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Organisation of the Biosynthetic Gene Cluster and Tailoring Enzymes in the Biosynthesis of the Tetracyclic Quinone Glycoside Antibiotic Polyketomycin

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Polyketomycin is a tetracyclic quinone glycoside produced by *Streptomyces diastatochromogenes* Tü6028. It shows cytotoxic and antibiotic activity, in particular against Gram-positive multi-drug-resistant strains (for example, MRSA). The polyketomycin biosynthetic gene cluster has been sequenced and characterised. Its identity was proven by inactivation of a α-ketoacyl synthase gene (*pokP1*) of the "minimal polyketide synthase II" system. In order to obtain valuable information about tailoring steps, we performed further gene-inactivation experiments. The generation of mutants with deletions in oxygenase genes (*pokO1*, *pokO2*, both in parallel and *pokO4*) and methyltransferase genes (*pokMT1*, *pokMT2* and *pokMT3*) resulted in new polyketomycin derivatives, and provided information about the organisation of the biosynthetic pathway.

Introduction

Streptomycetes are soil-dwelling Gram-positive filamentous bacteria of the order Actinomycetales and are known to produce a widespread variety of secondary metabolites. They are often clinically used as antibiotic, antifungal or anticancer agents.[1,2] The tetracyclic quinone glycoside polyketomycin (POK; **1**) was isolated independently from the culture broth of two strains, *Streptomyces* sp. MK277-AF1[3,4] and *Streptomyces diastatochromogenes* Tü6028.[5] POK inhibits the growth of Gram-positive bacteria, including multi-drug-resistant strains, for example methicillin-resistant *Staphylococcus aureus* (MRSA), as well as the growth of several tumour cell lines.[4] POK consists of two polyketide moieties: the decaketide-derived polyketomycinone (PON) and the tetraketide-derived 3,6-dimethylsalicylic acid (3,6-DMSA). These moieties are linked together by a disaccharide chain consisting of β-D-amicetose and α-L-axenose. Structurally related to POK are the antitumour agents dutomycin from *Streptomyces* sp. 1725,[6] the DNA-methyltransferase inhibitor DMI-2, which is a tautomer of dutomycin, from *Streptomyces* sp. strain No. 560[7] and the cervimycins, which were isolated from *S. tendae* HKI-179.[8,9] All of these compounds have a *p*-quinone in ring D in common.

PON belongs to the group of tetracyclic aromatic polyketides that are usually derived from type II-polyketide synthase (PKSII) metabolism.[10] After the formation of the tetracyclic backbone further reactions initiate the decoration of the tetracyclic skeleton of PON to form a richly substituted compound. Oxygenases, methyltransferases and glycosyltransferases are expected to be involved in these reactions.[11]

Herein we report the cloning, sequencing and functional analysis of the POK biosynthetic gene cluster in *S. diastatochromogenes* Tü6028. The identity of the POK biosynthetic gene cluster was confirmed by the generation of a non-producing mutant in which the α-ketoacyl synthase gene *pokP1* had been deleted. The inactivation of oxygenase genes (*pokO1*, *pokO2* and *pokO4)* and methyltransferase genes (*pokMT1, pokMT2* and *pokMT3*) led to nonproducing mutants or mutants that produce new POK pathway intermediates/shunt products of early or late biosynthetic steps. These investigations gave us valuable information about the process of POK formation, and resulted in a proposal for the entire biosynthetic pathway.

Results and Discussion

Screening and organisation of the POK gene cluster

A genomic cosmid library from *S. diastatochromogenes* Tü6028 was generated. Several cosmids, including cosmids CB30-4E08, CB30-6D20 and CB30-2A21 were isolated due to their capacity to hybridise to a PKS II gene probe and an NDP-glucose 4,6-dehydratase gene probe. The corresponding enzyme of the latter gene is involved in deoxysugar biosynthesis. A 52.2 kb segment of continuous DNA with an average G+C content of 72.3% were sequenced. Forty-one open-reading frames (Figure 1) were identified by computer-aided analysis. The putative functions of the deduced amino acid (aa) sequences are given in Table 1.

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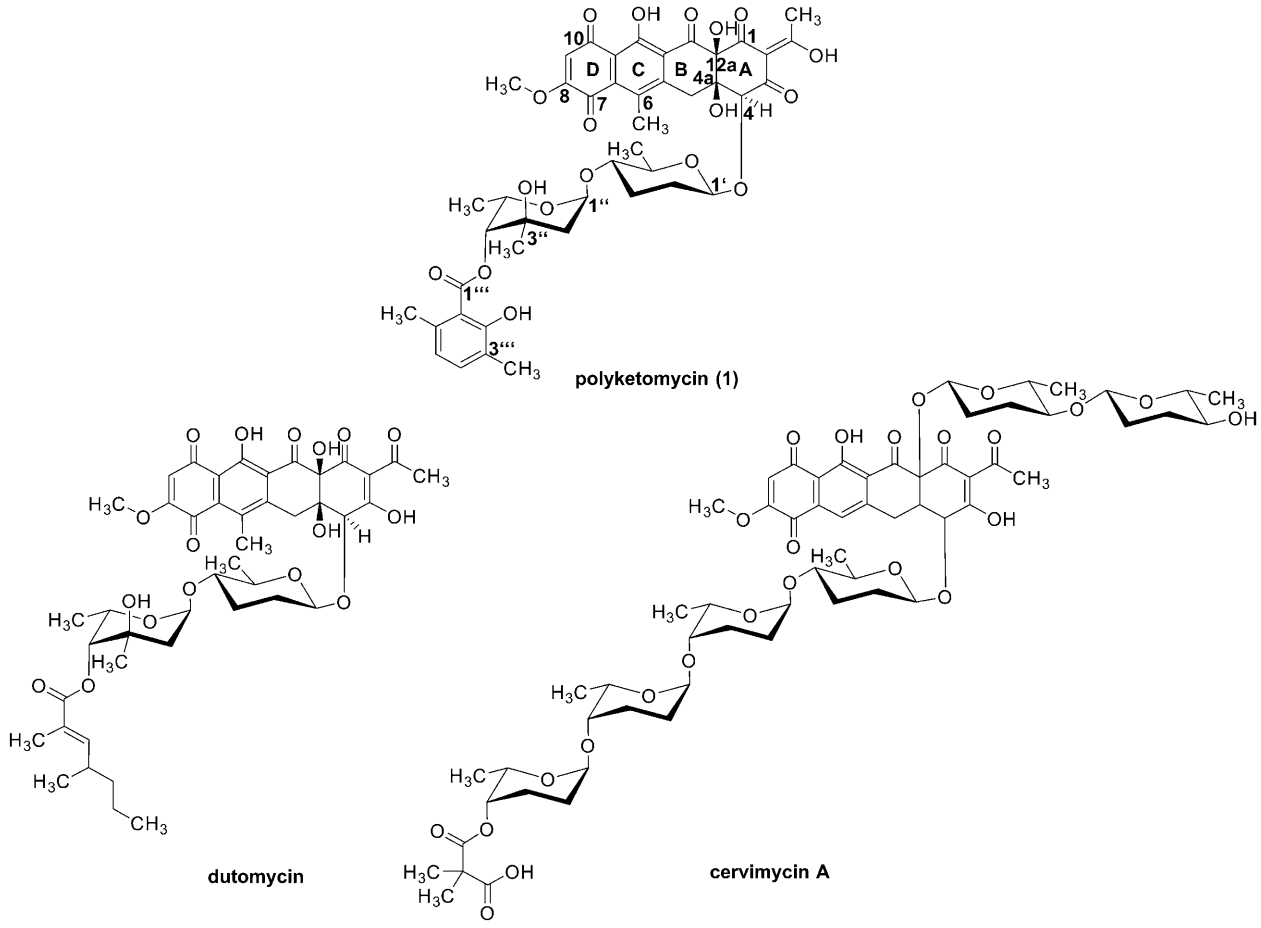


Genes involved in the biosynthesis of POK

Three genes, *pokP1*, *pokP2* and *pokP3* represent the minimal PKSII[12] in the cluster. Genes that encode cyclases/aromatases are *pokC1*, *pokC2* and *pokC3* and genes that putatively encode ketoreductases are *pokT1* and *pokT2*. The POK gene cluster also contains oxidoreductase-encoding genes (*pokO1* to *pokO4*) and methyltransferase genes (*pokS8*, *pokMT1*, *pokMT2* and *pokMT3*). Two genes, *pokGT1* and *pokGT2,* encode glycosyltransferases, and nine genes, *pokS1* to *pokS9,* are most probably involved in deoxysugar biosynthesis. The ORF *pokM1* codes for a 6-methylsalicylic acid (6-MSA) synthase, which belongs to the iteratively working type I PKS. The deduced aa sequence of *pokM2* resembles AviN, an enzyme that is involved in the avilamycin biosynthesis with an unknown function.[13,14] The attachment of 3,6-DMSA to the sugar side might be catalysed by PokM3, a putative AMP ligase, or by PokL, a putative acyl-CoA ligase. The POK gene cluster also contains *pokAC1*, *pokAC2* and *pokAC3*. The deduced proteins of these genes most probably provide malonyl-CoA, which is the extender unit for the PKS.[15]

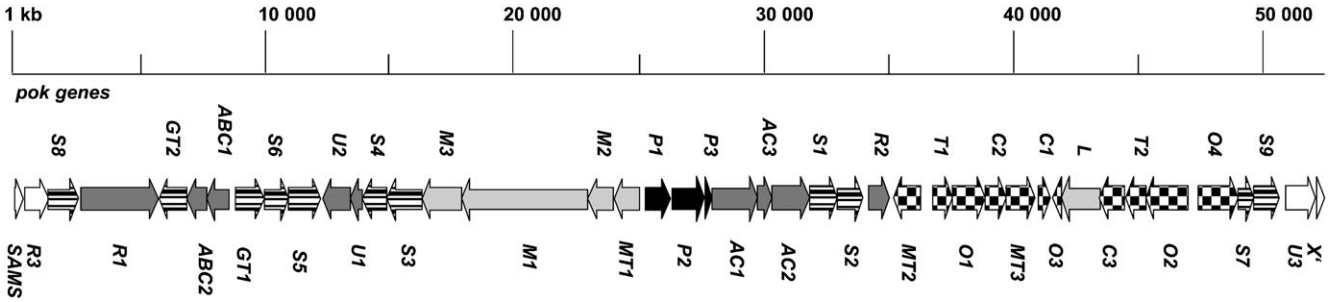


Figure 1. Genetic organisation of the polyketomycin biosynthetic gene cluster. Genes of the minimal PKSII system are highlighted in black, and genes encoding PKSII-derived polyketide modifying enzymes are checked. Genes that are involved in 3,6-DMSA synthesis are labelled in light grey and genes that are responsible for the dioxysugar biosynthesis and glycosyltransfer are striped. Genes that are putatively involved in providing essential precursors or regulation and resistance are displayed in dark grey. Genes that are most probably not involved in polyketomycin biosynthesis are shown in white.

Table 1. Deduced functions of ORFs located within the polyketomycin gene cluster of *Streptomyces diastatochromogenes* Tü6028.

ORF	aa	Most similar protein (identical aa [%])	Access. no.	Proposed function
pokSAMS′	>118[a]	SGR_6058 from *S. griseus* subsp. *griseus* NBRC 13350 (87)	YP_001827570	*S*-adenosylmethionine synthetase
pokR3	326	RemJ from *S. resistomycificus* (63)	CAE51179	kinase
pokS8	415	TylCIII from *S. fradiae* (66)	AAD41823	dNDP-hexose 3-C-methyltransferase
pokR1	1065	Nfa 34460 from *Nocardia farcinica* IFM 10152 (40)	BAD58294	transcriptional regulator
pokGT2	382	SpnG from *Saccharopolyspora spynosa* (35)	AAG23268	glycosyltransferase
pokABC2	269	ORFR2 from *S. rochei* F20 (41)	CAA75762	ABC transporter, transmembrane component
pokABC1	310	FRAAL5527 from *Frankia alni* ACN14a (60)	CAJ64160	ABC transporter, ATP-binding component
pokGT1	397	CmmGIV from *S. griseus* subsp. *griseus* (43)	CAE17547	glycosyltransferase
pokS6	342	Orf14 from *S. eurythermus* (47)	ABV49596	NDP-hexose 4-ketoreductase
pokS5	432	Lct46 from *S. rishiriensis* MJ773-88K4 (75)	ABX71129	NDP-hexose 3-dehydratase
pokU2	384	Francci3_4142 from *Frankia* sp. CcI3 (45)	YP_483219	acyl-CoA dehydrogenase
pokU1	173	SCO5092 from *S. coelicolor* A3 (2) (45)	NP_629242	dimerase
pokS4	333	ChlC4 from *S. antibioticus* (54)	AAZ77681	NDP-hexose 3-ketoreductase
pokS3	476	SimB3 from *S. antibioticus* Tu6040 (54)	AAK06810	dTDP-hexose 2,3-dehydratase
pokM3	551	MdpB2 from *Actinomadura madurae* (73)	ABY66018	6-methylsalicylic acid-AMP ligase
pokM1	1739	MdpB from *Actinomadura madurae* (65)	ABY66019	6-methylsalicylic acid synthase
pokM2	342	ChlB3 from *S. antibioticus* (49)	AAZ77676	β-ketoacyl-ACP synthase
pokMT1	347	MdpB1 from *Actinomadura madurae* (72)	ABY66020	C-methyltransferase
pokP1	422	Orf11 from *S. echinatus* (75)	ABL09959	α-ketoacyl synthase
pokP2	407	SimA2 from *S. antibioticus* Tu6040 (63)	AAK06785	β-ketoacyl synthase
pokP3	85	StfS from *S. steffisburgensis* (59)	CAJ42318	acyl carrier protein
pokAC1	579	ZhuF from *S.* sp. R1128 (61)	AAG30193	acetyl-CoA carboxylase, carboxyl transferase subunit
pokAC3	178	ZhuE from *S.* sp. R1128 (43)	AAG30192	biotin carboxylase carrier protein
pokAC2	470	AccC from *Frankia alni* ACN14a (65)	CAJ61842	acetyl-CoA carboxylase, biotin carboxylase subunit
pokS1	354	StrD from *S. griseus* subsp. *griseus* NBRC 13350 (64)	CAH94331	NDP-glucose synthase
pokS2	329	AviE1 from *S. viridochromogenes* Tü57 (70)	AAK83196	NDP-glucose 4,6-dehydratase
pokR2	270	SrrZ from *S. rochei* (47)	BAC76529	regulator of the SARP family
pokMT2	345	OxyF from *S. rimosus* (60)	AAZ78330	C-methyltransferase
pokT1	259	CmmTI from *S. griseus* subsp. *griseus* (46)	CAE17519	ketoreductase
pokO1	409	CmmOI from *S. griseus* subsp. *griseus* (57)	CAE17524	mono-oxygenase
pokC2	261	AknW from *S. galilaeus* (73)	AAF73459	cyclase
pokMT3	371	FdmN from *S. griseus* (54)	AAQ08925	O-methyltransferase
pokC1	150	CmmX from *S. griseus* subsp. *griseus* (58)	CAE17525	cyclase
pokO3	109	MtmOIII from *S. argillaceus* (45)	CAK50778	mono-oxygenase
pokL	495	CmmLII from *S. griseus* subsp. *griseus* (53)	CAE17553	acyl-CoA ligase
pokC3	314	StfQ from *S. steffisburgensis* (51)	CAJ42327	aromatase
pokT2	251	MtmTII from *S. argillaceus* (57)	CAA07756	ketoreductase
pokO2	541	MtmOII from *S. argillaceus* (53)	CAK50777	mono-oxygenase
pokO4	506	RubN from *S. collinus* (47)	AAM97364	mono-oxygenase
pokS7	204	EryBVII from *Saccharopolyspora erythraea* (72)	CAA72086	dNDP-hexose 3,5-epimerase
pokS9	319	DnmV from *S. peucetius* (49)	AAB63047	NDP-hexose 4-ketoreductase
pokU3	393	FadE14 from *S. avermitilis* MA-4680 (88)	BAC68977	acyl-CoA dehydrogenase
pokX1′	>96[a]	SCO7239 from *S. coelicolor* A3 (2) (91)	CAB94053	unknown function

[a] Incomplete ORF. aa = amino acid.

Genes putatively involved in regulation and resistance

Two putative regulator genes, *pokR1* and *pokR2* were found within the cluster. The protein PokR1 is similar to the transcriptional regulator Nfa34460 from *Nocardia farcinica* IFM10152[16] and PokR2 belongs to the *Streptomyces* antibiotic regulatory protein (SARP) family.[17] The corresponding enzymes of the two adjacent ABC transporter genes, *pokABC1* and *pokABC2* are most probably involved in the transport of POK out of the producer cell.

Genes probably not directly involved in POK biosynthesis or with unknown function

Six ORFs with unknown function are situated within the 52.2 kb sequenced genome fragment of *S. diastatochromogenes* Tü6028. At one border, there are the sequences of an incomplete S-adenosylmethionyl transferase gene (*pokSAMS*) and a putative adenosine kinase gene (*pokR3*). On the other border there are the sequences of *pokU3* (acyl-CoA dehydrogenase gene) and *pokX1* (coding for a hypothetical protein). The genes *pokU1*, which encodes a dimerase, and *pokU2*, which encodes a dehydrogenase are both located between sugar biosynthetic genes.

Inactivation of genes within the POK cluster

The generated mutants and major metabolites that were detected by HPLC–MS (Figure 2) are listed in Table 2. The deduced structures of the accumulating metabolites are presented in Figure 3.

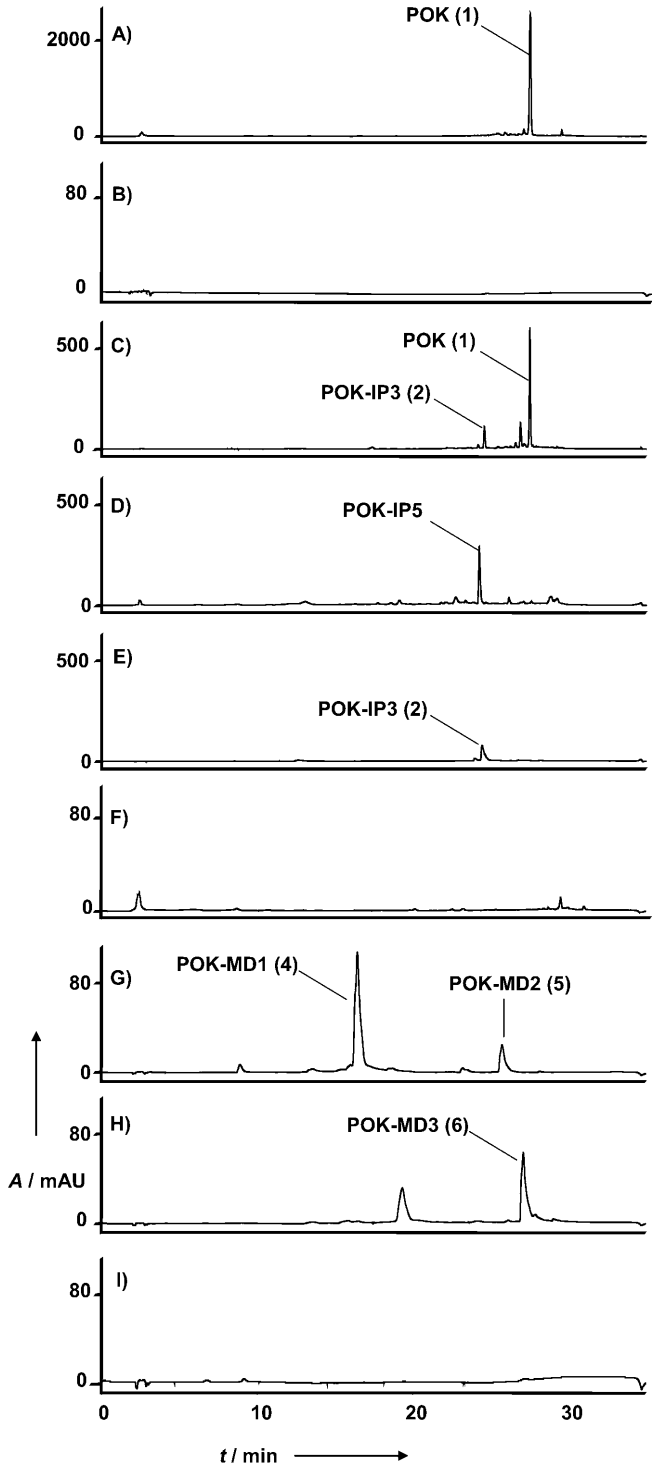


Figure 2. HPLC analysis (430 nm) of crude extracts of *Streptomyces diastatochromogenes* Tü6028 A) wild-type, B) ΔpokP1, C) ΔpokO1, D) ΔpokO2, E) ΔpokO1-ΔpokO2, F) ΔpokO4, G) ΔpokMT1, H) ΔpokMT2 and I) ΔpokMT3.

Table 2. Overview of the generated mutants of *Streptomyces diastatochromogenes* Tü6028.

Inactivation of gene(s)	Leads to a mutant with this property concerning the production profile
pokP1	non-POK producing[a]
pokO1	POK-IP3 (**2**), POK[b]
pokO2	POK-IP5[b,c]
pokO1 and *pokO2*	POK-IP3 (**2**)[b]
pokO4	non-POK producing[a]
pokMT1	POK-MD1 (**4**), POK-MD2 (**5**)[b]
pokMT2	POK-MD3 (**6**)[b]
pokMT3	non-POK producing[a]

[a] The strain was able to accumulate neither an intermediate nor a shunt product of POK. [b] Major metabolites are underlined. [c] Putatively identical to premithramycinone G (**3**).

Inactivation of *pokP1*

In order to confirm that we had indeed found the POK biosynthetic gene cluster, the putative α-ketoacyl synthase gene *pokP1* was selected for the first gene-deletion experiment. For deletion of *pokP1*, nearly the whole gene was replaced by the spectinomycin-resistance cassette *aadA*; this resulted in *S. diastatochromogenes* ΔpokP1, a non-POK-producing mutant.

Inactivation of *pokO1*, *pokO2* and *pokO4* and generation of a *pokO1–pokO2* double mutant

The biosynthetic gene cluster of POK contains three ORFs, *pokO1*, *pokO2* and *pokO4*; these encode putative FAD-dependent mono-oxygenases. In order to establish the function of these genes, mutants with deletion in *pokO1* (*S. diastatochromogenes* ΔpokO1), *pokO2* (*S. diastatochromogenes* ΔpokO2) and *pokO4* (*S. diastatochromogenes* ΔpokO4) and one mutant with deletions in *pokO1* and *pokO2* (*S. diastatochromogenes* ΔpokO1-ΔpokO2) were generated. Gene-deletion plasmids were constructed by using the Red/ET® recombineering method (see the Experimental Section). In all cases we were able to remove nearly the entire gene from the chromosome, but *S. diastatochromogenes* ΔpokO1 was still able to produce POK. Interestingly, PokO1 is similar to MtmOI and inactivation of *mtmOI* did not influence mithramycin biosynthesis in *S. argillaceus* ATCC12956.[18,19] In contrast to the wild-type strain, *S. diastatochromogenes* ΔpokO1 produced a small amount of a compound with a mass of 396 Da (*m/z* 395 $[M-H]^-$) which was named POK-IP3 (**2**; Figure 3).

POK could not be detected in the crude extract of *S. diastatochromogenes* ΔpokO2, but a new POK derivative, POK-IP5, with a mass of 452 Da (*m/z* 451 $[M-H]^-$) was generated by the mutant. Due to its instability, we were not able to isolate the new compound in adequate amounts for NMR spectroscopic analysis. A similar compound, premithramycinone G (**3**),[18] was observed after inactivation of *mtmOII*. This gene codes for an oxygenase (53% identical aa in comparison to PokO2) that plays an essential role in mithramycin biosynthesis. The structure of premithramycinone G was elucidated by Rohr and coworkers and is presented in Figure 3. POK-IP5 shows the same characteristics concerning the UV/visible spectrum, the same detected mass and the same stability problems as **3**.

S. diastatochromogenes ΔpokO1–ΔpokO2 neither produced POK nor POK-IP5. As in *S. diastatochromogenes* ΔpokO1, POK-IP3 was also accumulated in this mutant. For structure elucida-

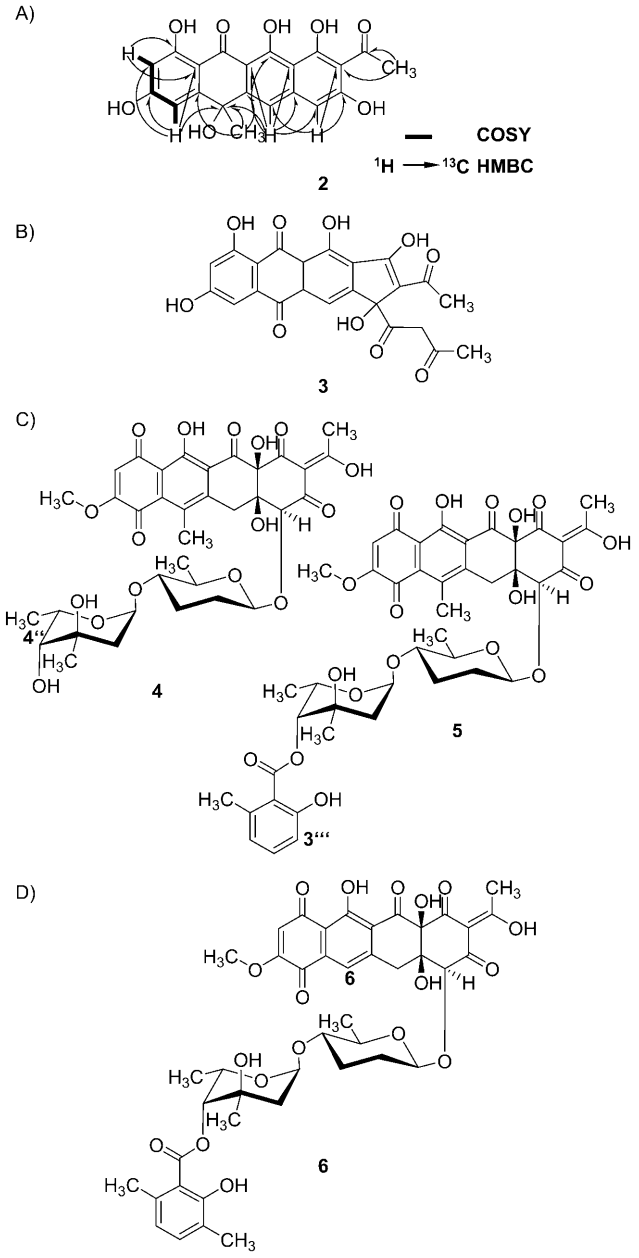


Figure 3. Structures of compounds generated in this study. A) POK-IP3 (**2**) (one *meta*-coupled $^{1}H,^{1}H$ COSY correlation and $^{2,3}J(CH)$ couplings are also shown), B) premithramycinone G[18] (**3**) probably identical to POK-IP5, C) proposed structure for POK-MD1 (**4**) and POK-MD2 (**5**) and D) POK-MD3 (**6**).

tion, POK-IP3 was isolated from a culture of *S. diastatochromogenes* ΔpokO1–ΔpokO2 (20 L). The dry orange powder of POK-IP3 has a molecular weight of $M_r = 396\ g\,mol^{-1}$ based on the deprotonated molecular ion peak at *m/z* 395.2 $[M-H]^-$. The high-resolution electrospray ionisation (HRESI) mass spectrometry (*m/z* 395.07731) revealed the molecular formula as $C_{21}H_{16}O_8$. The ^{1}H NMR spectrum of **2** showed two singlets at $\delta_H = 2.68$ and 1.49 ppm for two methyl groups. In the aromatic region two singlets at $\delta_H = 6.53$ and 7.28 ppm and furthermore two *meta*-coupled protons at $\delta_H = 6.84$ and 6.22 ppm were observed. Four out of six hydroxy groups were also detected in the ^{1}H NMR spectrum ($\delta_H = 6.05$, 10.77, 11.97 and 12.34 ppm). The ^{13}C NMR spectrum consisted of 21 signals. Three of them appeared in the aliphatic region: two methyl groups ($\delta_C = 32.9$ and 39.2 ppm) and one quaternary carbon at $\delta_C = 70.0$ ppm. The structure includes two carbonyl groups with signals at $\delta_C = 187.4$ and 203.8 ppm. The latter one together with one methyl group forms the short carbon chain. The residual 16 carbon atoms are part of the aromatic ring system. The other carbonyl group and the mentioned quaternary carbon complete this tetracyclic skeleton. The constitution of the carbons and their functional groups were deduced from 2D NMR spectroscopic studies ($^{1}H,^{1}H$ COSY, HSQC and HMBC; Table 3). A database search in SciFinder (Chemical Abstracts Service) did not yield any identical structure that has already been published; however it resembles saintopin E from a *Paecilomyces* sp.[20,21] and WJ119 from *S. coelicolor* CH999, which harbours oxytetracycline biosynthetic genes.[22] Zhang and co-workers proposed that the ring-C formation of WJ119 is the result of a spontaneous oxidation process, likely as a result of air oxidation during purification. We propose that POK-IP3 is also an oxidation product that is derived from an intermediate in the POK biosynthesis. Because POK-IP3 does not contain any of the four enzymatically introduced oxygen atoms, we conclude that PokO1 is the first oxygenase involved in the POK biosynthesis; it introduces the hydroxy group in position C4. PokO2 is able to substitute the function of PokO1, as indicated by POK production in *S. diastatochromogenes* ΔpokO1. It was verified by generating the double mutant *S. diastatochromogenes* ΔpokO1–ΔpokO2 as well as semi-complementation by introducing the native *pokO2* gene. To our knowledge, this is the

Table 3. ^{1}H NMR (600 MHz, $[D_6]$ DMSO) and ^{13}C NMR (150 MHz, $[D_6]$ DMSO) data as well as the 2D NMR (HSQC, HMBC) assignments of POK-IP3 (**2**).

Position	δ_C [ppm]	δ_H [ppm] (*J* [Hz])	HMBC ($^{1}H \rightarrow {}^{13}C$)
1	160.6		
2	109.1		
3	161.0		
4	101.3	6.53 (s)	C2, C3, C5
4a	142.4		
5	112.6	7.28 (s)	C4, C4a, C6, C11a, C12, C12a
5a	153.7		
6	70.0		
6a	149.3		
7	105.5	6.84 (d, 2.3)	C6, C8, C9, C10a
8	165.3		
9	101.4	6.22 (d, 2.3)	C10, C10a
10	164.3		
10a	106.3		
11	187.4		
11a	105.5		
12	166.9		
12a	107.3		
13	203.8		
14	32.9	2.68 (s)	C2, C13
15	39.2	1.49 (s)	C5a, C6, C6a

Visible hydroxy group signals: $\delta_H = 6.05$, 10.77, 11.97 and 12.34 ppm.

first description of a post-PKS tailoring oxygenase that is able to take over the function of another oxygenase. The biological activity of POK-IP3 against *Bacillus subtilis* COHN ATCC6051 and *S. viridochromogenes* Tü57 was tested by an agar diffusion assay, but it only showed moderate antibiotic activity.

Inactivation of *pokO4* resulted in a POK-non-producing mutant. In addition we were not able to detect any other intermediate of the biosynthesis. PokO4 is related to the C–C bond-cleaving Baeyer–Villiger mono-oxygenase MtmOIV of *S. argillaceus* ATCC12956 (48% identical aa),[23] and it might affect the length of the polyketide chain; this effect has already been reported for the oxygenase MtmOII[18] and also for the oxygenase OtcC, which is involved in the biosynthesis of oxytetracyclin.[24]

Inactivation of *pokMT1*, *pokMT2* and *pokMT3*

Four SAM-dependent methyltransferase genes (*pokS8, pokMT1, pokMT2 and pokMT3*) were detected in the *pok* cluster. Deletions were introduced into *pokMT1* (*S. diastatochromogenes* ΔpokMT1), *pokMT2* (*S. diastatochromogenes* ΔpokMT2) and *pokMT3* (*S. diastatochromogenes* ΔpokMT3). Further, a frame shift was introduced into *pokMT1* and *pokMT2*, and an in-frame deletion was introduced into *pokMT3*.

One major metabolite, POK-MD1 (**4**; Figure 3) with a quasi-molecular ion of *m/z* 715 $[M-H]^-$ was produced by *S. diastatochromogenes* ΔpokMT1. The mass difference between POK and POK-MD1 matches the mass of the 3,6-DMSA residue; this indicates that the transfer of the salicylic acid moiety occurs preferentially after its methylation in position 3. Additionally, one minor compound, POK-MD2 (**5**; Figure 3) was produced in a very small amount by *S. diastatochromogenes* ΔpokMT1; it had a quasi-molecular ion of *m/z* 849 $[M-H]^-$, which reflects a mass difference of 14 amu in comparison to that of POK. The fragmentation patterns of POK-MD2 and POK were compared, and the results indicated again that PokMT1 targets the salicylic acid moiety. This was confirmed by feeding experiments by using L-[*S*-methyl-^{13}C]-methionine coupled with ^{13}C NMR studies. The ^{13}C NMR spectrum of POK that was produced by the wt-strain showed four enriched carbon atoms (Table 4), whereas the mutant showed three enriched carbon atoms. The ^{13}C signal at $\delta_C = 15.85$ ppm, which was assigned to the 3‴-methyl group was not present; this demonstrates that POK-MD2 is the 3‴-demethylpolyketomycin. Like MdpB1, a methyltransferase that is involved in maduropeptin biosynthesis,[25] PokMT1 is responsible for the methylation of the 6-methylsalicylic acid in position 3.

Table 4. ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C) data of the detected enriched carbon atoms of POK-MD2 (**5**) and POK (**1**) after the feeding experiment with L-[*S*-methyl-^{13}C]-methionine.

Position	δ_C [ppm]	
	POK-MD2	POK
6-CH_3	16.6	16.7
8-OCH_3	56.9	56.9
3″-CH_3	25.7	25.7
3‴-CH_3	[a]	15.8

[a] No enriched carbon atom was detected at this chemical shift.

A novel compound POK-MD3 (**6**; Figure 3) was detected in *S. diastatochromogenes* ΔpokMT2. The UV/vis spectrum of POK-MD3 was nearly identical to the spectrum of POK. Just one maximum was lightly hypsochromically shifted from 445 to 436 nm. The corresponding quasi-molecular ion of *m/z* 849 $[M-H]^-$ reflected a mass difference of 14 amu in comparison to POK. For structure elucidation, POK-MD3 (**6**) was purified from a culture (3 L) of *S. diastatochromogenes* ΔpokMT2 and subjected to 1D (^{1}H,^{13}C) and 2D (^{1}H,^{1}H COSY, HSQC, HMBC) NMR spectroscopy (Table 5). The resulting spectra of the orange powder were compared to the assignments of POK published by Momose et al.[3] and Paululat et al.[5] Most striking was the absence of the signals from the methyl group linked to C6 in POK ($\delta_H = 2.6$ ppm and $\delta_C = 16.7$ ppm) and a considerable up-field shift of C6 ($\delta_C = 132.7$ ppm in POK) to 119.8 ppm in the ^{13}C NMR spectrum. The detection of an additional singlet signal at $\delta_H = 7.56$ ppm in the ^{1}H NMR spectrum that was coupled with C6 (observed in HSQC spectrum), indicated a newly generated aromatic proton at C6. Both clearly showed that POK-MD3 is 6-demethylpolyketomycin, and we can conclude that PokMT2 methylates C6 in the POK biosynthesis. The antibiotic activity of POK-MD3 against *Bacillus subtilis* COHN ATCC6051 was moderately reduced in comparison to the activity of POK; this indicates that the methyl group is critical for the antibiotic activity of POK.

No compound was detectable in *S. diastatochromogenes* ΔpokMT3. PokMT3 shares 54% identical aa with FdmN, an O-methyltransferase involved in the fredericamycin biosynthesis in *S. griseus*,[26] 51% with TcmN, which is an O-methyltransferase that is involved in the tetracenomycin D3 biosynthesis in *S. glaucescens*[27,28] and 49% with ElmNII, which is an O-methyltransferase that is involved in the elloramycin biosynthesis in *S. olivaceus*.[29] Based on the function of these proteins, PokMT3 is proposed to catalyse the methyl transfer to the phenolic hydroxy group in ring D of POK. In contrast to TcmN, a methyltransferase that also acts as cyclase, a typical cyclase motif could not be found in PokMT3. PokMT3 might just play a structural role, for example, as a linker to keep the minimal PKS enzymes (PokP1, PokP2 and PokP3) together and/or at a correct distance for a functional complex. As described above, *S. diastatochromogenes* ΔpokO1 produced the non-O-methylated but fully cyclised derivative POK-IP3 (**2**; Figure 3). We propose that methylation of the hydroxy group at C8, which is catalysed by PokMT3 occurs after the cyclisation process has been completed. We cannot totally rule out a polar effect on *pokC1*, which is located downstream of *pokMT3*, but POK production was restored in our complementation experiment, and this led to the formation of around 25% POK compared to the wt strain. This lower production rate is not unusual and is often observed when the production rate of complemented mutants is analysed.

Table 5. ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C) and ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C) as well as 2D NMR (HSQC, HMBC) spectroscopic assignments of POK-MD3 (**6**).

Position	δ_C [ppm]	δ_H [ppm] (m, *J*, I)[a]	HMBC ($^{1}H\rightarrow^{13}C$)	Position	δ_C [ppm]	δ_H [ppm] (m, *J*, I)[a]	HMBC ($^{1}H\rightarrow^{13}C$)
1	191.6[b]			1′	102.0	4.73 (dd, *J* = 8.0, 1.2 Hz, 1H)	
2	110.4			2′	30.1	1.65 (m)	
3	195.3					2.15 (m)	
4	73.2	4.48 (br s, 1H)	C3, C1′	3′	29.3	1.57 (m)	
4a	75.6					2.20 (m)	
4a-OH		2.85 (br s)		4′	78.0	3.19 (dt, *J* = 9.2, 4.4 Hz, 1H)	
5	36.7	3.38 (d, *J* = 17.4 Hz, 1H)	C4, C4a, C5a, C11a	5′	74.4	3.07 (m)	
		3.74 (d, *J* = 17.4 Hz, 1H)	C4a, C5a, C6, C11a, 12a	6′	17.9	0.70 (d, *J* = 6.0 Hz, 1H)	C4′, C6′
5a	149.9			1″	100.1	5.06 (br d, *J* = 3.8 Hz, 1H)	C2″
6	119.9	7.54 (s, 1H)	C5a, C7, C10a, C11a	2″	37.1	1.71 (br d, *J* = 14.4 Hz, 1H)	C1″
6a	133.7					1.98 (br d, *J* = 14.4, 4.0 Hz, 1H)	C1″
7	178.9			3″	68.7		
8	160.4			3″-CH_3	25.7	1.18 (s, 3H)	
8-OCH_3	56.9	3.95 (s, 3H)	C8	3″-OH		3.93 (br s)	
9	110.4	6.17 (s, 1H)	C7, C8, C10, C10a	4″	75.6	5.09 (br s, 1H)	
10	190.2			5″	62.4	4.49 (br q, *J* = 6.0 Hz, 1H)	C6″
10a	113.4			6″	16.8	1.15 (d, *J* = 6.2 Hz, 3H)	C5″
11	163.5			1‴	110.8		
11-OH		13.52 (br s)	C10a, C11, C11a	1‴-CO	171.4		
11a	123.1			2‴	161.7		
12	189.6[b]			2‴-OH		11.63 (s)	
12a	81.3			3‴	124.6		
12a-OH		4.96 (br s)	C4a, C12, C12a	3‴-CH3	15.9	2.22 (s, 3H)	C4‴
13	201.3			4‴	135.4	7.18 (d, *J* = 7.6 Hz, 1H)	C3‴-CH_3
13-CH_3	26.8	2.74 (s, 3H)	C2, C13	5‴	122.3	6.65 (d, *J* = 7.6 Hz, 1H)	6‴-CH_3
13-OH		18.04 (br s)		6‴	138.4		
				6‴-CH3	24.6	2.55 (s, 3H	5‴

[a] m = multiplicity (br = broad), I = intensity and *J* = coupling. [b] These assignments are interchangeable.

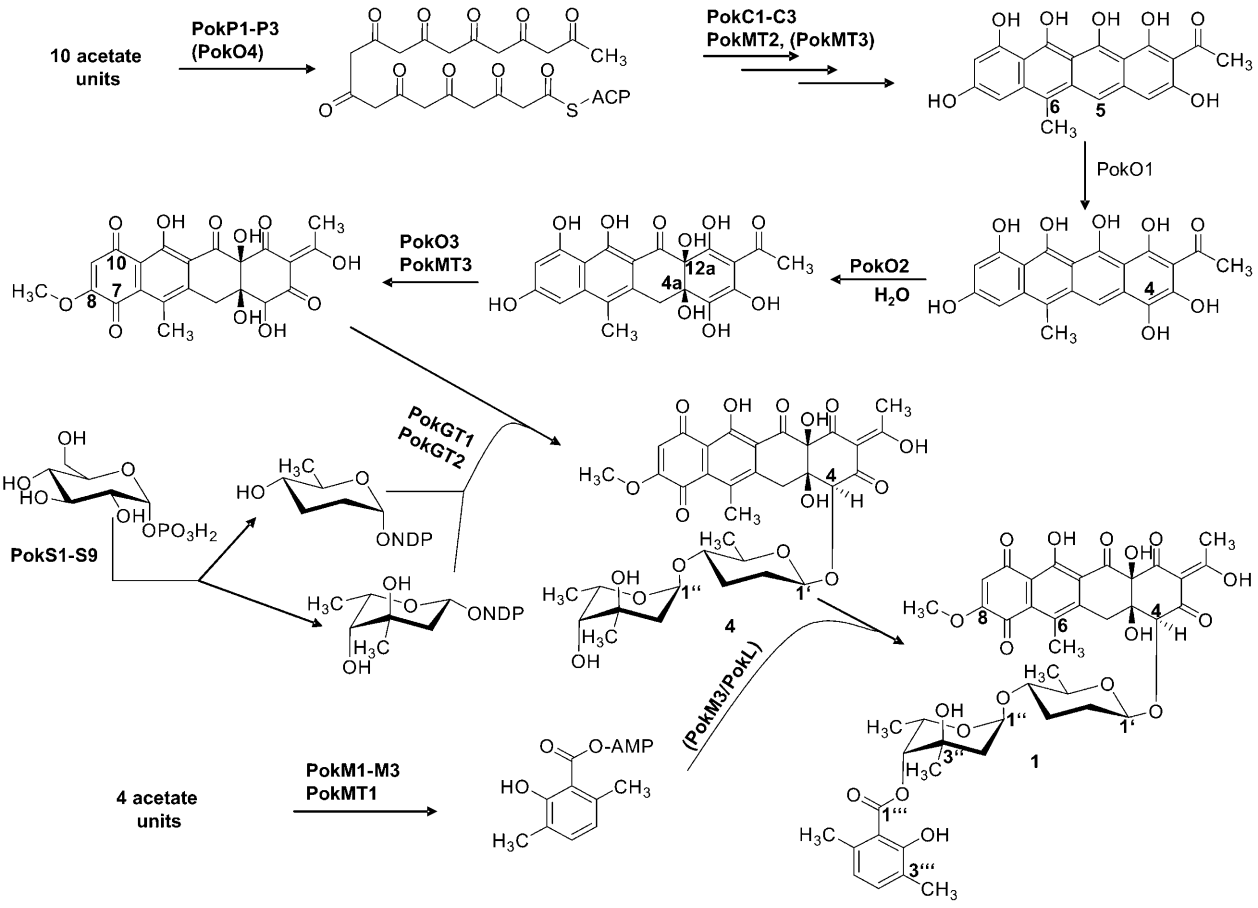
Complementation studies

Constructs pSET-pok28, pSET-pok29, pSET-pokMT1, pSET-pokMT2 and pSET-pokMT3 were used for complementation, and POK production could be restored in all cases. The deletion of *pokP1* was successfully complemented by integration of the cosmid CB30-6D20.

POK biosynthesis

The functional analysis of the POK gene cluster combined with the results from the inactivation experiments allows a comprehensive proposal for the biosynthesis of POK to be made (Scheme 1). The most important characteristics of POK biosynthesis are:

- PON, 3,6-DMSA, β-D-amicetose and α-L-axenose are building blocks that are connected to each other at the end of the biosynthesis.
- The 6-MSA is the substrate for PokMT1 rather than POK-MD2 (indicated by the low production of POK-MD2 and the higher production of POK-MD1 in *S. diastatochromogenes* ΔpokMT1).
- During PON biosynthesis, PokO4 is involved in the formation of a linear precursor that is afterwards cyclised (indicated by the POK non-producing mutant *S. diastatochromogenes* ΔpokO4), or it might be an inactive non-functional enzyme that is not used as an oxygenase but still has a structural role.
- In addition to its methyltransferase activity, PokMT3 might play a structural role that is important for the functional enzyme complex. The O-methylation that is catalysed by PokMT3 at the hydroxy group of C8 occurs after complete cyclisation (indicated by the structure of POK-IP3).
- The first methylation step during the PON biosynthesis occurs at C6 by the SAM-dependent C-methyltransferase PokMT2 (indicated by the presence of the appropriate methyl group in POK-IP3). The absence of the methyl group at C6 does not influence the enzyme activity of other biosynthetic enzymes.
- Methylation by PokMT2 precedes methylation by PokMT3, however, PokMT2 activity is not essential for POK biosynthesis (indicated by the formation of POK-MD3 in *S. diastatochromogenes* ΔpokMT2 and by the formation of Pok-IP3 in *S. diastatochromogenes* ΔpokO1−ΔpokO2).
- PokO2 is able to substitute the function of PokO1. This is indicated by POK production in *S. diastatochromogenes* ΔpokO1 and verified by the generation of the double mutant *S. diastatochromogenes* ΔpokO1−ΔpokO2.
- PokO2 most likely catalyses an epoxidation reaction followed by a *cis*-opening of the epoxide by water to yield two vicinal hydroxy groups in positions C4a and C12a in a putatively similar way as in the biosynthesis of tetracenomycin C.[30] This is indicated by the proposed structure of



Scheme 1. Proposal of polyketomycin biosynthesis. Enzymes that putatively influence the biosynthesis by just playing a structural role or whose function is unclear are given in brackets.

POK-IP5 that is produced by *S. diastatochromogenes* ΔpokO2, which is putatively identical to premithramycinone G.

- Oxygenation by PokO1 precedes oxygenation by PokO2 (indicated by the formation of POK-IP3 in *S. diastatochromogenes* ΔpokO1).
- The formation of the *p*-quinone in ring D, which is catalysed by the putative anthron oxygenase PokO3 is a late step during the PON biosynthesis (indicated by the structure of POK-IP3).

Conclusions

We have described the cloning and sequencing of the POK biosynthetic gene cluster. Seven genes of the cluster were deleted to give new POK derivatives. Interestingly, the oxygenase PokO2 is able to substitute the function of the oxygenase PokO1. The structures of new compounds POK-IP3, POK-IP5, POK-MD1, POK-MD2 and POK-MD3 give new insights into the biosynthesis of tetracyclic aromatic polyketides.

Experimental Section

General equipment: Preparative HPLC was conducted on a Waters 600 system. HPLC–MS analysis (analytical and preparative) was performed on an Agilent 1100 series system with an APCI mass detector. The detection wavelength range of the diode array was set to 200–500 nm. HRMS was executed on an APEX-IV-FTICR mass spectrometer (Bruker Daltonics). 1D and 2D NMR spectra were recorded on a Bruker Avance DRX400 (400 MHz for ^{1}H and 100 MHz for ^{13}C) instrument or on a Varian VNMRS600 (600 MHz for ^{1}H and 150 MHz for ^{13}C) instrument, respectively. Chemical shifts were referenced via the solvent signals. PCR was performed on a Gene Amp® PCR System 9700 (Applied Biosystems, Foster City, CA, USA).

General genetic manipulation and PCR: Standard molecular biology procedures were performed as previously described for *E. coli*[31] and *Streptomyces*.[32] Isolation of plasmid DNA from *E. coli*, DNA blunting/restriction/ligation and Southern hybridisation[33] were performed by following the protocols of the manufacturers of the kits, enzymes and reagents: Macherey & Nagel (Düren, Germany), QIAGEN (Hilden, Germany), Roche Diagnostics (Mannheim, Germany) and Promega (Mannheim, Germany). *Pfu*-Polymerase (Promega) was used for complementation and inactivation experiments. Mutants were verified by PCR by using *Taq* or Go*Taq*-polymerase (Promega). Oligonucleotide primers were purchased from Operon GmbH (Köln) and are listed in Table S1.

Bacteria strains, plasmids and culture conditions: *S. diastatochromogenes* Tü6028 wt and the mutants that were generated in this study, were cultivated in HA liquid medium (yeast extract 0.4%, malt extract 1% and glucose 0.4% in 1 L tap water, pH 7.3, prepared as solid or liquid medium). Subcloning and DNA manipulation were performed in *E. coli* XL1 Blue MRF$^-$ (Stratagene, La Jolla, CA, USA). Plasmids were passed through *E. coli* ET12567 (dam$^-$, dcm$^-$, hsdS$^-$, Cm^r),[34] including conjugation plasmid pUZ8002,[35] to generate unmethylated DNA for intergeneric conjugation with *S. diastatochromogenes* Tü6028. The cosmid library of POK was constructed by using cosmid vector pOJ436[36] and strain *E. coli* DH5α by Combinature Biopharm AG (Berlin, Germany). *E. coli* strains were grown on lysogeny broth (LB) medium that contained the appropriate antibiotic for selection under standard conditions.[31] The two strains, *Bacillus subtilis* (Ehrenberg) COHN ATCC6051 (American Type Culture Collection, 18th edition, 1992) and *S. viridochromogenes* Tü57[37] were used for testing antibiotic activity. The vectors pBluescript SK(−) (pBSK(−) (Stratagene), pLitmus28, pUC18 (both from New England Biolabs (Frankfurt, Germany)), pUC19 (a derivative of pUC18)[38] were utilised for cloning, pKC1132[36] (Eli Lilly and Company (Indianapolis, IN) was used for gene disruption and the vector pUWLoriT[39] and the integrative plasmid pSET-1cerm[40] were applied for complementation of the mutant strains. Plasmid pIJ773[41] served as a template of the [*aac(3)VI*+*oriT*] cassette and vector pSP1[42] for gene disruption by the Red/ET® recombineering method.[41] Vector pIJ785Spec is a modified copy of pIJ785 (http://streptomyces.org.uk/redirect/index.html) harbouring the resistance gene *aadA* instead of *aac(3)IV*. For genomic DNA isolation, *S. diastatochromogenes* Tü6028 was grown (16–30 h) in TSB$^+$ liquid medium (3% tryptic soy broth, 0.4% glycine, 10% sucrose in 1 L tap water).

Construction and screening of a *S. diastatochromogenes* Tü6028 genomic cosmid library: For the generation of a genomic cosmid library chromosomal DNA was partially digested with Sau3AI, ligated into cosmid pOJ436 that had been digested with HpaI and BamHI, and in vitro packaged with the Gigapack III Gold packaging extract kit according to the manufacturer's handbook (Stratagene). Based on the chemical structure of POK, the screening of the cosmid clones was accomplished with a strain-specific PKSII and an NDP-glucose 4,6-dehydratase gene probe, respectively, by using standard hybridisation procedures. Both gene probes were amplified by using oligonucleotide primers PKSII-for and PKSII-rev and 4,6-DH-for and 4,6-DH-rev (Table S1 in the Supporting Information), respectively. Cosmid CB30–6D20, which hybridised to both probes was sequenced. Two DNA fragments that were located on both ends of the cluster (30–6D20L and 30–6D20R, amplified by PCR using primers 30–6D20L-for and -rev and 30–6D20-R-for and -rev) were used as probes to screen for overlapping cosmids. Cosmids CB30–4E08 and CB30-2A21, which hybridised to one of these probes were also chosen for further sequencing experiments.

DNA sequencing and computer-aided sequence analysis: Nucleotide sequences were determined on an ABI sequencer at 4base Lab GmbH (Reutlingen, Germany) by using standard primers (M13 universal and reverse, T3 and T7) or customised internal primers. Computer-aided analysis was done with the DNASIS software package (version 2.1, 1995, Hitachi Software Engineering) and the FramePlot software at http://www.nih.go.jp/~jun/egi-bin/frameplot.pl.[43] Database comparison was performed with the BLAST search tools on the server of the National Center for Biotechnology Information (Bethesda, MD, USA).[44] To analyse PKS domains, the SEARCHPKS program http://www.nii.res.in/searchpks. Html), which is offered by the National Institute of Immunology (New Delhi, India) was used.[45] The sequence reported here has been deposited in the *GenBank* database (http://www.ncbi.nlm.nih.gov/Genbank) under the accession number FJ483966.

Construction of plasmids for gene inactivation: A 3.7 kb SacI fragment that contained *pokP1* was cloned into pUC19. The gene *pokP1* was replaced by the cassette *aadA* obtained from pIJ785-Spec by using the Red/ET® recombineering method. P7,3red-F and P7,3red-R were used as primers (Table S1). In a second step the *aac(3)IV* cassette of plasmid pHPΩ45aac[46] was cloned into the HindIII restriction site to yield pB-P3,7red. The deletion of all three oxygenase genes (*pokO1*, *pokO2* and *pokO4*) resulted from the replacement of the oxygenase-encoding sequence by the cassette [*aac(3)IV*+*oriT*] of vector pIJ773 by using the Red/ET recombineering method. For deletion of *pokO1* a 6.3 kb NcoI fragment from cosmid CB30–6D20 was cloned into pLitmus28 (pLit-pok20). The fragment was then cloned after EcoRI and XbaI restriction into pSP1. The primers F-pok20Red and R-pok20Red (Table S1) were used to replace *pokO1* by *aac(3)IV*+*oriT*. These primers were used to introduce NheI sites to both sites of the cassette, and then the cassette was removed by NheI restriction. This fragment was afterwards cloned in pKC1132 after EcoRI and XbaI restriction to yield pKC-pok20del.

An 8.8 kb NcoI fragment that contained the two ORFs *pokO2* and *pokO4* was cloned into pLitmus28. The fragment was then cloned after EcoRI and XbaI restriction into pSP1. Both genes were disrupted by using the Red/ET recombineering method with the corresponding primers: F-pok28Red and R-pok28Red for *pokO2* inactivation and F-pok29Red and R-pok29Red for *pokO4* inactivation (Table S1). In both cases the incorporated apramycin-resistance cassette was removed by using NheI and SpeI. Fragments were cloned into pKC1132 after EcoRI and XbaI restriction; this resulted in pKC-pok28del (for *pokO2* inactivation) and pKC-pok29del (for *pokO4* inactivation).

For the inactivation of the gene *pokMT1*, a 3.2 kb fragment that contained *pokMT1* was amplified by using primers MD1-F and MD1-R (Table S1). The PCR product was ligated into the EcoRI site of pUC19. A frame shift was introduced into *pokMT1* by BglII restriction and treatment with T4-DNA-polymerase. The mutated EcoRI fragment was cloned into pKC1132 yielding pKC1132-MT1del.

A 5.6 kb BamHI fragment that contained the gene *pokMT2* was cloned into pUC19. A frame shift was introduced into the gene by AscI restriction and treatment with T4-DNA polymerase. A 4.6 kb fragment that contained the mutated gene was cloned into pKC1132 to yield pKC1132-MT2del.

The inactivation construct of the gene *pokMT3* was obtained by amplification of two fragments (MT3JG1 and MT3JG2), one contained DNA that was located upstream of *pokMT3* and parts of *pokMT3* (MT3JG1), and the other one contained parts of *pokMT3* and DNA that were located downstream of *pokMT3* (MT3JG2). Primers JG1-F and JG1-R, and JG2-F and JG-2R (Table S1) were used, respectively. MT3JG1 was cloned into the XbaI-EcoRI sites of pBSK(−); this resulted in pBSK-MT3JG1. MT3JG2 was cloned into the EcoRI-HindIII sites of pBSK-MT3JG1; this resulted in pBSK-MT3JG1JG2. The DNA fragment that contained *pokMT3* with a 600 bp in-frame deletion was ligated into pKC1132 to create pKC1132-MT3del.

Generation of mutant strains of *S. diastatochromogenes* Tü6028: Gene-inactivation plasmids were transferred from *E. coli* ET12567 (pUZ8002) to *S. diastatochromogenes* Tü6028 by intergeneric conju-

gation as described for *S. cyanogenus* S136.[47] *S. diastatochromogenes* ΔpokO1 was used to generate *S. diastatochromogenes* ΔpokO1–ΔpokO2. For the generation of *S. diastatochromogenes* ΔpokP1, *S. diastatochromogenes* ΔpokO1, *S. diastatochromogenes* ΔpokO2, *S. diastatochromogenes* ΔpokO4, *S. diastatochromogenes* ΔpokO1–ΔpokO2, *S. diastatochromogenes* ΔpokMT1, *S. diastatochromogenes* ΔpokMT2 and *S. diastatochromogenes* ΔpokMT3 single cross-over mutants were screened for loss of vector-resistance as a consequence of a double cross-over event. Deletions within the genes were confirmed by PCR and/or Southern hybridisation.

Construction of plasmids for complementation: For complementation *pokO2* was amplified by PCR by using primers F-pok28komp and R-pok28komp (Table S1). The fragment was cloned into pSET-1cerm after EcoRI and XbaI restriction resulting in pSET-pok28. For complementation of *pokO4* a 3 kb BamHI fragment that contained the gene was cloned into pUWLoriT. The resulting plasmid was restricted with ClaI and XbaI and the fragment that contained *pokO4* was ligated in pSET-1cerm resulting in pSET-pok29.

For the complementation of the generated methyltransferase mutants, primers MT1-F and MT1-R, MT2-F and MT2-R, and MT3-F and MT3-R (Table S1) were used, and Cos30–6D20 was used as a template. PCR fragments were ligated into the MfeI and XbaI sites of pSET-1cerm to yield pSET-pokMT1, pSET-pokMT2, pSET-pokMT3.

The constructed plasmids were transferred into the respective mutants by intergeneric conjugation.

Biological properties: Antimicrobial activity was determined by the agar plate diffusion method on a paper disk (6 mm diameter). *Bacillus subtilis* COHN ATCC6051 and *S. viridochromogenes* Tü57 were used as Gram-positive test strains. Compounds were dissolved in MeOH (15 μL). After evaporation of the MeOH, discs were fixed on the test-plates and incubated over night at 37 °C.

Analysis of POK production and isolation of POK pathway intermediates/shunt products: Strains were cultivated in HA liquid medium for six days at 28 °C on a rotary shaker (180 rpm). Mycelia were separated from the supernatant by centrifugation. The pellet was extracted first with acetone (triple volume of the cell pellet volume) and afterwards mixed with water. The acetone was evaporated, and the residual aqueous phase was added to the supernatant followed by extraction with ethyl acetate (1:1). The ethyl acetate was evaporated to dryness and then dissolved in 50% acetonitrile (0.2 to 2 mL, adapted to the amount of the crude extract). Detection of compounds was performed by using HPLC-UV/HPLC-APCI-MS. The LC-system was equipped with a Zorbax XDB-C8 precolumn (12.5×4.6 mm, 5 μm) and a Zorbax XDB-C8 main-column (150×4.6 mm, 5 μm). A nonlinear 0.5% aq acetic acid/acetonitrile gradient over 30 min ranging from 20% to 95% was used (flow rate: 0.7 mL min^{-1}).

POK-IP3 was purified by using an Oasis® HLB 20/35cc (6 g) cartridge. POK-IP3 was eluted from the column by using a MeOH gradient. Further purification was performed on a preparative HPLC (Waters type 600 Controller) equipped with an XTerra® Prep C_{18} column system (7.8×100 mm, 5 μm). The 0.5% aq acetic acid/acetonitrile gradient ranged from 50 to 95% at a flow rate of 2.5 mL min^{-1}. The eluate of POK-IP3 was dried to afford an orange powder with an actual yield of 5 mg. For structure elucidation by NMR spectroscopy, POK-IP3 was dissolved in [D_6]DMSO.

For the isolation of POK-MD3 the crude extract was fractionated with a SepPak® C18 column (Waters Associates) and ascending concentrations of MeOH from 10 to 100% (in 10% steps). Fractions that contained POK-MD3 were pooled and applied to an Agilent 1100 system (see above) that was equipped with an Agilent® Zorbax SB-C18 column (150×9.6 mm, 5 μm) utilising mass-guided fraction collection. The 0.5% acetic acid/acetonitrile gradient ranged from 50 to 95% at a flow rate of 3.5 mL min^{-1}. Approximately 7.5 mg of POK-MD3 were isolated.

Structure elucidation of the generated POK derivatives: HRESI mass spectra were acquired by using a Micromass QTOF2 mass spectrometer. The chemical structure of POK-IP3 was reflected in 1D NMR (^{1}H (600 MHz), ^{13}C (150 MHz)) and 2D NMR (HSQC, HMBC, ^{1}H,^{1}H COSY) spectra on a Varian 600. ^{1}H (400 MHz), ^{13}C (100 MHz) and 2D NMR (HSQC, HMBC, ^{1}H,^{1}H COSY) spectra of POK-MD3, labelled POK-MD2 and labelled POK were recorded on a Bruker avance DRX400. Chemical shifts are expressed in δ values (ppm) by using the correspondent solvent as internal reference ([D_6]DMSO: $\delta_H = 2.49$, $\delta_C = 39.5$, [D_1]chloroform: $\delta_H = 7.26$, s; $\delta_C = 77.0$, t).

Feeding of L-[*S*-methyl-^{13}C]-methionine: HA medium (1.05 L in 5 flasks and 1.95 L in 13 flasks, respectively) was inoculated with a *S. diastatochromogenes* Tü6028 wt (36 h old) and a *S. diastatochromogenes* ΔpokMT1 seed culture (36 h old), respectively. The cultures were grown on a rotary shaker at 28 °C and 180 rpm. L-[*S*-methyl-^{13}C]-methionine (180 mg per litre of culture) was fed in equal portions after 21, 26, 33, 48 and 72 h. Cultures were harvested 6 days after inoculation. Compound isolation was performed as described above. The various methyl groups of l-[*S*-methyl-^{13}C]-methionine-labelled POK showed a specific incorporation rate of 31% for the methyl group at C6, 35% at C8, 40% at C3″, and 43% at C3‴, respectively. We were not able to determine the specific incorporation rates of the various methyl groups of POK-MD2 that were labelled by L-[*S*-methyl-^{13}C]-methionine, because the amount of POK-MD2 was very small. The unlabelled carbon atoms were nearly not visible, but in conjunction with an altered isotope ratio of the molecular ion in the MS spectrum of POK-MD2 after feeding of L-[*S*-methyl-^{13}C]-methionine, we can state that the observed carbon atoms belong indeed to POK-MD2. A labelling rate for $^{13}C_0 = 4.1\%$, $^{13}C_1 = 14.4\%$, $^{13}C_2 = 32.6\%$, and $^{13}C_3 = 33.2\%$ was detected for the molecular ion.

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Keywords: biosynthesis · methyltransferases · natural products · oxygenases · polyketides

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