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PAPER

Elucidation of post-PKS tailoring steps involved in landomycin biosynthesis†

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The functional roles of all proposed enzymes involved in the post-PKS redox reactions of the biosynthesis of various landomycin aglycones were thoroughly studied, both *in vivo* and *in vitro*. The results revealed that LanM2 acts as a dehydratase and is responsible for concomitant release of the last PKS-tethered intermediate to yield prejadomycin (**10**). Prejadomycin (**10**) was confirmed to be a general pathway intermediate of the biosynthesis. Oxygenase LanE and the reductase LanV are sufficient to convert **10** into 11-deoxylandomycinone (**5**) in the presence of NADH. LanZ4 is a reductase providing reduced flavin (FMNH) co-factor to the partner enzyme LanZ5, which controls all remaining steps. LanZ5, a bifunctional oxygenase–dehydratase, is a key enzyme directing landomycin biosynthesis. It catalyzes hydroxylation at the 11-position preferentially only after the first glycosylation step, and requires the presence of LanZ4. In the absence of such a glycosylation, LanZ5 catalyzes C5,6-dehydration, leading to the production of anhydrolandomycinone (**8**) or tetrangulol (**9**). The overall results provided a revised pathway for the biosynthesis of the four aglycones that are found in various congeners of the landomycin group.

Introduction

The landomycins are the largest sub-group of angucycline natural products, comprising a polyketide-derived benz[*a*]anthraquinone backbone, *e.g.*, landomycinone (**4**), anhydrolandomycinone (**8**), 11-deoxylandomycinone (**5**) or tetrangulol (**9**) and a deoxysugar chain of various lengths attached at the 8-position of the aglycone through an *O*-glycosidic linkage (Fig. 1A). A number of different congeners of this family have been isolated so far, among which landomycin A (**1**), isolated from *Streptomyces cyanogenus* S136,[1] and landomycin E (**2**), isolated from *Streptomyces globisporus*, were most intensively studied. Some of the landomycins show weak antibacterial activities,[2,3] but this group of angucyclines is most known for their remarkable antitumor activities,[1,4–6] with an interesting, apparently unique mode of action.[7] While other antitumor drugs with similar structure directly bind to DNA, the landomycins do not. They interfere most likely with steps of the DNA biosynthesis or repair enzymes, although the exact mechanism of action remains unclear.[8] Landomycin biosynthesis has been a subject of our studies from the very beginning.

Cloning and sequencing of chromosomal DNA of *S. cyanogenus* S136 (*lan* cluster) and of *S. globisporus* 1912 (*lnd* cluster) revealed genes, which encode PKS enzymes, deoxysugar biosynthetic enzymes, post-PKS tailoring enzymes and other enzymes involved in the regulation of biosynthesis and export of the antibiotic.[9,10] The post-PKS tailoring enzymes include oxygenases (LanE, LanZ5), reductases (LanV, LanZ4, LanO) and an oxidoreductase (LanM2) along with four glycosytransferases (LanGT1–4)].[11,12] The functional role of the LanGTs and timing of their actions have been established through targeted gene inactivation, complementation and cross-feeding experiments.[13–19] Incorporation studies involving the feeding of ^{13}C-labeled precursors revealed that the carbon backbone of landomycinone (**4**) is derived from 10 acetate and malonate units.[6] Further experiments involving ^{18}O-labeled molecular oxygen ($^{18}O_2$) and $CH_3C^{18}O^{18}OH$ indicated that only two of the six oxygen atoms of **4**, namely those at the 1- and 8-positions, originate from the polyketide building blocks, while all the others are incorporated from aerial oxygen through post-PKS tailoring reactions.[6] To provide more insight into this unusual oxygenation process, the suspected biosynthetic genes (*lndM2*, *lndZ4–lndZ5*, *lndE*, *lndV*) were inactivated sequentially from the landomycin E (**2**) biosynthetic gene cluster (*lnd* cluster, Fig. 1B).[9] A targeted inactivation of the proposed oxidoreductase *lndM2* (a homologue of *lanM2*) in the landomycin E producer *S. globisporus* 1912 led to the accumulation of 6-deoxygenated compounds (Fig. 1B) and

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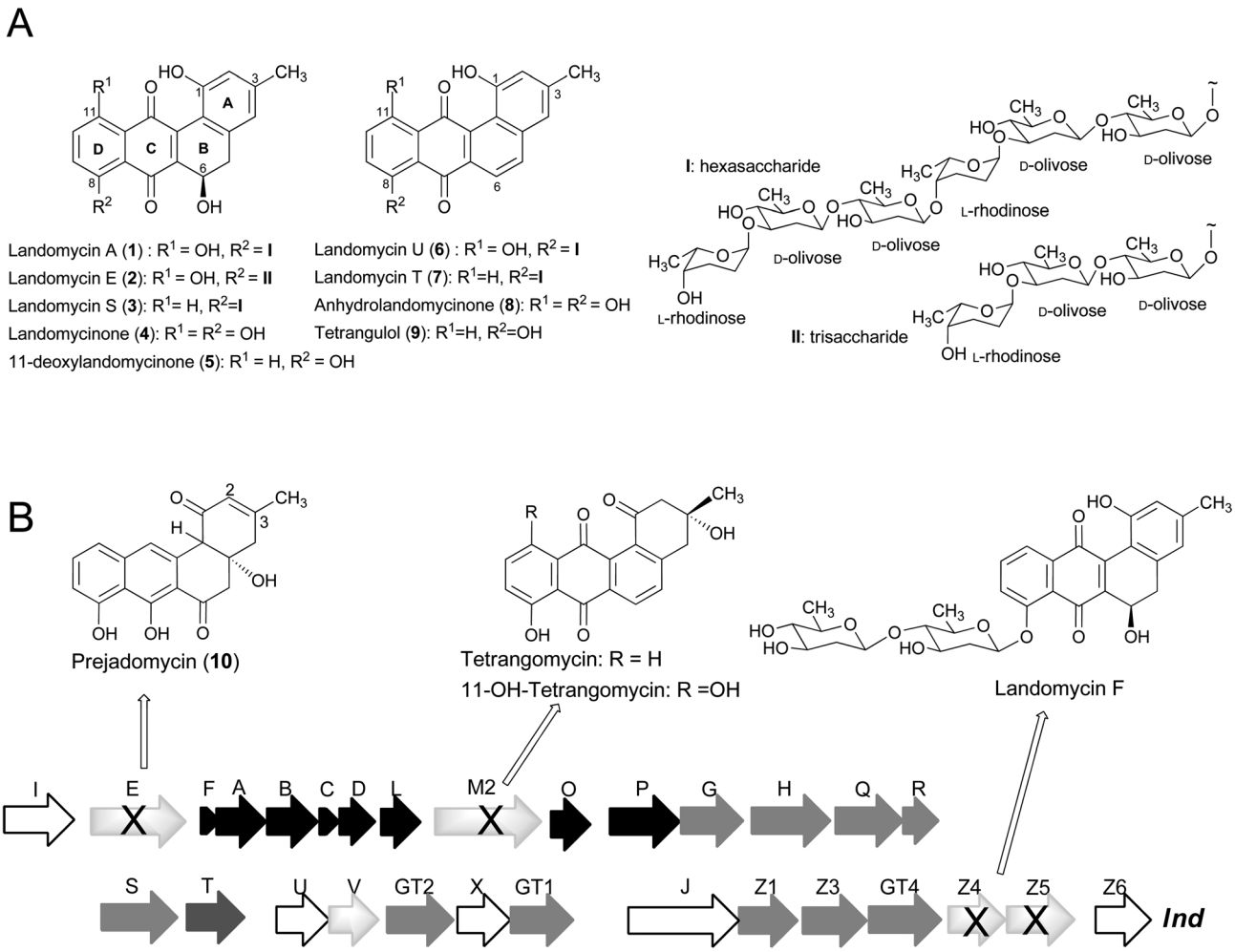


Fig. 1 **A**: Structures of representative landomycins, showing examples of two saccharide chains and all four different aglycones found in this group of anticancer agents. **B**: Predominantly accumulated compounds upon gene inactivation experiments (color scheme of *lnd* cluster: black = PKS genes, white = regulatory, dark gray = sugar pathway and attachment, light gray = oxidoreductases).

therefore indicated that the product of *lndM2* is responsible for the stereospecific incorporation of the hydroxyl group at 6-position of **4**.[20] Similarly, inactivation of *lndE* (homologue of *lanE*) led to the accumulation of a single metabolite, prejadomycin (**10**), suggesting LanE–LndE's functional role as a C-12 oxygenase.[21] Deletion of both *lndZ4* and *lndZ5* (*lanZ4* and *lanZ5* homologues, respectively) accumulated three landomycin derivatives, *e.g.* landomycin F, lacking the hydroxyl group at the 11-position.[22] This suggested that this pair of enzymes is responsible for the incorporation of oxygen at the 11-position of **4**. Although these studies provided preliminary information regarding the role of these enzymes, their exact substrates, co-factors, and the exact sequence of their actions remained ambiguous. In this context, we report here the characterization of these enzymes.

We used both, *in vivo* experiments utilizing minimal PKS enzymes and different combinations of post-PKS tailoring enzymes in a heterologous host, and *in vitro* utilizing individual isolated enzymes. A conversion of prejadomycin (**10**) to 11-deoxylandomycinone (**5**) is demonstrated *in vitro* utilizing a minimum number of isolated, overexpressed enzymes. Overall, this thorough study of function and substrates specificities of landomycin post-PKS tailoring enzymes led to a revised pathway for landomycin biosynthesis.

Results and discussion

Characterization of Lan-post-PKS enzymes (LanM2, E, V, Z4–Z5) *in vivo*

With the aim of elucidating the functional role of each of the Lan-post-PKS enzymes (LanM2, E, V, Z4 and Z5) and the timing of their actions, we first constructed expression cassettes, where individual genes encoding these enzymes were co-expressed with PKS genes alone, and then in groups. We took advantage of our earlier angucyclinone expression system, which contained the minimal PKS genes for the production of UWM6 (**16**).[23] A heterologous expression host, *S. lividans* TK64, was used for the co-expression.

LanM2 represents an oxidoreductase enzyme where the N-terminal 513 amino acid region showed strong similarity (>95% identity) to many FAD-dependent oxygenases or oxygenase components of oxidoreductases involved in the biosynthesis of secondary metabolites, *e.g.*, OvmOII (accession CAG14970,

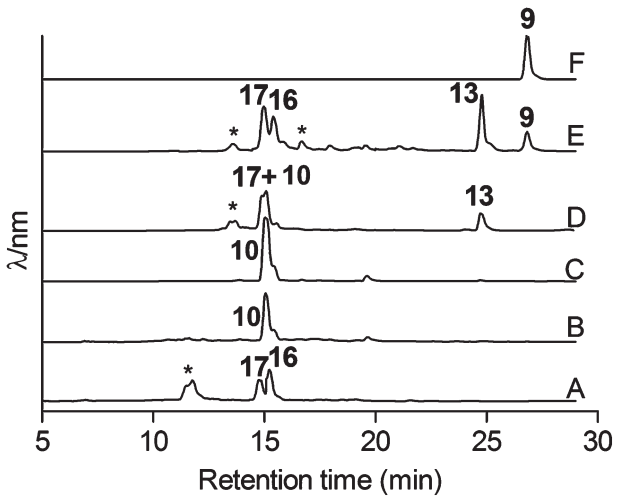


Fig. 2 HPLC analyses of the metabolites generated by co-expression of PKS and landomycin post-PKS oxygenase genes in *S. lividans* TK64. Trace A: the products of pRab4 (PKS enzymes only), **16** = UWM6, **17** = rabelomycin; trace B: products of *PKS–lanM2* (pLaox2), **10** = prejadomycin; trace C: *PKS–lanZ4–lanZ5–lanM2–lanV* (pLaox10); trace D: *PKS–lanZ4–lanZ5–lanM2–lanE* (pLaox6), **13** = dehydrorabelomycin; trace E: *PKS–lanZ4–lanZ5–lanM2–lanE–lanV* (pLaox12), **9** = tetrangulol; trace F: standard tetrangulol (**9**). The peaks labeled by asterisks represent minor metabolites not analyzed further.

94% identity), PgaM (AAK57530, 97%), KinOR (AAO65351, 95%) from the oviedomycin, gaudimycin and kinamycin gene clusters, respectively. The C-terminal 131 amino acids of LanM2 showed high homology (>90%) to reductases/reductase components of oxidoreductases such as Aur1I (ZP_07291694, 97%), BexM (ADI71448, 97% and Sim8 (AAL15586, 97%) from various Type II-PKS gene clusters. Co-expression of *lanM2* with the minimal set of PKS genes led to the accumulation of prejadomycin (**10**) instead of the usual product UWM6 (**16**) that is produced by the PKS enzymes alone (Fig. 2). This result suggested for the first time that LanM2 might catalyze a thioesterase-like decarboxylative 2,3-dehydration of the still ACP-tethered nascent angucycline **11**. Prejadomycin (**10**) was first found being accumulated by the *lndE*-deleted mutant of *S. globisporus*, and was proven to be an intermediate of the landomycin E biosynthetic pathway.[21]

LanZ4 and *lanZ5* are transitionally coupled genes in the *lan* cluster.[22] Homology searches in the database suggested that LanZ4 contains a typical NADPH-FMN binding domain and represented a homologue of NADPH-dependent FMN reductases (90–100% identity with reductases from a variety of bacteria). Similar searches for LanZ5 indicated that this enzyme was analogous to oxygenases, dehydrogenases and hydroxylases (80–95% identity) from various organisms. Deletion of either of their homologues from the *lnd*-cluster led to the removal of the hydroxyl group at 11-position of landomycin E, suggesting their mutual roles in the 11-hydroxylation step.[22] From a literature search we hypothesized that LanZ4 and LanZ5 are co-dependent, the former serving as a flavin regenerating reductase that provides reduced flavin co-factor to its partner LanZ5 consuming NADPH or NADH. Co-expression of the *lanZ4–lanZ5* pair with all the PKS genes did not reveal any difference in the production of metabolites (data are not shown), clearly indicating that the LanZ4–LanZ5 pair does not operate immediately after the PKS enzymes. Thus, the pair should operate late in the landomycin biosynthetic pathway.

Oxygenase LanE belongs to a super-family of enzymes with conserved nicotinamide adenine dinucleotide (NAD) binding domain. Homology searches revealed high amino acid sequence similarities (89–99%) to a number of oxygenases, including PgaE (99% identity), SchP10 (CAH10119, 98%), SimA7 (AAK06782, 98%), SaqE (ACP19351, 98%) from the gaudimycin, Sch47554, simocyclinone and saquayamycin biosynthetic gene clusters, respectively. Co-expression of *lanE* with the PKS genes led to the accumulation of solely rabelomycin (**17**), instead of predominantly UWM6 (**16**) generated by the PKS expression cassette pRab1 alone. This result indicated that LanE–LndE catalyzes the 12-oxidation step, and possibly the 4a,12b-dehydration, in the biosynthesis of landomycinone (**4**) and 11-deoxylandomycinone (**5**). Earlier feeding experiments suggested **17** to be a pathway intermediate for **4**.[20] But all previous results still left ambiguities, *e.g.*, which enzyme (LanM2 or LanE) acts first, after the assembly of the tetracyclic backbone by the PKS enzymes (Fig. 2).

LanV was another enzyme potentially participating in early post-PKS tailoring reactions. It harbors an NAD-binding domain and shows high homology to many reductases (90–100% amino acid identity) found in angucycline biosynthetic pathways such as SaqN (ACP19359, 99% amino acid sequence identity), PgaM (AAK57530, 100%), Sim8 (AAL15586, 100%) and AzicF (ADB02849, 96%) from the saquamycin, gaudimycin, simocyclinone and azicemicin biosynthetic pathways, respectively.[24–27] Previous complementation results suggested LanV to serve as a bifunctional aromatase/reductase, responsible for the aromatization of ring A as well as for the reduction of the 6-keto group of a postulated angucyclinone intermediate during landomycin biosynthesis.[28] However, co-expression of *lanV* with the PKS genes in *S. lividans* TK64 did not change the production of metabolites compared to that of the PKS enzymes alone. The results ruled out an early role of LanV during the biosynthesis of **4**.

To shed further light on the functional role of these post-PKS tailoring enzymes and their exact timing of action in the landomycin pathway, many other expression constructs were generated probing the co-expression of two or more post-PKS genes along with the PKS genes. Expression of *lanV* and *lanZ4–lanZ5* together with the PKS genes in *S. lividans* TK64 did not reveal any change in metabolite production compared to the control pRab4 (PKS enzymes only). Prejadomycin (**10**) was observed as the major metabolite when pRab4 was further complemented with *lanM2* and *lanZ4–lanZ5*, or with *lanM2* with *lanV* or with *lanM2* and all three other oxygenase genes together (Fig. 2). This confirmed that LanM2 likely catalyzes the first step of the post-PKS reactions of landomycin biosynthesis, and neither LanZ4–LanZ5 nor LanV catalyze the next step of the oxygenation cascade. Complementation of pRab4 with *lanE* and *lanZ4–lanZ5* or with *lanE*, *lanZ4–lanZ5* and *lanV* resulted in the accumulation of rabelomycin (**17**) as the major metabolite as observed earlier, when pRab4 was complemented with *lanE* alone. These results disfavor rabelomycin (**17**) as a substrate for either LanZ4–LanZ5 or LanV. However, when *lanE* was used to complement the PKS–LanM2 construct or the PKS–LanZ4–LanZ5–lanM2 construct, and accumulation of

dehydrorabelomycin (**13**) was observed (Fig. 2). These results reiterated the earlier functional assignment of LanE as 12-oxygenase. Production of tetrangulol (**9**) was observed in addition to **17**, **16** and **13** when *lanV* was added to the PKS–lanZ4–LanZ5–lanM2–lanE construct (Fig. 2). The results indicated that LanV might be responsible for the reduction of the 6-keto group of prejadomycin (**10**) to a secondary alcohol, which then might be removed through dehydration by either other Lan-pathway enzymes or by host enzymes, or spontaneously, leading to the formation of tetrangulol (**9**). The results also indicated that LanV operates after both LanM2 and LanE. Tetrangulol (**9**) was isolated earlier as a shunt product from the culture broth of the LndM2-deletion mutant *S. globisporus* 1912.[20]

Although these heterologous expression experiments provided some additional insights into the landomycin biosynthetic pathway, the results still did not provide a clear picture regarding the biosynthesis of the aglycones **4** and **5**. Production of **9** instead of the anticipated products (**4** or **5**) was indicative of the existence of secondary catalytic events. Earlier *in vivo* studies had demonstrated that angucycline post-PKS tailoring reactions were often accompanied by follow-up catalytic events by host enzymes, leading to the accumulation of shunt products.[20,29–31] Such shunt products, often with unexpected alterations in the structures, impose challenges for the clarification of biosynthetic pathways, since the separation of the real biosynthetic events from non-specific, spontaneous or host-contributed endogenous reactions is difficult or impossible. Thus, we further focused on *in vitro* enzyme assays involving purified enzymes and substrates which provide cleaner results and could complement and clarify the *in vivo* results.

Expression and characterization of LanZ4

To identify the functional role of LanZ4 and optimal conditions for its activity, the enzyme was produced in *E. coli* as a His-tagged protein and purified through immobilized metal affinity chromatography (IMAC) (Fig. S1†). The intense yellow color of the purified enzyme indicated the presence of bound flavin cofactor. HPLC analyses of the released cofactor with the standard compounds revealed that FMN is bound to the enzyme (Fig. S2†). The substrate specificity of LanZ4 was monitored by comparing its relative activities toward a variety of combinations of cofactors (FAD and NADPH, FAD and NADH, FMN and NADPH, and FMN and NADH). A rapid time-dependent decrease in absorption at $A_{340\ nm}$ was observed when the enzyme was incubated with NADPH and FMN (Fig. S3†). The rate of consumption of NADPH was dependent on the concentration of LanZ4 in the presence of excess amount of FMN (Fig. S4†). Although the experiment was performed with only a single enzyme concentration, the activity of LanZ4 with FAD and NADPH was fourfold less compared to LanZ4 with NADPH and FMN, indicating the latter pair as the preferred substrates. No change in $A_{340\ nm}$ was observed when NADH was used in these assays indicating the strict specificity of the enzyme towards NADPH (Fig. S3†). The results revealed that FMN and NADPH are the natural substrates of LanZ4. Steady-state kinetic assays were conducted using a fixed concentration of NADPH and various concentrations of FMN. The results revealed a typical Michaelis–Menten kinetic curve with K_m and

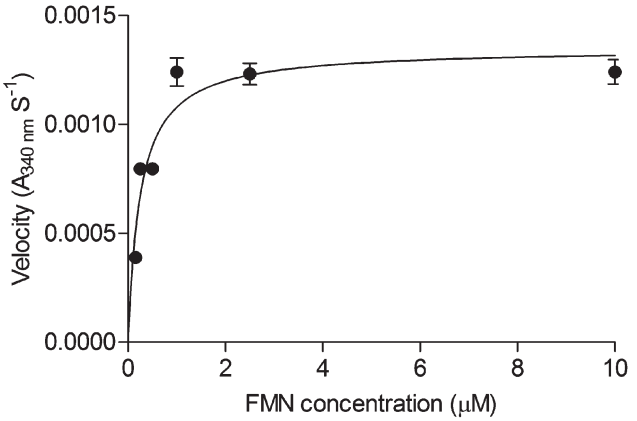


Fig. 3 Kinetics analysis of LanZ4. The curve was fitted to Michaelis–Menten kinetics to determine the kinetic parameters K_m and k_{cat}.

k_{cat} of 0.25 ± 0.047 μM and 0.332 s^{-1}, respectively (Fig. 3). However, we were unable to attain the substrate saturation condition, because the assays were conducted using a fixed concentration of FMN and various concentrations of NADPH (Fig. S5†).

Heterologous production and enzyme assay of LanZ5

LanZ5 was overexpressed as colorless His-tagged protein in *E. coli* and was subjected to several assays with a variety of substrates. No conversion was observed when LanZ4 and LanZ5 were incubated together with tetrangulol (**9**), prejadomycin (**10**), dehydrorabelomycin (**13**), UWM6 (**16**) or rabelomycin (**17**), in each case in the presence of FMN and NADPH. However, incubation of the enzyme pair with tetrangomycin (**14**) resulted in the consumption of the substrate with the concomitant production of a new compound. The observance of a UV maximum at 450 nm instead of at 398 nm (maximum of tetrangomycin) indicated a change of the chromophore, likely due to hydroxylation at ring D. Further comparison of the HPLC retention time and ESI-MS data revealed that the new peak represented 11-hydroxytetrangomycin (**15**) (Fig. S6†). The results were in good agreement with the earlier *in vivo* results where the homologous enzymes LndZ4 and LndZ5 of *S. globisporus* 1912 were found to catalyze the conversion of **14** to the pathway shunt product **15**.[20] Surprisingly, 11-deoxylandomycinone (**5**) was rapidly converted into tetrangulol (**9**) and into trace amounts of 11-hydroxylandomycinone (**8**) when incubated with this enzyme pair (Fig. 4, trace D). Removal of LanZ4 from the assay mixture slowed down the conversion, and yielded only tetrangulol (**9**, Fig. 4, trace C). NADPH was essential for all the reactions. The formation of mostly **9** instead of the expected 11-hydroxylated product landomycinone (**4**) suggested that LanZ5 does not prefer to hydroxylate **5**. Rather, a secondary dehydratase activity was observed catalyzing 5,6-dehydration. The binding of NADPH to the enzyme seems to provide the optimal conditions to catalyze also this dehydration reaction. The enzyme pair LanZ4–Z5 was inactive *in vitro* toward glycosylated landomycins, such as landomycin F (no 11-hydroxy group, two sugars, see Fig. 1B), landomycin V (no 11-hydroxy group, 5 sugars) or landomycin S (no 11-hydroxy group, 6 sugars). The 5,6-dehydration was also not

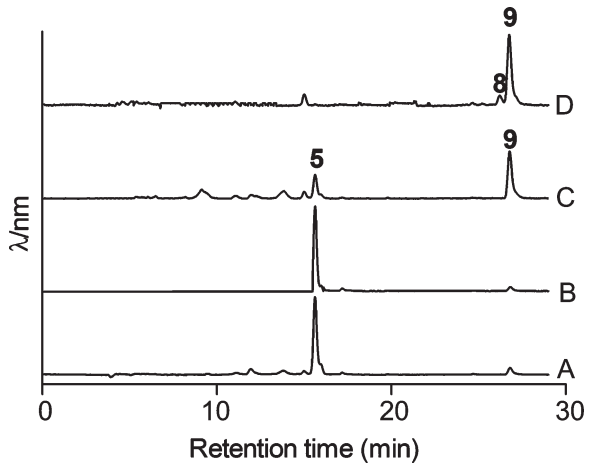


Fig. 4 HPLC traces demonstrating the conversion of 11-deoxylandomycinone (**5**) to tetrangulol (**9**) *in vitro*. Trace A: **4** and NADPH (control sample); trace B: **5** and LanZ5; trace C: **5**, LanZ5, NADPH; trace D: **5**, LanZ4, LanZ5, NADPH, FMN. **8** = anhydrolandomycinone.

observed when the enzyme pair LanZ4–Z5 was incubated with 11-hydroxylated, glycosylated landomycins, *e.g.*, landomycins A (11-hydroxy group, 6 sugars) and B (11-hydroxy group, 5 sugars). Thus, it can be concluded that the LanZ4–Z5 pair works preferentially after the first glycosylation to provide the 11-hydoxylation reaction, although a direct proof of this conclusion was not possible as the potential substrate landomycin H (**18**, one sugar residue, no 11-OH group) was not available for the assay. The 5,6-dehydration catalyzed by LanZ4–Z5 is a side reaction, leading to minor landomycin congeners with aromatic ring B, and only occurs in absence of sugar residues (Scheme 1).

Study of activities of LanM2 and LanV *in vitro*

The *in vivo* experiments (see above) suggested for the first time that LanM2 might be responsible for the 2,3 dehydration of the presumed PKS-tethered intermediate **11**. However, a different functional role for LanM2 was proposed earlier, namely the hydroxylation of tetrangomycin (**14**) at 6-position to generate rabelomycin (**17**), as well as its subsequent reduction to 11-deoxylandomycinone (**5**).[20] To clarify the exact functional role of LanM2, the enzyme was expressed in *E. coli* and purified. The intense yellow color of the enzyme indicated the presence of a bound flavin co-factor. Further analyses revealed FAD as the bound co-factor of LanM2 (Fig. S1†). Incubation of LanM2 individually with potential substrates, namely tetrangomycin (**14**), UWM6 (**16**), rabelomycin (**17**), dehydrorabelomycin (**13**), prejadomycin (**10**), tetrangulol (**9**), or 11-deoxylandomycinone (**5**), each in the presence of FAD and NAD^+ or $NADH^+$ or $NADP^+$ or NADPH did not reveal any conversion. Analogous experiments with purified enzyme LanV gave the same negative results (data not shown).

Assay of activity of LanE *in vitro*

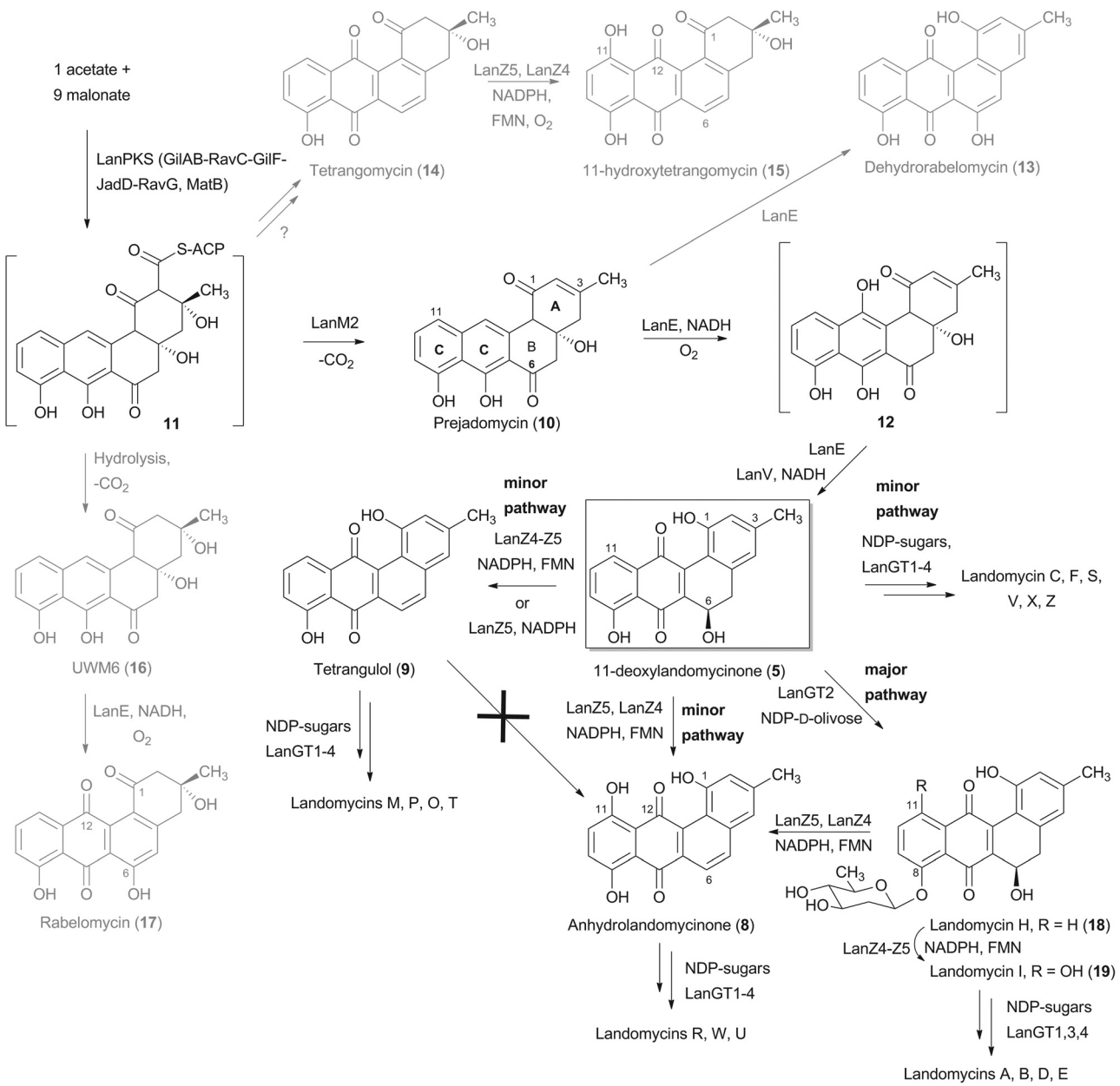
LanE–LndE was earlier proposed to be responsible for the 12-oxygenation reaction, however, its exact substrate remained ambiguous.[21] The *in vivo* co-expression results described above suggested that oxygenase LanE catalyzes the conversion of **10** into **13**. To clarify the exact functional role of LanE, the enzyme was overexpressed from *E. coli* BL21, and the purified enzyme was subjected to an assay, interrogating all available angucyclinones possibly involved in the landomycin pathway: compounds **17**, **16**, **14**, **13**, **10**, **9**, or **5**, each in the presence of a reduced co-factor (NADH or NADPH). Only two of these potential substrates were converted by LanE. As expected from the *in vivo* results, prejadomycin **10** was converted into dehydrorabelomycin **13**. In addition, UWM6 **16** was converted to rabelomycin **17**. The enzyme worked with either NADH or NADPH in the assay mixture (Fig. 5). The results reiterated the earlier conclusions which suggested that LanE was responsible for the introduction of the oxygen atom into the 12-position, of either **10** or **16**. However, to reach **13** and **17**, respectively, both conversions would also require oxidation in ring C (hydroquinone to quinone, likely spontaneous) and 4a,12b-dehydration (aromatization or rings A/B), in addition to the 12-oxygenation. The 4a,12b-dehydration is probably also catalyzed by LanE, but also may occur spontaneously, since neither of the LanE-products, **13** or **17**, reacted further with any of the other post-PKS tailoring enzymes, thus are presumably shunt products.

Synthesis of 11-deoxylandomycinone using a mixture of 5 enzymes (LanM2, LanE, LanZ4, LanZ5, and LanV)

Since our *in vitro* studies with individual enzymes did not provide an unambiguous sequence of events for the biosynthesis of the aglycones found in various landomycins, we used assays involving systematic variations of mixtures of two or more enzymes, a method for which we recently suggested the term "combinatorial biosynthetic enzymology", and which we applied successfully to unravel the complex post-PKS tailoring events in gilvocarcin biosynthesis.[32]

Here, UWM6 (**16**) was not converted when it was incubated with any combination mixture involving LanZ4, LanZ5, LanM2 and LanV. Inclusion of LanE in any of these combinations led to the production of rabelomycin (**17**), as observed earlier. Furthermore, a reaction of **17** with all five of these enzymes and cofactors failed to give any product. This confirmed that neither UWM6 (**16**) nor rabelomycin (**17**) was an intermediate of the landomycin biosynthetic pathway.

The incubation of prejadomycin (**10**) with LanZ4, LanZ5, LanM2 and LanV in the presence of FAD/FMN, NADH/NADPH did not reveal any product either (Fig. 5). Interestingly, the incubation of **10** with a mixture of just LanE and LanV in the presence of NADH showed a complete conversion of substrate **10** into 11-deoxylandomycinone (**5**, Fig. 5, trace G). However, the production of **5** was almost completely abolished in favor of dehydrorabelomycin (**13**, Fig. 5, trace F) as the sole product of the LanE–LanV mixture, when co-factor NADH was replaced by NADPH, confirming a strict specificity of enzyme LanV for NADH. These results suggested that LanV is responsible for the stereospecific reduction of C-6 carbonyl group of prejadomycin (**10**) to the *R*-configured secondary alcohol in **5**. This reaction of LanV was recently also observed by Metsä-Ketelä *et al.*[40] Adding LanZ4–Z5 to this mixture resulted in the formation of tetrangulol (**9**), while the addition of LanM2 did not lead to any change in the product profile (data not shown).



Scheme 1 Newly proposed pathway for landomycin biosynthesis. The pathway leading to the three different series of landomycins, with different aglycones, is depicted in black, while shunt pathways are shown in gray.

Discussion

The results garnered from rigorous *in vivo* and *in vitro* experiments have led to a major revision of the biosynthesis process towards various landomycins (Scheme 1). It has long been believed that UWM6 (**16**) would be the first angucyclinone intermediate and that the conversion of **16** to 11-deoxylandomycinone (**5**) might occur through a sequence involving the intermediates prejadomycin (**10**), tetrangomycin (**14**), and rabelomycin (**17**). The sequence **16** → **10** → **14** → **17** → **5** was based on earlier feeding studies and gene inactivations.[20,21] Our current results clearly demonstrated that neither **14**, **16** or **17** serve as intermediates of the biosynthesis towards **5**. Co-expression of PKS and *lan* genes encoding post-PKS tailoring enzymes led to the production of tetrangulol (**9**), which provided a basis for *in vitro* follow-up studies involving isolated enzymes. Such follow up studies demonstrated that prejadomycin (**10**) is the first verified angucyclinone intermediate. Prejadomycin (**10**) is converted into 11-deoxylandomycinone (**5**) through the activities of two enzymes, LanE and LanV. Our *in vitro* studies also proved that UWM6 (**16**) is a shunt product rather than an intermediate of the landomycin biosynthetic pathway. Exclusive production of prejadomycin (**10**) instead of UWM6 (**16**, the product of the PKS enzymes alone) through co-expression of LanM2 with the PKS enzymes indicated that LanM2 is the first tailoring enzyme, responsible for catalyzing the 2,3-dehydration step, the same reaction found for JadF in jadomycin biosynthesis.[27,29] Furthermore, it can be concluded that this elimination happens

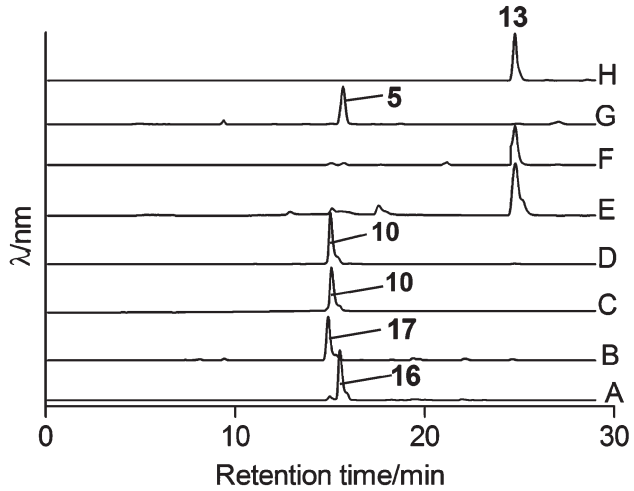


Fig. 5 HPLC analyses of the products generated through the activities of the Lan-oxygenases *in vitro*. Trace A: standard UWM6 (**16**); trace B: **16** with LanE, LanV and NADH, **17** = rabelomycin; trace C: standard prejadomycin (**10**); trace D: **10** with LanZ4, LanZ5, LanM2, LanV (NADH/NADPH and FAD/FMN were supplied); trace E: **10** with LanE, NADH; trace F: **10** with LanE, LanV, NADPH; trace G: **10** with LanE, LanV and NADH, **5** = 11-deoxylandomycinone; trace H: standard dehydrorabelomycin (**13**).

simultaneously with the cleavage of the angucyclinone **10** thereby generated from the ACP. This parallels the findings we recently established for the gilvocarcin and jadomycin biosyntheses,[32] and is corroborated by the fact that LanM2 cannot *in vitro* convert **16** into **10**. As suggested for GilOIV and JadF in the gilvocarcin and jadomycin biosynthesis, respectively, LanM2 might act instead on the enzyme-bound angucyclinone intermediate **11** (Scheme 1).[29,30,32] Note also that JadF shares significant amino acid (aa) sequence homology (62% aa-identity) with the oxygenase portion of LanM2. However, the functional role of the reductase portion of LanM2 remained uncertain. The subsequent mediated conversion of prejadomycin (**10**) to dehydrorabelomycin (**13**) by LanE (and of **16** to **17**) in both *in vivo* and *in vitro* assays provided clear evidence that LanE is a true oxygenase, with broader substrate specificity, and also suggests its role as 4a,12b-dehydratase. An identical conversion has been reported for PgaE in gaudimycin, for JadH in jadomycin, and most recently for GilOI in gilvocarcin biosynthesis.[29–32] The quantitative conversion of prejadomycin (**10**) to 11-deoxylandomycinone (**5**) through the combined activities of LanE and LanV unambiguously proved **10** as a true intermediate, while these two combined enzymes cannot further convert dehydrorabelomycin (**13**), suggesting that **13** is not an intermediate of the landomycin biosynthetic pathway. Instead, it can be proposed that LanE catalyzes the conversion of **10** to the unstable intermediate **12**, which then undergoes reduction of the carbonyl group in the 6-position (catalyzed by LanV) along with 4a,12b-dehydration leading to the aromatization of ring A, to produce 11-deoxylandomycinone (**5**). These interpretations also fit well with the earlier complementation studies, which indicated the crucial role of LanV in the aromatization of ring A and in the reduction of the 6-keto group.[28] In the absence of LanV (*i.e.* using LanE alone), intermediate **12** undergoes oxidation, dehydration and tautomerization to generate dehydrorabelomycin (**13**). The inability of the LanE–V mixture to catalyze any conversion of rabelomycin (**17**) or of tetrangomycin (**14**) suggested that neither **17** nor **14** are true intermediates of the landomycin pathway, as was proposed earlier.[20,21] The observed *in vivo* conversions of **17** to **5** and of **14** to **5** in earlier feeding experiments might be explained by alternate pathways involving host enzymes. The biosynthetic steps towards **14** still remain unclear.

The studies with overexpressed LanZ4 demonstrated that the enzyme is an FMN-NADPH reductase, supporting oxygenase LanZ5 by providing reduced cofactor $FMNH_2$. The conversion of tetrangomycin (**14**) to 11-hydroxytetrangomycin (**15**) through the collaborative activities of LanZ4 and LanZ5 indicated that these enzymes represent an example of a pair of co-dependent enzymes, in which LanZ5 is the partner which catalyzes the oxygenation reaction. The inability of the other enzymes (LanE, LanV and LanM2) to convert **15** to anhydrolandomycinone (**8**) suggested **15** to be a shunt product of the landomycin biosynthetic pathway. The LanZ4–Z5-mediated conversion of **14** to **15** could just reflect a relaxed substrate specificity of LanZ5. An identical conclusion had been drawn earlier, when inactivation of LndM2 in *S. globisporus* 1912 led to the accumulation of **15**.[20] This study also revealed a new route for the production of tetrangulol (**9**), where LanZ5 catalyzed 5,6-dehydration of 11-deoxylandomycinone (**5**). The finding that the pair LanZ4–Z5 could convert **5** to tetrangulol (**9**), but failed to hydroxylate the 11-position of **5** or of **9** to landomycinone **4** and anhydrolandomycinone **8**, respectively, combined with the fact that LanZ4–Z5 did not act on glycosylated landomycins, regardless whether these carried a hydroxyl group in 11-position or not, led us to the new hypothesis: the combined activities of LanZ4 and LanZ5 preferentially only act as hydroxylase for the 11-position after the first glycosylation step catalyzed by LanGT2, and convert the 11-hydroxylation of landomycin H (**18**) to landomycin I (**19**), which in turn is further glycosylated to produce the main series of 11-hydroxylated landomycins, *e.g.*, landomycin A (**1**), while the 11-hydroxylation of **5** to landomycinone **4** is a minor reaction. In the absence of the glycosyltransfer, LanZ5 mostly catalyzes 5,6-elimination, *i.e.* the conversion of **5** to **9**, and to a minor extent of **4** to **8**. Glycosylation of **8** then can lead to the production of recently discovered minor landomycin congeners, such as landomycin R, landomycin U and landomycin W, all of which contain anhydrolandomycinone (**8**) as aglycone.[8] LanZ5 is unable to catalyze 5,6-dehydration of saccharidal landomycins, like landomycin D[22] or landomycin A (**1**), which also supports the sequence of events suggested here. Glycosylation of tetrangulol (**9**) results in the production of other landomycin side products with tetrangulol aglycone, such as landomycins M, O, P and T (Scheme 1).[8,33] Clearly, the very last acting oxygenase of the landomycin pathway, LanZ5, is a key enzyme directing this pathway towards its preferred products, the landomycins with **4** as the aglycone moiety.

Experimental

Cloning and preparation of expression constructs

Genomic DNA of *S. cyanogenus* S136 was prepared following the standard protocol.[34] The oligonucleotide primers used in this

study (Fig. S1†) were obtained from Integrated DNA Technology Inc. Pfu-polymerase (Stratagene) was used to amplify genes (*lanM2*, *lanV*, *lanZ4* and *lanZ5*) from the genomic DNA of *S. cyanogenus* S136. The obtained products were purified and then cloned into ZeroBlunt-TOPO vector (Invitrogen). A few clones for individual genes were sequenced to confirm that no error has been incorporated during PCR amplification. Each gene was excised as *Nde*I/*Eco*RI fragment from the corresponding TOPO-clone and ligated at the identical sites of pET28a(+) expression vector (Novagen). PCR products of *lanE* were cloned directly into pET-30 EK/LIC vector utilizing the kit provided by Novagen. The constructs were then introduced into *E. coli* BL21 (DE3) expression host for the production of recombinant protein.

A dozen constructs (pLaox1–pLaox12) were generated for expression of *lanM2*, *lanE*, *lanZ4*, *lanZ5*, and *lanV* in the heterologous host *S. lividans* TK64 (Table S2†). Primers used for the amplification of these genes are listed in Table S1.† Each gene was amplified using PCR and cloned into TOPO-vector. *lanZ4* and *lanZ5* both were amplified together as a single fragment. *Spe*I and *Eco*RI were used to isolate *lanE*, *lanM2* and *lanZ4–lanZ5* fragments from their corresponding TOPO clones and ligated at *Nhe*I and *Eco*RI sites of the recently created UWM6 biosynthetic construct (pXY200-*gilABCP-jadD-ravG*).[23] *Nde*I and *Eco*RI were used to excise entire fragments spanning PKS genes and landomycin post-PKS genes. Thus isolated fragments were ligated at *Nde*I and *Eco*RI sites of the *E. coli-Streptomyces* shuttle vector pUWL201PW to create the constructs pLaox1-3 and pLaox7.[35] Similarly, the remaining seven constructs which included two to five landomycin pathway genes were created taking the advantage of compatibility of *Nhe*I and *Spe*I sites. These constructs were introduced into *S. lividans* TK64 for the production of secondary metabolites. Standard protocol was followed to transform protoplasts of *S. lividans* TK64.[36]

Bacterial strains and culture conditions

E. coli XL1 Blue MRF strain (Stratagene) was used as a host to amplify plasmid DNA. All *E. coli* strains were grown on Lauria Bertani (LB) agar medium or in liquid broth at 37 °C. Kanamycin (50 μg mL^{-1}), ampicillin (100 μg mL^{-1}), and apramycin (100 μg mL^{-1}) were supplemented in the medium to maintain plasmids whenever needed. The strain *S. lividans* TK64 was grown at 28 °C on M2 agar medium to produce spores and in SG liquid medium to produce secondary metabolites as described earlier.[23]

Production and analysis of secondary metabolites

Small chunk of the agar medium comprising *Streptomyces* spores were inoculated into an Erlenmeyer flask containing sterile SG-medium (100 mL) and thiostrepton (25 μg mL^{-1}). This seed culture was grown at 28 °C for 3 days in an incubator under shaking conditions (200 rpm). 2 mL of fully grown culture was transferred into the 100 mL SG-production medium supplemented with thiostrepton and then grown for 5 days. The culture broth was separated from the mycelial cake through centrifugation (4000 × g). Acetone was added into the mycelial fraction and sonicated for 10 min. The acetone extract was separated from the cell-debris through centrifugation and dried off using a rotary evaporator. The aqueous portion was mixed with the culture broth and the mixture (25 mL) was extracted with ethyl acetate (2 × 40 mL). The organic solvent was dried off under vacuum and methanol (5 mL) was used to dissolve the extract. The solution was filtered through the syringe filter (0.25 μM pore size) and the filtrate was subjected for HPLC-MS analysis. Metabolites were separated using a linear gradient of acetonitrile and water (solvent A = H_2O; solvent B = acetonitrile; 0–15 min 25% B to 100% B; 16–24 min 100% B; 25–26 min 100% to 25% B; 27–29 min 25% B). An analytical column from Waters Corporationn (Symmetry C18, 4.6 × 250 mm, 5 μm) was used for separation of metabolites while maintaining the flow rate at 0.5 mL min^{-1}. A photodiode array detector (Waters 2996) along with a Micromass ZQ 2000 mass spectrometer (Waters Corporation) equipped with an atmospheric pressure chemical ionization (APCI) probe was used to detect the molecular ions and analyze the compounds. Methanolic solution (0.1 mg mL^{-1}) of UWM6 (**16**), prejadomycin (**10**), tetrangulol (**9**), tetrangomycin (**14**), 11-hydroxytetrangomycin (**15**), rabelomycin (**17**), dehydrorabelomycin (**13**) and 11-deoxylandomycinone (**5**) were injected in HPLC-MS for references. Similarly, reference HPLC-MS data were acquired for glycosylated Las.

Production and purification of recombinant proteins

All the landomycin post-PKS enzymes with N-terminal hexahistidine tag were heterologously expressed using *E. coli* BL21 (DE3) host. Transformation of expression constructs into *E. coli* BL21 (DE3) and selection of colonies were carried out following the standard protocol.[34] A seed culture was prepared through inoculation of a single colony into LB liquid (20 mL) in a baffled Erlenmeyer flask. The medium was supplemented with kanamycin sulfate and the seed culture was grown for 4 hours at 37 °C. A liter of LB (100 mL × 10 flasks) was inoculated with 10 mL (1 mL in each flask) of the seed and the culture was grown at 37 °C until OD600 reached 0.5. Production of protein was then induced by supplying isopropyl-thio-galactopyranoside (IPTG, 0.02 mM final concentration). The culture was allowed to grow at 23 °C for 12 hours and then harvested *via* centrifugation (4000 × *g*, 15 min) to collect cell pellets. The pellets were washed twice with 20 mL of lysis buffer (50 mM KH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 7.6) and then disrupted in a French Press (Thermo Electron Corporation). Crude enzyme fraction was prepared through the centrifugation of the lysate (17 000 × *g*, 50 min) and purified through immobilized metal affinity chromatography (IMAC) column. Desalting of individual enzyme was carried out through several cycles of concentration and dilution using Amicon ultracentrifugation tubes. Purified enzymes were subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis (Fig. S1†). Enzyme concentration was determined using the traditional Bradford protein assay method.[37] The sizes of proteins observed on SDS-PAGE were in good agreement with the calculated sizes (LanZ4: 23.81 kDa; LanZ5: 43.45 kDa; LanE: 56.96 kDa; LanV: 28.35 kDa). LanM2 appeared as a slightly smaller protein (~55 kDa) as compared to the calculated MW (77.47 kDa). Such an inaccuracy of SDS-PAGE was observed earlier in our lab for several deoxysugar biosynthetic enzymes.[38]

Analysis of enzyme-bound co-factors

LanZ4 and LanM2 were eluted as intense yellow enzymes. To identify the enzyme bound cofactors, 1 mL of enzyme preparation (4 mg mL^{-1}) was boiled for 5 min. The precipitated enzyme was removed by centrifugation (12 000 × *g*, 5 min) and the yellow solution was subjected for HPLC analysis. Standard solutions (0.5 μM) of FAD and FMN were used for comparison. Carbopac PA1 (Dionex Inc.) analytical column (4 × 250 mm) was injected with 50 μL of each sample. A gradient program of ammonium acetate (500 mM) and water (ammonium acetate: 5 to 20% over 15 min, 20 to 60% over 20 min, 60 to 100% over 2 min, 100% over 3 min and 100% to 5% over 5 min and 5% over 15 min) was used to elute the compounds. The flow rate and detector were set to 1 mL min^{-1} and 267 nm, respectively. UV spectrum and HPLC retention time analyses revealed that FAD and FMN were bound with LanM2 and LanZ4, respectively (Fig. S2†).

Enzyme assays

Several metabolites were subjected for enzymatic conversion experiments. Standard solutions (100 μM) were prepared for rabelomycin (**17**), prejadomycin (**10**), UWM6 (**16**), tetrangomycin (**14**), 11-deoxylandomycinone (**5**), dehydrorabelomycin (**13**) in methanol. Each substrate was incubated with an individual enzyme or in a cocktail of multiple enzymes (Table S3†). Enzyme assays were carried out in 100 μL volume comprising a final concentration of 5 μM substrate, 20 μM NADH or NADPH, 2 μM of FAD or FMN and 0.5 μM of individual enzyme in 50 mM phosphate buffer (pH 6.5). Enzymes were replaced with buffer (50 mM pH 6.5) in control samples. Each assay mixture was incubated at 28 °C for 3 hours. The reactions were terminated by adding 200 μL of ethyl acetate. The ethyl acetate extract was separated and dried. Thus prepared extract was dissolved in methanol, filtered through membrane filter (0.25 μm pore size) and analyzed with HPLC-MS.

UV-spectrophotometer was used to determine the substrate preference of LanZ4 following a similar protocol reported earlier.[39] Reactions were carried in 1 mL volume. Enzyme (99.7 μg) was incubated with all four different combinations (NADPH and FAD, NADPH and FMN, NADH and FAD, and NADPH and FAD) using 160 μM of NADH or NADPH (final concentration) and 100 μM of flavins (FAD or FMN). Buffer was supplied in place of enzyme for control samples. UV absorbance at 340 nm was recorded in every 4 seconds. The data acquired from the machine was plotted using Graph Pad Prism version 5 to generate Fig. S3.† A clear preference of LanZ4 for FMN and NADPH was observed. The amount of LanZ4 was varied from 0.71 μg to 106.48 μg using FMN and NADPH which revealed concentration dependent depletion of NADPH in the assay mixture (Fig. S4†). For kinetic studies, assay was conducted in 1 mL volume comprising 96.8 μg of enzyme. The concentration of NADPH was fixed at 160 μM in all assay samples whereas the concentration of FMN was varied from 0.1–10 μM. Three replicates of each sample were used to minimize experimental errors. The data acquired from the UV-spectrophotometer was analyzed using Graph Pad Prism to determine K_m and k_{cat} values (Fig. 3). No substrate saturation condition for LanZ4 was observed when the concentration of FAD was fixed at 100 μM and the concentration of NADPH was varied from a final concentration of 1 μM to 600 μM (Fig. S5†).

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