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To be cited as: 10.1002/anie.201900290

Link to VoR: https://doi.org/10.1002/anie.201900290

Biosynthesis and ether bridge formation in nargenicin macrolides

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Abstract: The nargenicin family of antibiotics are macrolides containing a rare ether-bridged *cis*-decalin motif. Several of these compounds are highly active against multi-drug resistant organisms. Despite the identification of the first members of this family almost 40 years ago, the genetic basis for the production of these molecules and the enzyme responsible for formation of the oxa-bridge, remain unknown. Here, we identified the 85 kb nargenicin biosynthetic gene cluster from a human pathogenic *Nocardia arthritidis* isolate and show that this locus is solely responsible for nargenicin production. Further investigation of this locus revealed a putative iron-α-ketoglutarate dependent dioxygenase, which was found to be responsible for formation of the ether bridge from the newly identified deoxygenated precursor, 8,13-deoxynargenicin. Uncovering the nargenicin biosynthetic locus provides a molecular basis for the rational bioengineering of these interesting antibiotic macrolides.

The Golden Age of antibiotic discovery saw a rapid increase in the number and type of identified bioactive metabolites from bacteria and fungi. During this period broad spectrum antibiotics were the preferred candidates, and those with a narrow spectrum of activity were often shelved.[1] However, broad spectrum agents kill a large percentage of the host microbiota, which can lead to chronic intestinal problems and infections with other pathogens, most notably *Clostridium difficile*.[2,3] A potential solution to this problem is the development of narrow spectrum antibiotics.[1,4] One such compound, nargenicin A1, which was originally isolated in the late 1980s, has gained renewed interest in recent years with its unusual molecular target (DnaE) and activity primarily against staphylococci (including multi-drug resistant strains).[5–7] Indeed, several nargenicin-like antibiotics exist, all of which have a rare oxa-bridged decalin core as a key structural feature (Figure 1, see Figure S1 for nargenicin family compounds).[8–11] While members of this family have been isolated from actinomycetes across a range of environments and geographic locations, the genetic basis for the production of nargenicin has remained unknown for almost 40 years.[6,12–14] Also undefined has been the basis for the formation of the ether bridge in these compounds, although P450 hydroxylases have previously been suggested to be involved.[10]

Herein, we have identified the genetic basis for nargenicin biosynthesis in a human pathogenic *Nocardia* isolate and show that the nargenicin biosynthetic gene cluster is rare among sequenced bacteria. Furthermore, we show that a dioxygenase is directly responsible for the formation of the unusual ether bridge moiety through functionalization of an 8,13-deoxynargenicin precursor, and also show that the ether bridge is essential for the antibiotic activity of nargenicin.

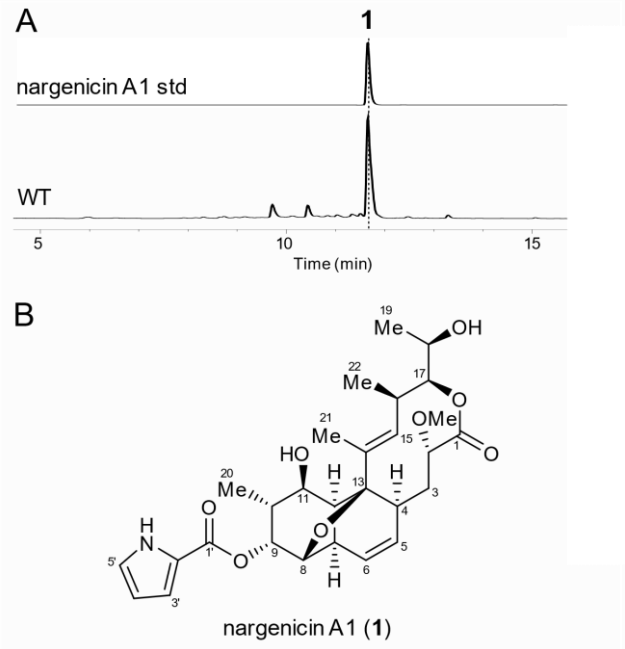

Figure 1. A) Comparison of HPLC traces of AUSMDU00012717 extract with authentic nargenicin A1 standard at 265nm. B) Structure of nargenicin A1.

As part of our discovery program for bioactive molecules from human pathogenic Actinomycetes, a *Nocardia arthritidis* isolate (ID: AUSMDU00012717) with antibacterial activity against *Staphylococcus aureus*, including methicillin resistant *S. aureus* isolates, was identified. Bioassay guided fractionation of ethyl acetate extracts from AUSMDU00012717 identified a major peak with UV maximum at 265nm (Figure 1) and a molecular formula of $C_{28}H_{37}O_8N$ by HR-ESIMS ([M+H] at *m/z* of 516.2608, calculated for $C_{28}H_{38}O_8N$ as 516.2597). Database searching suggested the compound was nargenicin A1 (also known as CP 47444).[6] Comparison of our active compound to commercially available, purified **1** showed that both peaks had the same retention time (Figure 1). Furthermore, **1** isolated from AUSMDU00012717 had 1H and 13C NMR spectra that matched previously published data (Figure S15, S16).[6]

Despite the identification of nargenicin and the related molecule nodusmicin almost four decades ago[6,15], the complete nargenicin biosynthetic gene cluster has not been identified.

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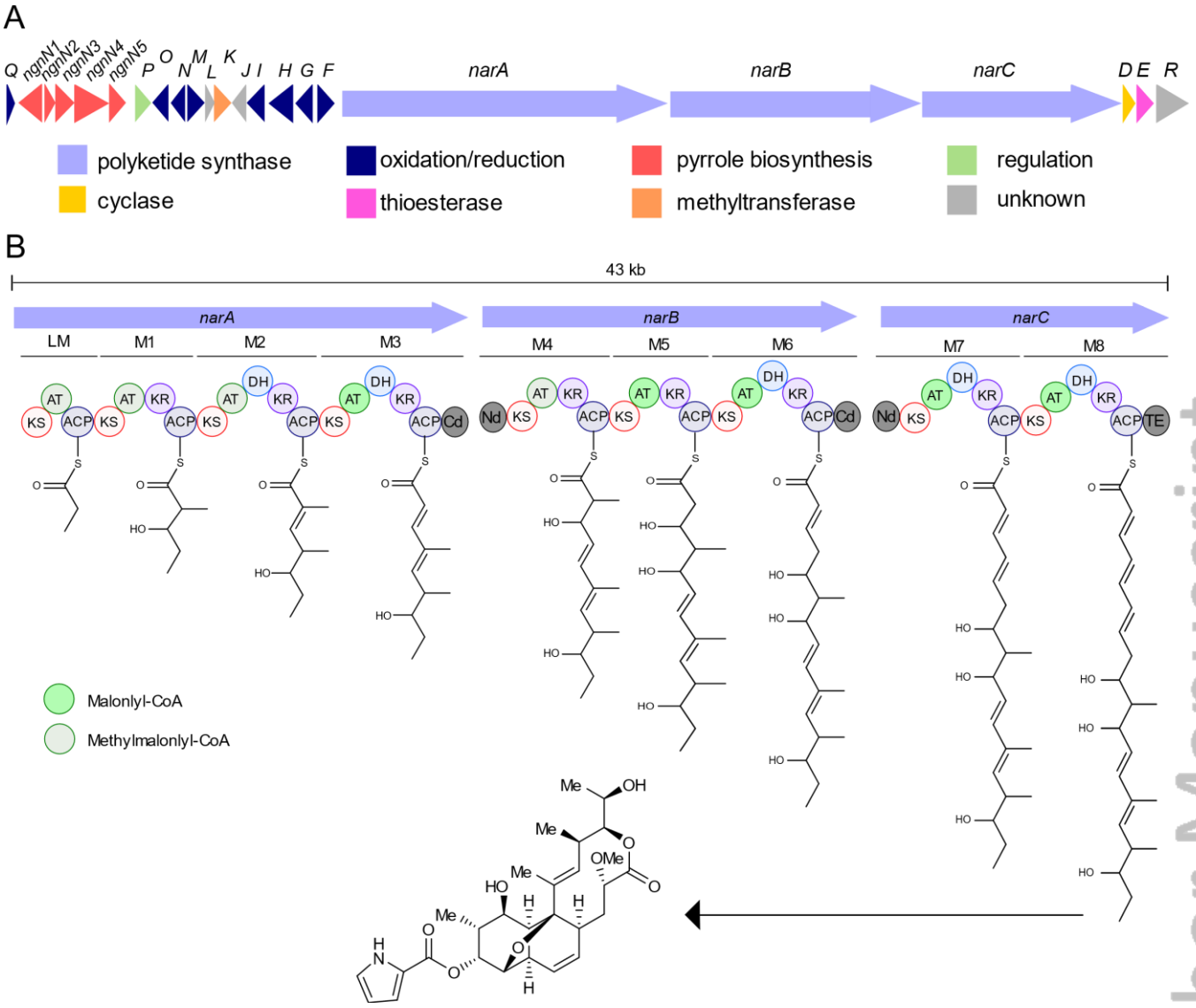

Figure 2. The *nar* biosynthetic locus in *N. arthritidis* AUSMDU00012717. A) Representation of the *nar* locus. Genes are colour coded according to the function of their predicted protein sequences. B) Module and domain predictions for NarA-C. The linear nargenicin precursor is released from the modular assembly line, cyclised, then modified to give the final molecule, nargenicin A1. Green circles represent malonate or methylmalonate specificity of the acyltransferase (AT) domains.

Early biosynthetic work based on ^{13}C labelling studies suggested that these compounds were polyketides.[15–18]. To investigate the biosynthesis of **1**, we sequenced the genome of *N. arthritidis* AUSMDU00012717 using a combination of Pacbio SMRT and Illumina DNA sequencing. Based on polyketide biosynthetic logic, we reasoned that the linear nargenicin precursor would be produced by a nine module PKS, followed by cyclisation and further processing reactions, including hydroxylation, methylation and the addition of the pyrrole moiety. AntiSMASH[19] analysis of the AUSMDU00012717 genome identified a candidate 85 kb locus, consisting of 28 open reading frames (ORFs), including three type I polyketide synthase (PKS) encoding genes (*narA-C*) (Figure 2). NarA-C are predicted to contain nine PKS modules, whose domain structure matches with that required to produce the linear nargenicin precursor, suggesting that this gene cluster was involved in nargenicin production (Figure 2).

Mining of the GenBank database using the *nar* locus identified three related gene clusters: the previously described streptoseomycin (*stm*) gene cluster,[10] a *stm*-like gene cluster from *Streptomyces* sp. NRRL S-350, as well as a putative *nar*-like gene cluster in *Nocardia tenerifensis* DSM44704 (Figure S2, Table S3). Although gene synteny and orthology suggests the *N. tenerifensis* gene cluster is most closely related to *nar*, the fragmented assembly of the PKS genes in the DSM44704 draft genome sequence did not allow for prediction of the

resulting molecule. However, a comparison of the module and domain structure of the Nar and Stm PKSs shows that they both encode nine PKS modules, although they differ in the specificity of the acyltransferase domain in module 4. Here, the Nar PKS selects methylmalonate, while the Stm PKS selects malonate, which correlates with the altered methylation status of C10 in both molecules (Figure S3).

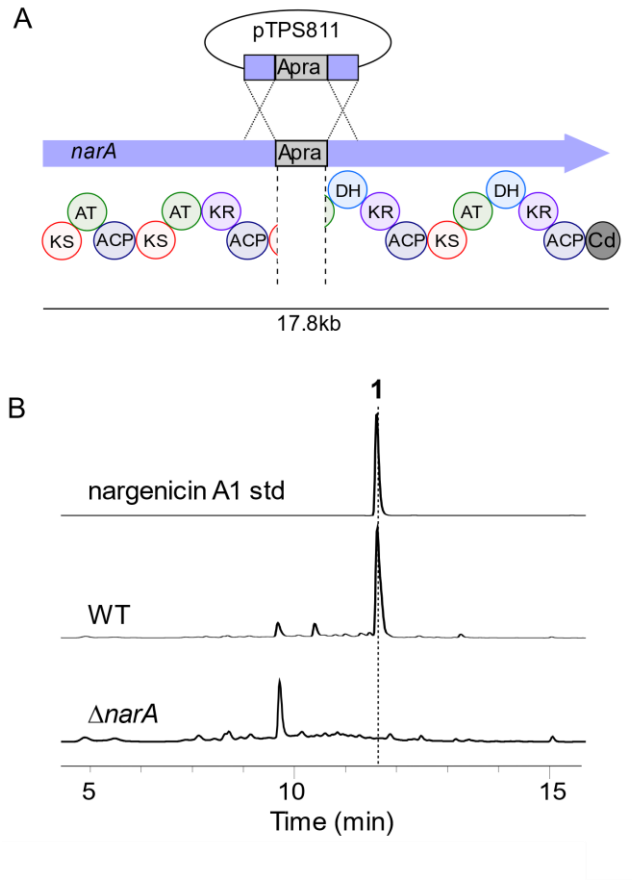

Figure 3. Inactivation of *narA* in *N. arthritidis* AUSMDU00012717. A) Graphic depiction of inactivation of narA with the non-replicative homologous recombination vector pTPS811. B) HPLC trace of ethyl acetate extracts from wild-type and ΔnarA strains, compared with a commercial nargenicin A1 standard. Traces are extracted from absorbance at 265nm.

Close scrutiny of the likely accessory proteins in the *nar* gene cluster revealed a number of putative oxidative and reductive enzymes, including flavin-dependent monoxygenases, short-chain dehydrogenases, P450 hydroxylases and a dioxygenase (Table S3). The majority of these proteins that are predicted to be required for production of the nargenicin core macrolactone are also conserved in the *stm* gene cluster (Figure S2). Nargenicin A1 is hydroxylated at C2 and C18 and both of these oxygen atoms are derived from molecular oxygen, meaning that their incorporation occurs as part of post-PKS processing.[18,20] The most likely candidates for these hydroxylation reactions are the two putative P450 hydroxylases (NarG and NarM), and the C2 position is likely to be further methylated by an encoded O-methyltransferase (NarK). Orthologous genes are present in the *stm* gene cluster and hydroxylation and methylation occur at equivalent positions of nargenicin A1 and streptoseomycin.[10] Interestingly, nargenicin possesses a C8-linked pyrrole moiety (absent in streptoseomycin), which correlates with the presence of predicted pyrrole-related genes (*ngnN2, ngnN3, ngnN4 and ngnN5*)[21] in the *nar* locus. These genes are absent from the *stm* gene cluster. Although a Diels-Alderase has been suggested to be involved in producing the decalin core of nargencin-like molecules via an intramolecular [4+2] cycloaddition[22], limited amino acid sequence homology between known Diels-Alderases makes their identification from sequence comparisons alone difficult.[23–29] However, the *nar* gene cluster does encode a putative SnoaL-like polyketide cyclase (*narD*). Although these enzymes are better known to function in aromatic polyketide biosynthesis, its presence here suggests a possible alternative pathway for the cyclisation of nargenicin.

In *S. aureus*, nargenicin binds to and inhibits DnaE, which is likely involved in synthesis of the lagging DNA strand.[5,30] While many Gram positive bacteria have two copies of *dnaE* (termed *polC* and *dnaE3*)[31], the *N. arthritidis* genome contains three copies, with the third copy being located within the *nar* locus (*narR*). Orthologues or *narR* are also seen in the *nar*-like gene clusters in *N. tenerifensis* and *Streptomyces sp.* NRRL S-350 (Figure S2). This suggests a potential mechanism of self-resistance using a "decoy" copy of *dnaE*, most likely caused by a prior gene duplication event, as reported for β-lactam resistance in other bacteria.[32]

Although our bioinformatic analysis strongly suggested the involvement of the *nar* locus in nargenicin biosynthesis, to confirm this we made a targeted deletion in *narA*, the first PKS encoding gene in the cluster, in the producing *N. arthritidis* strain (Figure 3). Construction of a Δ*narA* mutant in AUSMDU00012717 was performed by homologous recombination using the vector pTPS811 (Table S1), and whole genome sequencing of the Δ*narA* mutant confirmed that only *narA* was inactivated and no further mutations were present. HPLC-MS analysis of extracts from the Δ*narA* mutant (TPS8819, Table S1) showed loss of **1**, consistent with the *nar* gene cluster being responsible for nargenicin biosynthesis (Figure 3).

Having confirmed that the *nar* locus is responsible for nargenicin production, we then investigated the roles of the accessory genes within the *nar* gene cluster. A rare structural feature of nargenicin family antibiotics is a C8-C13-linked ether bridge, however, an enzyme responsible for the formation of this ether bridge has not been described from bacterial systems. The fungal alkaloid loline contains a C2-C7 ether bridge whose formation is catalyzed by LolO, an iron and α-ketoglutarate dependent (Fe-2OG) dioxygenase.[33,34] A putative dioxygenase with sequence homology to the same family is encoded in the *nar* gene cluster (NarN). To determine if NarN was involved in ether bridge formation, we inactivated *narN* by homologous recombination (Figure 4). HPLC analysis of the Δ*narN* strain showed loss of **1**, but resulted in a new peak with increased retention time (Figure 4). Purification and HR-ESIMS analysis of this peak revealed a *m/z* of 502.2800 [M+H] (predicted molecular formula $C_{28}H_{40}O_7N$, calcd as 502.2805),

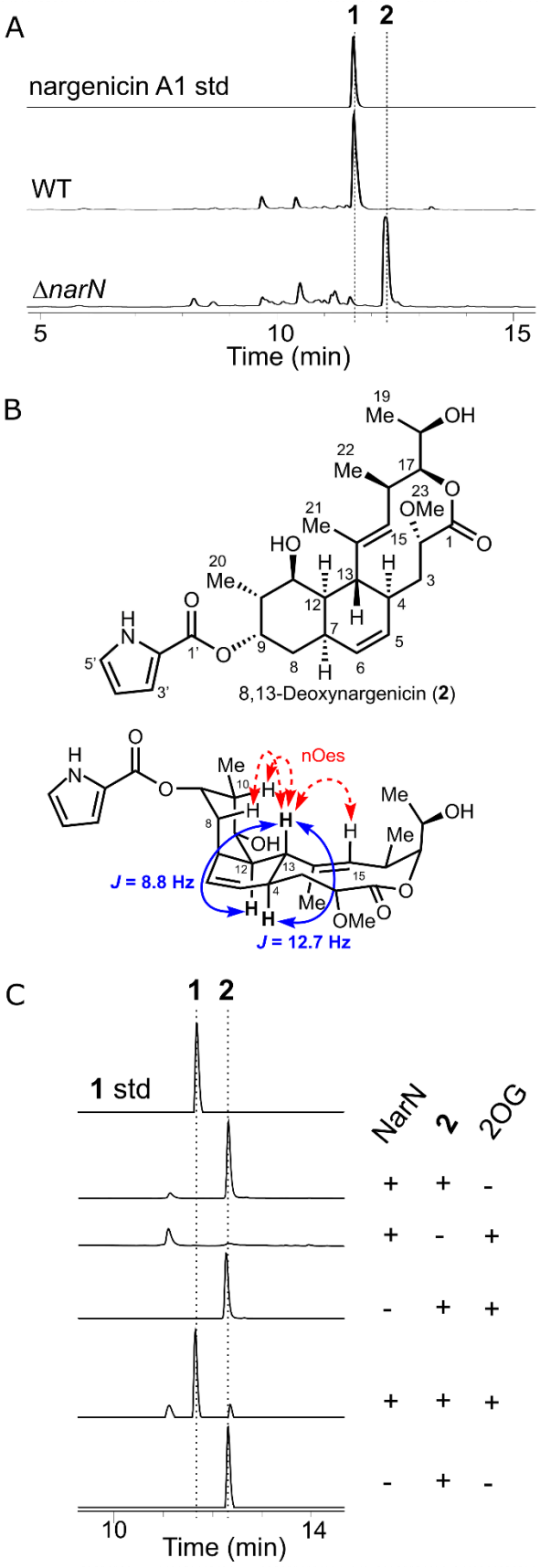

Figure 4. NarN is an oxa-bridge forming dioxygenase. A) HPLC traces at 265 nm showing WT extract versus Δ*narN* extract. B) Structure of 8,13-deoxynargenicin and key correlations supporting the C13 stereochemistry. C) NarN deoxynargenicin conversion assay showing HPLC traces at 265 nm. (+) or (–) indicates presence or absence of each reaction component, respectively. Conversion of **2** to **1** in the presence of Fe and 2OG was confirmed by HR-ESIMS.

corresponding to the loss of one oxygen atom and addition of two hydrogen atoms, consistent with a deoxygenated form of **1**.

Elucidation of the structure of the molecule by both 1D- and 2D-NMR spectroscopy revealed that the Δ*narN* strain produced a new major metabolite, 8,13-deoxynargenicin (**2**) {$[\alpha]_D^{22}$ +31.2 (c 0.42, CH2Cl2)} (Figure 4). The H13 H-H coupling constants 3JH13,H4 = 12.7 Hz and 3JH13,H12 = 8.8 Hz suggested a trans-diaxial relationship between H13 and H4/H12. In addition, NOESY cross peaks observed between H13 and H15, H8ax and H10 also supported the assignment of the C13 stereochemistry as depicted in Figure 4 (see Supporting Information for details). While previous studies have suggested that oxidation at C18 is the last step in nargenicin biosynthesis[15,18], the structure of **2** suggests that all oxidations are complete prior to ether bridge formation, or at least that these reactions are not inhibited by the absence of the C8-C13 oxa-bridge. Investigation of the antibiotic activity of 2 showed removal of the ether bridge abolished antibiotic activity (Table 1).

To confirm that NarN is indeed responsible for the installment of a C8-C13 oxa-bridge into **2**, we cloned *narN* from AUSMDU00012717 and produced purified, recombinant NarN from *E. coli*. In an *in vitro* deoxynargenicin conversion assay the addition of NarN to **2** in the presence of both Fe and 2OG resulted in the almost complete conversion of **2** to **1** (Figure 4C), which was confirmed at 265nm by HPLC-UV and by HR-ESIMS, with an observed ion at *m/z* 516.2591 [M+H] (calculated for $C_{28}H_{38}O_8N$ as 516.2597) corresponding to the nargenicin peak standard. However, in the absence of NarN or 2OG, there was no conversion of **2** to **1** by NarN, suggesting that NarN activity is dependent on 2OG and that NarN does indeed belong to the Fe-2OG dependent dioxygenase family.

Table 1. MIC values (μg/ml) for nargenicin A1 (**1**) and 8,13-deoxynargenicin (**2**).

	MIC (μg/ml)	
Strain[a]	**1**	**2**
Staphylococcus aureus Newman	0.25	>32
S. aureus JKD6008 (MDR)	0.25	>32
Escherichia coli DH10B	>32	>32
Streptococcus mutans	0.5	>32
Mycobacterium smegmatis mc^{2}155	4	>32

[a] See Table S1 for strain information

Further investigation of orthologous dioxygenases revealed a NarN ortholog encoded in the *stm* gene cluster (StmO3), however, amino acid sequence identity is only 53% (Table S3).

Given that NarN and StmO3 form oxa-bridges at different positions on the decalin scaffold in **1** and streptoseomycin (C8-C13 for NarN and C9-C13 for StmO3), these functional differences may be explained by amino acid sequence differences. An amino acid sequence comparison of NarN orthologues and known Fe-2OG utilizing dioxygenases showed that the ether bridge forming enzymes form individual clades within the broader dioxygenase family and that these enzymes are rare among bacteria with only four examples (Figure S4). While sequence similarity is known to be limited among dioxygenases due to the vast array of chemical transformations that they perform,[35–37] it appears that the ether-bridge forming dioxygenases are closely related. Further DNA sequencing of other producers of nargenicin family antibiotics is likely to reveal additional functional variants of this group of enzymes.

In conclusion, we have revealed the complete biosynthetic locus for **1**, a gene cluster that has remained hidden for almost four decades. We have shown that the *nar* locus is solely responsible for the production of **1** and have identified *nar*-like gene clusters from other Actinomycetes, not previously considered to produce nargenicin-like compounds. Although only four currently sequenced genomes from *Nocardia* and *Streptomyces* species contain nargenicin-like gene clusters, nargenicin family antibiotics have been also found from *Pseudonocardia*, *Saccharothrix*, *Actinoplanes*, *Nocardioides* and *Saccharopolyspora* species.[8,11,12,38] While genomes of the latter producing organisms are not yet publicly available, future sequencing of these organisms will allow for a comprehensive comparison of these PKS biosynthetic gene clusters. Here, we have shown for the first time that a dedicated bacterial dioxygenase is essential for ether bridge formation. Comparison of *nar*-like genomic loci has allowed for the identification of essential genes for the production of these macrocyclic, oxa-bridge polyketides. The *nar* biosynthetic locus uncovered here appears to provide a blueprint for the biosynthesis of members of this molecular family, and this work paves the way for heterologous expression and biosynthetic engineering studies on this group of potent, narrow-spectrum antibiotics to derive the next generation of nargenicin-like molecules.

Experimental Section

See Supporting Information.

Acknowledgements

We wish to thank Ching-Seng Ang, Shuai Nai and Nick Williamson at the Bio21 Mass Spectrometry and Proteomics Facility for helpful discussions and assistance with mass spectrometry. This work was supported by an Australian National Health and Medical Research Council grant GNT1105522.

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Keywords: nargenicin • ether bridge • oxygenation • polyketide

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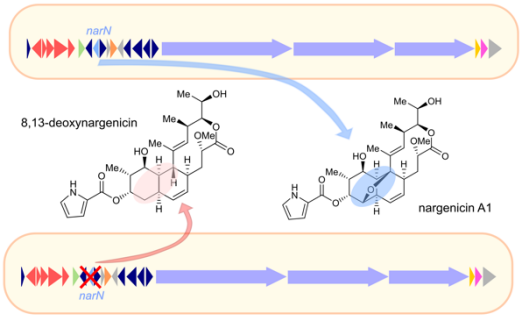
Entry for the Table of Contents

COMMUNICATION

Building (oxa-) bridges: The biosynthetic locus for the potent antibiotic nargenicin was identified from a human pathogenic *Nocardia* strain. Gene inactivation studies identified an unusual dioxygenase responsible for ether bridge formation, and removal of the ether bridge abolished nargenicin antibiotic activity.



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