Pyridoxal-5'-Phosphate-Dependent Enzyme GenB3 Catalyzes C-3',4'-Dideoxygenation in Gentamicin Biosynthesis

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Abstract

Background: C-3',4'-dideoxygenation structure in gentamicin can prevent deactivation by aminoglycoside 3'-phosphotransferase (APH(3')) in drug-resistant pathogens. However, the enzyme catalyzing the dideoxygenation step in the gentamicin biosynthesis pathway remains unknown.

Results: Here, we report GenP catalyzes 3′ phosphorylation of gentamicin biosynthesis intermediates JI-20A, JI-20Ba, and JI-20B. We further demonstrate that a pyridoxal-5′-phosphate (PLP)-dependent enzyme GenB3 uses these phosphorylated substrates to form 3',4'-dideoxy-4',5'-ene-6'-oxo products. The following C-6' transamination and GenB4 catalyzed reduction of 4',5' olefin lead to the formation of gentamicin C. To the best of our knowledge, GenB3 is the first PLP dependent enzyme catalyzing dideoxygenation in aminoglycoside biosynthesis.

Conclusions: This discovery solves the long-standing puzzle in gentamicin biosynthesis, also enriches the chemistry of PLP dependent enzymes. Interestingly, these results demonstrate that to evade APH(3') deactivation from the pathogens, the gentamicin producers evolved a smart strategy, which utilized their own APH(3') to activate hydroxyls as leaving groups for the 3',4'-dideoxygenation in gentamicin biosynthesis.

Keywords: gentamicin; C-3',4'-dideoxygenation; phosphotransferase; pyridoxal-5′-phosphate (PLP)-dependent enzyme

Background

Aminoglycoside antibiotics (AGAs) have been available for clinical use since the 1940s and have been shown to exhibit broad-spectrum activities[1]. However, the spread of resistance is limiting their use in clinic, which is primarily induced by aminoglycoside-modifying enzymes (AMEs). The amino and hydroxyl groups of AGAs are deactivation targets of AMEs[2,3]. Because deoxygenations remove the modifiable groups of AGAs, deoxy functionalities are natural defensive strategies that can prevent deactivation by the AMEs[4]. C-3' deoxygenation presenting in tobramycin, apramycin, and lividomycin can evade modification by aminoglycoside

3'-phosphotransferase (APH (3'))[5]. C-3',4'-dideoxygenation structural elements in gentamicin, fortimicin, and istamycin can prevent deactivation by APH(3') and adenyltransferase (ANT(4'))[6]. Some semi-synthetic antibiotics have utilized the dideoxygenation structure to increase their efficacies, such as dibekacin (3',4'-dideoxykanamycin B)[7], arbekacin (1-N-((S)-4-amino-2-hydroxybutyryl)-3',4'-dideoxy-kanamycin B)[8,9], and the newly approved sisomicin derivative, plazomicin, which retains antibiotic activity toward most AMEs[10,11].

The C-3' deoxygenation biosynthetic process has recently been dissected by several research groups[12-14]. A radical S-adenosyl-L-methionine (SAM) enzyme AprD4, along with AprD3, is responsible for the 3'-deoxygenation in apramycin biosynthesis[15]. Gentamicin is a broad-spectrum aminoglycoside bactericidal antibiotic featuring the C-3',4' dideoxygenation moity. As with other 2-deoxystreptamine-containing AGAs, significant progress has been made in gentamicin biosynthesis[16-20]. However, little detail of the featured C-3',4' dideoxygenation process is known. (Figure 1)

The APH(3') family catalyzes the transfer of the gamma-phosphoryl group from ATP to the 3'-hydroxyl group of AGAs. Hence, the APH gene is thought to have originated as a self-defense mechanism used by microorganisms that produce AGAs[21]. Genetic studies have shown that an APH(3') GenP is involved in the aminoglycoside dideoxy process in gentamicin biosynthesis[22], but details remain unknown for the dideoxy process. A previous biochemical study showed that GenP can phosphorylate C-3' of an unnatural substrate, kanamycin B[23]. Nevertheless, the enzymatic properties for its natural substrates have remained unclear. In the present study, we demonstrate that the phosphorylation catalyzed by GenP, is the first step in the gentamicin C-3',4'-dideoxy process. More importantly, we show that a PLP-dependent enzyme GenB3 uses the products of GenP to catalyze the dideoxygenation, forming 3',4'-dideoxy-4',5'-ene-6'-oxo products. The following transamination and reduction by GenB4 finish the gentamicin biosynthesis. (Figure 1)

Results and discussion

GenP starts the C-3',4'-dideoxy process in gentamicin biosynthetic pathway

To investigate the function of GenP in gentamicin biosynthesis, the intermediate metabolites of the biosynthetic pathway, JI-20A, JI-20Ba, and JI-20B, were used as substrates. They are the starting compounds for C-3',4'-dideoxy process in gentamicin biosynthesis. The results of high-performance liquid chromatography using evaporative light-scattering detection (HPLC-ELSD) are shown in Figure 2a. GenP converted all of these substrates into new products. Products of the reactions were analyzed by mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectrometry. The ESI-MS spectrum suggested that GenP catalyzed the formation of monophosphorylated products (Figure 2). Additionally, ^{1}H, ^{13}C, HHCOSY and ^{31}P NMR results of compound **2** confirmed that the product had a C-3'-OH phosphorylation moiety (Figure S1). Further experiments showed that GenP had promiscuous substrate specificities. In addition to phosphorylate the other two intermediate metabolites of the gentamicin biosynthetic pathway, X2 and G418, giving phosphorylated compound **4** and **5** (Figure S2), GenP can also phosphorylate kanamycin B as previously reported[15]. However, GenP has a higher specificity toward JI-20A and JI-20Ba (Table S1).

GenB3 completes the C-3',4'-dideoxygenation process

We then investigated the proteins with unknown function in gentamicin biosynthesis. Bioinformatic analysis showed that GenB3 is a PLP-dependent aminotransferase. GenB3 has an approximately 30% identity with other aminotransferases in gentamicin biosynthesis pathway, such as GenB1, GenB2, and other AGAs C-6' aminotransferases. To investigate the function of *gen*B3, gene-disruption experiments were performed. The disruption strain, Δ*gen*B3, did not produce C-3',4'-deoxygenated products, but instead it accumulated non-deoxygenated products, including JI-20Ba and JI-20B (Figure 3a). These gene-disruption results demonstrate that GenB3 is involved in the formation of gentamicin C-3',4'-dideoxygenation.

To further investigate the role of GenB3, the recombinant protein was expressed and purified in *E. coli*. GenB3 did not show obvious activity on JI-20Ba. We

suspected that C-3'-phosphorylation-JI-20Ba (**2**) might be the substrate of GenB3 and the phosphate group could be hydrolyzed in the experiments with disruption strain. Indeed, HPLC-ELSD analysis showed that GenB3 converted **2** to a new compound **6** (Figure 3c), which was characterized to be 6'-oxo-verdamicin by MS (Figure 3e), ^{1}H and ^{13}C NMR (Figure S3). The results demonstrate that GenB3 catalyzed the C-3'-dephosphation and C-4',5'-dehydratation of **2**. To gain more evidences on the keto group on C-6', $NaBH_4$ was added to the reaction mixture to reduce the keto group. HPLC-ELSD analysis showed that compound **6** disappeared after adding $NaBH_4$, two new peaks appeared as compounds **7** and **8** (Figure 3c). MS analysis revealed that their molecular masses were both 462, and were thus presumed to be C-6'-isomers of the hydroxyl group (Figure 3f, 3g).

When C-3'-phosphorylation-JI-20A (**1**) was used as a substrate, GenB3 catalyzed C-3'-dephosphation and C-4',5'-dehydratation to form **9** (Figure 3d). However, unlike with **6**, GenB3 further catalyzed the aminotransfer of C-6' to form sisomicin (**10**) (Figure 3h). This discrepancy may have been caused by the steric hindrance from the C-6' methyl group of compound **6**. Results of UV spectrometry confirmed that PLP of GenB3 was converted to pyridoxamine 5'-phosphate (PMP) during the enzymatic reaction (Figure 3b). Furthermore, no free ammonia was detected in the reaction solution (Figure S4). In addition, adding the amino acceptor, α-ketoglutarate, to the reaction mixture promoted GenB3-catalyzed reaction (Figure S5). These results indicate that the C-6' amino group of the substrate was transferred to PMP.

All of the tested substrates of GenB3 contain amino groups at both C-2' and C-6'. To identify which amino group is the functional group for PLP binding, we tested GenB3 activity toward substrates only containing one amino group at C-6' and C-2'. GenB3 did not catalyze C-2' amino group containing deoxygenation, but it did catalyze the C-6' amino group containing phosphorylation gentamicin B deoxygenation. Therefore, the C-6' amino group is the functional group for the GenB3-catalyzed reaction (Figure S6).

GenB3 first catalyzes the C-4',5'-dehydration in the C-3',4'-dideoxygenation

process

To further investigate the reaction mechanism of GenB3, the reaction condition was shifted from 30°C to 20°C, when C-3'-phosphorylation-JI-20Ba (**2**) was used as a substrate. A small amount of compound **11** was detected in the reaction (Figure 4a). MS results showed that it was a hydroxyl group at the 3' position of **11** (Figure 4c). We speculated that compound **11** was a reaction byproduct instead of an intermediate, because GenB3 did not further catalyze the 3'-hydroxyl deoxygenation of compound **11** with longer incubation time at 30°C. We assumed that the C-4',5' dehydration occurred first in the dideoxygenation process, after which the C-3' phosphate was removed. However, during unfavorable conditions for phosphate elimination, the C-3' phosphate bond may have been hydrolyzed, which would then form compound **11**. This speculative step is consistent with our *in vivo* results. Compound **11** was also detected in *gen*B4-disruption strains (Figure 4b). These results demonstrate that C-4',5' dehydration occurred first in the dideoxygenation process.

Plausiblemechanism of GenB3

Based on these results, we proposed the following mechanism for the GenB3 catalyzed dideoxygenation reaction (Scheme 1). As other PLP-dependent enzymes, the reaction starts with an external aldimine **12** generated from the 6'-amine of compound **2** with the internal aldimine of the enzyme with PLP[24]. The 4'-hydroxyl group of aldimine **12** may be deprotonated by a basic residue and then attacks the adjacent phosphate group on the 3'-C, forming a more stable cyclic phosphodiester. By this means, the 4'-hydroxyl was activated by the enzyme as a good leaving group, which is a prerequisite for a γ-elimination. A following deprotonation would convert the external aldimine **13** to the quinonoid **14**[25]. Then a classical PLP dependent γ-elimination can happen, giving 4'-deoxyl-4',5'-olefin quinonoid **16**[26-28]. The 3'-phosphate group facilitates the second dehydroxylation as a good leaving group to yield aldimine **17**. Protonation at 3'-C of aldimine **17** affords ketimine **18**. Hydrolysis of ketimine **18** would give keto product compound **6** and the cofactor as PMP. This mechanism was supported by the isolation of 3'-hydroxyl intermediate compound **11**

from GenB3 reaction, which should be the hydrolysis product of intermediate **16**.

GenB3-catalyzed products need further aminotransferation and reduction to produce components of gentamicin C. Although the intact C-6' aminotransferases, GenB1 and GenB2, existed in the *gen*B4 disruption strain, the main product of the *gen*B4-disruption strain was the 6'-oxo-containing compound **6** (Figure 4b), which indicated that the C-6' aminotransferase was inefficient for compound **6** *in vivo*. We have also proved that GenB4 could convert C-6'-amino-containing **6** (verdamicin C2 and verdamicin C2a) into gentamicin C2a, when GenB4 coupled with aminotransferases[29,30]. However, when we incubated **6** with purified GenB4, the C-4',5' olefin reduction was undetectable via HPLC-ELSD (Figure S7). These results demonstrate that the C-6' amino group was a prerequisite for GenB4-catalyzed C-4',5' olefin reduction.

Conclusions

In summary, the results presented here firmly demonstrate the functions of both GenP and GenB3 in gentamicin biosynthesis. GenB3 appears to be the first reported PLP-dependent enzyme catalyzing dideoxygenation in aminoglycoside biosynthesis. Interestingly, structurally related C-3' deoxygenation and C-3',4'-dideoxygenation of AGAs are catalyzed by distinct catalytic pathways. C-3' deoxygenation is catalyzed through a radical mechanism by the radical SAM dehydratase, AprD4, along with the reductase partner, AprD3[12-15]. Although an AprD3 homologue was identified in the gentamicin pathway, GenB3-catalyzed dehydration and 3'-phosphate elimination do not require a reductase partner. Instead, this process behaves in an "aminotransferase-like" manner, which is similar to that with dehydratase ColD from the biosynthetic pathway of L-colitose[31,32]. PLP-dependent amino-transferases GenB1, GenB2, GenB3, and GenB4 not only have the common promiscuous activity to catalyze C-6' aminotransfer as reported[29], but also have their unique functions in gentamicin biosynthesis, which demonstrates the diversity of PLP chemistry in enzymatic catalysis.

AMEs exist in both resistant pathogens and AGAs producers[33]. It has been

speculated that these enzymes may perform other metabolic functions[21]. In the present study, we have demonstrated that gentamicin producers evolve a smart strategy to evade APH(3') deactivation from pathogens. The gentamicin biosynthetic pathway utilizes its APH(3') to activate hydroxyls as leaving groups, then PLP-dependent enzyme GenB3 catalyzes dideoxygenation. The unveiling of the C-3',4'-dideoxygenation pathway of gentamicin may pave the way for dissection of other sugar dideoxygenation. As fortimicin and istamycin share the same C-3',4' dideoxygenation with gentamicin, and their biosynthetic gene clusters have identical enzymes with GenP and GenB3/GenB4[34], their deoxygenation pathways may be identical with one another.

The APH(3')-activated dideoxygenation pathway of gentamicin provides inspiration for chemical regiospecific removal of oxygen from sugars[35]. Since GenP, GenB3, and GenB4 catalyze C-3',4' dideoxygenation, they have the potential to be applied in combinatorial biosynthesis of C-3',4' dideoxygenation containing semisynthetic antibiotics, such as dibekacin and arkekacin[36], to develop novel anti-infective drugs against life-threatening pathogens and to yield an improved safety profile[37] Because C-3', C-4', and C-6' are frequently used chemical-modification sites for semi-synthesis of AGAs[38-40], GenP, GenB3, and GenB4 may be used for combined chemical and enzymatic syntheses of promising therapeutic leads.

Methods

Strains and culture conditions

E. coli Top10 was used as cloning hosts; *E. coli* ET12567 (pUZ8002) was used for *E. coli- Streptomyces* conjugation; *E. coli* BL21 (DE3) was used for protein expression. *E. coli* strains were grown in LB media (typtone 10 g, yeast extract 5 g, NaCl 10 g, 1 L of distilled water) at 37°C via antibiotic selection (100 μg/ml ampicillin, 50 μg/ml apramycin, 25 μg/ml chloramphenicol, 50 μg/ml kanamycin). *Pfu* DNA polymerase was obtained from Vazyme, while GC buffer and dNTPs were obtained from Takara. T4 DNA ligase and DNA Marker were purchased from Takara. Gene sequencing was performed by Synbio Technologies. *Micromonospora*

echinospora wild-type and mutant strains were grown in media (soluble starch 10 g, Wheat bran 10 g, $MgSO_4.7H_2O$ 5 g, $K_2HPO_4.3H_2O$ 0.3 g, KNO_3 1 g, NaCl 0.5g, $CaCO_3$ 1 g, Asparagine 0.02 g, 1 L, Agar 2.5 g) at 37°C to obtain *Streptomyces* spores. For collection of gentamicin, *Micromonospora echinospora* wild-type and mutant strains were cultured in seed cultures (soluble starch 15 g, Soybean powder 1 g, Glucose 1 g, KNO_3 0.5 g, $CaCO_3$ 23 g, 1 L) at 34°C for 36 h. Fermentation cultures (soluble starch 50 g, Soybean powder 35 g, Glucose 15 g, Corn powder 15 g, Peptone 15 g, $(NH_4)_2SO_4$ 0.5 g, KNO_3 0.5 g, $CoCl_2.6H_2O$ 0.01 g, $CaCO_3$ 26 g, 1 L) were conducted for 120 h.

Construction of gene-disruption plasmids

For gene disruption, about 2000-bp upstream and downstream of the gene were amplified from the genomic sequence (see list of Primers used in the Supplemental Information). PCR products (94°C for 5 min; 94°C for 1 min; 60°C for 45 s; 72°C for 2 min; 72°C for 10 min, 30 cycles) were cloned into the *E. coli-Streptomyces* vector pKC1139 or pD2925 (a plasmid derived from pIJ2925) to obtain the gene-disruption plasmids, pKCP (*gen*P) and pDB3 (*gen*B3). All of the plasmids were verified by sequencing. (Related plasmids are shown in Table S3)

Gene disruption of *gen*P, *gen*B3 and *gen*B4

To obtain the mutant strains of Δ*gen*P and Δ*gen*B3, the gene-disruption plasmids, pKCP and pDB3, were introduced into the wild-type strain by conjugation from *E. coli* ET12567 (pUZ8002, the gene disruption plasmid) to *Streptomyces.* Apramycin-resistant (Apr^R) colonies were screened and confirmed by PCR amplification with checking primers. The Apr^S colonies were selected from the initial Apr^R colonies and confirmed by PCR amplification with checking primers (Supplemental Figure S8).

Gene complementation of Δ*gen*P, Δ*gen*B3 and Δ*gen*B4

Complementation plasmids contained the complete fragment of the gene under the control of the P_{hrdB} promoter. The complementation plasmids were prepared by cloning *gen*P and *gen*B3 into pEAP1 (a plasmid derived from pSET152) under the control of the P_{hrdB} promoter. The complementation plasmid was introduced

individually into the mutant strain by conjugation. Complemented strains were based on erythromycin resistance (100 μg/ml) and were confirmed by PCR amplification.

Extraction, isolation, and purification of gentamicin-C complexes and intermediates

Fermentation products of wild-type and mutant strains were adjusted to a pH of 2.0 with H_2SO_4. The supernatant after centrifugation (5,000 r/min, 10 min, room temperature) was adjusted to a pH of 7.0 with NaOH. The fermentation broth was centrifuged again, and the supernatant was adsorbed by cationic resin D152 adsorption at 37°C for 3 h. The adsorbed resin was packed into a column. The column was washed with 10 times the column volume of 0.01 mM to 0.2 M $NH_3 \cdot H_2O$. The eluate was freeze-dried, re-dissolved in 1 ml of water, and filtered through a 0.22 μm microporous membrane before subjection to HPLC-ELSD analysis.

Construction of gene-expression plasmids in *E. coli*

*gen*P, and *gen*B3 genes were amplified from the DNA of *M. echinospora* by PCR amplification (see the list of primers used in Table S2). The PCR products were digested with *Nde*I and *Hin*dIII and were inserted into pET28a(+) to obtain gene-expression strains. Each plasmid was transformed into *E. coli* BL21 (DE3). The expression plasmids were verified by DNA sequencing.

Gene expressions and purifications of GenP and GenB3

E. coli BL21 (DE3) containing the expression plasmid was cultured in LB (50 μg/ml kanamycin) at 37°C to at an OD_{600} = 0.6–0.8 and was induced by isopropylthiogalactoside (IPTG, 0.1 mM) at 16°C for 20 h. The cells were collected by centrifugation and resuspended in 20 ml of binding buffer (0.5 M NaCl, 20 mM Tris-HCl at a pH of 8.0). The recombinant protein was obtained by sonication for 20 min (3 s on, 5 s off), the supernatant was obtained by centrifugation at 4°C for 20 min. The recombinant protein was purified by Ni^{2+}-charged His-Bind resin (GE Healthcare). Impurities were eluted by washing buffer A (0.5 M NaCl, 20 mM Tris-HCl, 20 mM imidazole), washing buffer B (0.5 M NaCl, 20 mM Tris-HCl, 40 mM imidazole) and washing buffer C (0.5 M NaCl, 20 mM Tris-HCl, 60 mM imidazole), The recombinant proteins were eluted by elution buffer (0.5 M NaCl, 20

mM Tris-HCl, 200 mM imidazole). Imidazole in the eluted proteins was removed by dialysis. The purified proteins were stored at -30°C.

Preparation of substrates for in-vitro catalytic reactions

Different reaction substrates were obtained by fermentation of different mutant strains. Gentamicin X2 was obtained from the fermentation broth of the *M. echinospora* Δ*gen*KΔ*gen*Q mutant strain. G418 was purchased from the Sigma. JI-20A was isolated from the fermentation broth of Δ*gen*KΔ*gen*P. JI-20Ba and JI-20B were isolated from the fermentation broth of *M. echinospora* Δ*gen*B1Δ*gen*P. Ver C2a was isolated from the fermentation broth of *M. echinospora* Δ*gen*B4. For the separation of these compounds, refer to Method 5.

GenP-catalyzed reaction with JI-20A, JI-20Ba, and JI-20B

The GenP activity assays contained substrate (200 μM), GenP (15.8 μM), ATP (10 mM), and $MgCl_2$ (10 mM) in 500 μl Tris-HCl buffer (50 mM, pH 8.0). Incubations were at 37°C 1 h and were quenched by addition of chloroform (500 μl), followed by centrifugation to remove proteins. The supernatants were filtered through 0.22 μm microporous membranes before subjection to HPLC-ELSD analysis.

Separation and purification of compound 1 and 2

In order to obtain a large amount of **1** and **2**, the volume of GenP-catalyzed reaction was expanded to 10 mL, including substrates (JI-20A or JI-20Ba, 4 mM), GenP (25 μM), ATP (100 mM), $MgCl_2$ (50 mM), and Tris-HCl buffer (50 mM, pH 8.0). Incubations were at 30°C overnight. The reaction was ended in a boiling water bath and centrifuged at 10,000 rpm for 10 min. The supernatant was then passed through a column of D152 (5 ml) at a flow rate of 0.2 ml/min and unbound compounds were discarded. The compound-bound column was washed with water (100 ml) followed by gradient elution from 0.01 M to 0.06 mM (50 ml). Every fraction was checked by HPLC-ELSD, then, the eluates of compound **1** or **2** with higher purity are combined and lyophilized.

GenB3-catalyzed reaction with compound 1 and 2

The GenB3 activity assays contained substrate (200 μM), GenB3 (25 μM) amino donor (L-Glu, 10 mM), PLP (1 mM), in 500 μl KPi buffer (50 mM, pH 8.0, KOH).

Incubations were at 30°C overnight and were quenched by addition of chloroform (500 μl), followed by centrifugation to remove proteins. The supernatants were filtered through 0.22 μm microporous membranes before subjection to HPLC-ELSD analysis.

GenP and GenB3 catalyzes dideoxygenation with JI-20Ba

For enzymatic assays, 500 μl of reaction solution was used containing substrate (JI-20Ba, 200 μM), GenP (15.8 μM), GenB3 (12.5 μM), ATP (10 mM), $MgCl_2$ (10 mM), PLP (1 mM), α-ketoglutarate (10 mM), and Tris-HCl buffer (50 mM, pH 8.0). Incubations were at 30°C overnight and were quenched by addition of chloroform (500 μl), followed by centrifugation to remove proteins. The supernatants were filtered through 0.22 μm microporous membranes before subjection to HPLC-ELSD analysis.

HPLC-ELSD analysis of gentamicin C complex and related intermediates

HPLC-ELSD analysis of mixtures was performed with a Welch C18 column (4.6 × 250 mm, 5 μm) connected to SofTA Model 300s ELSD. In the GenP-catalyzed reaction, X2, G418, JI-20A, JI-20Ba, JI-20B and gentamicin B1 were used as substrates, the mobile phase was 0.2 M TFA (1 ml/min). In the GenB3-catalyzed reaction, compound **1** and **2** were used as substrates, the mobile phase was 92:8 (0.2 M TFA: methanol; 0.8 ml/min). In the GenB4-catalyzed reaction, Ver C2a and compound **6** were used as substrates, the mobile phase was 92:8 (0.2 M TFA: methanol; 0.8 ml/min). The mobile phase analysis of the fermentation of the strains was 98:2 (0.2M TFA: methanol; 0.8 ml/min).

Mass Spectrometry, ^{1}H and ^{13}C NMR analyses

MS analysis of the molecular formulas of compounds was performed via a micrOTOF-Q operator. ^{1}H and ^{13}C NMR analyses of compounds were performed via a Bucker 600MHz. Compounds were purified to 5–10 mg through the cationic resin D152, and were dissolved in 500 μl of D_2O.

^{31}P NMR analyses

^{31}P NMR analysis of compound **2** was performed via a Bucker 400MHz. Compound **2** was purified to 3 mg through the cationic resin D152, and was dissolved

in 500 μl of D_2O.

Figure titles and legends

Figure 1. Biosynthetic pathway of gentamicin.

Figure 2. Catalytic activity of GenP. (a) HPLC-ELSD analysis of GenP reactions with gentamicin JI-20A, JI-20Ba and JI-20B. (b-d) MS analysis of **1**, **2**, and **3**. MW, molecular weight.

Figure 3. GenB3-catalyzed dideoxygenation. (a) HPLC-ELSD analysis of mutant-strain fermentation products, showing the (i) wild-type, (ii) *gen*B3-disruption strain (Δ*gen*B3), and (iii) Δ*gen*B3: *gen*B3 (complementation of the Δ*gen*B3 mutant). (b) Ultraviolet-visible absorption spectrum of GenB3-catalyzed reaction at different reaction times, where UV absorption was measured every 2 min from 300–500 nm. (c) HPLC-ELSD analysis of (i) **2** standard with L-Glu, (ii) GenB3-catalyzed reaction without L-Glu, (iii) GenB3-catalyzed reaction with L-Glu, and (iv) with $NaBH_4$ added after the reaction. (d) HPLC-ELSD analysis of (i) the GenB3-catalyzed reaction with **1** was found to produce **9**, and (ii) L-Glu was added, as well as (iii) an L-Glu-added sisomicin standard (Sis). (e–h) MS analysis of **6**, **7**, **8**, and **10**.

Figure 4. Discovery of **11** in GenB3-catalyzed reactions *in vivo* and *in vitro*. (a) HPLC-ELSD analysis of GenB3-catalyzed reactions *in vitro* with **2** at (i) 30°C and (ii) 20°C. (b) HPLC-ELSD analysis of *gen*B4-disruption strains identified compound **11**. (c) MS analysis of **11**.

Scheme 1. Proposed mechanism for the GenB3-catalyzed 3',4'-dideoxygenation of JI-20Ba.

Additional file

Figure S1. Structure Identification of compound **2**. (a) ^{1}H NMR and ^{13}C NMR of compound **2**. ^{1}H NMR (600 MHz, D_2O) δ 3.42-3.21 (m, 1H), δ 1.19 (d, J = 12.6, 1H), δ 2.91 (ddd, J_1=12.3, J_2=9.8, J_3=4.2,1H), δ 3.42-3.21 (m, 1H), δ 3.58 (dd, J_1=7.0, J_2=2.0, 1H), δ 3.54 (t, J=9.2, 1H), δ 5.3 (d, J=3.8, 1H), δ 2.85 (m, 1H), δ 4.00 (ddd, J_1=10.3, J_2=8.7, J_3=7.5, 1H), δ 2.85-2.81 (m, 1H), δ 3.74 (dd, J_1=10.1, J_2=1.9, 1H), δ 1.9 (dq, J_1=13.0, J_2=3.9, 1H), δ 4.96 (d, J=3.8, 1H), δ 3.93 (dd, J_1=12.8, J_2=3.4, 1H),

δ 3.42-3.21 (m, 1H), δ 3.88 (dd, J_1=10.8, J_2=3.9, 1H), δ 2.97(d, J=10.8, 1H), δ 1.24 (d, J=6.9, 3H), δ 2.63 (s, 3H), 1.14 (s, 3H). ^{13}C NMR (151 MHz, D_2O) δ 50.53, 34.07, 49.19, 86.5, 70.18, 74.08, 99.64, 54.17 (d, J=4.8), 75.18 (d, J=5.3), 72.29, 85.98, 46.39, 100.18, 67.33, 63.83, 70.74, 67.42, 15.78, 35.51, 21.11. (b) ^{1}H-^{1}H-COSY NMR of **2**. (c) ^{31}P NMR of **2** (4.49 ppm, zero point calibration is 85% phosphoric acid solution).

Figure S2. HPLC-ELSD analysis of GenP reactions with gentamicin X2 and G418. (i) X2 standard; (ii) GenP-catalyzed reaction with X2; (iii) G418 standard; (iv) GenP-catalyzed reaction with G418.

Figure S3. ^{1}H and ^{13}C NMR of compound **6**. ^{1}H NMR (600 MHz, D_2O) δ 3.31-3.01 (m, 1H), δ 1.18 (d, J=12.2,1H), 2.15 (ddd, J_1=10.3, J_2=9.5, J_3=4.0, 1H), δ 3.32-3.11 (m, 1H), δ 3.44 (d, J=4.2, 1H), δ 3.55 δ (dd, J_1=7.0, J_2=2.0, 1H), δ 3.23 (t, J=9.2, 1H), δ 5.35 (d, J=2.2, 1H), δ 3.10-3.02 (m, 1H), δ 1.95 (d, J=12.2, 1H), δ 2.20 (ddd, J_1=10.5, J_2=7.0, J_3=4.0, 1H), δ 4.95-4.86 (m, 1H), δ 5.06 (d, J=4.0, 1H), δ 3.82 (dd, J_1=10.8, J_2=3.4, 1H), δ 2.62-2.51 (m, 1H), δ 4.05 (dd, J_1=10.8, J_2=3.9, 1H), δ 3.31 (d, J=10.8, 1H), δ 1.26 (d, J=6.9, 3H), δ 2.64 (s, 3H), δ 1.16 (s, 3H). ^{13}C NMR (151 MHz, D_2O) δ 50.76, 35.78, 49.17, 99.54, 74.35, 86.31, 100.5, 58.16, 29.93, 99.3, 145.8, 197.4, 83.7, 72.1, 63.81, 71.08, 67.86, 25.49, 33.94, 21.23.

Figure S4. Ammonia analysis of GenB3-catalyzed reaction with **4**.

Figure S5. GenP and GenB3 catalyzes dideoxygenation. HPLC-ELSD analysis of (i) GenP and GenB3-catalyzed reaction (ii) GenP and GenB3-catalyzed reaction with L-Glu, and (iii) GenP and GenB3-catalyzed reaction with α-Ketoglutarate.

Figure S6. Identification of PLP-binding sites in GenB3-catalyzed reactions. (a) HPLC-ELSD analysis of reactions catalyzed by the following: (i) GenP with gentamicin B1; (ii) GenP and GenB3 with gentamicin B1; (iii) GenP with G418; and (iv) GenP and GenB3 with G418. (b) Schematic of the GenP and GenB3 *in-vitro* reaction with gentamicin B1. (c) MS analysis of **20**.

Figure S7. Dissection of the C-4',5' reduction process catalyzed by GenB4.

Figure S8. Schematic representation and confirmation by PCR amplication of inframe deletion of *gen*P and *gen*B3.

Table S1. Kinetic constants of GenP catalyzing phosphorylation of different substrates

Table S2. List of primers used in this study

Table S3. List of strains and plasmids used in this study

Availability of data and materials

The datasets and strains materials generated and analyzed during the current study are available from the corresponding author H. X. upon request.

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Author Contributions

X.N. and H.Z designed research; S.Z., X.C., Y.L., H.Z., and X.N. performed research; S.Z., X.C., Y.L., H.Z., X.N., and H.Z analyzed data; X.N., M.D. and S.Z. wrote the paper.

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Not applicable.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing financial interest.

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Figures

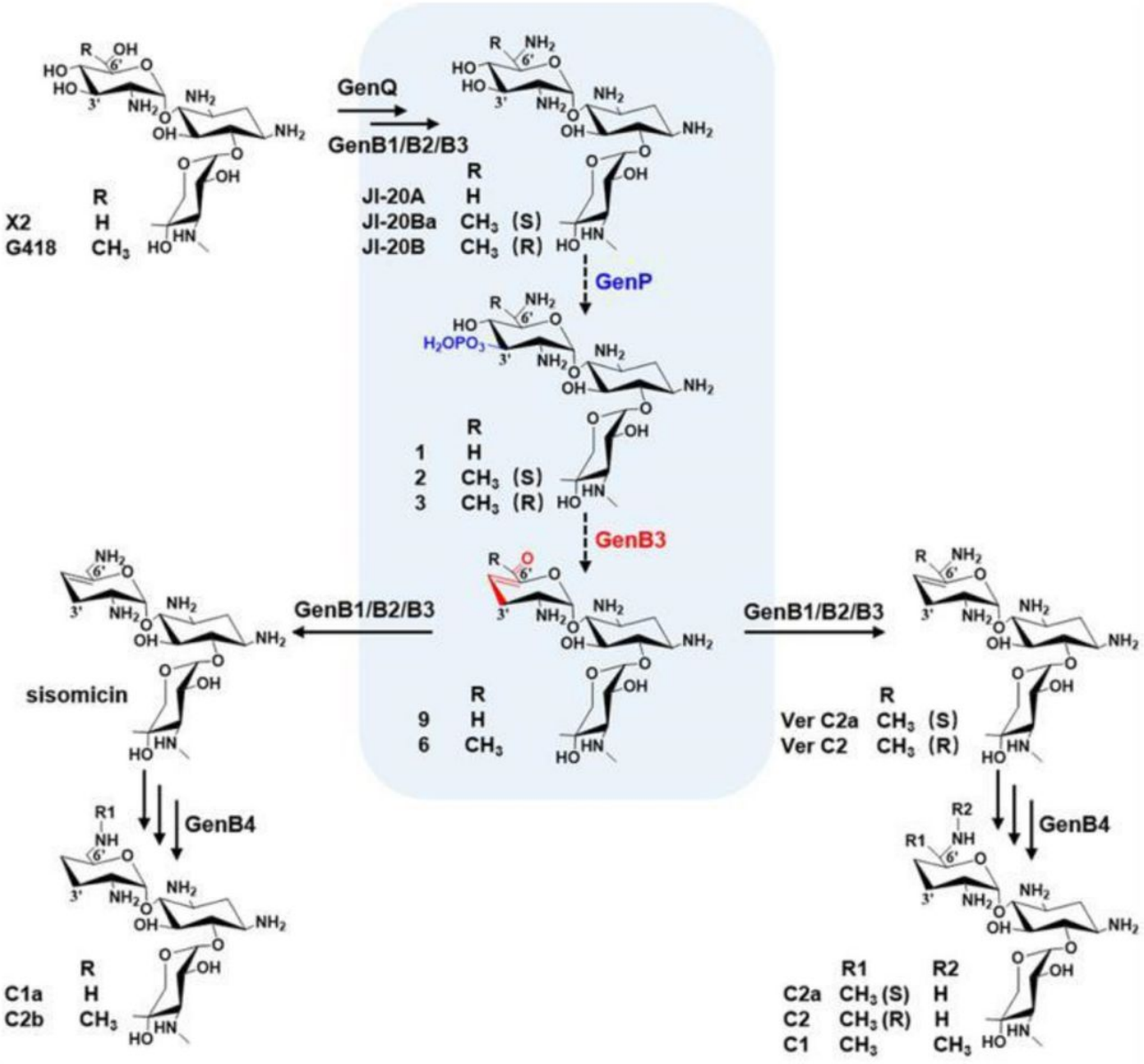

Figure 1

Biosynthetic pathway of gentamicin.

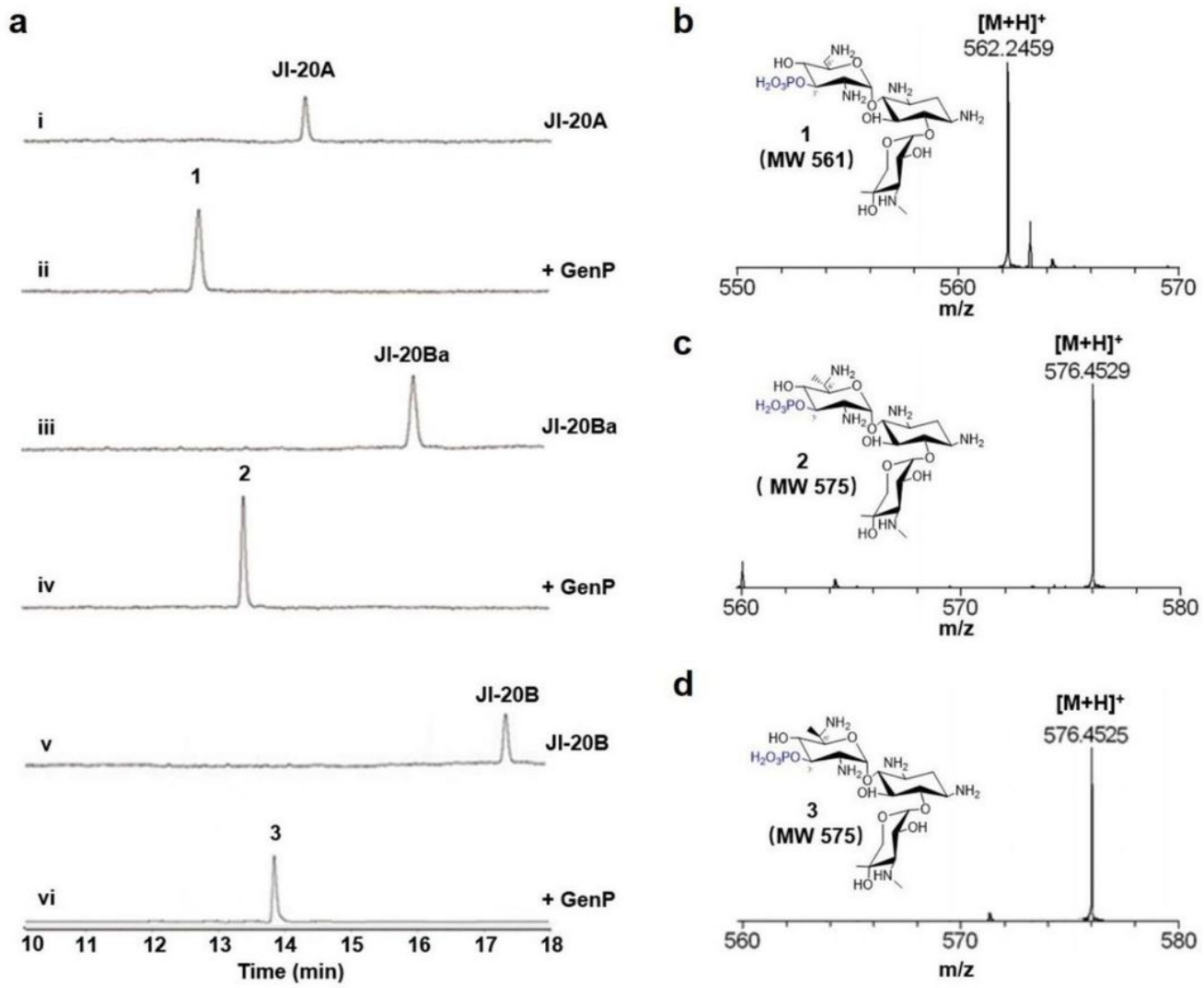

Figure 2

Catalytic activity of GenP. (a) HPLC-ELSD analysis of GenP reactions with gentamicin JI-20A, JI-20Ba and JI-20B. (b-d) MS analysis of 1, 2, and 3. MW, molecular weight.

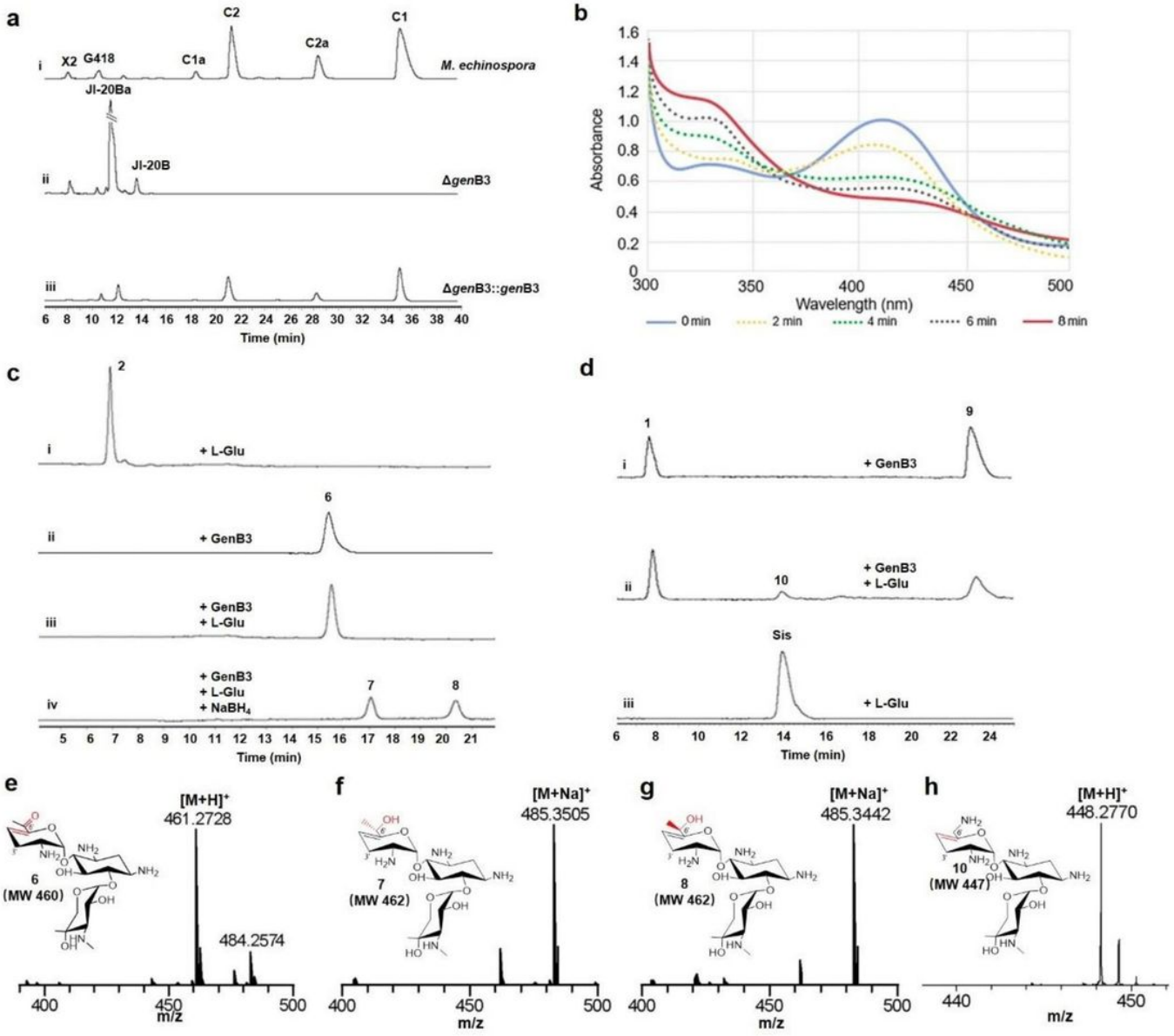

Figure 3

GenB3-catalyzed dideoxygenation. (a) HPLC-ELSD analysis of mutant- strain fermentation products, showing the (i) wild-type, (ii) genB3-disruption strain (ΔgenB3), and (iii) ΔgenB3: genB3 (complementation of the ΔgenB3 mutant). (b) Ultraviolet-visible absorption spectrum of GenB3-catalyzed reaction at different reaction times, where UV absorption was measured every 2 min from 300–500 nm. (c) HPLC-ELSD analysis of (i) 2 standard with L-Glu, (ii) GenB3-catalyzed reaction without L-Glu, (iii) GenB3-catalyzed reaction with L-Glu, and (iv) with NaBH4 added after the reaction. (d) HPLC-ELSD analysis of (i) the GenB3-catalyzed reaction with 1 was found to produce 9, and (ii) L-Glu was added, as well as (iii) an L-Glu-added sisomicin standard (Sis). (e–h) MS analysis of 6, 7, 8, and 10.

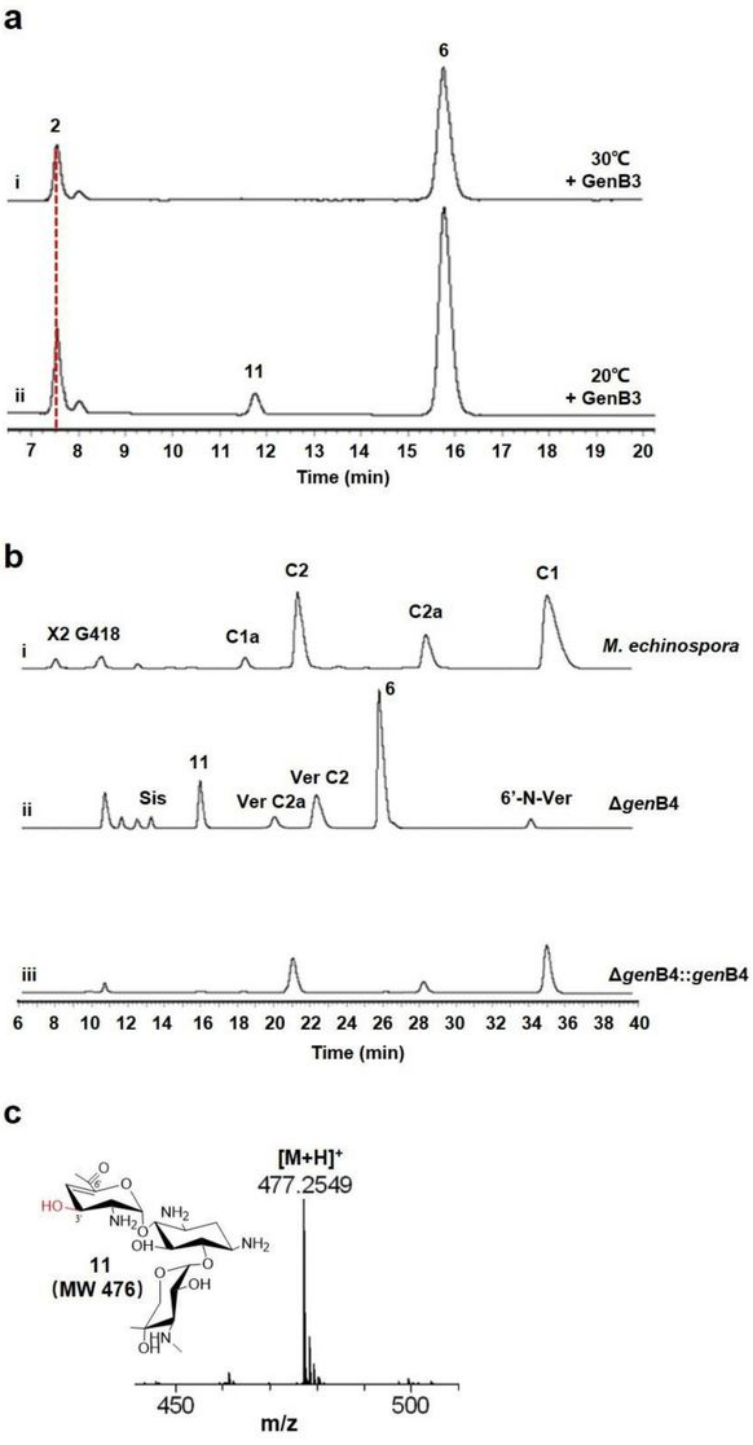

Figure 4

Discovery of 11 in GenB3-catalyzed reactions in vivo and in vitro. (a) HPLC-ELSD analysis of GenB3-catalyzed reactions in vitro with 2 at (i) 30°C and (ii) 20°C. (b) HPLC-ELSD analysis of genB4-disruption strains identified compound 11. (c) MS analysis of 11.

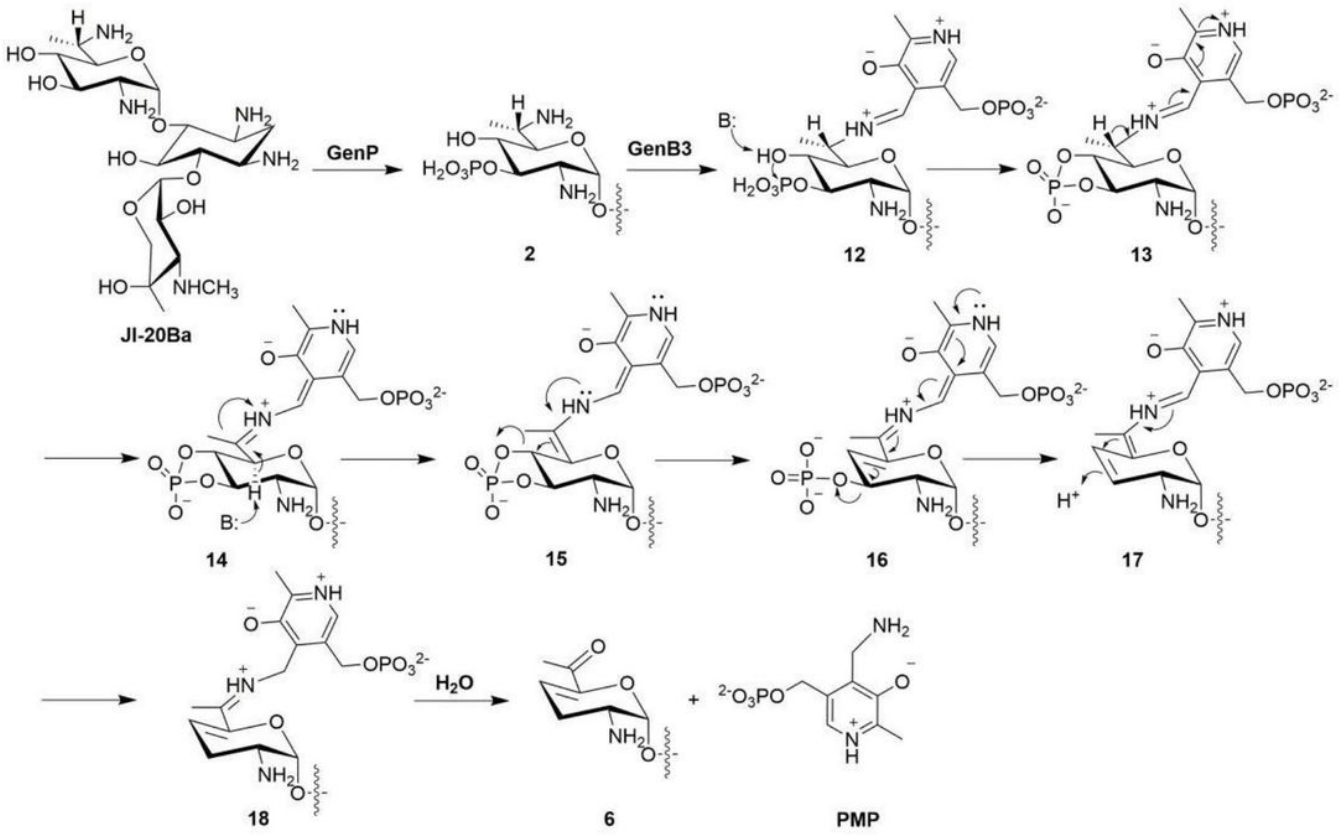

Figure 5

Scheme 1.Proposed mechanism for the GenB3-catalyzed 3',4'-dideoxygenation of JI-20Ba.

Supplementary Files

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