# Characterisation of a sugar epimerase enzyme involved in the biosynthesis of a vancomycin-group antibiotic

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**An enzyme involved in the biosynthesis of the 4-*epi*-vancosamine substituents of a vancomycin group antibiotic has been expressed and its role as a TDP-4-keto-6-deoxyglucose-3,5-epimerase demonstrated.**

The glycopeptide antibiotics vancomycin (**1**, Fig. 1) and teicoplanin are currently the drugs of choice for treatment of infections due to methicillin-resistant *Staphylococcus aureus*.[1] The continued rise of vancomycin-resistant bacteria has heightened the need for new therapeutic agents. Manipulation of the biosynthetic gene cluster for the glycopeptide chloroeremomycin (**2**, Fig. 1) may be a potential route to new antibiotics.[2–5] This approach will require further understanding of the biosynthetic pathway.

Chloroeremomycin consists of a crosslinked heptapeptide backbone, adorned with the unusual deoxy-sugar 4-*epi*-vancosamine (4-*e*-V) on residue 6, and the disaccharide glucosyl-4-*e*-V on residue 4. These substituents enhance antibacterial efficacy by promoting antibiotic dimerisation, which is important in the mode of action of this group of compounds.[6,7] Analysis of sequence data from the chloroeremomycin gene cluster revealed five putative proteins, ORFs 23–26 and ORF14, that had significant homologies (30–70% identity) with sugar biosynthesis enzymes in other antibiotic gene clusters, in particular those for erythromycin and daunomycin.[2,8–11] Given the ubiquitous deoxy-sugar precursor TDP-4-keto-6-deoxyglucose, homologies to enzymes identified in the biosynthetic pathways for daunosamine and mycarose allowed a probable route to 4-*e*-V to be deduced (Fig. 2).[2] This paper describes the expression of EvsA (previously referred to as ORF26) and demonstration of its role as the TDP-4-keto-6-deoxyglucose-3,5-epimerase in the 4-*e*-V biosynthetic pathway.

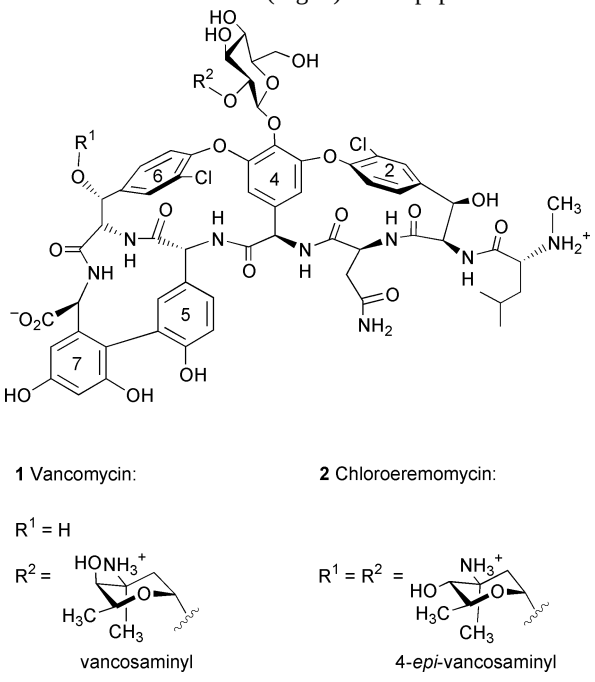


**Fig. 1** Structures of vancomcyin (**1**) and chloroeremomcyin (**2**).

EvsA consists of 205 amino acids and shows strong homology (~60% identity) with putative sugar 3,5-epimerase enzymes from the gene clusters of several antibiotics containing L-sugars, including those involved in the daunosamine pathway of *S. peucetius* (DnmU) and the mycarose pathway of *S. erythraea* (EryBVII).[8–11] It is also closely related to the enzyme RmlC from the rhamnose pathway of *E. coli*, *M. tuberculosis* and *S. enterica*, which has been shown to be a TDP-4-keto-6-deoxyglucose-3,5-epimerase.[12,13] The crystal structure of RmlC from *S. enterica* has been determined and the potential active site located, but as yet the residues responsible for catalysis have not been identified.[14] All the highly conserved residues present in this region are also present in EvsA. Although epimerisation at C-3 is not required in the biosynthesis of 4-*e*-V, it seems plausible that all these enzymes have evolved from a common ancestor capable of catalysing epimerisation at both positions 3 and 5, and thus EvsA is suggested to be the first enzyme in the 4-*e*-V pathway.

In order to confirm the proposed role of EvsA, the *evs*A gene was amplified by polymerase chain reaction (PCR) and cloned into the expression vector pET28a(+) (Novagen). The resulting plasmid was used to transform *E. coli* BL21(DE3) and the cells grown at 37 °C in LB medium with induction by isopropyl β-D-thiogalactoside (IPTG, 1 mM) to produce *N*-terminal $His_6$-tagged EvsA. The enzyme was purified using Novagen His-Bind Quick 900 cartridges and transferred to 50 mM HEPES, pH 7.6 using Millipore centrifugal filters. The relative molecular mass of the purified protein (which gave rise to a single band on an SDS-PAGE gel) was found to be 24.52 kDa (using ESI-MS) which was in excellent agreement with that calculated from the protein sequence (24.521 kDa).

The proposed substrate for EvsA, TDP-4-keto-6-deoxyglucose, was prepared enzymatically from TDP-glucose using RmlB (also known as RfbB), the TDP-glucose-4,6-dehydratase

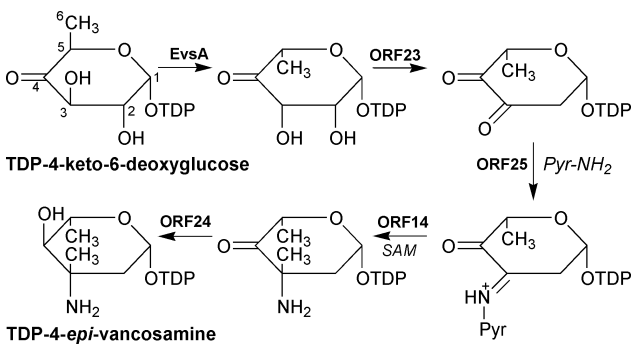


**Fig. 2** Proposed route for the biosynthesis of TDP-4-*epi*-vancosamine from TDP-4-keto-6-deoxyglucose. Putative enzymes involved in each step are indicated. Cofactors thought to be required are shown in italics. Abbreviations: Pyr-$NH_2$, pyridoxamine-5′-phosphate; SAM, S-adenosyl-methionine.

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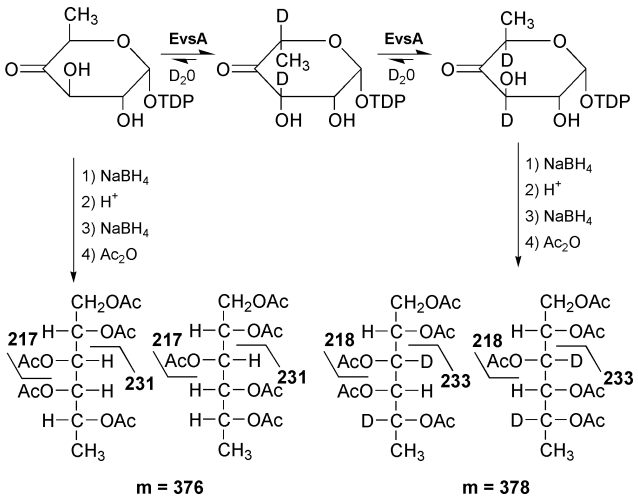


**Fig. 3** The equilibrium catalysed by EvsA and the products of the subsequent derivatisation reactions (with the masses of the molecular ions and their fragments).

from the rhamnose pathway.[15] Incubation of TDP-glucose (5 mg) with RmlB (1.6 mg) at 25 °C for 3 h resulted in essentially complete conversion to TDP-4-keto-6-deoxyglucose (monitored by observing the intensity of the UV absorption band of the product at 320 nm).

The potential epimerase activity of EvsA was assayed using a technique based on that described by Stern *et al.* when investigating RmlC, the 3,5-epimerase of the rhamnose pathway.[12] This study demonstrated that incubation of the enzyme with TDP-4-keto-6-deoxyglucose in $D_2O$ allowed for the incorporation of two deuterium atoms from the solvent at positions 3 and 5, and thus that RmlC was capable of catalysing the expected epimerisations.

Consequently, EvsA (10 μg in 50 mM HEPES, pH 7.6) was incubated with TDP-4-keto-6-deoxyglucose (10 μg) in $D_2O$ at

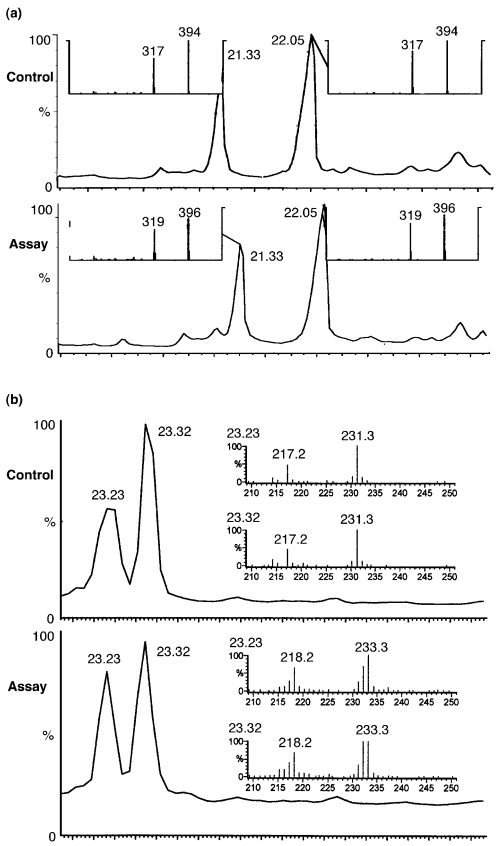


**Fig. 4** GC-MS traces [(a) chemical ionisation, (b) electron impact] of the alditol acetates produced from the assay with EvsA and the control without EvsA.

37 °C for 90 min in a total volume of 100 μl. Due to $[^1H]_2O$ in the enzyme and substrate solutions, the resulting concentration of $[^2H]_2O$ was about 80%. A reaction in which protein was replaced by buffer was used as a control. The reactions were stopped by addition of EtOH and the protein removed by centrifugation. The resulting 4-keto-sugar nucleotides were then reduced with $NaBH_4$, hydrolysed with trifluoroacetic acid, reduced again, and then acetylated for analysis by GC-MS as described by Stern *et al.* (Fig. 3).[12] The alditol acetates produced were dissolved in MeOH and analysed by ammonia chemical ionisation GC-MS. The traces for the GC-MS of the EvsA and control reactions are shown in Fig. 4(*a*). Mass analysis of the two peaks revealed that the molecular ions ($MNH_4^+$) present in the EvsA reaction are two mass units higher (*m*/*z* 396) than those in the control reaction (*m*/*z* 394).[16] The same difference is observed in the masses of the major fragment ion, which corresponds to loss of acetic acid and ammonia. Thus two deuterium atoms have been incorporated into TDP-4-keto-6-deoxyglucose from the solvent by the action of EvsA. In order to establish the positions of incorporation, the samples were also analysed using electron impact GC-MS, which gives fragments rather than the molecular ions (Fig. 3 and Fig. 4(*b*)). No deuterium incorporation was seen in the control but incorporation at both positions 3 and 5 occurred in the presence of EvsA.[17] These results confirm that EvsA is indeed an epimerase involved in the biosynthesis of 4-*e*-V at the stage suggested (Fig. 2) and that epimerisation is catalysed at both C-5 and C-3.

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## Notes and references

1 M. Foldes, R. Monro, T. C. Sorrell, S. Shankar and M. Toohey, *J. Antimicrob. Chemother.*, 1983, **11**, 21.
2 A. M. v. Wageningen, P. N. Kirkpatrick, D. H. Williams, B. R. Harris, J. K. Kershaw, N. J. Lennard, M. Jones, S. J. Jones and P. J. Solenberg, *Chem. Biol.*, 1998, **5**, 155.
3 P. J. Solenberg, P. Matsushima, D. R. Stack, S. C. Wilkie, R. C. Thompson and R. H. Baltz, *Chem. Biol.*, 1997, **4**, 195.
4 D. P. O'Brien, P. N. Kirkpatrick, S. W. O'Brien, T. S. Staroske, T. I. Richardson, D. A. Evans, A. Hopkinson, J. B. Spencer and D. H. Williams, *Chem. Commun.*, 2000, **1**, 103.
5 O. W. Choroba, D. H. Williams and J. B. Spencer, *J. Am. Chem. Soc.*, 2000, in the press.
6 J. P. Mackay, U. Gerhard, D. A. Beauregard, M. S. Westwell, M. S. Searle and D. H. Williams, *J. Am. Chem. Soc.*, 1994, **116**, 4581.
7 J. P. Mackay, U. Gerhard, D. A. Beauregard, R. A. Maplestone and D. H. Williams, *J. Am. Chem. Soc.*, 1994, **116**, 4573.
8 S. Gaisser, G. A. Bohm, J. Cortes and P. F. Leadlay, *Mol. Gen. Genet.*, 1997, **256**, 239.
9 R. G. Summers, S. Donadio, M. J. Staver, E. WendtPienkowski, C. R. Hutchinson and L. Katz, *Microbiology*, 1997, **143**, 3251.
10 S. L. Otten, X. C. Liu, J. Ferguson and C. R. Hutchinson, *J. Bacteriol.*, 1995, **177**, 6688.
11 S. L. Otten, M. A. Gallo, K. Madduri, X. C. Liu and C. R. Hutchinson, *J. Bacteriol.*, 1997, **179**, 4446.
12 R. J. Stern, T. Y. Lee, T. J. Lee, W. Yan, M. S. Scherman, V. D. Vissa, S. K. Kim, B. L. Wanner and M. R. McNeil, *Microbiology*, 1999, **145**, 663.
13 M. Graninger, B. Nidetzky, D. E. Heinrichs, C. Whitfield and P. Messner, *J. Biol. Chem.*, 1999, **274**, 25 069.
14 M. F. Giraud, G. A. Leonard, R. A. Field, C. Berlind and J. H. Naismith, *Nat. Struct. Biol.*, 2000, **7**, 398.
15 The enzyme RmlB was originally derived from *Salmonella enterica* LT2 and was overexpressed and purified from *E. coli* HB101/pYLR1 cells in the laboratory of H.-w. Liu.
16 The presence of just two major peaks (rather than four) corresponding to the expected masses of the ammoniated molecular ions is presumably due to the previously observed equilibrium position of the reaction catalysed by 3,5-epimerases, which lies strongly on the side of the *gluco*-configuration.[12]
17 This incorporation was not total as there are ions observed at 217 and 232 in the EvsA assay. This will be in part due to the presence of some $[^1H]_2O$ in the reaction mixture, but it is also possible that reaction of the substrate was not complete.