

Enzyme Catalysis

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Isotope Probing of the UDP-Apiose/UDP-Xylose Synthase Reaction: Evidence of a Mechanism via a Coupled Oxidation and Aldol Cleavage

Thomas Eixelsberger, Doroteja Horvat, Alexander Gutmann, Hansjörg Weber, and Bernd Nidetzky*

Abstract: The C-branched sugar D-apiose (Api) is essential for plant cell-wall development. An enzyme-catalyzed decarboxylation/pyranoside ring-contraction reaction leads from UDP- α -D-glucuronic acid (UDP-GlcA) to the Api precursor UDP- α -D-apiose (UDP-Api). We examined the mechanism of UDP-Api/UDP- α -D-xylose synthase (UAXS) with site-selectively ^2H -labeled and deoxygenated substrates. The analogue UDP-2-deoxy-GlcA, which prevents C-2/C-3 aldol cleavage as the plausible initiating step of pyranoside-to-furanoside conversion, did not give the corresponding Api product. Kinetic isotope effects (KIEs) support an UAXS mechanism in which substrate oxidation by enzyme-NAD⁺ and retro-aldol sugar ring-opening occur coupled in a single rate-limiting step leading to decarboxylation. Rearrangement and ring-contracting aldol addition in an open-chain intermediate then give the UDP-Api aldehyde, which is intercepted via reduction by enzyme-NADH.

Uridine 5'-diphosphate (UDP)- α -D-apiose (**1**) is the precursor of the C-branched pentose D-apiose [3-C-(hydroxymethyl)-D-glycero-tetrose; **2**].^[1] Compound **2** is present in the cell-wall polysaccharides rhamnogalacturonan II and apiogalacturonan, as well as in various secondary metabolites in plants.^[1–4] Sugar nucleotide **1** is derived from UDP- α -D-glucuronic acid (**3**) in a decarboxylation/pyranoside ring-contraction reaction catalyzed by UDP- α -D-apiose/UDP- α -D-xylose synthase (UAXS).^[5] The proposed mechanism of this

chemically intriguing biotransformation (Scheme 1) involves nicotinamide adenine dinucleotide (NAD⁺)-assisted oxidation at C-4 of substrate **3** and subsequent decarboxylation to give UDP- β -L-threo-pentopyranosid-4-ulose (**4**).^[5–7] Carbon skeleton rearrangement (**4**→**5**→**6**) then occurs probably via a retro-aldol/aldol reaction,^[6] and reduction of UDP- α -D-apiose 3'-aldehyde (**6**) by enzyme-NADH gives **1**.^[5–7] The alternative reaction product, UDP- α -D-xylose (**7**), is derived from **4**, also by NADH-dependent reduction. UDP- α -D-xylose synthase (UXS) is structurally and mechanistically related to UAXS, but lacks the ability to catalyze the pyranoside-into-furanoside conversion.^[8] A plausible point of divergence in the proposed pathways of UAXS and UXS is therefore intermediate **4**.

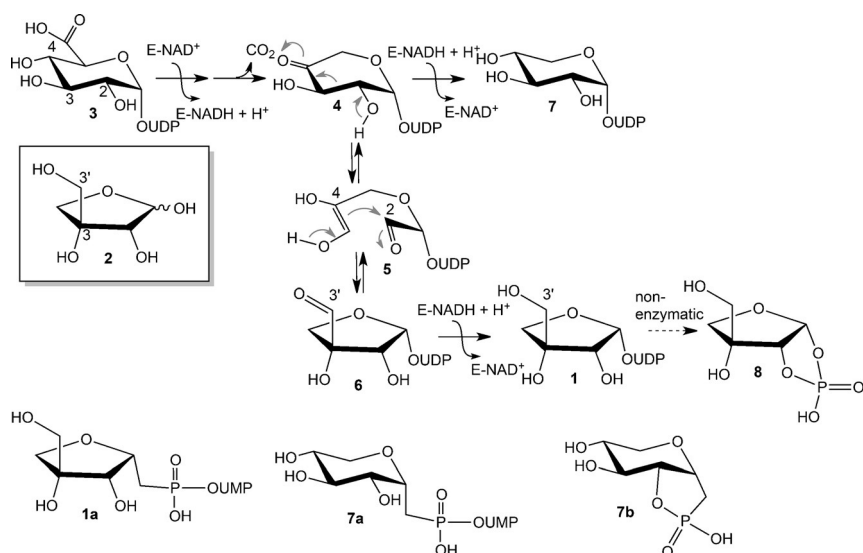
Its widespread acceptance in the literature notwithstanding,^[5–7] the mechanism of Scheme 1 raises disquiet in that it requires UAXS to recognize **4** equally for aldol ring cleavage and for reduction by NADH. How the enzyme distinguishes between these possibilities is not clear. Moreover, there is only indirect evidence in support of the retro-aldol/aldol route of conversion of **4** into **6**. A 2-deoxy-2-fluoro analogue of **3**, rendering impossible the C-2/C-3 aldol cleavage in a corresponding 2-fluoro derivative of **4**, was completely unreactive with UAXS.^[6a] A chemically stable phosphonate analogue of **1** (Scheme 1, **1a**) was converted by UAXS into the corresponding xylosyl compound. A xylose cyclic phosphonate (**7b**) instead of the expected product **7a** (Scheme 1) was formed. This was interpreted to involve an enzymatically deprotonated C-2 hydroxy group, which would also be involved in the “native” retro-aldol conversion of **4**.^[6b] The current study was performed to re-investigate the catalytic steps for conversion of substrate **3** into **1** and **7**. Evidence supporting an updated mechanism, involving retro-aldol ring opening prior to the decarboxylation, is presented.

Purified UAXS from *Arabidopsis thaliana* recombinantly expressed in *Escherichia coli* was used (Figure S1 in the Supporting Information). Reactions were performed at pH 8.5 because the enzyme activity was highest (Table S1, Supporting Information) and the ratio of product **1** to **7** maximized under these conditions. No intermediary products (e.g., **4**)^[6b,7] were released. Under the conditions used, α -D-apiofuranosyl-1,2-cyclic phosphate (**8**; Scheme 1) was spontaneously formed from **1**.^[5,7] Product **1** was therefore always detected as **8**. Site-selectively ^{13}C - or ^2H -labeled analogues of substrate **3** were synthesized from the corresponding isotopically labeled D-glucoses (Scheme S1, Figure S2–S11).^[9a] Unlabeled **3** was prepared identically and used as a reference. A 2-deoxy analogue of **3** was synthesized from 1,5-anhydro-2-deoxy-D-arabino-hex-1-enitol via 2-deoxy-D-glucose-1-phos-

[*] Dr. T. Eixelsberger, D. Horvat, Dr. A. Gutmann, Prof. Dr. B. Nidetzky
Institute of Biotechnology and Biochemical Engineering
Graz University of Technology, NAWI Graz
Petersgasse 12, 8010 Graz (Austria)
E-mail: bernd.nidetzky@tugraz.at
Prof. Dr. H. Weber
Institute of Organic Chemistry
Graz University of Technology, NAWI Graz
Stremayrgasse 9, 8010 Graz (Austria)
Prof. Dr. B. Nidetzky
Austrian Centre of Industrial Biotechnology (acib)
Petersgasse 14, 8010 Graz (Austria)

Supporting information (the coding gene used (Figure S29–S30); the preparation of the enzymes used; the substrate synthesis and characterization; the methods of analysis; and determination of the KIEs) and the ORCID identification number(s) for the author(s) of this article can be found under <http://dx.doi.org/10.1002/anie.201609288>.

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Scheme 1. The proposed mechanism of UAXS; E = enzyme.^[5–7]

phate, exploiting the reaction of cellobiose phosphorylase (Scheme S2, Figure S12–S18).^[9b] Each compound was isolated and its identity confirmed by ^1H and ^{13}C NMR spectroscopy. Purity was furthermore determined by capillary electrophoresis and HPLC.

In situ NMR analysis of enzymatic reactions of $[2-^{13}\text{C}]\text{-3}$ and $[3-^{13}\text{C}]\text{-3}$ demonstrated rearrangement of the carbon skeleton on formation of **1** as expected from Scheme 1. Despite being confirmatory mainly,^[5–7] the evidence was nonetheless crucial for it enabled the precise assignment of all the ^{13}C signals from substrate and products (Figure S19–S24). Reaction of unlabeled **3** in D_2O resulted in a single deuterium from solvent to be incorporated at C-4 of **1** and C-5 of **7** as result of the decarboxylation, consistent with literature.^[5d,e] Reactions of $[3-^2\text{H}]\text{-3}$ and $[4-^2\text{H}]\text{-3}$ both gave $[3'-^2\text{H}]\text{-1}$, as expected from Scheme 1,^[5e] however with clearly distinct $\text{C}^2\text{H}^1\text{H}$ groups at position 3' (Figure 1A, panels a,b; Figure S25). Because reduction of **6** by the hydrogen or deuterium abstracted from C-4 is stereospecific,^[5d,e] the absolute configuration at C-3' in **1** will be opposite in the two conversions. Irrespective of whether reaction of unlabeled **3** was examined in D_2O or reaction of $[3-^2\text{H}]\text{-3}$ in water, no $^1\text{H}/^2\text{H}$ exchange was observed at position 3' (Figure 1A, panels c,d; Figure S25). The result was important mechanistically for it validated the determination of kinetic isotope effects (KIEs) through the deuteration at C-3 of **3**. Studying the UAXS from parsley or duckweed, however, with a relatively complicated and indirect procedure of product analysis, other authors reported uptake of $0.5 \text{ mol } ^3\text{H mol}^{-1}$ in **1** at C-3'.^[5b,e] We rule out a similar exchange with protons from solvent in the enzymatic reaction under the conditions we used.

2-Deoxy-**3** was examined as a substrate of UAXS for it prevents the C-2/C-3 retro-aldol cleavage to initiate pyranoside-into-furanoside ring conversion. Compared to 2-deoxy-2-fluoro-**3** used earlier with the same rationale,^[6a] 2-deoxy-**3** features only weak electronic perturbation at the position 2,

thus rendering it a mechanistic probe of the enzyme in its own right. Because preliminary experiments suggested UAXS to be inactive towards 2-deoxy-**3**, we also tested UXS and demonstrated enzymatic conversion to give 2-deoxy-**7** as the product (Figure 1B, Figure S26–S28). Noticing that UAXS might be inhibited by UDP/UMP released from 2-deoxy-**3** due to decomposition, we re-examined UAXS under addition of alkaline phosphatase to hydrolyze the nucleosides present. Synthesis of a tiny amount of 2-deoxy-**7** was shown under these conditions (Figure 1B).

No evidence of 2-deoxy-**1** was found, as expected. However, while UXS exhibited substantial activity with 2-deoxy-**3** (Figure 1B; Figure S26), the UAXS activity was almost completely destroyed ($\leq 0.1\%$ of activity with **3**) on substituting the 2-OH in the substrate by a hydro-

gen atom. These results are important mechanistically showing that 2-deoxy-**3** was fully competent to undergo oxidative decarboxylation in the UXS-type reaction, plausibly via 2-deoxy-**4**. Assuming intermediate **4** to be the point of divergence in the UAXS and UXS reaction paths, the huge difference in activity of the two enzymes in forming 2-deoxy-**7** was somewhat counterintuitive. Note that only a small change in the reaction conditions (e.g. pH, ion type and concentration) is sufficient to shift the distribution of UAXS reaction products to favor **7**, while at the same time the overall activity is just moderately affected (data not shown; see also Ref. [5f]). We therefore hypothesized that the pyranoside ring-opening characteristic of UAXS might not be as clearly decoupled from the oxidation–decarboxylation common to both enzymatic conversions as the mechanistic proposal of Scheme 1 assumes. Intermediate **4** might be formed through a different path in UAXS than in UXS, and we designed a KIE study to address the problem. It will become clear below that the minute conversion of 2-deoxy-**3** by UAXS is probably a relic of UXS-type activity in this enzyme, which considering the relatedness of UAXS and UXS at the level of amino acid sequence^[10] is not a complete surprise.

We determined the effect of $[4-^2\text{H}]\text{-3}$ on the rate of substrate consumption (V_s). Direct comparison of V_s with *protio* and *deuterio* substrates at saturating concentration gave a large KIE of 2.72 ± 0.20 ($N = 5$). The KIE measured by intermolecular competition was also high and its value (2.47 ± 0.43) was similar to the KIE on k_{cat} (Table S2). Contrary to the directly determined KIE, which influences the catalytic constant (k_{cat}), the KIE from the competition experiment necessarily influences the second-order rate constant ($k_{\text{cat}}/K_{\text{ms}}$).^[11] Scheme 2 is used for interpretation of the data.

Whereas k_{cat} involves all the unimolecular steps of the reaction, $k_{\text{cat}}/K_{\text{ms}}$ includes only those steps up to the first irreversible step, which in UAXS is the decarboxylation. The large KIE on $k_{\text{cat}}/K_{\text{ms}}$ implies that hydride abstraction from substrate is partly rate limiting for the steps covered by $k_{\text{cat}}/$

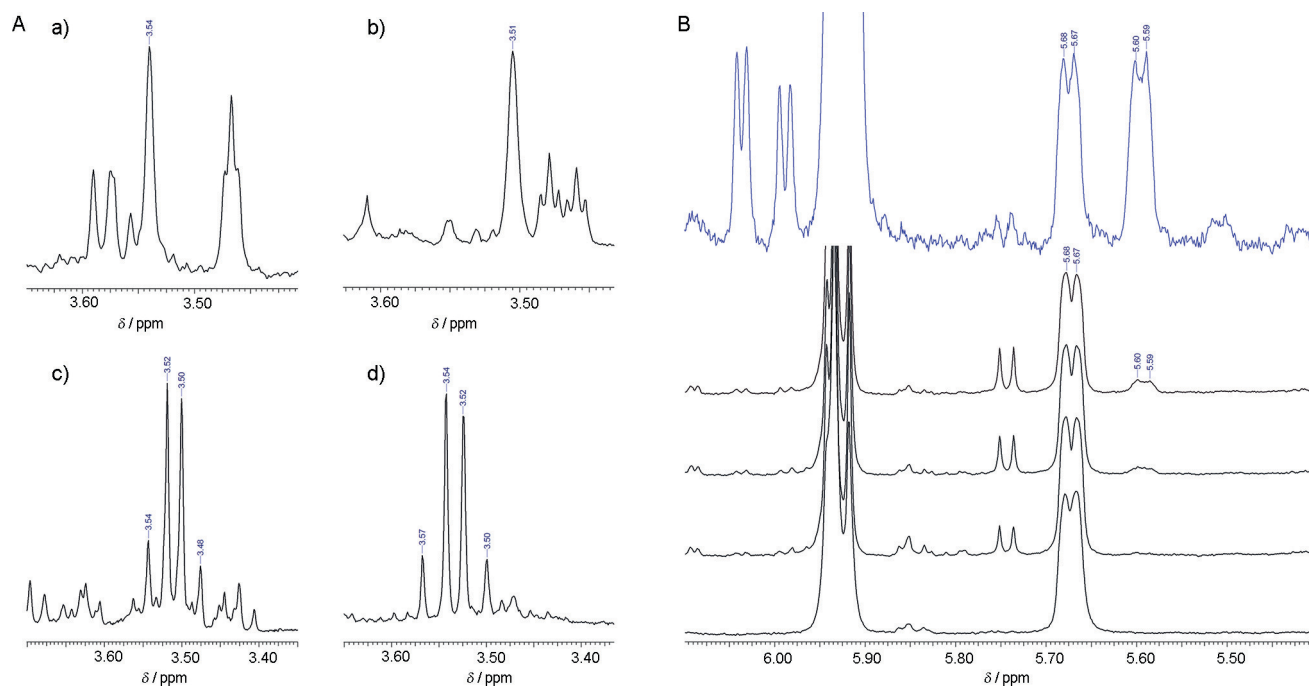
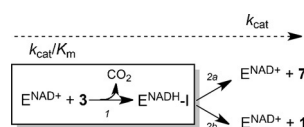


Figure 1. A) Formation of product **1** (detected as **8**) by UAXS. a), b) ^1H NMR signal of the 3'-H in **8** obtained from $[3\text{-}^2\text{H}]\text{-3}$ (a) and $[4\text{-}^2\text{H}]\text{-3}$ (b). Both reactions were carried out in H_2O (pH 8.5). c), d) ^1H NMR signal of 3'-H in **8** obtained from unlabeled **3** in D_2O (c) and H_2O (d). In panels (a)–(d), the product was analyzed directly from the reaction mixture. Enzyme: $20\ \mu\text{M}$; substrate **3**: $2\ \text{mM}$; $\text{pH(D)} = 8.5$. B) Formation of 2-deoxy-**7** by UAXS and UXS. In blue: the ^1H NMR spectrum of a reaction mixture of 2-deoxy-**3** ($\delta = 5.67\ \text{ppm}$, $5.68\ \text{ppm}$) converted partially into 2-deoxy-**7** ($\delta = 5.59\ \text{ppm}$, $5.60\ \text{ppm}$) by UXS. The black stack plot shows an in situ ^1H NMR experiment of the conversion of 2-deoxy-**3** ($\delta = 5.67\ \text{ppm}$, $5.68\ \text{ppm}$) by UAXS. It shows that 2-deoxy-**7** ($\delta = 5.59\ \text{ppm}$, $5.60\ \text{ppm}$) is formed in small amounts. UXS: $20\ \mu\text{M}$; UAXS: $100\ \mu\text{M}$; substrate 2-deoxy-**3**: $2\ \text{mM}$. Reactions were performed in D_2O ($\text{pD} = 8.5$) for 2 h (UXS) and over 12 h with spectra recording in 2 h intervals (UAXS).



Scheme 2. A minimal kinetic mechanism of UAXS is shown. “I” represents an enzyme-bound intermediate suggested to be the acyclic form **5**. A primary deuterium KIE could arise in steps 1, 2a and 2b. The sequence of reaction steps included in $k_{\text{cat}}/K_{\text{m}}$ is shown in the box.

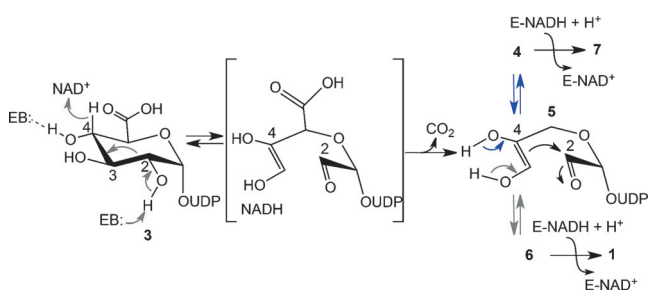
K_{mS} . The UAXS reaction involves three isotope-sensitive steps (Scheme 1 and Scheme 2) and each could contribute to the KIE on k_{cat} . We measured under rapid-mixing conditions by absorbance at $340\ \text{nm}$ the reduction of enzyme-bound NAD^+ . NADH did not accumulate in detectable amounts, which it would if the two hydride transfers from NADH were limiting for k_{cat} . This result together with the evidence that $k_{\text{cat}}/K_{\text{mS}}$ and k_{cat} were affected by a similar KIE, identified hydride abstraction from substrate to NAD^+ as major rate-determining step of the UAXS reaction. Although hydride transfers from enzyme- NADH were not slow steps of the overall reaction, the ratio of the products **1** and **7** was nonetheless affected by $[4\text{-}^2\text{H}]\text{-3}$. The ratio changed from a value of 1.84 with undeuterated **3** to a lower value of 1.48 with $[4\text{-}^2\text{H}]\text{-3}$ (Table S3). Therefore, this suggested a larger deuterium KIE on reduction of **6** than on reduction of **4**. Moreover, the result demonstrated the ability of UAXS to reversibly interconvert intermediates **4** and **6**, in good agree-

ment with the results of Liu and co-workers,^[6b] and to rapidly equilibrate their enzyme-bound forms.

Next, we determined in intermolecular competition experiments the effect from $[3\text{-}^2\text{H}]\text{-3}$ on $k_{\text{cat}}/K_{\text{mS}}$. We considered that a KIE would be a secondary one, arising from hybridization change at C-3 during sugar ring opening and closure. And additionally, because substrate oxidation was a rate-limiting step, a KIE different from unity would be possible only in the case that the reaction step(s) affecting C-3 happened within the rate-determining cascade of oxidation and decarboxylation. Starting from a substrate composed initially of roughly equal amounts of **3** and $[3\text{-}^2\text{H}]\text{-3}$, we determined by NMR spectroscopy at three different levels of conversion the $^1\text{H}/^2\text{H}$ isotope ratio at C-3 in the remaining substrate. A KIE of 1.20 ± 0.03 was obtained (Table S4), indicating that $[3\text{-}^2\text{H}]\text{-3}$ reacted significantly more slowly than **3**. The observed effect was in the upper region of the KIEs previously reported from enzyme-catalyzed aldol/retro-aldol reactions.^[12] It would be consistent with a fully developed secondary KIE resulting from complete $\text{sp}^3 \rightarrow \text{sp}^2$ hybridization change between the ground state and the rate-limiting transition state of a retro-aldol carbon-carbon bond cleavage for enzymatic pyranosyl ring opening. It may be noted that secondary deuterium KIEs of similar magnitude were observed in other enzymes catalyzing aldol reactions.^[12] Additionally we measured the $^1\text{H}/^2\text{H}$ isotope ratio at C-3' in **1** and C-3 in **7** at approximately 50% conversion and found its value of approximately 1.2 to be the same in both products

and to reflect exactly the corresponding isotope ratio (i.e. $3\text{-}^2\text{H}/3\text{-}^1\text{H}$) in the residual substrate (Table S5). These results indicate that reaction steps after the decarboxylation involving hybridization change at C-3, namely the reversible aldol addition to give **4** and the reduction of **6**, did not have a significant secondary KIE. Had there been a KIE in one of these steps, the isotope ratio in **1** and **7** would not have been the same, and would also have been different from that in the **3** converted.

Taken together, the primary and secondary KIEs also suggest a relative timing of substrate oxidation and ring opening. A distinctly slow retro-aldol reaction occurring after the oxidation at C-4 would likely make the hydride transfer to NAD^+ come to equilibrium. This scenario is inconsistent with the large primary KIEs observed. Therefore, a concerted transformation is supported in which hydride abstraction and ring opening take place coupled one to another in a single rate-determining reaction step (Scheme 3). Following decar-



Scheme 3. Updated mechanistic proposal for UAXS. “EB” indicates an enzyme base in the active site.

boxylation, ring closure would thus yield intermediates **4** and **6**, probably in rapid equilibrium, which are then reduced to products **7** and **1**, respectively.^[13] This way of product formation seems attractive as it avoids the dual use of intermediate **4** as substrate for enzymatic aldol cleavage and for NADH -dependent reduction. Selective stabilization of the acyclic intermediate after the decarboxylation could be a catalytic strategy of UAXS to facilitate rapid interconversion of the more stable cyclic forms **4** (in particular) and **6**. In this mechanistic scenario, formation of 2-deoxy-**7** from 2-deoxy-**3** in the absence of ring opening is considered a minor side activity of UAXS.

In summary, KIE and substrate analogue studies suggest an updated UAXS mechanism (Scheme 3). Aldol cleavage for pyranosyl ring opening is proposed to occur early in the reaction, concerted with the oxidation at C-4. The reactions of UAXS and UXS would not, therefore, proceed on the same path up to intermediate **4**.^[5–7] Formation of the two UAXS products **1** and **7** involves reduction by enzyme- NADH at distinct “exit points” of the aldol/retro-aldol rearrangement cycle in rapid equilibrium. The UAXS mechanism, although highly specialized, possesses fundamental significance, as other C-branched carbohydrates, such as L-streptose (3-C-formyl-5-deoxy-L-lyxose),^[14] L-dihydrostreptose (3-C-hydroxymethyl-5-deoxy-L-lyxose),^[14] D-hamamelose (2-C-hydroxymethyl-D-ribose),^[11] and aceric acid (3-C-carboxy-5-

deoxy-D-xylose)^[11] might be formed via mechanistically similar transformations.

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Conflict of interest

The authors declare no conflict of interest.

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