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Biosynthesis of Macrolactam BE-14106 Involves Two Distinct PKS Systems and Amino Acid Processing Enzymes for Generation of the Aminoacyl Starter Unit

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SUMMARY

BE-14106 is a macrocyclic lactam with an acyl side chain previously identified in a marine-derived *Streptomyces* sp. The gene cluster for BE-14106 biosynthesis was cloned from a *Streptomyces* strain newly isolated from marine sediments collected in the Trondheimsfjord (Norway). Bioinformatics and experimental analyses of the genes in the cluster suggested an unusual mechanism for assembly of the molecule. Biosynthesis of the aminoacyl starter apparently involves the concerted action of a distinct polyketide synthase (PKS) system and several enzymes that activate and process an amino acid. The resulting starter unit is loaded onto a second PKS complex, which completes the synthesis of the macrolactam ring. Gene inactivation experiments, enzyme assays with heterologously expressed proteins, and feeding studies supported the proposed model for the biosynthesis and provided new insights into the assembly of macrolactams with acyl side chain.

INTRODUCTION

Macrocyclic lactams (macrolactams) have been shown to have a wide range of activities, e.g., bactericidal (Zafriri et al., 1981), antiviral (Naruse et al., 1991), cytotoxic (Futamura et al., 2008), antifungal (Jakobi et al., 1996), and antiprotozoal (Jomon et al., 1972). BE-14106 is a macrolactam antibiotic exhibiting antibacterial, antifungal, and anticancer activity first described in 1992, when the compound was isolated from a *Streptomyces* strain designated as *S. spheroids* A14106 (Kojiri et al., 1992). A second *Streptomyces* producer of BE-14106 and 8-deoxy BE-14106 was described some years later (Takahashi et al., 1997). In both cases activity against cancer cell lines was shown, but the mechanism behind BE-14106's anticancer properties has not been elucidated. The structure of BE-14106 is shown in Figure 1 along with similar small-ring macrolactams.

Currently, there are no macrolactams in use as antineoplastic agents, but a derivative of the ansamycin antibiotic geldanamycin has completed phase I clinical trials (Nowakowski et al., 2006) and several more have been described that have anticancer activity. Vicenistatin was active against xenographed models of certain human colon cancers (Shindo et al., 1993), hitachimycin/stubomycin and its derivatives had inhibitory activity against murine tumors (Umezawa et al., 1981; Komiyama et al., 1982; Shibata et al., 1988, 1989a, 1989b), aureoverticillactam was found to have cytotoxic activity against several tumor cell lines (Mitchell et al., 2004), and leinamycin and its derivatives have shown antitumor activity against several types of tumors and cancer cell lines (Hara et al., 1989a, 1989b; Kanda et al., 1998, 1999, 2003; Bassett et al., 2004).

In the biosynthetic pathway for ansamycins (e.g., Rascher et al., 2003; Yu et al., 2002) 3-amino-5-hydroxybenzoic acid usually represents the starter unit, while amino acids or amino acid derivatives are used as starters for the synthesis of vicenistatin (Ogasawara et al., 2004), leinamycin (Cheng et al., 2003), and salinilactam (Udwary et al., 2007). The biosynthetic gene clusters for macrolactams often contain both polyketide synthases (PKS) and enzymes similar to nonribosomal peptide synthetase (NRPS) domains, and the biosyntheses appear to involve a cooperative action of these two types of enzyme systems. The biosynthesis of leinamycin starts with the activation of D-alanine by a discrete adenylation (A) domain and loading on a discrete peptidyl carrier protein (PCP) domain (Tang et al., 2007). The discrete A and PCP domains are thought to represent the loading module for the biosynthesis and chain elongation proceeds through one additional NRPS module and six PKS modules (Cheng et al., 2003). None of the PKS modules contain the usual acyltransferase (AT) domain for activation and loading of the building blocks and malonyl-CoA units are instead supplied in *trans* by a discrete acyltransferase. The PKS enzymes involved in the biosynthesis of vicenistatin are more conventional in the sense that all extender modules include the core β-ketosynthase (KS), AT, and acyl carrier protein (ACP) domains (Ogasawara et al., 2004). The loading module is represented by a single ACP domain, which presumably accepts the amino acid-derived starter 3-methylaspartate. The vicenistatin biosynthetic gene cluster encodes several enzymes assumed to be involved in synthesizing the starter, and Ogasawara et al. (2004) proposed several possible pathways for the generation of 3-methylaspartate from glutamate. In analogy to the vicenistatin biosynthesis,

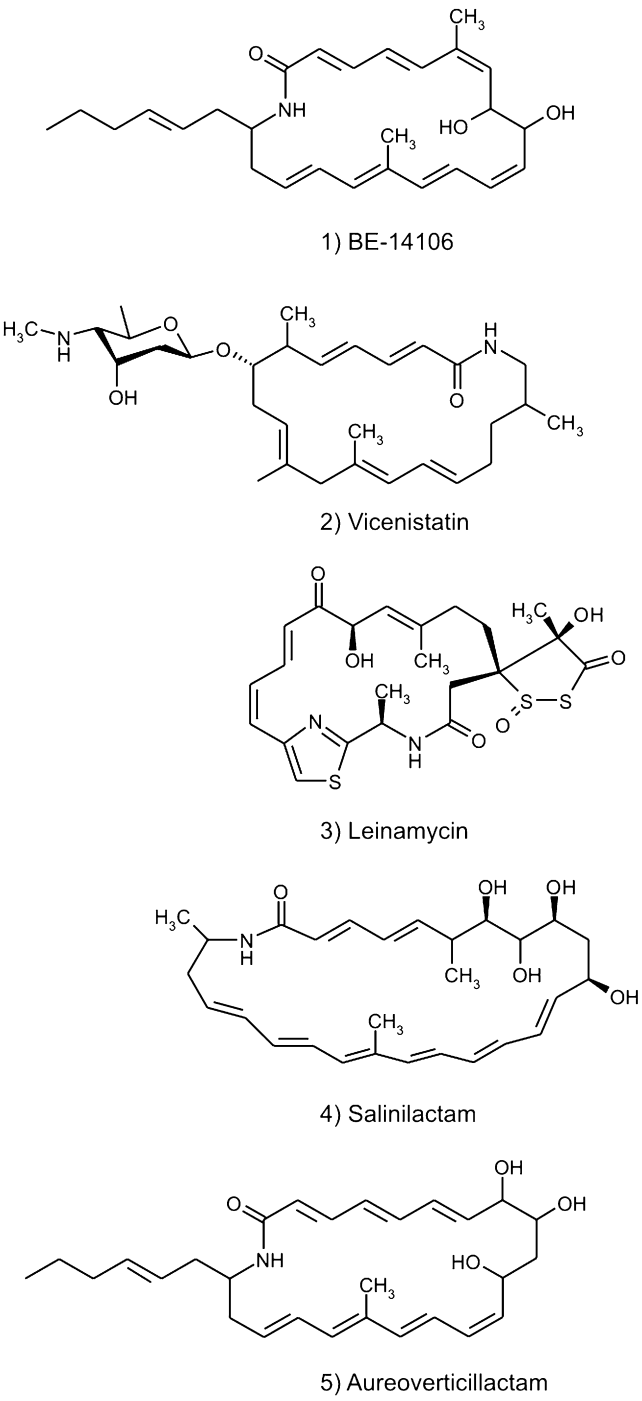

Figure 1. Structures of BE-14106 and Similar Small-Ring Macrolactams

salinilactam is synthesized by a PKS system using an amino acid as the starter. A lysine derivative was assumed to represent the starter; however, the details of the starter unit synthesis have not been elucidated (Udwary et al., 2007). Judging from the biosynthesis of these different macrolactams, nature has apparently generated several possible pathways for producing such compounds.

In this study we report the isolation of a BE-14106-producing *Streptomyces* strain from marine sediment, confirm and extend the knowledge about the antifungal and cytotoxic activity of BE-14106, describe the sequencing and analysis of the BE-14106 biosynthetic gene cluster, and propose a model for its biosynthesis.

RESULTS

Isolation of a New BE-14106 Producer from Marine Sediment

Streptomyces sp. DSM 21069 was isolated as a part of a screening effort aimed at identifying new antifungal compounds produced by marine actinomycetes. The screening included spectroscopic analysis of DMSO extracts coupled to an assay for activity against *Candida* strains (see Experimental Procedures). The screening resulted in the identification of a group of ~30 actinomycete isolates, including *Streptomyces* sp. DSM 21069, which could be assumed to produce non-polyene antifungal compounds based on the UV profile (lack of characteristic polyene absorption peaks) and the biological activity (comparable inhibition of both polyene-resistant and -sensitive strain).

DMSO extracts from *Streptomyces* sp. DSM 21069 and other isolates selected from this group were subjected to liquid chromatography fractionation, identification of bioactive fractions, and LC-MS-TOF analysis as described in Experimental Procedures. In LC-MS analysis of bioactive fractions from six extracts, significant peaks with a molecular mass corresponding to the macrolactam BE-14106 were identified. The molecular mass observed in the LC-MS-TOF analysis was within 1 ppm of the molecular mass given in Database of Natural Products (DNP; accurate mass 423.277344) (see Figure S1 available online). In addition, the DAD profiles of these extracts correlated well with the information given in DNP about the UV absorbance spectra of BE-14106. Although BE-14106 is apparently a tetraene (Figure 1), its UV profile does not have a polyene-characteristic three peak spectrum (data not shown), thus explaining why these extracts were not disqualified during the screening for non-polyene antifungals. The six isolates found to produce the BE-14106-like compound were morphologically similar and taxonomic characterization by analysis of 16S rDNA from three out of the six strains showed that they presumably represented replicates of the same strain (data not shown). *Streptomyces* sp. DSM 21069 was chosen for further work since it provided the highest yield of presumed BE-14106 (~1 g/liter in shake flasks).

The BE-14106-like compound was extracted to produce a crude product (see Experimental Procedures) and purified to ~99% purity by preparative HPLC. The biological assays using the yeast strains *Candida albicans* and *Candida glabrata* (see Experimental Procedures) determined that the lowest concentrations resulting in >50% inhibition of the two strains (IC_{50}) were 7.1 and 3.5 μM, respectively (Table 1).

The purified compound was further tested against human Jurkat-T lymphoma cells and normal rat kidney epithelial cells (NRK). The IC_{50} value for the compound based on the WST signal (metabolic activity) was 4.5 and 7.0 μM, respectively (Table 1), i.e., its potency was similar to that against the fungal strains. Importantly, the LC_{50} value based on the morphologically evident apoptosis was similar (data not shown), indicating that

Table 1. Biological Activity of BE-14106

Cell Line/Strain (ATCC no.)	Origin	IC_{50}[a] (μM)
Jurkat-T (TIB-152)	Human T cell lymphoma	4.5 ± 1.1
NRK (CRL-6509)	NRK	7.0 ± 1.4
C. albicans (CCUG3943)	Clinical isolate	7.1
C. glabrata (CCUG3942)	Clinical isolate	3.5

[a] The IC_{50} values for cell lines was determined by the WST-1 assay and the IC_{50} values for *Candida* strains were determined as the lowest concentration resulting in >50% reduction in growth (optical density at 660 nm) as compared to reference samples containing the solvent (DMSO).

the presumed BE-14106 acted by inducing apoptotic cell death rather than by merely arresting cell growth.

The identity of the purified compound as BE-14106 was then confirmed by NMR spectroscopy. The chemical shifts observed for both the ^{1}H and ^{13}C nuclei (Table S1) were almost identical to those earlier reported for BE-14106 (Kojiri et al., 1992). Since chemical shift is an extremely sensitive indicator of the nucleus environment, and thus of the structure of a molecule, the NMR results unambiguously confirmed that the compound isolated was authentic BE-14106.

Cloning of the BE-14106 Biosynthetic Gene Cluster

To elucidate the pathway for BE-14106 biosynthesis, the corresponding biosynthetic gene cluster was subsequently cloned from *Streptomyces* sp. DSM 21069 (see Supplemental Experimental Procedures for details). In essence, DNA fragments encoding parts of KS domains of modular PKS were PCR amplified, sequenced, and analyzed. This resulted in identification of one fragment encoding part of a KS domain with 73% identity to the VinP1 PKS involved in the biosynthesis of the macrolactam vicenistatin in *Streptomyces halstedii* (Ogasawara et al., 2004). To ensure that this DNA fragment belonged to the BE-14106 biosynthetic gene cluster, a gene inactivation experiment using this fragment was performed (see Supplemental Experimental Procedures), which resulted in complete abrogation of BE-14106 biosynthesis. Using this fragment, and subsequent chromosome "walking," four overlapping cosmids were identified in the DSM 21069 genomic library. End sequencing of the cosmids suggested that they encompassed the complete BE-14106 biosynthetic gene cluster. Next, KS-encoding DNA fragments were amplified from cosmids 2 and 3 using one cosmid primer and one degenerate primer for the KS domain. A 1.1 kb fragment was obtained for cosmid 2 and a 3.7 kb fragment for cosmid 3. Both fragments were cloned, sequenced, and used to construct gene inactivation vectors. The vectors were transferred into *Streptomyces* sp. DSM 21069 by conjugation and the transconjugants obtained were analyzed for production of BE-14106. This analysis demonstrated almost complete abrogation of BE-14106 biosynthesis when the 1.1 kb fragment was used, and >99% reduction of yield for the 3.7 kb fragment. Based on these results it was concluded that cosmids 1–4 contained the BE-14106 biosynthetic cluster and the four cosmids were fully sequenced.

Gene Organization within and around the BE-14106 Biosynthetic Gene Cluster

The genomic region represented by the sequenced cosmids encompasses 85,059 bp and contains, according to the FramePlot (Ishikawa and Hotta, 1999) and BLAST analyses, 27 complete and 1 incomplete open reading frames (*orf*s; Figure 2). Results of the bioinformatics analysis of predicted gene products with proposed functions are presented in Table 2. Homologs from the biosynthetic gene clusters of the structurally similar macrolactams vicenistatin and salinilactam have also been included in the table. Initially, based on the structural features of BE-14106 and general knowledge of the organization of secondary metabolite biosynthetic gene clusters, 21 of the identified *orf*s were suggested to be involved in the biosynthesis of this compound. The PKS genes in the cluster are represented by the genes *becA–G*, which are organized in four subclusters (Figure 2). Interestingly, both *becA* and *becC* are truncated at 3′ and 5′ ends, respectively, thus encoding truncated PKS proteins. Although this feature has been described for several other secondary metabolite gene clusters (e.g., Duitman et al., 1999; Kopp et al., 2005; Piel et al., 2004; Silakowski et al., 2001), we could not exclude that rearrangements might have occurred during cloning or there might have been mistakes in the assembly of the sequences. We therefore decided to verify the organization of the *becA-becC* region in the chromosome of *Streptomyces* sp. DSM 21069. For this purpose, a 4.4 kb DNA fragment encompassing the 3′ end of *becA* and the 5′ end of *becB* was amplified by PCR and subjected to restriction analysis. Data obtained (data not shown) were fully consistent with the proposed DNA sequence of this region, thus ruling out the possibility of rearrangements during cloning or mistakes in the assembly.

Besides the PKS genes presumably involved in the synthesis of the macrolactam ring and the C20-C25 acyl side chain (see below), the genes predicted to be involved in the activation and modification of the nitrogen-donating amino acid have been identified. These genes, *becJ*, *S*, *L*, and *K*, were localized at the center of the gene cluster and encode an AMP-dependent acyl-CoA synthetase/ligase, a discrete PCP, a discrete NRPS adenylation domain, and a discrete acyltransferase, respectively (see Discussion).

Genes *becH* and *becM* presumably encode transcriptional regulators that control the biosynthesis of BE-14106. BecH was homologous of several LuxR-type transcriptional regulators, e.g., GdmRI from the geldanamycin biosynthesis in *Streptomyces hygroscopius* (He et al., 2008) and NysRI from the nystatin biosynthesis in *Streptomyces noursei* (Sekurova et al., 2004). BecM showed homology to TetR-type transcriptional regulators and the closest match was Strop_2766, whose gene sequence is located close to the salinilactam biosynthetic gene cluster in *Salinispora tropica*.

In addition to the aforementioned *becK*, a second putative acyltransferase, *orf10*, located at the right flank of the cluster was identified. *orf10* encodes a homolog of PlsC-type phospholipids/glycerol acyltransferases and could potentially have a role in the biosynthesis of BE-14106. A gene inactivation experiment affecting *orf10* had, however, no effect on the BE-14106 production (data not shown).

A gene encoding thioesterase type II (TEII), *becQ*, was identified upstream of the *orf10* gene. Such thioesterases are often associated with PKS and NRPS clusters and are thought to be responsible for deblocking ACP/PCP domains that have been misacylated (Kim et al., 2002; Schwarzer et al., 2002). TEIIs

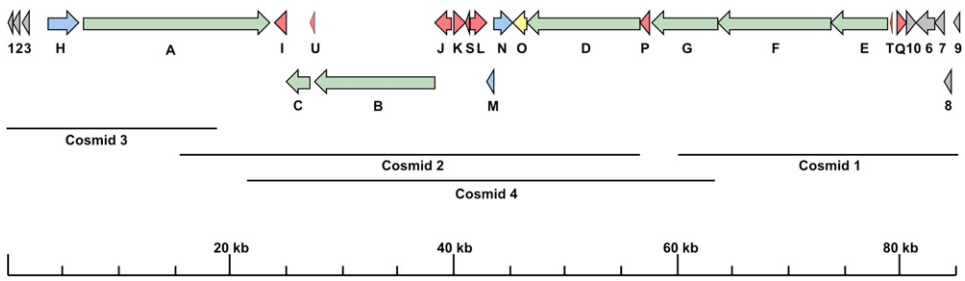

Figure 2. Organization of the BE-14106 Biosynthetic Gene Cluster in *Streptomyces* sp. DSM 21069

PKS, green; genes presumed to be involved in modification of aminoacyl starter, red; regulators/efflux pump, blue; post-PKS modification, yellow; not involved in biosynthesis of BE-14106, gray. Coverage of the four sequenced cosmids is shown.

have also been ascribed a role in the modification of starter amino acid units. In the chloroeremomycin and the nikkomycin biosynthesis, TEIIs have been suggested to be responsible for releasing the amino acid from the PCP domain after initial modification (Chen et al., 2001). BecQ is also homologous to Strop_2763, a putative thioesterase whose corresponding gene is located quite close to the salinilactam biosynthetic gene cluster in *Sal. tropica*.

The *becO* gene encoding a putative P450 monooxygenase was tentatively assigned the role of a C-8 hydroxylase responsible for the modification of the macrolactam ring.

The gene cluster contains one gene, *becN*, whose translation product might be involved in the BE-14106 transport or resistance mechanism. BecN resembles major facilitator superfamily-type efflux pumps and might be responsible for the efflux of BE-14106 from the cell. BecN was homologous to the product of Strop_2765, a gene located in close proximity to the salinilactam cluster in *Sal. tropica*.

Several genes whose products could not be assigned specific functions in the BE-14106 biosynthesis were found in the cluster. The translation product of *becT* is a putative protein of 95 amino acids with only poor homology to several hypothetical proteins. Based on the sequence analysis alone it was not possible to suggest a role for BecT in the BE-14106 biosynthesis. Three genes with unclear function, *becI*, *becP*, and *becU*, were found to encode a putative glycine oxidase/FAD-dependent oxidoreductase, L-amino acid amidase/proline iminopeptidase, and NRPS accessory protein, respectively. BecI was homologous to putative glycine oxidases from potential thiamin biosynthetic gene clusters in several *Streptomyces* genome sequences, but no *becI* homologs were found in the other characterized macrolactam biosynthesis gene clusters. BecP showed the highest homology to VinJ and Strop_2777 from the vicenistatin and salinilactam biosynthesis pathways, respectively (Ogasawara et al., 2004; Udwary et al., 2007). Both VinJ and Strop_2777 were thought to represent proline-specific peptidases, but functional roles in the biosynthesis were not assigned for them. BecU showed homology to several hypothetical proteins for which the corresponding genes were all located within clusters containing both NRPS- and PKS-encoding genes. The highest homology was to hypothetical protein SAV_606 from the genome sequence of *Streptomyces avermitilis* MA-4680, but the product presumably synthesized by the enzymes encoded by the tentative biosynthetic gene cluster has not been characterized (Ōmura et al., 2001).

Orfs1–3 and 6–9, located at the left and right flanks of the gene cluster, do not seem to have any roles in the BE-14106 biosynthesis, judging from the analysis of their translation products (Table 2).

Organization and Functional Assignments of the PKS Proteins

To assign specific roles for the seven PKS genes in the biosynthesis of BE-14106, their translation products were analyzed for domain organization and presence of intact active sites in the domains. BecA was found to contain a loading module, two additional complete modules, and one truncated module and was thought to be responsible for synthesizing the C20-C25 acyl side chain. As reported for several other type I modular PKS clusters (Kuhstoss et al., 1996; Kakavas et al., 1997; Waldron et al., 2001), the N-terminal domain of the loading module resembles a ketoacyl synthase (KS) domain, but has a glutamine residue instead of the usual conserved cysteine in the active site. Such KS^Q domains are thought to be responsible for chain initiation by decarboxylating the starter unit selected by the AT domain of the loading module (Bisang et al., 1999; Long et al., 2002). The AT domain of the BecA loading module appears to be specific for methylmalonyl-CoA with substrate-specific motifs as described by Reeves et al. (2001). With decarboxylation by the KS^Q domain, this leaves propionate as the first unit to be incorporated in the biosynthesis of the side chain. The AT domains of the extender modules 1, 2, and 3 are specific for malonyl-CoA. The third module, however, is truncated after the AT domain and lacks reductive and ACP domains.

The BE-14106 macrolactam ring is presumably synthesized by the PKS enzymes BecB, BecD, BecE, BecF, and BecG. BecB has an N-terminal ACP domain and in addition two complete PKS modules. Presumably, the N-terminal ACP domain represents a loading module for the initiation of the macrolactam ring synthesis. Loading modules consisting of only ACP domains have also been described in the biosynthesis of vicenistatin (Ogasawara et al., 2004) and salinilactam (Udwary et al., 2007). In both cases, the initiation of the biosynthesis might involve the loading of an amino acid starter unit on the loading module ACP domain. Upon loading of the aminoacyl starter (see below), the biosynthesis of the BE-14106 macrolactam ring is thought to proceed through modules 1–8. Besides the extender modules 1 and 2 represented by BecB, BecD constitutes modules 3 and 4, BecE module 5, BecF modules 6 and 7, and BecG module 8 as well as a C-terminal TE domain for

Table 2. Genes Identified in and around the BE-14106 Biosynthetic Gene Cluster and Their Putative Functions

Gene Designation	Product (Amino Acids)	Protein Homologs (GenBank Accession Numbers), % Positives/% Identity	Putative Function	Homologs from *vin* and *slm* Clusters
orf1	135	SCO7674 (NP_631713), 80/74	Putative secreted metal-binding protein	
orf2	199	SCO7673 (NP_631712), 74/60	Putative lipoprotein	
orf3	144	SGR_1584 (YP_001823096), 60/46	Hypothetical protein	
becH	951	Orf30 (AAX98205), 52/36	LuxR-type transcriptional regulator	Strop_2761
becA	5582	KijS1 (ACB46488), 62/49	Polyketide synthase type I	
becI	362	SGR_5394 (YP_001826906), 60/47	Glycine oxidase/FAD-dependent oxidoreductase	
becC	694	LasAVI (CAQ64691), 57/45	Polyketide synthase type I	
becU	187	SAV_606 (NP_821781), 57/47	Putative NRPS accessory protein	
becB	3527	Strop_2768 (YP_001159588), 66/55	Polyketide synthase type I	
becJ	532	Strop_2775 (YP_001159595), 73/58	AMP-dependent acyl-CoA synthetase/ligase	VinN, Strop_2775
becK	323	VinK (BAD08368), 66/48	Acyltransferase	VinK, Strop_2773
becS	78	VinL (BAD08369), 67/53	PCP	VinL, Strop_2776
becL	505	Strop_2774 (YP_001159594), 71/58	NRPS adenylation domain	VinM, Strop_2774
becM	198	Strop_2766 (YP_001159586), 65/48	TetR-type transcriptional regulator	Strop_2766
becN	524	Strop_2765 (YP_001159585), 70/50	Major facilitator superfamily-type efflux pump	Strop_2765
becO	411	Sare_1860 (YP_001536736), 55/38	P450 monooxygenase	
becD	3372	SSAG_05828 (EDX26173), 66/55	Polyketide synthase type I	
becP	313	VinJ (BAD08367), 79/65	Putative L-amino acid amidase/proline iminopeptidase	VinJ, Strop_2777
becG	1986	Orf17 (AAX98192), 64/52	Polyketide synthase type I	
becF	3377	LipPks2 (ABB05103), 64/53	Polyketide synthase type I	
becE	1631	Orf16 (AAX98191), 61/50	Polyketide synthase type I	
becT	95	Rv3281 (NP_217798), 59/40	Hypothetical protein, SimX2-like protein	
becQ	257	SAV_953 (NP_822128), 67/48	Thioesterase type II	Strop_2763
orf10	237	SGR_6303 (YP_001827815), 94/89	PlsC-type phospholipid/glycerol acyltransferase	
orf6	537	SAML1218 (CAJ90204), 85/75	Putative tripeptidylaminopeptidase	
orf7	256	SGR_6300 (YP_001827812), 78/72	Putative urease accessory protein	
orf8	231	SGR_6299 (YP_001827811), 92/87	Putative urease accessory protein	
orf9	incomplete	–	Putative urease accessory protein	

In addition to the closest homologs from GenBank, homologs from the vicenistatin (*vin*) and salinilactam (*slm*) clusters are also included.

the release and lactamization of the reduced aminoacyl chain. All AT domains in the BecB-G PKS system are specific for malonyl-CoA except AT2 of BecB and AT6 of BecF, which are specific for methylmalonyl-CoA. All modules (except the loading module) contain both KR and DH domains, but the DH domain in BecE appears to be inactive since the hydroxyl group at C-9 is intact. The DH domain of BecE has an intact conserved active site motif ($H(X_3)G(X_4)P$), but has a deletion in the C-terminal part of the domain of about 40 amino acids compared to DH domains from BecD, F, and G.

BecC is the second truncated PKS encoded by the gene cluster and consists only of the KR and ACP domains. The scheme described above involving the BecB-BecG enzymes leaves no role for the BecC protein in the biosynthesis of the macrolactam ring, thus suggesting its involvement in the biosynthesis at an earlier step (see below).

Enzymes Presumably Involved in the Biosynthesis of the Starter Unit

We propose that the BecI, BecJ, BecK, BecL, and BecS proteins may be involved in the activation or modification of an aminoacyl starter unit for the BE-14106 biosynthesis. Both BecL and BecJ display homology to the LuxE-type adenylate-forming protein superfamily, including acyl-CoA synthetases/ligases and NRPS. BecL and BecJ showed the highest homology to the enzymes Strop_2774 and Strop_2775, respectively, which were suggested to be involved in the biosynthesis of salinilactam (Udwary et al., 2007). The exact roles of Strop_2774 and Strop_2775 in the biosynthesis of salinilactam have not been elucidated, but they were presumed to be involved in the starter unit biosynthesis (Udwary et al., 2007). BecL was also found to be homologous to other presumed or validated amino acid adenylation enzymes, e.g., VinM from the biosynthesis of vicenistatin

(Ogasawara et al., 2004) and LnmQ from the leinamycin biosynthesis (Cheng et al., 2003). For VinM, the exact role in the biosynthesis has not been determined, although it was suggested that the enzyme activates the starter unit 3-methylaspartate (Ogasawara et al., 2004). LnmQ, on the other hand, was shown to directly activate D-alanine as D-alaninyl-AMP and load it onto an independent PCP domain, LnmP (Tang et al., 2007). An alignment of the protein sequences (Figure S2) of BecJ, BecL, Strop_2774, Strop_2775, VinM, and LnmQ show that they all contain the ten conserved core motifs of adenylating enzymes (Marahiel et al., 1997). According to the mechanism suggested by Reger et al. (2007), representatives of the LuxE superfamily perform two half-reactions, namely adenylation of a substrate and formation of a thioester using the adenylated substrate. The latter reaction may involve both coenzyme A (yielding acyl-CoA) or the pantheteine arm of the holo-PCP (yielding aminoacyl-PCP). Amino acid residues necessary for the two half-reactions are conserved in both BecL and BecJ, but these proteins share only 28% identity, suggesting that they might use different substrates, for example, amino acid and fatty acid. Phylogenetic analysis of BecJ, BecL, and their closest homologs (Figure S3) revealed that BecL (along with VinM and Strop_2775) clustered with LnmQ, which was proven to be specific for an amino acid substrate, while BecJ and Strop_2775 formed a separate clade. Based on these considerations, we suggest that BecL is responsible for activation of an amino acid, while BecJ uses a fatty acid-like substrate, acting as an acyl-CoA ligase. A bioinformatics analysis of BecL using NRPS predictor software (Rausch et al., 2005) was performed to obtain a possible hint on the amino acid starter for the BE-14106 biosynthesis. The analysis resulted in prediction of BecL being specific for Gly = Ala = Val = Leu = Ile with a specificity score 1.396. None of these amino acids could be easily fitted into the biosynthesis of BE-14106, although assumed specificity of BecL for glycine correlated with the presence of a glycine oxidase, BecI, shown to be essential for the biosynthesis (see below).

BecS is homologous to LnmP, the PCP protein accepting activated D-alanine from the LnmQ adenylation domain in the leinamycin biosynthesis (Tang et al., 2007), and also shares homology with VinL and Strop_2776 from the vicenistatin and salinilactam biosynthesis pathways, respectively. VinL was assigned as an aminoacyl carrier protein in the biosynthesis of vicenistatin, while Strop_2776 is thought to be one of nine accessory proteins involved in the starter unit synthesis of the salinilactam biosynthesis. It is likely that BecS plays a similar role in the BE-14106 biosynthesis and the enzyme might serve the function of a PCP domain for loading of an amino acid activated by BecL.

BecI represents a putative glycine oxidase/FAD-dependent oxidoreductase and may be involved in the modification of the aforementioned amino acid. BecI is homologous to several putative glycine oxidases from streptomycetes, some of which are encoded in clusters of thiamin biosynthesis genes. An alignment of protein sequences (Figure S4) for BecI, two glycine oxidases from streptomycetes, and the glycine oxidase ThiO from the thiamin biosynthesis pathway of *Bacillus subtilis* (Settembre et al., 2003) showed that although the overall sequence similarity between the streptomycete glycine oxidases and ThiO is not high (25%–28%), most of the active site residues determined for ThiO are conserved among all four enzymes. Moreover, larger areas surrounding the active site residues were also well conserved, as were the FAD-binding residues, thus pointing to a related function or mechanism for these proteins.

BecK represents a putative acyltransferase and showed the highest homology to VinK from the vicenistatin biosynthesis. VinK was suggested to be responsible for the transfer of the amino acid-derived starter unit to an ACP domain (Ogasawara et al., 2004). BecK was also homologous of Strop_2773, one of nine accessory proteins thought to be involved in the starter unit synthesis of the salinilactam biosynthesis. The substrate-specific motifs of BecK, VinK, and Strop_2773 appear to be rather special (see Table S2). Since the loading module of BecB lacks an AT domain, it is possible that BecK functions as an independent AT domain loading the starter unit onto the ACP domain of the BecB loading module. The unique substrate specificity motifs of BecK most likely reflect an unusual structure of the starter unit suggested for the BE-14106 biosynthesis (see below).

Gene Inactivation Experiments

In order to verify the roles of certain genes in the biosynthesis of BE-14106, a series of gene inactivation experiments was carried out. The gene inactivation experiment using PCR-amplified fragments from cosmid 2 and 3 (see above) involved the BecA module 2 KS, AT, and DH domain-coding regions. The production of BE-14106 was clearly affected in both mutants, although they produced trace amounts of BE-14106. The low level of production in the mutants may be a result of reversion to wild-type for some cells and subsequent production during fermentation as only single crossover mutants were constructed. Nonetheless, the experiment demonstrated an essential role for BecA in the biosynthesis of BE-14106.

BecI, BecC, and BecP had questionable roles in the biosynthesis of BE-14106, and the suggested role of BecO as a C-8 hydroxylase needed verification. Replacement vectors designed to introduce deletions into these genes were constructed (see Supplemental Experimental Procedures). Gene replacement mutants with in-frame deletions were obtained for all genes and verified by southern blot analyses (data not shown), and the BE-14106 production was evaluated using LC-MS of fermentation extracts. For the Δ*becO* mutant, the expected mass corresponding to the stoichiometric formula ($C_{27}H_{37}NO_2$) of the suggested 8-deoxy BE-14106 was found with 1.0 ppm difference from the theoretical mass (Figure S5). The role of BecO as a P450 monooxygenase hydroxylating BE-14106 was thus confirmed. The C-8 carbon represents the only likely target of BecO, since the hydroxyl group at the C-9 appears due to the lack of the DH domain activity in the BecE PKS involved in the biosynthesis of the macrolactam ring. LC-MS analyses of fermentation extracts from the Δ*becI*, Δ*becC*, and Δ*becP* mutants all showed complete absence of BE-14106 production and no putative BE-14106 analogs/precursors could be identified (data not shown).

Heterologous Expression and Enzyme Assays of BecI and BecP

To shed more light on the role of BecI and BecP in the biosynthesis of BE-14106, both enzymes were heterologously produced in *Escherichia coli* as recombinant proteins with N-terminal and

C-terminal His tags (see Experimental Procedures and Supplemental Experimental Procedures). All proteins were purified by nickel affinity chromatography and their purity and molecular weight were evaluated by SDS-PAGE (Figure S6). The apparent observed molecular weights of the recombinant proteins were close to those theoretically calculated (39.4 kDa for BecI and 35.6 kDa for BecP). The specificity of the purified proteins was judged by enzyme assays with different substrates. The highest activity was achieved with C-terminal His-tagged BecI and BecP recombinant proteins. BecI-6xHis was tested with glycine, L- and D-glutamine, L- and D-asparagine, and L- and D-lysine (Figure S7), which were judged to be the most likely candidates for the biosynthesis of the starter unit based on the structure of BE-14106. BecI-6xHis was able to utilize all substrates tested, but with modest activity. The highest activity was obtained with glycine (0.40 units/mg protein) and D-asparagine (0.21 units/mg protein). The overall low activity level may be due to the presence of the His tag or the need for an additional protein partner. Another possibility is that it may be necessary for the amino acid substrate to be tethered to the PCP domain, BecS, for BecI to achieve its full potential as a glycine/D-amino acid oxidase.

BecP-6xHis was initially only tested with L-proline-pNA (p-nitroanilide). Since good activity was obtained with this substrate, the enzyme assay was repeated with L-alanine-pNA and L-lysine-pNA as negative controls to verify BecP's assumed preference for L-proline. Surprisingly, L-Ala-pNA turned out to be a much better substrate for BecP. Considering the results from the BecI enzyme assay, the BecP assay was repeated a second time including also glycine-pNA as substrate. Gly-pNA turned out to be a slightly better substrate than L-Pro-pNA, but still less preferred than L-Ala-pNA. In an effort to investigate a potential role for BecP in the resistance mechanism, the enzyme was also incubated under the same assay conditions with purified BE-14106 as substrate. Reaction mixtures with and without enzyme were subjected to LC-MS for detection of any decomposition of BE-14106, but no difference was observed between the samples.

Feeding Studies Designed to Determine the Amino Acids Used in the BE-14106 Biosynthesis

Based on the enzymatic activity of the recombinant BecI enzyme assay, bioinformatics analysis of BecL, and the results of the gene inactivation experiments it seemed likely that glycine might be used as a starter for BE-14106 biosynthesis. However, at that moment we could not exclude the possibility that other amino acids, such as aspartate or glutamate, could be used as starters in macrolactam biosynthesis (Ogasawara et al., 2004). To determine the starter amino acid, we first performed feeding experiments where nine different unlabeled amino acids (glycine, D- and L-alanine, D- and L-asparagine, D- and L-glutamine, D- and L-aspartate, and D- and L-glutamate) were fed separately to the BE-14106 producer cultivated on Silantes growth media with ^{15}N- or ^{13}C-labeled amino acids (see Experimental Procedures for details). To achieve good growth of the cultures, two alternative nitrogen sources, ammonium sulfate or sodium nitrate, were used in these media. The feeding experiments were followed by LC-DAD-TOF analyses of the BE-14106 molecules produced under these conditions, which provided an estimate of incorporation of either ^{14}N or ^{12}C from the fed amino acids into the BE-14106 structure. In addition, production of BE-14106 under these conditions was assessed. The volumetric yield of BE-14106 without fed amino acids (control) was approximately 50 mg/liter in both ammonium- and nitrate-supplemented BeSi medium. None of the amino acids fed to the cultures had any significant effect on the production of BE-14106. Isotope distribution plots were also obtained in order to assess specificity of incorporation and to rule out cases where amino acids were efficiently metabolized (data not shown). Analysis of the data obtained clearly demonstrated that the nitrogen atom from glycine was most efficiently incorporated into the BE-14106 molecule on both ammonium- and nitrate-containing media (Figure 3) compared to the other amino acids tested, while the carbon atoms from glycine were not incorporated to any extent. Apparently, fed amino acids (except for L-aspartate), were more efficiently catabolized as nitrogen and carbon sources on the medium containing nitrate, as evidenced by the almost equal incorporation of their N and C atoms into the BE-14106 molecule (Figure 3B).

To further confirm our findings, ^{15}N- or ^{13}C-labeled glycine, L-asparagine, and L-glutamate (labeled D forms were not commercially available) were fed to cultures grown on unlabeled media, but under the same conditions as for the labeled media described above. Approximately 50% incorporation of ^{15}N from glycine into the BE-14106 molecule was demonstrated in these experiments when using either ammonium or nitrate as nitrogen source, while no significant ^{13}C incorporation could be detected (Figure S8). Interestingly, up to 20% of the BE-14106 molecules produced with labeled L-glutamate feeding contained ^{15}N from this amino acid. At the moment, we do not have a plausible explanation for this observation.

Taken together, the results from both the "reverse" and the "direct" feeding experiments clearly suggest that glycine is the preferred starter amino acid for the BE-14106 biosynthesis. Intriguingly, carbon atoms from this amino acid were not incorporated into the BE-14106 molecule. This fact correlates well with the assumed functions of BecA and BecC, which together represent four complete PKS modules assembling a polyketide chain from one propionate and three acetate units. Such a chain could only be represented by the C25-C17 part of the BE-14106 molecule, leaving no space for incorporation of carbons from any amino acid starter. Considering all the above, it seems likely that glycine is used merely as a nitrogen atom donor in the biosynthesis of the aminoacyl starter unit (see Discussion).

DISCUSSION

Based on the sequence analysis, gene inactivation experiments, enzyme assays, and feeding studies, we propose a model for the BE-14106 biosynthesis in *Streptomyces* sp. DSM 21069 (Figure 4). The biosynthesis is thought to start with loading of methylmalonyl-CoA on the first ACP domain of BecA. Decarboxylation by the KS^Q domain results in the propionyl starter unit (C25-C23), which is extended with three acetate units by modules 1 and 2 of BecA and the module 3 split between BecA and BecC (C22-C17). Modules split between individual PKS proteins have been described in several cases and they appear to be functional (Duitman et al., 1999; Kopp et al., 2005; Piel et al., 2004; Silakowski et al., 2001). The inactivation of the *becC* gene led to complete abrogation of BE-14106 production, indicating an essential role for BecC in the

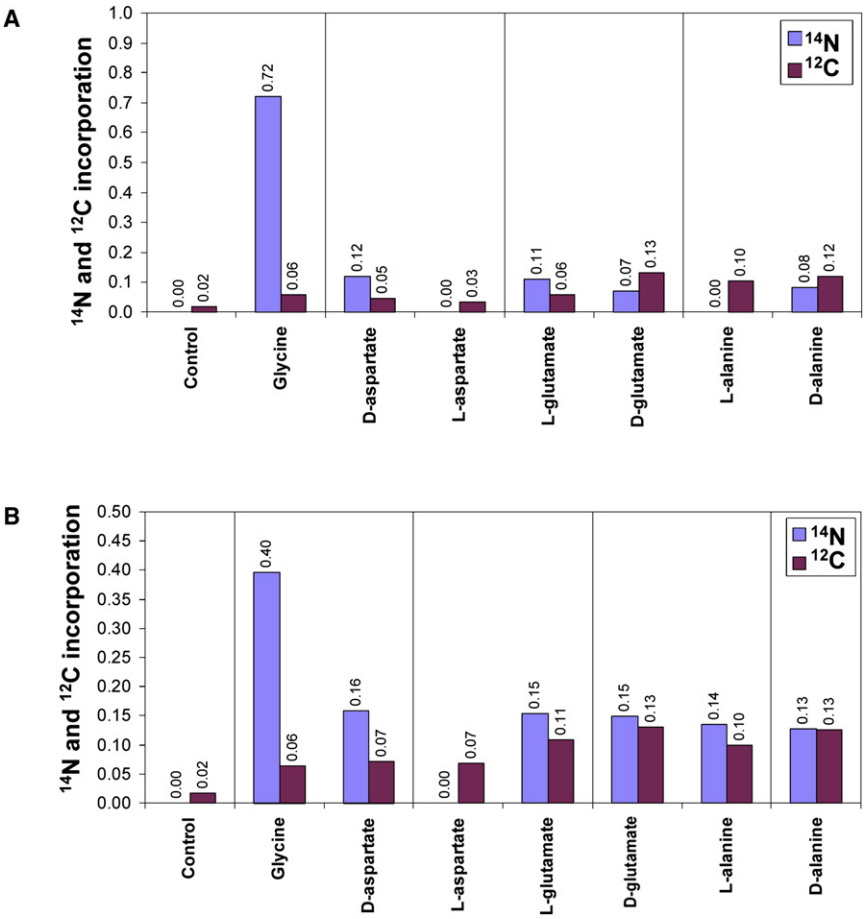

Figure 3. Incorporation of Nitrogen and Carbon Atoms from Unlabeled Glycine into the BE-14106 Molecule in the Feeding Experiments

Experiments were performed using the Silantes growth media supplemented with ammonium sulfate (A) or sodium nitrate (B).

biosynthesis. In parallel with the synthesis of the starter polyketide chain, glycine is assumed to be activated by the discrete NRPS adenylation domain, BecL, followed by loading of the glycine residue on the discrete PCP domain, BecS. Interaction between BecS and the BecA/BecC complex may be mediated by the putative NRPS accessory protein BecU, bringing the glycine residue and the polyketide chain into proximity. BecU homologs are encoded by genes situated close to NRPS- and PKS-encoding genes in several genomes (e.g., *Streptomyces avermitilis* MA-4680 and *Microscilla marina* ATCC 23134), indicating that such enzymes may participate in interactions between NRPS and PKS proteins. The feeding experiments clearly established that glycine is the preferred amino acid used for the biosynthesis of the aminoacyl starter, and the fact that only the nitrogen atom from glycine is used suggests a unique mechanism that must involve transfer of the nitrogen to the acyl chain. There exist several examples where an aminoacyl moiety is assembled by a PKS with the help of either an embedded aminotransferase domain (mycosubtilin biosynthesis; Aron et al., 2005) or through the extension of the acyl chain with an amino acid by an NRPS module (FK520 biosynthesis; Gatto et al., 2005). However, through the bioinformatics analyses of the gene cluster described here and ~15 kb DNA regions flanking the cluster on each end (data not shown), we were not able to identify any domains or proteins that could possess either aminotransferase or condensation activity. The importance of BecI for the biosynthesis of BE-14106 and its specificity toward glycine suggests that this enzyme is involved in the transfer of the nitrogen atom from glycine to the acyl chain assembled by BecA-BecC PKS.

Although the exact mechanism remains unclear at the moment and requires further elucidation, the scheme for the formation of the aminoacyl starter presented in Figure 4A seems plausible. We suggest that the acyl starter is assembled from one propionate and three acetate units by BecA-BecC PKS, and then aminated at C-19. Bearing in mind that BecI is homologous to glycine oxidases and the chemistry of the first half-reaction performed by this type of enzymes (Scrutton, 2004), BecI is likely to be involved in the amination using as a substrate glycin activated by BecL and tethered to BecS PCP. This is followed by a reduction, possibly involving KR domain of BecC, which leads to the appearance of the C21-C22 double bond. The resulting aminoacyl is likely to be released as a carboxylic acid via the action of the thioesterase BecQ. In the next step, the putative acyl-CoA ligase, BecJ, presumably activates the aminoacyl carboxylic acid to produce its CoA thioester. The resulting aminoacyl-CoA is assumed to be selected by the discrete acyltransferase BecK and transferred to the loading module ACP domain of the BecB PKS. The synthesis of the polyketide chain proceeds through condensation of six malonyl-CoA and two methylmalonyl-CoA units by BecB, BecD, BecE, BecF, and BecG. The TE domain of BecG hydrolyses the thioester bond, catalyzes the cyclization of the macrolactam ring, and releases the polyketide chain from the PKS complex. As a final step in the biosynthesis, the 8-deoxy BE-14106 is hydroxylated at C-8 by the P450 monooxygenase BecO.

The exact role of BecP, a putative iminopeptidase, remains obscure, although, judging from the gene inactivation experiment it is required for the BE-14106 biosynthesis. Supporting the role of BecP as an iminopeptidase is the fact that BecP was shown to cleave several different amino acid p-nitroaniline substrates, displaying the highest activity with L-Ala-pNA followed by Gly-pNA and L-Pro-pNA. It is thus tempting to speculate that BecP might be involved in the process of the aminoacyl formation together with BecI. The presence of BecP homologs in both the vicenistatin and salinilactam gene clusters (Table 2) indicates that this enzyme's function may be required for the biosynthesis of these macrolactams as well.

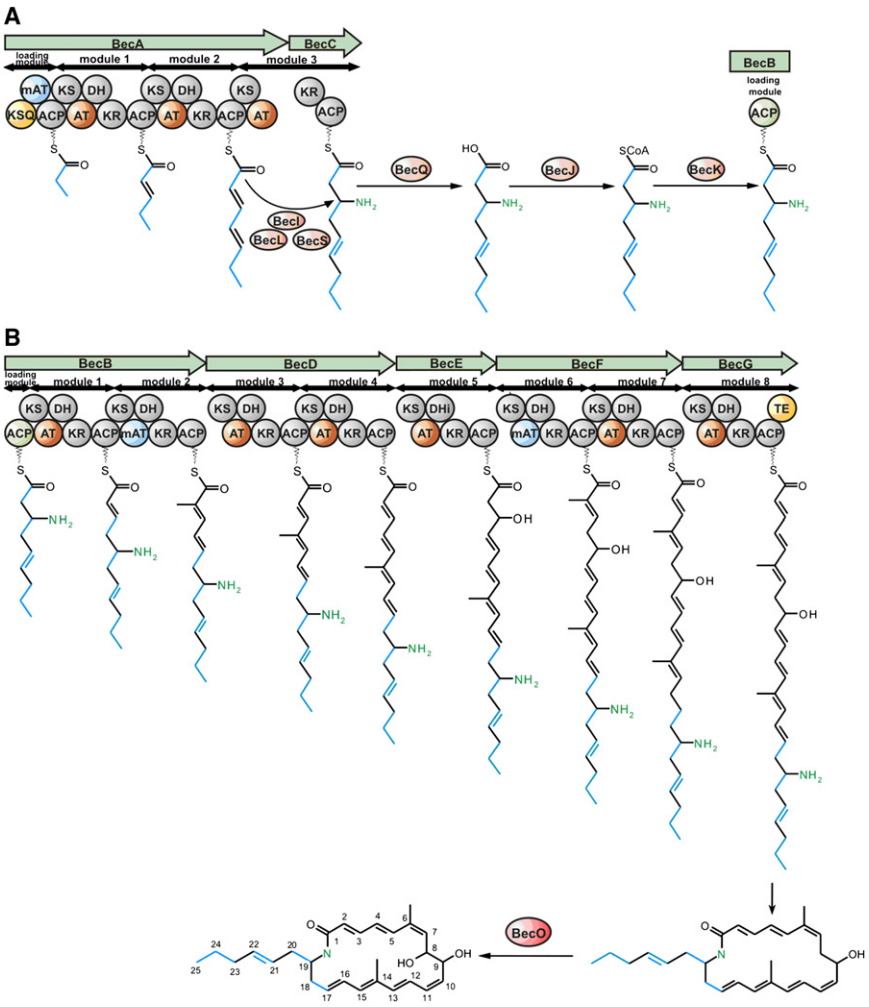

Figure 4. Proposed Model for the Biosynthesis of BE-14106

(A) Synthesis and activation of the acyl amino acid starter.

(B) Extension of the aminoacyl starter, cyclization, and C-8 hydroxylation. Malonyl-CoA-specific AT domains are shown in orange and methylmalonyl-CoA-specific AT domains are shown in blue.

SIGNIFICANCE

Cloning and analysis of the biosynthetic gene cluster for the cytotoxic macrolactam BE-14106 provided new insights into the biosynthesis of macrolactams, in particular those containing acyl side chains. Based on the bioinformatics analysis and experimental data, involvement of two distinct PKS systems, one functioning together with NRPS-related enzymes in the assembly of a starter and another in the synthesis of the ring, has been proposed. The synthesis of the polyketide starter and the manner in which it is aminated represents a new mechanism for generation of an aminoacyl starter unit for macrolactam biosynthesis. Understanding of the biosynthetic pathway, access to the genes, and demonstrated possibility to manipulate them open new possibilities for modification of the BE-14106 molecule and generation of novel analogs with improved pharmacological properties.

EXPERIMENTAL PROCEDURES

Isolation of Strains from Sediments and Screening for Antifungal Activity

Sediment samples were collected from different sites in the Trondheimsfjord and used for isolation of marine actinomycetes as described by Bredholt et al. (2008). The strain designated *Streptomyces* sp. DSM 21069 was isolated from a sediment sample collected by scuba divers at 6 m depth at position 63°26N 10°21E. The medium used for isolation of this strain was IM8 (Bredholt et al., 2008), supplemented with the antibiotics novobiocin (20 μg/ml), cycloheximide (50 μg/ml), and nystatin (75 μg/ml).

The collection of actinomycete strains obtained from sediments was investigated for production of antifungal activity using a set of different solidified production media. For *Streptomyces* sp. DSM 21069, growth at 25°C on medium PM2 (Bredholt et al., 2008) for 7 days resulted in strong antifungal activity. After the appropriate incubation time, the medium was dried and then extracted with DMSO. After filtration, the DMSO extracts were used as samples in a robotic bioassay procedure with the strains *Candida albicans* CCUG3943 and *Candida glabrata* CCUG3942 as indicator organisms as described by Jørgensen et al. (2009). The latter strain has a high level of resistance against polyene antibiotics, while the *C. albicans* strain is sensitive to polyenes. The medium used in the bioassay was AM19(B) (9.4 g/liter peptone [Oxoid], 4.7 g/liter yeast extract [Oxoid], 2.4 g/liter beef extract [Difco], 10 g/liter glucose [BDH], and distilled water).

Selected DMSO extracts displaying interesting bioactivity were fractionated using an Agilent 1100 series HPLC system equipped with a diode array detector (DAD) and a fraction collector. Each sample was fractionated in parallel using two different types of LC columns: Agilent ZORBAX Eclipse XDB-C18, 5 μm, 4.6 × 150 mm, and Agilent SB-CN, 3.5 μm, 4.6 × 75 mm. For both types of columns, a flow of 1 ml/min of a mixture of 0.005% formic acid in deionized water and acetonitrile was used as mobile phase. In both cases the concentration of acetonitrile was kept at 40% the first minute, then increased linearly from 40% to 95% during the next 9 min and kept at a concentration of 95% for the rest of the run. The fraction collector was used to collect 12 fractions of the eluent from 1 min until 13 min from injection.

The samples were dried in a vacuum centrifuge (Savant Speed-Vac) and dissolved in DMSO and the bioactivity of the fractions was determined in an agar diffusion assay using the two yeast strains described above as indicator organisms and AM19 as assay medium (equal to AM19B described above, with 15 g/liter agar and 10 g/liter NaCl). The fractions with bioactivity were analyzed using an Agilent 1100 series HPLC system connected to a DAD and a time-of-flight (TOF) mass spectrometer. The same columns and buffers were used in this analysis as described above for the fractionation step. Electrospray ionization was performed in the negative (ESI-) mode. The DAD plots were used to identify the approximate retention time of the bioactive compounds in the fractionation runs and in the LC-MS-TOF analysis. Molecular masses corresponding to significant peaks identified in bioactive samples from parallel fractionations (C18 and CN columns) were compared and molecular masses common to fractions from the C18 and CN columns were identified. These molecular masses (10 ppm window) were submitted to the online version of the Dictionary of Natural Products (http://dnp.chemnetbase.com/) in order to search for previously characterized compounds with bioactivity.

Production of BE-14106 and Deoxy-BE-14106

For production of BE-14106 and its analog, deoxy-BE-14106, DSM 21069 strains were grown in 500 ml baffled shake flasks with 100 ml 0.3× BPS medium (9 g/liter oatmeal, 1.5 g/liter malt extract, 0.9 g/liter yeast extract, 0.12 g/liter $MgSO_4x7H_2O$, 0.3 g/liter NaCl, 1.5 g/liter $CaCO_3$, 9.0 g/liter soluble starch, 11.0 g/liter MOPS, and 10.0 g/liter glucose [pH 7]). Each flask was supplemented with 3 g of glass beads (3 mm) to increase the shearing force. The flasks were incubated for 6 days at 25°C (225 rpm, orbital movement, 2.5 cm amplitude). The production cultures were inoculated with 3 vol% from a preculture cultivated for 2 days at 25°C (0.5× Tryptone Soya Broth supplemented with 20 g/liter glucose in 500 ml baffled shake flasks). The incubation conditions for the precultures were otherwise as described for the production cultures.

Feeding Experiments

For the feeding studies with DSM 21069, precultures were grown in shake flasks with glass beads in 0.5× TSB with glucose as described above. Ten milliliters of each preculture was harvested by centrifugation and the cells were washed with 40 ml BeDef medium, resuspended in 40 ml of the same, and inoculated with 3 vol% in Silantes OD2 medium (Silantes) and incubated for 1 day. The production and feedings were performed in deep well plates with one 3 mm glass bead and 600 μl medium in each well at 25°C, 80% humidity, 800 rpm with a 3 mm amplitude for 6 days. The experiments were performed in the BeSi medium consisting of 0.4 g/liter $MgSO_4x7H_2O$, 5.0 g/liter $CaCO_3$, 0.2 g/liter KH_2PO_4, and 536 ml/liter Silantes OD2 medium (Silantes pr. No. 103002, 102202, and 100002). The Silantes addition to the media represents a mix of 16 different amino acids at a total concentration of 0.26 mM. The minimal medium BeDef is a modified SMM medium (Takano et al., 1992) without casaminoacids and PEG and with double amount of phosphate. Both media were supplemented with 3 ml/liter trace mineral solution TMS1 (Borgos et al., 2006a), 10 g/liter glucose, and 0.34 g/liter $(NH_4)_2SO_4$ or 0.44 g/liter $NaNO_3$. A total of nine different unlabeled amino acids were tested in this feeding experiment. The amino acids were supplemented two times during cultivation (after 24 and 48 hr, 2.5 mM at each addition). Since many D forms of amino acids are not available as ^{15}N or ^{13}C labeled, we chose to first test a set of nine unlabeled amino acids (glycine, D- and L-alanine, D- and L-asparagine, D- and L-glutamine, D- and L-aspartate, and D- and L-glutamate) in media where all other carbon or nitrogen sources were either ^{15}N or ^{13}C labeled.

In addition, feeding studies using three available ^{15}N- and ^{13}C-labeled amino acids (glycine, L-glutamate, and L-aspartate) were performed using unlabeled Silantes medium. The conditions were the same as for the experiments with the labeled media. After cultivation, cultures were freeze-dried, extracted, and analyzed on LC-DAD-TOF to determine how the isotopic distribution of the mass spectra of BE-14106 was affected by the addition of different amino acids.

Purification of BE-14106 and Deoxy-BE-14106

BE-14106 was purified in a two step purification process. Culture broth was centrifuged and the pellet was freeze-dried. The dried pellet was extracted with methanol (240 ml/g pellet) and filtered to remove any cell residues. BE-14106 in the extract was precipitated with an equal amount of water. The precipitated product was washed with water and thereafter freeze-dried. Preparative LC-DAD-MS-guided purification was performed essentially as described previously (Bruheim et al., 2004; Borgos et al., 2006b) but with methanol (85%) instead of acetonitrile as the mobile phase organic constituent.

LC-MS Assisted Determination of Production Yield and Qualitative Analyses

Quantification of BE-141016 in fermentation broth was performed by extraction of the broth with an appropriate amount of methanol or DMSO followed by LC-TOF or LC-qTOF analyses performed essentially as described above. Concentrations of BE-14106 and deoxy-BE-14106 were determined by UV peak absorption at 291 nm using BE-14106 purified by preparative HPLC as a standard. Purity of BE-14106 was determined by assuming that molar extinction coefficients in the spectral regions of interest were the same for BE-14106 and the contaminating derivatives.

Confirmation of the Chemical Structure using NMR

Samples for NMR spectroscopy were prepared by dissolving the freeze-dried compound in deuterated d_6-DMSO to a concentration of about 1 mM. To enable a direct comparison with NMR assignments reported previously, the experimental conditions were analogous to those described earlier for BE-14106 (Kojiri et al., 1992). All NMR experiments were recorded at 298 K on a Bruker Avance 600 MHz spectrometer equipped with a 5 mm z-gradient TXI (H/C/N) cryogenic probe. Proton and carbon chemical shifts were referenced to TMS signal. To monitor the chemical structure of the investigated compound both one dimensional ^1H and two dimensional COSY and ^1H-^{13}C HSQC spectra were recorded.

IC_{50} Determination

The antifungal activity of purified BE-14106 was determined in a robotic bioassay procedure with the strains *Candida albicans* CCUG3943 and *Candida glabrata* CCUG3942. Serial dilutions (six parallel wells) of the purified compound was prepared in a 384 well plate and diluted samples were added to assay plates containing medium M19B inoculated with the test strains. Growth in the presence of various BE-14106 concentrations was compared to growth in reference wells where only solvent (DMSO) was added, and the concentration of BE-14106 resulting in 50% or more reduction in growth was determined. Assay conditions were otherwise as described by Jørgensen et al. (2009).

Cell Handling and Experimental Conditions for Cytotoxicity Testing

Jurkat-T human lymphoma cells (ATCC no. TIB-152) and NRK epithelial cells (ATCC no. CRL-6509) were cultured in DMEM (EuroClon) supplemented with 10% heat-inactivated fetal bovine serum (Cambrex) in 40 ml flasks (Nunc) in a humidified incubator (5% CO_2, 37°C). For cytotoxicity testing, the cells were seeded in 96 well tissue culture plates at 150,000 cells/ml (jurkat) or 50,000 cells/ml (NRK). The NRK cells were left in the incubator overnight to attach to the substratum before experiments. The cells were exposed to various concentrations of BE-14106 or vehicle for 22 hr before addition of the cell mass (viability) reporter dye WST-1 (Roche Diagnostics). Plates were read 2 hr after WST-1 addition and the cells were fixed in 2% formaldehyde (pH 7.4) with the DNA-specific dye Hoechst 33342 (Polysciences Inc) to assess apoptosis. Apoptotic surface and nuclear chromatin morphology were examined by phase contrast and UV microscopy (Zeiss axiover 35M) as described before (Sandal et al., 2001, 2002).

Bacterial Strains, Media, and Growth Conditions

Bacterial strains and plasmids that were used in this study are listed in Table S3. *Streptomyces* sp. DSM 21069 strains were maintained on ISP2 agar medium (Difco), soy flour mannitol medium, and Mineral agar 1 Gause (starch-soluble; 20 g/liter K_2HPO_4, 0.5 g/liter $MgSO_4$, 0.5 g/liter KNO_3, 1.0 g/liter NaCl, 0.5 g/liter $FeSO_4$, 0.01 g/liter agar, and 20.0 g/liter tap water). Liquid cultures were grown in Tryptone Soya Broth (Oxoid). *E. coli* strains were grown in Luriana-Bertani (LB) broth or on LB agar. For cloning, EZ cells were used for vectors with blue-white selection, ER2925 was used when Dam/Dcm methylation-sensitive restriction enzymes were needed, and DH5α for general cloning. M15 (pREP4) were used for the expression vectors pQE60/70. XL-Blue MR was used for construction of the genomic library. ET12567 (pUZ8002) was used for intergeneric transfer of pSOK201-based constructs to *Streptomyces* sp. DSM 21069. Antibiotics were supplemented to growth media at the following concentrations: 100 or 150 μg/ml ampicillin, 50 or 100 μg/ml apramycin, 20 μg/ml chloramphenicol, 25 or 50 μg/ml kanamycin, 30 μg/ml nalidixic acid.

Cloning and sequencing of the BE-14106 gene cluster is described in detail in the Supplemental Experimental Procedures.

Construction of Plasmids for Gene Inactivation Experiments and Expression of Recombinant Proteins

Construction of plasmids used in this study is described in the Supplemental Experimental Procedures and the plasmids are listed in Table S3. Recombinant His-tagged proteins were purified following the procedure described by Ortiz de Orué Lucana and Schrempf (2000), but with some modifications. DH5α was used as a host for the expression of N-terminal His tag proteins and M15 (pREP4) for the expression of C-terminal His tag proteins. Cultures

were grown in LB medium containing 100 μg/ml ampicillin (pQE2) or 100 μg/ml ampicillin and 25 μg/ml kanamycin (pQE60/70) and induced at OD_{600} = 0.6 to a final concentration of 1 mM IPTG. Cultures were harvested after 4 hr of growth (succeeding the induction) and stored at −80°C. Cells were resuspended in solution A (Ortiz de Orué Lucana and Schrempf, 2000) containing 10 mM imidazole and disrupted by sonication. Cellular debris was removed by centrifugation (10.000 × g, 25 min, 4°C) and 0.5 ml Ni-NTA Agarose (QIAGEN) (prewashed with solution A) was added to the cleared lysate. The mixture was left rotating at 4°C for 2 hr to allow proper binding of the recombinant proteins before being loaded on the column (QIAGEN). The column was washed twice with 10 ml solution A containing 20 mM imidazole. The recombinant proteins were eluted with 4× 0.5 ml solution A containing 300 mM imidazole. Glycerol was added to a final concentration of 15% and the eluates were stored at −80°C. Eluates, induced and preinduced culture samples, and lysates were analyzed by SDS-PAGE and the protein concentration was determined by use of Nano-Drop (A280) with BSA as a standard.

Enzyme Assays for BecI and BecP

BecI

Enzyme assay was performed as described by the Sigma-Aldrich procedure "Enzymatic Assay of D-AMINO ACID OXIDASE APOENZYME" (http://www.sigmaaldrich.com/etc/medialib/docs/Sigma/General_Information/d-amino_acid_oxidase_apo_reactivation_assay.Par.0001.File.dat/). Unit definition for the enzyme activity: one unit will oxidatively deaminate 1.0 μmol of substrate per minute at pH 8.3 at 25°C, in the presence of catalase.

BecP

Enzyme assay was performed as described by Uraji et al. (2007) with a substrate concentration of 1 mM.

ACCESSION NUMBERS

DNA sequences for the BE-14106 biosynthetic gene cluster and 16S rDNA of *Streptomyces* sp. DSM 21069 were deposited in the GenBank under accession numbers FJ872523 and FJ872524, respectively.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, eight figures, and four tables and can be found with this article online at http://www.cell.com/chemistry-biology/supplemental/S1074-5521(09)00324-X.

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