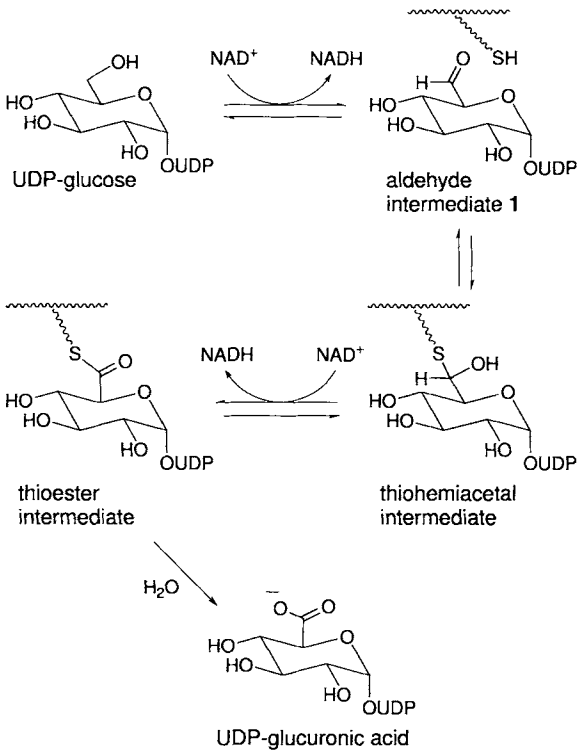
Uridine Diphospho-α-D-*gluco*-hexodialdose: Synthesis and Kinetic Competence in the Reaction Catalyzed by UDP-Glucose Dehydrogenase**

Robert E. Campbell and Martin E. Tanner*

UDP-glucose dehydrogenase catalyzes the irreversible NAD^+-dependent oxidation of UDP-glucose to UDP-glucuronic acid (Scheme 1).[1] In mammals the product of this reaction is used in the liver to tag waste metabolites for excretion,[2] and in the biosynthesis of glycosaminoglycans such as heparin[3] and hyaluronic acid.[4] In many strains of pathogenic bacteria, such as group A streptococci[5] and *Streptococcus pneumoniae* type 3,[6] it provides glucuronic acid, which is used in the construction of an antiphagocytic capsule. This capsule allows the bacteria to evade the immune system of the host and is thus responsible for their virulence.[7–9]

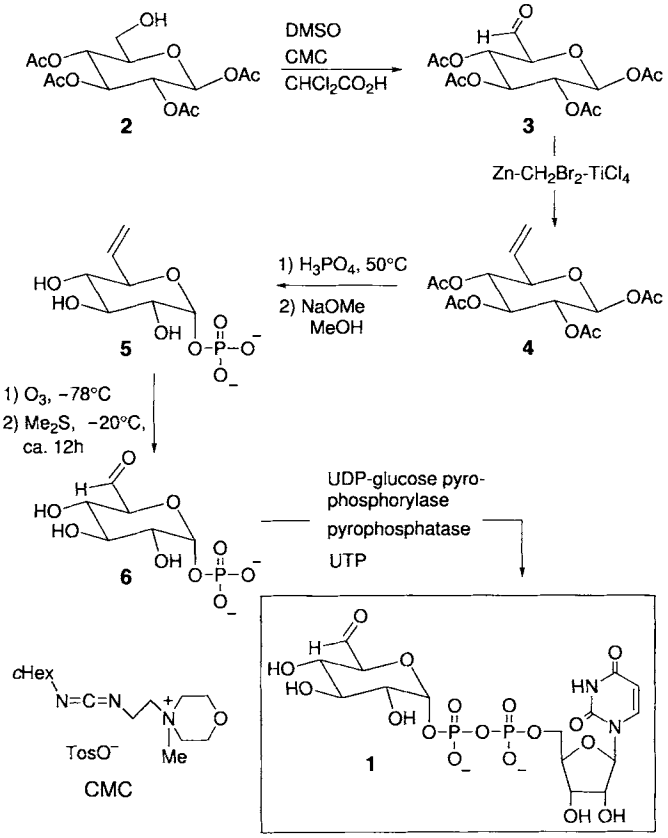


Scheme 1. Proposed mechanism for the reaction catalyzed by UDP-glucose dehydrogenase.

UDP-glucose dehydrogenase belongs to a small class of enzymes that are capable of carrying out the twofold oxidation of an alcohol to an acid without the release of an aldehyde intermediate.[1] The putative intermediate, uridine diphospho-α-D-*gluco*-hexodialdose (**1**), has therefore never been detected nor trapped with carbonyl-modifying reagents.[10–12] In previous work on the bovine liver enzyme it was suggested that the aldehyde is not an actual intermediate in the reaction and that the alcohol is converted directly to an enzyme-linked imine, which is held through a lysine side chain.[13] This is somewhat at odds with a report that only one solvent-derived oxygen atom is found in the product acid.[14] If an imine intermediate was formed one would expect both carboxylate oxygen atoms to come from solvent. In order to support the notion that **1** it is a tightly bound intermediate, we have synthesized it and measured the kinetic parameters that describe its oxidation by the enzyme.

Earlier work reported that **1** can be obtained from UDP-glucose with UDP-galactose 4-epimerase and galactose oxidase.[11, 13] This procedure tended only to work on a very small scale and required large amounts of expensive enzymes. Furthermore, the procedure neccesarily yielded an inseparable mixture of epimers at the C4 position, and no spectroscopic characterization of the product or indication of purity was reported. Therefore, we chose to carry out the stepwise synthesis of **1**.

A relatively recent paper by Müller and Schmidt[15] outlining the preparation of a dTDP-ketosugar prompted us to use an alkene as a protecting group for the sensitive aldehyde functionality (Scheme 2). The synthesis begins with an oxidation at C6 of the known tetraacetylated glucose **2**.[16] It is well documented



Scheme 2. Stepwise synthesis of **1** with an alkene as a protecting group for the sensitive aldehyde functionality.

that a facile β-elimination reaction occurs to give the α,β-unsaturated enone in these systems;[17] however, a Moffat oxidation is mild enough to minimize this elimination in related compounds.[18] The water-soluble coupling reagent CMC was used in place of *N*,*N*′-dicyclohexylcarbodiimide because the resulting urea derivative can easily be removed by extraction. We thus prepared aldehyde **3** and immediately used it without complete

[*] Dr. M. E. Tanner, R. E. Campbell
The University of British Columbia
Department of Chemistry
Vancouver, BC V6T 1Z1 (Canada)
Fax: Int. code +(604)822-2847
e-mail: mtanner@chem.ubc.ca

[**] This work was supported by The Natural Sciences and Engineering Research Council of Canada.

purification. The alkene was introduced with Lombardo's reagent,[19] and **4** isolated in an overall yield of 12% based on **2** (Table 1). The α-phosphate was prepared by a modified MacDonald reaction[20] in neat phosphoric acid at 50 °C. No β-anomer was detected. The acetyl groups were removed with sodium methoxide to give **5** in 39% yield. Deprotection of **5** to give **6** was achieved by ozonolysis at −78 °C followed by an overnight treatment with dimethylsufide at −20 °C. Aldehyde **6** was used directly in an enzymatic coupling reaction with UTP, UDP-glucose pyrophosphorylase, and pyrophosphatase.[21] It was expected that **6** could replace the normal substrate glucose-α-1-phosphate in the coupling reaction, since the steric differences between them are slight. Indeed, following the reaction with ion-paired reverse-phase HPLC[22] indicated that one equivalent of UTP was cleanly converted into **1**. The product was surprisingly stable and could be isolated in 44% yield (based on **5**) by a combination of anion-exchange (DE-52 resin) and size-exclusion (Bio-Gel P-2) chromatographies. The aldehydic proton appears as a doublet at $\delta = 5.17$ in the ^{1}H NMR spectrum taken in D_2O (Figure 1, Table 1). This indicates that more than 95% of the aldehyde is present as a hydrate.[23]

Table 1. Selected physical data for **1**, **4**, and **5**.

1 (disodium salt): ^{1}H NMR (400 MHz, D_2O): $\delta = 3.51$ (m, 2H, H2″, H4″), 3.74 (dd, $J = 9.5$, 9.5 Hz, 1H, H3″), 3.83 (dd, $J = 1.3$, 10.0 Hz, 1H, H5″), 4.16–4.42 (m, 4H, H3′, H4′, H5′), 4.34 (d, $J = 3.3$ Hz, 1H, H2′), 5.17 (d, $J = 1.3$ Hz, 1H, H6″), 5.62 (dd, J(H,H) = 3.4 Hz, J(H,P) = 7.6 Hz, 1H, H1″), 5.95 (m, 2H, H1′, H5), 7.91 (d, $J = 8.2$ Hz, 1H, H6); ^{31}P NMR (300 MHz, D_2O): $\delta = -12.45$ (d, J(P,P) = 20.9 Hz, 1P), −10.82 (d, J(P,P) = 20.9 Hz, 1P); HR-LSI MS [a] calcd for $C_{15}H_{20}N_2O_{17}P_2Na$ (nonhydrated monosodium salt): 585.01349; found: 585.01370.

4: ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 1.96$, 1.98, 2.00, 2.07 (4s, 12H, 4Ac), 3.99 (dd, $J = 7.1$, 9.8 Hz, 1H, H5), 4.93 (dd, $J = 9.7$, 9.7 Hz, 1H, H4), 5.09 (dd, $J = 9.6$, 8.3 Hz, 1H, H2), 5.23 (dd, $J = 9.5$, 9.5 Hz, 1H, H3), 5.26 (d, $J = 10.5$ Hz, 1H, H7-*trans*), 5.34 (d, $J = 17.2$ Hz, 1H, H7-*cis*), 5.69 (m, 1H, H6), 5.73 (d, $J = 8.3$ Hz, 1H, H1); DCI-MS (NH_3): m/z (%) = 362 (100, $[M+NH_4]^+$); elemental analysis calcd for $C_{15}H_{20}O_9$: C 52.33, H 5.85; found: C 52.36, H 5.82.

5 (monosodium salt): ^{1}H NMR (400 MHz, D_2O): $\delta = 3.32$ (dd, $J = 9.6$, 9.7 Hz, 1H, H4), 3.57 (dt, $J = 9.8$, 2.7 Hz, 1H, H2), 3.76 (dd, $J = 9.6$, 9.5 Hz, 1H, H3), 4.25 (dd, $J = 9.4$, 8.7 Hz, 1H, H5), 5.44 (m, 3H, H1, H7-*trans*, H7-*cis*), 5.86 (m, 1H, H6); ^{31}P NMR (300 MHz, D_2O): $\delta = 0.373$; HR-LSI MS [a] calcd for $C_7H_{12}O_8P$: 255.02698; found: 255.02786.

[a] High-resolution liquid secondary-ion mass spectrometry: negative-ion mode in a glycerin matrix.

Treatment of aldehyde **1** with a saturating amount of NAD^+ (500 μM) and the *Streptococcus pyogenes* UDP-glucose dehydrogenase[10] resulted in the quantitative production of UDP-glucuronic acid and NADH, as analyzed by ion-paired reverse-phase HPLC.[22] The kinetics and stoichiometry of the reaction were followed by monitoring NADH formation with UV spectroscopy at $\lambda = 340$ nm. For each molecule of **1** that was oxidized, one molecule of NADH was generated. The oxidation of **1** at 30 °C and pH 8.7 followed Michaelis–Menten kinetics with $k_{cat} = 1.0\ s^{-1}$ and $K_M = 14\ \mu M$.[24, 25] These compare well with values obtained for UDP-glucose measured under identical conditions ($k_{cat} = 1.2\ s^{-1}$ and $K_M = 14\ \mu M$).[10, 25] The agreement in the k_{cat} values indicates that the aldehyde is kinetically competent to be an intermediate in the normal reaction pathway.

We envision two possibilities for the oxidation process. The enzyme may catalyze the oxidation of only the minor, unhydrated form of the aldehyde present in solution, leaving the hydrated form to bind nonproductively in a competitive manner. Alternatively, the enzyme may operate on the hydrated form and catalyze its dehydration prior to oxidation. It seems unlikely that the oxidation proceeds directly through the hydrate, since mounting evidence[10, 26, 27] suggests that the second oxidation proceeds via a thioester intermediate in a manner analogous to the reaction catalyzed by glyceraldehyde 3-phosphate dehydrogenase.[28, 29] The bound aldehyde is likely in equilibrium with a covalently bound thiohemiacetal intermediate that is attached to an active-site cysteine residue (see Scheme 1).

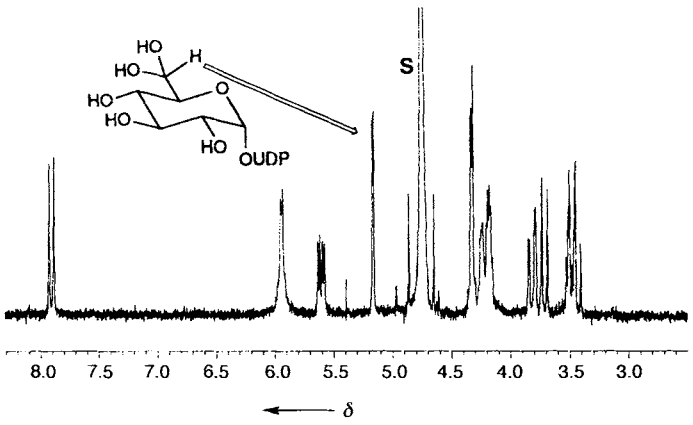


Figure 1. 200 MHz ^{1}H NMR spectrum of the hydrated form of **1** in D_2O. "S" indicates the signal for residual protiated solvent.

Received: January 7, 1997 [Z9970IE]
German version: *Angew. Chem.* **1997**, *109*, 1593–1595

Keywords: carbohydrates · enzyme catalysis · intermediates · nucleotides

[1] N. J. Oppenheimer, A. L. Handlon in *The Enzymes, Vol. 20* (Ed.: D. S. Sigman), Academic Press, San Diego, **1992**, pp. 453–504.
[2] G. J. Dutton, *Glucuronidation of Drugs and Other Compounds*, CRC Press, Boca Raton, FL, USA, **1980**.
[3] L. Kjellén, U. Lindahl, *Annu. Rev. Biochem.* **1991**, *60*, 443–475.
[4] *The Biology of Hyaluronan* (Eds.: D. Evered, J. Whelan), Wiley, Chichester, **1989**.
[5] B. A. Dougherty, I. van de Rijn, *J. Biol. Chem.* **1993**, *268*, 7118–7124.
[6] C. Arrecubieta, E. García, R. López, *J. Bacteriol.* **1996**, *178*, 2971–2974.
[7] D. A. Watson, D. M. Musher, *Infect. Immun.* **1990**, *58*, 3135–3138.
[8] E. R. Moxon, J. S. Kroll, *Curr. Top. Microbiol. Immunol.* **1990**, *150*, 65–86.
[9] M. R. Wessels, J. B. Goldberg, A. E. Moses, T. J. DiCesare, *Infect. Immun.* **1994**, *62*, 433–441.
[10] R. E. Campbell, R. F. Sala, I. van de Rijn, M. E. Tanner, *J. Biol. Chem.* **1997**, *272*, 3416–3422.
[11] G. L. Nelsestuen, S. Kirkwood, *J. Biol. Chem.* **1971**, *246*, 3828–3834.
[12] J. L. Strominger, E. S. Maxwell, J. Axelrod, H. M. Kalckar, *J. Biol. Chem.* **1957**, *224*, 79–90.
[13] A. B. Ordman, S. Kirkwood, *J. Biol. Chem.* **1977**, *252*, 1320–1326.
[14] J. G. Schiller, A. M. Bowser, D. S. Feingold, *Carbohydr. Res.* **1972**, *25*, 403–410.
[15] T. Müller, R. R. Schmidt, *Angew. Chem.* **1995**, *107*, 1467–1468; *Angew. Chem. Int. Ed. Engl.* **1995**, *34*, 1328–1329.
[16] D. D. Reynolds, W. L. Evans, *Organic Syntheses, Vol. 3* (Ed.: E. C. Horning), Wiley, New York, **1955**, pp. 432–434.
[17] R. W. Binkley, *J. Org. Chem.* **1977**, *42*, 1216–1221.
[18] D. Nicoll-Griffith, L. Weiler, *J. Chem. Soc. Chem. Commun.* **1984**, 659–661.
[19] L. Lombardo, *Tetrahedron Lett.* **1982**, *23*, 4293–4296.
[20] C. D. Warren, R. W. Jeanloz, *Biochemistry* **1973**, *12*, 5031–5037.
[21] A. N. Singh, J. S. Newborn, F. M. Raushel, *Bioorg. Chem.* **1988**, *16*, 206–214.
[22] I. Meynial, V. Paquet, D. Combes, *Anal. Chem.* **1995**, *67*, 1627–1631.
[23] D. Horton, M. Nakadate, J. M. J. Tronchet, *Carbohydr. Res.* **1968**, *7*, 56–65.
[24] Initial dismutation to an alcohol and an acid was observed during the oxidation of aldehydes by horse-liver alcohol dehydrogenase (G. T. M. Henehan, N. J. Oppenheimer, *Biochemistry* **1993**, *32*, 735–738). This does not occur with UDP-glucose dehydrogenase, since UDP-glucuronic acid, and not NADH, is released last in the kinetic mechanism [10]. No lag phase was observed during the oxidation of **1**.

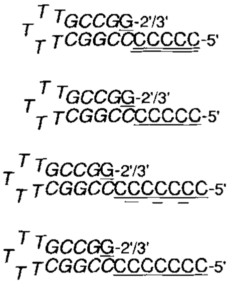
  0570-0833/97/3613-1521 $ 17.50+.50/0

[25] The kinetic data could be fit somewhat better if corrections were made for potential weak allosteric interactions. These corrections (Hill coefficient $n = 1.5$ for UDP-glucose and $n = 1.3$ for 1) would not change the values of k_{cat} by more than 10%.
[26] B. Franzen, C. Carrubba, D. S. Feingold, J. Ashcom, J. S. Franzen, *Biochem. J.* **1981**, *199*, 603–609.
[27] W. P. Ridley, J. P. Houchins, S. Kirkwood, *J. Biol. Chem.* **1975**, *250*, 8761–8767.
[28] C. Corbier, F. Della Seta, G. Branlant, *Biochemistry* **1992**, *31*, 12532–12535.
[29] J. I. Harris, M. Waters in *The Enzymes, Vol. 13* (Ed.: P. D. Boyer), Academic Press, New York, **1976**, pp. 1–49.

Activity of 2′,5′-Linked RNA in the Template-Directed Oligomerization of Mononucleotides**

Thazha P. Prakash, Christopher Roberts, and Christopher Switzer*

The ability of 2′,5′-linked nucleic acids to encode genetic information has been a matter of both experimental and theoretical interest. Whether 2′,5′-linked nucleic acids form helices had been debated[1] until relatively recently, when 2′,5′-linked oligonucleotides were found to associate.[2] A related issue is the possibility of forming chimeric helices. It has long been known that 2′,5′-linked oligoadenylate forms a triplex with natural oligouridylate.[3] This phenomenon has since been extended to other sequences and structures.[4] These findings raise the possibility of genetic-information transfer with 2′,5′-linked RNA or natural RNA with 2′,5′-linkage defects. Within the context of an enzymatic reaction, Lorsch et al.[5] demonstrated that reverse transcriptase reads through an RNA template bearing a 2′-linkage defect. In the context of non-enzymatic reactions, the ability of isomeric RNA to serve as a template was recently confirmed using the complex mixture of linear and cyclic 2′,5′-linked oligocytidylates that result from clay-catalyzed condensation of activated 5′-CMP.[6] Here we report the first mononucleotide oligomerizations allowing direct comparisons to be made between the properties of natural and 2′,5′-linked RNA templates.



1 2 3 4

Figure 1. Oligonucleotides used in this study. Double-underlined letters are 2′,5′-linked RNA. Single-underlined letters are 3′,5′-linked RNA. Italicized letters are 3′,5′-linked DNA.

Oligonucleotides used to explore template-directed synthesis are given in Figure 1. A schematic illustration of the template-directed reaction investigated in this work is given in Figure 2. The templates are modelled on hairpin-templates reported by Wu and Orgel.[7] As noted below and in earlier work,[7] incorporation of DNA into the stem and loop of the templates enables assay of product phosphodiester linkages (3′,5′ versus 2′,5′) with RNase. To assess whether the DNA stem perturbs the conformation of the 2′,5′-linked RNA tract in template 1, the CD spectra of 1 and a standard for the RNA tract alone, 2′,5′-C_{12}, were compared. The standard gave an A-helix spectrum, and 1 a spectrum consistent with the superposition of B-helix and A-helix spectra. These observations are consistent with the RNA tract in 1 maintaining its conformational integrity.

[*] Prof. C. Switzer, Dr. T. P. Prakash, C. Roberts
Department of Chemistry
University of California
Riverside, CA 92521 (USA)
Fax: Int. code +(909)787-4713
e-mail: switzer@citrus.ucr.edu

[**] This work was supported by the National Aeronautics and Space Administration [NAGW-4184].

Figure 2. General scheme by which the reaction between templates 1–4 (Figure 1) and 2-MeImpG occurs. The GMP units sequentially add to the 2′/3′-terminus of the template (G^n). G^{n+5} is the full-length product for templates 1 and 2, and G^{n+7} for templates 3 and 4.

Initially the effect of different divalent metal ions on oligomerization of the 2-methylimidazolide of guanosine-5′-phosphate (2-MeImpG) was explored with templates 1 and 2 (Table 1). By far, the most effective divalent metal ion in promoting oligomerization with the 2′,5′-linked RNA bearing template 1 was magnesium. Magnesium ion was also most effective in promoting oligomerization with the 3′,5′-linked RNA template 2, but other metal ions were also effective (notably mercury, zinc, and lead). It is not yet clear why 2′,5′-linked RNA is most active in the presence of Mg^{2+}, although it may be that the metal ion orders activated nucleotides on the template in a kinetically significant way. A representative autoradiogram showing oligomerization products obtained after eight days with 1 is given in Figure 3. On the basis of time-dependent data and comparison with the product of 2 (data not shown), the predominant product in lane 2 appears to result from incorporation of four ribonucleotides. To ensure that the observed reactions were indeed template dependent, 1 and 2 were incubated with 2-MeImpA. With this monomer no more than 10% of

Table 1. Oligomerization with templates 1 and 2 and different metal ions (100 mM in all cases) for eight days. Percentages refer to the amount of starting template remaining unconsumed by oligomerization. See the legend to Figure 3 for further details.

Metal ion	Template 1 [%]	Template 2 [%]
$MgCl_2$	27.4	1.44
$HgCl_2$	84.5	18.4
$ZnCl_2$	73.8	36.7
$CoCl_2$	76.6	61.4
$MnCl_2$	80.1	92.4
$UrO_2(NO_3)_2$	76.6	58.2
$Pb(NO_3)_2$	71.3	43.7
$Ni(NO_3)_2$	80.7	75.4

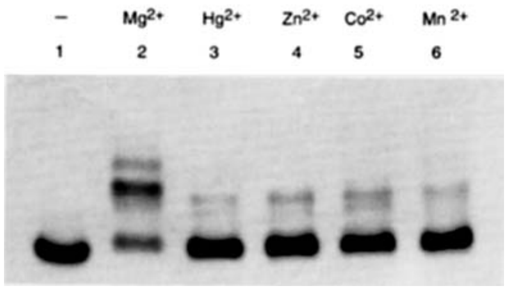


Figure 3. Autoradiogram of the products formed upon oligomerization of 2-MeImpG with template 1 in the presence of 100 mM divalent metal ion after eight days. All oligomerizations were conducted under the general conditions described by Wu and Orgel [7].