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Glycosylated Derivatives of Steffimycin: Insights into the Role of the Sugar Moieties for the Biological Activity

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Expression of the steffimycin gene cluster in Steptomyces albus *in combination with plasmids directing the biosynthesis of different neutral and branched-chain deoxyhexoses led to the identification of twelve new glycosylated derivatives of steffimycin with different degrees of decoration in the tetracyclic core. These experiments demonstrate the flexibility of* L*-rhamnosyltransferase StfG for recognition of a variety of* D*- and* L*-deoxyhexoses, harboring different degrees of deoxygenation as 2-deoxyhexoses, 2,6-deoxyhexoses, and 2,3,6-deoxyhexoses, and their attachment to 8-demethoxy-10-deoxysteffimycinone. In addition, the flexibility of 3′-O-methyltransferase OleY, from* Streptomyces*, for the methylation of deoxyhexoses attached to the steffimycin aglycone is shown by expression of* oleY *in* Streptomyces steffisburgensis*, leading to the isolation of 3′-O-methylsteffimycin. Analysis of the biological activities of these compounds against three human tumor cell lines—breast adenocarcinoma, non-small cell lung cancer, and colon adenocarcinoma—revealed two of them, 3′-O-methylsteffimycin and* D*-digitoxosyl-8-demethoxy-10-deoxysteffimycinone, to possess improved antitumor activities, showing GI_{50} values below 1.0 μM, while steffimycin's GI_{50} values fluctuate between 2.61 to 6.79 μM depending upon the cell line used. The antitumor activity data provide some insights into the structure–activity relationships of the new steffimycin derivatives, in relation to the configuration of hydroxy groups at positions C-3′ and C-4′ of the sugar moiety and positions C-8 and C-10 of the tetracyclic core.*

Introduction

Anthracycline antibiotics such as doxorubicin, daunorubicin, aclarubicin, epirubicin, pirarubicin, valrubicin, idarubicin, and amrubicin are nowadays in clinical use for the treatment of a wide variety of cancers such as acute myeloid leukemia, lymphomas, and a diversity of solid tumors including breast, small cell lung, cervical, head, and neck cancer.[1] They represent 5% of a total of 155 anticancer agents that have earned clinical approval during the last forty years,[1,2] the first three anthracyclines being natural products and the others being derived from natural products by semisynthetic modifications. In addition, around 400 anthracyclines from different sources such as screening and biosynthetic studies have been reported,[3] while 2000 additional analogues have been developed by structural modification of natural compounds or by total and semisynthesis.[1,4,5,6] However, several limitations exist, usually related to the clinical use of anthracyclines, in particular drug resistance and cardiac toxicity, which has prompted intensive research to develop new derivatives through biosynthetic studies or chemical synthesis.[1,5,6]

The cytotoxic activities of anthracyclines are mainly a result of DNA intercalation and interaction with the topoisomerase II–DNA complex, which results in the induction of DNA breakage, finally leading to apoptosis and cell death.[7] The sugar moieties of anthracyclines are critical for the anticancer activity, as is also the case for the biological activities of many other drugs,[8,9] since the aglycones themselves are usually inactive.[6] As regards the sugar moieties, studies directed towards investigation of the roles of the aminosugars in some anthracyclines have shown that the amino group in position C-3′ is not essential for cytotoxic activity and can be replaced by a hydroxy group, which results in reduced DNA-binding activity but is not an essential determinant for drug activity.[10] In addition, the presence and orientation of a hydroxy group at position C-4′ has been shown to be an important determinant for anthracycline activity. Consequently, Zhu and collaborators have proposed 2,6-dideoxyhexoses as a better choice for generating biologically active anthracycline derivatives through the synthesis of several daunorubicin analogues containing uncommon sugars.[6]

In recent years, with the isolation of a number of biosynthetic gene clusters and thanks to the advantages of the development and improvement of DNA recombinant technology and of the flexibility of enzymes involved in secondary metabolism, a new process to obtain new bioactive compounds—combina-

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torial biosynthesis[11–13]—is attracting great attention. In particular, great interest has been drawn to combinatorial biosynthesis with the use of genes involved in the biosynthesis of deoxyhexoses present in natural products produced by microorganisms.[8,14–16]

Steffimycin is a member of the anthracycline family. It had previously been reported to show a low inhibitory effect on the growth of mouse leukemia L1210[17] and P388 cells[18] but to be able to induce a high apoptotic response in HCT116 colon carcinoma cells expressing p53 by inducing DNA damage.[19]

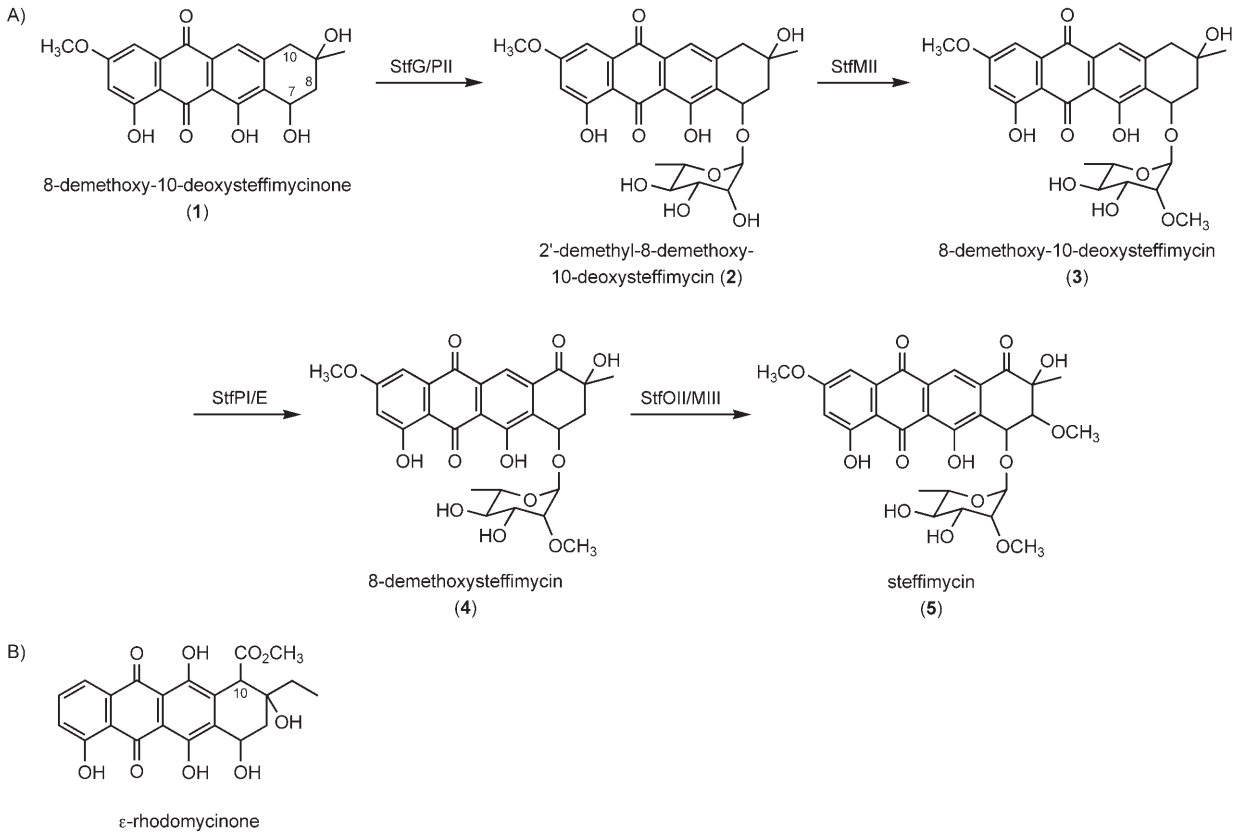
Recently we have reported the isolation and characterization of the biosynthetic gene cluster for the anthracycline steffimycin from *Streptomyces steffisburgensis*.[20] Here we describe the generation of twelve steffimycin derivatives by heterologous expression in *Streptomyces albus* of the steffimycin gene cluster and different combinations of genes directing the biosynthesis of several deoxysugars. We demonstrate the flexibility of L-rhamnose glycosyltransferase StfG and, in addition, we show the ability of 3′-*O*-methyltransferase OleY from the oleandomycin cluster to methylate deoxyhexoses attached to anthracyclinones. Analysis of the biological activities of those compounds against three human tumor cell lines is also reported.

Results and Discussion

Production of steffimycinone and its derivatives in *S. albus*

In a recent work we reported the expression of the steffimycin gene cluster (plasmid pEM4STFa) in the heterologous host *S. albus*.[20] The analysis of products accumulated by the corresponding recombinant strain (STFa) led to the identification of 8-demethoxy-10-deoxysteffimycinone, with an HPLC retention time of 21.25 min and *m/z* 371 $[M+H]^+$ (compound **1**, Scheme 1 A, Figure 1 A). In that experiment, no glycosylated compounds were detected, in agreement with the absence of genes involved in the biosynthesis of L-rhamnose, the 6-deoxysugar present in steffimycin, in the steffimycin gene cluster.[20] Compound **1** lacks the steffimycinone sugar moiety, as well as the methoxy group at the 8-position and the keto group at the 10-position.

Because of the absence of genes directing the biosynthesis of L-rhamnose in pEM4STFa and in *S. albus*, and also in order to verify that the steffimycin gene cluster contains all the genes required for the biosynthesis of this anthracycline, plasmid pRHAM was introduced into *S. albus* STFa, generating strain STFaRHAM. pRHAM has previously been shown to contain all genes necessary for the biosynthesis of L-rhamnose and, when introduced into a streptomycete host, to endow the host with the capability to synthesize L-rhamnose.[21] Cultures of strain STFaRHAM analyzed by HPLC showed the pro-



Scheme 1. A) Final steps in steffimycin biosynthesis, and B) structure of ε-rhodomycinone.

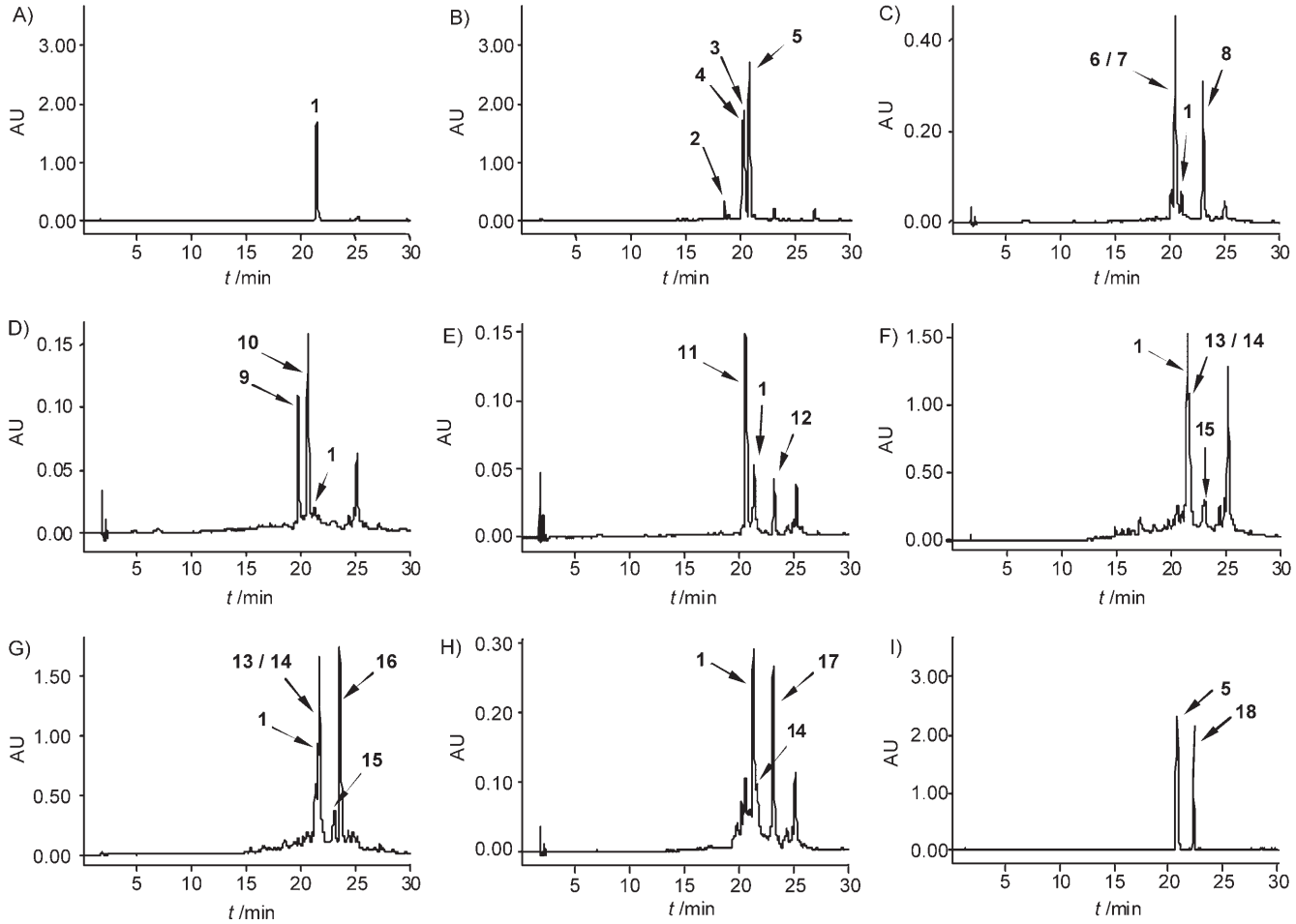


Figure 1. HPLC analyses of cultures of *S. albus* strains: A) STFa, B) STFaRHAM, C) STFaMP1B, D) STFaMP3B, E) STFaLN2, F) STFaLNBIV, G) STFaFL844, H) STFaFL942, and I) *S. steffisburgensis* strain STFLR14.

duction of different glycosylated metabolites with HPLC retention times and masses ($[M+H]^+$) of 18.7 min and 517 *m/z* (compound **2**), 20.1 min and 545 *m/z* (compound **4**), 20.3 min and 531 *m/z* (compound **3**), and 20.7 min and 575 *m/z* (compound **5**) (Figure 1 B). Molecular ions corresponding to fragments of those compounds lacking the sugar moiety were detected for each case, corresponding to 371, 385, 371, and 415 *m/z* ($[M+H]^+$), respectively. All these mass analyses are consistent with compounds **2**, **3**, **4**, and **5**, corresponding to 2′-demethyl-8-demethoxy-10-deoxysteffimycin, 8-demethoxy-10-deoxysteffimycin, 8-demethoxysteffimycin, and steffimycin, respectively (Scheme 1 A). These experiments demonstrated that pEM4STFa contains all the genes required for the tailoring modification steps and decoration of the sugar moiety that occurs after L-rhamnose attachment. In addition, production of steffimycin and intermediates by *S. albus* STFaRHAM showed similar titers to those obtained in *S. steffisburgensis* wild type (data not shown).

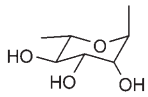
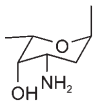
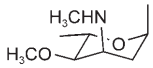
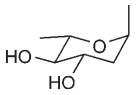
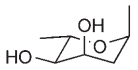
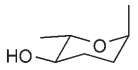
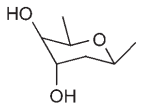
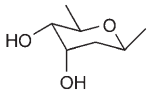
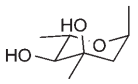
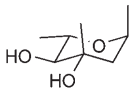
Taking advantage on the production of steffimycin in *S. albus* containing plasmids pEM4STFa and pRHAM, we addressed the possibility of generating novel derivatives of steffimycin by coexpression of plasmid pEM4STFa together with plasmids directing the biosyntheses of different 2,6-deoxysugars such as aminodeoxysugars, neutral deoxysugars, including 2,3,6-deoxysugars, and branched chain deoxysugars.

Plasmids directing the biosynthesis of aminodeoxysugars

Plasmids pWHM1910 and pWHM1919 contain genes involved in the biosynthesis of L-daunosamine in *S. peucetius* and a gene coding for the DnmS glycosyltransferase responsible for the attachment of L-daunosamine to ε-rhodomycinone during the biosynthesis of daunorubicin and doxorubicin.[22] The difference between pWMH1910 and pWHM1919 lies in the absence in the latter of *dnrH*, a gene coding for a glycosyltransferase that might govern the addition of a second L-daunosamine to the C-4′ hydroxy group of daunorubicin.[23] Plasmid pETAS contains genes involved in the biosynthesis of L-ristosamine in *S. longisporoflavus* during the biosynthesis of staurosporine.[24]

HPLC-MS analysis of *S. albus* containing these plasmids (strains STFa1910, STFa1919, and STFaETAS) showed no evidence of production of any glycosylated compound. This negative result might be the result of the inability of StfG to recognize L-aminosugars, although as described below, StfG possesses a certain degree of substrate flexibility, being able to attach

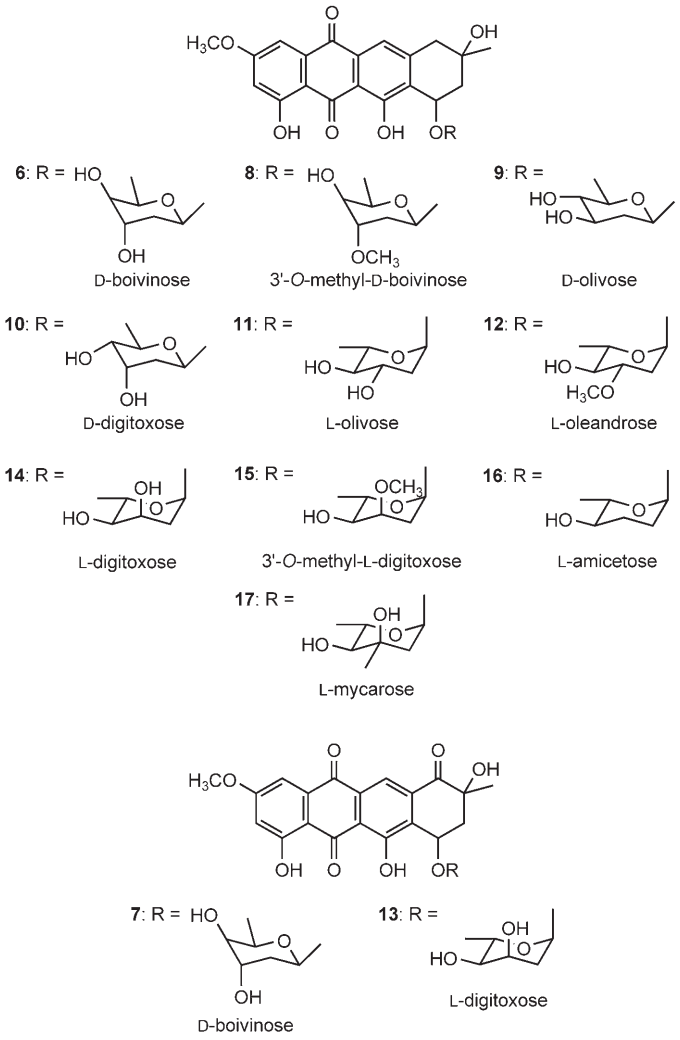
Table 1. Plasmids used in this work and 6-deoxyhexoses synthesized.

Plasmid	*S. albus* strain	Genes	6-Deoxyhexose
pEM4STFa[20]	STFa	steffimycin gene cluster	–
pRHAM[21]	STFaRHAM	*oleL, oleS, oleE, oleU*	
pWHM1910[22]	STFa1910	*dnrI, dnmJ, dnmV, dnmU, dnmZ, dnrH, dnmT, dnmW, dnmQ, dnmS*	
pWHM1919[22]	STFa1919	*dnrI, dnmJ, dnmV, dnmU, dnmZ, dnmT, dnmW, dnmQ, dnmS*	L-daunosamine
pETAS[24]	STFaETAS	*staMA, staJ, staK, staI, staE, staMB*	
pLN2[28]	STFaLN2	*oleV, oleW, oleU, oleY, oleL, oleS, oleE*	
pLNBIV[29]	STFaLNBIV	*oleV, oleW, eryBIV, oleY, oleL, oleS, oleE*	
pFL844[30]	STFaFL844	*oleV, oleW, eryBIV, oleY, oleL, oleS, oleE, urdQ*	
pMP1*BII[27]	STFaMP1B	*mtmE, mtmD, oleV, eryBII, oleU, oleY*	
pMP3*BII[27]	STFaMP3B	*mtmE, mtmD, oleV, eryBII, urdR, oleY*	
pFL942[31]	STFaFL942	*mtmE, mtmD, oleV, eryBII, eryBIV, eryBIII, eryBVII*	
pFL947[31]	STFaFL947	*mtmE, mtmD, oleV, oleW, eryBIV, mtmC, eryBVII*	
pLR14-b4[33]	LR14	*oleY*	–

neutral or branched chain deoxysugars to the aglycone 8-demethoxy-10-deoxysteffimycinone. This inability contrasts with previous reports of glycosyltransferases that normally transfer aminosugars and have been demonstrated also to transfer neutral deoxysugars. This is the case with glycosyltransferase StaG, involved in the attachment of the aminosugar L-ristosamine to the indolocarbazole staurosporine aglycone.[24] In strains STFa1910 and STFa1919 another glycosyltransferase, DnmS, is also present[22] (Table 1). The fact that no glycosylated compounds were produced points to a lack of recognition of 8-demethoxy-10-deoxysteffimycinone by DnmS. Structural differences between 8-demethoxy-10-deoxysteffimycinone and ε-rhodomycinone, the natural substrate of DnmS, could be the reason for the absence of sugar transfer. ε-Rhodomycinone possesses a carboxymethyl group in position C-10, required for the fourth ring cyclization reaction, a Claisen reaction, during the biosynthesis of ε-rhodomycinone.[25] The 10-carboxymethyl group is finally removed by esterase DnrP after the introduction of L-daunosamine by DnmS during daunorubicin biosynthesis.[26] In steffimycin biosynthesis no carboxymethyl group in position C-10 is present at any time, due to the operation of a different mechanism for the fourth ring cyclization.[20] It seems plausible that the C-10 carboxymethyl group of ε-rhodomycinone might be involved not only in the activation of fourth ring cyclization but also in the recognition of the aglycone by glycosyltransferase DnmS.

Plasmids directing the biosynthesis of neutral deoxysugars

For the generation of steffimycin derivatives containing D-deoxysugars, plasmids pMP1*BII and pMP3*BII were used (Table 1). Plasmid pMP1*BII directs the biosynthesis of the 2,6-dideoxyhexose D-boivinose.[27] From cultures of strain STFaMP1B (harboring pMP1*BII), three new compounds with HPLC retention times and masses ($[M+H]^+$) of 20.26 min and 501 *m/z* (compound **6**), 20.58 min and 515 *m/z* (compound **7**), and 23.7 min and 515 *m/z* (compound **8**) were isolated (Figure 1C). In two of them, compounds **6** and **8**, fragmentation ions with masses of 371 *m/z*, corresponding to 8-demethoxy-10-deoxysteffimycinone derivatives, were observed. In the third, compound **7**, a fragmentation ion of 385 *m/z* corresponding to 8-demethoxy-deoxysteffimycinone was observed.



Scheme 2. Proposed structures for the new glycosylated steffimycins.

According to the observed masses those compounds might correspond to D-boivinosyl-8-demethoxy-10-deoxysteffimycin (compound **6**), D-boivinosyl-8-demethoxysteffimycin (compound **7**), and 3′-*O*-methyl-D-boivinosyl-8-demethoxy-10-deoxysteffimycin (compound **8**; Scheme 2).

Plasmid pMP3*BII directs the biosynthesis of 2,6-deoxysugar D-digitoxose.[27] Two new compounds—**9** and **10**, with HPLC retention times of 19.73 and 20.66 min, respectively (Figure 1 D)—were detected in cultures of strain STFaMP3B (harboring pMP3*BII). Masses of 501 and 371 *m/z* ($[M+H]^+$) corresponding to the parent and fragmentation ions were observed for both of them. Plasmid pMP3*BII directs the biosynthesis of the intermediate NDP-2,6-dideoxy-D-glycero-4-hexulose, which is converted into D-digitoxose by 4-ketoreductase UrdR. In addition, as a result of C-3 to C-4 tautomerism leading to NDP-4-keto-2,6-dideoxy-D-glucose and UrdR activity, derivatives containing D-olivose can be also obtained, although in lower amounts.[27] In view of this previous information and of the relative amounts of compounds **9** and **10** produced, they might correspond to D-olivosyl-8-demethoxy-10-deoxysteffimycin and D-digitoxosyl-8-demethoxy-10-deoxysteffimycin, respectively (Scheme 2).

Three plasmids—pLN2, pLNBIV, and pFL844—directing the biosynthesis of different neutral L-deoxysugars were also used to generate steffimycin analogues. When plasmid pLN2, which directs the biosynthesis of the 2,6-deoxyhexose L-olivose,[28] was expressed in *S. albus* STFa (strain STFaLN2), production of two new compounds—**11** and **12**, with retention times and masses ($[M+H]^+$) of 20.45 min and 501 *m/z* and 23.08 min and 515 *m/z*, respectively—was detected by HPLC-MS (Figure 1 E). Since, in each case, a fragmentation ion with a mass of 371 *m/z* was observed, these compounds might correspond to L-olivosyl-8-demethoxy-10-deoxysteffimycin (compound **11**) and L-oleandrosyl-8-demethoxy-10-deoxysteffimycin (compound **12**), the latter resulting from the action of the OleY methyltransferase (Scheme 2).

Production of steffimycin derivatives containing the 2,6-deoxyhexose L-digitoxose was achieved with plasmid pLNBIV.[29] From cultures of strain STFaLNBIV, three new compounds with HPLC-MS retention times and masses corresponding to the parent and fragmentation ions ($[M+H]^+$) of 21.5 min and 515/385 *m/z* (compound **13**), 21.6 min and 501/371 *m/z* (compound **14**), and 23.02 min and 515/371 *m/z* (compound **15**) were isolated (Figure 1 F). These data are consistent with compounds **13**, **14**, and **15** being L-digitoxosyl-8-demethoxysteffimycin, L-digitoxosyl-8-demethoxy-10-deoxysteffimycin, and 3′-*O*-methyl-L-digitoxosyl-8-demethoxy-10-deoxysteffimycin, respectively (Scheme 2).

Plasmid pFL844[30] was used to obtain steffimycin derivatives containing the 2,3,6-deoxyhexose L-amicetose. Cultures of strain STFaFL844 showed the presence of a new peak corresponding to compound **16** (Figure 1 G), with an HPLC retention time of 23.54 min and masses of 485 *m/z* ($[M+H]^+$) for the parent ion and 371 *m/z* ($[M+H]^+$) for the fragmentation ion, that might correspond to L-amicetosyl-8-demethoxy-10-deoxysteffimycin (Scheme 2). In addition, three other peaks with the same retention times and masses as compounds **13**, **14**, and **15** were also observed (Figure 1 F and G). Plasmid pFL844 directs the biosynthesis of L-amicetose and it has been also shown to produce, although to a lesser extent, L-digitoxose through the agency of 4-ketoreductase EryBIV acting before the 3-dehydratase UrdQ removes the C-3 hydroxy group.[30] In order to allow in vitro antitumor tests, compounds **13**, **14**, and **16** were purified from cultures of strain STFaFL844. Compound **15** was not recovered in sufficient amount to be tested.

Plasmids directing the biosynthesis of branched-chain deoxysugars

Two plasmids harboring genes coding for the activities required for the biosynthesis of the branched-chain 2,6-deoxyhexoses L-mycarose (pFL942) and 4-deacetyl-L-chromose B (pFL947)[31] were also used. Only one of the *S. albus* strains generated, STFaFL942, showed the accumulation of a new glycosylated compound: compound **17**, with an HPLC retention time of 23.23 min and a mass of 515 *m/z* ($[M+H]^+$; Figure 1 H). Since the fragmentation ion showed a mass of 371 *m/z*

Table 2. In vitro grown inhibition of three human tumor cell lines by steffimycin and novel derivatives.

	Compound	Tumor cell line, GI_{50} [μM]		
		MDA-MB-231	NSCLC A549	HT29
5	steffimycin	4.87	2.61	6.79
6	D-boivinosyl-8-demethoxy-10-deoxysteffimycin	1.86	>10	>10
7	D-boivinosyl-8-demethoxysteffimycin	0.99	8.75	2.53
8	3′-*O*-methyl-D-boivinosyl-8-demethoxy-10-deoxysteffimycin	>10	14.8	>10
9	D-olivosyl-8-demethoxy-10-deoxysteffimycin	15.2	11	>10
10	D-digitoxosyl-8-demethoxy-10-deoxysteffimycin	0.34	0.59	0.28
11	L-olivosyl-8-demethoxy-10-deoxysteffimycin	>10	13	>10
12	L-oleandrosyl-8-demethoxy-10-deoxysteffimycin	17.3	14.8	>10
13	L-digitoxosyl-8-demethoxysteffimycin	4.80	5.39	8.19
14	L-digitoxosyl-8-demethoxy-10-deoxysteffimycin	2.72	15.7	9.14
16	L-amicetosyl-8-demethoxy-10-deoxysteffimycin	>10	>10	>10
17	L-mycarosyl-8-demethoxy-10-deoxysteffimycin	17.5	9.33	>10
18	3′-*O*-methylsteffimycin	0.98	1.16	0.81

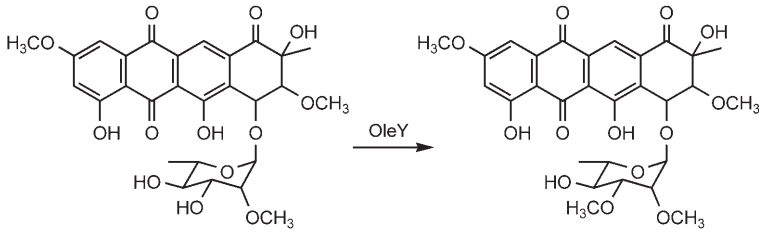
($[M+H]^+$), compound **17** might correspond to L-mycarosyl-8-demethoxy-10-deoxysteffimycin (Scheme 2). In addition, traces of L-digitoxosyl-8-demethoxy-10-deoxysteffimycin (compound **14**) were also detected. Both L-mycarose and L-digitoxose share the common intermediate NDP-2,6-dideoxy-D-glycero-4-hexulose, which upon C-3 methylation by EryBIII followed by C-4 ketoreduction leads to L-mycarose and in the absence of methylation affords L-digitoxose.[31] In the case of strain STFaFL947 no production of glycosylated metabolites was detected, probably due to the different configurations of methyl and hydroxy groups at position C-3 between L-mycarose and 4-deacetyl-L-chromose B (Table 2).

In all experiments described above, production of new glycosylated compounds fluctuated with respect to that observed in control strain STFaRHAM, between 35% for strain STFaFL844 and 7% for strain STFaLN2. However, flexibility of glycosyltransferase StfG is clearly demonstrated by the identification of twelve new glycosylated derivatives of 8-demethoxy-10-deoxysteffimycinone, harboring both D- and L-deoxyhexoses, with different degrees of deoxygenation at positions C-6′ (L-rhamnose), C-2′ and C-6′ (L-digitoxose, D-digitoxose, L-olivose, and D-boivinose), and C-2′, C-3′, and C-6′ (L-amicetose), and even with *C*-methylation at C-3′ (L-mycarose; Scheme 2). The flexibility of some anthracycline glycosyltransferases has been shown previously for AraGT, involved in the biosynthesis of aranciamycin and capable of recognizing 2,3,6-L-deoxyhexoses (L-rhodinose), 2,3,6-D-deoxyhexoses (D-amicetose), and branched chain 2,6-L-deoxyhexoses (L-axenose), in addition to its natural substrate L-rhamnose.[15,32] Another issue is why all the glycosylated compounds contain either 8-demethoxysteffimycinone or 8-demethoxy-10-deoxysteffimycinone as aglycone. In steffimycin biosynthesis, it is believed that StfPI and StfE introduce the keto group at C-10 after StfMII has methylated the C-2′ hydroxy group in L-rhamnose[20] (Scheme 1). It might be possible that for the correct activity of StfPI and StfE the presence of a methoxy group at C-2′ is required. None of the new D- or L-deoxysugar derivatives identified in this work has a hydroxy group available in C-2′ for methylation by StfMII. According to this, only two compounds (**7** and **13**), produced at low levels, harboring a keto group at C-10, have been identified (Scheme 2). The final step in steffimycin biosynthesis is performed by enzymes StfOII and StfMIII, which are believed to introduce the methoxy group at C-8[20] (Scheme 1 A). No derivatives of steffimycin containing decoration at C-8 have been identified, probably due to the absence of the keto group at C-10 in most of them, and also probably because of the low production of compounds containing the C-10 keto group: compounds **7** and **13**.

Expression of oleY in *S. albus* and *S. steffisburgensis*

As mentioned above, experiments with strains STFaMP1B, STFaLN2, and STFaFL844 showed the presence of compounds **8**, **12**, and **15** (Scheme 2), each of them containing a methyl group in the sugar moiety. In plasmids pMP1*BII, pLN2, and pFL844 only one gene—*oleY*—coding for an *O*-methyltransferase is present (Table 1). OleY has been reported to methylate the hydroxy group present at the C-3′ position of L-olivose in the 14-membered macrolide L-olivosyl-erythronolide B, leading to L-oleandrosyl-erythronolide B.[33] This methyltransferase has been shown to be able to methylate several sugar residues attached to macrolactone rings.[33] However, no methylation at the C-3′ hydroxy group has been observed when L-olivose or other deoxysugars are attached to 8-demethyl-tetracenomycin C, an aglycone found in several derivatives of elloramycin.[27,28] In elloramycin and its derivatives the sugar attachment is established at its aromatic 8-position[27,28] rather than at a secondary alcohol such as C-7 in steffimycin and other anthracyclines.

To verify that OleY is in fact capable of 3′-*O*-methylation in anthracycline compounds, a biotransformation experiment of steffimycin, which possesses a free hydroxy group at C-3′, was performed with strain LR14, harboring plasmid pLR14-b4 for *oleY* expression.[33] Analysis by HPLC showed the biotransformation of 40% steffimycin into a new compound, **18** (Scheme 3), with a retention time of 22.19 min and a mass of 589 *m/z* ($[M+H]^+$). A fragmentation ion with a mass of 415 ($[M+H]^+$) was detected. The masses observed fitted with a steffimycin derivative with an additional methyl group in the sugar moiety that might correspond to 3′-*O*-methylsteffimycin.



Scheme 3. Methylation of steffimycin by the 3′-*O*-methyltransferase OleY.

In addition, *oleY* was expressed in *S. steffisburgensis*. Cultures of strain STFLR14 showed the production of steffimycin (compound **5**) and 3′-*O*-methylsteffimycin (compound **18**) but no other steffimycin glycosylated intermediates harboring the C-3′ *O*-methyl group (Figure 1 l) were detected. These results confirm the capability of OleY to methylate steffimycin, suggesting that it methylates the final product more efficiently than it does other glycosylated intermediates.

Analysis of biological activity

The antitumor activities of the twelve new steffimycin derivatives obtained were tested against three human tumor cell lines—breast adenocarcinoma, non-small cell lung cancer, and colon adenocarcinoma—with steffimycin as reference compound (Table 2). Steffimycin showed moderate antitumor activity, with GI_{50} values between 2.61 and 6.79 μM depending upon the cell line (Table 2). The twelve new compounds tested can be divided into three different groups according to their level of antitumor activity with respect to steffimycin. The first group would include compounds with lower antitumor activity than steffimycin, their GI_{50} values being above 10 μM (compounds **6**, **8**, **9**, **11**, **12**, **16**, and **17**). The second group would comprise compounds with GI_{50} values similar to those shown by steffimycin (compounds **7**, **13**, and **14)**. The third group would include compounds **10** and **18**, showing antitumor activities between two and 24 times higher than steffimycin for each cell line (Table 2).

From the results described above and the predicted structures of the compounds it is clear that methylation of the hydroxy group at position C-3′ of 2′-*O*-methyl-L-rhamnose greatly increases the activity of steffimycin, as shown by compound **18** (3′-*O*-methylsteffimycin). In addition, the presence of D-digitoxose represents the best improvement in antitumoral activity, as is shown by compound **10** (D-digitoxosyl-8-demethoxy-10-deoxysteffimycin). In this compound, additional changes such as the absence of keto and methoxy groups at positions C-8 and C-10 might correspond to improvements in activity, since highly active anthracyclines such as daunorubicin, doxorubicin, or epirubicin lack both 8-methoxy and 10-keto groups or any other decoration at their 8- and 10-positions. However, on comparison of the GI_{50} values of compound **6** (D-boivinosyl-8-demethoxy-10-deoxysteffimycin) versus **7** (D-boivinosyl-8-demethoxysteffimycin) and those of **13** (L-digitoxosyl-8-demethoxysteffimycin) versus **14** (L-digitoxosyl-8-demethoxy-10-deoxysteffimycin), the presence of the keto group at C-10 seems to correspond to a small improvement in activity against tumor cell lines NSCLC A549 and HT29 (Table 2).

Other remarkable inferences, such as the importance of hydroxy group configuration at positions C-3′ and C-4′ of the sugar moiety, can be drawn from the antitumor activity data shown in this work. Compounds **9** (D-olivosyl-8-demethoxy-10-deoxysteffimycin) and **10** (D-digitoxosyl-8-demethoxy-10-deoxysteffimycin) have the same predicted structure save for the equatorial or axial configurations of their C-3′ hydroxy groups, which translated into better antitumoral activity for compound **10** with the axial configuration (Table 2, Scheme 2). In addition, compounds **6** (D-boivinosyl-8-demethoxy-10-deoxysteffimycin) and **10** (D-digitoxosyl-8-demethoxy-10-deoxysteffimycin), with the same predicted configuration at C-3′ hydroxy group but opposite ones at C-4′ (equatorial in compound **10**), and with no other difference between them show great differences in antitumor activity against all cell lines tested (Table 2, Scheme 2). The importance of these groups for improvement of anticancer activity in daunorubicin analogues has previously been shown by Zhu and collaborators.[6] In addition, the relevance of changes in the sugar moiety profile for anticancer activity in aranciamycin has recently been shown.[15]

The best antitumor activities have been gained with compounds harboring a neutral D-2,6-deoxyhexose—D-digitoxose in compound **10**—while the equivalent compound carrying a L-2,6-dexoxyhexose, L-digitoxose in compound **14**, presents an activity not different from that shown by steffimycin (Table 2, Scheme 2). However, in other cases, such as with compounds **9** and **11**, containing D-olivose and L-olivose, respectively, no such improvement in antitumor activity is correlated with the presence of the D-2,6-deoxysugar (Table 2, Scheme 2). Finally, no improvements in antitumor activity were observed in compounds containing either a neutral L-2,3,6-deoxyhexose, such as L-amicetose in compound **16**, or a branched chain L-2,3-deoxyhexose such as L-mycarose in compound **17** (Table 2, Scheme 2).

Structural elucidation

On the basis of the biological activity data and the availability of different compounds for comparison, the structures of compounds **10**, **13**, **16**, **17**, and **18** were elucidated by NMR and high-resolution MS (see physicochemical data below). These studies confirmed compound **10** as β-D-digitoxosyl-8-demethoxy-10-deoxysteffimycin. Characteristic for this compound were the missing 10-oxygen, which is replaced by two protons (a CH_2 signal is visible at $\delta = 44.4$ in the ^{13}C NMR spectrum), the missing 8-OMe group, the β-glycosidically bound sugar (large coupling constant of 1′-H), the 3′-H signal (only small e,a or e,e couplings), and the 4′-H with one large and one small coupling.

Compound **13** was identified as α-L-digitoxosyl-8-demethoxy-steffimycin. Characteristic for this compound were the missing 8-OCH_3 group and the sugar signals (the small coupling constant of 1′-H indicating an α-glycosidically bound sugar), the equatorial 3′-H with only small couplings besides the H,OH coupling of 6.5 Hz, and the 4′-H signal (one large a,a coupling of 9.5 Hz, a H,OH coupling of 7 Hz, and a 3 Hz e,a coupling with 3′-H).

Compound **16** was identified as α-L-amicetosyl-8-demethoxy-10-deoxysteffimycin. Its aglycon showed the same missing 10-CO group and 8-OCH_3 signal as found for compound **10**, and the sugar moiety shows characteristic signals for an α-glycosidically linked 2,3,6-trideoxysugar with equatorial 4′-OH group. Typical for this type of sugar are the complex overlapping multiplets of the 2′- and 3′-CH_2 group, which are visible as CH_2 signals in the ^{13}C NMR at typical chemical shifts (δ_C = 27.9 and 30.0 ppm), as well as the axial 4′H, which showed two large a,a couplings and two smaller couplings, one of them a H,OH coupling.

Compound **17** was identified as α-L-mycarosyl-8-demethoxy-10-deoxysteffimycin. While the aglycon moiety shows the same characteristics as its counterparts in compounds **10** and **16**, the sugar moiety was identified as an α-glycosidically 3-branched 2-deoxy-L-sugar. Typical for this was the small e,a coupling of 1′-H, the interrupted spin system, the additional 3′-CH_3 group, and the 4′-H signal as a doublet with only one large (9.7 Hz) a,a coupling.

Compound **18** was easily identified as 3′-*O*-methylsteffimycin because of its additional 3′-OCH_3 signal (at δ_H = 3.54 ppm) as the only difference relative to the ^1H NMR spectrum of steffimycin.

Experimental Section

Strains, culture conditions, and plasmids: *Streptomyces steffisburgensis* NRRL3193, steffimycin producer,[34] and *Streptomyces albus* J1074[35] were routinely grown on tryptone soya broth (TSB). Standard procedures were used for protoplasts formation and transformation.[36] After regeneration, clones were grown on agar plates containing A medium (MA) for sporulation.[37]

Plasmid pEM4STFa (Table 1) carrying the steffimycin gene cluster[20] was used to transform *S. albus* J1074 protoplasts, leading to strain STFa routinely selected with apramycin (25 μg mL^{-1}). Plasmids directing the biosynthesis of deoxysugars were used to transform *S. albus* STFa protoplasts, leading to the strains listed in Table 1 routinely selected with apramycin (25 μg mL^{-1}) and thioestrepton (50 μg mL^{-1}). Plasmid pLR14-b4[33] (Table 1) was introduced into *S. steffisburgensis* and *S. albus* by protoplast transformation, and transformants were selected with thiostrepton (50 μg mL^{-1}), leading to strains STFLR14 and LR14, respectively.

Biotransformation experiments: Liquid cultures of *S. albus* LR14 were grown as a seed culture in TSB (30 mL in 250 mL Erlenmeyer flasks). After 2 days incubation in a rotary incubator (30 °C; 250 rpm), the cultures (2.5%, *v/v*) were used to inoculate R5A liquid medium (25 mL). After two additional days of incubation with shaking, steffimycin dissolved in methanol (100 μg mL^{-1}) was added to the cultures. After one additional day of incubation the cultures were harvested for analysis.

Analysis of anthracycline production: Anthracycline production was assessed by growing *S. albus* or *S. steffisburgensis* strains on solid and liquid R5A medium[37] and analyzed by HPLC and HPLC-MS as described before[20] to verify the production of novel steffimycin derivatives.

Isolation of the new steffimycin glycosylated derivatives: Compounds produced by *S. albus* STFaFL844 were purified from 3 L cultures by the procedure described previously.[20] Compounds produced by *S. albus* strains STFaFL942, STFaMP1B, STFaMP3B, and STFaLN2 and by *S. steffisburgensis* STFLR14 were purified from solid R5A medium agar plates. Liquid cultures of each strain were grown as seed cultures in TSB as described above and were used to inoculate the agar plates. After 5 days at 30 °C, agar media were extracted with ethyl acetate and concentrated by evaporation with a rotavapor. The extracts were dissolved in methanol (4–5 mL) and then chromatographed as described previously.[20]

Physicochemical properties: NMR data were acquired on a Varian Inova 400 NMR spectrometer; assignments and multiplicities were based on 2D-experiments (H,H-COSY, HSQC and CIGAR-HMBC) and comparison with literature data. Signals not listed below were not observed.

β-D-Digitoxosyl-8-demethoxy-10-deoxysteffimycin (10): ^1H NMR (400 MHz, [D_6]acetone): δ = 1.26 (d, J = 6.9 Hz, 3H; 6′-H_3), 1.36 (s, 3H; 9-CH_3), 1.56 (dd, J_1 = 12.5 Hz, J_2 = 5.4 Hz, 1H; 8-H_A), 2.00 (dd, J_1 = 12.5 Hz, J_2 = 4.0 Hz, 1H; 8-H_B), 2.17 (ddd, J_1 = 13.0 Hz, J_2 = 3.0 Hz, J_3 = 3.0 Hz, 1H; 2′-H_A), 2.70 (ddd, J_1 = 13.0 Hz, J_2 = 3.0 Hz, J_3 = 3.0 Hz, 1H; 2′-H_B), 2.86 (d, J = 16.6 Hz, 1H; 10-H_A), 3.04 (d, J = 16.6 Hz, 1H; 10-H_B), 3.08 (dd, J_1 = 9.6 Hz, J_2 = 3.8 Hz, 1H; 4′-H), 3.14 ddd (J_1 = 3.8 Hz, J_2 = 3.0 Hz, J_3 = 3.0 Hz, 1H; 3′-H), 3.78 (dq, J_1 = 9.6 Hz, J_2 = 6.9 Hz, 1H; 5′-H), 3.99 (s, 3H; 2-OCH_3), 5.11 (dd, J_1 = 5.4 Hz, J_2 = 4.0 Hz, 1H; 7-H), 5.27 (dd, J_1 = 9.3 Hz, J_2 = 3.0 Hz, 1H; 1′-H), 6.79 (d, J = 2.5 Hz, 1H; 3-H), 7.27 (d, J = 2.5 Hz, 1H; 1-H), 7.48 (s, 1H; 11-H), 12.23 (br s, exchangeable by D_2O, 1H; 6-OH), 12.69 ppm (br s, exchangeable by D_2O, 1H; 4-OH); ^{13}C NMR (100.6 MHz, [D_6]acetone): δ = 18.2 (C-6′), 28.2 (9-CH_3), 38.5 (C-2′), 44.3 (C-8), 44.8 (C-10), 56.1 (2-OCH_3), 68.2 (C-9), 68.4 (C-5′), 69.5 (C-7), 72.1 (C-3′), 73.3 (C-4′), 100.3 (C-1′), 106.4 (C-3), 108.1 (C-1), 113.5 (C-11), 116.2 (C-4a), 120.4 (C-5a), 129.1 (C-6a), 133.2 (C-12a), 138.1 (C-11a), 147.5 (C-10a), 162.2 (C-6), 165.6 (C-4), 166.9 (C-2), 187.2 (C-12), 196.1 ppm (C-5); HR-FAB (*m/z* 500.1672; calcd. for $C_{26}H_{28}O_{10}$: 500.1682).

α-L-Digitoxosyl-8-demethoxysteffimycin (13): ^1H NMR (400 MHz, [D_6]acetone): δ = 1.26 (d, J = 6.2 Hz, 3H; 6′-H_3), 1.40 (s, 3H; 9-CH_3), 1.92 (dd, J_1 = 12.3 Hz, J_2 = 6.4 Hz, 1H; 8-H_A), 2.08 (dd, J_1 = 12.3 Hz, J_2 = 4.7 Hz, 1H; 8-H_B), 2.20 (ddd, J_1 = 13.0 Hz, J_2 = 3.5 Hz, J_3 = 3.5 Hz, 1H; 2′-H_A), 2.29 (ddd, J_1 = 13.0 Hz, J_2 = 3.0 Hz, J_3 = 1.0 Hz, 1H; 2′-H_B), 3.09 (ddd, J_1 = 9.5 Hz, J_2 = 7.0 Hz, J_3 = 3.0 Hz, 1H; 4′-H), 3.14 ddd (J_1 = 6.5 Hz, J_2 = 3.5 Hz, J_3 = 3.0 Hz, 1H; 3′-H), 3.35 (d, J = 6.5 Hz, exchangeable with D_2O, 1H; 3′-OH), 3.27 (d, J = 7 Hz, exchangeable with D_2O, 1H; 4′-OH), 3.83 (dq, J_1 = 9.5 Hz, J_2 = 6.5 Hz, 1H; 5′-H), 3.99 (s, 3H; 2-OCH_3), 5.01 (dd, J_1 = 6.4 Hz, J_2 = 4.7 Hz, 1H; 7-H), 5.27 (d, J = 3.5 Hz, 1H; 1′-H), 6.79 (d, J = 2.6 Hz, 1H; 3-H), 7.26 (d, J = 2.6 Hz, 1H; 1-H), 7.49 (s, 1H; 11-H), 12.23 (br s, exchangeable by D_2O, 1H; 6-OH), 12.80 ppm (br s, exchangeable by D_2O, 1H; 4-OH); ^{13}C NMR (100.6 MHz, [D_6]acetone): δ = 22.6 (C-6′), 28.2 (9-CH_3), 40.9 (C-2′), 48.9 (C-8), 61.2 (2-OCH_3), 70.1 (C-7), 72.5 (C-9), 73.4 (C-5′), 76.2 (C-3′), 78.3 (C-4′), 98.4 (C-1′), 105.6 (C-3), 108.1 (C-1), 113.3 (C-11), 122.7 (C-5a), 146.2 (C-10a), 161.2 (C-6), 163.7 (C-4), 167.0 (C-2), 187.8 (C-12), 196.2 (C-5) 199.2 ppm (C-10). HR-FAB (*m/z* 514.1464; calcd. for $C_{26}H_{26}O_{11}$: 514.1475).

α-L-Amicetosyl-8-demethoxy-10-deoxysteffimycin (16): ^{1}H NMR (400 MHz, [D$_6$]acetone): $\delta = 1.19$ (d, $J = 6.2$ Hz, 3H; 6′-H$_3$), 1.43 (s, 3H; 9-CH_3), 1.60 (dddd, $J_1 = 11.0$ Hz, $J_2 = 4.2$ Hz, $J_3 = 4.2$ Hz, $J_4 = 4.2$ Hz, 1H; 3′-H$_{eq}$), 1.68 (m, 1H; 2′-H$_A$), 1.72 (m, 1H; 3′-H$_{ax}$), 1.79 (m, 1H; 2′-H$_B$), 2.18 (dd, $J_1 = 12.1$ Hz, $J_2 = 5.7$ Hz, 1H; 8-H$_A$), 2.26 (dd, $J_1 = 12.1$ Hz, $J_2 = 3.2$ Hz, 1H; 8-H$_B$), 2.88 (d, $J = 17.3$ Hz, 1H; 10-H$_A$), 3.07 (d, $J = 17.3$ Hz, 1H; 10-H$_B$), 3.15 (dddd, $J_1 = 11.0$ Hz, $J_2 = 11.0$ Hz, $J_3 = 4.2$ Hz, $J_4 = 4.2$ Hz, 1H; 4′-H), 3.75 (dq, $J_1 = 11.0$ Hz, $J_2 = 6.2$ Hz, 1H; 5′-H), 3.98 (s, 3H; 2-OCH_3), 4.95 (d, $J = 4.2$ Hz, 1H; 4′-OH), 5.03 (dd, $J_1 = 5.7$ Hz, $J_2 = 3.2$ Hz, 1H; 7-H), 5.19 (br d, $J_1 = 2.7$ Hz, 1H; 1′-H), 6.78 (d, $J = 2.5$ Hz, 1H; 3-H), 7.25 (d, $J = 2.5$ Hz, 1H; 1-H), 7.47 (s, 1H; 11-H), 12.22 (br s, exchangeable by D$_2$O, 1H; 6-OH), 12.73 ppm (br s, exchangeable by D$_2$O, 1H; 4-OH); ^{13}C NMR (100.6 MHz, [D$_6$]acetone): $\delta = 17.8$ (C-6′), 27.9 (C-3′), 28.4 (9-CH_3), 30.0 (C-2′), 43.8 (C-8), 44.9 (C-10), 56.8 (2-OCH_3), 68.8 (C-9), 70.4 (C-5′), 69.4 (C-7), 72.4 (C-4′), 99.4 (C-1′), 106.4 (C-3), 108.1 (C-1), 113.6 (C-11), 116.1 (C-4a), 120.4 (C-5a), 131.2 (C-6a), 132.5 (C-12a), 138.2 (C-11a), 147.1 (C-10a), 162.9 (C-6), 165.6 (C-4), 167.1 (C-2), 187.1 (C-12), 193.8 ppm (C-5); HR-FAB (*m/z* 484.1735; calcd. for $C_{26}H_{28}O_9$: 484.1733).

α-L-Mycarosyl-8-demethoxy-10-deoxysteffimycin (17): ^{1}H NMR (400 MHz, [D$_6$]acetone): $\delta = 1.20$ (s, 3H; 3′-CH_3), 1.28 (d, $J = 6.8$ Hz, 3H; 6′-H$_3$), 1.45 (s, 3H; 9-CH_3), 1.95 (dd, $J_1 = 12.1$ Hz, $J_2 = 5.3$ Hz, 1H; 8-H$_A$), 2.13 (dd, $J_1 = 12.1$ Hz, $J_2 = 6.7$ Hz, 1H; 8-H$_B$), 2.15 (dd, $J_1 = 15.0$ Hz, $J_2 = 3.9$ Hz, 1H; 2′-H$_{ax}$), 2.26 (dd, $J_1 = 15.0$ Hz, $J_2 = 1.1$ Hz, 1H; 2′-H$_{eq}$), 2.82 (d, $J = 17.1$ Hz, 1H; 10-H$_A$), 3.09 (d, $J_1 = 9.7$ Hz, 1H; 4′-H), 3.31 (d, $J = 17.1$ Hz, 1H; 10-H$_B$), 3.81 (dq, $J_1 = 9.7$ Hz, $J_2 = 6.8$ Hz, 1H; 5′-H), 4.01 (s, 3H; 2-OCH_3), 5.01 (dd, $J_1 = 6.7$ Hz, $J_2 = 5.3$ Hz, 1H; 7-H), 5.39 (d, $J = 3.9$ Hz, 1H; 1′-H), 6.79 (d, $J = 2.5$ Hz, 1H; 3-H), 7.25 (d, $J = 2.5$ Hz, 1H; 1-H), 7.48 (s, 1H; 11-H), 12.21 (br s, exchangeable by D$_2$O, 1H; 6-OH), 12.77 ppm (br s, exchangeable by D$_2$O, 1H; 4-OH); ^{13}C NMR (100.6 MHz, [D$_6$]acetone): $\delta = 17.8$ (C-6′), 27.3 (3′-CH_3), 28.5 (9-CH_3), 35.1 (C-2′), 43.1 (C-8), 44.9 (C-10), 56.4 (2-OCH_3), 68.4 (C-9), 68.9 (C-7), 72.5 (C-3′), 76.6 (C-5′), 78.6 (C-4′), 100.8 (C-1′), 106.4 (C-3), 108.2 (C-1), 110.3 (C-4a), 113.7 (C-11), 120.6 (C-5a), 132.2 (C-6a), 132.3 (C-12a), 135.5 (C-11a), 146.9 (C-10a), 162.2 (C-6), 165.5 (C-4), 166.5 (C-2), 181.1 (C-12), 191.2 ppm (C-5); HR-FAB (*m/z* 514.1848; calcd. for $C_{27}H_{30}O_{10}$: 514.1839).

3′-*O*-Methylsteffimycin (18): ^{1}H NMR (400 MHz, [D$_6$]acetone): $\delta = 1.37$ (d, $J = 6.2$ Hz, 3H; 6′-H$_3$), 1.51 (s, 3H; 9-CH_3), 3.26 (dd, $J_1 = 3.2$ Hz, $J_2 = 1.5$ Hz, 1H; 2′-H), 3.32 (dd, $J_1 = 9.7$ Hz, $J_2 = 9.2$ Hz, 1H; 4′-H), 3.40 (s, 3H; 8-OCH_3), 3.50 (s, 3H; 2′-OCH_3), 3.54 (s, 3H; 3′-OCH_3), 3.65 (dd, $J_1 = 9.7$ Hz, $J_2 = 3.2$ Hz, 1H; 3′-H), 3.78 (dq, $J_1 = 9.7$ Hz, $J_2 = 6.2$ Hz, 1H; 5′-H), 4.05 (s, 3H; 2-OCH_3), 4.61 (d, $J = 2.3$ Hz, 8-H), 5.23 (d, $J_1 = 2.3$ Hz, 1H; 7-H), 5.61 (d, $J = 1.5$ Hz, 1H; 1′-H), 6.88 (d, $J = 2.8$ Hz, 1H; 3-H), 7.35 (d, $J = 2.8$ Hz, 1H; 1-H), 8.2 (s, 1H; 11-H), 12.20 (br s, exchangeable by D$_2$O, 1H; 6-OH), 12.78 ppm (br s, exchangeable by D$_2$O, 1H; 4-OH); HR-FAB (*m/z* 588.1863; calcd. for $C_{29}H_{32}O_{13}$: 588.1843).

Antitumor tests: The antitumor activities of the purified compounds were tested against human tumor cell lines of breast adenocarcinoma (MDA-MB-231), non-small-cell lung cancer (NSCLC A549), and colon adenocarcinoma (HT29). Quantitative measurement of cell growth and viability was carried out by a colorimetric type of assay, with use of the sulforhodamine reaction.[38]

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