Biosynthetic Investigation and Structural Diversity of Azoxyalkene Compounds

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Research objective

Actinomycetes produce a variety of secondary metabolites with remarkable biological activities and complex molecular architectures. In *Streptomyces rochei* 7434AN4, a multigene mutant accumulated the azoxyalkene compound KA57A (Figure 1A)[1]. This compound contains a unique azoxy bond, and genomic analysis[2] revealed no homologous genes for known N-N bond-forming enzymes, suggesting a novel enzyme. The strain also accumulated hydrazide compounds KA57D1 and KA57D2 (Figure 1B)[3]. Labeled amino acid incorporation experiments revealed that KA57-A originated from serine, KA57D1 from valine, and KA57D2 from isoleucine, indicating broad substrate specificity for the N-N bond-forming enzyme. This study aims to elucidate the biosynthetic machinery of azoxyalkene compounds through gene disruption analysis of the KA57A biosynthetic genes, enzyme conversion experiments of possible biosynthetic substrates, and screening for related biosynthetic genes.

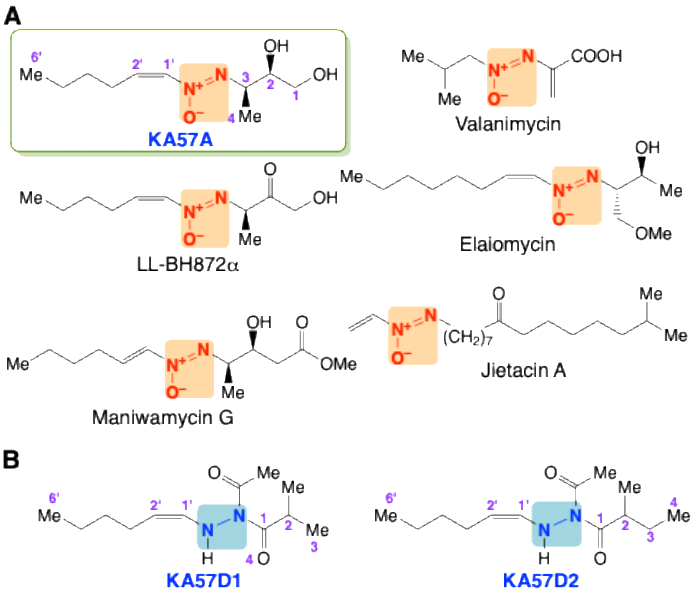

Figure 1. (A) Structures of azoxyalkene compounds
(B) Hydrazide-alkene compounds detected from *S. rochei* strain KA57

Methods

The putative biosynthetic pathway of KA57A is shown in Figure 2. First, hexylamine and serine are condensed followed by the rearrangement and oxidation of a hydroxylamine intermediate to form an azoxy ($N{=}N^{+}{-}O^{-}$) bond. Subsequent enzymatic steps yield 1',2'-dihydroKA57A, with C1'-C2' desaturation producing KA57A (Ref. 1). Furthermore, by comparing the biosynthetic gene clusters of KA57A, valanimycin, and maniwamycin (Figure

1)[4], we identified shared genes, including *N*-hydroxylase and kinase. In this study, we planned to comprehensively elucidate the biosynthetic pathway by analyzing the metabolites in various gene disruptants.

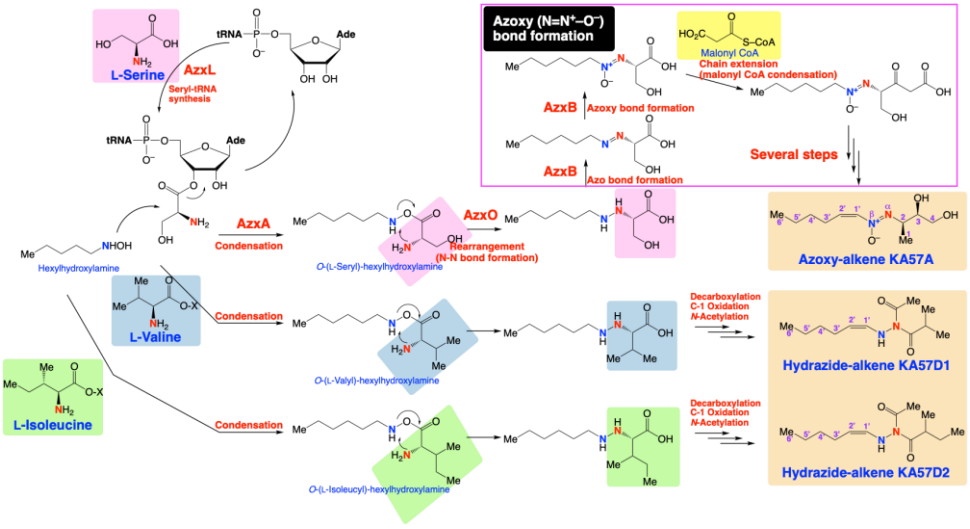

Figure 2. Biosynthetic pathway of azoxyalkene KA57A and hydrazide-alkenes KA57D1 and KA57D2

Results

The genome of *Streptomyces* sp. TOHO-M025, a maniwamycin producer, was determined by next-generation sequencing. Comparison of the KA57A biosynthetic gene (*azx*) cluster[2] with maniwamycin genes revealed shared genes, including *N*-hydroxylase (*M025-432*, *SRO_1832*) and kinase (*M025-426*, *SRO_1821*) (Table 1). Comprehensive gene disruption of these genes was performed, followed by metabolite analysis. Similar to valanimycin disruptants, the *N*-hydroxylase disruptant produced no azoxyalkenes,

Figure 1. Biosynthetic gene cluster for maniwamycin (*mwm*), KA57A (*azx*) , valanimycin (*vlm*)

mwm	start	stop	direction	*azx*	*vlm*	Putative function
M025-423	497081	498550	+			CoA transferase
M025-424	498603	499157	+			carboxymuconolactone decarboxylase family protein
M025-425	499258	500736	–	**SRO_1819**	**vlmK**	VlmK-like protein
M025-426	500736	501722	–	**SRO_1821**	**vlmJ**	diacylglycerol kinase
M025-427	502359	503438	+	**SRO_1848**		fatty acid desaturase
M025-428	503542	504951	+	**SRO_1846**		MFS transporter
M025-429	505043	505189	+	**SRO_1849**		hypothetical protein
M025-430	505245	506222	–	**SRO_1850**	**vlmA**	hypothetical protein
M025-431	506393	508000	+	**SRO_1831**		aldehyde dehydrogenase
M025-432	508032	509168	+	**SRO_1832**	**vlmH**	oxidoreductase
M025-433	509209	510498	+	**SRO_1833**		acetylornithine aminotransferase
M025-434	510508	510999	+	**SRO_1834**		cyclase
M025-435	511016	511543	+	**SRO_1835**	**vlmO**	VlmO-like protein
M025-436	511543	512109	+	**SRO_1836**	**vlmR**	NADPH - flavin oxidoreductase
M025-437	512202	513113	+	**SRO_1837**	**vlmB**	FAD/FMN-containing dehydrogenases
M025-438	513143	514126	+	**SRO_1838**		3-oxoacyl-ACP synthase
M025-439	514175	514402	+	**SRO_1839**		Acyl carrier protein
M025-440	514402	515622	+	**SRO_1840**		3-oxoacyl-ACP synthase
M025-441	515674	516945	+	**SRO_1843**	**vlmL**	serine-tRNA ligase
M025-442	517184	517345	+			IS110 family transposase
M025-443	517341	517625	–			hypothetical protein
M025-444	517735	518079	+			hypothetical protein
M025-445	518480	519715	–			Putative amino acid permease

mwm; maniwamycin biosynthetic genes
azx; KA57A biosynthetic genes
vlm; valanimycin biosynthetic genes

confirming its essential role in biosynthesis. Metabolite analysis of the kinase gene, *SRO_1821*, revealed that KA57A was produced in approximately 10% of the parental strain; this suggests that housekeeping (or other functional) kinases may substitutes for this gene. Trace metabolites in this disruptant were identified, and structural analysis is ongoing to further elucidate the biosynthetic mechanism.

Conclusion

I Recent studies have explored N-N bond formation in natural products, but no genes corresponding to pathways such as cremiomycin (nitrite production) or s56-p1 (hydrazone unit) were identified in the KA57A biosynthetic gene cluster of the *S. rochei* genome[1] or in maniwamycin-producing strains[4]. Nonetheless, the biosynthesis of azoxyalkene compounds valanimycin and azodyrecin has been reported recently[5, 6]. The intermediate formed by the condensation of hydroxylamine with an amino acid is isomerized by VlmO, followed by the formation of an azo group (N=N) and an azoxy group ($N=N^{+}–O^{-}$) by VlmB [5, 6]. This suggests the diversity of N-N bond-forming enzymes. In this study, we demonstrated that KA57A-producing bacteria utilize diverse amino acids to form hydrazides[3]. This strongly suggests substrate flexibility of the nitrogen-nitrogen bond-forming enzyme, and elucidating the molecular basis of this enzyme could enable the design of novel bioactive molecules that surpass natural products.

References

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