Characterization of the P450 Monooxygenase LobP1 as C-32 Hydroxylase in Lobophorin Biosynthesis

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Abstract

Lobophorins (LOBs) belong to a large family of spirotetronate antibiotics with antibacterial and antitumor activities. In this study, we demonstrated the function of LobP1, a P450 monooxygenase encoded in the LOB biosynthetic gene cluster, by in vivo deletion and in vitro biochemical assays. The disruption of lobP1 led to the isolation of three new LOBs derivatives (3-5) and three known ones (6-8) without the hydroxyl group at C-32. LobP1 was shown to have relatively broad substrate scope. Determing the kinetic parameters of LobP1 towards different substrates revealed that LobP1 preferred substrate with a nitrosugar. The major product LOB E (6) from the [?]lobP1 mutant displayed better cytotoxic activities against several cancer cell lines than LOB B, the C-32 hydroxlated counterpart.

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Comprehensive Summary

Lobophorins (LOBs) belong to a large family of spirotetronate antibiotics with antibacterial and antitumor activities. In th

Keywords
Lobophorins | Biosynthesis | Post modifications | P450 monooxygenase | Hydroxylation

Background and Originality Content

Lobophorins (LOBs) belong to a large group of spirotetronate antibiotics, which possess a pentacyclic aglycon embedded in the macrocyclic core scaffold and feature a tetronate moiety *spiro* -linked with a cyclohexene ring, displaying potential antibacterial and antitumor activities.[1] Different modifications, such as glycosylation, methylation and hydroxylation on the core scaffold endow LOBs with significant structural and bioactive diversity. To date, more than 30 LOBs have been reported.[2-16]

The structural complexity and pharmaceutical potency inspired a number of biosynthetic studies on LOBs and related spirotetronates to elucidate the formation of the pentacyclic aglycon[17-23] and the tailoring modification reactions,[8, 9, 13] such as the stepwise glycosylations and the sugar-*O* -methylation (Figure 1). LobG1 was characterized as the *O* -glycosyltransferase to attach a D-kijanose at C-17. LobG3 was demonstrated as a flexible glycosyltransferase that could not only attach the first two L-digitoxoses at C-9, but also add the D-kijanose or its variants at C-9. LobG2 was verified to attach the terminal digitoxose,[9, 13] while LobS1 was responsible for installing the methyl group at the terminal digitoxose[8] (Figure 1). However, the enzyme responsible for the C-32 hydroxylation has not been elucidated. Bioinformatic analysis shows that the P450 monooxygenase LobP1 is the most likely candidate responsible for catalyzing this reaction.

Figure 1 The structures of LOBs A (**1**) and B (**2**) and enzymes responsible for the glycosylation, methylation and possible hydroxylation in the post modifications.

Herein, we report (i) the isolation, structure elucidation and bioactivity evaluation of LOB derivatives from the *lobP1* -inactivated mutant; (ii) the functional characterization of LobP1 as the C-32 hydroxylase by both *in vivo* genetic experiments and *in vitro* biochemical assays; (iii) the investigation of the substrate scope and kinetic parameters of LobP1 towards different substrates. This study characterizes the catalytic features of LobP1 and provides important implications for the reaction timing of tailoring steps in LOB biosynthesis.

Results and Discussion

Genetic characterization of *lobP1*

Bioinformatics analysis shows that *lobP1* encodes a cytochrome P450 monooxygenase, sharing 70% identity with KijA3, an enzyme proposed to be responsible for the C-32 hydroxylation of kijanimicin.[18] Therefore, LobP1 was proposed to be the most likely candidate catalyzing the C-32 hydroxylation in LOB biosynthesis. To verify this hypothesis, *lobP1* was inactivated by insertional mutation in *Streptomyces* sp. SCSIO 01127 (Figure S1). The resulting [?]*lobP1* mutant abolished the production of LOBs A and B (**1** and **2**), but accumulated several LOB-related products (Figure 2). Subsequently, three new derivatives LOBs N1-N3 (**3** -**5**) were isolated from the [?]*lobP1* mutant (Figure 2, Figures S2-S4 and Table S3), together with three known ones LOBs E (**6**),[4] N (**7**)[15] and CR4 (**8**)[9, 13, 16] determined by comparing their NMR and HRESIMS data with those previously reported, respectively (Figure 2, Figures S5-S7).

LOB N1 (**3**) ($C_{60}H_{88}N_2O_{20}$,*m /z* 1155.5854 [M - H]$^-$, calcd 1155.5857; Figure S2) was highly similar to **6** .[4] The only difference was that the oxymethyl (δ_C/δ_H 57.4/3.41, CH_3-7_C) in **6** was absent in **3** , accordingly, the carbon signal of C-4_C was shielded from δ_C 88.2 (in $CDCl_3$) in **6** to δ_C 69.0 (in DMSO-d_6) in **3** . Thus, **3** was determined to be desmethyl derivate of **6** . The molecular formula of LOB N2 (**4**) was established to be $C_{61}H_{91}NO_{19}$(*m /z* 1140.6117 [M - H]$^-$, calcd 1140.6112, Figure S3) by HRESIMS. Inspection of the ^{1}H, ^{13}C, and 2D NMR data of **4** (Table S3) revealed that **4** only differed from **7** in the sugar D moiety: a 3-hydroxyl-D-kijanose (δ_C 70.7, C-3_D) in **4** , and a 3-amino-D-kijanose in **7** (δ_C 54.2, C-3_D). Therefore, **4** was characterized as 3-hydroxyl-D-kijanose derivative of **7** . The molecular formula of LOB N3 (**5**) was assigned as $C_{63}H_{94}N_2O_{20}$(*m /z* 1197.6325 [M - H]$^-$, calcd 1197.6327, Figure S4) by HRESIMS. The NMR data of **5** were highly similar to those of **4** except for the signals of an additional carbonyl (δ_C 172.6, C-10_D) and methyl (δ_C/δ_H 26.3/2.00, CH_3-11_D) groups existed in **5** . The methyl and carbonyl were found to belong to a *N* -methylcarbamoyl group by the COSY correlation between H_3-11_D/10_D-NH (δ_H 9.57) and

HMBC correlation from H_3-11_D to C-10_D, which was linked to 3_D-O by shielded C-3_D in**5** comparing to that in **4** and the NOESY correlation between H_3-11_D/H_3-7_D.

Compounds **3** -**8** were all confirmed as LOBs lacking the hydroxyl group at C-32. Besides, introduction of the BAC pCSG5661 (containing *lob* BGC with inactivated *lobP1* ; Figure S8) into *S. coelicolor* M1154 also abolished the production of the C32-hydroxylated LOBs **16** -**18** ,[13]but led to the non-hydroxylated LOBs (such as **10** , **12** ,**13** , and **15**) (Figure 2). These *in vivo* results confirmed LobP1 as the requisite C-32 hydroxylase.

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Figure 2 Metabolite analysis of [?]*lobP1* mutants and structures of LOB analogues. HPLC analysis of metabolite profiles of (i) SCSIO 01127, (ii) SCSIO 01127/[?]*lobP1* , (iii) M1154/pCSG5560 (containing *lob* BGC), (iv) M1154/pCSG5661 (containing *lob*BGC with disruption of *lobP1*).

In vitro **biochemical characterization of LobP1**

For *in vitro* biochemical characterization, LobP1 was produced as soluble His_6-tagged proteins in *E. coli* (Figure S9). The purified LobP1 was shown to convert **6** , the major product from the [?]*lobP1* mutant of *Streptomyces* sp. SCSIO 01127 (Figure 2), to the C-32 hydroxylated product **2** in the presence of ferrodoxin (Fdx) and ferrodoxin reductase (FdR) from cyanobacterium *Synechococcus elongatus*PCC7942,[24] in contrast, **6** remained unchanged in the absence of LobP1 (Figure 3, Figure S10). Subsequently, another five intermediates **3** -**5** , **7** -**8**from the [?]*lobP1* mutant of *Streptomyces* sp. SCSIO 01127 were assayed with LobP1. Interestingly, LOBs **3** -**5** and**7** could be converted by LobP1 to their putative hydroxylated products LOBs CR5 (**19**), CR1 (**20**),[7] CR6 (**21**) and A (**1**) (Figure 3), respectively, upon comparison with the standard or by LC-ESI-HRMS analyses (Figure S10-S11). However, no reaction of LobP1 with **8** was detected (Figure S12). Cumulatively, these*in vitro* biochemical assays further confirmed LobP1 as the LOB C-32 hydroxylase.

To probe the substrate scope of LobP1, 13 more LOBs,**9** -**15** (Figure 2) and **22** -**27** (Figure 3) were also assayed with LobP1. LOBs F (**22**) and G2-1 (**23**), previously isolated from the mutant [?]*lobG2* /*Streptomyces* sp. SCSIO 01127,[9] could be converted by LobP1 to putative hydroxylated products LOBs L2 (**28**) and L (**29**)[14] (Figure 3, Figure S10), respectively, by analyzing the LC-ESI-HRMS (Figure S11). However, LOBs**9** -**15** and **24** -**27** , previously isolated from *S. coelicolor* M1154/pCSG5560 (carrying the *lob* BGC) and *S. coelicolor* M1154/pCSG5561 (carrying the *lob* BGC with [?]*lobG1*),[13] could not react with LobP1 (Figure S12). Conclusively, LobP1 recognizes LOBs containing a sugar at C-17 and a tri- or di-saccharide chain at C-9 (**3** -**7** , **22** and **23**), while does not recognize LOBs with no sugar at C-17 (**8** and**24** -**27**), or LOBs with a monosaccharide at C-9 (**11** -**15**). Apparently, LobP1 displays relatively higher catalytic efficiency towards substrates with a trisaccharide at C-9 (*e.g.* **3** -**7**) than those with a disaccharide at C-9 (*e.g.* **22** and **23**) (Figure 3), indicating that the C-32 hydroxylation by LobP1 most likely occurs after the terminal digitoxosylation by LobG2 (Figure 1). Intriguingly, biochemical characterization showed that LobP1 failed to recognize LOBs with a monosaccharide at C-9, such as **11** , **13** and **14** , which was inconsistent with the facts that their corresponding C-32 hydroxylated counterparts **18** , **16** and **17** were quantitatively produced in *S. coelicolor* M1154/pCSG5560 (carrying the *lob* BGC) (Figure 2). This apparent *in vivo* and*in vitro* functional discrepancy of LobP1 led to the assumption that LobP1 might require native redox partners from *S. coelicolor*M1154 to perform *in vivo* C-32 hydroxylation on **11** ,**13** and **14** , since different redox partners may exert distinct effects on P450 enzyme reactions.[25]

We have previously shown that the methyltransferase LobS1 catalyzed the installation of the 7c-*O* -methyl group at the terminal L-digitoxose moiety of LOBs.[8] It was unclear about the reaction timing of LobS1 and LobP1 in the LOB biosynthetic pathway. For a better understanding, we compared kinetic parameters of LobP1 toward different substrates (Table 1 and Figure S13). It was shown that LobP1 displayed 100

times higher affinity (K_m) toward **6** than **7** , with thek_{cat}/K_m value toward**6** 30 times greater than **7** , suggesting a preference of LobP1 to LOBs with a nitrosugar over an amino sugar at C-17. LobP1 displayed a slightly higherk_{cat}/K_m value of**3** (7_C-OMe) than **6**(7_C-OH), indicating that 7_C-*O* -methylation by LobS1 might occur after the LobP1-catalyzed C-32 hydroxylation.

Table 1 Kinetic parameters of LobP1 toward different substrates

Substrate	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} μM^{-1})
3	1.74 ± 0.18	116.60 ± 1.90	67.20
6	1.15 ± 0.17	46.35 ± 1.23	40.30
7	131.20 ± 12.73	174.04 ± 7.46	1.33

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Figure 3 HPLC analysis of biochemical assays of LobP1 and the scheme for LobP1-catalyzed hydroxylation. A typical assay was performed in PBS buffer (50 mM, pH 7.6) at 30 for 1 h, containing the substrate 400 μ M, LobP1 1.8 μ M, Fdx/FdR 5 μ M each, NADPH 2 mM. Representative nonsubstrates (boxed) of LobP1 were also shown. Notably, although LobP1 could not recognize **11** , **13** , and **14** as substrates for C-32 hydroxylation, *in vivo*production of their hydroxylated counterparts **18** , **16** , and **17** , respectively, was observed (see Figure 2).

Antibacterial and cytotoxic activity evaluation of LOBs

LOBs obtained in this study were also evaluated for antibacterial activities against *Straphylococcus aureus* ATCC 29213, methicillin-resistant *Staphylococcus aureus* (MRSA) shhs-A1,*Bacillus subtilis* 1064 and *Micrococcus luteus* SCSIO ML01, and the results were summarized in Table 2. All the tested LOBs displayed significant activities against *B. subtilis* 1064 and*M. luteus* SCSIO ML01 with MIC values ranging from 0.125 to 8μ g mL^{-1}. Compounds **1** -**8** showed no activity or weak activities against *S. aureus* ATCC 29213 and MRSA, while compound **6** exhibited antibacterial activities against these two strains with MIC values of 16 μ g mL^{-1}. And compound **7** showed better antibacterial activities than its corresponding hydroxylated product**1** . In addition, Compounds **1** -**3** and**5** -**7** were tested for the cytotoxic activities against four human tumor cell lines: SF-268, MCF-7, HeG-2 and A549 (Table 2). Notably, compound **6** displayed overall better cytotoxic activities than other tested compounds, including its hydroxylated product **2** , with IC_{50} values of 11.94, 12.07, 3.11 and 4.34 μ M against SF-268, MCF-7, HeG-2 and A549, respectively.

Table 2 Antibacterial and cytotoxic activities of compounds**1** -**8**

	MIC ($\mu g \cdot mL^{-1}$)	MIC ($\mu g \cdot mL^{-1}$)	MIC ($\mu g \cdot mL^{-1}$)	MIC ($\mu g \cdot mL^{-1}$)	IC_{50} /μM	IC_{50} /μM	IC_{50} /μM	IC /μ
	B. Subtilis 1064	*S. Aureus* ATCC29213	MRSA shhs-A1	*M. Luteus* SCSIO ML01	SF-268	MCF-7	HepG-2	A5
1	8	>64	>64	0.5	>100	>100	>100	>1
2	0.25	>64	>64	0.125	44.56 ± 0.42	69.99 ± 2.03	28.16 ± 1.19	66. ±
3	0.5	32	64	0.125	17.16 ± 0.54	27.91 ± 1.21	21.21±1.75	23. ±
4	1	>64	>64	0.125	-	-	-	-
5	0.5	>64	>64	0.125	31.11 ± 4.46	55.75 ± 2.66	26.30 ± 3.87	25. ±

	MIC (μg·mL^{-1})	MIC (μg·mL^{-1})	MIC (μg·mL^{-1})	MIC (μg·mL^{-1})	IC_{50} /μM	IC_{50} /μM	IC_{50} /μM	IC /μ
6	0.25	16	16	0.125	11.94 ± 0.33	12.07 ± 0.13	3.11 ± 0.63	4.3 0.2
7	1	>64	>64	0.125	51.27 ± 0.76	72.77 ± 3.59	43.19±3.13	43 ±
8	8	64	>64	1	-	-	-	-
Vancomycin	0.125	0.5	1	0.125	-	-	-	-
TMP	0.125	2	>64	4	-	-	-	-
Ampicillin	0.125	0.125	64	0.125	-	-	-	-
Doxorubicin	-	-	-	-	1.94 ± 0.01	2.00 ± 0.04	2.16 ± 0.05	2.1 0.0

Conclusions

In summary, the P450 monooxygenase LobP1 was functionally characterized as the C-32 hydroxylase in the biosynthesis of LOBs by *in vivo*genetic disruption and *in vitro* biochemical assays. Inactivation of *lobP1* afforded three new LOBs (**3** -**5**) and three known LOBs (**6** -**8**). Notably, the major derivative**6** from the [?]*lobP1* mutant showed moderate cytotoxic activity, better than its hydroxylated derivative **2** . Investigations on the substrate scope reveal that LobP1 could recognize LOBs with di- or tri- saccharide chain at C-9 and a sugar at C-17 (**3** -**7** , **22** , **23**), but could not recognize LOBs with a monosaccharide at C-9 (such as **11** ,**13** , and **14**) in *in vitro* biochemical assays. The*in vivo* hydroxylation of compounds **16** -**18** may be achieved by LobP1 with the help of native redox partner. Further studies are warranted to explain their generation. The kinetic parameters of LobP1 toward different substrates indicated that the C-32 hydroxylation may occur before sugar-*O* -methylation. Up to now, the enzymes responsible for tailoring modifications in LOBs A and B, including sugar-*O* -methylation (LobS1) and C-32 hydroxylation (LobP1) have been biochemically characterized.

Experimental

Strains and culture conditions

All strains and plasmids used and constructed in this study are listed in Table S1. *Streptomyces* sp. SCSIO 01127[9] was cultured at 28 oC in 38# medium (yeast extract 4 g L^{-1}, glucose 4 g L^{-1}, maltose 5 g L^{-1}multivitamins 500 µL L^{-1}, sea salt 30 g L^{-1}, agar powder 15-20 g L^{-1}, pH 7.0 -7.5), and the heterologous recombinant host *Streptomyces coelicolor* M1154/pCSG5560[13] and the derivatives were cultured at 28 ºC in SFM medium (soybean powder 20 g L^{-1}, mannitol 20 g L^{-1}, agar powder 15-20 g L^{-1}) for growth and sporulation.*Escherichia coli* strains were grown in Luria-Bertani medium at 37 °C.

DNA isolation, manipulation and sequencing

DNA isolation and manipulation in *E. coli* and actinomycetes were carried out according to standard procedures.[26, 27] Primers used in this study (Table S2) were synthesized at the Shanghai Invitrogen Biotech Co., Ltd. DNA sequencing was performed at the Invitrogen Biotech Co., Ltd. (Guangzhou), and Chinese National Genome Center (Shanghai).

Disruption of *lobP1*

The lambda-RED-mediated gene replacement was performed as a standard procedure.[28] The gene disruption experiments in*Streptomyces* sp. SCSIO 01127 were carried out using the previously reported genetic manipulation system.[9] Details for *lobP1* -gene disruption in native strain *Streptomyces* sp. SCSIO 01127 were described in Figure S1. The disruption of *lobP1* in heterologous host *S. coelicolor* M1154 was conducted using the previously reported genetic manipulation system.[13] Details for the construction of [?]*lobP1* mutant *S. coelicolor*M1154/pCSG5661 were described in Figure S8.

Fermentation and metabolite analysis

The fermentation of all strains in this study were carried out using N1 medium (soybean powder 3 g L^{-1}, yeast extract 3 g L^{-1}, trehalose 1 g L^{-1}, L-proline 1 g L^{-1}, beef extract 3 g L^{-1}, glycerol 6 g L^{-1}, K_2HPO_4 0.3 g L^{-1}, $MgSO_4$*$7H_2O$ 0.5 g L^{-1}, $FeSO_4$*$7H_2O$ 0.5 g L^{-1}, $CaCO_3$ 2 g L^{-1}, sea salt 30 g L^{-1}, pH 7.2-7.4) in 250 mL flask by shaking at 200 rpm and 28 oC for 5-7 days. 5 mL of fermentation broth was extracted with 5 mL butanone, and the extract was then dried under vacuum. The residue was dissolved into 70 μ L of CH_3OH and subjected to HPLC analysis using a reversed-phase (Phenomenex Kinetex C18, 250 × 4.6 mm, 5 μ m) with UV detection at 265 nm on Agilent 1360 Infinity series workstation, the program was set as follows: solvent system (solvent A, 10% acetonitrile in water supplementing with 0.1% formic acid; solvent B, 90% acetonitrile in water); 5% B to 100% B (0-20 min), 100% B (20-30 min), 100% B to 5% B (30-32 min), 5% B (32-35 min), flow rate at 1 mL min^{-1}.

Fermentation, Extraction, and isolation

The scaled up fermentation was carried out using spores directly in 250 mL flask. Briefly, the spores of *Streptomyces* sp. SCSIO 01127/[?]*lobP1* was inoculated into a 250 mL flask containing 50 mL of N1 medium and grown at 28 ºC for 6 days at 200 rpm. Subsequently, the fermentation cultures were centrifuged (3900 rpm, 15 min) to yield the supernatant and mycelia cake. A total of 12 L culture was prepared for isolating intermediates from *Streptomyces* sp. SCSIO 01127/[?]*lobP1* . The metabolites in supernatants were absorbed by XAD-16 resins, and the collected resins were eluted with 6 L acetone. The mycelia cake was extracted four times with 4 L acetone. After evaporation of organic solvents under vacuum, the residues were combined and re-extracted each time with 1 L butanone for eight rounds. After drying under vacuum, the extract (8 g) was then subjected to normal phase silica gel column (100-200 mesh) and eluted with a gradient of $CHCl_3$/CH_3OH (1:0, 4:1, 2:1, 0:1, v/v, 600 mL) to yield 4 fractions (Fr.1 to Fr.4). Fr.2 was further separated by Sephadex LH-20, eluted with $CHCl_3$/CH_3OH (1:1, v/v) to obtain 4 sub-fractions (Fr.2.1 to Fr.2.4). Fr.2.2 was purified by MPLC with ODS column, eluted with a linear gradient under the following program: solvent system (solvent A, water supplementing with 0.1% formic acid; solvent B, acetonitrile); 0% B to 60 % B (0-40 min), 60% B to 85% B (40-80 min), 85% B to 100% B (80-100 min), 100% B (100-120 min), flow rate at 20 mL min^{-1} to yield 9 sub-fractions (Fr. 2.2.1 to Fr. 2.2.9). Fr. 2.2.2 was purified by semi-preparative HPLC to yield **3** (5 mg), **4** (4.5 mg), **5** (7.6 mg),**7** (8.1 mg), **8** (11.6 mg); Fr. 2.2.8 was purified by semi-preparative HPLC to afford **6** (305 mg).

Lobophorin N1 (**3**): white powder; [a]25 D-79.50 (c 0.08, CH_3OH); UV (CH_3OH)λ_{max} (log ϵ) 205 nm (4.36), 237 nm (3.83), 265 nm (3.72); ^{1}H NMR (700 MHz, DMSO-d_6) and ^{13}C NMR (175 MHz, DMSO-d_6) data: see Table S3; IR (film)ν_{max} 3360, 2922, 1730, 1631, 1543, 1060, 1012, 866 cm^{-1}; HRESIMS m/z 1155.5854 [M - H]$^-$ (calcd for $C_{60}H_{88}N_2O_{20}$, 1155.5857).

Lobophorin N2 (**4**): white powder; [a]25 D-87.25 (c 0.28, CH_3OH); UV (CH_3OH)λ_{max} (log ϵ) 205 nm (4.31), 235 nm (3.87), 266 nm (3.76); ^{1}H NMR (700 MHz, DMSO-d_6) and ^{13}C NMR (175 MHz, DMSO-d_6) data: see Table S3; IR (film)ν_{max} 3450, 2931, 1732, 1635, 1541, 1058, 995, 866 cm^{-1}; HRESIMS m/z 1140.6117 [M - H]$^-$ (calcd for $C_{61}H_{91}NO_{19}$, 1140.6112).

Lobophorin N3 (**5**): white powder; [a]25 D-88.48 (c 0.13, CH_3OH); UV (CH_3OH)λ_{max} (log ϵ) 205 nm (4.37), 235 nm (3.83), 265 nm (3.68); ^{1}H NMR (700 MHz, DMSO-d_6) and ^{13}C NMR (175 MHz, DMSO-d_6) data: see Table S3; IR (film)ν_{max} 2924, 2358, 1732, 1633, 1541, 1417, 1060, 1008, 866 cm^{-1}; HRESIMS m/z 1197.6325 [M - H]$^-$ (calcd for $C_{63}H_{94}N_2O_{20}$, 1197.6327).

Overexpression and purification of LobP1, Fdx and FdR

The *lobP1* gene was PCR amplified from the genomic DNA of*Streptomyces* sp. SCSIO 01127 using primers LobP1EF/ER (Table S2). PCR products were assembled with the linearized pET28a (digested by*Nde* I/*Bam* HI) using Single One Step Clone Kit (Vazyme Biotech) to afford pCSG5662 (Table S1) after sequencing confirmation. Expression of *lobP1* were carried out in *E. coli* Rosetta (DE3)/pCSG5662 following standard procedures. The purification of $(His)_6$-tagged LobP1 was performed by Ni^{2+}-NTA affinity chromatography

(GE Healthcare) with standard protocols. The purified and desalted LobP1 proteins were stored in storage buffer (50 mM HEPES buffer, 100 mM NaCl, 1 mM DTT, 10% glycerol, pH 7.6) at -80 °C until use. To obtain redox partners ferrodoxin (Fdx) and ferrodoxin reductase (FdR) from cyanobacterium*Synechococcus elongatus* PCC7942,[24] the two expression plasmids pET28b-fdx_1499 and pET28b-fdR_0978 (Table S1) were transformed into *E. coli* BL21 (DE3), respectively. The expression and purification of Fdx and FdR were carried out in the same way as that of LobP1.

Enzyme assays of LobP1

To assay the enzymatic activities of LobP1 towards various substrates, each 100 μ L of reaction mixtures were prepared to contain the corresponding LOB substrate (400 μ M), LobP1 (1.8 μ M), Fdx/FdR (each 5 μ M) and NADPH (2 mM), in PBS buffer (50 mM, pH 7.6). Reactions were performed at 30 °C for 1 h. The reactions were quenched by adding 100 μ L methanol. The enzyme assays were monitored via HPLC analysis under the condition described above. To determine the kinetic parameters of LobP1 toward LOB N1 (**3**), the enzyme assay contains LobP1 (1.25 nM), saturating Fdx/FdR (each 5μ M), NADPH (1 mM) and **3** (2.5, 5, 10, 20, 50, 75, 100μ M) in PBS buffer (50 mM, pH 7.6); for the substrate LOB E (**6**), the enzyme assay contains LobP1 (2.5 nM), saturating Fdx/FdR (5 μ M each), NADPH (1 mM) and **6** (1, 2.5, 5, 10, 20, 50, 100 μ M), in PBS buffer (50 mM, pH 7.6); for the substrate LOB N (**7**), the enzyme assay contains LobP1 (12.5 nM), saturating Fdx/FdR (each 5 μ M), NADPH (1 mM) and **7** (5, 10, 20, 40, 50, 100, 150, 200, 400 μ M), in PBS buffer (50 mM, pH 7.6);. Each reaction mixture (100 μ L) was performed in triplicates and was incubated at 30 °C for 5 min, quenched by adding 100μ L methanol. Kinetic parameters were calculated by nonlinear regression analysis using GraphPad Prism 6 software.

Antibacterial assays

Antimicrobial activities were measured against four indicator strains,*Bacillus subtilis* 1064, *Micrococcus luteus* SCSIO ML01,*Staphylococcus aureus* ATCC 29213 and MRSA shhs-A1, by the broth microdilution method. The methods for the culturing, diluting, sampling and recording were the same as previously described.[13]

Antitumor assays

The *in vitro* cytotoxic activities of LOBs were evaluated against four tumor cell lines, SF-268 (human glioma cell line), HepG2 (human liver carcinoma cell line), MCF-7 (human breast adenocarcinoma cell line) and A549 (human lung adenocarcinoma cell) by the SRB method.[29] The assays were performed as the same way previously described.[13]

Supporting Information

The supporting information for this article is available on the WWW under https://doi.org/10.1002/cjoc.2021xxxxx.

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The P450 monooxygenase LobP1 was characterized as a promiscuous hydroxylase catalyzing the C-32 hydroxylation in lobo