

Characterisation of *Streptomyces spheroides* NovW and revision of its functional assignment to a dTDP-6-deoxy-D-xylo-4-hexulose 3-epimerase

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Characterisation of recombinant *Streptomyces spheroides* NovW *in vitro* suggests that it is not a kinetically competent dual action dTDP-6-deoxy-D-xylo-4-hexulose 3,5-epimerase, but possesses only significant 3-epimerase activity.

The aminocoumarin antibiotic novobiocin (Fig. 1) is active against Gram-positive bacteria by virtue of its ability to inhibit the type II topoisomerase DNA gyrase.¹ It shares structural features in common with other *Streptomyces*-derived aminocoumarins, such as clorobiocin and coumermycin A₁. Data from several crystallographic^{2,3} and structure–activity relationship studies^{4–7} show that noviose, the sugar moiety of these antibiotics, is crucial for the binding and renders all three natural products competitive inhibitors of DNA gyrase with respect to ATP binding.

Following the identification of the *S. spheroides* gene cluster responsible for novobiocin production,⁸ a biosynthetic pathway leading to noviose was proposed (see Fig. 2, central pathway). Our attention was drawn to NovW, proposed to be the third enzyme of the pathway, which was thought to serve as a dTDP-6-deoxy-D-xylo-4-hexulose 3,5-epimerase. NovW has high sequence

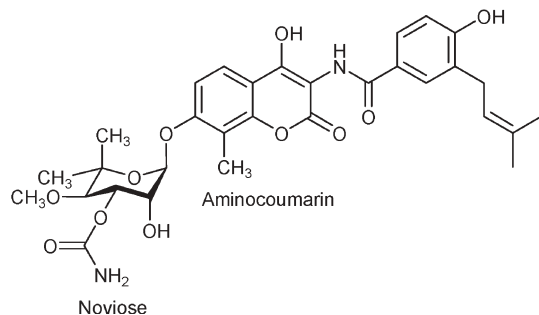


Fig. 1 Chemical structure of novobiocin.

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homology to the validated 3,5-epimerase RmlC^{9–11} from the dTDP-L-rhamnose pathway and to the 5-epimerase EvaD¹² from

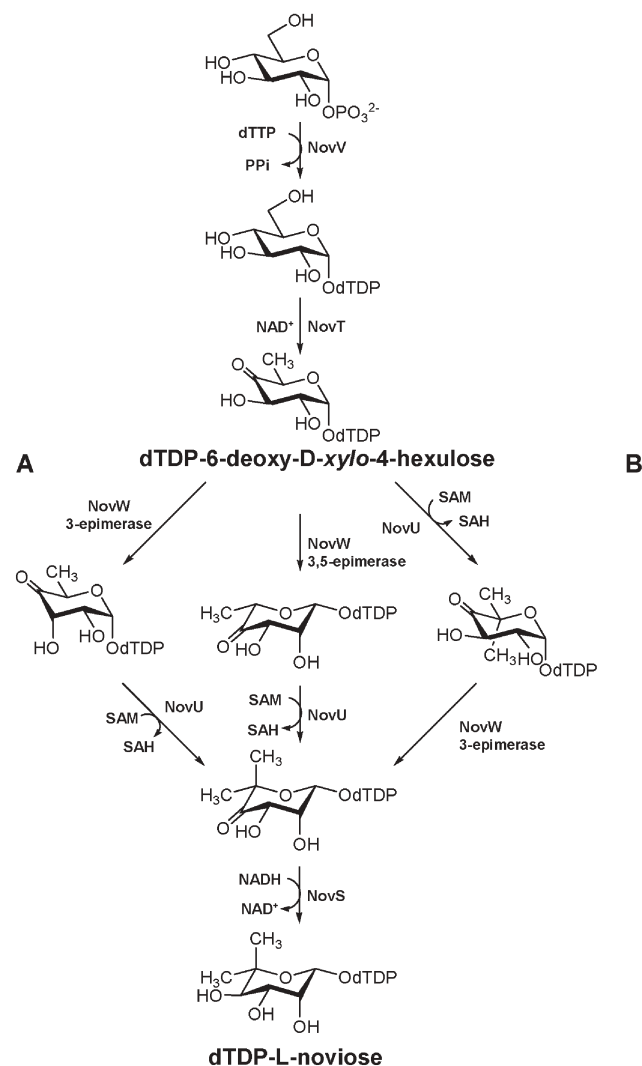


Fig. 2 Prospective biosynthetic pathways leading to dTDP-L-noviose. The route proposed by Steffensky *et al.*,⁸ with NovW serving as a 3,5-epimerase that precedes 5-methyltransferase NovU, is depicted in the central lane. An alternative route, A, shows NovW as a 3-epimerase preceding the NovU 5-methyltransferase. In route B, 5-methyltransferase NovU precedes 3-epimerase NovW. (SAM is S-adenosylmethioine; SAH is S-adenosylhomocysteine).

Table 1 Sequence identities for RmlC family epimerases^a

	EvaD <i>A. orientalis</i>	RmlC <i>S. enterica</i>	RmlC <i>M. tuberculosis</i>
NovW <i>S. spheroides</i>	46%	38%	49%
EvaD <i>A. orientalis</i>		33%	42%
RmlC <i>S. enterica</i>	33%		36%

^a Values generated using NCBI BLAST version 2.2.12.

the dTDP-epivancosamine pathway (see Table 1). Sequence-wise, NovW is more like *Mycobacterium tuberculosis* 3,5-epimerase RmlC than the *M. tb.* enzyme is to some other authenticated RmlC proteins (e.g. the *Salmonella* enzyme)(Table 1). Given the earlier misassignment of EvaD as a 3,5-epimerase,¹³ we were drawn to investigate NovW in more detail. We were further prompted to do so by the entirely plausible proposal from Thuy *et al.*¹⁴ that NovW is a 3,5-epimerase required to enable the subsequent action of C-5 methyltransferase NovU. However, since the methyltransferase reaction might occur with either retention or inversion of configuration at C-5, it is not clear that epimerisation at C-5 is necessarily required *en route* to dTDP-L-noviose. Moreover, the origin of the 5- and 5'-methyl groups has not been established. A combination of structural studies, solvent isotope incorporation and kinetic analysis were therefore employed to establish the true activity of NovW.¹⁵

Studies on RmlC¹¹ and EvaD¹² have implicated 4 key residues in the catalytic cycle. These 4 residues (His62, Lys72, Tyr132, Asp168§) are conserved in all RmlCs, EvaD and NovW. It is therefore not possible to deduce from amino acid sequence alone if NovW will have single or double epimerase activity. X-ray crystallographic analysis of active sites of the 3 epimerases (Fig. 3) reveals that His62, Asp168 and Lys72 overlap closely, while the side chain of Tyr132 adopts different conformations.¹⁶ In the case of EvaD and NovW, Tyr132 adopts the same orientation, which is distinct from that in RmlC. In previous comparative studies of RmlC and EvaD,¹² this observation was used to explain the difference in their activities: the orientation of Tyr132 establishing EvaD as a 5-epimerase and RmlC as a 3,5-double-epimerase. Therefore, based on the orientation of Tyr132, NovW would be expected to have only 5-epimerase activity. However, the structure of noviose clearly requires the action of a 3-epimerase *en route* from dTDP-D-glucose to dTDP-L-noviose (Fig. 2). Hence protein X-ray crystallographic analysis does not immediately identify the true function of NovW either.

Table 2 Kinetic parameters for double epimerase activity

	K_m /mM	k_{cat}/s^{-1}	$(k_{cat}/K_m)/s^{-1} mM^{-1}$
NovW <i>S. spheroides</i>	0.97 ± 0.01	0.020 ± 0.001	0.021
EvaD <i>A. orientalis</i> ¹²	0.27 ± 0.03	0.08 ± 0.01	0.33
RmlC <i>S. enterica</i> ¹⁷	0.71 ± 0.03	39 ± 6.6	54.9

Kinetic parameters (Table 2) for the double epimerisation activity of NovW acting on dTDP-6-deoxy-D-xylo-4-hexulose were measured by the coupled assay method used previously to assay RmlC^{11,17} and EvaD.¹² That is, the formation of 3,5-epimerised product was coupled with *in situ* reduction by RmlD, with concomitant NADPH consumption. The k_{cat} for NovW determined in this manner was nearly 3 orders of magnitude lower than for RmlC of *S. typhimurium* and 4 times lower than that of EvaD. On the other hand, K_m values for all 3 enzymes are broadly of the same magnitude. The k_{cat}/K_m ratio for these enzymes situates NovW and EvaD at a significantly lower level than RmlC (ca. 100–1000 fold). On this basis, the assignment of NovW as a dTDP-6-deoxy-D-xylo-4-hexulose 3,5-epimerase is implausible.

To get direct evidence for the site of NovW action on dTDP-6-deoxy-D-xylo-4-hexulose,¹⁸ solvent isotope incorporation experiments were performed, following the method employed by Stern *et al.*⁹ to study RmlC. Since the equilibrium position for RmlC⁹/EvaD/NovW action is very heavily in favour of substrate (data not shown), deuterium incorporation into substrate was monitored. Following a reduction–cleavage–reduction–acetylation work-up procedure, GC-MS analysis of NovW reactions performed in D₂O with dTDP-6-deoxy-D-xylo-4-hexulose showed two main peaks (6.57 min and 6.65 min) in the GC trace (Fig. 4). The peak at 6.57 min corresponded to the fucitol standard (axial product arising from 4-keto substrate reduction) and at 6.65 min to the quinovositol standard (equatorial product arising from 4-keto substrate reduction). MS analysis of both peaks gave characteristic 218/232 peaks arising from incorporation of a single deuterium atom specifically at position 3. GC-MS results for parallel experiments on RmlC showed two major GC peaks at the same retention times as for NovW reactions. However, MS analysis of both peaks revealed the 218/233 profile of deuterium incorporation at both positions 3 and 5, as expected for RmlC activity. These results clearly indicate that under conditions where an equimolar quantity of RmlC proved capable of effecting complete deuterium

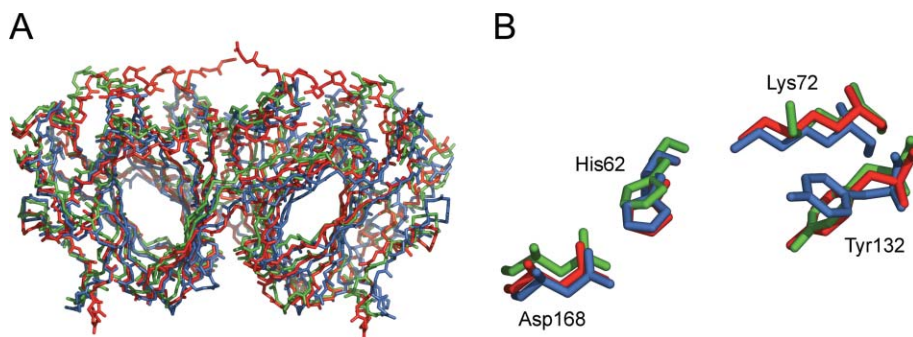


Fig. 3 (A) Overlap of sugar-nucleotide epimerase backbone structures: RmlC of *Salmonella typhimurium* in blue (PDB accession code 1DZR), NovW of *Streptomyces spheroides* in green (PDB accession code 2COZ) and EvaD of *Amylocalyptis orientalis* in red (PDB accession code 1OFN). (B) Overlap of the 4 main residues participating in the epimerization reaction.

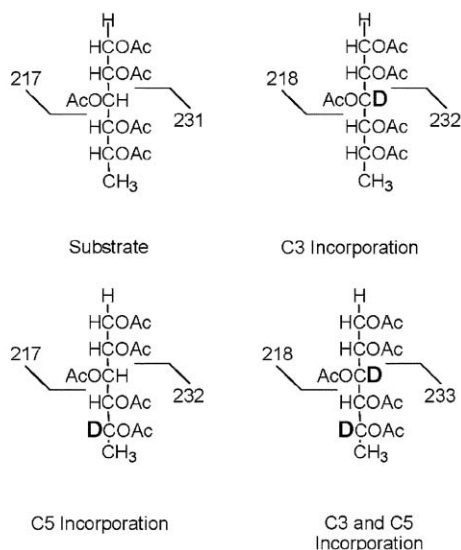
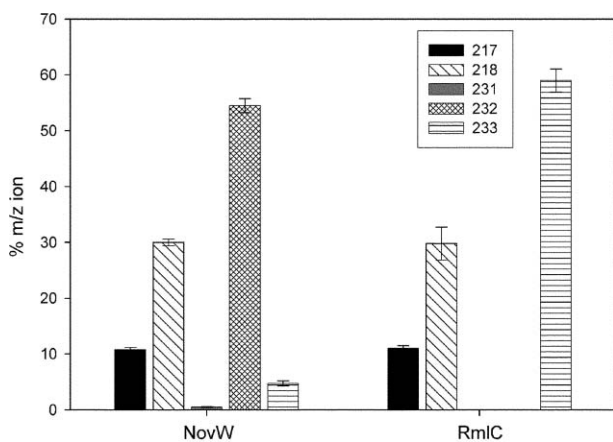


Fig. 4 Enzyme catalysed solvent deuterium incorporation into dTDP-6-deoxy-D-xylo-4-hexulose, as analysed by GC-MS of the corresponding alditol peracetates. For related data for EvaD see ref. 12.

incorporation from D₂O into dTDP-6-deoxy-D-xylo-4-hexulose at positions 3 and 5, NovW was only capable of effecting the single, specific incorporation of deuterium into position 3 of the same substrate.

In conclusion, we have demonstrated by kinetic characterisation and by deuterium incorporation analysis that, whilst NovW is a chemically competent dTDP-6-deoxy-D-xylo-4-hexulose 3,5-epimerise *in vitro*, kinetically it serves as only a 3-epimerase. These data highlight the need for caution when assigning function based solely on gene sequence similarities and/or protein crystal structure data.

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Notes and references

§ The residue numbering used herein corresponds to that of NovW from *S. spheroides*.

- 1 A. Maxwell and D. M. Lawson, *Curr. Top. Med. Chem. (Sharjah, United Arab Emirates)*, 2003, **3**, 283.
- 2 V. Lamour, L. Hoermann, J. M. Jeltsch, P. Oudet and D. Moras, *Acta Crystallogr., Sect. D*, 2002, **D58**, 1376.
- 3 R. J. Lewis, O. M. Singh, C. V. Smith, T. Skarzynski, A. Maxwell, A. J. Wonacott and D. B. Wigley, *EMBO J.*, 1996, **15**, 1412.
- 4 D. C. Hooper, J. S. Wolfson, G. L. McHugh, M. B. Winters and M. N. Swartz, *Antimicrob. Agents Chemother.*, 1982, **22**, 662.
- 5 B. Musicki, A. M. Periers, L. Laurin, D. Ferroud, Y. Benedetti, S. Lachaud, F. Chatreaux, J. L. Haesslein, A. Iltis, C. Pierre, J. Khider, N. Tessot, M. Airault, J. Demasse, C. Dupuis-Hamelin, P. Lassaigne, A. Bonnefoy, P. Vicat and M. Klich, *Bioorg. Med. Chem. Lett.*, 2000, **10**, 1695.
- 6 B. Musicki, A. M. Periers, L. Piombo, P. Laurin, M. Klich, C. Dupuis-Hamelin, P. Lassaigne and A. Bonnefoy, *Tetrahedron Lett.*, 2003, **44**, 9259.
- 7 D. Ferroud, J. Collard, M. Klich, C. Dupuis-Hamelin, P. Mauvais, P. Lassaigne, A. Bonnefoy and B. Musicki, *Bioorg. Med. Chem. Lett.*, 1999, **9**, 2881.
- 8 M. Steffensky, A. Muhlenweg, Z. X. Wang, S. M. Li and L. Heide, *Antimicrob. Agents Chemother.*, 2000, **44**, 1214.
- 9 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.
- 10 M. F. Giraud, G. A. Leonard, R. A. Field, C. Bernlind and J. H. Naismith, *Nat. Struct. Biol.*, 2000, **7**, 398.
- 11 C. Dong, L. L. Major, A. Allen, W. Blankenfeldt, D. J. Maskell and J. H. Naismith, *Structure*, 2003, **11**, 715.
- 12 A. B. Merkel, L. L. Major, J. C. Errey, M. D. Burkart, R. A. Field, C. T. Walsh and J. H. Naismith, *J. Biol. Chem.*, 2004, **279**, 32684.
- 13 P. N. Kirkpatrick, W. Scaife, T. M. Hallis, H. S. Liu, J. B. Spencer and D. H. Williams, *Chem. Commun.*, 2000, 1565.
- 14 T. T. Thu Thuy, H. C. Lee, C. G. Kim, L. Heide and J. K. Sohng, *Arch. Biochem. Biophys.*, 2005, **436**, 161.
- 15 N-terminal His-tagged NovW was produced by amplifying the *novW* gene from *S. spheroides* genomic DNA and cloning it into pET-15b (Novagen). NovW was coexpressed with GroES/GroEL¹⁹ chaperone system in *E. coli* BL21 (DE3) and was purified by Ni-NTA affinity chromatography (Hi-Trap column, Amersham), followed by size exclusion chromatography (HiLoadSM SuperdexSM 16/60, Amersham). Expression level: ca. 13 mg L⁻¹.
- 16 P. Jakimowicz, M. Tello, C. L. Freil Meyers, C. T. Walsh, M. J. Buttner, R. A. Field and D. M. Lawson, *Proteins: Struct., Funct., Bioinf.*, 2005, DOI: 10.1002/prot.20818.
- 17 M. Graninger, B. Nidetzky, D. E. Heinrichs, C. Whitfield and P. Messner, *J. Biol. Chem.*, 1999, **274**, 25069.
- 18 The reaction substrate, dTDP-6-deoxy-D-xylo-4-hexulose, was prepared enzymatically by overnight incubation of dTDP-glucose (10 mg) with dTDP-glucose-4,6-dehydratase (RmlB) (1.5 mg) and 1 mM NAD⁺ at 37 °C. Substrate purity was monitored by ¹H NMR spectroscopy; data were in accord with the literature.²⁰ D-Fucitol, D-quinovositol and L-rhamnitol were used as standards for alditol analysis by GC-MS.
- 19 H. Grallert and J. Buchner, *J. Struct. Biol.*, 2001, **135**, 95.
- 20 A. Naundorf and W. Klaffke, *Carbohydr. Res.*, 1996, **285**, 141.