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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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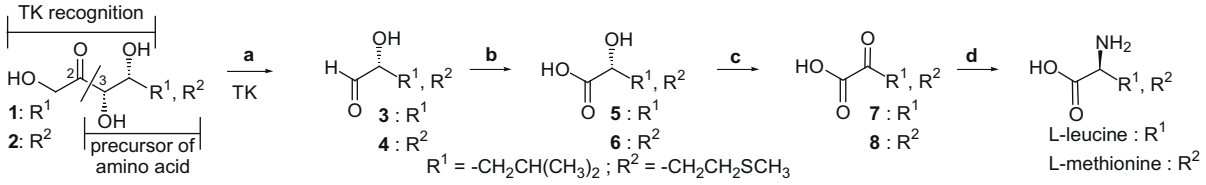

Figure 1. Putative in vivo pathway in *E. coli* auxotrophs from probes **1** and **2** leading to complementation via their corresponding amino acids.

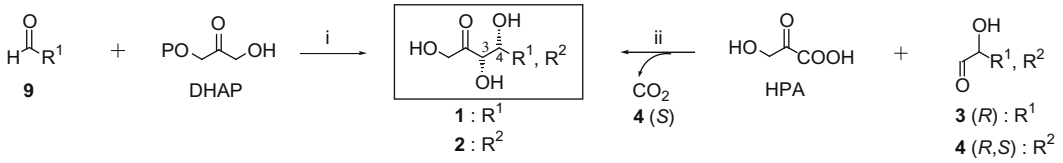

Figure 2. Syntheses of TK probes **1** and **2** from aldehydes **3**, **4** and **9**. (i) RAMA (30 units), pH 7.5, DHAP (125 mM), **9** (312 mM), **1** (37%); (ii) yeast TK (50 units), pH 7.5, HPA (120 mM), **3** (100 mM), **4** (200 mM), **1** (5%), **2** (47%).

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-

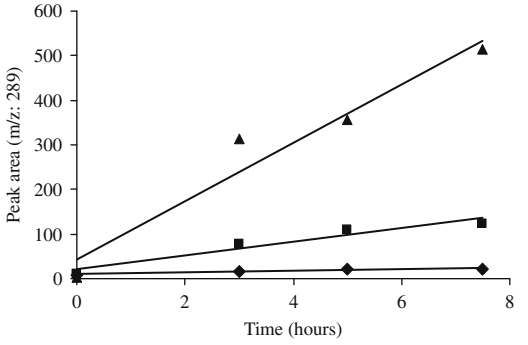

Figure 3. TK-dependent appearance of S-7-P from compounds 1 and 2. Control: (♦): R-5-P (10 mM), TK extract (2 units mL^{-1}), $AcONH_4$ 50 mM pH 7.2. TK reactions: R-5-P (10 mM), ThDP (2 mM), $MgCl_2$ (3 mM), TK extract (2 units mL^{-1}), $AcONH_4$ 50 mM pH 7.2; (■): compound **1** (10 mM); (▲): compound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

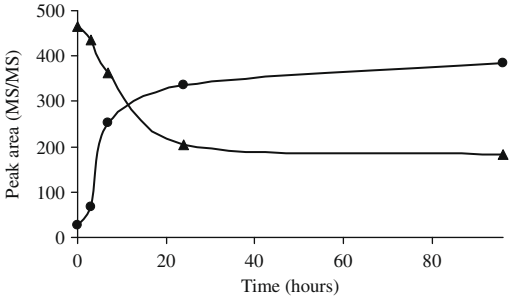

Figure 4. TK-dependent disappearance of compound **2** and appearance of α-hydroxyaldehyde **4**. TK reaction: R-5-P (10 mM), ThDP (2 mM), $MgCl_2$ (3 mM), TK extract (2 units mL^{-1}), $AcONH_4$ 50 mM pH 7.2; (▲): compound **2** (10 mM); (●): compound **4**.

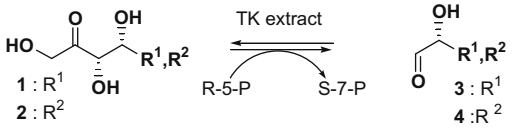

Figure 5. TK in vitro assay.

catalyzed synthesis of **2**. The sulfur atom present in the side chain of compound **2** may interact steadily with the residues of the TK active site in comparison with the apolar side chain of compound **1**.

In vivo complementation assays were carried out in growth experiments in liquid minimal saline medium (SM) with *E. coli* auxotrophs and the appropriate compounds **1** or **2** as well as the possible intermediates **5–8** in order to validate the four enzymatic steps a, b, c and d of the proposed pathway depicted in Figure 1. The genotypes of the bacterial strains are given in Supplementary data.

For step (b), since *E. coli* cells express various house-keeping oxidoreductases involved in the conversion of the highly reactive aldehydes,[12,13] we surmised that the hydroxyaldehydes generated intracellularly from compounds **1** and **2** should rapidly be converted into the corresponding α-hydroxyacids **5** or **6**.

The following sequential steps of α-hydroxyacid oxidation (c) and transamination of keto-acids (d) were investigated by testing the growth complementation of a leucine auxotroph and a methionine auxotroph with the corresponding α-hydroxy-acids **5** (*R* or *S* configuration) and **6** (racemic) and keto acids **7** and **8** as unique leucine or methionine sources. As shown in Table 1, the generation times registered with both α-hydroxyacids and α-ketoacids were very similar to that obtained with the amino acid. The results indicate that compounds **5, 7** and **6, 8** were actually converted into their corresponding amino acid, leucine or methionine, respectively, and that neither step of hydroxyacid oxidation or ketoacid transamination was limiting.

We then investigated whether D-*threo*-ketoses **1** and **2** could supply in vivo leucine or methionine auxotrophs with the required amino acid and, in positive cases, if the growth complementation was dependent on the expression of yeast TK activity. We first established that the expression of the yeast TKL1 gene in *E. coli* actually led to a functional TK. For that purpose, the yeast TKL1 gene was cloned into a multicopy plasmid and expressed in a TK-deficient *E. coli* mutant. Growth in SM with glucose or ribose as unique carbon source was monitored in a Bioscreen C apparatus. As shown in Table 2, the TK-deficient mutant harbouring the yeast TKL1 gene on a plasmid displayed a prototroph phenotype similar to that of a wild type *E. coli* strain.[14] These results confirmed the effective expression of TKL1 gene and the correct functioning of yeast TK in our *E. coli* genetic background. We then tested whether *E. coli* auxotrophs expressing the yeast TKL1 gene convert the synthesized compounds **1** and **2** into a usable amino acid source. For that purpose, leucine and methionine auxotroph mutants devoid of the chromosomal TK-encoding genes were constructed and transformed with a TKL1-harbouring plasmid or empty vector. The complementation of the amino acid requirement of the auxotrophs with compounds **1** or **2** was monitored in a Bioscreen C apparatus. In parallel, the stability of compounds **1** and **2** was verified under culture conditions in the absence of *E. coli*. As shown in Tables 3 and 4, leucine and methionine fulfilled equally well the metabolic requirement of both TK-deficient and TK-expressing bacteria. TK-expressing methionine auxotrophs displayed similar generation times when compound **2** or methionine were provided in culture medium. In contrast, compound **2** was less efficient than methionine in TK-deficient bacteria (Table 4). Thus, the growth of the methionine auxotroph with compound **2** seems directly correlated with the expression of the yeast TK. However, the generation time displayed by the TK-deficient methionine auxotroph indicates that compound **2** was still converted into methionine by a TK-independent pathway.

The results obtained with leucine auxotrophs indicate that compound **1** was a poor source of leucine (Table 3). This could be explained by the low efficiency of compound **1** cleavage by TK consistent with the results obtained in vitro as well as an inefficient transport across the bacterial cellular membrane.

Altogether, the results obtained in the present preliminary study validate the principle of the strategy we designed for an in vivo selection system for TK activity. First, we prepared new compounds combining a sugar moiety and the lateral chain of amino acids by chemoenzymatic ways. We showed in vitro that two such compounds (**1** and **2**) are donor substrates of yeast TK which releases the corresponding hydroxyaldehydes (**3** and **4**). In vivo, compounds **1** and **2** supplied *E. coli* auxotrophs with required leucine or methionine when the bacteria expressed TK activity. However, significant growth was registered even in the absence of any TK gene expressed in the cells. Work is currently in progress for the characterization of TK-independent compound **2** conversion, which may help to construct *E. coli* mutants displaying a reduced

Table 1
Growth of methionine or leucine *E. coli* auxotrophs

Genotype	Generation time for *E. coli* auxotrophs cultivated with indicated complement[a]					
	leu	**5**	**7**	met	**6** (rac)	**8**
ΔmetA	—	—	—	2h45	2h40	2h35
Δ (ara-leu)	7h00	6h30 (R) 7h30 (S)	6h00	—	—	—

[a] Bacteria were cultivated at 37 °C in minimum saline medium (SM) glucose 0.2% (w/v) with the indicated complement at 0.3 mM.

Table 2
Growth of TK-deficient and TK-expressing *E. coli*

Genotype	Generation time for *E. coli* strains cultivated with indicated carbon source and complements[a]			
	SM glucose	SM glucose ARO shi B6	SM ribose	SM ribose ARO shi B6
wt	4h00	3h35	22h40	22h40
Δ*tktA* Δ*tktB*	NG	7h20	NG	NG
Δ*tktA* Δ*tktB*TKL1+	4h20	4h20	30h50	28h40

[a] Carbon source concentration was 0.2% (w/v). ARO: aromatic amino acids: phe, tyr and trp (0.3 mM each). Shi: shikimic acid (0.3 mM). B6: pyridoxine (3 μM). wt: wild type. NG: no growth.

Table 3
Growth of leucine *E. coli* auxotrophs

Genotype	Generation time for leucine auxotrophs cultivated with indicated source of leucine[a]	
	Leu (0.25 mM)	**1** (2.5 mM)
Δ*tktA* Δ*tktB* Δ*leuABCD* pDRI5 TKL1+	6h30	125 h
Δ*tktA* Δ*tktB* Δ*leuABCD* empty vector pSP100	4h40	700 h

[a] Culture medium: SM glucose 0.2% supplemented with phenylalanine, tyrosine and tryptophan (0.3 mM each), shikimic acid (0.3 mM), pyridoxine (3 μM) and the indicated amino acid source.

Table 4
Growth of methionine *E. coli* auxotrophs

Genotype	Generation time for methionine auxotrophs cultivated with indicated source of methionine[a]	
	Met (0.25 mM)	**2** (2.5 mM)
Δ*tktA* Δ*tktB* Δ*metA* pGEN377 TKL1+	3h45	6h00
Δ*tktA* Δ*tktB* Δ*metA* empty vector pVDM18	5h45	20h15

[a] Culture medium: SM glucose 0.2% supplemented with phenylalanine, tyrosine and tryptophan (0.3 mM each), shikimic acid (0.3 mM), pyridoxine (3 μM) and the indicated amino acid source.

background. Our next step will apply the principle of our test by the use of new compounds displaying the more suitable side chain, leucine or methionine, and a sugar moiety to the selection of TK variants with improved or modified specificities.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.04.111.

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