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Formaldehyde as a Promising C₁ Source: The Instrumental Role of Biocatalysis for Stereocontrolled Reactions

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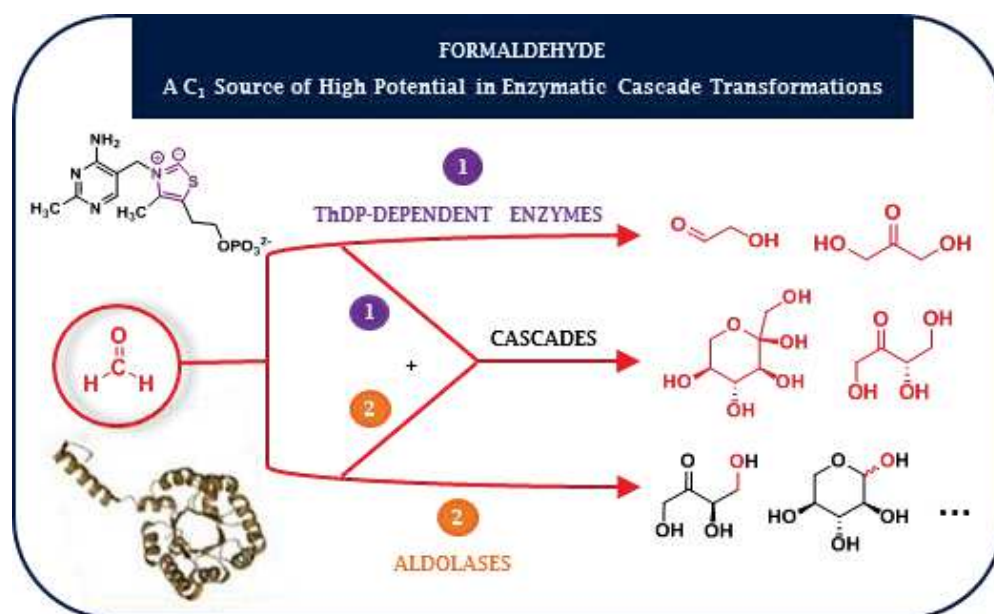
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ABSTRACT:

In the context of the depletion of fossil resources, formaldehyde is an emerging C₁ source exhibiting high and versatile reactivity, compared to the most studied C₁ molecules (CO₂, CO, HCOOH, and CH₄). In the present mini-review, we show that biocatalysis is an ideal approach to control the reactivity of formaldehyde and to use its great potential as a platform for the synthesis of value-added chiral products. The ability of aldolases and ThDP-dependent enzymes to catalyse stereocontrolled carbon-carbon bond formation with formaldehyde are shown to involve aldol and umpolung reactivity. The synergetic combination of i) enzyme discovery, ii) mechanism understanding, and iii) enzyme engineering provides highly stereospecific biocatalysts of increasing interest for synthetic chemistry.

KEYWORDS: formaldehyde, enzyme, aldolisation, umpolung reaction, carbon-carbon bond formation, selectivity, carbohydrate, cascade

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1. INTRODUCTION

1.1 Formaldehyde: discovery and achiral use

Butlerov discovered formaldehyde (HCHO) in 1859 when studying methylene acetate hydrolysis.¹ It was also the first polyatomic molecule detected in space by astrophysicists. For the chemical industry, formaldehyde is an important building block and more than 20 million tons of it are produced each year, mostly from partial oxidation of methanol.¹ Its price is indexed on that of methanol and on regional markets. Formaldehyde is used as a cross-linking agent in resins (e.g. phenol-, urea- and melamine-resins, etc.). In these condensation reactions, it is used as a hydroxymethylene or methylene source. It is noteworthy that its slow and constant release from the wood-derived products is responsible for indoor pollution.²⁻³

1.2 Scope of this review: aldol and umpolung reactions with formaldehyde

Beyond its large scale achiral industrial use, formaldehyde is implicated in chiral processes involving aldolisation and umpolung activation. The formose reaction (FR) is a particular case of umpolung, which consists in oligo- or polymerization of formaldehyde. Previous reviews describe chemocatalyzed i) asymmetric aldolisations of formaldehyde,⁴ as well as ii) FR.⁵⁻⁶ Chemo- and biocatalyzed aldol and umpolung reactions of carbonyl compounds were described as major C-C bond formation processes, as part of a larger review highlighting the complementary roles of chemo- and biocatalysis in retrosynthesis.⁷ The high potential of aldolase in aldol reaction was recently highlighted in detail.⁸⁻¹⁰

Because formaldehyde is of high interest as a C₁ source for the synthesis of value-added chemicals, the objective of the present review is to focus on this peculiar molecule and its biocatalytic transformations. Herein we thus provide an exhaustive overview of enzyme-catalyzed aldol and umpolung reactions with formaldehyde to generate complex chiral products.

The present publication highlights the importance of i) enzyme discovery, ii) mechanism understanding, and iii) enzyme engineering. The combination of these three approaches is instrumental in providing highly stereospecific biocatalysts dedicated to synthetic chemistry. Moreover, these selective systems are combined in cascade reactions without the need of protection/deprotection cycle nor isolation of intermediates to promote the generation of value-added products. With the examples developed herein, we wish to highlight the complementarity between synthetic chemistry and biochemistry.

1.3 Formaldehyde: a C₁ source of high potential

Formaldehyde transformation is highly rewarding because it potentially gives rise to an important gain in complexity: from a simple achiral C₁ building block, C-C bonds are formed and asymmetric carbon centers can be generated. In living organisms, formaldehyde is i) a key metabolite in aerobic methylotrophic organisms via its oxidation to the formyl level,¹¹⁻¹² and ii) the main source of methylene in carbon assimilation pathways.¹³ In the context of the depletion of fossil resources, an increasing interest is dedicated to C₁ chemistry.¹⁴⁻¹⁶ While the most studied C₁ molecules remain CO₂, CO, HCOOH, and CH₄, HCHO is an emerging C₁ source due to recent developments toward its production either from biomass¹⁷⁻¹⁹ or from CO₂.²⁰⁻²³ Contrarily to the cited C₁ molecules, formaldehyde is a particularly versatile and reactive substrate which offers great promise for the synthesis of value-added chiral products.

1.4 Formaldehyde: a challenging substrate

Formaldehyde is also challenging to use and transform: i) it is a gas under atmospheric conditions and is usually used *in vitro* as an aqueous solution –so-called formalin solution– featuring a rather ill-defined composition and exhibiting stability issues over time; and ii) formaldehyde is a symmetrical and very reactive molecule. While these challenges appear perfectly fitted for the high selectivity of enzymatic catalysis, the control of the high reactivity of formaldehyde is the most important issue to solve for biocatalytic transformation of HCHO. Undesired reaction of formaldehyde with nucleophilic residues leads to protein deactivation²⁴⁻²⁹ or even denaturation by cross-linking if formaldehyde reacts with two residues.³⁰⁻³⁴ We wish to show that the discovery and engineering of enzymes are instrumental to address this issue and impressive progress enable some enzymes to better “handle” formaldehyde.

1.5 Description of aldol and umpolung reactions: formaldehyde as a hydroxymethylating agent

In aldol reaction, formaldehyde reacts with an enolizable carbonyl function giving rise to a γ -hydroxycarbonyl. In this reaction the generated asymmetric carbon center is the C β of the carbonyl donor reactant and not the C γ arising from formaldehyde (Figure 1a). Few aldolases, described in part 2, are able to catalyze such reaction with formaldehyde.

Umpolung refers to polarity inversion. In the present case, this powerful method concerns the activation of the C α of a carbonyl function giving rise to a β -hydroxycarbonyl compound. The resulting product is enolizable and can subsequently be engaged in aldol reactions to increase the level of complexity of the final molecule (Figure 1b). When formaldehyde is used as an

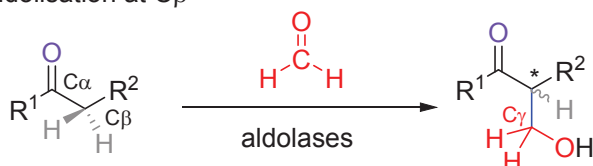
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3 acceptor, the C β center arises from formaldehyde. While the intermediate is not chiral, the final
4 product of the cascade is an asymmetric molecule in which the chiral center arises from
5 formaldehyde (Figure 1b). A very particular case of this umpolung + aldolisation cascade is if
6 formaldehyde (Figure 1b). A very particular case of this umpolung + aldolisation cascade is if
7 all carbonyl substrates are formaldehyde. The reaction is then called formose reaction (FR) and
8 leads to the *de novo* formation of carbohydrate derivatives (Figure 1c). These transformations
9 are described in part 3.
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Chiral transformations with formaldehyde: hydroxymethylation

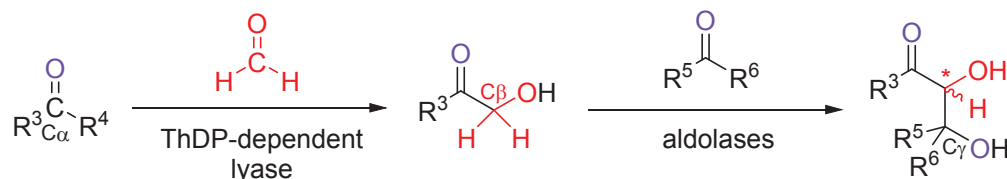


achiral, symmetrical, versatile and
reactive C₁ building block

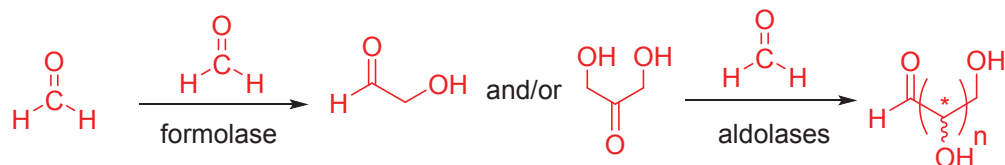
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22 a) Aldolisation at C β



29 b) Umpolung at C α followed by aldolisation at C β



37 c) Formose reaction: umpolung + aldolisation with R³ = R⁴ = R⁵ = R⁶ = H



44
45 **Figure 1.** Enzyme-catalyzed transformations of formaldehyde: a) aldol reaction, b) cascade of
46 umpolung and aldol reactions, c) formose reaction
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2. TRANSFORMATION OF FORMALDEHYDE BY ALDOLASES

52 Aldolases belong to the C-C bond forming lyase or transferase family and catalyze aldol
53 reactions. The general mechanisms of action of these classes of aldolase currently known are
54 presented hereafter (Figure 2).
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2.1 Mechanisms

Aldol reaction concerns the formation of a C-C bond between an enolizable carbonyl compound, referred as the donor substrate, and an acceptor. Enolization process generates a nucleophilic carbon atom, which adds to the electrophilic carbonyl function of the acceptor. The donor substrate can be activated according to three different mechanisms, presented in the general scheme in Figure 2. In each case, the C-C bond formation step is depicted. For the class I aldolases, the donor substrate is activated by a lysine residue, forming an enamine intermediate, while for the class II, a Lewis acidic metal co-factor promotes an enediolate form.^{8-9, 35-39} The third possible activation concerns hydroxymethyl pyridoxal 5'-phosphate (PLP)-dependent enzyme. The PLP co-enzyme activates the donor substrate to form a quinoide aldimine intermediate.⁴⁰⁻⁴¹ Structurally, formaldehyde cannot be a donor substrate because it is not enolizable. However, it is a good electrophile and thus a reactive acceptor for a nucleophilic attack from these three key intermediates. Furthermore, the small size and symmetry of formaldehyde are challenges for controlling its approach and ultimately its reactivity.

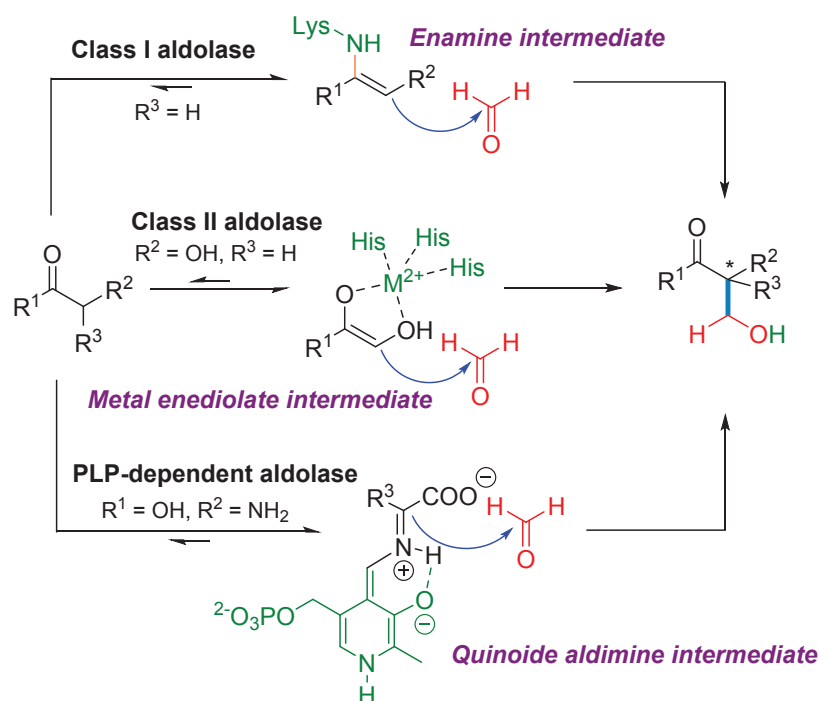


Figure 2. General scheme of aldolisation catalyzed by class I, class II and PLP-dependent aldolases

In each mechanism, the chiral center C β is generated during the formation of the C-C bond and the donor substrate is primarily activated by the catalytic center. Thanks to these bindings, the degree of freedom of the donor is reduced and the enzyme pocket orientates preferentially one face of the donor to the incoming acceptor to insure the stereocontrol. As a consequence, aldolases display high donor substrate specificity. The spatial arrangement of the incoming acceptor appears less constrained by the enzyme, leading to broader acceptor scopes. Formaldehyde is a good acceptor, however it may also interact with various nucleophilic residues. For instance, class I aldolase are particularly sensitive to formaldehyde concentration, because the catalytic lysine residue can react with formaldehyde which inhibits or inactivates the enzyme.

2.2 Transformation of formaldehyde by aldolases: impressive stereocontrolled reactions and scope improvement

Among the class I, class II and PLP-dependent aldolases, few of them have been shown to tolerate formaldehyde as acceptor substrate. These enzymes are presented hereafter. Although, their reactivities with other acceptor are mentioned, the focus is on the aldol reaction with formaldehyde. The product yields are mentioned when they were reported.

2.2.1 Pyruvate and phenylpyruvic acid-dependent aldolases

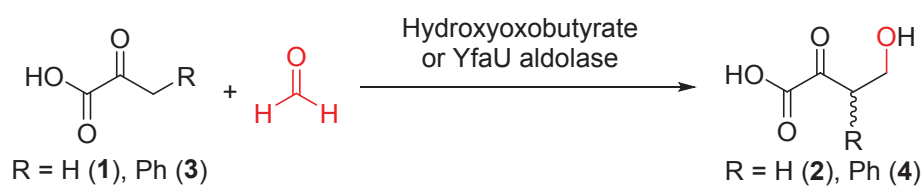


Figure 3. Pyruvate aldolase catalysis: synthesis of compounds 2 and 4

Hydroxyoxobutyrate aldolase (EC 4.1.2.1) was discovered in 1952 in mammalian organisms and extracted from beef liver.⁴² It was later reclassified in the enzyme family of 4-hydroxy-2-oxoglutarate aldolase (EC 4.1.3.16). It is a pyruvate-dependent class I aldolase, which catalyzes the aldol addition of pyruvic acid (1) to formaldehyde (Figure 3). The formation of α -keto- γ -hydroxybutyric acid (2) was evidenced by the γ -lactone form of the product. Formaldehyde

concentration is rate-determining but an excess of formaldehyde initiates side reactions (up to 0.073 M of formaldehyde is tolerated without activity loss). Phenylpyruvic acid (**3**) is also a donor substrate leading to α -keto- β -phenyl- γ -hydroxybutyric acid (**4**) as a product of the reaction (Figure 3). Despite further tests, no other donor substrates were found suitable with formaldehyde.

2-Keto-3-deoxy-L-rhamnonate class II aldolase (YfaU, EC 4.1.2.53) mutant of 2-keto-3-deoxy-6-phosphogluconate aldolase (KDP),⁴³⁻⁴⁴ was produced as a soluble fusion protein, with its natural cofactor Mg^{2+} . Compared to class I aldolases,⁴² this class II pyruvate aldolase was found to be more tolerant to higher formaldehyde concentration (up to 1.4 M is tolerated). The reaction of sodium pyruvate (**5**) with formaldehyde affords sodium 4-hydroxy-2-oxobutanoate (**6**) in quantitative yield (Figure 4). A double condensation of formaldehyde, in particular when Mg^{2+} is replaced by Ni^{2+} or Co^{2+} , affords 4-hydroxy-3-hydroxymethylene-2-oxobutanoate (**7**) in more than 60% yield (Figure 4a). *YfaU* was then used in an elegant tandem reaction in combination with (*S*) or (*R*) selective transaminases to produce amino-acids in a stereocontrolled way: (*S*) and (*R*)-homoserine (**9**) are obtained in 86 and >95% yield respectively as unique enantiomer (Figure 4b).⁴⁵

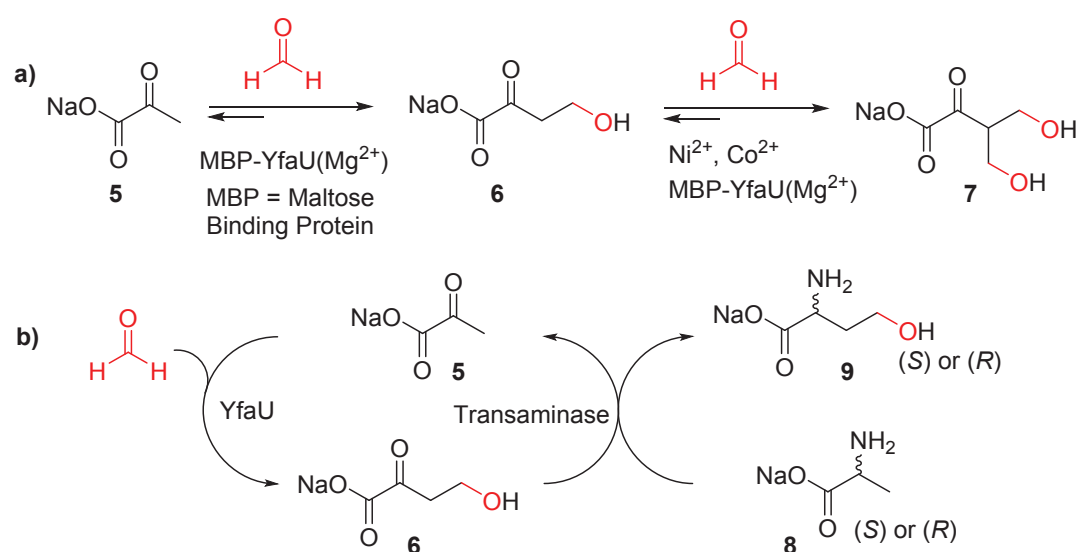


Figure 4. *YfaU* aldolase reaction with formaldehyde: a) synthesis of compound **7**, b) synthesis of compound **9**

2.2.2 D-Ribulose 5-phosphate-dependent aldolase

3-Hexulose phosphate synthase (HPS-aldolase, EC 4.1.2.43) is a class II aldolase found in aerobic methylotrophic bacteria and is involved in the ribulose monophosphate cycle. It

catalyzes the aldol reaction between D-ribulose 5-phosphate (**10**) and formaldehyde, yielding D-arabinose 3-hexulose 6-phosphate (**11**, Figure 5).⁴⁶ While compound **10** is the only suitable donor found over 20 tested, several other aliphatic and even aromatic aldehydes are acceptor substrates besides the natural acceptor formaldehyde.⁴⁷

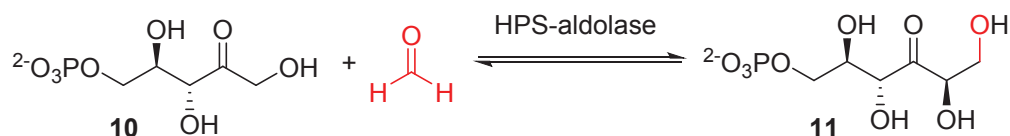


Figure 5. HPS-aldolase catalysis: synthesis of compound **11**

2.2.3 Pyridoxal 5'-phosphate-dependent aldolases

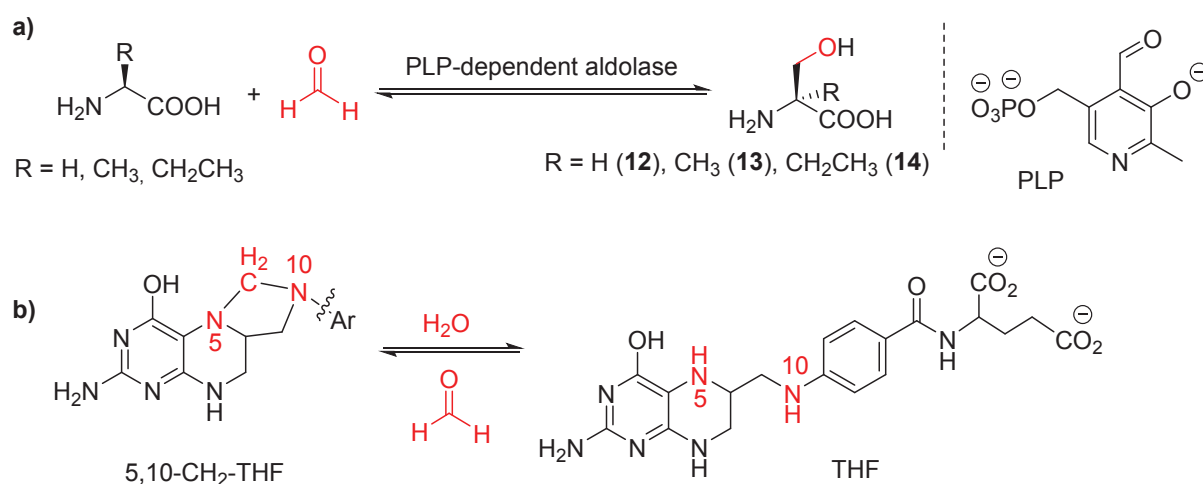


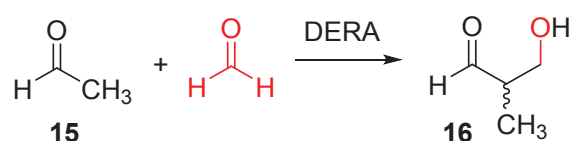
Figure 6. PLP-dependent aldol reaction: a) synthesis of compounds **12-14** and structure of PLP b) reversible THF reactivity toward formaldehyde

Serine hydroxymethyltransferase (SHMT, EC 2.1.2.1), found in prokaryotes and eukaryotes organisms, belongs to the glycine-dependent aldolase family.⁴⁸ This enzyme catalyzes the reversible aldol transformation of glycine into serine (**12**) (Figure 6a). Formaldehyde is generated in the enzyme from 5,10-CH₂-tetrahydrofolic acid (THF, Figure 6b).⁴⁰

α-Methylserine hydroxymethyltransferase (MSHMT, EC 2.1.2.7) of *E. coli* whole-cell extract catalyzes the formation of α-methyl-L-serine (**13**), in 73% yield, from formaldehyde and a slight excess of D-alanine (Figure 6a).⁴⁹

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3 **α -Methylserine aldolase** catalyzes the enantioselective formation of α -methyl-L-serine (**13**)
4 and α -ethyl-L-serine (**14**) in 95 and 96% *in situ* yield, respectively (Figure 6a).⁵⁰ The enzyme is
5 inhibited by the use of more than 10 mM of formaldehyde. In contrast with SHMT and
6 MSHMT, this enzyme is, *in vitro*, THF-independent.⁵⁰⁻⁵²
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10 11 12 13 2.2.4 Acetaldehyde-dependent aldolase



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Figure 7. DERA aldolase catalysis: synthesis of compound **16**

2-Deoxyribose-5-phosphate aldolase (DERA, EC 4.1.2.4)⁵³ is isolated from animal tissues
and microorganisms, and is currently commercially available. DERA from *E. coli* is a fairly
stable class I aldolase and is the first discovered aldolase able to use an aldehyde as donor
substrate. *In vivo*, DERA catalyzes the reversible aldol reaction between acetaldehyde and D-
glyceraldehyde-3-phosphate (D-G3P) to generate D-2-deoxyribose-5-phosphate. Based on TLC
analysis, formaldehyde and acetaldehyde (**15**) are presumed to react to afford 2-methyl-3-
hydroxy-propionaldehyde compound **16** (Figure 7).

2.2.5 DHAP-dependent-aldolases

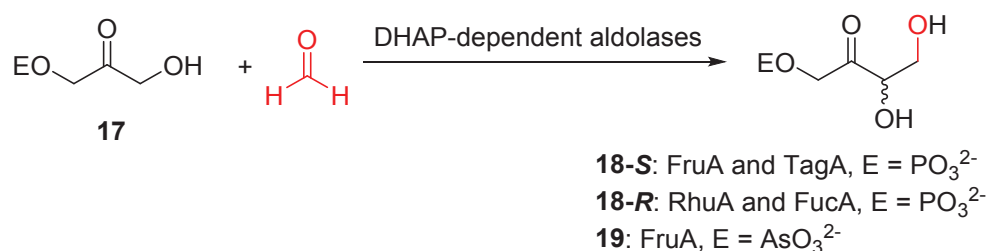


Figure 8. DHAP-dependent aldolase catalysis: synthesis of compounds **18** and **19**

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3 **D-Fructose-1,6-bisphosphate (FBP) aldolases (FruA, EC 4.1.2.13)** are dihydroxyacetone
4 phosphate (DHAP)-dependent aldolases. These glycolytic enzymes play a crucial role in
5 glycolysis, glycogenesis and fructose metabolism. Class I FBP aldolase extracted from rabbit
6 muscle, named RAMA, has been the most studied DHAP-dependent enzyme over the year. In
7 1989, RAMA was studied on a large variety of acceptor substrates. Among more than 50
8 different aldehydes, formaldehyde was shown to react with DHAP (**17**) to afford **18-S** (Figure
9 8).⁵⁴ Formaldehyde displays a good reactivity as an acceptor in RAMA-catalyzed aldol
10 condensation, similar to its natural acceptor D-G3P (relative reactivity v_{rel} of 105 and 100,
11 respectively). RAMA withstands organic co-solvents up to 10% DMSO or 20% EtOH without
12 significant loss of activity and is commercially available. It was the first aldolase exhibiting a
13 large scope of acceptors and thus the first to be used as a synthetic biocatalyst. However, two
14 main drawbacks remained: (i) deactivation of RAMA is fairly rapid in solution; (ii) the substrate
15 specificity of the donor is almost only limited to DHAP that is an unstable and rather expensive
16 substrate.⁵⁵ Subsequently, two other FruA from *Staphylococcus carnosus* and *Staphylococcus*
17 *aureus* were used in the same reaction.⁵⁶⁻⁵⁸ They exhibit higher thermo- and pH stability, and
18 solvent tolerance than RAMA. *S. carnosus* was furthermore shown to tolerate DHAA, the
19 arsenic analogue of DHAP leading to the synthesis of compound **19** (Figure 8).⁵⁹ A class II FBP
20 FruA from *E. coli* was also shown to catalyze the formation of **18-S** in 72% yield.⁶⁰

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35 **Tagatose 1,6-diphosphate aldolase (Tag A, EC 4.1.2.40):** in the same study, class I TagA
36 from *E. coli* and class I TagA from *Bacillus licheniformis*, were shown to afford **18-S** in 70 and
37 68% conversion rate, respectively.⁶⁰

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41 **Rhamnulose 1-phosphate aldolase (RhuA, EC 4.1.2.19) and Fuculose 1-phosphate aldolase**
42 **(FucA, EC 4.1.2.17)** are both bacterial aldolases from *E. coli*. Upon aldol reaction between
43 DHAP and formaldehyde, the other isomer, compound **18-R** is obtained in 81 and 73% yields
44 with RhuA and FucA, respectively. Both of these enzymes are stable and tolerate organic co-
45 solvent up to 30-50% of EtOH, DMF or DMSO.⁶¹ It was later reported that RhuA is able to
46 accept the inexpensive non-phosphorylated DHA as a donor substrate,^{8, 62} however, to our
47 knowledge, no reaction has been yet reported with formaldehyde. Only a theoretical model have
48 been considered for the aldol addition of DHA to formaldehyde.⁶³

54 55 **2.2.6 DHA and derivatives-dependent aldolase: FSA, mutation and cascade**

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57 **D-Fructose-6-phosphate aldolase (FSA, EC 4.1.2.n).** FSA has been discovered in 2001 from
58 gene *fsaA* of *E. coli* genome and is so far of unknown physiological function.⁶⁴ Although the
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3 amino acid sequence and the structure of FSA is evolutionary related to a transaldolase, this
4 lyase exhibits the reactivity of a class I aldolase. The quaternary structure of FSA reveals high
5 packing in a homodecamer form structure, which is believed to explain its high
6 thermostability.⁶⁵ Its subunit is folded into an α/β barrel. Its crystal structure with a
7 glyceraldehyde compound (**23**) in purple is depicted in Figure 9a. The lysine residue K85 is
8 covalently bond to compound **23** forming a hemiaminal *en route* to the formation of a Schiff
9 base (Figures 2 and 9b). The highlighted alanine residues, A165 and A129 in cyan, have been
10 subsequently subjected to mutagenesis to tune the enzyme properties (*vide infra*). FSA is
11 produced on large scale, tolerates co-solvents (10-20% of DMF, DMSO or acetonitrile) and is
12 stable at room temperature or lower. The discovery and use of this enzyme is remarkable
13 because i) it accepts several non-phosphorylated donors (dihydroxyacetone (DHA, **21**),
14 hydroxyacetone (HA, **22**), glycolaldehyde (**20**, GA), 1-hydroxy-2-butanone), and acceptors,<sup>66-
15 68</sup> and ii) it tolerates aldehyde *and* ketone donor substrate, larger ketols and bioisosteric ether.<sup>69-
16 70</sup> Consequently, FSA is the first enzyme able to catalyze the production of ketose *and* aldose
17 (Figures 10 and 11).

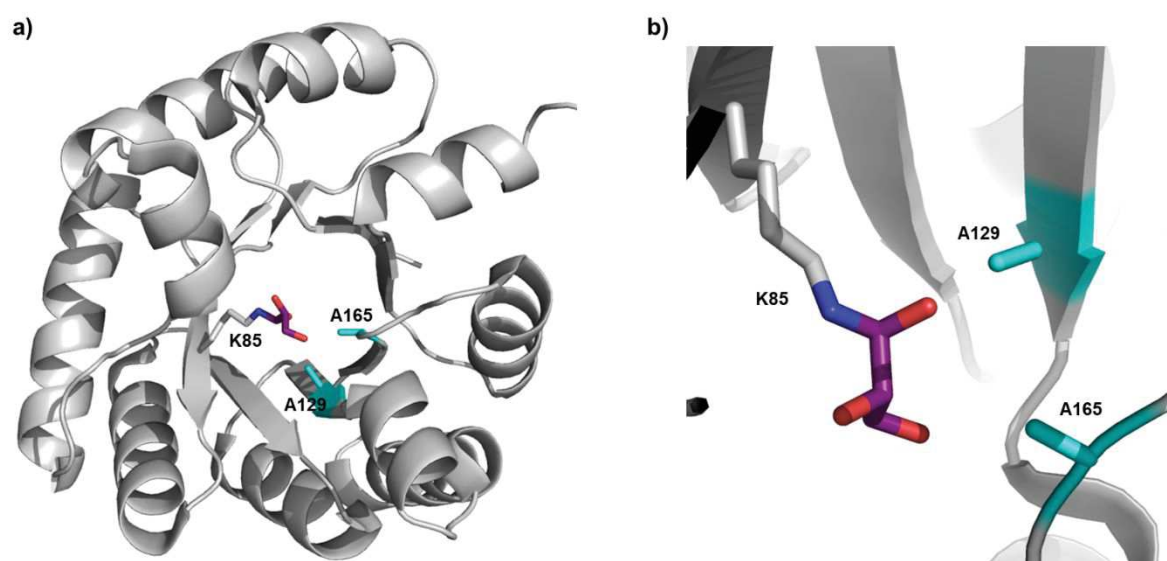
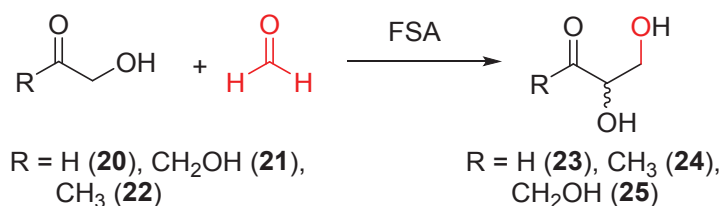


Figure 9. Views of the *E. coli* FSA (PDB ID: 1L6W): a) monomer and b) its active site with the catalytic lysine (K85) and glyceraldehyde (purple).⁶⁵ The figure was prepared using PyMol Molecular Graphics System, v0.99 (Schrödinger).



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Figure 10. FSA aldolase catalysis: synthesis of compounds **23-25**

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2.2.6.1 FSA-catalyzed aldol reaction of three donor substrates with formaldehyde: In 2009, FSA was shown to catalyze the aldol reaction of GA as donor substrate with formaldehyde to successfully afford quasi-quantitatively L-glyceraldehyde (**23**) as the only enantiomer (Figure 11).⁶⁹

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In 2011, FSA was immobilized on the inexpensive inorganic support Mg₂Al-NO₃ layered double hydroxide (LDH), with a loading of 90%.⁷¹ In the aldol addition of HA to formaldehyde, the LDH-FSA is reused up to four times with 70-61% conversion and 100-87% of total FSA activity. 3,4-dihydroxybutan-2-one (**24**) is obtained in 95% yield, as the only enantiomer (Figure 11).

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 K_M , k_{cat} and k_{cat}/K_M are the kinetic parameters used to compare the performances of different enzymes toward different substrates. According to the kinetic data presented in Table 1, DHA features less favorable kinetic parameters compare to HA with the wild-type FSA:^{67, 69, 72}

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i) The Michaelis constant K_M is indeed higher for DHA than for HA. K_M value reflects not only the affinity constant of the Michaelis complex (although it is often approximated to), but more precisely the catalytic steps up to the formation of the enzyme-substrate intermediate.

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ii) The catalytic constant k_{cat} (or turnover number) is 1 order of magnitude higher for HA than DHA.

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iii) Overall, the catalytic efficiency k_{cat}/K_M is of 145 and 4 M⁻¹.s⁻¹ for HA and DHA, respectively.

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In 2010, FSA was engineered to create FSA mono-mutant A129S to circumvent this lower reactivity toward DHA.⁷² As a result, the catalytic efficiency of FSA^{A129S} toward DHA increased 17-fold compared to the wild-type enzyme (2.9-fold decrease of the K_M value and 6.6-fold increase of k_{cat} , Table 1). The mutant incorporates a serine, which is thought to stabilize the DHA donor and disfavors the access of the aldehydic acceptor to the donor part of the active site. FSA^{A129S} catalyzes the reaction between DHA and formaldehyde to successfully afford L-

erythrulose (**25**) in 68% isolated yield as a unique enantiomer (Figure 11), despite poor kinetic parameters determined for formaldehyde (Table 1).

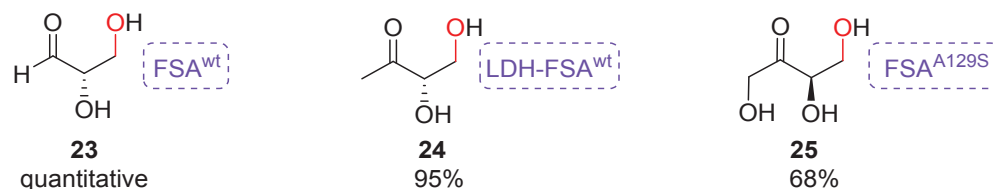


Figure 11. Products of FSA-catalyzed transformation of formaldehyde

Table 1. Steady-state kinetic parameters of aldol reactions catalyzed by FSA wild-type and A129S^{69, 72}

Substrate	FSA wild-type			FSA ^{A129S}		
	K_M (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_M (s ⁻¹ .mM ⁻¹)	K_M (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_M (s ⁻¹ .mM ⁻¹)
DHA ^a	32	116	4	11	760	69
HA ^a	17.4	2527	145	22	899	41
GA ^a	0.20	16.5	83	0.18	4.4	25
Formald ehyde ^b	nd	nd	nd	77	27.7	0.4

nd: not determined, ^a donor substrate; ^b acceptor substrate.

2.2.6.2 Cascade reaction with FSA

The access to orthogonal specificity of different enzymes and mutants enables cascade reactions leading to impressive complex transformations from simple building blocks. An efficient and elegant cascade reaction involving FSA and a kinase has been developed for the synthesis of four rare phosphorylated L-sugars (**27-30**, Figure 12a) from simple and achiral GA and formaldehyde.⁷³ The cascade reaction involves a first FSA-catalyzed aldol reaction between GA and formaldehyde affording L-glyceraldehyde (**23**), isolated in 72% yield. This expensive L-sugar is then phosphorylated thanks to glycerol kinase (GK), coupled with an ATP regenerating system affording the valuable L-glyceraldehyde-3-phosphate (**26**). The reaction of this substrate with four different donors leads to the phosphorylated sugars **27-30**.

The preparation of the rare L-xylopyranose (**31**) via stereoselective glycolaldehyde double addition to formaldehyde was recently reported (Figure 12b).⁷⁴ A structure-guided site directed mutagenesis of the acceptor-binding site was used to design a FSA mutant able to perform this C₁+C₂+C₂ addition.⁷⁵ Different mutations were tested and the double mutant A129T/A165G appeared to be the most efficient C₁+C₂+C₂ catalyst. Compound **31** was thus successfully prepared in 62% isolated yield as a unique enantiomer. Using a strategy of substrate confinement, the synthesis of **31** was further improved to an increased yield >95% by using FSA^{A129T/S166G}.⁷⁶

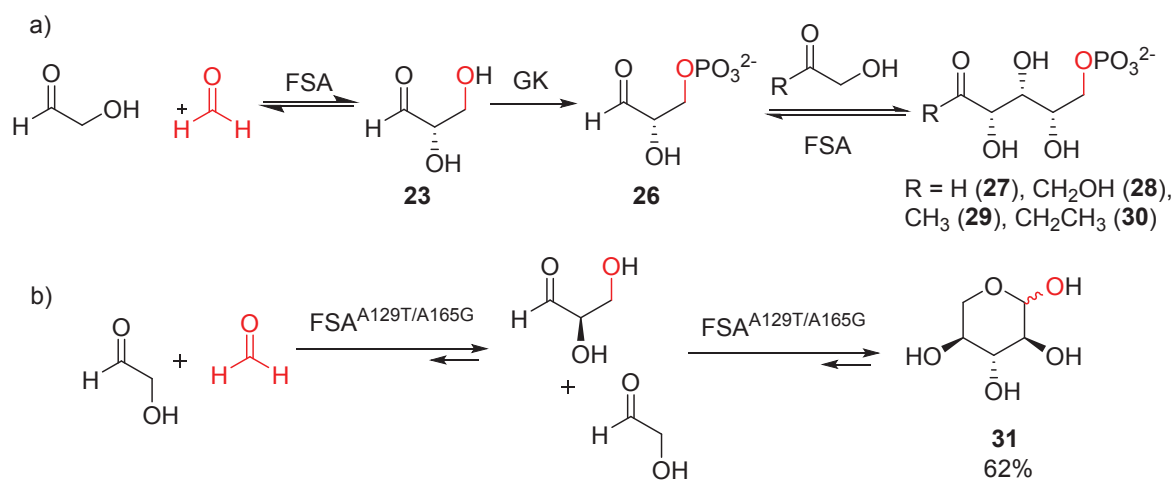
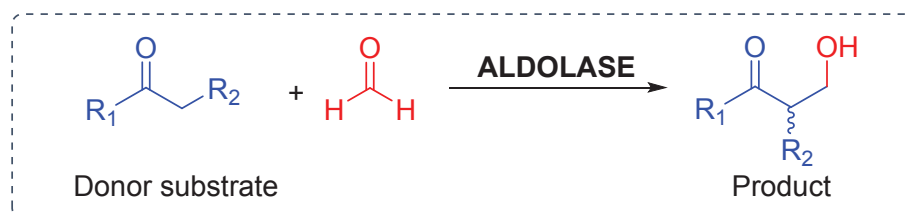


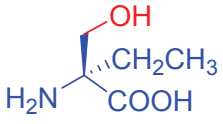
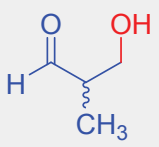
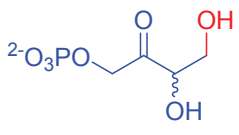
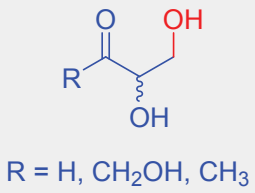
Figure 12. Cascade reactions involving FSA for the synthesis of complex structures: (a) synthesis of compounds **27-30**, (b) synthesis of compound **31**

Table 2 below is a summary of the presented aldolase-catalyzed reactions with formaldehyde along with the level of tolerance toward this acceptor substrate. The complexity of these transformations is impressive in absence of protective group. However, the use of such enzymes as synthetic biocatalysts would require further improvements.

Table 2. Summary of aldolase reactions with formaldehyde and tolerance toward this acceptor substrate.



PRODUCT	ALDOLASE	[HCHO] used in product synthesis (M)	Inhibitory [HCHO] (M)
	Hydroxyoxobutyrate aldolase ⁴²	0.073	0.146
	Hydroxyoxobutyrate aldolase ⁴² 2-Keto-3-deoxy-L-rhamnonate class II aldolase (YfaU) ⁴⁵	1	>1.4
	2-Keto-3-deoxy-L-rhamnonate class II aldolase (YfaU) ⁴⁵	1	>1.4
	3-Hexulose phosphate synthase (HPS-aldolase) ⁴⁶	0.000030	nd
	α -Methylserine hydroxymethyltransferase (MSHMT) ⁴⁹ α -Methylserine aldolase ^{50, 52, 77}	0.020 0.010	nd >0.010

	α -Methylserine aldolase ^{50, 52, 77}	0.010	>0.010
	2-Deoxyribose-5-phosphate aldolase (DERA) ⁵³	0.1	nd
	DHAP-dependent aldolase ^{54, 56-61}	0.040 - 0.2	nd
	D-Fructose-6-phosphate aldolase (FSA) ⁶⁹⁻⁷⁶	0.025	nd

nd: not determined.

3. UMPOLUNG ACTIVATION BY THIAMINE DIPHOSPHATE-DEPENDENT ENZYMES

The umpolung activation of a carbonyl compound refers to the polarity inversion of the electrophilic $C\alpha$. This is a powerful method for the formation of C-C bonds. The generated nucleophilic carbon adds to an electrophilic carbon center, such as the carbon of formaldehyde, giving rise to β -hydroxycarbonyl compounds (Figure 13a, step i)). However, only few species are able to perform such transformation. This field is dominated –but not limited to– carbene catalysts.⁷⁸ In Nature, thiamine diphosphate (ThDP) –derived from vitamin B1– is the only known catalyst able to perform umpolung activation of carbonyl functions, thanks to a carbene activation moiety.⁷⁹⁻⁸¹ ThDP is a co-enzyme able to bond in the active site of various enzymes and plays the key catalytic role. Three different ThDP-containing enzymes give rise to reactivity with formaldehyde: transketolase (TK), benzaldehyde lyase (BAL), and engineered formolase (FLS). As depicted in the general Figure 13, the obtained β -hydroxycarbonyl product does not feature any chiral center, but an enolizable carbonyl moiety. Subsequent aldol reaction leads to chiral compounds in which the chiral carbon center is the carbon of the formaldehyde acceptor substrate (Figure 13a, step ii)). A particular case of umpolung is when formaldehyde is the only

carbonyl substrate (*i.e.* both the donor and the acceptor), the reaction is then called formose reaction (FR, Figure 13b). The first enzyme-catalyzed formose reaction has been recently disclosed and later combined with aldolase to afford C₄ and C₆ carbohydrate with perfect stereocontrol. This represents an impressive gain in complexity starting from a simple achiral and symmetrical building block (*vide infra*).

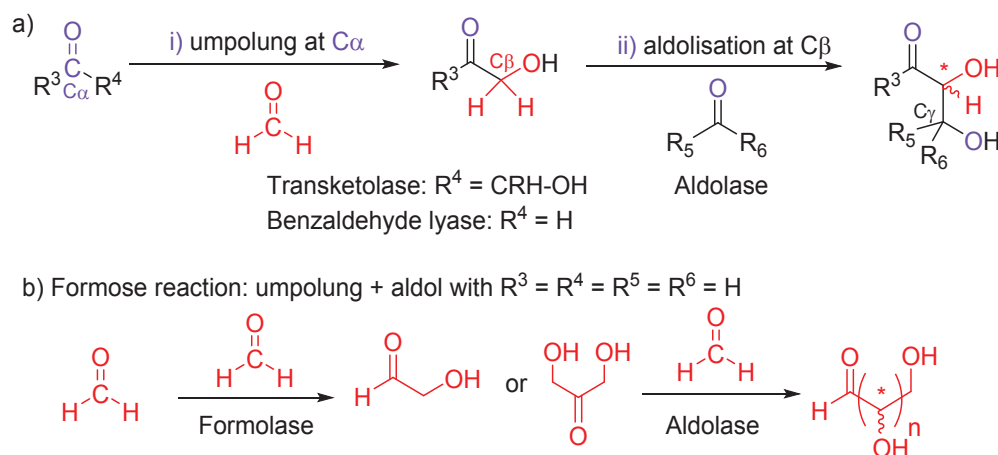


Figure 13. a) Umpolung and aldol cascade reactions, b) formose reaction

ThDP: an *in situ* generated active carbenic center

The exact mechanism of action of ThDP remained unclear until Breslow *et al.* reported that the catalysis is centered at the C-2 of the thiazolium moiety.⁸² NMR studies and deuterium exchange have shown that a glutamic residue favors the imino tautomer of ThDP leading to C-2 deprotonation (Figure 14a).⁸³⁻⁸⁴ Once deprotonation takes place, the anionic charge is stabilized by the adjacent cationic nitrogen atom, generating an ylide or what was later recognized as a carbene, a very rare and elusive species to be observed in biological system.⁸⁵⁻⁸⁶ More precisely, Breslow *et al.* reported this breakthrough while studying ThDP-catalyzed decarboxylation of pyruvate. They proved the generation of a nucleophilic acyl anion intermediate, which is now called Breslow intermediate (Figure 14b) and identified as the key species for the C-C bond formation. Indeed, thanks to the polarity inversion of the initial carbonyl function, the newly generated nucleophilic carbon can attack an electrophile moiety, leading to the carboligation between two initially electrophilic substrates. It is noteworthy that Breslow's discovery not only impacted the field of biocatalysis but also the development of carbenes as prototypical organocatalysts.⁸⁷⁻⁹⁰

Although, pyruvate decarboxylase enzymes using ThDP as co-factor have been involved in C-C bond formation, no example using formaldehyde have been reported so far. The related phenylpyruvate decarboxylase was tested with formaldehyde, however, no reactivity was detected.⁹¹

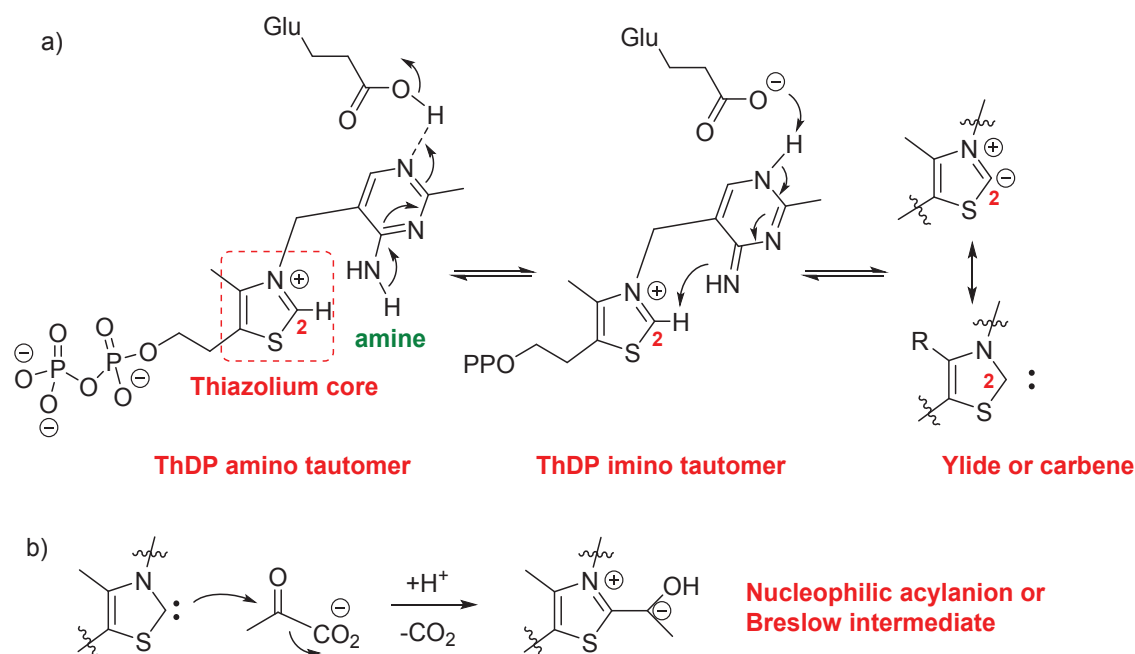
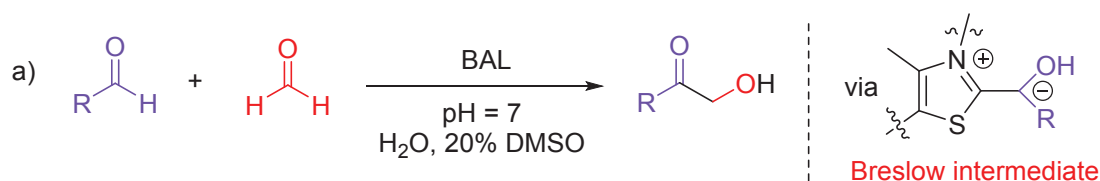


Figure 14. ThDP activation mode: a) carbene formation, b) Breslow intermediate generation

3.1 Benzaldehyde lyase (BAL, EC 4.1.2.38)

Benzaldehyde lyase is another type of lyase using ThDP as co-factor. It catalyzes C-C coupling between different benzaldehyde derivatives and formaldehyde in good to excellent yields with a scope of 11 different donors varying by aromatic groups R (Figure 15a).⁹² However, no reaction takes place in the case of fluorinated aromatic, indole and pyridinyl groups. To avoid benzoin condensation, an excess of formaldehyde is necessary. It is also reported that retro-benzoin condensation occurs only with the *R* isomer of benzoin (**32**). Thanks to the reaction with formaldehyde, kinetic resolution is performed to isolate the *S* benzoin enantiomer (Figure 15b).



R	Isolated Yield (%)	R	Isolated Yield (%)
Ph	94	4-ClC ₆ H ₄	88
4-MeC ₆ H ₄	94	4-OHC ₆ H ₄	89
4-MeOC ₆ H ₄	91	2-Furanyl	77
3-MeOC ₆ H ₄	92	4-Pyridinyl	<5
2-MeOC ₆ H ₄	68	3-Pyridinyl	<5
3-MeO-4-OHC ₆ H ₃	51	2-FC ₆ H ₄	<5
3-BrC ₆ H ₄	83	2,4-F ₂ C ₆ H ₃	15
2-ClC ₆ H ₄	77	Indole-3-carbaldehyde	No reaction

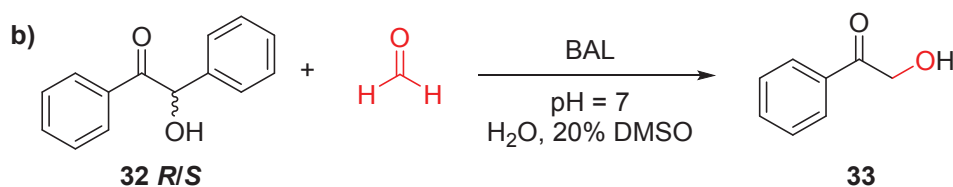
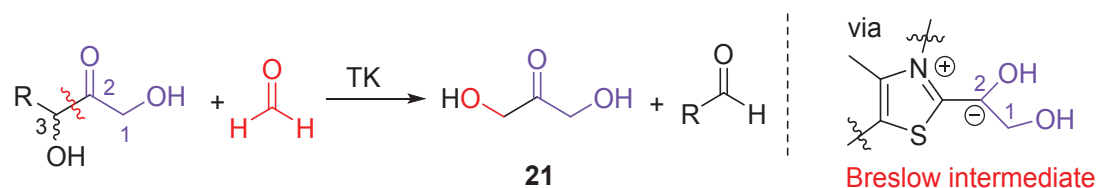


Figure 15. BAL-catalyzed umpolung reaction with formaldehyde: a) benzoin reaction, b) synthesis of compounds **33**

3.2 Transketolase (TK, EC 2.2.1.1)

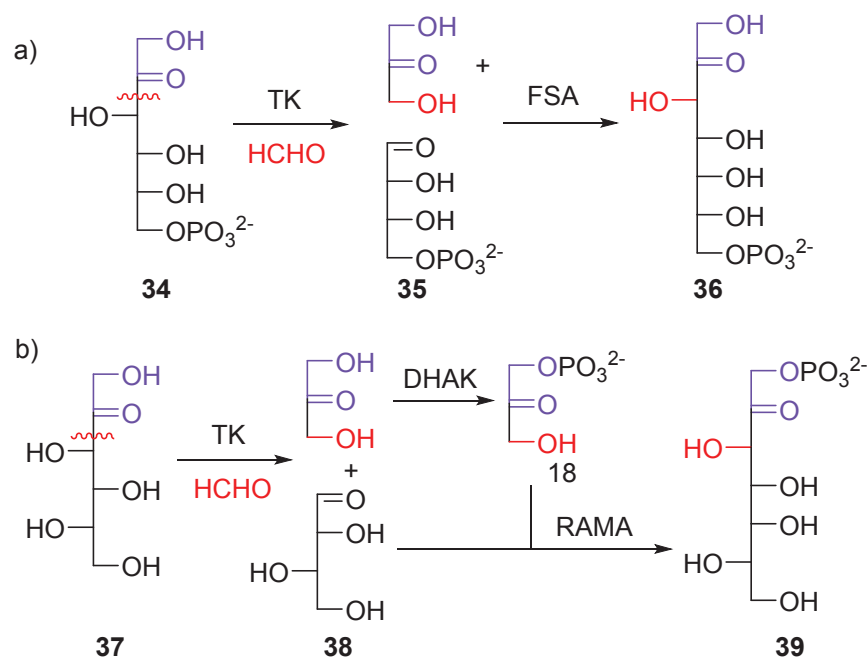
Transketolase is a key transferase in the natural pentose phosphate pathway. It is easily produced from *E. coli* and uses ThDP and Mg²⁺ (or Ca²⁺) as cofactor. The role of ThDP is to break the C-C bond between the C-2 and C-3 of a number of ketose and aldose sugars (Figure 16).⁹³ With this crucial step, TK catalyzes the reversible transfer of a hydroxyacetyl group to various carbonyl acceptors via the Breslow intermediate depicted in Figure 16.³⁸ This nucleophilic hydroxyacetyl group notably reacts with formaldehyde to afford DHA (**21**) and the complementary aldehydic fragment (Figure 16).⁹⁴ While the generated DHA is not chiral,

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3 it is only an intermediate that is further used as a C₃ synthon in asymmetric transformations
4 described hereafter in which the formed chiral center is the carbon atom arising from
5 formaldehyde (Figure 17).
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16 **Figure 16.** TK catalysis and activation mode: synthesis of compound **21**
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20 Two elegant cascade reactions use this general scheme to form DHA **21**.⁹⁵ The first cascade
21 uses successively TK and FSA (Figure 17a). TK catalyzes the breaking of D-fructose-6-
22 phosphate (**34**) with formaldehyde to generate DHA and D-erythrose-4-phosphate (**35**).
23 Concentration of formaldehyde has to be kept under 400 mM in order to avoid TK inhibition.
24 The two products are combined to afford the C₇ D-sedoheptulose-7-phosphate (**36**), in 70%
25 yield in a FSA-catalyzed aldol reaction. The second cascade involves a similar TK-catalyzed
26 C-C breaking from L-sorbose (**37**) and reaction with formaldehyde to provide L-threose (**38**)
27 and DHA (Figure 17b). Upon conversion of DHA into DHAP (**18**), L-galacto-2-heptulose-1-
28 phosphate (**39**) is efficiently prepared in 80% yield from the aldol reaction of DHAP and L-
29 threose catalyzed by RAMA.
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25 **Figure 17.** Cascade reactions involving TK transformation of formaldehyde into DHA
 26 intermediate: a) synthesis of compound 36, b) synthesis of compound 39
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31 3.3 Formolase

32 3.3.1 Formose reaction

33 FR generates carbohydrates from the oligomerization of formaldehyde. Since its first report in
 34 1861 by Butlerov, FR has attracted a large and continuous interest on a synthetic point of view⁵⁻⁶
 35 and in prebiotic chemistry.⁹⁶⁻⁹⁸ FR is a general term covering various type of reactions.
 36 Depending on the conditions and the catalyst used, the importance and occurrence of these
 37 reactions vary. FR catalyzed by inorganic base in the presence of co-activator gives rise to a
 38 complex mixture of carbohydrates due to the auto-catalytic nature of the process and of the
 39 multiple competitive reactions taking place (aldol, retro-aldol, Canizzaro, cross-Canizzaro,
 40 etc.).⁵⁻⁶ The use of carbene and in particular the pioneering work of Castell with thiamine
 41 enabled important progress in the selectivity for short chain carbohydrate.⁹⁹⁻¹⁰²
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51 The high specificity and selectivity of enzyme-catalyzed transformation represent a
 52 complementary approach to synthetic chemistry. It is particularly true with FR because it is an
 53 oligomerization process in which every product is a potential substrate. However, no enzyme
 54 is known to catalyze FR *in vivo*. The first engineered enzyme catalyzing FR leads selectively
 55 to C₂ and C₃ carbohydrates and was later used in a cascade reaction to afford C₄ and C₆
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3 carbohydrates with perfect stereocontrol (*vide infra*). These two examples are described
4 hereafter.
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6 7 **3.3.2 Formolase: first enzyme-catalyzed formose reaction leading to C₂ and C₃** 8 **carbohydrates** 9

10
11 In 2015, the first enzyme-catalyzed FR was reported. The engineered formolase (FLS) catalyzes
12 the di- and tri-merization of formaldehyde into GA and DHA, respectively (Figure 18, step a +
13 a').¹⁰³ The production of DHA and GA was monitored over time in function of the concentration
14 of introduced formaldehyde.¹⁰⁴ The product profile is shown to be concentration-dependent. At
15 high concentration of formaldehyde (10 mM), DHA is obtained as the major product. At lower
16 concentration of formaldehyde (0.2 mM), GA is obtained as the main product. The mechanism
17 of FR catalyzed by carbene is useful to understand these outcomes.¹⁰⁵⁻¹⁰⁶ ThDP activates
18 formaldehyde to afford the simplest Breslow intermediate i) (Figure 18). This acylanion adds
19 to a second equivalent of formaldehyde to afford another acylanion after proton migration from
20 C- to O-atom ii). At this stage, the newly formed C₂ compound can be released to afford GA
21 and complete step a. Since it is an acylanion, it can also add to a third molecule of formaldehyde
22 to generate the last intermediate iii) leading to DHA after release. This mechanism explains that
23 a higher concentration of formaldehyde drives the reaction toward DHA via intermediate iii),
24 whereas a lower concentration leads to GA. Additional investigations probed the involvement
25 of GA as a valid intermediate in the formation of DHA. The use of ¹³C-labeled formaldehyde
26 (0.2, 1 and 10 mM) and unlabeled GA (1 mM) gives rise to mix-labeled DHA. This proves that
27 FLS is able to accept free GA to form the final DHA moiety upon reaction with one equivalent
28 of formaldehyde. It is indeed possible that intermediate i) adds to the carbonyl of GA instead
29 of formaldehyde. Since formaldehyde is a better acceptor, this situation arises when
30 formaldehyde concentration is low. It is also possible that ThDP activates GA to form the
31 intermediate ii) affording DHA via intermediate iii).
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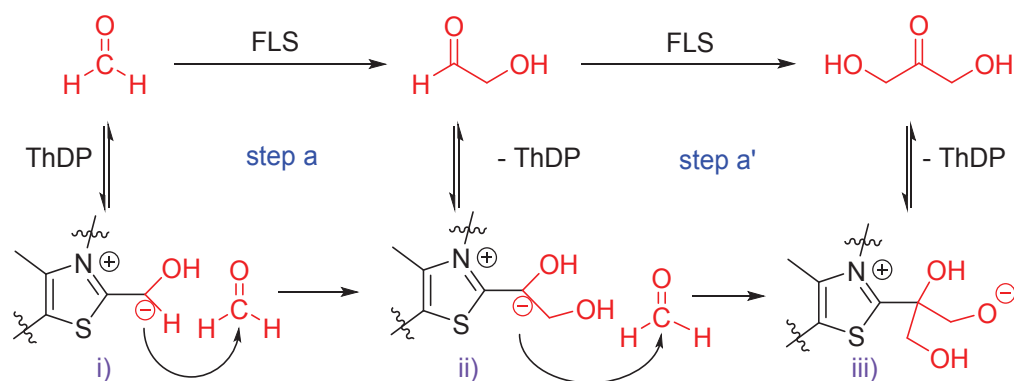


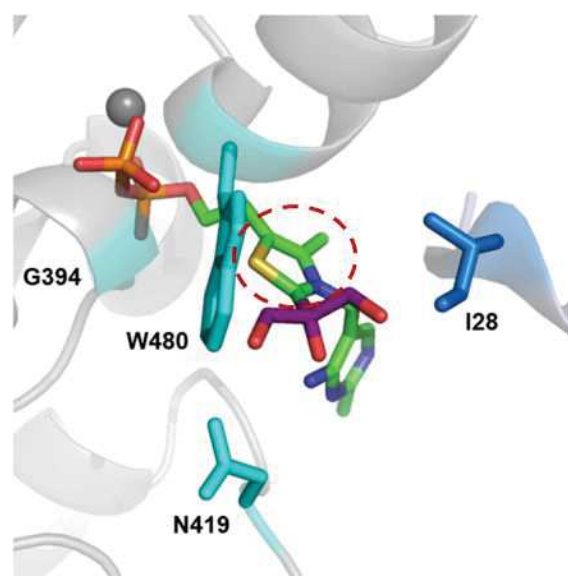
Figure 18. FLS-catalyzed formose reaction to GA and DHA

3.3.3 FLS: an engineered enzyme from ThDP-dependent BAL

FLS is an evolved enzyme that has been engineered from BAL from *Pseudomonas fluorescens biovar I*.¹⁰³ As presented in 3.1, BAL is a ThDP-dependent enzyme which activates benzaldehyde to catalyze benzoin condensation as its natural transformation. Its ability to activate formaldehyde was thus hypothesized. Indeed, wild-type BAL was shown to catalyze the oligomerization of formaldehyde into GA and DHA. However, catalytic efficiency for the FR ($k_{\text{cat}}/K_{\text{M}} = 50 \text{ mM}^{-1} \cdot \text{s}^{-1}$) was 36 000 times slower than for benzoin production. The active site was thus swimmingly engineered to accept formaldehyde as donor substrate instead of benzaldehyde with the introduction of seven mutations. Four mutations, A28I, A394G, G419N, and A480W, were introduced with computational redesign of the active site. Figure 19 features the active site of the enzyme with the thiazolium ring fragment (red circle) bonded to DHA intermediate (purple). The four mentioned mutations are indicated in blue. The A394G/A480W mutations were introduced to fill the gap in the active site to improve formaldehyde recognition. Reverting A28I and G419N induced about 2-fold and 4-fold decrease of FLS activity respectively. This suggests that new and productive interactions were created between the introduced isoleucine and asparagine and FR intermediate.¹⁰⁴ One extra mutation, L90T, was identified thanks to a round of computationally assisted site-directed mutagenesis. Finally, error-prone PCR highlighted two surface mutations, W89R and R188H. Thereby, FLS displays a 94-fold increase of the catalytic efficiency for the FR ($k_{\text{cat}}/K_{\text{M}} = 4700 \text{ mM}^{-1} \cdot \text{s}^{-1}$) compared to the initial BAL. In addition, no activity for the benzoin reaction at concentrations up to 10 μM of enzyme and 20 mM of benzaldehyde was detected for FLS.

Glycolaldehyde synthase (GALS) was recently engineered to produce GA from formaldehyde in 90% yield at 67 mM of formaldehyde.¹⁰⁷ An important feature of this work is the use of

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3 formaldehyde as a key C_1 synthon to produce Acetyl-CoA thanks to an enzyme cascade
4 reaction. The discovery of GAL5 is reminiscent of that of FLS because it was selected among
5 ThDP-dependent enzymes and further engineered through several rounds of mutagenesis. The
6 seven mutations used for FLS were notably tested. However, the final product is not chiral and
7 this work is not further described in the present review.
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33 **Figure 19.** Model of FLS active site (PDB ID: 4QQ8) with the four computationally-designed
34 mutations (blue) and the three-carbon intermediate (purple) linked to ThDP co-factor
35 corresponding to intermediate iii) of Figure 18.¹⁰³⁻¹⁰⁴ The 3D structure of the intermediate was
36 built and geometrically optimized by using Avogadro software, v1.1.1. The figure was then
37 prepared using PyMol Molecular Graphics System, v0.99 (Schrödinger).
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45 3.4 Cascade FLS/FSA reaction: toward chiral higher monosaccharides

46 The synthesized GA and DHA are not chiral molecules. In 2017, a cascade reaction used FLS
47 and FSA to generate chiral carbohydrates.¹⁰⁸ Surprisingly, FLS was active even in absence of
48 the ThDP co-factor. L-erythrulose (C_4 , **40**) and L-sorbose (C_6 , **41**) were selectively obtained in
49 66% and 43% isolated yield, respectively (Figure 20). Compound **40** is obtained from the FLS-
50 catalyzed formation of DHA (step a + a') followed by FSA^{A129S}-catalyzed transformation of
51 DHA with formaldehyde (step b). Compound **41** is obtained from the FLS-catalyzed formation
52 of GA (step a), followed by FSA-catalyzed transformation of GA with formaldehyde into L-
53 glyceraldehyde (step c). Further FSA^{A129S}-catalyzed transformation of L-glyceraldehyde with
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formaldehyde gave rise to **41** (step d). The latter strategy is an elegant example of symbiosis between FLS, FSA and the mono-mutant FSA^{A129S}.⁷² Wild-type FSA preferentially uses GA compared to DHA thus enabling step c to occur before its subsequent transformation into DHA in step a'. With favorable kinetics on DHA compared to GA (Table 1), the mono-mutant FSA^{A129S} is then added subsequently in a one pot-two step procedure.

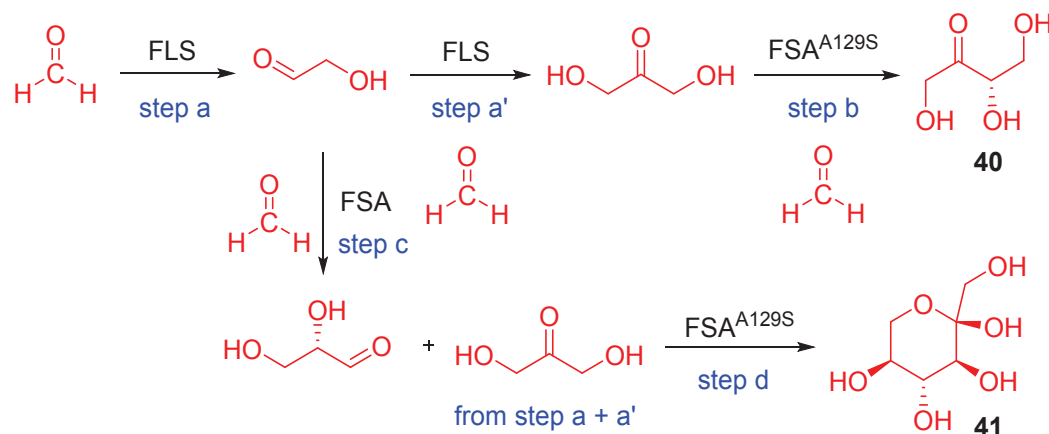


Figure 20. FLS/FSA cascade reaction: synthesis of compounds **40** and **41**

4. CONCLUSION

In the context of the depletion of fossil resources, formaldehyde is an emerging C₁ source.¹⁷⁻²³ Compared to the most studied C₁ molecules (CO₂, CO, HCOOH, and CH₄), formaldehyde is a particularly versatile and reactive substrate, making it a very difficult source to control but offering great promise for the synthesis of value-added chiral products.

The formose reaction is a perfect summary of the duality encountered in formaldehyde use: this remarkable reaction enables to build carbohydrates from formaldehyde as the only substrate. From this achiral and symmetrical building block, the gain in complexity is impressive with the generation of a carbon chain and chiral centers. Although those features have attracted interest in the fields of synthetic as well as prebiotic chemistry, understanding and control of this reaction remains largely unsolved challenges.¹⁰⁹

Biocatalyzed transformations are powerful answers to these specific issues, because enzymes afford high selectivity, stereospecificity and stereochemical complementarity.^{9,36} The examples described in this publication show that aldol and umpolung reactions leads to complex value-added products with perfect stereocontrol and without the need for protective groups. Aldolase catalysis has witnessed a large development from the first discoveries to the most recent

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3 examples. FSA and its mutants have notably enabled an increasing tolerance toward the use of
4 formaldehyde as an acceptor. The engineered FLS is another noticeable breakthrough achieving
5 the difficult task of controlling formaldehyde oligomerization. Aldol and umpolung are
6 combined in the last result described in the present paper: an impressive gain in complexity is
7 indeed reached with a FSA/FLS cascade catalyzing the transformation of the simple and achiral
8 formaldehyde substrate into a perfectly stereocontrolled C₄ and C₆ carbohydrates.
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12 These results are of high interest since the resultant β - and γ -hydroxycarbonyl compounds (in
13 particular carbohydrates) are biologically-relevant and thus of interest to the pharmaceutical
14 industry.¹¹⁰⁻¹¹²
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17 Finally, the present publication highlights the importance of i) enzyme discovery, ii) mechanism
18 understanding and iii) enzyme engineering. The combination of these three approaches is
19 instrumental in providing highly stereospecific biocatalysts dedicated to synthetic chemistry.
20 Although the scope of product is still relatively narrow, this perspective shows that newer
21 developments are expected to exploit the full potential of formaldehyde as a reactive C₁ source
22 for the generation of high value-added chemicals.
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