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Engineered biosynthesis of novel spinosyns bearing altered deoxyhexose substituents†

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Novel spinosyns have been prepared by biotransformation, using a genetically engineered strain of *Saccharopolyspora erythraea*, in which the β-D-forosamine moiety in glycosidic linkage to the hydroxy group at C17 is replaced by α-L-mycarose.

Spinosyns A **1** and D **2** (Fig. 1) are tetracyclic macrolide polyketides produced by *Saccharopolyspora spinosa*. They only differ in that **2** bears an additional methyl group at C6 and together they constitute the main active compounds of the potent agricultural insecticide Tracer™ (Dow AgroSciences) which displays a highly favourable environmental and mammalian toxicity profile.[1] Spinosyns contain tri-*O*-methylated L-rhamnose and D-forosamine moieties attached in glycosidic linkage to C9 and C17 respectively.[1] The selective removal of either of these deoxyhexose moieties results in almost complete loss of biological activity and *O*-demethylated compounds are also generally less active than **1** and **2**.[2,3]

The spinosyn biosynthetic gene cluster has been cloned from *S. spinosa* and sequenced, and the results have been used to formulate a proposed biosynthetic pathway.[4] Biosynthesis appears to involve the formation of a decaketide followed by intra-molecular cyclisation in an enzyme-mediated process resembling a Diels–Alder cycloaddition. The resulting tetracyclic aglycone is then modified by the addition of L-rhamnose to C9 with subsequent per-methylation of this moiety to yield spinosyn pseudoaglycones **3** and **4**.[4] The final biosynthetic step involves transfer of the forosamine moiety (from dTDP-D-forosamine) to the C17 position to yield the biologically active compounds **1** and **2**. The evident requirement of this glycosylation for biological activity prompted us to examine whether spinosyns bearing alternative glycosyl substituents at this position might be accessible by appropriate engineering of the biosynthetic pathway.[5]

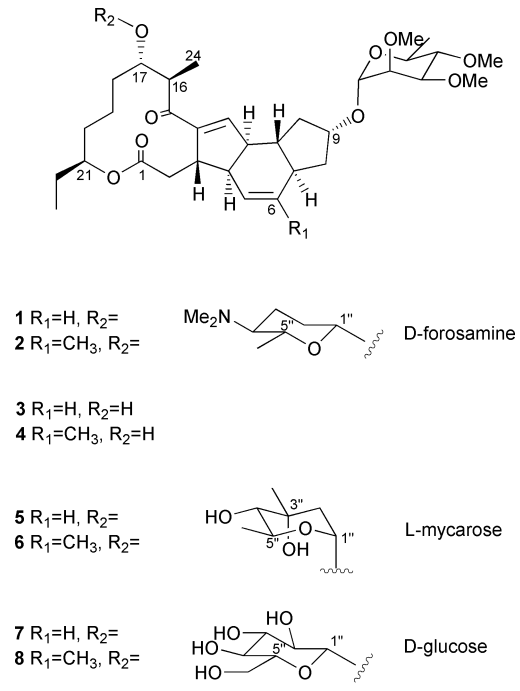

Fig. 1 Chemical structures of natural and 'non-natural' spinosyns.

We have recently described the construction of a defined biotransformation system using mutant strains of the erythromycin producer *Saccharopolyspora erythraea*.[6,7] Strain SGT2 has been genetically modified to remove the erythromycin polyketide synthase and both of the glycosyltransferase (GT) genes that govern addition of the L-mycarose and D-desosamine moieties to 6-deoxyerythronolide B. However, the strain remains capable of synthesising their activated deoxyhexose substrates. The transformation of *S. erythraea* SGT2 with plasmid vectors capable of (over)expressing exogenous GTs allows these GTs to be rapidly screened for their ability to attach activated deoxysugars to spinosyn aglycone substrates .[6,7] We describe (*vide infra*) the use of this approach to examine *spnP*, the gene that encodes the forosaminyl transferase involved in the final step of spinosyn biosynthesis.[4]

The *S. erythraea* strain SGT2pSGSpnP was created, which expresses *spnP*.[8] To assess whether the SpnP glycosyltransferase accepts activated deoxyhexoses present in the host strain as substrates, exogenous pseudoaglycones were added to growing cultures of *S. erythraea* SGT2pSGSpnP using procedures described previously.[6,7] When **4** was fed to cultures of *S. erythraea* SGT2pSGSpnP, and the fermentation broth extracted after an incubation time of 72 h, HPLC analysis indicated almost complete bioconversion of **4** to a major new spinosyn-like component that was not present in extracts from a control strain lacking *spnP*. Further LCMS analysis revealed that the parental mass was shifted from that of **4** by +144 mass units, as expected if L-mycarose were attached in glycosidic linkage to C17 of **4**. We also observed the production of a further spinosyn-like compound more polar than **4**. This was also detected in the control strain and therefore produced by a GT activity endogenous to *S. erythraea* SGT2.

To isolate enough of these materials for bioassay and for structural studies the experiment was repeated on a larger scale using a crude mixture of **3** and **4** (6.5 : 1 respectively; 170 mg total) as substrate. Extraction and analysis of the fermentation broth indicated that > 50% of substrate had been converted to a mixture of four novel spinosyn-like products **5**–**8**. The compo-

† Electronic supplementary information (ESI) available: ^{1}H and ^{13}C NMR data for compounds **5**–**8**. See http://www.rsc.org/suppdata/cc/b2/b200536k/

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nents were isolated by preparative scale reversed-phase chromatography and subjected to detailed spectroscopic analysis. Analysis of **5** (26 mg: isolated yield) and **6** (3.3 mg) by FTICR-MS/MS gave results consistent with the glycosidic addition of a $C_7H_{13}O_3$ moiety to C17 of **3** and **4** respectively. NMR experiments (COSY, HMBC, HMQC, NOE) allowed assignment of the pseudoaglycone moiety of these molecules, and clearly identified a mycarose spin system with appropriate connectivity, in particular a strong HMBC correlation which confirmed the C17 to C1″ linkage. The expected coupling constants were observed for an α-L-mycarosyl glycosidic linkage, and in addition NOE enhancements were observed for H1″ to H2a″, H2b″, H17 and H24, but significantly not for H1″ to H5″. Therefore, compounds **5** and **6** are 17-*O*-α-L-mycarosyl spinosyns A and D respectively.

Since SpnP normally accepts D-forosamine, and attaches it to the spinosyn nucleus in β-glycosidic linkage, we expected that D-desosamine would be the preferred deoxysugar substrate in the recombinant host strain. The addition of L-mycarose, a neutral L-deoxyhexose, to C17 of pseudoaglycones to give an α-glycosidic link was therefore surprising, but can be explained if SpnP treats the β-*O*-dTDP-L-mycarose **9** substrate as if it is a D-sugar. To do this **9** would be flipped to its higher-energy conformer **10**, which has the *O*-dTDP group in the axial position (Fig. 2). SpnP would then attach this equatorially to the pseudoaglycone to give **11** or **12**. The mycarose moiety of **11** or **12** then flips back to the more stable conformer to give **5** or **6**. Intriguingly, a recent patent application discloses spinosyns from a strain of *Saccharopolyspora pogona* whose structures reveal that both deoxyhexose and deoxyaminohexose moieties may be linked at C-17.[9] Our results make it likely that in *S. pogona* both types of substituent are introduced by the same glycosyltransferase. We also examined a number of other GTs, exogenous to *S. erythraea* SGT2 but capable of accepting either activated D-desosamine or L-mycarose as substrates (data not given); however, none were capable of glycosylating pseudoaglycones. The polar compounds **7** (15 mg) and **8** (0.2 mg) were analysed initially by FTICR-MS/MS, which indicated the glycosidic attachment of a $C_6H_{11}O_5$ moiety to C17 of **3** and **4** respectively. The presence of a pseudoaglycone core in these molecules was verified by NMR, and the data also clearly identified a glucose spin system with appropriate connectivity. Again, a strong HMBC correlation confirmed the C17 to C1″ linkage. Analysis of coupling constants together with NOE enhancements for H1″ to H3″, H5″, and H24 are consistent with the presence of a D-glucosyl moiety in β-glycosidic linkage as shown. Thus, compounds **7** and **8** are 17-*O*-β-D-glucosyl spinosyns A and D respectively. This confirms the presence in *S. erythraea* of a glucosyltransferase with broad substrate specificity towards the aglycone substrate.[6]

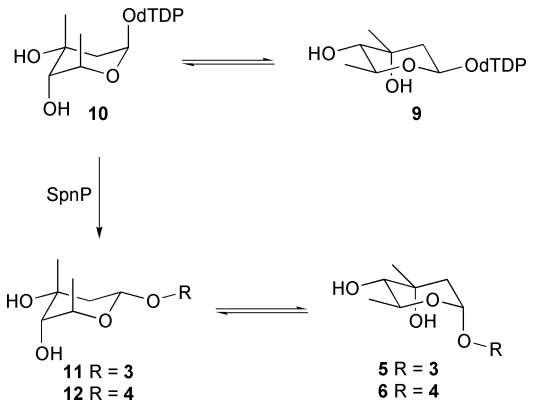

Fig. 2 Proposal for the recognition of an L-deoxyhexose, and its subsequent glycosidic attachment to C9 of **3** and **4**, by SpnP.

In summary, we have demonstrated that additional structural diversity can be readily introduced into agriculturally important polyketides by targeted glycosylation using a cloned glycosyltransferase and activated deoxysugars accumulated in a heterologous host strain. This in turn suggests that a systematic combinatorial approach using multiple sugar pathways and GTs could rapidly give rise to a library of spinosyn analogues.

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Notes and references

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