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Rationally Designed Glycosylated Premithramycins: Hybrid Aromatic Polyketides Using Genes from Three Different Biosynthetic Pathways

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Abstract: Heterologous expression of the *urdGT2* gene from the urdamycin producer *Streptomyces fradiae* Tü2717, which encodes a *C*-glycosyltransferase, into mutants of the mithramycin producer *Streptomyces argillaceus*, in which either one or all glycosyltransferases were inactivated, yielded four novel *C*-glycosylated premithramycin-type molecules. Structure elucidation revealed these to be 9-*C*-olivosylpremithramycinone, 9-*C*-mycarosylpremithramycinone, and their respective 4-*O*-demethyl analogues. In another experiment, both the *urdGT2* gene from *S. fradiae* and the *lanGT1* gene from *S. cyanogenus*, were coexpressed into a *S. argillaceus* mutant lacking the MtmGIV glycosyltransferase. This experiment, in which genes from three different organisms were combined, resulted in the production of 9-*C*-(olivo-1−4-olivosyl)premithramycinone. These results prove the unique substrate flexibility of the *C*-glycosyltransferase UrdGT2, which tolerates not only a variety of sugar-donor substrates, but also various acceptor substrates. The five new hybrid products also represent the first compounds, in which sugars were attached to a position that is normally unglycosylated. The successful combination of two glycosyltransferases in the latter experiment proves that the design of saccharide side chains by combinatorial biosynthetic methods is possible.

Introduction

Polyketides constitute a large and structurally diverse family of pharmaceutically important natural products.[1] Many polyketides have useful biological activities, and they have found clinical applications as antibiotics, antifungals, antiparasitics, anticancer, or immunosuppressant agents and also as herbicides and insecticides.[2] Although the chemical structures of polyketides are very diverse, all of them are assembled in a similar manner. The biosynthesis of the polyketide skeleton occurs through the condensation of short-chain carboxylic acids in a series of reactions catalyzed by polyketide synthases.[3] This condensation process generates carbon chains of varying length, with different side chains and reduction patterns that are differentially cyclized and subsequently modified to give the final structures. A number of polyketide structures contain sugar moieties attached to the aglycons. These sugar components usually participate in the molecular recognition of the cellular target, and they are therefore important, and often essential, for the biological activity.[4] Most of these sugars belong to the wide family of the 6-deoxyhexoses, which to date comprises more than 70 different deoxyhexoses, identified in a variety of natural product pathways.[5] Due to the importance of the sugars for biological activity, the idea has emerged of generating novel glycosylated derivatives by altering the glycosylation pattern of bioactive compounds. Recent evidence increasingly suggests some degree of flexibility of glycosyltransferases involved in the biosynthesis of secondary metabolites, and some examples have been reported in which foreign sugars have been transferred to aglycons.[6] In all of these examples, the attachment of the new

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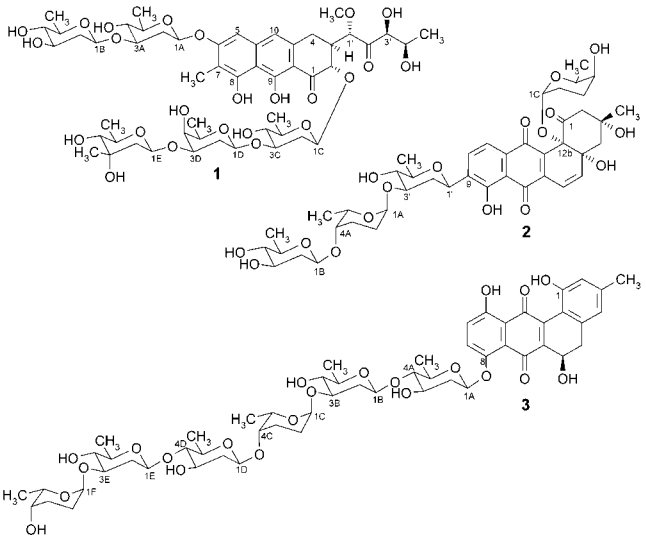
sugars took place at the same position of the aglycon as the natural sugar. However, as far as we know, there are no reports in the literature of secondary metabolism glycosyltransferases being used to attach a sugar moiety to a different position of the aglycon.

Here we report the rational design of novel glycosylated derivatives from two nonglycosylated tetracyclic premithramycin-type molecules (4-demethylpremithramycinone and premithramycinone) by expressing a glycosyltransferase gene from the urdamycin A pathway of *Streptomyces fradiae* into mithramycin nonproducing mutants of *S. argillaceus*. In addition, we also report the extension of this glycosyl moiety into a disaccharide chain by expressing another glycosyltransferase gene from the landomycin A pathway of *S. cyanogenus*. The latter experiment results in an aromatic hybrid polyketide which derives from a combination of genes from three different pathways.

Mithramycin (**1**), an aromatic polyketide produced by *S. argillaceus* ATCC 12956, is used for the treatment of certain cancers, such as testicular carcinoma or disseminated embryonal cell carcinoma as well as for the treatment of Paget's disease and to control hypercalcemia in patients with malignant diseases.[7a,b] Structurally, it is composed of a tricyclic aglycon with a C_5-side chain, a dissaccharide (D-olivose−D-olivose) located at the 6-position and a trisaccharide (D-olivose−D-oliose−D-mycarose), attached to the 2-position.[7c] It has been shown that the glycosylation steps of the biosynthesis of mithramycin occur on tetracyclic intermediates. First, the sugars of the trisaccharide are sequentially added to the premithramycinone aglycon. Then a previously formed D-olivosyl-D-olivose disaccharide is transferred intact into the pseudoaglycon.[8b,e] As one of the last steps in the mithramycin biosynthesis, an oxidative cleavage of the fourth ring of the fully glycosylated tetracyclic intermediate premithramycin B results in a tricyclic compound, which is the immediate precursor of mithramycin.[8] (Figure 1). A number of these tetracyclic biosynthetic intermediates of mithramycin have been isolated from different mutants and their structures have been elucidated.[6j,8] Two of them (4-demethylpremithramycinone and premithramycinone) contain no sugar moieties, others possess mono-, di-, or trisaccharides at the 6-position (premithramycins A1, A2, and A3).



Urdamycin A (**2**) is an angucycline polyketide produced by *S. fradiae* Tü2717 which also shows antitumor activity.[9] It consists of the aglycon aquayamycin, which includes a *C*-glycosidically linked D-olivose, and three additional *O*-glycosidically linked deoxyhexoses: two L-rhodinoses and another D-olivose. Four glycosyltransferase genes have been identified in the urdamycin gene cluster and specific functions have been assigned to each of them.[10] The UrdGT2 glycosyltransferase catalyzes the earliest glycosylation step in the urdamycin biosynthesis. This *C*-glycosyl transfer step requires an activated D-olivose as sugar donor substrate and an unknown angucyclinone as the acceptor substrate, possibly compound **4**, the product being compound **5**.[10a] The putative substrate (**4**) of UrdGT2 resembles to some extent the intermediates of the mithramycin biosynthetic pathway, particularly 4-demethylpremithramycinone (**6**) and premithramycinone (**7**, Figure 2). Thus, we anticipated that UrdGT2 might recognize and glycosylate these intermediates of the mithramycin biosynthetic pathway and thereby create novel glycosylated derivatives.

Results and Discussion

To test this hypothesis we expressed *urdGT2* under the control of the erythromycin resistance promoter (*ermE**p) in several mutants of *S. argillaceus* that accumulate different tetracyclic intermediates. The selected mutants had been generated by gene replacement of all four mithramycin glycosyltransferase genes (M3ΔMG mutant) or by gene replacements of single genes

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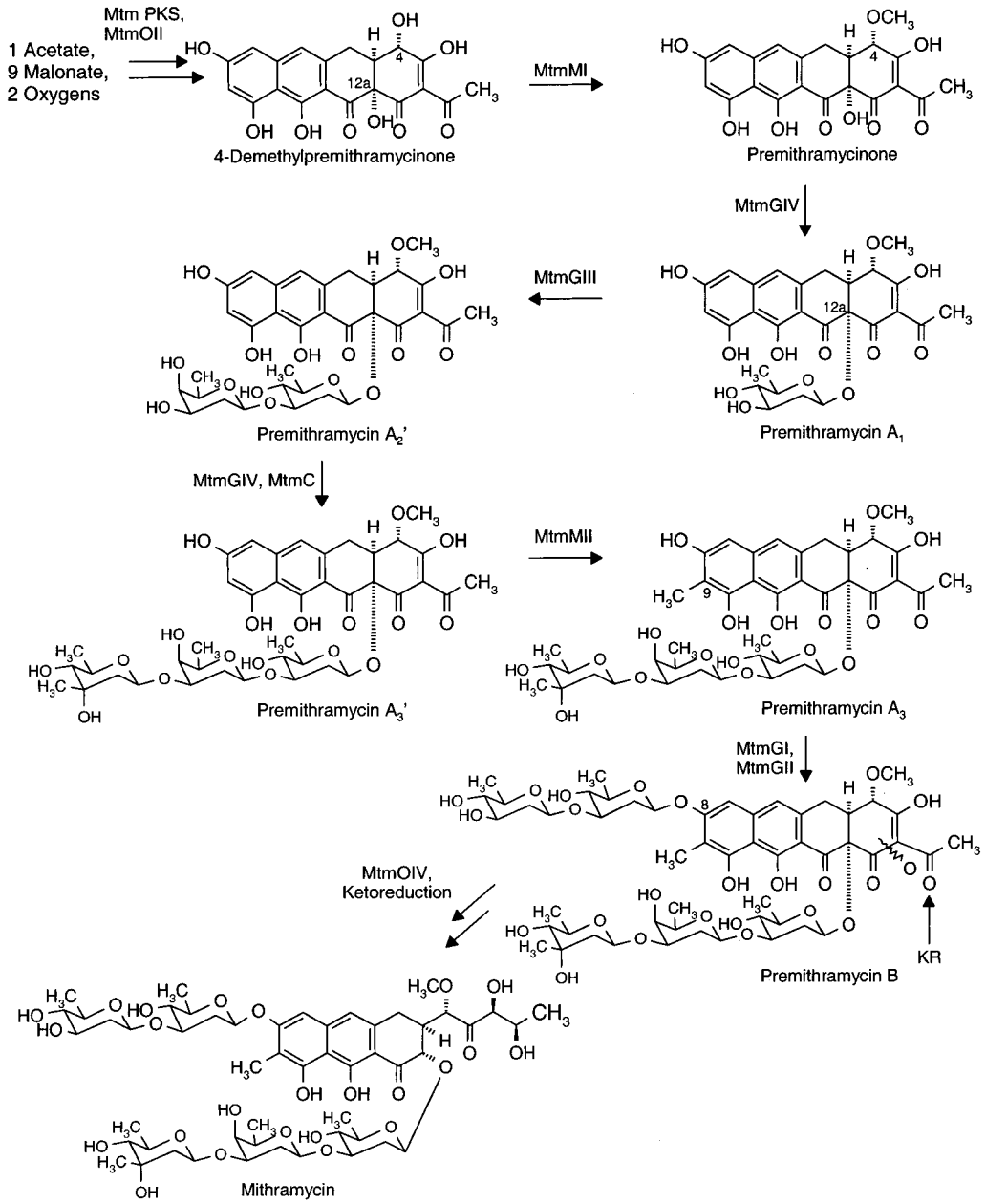

Figure 1. Late biosynthetic pathway to mithramycin showing the sequence of events of the post-PKS tailoring steps catalyzed by oxidoreductases (O = oxygenase; KR = ketoreductase) or group transferases (G = glycosyltransferase; M = methyltransferase). The newly added structural elements of each shown step are highlighted in bold.

encoding glycosyltransferases (strains M3G1, M3G2, M3G3, and M3G4 with mutations in *mtmGI*, *mtmGII*, *mtmGIII*, and *mtmGIV*, respectively).[8a−e] Expression of *urdGT2* in mutants M3G1, M3G2 and M3G3 did not lead to the production of any new compound as shown when cultures were extracted with ethyl acetate and analyzed by reverse phase HPLC. However, upon expression of *urdGT2* in M3ΔMG and M3G4 mutants, four new HPLC-peaks (two in each mutant) were observed with the characteristic absorption spectra of the tetracyclic premithramycin-type aglycons. The new compounds were isolated by preparative HPLC and their structures were elucidated using NMR and mass spectroscopy.

Mutant M3G4(*urdGT2*) yielded two compounds. Both compounds exhibit all of the typical NMR signals of a tetracyclic premithramycin-type aglycon, except for the 9-position. In all previously isolated molecules of such type, this position is substituted by either a proton, as in premithramycinone[8c] (**7**) and premithramycin A1[8b] (**8**), or by a methyl group, for example as in premithramycin A2[8b] (**9**) or premithramycin A3[8b] (**10**). The ^{1}H NMR data (Tables 1, 2) of the new compounds showed neither a 9-H nor a 9-CH_3 signal. Such a methyl group was also not observed in the ^{13}C NMR spectra, which revealed C-9 to be a quaternary carbon. This is all consistent with structures in which the 9-position is substituted by a different residue. Since the NMR data also indicated the presence of one deoxyhexose moiety in each of the compounds, a linkage of these deoxysugar moieties at 9-position seemed likely. Indeed, the ^{13}C NMR signals of C-9 (δ_C 111.5/111.4) and for C-1A (δ_C 72.5/70.2), the anomeric carbons of the sugar moieties, show the absence of a directly bound oxygen atom at C-9 and only

Table 1. ^{1}H NMR Data of 9-*C*-Olivosylpremithramycinone (**12**), 9-*C*-Mycarosylpremithramycinone (**14**), 9-*C*-Olivosyl-4-*O*-demethylpremithramycinone (**11**), 9-*C*-Mycarosyl-4-*O*-demethylpremithramycinone (**13**), and 9-*C*-Di-olivosylpremithramycinone (**15**) in Acetone-d_6 at 400 MHz,[a] δ in ppm Relative to Internal TMS

	δ, multiplicity (*J*/Hz)				
position	**12**[b]	**14**[c]	**11**[d]	**13**[e]	**15**[a,f,g]
4-H	4.18 br d (11.5)	4.18 br d (11.5)	4.42 br m	4.42 br m	4.24 br d (11.5)
4-OCH_3	3.59 br s	3.58 br s	-	-	3.58 br s
4a−H	2.68 br d (11.5)	2.72 br d (11.5)	2.70 ddd (11.5, 5, 3)	2.70 ddd (11.5, 4.5, 3)	2.72 ddd (11.5, 4.5, 3)
5-H	a: 3.48 ddd (16, 5, 2) e: 3.15 dd (16, 3)	a: 3.48 ddd (16, 5, 2) e: 3.14 dd (16, 3)	a: 3.45 ddd (16, 5, 1) e: 3.25 dd (16, 3)	a: 3.45 ddd (16, 4.5, 1) e: 3.25 dd (16, 3)	a: 3.49 ddd (16, 4.5, 1.5) e: 3.16 dd (16, 3)
6-H	7.00 br s	7.00 br s	6.99 br s	6.98 br s	7.02 br s
7-H	6.62 s	6.60 s	6.62 s	6.62 s	6.68 s
2′-H_3	2.61 s	2.62 s	2.61 s	2.63 s	2.62 s
1A-H	5.30 dd (12, 2)	5.62 dd (12, 2)	5.30 dd (12, 2)	5.62 dd (12, 2)	5.30 dd (12, 2)
2A-H	a: 1.80 ddd (13, 12, 12) e: 2.21 ddd (13, 5, 2)	a: 1.86 dd (13, 12) e: 1.99 dd (13, 2)	a: 1.80 dd (13, 12) e: 2.20 ddd (13, 5, 2)	a: 1.85 dd (13, 12) e: 2.00 dd (13, 2)	a: 1.76 ddd (13, 12, 12) e: 2.25 ddd (13, 5, 2)
3A-H	3.73 ddd (12, 9, 5)	-	3.73 ddd (12, 9, 5)	-	3.71 ddd (12, 9, 5)
3A-CH_3	-	1.26 s	-	1.25 s	-
4A-H	3.18 dd (9, 9)	3.23 br d (9.5)	3.17 dd (9, 9)	3.21 br d (9.5)	3.30 dd (9, 9)
5A-H	3.56 dq (9, 6.5)	3.84 dq (9.5, 6)	3.57 dq (9, 6.5)	3.83 dq (9.5, 6)	3.62 dq (9, 6.5) obsc
6A-H_3	1.40 d (6.5)	1.38 d (6)	1.40 d (6.5)	1.38 d (6)	1.41 d (6.5)

[a] Recorded at 500 MHz. br = broad; obsc = obscured by solvent or water. [b] OH signals (not assigned): 4.20, 4.30, 5.70 (3′-OH), 9.30, 10.20, 15.58, 18.18. [c] OH signals (not assigned): 3.60, 4.02, 5.70, 9.40, 10.00, 16.60, 18.20. [d] OH signals (not assigned, not all observed): 5.75, 9.30, 10.00. [e] OH signals (not assigned, not all observed): 5.80, 9.40, 9.98. [f] OH signals (not assigned, not all observed): 4.32, 4.58, 5.72, 9.13, 10.08. [g] Signals of the second olivose moiety: 4.75 dd (1B-H, J = 12, 2); 1.56 ddd (2B-H_a, J = 13, 12, 12); 2.25 ddd (2B-H_e, J = 13, 5, 2); 3.60 m obsc (3B-H); 2.99 dd (4B-H, J = 9, 9); 3.40 dq (5B-H, J = 9, 6.5); 1.29 d (6B-H_3, J = 6.5).

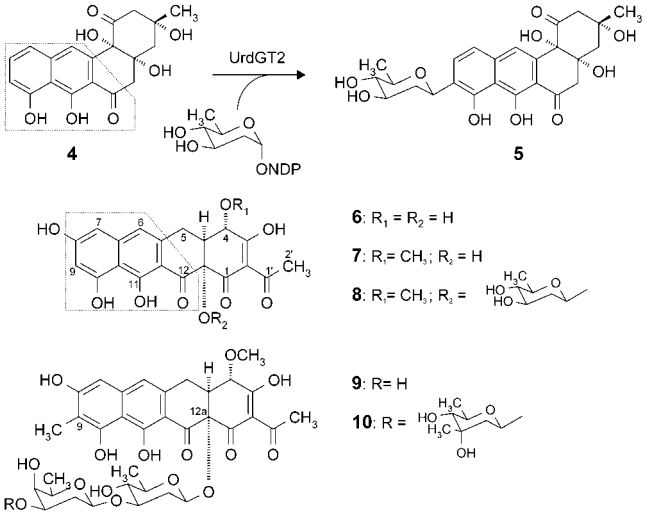

Figure 2.

one directly attached oxygen atom at the anomeric carbon C-1A, respectively. This clearly indicates a *C*-glycosidic linkage of the sugar building blocks at the 9-position. The sugar moiety of the major compound is olivose, as indicated by the H,H coupling constants, along with the other ^{1}H- and ^{13}C NMR data (see tables 1, 2), and mycarose for the minor compound. Representative signals for the latter are the 3A-methyl group (δ_H 1.26, δ_C 27.6) instead of a 3A-H signal, along with the simpler ^{1}H NMR signals for 2A-H and 4A-H, which both lack one a,a-coupling compared to the respective patterns in olivose. Both sugar moieties are β-glycosidically linked, as revealed by the large (12 Hz) coupling constants of the anomeric protons which appear at δ 5.30 (dd, J = 12 and 2 Hz) and δ 5.62 (dd, J = 12 and 2 Hz), respectively. Thus, the two compounds of mutant M3G4(*urdGT2*) are 9-*C*-olivosylpremithramycinone (**12**) and 9-*C*-mycarosylpremithramycinone (**14**). The yields for these compounds were 5.2 mg/L for **12** and 5.8 mg/l for **14**. The deduced molecular formula for the former compound is $C_{27}H_{28}O_{12}$ (544.5), which was confirmed by the APCI mass spectrum (*m*/*z* 543, M − H$^-$,100%). In conformity with structure **14**, the APCI mass spectrum shows a molecular ion at *m*/*z* 557 (M − H$^-$, 100%), proving the molecular formula of $C_{28}H_{30}O_{12}$ (558.5).

Compared with these two compounds, the two minor compounds found in mutant M3ΔMG(*urdGT2*) are almost identical in respect to all NMR data. Again, one of these minor compounds contains an olivose moiety, while the other contains a mycarose moiety. They differ from compounds **12** and **14** only in that they possess a 4-OH instead of a 4-*O*-methyl group. This is revealed in the NMR spectra, where the *O*-methyl signal (in compounds **12** and **14** at δ_H ca. 3.6/δ_C ca. 62) is missing. In agreement with this, the 4-H signals of both additional compounds shifted downfield from ca. δ 4.18 to δ 4.42, and the 4-H signals appear as a broad multiplet instead of as a broad doublet due to the additional H,OH coupling. A smaller downfield shift of 0.1 ppm can also be observed on the neighboring 5-H_e protons. Otherwise, the NMR data are almost identical with the corresponding 4-methyl ether analogues **12** and **14** (see Tables 1, 2). Thus, the NMR data allow us to suggest 9-*C*-olivosyl-4-*O*-demethylpremithramycinone (**11**) and 9-*C*-mycarosyl-4-*O*-demethylpremithramycinone (**13**) as the structures of these two minor compounds of mutant strain M3ΔMG-(*urdGT2*) with yields of 3.5 mg/L for **11** and 3.9 mg/L for **13**. As in the corresponding 4-methyl ether analogues, the sugar moieties in **11** and **14** are β-glycosidically linked, as can be deduced from the large (12 Hz) couplings constants of the anomeric protons (see Table 1).

The results prove two important facts about UrdGT2. This *C*-glycosyltransferase is not only capable of handling its normal sugar substrate NDP− D-olivose, but also NDP− D-mycarose, which is quite different from NDP− D-olivose with respect to its 3-methyl branch and in possessing an axial OH group instead of an equatorial one. In addition, these experiments illustrate again the interestingly wide substrate range of UrdGT2 regarding its acceptor substrate. Although it was noticed before that UrdGT2 can glycosylate a variety of angucyclinones and even the bicyclic quinone naphthazarine,[10a] the highly substituted

Table 2. ^{13}C NMR Data of 9-*C*-Olivosylpremithramycinone (**12**), 9-*C*-Mycarosylpremithramycinone (**14**), 9-*C*-Olivosyl-4-*O*-demethylpremithramycinone (**11**), 9-*C*-Mycarosyl-4-*O*-demethylpremithramycinone (**13**), and 9-*C*-Di-olivosylpremithramycinone (**15**) in Acetone-d_6 at 100.6 MHz, δ in ppm

	δ						δ				
position	**12**	**14**	**11**	**13**	**15**[a,b]	position	**12**	**14**	**11**	**13**	**15**[a,b]
1	197.2	197.0	n.o.	n.o.	197.2	12	191.8	191.2	192.0	192.0	191.0
2	112.0	112.0	111.0	112.2	111.4	12a	79.8	77.8	n.o.	n.o.	77.4
3	196.0	196.3	196.0	n.o.	196.0	1′	204.5	204.2	204.0	n.o.	204.6
4	77.3[1]	77.2[3]	78.0[4]	78.0	78.0	2′	28.0	28.0	28.0	28.0	28.2
4-OCH_3	61.7	62.0	–	–	61.8	1A	72.5	70.2[2]	72.5	70.3[5]	73.5
4a	44.8	44.8	46.0	46.0	45.0	2A	39.3	43.7	39.3	43.8	38.8
5	26.5	26.0	26.5	26.0	26.0	3A	78.0[1]	75.0[3]	78.0[4]	75.0	70.5
5a	135.3	135.0	136.0	135.0	135.5	3A-CH_3	∼	27.6	∼	27.5	?
6	118.3	118.1	118.5	118.3	118.2	4A	78.1[1]	77.3[3]	78.1[4]	77.2	88.0
6a	141.7	141.4	141.5	141.5	141.5	5A	74.2	72.0[2]	74.0	72.0[5]	76.0
7	105.0	104.9	105.0	104.8	105.0	6A	18.4	18.5	18.5	18.5	18.0
8	162.0	162.1	162.0	162.0	162.0	1B	–	–	–	–	102.0
9	111.5	111.4	111.5	111.3	110.9	2B	–	–	–	–	40.3
10	156.0	156.0	n.o.	n.o.	156.0	3B	–	–	–	–	71.3
10a	107.0	107.0	n.o.	107.0	107.0	4B	–	–	–	–	77.5
11	168.8	168.4	n.o.	n.o.	167.2	5B	–	–	–	–	72.7
11a	108.0	107.8	108.0	107.9	108.2	6B	–	–	–	–	17.9

[a] Assignments confirmed by HMBC/HSQC; n.o. = not observed. [1,2,3,4] assignments interchangeable. [b] Recorded at 125.7 MHz.

tetracyclic premithramycinone and its 4-demethyl analogue differ in their shape more drastically from the natural acceptor substrate than anything previously shown to be a substrate of UrdGT2. The fact that only premithramycinone and 4-demethylpremithramycinone glycosylated derivatives were isolated suggests that other mithramycin biosynthetic intermediates, such as premithramycin A1 (**8**), the principal product of mutant M3G3, are not good substrates for UrdGT2. Probably, the D-olivose moiety, which is attached at 12a-position in premithramycin A1,[8e] modifies the acceptor substrate to be recognizable by UrdGT2. On the other hand MtmGIV, the glycosyltransferase that catalyzes the 12a-*O*-glycosylation of premithramycinone to yield premithramycin A1,[8e] might be inhibited once the *C*-sugar unit has been linked to the 9-position (by UrdGT2).

The ability of UrdGT2 to transfer either a D-olivose or a D-mycarose moiety to the two nonglycosylated premithramycinones prompted us to design new experiments in which the possibility of extending the saccharide chain of the new compounds could be assayed. For this purpose we chose the M3G4 mutant as host and a GT-encoding gene of the landomycin A gene cluster. Landomycin A (**3**), which is produced by *S. cyanogenus* S136, contains an unusual hexasaccharide side chain consisting of four D-olivose and two L-rhodinose residues.[11] Four glycosyltransferase genes have been found in the landomycin cluster.[11d] LanGT1 was identified to be responsible for the linkage of the second sugar, a D-olivose, to the 4-OH group of the first sugar moiety of the hexasaccharide chain, which is also a D-olivose. In addition, it has been shown recently that LanGT1 displays some degree of acceptor substrate flexibility, since it was able to transfer a D-olivose moiety to the 4-position of the *C*-glycosidically linked D-olivose moiety of aquayamycin.[10d] Because of this flexibility, the *lanGT1* gene was chosen for the chain extension experiments.

Thus, the *lanGT1* gene was cloned downstream of *urdGT2* gene so that both genes were under the control of a unique *ermE** promoter. When they were coexpressed in mutant M3G4 and cultures of recombinant clones analyzed by HPLC, it was found that the peak corresponding to 9-*C*-olivosylpremithramycinone greatly decreased in favor of a new peak, while that corresponding to 9-*C*-mycarosylpremithramycinone did not change. The new compound was isolated and its structure determined by NMR and mass spectroscopy.

In contrast to the monoglycosylated structures obtained in the experiments described above, the NMR data of the new compound produced by mutant strain M3G4(*urdGT2*,*lanGT1*) clearly show the presence of two sugar moieties. The NMR data (Tables 1, 2) also indicate a premithramycinone moiety with a 4-*O*-methyl group. The exact analysis of the H,H coupling constants reveals that both sugar moieties are olivoses, one of which is *C*-glycosidically linked, the other *O*-glycosidically linked, as can be easily determined from the ^{13}C NMR shifts of the anomeric carbons (δ_C 73.5 and 102.0, respectively). The ^{13}C NMR chemical shift of C-9 (δ_C 110.9), and the fact that it is again a quaternary carbon, indicate the linkage of one of the olivoses at this position as a *C*-glycosidic moiety. This was further proven through the $^2J_{C-H}$ and $^3J_{C-H}$ long-range couplings between the anomeric proton of this olivose and carbons 8, 9, and 10 of the premithramycinone moiety observable in the HMBC spectrum. The HMBC spectrum also shows $^3J_{C-H}$ couplings between 4A-H and C-1B as well as between 1B-H and C-4A (Figure 3), which proves that the second olivose is connected to the 4A-OH group of the *C*-glycosidically linked olivose. Again the large coupling constants of the anomeric protons of both sugar moieties (both, $J_{1A-H/2A-Ha}$ and $J_{1B-H/2A-Ha}$, are 12 Hz) indicate a β-glycosidic linkage. Thus, the structure of the new compound detected in strain M3G4(*urdGT2*,*lanGT1*) is 9-*C*-(olivo-1-4-olivosyl)premithramycinone, that is, 9-*C*-diolivosylpremithramycinone (**15**). Its molecular formula $C_{33}H_{38}O_{15}$ (674.6) is confirmed by the APCI mass spectrum, which shows a molecular ion at *m*/*z* 674 (M^-, 70%) and some fragments. Since 9-*C*-diolivosylpremithramycinone (**15**) is the compound expected to result from the experiments as designed, it can be viewed as a rationally engineered hybrid compound.

The antitumor activities of the four compounds described here, 9-*C*-olivosylpremithramycinone, 9-*C*-mycarosylpremith-

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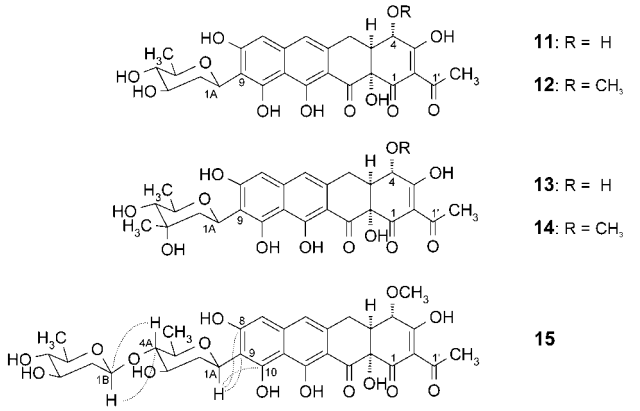

Figure 3.

ramycinone and their 4-demethylanalogues were evaluated against different tumor cell lines. Most of them showed very low activity, with 9-*C*-mycarosylpremithramycinone giving the best results. Growth percentages ranged from 52 to 110% with respect to 100% for the controls.[12]

Relaxed specificity for sugar cosubstrates has already been described for various glycosyltransferases. Recently, OleG2, an L-olivosyl transferase from *S.antibioticus*, was shown to be able to transfer L-rhamnose.[6f,h,13] TylM2, a glycosyltransferase from *S. fradiae*, was integrated into the chromosome of a triple mutant of *Saccharopolyspora erythraea*, which produced new hybrid antibiotics when the resulting strain was fed with tylactone.[15]

By deleting the gene encoding an *N*-methyltransferase contributing to the biosynthesis of D-desosamine and by recombining the D-desosamine genes with deoxysugar biosynthesis genes from the calicheamicin producer strain, it was also shown that glycosyltransferase DesVII is capable of recognizing and processing sugar substrates other than TDP-D-desosamine in the methmycin/neomethymycin pathway.[6d,15] Moreover, recombination of the desosamine genes with genes from the streptomycin producer showed that DesVII can also transfer L-sugars.[16]

The redesign and expression in *S. lividans* of deoxysugar genes of the avermectin producer *S. avermitilis* in *S. lividans* and combination with exogenously fed avermectin aglycon led to novel avermectin derivatives and showed that the *S. avermitilis* glycosyltransferase exhibits some flexibility toward the activated sugar-cosubstrate.[17] Another well characterized flexible glycosyltransferase is ElmGT from *S. olivaceus*, which is able to transfer a broad variety of L- and D-deoxysugars as well as a D-olivose disaccharide.[6a,c,i]

Glycosyltransferases that are flexible toward the alcohol acceptor substrate have also been described. For instance, expression of an *Antirhinum majus* UDP-glucose:flavonoid-3-*O*-glucosyltransferase transgene alters the flavonoid glycosylation in the lisianthus plant *Eustoma grandiflorum*.[18] Glycosyltransferase DesVII has the ability to catalyze the transfer of D-desosamine to both the 12- and 14-membered ring macrolactones of the picromycin/methymycin pathway.[19] Moreover, a D-desosamine containing polyketide library was generated by coexpression of D-desosamine biosynthetic genes, a glycosyltransferase gene from the picromycin/methymycin biosynthetic gene cluster and genes encoding genetically modified polyketide synthases in *S. lividans*.[20] These experiments show that the pikromycin desosaminyl transferase is quite tolerant of changes in the macrolactone acceptor substrate.

The glycosyltransferase UrdGT2 reported in this study showed relaxed specificity for both the sugar donor substrate and the acceptor substrate. Thus, UrdGT2 can be viewed as a very flexible enzyme with a high potential for generating novel *C*-glycosylated compounds. The coexpression of *urdGT2* and *lanGT1* resulting in the successful generation of a new compound adds another example of the design of novel compounds by combinatorial biosynthesis. In addition, it is a clear demonstration that altering the glycosylation pattern can be a convenient tool for increasing structural biodiversity from natural products.

Glycosyltransferase MtmGIV is responsible for the linkage of the first sugar moiety of the trisaccharide to the 12a-position of premithramycinone, thus converting premithramycinone (**7**) into premithramycin A1 (**8**).[8b,e] The fact that we could not detect 9-*C*-glycosylated products in all experiments where mutant strains were used that still contained the natural glycosyltransferase MtmGIV (strains M3G1, M3G2, and M3G3) of *S. argillaceus* suggests that possibly UrdGT2 cannot *C*-glycosylate the 9-position once a sugar is attached at the 12a-position. This is not really surprising, since in this situation the heterologously expressed glycosyltransferase UrdGT2 has to compete with a natural glycosyltransferase MtmGIV for the same substrate, premithramycinone (**7**). In addition, UrdGT2 also has to compete with MtmMII for the 9-position of the premithramycins. MtmMII is the methyltransferase, which in the natural mithramycin biosynthetic pathway C-methylates this 9-position.[8f] Although this step preferentially happens after completion of the trisaccharide chain, MtmMII was proven to be quite flexible in respect of its substrate, and can convert mono-, di- and triglycosylated premithramycins into their 9-methylated analogues.[8f]

The fact that no *C*-glycosylated (tricyclic) mithramycin-type molecules were found also suggests that the 9-*C*-glycosylated compounds are not good substrates for the ring-opening oxygenase MtmOIV.[8d]

Experimental Section

Microorganisms, Culture Conditions, and Plasmids. Mithramycin nonproducing mutants were used as transformation hosts: *S. argillaceus* M3ΔMG,[8d] M3G1 and M3G2,[8b] and M3G3 and M3G4.[8e] *Escherichia coli* XL1blue (Stratagene, Germany) was used as host for plasmid propagation. For sporulation on solid medium, they were grown at 30 °C on plates containing A medium.[8b] For protoplast transformation the

(12) Antitumor tests were evaluated in the three cell line system (lung NCI-H460, breast MCF7, and CNS SF-268), one dose primary anticancer assay by the National Cancer Institute, Bethesda, MD. For details, see http://dtp.nci.nih.gov.

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mutants were grown in YEME medium containing 17% sucrose. For growth in liquid medium, the organism was grown in TSB medium (trypticase soya broth, Oxoid). When plasmid-containing clones were grown, the medium was supplemented with the appropiate antibiotics: 50 μg/mL thiostrepton or 20 μg/mL tobramycin. Plasmids pMUN-*urdGT2*, pUWL-*urdGT2*, and pMUN-*lanGT1* have been previously described.[10d] Plasmid pUWL-*urdGT2*-*lanGT1* has been constructed during this work as described in ref 10d.

DNA Manipulation Techniques. Plasmid DNA preparations, restriction endonuclease digestions, alkaline phosphatase treatments, ligations and other DNA manipulations were according to standard procedures for *Streptomyces*.[21]

Isolation of New Compounds. Culture conditions and HPLC procedures for the purification of new compounds were as previously described.[8f]

Structure Elucidation and Characterization. NMR spectra were recorded in d_6-acetone/D_2O (95:5) on Varian Inova 400 and Bruker DMX 500 NMR instruments at 400 and 500 MHz, respectively, for ^{1}H and 100.6 and 125.7 MHz, respectively, for ^{13}C, using 1D spectra and 2D homo- and heteronuclear correlation experiments (^{1}H,^{13}C, DEPT, H,H-COSY, HSQC, and HMBC). The NMR data are listed in Tables 1 and 2. Negative-ion atmospheric pressure chemical ionization mass spectra (APCI-MS) were acquired at the Medical University of South Carolina, Regional Mass Spectroscopy Center using a Finnigan MAT LCQ. High-resolution (HR-APCI) mass spectra were acquired at the University of South Carolina, Department of Biochemistry and Chemistry facilities in Columbia, SC, using a VG70SQ double-focusing magnetic sector mass spectrometer. Retention times (t_R) were determined on a Waters HPLC system (Delta 600, M32 add-on single system, with a photodiode array detector model 996) using a Symmetry C18 (4.6 mm × 250 mm, Waters) reversed phase column with acetonitrile and 0.1% trifluoroacetic acid in water as solvent. A linear gradient from 10 to 100% acetonitrile in 30 min, at a flow rate of 1 mL/min was used. Detection was monitored at 280 nm. R_f values of the isolated compounds were determined on silica with a solvent mixture of chloroform/methanol/acetic acid/water (58:7:3.5:1). The UV spectra were recorded on a Beckman DU 650 spectrophotometer and IR spectra were obtained from a pure sample on KCl disks in a Mattson Genesis II FT FT-IR spectrometer. CD spectra were recorded on a AVIV circular dichroism spectropolarimeter (model 60DS) in a 1 cm cell.

11: 9-*C*-olivosyl-4-*O*-demethylpremithramycinone, $C_{26}H_{26}O_{12}$; APCI-MS *m*/*z* (relative intensity) $[M]^-$ 530 (40), $[M\text{-}OH]^-$ 513 (100); HR-APCI *m*/*z* $[M]^-$ calcd 530.1424, found 530.1428; t_R = 18.05 min; R_f = 0.21; UV (MeOH) λ_{max} (ϵ): 426 (6800), 322 (6700), 285 (32100), 239 (20,800) nm; (MeOH−HCl) λ_{max} (ϵ): 417 (7900), 327 (5300), 281 (27900), 231 (27000) nm; (MeOH−NaOH) λ_{max} (ϵ): 417 (10700), 278 (30100), 228 (19400) nm; FT-IR (KCl) ν 3297, 2970, 2910, 2832, 2363, 2338, 1676, 1636, 1580, 1522, 1448, 1420, 1337, 1270, 1162, 1125 cm^{-1}; CD (MeOH) λ_{extr} (Θ_D^{20}): 218 (−11500), 224 (−12700), 236 (−10600), 264 (−26500), 382 sh (37800), 469 (48400) nm.

12: 9-*C*-olivosylpremithramycinone, $C_{27}H_{28}O_{12}$; APCI-MS *m*/*z* (relative intensity) $[M - H]^-$ 543 (100); HR-APCI was unable to be obtained for this compound; t_R = 22.5 min; R_f = 0.57; UV (MeOH) λ_{max} (ϵ): 421 (18500), 326 (10400), 279 (65800), 223 (49000) nm; (MeOH−HCl) λ_{max} (ϵ): 415 (15700), 326 (9200), 278 (52500), 227 (59200) nm; (MeOH−NaOH) λ_{max} (ϵ): 421 (21900), 280 (57500) nm; FT-IR (KCl,) ν 3376, 2929, 2830, 2323, 1676, 1633, 1590, 1508, 1444, 1339, 1271, 1158, 1121 cm^{-1}; CD (MeOH) λ_{extr} (Θ_D^{20}): 227 (18000), 269 (−7146), 300 (5000), 312 (1800), 372 (43500), 433 (38000), 483 (47800) nm.

13: 9-*C*-mycarosyl-4-*O*-demethylpremithramycinone, $C_{27}H_{28}O_{12}$; APCI-MS *m*/*z* (relative intensity) $[M]^-$ 544 (40), $[M - OH]^-$ 527 (100); HR-APCI *m*/*z* $[M]^-$ calcd 544.1581, found 544.1575; t_R = 19.7 min; R_f = 0.30; UV (MeOH) λ_{max} (ϵ): 428 (9800), 324 (6700), 284 (30600), 231 (22000) nm; (MeOH−HCl) λ_{max} (ϵ): 417 (7400), 326 (5100), 281 (26700), 232 (27700) nm; (MeOH−NaOH) λ_{max} (ϵ): 417 (10100), 277 (28800) nm; FT-IR (KCl) ν 3332, 2973, 2930, 2359, 2332, 1684, 1635, 1590, 1525, 1448, 1427, 1373, 1333, 1275, 1165, 1125 cm^{-1}; CD (MeOH) λ_{extr} (Θ_D^{20}): 216 (−1400), 266 (−21800), 320 sh (2300), 391 sh (43000), 473 (53000) nm.

14: 9-*C*-mycarosylpremithramycinone, $C_{28}H_{30}O_{12}$; APCI-MS *m*/*z* (relative intensity) $[M]^-$ 558 (95), $[M - OH]^-$ 541 (60), $[M - OH - CH_3 - COCH_3]^-$ 483 (100); HR-APCI *m*/*z* $[M]^-$ calcd 558.1737, found 558.1750; t_R = 23.7 min; R_f = 0.57; UV (MeOH) λ_{max} (ϵ): 425 (11400), 324 (5500), 280 (38200), 239 (24500) nm; (MeOH−HCl) λ_{max} (ϵ): 419 (9800), 278 (6000), 280 (32000) 231 (33500) nm; (MeOH−NaOH) λ_{max} (ϵ): 417 (12900), 280 (36600) nm; FT-IR (KCl) ν 3332, 2970, 2932, 2835, 2370, 2334, 1675, 1636, 1590, 1517, 1448, 1424, 1372, 1330, 1272, 1162, 1124 cm^{-1}; CD (MeOH) λ_{extr} (Θ_D^{20}): 220 (−3400), 225 (−6900), 242 (−900), 269 (−18000), 281 (2000), 291 (−36400), 318 sh (−5100), 378 (40700), 423 (32600), 485 (51000) nm.

15: 9-*C*-diolivosylpremithramycinone, $C_{33}H_{38}O_{15}$; APCI-MS *m*/*z* (relative intensity) $[M]^-$ 674 (100), $[M - OH]^-$ 657 (60), $[M - OH - CH_3 - COCH_3]^-$ 599 (85); HR-APCI *m*/*z* $[M]^-$ calcd 674.2211, found 674.2195; t_R = 21.5 min; R_f = 0.53; UV (MeOH) λ_{max} (ϵ): 424 (11300), 326 (5800) 281 (38100), 241 (23800) nm; (MeOH−HCl) λ_{max} (ϵ): 418 (9400), 326 (5800), 280 (31900), 231 (31900) nm; (MeOH−NaOH) λ_{max} (ϵ): 419 (12500), 279 (35600) nm; FT-IR (KCl) ν 3337, 2970, 2929, 2370, 2341, 1683, 1634, 1577, 1521, 1448, 1424, 1373, 1334, 1291, 1163, 1124 cm^{-1}; CD (MeOH) λ_{extr} (Θ_D^{20}): 228 (9400), 262 (−4700), 300 sh (8800), 381 sh (58700), 470 (69500) nm.

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