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Biosynthesis of the Antitumor Chromomycin A_3 in *Streptomyces griseus*: Analysis of the Gene Cluster and Rational Design of Novel Chromomycin Analogs

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Summary

The biosynthetic gene cluster of the aureolic acid type antitumor drug chromomycin A_3 from *S. griseus* subsp. *griseus* has been identified and characterized. It spans 43 kb and contains 36 genes involved in polyketide biosynthesis and modification, deoxysugar biosynthesis and sugar transfer, pathway regulation and resistance. The organization of the cluster clearly differs from that of the closely related mithramycin. Involvement of the cluster in chromomycin A_3 biosynthesis was demonstrated by disrupting the *cmmWI* gene encoding a polyketide reductase involved in side chain reduction. Three novel chromomycin derivatives were obtained, named chromomycin SK, chromomycin SA, and chromomycin SDK, which show antitumor activity and differ with respect to their 3-side chains. A pathway for the biosynthesis of chromomycin A_3 and its deoxysugars is proposed.

Introduction

Chromomycins, mithramycin, olivomycins, chromocyclomycin, UCH9, and durhamycin A belong to the class of antitumor compounds called aureolic acids [1]. They inhibit growth and multiplication of several tumor cell lines and also act on gram-positive bacteria. More recently, chromomycin as well as mithramycin were also found to stimulate K562 cell erythroid differentiation [2], and both drugs were also suggested as neurological therapeutics [3] and for the treatment of HIV-1 [4]. The antitumor properties are ascribed to their inhibitory effects on replication and transcription processes during macromolecular biosynthesis by interacting, in the presence of Mg^{2+}, with G/C-rich nucleotide sequences located in the minor groove of DNA.

Structurally, the aureolic acid type compounds (with the exception of chromocyclomycin) contain a tricyclic chromophore (aglycon) with two aliphatic side chains attached at C-3 and C-7. The aglycon is synthesized through the condensation of one acetyl-CoA and nine malonyl-CoA units [5], and therefore this family of antibiotics belongs to the polyketide group of compounds. The aglycons are glycosylated by oligosaccharides of different length chain and sugar composition.

Chromomycin A_3 (Figure 1A) is the main component of a fermentation mixture produced by *S. griseus* subsp. *griseus* ATCC13273. Chromomycin A_3 possesses the same aglycon as mithramycin but differs in the glycosylation pattern. Mithramycin is produced by different streptomycete strains, and it contains a trisaccharide of D-olivose, D-oliose, and D-mycarose, and a disaccharide of D-olivoses, while chromomycin A_3 contains a trisaccharide of D-olivose (sugar C), D-olivose (sugar D), and 4-*O*-acetyl-L-chromose B (sugar E), and a disaccharide of 4-*O*-acetyl-D-oliose (sugar A) and 4-*O*-methyl-D-oliose (sugar B) attached at positions 2 and 6 of the aglycon, respectively. The carbohydrate moieties are major structural contributors to the biological activity of chromomycin. The acetoxy groups in sugars A and E of chromomycin contribute distinctively in the DNA complex formation by providing an additional H bond with the 2-amino groups of G bases and thus adding more specificity to the DNA binding.

Here, we report the cloning and characterization of the chromomycin A_3 gene cluster from *S. griseus* subsp. *griseus* ATCC13273. We also show a rational approach to generate novel chromomycin derivatives with antitumor activity by specific inactivation of the ketoreductase gene *cmmWI* responsible for the reduction of the 4′-keto group of the 3-side chain, which occurs at the last step of the biosynthesis.

Results

Cloning and Sequencing of the Chromomycin Biosynthetic Gene Cluster from *Streptomyces griseus* subsp. *griseus*

An *S. griseus* subsp. *griseus* total DNA library was constructed in the bifunctional cosmid pKC505. A total of 3300 recombinant colonies were screened by in situ colony hybridization using two different probes: (1) a heterologous probe (PKS probe; see Experimental Procedures for details) and (2) a homologous probe generated using a pair of oligonucleotides specifically designed to amplify ABC transporters involved in self-resistance in producer microorganisms (ABC probe) [6]. Thirteen cosmid clones were identified that either hybridized with the PKS probe (four clones), with the ABC probe (four clones), or with both probes (five clones). Preliminary evidence of the involvement of the cloned DNA region in chromomycin biosynthesis was obtained by expressing one of the ABC hybridizing clones, cosGR60, in the chromomycin-sensitive strain *S. albus*. Recombinant *S. albus* strains were shown to be able to grow in the presence of 100 μg/ml of chromomycin A_3, while control strains (only containing the vector pKC505) did not.

A DNA region of approximately 42 kb was sequenced.

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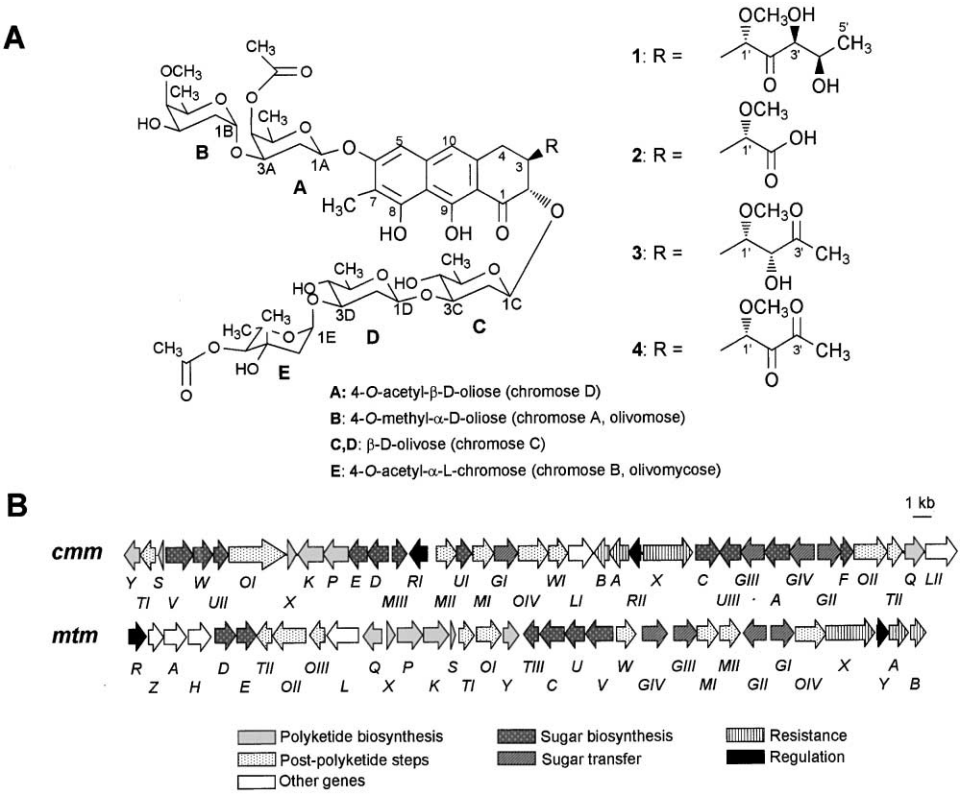

Figure 1. Structure of Chromomycin A_3 and Derivatives and Genetic Organization of the Chromomycin and Mithramycin Gene Clusters

(A) Chemical structures of chromomycin A_3 and its new derivatives generating by insertional inactivation: chromomycin A_3 (**1**), chromomycin SA (**2**), chromomycin SK (**3**), and chromomycin SDK (**4**). The stereochemistry of C-2′ in chromomycin SK was deduced from its NMR data in comparison with its analog mithramycin SK (see [12]).

(B) Genetic organization of the chromomycin A_3 gene cluster and its comparison with the mithramycin gene cluster.

The nucleotide sequence was deposited at the EMBL Nucleotide Sequence Database under the accession number AJ578458. The sequence was analyzed for putative open reading frames (ORFs) using the CODON-PREFERENCE program, and the deduced amino acid sequences were compared to proteins in databases using the BLAST program. Thirty-six complete ORFs were identified (Figure 1B and Table 1). All the genes were designated as *cmm*, with the exception of three ORFs designated as *cmr* for being related to chromomycin resistance.

PKS Genes

Several genes of the cluster probably participate in polyketide biosynthesis. Three genes (*cmmP*, *cmmK*, and *cmmS*) would code for the minimal PKS that would be responsible for the biosynthesis of the 20-carbon polyketide backbone of the chromomycin aglycon. Two of these genes (*cmmP* and *cmmK*) are located together in the central region of the cluster, while the acyl carrier protein gene (*cmmS*) is located more than 8 kb distant downstream. CmmP and CmmK are highly similar to KS_α (ketosynthase I) and KS_β (ketosynthase II) homologous proteins, respectively. It is notable that CmmK contains a glycine residue instead of the conserved glutamine residue that is present in most of KS_β proteins [7]. CmmS would be the third protein of the minimal *cmm* PKS complex and showed similarity to different ACPs.

Three additional enzymes (CmmQ, CmmX, and CmmY) would be involved in cyclization and aromatization processes. The *cmmQ* product would code for a bifunctional cyclase/aromatase that would participate in the cyclization and aromatization of the first ring. It is similar to various aromatases involved in type II polyketide biosynthesis, including the mithramycin [8] aromatase, that show a C-7/C-12 first ring closure as necessary for chromomycin. However, the localization of this gene in the *cmm* cluster is quite unusual since it is located at a site remote from the ketosynthase gene *cmmP*, at one end of the cluster. CmmX shows convincing similarity to MtmX from the mithramycin gene cluster [8], proposed to be a cyclase involved in the fourth ring closure in mithramycin biosynthesis. CmmY shows strong similarity to putative cyclases such as DpsY [9] and MtmY [10]. Based on these similarities, we propose roles for CmmX and CmmY as cyclases involved in fourth and second/third ring closures during the biosynthesis of chromomycin, respectively.

Post-Polyketide Genes

Three genes (*cmmOI*, *cmmOII*, and *cmmOIV*) have been identified that would code for proteins exhibiting signifi-

Table 1. Deduced Functions of the Open Reading Frames of the Chromomycin A_3 Cluster

Protein	aa	Proposed Function	Similar Protein, Pathway	Identity/ Similarity (%)	Accession No.
CmmA	422	*O*-acyltransferase	MdmB, midecamycin	37.7/58.8	A42719
CmmC	408	NDP-*C*-methyltransferase	OrfX, balhimycin	63.2/78.4	CAC48364
CmmD	355	NDP-glucose-synthase	LanG, landomycin	56.1/73.8	AAD13545
CmmE	326	NDP-4,6-dehydratase	RhoH, rhodomycin	64.4/76.7	BAB87836
CmmF	209	NDP-5-epimerase	CalS1, calicheamicin	54.3/74.5	AAM94768
CmmGI	393	Glycosyltransferase	MycB, mycinamicin	41.1/60.0	BAC57037
CmmGII	396	Glycosyltransferase	MtmGII, mithramycin	43.4/63.8	AAC64927
CmmGIII	397	Glycosyltransferase	MtmGIII, mithramycin	48.9/68.5	AAD55583
CmmGIV	421	Glycosyltransferase	MtmGIV, mithramycin	51.6/67.9	AAD55582
CmmK	422	KS_β	Sim3, simocyclinone	64.4/76.9	AAL15581
CmmLI	413	Acyl-CoA ligase	CloL, clorobiocin	36.6/55.1	AAN65228
CmmLII	528	Acyl-CoA ligase	MtmL, mithramycin	54.8/68.1	CAA07759
CmmMI	357	*O*-methyltransferase	MtmMI, mithramycin	49.0/66.7	AAD55585
CmmMII	342	*C*-methyltransferase	MtmMII, mithramycin	52.5/73.0	AAD55584
CmmMIII	250	*O*-methyltransferase	ElmMIII, elloramycin	61.4/76.5	AAG23269
CmmOI	415	Oxygenase[a]	MtmOI, mithramycin	57.7/75.1	CAA07766
CmmOII	557	Oxygenase	MtmOII, mithramycin	57.9/72.9	CAA07757
CmmOIV	506	Oxygenase	MtmOIV, mithramycin	58.7/72.3	AAC64929
CmmP	422	KS_α	UrdA, urdamycin	75.8/82.7	CAA60569
CmmQ	318	Aromatase	MtmQ, mithramycin	50.5/66.8	CAA07760
CmmRI	300	Transcriptional activator	Grhr2, griseorhodin	45.1/63.4	AAM33680
CmmRII	198	Transcriptional regulator	AclS, aclacinomycin	26.5/51.9	BAB72058
CmmS	83	ACP	LanC, landomycin	54.8/67.9	AAD13538
CmmTI	252	Ketoreductase	MtmTI, mithramycin	54.2/68.3	CAA07765
CmmTII	253	Ketoreductase	MtmTII, mithramycin	61.7/75.9	CAA07756
CmmUI	249	NDP-4-ketoreductase	LanR, landomycin	52.0/71.2	AAD13548
CmmUII	251	NDP-4-ketoreductase	UrdR, urdamycin	51.4/68.0	AAF72551
CmmUIII	356	NDP-4-ketoreductase	AveBIV, avermectin	59.9/74.7	BAC68658
CmmV	460	NDP-2,3-dehydratase	MtmV, mithramycin	52.0/69.9	CAB96551
CmmW	336	NDP-3-ketoreductase	Gra-orf26, granaticin	54.2/66.8	CAA09647
CmmWI	327	Ketoreductase	MtmW, mithramycin	62.5/77.4	AJ459240
CmmX	142	Cyclase	SnoO, nogalamycin	59.7/72.7	AAF01807
CmmY	258	Cyclase	AknW, aclacinomycin	67.6/81.5	AAF73459
CmrA	325	ATP binding protein	MtrA, mithramycin	54.2/70.1	AAC44357
CmrB	250	Membrane protein	MtrB, mithramycin	47.9/71.8	AAC44358
CmrX	826	UV-repair system	Dra0188	42.7/63.4	AAF12187

[a] This protein could be expressed as a fusion protein with 378 extra amino acids at the N terminus. This N-terminus region shows similarity to biotin carboxylases.

cant similarities to FAD-dependent monooxygenases involved in hydroxylations of different aromatic polyketides. The highest similarity of the *cmmOI* gene product was with the MtmOI oxygenase from the mithramycin cluster [10]. The most similar protein to CmmOII is MtmOII, which has been proposed to act on a tricyclic intermediate prior to the fourth ring cyclization to form 4-demethylpremithramycinone [10]. CmmOIV is more similar to MtmOIV, an oxygenase responsible for the oxidative fourth ring opening of premithramycin B [11]. CmmOII would be involved in the hydroxylation of chromomycin aglycon, and CmmOIV would be the oxygenase responsible for the fourth ring scission to generate the tricyclic aglycon with the pentyl side chain attached at C-3. This process, leading ultimately to the formation of chromomycin A_3, would also require the participation of a ketoreductase. Three ketoreductase genes have been identified in the cluster, *cmmTI*, *cmmTII*, and *cmmWI*. No role can be assigned at the moment for CmmTI and CmmTII (see below). CmmWI shows similarity to the aldo/keto reductase protein family (Pfam00248), which includes proteins reported to have oxidoreductase activity. This protein is also similar to MtmW, a ketoreductase that has been recently proven to be involved in the ketoreduction step affecting the 3-side chain in mithramycin biosynthesis [12].

The chromomycin aglycon contains two methyl groups. Two methyltransferase genes have been found, *cmmMI* and *cmmMII*. The deduced gene products are most similar to methyltransferases MtmMI and MtmMII from the mithramycin cluster [13]. CmmMI shows closer similarity to MtmMI, which is responsible for the *O*-methylation at the 4 position of 4-demethylpremithramycinone. CmmMII is more similar to MtmMII, which carries out the *C*-methylation step at the 9 position of tetracyclic intermediates. Thus, we propose CmmMI and CmmMII to be methyltransferases involved in 4-*O*-methylation and 9-*C*-methylation of tetracyclic intermediates, respectively.

Genes Involved in Glycosylation

Chromomycin A_3 contains five deoxysugars attached to the aglycon: two D-olivoses, one 4-*O*-acetyl-L-chromose, one 4-*O*-acetyl-D-oliose, and one 4-*O*-methyl-D-oliose. Several genes have been identified that could be involved in sugar biosynthesis. Two of them (*cmmD* and *cmmE*) are located together and would direct the first steps in deoxysugar biosynthesis. CmmD is very

similar to NDP-D-glucose synthases, and CmmE is very similar to NDP-D-glucose 4,6-dehydratases [14]. CmmD and CmmE are proposed to catalyze the first and second common steps in the biosynthesis of chromomycin deoxysugars.

Downstream of *cmmDE* genes, there are three other genes (*cmmV*, *cmmW*, and *cmmUI*). The *cmmV* product shows strong similarity to dTDP-2,3-glucose dehydratases involved in the C-2 deoxygenation step of other antibiotics [15, 16]. CmmW shows similarity to 3-ketoreductases, which render hydroxyl groups at *C*-3 with an equatorial configuration, as is the case of Gra-orf26 involved in D-olivose biosynthesis [15]. CmmV and CmmW could be the dehydratase and reductase enzymes involved in the 2-deoxygenation step occurring during biosynthesis of all chromomycin deoxysugars (sugars A–E). The third gene (*cmmUI*) would code for a 4-ketoreductase. There are two other genes (*cmmUII* and *cmmUIII*) whose products are similar to 4-ketoreductases and that are scattered in the cluster. CmmUI and CmmUII show highest similarities to UrdR, involved in the biosynthesis of D-olivose in the urdamycin pathway [17]. On the other hand, CmmUIII is more similar to 4-ketoreductases involved in L-sugar biosynthesis, such as AveBIV from the L-oleandrose biosynthetic pathway [18]. CmmUIII could be the 4-ketoreductase involved in the biosynthesis of 4-*O*-acetyl-L-chromose, and the two other ketoreductases (CmmUI and CmmUII) in the biosynthesis of the D-deoxysugars in chromomycin A_3.

Immediately downstream and convergently transcribed to *cmmUIII* is *cmmC*, which would code for a *C*-methyltransferase probably involved in the biosynthesis of 4-*O*-acetyl-L-chromose. CmmC shows high similarity to *C*-methyltransferases involved in branched-chain sugars biosynthesis from different clusters. It shows closer similarity to *C*-methyltransferases that will produce a *C*-methyl group at *C*-3 with an axial configuration [19]. Accordingly, CmmC could be the *C*-methyltransferase needed for the biosynthesis of 4-*O*-acetyl-L-chromose. There is another gene, *cmmF*, whose product resembles proteins proposed to act as 5- or 3,5-epimerases and that contains conserved domains for NDP-sugar-isomerases (Pfam00908). CmmF could play the role of an epimerase that is required for the d/l-switch in the biosynthesis of the 4-*O*-acetyl-L-chromose moiety.

Three chromomycin sugars are decorated by either a methyl (sugar B) or acetyl groups (sugar A and E) at their C-4 hydroxy groups. The *cmmMIII* gene would code for a protein that strongly resembles *O*-methyltransferases. These enzymes methylate sugars once they have been attached to the aglycons [20]. The *cmmA* product is similar to acyltransferases acting both on macrolide aglycons and on sugars already attached to macrolide aglycons, and it contains a conserved domain of an acyltransferase family (Pfam01757). We propose CmmMIII to be the methyltransferase responsible for the 4-*O*-methylation of the terminal d-oliose of the disaccharide chain, i.e., the generation of 4-*O*-methyl-D-oliose, and for CmmA the role of an acetyltransferase responsible for the transfer of one or both acetyl groups required for the decoration of the sugars A and E.

Four genes in the cluster would encode glycosyltransferases. The highest similarities of the corresponding proteins were found with mithramycin glycosyltransferases. The number of glycosyltransferases genes in chromomycin cluster does not correspond to the number of sugar moieties, i.e., one GT is missing. CmmGIV and CmmGIII are similar to MtmGIV and to MtmGIII, respectively, which have been shown to be responsible for transferring the first (D-olivose) and the second (D-oliose) deoxysugars of the trisaccharide to the aglycon [21]. According to these similarities, CmmGIV and CmmGIII could transfer the first and second D-olivose moieties of the trisaccharide, respectively. CmmGII is more similar to MtmGII [22], which suggests its involvement in the formation of the disaccharide. Since the amino acid sequence of CmmGI is equally similar to those of MtmGIII and MtmGI, it could be involved theoretically either in the formation of the disaccharide or in the transfer of the third deoxysugar of the trisaccharide chain.

Genes Involved in Regulation

Database searches revealed two genes with probable regulatory function in the *cmm* cluster, CmmRI and CmmRII. CmmRI resembles transcriptional activators of the *Streptomyces* antibiotic regulatory proteins (SARP) family. It contains a bacterial transcriptional activator domain (BAD) (Pfam03704) characteristic of this family of regulators. A second protein, which potentially has a regulatory function, is encoded by *cmmRII*. This gene could be cotranscribed with two other genes that form part of an ABC transporter system. It contains a putative conserved domain (COG1695) predicted in several transcriptional regulators.

Genes Involved in Self-Resistance

In the right part of the cluster, there are three genes (*cmrA*, *cmrB*, and *cmrX*) that could be involved in self-resistance to chromomycins. CmrA and CmrB are similar to ATP binding proteins and to membrane proteins, respectively, which form part of type I ABC transporter systems [6]. The *cmrX* gene product resembles one of the three subunits (UvrA) of ABC excision nuclease systems responsible for DNA repair. It was predicted that the ABC transporter system (CmrAB) and the CmrX protein would be involved in self-resistance to chromomycin.

Other Genes

CmmTI contains domains of enoyl-[acyl-carrier-protein] reductases (COG0623) and short chain dehydrogenases (Pfam00106). Its closest homolog is MtmTI from the mithramycin cluster [8], for which a clear role in mithramycin biosynthesis has not been established. It has been proposed that MtmTI together with MtmX plays some role in polyketide biosynthesis, probably in ring cyclizations. CmmTII produces significant alignments with short chain dehydrogenases (Pfam00106). It shows closer similarity with MtmTII from the mithramycin cluster, which seems to be not essential for mithramycin biosynthesis [10].

Two putative PKS accessory genes have been identified, *cmmLI* and *cmmLII*. They code for proteins similar

to acyl CoA ligases. They also contain conserved domains for AMP binding (Pfam00501) present in a number of enzymes that act via an ATP-dependent covalent binding of AMP to their substrates.

Rational Design of New Chromomycins

An experiment was carried out to prove the involvement of the *cmm* genes in chromomycin biosynthesis. This experiment was also designed to generate new chromomycin derivatives with novel but predicted structures and with potential antitumor activity. We chose the *cmmWI* gene that codes for a protein showing strong similarity to MtmW (62.5% identity) [12]. Inactivation of *mtmW* leads to a chemically unstable compound that can undergo various chemical reactions that consequently lead to the production of several novel mithramycin derivatives bearing shorter side chains attached at C-3 instead of the usual pentyl side chain, with one of them (mithramycin SK) showing a dramatically improved therapeutic index compared to the parent drug mithramycin [12]. We therefore envisaged that we could create analogous novel chromomycin derivatives that would differ from the corresponding mithramycin derivatives with respect to their sugar residues.

The *cmmWI* gene was in vitro mutated by inserting an apramycin resistance cassette in the same direction of transcription as the gene (Figure 2A). This construct was introduced into *S. griseus* by intergeneric conjugation with *E. coli* and transconjugants selected by their resistance to apramycin and then further tested for their sensitivity to thiostrepton as a consequence of a double crossover. Southern hybridization demonstrated the occurrence of a double recombination event (Figure 2B). A mutant strain (C60WI) was selected and tested for chromomycin production. No chromomycin A_3 was detected in cultures of C60WI, proving the involvement of this region in chromomycin biosynthesis. Instead, this mutant accumulated three new compounds (designated as chromomycin SA, SK, and SDK; Figures 2C and 1A), with chromomycin SK being the major accumulated compound. All three compounds showed the same UV absorptions as chromomycin A_3, suggesting that they all contain the tricyclic chromophore.

Structural Elucidation of Compounds Accumulated by Mutant C60WI

The three compounds accumulated by mutant C60WI were purified by preparative HPLC from cultures grown on R5A medium. Purification of the compounds was complicated since they were rather unstable. The amounts of purified compounds obtained from 100 plates were 28.2 mg for chromomycin SA, 34.8 mg for chromomycin SK, and 34 mg for chromomycin SDK, making a total of 97 mg. Under similar cultivation conditions, 169 mg chromomycin A_3 can be obtained.

Chromomycin A_3 (Figure 1A, 1)

Although the correct structure of chromomycin A_3 (**1**) was published already ca. 20 years ago, we needed to reassign the ^{1}H- and ^{13}C-NMR signals since we used d_5-pyridine as an NMR solvent, which has never been used before on chromomycins and causes significantly different NMR spectra compared to those recorded in other NMR solvents. This was necessary since this solvent proved to be the best for the generation of most of the NMR data of the new chromomycin derivatives (the chromomycin A_3 assignments are available as Supplemental Data with this article online for comparison). Our ^{13}C assignments of the aglycon signals correlate with the recently published data on durhamycin A [23], which were also recorded in pyridine. For better comparison with other aureolic acid drugs, we also used the standard ring labeling (A–E) of the sugar residues (see Figure 1A), which for unclear reasons was not used historically for chromomycin A_3.

Chromomycin SA (Figure 1A, 2)

The molecular ions identified in the negative fast atom bombardment mass spectrum at *m/z* 1123 ([M–H$^-$]) and in the positive FAB MS at *m/z* 1147 ([M+Na]$^+$) allowed the deduction of the molecular formula of $C_{54}H_{76}O_{25}$, which was also supported by the NMR data and confirmed by HR-ESI-MS (1123.4592 observed, calculated for $C_{54}H_{76}O_{25}$: 1123.4603). Compound **2** was obtained as yellow amorphous solid, which showed identical UV and similar IR data (see Experimental Procedures) as chromomycin A_3. The ^{1}H-NMR spectrum of compound **2** closely resembled that of chromomycin A_3, except that three characteristic signals of the pentyl side chain attached at C-3 of chromomycin A_3 were missing, namely 3′-H, 4′-H, and that of the terminal methyl group (5′-H_3). The ^{13}C-NMR spectrum showed only 54 signals, i.e., three signals less than **1**, due to the modified 3-side chain. Compared to the parent compound **1**, typical signals of the pentyl side chain are missing, including the terminal methyl group, the two hydroxylated methine carbons C-3′ and C-4′, and the C-2′ keto group, which normally represents the most downfield signal (δ 213.5). Instead, compound **2** has a new carbonyl further upfield at δ 179.7, which is typical for an acid carbonyl. Thus, the side chain of compound **2** consists only of two carbons, and structure **2** was deduced for this compound, called chromomycin SA, in analogy to the recently described mithramycin SA [12].

Chromomycin SK (Figure 1A, 3)

The molecular ions identified in the negative FAB mass spectrum at *m/z* 1151 ([M–H$^-$]) and in the positive FAB MS at *m/z* 1175 ([M+Na]$^+$) are in agreement with a molecular formula of $C_{56}H_{80}O_{25}$, which also was supported by the NMR spectra and the high-resolved ESI mass spectrum (observed, 1151.5053; calculated for $C_{56}H_{80}O_{25}$, 1151.4916). Compound **3** was obtained as yellow amorphous solid, which also showed almost identical UV and similar IR data (see Experimental Procedures) as chromomycin A_3.

As expected from the design of the inactivation experiment, the ^{1}H-NMR, although in general similar to those of compounds **1** and **2**, showed differences with respect to the signals of the 3-side chain. The terminal methyl group (δ 2.47) appeared as a singlet whose chemical shift indicates a carbonyl neighborhood. Two methine protons whose 3 Hz coupling indicated a direct neighborhood were identified as part of this side chain, namely 1′-H (a doublet of a doublet at δ 5.37, $J = 3$ and 1 Hz), and 2′-H (a doublet at δ 4.70, $J = 3$ Hz). Both of their chemical shifts indicated that these protons were attached to an oxygen-bearing carbon. All other signals

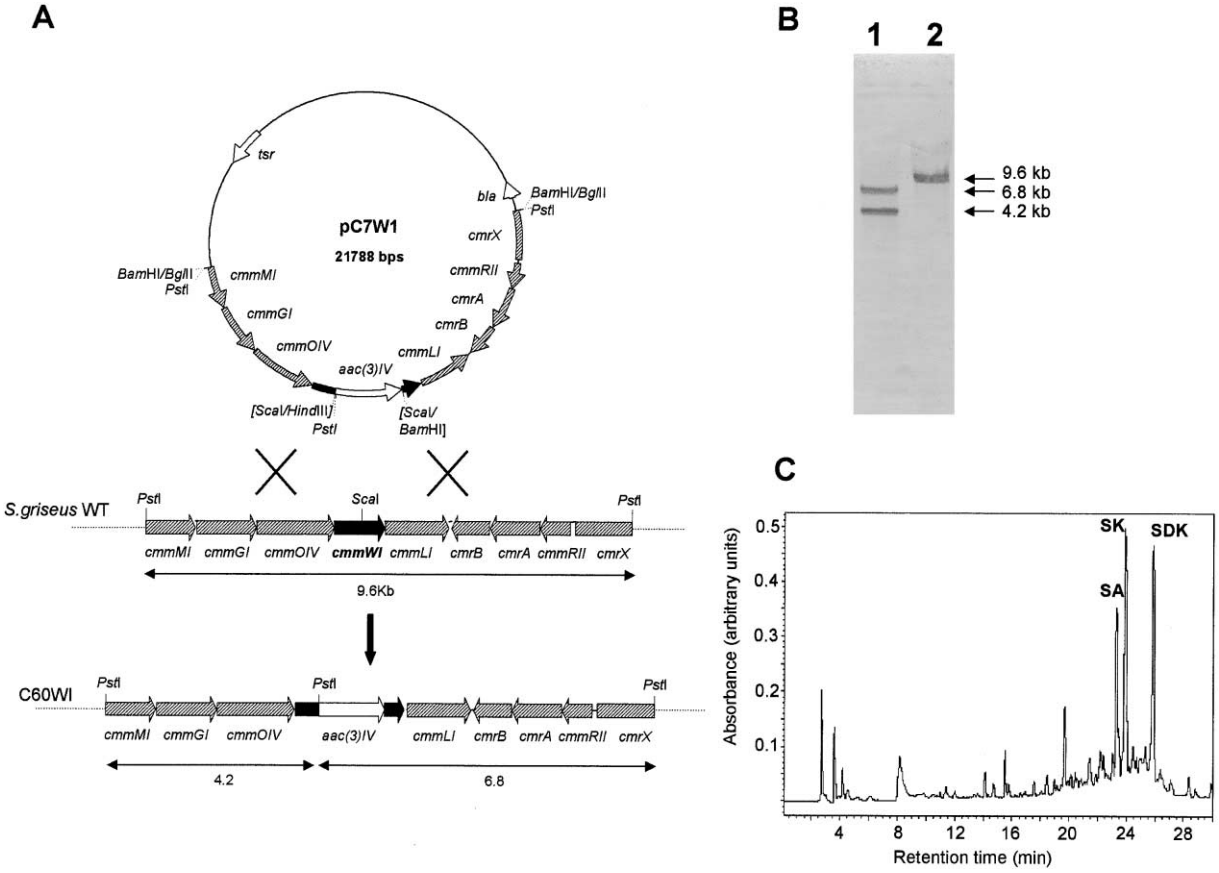

Figure 2. Generation and Analysis of Mutant C60WI

(A) Scheme representing the replacement in the chromosome of the wild-type *cmmWI* gene by the in vitro mutated one. *aac(3)IV*, apramycin-resistance gene; *tsr*, thiostrepton-resistance gene; *bla*, β-lactamase gene.

(B) Southern hybridization using the 9.6 kb PstI fragment as probe. Lane 1, PstI-digested chromosomal DNA from mutant C60WI; lane 2, PstI-digested chromosomal DNA from wild-type strain.

(C) HPLC analysis of a culture of mutant C60WI.

of the ^{1}H-NMR spectrum were very similar to those of compounds **1** and **2**, indicating that the sugars and the remaining aglycon was unchanged. The ^{13}C-NMR spectrum showed 56 signals, of which the most downfield-shifted signals at δ 207.2 was assigned to C-3′, since a long-range $^{2}J_{C\text{-}H}$ coupling with the 4′-H_3 singlet (δ 2.47) was observed in the HMBC spectrum. The other unique carbon, compared to the ^{13}C-NMR spectra of structures **1** and **2**, is the signal at δ 28.9, which could be assigned to C-4′, since its chemical shift indicates a carbonyl neighborhood, and its attached protons show a $^{3}J_{C\text{-}H}$ coupling with C-2′ (δ 76.2) in the HMBC spectrum. Thus, the side chain of compound **3** was unambiguously identified as identical with the butyl side chain recently described for mithramycin SK. In summary, the MS, ^{1}H-NMR, and ^{13}C-NMR data, in comparison with the data of chromomycin A_3 (**1**), reveal structure **3**, which is called chromomycin SK in analogy to mithramycin SK.

Chromomycin SDK (Figure 1A, 4)

The molecular ion identified in the negative fast atom bombardment mass spectrum at *m/z* 1149 ($M-H^{-}$) and the positive FAB MS at *m/z* 1173 $(M-Na)^{+}$ allowed the deduction of the molecular formula to be $C_{56}H_{78}O_{25}$, which is supported by 56 observed signals in the ^{13}C-NMR spectrum and the highly resolved ESI MS (observed, 1149.4783; calculated for $C_{56}H_{78}O_{25}$, 1149.4759). Compound **4** was isolated as yellow amorphous solid, which showed similar UV and IR spectra (see Experimental Procedures) with chromomycin A_3 as well as the above-described derivatives.

Again, the ^{1}H-NMR was similar to that of chromomycin A_3, except for the missing signals for 2′-H and 3′-H. Like for chromomycin SK (**3**), a downfield-shifted methyl group singlet at δ 2.43 was observable whose chemical shift and missing coupling indicates a carbonyl neighbor, assigned as 4′-H_3. The 1′-H signal (δ 5.50) appears as a doublet (J = 1Hz) like in chromomycin, also indicating a carbonyl neighbor. Combining all this information, it could be concluded that the 3-side chain consists of only four carbons, two being carbonyl groups. This was confirmed by the ^{13}C-NMR spectrum, which showed the two postulated carbonyl signals of C-2′ and C-3′ in the downfield region of the spectrum at δ 198.6 and 199.5, which is typical for a δ-dicarbonyl arrangement, along with the C-4′ methyl signal at δ 26.4 neighboring one of these carbonyls. In summary, the structure of **4** was deduced for the compound.

The structures of chromomycin SK, SA, and SDK (Fig-

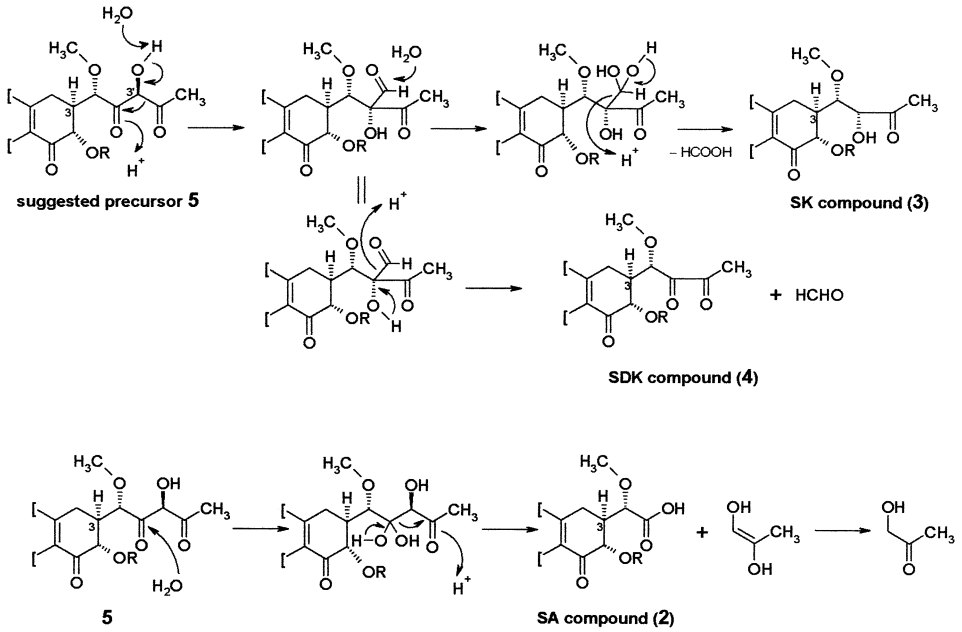

Figure 3. Formation of Chromomycin Derivatives

Formation of chromomycins SK (3) and SDK (4) through a Favorskii-like rearrangement and of chromomycin SA (2) through retro-aldol-type cleavage of the reactive product 5, proposed to be generated by the inactivation of the ketoreductase-encoding gene *cmmWI*.

ure 1A) were expected from our analogous previous experiments with mithramycin. As in the case of mithramycin, a β-diketo compound (5) is expected from the inactivation of ketoreductase CmmWI (Figure 3). The highly functional and thus reactive 3-side chain of 5, which contains two keto-functions in β position to each other separated by a carbon bearing another oxygen atom, triggers a Favorskii-like rearrangement for which an 1,2 acyl shift induced by deprotonation of the central alcohol can be envisaged. This is either followed by addition of water on the resulting aldehyde and consequent departure of formic acid (leading to chromomycin SK) or just by departure of formaldehyde (leading to chromomycin SDK). Chromomycin SA also gives indirect evidence for the labile structure 5, since its formation from 5 is possible through attack of water at the carbonyl adjacent to the methoxy group followed by retro-aldol cleavage to yield 2 and hydroxyacetone.

Antitumor Activity

The antitumor activity of the three novel chromomycin derivatives was tested against a variety of tumor cell lines (Table 2). Compilation of the average log (GI_{50}) values showed that the three compounds were clearly active, although the activity levels were lower than those for the natural chromomycin A_3 compound. However, when compared with mithramycin, the three compounds showed similar levels of activity.

Discussion

The chromomycin A_3 cluster of *Streptomyces griseus* subsp. *griseus* consists of 36 genes: 31 coding for structural proteins, 2 for pathway regulatory proteins, and 3 involved in self-resistance to chromomycin. Genes encoding most of the activities that are required for chromomycin biosynthesis were identified within the cluster. Exceptions are the absence of a fifth glycosyltransferase gene and the identification of only one sugar acyltransferase gene. Genes are organized in at least ten transcriptional units that, in most cases, include genes with no related roles in chromomycin biosynthesis (i.e., PKS genes together with sugar biosynthesis genes). It must be noted that genes involved in polyketide biosynthesis are scattered throughout the cluster. Particularly, it is noticeable that *cmmS* (ACP), *cmmY* (cyclase), and *cmmQ* (aromatase) are unusually located when compared to other polyketide clusters. A similar situation has been described for the medermycin cluster [24]. This genetic organization implies that the expression of the different genes involved in the biosynthesis of the chromomycin aglycon will need to be tightly coordinated.

It is predicted that the biosynthesis of the chromomycin aglycon would follow similar steps as that for mithramycin, since both compounds share the same aglycon structure (Figures 1A and 4). In the mithramycin pathway, the first stable compound is the 4-demethyl-premithramycinone, which is then *O*-methylated at the 4-position to generate premithramycinone [13]. Several genes could have a role in the biosynthesis of premithramycinone in the chromomycin A_3 pathway. The minimal PKS encoded by the *cmmP*, *cmmK*, and *cmmS* genes would synthesize the 20-carbon polyketide backbone that then would be folded and cyclized with the participation of the aromatase CmmQ, the cyclases CmmX and CmmY, and probably the reductase CmmTI. In addition, formation of 4-demethyl-premithramycinone would require the introduction of two oxygen atoms in the 4

Table 2. Antitumor Activity Tests of Chromomycin Derivatives Generated in This Work

Tumor Cell Lines		Log (GI_{50})[a]				
		Mithramycin	Chromomycin A_3	Chromomycin SA	Chromomycin SK	Chromomycin SDK
Prostate	DU-145	−7.77	−8.70	−7.72	−8.70	−7.93
	LN-caP	−8.82	−9.60	−7.84	−7.91	−7.84
Ovarian	IGROV	−7.72	−8.70	−7.70	−7.95	−7.84
Breast	SK-BR-3	−7.52	−8.40	−7.45	−7.78	−7.62
Melanoma	SK-MEL-28	−7.84	−8.71	−7.79	−7.96	−8.02
NSCL	A549	−7.40	−7.85	−7.39	−7.71	−7.77
Pancreas	PANC-1	−7.74	−8.68	−7.66	−7.94	−7.85
Colon	HT-29	−7.51	−8.33	−7.12	−7.67	−7.68
	LoVo	−7.82	−8.46	−7.69	−6.51	−7.79
Cervix	HELA	−8.05	−8.76	−7.79	−8.00	−7.92

For comparison, values for chromomycin A_3 and mithramycin are also shown.
[a] GI_{50}, 50% growth inhibition.

and 12a position, probably catalyzed by the oxygenase CmmOII. Methylation of 4-demethyl-premithramycinone catalyzed by the CmmMI methyltransferase would lead to the synthesis of premithramycinone.

Formation of premithramycinone would be followed by several glycosylation steps as well as the *C*-methylation at the 9 position. Four glycosyltransferases have been identified in the cluster. CmmGIV and CmmGIII would most probably catalyze the transfer of the first and second D-olivose units that form part of the trisaccharide chain attached at the 12a position of premithramycinone. CmmGII and probably also CmmGI would be involved in the formation of the disaccharide chain attached at 8 position. Transfer of the third deoxysugar of the trisaccharide could be carried out by any of these four glycosyltransferases, most likely by CmmGIV or, alternatively, by a not yet identified GT outside of the cluster. The same situation is found in the mithramycin cluster, in which only four glycosyltransferase genes have been identified for the transfer of five sugars, and there was no candidate for transferring the third sugar of the trisaccharide chain [21, 22]. However, indirect evidence suggests that MtmGIV is responsible for the transfer of both the first and the third sugar of the trisaccharide chain [25]. Thus, CmmGIV is also the most probable candidate to catalyze this glycosylation step. The CmmMII methyltransferase would catalyze a *C*-methylation that most probably would occur once the trisaccharide chain has been formed, as it happens during mithramycin biosynthesis [13]. By analogy with the mithramycin biosynthesis, one of the final steps in chromomycin A_3 biosynthesis will be the oxidative cleavage of the fourth ring of a fully glycosylated tetracyclic intermediate along with a decarboxylation followed by the ketoreduction of the thereby formed pentyl side chain. The result of these biosynthetic key steps is the formation of a fully glycosylated molecule with a tricyclic aglycon at whose 3 position the important highly functionalized pentyl side chain is attached. Candidates for catalyzing these reactions are the oxygenase CmmOIV and the ketoreductase CmmWI.

Chromomycin A_3 contains three unusual deoxysugar units: 4-*O*-acetyl-D-oliose, 4-*O*-methyl-D-oliose, and 4-*O*-acetyl-L-chromose. The *cmm* cluster contains all genes needed for the biosynthesis of these sugars (Figure 5). The first two enzymatic steps would be the activation of glucose-1-phosphate to NDP-d-glucose by the action of CmmD (NDP-glucose synthase) and further conversion to NDP-4-keto-6-deoxy-glucose by CmmE (NDP-glucose 4,6-dehydratase). Next, the C-2 deoxygenation of this last intermediate would occur, requiring a 2,3-dehydration and a 3-reduction step catalyzed by CmmV and CmmW, respectively. Accordingly, the reaction product of CmmW would be NDP-4-keto-2,6-dideoxy-d-glucose, which might be the last common intermediate in the biosynthesis of all chromomycin deoxyhexoses. Reduction of this intermediate at C-4 will produce NDP-D-olivose or NDP-D-oliose depending on the stereocontrol enforced by the involved ketoreductase. The 4-ketoreductases CmmUI and CmmUII are most probably involved in these processes. From NDP-4-keto-2,6-dideoxyglucose, the biosynthesis of L-olivomycose would require a C-3 methylation (catalyzed by CmmC), 5- or 3,5-epimerization (catalyzed by CmmF), and a 4-ketoreduction step (catalyzed by CmmUIII). The CmmC-catalyzed C-3 methylation would take place on a D-deoxyhexose with overall inversion of configuration at C-3, resulting in a methyl group with equatorial configuration. Epimerization at C-5 would occur next and most probably would be carried out by CmmF. Alternatively, the *C*-methylation could occur with retention of the 3-configuration (i.e., axial methyl group), and the epimerase would be a 3,5-epimerase. Once the different deoxyhexoses were transferred to the polyketide backbone or growing oligosaccharide chains, 4-*O*-methylation of sugar B and 4-*O*-acetylations of sugars A and E should occur to generate 4-*O*-methyl-D-oliose, 4-*O*-acetyl-D-oliose, and 4-*O*-acetyl-L-chromose, respectively. The CmmMIII methyltransferase would catalyze the *O*-methylation from D-oliose to 4-*O*-methyl-D-oliose, and the acetylations of sugars A and E would probably be catalyzed by the CmmA acyltransferase.

The *cmm* cluster also contains an ABC transporter system (*cmrAB*) and a UV-repair system (*cmrX*) that can contribute to self-resistance to chromomycin A_3. The involvement of these genes in chromomycin resistance was demonstrated by expressing a cosmid containing these genes in the chromomycin-sensitive strain *S. albus* that afterwards became chromomycin resistant. Although similar types of genes were also found in the mithramycin pathway, they do not protect *S. argillaceus* from chromomycin, suggesting a great specificity of these transporters [26].

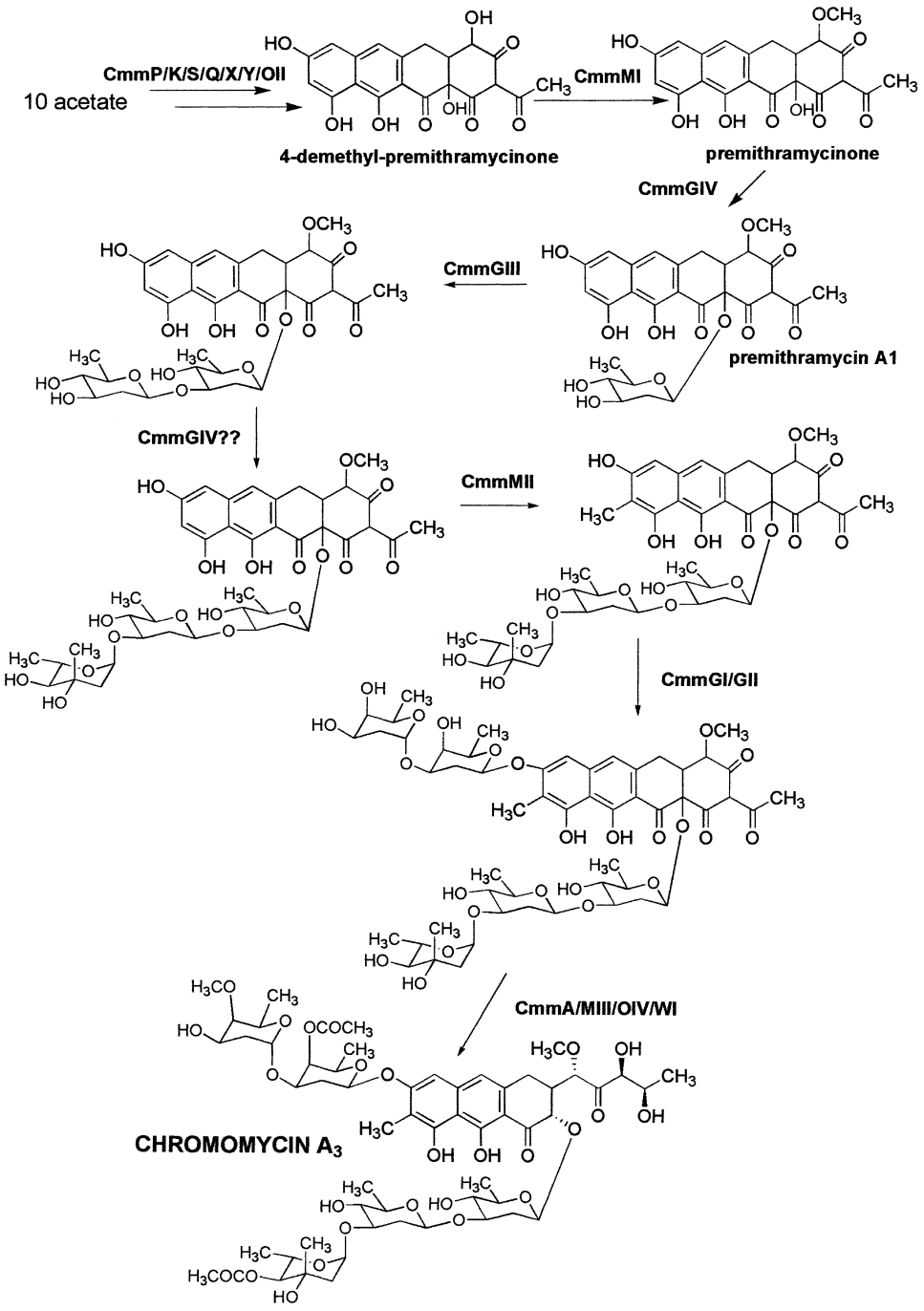

Figure 4. Proposed Pathway for the Biosynthesis of Chromomycin A_3

In many cases, genetic organization of biosynthetic clusters for structurally related antibiotics is quite similar. In this context, the striking correspondence in the organization of the aminocoumarin gene clusters is noticeable: the biosynthetic genes for each structural moiety of the aminocoumarin antibiotic are grouped together, and the order of the genes within each group is identical [27]. However, in the case of chromomycin A_3, an antibiotic closely related to mithramycin in its chemical structure, this similarity is not reflected at the level of the biosynthetic genes. Both gene clusters show very different genetic organization. This is especially noticeable in relation to genes involved in the biosynthesis of the aglycon. Thus, in the case of the mithramycin cluster, the genes required for the formation of 4-demethyl-premithramycinone are all grouped in the central part of the cluster, while in the chromomycin A_3 cluster these genes are scattered throughout the cluster. However, some other groups of genes, such as resistance genes, are organized in a similar way in both clusters. It has

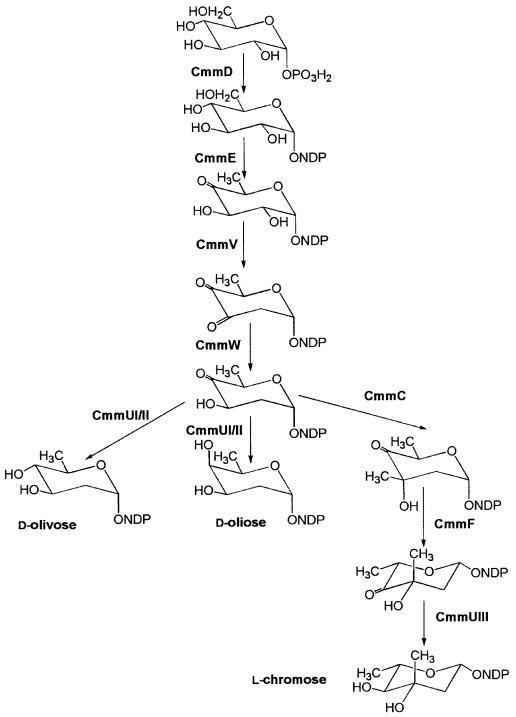

Figure 5. Proposed Pathways for the Biosynthesis of the Deoxysugars in Chromomycin A_3

been suggested that aromatic polyketide clusters could be transferred horizontally between different *Streptomyces* species, and thus very similar antibiotic clusters can be found in distantly related species [28]. In the case of the mithramycin and chromomycin clusters, if this transfer occurred, there were probably gene rearrangements through evolution that could be responsible for the differences in gene organization between these two clusters. On the other hand, we could find and putatively assign all genes found in the chromomycin pathway with the corresponding genes of the mithramycin pathway. Apparently, the enzymes performing the biosynthesis of such biosynthetically closely related molecules have to arrange themselves in a similar way so that the passing of the same or very similar intermediates can be achieved in the most efficient way, although the gene arrangement of the two pathways is totally different. Thus, one could suggest that the gene arrangement does not influence the arrangement of the proteins at all, and the organization of the biosynthetic enzymes seems to be a self-driven, autocatalytic process.

The involvement of the isolated cluster in chromomycin biosynthesis was unequivocally proven by two different approaches. First, it was proved that the cluster contained a chromomycin-resistance determinant. This is a strong indication that the cluster is actually involved in chromomycin biosynthesis, since in most antibiotic biosynthetic gene clusters resistance determinants are linked to biosynthetic genes. A further definite proof was obtained through the generation of a nonproducer mutant by specifically inactivating the *cmmWI* gene. We have recently shown that by inactivating an equivalent gene in the mithramycin cluster, active mithramycin derivatives were generated that differed in the length and structure of their side chains [12]. We reasoned that by inactivating *cmmWI* we could first prove the involvement of this gene in the biosynthesis of chromomycin A_3, and at the same time we could generate novel chromomycin derivatives with promising biological activity. This hypothesis turned out to be correct, since the C60WI mutant lost the capability of producing chromomycin A_3 and the three novel compounds showed interesting antitumor activity. They consisted of fully glycosylated chromomycins only differing from the parent compound in the structure and length of their side chains, as was anticipated. These results also confirm that CmmWI is the ketoreductase acting on the side chain of the aglycon.

Significance

The biosynthetic gene cluster of the antitumor antibiotic chromomycin A_3 from *Streptomyces griseus* subsp. *griseus* shows a different genetic organization to that of the closely structurally related mithramycin. Inactivation of a ketoreductase gene *cmmWI* involved in side-chain reduction has been proven to be an appropriate target to generate three novel and active chromomycin A_3 derivatives. Knowledge of the chromomycin A_3 cluster has allowed the identification of the biosynthetic pathways for novel deoxysugars, especially for 4-*O*-acetyl-*L*-chromose. In this context, several tailoring genes (acetylation, methylation) for sugar modification have been identified that could be useful genetical/biochemical tools for combinatorial biosynthesis, to generate novel derivatives from bioactive compounds.

Experimental Procedures

Microorganisms, Culture Conditions, and Plasmids

Streptomyces griseus subsp. *griseus* ATCC 13273, chromomycin A_3 producer, was used as donor of chromosomal DNA. For sporulation on solid medium, it was grown at 30°C on plates containing A medium [22]. For growth in liquid medium, the organism was grown either on TSB medium (trypticase soya broth, Oxoid) or in R5A medium [22]. *Streptomyces albus* J1074 (*ilv-1*, *sal-2*) [29] was used as host for expression of chromomycin genes. *Escherichia coli* ED8767 [30] was used to construct the gene library, and *E. coli* DH10B (Invitrogene) was used as host for subcloning. *E. coli* ET12567 (pUB307) [29] was used as donor for intergeneric conjugation. When plasmids containing clones were grown, the medium was supplemented with the appropriate antibiotics as follows: 5 or 25 μg/ml thiostrepton for liquid or solid cultures, respectively, 100 μg/ml ampicillin, 25 μg/ml apramycin, 20 μg/ml tobramycin, 25 μg/ml kanamycin, or 25 μg/ml chloramphenicol. The bifunctional (*E. coli-Streptomyces*) cosmid pKC505 [29] was used to construct the *S. griseus* subsp. *griseus* DNA library. pUC18, pHZ1358 [29], pUK21 [31], and pIJ2925 [29] were used for subcloning.

DNA Manipulation

Plasmid DNA preparations, restriction endonuclease digestions, alkaline phosphatase treatments, DNA ligations, Southern hybridization, and other DNA manipulations were performed according to standard techniques for *Escherichia coli* [30] and *Streptomyces* [29]. Preparation of *S. albus* protoplasts, transformatio, and selection of transformants were carried out as described [29]. Intergeneric conjugation from *E. coli* ET12567 (pUB307) to *S. griseus* subsp. *griseus* was performed as described [29].

Construction and Analysis of the Cosmid Library

A genomic library of *S. griseus* subsp. *griseus* total DNA was constructed in cosmid pKC505. The library was analyzed by in situ colony hybridization with two types of probes, a PKS and an ABC probe. The PKS probe consisted of a 1 kb BamHI fragment containing PKS genes (the 3′ end of *mtmP* and the 5′ end of *mtmK* of the mithramycin gene cluster) [8]. The ABC probe was generated by PCR amplification using two degenerate oligonucleotides specially designed to amplify ATP binding domains of ABC transporters in antibiotic producer microorganisms [6]. The synthetic olinonucleotides used were primer WA (5′-GATCGAATTCGGG(C)G(C)A(C)CA ACGGCGCG(C)GGCAAG-3′) and primer WB (5′- GATCAAGCTTGA GGC(T)CGG(T)TGGTG(C)GGC(T)TCGTC-3′) (EcoRI and HindIII sites in the respective primers are underlined).

DNA Sequencing

Sequencing was performed on double-stranded templates derived from different clones in pUC18 and by using the dideoxynucleotide chain termination method and the Cy5 AutoCycle Sequencing Kit (Amersham Pharmacia Biotech). Both DNA strands were sequenced with primers supplied in the kits or with internal oligoprimers (17-mer) using an ALF-express automatic DNA sequencer (Amersham Pharmacia Biotech). Computer-aided database searching and sequence analysis were carried out using the University of Wisconsin Genetics Computer Group program package (UWGCG) and the BLAST program.

Generation of Mutant C60W1

For the generation of mutant C60W1, plasmid pC7WI was constructed. A 9.6 kb PstI fragment containing *cmmMI*, *cmmGI*, *cmmOIV*, *cmmWI*, *cmmLI*, *cmrB*, *cmrA*, *cmmRII*, and the 5′ end of *cmrX* was subcloned into the PstI site of pUK21, generating pKW1. Then, an apramycin-resistance cassette was inserted as a blunt-ended HindIII-BamHI fragment into the unique ScaI site located within *cmmWI*, generating pKW1A. The insert from this plasmid was rescued as a HindIII-XbaI (using these sites from the polylinker) fragment and subcloned into the same sites of pIJ2925. Finally, the resultant construct was digested with BglII, and the insert flanked by two BglII sites was subcloned into the BamHI site of pHZ1358, generating pC7W1. In this construct, the apramycin-resistance gene is transcribed in the same direction as *cmmWI*. pC7W1 was introduced by intergeneric conjugation into *S. griseus*, and apramycin-resistant, thiostrepton-sensitive transconjugants were selected for further characterization.

Determination of Minimal Inhibitory Concentrations

Susceptibility to chromomycin A_3 was tested by determining the minimal inhibitory concentration (MIC). This was carried out by replica plating the strains on medium A agar plates containing different concentrations of chromomycin A_3.

Detection and Purification of Compounds

One hundred plates of R5A solid medium were inoculated with spores of *S. griseus* C60WI and incubated for 8 days at 28°C. Agar cultures were removed from the plates, placed in three 2 liter Erlenmeyer flasks, covered with ethyl acetate, and extracted for 3 hr at 30°C and 150 rpm. The organic extracts were evaporated in vacuo, and the extraction procedure was repeated twice. The pooled extracts were redissolved in 6 ml of a mixture of DMSO and methanol (50:50) and chromatographed in a μBondapak C18 radial compression cartridge (PrepPak Cartridge, 25 × 100 mm, Waters). A mixture of acetonitrile and 0.1% trifluoroacetic acid in water (55:45) at 10 ml/min was used for elution in isocratic conditions. Peaks corresponding to chromomycin SA, SK, and SDK were collected on 0.1 M potassium phosphate buffer (pH 7.0). Each of these resulting solutions were diluted 4-fold with water, applied to a solid-phase extraction column (Sep-Pak Vac C18, Waters), washed with water to eliminate salts, and the retained material was eluted with methanol. Chromomycins SA and SK were repurified as above, except that the acetonitrile in the mobile phase was reduced to 50%. Final purification for chromomycin SK and SDK was achieved through chromatography on Sephadex LH 20 (MeOH, column 100 × 2.5 cm). The purified compounds were finally redissolved in a small volume of tert-butanol and lyophilized.

Physicochemical Properties of the New Compounds

General

The optical rotations were measured using a Perkin-Elmer 241 polarimeter, and the fast atom bombardment (FAB) and electrospray ionization mass spectra (ESI MS) were acquired with a Finnigan MAT LCQ mass spectrometer. UV spectra were recorded on a Varian CARY50 spectrophotometer, and the IR spectra were obtained from pure samples pressed in KBr disks using a Bio-Rad FTS3000MX FT IR. All NMR data were recorded on a Varian Inova 400 instrument at B_0 9.4 T.

Chromomycin SA

$[\alpha]^{23}_D$ (c = 0.0052, MeOH): + 12°. MS: FAB (−ve) *m/z* 1123; FAB (+ve) *m/z* 1147 (M^++Na); Ion Spec HR ESI calcd. for $C_{54}H_{75}O_{25}$ 1123.4603, found 1123.4592. IR ν_{max} (KBr): 3426 (OH), 2920 (CH), 1690 (C = O), 1650, 1630, 1450 (C = C), 1430, 1170, 1105, and 1051 cm^{1-}. UV λ_{max}^{MeOH} (ϵ): 430 (5900), 317 (6100), and 281 (32300) nm. NMR data: see Supplemental Data available with this article online.

Chromomycin SK

$[\alpha]^{23}_D$ (c= 0.0026, MeOH): + 42°. MS: FAB (−ve) *m/z* 1151; FAB (+ve) *m/z* 1174 (M^++Na); Ion Spec HR ESI calcd. for $C_{56}H_{79}O_{25}$ 1151.4916, found 1151.5063. IR ν_{max} (KBr): 3422 (OH), 2930 (CH), 1734, 1700sh (C = O), 1630, 1447 (C = C), 1373, 1169, 1121, and 1044 cm^{1-}. UV λ_{max}^{MeOH} (ϵ): 422 (10700), 316 (11500), and 285 (39900) nm. NMR data: see Supplemental Data available with this article online.

Chromomycin SDK

$[\alpha]^{23}_D$ (c = 0.0028, MeOH): + 48°. MS: FAB (−ve) *m/z* 1149; FAB (+ve): *m/z* 1172 (M^++Na); Ion Spec HR ESI calcd. for $C_{56}H_{77}O_{25}$ 1149.4759, found 1149.4783. IR ν_{max} (KBr): 3428 (OH), 2930 (CH), 1740, 1710 (C = O), 1632, 1447 (C = C), 1374, 1170, 1121, and 1071 cm^{1-}. UV λ_{max}^{MeOH} (ϵ): 420 (64000), 317 (7900), and 284 (32200) nm. NMR data: see Supplemental Data available with this article online.

Antitumor Tests

The antitumor activity of the compounds was tested against a variety of tumor cell lines. Quantitative measurement of cell growth and viability was carried out by using a colorimetric type of assay using sulforhodamine reaction [32].

Supplemental Data

Two tables showing ^{1}H-NMR data of compounds chromomycins SA (**2**), SK (**3**), and SDK (**4**) in comparison with chromomycin A_3 (**1**) at 400 MHz in d_5-pyridine and ^{13}C-NMR data of chromomycins SA (**2**), SK (**3**), and SDK (**4**) in comparison with chromomycin A_3 (**1**) at 100.6 MHz δ in ppm are available online at http://www.chembiol.com/cgi/content/full/11/1/21/DC1.

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Accession Numbers

The nucleotide sequence of the chromomycin gene cluster has been deposited at the EMBL Nucleotide Sequence Database under the accession number AJ578458.