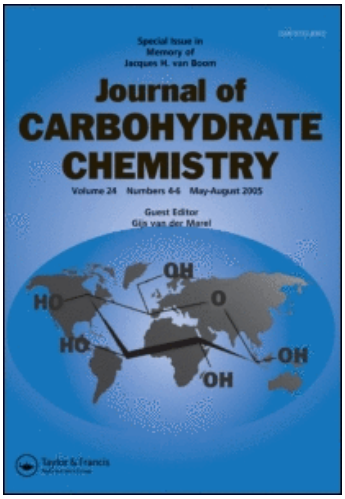

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D-XYLOSE (D-GLUCOSE) ISOMERASE (EC 5.3.1.5): OBSERVATIONS AND COMMENTS CONCERNING STRUCTURAL REQUIREMENTS OF SUBSTRATES AS WELL AS MECHANISTIC FEATURES

Herwig Häusler[a]; Hansjörg Weber[a]; Arnold E. Stütz[a]
[a] Glycogroup, Institut für Organische Chemie, Technische Universität Graz, Graz, Austria

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D-XYLOSE (D-GLUCOSE) ISOMERASE (EC 5.3.1.5): OBSERVATIONS AND COMMENTS CONCERNING STRUCTURAL REQUIREMENTS OF SUBSTRATES AS WELL AS MECHANISTIC FEATURES

Herwig Häusler, Hansjörg Weber, and Arnold E. Stütz*

Glycogroup, Institut für Organische Chemie, Technische Universität
Graz, Stremayrgasse 16, A-8010 Graz, Austria

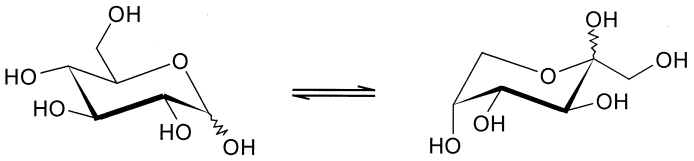
ABSTRACT

In the course of investigations into the applicability of xylose isomerase as a reagent for carbohydrate synthesis, some questions raised in context with structural requirements for productive substrates as well as mechanistic considerations have been addressed. Amongst the interesting findings obtained so far is the observation that the enzyme requires two vicinal hydroxyl groups, either at positions C-3 and C-4 or at C-4 and C-5, for productive binding/catalysis. Furthermore, two apparently different reaction pathways occur in parallel when certain aldopentoses as well as selected aldohexoses are offered as substrates, this explaining the recently observed formation of epimeric aldoses together with the expected ketoses observed upon extended reaction times.

INTRODUCTION

D-Xylose (D-glucose) isomerases are large scale industrial products employed for the multi-ton conversion of D-glucose into high fructose corn syrup (Scheme 1).[1] Several strains of *Streptomyces*, *Bacillus* and *Lactobacillus* as well as other microorganisms were discovered to produce these enzymes.[2]

Their pH-optimum was determined to be around pH 8 and the range of activity spans from pH 5 to pH 12. Enzyme activities from a variety of sources were found to be dependent on either magnesium, manganese, or cobalt ions while cal-

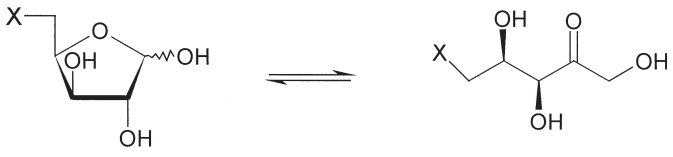


Scheme 1.

cium and some divalent transition metal ions such as copper, iron, and nickel were found to inhibit their activities. Xylose isomerases are exceptionally stable enzymes and can be employed at temperatures between 70 and 85°C for limited periods of time.[1] However, enzyme stability is found to decrease dramatically in this temperature range. Consequently, industrial processes are conducted around 60°C. Recently, enzymes allowing higher reaction temperatures of up to 95°C have been reported.[3]

D-Xylose isomerases found in microorganisms such as *Lactobacillus brevis*[4] and *Bacillus coagulans*[5] were also able to convert D-ribose into D-ribulose. A further extension of substrates was reported for D-xylose isomerase from *Streptomyces albus*, which was also able to isomerise D-allose, L-arabinose and L-rhamnose into the corresponding ketoses.[6]

Due to their high tolerance towards non-natural substrates, several interesting applications of xylose isomerases in carbohydrate and natural products chemistry have been realised.[7,8,9,10] Transition metal ion mediated catalysis of a hydride shift from C-2 of the open-chain aldose to C-1 of the corresponding open-chain ketose and *vice versa* is the currently widely accepted mechanism of this family of enzymes (Scheme 2).[11] However, despite many efforts, this reaction pathway has remained a matter of discussion.[12] In earlier investigations it was found that typical representatives of microbial xylose isomerases were able to convert C-5-modified D-xylose (**1**) [8a] and D-ribose (**2**) derivatives[10] into the corresponding ketopentoses (**3** and **4**, respectively). In addition, a wide range of D-glucose derivatives modified at positions C-3 (**5**),[13] C-5 (**6**),[9a,13] or C-6 (**7**)[7,13] as well as dimodified derivatives bearing non-natural substituents at C-3 and C-6 (**8**)[8] or C-5 and C-6 (**9**) are accepted as substrates.[9b,c] In the L-series, C-5-modified L-idofuranoses (**10**)[9a] as well as L-talofuranoses (not depicted)[10b] were discovered to be substrates yielding the corresponding ketohexoses (**11**–**16**, Scheme 2).



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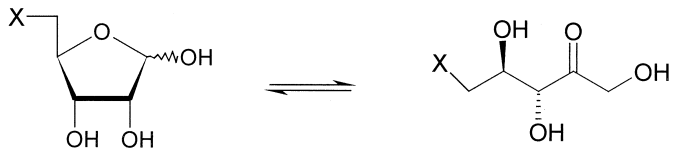

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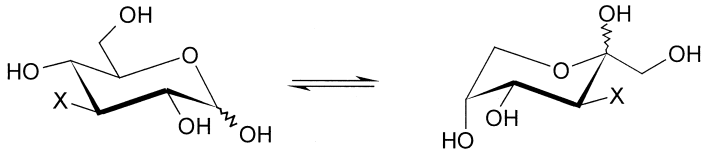

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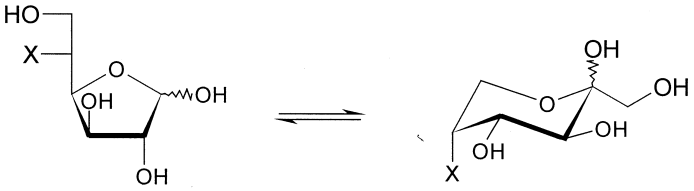

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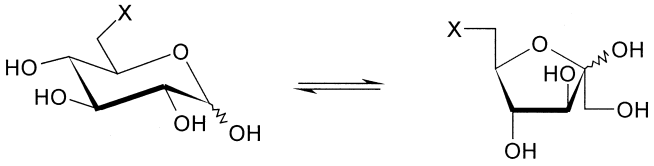
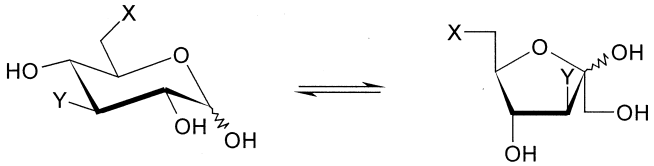


7 **13**



8 **14**

Scheme 2.

(continued)

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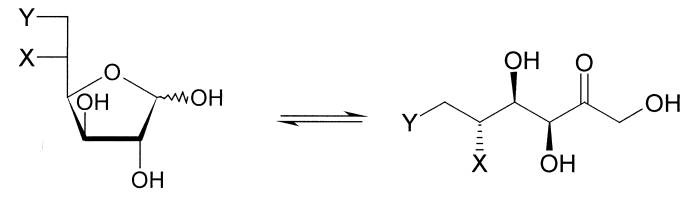

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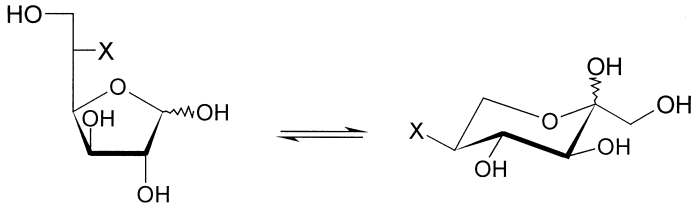
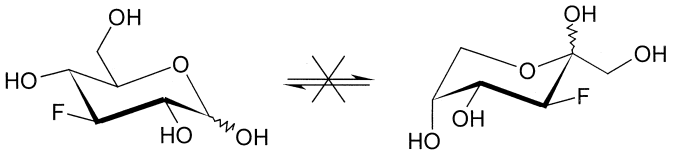


10 **16**

X, Y: for example, OMe, OBn, H, F, N_3

Scheme 2. Continued.

Furthermore, it was found that neither 3-deoxyfluoro-D-glucose (**17**) nor the corresponding ketose, 3-deoxyfluoro-D-fructose (**18**), were isomerised[8a,11h] in contrast to, for example, 3-deoxy-D-ribohexose ("3-deoxy-D-glucose") which gave around 40% conversion into the corresponding ketose.[13]



17 **18**

In recent work it was observed that the isomerisation of D-ribose (**19**) in the presence of high enzyme concentrations (soluble D-xylose isomerase from *Streptomyces rubiginosus* as well as Sweetzyme T, an immobilised form of the enzyme from *Streptomyces murinus*) and after reaction times of at least 12 hours not only led to the expected D-ribulose (**20**) but gave also considerable amounts of D-arabinose (**21**) which practically linearly increased with the reaction time (Scheme 3).[12a]

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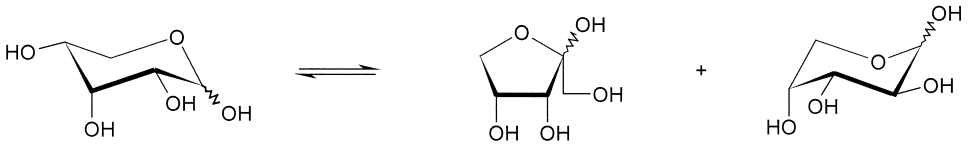


19 **20** **21**

Scheme 3.

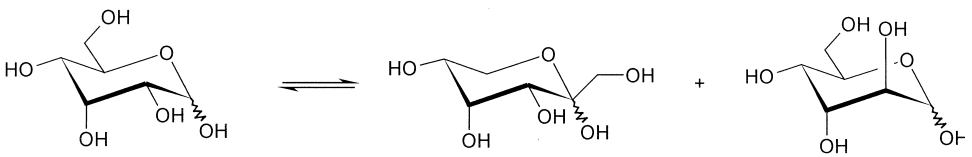
Other sugars such as L-ribose and D-lyxose behaved in a similar manner furnishing the corresponding aldose epimers at C-2 as side products.[12a] These findings were interpreted as being caused by an ene-diol pathway based enzyme mechanism. In the hexose series, D-psicose (**23**) and D-altrose (**24**) were found to be formed when D-allose (**22**) was employed as substrate (Scheme 4).[12b]

D-Idose (**25**) was rapidly converted into D-sorbose (**26**) and the same was true when D-gulose (**27**) was exposed to the soluble enzyme (Scheme 5).[12b]

RESULTS AND DISCUSSION

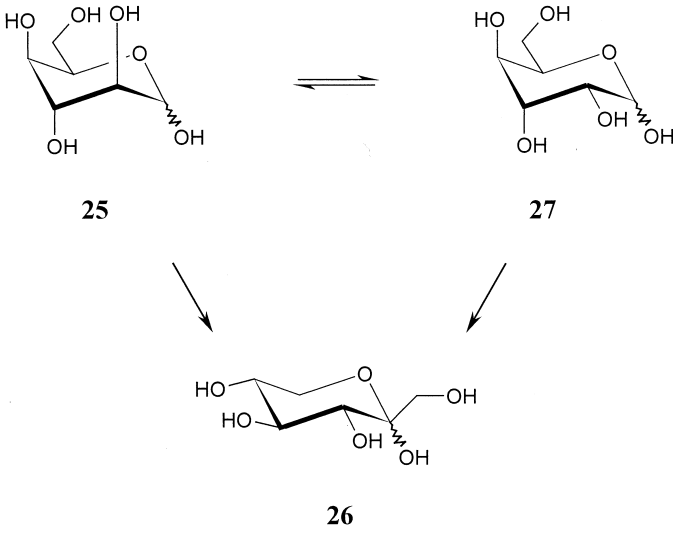
Structural Requirements of Substrates

From the increasing number of natural and, in particular, non-natural substrates of D-xylose isomerase (EC 5.3.1.5), a high tolerance of this enzyme towards structural variations is clearly evident. X-ray data of enzymes from various sources show that O-1, O-2, O-4 and O-5 of D-xylose as well as of D-glucose are involved in interactions with the active site during the catalytic process.[14] O-3 of both sugars as well as O-6 of D-glucose apparently do not take part in the reaction. D-Allose can be accepted depending on the source of the xylose isomerase employed.[6,10b,12b] In the range of substrates lacking hydroxyl groups and/or bearing non-natural substituents such as fluoro or azido groups at one or more positions the following trends have been observed: Modifications at C-6[7] such as deoxy, azidodeoxy or deoxyfluoro as well as modifications at C-3[13] (other than deoxyfluoro)[8a,11h] and their combinations[8] are tolerated. (Apart from 3-deoxyfluoro-D-allose which behaves like deoxyfluoro-D-glucose, to the best of our knowledge, D-allose derivatives with non-natural substituents at C-3 have not been probed, to date. Nevertheless it is



22 **23** **24**

Scheme 4.



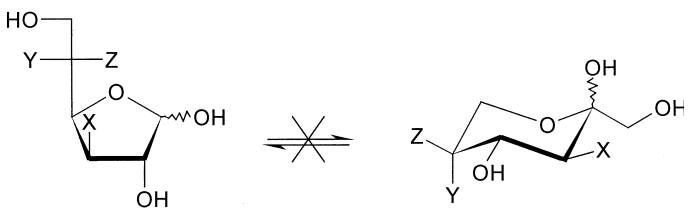
25 27

26

Scheme 5.

fairly safe to assume that most of such compounds would also be substrates, provided 5-OH is present—*vide infra*).

Modifications at C-5 (other than 5-epi[13]) are a special case as both the D-*gluco* as well as the L-*ido* configured aldofuranoses are quantitatively converted into the corresponding thermodynamically distinctly more stable ketopyranoses.[9a,13] The latter, for the same reason, cannot be transformed into the corresponding aldofuranoses (neither with the enzyme nor by other means of catalysis such as common basic or acidic conditions). Isomerisation was not found to take place with any 3,5-dimodified D-gluco- (**28**) or L-idofuranose (**29**) probed thus far (Scheme 6; X,Y or X,Z: various combinations of OBn, OMe, H, F, N_3).[15] These sugars remained stable for several hours or, in case of simple deoxy derivatives such as 3,5-dideoxy-D-*erythro*-hexofuranose, decomposed during extended reaction times. The same was found true for 3,5-dimodified D-xylose derivatives. For example, 3,5-di-*O*-methyl-D-xylofuranose (**30**),[16] did not react. Steric reasons for this failure are not obvious because 3-*O*-methyl-D-glucose,[13] 6-azido-6-deoxy-3-



28: Z = H: 29: Y = H

Scheme 6.

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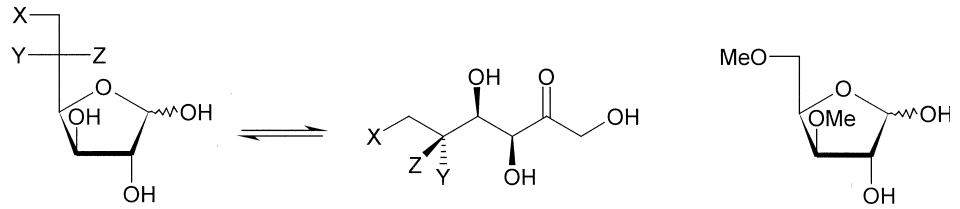


X, Y, Z: Any combination of H, F, N_3 **30**

Scheme 7.

O-methyl-D-glucose[8] as well as 5-*O*-benzyl-D-glucofuranose[9a] had successfully been isomerised.

5,6-Dimodified aldoses of both the D-*gluco* as well as the L-*ido* series isomerise to the corresponding open-chain ketoses which are usually preponderant in the equilibrium (Scheme 7).[9b,c]

In the light of the above observations it is safe to conclude that—apart from the intact functionalities at C-1 and C-2—two vicinal hydroxyl groups, the absolutely essential[13] 4-OH together with *either* 3-OH *or* 5-OH, are the minimum requirement for productive binding/catalysis. 5-OH is believed to be coordinated by a "ring-opening base" such as a histidine but it could be demonstrated that this interaction is not necessary for a catalytically competent enzyme.[11i] Apparently, with C-5-modified substrates, a change of coordination to the active site, now involving O-3, occurs.

Thus, according to our current level of understanding, 3-OH must be present for productive interaction with the enzyme when 5-OH is missing.

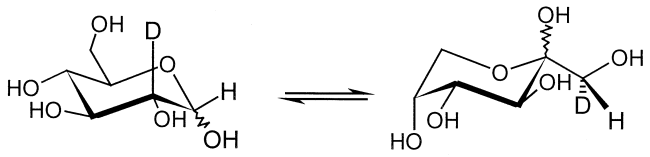
Observations Related to Side Product Formation and Mechanistic Considerations

A reinvestigation into early work on mechanistic details with ^{1}H NMR spectrometric methods employing D-(2-^{2}H)glucose (**31**) and D-(1-^{2}H)glucose (**33**) confirmed observations and conclusions made by Bock and co-workers.[13] Namely, the *pro-R* hydrogen at C-1 of D-fructose or the corresponding deuterium in its (1-^{2}H)-derivative **32** is shuttled between C-1 and C-2 of D-glucose in a perfectly stereoselective manner (Scheme 8).

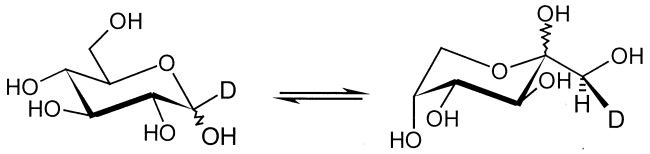
The formation of the respective 1-*C*-deuterated D-fructose derivative could be followed by direct measurements of the reaction mixtures employing solvent suppression methodology (Figure 1).

In an attempt to gain additional information about the interesting reported formation[12a] of D-arabinose from D-ribose, we employed commercially available D-(2-^{2}H)ribose (**34**)[17] as substrate.

When exposed to Sweetzyme T, this pentose gave the expected (1-^{2}H)ribulose **35**. Applying one equivalent by weight of immobilised enzyme, the apparent



31 **32**



33

Scheme 8.

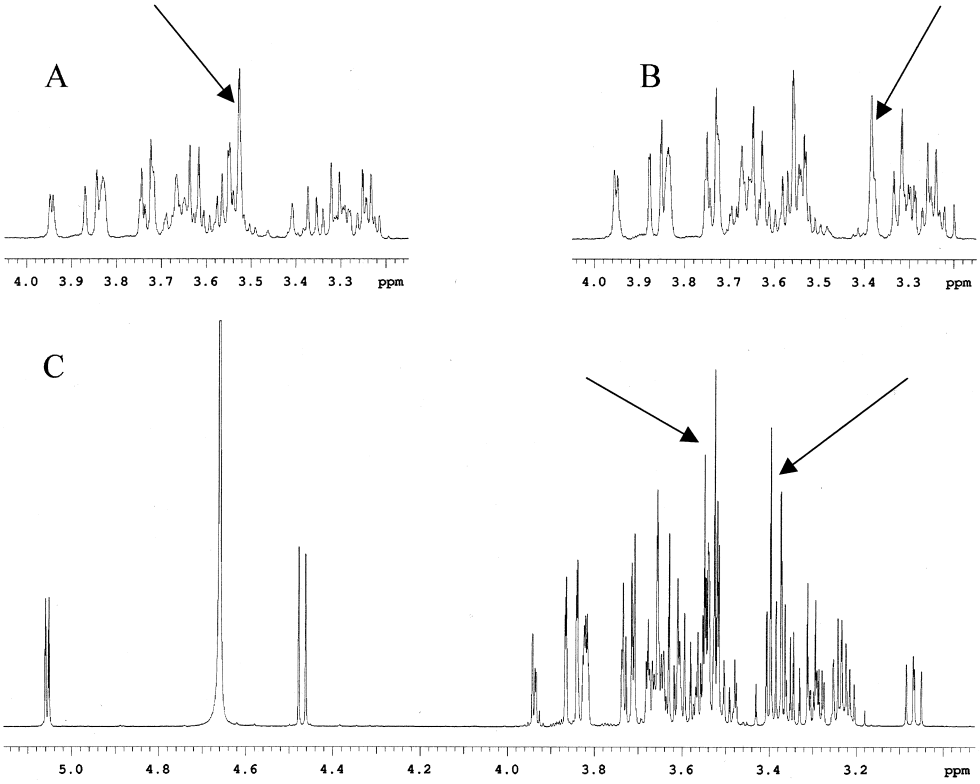


Figure 1. **A)** Isomerisation of (1-^{2}H)-D-glucose. **B)** Isomerisation of (2-^{2}H)-D-glucose. **C)** Isomerisation of D-glucose/D-fructose. Arrows indicate the signals of proton(s) of **A)** the 1*S*, **B)** the 1*R*, **C)** the unsubstituted β-D-fructopyranose tautomer.

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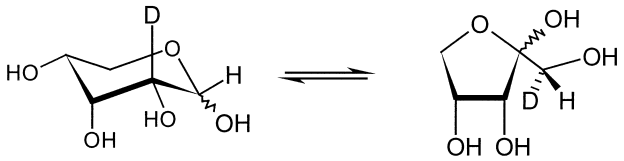
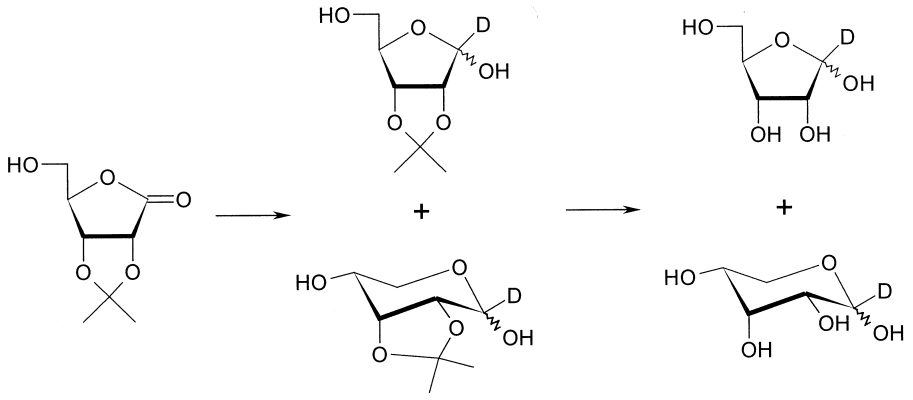


34 **35**

Scheme 9.

equilibrium was reached after 6–8 h. As with D-fructose, the 1-*pro-R* hydrogen was observed to be shifted between C-1 of D-ribulose and C-2 of D-ribose. Arabinose could not be found under these conditions after up to 25 h. Applying ten equivalents by weight of immobilised enzyme, formation of minute amounts of D-arabinose (**21**) could be detected (^{1}H NMR) after 10 h, the proportion of which was found to extremely slowly increase with the reaction time. Interestingly, this product was identified as a mixture of (2-^{2}H)arabinose (H-1 as singlets at 5.06 ppm and 4.35 ppm, respectively) and non-deuterated material [H-1 appearing as doublets (α, 3.6 Hz; β, 7.8 Hz)], slightly favouring the deuterium free sugar (Figure 2). When the enzyme was inhibited by the presence of a large excess of calcium chloride (Ca^{2+}, K_i: 3 μM),[1a,6,11d] neither ribulose nor arabinose were produced from D-ribose over several (up to 30) hours. The same was true when Sweetzyme T was deactivated/denatured[1] by heat treatment (exposed to distilled water at 96°C for 1.5 hours) prior to the addition of substrates.

Interestingly, (1-^{2}H)ribose (**36**), prepared by $NaBD_4$ reduction of 2,3-*O*-isopropylidene-D-ribonolactone in D_2O and subsequent acidic deprotection according to Scheme 10; (>90% isotopic purity by ^{1}H NMR) slowly gave a mixture of D-arabinose and its (2-^{2}H)-derivative, the composition of which strongly favoured (2-^{2}H)arabinose after 72 h (singlets at 5.06 and 4.35 ppm, respectively, Figure 2).



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Scheme 10.

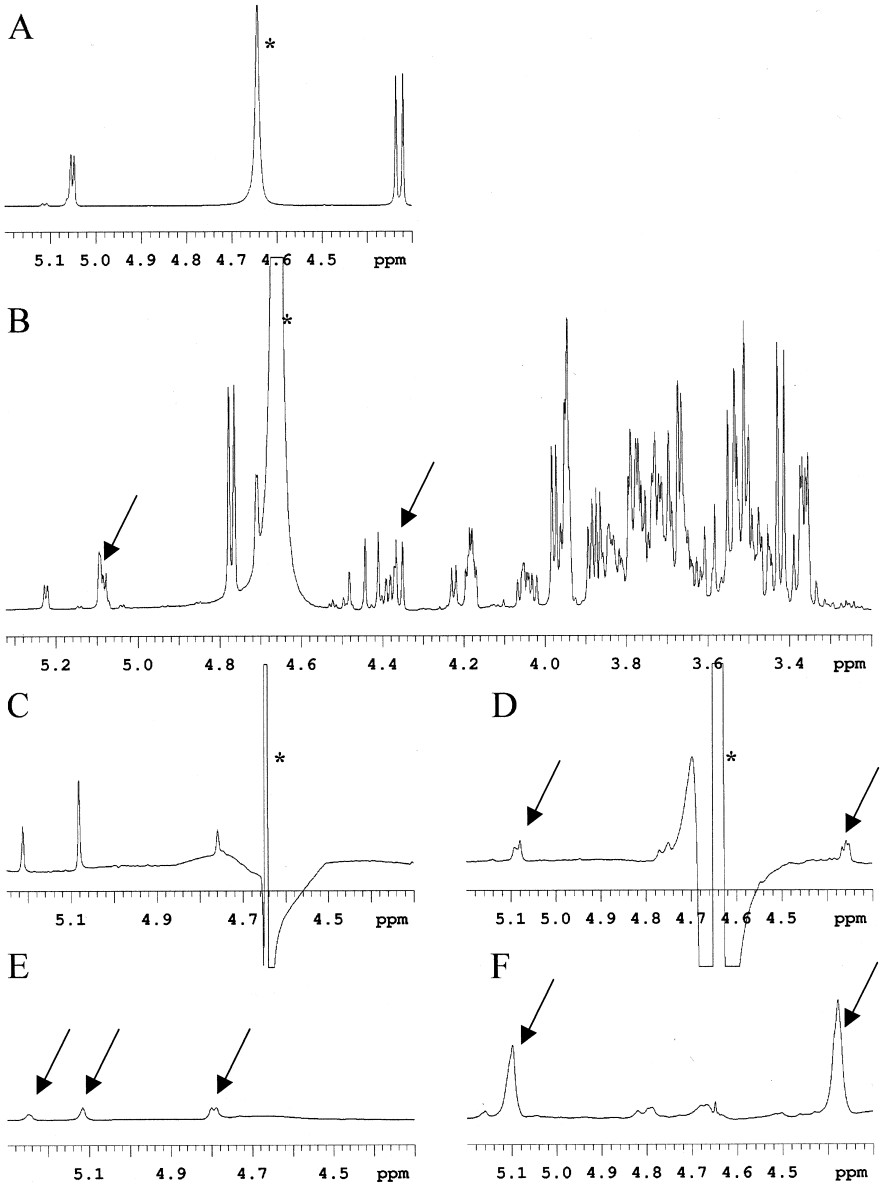

Figure 2. **A)** D-ribose, **B)** Isomerisation of D-ribose with D-arabinose formed (arrows indicate anomeric protons), **C)** D-(2-^{2}H)ribose, **D)** isomerisation of D-(2-^{2}H)ribose with emerging signals of D-arabinose and D-(2-^{2}H)arabinose (arrows), **E)** (1-^{2}H)-D-ribose with residual nondeuterated D-ribose (arrows), **F)** isomerisation of D-(1-^{2}H)ribose with anomeric proton of D-(2-^{2}H)arabinose emerging.
* residual water signal

CONCLUSIONS

The results obtained indicate that the "natural", biochemical reaction proceeds fairly rapidly (as compared to any of the side reactions observed by us and others) by a highly stereoselective intramolecular hydrogen transfer from C-2 of

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the ketose to C-1 of the aldose for both the D-glucose/D-fructose as well as the D-ribose/D-ribulose systems as shown with deuterium substituted substrates. This can more easily be rationalised by an intramolecular hydride transfer as opposed to proton transfer in an ene-diol intermediate which would require strict exclusion of water from the active site to allow for *quasi*-intramolecular $^2H^+$ transfer.

The observed lack of reactivity of 3-deoxyfluorosugars **17** and **18** provides additional support for a "hydride shift related mechanism" in the natural substrates as proton abstraction would be facilitated by the strong inductive effect of the fluorine substituent whereas hydride abstraction from C-2 in 3-deoxyfluoro-D-glucose would occur less likely in the presence of the increased positive partial charge at this carbon as compared to D-glucose. The hydride shift from C-1 of 3-deoxyfluoro-D-fructose to C-2 to form the corresponding aldose, on the other hand, would meet difficulties due to the lack of stabilisation of developing positive charge at C-2 in the proposed[14] transition state.

A fraction of D-arabinose produced from D-ribose in the presence of active enzyme—as concluded in the original account[12a]—was apparently formed by a Lobry de Bruyn—Alberda van Ekenstein rearrangement *via* an ene-diol intermediate as could be demonstrated by the complete loss of deuterium substitution from a proportion of the aldose substrate. Such a reaction could possibly occur by interaction with only one of the two metal ions in the active site. (L-Fucose isomerase, EC 5.3.1.3, for example, contains only one metal ion in its active site and has been reported to catalyse the L-fucose/fuculose transformation along the ene-diol pathway).[18] Conceivably, a general basic environment (such as "basic pockets") on the enzyme's surface and not related to the active site could also be responsible for the epimerisation reaction of D-ribose into its thermodynamically more stable epimer taking into account the prolonged reaction periods employed. Nonetheless, the observed lack of arabinose formation with intact but Ca^{2+} inhibited enzyme does not support this hypothesis.

Disregarding the position of deuterium substitution in the starting D-ribose (C-1 or C-2), a considerable amount of D-arabinose formed contained a deuterium atom at C-2 (Figure 2). This was the main product when D-(1-^{2}H)ribose was the substrate and the minor fraction of D-arabinose formed when D-(2-^{2}H)ribose was isomerised. As none of the feasible ene-diol based reactions and reaction sequences from D-(1-^{2}H)- as well as (2-^{2}H)ribose or (1*R*)- and (1*S*)-D-(1-^{2}H)ribulose would yield this compound, it can be assumed to stem from "production errors" in the active site "occasionally" occurring during isomerisation of the 1-*C*-deuterated ribuloses and becoming statistically significant by accumulation upon extended reaction times. In the D-arabinose formation reactions from both deuterated "intermediates" [i.e., (1*R*)- and (1*S*)-D-(1-^{2}H)ribulose, respectively] the stereochemically "wrong" *pro-S* hydrogen (deuterium) is preferably transferred to the "wrong" *si* face of the ketose carbonyl group leading to more non-deuterated arabinose from the (1*R*)-epimer of (1-^{2}H)ribulose [selectively produced from (2-^{2}H)ribose] and distinctly more (2-^{2}H)arabinose from the corresponding (1*S*)-epimer [stemming from 1-^{2}H)ribose]. It can be envisaged that this occurs by the *normal* enzymatic process on a differently, "upside down" coordinated D-ribulose (Scheme 11).

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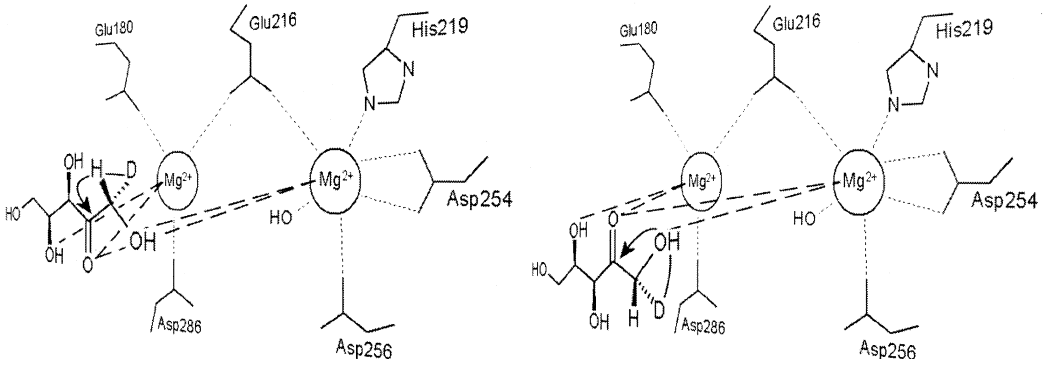


Scheme 11. Outline of interaction[11] of D-xylose isomerase with D-xylulose (right) and "upside-down" binding mode of L-xylulose (and other suitable sugars) as proposed in this section (left). Arrows indicate the hydride/deuteride transfer to the *re*-face (right) and the *si*-face (left) by the same enzymatic process (the dotted lines between Mg ions and O-2 as well as O-4 outline catalytic interactions).

Following this hypothesis, L-lyxose (**37**) should fit even better in the "upside down" mode as the hydroxyl group at C-3 would occupy the same space as the corresponding hydroxyl groups in D-xylose and D-glucose bound in the "normal" orientation.

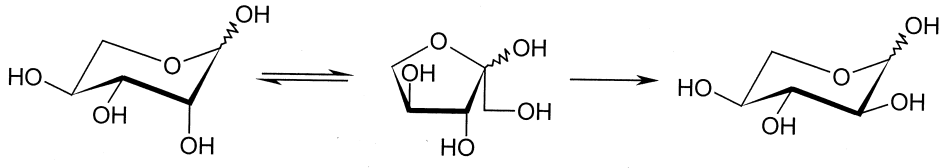
Gratifyingly, isomerisation of L-lyxose (**37**) gave L-xylulose (**38**) and, subsequently, upon extended reaction times, L-xylose (**39**, Scheme 12, Figure 3).

This hitherto unreported interconversion occurred somewhat faster than the formation of D-arabinose from D-ribose further substantiating the above drawn conclusions.

Additional support for the proposed laterally inverted mode of coordination to the enzyme's active site can also be found in the recently reported successful isomerisation of D-gulose (**27**), the (5*R*)-homologue of L-lyxose, into D-sorbose.[12b] as well as in the isomerisation of L-rhamnose.[6]

Collection of additional data including the measurement of isotope effects is clearly indicated to validate this somewhat preliminary interpretation.

The reported formation[12b] of thermodynamically strongly favoured D-sorbose (**26**) from energy-rich D-idose (**25**), attributed to xylose isomerase catalysed



37 **38** **39**

Scheme 12.

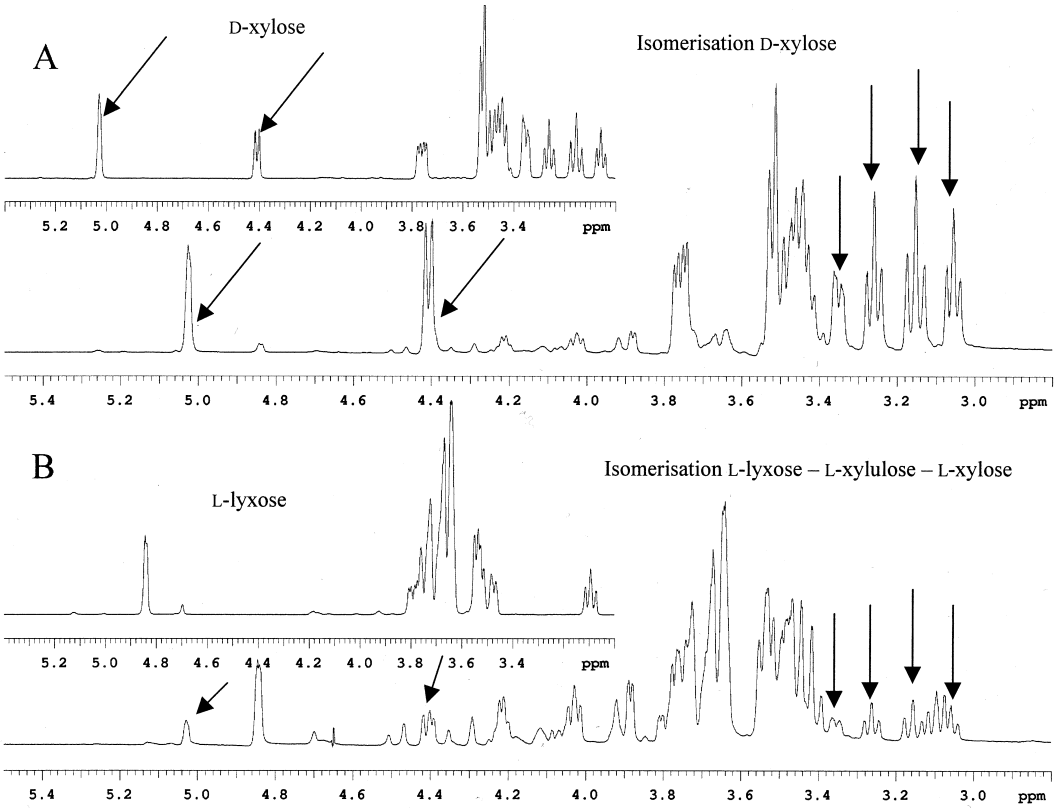


Figure 3. **A)** Spectra of d-xylose (insert) and of the isomerisation of D-xylose (short reaction time). **B)** Spectra of L-xylose (insert) and of the isomerisation, via L-xylulose, into L-xylose. Arrows indicate characteristic matching xylose signals.

isomerisation, would have to be interpreted with care. The irreversible conversion of idose to sorbose has been found to be a spontaneous process [19] even occurring with pure material *in substantia* thus limiting the shelf life of (either enantiomer of) idose. Consequently, for this particular substrate, further experiments are deemed to be important to distinguish between genuine enzymatic catalysis and spontaneous isomerisation under the reaction conditions.

EXPERIMENTAL

General Methods

TLC was performed on precoated aluminum plates (Merck 5554) employing 5% vanillin/sulfuric acid as well as ceric ammonium molybdate as staining agents. For column chromatography, silica gel 60, 230–400 mesh (Merck 9385), was used. ^{1}H NMR spectra were recorded on a Varian INOVA 500 operating at 499.925 MHz employing a transmitter presaturation pulse sequence with a presaturation delay of 1.5 s. Eight scans were accumulated starting with eight steady state scans.

^{13}C NMR spectra were recorded at 75.47 or 50.29 MHz. Residual non-deuterated solvent was used as internal standard for determination of chemical shifts.

General Procedure for Isomerisation Reactions

To a 3–5% solution of the respective aldose, immobilised glucose isomerase (Sweetzyme T from Novo Nordisk, pre-washed with distilled water, 0.5 eq by weight) and aqueous $MgSO_4$ solution (1 g/100 mL, 5 drops) were added and the mixture was stirred or spun on a rotary evaporator at 65°C. For the D-glucose/D-fructose interconversions the reaction time was 1.5 h, the D-ribose/D-ribulose reactions were monitored for 10 to 25 h.

Experiments with Ca^{2+} Inhibited Enzyme

The reactions were performed following the General Procedure but instead of $MgSO_4$, $CaCl_2$ (0.25 eq by weight of starting material) was employed.

Experiments with Denatured Enzyme

Heat treated isomerase (1.5 h at 96°C in distilled water) was employed in the General Procedure. The reactions were monitored for 24 h.

3,5-Di-*O*-methyl-D-xylofuranose (30). To a 5% solution of 1,2-*O*-isopropylidene-α-D-xylofuranose[16]] (700 mg, 3.68 mmol) in THF/DMF (3:1, v/v), sodium hydride (55–65% suspension in mineral oil, 2.5 eq per hydroxyl group) and iodomethane (1.2 eq per hydroxyl group) were added and the mixture was stirred at ambient temperature for 16 h. Excess methanol was added and the mixture was partitioned between dichloromethane and 5% aqueous HCl. The organic layer was washed with aqueous sodium bicarbonate, dried (Na_2SO_4), filtered and concentrated under reduced pressure. A 5% solution of the crude product in 50% aqueous acetonitrile was stirred with Amberlite IR 120 [H^+] at 45°C for 60 h. The resin was removed by filtration and the crude product was purified by chromatography on silica gel to give compound **21** (426 mg, 65%) as a 1:1-mixure of anomers, as estimated from the integral over H-1/α (d, 4 Hz) and H-1/β (s). ^{13}C NMR (D_2O) δ 102.0, 95.5 (C-1, α/β), 84.8, 84.75, 79.5, 77.5, 74.3 (C-2 to C-4, α/β), 71.7, 70.9 (C-5, α/β), 58.65, 58.6, 58.4, 58.0 (OMe).

D-(1-^{2}H)ribose (36). To a 1.5% solution of 2,3-*O*-isopropylidene-D-ribonolactone (100 mg, 0.55 mmol) in dry THF, $NaBD_4$ (1.1 eq) was added and the reaction mixture was stirred at 0°C for 3 h when TLC indicated completed conversion into a single, more polar product. After addition of a few drops of acetone, the reaction mixture was treated with ion exchange resin Amberlite IR 120 [H^+]. The resin was removed by filtration and the filtrate was concentrated under re-

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duced pressure. Chromatography of the residue (cyclohexane/ethyl acetate 2:1, v/v) gave 87 mg (85%) of 2,3-*O*-isopropylidene-D-(1-2H)ribose. ^{13}C NMR ($CDCl_3$) δ 112.3 (isop), 103.1, 94.3, 88.0 (2 C), 87.2, 82.0, 87.1 (2 C), 70.2, 63.8 (2 C); 29.5, 26.6, 26.4, 24.9 (isop).

A 1.5% solution of this intermediate (61 mg, 0.33 mmol) in acetonitrile/D_2O (3:1, v/v) was stirred with resin IR 120 [H^+] at 40°C for 16 h. The resin was removed by filtration and the filtrate was concentrated to give compound **36** (28 mg, 59%). ^{13}C NMR (D_2O) δ 93.9, 71.1, 69.1, 67.3, 63.1 (pyranose); 101.0, 82.6, 75.3, 70.1, 62.1 (furanose).

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