Cryptic phosphorylation in nucleoside natural product biosynthesis

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Kinases are annotated in many nucleoside biosynthetic gene clusters but generally are considered responsible only for self-resistance. Here, we report an unexpected 2′-phosphorylation of nucleoside biosynthetic intermediates in the nikkomycin and polyoxin pathways. This phosphorylation is a unique cryptic modification as it is introduced in the third of seven steps during aminohexuronic acid (AHA) nucleoside biosynthesis, retained throughout the pathway’s duration, and is removed in the last step of the pathway. Bioinformatic analysis of reported nucleoside biosynthetic gene clusters indicates the presence of cryptic phosphorylation in other pathways and the importance of functional characterization of kinases in nucleoside biosynthetic pathways in general. This study also functionally characterized all of the enzymes responsible for AHA biosynthesis and revealed that AHA is constructed via a unique oxidative C–C bond cleavage reaction. The results indicate a divergent biosynthetic mechanism for three classes of antifungal nucleoside natural products.

Nucleoside natural products constitute an important class of molecules with diverse structures and biological activities useful in medicine, agriculture and research1,2. While approximately 29 biosynthetic gene clusters (BGCs) have been reported3–5, characterization of these pathways is frequently challenging because hydrophilic biosynthetic intermediates do not accumulate or are difficult to detect in gene disruption experiments. Notably, these challenges limited our understanding of how the conserved nucleoside moiety in antifungal peptidyl nucleosides is biosynthesized, even though the BGCs have been known for more than a decade6,7.

Peptidyl nucleosides have wide-ranging applications in medicine (nikkomycin Z, 1)8 and agriculture (polyoxin D, 2)9. Fig. 1a, and these compounds exhibit antifungal activities through selective inhibition of citatin synthesis10. In contrast to many antifungal compounds, peptidyl nucleosides exhibited no side effects in humans during dose-finding clinical trials8 and the United States Environmental Protection Agency does not regulate polyoxin residue on agricultural products (EPA reg. no. 68173-1). Thus, peptidyl nucleosides represent an attractive platform for the development of new antifungals.

Peptidyl nucleosides are characterized by a nonproteinogenic amino acid ligated to a conserved six-carbon nucleoside, aminohexuronic acid (AHA, 3). The biosynthesis of peptidyl nucleosides has been studied for more than three decades, and the BGCs for nikkomycins11 and polyoxins were reported in the 1990s and 2000s, respectively. Still, our understanding of the biosynthesis of AHA is limited to the first two steps: (1) coupling of uracil 5′-monophosphate (UMP, 4) and phosphoenol pyruvate to form enolpyruvyl-UMP (EP-UMP, 5) by NikO/PolA (Fig. 1a and Supplementary Table 1)12 and (2) a radical-mediated cyclization of EP-UMP into octosyl acid 5′-phosphate (5′-OAP, 6)13,14. Subsequent steps in AHA biosynthesis remain uncharacterized.

Four enzymes (PolJ/NikL, PolK/NikM, PolD/NikI and PolI/NikK) were previously proposed to convert 5′-OAP into AHA based on their conservation in both the nikkomycin and polyoxin pathways (Fig. 1a)14. While a previous study reported14 that PolJ dephosphorylated 5′-OAP to yield octosyl acid (7), PolJ also catalyzed dephosphorylation of EP-UMP at apparently comparable efficiency, which left ambiguity about the relevance of these observations to AHA biosynthesis. Furthermore, while gene knockout strains for all four enzymes have been reported10–11, no biosynthetic intermediates were described. Therefore, the mechanism for 5′-OAP’s transformation into AHA remained unknown.

Here, we report that AHA biosynthesis (Fig. 1b) proceeds through an unexpected and cryptic 2′-phosphorylation catalyzed by a kinase (PolQ2). Previous proposals suggested that PolQ2 and its homologs only participate in the export of the final metabolite. However, our characterizations revealed that PolQ2 catalyzes an ATP-dependent 2′-phosphorylation of 5′-OAP to yield octosyl acid 2′,5′-bisphosphate (OABP, 8). This finding permitted the in vitro reconstitution of AHA biosynthesis, where OABP is dephosphorylated at the 5′-position, followed by conversion of the octosyl acid backbone into the AHA backbone via hydroxylation, transamination, and oxidative C–C bond cleavage (Fig. 1b). Finally, the amide ligase, NikS, couples AHA 2′-phosphate (AHAP, 9) to 4′-hydroxy-2′-pyridinyl homothreonine (HPHT, 10), the N-terminal amino acid of nikkomycin Z, to yield nikkomycin Z 2′-phosphate (11). Gene knockout studies verified the physiological relevance of these in vitro characterizations. This unexpected 2′-phosphorylation explains why biosynthetic intermediates may have been overlooked in previous gene disruption studies. A comparison of nucleoside BGCs suggests the conservation of kinases in ~60% of reported nucleoside natural product biosynthetic pathways. While most of these kinases are uncharacterized, cryptic phosphorylation may be relevant to the biosynthesis of many other nucleoside natural products.

Results

Search for the enzyme that acts on 5′-OAP. We initially hypothesized that the four enzymes (PolJ/NikL, phosphatase, PolI/NikK aminotransferase, PolD/NikI α-ketoglutarate (α-KG) dependent oxygenase and PolK/NikM α-KG-dependent oxygenase; Supplementary Table 1) conserved between the nikkomycin and polyoxin pathways may be responsible for the transformation of 5′-OAP into AHA. To test this hypothesis, we initially focused

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Selective 5′-dephosphorylation of OABP by PolJ. Of the remaining conserved enzymes, only the phosphatase PolJ produced a new compound from OABP (Fig. 2b and Extended Data Fig. 3), which migrated closely to 5′-OAP on HPAEC. The same product was formed by His-tagged and MBP fusion forms of PolJ. PolJ’s product was structurally characterized by high-resolution mass spectrometry (HRMS) and nuclear magnetic resonance (NMR) (Supplementary Note), which were consistent with OABP. PolQ2 demonstrated a turnover rate of ~0.1–0.21 min⁻¹ with 5′-OAP as the substrate (Supplementary Fig. 2), which may be compared to 6.8 min⁻¹ reported for NikO12 and ~0.3 min⁻¹ for NikI/PoH15. No activity was observed with EP-UMP despite prolonged incubation (12 h, Extended Data Fig. 2). Thus, PolQ2 specifically acts on 5′-OAP, and the observed turnover rate suggested its relevance to AHA biosynthesis.

Selective 5′-dephosphorylation of OABP by PolJ. Of the remaining conserved enzymes, only the phosphatase PolJ produced a new compound from OABP (Fig. 2b and Extended Data Fig. 3), which migrated closely to 5′-OAP on HPAEC. The same product was formed by His-tagged and MBP fusion forms of PolJ. PolJ’s product was isolated and structurally characterized by HRMS and
Oxidation of 2′-OAP by PolK. When assayed with 2′-OAP, both oxygenases, PolD and PolK, yielded products in an α-KG-dependent fashion. PolD yielded a single product that migrated very early on HPAEC (Fig. 3a), while PolK assay yielded two identical products in a ~1:1 ratio that migrated very closely to 2′-OAP (Fig. 3b). PolD’s product was isolated and characterized by liquid chromatography–high-resolution tandem mass spectrometry (LC–HRMS/MS), which was consistent with heptosyl acid 2′-phosphate (2′-HAP, 13 and Supplementary Note). However, this compound was not converted to any advanced intermediates by phosphatase PolJ, aminotransferase NikK or oxygenase PolD (Extended Data Fig. 4). When 2′-OAP was assayed in the presence of both oxygenases PolK and PolD, 2′-HAP was not formed, and only PolK’s products were observed (Fig. 3a). These observations suggest that 2′-HAP is likely an off-pathway product that is irrelevant to AHA biosynthesis.

PolK’s two products were isolated for further characterization. The product with a later retention time on HPAEC (2.2 min in Fig. 3b) was stable and characterized by LC–HRMS and NMR analyses, which were consistent with 5′-keto-octosyl acid 2′-phosphate (KOAP, 14 and Supplementary Note). In contrast, the product with an earlier retention time (2.0 min in Fig. 3b) exhibited limited chemical stability. Interpretation of the 1H NMR spectra of a mixture of this product and its degraded compounds were challenging, but the absence of 1H signals at 2.0–2.5 ppm suggested an oxidation of C6′ (Supplementary Note). Based on the exact mass of this compound determined by LC–HRMS (+31.989 Da from 2′-OAP, Supplementary Note) and its acceptance by the pyridoxal phosphate- (PLP-) dependent aminotransferase NikK (Fig. 3c, see below for details), which requires the presence of a ketone, the structure of this compound was inferred as a hydrated form of 6′-hydroxy-5′-keto-octosyl acid 2′-phosphate (HKOAP, 15). While the stereochemistry of C6′ of HKOAP cannot be assigned, it is likely identical to that of AHKOAP described below. Recombinant NikK, the homolog of PolK in the nikkomycin pathway, also catalyzed the formation of HKOAP and KOAP (Fig. 3b), suggesting that the mechanism of AHA biosynthesis is likely conserved between the two pathways.

No substantial amount of HKOAP was formed when isolated KOAP was incubated with oxygenase PolK for 20h (Extended Data Fig. 5), suggesting that KOAP is not an intermediate of HKOAP formation. When HKOAP and KOAP were individually incubated with NikK aminotransferase in the presence of l-Glu or l-Phe as an amino donor, only HKOAP was converted to another compound (Fig. 3c). Neither HKOAP nor KOAP was a substrate of the other oxygenase, PolD (Fig. 3a and Extended Data Fig. 6). These observations suggested that HKOAP is an on-pathway intermediate that is aminated by NikK. A C4′-epimer of dephosphorylated KOAP (octosyl acid C, Supplementary Fig. 4)10 and a 6′-hydroxylated and dephosphorylated 2′-OAP (nikkomycin S8a, Supplementary Fig. 4)11 were previously isolated from culture media of Streptomyces cacaoi and Streptomyces tendae, respectively, suggesting the relevance of our in vitro observations to both polyoxin and nikkomycin biosynthesis and previous literature in the field.

HKOAP is selectively aminated by NikK. As described above, the aminotransferase NikK selectively accepts HKOAP to form a less anionic molecule. LC–HRMS and Fourier transform infrared (FTIR) analyses of this compound (Supplementary Note) were consistent with the conversion of a ketone of HKOAP into an amine. However, characterization by NMR was challenging due to broadened 1H NMR signals and a slow T1 relaxation (0.5–1.0 s, Supplementary Note), likely from a slow conformational equilibrium or extended H-bond interactions in solution. We consequently characterized a dephosphorylated analog. On dephosphorylation using calf intestinal alkaline phosphatase (CIP) as confirmed by LC–HRMS (Supplementary Note), the resulting compound...
Fig. 3 | Transformation of 2′-OAP into AHAP. a, PolK and PolD assays with 2′-OAP. Shown are HPAEC chromatograms at 270 nm for an assay with PolK (trace (i)), an assay with PolD and PolK (ii), an assay with PolD (iii) and a control with heat-inactivated PolD (iv). The peaks shown with asterisks are an unknown contaminant associated with both PolD and PolK, which has λ<sub>max</sub> at 247 nm and is not a uracil-related compound. The peaks shown with asterisks are an unknown contaminant associated with both PolD and PolK, which has λ<sub>max</sub> at 247 nm and is not a uracil-related compound. b, PolK and NikM assays with 2′-OAP. Shown are HPAEC chromatograms at 260 nm for an assay with PolK (trace (i)), an assay with NikM (ii), a control without the enzyme (iii) and a control PolK assay without α-KG (iv). c, NikK assays with HKOAP or KOAP. Shown are HPAEC chromatograms at 260 nm for a NikK assay with HKOAP (trace (i)), a NikK assay with KOAP (scaled 1/6x) (ii), a control with HKOAP without NikK (iii) and a control with HKOAP without L-Glu (iv). d, PolD assays with AHOAP. Shown are HPAEC chromatograms at 260 nm for the PolD assay in the complete condition (trace (i)), a control without the enzyme (ii) and a control without α-KG (iii). All enzyme activities were qualitatively reproducible in at least three independent assays in multiple enzyme preparations.
We did not detect KHA formation (Supplementary Fig. 5), suggesting α
Fig. 6a). The NikS product was converted to nikkomycin Z on treat-
ment with phosphorylated nikkomycin Z (Fig. 4a,b and Supplementary
characterization of these enzymes, we propose the structure of NikK's product as
HKOAP into AHOAP. PolD catalyzes oxidative C–C bond cleavage reaction in AHA biosynthesis.
NikK was previously proposed to catalyze a transamination of ketohexuronic acid (KHA, 17, Fig. 1a) to AHA. Thus, we tested this hypothesis by incubating AHA with NikK in the presence of α-KG. We did not detect KHA formation (Supplementary Fig. 5), and NikK does not catalyze the transamination between AHA and KHA. These observations suggest that the physiological function of NikK is likely the transformation of HKOAP into AHOAP.

PolD catalyzes oxidative C–C bond cleavage of AHOAP. When AHOAP was assayed with oxygenase PolD in the presence of α-KG and Fe²⁺, PolD efficiently converted AHOAP into another compound that migrated earlier on HPAEC (Fig. 3d). NMR and LC–HRMS characterization of this product (Supplementary Note) was consistent with AHAP (9), and treatment of this product with CIP yielded AHA. PolD catalyzed the conversion of AHOAP into AHAP at least 150-fold faster than the conversion of 2'-OAP into 2'-HAP under similar conditions (Extended Data Fig. 8), indicating that the former is likely relevant to AHA biosynthesis. These observations support the idea that PolD catalyzes a unique oxidative C–C bond cleavage reaction in AHA biosynthesis.

NikS amide ligase requires 2'-phosphorylation. With the in vitro reconstitution of AHAP biosynthesis, we investigated the coupling of AHAP with HPHT by NikS. When AHAP and HPHT were incubated with NikS in the presence of ATP and MgCl₂, we observed the formation of a new peak with the molecular weight consistent with phosphorylated nikkomycin Z (Fig. 4a,b and Supplementary Fig. 6a). The NikS product was converted to nikkomycin Z on treatment with CIP (Fig. 4b,c and Supplementary Fig. 6b). NikS did not catalyze the ligation of AHA with HPHT even after prolonged incubation (Fig. 4b,c). These observations provide strong evidence that NikS selectively conjugates HPHT with AHAP suggesting the importance of 2'-phosphate for substrate recognition. Furthermore, these data suggest that at least in nikkomycin biosynthesis, AHA is likely a shunt metabolite.

Genetic characterization of polQ2, nikL and nikK. The in vitro characterization of these seven enzymes (PolQ2, PolJ, PolK/NikM, NikK, PolD and NikS) suggested the presence of an unexpected cryptic phosphorylation during AHA biosynthesis. To test the physiological relevance of these observations, we performed genetic knockout studies for polQ2, nikL and nikK. Due to the high titer of nikkomycin Z (~0.15 mg ml⁻¹, isolated yield), we primarily focused on the nikkomycin pathway, except for the polQ2 kinase (Fig. 5a). A selective knockout of kinase activity would be challenging in the nikkomycin pathway because the corresponding kinase (NikN) is fused with a putative MFS transporter. The polQ2 gene was disrupted in-frame by homologous recombination in S. cacaoi. The resulting polQ2 disruptant (ΔpolQ2) was then characterized for polyoxin production using LC–HRMS. Under conditions that the S. cacaoi wildtype (WT) strain produced polyoxins, S. cacaoi ΔpolQ2 did not (Extended Data Fig. 9). Complementation of S. cacaoi ΔpolQ2 with the WT polQ2 gene restored polyoxin production (Supplementary Fig. 7a and Extended Data Fig. 9). These observations are consistent with the essential function of PolQ2 in polyoxin biosynthesis.

The nikL and nikK genes were individually disrupted in S. tendae with a kanamycin resistance gene (kan) replacement. The resulting disruptants S. tendae ΔnikL::kanR and ΔnikK::kanR did not produce detectable amounts of nikkomycin Z (Supplementary Fig. 7b), confirming that these genes are essential for nikkomycin biosynthesis. Genetic complementation of ΔnikK::kanR with the WT nikK gene restored nikkomycin Z production (Supplementary Fig. 7b).

We searched for the characterized biosynthetic intermediates in the culture media of the gene disruptants, and observed accumulation of 5'-OAP (~0.25 mg ml⁻¹ isolated yield) and OABP (~0.03 mg ml⁻¹, LC–MS quantitation) in ΔnikL::kanR, and 2'-OAP (~0.2 mg ml⁻¹ isolated yield) and KOAP (~0.1 mg ml⁻¹ isolated yield) in ΔnikK::kanR mutants (Fig. 5b). These accumulated metabolites were isolated and characterized by LC–HRMS (Fig. 5c,d) and NMR (Supplementary Note). OABP accumulation in ΔnikL is consistent with the proposed function of NikL (Fig. 5a), and its relatively low accumulation is consistent with OABP's inability to passively diffuse through the membrane due to its strong negative charge. LC–MS analysis also revealed that the ΔnikK::kanR strain accumulated trace amounts of HKOAP (~0.01 mg ml⁻¹, LC–MS quantitation, Fig. 5e), which is consistent with the in vitro function of NikK (Fig. 5a). The trace amount of HKOAP accumulation likely derives from HKOAP's limited stability as described above. The accumulation of HKOAP and KOAP is also consistent with the in vitro function of NikM, which promiscuously catalyzed the conversion of 2'-OAP into HKOAP and KOAP (Fig. 5a). Therefore, the results from the gene knockout studies were consistent with the in vitro assays and provide in vivo support for the proposed AHA biosynthetic mechanism involving unexpected cryptic phosphorylation.

Roles of kinases in nucleoside BGCs. To investigate the potential generality of cryptic phosphorylation, we surveyed reported nucleoside natural product BGCs and identified kinases in 17 of 29 reported BGCs (Supplementary Fig. 8). Nine of these kinases belong to the P-loop NTPase superfamily, including MalE (malayamycin, Fig. 6a), PumH (pseudouridimycin)², Mur28 (muraymycins)²⁴ and Cpz12 and Cpz27 (caprazamycins)²⁵. The malayamycin² and pseudouridimycin² BGCs contain other AHA biosynthetic enzymes or NikS homologs (Fig. 6b). Mur28 kinase from the muraymycin pathway catalyzes the 3'-OH phosphorylation of a GlyU-ADR disaccharide biosynthetic intermediate, and homologous kinases are conserved among GlyU-ADR containing nucleoside BGCs (caprazamycins²⁷, A-90289 (ref. ²⁸) and muramidinomycin²). A 3'-phosphorylated caprazamycin precursor has been isolated. More recently, while this manuscript was in revision, one of the muraymycin biosynthetic enzymes (Mur24) was reported to act specifically on 3'-phosphorylated GlyU-ADR²⁹. Therefore, these pathways likely involve cryptic phosphorylation. Eight other nucleoside BGCs contain putative kinases in families related to phosphorylation of nucleosides and carbohydrates (Supplementary Fig. 8), and thus it is still conceivable that these kinases catalyze phosphorylation of carbohydrates or nucleoside/nucleotide biosynthetic intermediates. While some of these kinases may have differing roles, such as self-resistance as previously proposed, these analyses and the PolQ2's essential role in AHA biosynthesis suggest the importance of the functional characterization of kinases in nucleoside BGCs to understand their biosynthetic mechanisms in general.

Discussion. This report describes the mechanism of AHA biosynthesis in peptidyl nucleoside biosynthetic pathways and the in vitro preparation of nikkomycin Z 2'-phosphate (Fig. 1b). In particular, we demonstrate that the 2'-phosphorylation is introduced by a P-loop NTPase kinase PolQ2 (NikN) and is retained for the pathway's duration. Previously, PolQ2/NikN kinases were thought to be involved exclusively in the export of the final metabolites since no additional phosphorylation beyond 5'-OAP was immediately

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APPARENT, and these kinases are associated with the putative MFS transporter. In contrast, our in vitro and in vivo characterizations of PolQ2 and the downstream enzymes reveal that the kinase is essential for AHA biosynthesis and is responsible for cryptic phosphorylation at the 3'-OH.

The discovery of 3'-phosphorylation in AHA biosynthesis has important implications for our general understanding of nucleoside natural product biosynthesis. Phosphorylated intermediates behave differently from nucleosides and can be easily missed when characterizing biosynthetic pathways. AHA and its precursors were not reported in previous gene knockout studies14–17 likely because phosphorylation was not expected. Based on our bioinformatics analysis suggesting the potential abundance of such cryptic phosphorylation, this may be the case for other nucleoside natural product pathways as well. Seventeen of the 29 reported nucleoside BGCs contained kinases, of which nine belonged to the P-loop kinase superfamily. In particular, cryptic phosphorylation is likely involved in the biosynthetic pathways of malayamycin, pseudouridimycin, and GlyU-ADR-containing nucleosides (for example, caprazamycins). Therefore, these findings indicate the importance of functional characterization of kinases in nucleoside BGCs in general.

Previously reported cryptic modifications of biosynthetic intermediates usually serve chemical roles; for example, cryptic chlorination installs a leaving group for subsequent cyclopropanation22. Cryptic acylation is found in the biosyntheses of arginine33, butirosin4,35 and the polyoxin N-terminal amino acid, carbamoylpolyoxamic acid36 (CPOA3, 18), and is thought to function as a protecting group. Transient phosphorylation has been reported to act as a leaving group for dehydration in the biosynthesis of ribosomally synthesized posttranslationally modified peptides37. Compared to these precedents, cryptic phosphorylation in AHA biosynthesis is unique since phosphorylation is introduced early in the pathway, retained for the pathway’s duration and has no apparent chemical role.

Consequently, cryptic phosphorylation in AHA biosynthesis is more similar to the recent reports of loading biosynthetic intermediates to an acyl carrier protein during mitomycin biosynthesis38,39, where the function of the acyl carrier protein was not immediately apparent. We propose two possible biological functions for phosphorylation in AHA biosynthesis. The first is to facilitate specific recognition by downstream enzymes. With the exception of nicotinamide adenine dinucleotide 3'-phosphate, nucleoside 3'-phosphate is rare in nature, suggesting that 3'-phosphorylation may serve as a specific recognition tag. Since peptidyl nucleosides have substantial variations in their nucleobase structures that the AHA biosynthetic machinery must accommodate, 3'-phosphorylation may be a mechanism to produce structurally diverse natural products while maintaining the crucial specificity for efficient metabolic flux. A second possible function is to prevent the diffusion of intermediates to the extracellular space. In fact, no phosphorylated intermediates were observed in our MS analysis of WT S. tendae. Even in gene knockout strains, the amount of OABP in the culture media was 25-fold less than 5'-OAP, suggesting that the additional phosphate is rare in nature, suggesting that 3'-phosphorylation may serve as a specific recognition tag. Since peptidyl nucleosides have substantial variations in their nucleobase structures that the AHA biosynthetic machinery must accommodate, 3'-phosphorylation may be a mechanism to produce structurally diverse natural products while maintaining the crucial specificity for efficient metabolic flux. A second possible function is to prevent the diffusion of intermediates to the extracellular space. In fact, no phosphorylated intermediates were observed in our MS analysis of WT S. tendae. Even in gene knockout strains, the amount of OABP in the culture media was 25-fold less than 5'-OAP, suggesting that the additional phosphate group minimizes its diffusion/export to the extracellular media. Thus, 3'-phosphorylation may allow efficient metabolic flux during peptidyl nucleoside biosynthesis.

While the current study revealed most of the steps in nikkomycin biosynthesis, the mechanism of dephosphorylation of nikkomycin Z 3'-phosphate remains unknown. Nikkomycin Z 3'-phosphate is not detectable in WT S. tendae, suggesting that a mechanism for dephosphorylation exists. Two possibilities are conceivable: (1) promiscuous activity of phosphatase NikL or (2) a reverse reaction of the NikN kinase domain. In the latter case, it is possible that dephosphorylation is coupled to the export of nikkomycin Z through the MFS transporter. Such a mechanism may explain the
absence of detectable accumulation of nikkomycin Z 2′-phosphate and the genetic association of the kinase and MFS transporter.

This study also revealed the functions of the two α-KG-dependent dioxygenases, PolK and PolD. PolK catalyzes the dual hydroxyl-ation, while PolD catalyzes an oxidative C–C bond cleavage. Based on these observations, we propose a divergent mechanism for the biosynthesis of the structurally related antifungal nucleosides malayamycins and ezomycins. Ezomycin A1 (Extended Data Fig. 10) contains a C8 bicyclic sugar, while malayamycins contain a C7 bicyclic sugar (Fig. 6a); AHA in the peptidyl nucleosides contains 2′-OAP (Fig. 1a). Together with our studies, this earlier work will yield AHAP. Alternatively, a processive oxidative decarboxylation could transform AHOAP into AHAP (Supplementary Fig. 9b). Finally, although unprecedented, it is possible that PolD generates ketoacid intermediates, followed by their oxidative decarboxylation by a mechanism analogous to the oxidative cleavage of α-KG into succinate (Supplementary Fig. 9c). Mechanistic studies are currently underway to distinguish these possibilities.

Our study also revises the previously proposed function of NickS⁴⁰. Initial proposals suggested that NickS loads HPHT or its precursor onto NikT’s acyl carrier domain⁴¹. More recently, NickS was proposed to catalyze an ATP-dependent amide ligation between AHA and HPHT⁴². However, no in vitro functional characterization was reported. Our results demonstrate that NickS does not catalyze ligation between HPHT and AHA, and instead ligates HPHT and AHAP to form nikkomycin Z 2′-phosphate. Therefore, AHAP is likely the physiological substrate of NickS, and AHA is an off-pathway shunt metabolite. In a recent report for PolG⁴³, the NickS homolog in the polyoxin pathway, recombinant PolG ligates AHA and CPOAA (Fig. 1a). Together with our studies, this earlier...
In conclusion, the identification of unexpected cryptic 2'-phosphorylation catalyzed by a P-loop NTPase kinase, PoiQ2/ NikN, resolved long-standing mysteries regarding AHA biosynthesis. The presence of related kinases in numerous nucleoside biosynthetic pathways suggests the relevance of cryptic phosphorylation beyond the nikkomycin/polyoxin pathways and the importance of functionally characterizing these kinases in order to understand nucleoside natural product biosynthesis.

Online content

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PolK functional characterization were not detected with either tag. or MBP fusion proteins were used in the assays without removal of the His-tag. PolK2, NikK and NikM were expressed as N-terminal fusion with His-tagged expressions, we screened for expression conditions and tags. As a consequence, Periplasmic fractions were washed with Buffer A (50 mM Tris pH 7.6, 150 mM NaCl, 10% glycerol), frozen with liquid nitrogen and stored at −20 °C. Trypticolysis was incubated with Ni-NTA agarose (Genese Scientific, 20 mL equilibrated in Buffer B supplemented with 40 mM imidazole) for 1 h at 4 °C. The resin was subsequently packed into a column, and the column was washed with 10 column volumes (CV) of Buffer B supplemented with 200 mM imidazole.

Methods

General microbiology and cloning methods. The strains and plasmids used in this study are listed in Supplementary Tables 2 and 3, respectively. Escherichia coli and Streptomyces were purchased from commercial sources without further purification. NMR spectra were performed using a Shimadzu LC system (comprising a solvent degasser, two LC-TOF) with dual ESI source and an Agilent Technologies hydrophilic interaction chromatography Plus column or on a reverse-phase LC–ESI–MS/MS system of a Dionex ICS-5000+ system consisting of a Dionex ICS-5000+ SP pump, Dionex ICS-5000+ DC column oven, Dionex AS-AP autosampler and Dionex PDA detector array with a DNASpec PA100 4 × 250 mm column (Thermo Scientific). LC–HRMS data was collected with an Agilent Technologies 1200 Series Scientific) with dual ESI source and an Agilent Technologies high-resolution TripleTOF5600 mass spectrometer (Sciex). LC–HRMS data was collected with an Agilent Technologies 1200 Series Scientific). LC–HRMS data was collected with an Agilent Technologies 1200 Series Scientific). LC–HRMS data was collected with an Agilent Technologies 1200 Series Scientific).

General methods of protein expression and purification. In general, polyoxin homologs (PolQ2 kinase, PolJ phosphatase, PolD oxygenase and PolK oxygenase) were characterized as they were more soluble and stable than nikkomycin homologs. The exceptions were NikK aminotransferase and NikSamide ligase. NikK was chosen as its aminotransferase activity toward proteinogenic amino acids was previously reported. NikK was chosen as its aminotransferase activity toward proteinogenic amino acids was previously reported. NikK was chosen as its aminotransferase activity toward proteinogenic amino acids was previously reported. NikK was chosen as its aminotransferase activity toward proteinogenic amino acids was previously reported. NikK was chosen as its aminotransferase activity toward proteinogenic amino acids was previously reported. NikK was chosen as its aminotransferase activity toward proteinogenic amino acids was previously reported.

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PolQ activity assays. His-Pol or MBP-Pol (10 µM) was incubated with 100 µM of S′-OAP or OAPB in Buffer A supplemented with 20 mM MgCl₂ at 25 °C. After 15, 30, 60, 120, and 240 min, aliquots (10 µl) were diluted four times in water, boiled quenched at 95 °C for 2 min and were clarified by centrifugation. Then, 12 µl of the supernatant was injected into and analyzed by HPAEC using a DNA-Pac PA100 4 × 250-mm column (Thermo Scientific) under identical conditions to those described for PolQ2. Chromatography was monitored by UV absorbance at 260 nm.

PolD, PolK and NIKM activity assay. His-PolD (10 µM), His-PoK (40 µM) or MBP-NIKM (40 µM) was incubated with 100 µM of substrate (2′-OAP, OAPB, AHOO or KOAP), 100 mM (NH₄)₂SO₄, 6.0 mM, 1 mM ascorbate and 2 mM α-KG in oxygen saturated Buffer A at 25 °C. After 15, 50, 120 and 1,080 min, aliquots (10 µl) were collected, diluted five times in dH₂O, boiled quenched for 2 min at 95 °C and were clarified by centrifugation. Then, 12 µl of the supernatant was injected into and analyzed by HPAEC (for AHOO, ZOP and OAPB) using a DNA-Pac PA100 4 × 250-mm column (Thermo Scientific). KOAP assays were analyzed by HPLC using an Xbridge Amide (Waters) column. Chromatography was monitored by UV absorbance at 260 nm.

NIKK activity assay. MBP-NIKK (10 µM) was incubated with HKOAP (100 µM) or KOAP (500 µM) in the presence of l-Glu (3 mM) or l-Phe (25 µM) at 25 °C. After 15, 60, 120 and 1,080 min, aliquots (10 µl) were collected, diluted five times in dH₂O, boiled quenched for 2 min at 95 °C and were clarified by centrifugation. Then, 12 µl of the supernatant was injected into and analyzed using HPAEC equipped with an Xbridge Amide (Waters) column under isocratic conditions: 25% 100 mM ammonium acetate pH 4.875% MeCN. UV absorbance was monitored at 280 nm. Identical samples were submitted for LC–HRMS analysis with or without CIP treatment, and the MS data were analyzed by MassHunter B.07.00 (Agilent Technologies).

Construction of PolQ2 in-frame deletion mutants. PCR-based homologous recombination and temperature sensitivity selection were used to create Streptomyces deletion mutants (Supplementary Fig. 14). Gene deletion was accomplished using pKC1139 (ref. 1) as a temperature sensitive Streptomyces expression vector一同，E. coli shuttle vector that was modified from a previously reported protocol10. For PolQ2 deletion, markerless in-frame deletion was performed. The 5′ flanking region of PolQ2 (5′-polQ2) was amplified with primers KA16F and KA16R (Supplementary Table 4) to generate a 1,294-bp DNA fragment with terminal EcoRI and Ndel sites. The 3′ flanking region of PolQ2 (3′-polQ2) was amplified with primers KA21F and KA21R to generate a 1,431-bp DNA fragment with terminal Ndel and HindIII sites. Then, the PCR products of 5′-polQ2 and 3′-polQ2 were individually cloned into pTE1.2 blunt cloning vector (Supplementary Fig. 4). The flanking fragments were digested from pTE1.2 and pET3/5′-polQ2 and pET3/3′-polQ2, at their restriction sites described above and subcloned into pKCI139 digested with EcoRI and HindIII to create pKCI139PolQ2. The resulting plasmid was introduced into E. coli ET12567/pUZ8002 (ref. 10) by a CaCl₂ transformation and was grown at 30 °C for 24 h. The resulting plasmid was digested with Ndel and HindIII and the fragment was subcloned into the Ndel and HindIII site of pMPL257 (ref. 10) to yield pML257n3k. The Ndel and HindIII site downstream of constitutive ermB promoter, allowing constitutive expression of n3k. Intergeneric conjugation of E. coli ET12567/pUZ8002 carrying the pML257n3k with S. tendae ΔnikKΔkan was carried out as described above using the spore suspension that was heat shocked at 37 °C for 30 min. S. tendae Δex-conjugants were selected on MS agar overlaid with 10 µg ml⁻¹ of thiostrepton and 25 µg ml⁻¹ of naldixic acid, grown at 28 °C for 5–7 d. The resulting recombinant strain, S. tendae ΔnikKΔkan+pUWL201PW7 was streaked on the same media containing 30 µg ml⁻¹ of thiostrepton and 25 µg ml⁻¹ of naldixic acid until the ex-conjugants were free from E. coli cells. The recombinant strains were confirmed by colony PCR using primers LIC-MBP-polQ2-F and LIC-MBP-polQ2-R (Supplementary Table 4).

Construction of nikK and nikL disruption mutants. ΔnikK and ΔnikL mutants were created and characterized using the targeted gene with a kanamycin resistant gene. (Supplementary Fig. 16). The replacement fragments contained the target’s 5′ flanking region, a kanamycin resistant gene, and the target’s 3′ flanking region, respectively, and were amplified using overlapping PCR. Primers and fragments amplified for ΔnikK and ΔnikL knockout mutants are shown in Supplementary Table 4 and Supplementary Fig. 16. Cloning and conjugation were performed as described above. Selection of double crossover ex-conjugants was performed by screening for apramycin sensitivity and kanamycin resistance. Verification of the resulting mutants was performed by Streptomyces colony PCR using primer pairs HSU-nik11 and HSU-nik15 for S. tendae ΔnikKΔkan, and HSU-Nik16 and HSU-Nik22k for S. tendae ΔnikKΔkan (Supplementary Table 4 and Supplementary Fig. 15b).

Genetic complementation of S. cacaoi ΔnikKΔpolQ2 with WT polQ2 gene. The polQ2 gene was subcloned from pET28b/polQ2 into the Ndel/HindIII site of pUWL201PW7 (Streptomyces multi-copy expression vector, pUWL201PW7, with oriT inserted into the PsI site) creating pUWL201PW7/polQ2. The resulting strains were streaked on the same media containing 30 µg ml⁻¹ of thiostrepton and 25 µg ml⁻¹ of naldixic acid, grown at 28 °C for 5–7 d. The resulting recombinant strain, S. cacaoi ΔnikKΔpolQ2+pUWL201PW7 was streaked on the same media containing 30 µg ml⁻¹ of thiostrepton and 25 µg ml⁻¹ of naldixic acid until the ex-conjugants were free from E. coli cells. The recombinant strains were confirmed by colony PCR using primers LIC-MBP-polQ2-F and LIC-MBP-polQ2-R (Supplementary Table 4).

HPCAEC and LC–HRMS analysis of S. tendae and S. cacaoi culture supernatants. S. cacaoi and S. tendae strains were grown in fermentation medium P (40 g–1) of mannitol, 10 g–1 of glycerol, 10 g–1 of soluble starch, 20 g–1 of soy peptone, 10 g–1 of yeast extract, 5 g–1 of ammonium sulphate, 3.75 g–1 of FeSO₄·7H₂O, 6.9 g·1 of NaH₂PO₄·H₂O, 8.7 g·1 of K₂HPO₄ or medium N (431 g·1 of mannitol, 12 g·1 of soluble starch, 20 g·1 of soy peptone, 10 g·1 of yeast extract, 3.75 mL·1 of FeSO₄·7H₂O, respectively). A 1:1 mixture of TSF and the fermentation medium P or N (total 10 ml) in 50 ml tube with 2 cm of spring TSB:YEME (a mixed media containing all the ingredients for TSB and yeast extract–malt extract (YEME) media with additions of 5 g·l of Bacto-glucose, 10 g·l of Bacto-peptone, 5 g·l of ammonium sulphate, 3.75 g·l of FeSO₄·7H₂O, 6.9 g·l of NaH₂PO₄·H₂O, 8.7 g·l of K₂HPO₄ or medium N (431 g·l of mannitol, 12 g·l of soluble starch, 20 g·l of soy peptone, 10 g·l of yeast extract, 3.75 mL·l of FeSO₄·7H₂O, respectively). The resulting strains were streaked on the same media containing 30 µg ml⁻¹ of thiostrepton and 25 µg ml⁻¹ of naldixic acid, grown at 28 °C for 5–7 d. The resulting Streptomyces colony liquid cultures (0.5–2.0 cm) were then diluted 400× in 50 ml of the fermentation medium P or N containing 100 mM PIPES buffer pH 6.0 in 250 ml baffled flasks containing stainless steel springs with the starting OD₆₀₀ = 0.1. The cultures were grown at 28 °C with shaking at 225 r.p.m. During the fermentation,
the pH was monitored. The pH was at ~6.5 between days 1–5, and increased to ~7.0 on day 6–7. After 5–7 d of culture, the culture media were collected, cleared by centrifugation at 14,000 r.p.m., 4°C for 20 min to remove mycelia and analyzed by LC–HRMS. Nikkomycins were chromatographed on an Agilent Hypersil column of length 15 cm with a linear gradient of 15–50% A for 20 min at a flow rate of 0.5 ml min⁻¹. Polyoxins and AHA biosynthetic intermediates were chromatographed on a Waters Xbridge Amide column at 40 °C using solvents A (10 mM NH₄OAc pH 10.0) and B (acetonitrile): with a linear gradient of 15–50% A for 20 min at a flow rate of 0.5 ml min⁻¹. The elution was monitored by UV absorption at 254 nm as well as ESI–TOF–MS. The MS data were analyzed by MassHunter (Agilent Technologies).

**Data availability**

The authors declare that all the data supporting the findings of this study are available within the paper, Extended Data and Supplementary Information. All plasmids, analytical amounts of reported compounds, and raw data are available upon request. Sequences are deposited at National Center for Biotechnology Information under accession nos.: PolQ2, ABX24486; PolJ, ABX24494; PolK, ABX24493; PolD, ABX24500; NikL, CAC80910; NikM, CAC80911; NikK, CAC80909; NikS, CAC11141.

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

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**Author contributions**

M.M.D., A.T., H.S. and K.Y. designed the experiments. M.M.D. performed the in vitro enzyme characterizations. M.M.D. performed the biosynthetic intermediate purification and characterization experiments. A.T. and H.S. performed the microbiology and gene characterization experiments. M.M.D., A.T., H.S. and K.Y. designed the experiments. M.M.D. performed the in vitro enzyme characterizations. A.T. and H.S. performed the microbiology and gene characterization experiments. M.M.D. and A.T. analyzed the microbial metabolites. M.M.D., A.T. and K.Y. wrote the manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

Extended data is available for this paper at https://doi.org/10.1038/s41589-020-00656-8. Supplementary information is available for this paper at https://doi.org/10.1038/s41589-020-00656-8.

Correspondence and requests for materials should be addressed to K.Y.

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Extended Data Fig. 1 | Activity of PolD and PolK with 5′-OAP. Shown are HPLC chromatograms at 260 nm for a full reaction with PolK, a full reaction with PolD, a control without enzyme, and a control without α-KG. No consumption of 5′-OAP was detected. The results were reproducible in at least two independent assays.
Extended Data Fig. 2 | Activity of PolQ2 with EP-UMP. Shown are HPAEC chromatograms at 260 nm for a control without the enzyme (trace i) and the complete reaction (trace ii). No consumption of EP-UMP is detected. The results were reproducible in at least two independent assays.
Extended Data Fig. 3 | Activity of PolD, PolK, and NikM with OABP. Shown are HPAEC chromatograms at 260 nm for a control without the enzyme (trace i), an assay with PolD (ii), an assay with PolK (iii), and an assay with NikM (iv). No consumption of OABP was observed after 24 hours. The results were reproducible in at least two independent assays.
Extended Data Fig. 4 | Activity assays of PolJ, NikK, and PolK with 2′-HAP. a. PolJ phosphatase (10 μM) was incubated with 2′-HAP (100 μM) in 50 mM Tris pH 8.0 supplemented with 10 mM MgCl₂ at 25°C for 18 hrs. b. NikK aminotransferase (30 μM) was incubated with 2′-HAP (200 μM) in 200 mM Tris pH 9.0 supplemented with 1 mM MgCl₂ and 10 mM L-Glu at 25°C for 2 hrs. c. PolK oxygenase (25 μM) was incubated with 2′-HAP (200 μM) in 150 mM NaCl, 50 mM Tris pH 7.5 supplemented with 1 mM Fe²⁺, 1 mM ascorbate, 200 μM α-KG at 25°C for 18 hrs. No product formation was detected. The results were reproducible in at least two independent assays.
Extended Data Fig. 5 | Activity assays of PolK with KOAP. PolK (40 μM) was incubated with KOAP (100 μM) in the presence of 100 μM Fe^{2+}, 2 mM ascorbate, and 1 mM α-KG for 20 hours at 25 °C. Even after prolonged incubation (20 hours), no product is observed. The results were reproducible in at least two independent assays.
Extended Data Fig. 6 | Activity of PolD with KoAP. PolD (40 μM) was incubated with KoAP (100 μM) in the presence of 100 μM Fe(II), 1 mM ascorbate, and 2 mM α-KG for 2 hours at 25 °C. No product formation or KoAP consumption. For comparison, under similar conditions, PolD completed the conversion of AHOAP to AHAP in <15 minutes. The results were reproducible in at least two independent assays.
Extended Data Fig. 7 | Stereochemistry of AHOAP. Coupling constants for possible stereochemistry of C4', C5', C6' and C7'. Experimental evidence of $J_{H3'-H4'} = 10.7\, \text{Hz}$, $J_{H4'-H5'} = 3.5\, \text{Hz}$, $J_{H5'-H6'} = 3.5\, \text{Hz}$ and $J_{H6'-H7'} = 2.1\, \text{Hz}$ for AHOA is most consistent with the stereochemistry of 4'R, 5'R, 6'R, 7'S, indicating that AHOA and AHOAP have 4'R, 5'R, 6'R, 7'S stereochemistry. Dihedral angles were calculated with ChemDraw Professional v19.0 and ChemDraw 3D v19.0 (PerkinElmer Informatics).
Extended Data Fig. 8 | Comparison of the rates of reactions between PolD + AHOAP vs. PolD + 2′-OAP. The PolD assays with AHOAP were performed with 10 µM PolD, 0.1 mM Fe²⁺, 2 mM ascorbate, 100 µM AHOAP in 150 mM NaCl, 50 mM Tris pH 7.6, 10% glycerol. The PolD assays with 2′-OAP were performed with 30 µM PolD, 1 mM ascorbate, 0.5 mM Fe²⁺, 200 µM 2′-OAP in 150 mM NaCl, 50 mM Tris pH 7.6.
Extended Data Fig. 9 | LC-HRMS analysis of culture media of *S. cacao* ΔpolQ2, wt, and ΔpolQ2 + polQ2. Shown are EICs (calculated m/z for [M+H]⁺ ± 5 ppm) for polyoxins A, B, D, F, G, H, I, and J. No polyoxin production was detected in ΔpolQ2 (a), polyoxin A, B, F, and G were found in the wt strain (b), and polyoxin A and F were detected in the ΔpolQ2 + polQ2 strain (c). The observations were reproducible for 2-3 different clones for each mutant strain. The culture was repeated twice for each clone.
Extended Data Fig. 10 | Proposed divergent biosynthesis of antifungal nucleoside natural products. Proposed tailoring of sugar size by oxidative C-C bond cleavage by PolD homologs.
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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection: MS data were collected and analyzed by MassHunter B.07.00 (Agilent Technologies) or Analyst TF1.5 software (Sciex, Framingham, MA).

Data analysis: NMR data were analyzed by SpinWorks 4.2.8.0. The FTIR data were analyzed by Spectrum 10.5.2.636 [PerkinElmer], and Origin 2017 [64-bit] SR2 b9.4.2.380 [OriginLab].

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| Sample size | All the enzyme assays were performed with at least two different preparation of the enzymes. Gene disruption mutants were characterized for at least two different clones. |
| Data exclusions | We did not exclude any data. |
| Replication | The reported data were confirmed for reproducibility by repeating the experiments with the enzymes with different preparations or different clones of gene disruption mutants. |
| Randomization | This is not relevant to our study because we are characterizing specific enzymes in the PN biosynthesis. |
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