Mechanism of the 2-Deoxygenation Step in the Biosynthesis of the Deoxyhexose Moieties of the Antibiotics Granaticin and Oleandomycin

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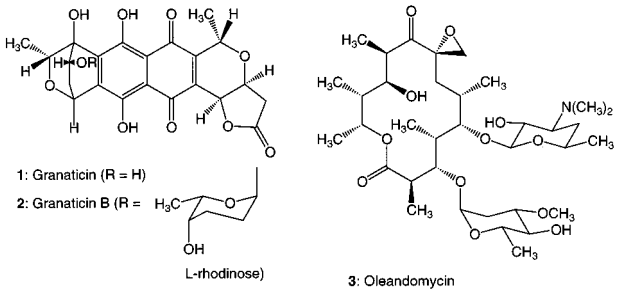
Deoxyhexoses are prominent structural components of many antibiotics[1] and of the antigenic determinants of certain pathogenic bacteria.[2] They are derived from glucose-1-phosphate by transformations which take place at the level of the sugar diphosphonucleotides, e.g., starting from dTDP-glucose or CDP-glucose.[3,4] The first reaction involves removal of the oxygen function at C-6, catalyzed by NDP-hexose 4,6-dehydratases, which generate the corresponding NDP-4-keto-6-deoxyhexoses.[5] In the formation of the antigenic determinant deoxyhexoses the resulting CDP-4-keto-6-deoxyglucose is then further deoxygenated at C-3 by the action of a pyridoxamine phosphate (PMP) and iron−sulfur cluster-containing enzyme, E_1, and an electron-transfer protein, E_3, to give CDP-4-keto-3,6-dideoxyglucose.[3] This reaction has been studied extensively[6,7] and involves a novel PMP-mediated radical reaction.[8] In the antibiotic deoxy sugars a common modification following 6-deoxygenation is the loss of the oxygen function from C-2, but very little is known about this process or the enzyme(s) catalyzing it.[3,4]

In our studies on antibiotic biosynthesis[9] we have examined the formation of the sugar moieties of granaticin (**1**) and granaticin B (**2**) from *Streptomyces violaceoruber* Tü22.[10] **1** contains a D-glucose-derived[11,12] 2,6-dideoxy-D-hexose uniquely 1,4-linked to a benzoisochromane quinone chromophore, and **2** carries an additional glycosidically linked L-rhodinose. The involvement of the dTDP-glucose 4,6-dehydratase reaction in **1** formation has been established,[11,12] which allowed us to clone and sequence several genes involved in the formation of these sugar moieties.[13]

In recent work on the sequence of the granaticin biosynthetic gene cluster[14] we identified several additional genes which, based on sequence homology, were likely to code for enzymes involved in deoxy sugar formation. In particular *gra* orf23, a homologue of *ascC* and *rfbH*,[15] genes encoding the 3-deoxygenation enzyme E_1, was a good candidate to encode the enzyme catalyzing the 2-deoxygenation step. However, when gene inactivation experiments showed that *gra* orf23 is not involved in the formation of the deoxy sugar moiety of **1**,[16] we were left with only one other candidate gene, *gra* orf27. This gene is homologous to genes in other 2,6-dideoxyhexose biosynthetic gene clusters, e.g., *eryBVI* from *Saccharopolyspora erythraea*[17] and *dnmT* from *S. peucetius*,[18] which have been proposed as the 2-deoxygenation genes in the formation of the erythromycin and daunomycin sugar moieties. We have found a homologous gene, *Tü99* orf10, in a cluster of sugar biosynthesis genes associated with a type I PKS gene, which we isolated from *S. antibioticus Tü99*.[19] This cluster was originally assumed to encode formation of the two D-olivose moieties of chlorothricin,[20] an antibiotic biosynthesized by this strain.[21−23] However, sequence comparison with three recently published genes[24] suggests that it, in fact, represents part of the oleandomycin (**3**) biosynthesis cluster. Both *gra* orf 27 and *Tü99* orf10 are flanked by genes, *gra* orf26 and *Tü99* orf11, whose deduced amino acid sequences show homology to that of a gene, *rdmF*, of unknown function in the rhodomycin biosynthetic gene cluster[25] and to glucose−fructose oxidoreductase from *Zymomonas mobilis*.[26] We therefore decided to express these genes in *Escherichia coli* and to test the enzymatic functions of their products.



Each of the 4 genes, *gra* orf 27 and 26 and *Tü99* orf 10 and 11, was amplified from the cosmids carrying them by PCR using primers which introduced suitable restriction sites for cloning into expression vector pRSET B (Invitrogen). They were then expressed in *E. coli* BL21(DE3)/pLysS under the control of the T7 promoter either as such or as His_6-tagged fusion proteins. The culture and induction conditions were optimized for each enzyme to obtain a maximum amount of soluble protein. The His_6-tagged proteins were purified on Ni-NTA affinity columns.[27] Incubations

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(27) Gra Orf26 and Tü99 Orf11 were quite unstable, losing activity within an hour of cell disruption, but were stabilized somewhat in the presence of PMSF.

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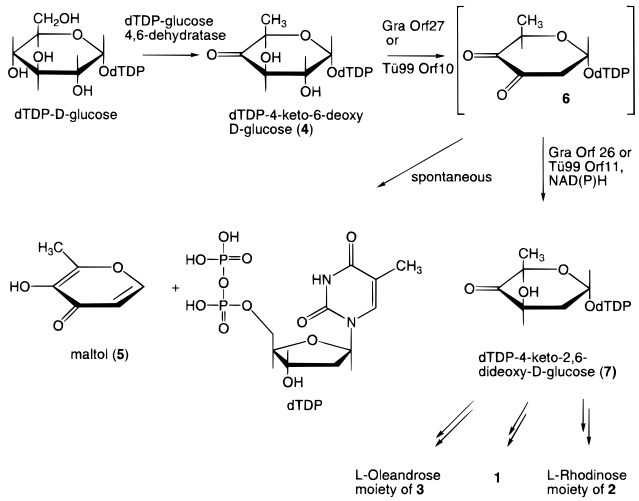
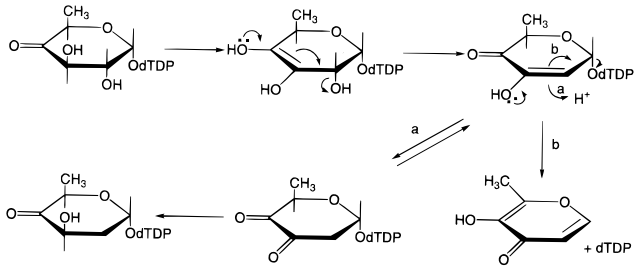
Scheme 1



of the various proteins with dTDP-4-keto-6-deoxyglucose (**4**) plus, in the case of Gra Orf26 and Tü99 Orf11, NADPH were analyzed by capillary electrophoresis (CE).[28]

Incubation of Gra Orf27 with **4**[29] gave dTDP (t_{ret} = 6.85 min) and a compound (t_{ret} = 3.12 min) identified by its NMR spectrum and by comparison with an authentic sample as maltol (3-hydroxy-2-methyl-*4H*-pyran-4-one) (**5**). The same two compounds were obtained upon incubation of **4** with Tü99 Orf10. Evidently the enzymatic reaction produces an unstable compound, presumably dTDP-3,4-diketo-2,6-dideoxyglucose (**6**) or its 2,3-enol, which undergoes facile elimination of dTDP. We reasoned that in the normal biosynthesis such an intermediate may be stabilized by immediate reduction of the 3-keto group and examined whether Gra Orf26 or Tü99 Orf11 catalyzes this reaction. Incubation of **4** with Gra Orf 26 and NAD(P)H alone gave no reaction, but addition of Gra Orf 27[30] resulted in the almost quantitative conversion into a new compound, a sugar nucleotide of t_{ret} = 4.80 min.[31] This compound also underwent elimination of dTDP, particularly upon attempts at purification by ion-exchange chromatography, but was stable enough to allow purification by gel filtration (Sephadex G15, water) to a sample which was mainly contaminated with some dTDP. Analysis of this material by ES-MS and 1D and 2D ^{1}H NMR[32] revealed its structure to be that of dTDP-4-keto-2,6-dideoxyglucose (**7**) (Scheme 1). The same compound was formed upon incubation of **4** with Tü99 Orf10 + 11 and NADPH, and also with the combination of Tü99 Orf10 + Gra Orf 26 or Gra Orf27 + Tü99 Orf11 and NADPH. NADPH was the better reductant, but NADH could substitute for it in these reactions.

Scheme 2



These results establish the mode of removal of the 2-OH group in the biosynthesis of 2,6-dideoxyhexoses such as those found in **1**, **2**, and **3** and probably other antibiotics as well. Gra Orf27 or Tü99 Orf10 act as dTDP-4-keto-6-deoxyglucose 2,3-dehydratases which convert **4** into **6**, probably by the mechanism shown in Scheme 2. **6** is reductively captured by the 3-ketoreductases, Gra Orf26 or Tü99 Orf11, to give **7** but in their absence decomposes to dTDP and **5** (Scheme 1). The notable instability of **6** suggests that these 2,3-dehydratases must operate in concert with another enzyme to trap their product. In the present case the trapping occurs through ketoreduction; in the biosynthesis of 2,3,6-trideoxy-3-aminohexoses, such as daunosamine,[33] it may occur through transamination. The likely mechanism established here for the 2-deoxygenation of hexose nucleotides (Scheme 2) is fundamentally different from that of the 3-deoxygenation of CDP-4-keto-6-deoxyglucose, but has analogy in other reactions of carbohydrates, e. g., in the first step of the conversion of ribulose 5-phosphate into 3,4-dihydroxybutanone 4-phosphate, a precursor of riboflavin.[34]

Compound **7** is proposed to be the substrate for the connection of the sugar moiety to the aromatic core of granaticin. A further 3,5-epimerization and 4-ketoreduction is necessary to convert it into dTDP-L-olivose, the precursor of the L-oleandrose moiety of **3**. These two steps may be mediated by *Tü99* orf9 and orf6, homologues of *strM* and *strL* from *Streptomyces griseus*,[35] respectively. Since there is not a second set of 2-deoxygenation genes in the granaticin biosynthetic gene cluster, **7** is probably also the precursor of the L-rhodinose moiety present in granaticin B.

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(28) A P/ACE System 5010 with a P/ACE UV absorbance detector and System Gold software (version 8.1) from Beckman Coulter was used for the CE experiments. The capillary cartidge contained a 57 cm long (50 cm to detector) 75 μm i.d. fused silica capillary. The run buffer was 12.5 mM sodium borate and 16 mM boric acid in demineralized water. The run voltage was 30 kV (normal polarity), and the runs were performed at 25 °C. Samples were injected by pressure (1 s) and detected at 280 nm.

(29) Ten μL of a 50× concentrated crude extract of 36 h induced cells (*E. coli* BL21 DE3 pLysS) containing Gra orf27 was incubated with 0.1 mg of **4** in 40 μL of 10 mM potassium phosphate buffer (pH 7.2) for 30 min at 28 °C. The reaction mixture was heated in boiling water for 2 min, cleared by centrifugation and analyzed by CE.

(30) Ten μL each of a 50× concentrated crude extract of 36 h induced cells (*E. coli* BL21 DE3 pLysS) containing Gra orf27 and Gra orf26 was incubated with 0.1 mg of **4** in 30 μL of 10 mM potassium phosphate buffer (pH 7.2) in the presence of 1 mM NAD(P)H for 30 min at 28 °C. The reaction mixture was heated in boiling water for 2 min, cleared by centrifugation, and analyzed by CE.

(31) dTDP-glucose t_{ret} = 4.63 min, dTDP-4-keto-6-deoxyglucose t_{ret} = 4.72 min.

(32) ESI-MS (negative ion mode): *m*/*z* 529 (32, $M^{-2} + H^+$), 547 (100, $M^{-2} + H_2O + H^+$), 551 (11, $M^{-2} + Na^+$), 569 (22, $M^{-2} + H_2O + Na^+$); ^{1}H NMR (dideoxysugar domain; 500 MHz, D_2O, DHO = 4.63 ppm) δ (ppm): 5.48 (ddd, J = 6.8, 3.1 and 1.2 Hz, 1H, 1-H), 3.92 (q, J = 6.8 Hz, 1H, 5-H), 3.89 (dd, J = 12.4 and 4.9 Hz, 1H, 3-H), 2.03 (ddd, J = 13.6, 4.9 and 1.2 Hz, 1H, 2-H_e), 1.68−1.76 (m, 1H, 2-H_a), 1.08 (d, J = 6.8 Hz, 3H, 6-H).

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