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Biosynthesis of cervimycin C, an aromatic polyketide antibiotic bearing an unusual dimethylmalonyl moiety

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Cervimycin C is the major component of an antibiotic complex produced by *Streptomyces tendae* HKI-179 consisting of a tetracycline-type aglycon, six tridesoxysugars and a rare dimethylmalonyl moiety. The biosynthetic origin of cervimycin was studied by molecular studies and feeding experiments, which reveal that the dimethylmalonate unit is not derived from malonate, but from valine.

Introduction

In the course of our screening program for novel antibiotics, we recently identified a complex of aromatic polyketide glycosides from *Streptomyces tendae* HKI-179, a strain isolated from the *Grotta dei Cervi*, Italy.[1] The complex, named cervimycin A–D after its origin, exhibits strong antibacterial activity, even against vancomycin-resistant *Enterococci*.[2] The major component of this mixture, cervimycin C (CerC, **1**), consists of a carbamoyl-substituted tetracyclic system reminiscent of the tetracyclines,[3] and six tridesoxysugar moieties. The most unusual structural feature, however, is the rare dimethylmalonyl unit attached to the 4′ terminus of the tridesoxyhexose tetramer (Fig. 1)‡. Interestingly, despite its similarity to the tetracyclines, the cervimycins exhibit a different antibacterial mode of action, which dramatically depends on the substitution pattern (K. Herold *et al.* unpublished results). For this purpose, we want to gain insight into the biosynthesis of this complex molecule and to set the basis for genetically engineering more active and less cytotoxic antibiotics against multiresistant pathogens.

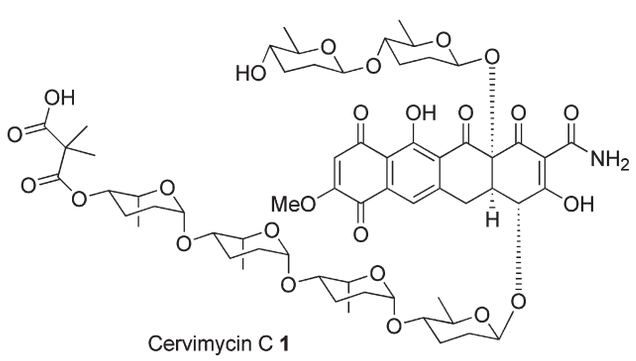

Fig. 1 Structure of cervimycin C (absolute configuration not determined).

Results and discussion

A series of labeling experiments was undertaken to address the biosynthetic origin of the cervimycin aglycons. Surprisingly, when 1-^{13}C-, 2-^{13}C- and 1,2-^{13}C-acetate were added to the fermentation broth, none of the precursors were incorporated, possibly because simple carboxylic acids are not taken up by the bacterium. Conversely, feeding experiments with the corresponding 2-^{13}C-, 1,2-^{13}C- and 1,2,3-^{13}C-malonate probes were successful (see Table 1). As in oxytetracycline[4] and lysolipin[5] biosynthesis, the starter (C3) malonyl unit is intact.[6] The pattern of ^{13}C incorporation is comparable with tetracycline-type polyketides, where malonamate undergoes eight Claisen condensations (Scheme 1).[4,6] The data clearly supports a *Streptomyces* (S)-type polyketide folding as opposed to the fungal (F)-type folding of the structurally related metabolite viridicatumtoxin, which is produced by a variety of *Penicillium* species.[7]

The structure and labeling pattern of the cervimycin aglycon is very suggestive for the involvement of a type II (aromatic) polyketide synthase (PKS), which generally consists of two iteratively used ketosynthase subunits, KS_α and KS_β, as well as an acyl carrier protein (ACP).[8] For targeting the cervimycin (*cer*) PKS by PCR, we employed a series of degenerate primers that are specific for genes encoding ketosynthase (KS) subunits of aromatic PKS. With these primers, however, we were only able to amplify a fragment of an angucycline-type PKS from the *S. tendae* HKI-179 genomic DNA template, as sequence comparisons reveal. Most likely, this PKS is involved in the formation of the rabelomycin-type red pigment of *S. tendae* (K. Herold *et al.* unpublished results). Since these primers failed to amplify the *cer* KS_α, we designed new sets of oligonucleotides with higher specificity for tetracycline-type PKS. Alignment of KS_α amino acid sequences from the tetracycline producers *S. rimosus*, *S. albofaciens and S. aureofaciens* (GenBank accession nos P43678, BAA92277 and BAB12566, respectively) revealed highly conserved motifs, from which primers cer-F1, cer-F2, cer-R1, and cer-R2 were deduced (Table 1). By PCR a 0.8 kb DNA fragment was successfully amplified using cer-F1 and cer-R2. Cloning and sequencing of this fragment revealed high homology of the deduced amino acid sequence with KS_α sequences from the database, in particular with the KS from *S. albofaciens* (identities = 79%, positives = 89%). The involvement of this ketoacyl synthase in cervimycin biosynthesis was confirmed by inactivation. For this purpose, the 0.8 kb fragment was ligated into suicide vector pOJ260.[9] The resulting plasmid, pXU01, was transferred into *S. tendae* HKI-179 by polyethylene glycol (PEG)-induced protoplast transformation.[10] Selection for apramycin resistance ($apra^R$) yielded a single crossover mutant, *S. tendae* HKI-179::pXU01, as confirmed by PCR with specific primers for the $apra^R$-cassette. HPLC-MS analysis of the extract obtained from the fermentation broth of this mutant clearly showed that this strain is incapable of producing cervimycin C (Fig. 2).

The polyketide aglycon is subject to a series of tailoring reactions, such as methylation, oxygenation, glycosylation with the tridesoxysugars and acylation with an unprecedented dimethylmalonyl unit. While there is no information on the formation of a "stand-alone" dimethylmalonate, a few polyketide metabolites, such as epothilone,[11] resistomycin,[12] and benastatin,[13] bear integrated *gem*-dimethyl moieties that are synthesized by (bis)methylation of (methyl)malonate units, most likely after the malonyl transfer has occurred. Accordingly, the formation of the cervimycin dimethylmalonyl unit could be rationalized. Most surprisingly though, our feeding experiments reveal that the labeled methyl group of fed ^{13}C-methionine can only be detected at the C11 methoxy group and that the ^{13}C-labeled malonate probes are not incorporated either. This result clearly indicates that the dimethylmalonyl moiety is neither derived from malonate nor from methylmalonate by (bis)methylation. An alternative model for the synthesis of dimethylmalonate could be carboxylation of an

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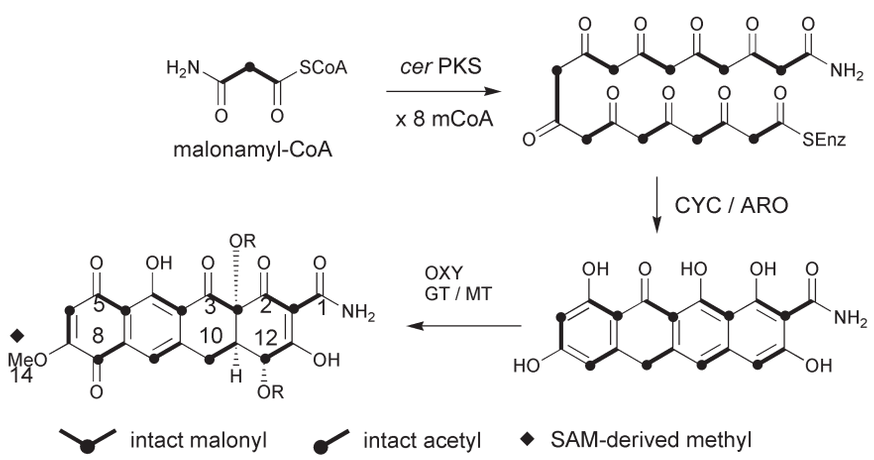
Table 1 Incorporation of labeled probes into **1**. (a) 2-^{13}C-malonic acid; (b): 1,3-$^{13}C_2$-malonic acid; (c) 1,2,3-$^{13}C_3$-malonic; and (d) L-methionine-(methyl-^{13}C); n.d.: not determined

C-atom	^{13}C NMR δ (ppm)	(a) %	(b) %	(c) %/$^1J_{CC}$ (Hz)	(d) %
1	192.0	0.75	**10.84**	n.d.	1.01
2	101.1	**8.16**	1.42	n.d.	0.96
3	194.8	0.65	**10.83**	**13.22**/45	1.02
4	74.1	**7.14**	1.28	**12.22**/45	1.00
4α	43.4	0.96	**14.77**	**12.56**/35	0.83
5	28.6	**6.67**	1.52	**14.58**/35	0.80
5α	153.3	0.77	**14.40**	**14.09**/57	1.07
6	120.2	**8.02**	1.02	**13.90**/57	0.97
6α	134.0	1.06	**8.60**	**13.51**/56	1.11
7	179.1	**12.77**	0.55	**16.43**/56	1.48
8	161.30	1.05	**11.03**	**13.83**/71	1.36
9	110.5	**7.80**	1.10	**12.78**/71	1.10
10	190.6	0.89	**9.44**	**12.46**/55	1.14
10α	113.0	**11.0**	0.63	**15.27**/55	1.15
11	163.3	0.89	**10.98**	**13.86**/68	1.11
11α	125.6	**8.50**	0.54	**14.65**/68	1.28
12	189.2	0.77	**9.75**	**13.49**/45	0.98
12α	86.2	**8.61**	0.47	**15.60**/45	1.04
13	174.6	0.94	**10.13**	**15.11**/63	1.13
14	56.8	0.99	3.88	n.d.	**77.14**
1′	173.4	1.04	0.86	n.d.	1.12
2′	50.7	0.99	0.82	n.d.	1.12
3′	23.3	1.11	1.00	n.d.	1.22
4′	23.4	1.04	0.49	n.d.	1.05
5′	175.52	0.71	n.d.	n.d.	0.80



Scheme 1 Biosynthesis of the polyketide aglycon; CYC: cyclase, ARO: aromatase, OXY: oxygenase, MT: methyl transferase, GT: glycosyl transferase.

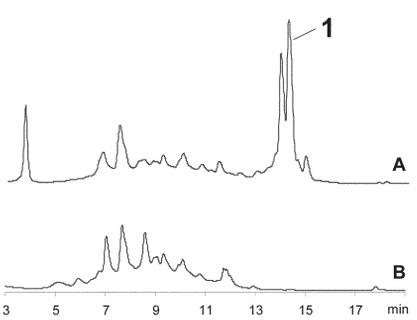
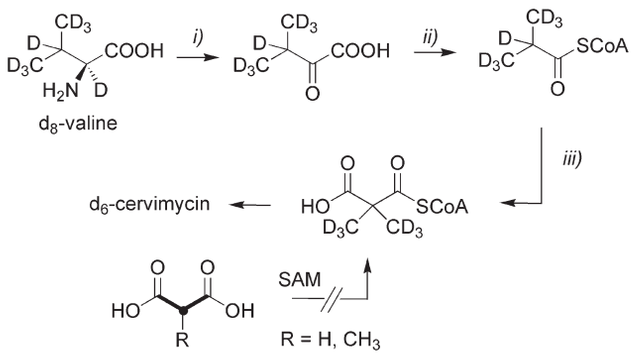


Fig. 2 HPLC profiles of extracts from (A) wild type (S. tendae HKI 19024) and (B) mutant (S. tendae HKI-179::pXU01).

isobutyrate entity. In bacterial metabolism, branched short-chain carboxylic acids, which may serve as starter units for fatty acid synthases[14] and PKS,[6] arise from the catabolism of leucine, isoleucine and valine *via* their respective α-keto acids.[6,15,16] Important examples of this scheme include the biosyntheses of the branched avermectin primers[17–19] and the unusual 4-methylvaleryl starter of the tetrahydroanthracenone derivative R1128C.[20] In order to address this possibility, d_8-labeled valine was administered to the fermentation broth and the extract was analyzed by MS. In spite of the relatively low incorporation rate of the probe (9.4%), ESI-MS^n studies with native and labeled cervimycin C clearly show that the dimethylmalonyl unit of **1** is in fact derived from valine. Observed pseudo molecular ions and pseudo daughter ions are displayed in Fig. 3. In fact, d_8-valine is incorporated with the loss of two deuterium atoms, as the M + 6 shift of the pseudo molecular ions 1250 and 1256 (M + Na) for **1** and d_6-**1** indicate. Since the tetrasaccharide fragment bearing the dimethylmalonyl is labeled (M + Na: 593/599), as opposed to the complementary fragment consisting of polyketide aglycon and two tridesoxyhexoses (M + Na: 680), the d_6-label can undoubtedly be assigned to the dimethylmalonyl unit (Fig. 3). The loss of two deuterium atoms of d_8-valine perfectly matches with the proposed transamination-decarboxylation scheme of amino acid degradation.[6,15,16] The first deuterium label is lost at C-2 during the transamination-dehydrogenation sequence, a process usually catalyzed by an amino acid transaminase,[18] yielding d_7-ketovaleriate. After decarboxylation of d_7-ketovaleriate, putatively promoted by the branched-chain α-keto acid dehydrogenase (BCDH) complex, the resulting thioester may be subject to carboxylation. This reaction can only take place with loss of the C-2 deuterium label (Scheme 2). Finally, the activated d_6-dimethylmalonyl unit could be loaded onto the sugar residue by a specialized acyl transferase.



Scheme 2 Proposed biosynthesis of the dimethylmalonyl unit from valine. i) transamination; ii) ketothiolation-decarboxylation; iii) carboxylation; methyl groups are not derived from SAM.

In summary, we have established the biosynthetic origin of the cervimycin aglycon carbons by isotope labeling experiments as well as molecular studies. We have designed degenerate primers that are specific for a tetracycline-type PKS and targeted the PKS involved in cervimycin biosynthesis by PCR. The identity of the *cer* PKS was confirmed by gene inactivation. Finally, labeling experiments unequivocally reveal that the unusual dimethylmalonyl unit is neither derived from malonate nor from SAM, but from valine. The loss of specific deuterium labels provides strong evidence for a transamination-decarboxylation-carboxylation sequence. Additional work is underway to clone and sequence the entire set of genes involved in the biosynthesis of this potent antibacterial agent for further functional studies as well as for engineering novel bioactive derivatives.

Experimental

Bacterial strains and culture conditions

For cervimycin production, *Streptomyces tendae* HKI-179 was grown in 1 l Erlenmeyer flasks with 500 ml liquid medium consisting of 2.5% glucose, 0.75% cornsteep (FA.), 0.025% KH_2PO_4, 0.5% NaCl at 28 °C for 48 h on a rotary shaker at 160 rpm. For protoplast preparation, *S. tendae* HKI-179 was cultured in YEME.[10] Protoplasts were grown on regeneration medium R5[10] and selected for apramycin resistance. *E. coli* XL1-Blue was used as standard cloning host and grown in LB medium supplemented with ampicillin (100 μg ml^{-1}) for selection.

Labeling experiments

All labeled compounds were purchased from ISOTEC with 99% ^{13}C-atom and 98% ^{2}H-atom purity. 500 ml shaking cultures were inoculated with 5 vol% of a 30 h old seed culture. Precursors were added to each 500 ml culture by pulse feeding as sterile solutions (5 ml) at 14, 17, 19 and 21 h after incubation in the following concentrations (total volume of fermentation). a: 1.8 mmol l^{-1} 2-^{13}C-malonic acid (4 l), b: 1.8 mmol l^{-1} 1,3-$^{13}C_2$-malonic acid (4 l), c: 1.7 mmol l^{-1} 1,2,3-$^{13}C_3$-malonic acid (4 l), d: 1.6 mmol l^{-1} L-methionine-(methyl-^{13}C), e: 0.2 mmol l^{-1} d_8-L-valine (1 l). The cultures were harvested after 48 hours and separated into mycelium and supernatant by centrifugation. The dried combined extracts of mycelium (MeOH extract, re-extraction with ethyl acetate) and supernatant (ethyl acetate extract) were applied to a sephadex column to obtain a crude cervimycin mixture. The cervimycin complex was separated by preparative reverse phase HPLC (Shimadzu HPLC-system, ProntoSIL column 120-15-C_{18}AQ, 250 × 20 mm, 15 μm particle size, eluted with ACN (83%)/0.01 mol aqueous ammonium acetate (pH = 5.0) with a gradient ranging from 20% to 99.5% ACN (83%) during 25 min at a flow rate of 25.0 ml min^{-1} (UV detection at 247 nm). Yields of labeled **1**: a: 66.4 mg, b: 48.3 mg, c: 51.0 mg, d: 36.4 mg, e: 11.2 mg. The labeled cervimycin was analyzed by NMR and ESI-MS and ESI-MS^n. ESI-MS spectra were recorded on a VG Quattro of Fisons Instruments equipped with electrospray ion source. A Finnigan LCQ benchtop mass spectrometer equipped with electrospray ion source and ion-trap mass analyzer was used for ESI-CID-MS^n experiments. Fragmentation (CID-MS^n) of pseudomolecular ions ($[M + Na]^+$) was conducted by collision-induced dissociation using helium as collision gas (Spray voltage: 5 kV, capillary temperature: 200 °C. NMR spectra of pure labeled **1** in pyridine were recorded with a Bruker Avance DPX 300 and DRX 500.

PCR targeting and inactivation of the *cer* PKS

DNA isolation, plasmid preparation, restriction digests, gel electrophoresis and ligation reactions were conducted according to standard methods. Restriction enzyme-digested DNA fragments were recovered from agarose gel by the GFX PCR DNA and Gel Band Purification Kit (Amersham). Two fragments of expected sizes (0.8 kb and 0.7 kb) were amplified with primer-pairs cer-F1 (5′-ATGACSATSAGYCTSGACCG-3′)/cer-R2 (5′-AGSGGSACGTAGTCSAGG-3′), and cer-F2 (5′-ACTTC-GTSCCSTCSTCSATGG-3′)/cer-R2 (5′-AGSGGSACGTAGTC-SAGG-3′), respectively, from a *S. tendae* HKI-179 genomic DNA template (with S = C or G; Y = C or T). These two fragments were cloned using the pGEM-T Easy Vector System (Promega) and sequenced. Inactivation of KS_α of cervimycin was performed by insertional disruption *via* single crossover. The 0.8 kb KS_α fragment was excised with *Eco*RI and ligated into the *Eco*RI sites of suicide plasmid pOJ260.[9] DNA of the resulting plasmid (pXU01) was denatured and introduced into *S. tendae* HKI-179 by PEG-mediated protoplast transformation.[10] Selection with 40 μg ml^{-1} apramycin yielded clone *S. tendae* HKI-179::pXU01. Integration of the plasmid was confirmed by PCR amplification of the apramycin cassette from genomic DNA of the mutant, with pOJ260 and genomic DNA of *S. tendae* HKI-179 as positive and negative controls, respectively.

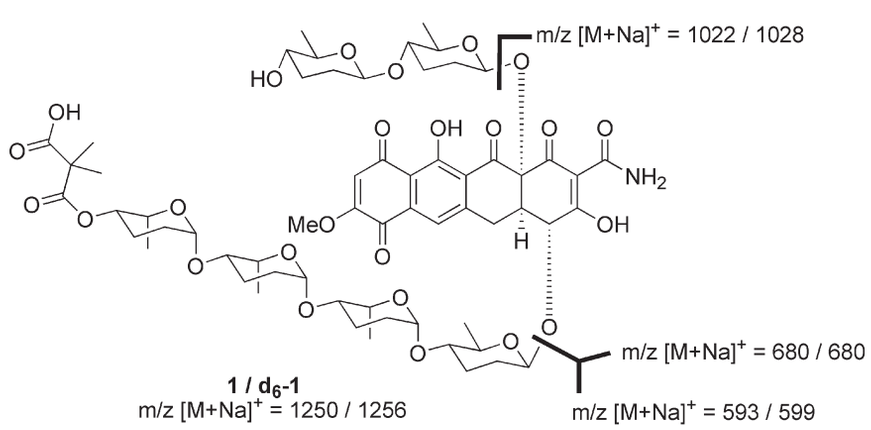


Fig. 3 Results of MS^n experiments: pseudo daughter ions of **1**/d_6-**1**.

Acknowledgements

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References and footnotes

‡ Database searches suggest that cervimycin C is closely related to or identical with compound A2121A from the patent literature: Japanese Patent, 1994, 94 339 395 (CA 122, 237919g).

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