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Biosynthesis of the Antitumor Agent Chartreusin Involves the Oxidative Rearrangement of an Anthracyclic Polyketide

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Summary

Chartreusin is a potent antitumor agent with a mixed polyketide-carbohydrate structure produced by *Streptomyces chartreusis*. Three type II polyketide synthase (PKS) gene clusters were identified from an *S. chartreusis* HKI-249 genomic cosmid library, one of which encodes chartreusin (*cha*) biosynthesis, as confirmed by heterologous expression of the entire *cha* gene cluster in *Streptomyces albus*. Molecular analysis of the 37 kb locus and structure elucidation of a linear pathway intermediate from an engineered mutant reveal that the unusual bis-lactone aglycone chartarin is derived from an anthracycline-type polyketide. A revised biosynthetic model involving an oxidative rearrangement is presented.

Introduction

Chartreusin (**1**, Figure 1) is an aromatic polyketide glycoside isolated from *Streptomyces chartreusis* that consists of fucose, digitalose, and an unusual bislactone aglycone, named chartarin [1, 2]. While chartreusin was first investigated because of its antibacterial activity [1], further studies revealed a significant chemotherapeutic activity against various tumor cell lines, such as murine P388 and L1210 leukemia, and B16 melanoma cells [3]. Unfortunately, development of chartreusin has been hampered by its unfavorable pharmacokinetics due to rapid biliary excretion and slow gastrointestinal absorption [3].

Given the remarkably high antitumor activity in vitro, there is considerable interest in addressing these pharmacokinetic shortcomings by accessing natural and semisynthetic chartreusin analogs with improved properties in vivo [4]. A natural derivative of **1**, elsamicin A or elsamitrucin (**2**, Figure 1), produced by an unidentified actinomycete strain, has improved water solubility due to the amino sugar moiety [5]. Both elsamicin A and a semisynthetic derivative of chartreusin, IST-622 (Figure 1), which appears to be a pro-drug with more suitable pharmacokinetic properties, have reached phase II clinical trials [6]. Pharmacological studies revealed that chartreusin and its derivatives exert their antitumor activities through binding to DNA [7], radical-mediated single-strand breaks, and inhibition of topoisomerase II [8]. Chartreusin and elsamicin A recognize almost the same G+C-rich DNA sequences, and analogs modified in the disaccharide portion of **1** retained antileukemic effects in vivo [4]. Consequently, there is strong evidence that the unusual polycyclic aromatic aglycone, named chartarin (**3**), represents a key element for bioactivity [9, 10].

Early ^{13}C-labeling experiments clearly revealed that the coumarin-related aglycone **3** of chartreusin and elsamicin is derived entirely from acetate and is thus supposedly of polyketide origin [11–13]. However, to date, the exact biosynthetic pathway to chartarin has remained unresolved, as the labeling pattern of acetate precursors is atypical for a polyketide metabolite (Figure 1). It was suggested that two individual polyketide chains are fused during the biosynthesis [14]; however, in the currently accepted biosynthetic model, an undecaketide-derived benzopyrene intermediate, **11**, is believed to undergo a series of oxidative C-C bond fissions and rearrangements [11, 12] (see below) reminiscent of the biosynthesis of gilvocarcin [15]. It should be mentioned that the formation of chrymutasin (**4**) [16], a metabolite of a randomly generated *S. chartreusis* mutant, cannot be rationalized with the current model.

Here, we report on the cloning, sequencing, and heterologous expression of a gene cluster encoding chartreusin biosynthesis to set the ground for genetically engineering analogs with improved pharmacokinetic properties. In addition, based on sequence analyses and structural elucidation of a pathway intermediate from a blocked mutant, we present a revised biosynthetic model for chartreusin biosynthesis.

Results

Identification, Cloning, and Heterologous Expression of the Chartreusin Biosynthetic Gene Cluster

Despite its highly unusual ring system, the aromatic structure and the labeling pattern suggest the involvement of an aromatic (type II) polyketide synthase in chartreusin biosynthesis, as well as tailoring enzymes that catalyze an unprecedented, to our knowledge, rearrangement of a yet unidentified polyphenolic precursor. An *S. chartreusis* HKI-249 genomic cosmid library was constructed by using the integrative shuttle cosmid vector pOJ436 [17]. The library consisting of 2324 clones was screened with a ketosynthase (KS_α) probe by dot blot/colony hybridization [18]. Nineteen positive cosmids were subjected to restriction mapping and Southern hybridization, revealing three individual loci. For characterization of the three putative type II PKS gene clusters (*pks1–3*), spot sequencing, heterologous expression, and inactivation experiments were undertaken. Heterologous expression of cosmid pSC6M16

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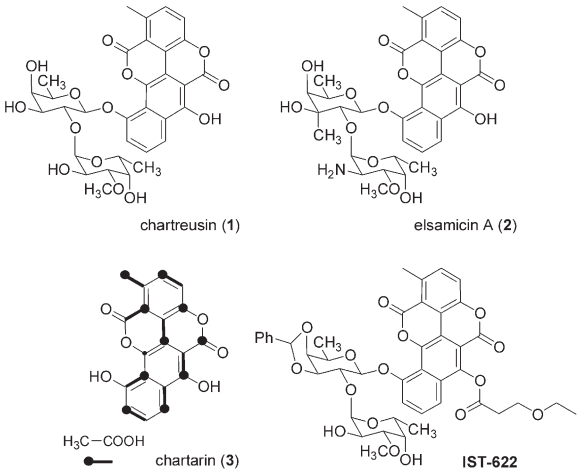

Figure 1. Structures of the Potent Antitumor Agents Chartreusin, Elsamicin, and IST-622, and Acetate Labeling Pattern of the Aglycone Charartin

conferred on the host *Streptomyces lividans* ZX1 [19] the ability to produce a blue-gray pigment, which is also formed by *S. chartreusis* HKI-249 when grown on R2YE plates. Thus, cluster *pks1* might encode a spore pigment biosynthetic pathway. Spot sequencing of cosmid pSC2N16 and homology searches revealed that cluster *pks2* codes for the biosynthesis of an angucycline-type metabolite. However, to date, all attempts to express the cluster in a heterologous host have failed, and genomic disruption by insertional mutation did not result in an altered metabolic profile. Thus, it is likely that cluster *pks2* is silent under standard cultivation methods. All cosmids bearing cluster *pks3*, which strongly resembled an anthracycline-type gene cluster by spot sequencing, were introduced into *S. lividans* ZX1 [19] and *Streptomyces albus* [20] by PEG-induced protoplast transformation and intergenic conjugation, respectively. While none of the *S. lividans* transformants produced any chartreusin, the metabolite was unequivocally detected by its characteristic blue fluorescence on TLC plates in an extract of the transconjugant *S. albus*::pSC5P21. The identity of **1** produced by the recombinant strain was further established by HPLC/MS by using an authentic sample, as well as by MS^n analyses (Figure 2). Since cosmid pSC5P21 appeared to contain all genes necessary to encode chartreusin (*cha*) biosynthesis, the entire insert DNA (~40 kb) was subjected to shotgun sequencing. All remaining gaps were filled by targeted subcloning and primer walking, except for a probable terminator region (<140 bp) between *chaX* and *chaK*, which could not be resolved by any means. The nucleotide (nt) sequence was deposited at the EMBL database under the accession numbers AJ786382 and AJ786383.

Architecture of the Genomic Region Involved in Chartreusin Biosynthesis

Analysis of the sequence for open reading frames (ORFs) with the FRAMEPlot program [21] and alignment

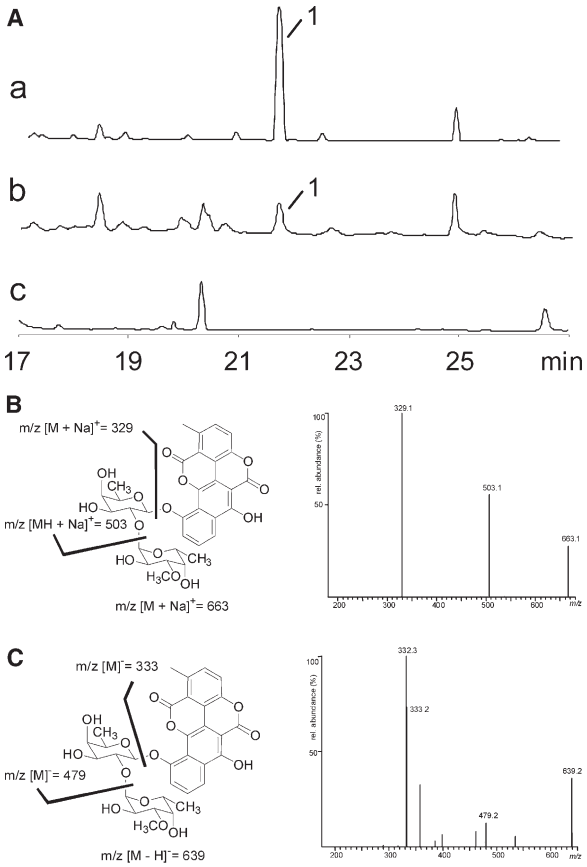

Figure 2. Detection of Chartreusin in Wild-Type and Recombinant Strains

(A) HPLC profiles of crude extracts from (a) wild-type *S. chartreusis*, (b) *S. albus*/pSC5P21, and (c) host *S. albus*.

(B and C) MS^n analyses (in positive [B] and negative [C] modes) of chartreusin (1) produced by *S. albus*/pSC5P21.

with related sequences in the database revealed the architecture of the entire ~37 kb chartreusin (*cha*) biosynthesis gene cluster, which is flanked by genes involved in primary metabolism and housekeeping. Based on homology searches, 35 open reading frames (ORFs) that consist of 27 probable structural genes and 8 genes for regulation and resistance were detected. The gene organization of the gene cluster is graphically presented in Figure 3, and the results of the sequence analyses are summarized in Table 1. Putative functions of deduced gene products were assigned by sequence comparisons with database proteins (BLAST and PROSITE searches [22]).

Regulatory and Resistance Genes

Three probable regulatory genes were located on the *cha* gene cluster. ORF *chaR1* is similar to a variety of pathway-specific SARP regulator genes from other secondary metabolite biosynthesis gene clusters, such as *grhR2* from *Streptomyces* sp. JP95 [23] and *aknO* from *Streptomyces galilaeus* [24]. A second protein with potential regulatory function is encoded by *chaR2* and resembles several putative regulatory proteins from

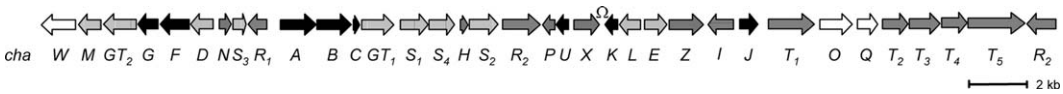

Figure 3. Organization of the *cha* Biosynthesis Gene Cluster in *S. chartreusis*

Each arrow indicates the direction of transcription and the relative sizes of the ORFs deduced from analysis of the nucleotide sequence. Black ORFs, minimal PKS and cyclase/aromatase genes; light-gray ORFs, ketoreductase genes; dark-gray ORFs, oxidoreductase and methyl transferase genes; checked ORFs, probable regulatory and resistance genes; hatched ORFs, genes putatively involved in deoxysugar biosynthesis and attachment; white ORFs, genes of unknown function.

Streptomyces coelicolor [25] and *Streptomyces avermitilis* [26], which all contain a pfam06036 motif. Proteins of this family are thought to be involved in sporulation. The deduced gene product of the third candidate regulator gene (*chaR3*) resembles putative LacI family transcriptional regulators, which have been identified in the genomes of *S. avermitilis* [26] and *S. coelicolor* [25].

Several genes, *chaT1–5*, that are probably involved in export and self-resistance were identified in the right-hand side of the cluster. The deduced protein of *chaT1* shows a motif of sugar transporters (pfam00083.11), such as the drug transporter of *Mycobacterium tuberculosis* (accession no. CAA15906). Another closely related protein is a transmembrane efflux protein from *S. avermitilis* [26]. The deduced gene products of ORFs *chaT2–5*, which are clustered together, are similar to components of ATP binding cassette (ABC) ion transporters from *Kineococcus radiotolerans* (see Table 1). Based on sequence homologies, the gene product of *chaT2* presumably functions in surface adhesion, and ChaT3 and ChaT4 would represent the ATPase and permease components, respectively. ChaT5 has the highest similarity to an ion-transporting ATPase from *S. coelicolor* (accession no. CAA22769). ABC transporters are known to confer self-resistance to the polyether ionophore tetronasin to *Streptomyces longisporoflavus* [27] and have also been implicated in polyene macrolide resistance [28].

Genes Encoding a Type II Polyketide Synthase

Bacterial type II (aromatic) PKSs are typically composed of a single set of iteratively used individual proteins. A β-ketoacyl synthase (KS_α), a so-called chain length factor (CLF, or KS_β), and an acyl carrier protein (ACP), which acts as an anchor for the growing polyketide chain, are responsible for the formation of the poly-β-keto chains, and are thus referred to as a minimal PKS. Three ORFs encoding the chartreusin minimal PKS are located centrally in the *cha* gene cluster. All deduced gene products are closely related to known aromatic PKS components. However, the deduced gene products of the two ketosynthase subunits, ChaA and ChaB, are most similar to PKS involved in the biosynthesis of decaketides, such as tetracenomycin [29], nogalamycin [30], and aklavinone [31], and not to PKSs involved in making longer polyketide chains, as would be predicted from the current biosynthetic scheme.

Other PKS Genes

The deduced gene products of *chaD*, *chaE*, and *chaL* show a distinct dehydrogenase motif (pfam00106), and the highest scores are with ketoreductases involved in the processing of poly-β-keto intermediates. ChaD and ChaE, which share 54.8% sequence identity, resemble C-9 ketoreductases, such as AknA [31]. ChaL also belongs to the family of ketoreductases; however, it is more related to C-17 ketoreductases, which are represented by AknU [32], DnrH [33], and SnoaF [34].

Candidate genes encoding cyclases and aromatases that are involved in the directed folding of the nascent polyketide chain are *chaF*, *chaG*, *chaJ*, and *chaK*. ChaF is similar to a family of proteins that is known to catalyze the dehydratation/aromatization of the A-ring of anthracyclic polyketides, including AknE1, RdmK, and SnoaE [30, 31]. The deduced 231 aa gene product of *chaG* shows a conserved motif (HxGTHxDxPxH) that is likely to form part of the active site of cyclases (pfam04199.6). The closest relatives of ChaG are cyclases required for complete cyclization of the second and, most probably, also the third ring of linear polyketides [35], such as CmmY (chromomycin) [36], SnoaM (nogalamycin) [34], and AknW (aclarubicin) [24].

Two genes, *chaJ* and *chaK*, probably encode two copies of ester cyclases similar to the nogalonic acid methyl ester transferase, SnoaL, and functionally identical cyclases, DnrD, RdmA, DauD, and AknH, from related anthracycline pathways [37]. These enzymes are known to catalyze an aldol-type cyclization of the fourth (D) ring [38].

Probable Post-PKS Genes

The deduced gene product of *chaI* resembles SAM-dependent methyl transferases (COG0500.1) from secondary metabolite pathways. ChaI shows the highest sequence identity to aklanonic acid methyl transferases (AknG) from the *Streptomyces galilaeus* aklavinone (69% identity) [31] and the *Streptomyces peucetius* daunorubicin pathways (59% identity) [39], as well as to the nogalonic acid methyl transferase SnoaC from *Streptomyces nogalater* (56% identity) [40].

In the *cha* biosynthetic gene cluster, four putative oxygenases are encoded that are putatively involved in tailoring, disruption, and rearrangement of the aromatic polyketide ring system of a chartarin precursor. The deduced 106 aa gene product of *chaH* resembles anthrone oxygenases (pfam03992.6). Related enzymes, which probably occur as multimers, are involved in the biosynthesis of aklavinone AknX (BAB72044.1) [31] and nogalamycin SnoaB (CAA12015.1) [30]. These oxygenases are responsible for the introduction of the quinone oxygen at C-12 of aklanonic acid and nogalonic acid, respectively. ChaX shares the highest similarity with putative hydroxylases AclQ (accession no. BAB72056,

Table 1. Proposed Functions of Deduced *cha* Gene Products

Deduced Protein	Size (aa)	Proposed Function	Sequence Similarity (Protein, Origin)	Similarity/ Identity, %	Accession Number
ChaW	433	Unknown	*Nostoc* sp. PCC 7120	46/29	NP_488388
ChaM	257	SAM-dependent methyl transferase	*Ralstonia eutropha*	54/39	ZP_00168466
ChaGT2	373	Glycosyl transferase	*Crocosphaera watsonii*	61/45	ZP_00176915
ChaG	231	Cyclase (2^{nd} + 3^{rd} ring)	CmmY, *Streptomyces griseus* subsp. *griseus*	70/59	CAE17518
ChaF	318	Cyclase/Aromatase	AknE1, *Streptomyces galilaeus*	68/56	BAB72042
ChaD	260	C-9 Keto reductase	AknA, *Streptomyces galilaeus*	78/71	BAB72043
ChaN	145	Unknown	SnoO, *Streptomyces nogalater*	51/41	AAF01807
ChaS3	163	NDP-4-Keto-6-deoxy-D-glucose reductase	*Thermobifida fusca*	45/30	ZP_00293833
ChaR1	216	SARP family regulator	Orf71, *Streptomyces rochei*	72/58	NP_851493
ChaA	422	Ketoacyl synthase	TcmK, *Streptomyces glaucescens*	75/66	P16538
ChaB	408	Chain length factor	Snoa2, *Streptomyces nogalater*	71/62	CAA12018
ChaC	85	Acyl carrier protein	NcnC, *Streptomyces arenae*	60/42	AAD20269
ChaGT1	381	Glycosyl transferase	CloM, *Streptomyces roseochromogenes* subsp. *oscitans*	53/39	AAN65229
ChaS1	352	NDP-Hexose synthase	LanG, *Streptomyces cyanogenus*	66/50	AAD13545
ChaS4	310	Nucleoside-diphosphate-sugar epimerase	SpcI, *Streptomyces netropsis*	48/37	AAD45554
ChaH	106	Anthrone oxygenase	SnoaB, *Streptomyces nogalater*	62/51	CAA12015
ChaS2	332	NDP-D-Glucose 4,6-dehydratase	*Corynebacterium glutamicum*	69/56	BAB97727
ChaR2	437	Putative transcriptional regulator	*Streptomyces avermitilis*	36/20	NP_825027
ChaP	130	Dioxygenase	*Ralstonia eutropha*	68/51	ZP_00166028
ChaU	137	Ester cyclase	AclR, *Streptomyces galilaeus*	77/59	BAB72057
ChaX	287	Hydroxylase	AclQ, *Streptomyces galilaeus*	60/55	BAB72056
ChaK	147	Ester cyclase	RdmA, *Streptomyces purpurascens*	77/67	AAA83420
ChaL	246	C-17 Keto reductase	DauE, *Streptomyces* sp. C5	71/58	AAB08021
ChaE	261	C-9 Keto reductase	AknA, *Streptomyces galilaeus*	71/59	BAB72043
ChaZ	395	FAD-dependent monooxygenase	*Photorhabdus luminescens* subsp. *laumondii*	52/35	CAE14036
ChaI	286	SAM-dependent methyl transferase	AknG, *Streptomyces galilaeus*	69/59	BAB72050
ChaJ	138	Ester cyclase	DnrD, *Streptomyces peucetius*	75/59	AAA99000
ChaT1	542	Drug transporter transmembrane efflux protein	*Mycobacterium tuberculosis*	50/34	NP_215766
ChaO	387	Isovaleryl-CoA dehydrogenase	*Frankia* sp. EuIK1	86/78	AAL02422
ChaQ	247	Hypothetical protein	*Streptomyces avermitilis*	68/54	BAC70751
ChaT2	307	ABC-type metal ion transport system	*Kineococcus radiotolerans*	67/56	ZP_00227783
ChaT3	268	Mn/Zn transport systems, ATPase component	*Kineococcus radiotolerans*	71/56	ZP_00227784
ChaT4	288	ABC-type Mn2+/Zn2+ transport systems, permease components	*Kineococcus radiotolerans*	86/72	ZP_00227785
ChaT5	686	Putative cation-transporting ATPase	*Streptomyces coelicolor* A3(2)	77/69	CAA22769
ChaR3	354	Putative LacI-family transcriptional regulator	*Streptomyces avermitilis*	59/45	BAC68741

55% identity) from *S. galilaeus* and SnoaW (accession no. AAF01810, 47% identity) from *S. nogalater*. The function of these proposed monooxygenases has not been established to date. A putative FAD-dependent monooxygenase with a conserved C-terminal domain (pfam01360.11) is encoded by *chaZ*. The probable 395 aa enzyme is related to an unnamed hypothetical protein from the entomopathogenic bacterium *Photorhabdus luminescens* (NP_929024.1), whose function is unknown [41]. The deduced 130 aa gene product of *chaP* belongs to the superfamily of vicinal oxygen chelate fold (VOC) oxidoreductases. It shows a conserved domain (pfam00903.11) that is found in a variety of structurally related metalloproteins, including the bleomycin resistance protein, glyoxalase, and ring-cleaving dioxygenases. The closest relative of ChaP is a lactoylglutathione lyase from *Ralstonia eutropha* JMP134 (accession no. ZP_00166028.1, 51% identity).

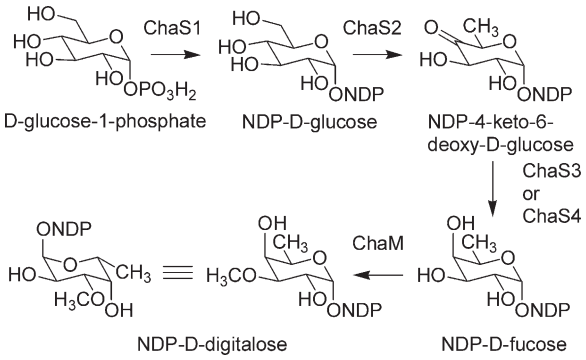

Figure 4. Proposed Pathway for the Biosynthesis of the 6-Deoxysugars in Chartreusin

Genes Encoding Biosynthesis and Attachment of Deoxyhexoses

Chartreusin contains two deoxysugars, D-fucose and D-digitalose, that are attached to the chartarin aglycone. Several genes that are possibly involved in sugar biosynthesis and attachment [42] have been detected in the *cha* gene cluster. Activation of glucose 6-phosphate to the corresponding NDP-hexose is putatively catalyzed by the deduced 352 aa gene product of *chaS1* (Figure 4), which is similar to a variety of NDP-D-glucose synthases from Streptomycetes, e.g., LanG (landomycin) from *Streptomyces cyanogenus* (accession no. AAD13545) and VinA (vicenistatin) from *Streptomyces halstedii* (accession no. BAD08355). The deduced gene product of *chaS2*, which incorporates an epimerase motif (pfam01370.11), seems to belong to the NAD-dependent epimerase/dehydratase family of enzymes. ChaS2 has the highest sequence identity to a dTDP-D-glucose 4,6-dehydratase from *Corynebacterium glutamicum* (accession no. BAB97727).

The subsequent conversion of NDP-4-keto-6-deoxy-D-glucose to NDP-D-fucose is putatively catalyzed by the deduced 163 aa gene product of *chaS3*, a probable NDP-glucose-4-keto-6-deoxy-D-glucose reductase of the GtrA family (pfam04138.6). Related membrane bound proteins are known to take part in the biosynthesis of NDP-D-fucose in the context of serotype-specific polysaccharide antigen formation, as by *Actinobacillus actinomycetemcomitans* Y4 [43]. Curiously, in the *cha* gene cluster, a second candidate gene for a 4-epimerase can be found. ORF *chaS4* codes for a protein that shows a dehydrogenase motif (COG1028.1) at its N terminus and an epimerase motif at its C terminus (pfam01370.11). ChaS4 is most similar to several NAD-dependent NDP-hexose-4-epimerases, such as SpcI (spectinomycin) from *Streptomyces netropsis* (accession no. AAD45554, 37% identity), which is implicated in the biosynthesis of the fucose-derived aminoglycoside spectinomycin.

ChaS1–4 would be sufficient for the biosynthesis of NDP-D-fucose. For the synthesis of D-digitalose, which is equivalent to 3-O-methylated fucose, an O-methyl transferase is required, which is most likely encoded by *chaM*. The deduced 257 aa sequence of ChaM clearly reveals an SAM-dependent methyl transferase motif (COG0500.1) and shows 54% identity to a probable SAM-dependent methyltransferase from *Ralstonia eutropha* JMP134 (accession no. ZP_00168466).

Two genes in the *cha* cluster putatively encode glycosyltransferases. Upstream of *chaS1* is a gene, *chaGT1*, that codes for a glycosyl transferase (COG1819.1, COG1819) with marked similarity to a variety of enzymes that catalyze the attachment of deoxyhexoses to aromatic polyketide aglycones. Among the closest relatives are the glycosyltransferases CmmGII (chromomycin) from *Streptomyces griseus* subsp. *griseus* [36] (accession no. CAE17548, 39% identity) and NovM (novobiocin) from *Streptomyces spheroides* [44] (accession no. AAF67506, 34% identity) as well as CouM (coumermycin) from *Streptomyces rishiriensis* [45] (accession no. AAG29785, 38% identity), the latter of which glycosylates coumarin-type aglycones.

The second glycosyltransferase required for the transfer of the second sugar moiety (NDP-digitalose) is putatively encoded by *chaGT2*. It should be pointed out that ChaGT2 (pfam00535.11) shows only very low similarity to glycosyl transferases from known secondary metabolite pathways. Its closest relative (45% identity) is a glycosyltransferases (accession no. ZP_00176915.2) involved in cell wall biogenesis of *Crocosphaera watsonii* WH 8501.

Genes of Unknown Function

ChaN is related to a family of proteins found in several type II PKS pathways, including AknV (*S. galilaeus*) [24] and SnoO (*S. nogalater*) [34]. Although it has been suggested that these enzymes may participate in the glycosylation of aclacinomycins, ChaN shows the conserved motif (cd00667.2) of the β subunit of aryl ring hydroxylating dioxygenases. However, only a structural role has been attributed to this subunit. Interestingly, ChaN is also similar to the C terminus of AknE1 from *S. galilaeus*, a known cyclase-aromatase. ORF *chaO* codes for a protein with very high similarity to an isovaleryl-CoA dehydrogenase from a *Frankia* sp. (accession no. AAL02422, 78% identity). Its function in the context of chartreusin biosynthesis remains unclear. ChaW and ChaQ do not have any conserved motifs. They resemble hypothetical proteins from *Nostoc* sp. PCC7120 (accession no. NP_488388) and *S. avermitilis* MA-4680 (accession no. NP_824216), respectively.

Inactivation of chaZ and Identification of a Linear Polyketide

The deduced function of *cha* PKS gene products strongly suggests that the biosynthesis of the polyketide aglycone chartarin shares common steps with anthracycline-type aromatic polyketides. Consequently, the chartreusin and anthracyclin pathways would branch at a specific biosynthetic step. We assumed that the putative FAD-dependent oxygenase ChaZ would play a crucial role in chartarin formation and aimed at an inactivation of *chaZ*. This goal was achieved by double crossover in *E. coli* by using the λred system [46–48]. After excision of *chaZ* from the *cha* gene cluster on cosmid pSC5P21 and confirmation

of the deletion by restriction mapping, the resulting cosmid (pXU-C01) was introduced into *S. albus* by intergenic conjugation with *E. coli* [20]. Analysis of a plate culture of the transconjugant *S. albus*::pXU-C01 revealed that chartreusin production was fully abolished in the mutant. Instead, an orange fluorescent metabolite was produced. Isolation of this compound from the extract of a medium-scale fermentation (10 l) by column chromatography and preparative HPLC provided sufficient material for full structural elucidation. From ESI HR-MS and NMR data a molecular composition of $C_{21}H_{14}O_6$ was deduced. Except for aromatic proton signals, the ^{1}H-NMR spectrum of **8** showed two chelated hydroxyl protons at 13.73 and 12.21 ppm, as well as one methyl and one methoxy group. The absence of any methylene protons, as well as a H-H correlation and coupling pattern accounted for a linear fully aromatic polyketide. Database and literature searches revealed that **8** is identical with resomycin C, an aromatic polyketide that was only recently isolated in the Laatsch group from *Streptomyces* sp. isolate GW71/2497 [49]. Strikingly, resomycin is only formed by the strain under specific cultivation methods, while it usually produces large quantities of chartreusin.

Discussion

Nature only provides a few different core polyketide structures in aromatic polyketides by varying the chain length, position of the ketoreduction, and regiochemistry of the cyclization events. All bacterial aromatic polyketide folds are either linear or angucyclic, except for resistomycin, which bears a unique discoid carbocyclic system [50]. The large structural diversity within the family of aromatic polyketides is rather dictated by the employment of different starter units [51], and by tailoring enzymes [52], such as oxygenases, methylases, and glycosyl transferases. Oxidative rearrangement reactions may result in entirely novel polyketide structures. Dramatic examples are the enterocin [53] and griseorhodin [23] pathways, in which the rearrangement results in nonplanar polyketide ring systems. The pentacyclic aromatic bislactone chartarin, which is relevant for the significant antitumor activities of chartreusin and elsamicin, represents another important example of a rearrangement of a regular polyketide, and the enzymes involved in the elaboration of such unusual polyketide structures may provide new opportunities for combinatorial biosynthesis approaches.

Chartreusin Shares Early Biosynthetic Steps with Anthracyclines

The current model for chartreusin biosynthesis involves a benzopyrene precursor that is derived from an undecaketide (Figure 5). However, the high sequence similarity of the ketosynthase subunits ChaA and ChaB with PKS enzymes from the tetracenomycin and nogalamycin pathways strongly suggested that chartarin is rather derived from a decaketide progenitor. We could unequivocally prove this assumption by inactivation of an assumed key enzyme, ChaZ, as the resulting mutant produces the anthracyclic polyketide **8** in lieu of chartreusin. All deduced *cha* gene products are in line

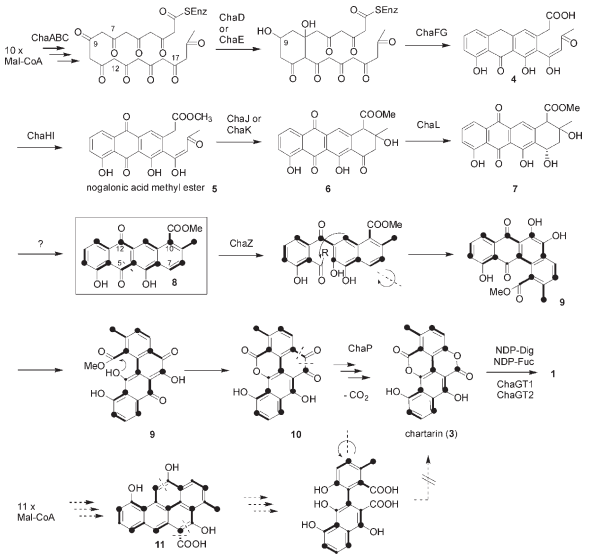

Figure 5. Revised Biosynthetic Model for the Chartreusin Aglycone, Chartarin

The linear polyketide metabolite resomycin C (**8**) was isolated from the *chaZ* null mutant. The previously proposed pathway via benzopyrene **11** is shown for comparison.

with this finding (Figure 5). The decaketide would be subject to ketoreduction at C-13, catalyzed by one of the two C-9 ketoreductase candidates that are encoded by *chaD* and *chaE*, respectively. The ketoreduction most likely occurs on the A-ring after 7/12 cyclization, which is probably catalyzed by the ketoacyl synthase. Aromatization of the A-ring would be catalyzed by the putative aromatase ChaF, which strongly resembles AknE1, RdmK, and SnoaE from anthracycline pathways [30]. Formation of the linear polyketide backbone is then promoted by ChaG. Similar cyclases are involved in the cyclization of the second and, most probably, also the third ring of chromomycin, nogalamycin, and aclarubicin [30, 36, 54]. The next steps would involve the oxidation of the proposed nogalic acid intermediate by the putative anthrone oxygenase ChaH [31], and methylation of the carboxyl group by ChaI, which is strongly related to the SAM-dependent aklanonic acid and nogalonic acid methyl transferases. Upon methylation, the fourth ring would be formed by an aldol-type cyclization catalyzed by ChaJ or ChaK, which both have similarity to ester cyclases from related anthracycline pathways, such as SnoaL and AknH [37, 38]. Finally, the putative C-17 ketoreductase ChaL would reduce the keto group, a reaction analogous to those catalyzed by AknU [55], DnrH [56], and SnoaF [54], yielding the anthracyclic aglycone of nogalamycin, auramycinone (**7**). The deduced gene products of *chaA-L* would be sufficient for the biosynthesis of an anthracyclin-type polyketide, such as **7**. Compound **8** could be derived from **7** by dehydration. However, it remains unclear which enzymes are involved in the aromatization of the D-ring. Interestingly, **8** is identical to a recently identified metabolite, resomycin C, of another chartreusin producer [49]. Since resomycin C is only

formed when the strain is cultivated under specific conditions, it seems that, under these conditions, ChaZ is produced at lower titers. With the finding that chartreusin is derived from an anthracyclin, the current biosynthetic model for **1** needed to be revised.

New Model for the Biosynthesis of Chartarin via Rearrangement of an Anthracyclic Decaketide

Undoubtedly, the gene product of *chaZ* plays a crucial role in the rearrangement of a linear polyketide intermediate, since deletion of *chaZ* results in the accumulation of **8** and prevents any substrates from being made for downstream reactions. The incorporation pattern suggests that the first ring cleavage could occur adjacent to the anthraquinone carbonyl of **8** (Figure 5). The putative FAD-dependent monooxygenase ChaZ could cleave this carbon-carbon bond in a Baeyer-Villiger-type oxygenation. According to the labeling pattern, a new C-C bond would be formed between the carbonyl and the unsubstituted carbon of the C ring. Depending on the nature of R, the ring fusion would occur by formylation or acylation. A hydroxyl group could be introduced either during the ring cleavage or by hydroxylation of the angucyclic intermediate by the putative hydroxylase ChaX, but the timing of hydroxylation cannot be predicted as yet. Unfortunately, biotransformation experiments with resomycin C by using a *chaABC* null mutant have been unsuccessful to date. Thus, at this stage, it cannot be ruled out that resomycin C (**8**) rather represents a shunt product, while **6** or **7** are the true substrates of ChaZ. In this case, aromatization of ring D would take place later in the biosynthetic pathway. However, once the framework of **9** has been established, the quinone oxygen, probably in the enol form, could attack the methyl ester carbonyl with substitution of methanol and form the first lactone ring. The proposed intermediate, **10**, that results from this intramolecular ring closure could be the substrate of another unprecedented rearrangement reaction. The best candidate for such a reaction is the putative dioxygenase ChaP. The rather small enzyme belongs to the vicinal oxygen chelate (VOC) superfamily of enzymes. Metalloenzymes of this type are known to catalyze a variety of reactions, including isomerizations (glyoxalase I), nucleophilic substitutions (fosfomycin resistance), and oxidative cleavage of C-C bonds [57]. The latter comprise the structurally and mechanistically fascinating extradiol dioxygenases, 4-hydroxyphenylpyruvate dioxygenase, 2,3-dihydroxybiphenyl dioxygenase, and related *meta*-cleavage dioxygenases [58]. In a similar fashion, ChaP might disrupt the dione moiety of **10** by attack of a dioxygen species and loss of carbon dioxide. Subsequent lactone formation would ultimately furnish the chartarin aglycone. Elucidation of all pathway intermediates as well as mechanistic studies are in progress in order to gain a deeper insight into this intriguing rearrangement cascade.

Molecular Basis for an Unusual Polyketide Glycosylation Pattern

Besides the rearranged chartarin ring system, another remarkable feature of chartreusin is the unusual 2-glycosylation pattern of the fucose moiety. In the *cha* cluster, all genes required for the biosynthesis of the activated 6-deoxysugars NDP-D-fucose and NDP-D-digitalose can be detected (*chaS1–S4* and *chaM*, Figure 4). ChaGT1 is highly similar to a variety of glycosyltransferases involved in glycosylation of polyketide aglycones, and it is thus considered to catalyze the attachment of fucose to chartarin. The second glycosyltransferase required for the transfer of the second sugar moiety (NDP-digitalose) is putatively encoded by *chaGT2*. Since this enzyme would represent a 2-glycosyl transferase, which is unprecedented in secondary metabolism, ChaGT2 unsurprisingly shows only very low sequence similarity to glycosyl transferases from known secondary metabolite pathways. It is more closely related to glycosyltransferases involved in polysaccharide and cell wall biogenesis, where 1,2-glycosidic linkages are more common. The enzymes involved in the glycosylation of chartarin would be the preferred targets for engineering chartreusin analogs with improved solubility.

In conclusion, we have identified the chartreusin biosynthesis gene cluster out of three type II PKS gene clusters from the genome of *S. chartreusis* HKI-249. The *cha* cluster has been fully sequenced, and its identity was confirmed by heterologous expression. All genes relevant for polyketide and sugar biosynthesis have been assigned. Sequence comparisons and knock-out of a key pathway enzyme, ChaZ, provide the strongest evidence that chartreusin is derived from an anthracyclic precursor. On the basis of this information and on earlier isotope labeling results, a revised biosynthetic model involving an unprecedented, to our knowledge, rearrangement of an anthracyclic ring system is proposed. Our work sets the basis for engineering chartreusin analogs with improved pharmacological properties and studying the enzymology of the intriguing tailoring reactions. These experiments are currently underway in our laboratory.

Significance

The polyketide glycoside chartreusin (*cha*) produced by *Streptomyces chartreusis* is a very potent antitumor agent in vitro. Its development as a drug, however, is limited by its unfavorable pharmacokinetics due to its low solubility. Cloning, sequencing, and heterologous expression of the entire *cha* biosynthesis gene cluster now sets the basis for rationally engineering analogs with improved solubility.

Furthermore, the polyketide aglycone chartarin, which is relevant for antitumor activity, has a unique pentacyclic bis-lactone structure. Analysis of the *cha* gene cluster and isolation of resomycin C from a rationally designed mutant proved that chartarin is derived from an anthracyclic progenitor by an oxidative rearrangement sequence. A revised biosynthetic model rationalizing the irregular acetate-labeling pattern of chartreusin is presented. Considering that only relatively few polyketide ring systems are realized in nature, the chartreusin pathway represents another important example of the elaboration of unusual cyclization patterns. Thus, the *cha* tailoring enzymes, including unusual ring-cleaving oxidoreductases and

a putative 2-glycosyl transferase, are not only intriguing from a mechanistic point of view, but are also promising candidates for combinatorial biosynthetic approaches.

Experimental Procedures

Bacterial Strains and Culture Conditions

The chartreusin producer *Streptomyces chartreusis* HKI-249, obtained from the HKI strain collection, was used as the source of DNA in the construction of the genomic DNA library. *S. lividans* ZX1 [19], kindly provided by Prof. Zhou, and *S. albus* [19], kindly provided by Prof. Salas, served as host strains for heterologous expression experiments. For chartreusin production, wild-type and mutant strains were cultivated in MS medium (mannitol soya flour medium) for 5 days at 28°C with shaking. *S. lividans* ZX1 was cultured on R5 agar and YEME liquid medium [20] for protoplast transformation and on MS (mannitol soya flour) [23] for all other experiments. Transformants were selected with apramycin or spectinomycin (Sigma) at 50 μg/ml in both solid and liquid medium. *E. coli* strains DH5α and XL1 blue served as hosts for library construction and routine subcloning, respectively [59]. For intergenic conjugation, *E. coli* ET12567 containing the RP4 derivative pUZ8002 was used [20]. *E. coli* strains were grown in LB medium supplemented with ampicillin (100 μg/ml), or with apramycin (50 μg/ml) for selection of plasmids [59].

Plasmids and General DNA Procedures

DNA isolation, plasmid preparation, restriction digests, gel electrophoresis, and ligation reactions were conducted according to standard methods [20, 59]. pBluescript II SK(-) [60] was the routine vector for subcloning and preparation of DNA templates for sequencing. The integrative *E. coli-Streptomyces* shuttle vector pOJ436 [17] was used for all expression experiments in *Streptomyces*. Restriction enzyme-digested DNA fragments were recovered from agarose gel by the GFX PCR DNA and Gel Band Purification Kit (Amersham). For Southern blot hybridization, the DIG DNA Labeling and Detection Kit (Roche) was used. Sequencing of pSC5P21 was performed with a shotgun approach. Remaining gaps were filled by targeted subcloning and primer walking.

Construction and Screening of an *S. chartreusis* HKI-249 Genomic Cosmid Library

An *S. chartreusis* HKI-249 genomic cosmid library was constructed in *E. coli* DH5α by using pOJ436 as a cosmid vector [17]. For DNA extraction, mycelium was embedded in agarose, then the DNA was partially digested and isolated, yielding fragments with an average size greater than 35 kb. Robotically produced high-density colony arrays (Hybond N$^+$; Amersham Pharmacia) were utilized for the screening of 2304 cosmid clones with a strain-specific Type II PKS probe by following standard hybridization procedures.

Inactivation of *chaZ*

The *chaZ* null mutant was constructed by using the λred system [46–48]. To amplify the extended streptomycin and spectinomycin resistance gene (*aadA*) flanked by FRT sites (FLP recognition targets) and 39 nt from sense/antisense strands ending in start/stop codons, two long primers, chaZ-P.T.L: 5′-TCGCCGCGCCGCCGAACA GTGGGGAGGCGGGGGCCGATGATTCCGGGGATCCGTCGACC-3′ (59 nt), and chaZ-P.T.R: 5′-GAGGGCGTCCGCTGTCGAGTGGGTT CCGTGTGCCGGTCATGTAGGCTGGAGCTGCTTC-3′ (58 nt), were designed. The PCR product was introduced into *E. coli* BW25113/pIJ790 containing cosmid pSC5P21 with concomitant substitution of the *chaZ* gene by the extended antibiotic resistance cassette. The inserted cassette was removed through expression of the FLP-recombinase in *E. coli*, yielding a 81 bp "scar" in the preferred reading frame. The resulting plasmid, pXU-C01, was then introduced into *S. albus* by intergenic conjugation by using *E. coli* ET12567 containing the RP4 derivative pUZ8002. The transconjugant *S. albus*::pXU-C01 was selected for its apramycin resistance.

Fermentation and Isolation of Metabolites

Chartreusin was extracted with ethyl acetate from chopped agar plates or from liquid culture broths of wild-type and recombinant strains. Extracts were concentrated in vacuo and redissolved in MeOH. Chartreusin was identified by comparison of the extracts with an authentic sample by thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), and mass spectrometry (MS). HPLC analysis was carried out on a GROM-Sil ODS 0 AB column (3 μm particle size, 250 × 4.6 mm) by using an acetonitrile/0.1% TFA gradient (0.5%–100% in 30 min) at a flow rate of 1.0 ml/min and UV detection at 200–800 nm (max plot). Fragmentation (CID-MS^n) of pseudomolecular ions ([M+Na]$^+$) was conducted by collision-induced dissociation by using helium as the collision gas (Spray voltage: 5 kV, capillary temperature: 200°C.

For medium-scale fermentation, *S. albus*::pXU-C01 was grown in 20 liter baffled Erlenmeyer flasks, each containing 500 ml MS (mannitol soya flour) medium supplemented with 50 μg/ml apramycin, at 28°C in a rotary shaker. The culture was harvested after 6 days and was extracted with ethyl acetate. The crude extract was separated on silica, and fractions containing **8** were further purified by preparative HPLC. Spectroscopic data for **8**:

^{1}H-NMR (300 MHz, $CDCl_3$): δ 13.73 (s, 1H, 6-OH), 12.21 (s, 1H, 4-OH), 8.49 (d, *J* = 8.6 Hz, 1H, 7-H), 8.25 (s, 1H, 11-H), 7.88 (dd, *J* = 7.5, 1.1 Hz, 1H, 1-H), 7.68 ("t," *J* = 7.7 Hz, 1H, 2-H), 7.53 (d, *J* = 8.6 Hz, 1H, 8-H), 7.29 (dd, *J* = 7.7, 1.1 Hz, 1H, 3-H), 4.09 (s, 3H, OCH_3), (3.69, 3.47,) 2.55 (s, 3H, 9-CH_3) ppm. IR (crystal powder) ν 2956, 2916, 1727, 1492, 1378, 1262, 1136, 1028 cm^{-1}. ESI-MS (m/z): 747.6 (2x M+Na)$^+$ (100), 363.5 (M+H)$^+$ (12). HRMS calcd. for $C_{21}H_{14}O_6$ (M+H): 363.0869, observed: 363.0858. **8** is in all respects identical to the previously described resomycin C [49].

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Accession Numbers

The nucleotide sequence of the chartreusin biosynthesis gene cluster has been deposited at the EMBL Nucleotide Sequence Database under the accession numbers AJ786382 and AJ786383.