Puromycin A, B and C, cryptic nucleosides identified from *Streptomyces alboniger* NRRL B-1832 by PPtase-based activation

Xiaoli Yan [a, 1], Benyin Zhang [b, 1], Wenya Tian [a], Qi Dai [a], Xiaoqin Zheng [a], Ke Hu [a], Xinxin Liu [a], Zixin Deng [a], Xudong Qu [a, c, *]

[a] *Key Laboratory of Combinatorial Biosynthesis and Drug Discovery Ministry of Education, School of Pharmaceutical Sciences, Wuhan University, China*
[b] *State Key Laboratory of Plateau Ecology and Agriculture, Qinghai University, China*
[c] *Jiangshu National Synergetic Innovation Center for Advanced Materials (SICAM), China*

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ABSTRACT

Natural product discovery is pivot for drug development, however, this endeavor is often challenged by the wide inactivation or silence of natural products biosynthetic pathways. We recently developed a highly efficient approach to activate cryptic/silenced biosynthetic pathways through augmentation of the phosphopantetheinylation of carrier proteins. By applying this approach in the *Streptomyces alboniger* NRRL B-1832, we herein identified three cryptic nucleosides products, including one known puromycin A and two new derivatives (puromycin B and C). The biosynthesis of these products doesn't require the involvement of carrier protein, indicating the phosphopantetheinyl transferase (PPtase) indeed plays a fundamental regulatory role in metabolites biosynthesis. These results demonstrate that the PPtase-based approach have a much broader effective scope than the previously assumed carrier protein-involving pathways, which will benefit future natural products discovery and biosynthetic studies.

1. Introduction

Natural products are a major source for drug discovery and development. It is revealed that more than 50% small molecule drugs (including pesticides) are from natural products or their derivatives [1,2]. Although the discovery of natural products is pivot for drug development, the rate of identification of useful molecules has been steadily declined since from the late of last century [2,3]. One of major reason for that is the widely inactivation and silence of microbial biosynthetic pathways [2,4]. It is estimated that more than 90% of microbial metabolites in the genome are indeed not encoded under normal laboratory culturing conditions due to the absence of essential regulation signals to trigger their biosynthesis [5]. Metabolites biosynthesis depends on orchestrated regulations of biomachineries involving translation from DNA to working

proteins [6]. The inactivation or silence of natural products biosynthesis is majorly resulted by missing or improper regulations during that process [2,4–6].

So far a few of strategies targeting on altering epigenetic (eukaryote only) [7,8], transcriptional [9] and translational regulation [10,11] as well as elicitation [12–14] have been developed to successfully activate natural products biosynthesis. Very recently, we developed an additional approach from the aspect of protein-modification level regulation to awake cryptic/silenced biosynthetic pathways [15]. Biosynthesis of polyketide (PK), nonribosomal peptide (NRP) and fatty acid (FA) as well as a few primary metabolites including lysine and tetrahydrofolate are depended on a conserved protein modification that requires phosphopantetheinyl transferase (PPtase) to convert carrier proteins (CPs) from inactive *apo*-form into active *holo*-form [16]. By augmentation of the phosphopantetheinylation *in vivo*, we observed a significantly high number of strains (70%) from 33 tested *Actinomycetes* produce activated metabolites [15]. Isolation of activated products from two strains resulted to successfully identify two groups of cryptic polyketide products, confirming its high efficiency to activate CP involving metabolites [15]. By continued application of this approach, we herein confirmed that the effective scope of this

* Corresponding author. Key Laboratory of Combinatorial Biosynthesis and Drug Discovery Ministry of Education, School of Pharmaceutical Sciences, Wuhan University, China.
E-mail address: quxd@whu.edu.cn (X. Qu).
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[1] These authors contributed equally.

PPtase-activation approach can be indeed extended to non-CP involving pathways. Overexpression of the broad-selective PPTases into the *Streptomyces alboniger* NRRL B-1832 resulted in activation and identification of three nucleosides products puromycins (A-C) whose biosynthesis doesn't require involvement of CP. This result demonstrates the very broad-utility of this approach which will benefit further natural products discovery and biosynthetic studies.

2. Materia and methods

2.1. General experimental details

NMR spectra were measured on a Bruker Avance DRX-600 spectrometer ([1]H: 600 MHz, [13]C: 150 MHz). HPLC Analysis was carried out on an SHIMADZU LC-20A Prominence HPLC system with a column of Diamonsil (C18 (2), 5 μm, 250 × 4.6 mm, Dikma Technologies Inc.). Semipreparative HPLC was performed with a ZOR-BAX SB-C18 5 μm column (9.4 × 250 mm); HPLC-MS analysis was carried out on a Thermo Instruments HPLC system connected to LCQ Fleet electrospray ionization (ESI) mass spectrometer (ThermoFisher Scientific Inc.). HPLC-ESI-high resolution MS (HPLC-ESI-HRMS) analysis was carried out on ESI-LTQ Orbitrap (ThermoFisher Scientific Inc.). Sephadex LH-20 (25−100 μm, Pharmacia Biotek, Denmark) was used for column chromatography. All solvents used were analytical or HPLC grade. *S. alboniger* was cultivated either on the MS agar (agar 2 g, mannitol 2 g, soya flour 2 g in 100 mL tap water) or tryptic soy broth (TSB) for growth.

2.2. Construction of S. alboniger NRRL B-1832-PPTase and the control strain

Following the previous developed procedure, the empty vector pIB139 and pWHU2449 [15] which carries the gene cassette *ermE**-*sfp-svp* were introduced into *S. alboniger* NRRL B-1832 through conjugation to yield the control strain and PPTase overexpression strains, respectively [15].

2.3. Fermentation and metabolites profile analysis

S. alboniger NRRL B-1832 and recombinant strain *S. alboniger* NRRL B-1832-PPTase were cultured in media TSB for 2 days, then inoculated in IWL-4 medium (soluble starch 1 g, K_2HPO_4 0.1 g, $MgSO_4$ 0.1 g, NaCl 0.1 g, $(NH_4)_2SO_4$ 0.2 g, $CaCO_3$ 0.2 g, $FeSO_4$ 0.1 mg, $MnCl_2·6H_2O$ 0.1 mg, $ZnSO_4$ 0.1 mg, yeast extract 5.0 mg, Tryptone 100 mg, in 100 mL ddH_2O, pH = 7.2; yeast extract and tryptone were purchased from Angel Yeast Co. Ltd.) and cultured for 5 days at 28 °C. After fermentation, the liquid culture broth was extracted with the equal volume ethyl acetate under sonication for 20 min. Organic phase was transferred and dried by vacuum at low temperature (30 °C). Metabolites were subsequently redissolved by 1 mL ethanol and filtrated by a 0.22 μm membrane to remove particles before HPLC or HPLC-MS analysis. For sample analysis, a SHIMADZU LC-20A Prominence HPLC system was used. The flow-rate was 1 mL min⁻¹ and the column temperature was maintained at 25 °C. The mobile phase consisted of a mixture of acetonitrile (B) and 5 mM NH_4Ac aqueous (A). The gradient elution was employed as follows: 5−100% (v/v) B at 0−30 min; 100% B at 30−33 min; 100-5% B at 33−34 min; 5% B at 34−40 min.

2.4. Cytotoxic activity assay

Two human cancer cell lines (HL60, NB4) were seeded at a density of $3-5 × 10^3$ per well in 96-well plate and incubated overnight, and then treated with compounds **1**−**3** in various concentrations, solvent DMSO (<0.1%) was used as a negative control, doxorubicin (2 μM) was used as a positive control. After 48 h treatment, the viability was determined using a CCK-8 kit according to the manufacturer's instructions. The 50% inhibiting concentration (IC50, μM) was calculated by SPSS software version 13.0.

3. Results

3.1. Activation and isolation of metabolites in S. alboniger NRRL B-1832

To activate metabolites in the *S. alboniger* NRRL B-1832, two broad-selective PPtase genes, *sfp* and *svp* from the *Bacillus subtilis* and *S. verticillus* respectively [15] were introduced in a way of *ermE**-*sfp-svp* (ermE* is a strong constitutive promotor) into the strain through conjugation (see 2.2). Analysis of the fermentation broth of the wild-type, control strain which has an empty pIB139 integrated in the genome, and PPTase strains revealed that a number of new peaks were activated and produced by overexpression of the PPTase genes (Fig. 1b). To isolate the major products, a large scale fermentation was employed. NRRL B-1832-PPTase was cultured in IWL-4 medium (100 × 100 mL) within 300 mL baffled shake flasks, each containing a loop of a 20 cm spring (diameter 1 cm) at 28 °C and 200 rpm for 5 days. The culture broth was extracted three times with equal volume ethyl acetate at room temperature. The filtrate was evaporated *in vacuo* to obtain crude extract, which was further subjected to Sephadex LH-20 gel column (20 × 1800 mm) separation and eluted successively with methanol. The eluted fractions containing compounds **1** (6.3 mg), **2** (3.3 mg) and **3** (4.2 mg) were combined and semi-prepared on an SHIMADZU LC-20A Prominence HPLC system using Agilent ZOR-BAX SB-C18 (5 μm, 250 × 9.4 mm, Agilent Technologies Inc.) at a flow rate of 3 mL min⁻¹ over a 40 min gradient program with 5 mM NH_4Ac as eluent A.

3.2. Structural characterization of puromycin A-C

Compound **1** was obtained as a white amorphous powder. Interpretation of the ^{1}H, ^{13}C NMR, HSQC, HMBC and COSY spectroscopic data, and ESI-HRMS data showed compound **1** was puromycin [17], which is an aminonucleoside antibiotic reported in 1952 [18]. It is a broad spectrum secondary metabolite active against Gram-positive bacteria, protozoans, and mammalian cells, including tumor cells [19] by inhibiting both 70s and 80s ribosomes [20].

Compound **2** was obtained as a white amorphous powder. The ^{1}H and ^{13}C NMR spectrum of **2** are similar to those of **1**, showed the presence of structural skeleton of puromycin. However, in the ^{1}H NMR spectrum of **2**, there appears a methyl signal at δ_H 1.95, meanwhile, two carbon signal at δ_C 21.8 and 170.9 appears in the ^{13}C NMR spectrum. The HMBC correlations of proton at δ_H 1.95 to carbons at δ_C 54.9 and 170.9 suggested the presence of an acetyl group linked at amino of tyrosine group. On the basis of these data and ESI-HRMS m/z 514.2432 $[M + H]^+$ (calcd for 514.2409, $C_{24}H_{32}N_7O_6$), the compound **2** was shown in Fig. 1a and named puromycin B (Fig. 1a and Table 1).

Compound **3** was obtained as a white amorphous powder. ESI-HRMS data showed the mass 556.2544 $[M + H]^+$ (calcd for 556.2514, $C_{26}H_{34}N_7O_7$). The molecular weight of **3** is 43 greater than **2**, suggesting it bears an additional acetyl group, which is also revealed from the comparison to ^{1}H NMR and ^{13}C NMR spectrum of 2, there appear additional signals for methyl group at δ_H 1.99 and two carbons at δ_C 20.1 and 169.6. We thereby confirmed that another acetyl group was linked to the glycosyl group by the HMBC
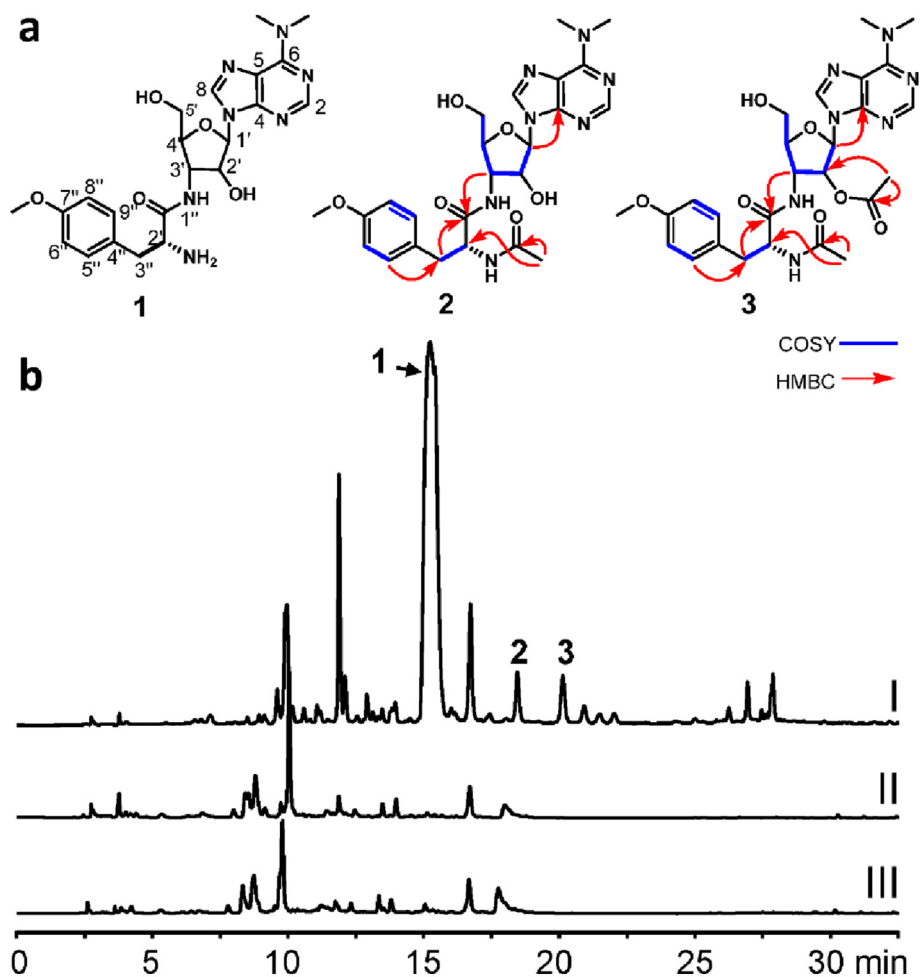
Fig. 1. Activation of puromycin biosynthesis in *S. alboniger* NRRL B-1832 by overexpression of the PPtases. (a) Structures of compounds **1**–**3** and key ^{1}H-^{1}H COSY and HMBC correlations for **2** and **3**; (b) HPLC traces show the metabolites profiles in the PPtase strain (I), control strain (II) and wild-type strain (III).

Table 1
NMR data of compounds **2** and **3** in a mixed solvent of DMSO-d_6 and CD$_3$OD-d_4 (ration is 1:1).

Position	2		3	
	δ_H (multi, J in Hz)	δ_C	δ_H (multi, J in Hz)	δ_C
2	8.28, s	152.0	8.30, s	152.2
4		149.8		150.0
5		120.3		120.0
6		154.9		154.7
8	8.41, s	138.0	8.18, s	138.2
6-N-(CH$_3$)$_2$	3.56, brs	37.9	3.56, brs	39.7
1′	6.05, d (3.1)	90.3	6.05, d (3.1)	87.0
2′	4.63−4.65, m	73.7	4.73, t (6.7)	74.3
3′	4.60−4.62, m	50.9	4.48−4.50, m	49.4
4′	4.05, ddd (7.2, 3.4, 2.3)	83.7	3.93 ddd (7.2, 3.4, 2.3)	83.6
5′	3.84, dd (12.4, 2.2)	61.2	3.62, dd (12.4, 2.2);	61.2
	3.62, dd (12.4, 3.5)		3.44, dd (12.4, 3.5)	
1″		172.4		172.0
2″	4.68, dd (8.4, 6.5)	55.0	4.49, dd (8.4, 6.5)	54.9
3″	3.07, dd (13.8, 6.5)	37.3	2.87, dd (13.8, 6.5)	37.1
	2.91, dd (13.8, 8.4)		2.71, dd (13.8, 6.5)	
4″		129.5		129.7
5″, 9″	7.26, d (8.4)	130.4	7.13, d (8.4)	130.4
6″, 8″	6.93, d (8.4)	113.8	6.80, d (8.4)	113.7
7″		158.6		158.4
7″-O-CH$_3$	3.83, s	54.8	3.80, s	54.5
2″-NH-CO		170.9		170.0
2″-NH-CO-CH$_3$	1.95, s	21.8	1.77, s	22.1
2′-O-CO				169.6
2′-O-CO-CH$_3$			1.99, s	20.1

Table 2
Cytotoxic activity of compounds **1**–**3**.

Compound	HL60 (IC50, μM)	NB4 (IC50, μM)
1	0.11	0.03
2	>100	>100
3	>100	>100

correlations from the proton at δ_C 1.99 to the carbons at δ_C 169.6 and δ_C 74.3. Thus, the compound **3** was shown in Fig. 1a and named puromycin C (Fig. 1a and Table 1).

2.3. Bioactivity assay of puromycin A-C

All of the isolated compounds were further tested for their cytotoxicity against two human cancer cell lines (HL60, NB4) (Table 2). It was observed that compound **1** exhibited powerful cytotoxic activity to HL60 and NB4 with IC_{50} values of 0.11 and 0.03 μM, respectively, while two other new compounds **2**, **3** showed no apparent activity.

3. Discussion

The biosynthetic pathway of puromycin has been extensively studied [21—25]. Its biosynthesis starts from the conversion of ATP. Through the sequentially catalysis of Pur10, Pur4, Pur7 and Pur3, ATP was converted to the pivot intermediate 3′-amino-3′-dA. 3′-amino-3′-dA was further accepted by Pur6 and condensed with the carbonyl group of tyrosine to form the amide intermediate. Following with post-modification by tailoring enzymes (Pac, Pur5, DmpM, NapH), this intermediate was finally converted into puromycin A. Surprisingly, from the biosynthetic pathway, the puromycin biosynthetic machinery indeed doesn't have any acyl carrier protein. Thus the production of compounds **1**–**3** must be activated through a different mechanism from oviedomycin [15].

Except for CP-involving products, phosphopantetheinylation is also essential for the biosynthesis of lysine and tetrahydrofolate [26,27]. These central primary molecules including HSCoA play very important roles in metabolite biosynthesis, and their variation will influence many secondary metabolite biosynthesis pathways. More so, the variation of FA, PK and NRP which are used as components of the cell membrane, signal transmitters (e.g., quorum sensor) or nutrients scavengers (e.g., siderophores) can also cause physiological change and metabolic flux variation to stimulate other secondary metabolites biosynthesis [16,28]. We observed NRRL B-1832-PPtase has a slightly slow growth comparing to its wild-type strain (data not shown). This indicates the activation of *pum* may indeed result from the effect of primary metabolites production, e. g. FA biosynthesis. The activation of non-CP involving metabolites suggests that PPtase plays a fundamental role in regulation of primary and secondary metabolites biosynthetic networks through direct impact on HSCoA consumption and subsequently influence on HSCoA-related cascades. These results demonstrate that PPtase approach is broadly useful for switching on cryptic/silenced pathway through a diverse action of mode that can not only activate PK/NPR/FA but also other type of pathways.

4. Conclusion

In summary, we have identified three cryptic puromycin products from the *S. alboniger* NRRL B-1832 by PPtase-based activation. The two of puromycin products (B and C) are new and their activity were accessed which show no apparent activity to cancer cell lines. The successful activation of non-carrier protein involving pathways

confirmed that the PPtase-based approach indeed have a much broader effective scope than previously assumed. These results not only broaden the utility of this approach, but also suggest a fundamental role of the PPtase in metabolites biosynthesis regulation, which will benefit future natural products discovery and biosynthetic studies.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.synbio.2018.02.001.

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