Artificial Fusions between P450 BM3 and an Alcohol Dehydrogenase for Efficient (+)-Nootkatone Production

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Methods

Construction of the fusion constructs

The following primers were used for the overlap-extension PCR of the cyp102A1-AI (coding for P450 BM3 AI) and syadh genes (Table S1). In the first reaction step, sequences were amplified with pET28a(+) - p450 bm3 and pET28a(+) - syadh as templates with the flanking primers ADH-fwd NheI / ADH fwd XhoI and BM3 fwd NheI / BM3 rev Xhol and the remaining primers that introduce overlapping sequences of the fusion site (designated with the respective names of the fusion constructs). The PCR was conducted with the temperature gradient depicted in Table S2 with 35 cycles. The same temperature gradient was utilized for the overlap extension in 10 cycles but different annealing temperatures for each fusion construct as depicted in Table S1. Final amplification was conducted with 20 cycles with the temperature gradient depicted in Table S2 after addition of the flanking primers.
Table S1 Primer list for the overlap-extension PCR of p450 bm3 and syadh. Annealing temperatures differed according to the reaction steps.

| Name | Sequence 5´ - 3´ | Annealing temperature [°C] |
|------|------------------|---------------------------|
| ADH fwd Nhel | CTAGCTAGCATGACCACGCTTCCGACT | 64 |
| BM3 rev Xhol | CCGCTCGAGTTACCAGCCACACGT | 64 |
| ADH rev Xhol | CCGCTCGAGTCAGGCTTCCGCGCGATA | 64 |
| BM3 fwd Nhel | CTAGCTAGCATGAAATAGCCTCAGC | 64 |
| SB ADH rev | CTTTCTTTAATTGTCATGCTTCGCGCGATAGC | 71 |
| SB BM3 fwd | TATCGGCCGGAAGCCATGACAATTAAAGAAATGCTCAGCC | 71 |
| SB-AA ADH rev | CGCTGCCGCTGGCTGCTGCGCGGCTTCCGCGCGATAGC | 72 |
| SB-AA BM3 fwd | GCAGCAGCCGAGCGACGCGGCAATGACAATTAAAGAAATGCTCAGCC | 72 |
| SB-EA ADH rev | CGCTGCCTCCTTGGCCTGCCTCCTCCGCGCGATAGC | 69 |
| SB-EA BM3 fwd | GCGGCAAAGGGAAGGCAAGCGGCAATGACAATTAAAGAAATGCTCAGCC | 69 |
| SB-GS ADH rev | ACCACCTCCAGATCCACCGCCACCGGCTTCCGCGCGATAGC | 72 |
| SB-GS BM3 fwd | GGTGGATCTGGAGGTGGTGGCTCTATGACAATTAAAGAAATGCTCAGCC | 72 |
| BS ADH fwd | AAGACGTGGTGGCTGAGTGAACCACGCTTCCGACT | 72 |
| BS BM3 rev | AGTGCGACCGTGCTGTATCCACCGCGCCACCTT | 72 |
| BS-AA ADH fwd | GCAGCAGCCGAGCGACGCGGCAATGACCACGCTTCCGACT | 72 |
| BS-AA BM3 rev | CGCTGCCGCTGGCTGCTGCGCGGCTTCCGCGCGATAGC | 72 |
| BS-EA ADH fwd | GCGGCAAAGGGAAGGCAAGCGGCAATGACCCACGCTTCCGACT | 69 |
| BS-EA BM3 rev | CGCTGCCTCCTTGGCCTGCCTCCTCCGCGCGATAGC | 69 |
| BS-GS ADH fwd | GGTGGATCTGGAGGTGGTGGCTCTATGACCACGCTTCCGACT | 72 |
| BS-GS BM3 rev | ACCACCTCCAGATCCACCGCCACCTT | 72 |

Table S2 Temperature gradient for the thermal cycler for overlap-extension PCR of p450 bm3 and syadh.

|          | Temperature [°C] | Time [s] |
|----------|-----------------|---------|
| Initial denaturation | 98 | 30 |
| Denaturation | 98 | 10 |
| Annealing | 64 / ref. Table S1 | 15 |
| Elongation | 72 | 45 |
| Final elongation | 72 | 300 |
Heterologous expression and purification of the fusion constructs

Sonication of the E. coli cells that harbored the fusion constructs were conducted on ice in four cycles of 45 s sonication and 1 min pause (Branson sonifier, BRANSON Ultrasonics Corporation, output control: 4, duty cycle: 40%). The clarified lysate was loaded onto a 5 mL HisTrap FF Crude column (Cytiva) connected to an ÄKTA prime (Cytiva) chromatography system. Contaminating proteins were washed off the column with 5 column volumes of a solution of KPi (50 mM pH 7.5), NaCl (500 mM) and imidazole (40 mM). The fusion constructs were eluted using a solution of KPi (50 mM pH 7.5), NaCl (500 mM) and imidazole (250 mM). Subsequently, the solution that contained the fusion constructs was concentrated by centrifugal filtration (Sartorius, Vivaspin 15 MWCO 10 kDa) at 4°C and 4000 x g. For the size exclusion chromatography (Superdex 200 Increase 10/300 GL, ÄKTA purifier chromatography system (Cytiva)), a solution containing KPi (50 mM pH 7.5), NaCl (125 mM) and 5% glycerol was utilized. After column equilibration, 500 µL of the protein solution were injected and purification was conducted at a flow rate of 0.75 mL min⁻¹. The fractions containing the protein were collected, unified and concentrated by centrifugal filtration for storage at -20°C.

Calibration curve for the quantification of (+)-valencene, nootkatol and nootkatone by GC-MS

To quantify the products after conversion of (+)-valencene, a calibration curve was created through the addition of (+)-valencene, nootkatol and (+)-nootkatone (solubilized in DMSO) in known concentrations to Tris-HCl (50 mM pH 7.5). With nootkatol, concentrations ranged between 0.1, 0.25, 0.5, 0.75, 1, 1.5, 3, 4, 5, 6 mM. With (+)-valencene and (+)-nootkatone, concentrations of 0.1, 0.25, 0.5, 0.75, 1.25, 2, 3, 4, 5, 8 mM were utilized. After addition of the internal standard (R)-(-)-carvone (0.5 mM) to the reaction mixtures, 2 reaction volumes of ethyl acetate were added and the products extracted through vigorous shaking for 30s. Samples were centrifuged for 5 min at 18 000 x g and the organic phase was extracted for analysis by GC/MS

Product extraction and analysis

Products of the reactions were analyzed with the GC/MS-QP2010 Plus system (Shimadzu) connected to a FS-Supreme-5 column (30 m × 0.25 mm × 0.25 µm CS-Chromatographie Service GmbH). The interface temperature was set to 285°C and the injection temperature was set to 250°C. A temperature of 200°C was set for the ion source. Samples (0.5 µL) were measured in split mode which ranged from 10 to 30 and the total ion current was detected (TIC). An m/z range from 40-300 was utilized. The temperature gradient started at 130°C with a hold time of 3 min, ramped up to 260°C in 10°C increments per minute and in the final step to 300°C in a 40°C increment over 1 min.
Results

Determination of specific activity of SyADH with various substrates

Table S1 Specific activity of SyADH measured with the substrates 2-octanol, 2-pentanol, 2-propanol, ethyl-3-hydroxybutyrate and nootkatol.
The reaction mixture in KPi (50 mM, pH 7.5) consisted of the respective substrates (500 µM), NADP+ (1 mM) and purified SyADH (50 nM).
NADPH generation was measured at 340 nm in deepwell plates and the slope of the initial reaction rate was used to determine specific activity with the extinction coefficient of NADPH ($\varepsilon_{340}$ = 6.22 mM$^{-1}$ cm$^{-1}$).

| Substrate         | mU mg$^{-1}$ |
|-------------------|--------------|
| 2-octanol         | 1508 ± 141   |
| 2-pentanol        | 324 ± 11     |
| 2-propanol        | 14 ± 1       |
| ethyl-3-hydroxybutyrate | 10 ± 0.5   |
| nootkatol         | 104 ± 4.2    |

Conversion of trans- and cis-nootkatol by SyADH

Figure S1. Conversion of trans- and cis-nootkatol by SyADH over 6h. Reaction mixtures in KPi (50 mM pH 7.5) consisted of nootkatol (0.2 mM), NADP+ (0.5 mM) and 50 µL of clarified E. coli lysate containing SyADH. Reaction mixtures were incubated at 25°C and 400 rpm in an Eppendorf thermomixer for 2, 4 and 6h. After addition of the internal standard (R)-(-)-carvone (50 µM), the products were extracted with 1 reaction volume of ethyl acetate with vigorous shaking for 30 s. After centrifugation at 18 000 x g for 5 min, the organic phase was extracted, evaporated and the remaining products were resuspended in 50 µL ethyl acetate and analyzed by GC/MS.
Reduction of (+)-nootkatone by SyADH

**Figure S2.** Reduction of (+)-nootkatone to trans- and cis-nootkatol upon NADPH addition over 4h. Reaction mixtures contained Tris-HCl (50 mM pH 7.5), (+)-nootkatone (0.5 mM) solubilized in DMSO (fc. 2% v/v), NADPH (2 mM) and SyADH (11 µM). Reaction samples were incubated at 25°C with 2 rpm overhead shaking for 4h. The internal standard (R)-(-)-carvone (0.5 mM) was added and the samples were extracted with 2 volumes of ethyl acetate by vigorous shaking for 30 s. The samples were centrifuged at 18 000 x g for 5 min, the organic phase was extracted and analyzed by GC/MS. Shaking conditions were changed compared to Figure S1 because slow rotation in an overhead shaker was found more advantageous for the two-enzyme oxidation of valencene.

Product distribution of (+)-valencene conversion by separate and fused enzymes either in purified form or in *E. coli* lysates

**Figure S3.** Total product distribution (%) after conversion of (+)-valencene with fusion constructs and separate enzymes either as (A) purified enzymes or (B) clarified *E. coli* cell lysates. (A) Reaction mixtures consisted of (+)-valencene (1 mM) dissolved in DMSO (fc. 2% v/v), NADPH (3 mM), catalase (150 U), enzymes (0.5 µM) in Tris-HCl (50 mM, pH 7.5). (B) Reactions consisted of (+)-valencene (2 mM) dissolved in DMSO (fc. 2% v/v), NADP⁺ (0.5 mM), isopropanol (10 mM), catalase (150 U), enzymes (1 µM) in Tris-HCl (50 mM, pH 7.5).
(+)-Valencene conversion based on cofactor regeneration with fused and separate enzymes with different cosubstrates for SyADH

Figure S4. Concentration of oxidation products after (+)-valencene (1 mM) conversion with NADP⁺ (0.5 mM), purified SB-GS, BS-GS and separate enzymes (0.5 µM) based on cofactor regeneration with isopropanol, ethyl-3-hydroxybutyrate (Hydroxybutyrate) and 2-pentanol (10 mM). The reaction mixtures were incubated for 4h at 25°C and 2 rpm overhead shaking. Following the addition of (R)-(-)-carvone (0.5 mM), samples were extracted with 2 reaction volumes of ethyl acetate with vigorous shaking for 30s. Following the centrifugation at 5 min and 18 000 x g, the organic phase was extracted and analyzed by GC/MS. The standard deviation of triplicate measurements was calculated to depict the error bars.
Conversion of (+)-valencene in the presence of rising 2-pentanol concentrations

**Figure S5** Conversion of (+)-valencene (1 mM) in Tris-HCl (50 mM pH 7.5) with NADP⁺ (0.5 mM), catalase (150 U) and purified enzymes (0.5 µM). The cosubstrate 2-pentanol was added in concentrations of 10 mM, 50 mM and 150 mM. Incubation of the samples was conducted at 25°C and 2 rpm overhead shaking for 20h. The internal standard (R)-(-)-carvone (0.5 mM) was added the reaction mixtures and samples were extracted with 2 reaction volumes of ethyl acetate by vigorous shaking for 30s. The samples were centrifuged at 18,000 x g, the organic phase was extracted and analyzed by GC/MS. The error bars represent the standard deviation of triplicate measurements.
Influence of rising (+)-valencene concentration on specific activity of SyADH with 2-octanol

Figure S6 Conversion of 2-octanol (0.5 mM) by purified SyADH (50 nM). The reactions were conducted in KPi (50 mM pH 7.5) and contained NADP⁺ (1 mM). (+)-Valencene was solubilized in DMSO (fc. 2% v/v) and added in concentrations of 0.5, 1, 2, 4, 7 and 10 mM. Generation of NADPH during 2-octanol conversion was measured in deepwell plates at 340 nm. The slope of the initial reaction rate was used to calculate the specific activity of SyADH with the extinction coefficient of NADPH ($\varepsilon_{340} = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$). Measurements were conducted in triplicate and the standard deviation was calculated and depicted as the error.
Conversion of (+)-valencene solubilized in methyl-β-cyclodextrin with separate and fused lysates

Figure S7. Total product concentrations and product distributions of reactions with fusion constructs and separate enzymes over (A + B) 24h and (C+D) 48h. Reactions consisted of (+)-valencene (10 mM) dissolved in methyl-β-cyclodextrin (fc. 2% v/v), NADP⁺ (0.5 mM), isopropanol (30 mM), catalase (150 U) and enzymes (5 µM). Reactions were conducted in Tris-HCl (50 mM, pH 7.5) to which the enzymes were added in KPi buffer (50 mM, pH 7.5, 300 mM NaCl) equal to 33% of the reaction volume. Product concentrations were measured over 4, 8, 16, 24 and 48h. Error bars represent the standard deviation of triplicate measurements.