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BIOCATALYSIS - SHORT COMMUNICATION

In vitro reconstitution of the cyclosporine specific P450 hydroxylases using heterologous redox partner proteins

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Abstract The cytochrome P450 enzymes (CYPs) CYP-sb21 from *Sebekia benihana* and CYP-pa1 from *Pseudonocardia autotrophica* are able to hydroxylate the immunosuppressant cyclosporin A (CsA) in a regioselective manner, giving rise to the production of two hair-stimulating agents (with dramatically attenuated immunosuppressant activity), γ-hydroxy-*N*-methyl-L-Leu4-CsA (CsA-4-OH) and γ-hydroxy-*N*-methyl-L-Leu9-CsA (CsA-9-OH). Recently, the in vitro activity of CYP-sb21 was identified using several surrogate redox partner proteins. Herein, we reconstituted the in vitro activity of CYP-pa1 for the first time via a similar strategy. Moreover, the supporting activities of a set of ferredoxin (Fdx)/ferredoxin reductase (FdR) pairs from the cyanobacterium *Synechococcus elongatus* PCC 7942 were comparatively analyzed to identify the optimal redox systems for these two CsA hydroxylases. The results suggest the great value of cyanobacterial redox partner proteins for both academic research and industrial application of P450 biocatalysts.

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Introduction

Cytochrome P450 enzymes (CYPs) originated from actinomycetes play important roles in biosynthesis of diverse bioactive natural products [14] and bioconversion of xenobiotic compounds [3]. We have continuously been searching for biocatalysts that are capable of mediating the conversion from the immunosuppressant cyclosporine A (CsA) to the site-specific hydroxylation products γ-hydroxy-*N*-methyl-L-Leu4-CsA (CsA-4-OH) and γ-hydroxy-*N*-methyl-L-Leu9-CsA (CsA-9-OH) with significant hair-stimulating effects, but reduced immunosuppressive activity (Fig. 1) [6]. As part of these efforts, two P450 enzymes CYP-sb21 and CYP-pa1 were identified from *Sebekia benihana* and *Pseudonocardia autotrophica*, respectively [2, 7, 8]. Interestingly, in vivo bioconversion results [8] revealed that CYP-sb21 primarily hydroxylates the tertiary C–H bond of the side chain of the fourth methyl leucine, whereas CYP-pa1 exhibits preference for the ninth methyl leucine position. The protein sequence alignment of CYP-sb21 and CYP-pa1 showed 55/67% of identity/similarity. Recently, CYP-sb21 and CYP-pa1 were named CYP107Z14 and CYP107Z15, respectively, based on the nomenclature system developed by Nelson [12].

Most CYPs require redox partner proteins to sequentially transfer two electrons form NAD(P)H to their heme–iron reactive center for dioxygen activation [15]. The functionalities of many P450 enzymes have been identified; however, their physiologically associated redox partners remain largely unknown. Therefore, it often becomes

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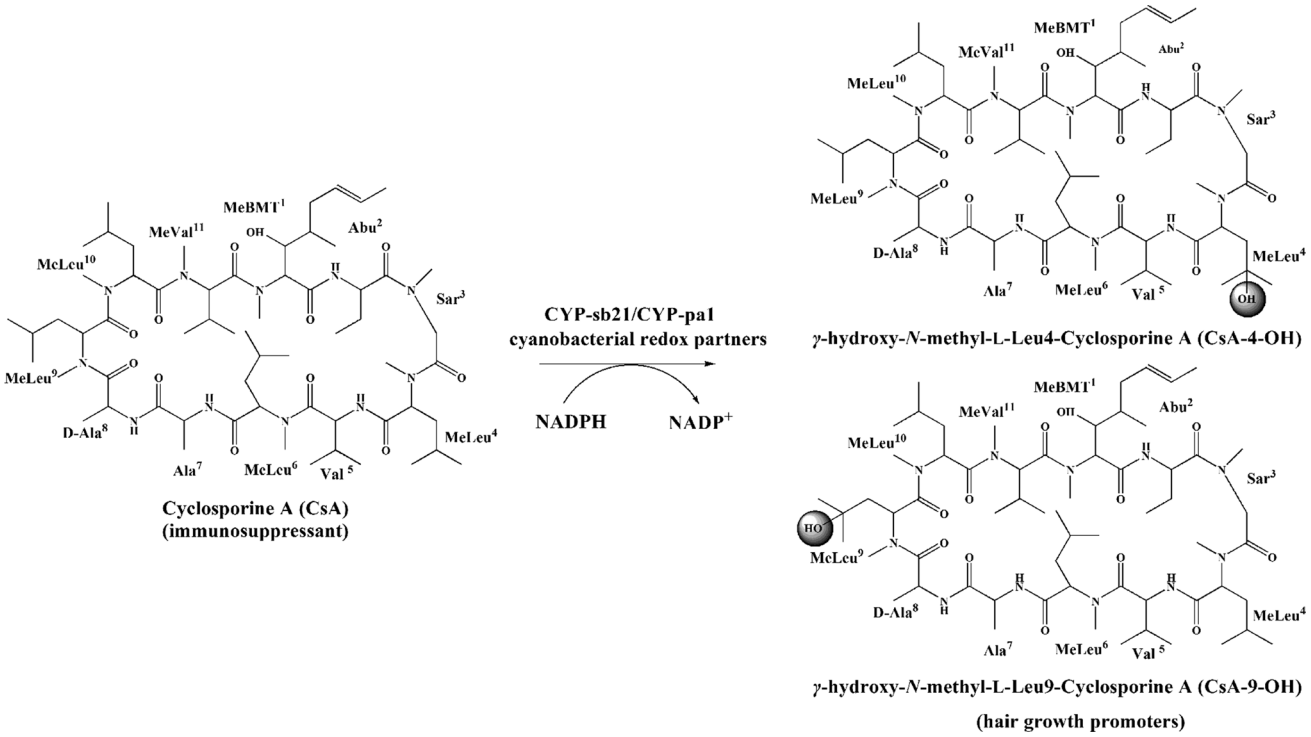


Fig. 1 Bioconversion of CsA to CsA-4-OH and CsA-9-OH by CYP-sb21 or CYP-pa1 using cyanobacterial Fdx/FdR as redox partners

necessary to construct hybrid P450 reaction systems by using surrogate redox partners derived from heterologous hosts [18]. For example, Agematu et al. screened the activity of 213 bacterial CYPs in *Escherichia coli* and found 24 P450 enzymes that are able to stereoselectively hydroxylate testosterone by co-expressing the putidaredoxin gene *pdx* and the putidaredoxin reductase gene *pdR* from *Pseudomonas putida* ATCC17453 [1]. The oleic acid hydroxylation activity of CYP105D5 from *Streptomyces coelicolor* A3(2) could be supported by either Pdx/PdR from *P. putida* or flavodoxin/flavodoxin reductase (Fld/FlR) from *E. coli* [4]. Recently, we found that the P450 peroxygenase $OleT_{JE}$ (CYP152L1) from *Jeotgalicoccus* sp. ATCC 8456 is capable of decarboxylating long-chain fatty acids to terminal alkenes when partnering with RhFRED from *Rhodococcus* sp. NCIMB 9784 [9] or Fld/FlR from *E. coli* [10]. Of particular importance, alternative redox partners not only influence catalytic efficiency and/or product distribution, but also could change the type and selectivity of P450 reactions [4, 10, 18].

The native preferred redox partner proteins of CYP-sb21 and CYP-pa1 remain unclear. Nonetheless, we successfully utilized the ferredoxin Fdx_1499 and the ferredoxin reductase FdR_0978 from the cyanobacterium type strain *Synechococcus elongatus* PCC 7942 to reconstitute the in vitro activity of CYP-sb21 [11]. Interestingly, this cyanobacterial strain has at least seven different ferredoxin genes (*fdx_0338*, *fdx_0698*, *fdx_0814*, *fdx_0898*, *fdx_1499*, *fdx_1749*, and *fdx_2581*), as well as a single ferredoxin reductase gene (*fdR*_0978). Thus, in this study, we comparatively tested the ability of these Fdxs to support the in vitro activity of CYP-sb21 and CYP-pa1. As a result, these two analogous P450 enzymes showed distinct activity and regioselectivity against CsA when partnered by different Fdx/FdR pairs. Use of the optimal redox partners should enable development of better P450-based CsA biotransformation systems.

Materials and methods

Materials

Antibiotics and bovine heart cytochrome *c* were obtained from Solarbio (Beijing, China). Chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). The codon-optimized *cyp-sb21* and *cyp-pa1* were synthesized by Cosmo Genetech (Seoul, Korea), and six out of seven PCC

7942 ferredoxin genes (except *fdx_1499*) were synthesized by GENEWIZ (Beijing, China). The recombinant His-tagged proteins were purified using nickle–nitrilotriacetic acid (Ni–NTA) resin (Qiagen, Valencia, CA, USA), Amicon Ultra centrifugal filters (Merck Millipore, Billerica, MA, USA), and PD-10 desalting columns (GE Healthcare, Piscataway, NJ, USA). CsA, CsA-4-OH, and CsA-9-OH were prepared as previously reported [2, 8].

Construction of expression vectors

DNA manipulations were carried out by following standard procedures. The DNA sequences of *cyp-sb21* (GenBank Accession No. AGE14538), *cyp-pa1* (KJ433554), and *fdxs*/*fdR* (WP_011243487 for *fdx_0338*, WP_011243144 for *fdx_0698*, WP_011243037 for *fdx_0814*, WP_011377779 for *fdx_0898*, WP_011244794 for *fdx_1499*, WP_011244652 for *fdx_1749*, WP_011243839 for *fdx_2581* and WP_011242878 for *fdR_0978*) were retrieved from *S. benihana* KCTC9610, *P. autotrophica* KCTC9441, and *S. elongatus* PCC 7942, respectively. The pET28b-*cyp-sb21*, pET28b-*fdx_1499*, and pET28b-*fdR_0978*, were constructed previously [11]. The codon-optimized *cyp-pa1* gene was inserted between the restriction sites *Nde*I/*Eco*RI of pET-28b to afford pET-28b-*cyp-pa1*. The six synthesized *fdx* genes were individually subcloned into pET-28b (*Nde*I/*Xho*I). All constructs were confirmed through DNA sequencing by Sangon Biotech (Shanghai, China).

Expression, purification, and spectral characterization of CYP-sb21, CYP-pa1 and redox proteins

All recombinant His-tagged proteins were overexpressed in *E. coli* BL21 (DE3) and purified by Ni–NTA affinity chromatography [9]. The functional concentrations of CYP-sb21 and CYP-pa1 were determined by CO-bound reduced difference spectra using the extinction coefficient ($\varepsilon_{450–490nm}$) of 91,000 M^{-1} cm^{-1} [13]. The Fdx and FdR concentrations were determined by Bradford assay using bovine serum albumin (BSA) as standard.

In vitro reconstitution of the CsA hydroxylation activity of CYP-sb21 and CYP-pa1

The standard reaction contained 10 μM P450 enzyme, 10 μM Fdx, 10 μM FdR, 200 μM CsA (diluted from 20 mM stock solution in methanol), 5 U glucose dehydrogenase (GDH) and 20 mM glucose as NADPH regeneration system [5], and 1 mM NADPH in 100 μl of reaction buffer (50 mM NaH_2PO_4, 10% glycerol, pH 7.4). The reaction was quenched by addition of 100 μl methanol after incubation at 30 °C for 16 h. The reaction samples were high-speed centrifuged to remove precipitated proteins, and the supernatants were analyzed by HPLC with an ODS HYPERSIL C18 reverse phase column (5 μm, 4.6 × 150 mm, Thermo) in a gradient system consisting of 25% methanol in deionized water (solvent A) and 100% acetonitrile (solvent B). One cycle of buffer B gradient was programmed as follows: 45% B for 4 min, 45–61% B over 16 min, 61–100% B over 20 min, and 45% B for 5 min. The detection wavelength was 210 nm, and the flow rate was 1.0 ml/min.

LC-Q-TOF/MS analysis of products

Liquid chromatography-quadrupole time of flight mass spectrometry (LC-Q-TOF/MS) was recorded on a maXis UHR-TOF system (Bruker Daltonik, Germany) using a Thermo Scientific Hypersil GOLD column (5 μm, 2.1 mm × 100 mm) at 30 °C with H_2O + 25% MeOH as solvent A and acetonitrile as solvent B. The gradient elution profile was as follows: 0–4 min, 40% B; 4–15 min, 40–61% B; 15–32 min, 61–100% B; and 32–35 min, 40% B. The flow rate was set as 0.2 ml/min.

Results and discussion

Protein expression, purification and spectral characterization

The heterologous expression of two P450 genes, one FdR gene, and seven Fdx genes was conducted in *E. coli* BL21 (DE3). We were able to express CYP-sb21, CYP-pa1, FdR_0978, and Fdx_1499 in soluble form and in good quantities. However, direct expression of the native genes of *fdx_0338*, *fdx_0698*, *fdx_0814*, *fdx_0898*, *fdx_1749*, and *fdx_2581* led to non-expression or poor quantity of proteins. Thus, these DNA sequences were codon optimized to suit the codon preference of *E. coli*, by which the six *fdx* genes were successfully expressed and purified.

The CO-bound reduced difference spectra of CYP-sb21 and CYP-pa1 showed a characteristic peak at 450 nm, which was indicative of functional P450 enzymes (Fig. 2a, b). FdR_0978 displayed the typical UV–Vis spectrum of a ferredoxin reductase [17] with the local maximal peaks at 392 and 458 nm, and a pronounced shoulder at 492 nm (Fig. 2c).

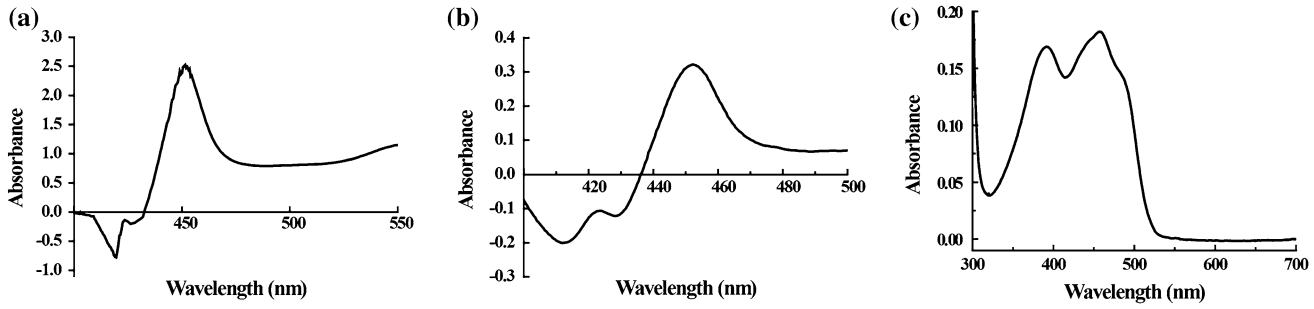


Fig. 2 CO-bound reduced difference spectra of purified CYP-sb21 (**a**) and CYP-pa1 (**b**), and the UV–Vis absorption spectrum of FdR_0978 (**c**)

In vitro reconstitution of the activity of CYP-pa1 and CYP-sb21

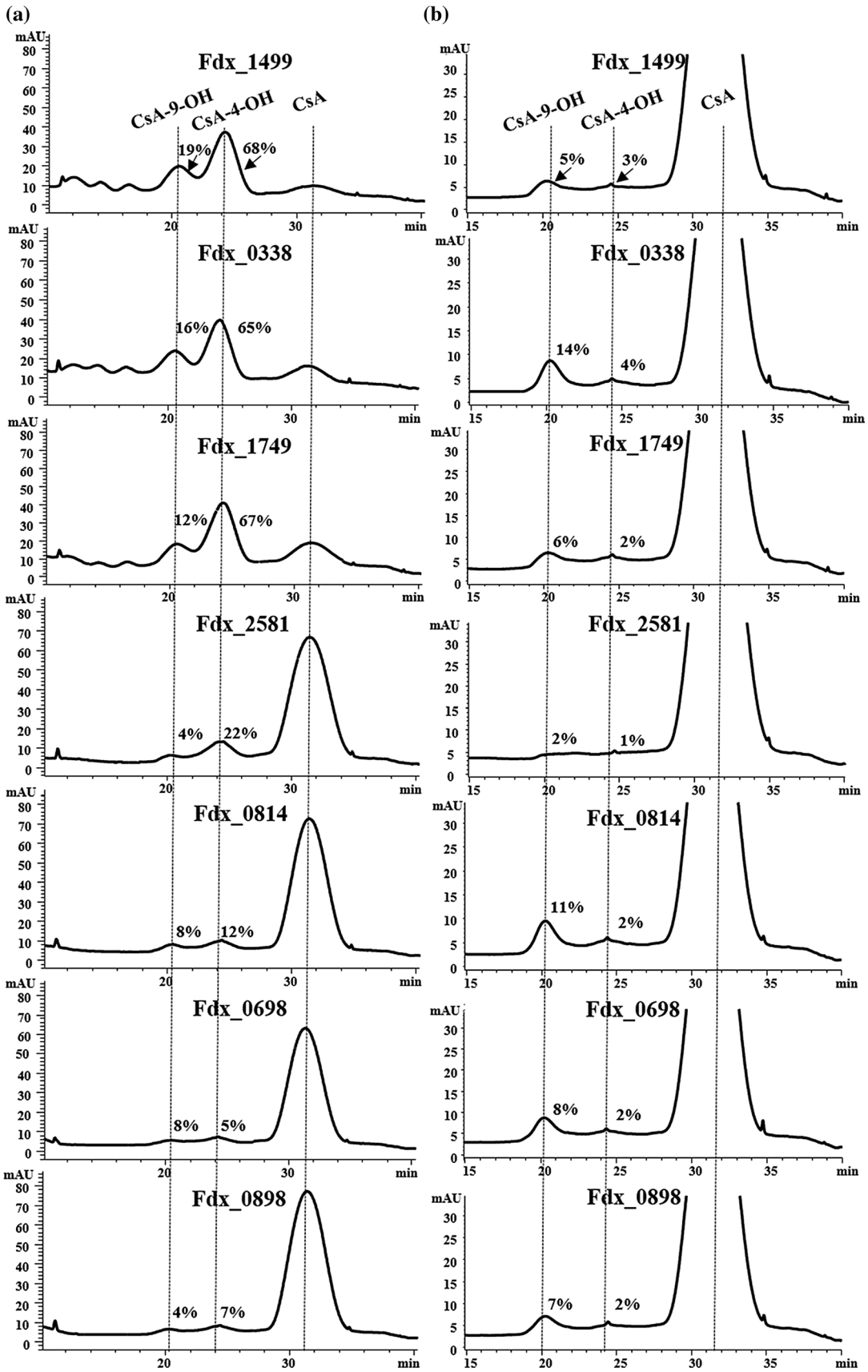
In our previous study [11], the highest conversion of CsA by CYP-sb21 was achieved when using Fdx_1499 and FdR_0978 as redox partners. Since the protein sequence of CYP-pa1 is 55/67% identical/similar to that of CYP-sb21, we speculated that this redox partner system could also support the activity of CYP-pa1. As expected, CsA was converted into CsA-9-OH (5%) and CsA-4-OH (3%) by CYP-pa1/Fdx_1499/FdR_0978 in the presence of NADPH and the GDH/glucose based NADPH regeneration system, albeit in very low yield (Fig. 3). The identity of compounds was confirmed by comparison of their retention times with those of authentic standards and high resolution mass spectrometry (HRMS, Fig. 4). The in vitro hydroxylation site preference of CYP-pa1 is consistent with the previous in vivo results [2], confirming that this P450 enzyme is primarily a CsA-9-OH synthase. The low conversion ratio could be attributed to the high Fdx selectivity of CYP-pa1. Consistently, when CYP-pa1 was heterologously expressed in *Streptomyces coelicolor*, the conversion of CsA dramatically decreased relative to the same conversion in the native host *P. autotrophica*, which likely resulted from the lack of suitable redox partners [2]. The CsA hydroxylation catalyzed by CYP-sb21/Fdx_1499/FdR_0978 was analyzed as a control. Both CsA-4-OH (major product, 68% conversion) and CsA-9-OH (minor product, 19% conversion) were produced from CsA, which is consistent with the previous result [16].

The supporting activity of different cyanobacterial redox partners to CYP-pa1 and CYP-sb21

The low supporting activity of Fdx_1499/FdR_0978 to CYP-pa1 motivated the search for better redox partner proteins. Through genome mining, we found at least seven Fdx encoding genes (*fdx_0338*, *fdx_0698*, *fdx_0814*, *fdx_0898*, *fdx_1499*, *fdx_1749*, and *fdx_2581*) from the genome of *S. elongatus* PCC7942 (ASM1252v1). Upon expression and purification of the seven Fdxs and the single FdR_0978, we comparatively investigated the supporting activity of each Fdx (paired with FdR_0978), to CYP-pa1 and CYP-sb21. As shown in Fig. 3, all tested redox systems were capable of supporting the CsA hydroxylating activity of CYP-pa1 and CYP-sb21. However, no Fdxs supported the hydroxylating activity of CYP-pa1 well since the CsA conversion ratios only ranged from 3 to 18%. The optimal redox system for CYP-sb21 and CYP-pa1 turned out to be Fdx_1499/FdR_0978 (87% conversion) and Fdx_0338/FdR_0978 (18% conversion), respectively. For CYP-sb21, Fdx_0338 and Fdx_1749 also showed suboptimal activity. Taken together, the Fdx selectivity of CYP-pa1 appeared to be higher than that of CYP-sb21. These findings suggest that it could be more challenging to use heterologous redox partner proteins to engineer a CYP-pa1 based CsA-9-OH production system than we did for the CYP-sb21 based CsA-4-OH production system [11]. It is important to identify the native redox partner proteins from *P. autotrophica* to obtain the optimal activity of CYP-pa1, which is currently being investigated in our laboratories. Moreover, in all cases, CYP-pa1 and CYP-sb21 preferred hydroxylation at the ninth and the fourth *N*-methyl leucine, respectively, which indicates that the different choice of Fdx did not reverse the regioselectivity of these two CsA hydroxylases.

Fig. 3 HPLC analysis of CsA hydroxylation by CYP-sb21 (**a**) and CYP-pa1 (**b**) using different types of cyanobacterial Fdx paired with FdR_0978 as redox partners

With respect to application potential, CsA-4-OH is a better hair growth promoter since it has a similar hair-stimulating effect; however, it has ten times less immunosuppressive activity than CsA-9-OH [16]. We previously conducted domain swapping between CYP-sb21 and CYP-pa1, and found that the second domain (135–228 amino acid) could affect the regioselectivity, and that swapping of this domain could increase the ratio of CsA-4-OH/CsA-9-OH


(a)
(b)
Fdx_1499
CsA-9-OH
CsA-4-OH
CsA
19%
68%
5%
3%
Fdx_0338
16%
65%
14%
4%
Fdx_1749
12%
67%
6%
2%
Fdx_2581
4%
22%
2%
1%
Fdx_0814
8%
12%
11%
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Fdx_0898
4%
7%
7%
2%
mAU
min

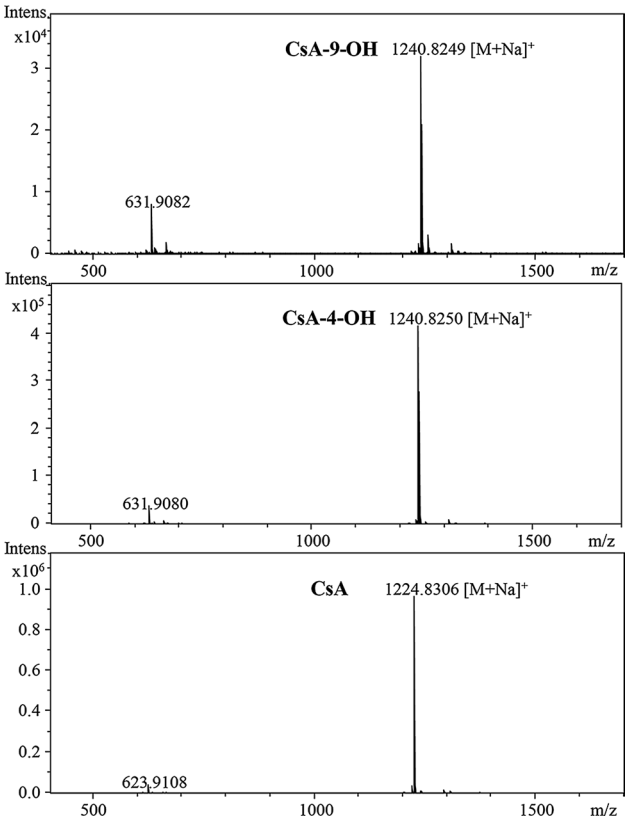

Fig. 4 High-resolution mass spectrometry (HRMS) of CsA-9-OH ($[M + Na]^+$: *obs.* 1240.8249, *calc.* 1240.8255 for $C_{62}H_{111}N_{11}O_{13}Na^+$), CsA-4-OH ($[M + Na]^+$: *obs.* 1240.8250, *calc.* 1240.8255), and CsA ($[M + Na]^+$: *obs.* 1224.8306, *calc.* 1224.8311 for $C_{62}H_{111}N_{11}O_{12}Na^+$)

[16]. The results of this and previous studies [11] indicate that the selection of Fdx could also change product distribution and total conversion. Taken together, these results have laid the foundation for engineering a strict P450 CsA-4-OH synthase for future industrial applications.

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