C-10), 39.77 (C-8a), 37.61 (C-1), 37.10 (C-12), 35.64 (C-9), 32.13, 29.13 (C-2, C-6), 23.65 (6-Me), 14.29 (2-Me); MS (ES) 347 (MNa^+, 100), 671 ($2MNa^+$, 25).

14. For incorporation of monacolin J (**7**) into **1**, a solution of **7** (0.1 mL of 30 mg in 1.2 mL of ethanol) was added to growing cultures of *A. terreus lovC* disruptant (1 L) every 6 h for 3 days, starting 16 h after the transfer. The cultures were then grown for an additional 3 days. The wet mycelia (45 g/L) were vacuum filtered, and the acidified filtrate (pH 2, 2 N HCl) was extracted with EtOAc (3×350 mL). The combined organic layers were washed with brine and dried (Na_2SO_4). After evaporation of the solvent, the residue was redissolved in toluene (60 mL) and heated under reflux with a Soxhlet containing calcium hydride for 1 h. The toluene was evaporated in vacuo, and the residue (0.28 g) was purified by HPLC on a µBondapak radial compression reverse-phase C_{18}, 25×100 mm, 10 µm, 125 Å column (Millipore Waters, Mississauga, ON). The flow rate was 15 mL/min, and the gradient was kept at 40% acetonitrile in water and 0.1% TFA for 10 min, raised from 40 to 49% over 11 min, kept at 49% for 8 min, raised from 49 to 56% over 8 min, then raised from 56 to 95% over 2 min, and kept at 95% for 3 min. The UV detector was set at 220 nm. Retention time was 17.2 min for **1** and 5.1 min for **7**. The yield of lovastatin (**1**) was about 25 mg/L of cultures (70% from monacolin J), with 5 mg/L of recovered monacolin J (**7**) (15%). The incorporation of 6-desmethylmonacolin J (**15**) into compactin (**2**) was done similarly except for the HPLC gradient which was kept at 30% acetonitrile in water and 0.1% TFA for 5 min, raised from 30 to 49% over 16 min, raised from 49 to 95% over 8 min, and kept at 95% for 5 min. The UV detector was set at 238 nm. Retention time was 20.5 min for **2**. Further purification of **2** was achieved by filtration through a silica gel SEP-PAK® cartridge (Millipore Waters) with 50% EtOAc in hexanes, and a Plus C_{18} SEP-PAK® cartridge using MeOH. The yield of **2** was about 11 mg/L of cultures (45% from the 25 mg of **15**). The incorporation of 4a,5-dihydromonacolin L (**3**) as above gave **1**: 6 mg/L of culture (40% from the 12 mg of **3**).

15. A solution of compactin (**2**) (55 mg, 0.13 mmol) and lithium hydroxide monohydrate (60 mg, 1.3 mmol) in water (15 mL) was heated to reflux for 1 day. The mixture was cooled in ice, acidified to pH 1 (2 N HCl), and extracted with EtOAc (5×30 mL) and $CHCl_3$ (4×20 mL). The combined organic layers were dried (Na_2SO_4) and concentrated in vacuo. To ensure that **15** was lactonized, toluene (60 mL) was added to the residue and the solution was heated under reflux with a Soxhlet containing calcium hydride for 1 h. The toluene was removed in vacuo and the compound was purified by silica gel flash column chromatography (100% EtOAc, R_f 0.12) to afford **15** as an oil (30 mg, 75%). $[\alpha]_D^{25}$ +780 (*c* 0.15, $CHCl_3$); IR ($CHCl_3$ cast) 3407 (br s), 2928 (s), 1710 (s), 1256 (s), 1075 (s), 754 (s); ^1H NMR (500 MHz, $CDCl_3$) δ 5.92 (d, 1H, *J* = 9.6 Hz, H-4), 5.71 (dd, 1H, *J* = 9.6, 6.0 Hz, H-3), 5.52 (br d, 1H, *J* = 2.1 Hz, H-5), 4.69 (m, 1H, H-11), 4.52 (m, 1H, H-13), 4.21 (br s, 1H, H-8), 2.67 (dd, 1H, *J* = 17.7, 5.0 Hz, H_a-14), 2.59 (ddd, 1H, *J* = 17.7, 3.7, 1.7 Hz, H_b-14), 2.31 (m, 2H, H-2, H_a-6), 2.14 (m, 2H, H_b-6, H-8a), 1.96 (m, 2H, H_a-7, H_a-12), 1.82–1.62 (m, 5H, H-1, H_b-7, H_a-9, H_a-10, H_b-12), 1.53–1.39 (m, 2H, H_b-9, H_b-10), 0.88 (d, 3H, *J* = 7.0 Hz, 2-Me); ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.81 (C-15), 133.27 (C-4a), 133.01 (C-3), 128.33 (C-4), 123.65 (C-5), 76.22 (C-11), 64.42 (C-8), 62.56 (C-13), 38.75 (C-8a), 38.51 (C-14), 36.39 (C-1), 36.16 (C-7), 32.63 (C-9), 30.77 (C-2), 29.09 (C-12), 23.79 (C-10), 20.29 (C-6), 13.87 (2-Me); MS (ES) calcd for $C_{18}H_{26}O_4Na$ 329.1729, found 329.1725 (MNa^+, 100), 307.2 (MH^+, 10).