DOI: 10.1002/cbic.201000085

Biosynthesis of the Myxobacterial Antibiotic Corallopyronin A

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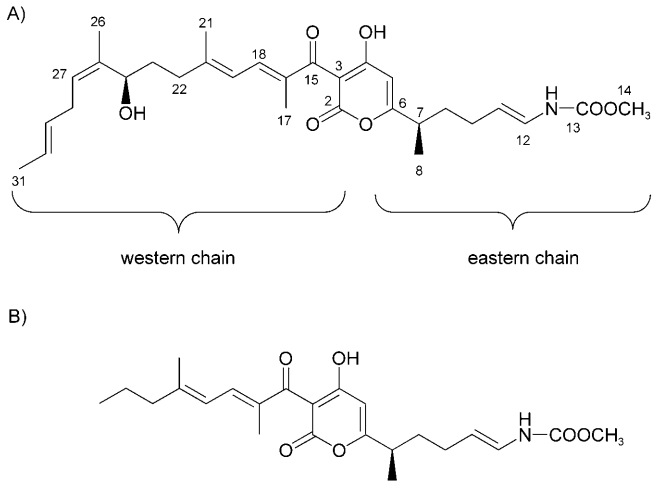
Corallopyronin A is a myxobacterial compound with potent antibacterial activity. Feeding experiments with labelled precursors resulted in the deduction of all biosynthetic building blocks for corallopyronin A and revealed an unusual feature of this metabolite: its biosynthesis from two chains, one solely PKS-derived and the other NRPS/PKS-derived. The starter molecule is believed to be carbonic acid or its monomethyl ester. The putative corallopyronin A biosynthetic gene cluster is a trans-AT-type mixed PKS/NRPS gene cluster, containing a β-branching cassette. Striking features of this gene cluster are a NRPS-like adenylation domain that is part of a PKS-type module and is believed to be responsible for glycine incorporation, as well as split modules with individual domains occurring on different genes. It is suggested that CorB is a trans-acting ketosynthase and it is proposed that it catalyses the Claisen condensation responsible for the interconnection of the two chains. Additionally, the stereochemistry of corallopyronin A was deduced by a combination of a modified Mosher's method and ozonolysis with subsequent chiral GC analyses.

Introduction

As a result of the dramatic increases in resistance towards therapeutically used antibiotics, the evaluation of new chemical entities with antibiotic properties is an urgent medical priority. Natural products and their derivatives have been at the centre of antibiotic research since the discovery of the penicillins, and make up more than two thirds of all antibiotic drugs applied in medical treatments.[1,2] The recent introduction of the acyldepsipeptide daptomycin as a member of a new class of antibiotics clearly demonstrates the medical potential of microbial metabolites.[3]

Corallopyronin A (Scheme 1) is a myxobacterial compound with potent antibacterial activity.[4,5] In a project focussing on the evaluation of new structural types of antibiotics, we obtained corallopyronin A from a strain of the gliding bacterium *Corallococcus coralloides*. Investigation of the antibiotic activity of corallopyronin A towards *Staphylococcus aureus*, including methicillin-resistant strains (MRSA), resulted in MIC values of 0.25 μg mL^{-1}, thus confirming the excellent in vitro activity of this natural product. The effect of corallopyronin A towards eukaryotic cells is marginal and no toxicity in mice was reported up to 100 mg kg^{-1}.[4,5]

The mode of action of corallopyronin A involves the selective inhibition of bacterial DNA-dependent RNA polymerase



Scheme 1. Structures of A) corallopyronin A and B) myxopyronin A.

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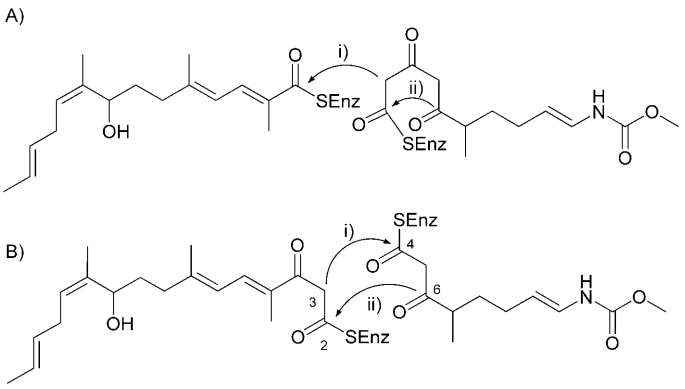
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cbic.201000085: NMR spectra of corallopyronin A; results and NMR data of labelling experiments; information about primers used in PCR studies; alignments of the conserved regions of KS domain, DH domain, KR domain, ACP domain and PCP domain; 16S rDNA determined for Corallococcus coralloides BO35. Results and NMR data of modified Mosher's method. GC-MS runs after ozonolysis.

(RNAP);[4,5] most importantly, activity towards rifampin-resistant mutants of *S. aureus* is retained.[6] X-ray analysis of bacterial RNAP in complexation with myxopyronin A or desmethyl-myxopyronin (dMyx), both structurally closely related to corallopyronin A (Scheme 1), and biochemical data showed that these compounds interact with the target enzyme in an otherwise unprecedented way, different from that of known RNAP inhibitors such as the rifamycins.[7,8] Mukhopadhyay et al.[7] showed in 2008 that myxopyronin A interacts with the RNAP "switch region", the hinge that mediates opening and closing of the RNAP active centre cleft, thus preventing interaction of RNAP with promoter DNA. In 2009 Belogurov et al.[8] solved the structure of dMyx in complexation with a *Thermus thermophilus* RNAP holoenzyme and found that the antibiotic binds to a pocket deep inside the RNAP clamp head domain, which interacts with the DNA template in the transcription bubble.

Myxobacteria are a prolific source of structurally most unusual natural products, many of which have been found to possess prominent pharmacological activity.[9] Several of these compounds have antibacterial activity: sorangicin A, ripostatin B and etnangien, for example, have been at the centre of recent investigations.[10–12] Antifungal activity was found, for example, for the isochromanone derivatives ajudazole A and B[13] and the cyclic hexapeptides pedein A and B.[14] Most of the products are synthesized by multienzyme complexes composed of polyketide synthases (PKSs), non-ribosomal peptide synthetases (NRPSs) and hybrids of these. The ability to produce a wide range of compounds with high structural diversity is further increased by the presence of enzymes introducing β-branching and post-PKS and -NRPS reactions such as hydroxylation, methylation, isomerization of double bonds and halogenation. For the synthesis of the aromatic electron transport inhibitor stigmatellin, for example, the myxobacterium *Stigmatella aurantiaca* uses PKS-embedded methyltransferases (MTs), as well as a "standalone" *O*-MT and a cytochrome P450 monooxygenase for post-PKS reactions.[15]

The goal of the current investigation was to decipher the biosynthesis of corallopyronin A both at the biochemical and at the genetic level. On the basis of its chemical structure it was proposed that this antibiotic was the product of a mixed PKS and NRPS biosynthetic machinery. Feeding experiments with labelled precursors allowed us to deduce all building blocks for corallopyronin A, and revealed its biosynthesis from two chains, one solely PKS-derived and the other NRPS/PKS-derived, as a striking feature of this metabolite (Scheme 2 and Figure 1, below). There are only a few reported examples of molecules derived from two PKS chains; examples include dialkylresorcinol,[16] the 4-hydroxy-2-alkylquinoline (HAQ) derivative 3,4-hydroxy-2-heptylquinoline (PQS)[17] and bacterial stilbenes.[18] It is suggested that a trans-acting ketosynthase (KS)—CorB—catalyses the Claisen condensation responsible for the connection of the two chains (Figure 1, below). The proposed starter molecule for corallopyronin A seems to be carbonic acid or its methyl ester (C_1 building block). This appears to be extremely rare in the biosynthesis of secondary metabolites; to the best of our knowledge no such starter unit has yet been found for any other natural product. Some unusual arrangements at the genetic level were found, including a NRPS-like adenylation (A) domain in *corI* that is part of a PKS-type module, as well as so-called split modules with individual domains occurring on different proteins.



Scheme 2. A) and B) Proposed mechanisms for the formation of corallopyronin A from two polyketide chains through i) a Claisen-type reaction, and ii) lactonization.

Results

Analysis of the stereochemistry of corallopyronin A

The stereochemistry of corallopyronin A was established by a combination of a modification of Mosher's method and ozonolysis with subsequent chiral GC analyses. By the modification of Mosher's method the absolute configuration of the secondary alcohol group at the chiral centre C-24 was assigned as *R* (Figures S10–12 in the Supporting Information). To determine the absolute configuration at C-7, corallopyronin A was treated with ozone and H_2O_2, resulting in the formation of 2-methylglutaric acid, which after derivatization was shown by chiral GC analysis to be *R*-configured (Figure S13 in the Supporting Information).

Biosynthetic studies with labelled precursors

The proposed building blocks of corallopyronin A are predominantly malonyl-CoA units (derived from acetate in feeding experiments) for the formation of the basic polyketide skeleton. Malonyl-CoA is also regarded as the precursor for some of the methyl branches, whereas other methyl groups may be derived from *S*-adenosyl-L-methionine (SAM). The amino acid glycine is believed to be the source of the carbamoyl nitrogen atom. Incubation experiments with ^{13}C- and ^{15}N-labelled precursors allowed us to deduce the incorporation of the corresponding building blocks into corallopyronin A. As a prerequisite for detection of the isotope label, the assignment of ^{1}H and ^{13}C NMR data was necessary (Table 1, Figures S1 and S2 in the Supporting Information) and some corrections relating to the original publications were made [that is, the assignment of the ^{13}C NMR resonances for carbons C-29 ($\delta = 130.9$ ppm) and C-30 ($\delta = 126.1$ ppm) was reversed[4,5]].

Table 1. NMR spectroscopic data for corallopyronin A in [D_4]MeOH.

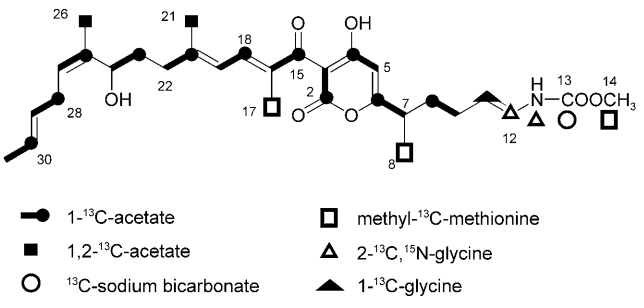
No.	δ_C	multiplicity	δ_H (J [Hz])	No.	δ_C	multiplicity	δ_H (J [Hz])
2	168.1	qC		17	11.7	CH_3	1.97, s
3	102.7	qC		18	137.7	CH	7.37, brd (11.7)
4	181.3	qC		19	122.8	CH	6.32, d (11.7)
5	107.8	CH	5.77, s	20	148.8	qC	
6	168.4	qC		21	17.3	CH_3	1.85, s
7	38.7	CH	2.55, m	22	37.9	CH_2	2.22, m
8	18.7	CH_3	1.25, d (7.0)	23	34.3	CH_2	a: 1.65, m; b: 1.80, m
9	36.0	CH_2	a: 1.58, m; b: 1.79, m	24	69.5	CH	4.55, t (7.0)
10	28.5	CH_2	2.04, m	25	138.5	qC	
11	111.0	CH	5.11, dt (14.3, 7.0)	26	17.7	CH_3	1.74, d (1.1)
12	125.7	CH	6.43, d (14.3)	27	126.4	CH	5.28, t (7.7)
13	156.8	qC		28	31.5	CH_2	2.75, m
14	52.7	CH_3	3.69, s	29	130.9	CH	5.44, m
15	201.3	qC		30	126.1	CH	5.44, m
16	136.5	qC		31	18.1	CH_3	1.66, d (4.8)

In a first experiment, addition of 1-[^{13}C]acetate to the culture medium led to enhancements of the ^{13}C NMR signals of C-2, C-4, C-6, C-9, C-15, C-18, C-20, C-23, C-25, C-28 and C-30 (Figure S3 in the Supporting Information, incorporation rates Table S1 in the Supporting Information). Feeding experiments with 1,2-[^{13}C]acetate (Figure S4 in the Supporting Information) showed incorporation of the label in the polyketide chain as expected from the first experiment and thus indicated the presence of 11 intact acetate building blocks. From these results it became clear, however, that the acetate units do not arrange in the form of a continuous PKS/NRPS chain that could then cyclize to give the pyrone ring. Rather, the labelling pattern suggests the formation of two chains: either an octaketide (C-2 through C-15 to C-31) and a nitrogen-containing triketide (C-4 to C-10), or a heptaketide (C-15 to C-31) and a nitrogen-containing tetraketide (C-2 to C-10), which would be connected through a Claisen-type reaction and lactonization. In this way a branched and cyclized PKS/NRPS chain could be formed (Scheme 2).

The origin of the methyl groups was deduced from feeding experiments employing 1,2-[^{13}C]acetate and SAM. Feeding with 1,2-[^{13}C]acetate resulted in enhanced ^{13}C NMR signals for C-26 ($\delta = 17.7$ ppm) and C-21 ($\delta = 17.3$ ppm), which appeared as singlet resonances, giving confirmation that these methyl groups are derived from C-2 of acetate (Figure S4 in the Supporting Information). From these results it is possible to propose a β-branching reaction during biosynthesis, which would incorporate the corresponding methyl branches into the growing polyketide chain. This suggestion is also supported by the position of the methyl branches at the C-1 carbon of the former acetate building block.[19,20] The corresponding ^{13}C NMR spectrum did not contain enhanced signals for C-17 and C-8, suggesting that these methyl groups do not derive from acetate. Instead, feeding with [^{13}C]methyl-L-methionine gave clear labelling of the methyl group carbons C-8 ($\delta = 18.7$ ppm) and C-17 ($\delta = 11.7$ ppm), which showed that these methyl branches are incorporated by MTs (Figure S6 in the Supporting Information). This suggestion is also supported by the position of the corresponding methyl branches at C-2 of the former acetate building blocks. Incubation with 2-[^{13}C][^{15}N]glycine resulted in a strongly enhanced ^{13}C NMR signal for C-12, providing evidence that C-12 ($\delta = 125.7$ ppm) is derived from C-2 of glycine. Feeding with 1-[^{13}C]glycine gave a clear labelling of C-11 ($\delta = 111.0$ ppm; Figure S5A and B in the Supporting Information). These results indicate that C-11, C-12 and the nitrogen originate from glycine. The enhanced ^{13}C NMR signal for C-11 observed on feeding of the bacterial culture with 1-^{13}C-labelled acetate (Figure S3 in the Supporting Information) may be explained by the action of primary metabolism, with use of acetate for the formation of phosphoglycerate, which is subsequently transformed into serine and then into glycine. Consequently, doubly labelled acetate gave rise to enhanced ^{13}C NMR signals for C-11 and C-12.

At this point the origins of all carbons and the nitrogen atom have been explained, except for the metabolic origin of the carbamoyl carbon C-13 and the methyl ester carbon C-14. After feeding with 1,2-[^{13}C]acetate, C-13 ($\delta = 156.8$ ppm) is weakly labelled (Figure S4 in the Supporting Information) and so is probably indirectly derived from a cleaved acetate unit. Because feeding with 1-^{13}C-labelled acetate also produced an enhancement in the ^{13}C NMR signal of C-13, acetate could be involved in the formation of the ultimate C_1-building block, in which C-1 and C-2 of acetate would be randomized. Finally, labelling experiments with [^{13}C]bicarbonate produced a ^{13}C NMR spectrum with the ^{13}C NMR signal of C-13 greatly enhanced (Figure S7 in the Supporting Information). It thus appears that carbonic acid, probably derived from acetate, could be the origin of C-13 in corallopyronin A. After feeding with [^{13}C]-methyl-methionine, the methyl ester carbon C-14 is strongly labelled, making it plausible that this methyl group is incorporated through the action of an *O*-MT (Figure S6 in the Supporting Information).

Scheme 3 illustrates the building blocks for corallopyronin A as deduced from incorporation studies. The biochemistry of the PKS and NRPS pathways explains most of the functionality within this molecule, such as the keto functions at C-15, C-2 and C-4. Because of its position, however, it is suspected that



Scheme 3. Building blocks for corallopyronin A as deduced from incorporation studies with labelled precursors.

the hydroxy group at C-24 is incorporated in a post-PKS reaction. Likewise, the double bonds $\Delta^{25,27}$ and $\Delta^{11,12}$ appear to have changed position relative to their locations in regular PKS/NRPS systems, although exceptions to this have recently been described.[21–24]

Analysis of the corallopyronin A biosynthetic gene cluster

Feeding experiments showed that the core structure of corallopyronin A is the product of a mixed PKS/NRPS biosynthetic machinery. Investigations with ^{13}C-labelled SAM, as well as the positions of the corresponding methyl functionalities (i.e., α- or β-branching or $-OCH_3$) in the molecule, clearly showed that methyl and methoxy groups are incorporated by two mechanisms: from SAM dependent by MTs and by the successive action of a β-branching cassette. From alignments made with genes of the biosynthetic gene clusters of myxovirescin,[25] chivosazol,[26] pederin[27] and leinamycin,[28] conserved motives for hydroxymethylglutaryl-CoA synthase-like (HMG-CoA synthase-like, HCS-like) enzymes, ketosynthase (KS) and A domains were deduced. From these studies degenerate primers for conserved regions of the corresponding domains were constructed and used for PCRs with genomic DNA of *C. coralloides* B035. PCR with KS primers resulted in the amplification of part of a KS domain of a type I PKS, which bioinformatic analysis suggested belonged to the trans-acyltransferase (AT) type.[27,29] Amplification with NRPS primers yielded gene fragments of A domains specific for glycine, as predicted from in silico analysis of substrate specificity[30,31] (program: NRPSpredictor[32]). PCR also gave sequences for a HCS-like gene. The fact that HCS genes often occur together with trans-AT PKS gene clusters[27,28,33,34] gave rise to the assumption that the corallopyronin A gene cluster might also contain both of these features.

A genomic library containing 2.304 cosmids (average size of insert DNA is 40 kb), representing eight- to tenfold coverage of the suspected genome of the corallopyronin A producer strain (estimated genome size of 10 Mbp), was constructed. To obtain homologous and heterologous probes for screening of the genomic library of *C. coralloides* B035, PCRs with degenerate primers and genomic DNA of *C. coralloides, Myxococcus xanthus* DK1622 and *Chondromyces crocatus* Cmc5 were performed. In a first set of experiments the library was probed by Southern hybridization with homo- and heterologous NRPS- and PKS-type probes for the presence of sequences for A and KS domains. A total of 23 cosmids hybridized with both probe types. Restriction and sequencing analysis revealed that the cosmids belonged to at least two distinct gene clusters. To distinguish these cosmids further, the focus of the screening effort was subsequently placed on sequences coding for enzymes responsible for structural characteristics of corallopyronin A that were regarded as specific for this compound: namely an A domain for glycine activation, a HCS, and KS domains of trans-AT PKSs.

PCR analysis with subsequent sequencing of the 23 cosmids with primers for A domains yielded five cosmids (FJ7, CD6, DE22, EB5, FH7) bearing A domains predicted to activate glycine. These cosmids were further screened by Southern hybridization with homologous HCS and KS probes derived from the previously obtained amplicons, yielding one cosmid (FJ7) that hybridized with both probes. All these features were in good agreement with the corallopyronin A structure, so it was predicted that cosmid FJ7 specified part of the corresponding biosynthetic gene cluster. Cosmid FJ7 was subsequently end-sequenced to obtain insights into the outer parts of the cloned fragments, and yielded sequences for a trans-AT gene and a KS domain. To obtain more sequence information, fragments of cosmid FJ7 were subcloned. Overall 20 subclones were sequenced, resulting in sequences that showed high similarity to genes coding for trans-ATs, HCS and KS domains, which confirmed our results from PCR and hybridization experiments. Further cosmids that covered the remaining part of the gene cluster were isolated by chromosome walking with the aid of PCR primers derived from insert ends. Upon sequencing, cosmid FJ7 was found to contain 39 kbp of information putatively related to the biosynthesis of corallopyronin A: namely genes for a trans-AT/ER (*corA*), a free-standing KS (*corB*), a β-branching cassette (*corC-G*) and an *O*-MT (*corH*), as well as part of the putative PKS/NRPS genes *corI* and *corJ*. From the sequence information of two further cosmids (AM24 and BA5), two more putative PKS genes (*corK* and *corL*) could be identified, as well as genes encoding thioesterase (TE) and tailoring enzymes (*corM–O*). All three cosmids together contained 65 kbp of sequence encoding the putative biosynthetic gene cluster of corallopyronin A.

The putative corallopyronin A gene cluster consists of 16 open reading frames (ORFs), all transcribed in the same direction. It shows an overall GC content of 72.8%, which fits well with other myxobacterial genes. Functions for the corresponding proteins were predicted by use of the program CLUSEAN (Table 2 and Figure 1).[35] From these 16 ORFs, 15 could be attributed directly to specific biosynthetic steps. Only for the putative product of *orf1* no function could be predicted, the closest homologue being a hypothetical protein of *Sorangium cellulosum* (Table 2).

In silico analysis of the sequence resulted in the identification of only one AT, encoded by *corA*. BlastX alignments showed significant identities—in the 50% range—to ATs of mupirocin,[36] disorazole,[37] bacillaene[38] and difficidin.[33] Conserved regions include the active site motif GHSXG,[39] which in the case of CorA is GHSLG (Figure 2). Approximately 100 residues downstream of the active site serine, the YASH[40,41] or HAFH[39] motifs are usually regarded as indicative of methyl malonate or malonate activation, respectively. In the case of CorA, an AAFH sequence can be found, including the most significant phenylalanine residue (Figure 2), thus suggesting that only malonyl-CoA is used as an extender unit. Whereas the N-terminal part of CorA has a high similarity to trans-ATs, the C-terminal part has a high similarity to reducing enzymes (52/71% identity/similarity to MmpIII from *Burkholderia thailandensis*, a 2-nitropropane dioxygenase (NPD)-like domain, associated with the ACP S-malonyltransferase FabD). It is thus suggested that CorA has a trans-ER function. CorB is a KS of the β-ketoacyl-acyl carrier protein synthase (KAS) III-type, defined by its catalytic cysteine-histidine-asparagine (CHN) triad.[42] In a Clus-

Table 2. Proteins present in the putative corallopyronin A biosynthetic gene cluster and their proposed functions.

Protein	Length (aa)	Putative function	Highest identity/similarity [%] to:	Accession no.
CorA	763	acyltransferase and enoyl reductase	53/73 *Bacillus amyloliquefaciens* FZB42 (DfnA)	YP_001421800.1
CorB	335	β-ketoacyl-ACP-synthase I	47/68 *Pelodictyon phaeoclathratiforme* BU-1	YP_002017776.1
CorC	83	acyl carrier protein	40/59 *Clostridium papyrosolvens* DSM 2782	ZP_05496376
CorD	423	ketosynthase	45/66 *Kordia algicida* OT-1	ZP_02163869.1
CorE	410	HMG-CoA synthase	55/70 *Burkholderia thailandensis* E264	YP_439864.1
CorF	261	enoyl-CoA hydratase/isomerase	41/59 *Cyanothece* sp. PCC 7425	YP_002483587.1
CorG	254	enoyl-CoA hydratase/isomerase	48/62 *Kordia algicida* OT-1	ZP_02163869.1
CorH	286	methyltransferase	37/49 *Mycobacterium abscessus*	YP_001705054.1
CorI	3883	hybrid PKS/NRPS	42/54 *Streptomyces hygroscopicus* ATCC 53653	ZP_05519322.1
CorJ	3817	trans-AT type PKS	35/54 *Bacillus thuringiensis*	ZP_04093843.1
CorK	4068	trans-AT type PKS	40/53 *Sorangium cellulosum* (DszA)	AAY32964.1
CorL	4981	putative type I PKS	36/48 *Streptomyces griseus* subsp. *griseus* NBRC	YP_001828297.1
Orf1	573	hypothetical protein	53/72 *Sorangium cellulosum*	YP_001615316.1
CorM	240	thioesterase	49/64 *Nostoc punctiforme* PCC 73102	YP_001864469.1
CorN	724	3-hydroxyacyl CoA-dehydrogenase/enoyl CoA hydratase	44/58 *Sorangium cellulosum*	YP_001618204.1
CorO	449	cytochrome P450	44/63 *Haliangium ochraceum* DSM 14365	YP_003269864.1

talW alignment with the other KS domains of the cluster it shows the highest homology to CorD (Figure S9 in the Supporting Information).

The successive action of several enzymes encoded in a so-called "β-branching cassette" is known to incorporate methyl groups into a growing PKS chain. In this process an ACP is loaded with malonyl-CoA, which is decarboxylated by the action of a KS_S (that is, a KS with a specific cysteine-to-serine exchange in the catalytic triad). The resulting ACP-bound acetate is used as a substrate by the HCS, together with the similarly ACP-bound β-keto ester on the PKS assembly line. This results in an ACP-bound intermediate on the PKS assembly line, which after dehydration and decarboxylation yields a β-branch. The described proteins—namely ACP, KS_S, HCS, a decarboxylase and an isomerase, the last two annotated as enoyl-CoA hydratase/isomerase (ECH) or crotonase (CR)—can either be encoded by five freestanding genes or may be located within larger, multidomain-encoding genes.[19,20,43]

The *corC–G* region exactly reflects the former case: that is, a freestanding arrangement. BlastX analysis revealed an identity of approximately 50% of CorE to many proteins with HCS activity. ClustalW alignments of CorE with the proteins JamH, PedP, TaC and MupH showed that CorE shares the conserved catalytic triad (Figure 2).

CorH shows 37% identity to an *O*-MT from *Mycobacterium ulcerans*. BlastX alignments also revealed CorH to have 27% identity to MelK from the melithiazol gene cluster of the myxobacterium *Melittangium lichenicola*,[44] which was biochemically characterized as an *O*-MT.[45] It is thus assumed that *corH* encodes an *O*-MT. Further MT domains were identified in modules 5 and 6′, which carry the conserved motif EXGXG for C-MT (EVGAG for both) and show an overall identity of 36% to each other.

CorI contains regions identified as A domains, and BlastX analysis showed approximately 40% identity at the protein level to A domains of BaeJ, Ta1, OnnI and NosC. With the aid of the program NRPSpredictor[32] it was proposed that the A domain of CorI activates glycine, as is evident from the ten amino acid residues—DILQLGLIWK—within the substrate binding pocket, which are known to convey substrate specificity.[30,46]

Altogether, the putative corallopyronin A gene cluster encodes 15 KSs (including CorB and the one from the β-branching cassette), most of which are highly similar to KSs in type I PKS modules (Figure S9 in the Supporting Information). All KS domains encoded by the NRPS/PKS genes *corI–L* are characterized by the highly conserved CHH catalytic triad,[47] except for KS_0 in module 5, which has a mutation to CAH in the catalytic triad, therefore supposedly being inactive.[48] Ketoreductase (KR) domains are characterized by the consensus Rossmann fold motif GxGxxG(A)xxxA, which is required for NADP(H) binding.[40] Almost all KRs of the putative corallopyronin A gene cluster exhibit this typical NADPH-binding motif. An exception to this, however, is the KR at the N-terminal end of CorI, which does not have this motif and so is assumed to be inactive. ClustalW alignments also clearly identified dehydratase (DH) domains in the PKS, through their conserved LxxHxxxGxxxxP active site motif, in which H and G form the catalytic diad required for the enzymatic activity.[49] The sequences of all acyl-carrier protein (ACP) domains were checked for the presence of the conserved serine residue as the putative 4′-phosphopantetheine binding site.[50] The essential serine was found in all ACP domains, except for the doubled ACPs in modules 6, 3′ and 7′, in which in each case only one of the two carries the conserved serine residue (Figure S9 in the Supporting Information). It can therefore be assumed that these ACP-like domains are inactive.

In CorI the A domain stands alone amidst PKS domains: that is, upstream a KS is found instead of the canonical condensation domain, whereas the downstream carrier protein exhibits more similarity to ACPs than to PCPs. To evaluate the functionality of the A domain it was expressed as a recombinant protein in *E. coli* with a length of 583 amino acids, including a His_6-tag. The fusion protein was successfully obtained from plasmid pET28-Cc-Ad. Purification by Ni-NTA affinity chromatography yielded purified protein, as determined by SDS-page gel electrophoresis (data not shown). By the γ-$^{18}O_4$-ATP pyrophosphate exchange assay[51] the activity of the A domain to-

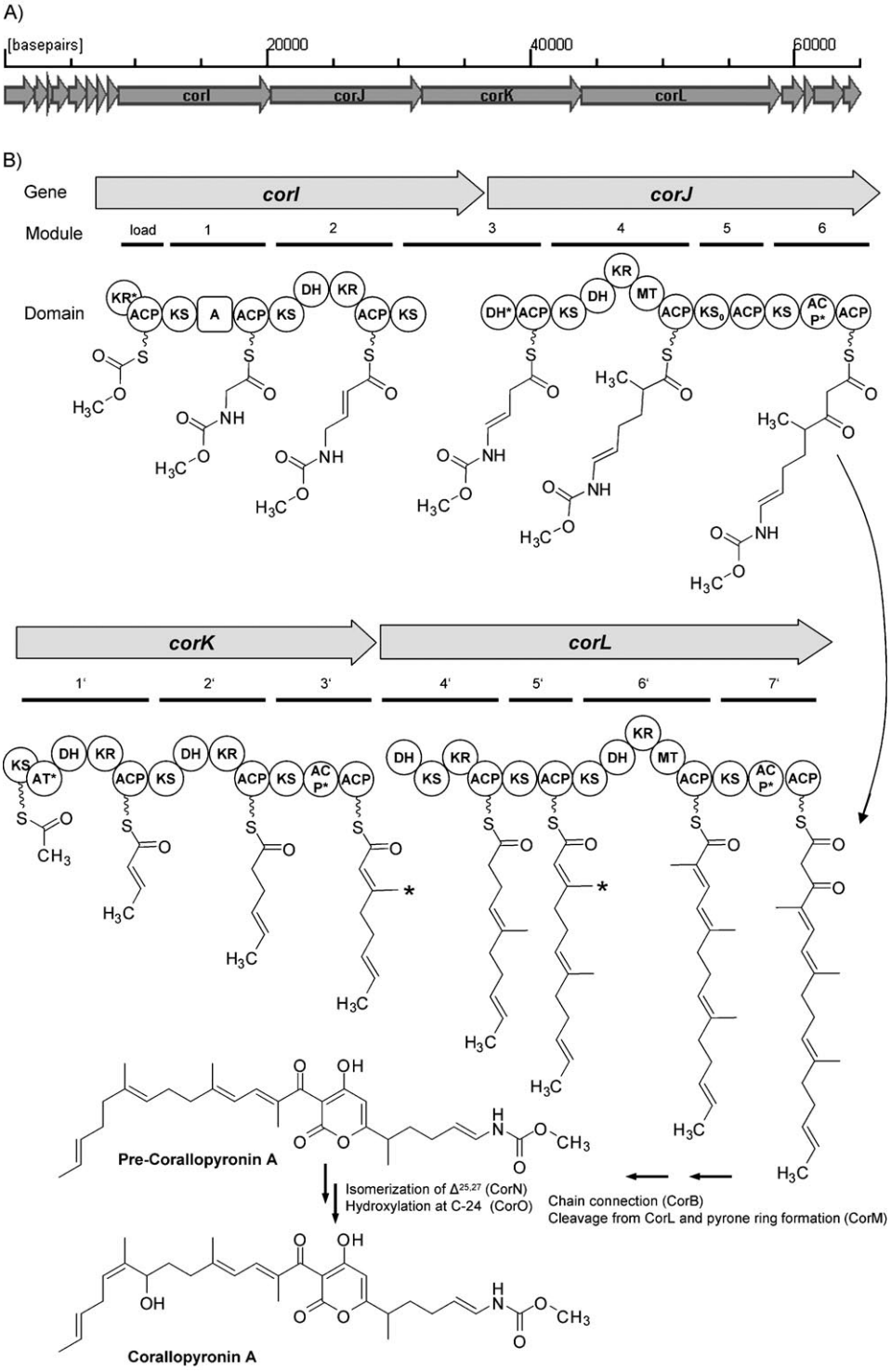


Figure 1. A) Complete putative corallopyronin A gene cluster. B) Hypothetical corallopyronin A biosynthesis pathway. A: adenylation domain; ACP: acyl carrier protein; DH: dehydratase domain; KR: ketoreductase domain; KS: ketosynthase domain; KS_0: inactive KS; MT: methyltransferase domain. The AT*, ACP* and KR* domains are degraded. DH* marks a putative "shift" domain. The black arrow indicates that the chains from modules 6 and 7′ have to be connected to form the pyrone ring. The asterisk (*) marks the carbons introduced by the β-branching cassette.

wards various amino acids was determined. Activity was found towards glycine and threonine (Figure 3), confirming the functionality of the *corI*-encoded A domain. With the other amino acids tested, no significant ATP/PP_i exchange was detectable.

Discussion

The putative corallopyronin A gene cluster is a trans-AT-type mixed PKS/NRPS biosynthetic gene cluster containing a β-branching cassette. All PKS modules of the biosynthetic gene cluster lack ATs. The only exception was the first KS domain of CorK with a degraded AT domain, the closest homologue of which is the first KS of the disorazole gene cluster.[37,52] Only one trans-AT gene, *corA,* is therefore located in the putative corallopyronin A cluster (Figure 1). It can be proposed that *corA* is the first gene within the putative cluster because all genes presumably required for the biosynthesis of corallopyronin A are located downstream of *corA*. Sequence information revealed an ORF in the opposite direction upstream of *corA* (after a gap of $>$300 bp); this ORF showed similarity to hypothetical proteins.

A noteworthy fact is the occurrence of a further standalone (and thus also "trans-acting") protein with similarity to KSs (CorB) encoded directly downstream of the trans-AT/ER. The proteins encoded by the following genes—*corCDEFG* (coding for an ACP, a KS, a HCS, a decarboxylase and an isomerase, respectively)—seem to form a functional unit. Previous functional studies with the corresponding proteins of the curacin A[19] and bacillaene[20] pathways revealed that these enzymes catalyse an aldol addition of a malonyl-CoA-derived acetyl group onto a β-keto function present in the growing polyketide chain, followed by dehydration and decarboxylation. It is thus proposed that the proteins encoded by the *corCDEFG* genes in the putative corallopyronin A cluster form a β-branching cassette. Accordingly, in the proposed corallopyronin A biosynthesis the β-keto synthase CorD, with the cysteine-to-serine active-site substitution (KS_S), would catalyse decarboxylation of an ACP (CorC)-bound malonyl resi-

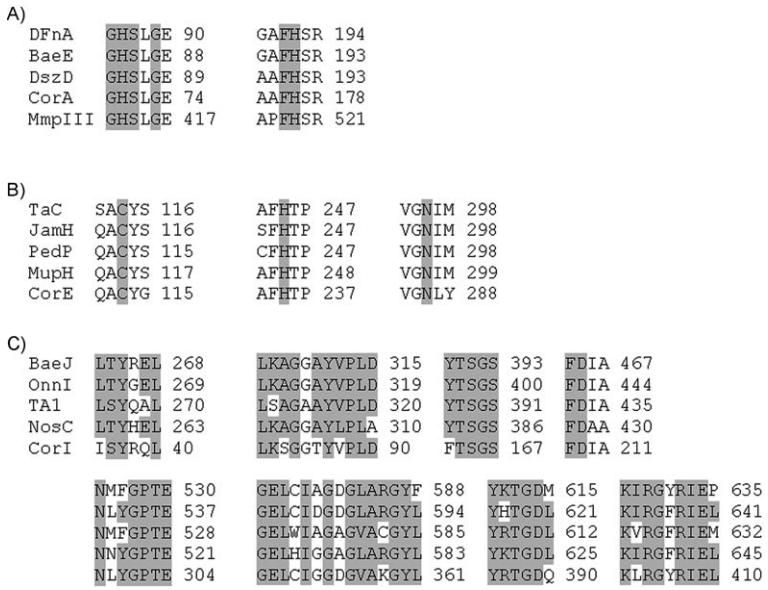


Figure 2. Alignment of the conserved regions of A) trans-ATs, B) HMG-CoA synthases, and C) A domains. The numbers indicate amino acid positions within sequences. Accession numbers of AT homologues: MmpIII (AAM12912), DszD (AAY32968), BaeA (YP_001421287) and DfnA (YP_001421800). Accession numbers of HMG-CoA-synthase homologues: JamH (AAS98779), PedP (AAW33975), TaC (ABF90176) and MupH (AAM12922). Accession numbers of A domain homologues: BaeJ (YP_001421292), Ta1 (CAB38084), OnnI (AAV97877) and NosC (AAF17280). Conserved residues are highlighted in grey.

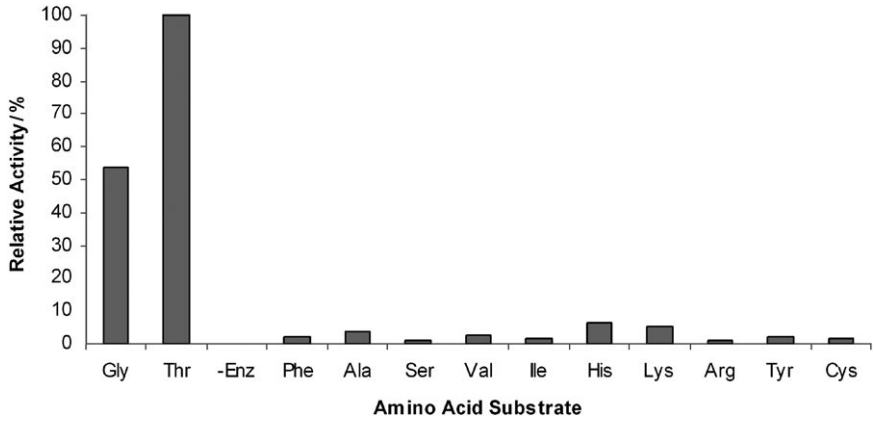


Figure 3. Substrate-dependent ATP/PP_i exchange activity for the A domain.

due, and the HCS CorE would add the resulting acetate to C-20 and C-25, respectively. It is proposed that CorF catalyses dehydration and CorG decarboxylation, eventually resulting in the presence of methyl groups CH_3-21 and CH_3-26. Such a set of genes (*corC–G*), encoding proteins with the described functions, has previously been found in gene clusters related to β-branched secondary metabolites such as bacillaene, curacin A, jamaicamide, mupirocin, myxovirescin, pederin and etnangien.[12,19,25,33,36,43,53]

The occurrence of several single modules on separate proteins (split modules, first described in the myxalamid gene cluster [54]) accounts for the complexity of the corallopyronin A gene cluster. This situation is encountered in module 3, where the KS is the last domain on CorI, whereas the DH is present on CorJ. Although extremely rare in "conventional" (*cis*-AT) PKSs, split modules are ubiquitous in trans-AT systems. To date, nothing is known about the biosynthetic relevance of such architectures. In the interface between CorK and CorL the arrangement of the domains is highly unusual. Module 4′ (on *corL*) has the assembly DH-KS-KR-ACP. Together with the tandem-ACP located as the last domains on CorJ, both unusual modules are encoded up- and downstream of *corK*. It can be speculated that this may be a reflection of the evolutionary development of the cluster. Comparison of the chemical structures of corallopyronin A and myxopyronin A (Scheme 1) shows that the western chain of corallopyronin A is three C_2-building blocks longer than that of myxopyronin A. Accordingly, the *corK* gene in the corallopyronin A gene cluster encodes three KS domains. It may be hypothesized that the corallopyronin A biosynthetic gene cluster has evolved by the integration of *corK* in the myxopyronin A biosynthetic gene cluster, and the unusual arrangement of module 4′ might be a relic of this integration event.

Biosynthesis of the eastern chain

The eastern, nitrogen-containing chain of corallopyronin A bears a most unusual α,β-unsaturated methyl carbamate functionality. NMR measurements after feeding experiments with labelled precursors clearly show that both C-12 and C-11 arise from the amino acid glycine, as does the nitrogen atom of the carbamate moiety. It is suggested that the methionine-derived CH_3-14 is incorporated into the molecule by the *O*-MT CorH. Feeding studies with precursor molecules further revealed that the resonance signal for C-13 is enhanced in NMR spectra after feeding of labelled sodium bicarbonate, but also, albeit more weakly, after feeding with glycine and acetate. This result can be explained by taking into account that in primary metabolism glycine can be degraded to acetate and CO_2 via pyruvate, resulting in C_1 units. Because glycine and acetate are linked metabolically in that acetate is used for the formation of phosphoglycerate, which is then transformed into serine and subsequently into glycine, the incorporation of acetate-derived carbons in the C-11 and C-12 positions of corallopyronin A may also be explained. For C-13 the incorporation of a C_1-building block originating from C_1-metabolism is proposed. Recent in vivo biochemical investigations into the origin of the ureido linkage of the NRPS-product

syringolin A revealed integration of either bicarbonate or carbon dioxide,[55] which was verified by in vitro characterization of SylC, showing that the incorporation of the C1-unit is mediated by this enzyme.[56]

The incorporation of sodium bicarbonate may start with a reaction similar to the first step in malonyl-CoA or carbamoyl phosphate biosynthesis in fatty acid and pyrimidine biosynthesis or the urea cycle, respectively. In a first reaction step the responsible enzymes—acetyl-CoA carboxylase or carbamoyl phosphate synthetase, respectively—catalyse the transfer of a phosphate group from ATP to HCO_3^- to give carbonyl phosphate. A similar reaction has been described for purine biosynthesis in *E. coli*, in which PurK transforms HCO_3^- into carbonyl phosphate, which is required for the carboxylation of aminoimidazole ribonucleotide by PurE.[57] The presence of a homologous gene to the carbamoyl phosphate synthetase in *C. coralloides* strain B035 was shown by amplification of a fragment from its genomic DNA by degenerate PCR and subsequent sequencing (data not shown). In addition, a *purK* gene was identified in the myxobacterium *M. xanthus*, the genome sequence of which is available (GenBank ID: ABF86046.1), which thus makes it likely that *C. coralloides* also has this enzyme.

Carbonyl phosphate may act as starter unit for corallopyronin A biosynthesis. For the transfer to the first ACP encoded in *corI* a transferase-like enzyme can be assumed, though no such enzyme is encoded within the biosynthetic gene cluster. It may be a case of self-loading, as already described for ACPs.[58] Because this intermediate might be rather unstable, especially after its reaction with the glycine moiety, it is suggested that the *O*-MT CorH might attach the methyl group CH_3-14 to the carbonic acid immediately, or alternatively to the carbamate moiety after Claisen condensation of the C_1 building block with glycine. An analogous reaction to the proposed Claisen condensation of carbonic acid or its methyl ester and glycine—that is, nucleophilic attack of a nitrogen atom at the activated carbonate carbon—takes place in carbamoyl phosphate synthesis. In corallopyronin A biosynthesis this condensation is thought to be catalysed by the first KS encoded on *corI*. The second carrier protein domain encoded on this gene, exhibiting more similarity to ACPs than to PCPs, is loaded with the amino acid glycine, thereby functioning as a PCP domain. A degenerate KR domain is present at the N-terminal end of CorI, but it remains a matter of speculation whether this domain can accomplish any function, because the typical Rossmann fold for NAD(P)-binding is missing.

The domain organization of CorI was analysed as (KR)-ACP-KS-A-PCP/ACP-KS-DH-KR-ACP-KS and once again demonstrates that myxobacteria in particular use mixed PKS/NRPS hybrids and exhibit extraordinary diversity in the arrangement of different modules, as already reviewed.[34] Because the assembly of NRPS and PKS biosynthetic machineries is highly convergent, it can be assumed that this new arrangement with mixed NRPS/PKS domains within one module is nevertheless functional. This is supported by in silico analysis of the first KS of CorI, which showed that the conserved active-site cysteine and two histidine residues are present, making the full functionality of this protein likely. In addition, the functionality of the A domain was confirmed in an ATP/PP_i exchange assay in which the heterologously expressed protein showed activity with the amino acids threonine and glycine, the latter being the substrate in the biosynthesis of corallopyronin A, as predicted by the feeding experiments. The fact that the amino acid threonine appeared to be the better substrate for the A domain protein may be explained by a somewhat different specificity of a single, heterologously expressed autonomous protein in relation to a protein embedded in the PKS/NRPS multienzyme complex. A further reason, possibly explaining the bias towards threonine, might be the chemical structure of the first intermediate of the western chain: that is, the methylated carbamate residue, which shows some similarity to the amino acid threonine. Because no corallopyronin intermediate with an incorporated threonine has to date been identified, it can be assumed that, if threonine is activated in vivo, this elongation intermediate is not further processed. Either the binding to the following carrier protein is inefficient or the following KS domain does not process the intermediate. However, the ability of the A domain to activate glycine in vitro is established.

A peculiarity of corallopyronin A biosynthesis is the reduction of the carbonyl group of the incorporated glycine moiety through the action of a KR domain and the subsequent dehydration by a DH domain in module 2. The resulting carbon-carbon double bond of the elongated intermediate would be expected to be positioned between C-10 and C-11 (α,β). In corallopyronin A, however this double bond is located between C-11 and C-12 (β,γ). From this it has to be concluded either that a direct β,γ-dehydration takes place, or that an α,β-double bond is isomerized subsequent to the dehydration event. Recently, it was reported that both pathways can be encountered during biosynthetic processes. In the first case the unsaturation is introduced with a concomitant double bond shift, leading to a β,γ-dehydration as shown for the biosynthesis of bacillaene and rhizoxin.[23,24] The second case—that is, the double bond is firstly formed by an α,β-dehydration and then shifted to the β,γ-position—has also been observed in rhizoxin biosynthesis.[24] For the biosynthesis of corallopyronin A, we assume that the formation of a double bond between C-11 and C-12 may be facilitated by the DH domain in module 3, which seems not involved in chain elongation. It cannot be completely excluded, however that the required isomerase activity might be provided by the DH domain of module 2. In rhizoxin biosynthesis, the β,γ-dehydration is mediated by a DH* domain, which deviates in sequence alignment from those of classical DH domains. The Hx_3Gx_4P motif is mutated to Hx_9, for example, whereas the DxxxQ/H motif is completely absent. In the corresponding DH* of the putative corallopyronin A cluster the Hx_3Gx_5 motif is still found (Figure S9 in the Supporting Information), but the DxxxQ/H-motif is also missing. This supports the suggestion that DH* is responsible for the formation of the C-11/C-12 double bond and this rare feature agrees perfectly with the β,γ-double bond in the structure of corallopyronin A.

The architecture of module 4, made up of KS-DH-KR-MT-ACP domains, suggests a complete reductive cycle, resulting in the formation of a single carbon-carbon bond, even though an ER domain, which would usually perform the third reduction step,

is missing. The lack of such domains is a common peculiarity of trans-AT PKSs,[34,59] and so fits a known pattern. With PksE from *B. subtilis*, made up of an AT domain along with an ER domain, the first trans-ER utilized in PK biosynthesis was identified.[60] Several saturated carbon-carbon bonds were identified in corallopyronin A, even though *cis*-ER domains are absent in all modules. This suggests a trans-enoyl reduction, for which CorA would be the appropriate candidate, because CorA has the same assembly as found in PksE. The MT encoded in module 4 accounts for the introduction of the methyl group CH_3-8 from SAM onto a nucleophilic α-carbon. Because of a mutation in the KS_0 domain, the adjacent module 5 is not involved in polyketide chain elongation (Figure S9 in the Supporting Information), although from bioinformatic analysis the ACP domain in module 5 seems to be functional and is probably involved in passing on the polyketide chain for further extension. Module 6, however, being composed of KS-ACP domains, would be responsible for the next chain elongation, resulting in the complete eastern chain.

Biosynthesis of the western chain

The western chain of corallopyronin A is exclusively PKS-derived. Apart from basic PKS-type reactions, however the hydroxy group at C-24 must be incorporated and the $\Delta^{25,27}$ double bond is not at the expected position, which might be the result of an isomerization reaction. Feeding studies suggest that the biosynthesis of the western chain starts with the fusion of an acetyl-S-ACP with malonyl-S-ACP. CorK begins with module 1′ and is composed of KS-(pseudo AT)-DH-KR-ACP domains, in which a typical loading module is missing. The same situation is reflected in the first module of the disorazole PKS cluster, in which a crotonyl intermediate is produced, identically to what has been proposed for corallopyronin A biosynthesis.[37,53] The acetyl starter would therefore be directly loaded onto the KS domain and the reductive domains in this module correspond to the double bond between C-29 and C-30. Module 2′ shows the domain assembly KS-DH-KR-ACP, and even though an ER domain is missing, as in module 4, a single bond between C-28 and C-27 is eventually found in the molecule. Module 3′, simply composed of KS-ACP domains, is viewed as responsible for the introduction of another C_2-moiety. It is suggested that the first β-branching takes place on this ACP domain. CH_3-26 is therefore finally incorporated, also giving rise to a double bond between C-25 and C-24. Module 4′, introducing the carbons C-22 and C-23, contains a DH and a KR domain, but an ER domain, required for the complete reduction to a single bond, is again missing. On module 5′ (KS-ACP) a further C_2-moiety is added and the second β-branching takes place. In both β-branching reactions in corallopyronin A biosynthesis a methyl group is introduced (CH_3-26 and CH_3-21). Because only one β-branching cassette is encoded in the putative gene cluster, it is assumed that the appropriate enzymes are responsible for both branching events in corallopyronin A biosynthesis. In contrast, in myxovirescin biosynthesis it is presumed that because of the two different substrates in the two β-branching events (resulting in either a methyl or an ethyl side group), two different ACPs and ECHs, respectively, are needed.[61] Module 6′ (KS-DH-KR-MT-ACP) is in perfect agreement with an α-methylated olefinic unit and module 7′, which is composed of KS-ACP domains, again corresponds to the β-keto-derived moiety present in corallopyronin A, which completes the chain-elongation process. These deductions support the proposed pathway for corallopyronin A biosynthesis, because the appropriate number of chain-elongation steps, the incorporation of methyl groups through SAM-dependent mechanisms and the β-branching events match the proposed model (Figure 1B).

Two polyketide chains form corallopyronin A

According to the sequence data, two assembly lines—a NRPS/PKS system and a PKS system—generate two chains with the expected chemical features for the formation of corallopyronin A, apart from the hydroxy group at C-24 and the adjacent double bond $\Delta^{25,27}$ (Figure 1).

For the biosynthesis of the complete corallopyronin A molecule, the western and eastern chains have to be connected. For the proposed Claisen reaction a KS-type enzyme has to be expected, pointing towards the involvement of the KS CorB. This "standalone" KS could perform such a reaction in trans. Scheme 2B shows the putative chain-connecting reaction, involving the C-4 carbonyl group on one side and the C-3 methylene functionality on the other. The pyrone ring would be formed after keto–enol tautomerism of the carbonyl group at C-6 to give an enol functionality, which would undergo lactonization with the carboxyl moiety at C-2. The thioesterase (TE) CorM would be responsible for cleaving the molecule from CorL and pyrone ring formation, because TEs are usually accountable for the release of the product from the biosynthetic enzyme and in some cases for lactone formation. A mechanism similar to the one described here for corallopyronin A was previously proposed for the biosynthesis of the dialkylresorcinol analogue 2-hexyl-5-propyl-alkylresorcinol (HPR).[16] In this case it was suggested that DarA catalyses a head-to-head condensation between two β-ketoacyl thioester intermediates, which results in a dioxocyclohexene intermediate, and finally in the aromatic resorcinol moiety of HPR. Through studies with labelled precursors it was shown in another example that 4-hydroxy-2-alkylquinolines (HAQs), including the compound 3,4-hydroxy-2-heptylquinoline (PQS), are synthesized through head-to-head condensations of anthranilic acid and β-keto fatty acids.[17] In both models it was proposed that the condensation step should be a Claisen condensation, as suggested here for the formation of the pyrone ring in corallopyronin A. The PQS gene cluster also harbours KSs (PqsB and PqsC) that should be able to perform the Claisen condensation of the two chains. In an alignment of these proteins with CorB, it was evident that none of the three carries the catalytic triad known for classical KSs (Figure S9 in the Supporting Information). For PqsB no conserved residues could be detected. However, CorB and PqsC harbour the catalytic diad (the complete triad in the case of CorB) specific for ketoacyl-acyl carrier protein syntha-

se III (KASIII) enzymes, which initiate the elongation in type II fatty acid synthase systems.[42]

Reactions comparable to those proposed for corallopyronin A pyrone ring formation have been described for the myxobacterial metabolites stigmatellin, with a xanthone nucleus, and for the isochromanone derivatives ajudazole and psymberin.[13,15,62] In stigmatellin synthesis it is proposed that in a first step the aromatic ring is formed by the catalytic activity of the last domain of StiJ (designated cyclase domain), followed by the formation of a chromone ring. In the case of ajudazole and psymberin biosynthesis the gene cluster in question encodes a TE domain, which may be involved in product release and lactone ring formation.

In our model (Figure 1) it is proposed that CorB, performing a ketosynthase reaction, and CorM, with thioesterase activity (Table 2), are responsible for pyrone ring formation and release of the corallopyronin A precursor pre-corallopyronin A from CorL. The corresponding mass of this molecule (*m/z* 512 $[M+H]^+$) is detectable in extracts of *C. coralloides* fermentations by LC/MS measurements. The isomerization of the double bond from the $\Delta^{24,25}$ position to the $\Delta^{25,27}$ position may be catalysed by CorN, which, according to sequence analysis, belongs to the crotonase/enoyl CoA hydratase superfamily. This superfamily contains a diverse set of enzymes that play important roles in fatty acid metabolism, isomerases also among them, so it is assumed that CorN catalyses the double bond shift. CorO, which has a similarity to cytochrome P450 proteins, is probably accountable for hydroxylation at C-24. With the last two post-PKS modifications the biosynthesis of corallopyronin A would be completed.

Experimental Section

General procedures: HPLC was performed with a Merck–Hitachi system fitted with an L-6200 A pump, an L-4500 A photodiode array detector, a D-6000 A interface with D-7000 HSM software, and a Rheodyne 7725i injection system. GC-MS analyses were performed with a Perkin–Elmer AutoSystem XL and a TurboMass spectrometer. ^{1}H, ^{13}C, COSY, HSQC and HMBC NMR spectra were recorded in [D_4]MeOH with a Bruker Avance 300 DPX or 500 DRX spectrometer operating at 300 or 500 MHz, respectively, for ^{1}H and at 75 or 125 MHz, respectively, for ^{13}C. Spectra were calibrated to residual solvent signals with resonances at $\delta_{H/C}$ = 3.35/49.0 ppm ([D_4]MeOH). Optical rotations were obtained with a Jasco DIP 140 polarimeter. LRESIMS measurements were performed with an API 2000, Triple Quadrupole LC/MS/MS, Applied Biosystems/MDS Sciex and ESI source.

Biological material: The myxobacterial strain was isolated from a soil sample collected in Remonchamps, Belgium. Small amounts of the sample were applied onto WCX-*E. coli*-agar plates on which the strain was isolated. It was transferred on VY/2 agar plates until an axenic culture was obtained. The morphology of the swarm and the microscopic appearance of the vegetative cells led to the conclusion that the strain was *Corallococcus coralloides* (strain B035 of the culture collection of the Institute for Pharmaceutical Biology). The result of the 16S rDNA sequencing analysis supports these findings (see the Supporting Information). Stock cultures of the strain are kept at −80 °C.

Cultivation and isolation procedure: Cultivation was performed in ten 5 L Erlenmeyer flasks, each containing a casitone medium (1.5 L, MD1 medium, supplemented with 0.2% glucose) with Amberlite XAD-16 (2%, Fluka, Germany). MD1 medium consists of casitone (3 g L^{-1}), $CaCl_2 \cdot 2H_2O$ (0.7 g L^{-1}) and $MgSO_4 \cdot 7H_2O$ (2 g L^{-1}). After autoclaving, trace element solution (0.3%) and vitamin B12 (0.1%) were added. The flasks were inoculated with a preculture (same medium, 200 mL) and shaken on a rotary shaker (140 rpm) at 30 °C for 10–14 days. At the end of the cultivation, the bacterial cells and adsorber resin were separated from the culture broth by centrifugation and extracted with acetone (6×500 mL).

After removal of the solvent, the residue was suspended in water (250 mL) and extracted three times with ethyl acetate (250 mL). The ethyl acetate layers were combined and dried (0.8 g crude extract). Separation of this extract was achieved by vacuum liquid column chromatography over Polygoprep 60–50 RP (Macherey–Nagel; about 30 g) by consecutive employment of methanol/water mixtures as eluents (gradient from 20:80 to 100:0) to provide nine fractions. ^{1}H NMR spectroscopic analysis indicated that fractions 6 (70:30, 17.4 mg), 7 (80:20, 33.5 mg) and 8 (90:10, 120 mg) contained corallopyronin A. These fractions were subjected to semipreparative RP-HPLC (column: Macherey–Nagel, Nucleodur Sphinx RP, 250×4.6 mm, 5 μm), eluent: methanol/water (60:40), flow rate: 1.5 mL min^{-1}). The retention time of corallopyronin A was 15 min. The separation yielded 10 mg of corallopyronin A.

Biosynthetic studies: For the feeding experiments, strain B035 was cultivated in the same liquid medium as described above. The preculture was grown for seven days in medium (3×200 mL), which was the inoculum for the main culture (3×1 L) to which Amberlite XAD-16 (2%) was added. After 48 h the labelled compounds were added in the form of sterile filtered solutions {1,2-[^{13}C]$_2$-sodium acetate (1 g), 1-[^{13}C]sodium acetate (1 g), 2-[^{13}C][^{15}N]glycine (0.5 g), 1-[^{13}C]glycine (1 g), methyl-[^{13}C]methionine (1 g), [^{13}C]sodium bicarbonate (1 g), Cambridge Isotope Laboratories}. The isolation of labelled corallopyronin A was carried out according to the isolation procedure mentioned above.

Preparation of the (*R*)- and (*S*)-MTPA esters: Corallopyronin A (10 μmol) was dissolved in pyridine (0.4 mL,100%) and the appropriate MTPA-Cl (50 μmol) was added. After 10 min the reaction was quenched with cooled water (1 mL). The MTPA ester was extracted by liquid–liquid partitioning with ethyl acetate (5×1 mL) and purified by HPLC with the Merck–Hitachi system. The separation was performed with a RP18 column (Knauer Eurospher, 5 μm, 250×8 mm) and a mobile phase (1.5 mL min^{-1}) consisting of MeOH/H_2O 90:10. The $\Delta^{(S-R)}$ values between (*S*)- and (*R*)-MTPA esters were recorded with the help of both ^{1}H NMR and ^{1}H,^{13}C HSQC spectra.

Ozonolysis of corallopyronin A: A stream of O_3 was bubbled through a MeOH solution of corallopyronin A (5 mg in 2 mL) for 20 min at room temperature. The reaction was quenched with five drops H_2O_2 (30%). The solution was shaken for 60 min at room temperature and the methanol was removed under a stream of nitrogen. The resulting residue was subjected to chiral GC analysis as outlined below.

Chiral GC: The residue from the ozonolysis and the standards (*S*)-(+)-2-methylglutaric acid and (*R*)-(−)-2-methylglutaric acid (1 mg each) were treated at 110 °C with isopropyl alcohol (500 μL) and acetyl chloride (150 μL) for 1 h to create the isopropyl esters. After removal of the solvent the dry residues were resolved in dichloromethane (1 mL). These solutions (1 μL) were analysed by GC-MS with a Perkin–Elmer CyclosilB column (30 m×0.25 mm, 0.25 μm, program rate: column temperature held at 50 °C for 5 min; 50 °C to

180 °C at 3 °C min^{-1}; flow: 1.0 mL min^{-1}; Inj.: 190 °C). The retention times of the (*S*)-(+)-2-methylglutaric acid isopropyl ester and (*R*)-(−)-2-methylglutaric acid isopropyl ester were 35.63 min and 35.76 min, respectively.

Corallopyronin A: Slightly yellow film; $[\alpha]_D^{24}$ −92.3 (c = 0.2, MeOH); ^{1}H and ^{13}C NMR data see Table 1; LRESIMS m/z 526 $[M-H]^-$.

Analysis of the biosynthetic gene cluster

General procedure: *C. coralloides* strain B035 was grown in liquid MD1 + Glucose medium at 30 °C (140 rpm) for five days. *E. coli* XL1Blue was used as a host for routine cloning and construction of targeting plasmids. *E. coli* strains were grown in Luria Bertani (LB) medium supplemented with ampicillin at a final concentration of 100 μg mL^{-1}.

All restriction enzymes and T4 DNA ligase were purchased from MBI Fermentas. *Taq* DNA-Polymerase and dNTPs for PCR were obtained from Promega. Oligonucleotides were purchased from MWG-Biotech-AG, Germany. Gel electrophoresis materials were supplied from Neolab, Germany. All other materials and substances were supplied by Roth, Germany.

Plasmid DNA and chromosomal DNA isolation, *E. coli* strain transformation, colony and Southern hybridization were performed by standard methods.[63] DNA fragments were isolated from agarose gels with the QIAquick Gel Extraction Kit (Qiagen). For subcloning and preparation of DNA templates for sequencing the vectors pGEMT (Promega, Germany) and pCR 2.1 (Invitrogen) were used. Plasmid-DNA purification was performed with the GeneJET Plasmid Miniprep Kit (Fermentas). For Southern blot hybridizations, the DIG DNA labelling and detection kit (Roche, Germany) was used.

PCR was carried out with *Taq* DNA Polymerase (Promega). DMSO was added to the reaction mixture to a final concentration of 5%. Conditions for amplification using a Thermoycler TGradient (Biometra, Germany) were as follows: denaturation 120 s at 95 °C; annealing 45 s at 48–60 °C; extension 45 s at 72 °C; 30 cycles and a final extension for 5 min at 72 °C. PCR products were purified with the QIAquick PCR Purification Kit (Qiagen).

Construction of the cosmid library: Chromosomal DNA of a culture of *C. coralloides* B 035 (100 mL) was partially digested with Bsp43I (SauIIIAI), dephosphorylated, and then ligated into SuperCos 1 (Stratagene) pre-digested with XbaI, dephosphorylated and restricted with BamHI. *E. coli* Sure strain and Gigapack III XL packaging extract (Stratagene) were used for library construction according to the manufacturer's instructions.

Screening of the cosmid library: 2304 colonies were transferred into 384-well microtitre plates. For colony hybridization, the colonies were transferred twice onto a 22.2 × 22.2 cm nylon membrane with a Qbot robot (Genetix, New Milton, UK).

Probes used for Southern hybridization were labelled with Dig-11-dUTP with the aid of the DIG-High Prime Kit according to the manufacturer's recommendations (Roche). For hybridization with heterologous DNA probes 60 °C was used as hybridization temperature. For hybridization with homologous DNA probes the temperature was adjusted to 68 °C. The nylon membrane with the transferred cosmid DNA fragments was prehybridized in standard pre-hybridization buffer with formamide for at least 4 h at 30 °C for homologous probes and at 42 °C for heterologous probes. After removal of this solution the membrane was hybridized with the prepared DIG-DNA probe at hybridization temperature for at least 16 h in a hybridization oven (Heraeus–Sepatech, Germany). After stringent washing steps, detection was performed with CSPD-Star as substrate (Roche).

Degenerate PKS primers were deduced from conserved sequence motifs of PKS type I KS domains, yielding fragments of 700 bp in the PCR. Degenerate NRPS primers were deduced from conserved sequence motifs of A3 and A8 motifs within the A domain. These primers led to the amplification of 1200 bp PCR products. Homologous and heterologous KS and adenylation fragments were amplified from the genomes of *C. coralloides* B035, *M. xanthus* DK1622 and *C. crocatus* Cmc5 with the degenerate oligonucleotides.

Primers are all listed in Table S2 in the Supporting Information. Primers for KS:[64] Ksd1 and KSup; for A domains: A3rev.1 and LGDD.S; for HMG-CoA-synthase fragments: hmgrev09 and hmgfw09; for trans-AT-PKS fragments: tatfw and tatrev.

PCR for homologous probes for HMG-CoA synthase and trans-AT-KS fragments were performed with genomic DNA from *C. coralloides* B035. HMG-CoA-synthase and trans-AT primers both yielded fragments of approximately 450 bp. The genomic library was screened by use of these PCR products as hybridization probes. Primers used for identification of overlapping cosmids: Fj7fw, Fj7rev; fragment size approximately 450 bp and Am24fw, AM24rev; fragment size approximately 1100 bp.

Restriction analysis of cosmids: Restriction analysis was performed with the cosmids that were positive in the Southern hybridization experiments, with use of the restriction enzymes ApaI, PstI and EcoRI. Incubation of the cosmids with the enzymes and appropriate buffers was conducted at 37 °C overnight. For analysis the restriction reaction was applied to 1% agarose gels.

Sequencing of cosmids: The sequence data were obtained from GATC Biotech AG (Konstanz, Germany). Samples were automatically sequenced with an ABI 3730xl DNA Analyser (Applied Biosystems). The nucleotide sequence has been deposited in GenBank under the accession number HM071004.

Bioinformatic analysis of sequences: The software programs Vector NTI, DNASTAR Lasergene 8, CLUSEAN and Artemis were used to analyse DNA sequences. The Blast program provided by NCBI was used for sequence data analysis. The multiple alignments of nucleotide sequences were generated with the program ClustalW powered by EMBL.

Expression and purification of the adenylation domain: The A domain was amplified from chromosomal DNA of *C. coralloides* with the primers A-domain up and A-domain down (Table S2 in the Supporting Information) with the introduced EcoRI and HindIII recognition sites. The resulting 1598 bp fragment was cloned into pGEM-T (Promega), and then subcloned into expression vector pET28a (Novagen). Plasmid pET28-Cc-Ad was then transformed into *E. coli* BL21 cells and sequenced. Positive clones were selected with kanamycin (50 μg mL^{-1}). The cells were grown in LB medium at 37 °C until an OD_{600} of 0.6–0.8 was reached, and protein expression was then induced with IPTG (20 nM). Growth was then allowed to continue at 20 °C overnight. Cells were harvested and resuspended in lysis buffer [NaH_2PO_4 (50 mM), NaCl (300 mM), imidazole (10 mM), pH 8.0]. After disruption of the cells by incubation with lysozyme (final concentration of 1 mg mL^{-1}), cell debris was removed by centrifugation for 10 min at maximum speed in a table-top centrifuge. The supernatant containing the His-tagged protein was bound to Ni-NTA spin columns (Qiagen), and the pure A domain was then eluted with elution buffer [NaH_2PO_4 (50 mM), NaCl (300 mM), imidazole (300 mM), pH 8.0], according to the manufacturer's instructions.

Adenylation enzyme characterization by γ-$^{18}O_4$-ATP pyrophosphate exchange: In the assay the isotopic back exchange of unlabelled pyrophosphate into γ-$^{18}O_4$-labelled ATP was measured.[51] Reactions were performed in buffer (6 μL) containing $MgCl_2$ (5 mM), PP_i (5 mM), γ-$^{18}O_4$-ATP [or ^{16}O-ATP(1 mM), as a positive control], amino acid (1 mM) and Tris-HCl (20 mM, pH 7.5). The reactions were carried out for 2 h at room temperature after addition of the purified A domain, and were then stopped by the addition of the same volume of 9-aminoacridine in acetone (10 mg mL^{-1}). As a negative control, buffer was added to the reaction mixture instead of enzyme. The samples were then analysed by MALDI-TOF MS.

Acknowledgements

We thank E. Neu, A. Rosenbaum and S. Felder for technical assistance. Prof. G. Bierbaum for the assessment of antibiotic activity, and Dr. T. Weber for providing the program CLUSEAN. Research in R.M.'s laboratory was funded by the German Research Foundation (DFG) and the Bundesministerium für Bildung und Forschung (BMBF). We are grateful to the DFG for providing the funding for this work (FOR 845).

Keywords: antibiotics · biosynthesis · *Corallococcus coralloides* · corallopyronin A · myxobacteria · polyketides

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Received: February 11, 2010
Published online on May 25, 2010

