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Combining sugar biosynthesis genes for the generation of L- and D-amicetose and formation of two novel antitumor tetracenomycins†

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L- and D-stereoisomers of amicetose were generated by combining sugar biosynthesis genes from four different antibiotic gene clusters and both sugars were transferred to the elloramycin aglycone by the sugar flexible ElmGT glycosyltransferase.

Many natural products are glycosylated compounds. These saccharide moieties are essential for biological activity. Sugar transfer to the aglycone is one of the late steps during biosynthesis. The sugar domains of natural product drugs are mainly 6-deoxyhexoses (6DOH) and more than 80 different 6DOH have been described. Biosynthesis of 6DOH takes place through the common intermediate NDP-4-keto-6-deoxy-D-glucose (NDP stands for nucleosyldiphosphate), which suffers further modifications (deoxygenations, epimerizations, methylations, transaminations, *etc.*) resulting in a great variety of DOH structures.[1–3]

Remarkable efforts have been carried out in the last years for altering the glycosylation pattern of bioactive compounds in order to generate novel glycosylated derivatives. This has been facilitated by the "substrate flexibility" shown by some glycosyltransferases involved in the biosynthesis of different antibiotics by actinomycetes.[4–7] However, the extension of these studies to a large number of DOH is limited by the absence of the selected NDP-sugar in the host strain. Therefore, designing genetic or biochemical systems to generate specific NDP-sugars within the organism harboring the glycosyltransferase to be tested is a challenge for combinatorial biosynthesis. Some advances have been recently achieved in *in vitro* studies through glycorandomization[8] and by enzymatic assays of some glycosyltransferases.[9] However, a complete *in vivo* natural "assembly line" for 6DOH biosynthesis is desirable. This relies on a deeper understanding of 6DOH biosynthetic pathways and the use of combinatorial biosynthesis using 6DOH genes to generate *à la Carte* NDP-activated 6DOH that could serve as substrates for "donor-flexible" glycosyltransferases.

L- and D-amicetose are two deoxysugars for which no biosynthetic pathway has been identified so far. Here we report the use in combinatorial biosynthesis of 6DOH biosynthetic genes from four different antibiotic-producing organisms in constructing plasmids directing the biosynthesis of these two stereoisomers of amicetose. We also show that the glycosyltransferase ElmGT from the elloramycin gene cluster is unselective toward nucleotide-activated D- and L-amicetoses which results in the generation of two novel glycosylated tetracenomycin derivatives.

L-Amicetose is a 2,3,6-trideoxyhexose (2,3,6DOH), which is present in amicenomycin antibiotics and differs from the more common 2,3,6DOH L-rhodinose in the stereochemistry at C-4. To generate a plasmid directing L-amicetose biosynthesis, we used as starting construct pLN2 (Fig. 1), which has been shown to direct the biosynthesis of L-olivose.[6] Since L-olivose only differs from L-amicetose by the presence of a hydroxyl group at C-3, we anticipated that the introduction of a gene coding for a 3-dehydratase in pLN2 could render L-amicetose. We therefore incorporated the *urdQ* gene from the urdamycin biosynthetic cluster[10] and the construct, pFL842 (Fig. 1), was introduced into *Streptomyces lividans* 16F4. This is a strain harboring cos16F4, which contains part of the elloramycin biosynthetic gene cluster from *Streptomyces olivaceus* Tü2353. Its expression in streptomycete hosts generates 8-demethyl-tetracenomycin C[5] (Fig. 2). This cosmid also contains a gene for the sugar flexible glycosyltransferase ElmGT[5]. Upon incubation of *S. lividans* 16F4 (pFL842) and analysis of cultures by HPLC-MS, formation of L-amicetosyl-tetracenomycin C was not observed but only L-olivosyl-tetracenomycin C was detected. A possible explanation could be that the OleU 4-ketoreductase in pFL842, which usually acts on a 2,6-dideoxyhexose (2,6DOH), was unable to reduce a 2,3,6-trideoxy intermediate. Consequently, we constructed another plasmid carrying a different 4-ketoreductase. We incorporated the *urdQ* gene into pLNBIV,[6] (Fig. 1) in which *oleU* has been substituted by *eryBIV* from the erythromycin pathway.[11] This reductase has been shown to be quite flexible in recognizing different 6DOH biosynthetic intermediates (axial or equatorial C-3

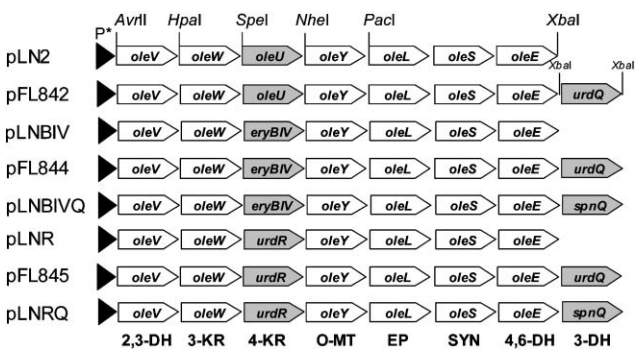

Fig. 1 Sugar plasmids showing the different gene cassettes: P*, *ermE* promoter; DH, dehydratase; KR, ketoreductase; MT, methyltransferase; EP, 3,5-epimerase; SYN, synthase.

† Electronic supplementary information (ESI) available: Experimental details, NMR data of compounds. See http://www.rsc.org/suppdata/cc/b4/b417815g/

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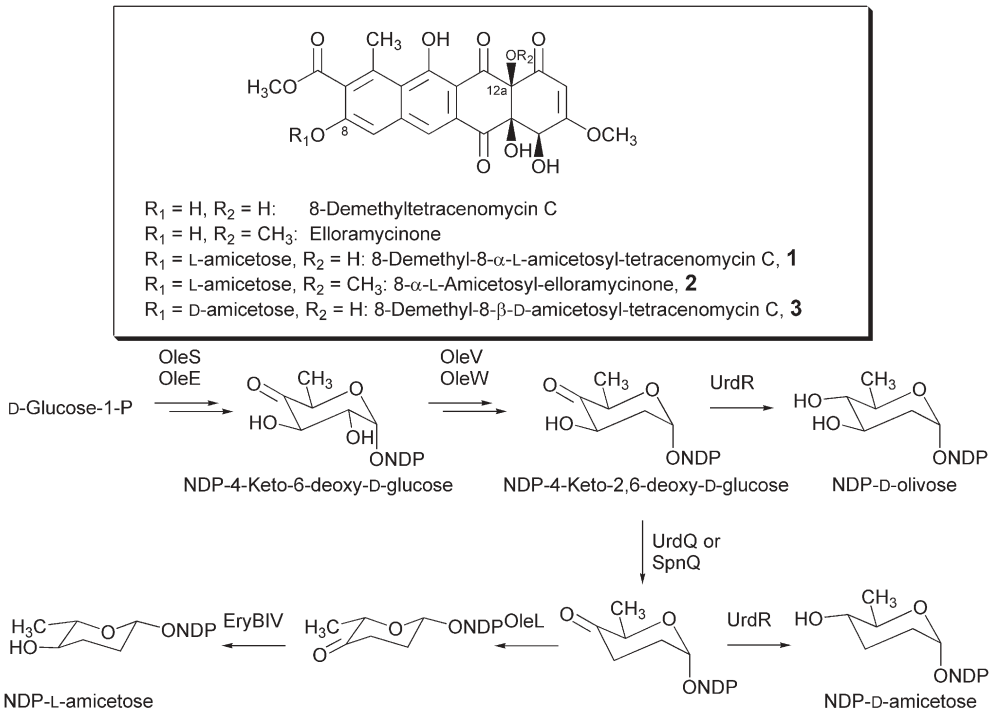

Fig. 2 Chemical structures of novel tetracenomycins and proposed pathways for the biosynthesis of NDP-deoxysugars.

hydroxyl groups).[6] Upon introduction of this construct pFL844 (Fig. 1) into *S. lividans* 16F4, formation of two novel compounds were detected by HPLC-MS, with elution times of 18.1 min and 20.2 min, and representing approximately 36.6% and 18.9% of all tetracenomycins, respectively. The major compound **1** showed an *m*/*z* value in positive mode of 573 (and a fragment *m*/*z* 459 corresponding to the aglycon 8-demethyl-tetracenomycin C). After purification (7 mg) and structural elucidation by NMR, its structure was confirmed to be the novel compound L-amicetosyl-tetracenomycin C (Fig. 2) (see ESI†). The second minor compound **2** showed an *m*/*z* value in positive mode of 587 (and its fragment *m*/*z* 473 corresponding to the aglycon elloramycinone), which is in accordance with the presence of an extra methyl group in the aglycone and probably corresponds to L-amicetosyl-elloramycinone (Fig. 2).

D-Amicetose is another 2,3,6DOH present in amicetin and polyketomycin that only differs from D-olivose by the absence of a C-3 hydroxyl group. In order to construct a plasmid directing the biosynthesis of D-amicetose, we cloned the *urdQ* 3-dehydratase into pLNR (Fig. 1), which has been shown to direct the biosynthesis of D-olivose.[6] After introducing the resultant construct pFL845 (Fig. 1) into *S. lividans* 16F4, the formation of a novel compound eluting at 18.7 min (representing about 30.3% of all tetracenomycins) was detected with an *m*/*z* value in positive mode of 573 (with a fragmentation ion of *m*/*z* 459 corresponding to 8-demethyl-tetracenomycin C). 10 mg of this compound were purified and NMR analysis demonstrated it was actually the novel compound D-amicetosyl-tetracenomycin C **3** (Fig. 2) (see ESI†).

D- and L-amicetosyl derivatives were also generated when *urdQ* was replaced by *spnQ* in pFL845 (pLNRQ) and in pFL844 (pLNBIVQ) (Fig. 1), respectively. The *spnQ* from the spinosad biosynthetic gene cluster in *Saccharopolyspora spinosa*[12] has been proposed to code for a 3-dehydratase involved in the biosynthesis of the 2,3,4,6-tetradeoxy-4-aminosugar D-forosamine. These amicetosyl derivatives showed the same retention times and *m*/*z* values as **1** and **3**, and represented about 25% and 12.6%, respectively.

In summary, we have shown that combinatorial biosynthesis of sugar biosynthesis genes can be efficiently used to endow a streptomycete host with the capability of synthesizing the L- and D-stereoisomers of amicetose. For constructing the plasmids shown above, we combined genes involved in the biosynthesis of four 6DOH from different antibiotic-producing streptomycetes: L-olivose and D-olivose (2,6DOH), L-mycarose (2,6-dideoxy-3-methyl-L-hexose), L-rhodinose (2,3,6DOH) and D-forosamine (2,3,6-trideoxy-4-amino-D-hexose). Formation of the two amicetosyl derivatives of tetracenomycin C also provides further information on 6DOH biosynthesis (Fig. 2). We have shown that the EryBIV and UrdR 4-ketoreductases are able to act on trideoxyhexose intermediates in addition to their respective normal dideoxyhexose substrates, thus showing some substrate flexibility. In addition, the fact that both the L- and D-amicetosyl derivatives were produced either using the 3-dehydratases SpnQ (from a D-sugar pathway) or UrdQ (from an L-sugar pathway) suggests that in both cases the 3-dehydration event occurs on the same intermediate, before C-5 epimerization. Formation of the two novel compounds **1** and **3** extend the sugar substrate flexibility of the ElmGT glycosyltransferase, which has been previously shown to incorporate D-glucose, 6DOH (L-rhamnose), 2,6DOH (L-olivose, L-digitoxose, D-olivose, D-mycarose), and 2,3,6DOH (L-rhodinose).

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