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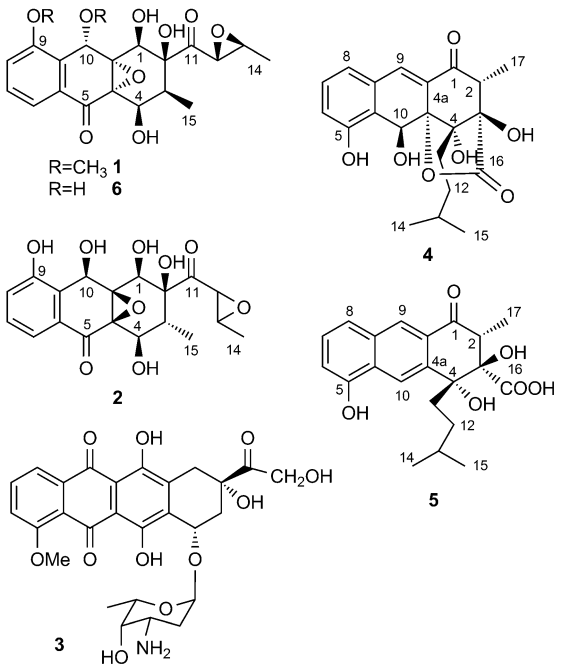
Cloning and Heterologous Expression of Three Type II PKS Gene Clusters from *Streptomyces bottropensis*

Xiaohui Yan,[a] Katharina Probst,[a] Anton Linnenbrink,[a] Moritz Arnold,[b] Thomas Paululat,[c] Axel Zeeck,[b] and Andreas Bechthold*[a]

Mensacarcin is a potent cytotoxic agent isolated from *Streptomyces bottropensis*. It possesses a high content of oxygen atoms and two epoxide groups, and shows cytostatic and cytotoxic activity comparable to that of doxorubicin, a widely used drug for antitumor therapy. Another natural compound, rishirilide A, was also isolated from the fermentation broth of *S. bottropensis*. Screening a cosmid library of *S. bottropensis* with minimal PKS-gene-specific primers revealed the presence of three different type II polyketide synthase (PKS) gene clusters in this strain: the *msn* cluster (mensacarcin biosynthesis), the *rsl* cluster (rishirilide biosynthesis), and the *mec* cluster (putative spore pigment biosynthesis). Interestingly, luciferase-like oxygenases, which are very rare in *Streptomyces* species, are enriched in both the *msn* cluster and the *rsl* cluster. Three cosmids, cos2 (containing the major part of the *msn* cluster), cos3 (harboring the *mec* cluster), and cos4 (spanning probably the whole *rsl* cluster) were introduced into the general heterologous host *Streptomyces albus* by intergeneric conjugation. Expression of cos2 and cos4 in *S. albus* led to the production of didesmethylmensacarcin (DDMM, a precursor of mensacarcin) and the production of rishirilide A and B (a precursor of rishirilide A), respectively. However, no product was detected from the expression of cos3. In addition, based on the results of isotope-feeding experiments in *S. bottropensis*, a putative biosynthesis pathway for mensacarcin is proposed.

Introduction

Mensacarcin (**1**) is a potent antitumor agent that was first isolated from the culture broth of *Streptomyces bottropensis* (formerly named *Streptomyces.* sp. Gö C4/4, found in a soil sample by the cafeteria (Mensa) at the University of Göttingen) in 1998.[1] It has nine stereogenic centers including two epoxide groups (Scheme 1) and is closely related to cervicarcin (**2**), an antitumor antibiotic produced by *Streptomyces ogaensis*.[2–4] In an antitumor activity assay performed by Beil,[5] mensacarcin showed an average total growth inhibition (TGI) value of 1.3 μM against 60 cell lines, which is comparable to that of doxorubicin (**3**; TGI = 1.9 μM), one of the most widely used agents for antitumor therapy.[6] Furthermore, mensacarcin displays cytostatic and cytotoxic activity against the doxorubicin-resistant human gastric carcinoma cell line KATO III.[5] Cytotoxicity studies with different mensacarcin analogues indicated that the hydroxylation pattern of ring C and the side chain play an important role in the cytostatic effect.[7] Despite all the investigations on the biological activities of mensacarcin, the exact mechanism of action at the molecular level still remains unknown.



Scheme 1. Structures of mensacarcin (**1**), cervicarcin (**2**), doxorubicin (**3**), rishirilide A (**4**), rishirilide B (**5**), and DDMM (**6**).

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Rishirilide A (**4**) and B (**5**) are α_2-macroglobulin inhibitors, first found in culture broth of *Streptomyces rishiriensis* OFR-1056 in 1984.[8] They both have a tricyclic, partial-aromatic backbone with a C-4 isopentyl side chain. Rishirilide A and B were found to inhibit α_2-macroglobulin with an IC_{50} of 100 $\mu g\,mL^{-1}$ for (**4**) and 35 $\mu g\,mL^{-1}$ for (**5**). α_2-Marcoglobulin is a tetrameric serum glycolprotein that irreversibly inactivates a variety of proteinases by a unique trapping mechanism.[9,10] Inhibitors of α_2-macroglobulin are very useful in the prevention and treatment of fibrinolytic accentuation-induced thrombosis.[11,12] Rishirilide B was also observed to inhibit glutathione S-transferase.[13] As glutathione S-transferase can lead to enhanced resistance toward many anticancer drugs,[14–16] rishirilide B might be helpful in anticancer therapies. Interestingly, Arnold[17] also isolated rishirilide A from the culture of *S. bottropensis*.

Typically, type II polyketide synthase (PKS) consists of three "minimal PKS" proteins: ketosynthase α (KS_α), ketosynthase β/chain length factor (KS_β/CLF), and acyl carrier protein (ACP). These enzymes are responsible for the assembly of the polyketide chains, which are modified by the polyketide ketoreductase, aromatases, and cyclases to form the aromatic rings.[18] Finally, the intermediates are transformed into fully decorated natural products by tailoring enzymes, for example, oxygenases, glycosyltransferases, and methyltransferases.[19,20] These tailoring reactions can affect both the physicochemical properties and the biological activities of the compounds. Consequently, the recombining of genes from different biosynthetic pathways has a huge potential for generating new compounds with novel and improved properties.[21,22]

In this paper we report the screening, sequence analysis, and heterologous expression of three type II PKS gene clusters from *S. bottropensis*. The *mec* cluster is believed to encode proteins for polyketide spore pigment production, while the *rsl* and *msn* clusters are proven to be responsible for the biosynthesis of rishirilides (**4** and **5**) and mensacarcin (**1**), respectively. Furthermore, feeding experiments with [^{13}C] and [$^{18}O_2$]gas-labeled precursors in *S. bottropensis* clearly provided us with a possible biosynthetic pathway for mensacarcin.

Results and Discussion

Production of mensacarcin and rishirilide A by *S. bottropensis*

Mensacarcin (**1**) was produced in shake flasks under standard conditions. After neutral extraction, the lyophilized culture filtrate was chromatographed on silica gel. The active fractions were collected and formed white crystals in ethyl acetate/*n*-pentane (purified yield 60 $mg\,L^{-1}$). The ^{1}H and ^{13}C NMR spectra of mensacarcin are summarized in the Experimental Section. Its full structure was obtained from the 2D-NMR data and a crystal structure, combined with chemical derivatization. Production of rishirilide A by *S. bottropensis* was observed in medium Nr.1153, in which no mensacarcin was produced. After purification on silica gel and sephadex LH-20, a white solid was isolated (yield 5 $mg\,L^{-1}$). The ^{1}H and ^{13}C NMR data of this compound (Table 1) were in good accordance to those for rishirilide A reported by H. Iwaki,et al.[8] A combination of the NMR, UV, and mass and IR spectra was used to deduce the identity of this compound to be rishirilide A. However, production of rishirilide by *S. bottropensis* was accomplished only once—no further production was successful.

Table 1. ^{13}C and ^{1}H NMR spectral data of rishirilide A (**4**) and B (**5**)

Pos.	Rishirilide A[a]		Rishirilide B[a]	
	δ_c (ppm)[b]	δ_H (J [Hz])[c]	δ_c (ppm)[d]	δ_H (J [Hz])[e]
1	199.4		197.3	
2	50.7	2.77 (q, 7.0)	35.1	2.95 (q, 6.6)
3	81.8		77.0	
4	82.1		83.5	
4a	85.4		140.6	
5	156.8		153.2	
6	120.7	6.89 (d)	119.6	6.92 (d, 7.4)
7	131.0	7.24 (dd, 8.0)	125.6	7.28 (dd, 7.4)
8	123.8	6.89 (d)	119.8	7.45 (d, 7.4)
8a	131.7		132.4	
9	139.6	7.57 (s)	126.4	8.27 (s)
9a	131.9		126.1	
10	64.6	5.49 (s)	109.8	8.25 (s)
10a	123.2		130.2	
11	31.0	1.49–1.70,2.52 (m)	48.0	1.49–1.63 (m)
12	32.6	1.49–1.70 (m)	31.2	2.15–2.30 (m)
13	30.0	1.41 (m)	29.0	1.21–1.33 (m)
14	23.1	0.89 (d, 6.0)	22.8	0.76 (d, 6.6)
15	22.7	0.86 (d, 6.0)	22.5	0.65 (d, 6.6)
16	177.5		174.2	
17	12.6	1.21 (d, 8.0)	10.2	1.17 (d, 6.6)

[a] Rishirilide A was isolated from *S. bottropensis*, rishirilide B was isolated from *S. albus*::cos4. [b] and [c] in CD_3OD. [b] 75.5 MHz; [c] 300 MHz. [d] and [e] in [D_6]DMSO. [d] 100 MHz; [e] 400 MHz.

Cloning of the three type II PKS biosynthetic gene clusters from *S. bottropensis*

A genomic cosmid library of *S. bottropensis* was constructed and screened by using a strain-specific ketosynthase α gene probe.[23] Cosmids hybridizing to the probe could be arranged in three groups with overlapping DNA sequences; this indicated the existence of three type II PKS gene clusters in the genome of *S. bottropensis*. The three gene clusters were named *mec*, *msn*, and *rsl*. Cosmid mapping and end-sequencing of cosmid subclones provided a first insight into the sizes and organization of the gene clusters. One cosmid (cos4, harboring probably the whole *rsl* gene cluster) and another (cos2, covering most of the *msn* genes) were sequenced by a combined approach of end-sequencing and primer walking with subclones from overlapping cosmids. The *mec* cluster is located in cosmid cos3. Because of its limited size (8.8 kb), the full sequence of the *mec* cluster was determined by the primer walking method.

The *mec* cluster is a spore pigment biosynthetic gene cluster

Analysis of the nucleotide sequence of the *mec* cluster revealed eight ORFs that encode proteins with very high homol-

ogy (>94% identity) to the spore pigment biosynthesis proteins from *Streptomyces scabies* 87.22 (see the Supporting Information).[24] Moreover, the *mec* cluster displayed strikingly similarity in gene organization (Figure 1) to the spore pigment biosynthetic gene clusters from *Streptomyces collinus* DSM 2012 (unpublished data), *Streptomyces avermitilis* MA-4680,[25] *Streptomyces coelicolor*,[26] and other *streptomyces* strains.[27–29] From these results it is reasonable to propose that the *mec* cluster is responsible for the biosynthesis of spore pigment in *S. bottropensis*.

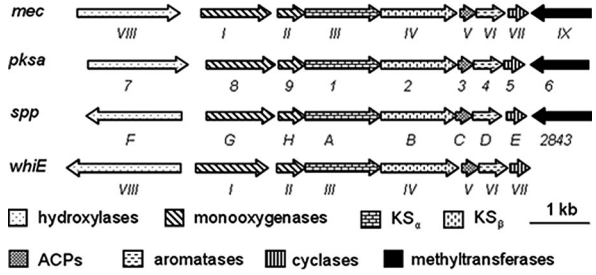


Figure 1. Alignment of the *mec* cluster with other spore pigment gene clusters from *Streptomyces* species. *pksa*: putative spore pigment polyketide synthase cluster from *Streptomyces collinus* DSM2012 (unpublished); *spp*: putative spore pigment biosynthetic gene cluster from *Streptomyces avermitilis* MA-4680; *whiE*: spore pigment cluster from *Streptomyces coelicolor* A3(2); KS: ketosynthase; ACP: acyly carrier protein.

Although ectopic expression of the *whiE-*, the *sch-* and the *cur-* spore pigment gene clusters led to the isolation of polycyclic aromatic polyketides,[30] characterization of the spore pigments from either *S. bottropensis* or *S. albus* (cos3) was unsuccessful. *S. bottropensis* can form gray spores on the MS plate, while the color of the spores from its Δ*mec III* mutant were yellow. This clearly proves that the *mec* cluster is responsible for the biosynthesis of spore pigment in *S. bottropensis*.

Heterologous expression of cos2 and cos4 in *S. albus*

Cosmids co2 and cos4 were introduced into *S. albus* by intergeneric conjugation, and their production profiles were analyzed by HPLC/ESI-MS at 254 nm. In the chromatogram of the *S. albus*::cos2 extract, a new compound with molecular ions of *m/z* 391 $[M-H]^-$ and *m/z* 427 $[M+Cl]^-$ was spotted at 9.9 min (Figure 2C, see also the Supporting Information). Comparison of the UV and mass spectra of this compound to those of the production profiles of *S. bottropensis* wild type revealed the identity of this compound as didesmethylmensacarcin (DDMM, **6**), a biosynthetic precursor of mensacarcin.[17] The successful production of DDMM in *S. albus*::cos2 suggests that the *msn* gene cluster is responsible for the biosynthesis of mensacarcin. The production of DDMM instead of mensacarcin in *S. albus*::cos2 could be attributed to the fact that the genes encoding methyltransferase (*MsnM6*) or enzymes for *S*-adenosyl-L-methionine regeneration (*msnM3–msnM*) were not located on cos2.

From the chromatogram of the *S. albus*::cos4 extract, two novel UV-active peaks could be detected (Figure 2B). In negative mode ESI-MS, these two substances displayed masses of *m/z* 387.3 (20.6 min) and *m/z* 371.3 (22.9 min; see the Supporting Information). Determination of the major compound as rishirilide B was based on ESI-MS and NMR spectral data (Table 1).[8] Rishirilide A, the minor compound, was identified by HPLC/MS. Therefore, the *rsl* cluster is responsible for the biosynthesis of rishirilide A. In an attempt to check the reproducibility of rishirilide A and B by heterologous expression, cos4 was also introduced into another heterologous host, *Streptomyces lividans* 1326. The resulting strain (*S. lividans*::cos4) was cultivated in several different media (SG, HA, and NL19+), and all produced rishirilide A and B (Supporting Information).

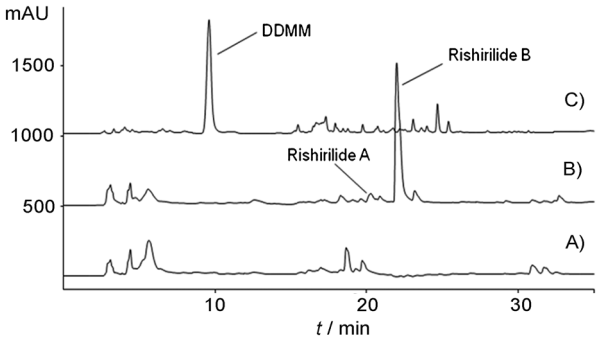


Figure 2. HPLC analysis (254 nm) of the product profiles of *S. albus* and its mutants. A) *S. albus* wild-type, B) *S. albus*::cos4, C) *S. albus*::cos2.

Analysis of the rishirilide biosynthetic gene cluster

Cosmid cos4 spans 46 kb and contains 42 putative ORFs. The first 11 genes encode peptides with high similarity (>77% identity) to proteins for the biosynthesis of the exopolysaccharide in *Streptomyces sp.* 139;[31] therefore, they are assumed to be involved in *S. bottropensis* exopolysaccharide production. The last three ORFs in cos4 encode two peptides that are similar to putative transcription anti-termination regulator from *Streptomyces ambofaciens* (ATCC 23877)[32] and one hypothetic protein. The rishirilide biosynthesis cluster is located in the middle of cos4. It contains 28 ORFs, including 20 structural genes, four regulatory genes, and four genes related to the secretion of rishirilides (Supporting Information). The position and organization of the *rsl* cluster in cos4 is presented in Figure 3.

The putative functions of the 20 structural genes were assigned based on homology with known aromatic PKS genes. Similar to the biosynthetic gene clusters of R1128,[33] daunorubicin,[34] and aclacinomycins,[35] *rslA* and *rslK4* encode an acyltransferase and a starter unit specific for β-ketoacyl/ACP synthase III (KSIII); these are homologous to *AknF* and *AknE2* from the aclacinomycin biosynthesis pathway and to the *dpsC* and *dpsD* genes from the daunorubicin biosynthesis pathway. A cell-free experiment showed that DpsC, a type III ketosynthase, together with an acyltransferase (DpsD), plays a primary roles in the selection of the starter unit and in the initiation of daunorubicin polyketide biosynthesis.[36] In the biosynthesis of

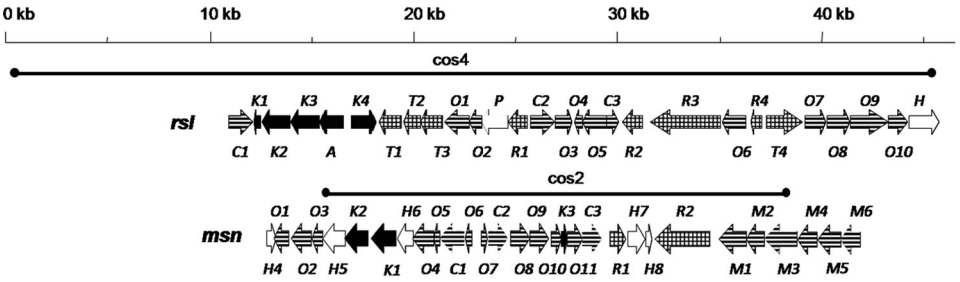


Figure 3. Organizations of the *rsl*- and the *msn*- biosynthetic gene clusters. The locations of the clusters in the cosmids are also indicated, with genes for polyketide chain biosynthesis (black), polyketid modifying (striped), and enzymes for regulation and reisistance (chequered). Genes that are probably not involved in mensacarcin and rishirilide biosynthesis are displayed in white.

aclacinomycins, the products of *AknE2* and *AknF* also cooperate to provide the propionyl-CoA starter unit.[35] Because of their high similarities to AknE2 and AknF, RslK4 and RslA are believed to process the unusual isobutyryl-CoA starter unit and form the ACP-bound intermediate, thus resembling the biosynthesis pathway of R1128C, an agent with the same side chain as rishirilides. The intermediate is subsequently transferred onto the KS_α/KS_β heterodimer (encoded by *rslK3* and *rslK2*) and elongated by seven malonyl-CoA extender units to generate the complete polyketide chain. ACP (encoded by *rslK1*) and a malonyl-CoA/ACP malonyltransferase (MAT), probably from the endogenous fatty acid biosynthetic pathway, also participate in the chain extension reactions. Aromatization and cyclization of the polyketide chain are catalyzed by RslC1, RslC2, and RslC3.

There are ten putative oxidoreductases in the rishirilide gene cluster. RslO4, RslO8, and RslO10 show high similarity to the well studied proteins SnoaB,[37] ActVI-ORF2[38] and AknA,[39] respectively, thereby implying that they possibly decorate the rishirilide polyketide by post-PKS modifications (like the homologous three enzymes). RslO1 and RslO6 are similar to proteins from the bacterial luciferase-like monooxygenase family. Such enzymes are very rare in *streptomyces* species. Their functions in the biosynthesis of secondary metabolites will be of great interest.

Notably, there were altogether four regulators in the rishirilide cluster. The first three (RslR1, RslR2, and RslR3) are SARP (*Streptomyces* Antibiotic Regulatory Protein) family regulators. Because most reported antibiotic biosynthesis clusters from Streptomyces harbor only one SARP-encoding gene,[40] the occurrence of 3 SARP-type regulators in the rishirilide gene cluster is quite unusual. This might help to explain the nonreproduciblility of rishirilide A (**4**) production in *S. bottropensis*. RslR4 showed similarity to transcriptional regulators of the MarR family, which are responsible for multiple antibiotic resistance.[41] Adjacent to the polyketide synthases are three ABC transporter genes, encoding a substrate binding protein (RslT1), an ATP-binding protein (RslT2), and a trans-membrane protein (RslT3). Another transporter peptide (RslT4) showed 39% identity to the resistance protein chlG in the chlorothricin gene cluster.[42] These proteins are probably involved in the export of and resistance to rishirilides.

Analysis of the mensacarcin biosynthetic gene cluster

Sequence analysis of the *msn* cluster revealed 34 ORFs, with 23 structural genes, two regulatory genes, and nine genes encoding hypothetical proteins of unknown function (Supporting Information). The products of *msn*K1, *msn*K2, and *msn*K3 form the "minimal" PKS II complex (KS_α/KS_β/ACP) for mensacarcin biosynthesis. Because MsnK3 is separate from the other two genes, the mensacarcin PKS also belongs to the rare disconnected minimal PKS.[43,44] Three putative cyclases (MsnC1, MsnC2, and MsnC3) in the *msn* cluster cyclize the highly unstable open polyketide chain formed by the minimal PKS. MsnC2 displays high similarity to ActIV (52% identity), a cyclase in the actinorhodin biosynthesis cluster.[45] MsnC1 and MsnC3 are similar to bifunctional cyclases/dehydratases like Med-ORF19 and RdmK,[46,47] thereby providing a strong hint that these genes may analogously catalyze aromatization of the A-ring. Furthermore, three ketoreductases (MsnO1, MsnO10, and MsnO11) were found within this cluster. MsnO1 and MsnO11 show high similarities to BenL from the benastatin pathway[48] and ActIII from the actinorhodin pathway,[49] respectively, but no protein similar to MsnO10 was discovered. Like their orthologues, these ketoreductases might be involved in the reduction of keto groups that determine the orientation of the polyketide chain and help to form the rings.

The highly oxygenated structure of the mensacarcin backbone is consistent with the existence of many putative oxygenase-encoding genes in the *msn* cluster. Among these, MsnO2, MsnO4, and MsnO8 display high similarities to subdomains of the luciferase of *Vibrio harveyi*, which is responsible for bioluminescence.[50] Analysis of conserved domains in MsnO8 revealed a coenzyme F_{420}-dependent N_5,N_{10}-methylene-tetrahydromethanopterin (methylene-H_4MPT)-reductase motif (COG 2141). This is consistent with the observation that luciferase-like oxidoreductases use F_{420}, while the bacterial luciferases use flavin mononucleotide (FMN), as coenzyme.[51] However, the COG2141 domain was not found in MsnO2 or MsnO4. The deduced product of *msnO3* resembles Gra-orf34, a flavin reductase from the granaticin biosynthesis pathway.[52] MsnO5, MsnO6, and MsnO7 are putative cofactor-independent monooxygenases. Their orthologues, (e.g., AknX[53] and SnoaB)[54] are proposed to assemble as multimers and introduce the quinine oxygen. MsnO9 is highly similar to an NADPH:quinone oxidoreductase, GrhO7, which collaborates with GrhO3 to form the epoxy moiety in griseorhodin A.[55] As the mensacarcin molecule contains two epoxy moieties, it is proposed that MsnO9 is responsible for the introduction of the epoxy moiety. However no protein similar to GrhO3 was found in the *msn* cluster.

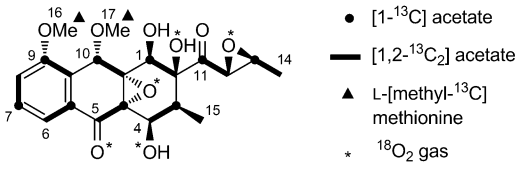
Mensacarcin has four methyl groups. MsnM6, the only methyltransferase in the *msn* cluster, is proposed to be responsible

for the methylation of 9- and 10-OH. This hypothesis was proved by feeding experiments with L-[methyl-^{13}C] methionine (discussed below). Because methyltransferases usually show high regiospecificity,[56] it is very interesting that MsnM6 can catalyze the transfer of two methyl residues at different positions of the molecule. Beside MsnM6, there are five more genes in the cluster, encoding proteins that are necessary for the regeneration of *S*-adenosyl-L-methionine (SAM; MsnM1–MsnM5). Enzymes involved into the utilization and regeneration of SAM were also found in some biosynthesis pathways of methylated secondary metabolites.[48,57,58]

Biosynthesis studies of mensacarcin in *S. bottropensis*

In order to identify the building blocks of mensacarcin, feeding experiments with [1-^{13}C]acetate, [1,2-$^{13}C_2$]acetate, L-[methyl-^{13}C]methionine and $^{18}O_2$ gas were conducted. The [^{13}C]acetate feeding results showed the presence of nine intact acetate units as well as one acetate-derived methyl group (C-15). Thus it could be deduced that the mensacarcin polyketide chain is synthesized by the type II PKS enzymes complex (MsnK1–3) from an acetyl-CoA starter unit and nine malonyl-CoA extender units. The enhancement of the ^{13}C NMR signal of C-15 by incorporated [1,2-$^{13}C_2$]acetate suggested the occurrence of a decarboxylation reaction at this position. Feeding studies with L-[methyl-^{13}C]methionine resulted in enhancement of the ^{13}C NMR signal at C-16 and C-17, which implies that these two methyl groups were derived from SAM (Scheme 2).

The high oxygen content of mensacarcin and the enrichment of different oxygenases in the *msn* cluster are intriguing.



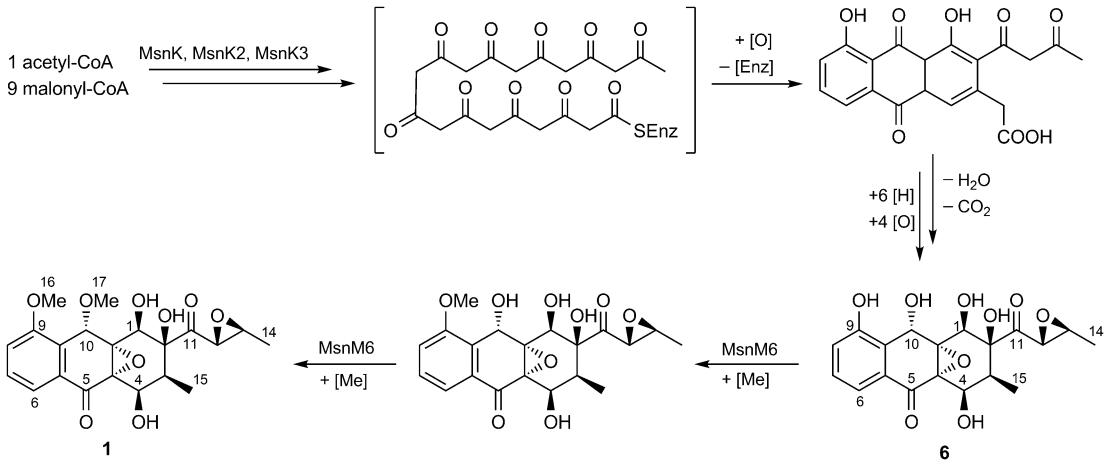
Scheme 2. Labeling patterns of mensacarcin with different precursors.

Table 2. ^{13}C and [$^{18}O_2$] incorporation into mensacarcin together with coupling constants after feeding with [1-^{13}C]acetate (I), [1,2-$^{13}C_2$]acetate (II), L-[methyl-^{13}C]-methionine (III) and [$^{18}O_2$] gas (IV).

Pos.	δ_c (ppm)[a]	I[b]	II J_{CC} [Hz][c]	III[d]	IV $\Delta\delta$ (ppb)	IV $^{16}O/^{18}O$ [%]
1	70.0	11.9	37.5	0		
2	83.2	−0.5	37.5		27	58:42
3	31.5	13.4	37.5			
4	68.3	−0.3	37.5		21	54:46
4a	63.2	12.2	55.3		25	51:49
5	192.6	−0.4	55.3		42	87:13
5a	130.2	10.9	59.3			
6	119.3	−0.4	59.3			
7	129.5	15.9	56.5			
8	116.8	−0.4	56.5			
9	158.8	13.1	69.5			
9a	127.9	−0.5	69.5			
10	69.4	12.9	49.9			
10a	66.3	−0.5	49.9		38	53:47
11	208.8	14.8	53.2			
12	58.0	−0.1	53.2		28	53:47
13	56.9	15.9	44.6		34	51:49
14	17.7	0.0	44.6			
15	11.0	−0.2		0		
16	56.0	0.0		49.1		
17	59.2	0.0		54.3		

[a] Chemical shifts are referred to residual $CDCl_3$. [b] Relative enrichments were normalized to the peak intensity of the C-2′ signal. [c] Relative enrichments/coupling constants based on ^{13}C NMR data measured in $CDCl_3$. [d] Relative enrichments were normalized to the C-1′ signal because it had no ^{13}C incorporation when L-[methyl-^{13}C]-methionine was used.

In an attempt to determine the bioincorporation of the oxygen atoms into mensacarcin, production of mensacarcin was also performed in an [$^{18}O_2$]-enriched atmosphere. The isolated product displayed enhanced α-isotopic shifts in the ^{13}C NMR spectrum for C-2, C-4, C-5, C-4a/C-10a, and C-12/C-13 (Table 2). This suggests that the five oxygen atoms connected to the above carbons are introduced into mensacarcin by oxygenases, while the other oxygen atoms possibly derive from the starter and extender units in the polyketide biosynthesis (Scheme 2). From these results, a putative biosynthesis pathway for mensacarcin is proposed (Scheme 3).



Scheme 3. Proposed biosynthetic pathway for mensacarcin (**1**).

Conclusion

Mensacarcin and rishirilide A were both isolated from the fermentation broth of *S. bottropensis*; however, production of the latter was not repeatable due to a still unknown problem. Searching of the type II ketosynthase genes in the cosmid library of *S. bottropensis* led to the discovery of three polyketide biosynthesis gene clusters. The genes in the *mec* cluster showed very high similarities to the genes from a spore pigment biosynthetic cluster. Although heterologous expressions of cos2 only produced didesmethylmensacarcin (**6**), an intermediate product of mensacarcin synthesis, expression of the *rsl* cluster in *S. albus* and *S. lividans* yielded rishirilide A (**4**) and B (**5**).

These results provide the foundation for investigating the biosynthesis of these two natural compounds. With the help of isotope feeding experiments, the origin of the building blocks for the backbone and the decoration groups of mensacarcin were clarified.

Experimental Section

Construction of cosmid library and screening of the type II PKS clusters: A genomic library of *S. bottropensis* was constructed by Combinature Biopharm AG (Berlin, Germany) according to previously described protocols.[59] Screening of the cosmid library was performed by standard hybridization and detection methods. The ketosynthase gene probes for cosmid hybridization were generated by using the KS forward primer 5′-TSG CST GCT TGG AYG CSA TC-3′ and KS reverse primer 5′-TGG AAN CCG CCG AAB CCG CT-3′ according to Metsä-Ketelä et al. (S = C or G; Y = C or T; B = C, G or T and N = A, T, C or G).[23] Cosmids with significant hybridization signals were further analyzed by the pattern of BamHI digestion. Among them, three representative cosmids (cos2, cos3, and cos4) were sequenced by shotgun sequencing and primer-walking. ORFs were predicted by using the Frame Plot 3.0 beta program http://watson.nih.go.jp/~jun/cgi-bin/frameplot-3.0b.pl, and the deduced proteins were analyzed by comparing with other known proteins in the NCBI database.

Heterologous expression of the three type II PKS clusters: The transfer of cos2, cos3 and cos4 from *E. coli* ET12567(pUZ8002) into *S. albus* J1074 and *S. lividans* 1326 was accomplished by intergeneric conjugation using standard protocols, to obtain *S. albus*::cos2, *S. albus*::cos3, *S. albus*::cos4, and *S. lividans*::cos4.[60] Integration of the cosmids was confirmed by apramycin resistance and PCR. Overnight cultures of *S. albus* wild-type and mutants (10 mL each) were inoculated into HA liquid medium (100 mL, yeast extract (4 gL^{-1}), malt extract (10 gL^{-1}), glucose (4 gL^{-1}), pH 7.2)) and cultured at 28 °C, 180 rpm for five days. For other media, the components were SG: soya peptone (10 gL^{-1}), glucose (20 gL^{-1}), L-valine (2.34 gL^{-1}), $CaCO_3$ (2.0 gL^{-1}), pH 7.2; and NL19+: mannitol (20 gL^{-1}), soya flour (20 gL^{-1}), L-valine (2.34 gL^{-1}), pH 7.5.

Production of mensacarcin and rishirilide A in *S. bottropensis*: Mensacarcin was produced in oatmeal medium (oatmeal (20 gL^{-1}), 2.5 mLL^{-1} trace element solution ($ZnCl_2$ (40 mgL^{-1}), $FeCl_3 \cdot 6H_2O$ (200 mgL^{-1}), $CuCl_2 \cdot 2H_2O$ (10 mgL^{-1}), $MnCl_2 \cdot 4H_2O$ (10 mgL^{-1}), $Na_2B_4O_7 \cdot 10H_2O$ (10 mgL^{-1}), $(NH_4)Mo_7O_{24} \cdot 4H_2O$ (10 mgL^{-1}), pH 7.8) at 28 °C. One eighth of an agar plate, well-covered with *S. bottropensis*, was inoculated into a 250 mL Erlenmeyer flask containing oatmeal medium (100 mL) and grown for 48 h. Fermentations of mensacarcin were carried out in parallel in two 2 L fermenters (containing 1 L oatmeal medium) and one 5 L fermenter (containing 3 L medium), with the 48 h preculture (10% *v/v*). Fermentations in the 2 L fermenters were conducted with stirring (500 rpm) and aeration (5 vvm) for 60–72 h. For the 5 L fermenter, the aeration was 3 vvm and the fermentation time was 72 h. In the 2 L fermenters, an average yield of 56.2 mgL^{-1} was obtained; in the 5 L fermenter, the yield was 32.3 mgL^{-1}.

For the production of rishirilide A, *S. bottropensis* was cultured in Nr. 1153 medium (glycerol (12.5 gL^{-1}), arginine (1 gL^{-1}), $MgSO_4 \cdot 7H_2O$ (0.5 gL^{-1}), NaCl (1 gL^{-1}), with supplementation solution (5 mLL^{-1}: $CaCl_2 \cdot 2H_2O$ (3 gL^{-1}), Fe^{III} citrate (1 gL^{-1}), $MnSO_4$ (0.2 gL^{-1}), $ZnCl_2$ (0.1 gL^{-1}), $CuSO_4 \cdot 5H_2O$ (25 mgL^{-1}), $Na_2B_4O_7 \cdot 10H_2O$ (20 mgL^{-1}), $CoCl_2$ (4 mgL^{-1}), $Na_2MoO_4 \cdot 2H_2O$ (10 mgL^{-1})), pH 7.3). The 48 h preculture was inoculated into a 3 L fermenter containing medium (2 L), with stirring (500 rpm) and aeration (1 vvm). The fermentation was performed at 28 °C for 113 h. The yield of rishirilide A was 5 mgL^{-1}.

Feeding of isotope-labeled materials: For the feeding of ^{13}C-labelled compounds, sodium [1-^{13}C]acetate (750 mg), sodium [1,2-$^{13}C_2$]acetate (450 mg), and L-[methyl-^{13}C]methionine (380 mg; 99% ^{13}C purity; Chemotrade, Düsseldorf, Germany) were dissolved in sterile water (100 mL) and adjusted pH to 7.0. After the 48 h precultivation as described above, the 100 mL *S. bottropensis* broth was added to sterile oatmeal medium (900 mL) in a 2 L fermenter and cultivated for 24 h (500 rpm, 3 vvm). Then the aqueous solution (100 mL) containing the labeled precursors was continuously fed into the fermenter (5 mLh^{-1}). When the feeding was finished, the fermentation was conducted for extra 48 h.

Feeding of [$^{18}O_2$] to mensacarcin was performed in a closed apparatus as described previously.[61] One Erlenmeyer flask contained sterile water (100 mL). Four flasks contained sterile oatmeal medium (100 mL) and were inoculated with sections of well-covered agar (1 cm^2) from a *S. bottropensis* plate. Bacteria were cultured at 28 °C with stirring (250 rpm) and aeration (5 vvm). After 35 h, the apparatus was flushed with nitrogen (3 min), then the nitrogen was drawn out and [$^{18}O_2$] gas was introduced into the system for 45 h. The yield of ^{18}O-labeled mensacarcin was 40 mgL^{-1}.

Isolation and identification of compounds: After fermentation, cultures were extracted twice with equal volumes of ethyl acetate. The organic extracts were dried in vacuo and dissolved in methanol. The preparative isolation of mensacarcin was done by silica gel flash chromatography (column 30 × 2 cm) with dichlormethane/methanol (97:3) as solvent. Purification of rishirilide A and B was performed by a combination of flash chromatography in silica gel (chloroform/methanol, 95:5) and Sephadex LH-20 (methanol, 40.0 × 2.3 cm). HPLC/MS analysis was performed on an Agilent 1100 series LC/MS system with electrospray ionization (ESI) and detection in the positive and negative modes. The LC system was equipped with a Zorbax SB C-18 column (5 μm particle size, 4.6 × 150 mm; Hewlett Packard), maintained at room temperature. Detection wavelength of the diode array was 254 nm. The gradient profile consisted of solvent A (acetic acid (0.5% *v/v*)) and solvent B (acetic acid (0.5% *v/v*) in acetonitrile). An initial hold (6 min, 20% B) was followed by linear gradients (30% B after 7 min, 95% B after 25 min, 95% B after 28 min, 20% B after 30 min) and held at 20% B for 5 min. The solvent flow was 0.5 mLmin^{-1}.

^1H NMR data and other physic-chemical properties of mensacarcin (1): ^1H NMR (300 MHz, $CDCl_3$): $\delta = 1.07$ (d, $J = 7.5$ Hz, 3H; 15-H_3), 1.44 (d, $J = 5.0$ Hz, 3H; 14-H_3), 2.28 (dq, $J = 7.5$, 3.0 Hz, 1H; 3-H),

3.04 (dq, $J=5.0$, 2.0 Hz, 1H; 13-H), 3.57 (d, $J=10$ Hz, 1H; 4-OH), 3.64 (d, $J=4.5$ Hz, 1H; 1-OH), 3.70 (s, 3H; 17-H_3), 3.92 (d, $J=2.0$ Hz, 1H; 12-H), 3.92 (s, 3H; 16-H_3), 4.43 (d, $J=10.0$ Hz, 1H; 1-H), 4.70 (dd, $J=4.5$, 3.0 Hz, 1H; 4-H), 5.02 (s, 1H; 2-OH), 5.48 (s, 1H; 10-H), 7.18 (dd, $J=8.0$, 1.0 Hz, 6-H), 7.40 (dd, $J=8.0$, 8.0 Hz, 1H; 7-H), 7.62 (dd, $J=8.0$, 1.0 Hz, 1H;8-H) ; IR (KBr): $\nu_{max}=3438$, 3297, 2940, 2842, 1710, 1682, 1582 cm^{-1}; UV (MeOH): λ_{max} (ε) = 226 nm (16400), 263 nm (8820), 319 (2940). (MeOH/HCl): λ_{max} (ε) = 226 nm (16300), 262 (7260), 320 (2870). (MeOH/NaOH): λ_{max} (ε) = 226 nm (1530), 261 (6440), 320 (2310). CD (MeOH): λ_{extr} ($\Delta\varepsilon$) = 219 nm (−4.8), 258 (3.7), 336 (−2.4).

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Keywords: biosynthesis · genomics · mensacarcin · rishirilide · spore pigment · type II PKS cluster

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