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Identification of the Function of Gene *lndM2* Encoding a Bifunctional Oxygenase-Reductase Involved in the Biosynthesis of the Antitumor Antibiotic Landomycin E by *Streptomyces globisporus* 1912 Supports the Originally Assigned Structure for Landomycinone

Lili Zhu[†], **Bohdan Ostash**[‡], **Uwe Rix**[†], **Mohammad Nur-e-Alam**[†], **Almuth Mayers**[§], **Andriy Luzhetskyy**[§], **Carmen Mendez**[‖], **Jose A. Salas**[‖], **Andreas Bechthold**[§], **Victor Fedorenko**[*,‡], and **Jürgen Rohr**[*,†]

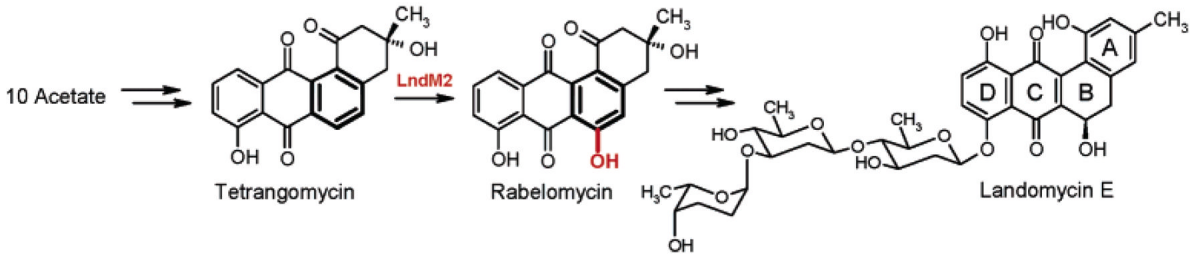
[†]Department of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, 725 Rose Street, Lexington, Kentucky 40536-0082

[‡]Department of Genetics and Biotechnology, Ivan Franko National University of L'viv, Grushevskyy st. 4, L'viv 79005, Ukraine

[§]Faculty of Pharmaceutical Biology, Albert-Ludwigs-University of Freiburg, Stefan-Meier-Strasse 19, 79104 Freiburg, Germany

[‖]Departamento de Biología Funcional e Instituto Universitario de Oncología del Principado de Asturias (I.U.O.P.A), Universidad de Oviedo, 33006 Oviedo, Spain

Abstract



The angucycline antibiotic family of the landomycins displays potent antitumor activity. To elucidate early post polyketide synthase (PKS) tailoring steps of the landomycin E biosynthetic pathway in *Streptomyces globisporus* 1912, the mutant *S. globisporus* M12 was prepared through gene replacement experiment of *lndM2*. It encodes an enzyme with putative oxygenase and reductase domains, according to sequencing of the gene and its counterpart *lanM2* from *S. cyanogenus* S136 landomycin A biosynthetic gene cluster. The isolation of the novel shunt products 11-hydroxytetrangomycin and 4-hydroxytetrangomycin along with the well-known angucyclines tetrangomycin and tetrangulol from the culture of *S. globisporus* M12 provides evidence for the

jrohr2@uky.edu.

Supporting Information Available: Original NMR spectra obtained after the acetate feeding experiments on landomycin A and a newly generated ^{1}H NMR spectrum of natural landomycinone supporting the original landomycin structure. This material is available free of charge via the Internet at http://pubs.acs.org.

involvement of *lndM2* in the early biosynthetic pathway of the landomycins, in particular in the formation of the alicyclic 6-hydroxy function of the landomycin aglycon. We therefore propose LndM2 to be responsible for both hydroxylation of the 6-position and its subsequent reduction. These reactions are necessary before the glycosylation reactions can occur. The results are in agreement with the originally published structure of landomycin but do not support the recently suggested revised structure.

Introduction

The landomycins, an angucycline family of antibiotics possessing potent anticancer activities, have been studied widely ever since their first discovery in 1990 as products of *Streptomyces cyanogenus* S136.1,[2] Over the past decade, two gene clusters encoding landomycin biosyntheses, namely, (i) the hexasaccharidal landomycin A (**2**, Scheme 1) produced by *S. cyanogenus* S136 (*lan*) and (ii) the trisaccharidal landomycin E (**1**) produced by *S. globisporus* 1912 (*lnd*, Scheme 1) have been cloned and sequenced.[3–5] The studies revealed that the clusters display an extraordinarily high degree of identity with regard to both the gene cluster organization and the DNA sequence of the respective genes.[3,4,6] However, progress in elucidating their biosynthetic pathways was hampered significantly by the refractory nature of both strains toward genetic manipulation. Therefore, previous experiments had been limited to the heterologous expression of selected biosynthetic genes in other polyketide-producing strains, such as the urdamycin A (**3**) producer *S. fradiae* TÜ 2717, which led to the identification of the glycosyltransferase genes *lanGT1* and *lanGT4* as D-olivosyl- and L-rhodinosyltransferases, respectively.[7,8]

Only recently, successful initial gene inactivation experiments were performed in *S. globisporus* by intergeneric conjugation,[9] which further led to the identification of the function of a regulatory enzyme encoded by *lndI*[6] and the cyclases encoded by *lndF* and *lndL*,[10] which are both involved in the formation of the angucycline framework of landomycin E (**1**).

Particularly puzzling are the oxygenation steps of landomycin biosynthesis, since ^{18}O-labeling experiments revealed that only two of the six oxygen atoms of the aglycon, namely, 1-O and 8-O, derived from acetate building blocks, whereas all others appeared to be introduced by molecular oxygen (Scheme 2).[2,11] Only recently we could identify the reductase/oxygenase pair LndZ4/Z5 as being responsible for the introduction of the 11-hydroxy function of the landomycin aglycon.[12] The incorporation experiments left some ambiguity particularly with respect to the oxygen in 6-position, because the 6-oxygen, although in a suitable position for being acetate-derived, clearly was not ^{18}O-labeled by [1-^{13}C,$^{18}O_2$] acetate, while the fermentation in an $^{18}O_2$-enriched atmosphere yielded anhydrolandomycin A (**4**) lacking the 6-oxygen (Scheme 2).[2]

In this context it is interesting that Roush et al. recently suggested a revised structure of landomycin A with the hydroxy group in the 5-position instead of in the 6-position.[13] This suggestion was based on the fact that the ^{1}H and UV spectroscopic data of the synthetic landomycinone[13] did not match the data provided for hydrolyzed landomycinone from natural landomycin A.[1]

Previous sequence alignment studies revealed two other putative oxygenase-encoding genes to be present in both landomycin gene clusters, namely, *lanE/lndE* and *lanM/lndM*.[3–5,14] The similarity of *lanE* (and therefore also *lndE*) with *urdE*[14–16] suggested that this gene is most likely involved in the generation of the *p*-quinone moiety. Therefore, the putative oxygenases LanM/LndM were the best candidates for being responsible for the 6-oxygenation in landomycin biosynthesis. In course of this work we have found sequencing errors in the published *lanM* sequence. Further careful sequencing revealed that genes *lanN* and *lndN*

appeared to form single open reading frames with *lanM* and *lndM*, respectively. These revised genes are referred to here as *lanM2* and *lndM2*. Here we also describe the generation of the *S. globisporus* M12 mutant strain, in which the putative oxygenase-reductase encoding gene *lndM2* has been inactivated. We expected both corresponding enzymes to be involved in the generation of the secondary alcohol function in the 6-position of the landomycin aglycon, an early post-PKS tailoring event in landomycin biosynthesis.

Results and Discussion

Probing *lanM2/lndM2* Functions, Complementation Studies, and lndM2 Disruption

Initially we tried to obtain clues regarding the C-6-oxygenation through heterologous expression of *lanM/lndM* and *lanN/lndN* genes into the urdamycin producer *S. fradiae* Tü2717. In the course of *lanM* and *lanN* PCR cloning we uncovered that in fact there are no two separate *lanM* and *lanN* genes in the *S. cyanogenus* S136 *lan* cluster but a single *orf* encoding an oxygenase-reductase protein, now designated as *lanM2*. The same was shown for the landomycin E gene cluster in *S. globisporus* 1912. Genes *lanM2* (2232 bp) and *lndM2* (2250 bp) share 79% of identity at the level of nucleotide sequences, and their probable translation products display significant end-to-end similarity (60–65%) to several oxygenase-reductase encoding genes involved in biosynthesis of angucycline/angucyclinone compounds from *S. aureofaciens*,[17a] *S. sp*. PGA64,[17b] PD116740,[18] and kinamycin,[19] which is also biosynthesized via an angucyclinone.

Former *lanN/lndN* genes were thought to encode putative short chain alcohol dehydrogenases, putatively involved in one of the reduction steps during landomycin biosynthesis (Scheme 2).[3] With the revised sequences in hand, we can put LanM2/LndM2 proteins in the group of UrdM-like bifunctional reductases-oxygenases,[15,20] which are common for angucycline biosynthesis. Indeed, in most cloned gene clusters for angucycline biosynthesis we encounter *lanM2/lndM2* homologues.

Genes *lanM2/lndM2* were expressed in the wild-type urdamycin producer, and *lanM2* was also expressed in the *urdM* disrupted strain Δ*urdM*,[14,20] in the AX strain (lacks genes encoding the glycosyltransferases UrdGT1a, UrdGT1b, UrdGT1c), and in the A0 strain (lacking all four glycosyltransferase genes of the urdamycin pathway – UrdGT1a, UrdGT1b, UrdGT1c, and UrdGT2).[8,21] We could not detect any changes in the production spectrum of *S. fradiae* and its mutant strains upon expressing either *lndM2* or *lanM2*. Thus the LanM2/LndM2 protein recognizes neither urdamycin A nor any of its intermediates as a substrate.

The suicide plasmid pTM7hy9 (Table 1) has been transferred into the landomycin E producer *S. globisporus* 1912. Apramycin-sensitive (Am^s) and hygromycin-resistant (Hy^r) transconjugants were selected with double crossover between homologous sequences in *S. globisporus* chromosome and the plasmid. This corresponds to a replacement of the intact chromosomal copy of *lndM2* by the disrupted allele *lndM2::hyg*. Disruption of the *lndM2* gene in one Am^s Hy^r clone marked as M12 has been confirmed by PCR analysis.

Preliminary TLC analysis of M12 mutant extracts showed an altered landomycin production pattern. The introduction of gene *lndM2* or *lanM2* into the M12 mutant restored the production of landomycin E at the wild-type level. Thus, the possibility of a polar effect on downstream genes caused by the *lndM2* disruption was ruled out. These complementation experiments also prove that the corresponding LndM2 and LanM2 proteins possess the same function for the biosynthesis of landomycin E and A, respectively, and that failure to express *lanM2/lndM2* genes in *S. fradiae* mutants is not caused by errors in course of gene cloning procedures.

Structure Elucidation of the M12 Mutant Products

Cultivation of the *S. globisporus* M12 mutant strain and subsequent TLC and HPLC/MS analysis of the crude extract showed three major products and trace amounts of a fourth, purple compound. The major compound, produced in amounts of 2 mg/L, displayed a molecular weight of 322 g/mol, based on a protonated molecular ion peak at *m/z* = 323 in the positive mode APCI-MS spectrum. Purification of this metabolite yielded a yellow solid, whose IR and UV spectra (e.g., a characteristic UV absorption maximum at λ = 267 nm) revealed a hydroxy-anthraquinone chromophore. Considering the mass, this compound was thought to be most likely tetrangomycin (**5**),[22,23] which was confirmed by the ^{1}H NMR and ^{13}C NMR spectra. The second and third major compounds, both orange solids produced in amounts of 1 mg/L (**6**) and 0.5 mg/L (**7**), respectively, displayed similar UV spectra as **5**, with only a small hypsochromic shift observed for the lower absorption maximum at λ = 262 nm. The APCI mass spectrum revealed that both of their molecular weights were the same, namely, 338 g/mol, which indicated them to be isomeric compounds containing one more oxygen atom compared with **5**. Subsequent NMR analysis (Table 2 and Table 3) of **6** revealed two aromatic AB systems, one of them in the 9/10-position in ring D instead of the ABC-system observed there for compound **5**. This rendered structure **6** as 11-hydroxytetrangomycin. The isomeric compound **7** was determined as 4-hydroxytetrangomycin based on the following ^{1}H NMR facts: both aromatic systems, the ABC system of ring D as well as the AB system of ring B, are like in **5**, whereas significant differences were observed for ring A. In compound **7**, 4-H appears as a downfield shifted doublet (δ 4.71 d, *J* = 7.5 Hz), and the AB-system of 2-H_2 shows only the geminal coupling of 16 Hz and lacks the longrange W-coupling observed between the two equatorial 2-H and 4-H protons of **5**. A new signal representing 4-OH is found, which shows a H,OH-coupling with 4-H of 7.5 Hz. In addition, a 2D-NOESY shows strong correlations between 4-H and 3-CH_3, respectively, which also renders the stereochemistry of C-4 as *R*. The ^{13}C NMR showing all 19 carbons is also in agreement with structure **7**. Indicative for the hydroxylated carbon 4 is its downfield shift to δ 74.3 compared to tetrangomycin (δ_{C-4} 43.5). Finally the purple trace compound was identified as tetrangulol (**8**), which is known as the decomposition product of **5**. The accumulation of tetrangomycin (**5**), 11-hydroxytetrangomycin (**6**), and 4-hydroxytetrangomycin (**7**) as major metabolites of the *S. globiporus* M12 raises the question of how these compounds are involved in the biosynthetic pathway towards the landomycins, either as intermediates or shunt products. Compared to the final product landomycins E (**1**) and later intermediates of the pathway, all sugar moieties were missing. Moreover, all three main products accumulated by *S. globisporus* M12 lack the 6-OH group and possess an aromatic B-ring. Although the absence of the 6-OH function in **5, 6**, and **7** could be hypothetically explained by elimination of water from ring B, it is known that this hydroxyl function in 6-position is rather stable.[2] Therefore, the missing 6-OH group is most likely a result from the inactivation of oxygenase LndM2, and thus LndM2 can now be assigned as the most likely candidate responsible for the introduction of the 6-OH function. The fact that *S. globisporus* M12 does not produce any glycosylated metabolites suggests that the 6-oxygenation and de-aromatization of ring B are crucial early biosynthetic steps, shaping the landomycin aglycon for its recognition by the first glycosyltransferase, Lnd/LanGT2.[12] However, at this stage it could not be distinguished whether the monooxygenation of the 6-position is preceded or followed by a reduction step in order to yield the partly saturated ring B. This reduction step is most likely catalyzed by the reductase domain of LndM2, which therefore acts either as a ketoreductase (Scheme 4, path A via rabelomycin) or as saturase (or enereductase, path B, Scheme 4), the former being more likely than the latter as a result of the sequential arrangement in the gene *lndM2*. Whether path A or path B is taken also distinguishes which enzyme subfunction provides the stereochemistry of the only chiral center of landomycinone, the oxygenase or the reductase domain of oxidoreductase LndM2.

Feeding Experiments

All of the four acquired M12-metabolites (**5–8**) as well as another related compound, rabelomycin (**9**),[24] were fed to an early block mutant of the landomycin biosynthesis to check whether they were intermediates of landomycin E biosynthesis. Rabelomycin (**9**) was isolated from the mutant *S. fradiae* ΔM.[14,20] The feeding experiments were carried out using the mutant *S. globisporus* F133[10] in which the PKS-associated fourth ring cyclase gene *lndF* was inactivated. Thus, this mutant cannot produce any correctly cyclized polyketides[10] but can still express all downstream enzymes necessary for the landomycin E production when fed with an intermediate containing a completed polyketide moiety. Strain *S. globisporus* F133 was grown for 24 h, and compounds **5–9** were then fed in a single portion as solid powders (1 mg each). HPLC monitoring (every 24 h) showed that only the feeding of tetrangomycin (**5**) and of rabelomycin (**9**) led to the production of landomycin E in *S. globisporus* F133 48 h after the feeding, while the feeding of compounds **6, 7**, and **8** did not lead to any landomycin E production (the fed compounds **6–8** were still detectable after 72 h). The disappearance of the fed metabolites **5** and **9** as well as the appearance of landomycin E (**1**) was monitored by HPLC-MS; a peak not observable in a control experiment (in which no compound was added) after feeding with tetrangomycin or rabelomycin was identified to be landomycin E according to its HPLC relative retention time and its UV and MS spectra.[12] No such transformation was detected in the corresponding cultures after feeding with the same amount of the other three compounds. Accordingly, it was concluded that tetrangomycin (**5**) and rabelomycin (**9**) are intermediates in the biosynthesis of landomycin E, while the other three metabolites (compounds **6–8**) yielded by *S. globisporus* M12 strain are shunt products. This also proves the validity of the left pathway (path A) shown in Scheme 4. In a second control experiment we fed rabelomycin (**9**) to *S. globisporus* M12. In contrast to *S. globisporus* F133, the *S. globisporus* M12 strain was unable to convert rabelomycin into landomycin E, suggesting that both activities of the oxidoreductase LndM2 were destroyed in the M12 mutant.

Conclusions

In summary, the inactivation of the *lndM2* gene resulted in four products, namely, tetrangomycin (**5**), which we assume to be a biosynthetic intermediate of the pathway, and the three other products **6**, **7** and **8**, which we assume to be shunt products of the pathway. Two of the discovered compounds, namely, 11-hydroxytetrangomycin (**6**) and 4-hydroxytetrangomycin (**7**), are new natural products. The structures of these accumulated products suggest that the putative flavoprotein LndM2 acts as an oxidoreductase, whose oxygenase activity is responsible for the attachment of the 6-oxygen, thereby producing rabelomycin (**9**) as an intermediate, which then is reduced to **11**, catalyzed by the reductase activity of the same enzyme LndM2. This view is further supported by the feeding experiment of rabelomycin (**9**) to *S. globisporus* M12, in which the mutant strain was unable to convert the intermediate **9**. This suggests that the needed reductase activity is provided by LndM2 (which is inactivated here) and not by any other enzyme.

Whereas the formation of tetrangulol (**8**) is most likely due to a spontaneous dehydration and aromatization of ring A of **5**[22,23] and the formation of shunt product **6** can be explained by a certain flexibility of the enzymes LndZ4/Z5, which are responsible for the 11-hydroxylation,[12] the formation of angucyclinone **7** cannot readily be explained, because a 4-hydroxylation has never been observed before in any of the landomycins, neither from *S. globisporus* nor from *S. cyanogenus*. Nevertheless, this shunt pathway toward **7** might be caused by one of the two remaining oxygenases of the *lnd* gene cluster, e.g., LndE, or more likely by an oxygenase of *S. globisporus*, which is not part of the *lnd* pathway. It is likely that all three observed shunt pathways occur only because of the accumulation of tetrangomycin (**5**), which normally is quickly converted to **9** and on to **11**, etc., before the final glycosylation reactions set in.

Although the suggested revised structure of landomycinone[13] is in agreement with the above summarized ^{18}O-incorporation data (see Scheme 2), the revised landomycinone structure would require an incorporation of the third acetate building block against all other acetate building blocks, which is biochemically most unlikely, since the earlier incorporation experiments clearly prove that the oxygen-bearing carbon of ring B is enriched by [1-^{13}C]-acetate (see also Supporting Information). Therefore, in agreement with the typical polyketide head-to-tail condensation of acetate units, this oxygen-bearing carbon was assigned as C-6, and this ^{13}C-assignment was proven by long-range C–H couplings and an INADEQUATE experiment after feeding of doubly ^{13}C-labeled acetate (see also Supporting Information).[2] The results described in this publication also support the originally assigned landomycin structure and in addition solve the mystery caused by the ^{18}O-incorporation experiments, because the incorporation of tetrangomycin and especially of rabelomycin suggest that the biosynthesis toward landomycinone proceeds through an angucyclinone with an aromatic ring B (tetrangomycin), whose 6-position (!) is then (re)-hydroxylated by LndM2 to rabelomycin (Scheme 4). Note also that the ^{1}H NMR data of recently produced natural landomycinone match perfectly those of the synthetic landomycinone.13,[25] The result that the landomycin biosynthesis proceeds via angucyclinone intermediates with aromatic B-rings (tetrangomycin, rabelomycin) also explains why all attempts to complement *S. fradiae* mutants with the *lndM2* gene failed, since no compound with an aromatic ring B can be an intermediate of urdamycin A biosynthesis.

Experimental Section

Bacterial Strains and Culture Conditions

The bacterial strains and plasmids used in this work are listed in Table 1. *Escherichia coli* strains were grown at 37 °C in LB, 2×TY or TB[26] for routine applications. *S. globisporus* strains were grown at 30 °C. Polyketide production of *S. globisporus* and *S. fradiae* strains was examined in SG and NL111v, respectively.5,8 For total and plasmid DNA isolation, streptomycete strains were grown in YEME for 48 h.[27] Solid oatmeal medium (OM)[8] was used for *S. globisporus* sporulation and plating of *E. coli–Streptomyces* matings. Where required, *S. globisporus* and *E. coli* strains were grown in the presence of ampicillin, apramycin, spectinomycin, kanamycin, chloramphenicol, nalidixic acid, and hygromycin at concentrations specified.[27]

DNA Manipulations

Genomic and plasmid DNA from *Streptomyces* and plasmid DNA from *E. coli* was isolated using standard protocols.[26,27] *E. coli* transformation, *S. fradiae* protoplast transformation, and intergeneric *E. coli–Streptomyces* matings were performed following established protocols.[9,26,27] Restriction endonucleases, the Klenow fragment, bacterial alkaline phosphatase, T4 DNA ligase, and *Pfu* polymerase were purchased from standard commercial sources (MBI Fermentas, NEB, Amersham Biosciences) and used according manufacturer's instructions. PCR reactions were performed using thermal cycler MiniCycler (MJ Research).

DNA Sequencing and Revision of *lanM,N/lndM,N* Sequences

Nucleotide sequences were determined by the Sanger method using automatic laser fluorescence sequencer (Vistra 725, Amersham-Pharmacia Biotech). Sequencing reactions were completed using a thermosequenase cycle sequencing kit (Amersham-Pharmacia Biotech). Sequences were analyzed using DNASIS software (version 2.1 Hitachi Software Engineering). Positions 8767 (A) and 8772 (T) of published sequence of *S. cyanogenus* S136 *lan* cluster[3] (accession number AF080235) do not exist. Thus, the *lanM* open reading frame (*orf*) reaches up to position 9469, comprising former putative reductase gene *lanN*. This revised *orf* was designated *lanM2*. Complete sequencing of the respective fragment of the *S.*

globisporus 1912 *lnd* cluster revealed the same gene organization (Figure 1), and the *orf* containing former *lndM* and *lndN* genes was named *lndM2*. Both strands of *lanM2* and *lndM2* genes were sequenced three times to avoid any errors. GenBank accession numbers to *lanM2* and *lndM2* sequences are AY640376 and AY640377, respectively.

Construction of Plasmids. A. For Expression Experiments

Gene *lndM2* was excised as 2.5-kb *Sac*I-*Eco*RV fragment from pKK3 (Figure 1), blunt ended with Klenow polymerase, and cloned into *Eco*RV site of expression vector pKC1218E[12] under the control of the *ermE** promoter to yield pKC1218ElndM2. The reductase part of the *lndM2* gene and the *lndO* gene encoding a putative reductase[5] were retrieved as 2.5-kb *Pst*I fragment from pKK3 and cloned into respective site of pKC1218E to give pKC1218E-NO9. Gene *lanM2* was amplified from cosmid H2-26 with primers LanM2forward, 5′-AGGGAA*CAATTG*CAGACGGCCCTGAGC, and LanM2reverse, 5′-TCCTGCGACATGAGTGTC*CATATG*GAC. In LanM2forward, nucleotides AT (typed in italics) in positions 9, 10 are introduced instead of GC in H2-26 in order to generate a site for the *Mun*I restrictase. In a similar way an *Nde*I site was generated in LanM2reverse (both sites are underlined in the oligonucleotide sequence above). The PCR product has been sequenced and cloned into the pMUNII vector to give pMUN-lanMneu. Gene *lanM2* was excised as 2.5-kb *Hin*dIII-*Xba*I fragment from pMUN-lanMneu and cloned into pUWL201[8] under the *ermE** promoter (pUWLlanM2). It was also cloned as 2.9-kb *Pvu*II fragment into pKC1218E (pKC1218ElanM2).

B. For *lndM2* Disruption

A 3.5-kb *Sac*I fragment from pKK3 (containing the whole *lndM2*, *lndO* and the 5′-terminus of *lndP*; Figure 1) was subcloned into *Sac*I-digested pBlueΔPstI. Protruding 3′-ends of the *Pst*I site (883 bp downstream of the *lndM2* start codon) in pBM7 were digested with the Klenow fragment. The hygromycin resistance gene *hyg* (encoding hygromycin B phosphotransferase27), retrieved as *Eco*RV fragment from plasmid pHYG1 (see Table 1), has been inserted into the blunted *Pst*I site (resulting plasmid is pBM7hy1). Internally to the *Eco*RV site at the 5′-end of the *hyg* gene is a *Pst*I site derived from a multiple cloning site of the vector DNA, from which the *hyg* gene was previously subcloned. Restriction of pBM7hy1 with *Sac*I (located at the 5′-end of *lndM2*) and *Pst*I (5′-end of the *hyg* gene) showed the expected 900-bp fragment, confirming the *hyg* gene to be cloned in the same transcriptional orientation as the *lndM2* gene. A 5.1-kb *Sac*I fragment with *lndM2::hyg* was excised from pBM7hy1, treated with the Klenow fragment and cloned into an *Eco*RV digested pTNK to set up the final construct pTM7hy9. Replacement of *lndM2* with disrupted allele *lndM2::hyg* was confirmed by PCR amplification of *lndM2::hyg* from the *S. globisporus* mutant genome using primers for the ends of the remaining oxygenase halve of *lndM2*: LNDM5, 5′-TCGCCAAGGACTACGCCG (T_m = 60 °C) and LNDM3, 5′-TCACCAGTGCCGTCCGC (T_m = 58 °C).

Metabolite Analysis

Large-scale liquid *S. globisporus* M12 cultures were grown at 30 °C in a GFL 3033 shaking incubator from GFL (Burgwedel, Germany) for 3 days to generate four metabolites. The crude ethyl acetate extract of the main cultures was purified by chromatography (column 25 cm × 2.5 cm, silica gel with 50–100 μm particle size from Macherey & Nagel, Düren, Germany). For the TLC analysis SilG-25 and silica 60 F_{254} silica gel plates (Merck, Darmstadt, Germany) with UV fluorescence indicator were used. HPLC-atmospheric pressure chemical ionization (APCI)-MS analysis was performed on a Waters Alliance 2695 instrument coupled with a Waters 2996 photodiode array detector and a Waters/Micromass ZQ mass spectrometer. The HPLC-MS analysis was carried out on a Waters Symmetry C_{18} (4.6 mm × 50 mm, 5 μm

particles) column at a flow rate of 0.5 mL/min, with the following gradient: 0–3 min 25% B, 3–10 min linear to 100% B, 10–12 min linear to 25% B (solvent A 99.9% H_2O, 0.1% formic acid; solvent B 100% acetonitrile). For the detection a Waters 996 photodiode array detector was used set to scan from 200 to 500 nm.

The structures of the major three metabolites tetrangomycin (**5**), 11-hydroxytetrangomycin (**6**), and 4-hydroxytetrangomycin (**7**) were identified by NMR spectroscopy supported by UV and MS. NMR data were acquired on a Varian Inova-400 instrument at a magnetic field strength of B_0 9.4 T. For further details see Table 2 and Table 3; the reported chemical shifts are given in ppm relative to internal TMS. The structure of a trace amount metabolite, tetrangulol (**8**), was determined by HPLC-MS (see text). Urdamycin production, monitored by TLC and HPLC-MS, is described elsewhere.[8]

Feeding Experiments

A 1 mg portion of each of the five compounds **5–9** was added to a 50-mL liquid culture of *S. globisporus* F133, and the landomycin E production was monitored by HPLC-MS after 48 h. Standard control experiments were carried out analogously, without feeding of compounds **5–9**. As another control experiment, 1 mg of rabelomycin (**9**) was added to a 50-mL liquid culture of *S. globisporus* M12, and no landomycin E production was detectable by HPLC-MS after 48 h. he HPLC-MS conditions were the same as described above.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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25. The discrepancy in the ^{1}H NMR and UV data between natural and synthetic landomycinone can at this point only be speculated on and might be just due to a typo in the original publication.[1] Natural landomycinone might also have been contaminated with metal ions from the fermentation process, since a chelate formation is possible, particularly in the 6-O, 7-O, 8-O region. In fact, recently produced natural landomycinone that was purified including an ion-exchange step showed ^{1}H NMR data (see also Supporting information) identical to those of the synthetic compound,[13] but no additional data regarding the stereocenter C-6[1] were generated.
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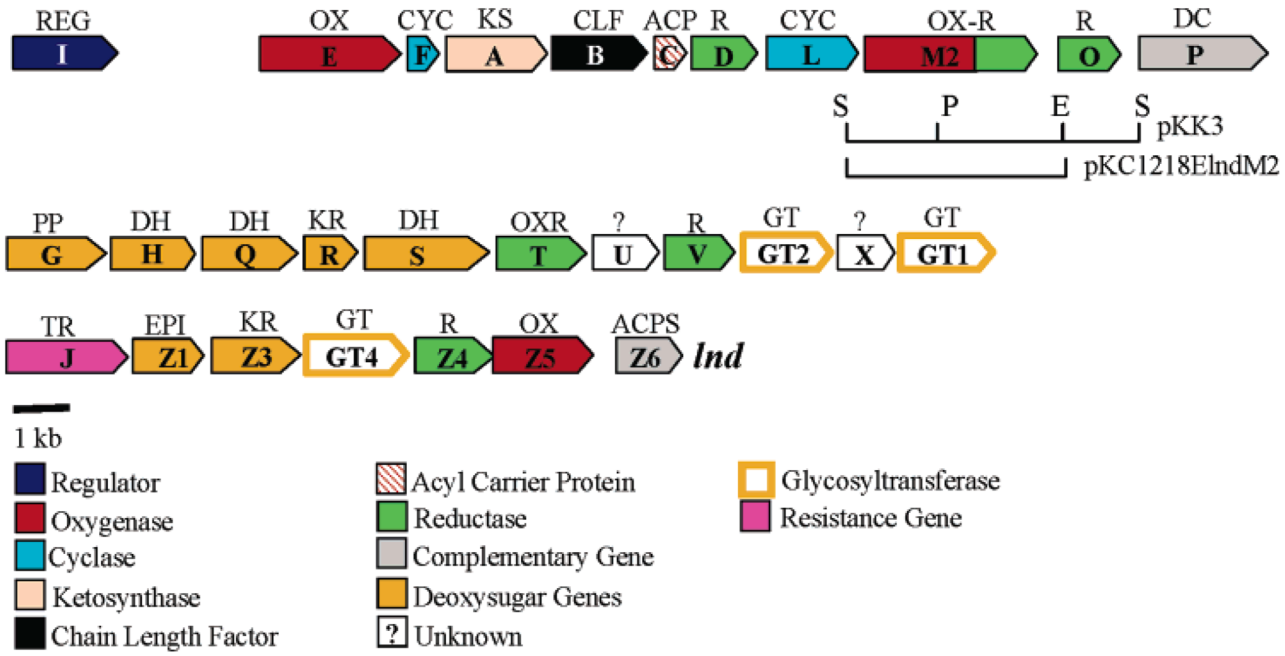
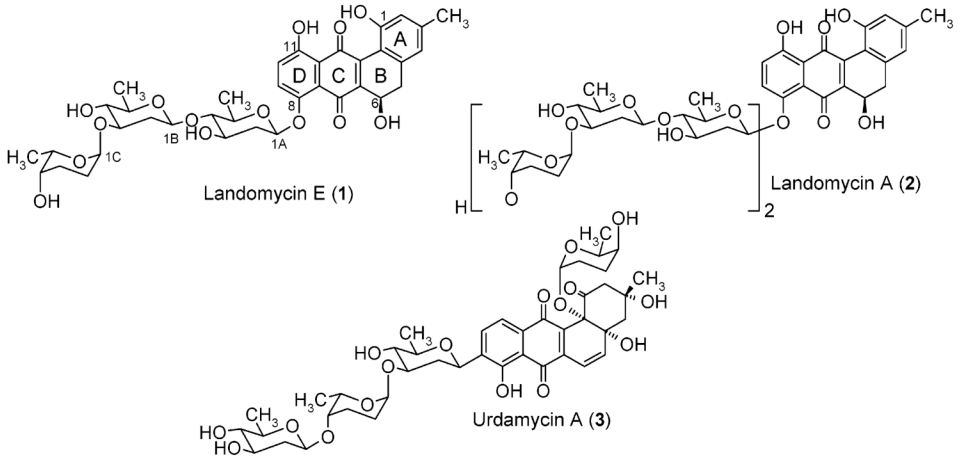


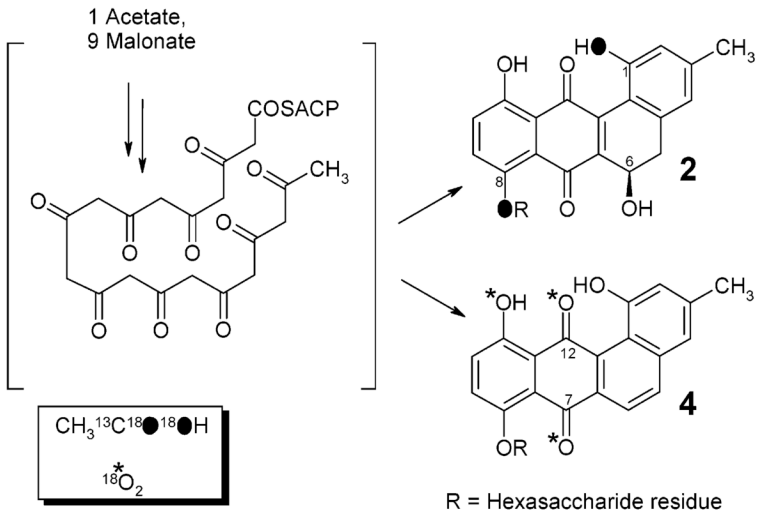
FIGURE 1.

Biosynthetic gene cluster of landomycin E (**1**) in *S. globiporus* 1912 (*lnd*). Relevant restriction sites are shown on plasmid pKK3, used in the course of plasmid construction (S, *Sac*I; P, *Pst*I; E, *Eco*RV). The gene organization of the *S. cyanogenus* S136 *lan* cluster in the studied region is essentially the same. So far, only the *lndL*, *lndF*, the *lndGT* functions, and the functions of *lndZ4* and *lndZ5* were confirmed by knockout and/or complementation experiments, whereas all others have so far been deduced only from in silico analysis.[3,6]



SCHEME 1.

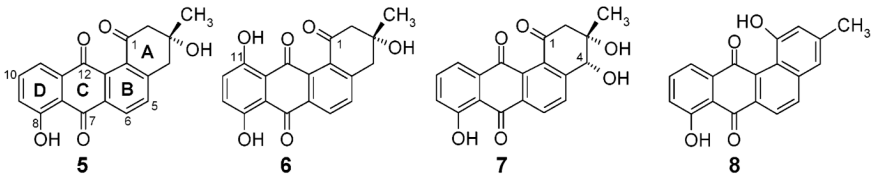
Chemical Structures of Angucycline Antibiotics Landomycin E (1) from *S. globisporus* 1912, Landomycin A (2) from *S. cyanogenus* S136, and Urdamycin A (3) from *S. fradiae* TÜ 2717.



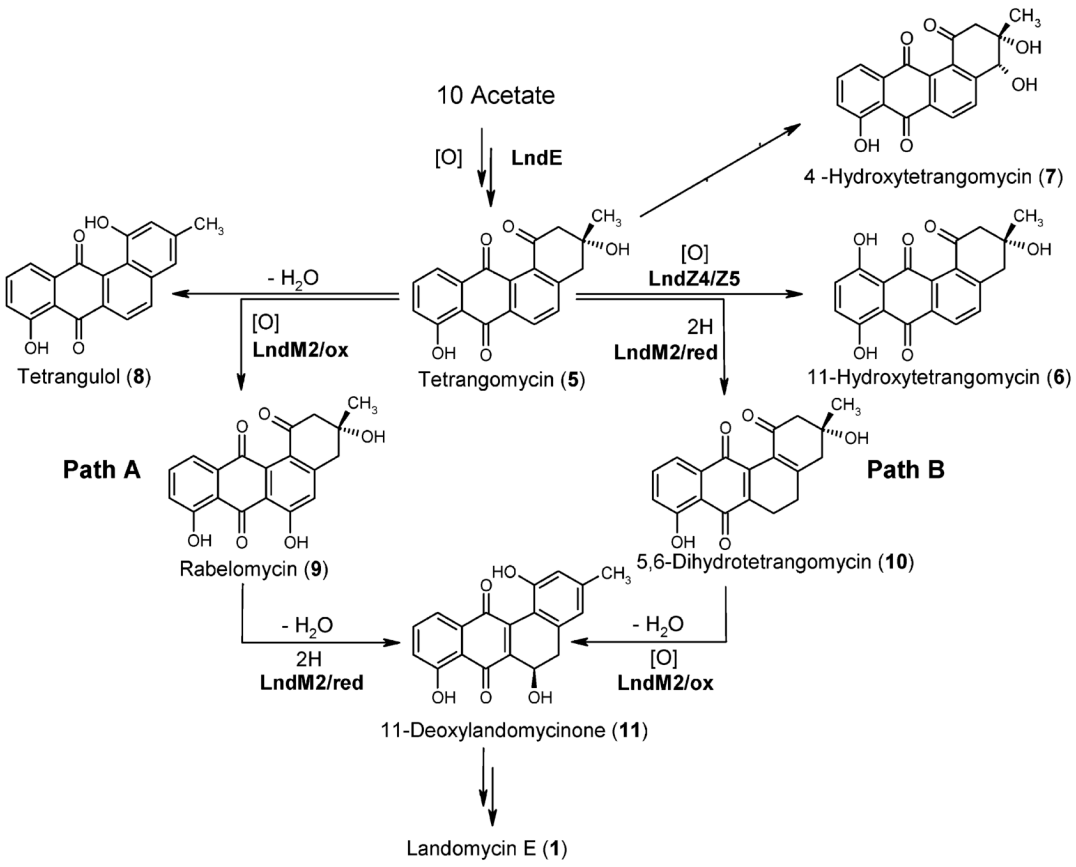
SCHEME 2.

Results from Earlier Incorporation Experiments with ^{18}O-Labeled Precursors on the Landomycin A Producer *S. cyanogenus*-136a

[a]^{18}O-Enrichment in positions 1 and 8 was observed upon feeding with [1-^{13}C,$^{18}O_2$]acetate (**2**), and 5,6-anhydrolandomycin A (**4**) was isolated after a fermentation under an ^{18}O-enriched atmosphere and shows ^{18}O-enrichment in the 7-, 11-, and 12-positions. From these experiments it was unclear where the oxygen in the 6-position derives from.[2]



SCHEME 3.
Chemical Structures of the Metabolites Accumulated by the M12 Strain



SCHEME 4.

Hypothetical Representation of Early Oxygenation and Reduction Steps during the Biosynthesis of Landomycin E (1); Path A Turned Out To Be Correct

TABLE 1

Strains and Plasmids Used in This Work

bacterial strain or plasmid	description	source or reference
E. coli DH5α	routine cloning procedures	MBI Fermentas
E. coli ET12567 (pUB307)	*dam*-13::Tn9(Cm^r) *dcm*–6 *hsd*M; harbors conjugative plasmid pUB307; Cm^r, Km^r; used to introduce recombinant plasmids into *S. globisporus* strains	C. P. Smith, UMIST, U.K.
S.globisporus 1912	landomycin E producer	ref 5
S. globisporus M12	*lndM2* disruption mutant (*lndM2::hyg*)	this work
S. globisporus F133	mutant carrying fourth ring cyclase *lndF* gene disruption (*lndF::aphII*)	ref 10
S. fradiae Tü 2717	urdamycin producer	refs 16 and 28
S. fradiae ΔurdM	mutant with oxygenase *urdM* gene disruption	ref 20
S.fradiae AX	mutant with disrupted glycosyltransferase (GT) genes *urdGT1a*, *urdGT1b*, *urdGT1c*	ref 8
S.fradiae AO	mutant lacking all 4 GT genes of the *urd* cluster	ref 21
pBluescriptIIKS(+)	general purpose *E. coli* cloning vector	MBI Fermentas
pBlueΔPstI	pBluescriptIIKS(+) derivative with *Pst*I site removed from polylinker	ref 6
pMUNII	general purpose *E. coli* cloning vector	ref 8
pKC1218E	*E. coli/Streptomyces* shuttle expression vector with P_{ermE} and SCP2* replicon, derivative of pKC1218 [12]; Am^r	ref 12
pUWL201	*E. coli* / *Streptomyces* shuttle expression vector with P_{ermE} and pIJ101 replicon; Th^r	ref 8
pTNK	conjugative suicide vector; Am^r	ref 9
H2-26	cosmid pOJ446 [12] carrying the entire *lan* cluster of *S. cyanogenus* S136	ref 3
pHYG1	pLitmus38 containing hygromycin resistance cassette hyg	C. Olano, Universidad de Oviedo, Spain
pKK3	pBluescriptIIKS(+) carrying 3.5 kb *Sac*I fragment of *lnd* cluster with genes *lndLlndM2lndO*	ref 5
pMUN-lanMneu	pMUNII carrying *lanM2* gene	this work
pUWLlanM2	pUWL201 carrying *lanM2* gene	this work
pKC1218ElanM2	pKC1218E carrying *lanM2* gene	this work
pKC1218ElndM2	pKC1218E carrying *lndM2* gene	this work
pKC1218E-NO9	pKC1218E carrying “reductase” halve of *lndM2* gene and *lndO* gene	this work
pBM7	pBlueΔPstI with 3.5 kb *Sac*I fragment from pKK3	this work
pBM7hy1	pBM7 with *hyg* cassette cloned into unique *Pst*I site within *lndM2* gene	this work
pTM7hy9	pOJ260 carrying 5.1 kb *Sac*I fragment with disrupted allele of *lndM2* gene *lndM2::hyg*	this work

TABLE 2

^{1}H NMR (400 MHz, DMSO-d_6, TMS) of New Compounds 11-Hydroxytrangomycin (6) and 4-Hydroxytetrangomycin (7) in Comparison with Tetrangomycin (5)

	δ ^{1}H (ppm) multiplicity (*J*, Hz)		
position	**5**	**6**	***7*** [b]
$2H_a$	3.06 d (14)	3.06 d (14)	2.79 d (16)
$2H_e$	2.74 dd (14, 2)	2.80 dd (14, 2)	3.05 d (16)
3-OH[a]	5.02 s (br)	5.05 s (br)	4.85 s
$3\text{-}CH_3$	1.35 s	1.49 s	1.34 s
$4H_a$	3.07 dd (16, 2)	3.07 dd (16, 2)	4.71 d (7.5)
$4H_e$	3.23 d (16)	3.22 d (16)	
4-OH[a]			5.97 d (7.5)
5	7.74 d (8)	7.78 d (8)	8.08 d (8)
6	8.23 d (8)	8.26 d (8)	8.34 d (8)
8-OH[a]	12.09 s (br)	11.84 s (br)	12.10 s (br)
9	7.36 dd (8, 1)	7.42 d (9)	7.36 dd (8, 1)
10	7.80 ddd (8, 8, 1)	7.44 d (9)	7.80 ddd (8, 8, 1)
11	7.52 dd (8, 1)		7.52 dd (8,1)
11-OH[a]		12.41 s (br)	

[a] Exchangeable with D_2O.

[b] The stereochemistry of this structure has been verified by 2D-COSY and 2D-NOESY experiments.

TABLE 3

^{13}C NMR (100 MHz, DMSO-d_6, TMS) of New Compounds 11-Hydroxytrangomycin (6) and 4-Hydroxytetrangomycin (7) in Comparison with Tetrangomycin (5)

	Δ ^{13}C (ppm)		
position	**5**	**6**	**7**
1	196.6	197.4	195.6
2	53.1	53.4	51.8
3	71.6	71.6	73.4[a]
3-CH_3	29.7	29.8	26.4
4	43.5	43.5	74.3[a]
4a	149.1	149.1	152.2
5	134.1	134.9	131.8
6	128.5	128.4	128.7
6a	132.7	128.5	133.1
7	186.7	185.7	186.7
7a	115.3	112.5	115.4
8	160.6	155.3	160.7
9	123.1	133.1	123.2
10	137.1	133.4	137.1
11	118.4	156.1	118.5
11a	135.4	113.7	135.1
12	182.7	186.0	182.6
12a	135.1	129.2	134.7[a]
12b	135.3	136.1	134.8[a]

[a]Assignments interchangeable.