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## Isolation and structure determination of two new nosiheptide-type compounds provide insights into the function of the cytochrome P450 oxygenase NocV in nocathiacin biosynthesis†

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Thiopeptides, which are a class of sulfur-rich, ribosomally synthesized and post-translationally modified peptides (RiPPs), have great potential in the treatment of diseases caused by oral pathogens. Nocathiacin I (NOC-I) and nosiheptide (NOS) are two structurally similar thiopeptide members that feature an indolic side ring. In the structure of NOC-I, this side ring is further rigidified through the formation of an ether linkage; however, the related biosynthetic process remains poorly understood. Here, we report that NocV, a cytochrome P450 protein found to be unique in the biosynthetic pathway of NOC-I, is responsible for the establishment of the intramolecular ether linkage through two oxidation steps. This observation benefited from the heterologous overexpression of the gene *nocV* in an engineered *Streptomyces* strain producing the bicyclic NOS intermediate NOS1260, and subsequent isolation and structure characterization of two functionalized products. The product NOS-V1 contains a new hydroxyl group at C $\alpha$  of the residue Glu6, in contrast with the other product NOS-V2, in which this hydroxyl group is further coupled with the C4 methyl group of the indolic moiety to form an ether linkage. These findings provide insights into the catalytic logic of NocV in the biosynthesis of NOC-I, during which this cytochrome P450 protein appears to act in tandem on two positions in NOS1260 by selectively hydroxylating Glu6 and then oxidatively coupling the indolic moiety. Rigidifying the side ring via ether bond formation has a positive impact on the antibacterial properties of NOS-type thiopeptides, evidenced by the improved activity of NOC-V2 against various tested oral pathogens compared with NOS1260.

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## Introduction

Thiopeptides are a class of sulfur-rich, ribosomally synthesized and post-translationally modified peptides (RiPPs), characterized by a macrocyclic framework that contains a six-membered

heterocycle domain central to multiple azoles and dehydro-amino acids.<sup>1</sup> Similar to many other RiPPs, a thiopeptide originates from a precursor peptide consisting of an N-terminal leader peptide (LP) and a C-terminal core peptide (CP). The CP undergoes a series of post-translational modifications (PTMs) in a manner either dependent or independent of the LP to produce mature products.<sup>2</sup> Most members of thiopeptides exhibit potent activity against drug-resistant Gram-positive bacteria; however, poor water solubility and low bioavailability limit their clinical use.<sup>3</sup> Previously, we reported the promising activities of the thiopeptide members thiostrepton (TSR) and siomycin (SIO), as well as their analogs, against oral pathogens, indicating the potential application of thiopeptides for the treatment of human oral diseases.<sup>4</sup>

Nocathiacin I (NOC-I) and nosiheptide (NOS) are two thiopeptides with an indolic moiety appended to the characteristic macrocyclic framework through two ester linkages.<sup>5–7</sup> NOC-I shares an overall similarity with NOS in the molecular skeleton but is more functionalized. As a unique glycosylated thiopeptide, NOC-I shows good water solubility, which is largely attrib-

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uted to glycosylation of the Glu6  $\gamma$ -hydroxyl with a rare sugar moiety.<sup>8</sup> Besides, the aglycone of NOC-I bears additional structural modifications, such as *O*-methylation of the residue Thr4, *N*-hydroxylation of the indolic moiety, and rigid constraint of the indole-possessing side ring *via* an ether bond. Comparative analysis of the *noc* and *nos* biosynthetic gene clusters (BGCs) shows significant similarity in their gene sequence and arrangement,<sup>9,10</sup> suggesting great similarity in the biosynthesis of NOC-I and NOS. Apart from homologous genes, the *noc* BGC contains several additional genes that may be involved in PTMs to afford the specific functionalization of NOC-I (Fig. 1A). Systematic studies have revealed in depth the

biosynthetic pathway of NOS,<sup>11–16</sup> including the LP-independent hydroxylation steps catalyzed by two cytochrome P450 oxygenases, NosB and NosC.<sup>17</sup> In addition to the NosB and NosC homologs NocB and NocC, three other P450 oxygenases, NocT, NocU, and NocV, are supposed to be functional in multiple oxidative routes towards the maturation of NOC-I (Fig. 1B). Unfortunately, the NOC-producing strain *Nocardia* sp. is resistant to available genetic manipulation approaches,<sup>9</sup> making the biosynthetic studies of NOC-I largely delayed. Given the high similarity of the molecular skeleton of NOS and NOC-I, the unclear biosynthetic pathway of NOC-I can be elucidated *via* heterologous expression of the *noc* genes in the NOS-



**Fig. 1** BGCs and biosynthetic pathways of NOC-I and NOS. (A) Organization of the *noc* BGC in comparison with the *nos* BGC.<sup>9,10</sup> The deduced functions of biosynthetic genes are labeled in pattern, and the sequence homologies are indicated by a dashed line for each pair. (B) Proposed biosynthetic pathway of NOC-I and NOS.<sup>11–17</sup> Precursor peptides of NocM for NOC-I and NosM for NOS go through ordered PTMs either dependent or independent of the LPs. The structural differences are colored red (for NOC-I) or blue (for NOS).

producing strain *Streptomyces actuosus*. Here, we apply an NOS biosynthetic machinery to explore the function of the P450 oxygenase NocV and evaluate the antibacterial activities of the resulting NOS framework-containing analogs against oral pathogens.

## Results and discussion

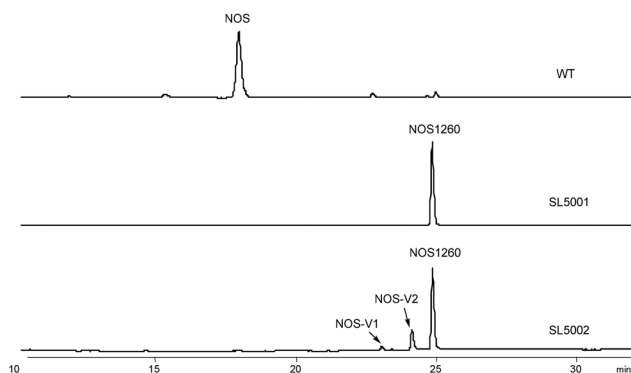
Considering the multi-substrate specificity of NosB and NosC in conjunction with NosA in the maturation of NOS,<sup>17</sup> we constructed a *Streptomyces actuosus* mutant strain, SL5001, in which the contiguous genes *nosA*, *nosB*, and *nosC* were deleted simultaneously. In SL5001, the PTMs performed during NOS biosynthesis can be simplified, leading to accumulation of a key intermediate, NOS1260 (Fig. 2). NOS1260 is the first intermediate possessing the NOS-type thiopeptide framework after LP release. Structure characterization of NOS1260 confirmed the presence of the unoxxygenated Glu6 and six-membered heterocycle core, as well as the bis-dehydroalanine (Dha) tail.

To investigate the *in vivo* function of NocV, we heterologously expressed *nocV* in the engineered NOS biosynthetic system. A plasmid consisting of two copies of *nocV* was introduced into the engineered *S. actuosus* strain SL5001 *via* intergeneric conjugation. The resulting recombinant strain, SL5002, was capable of producing two new compounds, nosiheptide V1 (NOS-V1) and nosiheptide V2 (NOS-V2), based on HPLC analysis (Fig. 2). Both compounds showed UV absorption patterns similar to that of NOS1260, indicating that they were NOS-derived analogs (Fig. S1†). HR-ESI-MS ion data further established the molecular formulas of these two compounds as  $C_{54}H_{45}N_{13}O_{13}S_6$  ( $m/z$  1276.1652 [ $M + H$ ]<sup>+</sup>, calcd 1276.1657) and  $C_{54}H_{44}N_{13}O_{13}S_6$  ( $m/z$  1274.1493 [ $M + H$ ]<sup>+</sup>, calcd 1274.1500), suggesting that NOS-V1 and NOS-V2 were generated *via* introduction of a hydroxyl and an oxygen atom into NOS1260, respectively (Fig. S2 and S5†).

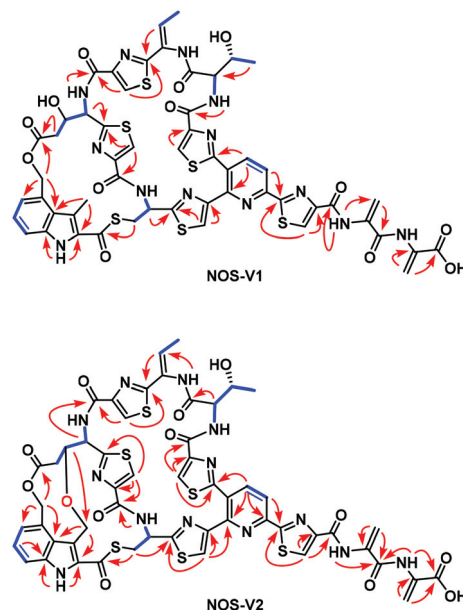
The recombinant strain SL5002 was subjected to large-scale fermentation to accumulate target products for structure eluci-

dation. A total of 5.6 mg NOS-V1, 11.1 mg NOS-V2, and 46.2 mg NOS1260 were isolated to high purity and subjected to 1D and 2D NMR (<sup>1</sup>H and <sup>13</sup>C NMR, <sup>1</sup>H–<sup>1</sup>H COSY, HSQC, HMBC, and ROESY) experiments (Fig. S4 and S7†). In comparison to the NMR spectra of NOS1260, the 1D and 2D NMR spectra of NOS-V1 clearly revealed the presence of an additional hydroxyl group: (i) the H<sub>2</sub>( $\delta_H$  2.38)–H<sub>3</sub>( $\delta_H$  4.46)–H<sub>4</sub>( $\delta_H$  5.28) spin–spin coupling system in the residue Glu6; (ii) the HMBC correlations from H-2 ( $\delta_H$  2.38) to C-1 ( $\delta_C$  169.4), C-3 ( $\delta_C$  68.2) and C-4 ( $\delta_C$  54.1); and (iii) the <sup>1</sup>H–<sup>1</sup>H coupling constant <sup>3</sup>*J*<sub>H-3/H-4</sub> (9.0 Hz) and the H-3/H<sub>3-3'</sub> (Ind) ROESY correlation indicated that HO-3 (Glu6) was  $\alpha$ -oriented. Meanwhile, careful analysis of the NMR spectra of NOS-V2 revealed that the structural features of NOS-V2 were similar to those of NOS1260, differing only in the oxygenation in Glu6 C-3 ( $\delta_C$  78.8 and  $\delta_H$  4.06) and the 2,4-dimethylindolic acid (DMIA) moiety C-3' ( $\delta_C$  64.0 and  $\delta_H$  4.77 (Ha), 4.06 (Hb)), resulting in formation of an oxygen ether bond. The findings were demonstrated by HMBC correlations from H-3 ( $\delta_H$  4.06, Glu6) to C-3' ( $\delta_C$  64.0, indole). Therefore, the chemical structures of NOS-V1 and NOS-V2 were elucidated, featuring a hydroxyl group of Glu6 at the  $\alpha$ -position and an ether bond between Glu6 and the indole 3-methyl, respectively (Fig. 3, S3, S6 and Table S3, S4†).

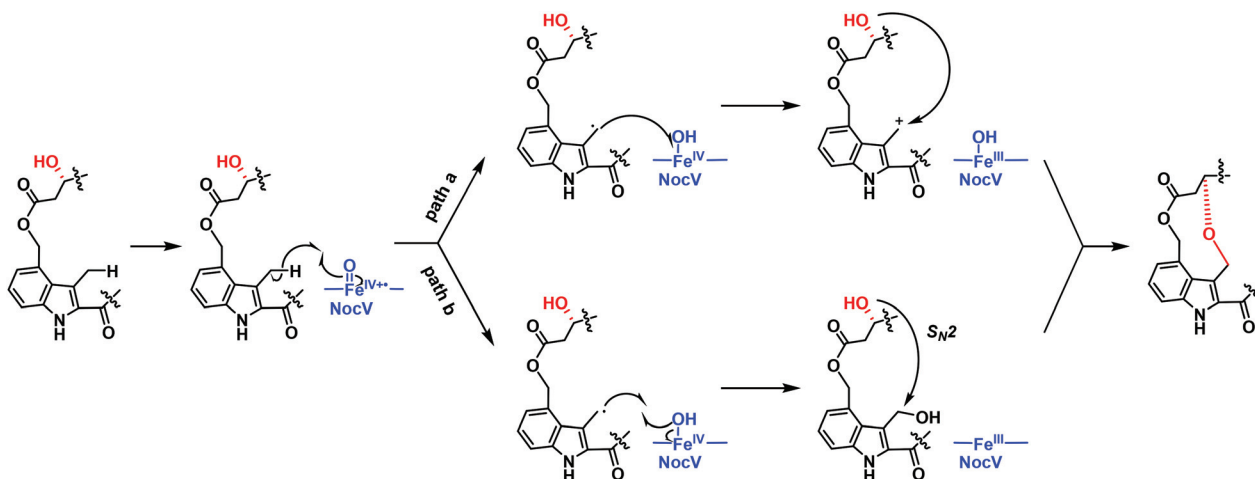
The structure characterization of both NOS-V1 and NOS-V2 supported the notion that NocV acts as a cytochrome P450 oxygenase that catalyzes the two-step oxidation of Glu6 and the indolic moiety to form a hydroxyl group and an ether bond. However, the catalytic logic of NocV in NOS biosynthesis remained unclear. We thus overexpressed and purified NocV along with another cytochrome P450 protein, NocB (Fig. S8†), whose counterpart in NOS biosynthesis has been characterized for *in vitro* assays.<sup>17</sup> NocV and NocB, in the C-terminally 6×



**Fig. 2** HPLC analysis of the fermentation products from the wild-type NOS-producing strain *Streptomyces actuosus* ATCC25421, the mutant strain SL5001, and the recombinant strain SL5002. The NOS framework-containing compounds were characterized as NOS1260, NOS-V1, and NOS-V2.



**Fig. 3** <sup>1</sup>H–<sup>1</sup>H COSY (—) correlations, key HMBC (H → C) correlations of NOS-V1 and NOS-V2.



**Fig. 4** Proposed mechanism for the NocV-catalyzed ether bond formation. The methyl group of indole was activated by an  $\text{Fe}^{\text{IV}}=\text{O}$  radical cation to form an allyl radical and an  $\text{Fe}^{\text{IV}}-\text{OH}$  species. Through path a, the  $\text{Fe}^{\text{IV}}-\text{OH}$  species reduced the allyl radical to an allyl cation, which was then captured by the hydroxyl group and formed the ether bond. Through path b, the allyl radical abstracted the hydroxyl radical to form a dihydroxyl group intermediate and an  $\text{Fe}^{\text{III}}$  species. The  $\alpha$ -OH substituted the newly formed hydroxyl to construct the ether bond.

His-tagged form, showed typical characteristics of cytochrome P450 proteins in CO binding difference spectra (Fig. S9†). Although three different electron transfer systems were tested, the *in vitro* activity of NocV could not be detected, using either NOS1260 or NOS-V1 as the substrate (Fig. S10†). As a reference, NocB was capable of catalyzing the hydroxylation of Glu6 at  $\text{C}_\gamma$ , using NOS1260, NOS-V1, and NOS-V2 as the substrates (Fig. S10†). These results suggest that NocV might not match the provided electron transfer systems; however, the possibility that NOS1260 and NOS-V1 are not the real substrates of NocV cannot be excluded at this time. NocV may catalyze the oxidation reaction in an LP-dependent manner that occurs in the early stage of PTMs. Based on existing precedents of cytochrome P450 proteins, such as those in the biosynthesis of aureothin,<sup>18,19</sup> platensimycin,<sup>20</sup> and paspalicine,<sup>21</sup> we proposed an enzymatic mechanism for the NocV-catalyzed ether bond formation. First, NocV functions as a hydroxylase to oxygenate the residue Glu6 in a stereoselective manner. Then, NocV abstracts a hydrogen atom from the C4 indole methyl group, giving rise to a carbocation. Eventually, NOS-V2 is produced *via* a cyclization by coupling the Glu6  $\alpha$ -hydroxyl and the indole carbocation. Alternatively, the ring closure might

occur through nucleophilic substitution *via* a bis-hydroxylated intermediate (Fig. 4).

NOC-I exhibits significantly higher biological activity than NOS.<sup>8</sup> This is undoubtedly attributed to the glycosylation modification. Meanwhile, it may also be due to the contribution of the side-ring ether bond that endows molecular rigidity. NOS-V1, NOS-V2, NOS, and NOS1260 were thus evaluated for their activities against a panel of oral pathogens, including Gram-positive cariogenic bacteria (*Streptococcus mutans*, *Lactobacillus acidophilus*, *Actinomyces viscosus*, *Enterococcus faecalis*) and Gram-negative periodontal bacteria (*Fusobacterium nucleatum*, *Porphyromonas gingivalis*) (Table 1). Compared with NOS1260, NOS-V2 showed markedly improved activity against *Streptococcus mutans*, *Actinomyces viscosus*, *Enterococcus faecalis*, and *Porphyromonas gingivalis* with significantly reduced minimum inhibitory concentration (MIC) values. NOS-V2 presents a side-ring ether bond that distinguished it from NOS1260, indicating that the increase in molecular rigidity has a positive impact on the biological activity of NOC-I. Among the tested compounds, NOS exhibited the best antibacterial activity in most cases, suggesting that the PTMs in NOS biosynthesis, including hydroxylation of the NOS

**Table 1** MIC values of NOS-V1, NOS-V2, NOS, and NOS1260 against oral pathogens. Sodium fluoride (NaF) and chlorhexidine (CHX) were chosen as control drugs for the tests of Gram-positive cariogenic bacteria. Levofloxacin (LVX) and minocycline hydrochloride (MCC) were chosen as control drugs for tests against Gram-negative periodontal bacteria

Oral pathogen	MIC ( $\mu\text{g mL}^{-1}$ )							
	NOS1260	NOS-V1	NOS-V2	NOS	NaF	CHX	LVX	MCC
<i>Streptococcus mutans</i> UA159	0.052	0.065	0.039	0.009	3000	1.66	n.d.	n.d.
<i>Lactobacillus acidophilus</i> ATCC 4356	0.026	0.104	0.032	0.032	12 000	2.5	n.d.	n.d.
<i>Actinomyces viscosus</i> ATCC 19246	0.039	0.104	0.005	0.001	4000	1.25	n.d.	n.d.
<i>Enterococcus faecalis</i> ATCC 29212	0.312	0.729	0.032	0.104	>12 000	5	n.d.	n.d.
<i>Fusobacterium nucleatum</i> ATCC 25286	10	10	10	5	n.d.	n.d.	1.25	0.13
<i>Porphyromonas gingivalis</i> ATCC 33277	0.208	0.417	0.078	0.020	n.d.	n.d.	0.208	0.13



framework and removal of one Dha tail, were of great significance for biological activity.

## Conclusions

Benefiting from the high similarity of the NOC and NOS biosynthetic machinery,<sup>9,10</sup> we studied the *in vivo* catalytic function of the cytochrome P450 oxygenase NocV in an engineered NOS-producing strain. Structure elucidation of the resultant products revealed the generation of two new compounds, NOS-V1 and NOS-V2, with a hydroxyl group and an ether bond in the side ring of the NOS framework, respectively. Although the *in vitro* enzymatic activity assays did not determine the real substrate of NocV, we can speculate that NocV catalyzes a two-step oxidation of Glu6 and the indolic moiety. NOS-V2, containing an ether bond in the side ring, showed better activity against oral pathogens than its parental molecule, indicating the importance of molecular rigidity in the biological activities of natural products. Studies on NOC biosynthesis have been greatly limited due to the resistance of the producing strain to the available genetic manipulation approaches.<sup>9</sup> Here, we revealed the catalytic function of NocV, filling in a blank in NOC biosynthetic research. Development of thiopeptides into oral medicines could effectively avoid problems such as poor water solubility and low bioavailability. Therefore, advances in biosynthetic studies of NOC-I will promote application of thiopeptides as dental drugs.

## Conflicts of interest

There are no conflicts to declare.

## Acknowledgements

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