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Study of the Specificity of a Spinach Transketolase on Achiral Substrates

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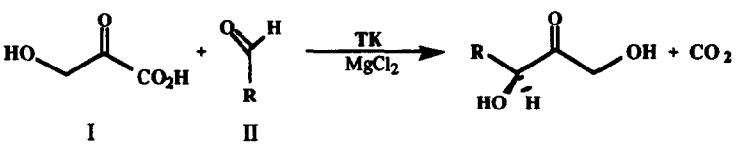
Abstract : The behaviour and the specificity of a spinach transketolase towards achiral aldehydes was studied. We have shown that these aldehydes are accepted by the enzyme and yield the corresponding saccharides with good enantiomeric excess.

Transketolase (TK) has proved to be a very efficient biocatalyst for the synthesis of sugar derivatives[1,2]. This enzyme is involved *in vivo* in the building of sugars by carbon-carbon bond formation, and in particular by an aldolisation type reaction, though it is not strictly an aldolase. It catalyses the reversible transfer of a hydroxyacetyl group from a ketose donor to an aldose acceptor **II** in the presence of thiamine pyrophosphate and magnesium. This enzyme is a useful catalyst for the synthesis of ketoses when hydroxypyruvate **I** is used as the donor substrate, since then the condensation becomes irreversible (Scheme 1).



I II

Scheme 1

We[3-5], and other authors[6,7], have shown that the reaction is diastereogenic with chiral α-hydroxylated aldehydes; the asymmetrical carbon formed always has the S configuration.

Here, we report a study of the behaviour of spinach transketolase towards some achiral derivatives of acetaldehyde and propionaldehyde.

First, we measured the activity of the spinach transketolase in the presence of chloro- **5**, methylthio- **4**, and methoxyacetaldehyde **3**, and methylthiopropionaldehyde **7** (Table 1).

Table 1

Aldehyde		Concentration (mM)	Relative activity
$CHO\text{-}CH_3$	**1**	100	0.12
$CHO\text{-}CH_2OH$	**2**	100	1.00
$CHO\text{-}CH_2OCH_3$	**3**	100	0.32
$CHO\text{-}CH_2SCH_3$	**4**	100	0.35
$CHO\text{-}CH_2Cl$	**5**	50	0.23
$CHO\text{-}CH_2\text{-}CH_3$	**6**	100	0.05
$CHO\text{-}CH_2\text{-}CH_2SCH_3$	**7**	100	0.24
$CHO\text{-}CH_2\text{-}CH_2OCH_3$	**8**	100	0.31

Aldehyde initial reaction rates are expressed relative to the initial reaction rate for glycolaldehyde **2**, the most reactive substrate. The activity of the enzyme for glycolaldehyde is 0.43 μmole/min/ml.

The results presented in table 1 confirm those obtained previously[8], i.e. that the presence of a hydroxyl group α to the aldehyde function in the 2-carbon substrates strongly favours reactivity: The relative reactivity of transketolase for glycolaldehyde **2** was 1 and it falls to 0.12 for acetaldehyde **1**. However, the presence of a hydroxyl α to the aldehyde function is not compulsory since the enzyme is still active towards:

- α-deoxyaldehydes, e.g. acetaldehyde **1** and propionaldehyde **6**, though weakly so,
- aldehydes in which the proton of the α hydroxyl is replaced by a methyl group, e.g. methoxyacetaldehyde **3**,
- aldehydes in which the hydroxyl group on the α carbon is replaced by methylthiol, e.g. methylthioacetaldehyde **4**,
- aldehydes in which an α hydroxyl group is replaced by chlorine, e.g. chloroacetaldehyde **5**.

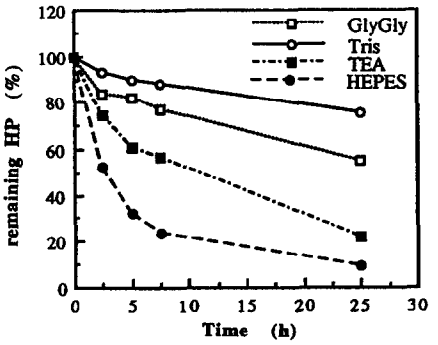
Aldehydes **3**, **4**, **5** are better substrates for the enzyme than acetaldehyde; an electronegative atom in the α position therefore appears to favour reactivity. The presence of a β heteroatom in the 3-carbon aldehydes is also conducive to reactivity (**7** and **8** compared with **6**).

All these achiral aldehydes are sufficiently reactive to transketolase to be potential substrates for ketose synthesis.

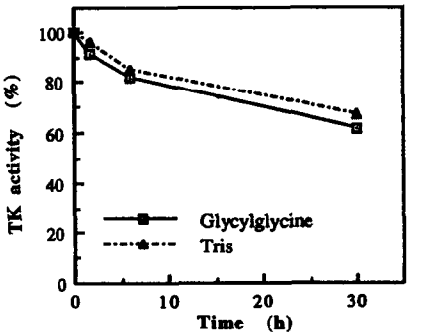
As the activities were measured at high concentrations, it was important, before attempting ketose synthesis under such conditions, to determine whether hydroxypyruvate would be sufficiently stable. The condensation of hydroxypyruvate anion and an aldehyde catalysed by transketolase consumes one proton. The resulting increase in pH is partially offset by reaction of CO_2 (from decarboxylation of hydroxypyruvate) with hydroxide to form bicarbonate. Despite this, the pH of the mixture will exceed 7.5, the optimun pH for transketolase. The pH can be regulated effectively with a buffer. We determined the stability of 100 mM hydroxypyruvate in 100 mM concentrations of four buffer systems (Scheme 2). In all cases, significant

decomposition of hydroxypyruvate occurred within hours . However, hydroxypyruvate was much more stable in Tris and glycylglycine than in triethanolamine and Hepes buffers. Accordingly, we studied the behaviour of transketolase in the first two buffers (Scheme 3). The enzyme activity fell more slowly in Tris and therefore this buffer was therefore chosen for the enzyme-catalysed synthesis.

Scheme 2

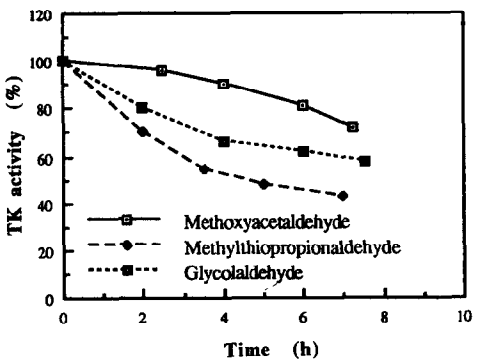


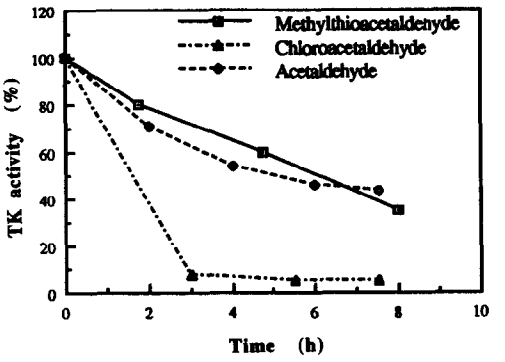
Scheme 3



Lastly, we studied the stability of transketolase in the presence of the above aldehydes at 100 mM (Scheme 4). This revealed that chloroacetaldehyde **5**, despite its reactivity, would not readily yield 4-deoxy-4-chloro-L-erythrulose because of its inhibiting effect on the transketolase activity. Transketolase was stable in the presence of methoxyacetaldehyde, but less so in the presence of aldehydes **4** and **7**. As these last two were reactive substrates, we undertook the synthesis of the corresponding saccharides using a large excess of transketolase to ensure a fast reaction.

Scheme 4





For the synthesis of sugar analogues, we condensed hydroxypyruvate (7 mmoles, 100 mM) onto aldehydes **3**, **4** and **7** (7 mmoles, 100 mM) in the presence of spinach transketolase (250 U) and its cofactors.

The reaction was carried out in Tris buffer which provided optimal hydroxypyruvate and transketolase reactivity.

The saccharides obtained from the enzyme reaction were identified by ^{1}H and ^{13}C NMR. Experimental results are given in table 2.

Table 2

R	Cetoses	Yield	Optical rotation	Enantiomeric excess
OH	L-erythrulose	70 %	+7 [3]	64 % [8]
H	4-deoxy-L-erythrulose	30 %	+4 [4]	70 % [8]
OCH_3	4-deoxy-4-methoxy L-erythrulose	45 %	+ 3	60 %
SCH_3	4-deoxy-4-methylthio-L-erythrulose	46 %	- 5	64 %
CH_2OH	D-xylulose	57 %	-30	>95 % [8]
CH_2SCH_3	4,5-dideoxy-4-methylthio-D-xylulose	20 %	- 2	76 %

Optical purity was determined on trifluoroacetates derivatives of saccharides by Gas Chromatography. Enantiomers were separated on a Lipodex E chiral column (modified γ-cyclodextrin). The proportions were measured and the enantiomeric excess calculated.

In order to take into consideration what we know about enzyme specificity and our work on L-erythrulose[3] and 4-deoxy-L-erythrulose[4], we suggest a (S) configuration for the asymetric carbon of the major product and a (R) configuration for the minor. The retention time for the (R) enantiomer was smaller than for the (S). For example, the retention time of (R)-4-deoxy-4-methylthio-L-erythrulose was 1456 sec, and it was 1492 sec for the (S). The (R) and (S)-4,5-dideoxy-4-methylthio-D-xylulose retention times were 1422 and 1468 sec, and for (R) and (S) 4-deoxy-4-methoxy-L-erythrulose 1478 and 1516 sec.

Conclusion:

Transketolase thus accepts 2- and 3-carbon aldehyde substrates with no α hydroxyl.

Measurement of enantiomeric excesses showed that transketolase conserved high stereospecificity, though this was slightly reduced with the achiral 2-carbon aldehydes **3** and **4**. The predominant enantiomer had configuration 3 (S) for L-erythrulose and 4-deoxy-L-erythrulose, so we assigned the 3(S) configuration to the major product obtained with the other achirals substrates .

For the 3-carbon aldehyde **7** bearing a β heteroatom, the value of the enantiomeric excess shows that the enzyme loses stereoselectivity when the asymmetric carbon is created; the enantiomeric excess was only 76 % with 4-methylthio-propionaldehyde **7** whereas it was 100% with glyceraldehyde (leading to D-xylulose). However this loss was less than that occurring with methylthioacetaldehyde **4**.

Experimental section

Measurement of relative reaction rates of aldehyde substrates:

The rates of the TK reactions were determined by following the disappearance of lithium β-hydroxypyruvate from the reaction mixture. The reaction was carried out in Gly-Gly buffer (100 mM, 1mL, pH 7.5) with TPP (0.02 mM), $MgCl_2$ (1 mM), β-hydroxypyruvate (100 mM) and aldehyde (100 mM). The reaction was started by the addition of TK (1 U). At times 0 and 5 min, 20 µL aliquots of solution were withdrawn from the reaction mixture. The quantity of β-hydroxypyruvate in each sample was determined spectrophotometrically by measuring the change in absorbance of NADH at 340 nm due to reduction of residual β-hydroxypyruvate by NADH and lactate dehydrogenase.

A spectrophotometric cuvette was loaded with 1 mL of Triethanolamine buffer 0.1 M, pH 7.5 containing 20 µL of the previous aliquot and 0.2 mM of NADH. 25 U of LDH was then added to the cuvette. From the decrease in absorbance at 340 nm, the amount of β-hydroxypyruvate in the sample was mesured and the reaction rate calculated.

Measurement of transketolase stability with aldehyde substrates:

The stability of the enzyme was determined by following the disappearance of transketolase activity from the reaction mixture. The reaction was carried out in Tris buffer (100 mM, 2,8 mL) with aldehydes (100mM) and transketolase (15 U). The pH of the solution was adjusted to 7.5 and the mixture was kept stirred at 25°C.

At times 0, 2, 4...10 hours, 10 µL aliquots of solution were withdrawn from the reaction mixture to measure transketolase activity. A spectrophotometric cuvette was loaded with 1 mL of glycylglycine buffer 0.1 M, pH 7.5 containing 0.2 mM of NADH, 1 mM of D-xylulose-5-phosphate, 2.5 mM of ribose-5-phosphate, 1 mM of $MgCl_2$, 0.2 mM of TPP, 10 U of GDH, 1 U of TPI.10 µL of the previous aliquot was then added to the cuvette to start the reaction.

General procedure for the transketolase-catalysed condensation of lithium β-hydroxypyruvate and aldehydes:

In a flask equipped with a magnetic stirbar was placed 40 mL of Tris buffer 0.1 M containing hydroxypyruvate (100 mM), aldehyde (100 mM), thiamine pyrophosphate (1.5 mM), $MgCl_2$ (3 mM). The pH was adjusted to 7.5 with a few drops of 1 N HCl. TK (250 U) was then added. The mixture was deoxygenated with nitrogen and left at 25°C in the dark, with slow stirring. The progress of the reaction was followed by TLC (using 20 % methanol in chloroform) and consumption of hydroxypyruvate . When the reaction was judged to be complete, ethanol (3 vol.) was added and the precipitate discarded. The solution was concentrated under reduced pressure and desalted by chromatography on cation exchanger (H^+, DOWEX 50 W-X2). The fractions containing the sugar were pooled, adjusted to pH 6.5 with anion exchanger (OH^-, Amberlite IRA 93) and concentrated. Chromatography of the residue on silicagel (elution with chloroform-methanol 20:1) gave the deoxysugar as a syrup.

4-deoxy-4-methylthio-L-erythrulose: (46 %)

^1H NMR ($CDCl_3$, 300 MHz) δ 2.12 (s, 3H, CH_3); 3.35 (d, 1H, J = 13 Hz, CH_2OH); 3.47 (d, 1H, J = 13 Hz, CH_2OH); 3.98 (d, 2H, J = 2.3 Hz, CH_2-S); 4.59 (t, 1H, J = 2.3 Hz, CHOH); 3.62 (wide peak, 1H, OH); 2.95 (wide peak, 1H, OH).

^{13}C NMR ($CDCl_3$, 300 MHz), δ 15.96 ($\underline{C}H_3$); 38.8 ($\underline{C}H_2$); 64.0 ($\underline{C}H_2OH$); 76.1 ($\underline{C}HOH$); 205.1 ($\underline{C}O$).
$[\alpha]_J^{25}$= - 5 (C = 0.018, $CHCl_3$).

4-deoxy-4-methoxy-L-erythrulose: (45 %)
^1H NMR (CD_3OD, 300 MHz) δ 3.42 (s, 3H, $C\underline{H}_3$); 3.67 (dd, 1H, J = 3.2 et 10.8 Hz, $C\underline{H}_2$-OCH_3); 3.77 (dd, 1H, J = 3.2 et 10.8 Hz, $C\underline{H}_2$-OCH_3); 4.40 (t, 1H, J = 4.5 Hz, $C\underline{H}OH$); 4.49 (d, 1H, J = 4.5 et 16.4 Hz, $C\underline{H}_2OH$); 4.58 (d, 1H, J = 16.4 Hz, $C\underline{H}_2OH$).
^{13}C NMR (CD_3OD, 300 MHz), δ 63.8 ($\underline{C}H_3$); 71.4 ($\underline{C}H_2OCH_3$); 79.1 ($\underline{C}H_2OH$); 80.3 ($\underline{C}HOH$); 215.9 ($\underline{C}O$).
$[\alpha]_J^{25}$= + 3 (C = 0.017, MeOH).

4,5-dideoxy-5-methylthio-D-xylulose: (20%)
^1H NMR ($CDCl_3$, 300 MHz) δ 1.90 (m, 2H, J=7.1 Hz, $C\underline{H}_2$-CHOH); 2.13 (s, 3H, $C\underline{H}_3$); 2.60 (m, 2H, J=7.1 Hz, $C\underline{H}_2$-S); 3.68 (d, 1H, J = 8.6 Hz, $C\underline{H}_2OH$); 3.93 (d, 1H, J= 8.6 Hz, $C\underline{H}_2OH$); 4.64 (t, 1H, J = 7,1 Hz, $C\underline{H}OH$).
^{13}C NMR ($CDCl_3$, 300 MHz), δ 15.8 ($\underline{C}H_3$); 29.4 ($\underline{C}H_2$-CHOH); 34.5 ($\underline{C}H_2$-S); 71.1 ($\underline{C}H_2OH$); 97.1 ($\underline{C}HOH$); 213.6 ($\underline{C}O$).
$[\alpha]_J^{25}$= - 2 (C = 0.02 , $CHCl_3$).

General procedure for enantiomeric excess determination:

The enantiomeric excesses were determined by Gas Chromatography (GC) using an instrument equipped with a flame ionisation detector and a 25m x 0.25mm capillary column coated with Lipodex E (modified γ-cyclodextrin, Macherey-Nagel). The carrier gas was hydrogen at 65 kPa. The oven temperature was 90°C for 5 min, then 90°C to 170°C at 4°C/min and finally 170°C for 5min.

The determinations were made with trifluoroacetate derivatives of saccharides, which were obtained in the following way : In a flask (1 mL) equipped with a refrigerant was placed a solution of 0.1 mg of deoxysugar in 0.1 mL of pyridine and 0.1 mL of trifluoroacetic anhydride. The mixture was heated for 1 hour at 60-70°C. A solid derivative was obtained.

This solid was dissolved in chloroform and 3µl of the solution (1mg/mL) were directly injected in the CPG.

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