Supporting Information

Complete Switch of Reaction Specificity of an Aldolase by Directed Evolution In Vitro: Synthesis of Generic Aliphatic Aldol Products
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| Section                                                                 | Page |
|------------------------------------------------------------------------|------|
| 1. Materials                                                           | 4    |
| 2. General procedure for mutagenesis and transformation               | 4    |
| 3. Correlated mutation analysis using 3DM                              | 6    |
| 4. Site directed mutagenesis and library creation                      | 9    |
| 5. Cell growth                                                         | 10   |
| 6. TLC screening of FSA D6H/N28L and D6H/N28T against D6H             | 11   |
| 7. TLC screening of N28X libraries                                      | 12   |
| 7.1 Screening of 96-well plates by thin layer chromatography           | 12   |
| 7.2 Screening of single-site FSA D6L/N28X library                      | 12   |
| 7.3 Screening of single-site FSA D6E/N28X library                      | 13   |
| 7.4 Screening of single-site FSA D6H/N28X library                      | 14   |
| 8. TLC screening of double-site FSA D6X/T26X library                   | 15   |
| 8.1 Primary screening for activity against acetone                      | 15   |
| 8.2 Rescreening                                                        | 17   |
| 8.3 Screening with n-butanal                                           | 18   |
| 9. Sequencing results from the D6X/N28X library screenings              | 19   |
| 10. High performance TLC screening with acetone and propanal          | 21   |
| 11. High performance TLC analysis for propanal self aldolization       | 23   |
| 12. High performance TLC analysis for acetone and isopentanal          | 25   |
| 13. Synthesis and NMR spectroscopic analysis                          | 27   |
| 13.1 Synthesis of (R)-4-hydroxyhexan-2-one                              | 27   |
| 13.2 Synthesis of (R)-4-hydroxy-6-methylheptan-2-one                   | 28   |
| 13.3 Synthesis of (2R,3R)-2-methylpentane-1,3-diol                     | 29   |
| 13.4 Synthesis of (3R)-2-methylpentane-1,3-diol                        | 30   |
| 13.5 Synthesis of (4R,5R)-4-ethyl-2,2,5-trimethyl-1,3-dioxane           | 31   |
| 13.6 Synthesis of (4R)-4-ethyl-2,2,5-trimethyl-1,3-dioxane             | 33   |
| 13.7 Synthesis of (4R,5R)-5-ethyl-2,2-dimethyl-4-propyl-1,3-dioxane    | 35   |
| 13.8 Synthesis of (4R)-5-ethyl-2,2-dimethyl-4-propyl-1,3-dioxane with FSA | 37   |
| 13.9 Synthesis of racemic 4-hydroxyhexan-2-one                         | 38   |
| 14. GC Analysis                                                        | 39   |
| 14.1 Relative activity of different FSA variants in the formation of 4-hydroxyhexan-2-one | 39   |
| 14.2 Enantioselectivity of FSA D6E in the synthesis of 4-hydroxyhexan-2-one | 40   |
| 14.3 Substrate selectivity of FSA variants by GC analysis              | 42   |
| 14.4 Kinetic analysis of propanal/butanal homoaldolization by FSA variant D6A/T26L | 45   |
| 15. GC-MS                                                               | 46   |
| 15.1 GC-MS (EI) analysis of (4R,5R)-4-ethyl-2,2,5-trimethyl-1,3-dioxane | 46   |
| 15.2 GC-MS (EI) analysis (EI) of (4R)-4-ethyl-2,2,5-trimethyl-1,3-dioxane | 47   |
| 15.3 GC-MS (EI) analysis of (4R,5R)-5-ethyl-2,2-dimethyl-4-propyl-1,3-dioxane | 48   |
| 16. D-F6P assay for inhibitor identification                            | 49   |
| 17. References                                                         | 50   |

Author Contributions 50
1 Materials

Media components and all chemical reagents were purchased from Sigma-Aldrich and used without purification, except for aldehydes, which were freshly purified by distillation prior to use. Organic solvents were of analytical grade. HPTLC analysis was performed on silica gel F254 plates (Merck) using a CAMAG Automatic TLC Sampler 4 and CAMAG TLC Scanner 4. Screening consumables (96-well microtiter plates, acetate foil, pipette tips) were purchased from Sarstedt. Plates were incubated in an Infors Ecotron shaker. Synthetic oligonucleotides were purchased from Sigma-Aldrich. Triose phosphate isomerase/glycerol-3-phosphate dehydrogenase (mix from rabbit muscle) and NADH were purchased from Sigma-Aldrich. Proteinase K (Tritirachium album) came from Serva. Antibiotics, acrylamide-bisacrylamide and buffer components were from Carl Roth. Milli-Q grade water was used for preparations of buffers, whereas other assay solutions were obtained from an Arium Pro Ultrapure Water Purification System (Sartorius Stedim Biotech). Bacterial strains, oligonucleotides and plasmids used in this study are listed in Table 1. The plasmid pET16b fsa containing the FSA gene for expression (gene code, fsa, formerly termed mipB [1]) was constructed using routine cloning procedures. Gene sequencing was performed by GATC Biotech AG. Protein concentration was determined according to the Bradford method using commercial reagent (Carl Roth).

2 General procedure for mutagenesis and transformation

Mutagenesis of d-Fructose-6-phosphate aldolase (fsa) was performed by using the QuikChange site-directed mutagenesis kit (Agilent), using the plasmids pET16b fsa or pQE40 fsa as template. The specific combination of oligonucleotides and templates used are compiled in Table 1. Competent cells of E. coli XL10 (Agilent) were used for transformation and plasmid preparation, and E. coli strain BL21 (DE3) [2] was used for protein expression. Plasmid DNA was isolated using the GenElute™ HP Plasmid Miniprep Kit (Sigma-Aldrich). The expected mutations in the gene sequence were confirmed by DNA sequencing.

| Plasmids     | Relevant genetic characteristics | Origin    |
|--------------|-----------------------------------|-----------|
| pQE40        | Prs, Ampr, ColE1 ori               | Quiagen   |
| pQE40 fsa D6E| fsa D6E gene cloned in pQE40      | This study|
| pQE40 fsa D6H| fsa D6H gene cloned in pQE40      | This study|
| pQE40 fsa D6H/N28D| fsa D6H/N28D gene cloned in pQE40 | This study|
| pQE40 fsa D6H/N28L| fsa D6H/N28L gene cloned in pQE40 | This study|
| pQE40 fsa D6H/N28T| fsa D6H/N28T gene cloned in pQE40 | This study|
| pQE40 fsa D6H/N28X| fsa D6H/N28X gene cloned in pQE40 | This study|
| pQE40 fsa D6H/N28X| fsa D6H/N28X gene cloned in pQE40 | This study|
| pET16b     | Pr7, Ampr, pBR322ori | Novagen          |
|------------|----------------------|------------------|
| pET16b fsa D6A | fsa D6A gene cloned in pET16b | This study |
| pET16b fsa D6A/T26I | fsa D6A/T26I gene cloned in pET16b | This study |
| pET16b fsa D6L | fsa D6L gene cloned in pET16b | This study |
| pET16b fsa D6L/N28X | fsa D6L/N28X gene cloned in pET16b | This study |
| pET16b fsa D6X/T26X | fsa D6X/N28X gene cloned in pET16b | This study |
| Strains    | Relevant genotype    | Origin           |
| XL10 Gold  | TetrD(mcrA)183 D(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte [F´ proAB lacIqZD7M15 Tn10 (Tet) Amy Cam] | Agilent |
| Bl21 (DE3) | fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS λ DE3 = λ sdamHo ΔEcoRI- B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5 | New England BioLabs |

**Oligonucleotides**

| Oligonucleotide sequences (5’ → 3’) | Origin |
|-------------------------------------|--------|
| D6L forward                         | This study |
| D6L reverse                         | This study |
| D6A reverse                         | This study |
| D6A forward                         | This study |
| D6E forward                         | This study |
| D6E reverse                         | This study |
| D6X reverse                         | This study |
| D6X forward                         | This study |
| T26X reverse                        | This study |
| T26X forward                        | This study |
| T261I forward                       | This study |
| T261 reverse                        | This study |
| N28X reverse                        | This study |
| N28X forward                        | This study |
| N28T forward                        | This study |
| N28T reverse                        | This study |
| N28L forward                        | This study |
| N28L reverse                        | This study |
| N28D forward                        | This study |
| N28D reverse                        | This study |
3 Correlated mutation analysis using 3DM

The 3DM database was created for the aldolase superfamily by first superpositioning of all published aldolase structures to determine a common core of structurally equivalent positions, followed by subfamily alignment of all protein sequences with >30% sequence identity, then merging all subfamilies into a fully aligned database according to their core residue positions. The database was interrogated for correlated mutations for the D6 and T26 sites, separately for collections containing (1) all aldolases, (2) all FSA-type enzymes, (3) all enzymes comprising a mechanistic water molecule for nucleophile activation/Schiff-base formation and release, and (4) all enzymes having a Schiff-base forming K78 residue.

### Table: CMA scores for positions 6 and 32

| % | A | C | D | E | F | G | H | I | K | L | M | N | P | Q | R | S | T | V | W | Y |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 0.11 | 0.43 | 0.00 | 0.11 | 0.39 | 0.10 | 0 | 3.83 | 0 | 2.03 | 0.06 | 0.02 | 0.01 | 0 | 0.09 | 0.02 | 7.19 | 0.00 | 0.33 |
| C | 0 | 0 | 0.03 | 0.27 | 0 | 0 | 0 | 0 | 0.00 | 0 | 0.10 | 0.00 | 0.00 | 0 | 0.06 | 0 | 0.00 | 0.06 | 0.01 |
| D | 0.04 | 0.02 | 0.01 | 0.01 | 0.00 | 0.01 | 0 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0 | 0.01 | 0.01 | 0.01 |
| E | 0.00 | 0 | 0.01 | 0.01 | 0.00 | 0.01 | 0 | 0 | 0 | 0 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0 | 0.01 | 0.01 | 0.01 |
| F | 0.02 | 0.01 | 0 | 0 | 0.02 | 0.02 | 0 | 0 | 0 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0 | 0.01 | 0.02 | 0.00 |
| G | 0.01 | 0.00 | 0.00 | 0.02 | 0 | 0.00 | 0 | 0.02 | 0 | 5.13 | 0.00 | 0 | 0.00 | 0.01 | 3.57 | 0 | 0.01 | 0.01 | 0.01 |
| H | 0 | 0 | 0 | 0.00 | 0 | 0 | 0 | 0 | 0 | 0.00 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| I | 0.00 | 0 | 0.00 | 2.35 | 0 | 0 | 0 | 0 | 0 | 0.00 | 0 | 0.06 | 0.01 | 0 | 0 | 0.12 | 0 | 0.00 | 0.01 |
| J | 0 | 0.00 | 0 | 0.00 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| K | 0 | 0.00 | 0 | 0.00 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| L | 0.00 | 0 | 0.00 | 0 | 0.00 | 0 | 0 | 0 | 0 | 0 | 0 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0 | 0.01 | 0.03 | 0.00 |
| M | 0 | 0.01 | 0 | 0.00 | 0 | 0 | 0 | 0 | 0.00 | 0 | 0 | 0.00 | 0 | 0 | 0 | 0.06 | 0 | 0.00 | 0.00 |
| N | 1.75 | 0.04 | 0.14 | 0.23 | 0 | 1.11 | 0.08 | 0.09 | 0 | 0.00 | 0.00 | 0.07 | 0.35 | 0.01 | 0.06 | 0.30 | 0.05 | 0.00 | 0.00 |
| P | 2.31 | 0.16 | 0.32 | 2.01 | 0.75 | 0.03 | 0 | 1.60 | 0.01 | 1.73 | 1.74 | 0.10 | 0 | 0 | 0.44 | 0.10 | 2.07 | 0 | 0.01 |
| Q | 0 | 0 | 0.01 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| R | 0 | 0.00 | 0 | 0.01 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| S | 0.02 | 0.36 | 0 | 0.00 | 0 | 0.02 | 0 | 0.00 | 0 | 0 | 0 | 0 | 0.02 | 0.02 | 0.00 | 0 | 0 | 0 | 0 |
| T | 0.23 | 6.55 | 0.02 | 12.43 | 0.02 | 0.01 | 0 | 0.01 | 0.00 | 0.03 | 0.17 | 0.00 | 0 | 0.05 | 0.00 | 0.01 | 0.02 | 0.06 | 0.08 |
| V | 0.02 | 0.02 | 0.03 | 12.73 | 0 | 0.00 | 0 | 0.19 | 0.01 | 0.08 | 0.00 | 0.00 | 0 | 0.09 | 0.00 | 0.00 | 0.01 | 0.01 | 0.04 | 0.00 |
| W | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Y | 0.26 | 0 | 0 | 0.01 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
CMA score for positions 6 and 32 is: 0.00

This graph shows the occurrences of amino acid couples of positions 6 and 32

| % | A  | C  | D  | E  | F  | G  | H  | I  | K  | L  | M  | N  | P  | Q  | R  | S  | T  | V  | W  | Y  |
|---|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| A | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  |
| C | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  |
| D | 0.02 | 0.16 | 0.04 | 0 | 0 | 0 | 0.05 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0 | 0 | 0.02 | 0.02 | 0.09 | 98.26 | 0.02 | 0 | 0 |
| E | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  |
| F | 0  | 0.02 | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  |
| G | 0  | 0  | 0  | 0.02 | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  |
| H | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  |
| I | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  |
| K | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  |
| L | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  |
| M | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  |
| N | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  |
| P | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  |
| Q | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  |
| R | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  |
| S | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  |
| T | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  |
| V | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  |
| W | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  |
| Y | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  |
Figure 1. Correlation analysis for positions 6 and 32 (3DM core numbering corresponding to D6 and T26 in FSA from *E. coli*) using the 3DM aldolase database. A) Full aldolase dataset; B) FSA subset; C) FSA enzymes featuring a catalytic water triade; D) all sequences featuring a lysine residue at position 78.
4 Site directed mutagenesis and library creation

The mutant *fsa* D6A was obtained using pET16b *fsa* as templates with the mutagenesis primers FSA D6A. BL21 (DE3) cells were transformed with this plasmid to heal the plasmid nicks. One of the colonies was cultivated in LB media overnight for plasmid isolation. The mutant *fsa* D6A/T26I was obtained using this pET16b *fsa* D6A as templates in a new PCR with the mutagenesis primers FSA T26I. The mutant *fsa* D6L was obtained using pET16b *fsa* as templates and the mutagenesis primers FSA D6L.

The library D6L/N28X was created with the N28X primer mixture and pET16b *fsa* D6L as template. The library D6E/N28X was created with the N28X primer mixture and pQE40 *fsa* D6E as template. The library D6H/N28X was created with the N28X primer mixture and pQE40 *fsa* D6H[3] as template. The variants D6H/N28D, D6H/N28L and D6H/N28T were created with the N28D, N28L or N28T primer mixtures and pQE40 *fsa* D6H[3] as template.

To create the D6X/T26X double site library pET16b *fsa* was used as a template in a PCR reaction with the D6X primer mixture. XL10 Gold cells were transformed with the resulting mix to heal the plasmid nicks and grown in LB media. From the mixed culture plasmid DNA was isolated to be used as a template in a second PCR reaction with the T26X primer mixture. The resulting plasmid mixture was sequenced for confirmation of the mutagenesis (Figure 1.) and used to transform BL21 (DE3) cells to create the final library.

![Electropherogram](image)

**Figure 2.** Electropherogram of the *fsa* D6X/T26X plasmid mix. Codon triplets are marked with a grey rectangle, the corresponding amino acids and their numbering is listed underneath. Mutated triplets are marked with an orange rectangle.
5 Cell growth

96-well master plate creation

E. coli strain BL21 (DE3) was transformed with the plasmid mixture and spreaded on an agar plate. The colonies were picked into a 96-well plate and grown in LB media (130 µL/well) at 30 °C for 18 h at 230 rpm. The media was mixed with glycerol (15 %) to create a master plate which was stored at -80 °C.

96-well protein expression to create a screening plate

A fresh 96-deepwell plate with LB-medium (950 µl/well) was inoculated from the master plate and incubated at 30 °C and 230 rpm for 18 h. The cells were centrifuged (2540 × g for 30 min), the supernatant was discarded, and cells resuspended in LB media (950 µL/well) containing IPTG (0.5 mM) at 2500 rpm. After 18 h incubation at 30 °C (230 rpm), cells were harvested (2540 × g for 30 min) and suspended in TEA buffer (50 mM, pH 8.5, 500 µL/well).

Cell growth in Eppendorf vials

In an Eppendorf vial (2 mL) LB media (1.6 mL) was inoculated with BL21 (DE3) cells containing the appropriate expression plasmid and cultivated for 18 h at 37 °C. After centrifugation (16000 × g, 30 sec) the cell pellets were resuspended in LB media (1.6 mL) containing IPTG (0.5 mM). The vials were incubated for 6 h (37 °C) and then centrifuged (16000 × g, 30 sec).

Cell growth in shake flasks

E. coli strain BL21 (DE3) was transformed with the appropriate expression plasmid. Cells were grown in auto induction media[4] (AI; 4 L) containing ampicillin (100 mg L⁻¹) for 20 h at 37 °C. Cells were harvested, suspended in TEA buffer (300 mL, 50 mM, pH 8.5) and centrifuged (2540 × g for 30 min). The cell pellet was resuspended in TEA buffer (200 mL, 50 mM, pH 8.5) and then lyophilized.

Protein expression and purification for preparative use

For expression of the FSA wt and the mutant proteins E. coli strain BL21 (DE3) was transformed with the corresponding plasmids. Cells were grown in AI medium (4 L) containing ampicillin (100 mg L⁻¹) for 22 h at 37 °C. Cells were harvested by centrifugation (2254 × g, 30 min) and suspended in 200 mL buffer solution (50 mM TEA, pH 8.5 for synthesis; 50 mM GlyGly, pH 7.0 for inhibition studies) containing lysozyme (1600 kU). Cell suspension was frozen at -20 °C. After thawing up to room temperature DNAse (800 U) and DTT (2 mM) were added. After incubation for 0.5-1 h cellular debris was removed by centrifugation (2540 × g for 30 min). The clear supernatant was purified by heat-shock treatment (70 °C, 30 min) followed by centrifugation (16000 × g for 10 min). The supernatant was separated and lyophilized.
6 TLC screening of FSA D6H/N28L and D6H/N28T against D6H

The cells grown in an Eppendorf vial were resuspended in TEA buffer (0.5 mL, 50 mM, pH 8.0) and mixed with a stock solution of acetone and propanal for final concentrations of 1230 mM and 75 mM.

Figure 3. Exemplary TLC screening of variants D6H/N28T and D6H/N28L against D6H and FSA wt as positive controls with acetone and propanal, TLC development with DCM/MeOH, 15:1. Staining with anisaldehyde solution.[5]

Figure 4. Exemplary TLC screening of variant D6H/N28D with acetone and propanal, TLC development with cyclohexane/EtOAc, 1:1. Staining with anisaldehyde solution.
7 TLC screening of N28X libraries

7.1 Screening of 96-well plates by thin layer chromatography

The cell suspensions in the 96-well screening plate were mixed with a stock solution of acetone and propanal for final concentrations of 1230 mM and 75 mM. After incubation for 6 h (37 °C, 1200 rpm) samples of 2.3 µL from each well were removed and analysed by TLC (EtOAc/cyclohexane, 1:1). Staining was performed with anisaldehyde solution.

7.2 Screening of single-site FSA D6L/N28X library

Figure 5. TLC screening of the D6L/N28X library with acetone and propanal, TLC development with cyclohexane/EtOAc, 1:1. Staining with anisaldehyde solution. All positive wells contain FSA (D6L/N28); weak spots caused by mixed cultures.
7.3 Screening of single-site FSA D6E/N28X library

**Figure 6.** TLC screening of the D6E/N28X library with acetone and propanal, TLC development with cyclo-hexane/EtOAc, 1:1. Staining with anisaldehyde solution. All positive wells contain FSA (D6E/N28); weak spots caused by mixed cultures.
7.4 Screening of single-site FSA D6H/N28X library

Figure 7. TLC screening of the D6H/N28X library with acetone and propanal, TLC development with cyclohexane/EtOAc, 1:1. Staining with anisaldehyde solution. All positive wells contain FSA (D6H/N28); weak spots caused by mixed cultures.
8 TLC screening of double-site FSA D6X/T26X library

8.1 Primary screening for activity against acetone

The cell suspensions in the 96-well screening plate were mixed with a stock solution of acetone and propanal for final concentrations of 1230 mM and 75 mM. After incubation for 6 h (37 °C, 1200 rpm) samples of 2.3 µL from each well were removed and analysed by TLC (CHCl₃/MeOH, 15:1). Staining was performed with anisaldehyde solution.

Figure 8. TLC screening of the D6X/T26X library with acetone and propanal, TLC development with CHCl₃/MeOH, 1:15. Staining with anisaldehyde solution. Variants with green rectangles were picked for the rescreening master plate; variants with orange rectangles were sequenced but accidentally not included for rescreening.
Figure 9. TLC screening of the D6X/T26X library with acetone and propanal, TLC development with CHCl₃/MeOH, 1:15. Staining with anisaldehyde solution. Variants with green rectangles were picked for the rescreening master plate; the variant with orange rectangles were sequenced but accidentally not continued for rescreening (duplicate entries).

All the variants marked in green were collected from the master plates to create a new rescreening master plate. From this master hit plate new screening plates were produced.
8.2 Rescreening

The cell suspensions in the 96-well hit plate were mixed with a stock solution of acetone and propanal for final concentrations of 1230 mM and 75 mM. After incubation for 6 h (37 °C, 1200 rpm) samples of 2.3 µL from each well were removed and analysed by TLC (EtOAc/cyclohexane, 1:1). Staining was performed with anisaldehyde solution.

Figure 10. TLC screening of the D6X/T26X library with acetone and propanal, TLC development with cyclohexane/EtOAc, 1:1. Staining with anisaldehyde solution.
8.3 Screening with \( n \)-butanal

The cell suspensions in the 96-well hit plate were mixed with \( n \)-butanal for a final concentration of 110 mM. After incubation for 6 h (rt, 1200 rpm) samples of 2.3 \( \mu \)L from each well were removed and analysed by TLC (EtOAc/cyclohexane, 1:1). Staining was performed with anisaldehyde solution.

*Figure 11.* TLC screening of the D6X/T26X library with \( n \)-butanal. TLC development with cyclohexane/EtOAc, 1:1. Staining with anisaldehyde solution.
# Sequencing results from the D6X/N28X library screenings

## Table 2. Mutations collected in the rescreening hit plate of the D6X/T26X library

| position | mutation D6X/T26X | position | mutation D6X/T26X | position | mutation D6X/T26X |
|----------|------------------|----------|------------------|----------|------------------|
| A01      | D6E              | C09      | D6V              | F05      | D6E              |
| A02      | D6H              | C10      | D6P              | F06      | D6A              |
| A03[a]   | mix              | C11      | D6E              | F07      | D6E              |
| A04      | D6L              | C12      | D6E              | F08      | D6H              |
| A05      | D6L              | D01      | D6A              | F09      | D6A              |
| A06      | D6H              | D02      | D6A              | F10      | D6V              |
| A07      | D6P              | D03      | wt               | F11      | D6A              |
| A08      | D6H              | D04      | D6L              | F12      | D6A              |
| A09      | D6V              | D05      | D6L              | G01      | D6A              |
| A10      | D6A              | D06      | D6A              | G02      | D6L              |
| A11      | D6L              | D07      | wt               | G03      | wt               |
| A12      | D6L              | D08      | Wt               | G04      | D6H              |
| B01      | D6Q              | D09      | D6A              | G05      | D6H              |
| B02      | D6H              | D10      | D6A              | G06      | D6V              |
| B03      | D6L              | D11      | D6A              | G07      | D6A              |
| B04      | D6A              | D12      | D6P              | G08      | D6E              |
| B05      | D6E              | E01      | D6Q              | G09      | D6A              |
| B06      | D6E              | E02      | Wt               | G10      | D6A              |
| B07      | D6V              | E03      | D6V              | G11      | D6V              |
| B08      | D6H              | E04      | D6E              | G12      | D6V              |
| B09      | D6A              | E05      | D6A              | H01      | D6H              |
| B10      | D6E              | E06      | D6H              | H02      | D6P              |
| B11[b]   | wt               | E07      | D6V              | H03      | D6A              |
| B12      | D6L              | E08      | wt               | H04      | D6H              |
| C01      | D6H              | E09      | D6E              | H05      | D6V              |
| C02      | D6A              | E10      | D6A              | H06      | D6E              |
| C03      | D6L              | E11      | D6A              | H07      | D6L              |
| C04      | D6V              | E12      | D6P              | H08      | D6V              |
| C05      | D6A              | F01      | D6E              | H09      | D6A              |
| C06      | D6L              | F02      | wt               | H10      | D6A              |
| C07      | D6E              | F03      | D6L              | H11      | D6A              |
| C08      | D6A              | F04      | D6L              | H12      | D6Q              |

[a] = mixture of variants;  
[b] = false positive (FSA wt);  
[c] = start codon mutated.  
Color code for variants: green selective for 4-hydroxyhexan-2-one, blue for 3-hydroxy-2-methylpentanal, black non-selective, red inactive.

All unique positive mutants were collected in one 96-well master plate to build the hit library for subsequent screenings.
Table 3. Mutations collected in the hit library master plate

| Line position | A       | B       | C       | D       | E       | F       |
|---------------|---------|---------|---------|---------|---------|---------|
| 1             | D6Q/T26L | D6Q/T26L | D6A/T26A | D6P/T26I | D6H     | D6A/T26A |
| 2             | D6H/T26L | D6H/T26L | D6L     | D6L     | wt      | wt      |
| 3             | D6L/T26L | D6L/T26L | D6V/T26L | D6V/T26L |         |         |
| 4             | D6A/T26V | D6A/T26V | D6E/T26A | D6E/T26A |         |         |
| 5             | D6E     | D6E     | D6A     | D6A     |         |         |
| 6             | D6E/T26L | D6E/T26L | D6V/T26V | D6V/T26V |         |         |
| 7             | D6V/T26A | D6V/T26A | T26V    | T26V    |         |         |
| 8             | D6E     | D6H     | D6L/T26A | D6L/T26A |         |         |
| 9             | D6A/T26L | D6A/T26L | T26L    | T26L    |         |         |
| 10            | D6E/T26V | D6E/T26V | D6A/T26I | D6A/T26I |         |         |
| 11            | D6L/T26V | D6H/T26A | D6P/T26L | D6P/T26L |         |         |
| 12            | D6H/T26A | D6L/T26V | D6Q/T26I | D6Q/T26I |         |         |
High performance TLC screening with acetone and propanal

The cell suspensions in the 96-well hit plate were mixed with a stock solution of acetone and propanal for final concentrations of 1230 mM and 75 mM. After incubation for 6 h (37 °C, 1200 rpm) cells were centrifuged (2250 × g, 30 min) and samples of 50 µL/well were transferred to a new plate and mixed with 50 µL MeOH per well. After shaking (1200 rpm) for 20 sec, the plate was centrifuged (2250 × g, 30 min). A sample from the liquid phase of each well (4 µL) was sprayed with 6 mm band width on TLC plates, which were developed with EtOAc/cyclohexane (1:1, v/v). Staining was performed by dipping into an anisaldehyde reagent solution followed by heating in an oven at 110 °C for 90 sec. Plates were densitometrically analysed at 600 nm using the CAMAG-Scanner.

Figure 12. HPTLC screening of the D6X/T26X hit library with acetone and propanal, TLC development with cyclohexane/EtOAc, 1:1. Staining with anisaldehyde solution.
### Table 4. Densitometric analysis at 600 nm

| Lane A | Mutation | Color density | Lane C | Mutation | Color density |
|--------|----------|---------------|--------|----------|---------------|
| 1      | D6Q/T26L | 1806          | 1      | D6A/T26A | 23472         |
| 2      | D6H/T26L | 2329          | 2      | D6L      | 26092         |
| 3      | D6L/T26L | 2578          | 3      | D6V/T26L | 6992          |
| 4      | D6A/T26V | 6619          | 4      | D6E/T26A | 25200         |
| 5      | D6E      | 13941         | 5      | D6A      | 27249         |
| 6      | D6E/T26L | 5473          | 6      | D6V/T26V | 2807          |
| 7      | D6V/T26A | 13201         | 7      | T26V     | 11198         |
| 8      | D6H      | 14976         | 8      | D6L/T26A | 21022         |
| 9      | D6A/T26L | 3557          | 9      | T26L     | 8155          |
| 10     | D6E/T26V | 5671          | 10     | D6A/T26I | 3585          |
| 11     | D6L/T26V | 6246          | 11     | D6P/T26L | 1373          |
| 12     | D6H/T26A | 13333         | 12     | D6Q/T26I | 3928          |

### Analysis from rescreening

| Position | mutation | Color density at R<sub>f</sub> 0.1 | Density difference % related to D6E |
|----------|----------|------------------------------------|------------------------------------|
| A5       | D6E      | 15168                              | 100                                |
| A8       | D6H      | 16830                              | 111                                |
| F2       | wt       | 3924                               | 26                                 |
| D2       | D6L      | 18365                              | 121                                |
| D5       | D6A      | 15150                              | 100                                |
| A7       | D6V/T26A | 9083                               | 60                                 |
| A12      | D6H/T26A | 7489                               | 49                                 |
| C1       | D6A/T26A | 16521                              | 109                                |
| D4       | D6E/T26A | 3799                               | 25                                 |
| D7       | T26V     | 604                                | 4                                  |
| D8       | D6L/T26A | 5500                               | 36                                 |
High performance TLC analysis for propanal self aldolization

The cell suspensions in the 96-well hit plate were mixed with propanal for a final concentration of 75 mM. After incubation for 6 h (37 °C, 1200 rpm) cells were centrifuged (2250 × g, 30 min) and samples of 50 μL/well were transferred to a new plate and mixed with 50 μL MeOH per well. After shaking (1200 rpm) for 20 sec, the plate was centrifuged (2250 × g, 30 min). A sample from the liquid phase of each well (4 μL) was sprayed with 6 mm band width on TLC plates, which were developed with EtOAc/cyclohexane (1:1, v/v). Staining was performed by dipping into an anisaldehyde reagent solution followed by heating in an oven at 110 °C for 90 sec. Plates were densitometrically analysed at 615 nm using the CAMAG-Scanner.

Figure 13. HPTLC screening of the D6X/T26X hit library with propanal, TLC development with cyclohexane/EtOAc, 1:1. Staining with anisaldehyde solution.
### Table 5. Densitometrical analysis at 615 nm

| Line A | Mutation     | Color density | Line C | Mutation     | Color density |
|--------|--------------|---------------|--------|--------------|---------------|
| 1      | D6Q/T26L     | 10168         | 1      | D6A/T26A     | 12428         |
| 2      | D6H/T26L     | 7092          | 2      | D6L          | 2012          |
| 3      | D6L/T26L     | 2385          | 3      | D6V/T26L     | 6550          |
| 4      | D6A/T26V     | 10373         | 4      | D6E/T26A     | 12337         |
| 5      | D6E          | 5563          | 5      | D6A          | 8854          |
| 6      | D6E/T26L     | 10249         | 6      | D6V/T26V     | 223           |
| 7      | D6V/T26A     | 7608          | 7      | T26V         | 5250          |
| 8      | D6H          | 682           | 8      | D6L/T26A     | 1079          |
| 9      | D6A/T26L     | 1066          | 9      | T26L         | 7117          |
| 10     | D6E/T26V     | 5852          | 10     | D6A/T26I     | 5792          |
| 11     | D6L/T26V     | 643           | 11     | D6P/T26L     | 4402          |
| 12     | D6H/T26A     | 11431         | 12     | D6Q/T26I     | 6309          |

### Analysis from rescreening

| position | Mutation     | Color density | Density % relative to DERA F200I |
|----------|--------------|---------------|----------------------------------|
| A1       | D6Q/T26L     | 10366         | 103                              |
| A6       | D6E/T26L     | 9937          | 99                               |
| A12      | D6H/T26A     | 8708          | 87                               |
| C1       | D6A/T26A     | 13584         | 136                              |
| C4       | D6E/T26A     | 14817         | 148                              |
| F1       | D6P/T26I     | 10674         | 107                              |
| F2       | wt           | 1095          | 11                               |
| H3       | DERA F200I   | 10016         | 100                              |
| A8       | D6H          | 840           | 8                                |
| A4       | D6A/T26V     | 14025         | 140                              |
High performance TLC analysis for acetone and isopentanal

The cell suspensions in the 96-well hit plate were mixed with acetone and isopentanal for final concentrations of 1230 mM and 75 mM, respectively. After incubation for 6 h (37 °C, 1200 rpm) cells were centrifuged (2250 × g, 30 min) and samples of 50 µL/well were transferred to a new plate and mixed with 50 µL MeOH per well. After shaking (1200 rpm) for 20 sec, the plate was centrifuged (2250 × g, 30 min). A sample from the liquid phase of each well (4 µL) was sprayed with 6 mm band width on TLC plates, which were developed with EtOAc/cyclohexane (1:1, v/v). Staining was performed by dipping into an anisaldehyde reagent solution followed by heating in an oven at 110 °C for 90 sec. Plates were densitometrically analysed at 450 nm using the CAMAG-Scanner.

Figure 14. HPTLC screening of the D6X/T26X hit library with acetone and isopentanal, TLC development with cyclohexane/EtOAc, 1:1. Staining with anisaldehyde solution.
Table 6. Densitrometrical analysis at 450 nm

| Line B | Mutation   | Color density | Line C | Mutation   | Color density |
|--------|------------|---------------|--------|------------|---------------|
| 1      | D6Q/T26L   | 510           | 1      | D6A/T26A   | 2505          |
| 2      | D6H/T26L   | 432           | 2      | D6L        | 12757         |
| 3      | D6L/T26L   | 1013          | 3      | D6V/T26L   | 547           |
| 4      | D6A/T26V   | 2416          | 4      | D6E/T26A   | 6398          |
| 5      | D6E        | 8534          | 5      | D6A        | 7422          |
| 6      | D6E/T26L   | 2811          | 6      | D6V/T26V   | 352           |
| 7      | D6V/T26A   | 6472          | 7      | T26V       | 5000          |
| 8      | D6H        | 17840         | 8      | D6L/T26A   | 3196          |
| 9      | D6A/T26L   | 917           | 9      | T26L       | 3044          |
| 10     | D6E/T26V   | 4424          | 10     | D6A/T26I   | 706           |
| 11     | D6H/T26A   | 5770          | 11     | D6P/T26L   | 321           |
| 12     | D6L/T26V   | 2455          | 12     | D6Q/T26I   | 3193          |

Analysis from rescreening

| position | Mutation   | Color density | Density % relative to D6H |
|----------|------------|---------------|----------------------------|
| E2       | wt         | 5633          | 23                         |
| F1       | D6A/T26A   | 2505          | 10                         |
| F2       | wt         | 7060          | 29                         |
| C2       | D6L        | 21882         | 89                         |
| A8       | D6H        | 24484         | 100                        |
| A5       | D6E        | 13834         | 57                         |
| C5       | D6A        | 9717          | 40                         |
| A7       | D6V/T26A   | 7238          | 30                         |
13 Synthesis and NMR spectroscopic analysis

13.1 Synthesis of (R)-4-hydroxyhexan-2-one

In the reaction vessel lyophilized whole cells of *E. coli* BL21, previously cultivated for expression of FSA D6E (250 mg, equivalent to 15 mg of pure FSA) were suspended in PBS buffer (15 mL, pH 7.0). Acetone (4 mL, 54.5 mmol), freshly distilled propanal (1 mL, 13.9 mmol), and DTT (2 mM) were added. The suspension was shaken on a rotary shaker at 21 °C for 4 days, then centrifuged at 3087 × g for 20 min. The supernatant was extracted with diethyl ether (3 x 20 mL), and the combined organic layers were dried over MgSO₄, filtered, and the solvent evaporated under vacuum. The oily residue containing almost pure product (550 mg, 33.9%) was analyzed by NMR spectroscopy.

![Figure 15. 1H NMR spectrum of (R)-4-hydroxyhexan-2-one from acetone and propanal by FSA D6E catalysis](image)

1H NMR (300 MHz, MeOD): δ = 1.05 (t, 3H, J₆,₅ = 7.5 Hz, H-6), 1.58 (m, 2H, H-5), 2.29 (s, 3H, H-1), 2.68 (d, 2H, J₃,₄ = 6.4 Hz, H-3), 4.06 (tt, 1H, H-4). 13C NMR (75 MHz, MeOD): δ = 10.24 (C-6), 30.80 (C-1), 30.93 (C-5), 51.23 (C-3), 70.05 (C-4), 210.80 (C-2).
13.2 Synthesis of (R)-4-hydroxy-6-methylheptan-2-one

In the reaction vessel lyophilized whole cells of *E. coli* BL21, previously cultivated for expression of FSA D6E (191 mg, equivalent to 11 mg of pure FSA) were suspended in PBS buffer (15 mL, pH 7.0). Acetone (4 mL, 54.5 mmol), freshly distilled isopentanal (1 mL, 9.3 mmol), and DTT (2 mM) were added. The suspension was shaken on a rotary shaker at 21 °C for 4 days, then centrifuged at 3087 × g for 20 min. The supernatant was extracted with diethyl ether (3 x 20 mL), and the combined organic layers were dried over MgSO₄, filtered, and the solvent evaporated under vacuum. The oily residue, containing almost pure product (284 mg, 21.2%), was analyzed by NMR spectroscopy.

![Figure 16. 1H NMR spectrum of (R)-4-hydroxy-6-methylheptan-2-one from acetone and isovaleraldehyde](image)

1H NMR (300 MHz, CDCl₃): \( \delta = 0.87 \) (d, 6H, \( J_{6,1'7} = 6.6 \) Hz, H-1’/7), 1.09 (ddd, 1H, \( J_{5a,5b} = 13.7, J_{4,5b} = 4.4, J_{3b,6} = 8.6 \) Hz, H-5b), 1.42 (dd, 1H, \( J_{5a,5b} = 13.7, J_{4,5a} = 9.0, J_{3a,6} = 5.5 \) Hz, H-5a), 1.74 (m, 1H, H-6), 2.13 (s, 3H, H-1), 2.47 (dd, 2H, \( J_{3a,3b} = 17.6, J_{3a,4} = 8.4 \) Hz, H-3a), 2.57 (dd, 2H, \( J_{3a,3b} = 17.6, J_{3b,4} = 3.6 \) Hz, H-3b), 4.08 (dddd, 1H, \( J_{4,5a} = 8.9 \) Hz, H-4). 13C NMR (75 MHz, CDCl₃): \( \delta = 22.06 \) (C-6), 23.35 and 24.39 (C-7/1’), 30.84 (C-1), 45.58 (C-5), 50.57 (C-3), 65.69 (C-4), 210.15 (C-2).
13.3 Synthesis of (2R,3R)-2-methylpentane-1,3-diol

The enzyme catalyst FSA D6A/T26I (35 mg) was dissolved in triethanolamine buffer (100 mL; 50 mM, pH 7.4) containing DTT (2 mM). After addition of freshly distilled propanal (1500 µL, 20.9 mmol) the vessel was stoppered and reaction mixture stirred at 20 °C for 3 days. Then CaCl₂ solution was added (1 mM final concentration) followed by proteinase K (51 U)⁶, and the pH adjusted to 7.0 using sat. aq NaHCO₃ solution. This mixture was incubated at 55 °C for 2 h during which the turbid solution became clear. The product was extracted with ethyl acetate (4 × 60 mL) with TLC control. The combined organic layers were dried (MgSO₄), filtered, and the solvent evaporated at 40 °C under vacuum. The oily residue was purified by silica column chromatography using cyclohexane–ethyl acetate (3:1) as eluent. The aldehyde (1 eq) was dissolved in MeOH (1.4 mL per mmol aldehyde) and treated portion wise with NaBH₄ (1.5 eq per mmol) with stirring at room temperature. After 2 h, 2 volumes of brine were added and a solid precipitated. The mixture was slowly treated with deionized water until the turbidity disappeared (ca. 4 mL/mL MeOH). The solution was extracted with ethyl acetate (6 × 20 mL; TLC control). The combined organic phases were dried (MgSO₄), filtered and concentrated under vacuum at 40 °C to give 130 mg pure product (10.6 %).

**Figure 17.** ¹H NMR spectrum of (2R,3R)-2-methylpentane-1,3-diol

¹H NMR (300 MHz, CDCl₃): δ = 0.79 (d, 3H, J = 7.05 Hz, H-5), 0.84 (t, 3H, J = 7.45 Hz, H-2'), 1.35 (m, 2H, H-4), 1.65 (m, 1H, H-2), 3.50 (m, 2H, H-3), 3.58 (m, 1H, H-1), 3.96 (brs, 2H, OH).

¹³C NMR (75 MHz, CDCl₃): δ = 9.90 (C-5), 10.57 (C-2'), 26.71 (C-4), 38.75 (C-2), 66.19 (C-1), 74.96 (C-3).
13.4 Synthesis of (3R)-2-methylpentane-1,3-diol

The enzyme catalyst FSA D6A/T26L (15 mg) was dissolved in triethanolamine buffer (200 mL, 50 mM, pH 7.4) containing DTT (2 mM). After addition of freshly distilled propanal (1.6 g, 27.89 mmol) the vessel was stoppered and reaction mixture stirred at 20 °C for 3 days. Work-up was performed as above to furnish the aldol product as an oily residue. The residue was dissolved in alkaline buffer (pH 9.0), allowed to stand for 10 min, and then reduced with NaBH₄ and worked up as above. The yellow oily residue gave the title compound (7.7 %) as a mixture of diastereoisomers, which was analyzed by NMR spectroscopy. The ratio of (2R,3R) to (2S,3R) or syn/anti was found to be ca. 1:2.

![Figure 18. 1H NMR spectrum of (3R)-2-methylpentane-1,3-diol](image)

(2R,3R)-2-methylpentane-1,3-diol

1H-NMR (300 MHz, CDCl₃): δ = 0.88 (m, 6H, H-1, H-5), 1.43 (m, 2H, H-4), 1.72 (m, 1H, H-2), 3.54 (m, 1H, H-3), 3.7-4.06 (m, 2H, H-1).

(2S,3R)-2-methylpentane-1,3-diol

1H-NMR (300 MHz, CDCl₃): δ = 0.88 (m, 6H, H-1, H-5), 1.43 (m, 2H, H-4), 1.95 (m, 1H, H-2), 3.7-4.06 (m, 3H, H-1, H-3).
13.5 Synthesis of (4R,5R)-4-ethyl-2,2,5-trimethyl-1,3-dioxane

A sample of the diol was taken up in dry acetone (2.5 mL) followed by addition of dimethoxypropane (2.5 mL). To this was added molecular sieves (250 mg; 3 Å) and a catalytic quantity of p-TosOH. The vessel was closed with a septum and stirred at 20 °C for 2.5 h. After addition of satd NaHCO₃ solution (2.5 mL) the mixture was extracted using diethyl ether (3 × 5 mL). The combined organic phases were dried (MgSO₄), filtered and concentrated under vacuum to furnish the acetal as an oil (300 mg, 29.9 %).

![Figure 19. 1H NMR spectrum of (4R,5R)-4-ethyl-2,2,5-trimethyl-1,3-dioxane](image)

1H-NMR (300 MHz, CDCl₃/C₆D₆ 7:3): δ = 1.54 (t, 3H, J₃',₄' = 7.4 Hz, H-4'), 1.73 (d, 3H, J₅,₅' = 6.8 Hz, H-5'), 1.83 (m, 1H, H-5), 1.95 (ddq, 1H, H-3'a), 2.03 (s, 3H, H-1'), 2.14 (s, 3H, H-2'), 2.20 (ddt, 1H, J₃',₄' = 7.4 Hz, H-3'b), 4.16 (dd, J₅,₆b = 1.66 Hz, J₆a,₆b = 11.40 Hz, 1H, H-6a), 4.32 (ddd, 1H, J = 2.5, 3.5, 7.7 Hz, H-4), 4.56 (dd, J₅,₆a = 2.8, J₆a,₆b = 11.40 Hz, 1H, H-6b). 13C-NMR (75 MHz, CDCl₃/C₆D₆ 7:3): δ = 9.56 (C-4'), 10.33 (C-5'), 18.87 (C-1'), 29.81 (C-3'), 29.80 (C-2'), 31.31 (C-5), 66.65 (C-6), 72.82 (C-4), 98.22 (C-2).
Figure 20. NOESY spectrum of (4R,5R)-4-ethyl-2,2,5-trimethyl-1,3-dioxane shows a coupling between H-4 and H-5 proving their syn configuration.
13.6 Synthesis of (4R)-4-ethyl-2,2,5-trimethyl-1,3-dioxane

The crude diastereomeric mixture of (3R)-2-methylpentane-1,3-diol synthetized above (1570 mg, 13.29 mmol) was taken up in dry acetone (38 mL) followed by addition of dimethoxypropane (38 mL). To this was added molecular sieves (7 g; 3 Å) and a catalytic quantity of p-TosOH. The vessel was closed with a septum and stirred at 20 °C for 2.5 h. After addition of satd NaHCO₃ solution (38 mL) the mixture was extracted with diethyl ether (3 × 80 mL).[7] The combined organic phases were dried (MgSO₄), filtered and concentrated under vacuum to furnish the acetal as an oil (384 mg, 18.2 %).

Figure 21. $^1$H NMR spectrum of (4R)-4-ethyl-2,2,5-trimethyl-1,3-dioxane

(4R,5S)-4-ethyl-2,2,5-trimethyl-1,3-dioxane (anti, major constituent)

$^1$H-NMR (300 MHz, CDCl₃): $\delta = 0.72$ (d, 3H, $J_{5,5'} = 6.7$ Hz, H-5'), 0.90 (t, 3H, $J_{3',4'} = 7.4$ Hz, H-4'), 1.32 (m, 1H, H-3'a), 1.37 (s, 3H, H-1'), 1.40 (s, 3H, H-2'), 1.46 (m, 1H, H-3'b), 3.35 (ddd, $J = 2.7$, 8.0, 10.4 Hz, 1H, H-4), 3.46 (dd, 1H, $J_{6a,6b} = J_{5,6b} = 11.3$ Hz, H-6b), 3.66 (dd, $J_{6a,6b} = 11.6$, $J_{5,6a} = 5.1$ Hz, 1H, H-6a). $^{13}$C-NMR (75 MHz, CDCl₃): $\delta = 9.39$ (C-4'), 12.81 (C-5'), 19.31 (C-1'), 24.58 (C-3'), 25.91 (C-2'), 33.69 (C-5), 66.25 (C-6), 76.26 (C-4), 98.23 (C-2).
Published NMR spectra for the (4R,5S)-configured acetal prove the assignments.

**Figure 22.** Comparison of $^1$H NMR spectra for diastereomeric acetonides. a) Published spectrum for (4R,5S)-4-ethyl-2,2,5-trimethyl-1,3-dioxane; b) published spectrum for diastereomeric mixture of (4R)-4-ethyl-2,2,5-trimethyl-1,3-dioxane; c) spectrum of (4R,5R)-4-ethyl-2,2,5-trimethyl-1,3-dioxane from FSA-catalyzed enzymatic synthesis.
13.7 Synthesis of \((4R,5R)-5\)-ethyl-2,2-dimethyl-4-propyl-1,3-dioxane

The enzyme catalyst FSA D6A/T26L (35 mg) was dissolved in triethanolamine buffer (100 mL; 50 mM, pH 7.4) containing DTT (2 mM). After addition of freshly distilled butanal (400 µL, 20.9 mmol) the vessel was stoppered and reaction mixture stirred at 20 °C for 3 days. Then CaCl₂ solution was added (1 mM final concentration) followed by proteinase K (51 U), and the pH adjusted to 7.0 using satd aq NaHCO₃ solution. This mixture was incubated at 55 °C for 2 h during which the turbid solution became clear. The product was extracted using ethyl acetate (4 x 60 mL) with TLC control. The combined organic layers were dried (MgSO₄), filtered, and the solvent evaporated at 40 °C under vacuum. The oily residue was purified by silica column chromatography using cyclohexane–ethyl acetate (3:1) as eluent to give pure product (90 mg, 26.9%).

For stereochemical analysis, the aldehyde (1 eq) was dissolved in MeOH (1.4 mL per mmol aldehyde) and treated portion wise with NaBH₄ (1.5 eq per mmol) with stirring at room temperature. Work-up and acetal protection using dimethoxypropane in acetone with acid catalysis was essentially performed as above. Extraction of product was performed using cyclohexane, and purification was achieved by silica gel column chromatography (ethyl acetate–cyclohexane 1:10).
Figure 23. $^1$H NMR spectrum of (4R,5R)-5-ethyl-2,2-dimethyl-4-propyl-1,3-dioxane

$^1$H-NMR (300 MHz, CDCl$_3$/C$_6$D$_6$ 7:3): $\delta = 1.31$ (t, 6H, $J = 7.5$ Hz, H-5',-7'), 1.36 (m, 1H, $J = 10.5$ Hz, H-5), 1.66 (m, 2H, H-4a',-6a'), 1.72-1.85 (m, 2H, H-4b',-6b'), 1.77 (s, 3H, H-1'), 1.78 (s, 3H, H-2'), 1.90 (m, 1H, H-3a'), 2.12 (m, 1H, $J = 3.3$ Hz, H-3b'), 4.17 (dd, 1H, $J_{5,6a} = 1.75$ Hz, $J_{6a,6b} = 11.75$ Hz, H-6a), 4.24 (m, 2H, H-4, -6b). $^{13}$C-NMR (300 MHz, CDCl$_3$): $\delta = 12.06$ (C-7'), 14.06 (C-5'), 16.15 (C-4'), 18.83 (C-6'), 19.15 (C-1'), 29.79 (C-2'), 34.88 (C-3'), 39.11 (C-5), 62.63 (C-6), 71.94 (C-4), 98.44 (C-2). **ESI-MS**: $m/z$ [M]$^+$ calculated for C$_{11}$H$_{22}$O$_2$ 186.1614, found 186.1617.
13.8 Synthesis of (4R)-5-ethyl-2,2-dimethyl-4-propyl-1,3-dioxane with FSA

The diastereomer mixture was created by epimerization of C5 using base treatment of the aldol product in solution as above, followed by standard reduction and acetal protection (yield 93 mg, 73.5 %).

**Figure 24.** $^1$H NMR spectrum of diastereomeric mixture of (4R)-5-ethyl-2,2-dimethyl-4-propyl-1,3-dioxane
13.9 Synthesis of racemic 4-hydroxyhexan-2-one

A gas washing bottle was filled with propanal (12 mL), then connected to a second gas washing bottle filled with acetone (94 mL) and methanolic KOH solution (1 M, 6 mL) at –5 °C. A gentle stream of nitrogen was passed through the propanal bottle in order to slowly carry the aldehyde over into the acetone solution. When the propanal had completely evaporated, a solution of oxalic acid (300 mg in 10 mL MeOH, 2.38 mmol) was added to the reaction mixture for neutralization, upon which potassium oxalate precipitated. After filtration, remaining acetone was evaporated under vacuum. The remaining liquid was distilled at 10 mbar to give a mixture of 4-hydroxyhexan-2-one and 4-hydroxy-4-methylpentan-2-one (from self-aldol addition of acetone), which was used as a GC reference sample without further purification.

Figure 25. $^1$H NMR spectrum of 4-hydroxyhexan-2-one and 4-hydroxy-4-methylpentan-2-one

$^1$H-NMR (300 MHz, CDCl$_3$): $\delta = 0.89$ (t, 3H, $J$(6/5) = 7.5 Hz, H-6), 1.37-1.48 (m, 2H, $J = 7.4; 2.0$ Hz, H-5), 2.12 (s, 3H, H-1), 2.52 (d, 2H, H-3), 3.91 (m, 1H, H-4). $^{13}$C-NMR (75 MHz, CDCl$_3$): $\delta = 9.80$ (C-6), 29.39 (C-1), 30.76 (C-5), 49.65 (C-3), 68.90 (C-4), 209.87 (C-2).
14 GC Analysis

14.1 Relative activity of different FSA variants in the formation of 4-hydroxyhexan-2-one

The *fsa* mutants D6L, D6L/T26A, D6A and D6E from the hit library were cultivated in 1.6 mL LB media (18 h, 30 °C, 900 rpm). After centrifugation (16000 × g, 30 sec) the cell pellets were resuspended in 1.6 mL LB media containing IPTG (0.5 mM) and incubated for 6 h (37 °C, 900 rpm). The cells were collected by centrifugation (16000 × g, 30 sec) and resuspended in TEA buffer (2 mL, 50 mM, pH 8.3), then propanal (40 mM) and acetone (680 mM) were added and the mixtures were incubated at room temperature. After periods of 40, 90 and 140 min the cells were removed by centrifugation (16000 × g, 30 sec) and an aliquot of the reaction volume (100 µL) was mixed with MeOH (900 µL). After 5 min this mixture was again centrifuged (16000 × g, 30 sec) and analyzed by GC.

![Graph showing relative activity of different FSA variants](image)

**Figure 26.** Relative activity of different FSA variants in the production of 4-hydroxyhexan-2-one

| FSA variant | Relative activity in production of 4-hydroxyhexan-2-one in % |
|-------------|------------------------------------------------------------|
| D6L         | 160                                                        |
| D6A         | 155                                                        |
| D6E         | 100                                                        |
| D6L/T26A    | 116                                                        |

**Table 7.** Relative activity of different FSA variants in the production of 4-hydroxyhexan-2-one
14.2 Enantioselectivity of FSA D6E in the synthesis of 4-hydroxyhexan-2-one

Analytical samples of enzymatically prepared (4R)-hydroxyhexan-2-one and of racemic 4-hydroxyhexan-2-one (mixture with 4-hydroxy-4-methylpentan-2-one) and were dissolved in MeOH (1 µL each in 999 µL MeOH) and analysed by GC on a chiral stationary column.

| Parameters               | Value                        |
|--------------------------|------------------------------|
| Injector temperature:   | 230 °C                       |
| Detector temperature:   | 230 °C                       |
| Start temperature       | 80 °C                        |
| Final temperature:      | 230 °C                       |
| Isothermal phase        | none                         |
| Temperature gradient    | 4 °C min⁻¹                   |
| Detector:               | Flame ionization detector    |
| Column:                 | Rt-βDEXsa™ (Restek), 0.25 mm x 30 m |
| GC device               | GC 8000 Series FIONS Instrument |
| Carrier gas:            | Nitrogen                     |

Figure 27. GC analysis of racemic 4-hydroxyhexan-2-one (Rt: 19 min), mixture with 4-hydroxy-4-methylpentan-2-one (Rt: 15.4 min)
Figure 28. GC analysis of enzymatically produced (4R)-hydroxyhexan-2-one (Rt: 19 min)
14.3 Substrate selectivity of FSA variants by GC analysis

Cells grown in an Eppendorf vial for expression of the appropriate FSA variant were resuspended in TEA buffer (0.5 mL, 50 mM, pH 8.3) and mixed with a stock solution of acetone and propanal for final concentrations of 680 mM and 40 mM, respectively. After incubation at 37 °C (1 h, 900 rpm), the suspension was centrifuged (16000 × g, 30 sec) and an aliquot of the supernatant (100 µL) was diluted with MeOH (900 µL). After 5 min this solution was centrifuged (16000 × g, 30 sec) and analysed by GC.

| Parameters       | Values                       |
|------------------|------------------------------|
| Injector temperature: | 200 °C                      |
| Detector temperature: | 230 °C                      |
| Start temperature: | 100 °C                      |
| Isothermal phase: | 2 min                       |
| Final temperature: | 200 °C                      |
| Temperature gradient: | 20 °C min⁻¹              |
| Detector:         | Flame ionization detector    |
| Column:           | Rt-βDEXsa™ (Restek), 0.25 mm x 30 m |
| GC device         | GC 8000 Series FISONS Instrument |
| Carrier gas:      | Nitrogen                     |
Figure 29. GC analysis of the FSA D6L catalyzed reaction with acetone and propanal after 1 h reaction time at 37° C.

Figure 30. GC analysis of the FSA D6H/T26L catalyzed reaction with acetone and propanal after 1 h reaction time at 37° C.
Table 8. Product analysis by GC for reactions catalyzed by different FSA variants with acetone and propanal\(^\text{[a]}\)

| Variant       | \(T_R\) of the GC area [min] | Selectivity | \(\text{ratio of 3.40/3.97}\) | \(\text{ratio of 3.97/3.40}\) |
|---------------|-------------------------------|-------------|-------------------------------|-------------------------------|
|               |                               | variant     | area integrals (*10^3)        |                               |
| D6L           | 3.40                          | 510         | 27                            | 18.27                         | 0.05                          |
| D6H           | 3.97                          | 229         | 7                             | 28.78                         | 0.03                          |
| D6L/T26A      |                               | 198         | 11                            | 17.04                         | 0.06                          |
| D6L/T26V      |                               | 75          | 14                            | 5.12                          | 0.20                          |
| D6A/T26L      |                               | 45          | 376                           | 0.12                          | 8.27                          |
| D6E/T26L      |                               | 79          | 335                           | 0.24                          | 4.21                          |
| T26L          |                               | 64          | 257                           | 0.25                          | 3.98                          |
| D6H/T26L      |                               | 23          | 234                           | 0.10                          | 10.09                         |
| D6P/T26I      |                               | 30          | 212                           | 0.14                          | 7.03                          |
| D6E           |                               | 191         | 200                           | 0.95                          | 1.05                          |
| D6A/T26I      |                               | 15          | 181                           | 0.09                          | 11.60                         |
| D6P/T26L      |                               | 14          | 158                           | 0.09                          | 10.63                         |
| D6A/T26A      |                               | 21          | 158                           | 0.13                          | 7.52                          |
| D6V T26L      |                               | 43          | 120                           | 0.36                          | 2.76                          |
| D6E/T26V      |                               | 35          | 117                           | 0.30                          | 3.32                          |
| T26V          |                               | 29          | 107                           | 0.27                          | 3.66                          |
| D6Q T26I      |                               | 1           | 92                            | 0                             | 1.87                          |
| wt            |                               | 23          | 14                            | 1.66                          | 0.60                          |

[a] Color code: \textbf{blue} = selective for 3-hydroxy-2-methylpentanal, \textbf{green} = selective for 4-hydroxyhexan-2-one
14.4 Kinetic analysis of propanal / butanal homoaldolization by FSA variant D6A/T26L

Lyophilized FSA D6A/T26L (21 mg) was dissolved in 200 mL of TEA buffer (50 mM, pH 7.4) containing DTT (2 mM). The solution was divided into two equal portions; one was charged with propanal (1.6 mL, 223 mM), the other with n-butanal (2.0 mL, 221 mM). Both reaction mixtures were gently stirred at room temperature. At certain intervals, samples (100 µL) were withdrawn from each vessel, diluted with MeOH (900 µL), centrifuged and analyzed for product formation by GC.

Table 9. GC analysis of kinetic profiles for FSA catalyzed homoaldolization of propanal and butanal

| Time [min] | GC area 4 | GC area 12 |
|------------|-----------|------------|
| 0          | 0         | 0          |
| 128        | 214079    | 72395      |
| 229        | 334092    | 116938     |
| 1405       | 449323    | 170611     |
| 1778       | 445468    | 354952     |
| 2865       | 481846    | 427084     |
| 3215       | 482494    | 462616     |
| 4269       | 568304    | 463417     |

Figure 31. Kinetic profiles for conversion of propanal and butanal catalyzed by FSA variant D6A/T26L.
15 GC-MS

15.1 GC-MS (EI) analysis of (4R,5R)-4-ethyl-2,2,5-trimethyl-1,3-dioxane

(4R,5R)-4-ethyl-2,2,5-trimethyl-1,3-dioxane, produced with D6A/T26L was solved in MeOH (1 µl in 999 µl MeOH) and analysed through GC on a chiral column.

Figure 31. GC-MS analysis of (4R,5R)-4-ethyl-2,2,5-trimethyl-1,3-dioxane produced with FSA D6A/T26L
15.2 GC-MS (EI) analysis (EI) of (4R)-4-ethyl-2,2,5-trimethyl-1,3-dioxane

An unpurified sample of (4R)-4-ethyl-2,2,5-trimethyl-1,3-dioxane (also containing the mixed acetal (E)-(1-methoxy-1-methylethoxy)-2-methyl-pent-2-ene) was dissolved in MeOH (1 μL in 999 μL MeOH) and analysed by GC on a chiral column.

Figure 32. GC-MS analysis (EI) of (4R)-4-ethyl-2,2,5-trimethyl-1,3-dioxane. The peak shown in dark color corresponds to the mixed acetal (E)-(1-methoxy-1-methylethoxy)-2-methyl-pent-2-ene).
15.3 GC-MS (EI) analysis of \((4R,5R)-5\text{-ethyl}-2,2\text{-dimethyl-4-propyl-1,3-dioxane}\)

\((4R,5R)\)-4-Ethyl-2,2,5-trimethyl-1,3-dioxane, produced by catalysis with D6A/T26L, was dissolved in MeOH (1 µL in 999 µL MeOH) and analysed by GC on a chiral stationary column.

**Figure 33.** GC-MS analysis of \((4R,5R)-5\text{-ethyl}-2,2\text{-dimethyl-4-propyl-1,3-dioxane}\) produced with FSA D6A/T26L
**D-F6P assay for inhibitor identification**

Assay components D-F6P (30 mM), NADH (125 mM), triosephosphate isomerase (13.45-38.4 U mL\(^{-1}\)) and glycerol-3-phosphate-dehydrogenase (1.45-3.82 U mL\(^{-1}\)) were dissolved in GlyGly buffer (50 mM, pH 7.0) in a 1 mL cuvette at room temperature. Absorbance change was monitored at 340 nm. After 60 sec equilibration FSA wt (175 µg mL\(^{-1}\)) was added and monitoring was continued. After another 140 sec potential effectors were added (Table 9) and the measurement continued.

| Compound          | Assay concentration [mM] | Residual activity [%] |
|-------------------|--------------------------|-----------------------|
| Acetoin           | 10                       | 95                    |
| Pyruvate          | 10                       | 91                    |
| Isobutyraldehyde | 10                       | 90                    |
| Acetone           | 40                       | 87                    |
| L-Glyceraldehyde | 10                       | 65                    |
| Hydroxyacetone   | 40                       | 57                    |
| 3-Hydroxypropanal| 10                       | 48                    |
| Dihydroxyacetone | 40                       | 47                    |
| Propanal          | 10                       | 42                    |
| Formaldehyde      | 10                       | 40                    |
| Acetaldehyde      | 10                       | 18                    |

**Figure 34.** Influence of different ketones on the FSA activity
Figure 35. Influence of different aldehydes on the FSA activity

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18 Author Contributions

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