



Characterization of Nocardithiocin Derivatives Produced by Amino Acid Substitution of Precursor Peptide notG

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Abstract

Nocardithiocin is a thiopeptide compound produced by the pathogenic actinomycete *Nocardia pseudobrasiliensis* that displays activity against Gram-positive bacteria including drug-resistant *Mycobacterium tuberculosis*. However, the clinical use of nocardithiocin is hampered by light sensitivity and low water solubility. To improve these properties, the sixth amino acid of core peptide was substituted for other 19 amino acids by modifying the gene sequence of precursor peptide. Ten amino acids were successfully substituted to produce 1–3 novel compounds, of which six were the expected nocardithiocin derivatives. The compounds were tested for their stability, solubility, and antimicrobial activity. Of the 17 compounds produced, ten displayed antibiotic activity and two of them improved MIC value to bacteria. Furthermore, nocardithiocin and all derivatives were stable to the light. The substitution of amino acid sequence in precursor peptide could lead the successful production of nocardithiocin derivatives and alter its property. This report represents the first step of nocardithiocin utilization, and amino acid substitution technique can be used to create desirable nocardithiocin derivatives.

Keywords Thiopeptides · Nocardithiocin · Antimicrobial · *Mycobacterium tuberculosis* · Gene modification

Introduction

Thiopeptides are natural products derived from ribosomally synthesized post-translationally modified peptides (RiPPs) that possess a variety of biological properties including antimicrobial, antiparasitic, and anticancer activities (Li and Kelly 2010; Just-Baringo et al. 2014a). However, their poor pharmacokinetic properties, especially low water solubility, hampers their clinical use. To improve their properties, derivatives have been generated by several ways such as chemical synthesis (Nicolaou et al. 2003; Gross et al. 2013; Just-Baringo et al. 2014b), semi-synthesis (Naidu et al. 2006; Xu et al. 2013), and genetic modification (Bowers et al. 2010; Li et al. 2011, 2012; Young et al. 2012; Zhang and Kelly 2015) (Just-Baringo et al. 2014c). Based on the biosynthetic pathway of RiPPs compounds, DNA sequence of precursor peptide is directly related to their peptide structure (Arndt et al. 2009; Kelly et al. 2009; Morris et al. 2009). So the derivatives production of thiopeptides were achieved relatively easy by modifying the gene sequence of core peptide region among precursor peptide. The increasing information of biosynthetic genes and recent progress in the development of genetic tools and techniques have made

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thiopeptides more attractive as novel lead compounds for pharmaceutical agents.

Nocardithiocin is a thiopeptide compound produced by *Nocardia pseudobrasiliensis* that displays antimicrobial activity against Gram-positive bacteria, especially acid-fast bacteria (Fig. 1a) (Mukai et al. 2009). Impressive antimicrobial activity against drug-resistant *Mycobacterium tuberculosis* has resulted in significant attention to the gene cluster responsible for nocardithiocin biosynthesis (Sakai et al. 2015). Gene disruption and expression analysis revealed a 15.2 kb cluster of 12 genes including precursor peptide (*notG*), as well as post-transcriptional modification enzymes that modify the peptide structure (Fig. 2a).

Like other thiopeptides, nocardithiocin is poorly soluble in water and light sensitivity was reported (Mukai et al. 2009). Herein, we attempted to improve its properties by genetic modification without diminishing its antibiotic activity. Although the actual mode of action of nocardithiocin is not elucidated yet, a number of 26-membered macrocycles thiopeptides including thiostrepton, noshiheptide, and micrococcin have been reported that interaction with ribosomal protein L11 as well as 23S rRNA through a Threonine residue (highlighted in red in Fig. 1a) cause antimicrobial activity (Bowers et al. 2010; Acker et al. 2009; Tran et al.

2017). Residues S₁, C₉, S₁₀, C₁₁, and T₃ of nocardithiocin precursor peptide were thought to be related to retention of the macrocyclic structure and antimicrobial activity, respectively (Fig. 2b). In the present study, the sixth amino acid (V₆) of the core peptide which exist at distal position from pyridine ring was selected to replace with the other 19 amino acids and the properties and antimicrobial activity of the derivatives were assessed.

Materials and Methods

Strains and Cultivation Conditions

Nocardithiocin producer *N. pseudobrasiliensis* IFM 0761 was obtained from Medical Mycology Research Center (MMRC), Chiba University, Japan. *Escherichia coli* DH5 α was used for construction and propagation of vectors. *N. pseudobrasiliensis* strains were normally cultivated at 37 °C in brain–heart infusion (Becton Dickinson and Company, Tokyo, Japan) supplemented with 1% glucose and 1% glycerol (BHIgg) broth or 2% agar plates. For the nocardithiocin production, strains were cultivated at 30 °C in Czapek–Dox (CD) medium consisting of 0.6% NaNO₃, 0.052% KCl,

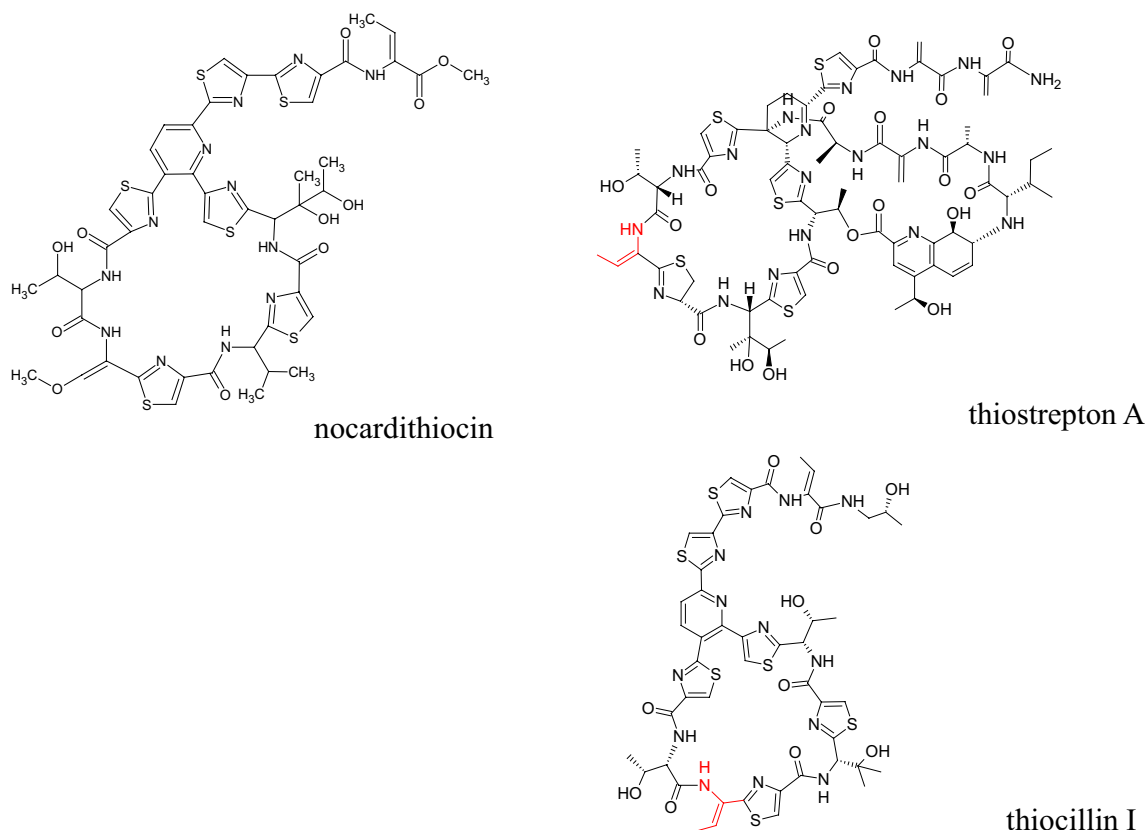


Fig. 1 Structure of nocardithiocin and other thiopeptides

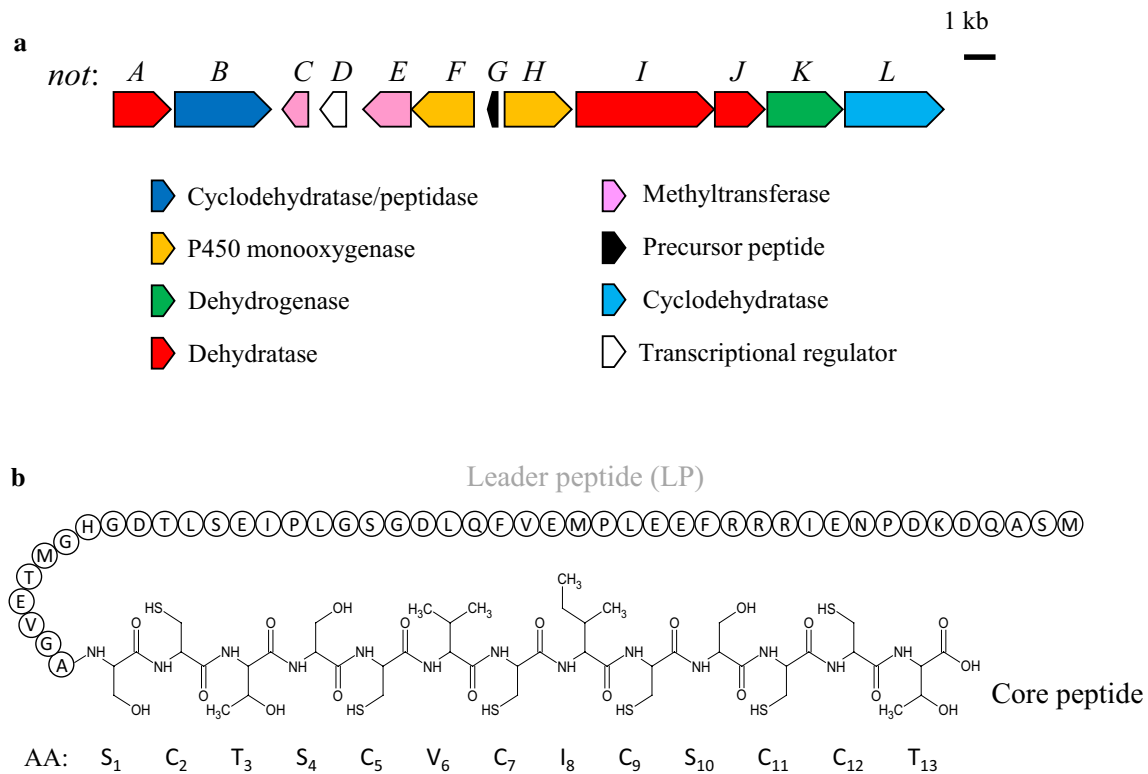


Fig. 2 **a** Nocardithiocin biosynthetic gene cluster showing the 12 genes *notA*–*notL*. **b** Amino acid sequence of *notG*. The amino acid in the sixth position (valine, in red) was replaced by the other 19 amino acids

0.152% KH_2PO_4 , 1% glucose, 2 mM MgSO_4 and trace elements.

Deletion of *notG* in *Nocardia pseudobrasiliensis*

The *notG* gene was removed from *N. pseudobrasiliensis* genome by the double cross-over method, leaving the start and stop codon to retain in-frame translation of the rest of the biosynthetic genes. The *notG* disruption vector was constructed using upstream and downstream *notG* fragments that were amplified by PCR using *notG*-upF (tgctgcaggtcgagcgtcccaggtgacc) and *notG*-upR (acgggactactacatgacactatccttct), and *notG*-downF (gatagtgtcatgtagtagtcccgtcgtggg) and *notG*-downR (tcctctagagtcgacgaacaacctggcatc), primer pairs, with *N. pseudobrasiliensis* IFM 0761 genomic DNA as a template. Underlined sequences correspond to overlaps used for recombination. Both up- and down-stream fragments were ligated into the *HincII* site of the pNV18.1 vector using the GeneArt Seamless Cloning and Assembly Enzyme Mix (Life Technologies, Tokyo, Japan) according to the manufacturer's protocol.

Transformation of *N. pseudobrasiliensis* was conducted as described previously (Sakai et al. 2015). The first cross-over strains were grown on BHIgg plates containing 100 $\mu\text{g}/$

ml neomycin, and further cultivated in BHIgg liquid medium without neomycin for 3 days. Cultures were spread onto BHIgg agar medium without neomycin, and resulting colonies were picked and inoculated into BHIgg plates with and without neomycin. Colonies that grew only on BHIgg plates without neomycin were selected as double cross-over strains. Deletion of *notG* in the double cross-over strains was confirmed by PCR, Southern blotting, and DNA sequencing.

Expression of Site-Directed *notG* Mutants

Native and mutant *notG* expression vectors were constructed by joining PCR fragment of the *notG* fragment to the native promoter and *nmrB* terminator. Site-directed mutagenesis of *notG* was conducted by PCR using *notG*-nPF/V60X-R and V60X-F/*notG*-R-TrrnB primer pairs with *N. pseudobrasiliensis* genomic DNA as the template (X indicates any amino acid). The terminator sequence was amplified using the *notG*-TeenBF/TrrnB-R primer pair with the pRU1701 vector as template. All fragments were ligated into the *HincII* site of pNV18.1 using the GeneArt Seamless Cloning and Assembly Enzyme Mix. All expression vectors were confirmed by sequencing, and primers used for construction are listed in Table S1.

Detection and Purification of Nocardithiocin Derivatives

Constructed strains (the $\Delta notG$ strain containing a native or mutant *notG* expression vector) were pre-cultured in BHIgg medium containing neomycin for 3 days at 37 °C, and 1% of each pre-culture was inoculated into CD medium containing neomycin and cultivated for 7 days at 30 °C. After cultivation, the same volume of methanol was added to the culture and incubated for at least 1 h. Samples were filtered to remove bacterial cells, and filtrates were evaporated using a rotary evaporator. The remaining water layer was extracted with ethyl acetate, and the ethyl acetate extract was dried in vacuo.

Crude extracts were analyzed to examine the production of nocardithiocin and its derivatives by HPLC (RF-20A/RF-20Ax, Shimadzu Corp., Kyoto, Japan) with a WakoPack Wakosil-II 3C18 HG column and a gradient elution of 10–100% acetonitrile in water over 50 min at a flow rate of 1 ml/min. Eluates were monitored using a fluorescence detector at an extinction wavelength of 350 nm and an emission wavelength of 450 nm (RF-20A/RF-20Axs, Shimadzu Corp.) and a mass spectrometer (LCMS-2020, Shimadzu Corp.). Nocardithiocin derivatives were purified from crude extracts by preparative HPLC using a Cosmosil 5C18 MS-II column (10 × 150 mm, NACALAI TESQUE, Inc., Kyoto, Japan) with a gradient elution of 30–60% acetonitrile in water over 20 min followed by 100% acetonitrile for 5 min, monitored by a fluorescence detector.

Structural Confirmation

The crude extracts were first analyzed by LC–MS and the structure of nocardithiocin derivatives were determined by LC–MS/MS analysis. LC–MS/MS was conducted using TripleTOF 6600 hybrid quadrupole-time-of-flight tandem mass spectrometer (Sciex, Concord, Canada) equipped with an electrospray ionization interface. An ODS-80Ts column (5 μm, 2.0 × 150 mm, TOSOH, Tokyo, Japan) was used with mobile phases 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). LC gradient condition was initially 10% B to 100% B (30 min) and 10% (40 min) at flow rate of 0.2 ml/min. Information dependent acquisition was performed in positive ion mode. MS spectra were acquired from m/z 100 to m/z 2000. The 10 most abundant ions were selected for subsequent fragmentation, and MS/MS spectra were acquired from m/z 100 to m/z 2000.

Testing Light and Temperature Stability of Nocardithiocin and Its Derivatives

Purified compounds were dissolved in DMSO at a final concentration of 0.5 mg/ml and divided into three parts. One

part was covered with foil and kept in the dark, one part was placed under a fluorescent lamp (light) for 4 days at room temperature, and the final part was stored at 4 °C in the dark. Samples were analyzed by HPLC to quantify the amount of remaining nocardithiocin derivatives. Light stability was calculated by comparing the HPLC peak area of samples stored in the light and dark at room temperature. Similarly, temperature stability was calculated by comparing the dark samples stored at room temperature (30 °C, in summer) and 4 °C for 4 days.

Testing Solubility of Nocardithiocin and Its Derivatives

Purified dried samples were dissolved in DMSO or water at a final concentration of 1 mg/ml and incubated overnight. Insoluble matter was removed by centrifugation, and soluble compounds were quantified using HPLC by comparing the peak areas of compounds in DMSO (completely dissolved 100%) and water.

Testing Antimicrobial Activity

Antimicrobial activity against several bacteria and fungi (*Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Micrococcus luteus*, *Aspergillus niger*, *Trichophyton mentagrophytes*, *Candida albicans*, and *Cryptococcus neoformans*) was determined as described previously (Nagai et al. 1993) using the microbroth dilution method. These strains were obtained from the Medical Mycology Research Center (MMRC), Chiba University. The minimum inhibitory concentration (MIC) against mycobacteria was determined using the Clinical Laboratory and Standards Institute (CLSI) protocol (M24-A2). Mycobacterial strains were obtained from the National Institute of Infectious Diseases (NIID). MIC and IC₅₀ (half maximal inhibitory concentration) values were determined for antimicrobial activity against bacteria and fungi, respectively.

Results

Deletion of *notG* in *N. pseudobrasiliensis*

Before expressing the mutant *notG* gene, native *notG* which encode the precursor peptide was removed from the *N. pseudobrasiliensis* IFM 0761 genome (Fig. 2). Deletion of *notG* in $\Delta notG$ strains was confirmed by PCR and sequencing (data not shown). HPLC analysis confirmed the absence of nocardithiocin production in $\Delta notG$ strains (Fig. 3). Three $\Delta notG$ strains were checked and all of them exhibited the same morphology, so one of them was selected as the host for production of nocardithiocin derivatives.

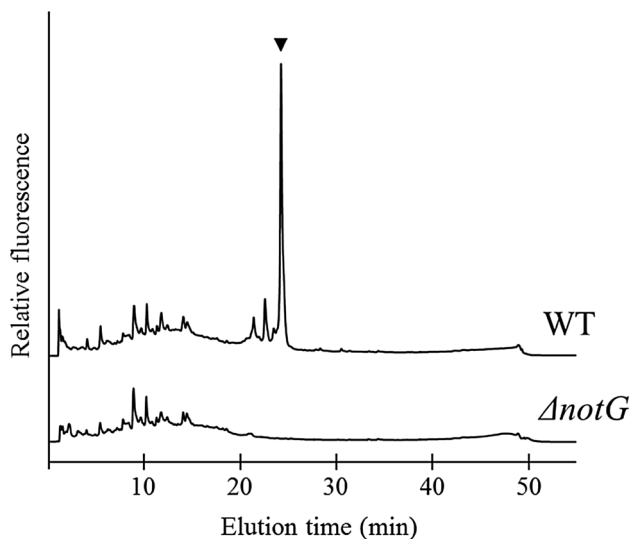


Fig. 3 HPLC analysis of nocardithiocin production in wild-type (WT) and $\Delta notG$ strains. The nocardithiocin peak is indicated by an arrowhead

Production of Nocardithiocin Derivatives

The native *notG* expression vector and 19 mutant *notG* expression vectors with the sixth amino acid (V_6) replaced by the other 19 amino acids (Fig. 2b) were each transformed into the $\Delta notG$ strain, and transformants were cultivated on agar plates. Several colonies were picked to confirm the production of nocardithiocin or its derivatives by HPLC, and the highest producer was selected for subsequent experiments. As shown in Fig. 4, several fluorescence peaks were detected that appeared to be nocardithiocin derivatives. Peaks were significant for ten mutants (A, F, L, I, M, Y, S, T, N, and Q substitutions), indicating successful production of nocardithiocin derivatives. Substitution with A, L, M, S, T, or Q resulted in two or three clear fluorescence peaks with slightly different m/z values, were numbered in order of elution time in Fig. 4. The information of replaced amino acid and MS data from the fluorescence peaks are summarized in Table 1.

Structural Confirmation of Nocardithiocin Derivatives

Nocardithiocin and its derivatives were purified by preparative HPLC. As shown in Table 1, peptides in which the sixth amino acid was replaced by F, M, Y, and N did not yield the expected m/z values, whereas peptides containing A, L, I, S, T, and Q matched the expected m/z values, although peptides with A, L, S, T, and Q produced additional unknown peaks. To further confirm the structure of these compounds, LC-MS/MS analysis was performed and the MS/MS results and proposed structures are shown in Fig. S1 and Table S2.

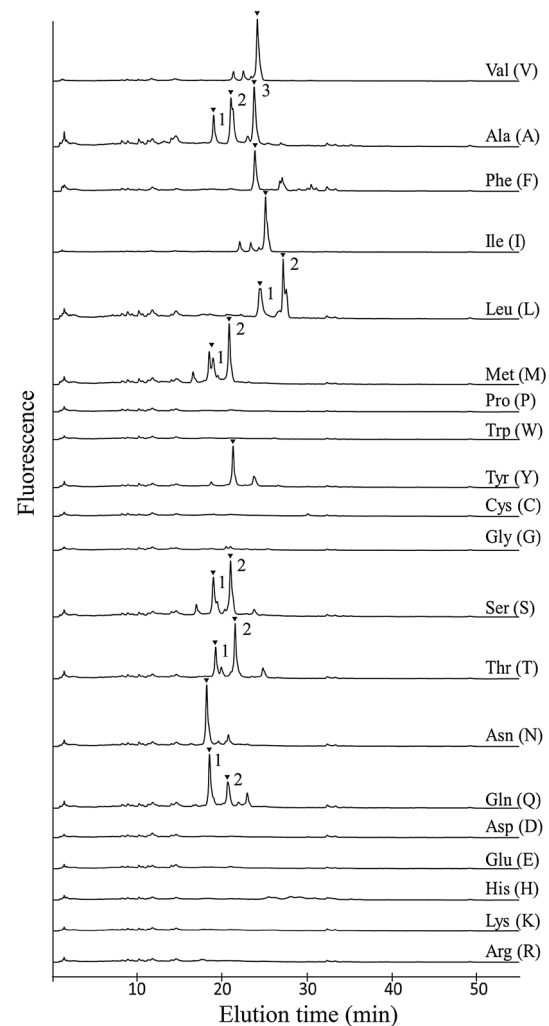


Fig. 4 HPLC analysis of transformants. Val (V) indicates native nocardithiocin, and replacement amino acids are shown on the right. Arrowheads indicate purified peaks corresponding to nocardithiocin derivatives and are numbered according to the order of elution

Compounds with the expected m/z values (A3, L2, I, S2, T2, and Q2) produced fragmentation patterns comparable to that of nocardithiocin (i.e., fragment ions were observed with the expected mass shifts). Therefore, these compounds were assumed to be expected nocardithiocin derivatives. The different fragmentation pattern of the other compounds with that of nocardithiocin indicating that they have other structures than expected one amino acid substituted derivatives. These non-expected derivatives were divided into two groups based on their MS/MS fragmentation patterns. Group 1 consists of peptides A1, F, L1, M1, M2, T1, and N, all of which keep the similar structure to nocardithiocin and its expected derivatives. However for example, A1 and M1 have +6 and +4 m/z value different from expected derivative, respectively. This may be caused by the failure of thiazole ring formation next to the substituted amino acid. And the

Table 1 Aminoacids and LC–MS result

6th Amino acid	Side chain (–R)	Theoretical		Measured <i>m/z</i> value		
		Chemical formula of derivatives	Exact mass	Peak 1	Peak 2	Peak 3
Hydrophobic						
Nonpolar						
Val (V)	–CH(CH ₃) ₂	C ₄₈ H ₄₈ N ₁₂ O ₁₁ S ₆	1161.1963	<i>1160.9</i>	–	–
Ala (A)	–CH ₃	C ₄₆ H ₄₄ N ₁₂ O ₁₁ S ₆	1133.3054	1138.9	1120.85	<i>1132.85</i>
Phe (F)	–CH ₂ C ₆ H ₅	C ₅₂ H ₅₀ N ₁₂ O ₁₁ S ₆	1209.4013	1196.9	–	–
Leu (L)	–CH ₂ CH(CH ₃) ₂	C ₄₉ H ₅₀ N ₁₂ O ₁₁ S ₆	1175.3851	1162.9	<i>1174.9</i>	–
Ile (I)	–CH(CH ₃)CH ₂ CH ₃	C ₄₉ H ₅₀ N ₁₂ O ₁₁ S ₆	1175.3851	<i>1174.9</i>	–	–
Met (M)	–CH ₂ CH ₂ SCH ₃	C ₄₈ H ₄₈ N ₁₂ O ₁₁ S ₇	1193.4235	1196.85	1208.85	–
Pro (P)	–CH ₂ CH ₂ CH ₂ [–]	C ₄₈ H ₄₆ N ₁₂ O ₁₁ S ₆	1159.3426	–	–	–
Trp (W)	–CH ₂ C ₈ H ₆ N	C ₅₄ H ₄₉ N ₁₃ O ₁₁ S ₆	1248.4374	–	–	–
Hydrophilic						
Polar uncharged						
Tyr (Y)	–CH ₂ -C ₆ H ₄ OH	C ₃₂ H ₅₀ N ₁₂ O ₁₂ S ₆	1225.4007	1212.95	–	–
Cys (C)	–CH ₂ SH	C ₄₆ H ₄₄ N ₁₂ O ₁₁ S ₇	1165.3704	–	–	–
Gly (G)	–H	C ₄₅ H ₄₂ N ₁₂ O ₁₁ S ₆	1119.2788	–	–	–
Ser (S)	–CH ₂ OH	C ₄₆ H ₄₄ N ₁₂ O ₁₂ S ₆	1149.3048	1138.85	<i>1148.85</i>	–
Thr (T)	–CH(OH)CH ₃	C ₄₇ H ₄₆ N ₁₂ O ₁₂ S ₆	1163.3313	1150.85	<i>1162.9</i>	–
Asn (N)	–CH ₂ CONH ₂	C ₄₇ H ₄₅ N ₁₃ O ₁₂ S ₆	1176.3301	1163.85	–	–
Gln (Q)	–CH ₂ CH ₂ CONH ₂	C ₄₈ H ₄₇ N ₁₃ O ₁₂ S ₆	1190.3567	1179.9	<i>1189.9</i>	–
Negative charge						
Asp (D)	–CH ₂ COOH	C ₄₇ H ₄₄ N ₁₂ O ₁₃ S ₆	1177.3149	–	–	–
Glu (E)	–CH ₂ CH ₂ COOH	C ₄₈ H ₄₆ N ₁₂ O ₁₃ S ₆	1191.3414	–	–	–
Positive charge						
His (H)	–CH ₂ -C ₃ H ₃ N	C ₄₉ H ₄₆ N ₁₄ O ₁₁ S ₆	1199.3667	–	–	–
Lys (K)	–(CH ₂) ₄ NH ₂	C ₄₉ H ₅₁ N ₁₃ O ₁₁ S ₆	1190.3997	–	–	–
Arg (R)	–(CH ₂) ₃ NH-C(NH)NH ₂	C ₄₉ H ₅₁ N ₁₅ O ₁₁ S ₆	1218.4131	–	–	–

Italic values indicating the expected calculated MS value

"–" Indicates that no obvious LC–MS peak was observed

other F, L1, Y, T1 and N showed – 12 *m/z* value difference with expected derivatives and M2 showed – 12 *m/z* value difference with M1 may indicating the defective of methyl-transferase activity at S₄. Group 2 is composed of A2, S1, and Q1 peptides whose fragment patterns were completely different from nocardithiocin and expected derivatives, so we failed to determine their structures but the compounds in this group showed same fragment at lower *m/z* values (≤ 736).

Properties of Nocardithiocin Derivatives

The light sensitivity of purified compounds was tested and compared with that of native nocardithiocin. In a previous study, nocardithiocin was reported to be unstable in the light (Mukai et al. 2009). However, the results of the present study (Fig. 5a) suggest that neither nocardithiocin nor

its derivatives (including unexpected compounds) were light sensitive. Additionally, the stability at room temperature was also examined, and nocardithiocin and its derivatives were shown to be stable (Fig. 5b).

Since the solubility of thiopeptide compounds is very low (Li and Kelly 2010; Just-Baringo et al. 2014b), we tested the solubility of nocardithiocin and its derivatives. Compared the solved amount in DMSO (completely soluble), only 0.14% (1.2 μ M) of nocardithiocin was soluble in water. All nocardithiocin derivatives showed a slight improvement in solubility in water (Fig. 6), and Q2 was the most soluble among expected derivative (3.07%; 25.8 μ M).

Antimicrobial Activity of Nocardithiocin Derivatives

The antimicrobial activity of nocardithiocin derivatives was tested against several bacteria and fungi (Table 2). Expected nocardithiocin derivatives A3, L2, I, S2, T2, and Q2 retained

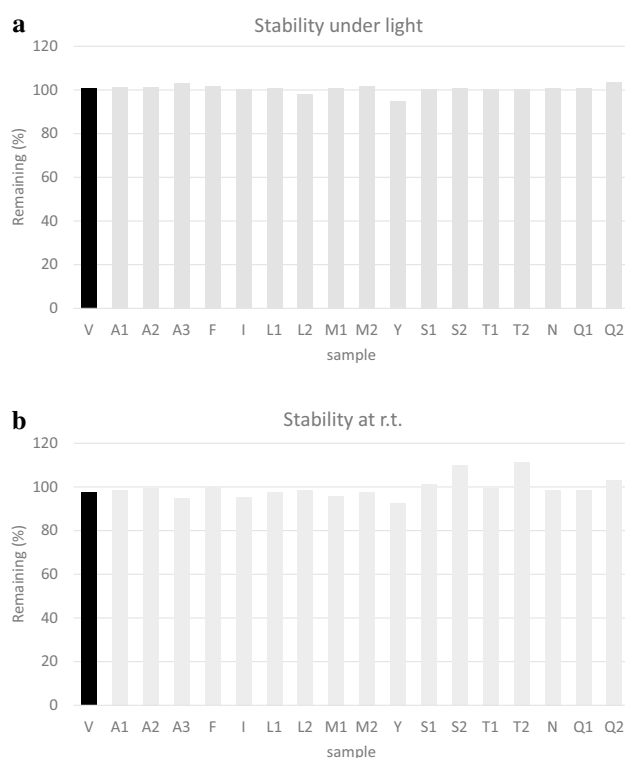


Fig. 5 Analysis of stability tests. **a** Stability of nocardithiocin and purified compounds in the presence of light. The percentage of the compound remaining was calculated by comparing the peak area following storage in the light and dark. **b** Stability of nocardithiocin and purified compounds stored at room temperature. The percentage of the compound remaining was calculated by comparing the peak area following storage at °C and room temperature (30 °C, in summer) for 4 days. Nocardithiocin and its derivatives are indicated by orange bars, and compounds belonging to groups 1 and 2 are indicated by green and blue bars, respectively

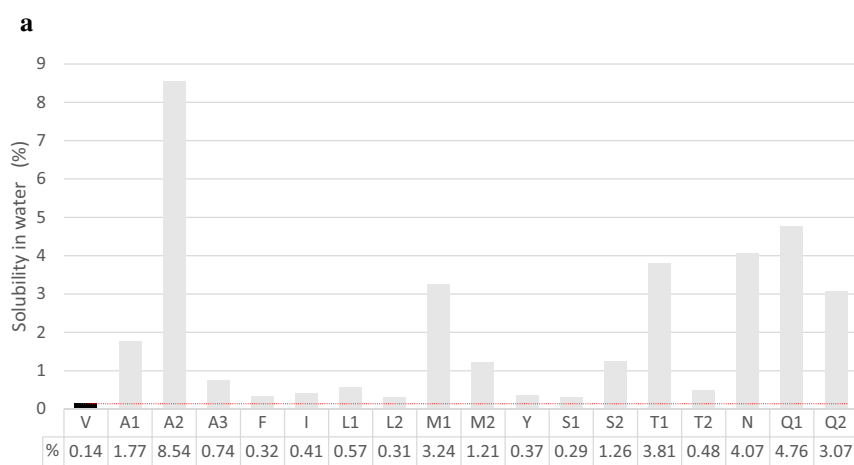


Fig. 6 Solubility of nocardithiocin and purified compounds in water. **a** The level of solubility in DMSO was assigned a value of 100%, and peak areas of compounds dissolved in water were compared with peak areas of compounds dissolved in DMSO. **b** The concentration

of dissolved compound was calculated using m/z values. Nocardithiocin and its derivatives are indicated by orange bars, and compounds belonging to groups 1 and 2 are indicated by green and blue bars, respectively

antimicrobial activity, but compounds with unexpected structures lost its activity, except for compound M1. Furthermore, expected derivatives and compounds M1 and M2 exhibited activity against drug-resistant *Mycobacterium tuberculosis* strains (Table 3). In most cases, native type of nocardithiocin showed stronger antimicrobial activity than its derivatives, but compound I displayed four times and two times greater activity against *Bacillus subtilis* and *Micrococcus luteus*, respectively. And compound L2 showed two times higher activity against *M. luteus* (Table 2).

Discussion

Nocardithiocin is a thiopeptide compound produced by *N. pseudobrasiliensis* (Mukai et al. 2009) that displays powerful activity against drug-resistant *M. tuberculosis*, making it a promising novel antibacterial drug. However, its clinical use is hampered by low water solubility and light sensitivity, so the property should be improved for further application. Identification of the nocardithiocin biosynthesis gene cluster (Sakai et al. 2015) revealed that it is synthesized through modification of a ribosomally translated precursor peptide, similar to other thiopeptides (Wieland Brown et al. 2009; Liao et al. 2009). This biosynthetic feature prompted us to modify the nocardithiocin structure by altering the amino acid sequence of the precursor peptide to improve its properties.

Firstly, we removed the native *notG* gene encoding the precursor peptide from the genome of *N. pseudobrasiliensis*, and the *notG* deletion strain was used as the host for nocardithiocin derivative production. The

Table 2 Antimicrobial activity of nocardithiocin and its derivative

Test organisms	MIC and IC ₅₀ values for bacteria and fungi, respectively (µg/ml)																	
	V	F	Y	A1	A2	A3	L1	L2	I	M1	M2	S1	S2	T1	T2	N	Q1	Q2
<i>Escherichia coli</i>	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32
<i>Staphylococcus aureus</i>	4	>32	>32	>32	>32	>32	>32	16	16	>32	>32	>32	>32	>32	16	>32	>32	>32
<i>Bacillus subtilis</i>	4	>32	>32	>32	>32	>32	>32	16	1	>32	>32	>32	>32	>32	>32	16	32	>32
<i>Micrococcus luteus</i>	32	>32	>32	>32	>32	>32	>32	16	16	>32	>32	>32	>32	>32	>32	>32	>32	>32
<i>Gordonia bronchialis</i>																		
IFM 0150	2	>32	>32	>32	>32	>32	>32	16	8	>32	>32	>32	>32	>32	32	>32	>32	>32
<i>Aspergillus niger</i>																		
IFM 62678	8	>32	>32	>32	>32	32	>32	16	32	32	>32	>32	>32	>32	>32	>32	>32	>32
<i>Candida albicans</i>																		
IFM 62680	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32
<i>Cryptococcus neoformans</i>																		
IFM 62681	32	>32	>32	>32	>32	>32	>32	>32	32	>32	>32	>32	>32	>32	>32	>32	>32	>32

The italic values indicated the stronger activity than nocardithiocin

mutant fragment of *notG* were prepared by PCR-based site directed mutagenesis method and expressed under the *notG* native promoter, because production of derivatives under the control of constitutive promoter *PermE* (Labes et al. 1997) was much lower (Fig. S2) than native *notG* promoter. Expression of *notG* under the control of *PermE* was actually higher than the native promoter, suggesting that coordinated regulation with other genes in the cluster is important for optimal nocardithiocin production. Therefore, further improvement of nocardithiocin production may be achieved by modifying the expression levels of the transcriptional regulator *notD* in the gene cluster.

Among the 19 amino acid substituted derivatives, 10 amino acid produced clear HPLC fluorescent peaks under nocardithiocin detection condition, but nine (P, W, C, G, D, E, H, K, and R) did not show any significant compound production (Fig. 3). The imino acid structure of P, the large side chain of W, and the small side chain of G may prevent the enzymatic reaction(s) required to synthesize nocardithiocin derivatives. Similarly, a negative (D and E) or positive (H, K, and R) charge could disrupt derivative synthesis. Finally, the sulfur-containing side chain of C could form a thiazole ring following the action of cyclodehydratase, resulting in three adjacent thiazoles at positions C₅ and C₇, which could explain the failure to synthesize this derivative. Several reports showed the preference of amino acids depending on the replaced position in thiopeptides (Young et al. 2012; Zhang et al. 2012; Tran et al. 2017).

Substitution with A, L, M, S, T, or Q resulted in the production of two or three major compounds, implying that nocardithiocin biosynthetic enzymes or other enzymes might have the ability to produce diverse compounds from nocardithiocin precursor peptides with altered amino acid sequences. Other than the compound M2, expected

nocardithiocin derivatives eluted later in the elution profile (Table 1). Comparison of the MS/MS fragmentation patterns with that of nocardithiocin (V) confirmed that A3, L2, I, S2, T2, and Q2 were the expected derivatives (Fig. S1). Although the exact structures have not been determined, unexpected compounds were classified into two groups based on their MS/MS fragmentation patterns. The fragmentation pattern of group 1 compounds suggested that their similar structure with nocardithiocin derivatives because several MS/MS fragments were coincident with those of nocardithiocin (Table S2). The speculated structure of group 1 compounds from MS/MS spectrum indicated the partial or defective reaction of thiazole formation or addition of methyl group (Fig. S1). The size of amino acid residue may cause this incomplete reaction. The properties of the purified compounds were tested, and none of them displayed the previously reported light sensitivity (Mukai et al. 2009). Although nocardithiocin and its derivatives in crude samples appeared as single peaks in the HPLC analysis, purified samples contained two separated peaks (Fig. S3). After single peaks were collected by HPLC, collected samples showed two separate peaks with similar proportions in the 1st HPLC. This phenomenon could have been interpreted as light-induced degradation in the previous report. Furthermore, the previous study used a glass tubes for storing nocardithiocin samples, and nocardithiocin may be adsorbed to glass tubes due to its hydrophobic and basic nature.

Nocardithiocin and produced compounds were not degraded under light irradiation, so light sensitivity remains as major limitation of clinical use. However the produced derivatives remained poorly soluble in water (Fig. 5). In previous report, water-soluble analogues of nocardithiocin prepared by semi-synthesis retained antimicrobial activity (Xu et al. 2013). In that report, modification of side chains

Table 3 Antimicrobial activity against *Mycobacterium tuberculosis* strains

Strains	Drug resistance MIC ($\mu\text{g/ml}$)																				
	V	F	Y	A1	A2	A3	L1	L2	I	M1	M2	S1	S2	T1	T2	N	Q1	Q2	RFP	SM	INH
H37Rv	0.25	> 32	> 32	> 32	> 32	4	> 32	1	1	> 32	4	> 32	8	> 32	1	> 32	> 32	16	0.125	0.5	1
NIHJ 3002	0.125	> 32	> 32	> 32	> 32	4	> 32	1	1	> 32	4	> 32	8	> 32	1	> 32	> 32	8	0.125	0.5	1
NIHJ 3003	0.125	> 32	> 32	> 32	> 32	2	> 32	0.5	0.5	> 32	2	> 32	4	> 32	0.5	> 32	> 32	8	32	0.5	1
NIHJ 3004	0.25	> 32	> 32	> 32	> 32	32	> 32	2	1	> 32	8	> 32	8	> 32	2	> 32	> 32	8	0.125	> 32	0.5
NIHJ 3005	0.125	> 32	> 32	> 32	> 32	4	> 32	1	0.25	> 32	4	> 32	8	> 32	2	> 32	> 32	4	0.125	0.5	> 32
NIHJ 3012 (clinical isolate)	1	> 32	> 32	> 32	> 32	16	> 32	4	4	> 32	16	> 32	32	> 32	4	> 32	> 32	32	0.125	1	1
NIHJ 3014 (clinical isolate)	0.125	> 32	> 32	> 32	> 32	2	> 32	0.5	0.5	32	2	> 32	2	> 32	0.5	> 32	> 32	4	> 32	0.25	0.25
NIHJ 3019 (clinical isolate)	0.125	> 32	> 32	> 32	> 32	1	> 32	0.5	0.25	32	2	> 32	2	> 32	0.25	> 32	> 32	4	32	> 32	> 32

PZA pyrazinamide, RFP rifampicin, SM streptomycin, INH isoniazid

at the tail end of the peptide improved its water solubility, and similar modifications could also enhance the water solubility of nocardithiocin derivatives without diminishing antimicrobial activity.

The antimicrobial activity of nocardithiocin and its derivatives was tested against several microbes. Among the 17 successfully purified compounds, 10 showed some antimicrobial activity. While native nocardithiocin displayed higher activity than most of its derivatives, compounds L2 and I displayed slightly higher antimicrobial activity against Gram-positive bacteria (Table 2). Young et al. (2012) also reported the amino acid replacement of G37468 (T2C) increased twofold improved activity against *B. subtilis* implying the potential of amino acid replacement of thiopeptide compounds for improvement of its antimicrobial activity. Compounds L2, I, and T2 that exhibited relatively strong antimicrobial activity share similar side chains at the sixth position to that present in nocardithiocin (V_6), and similarly low water solubility. This result indicated that a branched methyl group at the sixth position of nocardithiocin makes some contribution to its antimicrobial activity. Although the sixth amino acid was chosen for mutation based on the assumption that it is not crucial for antimicrobial activity, substitution clearly gave a significant impact to antimicrobial activity. The prepared compounds which have different structure with expected nocardithiocin derivatives did not display clear antimicrobial activity may implying the importance of methyl group on S_4 or proper thiazole ring formation for nocardithiocin activity.

In this report, among 13 amino acid residue of nocardithiocin, only the sixth amino acid was substituted, and many more derivatives could be prepared by replacing other position of amino acid residue(s) and/or by using unusual amino acids other than the 19 canonical amino acids found in native proteins (Luo et al. 2016). The relationship of structure–activity could not be deeply analyzed yet, increased information will facilitate the rational design of nocardithiocin derivatives with desirable properties in future studies (Wolf et al. 2014).

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Compliance with Ethical Standards

Conflict of interest All the authors declare that they have no conflict of interest.

Research Involving Human Participants and/or Animals This article does not contain any studies with human participants or animal performed by any of the authors.

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