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Relation	

Isolation and Biosynthesis of an Azoxyalkene Compound Produced by a Multiple Gene Disruptant of *Streptomyces rochei*

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Abstract

Streptomyces rochei 7434AN4 predominantly produces lankacidin and lankamycin under normal culture conditions, suggesting that other biosynthetic gene clusters for secondary metabolites are silent. To exploit silent metabolites of strain 7434AN4, we constructed mutant KA57, a multiple disruptant of the transcriptional repressor gene *srrB* together with the biosynthesis genes for both antibiotics. Mutant KA57 accumulated a compound (KA57-A) with a strong UV absorption at 235 nm, which was not detected in the parent strain or other mutants. Various spectroscopic analyses revealed that KA57-A is an azoxyalkene compound of a molecular formula of $C_{10}H_{20}N_2O_3$ with the **R** configuration at C-2. Biosynthesis of KA57-A was also studied by feeding with labeled acetates, amino acids, and 1-hexylamine. The hexenyl moiety (C1'-C6') was derived from fatty acid, while the 3-amino-butan-1,2-diol moiety (C1-C4) was derived from C-2 of acetate (C1) and serine (C2-C4). Incorporation of [1,1- 2H_2]1-hexylamine indicated that C1'-C2' dehydrogenation occurs at the final step of biosynthesis.

Introduction

The filamentous and saprophytic soil bacteria, *Streptomyces*, produce the majority (nearly 70%) of commercially available antibiotics.^[1,2] To date, genome sequencing projects of many *Streptomyces* species have been completed. *Streptomyces* carry a 6- to 10-Mb linear chromosome and have a genetic potential to produce over 20 secondary metabolites, including polyketides, non-ribosomal peptides, terpenoids, aminoglycosides, and other classes of

metabolites.^[3] However, most of the biosynthetic gene clusters for secondary metabolites are expressed poorly, or not expressed at all, under laboratory growth conditions. Several strategies to activate silent biosynthetic gene clusters have been reported for natural product discovery.^[4,5]

Streptomyces rochei 7434AN4 produces two structurally unrelated polyketide antibiotics, lankacidin **(1)** and lankamycin **(2)** (Scheme 1), and carries three linear plasmids pSLA2-L, -M, and -S.^[6] The two antibiotics attracted great attention because they inhibit peptide synthesis synergistically by binding to the neighboring sites in the large ribosomal subunit.^[7,8] Nucleotide sequencing of the largest plasmid pSLA2-L (210,614 bp) together with extensive mutational analysis revealed that the biosynthetic gene clusters for lankacidin (*lkc*) and lankamycin (*lkm*) were located on pSLA2-L.^[9] This plasmid contains two additional biosynthetic clusters for a cryptic type-II polyketide (*roc*) and a carotenoid (*crt*). Furthermore, pSLA2-L carries many regulatory genes, including a biosynthetic gene *srrX* for the signaling molecules termed SRBs (*S. rochei* butenolides) and three *tetR*-type receptor genes (*srrA*, *srrB*, and *srrC*). The signaling molecules and their receptors play a pivotal role in the production of secondary metabolites in many *Streptomyces* species.^[10] Gene inactivation analysis of *srrX* and three repressor genes, *srrA*, *srrB*, and *srrC* revealed that *srrX* and *srrA* constitute the signaling molecules/receptor system for lankacidin and lankamycin production in *S. rochei*.^[11] Remarkably, disruption of *srrB* increased the production of **1** and **2** (2- and 8-fold improvement, respectively), suggesting that *srrB* has a negative function on antibiotic production. *SrrB* belongs to the signaling-molecule receptor family with high pI value (~10), which

act as negative regulators for antibiotic production. They are exemplified by TylQ for tylosin,^[12] BarB for virginiamycin,^[13] AlpW for kinamycins,^[14] and ScbR2 for an unidentified polyketide with antimicrobial activity.^[15] Disruption of some biosynthetic pathways in the producer organism can occasionally increase other secondary metabolite production.^[16,17] Recently we reported that mutant of *lkcA*, a non-ribosomal peptide synthase (NRPS)/polyketide synthase (PKS) hybrid gene for lankacidin biosynthesis, produced a large amount of three polyketides, pentamycin, citreodiol, and *epi*-citreodiol (250-fold for pentamycin and 5-fold for citreodiols compared with the parent), although the mechanism of the complex metabolic changes remains unclear.^[18] Hence, we expect that multiple mutations on both lankacidin and lankamycin biosynthetic genes together with a regulatory gene will activate silent secondary biosynthetic gene clusters in *S. rochei*.

Here we report the isolation of an azoxyalkene compound KA57-A from a disruptant strain of three genes, *srrB*, *lkcF-KR1* (a ketoreductase domain of *lkcF* for lankacidin biosynthesis), and *lkmE* (a type-II thioesterase gene *lkmE* for lankamycin biosynthesis). Biosynthesis of KA57-A was also studied by feeding of labeled acetates, amino acids, and 1-hexylamine, which is described in this paper.

Results

Isolation and structural elucidation of azoxyalkene KA57-A from the *srrB-lkmE-lkcF-KR1* triple mutant KA57.

To activate silent secondary metabolite biosynthetic clusters in *S. rochei*, we

performed multiple mutations on both lankacidin and lankamycin gene clusters together with the transcriptional repressor *srrB*. Mutation targets on the *lkc* and *lkm* clusters were the lankacidin PKS gene *lkcF-KR1* and the type-II thioesterase gene *lkmE*, whose inactivation resulted in abolishment of lankacidins and 70% decrease of lankamycin production, respectively.^[19,20] To construct a triple mutant KA57 ($\Delta srrB \cdot \Delta lkmE \cdot lkcF-KR1^{Y343F}$), plasmid pKAR1020^[19] carrying *lkcF-KR1*^{Y343F} mutation was transformed into protoplasts of the *srrB-lkmE* double mutant NS01.^[20] The third mutation in strain KA57 was confirmed by Southern hybridization analysis. A 2.9-kb PvuI fragment in strain NS01 was changed to the 1.2- and 1.7-kb PvuI-PstI fragments in strain KA57 (Figure S1; Supplemental data). Metabolites of strain KA57 were analyzed by TLC and HPLC (Figure 1). Strain KA57 was defective in lankacidin production due to the inactivation of *lkcF-KR1* domain. Regarding to lankamycin production, strain KA57 produced a similar level of lankamycin and its derivatives compared with strain NS01. Remarkably, strain KA57 accumulated a UV-active compound (**3**), which was not detected in any other single or double mutants as well as the parent strain 51252 (Figures 1A and 1B). This compound, named KA57-A, was successively purified by Sephadex LH20 with MeOH, and silica gel chromatographies with two different solvent systems of CHCl₃-MeOH (50:1, v/v) and toluene-EtOAc (2:1, v/v). KA57-A was detected at 10.5 min on HPLC with a maximum UV absorbance at 238 nm, which differed from that of lankacidin C ($\lambda_{max}=227$ nm) (Figure 1C). The molecular formula of KA57-A was determined by high resolution ESI-MS to be C₁₀H₂₀N₂O₃. Its ¹H and ¹³C NMR assignments were summarized in Table 1. The ¹³C NMR spectrum revealed the presence of 10

carbons, which were classified into two methyl, four methylene, and four methine carbons by DEPT spectrum. In its ^1H NMR, a triplet methyl proton ($\delta_{\text{H}}=0.92$ ppm), a doublet methyl proton ($\delta_{\text{H}}=1.27$ ppm), three deshielded protons ($\delta_{\text{H}}=3.55\text{-}3.56$, 3.73 , 4.28 ppm), and two olefinic protons ($\delta_{\text{H}}=5.74$ and 6.73 ppm) were observed. The coupling constant between two olefinic protons was 9.2 Hz, indicating the presence of a *cis* C=C double bond. All the spectral data agreed well with the reported data of (*Z*)-2-(3,4-dihydroxybutan-2-yl)-1-((*Z*)-hex-1-en-1-yl)diazene 1-oxide (**3**; Scheme 1).^[21] This compound contains unique azoxy ($\text{N}=\text{N}^+-\text{O}^-$) chromophore, and exhibited a weak antibiotic activity against *Rhodotorula* sp. Although the C-3 configuration has been reported to be *R* from its CD spectra,^[21] the stereochemistry at C-2 remained to be elucidated. Therefore, the C-2 configuration in **3** was determined from the $\Delta\delta$ ($=\delta_{\text{S}}-\delta_{\text{R}}$) value of its (*R*) and (*S*)- α -methoxy- α -trifluoromethyl- α -phenylacetate (MTPA) derivatives according to modified Mosher method.^[22] The H-1 methylene protons exhibited negative values (-10 and -20 Hz), while the characteristic H-3, H-1', and H-2' proton signals showed positive values ($+5$, $+25$, and $+15$ Hz, respectively). The C-2 configuration in **3** was thus deduced to be *R*. This is the first discovery of **3** from *Streptomyces* species.

Biosynthetic origin of KA57-A

Naturally occurring azoxyalkene compounds constitute a fascinating group of secondary metabolites with a vast array of structural diversity; for example, elaiomycin,^[23] LL-BH872 α ,^[24] valanimycin,^[25] jjetacin,^[26] maniwamycins,^[27] and elaiomycins D and E (Scheme S2).^[28] The well-studied azoxy-bearing natural

products are elaiomycin and valanimycin. According to isotope labeling studies, the elaiomycin skeleton is assembled from *n*-octylamine, L-serine, and C-2 of acetate.^[29,30] The azoxy antibiotic valanimycin is synthesized from L-serine and L-valine, and its biosynthetic genes were extensively analyzed.^[31] To investigate the biosynthetic origin of KA57-A, we carried out feeding experiments with isotope-labeled precursors including sodium [1-¹³C]acetate, sodium [2-¹³C]acetate, DL-[2,3,3-²H₃]serine, [1,1-²H₂]1-hexylamine, L-[¹⁵N]serine, and [¹⁵N]1-hexylamine.

The hexenyl moiety (C1'-C6') in **3** seemed to be derived from malonyl CoA by a fatty acid biosynthetic pathway. Thus, [1-¹³C] and [2-¹³C]acetate were fed to the culture of strain KA57, and the resultant KA57-A was purified as mentioned above. As anticipated, the C-1 label of acetate was incorporated into the C-1', -3', and -5' positions of KA57-A, while the C-2 label of acetate was into C-2', -4', and -6' positions (Table 1). Remarkably, a high degree of ¹³C enrichment at C-1 of **3** ($\delta_c=63.6$ ppm) was observed in the feeding with sodium [2-¹³C]acetate.

A possible biosynthetic origin of the 2-aminopropanol moiety (C2-C4) was alanine based on its chemical structure, however, no incorporation of [3-¹³C]alanine into C-4 was observed (data not shown). We then considered serine as a second candidate for the origin of C2-C4. After feeding of DL-[2,3,3-²H₃]serine to the culture, the resultant KA57-A was analyzed by ²H NMR (Figure 2A-ii). A distinct deuterium signal was detected at $\delta_D=1.27$ ppm, corresponding to the C-4 methyl of KA57-A. This labeling pattern indicated that serine is a biosynthetic precursor for **3**, and its H-2 proton (open circle in Figure 2B) was eliminated during dehydration process in KA57-A biosynthesis.

Feeding of [1,1-²H₂]1-hexylamine gave a notable insight into the tailoring biosynthetic step of KA57-A. The resultant KA57-A was purified in a similar way, however, an inseparable compound appeared at the same R_f value on TLC (KA57-A showed a light green spot, while this contaminant did a pink spot when baked with anisaldehyde-H₂SO₄). Our further effort to separate them was unsuccessful even by flash chromatography on silica gel with various combinations of organic solvents. Because of the limited amounts available, further analysis was carried out as a mixture. The ²H NMR spectrum showed two deuterium signals at δ_D=6.78 and 4.14 ppm (Figure 2A-iii). The former signal corresponded well to H-1' of **3** (δ_H=6.74 ppm), suggesting the presence of [1'-²H]-**3** in this mixture. Its presence was further supported by detection of a molecular ion peak at [M+Na]⁺=240.1424 (calcd. for C₁₀H₁₉²HN₂O₃Na, [M+Na]⁺=240.1429) in its ESI-MS spectrum (Figure 2C-i). To our surprise, an additional molecular ion peak ([M+Na]⁺=243.1645) was also observed at four mass units larger than that of unlabeled **3** ([M+Na]⁺=239.1368) (Figure 2C-i). Thus, electrospray ionization tandem mass spectrometry with collision-induced dissociations (ESI-CID-MS/MS) was applied for monitoring the isotope incorporation into **3** and/or its derivative. ESI-CID-MS/MS spectra for three parent ions at *m/z* 239, 240, and 243 were shown in Figure 2D. When the molecular ion at *m/z* 239 for unlabeled **3** was selected as a parent ion, a characteristic fragment ion [C₈H₁₄N₂Na]⁺ was observed at *m/z* 161 due to the loss of both an ethyleneglycol cation (61 amu) and a hydroxyl anion (17 amu) from unlabeled **3** (hashed arrow in Figure 2D-i). An additional fragment ion [C₄H₉NO₂Na]⁺ was also observed at *m/z* 126, which could be caused by

cleavage of the N=N bond in the azoxyalkene moiety (solid arrow in Figure 2D-i). ESI-CID-MS/MS analysis of [1-²H]-**3** (*m/z* 240) showed two major fragment ions at *m/z* 162 together with *m/z* 126 (Figure 2D-ii). The former fragment ion corresponded to [C₈H₁₃²HN₂Na]⁺, which further supported the specific incorporation of one deuterium atom into **3**. ESI-CID-MS/MS spectrum for *m/z* 243 showed a distinct fragment peak at *m/z* 165 (Figure 2D-iii). Based on high resolution ESI-MS analysis, the parent (*m/z* 243) and its fragment (*m/z* 165) ions were established to be [C₁₀H₂₀²H₂N₂O₃Na]⁺ and [C₈H₁₄²H₂N₂Na]⁺, respectively. Hence, this contaminant obtained by feeding of [1,1-²H₂]1-hexylamine was presumably assigned to be [1',1'-²H₂]-1,2-dihydro-KA57-A ([1',1'-²H₂]-**4**). A deuterium signal at δ_D=4.14 ppm in Figure 2A-iii showed a similar chemical shift with the saturated methylene proton at CH₂-N(-O⁻)=N in valanimycin (δ_H=4.14 ppm),^[25] supporting the presence of [1',1'-²H₂]-**4** in this mixture. Compound **4** was not detected in the fermentation broth obtained by feeding of unlabeled 1-hexylamine (Figure 2C-ii), indicating that accumulation of [1',1'-²H₂]-**4** was caused by a deuterium-isotope effect on the C1'-C2' dehydrogenation step. The lack of a fragmentation ion at *m/z* 126 in ESI-CID-MS/MS spectrum for *m/z* 243 (Figure 2D-iii) suggests that the cleavage of the N=N bond does not occur without a conjugated C-1'/C-2' double bond.

To investigate the origin of nitrogen atoms of KA57-A, ¹⁵N-labeled L-serine and 1-hexylamine were independently fed to the culture of strain KA57. Compound **3** obtained by feeding of L-[¹⁵N]serine showed a ¹⁵N-¹H long range HMBC correlation from H-4 to α-nitrogen atom N_α (δ_N=357 ppm), while **3** obtained by feeding of [¹⁵N]1-hexylamine showed a ¹⁵N-¹H long range HMBC

correlation from H-2' to β -nitrogen atom N β ($\delta_N=336$ ppm). All the feeding experiments mentioned above clearly indicated that KA57-A is assembled from 1-hexylamine (for C1'-C6' and N β), serine (for C2-C4 and N α), and C-2 of acetate (for C1) (Figure 2B).

Discussion

In this study, we analyzed the structure and biosynthetic origin of the UV-active compound KA57-A (**3**) produced by the triple mutant KA57 ($\Delta srrB$ - $lkcF$ - $KR1^{Y343F}$ - $\Delta lkmE$) of *S. rochei*. Compound **3** contains a dipolar azoxy (N=N⁺-O⁻) moiety that is a rare functional group among natural products (Scheme S2).^[32] This compound was originally isolated from an aerobic actinomycete, *Actinomadura* sp. A7.^[21]

Manipulation of regulatory pathways, by overexpression of pathway-specific activator genes or by inactivation of transcriptional repressor genes, is the most intuitive approach to activate silent biosynthetic gene clusters.^[4,5] Especially, the latter approach provides an opportunity to activate silent biosynthetic gene clusters in wild-type microorganisms. A novel antibacterial activity of an uncharacterized product was detected in *S. coelicolor* after deleting the pseudorepressor gene *scbR2*, which is located within the *cpk* gene cluster.^[15] In *Streptomyces ambofaciens*, inactivation of the *tetR*-type transcriptional repressor gene *alpW* led to a detectable production of kinamycins.^[14] In *Kitasatospora setae*, deletion of the repressor gene *ksbC* resulted in lowered production of bafilomycin and enhanced production of a novel β -carboline alkaloid named kitasetaline.^[33] In the case of *S. rochei*, mutation of *srrB* resulted

in overproduction of lankacidin and lankamycin, however, no other secondary metabolites were detected in **this** single mutant.^[11] Our preliminary experiment indicated that *srrB* is involved in the signaling pathway for lankacidin and lankamycin production, and serves as a late stage regulator for controlling their titers in *S. rochei* (unpublished results).

Blockage of the competing biosynthetic pathways is also a versatile approach to obtain secondary metabolites of interest.^[16] In the case of nanchangmycin production by *Streptomyces nanchangensis*, its titer was improved threefold by gene inactivation of the uncharacterized PKS cluster.^[17] This mutation possibly affected precursor supply for nanchangmycin in this microorganism. In *S. rochei*, disruption of the NRPS/PKS hybrid gene *lkcA*, which is responsible for the early stage of lankacidin biosynthesis, caused a large production of three polyketides, pentamycin, citreodiol, and *epi*-citreodiol.^[18] This case, however, seems different from that in *S. nanchangensis*, because their overproduction occurred only in the *lkcA* mutant, not in other *lkc*-PKS mutants. Analogously, compound **3** was detected only in strain KA57, not in any other *S. rochei* mutants harboring mutations on *lkc*, *lkm*, and their regulatory genes hitherto constructed. The mechanism of **these** complex metabolic changes in the triple mutant KA57 remains to be clarified. It is also noteworthy that triple mutations on the linear plasmid pSLA2-L caused production of **3**, whose biosynthetic genes are possibly coded on the *S. rochei* chromosome. Thus, combined manipulation of regulatory gene(s) together with major biosynthesis clusters will become one of new momentum to activate cryptic biosynthetic gene clusters for natural product discovery.

According to the feeding experiments with isotope-labeled precursors, KA57-A is assembled from 1-hexylamine (for C1'-C6' and N β), serine (for C2-C4 and N α), and C-2 of acetate (for C1). The possible KA57-A biosynthetic pathway is summarized in **Scheme 2**. One of the interesting features in KA57-A biosynthesis is an unusual biosynthetic pathway of a 3-aminobutane-1,2-diol moiety (C1-C4 unit), which is mechanistically analogous to elaiomycin biosynthesis.^[30] An activated serine residue receives a Claisen condensation with a malonyl CoA extender unit (Step 1) to yield a β -keto ester conjugate, which is then decarboxylated (Step 2). The resultant is converted to a possible biosynthetic intermediate harboring a 3-amino-1,2,4-triol moiety (Step 3) by two redox reactions including C-1 hydroxylation and C-2 reduction, whose order remains unclear.

The incorporation pattern of [2,3,3- $^2\text{H}_3$]serine (Figure 2A-ii) revealed that a dehydration reaction took place at C3-C4 positions in KA57-A biosynthesis (Step 4). The draft genome data of *S. rochei* 7434AN4 revealed the presence of the valanimycin biosynthetic gene (*vIm*) homologs, *vImJ* and *vImK*, possibly encoding kinase and dehydratase, respectively. In valanimycin biosynthesis, a hydroxyl group of serine residue is phosphorylated by *VImJ*, followed by a subsequent phosphate elimination by *VImK* to give a dehydroalanine moiety.^[31] It seems likely that *vImJ/vImK* homologous genes are responsible for C3-C4 dehydration in KA57-A biosynthesis (Step 4) rather than direct dehydration by a single enzyme.

Detection of [1',1'- $^2\text{H}_2$]-**4** in the feeding of [1,1- $^2\text{H}_2$]1-hexylamine (Figure 2C-i) gave us a notable insight for KA57-A biosynthesis. Compound **4** is possibly

synthesized from a C3,C4-dehydrated intermediate above mentioned by a stereospecific reduction (Step 5), however, **4** has not been detected in the culture of strain KA57 with the feeding of unlabeled 1-hexylamine (Figure 2C-ii). Our further effort to obtain **4** was unsuccessful even in the large-scale fermentation of strain KA57 (~20 L). Chromatographic isolation of [1',1'-²H₂]-**4** from the mixture of unlabeled and [1'-²H]-labeled **3** was also unsuccessful. These observations could be explained by a strong deuterium-isotope effect on the C-H/D bond cleavage at C-1' in **4**. The presence of a fully saturated hexyl side chain in [1',1'-²H₂]-**4** indicated that C1'-C2' dehydrogenation occurred at the final step in KA57-A biosynthesis (Step 6). By analogy to the valanimycin biosynthetic pathway,^[31] O-(L-seryl)-hexylhydroxylamine (Scheme 2) is the first intermediate synthesized from 1-hexylamine and L-serine, although we have not yet detected it in *S. rochei*. Further genetic analysis is necessary to characterize the gross biosynthetic machinery of azoxyalkene compounds including the unique azoxy bond formation.

Experimental Section

Strains and culture conditions: *S. rochei* wild-type strain 7434AN4 and strain 51252 that carries only pSLA2-L were described previously.^[6,9] Strains KA07 ($\Delta srrB$), KA35 (*Ikcf-KR1*^{Y343F}), and NS01 ($\Delta srrB \cdot \Delta lkmE$) were constructed previously.^[11,19,20] YEME liquid medium (0.3% yeast extract, 0.5% polypeptone, 0.3% malt extract, 1% D-glucose, and 34% sucrose) was used for preparation of *S. rochei* protoplasts.^[34] Protoplasts were regenerated on R1M agar medium.^[35] YM medium (0.4% yeast extract, 1.0% malt extract, and 0.4% D-glucose, pH 7.3)

was used for secondary metabolite production. *Escherichia coli* XL1-Blue (Agilent Technologies, Santa Clara, CA, USA) was used for construction of targeting plasmids. *E. coli* strains were grown in Luria Bertani (LB) medium supplemented with ampicillin (100 $\mu\text{g mL}^{-1}$) when necessary. Sodium [1- ^{13}C]acetate (99 atom% ^{13}C), sodium [2- ^{13}C]acetate (99 atom% ^{13}C), and L-[^{15}N]serine (98 atom% ^{15}N) were purchased from Isotec (Miamisburg, OH, USA). DL-[2,3,3- $^2\text{H}_3$]serine (98 atom% ^2H) and DL-[3- ^{13}C]alanine (99 atom% ^{13}C) were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Synthetic procedures of [1,1- $^2\text{H}_2$]1-hexylamine hydrochloride (98 atom% ^2H) and [^{15}N]1-hexylamine hydrochloride (49 atom% ^{15}N) were described in Supplemental data.

Construction of strain KA57: Plasmid pKAR1020^[19] carrying Y343F mutation in *lkcF-KR1* was transformed into protoplasts of *S. rochei* NS01. Transformants were grown in YM liquid medium supplemented with thiostrepton (10 $\mu\text{g mL}^{-1}$) to give the plasmid-integrated (single crossover) strain. The thiostrepton-resistant colonies were subjected to a sequential cultivation in YM medium without thiostrepton to give a triple mutant KA57 ($\Delta\text{srrB}\cdot\Delta\text{lkmE}\cdot\text{lkcF-KR1}^{\text{Y343F}}$). Disruption was confirmed by Southern blot analysis (Figure S1).

In a similar manipulation, strain KA36 ($\Delta\text{srrB}\cdot\text{lkcF-KR1}^{\text{Y343F}}$) was constructed by transformation of pKAR1020 into protoplasts of *S. rochei* KA07.

Detection of metabolites: Each 100 mL of cell culture in a 500-ml Sakaguchi flask was incubated at 28°C for 3 days. The culture broth was extracted twice

with equal volume of EtOAc. The combined organic phase was dried (Na_2SO_4), filtered, and concentrated to dryness. The crude residue was analyzed by high-performance liquid chromatography (HPLC) and thin layer chromatography (TLC). The crude extract was dissolved in acetonitrile and applied on a COSMOSIL Cholester column (4.6 x 250 mm, Nacalai Tesque, Japan) with elution of a mixture of acetonitrile-10 mM sodium phosphate buffer (pH 8.2) (3:7, v/v) at a flow rate of 1.0 mL min^{-1} . The eluent was monitored with a JASCO MD-2010 multiwavelength photodiode array detector at a scan range of 200-600 nm. Lankacidin C (**1**) and azoxyalkene (**3**) were detected at 9.5 and 10.5 min, respectively. TLC (silica gel 60 F₂₅₄, Merck) was developed with a mixture of CHCl_3 -MeOH (20:1, v/v), visualized by a 254-nm UV lamp, and then baked after spraying with anisaldehyde- H_2SO_4 .

Isolation of metabolites: The crude extract of strain KA57 from the three-days culture was firstly purified by Sephadex LH-20 (1.5 x 40 cm; GE Healthcare) with methanol. The fractions containing **3** ($R_f = 0.5$ in CHCl_3 -MeOH = 15:1) were combined, and then purified by a series of silica gel column chromatographies with two different solvent systems of CHCl_3 -MeOH (50:1, v/v) and toluene-EtOAc (2:1, v/v). Average yield of **3** was 3.3 mg L^{-1} .

Compound **3**: $[\alpha]_{\text{D}}^{25} = -40$ ($c=0.35$, MeOH) [Ref. 21]; $[\alpha]_{\text{D}}^{25} = -30$ ($c=0.2$, MeOH). HRMS (positive ESI): m/z calcd for $\text{C}_{10}\text{H}_{20}\text{N}_2\text{O}_3\text{Na}$: 239.1373 $[\text{M}+\text{Na}]^+$; found: 239.1364. The ^1H and ^{13}C NMR assignments are listed in Table 1.

The absolute configuration at C-2 was established by modified Mosher method.^[22] The difference in chemical shift ($\Delta\delta$) was obtained by subtracting the

δ value for its (*R*)- α -methoxy- α -trifluoromethyl- α -phenylacetate (MTPA) ester from that for the (*S*)-MTPA ester (δ_S - δ_R).

Feeding experiments: Isotope-labeled substrates were independently fed at 12 h period to two-liter cultures of strain KA57 (20 x 100 mL in the 500-mL Sakaguchi flasks). Isotope-labeled substrates used in this study were sodium [1-¹³C]acetate (1.0 g), sodium [2-¹³C]acetate (1.0 g), DL-[3-¹³C]alanine (100 mg), DL-[2,3,3-²H₃]serine (100 mg), [1,1-²H₂]1-hexylamine hydrochloride (200 mg), L-[¹⁵N]serine (100 mg), and [¹⁵N]1-hexylamine hydrochloride (50 mg). After cultivation for additional 2.5 days at 28°C, the resultant isotope-labeled KA57-A was isolated as above mentioned.

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Table 1. NMR data for **3** in CDCl₃.^[a]

Position	δ_{H} (Mult., J [Hz]) ^[b]	δ_{C} (Mult.) ^[b]	$\square\square\square\square\square\square\square\square\square\square\square\square\square\square\square\square\square$ $\square\square\square\square$ ^[c]	
			[1- ¹³ C]acetate	[2- ¹³ C]acetate
1	3.56 (dd, 5.5 and 11.5) 3.68 (dd, 3.5 and 11.5)	63.6 (t)	1.0	2.5
2	3.75 (m)	74.1 (d)	1.6	0.7
3	4.30 (dt, 7.0 and 6.5)	57.6 (d)	1.1	0.9
4	1.28 (d, 7.0)	13.4 (q)	1.0	1.0
1'	6.74 (dt, 9.0 and 1.5)	135.5 (d)	2.5	1.5
2'	5.74 (dt, 9.0 and 7.5)	134.8 (d)	1.1	2.4
3'	2.64 (m)	26.9 (t)	2.7	1.3
4'	1.46 (m)	31.1 (t)	1.1	3.0
5'	1.36 (m)	22.3 (t)	3.0	1.3
6'	0.92 (t, 7.0)	13.8 (q)	1.4	3.3

[a] Spectra were recorded at 500 MHz for ¹H and 125 MHz for ¹³C.

[b] The residual solvent signal ($\delta_{\text{C}} = 77$ ppm) and internal standard tetramethylsilane ($\delta_{\text{H}} = 0$ ppm) were used as a reference.

[c] Peak intensities were normalized to the ¹³C signal of C-4.

Figure and Scheme legends

Scheme 1

Structures of lankacidin C (**1**), lankamycin (**2**), and KA57-A (**3**) isolated from *Streptomyces rochei* strains 51252 (parent) and KA57 ($\Delta srrB \cdot \Delta lkmE \cdot lkcF \cdot KR1^{Y343F}$).

Scheme 2

Proposed biosynthetic pathway of **3**. Bold letters indicate hydrogen atoms from C2 and C3 of serine. Italic letters indicate hydrogen atoms from C-1 of 1-hexylamine.

Figure 1

Analysis of metabolites produced by strain KA57. A) Silica gel TLC analysis of metabolites from various strains above mentioned: i) when irradiated with UV lamp at 254 nm, and ii) stained with anisaldehyde. TLC plate was developed with a mixture of CHCl_3 -MeOH (15:1, v/v). B) HPLC chromatograms obtained by a photodiode array detector. i) strain 51252 (parent); ii) strain KA07 ($\Delta srrB$); iii) strain KA36 ($\Delta srrB \cdot lkcF \cdot KR1^{Y343F}$), iv) strain NS01 ($\Delta srrB \cdot \Delta lkmE$); v) strain KA57 ($\Delta srrB \cdot \Delta lkmE \cdot lkcF \cdot KR1^{Y343F}$); vi) purified KA57-A (**3**). The crude extract was applied on a COSMOSIL Cholester column (4.6 x 250 mm, Nacalai Tesque) and eluted with a mixture of acetonitrile-10 mM sodium phosphate buffer (pH 8.2) (3:7, v/v) at a flow rate of 1.0 mL min⁻¹. C) The UV-visible spectra of compounds **1** (i) and **3** (ii).

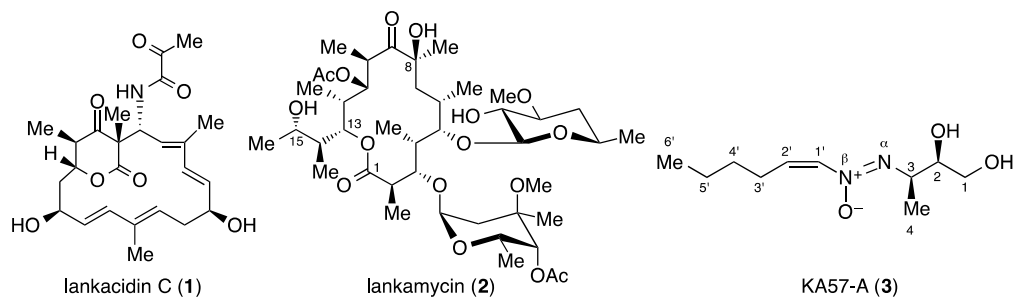
Figure 2

Biosynthetic origin of KA57-A. A) ²H-NMR spectrum of: i) unlabeled **3**, ii) labeled **3** obtained by feeding of DL-[2,3,3-²H₃]serine, iii) a labeled mixture of **3** and 1',2'-dihydro-KA57-A (**4**) obtained by feeding of [1,1-²H₂]1-hexylamine, and iv) ¹H-NMR spectrum of unlabeled **3**. B) Proposed biosynthetic origin of KA57-A. C) ESI mass spectrum of a mixture containing KA57-A and its derivatives obtained by feeding of: i) [1,1-²H₂]1-hexylamine, and ii) unlabeled 1-hexylamine. Chemical structures of [1'-²H]-**3** and [1',1'-²H₂]-**4** were also shown in this panel. D) ESI-CID-MS/MS spectrum of parent ions: i) *m/z* 239, ii) *m/z* 240, and iii) *m/z* 243 obtained by feeding of [1,1-²H₂]1-hexylamine.

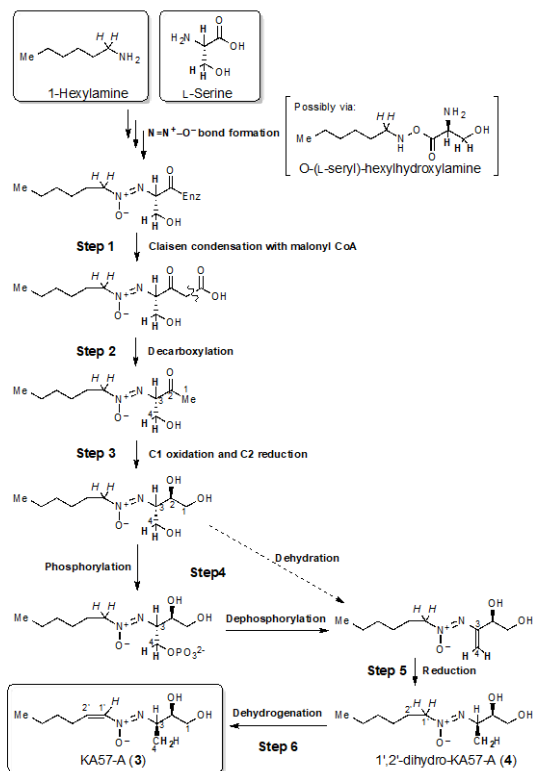
Table of Contents:

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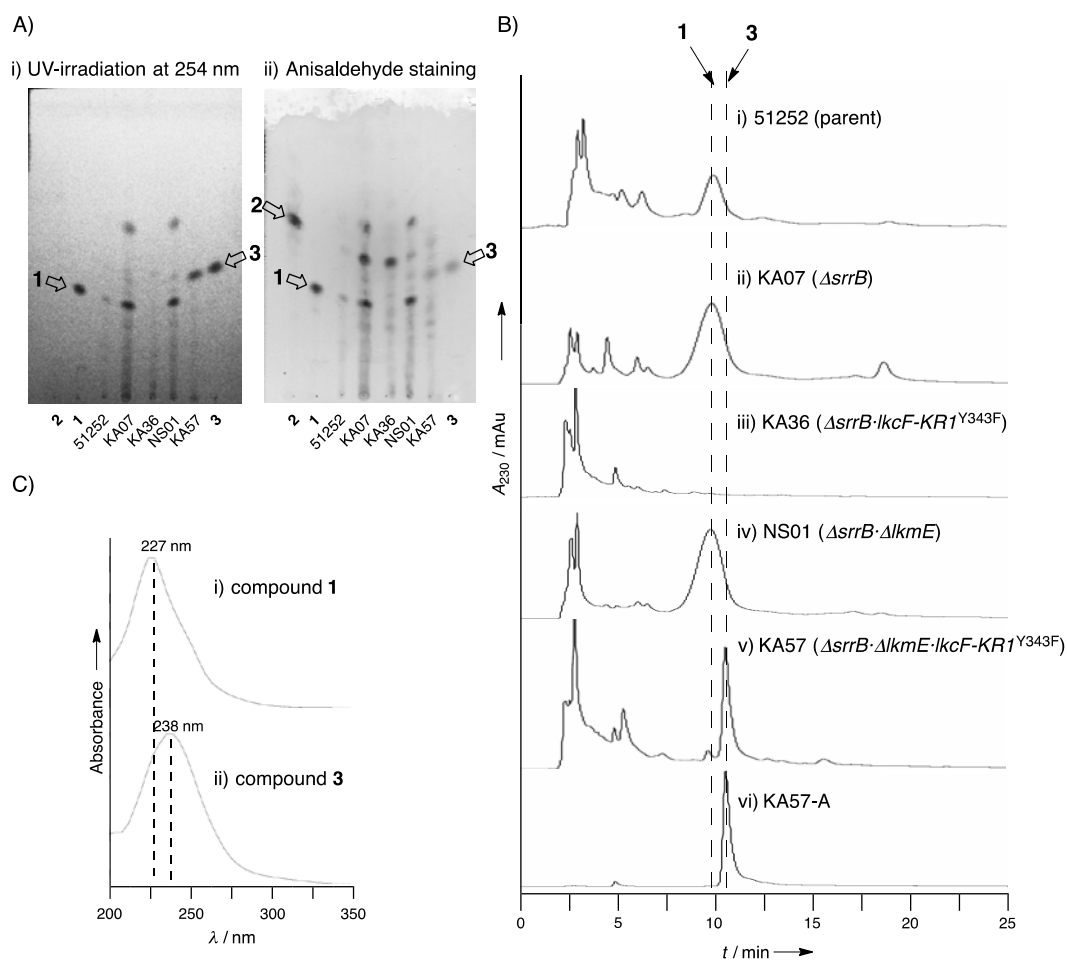


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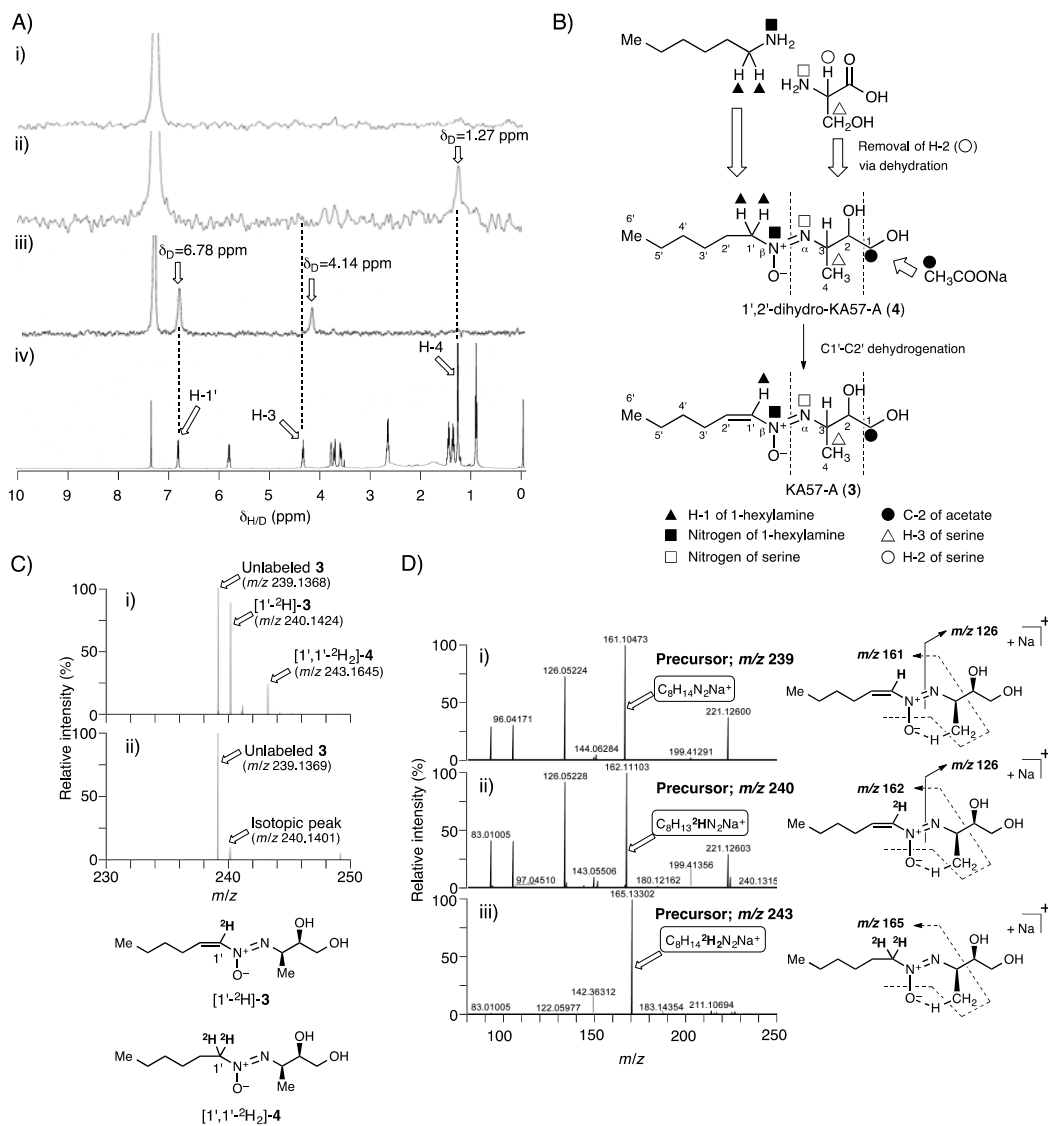


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