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NosA Catalyzing Carboxyl-Terminal Amide Formation in Nosiheptide Maturation via an Enamine Dealkylation on the Serine-Extended Precursor Peptide

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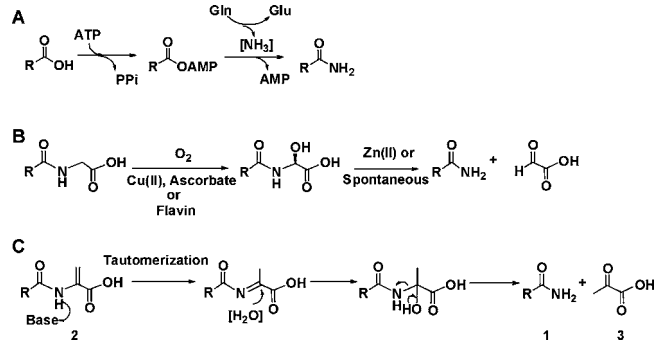
Abstract: The carboxyl-terminal amide group has been often found in many bioactive peptide natural products, including nosiheptide belonging to the over 80 entity-containing thiopeptide family. Upon functional characterization of a novel protein NosA in nosiheptide biosynthesis, herein we report an unusual C-terminal amide forming strategy in general for maturating certain amide-terminated thiopeptides by processing their precursor peptides featuring a serine extension. NosA acts on an intermediate bearing a bis-dehydroalanine tail and catalyzes an enamide dealkylation to remove the acrylate unit originating from the extended serine residue.

The amidation catalyzed by asparagine synthetase-like proteins usually utilizes the γ-carboxamide of Gln to *in situ* generate a nascent ammonia, attack of which to the activated carboxylate results in amide formation with Glu, AMP, and PPi as the coproducts (Scheme 1A).[1] For many peptide natural products, carboxyl-terminal amides can be generated endogenously (Scheme 1B), as shown in the biosynthesis of ribosomally synthesized animal hormones and nonribosomally microbial metabolites such as melithiazols.[2] Their amide moieties arise from the oxidative cleavage of C-terminal Gly-extended peptides accompanied by glyoxylate production,[3] during which the α-hydroxylation of Gly followed by dealkylation is divalent ion or flavin dependent. Distinct from these well established strategies, we here report a new way to furnish the C-terminal amide in general for maturating certain thiopeptides, based on *in vivo* and *in vitro* characterization of a novel enzyme NosA that acts on the Ser-extended precursor peptide in nosiheptide (**1**) biosynthesis.

Nosiheptide is one of the parent compounds in the thiopeptide family that contains over 80 clinically interesting entities.[4] The characteristic macrocyclic core of **1** consists of a hydroxypyridine central to multiple thiazoles, dehydroamino acids, and an amide-terminated peptide chain (Figure 1). We have recently unveiled that **1** has a ribosomal origin as other members newly to be characterized biosynthetically,[5,6] showing complex posttranslational modifications on the precursor peptide NosM. The structural peptide of NosM, consistent with the amino acids constituting the peptide backbone, possesses an extended Ser residue proven to be the nitrogen source of the terminal amide.[7] Functional assignment of biosynthetic genes in the *nos* cluster allows for identifying a hypothetic gene, *nosA*, which encodes a 151-aa protein likely

Scheme 1. Variable Mechanisms for Carboxyl-Terminal Amide Formation



involved in the late-stage tailoring to give **1**.[5] Whether or not NosA catalyzes C-terminal amide formation remains elusive, given that all NosA homologues in the database are annotated as unknown proteins.

We first validated the role of NosA in **1** maturation by gene inactivation in the producing strain *Streptomyces actuosus*. To exclude the potential effects on downstream gene expression, *nosA* mutant strain SL4008 was generated by in-frame deletion. SL4008 completely lost the ability to produce **1**; however, it produced a distinct compound (**2**) that exhibits the UV absorption pattern quite similar to that of **1** (Figure 2, II). *In trans* expression of *nosA* in SL4008 led to the complete conversion of **2** to **1** in the resulting recombinant strain SL4010 (Figure 2, III), confirming the indispensability of *nosA* in **1** biosynthesis. For structural elucidation, **2** was purified and then subjected to spectroscopic analysis (Figures

VDAAHLSDLDIDALEISEFLDESRLEDSEVVAKVMSA-**SCTTCECCCSCSS*** **NosM**

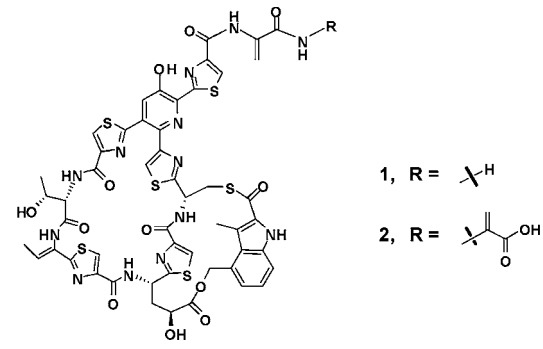


Figure 1. Precursor peptide NosM and structures of **1** and **2**. The structural peptide is shown in bold. Asterisk indicates the extended Ser.

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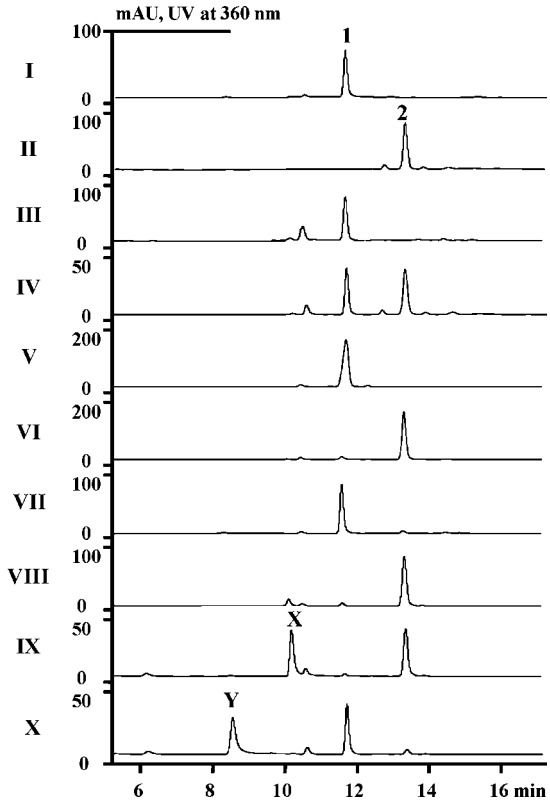

Figure 2. Validation of NosA as a terminal amide-forming protein. *In vivo* **1** or **2** production in *S. actuosus* strains, including the wild type (I); *nosA* mutant strain SL4008 (II); SL4010, a SL4008 derivative carrying *nosA* (III); and SL4009, a SL4008 derivative carrying *nocA* (IV). Using standard **1** (V) and purified **2** (VI) as the controls, *in vitro* conversion of **2** to **1** catalyzed by active NosA (VII) and NosA inactivated by heating (VIII); transformation of **2** to **X** by incubation at pH 9.0 for 12 h in the absence of NosA (IX); and transformation of the mixture of **2** and **X** respectively to **1** and **Y** in the presence of NosA (X).

S1−S3). HR-ESI-MS analysis established the molecular formula as $C_{54}H_{45}N_{13}O_{14}S_6Na$ (*m*/*z* $[M + Na]^+$ 1314.1467, 1314.1431 calculated), and extensive MS/MS analyses suggested the only difference of **2** from **1** in the C-terminal functionality of the peptidyl side chain. The ^1H, ^{13}C, and 2D NMR spectra (including HSQC and HMBC correlations) finally established that **2** is a new biosynthetic intermediate of **1** that bears a bis-dehydroalanine tail (Figure 1). These findings strongly supported the involvement of *nosA* in amide formation by removing the terminal acrylate unit of **2** to give **1**.

The counterparts of *nosA* were also found for biosynthesizing some amidated thiopeptides (Figures S4 and S5), as *nocA* (64% identity) for nocathiacin and *tpdK* (34% identity) for GE2270A.[6d,f] Similar to NosM in **1** biosynthesis, their precursor peptides contain one (Ser for NocM) or two (Ser-Ala for TpdA) extended amino acid(s) at the C-terminus in addition to the sequence for forming the thiopeptide backbone. We therefore carried out heterologous complementation to examine if the Cα−N cleavage on the dehydroalanine unit (derived from Ser) is common for maturating these thiopeptides. *nocA* was introduced into SL4008, yielding the recombinant strain SL4009 for product detection. Remarkably, about 50% of **2** was converted to **1** (Figure 2, IV), ascertaining the functional identity of *nocA* to *nosA*. This interchangeability indicates a strategy in general to afford a C-terminal amide by processing precursor peptides that feature a Ser residue extension.

To characterize NosA as a novel protein for C-terminal amide formation, we next explored its cofactor dependence and subsequent chemical conversion. NosA was overproduced in *Escherichia coli* BL21(DE3) in a 6 × His tagged form and then purified to homogeneity for *in vitro* studies (Figure S6A). The resulting protein was subjected to MALDI-TOF MS analysis, exhibiting an M^+ ion at *m*/*z* = 18 302.405 (Figure S6B). Taking the calculated molecular weight 18 318.46 and UV-absorptions into account, it is unlikely that any cofactor binds to NosA. In the presence of NosA, the conversion of **2** to **1** efficiently took place (Figure 2, VII), requiring no cofactor. To probe the catalytic chemistry, 2,4-dinitrophenylhydrazine was added in the NosA-catalyzed reaction for derivatization, leading to identification of the coproduct pyruvate (**3**, corresponding to the derivative 2-(2,4-dinitrophenylhydrazono)-propanoic acid) (Figure S7). These results validated that NosA acts on **2**, catalyzing the amide moiety formation via a Cα−N bond cleavage to afford **1** by eliminating the terminal acrylate moiety. The characteristic thiopeptide framework might be necessary for substrate recognition, since 2-acetamidoacrylic acid, a mimic to the peptidyl tail of **2**, cannot be turned over by NosA to generate **3**.

For analyzing the NosA-catalyzed reaction *in vitro*, we evaluated the stability of the substrate **2** before the condition optimization. **2** was stable at pH from 3.0 to 8.0 (Figure S8). By contrast, the incubation at pH 9.0 resulted in a time-dependent transformation of **2** to a major distinct compound (**X**, HR-ESI-MS *m*/*z* $[M + H]^+$ calcd 1310.1717 for $C_{54}H_{48}N_{13}O_{15}S_6$, found 1310.1765), which, with the 18-Da increase in molecular weight, could be a hydrated or side-ring-hydrolyzed product of **2** (Figure 2, IX, and Figure S9). **X** can be efficiently converted by NosA to **Y** (HR-ESI-MS *m*/*z* $[M + H]^+$ calcd 1240.1662 for $C_{51}H_{46}N_{13}O_{13}S_6$, found 1240.1725), a putative acrylate-removed analogue (Figure 2, X), excluding the possibility of H_2O addition onto the terminal dehydroalanine unit. The mixtures containing **X** and **Y**, respectively, were therefore subjected to sulfhydryl derivatization by addition of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (Figure S10), showing the generation of their corresponding derivatives **X′** (ESI-MS *m*/*z* $[M + H]^+$ calcd 1507.1, found 1506.6) and **Y′** (ESI-MS *m*/*z* $[M + H]^+$ calcd 1437.1, found 1436.8). This indicated the cleavage of the thioester bond by hydrolysis at high pH to give a free sulfhydryl group. Further, MS/MS analysis supported the deduced structures of **X** and **Y** (Figures S11 and S12), both of which are indole side-ring-opened. The finding that NosA tolerates **X** as the substrate is consistent with the hypothesis that the main thiopeptide core plays a key structural role in the enzyme action, as a similar modification for maturation could be involved in the biosynthesis of the monocyclic thiopeptide GE2270A.[6d]

Thus, the pH dependence of NosA-catalyzed conversion was investigated by using **2** as the substrate in the reaction with buffers varying from 4.0 to 8.5, displaying an optimal activity at pH 8.0 (Figure S13). The addition of EDTA into the reaction mixture did not afford a significant change in enzymatic activity, suggesting the metal ion independence of NosA. Under the optimized conditions, the steady-state kinetic parameters were measured, showing a conversion with a k_m value of 93.7 ± 0.7 μM for **2** and a k_{cat} value of 1200 ± 30 min^{-1} (Figure S14).

We thereby proposed that the NosA-catalyzed reaction may proceed via an enamide dealkylation: (1) specific tautomerization of the terminal dehydroalanine unit of **2** to the corresponding methyl imine is followed by nucleophilic attack of H_2O to generate the terminally hydrated intermediate, and (2) subsequent cleavage of the Cα−N bond yields **1** with pyruvate production (Scheme 1C). Consistent with previous studies in chemical synthesis of thiostrepton,[8] the reaction can be base-initiated, whereas acidic conditions would make tautomerization of enamide to the labile imine

difficult and lead to a dramatic decrease in NosA activity (Figure S13). The NosA chemistry is nonoxidative and takes advantage of the terminal enamide moiety generated from the extended Ser by the dehydratase pair NosE and NosD that act at the early stage posttranslational modifications on the precursor peptide NosM.[6] This strategy is clearly distinct from that for maturation of the thiopeptides originating from the non-Ser-extended precursor peptides, such as thiostrepton and siomycin (Figure S5),[7b-d] which requires an amidotransferase (TsrT/TsrC or SioC belonging to the asparagine synthetase family) to amidate the free carboxylic acid (Scheme 1A). Though terminal amide formation is often of importance to bioacitivity,[9] **2** showed a activity comparable to that of **1** against the test strain *Bacillus subtilis* (Table S2).

In conclusion, we have characterized that NosA in a cofactor-independent manner catalyzes an enamide dealkylation that may be common for maturating the thiopeptides nosiheptide, nocathiacin, and GE2270A, by processing the Ser-extended precursor peptide to furnish the terminal amide moiety with pyruvate production. The NosA chemistry is mechanistically distinct from known C-terminal amide forming proteins. Taking advantage of the substrate tolerance, it can be exploited by combinatorial biosynthesis for structural diversity in thiopeptide natural products.

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Supporting Information Available: Experimental procedures for gene inactivation and complementation, characterization of reactions and compounds, protein expression and analysis, sequence analysis, and bioassay; supplementary tables; and supplementary figures. These materials are available free of charge via the Internet at http://pubs.acs.org.

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