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Functions of Genes and Enzymes Involved in Phenalinolactone Biosynthesis

Martina Daum,[a] Hans-Jörg Schnell,[a] Simone Herrmann,[a] Andreas Günther,[a] Renato Murillo,[b] Rolf Müller,[c] Philippe Bisel,[d] Michael Müller,[d] and Andreas Bechthold*[a]

Phenalinolactones are novel terpene glycoside antibiotics produced by *Streptomyces* sp. Tü6071. Inactivation of three oxygenase genes (plaO2, plaO3 and plaO5), two dehydrogenase genes (plaU, plaZ) and one putative acetyltransferase gene (plaV) led to the production of novel phenalinolactone derivatives (PL HS6, PL HS7, PL HS2 and PL X1). Furthermore, the exact biosynthetic functions of two enzymes were determined, and their in vitro activities were demonstrated. PlaO1, an Fe^{II}/α-ketoglutarate-dependent dioxygenase, is responsible for the key step in γ-butyrolactone formation, whereas PlaO5, a cytochrome P450-dependent monooxygenase, catalyses the 1-C-hydroxylation of phenalinolactone D. In addition, stable isotope feeding experiments with biosynthetic precursors shed light on the origin of the carbons in the γ-butyrolactone moiety.

Introduction

Terpenoid natural products with antibiotic properties isolated from bacteria are extremely rare, although the natural pool of terpenes offers great chemical diversity.[1,2] Among prokaryotes, actinomycetes strains are known to produce a relatively large number of isoprenoid compounds.[3,4] Novobiocin was isolated from *Streptomyces niveus*[5] as the first antibiotic with a terpenoid side chain, and was followed by the pentalenolactone from a *Streptomyces* sp., exhibiting a pure terpenoid structure.[6] Only a few basic structures have been added to this limited pool, most of them prenylated scaffolds of different biosynthetic origin rather than pure terpenoid compounds.[1] Phenalinolactones (PLs, Scheme 1) are antibiotics with a terpenoid scaffold, which is richly functionalized and decorated by multiple modifying enzymatic steps. The main metabolites isolated from fermentation extracts of *Streptomyces* sp. Tü6071 are the phenalinolactones (PLs) PL A to PL D (Scheme 1), whereas minor compounds are PL D2 and PL A2 (see the Supporting Information).

Gebhardt and Fiedler isolated the strain in 2001 and demonstrated antibacterial activity of the PLs against a selection of Gram-positive pathogens.[7] Meyer and Zeeck elucidated the structures of the PLs in 2003, revealing that they constitute a unique assembly of novel structural elements and biosynthetic interest.[8] In 2006, Dürr and co-workers reported the cloning and sequencing of the PL biosynthetic gene cluster from *Streptomyces* sp. Tü6071.[9] It was shown by feeding D-[1-^{13}C]glucose that the isopentenyl diphosphate (IPP) units in the terpene scaffold of the PLs are synthesized by the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway,[9–11] as had also previously been shown for brasilicardin A from *Nocardia terpenica*, the closest structural relative of the PLs.[12] Recently the PL biosynthetic gene cluster was reconstituted from two cosmids and heterologously expressed in several *Streptomyces* strains (for example, *Streptomyces lividans*), yielding PL A and PL D in each case. These data indicated that all genes necessary for PL production are localized on these cosmids.[13] We now report further feeding experiments with ^{13}C-labelled precursors and the deletion of additional genes from the gene cluster, in work carried out in order to obtain more information about the biosynthesis of PLs. We also report on the in vitro analysis of PlaO1 and PlaO5.

Results and Discussion

Isotope feeding experiments

The results of the feeding experiments are summarized in Table 1. In contrast to the previously described feeding experiment[9] we focused on ^{13}C incorporation into the γ-butyrolactone, the pyrrole and the sugar moiety.

[a] *Dr. M. Daum,[+] Dr. H.-J. Schnell,[+] S. Herrmann, A. Günther, Prof. Dr. A. Bechthold*
Albert-Ludwigs-Universität, Institut für Pharmazeutische Wissenschaften
Pharmazeutische Biologie und Biotechnologie
Stefan-Meier-Strasse 19, 79104 Freiburg (Germany)
Fax: (+49) 761-203-8383
E-mail: andreas.bechthold@pharmazie.uni-freiburg.de

[b] *Prof. Dr. R. Murillo*
Escuela de Quimica and CIPRONA, Universidad de Costa Rica
San José (Costa Rica)

[c] *Prof. Dr. R. Müller*
Institut für Pharmazeutische Biotechnologie, Universität des Saarlandes
66123 Saarbrücken (Germany)

[d] *P. Bisel, Prof. Dr. M. Müller*
Albert-Ludwigs-Universität, Institut für Pharmazeutische Wissenschaften
Pharmazeutische und Medizinische Chemie
79104 Freiburg (Germany)

[+] *These authors contributed equally to this work.*

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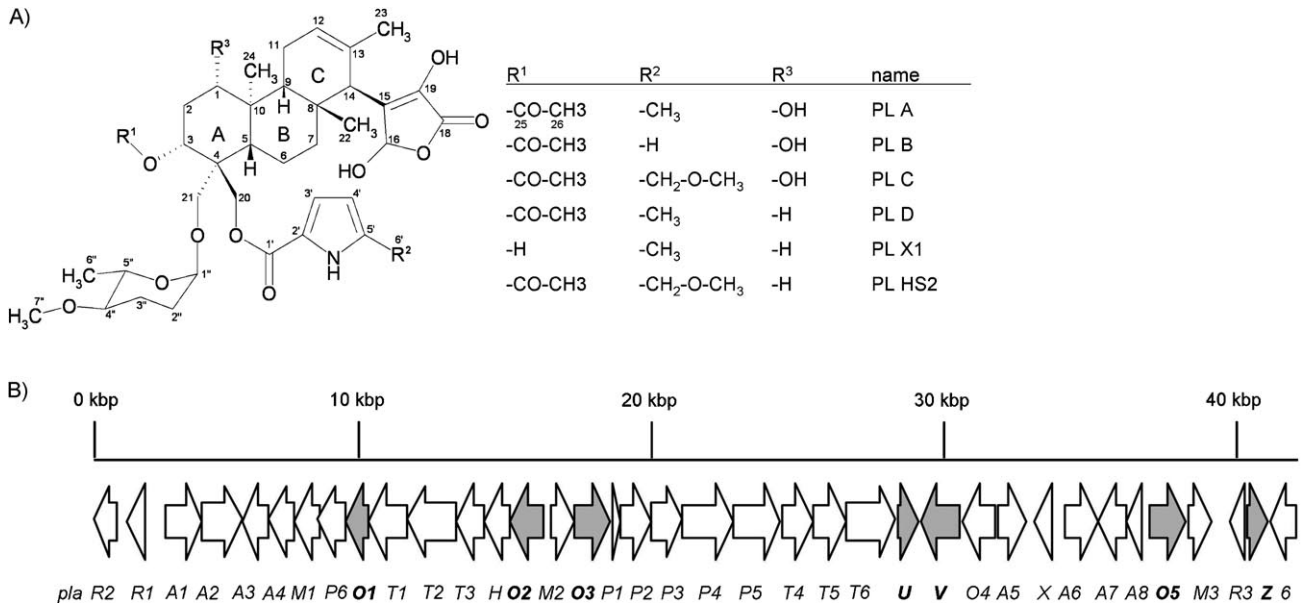


Scheme 1. A) Chemical structures of the main metabolites PL A, PL B, PL C and PL D produced by the wild type of *Streptomyces* sp. Tü6071 and of the major metabolites PL X1 and PL HS2 isolated after fermentation of the *plaV* mutant and the *plaO5* mutant, respectively. B) Scheme of the *pla* biosynthetic gene cluster,[9] with genes that were the subject of this study highlighted in grey.

The ^{13}C NMR analysis of PL D isolated from the [2-^{13}C]pyruvate feeding experiment showed the expected enrichment of carbons C-4, C-8, C-10 and C-13 as the result of the MEP pathway, confirming the previously described ^{13}C incorporation pattern of the terpenoid backbone of the feeding experiment with D-[1-^{13}C]glucose.[9] Additionally C-25 was enriched, due to the metabolism to [1-^{13}C]acetate.

The structure of PL CD6 (Scheme 2), which contains the direct precursor of the γ-butyrolactone moiety (see below), suggested that pyruvate could putatively deliver the required carbon atoms.[9] However, no enrichment of C-19 of the γ-butyrolactone after [2-^{13}C]pyruvate feeding was detectable in this study. This could be explained either by a very low incorporation rate of [2-^{13}C]pyruvate into the γ-butyrolactone or, more probably, by the alternative scenario that another compound and not pyruvate is the precursor of the γ-butyrolactone moiety.

D-[U-$^{13}C_6$]Glucose was also fed to cultures of *Streptomyces* sp. Tü6071. The carbons that would be expected to arise from D-glucose were enriched. In particular, the INADEQUATE spectrum of PL D derived from the D-[U-$^{13}C_6$]glucose feeding experiment showed a cross peak between $\delta_c = 169.1$ and $\delta_c = 138.8$; this indicated that carbons C-18 and C-19 arise intact from D-glucose. Carbon C-16 was also slightly enriched, as was also the case after feeding of D-[1-^{13}C]glucose. The ^{13}C NMR spectrum showed ^3J and ^2J correlations (indicated by satellites around the main peak) between carbons C-16 and C-18 and C-16 and C-19, respectively. The detected couplings between these carbons (C-16, C-18 and C-19) led to the conclusion that all three carbons have their origin in one and the same glucose molecule. Nevertheless the identity of the exact carbon building block remains elusive.

The ^{13}C NMR analysis of PL D isolated after feeding of L-[$^{13}CH_3$]methionine showed that carbons C-6′ and C-7′′ were enriched, indicating the involvement of SAM-dependent methyltransferases in the biosynthesis, putative candidate proteins for catalysis of these transformations are PlaP5 and PlaM1.

The ^{13}C NMR analysis of PL A isolated after feeding of [1-^{13}C]-acetate (not listed in Table 1) showed strong enrichment of carbon C-25, indicating the involvement of an acetyltransferase. A weak enrichment of C-1′ and C-5′ was also detected, which can be explained by incorporation of [1-^{13}C]acetate into L-proline as precursor of the pyrrole moiety.[14,15]

Generation of mutant strains

The biosynthetic gene cluster of the PLs contains five oxygenase genes. The inactivation of two of these genes (*plaO1* and *plaO4*) has already been described.[9] In this manuscript the deletion of *plaO2*, *plaO3* and *plaO5* and the analysis of products generated by these mutants are presented. Furthermore, we generated mutants with deletions in *plaU*, *plaV* and *plaZ*. All mutations were achieved by deletion of an internal DNA fragment from the corresponding gene. Inactivation plasmids were introduced into the wild-type (wt) chromosome by means of homologous recombination. The single- and double-crossover recombination events were documented by PCR analysis and in the cases of *plaO2*, *plaU* and *plaZ* also by Southern hybridization. All mutants complemented *in trans* with a full-length copy of the corresponding gene under the control of the constitutive promotor *ermE**. Production of the main wild-type metabolites was restored in each mutant with different chemical phenotype.

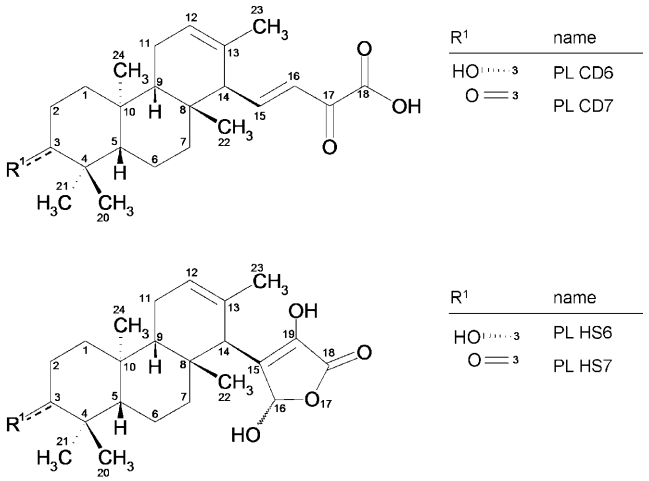
Table 1. ^{13}C incorporation into PL D detected after feeding experiments with listed stable isotopes.

Position PL D	δ_C (ppm)[a]	D-[1-^{13}C] glucose[b]	D-[U-$^{13}C_6$] glucose	L-[$^{13}CH_3$] methionine	[2-^{13}C] pyruvate
1	38.2		3.0[c]		
2	23.4	4.0	[d]		
3	73.9		[d]		
4	45.1		[d]		3.3
5	49.1		[d]		
6	20.7	3.9	[d]		
7	40.5		3.9[c]		
8	39.2		[d]		2.7
9	54.7		[d]		
10	38.4		[d]		2.4
11	23.1	4.8	[d]		
12	122.2		3.7[c]		
13	132.4		[d]		2.8
14	40.2		[d]		
15	129.8	4.5	[d]		
16	98.5	4.6	[d]		
18	169.1		[d]		
19	138.8		[d]		
20	62.6		4.5[c]		
21	68.6	4.7	[d]		
22	27.6	3.7	[d]		
23	22.1	3.9	[d]		
24	14.3	4.2	[d]		
25	170.5		[d]		4.5
26	21.3	4.7	[d]		
1′	160.3				
2′	120.9				
3′	116.6				
4′	109.1				
5′	134.1		[e]		
6′	13.2	2.6	[e]	37.6	
1″	98.1	11.4	[d]		
2″	29.3		[d]		
3″	23.8		[d]		
4″	81.6		[d]		
5″	68.8		[d]		
6″	17.7	3.2	[d]		
7″	56.7	2.6	[e]	39.6	

[a] Chemical shifts were referenced to residual $CHCl_3$. [b] The results of the feeding experiment with D-[1-^{13}C]glucose relating to the diterpene scaffold of PL D have already been published.[8] We have now focused on ^{13}C incorporation into the γ-butyrolactone, the pyrrole and the sugar moiety. [c] Slight satellites around the main peaks due to correlations between enriched carbons were detected and consequently the enrichment of ^{13}C could not be determined exactly. [d] Couplings to neighbouring carbons led to signal splitting; the exact ^{13}C enrichment could not be determined due to this. [e] Slightly heightened signals, possibly due to incorporation of [1,2-^{13}C]acetate derived from glycolysis of D-[U-$^{13}C_6$]glucose in L-methionine and/or L-proline biosynthesis.

Generation of a *plaO2* mutant

The mutant *Streptomyces* sp. Tü6071Δ*plaO2* was generated by inactivation of *plaO2*, which was achieved by deletion of a 300 bp internal fragment. Analysis of the fermentation extract by HPLC/electrospray ionization (ESI)-MS revealed that the production spectrum was identical to that of the wild-type strain with PL A as major metabolite, indicating that PlaO2 is not essential in the biosynthesis of so far known PL derivatives.



Scheme 2. Chemical structures of PL CD6 and PL CD7[8] and of PL HS6 and PL HS7 (this study).

Generation of a *plaO3* mutant and structure elucidation of PL HS6 and PL HS7

The mutant *Streptomyces* sp. Tü6071Δ*plaO3* was generated by inactivation of *plaO3*, which was achieved by deletion of a 264 bp internal fragment. The production profile was analysed by HPLC/ESI-MS at the detection wavelength of 254 nm. Two novel compounds, named PL HS6 and PL HS7, with molecular ions of *m/z* 389 $[M-H]^-$ and *m/z* 387 $[M-H]^-$, respectively, could be detected. The mass difference of 2 amu, matching the mass of two protons, suggested that PL HS6 and PL HS7 constitute a redox pair of a common basic structure, as had also been reported for PL CD6 and PL CD7. For structure elucidation, both derivatives were purified from a scaled-up fermentation and subsequently subjected to high-resolution ESI-MS. The observed molecular ions were *m/z* 391.2481 $[M+H]^+$ for PL HS6 and *m/z* 389.2324 $[M+H]^+$ for PL HS7, indicating molecular formulae of $C_{23}H_{35}O_5$ $[M+H]^+$ (calculated: *m/z* 391.2479) and $C_{23}H_{33}O_5$ $[M+H]^+$ (calculated: *m/z* 389.2422), respectively. Subsequent NMR analysis (Experimental Section) identified PL HS6 as the unmodified diterpenoid core of the PLs extended with the γ-butyrolactone moiety attached to C-15 (Scheme 2). The signals for the methyl groups at C-20, C-21, C-22, C-23 and C-24 could be assigned on the basis of their corresponding ^3J correlations to the core carbon atoms observed in the HMBC spectrum. The characteristic signals of the γ-butyrolactone portion were clearly visible and were compared with the corresponding signals of PL D. The chemical shifts of the signals of the "side chain" carbons in PL HS6 were nearly identical to those of the corresponding signals of the main PL metabolites. The presence of the OH function at C-3 is reflected in the chemical shift of $\delta_H = 3.13$ for H-3, and is consistent with its attachment to a carbon bearing a heteroatom. The axial position of H-3 could be determined from the coupling constant values of 11.5 Hz and 5 Hz, indicating axial-axial and axial-equatorial coupling to H_{ax}-2 and H_{eq}-2, respectively. The 3-OH group is therefore in the equatorial position, like the 3-*O*-acetyl substituent in wild-type PLs such as PL A and PL D.

A further characteristic is the olefinic proton signal of H-12, which appears as an unresolved broad multiplet due to two allylic couplings to H-23 and H-14 and a ^3J coupling to H-11. The initial assumption that PL HS7 is the oxidized variant of PL HS6 was clearly confirmed by ^{1}H and ^{1}H/^{1}H COSY analysis of PL HS7. The absence of the H-3 signal documents the oxidation of the secondary alcohol at C-3 to the keto function. This is further reflected in the downfield shifts of the neighbouring H-2$_{a/b}$ methylene signal from $\delta_H = 1.60$ in PL HS6 towards $\delta_H = 2.45$ in PL HS7 and of the signal of carbon C-3 from $\delta_C = 79.6$ towards $\delta_C = 220.8$, representing a typical value for a carbonyl group.

Generation of a *plaO5* mutant and structure elucidation of PL HS2

The mutant *Streptomyces* sp. Tü6071Δ*plaO5* was generated by inactivation of *plaO5*, which was achieved by deletion of a 363 bp internal fragment. The production profile was analysed by HPLC/ESI-MS at the detection wavelengths of 254 nm and 280 nm. One major compound with a retention time of 24.4 min and a molecular ion of *m/z* 698 $[M-H]^-$ was identified as PL D. No PL A was detectable. In addition, several new derivatives—named PL X1, PL HS2 to HS5 and PL HS8 to HS11 (Scheme 1 and Supporting Information)—were detected. PL X1 and PL HS2 (Scheme 1) were purified and subsequently analysed by NMR spectroscopy (Experimental Section; PL X1 see also below), and all other compounds were characterized by mass spectroscopy. The mass difference between PL HS2 (*m/z* 728 $[M-H]^-$) and PL D (*m/z* 698 $[M-H]^-$) corresponds to one additional methoxy group (+30 amu). Additionally, its expected molecular formula of $C_{39}H_{56}O_{12}N$ was confirmed by high-resolution mass spectrometry (*m/z* 730.3776 $[M+H]^+$; calculated *m/z* 730.3797 $[M+H]^+$). Subsequent NMR analysis showed that the signal for the C-6′ methyl group ($\delta_H = 2.33$, $\delta_C = 13.2$) of the pyrrole moiety of PL D was missing and instead signals for a methylene group (C-6′, $\delta_H = 4.45$, $\delta_C = 67.0$) and a methoxy group (C-7′ $\delta_H = 3.38$, $\delta_C = 58.1$) were present. Therefore, PL HS2 was identified as a PL D derivative with a methoxy group at position 6′. An atmospheric pressure chemical ionization (APCI) mass detector was used to determine the masses of PL HS3 (*m/z* 712 $[M-H]^-$), PL HS4 (*m/z* 712 $[M-H]^-$), PL HS5 (*m/z* 742 $[M-H]^-$), PL HS8 (*m/z* 686 $[M-H]^-$), PL HS9 (*m/z* 700 $[M-H]^-$), PL HS10 (*m/z* 670 $[M-H]^-$) and PL HS11 (*m/z* 728 $[M-H]^-$). The resulting masses provide confirmation of the corresponding chemical structures (see the Supporting Information).

Generation of a *plaV* mutant and structure elucidation of PL X1

The mutant *Streptomyces* sp. Tü6071Δ*plaV* was generated by inactivation of *plaV* as described in the Experimental Section. The production profile was analysed by HPLC/ESI-MS at the detection wavelengths of 254 nm and 280 nm. PL X1 was observed as a major metabolite at $t_R = 22.7$ min, with a molecular ion of *m/z* 656 $[M-H]^-$. The purified PL X1 was analysed by NMR spectroscopy. The ^{13}C and ^{1}H NMR spectra clearly revealed a PL derivative like PL D, but lacking the ^{13}C signals for the acetyl group at C-3. In addition the signals detected for C-2, C-3 and C-4 in the ^{13}C and ^{1}H spectra were shifted (C-2: $\delta_H =$ 1.62–1.72, m, $\delta_H = 25.7$; C-3: $\delta_H = 3.49$, m, $\delta_C = 72.3$; C-4: $\delta_C =$ 46.3). An additional proton signal was observed at $\delta_H = 5.44$, confirming the presence of a hydroxy group at C-3. Consequently, PL X1 is 3-deacetyl-PL D (Scheme 1). This was supported by high-resolution mass spectrometry. The observed molecular ion was *m/z* 658.3570 $[M+H]^+$, revealing the expected molecular formula of $C_{36}H_{52}O_{10}N$ (calcd: *m/z* 658.3586 $[M+H]^+$). The configuration of 3-OH could not be determined because the ^{1}H signal of 3-H appears as an unresolved multiplet, putatively due to additional correlations with other neighbouring protons (from 3-OH, C-1, C-20 or C-21). However, we propose that 3-OH is in the equatorial position, as are the 3-OH in PL HS6 and the 3-acetyl group in PL D. Additionally a second compound, PL MD1 ($t_R = 18.7$ min), was also produced by this mutant but in very small amounts, and so it was characterized by mass spectroscopy. The mass difference between PL MD1, with a molecular ion of *m/z* 528 $[M-H]^-$, and PL X1 led to the assignment of PL MD1 (see the Supporting Information) as the aglycone of PL X1 (−128 amu; Scheme 2). The two detected deacetyl-PL intermediates indicate that PlaV transfers the acetyl group to 3-OH. It is notable that the encoding gene is not similar to acetyltransferase genes. Instead, the corresponding protein sequence is somewhat similar (around 40% similar amino acids relative to different enzymes) to serine-β-lactamases,[16] which are responsible for resistance against several β-lactam antibiotics. They hydrolyse the β-lactam ring with the help of a serine residue located in the active site. The typical SxxK serine β-lactamase motif[16,17] is also present in PlaV, indicating a similar enzymatic mechanism. The catalytic importance of S66 and K69 in the SxxK motif was confirmed by a site-directed mutagenesis experiment. Both encoding triplets were exchanged (combined and separately) in *plaV* and introduced in the *plaV* mutant through the use of the integrative vector pSET-1cerm. In comparison to the complementation with the native gene, no production or production of only traces of acetylated PLs was observed.

Generation of a *plaZ* mutant, a *plaU* mutant and a *plaU-plaZ* double mutant

Mutants were generated by inactivation of *plaZ* (deletion of a 520 bp internal fragment), *plaU* (deletion of a 156 bp internal fragment) and both genes in tandem (Experimental Section). Analysis of the mutant extracts by HPLC/ESI-MS revealed that the product spectrum was identical to that of the wild type; this indicates that these dehydrogenase genes are not directly involved in the biosynthesis of the known PL derivatives.

Studies on the function of PlaO1

Inactivation of *plaO1* resulted in the formation of PL CD6, a compound with an almost unmodified diterpenoid core, but with an extended C_4 side chain at C-14.[8] In order to study whether PlaO1 oxidizes only the side chain of PL CD6 or cataly-

ses the multi-stage formation of the entire γ-butyrolactone moiety, we overexpressed PlaO1 in *E. coli* as a N-His_6 fusion protein. Incubations of purified protein were performed in the presence of the substrate PL CD6 and the cofactors Fe^{II} and α-ketoglutarate. As shown in Figure 1, a new compound was

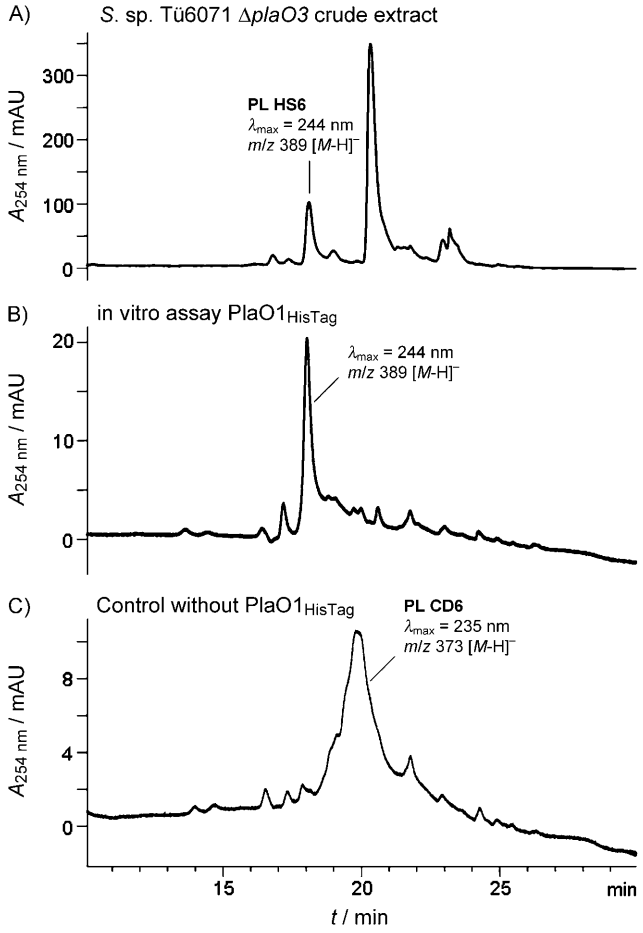


Figure 1. In vitro activity of PlaO1. A) PL HS6 (Scheme 2) was detected in the crude extract of *Streptomyces* sp. Tü6071Δ*plaO3*. B) When PL CD6 was incubated with $PlaO1_{HisTag}$ PL HS6 was produced. C) PL HS6 was not detectable in the absence of $PlaO1_{HisTag}$.

produced and identified as PL HS6, due to its identical UV absorption, mass spectra and HPLC retention time. Hence, the single enzyme PlaO1 is able to catalyse the entire formation of the γ-butyrolactone moiety with PL CD6 as substrate.

Studies on the function of PlaO5

The attachment of the hydroxy group at position C-1 is catalysed by PlaO5 as indicated by results of gene inactivation experiments as described above. PlaO5 was overexpressed in *E. coli* as an N-His_6 fusion protein. Because ferredoxin and the ferredoxin:$NADP^+$ reductase from the PL producer were not available, we used spinach ferredoxin and the spinach ferredoxin:$NADP^+$ reductase in the in vitro assay.[18–20] High concentrations of NADPH and $NADP^+$ and an efficient NADPH regenerating system were used.[18] Incubations were performed with an extract containing PL D and PL D2. As shown in Figure 2, two new compounds were obtained and found to be identical to PL A, the 1-hydroxy derivative of PL D, and PL A2, the 1-hydroxy derivative of PL D2.

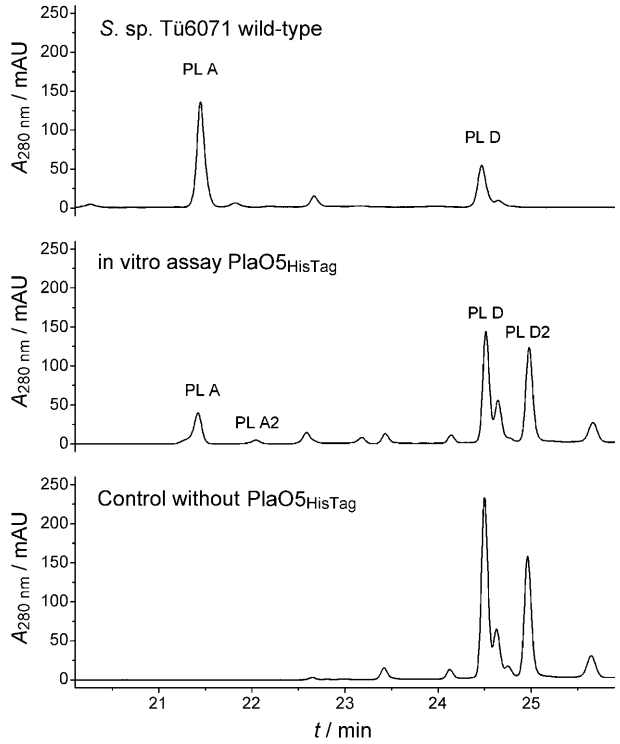
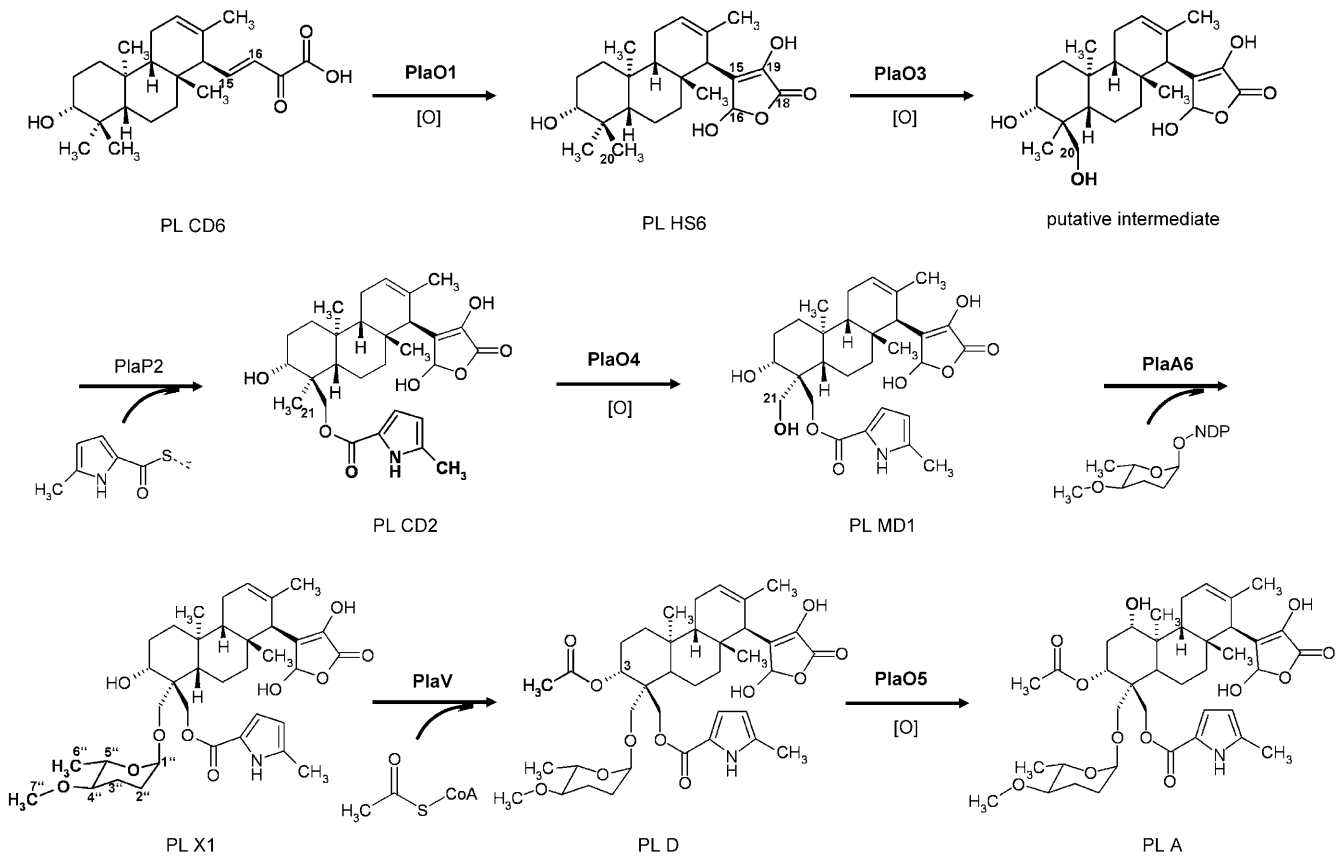


Figure 2. In vitro activity of PlaO5. A) PL A and PL D were detected in the crude extract of *Streptomyces* sp. Tü6071. B) When a mixture of PL D and PL D2 was incubated with $PlaO5_{HisTag}$, PL A and PL A2 were produced. C) No PL A and PL A2 were detectable in the reaction sample without incubation of $PlaO5_{HisTag}$.

Conclusions

PLs are tricyclic terpene metabolites from *Streptomyces* sp. Tü6071 with antibacterial activity. The results of the feeding experiments with ^{13}C-labelled biosynthetic precursors and gene-inactivation and -expression experiments enable a comprehensive understanding of the enzymatic functionalization and decoration of the terpenoid scaffold during PL biosynthesis (Scheme 3).

The incubation of PlaO1 with PL CD6 proves that this unique Fe^{II}-α-ketoglutarate-dependent dioxygenase enables the entire formation of the γ-butyrolactone. PL HS6 was accumulated due to inactivation of *plaO3*, so the activity of PlaO3 is required as the following enzymatic step. Then the pyrrole moiety has to be transferred to the C20–OH group in a step putatively catalysed by PlaP2, as indicated by its presence in the PL CD2 molecule.[9] Subsequently the second hydroxylation takes place at C-21, resulting in PL MD1, which is afterwards glycosylated by the glycosyltransferase PlaA6 to afford PL X1.[12] The presence of these secondary metabolites as a consequence of the inactivation of *plaV* provided evidence



Scheme 3. Biosynthetic pathway from PL CD6 to PL A. The functions of enzymes written in bold are experimentally corroborated.

that PlaV acts as the 3-*O*-acetyltransferase. Because the inactivation of *plaA6*[12] leads to PL E, an acetylated PL MD1 derivative, we can conclude that the 3-*O*-acetylation takes place independently of the glycosylation state of the PL intermediate. Nevertheless, we assume that PL X1 is the favoured substrate of PlaV because of a substantially higher production level than that of PL MD1. Finally the 1-*C*-hydroxylation of PL D gives PL A in a step catalysed by the enzyme PlaO5. This was corroborated by the in vitro assay with recombinant PlaO5 and non-hydroxylated PLs as substrates and also by the inactivation of the associated gene.

Experimental Section

Bacterial strains and culture conditions: *Streptomyces* sp. Tü6071 was grown in NL-19 [soy flour (2%), mannitol (2%), pH 7.2] or NL-111 [lab lemco meat powder (2%), $CaCO_3$ (1%), malt extract (10%), pH 7.2] liquid media dispensed in double-baffled flasks. The cultures were maintained at 28 °C and 180 rpm on a rotary incubator and harvested after 6–8 days. *E. coli* strains XL 1 blue MRF′ (Stratagene), ET12567 (*dam*$^-$, *dcm*$^-$, *hsd*S, Cm^R)[21] including conjugation plasmid pUZ8002,[22] and BL21 (DE3)/pLysS (Novagen) were cultured in LB medium at 37 °C with the appropriate antibiotic selection at final concentrations of 100 μg mL^{-1} carbenicillin, 30 μg mL^{-1} kanamycin, 35 μg mL^{-1} apramycin and 25 μg mL^{-1} chloramphenicol.

Analysis of PL production and isolation of derivatives: To assess PL production of *Streptomyces* sp. Tü6071 and its mutants, NL-19 or NL111 liquid medium (20 mL) was inoculated from a sporulated plate culture and grown as described above. After 6–8 days, an aliquot (1 mL) of the culture was extracted with ethyl acetate (1 mL). The evaporated residue was dissolved and subsequently analysed by HPLC/MS ESI and APCI as previously described.[9] For upscaling of PL production, NL19 liquid medium (3.5 L) was dispensed equally into 25 double-baffled 500 mL Erlenmeyer flasks and inoculated with two-day-old seed cultures. After 6–8 days of cultivation the crude extract was obtained and fractionated as described previously.[9] Fractions containing PL derivatives were pooled and applied to an Agilent 1100 system with a semipreparative Zorbax Eclipse XDB-C18 column (5 μm particle size, 9.4×150 mm) utilizing UV detection or mass guided-fraction collection by APCI-MS or ESI-MS. The gradient profile consisted of solvent A [acetic acid (0.5%, *v/v*) in H_2O] and solvent B [acetic acid (0.5%, *v/v*) in acetonitrile] and was adjusted to the retention characteristics of the PL derivative concerned. The solvent flow rate was 3.0 mL min^{-1}. PL derivatives were yielded in around 1 mg per L fermentation broth.

Structure elucidation: To verify the identities of the compounds, a LC-coupled FT-Orbitrap-MS analysis was performed. Analysis was carried out with an Accella UPLC system (Thermo Electron Corporation) that was coupled to a LTQ Orbitrap Mass Spectrometer (Thermo Fisher Scientific) operating in negative-ionization mode at a scan range of *m/z* 100–2000. A Hypersil Gold column (2.1Q50 mm; Thermo Fisher Scientific) was used for separation with a solvent system consisting of formic acid (0.1%, *v/v*) in water (A) and formic acid (0.1%, *v/v*) in acetonitrile (B). A gradient of 5–95% B was applied over 10 min. Measurements were carried out in single-ion mode (SIM). NMR spectra were recorded at ^{1}H resonance

frequencies of 500 MHz (Bruker DRX 500) and 400 MHz (Bruker DRX 400). Standard parameters were used for 1D and 2D NMR spectra, which included ^{1}H, ^{13}C, $^{1}H/^{1}H$-correlated spectroscopy (COSY), heteronuclear single quantum coherence (HSQC), heteronuclear multiple bond correlation (HMBC) and Incredible Natural Abundance DoublE QUAntum Transfer Experiment (INADEQUATE). All chemical shifts were referenced to residual $CHCl_3$ (PL HS2 and PL X1) or methanol (PL HS6 and PL HS7).

PL HS2: ^{1}H NMR ($CDCl_3$): $\delta = 0.98$ (s, H-22, C-9, C-14), 1.05–-1.15 (m, H-1a, H-7a, H-9, C-8, C-12, C-14), 1.16 (s, H-24, C-1, C-5, C-9), 1.23 (d, $J = 6.1$ Hz, H-6″), 1.30–1.36 (m, H-5), 1.50–-1.78 (m, H-2a, H-2b, H-7b, H-2″a, H-3″a, H-3″b, H-6a), 1.60 (s, H-23, C-12, C-13), 1.80–-1.92 (m, H-1b, H-6b, H-2″b), 2.03 (s, H-26, C-25), 2.07–-2.17 (m, H-11a), 2.24–-2.36 (m, H-11b), 3.00 (ddd, $J = 10.8$, 4.5, 4.5 Hz, H-4″), 3.38 (s, H-7′, C-6′), 3.40–-3.43 (m, H-21a, H-14), 3.50 (s, H-7″, C-4″), 3.81–-3.90 (m, H-5″), 3.93 (d, $J = 11.8$ Hz, H-20a), 4.41 (d, $J = 9.6$ Hz, H-21b), 4.45 (s, H-6′, C-7′), 4.61 (dd, $J = 4.7$, 2.3 Hz, H-1″), 4.77 (d, $J = 11.7$ Hz, H-20b), 5.00 (dd, $J = 12.1$, 4.6 Hz, H-3), 5.53 (brs, H-12), 5.86 (d, $J = 4.8$ Hz, H-16), 6.16 (t, $J = 3.0$ Hz, H-4′), 6.86 (dd, $J = 3.5$, 2.6 Hz, H-3′), 7.17 (d, $J = 5.5$ Hz, 16-OH), 9.34 ppm (brs, NH); ^{13}C NMR ($CDCl_3$): $\delta = 14.2$ (C-24), 17.7 (C-6″), 20.7 (C-6), 21.3 (C-26), 22.1 (C-23), 23.1 (C-11), 23.3 (C-2), 23.8 (C-3″), 27.6 (C-22), 29.3 (C-2″), 38.2 (C-1), 38.3 (C-10), 39.3 (C-8), 40.2 (C-14), 40.5 (C-7), 45.0 (C-4), 49.1 (C-5), 54.7 (C-9), 56.7 (C-7″), 58.1 (C-7′), 62.9 (C-20), 67.0 (C-6′), 68.6 (C-21), 68.7 (C-5″), 73.9 (C-3), 81.6 (C-4″), 98.1 (C-1″), 98.5 (C-16), 109.6 (C-4′), 115.8 (C-3′), 122.1 (C-12), 122.4 (C-2′), 129.9 (C-15), 132.4 (C-13), 134.1 (C-5′), 138.9 (C-19), 160.5 (C-1′), 169.1 (C-18), 170.3 ppm (C-25).

PL HS6: ^{1}H NMR (CD_3OD): $\delta = 0.79$ (s, H-21, C-3, C-4, C-5, C-20), 0.83–-0.90 (m, H-5), 0.90–-1.00 (m, H-1a, C-9), 0.97 (s, H-20, C-3, C-4, C-5, C-21), 0.99* (s, H-22, C-9, C-14), 0.99 (s, H-24, C-9), 1.03–-1.10 (m, H-9, C-8/10, C-11, C-14, C-24), 1.14–-1.25 (m, H-7a), 1.47–-1.60 (m, H-6ab), 1.59 (s, H-23, C-12, C-13, C-14), 1.55–-1.68 (m, H-2ab), 1.72–-1.79 (m, H-7b), 1.83 (ddd, $J = 12.9$, 3.8, 3.5 Hz, H-1b), 2.00–-2.13 (m, H-11a, C-8/10, C-12, C-13), 2.19–-2.34 (m, H-11b), 3.13 (dd, $J = 11.5$, 5.0 Hz, H-3, C-4, C-20, C-21), 3.32 (s, H-14), 5.47 (brs, H-12), 5.95 ppm (s, H-16); ^{13}C NMR (CD_3OD): $\delta = 14.8$ (C-24), 16.4 (C-21), 19.2 (C-6), 22.7 (C-23), 23.8 (C-11), 28.1 (C-2), 28.8 (C-20), 29.0 (C-22), 39.4** (C-10), 40.2** (C-8), 40.3 (C-1), 40.3 (C-4), 41.6 (C-7), 41.9 (C-14), 56.4 (C-9), 57.4 (C-5), 79.6 (C-3), 100.3 (C-16), 123.3 (C-12), 131.8 (C-15), 134.1 (C-13), 142.0 (C-19), 170.5 ppm (C-18). * Indirectly observed through HMBC, ** assignments are interchangeable.

PL HS7: ^{1}H NMR (CD_3OD): $\delta = 1.02$ (s, H-24, C-1, C-5, C-9), 1.05 (s, H-22, C-7, C-9, C-14), 1.08 (s, H-21, C-3, C-4, C-5, C-20), 1.12 (s, H-20, C-3, C-4, C-5, C-20), 1.23–-1.35 (m, H-7a, H-9), 1.40–1.75 (m, H-6ab, H-1a, C-3, H-5, C-24), 1.62 (s, H-23, C-12, C-13, C-14), 1.77–-1.84 (m, H-7b), 2.00–-2.16 (m, H-1b, C-3, H-11a, C-12), 2.35–-2.60 (m, H-2ab, C-3H-11b), 3.38 (brs, H-14), 5.51 (brs, H-12), 5.99 ppm (s, H-16); ^{13}C NMR (CD_3OD): $\delta = 15.6$ (C-24), 20.5 (C-6), 21.6 (C-21), 22.7 (C-23), 24.0 (C-11), 27.8 (C-20), 28.7 (C-22), 35.0 (C-2), 38.9 (C-10), 40.0 (C-8), 40.5 (C-7), 40.6 (C-1), 41.5 (C-14), 48.2* (C-4), 55.2 (C-9), 56.2 (C-5), 100.1 (C-16), 123.0 (C-12), 131.4 (C-15), 134.0 (C-13), 142.1 (C-19), 170.4 (C-18), 220.8 ppm (C-3). * Indirectly observed through HMBC.

PL X1: ^{1}H NMR ($CDCl_3$): $\delta = 0.92$–1.01 (m, H-1a, C-24), 1.03 (s, H-22, C-7, C-8, C-14), 1.08–1.14 (m, H-9, C-8, C-10, C-11, C-12, C-14, C-24), 1.16 (s, H-24, C-1, C-5, C-9, C-10), 1.17–1.20 (m, H-7a), 1.25 (d, $J = 5.8$ Hz, H-6″, C-4″, C-5″, C-2″), 1.26 (brd, $J = 11$ Hz, H-5, C-10), 1.58–1.62 (m, H-3″a, C-1″), 1.62 (brs, H-23, C-12, C-13, C-14), 1.62–1.72 (m, H-2), 1.70–1.76 (m, H-7b), 1.74–1.78 (m, H-2″a), 1.79–1.82 (m, H-6a), 1.82–1.89 (m, H-2″b), 1.83–1.89 (m, H-6b), 1.84–1.92 (m, H-1b), 2.06–2.10 (m, H-3″b), 2.12–2.18 (m, H-11a), 2.28–2.34 (m, H-11b, C-13), 2.35 (s, H-6′, C-4′, C-5′), 3.03 (ddd, H-4″), 3.46 (brs, H-14), 3.49 (m, H-3, C-4, C-5), 3.52 (s, H-7″), 3.55 (d, $J = 9.5$ Hz, H-21a, C-4, C-5), 3.88 (dq, H-5″), 4.35 (d, $J = 12$ Hz, H-20a, C-5, C-3, C-1′), 4.36 (d, $J = 9.5$ Hz, H-21b, C-4, C-5, C-20), 4.79 (d, $J = 12$ Hz, H-20b, C-5, C-3, C-1′), 4.61 (d, $J = 2.4$ Hz, H-1″, C-3″, C-5″), 5.44 (s, 3-OH), 5.56 (brs, H-12), 5.91 (d, $J = 5.8$ Hz, H-16, C-18, C-19), 6.01 (d, $J = 2.4$ Hz, H-4′, C-2′, C-5′), 6.82 (dd, $J = 2.4$, 2.0 Hz, H-3′, C-2′, C-5′), 7.20 (d, $J = 5.8$ Hz, 16-OH, C-15, C-16), 8.87 ppm (brs, NH); ^{13}C NMR ($CDCl_3$): $\delta = 13.2$ (C-6′), 14.2 (C-24), 17.7 (C-6″), 20.7 (C-6), 22.1 (C-23), 23.1 (C-11), 23.8 (C-3″), 25.7 (C-2), 27.7 (C-22), 29.4 (C-2″), 38.3 (C-10), 38.6 (C-1), 39.3 (C-8), 40.2 (C-14), 40.7 (C-7), 46.3 (C-4), 49.2 (C-5), 54.8 (C-9), 56.7 (C-7″), 64.6 (C-20), 67.9 (C-21), 68.4 (C-5″), 72.3 (C-3), 81.7 (C-4″), 98.0 (C-1″), 98.6 (C-16), 109.4 (C-4′), 116.5 (C-3′), 120.6 (C-2′), 122.2 (C-12), 129.9 (C-15), 132.3 (C-13), 134.6 (C-5′), 138.8 (C-19), 161.8 (C-1′), 169.1 ppm (C-18).

Feeding of labelled precursors and quantification of enrichment: Culture (1 L) in seven flasks was inoculated with a 32 h-old *Streptomyces* sp. Tü6071 seed culture and was grown in a rotary incubator at 28 °C and 180 rpm. L-[$^{13}CH_3$]Methionine (200 mg), [1-^{13}C]acetate (250 mg), D-[U-$^{13}C_6$]glucose (263 mg), [2-^{13}C]pyruvate (467 mg) and [U-^{13}C] amino acid mixture (250 mg) were fed to the cultures. Compounds were fed in equal portions after 114, 138 and 162 h. In the case of D-[U-$^{13}C_6$]glucose four equal portions were given to the cultures after 90, 114, 138 and 162 h. The fermentation was harvested eight days after inoculation. The extraction and isolation of labelled PL D was performed as described above. The feeding experiment with D-[1-^{13}C]glucose had been carried out previously and the relative ^{13}C enrichments were quantified as described previously.[9]

Generation of mutant strains of *Streptomyces* sp. Tü6071: DNA manipulation[23] was carried out with *E. coli* XL-1 Blue MRF′ as host strain. To generate mutants with deletion in *plaO3, plaO5, plaU, plaV* and *plaZ* by means of insert-directed homologous recombination, the target genes were amplified by PCR, including flanking DNA of minimum 1.0 kbp up- and downstream of the start and stop codon, respectively, or subcloned from the appropriate cosmid. Restriction sites were introduced to enable cloning of the fragments. Cosmids 3–1O12 and 10–4D08 were used as templates. The primer sequences and a protocol for the generation of the inactivation plasmids are described in the Supporting Information. The final pKC1132[24]-derived plasmids were introduced into *Streptomyces* sp. Tü6071 by intergeneric conjugation with the methylation-deficient donor strain *E. coli* ET12567/pUZ8002 as previously described.[8] Exconjugants were selected for the integration of the vector with apramycin. Resistant colonies (single crossover) were grown nonselectively on HA agar plates for several generations. Spores of single colonies were dissected by replica plating for individuals that had lost the vector (double crossover). Single- and double-crossover recombination events were screened by PCR (see the Supporting Information). In the case of the double mutant *Streptomyces* sp. Tü6071Δ*plaU*-Δ*plaZ* the method of insertional inactivation through a single crossover[25] was applied. The gene *plaZ* was amplified by PCR with exclusion of the regions around the start and stop codons, ligated into pKC1132 and afterwards introduced into the mutant *Streptomyces* sp. Tü6071Δ*plaU*. Resistant colonies indicated the single-crossover mutant. PCR was performed to confirm the correct introduction of the plasmid (see the Supporting Information). The cultivation was accomplished in the presence of apramycin to avoid the loss of the plasmid.

Complementation of *Streptomyces* sp. Tü6071 mutant strains: Mutant strains were complemented with pSET-1cerm-derived constructs (see the Supporting Information) as previously described.[8]*

Site-directed mutagenesis of *plaV*: The encoding triplets for the suggested catalytically active amino acid residues S66 (AGC) and K69 (AAA) were exchanged by codon GCC (translation to A). The exchange of K69 was accomplished by use of the QuikChange Site-Directed Mutagenesis Kit (Stratagene), primers K69Ex-F and -R, and the plasmid pMD-Vco (Supporting Information) as template revealing in pMD-VcoKEx. For the exchange of the triplet of S66 and the combination of both triplets in parallel, pMD-Vco was restricted by NcoI and NotI and a 730 bp internal fragment of *plaV* was replaced by synthetically produced fragments (Mr. Gene) with the appropriate mutation to obtain pMD-VcoSEx (exchange of the S66 triplet) and pMD-VcoSKEx (exchange of both triplets). Plasmids were introduced in the mutant *Streptomyces* sp. Tü6071Δ*plaV* by intergeneric conjugation and the extract of the production culture was analysed by HPLC/MS in comparison with the mutant that was complemented with pMD-Vco carrying the native gene *plaV*.

Expression and purification of PlaO1 and PlaO5: Primer sequences and a protocol for the generation of the expression plasmids are described in the Supporting Information. *E. coli* BL21(DE3)/pLysS was transformed with the plasmids pRSET-O1 or pRSET-O5, respectively, and incubated at 37 °C on LB medium containing chloramphenicol and carbenicillin. A seed culture with appropriate antibiotics was inoculated with a single colony and cultured overnight. This seed culture (3 mL) was used to inoculate LB liquid medium (100 mL) and grown to OD_{600} 0.6 at 37 °C and 180 rpm. Isopropyl β-D-thiogalactopyranoside (IPTG, 1 mM, Roth) was then added for induction and the expression culture was incubated for 5 h at 25 °C and 180 rpm. The cells were harvested by centrifugation and cell lysis was achieved by mechanical cell disruption (French®Pressure Cell Press) after resuspension in lysis buffer [NaCl (300 mM), imidazole (10 mM), phosphate buffer (50 mM), pH 8.0, 4 mL]. After centrifugation, the supernatant fraction was used for purification. The protein was bound to Ni-NTA agarose (Qiagen) and loaded on a column. The column was washed twice with washing buffer containing imidazole (20 mM and 40 mM, respectively). The protein was eluted with elution buffer containing imidazole (250 mM). Roti®Spin MIDI-15 columns (Roth) for recombinant PlaO1 or MIDI-30 columns for recombinant PlaO5 were used to desalt and resolve the protein in Tris buffer (50 mM, pH 7.5) or sodium phosphate buffer (10 mM, pH 7.5), respectively.

Expression and purification of spinach ferredoxin: The plasmid pET14-SpFd was provided by Prof. J. Robinson (Institute of Organic Chemistry, University of Zürich). The procedure was analogous to that used for the expression of PlaO5 with a small difference: $FeSO_4$ (1 M) was added to the expression culture, which was cultivated upon an OD_{600} 1.2 and then induced with IPTG. After induction, the incubation conditions were changed to 30 °C and 180 rpm. Harvesting and cell disruption was applied analogously to PlaO1 and PlaO5. Additionally, dithiothreitol (DTT, 1 mM) and phenylmethanesulfonyl fluoride (PMSF, 1 mM) were added to the lysis buffer. Centrifugation, binding to Ni-NTA agarose and washing the protein were done as described above. Roti®Spin MIDI-3 columns (Roth) were used for desalting. Spinach ferredoxin was stored at −20 °C in sodium phosphate buffer (pH 7.0, 10 mM)/glycerol (15%, *v*/*v*).

In vitro assay of PlaO1: The in vitro assay was accomplished in the presence of Tris buffer (pH 7.5, 50 mM) and saturating concentrations of the *Streptomyces* sp. Tü6071Δ*plaO1* crude extract, including the substrate PL CD6 in a total volume of 500 μL. The assay mixture also contained the purified $PlaO1_{HisTag}$ (0.12 mg), α-ketoglutaric acid (2 mM), $FeSO_4$ (1 mM) and ascorbic acid (1 mM). After overnight incubation at 28 °C and acidification, the products were extracted twice with an equal volume of ethyl acetate. The separated organic phase was dried (Speed®Vac) and the residue was dissolved in methanol (10 μL) and afterwards mixed with DMSO (50%, 190 μL) and analysed by HPLC/MS as previously described for assessment of PL production.

In vitro assay of PlaO5: Standard analytical enzyme assays (500 μL) were performed at 37 °C for 2 h. One incubation mixture contained PlaO5 (0.2 mg), $NADP^+$ (1 μM), NADPH (1 μM), glucose-6-phosphate (5 μM), glucose-6-phosphate dehydrogenase (0.5 units), engineered spinach ferredoxin (28.5 μg), spinach ferredoxin reductase (0.05 units), PL D substrate (0.1 μM) and sodium phosphate buffer (10 mM, pH 7.5). Reactions were stopped by the addition of ethyl acetate (500 μL) and the systems were vortex-mixed and subjected to centrifugation (14000 rpm, 5 min, at room temperature). The organic phase was removed and dried (Speed®-Vac). The residue was dissolved in methanol (200 μL) and analysed by HPLC/MS. The $NADP^+$, glucose-6-phosphate and glucose-6-phosphate dehydrogenase constituted an NADPH regenerating system.[17,18]

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