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Generation of New Landomycins by Combinatorial Biosynthetic Manipulation of the LndGT4 Gene of the Landomycin E Cluster in *S. globisporus*

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Summary

A 3 kb DNA fragment from the *Streptomyces globisporus* 1912 landomycin E (LaE) biosynthetic gene cluster (*lnd*) was completely sequenced. Three open reading frames were identified, *lndGT4*, *lndZ4*, and *lndZ5*, whose probable translation products resemble a glycosyltransferase, a reductase, and a hydroxylase, respectively. Studies of generated mutants from disruption and complementation experiments involving the *lndGT4* gene allowed us to determine that LndGT4 controls the terminal L-rhodinose sugar attachment during LaE biosynthesis and that LndZ4/LndZ5 are responsible for the unique C11-hydroxylation of the landomycins. Generation of the novel landomycins F, G, and H in the course of these studies provided evidence for the flexibility of *lnd* glycosyltransferases toward their acceptor substrates and a basis for initial structure-activity relationships within the landomycin family of antibiotics.

Introduction

Landomycins (Las) are a family of angucycline antitumor polyketides possessing a unique phenylglycoside moiety in their structures. All Las identified to date share the same aglycon (landomycinone **1**) and vary only in their oligosaccharide chain [1] (Figure 1). Antitumor activities of these Las appear to depend on the length of the deoxysugar moiety [2], and landomycin A **2** (LaA), the most active compound of the family so far, contains the longest saccharide chain, consisting of six sugars. However, the influence of the aglycon structure on the antitumor activity has not yet been studied because Las with modified polyketide backbones were not available.

There are two La-producing strains: *Streptomyces cyanogenus* S136 (principal product, LaA **2**; Figure 1) and *S. globisporus* 1912 (major product, landomycin E **3**, LaE; Figure 1). Gene clusters for LaA **2** and LaE **3** biosyntheses (*lan* and *lnd* clusters, respectively) have been cloned from the respective strains [3, 4], and both clusters are very similar at the level of gene organization and nucleotide sequences. Recently, it was shown that intergeneric *E. coli-Streptomyces* conjugation is an effective way to introduce plasmid DNA into La-producing strains, which up to this point had proven to be refractory to genetic manipulation [5, 6]. This paved the way toward the generation of novel La derivatives (potentially more active/less toxic) via gene disruption and heterologous expression experiments. Also, in-depth studies of La biosynthetic genes will allow the harnessing of them for combinatorial biosynthesis of novel bioactive compounds.

We are interested primarily in studying the post-PKS ("tailoring") steps of La biosynthesis, since they contribute considerably to the biological activity of many polyketide antibiotics [7]. Particularly, we would like to learn more about the glycosylation and oxygenation steps of La biosynthesis. Only four genes encoding glycosyltransferases (GTs) have been identified in the *lan*-cluster of *S. cyanogenus* [3], and it is not yet understood how these four GTs can assemble a hexasaccharide chain. In the *lnd*-cluster of *S. globisporus* 1912, three GT-encoding genes were found by partial sequencing, putatively controlling the three glycosylation steps necessary for LaE **3** formation [4]. Thus, LaE **3** biosynthesis can be considered a simple model for the first three sugar attachments to landomycinone **1**, and an unambiguous assignment of functions to all *lnd*-gene-encoding GTs will help to elucidate the enigmatic "six sugars, four GT genes" situation that occurs in LaA **2** biosynthesis. The introduction of oxygens into the landomycinone precursor also remains poorly understood, and studying its respective genes is of great practical value.

Here, we report the sequencing of three genes from the *S. globisporus* 1912 *lnd*-cluster coding for a GT (*lndGT4*), a reductase (*lndZ4*), and an oxygenase (*lndZ5*). Functions of the three enzymes encoded by these genes can now be suggested based on gene disruption and complementation experiments along with the analyses of the structures of novel Las produced by a *S. globisporus lndGT4* disruption mutant. This is also the first report on the generation of landomycin derivatives with modified polyketide frameworks due to the ceased expression of an oxygenase in context with the *lndGT4* disruption.

Results

Sequencing and Analysis of Genes *lndGT4*, *lndZ4*, and *lndZ5* from the *S. globisporus* 1912 *lnd* Cluster

It has been previously shown that plasmid pBX81 carries genes *lndJ* and *lndZ1* and lacks *lanGT3* and *lanZ2* homo-

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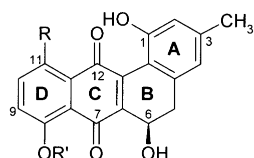
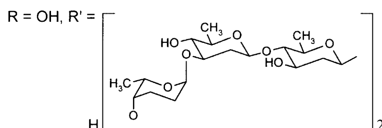


Figure 1. Structures of Landomycin Family Members

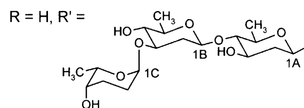
R = OH, R' = H Landomycinone **1**

R = OH, R' =



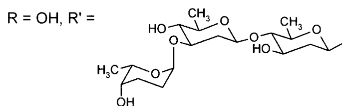
Landomycin A **2**

R = H, R' =



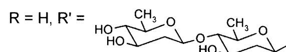
Landomycin G **5**

R = OH, R' =



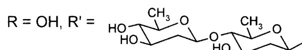
Landomycin E **3**

R = H, R' =



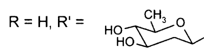
Landomycin F **6**

R = OH, R' =



Landomycin D **4**

R = H, R' =



Landomycin H **7**

logs [4] (Figure 2). Further sequencing of pBX81 allowed the identification of three open reading frames (ORFs), marked as *lndGT4*, *lndZ4*, and *lndZ5*, with characteristics of *Streptomyces* genes (overall G + C content of 72% and a high bias toward G and C at the third codon position). Gene *lndGT4* encodes a hypothetical protein of 416 amino acids (aa) with the highest similarity to LanGT4 (87.5%), the L-rhodinosyltransferase from the *S. cyanogenus* S136 *lan*-cluster [3], UrdGT1a (69.8%), involved in the L-rhodinose attachment to the C-12b position of aquayamycin [8], and AclK, a GT from the *S. galilaeus* aclacinomycin biosynthetic gene cluster (69.8%) [9]. The putative LndGT4 protein contains all of the motifs suggested to be essential for GTs to transfer L-rhodinose to the acceptor substrate [10]. These data strongly indicate that LndGT4 is responsible for the last deoxysugar transfer during LaE **3** biosynthesis.

The stop codon of *lndZ4* overlaps with the start codon of *lndZ5* (four nucleotides). The putative translation product of *lndZ4* (187 aa) shows 81% similarity to LanZ4, a putative reductase from the *S. cyanogenus* S136 landomycin cluster [3], and 69% to the putative reductases UrdO and SimA10 from the urdamycin and simocyclinone producers *S. fradiae* Tü2717 and *S. antibioticus* Tü64, respectively [8, 11]. Gene *lndZ5* codes for a putative protein of 391 aa, which shows 81% similarity to LanZ5, a putative oxygenase from the LaA **2** producer, 68% to NcnH, a hydroxylase involved in naphthocyclinone biosynthesis (*S. arenae*) [12], and 62% to the ActVA-5 oxygenase from the actinorhodin cluster in *S. coelicolor* [13].

Generation and Characterization of an *S. globisporus* Mutant with a Disrupted *lndGT4* Gene

A mutant affecting the *lndGT4* gene was generated by gene replacement. Suicide plasmid pTGT4.3, containing the apramycin resistance (Am^r) cassette and the *lndGT4* gene interrupted with the *aadA* (spectinomycin resistance, Sp^r) gene, was introduced into *S. globisporus* 1912 by conjugation (Figure 3). Approximately 300 Sp^r exconjugants were obtained and screened for apramycin sensitivity, and from 10 selected Sp^r Am^s colonies (the consequence of a double crossover) one, marked as GT4.1, was chosen for further studies. Chromosomal mutation in strain GT4.1 was analyzed by Southern hybridization. An 8.1 kb BamHI-XhoI fragment (from pBX81; Figure 2) was used as a probe against BamHI-digested chromosomal DNA. Hybridization analysis of the wild-type *S. globisporus* showed the expected 10 kb fragment. When chromosomal DNA from the GT4.1 mutant was treated similarly, a 12 kb fragment was detected, indicating that the wild-type copy of *lndGT4* was replaced by the mutated one (Figure 3). Restriction analysis of pTGT4.3 with SphI showed the resistance cassette to be incorporated in the same orientation as *lndGT4*.

According to sequence analysis, *lndGT4* should control the last deoxysugar attachment during LaE **3** biosynthesis. Thus, we expected the *lndGT4*-minus mutant to accumulate landomycin D **4** (LaD), which contains only a disaccharide chain, and possibly 8-D-olivosyl-landomycin **8** (Figures **1** and 4). Surprisingly, the GT4.1 cultures produced two novel compounds, named landomycins F **6** and H **7**, which displayed UV spectra clearly different from those of the known landomycins. The NMR analysis showed that both compounds lack the third sugar (see below), confirming the involvement of *lndGT4* in the attachment of the third sugar.

Complementation Studies of the GT4.1 Mutant

Complementation of mutant GT4.1 was achieved through expressing the *lndGT4* gene (plasmid pKC1218ElndGT4.5) into this mutant. In addition, whether the GT4.1 mutant

Figure 2. Organization of the *lnd* Gene Cluster and of Constructs Leading to the Inactivation of LndGT4 and Its Downstream Enzymes as Well as Fragments Used for Complementation Experiments

(A) Organization of the *S. globisporus* 1912 *lnd* cluster.

(B) The downstream *lnd* gene cluster fragment and its comparison with that from *S. cyanogenus* S136 (*lan* cluster). DNA fragments used for the generation of different constructs are shown. The black triangle on the pTGT4.3 plasmid marks the disrupted *lndGT4* gene with the 2 kb spectinomycin resistance cassette *aadA*. Relevant restriction sites are marked above *lnd* cluster (B, BamHI; S, SacI; P, PstI; Pv, PvuII; Bg, BglII; X, XhoI; Sp, SphI).

could also be complemented with the *lanGT4* gene from *S. cyanogenus* (plasmid pSElanGT4.3) was assayed. The resulting strains produced the same novel compound, named landomycin G **5**, with R_f and R_t values similar to those of LaE **3** but a different UV spectrum. This indicates that both genes play the same role and that other enzyme function(s), in addition to the LndGT4 glycosyltransferase, were affected in mutant GT4.1. Since the *aadA* cassette from pHP45Ω is flanked with phage T4 transcriptional and translational terminators, its insertion into *lndGT4* could have caused a polar effect on downstream genes. To prove this, further complementation experiments were conducted concerning the reductase (*lndZ4*) and oxygenase (*lndZ5*) encoding genes immediately downstream of *lndGT4*. Expression of genes *lndGT4lndZ4lndZ5* (plasmid pKC1218EBX2) in GT4.1 was sufficient to fully restore the LaE **3** production, and strain GT4.1 complemented with only the oxygenase- and reductase-encoding genes *lndZ4lndZ5* (plasmid pKC1218EBX3) produced LaD **4** [30, 31]. However, the separate expression of *lndZ5* (or *lanZ5*) (plasmids pKC1218EBX4 and pKC1218ElanZ5, respectively; Figure 2) or *lndZ4* (plasmid pKC1218EBX5) in strain GT4.1 did not alter its production profile. These results clearly show that the expression of the downstream genes *lndZ4lndZ5* was also affected in the GT4.1 mutant.

Structural Elucidation of the Novel Landomycins

The known landomycins (LaE **3**, LaD **4**) were identified through TLC, HPLC, and MALDI-TOF, while the structures of the novel landomycins F **6**, G **5**, and H **7** were determined through mass spectrometry in combination with NMR and UV spectroscopy.

All three novel compounds were clearly distinguishable from known Las by their light orange color, which indicated an altered aglycon moiety, compared to the typical naphthazarine chromophore, which is dark red. This observation was confirmed by the obtained UV spectra, which for all three new compounds displayed significant hypsochromic shifts of the absorption maximum found in landomycins, usually between $\lambda = 440$ and 450 nm to around $\lambda = 400$ nm.

MS analysis of LaG **5** showed a negative mode molecular ion peak at $m/z = 695$, which suggested that LaG

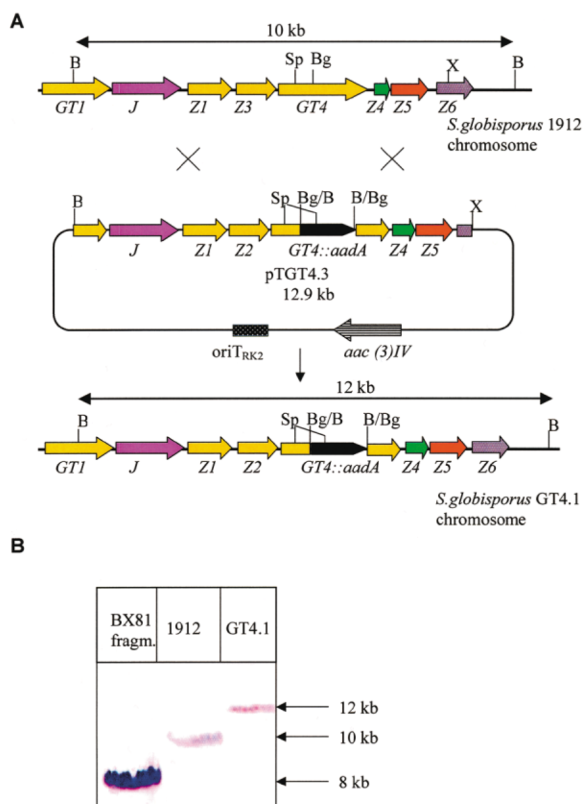


Figure 3. A Scheme Representing the Replacement Event in the Chromosome of Wild-Type *S. globisporus* Strain by a Double Crossover to Construct Mutant GT4.1

(A) Scheme of *lndGT4* gene replacement in *S. globisporus*. Relevant restriction sites are shown (B, BamHI; Bg, BglII; Sp, SphI; X, XhoI). *aac (3)IV*, apramycin resistance gene; oriT$_{RK2}$, origin of conjugative DNA transfer of plasmid RK2.

(B) Southern analysis showing the gene replacement event. The total wild-type and mutant DNA was digested with BamHI.

5 is a deoxygenated analog of LaE **3**, since it has a 16 amu lower mass. Subsequent high-resolution ESI-MS confirmed the corresponding molecular formula of $C_{37}H_{44}O_{13}$. Likewise, LaF **6** and LaH **7** were identified as being the deoxy-derivatives of the originally expected LaD **4** and a thus far not found monoglycosylated landomycin, respectively.

The NMR spectra of LaG **5** were widely identical to those of LaE **3** (Tables 1 and 2). However, a major difference was found in the aromatic ring D of the aglycon, which displayed an additional proton signal at $\delta = 7.72$ in the ^{1}H-NMR compared to LaE **3**, as obvious from integration. Thus, instead of the two typical doublets found for 9-H and 10-H in the usual landomycins, three signals were observed that overlap and couple to each other and appear as a higher-order system. Consistently, the signal for C-11 at $\delta = 151.2$ in the ^{13}C-NMR of LaE **3** is replaced in LaG **5** by a signal resonating at $\delta = 122.1$. Similar D-ring signals were observed for LaF **6** and LaH **7** (Tables 1 and 2). The most significant differences between these compounds and LaG **5** lie in the shortened deoxysugar chains. That is, LaF **6** is lacking the ^{1}H- and ^{13}C-NMR signals of the L-rhodinosyl moiety, most notably the anomeric signal found in LaG at $\delta_H = 4.97/\delta_C = 95.5$, while the spectra of LaH **7** display only one set of anomeric signals, found at $\delta_H = 5.33/\delta_C = 99.5$, along with the remaining signals of a D-olivosyl moiety. LaH **7**, therefore, is the first proven example of a landomycin carrying only one deoxysugar moiety.

Anticancer Assays of the Landomycins

MTS cell proliferation assays were performed on NCI-H460 (NSCLC) human lung cancer and MCF-7 breast cancer cell lines. The three novel landomycins F **6**, G **5**, and H **7** were assayed in comparison with their 11-oxygenated analogs landomycins D **4** and E **3** as well as LaA **2** to obtain further structure-activity relationships within the landomycin family pertaining to the length of the deoxysugar chain and the 11-hydroxy group. The results of these assays are listed in Table 3 and discussed below.

Discussion

The LaE **3** biosynthetic gene cluster was retrieved from a *S. globisporus* 1912 phage library using urdamycin PKS gene probes. Partial sequencing of the *lnd* cluster revealed that it is widely congruent with the *lan* cluster of *S. cyanogenus* [4, 14]. Disruptions of various *S. globisporus* 1912 *lnd* genes putatively governing the polyketide framework formation abolished LaE **3** production [5, 14, 15]. These data provided evidence that the cloned genes were indeed required for LaE **3** biosynthesis. Functions of *lan* genes for GTs were previously probed solely via heterologous expression experiments, since a system for gene disruption in *S. cyanogenus* S136 was not available [16]. Two GTs, namely LanGT1 and LanGT4, were shown to recognize foreign acceptor substrates from the urdamycin and mithramycin pathways [16, 17].

We were interested in applying gene replacement techniques to *lnd* genes controlling oxygenation and glycosylation steps of the initial polyketide backbone, thus leading to novel bioactive compounds. In this study, we aimed at the GT gene *lndGT4*, a *lanGT4* homolog. To disrupt *lndGT4,* the spectinomycin resistance cassette *aadA* was used. The *S. globisporus* mutant with a replaced *lndGT4* gene was expected to produce landomycin D **4**. However, the mutant GT4.1 was shown to accumulate two novel compounds, LaF **6** and LaH **7**. LaF **6** is 11-deoxylandomycin D and LaH **7** is the monoglycosylated 11-deoxylandomycinone. Expression of *lndGT4/lanGT4* in the GT4.1 mutant led to the production of landomycin G **5** (LaG), i.e., 11-deoxyLaE (meaning that LndGT4 controls the attachment of the terminal deoxysugar L-rhodinose during LaE **3** production), but the reconstitution of the 11-OH group was not achieved by this complementation. These results suggest that *lndGT4* disruption had a polar effect on downstream *lnd* genes. Additional complementation experiments showed that expression of the *lndZ4lndZ5* genes is affected in the GT4.1 mutant. From the structures of the novel landomycins and complementation studies, we can deduce that the gene pair *lndZ4lndZ5* must be involved in the C11-hydroxylation of lando-

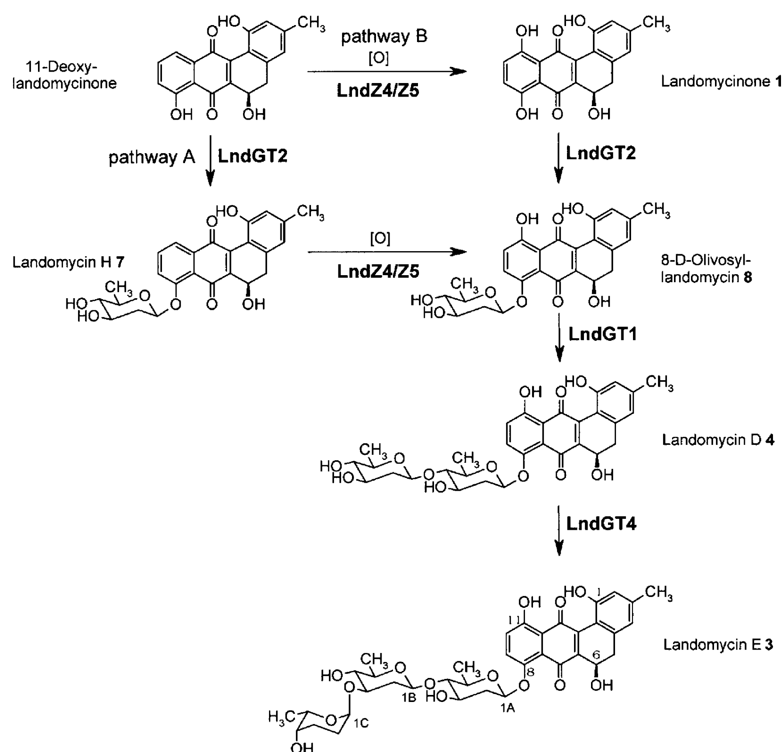


Figure 4. Proposed Late Biosynthetic Pathway of Landomycin E 3 in *S. globisporus* 1912
Pathway A is favored.

Table 1. ^{1}H NMR Data of Landomycins E 3, F 6, G 5, and H 7

Position	LaE	LaF	LaG	LaH
2	6.62 s	6.63 s (br)	6.62 s	6.63 s
3-CH_3	2.30 s	2.30 s	2.30 s	2.30 s
4	6.71 s	6.71 s (br)	6.71 s	6.70 s
5 α	2.85 dd (16.5,3.5)	2.86 dd (17,4)	2.86 dd (16.5,4)	2.85 dd (16.5,4)
β	2.99 dd (16.5,2)	3.02 dd (17,2.5)	3.03 dd (16.5,2.5)	3.02 dd (16.5,2.5)
6	5.14 m	5.20 dd (4,2.5)	5.20 dd (4,2.5)	5.19 dd (4,2.5)
9	7.49 d (9.5)	7.55 m[a]	7.54 m[a]	7.54 dd (7,3)
10	7.22 d (9.5)	7.72 m[a]	7.71 m[a]	7.70 dd (7.5,7)
11	-	7.73 m[a]	7.72 m[a]	7.71 dd (7.5,3)
1A	5.13 dd (10,1.5)	5.34 dd (9.5,2)	5.34 dd (9.5,2)	5.33 dd (9,2)
$2A_{ax}$	1.81 ddd (12,12,10)	1.93 ddd (12,12,9.5)	1.92 ddd (12,12,9.5)	1.95 ddd (12,12,9)
$_{eq}$	2.57 ddd (12,5,1.5)	2.52 ddd (12,5,2)	2.52 ddd (12,5,2)	2.46 ddd (12,5,2)
3A	3.60-3.69 m	3.73 ddd (12,8.5,5)	3.70 ddd (12,9,5)	3.64 ddd (12,9,5)
4A	3.11 dd (9,9)	3.17 dd (8.5,8.5)	3.17 dd (9,9)	3.06 dd (9,9)
5A	3.39-3.47 m	3.52-3.60 dq (8.5,6)	3.54 dq (9,6)	3.44 dq (9,6)
5A-CH_3	1.28 d (6)	1.32 d (6)	1.32 d (6)	1.32 d (6)
1B	4.64 dd (9.5,1.5)	4.68 dd (10,2)	4.67 dd (9.5,2)	-
$2B_{ax}$	1.39-1.49 m	1.55 ddd (12,12,10)	1.46 ddd (12,12,9.5)	-
$_{eq}$	2.35 ddd (12,5,1.5)	2.22 ddd (12,5,2)	2.37 ddd (12,5,2)	-
3B	3.60-3.69 m	3.52-3.60 ddd (12,9,5)	3.65 ddd (12,9,5)	-
4B	3.07 dd (9,9)	2.95 dd (9,9)	3.08 dd (9,9)	-
5B	3.39-3.47 m	3.39 dq (9,6)	3.44 dq (9,6)	-
5B-CH_3	1.35 d (6)	1.34 d (6)	1.36 d (6)	-
1C	4.95 s (br)	-	4.97 s (br)	-
$2C_{ax}$	1.39-1.49 m	-	1.46 m	-
$_{eq}$	2.00-2.15 m	-	2.00-2.20 m	-
$3C_{ax}$	1.66 m	-	1.67 m	-
$_{eq}$	2.00-2.15 m	-	2.00-2.20 m	-
4C	3.55 s (br)	-	3.54 s (br)	-
5C	4.19 dq (6.5,1.5)	-	4.20 dq (7,1.5)	-
5C-CH_3	1.14 d (6.5)	-	1.15 d (7)	-

[a] Higher-order signal.

Table 2. ^{13}C NMR Data of Landomycins E **3**, F **6**, G **5**, and H **7**

Position	LaE	LaF	LaG	LaH
1	156.3	157.0	157.0	157.0
2	117.8	117.5	117.5	117.6
3	142.9	143.9	143.9	143.9
3-CH_3	21.4	21.7	21.6	21.7
4	123.1	123.2	123.2	123.3
4a	139.3	139.1	139.1	139.1
5	37.7	38.0	37.9	38.0
6	60.2	59.9	59.9	59.9
6a	145.1	142.1	142.2	142.2
7	182.4	183.6	183.6	183.6
7a	120.3	122.0	122.0	121.9
8	151.2	157.8	157.8	157.9
9	131.4	124.6	124.5	124.4
10	126.0	135.7	135.7	135.8
11	158.3	122.1	122.1	122.0
11a	116.8	137.6	137.6	137.6
12	191.1	187.6	187.6	187.7
12a	140.5	143.3	143.2	143.2
12b	116.2	115.1	115.1	115.1
1A	99.9	99.4	99.4	99.5
2A	39.1	39.0	39.0	40.1
3A	70.0	70.7	70.7	72.1
4A	88.7	88.4	88.4	78.4
5A	71.6	72.2	72.2	73.9
5A-CH_3	18.4	18.2	18.4	18.5
1B	101.8	102.2	102.1	-
2B	37.4	40.6	37.3	-
3B	78.0	72.1	76.5	-
4B	76.0	78.2	76.3	-
5B	73.2	73.8	73.7	-
5B-CH_3	18.1	18.2	18.2	-
1C	96.2	-	95.5	-
2C	24.8	-	25.0	-
3C	26.4	-	26.6	-
4C	67.3	-	68.1	-
5C	68.0	-	68.1	-
5C-CH_3	17.6	-	17.6	-

mycin. Although the precise sequence of reactions remains speculative, it is likely that the C11-hydroxylation occurs directly after the attachment of the first D-olivose (Figure 4, path A) and not prior to this glycosylation step (Figure 4, path B).

At this stage, we also cannot sufficiently explain the role of the putative reductase LndZ4 for the oxygenation of the C11-position of LaE **3**. Little is known about LndZ5's class of oxygenases, but possible scenarios include a supporting or reconstituting function of LndZ4 (e.g., cofactor regeneration) required directly for the catalytic cycle or a necessary structural protein-protein interaction in the enzyme cluster. Further studies of

Table 3. Anticancer Activity Assays of Novel Landomycins F **6**, G **5**, and H **7** Compared to Previously Described Landomycins

Landomycin **X**	Anticancer IC_{50} (μM)	
(No. of Sugars)	NCI-H460 (Lung)	MCF-7 (Breast)
A (6)	10.4 ± 6.0	2.6 ± 0.4
E (3)	29.0 ± 6.3	2.8 ± 1.7
D (2)	105.3 ± 46.2	5.6 ± 1.8
G (3)	18.5 ± 12.3	3.1 ± 0.2
F (2)	111.0 ± 57.5	15.9 ± 3.0
H (1)	49.2 ± 10.3	4.6 ± 0.3

genes *lndZ4lndZ5* and the respective proteins are of great interest, since among the angucyclines only the landomycins possess a C11-hydroxylated scaffold [1]. Harnessing the angucycline C11-hydroxylase gene for combinatorial biosynthesis will greatly enhance the diversity of engineered polyketides. Our data show that the *lndGT4lndZ4lndZ5* genes are within one transcriptional unit and that the downstream gene *lndZ6* [4], encoding a putative holo-ACP-synthase with the highest similarity (80%) to *jadM* from the jadomycin biosynthetic pathway in *S. venezuelae* [18], is probably transcribed from its own promoter. This is apparent from our complementation experiment of mutant GT4.1 with genes *lndGT4lndZ4lndZ5*, which showed, through the reconstitution of LaE **3** biosynthesis, that there was no further polar effect on the downstream-located *lndZ6* gene.

Two pathways to the hexasaccharide chain of LaA **2** were suggested: (1) step-by-step addition of activated monodeoxysugar units or (2) attachment of a disaccharide unit (D-olivosyl-D-olivose) to landomycinone **1**, then addition of L-rhodinose, and repetition of this two-step process to produce the hexasaccharide moiety [19]. The identification of di-, tri-, and especially the monosaccharide LaH **7** in this study strongly supports the first pathway for LaE **3** biosynthesis. Taking into account the high similarity of the *lnd* and *lan* clusters, we tentatively propose that the same mechanism applies to LaA **2** biosynthesis during attachment of the first three sugars (Figure 4).

The successful expression of *lanGT4* in the GT4.1 mutant proved the functional interchangeability of *lan* and *lnd* GTs. Of special interest is the fact that the *lnd* GTs (at least LndGT1 and LndGT4) recognized acceptor substrates lacking the C11-hydroxyl group. This could be inferred from the structures of LaF **6**, G **5**, and H **7**. Although there is no direct experimental evidence for the function of LndGT2, indirect evidence exists: since *lndGT1* has a striking resemblance to *lanGT1*, whose function was clearly established through gene combination experiments [16, 17], it can be safely assumed that LndGT1 is the olivosyltransferase, which adds the second olivose to the saccharide chain. The role of LndGT4 was unambiguously established in the work described here. Thus, it is only logical to assume that the only remaining GT, LndGT2, is responsible for starting the oligosaccharide chain by transferring the first olivose to the aglycon. Considering the similarity of LndGT2 with LanGT2 and the demonstrated high substrate specificity of LanGT2 [16], it would be surprising if LndGT2 were flexible regarding its aglycon acceptor substrate. Thus, we favor a sequence of events in which the first glycosylation step occurs before the hydroxylation of the 11 position (Figure 4, path A). If the 11-hydroxylation occurs first, rings C and D would become symmetrical, and LndGT2 might have problems distinguishing between 8-OH and 11-OH, resulting in landomycins with glycosidic moieties attached to 11-OH, which cannot be observed.

The generation of these novel landomycins opened the way to further examine structure-activity relationships of the landomycins, not only with regard to the glycosylation pattern but also to the La aglycon. Thus, from our data it is apparent that antitumor activity of

the landomycins generally increases with the length of the deoxysugar chain (with the exception of LaH **7**), as has been proposed before [2], making LaA **2** the most potent congener (Table 3). Unfortunately, the impact of the 11-hydroxy group is harder to assess. Comparison of LaE **3** and its 11-deoxy analog LaG **5** does not show any significant difference. On the other hand, an approximately 3-fold decrease in antitumor activity is observed for the diglycosidic Las if the 11-hydroxy group is missing. It is therefore possible that this hydroxy function has an activity-increasing effect similar to what has been described for the 11-hydroxy group of the anthracycline antitumor antibiotic doxorubicin [20]. To get a more complete understanding of the importance of the 11-hydroxy function in the Las, it appears necessary to also generate 11-deoxy-LaA and the thus far not isolated 8-D-olivosyl-landomycin (Figure 4; **8**) and evaluate their cytotoxicity profiles. An isolation of 8-D-olivosyl-landomycin **8** would also allow the further evaluation of the surprisingly high activity of LaH **7**. Since the length of the sugar chain was found to be important for the antitumor activity of the Las, the monoglycoside LaH **7** would have been expected to be a weaker drug than LaD **4** and to display only marginal activity. But this line of reasoning apparently holds true only for the larger congeners, which in turn might indicate a different mechanism of action for Las with only one or no deoxysugar moieties. This aspect might be of significant importance with regard to the high toxicity displayed by LaA **2** and opens the possibility of finding a novel La analog with an improved toxicity profile. Therefore, the data obtained will be of great practical interest for future attempts at engineering novel antitumor agents via combinatorial biosynthesis [21].

Significance

The *lndGT4* gene controls the transfer of the terminal deoxysugar L-rhodinose during LaE biosynthesis in *S. globisporus* 1912. Apparently, a joint action of the LndZ4 reductase and the LndZ5 oxygenase is required for the C11-hydroxylation of the landomycins. The Lnd GTs have flexibility in their substrate specificity. The *lndGT*s and the C11-hydroxylation determinants *lndZ4lndZ5* are potentially valuable tools for engineered biosynthesis of novel polyketides. Three novel landomycins, F, G, and H, have been generated, lacking the C11-hydroxyl group and carrying sugar chains of different lengths, thereby providing evidence for a stepwise glycosylation sequence in La biosynthesis. Their availability allowed the first structure-activity relationship studies of the landomycin family of antitumor antibiotics with regard to the aglycon moiety.

Experimental Procedures

Microorganisms, Culture Conditions, and Vectors

S. globisporus 1912 was obtained from B. Matselyukh (Institute of Microbiology and Virology, Kyyiv, Ukraine). *E. coli* DH5α (MBI Fermentas, Lithuania) was used as a host for subcloning. *E. coli* ET 12567 carrying pUB307 (*dam*$^-$ *dcm*$^-$*hsdS*$^-$ Cm^{R}Km^R) [22], which was used to perform intergeneric conjugations, was a gift from C.P. Smith (University of Manchester, UMIST, United Kingdom). Plasmids pBluescriptIIKS$^-$/SK$^-$ and pUC18 were used as vectors for subcloning experiments and DNA sequencing. The conjugative vector pTNK [5] was used for gene replacement. Plasmid pHP45Ω [23] was kindly provided by J.-L. Pernodet (Institute de Genetique et Microbiologie, Orsay, France), and it was used as a source for the spectinomycin resistance gene *aadA*. For gene expression in *S. globisporus* strains, the integrative pSET152 [24] and the replicative pKC1218E (kindly provided by C. Olano, Universidad de Oviedo, Spain) plasmids were used. pKC1218E is a pKC1218 [24] derivative with the strong constitutive promoter *ermEp** of the erythromycin resistance gene *ermE* cloned into the polylinker of pKC1218. Subclones of the *lnd* cluster were provided by K. Pankevych and H. Krügel (Hans Knöll Institute for Natural Products Research, Jena, Germany). Plasmids pMun2-lanZ5 and pUWL201-1, containing *lanZ5* and *lanGT4* genes, respectively, were gifts from A. Bechthold (Albert-Ludwigs-Universität, Freiburg im Breisgau, Germany). For sporulation, *S. globisporus* strains were grown on oatmeal solid medium at 30°C [5]. *E. coli* strains were grown under standard conditions [25]. When antibiotic selection was needed, 25 μg/ml of apramycin or chloramphenicol, 100 μg/ml of ampicillin, 200 μg/ml of spectinomycin, and 50 μg/ml of kanamycin or nalidixic acid were used.

General Genetic Manipulations

Routine methods were performed as described [25, 26]. Intergeneric *E. coli*-*Streptomyces* conjugation was used to introduce plasmid DNA into *S. globisporus* strains. Matings were performed according to [5]. Enzymatic manipulation of DNA and Southern hybridizations were carried out according to manufacturer's directions (Stratagene, NEB, MBI Fermentas, Boehringer Mannheim). Oligonucleotide primers were purchased from Sigma-Genosys. PCR was performed in a thermocycler MiniCycler (MJ Research).

DNA Sequencing and Computer-Assisted Sequence Analysis

Sequencing was carried out via the dideoxynucleotide chain termination method [27] and the Cy5 AutoCycle Sequencing Kit (Pharmacia Biotech). Both DNA strands were sequenced with standard primers or with internal oligoprimers via an ALF-Express automatic DNA sequencer. Database searches were performed using the University of Wisconsin Genetics Computer Group programs and the BLAST 2.0 program [28, 29].

Generation of Mutant GT4.1

To generate this mutant, plasmid pTGT4.3 was constructed as follows. First, pBX81, which contains *lndJ* to *lndZ5* genes, the 3′ end of *lndGT1* and the 5′ end of *lndZ6*, was digested with BglII, the *aadA* gene was inserted into this site as a BamHI fragment, generating pBXA37. The unique BglII site of pBX81 is located 714 bp downstream of the *lndGT4* start codon. Then, the BamHI-XhoI fragment containing the disrupted *lndGT4* allele was excised from pBXA37, treated with the Klenow fragment, and ligated into the EcoRV site of the suicide vector pTNK to set up the final construct pTGT4.3 (Figures 2 and 3). Restriction of this plasmid with SphI showed the expected 500 bp fragment, confirming the *aadA* gene to be incorporated in the same orientation as *lndGT4*. pTGT4.3 was then introduced into *S. globisporus* by conjugation, and gene replacement was confirmed by Southern analysis and PCR amplification.

Generation of Gene Expression Constructs

The *lanGT4* gene with the *ermEp** promoter was excised as a KpnI-XbaI fragment from pUWL201-1, treated with the Klenow fragment, and ligated into the EcoRV site of pSET152 to give pSElanGT4.3. The *lanZ5* gene was excised from pMun2/lanZ5 as a PvuII-BglII fragment and cloned downstream of ErmEp* into EcoRV-BglII-digested pKC1218E to create pKC1218ElanZ5. The *lndGT4* gene was obtained from the *S. globisporus* 1912 chromosome by PCR. Primers used for amplification were 5′-GAATTCTCAGCGGCGGTGACGGGCC and 5′-TCTAGAACAAGCGTCCGGTACCCG. Sites for EcoRI and XbaI in BO-GT4RP and BO-GT4UP, respectively, are underlined. Purified PCR product was cloned into the EcoRV site of pBluescriptIIKS$^-$ (pBSKlndGT4.5) and sequenced to confirm its identity with *lndGT4*. Finally, *lndGT4* was excised as an XbaI-EcoRI fragment from pBSKlndGT4.5 and cloned into the corresponding sites of pKC1218E, giving pKC1218ElndGT4.5.

Genes *lndGT4lndZ4lndZ5* were cut as a SacI-XhoI fragment from pBX81, blunted, and cloned into the EcoRV site of pKC1218E (generating plasmid pKC1218EBX2; Figure 2). Plasmid pKC1218EBX2 was digested with PstI, and the major fragment was religated to give plasmid pKC1218EBX3 (Figure 2) expressing genes *lndZ4lndZ5*. A PstI-SphI fragment with the *lndZ4* gene was retrieved from pKC1218EBX3 and cloned into EcoRV-digested pKC1218E to set up pKC1218EBX5. The *lndZ5* gene was excised as a PvuII-XhoI fragment from pBX81, blunt ended, and cloned into the EcoRV site of pKC1218E, leading to plasmid pKC1218EBX4 (Figure 2).

Production and Purification of Landomycins

The *S. globisporus* 1912 wild-type strain and mutants were cultivated in liquid SG medium [14] supplemented with the appropriate antibiotics. This preculture was grown at 30°C for 24 hr with vigorous shaking and was subsequently used to inoculate the main culture of the same composition, which was harvested after 96 hr of shaking as above. The culture broth was extracted three times with equal volumes of ethyl acetate. Extracts were dried in vacuo, dissolved in methanol, and examined by TLC, HPLC [14, 30–32], and HPLC/MS. Purification was achieved by semipreparative HPLC and subsequent column chromatography on Sephadex LH-20 (methanol). HPLC/MS was performed on a Waters Alliance 2695 system with Waters 2996 photodiode array detector and a Micromass ZQ 2000 mass spectrometer equipped with an APCI ionization probe (solvent A, 0.1% formic acid in H_2O; solvent B, acetonitrile; flow rate, 0.5 ml/min; 0–6 min 75% A and 25% B to 100% B [linear gradient], 6–7 min 100% B, 7–7.5 min 100% B to 75% A and 25% B [linear gradient], 7.5–10 min 75% A and 25% B). Semipreparative HPLC was done on a Waters Delta 600 instrument with a Waters 996 photodiode array detector (solvent A, 0.1% trifluoroacetic acid in H_2O; solvent B, acetonitrile; flow rate, 10.0 ml/min; 0–20 min 50% A and 50% B to 100% B [linear gradient], 20–23 min 100% B, 23–23.5 min 100% B to 50% A and 50% B [linear gradient], 23.5–29.5 min 50% A and 50% B). The columns were Waters Symmetry C_{18}, 4.6 $\times$ 50 mm, particle size 5 μm (HPLC/MS) and Waters SymmetryPrep C_{18}, 19 $\times$ 150 mm, particle size 7 μm. Yields were as follows: Landomycin F **6**, 5 mg/l; Landomycin G **5**, 6 mg/l; Landomycin H **7**, 4.5 mg/l.

Chemical Analysis of Novel Landomycins

MALDI-TOF analysis was performed on a Voyager STR-DE machine [32], and high-resolution MS was performed on an IonSpec HiResESI FT-MS spectrometer. NMR data were acquired either on a Varian Inova-300 (300 MHz) or a Varian Inova-400 (400 MHz) spectrometer. UV spectra were generated on a Varian CARY50 Scan UV-visible instrument. LaF **6**: M = 582 g/mol ($C_{31}H_{34}O_{11}$); UV/Vis (methanol): λ_{max} (ϵ) = 395 (2300), 288 (7000), 265 (9700); APCI-MS (neg. mode): m/z = 581 (100) $[M\text{-}H]^-$, 321 (55) $[M\text{-}H\text{-}C_{12}H_{20}O_6]^-$, 304 (50) $[M\text{-}H_2O\text{-}C_{12}H_{20}O_6]^-$, HR-ESI-MS (pos. mode): m/z = calculated for $C_{31}H_{34}O_{11}Na$: 605.1993, found: 605.2010. LaG **5**: M = 696 g/mol ($C_{37}H_{44}O_{13}$); UV/Vis (methanol): λ_{max} (ϵ) = 402 (2800), 289 (6400), 266 (9100); APCI-MS (neg. mode): m/z = 695 (100; HR-ESI-MS: calculated for $C_{37}H_{43}O_{13}$: 695.2709, found: 695.269) $[M\text{-}H]^-$, 677 (3) $[M\text{-}H\text{-}H_2O]^-$, 535 (4) $[M\text{-}H\text{-}rhodinose\text{-}HCHO]^-$, 321 (75) $[M\text{-}H\text{-}trisaccharide]^-$, 303 (22) $[M\text{-}H\text{-}H_2O\text{-}trisaccharide]^-$, 291 (10) $[M\text{-}H\text{-}trisaccharide\text{-}HCHO]^-$. LaH **7**: M = 452 g/mol ($C_{25}H_{24}O_8$); UV/Vis (methanol): λ_{max} (ϵ) = 399 (3100), 289 (7000), 264 (9600); APCI-MS (neg. mode): m/z = 451 (100; HR-ESI-MS: calculated for $C_{25}H_{23}O_8$: 451.1398, found: 451.139) $[M\text{-}H]^-$, 433 (8) $[M\text{-}H\text{-}H_2O]^-$, 321 (47) $[M\text{-}H\text{-}C_6H_{10}O_3]^-$, 303 (13) $[M\text{-}H\text{-}H_2O\text{-}C_6H_{10}O_3]^-$, 291 (32) $[M\text{-}H\text{-}HCHO\text{-}C_6H_{10}O_3]^-$. For ^1H-NMR and ^{13}C-NMR data of LaF **6**, G **5**, and H **7**, see Tables 1 and 2.

Cancer Cell Proliferation Assays (MTS Assays)

Cells of the human cancer cell lines NCI-H460 (lung, NSCLC) and MCF-7 (breast) were seeded at the appropriate cell count of 3 $\times$ 104 and 1 $\times$ 10^4, respectively, per well into 96-well plates in a total volume of 100 μl and allowed to attach to the wells overnight. Test compounds were dissolved in DMSO (10 mM stock solution), diluted with complete medium, and then added as 100 μl aliquots to the appropriate total concentration of 100, 20, 4, 0.8, 0.16, and 0.032 μM, respectively. The drug-containing solution was removed after 24 hr (NCI-H460) or 48 hr (MCF-7) and replaced with 100 μl of complete medium. Twenty microliters of the MTS/PMS reagent solution, containing 333 μg of MTS and 25 mM of PMS in Dulbecco's phosphate buffered saline (DPBS), was added to the cells. Absorbance was measured at 490 nm after incubation at 37°C and 5% CO_2 for 1–4 hr. Each assay was repeated twice, and the error margins of each result are given in Table 3.

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References

1. Krohn, K., and Rohr, J. (1997). Angucyclines: Total syntheses, new structures and biosynthetic studies of an emerging new class of antibiotics. Top. Curr. Chem. *188*, 127–195.
2. Rohr, J., Wohlert, S.-E., Oelkers, C., Kirsching, A., and Ries, M. (1997). Biosynthetic short activation of the 2,3,6-trideoxysugar L-rhodinose. Chem. Commun. *7*, 973–974.
3. Westrich, L., Domann, S., Faust, B., Bedford, D., Hopwood, D.A., and Bechthold, A. (1999). Cloning and characterization of a gene cluster from *Streptomyces cyanogenus* S136 probably involved in landomycin A biosynthesis. FEMS Microbiol. Lett. *170*, 381–387.
4. Fedorenko, V., Basiliya, L.I., Pankevych, K.O., Dubitska, L.P., Ostash, B.O., Luzhetskyy, A.M., Gromyko, O.M., and Krügel, H. (2000). Genetic control of antitumor antibiotics-polyketides biosynthesis by actinomycetes. Bull. Inst. Agr. Microbiol. (Ukr.) *8*, 27–31.
5. Luzhetskyy, A.M., Ostash, B.O., and Fedorenko, V.O. (2001). Intergeneric conjugation *Escherichia coli – Streptomyces globisporus* 1912 with using of integrative plasmid pSET152 and its derivatives. Russ. J. Genet. *37*, 1123–1129.
6. Luzhetskyy, A., Fedoryshyn, M., Hoffmeister, D., Bechthold, A., and Fedorenko, V.A. (2002). Gene cloning system for *Streptomyces cyanogenus* S136. Visn. Lviv Univ. Ser. Biol. *29*, 62–68.
7. Rix, U., Fischer, C., Remsing, L.L., and Rohr, J. (2002). Modification of post-PKS tailoring steps through combinatorial biosynthesis. Nat. Prod. Rep. *19*, 542–580.
8. Trefzer, A., Hoffmeister, D., Künzel, E., Stockert, S., Weitnauer, G., Westrich, L., Rix, U., Fuchser, J., Bindseil, K.U., Rohr, J., et al. (2000). Function of glycosyltransferase genes involved in urdamycin A biosynthesis. Chem. Biol. *7*, 133–142.
9. Raty, K., Hautala, A., Torkkell, S., Kantola, J., Mantsala, P., Hakala, J., and Ylihonko, K. (2002). Characterization of mutations in aclacinomycin A-non-producing *Streptomyces galilaeus* strains with altered glycosylation patterns. Microbiol. *148*, 3375–3384.
10. Hoffmeister, D., Ichinose, K., and Bechthold, A. (2001). Two sequence elements of glycosyltransferases involved in urdamycin biosynthesis are responsible for substrate specificity and enzymatic activity. Chem. Biol. *8*, 557–567.
11. Trefzer, A., Pelzer, S., Schimana, J., Stockert, S., Bihlmaier, C., Fiedler, H.P., Welzel, K., Vente, A., and Bechthold, A. (2002). Biosynthetic gene cluster of simocyclinone, a natural multihybrid antibiotic. Antimicrob. Agents Chemother. *46*, 1174–1182.
12. Brünker, P., Sterner, O., Bailey, J.E., and Minas, W. (2001). Heterologous expression of the naphthocyclinone hydroxylase gene

from *Streptomyces arenae* for production of novel hybrid polyketides. Antonie Van Leeuwenhoek *79*, 235–245.

13. Sciara, G., Kendrew, S.G., Miele, A.E., Marsh, N.G., Federici, L., Malatesta, F., Schimperna, G., Savino, C., and Vallone, B. (2003). The structure of ActVA-Orf6, a novel type of monooxygenase involved in actinorhodin biosynthesis. EMBO J. *22*, 205–215.
14. Rebets, Y., Ostash, B., Luzhetskyy, A., Hoffmeister, D., Braña, A., Mendez, C., Salas, J.A., Bechthold, A., and Fedorenko, V. (2003). Production of landomycins in strains *Streptomyces globisporus* 1912 and *S. cyanogenus* S136 is regulated by genes encoding putative transcriptional activators. FEMS Microbiol. Lett. *222*, 149–153.
15. Ostash, B., Rebets, Yu., Yuskevich, V., Luzhetskyy, A., Tkachenko, V., and Fedorenko, V. (2003). Targeted disruption of *Streptomyces globisporus* 1912 *lndF* and *lndL* cyclase genes involved in antitumor landomycin E biosynthesis. Folia Microbiol. (Praha) *48*, 484–488.
16. Trefzer, A., Fischer, C., Stockert, S., Westrich, L., Künzel, E., Girreser, U., Rohr, J., and Bechthold, A. (2001). Elucidation of the function of two glycosyltransferase genes (*lanGT1* and *lanGT4*) involved in landomycin biosynthesis and generation of new oligosaccharide antibiotics. Chem. Biol. *8*, 1239–1252.
17. Trefzer, A., Blanco, G., Remsing, L., Künzel, E., Rix, U., Lipata, F., Braña, A.F., Méndez, C., Rohr, J., Bechthold, A., et al. (2002). Rationally designed glycosylated premithramycins: Hybrid aromatic polyketides using genes from three different biosynthetic pathways. J. Am. Chem. Soc. *124*, 6056–6062.
18. Wang, L., McVey, J., and Vining, L.C. (2001). Cloning and functional analysis of a phosphopantetheinyl transferase superfamily gene associated with jadomycin biosynthesis in *Streptomyces venezuelae* ISP5230. Microbiol. *147*, 1535–1545.
19. Kirsching, A., Bechthold, A., and Rohr, J. (1997). Chemical and biochemical aspects of deoxysugars and deoxysugar oligosaccharides. Top. Curr. Chem. *188*, 1–84.
20. Filippini, S., Solinas, M.M., Breme, U., Schluter, M.B., Gabellini, D., Biamonti, G., Colombo, A.L., and Garofano, L. (1995). *Streptomyces peucetius* daunorubicin gene, *dnr*F: Sequence and heterologous expression. Microbiol. *141*, 1007–1016.
21. Méndez, C., and Salas, J.A. (2001). Altering the glycosylation pattern of bioactive compounds. Trends Biotechnol. *19*, 449–456.
22. Flett, F., Mersinias, V., and Smith, C.P. (1997). High efficiency intergeneric conjugal transfer of plasmid DNA from *Escherichia coli* to methyl-DNA-restricting streptomycetes. FEMS Microbiol. Lett. *155*, 223–229.
23. Blondelet-Rouault, M.-H., Weiser, Y., Lebrihi, A., Branny, P., and Pernodet, J.L. (1997). Antibiotic resistance gene cassettes derived from the Ω interposon for use in *Escherichia coli* and *Streptomyces*. Gene *190*, 315–317.
24. Bierman, M., Logan, R., O'Brien, K., Seno, E.T., Rao, R.N., and Schoner, B.E. (1992). Plasmid cloning vectors for the conjugal transfer of DNA from *E. coli* to *Streptomyces spp*. Gene *116*, 43–49.
25. Sambrook. J., and Russel, D.W. (2001). Molecular Cloning: A Laboratory Manual, Third Edition (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).
26. Kieser, T., Bibb, M.J., Buttner, M.J., Chater, K.F., and Hopwood, D.A. (2000). Practical *Streptomyces* Genetics (Norwich, United Kingdom: The John Innes Foundation).
27. Sanger, F., Nicklen, S., and Coulson, A.R. (1977). DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA *74*, 5463–5467.
28. Devereux, J., Haeberli, P., and Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. *12*, 387–395.
29. Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. *25*, 3389–3402.
30. Henkel, T., Rohr, J., Beale, J., and Schwenen, L. (1990). Landomycins, new angucycline antibiotics from *Streptomyces* sp. Structural studies on landomycins A-D. J. Antibiot. (Tokyo) *43*, 492–503.
31. Weber, S., Zolke, C., Rohr, J., and Beale, J.M. (1994). Investigations of the biosynthesis and structural revision of landomycin A. J. Org. Chem. *59*, 4211–4214.
32. Fernandez-Lozano, M.J., Remsing, L.L., Quiros, L.M., Braña, A.F., Fernandez, E., Sanchez, C., Méndez, C., Rohr, J., and Salas, J.A. (2000). Characterization of two polyketide methyltransferases involved in the biosynthesis of the antitumor drug mithramycin by *Streptomyces argillaceus*. J. Biol. Chem. *275*, 3065–3074.
33. Rodriguez, L., Aguirrezabalaga, I., Allende, N., Braña, A.F., Méndez, C., and Salas, J.A. (2002). Engineering deoxysugar biosynthetic pathways from antibiotic-producing microorganisms. A tool to produce novel glycosylated bioactive compounds. Chem. Biol. *9*, 721–729.

Accession Numbers

The sequences of the *lnd* genes were submitted to GenBank with accession numbers AY443343, AY443344, and AY443345 for *lndGT4*, *lndZ4*, and *lndZ5*, respectively, and AY528820 and AY523868 for lndGT1 and a partial sequence of lndGT2, respectively.