Engineered biosynthesis of hybrid macrolide polyketides containing D-angolosamine and D-mycaminose moieties†

Ursula Schell,‡[a] Stephen F. Haydock,‡[b] Andrew L. Kaja,[a] Isabelle Carletti,[a] Rachel E. Lill,[a] Eliot Read,[a] Lesley S. Sheehan,[a] Lindsey Low,[a] Maria-Jose Fernandez,[b] Friederike Grolle,[b] Hamish A. I. McArthur,[c] Rose M. Sheridan,[a] Peter F. Leadlay,[b] Barrie Wilkinson*[a] and Sabine Gaisser*[a,d]

Received 9th May 2008, Accepted 24th June 2008
First published as an Advance Article on the web 28th July 2008
DOI: 10.1039/b807914e

The glycosylation of natural product scaffolds with highly modified deoxysugars is often essential for their biological activity, being responsible for specific contacts to molecular targets and significantly affecting their pharmacokinetic properties. In order to provide tools for the targeted alteration of natural product glycosylation patterns, significant strides have been made to understand the biosynthesis of activated deoxysugars and their transfer. We report here efforts towards the production of plasmid-borne biosynthetic gene cassettes capable of producing TDP-activated forms of D-mycaminose, D-angolosamine and D-desosamine. We additionally describe the transfer of these deoxysugars to macrolide aglycones using the glycosyl transferases EryCIII, TylMII and AngMII, which display usefully broad substrate tolerance.

Introduction

The elucidation of high-resolution X-ray crystal structures for clinically important antibiotics bound to their ribosomal targets has revealed key molecular interactions and provided insights into how such templates might be adapted through structure-aided drug design to provide modified derivatives with activity against drug-resistant organisms.[1] Macrolide antibiotics consist of a core aglycone, of polyketide origin, to which deoxysugar (most commonly deoxyhexose) moieties are attached. It is the deoxysugar moieties which confer potent and specific antibacterial activity through key contacts to the ribosome. Indeed, the importance of deoxysugar components for the activity of multiple types of natural products has driven an increasing interest in understanding the mechanisms and pathways by which they are biosynthesised, and in characterising the substrate range and tolerances of the glycosyltransferases (GTs) which attach them to their acceptor substrates. This has led to the development of powerful methods for the manipulation of deoxysugar biosynthesis and the production of new 'non-natural natural products' through the application of glycosylation engineering.[2,3] In particular, the utility of biosynthetic gene cassettes for deoxysugar production in heterologous *Streptomyces* hosts has been clearly demonstrated by Salas, Méndez and co-workers.[4]

We have previously described alteration of the glycosylation pattern of the macrolide antibiotics erythromycin (**1**), tylosin (**2**), and oleandomycin as well as the insecticidal spinosyns (**3**) (see Fig. 1).[5–8] Here we report the successful use of biosynthetic gene cassettes for the heterologous production and transfer of several deoxyhexoses to polyketide aglycones in place of the natural substituents. These are changes of potential relevance for generating compounds with altered and desirable activities. Such experiments have to some degree been previously hampered by incomplete information about the biosynthetic pathways to activated deoxyaminohexoses. For example, in the biosynthesis of TDP-D-mycaminose (**4**) (see Fig. 2), the 3,6-dideoxy-3-aminosugar component of **2**, it was originally proposed that the *tylMIII* gene from the biosynthetic gene cluster encoded a 3,4-ketoisomerase responsible for converting TDP-4-keto-6-deoxy-D-glucose (**5**) to its 3-keto isomer (**6**).[9] This assignment was based on its significant sequence similarity to genes governing TDP-D-desosamine (**7**) biosynthesis in the erythromcyin biosynthetic gene cluster.[10] Studies reported by Liu and colleagues have since shown that the biosynthetic route to TDP-D-desosamine does not require 3,4-keto tautomerisation.[11] Moreover, it has now been shown that TylMIII and homologues from other deoxyaminohexose biosynthetic gene clusters are actually involved in the glycosylation process itself, forming a complex with, and enhancing the activity of, the cognate GT.[12–14] The 3,4-ketoisomerase required for TDP-D-mycaminose production is actually encoded by *tyl1a*, a previously unassigned open reading frame (ORF) within the tylosin biosynthetic gene cluster of *Streptomyces fradiae* ATCC19609.[15,16] This finding stemmed from the observation that a homologue of *tyl1a* (*fdtA*) is required for TDP-3-acetamido-3,6-dideoxy-α-D-galactose biosynthesis in *Aneurinibacillus thermoaerophilus* L420-91^T, and is responsible for the production of TDP-6-deoxy-D-xylohex-3-ulose from TDP-6-deoxy-D-xylohex-4-ulose.[17] Liu and co-workers have used this insight to produce, *in vivo*, macrolide analogues bearing a D-mycaminose moiety in place of the natural

[a]*Biotica Technology Ltd, Chesterford Research Park, Little Chesterford, Saffron Walden, Essex, CB10 1XL, UK. E-mail: barrie.wilkinson@biotica.com*
[b]*Department of Biochemistry, University of Cambridge, 80 Tennis Court Road, Cambridge, CB2 1GA, UK*
[c]*Bioprocess Research, Central Research Division, Pfizer Inc., Groton, CT, 06340, USA*
[d]*Hochschule Biberach, Karlstraße 11, 88400, Biberach/Riß, Germany. E-mail: gaisser@fh-biberach.de*

† Electronic supplementary information (ESI) available: Details of the construction of gene cassettes, isolation of conversion vectors, and construction of strains. See DOI: 10.1039/b807914e

‡ These authors made equal contributions.

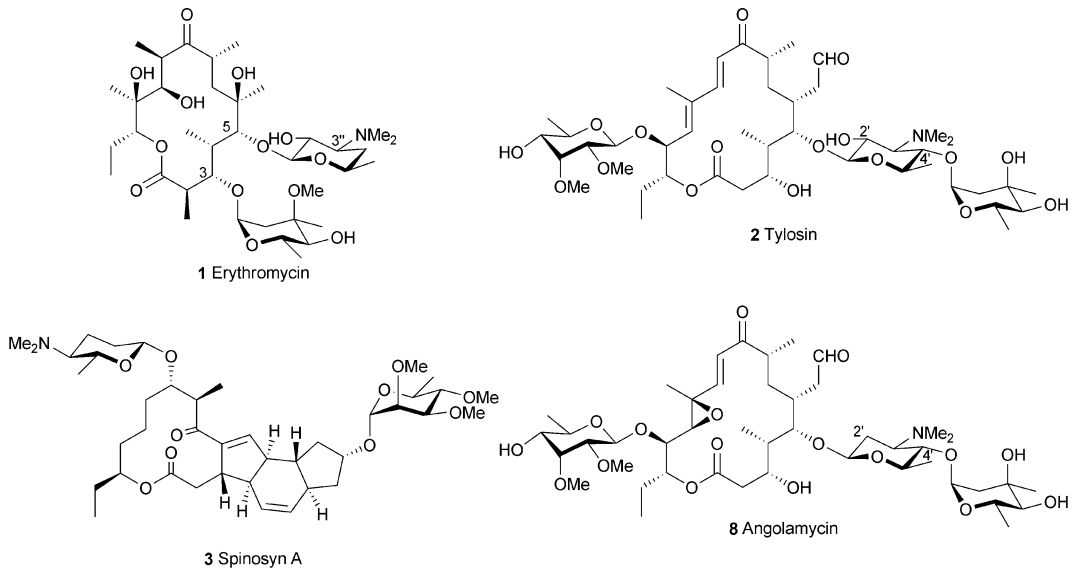

Fig. 1 Structures of selected polyketide macrolides.

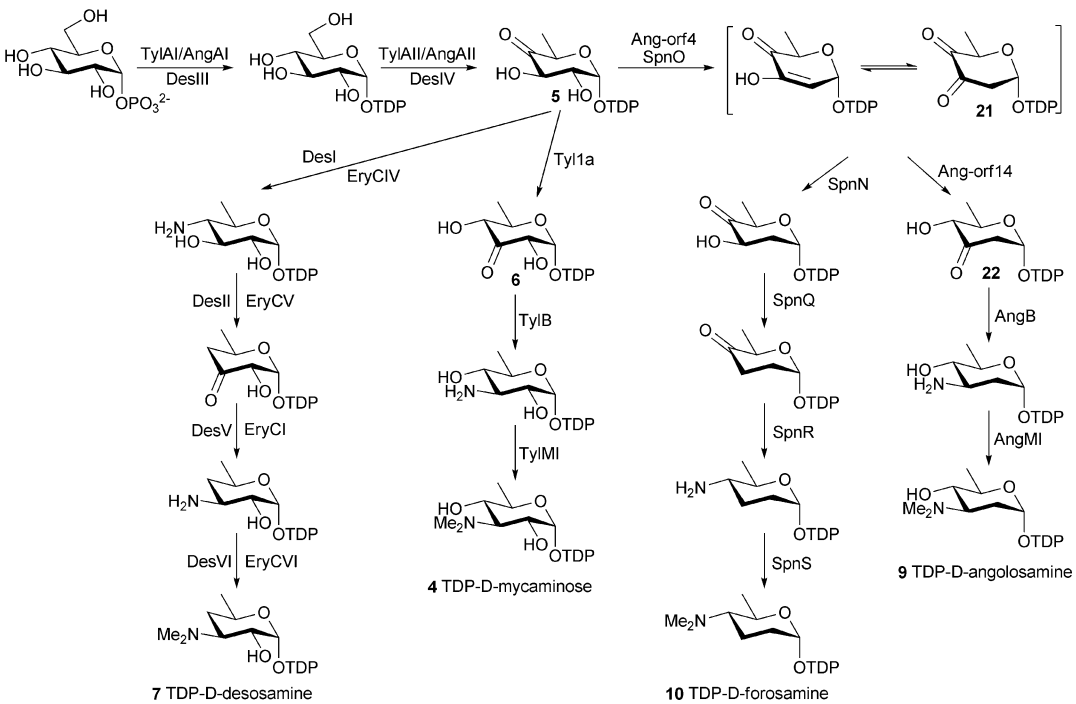

Fig. 2 Biosynthetic pathways to the TDP-D-deoxyaminohexoses discussed. The proteins from each pathway responsible for the different chemical steps are given (Des/Ery, desosamine pathways; Tyl, mycaminose pathway; Ang, angolosamine pathway; Spn, forosamine pathway).

D-desosamine.[15] The requirement of *tyl1a* for TDP-D-mycaminose biosynthesis was independently described in an international patent application.[18]

Herein we report the cloning and sequencing of the biosynthetic gene cluster from *Streptomyces eurythermus* ATCC23956 encoding production of the 16-membered macrolide antibiotic angolamycin (**8**).[19] **8** exhibits relatively weak antibacterial activity but is interesting due to the presence of an unusual 3-amino-2,3,6-trideoxy-D-hexose moiety derived from TDP-D-angolosamine (**9**). Further, we describe the construction of biosynthetic gene cassettes that confer upon heterologous hosts the ability to synthesise TDP-D-mycaminose, TDP-D-desosamine and TDP-D-angolosamine, and examine the ability of several cloned GTs to transfer these and other deoxyhexoses to polyketide aglycones to produce a range of novel macrolides.

Results

Provision of genes for the assembly of biosynthetic gene cassettes

The genes responsible for TDP-D-mycaminose biosynthesis were obtained from cloned DNA of the fully-sequenced tylosin

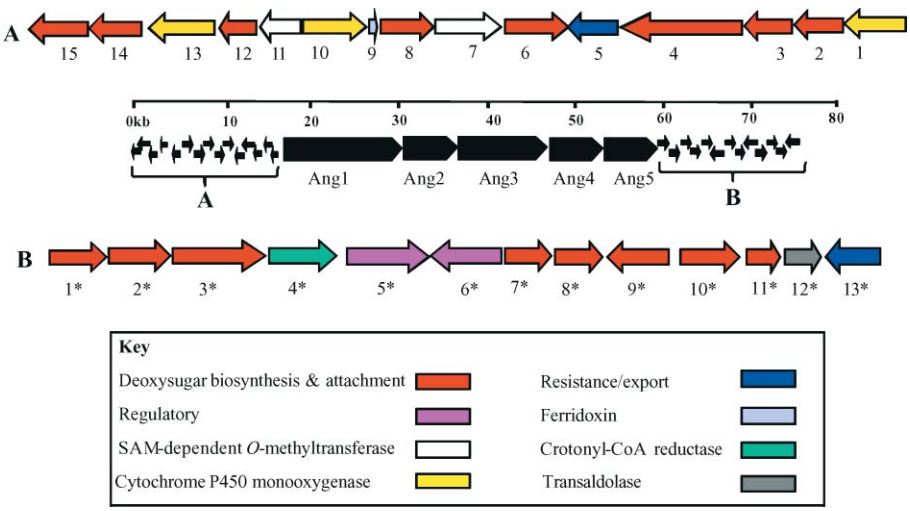

Fig. 3 Representation of the angolamycin biosynthetic gene cluster: The diagrams A and B (above and below the PKS gene cluster) show enlargement of the PKS flanking regions.

biosynthetic gene cluster of *S. fradiae* ATCC19609.[9] The genes required for the production and transfer of TDP-D-desosamine were obtained from the fully-sequenced erythromycin gene cluster of *Saccharopolyspora erythraea* NRRL2338.[10,20] We additionally used genes from the spinosyn biosynthetic gene cluster of *Saccharopolyspora spinosa* NRRL18395 involved in biosynthesis of the deoxyaminosugar TDP-D-forosamine (**10**) (Fig. 2).[21] To obtain the genes required for TDP-D-angolosamine biosynthesis we identified, cloned and sequenced the biosynthetic gene cluster for angolamycin (see below).

Identification, cloning and sequence analysis of the angolamycin biosynthetic gene cluster

S. eurythermus ATCC23956 was obtained from the American Type Culture Collection (Manassas, Virginia, USA). Initial identification of the angolamycin biosynthetic cluster was achieved by end-sequencing 768 clones from a *S. eurythermus* cosmid library generated in Supercos (Stratagene). Six clones were identified by BLAST searching which exhibited very high sequence homology to the genes of the tylosin biosynthetic cluster. The complete DNA sequence of four of these cosmids, designated 2C3, 2D8, 4h12 and 5B2 respectively, was obtained by the assembly of overlapping *Sau*3A fragments obtained from partial digestion of the parent cosmid. Remaining sequence gaps were filled using 21mer synthetic oligonucleotides as primers. These four cosmid clones were found to span an entire gene cluster containing modular polyketide synthase (PKS) genes with striking similarity to those of tylosin biosynthesis.[9] The angolamycin biosynthetic gene cluster sequence has been deposited as EU038272, EU220288 and EU232693.

The putative angolamycin (**8**) biosynthetic cluster lies within a 77 kbp locus (Fig. 3/Table 1). The biosynthesis of the polyketide backbone of **8** is governed by 5 large ORFs, designated *ang1* to *ang5*, which are co-transcribed and which encode, respectively, the multimodular PKS polypeptides Ang1 to Ang5. Examination of their encoded acyltransferase (AT) domains allowed the nature of the extender units (malonyl-, methylmalonyl-, or ethylmalonyl-CoA) to be readily deduced.[22] Examination of the β-keto group processing domains present (β-ketoreductase (KR), dehydratase (DH) and enoylreductase (ER)) allowed the level of reduction to be deduced for each such extension. This analysis showed that the constitution of Ang1–5 is wholly consistent with a role in the assembly of tylactone (**11**). Ang1 encompasses the loading domain and extension modules 1 and 2, Ang2 encompasses module 3, Ang3 encompasses modules 4 and 5, Ang4 encompasses module 6, and Ang5 encompasses module 7 plus the C-terminal thioesterase (TE) domain required for cyclisation and release of the polyketide chain from the PKS. This arrangement of enzyme domains is exactly analogous to that found in the tylosin PKS.[9]

Flanking the PKS-encoding genes are other ORFs involved in the biosynthesis of **8**. Numerous ORFs could be identified by comparison to sequences in published databases and by direct comparison with genes from the tylosin biosynthetic gene cluster. The proposed functions of these putative ORFs together with the best database matches are shown in Table 1 and include those required for TDP-D-angolosamine biosynthesis. The genes *orf15* and *orf13** most probably form the boundaries of the gene cluster. Upstream of *orf15* there is a strong RNA terminator structure, adjacent to genes encoding another distinct modular PKS. Downstream of *orf13** there is a non-coding region of about 1 kbp, followed by genes whose products appear to be involved in sporulation (data not shown).

A pathway for TDP-D-angolosamine biosynthesis is shown in Fig. 2 and is based on our sequencing and heterologoues expression experiments presented here. This is consistent with published data for the hedamycin[23] and medermycin[24] biosynthetic gene clusters. These polyketides also contain D-angolosamine moieties. The genes *angAI*, *angAII*, *angMIII*, *angB* and *angMI* seem to be exact counterparts of *tylAI*, *tylAII*, *tylMIII*, *tylB* and *tylMI* involved in TDP-D-mycaminose biosynthesis. The gene *ang*-ORF14 was identified to encode a TDP-4-keto-6-deoxyhexose reductase, the counterpart of *med*-ORF14 and *hedN* from the medermycin and hedamycin biosynthetic gene clusters respectively.

Table 1 Proposed functions of genes encoded by the angolamycin biosynthetic gene cluster

ORF	No. of amino acids	Best match in database	% Identity	Organism	Proposed function	Tylosin orthologue
15 (AngB)	373	Q7wt26	77	*Streptomyces* sp AM-7161	NDP-Hexose aminotransferase	TylB
14	333	Q67g46	49	*Streptomyces griseoruber*	NDP-Hexose 4-ketoreductase	
13	415	Q331r5	66	*Streptomyces* sp. KCTC0041BP	Cytochrome P450 monooxygenase	
12	211	Q9s4d4	72	*Streptomyces fradiae*	NDP-Hexose 3,(5)-epimerase	TylJ
11	272	Q9s4d5	79	*Streptomyces fradiae*	*O*-Methyltransferase	TylF
10	385	Q9s4d6	63	*Streptomyces fradiae*	Cytochrome P450 monooxygenase	TylH1
9	79	Q9shq2	51	*Streptomyces fradiae*	Flavodoxin	Tylodoxin
8	331	Q5sfa6	63	*Streptomyces fradiae*	NDP-Hexose 4-ketoreductase	TylD
7	395	Q9zhq4	71	*Streptomyces fradiae*	*O*-Methyltransferase	TylE
6	422	Q9zhq5	69	*Streptomyces fradiae*	Glycosyltransferase (deoxyallose)	TylN
5	283	Q9zhq6	57	*Streptomyces fradiae*	Ribosomal methylase (resistance)	
4 N-terminal	787	Q9jn57	63	*Streptomyces fradiae*	Type II thioesterase	
C-terminal		Q76kz0	60	*Streptomyces halstedii*	NDP-Hexose 2,3-dehydratase	tylCVI
3 (AngAII)	332	Q54144	70	*Streptomyces fradiae*	NDP-Hexose 4,6-dehydratase	tylAII
2 (AngAI)	295	Qfsfd2	74	*Streptomyces bikiniensis*	NDP-Hexose synthase	tylAI
1	414	Q59910	80	*Streptomyces fradiae*	Cytochrome P450 monooxygenase	
1* (AngMIII)	447	P95746	45	*Streptomyces fradiae*	Activation of AngMII	TylMIII
2* (AngMII)	424	P95747	67	*Streptomyces fradiae*	Glycosyltransferase (angolosamine)	TylMII
3* (AngMI)	240	P95748	65	*Streptomyces fradiae*	*N*-Methyltransferase	TylMI
4*	455	Q53865	93	*Streptomyces collinus*	Crotonyl-CoA reductase	
5*	641	Q00509	36	*Streptomyces ambofaciens*	Regulatory (SrmR)	
6*	447	Q9jn56	53	*Streptomyces fradiae*	Regulatory	TylR
7*	333	Q9xc69	54	*Streptomyces fradiae*	NDP-Hexose 4-ketoreductase	TylCIV
8*	334	033936	67	*Saccharopolyspora erythraea*	4-Keto-6-deoxy NDP-kexose 2,3 reductase	TylCII
9*	410	Q9xc68	78	*Streptomyces fradiae*	NDP-Hexose 3-C-methyltransferase	TylCIII
10*	404	Q9xc67	64	*Streptomyces fradiae*	Glycosyltransferase	TylCV
11*	202	Q9xc66	60	*Streptomyces fradiae*	NDP-hexose 3,(5)-epimerase	TylCVII
12*	388	Q82m93	29	*Streptomyces avermitilis*	Transaldolase	
13*	577	Q60248	65	*Streptomyces halstedii*	Exporter/resistance	

Heterologous expression hosts

We previously described an *in vivo* glycosylation system based upon defined *S. erythraea* mutant strains.[5–8] In the present study additional *S. erythraea* mutant strains were constructed. *S. erythraea* strain SGQ2 (BIOT-2175) is a quadruple mutant lacking the *eryA* PKS genes, the two GTs *eryCIII* and *eryBV*, and *eryCVI*, an *N*,*N*-dimethyltransferase gene required for the biosynthesis of TDP-D-desosamine. All of these are essential for erythromycin (**1**) biosynthesis and were successively mutated by

introducing deletions into the chromosome. *S. erythraea* Q42/1 (BIOT-2166) is a derivative of SGQ2 in which the biosynthesis of TDP-L-mycarose (an additional deoxyhexose required for the biosynthesis of **1**) was disrupted by integration of a suicide vector containing an internal fragment of *eryBVI*, a gene essential for its biosynthesis. *S. erythraea* LB1 (BIOT-2634) is deleted in almost the entire erythromcyin biosynthetic gene cluster, leaving only the *ermE* and *eryCI* genes on one flank and the gene *eryK* encoding a cyp450-dependent monooxygenase on the other flank. The host strains described here all retain the *ermE* gene, which confers resistance to macrolide antibiotics. A detailed description of host preparation is given in the ESI† (also see ref. 18).

Three different GTs were cloned and separately introduced into these host strains as required. When combined with appropriate aglycone substrates, these acted as reporter systems for the production of activated deoxyhexoses and their subsequent transfer to aglycone acceptor substrates. The first two GTs studied were TylMII from *S. fradiae* and AngMII from *S. eurythermus* (described above) both of which use tylactone (**11**) (see Fig. 4) as their natural acceptor substrate. During tylosin biosynthesis TylMII (in combination with TylMIII) catalyses transfer of D-mycaminose to the C5-hydroxyl group of **11** to give 5-*O*-β-D-mycaminosyltylactone (**12**) (Fig. 4). AngMII (plus AngMIII; this study) is proposed to catalyze the analogous transfer of a D-angolosamine moiety to **11** during biosynthesis of angolamycin. The third GT utilized was EryCIII which (with EryCII) catalyses attachment of D-desosamine to the C5-hydroxy group of 3-*O*-α-L-mycarosylerythronolide B (**13**) during erythromycin biosynthesis.[2]

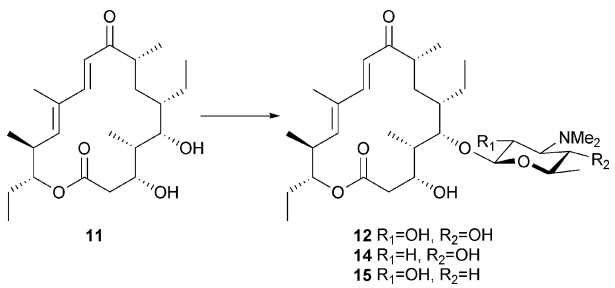

Fig. 4 Biotransformation of tylactone (**11**) to 5-*O*-β-D-angolosaminyltylactone (**14**) (BIOT-2945/2808) and 5-*O*-β-D-mycaminosyltylactone (**12**) (BIOT-2879/3007); the structure of the previously reported[5] 5-*O*-β-D-desosaminyltylactone (**15**) is given for comparison.

Construction of D-deoxyaminohexose biosynthetic gene cassettes

Biosynthetic gene cassettes were assembled in an integrative expression vector using the cloning strategy outlined in detail in the ESI†. This is an advancement of a procedure described previously.[7] In order to incorporate each gene into a cassette it was amplified with an *Nde*I restriction site overlapping the start codon and an *Xba*I restriction site adjacent to the stop codon. These PCR products were then cloned into the conversion vector pSGLit1 which was employed to introduce a Shine–Dalgarno sequence/ribosome binding site and to incorporate an *Xba*I site at the 5′-end of the gene that is sensitive to *dam*$^-$ methylation. DNA from the resulting plasmids was isolated from a *dam*$^-$ strain background, the gene isolated by *Xba*I digestion, and incorporated into the gene cassette after *Xba*I digests. The presence of a single methylation sensitive *Xba*I site allows for easy determination of the direction of integration by restriction digest. A schematic overview of the strategy is provided in the ESI as Fig. S1. This methodology is extremely flexible and has been used extensively in other systems, for example the combinatorial assembly of libraries of rapamycin post-PKS gene cassettes.[25]

Production of novel macrolides containing D-angolosamine

A biosynthetic gene cassette for the production of TDP-D-angolosamine (**9**) was assembled using genes from both the angolamycin and the spinosyn biosynthetic gene clusters. Ang-ORF4, a putative TDP-4-keto-6-deoxyhexose-2,3-dehydratase, appears to be present as the C-terminal domain of a protein whose N-terminal portion houses a thioesterase activity. To avoid uncertainty being introduced by use of this apparently bifunctional protein we substituted *ang*-ORF4 with *spnO*, which encodes the counterpart gene required for TDP-D-forosamine (**10**) biosynthesis in the spinosyn pathway of *S. spinosa* (see Fig. 2),[21] and whose function has been confirmed.[26] A gene cassette was thus constructed comprising *angAI*, *angAII*, *angMIII*, *spnO*, *ang*-ORF14, *angB*, *angMI* and *angMII*, and this cassette was used to transform *S. erythraea* Q42/1 to give strain BIOT-2945. The functions of these genes are described in Tables 1 and 2. Tylactone (**11**) was added to growing cultures of BIOT-2945 and after five days the fermentation broth was extracted with organic solvent. LCMS analysis of the extracts revealed the presence of a new **11**-derived metabolite which was not present when **11** was

Table 2 Function of deoxysugar biosynthetic genes utilised/discussed

Gene	Strain	Accession number	Proposed function
tylAI	*Streptomycs fradiae*	U08223	TDP-D-glucose synthase
tylAII	*Streptomycs fradiae*	U08223	TDP-D-glucose dehydratase
tylMI	*Streptomycs fradiae*	X81885	*N*,*N*-Dimethyltransferase
tylMII	*Streptomycs fradiae*	X81885	Glycosyltransferase
tylMIII	*Streptomycs fradiae*	X81885	Glycosyltransferase ancillary protein
tylB	*Streptomycs fradiae*	U08223	Aminotransferase
tylIa	*Streptomycs fradiae*	U08223	3,4-Ketoisomerase
eryBV	*Saccharopolyspora erythraea*	Y11199	Glycosyltransferase
eryBVI	*Saccharopolyspora erythraea*	AM420293	NDP-hexose 2,3-dehydratase
eryCII	*Saccharopolyspora erythraea*	Y14332	Glycosyltransferase ancillary protein
eryCIII	*Saccharopolyspora erythraea*	Y14332	Glycosyltransferase
eryCVI	*Saccharopolyspora erythraea*	Y11199	*N*,*N*-Methyltransferase
ermE	*Saccharopolyspora erythraea*	X51891	Methylase (erythromycin resistance)
spnO	*Saccharopolyspora spinosa*	AY007564	NDP-hexose 2,3-dehydratase

fed to the control strain *S. erythraea* Q42/1. The new compound (**14**) contained a deoxyaminohexose whose fragment mass (158 amu) corresponded to the presence of a D-angolosamine moiety. The new product represented ~50% of the tylactone (**11**) supplied. The experiment was then repeated using a gene cassette in which the GT-encoding *angMII* was replaced with *tylMII* to give strain BIOT-2808, and the same novel macrolide (**14**) was produced with similar efficiency from the added **11**. A more polar analogue was also observed in each case whose LCMS data was consistent with this being 5-*O*-β-D-glucosyltylactone produced by an unidentified native D-glucosyltransferase of broad substrate range as described previously.[5]

To confirm the identity of **14** the biotransformation using strain BIOT-2808 was scaled up to provide 20 mg of purified compound. Its structure was confirmed as 5-*O*-β-D-angolosaminyltylactone (Fig. 4) using multi-dimensional NMR experiments. The data are reported in the experimental section, but key features are the lack of an oxygen substituent at C-2′ and a hydroxyl group at C-4′. Glycosidic linkage between C-5 and C-1′ was firmly established through observation of the appropriate correlation in the HMBC spectrum. The analysis was aided by comparison to the equivalent D-desosamine[5] (**15**) and D-mycaminose[27] (**12**) containing analogues reported previously. This result confirmed that the assembled gene cassette was indeed capable of producing TDP-D-angolosamine (**9**) in the heterologous host strain, and also that the function of AngMII during **8** biosynthesis is the transfer of a D-angolosamine moiety to the hydroxy group at C-5 of tylactone.

To examine whether AngMII could transfer D-angolosamine to other macrolide aglycones, erythronolide B (**16**) (the aglycone of the erythromycin biosynthetic pathway) and 3-*O*-α-L-mycarosylerythronolide B (**13**) (the subsequent intermediate of erythromycin biosynthesis) were added separately to growing cultures of strain BIOT-2945. A new macrolide product was observed (>90% bioconversion) only with **16** as substrate. Following scale-up, analysis of the purified compound using a combination of LCMS and NMR methods was straightforward and allowed its structure to be confirmed as 3-*O*-β-D-angolosaminylerythronolide B (**17**) (Fig. 5A). The D-angolosamine moiety was readily assigned, and HMBC data clearly located the glycosidic linkage as C3–C1′. This result demonstrates that AngMII is reasonably relaxed in choosing its aglycone acceptor substrate. Glycosylation at the C3-hydroxyl group to yield **17** was unanticipated as AngMII normally transfers a D-angolosamine residue to the C5-hydroxyl group of its native substrate tylactone.

Previous work has suggested that, in general, GTs are more specific for their acceptor substrate than for the deoxysugar donor substrate. Therefore, to examine the possibility of transferring D-angolosamine to **13**, and produce modified erythromycin analogues, *angMII* was substituted within the biosynthetic gene cassette by the GT *eryCIII*. This cassette was then transferred into *S. erythraea* Q42/1 to give strain BIOT-2943. This host strain contains *eryCII* whose gene product is the activator partner protein for EryCIII.[18,28] Analysis by LCMS/MS indicated a series of erythromycin analogues which were not present in extracts when **13** was not fed, nor when **16** was fed. The LCMS profile of these compounds was consistent with the transfer to **13** of a deoxyaminohexose with the appropriate MS characteristics consistent for D-angolosamine (loss of 158 amu by MS/MS). The LCMS data for the erythromycin analogue **18** (Fig. 6) is

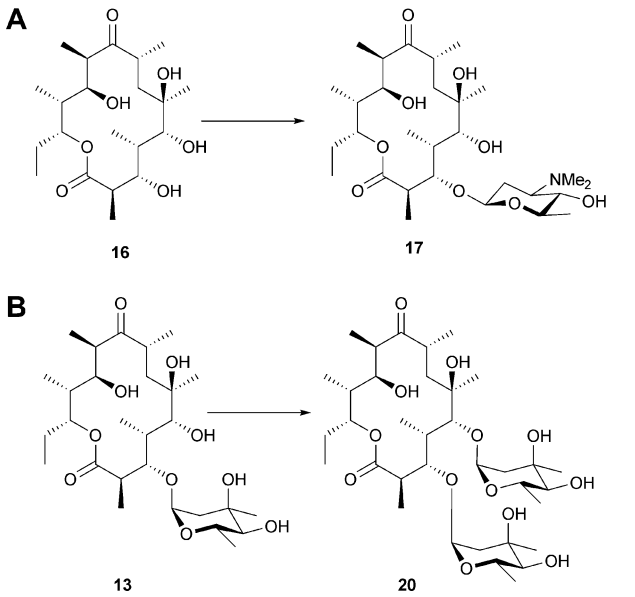

Fig. 5 A. Biotransformation of erythronolide B (**16**) to 3-*O*-β-D-angolosaminylerythronolide B (**17**) (BIOT-2945); B. Biotransformation of 3-*O*-α-L-mycarosylerythronolide B (**13**) to 3,5-bis-*O*-α-L-mycarosylerythronolide B (**20**) (BIOT-2191).

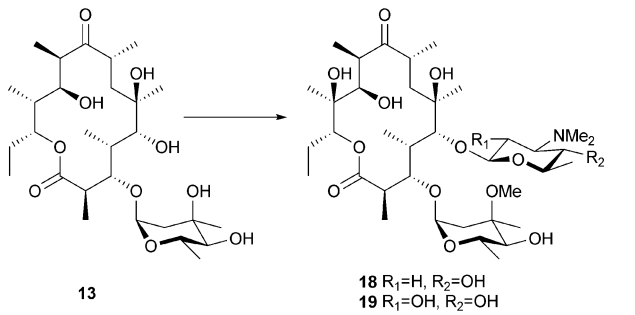

Fig. 6 Biotransformation of 3-*O*-α-L-mycarosylerythronolide B (**13**) to erythromycin analogues in which the 5-*O*-β-D-desosamine moiety has been replaced with D-angolosamine (**18**) and D-mycaminose (**19**) by engineered *S. erythraea* strains expressing *eryCII/CIII* and producing TDP-forms of the D-deoxyhexoses in place of TDP-D-desosamine (**7**) (BIOT-2943 & -3010/2794 respectively).

provided in the experimental section. Unfortunately the yield of this biotransformation was poor (~5% total conversion) and the compound was not isolated in sufficient yield and purity for complete NMR analysis. The poor yield is presumably because of a restricted specificity of EryCIII for TDP-D-angolosamine, or of the efficiency of EryCII/III to catalyse its transfer.

Production of novel macrolides containing D-mycaminose

A biosynthetic gene cassette for the production of TDP-D-mycaminose (**4**) was assembled using the same approach as described above. This cassette contained the genes *tylAI*, *tylAII*, *tylMIII*, *tylB*, *tyl1a*, *tylMI* and *tylMII* and was used to transform *S. erythraea* Q42/1 to give strain BIOT-2879. Tylactone (**11**) was added to growing cultures and after five days the fermentation broth was extracted and examined by LCMS/MS. A new compound was produced only on addition of **11** (~50% bioconversion) whose retention time and MS/MS spectrum (loss of a 176 amu

fragment) was identical with that of an authentic sample[27] of the anticipated product 5-*O*-β-D-mycaminosyltylactone (**12**) (see Fig. 4). The same gene cassette was also used to transform *S. erythraea* LB1 (lacking essentially all of the erythromycin gene cluster) to give strain BIOT-3007. The efficient bioconversion of **11** to **12** by this strain was also confirmed by LCMS/MS.

We then examined the possibility that EryCIII could transfer D-mycaminose to 3-*O*-α-L-mycarosylerythronolide B (**13**) in order to produce the equivalent erythromycin analogues, *e.g.* **19**. Such compounds would contain an additional 4″-hydroxy moiety and have been reported previously, including through semi-synthesis starting from erythromycin.[28,29] Thus, the *tylMII* gene at the 3′-end of the TDP-D-mycaminose biosynthetic gene cassette described above was replaced by *eryCIII*. This cassette was then used to transform *S. erythraea* Q42/1 to give strain BIOT-3010. After addition of **13** to growing cultures, LCMS/MS analysis showed the production of new compounds formed by the addition of a D-mycaminose moiety (*e.g.* **19**; Fig. 6). This indicated that D-mycaminose was transferred to yield a mixture of the corresponding erythromycin analogues A–D. Unfortunately the efficiency of this bioconversion was low, with the majority (~90%) of the substrate **13** remaining at the end of the biotransformation. When the gene cassette was used to transform *S. erythraea* LB1, which does not contain *eryCII* encoding the activator protein for EryCIII, bioconversion of **13** was not observed.

Unexpected production of 5-des-*O*-β-D-desosaminyl-5-*O*-β-D-mycaminosylerythromycin A (19)

As demonstrated by the production of **12**, the biosynthetic gene cassette containing the 3,4-ketoisomerase *tyl1a* and other *tylM* genes are sufficient for efficient TDP-D-mycaminose biosynthesis in both *S. erythraea* mutants Q41/1 and LB1. Further, when the GT *tylMII* is replaced with *eryCIII*, transfer of D-mycaminose to 3-*O*-α-L-mycarosylerythronolide B (**13**) was observed, although the production level of erythromycins bearing a D-mycaminose moiety in place of D-desosamine at C5 was low.

In the course of this work we also assembled a gene cassette which contained the majority of the genes for TDP-D-angolosamine biosynthesis, *angAI*, *angAII*, *ang*-ORF14, *angMIII*, *angB* and *angMI*, but not the gene *ang*-ORF4 which encodes the putative TDP-4-keto-6-deoxyhexose-2,3-dehydratase. This gene cassette was transferred into the host strain *S. erythraea* SGQ2, which still contains a functional copy of the native TDP-4-keto-6-deoxyhexose-2,3-dehydratase-encoding gene *eryBVI* whose gene product is responsible for 2-deoxygenation during TDP-L-mycarose biosynthesis. The gene cassette additionally contained the GT-encoding gene *eryCIII* (*eryCII* was present in the genome). When the resulting strain *S. erythraea* BIOT-2794 was fermented in the presence of 3-*O*-α-L-mycarosylerythronolide B (**13**), analysis by LCMS/MS indicated the surprising presence of compounds consistent with **19**. The bioconversion efficiency (~20%) was significantly greater than that observed in the previous example and, after scale-up, **19** (15 mg) was purified and its structure confirmed by NMR analysis; the data for erythromycin, 5-*O*-β-D-mycaminosyltylactone (**12**) and related compounds aided the assignment. Significantly, no metabolites could be observed by LCMS/MS containing a D-angolosamine moiety in place of D-mycaminose.

EryCIII transfers L-mycarose to 3-*O*-α-L-mycarosylerythronolide B (13)

Previous analysis of strains lacking in TDP-D-desosamine (**7**) biosynthesis showed elevated levels of a compound with MS/MS characteristics consistent with the addition of a second L-mycarose moiety to **13**.[18] To confirm the identity of this compound, and to determine whether EryCIII was responsible for the biosynthesis of this compound, the plasmid pSGeryCIII was introduced *in trans* into *S. erythraea* SGQ. pSGeryCIII contains *eryCIII* under the control of a strong promoter. *S. erythraea* SGQ2, which lacks both native erythromycin pathway GTs, was unable to biotransform exogenously added **13** whereas *S. erythraea* SGQ2/pSG*eryCIII* (BIOT-2191) converted ~50% of exogenous **13** to the described compound. This biotransformation was scaled up and the new compound was isolated. NMR analysis clearly identified this as 3,5-bis-*O*-α-L-mycarosylerythronolide B (**20**) (Fig. 5B).

Discussion

In order to modify the glycosylation pattern of macrolide antibiotics through engineered biosynthesis, it is important to understand both the biosynthetic pathways leading to the activated deoxyhexose components and the substrate tolerance of the cognate GTs. The majority of published studies have focused on the 14-membered macrolides erythromycin (**1**), oleandomycin and picromycin, and the 16-membered macrolide tylosin (**2**).[2] As a result, the biosynthetic routes to TDP-D-desosamine (for **1**, oleandomycin and pikromycin), TDP-L-mycarose (**1** and **2**), TDP-D-mycaminose (for **2**), and TDP-D-forosamine (for **3**) are relatively well understood (see Fig. 2). These studies have led to the identification of individual enzyme functions.

The identification and cloning of the angolamycin (**8**) biosynthetic gene cluster, in combination with the experiments reported here, is consistent with a pathway for the biosynthesis of TDP-D-angolosamine (**9**) proposed elsewhere[23,24] (Fig. 2). The common intermediate TDP-4-keto-6-deoxy-D-glucose (**5**) first undergoes C2-deoxygenation through the action of a TDP-4-keto-6-deoxy-D-glucose 2,3-dehydratase (believed to be Ang-ORF4 in *S. erythermus*). The resulting compound **21** is then reduced by Ang-ORF14, a deoxyhexose 4-ketoreductase, to give **22**. The amino function is introduced at C3 by the aminotransferase AngB, followed by *N*,*N*-dimethylation catalysed by AngMI to yield TDP-D-angolosamine (**9**). It is noteworthy that several of the *ang* genes are clear counterparts to those required for TDP-D-mycaminose production during tylosin biosynthesis. It is now understood that among the group of TDP-D-deoxyaminohexoses discussed here only the biosynthesis of TDP-D-mycaminose (**4**) requires a 3,4-ketoisomerase activity, and that this activity is encoded by *tyl1a* (see Fig. 2).[15,16,18] For biosynthesis of TDP-D-desosamine (**7**) and TDP-D-angolosamine (**9**), generation of the 3-keto functionality required for introduction of an amino group originates *via* the action of a TDP-4-keto-6-deoxyhexose-2,3-dehydratase (biosynthesis of **9**) or a novel 4-deoxygenation mechanism involving C4-aminotransfer and a deaminase activity (biosynthesis of **7**).[11] We used this combined information to prepare biosynthetic gene cassettes which, when expressed in an appropriate host strain, confer upon the resulting mutant the ability to biotransform various polyketide aglycones to produce

novel macrolide structures. We believe that these gene cassettes will have utility in systems other than those based on *S. erythraea*. The construction of biosynthetic gene cassettes extended our cloning strategy in which each gene is cloned using identical restriction sites by virtue of iterative conversion of introduced *Xba*I restriction sites to a methylation-sensitive sequence.[7,18,25] This approach allows the same gene fragments to be used in the construction of multiple cassettes without the limitations of restriction site compatibility, and thus combinatorial assembly processes are possible. Additionally, this method provides each gene with an individual ribosome binding site.

When introduced into both *S. erythraea* strains Q42/1 and LB1 the biosynthetic gene cassette for TDP-D-mycaminose (**4**), containing the cognate GT pairing of *tylMII*/*tylMIII* or the hybrid pair *tylMII*/*angMIII*, conferred the ability to efficiently produce and transfer D-mycaminose to exogenously added tylactone (**11**) as anticipated (this is the native aglycone (acceptor) for both of these GTs). Similarly, the cassette for TDP-D-angolosamine (**9**) in combination with *angMII*/*angMIII* was able to efficiently produce this D-deoxyaminohexose and transfer it to **11**. These data confirmed our biosynthetic gene cassette approach. Similarly, a cassette was assembled for TDP-D-desosamine (**7**) biosynthesis including the GT pairing of *eryCII*/*eryCIII*, and its effectiveness was verified by transformation into *S. erythraea* LB1, which lacks almost the entire erythromycin biosynthetic gene cluster (data not shown). Addition of exogenous 3-*O*-α-L-mycarosylerythronolide B (**13**) resulted in quantitative biotransformation to erythromycin C as anticipated.

As described in this paper, all three GTs studied displayed significant substrate flexibility and utilized both 'non-native' TDP-deoxyhexose (donor) *and* polyketide aglycone (acceptor) substrates. TylMII and AngMII were both able to accept and transfer TDP-D-angolosamine to tylactone (**11**). TylMII has previously been shown to efficiently transfer D-desosamine to **11**.[5] Thus TDP-D-mycaminose (**4**), TDP-D-desosamine (**7**) and TDP-D-angolosamine (**9**) are all effective deoxysugar donor substrates for this enzyme. In addition, we found that TylMII is able to transfer non-amino L-configured deoxyhexoses to **11** (Fig. 7). During our investigations of biosynthetic gene cassette assembly we expressed *tylMII* in a background lacking the aminotransferase *angB*. When exogenous **11** was added to this strain, several novel compounds were produced. Interestingly, two of these were only produced when the GT helper gene *tylMIII* was co-expressed with *tylMII*, even though the counterpart gene *eryCII* from the erythromycin biosynthetic gene cluster was present in the genome. The total bioconversion yield of these experiments was between 20–50% of the added material at an analytical scale. After scale-up biotransformation, 5 mg of the major new compound was isolated and detailed NMR analysis confirmed it as 5-*O*-α-L-rhamosyltylactone (**23**) (see experimental section). The MS spectrum of the closely eluting minor new metabolite was identical to that of **23** and most likely an analogue with a 6-deoxyhexose moiety attached at C5 (possibly D-quinovose although sufficient material was not obtained for complete characterization). The strain also produced the previously reported 5-*O*-β-D-glucosyltylactone, which arises through the action of an uncharacterized host GT.[5] The TDP-L-rhamnose required for **23** biosynthesis is believed to arise from the background metabolism of the *S. erythraea* mutant host as described previously.[5,30] The synthesis of **23** was reported previously during an *in vitro* study concerning the substrate flexibility of the DesVII/DesVIII GT pairing.[13]

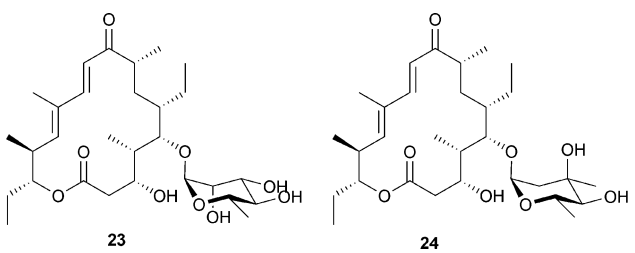

Fig. 7 Structure of 5-*O*-α-L-rhamnosyltylactone (**23**) and 5-*O*-α-L-mycarosyltylactone (**24**).

In addition to TDP-L-rhamnose, TylMII is also capable of utilizing TDP-L-mycarose to make 5-*O*-α-L-mycarosyltylactone (**24**) (Fig. 7). This material was observed at low levels in several biotransformation experiments when tylactone (**11**) was fed to *S. erythraea* mutants competent in TDP-L-mycarose (but not TDP-D-desosamine) biosynthesis and which contained *tylMII* and either *tylMIII* or *angMIII*. We were able to confirm the presence of **24** through the use of LCMS analysis and comparison to an authentic sample.[27] 5-*O*-α-L-Mycarosyltylactone (**24**) is a known and significant product, along with tylactone (**11**), of *S. fradiae* mutants deficient in TDP-D-mycaminose biosynthesis.[27] Thus, TylMII shows a remarkably broad substrate tolerance towards its deoxysugar donor substrate.

In addition to its natural 16-membered aglycone (acceptor) substrate **11**, AngMII was able to transfer D-angolosamine to the C3-hydroxyl group of the 14-membered macrolide erythronolide B (**16**). The corresponding activity has also been observed for TylMII, in which an *S. fradiae* mutant blocked in tylactone production[31] biotransformed **16** to give 3-*O*-β-D-mycaminosylerythronolide B (unpublished results[32]). The ability of macrolide GTs to act on structurally related aglycones of varying size may be a general phenomenon, and is a notable characteristic of DesVII, which naturally transfers D-desosamine to methonolide and narbonolide (12- and 14-membered aglycones respectively) during methymycin/pikromycin biosynthesis.[12] DesVII can also glycosylate the 16-membered tylactone **11** both *in vitro*[13] and *in vivo*.[33]

The biosynthetic gene cassettes for TDP-D-mycaminose (**4**) and TDP-D-angolosamine (**9**) were also used in combination with *eryCIII*. We were gratified to observe that EryCIII transferred both of these D-deoxyaminohexoses to its native substrate 3-*O*-α-L-mycarosylerythronolide B (**13**), although the transfer was inefficient. These new macrolides were not produced in sufficient yield for NMR characterization but LCMS/MS data was consistent with the production of the anticipated erythromycin analogues. As the biosynthetic gene cassettes utilized had been shown to be efficient for the production of their respective D-deoxyhexoses, we speculated that EryCIII may have limited tolerance towards these substrates. Surprisingly, in a related experiment we were able to produce the D-mycaminose-containing erythromycin derivative **19** in an improved, albeit still modest, yield. In this instance, a biosynthetic gene cassette was prepared which contained the majority of the genes required for TDP-D-angolosomine (**9**) biosynthesis, but lacked the TDP-4-keto-6-deoxyhexose-2,3-dehydratase-encoding gene (neither *ang*-ORF4

nor the heterologous *spnO* gene were included). This was transformed into *S. erythraea* SGQ2, which contains *eryBVI*, the native TDP-4-keto-6-deoxyhexose-2,3-dehydratase required for TDP-L-mycarose biosynthesis, and this cassette also contained *eryCIII*. This mutant was examined for its ability (or not) to biosynthesise **9**. When **13** was added to fermentations we were surprised to observe reasonable bioconversion to give novel erythromycins with an LCMS/MS profile consistent with D-mycaminosylated products. After scale-up and purification the major new product was shown to have a structure consistent with **19**. This result was surprising as the mutant does not contain a 3,4-ketoisomerase activity which is believed to be essential for TDP-D-mycaminose production. No metabolites containing a D-angolosamine moiety could be observed, and the host strain itself was not capable of producing **19** when fed **13**. We have no current explanation for this result but did observe that while a mutant transformed with an identical gene cassette lacking only *ang*-ORF14, a TDP-deoxyhexose C4-ketoreductase, was also able to produce **19**, its biotransformation capacity was much reduced.

The ability of EryCIII to utilize TDP-D-mycaminose (**4**) as a substrate *in vitro* has been reported elsewhere, although the activity with this substrate appeared significantly lower than with the native TDP-D-desosamine (**7**).[28] The tolerance of EryCIII towards alternative deoxyhexose templates appears relatively broad as we also demonstrated its ability to utilize TDP-D-angolosamine (**9**), and, surprisingly, a TDP-L-deoxyhexose (TDP-L-mycarose) and convert 3-*O*-α-L-mycarosylerythronolide B (**13**) to 3,5-bis-*O*-α-L-mycarosylerythronolide B (**20**) when expressed in BIOT-2191. This is consistent with observations that **20** is a direct fermentation product of an *S. erythraea* strain disrupted in the biosynthesis of TDP-D-desosamine.[18]

This apparent general ability of macrolide GTs to accept both TDP-D-deoxyaminohexoses and TDP-L-deoxyhexoses was highlighted by us previously during studies of SpnP, a GT whose native deoxysugar substrate is TDP-D-forosamine (**10**) during spinosyn (**3**) biosynthesis.[6] SpnP was shown to accept TDP-L-mycarose when expressed in a *S. erythraea* mutant and to produce novel spinosyn analogues. We proposed that SpnP may recognize the $^4C_1$ conformation of TDP-L-mycarose in which the hexose ring 'flips' to an alternative conformation where the β-TDP moiety sits in an axial conformation and would be more readily recognized by SpnP (D-deoxyhexoses favour the $^4C_1$ conformation). Similar arguments have been forwarded when discussing StaG required for staurosporine biosynthesis, which also accepts L- and D-deoxyhexoses.[34] Further publications demonstrate that GTs in other engineered systems will accept both NDP-D- and L-deoxyhexose substrates; notably ElmGT involved in elloramycin biosynthesis[35] and VinC involved in vicenistatin biosynthesis.[36]

In summary, a procedure for the generation of biosynthetic gene cassettes targeted towards the production of important D-deoxyaminohexoses has been described. Such gene cassettes can be expressed in appropriate heterologous hosts and, in combination with macrolide GTs and their ancillary proteins. The resulting organisms can be utilized as effective tools for the generation of hybrid macrolide antibiotics. The macrolide GTs EryCIII, AngMII and TylMII all exhibit usefully broad substrate tolerance, accepting a range of TDP-D- and L-deoxyhexose donors and several acceptor substrates.

Experimental

General methods

All solvents used were HPLC grade. LCMS/MS analysis was performed on an Agilent HP1100 HPLC system in combination with a Bruker Daltonics Esquire 3000+ ion trap mass spectrometer fitted with an electrospray source. The MS was operated in both positive and negative ion modes. UV analysis was performed at 210, 258 and 280 nm on an Agilent DAD detector. High resolution mass spectra were acquired on a Bruker BioApex II FTICR mass spectrometer. NMR spectra were recorded on a Bruker Avance 500 spectrometer at 298 K operating at 500 MHz and 125 MHz for ^1H and ^{13}C respectively. Standard Bruker pulse programs were used to acquire the ^1H–^1H COSY, APT, HMQC and HMBC spectra. Coupling constants are given in Hertz. NMR experiments were referenced to the residual resonance of the solvent.

Escherichia coli XL1-Blue MR (Stratagene), *E. coli* DH10B (GibcoBRL) and *E. coli* ET12567[37] were grown in 2TY medium as described elsewhere.[38] Vectors pUC18, pUC19 and Litmus 28 were obtained from New England Biolabs. *E. coli* transformants were selected with ampicillin (100 μg cm^{-3}). *S. erythraea* NRRL2338 strains were cultured as described previously[20] unless expressly described otherwise below. Expression vectors in *S. erythraea* were derived from plasmid pSG142.[7] Plasmid-containing *S. erythraea* were selected with thiostrepton (40 μg cm^{-3}) or apramycin (50 μg cm^{-3}). DNA manipulations, PCR and electroporation procedures were carried out using standard procedures.[38] Protoplast formation and transformation procedures of *S. erythraea* were as described previously.[10,20] Southern hybridizations were carried out with probes labelled with digoxigenin using the DIG DNA labelling kit (Boehringer, Mannheim). DNA sequencing was performed in the Department of Biochemistry DNA Sequencing Facility at the University of Cambridge. An Applied Biosystems 800 molecular biology CATALYST robot was used to apply Taq dideoxy terminator sequencing reactions (Big Dye Terminator kit, ABI) to an ABI 373A sequencer according to manufacturers' protocols.

Cloning and sequencing of the angolamycin (8) biosynthetic gene cluster

S. eurythermus ATCC23956 was propagated in tryptic soy broth (TSB; Difco) at 30 °C. Supercos (Stratagene) was used for construction of the cosmid library and transfections were performed in *E. coli* XL-Blue MR (Stratagene USA). Sub-cloning from recombinant cosmids was into plasmid pSG397[39] and recombinants were selected by growth in media supplemented with chloramphenicol (50 μg cm^{-3}).

Total DNA was isolated from *S. eurythermus* by 'Procedure B' from the John Innes *Streptomyces* manual.[40] For the generation of the cosmid library, total DNA was partially digested with *Sau*3A, dephosphorylated with shrimp alkaline phosphatase, ligated directly into pSuperCos and packaged with Gigapack Gold packaging extract (Stratagene) without size fractionation. All procedures were performed in accordance with the manufacturer's recommendations. Identification of the putative angolamycin biosynthetic cluster was by direct sequencing of cosmid DNA. Briefly, 768 colonies of the *S. eurythermus* cosmid library were individually grown in TSB cultures (1 cm^3), shaken

in eight 96-well plates for 24 h at 37 °C. An aliquot of each culture (0.05 cm^3) was transferred to corresponding labelled wells on a 96-well microtitre plate using a multi-channel pipette, and stored at −80 °C after addition of an equal volume of 40% glycerol (v/v). These stock suspensions were later used to inoculate larger volumes of culture once individual target clones had been identified from sequence data. Small scale DNA preparations were made from the remainder of each culture (0.9 cm^3; done as recommended by ABI) and the sequence of the ends of each insert was obtained using T3 and T7 primers as described below. Six clones were identified by BLAST searching with very high sequence homology to the genes of the tylosin biosynthetic cluster. Liquid cultures of these clones were grown in 2 × LB medium with appropriate antibiotic selection, and cosmid DNA was prepared from them using Qiagen midi-prep DNA purification kits. The complete DNA sequence of four of these cosmids, designated 2C3, 2D8, 4h12 and 5B2 respectively, was obtained by assembly of overlapping *Sau*3A fragments obtained from partial digestion of the parent cosmid. Briefly, DNA fragments of 2–5 kbp from the *Sau*3A digest were eluted from an agarose gel using the Gene Clean kit (Bio101, BioRad) and sub-cloned into pSG397. Remaining sequence gaps were filled using 21mer synthetic oligonucleotides as primers. Plasmid sequencing was perfomed using pUC forward and reverse primers. SeqEd v 1.0.3 was used for sequence editing. Sequence assembly employed the GAP (Genome Assembly Program) version 4.2.[41] DNA sequence translations were performed with the GCG software package. Database searches used the BLAST algorithm.

Analytical chemistry

An aliquot of fermentation broth was shaken vigorously with an equal volume of methanol (20 min) and then clarified by centrifugation. Supernatants were analysed by LCMS/MS and chromatography was achieved over base-deactivated Luna C_{18} reversed-phase silica (5 micron particle size) using a Luna HPLC column (250 × 4.6 mm; Phenomenex (Macclesfield, UK)) heated at 40 °C. Gradient elution was from 25% mobile phase B to 75% mobile phase B over 19 min at a flow rate of 1 cm^3 min^{-1}. Mobile phase A was CH_3CN–H_2O (1 : 9), containing 10 mM ammonium acetate and 0.15% formic acid; mobile phase B was CH_3CN–H_2O (9 : 1), containing 10 mM ammonium acetate and 0.15% formic acid.

General procedure for analytical scale biotransformation

This was based on published procedures.[20,42]

General procedure for scale up of biotransformations

S. erythraea strains were grown in TSB seed medium and then sucrose–succinate (SSDM) production media using protocols described previously.[20,42] For a typical experiment the production stage consisted of Erlenmeyer flasks (2 dm^3) each containing 400 cm^3 of SSDM medium (5% inoculum from seed). Substrate aglycones were added as methanol solutions to achieve a final concentration of 50 mg cm^{-3}. These were added after the production medium had been growing for 24 h.

Extraction and purification of new macrolides

The following general procedure was used unless described otherwise below. The biotransformation broth was clarified by centrifugation. The supernatant was adjusted to pH 9.0 (5 M NaOH) and extracted three times with an equal volume of ethyl acetate. The cell pellet was extracted twice with an equal volume of acetone–methanol (1 : 1). The organic extracts were combined and the solvents removed under reduced pressure. The resulting aqueous fraction was extracted three times with an equal volume of ethyl acetate and the combined organic solvent removed under reduced pressure to leave a crude oily extract. This was dissolved in methanol and chromatographed over base-deactivated Luna C_{18} reversed-phase silica (5 micron particle size) using a Luna HPLC column (250 × 21 mm; Phenomenex (Macclesfield, UK)). A Gilson 315 binary HPLC system was used to deliver a linear gradient of CH_3CN–H_2O (3 : 7) containing 10 mM ammonium acetate to CH_3CN–H_2O (7 : 3) containing 10 mM ammonium acetate at 21 cm^3 min^{-1} over 20 min.

Production and isolation of 3,5-bis-*O*-α-L-mycarosylerythronolide B (20)

S. erythraea SGQ2/pSGeryCIII (BIOT-2191) was grown initially in TSB and this seed then used to inoculate SSDM medium (4 dm^3; 5% inoculum) in a Applikon 7 dm^3 bioreactor (30 °C; initial pH 6.0–6.4; Rushton turbine agitator, tip speed 0.9–2.7 m s^{-1}; airflow 0.75 L L^{-1} min^{-1}). **13** (100 mg, 0.183 mmol) dissolved in methanol (1 cm^3) was added after 24 h. After a further 46–68 h the fermentation was harvested and clarified by centrifugation. The supernatant was applied to a column (16 × 15 cm) of Diaion® HP20 resin (Supelco), washed with 10% acetone in water (2 × 2 dm^3), and then eluted with acetone (3.5 dm^3). The cells were extracted twice with an equal volume of acetone–methanol (1 : 1). The organic extracts were combined and the solvents removed under reduced pressure. The resulting aqueous fraction was extracted three times with an equal volume of ethyl acetate and the combined organic solvent removed under reduced pressure to leave a crude oily extract. This was dissolved in methanol and chromatographed over base-deactivated Luna C_{18} reversed-phase silica (5 micron particle size) using a Luna HPLC column (250 × 21 mm; Phenomenex (Macclesfield, UK)). A Gilson 315 binary HPLC system was used to deliver the mobile phase at 21 cm^3 min^{-1}. Elution involved a linear gradient of 32.5% B to 63% B was used to initially to partially purify the sample. Fractions containing **20** were combined and finally purified by isocratic elution with 30% B. Mobile phase A was 20 mM ammonium acetate and mobile phase B was CH_3CN.

3,5-Bis-*O*-α-L-mycarosylerythronolide B (20)

Isolated yield, 15.8% (20 mg, 0.029 mmol). δ_H (500 MHz; CD_3OD) 0.88 (dd, *J* 7.5 & 7.3, C(15)Me), 0.92 (d, *J* 7.2, C(12)Me), 0.97 (d, *J* 7.0, C(10)Me), 1.01 (d, *J* 7.7, C(4)Me), 1.12 (d, *J* 7.0, C(8)Me), 1.20 (d, *J* 7.0, C(2)Me), 1.20 (s, C(3″)Me), 1.22 (s, C(3′)Me), 1.23 (d, *J* 6.4, C(6″)Me), 1.29 (d, *J* 6.2, C(6′)Me), 1.38 (dd, *J* 14.9 & 4.1, C(7a)H), 1.45 (s, C(6)Me), 1.49 (dqd, *J* 17.1, 7.5 & 4.5, C(14a)H), 1.64 (dqd, *J* 10.2, 7.2, 1.0, C(12)H), 1.74 (ddq, *J* 17.1, 9.8 & 7.3, C(14b)H), 1.81 (dd, *J* 14.5 & 4.4, C(2″a)H), 1.86 (dd, *J*

14.5 & 4.6, C(2′a)H), 2.00 (dd, *J* 14.9 & 9.0, C(7b)H), 2.05 (dd, *J* 14.5 & 1.1, C(2″b)H), 2.12 (dd, *J* 14.5 & 1.5, C(2′b)H), 2.20 (qdd, *J* 7.7, 7.3 & 1.4, C(4)H), 2.78 (m, C(8)H), 2.93 (dq, *J* 10.2 & 7.0, C(2)H), 2.97 (d, *J* 9.4, C(4′)H), 2.97 (d, *J* 9.8, C(4″)H), 2.99 (qd, *J* 7.0 & 1.5, C(10)H), 3.63 (d, *J* 7.3, C(5)H), 3.92 (dq, *J* 9.8 & 6.4, C(5″)H), 3.94 (dd, *J* 10.2 & 1.5, C(11)H), 3.97 (dd, *J* 10.2 & 1.4, C(3)H), 4.09 (dq, J 9.4 & 6.2, C(5′)H), 4.98 (d, *J* 4.6, C(1′)H), 5.08 (d, *J* 4.4, C(1″)H), 5.38 (ddd, *J* 9.8, 4.5 & 1.0, C(13)H); δ_C (125 MHz; $CDCl_3$) 9.5 (C10-Me), 9.8 (C12-Me), 10.4 (C4-Me), 11.0 (C15), 16.5 (C2-Me), 18.2 (C6″), 18.8 (C8-Me), 19.1 (C6′), 26.5 (C7″), 26.8 (C7′), 27.0 (C14), 27.8 (C6-Me), 39.3 (C7), 39.7 (C4), 41.2 (C10), 41.8 (C2″), 41.8 (C′2), 42.1 (C12), 44.8 (C8), 46.1 (C2), 67.0 (C5″), 67.3 (C5′), 70.5 (C3′), 70.8 (C11), 71.3 (C3″), 74.7 (C6), 76.2 (C13), 77.7 (C4″), 77.9 (C4′), 84.8 (C3), 87.4 (C5), 99.0 (C1), 100.5 (C1″), 177.0 (C1), 220.4 (C9); MS (ES) *m/z* 1403.1 $[2M + Na]^+$, 713.3 $[M + Na]^+$, 672.7 $[M - H_2O + H]^+$, 529 $[M - H_2O -$ mycarose $+ H]^+$.

5-*O*-β-D-Mycaminosyltylactone (12)

The identity of this compound was verified *versus* an authentic standard.[27] λ_{max}(DAD)/nm 281; MS (ES) *m/z* 568.4 $[M + H]^+$, 550.3 $[M - H_2O + H]^+$, 411.4 $[M -$ myc $+ H]^+$, 174.0 [myc − OH]$^+$: (myc, mycaminose).

5-*O*-β-D-Angolosaminyltylactone (14)

Isolated yield, 17.9% (20 mg, 0.036 mmol). This was purified from 1.6 dm^3 of biotransformation broth (80 mg (0.203 mmol) of **11** was used as substrate). λ_{max}(DAD)/nm 281; δ_H (500 MHz; $CDCl_3$) 0.83 (t, *J* 7.2, C(6)CH_2CH_3), 0.91 (d *J* 7.2, C(4)Me), 0.91 (t, *J* 7.2, C(17)H), 1.05 (d, *J* 6.5, C(14)Me), 1.15 (d, *J* 6.8, C(8)Me), 1.30 (d, *J* 6.0, C(6′)H), 1.45 (m, C(7a)H), 1.48 (m, C(2′a)H), 1.55 (m, C(7b)H), 1.55 (m, C(16a)H), 1.55 (m, C(6)$CH_2$$CH_3$), 1.56 (m, C(4)H), 1.76 (s, C(12)Me), 1.82 (m, C(16b)H), 1.91 (d, *J* 16.8, C(2a)H), 2.05 (ddd, *J* 10.4, 3.9 & 1.6, C(2′b)H), 2.46 (dd, *J* 16.8 & 10.5, C(2b)H), 2.48 (s, C(3″)NMe_2), 2.68 (m, C(6)H), 2.70 (m, C(8)H), 2.70 (m, C(14)H), 2.89 (td, *J* 10.4 & 3.9, H(3′)H), 3.16 (dd, *J* 9.6 & 9.0, C(4′)H), 3.26 (dq, *J* 9.6 & 6.0, C(5′)H), 3.68 (dd, *J* 10.5 & 1.2, C(3)H), 3.76 (d, *J* 10.3, C(5)H), 4.41 (d, *J* 8.6, C(1′)H), 4.68 (td, *J* 9.7 & 2.4, C(15)H), 5.60 (d, *J* 10.4, C(13)H), 6.26 (d, *J* 15.5, C(10)H), 7.27 (d, *J* 15.5, C(11)H); δ_C (125 MHz; $CDCl_3$) 9.4 (C17), 9.7 (C4-Me), 11.8 (C6-$CH_2$$CH_3$), 13.0 (C12-Me), 16.1 (C14-Me), 17.1 (C8-Me), 17.7 (C6′), 21.0 (C6-$CH_2$$CH_3$), 24.7 (C16), 28.0 (C2′), 33.6 (C7), 38.3 (C14), 38.7 (C6), 39.4 (C3″-NMe_2), 39.8 (C2), 40.4 (C4), 45.0 (C8), 65.8 (C3′), 66.9 (C3), 70.5 (C4′), 73.2 (C5′), 78.8 (C15), 80.7 (C5), 101.0 (C1′), 118.3 (C10), 133.5 (C12), 145.4 (C13), 157.7 (C11), 174.4 (C1), 203.9 (C9); MS (ES) *m/z* 552.3 $[M + H]^+$, 534.3 $[M - H_2O + H]^+$, 395.4 $[M -$ ang $+ H]^+$, 158.2 [ang − OH]$^+$: (ang, angolosamine).

3-*O*-β-D-Angolosaminylerythronolide B (17)

Isolated yield, 36% (30 mg, 0.054 mmol). This was purified from 1.2 dm^3 of biotransformation broth (60 mg (0.149 mmol) of **11** was used as substrate). δ_H (500 MHz; $CDCl_3$) 0.89 (d, *J* 7.7, C(12)Me), 0.90 (d, *J* 7.7, C(15)H), 0.98 (d, *J* 7.7, C(10)Me), 1.06 (d, *J* 6.7, C(4)Me), 1.16 (d, *J* 6.1, C(8)Me), 1.19 (d, *J* 6.9, C(2)Me), 1.30 (s, C(6)Me), 1.34 (d, *J* 6.0, C(6′)H), 1.44 (dd, *J* 14.6 & 5.4, C(7a)H), 1.49 (m, C(2′a)H), 1.51 (m, C(14a)H), 1.69 (m, C(12)H), 1.71 (qd, *J* 7.2 & 2.2, C(14b)H), 1.92 (dd, *J* 14.6 & 5.4, C(7b)H), 1.99 (m C(4)H), 2.00 (m, C(2′b)H), 2.48 (td, *J* 10.2 & 3.5, C(3′)H), 2.69 (m, C(8)H), 2.81 (dq, *J* 10.5 & 6.7, C(2)H), 2.91 (bq, *J* 6.6, C(10)H), 3.03 (dd, *J* 9.5 & 9.5, C(4′)H), 3.34 (dq, *J* 8.7 & 6.0, C(5′)H), 3.66 (dd, *J* 10.5 & 10.5, C(3)H), 3.69 (bs, C(5)H), 3.78 (d, *J* 10.0, C(11)H), 4.61 (dd, *J* 9.2 & 1.6, C(1′)H), 5.40 (dd, *J* 9.5 & 9.3, C(13)H); δ_C (125 MHz; $CDCl_3$) 8.3 (C4-Me), 8.5 (C10-Me), 9.1 (C15), 10.4 (C12-Me), 15.2 (C2-Me), 16.9 (C8-Me), 17.5 (C6′), 26.6 (C6-Me), 25.8 (C14), 27.0 (C2′), 36.5 (C4), 38.3 (C7), 40.1 (C10), 40.2 (C12), 43.4 (C8), 44.5 (C2), 65.2 (C3′), 70.3 (C4′), 70.6 (C11), 73.9 (C5′), 75.2 (C6), 75.6 (C13), 81.5 (C5), 89.7 (C3), 103.0 (C1′), 176.3 (C1), 217.8 (C9); MS (ES) *m/z* 582.5 $[M + Na]^+$, 560.5 $[M + H]^+$, 425.3 $[M -$ ang $+ Na]^+$, 158.0 [ang − OH]$^+$: (ang, angolosamine).

5-Des-*O*-β-D-desosaminyl-5-*O*-β-D-angolosaminylerythromycin A (18)

MS (ES) *m/z* 734.4 $[M + H]^+$, 716.5 $[M - H_2O + H]^+$, 576.4 $[M -$ cla $+ H]^+$, 558.3 $[M -$ cla $- H_2O + H]^+$, 158.2 [ang − OH]$^+$: (cla, cladinose; ang, angolosamine). Additional peaks for the erythromycin B analogue and other minor components were also observed.

5-Des-*O*-β-D-desosaminyl-5-*O*-β-D-mycaminosylerythromycin A (19)

Isolated yield, 7.1% (15 mg, 0.013 mmol). This was purified from 4 dm^3 of biotransformation broth (100 mg (0.183 mmol) of **13** was used as substrate). δ_H (500 MHz; $CDCl_3$) 0.83 (dd, *J* 7.4 & 7.4, C(15)H), 1.03 (d, *J* 7.4, C(4)Me), 1.12 (s, C(12)Me), 1.14 (d, *J* 7.0, C(10)Me), 1.16 (d, *J* 7.0, C(8)Me), 1.18 (d, *J* 7.1, C(2)Me), 1.23 (s, C(3′)Me), 1.27 (d, *J* 6.2, (C(6′)H), 1.29 (d *J* 6.1, C(6″)H), 1.44 (s, C(6)Me), 1.47 (dqd, *J* 14.3, 11.0 & 7.2, C(14b)), 1.55 (dd, *J* 15.2 & 4.8, C(2′b)H), 1.66 (dd, *J* 14.8 & 2.2, C(7a)H), 1.82 (dd, *J* 14.8 & 11.4, C(7b)H), 1.91 (ddq, *J*, 14.3, 7.5, & 2.2, C(14a)H), 2.00 (m, C(4)H), 2.32 (dd, *J* 15.2 & 0.9, C(2′b)H), 2.48 (dd, *J* 10.4 & 10.3, C(3″)H), 2.58 (s, C(3″)NMe_2), 2.69 (dqd, *J* 11.3, 7.0 & 2.2, C(8)H), 2.83 (dq, *J* 9.7 & 7.1, C(2)H), 3.01 (d, *J* 9.3, C(4′)H), 3.06 (qd, *J* 6.9 & 1.3, C(10)H), 3.09 (dd, *J* 9.9 & 9.0, C(4″)H), 3.29 (s, C3′)OMe), 3.31 (dq, *J* 9.0 & 6.1, C(5″)H), 3.53 (d, *J* 6.8, C(5)H), 3.56 (dd, *J* 10.4 & 7.3, C(2″)H), 3.81 (d, *J* 1.3, C(11)H), 3.91 (dd, *J* 9.7 & 1.6, C(3)H), 3.99 (dq, *J* 9.3 & 6.2, C(5′)H), 4.43 (d, *J* 7.4, C(1″)H), 4.87 (d, *J* 4.8, C(1′)H), 5.04 (dd, *J* 11.0 & 2.3, C(13)H); δ_C (125 MHz; $CDCl_3$) 9.7 (C4-Me), 10.6 (C15), 12.0 (C10-Me), 16.0 (C2-Me), 16.2 (C12-Me), 18.1 (C6″), 18.3 (C8-Me), 18.5 (C6′), 21.1 (C14), 21.4 (C3′-Me), 26.6 (C6-Me), 34.9 (C2′), 38.0 (C10), 38.5 (C7), 39.1 (C4), 41.7 (C3″-NMe_2), 44.9 (C2), 44.9 (C8), 49.4 (C3′-OMe), 65.6 (C5′), 68.9 (C11), 70.2 (C4″), 70.6 (C3″), 71.3 (C2″), 72.8 (C3′), 72.9 (C5″), 74.6 (C12), 74.8 (C6), 76.8 (C13*), 77.8 (C4′), 80.0 (C3), 85.4 (C5), 96.4 (C1′), 103.3 (C1″), 175.4 (C1), 221.6 (C9). (* This carbon was assigned from the HMQC spectrum); MS (ES) *m/z* 750.5 $[M + H]^+$, 732.5 $[M - H_2O + H]^+$, 592.4 $[M -$ cla $+ H]^+$, 574 $[M -$ cla $- H_2O + H]^+$, 174.1 [myc − OH]$^+$: (cla, cladinose; myc, mycaminose); HRMS (ES) *m/z* 750.4654 calcd for $C_{37}H_{68}NO_{14}$ requires 750.4634.

5-*O*-α-L-Rhamnosyltylactone (23)

Isolated yield, 5 mg. λ_{max}(DAD)/nm 281; δ_H (500 MHz; $CDCl_3$) 0.89 (t, *J* 7.5, C(6)CH_2CH_3), 0.91 (d, *J* 6.8, C(4)Me), 0.92 (t, *J* 7.4, C(17)H), 1.05 (d, *J* 6.5, C(14)Me), 1.19 (d, *J* 6.8, C(8)Me), 1.25 (m, C(6)CH_2CH_3), 1.26 (d, *J* 6.0, C(6′)H), 1.53 (m, C(16a)H), 1.55 (m, C(4)H), 1.77 (s, C(12)Me), 1.83 (ddd, *J* 14.3, 7.4 & 2.5, C(16b)H), 1.91 (d, *J* 16.8, C(2a)H), 2.45 (dd, *J* 16.8 & 10.3, C(2b)H), 2.70 (m, C(8)H), 2.70 (m, C(14)H), 3.46 (dd, *J* 9.4 & 9.4, C(4′)H), 3.67 (m C(3)H), 3.68 (dd, *J* 9.4 & 3.1, C(3′)H), 3.74 (m, C(5)H), 3.76 (d, *J* 9.4, C(5′)H), 4.03 (dd, *J* 3.1 & 1.5, C(2′)H), 4.68 (td, *J* 8.9, C(15)H), 4.73 (bs, C(1′)H), 5.61 (d, *J* 10.3, C(13)H), 6.26 (d, 13.7, C(10)H), 7.28 (d, *J* 13.7, C(12)H); δ_C (125 MHz; $CDCl_3$) 9.5 (C17), 9.5 (C4-Me), 12.1 (C6-CH_2CH_3), 12.9 (C12-Me), 16.1 (C14-Me), 17.2 (C8-Me), 17.2 (C6′), 21.9 (C6-CH_2CH_3), 24.7 (C16), 33.8 (C7), 38.4 (C14), 38.7 (C6), 39.8 (C2), 40.8 (C4), 44.9 (C8), 67.0 (C3), 68.6 (C5′), 71.1 (C2′), 71.9 (C3′), 73.1 (C4′), 79.2 (C15), 83.4 (C5), 102.6 (C1′), 118.4 (C10), 136.7 (C12), 145.5 (C13), 148.1 (C11), 174.3 (C1), 204.0 (C9); MS (ES) *m/z* 563.5 $[M + Na]^+$, 545.4 $[M - H_2O + Na]^+$, 579.5 $[M + K]^+$, 561.5 $[M - H_2O + K]^+$.

5-*O*-α-L-Mycarosyltylactone (24)

The identity of this compound was verified *versus* an authentic standard.[27] λ_{max}(DAD)/nm 281; MS (ES) *m/z* 561.5 $[M + Na]^+$, 543.5 $[M - H_2O + Na]^+$.

Acknowledgements

Professor Eric Cundliffe (University of Leicester) is acknowledged for numerous stimulating discussions regarding this work. We thank the staff of the DNA sequencing facility at the Department of Biochemistry, University of Cambridge. Tylactone, 5-*O*-β-D-mycaminosytylactone, 23-hydroxy-5-*O*-β-D-mycaminosyltylactone and 5-*O*-α-L-mycarosyltylactone were kind gifts of the ex-Bioprocess Research Group of GlaxoWellcome (now GSK).[23,27] Erythronolide B and 3-*O*-α-L-mycarosylerythronolide B were kind gifts of Pfizer (Groton, USA). The work was supported in part by a Marie Curie Industry Host Fellowship to Biotica from the European Community (TORINO, QLK2-CT-2001–60023), and a project grant to P.F.L. from the European Community (GENOVA, QLRT-1999–00095).

References

1 F. Schlünzen, R. Zarivach, J. Harms, A. Bashan, A. Tocilj, R. Albrecht, A. Yonath and F. Franceschi, *Nature*, 2001, **413**, 814–821; D. Tu, G. Blaha, P. B. Moore and T. A. Steitz, *Cell*, 2005, **121**, 257–270.
2 C. Méndez and J. A. Salas, *Trends Biotechnol.*, 2001, **11**, 449–456.
3 C. J. Thibodeaux, C. E. Melançon III and H. -W. Liu, *Nature*, 2007, **446**, 1008–1016; S. Blanchard and J. S. Thorson, *Curr. Opin. Chem. Biol.*, 2006, **10**, 263–271.
4 L. Rodriguez, I. Aguirrezabalaga, N. Allende, A. F. Braña, C. Méndez and J. A. Salas, *Chem. Biol.*, 2002, **9**, 721–729.
5 S. Gaisser, J. Reather, G. Wirtz, L. Kellenberger, J. Staunton and P. F. Leadlay, *Mol. Microbiol.*, 2000, **36**, 391–401.
6 S. Gaisser, C. J. Martin, B. Wilkinson, R. M. Sheridan, R. E. Lill, A. J. Weston, S. J. Ready, C. Waldron, G. D. Crouse, P. F. Leadlay and J. Staunton, *Chem. Commun.*, 2002, 618–619.
7 S. Gaisser, R. Lill, J. Staunton, C. Méndez, J. A. Salas and P. F. Leadlay, *Mol. Microbiol.*, 2002, **44**, 771–781.
8 S. Gaisser, R. Lill, G. Wirtz, F. Grolle, J. Staunton and P. F. Leadlay, *Mol. Microbiol.*, 2001, **41**, 1223–1231.
9 E. Cundliffe, *Actinomycetologica*, 1999, **13**, 68–75.
10 K. Salah-Bey, M. Doumith, J. -M. Michel, S. F. Haydock, J. Cortés, P. F. Leadlay and M.-C. Raynal, *Mol. Gen. Genet.*, 1998, **257**, 542–553.
11 L. Zhao, S. Borisova, S.-M. Yeung and H.-W. Liu, *J. Am. Chem. Soc.*, 2001, **123**, 7909–7910; P.-H. Szu, X. He, L. Zhao and H.-W. Liu, *Angew. Chem., Int. Ed.*, 2005, **44**, 6742–6746.
12 S. A. Borisova, L. Zhao, C. E. Melançon III, C. L. Kao and H. -W. Liu, *J. Am. Chem. Soc.*, 2004, **126**, 6534–6535; C. E. Melançon III, H. Takahashi and H.-W. Liu, *J. Am. Chem. Soc.*, 2004, **126**, 16726–16727.
13 S. A. Borisova, C. Zhang, H. Takahashi, H. Zhang, A. W. Wong, J. S. Thorson and H.-W. Liu, *Angew. Chem., Int. Ed.*, 2006, **45**, 2748–2753.
14 C. Leimkuhler, M. Fridman, T. Lupoli, S. Walker, C. T. Walsh and D. Khane, *J. Am. Chem. Soc.*, 2007, **129**, 10546–10550.
15 C. E. Melançon III, W.-I. Yu and H.-W. Liu, *J. Am. Chem. Soc.*, 2005, **127**, 12240–12241.
16 C. E. Melançon III, L. Hong, J. A. White, Y.-N. Liu and H.-W. Liu, *Biochemistry*, 2007, **46**, 577–590.
17 A. Pfoestl, A. Hofinger, P. Kosma and P. Messner, *J. Biol. Chem.*, 2003, **278**, 26410–26417.
18 *PCT Appl.* WO 054265, 2005.
19 R. Corbaz, L. Ettlinger, E. Gäumann, W. Keller-Schierlein, L. Neipp, V. Prelog, P. Reusser and H. Zähner, *Helv. Chim. Acta*, 1955, **38**, 1202–1209; A. Kinumaki and M. Suzuki, *J. Antibiot.*, 1972, **25**, 480–482; R. Okamoto, M. Tsuchiya, H. Nomura, H. Iguchi, K. Kiyoshima, S. Hori and T. Inui, *J. Antibiot.*, 1980, **33**, 1309–1315.
20 S. Gaisser, G. A. Böhm, J. Cortés and P. F. Leadlay, *Mol. Gen. Genet.*, 1997, **256**, 239–251.
21 C. Waldron, P. Matsushima, P. R. Rosteck Jr., M. C. Broughton, J. Turner, K. Madduri, K. P. Crawford, D. J. Merlo and R. H. Baltz, *Chem. Biol.*, 2001, **8**, 487–499.
22 S. F. Haydock, J. F. Aparicio, I. Molnar, T. Schwecke, L. E. Khaw, A. König, A. F. A. Marsden, I. S. Galloway, J. Staunton and P. F. Leadlay, *FEBS Lett.*, 1995, **374**, 246–248; C. D. Reeves, S. Murli, G. W. Ashley, M. Piagentini, C. R. Hutchinson and R. McDaniel, *Biochemistry*, 2001, **40**, 15464–15470; F. Del Vecchio, H. Petkovic, S. G. Kendrew, L. Low, B. Wilkinson, R. Lill, J. Cortes, B. A. M. Rudd, J. Staunton and P. F. Leadlay, *J. Ind. Microbiol. Biotechnol.*, 2003, **30**, 489–494.
23 T. Bililign, C.-G. Hyun, J. S. Williams, A. M. Czisny and J. S. Thorson, *Chem. Biol.*, 2004, **11**, 959–969.
24 K. Ichinose, M. Ozawa, K. Itou, K. Kunieda and Y. Ebizuka, *Microbiology*, 2003, **149**, 1633–1645.
25 PCT., WO007709, 2004;M. A. Gregory, H. Petkovic, R. E. Lill, S. J. Moss, B. Wilkinson, S. Gaisser, P. F. Leadlay and R. M. Sheridan, *Angew. Chem., Int. Ed.*, 2005, **44**, 4757–4760; M. A. Gregory, H. Hong, R. E. Lill, S. Gaisser, H. Petkovic, L. Low, L. S. Sheehan, I. Carletti, S. J. Ready, M. J. Ward, A. L. Kaja, A. J. Weston, I. R. Challis, P. F. Leadlay, C. J. Martin, B. Wilkinson and R. M. Sheridan, *Org. Biomol. Chem.*, 2006, **4**, 3565–3568.
26 L. Hong, Z. Zhao, C. E. Melançon III, H. Zhang and H.–W. Liu, *J. Am. Chem. Soc.*, 2008, **130**, 4954–4967.
27 Authentic tylactone, 5-*O*-β-D-mycaminosyltylactone, 23-hydroxy-5-*O*-β-D-mycaminosyltylactone and 5-*O*-α-L-mycarosyltylactone were obtained following patent procedures: *US Pat.* 4423148, 1983; *US. Pat.* 4440857, 1984.
28 Y. Yuan, H. S. Chung, C. Leimkuhler, C. T. Walsh, D. Kahne and S. Walker, *J. Am. Chem. Soc.*, 2005, **127**, 14128–14129.
29 P. H. Jones, K. S. Iyer and W. E. Grundy, *Antimicrob. Agents Chemother.*, 1969, **9**, 123–130; *US Pat.* 3598805, 1971.
30 M. Doumith, R. Legrand, C. Lang, J. A. Salas and M.-C. Raynal, *Mol. Microbiol.*, 1999, **34**, 1039–1048.
31 *US Pat.* 5,149,638, 1992.
32 J. Velasco, M. A. Hayes, R. H. Hall, B. A. M. Rudd, B. Wilkinson, A. P. Blackaby, M. J. Dawson and J. Cortés, unpublished data (manuscript in preparation).
33 W. S. Jung, S. K. Lee, J. S. J. Hong, S. R. Park, S. J. Jeong, A. R. Han, J. K. Sohng, B. G. Kim, C. Y. Choi, D. H. Sherman and Y. J. Yoon, *Appl. Microbiol. Biotechnol.*, 2006, **72**, 763–769.
34 A. P. Salas, L. Zhu, C. Sánchez, A. F. Braña, J. Rohr, C. Méndez and J. A. Salas, *Mol. Microbiol.*, 2005, **58**, 17–27.

35 F. Lombó, M. Gibson, L. Greenwell, A. F. Braña, J. Rohr, J. A. Salas and C. Méndez, *Chem. Biol.*, 2004, **11**, 1709–1718.
36 A. Minami and T. Eguchi, *J. Am. Chem. Soc.*, 2007, **129**, 5102–5107.
37 F. Flett, V. Mersinias and C. P. Smith, *FEMS Microbiol. Lett.*, 1997, **155**, 223–229.
38 J. Sambrook, E. F. Fritsch and T. Maniatis, *Molecular cloning: a laboratory manual*, Cold Spring Harbor Laboratory Press, New York, 2nd edn, 1989.
39 T. Hashimoto-Gotoh, K. Yasojima and A. Tsujimura, *Gene*, 1995, **167**, 333–334.
40 T. Kieser, M. J. Bibb, M. J. Buttner, K. F. Chater and D. A. Hopwood, *Practical Streptomyces Genetics*, The John Innes Foundation, Norwich, 2000.
41 J. K. Bonfield, K. Smith and R. Staden, *Nucleic Acids Res.*, 1995, **23**, 4992–4999.
42 P. Caffrey, D. J. Bevitt, J. Staunton and P. F. Leadlay, *FEBS Lett.*, 1992, **304**, 225–228.