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COMMUNICATION

A fluoro analogue of UDP-α-D-glucuronic acid is an inhibitor of UDP-α-D-apiose/UDP-α-D-xylose synthase†‡

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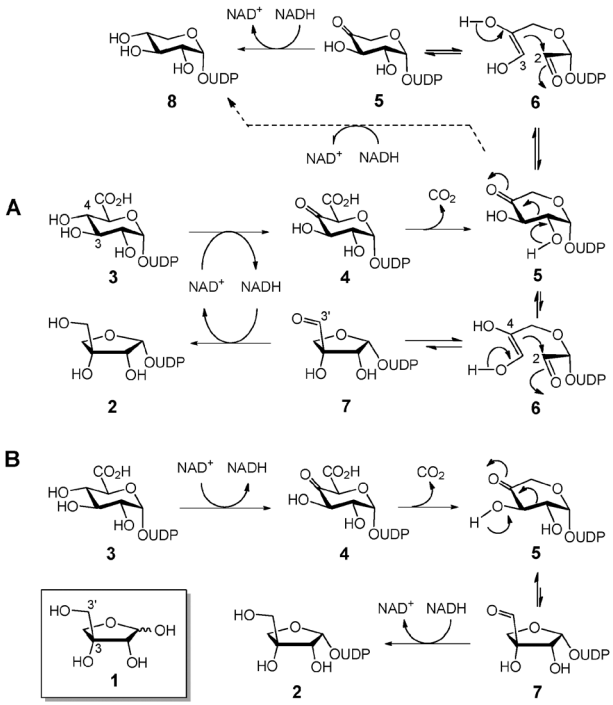
UDP-2F-glucuronic acid was synthesized and analyzed as a mechanistic probe to investigate the ring contraction step catalyzed by UDP-D-apiose/UDP-D-xylose synthase (AXS).

D-Apiose (3-*C*-hydroxymethyl-D-erythrose, **1**) is a sugar found in the pectic polysaccharides rhamnogalacturonan-II (RG-II)[1] and apiogalacturonan,[2] which are components of the plant cell-wall. It is a five-carbon furanose sugar carrying a hydroxymethyl group at *C*-3. This branched-chained furanose structure is rare in nature and has been observed in only a small number monosaccharides, such as streptose and hamamelose, in addition to apiose. The D-apiose residues are important for plant growth and development, because they crosslink the RG-II polysaccharides through the formation of a borate tetraester.[3] The significance of such crosslinks is demonstrated by the dwarfed phenotype of *mur1* mutated plants, in which the L-fucose residue within RG-II is replaced by L-galactose thereby inhibiting the crosslinking of the polysaccharides.[3]

The building block of D-apiose in the cell-wall polysaccharides is UDP-D-apiose (**2**),[4,5] which is derived directly from UDP-α-D-glucuronic acid (UDP-GlcA, **3**). The conversion of UDP-GlcA to UDP-apiose is catalyzed by a single enzyme, UDP-D-apiose/UDP-D-xylose synthase (AXS).[5] This transformation is NAD^+-dependent and UDP-D-xylose (**8**) can also be formed during the reaction.[5] The proposed reaction sequence (Scheme 1) involves oxidation of **3** by NAD^+ to form UDP-4-ketoglucuronate (**4**), which then undergoes decarboxylation to afford a 4-ketoxylose intermediate (UDP-4-KX, **5**). Subsequent ring contraction (**5** → **7**) followed by reduction at *C*-3′ by NADH yields the UDP-D-apiose product.

Two plausible mechanisms for the rearrangement step have been proposed.[6] As shown in Scheme 1, bond-cleavage between *C*-2/*C*-3 of **5** in a retroaldol reaction (mechanism A) could yield an enediol intermediate (**6**), which, after recyclization between *C*-4/*C*-2, would give **7**. Since these steps are reversible,



Scheme 1 Two proposed mechanisms for the biosynthesis of UDP-D-apiose catalyzed by AXS.

reduction of the resulting aldehyde moiety in **7** by NADH is essential to drive the reaction to completion and to regenerate the NAD^+ coenzyme. The formation of UDP-apiose (**2**) and UDP-xylose (**8**) during catalysis can be rationalized by the fact that both *C*-3 and *C*-4 in **6** could act as the nucleophile in the recyclization step.

A pathway involving a 1,2-bond shift (**5** → **7**, mechanism B) is also conceivable for the ring contraction step. In contrast to the stepwise retroaldol–aldol route, the C–C bond migration in this concerted mechanism would require deprotonation of *O*-3 and bypass formation of a discrete intermediate such as **6**. In both mechanisms, however, the formation of UDP-xylose (**8**) could result from a premature "capture" of the decarboxylated 4-keto intermediate **5** by NADH, prior to the rearrangement step.

We envisioned that a fluorinated analogue of UDP-GlcA, UDP-2-fluoro-2-deoxyglucuronic acid (UDP-2F-GlcA, **9**), could be a useful probe to study the mechanism of AXS. As depicted in Scheme 2, upon incubation, compound **9** may be

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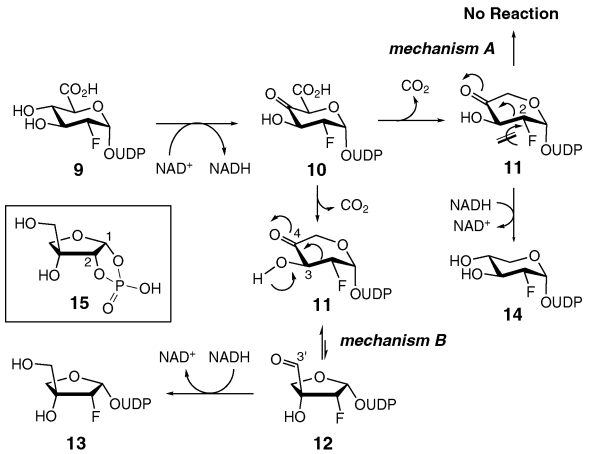
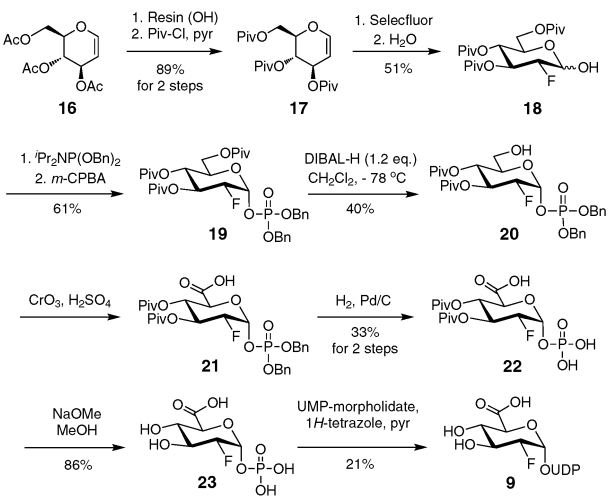


Scheme 2 Mechanistic scenario for UDP-2F-GlcA.

converted to a 4-ketointermediate (**11**) by oxidative decarboxylation. If AXS employs the 1,2-shift mechanism (mechanism B), then compound **11** may be turned over to give UDP-2F-apiose (**13**) as the product. However, if the retroaldol/aldol mechanism (mechanism A) is operative, no apiose product is expected due to the absence of a hydroxyl group at the *C*-2 position of **11**. A likely product in this case is the fluorinated xylose analogue **14**. Thus, product analysis of the incubation mixture could shed light on the mechanism of the AXS-catalyzed reaction.

Synthesis of compound **9** starting from the commercially available tri-*O*-acetyl-D-glucal (**16**) was accomplished following the reaction sequence shown in Scheme 3. According to a literature procedure,[8] the *O*-acetyl protecting groups in **16** were first replaced by sterically larger pivaloyl groups (**16** → **17**) to enhance the stereoselectivity of the subsequent *C*-2 fluorination reaction, which was carried out using Selectfluor.[9] A dibenzyl phosphate group was then introduced at *C*-1 by treatment of **18** with dibenzyl *N*,*N*-diisopropylphosphoramidite and 3-chloroperbenzoic acid (*m*-CPBA).[10] Selective deprotection of the primary pivaloyl group of **19** by reduction with DIBAL-H, followed by Jones oxidation and hydrogenation, yielded the phosphate compound **22**. The remaining pivaloyl groups were



Scheme 3 Synthetic scheme for compound **9**.

deprotected using NaOMe, and the resulting monophosphate **23** was coupled to UMP using UMP-morpholidate[11] to complete the synthesis of **9** (overall yield after 10 steps: 7%).

To test whether UDP-2F-GlcA (**9**) can be processed by AXS, UDP-2F-GlcA (400 μM) was incubated with AXS (10 μM) and NAD^+ (400 μM) in 50 mM Tris buffer (pH 8.0) at room temperature. To our disappointment, no new peak was observed by HPLC over a 40 min incubation period (Fig. S1, ESI‡). To determine whether UDP-2F-GlcA actually binds to AXS, a competition assay was performed, in which UDP-GlcA (**3**, 400 μM) was incubated with AXS (same conditions mentioned above) in the presence of two different concentrations of **9** (400 μM and 4 mM). An identical incubation without **9** was also carried out as a control. As shown in Fig. S2–S4 (ESI‡), UDP-2F-GlcA (**9**) showed inhibition of AXS against **3** in a concentration-dependent manner. These results suggest that compound **9** can be recognized by AXS and likely binds to the same active site.

The fact that UDP-2F-GlcA is not a substrate for AXS makes it unsuitable for the proposed mechanistic studies of AXS (Scheme 1). The inability of AXS to effect the initial dehydrogenation of the *C*-4 hydroxyl group of **9** is likely a consequence of inductive destabilization of the partial positive charge developed at *C*-4 during the oxidation step by the 2-fluoro substituent, although we had hoped to avoid such a destabilization by designing the 2-F rather than a 3-F analogue. Poor positioning of **9** in the active site of AXS could also prevent efficient hydride transfer.

Although no new product was found when **9** was incubated with AXS, an apparent shift of reaction flux was noted when **9** was included in the incubation with UDP-GlcA (**3**). As shown in Fig. 1, two product peaks could be detected by HPLC of the reaction of AXS with **3**. The peak with a retention time of 17 min corresponds to UMP, which derives from the decomposition of UDP-D-apiose (**2**) to form apiose 1,2-cyclic phosphate (**15**, $t_{\frac{1}{2}} \approx 100$ min).[7] The second peak appearing at 25 min

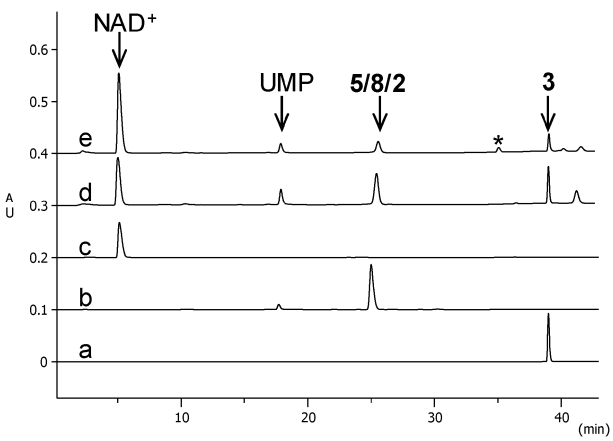


Fig. 1 HPLC traces of the AXS reaction. (a) UDP-GlcA (**3**) standard, (b) UDP-xylose (**8**) standard, (c) NAD^+ standard, (d) the reaction mixture containing 10 μM AXS, 400 μM NAD^+ and 400 μM UDP-GlcA (**3**) in 50 mM Tris buffer (pH 8.0) was incubated for 12 h, (e) excess H_2NOH was added to (d) and the mixture was incubated for an additional 3 h. The 35 minute peak (*) was isolated and analyzed by MS.

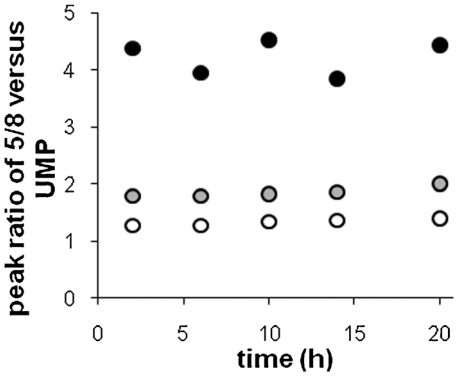
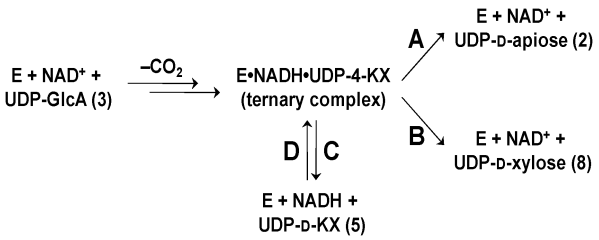


Fig. 2 The ratio of **5**/**8** *versus* UMP produced by AXS as determined by HPLC peak integrations is dependent on the concentration of the inhibitor, UDP-2F-GlcA (black: 4 mM **9**, grey: 400 μM **9**, white: no inhibitor).



Scheme 4 Partitioning of the E·NADH·UDP-4-KX ternary complex.

corresponds to a mixture of three components: UDP-D-xylose (**8**), the residual UDP-apiose (**2**), and UDP-4-KX (**5**), the latter of which can be released from the active site during turnover. A similar phenomenon has also been reported for several other NAD^+-dependent enzymes such as UDP-galactose 4-epimerase,[12] CDP-tyvelose 2-epimerase,[13] and RsU4kpxs from *Ralstonia solanacearum*.[14] Partial separation of the components of this peak was achieved by treatment of the reaction mixture at the end of incubation with hydroxylamine, which reacts with **5** to afford the corresponding 4-oxime adduct (see Fig. 1e and ESI‡). Since **5** and **8** are relatively stable while **2** is readily decomposed to UMP and **15**, analysis of the peak integrations of the HPLC peaks at 25 and 17 min provided an estimate of the relative concentrations of **2**/**5**/**8** (mainly **5** and **8**), and **2**, respectively. As shown in Fig. 2, the ratio of the two peaks at 25 and 17 min increases in the presence of higher concentrations of inhibitor **9**, suggesting an increase in the partitioning between direct reduction of UDP-4-KX (**5**) to form UDP-D-xylose (**8**) *versus* the rearrangement of UDP-4-KX to UDP-apiose (**2**, Scheme 4, B *vs.* A). However, model analysis indicates that it is more likely a consequence of simple competitive inhibition by **9** *versus* both the normal substrate (**3**) and the released UDP-4-KX (**5**) intermediate. In this scenario, partitioning of the ternary complex containing UDP-4-KX between release of UDP-4-KX *versus* formation of products (Scheme 4, C *vs.* A,B) would be unaffected by the presence of **9**. However, this would not be true for the binding back of free UDP-4-KX to complete the reaction (Scheme 4, D), which would have to compete with **9** to do so. This would then result in an increase in the HPLC peak ratio observed during the time course of the experiment.

In summary, we report the synthesis and analysis of UDP-2F-GlcA (**9**) as a potential mechanistic probe to investigate the mechanism of ring contraction catalyzed by AXS. Although compound **9** turned out not to be a substrate for AXS, it was identified as an inhibitor. The observed change in the distribution of products and the 4-keto-intermediate (**5**) as a function of inhibitor concentration is most likely the result of competitive inhibition between **9** and both **5** as well as **3**. This observation is reminiscent of the ability of *myo*-inositol 1-phosphate synthase to bind redox-altered mechanistic intermediates and analogues with the unproductive redox form of the nicotinamide cofactor to form dead-end complexes competitively *versus* the substrate.[15] Attempts to gain greater insight regarding the mode of inhibition by UDP-2F-GlcA in order to better understand the mechanism of the AXS-catalyzed reaction are in progress.

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