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Novel Hybrid Tetracenomycins through Combinatorial Biosynthesis Using a Glycosyltransferase Encoded by the *elm* Genes in Cosmid 16F4 and Which Shows a Broad Sugar Substrate Specificity

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Abstract: Cosmid 16F4 contains 25 kb of the elloramycin biosynthetic pathway of *Streptomyces olivaceus* Tü2353. Transformation of this cosmid into a polyketide synthase (PKS)-deleted mutant of the urdamycin producer, *Streptomyces fradiae* Tü2717/ΔPKS and into the mithramycin producer *Streptomyces argillaceus* ATCC 12956 resulted in the production of several novel glycosylated tetracenomycins. Four of the structures of these elloramycin analogues (**3**, **5**−**7**) were elucidated. They carry various deoxysugar moieties (D-olivose, L-rhodinose, D-mycarose, and a disaccharide consisting of two 1,3-linked D-olivoses) attached at C-8-O of the same aglycon, 8-demethyltetracenomycin C (**4**)**.** The transfer of the sugars is not catalyzed by glycosyltransferases of the *S. fradiae* or *S. argillaceus* strains since the novel hybrid tetracenomycins are also produced by a *S. argillaceus* mutant carrying cosmid 16F4 but lacking all the known mithramycin glycosyltransferases. Furthermore, a *Streptomyces lividans* strain containing cosmid 16F4 produced the novel tetracenomycins only when a second plasmid containing the cloned mithramycin sugar biosynthetic genes but lacking glycosyltransferase genes was also present. The glycosyl transfer therefore must be catalyzed by an elloramycin glycosyltransferase encoded by cosmid 16F4**.** Apparently, this glycosyltransferase is able to catalyze the glycosylation of 8-demethyltetracenomycin C (**4**, = 12a-demethylelloramycinone) using various D- and L-sugars including a disaccharide. Its future use for combinatorial biosynthetic approaches is discussed.

Introduction

Combinatorial biosynthesis is a novel strategy to produce new "unnatural" or hybrid natural products through targeted modifications or recombinations of selected biosynthetic genes.[1−7] Most of the research conducted has been on polyketides, and several novel compounds have already been produced. While the target genes of such approaches are usually those encoding the polyketide synthase, our attempts focus on genes coding for post-polyketide modifications, that is, for enzymes such as oxidoreductases and group transferases, since they can cause a dramatic change of the chemical structures as well as the biological activities of parent natural products.[7−17]

Deoxysugar moieties and oligosaccharides derived from them are more and more recognized as important structural elements contributing to the general mechanism of action of bioactive drugs, for example, antibiotics and anticancer agents such as anthracyclines, angucyclines, aureolic acid antibiotics, avermectins, enediynes, macrolides, and pluramycins.[18−20] Thus,

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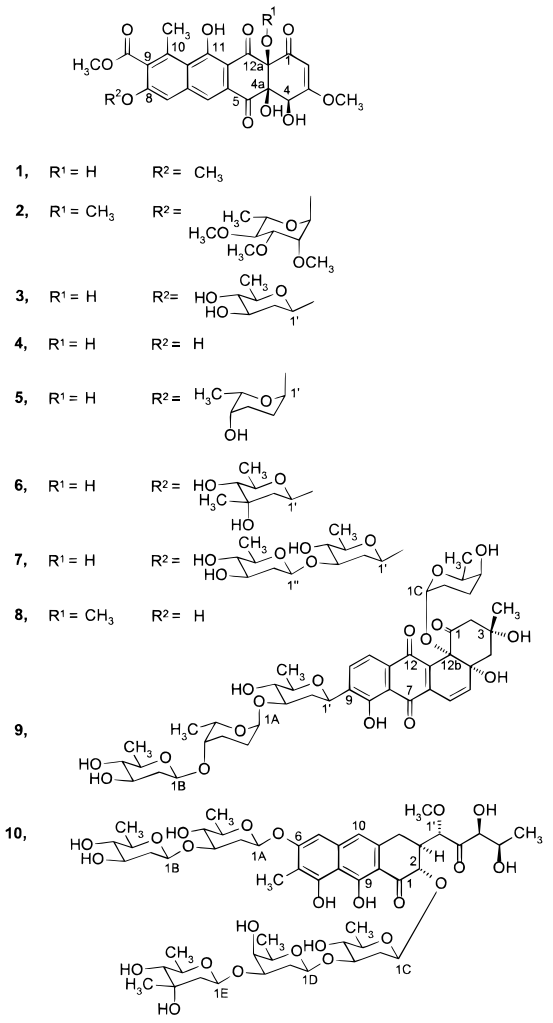

Figure 1. Structures of tetracenomycin C (**1**), elloramycin (**2**), 8-demethyl-8-β-D-olivosyl-olivosyltetracenomycin C (**3**), 8-demethyltetracenomycin C (**4**), 8-demethyl-8-α-L-rhodinosyltetracenomycin C (**5**), 8-demethyl-8-β-D-mycarosyltetracenomycin C (**6**), 8-demethyl-8-β-D-olivosyl-3′-1″−D-β-olivosyltetracenomycin C (**7**), elloramycinone (**8**), urdamycin A (**9**), and mithramycin (**10**).

it will be important to carry out research on the possibilities to add such deoxysugar moieties to various aglyca, and enzymatic glycosyl transfer steps are crucial for that purpose.

Recently, we were able to produce novel tetracenomycins through combinations of the tetracenomycin (*tcm*, tetracenomycin C, **1**) and elloramycin (*elm*, elloramycin A, **2**) gene clusters using the urdamycin (*urd*, urdamycin A, **9**) (see Figure 1) producer *Streptomyces fradiae* Tü2717 as the host organism.[10] Most interesting is 8-demethyl-8-β-D-olivosyltetracenomycin C (olivosyltetracenomycin C, **3**) resulting from the transformation of cosmid 16F4 into the urdamycin producer, since this hybrid molecule consists of 8-demethyltetracenomycin C (**4**), deriving from the elloramycin (**2**) biosynthesis and a D-olivose moiety from the urdamycin (**9**) biosynthesis which is connected at the 8-position. Cosmid 16F4 contains a 25-kb insert of the *elm* gene cluster, including the PKS, all oxygenases, and most of the methyltransferase-encoding genes from the elloramycin producer *Streptomyces olivaceus* Tü2353. Although it has not been fully characterized, the earlier and the current experiments indicate that all genes controlling the biosynthetic formation of 8-demethyltetracenomycin C (**4**) are present on this cosmid, while the genes encoding the biosynthesis of the permethyl-L-rhamnose moiety of elloramycin and the 12a-*O*-methyltransferase are completely or partially missing.[10,21] Here we present experimental evidence for the existence of a glycosyltransferase in cosmid 16F4 which shows a broad sugar substrate specificity.

Results

One important ambiguity regarding the formation of olivosyltetracenomycin C (**3**)[10] could not be solved with the previous experiments, namely, whether a putative *elm* glycosyltransferase (GT) encoded in cosmid 16F4 or one of the *urd* GTs from the *S. fradiae* host is responsible for the linkage of an NDP (= nucleosyl diphosphate) activated D-olivose to 8-demethyltetracenomycin C (**4**). We addressed this question by a series of experiments. *S. fradiae* Tü2717/ΔPKS is a mutant in which the *urdF*−*C* genes encoding the PKS and a cyclase were deleted.[22] When we transformed this mutant with cosmid 16F4, we expected an increased production yield for olivosyltetracenomycin C and the production of the other possible hybrid glycoside, L-rhodinosyltetracenomycin C (**5**), since besides D-olivose, L-rhodinose is the other deoxysugar moiety of urdamycin A (**9**), the principal product of *S. fradiae* Tü2717. Indeed, the production of olivosyltetracenomycin (**3**) was increased 5- to 8-fold, at a production rate of between 50 and 80 mg/L. In addition, 8-demethyl-8-α-L-rhodinosyltetracenomycin C (**5**) could be isolated in yields ranging between 5 and 10 mg/L. The structure of **5** was elucidated with the help of the negative ion FAB-MS (*m*/*z* 571, 100%, M − H$^-$) and various NMR data (see Tables 1, 2, and Figure 2) indicating the L-rhodinose moiety besides the structural elements of the aglycon. The position of the sugar moiety was not directly deducible from $^3J_{C-H}$ couplings (HMBC, Figure 2), but the H,H-NOESY NMR spectrum showed a significant NOE effect between 7-H and 1′-H. The α-glycosidic linkage follows from the coupling pattern of the 1′- and 2′-H atoms (^{1}H NMR spectrum).

Similarly, we transformed the mithramycin producer *Streptomyces argillaceus* ATCC 12956 with cosmid 16F4. Mithramycin (**10**) contains three different deoxysugar moieties, D-olivose, D-oliose, and D-mycarose.[23] In an analogy to the experiments with the urdamycin producer described above, we additionally used a mutant of *S. argillaceus* as a host in which part of the β-ketoacylsynthase (*mtmP*) and of the chain length factor (*mtmK*) genes of the PKS were deleted (PK$^-$ mutant).[24] These experiments indeed resulted in the production of 8-demethyltetracenomycin C (**4**) and moreover in three novel hybrid antibiotics, 8-demethyl-8-β-D-olivosyltetracenomycin C (**3**), 8-demethyl-8-β-D-mycarosyl-tetracenomycin C (**6**), and 8-demethyl-8-β-D-olivo-3′-1″-β-D-olivosyltetracenomycin C (**7**). Using the *S. argillaceus* wild-type strain as a host gave yields

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Table 1. ^{13}C NMR Data of **3**, **4**, **5**, **6** and **7** in Methanol-d_4[a]

	3	**4**	**5**	**6**	**7**[b]
1	193.0, s	193.5, s	193.0, s	193.8, s	192.9, s
2	100.7, d	100.7, d	100.8, d	100.7, d	100.8, d
3	176.0, s	176.1, s	175.6, s	176.0, s	175.8, s
3-OMe	57.6, q	57.5, q	57.6, q	53.0, q	57.5, q
4	70.7, d	70.7, d	70.7, d	70.7, d	70.7, d
4a	85.8,[d] s	84.9,[c] s	84.5,[d] s	84.5,[d] s	80.6,[c] s
5	193.1,[e] s	195.0, s	194.0,[e] s	194.8,[e] s	196.7,[d] s
5a	141.4, s	142.0, s	141.5, s	141.6, s	141.4, s
6	122.0, d	120.6, d	121.6, d	121.8, d	121.9, d
6a	129.3, s	129.3, s	121.0, s	122.5, s	129.4, s
7	112.4, d	112.4, d	112.2, d	112.3, d	112.1, d
8	156.1, s	158.9, s	155.6, s	156.5, s	156.0, s
9	130.6, s	130.4, s	130.9, s	129.6,[c] s	130.5, s
9-C=O	169.3, s	168.7, s	169.7, s	169.4, s	167.9, s
9-OMe	53.1, q	53.0, q	53.0, q	57.6, q	53.1, q
10	139.1, s	139.6, s	139.0, s	139.1, s	139.5, s
10-CH_3	21.1, q	21.2, q	21.1, q	139.1, q	21.1, q
10a	122.3, s	121.0, s	129.5, s	130.7,[c] s	122.3, s
11	167.5, s	170.3, s	168.0, s	167.8, s	168.7, s
11a	110.5, s	109.4, s	110.5, s	110.0, s	110.4, s
12	194.8,[e] s	197.7, s	196.0,[e] s	198.3,[e] s	198.4,[d] s
12a	84.5,[d] s	84.5,[c] s	84.8,[d] s	84.5,[d] s	84.6,[c] s
1′	98.1, d		97.0, d	97.9, d	97.7, d
2′	39.9, t		24.4, t	44.3, d	37.6, t
3′	71.6, d		26.4, t	71.7, s	80.6, d
3′-CH_3				27.1, q	
4′	78.1, d		67.3, d	77.6, d	76.2, d
5′	73.7, d		69.3, d	72.3, d	73.5, d
6′	18.3, q		17.4, q	18.6, q	18.1, q

[a] δ in ppm relative to internal TMS, 75.5 MHz, multiplicities through APT. [b] Signals of the second sugar of **7**: 100.0 (d, C-1″), 40.6 (t, C-2″), 72.1 (d, 3″), 78.2 (d, C-4″), 73.6 (d, C-5″), 18.4 (C-6″). [c] Assignments interchangeable.

higher than using its PK$^-$ mutant. The heretofore unknown compounds **6** and **7** were partially hidden (TLC, HPLC) under **4** and **3**, respectively, but could be separated from these known molecules by chromatography on Sephadex LH20.

Mycarosyl-tetracenomycin C (**6**) was identified from its mass spectrum (negative ion FAB-MS, *m/z* 601, 100%, M $-$ H$^-$, corresponding to $C_{29}H_{30}O_{14}$) and the $^1H-$ as well as ^{13}C NMR data (Tables 1 and 2) indicating the D-mycarose moiety in addition to the typical structural elements of the aglycon. The β-glycosidic linkage follows from the coupling pattern of the 1′- and 2′-H atoms (^1H NMR spectrum). The structure of the diolivosyltetracenomycin C (**7**) followed from the negative FAB mass spectrum (*m/z* 717, M $-$ H$^-$, corresponding to $C_{34}H_{38}O_{17}$), the UV data, and the analyses of various NMR data (Tables 1 and 2), indicating the aglycon as well as two olivose moieties.

It was not possible to deduce the position of the sugar moieties of **6** and **7** directly from $^3J_{C-H}$ couplings or a NOESY experiment, but the NMR data compared to those of **4** showed significant indirect evidence for an 8-O-linkage. In addition, the alkaline UV spectra of 8-demethyltetracenomycin C (**4**) as well as that of elloramycinone (**8**)[25,26] show a significant maximum at λ 315 nm which is caused by the free phenolic 8-hydroxy group. This UV maximum is lacking in all 8-*O*-methyl or 8-*O*-deoxysugar compounds such as **1** to **3** and **5** to **7**. The interglycosidic 1″,3′-β-linkage between both olivose moieties in **7** was deduced from the signal pattern of 1″-H in the ^1H NMR spectrum and the significant downfield shift observed for C-3′ in the ^{13}C NMR spectrum (Tables 1 and 2). This downfield shift is not observed for C-3′ in compound **3** in which this position is not further linked. The unambiguous assignments of all protons and carbons within the aglycon as well as the single sugar moieties were confirmed through H,H-COSY and HMBC experiments (Figures 3 and 4).

We conclude that the combination of gene sets from the elloramycin/urdamycin and elloramycin/mithramycin clusters can generate novel tetracenomycins with different patterns of glycosylation either presenting D-sugars (D-olivose or D-mycarose), L-sugars (L-rhodinose), or disaccharides (D-olivosyl-1,3-D-olivose). However, it cannot be decided from these experiments whether the sugar transfer is carried out by a sugar-specific GT present in the respective hosts (*S. fradiae* or *S. argillaceus*) or by a sugar-flexible GT present in cosmid 16F4.

To answer this question, we carried out two new experiments. (a) Since 8-demethyltetracenomycin C (**4**) is the most obvious substrate for the GT, we fed 12 mg of **4** to *S. fradiae* Tü2717, *S. fradiae* Tü2717/ΔPKS, and *S. argillaceus* cultures and after incubation analyzed for the presence of glycosylated tetracenomycins. In no case (control through TLC and HPLC) could any of the glycosylated products be observed.

(b) Plasmid pWHM1026 contains the entire tetracenomycin C gene cluster and also produces 8-demethyltetracenomycin C (**4**).[27] If not **4** itself, one of its biosynthetic predecessors serves as a substrate for the glycosylation; this unknown intermediate is most likely also produced by the pWHM1026 gene products. However, we had already observed earlier that *S. fradiae* (pWHM1026) did not yield any glycosylated hybrid tetracenomycins.[10] Thus *S. argillaceus* was also transformed with plasmid pWHM1026. However, the resulting recombinant strain *S. argillaceus* (pWHM1026) also did not produce any of the hybrid tetracenomycins described above. Since pWHM1026 still bears the genes for the 8-*O*-methyltransferase which might have interfered with a possible glycosyl transfer step, a control experiment using plasmid pWHM1026Δ*tcmO* was carried out. In this particular plasmid, the *tcmO* gene encoding the 8-*O*-methyltransferase was deleted.[28] The resulting recombinant strain *S. argillaceus* (pWHM1026Δ*tcmO*) produced 8-demethyltetracenomycin, but also did not produce any of the glycosylated tetracenomycins.

These experiments strongly suggest that the GT responsible for the sugar transfer is not encoded by the genome of the host strains used for the transformation experiments (*S. fradiae* or *S. argillaceus*) but rather is encoded by the cosmid 16F4 DNA. To substantiate this hypothesis, two additional experiments were carried out in which we tested the capability of generating glycosylated tetracenomycins by strains in which none of the host's known GT genes were present.

(a) Cosmid 16F4 was introduced into a *S. argillaceus* mutant (M3ΔMG2) in which all the glycosyltransferase genes (and two methyltransferase genes) involved in the mithramycin biosynthesis have been deleted.[29] The resultant recombinant strain produced the same glycosylated products (**3**, **6**, and **7**) as the wild-type strain transformed with cosmid 16F4.

(b) *Streptomyces lividans* TK21 was transformed with cosmid 16F4 and yielded only 8-demethyltetracenomycin C (**4**), as expected.[21] This strain was then transformed with a second plasmid (pFLOS) containing deoxysugar biosynthetic genes from the mithramycin producer *S. argillaceus*, namely *mtmD*, *mtmE*, *mtmU*, *mtmV*, *mtmW*, and *mtmC*, but not any GT-encoding gene.[30] The resulting recombinant strain produced all the glycosylated tetracenomycins (**3**, **6**, and **7**) described above.

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Table 2. ^{1}H NMR Data (300 MHz, CD_3OD) of **3**, **4**, **5**, **6**, and **7**

	3	**4**	**5**	**6**	**7**[b]
2-H	5.59 s	5.60 s	5.60 s	5.60 s	5.60 s
3-OMe	3.80 s	3.81 s	3.80 s	3.80 s	3.80 s
4-H	4.88 s	4.88 s	4.80 s	4.89 s	4.90 s
6-H	7.92 s	7.15 s	7.94 s	7.99 s	7.89 s
7-H	7.78 s	7.78 s	7.60 s	7.54 s	7.41 s
9-OMe	3.92 s	3.93 s	3.92 s	3.93 s	3.92 s
10-H_3	2.77 s	2.78 s	2.84 s	2.81 s	2.77 s
1′-H	5.43 dd (11,1)		5.88 s	5.67 dd (10,2)	5.45 dd (11,1)
2′$_a$-H	1.70 ddd (11,11,10)		1.68 d (15)	1.80 dd (13,10)	1.71 ddd (11,11,10)
2′$_e$-H	2.30 ddd (10,4,1)		2.22 m	2.08 dd (13,2)	2.38 ddd (10,4,1)
3$_a$′-H	3.68 ddd (11,6,4)		1.82 d (15)		3.85 ddd (11,6,4)
3$_e$′-H			2.08 m		
3′-H_3				1.29 s	
4′-H	2.98 dd (7,6)		3.60 s (br)	3.05 d (11)	3.08 dd (7,6)
5′-H	3.56 dd (7,7)		3.88 q (6)	3.95 dd (11, 7)	3.60 dq (7, 6)
6′-H_3	1.32 d (6)		1.05 q (6)	1.30 d (7)	1.32 d (6)

[a] δ in ppm relative to TMS (J in Hz). [b] Signals of the second sugar of **7**: 4.75 (dd, J = 11,1 Hz, 1″-H), 1.56 (ddd, J = 11,11,10 Hz, 2″-H$_a$), 2.18 (ddd, J = 10, 4, 1 Hz, 2″-H$_e$), 3.57 (ddd, J = 11, 6, 4 Hz, 3″-H), 2.95 (dd, J = 7, 6 Hz, 4″-H), 3.37 (dq, J = 7, 6 Hz, 5″-H), 1.30 (d, J = 6 Hz, 6″-H$_3$).

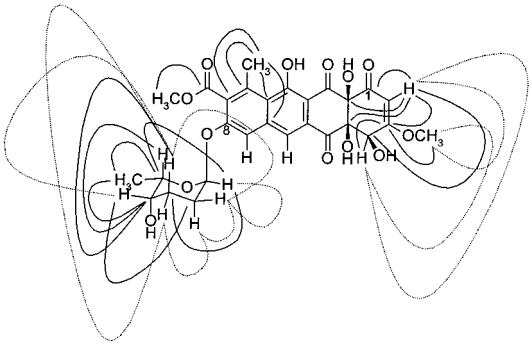

Figure 2. HMBC (solid lines) and H,H-COSY (dotted lines) couplings of 8-demethyl-8-α-L-rhodinosyltetracenomycin C (**5**).

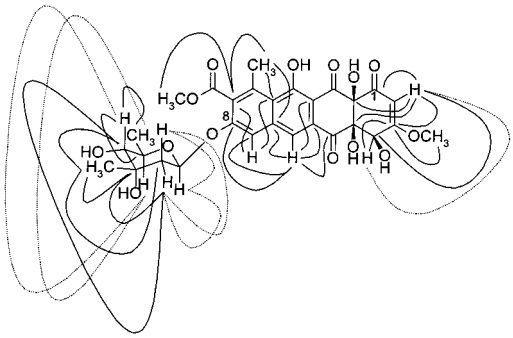

Figure 3. HMBC (solid lines) and H,H-COSY (dotted lines) couplings of 8-demethyl-8-β-D-mycarosyltetracenomycin C (**6**).

These experiments showed that glycosylated tetracenomycins can be generated by combining genes of the elloramycin and mithramycin clusters in the absence of any mithramycin GT.

Discussion

The results obtained strongly support the hypothesis that a GT gene is present in cosmid 16F4 which is responsible for the transfer of the deoxysugars in urdamycin as well as mithramycin to 8-demethyltetracenomycin C (**4**) and that extant *urd* and *mtm* GT genes are not capable of performing these reactions. The 16F4-GT appears to be selective concerning its aglycon substrate and appears to glycosylate only its phenolic 8-OH group, but it shows a remarkably flexible sugar substrate specificity ranging from D-sugars (D-olivose and D-mycarose) to L-sugars (permethyl-L-rhamnose, the natural sugar moiety of

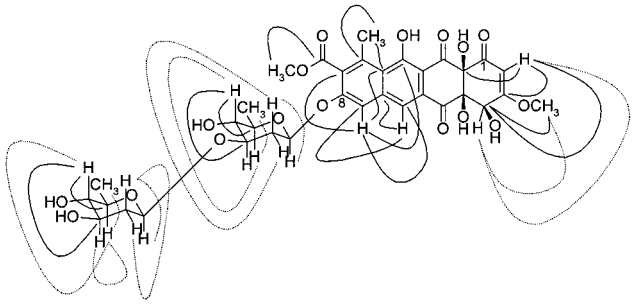

Figure 4. HMBC (solid lines) and H,H-COSY (dotted lines) couplings of 8-demethyl-8-β-D-olivosyl-3′-1″−D-β-olivosyltetracenomycin C (**7**).

elloramycin, and L-rhodinose), and to disaccharides (D-olivosyl-1,3-D-olivose). Therefore, this GT may turn out to be a useful tool for certain combinatorial biosynthetic approaches.

Using the PKS$^-$ mutant *S. fradiae* Tü2717/ΔPKS as a host, the *urd* GTs were no longer able to transfer their deoxysugar moieties to their natural positions since the natural aglycon moiety cannot be biosynthesized by this mutant. Thus, the activated sugars were only directed toward the hybrid tetracenomycin biosyntheses, resulting in increased yields of olivosyltetracenomycin C (**3**) and rhodinosyltetracenomycin C (**5**). The latter was detected only when the PKS$^-$ mutant was used. However, in the case of *S. argillaceus*, the yields of the hybrid tetracenomycins **3**, **6**, and **7** were higher using the wild-type strain than its PK$^-$ mutant. The reasons for this are unclear. It may be that the promotor region of the gene directing deoxysugar biosynthesis is also negatively affected in this mutant.

One of the authors (S. R.) observed that an 8-D-oliosyltetracenomycin C could not be found when cosmid 16F4 was transformed into *S. argillaceus*,[31] despite the fact that D-oliose is one of the deoxysugar moieties (sugar D) of mithramycin (**10**).[23] However, in context with our biosynthetic studies on mithramycin (**10**),[30] we assume that the oliose moiety is formed through a 4-epimerization of a former D-olivose moiety once the second olivose of the final trisaccharide has been transferred. We reported earlier[32] that our studies also gave significant evidence for the formation of a disaccharide consisting of two 1,3-linked D-olivose moieties prior to its linkage at the phenolic OH group of a mithramycin biosynthetic precursor. The

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trisaccharide chain, in contrast, is supposed to be formed by a sequential transfer of single, activated deoxysugar moieties. This may explain why only this diolivosyl-tetracenomycin C (**7**) and no other di- or trisaccharyl analogues were produced in *S. argillaceus* containing cosmid 16F4.

Experiments now in progress are focused on the isolation and characterization of the gene encoding the expected sugar-flexible GT from cosmid 16F4 DNA and its utilization for the transfer of a variety of deoxysugars to amenable aglycons. Recently, by using oligoprimers designed on the basis of well conserved amino acid regions found in various glycosyltransferases, a gene encoding a glycosyltransferase was found within the ca. 9.5 kB *Bam*HI fragment which is located at the right-hand side of cosmid16F4,[21] that is, downstream from the region where the tetracenomycin homologous genes were previously reported. Although the sequencing is still in progress, there were also three methyltransferase genes detected, which are presumably responsible for the methylation of the L-rhamnose moiety of elloramycin (**2**), and only the one mentioned gene encoding a glycosyltransferase.[33]

Initial anticancer activity assays with compounds **3**−**6** showed these compounds to be inactive.[34]

Experimental Section

Bacterial Strains and Plasmids. *S. fradiae* Tü2717 and *S. argillaceus* ATCC 12956 are wild-type strains, producing urdamycin (**9**) and mithramycin (**10**), respectively. *S. fradiae* Tü2717/ΔPKS was generated by deletion of the urdamycin PKS of the wild-type strain *S. fradiae* Tü 2717 as described elsewhere.[22] The *S. argillaceus* PK$^-$ mutant was generated by deleting most of the mithramycin PKS genes.[24] *S. argillaceus* M3ΔMG2 is a non-mithramycin producer mutant in which a DNA region containing two methyltransferase and four GT genes has been replaced by an erythromycin resistance cassette.[29] *S. lividans* TK21 is a non-polyketide producing strain.[35] Cosmid 16F4 contains a 25-kb DNA fragment from the elloramycin producer *S. olivaceus* Tü 2353.[21] Plasmid pWHM1026 contains the complete *tcm* gene cluster from *Streptomyces glaucescens* GLA.O.[21] Plasmid pFLOS contains a set of genes from the mithramycin cluster involved in the biosynthesis of the three D-sugars in mithramycin.[36] Protoplast transformation of the various wild-type and mutant strains was performed essentially as described.[35]

Cultivation and Fermentation. *S. fradiae* Tü2717/ΔPKS(pWHM1026) and *S. fradiae* Tü2717/ΔPKS(16F4) were grown on agar plates (M_2CaO; 21% agar, 10% malt extract, 4% glucose, 4% yeast extract, 1% calcium carbonate) for 6 days at 28 °C. Spores from these agar plates were used to inoculate a soy/glucose production medium (2% soybean meal, 2% glucose, pH 7.2) and grown for 72 h. All *S. argillaceus* strains were grown for production on agar plates (A-medium: 21% agar, 21% MOPS (= 3-(*N*-morpholine)propanesulfonic acid), 5% glucose, 0.5% yeast extract, 0.5% meat extract, 1% casamino acids, pH 7.0) for 120 h at 30 °C. Strains containing cosmid 16F4 were grown in the presence of apramycin (25−50 μg/mL). Strains containing pWHM1026, pWHM1026Δ*tcm*O, and pFLOS were grown in the presence of 25−50 μg/mL thiostrepton.

For the feeding experiment with 8-demethyltetracenomycin C (**4**), *S. argillaceus* was incubated for 6 days at 28 °C on agar plates (A-medium), and *S. fradiae* and *S. fradiae* Tü2717/ΔPKS on agar plates (M_2CaO) at 28 °C for 4 and 5 days, respectively. Pieces (approximately 3 mm^2) from these agar plates were then used to inoculate liquid cultures of the *S. fradiae* strains. *S. argillaceus* was cultivated on agar plates for 140 h at 30 °C using R2 medium[37] (25 μg/mL thiostrepton was added). *S. fradiae* Tü2717 and *S. fradiae* Tü2717/ΔPKS were shaken for 72 h at 28 °C and 30 °C, respectively, in soy/glucose medium (25 μg/mL erythromycin were added to the *S. fradiae* Tü2717/ΔPKS fermentation). All liquid fermentations were carried out with 100 mL of media in 250-mL triple-baffled Erlenmeyer flasks.

Isolation of the Products of *S. fradiae* and *S. argillaceus* Strains. The cultures of the strains *S. fradiae* Tü2717/ΔPKS(16F4) and *S. fradiae* Tü2717/ΔPKS(pWHM1026) were individually filtered with Celite (100 g/L), and the mycelia were extracted for 15 min using a water−acetone mixture (1:1) in an ultrasonic bath. The combined culture filtrate and mycelial extracts were extracted three times with ethyl acetate at pH 7 and evaporated to dryness. To isolate the products of all *S. argillaceus* strains, the agar plates were disected into small pieces and extracted with a mixture of ethyl acetate and acetone (1:1). The resulting crude extracts were purified by chromatography on silica gel (CH_2Cl_2/CH_3OH, 9:1) and Sephadex LH20 (CH_3OH). From *S. fradiae* Tü2717/ΔPKS(16F4) 70 mg/L of 8-demethyltetracenomycin C (**4**), 50 mg/L of 8-demethyl-8-β-D-olivosyltetracenomycin C (**3**) and 8 mg/L of 8-demethyl-8-α-L-rhodinosyltetracenomycin C (**5**) were obtained. Eighty agar plates with *S. argillaceus* (16F4) yielded 70 mg of 8-demethyltetracenomycin C (**4**), of 29 mg 8-demethyl-8-β-D-olivosyltetracenomycin C (**3**), 20 mg of 8-demethyl-β-D-mycarosyltetracenomycin C (**6**), and 23 mg of 8-demethyl-8-β-D-olivosyl-3′-1″-β-D-olivosyltetracenomycin C (**7**).

Feeding Experiments with 8-Demethyltetracenomycin C (4). The following small portions of 8-demethyltetracenomycin C (**4**) were added to cultures of *S. fradiae* Tü2717 and *S. fradiae* Tü2717/ΔPKS cultures: 3 mg/flask at 24 and 32 h after inoculation and 4 mg/flask at 40, 48, and 56 h after inoculation, respectively; 4 mg/flask of **4** were fed to the *S. argillaceus* cultures 104, 115, and 126 h after inoculation. The cultures were worked up as described above, and the resulting crude mixtures were compared by TLC and HPLC to pure samples of tetracenomycin C (**1**, R_{rel} = 13.4 min), 8-demethyl-8-β-D-olivosyltetracenomycin C (**3**, R_{rel} = 12.3 min), 8-demethyltetracenomycin C (**4**, R_{rel} = 12.9 min), 8-demethyl-8-α-L-rhodinosyltetracenomycin C (**5**, R_{rel} = 12.7 min), 8-demethyl-8-β-D-mycarosyltetracenomycin C (**6**, R_{rel} = 13.8 min), and 8-demethyl-8-β-D-olivosyl-3′-1″-β-D-olivosyltetracenomycin C (**7**, R_{rel} = 12.9 min), urdamycin A (**9**, R_{rel} = 14.1 min) and mithramycin (**10**, R_{rel} = 16.4 min). HPLC conditions were the following: solvent speed, 1.5 mL/min; eluent system, linear gradient H_2O/CH_3OH (95:5) → (2:8) during for 13.5 min and continued at (2:8) for 2.5 min.

Instruments. The NMR spectra were recorded in d_4-methanol or d_6-acetone at a field strength of 7.05 T. The new structures were elucidated with the help of various 1D and 2D NMR spectra, including several homo- and heteronuclear correlation experiments such as H,H-COSY, HMQC, HMBC, and NOESY. HPLC conditions were as follows: Kontron system 450; precolumn, RP-8 silica, 25−40 μm; column, Kontron spherisorb ODS 10 μm RP-18 silica.

8-Demethyl-8-α-L-rhodinosyltetracenomycin C (5): MW = 572.52 ($C_{28}H_{28}O_{13}$); neg. FAB-MS *m*/*z* (%) 571 (100) [(M−H)$^-$]; MP 128 °C; IR (KBr) ν = 3660−2075 (br), 2920, 2838, 2367, 1718, 1601, 1460, 1377, 1260, 1230, 1107, 965, 894, 830 cm^{-1}; UV (CH_3OH) λ_{max} nm (ϵ) 200 (8500), 248 (13900), 286 (13900), 412 (6000), 432 (5600); (CH_3OH/HCl) λ_{max} nm (ϵ) 200 (11700), 239 (12700), 287 (21300), 389 (5600), 407 (6000); (CH_3OH/NaOH) λ_{max} nm (ϵ) 209 (143000), 241 (112600); CD (c = 4.5 × 10^{-5}mol/L, CH_3OH) λ_{extr} nm ([Θ][26]) 210.8 (400), 224.4 (−3800). 256.4 (10000), 287.3 (1300), 298.6 (2200), 344.2 (−1000); $[\alpha]^{20}_D$ −25 (c = 0.001, CH_3OH); R_f 0.35 (CH_2Cl_2/CH_3OH, 9:1); NMR data, see Tables 1 and 2.

8-Demethyl-8-β-D-mycarosyltetracenomycin C (6): MW = 602.54 ($C_{29}H_{30}O_{14}$); neg. FAB-MS *m*/*z* (%) 601 (100) [(M−H)$^-$]; MP 144 °C; IR (KBr) ν = 3688−3120 (br), 2931, 1716, 1600, 1462, 1439, 1376,

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(34) Proliferation inhibition assays were carried out with 8-deoxytetracenomycin C (**4**) and the hybrid tetracenomycins **3**, **5**, and **6** using four different cancer cell lines (lung carcinoma A549, breast carcinoma MDA-MB 231, melanoma SK-Mel 30, and plate epithelial carcinoma KB) under standard conditions.[38] All compounds were inactive (IC_{50} > 4 μg/mL); further assays are planned.

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1260, 1231, 1116, 1064, 828 cm^{-1}; UV (CH_3OH) λ_{max} nm (ϵ) 209 (31600), 237 (45400), 285 (77900), 388 (20900), 406 (22500); (CH_3OH/HCl) λ_{max} nm (ϵ) 213 (21100), 238 (21900), 286 (30800), 388 (45300), 406 (84100); (CH_3OH/NaOH) λ_{max} nm (ϵ): 206 (42800), 252 (67000), 437 (23800); CD ($c = 4.8 \times 10^{-5}$mol/L, CH_3OH) λ_{extr} nm ($[\Theta]^{26}$) 207.1 (−1200), 211.8 (−200), 221.3 (−2900), 231.0 (−700), 261.2 (23800), 280.0 (1800), 295.5 (2100), 341.8 (−4100); $[\alpha]^{20}_D$ 10 ($c = 0.02$ in CH_3OH); R_f 0.33 (CH_2Cl_2/CH_3OH, 9:1); NMR data, see Tables 1 and 2.

8-Demethyl-8-β-D-olivosyl-3′-1″−D-β-olivosyltetracenomycin C (7): MW = 718.66 ($C_{34}H_{38}O_{17}$); neg. FAB-MS *m*/*z* (%) 718 (100) [$(M)^-$], 717 (33) [$(M-H)^-$]; MP 128 °C; IR (KBr) ν = 3650−3100 (br), 2931, 1731, 1602, 1460, 1372, 1267, 1232, 1112, 1061, 1003, 997, 838 cm^{-1}; UV (CH_3OH) λ_{max} nm (ϵ) 203 (26000), 235 (29900), 285 (46600), 386 (12400), 406 (13600); (CH_3OH/HCl) λ_{max} nm (ϵ) 203 (24000), 214 (24000), 226 (29400), 285 (50800), 388 (12900), 408 (13600); (CH_3OH/NaOH) λ_{max} nm (ϵ) 205 (28100), 253 (42200), 440 (14100); CD ($c = 3.2 \times 10^{-5}$mol/L, CH_3OH) λ_{extr} nm ($[\Theta]^{26}$) 207.0 (2300), 217.0 (−5100), 235.6 (−2600), 239.2 (−5500), 261.2 (40100), 341.8 (−7000); R_f 0.24 (CH_2Cl_2/CH_3OH, 9:1); NMR data, see Tables 1 and 2.

Anticancer Tests. The substances **3**−**6** were dissolved in DMSO to a final concentration of 20 mg/mL and further diluted (1:200, 1:2000, and 1:20,000) with RPMI-1640 medium.[38] Fifty microliters μL of these solutions were pipetted into 200 μL of cell cultures[34] in microtiter plates (MTPs). The cells had been incubated for 24 h to adhere to the MTPs. After 72 h of incubation at 37 °C and 5% CO_2, cell proliferation was analyzed with the sulforhodamine B assay.[38] The IC_{50} values were calculated from the from the inhibition of cell proliferation at different concentrations.

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