Single-step fermentative production of the cholesterol-lowering drug pravastatin via reprogramming of Penicillium chrysogenum

Kirsty J. McLeanb1, Marcus Hansa1, Ben Meijrinkb2, Wibo B. van Scheppeningb, Aad Vollebregtb, Kang Lan Teea, Jan-Metske van der Laanb2, David Leysa, Andrew W. Munroa2, and Marco A. van den Bergb2

The cholesterol-lowering blockbuster drug pravastatin can be produced by stereoselective hydroxylation of the natural product compactin. We report here the metabolic reprogramming of the antibiotics producer Penicillium chrysogenum toward an industrial pravastatin production process. Following the successful introduction of the compactin pathway into the β-lactam-negative P. chrysogenum DS50662, a new cytochrome P450 (P450 or CYP) from Amycolatopsis orientalis (CYP105AS1) was isolated to catalyze the final compactin hydroxylation step. Structural and biochemical characterization of the WT CYP105AS1 reveals that this CYP is an efficient compactin hydroxylase, but that predominant compactin binding modes lead mainly to the ineffective epimer 6-epi-pravastatin. To avoid costly fractionation of the epimer, the enzyme was evolved to invert stereoselectivity, producing the pharmacologically active pravastatin form. Crystal structures of the optimized mutant P450prava fused to a redox partner in compactin-producing P. chrysogenum yielded more than 6 g/L pravastatin at a pilot production scale, providing an effective new route to industrial scale production of an important drug.

Significance

Statins are successful widely used drugs that decrease the risk of coronary heart disease and strokes by lowering cholesterol levels. They selectively inhibit the key regulatory enzyme of the cholesterol synthesis pathway, thus lowering levels of plasma LDL (bad) cholesterol. Pravastatin is one of the leading and most effective statins, derived from the natural product compactin. However, pravastatin production involves a costly two-step fermentation process and in drug/xenobiotic detoxification and metabolism (10). In addition, nonhuman CYPs have increasing importance in production of antibiotics and other agents beneficial to human health (11). Among the statins, pravastatin has the lowest potential for drug interactions (e.g., with antibiotics) as it is not extensively metabolized by human CYPs (12). However, low P450sca-2 activity during biotransformation, compactin toxicity, and low pravastatin yield hamper large-scale manufacturing.

Statins inhibit 3β-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, the enzyme catalyzing the rate-limiting step in cholesterol biosynthesis. Statins reduce “bad” plasma (LDL) cholesterol levels and are thus effective against hypercholesterolemia. Several statins are on the market; the most prominent being the completely synthetic atorvastatin (Lipitor), the semisynthetic simvastatin (Zocor), and pravastatin (Pravachol) (1, 2). Atorvastatin was identified by a Pfizer R&D pharmaceutical screening program using entirely unnatural lead structures (3); simvastatin and pravastatin, discovered by Merck and Sanofi, respectively, are examples of synthetic variants of the naturally occurring statins lovastatin and compactin, and have superior pharmacokinetic properties (4, 5). Lovastatin and compactin are produced by filamentous fungi such as Aspergillus terreus and Penicillium chrysogenum, respectively (Fig. 1A). In recent years, their biosynthetic gene clusters were identified and comprise a set of nine proteins, including a HMG-CoA reductase, a transporter, and a transcriptional activator. Two large polyketide synthases produce the nonaketide precursor and methylbutyrate side chain. Additional oxido-reductase and hydroxylases enzymes lead to the production of lovastatin and compactin (6, 7). Simvastatin (Fig. 1B) is a semisynthetic variant of lovastatin wherein the methylbutyrate side chain is converted into a dimethylbutyrate moiety. This outcome can be achieved by direct methylation or by deacetylation and subsequent addition of a de novo chemically synthesized dimethylbutyrate group (8). Pravastatin (Fig. 1B) is obtained after stereoselective hydroxylation of compactin at the C-6 position. Industrially, this is achieved by a two-step production process. First, fermentation of P. citrinum produces compactin. Next, the statins are purified and the lactone ring is opened by addition of sodium hydroxide. After neutralization, the open lactone form of compactin is converted to pravastatin in a biotransformation step using the bacterium Streptomyces cavescens. This organism contains a cytochrome P450 enzyme (P450 or CYP) that is capable of hydroxylating compactin stereospecifically at C-6 with an overall yield of 70% (P450sca-2) (9). CYPs are hemoproteins of biotechnological interest due to their ability to perform regio- and stereoselective oxygen insertion chemistry. They are best known for their pivotal roles in human steroid synthesis and in drug/xenobiotic detoxification and metabolism (10). In addition, nonhuman CYPs have increasing importance in production of antibiotics and other agents beneficial to human health (11). Among the statins, pravastatin has the lowest potential for drug interactions (e.g., with antibiotics) as it is not extensively metabolized by human CYPs (12). However, low P450sca-2 activity during biotransformation, compactin toxicity, and low pravastatin yield hamper large-scale manufacturing.
Here, we present a radically different approach: development of a one-step, fully fermentative pravastatin production process. The industrial high producing P. chrysogenum strain DS17690 was genetically reprogrammed by introducing the full compactin biosynthetic pathway and an evolved compactin hydroxylase CYP from Amycolatopsis orientalis, leading to an efficient pravastatin producing strain.

**Results**

**P. chrysogenum as a Platform for Compactin Production.** The β-lactam antibiotics producer P. chrysogenum is a well-developed industrial microorganism that underwent numerous rounds of classical strain improvement, leading to current penicillin production titers of more than 50 g/L (14). Because these accumulated mutations contributed to the industrial robustness and the high flux from glucose to secondary metabolites, we speculated that this highly adapted organism would be suitable for producing other secondary metabolites. To produce other pharmaceuticals in this species, an isolate completely devoid of β-lactam antibiotics was needed. Deleting all penicillin biosynthetic genes in a high producing strain led to such a host (15). To test whether the deletions had any adverse effect on secondary metabolite production potential, we reintroduced the biosynthetic genes, resulting in the recovery of penicillin production (Table S1). Next, the complete compactin (ML-236B) gene cluster from P. citrinum (7) was subcloned on three plasmids (Fig. S1). All nine mlc genes were kept under control of their natural P. citrinum promoter and terminator regions, assuming that these would function similarly in P. chrysogenum. Transformants were selected on acetamide due to cotransformation of an acetamide expression cassette. As DNA integration in P. chrysogenum occurs almost exclusively via the nonhomologous end joining (NEHEJ) pathway, all fragments randomly integrate in the genome. Successful integration was confirmed by PCR and Southern blot analyses (SI Materials and Methods). Stable transformants harboring all DNA fragments were cultivated in synthetic medium and analyzed for compactin to pravastatin conversion, and the actinomycete showed the greatest hydroxylation activity (up to 100%; Fig. 2B). These results confirm that the P. chrysogenum β-lactam free platform strain retains its features for high secondary metabolite productivities, even for heterologous products. Moreover, the P. citrinum promoters are very efficient in P. chrysogenum and do not underperform compared with known strong homologous promoters such as pbcb (Table S2).

**Deletion of Esterase Activity Improves Compactin Yield in P. chrysogenum.** A surprisingly high ratio of deacylated (ML-236A) vs. acylated (compactin) statins (Fig. 1) in P. chrysogenum (up to 60%; Fig. 2C) suggested an imbalance in the introduced pathway or a competing activity. Initial experiments confirmed in vivo cleavage of the methylbutyrate side chain of compactin (SI Results), most likely by an inducible esterase or lipase. Due to numerous candidate genes in the P. chrysogenum genome (>200) (16), a biochemical approach was used to identify the responsible enzyme. Compactin producing P. chrysogenum strains were cultivated under statin deacylating (induced by using urea as nitrogen source; SI Results) and control (i.e., noninducing, by using yeast extract as nitrogen source) conditions (Fig. 2B). Proteins from cell lysates were fractionated by hydrophobic interaction chromatography and fractions with high compactin deacylation activity analyzed by SDS-PAGE. Relevant bands were characterized by MS analysis after tryptic digestion. A protein encoded by Pc15g00720 (GenBank accession no. CAP82958.1), a putative esterase, was only detectable in samples with increased statin deacylation. Deleting this gene reduced statin deacylation to an absolute minimum, even after cultivation with urea as sole N source (Fig. S2).

**Identification of an A. orientalis Compactin Hydroxylase.** The S. carophilus P450sca-2 enzyme (9) functions using NADPH-ferredoxin oxoreductase and ferredoxin reduct partners. Attempts to express P450sca-2 directly in our P. chrysogenum compactin-producing strain did not lead to detectable pravastatin (Table S2). Several eukaryotic and prokaryotic systems capable of hydroxylation of compactin have been reported (17–22), but none give 100% conversion nor show real improvements over S. carophilus. Moreover, as the natural P450sca-2 reduct partners are unknown and we required compactin hydroxylase functionality in a fungal host, we set out to isolate a new hydroxylating enzyme that is functional in eukaryotes. Several microorganisms were screened for compactin to pravastatin conversion, and the actinomycete A. orientalis showed the greatest hydroxylation activity (up to 100%; SI Materials and Methods). To clone the relevant gene, a genome library was constructed and screened for compactin hydroxylation.

**Fig. 1.** Structures of relevant statins and derivatives. (A) Naturally produced statins (i, compactin; ii, lovastatin). (B) Semisynthetic pharmaceutical statins (i, pravastatin (Pravachol); ii, simvastatin (Zocor)). (C) Alternative compactin oxidation products (i, 6-epi-pravastatin; ii, 3-α-iso-pravastatin). (D) Decayed derivatives (i, deacylated compactin (ML-236A); ii, deacylated pravastatin).

**Fig. 2.** Statin production levels in Penicillium strains. Red, ML-236A; blue, compactin. (A) Left bar, P. citrinum NRRL 8082; other bars, P. chrysogenum strains. (B) Influence of nitrogen source on ratio of deacylated (ML-236A) to acylated (compactin) statin. (a) Sodium nitrite; (b) yeast extract; (c) arginine; (d) lysine; (e) urea; (f) acetamide; (g) ammonium sulfate; (h) hydroxyamine; (i) peptone; (j) ammonium nitrate.
Two positive clones were isolated, and sequence analysis revealed a complete ORF with 40–60% amino acid identity to known CYP enzymes in one of the clones (Fig. S3), containing a conserved heme binding motif around the cysteine ligand to the CYP heme iron (FGGGHHQCLG). This ORF was cloned into the Escherichia coli expression vector pBAD-DEST49 under control of the minimal arabinose (araBAD) promoter, and activity assays confirmed the CYP enzyme as a compactin hydroxylase (cmpH; accession no. KF751385; CYP105AS1; Fig. S3).

Expression of CYP105AS1 in P. chrysogenum Leads to epi-Pravastatin.

One-step fermentative production of pravastatin requires the A. orientalis CYP105AS1 to be functional in compactin-producing P. chrysogenum. Various publications describe the use of reductase domains from self-sufficient CYPs, such as the RhF from Rhodococcus sp. and BM3 from Bacillus megaterium, for activation of type I CYP enzymes (23–25). Such hybrid fusion enzymes may catalyze oxidation reactions in absence of other oxidoreductase/ferredoxin partners. A codon-optimized version of CYP105AS1 was fused in frame to the RhF reductase (FMN- and 2Fe-2S cluster-binding) domain, and the resulting hybrid gene was expressed under the strong pcbC promoter of P. chrysogenum (Fig. S4). On introduction to P. chrysogenum compactin-producing strains, 19 of 192 transformants were identified that produced significant amounts of pravastatin (up to 550 mg/L; Fig. S3B).

The conversion activity of CYP105AS1 expressed in P. chrysogenum was good, but not complete: ~90% of the secreted statin was pravastatin (Fig. 3B; WT and WT-CpO). However, detailed analyses with NMR and liquid chromatography (LC)-MS/MS revealed that, although the correct compactin C-6 was hydroxylated, the wrong stereoisomer 6-epi-pravastatin (Fig. 1C) was produced almost exclusively. This isomer was also reported for E. coli and P. chrysogenum strains harboring CYP105AS1 produced 98–100% 6-epi-pravastatin and maximally only ~2% pravastatin (Table S3 and Fig. 3B). Although producing the undesired epimer, the transformants demonstrated that functional expression of the self-sufficient bacterial CYP in P. chrysogenum is sufficient to produce high amounts of hydroxylated statin.

Evolution of CYP105AS1 Toward an Efficient Pravastatin Synthase.

The CYP105AS1 structure was solved to 2.05 Å using molecular replacement with the vitamin D hydroxylase CYP105A1 (26) [Protein Data Bank (PDB) ID code 3ABA; 44% sequence identity]. The final structure contains residues Glu10 to Asp400 with the exception of the polypeptide stretch from Ser77 to Ala85, with an RMSD of 1.38 Å for 355 Cα atoms (Fig. 4), and is most similar to the filipin 1CYP105P1 complex (27) (PDB ID code 2ZBY; 42% sequence identity). The disordered nature of the BC-loop stretch and the open position of the FG-loop reorientates the BC-loop region (with the Pro82-Ala88 region now disordered) and reorganizes the ligand-free state, as is observed for other CYP105 members (including CYP105P1). Extensive attempts to obtain a cocrystal structure of CYP105AS1 with compactin/pravastatin revealed uninterpretable electron density in the active site (Fig. S4), suggesting there is ample space for the substrate to occupy multiple configurations. We hypothesized that minor changes in protein structure could influence and/or restrict the conformational landscape of the substrate to achieve different stereochemistry, resulting in increased pravastatin production at the expense of 6-epi-pravastatin. To this end, the CYP105AS1 gene was mutated by error-prone PCR so that each clone contained two to three mutations on average. PCR products were cloned in pBAD-DEST under control of arabinose induction. Per round, 5,000 clones were analyzed for compactin hydroxylation, focusing on the ratio of pravastatin to 6-epi-pravastatin. After a single round of error-prone PCR, mutants with significantly improved pravastatin:6-epi-pravastatin ratios were identified (Table S3).

Although CYP105AS1 (WT) hydroxylates compactin with a pravastatin:6-epi-pravastatin ratio of 3:97, the best mutant (#6) had a ratio of 48:52. Sequence analysis of mutants with the highest pravastatin:6-epi-pravastatin ratios revealed that, in most cases, a single amino acid change altered stereoselectivity. Interestingly, in addition to active site mutations, outer shell mutations were also identified. Furthermore, expression mutants were isolated (#1; I233T) that did not alter the ratio but improved activity fourfold. The 10 best mutants were taken as templates and site-saturated fusion PCR used to achieve all possible combinations. Screening these allowed isolation of mutants with corresponding pravastatin:6-epi-pravastatin ratios up to 96:4 (Table S3). Thus, with only two rounds of mutagenesis and three to five amino acid changes, the ratio of the compactin hydroxylation products, 6-epi-pravastatin was completely inverted, and CYP105AS1 was engineered into a pravastatin synthase (P450prava).

Characterization of P450prava Explains Enantiomeric Specificity. To characterize in vitro the influence of selected mutations on compactin affinity and CYP structure, CYP105AS1, five individual point mutants and combinations thereof (Table S4) were expressed and purified. UV-visible (UV-Vis) spectroscopy revealed a typical CYP heme spectrum for WT CYP105AS1, with a Soret maximum at 419 nm, and a Q (Q) bands at 570/537 nm, respectively. Subtle variations were observed for mutant forms (Table S4). Both compactin and pravastatin induced Soret shifts (toward 395 nm) through displacement of the heme iron axial water ligand, causing a low spin (LS) to high spin (HS) shift of the ferric heme iron. CYP105AS1 did not fully convert to HS on saturation with compactin, displaying an ~47% conversion and a Kd value of 29.3 ± 1.1 μM (Table S4). The compactin-saturated WT CYP105AS1 spectrum has a prominent HS shoulder at 395 nm, and a LS Soret peak shifted from 419 to 417 nm. All individual mutations improve both compactin affinity and the extent of HS shift and had additive effects. P450prava displays the best Kd (1.4 ± 0.4 μM), which is a 21-fold improvement over WT CYP105AS1, with near full conversion to the substrate-bound HS form and a Soret maximum at 393 nm (Fig. S5).

The cocrystal structure of P450prava with compactin at 1.8 Å led to a readily interpretable electron density for the ligand (Fig. 4). Comparison with the CYP105AS1 open structure shows that compactin binding to P450prava reorientates the BC-loop region (with the Pro82-Ala88 region now disordered) and reorganizes the FG helices. This closing motion shields the ligand and active site from bulk solvent, as previously observed in other CYP105 enzymes (26, 27). Compactin displaces the aqua-sixth ligand from the heme iron and is bound in an active conformation with the C6 carbon placed within 4.7 Å of the heme iron (Fig. 4B and D). Direct protein-ligand interactions are limited to hydrophobic contacts with residues from the BC-loop (Phe76, Pro80, Thr95, Thr95), the FG-loop (Met179, Val180, Val181), the I-helix

![Fig. 3. Expression and evolution of the A. orientalis CYP105AS1. (A) Schematic representation of the self-sufficient CYP105AS1 expression construct. (B) Statin production in P. chrysogenum transformants.](image-url)
Expression of P450Prava in a Compactin Production Strain. P450Prava with a pravastatin:6-epi-pravastatin ratio of 96:4 was fused to the Rhf reductase and randomly integrated in the best P. chrysogenum compactin strain. The resulting transformants produced large amounts of pravastatin; the highest isolate produced 688 mg/L (Fig. S5). Most importantly, the improved stereoselectivity ratio pravastatin:6-epi-pravastatin of the mutant P450Prava, as observed in E. coli was also confirmed in all P. chrysogenum transformants.

To assess industrial performance of the P450Prava, P. chrysogenum strain, 10-L fed-batch fermentations were carried out (SI Materials and Methods). After a 200-h fermentation time, the strain produced more than 6 g/L pravastatin (Fig. S7), with only minor amounts (<0.5 g/L) of deacylated statins and compactin.

Discussion

Process improvement of existing pharmaceuticals is mostly incremental, involving classical strain and process improvement for fermentation processes and optimizing yields for chemical processes. For the semisynthetic molecule pravastatin, process improvement was also limited to classical strain improvement and to the cloning of new hydroxylases. Here, we applied a radical approach and completely redesigned the pravastatin production process. We hypothesized that using existing industrial fermentation strains would shorten lead times and increase opportunities to obtain desired strains and product levels. We used an antibiotic-producing strain of P. chrysogenum as a chassis. P. chrysogenum is well known for β-lactam production, but this is an undesirable feature when producing other pharmaceuticals (29). Therefore, we used a variant in which the β-lactam biosynthetic genes are removed (15), essentially putting into practice the suggestion made by Jami et al. (30) to use P. chrysogenum for other biotechnological purposes. Reintroduction of the penicillin biosynthetic genes proved that all capabilities were retained in this mutant, and introducing the compactin cluster demonstrated that beneficial mutations are not restricted to a single compound of interest but have general advantages for production of secondary metabolites. These results demonstrated that regulatory mechanisms from the donor species, P. citrinum, were fully functional in the recipient P. chrysogenum. Although not often mentioned in the literature, degradation of statins via cleavage of the methylbutyrate side chain was already reported (31, 32). However, in the P. chrysogenum background, this reaction was much more pronounced, and efficient pravastatin production requires strict control via the nitrogen source or by deleting the relevant esterase, encoded by Pc15g00720. It is tempting to speculate that non–statin-producing fungi like P. chrysogenum have evolved this esterase in nature to protect themselves from the antifungal properties of statins (33), whereas statin-producing species solved this by an additional gene copy of the target enzyme HMG-CoA reductase and a specific statin transporter (34); however, evidence supporting such hypotheses is lacking.

Initial attempts to use the available P450ocs-2 to hydroxylate compactin directly in fungi were unsuccessful, and we attempted several strategies to improve its function. The S. carbolphihsis genome was sequenced and identified a ferredoxin and several ferredoxin oxidoreductases, which were cointroduced with both native and codon-optimized P450ocs-2 gene variants (under control of strong P. chrysogenum promoters) into compactin producing strains, but these approaches did not lead to pravastatin production (Table S2). Several studies have engineered CYPs by fusing them to reductase modules in efforts to enhance their catalytic rates (25, 35). However, fusion constructs between P450ocs-2 and the P450BM3 reductase or the reductase module of the Rhodococcus Rhf P450-reductase fusion enzyme also did not result in compactin to pravastatin conversion. Recently, P450ocs-2 was successfully mutated and expressed in E. coli (36, 37). Interestingly, the authors did not select a fusion to the Rhf reductase as the reductase crystal structure is unknown (38), but selected the Pseudomonas putida reductase/putidaredoxin system (38, 39), leading to 377.5 mg/L pravastatin from this three-component redox system.

In contrast, our search for novel CYPs led to the isolation of A. orientalis CYP105AS1, which readily hydroxylates compactin in E. coli. A. orientalis was included in our screen as it was reported to contain broad substrate-range CYPs (40). Although stand-alone CYP105AS1 showed low activity in P. chrysogenum, it was successfully expressed fused to Rhf reductase, producing a fully functional enzyme both in E. coli and P. chrysogenum.

The CYP105AS1 structure reveals an open conformation and suggests multiple compactin binding modes rather than a distinct enzyme-substrate complex that could guide rational engineering for improved stereoselectivity. CYP mutations often result in novel product mixes and therefore new specificities (41), and we successfully realigned CYP105AS1 stereoselectivity in two mutation rounds, without compromising expression levels. Most
beneficial mutations were identified in the active site above the heme (42). The P450Prava/compactin structure reveals how conformational freedom of the substrate is restricted to essentially a single conformation, where substrate is positioned with the p450 binding pocket and the compactin C-6 carbon close to the heme iron, explaining the enantiomeric excess of the mutant. Three mutations, in particular, appear to reshape the active site, enhancing ligand affinity and redefining the conformational landscape. This structural change explains the significant difference in regio- and stereoselectivity compared with CYP105AS1, also outperforming P450sca-2 (9). P450sca-2 produces 10% 6-epi-pravastatin (9), whereas the best CYP105AS1 mutants produce only 4%; this is a decisive difference when considering industrial production at a several-ton scale. A second unwanted byproduct of hydroxylating, 3α,iso-pravastatin (Fig. 1C; previously reported as 6α,iso-ML-236B) (9, 31), was hardly observed in our experiments. The production titers (>6 g/L) obtained by our engineered strain (Fig. S7) are much better than the classical two-step pravastatin production, which yields 2–3 g/L pravastatin (20, 43). Pravastatin secretion was fully operational in P. chrysogenum when all compactin biosynthetic genes and the P450Prava mutant were randomly integrated. These findings suggest that the P. citrinum protein MlcE is also heterologously functional as a transporter for all statins studied, including pravastatin.

To our knowledge, our results are the first example of harnessing the potential of a previously improved industrial production strain in a more generic way: the generation of a platform strain, harboring beneficial mutations, which can be used for rapid development of novel production strains for unrelated chemicals.

Materials and Methods

Additional procedures can be found in SI Materials and Methods.

Strains. E. coli DH10B and TOP10 (Invitrogen) were used for gene cloning. P. chrysogenum strains used were laboratory strain Wisconsin 54-1255 (44), the compactin producer DS47274 (15), the single penicillin gene cluster derivative DS47274′ (15), and the β-lactam free platform strain DS50662 (15). For compaction reference fermentations and genomic DNA isolation, P. citrinum NRRLB8082 was used (NRRL).

Media. P. chrysogenum was cultivated for 168 h at 25 °C in synthetic medium (SI Materials and Methods) at 250 (shake flask) or 400–550 rpm (microtiter plate, MTP). To produce penicillin V, 2 g/L phenoxyacetic acid was added. For compactin reference fermentations and genomic DNA isolation, P. citrinum strains used were laboratory strain Wisconsin 54-1255 (44), Strains DH10B and TOP10 (Invitrogen) were used for gene cloning. Four different concentrations of manganese salt were evaluated. Libra 300 Lysis Buffer (100 mM Tris, pH 8.0), 0.5 mM EDTA, and 1 mM β-mercaptoethanol were used. Samples were washed with 200 μL of methanol for LC-MS analysis.

Construction of a Compacting Producing P. chrysogenum Strain. The P. citrinum compactin gene cluster was cloned in three parts. The central and 3′-end (14 and 6 kb) were readily PCR amplified and cloned using Gateway technology (Invitrogen) into entry vectors pDONR-P4-P1R and pDONR221, respectively (Fig. S1). The 18-kb fragment was cloned in two steps. First, the 10- and 8-kb fragments were cloned separately in pCR2.1 TOPO T/A (Invitrogen) and combined afterward via compatible restriction ends. The small piece of remaining and interfering oligonucleotide sequence in mlcA was removed via exchange of an internal genomic PCR amplified mlcA fragment (4.1-kb Acc65I fragment). Ligation of all fragments produced the full-length 18-kb in pDONR412eo. All PCR amplified fragments were verified via sequencing. Linearized plasmids were transformed to P. chrysogenum as described previously (15).

LC-MS Analysis of Statins. A Waters Acquity ultraperformance liquid chromatography (UPLC) coupled to a Micromass ZQ 2000 mass spectrometer and controlled by Waters Masslynx software was used for LC-MS analysis. LC separation was performed on a Waters Acquity UPLC ethylene bridged hybrid Phenyl column with dimensions 50 × 2.1-mm ID (particle size, 1.7 μm) and with a flow of 0.5 μL/min and temperature of 60 °C. The binary mobile phase consisted of water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B) (Table 1). The samples were kept at 10 °C and injected with a 2-μL sample loop, a needle volume of 30 μL, and at a syringe draw rate of 30 μL/min. The full loop injection was used with an off-filler factor of 5.6 and loop offline after 0.1 min for the Load Ahead option. As needle wash, 400 μL acetonitrile/water (50:50) was used.

The MS was operated with an Electrospray source in the positive mode (ES+). A dwell time between the capillary at 3.00 kV and a cone voltage of 35 V. The source temperature was 120 °C, and the desolvation temperature was 360 °C. The desolvation gas flow was 600 L/h, and the cone gas flow was 50 L/h. The MS settings were as follows: extractor, 3 V; RF lens, 0.3 V; LM resolution, 15;0; HM resolution, 15;0; and cone voltage, 20 V. The source temperature was 120 °C, and the desolvation temperature was 360 °C. The 6 g/L B (ES+) was used.

Table 1. HPLC time table gradient for the separation of statins

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The MS was operated with an Electrospray source in the positive mode (ES+) with the capillary at 3.00 kV and a cone voltage of 35 V. The source temperature was 120 °C, and the desolvation temperature was 360 °C. The desolvation gas flow was 600 L/h, and the cone gas flow was 50 L/h. The MS settings were as follows: extractor, 3 V; RF lens, 0.3 V; LM resolution, 15;0; HM resolution, 15;0; and cone voltage, 20 V. The source temperature was 120 °C, and the desolvation temperature was 360 °C. The 6 g/L B (ES+) was used.

Evolution of CYP105AS1: Construction of an Error-Prone Library. Mutants of CYP105AS1 were generated using the Diversify PCR Random Mutagenesis Kit (Clontech) followed by Gateway cloning. Four different concentrations of manganese salt were evaluated. Libraries were constructed in a 15-μL BP reaction using 100 ng pDONR221, 3 μL BP Clonase II enzyme mix, and Tris-EDTA buffer (pH 8.0) and incubated for 4 h at 25 °C. Subsequent transfer to the expression vector [10 μL BP mix, 2 μL (150 ng/mL) pBAD-DEST49, 3 μL LR clonase II] was done for 2 h at 25 °C. The reaction was stopped by addition of 4 μg proteinase K (10 min, 37 °C). One microliter was transferred to E. coli TOP10, and 48 individual clones of each library were picked to determine mutation frequency.

Screening for Improved Pravastatin-6-epi-Pravastatin Conversion Ratio. Five thousand individual clones from the error-prone library, with an average of two to three nucleotide mutations per insert, were used to inoculate 96-MTP containing 200 μLJelluria-Bertani (LB) medium and 100 μg/mL ampicillin and grown overnight at 37 °C. Subsequently, 40 μL of the overnight culture was transferred to deep well 96-MTP filled with 500 μL LB + 100 μg/mL ampicillin and 1 mM 8-aminolevulinic acid plates were grown for 4 h at 30 °C, 300 rpm. Subsequently, 0.01% arabinose was added, followed by overnight incubation at 22 °C, 300 rpm. Cells were spun down and resuspended in 300 μL LB medium containing 100 mM phosphate buffer (pH 6.8), 0.2% glucose, 0.2 g/L compcatin, 1 mM 8-aminolevulinic, and 0.01% arabinose. After overnight incubation at 30 °C, 300 rpm, cells were spun down, and supernatant was extracted with an equal volume of methanol for LC-MS analysis.

Second Evolution Round: Site Saturation and Error-Prone PCR. DNA sequences encoding improved enzymes were PCR amplified, purified, and pooled in equimolar concentrations (10 ng/mL each). Using this as template DNA, site-saturation PCR was done for six positions (Table S5). DNA fragments were isolated, mixed in equimolar amounts, and used in a fusion PCR under error-prone PCR conditions. No additional PCR primers were added. The resulting library was cloned in pBAD-DEST49 as above.

Crystallization and Structural Determination of CYP105AS1 (WT) and the P450Prava Mutant. Pure WT CYP105AS1 and P450Prava mutant proteins (10–13 mg/mL) were crystallized in 10 mM Tris and 50 mM NaCl, pH 7.5.
Compaction (in DMSO) was added to the protein to a final concentration of 1 mM with ≤2% (vol/vol) DMSO for crystallization studies. Protein was centrifuged at 15,000 × g for 10 min. 4°C immediately before crystallization to remove any precipitate. Initial screens were performed using a Mosquito liquid handling robot (TTP LabTech) and soluble protein 96-well screens (Molecular Dimensions). Sitting drops (400 nL) were made by mixing equal volumes of protein solution and mother liquor and incubating at 4 °C. Crystallization conditions were identified using the Clear Screen Strategy I (CSS-1), Morpheus, and PACT premier screens (Molecular Dimensions). Crystal microscopy seeding proteins were used to improve crystal forms (46). Microseed stocks were prepared by using the Seed Bead kit (Hampton Research) with crystals in 50 μL of mother liquor from the same condition. Sitting drops were set up as before, including the microseed at a ratio of 4:1 protein to microseed before mixing with mother liquor. Diffraction quality crystals of the CYP105AS1 WT protein were obtained with 0.2 M ammonium chloride, 0.1 M Mes, pH 6.0, and 20% (wt/vol) PEG 6000, or 0.3 M sodium acetate, 0.1 M Tris, pH 8.5, 10% (wt/vol) PEG 8000, and 10% (wt/vol) PEG 1000. Diffraction quality crystals of the P450prava compactin-bound mutant were obtained using 100 mM Bis-Tris propane at pH 6.5, 200 mM sodium bromide, and 22.5% (wt/vol) PEG 3350. Single crystals were soaked in mother liquor supplemented with 10% (vol/vol) PEG 3350 as cryoprotectant and flash-cooled by plunging frozen in liquid nitrogen. Diffraction data were collected at Diamond synchrotron beamlines. Data were reduced/scaled with the XDS suite (47) and processed using the CCP4 suite (48). The WT CYP105AS1 structure was solved using molecular replacement with the CYP105A1 structure as a search model (PDB ID code 2ZBY) (28), and the P450prava mutant was solved using the WT CYP105A1 structure. Structures were refined using Refmac5 (48) and COOT (49). Data and final refinement statistics are shown in Table S6.

ACKNOWLEDGMENTS. We acknowledge access to Diamond beamlines and Colin Levy for assistance with synchrotron X-ray data collection. We thank Marina Golovanova, Rolf Poldermans, Erwin Talens, and Paul Klaassen, and Illumina Antwerp for technical support. These studies were funded by the Biotechnology and Biological Sciences Research Council Industrial Partnership Award Research Grant BB/G014329/1 with DSM (to A.W.M., D.L., and K.J.M.). This work was sponsored by DSM Anti-infectives ( presently DSM Sinochem Pharmaceuticals).


