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A Type I/Type III Polyketide Synthase Hybrid Biosynthetic Pathway for the Structurally Unique *ansa* Compound Kendomycin

Silke C. Wenzel, Helge B. Bode, Irene Kochems, and Rolf Müller*[a]

Kendomycin is a bioactive polyketide that is produced by various Streptomyces *strains. It displays strong antibiotic activities against a wide range of bacteria and exhibits remarkable cytotoxic effects on the growth of several human cancer cell lines. In this study we cloned the corresponding biosynthetic locus from the producer* Streptomyces violaceoruber *(strain 3844-33C). Our analysis shows that a mixed type I/type III polyketide synthase pathway is responsible for the formation of the fully carbogenic macrocyclic scaffold of kendomycin, which is unprecedented among all of the* ansa *compounds that have been isolated so far. Heterologous expression of a gene set in* Streptomyces coelicolor *shows that 3,5-dihydroxybenzoic acid is an intermediate in the starter unit biosynthesis that is initiated by the type III polyketide synthase. The identification of the kendomycin biosynthetic gene cluster sets the stage to study a novel chain termination mechanism by a type I PKS that leads to carbocycle formation and provides the starting material for the heterologous expression of the entire pathway, and the production of novel derivatives by genetic engineering.*

Introduction

Microorganisms produce an amazing number of metabolites with useful biological activities. These compounds are characterized by an enormous structural diversity that is not obtainable from alternative sources, which explains the still dominant role of natural products in the discovery process of drug leads.[1] The structurally unique polyketide kendomycin (also known as (−)-TAN 2162) is produced from various *Streptomyces* strains including *Streptomyces violaceoruber*[2–4] and shows promising biological activities. It acts as a potent endothelin receptor antagonist,[2,3] as an antiosteoporotic compound,[5] and has also been found to display strong antibiotic activities against a wide range of bacteria, including multidrug-resistant *Staphylococcus aureus* (MRSA) strains.[4] In vitro cytotoxicity tests revealed that kendomycin exhibits remarkable cytotoxic effects on the growth of multiple human cancer cell lines, and has a potency that is comparable to that of the clinically used anticancer drugs doxorubicin and cisplatin.[4] Recent studies on the mode of action of kendomycin in mammalian cells have shown that the compound mediates its cytotoxic effects, at least in part, through proteasome inhibition.[6] Structurally, kendomycin represents an aliphatic *ansa* system in which a highly substituted pyran ring is attached to a unique quinone methide chromophore. This fully carbogenic scaffold is unprecedented among all of the *ansa* compounds that have been isolated so far. Its novel molecular architecture along with the impressive biological profile renders kendomycin an important subject of (bio)chemical and molecular studies. Diverse synthetic approaches to this unique natural product have been reported, including two total syntheses.[7–13]

To elucidate the biosynthetic origin of kendomycin, stable isotope-labeling experiments were performed with the bacterial producer *S. violaceoruber* (strain 3844-33C).[4,14] The obtained data suggest that kendomycin is a product of a type I polyketide synthase (PKS) system. Those giant multienzyme systems accommodate a distinct set of catalytic domains to assemble coenzyme A (CoA)-activated short-chain carboxylic acids in a manner similar to fatty acid biosynthesis.[15,16] For each chain propagation step a minimal set of three domains is required: an acyl transferase (AT) domain for extender unit selection and transfer, an acyl carrier protein (ACP) for the covalent binding of the extender unit to the enzyme complex, and a ketoacyl synthase (KS) domain for the decarboxylative condensation with the growing polyketide chain. The resulting β-keto acid might subsequently be processed by β-ketoacyl reductase (KR), β-hydroxyacyl dehydratase (DH), enoyl reductase (ER) and methyl transferase (MT) domains. The catalytic domains that are required for the incorporation of a single polyketide unit are grouped into modules, and the order and architecture of the modules usually reflect the chain length as well as degree of reduction of the resultant polyketide. Whereas the first module initiates the biosynthesis by loading the starter unit, the last module usually contains an additional termination domain, for example, a thioesterase (TE) domain, to catalyze the release of the polyketide chain from the enzyme complex. Kendomycin biosynthesis seems to be unique in both aspects (start and termination) because polyketide chain formation most likely involves the recruitment of an unusual starter unit

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as well as a so-far unknown termination step.[4,14] Based on results from feeding experiments and the presence of a fully carbogenic *ansa* framework in kendomycin, it was concluded that the macrocyclization occurs via C–C bond formation after decarboxylation of the last extender unit rather than through the common TE-mediated macrolactone formation by intramolecular cyclization. In addition, the labeling studies suggested that a benzoic acid derivative possibly generated through a type III PKS-dependent pathway is used as a starter unit from the type I PKS biosynthetic machinery.[4] Type III PKSs (also known as chalcone synthase-like PKS) are iteratively operating condensing enzymes that act directly on acyl-CoA substrates and are usually independent of ACPs.[17] Originally assigned to plant secondary metabolism ("chalcone synthases"), these enzymes were later reported to be involved in the biosynthesis of bacterial compounds as well. The corresponding products are typically small aromatic metabolites like 1,3,6,8-tetrahydroxynaphthalene (THN) and its spontaneous oxidation product flaviolin, 2,4-diacetylphloroglucinol (2,4-DAPG) or 3,5-dihydroxyphenylglycine (3,5-DHPG), just to name a few.[18–23]

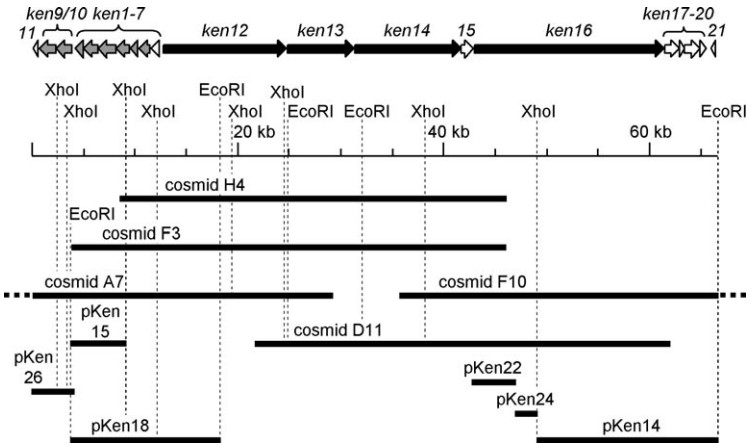


Figure 1. Map and gene arrangement of the kendomycin (*ken*) biosynthetic gene cluster in *S. violaceoruber* strain 3844–33C. The inserts of the cosmids and plasmids are indicated by black bars. The restriction sites of the enzymes *Eco*RI and *Xho*I, which were used for subcloning of fragments, are illustrated by dotted lines. Genes are shown as arrows, which also indicate the direction of transcription. Type I PKS-encoding genes are shown in black, whereas genes that are assumed to be involved in the biosynthesis of the aromatic starter unit are highlighted in grey. The proposed functions of the encoded proteins are listed in Table 1.

Kendomycin's potential as a medicinal agent, its unique structural topography, and the interesting questions concerning its biosynthesis encouraged us to identify and characterize the corresponding biosynthetic machinery. Here, we describe the identification of the kendomycin biosynthetic gene cluster by hybridizing a cosmid library from the producer strain *S. violaceoruber* (strain 3844-33C) with type I and type III PKS-specific probes. Sequence analysis and gene inactivation studies revealed that a type I/type III PKS hybrid system is indeed responsible for the formation of the unique polyketide. A set of genes involved in the biosynthesis of the unusual starter unit was subcloned and heterologously expressed in *Streptomyces coelicolor*; this indicates that 3,5-dihydroxybenzoic acid is an intermediate during type III PKS-initiated starter unit biosynthesis.

Results and Discussion

Identification of the kendomycin biosynthetic gene cluster in *Streptomyces violaceoruber*

The chemical structure of kendomycin and the labeling pattern from previous feeding studies[4,14] led us to assume that both type I and type III PKSs are involved in the biosynthesis. To identify the kendomycin gene cluster, a genomic library of the producer organism *S. violaceoruber* (strain 3844-33C) was constructed and initially screened with a KS-specific probe to detect type I PKS pathways. Positive cosmids were subsequently hybridized by using a heterologous probe based on the *dpgA* (encodes a type III PKS) and *dpgB–D* genes from the balhimycin pathway of *Amycolatopsis mediterranei*.[22] Five cosmids (including cosmids A7, F3, and H4; see Figure 1) gave strong signals with both probes and were further analyzed by end sequencing. Cosmid H4, which was found to encode a type I PKS at the T3 end and a protein homologous to DpgC[22] at the T7 end, was finally completely sequenced. Detailed sequence analysis indicated that the 36 kb insert from cosmid H4 does not contain the complete kendomycin biosynthetic pathway. Cosmids harboring fragments that overlap with the T7 end of cosmid H4 had already been identified in the hybridization experiment by using the *dpgA–D* probe (cosmid F3 and A7, see Figure 1). To detect cosmids that overlap with the T3 end of cosmid H4, the initial KS cosmid sublibrary was hybridized with a probe that was homologous to the T3 end of cosmid H4. Five cosmids (including cosmid D11 and F10; see Figure 1) gave strong signals and were further analyzed by their restriction pattern and by end sequencing. Fragments upstream and downstream of the cosmid H4 insert were subcloned into conventional cloning vectors; this resulted in pKen14, pKen15, pKen22, pKen24, and pKen26 (Figure 1 and Table S1 in the Supporting Information), which were completely sequenced. To prove the involvement of the identified gene cluster in kendomycin formation, a type I PKS-encoding gene (*ken12*) was inactivated by insertional mutagenesis. The resulting mutant strain *S. violaceoruber*::pKen41 was found to be unable to produce kendomycin, in contrast to the wild-type strain that was cultivated under identical conditions (see Figure 2).

Sequence analysis of the kendomycin biosynthetic gene cluster

Detailed analysis of the 66 kb kendomycin (*ken*) biosynthetic gene cluster (GenBank accession no. AM992894) revealed 20 open-reading frames (ORFs) that are organized into four putative operons (*ken1–7, ken9–10*, *ken12–16* and *ken17–20*) and two single genes (presumably of regulatory function) flanking both sides of the gene cluster. For a detailed description of the

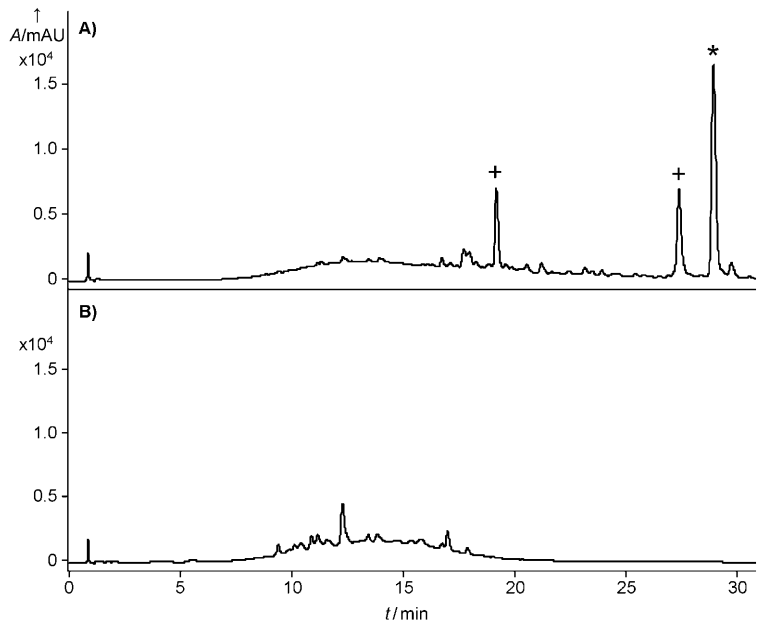


Figure 2. Phenotypic analysis of the *ken12* inactivation mutant in comparison to the wild-type strain. HPLC chromatograms of the A) *S. violaceoruber* (strain 3844-33C) extract and B) *S. violaceoruber*::pKen41 extract are shown. The peak that corresponds to kendomycin is marked with an asterisk and could only be detected in the wild-type extract (A). The two compound peaks that are marked with plus symbols represent potential kendomycin derivatives/biosynthetic intermediates (UV detection at 250–600 nm).

identified genes and the deduced function of the encoded proteins see Table 1. The four type I PKSs that are encoded by *ken12–14* and *ken16* harbor one loading module and eight modules for the incorporation of extender units. The domain organization of the loading module (CoA ligase (CL)-X-ACP) is unusual because a domain of unknown function (X) is inserted between the CL and the ACP domain (see below). All elongation modules of the kendomycin pathway incorporate the minimal set of KS, AT, and ACP domains, and analysis of the respective active sites suggests that all domains, with the exception of the DH from module 2, are functional. In silico analysis of the AT domain substrate specificity was performed according to Yadav et al.[24] Among the eight AT domains that are present in elongation modules, six AT domains have predicted specificity for methylmalonyl-CoA (AT1, AT2, AT4, AT5, AT6 and AT7), whereas two (AT3 and AT8) are expected to select malonyl-CoA. Interestingly, the sequences of the methylmalonyl-CoA-activating domains are almost 90% identical, which results in large DNA repeats within the PKS-encoding region of the gene cluster. This might indicate its evolutionary origin by gene duplication as described recently for similar systems.[25,26] Some of the modules additionally contain reductive domains (KR, DH, and/or ER); module 3 harbors a KR domain, modules 1, 2, and 5 include a DH-KR didomain and modules 4, 6, and 7 incorporate a complete reductive loop DH-ER-KR. In silico analysis of the KR domain stereochemistry was performed according to Caffrey.[27–29] The typical LDD motif (residues 93–95) for B-type reduction, which is often accompanied by P144 and N148, could be detected in all KR domains except for KR3, which shows the A-type motif W141. The last module (module 8) harbors a TE domain, which is usually required for the release of the processed polyketide chain from the multienzyme complex.[16] An additional gene (*ken15*), which encodes a protein with homology to FAD-dependent monooxygenases, is integrated into the type I PKS operon (*ken12–16*). A set of four genes is located (*ken17–20*) further downstream and encodes a putative lipase (Ken17), a protein (Ken18) with weak homology to an enediyne-binding protein (MdpA) from *Actinomadura madurae*,[30] a putative oxidoreductase (Ken19) as well as a probable integral membrane protein (Ken20). The following gene (*ken21*) is transcribed in the opposite direction and encodes a putative MarR family transcriptional regulator.[31] Upstream of the type I PKS-encoding genes (see Figure 1), a set of 10 genes (*ken1–7* and *ken9–11*) is putatively transcribed in reverse orientation. *ken1* encodes a protein with homology to type II thioesterases. The following three genes (*ken2–4*) as well as *ken7* show high similarity to the *dpgABCD* operon from *A. mediterranei*;[22] these encode a type III PKS (Ken2/DpgA), two enoyl-CoA hydratases (Ken3/DpgB and Ken7/DpgD), and a dioxygenase (Ken4/DpgC). Two additional genes, *ken5* and *ken6*, are inserted into this putative transcriptional unit (*ken1–7*). The corresponding proteins show homology to a benzoylformate decarboxylase and a benzaldehyde dehydrogenase from the mandelate degradation pathway of different *Pseudomonas* strains.[32,33] Further downstream, two putative modifying enzymes, a methyl transferase (Ken9), as well as a pyrroloquinoline quinone (PQQ)-dependent protein (Ken10) are encoded. A regulatory gene (*ken11*) is located directly after the putative *ken9/10* operon. The proposed role of the identified genes in the kendomycin biosynthesis is discussed below.

Biosynthesis of the kendomycin starter unit

An interesting feature of the kendomycin biosynthetic gene cluster is the presence of type I PKSs as well as a type III PKS protein-encoding gene. The latter is assumed to initiate the biosynthesis of the unusual starter unit, which most likely resembles a highly substituted benzoic acid derivative.[4,14] The type III PKS Ken2 as well as Ken3, Ken4 and Ken7 show high similarity to the *dpgA–D* gene products from *A. mediterranei*,[22] which are involved in the formation of 3,5-dihydroxyphenylglycine (3,5-DHPG). This nonproteinogenic amino acid is incorporated in several glycopeptide antiobiotics, for example, balhimycin, chloroeremomycin and vancomycin.[22,23,34] The Ken2 homologue DpgA represents a type III PKS, which condenses four malonyl-CoA units to produce 3,5-dihydroxyphenylacetyl-CoA (3,5-DHPA-CoA; see Scheme 1).[22,23,34] Two enoyl CoA hydratases (DpgB and DpgD, similar to Ken3 and Ken7, respectively) were described to increase production yields, and it was speculated that their hydratase/dehydratase activity facilitates the aromatization process.[34] It was demonstrated, however that both enzymes (DpgB and DpgD) are not crucial for 3,5-

Table 1. Genes that were identified in the sequenced region and the proposed function of the encoded proteins.

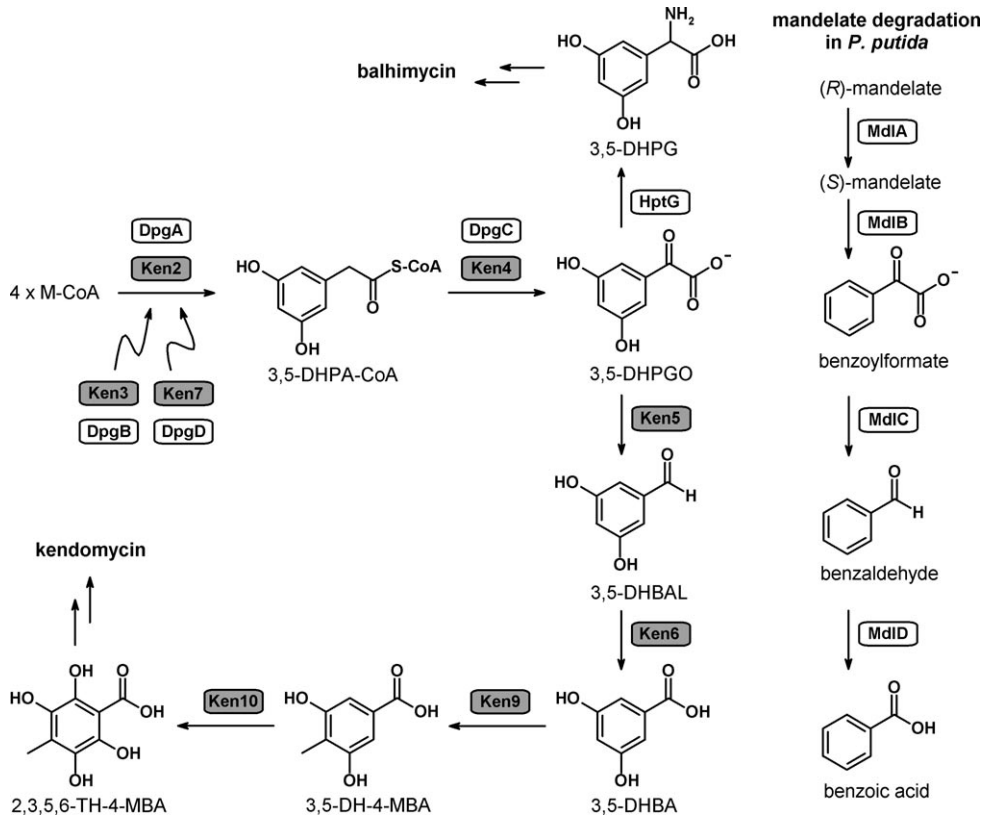
Name	Gene Localization	GC [%]	Size [aa]	Encoded protein Proposed function (domain arrangement)
ken11	617–177	73.0	146	transcriptional regulator
ken10	2377–746	74.6	543	PQQ-dependent enzyme
ken9	3933–2374	75.8	519	methyl transferase
ken7	5124–4327	74.0	265	enoyl-CoA hydratase/isomerase, DpgD homologue
ken6	6590–5121	76.3	489	benzaldehyde dehydrogenase
ken5	8278–6587	75.3	563	benzoylformate decarboxylase
ken4	9651–8275	75.2	458	dioxygenase, DpgC homologue
ken3	10429–9713	75.9	238	enoyl-CoA hydratase/isomerase, DpgB homologue
ken2	11657–10557	66.9	366	type III PKS, DpgA homologue
ken1	12562–11711	72.7	283	type II thioesterase
ken12	12831–24752	72.8	3973	type I PKS module 4-5 (KS-AT-DH-ER-KR-ACP-KS-AT-DH-KR-ACP)
ken13	24785–31303	71.1	2172	type I PKS module 6 (KS-AT-DH-ER-KR-ACP)
ken14	31342–41685	73.3	3447	type I PKS module 7-8 (KS-AT-DH-ER-KR-ACP-KS-AT-ACP-TE)
ken15	41682–42869	73.9	395	FAD-dependent monooxygenase
ken16	42937–61329	74.1	6130	type I PKS loading module + modules 1–3 (CL-X-ACP-KS-AT-DH-KR-ACP-KS-AT-DH-KR-ACP-KS-AT-KR-ACP)
ken17	61490–62833	71.4	447	lipase
ken18	62863-63318	72.6	151	hypothetical protein with weak homology to an endiyne-binding protein
ken19	63315–64868	73.2	517	FAD-dependent oxidoreductase
ken20	64876–65466	77.3	196	integral membrane protein
ken21	66387–65944	73.2	147	transcriptional regulator

Abbreviations: PKS, polyketide synthase; KS, ketosynthase; AT, acyltransferase; DH, dehydratase; ER, enoylreductase; KR, ketoreductase; ACP, acyl carrier protein; TE, thioesterase; CL, CoA-ligase; X, domain of unknown function that is possibly involved in quinone formation.

DHPA-CoA formation. The α-carbon of 3,5-DHPA-CoA is subsequently oxidized by DpgC (Ken4 homologue), which also acts as thioesterase to release 3,5-dihydroxyglyoxylate (3,5-DHPGO) as the free acid.[22,34,35] Due to the high similarity of the *ken2–4* and *ken7* gene products to DpgA–D, 3,5-DHPGO is also assumed to be an intermediate in kendomycin starter unit biosynthesis; however, the 3,5-DHPG pathway contains an additional transaminase (HpgT) that is missing in the kendomycin pathway. HpgT is required for the final conversion of 3,5-DHPGO to 3,5-DHPG (see Scheme 1). In contrast, two other genes (*ken5* and *ken6*) are grouped together with the 3,5-DHPGO biosynthetic genes within the kendomycin pathway. The encoded proteins show similarity to benzoylformate decarboxylases (Ken5) and benzaldehyde dehydrogenases (Ken6) from *Pseudomonas* strains. Both enzymes are involved in the mandelate degradation pathway, which enables pseudomonads to use mandelate as a sole carbon source.[32,33] In four reaction steps the compound (*R*)-mandelate is converted into benzoic acid (see Scheme 1), which can subsequently be metabolized through the β-ketoadipate pathway and the tricarboxylic acid cycle. The well-characterized benzoylformate decarboxylase (MdlC) from *Pseudomonas putida* ATCC 12633 is a thiamine-diphosphate-dependent enzyme, which catalyzes the nonoxidative conversion of benzoylformate into benzaldehyde and carbon dioxide.[33,36] The subsequent oxidation of the aldyhyde group by the NAD-dependent benzaldehyde dehydrogenase (MdlD) leads to the formation of benzoic acid.[32] In analogy to these reaction steps, we propose that the MdlC homologue Ken5 transforms 3,5-DHPGO into 3,5-dihydroxybenzaldehyde (3,5-DHBAL), which is subsequently oxidized by the MdlD homologue Ken6 to the corresponding acid (3,5-dihydroxybenzoic acid, 3,5-DHBA; see Scheme 1). Because two additional biosynthetic genes (*ken9* and *ken10*) are located downstream of *ken7*, we assume that the starter unit is further modified before entering the type I PKS assembly process. Ken9 harbors the conserved motif of SAM-dependent methyl transferases and is most likely responsible for the C-methylation of 3,5-DHBA in the *para* position. The resulting 3,5-dihydroxy-4-methyl-benzoic acid (3,5-DH-4-MBA) might then be further modified by Ken10. Analysis of its protein sequence revealed a single repeat of a β-propeller fold that is typical for quinoproteins.[37] This enzyme family uses PQQ[38] as a prosthetic group and is involved in diverse reactions (oxidations, hydroxylations, transaminations, decarboxylations, or hydrations).[39] Based on this, we assume that Ken10 hydroxylates 3,5-DH-4-MBA in both (chemically equivalent) *ortho*-positions to give rise to a fully substituted benzoic acid derivative (2,3,5,6-tetrahydroxy-4-methyl-benzoic acid, 2,3,5,6-TH-4-MBA; see Scheme 1), which after activation by the corresponding CoA ligase in Ken16 is loaded as starter unit onto the type I PKS complex.

Heterologous expression of starter unit biosynthetic genes

Based on the in silico analysis of the kendomycin biosynthetic gene cluster, we compiled a possible pathway for the generation of the unusual type I PKS-derived starter unit (see Scheme 1). Herein, 3,5-DHBA was assumed to be one of the central intermediates and Ken2–7 are suggested to be involved in its production. To gain biochemical evidence for the proposed function of these enzymes and to confirm the structure of the starter unit precursor, we aimed to heterologously express the corresponding set of genes. For this, a 14.5 kb fragment from cosmid F3 that harbors the complete *ken1–7* operon (and additional sequence of the 5′ end of *ken12*) was subcloned into the integrative *E. coli/Streptomyces* shuttle vector pSET152. The resulting plasmid pKen18 was subsequently introduced into two different streptomycetes, *S. lividans* TK24 and *S. coelicolor* A3(2), to generate the mutants *S. lividans*::pKen18 and *S. coelicolor*::pKen18. Mutant strains that harbor the empty expression vector (*S. lividans*::pSET152 and *S. coelicolor*::pSET152) were generated in parallel. After growing the recombinant strains in 50 mL cultures, the cells were extracted, and the extracts were analyzed for the presence of 3,5-DHBA by high-performance liquid chromatography–mass spectrometry (HPLC–MS). The obtained data were compared to an authentic 3,5-DHBA reference substance (see Figure 3). As expected, no 3,5-DHBA is produced from the



Scheme 1. Biosynthesis of the kendomycin starter unit compared to 3,5-DHPG biosynthesis in *A. mediterranei*[22] and the mandelate degradation pathway from *P. putida*.[32,33] The first two steps from the type III PKS-initiated kendomycin starter unit biosynthesis are identical to the 3,5-DHPG assembly process and the involved proteins from both pathways (Ken2–4,7/DpgA–D) show a high degree of similarity to each other. The produced 3,5-DHPGO, which is further transaminated in the 3,5-DHPG pathway is degraded to 3,5-DHBA by Ken5 and Ken6, which perform similar reactions as their homologues (MdlC and MdlD) from the mandelate degradation pathway. Two additional modification steps (catalyzed by Ken9 and Ken10) are most likely conducted to generate the putative, fully substituted aromatic starter unit (2,3,5,6-TH-4-MBA), which is loaded onto the type I PKS complex.

mutant strains that harbor the empty expression vector. By using HPLC–MS analysis, 3,5-DHBA could be clearly detected in *S. coelicolor*::pKen18 (production yield: ~30 $\mu g\,L^{-1}$), but not in *S. lividans*::pKen18. The lack of 3,5-DHBA production in the *S. lividans* mutant might be explained by the fact that the heterologous and native promotor of the *ken1–7* operon is not functional in this host strain. Indeed, the kendomycin-producer strain *S. violaceoruber* is genetically more closely related to *S. coelicolor* than to *S. lividans* (D. Hopwood, personal communication). Therefore, it seems likely that the native promotor structures of the *ken* genes are more effective in *S. coelicolor* than in *S. lividans*, which is consistent with our results. The successful heterologous expression of the *ken1–7* operon in *S. coelicolor* strongly suggests however, that the encoded enzymes are responsible for the formation of 3,5-DHBA (although background activity of host enzymes cannot be excluded) and provides further evidence that this product is an intermediate in kendomycin starter unit biosynthesis. As mentioned above, the role of DpgA–D in the biosynthesis of 3,5-DHPGO was already intensively analyzed in previous studies.[22,23,34,35] As these proteins are highly similar to Ken2–4 and Ken7, the detection of 3,5-DHBA after heterologous expression of the complete *ken1–7* operon strongly suggests that the additionally expressed proteins Ken5 and Ken6 are able to convert 3,5-DHPGO into 3,5-DHBA, which confirms their proposed functions as 3,5-DHPGO decarboxylase (Ken5) and 3,5-DHBAL dehydrogenase (Ken6). Attempts to detect the fully substituted benzoic acid derivative 2,3,5,6-TH-4-MBA after heterologous expression of the complete set of starter unit biosynthetic genes with or without the putative transcriptional regulator (*ken1–7, 9–10* or *ken1–7, 9–11*) failed so far (SCW and RM, unpublished). We speculate that the final yield of the product might be below the detection limit and/or that the cofactors required for Ken9 and Ken10 activity (SAM and PQQ) are not provided in sufficient amounts by the host strains. Further experiments to clarify this question (for example, exchange of the native promotors against strong promotors that are functional in the hosts and metabolic engineering of the host strains) are currently underway. However, there might also be the possibility that Ken9 and/or Ken10 act at a later stage during kendomycin assembly instead of modifying the aromatic starter unit directly.

Kendomycin type I PKS assembly line

The kendomycin type I PKS complex consists of four multifunctional enzymes (Ken12–14 and Ken16) that are encoded on

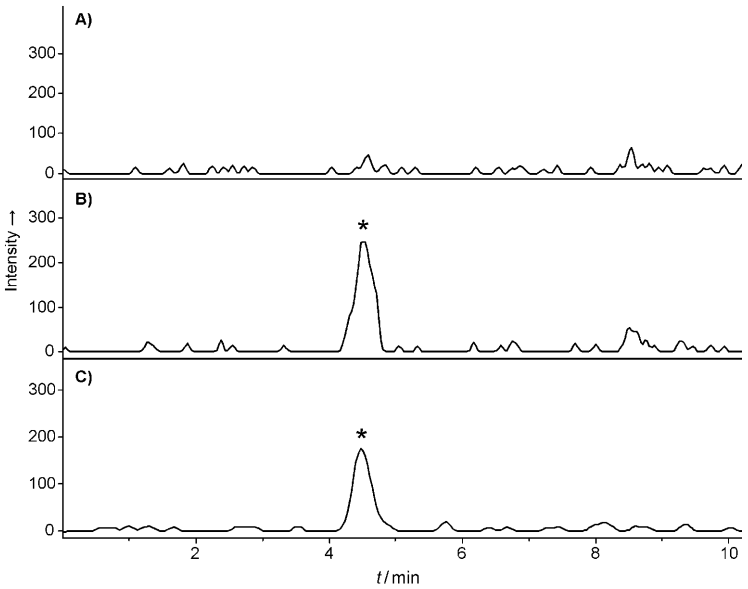
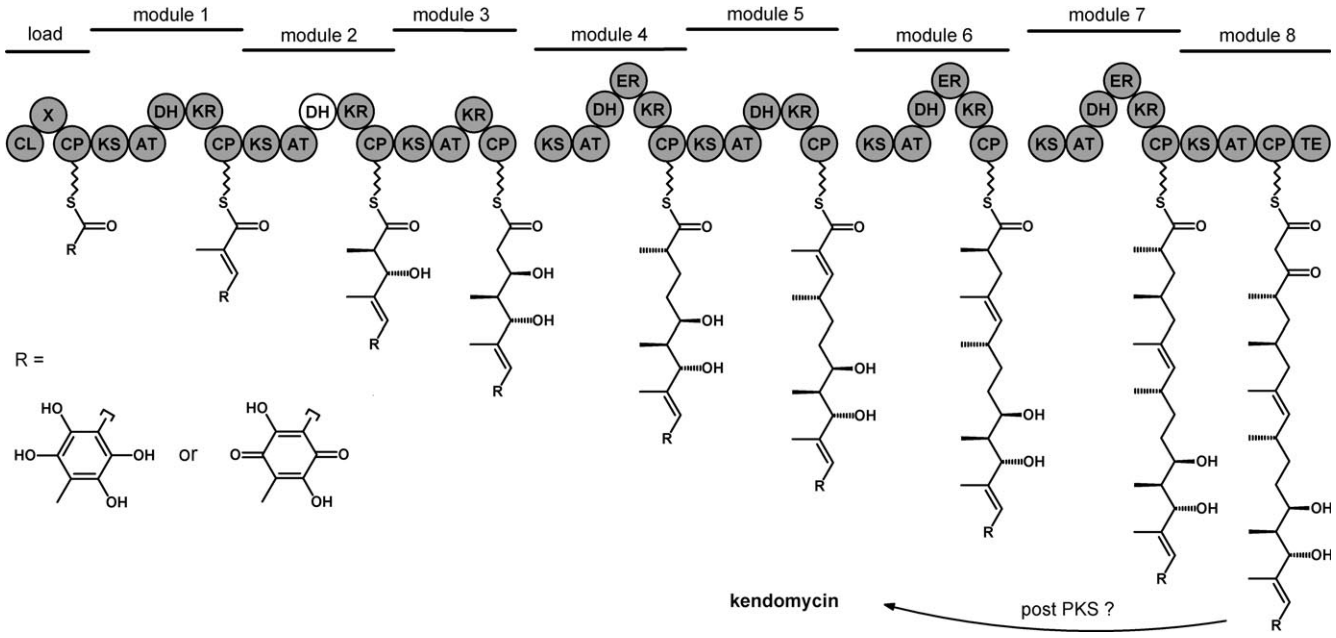


Figure 3. Phenotypic analysis of the *S. coelicolor* host strain that expresses the kendomycin starter unit biosynthetic genes in comparison to the *S. coelicolor* strain that harbors the empty pSET152 expression vector. HPLC diagrams of extracts from *S. coelicolor*::pSET152 A) and *S. coelicolor*::pKen18 B), which were analyzed for the production of 3,5-DHBA, in comparison to 3,5-DHBA authentic reference substance C) The chromatograms show the obtained profiles after selective extraction of negative ions within a mass range of 153.5 +/−0.5. 3,5-DHBA (marked with an asterisk) could only be detected in the host strain that expresses the kendomycin genes (B), but not in the strain with the empty expression vector (A).

one putative operon that is comprised of *ken12–ken16* and harbors one additional gene (*ken15*) that encodes a FAD-dependent monooxygenase. The presence of eight elongation modules in total corresponds with the incorporation of eight extender units (six methylmalonyl-CoA and two malonyl-CoA molecules), which was deduced from previous feeding studies.[4] Based on the results from the in silico AT domain specificity analysis and the presence of different sets of reductive domains within the modules, the assembly line can be assigned as follows: Ken16 (loading module and modules 1–3), Ken12 (modules 4–5), Ken13 (module 6) and Ken14 (modules 7–8 + TE; see Scheme 2). Formation of the kendomycin polyketide core is initiated by activation and covalent binding of the type III PKS-derived starter unit, which is most likely the fully substituted benzoic acid derivative 2,3,5,6-TH-4-MBA (as discussed above). The generation of such unusual starter units and their recruitment by bacterial PKS enzymes was recently reviewed.[40] In contrast to the standard starter units acetyl-CoA or propionyl-CoA, these precursors generally represent more complex structures, which are—in most cases—not provided by housekeeping enzymes of the producer strain, but by a specific set of genes (as discussed above for kendomycin starter unit formation). Among the PKS starter units that are known to date, a number of different benzoic acid derivatives have been identified: benzoate, aminobenzoates, and aminohydroxybenzoates, just to name a few. Interestingly, dihydroxybenzoic acid is speculated to act as starter in quinolidomicin A1 biosynthesis.[40] The free acid group of these precursors has to be activated before loading onto the PKS complex, which can be accomplished by a CoA ligase (CL) domain located within the loading module of the assembly line. The *ken* PKS-loading



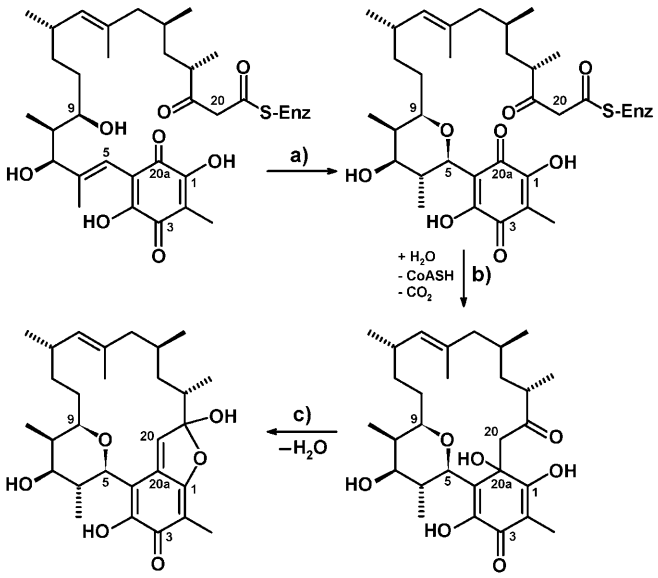
Scheme 2. Kendomycin type I PKS biosynthesis. The type I PKS assembly line consists of four multifunctional proteins (Ken12–14 and Ken16). The DH domain from module 2 is most likely inactive. The fully substituted benzoic acid derivative 2,3,5,6-TH-4-MBA (which is provided by the type III PKS pathway; see Scheme 1) is assumed to be loaded as a starter unit after activation as a CoA ester. Currently, it is not clear whether the starter unit is oxidized to the corresponding benzoquinone derivative by the X domain of the loading module, or if this oxidation happens later during the biosynthesis.

module harbors such a CL domain to select and activate the benzoic acid derivative as a starter unit, which is then transferred and covalently bound to the ACP domain. Interestingly, a third domain of unknown function (X), which shows similarity to KR domains, is inserted between the CL and the ACP domain of the loading module. Sequence analysis reveals that the X domain harbors the conserved sequence motif of the short-chain dehydrogenases/reductases protein family (which is consistent with the observed similarity to KR domains). Most of the members of this family are known to be NAD or NADP-dependent oxidoreductases and possess at least 2 domains (see Interpro entry IPR002198). The first of these binds the coenzyme (often NAD) and the second domain interacts with the substrate. Little sequence similarity was found in the coenzyme-binding domain, although there is a large degree of structural similarity. The conserved NADPH-binding site that is described for KR domains[41] could not be identified in the X domain. However, this does not exclude the presence of another NAD(P)-binding site or a binding site for an alternative cofactor. Thus, the X domain might be the oxidoreductase/dehydrogenase that is involved in quinone formation after loading the aromatic starter unit onto the PKS complex. This speculation is further supported by the observation that the X domain is also present in the PKS-loading modules of the benzoquinone ansamycins geldanamycin[42] and herbimycin,[43] whereas it is absent in loading modules from assembly lines that activate similar aromatic starter units but that produce "non-quinone" compounds (for example, ansamitocin,[44] or soraphen[45]). In current biosynthetic models for geldanamycin and herbimycin, however, the X domain is assumed to be redundant because the starter moiety is speculated to be oxidized after macrolide formation.[43] Whether the X domain is a novel PKS-domain-type with oxidoreductase/dehydrogenase activity or just an inactive KR domain remains to be elucidated.

The starter unit (or the corresponding benzoquinone derivative) is then further processed by the eight elongation modules, which contain different sets of domains to (partially) reduce the β-keto group of the elongated polyketide chains (see Scheme 2). Based on the sequence analysis, all reductive domains (with the exception of the DH domain from module 2) seem to be functional. This DH domain does not contain the conserved histidine (H) or the conserved glycine (G) residue from the consensus motif that was identified by Donadio et al. (HxxxGxxxxP).[46] Although single mutations can be found in the DH core regions from other modules as well (DH1 lacks the conserved glycine and DH6 lacks the conserved proline) these domains are assumed to be functional because similar substitutions in modules Nid3, Rif10, Mta1, and Mta2 from the niddamycin, rifamycin, and myxothiazol PKS do not lead to a loss of the DH activity.[41,47,48] In contrast to the DH domains, none of the annotated KR or ER domains show any mutations in the highly conserved core regions. The identified diagnostic residues for A-type reduction in KR3 and for B-type reduction in all other KR domains[27–29] correlates well with the stereochemistry of the C7 hydroxyl group, the pyran ring oxygen (derived from the C9 hydroxyl group), as well as the *E* configuration of the C13/C14 double bond in the kendomycin structure. After seven elongation steps the last extender unit (malonyl-CoA) is incorporated by module 8, which harbors a C-terminal TE domain. In silico analysis of this domain has shown high similarity to macrocycle-forming TEs such as the DEBS TE, whose crystal structure was recently solved by Stroud and colleagues.[49,50] Its overall tertiary architecture belongs to the α/β-hydrolase family. The TE has an unusual substrate channel that passes through the entire protein and its active site (Asp169-His259-Ser142) is located in the middle of the channel. The catalytic triad together with most of the conserved residues from secondary structural features (which are assumed to form the active site, to line the channel and to be important for the correct fold) could be identified in the kendomycin TE. Its high similarity to macrocycle-forming TE domains was surprising, as the release of the polyketide chain as a macrolactone is not consistent with the carbocyclic *ansa* framework of kendomycin. However, a few variations from the postulated TE consensus were detected in the Ken-TE (Figure S1) and further evaluation is necessary to determine if these minor differences lead to an altered release mechanism (for example, hydrolysis under formation of the free acid instead of macrolactonization), or even to the inactivity of the domain. Alternatively, conventional macrocyclization occurs, and subsequently the resulting lactone is reopened, which could, for example, be catalyzed by the putative lipase encoded by *ken17*.

Formation of the kendomycin *ansa* framework

To form the unique macrocyclic scaffold of kendomycin, the fully elongated polyketide chain that is bound to module 8 needs to undergo two major modifications: pyran ring formation between C5 and C9, which can also occur during the assembly process or after the macrocyclization step, as well as cyclization via C–C bond formation between C20 and the *ortho*-position of the aromatic starter moiety. A putative pathway and reaction mechanism for these transformations was postulated previously,[4] and is shown in Scheme 3 with minor modifications (which result from the analysis of the reductive domains in the type I PKS assembly line described herein). Formation of the pyran ring might be initiated by attack of C9 hydroxyl group to C5, as previous feeding studies revealed that the oxygen originates from a propionate extender unit.[14] A couple of different biosynthetic mechanisms for the generation of pyran rings or similar heterocycles have already been discussed in the literature (for example, jerangolid/ambruticin,[51] kirromycin[52] or aureothin[53] biosynthesis). Based on our current data however, it is hardly possible to propose a likely scenario for kendomycin biosynthesis. The supposed benzoquinone structure of the oxidized starter unit (which might be generated by the X domain from the loading module or later on by another dehydrogenase activity, for example, Ken19) would facilitate the ring closure by an aldol condensation with the "carboxy terminus" of the polyketide chain. The loss of C1 from the last incorporated malonate unit (most likely through carbon dioxide elimination) during cyclization was unambiguously proven by previous labeling studies.[14] Further elimination of water from the cyclization product (which might occur



Scheme 3. Formation of the unique macrocyclic scaffold of kendomycin. The polyketide chain that is generated from the type I PKS assembly line (see Scheme 2) needs to undergo further modifications to form the kendomycin quinone methide chromophore (see text). A mechanism for these modifications was proposed previously by Bode and Zeeck[4] and is shown here with minor modifications. a) pyran ring formation, b) macrocyclization and c) dehydration and hemiacetal formation.

spontaneously) and hemiacetal formation between the hydroxy group at C1 and the carbonyl group at C19 would finally lead to the unique quinone methide chromophore of kendomycin (see Scheme 3). In addition to the genes for the starter unit biosynthesis (*ken2–9*), type I PKS assembly (*ken12–14*, *ken16*) and regulation (*ken10*, *ken21*), six additional genes (*ken1*, *ken15* and *ken17–20*) could be identified within the kendomycin gene cluster region. Ken20 shows similarity to integral membrane proteins from *S. coelicolor* A3(2) and *S. erythraea* NRRL 2338 and has probably no biosynthetic function in kendomycin assembly. The gene product of *ken1* shows high homology to type II thioesterases. These enzymes are often associated with type I PKS or nonribosomal peptide synthetase (NRPS) megasynthetases and are assumed to serve an editing function, and for example, remove aberrantly loaded substrates from the assembly line.[54,55] Interestingly, the protein that is encoded by *ken18* shows poor similarity to MdpA from *Actinomadura madurae*, which was recently characterized as a new type of endiyne apo protein.[30] These proteins are assumed to be required to protect and transport the highly labile endiyne chromophore, which acts as the cytotoxic component by reacting with DNA, and leading to strand cleavage.[30,56] Based on its similarity to MdpA, it can be speculated that Ken18 forms a complex with kendomycin, or more likely with a biosynthetic precursor during/after the post-PKS modifications. The remaining three genes (*ken15*, *ken17* and *ken19*) encode a putative FAD-dependent monooxygenase (Ken15), a putative lipase (Ken17), as well as a putative FAD-dependent oxidoreductase (Ken19), and might be involved in the modification of the kendomycin polyketide chain. In particular, Ken19, which harbors a berberine bridge enzyme (BBE)-like domain in addition to the FAD-binding domain, is a promising candidate for the catalysis of the macrocyclization process. This domain is found in the berberine bridge enzymes and berberine bridge-like enzymes, which are involved in the biosynthesis of numerous isoquinoline alkaloids.[57] They catalyze the transformation of the *N*-methyl group of (*S*)-reticuline into the C8 berberine bridge carbon of (*S*)-scoulerine (and consequently the formation of a C–C bond). The same domain arrangement could be detected in SpnJ from *Saccharopolyspora spinosa*[58] and EncM from *Streptomyces maritimus*,[59] for instance. Although Ken19 shows only weak overall sequence similarity to EncM (30% identity, 40% homology), it should be mentioned that this enzyme (in which only a FAD and a BBE domain could be detected) exhibits a remarkable set of catalytic properties. EncM is involved in enterocin biosynthesis and not only catalyzes a Favorskii-like oxidative rearrangement, but also must facilitate two aldol condensations as well as two heterocycle-forming reactions.[60] SpnJ shows higher sequence homology to Ken19 (48% identity and 60% homology) and is involved in spinosad biosynthesis, which includes the formation of three intramolecular carbon–carbon bonds to build the unusual *as*-indacene skeleton. Recent studies have shown that SpnJ is responsible for the oxidation of a hydroxyl group (C15) after TE-mediated macrolactone formation;[61] this was suggested earlier by Martin et al. according to evidence from spinosyn PKS heterologous expression experiments.[62] Oxidation is required for the subsequent cyclizations (possibly through a Diels–Alder mechanism) and thus the involvement of SpnJ in the cross-bridging reactions cannot be excluded (although there is no evidence for this based on the in vitro data). Interestingly, the spinosad biosynthetic gene cluster also encodes a putative lipase (SpnM), which is assumed to play a role in the polyketide bridging reactions.[58] Although Ken17 belongs to another class of lipases (platelet-activating factor acetylhydrolase, or PAF-AH, a subfamily of phospholipases A2), it might be involved in the polyketide bridging reactions as well, and might thus be essential for the formation of the unique macrocyclic scaffold of kendomycin. However, the precise functions of the putative kendomycin-modifying enzymes (Ken15, Ken17 and Ken19) are difficult to predict by in silico analysis alone, because the biochemical mechanism(s) of pyran ring formation and especially the unique macrocyclization are not yet understood. In addition, similar types of reactions have not been well studied in other secondary metabolite-producing microorganisms, which makes the assignment of putative functions through the study of sequence similarities difficult or even impossible. Furthermore, it can not be excluded that additional genes involved in the kendomycin biosynthesis are encoded in the flanking regions of the sequenced *ken* cluster or are located somewhere else in the chromosome.

Clearly, future experimental studies are required to gain deeper insights into the mechanisms and enzyme activities that are involved in the biosynthesis of the unique macrocyclic scaffold of kendomycin. Because the natural producer is very difficult to mutagenize and no protocols for markerless gene deletions in the kendomycin producer could be established to date (SCW, RM, unpublished), a heterologous expression sys-

tem for the entire biosynthetic gene cluster is currently being developed in our group.

Genes involved in regulation and resistance

Two genes that are possibly involved in regulation could be identified in the *ken* biosynthetic gene cluster. The *ken11* gene product shows homology to putative transcriptional regulators of the HxlR family, which harbor a characteristic HxlR-type HTH domain (see Interpro entry IPR002577). The domain is named after HxlR, a DNA-binding protein that acts as a positive regulator of the formaldehyde-inducible *hxlAB* operon in *Bacillus subtilis*.[63] At the opposite end of the gene cluster is located *ken21*, which encodes a putative transcriptional regulator that belongs to the MarR family. MarR-type regulators contain a DNA-binding, winged helix–turn–helix (wHTH) domain (MarR-type HTH domain) and are named after *E. coli* MarR, a repressor of the *mar* operon. The Mar proteins are involved in multiple antibiotic resistance and represent a nonspecific resistance system.[31] A large number of compounds induce transcription of the *mar* operon. This is thought to be due to the binding of the compounds to MarR, which leads to a loss of DNA-binding activity. With MarR repression lost, transcription of the operon proceeds. Based on this, the putative MarR-type regulatory protein Ken21 could control antibiotic (and kendomycin) resistance in the producer strain *S. violaceoruber*.

Conclusion

Kendomycin is a structurally unique polyketide with an impressive biological activity profile. Its novel molecular architecture represents an attractive lead structure for further development in cancer chemotherapy. Furthermore, the compound might also serve as a useful tool to study novel aspects of proteasome-mediated cell biology.[6] However, kendomycin has a complex structure and thus its synthesis by standard synthetic routes is highly laborious. The identification of the kendomycin biosynthetic machinery represents an important step towards the improvement of the production yields and the generation of novel derivatives. Future development of a heterologous expression system for the entire biosynthetic gene cluster will be crucial to engineer the pathway and to gain deeper insights into the formation of the unique type III/type I PKS derived macrocyclic scaffold.

Experimental Section

Bacterial strains and general methods: *E. coli* strains were cultured in Luria Broth (LB) at 37 °C. Streptomyces strains *S. violeoruber* (strain 3844-33C)[64] *S. lividans* TK24[65] as well as *S. coelicolor* A3(2)[66] and their descendants were grown in tryptic soy broth (TSB) medium or mannitol soya (MS) medium[67] at 30 °C. The antibiotics apramycin sulfate (60 $\mu g\,mL^{-1}$), kanamycin sulfate (60 $\mu g\,mL^{-1}$), ampicillin (100 $\mu g\,mL^{-1}$), chloramphenicol (25 $\mu g\,mL^{-1}$), and zeocin (25 $\mu g\,mL^{-1}$) were added where appropriate. pBluescript II SK+ or pBC SK+ (Stratagene, La Jolla, CA, USA) and pCR-XL-TOPO (Invitrogen, Carlsbad, CA, USA) were used as routine vectors for subcloning. The *Streptomyces* knock-out vector pKC1132[67] was used for the gene inactivation experiment, and the *E. coli/Streptomyces* shuttle vector pSET152[67] was used for the expression studies in streptomycetes. Plasmids that were generated in this study are summarized in Table S1. DNA isolation, plasmid preparation, restriction digestions, gel electrophoresis, and ligation reactions were carried out according to standard methods.[68]

Construction and screening of a cosmid library: Genomic DNA was isolated from *S. violaceoruber* (strain 3844-33C) by using an established protocol.[67] The DNA was partially digested with *Sau*3A by using a serial dilution method, and ligated in *Bam*HI-hydrolysed SuperCos-1 (Stratagene). Ligations were packaged by using the Gigapack III Gold Packaging Extract (Stratagene) and transfected into *E. coli* SURE (Stratagene) according to the manufacturer's protocol. 2300 colonies were picked in 96-well microtiter plates and cultivated in LB medium that was amended with ampicillin (50 $\mu g\,mL^{-1}$) at 37 °C over night. Duplicates of the clones were transferred onto nylon membranes (Roche Molecular Biochemicals, Mannheim, Germany) as described in the DIG systems user guide for filter hybridization. For the screening of the cosmid library with homologous probes, degenerate oligonucleotides (EAKSC 5′-MGI-GARGCIYTIGCIATGGAYCCICARCARMG-3′ and LCK5NC 5′-GGRT-CNCCIARYTGIGTICCIGTICCRTGIGC-3′) and Taq DNA polymerase (GIBCO BRL, Eggenstein) were used to amplify ketosynthase (KS) fragments of approximately 750 bp in size from genomic DNA of *S. violaceoruber* strain 3844–33C. PCR conditions were as follows: initial denaturation 5 min at 97 °C; denaturation 30 s at 97 °C; annealing 30 s at 55 °C; extension 50 s at 72 °C; 30 cycles; addition of 5% dimethylsulfoxide to the PCR mixture. The generated product was labeled with 50 μCi α-^{33}P-dCTP (Hartmann Analytik, Braunschweig, Germany) and used for hybridization experiments in a buffer that contained 50% formamide at 42 °C with stringent washes at 68 °C. For the detection, Fuji imaging plates and a phosphoimager were used. From approximately 2300 cosmid clones, 96 gave strong signals. Cosmid DNA from all 96 clones was isolated and electroporated into *E. coli* XL1-Blue MRF′ (Stratagene) according to standard protocols.[68] After reisolation from *E. coli* XL1-Blue MRF′, the cosmid DNA was hydrolyzed with EcoRI/BamHI. Southern analysis was performed by using a heterologous probe based on the *dpgA*, *dpgB*, *dpgC* and *dpgD* genes from the balhimycin pathway of *Amycolatopsis mediterranei*.[22] The probe was generated by EcoRI/ClaI restriction of pVP5,[22] gel purification of the 0.96 and 2.95 kb fragments by using the Nucleospin Extract Kit (Macherey–Nagel, Düren, Germany), and labeling of the fragments with 50 μCi α-^{33}P-dCTP (Hartmann, Braunschweig, Germany). From the 96 cosmids, five gave strong signals after hybridization with the *dpgA-dpgB-dpgC-dpgD* probe (cosmid A7, C7, E12, F3, and H4). DNA from these five cosmids was isolated by using the Nucleospin Plasmid Kit (Macherey–Nagel, Düren, Germany), submitted for end sequencing, and the data were analyzed by BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). Cosmid H4 was completely sequenced (GATC Biotech, Konstanz, Germany). To detect cosmids that overlapped with the T3 end of cosmid H4, the sublibrary that contained the 96 KS cosmids (see above) was hybridized with a probe homologous to the T3 end of cosmid H4. The probe was generated by PCR by using the oligonucleotides H4,T3.3 (5′-CTG TTC GCG CCA GGT CAC-3′) and H4,T3.4 (5′-GTC CGC GAC GGC TGG TAC-3′) and HotStarTaq DNA polymerase (Qiagen, Hilden, Germany); PCR conditions were as follows: initial denaturation and activation of the DNA polymerase 15 min at 95 °C; denaturation 20 s at 94 °C; annealing 30 s at 57 °C; extension 40 s at 72 °C; 32 cycles. The 456 bp PCR product was gel purified by using the Nucleospin Extract Kit (Macherey–Nagel). For the hybridization experiment the DIG High Prime DNA labeling and detection Kit (Boehringer, Mann-

heim, Germany) was used. Strong signals could be detected for the cosmids B9, D11, F3, F10, and G2. DNA from these cosmids was isolated by using the Nucleospin Plasmid Kit (Macherey–Nagel) and submitted for end sequencing. Fragments upstream and downstream to the cosmid H4 insert were subcloned for sequencing (pKen14, pKen15, pKen22, pKen24, pKen26; see Figure 1 and Table S1). Sequencing was performed after in vitro transposon mutagenesis by using GPS-1 (NEB, Beverly, USA) according to the manufacturer's protocol and/or by primer walking. The complete nucleotide sequence of the kendomycin biosynthetic gene cluster was assembled by using the Lasergene software package (DNASTAR Inc.). Sequence analysis was performed by using FramePlot 2.3.2 (http://www.nih.go.jp/~jun/cgi-bin/frameplot.pl), BLAST (http://www.ncbi.nlm.nih.gov/BLAST/), Pfam 5.5 (http://www.mrc-lmb.cam.ac.uk/genomes/madanm/pres/pfam1.htm and the PKS/NRPS Analysis Web-server (http://www.tigr.org/jravel/nrps/). The nucleotide sequence that is reported here is available under accession number AM992894 (EMBL database).

Inactivation of the kendomycin biosynthetic gene cluster: After sequencing of cosmid H4, two oligonucleotides (Ken14: 5′-GCC ACC CCC GGA GTC TTC-3′ and Ken15: 5′-CAC AGC AGC CGG AAC CAC-3′) were designed and used to amplify a PKS fragment from *ken12*. The amplified fragment (1.25 kp) was cloned into pCR2.1-TOPO (Invitrogen) to give in plasmid pKen19. After *Eco*RI restriction the corresponding fragment was ligated into the knock-out vector pKC1132, which was introduced into *S. violaceoruber* strain 3844–33C by conjugation according to the procedure that was described in ref. [67], but by using fresh mycelium instead of spores. The introduced plasmids were integrated into the genome by homologous recombination; this led to the gene disruption mutant *S. violaceoruber*::pKen41. The integration of the plasmid was verified by genomic Southern blot analysis by using a fragment from the apramycin gene as a probe (data not shown). For phenotypic analysis, the mutant strain as well as *S. violaceoruber* wild-type were cultivated in 50 mL TSB liquid medium that contained 2% XAD for 4 days at 30 °C. The cells together with the XAD were harvested by centrifugation and extracted with acetone. To compare the spectrum of secondary metabolites that were produced by the mutant with that of the wild-type strain, concentrated MeOH extracts were analyzed by HPLC–MS (conditions see below).

Heterologous expression of the *ken1–7* operon in *Streptomyces* strains: A 14.5 kb EcoRI fragment was subcloned into the *E. coli*/*Streptomyces* shuttle vector pSET152[69] that featured an *oriT* RK2 for conjugation, the apramycin-resistance gene *aac(3)IV* for selection and the ΦC31-derived attachment site (*attP* ΦC31) and integrase (*int* ΦC31) for site-specific integration into the streptomycete chromosome. The resulting plasmid pKen18, which harbored the genes *ken1–7* (and the 5′ end of *ken12*) as well as the empty expression vector pSET152 was transferred into *S. lividans* TK24 and *S. coelicolor* A3(2) by conjugation by using an established protocol.[67] The obtained conjugants were selected and cultivated on MS medium[67] that had been amended with apramycin (60 μg mL^{-1}). Successful integration of the expression construct into the *Streptomyces* chromosome was analyzed by PCR by using the oligonucleotides apra_for (5′-GTGCAATACGAATGGCGAAA-3′) and apra_rev (5′-TCAGCCAATCGACTGGCGAG-3′) to amplify a 777 bp fragment from the apramycin-resistance gene. HotStarTaq DNA polymerase (Qiagen) was used, and the PCR conditions were as follows: initial denaturation and activation of the DNA polymerase 15 min at 95 °C; denaturation 20 s at 94 °C; annealing 30 s at 56 °C; extension 60 s at 72 °C; 32 cycles. After cultivation of the mutant strains for 4 days at 30 °C, the mycelium was separated by centrifugation and extracted with acetone. The extract was evaporated, redissolved in methanol, and analyzed for the production of 3,5-dihydroxybenzoic acid.

HPLC–MS analysis of the extracts: High-performance liquid chromatography–mass spectrometry (HPLC–MS) was used to analyze the mutant extracts in comparison to an extract from the wild-type (or the mutant that harbored the empty expression vector) that was grown under the same conditions. An Agilent 1100 series solvent delivery system that was coupled to Bruker HCTplus ion trap mass spectrometer was used. Chromatographic separation was carried out on an RP column Nucleodur C18 (125 by 2 mm, 3 μm particle size; Macherey and Nagel) that was equipped with a precolumn C18 (8×3 mm, 5 μm). For the analysis of the kendomycin knock-out mutant (*S. violaceoruber*::pKen41) the mobile phase gradient (solvent A: H_2O + 0.1% formic acid and solvent B: acetonitrile + 0.1% formic acid) was linear from 5% B at 2 min to 95% B at 32 min, followed by 4 min with 95% B at a flow rate of 0.4 mL min^{-1}. Diode-array detection was carried out at 200–600 nm and mass detection was performed in the positive and negative-ionization mode within a range of 100–1100 amu. For the analysis of the host strains that contained the kendomycin starter unit biosynthetic genes (*S. coelicolor*::pKen18 and *S. lividans*::pKen18), the mobile-phase gradient (solvent A: water + 0.1% formic acid and solvent B: acetonitrile + 0.1% formic acid) was linear from 5% B at 2 min to 10% B at 5 min and from 10% B at 5 min to 95% B at 9 min, followed by 1 min with 95% B at flow rate of 0.4 mL min^{-1}. Diode-array detection was carried out at 254 nm, and mass detection was done in negative-ionization mode. 3,5-Dihydroxybenzoic acid was identified by comparison to the retention time and the MS^2 pattern of an authentic reference standard (*m/z* 153 $[M-H]^-$; MS^2: *m/z* 109 $[M-H-CO_2]^-$). Quantification was carried out in manual MS^2 mode. Ions of *m/z* 153 $[M-H]^-$ were collected and subjected to fragmentation. Peak integration of the characteristic fragment ions *m/z* 109 was carried out by using the Bruker QuantAnalysis v1.6 software package. A calibration curve was established from serial dilutions of 3,5-dihydroxy benzoic acid down to 1 μg mL^{-1}.

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Keywords: biosynthesis · kendomycin · natural products · polyketides · *Streptomyces* · type III PKS

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