Mechanistic Studies of the Biosynthesis of 3,6-Dideoxy Sugars: Stereochemical Analysis of C-3 Deoxygenation†,[1]

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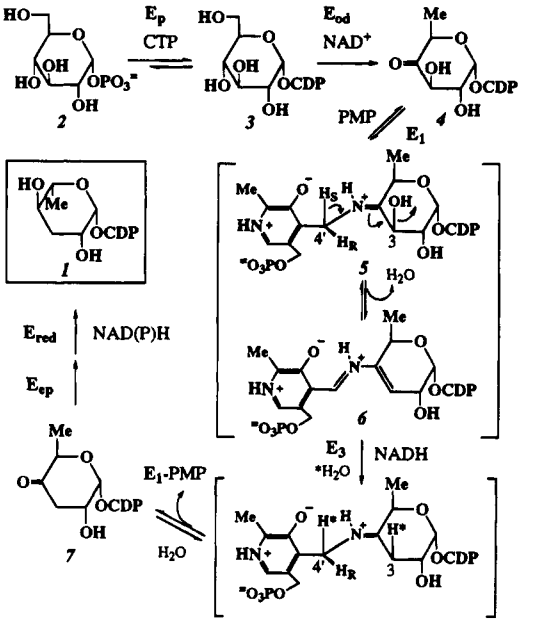
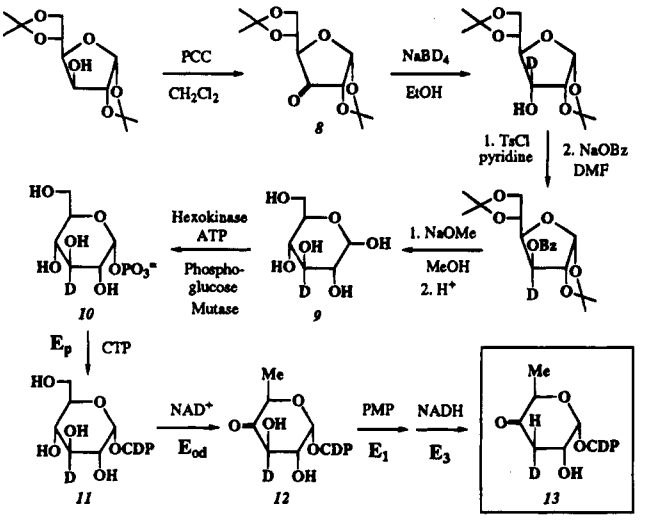
Deoxy sugars are ubiquitous in nature and contribute to a diverse repertoire of biological activities.[2] Among the vast number of dideoxyhexoses found in nature, the 3,6-dideoxyhexoses have attracted particular attention due to their high immunogenicity.[3] Investigation of the formation of CDP-ascarylose, a 3,6-dideoxy-L-*arabino*-hexopyranose (**1**), has established the general biosynthetic pathway of this class of monosaccharides,[4] in which the initial steps involve the conversion of α-D-glucose 1-phosphate (**2**), via CDP-D-glucose (**3**), to **4** (Scheme 1).[5] This stable intermediate is then converted to **7** in two consecutive enzymatic reactions mediated by CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose-3-dehydrase (E_1), a pyridoxamine 5′-phosphate (PMP)-linked iron−sulfur-containing catalyst,[6] and CDP-6-deoxy-$\Delta^{3,4}$-glucoseen reductase (E_3), a [2Fe-2S]-containing flavoprotein.[7] Early studies have shown that E_1-catalyzed C−O bond cleavage is in fact a reversible dehydration.[6c] Since the deprotonation and reprotonation at C-4′ of the cofactor−substrate adduct (**5/6**) are both *pro-S* stereospecific, it has been suggested that E_1 has the *si* face (C-4′) of this complex exposed to solvent and accessible to active-site catalytic groups.[6c] While these C-4′ stereochemical studies support the role postulated for the PMP cofactor in the proposed mechanism, it should be noted that the stereochemical course of C-3 displacement has never been determined. Thus, whether the active site of E_1 indeed shares features similar to those of other PLP/PMP-linked enzymes in controlling the orientation of the cofactor−substrate complex remains in question. Reported herein are the results of our recent stereochemical analysis of this displacement and the conclusion concerning the overall course of this unique deoxygenation. In addition, the implications of these results on the biosynthesis of other deoxy sugars are discussed.

Scheme 1



Scheme 2



Our original plan to determine the C-3 stereochemistry relied on the conversion of the E_1/E_3 product **7** to a mixture of perbenzoylated methyl glycosides which, after HPLC separation, were subjected to NMR analysis and the results compared with those earlier acquired with labeled standards.[8] However, despite numerous attempts, the low amounts of samples retrieved from such lengthy manipulation had rendered this approach impractical. Hence, a strategy for examining the E_1/E_3 product directly without further derivatization was adopted. Crucial to the stereochemical assignment is the availability of the C-3 deuterium-labeled substrate **12**, which was prepared by following a sequence delineated in Scheme 2. Most of the steps were based on well-documented procedures,[5,9,10] in which the labeling at C-3 was introduced by the reduction of **8** with sodium

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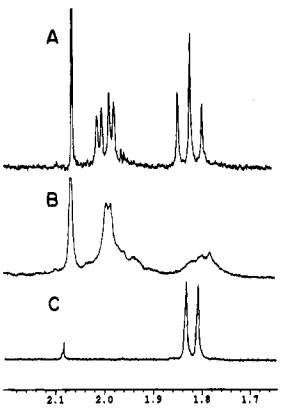

Figure 1. ^{1}H NMR spectra showing the C-3 methylene region of CDP-3,6-dideoxy-D-*glycero*-D-*glycero*-4-hexulose (**7**): (A) unlabeled standard, (B) **13** derived from incubation of **11** in normal buffer, and (C) **7** derived from incubation of **3** in deuterated buffer. Although the H-3_{eq} signal in spectrum B is relatively broad, its disappearance in spectrum C clearly indicates that the displacement is highly stereospecific with retention of configuration.

borodeuteride.[11] Conversion of the labeled glucose to its CDP derivative was accomplished by a commonly used method to make nucleoside diphosphohexoses. As shown in Scheme 2, treatment of **9** with hexokinase, phosphoglucomutase, and inorganic pyrophosphatase in the presence of α-D-glucose 1,6-diphosphate gave **10**, which was then incubated with CTP and the purified E_p[5] to furnish the desired CDP-[3-^{2}H]-D-glucose (**11**). Upon incubation with E_{od},[4d,5] **11** was swiftly transformed to the requisite substrate **12**. While **12** could be isolated by C_{18} HPLC using a 0−30% linear gradient between buffers A (20 mM triethylamine phosphate, pH 5.5) and B (50% A and 50% acetonitrile), the purified sample was complicated by partial racemization. Fortunately, the nearly quantitative conversion later achieved by using excess E_{od} rendered this step unnecessary. Thus, **12** generated *in situ* was used directly in E_1/E_3 incubation.[12]

A typical mixture consisting of CDP-D-glucose (15 μmol) and E_{od} (0.9 mg, 99 units)[4d,5] in 800 μL of 50 mM potassium phosphate buffer (pH 7.0) was incubated for 45 min at 25 °C. To this solution was added E_1 (5.8 mg, 330 units),[4d,6e] E_3 (0.17 mg, 5 units),[7d] NADH (15 μmol), PMP (100 nmol), and the incubation was continued for another 90 min at 25 °C. The enzymes were then removed by Centricon-10, the filtrate was lyophilized, and the dry residue was redissolved in deuterated water for NMR analysis.[13] As shown in Figure 1A, the two C-3 diastereotopic hydrogens of the unlabeled species are well resolved as two multiplets at δ 1.99 (dd, J = 12.5, 5.0 Hz) and 1.82 (t, J = 12.5 Hz).[14] The characteristic splitting patterns and coupling constants allowed unambiguous assignment of the downfield peak as H-3_{eq} and the upfield signal as H-3_{ax}. Interestingly, the upfield resonance was greatly diminished in the NMR of the sample derived from incubation of **12** with E_1/E_3 (Figure 1B), and the downfield peak was simplified to a broad doublet. A complementary experiment was also performed in deuterated phosphate buffer (pD 6.6) with all of the enzyme solutions exchanged via repetitive Centricon concentration prior to actual incubation. As expected, the δ 1.99 signal of the product **13** disappeared, concurrent with the δ 1.82 peak changing to a doublet (J = 12.5 Hz) (Figure 1C). These results evidently established that the C-3 displacement proceeds with retention of configuration. The same stereochemical preference has been noted for all PLP-dependent enzymes that catalyze β-elimination/replacement reactions.[15]

Previous stereochemical studies of E_1-catalyzed C-4′ deprotonation/reprotonation of **5/6** have revealed that E_1 has the *si* face (C-4′) of the complex **5/6** exposed to solvent and accessible to active-site catalytic groups.[6c] The retention of configuration at C-3 revealed in this work clearly indicates that the departure of the hydroxy group and the addition of a solvent hydrogen at C-3 occur at the same side. This result lends further credence for a single reactive face of **5/6** within E_1's active site. The bond cleavage and formation at C-3 and C-4′ are thus expected to occur suprafacially on the same (*si*) face of the Schiff base complex (**5/6**). As a result, the dehydration catalyzed by E_1 is likely a *syn* elimination, and the conjugated π-system in the nascent product **6** may have a cisoid geometry.[15] The stereochemical congruity of E_1 and other PLP/PMP enzymes suggests that E_1 behaves as a normal coenzyme B_6-dependent catalyst and its mediated dehydration follows the well-established PLP/PMP cofactor chemistry. This result also supports Dunathan's hypothesis that this class of enzymes, regardless of the catalytic diversity within the group, has evolved from a common progenitor.[16] It is worth noting that mechanistic studies related to the biosynthesis of other dideoxyhexoses are scarce.[4e] Limited information is currently available only for 2,6-dideoxy-D-*threo*-4-hexulose of granaticin[17] and 2,6-dideoxy-D-*arabino*-hexose of chlorothricin.[18] However, the observation of the formation of these two 2,6-dideoxy sugars with opposite stereochemical modes of OH replacement by H at C-2 suggests that C-2 deoxygenases may lack the stereochemical convergence found for PLP/PMP-linked enzymes. Instead, these C-2 deoxygenases may be divided into at least two classes.[18,19] The possible existence of two classes implies that the postulated mechanism of the 2,6-dideoxyhexose formation may no longer be solely grounded on the 3,6-dideoxy sugar pathway. Interestingly, this conclusion derived from stereochemical arguments appears to be concordant with an early mechanistic conjecture based on comparison of the ascarylose gene cluster with those of 2,6-dideoxyhexose-containing antibiotics.[20]

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(12) NMR of **4** (D_2O): δ 7.83 (1H; d, J = 7.5 Hz; cytidine-H) and 6.00 (1H; d, J = 7.5 Hz; cytidine-H); 5.85 (1H; d, J = 4.5 Hz; ribose H-1); 5.42 (1H; dd, J = 7.0, 3.5 Hz; H-1); 4.21 (1H; dd, J = 5.5, 5.0 Hz; ribose H-2); 4.17 (1H; dd, J = 5.0, 4.5 Hz; ribose H-3); 4.16−4.13 (2H; m; ribose H-4, 5); 4.09−4.06 (1H; m; ribose H-5); 3.96 (1H; q, J = 6.5 Hz; H-5); 3.64 (1H; d, J = 10.0 Hz; H-3); 3.49 (1H; ddd, J = 10.0, 3.5, 3.0 Hz; H-2); 1.08 (3H; d, J = 6.5 Hz; 5-Me).

(13) It was essential to use excess enzymes to curtail the incubation time and to trim further sample manipulation to minimize possible racemization at carbons 3 and 5. While the product **7** was separable from **4** on C_{18} HPLC under conditions described in the text, it was unfortunately eluted along with NAD^+. However, **7** and NAD^+ exhibited base-line separation on a Partisil SAX 10 column eluted with 30 mM potassium phosphate buffer, pH 7.0. Thus, the progress of the reaction could be monitored by the combination of the above two HPLC assays.

(14) NMR of **7** (D_2O): δ 7.84 (1H; d, J = 7.5 Hz; cytidine-H) and 5.99 (1H; d, J = 7.5 Hz; cytidine-H), 5.85 (1H; d, J = 4.0 Hz; ribose H-1); 5.36 (1H; dd, J = 7.0, 3.5 Hz; H-1); 4.44−4.07 (5H; m; ribose H-2, 3, 4, 5); 3.89 (1H; q, J = 6.5 Hz; H-5); 3.77−3.73 (1H; m; H-2); 1.99 (1H; dd, J = 12.5, 5.0 Hz; H-3_{eq}); 1.82 (1H; t, J = 12.5 Hz; H-3_{ax}); 1.03 (3H; d, J = 6.5 Hz; 5-Me). Since NAD^+ was not removed from the NMR sample, its signals were also discernible.

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