

# Auroramycin, a potent antibiotic from *Streptomyces roseosporus* by CRISPR-Cas9 activation

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**Abstract:** Silent biosynthetic gene clusters represent a potentially rich source for new bioactive compounds. We report the discovery, characterization and biosynthesis of a novel doubly glycosylated 24-membered polyene macrolactam from a silent biosynthetic gene cluster in *Streptomyces roseosporus* using the CRISPR-Cas9 gene cluster activation strategy. Structural characterization of this polyketide, named auroramycin, revealed a rare isobutyrylmalonyl extender unit and a unique pair of aminosugars. Relative and absolute stereochemistry were determined using a combination of spectroscopic analyses, chemical derivatization and computational analysis. The activated gene cluster for auroramycin production was also verified by transcriptional analyses and gene deletions. Finally, auroramycin exhibited potent anti-methicillin-resistant *Staphylococcus aureus* (anti-MRSA) activity towards clinical drug-resistant isolates.

Streptomycetes have been a prolific source of bioactive natural products with relevant biomedical applications, as epitomized by anthelmintic avermectin and antibiotic streptomycin.<sup>[1-5]</sup> Advances in genome sequencing have revealed that we have barely harnessed the biosynthetic potential of these organisms, with a significant number of cryptic biosynthetic gene clusters (BGCs), many of which are expected to be silent under normal laboratory conditions.<sup>[6]</sup> Activating the expression of these silent BGCs will provide unprecedented access to potentially

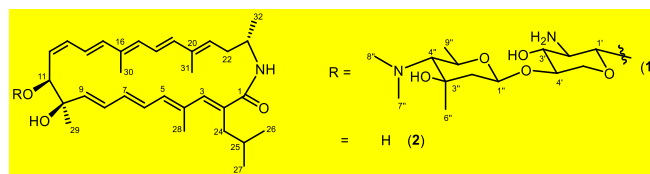
useful bioactive molecules and enable exploration of new chemical structure space.<sup>[7]</sup>

As an industrial producer of daptomycin<sup>[8]</sup>, *Streptomyces roseosporus* has been studied for decades but its biosynthetic potential has only recently been explored. Advances in mass spectrometry and networking analyses identified additional peptidyl antibiotics produced by *S. roseosporus* – arylomycins, napsamycins, and stenothricins.<sup>[9,10]</sup> Complementary molecular approaches to activate the expression of silent BGCs further expanded the secondary metabolite repertoire of *S. roseosporus* to include uridyl peptidyl,<sup>[11]</sup> phosphonate and polycyclic macrolactam compounds.<sup>[6]</sup> With >20 BGCs predicted from the *S. roseosporus* genome,<sup>[12]</sup> it is clear that we have yet to fully realize its biosynthetic potential.

Using a CRISPR-Cas9 gene cluster activation strategy,<sup>[12]</sup> we sought to activate a silent type I polyketide synthase (PKS) cluster in *S. roseosporus* NRRL 15998 that is homologous to the incednine BGC in *Streptomyces* sp. ML694-90F3.<sup>[13]</sup> Introduction of a 97 bp *kasO*\* promoter drove constitutive expression of *aurR1* (Figure S1), which encodes a *Streptomyces* LAL (large ATP binding members of the LuxR family) transcriptional activator. Additionally, we observed upregulated expression of the 95 kb BGC (Figure S22) and distinct metabolites that were not observed for the wild type *S. roseosporus* strain (Figure S2). Here, we describe the isolation, structural elucidation, proposed biosynthetic pathways, and biological activities of a structurally unique compound, named auroramycin (**1**) as well as its aglycon **2**.

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**Figure 1.** Structure of auroramycin (**1**) and its aglycon **2**.

Auroramycin (**1**) was obtained as a pale yellow/off-white solid (~52.9 mg/L) from the cultured agar plates of the producing strain by ethyl acetate partition, followed by trituration using ethyl acetate and methanol due to the acid and light sensitivity profile of **1**. The molecular mass of **1** was determined by HRMS, which gave  $m/z$  of 794.5319  $[M+H]^+$  and  $m/z$  of 397.7696  $[M+2H]^{2+}$ , suggesting a molecular formula of  $C_{46}H_{71}N_3O_8$ . Characteristic UV absorption at  $\lambda_{max} = 324, 311$  nm were indicative of a polyene moiety. IR absorption at 1631, 1521  $cm^{-1}$  suggested the presence of an amido group.

The assignable NMR spectra of **1** was obtained when the sample was dissolved in a mixture of  $CD_2Cl_2/CD_3OD$  (5:1). Analyses of the  $^1H$ ,  $^{13}C$  NMR, HMQC and DEPT spectra revealed that **1** contained 46 carbons, including one carbonyl, four

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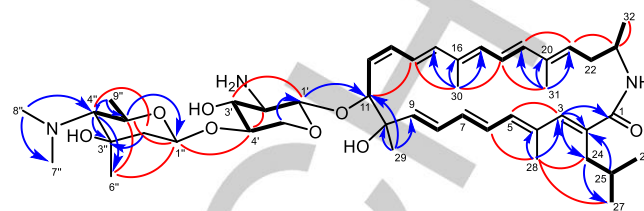
quaternary  $sp^2$ , two quaternary  $sp^3$ , fourteen  $sp^2$  methines, ten  $sp^3$  methines, four methylenes, and eleven methyl carbons.

The structure of **1** was elucidated by  $^1H$ - $^1H$  COSY, NOE and HMBC (Figure 1, 2). Detailed analysis of COSY spectrum revealed seven groups of  $^1H$ - $^1H$  connectivities belonging to: H-5 to H-9, H-11 to H-14, H-17 to H-19, H-21 to H-23 and methyl H-32, H-24 to H-27, H-1' to H-5', and also H-4'' to H-5'' and H-9''. The connectivities of C-5 to C-1 carbonyl were established through a combination of HMBC correlations (from C-28 to C-3 and C-4; from C-3 to C-1, C-2 and C-24; from C-25 to C-3) and NOEs interactions (a) between H-3 and H-5, (b) between H-28 and H-6, H-24. The linkage of the partial structures between C-5 to C-9 and C-11 to C-14 was established by HMBC correlations from H-29 to C-9 (weak), C-10 and C-11, of which C-10 is a quaternary oxygenated carbon. The structural linkage between C-14 to C-17 was established through HMBC correlations from H-30 to C-15, C-16 and C-17, and NOE correlations from H-30 to H-14 and H-18. Similarly, the linkage between C-19 and the partial structures of C-21 to C-32 was established through HMBC correlations from H-31 to C-19, C-20 and C-21, and NOEs correlations from H-31 to H-18, and from H-19 to H-21. Finally, a HMBC correlation between C-23 and C-1 as observed in the characterization of auroramycin aglycon (**2**) (Table S1 and Figure S17) established the ring closure across the macrolactam moiety. Geometry of all the olefins except C12-C13 were all determined to be *E* based on the NOEs observed for aglycon **2** (Figures S18). The olefin geometry for C12-C13 has been assigned as *Z* based on key NOE interactions: (a) between H-11 and H-14, (b) between C-12 and H-13 as observed in the aglycon **2**. The linkage between the macrolactam at C-11 and the glycoside at C-1' was established by HMBC and NOE.

The partial structures and relative stereochemistry of the two aminosugars were further deduced by HMBC and NOEs correlations as depicted in Figure 2. NOEs correlations between H1' and H3' suggest that the relative stereochemistry of C1'-O and C3'-OH is *syn* to each other. The relative stereochemistry of C2'-NH<sub>2</sub> and C4'-O is *syn* to each other by the same NOEs observations. Thus, the relative stereochemistry of the adjacent C2'-NH<sub>2</sub> and C3'-OH is *anti* for the inner amino sugar. The connectivities of C-1'' to C-5'' and C-4'' to *N*-methyl groups were established through HMBC correlations (from C-1'' to C-2''; from C-2'' to C-3''; from C-4'' to C-3''; from C-5'' to C-1''; from C-7''/C-8'' to C-4''; from C-7'' to C-8''). NOEs interactions (a) between H-6'' methyl and H-1'', H-5'', (b) between H-4'' and one of the H-2'' suggest that C-9'' methyl is on the opposite plane to both C-9'' methyl C4''-N. Finally, the linkage between C-4'' of the inner amino sugar and C-1'' of the outer amino sugar was established by HMBC and NOE.

The absolute configuration of the C-11 position of aglycon **2**<sup>[14]</sup> was determined by a modified Mosher's method. Both the *R*-MPA ester (**3**) and *S*-MPA ester (**4**) of **2** were prepared and subjected to  $^1H$  NMR analyses. Whilst both conformers (*sp* and *ap*) in each of **3** and **4** were observable in the  $^1H$  NMR spectra at 25 °C, integrations of the  $\delta_H$  signals of C-29 methyl and C-12 methine allowed the establishment of the major *sp* conformer in both **3** and **4**. The  $\Delta\delta$  values of the protons, thus elucidated the absolute configuration at C-11 to be *S* (Figure S19). The remaining stereochemistry of C-10 and C-23 was further established by NOE experiments and supported by computational analysis (Figure S20) and biosynthesis prediction (*vide infra*). Based on the observed NOEs between H-8 and H-29, H-11 and H-29, and H-11 and H-14, the relative stereochemistry at C-10 and C-11 is indicative to be *R*\* and *S*\*, respectively, which was supported by the Spartan program. The remaining stereocenter, C-23 was elucidated by the surrounding NOEs to be *S*\*, which

was supported by biosynthesis prediction and homology to precedent starter units. Thus, the overall stereochemistry of the three chiral centers of the aglycon **2** was 10*R*, 11*S* and 23*S*.



**Figure 2.** Gross structures of **1** determined by 2D-NMR spectra (COSY – bold lines, key HMBC correlations – arrows, key NOESY correlations – red curly lines).

Due to high GC-content and repetitive sequences of the PKS genes, assembly of the auroramycin BGC was incomplete in the deposited genome sequence (accession no: NZ\_DS999644.1). The missing BGC sequences was rectified by genome sequencing using PacBio RS II and verified by comparison to the transcriptome of the activated strain (Figure S22). The final 95,454 bp BGC consists of 10 PKS modules on 5 genes and an additional 33 genes involved in post-PKS modifications and biosynthesis of the 3-aminobutyrylate starter unit, isobutyrylmalonyl-CoA (*ibmCoA*) and sugars (Table S4). The order of reactions (chain length, dehydration, and incorporation of different extender units, Figure 3) aligns with the chemical structures. Aside from a loading acyl carrier protein (ACP) and thioesterase (TE), all modules except module 6 contain both dehydratase (DH) and ketoreductase (KR) domains. The standalone module 6 only has a KR tailoring domain. The *trans* double bond configuration correlates with KR1-4 and KR7-10 as B type, whilst KR5 and KR6 are predicted to be of A type stereochemistry (Figure S23).<sup>[15]</sup>

Consistent with the chemical structure and predicted module order, acyltransferases (ATs) 1, 3, 6 are methylmalonyl-CoA (*mmCoA*) specific and ATs 2, 4, 5, 7 and 8 are malonyl-CoA (*mCoA*) specific, based on the conserved YASH and HAFH motifs.<sup>[16]</sup> Due to presence of atypical motifs in AT9 and 10, a phylogenetic tree was constructed with ATs from similar macrolactam biosyntheses; incednine (methoxymalonyl-CoA loading), vicanistatin and divergolide (*ibmCoA* and ethylmalonyl-CoA loading) BGCs.<sup>[17]</sup> AT9 is highly similar to *mmCoA*-specific *IndAT5* (79%/85%, identity/similarity, HAAH motif) and AT10 is predicted to be *ibmCoA* specific due to closest similarity with *IndAT10* and lack of H/Y similar to ethylmalonyl-CoA and *ibmCoA* loading ATs from divergolide BGC (Figure S24). These predictions agree with the elucidated chemical structure.

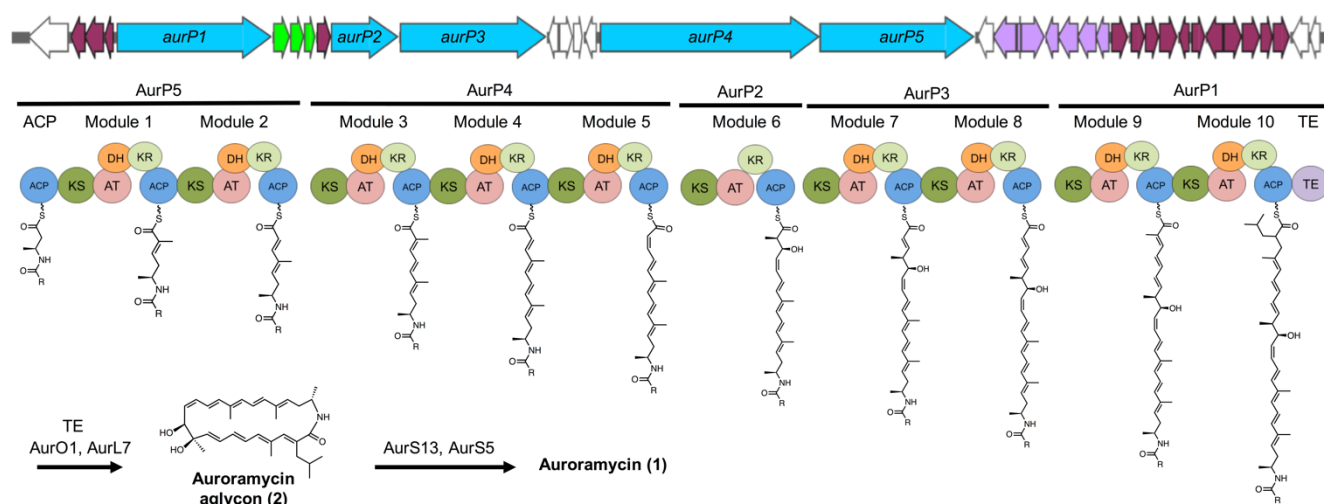
High similarity of biosynthetic enzymes with the incednine BGC<sup>[13]</sup> led us to propose similar pathways for 3-aminobutyryl-ACP and UDP-xylosamine biosyntheses (Figures S25, S26). In the latter, the absence of a methyltransferase results in UDP-xylosamine formation instead of an UDP-methylxylosamine sugar. However, there are also distinct differences compared to the incednine BGC, including the biosynthesis of a rare extender unit, *ibmCoA*, and also a novel NDP-3,5-*epi*-lemonose sugar. *ibmCoA* extender unit is predicted to be catalysed via a conserved three-gene operon found within the BGC (Figure S27). Although this operon can be found in other streptomycetes, the only verified examples of *ibmCoA* production and incorporation were previously observed within *Streptomyces* sp. HKI0576 for both germicidins and divergolide biosynthesis, *divRST*.<sup>[18]</sup> The operon encoding biosynthetic enzymes for the second sugar, contains

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similar enzymes for TDP-*N*-demethylforosamine production in the incednine BGC. However, absence of NDP-hexose-3,4-dehydratase (IndS12) and addition of NDP-hexose 3-C-methyltransferase (AurS11) form instead a unique NDP-3,5-*epi*-lemonose sugar from D-glucose-1-phosphate (Figure S28). Finally, although 4 glycosyltransferases are observed, only AurS5 and AurS13 are predicted to be involved in glycosylation as AurS4, S10 are truncated.<sup>[13]</sup> Deletion of a 13 kb region, consisting of

AurS5-15, within the activated gene cluster produced the auroramycin aglycon **2** as predicted (Figure 3, Figure S2).

Overall, while the 24-membered polyene macrolactam ring structure of auroramycin is similar to silvalactam<sup>[19]</sup> and incednine<sup>[20]</sup>, there are a number of key discerning features including the use of a relatively rare isobutyrylmalonyl extender unit, and the presence of a unique disaccharide comprising of a novel 3,5-*epi*-lemonose sugar.



**Figure 3.** Proposed enzymes and biosynthetic pathways for auroramycin biosynthesis. Gene arrangement of BGC; blue for PKS, red for sugar biosynthesis enzymes, green for extender unit, purple for starter unit and white for transporters, regulators and TE.

**Table 1.** *In vitro* anti-bacterial activity of auroramycin (1) and its aglycon 2.

Organism/Species	Strain ID	10% Serum	MIC ( $\mu\text{g/mL}$ ) <sup>[a]</sup>		
			1	2	Van.
<i>Staphylococcus aureus</i> (MRSA)	Clinical N216	-	1-2	>128	1
		+	0.5	NT	NT
<i>Staphylococcus aureus</i> (VI-MRSA)	Clinical Z172	-	2-4	NT	4
		+	0.25	NT	NT
<i>Enterococcus faecalis</i> (VRE)	ATCC 51299	-	1	NT	>8
		+	0.25-0.5	NT	NT

[a] Minimum inhibitory concentration (MIC) values were determined by broth microdilution in duplicate experiments, measured after 24h at 35°C. NT, not tested. Van. refers to vancomycin.

As a growing group of natural products found only in actinomycetes, polyene macrolactams have been reported to possess anti-bacterial, anti-cancer, anti-inflammatory

properties.<sup>[13,17,21]</sup> Auroramycin displayed potent antibacterial activity against Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA) (Table 1) but no activity (MIC >128  $\mu\text{g/mL}$ ) against Gram-negative bacteria (data not shown). The anti-MRSA activity of auroramycin is comparable to vancomycin against two clinical MRSA isolates, one of which is a vancomycin-intermediate (VI-MRSA) strain that is also daptomycin non-susceptible. Auroramycin also exhibited potent antibacterial activity against a vancomycin-resistant *Enterococcus faecalis* (VRE) strain. Interestingly, the anti-MRSA and anti-VRE activities of auroramycin were potentiated in 10% serum. These results highlight the potential of auroramycin as an alternative to current antibiotics, especially with the recent rapid emergence of resistant bacteria. Notably, the bioactivity of auroramycin is dependent upon its novel disaccharide unit as the aglycon did not exhibit any activity against Gram-positive bacteria (Table 1).

In summary, we discovered a potent glycosylated polyene macrolactam antibiotic by inducing the expression of a 95 kb BGC in *S. roseosporus*. Inclusion of a rare isobutyl extender unit and a unique disaccharide comprising of a novel 3,5-*epi*-lemonose sugar differentiates it from other 24-membered polyene macrolactams. Silent BGCs, including those in well-characterized bacteria strains, continue to be valuable sources of new bioactive molecules. Complementary to other BGC activation approaches

such as co-cultivation and heterologous expression,<sup>[6,7]</sup> our CRISPR/Cas-9-mediated approach allows the rapid exploration of silent BGCs in native hosts and facilitates natural product discovery efforts.

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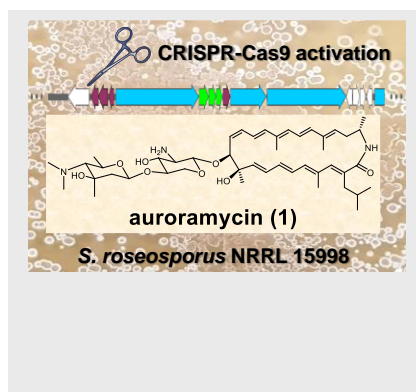
**Keywords:** Natural products • silent biosynthetic gene cluster • genome mining • polyene macrolactam • glycosylation

- [1] J. W. H. Li, J. C. Vederas, *Science* **2009**, *325*, 161-165.
- [2] D. J. Newman, G. M. Cragg, *J. Nat. Prod.* **2016**, *79*, 629-661.
- [3] M. M. Zhang, Y. Qiao, E. L. Ang, H. Zhao, *Expert Opin. Drug Discov.* **2017**, *12*, 475-487.
- [4] W. C. Campbell, *Curr. Pharm. Biotechnol.* **2012**, *13*, 853-865.
- [5] A. Schatz, E. Bugle, S. A. Waksman, *Proc. Soc. Exp. Biol. Med.* **1944**, *55*, 66-69.
- [6] H. Ren, B. Wang, H. Zhao, *Curr. Opin. Biotechnol.* **2017**, *48*, 21-27.
- [7] P. J. Rutledge, G. L. Challis, *Nature Rev. Microbiol.* **2015**, *13*, 509-523.
- [8] V. Miao, M. F. Coeffet-LeGal, P. Brian, R. Brost, J. Penn, A. Whiting, S. Martin, R. Ford, I. Parr, M. Bouchard, C. J. Silva, S. K. Wrigley, R. H. Baltz, *Microbiology* **2005**, *151*, 1507-1523.
- [9] W.-T. Liu, R. D. Kersten, Y.-L. Yang, B. S. Moore, P. C. Dorrestein, *J. A. Chem. Soc.* **2011**, *133*, 18010-18013.
- [10] W.-T. Liu, A. Lamsa, W. R. Wong, P. D Boudreau, R. Kersten, Y. Peng, W. J. Moree, B. M. Duggan, B. S. Moore, W. H. Gerwick, R. G. Linington, K. Pogliano, P. C. Dorrestein, *J. Antibiot.* **2014**, *67*, 99-104
- [11] L. Jiang, L. Wang, J. Zhang, H. Liu, B. Hong, H. Tan, G. Niu, *Sci. Rep.* **2015**, *5*, 14111.
- [12] M. M. Zhang, F. T. Wong, Y. Wang, S. Luo, Y. H. Lim, E. Heng, W. L. Yeo, R. E. Cobb, B. Enghaid, E. L. Ang, H. Zhao, *Nature Chem. Biol.* **2017**, *13*, 607-611.
- [13] M. Takaishi, F. Kudo, T. Eguchi, *J. Antibiot.* **2013**, *66*, 691-699.
- [14] The CD spectrum of **2** was consistent with that of **1**. See Figure S20.
- [15] A. T. Keatinge-Clay, *Chem. Biol.* **2007**, *14*, 898-908.
- [16] B. J. Dunn, C. Khosla, *J. Royal Soc. Interface* **2013**, *10*:20130297.
- [17] Y. Ogasawara, K. Katayama, A. Minami, M. Otsuka, T. Eguchi, K. Kakinuma, *Chem. Biol.* **2004**, *11*, 79-86.
- [18] Z. Xu, L. Ding, C. Hertweck, *Angew. Chem.* **2011**, *123*, 4763-4766; *Angew. Chem. Int. Ed.* **2011**, *50*, 4667-4670.
- [19] D. Schulz, J. Nachtigall, U. Geisen, H. Kalthoff, J. F Imhoff, H.-P. Fiedler, R. D. Süßmuth, *J. Antibiot.* **2012**, *65*, 369-372.
- [20] Y. Futamura, R. Sawa, Y. Umezawa, M. Igarashi, H. Nakamura, K. Hasegawa, M. Yamasaki, E. Tashiro, Y. Takahashi, Y. Akamatsu, M. Imoto, *J. Am. Chem. Soc.* **2008**, *130*, 1822-1823.
- [21] M. Nazare, H. Waldmann, *Chem. Eur. J.* **2001**, *7*, 3363-3376.

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A novel potent anti-methicillin-resistant *Staphylococcus aureus* (anti-MRSA) doubly glycosylated 24-membered polyene macrolactam, named auroramycin, was obtained from *Streptomyces roseosporus* using a CRISPR-Cas9 gene cluster activation strategy.



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