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# Activation of Natural Products Biosynthetic Pathways *via* a Protein Modification Level Regulation

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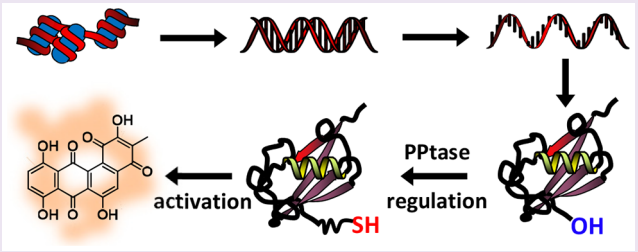
Supporting Information

**ABSTRACT:** Natural products are critical for drug discovery and development; however their discovery is challenged by the wide inactivation or silence of microbial biosynthetic pathways. Currently strategies targeting this problem are mainly concentrated on chromosome dissembling, transcription, and translation-stage regulations as well as chemical stimulation. In this study, we developed a novel approach to awake cryptic/silenced microbial biosynthetic pathways through augmentation of the conserved protein modification step-phosphopantetheinylation of carrier proteins. Overexpression of phosphopantetheinyl transferase (Pptase) genes into 33 *Actinomycetes* achieved a significantly high activation ratio at which 23 (70%) strains produced new metabolites. Genetic and biochemical studies on the mode-of-action revealed that exogenous PPtases triggered the activation of carrier proteins and subsequent production of metabolites. With this approach we successfully identified five oviedomycin and halichomycin-like compounds from two strains. This study provides a novel approach to efficiently activate cryptic/silenced biosynthetic pathways which will be useful for natural products discovery.




Natural products have long been appreciated for their critical role in drug discovery and development due to their diverse bioactivities.[1] It is revealed that more than 50% of small molecule drugs (including pesticides) are from natural products or their derivatives.[1,2] However, the rate of discovery of useful compounds from natural sources has declined progressively since 1970; thus, how to effectively discover natural products nowadays has been becoming a central question in the field of pharmaceuticals and its related fields.[1,2] Recently, the increasing number of sequenced genomes has indicated that microorganisms indeed have a far greater potential to produce metabolites than those previously isolated. It is revealed that more than 90% of microbial metabolites have not yet been accessed due to the inactivation or silencing of their biosynthetic machineries under normal laboratory culturing conditions.[3] One of the major reasons for this inactivation is the absence of the essential regulatory signals to trigger these pathways.

The biosynthesis of natural products from biosynthetic gene clusters depends on orchestrated regulations, including dissembling of chromosome to chromatin (eukaryotic microbe only), transcription of relevant genes to mRNAs, translation of mRNA to proteins, and modification of proteins to an active form which performs the biosynthesis. Currently, different strategies targeting chromosome dissembling,[4,5] transcription,[6] and translation stages[7,8] as well as others[2,9−12] have been developed to successfully activate cryptic or silent biosynthetic pathways. Protein modification is one of the major regulation stages; however thus far there are no methods that have been described for natural products activation.

We noticed that in the biosynthesis of three large natural product families, termed polyketide (PK), nonribosomal peptide (NRP), and fatty acid (FA), there is a conserved protein modification step, in which the encoded carrier proteins (*apo* form) of their synthases are phosphopantetheinylated by phosphopantetheinyl transferase (PPtase) and converted into an active form (*holo* form).[13] Interestingly, PPtases are also able to efficiently catalyze acyl-phosphopantetheinylation of the carrier proteins by accepting acyl-CoAs instead of HSCoA.[13,14] This misacylated acyl-phosphopantetheinylation is detrimental for metabolite biosynthesis, and the acyl group is required to be further removed by additional editing enzymes.[15] The promiscuity of PPtase, accepting both free CoA and acyl-CoA, contributes to activating and deactivating metabolite

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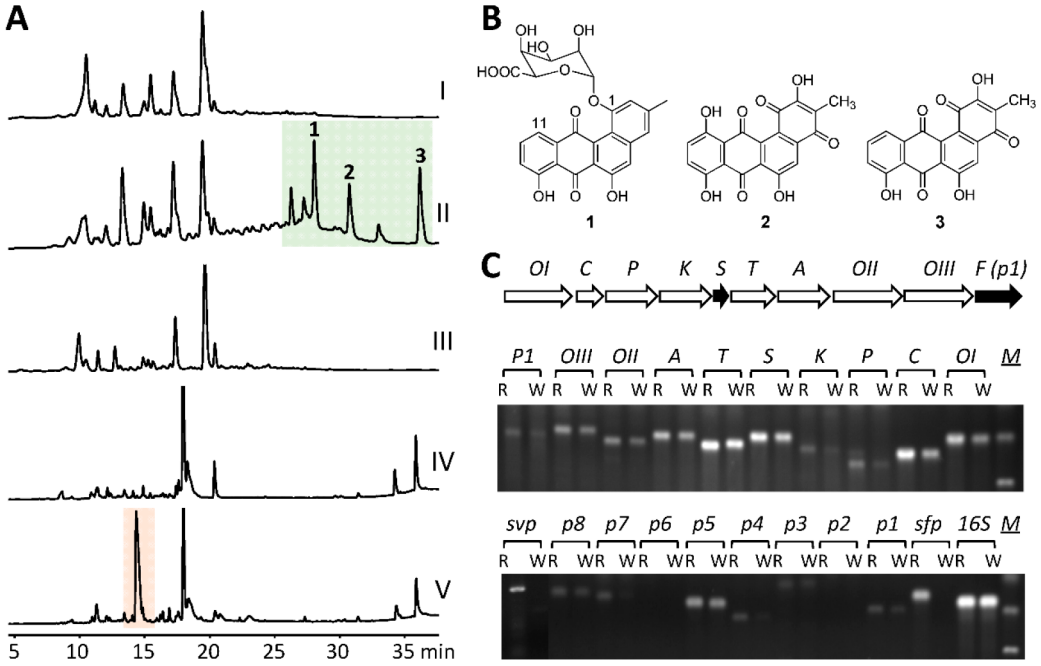


**Figure 1.** Activation and deactivation of metabolite biosynthesis by overexpression of PPtase. (A) HPLC analysis of metabolite profiles in NRRL 3238 wild-type (I), NRRL 3238-PPtase (II), mWHU2466 (NRRL 3238-PPtase-Δ*ovmK*) (III), ATCC 27064-PPtase strain (IV), and ATCC 27064 wild-type (V). Activated and deactivated metabolites are indicated by green and orange, respectively. (B) Isolated and characterized activated compounds (**1**−**3**) from NRRL 3238-PPtase. (C) RT-PCR analysis of the *ovm* biosynthetic genes, PPtase genes, and housekeeping 16S RNA gene in the wild-type and PPtase strains of NRRL 3238. R and W designate recombinant PPtase strain and wild-type strain, respectively.

biosynthesis, suggesting it could be a protein modification-level regulator, and augmentation of its activity may be able to turn on/off the biosynthesis of natural products. Each microbial genome typically contains 1−3 PPtases whose individual functions are often specific to a set of biosynthetic gene pathways.[16] Given that (i) microbial genomes, particularly *Actinomycetes*', are genetically unstable,[17] which can cause inactivation of the PPtase gene by occasional mutation or loss during propagation;[18] (ii) biosynthetic gene clusters often arise from interspecies horizontal transfer[19] whose carrier proteins may not be recognized by the endogenous PPtase; (iii) turning down robustly expressed secondary biosynthetic pathways (by PPtase) can activate the silenced pathways;[20] and (iv) regulating primary molecule synthesis (by PPtase) will cause substrate flux and physiological change[21] which can stimulate the activation of silenced pathways, we hypothesize that alteration of the PPtase activity in microbes is able to activate the biosynthesis of cryptic or silenced natural products.

In order to confirm this assumption, we chose two widely used broad-selective PPtase genes, *sfp*[18] from *Bacillus subtilis* and *svp*[22] from *Streptomyces verticillus*, to be overexpressed in *Actinomycetes* strains. *Sfp* and *svp* were constructed in a way to ensure sufficient phosphopantetheinylation activity (*ermE*-sfp-svp*, where *ermE** is a strong constitutive promotor). This gene cassette, carried by an integrative vector (pIB139), was introduced into two laboratory-housed stains *S. antibioticus* NRRL 3238 and *S. clavuligerus* ATCC 27064. Analysis of the fermentation broth revealed six new peaks in the NRRL 3238-PPtase strain (Figure 1A, trace II). In the ATCC 27064 strain, a major peak (at 14.8 min) disappeared after integration with PPtases (Figure 1A, trace IV). These results are in agreement with our assumption about the regulatory role of PPtase. The compounds produced by NRRL 3238-PPtase show an identical pattern of UV absorbance, suggesting that they possess a similar scaffold. By scaling up the fermentation, three major products (**1**−**3**) were further isolated, characterized, and revealed to be oviedomycin B (**1**), oviedomycin C (**2**), and oviedomycin A (**3**), falling into the family of angucycline[23] (Figure 1B). Oviedomycin B (**1**) and oviedomycin C (**2**) are new and show moderate anticancer activity against HL60 and NB4 cell lines (Table S1). Interestingly, these new compounds feature a number of special characteristics, including the unusual C-1 glycosylation (in **1**) and C-11 hydroxylation (in **2**), which were only observed in the amycomycin B and landomycins (including hydroxytetrangomycin), respectively, from the entire angucycline family (>500 members).[23] Furthermore, the unique glucuronic acid moiety (in **1**) is rare in secondary metabolites, and to the best of our knowledge such a glycosyl moiety has not been observed in microbial PKs. Taken together, these results confirm this PPTase-based method can be a promising tool for activating cryptic/silenced pathways to discover novel structures.

We are next interested in the relationship of the exogenous (Sfp, Svp) and endogenous PPtase activation of oviedomycin biosynthesis. Oviedomycin A (**3**) biosynthesis is known in *S. antibioticus* ATCC 11891 to be programed by a type II PKS system.[24] By sequencing the genome of NRRL 3238, we discovered an identical pathway (Figure 1C and Table S2) to that in ATCC 11891. Involved in the biosynthesis of **1**−**3**, other uncharacterized minor components were also confirmed by inactivation of the ketosynthase gene *ovmK* (Figure 1A, trace III). Intriguingly, genes encoding additional C-1 glucuronylation and C-11 hydroxylation for synthesis of **1** and **2** were not found in or flanking the gene cluster, suggesting these modifications are derived from discrete biosynthetic genes or cross talking between other pathways. To verify whether any other factors on the transcription stage were involved in *ovm* activation, we performed RT-PCR analysis on the tran-



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scriptome of the gene cluster (Figure 1C). Factors affecting translation were not considered because no apparent effect would be introduced by PPtase overexpression. It is revealed that all the essential genes in the nonproducing strain (wild type) were transcribed at an identical level to the producing strain (PPtase strain, Figure 1C). This indicates that transcription regulation was not responsible for *ovm* activation. Further analysis of the NRRL 3238 genome revealed a complex PPtase network comprising eight PPtase genes (Figure S1) consisting of two type I (*P7* and *P8*, specific for fatty acid synthase) and six type II PPtase (*P1*−*P6*, specific for secondary metabolite biosynthesis). *P1* is located at the boundary of *ovm* in NRRL 3238 and is also designated as *ovmF* in ATCC 11891.[24] PPtase genes in the wild type and PPtase strains exhibit similar transcription patterns to the transcriptome of the *ovm* biosynthetic gene cluster. Four type II PPtase (P1, P3, P4, P5) and two type I PPtase (P7, P8) are transcribed at a similar level under the oviedomycin production condition (Figure 1C). To verify their phosphopantetheinylation activity, all of the transcribed PPtases (P1, P3, P4, P5, P7, P8) and the carrier protein OvmS were overexpressed in *Escherichia coli*. P3 and P5 produced an insoluble inclusion body, whereas P1, P4, P7, P8, and OvmS were successfully purified. An *in vitro* assay revealed that P4, P7, and P8 have poor activity. P1 is as efficient as Sfp and Svp in catalyzing the phosphopantetheinylation of OvmS (Figure S2), suggesting it is naturally competent to activate the OvmS *in vivo*. These data together show that the silence of *ovm* is not due to the catalytic activity of endogenous PPtase nor transcription or translation of the other essential genes in the pathways. Analysis of *p1* and *ovmS* transcripts and quantitative comparison of transcript abundance showed that *p1* was transcribed approximately 20-fold less than its counterpart *ovmS* (Figure 1C). Enhancing its transcription intensity by overexpression (under the driven of *ermE**) can also stimulate the production of **1**−**3** (Figure S3). These results suggested that oviedomycins can still be produced in the wild type strain, but the significant undersupply of PPtase (P1) reduces the oviedomycins production below detection, resulting in the so-called silenced status. Overexpression of exogenous PPtase (Sfp and Svp) reached a similar level to that of OvmS (Figure 1C) and as such dramatically boosted phosphopantetheinylation activity, triggering the activation of OvmS and subsequent production of oviedomycins.

Finally, we were interested in the broad applicability of this approach. The PPtase genes (*ermE*-sfp-svp*) were introduced into an additional 31 *Actinomyctes* strains, which includes 29 *Streptomyces* and two other strains. PPtase strains were fermented on mannitol soya flour (MS) agar media and extracted on the seventh day. HPLC analysis identified that 12 PPtase strains, including the well-studied model strain *S. albus* J1074, produced new peaks, with most strains generating more than two new peaks (Figure S4 and Table S3). When the fermentation medium was changed to IWL-4 or YMG media, three of the activated strains identified on MS agar media and an additional nine PPtase strains generated new peaks compared to control strains (Figure S4 and Table S3). Interestingly, the HPLC profiles sourced from the metabolites of the same activated strains under different fermentation media consist of both overlapped and shifted peaks. Numerous observed peaks were either dramatically increased in intensity or eliminated/diminished in the PPtase strains (Figure S4 and Table S3). New and more intense peaks were detected in the fermentation products of 22 of the 33 strains tested, and one strain resulted in increased yields of metabolites. The activation of 23 out of 33 strains tested (70%) demonstrates the broad utility and adaptability of this approach, which in combination with other approaches could deliver a synergistic effect on metabolite regulation. To further confirm the production of structural variety, we scaled up the fermentation of *S. ghanaensis* CGMCC 4.1967-PPtase to isolate the significantly activated compounds (Figure 2). Compounds **4**−**8** have identical UV

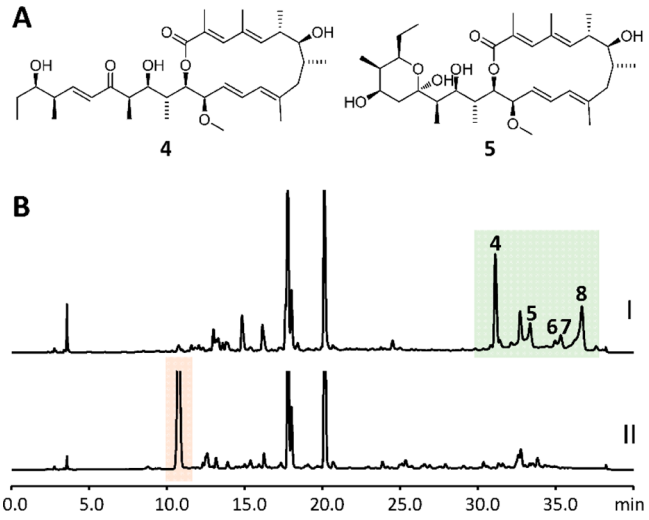


**Figure 2.** Activation of halichomycin and its analogues' biosynthesis in *S. ghanaensis* CGMCC 4.1967. (A) Structures of halichomycin (**4**) and defumarylhygrolidin (**5**). (B) HPLC traces show the metabolites profiles in the CGMCC 4.1967-PPtase strain (I) and wild-type strain (II). Activated and deactivated metabolites are indicated by green and orange, respectively.

patterns, indicating they belong to the same family. Peaks **4** and **5** were characterized and revealed to be halichomycin and defumarylhygrolidin, respectively. These compounds have been identified in several entophytic strains and show potent antifungal activity;[25,26] however their production in *S. ghanaensis* has not been previously identified. Peaks 6−8 were either isolated as a mixture (6 and 7) or unstable (8), thus it was not possible to confirm their identities. These results together demonstrate that the PPtase approach is broadly useful for switching on cryptic/silenced pathways and can be used to identify additional natural products.

PK, NRP, and FA are three large families of products which constitute >50% of bacterial metabolites. In addition to these metabolites functioning for defensive purposes and components of the cell membrane, a number of these products act as signal transmitters (*e.g.*, quorum sensor), nutrients scavengers (*e.g.*, siderophores), and fitness enhancers (*e.g.*, pigments) which regulate not only primary metabolism but also secondary metabolite biosynthesis.[13,27] Moreover, PPtase is involved in the biosynthesis/anabolism of central primary molecules, including lysine and tetrahydrofolate by using HSCoA to catalyze the phosphopantetheinylation of their carrier proteins.[13] These primary compounds play very important roles in metabolite biosynthesis, and their variation can influence many secondary metabolite biosynthesis pathways. In addition to directly regulating metabolite biosynthesis, PPtase modulation is also able to influence other PPtase expression. It was revealed that inhibition of the PPtase (AcpS) in *S. coelicolor* is able to improve the yield of actinorhodin by triggering the


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upregulation of another PPtase (Sfp).[28] Thus, through direct impact on HSCoA consumption and subsequent influence on HSCoA-related cascades in the sophisticated metabolic network, PPtase acts as a fundamental regulator in the biosynthesis of many secondary metabolites. The augmentation of PPtase activity disequilibrated the homeostatic biosynthesis of these HSCoA related products, which leads to the activation or inactivation of many secondary metabolite biosynthesis pathways. In the 33 strains tested, 11 PPtase strains (33%) show eliminated or diminished metabolites, while 23 strains (70%) resulted in an increased or activated production of metabolites. This suggests that bidirectional regulation from enhancing the phosphopantetheinylation activity occurs frequently, albeit with a bias to activation. It is worth noting that the synergistic combination between PPtase regulation and other parameters, such as growth conditions and temperature, can be applied to produce new metabolite profiles, making this PPtase-based approach effective for the activation of cryptic/silenced pathways. Finally, unlike the approaches requiring genome information, this approach of delivering PPtase into bacteria through conjugation is sequence-independent and highly efficient, which will adapt very well for high throughput purposes.

In summary, we have shown the significance of modulating the phosphopantetheinylation modification of carrier protein on metabolite biosynthesis. Overexpression of PPtase into *Actinomycetes* can efficiently activate silenced/cryptic metabolites. By extending this approach into a large sample collection, a total of 23 of 33 tested *Actinomycetes* strains generated increased or activated products compared to controls. This very high ratio (70%) demonstrated the broad utility and adaptability of this approach in discovery of silenced/cryptic natural products. With these results, we successfully identified five previously unproduced polyketide compounds including two novel structures from two strains. Our work filled a gap in the protein modification-level regulation on pathway activation, which will be useful for the discovery and study of secondary metabolite production.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.7b00225.

Experimental methods and procedures; supplementary tables providing cytotoxic activity data, deduced ORFs, bacterial strains and plasmids, primers used, and NMR data; figures providing PPtase genes analysis, *in vitro* analysis of the PPlation activity, HPLC analysis of the metabolites change in PPtase strains, electrophoretic analysis of recombinant proteins, PCR verification of the ovmK disrupted mutant, and NMR spectra (PDF)

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### Notes

The authors declare no competing financial interest.

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