Pyruvate Aldolases Catalyze Cross-Aldol Reactions between Ketones: Highly Selective Access to Multi-Functionalized Tertiary Alcohols

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Victor Laurent,[†] Léa Gourbeyre,[†] Alexandre Uzel,[†] Virgil Hélaine,[†] Lionel Nauton,[†] Mounir Traïkia,[†] Véronique de Berardinis,[‡] Marcel Salanoubat,[‡] Thierry Gefflaut,[†] Marielle Lemaire[†] and Christine Guérard-Hélaine*[†]

[†] Université Clermont Auvergne, CNRS, SIGMA Clermont, Institut de Chimie de Clermont-Ferrand, 63000 Clermont-Ferrand, France

[‡] Génomique Métabolique, Génoscope, Institut François Jacob, CEA, CNRS, Univ Evry, Université Paris-Saclay, 91057 Evry, France

ABSTRACT: Tertiary alcohols are widely represented in nature and among bioactive molecules. Their importance is attested by the continuous efforts to meet the challenge of their stereoselective synthesis. In this context, we propose an enzymatic approach, involving class II pyruvate aldolases. These enzymes are shown to catalyze selective cross-aldol reactions between pyruvic acid or derivatives as nucleophiles and a series of ketones as electrophiles. This catalytic activity is exemplified by the highly stereoselective preparation of seven branched ketols with good yields. One of them was readily converted into a constrained 4-hydroxyproline analogue in a multi-enzymatic one-pot one-step process.

KEYWORDS: pyruvate aldolase, tertiary alcohol, proline analogue, cascade reaction, transaminase

Tertiary alcohols are important motifs in pharmaceuticals, as well as being fine and specialty chemicals, such as chiral ligands,[1–3] building blocks in asymmetric synthesis,[4–6] and solvents.[6–9] Many bioactive compounds with these moieties are known, such as tramadol (analgesic), erythromycin (antibiotic), doxorubicin (antitumor agent), and ethynylestradiol (estrogen), along with many ethers, esters, and acetals derived from tertiary alcohols. However, the challenges to surmount for their synthesis are as high as the demand for enantiopure tertiary alcohols.[7,9] Recently, DHAP-dependent class II aldolases were shown to be active with ketones as electrophile substrates, thus allowing the stereoselective synthesis of a series of tertiary alcohols.[10] Therefore, we examined whether other members of the aldolase family display the same catalytic power. We assumed that this exceptional activity could be shared with other class II aldolases, as the enolate intermediate[11] formed in the active site of class-II aldolases should be more nucleophilic than the enamine intermediate characteristic of class I aldolases. Among the aldolase family, pyruvate aldolases may be good candidates for this purpose, as some of them belong to the class II aldolases. In addition, they have been well studied [11,12] because of their role in metabolism and their ability to provide chiral synthons with high potential in total synthesis. Until the discovery of DHAP-dependent aldolases in 2018,[10] 4-hydroxy-4-methyl-2-oxoglutarate/4-carboxy-4-hydroxy-2-oxoadipate aldolases[13,14] were the sole aldolases able to catalyze the formation of tertiary alcohols. They naturally catalyze the self-addition of pyruvate or the addition of pyruvate onto oxaloacetate. Nevertheless, this type of reaction is limited by the substrate scope of the enzymes.[13,14] The sole example of cross-coupling of α-keto acids at a preparative scale is the development of the enzymatic synthesis of monatin, a natural sweetener.[11,15,16] Recently, a collection of 21 class-II pyruvate aldolases from biodiversity, including RhmA, GarL and HpcH from *E. coli*, were shown to display high synthetic potential due to their exceptional tolerance towards nucleophile substrates, such as hydroxypyruvate or ketobutyrate.[17,18] This property remains rare as aldolases are generally named according to their strict specificity for their nucleophilic substrate. Here, we further explored the catalytic potential of this enzyme set by testing aldol reactions with various ketones as electrophiles in combination with pyruvic acid or derivatives.

First, the enzyme collection was assayed under standard conditions (1 mg/mL) with pyruvic acid **1** (20 mM) as the unique substrate to assess the self-addition reaction. Aldol formation was not observed with any pyruvate aldolase. Subsequently, three simple ketones (80 mM) were evaluated as potent electrophiles with **1** (20 mM) (Figure 1). These ketones could be classified into two groups: not activated (acetone: **4**), activated by either one hydroxyl (hydroxyacetone: **5**) or two hydroxyls (dihydroxyacetone: **6**). All tested enzymes displayed the same activity profile: an

aldol adduct was exclusively formed with ketone **6**, with complete conversion of **1** within 2 to 5 h (see SI). The two other less electrophilic ketones **4** and **5** did not undergo any significant reaction under our analytical conditions. Next, in order to select the most promising enzymes with the largest application scopes, ketones **4** to **6** were assayed with 2-oxobutyric acid **2** or hydroxypyruvic acid **3** as nucleophile substrates (20 mM), as these enzymes are known to accept them as nucleophiles with an aldehyde as electrophile.[17,18] Again, as described for **1**, the self-addition reactions of keto acids **2** and **3** was first assessed. Although no reaction was observed with **2** as a unique substrate (see SI), partial or complete conversion of **3** was observed with several enzymes. Given that the hydroxyl group of **3** provides an activating effect, the self-addition of **3** is consistent with the results obtained with ketones **4** to **6**. Consequently, **3** was not further used as a nucleophile in this study. Substrate **2** was then combined with ketones **4** to **6** in the presence of the various aldolases. As already observed with **1** as nucleophile, an aldolisation product was obtained only with ketone **6**. However, different reactivity profiles were observed. Indeed, only six enzymes were able to completely convert **2** into the corresponding tertiary alcohol in a few hours (see SI): Uniprot Id. Q0K203, B4XH86, A5VAX1, A9E0U5, A3SLS0, and I7DKY0 (deleted entry in UniProtKB, currently given by the UniParc ID UPI000160F944). Q0K203 was shown in a previous study to display very low stereoselectivity with **2**[18] and was discarded for the following experiments. Hence, five pyruvate aldolases were assayed in aldolisation reactions using **1** and **2** as nucleophiles combined with three other activated ketones as potent electrophile substrates: L-erythrulose **7**, D-fructose **8,** and 1,3-difluoroacetone **9** (Figure 1).

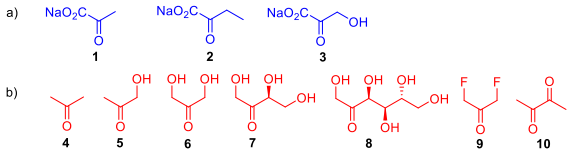


Figure 1. Nucleophiles a) and electrophiles b) evaluated with class II pyruvate aldolases from biodiversity.

There was no aldol addition observed between **1** and **8**, regardless of the enzyme tested. Such lack of reactivity with **8** may result from the accommodation limit of the substrate binding sites or the favored cyclisation of **8**, protecting the ketone from nucleophilic addition. Ketone **2** was therefore not tested with this electrophile. Conversely, the acyclic ketone **7** proved to behave as a substrate of the tested aldolases, although activity was less than that observed for the shorter ketone **6**. Aldolases B4XH86 and A5VAX1 were the most efficient catalysts for the aldolisation between **1** and **7,** with almost 80% conversion in 2 h (Figure 2). There was an additional decrease in aldolase activity when nucleophile **2** was used in combination with **7**. Nevertheless, approximately 80% conversion could be achieved in 30 h by A5VAX1 and B4XH86 with this substrate couple. For the aldol reaction of **1** with **9**, complete conversion of **1** occurred in three hours with four of the selected enzymes (all except I7DKY0, Figure 2). The enzyme activity was lower when **2** was used as a nucleophile in combination with **9**, for which the conversion rate never exceeded 50% after 48 h (data not shown).

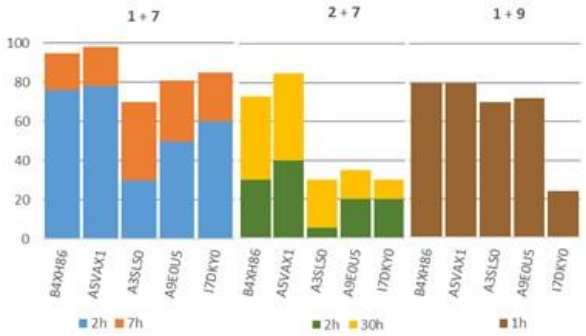


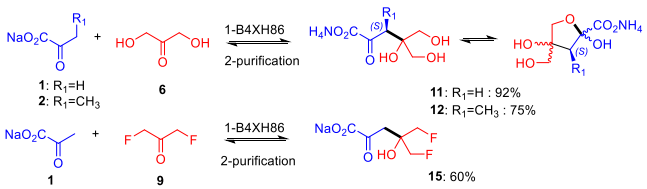
Figure 2. Conversion of nucleophiles **1** or **2** (20 mM) in the presence of ketones **7** or **9** (80 mM) with the selected aldolases

To note, the best performing above-mentioned enzymes were able to convert the considered electrophile ketones with an acceptable rate relative to that of structurally close aldehydes. Indeed, for example, DHA was converted only two times slower than D-glyceraldehyde using B4XH86 and **2** as nucleophile.

The promising analytical data obtained with ketones **6, 7**, and **9**, prompted us to carry out preparative syntheses at the 50-mg scale using a concentration of 20 mM for the nucleophiles and 80 mM for the electrophiles, to isolate aldol adducts for their full characterization and stereoselectivity assignments.

No asymmetric carbon was formed for the aldol reaction **1** + **6** (Scheme 1), so all five enzymes were expected to give the same aldol. B4XH86, one of the most promising aldolases, was thus chosen for this synthesis. The achiral aldol **11** was isolated with a 92 % yield after 8 h reaction and anion exchange chromatography for purification. NMR analysis showed that **11** exists in solution as an equimolar mixture of furanose derivatives.

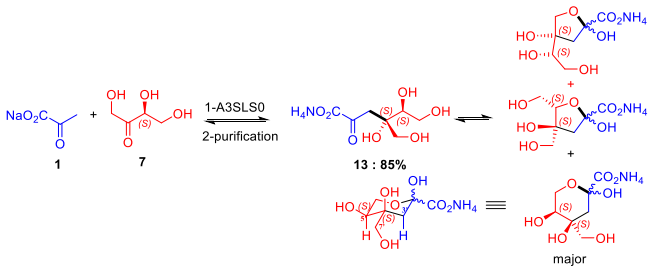
Aldolisation of **2** and **6** led to the formation of one asymmetric center on carbon 3 of aldol **12** (Scheme 1). The five selected enzymes were 3S specific when using **2** as nucleophile (see previous study[18] or below for aldol reaction **2** + **7** concerning A3SLS0). Thus, the five enzymes again yielded the same aldol. B4XH86 was again chosen as catalyst and product **12** was obtained after 12 h with a yield of 75%. Aldol **12** was observed in solution as two major furanose forms with roughly the same ratio.



Scheme 1. Aldolase-catalyzed reactions of pyruvate 1 or 2-ketobutyrate 2 with dihydroxyacetone 6 and difluoroacetone 9.

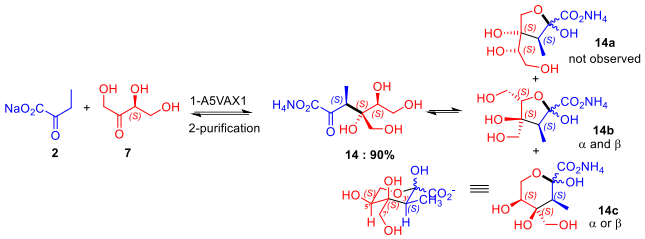
Aldol reaction **1** + **7** (Scheme 2) resulted in the formation of only one chiral center at C4 of aldol **13**. In theory, a non-

stereoselective enzyme could lead to the production of a total of twelve cyclic aldols, *i.e.* two epimers in the fourth position with three possible cyclic forms and potential alpha/beta anomerism (Scheme 2). Therefore, DEPT ^{13}C NMR experiments were first performed to focus on the tertiary carbon of the various forms (see SI). These analyses revealed that the most active enzymes, B4XH86 and A5VAX1, displayed poor stereoselectivity, with a mixture of nine forms observed by NMR. Conversely, with the most stereoselective aldolase A3SLSo, only six forms were observed, suggesting a possible unique configuration at C4. This assumption was confirmed by NMR studies performed on the decarboxylated aldol product. This decarboxylation reaction was run with H_2O_2 in a buffered solution (pH 7.5 to avoid the lactonization observed without buffer) and led to the formation of only one linear stereoisomer (> 95% by ^1H NMR). Subsequently, 2D NOESY NMR experiments revealed the (4*S*) configuration of **13**, as shown by correlations between H3, H7, and H5 of the major pyranose form (see SI). Finally, although A3SLSo was analytically found to be the least effective aldolase for catalyzing the coupling **1** + **7**, the reaction was fast enough (15 h) to selectively provide the aldol (4*S*, 5*S*)-**13** at an 85% yield after purification by anion exchange chromatography.



Scheme 2. Aldolase-catalyzed reaction of pyruvate with L-erythrulose: 1 + 7.

Aldol reaction **2** + **7**, was also thoroughly examined (Scheme 3). As observed at the analytical scale, reactions with **2** as nucleophile were much slower than those run with **1**. Consequently, aldolases A5VAX1 and B4XH86, which gave the best conversions in a reasonable time (24 h), were selected for preparative-scale syntheses. After purification on an anionic exchange resin, aldol **14** was isolated in 90% and 74% yields, respectively. From the twelve theoretical cyclic forms that could be obtained (strict 3*S* configuration), in contrast from **1**, only three appeared on the NMR spectra for both enzymes, 2 furanoses **14b** and one pyranose **14c** (Scheme 3), all in the same proportion. Additional 2D NOESY NMR experiments allowed to assign the configurations (3*S*, 4*S*, 5*S*) to the aldol formed for both enzymes. As expected, the configuration at C4 was the same for both aldols **13** and **14**, resulting from the similar addition mode on the *re* face of **7**. The reaction was also performed under the same conditions with A3SLSo to assess its stereoselectivity at carbon 3, as no previous studies had been conducted with this enzyme. An identical NMR spectrum was obtained as that for A5VAX1, also showing a 3*S* preference with **2**. However, the reaction did not exceed 50% conversion after 48 h.



Scheme 3. Aldolase-catalyzed reaction of 2-ketobutyrate with L-erythrulose: 2 + 7.

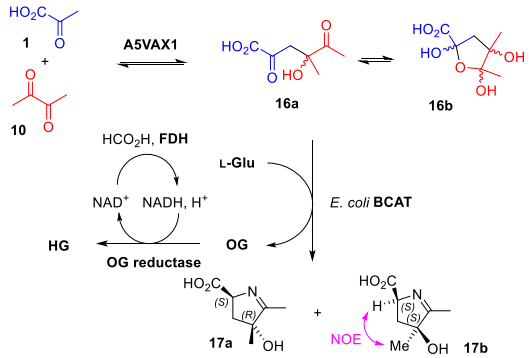
The reaction of **1** with symmetrical difluoroketone **9** also led to an achiral aldol product **15** (Scheme 1). The aldol reaction **1** + **9** was thus also performed with B4XH86, previously identified in analytical experiments as one of the most active aldolases with this substrate couple. However, the reaction and purification process were hampered by the volatility of **9** and the limited stability (degradation on ion exchange resin) of **15** (see SI). Nevertheless, aldol **15** could be isolated at a yield of 60% after 7 h.

We applied the methodology for the highly efficient preparation of a new constraint dehydro-proline analogue, to demonstrate the usefulness of this new route to tertiary alcohols. L-proline is an amino acid of particular interest due to its involvement in peptide and protein folding. Many proline analogues have been included in peptides and mimetics behaving as ligands for various therapeutic targets in the field of cancer, neuropathic pain, and autoimmune diseases.[19] Furthermore, naturally occurring 4-hydroxy-L-proline is found in plant and animal proteins, such as collagen and gelatin,[20] or in peptide antibiotics produced by several microorganisms.[21] It has also been shown to display antioxidant properties in halotolerant and halophilic microorganisms,[22] and has been used in polyamides as a porogen filler for bone reconstruction.[23]

The new targeted constrained hydroxyproline analogue **17** was readily prepared in a one-pot one-step process, including an enzymatic transamination of aldol **16** coming from the aldolisation reaction **1** + **10** (Scheme 4). The aldolisation step was preliminary studied with the five previously selected aldolases. B4XH86 and A5VAX1 gave the best results at an analytical scale (total conversion of **1** in less than half an hour), A3SLSo being the sole inactive enzyme under our experimental conditions. The aldol reaction was first performed using A5VAX1 as catalyst. As observed for **15**, purification by anion exchange chromatography led to partial degradation of **16**. Consequently, the excess diketone **10** was simply removed by liquid extraction and aldol **16** was thus obtained at a yield of 51% in a linear form **16a** and a cyclic hydrate **16b** in the same proportion. Subsequently, aldol **16** was used as a substrate for branched chain aminotransferase (BCAT) from *Escherichia coli* in the presence of glutamic acid (Glu) as amino donor. BCAT is a well-known enzyme that has already been used in many studies to prepare various amino acids of the L-series with very high stereoselectivity.[24–26] Thus, as expected, aldol **16** was converted into the corresponding α-amino acid, which spontaneously underwent cyclization to give a mixture of **17a** and **17b** (Scheme 4). Cyclic imines were the only forms observed by NMR, suggesting that the

cyclization could afford an efficient equilibrium shift of the transamination, as reported in similar cases using diamino or dicarbonyle derivatives as transamination substrates.[27–29] In our case, it appeared that conversion of **16** did not exceed 60% (see SI). Nevertheless, assuming that BCAT exclusively produces amino acids of the L-series, the formation of **17a** and **17b** in equimolar quantities indicates that aldolisation was not stereoselective and led to racemic **16a**.

Given that such a lack of enantioselectivity could be the result of a thermodynamic control of the aldol reaction, A5VAX1 and BCAT were combined in a one pot one step process to favor kinetic control and a possible improvement in stereoselectivity. Transamination was thus made irreversible through its coupling with the reduction of the coproduct 2-oxoglutaric acid (OG) formed from Glu. This reaction was catalyzed by an OG-reductase[30,31] in the presence of NADH, which was itself irreversibly regenerated using the formate/formate dehydrogenase (FDH) system.[32] These cascade reactions were achieved by adjusting the relative amounts of A5VAX1 and BCAT to slow down the aldolisation reaction, while trying to minimize the side transamination reaction between **1** and Glu, leading to the formation of alanine. Finally, the pyrroline **17a** and **17b** were isolated after 8 h at a yield of 57% at a 25/75 ratio using an A5VAX1/BCAT ratio of 1/40 w/w in this multi-enzyme one pot process, with 20, 80 and 20 mM **1**, **10,** and Glu, respectively. The use of B4XH86 in the same one step process gave a 35/65 ratio of **17a** and **17b** (purification was not performed). The cis (*S*, *S*) configuration of the major isomer **17b** could be unambiguously assigned by NOESY ^{1}H NMR experiments (Scheme 4).



BCAT = branched chain aminotransferase, **FDH** = formate dehydrogenase, **Glu** = Glutamic acid, **OG** = 2-oxoglutaric acid, **HG** = 2-hydroxyglutaric acid

Scheme 4. Multienzymatic cascade synthesis of 4-hydroxy proline analogues 17a and 17b.

In conclusion, we highlight a second class II family of aldolases, namely pyruvate-aldolases, which are able to use ketones as electrophiles. However, the ketone substrate spectrum of these biocatalysts is more restricted than that of the Rhamulose-1-phosphate aldolases previously described,[10] with selective activity towards the more highly activated ketones. Nevertheless, compared to DHAP aldolases, which are highly specific for their nucleophile substrate, pyruvate aldolases display significant activity with pyruvate analogues, thus opening access to structural diversity. This wide scope of application was illustrated in this study by the stereoselective preparation of seven new compounds isolated in moderate to very good yields (60-92%). Finally, a one-pot one-step cascade process, involving four enzymes, was designed for the highly efficient and stereoselective synthesis of a new constrained dehydroproline analogue.

AUTHOR INFORMATION

Corresponding Author

* E-mail: christine.helaine@uca.fr.

ORCID

Christine Guérard-Hélaine : 0000-0002-2311-7514

Notes

The authors declare no competing financial interest.

ASSOCIATED CONTENT

Supporting Information.
The supporting information is available free of charge on the ACS Publications Website at DOI:
Details on material, methods, experimental procedures and spectral data.

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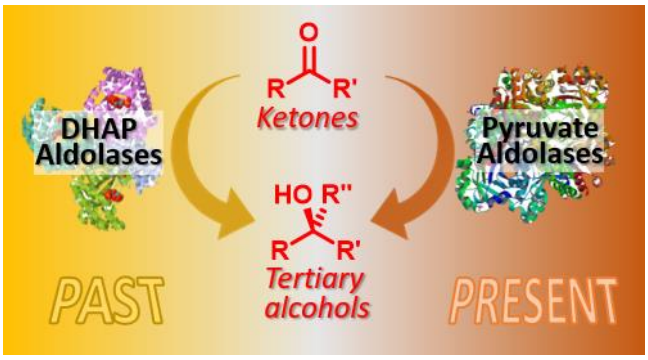
DHAP
Aldolases
O
R
R'
Ketones
HO R''
R
R'
Tertiary
alcohols
Pyruvate
Aldolases
PAST
PRESENT