Glycosylation engineering of spinosyn analogues containing an L-olivose moiety†

Sabine Gaisser,‡[a] Isabelle Carletti,[a] Ursula Schell,[a] Paul R. Graupner,[b] Thomas C. Sparks,[b] Christine J. Martin[a] and Barrie Wilkinson*[a]

Received 6th January 2009, Accepted 5th February 2009
First published as an Advance Article on the web 12th March 2009
DOI: 10.1039/b900233b

Biosynthetic genes encoding proteins involved in the first steps of deoxyhexose biosynthesis from D-glucose-1-phosphate were expressed in *Saccharopolyspora erythraea.* The resulting mutant was able to accumulate and utilise TDP-L-olivose. Co-expression of the spinosyn glycosyl transferase SpnP in the resulting mutant endowed upon it the ability to biotransform exogenously added spinosyn aglycones to yield novel spinosyn analogues.

Introduction

The glycosylation of many natural products is essential for their biological activity. This has led to the development of methods for the manipulation of deoxysugar biosynthesis and the production of new 'non-natural, natural products' through the application of glycosylation engineering.[1] Amongst *in vivo* methods of note are the diversion of native pathways by expressing heterologous biosynthetic and/or glycosyltransferase (GT) genes in a deoxysugar producing organism, and the use of biosynthetic gene cassettes which encode all the genes necessary for activated deoxysugar biosynthesis along with an appropriate GT. The utility of this latter approach in heterologous *Streptomyces* hosts has been elegantly demonstrated by Salas, Méndez and co-workers.[2] Use of *in vitro* glycorandomization has also been described whereby chemo-enzymatic methods provide additional scope for the introduction of chemical diversity. The power of this method was neatly exemplified by Thorson and co-workers who introduced azide functions to activated sugars allowing subsequent efficient modification using 'click' type chemistry.[3] More recently the description of reversible GT function in order to liberate, from natural products, their deoxysugars and replace them with alternatives has been made.[4,5]

We have reported several examples of *in vivo* methods similar to those described above.[6,7] In the course of extending these efforts we examined the effect of expressing various biosynthetic gene cassettes in mutant strains of *Saccharopolyspora erythraea.*[8] The strain *S. erythraea* SGT2 (BIOT-0535) is a triple mutant in which the erythromycin polyketide synthase as well as both GTs required for the transfer of L-mycarose (*eryBV*) and D-desosamine (*eryCII*) moieties during erythromycin biosynthesis have been deleted.[6] It retains however the ability to produce the TDP-activated forms of these two deoxysugars, and has been used as a heterologous expression host to generate altered spinosyn analogs.[7] The spinosyns are a complex of doubly glycosylated polyketide natural products, and spinosyns A **1** and D **2** (Fig. 1) form the main active components of the potent and environmentally favourable insecticide, spinosad (Dow AgroSciences).[9,10] The spinosyns contain L-rhamnose (in per-*O*-methylated form) and D-forosamine moieties linked glycosidically at C9 and C17 respectively. SpnP is the GT responsible for transfer of D-forosamine to C17 of the spinosyn pseudoaglycone (PsA, **3**) during the final step of spinsoyn biosynthesis. When *spnP* was expressed in *S. erythraea* SGT2 (strain BIOT-1488) and PsA **3** was fed to this growing strain, the transfer of L-mycarose (rather than D-desosamine) was observed, yielding

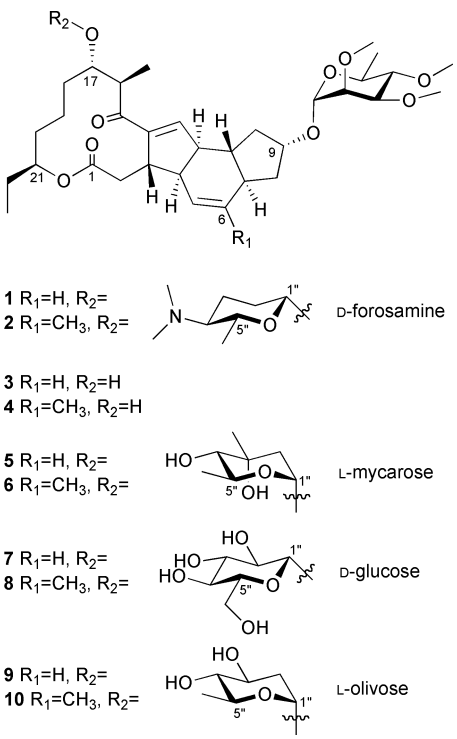

Fig. 1 Structures of naturally occurring and biosynthetically engineered spinosyns.

[a]*Biotica Technology Ltd, Chesterford Research Park, Cambridge, UK CB10 1XL. E-mail: barrie.wilkinson@biotica.com*
[b]*Dow AgroSciences, Indianapolis, IN, 46268, USA*

† Electronic supplementary information (ESI) available: General methods, plasmid construction details, gene sequences and NMR data for product **9**. See DOI: 10.1039/b900233b

‡ Current address: Hochschule Biberach, Karlstraße 11, 88400 Biberach/Riß, Germany

Table 1 Catalytic functions of the deoxyhexose biosynthetic enzymes discussed

Gene product(s)	Function	Homologue in TDP-L-olivose pathway
TylAI	D-glucose-1-phosphate thymidyltransferase	OleS
TylAII	TDP-D-glucose 4,6-dehydratase	OleE
TylX3, EryBVI, SpnO	2,3-dehydratase	OleV
TylCI, EryBII	3-oxo-reductase	OleW
TylCIII, EryBIII	3-*C*-methyltransferase	Not required
TylK, EryBVII	5-epimerase	OleL
TylCII, EryBIV	4-oxo-reductase	OleU
SpnN	3-oxo-reductase	n/a
SpnP	Spinosyn TDP-D-forosamine transferase	n/a

17-des-β-*O*-D-forosamine-17-α-*O*-L-mycarosylspinosyns A **5** and D **6** in good yield.[7] Additionally, compounds derived from the transfer of D-glucose were co-produced (17-des-β-*O*-D-forosamine-17-β-*O*-D-glucosylspinosyns A **7** and D **8**). These compounds are formed by the action of an endogenous host GT, as they were also produced by biotransformation using the untransformed host organism *S. erythraea* SGT2.

Results

In the current study we expressed in *S. erythraea* SGT2 a biosynthetic gene cassette (pSG146_T28) comprising the following genes: *tylAI*, *tylAII*, *spnO*, *spnN* and *spnP* (this was assembled using standard methodologies described by us elsewhere[8]). The functions of the gene products discussed in this paper are described in Table 1. The two tylosin pathway (*tyl*) genes are always present in our gene cassettes to ensure a sustained supply of the common deoxysugar precursor TDP-4-keto-6-deoxy-D-glucose **11** (Fig. 2) from D-glucose-1-phosphate.[8] The gene *spnO* is a homologue of *eryBVI* involved in TDP-L-mycarose biosynthesis in *S. erythraea*, and its gene product SpnO is a 2,3-dehydratase which acts upon **11** to generate TDP-3,4-diketo-2,6-dideoxy-D-glucose (**12**), a common intermediate of both the TDP-L-mycarose, TDP-L-olivose and TDP-D-forosamine pathways.[2,11,12] SpnN functions as a 3-ketoreductase which converts **12** to TDP-4-keto-2,6-dideoxy-D-glucose (**14**) during spinosyn biosynthesis.[12] **14** is the 3-epimeric form of **13**, which is itself an intermediate of the TDP-L-mycrose and TDP-L-olivose pathways (Fig. 2). The effect of expressing the GT encoding gene *spnP* in *S. erythraea* SGT2 is described above (transfer of L-mycarose to the 17-hydroxyl group of PsA).[7]

Introduction of pSG146_T28 into *S. erythraea* SGT2 gave strain BIOT-2134. PsA **3** was added to growing cultures of BIOT-2134 after one day, and after four further days of growth the supernatant was extracted and analyzed by LCMS. In addition to the previously described compounds **5** (~30% of total spinosyn complex) and **7** (~20% of complex), a new product **9** was observed eluting between PsA (**3**) and **5**. This represented ~35% of the spinosyn product complex and its MS spectrum was consistent with the glycosidic attachment of a 2,6-dideoxysugar to C17 of **3** (m/z = 721.5, $[M+H]^+$). A second new (minor) component was also observed which co-eluted with **3** and which represented ~5% of the spinosyn complex (untransformed PsA made up the balance of the spinosyn complex). We were unable to resolve sufficient of the minor component for structure elucidation, but did identify its mass ion (m/z = 737.5, $[M+H]^+$) which is consistent with the attachment of a 6-deoxyhexose such as L-rhamnose; the activated form of TDP-L-rhamnose is known to be available for glycosylation in *S. erythraea* strains.[8] LCMS data indicating the production of spinosyn D analogues including **10** bearing a 2,6-dideoxyhexose (m/z = 735.5, $[M+H]^+$) were obtained when its pseudoaglycone **4** was biotransformed using BIOT-2134.

Biotransformation was then run at a larger scale (120 mg of **3** was fed to 1.2 dm^3 of culture), and after four days this was extracted and the new product **9** was isolated (16 mg) using standard chromatographic techniques. High-resolution ESMS confirmed the molecular formulae of **9** as $C_{39}H_{60}O_{12}$. Analysis of the accumulated NMR data was straightforward when compared to similar compounds and clearly indicated the glycosidic linkage of a 2,6-dideoxysugar to C17 of **3** as shown in Table S2. In comparison to spinosyn A, the anomeric proton (H1″) is shifted

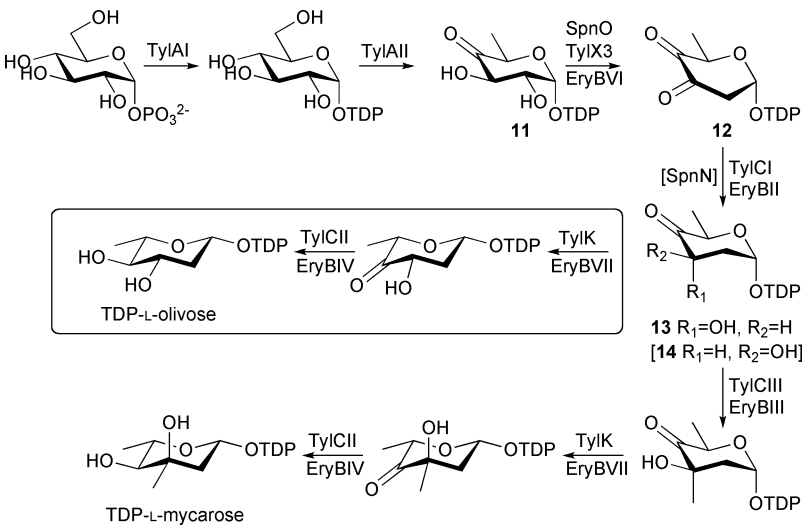

Fig. 2 Proposed biosynthesis of TDP-L-olivose in BIOT-2134 *via* a shunt of the TDP-L-mycarose pathway. Homologues from the erythromycin (ery), tylosin (tyl) and spinosyn pathways (spn) are shown. The putative 3-*epi* product of SpnN (**14**) is presented in brackets.

downfield from 4.5 to 5.0 ppm. In addition, the coupling constants for H1″-H2″ are different from spinosyn A (3.6 & <0.8 Hz *vs.* 9.2 & 2.0 Hz) indicating no axial–axial coupling, whereas the couplings for H3″-H4″ and H4″-H5″ (9.2 & 9.7 Hz respectively) are suggestive of axial couplings. Taken together these data are consistent with the glycosidic linkage of an L-olivose moiety attached in the *alpha* configuration. Subsequent through space (noe) experiments were performed, irradiating at H1″. For *beta* attachment of this sugar a through space correlation should be seen between H1″ and H3″ and H5″, whereas for *alpha* attachment no such interaction should be seen. As anticipated, noes were only observed between H1″ and $H2_{eq}$″ as well as between H1″ and H17 and H24 (see Fig. 3). Additional noes were observed in a noesy experiment which included between H6″ and $H4_{ax}$″, and between $H2_{ax}$″ and $H4_{ax}$″. In conclusion, the combined NMR data are consistent with the structure of **9** being 17-des-*β*-*O*-D-forosamine-17-*α*-*O*-L-olivosylspinosyn A.

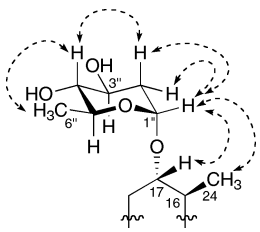

Fig. 3 Key through space (noe) correlations observed for the α-L-olivosyl moiety of **9**.

The biological activity of the L-olivosyl derivative **9** was examined using a topical bioassay against larvae of the beet armyworm, *Spodoptera exigua* (Table 2). In these assays **9** was inactive at 10 μg per larva. Likewise, **9** was only weakly active in a more sensitive injection bioassay (Table 2).

Discussion

The introduction into *S. erythraea* SGT2 of *tylAI*, *tylAII* and *spnO* on pSG146_T28 represents the expression of homologues of native genes and as such has the potential to increase the flux of substrates through the TDP-L-mycarose biosynthetic pathway. As the major new product **9** bears an L-olivose moiety, then SpnN is unlikely to be involved in its biosynthesis as **14**, the product of its function, is epimeric at C3 with respect to L-olivose (the 3-epimeric form of L-olivose is L-digitoxose).[12] Thus, the most likely scenario is that increased metabolic flux allows a shunt of the TDP-L-mycarose pathway to occur, providing significant quantities of TDP-L-olivose *in vivo* which can be utilized by SpnP. Such a shunt pathway was described *in vitro* for TDP-L-mycarose pathway enzymes of the tylosin producer *Streptomyces fradiae*,[13] but can be equally applied to their homologues required for erythromycin biosynthesis in *S. erythraea* (Fig. 2). In the published *in vitro* study, the action of the substrate 'relaxed' 5-epimerase TylK (homologue of EryBVII) upon the accumulated TDP-L-mycarose biosynthesis intermediate **13** is followed by action of the 4-ketoreductase TylCII (EryBIV homologue) to give TDP-L-olivose (Fig. 2).[13] This shunt pathway is analogous to the known TDP-L-olivose pathway required for oleandomycin biosynthesis in *Streptomyces antibioticus* ATCC11891.[11] As we were unable to observe any other 2,6-dideoxyhexose congeners of the novel spinosyn (**9**), we assume that at least one of EryBVII, EryBIV or EryBV are unable to utilize **14**, or any other 3-epimeric intermediates which might arise through the function of SpnN.

Table 2 Insecticidal activity of selected spinosyns and spinosyn derivatives

	Compound	Se inject[a]	Se[b] LC_{50} μg/larva	Tn[b] LC_{50} μg/larva	Tu[b] LC_{50} ppm
1	spinosyn A	100%	0.43	0.03	5[c]
2	spinosyn D	100%	–	–	11[c]
3	C17-PSA spinosyn A	0%	–	–	100[c]
5	L-mycarose spinosyn A	–	1	0.43	50
6	L-mycarose spinosyn D	–	1	0.74	50
7	D-glucose spinosyn A	–	1	4	50
9	L-olivose spinosyn A	17%	10	–	–

[a] Se inject – *Spodoptera exigua* injection assay, % mortality at 10 μg/larva. [b] Se–*Spodoptera exigua*, Tn–*Trichoplusia ni*, Tu–*Tetranychus urticae.* [c] Data adapted from reference 10.

The ability of SpnP and other GTs to accept L-configured sugars in contrast to their natural D-configured substrates appears quite common, and it has been hypothesized that these L-sugars adopt the higher energy $^4C_1$ conformation favored by TDP-D-sugars in order to be recognized by these GTs.[7,8] It was additionally shown that among the related 21-butenylspinosyns produced by *Saccharopolyspora pogona*, analogues with L-deoxysugars attached in the *alpha* configuration are naturally produced alongside those with D-forosamine residues with *beta* attachment at C17, providing additional evidence of the flexibility of SpnP like enzymes.[14]

We assessed the biological activity of **9**, along with the previously reported[7] spinosyn analogues **5**–**7**. Compared to spinosyn A (**1**), all of the D-forosamine replacement analogs (**5**–**7**,**9**) were much less active. The most active analog in this series was the L-mycarose derivative **5**, which exhibited a modest level of insecticidal activity against the cabbage looper (Tn); 14-fold less active than spinosyn A (**1**) (Table 2). While these replacements for D-forosamine provided no improvement in insecticidal activity, alteration or replacement of the other sugar at C9 (L-rhamnose) has been shown to provide substantial improvements in the biological activity of the spinosyns.[10,15,16]

In summary, we have shown that diverting intermediates of TDP-L-mycarose biosynthesis through the over-expression of sugar biosynthesis genes allows the production of TDP-L-olivose in *S. erythraea* SGT2. TDP-L-olivose then acts as a substrate of the spinosyn GT SpnP, albeit in competition with TDP-L-mycarose. This allows (co)biosynthesis of the new compound 17-des-*β*-*O*-D-forosamine-17-*α*-*O*-L-olivosylspinosyn A (**9**) which was purified, structurally characterized and its biological activity assessed in insecticidal assays. These results further exemplify the potential of glycosylation engineering as a robust method for the structural diversification of complex and valuable natural products.

Experimental section

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 positive ion mode. UV analysis was performed at 210, 245 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 ^{1}H and ^{13}C respectively. Standard Bruker pulse programs were used. Coupling constants are given in Hertz. NMR experiments were referenced to the residual resonance of the solvent.

Biotransformation, isolation and analytical procedures

BIOT-2134 was grown in SSDM media as described previously.[6–8] Fermentation broths were adjusted to pH~10 with 20% ammonia and extracted with an equal volume of ethyl acetate. Analytical samples were extracted once, the solvent removed under reduced pressure, and then dissolved in methanol prior to analysis by LCMS as described previously.[8,17] The production run (1.2 dm^3) was extracted twice, and the solvent removed under reduced pressure. The aqueous residue was extracted twice with a half-volume of ethyl acetate, and the combined solvents removed. The residue (~100 mg) was dissolved into the minimum amount of 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 30–70% acetonitrile in water, eluting at 21 cm^3 min^{-1}. **9** eluted at ~24 min, and was isolated as an amorphous, off white solid (15 mg). λ_{max} (DAD) 245 nm; LCMS (ES) m/z 721.5 $[M+H]^+$; HRMS (ES) m/z 738.4426 $[M+NH_4]^+$ (Δ = 0.4 ppm), 743.3982 $[M+Na]^+$ (Δ = 0.7 ppm); NMR data is presented in Table S2.† The purity of this compound was verified to be >95% by HPLC analysis observing the UV absorbance at three wavelengths (210, 254 and 280 nm), the MS response across the range m/z 100–1500 in both positive and negative modes, and by inspection of the ^{1}H and ^{13}C NMR spectra.

Plasmid and strain construction

Construction of plasmid pSG146_T28 was achieved using a variation of the gene cassette assembly method described previously,[8] and is described in detail in the ESI.† Protoplast transformation of *S. erythraea* SGT2 with pSG146_T28 to generate strain BIOT-2134 was achieved as described previously.[17]

Insect control assays

The spinosyn derivatives were screened against several insects. Whole plant assays with the two-spotted spider mites (*Tetranychus urticae*) were performed as described previously.[15] Topical bioassays using beet armyworm (*Spodoptera exigua*) and cabbage looper larvae (*Trichoplusia ni*) were conducted using second instar larvae placed in a 6-well microtiter plate containing a cube of artificial diet. Larvae were topically treated with 1 μL of the test compound (dissolved in acetone) at selected dosages. Controls received acetone only. There were six larvae per treatment, each treatment was run twice. Larvae were held at 27 °C in constant light. Mortality was determined at 4–5 days post treatment. The lethal concentration for 50% of the larvae (LC_{50}) was determined using probit analysis.[18] Injection bioassays were conducted using fourth instar *S. exigua* larvae injected (0.5 μL/larva) with selected dosages of the compound (dissolved in acetone) along the side of the abdomen using a 10 μL Hamilton syringe (SN1701). Treated larvae were held individually (one larva/well) in a 6-well microtiter plate containing a cube of artificial diet. There were six larvae per treatment, each treatment was run twice. Larvae were examined for symptoms of intoxication at selected intervals after treatment.

Acknowledgements

The work was supported in part by a Marie Curie Industry Host Fellowship to Biotica from the European Community (TORINO, QLK2-CT-2001–60023). We thank the staff of the DNA sequencing facility at the Department of Biochemistry, University of Cambridge, Dr. Laura Karr and D'Lee McCormick (Dow AgroSciences) for providing some of the bioassay data, and Dr Clive Waldron for helpful discussion of the manuscript.

References

1 C. J. Thibodeaux, C. E. Melançon and H.-W. Liu, *Nature*, 2007, **446**, 1008–1016.
2 L. Rodríguez, I. Aguirrezabalaga, N. Allende, A. F. Braña, C. Méndez and J. A. Salas, *Chem. Biol.*, 2002, **9**, 721–729.
3 X. Fu, C. Albermann, J. Jiang, J. Liao, C. Zhang and J. S. Thorson, *Nat. Biotechnol.*, 2003, **21**, 1467–1469.
4 A. Minami, K. Kakimuma and T. Eguchi, *Tetrahedron Lett.*, 2005, **37**, 6187–6190.
5 C. Zhang, B. R. Griffith, Q. Fu, C. Albermann, X. Fu, I. K. Lee, L. Li and J. S. Thorson, *Science*, 2006, **313**, 1291–1294.
6 S. Gaisser, J. Reather, G. Wirtz, L. Kellenberger, J. Staunton and P. F. Leadlay, *Mol. Microbiol.*, 2000, **36**, 391–401.
7 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.
8 U. Schell, S. F. Haydock, A. L. Kaja, I. Carletti, R. E. Lill, E. Read, L. S. Sheehan, L. Low, M.-J. Fernandez, F. Grolle, H. A. I. McArthur, R. M. Sheridan, P. F. Leadlay, B. Wilkinson and S. Gaisser, *Org. Biomol. Chem.*, 2008, **6**, 3315–3327.
9 G. D. Thompson, R. Dutton and T. C. Sparks, *Pest Manag. Sci.*, 2000, **56**, 696–702.
10 V. L. Salgado, and T. C. Sparks, in *Comprehensive Insect Molecular Science*, ed. L. I. Gilbert, K. Iatrou, and S. Gill, Elsevier, 2005, vol. 6. *Control*, pp. 137–173.
11 I. Aguirrezabalaga, C. Olano, N. Allende, L. Rodriguez, A. F. Braña, C. Méndez and J. A. Salas, *Antimicrob. Agents Chemother*, 2000, **44**, 1266–1275.
12 L. Hong, Z. Zhao, C. E. Melançon, H. Zhang and H.-W. Liu, *J. Am. Chem. Soc.*, 2008, **130**, 4954–4967.
13 H. Takahashi, Y. N. Liu, H. Chen and H.-W. Liu, *J. Am. Chem. Soc.*, 2005, **127**, 9340–9341.
14 PCT., WO019840, 2001.
15 G. D. Crouse, T. C. Sparks, J. Schoonover, J. Gifford, J. Dripps, T. Bruce, L. L. Larson, J. Garlich, C. Hatton, R. L. Hill, T. V. Worden and J. G. Martynow, *Pest Manag. Sci.*, 2001, **57**, 177–185.
16 P. B. Anzeveno, and F. R. Green, in *Synthesis and Chemistry of Agrochemicals VI*, ed. D. R. Baker, J. G. Fenyes, G. P. Lahm, T. P. Selby, and T. M. Stevenson, American Chemical Society, Washington D.C., 2002, pp. 262–276.
17 S. Gaisser, G. A. Böhm, J. Cortés and P. F. Leadlay, *Mol. Gen. Genet.*, 1997, **256**, 239–251.
18 D. J. Finney, *Probit Analysis*, 3rd Ed, Cambridge University Press, New York, NY, USA, p. 333, 1971.

