A Journal of the Gesellschaft Deutscher Chemiker

Angewandte International Edition GDCh Chemie

www.angewandte.org

Accepted Article

Title: Complete switch of reaction specificity of an aldolase by directed evolution in vitro: Synthesis of generic aliphatic aldol products

Authors: Wolf-Dieter Fessner, Sebastian Junker, Raquel Roldan, Henk-Jan Joosten, and Pere Clapés

This manuscript has been accepted after peer review and appears as an Accepted Article online prior to editing, proofing, and formal publication of the final Version of Record (VoR). This work is currently citable by using the Digital Object Identifier (DOI) given below. The VoR will be published online in Early View as soon as possible and may be different to this Accepted Article as a result of editing. Readers should obtain the VoR from the journal website shown below when it is published to ensure accuracy of information. The authors are responsible for the content of this Accepted Article.

To be cited as: *Angew. Chem. Int. Ed.* 10.1002/anie.201804831
Angew. Chem. 10.1002/ange.201804831

Link to VoR: http://dx.doi.org/10.1002/anie.201804831
http://dx.doi.org/10.1002/ange.201804831

WILEY-VCH

10.1002/anie.201804831

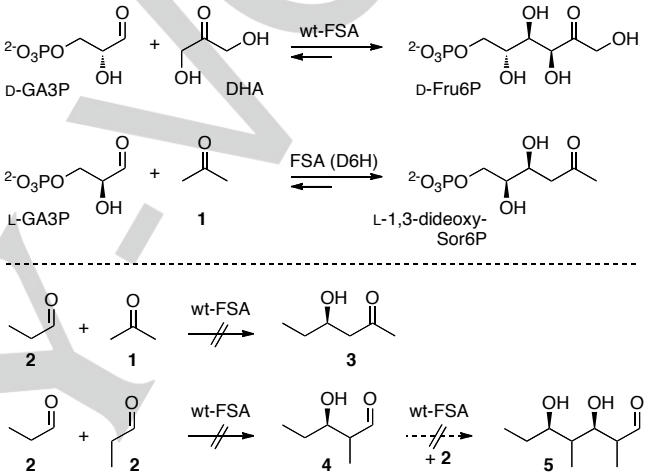
WILEY-VCH

Complete switch of reaction specificity of an aldolase by directed evolution *in vitro*: Synthesis of generic aliphatic aldol products

Sebastian Junker,[a] Raquel Roldan,[b] Henk-Jan Joosten,[c] Pere Clapés[b] and Wolf-Dieter Fessner*[a]

Abstract: A structure-guided engineering of fructose-6-phosphate aldolase was performed to expand its substrate promiscuity toward aliphatic nucleophiles, *i.e.*, unsubstituted alkanones and alkanals. A "smart" combinatorial library was created targeting residues D6, T26 and N28 that form a binding pocket around the nucleophilic carbon atom. Double-selectivity screening was executed by high-performance TLC that allowed simultaneous determination of total activity as well as a preference for acetone versus propanal as competing nucleophiles. D6 turned out to be the key residue that enabled activity with non-hydroxylated nucleophiles. Altogether 25 single- and double-site variants (D6X and D6X/T26X) were discovered that show useful synthetic activity and a varying preference for ketone or aldehyde as the aldol nucleophiles. Remarkably, all of the novel variants had completely lost their native activity for cleavage of fructose 6-phosphate.

Aldolases and transaldolases are specialized on sugar phosphates, yet highly interesting biocatalytic tools for chemical synthesis because of their precise stereoselectivity in the carboligation step and their activity under very mild reaction conditions.[1,2] For example, fructose-6-phosphate aldolase (FSA) from *E. coli* is a thermostable Class I enzyme, which reversibly catalyses the highly stereoselective addition of dihydroxyacetone (DHA) to D-glyceraldehyde 3-phosphate (D-GA3P) resulting in the formation of D-fructose 6-phosphate (D-Fru6P; Scheme 1).[3-5] Like other aldolases, FSA accepts a broad range of aldehydes as the electrophilic aldol component.[2,5,6] However, while most aldolases are quite specific for their nucleophilic substrate, FSA tolerates some structural variation of the DHA nucleophile and also converts hydroxyacetone, hydroxybutanone and hydroxyethanal.[6] Recently, we demonstrated that designed minimal mutations in the FSA active site could further open up the nucleophile tolerance for large ketols at least up to the size of 1-hydroxyheptan-2-one.[7] Lifting the absolute requirement for α-hydroxylation in the ketol nucleophiles, residual activity with generic aliphatic ketone substrates could be detected for wild-type FSA and its D6H variant by using a fluorogenic assay principle in aldol cleavage direction.[8] In direction of aldol synthesis, however, products from unsubstituted aliphatic nucleophiles (simple ketones and aldehydes) could only be isolated when applying L-GA3P as the electrophile with catalysis by FSA(D6H), presumably because phosphorylation confers high substrate binding affinity.



Scheme 1. Native use of DHA nucleophile by wild-type FSA and promiscuous tolerance for acetone of the D6H variant with phosphorylated aldehyde electrophile.[8] Catalyzed addition of unsubstituted nucleophiles (acetone, propanal) to generic aliphatic electrophiles is yet unknown.

An aldolase having the ability to utilize generic aliphatic, non-hydroxylated substrates both as electrophilic *and* nucleophilic components would constitute a highly flexible catalyst for a plethora of synthetic opportunities toward the construction of chiral building blocks by Green Chemistry principles. The range of addressable targets from such reactions, such as the homo aldol products of aldehydes (e.g., **4**, **5**), are common structural motifs in natural products like polyketides[9] or terpenoids,[10] but are also important as intermediates in the production of pharmaceuticals[11] and industrial bulk chemicals such as the Guerbet-type compounds.[12] Here we present the engineering by a directed evolution approach of novel FSA variants, which selectively use acetone (**1**) or propanal (**2**) as nucleophilic substrates with high carboligation stereoselectivity.

From an inspection of the enzyme active site, using the X-ray structure of FSA with a model of the D-Fru6P substrate bound to the catalytic lysine (K85),[8] it is evident that the 3-OH group in the nucleophile moiety interacts with residue D6 via hydrogen bonding and loosely contacts the backbone amide group of N28 (Fig. 1). D6 also forms a hydrogen bond with 5-OH of Fru6P. In addition, T26 donates a hydrogen bond to D6, which aligns it for an optimal substrate interaction. Replacing these polar residues by hydrophobic ones would interrupt the stabilizing contacts and thereby should improve an alternative binding of non-hydroxylated nucleophiles such as acetone or

[a] S. Junker, Prof. Dr. W.-D. Fessner
Institut für Organische Chemie und Biochemie
Technische Universität Darmstadt
Alarich-Weiss-Str. 4, 64287 Darmstadt, Germany
E-mail: fessner@tu-darmstadt.de

[b] Raquel Roldan, Prof. Dr. Pere Clapés
Instituto de Química Avanzada de Cataluña-IQAC-CSIC, Jordi Girona 18-26, 08034 Barcelona, Spain

[c] Dr. Henk-Jan Joosten
Bio-Prodict, Nieuwe Marktstraat 54e, 6511 AA Nijmegen, The Netherlands

Supporting information for this article is given via a link at the end of the document.

Accepted Manuscript

This article is protected by copyright. All rights reserved.

aliphatic aldehydes. The side chain of N28, however, donates a hydrogen bond to the 4-OH group, which stabilizes the incipient oxyanion formation at the aldehyde carbonyl group upon aldol attack and render mutation of N28 problematic.[13]

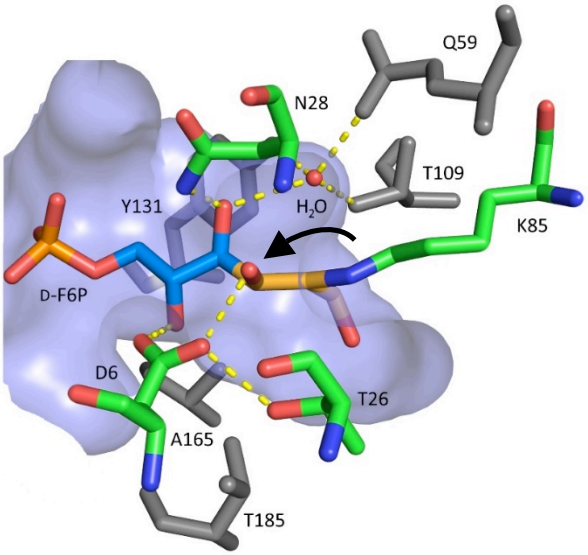


Figure 1. PyMOL[15] model of the active site of the wild-type FSA from *E. coli*.[8]The X-ray structure of FSA (PDB entry 1l6w)[4] was aligned with the D-F6P liganded in a highly similar transaldolase complex (PDB entry 3s1v).[14] The "essential" 3-OH group is highlighted by an arrow.

To limit the screening efforts we tested individual rational replacements for a first orientation. Indeed, single variants D6A, D6L, D6E and D6H[8] were found to be active with acetone as nucleophile and GA3P as electrophile. Exploratory saturation N28X in FSA variants D6E, D6H and D6L abolished activity.

Thus, simultaneous site saturation mutagenesis was performed on the two remaining sites. To reduce the library to a manageable size a smart construction approach was followed by eliminating mutations unlikely to yield positive hits.[16] A structure-based sequence alignment for the aldolase superfamily from Bio-prodict (3DM database)[17] delivered the amino acid distribution at positions that are structurally conserved within the whole superfamily. By encoding only the 3DM-derived subset instead of all 20 proteinogenic amino acids the library size can be significantly reduced. While the D6/T26 motif is highly conserved, an analysis of the correlated mutation data[18] of the 3DM database showed that there is a functional connectivity between the amino acids. Variations occur only at very low frequency, *e.g.*, for the entire TalI family A/I 19.85%; A/L 9.25%; D/A 0.27%, and for FSA-type enzymes L/T 0.74%; D/C 0.56%; D/D, D/S, P/T, R/T each 0.19%. Therefore, we created a combinatorial library where Asp6 was mutated by using the restricted SHH set coding for A, D, Q, E, H, L, P, and V, and T26 by using the VYT set coding for A, I, L, P, T and V (8 x 6 = 48 variants requiring the screening of 200 clones for 95% coverage).[19] Also, unusual solutions revealed by the 3DM software tool, such as uncommon but allowed variants D6A, D6L and D6P as well as the D6A/T26I and D6A/T26L combinations, are included in this setting.

Library screening was performed with whole cells in aqueous medium containing a mixture of acetone and propanal (**1**:**2** = 17:1, v:v) as competitive substrates. Although the aldehyde was essential to serve as the electrophile, the ketone proportion was strongly increased to compensate for its lower reactivity with the catalytic lysine. Samples were periodically analyzed by TLC for product formation. Roughly 55% of the clones yielded one or two new product spots (Figure 2) with intensities stronger than wild-type, whereas others barely produced detectable product amounts or were inactive.

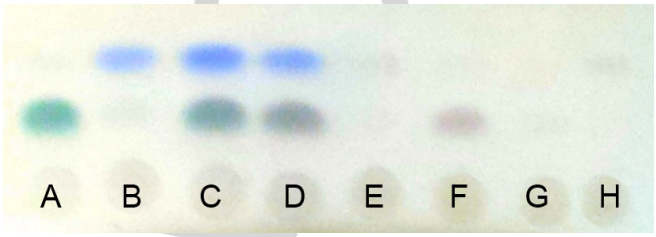


Figure 2. Exemplary TLC analysis using acetone and propanal as substrates catalysed by hit plate candidates having different activity and nucleophile selectivity. Anisaldehyde staining reveals a blue spot corresponding to the homo-aldol **4**, and a green spot for the cross-aldol **3**. Lanes correspond to variant sequences A = D6H, B = D6H/T26L, C = D = D6A/T26A (duplicate clone), E = T26I, F = wt, G = D6L/T26I, H = D6P/T26L. Image contrast was digitally improved for visualization.

Re-screening and sequencing furnished a total set of 25 unique genetic variants. The latter were further characterized for their relative kinetic activities and for their substrate selectivity against **1** and **2** by automated high-performance TLC (HPTLC) analysis using densitometrical product quantification. We found that HPTLC is a medium-throughput screening method, which is well suited for the direct screening of aldol product formation, allowing for simultaneous analysis of both activity and selectivity. It is complementary to the typical inverse determination of aldolase activity by aldol cleavage assays[3] albeit limited to endpoint measurements. The HPTLC method was validated by a comparison to standard GC results, using reactions catalyzed by FSA variants D6E, D6L, D6A and D6L/T26A.

Among the 25 unique hits, 16 (64%) showed a clear competitive preference for one over the other nucleophile (nucleophile preference >3:1), while the other 9 (36%) produced both products **3** and **4** in similar quantities (Scheme 2). The 16 selective variants were further analyzed by GC for an accurate determination of their nucleophile selectivity (Figure 3). The analysis shows that the two positions (D6/T26) selected for mutagenesis are indeed the expected hot spots for changing the catalytic properties of the enzyme, and that the "smart library" strategy paid off with a very high proportion of positive hits (25 out of 48 library members, 52% hit rate). The conditions chosen for substrate competition facilitated a direct evaluation of both activity and nucleophile selectivity, which could be read out using conventional medium-throughput TLC screening.

Clearly, non-hydroxylated nucleophiles become favored if the hydrogen-bonding pattern directed at the 3-OH group are deleted via non-polar D6 replacement (D6A, D6V, D6L, D6P), or

Accepted Manuscript

This article is protected by copyright. All rights reserved.

if mutant residues are incompatible with the hydrogen-bonding network and occupy an additional volume (D6H, D6E, D6Q). While under the specific conditions many active variants appeared rather non-selective (e.g., D6E), some variants showed very high complementary selectivity for either of the aldehyde or ketone nucleophiles. A detailed analysis of the selective candidates revealed that the known D6H variant[8] was the most selective catalyst for **1**, similar to the higher active D6L variant (Figure 3). However, most of the selective variants preferred the more reactive **2** as nucleophile, despite of its much lower assay concentration. Interestingly, the primary preference introduced by the D6X replacement can be strongly shifted towards improved preference for **2** by T26X exchanges, particularly with residues having increasing hydrophobic volume (A<<V<L<I). The effect is observed for D6E, D6L, D6H, and is particularly effective for D6H, where a strong switch of preference from **1** (D6H) to **2** (D6H/T26L) takes place, which both show similar rates. A synonymous trend is seen for D6A or D6P but to a lesser extent because these are already quite selective for **2**. While the T26V and T26I are mostly accompanied by lower rates, the T26L mutants typically show higher rate and thus represent the best choice with a compromise in rate and selectivity. The highest selectivity for **2** was determined for the D6Q/T26I variant but only at a rather low rate. Surprisingly, the simple T26L mutation alone results in significantly increased rate and selectivity for **2**. Notably, proline is tolerated in the D6 position but is not tolerated to replace T26; usually, introduction of proline is cumbersome because of conformational restrictions.

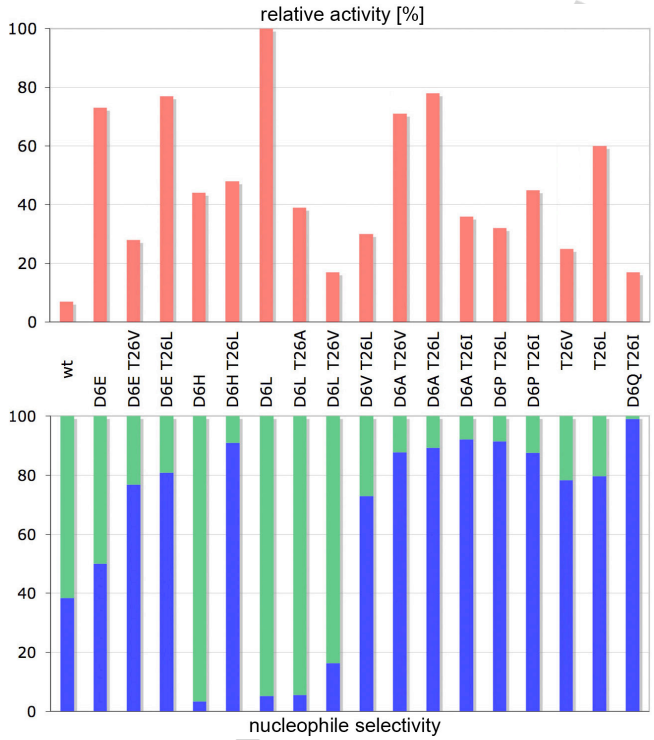
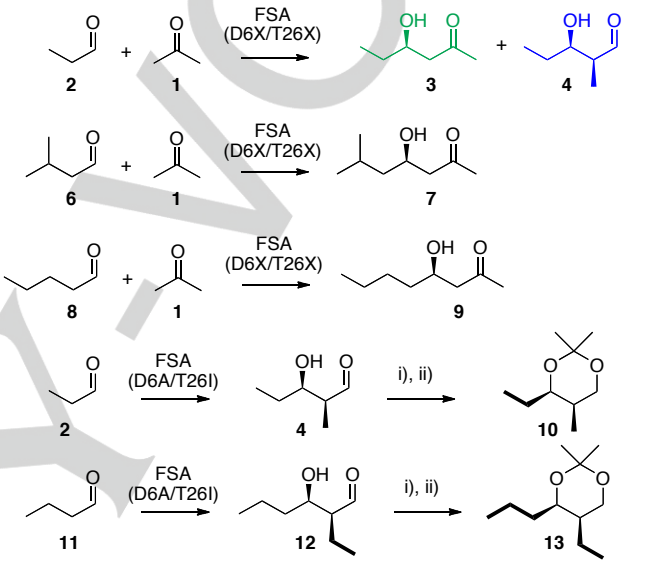


Figure 3. Activity and competitive nucleophile bias of selective FSA variants, in comparison to wild-type and the D6E variant as a non-selective example. Relative cumulative rates (GC peak sum for products **3** + **4**) are based on most active variant D6L. Green bars indicate preference for **1** (major product **3**) as nucleophile, blue bars for **2** (major product **4**).

The very high selectivity of several variants to utilize **2** as nucleophile was unexpected, given its low concentration in comparison to excessive **1**. Separate experiments were conducted to determine relative catalytic activities in the absence of nucleophile competition, firstly by using **2** as the sole substrate (Figure 4, left); as a reference enzyme we used the DERA from *E. coli* (F200I variant[20]), which so far is the only enzyme known to accept **2** as a non-native nucleophile.[21] A complementary screen was performed by using **1** in the presence of an aldehyde electrophile (**6**) that itself is unreactive for self aldolization (Figure 4, right).



Scheme 2. Reactions used for screening the nucleophile preference of the D6X/T26X library. Stereoselectivity was analyzed *via* cyclic acetal formation. Reaction conditions: i) $NaBH_4$, MeOH ii) 2,2-dimethoxypropane, H^+.

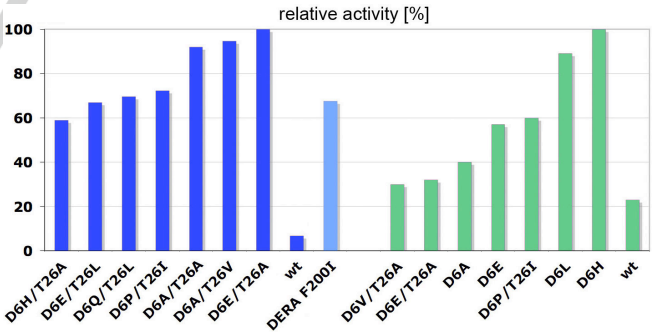


Figure 4. Blue bars: rate of formation of **4** by self-aldolization of **2** catalyzed by FSA variants most active in primary screenings; wt-FSA and DERA from *E. coli* (light blue) were included for reference. Green bars: rate of formation of **11** by addition of **1** to **6** using FSA variants most active on **1**. For reaction conditions see Supporting Info.

In the first tests, most active FSA variants, independent of their nucleophile selectivity, were screened for their rate of formation of **4**. The concentration of **2** had to be limited (83 mM) because of its denaturation effect on the enzyme at higher substrate concentrations. Whereas wild-type FSA showed barely any

Accepted Manuscript

This article is protected by copyright. All rights reserved.

detectable product formation, several variants displayed good activity. Several new FSA variants (D6A/T26A, D6A/T26V, D6E/T26A) were found to have activity superior to the DERA reference. A distinctive feature is that reactions catalyzed by FSA variants stop after the first addition to form **4**, while DERA catalyzes a consecutive addition to give a trimer of type **5**, which can cyclize into a lactol form.[21]

In the second test series, variants selective for **1** were investigated for their relative rates. As the electrophile, isopentanal (**6**) was employed when isobutanal proved unreactive with common nucleophiles; both did not show any reactivity for self-aldolization even at high enzyme concentrations, probably because of their steric bulkiness. The corresponding aldol products are acyclic and generated by a bimolecular synthetic process, thus thermodynamically unfavorable,[22] but acetone can be used at rather high concentration to drive the equilibrium towards product formation because FSA is quite stable to organic cosolvents. Screening for the formation of **7** under these non-competitive conditions showed that only the single mutants D6L and D6H have highest activity, whereas mutant combinations caused lower rates.

Unfortunately, for none of the reactions studied we could find a reliable *in vitro* assay to perform steady-state kinetic measurements with purified enzymes in direction of synthesis. Probably owing to its low electrophilic nature, acetone has only low binding affinity to FSA and no K_M could be estimated. Clearly, rate differences reported are not due to different expression levels, which were checked by SDS-PAGE, nor to different protein stabilities, which were checked by differential scanning fluorimetry.[7]

The novel activities and selectivities most likely are due to an improved binding of hydrophobic nucleophiles because the main catalytic mechanism of the Class I aldolase, involving covalent Schiff base formation at K85 and tyrosine residues acting as an acid-base catalyst,[22,23] remains intact. A major effect is caused by replacements of D6 that interrupt the hydrogen-bonding network for recognition of the 3-OH group and occupy a larger space than the native aspartate (D6L, D6H, D6E, D6Q; except for D6A). Especially, D6H and D6L show good activity with **1**, because the compact remaining space seems inappropriate to bind the larger alkyl moiety of **2**. Considering the large excess of **1** in the assay solution, the collective data indicate that all other variants essentially have a preference for **2**, which can be further enhanced by hydrophobic replacements of T26. Apparently, the side chain volumes of T26V, T26I and T26L restrict the most distant cavity space of the active site where C1 of the substrate is located. Thereby, binding of the C1 moiety of a ketone nucleophile becomes obstructed in favor of a smaller aldehyde moiety. Among the positive T26 variants the T26L mutation seems to be the most adaptive, while T26I needs to be combined with D6A replacement to compensate for its steric bulkiness.

Stereoselectivity is a critical issue when the electrophile C=O is not firmly positioned and can be attacked from both faces.[5] However, product **3** obtained on a preparative scale (34% yield) showed high enantiomeric purity (*ee* >95%) by chiral GC.[24] Similar results were obtained by preparative addition of **1** to **6** and to the straight-chain isomer **8**, yielding aldol products **7** and **9**, respectively.

(2*S*)-Configuration can be anticipated for steric reasons when using **2** as a nucleophile; however, as this was never rigorously proven for the corresponding DERA reaction,[21] there was no reference available. Thus, product **4** obtained on preparative scale (21% yield) was converted into the cyclic derivative **10** for stereochemical analysis (Scheme 2). NMR analysis clearly demonstrated the *cis*-(2*S*,3*R*) arrangement of the alkyl substituents, and chiral GC analysis showed the presence of only a single enantiomer (>99% *ee*). As a control, aldehyde **4** was exposed to alkaline conditions before being processed, which yielded a mixture of *cis* and *trans* diastereomers.[25] Thus, the native (3*S*,4*R*)-stereoselectivity of the wild-type FSA was fully retained with non-hydroxylated, hydrophobic substrate analogs. Gratifyingly, FSA variants D6A/T26L and D6A/T26I were even active with butanal (**11**) to form the aldol dimer **12** with about one third of the rate for **4** and with the same diastereoselectivity, as proven by a corresponding analysis of the derivative **13**. The larger homolog **8** did not lead to aldol formation. Both compounds **4** and **12** are formal precursors to industrially important Guerbet-type compounds, which are used as bulk solvents/plasticizers.[12] It is tempting to speculate that enzymatic routes to such man-made bulk materials[26] could be realized using FSA-derived catalysts in a biotechnology process.

In summary, following a single smart library strategy it was possible to identify a collection of new FSA variants having novel activities and distinct preferences for the nucleophilic substrate, and that are promising for possible use in the stereospecific synthesis of chiral building blocks. These enzymes significantly expand the product space hitherto addressable by enzymatic carboligation.[1,2] Remarkably, the synthesis of all generic aldol products such as **3**, **4**, **7**, **9** or **12** — stripped from the abundant polar functionalization of native substrates — could also be performed conveniently using recombinant whole cell catalysis because such substrates and products seem to permeate quite well across the cell wall. It is appropriate to stress that all new enzyme variants completely lost the native FSA activity for cleavage of Fru6P, nor do they show residual synthetic activity with hydroxylated nucleophiles.

This study shows that a complete switch in reaction specificity of an aldolase can be realized by focusing directed evolution on a confined hot spot of substrate binding. By exploiting existing knowledge on enzyme structure-function relationships and exhaustive bioinformatic analysis of protein databases, smart combinatorial libraries can be rapidly designed that offer very high hit rates. We could also show that parallel, automated HPTLC analysis is a valuable medium-throughput screening tool that is well suited for such a highly focused approach. We believe that the direct monitoring of product formation is more flexible, and furnishes more appropriate data for an evaluation of the synthetic capacity of carboligation catalysts, as compared to the conventional indirect monitoring of fragments arising from aldol cleavage.

Accepted Manuscript

This article is protected by copyright. All rights reserved.

Experimental Section

For experimental details, see Supporting Info..

Acknowledgements

This project received funding from the European Union's Horizon 2020 research and innovation program under grant no. 635595 (*CarbaZymes*), the Spanish Ministerio de Economía y Competitividad (MINECO), and the Fondo Europeo de Desarrollo Regional (FEDER) (grant no. CTQ2015-63563-R to P.C.).

Keywords: biocatalysis • fructose-6-phosphate aldolase • HPTLC screening • protein engineering • stereoselectivity

[1] a) M. Brovetto, D. Gamenara, P. Saenz Mendez, G. A. Seoane, *Chem. Rev.* **2011**, *111*, 4346; b) M. Mueller, *Adv. Synth. Catal.* **2012**, *354*, 3161; c) C. L. Windle, M. Mueller, A. Nelson, A. Berry, *Curr. Opin. Chem. Biol.* **2014**, *19*, 25.

[2] a) P. Clapés, W.-D. Fessner, G. A. Sprenger, A. K. Samland, *Curr. Opin. Chem. Biol.* **2010**, *14*, 154; b) W.-D. Fessner, in *Enzyme Catalysis in Organic Synthesis (3rd ed), Vol. 2*, (Eds.: K. Drauz, H. Groeger, O. May), Wiley-VCH, Weinheim, **2011**, p. 857; c) P. Clapés, X. Garrabou, *Adv. Synth. Catal.* **2011**, *353*, 2263.

[3] M. Schürmann, G. A. Sprenger, *J. Biol. Chem.* **2001**, *276*, 11055.

[4] S. Thorell, M. Schurmann, G. A. Sprenger, G. Schneider, *J. Mol. Biol.* **2002**, *319*, 161.

[5] A. K. Samland, M. Rale, G. A. Sprenger, W.-D. Fessner, *ChemBioChem* **2011**, *12*, 1454-1474.

[6] a) J. A. Castillo, J. Calveras, J. Casas, M. Mitjans, M. P. Vinardell, T. Parella, T. Inoue, G. A. Sprenger, J. Joglar, P. Clapés, *Org. Lett.* **2006**, *8*, 6067; b) M. Sugiyama, Z. Hong, P.-H. Liang, S. M. Dean, L. J. Whalen, W. A. Greenberg, C.-H. Wong, *J. Am. Chem. Soc.* **2007**, *129*, 14811; c) A. L. Concia, C. Lozano, J. A. Castillo, T. Parella, J. Joglar, P. Clapés, *Chem. Eur. J.* **2009**, *15*, 3808; d) X. Garrabou, J. A. Castillo, C. Guérard-Hélaine, T. Parella, J. Joglar, M. Lemaire, P. Clapés, *Angew. Chem. Int. Ed.* **2009**, *48*, 5521; e) M. Rale, S. Schneider, G. A. Sprenger, A. K. Samland, W.-D. Fessner, *Chem. Eur. J.* **2011**, *17*, 2623.

[7] D. Gueclue, A. Szekrenyi, X. Garrabou, M. Kickstein, S. Junker, P. Clapés, W.-D. Fessner, *ACS Catal.* **2016**, *6*, 1848.

[8] R. Roldan, I. Sanchez-Moreno, T. Scheidt, V. Helaine, M. Lemaire, T. Parella, P. Clapés, W.-D. Fessner, C. Guerard-Helaine, *Chem. Eur. J.* **2017**, *23*, 5005.

[9] J. Piel, *Nat. Prod. Rep.* **2010**, *27*, 996.

[10] P. K. Ajikumar, K. Tyo, S. Carlsen, O. Mucha, T. H. Phon, G. Stephanopoulos, *Mol. Pharm.* **2008**, *5*, 167.

[11] J. M. Patel, *J. Mol. Catal. B Enzym.* **2009**, *61*, 123.

[12] J. Falbe, H. Bahrmann, W. Lipps, D. Mayer, G. D. Frey, in *Ullmann's Encyclopedia of Industrial Chemistry*, Wiley-VCH, Weinheim, **2013**, p. 1.

[13] U. Schorken, S. Thorell, M. Schurmann, J. Jia, G. A. Sprenger, G. Schneider, *Eur. J. Biochem.* **2001**, *268*, 2408.

[14] A. Lehwess-Litzmann, P. Neumann, C. Parthier, S. Luedtke, R. Golbik, R. Ficner, K. Tittmann, *Nat. Chem. Biol.* **2011**, *7*, 678.

[15] W. L. DeLano, *Schrödinger LLC.,* **2002**, *http://www.pymol.org.*

[16] A. Nobili, M. G. Gall, I. V. Pavlidis, M. L. Thompson, M. Schmidt, U. T. Bornscheuer, *FEBS J.* **2013**, *280*, 3084.

[17] R. K. Kuipers, H.-J. Joosten, W. J. H. van Berkel, N. G. H. Leferink, E. Rooijen, E. Ittmann, F. van Zimmeren, H. Jochens, U. Bornscheuer, G. Vriend, V. A. P. Martins dos Santos, P. J. Schaap, *Proteins Struct. Funct. Bioinf.* **2010**, *78*, 2101.

[18] T. van den Bergh, G. Tamo, A. Nobili, Y. Tao, T. Tan, U. T. Bornscheuer, R. K. P. Kuipers, B. Vroling, R. M. de Jong, K. Subramanian, P. J. Schaap, T. Desmet, B. Nidetzky, G. Vriend, H.-J. Joosten, *PLoS One* **2017**, *12*, e0176427.

[19] A. E. Firth, W. M. Patrick, *Nucleic Acids Res.* **2008**, *36*, W281.

[20] S. Jennewein, M. Schuermann, M. Wolberg, I. Hilker, R. Luiten, M. Wubbolts, D. Mink, *Biotechnol. J.* **2006**, *1*, 537.

[21] H. J. M. Gijsen, C.-H. Wong, *J. Am. Chem. Soc.* **1995**, *117*, 7585.

[22] C. Zeymer, R. Zschoche, D. Hilvert, *J. Am. Chem. Soc.* **2017**, *139*, 12541-12549.

[23] V. Sautner, M. M. Friedrich, A. Lehwess-Litzmann, K. Tittmann, *Biochemistry* **2015**, *54*, 4475-4486.

[24] A. Knorr, A. Weissernborn, US1714378, **1929**.

[25] M. Haeckh, M. Mueller, S. Luedeke, *Chem. Eur. J.* **2013**, *19*, 8922.

[26] M. Biermann, D. Bakonyi, W. Hummel, H. Groeger, *Green Chem.* **2017**, *19*, 405.

Accepted Manuscript

WILEY-VCH

This article is protected by copyright. All rights reserved.

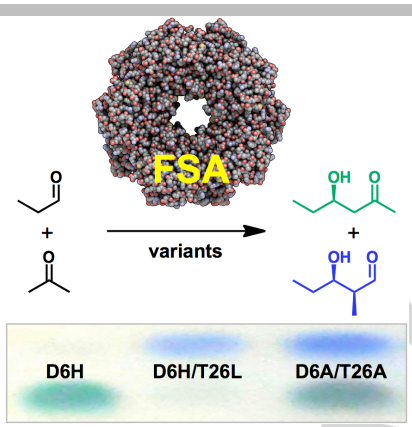
10.1002/anie.201804831

WILEY-VCH

Entry for the Table of Contents

COMMUNICATION

A “smart library” strategy identified D6/T26 as the key residues in fructose-6-phosphate aldolase (FSA) to expand its substrate promiscuity toward unsubstituted alkanones and alkanals as non-natural nucleophiles. Double-selectivity HPTLC screening allowed simultaneous determination of total activity as well as preference for competing nucleophiles. Remarkably, all of the novel variants had completely lost their native activity for cleavage of fructose 6-phosphate.



*S. Junker, R. Roldan, H.-J. Joosten, P. Clapés and W.-D. Fessner **

Page No. – Page No.

Complete switch of reaction specificity of an aldolase by directed evolution *in vitro*: Synthesis of generic aliphatic aldol products

WILEY-VCH
Accepted Manuscript

This article is protected by copyright. All rights reserved.