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ARTICLE

Cloning and heterologous expression of P450Lent4B11, a novel bacterial P450 gene, for hydroxylation of an antifungal agent sordaricin

Motoi Ueno[1] · Motoo Kobayashi[2] · Akihiko Fujie[3] · Takashi Shibata[4]

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Abstract

Microbial transformation is known to be one of promising options to add functional groups such as a hydroxyl moiety to active base compounds to generate their derivatives. Sordaricin, a diterpene aglycone of the natural product sordarin, is an antifungal agent to selectively inhibit fungal protein synthesis by stabilizing the ribosome/EF-2 (elongation factor 2) complex. We screened actinomycetes to catalyze hydroxylation of sordaricin on the basis that the hydroxyl moiety would make it easier to generate derivatives of sordaricin. As a result of the screening, 6-hydroxylation of sordaricin was found to be catalyzed by *Lentzea* sp. 7887. We found that the cytochrome P450 inhibitor metyrapone inhibited this reaction, suggesting that a cytochrome P450 may be responsible for the biotransformation. As a next step, we cloned multiple cytochrome P450 genes, one of which were named P450Lent4B11, using degenerate PCR primers. The expressed cytochrome P450 derived from the P450Lent4B11 gene provided a different absorbance spectrum pattern from original one when it was incubated with sordaricin. Moreover, in cell-free conditions, the corresponding cytochrome P450 displayed the 6-hydroxylation activity toward sordaricin. Taken together, these results indicate that P450Lent4B11, derived from *Lentzea* sp. 7887, should be responsible for catalyzing 6-hydroxylation of sordaricin.

Introduction

Invasive fungal infections are common problems especially in immunocompromised patients [1, 2]. Although a number of antifungal agents are used, clinical outcomes are often unsatisfactory because of their side effects or their narrow antifungal spectra [3, 4].

Sordaricin (Fig. 1a) is a diterpene aglycone of the natural product sordarin (Fig. 1b) isolated from *Sordaria araneosa* in the early 1970s [5]. Sordaricin was produced by the acid hydrolysis of sordarin [5]. These sordarin family of compounds, characterized by a unique tetracyclic diterpene core, exhibit antifungal activity through stabilizing the ribosome/EF-2 (elongation factor 2) complex [6].

The sordaricin derivatives were intensively generated, and were biologically evaluated in order to obtain the derivatives with pharmacologically better profiles [7–9].

The sordaricin analog 6-hydroxysordaricin (Fig. 1c), with a functional hydroxyl moiety added by biotransformation, made it easier to produce further sordaricin derivatives and to know the structure activity relationship [10].

Also, cloning of a gene responsible for this hydroxylation would enable us to try to enhance the biotransformation efficiency through genetic approaches such as overexpression or gene manipulation.

Here, we report that the 6-hydroxylation of sordaricin was catalyzed by *Lentzea* sp. 7887 [11], and that the reaction was attributed to a cytochrome P450. We therefore further attempted to clone a gene encoding cytochrome P450, which was responsible for this hydroxylation.

Supplementary information The online version of this article (https://doi.org/10.1038/s41429-020-0310-9) contains supplementary material, which is available to authorized users.

✉ Motoi Ueno
uenomotoi@gmail.com

[1] Pharmacology Research Laboratories, Astellas Pharma Inc., 21 Miyukigaoka, Tsukuba-shi, Ibaraki 305-8585, Japan

[2] Research Planning and Administration, Astellas Pharma Inc., 21 Miyukigaoka, Tsukuba-shi, Ibaraki 305-8585, Japan

[3] Research Management, Astellas Pharma Inc., 21 Miyukigaoka, Tsukuba-shi, Ibaraki 305-8585, Japan

[4] Pharmaceutical Science and Technology Labs, Astellas Pharma Inc., 5-2-3 Tokodai, Tsukuba, Ibaraki 300-2698, Japan

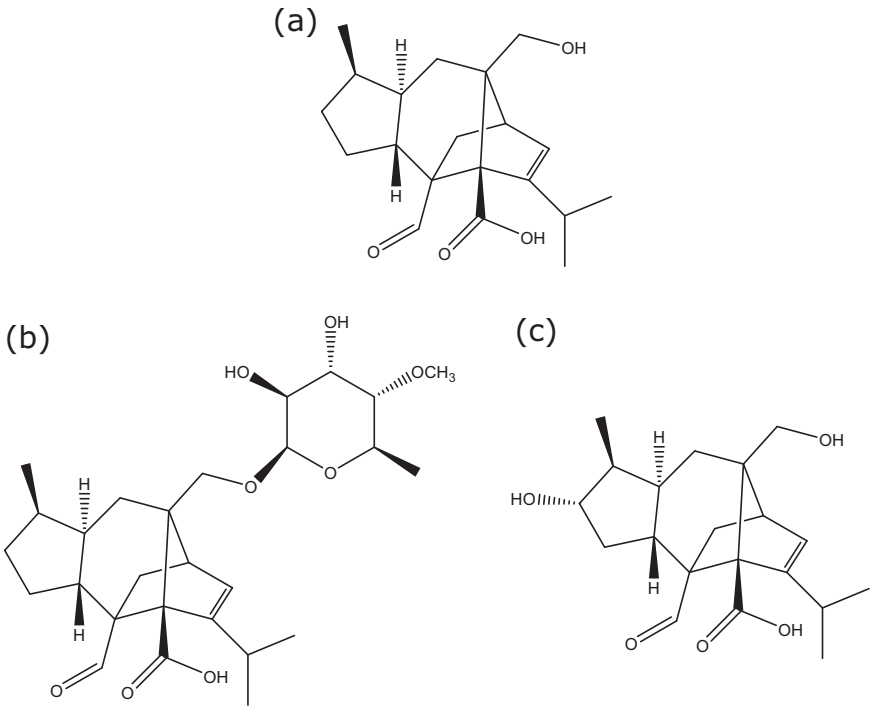

Fig. 1 Structures of (**a**) sordaricin, (**b**) sordarin, and (**c**) 6-hydroxysordaricin

Materials and methods

Chemicals

Sordaricin and 6-hydroxysordaricin were obtained from Dr Hanadate (Astellas Pharma.). Structure of 6-hydroxysordaricin has been confirmed by NMR analysis before use (Data not shown). D-Camphor was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Exploration of actinomycetes catalyzing the hydroxylation of sordaricin

Approximately 50 actinomycetes strains, reported to be involved in production of natural products or bioconversion of base compounds, were applied to the biotransformation screening.

Each strain was inoculated into a sterilized seed culture medium (pH 7.0, 100 ml) containing glucose 0.5%, sucrose 0.5%, oatmeal (Snow Brand Milk Products, Tokyo, Japan) 0.5%, yeast extract (Becton Dickinson, New Jersey, USA) 0.2%, peptone (Kyokuto, Tokyo, Japan) 0.5%, peanut powder (Masuda, Chiba, Japan) 0.5%, humic acid (IIC, Chiba, Japan) 0.01%, Tween 80 0.1%, and $CaCO_3$ 0.2% in 225-ml Erlenmeyer flasks. The inoculated flask was shaken on a rotary shaker (220 rpm, 5.1-cm throw) at 30 °C for 3 days. The seed culture (2%) was inoculated into a sterilized culture medium (pH 6.5, 100 ml) containing glycerol 6.0%, soybean flour (Matsumoto, Osaka, Japan) 1.0%, and corn steep liquor (Nihon Shokuhin Kako, Tokyo, Japan) 3.0% in 225-ml Erlenmeyer flasks. The expression culture was conducted at 30 °C for 3 days on a rotary shaker (220 rpm, 5.1-cm throw).

The biotransformation reaction was initiated by adding sordaricin (final concentration; 100 μg ml^{-1}) to the broth. After a further 24 h of incubation, an equal amount of acetone was added to the mixture to stop the reaction. The broth was centrifuged to obtain the supernatant (5000 × *g*, 10 min), which was extracted using an equal volume of ethyl acetate. The resultant extract was evaporated and dissolved with methanol.

To measure the biotransformation activity, both the substrate sordaricin and the converted product 6-hydroxysordaricin were monitored by HPLC analysis using LC-20AD system (Shimadzu, Kyoto, Japan) and Capcell Pak Phenyl type UG120 column (150 × 4.6 mm, 5 μm) (Shiseido, Tokyo, Japan). The mobile phase was 20-min linear gradient from 5 to 100% CH_3CN in 0.05% trifluoroacetic acid (TFA). The flow rate was 1.0 ml min^{-1}. The absorbance wavelength was 210 nm. The retention times for sordaricin and 6-hydroxysordaricin, were 12.4 min and 8.5 min, respectively. To investigate an involvement of cytochrome P450 in this reaction, a cytochrome P450 inhibitor metyrapone (Sigma-Aldrich) was added to the reaction system at various concentrations.

Cloning of cytochrome P450 gene fragments from Lentzea sp. 7887

To clone cytochrome P450 gene fragments (~300 bp), degenerate primers were constructed (forward, 5'-TSGCS

GGSCACGAGACSACSG-3'; reverse, 5'-GCACTGGTG-SACSCCGAASCCGAA-3'). Polymerase chain reaction (PCR) was performed using KOD Dash (Toyobo, Tokyo, Japan) on Perkin-Elmer DNA thermal cycler (Yokohama, Japan). PCR conditions were as follows: 98 °C for 4 min, 30 cycles of 98 °C for 30 s and 72 °C, and, finally, 72 °C for 7 min.

The amplified DNA fragments were ligated to pCR®2.1-TOPO® (Life Technologies, Carlsbad, California, USA) according to the manufacturer's protocol and subsequently introduced into *Escherichia coli* TOP10 (Life Technologies). Plasmids were prepared, from the ampicillin resistant clones, using a QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany), and subsequently a sequence analysis was conducted using ABI PRISM® 3100 Genetic Analyzer (Life Technologies).

Construction of genomic cosmid library for Lentzea sp. 7887

Genomic DNA of *Lentzea* sp. 7887, which was prepared using QIAGEN® Genomic-tip system (Qiagen), was partially digested with an appropriate concentration of *Sau*3AI to produce DNA fragments of ~40 kb in length. The digested DNA fragments were ligated into pFD666 cosmid vector and packed into a lambda phage using Gigapack® III XL packaging extract (Stratagene, La Jolla, CA, USA) according to the manufacturer's protocol. XL-1 Blue MRF' (Stratagene) was transformed with the recombinant phage, which was resistant against 50 μg ml^{-1} of ampicillin. The cosmids were prepared from the resultant recombinant clones through the same procedure as that for the plasmid preparation.

PCR screening to identify cosmid clones with an entire cytochrome P450 gene

To obtain an entire cytochrome P450 gene from the cosmid library, PCR was performed using authentic primers constructed based on the cloned cytochrome P450 gene fragments. When a DNA fragment whose size was ~300 bp was amplified, the corresponding cosmid was called "positive cosmid clone". To identify a whole cytochrome P450 open reading frame, a sequence analysis using the "positive cosmid clone" was conducted by a primer walking method [12].

Heterologous expression and purification of cytochrome P450 genes

The identified cytochrome P450 genes were amplified using the positive cosmid clone as a PCR template. The PCR primers with additional restriction sites (*Bam*HI site at the 5' end and *Eco*RI site at the 3' end) were constructed. The PCR was then performed using KOD-Plus (Toyobo) at 98 °C for 30 s, 60 °C for 30 , and 74 °C for 2 min for amplification (25 cycles) and at 68 °C for 7 min for extension (1 cycle). The amplified PCR products were introduced into pCR-TOPO® vector (Life Technologies) and subsequently digested with *Bam*HI / *Eco*RI. This digested product was ligated into a previously *Bam*HI / *Eco*RI-digested expression vector pTrcHisB (Life Technologies) using Ligation High (Toyobo). The resultant expression plasmids were introduced into *E. coli* DH5α.

The obtained transformants harboring the corresponding cytochrome P450 gene were inoculated into a sterilized seed culture medium (40 ml) containing Bacto yeast extract (Becton Dickinson) 0.5%, Bacto tryptone (Becton Dickinson) 1.0%, NaCl 1.0% and ampicillin 50 μg ml^{-1}, pH 7.0 in 100-ml Erlenmeyer flasks. The inoculated flasks were shaken on a rotary shaker (150 rpm) at 37 °C for 15 h and then inoculated (10%) into a sterilized expression culture medium super broth (200 ml) containing yeast extract (Becton, Dickinson) 1.5%, Bacto tryptone (Becton, Dickinson) 2.5%, NaCl 0.5% and ampicillin 100 μg ml^{-1}, pH 7.0 in 500-ml Erlenmeyer flasks. The flasks were incubated at 37 °C on a rotary shaker (150 rpm). Six hours later, isopropyl β-D-1-thiogalactopyranoside (IPTG) at 1 mM and 5-aminolevulinic acid at 80 μg ml^{-1} were added to the culture to induce the cytochrome P450 genes. After a further 3-day cultivation at 25 °C, the cells were collected (10,000 × *g*, 10 min.) and sonicated on ice with an Ultrasonic homogenizer VP-050 (Taitec, Saitama, Japan) at 60% maximum amplitude for 4 min. The expressed cytochrome P450s were then purified using Ni-NTA Superflow (Qiagen) according to the manufacturer's protocol. The resultant recombinant cytochrome P450s were applied to a 10% polyacrylamide gel (Bio-Rad, Tokyo, Japan) after adjusting total protein levels. Mark 12™ Unstained Standard (Life Technologies) was used as a molecular marker. After the electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 (Bio-Rad).

Measurement of absorbance spectra for cytochrome P450s

Absorbance spectra (350–500 nm) for the cytochrome P450-rich fractions (50 μM) incubated with 500 μM of sordaricin was compared with the cytochrome P450-rich fractions alone through the standard procedure [13]. The incubation was conducted at 30 °C for 5 min. The difference spectra observed among multiple conditions was recorded using SpectraMax M2 (Molecular Devices, Silicon Valley, California, USA). As a control, P450cam protein [14] was prepared as follows; Genomic DNA from *Pseudomonas putida* was prepared using QIAGEN® Genomic-tip System

(Qiagen) and subsequently used as a PCR template. The camC gene encoding the cytochrome P450cam was amplified using primers with additional restriction sites (*Bam*HI site at the 5' end and EcoRI site at the 3' end). As for the PCR, the expression and the purification, we took the same procedure as that for the cloned cytochrome P450s derived from *Lentzea* sp. 7887. The obtained cytochrome P450cam and D-camphor were incubated together to examine the spectra as a control condition.

Biotransformation assay under cell-free conditions using the obtained P450s

To start the biotransformation reaction, the obtained cytochrome P450s were incubated with NADH 2 mM, NADPH 2 mM, $MgCl_2$ 5 mM, glucose-6-phosphate (Sigma-Aldrich) 10 mM, glucose-6-phosphate dehydrogenase (Sigma-Aldrich) 1 unit ml^{-1}, ferredoxin from spinach (Sigma-Aldrich) 0.8 mg ml^{-1}, ferredoxin-NADP + reductase from spinach (Sigma-Aldrich) 0.2 units ml^{-1}, and sordaricin 500 µg ml^{-1}. The incubation time was 1 h. Detection of the converted product was carried out by LC/MS using the Agilent HP1100 series LC/MSD system (Hewlett-Packard, Palo Alto, CA, USA) and Mightysil RP-18GP column (3.0 × 50 mm, 3 µm) (Kanto, Tokyo, Japan). The mobile phase was 12-min linear gradient from 5 to 100% CH3CN in 0.05% TFA. The flow rate was 0.2 ml min^{-1}. The temperature was 50 °C. The fragmentor voltage was 160 V. The analytical mode was "selected-ion monitoring mode", which enabled us to detect the sordaricin analogs specifically and quantitatively. Retention times of sordaricin and 6-hydroxysordaricin, were 11.5 min and 1.9 min, respectively.

Results

Screening of actinomycetes strains adding a hydroxyl moiety to sordaricin

Our actinomycetes library was employed to search for strains adding a hydroxyl moiety to sordaricin by whole-cell biotransformation. As a result of the screening, *Lentzea* sp. 7887 was found to catalyze 6-hydroxylation. This reaction was completely inhibited by a cytochrome P450 inhibitor metyrapone (Fig. 2), which suggested that a cytochrome P450 might be responsible for the hydroxylation catalyzed by *Lentzea* sp. 7887.

Cloning of cytochrome P450 genes from Lentzea sp. 7887

We constructed degenerate PCR primers based on two conserved regions in cytochrome P450 genes, K-helix, and

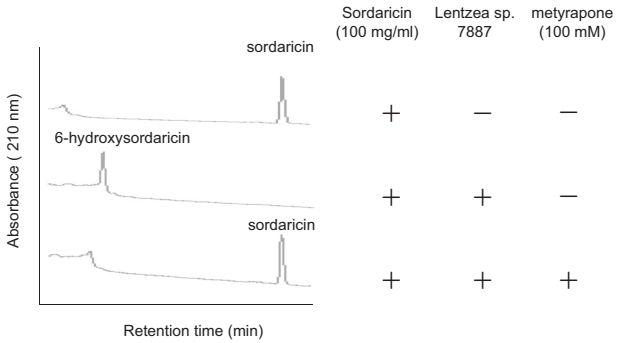

Fig. 2 Biotransformation of sordaricin to 6-hydroxysordaricin catalyzed by *Lentzea* sp. 7887. 6-hydroxylation of sordaricin catalyzed by *Lentzea* sp. 7887 was monitored by HPLC. (Upper chromatogram); only sordaricin was detected prior to the reaction. (Middle chromatogram); 6-hydroxylsordaricin was mainly detected when sordaricin was incubated with *Lentzea* sp. 7887. (Lower chromatogram); the 6-hydroxylation of sordaricin was inhibited in the presence of a cytochrome P450 inhibitor metyrapone

heme binding motifs [15, 16]. With these primers, PCR was performed using genomic DNA from *Lentzea* sp. 7887. Consequently, four amplified DNA fragments, with their sizes ~300 bp, were obtained. Subsequent sequence analysis demonstrated that each amplified DNA was a part of cytochrome P450.

Partially digested genomic DNA fragments from *Lentzea* sp. 7887 were inserted into cosmid vector pFD666 and then packaged into *E. coli* XL-1 Blue MRF', which provided a genomic DNA library of *Lentzea* sp. 7887.

To obtain positive cosmid clones encoding an entire cytochrome P450 gene, we carried out a PCR screening using authentic primers which were constructed based on the cloned cytochrome P450 fragments.

As a result, four positive cosmid clones encoding an entire cytochrome P450 gene were obtained. These four cytochrome P450 genes were named P450Lent1G3, P450Lent4B11, P450Lent6G12, and P450Lent1C7, respectively. All the four genes included K-helix and heme binding motif, both of which were typical motifs of cytochrome P450 (Fig. 3). These four genes have been submitted to the DDBJ databases under accession no. AB739030, AB739031, AB739032, and AB739033, respectively (Fig. S1).

Heterologous expression of the cloned cytochrome P450 genes

The cloned cytochrome P450 genes were inserted into an expression vector pTrcHisB. The resultant expression plasmids were introduced into *E. coli* DH5α. The transformants were grown in an expression culture medium super broth at 37 °C, and 6 h later isopropyl β-D-1-thiogalactopyranoside (IPTG) and 5-aminolevulinic acid were added to the culture

to induce the cytochrome P450 genes. After a further 3-day cultivation at 25 °C, the cells were lysed, and the expressed cytochrome P450 proteins were purified using Ni-NTA Superflow (Qiagen). Each cytochrome P450 protein expressed by these procedures was found to be considerably pure as confirmed by SDS-PAGE (Fig. 4a).

Absorbance spectra for the cytochrome P450s in the presence of sordaricin

It is reported that an absorbance spectrum for a cytochrome P450 will be affected if the cytochrome P450 is incubated with its substrate [13]. D-Camphor is known to be a substrate for cytochrome P450cam [14]. We incubated D-camphor and cytochrome P450cam together, and observed that the spectrum for the cytochrome P450cam was affected by existence of D-camphor (Fig. 4b).

Clone	K-helix	Heme binding motif
P450Lent 1G3	**E**LL**R**	FGH**G**IHQ**C**LG
P450Lent 4B11	**E**LM**R**	FGH**G**IHQ**C**LG
P450Lent 6G12	**E**LV**R**	FGH**G**LHH**C**IG
P450Lent 1C7	**E**LI**R**	FGM**G**IHY**C**LG

Fig. 3 Alignment of the deduced amino acid sequences in K-helix and heme binding motifs of the four cloned cytochrome P450 genes. All the four cytochrome P450 genes were found to encode common amino acids in both K-helix and heme binding motif. The conserved amino acids were represented as bolds

Subsequently, we measured absorbance spectra for the expressed cytochrome P450s with or without sordaricin. When the P450Lent4B11 expression product was incubated with sordaricin, the spectrum pattern was changed (Fig. 4c), which suggested that P450Lent4B11 gene might be responsible for the 6-hydroxylation of sordaricin.

Hydroxylation of sordaricin by P450Lent4B11 in a cell-free condition

We constructed a cell-free biotransformation system consisting of cytochrome P450s and other essential components such as electron transfer partners to examine the involvement of the P450Lent4B11 in the 6-hydroxylation of sordaricin. After 1 h incubation, the P450Lent4B11 expression product specifically catalyze 6-hydroxylation of sordaricin (Fig. 5). The conversion ratio was 21.3 ± 1.3%. This result indicates that P450Lent4B11 should be responsible for the 6-hydroxylation of sordaricin.

Discussion

Fungal pathogens cause life-threatening infections especially in immunocompromised patients receiving immunosuppressive agents for solid organ or hematopoietic stem cell transplantation [17]. Coupled with widespread use of immunosuppressive agents and organ transplantation,

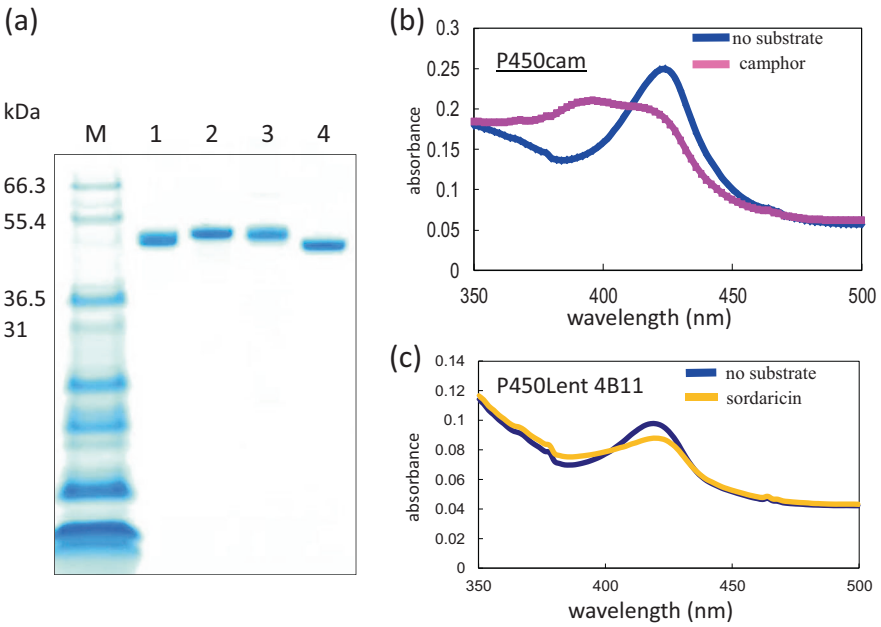

Fig. 4 Heterologous expression and substrate-binding difference spectra of the cloned P450 genes. **a** The SDS-PAGE analysis of the expressed and the purified cytochrome P450 proteins derived from *Lentzea* sp. 7887. The molecular marker was loaded in lane M. In lane 1–4, following proteins were loaded respectively; lane 1; P450Lent1G3, lane 2; P450Lent4B11, lane 3; P450Lent6G12, lane 4; P450Lent1C7. **b** The substrate-binding difference spectra for P450cam. D-Camphor, a substrate for P450cam, was used as a positive control. **c** The substrate-binding difference spectra for P450Lent4B11. The spectrum pattern for P450Lent4B11 was affected by sordaricin

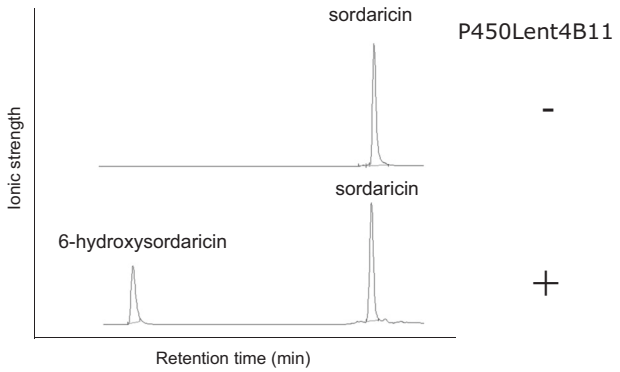

Fig. 5 6-Hydroxylation of sordaricin catalyzed by P450Lent4B11 in a cell-free biotransformation. 6-hydroxylation of sordaricin catalyzed by P450Lent4B11 in a cell-free condition was monitored by LC/MS. 6-hydroxysordaricin was produced only in the presence of P450Lent4B11

emergence of multi-drug resistant *Candida auris* [18] highlight the urgent need for novel therapeutic options.

Sordaricin might be a key molecule to combat such fungal species because it targets one of the most critical cellular process in fungi, protein synthesis, by inhibiting the function of the eukaryotic elongation factor 2 (eEF2), which is a target unaddressed by current clinical antifungals [6].

6-hydroxysordaricin might be a key intermediate to generate sordaricin derivatives with pharmacologically better profile because the antifungal activities of the sordaricin derivatives originated from 6-hydroxysordaricin proved to be changeable, with depending on the modification [10]. For example, when fluoro moiety was substituted for hydroxy moiety at C6 position of 6-hydroxysordaricin related base compound, the antifungal activity toward *Candida albicans* A28235 was maintained whereas in the case of substitution with *O*-hexyl moiety, the antifungal activity was lacked [10].

6-hydroxysordaricin was produced by biotransformation whereas it could not be produced by synthetic approach because C6 position of sordaricin does not have any functional groups, which shows that the biotransformation should be one of powerful approaches to generate its derivatives.

Of various enzymes involved in biotransformation, a wide range of bacterial cytochrome P450 enzymes are known to function as biocatalysts that are capable of adding functional groups such as a hydroxyl moiety by activating an inert C-H bond of base compounds [19–21]. These features of cytochrome P450s enable to generate their derivatives more easily and to further examine their structure activity relationships. As one example, P450Um-1 was reported to catalyze hydroxylation of the immunosuppressive agent AS1387392 to AS1429716, which may facilitate the synthesis of more derivatives [22].

From industrial point of view, cloning of responsible cytochrome P450 genes for the biotransformation is an important approach because it would be possible to manipulate cytochrome P450 genes to enhance their biotransformation efficiency or to modulate its specificity. For example, CYP105A1 with R73A/R84A mutation was reported to enhance its vitamin D3 hydroxylation by 400-fold [23]. As another example, CYP260A1 with S276N mutation was reported to convert progesterone more predominantly into 1α-hydroxy-progesterone [24].

As a first step to clone bacterial cytochrome P450 genes, it would be important to obtain original bacterial strains catalyzing oxidative activities toward some base compounds. In order to screen such strains of all diverse candidates, universal cultivation conditions should be ideally employed. In this regard, our cultivation protocol has been found to cultivate a wide range of strains (data not shown). Also, with regards to the biotransformation conditions, we referred to the previous similar experiments [22].

In the previous effort, 6-hydroxysordaricin was also produced by biotransformation using *Nocardia orientalis* SC4044 [10], which means that taxonomically different two strains would catalyze the same biotransformation. If the responsible gene is cloned from *N. orientalis* SC4044 as well, it would give us more insight into what sequence is conserved for the hydroxylation of sordaricin.

Detection of the converted product 6-hydroxysordaricin in our cell free system was carried out using a highly sensitive and selective mode of LC/MS in which sordaricin analogs can be specifically and quantitatively detected, which enabled us to monitor the biotransformation activity of P450Lent4B11.

Lentzea sp. 7887 was reported to catalyze hydroxylation of FR901459, a related compound of cyclosporin A [11]. Therefore, we examined whether P450Lent4B11 could hydroxylate the cyclic peptide FR901459. However, such hydroxylation activity was not observed (data not shown), which might suggest that P450Lent4B11 selectively hydroxylate the C6 position of sordaricin.

In order to make full use of P450Lent4B11 from industrial perspective, the biotransformation efficiency should be ideally optimized. For the functional expression of bacterial cytochrome P450s in heterologous systems, appropriate redox partners must be expressed at the same time [25, 26]. Also, utilization of a suitable heterologous expression system might be one of the useful approaches [27].

Application of these efforts to P450Lent4B11 would effectively build a platform to generate sordaricin based derivatives with more potent antifungal activities and broader antifungal spectrum.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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