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Amino acid precursors for the detection of transketolase activity in *Escherichia coli* auxotrophs

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ABSTRACT

Probes were developed for the in vivo detection of transketolase activity by the use of a complementation assay in *Escherichia coli* auxotrophs They combine the D-*threo* ketose moiety recognised by transketolase and the side chain of leucine or methionine. These compounds were donor substrates of yeast transketolase leading to the release of the corresponding α-hydroxyaldehydes which could be converted in *E. coli* by a cascade of reactions into leucine or methionine required for cellular growth.

Thiamine diphosphate (ThDP)-dependent enzymes have the potential of both breaking and forming C–C bonds.[1,2] Among ThDP enzymes, transketolase (TK) is one of the most widely used in organic synthesis.[3,4] The enzyme appears highly specific for ketol donor substrates, stereospecific (the newly formed asymmetric centre has *S* configuration) and enantioselective towards hydroxyaldehyde substrates with the (*R*) configuration. The fact that TK specifically catalyses the irreversible transfer of a ketol unit from α-hydroxypyruvic acid to an aldehyde to generate a D-*threo* (3*S*,4*R*) ketose makes it an ideal tool for synthetic purposes.

The current challenge is to modify the properties of TK in order to extend the synthetic potential of this enzyme.[5] The constitution of large combinatorial libraries of TK mutants would widen the possibilities of modifying the substrate specificity of the enzyme. However, an effective selection or screening system is an absolute prerequisite for identifying evolved enzyme variants that display improved properties. Spectrophotometric and colorimetric screening tests have already been developed for this type of enzyme.[6]

In order to detect modifications of the stereospecificity of mutant TK, we developed tests based on the detection of coumarine[7] or tyrosine[8] from stereochemical probes. However, these in vitro assays are unsuitable for screening large libraries of mutant enzymes because each enzyme variant has to be first produced and purified separately in order to determine its catalytic properties.

We elaborated the new principle of an in vivo selection test, which may enable the screening of large libraries of mutant enzymes and the direct selection of transketolase activities on non natural substrates in *Escherichia coli*. Our strategy links the catalytic activity of the TK variants to the release of a survival factor, which provides a growth advantage for the bacterial host. The principle of the assay makes use of specially designed and synthesized compounds which combine a sugar moiety potentially recognised by TK mutants and the side chain of an amino acid. The cleavage of the C2–C3 bond of these compounds by TK releases an α-hydroxyaldehyde which is converted in vivo into an L-amino acid absolutely required for the growth of the *E. coli* auxotroph host cells (Fig. 1).

In this Letter, we report the proof of concept of this complementation assay using wild type yeast TK and compounds **1** and **2**, respectively, precursors of L-leucine and L-methionine according to Figure 1. We chose compounds **1** and **2** because of (i) their differences in polarity and steric hindrance, (ii) their easy access by chemoenzymatic syntheses and (iii) the tightness and stability of leucine and methionine *E. coli* auxotrophs. Herein, we report the syntheses of compounds **1** and **2** which display the D-*threo* configuration recognized by the wild type yeast TK and harbour the side chain of leucine or methionine and the validation of enzymatic steps a, b, c, d of the proposed pathway.

The syntheses of compounds **1** and **2** were carried out chemoenzymatically using yeast TK[4,9] in order to generate asymmetric centers (3*S*,4*R*) in a straightforward way (Fig. 2). TK transfers a

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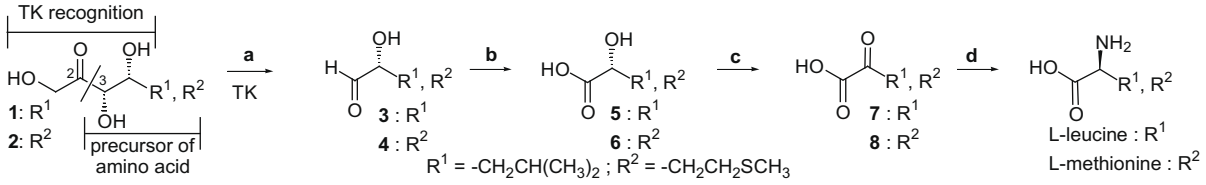

Figure 1. Putative in vivo pathway in *E. coli* auxotrophs from probes **1** and **2** leading to complementation via their corresponding amino acids.

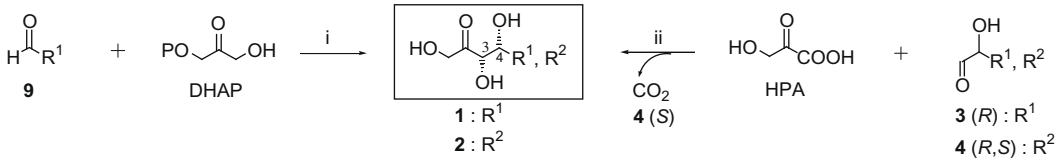

Figure 2. Syntheses of TK probes **1** and **2** from aldehydes **3**, **4** and **9**. (i) RAMA (30 units), pH 7.5, DHAP (125 mM), **9** (312 mM), **1** (37%); (ii) yeast TK (50 units), pH 7.5, HPA (120 mM), **3** (100 mM), **4** (200 mM), **1** (5%), **2** (47%).

two carbon unit from hydroxypyruvate (HPA), donor substrate which makes the reaction irreversible by cleavage of the C2–C3 bond and CO_2 release. The α-hydroxylated aldehydes **3** and **4** required as acceptor substrates were chemically synthesized.[10] The differences in yields of the TK-catalyzed reactions (47% with **3** vs 5% with **5**) may be explained by the distinctive steric hindrance and hydrophobic properties of these compounds. Because TK reaction is reversible, the feasibility of the syntheses may inform on the TK ability to accept compounds **1** and **2** as donor substrates. So, we might expect compound **2** to be a better donor substrate than compound **1**. For the synthesis of compound **1**, better yields (35%) were obtained using fructose-1,6-bisphosphate aldolase from rabbit muscle (RAMA)[11] that leads to the D-*threo*-ketose moiety in the presence of dihydroxyacetone phosphate (DHAP) as donor substrate and aldehyde **9** (commercially available) as acceptor.

We carried out in vitro experiments with wild type TK in the presence of compounds **1** and **2** as donor substrates and D-ribose-5-phosphate (R-5-P), the natural acceptor substrate of the enzyme. The TK-catalysed C_2–C_3 bond cleavage from ketoses **1** and **2** and subsequent transfer of the hydroxyacetyl group to R-5-P would lead to the formation of D-sedoheptulose-7-phosphate (S-7-P) and α-hydroxyaldehydes **3** and **4**, respectively (Fig. 5).

LC/MS monitoring was found to be the most suitable analytical technique to follow the appearance of the products (S-7-P, **3** or **4**) and the disappearance of both donor (**1** or **2**) and acceptor (R-5-P) at the same time. Both ketoses **1** and **2** were checked to be chemically stable after 96 h in reaction buffer in the presence of the acceptor R-5-P without TK. A slow appearance of S-7-P was monitored over time in reaction buffer with TK extract in the absence of compound **1** or **2** (control in Fig. 3). The presence of residual endogenous donor substrates in TK extracts could be responsible for this background noise.

In the presence of R-5-P, TK extract and compound **2** we observed an increase of S-7-P four-fold higher than from compound **1** compared to the control after 5 h incubation (Fig. 3). MS/MS experiments enabled us to follow the conversion of compound **2** at $m/z = 129$ concomitantly with the release of α-hydroxyaldehyde **4** at $m/z = 117$ (Fig. 4). Due to the lack of signal in MS for the low mass α-hydroxyaldehyde **3** using the electrospray source working in positive (ESI+) or negative (ESI−) mode, we were unable to cross confirm the results for ketose **1** and the parallel appearance of α-hydroxyaldehyde **4**. Altogether, these results strongly suggest that ketoses **1** and **2** are donor substrates for TK and lead in both cases to the corresponding α-hydroxyaldehydes **3** and **4**. Furthermore, compound **2** seems to be a better donor substrate than compound 1. This result correlates with the better yield obtained for the TK-

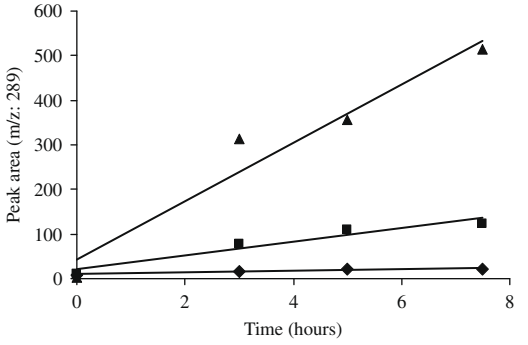

Figure 3. TK-dependent appearance of S-7-P from compounds 1 and 2. Control: (♦): R-5-P (10 mM), TK extract (2 units mL^{-1}), $AcONH_4$ 50 mM pH 7.2. TK reactions: R-5-P (10 mM), ThDP (2 mM), $MgCl_2$ (3 mM), TK extract (2 units mL^{-1}), $AcONH_4$ 50 mM pH 7.2; (■): compound **1** (10 mM); (▲): compound **2** (10 mM).

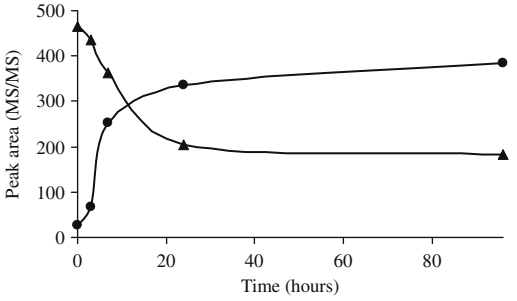

Figure 4. TK-dependent disappearance of compound **2** and appearance of α-hydroxyaldehyde **4**. TK reaction: R-5-P (10 mM), ThDP (2 mM), $MgCl_2$ (3 mM), TK extract (2 units mL^{-1}), $AcONH_4$ 50 mM pH 7.2; (▲): compound **2** (10 mM); (●): compound **4**.

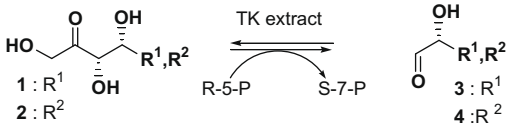

Figure 5. TK in vitro assay.