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Cloning and Characterization of the Biosynthetic Gene Cluster of 16-Membered Macrolide Antibiotic FD-891: Involvement of a Dual Functional Cytochrome P450 Monooxygenase Catalyzing Epoxidation and Hydroxylation

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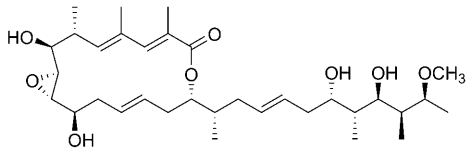
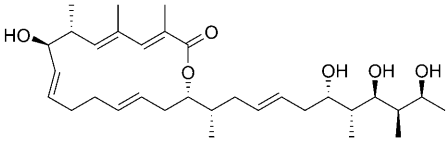
FD-891 is a 16-membered cytotoxic antibiotic macrolide that is especially active against human leukemia such as HL-60 and Jurkat cells. We identified the FD-891 biosynthetic (*gfs*) gene cluster from the producer *Streptomyces graminofaciens* A-8890 by using typical modular type I polyketide synthase (PKS) genes as probes. The *gfs* gene cluster contained five typical modular type I PKS genes (*gfsA*, *B*, *C*, *D*, and *E*), a cytochrome P450 gene (*gfsF*), a methyltransferase gene (*gfsG*), and a regulator gene (*gfsR*). The gene organization of PKSs agreed well with the basic polyketide skeleton of FD-891 including the oxidation states and α-alkyl substituent determined by the substrate specificities of the acyltransferase (AT) domains. To clarify the involvement of the *gfs* genes in the FD-891 biosynthesis, the P450 *gfsF* gene was inactivated; this resulted in the loss of FD-891 production. Instead, the *gfsF* gene-disrupted mutant accumulated a novel FD-891 analogue 25-*O*-methyl-FD-892, which lacked the epoxide and the hydroxyl group of FD-891. Furthermore, the recombinant GfsF enzyme coexpressed with putidaredoxin and putidaredoxin reductase converted 25-*O*-methyl-FD-892 into FD-891. In the course of the GfsF reaction, 10-deoxy-FD-891 was isolated as an enzymatic reaction intermediate, which was also converted into FD-891 by GfsF. Therefore, it was clearly found that the cytochrome P450 GfsF catalyzes epoxidation and hydroxylation in a stepwise manner in the FD-891 biosynthesis. These results clearly confirmed that the identified *gfs* genes are responsible for the biosynthesis of FD-891 in *S. graminofaciens*.

Introduction

FD-891 is a 16-membered macrolide antibiotic isolated from the culture broth of *Streptomyces graminofaciens* A-8890 that is known to induce morphological change of human promyelocytic leukemia (HL-60) cells.[1] FD-891 also showed strong cytocidal activities against mice leukemia cells P388 and L1210, but not antibacterial activities against Gram-positive and Gram-negative bacteria. Among various human cancer cell lines, four leukemia cell lines, HL-60, Jurkat, THP-1, and U-937 were highly sensitive to FD-891.[2] Further biochemical studies of FD-891 in Jurkat cells suggested that FD-891 strongly induces apoptosis of Jurkat cells through mitochondrial release of cytochrome c as well as caspase-9 processing triggered by caspase-8 activation. This apoptosis-signaling pathway caused by FD-891 was thus distinct from the other caspase-dependent apoptosis by chemotherapic drugs such as doxorubicin, etoposide, and vincristine. Cell cycle arrest at G_2/M phase by FD-891 was also suggested to contribute to the FD-891-induced apoptosis.[3] FD-891 also prevents cytotoxic T lymphocyte (CTL)-mediated killing pathway as an immunosuppressive activity.[4] However, in contrast to a structurally related concanamycin A, which blocks the perforin-dependent killing pathway in CTL-mediated cytotoxicity, FD-891 did not affect the vacuolar acidification and only slightly decreased perforin activity. Further biochemical studies illustrated that FD-891 inhibits CTL-



FD-891



FD-892

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target cell conjugate formation with the T-cell receptor (TCL) down regulation. However, the molecular target(s) of FD-891 still remains unclear.

Structurally related FD-892, which lacks the epoxide at C8-C9, the hydroxyl group at C10, and the 25-*O*-methyl group of FD-891, was also isolated from the same microorganism and showed the same induction of morphological change of HL-60 as FD-891.[1] FD-892 is thus presumed to be a biosynthetic intermediate of FD-891. However, FD-892 showed very weak cytocidal activities against HL-60, P388, and L-1210, probably due to the lack of an epoxide ring compared with FD-891. Therefore, the molecular targets of FD-891 and FD-892 appear to be partly different to induce distinct signaling pathways in HL-60. Unfortunately, the FD-891 producer *S. graminofaciens* A-8890 does not accumulate FD-892 anymore under the laboratory culture conditions, and the biochemical studies of the FD-892 are suspended at the moment.[5]

Although two groups established the total synthesis of FD-891 and its analogue,[6,7] the bioengineering approach is an alternative and attractive way to provide such structurally related FD-891 analogues, including FD-892, for biochemical study because the fermentation of the producer microorganisms is well established. FD-891 is presumed to be constructed by typical modular type I polyketide synthase (PKS) followed by epoxidation, hydroxylation, and methylation. Although genetic manipulation of PKS genes is indeed an attractive methodology to create novel polyketide molecules,[8,9] the genes for the tailoring enzymes can be more easily inactivated to create biosynthetic intermediates that were not accumulated in the wild-type producer strains.[10] Furthermore, such enzymes can be utilized for the in vitro transformation to create diverse structures of analogous compounds. Glycosyltransferase is one of the most applicable enzymes for the purpose, and thus glycorandomization methodology are widely accepted to create biologically relevant glycosides.[11] If we can control the order of the modification reactions in the FD-891 biosynthesis, seven FD-891 analogues, including FD-892, can be logically created through engineering of biosynthetic genes/enzymes. We thus tried to clone the FD-891 biosynthetic genes and to produce novel FD-891 analogues with biosynthetic technology.

Results

Identification of the gene cluster

The FD-891 biosynthetic gene cluster was presumed to contain typical modular type I PKS genes in Actinomycetes. Thus in order to identify typical β-ketoacylsynthases (KS) genes, which are highly conserved in many known type I PKSs for bacterial macrolides such as erythromycin and rapamycin, PCR by using the previously reported primers KSMA-F and KSMB-R[12] was carried out by using genomic DNA of *S. graminofaciens* A-8890 as a template. The expected size of the DNA fragment was successfully amplified and subsequently cloned by a standard genetic operation. As we anticipated, the DNA sequences of the PCR products showed significant similarity to the known type I KS genes (data not shown). The cloned KS genes were then used as a DNA probe to identify continuous PKS gene cluster over 65 kbp, which was estimated from the number of the extension units of FD-891 by assuming the average size of a gene for one module of type I PKS is 5 kbp. A pOJ446-based cosmid library with the randomly *Sau*3AI digested genome DNA of *S. graminofaciens* A-8890 was constructed and further screened by hybridization with the DIG-labeled KS DNAs. As a result, one of the KS gene probes gave a gene cluster containing total 69 kbp of five modular type I PKS genes (*gfsA*, *B*, *C*, *D*, and *E*), a cytochrome P450 monooxygenase gene (*gfsF*), a methyltransferase gene (*gfsG*), and a NarL family two-component response regulator gene (*gfsR*),[13] which could be activated by a certain sensor protein in the microorganism (Figure 1 and Table 1). There is no open-reading frame (ORF) over 1 kbp outside of the gene cluster; this suggests that this gene cluster is one operon for the biosynthesis of this particular polyketide. We then named this gene cluster as *gfs* cluster from *S. graminofaciens*.

PKS genes: *gfsA*, *B*, *C*, *D*, and *E*

Five PKS genes, *gfsA, B, C, D*, and *E,* were identified in the *gfs* cluster spanning 68.5 kbp. The domain structures of each PKS (GfsA, GfsB, GfsC, GfsD, and GfsE) were predicted by comparison with known modular type I PKSs through Pfam analysis[14] as shown in Figure 2. As a result, the modular structure of the PKSs (GfsA, 5 modules; GfsB, 3 modules; GfsC, 1 module; GfsD 2 modules; GfsE, 2 modules) was clearly determined. By com-

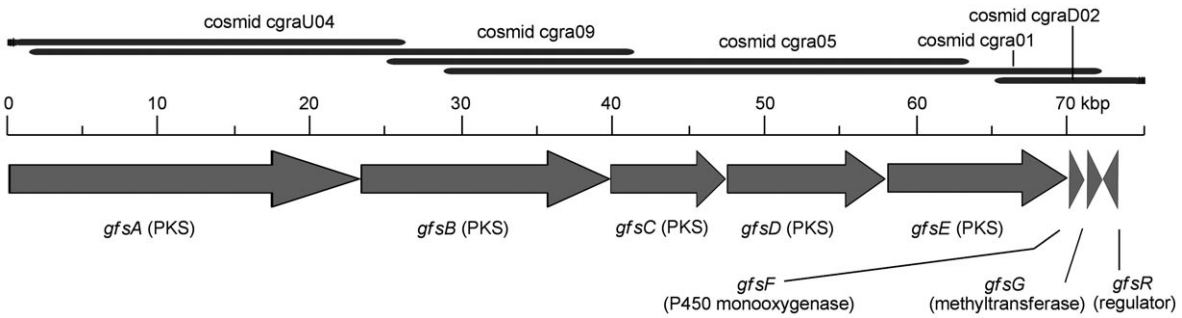


Figure 1. The 74.3 kb region of the *Streptomyces graminofaciens* A-8890 chromosome containing the FD-891 biosynthetic gene cluster, showing open reading frames (ORFs) and cosmid boundaries.

Table 1. Deduced functions of ORFs in the FD-891 biosynthetic gene cluster.

Gene	Size[a]	Homologue (biosynthetic product, identity [%]/similarity [%])	Predicted function in FD-891 biosynthesis
gfsA	7480	polyketide synthase PimS1 (pimaricin, 54/66)	PKS loading, module 1–4
gfsB	5571	polyketide synthase NysJ (nystatin, 51/62)	PKS modules 5–7
gfsC	2228	polyketide synthase Orf17 (ECO-02301, 63/74)	PKS module 8
gfsD	3639	polyketide synthase PteA2 (filipin, 53/65)	PKS modules 9–10
gfsD	3799	polyketide synthase Orf17 (ECO-02301, 51/63)	PKS modules 11–12
gfsE	414	P450 monooxygenase Mflv2418 (57/70)	P450 monooxygenase
gfsF	278	Methyltransferase AtM (AT2433, 57/70)	methyltransferase
gfsG	243	NarL family two-component response regulator	transcriptional regulator

[a] Numbers are in amino acids.

parison with the domain structures of the PKSs comprising of β-ketosynthase (KS), acyltransferase (AT), β-ketoreductase (KR), dehydratase (DH), enoylreductase (ER) and acyl carrier protein (ACP), and the chemical structure of FD-892, the catalytic order of the modular PKSs has been predicted as shown in Figure 2. The probable substrate specificity of AT domains for malonyl CoA (MT) or methylmalonyl CoA (MMT),[15,16] the existence of KS_Q domain[17] for lording of the starter unit in the front of the first module in GfsA, and a termination domain thioesterase (TE) in the tail of GfsE also agreed well with the chemical structure of FD-892. Docking domain analysis of PKSs based on the indicative amino acid residues suggested specific interaction of GfsA_C terminus (H1d)/GfsB_N terminus (T1d), GfsB_C terminus (H1b)/GfsC_N terminus (T1b) and GfsD_C terminus (H1c)/GfsE_N terminus (T1c).[18,19] GfsC_C terminus (H1d-like) and GfsD_N terminus (T1b-like) showed a different type of combination for the interaction, which should be distinguished from the other PKS docking domains (see the Supporting Information). In addition, the straightforward PKS gene organization, *gfsA*, *B*, *C*, *D*, and *E* seemed to be reasonable to form the appropriate quaternary structural organization of PKSs to construct this particular polyketide molecule.

One exception was found in module 10, because an unnecessary DH domain exists judging from the chemical structure of FD-892. The functionality at the corresponding C7 position should remain a hydroxyl group. However, the DH10 is quite similar to the other active DH domains in Gfs PKSs and does not show any indication of being an inactive DH domain. The stereochemistry of α-substituent determined by the preceding KR domain is known to be critical for the following dehydration reaction catalyzed by the DH domain.[20] Stereospecificity of the KR domains in the front of the active DH domains have been speculated by comparison with those of the other KR domains indicating that 2*R*-methyl and *3R*-hydroxyl functionality could be formed.[21,22] Because KR10 should afford 2*S*-methyl and *3R*-hydroxyl functionality judging from the stereochemistry of FD-891, DH10 might not accept the elongated polyketide intermediate having an *S* configuration of the α-substituent for dehydration, and thus the skipped substrate would be processed by the next module, module 11. The catalytic region of KR10 domain has two unique amino acid residues compared with the other B1 type KR domains (KR3, KR4, KR5, KR6, KR7, KR8, KR9, KR11, and KR12) indicating a B2-type KR domain (Supporting Information).[21] The KR1 domain belongs to the A1-type KRs that produces 2*R*-methyl and *3S*-hydroxyl configuration and KR2 domain belongs to A2-type KRs that produces the 2*S*-methyl and *3S*-hydroxyl configuration. The stereochemistry of the enoyl reduction catalyzed by the ER5 domain was also predicted to be the 2*S* configuration because the unique tyrosine residue exists.[23] Overall, the domain structure of Gfs PKSs agreed well with the chemical structure of FD-891, including its stereochemistry. Therefore, the identified *gfs* gene cluster was strongly supported to be responsible to the FD-891 biosynthesis.

Tailoring enzymatic genes: *gfsF* and *gfsG*

The deduced *gfsG* gene product showed moderate similarity to various *S*-adenosyl methionine (SAM)-dependent methyltransferase, such as AtM in the indolocarbazoles antibiotic AT2433 biosynthesis,[24] RebM in the rebeccamycin biosynthesis,[25] MitM in the mitomycin C biosynthesis,[26] and AveD in the avermectin biosynthesis.[27] Thus, GfsG seemed to catalyze a methylation of the hydroxyl group at C-25 of FD-892 or the other related biosynthetic intermediates.

The deduced *gfsF* gene product showed high similarity to various cytochrome P450 in Actinomycetes and belongs to the CYP105 family.[28] Cytochrome P450 contains a heme–iron complex in the active site and catalyzes various oxidation reactions, including hydroxylation and epoxidation. TylHI[29] and NysN[30] belong to the CYP105 family and reportedly catalyze the oxidations of polyketide skeletons in each biosynthetic pathway. Thus, GfsF was also presumed to catalyze the oxidations in the FD-891 biosynthesis, which should contain epoxidation at C8-C9 and hydroxylation at C10. However, only GfsF was encoded in the *gfs* gene cluster as a potential oxygenase; this suggests that it might catalyze both epoxidation and hydroxylation. Alternatively, GfsF could catalyze either of the oxidations, and another enzyme outside of the *gfs* gene cluster might be responsible for the remaining oxidation. To investigate these hypothetical functions of GfsF, the *gfsF* gene inactivation study and enzymatic characterization of the GfsF protein were carried out.

Inactivation of the *gfsF* gene

According to a conventional method,[31] we inactivated the *gfsF* gene in *S. graminofaciens* by the insertion of apramycin-resistant *aac(3)IV* gene derived from pOJ446 (see the Experimental Section for details). The resultant ΔgfsF strain of *S. graminofaciens* did not produce FD-891; this confirms that this gene is involved in the biosynthesis of FD-891 (Figure 3). Instead, this mutant accumulated a FD-891-analogous compound as the

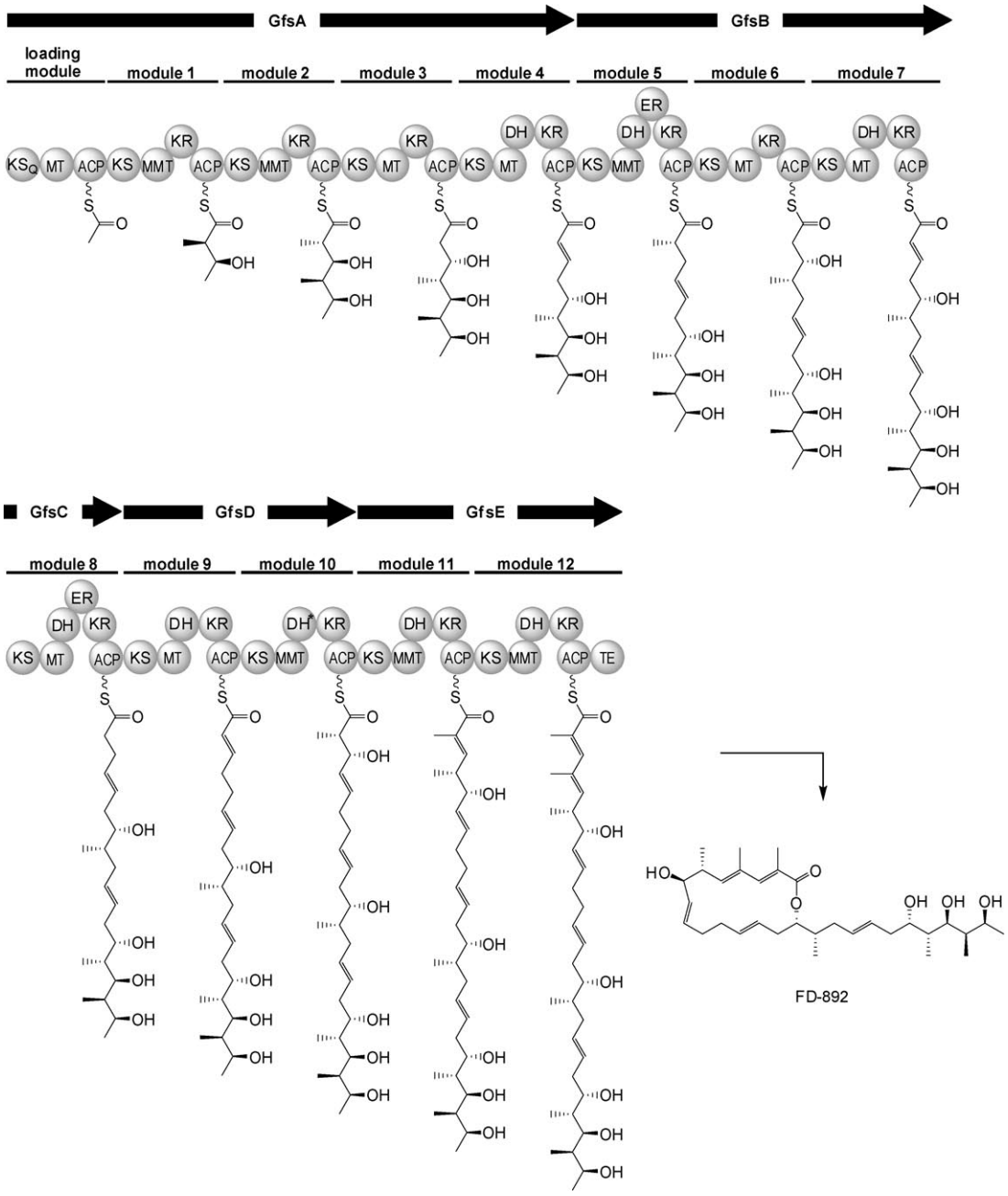


Figure 2. FD-891 modular polyketide synthase (PKS) encoded by *gfsA–E*, showing loading module and modules 1–12 and predicted ACP-bound polyketide intermediates. KS: β-ketosynthase, MT: malonyltransferase, MMT: methylmalonyltransferase, KR: β-ketoreductase, DH: dehydratase, ER: enoylreductase, ACP: acyl carrier protein and TE: thioesterase. The DH10 domain of module 10 indicated as an asterisk should not work in the biosynthesis of FD-891.

major product, judging from the HPLC-photodiode array (PDA) analysis. From the precipitate of 1.2 L culture of ΔgfsF strain, 280 mg of the novel compound was isolated by silica gel chromatography (2×), and its molecular formula was established to be $C_{33}H_{54}O_6$ by positive HR-FAB-MS indicating the loss of two oxygen atoms from FD-891.

The ^{1}H NMR spectrum showed three singlet methyl groups [δ_H = 1.95, 2.03, and 3.34 ppm], five doublet methyl groups [δ_H = 0.78 (d, J = 7.0 Hz, 3H), 0.92 (d, J = 7.1 Hz, 3H), 0.93 (d, J = 6.8 Hz, 3H), 1.15 (d, J = 6.7 Hz, 3H), and 1.18 ppm (d, J = 6.3 Hz, 3H)], and eight olefinic protons [δ_H = 7.20 (m, 1H), 5.77 (ddd, J = 6.8, 8,4, 15.4 Hz, 1H), 5.53 (m, 2H), 5.47 (m, 1H), 5.40 (d, J = 10.1 Hz, 1H), 5.35 (dd, J = 8.0, 15.4 Hz, 1H), and 5.30 ppm (m, 1H)]. The methyl group at 3.34 ppm appeared to be methoxy group. The ^{13}C NMR and DEPT spectra exhibited 33 carbon resonances, consisting of three quaternary carbons, including an ester carbonyl carbon (δ_C = 169.6 ppm), and two olefinic carbons (δ_C = 123.1 and 132.4 ppm), eight methyls including methoxy carbon (δ_C = 56.0 ppm), five methylenes, and seventeen methines, including five oxymethines (δ_C = 82.6, 78.8, 78.1, 76.4, and 72.9 ppm), and eight olefinic carbons (δ_C = 144.27, 144.34, 133.2, 133.1, 132.7 130.6, 129.5, and 125.7 ppm). These NMR spectral data of this compound are quite similar to those of FD-892 except for the existence of methoxy group. Further

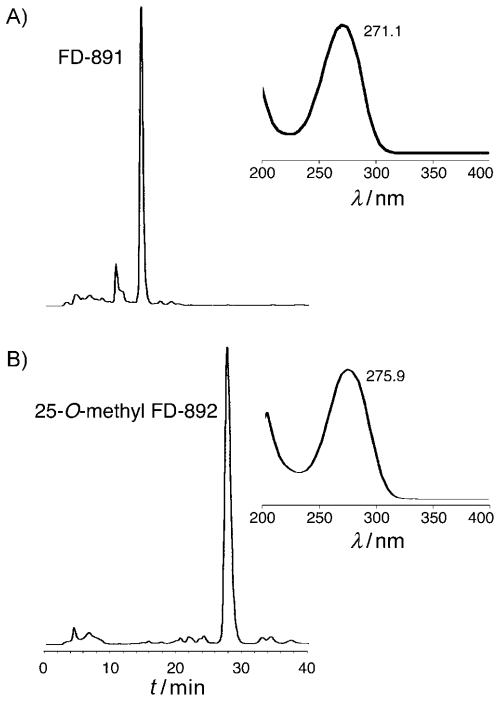


Figure 3. HPLC analysis of crude extracts from the culture of *S. graminofaciens*. A) The ethyl acetate extract of precipitate from the wild-type *S. graminofaciens* culture, and B) the ethyl acetate extract of precipitate from ΔgfsF *S. graminofaciens* culture.

NMR spectroscopic analysis, including COSY, HMQC, and HMBC, indicated that the chemical structure of the novel FD-891 analogue from the *gfsF* gene-disrupted mutant was determined as 25-*O*-methyl-FD-892 (Figure 4 A).

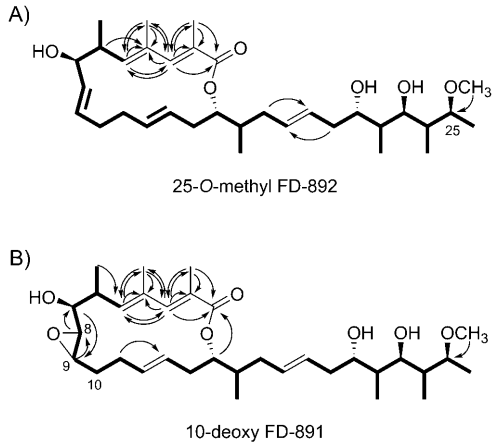


Figure 4. A) 25-*O*-methyl-FD-892 from ΔgfsF *S. graminofaciens* culture, and B) 10-deoxy-FD-891 in the GfsF reaction. Arrows indicate the HMBC correlations and bold lines indicate the spin couplings detected in the $^{1}H,^{1}H$ COSY spectrum.

GfsF reaction with 25-*O*-methyl-FD-892

The 25-*O*-methyl-FD-892 that accumulated in the ΔgfsF strain was presumed to be a substrate of GfsF P450 monooxygenase, although it was still unclear whether GfsF catalyzes both the epoxidation at C8–C9 and the hydroxylation at C10 of this molecule, or one of these reactions. Thus, we attempted to express GfsF in *E. coli* and have investigated its enzymatic reaction. The vast majority of the P450 enzymes require two-electron reduction by redox partner proteins such as NADPH and NADH.[32,33] However, because no such protein was encoded at the adjacent region of the *gfs* gene cluster in chromosome of *S. graminofaciens*, a set of known heterologous putidaredoxin and putidaredoxin reductase derived from *Pseudomonas putida* were coexpressed with the *gfsF* gene to reconstitute the expected oxidation reactions.[34] This coexpression system reportedly worked well with heterologous P450 enzymes.[35] The GfsF expressing *E. coli* coexpressed with putidaredoxin and putidaredoxin reductase was reacted with 25-*O*-methyl-FD-892 in the appropriate reaction buffer, and the reaction products were analyzed by HPLC (Figure 5). As a result, the GfsF reaction afforded FD-891 accompanied by a probable reaction intermediate. This result clearly verified that GfsF consecutively catalyzes both epoxidation and hydroxylation as a dual functional P450 monooxygenase in the biosynthesis of FD-891. The novel FD-891 analogue in the GfsF reaction was also isolated, and its molecular formula was established to be $C_{33}H_{54}O_7$ by positive HR-FAB-MS; this indicates either an epoxidized or hydroxylated compound.

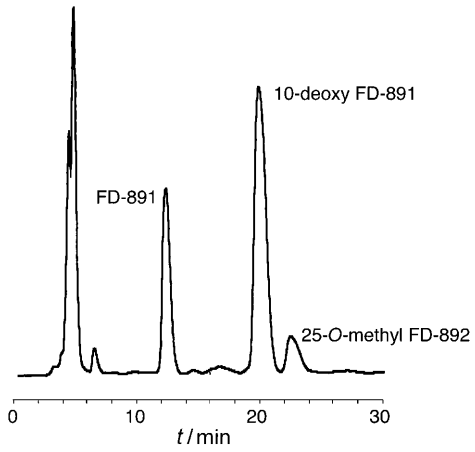


Figure 5. HPLC analysis of GfsF reaction from 25-*O*-methyl-FD-892 coupled with putidaredoxin and putidaredoxin reductase.

The ^{1}H NMR spectrum showed three singlet methyl groups [$\delta_H = 2.02$, 2.08, and 3.34 ppm], five doublet methyl groups [$\delta_H = 0.79$ (d, $J = 7.0$ Hz, 3H), 0.90 (d, $J = 6.5$ Hz, 3H), 0.92 (d, $J = 7.1$ Hz, 3H), 1.14 (d, $J = 7.2$ Hz, 3H), and 1.18 ppm (d, $J = 6.3$ Hz, 3H)], and six olefinic protons [$\delta_H = 5.60$ (m, 5H) and 7.32 ppm (m, 1H)]. In the ^{1}H NMR spectrum, two olefinic protons of 25-*O*-methyl-FD-892 disappeared and instead two protons of oxymethines appeared at $\delta = 2.91$ and 3.13 ppm indicating that one double bond was epoxidized by GfsF. The ^{13}C NMR spectrum exhibited 33 carbon resonances consisting of an ester carbonyl carbon ($\delta_C = 169.0$ ppm), eight olefinic carbons ($\delta_C =$ 144.2, 142.1, 135.5, 132.9, 130.1, 129.5, 125.7, and 124.4 ppm), eight oxygenated carbons (82.7, 78.4, 76.5, 73.1, 71.3, 57.2, 56.0, and 53.7 ppm), nine of which were methylenes and methines (39.65, 39.63, 36.8, 36.0, 35.8, 34.6, 34.2, and 29.2 ppm), and seven methyls ($\delta_C = 16.6$, 16.4, 16.2, 15.5, 12.6, 11.6, and

4.9 ppm); this suggests that one double bond was converted to one epoxide functionality at $\delta = 57.2$ and 53.7 ppm. Further NMR spectroscopic analysis clearly indicated that the novel FD-891 analogue in the GfsF reaction is 10-deoxy-FD-891 as shown in Figure 4B.

Then, the isolated 10-deoxy-FD-891 was reacted again with the GfsF reaction system, and this substrate was efficiently converted to FD-891. These results clearly indicated that GfsF first catalyzes the epoxidation at C8-C9 and then the hydroxylation at C10 to convert 25-*O*-methyl-FD-892 into FD-891 in a stepwise manner. The stereochemistry of 10-deoxy-FD-891 and 25-*O*-methyl-FD-892 should be as same as those of FD-891.

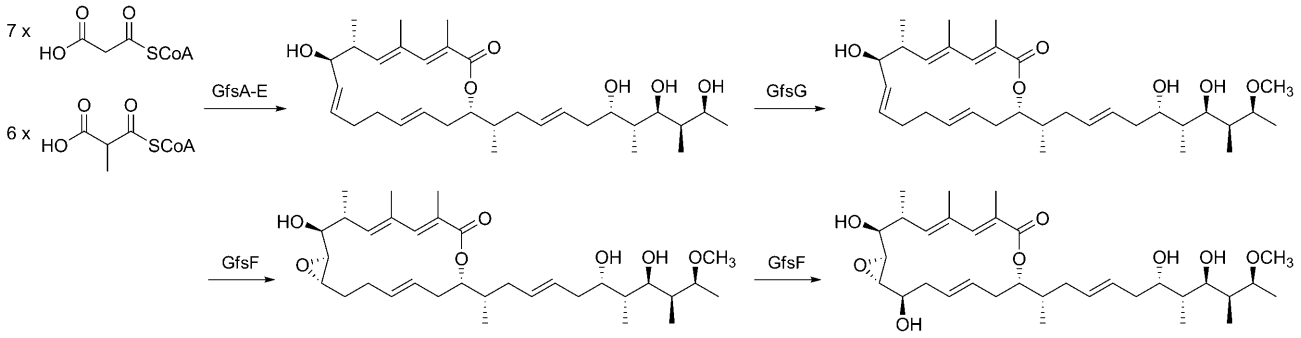
Discussion

We identified the FD-891 biosynthetic gene cluster from the producer strain *S. graminofaciens* A-8890 by screening with a KS probe, which was turned out to be the KS7 domain in module 7 (Figure 1 and Table 1). Among the PCR-amplified KS genes with genomic DNA of *S. graminofaciens* as the template, the KS3 domain was also found to be amplified by the PCR. The same PCR also afforded a probable concanamycin PKS gene (77% identity to ConE),[36] which was anticipated to be amplified because this strain reportedly produces concanamycin.[37] Three other KS genes were also amplified, indicating that this strain has the potential to produce unidentified polyketides.

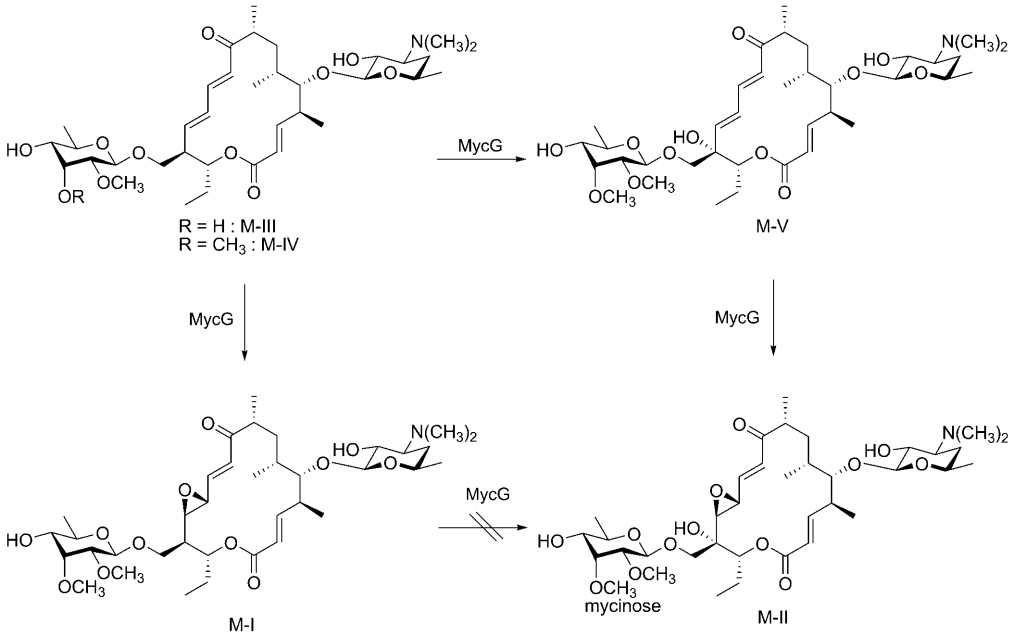
The modular structures of GfsA, GfsB, GfsC, GfsD, and GfsE clearly indicated the proposed polyketide assembly line to construct FD-892 as shown in Figure 2. Also, the *gfsF* gene inactivation experiment and the GfsF enzymatic activity revealed the order of post-PKS tailoring steps as shown in Scheme 1. Thus, the hydroxyl group at C25 of FD-892 should be methylated by a methyltransfearse GfsG to give 25-*O*-methyl-FD-892, which is first epoxidized and then hydroxylated to afford FD-891 in a stepwise manner by a P450 monooxygenase GfsF. In the proposed biosynthetic pathway, it is still unclear how GfsF distinguishes between FD-892 and 25-*O*-methyl-FD-892, because the oxidized moiety (C8, C9, and C10) is quite far from C25, which is the only difference between these two molecules. To verify the substrate specificity of GfsF, the *gfsG* gene-disrupted mutant is currently under construction, assuming that the mutant would accumulate FD-892 as discussed later.

The recognition mechanism of the dual functional P450 monooxygenase GfsF is an intriguing issue in the biosynthesis of FD-891. GfsF first catalyzes the regio- and stereospecific epoxidation of 25-*O*-methyl-FD-892 from the *Si*-face of C8–C9 double bond. Then, the *pro-R* hydrogen atom of 10-deoxy-FD-891 is abstracted by GfsF to form a probable substrate hydoxyl–iron–heme complex, which leads to FD-891. Initially, we speculated that the C10 position would be hydroxylated at first, because an allylic radical species is a likely intermediate. However, this is not the case. The electron-rich olefinic bond reacts with the ferryl-oxo intermediate (called compound I) in the active site of GfsF at first leading to the epoxide formation. The conformation of the first reaction product 10-deoxy-FD-891 is somehow modulated and the *pro-R* hydrogen atom would be stereospecifically abstracted by the ferryl-oxo intermediate.[38] Therefore, overall, modulation of substrate conformations appears to be critical for the stereospecific reactions catalyzed by GfsF. Thus, the C10 carbon of 25-*O*-methyl-FD-892 should not be reacted with the ferryl-oxo intermediate in GfsF, even though it can be closely positioned. Detailed study for the recognition mechanism of this unique dual functional P450 monooxygenase GfsF is an intriguing issue in enzymatic chemistry and should shed a light on the future enzymatic application as a biocatalyst.[39,40]

MycG is a recently characterized dual-functional P450 monooxygenase in the mycinamicin biosynthesis in *Micromonospora griseorubida*.[41] MycG catalyzes stereospecific hydroxylation at C14 of mycinamicin IV (M-IV) and epoxidation at C12–C13 of M-V (Scheme 2). The order of oxidations catalyzed by MycG is opposite to that of GfsF in FD-891 biosynthesis. Thus, in the MycG reaction, conformation of the first hydroxylated product M-V seems to be modulated to react with MycG again, resulting to the epoxidation at C12–C13 for completion of the M-II biosynthesis. The apparently opposite reaction scheme observed in the GfsF and MycG reactions is an intriguing issues how the enzymes distinguish the structure of substrates. MycG reportedly catalyzes the epoxidation at C12-C13 of M-IV to give M-I, which is not converted to M-II by MycG (Scheme 2, bottom). Therefore, only a subtle difference of conformation of substrates and/or functional groups appears to be critical for recognition by this unique enzyme. In addition, two desmetylated mycinosyl analogues M-III and M-VI cannot be accepted by MycG, suggesting that the dimethyl functionality in the mycinose moiety of M-IV is critical for the recognition of MycG.



Scheme 1. Proposed biosynthetic pathway for FD-891.



Scheme 2. A dual functional P450 monooxygenase MycG involved in mycinamicin biosynthesis.

Accordingly, in the case of GfsF reaction, the methyl functionality on the hydroxyl group at C25 of 25-*O*-methyl-FD-892 might be important for the recognition of GfsF. Thus, we are now constructing the methyltransferase *gfsG* gene disrupted mutant to produce FD-892, which is not accumulated in wild type and also express the GfsG to create various FD-891 analogues by combination of genetic engineering and tailoring enzymatic transformations.

Conclusions

We have produced two novel FD-891 analogues, 25-*O*-methyl-FD-892 and 10-deoxy-FD-891 through biosynthetic technology. These FD-891 analogues show unique bioactivity against cancer cell lines and thus chemical biological studies of these compounds are now in progress. Although the reaction order of the oxidation catalyzed by P450 GfsF restricts the number of novel FD-891 analogues, GfsF itself can be engineered to change its specificity. For this purpose, a detailed enzymatic reaction analysis of these biosynthetic enzymes would be necessary. Studies of the relatively simple macrolide antibiotic FD-891 biosynthetic pathway could provide an important solution to other problems in the biosynthetic technology.

Experimental Section

Bacterial strains and growth conditions: *Streptomyces graminofaciens* A-8890 was used as the FD-891 producer strain and source of the FD-891 biosynthetic (*gfs*) genes.[1] *S. graminofaciens* A-8890 was maintained on ISP2 (0.4% yeast extract, 1% malt extract, 0.4% glucose, pH 7.3) agar medium (2.0% agar, 28 °C, 3 d) and the spores were used for a seed culture in ISP2 liquid medium (28 °C, 200 rpm, 4–6 d). Genomic DNA was extracted from the cultured cells in 100 mL of ISP2 liquid medium (28 °C, 200 rpm, 4 d) according to a standard method.[31] The FD-891 production medium contained potato starch 3%, soya flake 1.5%, yeast extract 0.2%, corn steep liquor 0.5%, NaCl 0.3%, 0.05% $MgSO_4 \cdot 7H_2O$, 0.0005% $CoCl_2 \cdot 6H_2O$, and 0.3% $CaCO_3$ (pH 7.1). The seed cultures of disrupted mutants (1 mL) were inoculated to the medium (100 mL) in a 500 mL baffled flask, and further cultivation was continued for five days at 28 °C, 200 rpm.

Escherichia coli DH5α was routinely used for plasmid preparation. *E. coli* XL-1 Blue MRF' was used for cosmid manipulations. *E. coli* ET12567 was used for preparation of nonmethylated plasmids for transformation of *S. graminofaciens*. *E. coli* BL21(DE3) was used for the *gfsF* gene expression. Luria–Bertani (LB) medium supplemented with standard amounts of antibiotic as required (50 μg mL^{-1} ampicillin for LITMUS28 and pT7-Blue) was used for the culture of *E. coli*. Genetic manipulation in *E. coli* was carried out according to a standard manner.[42]

Preparation of DNA probe to identify the gfs gene cluster: Genomic DNA of *S. graminofaciens* A-8890 was used as a template for the PCR amplification of typical type I PKS KS genes with KSMA 5′-TSGCSATGGACCCSCAGCAG-3′ and KSMB 5′-CCSGTSCCGTGSGCCTCSAC-3′ as primers (S: a mixture of C and G).[12] PCR conditions were 94 °C, 5 min for denaturing, 30 cycles of 94 °C, 30 s, 50 °C, 30 s, 72 °C, 40 s for extension of DNA in 2× GC buffer I (5 μL), dNTP (1.6 μL; 2.5 mM each), 50 μM KSMA (0.2 μL), 50 μM KSMB (0.2), distilled H_2O (0.8 μL), LA-Taq DNA polymerase (TaKaRa; 0.1 μL), and template DNA (2.1 μL; 2.4 ng μL^{-1} of chromosomal DNA). The amplified PCR products were subcloned with the T vector of pT7-blue (TaKaRa) to obtain plasmids containing a partial sequence of type I PKS KS genes. The DNA sequences of the cloned plasmids were analyzed by using a DNA sequencer LONG READER 4200 (Li-Cor) and SequiTherm EXCEL II DNA Sequencing Kit-LC (for 66 cm gels, Epicentre Biotechnologies) with M13 Fwd (−29)/IRDye 800 and M13 Reverse (−29)/IRDye 800 (Li-Cor) according to the manufacturer's protocol.

Identification of the FD-891 gene (*gfs*) cluster: The chromosomal DNA of *S. graminofaciens* was partially digested with *Sau*3AI and

the restriction enzyme was denatured with the phenol-chloroform extraction. After ethanol precipitation, the digested DNA fragments of the chromosomal DNA were treated with calf intestine alkaline phosphatase (CIAP) at 50 °C and CIAP were denatured with the phenol–$CHCl_3$extraction. A cosmid vector pOJ446 was separately digested with *Hpal*, and after the treatment of CIAP, followed by digestion with *Bam*HI. After the phenol–$CHCl_3$ extraction and the EtOH precipitation, the resulting vector DNA was dissolved with the TE buffer. The digested pOJ446 and the partially digested chromosomal DNA were ligated by DNA Ligation kit ver. 2 (TaKaRa) at 4 °C overnight. After EtOH precipitation, the ligated DNA was dissolved in the TE buffer. The resulting ligation mixture was packaged into λ phage followed by phage transfection to *E. coli* XL1 Blue MRF′ by using with Gigapack III XL Packaging Extract (Stratagene) according to the manufacturer's protocol. The host strain *E. coli* XL1 Blue MRF′ was cultured in LB (3 mL) containing 10 mM $MgSO_4$ and 0.2% maltose by OD_{600} 0.5–1. The culture was centrifuged, and the resulting wet cells were suspended in a 10 mM $MgSO_4$ up to $OD_{600}=1$ for transfection.

A cosmid library of 2960 clones in *E. coli* grown on 50 μg mL^{-1} geneticin-containing LB medium was screened by the hybridization with a Dig-labeled DNA probe, which was made by the DIG DNA Labeling Kit (Roche). Among several KS genes obtained by using the above-mentioned PCR, KS5 (which was turned out to be a partial KS gene in module 7 of *gfsB*) was used as a DNA probe to identify type I PKS gene containing clusters. Hybridization was carried out with the DIG Nucleic Acid Detection Kit and NBT/BCIP solution (Roche) according to the manufacturer's protocol.

Thirty-eight positive clones were cultured and the corresponding cosmids were extracted by using a standard protocol. The cosmids were further screened by using the above-mentioned PCR and Southern hybridization with the same probe to obtain target cosmid cgra05, which contained the target partial KS gene. Overlapping cosmids, cgra09, cgra01, cgraU04, and cgraD02 spanning an entire PKS gene cluster were subsequently identified by chromosomal walking.

Sequencing and analysis: Because cosmid cgra09 (3663~41679) and cgra01 (29211~72473) seemed to contain the majority of a gene cluster (74321 bp), each of the two cosmids was randomly sequenced by a shotgun sequence method on double-stranded DNA templates with more than tenfold coverage and a minimum of three times each portion of the DNA sequence (Shimadzu Biotech, Kyoto, Japan). Further, several DNA fragments from cgraU04 (U04KpnI04, U04EcoRI05, U04BssHII03, and U04NcoI03) and cgraD02 (D02PstI04, D02BamHI09, D02BamHI10, and D02KpnI06) were subcloned into the plasmid vector LITMUS28 and sequenced on double-stranded DNA templates by using a LONG READER 4200 (Li-Cor). As a result, a 74321 bp of DNA sequence containing a type I PKS gene cluster was determined. ORFs were determined by FramePlot analysis (http://www.nih.go.jp/~jun/cgi-bin/frameplot.pl)[43] and BLAST homology search by using the NCBI BLAST server. The domain structure of PKS was analyzed by Pfam program[14] by using the Sanger institute server (http://pfam.sanger.ac.uk/). The determined DNA sequence data of the gene cluster in *S. graminofaciens* A-8890 has been deposited to the DDBJ databases under accession number AB469193.

Inactivation of the *gfsF* gene: *Nco*I digested fragment (7.5 kbp) from cgraD02 was subcloned into LITMUS28. From the plasmid, 3.4 kb of the *Bss*HII-digested fragment was recovered and treated with Klenow fragment. The resultant blunt-ended DNA was ligated with *E. coli--Streptomyces* shuttle vector pWHM3, which was treated with *SphI* and *Bam*HI, Klenow fragment, and bacterial alkaline phosphatase (BAP) in advance. The resultant plasmid pW-D2N3-Bs was digested with *PstI* and further treated with the Klenow fragment and BAP. An apramycin-resistance gene *aac(3)IV* cassette was inserted into the blunt-ended site, to obtain plasmid pWHM3-df. An *Eco*RI-*PstI* digested fragment containing the *aac(3)IV* gene derived from pOJ446 was subcloned into the corresponding site of pBluescript II KS+. From the plasmid, *Eco*RV-*Sma*I fragment was recovered and used as the *aac(3)IV* cassette.

Non-methylated plasmid pWHM3-df was recovered from *E. coli* ET12567 transformant and used for transformation of *S. graminofaciens*. Preparation of protoplasts of *S. graminofaciens* and transformation were performed according to a standard protoplast method.[31] The transformants of *S. graminofaciens* harboring pWHM3-df were inoculated on R5 agar medium containing 40 μg mL^{-1} apramycin and 16 μg mL^{-1} thiostrepton at 28 °C. Appropriate transformants were then cultured in ISP2 liquid medium (3 mL) containing 5 μg mL^{-1} of thiostrepton at 28 °C for six days. The seed cultures were spread on R5 agar medium containing 50 μg mL^{-1} of apramycin. After 10 days' culture at 28 °C, several colonies were inoculated in 3 mL of SK No.2 liquid medium containing 12.5 μg mL^{-1} of apramycin at 28 °C for 5 d. The cultures were again spread on R5 agar medium containing 50 μg mL^{-1} of apramycin and further cultured for 4 d at 28 °C; 148 colonies were screened on R5 agar medium containing 50 μg mL^{-1} of apramycin or 25 μg mL^{-1} of thiostrepton or no antibiotics. As a result, 32 thiostrepton-sensitive and apramycin-resistant strains were obtained. The *Bam*HI digested genomic DNAs of the strains were used for Southern hybridization with a DIG-labeled probe to confirm the insertion of *aac(3)IV* cassette in the expected site by double crossover homologous recombination. The probe DNA was derived from 865 bp of *Apa*I-digested DNA fragment in the *gfsF* gene. The desired *gfsF* gene mutants of *S. graminofaciens* were stored as spore suspension at −30 °C.

Isolation of 25-*O*-methyl-FD-892 from the *gfsF* gene mutant: Culture broth (1.2 L) of ΔgfsF was centrifuged to separate supernatant and precipitate at 7000 rpm for 20 min. The precipitate was extracted with acetone. After removal of acetone by rotary evaporation, the aqueous residue was extracted with EtOAc. The combined organic layer was dried on anhyd Na_2SO_4, and the solvent was removed by rotary evaporation. The extract (yellow syrup) was purified twice by silica gel chromatography (silica gel 60N, 63–210 μm; Kanto Chemical, Tokyo, Japan; first: 1.5×10 cm gel with hexane/acetone (1:1), second 1.5×20 cm gel with hexane/EtOAc (1:4)) to obtain 282 mg of 25-*O*-methyl-FD-892. ^{1}H and ^{13}C NMR spectra were taken by DRX-500 (Bruker) or ECX500 (JEOL) spectrometer, and FAB-MS spectra were measured by JMS-700 (JEOL). The assignments of ^{1}H and ^{13}C NMR spectroscopy signals are shown in the Supporting Information. HR-FAB-MS (positive, glycerol): *m/z*: calcd for $C_{33}H_{55}O_6$: 547.3999 $[M+H]^+$, found: 547.3979.

Expression of GfsF: The *gfsF* gene was amplified by PCR with the primers 891P450-N: 5′-AACATATGACCGACACGACACTC-3′ and 891P450-C: 5′-GAA CTA GTG GCA GGG CGG GGC-3′. PCR conditions were 30 cycles of 98 °C, 10 s, 50 °C, 5 s, 72 °C, 90 s for extension of DNA in 5× PrimeSTAR buffer (2 μL), 0.8 μL of dNTP (2.5 mM each), DMSO (0.5 μL), 0.1 mM 891P450-N (0.1 μL), 0.1 mM 891P450-C (0.1 μL), PrimeSTAR polymerase (TaKaRa; 0.1 μL), template DNA (0.1 μL; *gfsF* gene containing plasmid derived from cgraD02, 2.2 μg mL^{-1}), and H_2O (6.3 μL). The amplified PCR product was subcloned into the LITMUS28. After digestion of LT28 with *Eco*RV, we dephosphorylated the vector with BAP. After confirmation of the DNA sequence, an appropriate plasmid was digested with *NdeI*

and *SpeI*, and the resulting DNA fragment was inserted into the corresponding site of pCYP-camAB[44] to obtain pGfsF-camAB. The pGfsF-camAB was introduced into *E. coli* BL21(DE3) by a standard chemical transformation. The *E. coli* harboring pGfsF-camAB was precultured in LB medium (3 mL) with 100 $\mu g\,mL^{-1}$ of ampicillin at 37 °C overnight and then 1 mL of the culture was used for the main culture in M9 mix medium (6.78 $g\,L^{-1}$ Na_2HPO_4, 3 $g\,L^{-1}$ KH_2PO_4, 0.5 $g\,L^{-1}$ NaCl, 1 $g\,L^{-1}$ NH_4Cl, 1% casamino acid, 0.4% glucose, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$ were autoclaved and then mixed with 0.1 mM $FeSO_4$ 20 $mg\,L^{-1}$ thymine, 80 $mg\,L^{-1}$ 5-aminolevulinic acid) supplemented with 100 $\mu g\,mL^{-1}$ ampicillin. The main culture was carried out at 37 °C by $OD_{600}=0.7$, and a final 0.1 mM isopropyl β-D-thiogalactoside was then added for induction of overexpression. The culture was continued at 22 °C overnight and the cells were harvested by centrifugation (2300 *g* for 10 min). The wet cells (2.3 g from 100 mL culture) were suspended in CV-2 mM DTT buffer [25 mL; 50 mM NaH_2PO_4, 1 mM EDTA, 10% glycerol, 1 mM glucose (pH 7.3) were autoclaved, and then mixed with 2 mM DTT] and were stored at −80 °C.

GfsF reaction with 25-*O*-Methyl-FD-892: 25-*O*-Methyl-FD-892 (10 mg) was dissolved in DMSO (1 mL) and added to the above-mentioned GfsF-containing reactant (100 mL) in a baffled flask. After 24 h incubation at 28 °C, 200 rpm, the reaction product was extracted with EtOAc (3×100 mL). The combined organic layers were dried on anhyd Na_2SO_4, and the solvent was removed by rotary evaporator. The crude extract was purified by a standard silica gel chromatography (1.0×20 cm gel with hexane/acetone (1:1)) and a preparative TLC with hexane/EtOAc (1:4; $R_f=0.3$) to obtain 2.6 mg of 10-deoxy-FD-891. The assignments of ^1H and ^{13}C NMR signals are shown in the Supporting Information. HR-FAB-MS (positive, glycerol): *m/z*: calcd for $C_{33}H_{55}O_7$: 563.3948 $[M+H]^+$, found: 563.3942.

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[1] M. Seki-Asano, T. Okazaki, M. Yamagishi, N. Sakai, K. Hanada, K. Mizoue, *J. Antibiot.* **1994**, *47*, 1226 – 1233.
[2] S. Inaba, T. Eguchi, A. Motegi, K. Mizoue, T. Usui, K. Nagai, T. Kataoka, *J. Antibiot.* **2009**, *62*, 507 – 512.
[3] J. Garcia-Fortanet, J. Murga, M. Carda, J. A. Marco, R. Matesanz, J. F. Diaz, I. Barasoain, *Chem. Eur. J.* **2007**, *13*, 5060 – 5074.
[4] T. Kataoka, A. Yamada, M. Bando, T. Honma, K. Mizoue, K. Nagai, *Immunology* **2000**, *100*, 170 – 177.
[5] T. Eguchi, K. Yamamoto, K. Mizoue, K. Kakinuma, *J. Antibiot.* **2004**, *57*, 156 – 157.
[6] J. Garcia-Fortanet, J. Murga, M. Carda, J. A. Marco, *Org. Lett.* **2006**, *8*, 2695 – 2698.
[7] M. T. Crimmins, F. Caussanel, *J. Am. Chem. Soc.* **2006**, *128*, 3128 – 3129.
[8] C. D. Reeves, E. Rodriguez, *Methods Enzymol.* **2009**, *459*, 295 – 318.
[9] S. Peiru, H. Gramajo, H. G. Menzella, *Methods Enzymol.* **2009**, *459*, 319 – 337.
[10] C. Olano, C. Mendez, J. A. Salas, *Nat. Prod. Rep.* **2010**, *27*, 571 – 616.
[11] C. J. Thibodeaux, C. E. Melancon, III, H. W. Liu, *Angew. Chem.* **2008**, *120*, 9960 – 10007; *Angew. Chem. Int. Ed.* **2008**, *47*, 9814 – 9859.
[12] M. Otsuka, K. Ichinose, I. Fujii, Y. Ebizuka, *Antimicrob. Agents Chemother.* **2004**, *48*, 3468 – 3476.
[13] A. E. Maris, M. R. Sawaya, M. Kaczor-Grzeskowiak, M. R. Jarvis, S. M. Bearson, M. L. Kopka, I. Schroder, R. P. Gunsalus, R. E. Dickerson, *Nat. Struct. Biol.* **2002**, *9*, 771 – 778.
[14] R. D. Finn, J. Mistry, J. Tate, P. Coggill, A. Heger, J. E. Pollington, O. L. Gavin, P. Gunasekaran, G. Ceric, K. Forslund, L. Holm, E. L. Sonnhammer, S. R. Eddy, A. Bateman, *Nucleic Acids Res.* **2010**, *38*, D211 – 222.
[15] G. Yadav, R. S. Gokhale, D. Mohanty, *J. Mol. Biol.* **2003**, *328*, 335 – 363.
[16] L. Tang, Y. J. Yoon, C. Y. Choi, C. R. Hutchinson, *Gene* **1998**, *216*, 255 – 265.
[17] C. Bisang, P. F. Long, J. Cortes, J. Westcott, J. Crosby, A. L. Matharu, R. J. Cox, T. J. Simpson, J. Staunton, P. F. Leadlay, *Nature* **1999**, *401*, 502 – 505.
[18] M. Thattai, Y. Burak, B. I. Shraiman, *PLoS Comput. Biol.* **2007**, *3*, 1827 – 1835.
[19] K. J. Weissman, *ChemBioChem* **2006**, *7*, 485 – 494.
[20] A. Keatinge-Clay, *J. Mol. Biol.* **2008**, *384*, 941 – 953.
[21] A. T. Keatinge-Clay, *Chem. Biol.* **2007**, *14*, 898 – 908.
[22] C. R. Valenzano, R. J. Lawson, A. Y. Chen, C. Khosla, D. E. Cane, *J. Am. Chem. Soc.* **2009**, *131*, 18501 – 18511.
[23] D. H. Kwan, Y. Sun, F. Schulz, H. Hong, B. Popovic, J. C. Sim-Stark, S. F. Haydock, P. F. Leadlay, *Chem. Biol.* **2008**, *15*, 1231 – 1240.
[24] Q. Gao, C. Zhang, S. Blanchard, J. S. Thorson, *Chem. Biol.* **2006**, *13*, 733 – 743.
[25] H. Onaka, S. Taniguchi, Y. Igarashi, T. Furumai, *Biosci. Biotechnol. Biochem.* **2003**, *67*, 127 – 138.
[26] Y. Mao, M. Varoglu, D. H. Sherman, *Chem. Biol.* **1999**, *6*, 251 – 263.
[27] H. Ikeda, T. Nonomiya, M. Usami, T. Ohta, S. Omura, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 9509 – 9514.
[28] D. C. Lamb, H. Ikeda, D. R. Nelson, J. Ishikawa, T. Skaug, C. Jackson, S. Omura, M. R. Waterman, S. L. Kelly, *Biochem. Biophys. Res. Commun.* **2003**, *307*, 610 – 619.
[29] R. Fouces, E. Mellado, B. Diez, J. L. Barredo, *Microbiology* **1999**, *145*, 855 – 868.
[30] T. Brautaset, O. N. Sekurova, H. Sletta, T. E. Ellingsen, A. R. Strøm, S. Valla, S. B. Zotchev, *Chem. Biol.* **2000**, *7*, 395 – 403.
[31] T. Kieser, M. J. Bibb, M. J. Buttner, K. F. Chater, D. A. Hopwood, *Practical Streptomyces Genetics*, The John Innes Foundation, Norwich, **2000**.
[32] A. W. Munro, H. M. Girvan, K. J. McLean, *Nat. Prod. Rep.* **2007**, *24*, 585 – 609.
[33] I. G. Denisov, T. M. Makris, S. G. Sligar, I. Schlichting, *Chem. Rev.* **2005**, *105*, 2253 – 2277.
[34] J. A. Peterson, M. C. Lorence, B. Amarneh, *J. Biol. Chem.* **1990**, *265*, 6066 – 6073.
[35] Y. Fujii, S. Hirosue, T. Fujii, N. Matsumoto, H. Agematu, A. Arisawa, *Biosci. Biotechnol. Biochem.* **2006**, *70*, 2299 – 2302.
[36] S. F. Haydock, A. N. Appleyard, T. Mironenko, J. Lester, N. Scott, P. F. Leadlay, *Microbiology* **2005**, *151*, 3161 – 3169.
[37] M. Seki-Asano, Y. Tsuchida, K. Hanada, K. Mizoue, *J. Antibiot.* **1994**, *47*, 1234 – 1241.
[38] J. A. Krauser, F. P. Guengerich, *J. Biol. Chem.* **2005**, *280*, 19496 – 19506.
[39] V. B. Urlacher, S. Eiben, *Trends Biotechnol.* **2006**, *24*, 324 – 330.
[40] R. Bernhardt, *J. Biotechnol.* **2006**, *124*, 128 – 145.
[41] Y. Anzai, S. Li, M. R. Chaulagain, K. Kinoshita, F. Kato, J. Montgomery, D. H. Sherman, *Chem. Biol.* **2008**, *15*, 950 – 959.
[42] J. Sambrook, E. F. Fritsch, T. Maniatis, *Molecular Cloning. A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, New York, **1989**.
[43] J. Ishikawa, K. Hotta, *FEMS Microbiol. Lett.* **1999**, *174*, 251 – 253.
[44] H. Agematu, N. Matsumoto, Y. Fujii, H. Kabumoto, S. Doi, K. Machida, J. Ishikawa, A. Arisawa, *Biosci. Biotechnol. Biochem.* **2006**, *70*, 307 – 311.

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