Supporting Information

C=C-Ene-Reductases Reduce the C=N Bond of Oximes

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General

The 1H- and 13C-NMR spectra were recorded using a 300 MHz instrument. Chemical shifts are given in parts per million (ppm) and coupling constants (J) are reported in Hertz (Hz). Melting points were determined in open capillary tubes and are uncorrected. Thin layer chromatography was carried out on silica gel 60 F254 plates and compounds were visualised either by dipping into basic permanganate reagent (10 g/L KMnO4, 50 g/L Na2CO3, 0.85 g/L NaOH, in H2O), or by UV. High Resolution Electrospray Ionization Mass Spectrometry (HR-ESMS) measurements were performed on a Q-Exact Hybrid Quadrupole-Orbitrap MS after flow injection with a Dionex Ultimate 3000 series HPLC-system (Thermo Fisher Sci., Erlangen, Germany). The mobile phase was methanol containing 0.1 % (v/v) formic acid delivered with a flow rate of 0.2 mL/min, and the injection volume was set to 10 µL.

The HR-MS was furnished with a HESI-II atmospheric pressure electrospray ionization source (ES), using nitrogen as nebulizer and drying gas. Measurements were performed in positive or negative ionization mode, the spray voltage was +3.5 kV or -2.5 kV, capillary temperature 250 °C, sheath gas flow rate 45 AU, auxiliary gas temperature 400 °C, auxiliary gas flow rate 10 AU, and the resolution was 70,000 (FWHM). The observed mass range was set to m/z 100-350 with data dependent fragmentation at various normalized collision energies (NCE: 20, 30, 40) and subsequent recording of MS/MS fragment ions. Recorded mass differences were always <3 ppm compared to calculated masses.

Unless otherwise noted, reagents and organic solvents were obtained from commercial suppliers in reagent grade quality and used without further purification.

For GC-FID analysis: conversions were measured on an Agilent 7890A GC with FID detector and Agilent J&W HP-5 column (30 m, 320 µm, 0.25 µm) with helium as carrier gas. Conversions were calculated from the ratios between internal standard (tetramethylpyrazine, 10 mM) and product areas. Injection volume: 1 µL, split ratio 90:1. GC parameters: injector 300 °C, flow 31.202 cm/sec, 1.1913 mL/min.

Temperature programme (standard): 100 °C, hold 0.5 min, ramp 10 °C/min to 300 °C.

Temperature programme (highboilers): 200 °C, hold 0.5 min, ramp 5 °C to 300 °C.

For quantification of remaining oxime, the method was adapted in the following manner: injection volume: 3 µL, split ratio 50:1, temperature programme (standard) was left unchanged.

Chiral HPLC analysis was performed on a Shimadzu HPLC system using a Daicel Chiralpak IC column as chiral stationary phase, n-heptane/2-PrOH 90:10 as eluent, flow rate 1 mL/min, oven temperature 30 °C, 30 min.

General procedure for analytical scale biotransformations

In a 2 mL microcentrifuge tube 100 µg of purified ene reductase, NADPH (stock solution: 10 mM, final concentration: 0.5 mM), glucose (stock solution: 1.0 M, final concentration: 50 mM) and lyophilized GDH preparation (2 mg) were mixed in 50 mM phosphate buffer, pH 7.5. The reactions were started by adding the oxime substrate [final concentration: 10 mM, 5% (v/v) DMSO, total reaction volume: 500 µL]. Samples were shaken at 30 °C, 120 rpm for 24 h in a shaking incubator. The biotransformations were extracted twice with 0.5 mL ethyl acetate containing 10 mM tetramethyl pyrazine as internal standard and the organic phase was dried over MgSO4, centrifuged (13000 rpm, 1 min) and the supernatant transferred to a glass vial for GC-FID measurements.

General procedure for cascade biotransformations

In a 2 mL microcentrifuge tube 100 µg of purified ene reductase (added from a stock solution at varied concentration depending on the specific enzyme in buffer), NADPH (stock solution: 10 mM, final concentration: 0.5 mM), NADH (stock solution: 10 mM, final concentrations: 0.5 mM), ADH-A (heat-purified CFE, 1 mg), glucose (stock solution: 1.0 M, final concentration: 50 mM) and lyophilized GDH preparation (2 mg) were mixed in 50 mM phosphate buffer, pH 7.5. The reactions were started by adding the oxime substrate [final concentration: 10 mM, 5% (v/v) DMSO, total reaction volume: 500 µL]. Samples were shaken at 30 °C, 120 rpm for 24 h in a shaking incubator. A solution of benzoyl chloride (30 mM) in EtOAc containing 10 mM acetanilide as internal standard was added and the mixtures were shaken for 45 minutes (900 rpm, 30 °C). The layers were separated and the aqueous phase extracted once more with EtOAc containing 10 mM acetanilide. The organic phases were combined, dried over Na2SO4, centrifuged (13000 rpm, 1 min) and the supernatant transferred to a glass vial for HPLC-UV measurements.
General procedure for imine reductase screening

In a 2 mL microcentrifuge tube 2 mg of lyophilized imine reductase cell free extract, NAD(P)H (stock solution: 20 mM, final concentration: 0.5 mM), glucose (stock solution: 500 mM, final concentration: 50 mM) and lyophilized GDH preparation (1 mg) were mixed in 100 mM Tris-HCl buffer, pH 7.5. The reactions were started by adding the oxime substrate (final concentration: 10 mM, 5 % (v/v) DMSO, total reaction volume: 500 µL). Samples were shaken at 30 °C, 120 rpm for 24 h in a shaking incubator. The biotransformations were extracted with 1 mL ethyl acetate containing 10 mM tetramethyl pyrazine as internal standard and the organic phase was dried over MgSO₄, centrifuged (13000 rpm, 1 min) and the supernatant transferred to a glass vial for GC-FID measurements.

Table S1. Imine reductases screened for oxime reduction activity

| Entry | pEG Number | Uniprot Accession Number | Source Organism |
|-------|------------|--------------------------|-----------------|
| 1     | 381        | M4ZS15                   | *Streptomyces* sp. GF3546 |
| 2     | 375        | W7VJL8                   | *Micromonospora* sp. M42 |
| 3     | 380        | I8QLV7                   | *Frankia* sp. QA3 |
| 4     | 376        | V7GV82                   | *Mesorhizobium* sp. L2C089B000 |
| 5     | 382        | D2PR38                   | *Kribbella flavida* (DSM 17836) |
| 6     | 384        | K0F8R0                   | *Nocardia brasiliensis* ATCC 700358 |

a Internal plasmid number (pEG stands for plasmid of the elk group)

Procedure for control experiments

To prove the role of the ERs in oxime reduction and pyrazine formation, a series of control experiments was performed. The procedure corresponds to the one described under “General procedure for analytical scale biotransformations”, with the adaptations and results as listed in Table S2. NB: The ER used was OYE3.

Table S2. Control experiments to verify ER activity for oxime reduction

| Entry | Omitted ingredient | Added ingredient | Conv. (%) |
|-------|--------------------|------------------|-----------|
| 1     | Ene-reductase      | -                | 0         |
| 2     | NADPH              | -                | 0         |
| 3     | GDH                | -                | 8         |
| 4     | Glucose            | -                | 0         |
| 5     | Ene-reductase      | FMN (1 mM)       | 0         |
| 6     | Ene-reductase, GDH, glucose | FMN (1 mM), NADPH (40 mM) | 0         |
| 8     | Ene-reductase, GDH, glucose | NADPH (40 mM) | 0         |
Procedure for control experiment of oxime 1a and ADH-A

To prove that oxime 1a is not a substrate for ADH-A, a biotransformation was performed: heat-precipitated ADH-A CFE (1 mg), oxime 1a (10 mM), NADH (0.5 mM), Glucose (50 mM), GDH (2 mg), DMSO (5% v/v) in phosphate buffer (pH 7.5, 50 mM) total volume: 500 μL. The reactions were shaken for 24 h at 30 °C and 120 rpm and analysed by TLC using a synthetic sample of the oxime alcohol as reference. (SiO2, EtOAc/cyclohexane 1:2, Rf oxime: 0.42, Rf alcohol 0.23, KMnO4 stain). No alcohol was detected, proving that the ADH-A does not reduce the ketone functionality of oxime 1a.

Synthesis of oxime substrates

General procedure A:
To a stirred solution of the β-keto-ester or diketone (1.0 eq) in glacial acetic acid (5-10 mL) a solution of NaNO2 in water (1.5 eq in 5 mL H2O) was added dropwise. The reaction temperature was kept at 0 °C in an ice-water bath. The reaction was stirred until full conversion was detected via TLC. Afterwards the mixture was diluted with water (20 mL) and extracted with EtOAc (3 x 20 mL). The combined organic phases were washed with a saturated NaHCO3 solution (2 x 20 mL) and dried over Na2SO4. The remaining solvent was removed under reduced pressure to yield the crude product.

General procedure B:
To a stirred solution of the β-keto-ester or diketone (1.0 eq) in 15 % H2SO4 (5-10 mL) a solution of NaNO2 in water (1.5-2.0 eq) was added dropwise. The reaction temperature was kept at 0 °C in an ice-water bath. The reaction was stirred until full conversion was detected via TLC. Afterwards the mixture was diluted with water (10 mL) and neutralized with solid Na2CO3. The solution was extracted with EtOAc (3 x 20 mL) and the combined organic phases were dried over Na2SO4. The remaining solvent was removed under reduced pressure to yield the crude product.

General procedure C:
To a stirred solution of the diketone (1.0 eq.) in 25 mL 15 % KOH, NaNO2 (1.2 eq.) was added and the mixture cooled to 0 °C in an ice-water bath. 15 % H2SO4 (40 mL) was added dropwise over a period of 30 min and the reaction was allowed to stir at the low temperature for 30 min. After that time the formed precipitate was filtered off and dried under reduced pressure.

Ethyl-2-(hydroxyimino)-3-oxobutanoate (1a)
Oxime 1a was obtained from ethyl-3-oxobutanoate via general procedure A as a light yellow oil. The product was purified by flash chromatography. Yield 80% (3.56 g), TLC Rf = 0.35 (silica, EtOAc/hexanes 1:2). 1H-NMR (300 MHz, CDCl3): δH [ppm]= 9.64 (s, 1H), 4.40 (q, J = 7.1 Hz, 2H), 2.46 (d, J = 21.9 Hz, 3H), 1.37 (t, J = 7.1 Hz, 3H), 13C-NMR (75 MHz, CDCl3): δC [ppm]= 193.8, 161.6, 151.1, 62.5, 25.38, 13.9.

Allyl-2-(hydroxyimino)-3-oxobutanoate (1b)
Oxime 1b was obtained from allyl-3-oxobutanoate via general procedure A as a bright yellow oil. The product was purified via flash chromatography. Yield 90% (1.11 g), TLC Rf 0.23 (silica, EtOAc/hexanes 1:4), \(^{1}H\)-NMR (300 MHz, CDCl\(_3\)): \(\delta_H[ppm]=9.46\) (s, 1H), 5.94 (m, 1H), 5.35 (m, 2H), 4.81 (dt, 2H), 2.45 (d, 3H), \(^{13}C\)-NMR (75 MHz, CDCl\(_3\)): \(\delta_C[ppm]=193.9, 161.4, 151.1, 130.9, 130.7, 120.1, 119.6, 67.1, 66.8, 30.4, 25.5\).

2-Methoxyethyl-2-(hydroxyimino)-3-oxobutanoate (1c)
Oxime 1c was obtained from 2-methoxyethyl-3-oxobutanoate via general procedure A as a colorless oil which solidified upon storage (4 °C). Yield 94% (71.28 mg), TLC Rf 0.18 (silica, EtOAc/cyclohexane 1:3), \(^{1}H\)-NMR (300 MHz, CDCl\(_3\)): \(\delta_H[ppm]=4.49 – 4.44\) (m, 2H), 3.77 – 3.72 (m, 2H), 3.46 (s, 3H), 2.38 (s, 3H), 13C-NMR (75 MHz, CDCl\(_3\)): \(\delta_C[ppm]=193.8, 161.8, 150.8, 70.3, 64.6, 59.3\); HR-MS: m/z = 188.0562 [M-H]- (calcd. 188.0564).

Butyl-2-(hydroxyimino)-3-oxobutanoate (1d)
Oxime 1d was obtained from butyl-3-oxobutanoate via general procedure A as a yellow oil. Yield 96% (1.80 g), TLC Rf 0.28 (silica, EtOAc/hexanes 1:4), \(^{1}H\)-NMR (300 MHz, CDCl\(_3\)): \(\delta_H[ppm]=9.57\) (s, 1H), 4.32 (t, 2H), 2.40 (s, 3H), 1.74 – 1.62 (m, 2H), 1.47 – 1.33 (m, 2H), 0.93 (t, 3H), 13C-NMR (75 MHz, CDCl\(_3\)): \(\delta_C[ppm]=193.9, 161.9, 151.3, 66.4, 30.5, 25.5, 19.0, 13.7\); HR-MS: m/z = 228.1243 [M-H]- (calcd. 228.1241).

Heptyl-2-(hydroxyimino)-3-oxobutanoate (1e)
Oxime 1e was obtained from heptyl-3-oxobutanoate via general procedure A as a yellow to brown oil. Yield 92% (2.01 g), TLC Rf 0.47 (silica, EtOAc/hexanes 1:4), \(^{1}H\)-NMR (300 MHz, CDCl\(_3\)): \(\delta_H[ppm]=9.43\) (s, 1H), 4.31 (t, 2H), 2.41 (s, 3H), 1.80 – 1.60 (m, 2H), 1.45 – 1.17 (m, 8H), 0.88 (dd, 3H), 13C-NMR (75 MHz, CDCl\(_3\)): \(\delta_C[ppm]=200.1, 161.3, 143.5, 135.3, 129.3, 126.3, 121.4, 26.4\).

Benzyl-2-(hydroxyimino)-3-oxobutanoate (1f)
Oxime 1f was obtained from benzyl-3-oxobutanoate via general procedure A as a colorless oil. The product was purified via flash chromatography and solidified upon storage at 4 °C. Yield 80% (1.07 g, m.p.: 78-79 °C, lit.:\(^{[1]}\) 81-82 °C), TLC Rf 0.28 (silica, EtOAc/hexanes 1:4), \(^{1}H\)-NMR (300 MHz, CDCl\(_3\)): \(\delta_H[ppm]=9.26\) (s, 1H), 7.37 (m, 5H), 5.35 (s, 2H), 2.43 (d, 3H), 13C-NMR (75 MHz, CDCl\(_3\)): \(\delta_C[ppm]=200.1, 161.5, 151.1, 134.6, 128.8, 128.5\).

2-(Hydroxyimino)-3-oxo-N-phenylbutanamide (1g)
Oxime 1g was obtained from 3-oxo-N-phenylbutanamide via general procedure A as yellow crystals. The crude product was purified by column chromatography to yield bright yellow crystals. Yield 78% (1.60 g, m.p.: 78-79 °C, lit.:\(^{[2]}\) 81-82 °C), TLC Rf 0.32 (silica, EtOAc/cyclohexane 1:2), \(^{1}H\)-NMR (300 MHz, CDCl\(_3\)): \(\delta_H[ppm]=11.0\) (s, 1H), 7.72 - 7.16 (m, 5H), 2.60 (s, 3H), 13C-NMR (75 MHz, CDCl\(_3\)): \(\delta_C[ppm]=200.1, 161.3, 143.5, 135.3, 129.3, 126.3, 121.4, 26.4\).

Ethyl-2-(hydroxyimino)-3-oxopentanoate (1h)
Oxime 1h was synthesized from ethyl-3-oxopentanoate according to general procedure A. The compound was obtained as yellow oil and used without further purification (1.17 g, 94%). \(^{1}H\)-NMR (300 MHz, CDCl\(_3\)): \(\delta_H[ppm]=9.36\) (s, 1H), 4.36 (dq, J = 12.0, 7.1 Hz, 2H), 2.89 – 2.69 (m, 2H), 1.34 (td, J = 7.1, 3.3 Hz, 3H), 1.14 (dt, J = 13.0, 7.3 Hz, 3H), 13C-NMR (75 MHz, CDCl\(_3\)): \(\delta_C[ppm]=200.5, 196.5, 161.8, 150.5, 62.7, 62.4, 36.2, 31.2, 14.0, 7.44, 6.64\).

Ethyl-2-(hydroxyimino)-3-oxoheptanoate (1i)
Oxime 1i was synthesized from ethyl-3-oxoheptanoate according to general procedure A. The product was purified via flash chromatography (EtOAc/hexanes = 1:3, TLC Rf = 0.31) to yield the title compound as pale-yellow oil (902 mg, 69%). \(^{1}H\)-NMR (300 MHz, CDCl\(_3\)): \(\delta_H[ppm]=9.31\) (s, 1H), 4.40 (q, J = 7.1 Hz, 2H), 2.80 (t, J = 7.4 Hz, 2H), 1.71 – 1.57 (m, 2H), 1.44 – 1.31 (m, 5H), 0.93 (dd, J = 9.1, 5.6 Hz, 3H), \(^{13}C\)-NMR (75 MHz, CDCl\(_3\)): \(\delta_C[ppm]=196.1, 161.7, 150.8, 62.4, 37.5, 25.7, 22.3, 14.0, 13.8\); HR-MS: m/z = 200.0927 [M-H]- (calcd. 200.0928).
Ethyl-2-(hydroxyimino)-3-oxo-3-phenylpropanoate (1j)

Oxime 1j was obtained from ethyl-3-oxo-3-phenylpropanoate according to general procedure A. The substrate precipitated, was filtered under vacuum and washed with ice-cold H₂O (2 x 10 mL). The product was obtained as colorless powder which was dried under vacuum. Yield 80% (1.04 g, m.p.: 118-120 °C, lit.: [3] 121-122 °C), TLC Rₑ [silica, EtOAc/hexanes 1:4] = 0.22 and 0.136. The product showed distinct formation of cis/trans isomers. ¹H-NMR (300 MHz, CDCl₃): isomer 1: δ_H [ppm] = 9.64 (s, 1H), 7.88 (dt, 2H), 7.70 – 7.60 (m, 1H), 7.51 (t, 2H), 4.29 (q, J = 7.1 MHz, 2H), 1.23 (t, J = 7.1 MHz, 3H) isomer 2: δ_H [ppm] = 10.40 (s, 1H), 8.02 – 7.96 (m, 2H), 7.58 (dt, 1H), 7.48 – 7.41 (m, 2H), 4.39 (t, J = 7.1 MHz, 2H), 1.34 (t, J = 7.1 MHz, 3H). ¹³C-NMR (75 MHz, CDCl₃): δ_C [ppm] = 190.0, 149.8, 130.5, 129.3, 129.2, 128.6, 62.8, 14.0.

3-(Hydroxyimino)pentane-2,4-dione (1k)

Oxime 1k was obtained from pentane-2,4-dione according to general procedure B. The product was purified via flash chromatography (EtOAc/hexanes = 1:3, Rₑ = 0.22) to yield the title compound as a white crystalline solid (1.51 g, 59%); m.p.: 74-75°C, lit.: [4] 73-75°C. ¹H-NMR (300 MHz, CDCl₃): δ_H [ppm] = 10.2 (s, 1H), 2.42 (d, J = 3.0 Hz, 6H). ¹³C-NMR (75 MHz, CDCl₃): δ_C [ppm] = 199.6, 195.5, 155.5, 30.8, 25.9.

3-(Hydroxyimino)-6-methylheptane-2,4-dione (1l)

Oxime 1l was obtained from 6-methylheptan-2,4-dione via general procedure A as a bright yellow oil. The product was purified via flash chromatography (EtOAc/hexanes = 1:5), TLC Rₑ = 0.22 (silica, EtOAc/hexanes 1:5). ¹H-NMR (300 MHz, CDCl₃): δ_H [ppm] = 2.66 (d, J = 6.7 MHz, 2H), 2.40 (d, 3H), 2.20 (dpp, J = 6.7 MHz, 1H), 0.95 (dd, J = 6.7 MHz, 6H), ¹³C-NMR (75 MHz, CHCl₃): δ_C [ppm] = 201.8, 199.9, 197.8, 156.3, 52.3, 47.0, 31.0, 26.0, 25.1, 23.6, 22.7; HR-MS: m/z = 170.0822 [M-H]- (calcd. 170.08227).

2-(Hydroxyimino)-1-phenylbutane-1,3-dione (1m)

Oxime 1m was obtained from 1-phenylbutane-1,3-dione according to general procedure A. The precipitate was filtered off, washed with ice-cold H₂O (2 x 10 mL) and dried under reduced pressure. Yield 71% (834 mg, m.p.: 118-121 °C, lit.: [5] 127-128.5 °C), TLC Rₑ = 0.22 (silica, EtOAc/hexanes 1:4), ¹H-NMR (300 MHz, acetone-d₆): δ_H [ppm] = 11.95 (s, 1H), 7.87 – 7.81 (m, 2H), 7.73 – 7.66 (m, 1H), ¹³C-NMR (75 MHz, CDCl₃): δ_C [ppm] = 195.8, 193.2, 156.9, 135.8, 135.2, 129.9, 129.5, 25.7.

2-(Hydroxyimino)-5,5-dimethylcyclohexane-1,3-dione (1n)

Oxime 1n was obtained from 5,5-dimethylcyclohexane-1,3-dione according to general procedure C. The product was obtained as brown solid and used without further purification. Yield 71% (1.18 g, m.p.: 78-80 °C, lit.: [4] 85-88°C). ¹H-NMR (300 MHz, CDCl₃): δ_H [ppm] = 2.69 (d, J = 6.2 Hz, 4H), 1.14 (s, 6H). ¹³C-NMR (75 MHz, CDCl₃): δ_C [ppm] = 191.9, 191.4, 145.8, 52.6, 52.3, 30.4, 28.5.

Preparative scale biotransformations

Preparative scale biotransformations of pyrazines, general procedure:

Lyophilized E. coli cells (1.5 g) containing the overexpressed ene reductase (OYE-2 for 1a, FOYE for 1b, 1c, and 1g, OYE-3 for 1d, 1e and 1f, XenA for 1h) were rehydrated in phosphate buffer (190 mL, 50 mM, pH 7.5) in a 500 mL Erlenmeyer flask for 30 min at 30°C, 120 rpm. After that time NADPH (80 mg, final concentration: 0.5 mM), glucose (2 g, final concentration: 50 mM), GDH (400 mg) and the substrate (2 mmol in 10 mL DMSO (5% (v/v)) were added and the reactions were shaken at 30 °C, 120 rpm for 24 h. Afterwards, the suspension was extracted with ethyl acetate (4x 50 mL) and the combined organic layers dried over Na₂SO₄. The solvent was removed under reduced pressure to yield the crude pyrazine products which were subjected to further purification.
Diethyl 3,6-dimethylpyrazine-2,5-dicarboxylate (2a)
Pyrazine 2a was purified via flash chromatography (EtOAc/cyclohexane = 1:3, \( R_f = 0.32 \)) and obtained as colorless solid (155 mg, 61%); m. p.: 84-86 °C, lit.\[6\] 83 °C. \(^1\)H-NMR (300 MHz, CDCl\(_3\)): \( \delta_H [ppm]= \) 4.50 (q, \( J = 7.1, 4H \)), 2.81 (s, 6H), 1.45 (t, \( J = 7.1 \) Hz, 6H). \(^{13}\)C-NMR (75 MHz, CDCl\(_3\)): \( \delta_C [ppm]= \) 165.1, 150.8, 144.1, 62.4, 22.3, 14.2.

Diallyl 3,6-dimethylpyrazine-2,5-dicarboxylate (2b)
Pyrazine 2b was purified via flash chromatography (EtOAc/cyclohexane = 1:4, \( R_f = 0.39 \)) and obtained as light yellow solid (117 mg, 42%); m. p.: 58-59 °C. \(^1\)H-NMR (300 MHz, CDCl\(_3\)): \( \delta_H [ppm]= \) 6.08 (m, 2H), 5.47 (ddd, \( J = 17.2, 2.8, 1.4 \) Hz, 2H), 5.36 (ddd, \( J = 10.4, 2.3, 1.1 \) Hz, 2H), 4.93 (dt, \( J = 5.9, 1.3 \) Hz, 4H), 2.82 (s, 6H). \(^{13}\)C-NMR (75 MHz, CDCl\(_3\)): \( \delta_C [ppm]= \) 164.7, 151.0, 143.9, 131.3, 119.7, 66.9, 22.4; HR-MS: \( m/z = 277.1181 [M+H]^+ \) (calcd. 277.1183).

Bis(2-methoxyethyl) 3,6-dimethylpyrazine-2,5-dicarboxylate (2c)
Pyrazine 2c was purified via flash chromatography (EtOAc/cyclohexane = 1:1, \( R_f = 0.30 \)) and obtained as colorless solid (164 mg, 53%); m. p.: 66-67 °C. \(^1\)H-NMR (300 MHz, CDCl\(_3\)): \( \delta_H [ppm]= \) 4.60 - 4.52 (m, 4H), 3.80 - 3.71 (m, 4H), 3.41 (s, 6H), 2.78 (s, 6H). \(^{13}\)C-NMR (75 MHz, CDCl\(_3\)): \( \delta_C [ppm]= \) 164.7, 151.0, 143.9, 70.1, 65.0, 59.0, 22.2; HR-MS: \( m/z = 313.1393 [M+H]^+ \) (calcd. 313.1394).

Dibutyl 3,6-dimethylpyrazine-2,5-dicarboxylate (2d)
Pyrazine 2d was purified via flash chromatography (EtOAc/cyclohexane = 1:5, \( R_f = 0.35 \)) and obtained as colorless solid (190 mg, 62%); m. p.: 67-68 °C. \(^1\)H-NMR (300 MHz, CDCl\(_3\)): \( \delta_H [ppm]= \) 4.42 (t, \( J = 6.8 \) Hz, 4H), 2.68 (s, 6H), 1.85 - 1.72 (m, 4H), 1.54 - 1.39 (m, 4H), 0.97 (t, \( J = 7.4 \) Hz, 6H). \(^{13}\)C-NMR (75 MHz, CDCl\(_3\)): \( \delta_C [ppm]= \) 165.2, 150.6, 144.2, 66.2, 30.6, 22.3, 19.1, 13.7; HR-MS: \( m/z = 309.1809 [M+H]^+ \) (calcd. 309.1809).

Diheptyl 3,6-dimethylpyrazine-2,5-dicarboxylate (2e)
Pyrazine 2e was purified via flash chromatography (EtOAc/cyclohexane = 1:5, \( R_f = 0.33 \)) and obtained as light yellow solid (223 mg, 57%); m. p.: 55-56 °C. \(^1\)H-NMR (300 MHz, CDCl\(_3\)): \( \delta_H [ppm]= \) 4.41 (t, \( J = 6.9 \) Hz, 4H), 2.78 (s, 6H), 1.89 - 1.71 (m, 4H), 1.50 - 1.19 (m, 17H), 0.88 (dd, \( J = 8.8, 4.9 \) Hz, 6H). \(^{13}\)C-NMR (75 MHz, CDCl\(_3\)): \( \delta_C [ppm]= \) 164.7, 151.0, 143.9, 66.5, 31.7, 28.9, 25.8, 22.5, 22.3, 14.0; HR-MS: \( m/z = 393.2744 [M+H]^+ \) (calcd. 393.2748).

Dibenzyl 3,6-dimethylpyrazine-2,5-dicarboxylate (2f)
Pyrazine 2f was purified via flash chromatography (EtOAc/cyclohexane = 1:5, \( R_f = 0.29 \)) and obtained as colorless solid (176 mg, 50%); m. p.: 106-107 °C, lit.:\[7\] 108 °C. \(^1\)H-NMR (300 MHz, CDCl\(_3\)): \( \delta_H [ppm]= \) 7.52 - 7.33 (m, 10H), 5.45 (s, 4H), 2.76 (s, 6H). \(^{13}\)C-NMR (75 MHz, CDCl\(_3\)): \( \delta_C [ppm]= \) 164.7, 151.0, 143.9, 135.1, 128.7, 128.6, 128.5, 67.9, 22.3; HR-MS: \( m/z = 377.1496 [M+H]^+ \) (calcd. 377.1496).

3,6-Dimethyl-N,N-diphenylpyrazine-2,5-dicarboxamide (2g)
Pyrazine 2g was purified via flash chromatography (EtOAc/cyclohexane = 1:3, \( R_f = 0.48 \)) and obtained as yellow crystals (121 mg, 35%); m. p.: 225-228 °C. \(^1\)H-NMR (300 MHz, pyridine): \( \delta_H [ppm]= \) 11.08 (s, 2H), 7.45 (t, \( J = 7.9 \) Hz, 4H), 7.21 (dd, \( J = 10.6, 4.2 \) Hz, 3H), 2.90 (s, 6H). \(^{13}\)C-NMR (75 MHz, pyridine): \( \delta_C [ppm]= \) 164.1, 145.4, 140.2, 130.3, 125.6, 121.8, 23.4.

Diethyl 3,6-diethylpyrazine-2,5-dicarboxylate (2h)
Pyrazine 2h was purified via flash chromatography (EtOAc/cyclohexane = 1:6, \( R_f = 0.40 \)) and obtained as colorless solid (110 mg, 39%); m. p.: 62-63 °C. \(^1\)H-NMR (300 MHz, CDCl\(_3\)): \( \delta_H [ppm]= \) 4.48 (q, \( J = 7.1 \) Hz, 4H), 3.06 (q, \( J = 7.5 \) Hz, 4H), 1.43 (t, \( J = 7.1 \) Hz, 6H), 1.31 (t, \( J = 7.5 \) Hz, 6H). \(^{13}\)C-NMR (75 MHz, CDCl\(_3\)): \( \delta_C [ppm]= \) 165.4, 154.6, 144.5, 62.3, 28.1, 14.2, 13.6; HR-MS: \( m/z = 281.1496 [M+H]^+ \) (calcd. 281.1496).
Synthesis of liguzinediol

Pyrazine ester 2d (204 mg, 0.66 mmol, 1.0 eq.) was added to a 100 mL round bottom flask and dissolved in MeOH. Sodium methoxide (1.8 mg, 5 mol%) was added and the reaction was stirred at room temperature for 5 min. Sodium borohydride (150 mg, 4.0 mmol, 6.0 eq.) was added at once to the reaction and the mixture was stirred at room temperature for 5 h. Excess metal hydride was quenched with water and the mixture was extracted with ethyl acetate (4x 15 mL). The combined organic phases were dried over Na₂SO₄ and the solvent was removed under reduced pressure. The crude product was purified via flash chromatography (CHCl₃/MeOH = 10:1) to obtain the title compound as orange oil (80 mg, 72%).

1H-NMR (300 MHz, CDCl₃): δH [ppm] =4.43 (s, 4H), 4.22 (s, 2H), 2.47 (s, 6H). 13C-NMR (75 MHz, CDCl₃): δC [ppm]= 148.9, 146.1, 61.0, 19.2.

Synthesis of the N-benzoyl threonine ethyl ester isomers

N-Benzoyl-L-threonine
A solution of L-threonine (1.19 g; 10 mmol, 1 eq) in aqueous NaOH (1 M, 21 mL; 21 mmol; 2.1 eq) was cooled to 0 °C and a solution of benzoyl chloride (1.40 mL; 12 mmol; 1.2 eq) in 1,4-dioxane (10 mL) was carefully added. After thirty minutes, the cooling was removed and the mixture was stirred at room temperature overnight. Then, the 1,4-dioxane was removed under reduced pressure (40 °C) and, when needed, the pH of the solution was adjusted to approx. pH 1 using concentrated hydrochloric acid. The suspension was extracted with EtOAc (3x 25 mL), the combined organic layers dried over Na₂SO₄, filtered and solvent was removed under reduced pressure (40 °C). The obtained white solids were stirred in cyclohexane (40 mL) for thirty minutes to dissolve any residual benzoic acid and then filtered. The residue was triturated with pentane to obtain white solids (1.67 g; 7.5 mmol; 75%). 1H NMR (300 MHz, Methanol-d₄) δ 7.99 – 7.87 (m, 2H), 7.65 – 7.44 (m, 3H), 4.69 (d, J = 3.1 Hz, 1H), 4.45 (qd, J = 6.4, 3.1 Hz, 1H), 1.28 (d, J = 6.5 Hz, 3H). 13C NMR (75 MHz, Methanol-d₄) δ 172.39, 169.08, 133.80, 131.62, 128.27, 127.01, 67.24, 58.25, 19.26.

N-Benzoyl-D-threonine
Same procedure as above leading to a white solid (1.70 g; 7.6 mmol; 76%). 1H NMR (300 MHz, Methanol-d₄) δ 7.91 (dt, J = 7.0, 1.5 Hz, 1H), 7.69 – 7.40 (m, 2H), 4.69 (d, J = 3.1 Hz, 1H), 4.45 (qd, J = 6.4, 3.1 Hz, 1H), 1.28 (d, J = 6.4 Hz, 2H). 13C NMR (75 MHz, Methanol-d₄) δ 172.40, 169.09, 133.80, 131.63, 128.27, 127.02, 67.25, 58.25, 19.27.

N-Benzoyl-L-allothreonine
A solution of L-allo-threonine (250 mg; 2.1 mmol, 1 eq) in 1 M aqueous NaOH (4.4 mL; 4.4 mmol; 2.1 eq) was cooled to 0 °C and a solution of benzoyl chloride (0.3 mL; 2.52 mmol; 1.2 eq) in 1,4-dioxane (2 mL) was carefully added. After thirty minutes, the cooling was removed and the mixture was stirred at room temperature overnight. Then, the 1,4-dioxane was removed under reduced pressure (40 °C) and, when needed, the pH of the solution was adjusted to approx. pH 1 using concentrated hydrochloric acid. The suspension was extracted with EtOAc (3x 10 mL), the combined organic layers dried over Na₂SO₄, filtered and solvent was removed under reduced pressure (40 °C). The obtained oil was treated with cyclohexane, but contained too much water to precipitate the pure product. After removing the solvent under reduced pressure (40 °C), the oil was redissolved in EtOAc (50 mL) and dried over a substantial amount of Na₂SO₄, giving 760 mg of solid crude product after removing the solvent. This crude product was used without further purification or analysis.

N-Benzoyl-D-allothreonine
A solution of D-allo-threonine (100 mg; 0.84 mmol, 1 eq) in 1 M aqueous NaOH (1.76 mL; 1.76 mmol; 2.1 eq) was cooled to 0 °C and a solution of benzoyl chloride (117 μL; 1.0 mmol; 1.2 eq) in 1,4-dioxane (1 mL) was carefully added. After thirty minutes, the cooling was removed and the mixture was stirred at room temperature overnight. Then, the 1,4-dioxane was removed under reduced pressure (40 °C) and,
when needed, the pH of the solution was adjusted to approx. pH 1 using concentrated hydrochloric acid. The suspension was extracted with EtOAc (3x 5 mL), the combined organic layers dried over Na₂SO₄, filtered and solvent was removed under reduced pressure (40 °C). The obtained oil was treated with cyclohexane, stirred overnight and then filtered. The white sticky residue was recovered from the filter by dissolving it in MeOH to yield 158 mg of crude product as a yellow/brown oil, which was used without further purification or analysis.

The synthesis of the ester was performed according to a protocol from literature.[9]

**N-Benzoyl-L-threonine ethyl ester**

To a solution of the carboxylic acid (893 mg; 4.0 mmol; 1.0 eq) in anhydrous DMF (22 mL) was added K₂CO₃ (1.55 g; 11.2 mmol; 2.8 eq) and ethyl iodide (900 μL; 11.2 mmol; 2.8 eq). The suspension was flushed with argon for at least fifteen minutes, and the reaction was stirred at room temperature overnight. The mixture was filtered, the filtrate poured into water (80 mL) and extracted with EtOAc (3x 100 mL). The combined organic layers were washed with brine (2x 100 mL) and dried over Na₂SO₄, and solvent was removed under reduced pressure (40 °C) to yield 1.57 grams of crude product. The mixture was purified by column chromatography (SiO₂; EtOAc/cyclohexane 1:3, Rf 0.1) to yield the desired product as a yellow oil that crystallised upon standing in 75% yield (758 mg; 3.02 mmol). 1H NMR (300 MHz, Chloroform-d) δ 7.96 – 7.78 (m, 2H), 7.56 – 7.47 (m, 1H), 7.42 (dd, J = 8.2, 6.5 Hz, 2H), 7.11 (d, J = 8.8 Hz, 1H), 4.80 (dd, J = 8.8, 2.5 Hz, 1H), 4.44 (qd, J = 6.4, 2.5 Hz, 1H), 4.23 (q, J = 7.1 Hz, 2H), 2.92 (s, 1H), 1.29 (t, J = 7.0 Hz, 6H). 13C NMR (75 MHz, Chloroform-d) δ 171.16, 168.07, 133.74, 131.88, 128.59, 127.23, 68.27, 61.80, 57.82, 20.10, 14.14.

**N-Benzoyl-D-threonine ethyl ester**

To a solution of the carboxylic acid (893 mg; 4.0 mmol; 1.0 eq) in anhydrous DMF (22 mL) was added K₂CO₃ (1.55 g; 11.2 mmol; 2.8 eq) and ethyl iodide (900 μL; 11.2 mmol; 2.8 eq). The suspension was flushed with argon for at least fifteen minutes, and the reaction was stirred at room temperature overnight. The mixture was filtered, the filtrate poured into water (80 mL) and extracted with EtOAc (3x 100 mL). The combined organic layers were washed with brine (2x 100 mL) and dried over Na₂SO₄, and solvent was removed under reduced pressure (40 °C) to yield 3.38 grams of crude product. The mixture was purified by column chromatography (SiO₂; EtOAc/cyclohexane 1:2 to 1:1, Rf 0.1) to yield the desired product as a yellow oil that crystallised upon standing in 72% yield (720 mg; 2.78 mmol). 1H NMR (300 MHz, Chloroform-d) δ 7.90 – 7.81 (m, 2H), 7.57 – 7.47 (m, 1H), 7.43 (dd, J = 8.2, 6.5 Hz, 2H), 7.10 (d, J = 8.8 Hz, 1H), 4.80 (dd, J = 8.8, 2.6 Hz, 1H), 4.44 (qd, J = 6.4, 2.6 Hz, 1H), 4.23 (q, J = 7.1 Hz, 2H), 2.92 (s, 1H), 1.36 – 1.22 (m, 6H). 13C NMR (75 MHz, Chloroform-d) δ 171.20, 168.03, 133.79, 131.85, 128.58, 127.24, 68.25, 61.77, 57.84, 20.12, 14.14.

**N-Benzoyl-L-allo-threonine ethyl ester**

To a solution of the crude carboxylic acid (760 mg) in anhydrous DMF (11 mL) was added K₂CO₃ (1.55 g; 11.2 mmol; 2.8 eq) and ethyl iodide (900 μL; 11.2 mmol; 2.8 eq). The suspension was flushed with argon for at least fifteen minutes, and the reaction was stirred at room temperature overnight. The mixture was filtered, the filtrate poured into water/brine 1:1 (60 mL) and extracted with EtOAc (3x 60 mL). The combined organic layers were washed with brine (2x 100 mL), dried over Na₂SO₄, and solvent was removed under reduced pressure (40 °C) to yield 590 mg of crude product. The mixture was purified by column chromatography (SiO₂; EtOAc/cyclohexane 1:2, Rf 0.18) to yield the desired product as a yellow oil that crystallised upon standing in 18% yield over two steps (94 mg; 0.374 mmol). 1H NMR (300 MHz, Chloroform-d) δ 7.90 – 7.79 (m, 2H), 7.59 – 7.49 (m, 1H), 7.45 (dd, J = 8.2, 6.5 Hz, 2H), 7.24 (d, J = 7.1 Hz, 1H), 4.86 (dd, J = 8.2, 6.5 Hz, 2H), 4.40 – 4.19 (m, 3H), 1.32 (t, J = 7.1 Hz, 3H), 1.24 (d, J = 6.4 Hz, 3H). 13C NMR (75 MHz, Chloroform-d) δ 170.33, 168.26, 133.31, 132.08, 128.65, 127.25, 69.26, 62.07, 58.88, 18.79, 14.16.

**N-Benzoyl-D-allo-threonine ethyl ester**

To a solution of the crude carboxylic acid (760 mg) in anhydrous DMF (11 mL) was added K₂CO₃ (813 mg; 5.88 mmol; 2.8 eq) and ethyl iodide (473 μL; 5.88 mmol; 2.8 eq). The suspension was flushed with argon for at least fifteen minutes, and the reaction was stirred at room temperature overnight. The mixture was filtered, the filtrate poured into water/brine 1:1 (60 mL) and extracted with EtOAc (3x 60 mL). The combined organic layers were washed with brine (2x 100 mL), dried over Na₂SO₄, and solvent was removed under reduced pressure (40 °C) to yield 590 mg of crude product. The mixture was purified by column chromatography (SiO₂; EtOAc/cyclohexane 1:2, Rf 0.18) to yield the desired product as a yellow oil that crystallised upon standing in 18% yield over two steps (94 mg; 0.374 mmol). 1H NMR (300 MHz, Chloroform-d) δ 7.90 – 7.79 (m, 2H), 7.59 – 7.49 (m, 1H), 7.45 (dd, J = 8.2, 6.5 Hz, 2H), 7.24 (d, J = 7.1 Hz, 1H), 4.86 (dd, J = 8.2, 6.5 Hz, 1H), 4.40 – 4.19 (m, 3H), 1.32 (t, J = 7.1 Hz, 3H), 1.24 (d, J = 6.4 Hz, 3H). 13C NMR (75 MHz, Chloroform-d) δ 170.33, 168.26, 133.31, 132.08, 128.65, 127.25, 69.26, 62.07, 58.88, 18.79, 14.16.

**N-Benzoyl-D-allo-threonine ethyl ester**

To a solution of the crude carboxylic acid (158 mg) in anhydrous DMF (5 mL) was added K₂CO₃ (325 mg; 2.35 mmol; 2.8 eq) and ethyl iodide (189 μL; 2.35 mmol; 2.8 eq). The suspension was flushed with argon for at least fifteen minutes, and the reaction was stirred at room temperature overnight. The mixture was filtered, the filtrate poured into water/brine 1:1 (30 mL) and extracted with EtOAc (3x 30 mL). The combined organic layers were washed with brine (2x 50 mL), dried over Na₂SO₄, and solvent was removed under reduced pressure (40 °C) to yield 124 mg of crude product. The mixture was purified by
column chromatography (SiO2; EtOAc/cyclohexane 1:2, Rf 0.18) to yield the desired product as a yellow oil that crystallised upon standing in 10% yield over two steps (23 mg; 0.091 mmol). 1H NMR (300 MHz, Chloroform-d) δ 7.90 – 7.81 (m, 2H), 7.60 – 7.50 (m, 1H), 7.50 – 7.41 (m, 2H), 7.21 (d, J = 6.9 Hz, 1H), 4.87 (dd, J = 7.0, 3.2 Hz, 1H), 4.30 (pd, J = 6.8, 2.5 Hz, 3H), 2.97 (s, 2H), 1.34 (t, J = 7.2 Hz, 3H), 1.24 (d, J = 6.5 Hz, 3H). 13C NMR (75 MHz, Chloroform-d) δ 170.30, 168.30, 133.29, 132.11, 128.68, 127.24, 69.33, 62.11, 58.91, 18.75, 14.17.

Synthesis of ethyl 3-hydroxy-2-(hydroxyimino)butanoate
A solution of oxime 1a (318 mg; 2.0 mmol; 1.0 eq) in absolute ethanol (10 mL) was cooled to 0 °C and sodium borohydride (76 mg; 4.0 mmol; 2.0 eq) was added in one portion. The bubbling solution quickly turned white and then lightly yellow. TLC analysis (SiO2, EtOAc/Cy 1:1, Rf alcohol 0.33) showed full consumption of the oxime within ten minutes, and only one product formed. The reaction mixture was quenched by addition of water (5 mL) and then 1 M HCl (aqueous, 5 mL) while still cooled to 0 °C. The mixture was extracted with EtOAc (3x 25 mL), the combined organic layers dried over Na2SO4, filtered and solvent was removed under reduced pressure to yield the pure oxime alcohol in 50 % yield (161 mg; 1.0 mmol) as a slightly yellow oil. 1H NMR (300 MHz, Chloroform-d) δ 5.14 (q, J = 6.8 Hz, 1H), 4.32 (q, J = 7.2 Hz, 2H), 1.48 (d, J = 6.8 Hz, 3H), 1.35 (t, J = 7.1 Hz, 4H). 13C NMR (75 MHz, Chloroform-d) δ 163.03, 153.28, 63.25, 62.14, 20.79, 13.94. 13C NMR (75 MHz, Chloroform-d) δ 163.03, 153.28, 63.25, 62.17 (d, J = 4.1 Hz), 20.79, 13.94.

Gene synthesis, subcloning and protein expression

The gene sequence encoding the investigated FOYE was obtained from the Genbank database entry associated with the corresponding protein sequence entry in the UniProt database. Synthetic genes were obtained from Invitrogen as linear double-stranded strings optimized for expression in E. coli containing an Ndel and XhoI restriction site on the 5’ and the 3’ end, respectively for subcloning into a pET28a(+) vector. Successful insertion of the target gene into the vector was confirmed by DNA sequencing. The protein of interest was expressed in E. coli BL21(DE3).

Protein expression and Protein Purification of FOYE

The expression plasmid of FOYE was transformed into chemically competent E. coli BL21(DE3) cells following the supplier's manual. A single colony of the transformation plate was used for an over-night culture (10 mL LB medium, 50 µg/mL kanamycin) which was incubated at 30 °C, 120 rpm in a shaking incubator. 2 mL of the over-night culture were used on the next day to inoculate the main culture (300 mL LB medium, 50 µg/mL kanamycin). The resulting culture was incubated at 37 °C, 120 rpm until an OD600 of around 0.6-0.8 was reached (3-4 hours). Protein expression was then induced with isopropyl-β-D-thiogalactopyranosid (IPTG) at a final concentration of 0.05 mM for 18 h at 25°C and 120 rpm. Cells were harvested via centrifugation (5000 rpm, 4 °C, 20 min) and the supernatant discarded. The pellet was resuspended in 100 mM Tris-HCl buffer, pH 7.5 and transferred to a 50 mL plastic tube. The suspension was centrifuged again (5000 rpm, 4 °C, 20 min) and the pellet stored at -20 °C until further use.

The cell pellet was thawed and resuspended in HisTrap buffer A (10 mL/g cell weight, Tris-HCl buffer, 100 mM, pH 7.5, 50 mM imidazole) supplemented with 1 mg/mL lysozyme and 1 mg FMN for cell disruption. This suspension was incubated at 30 °C, 120 rpm for 45 min and then cooled to 0 °C in an ice-water bath. Cell disruption was performed by ultrasonication using a Branson Digital Sonifier 250 at 30% amplitude, 2 s pulse, 4 s pause, for a total pulse time of 3 min (90 cycles). Cell debris was removed by centrifugation (16000 rpm, 4 °C, 20 min) and the supernatant used for protein purification via immobilized metal affinity chromatography (IMAC) as described below.

A 5 mL HisTrap FF column was equilibrated with 10 column volumes (CV) of HisTrap buffer A (Tris-HCl buffer, 100 mM, pH 7.5, 50 mM imidazole). The supernatant obtained from the cell disruption was filtered through a 0.45 µm syringe filter prior to the application onto the column. Unbound proteins were removed from the HisTrap column by washing with 10 CV of HisTrap buffer A. The protein of interest was then eluted with 5-10 mL of HisTrap buffer B (Tris-HCl buffer, 100 mM, pH 7.5, 500 mM imidazole) and concentrated using a Sartorius VivaSpin 20 centrifugal filter with a molecular weight cut-off of 10 kDa.
The concentrated protein solution was desalted using a GE Healthcare PD 10 desalting column with Tris HCl buffer, 100 mM, pH 7.5 as the eluent. Protein concentration of the desalted sample was determined using the Biorad Bradford assay following the supplier’s instructions.

For exchange of the protein into phosphate buffer (50 mM, pH 7.5), the purified protein was loaded onto a PD10 column and eluted following the gravity protocol described by the supplier.

All other enzymes were expressed as reported previously.[10]

### Table S3: Ene-reductases investigated in this paper

| Enzyme | pEG Number | Literature |
|--------|------------|------------|
| OYE-1  | 360        | [11]       |
| OYE-2  | 361        | [12]       |
| OYE-3  | 362        | [12]       |
| OPR-3  | 367        | [13]       |
| XenA   | 145        | [14]       |
| FOYE   | 369        | [15]       |

**Figure S1.** SDS-PAGE of FOYE (lines 6-9). 5: Protein standard, 6: Purified fraction, 7: Supernatant, 8: Pellet, 9: Flow-through.

**Figure S2:** SDS-PAGE of ADH-A after heat-precipitation. 1: Protein standard, 2 and 3: ADH-A (in different amounts).
Analytics

The retention times of various products analysed on GC-FID can be found in Table S4.

| Compound | Method            | Retention time (min) |
|----------|-------------------|----------------------|
| Pyrazine 2a | Standard          | 11.1                 |
| Pyrazine 2b | Standard          | 12.8                 |
| Pyrazine 2c | Standard          | 15.2                 |
| Pyrazine 2d | Highboilers       | 6.2                  |
| Pyrazine 2f | Highboilers       | 13.8                 |
| Pyrazine 2g | Highboilers       | 17.2                 |
| Pyrazine 2h | Standard          | 20.2                 |
| Oxime 1h  | Standard (increased injection volume and split ratio) | 11.9 |
| Tetramethylpyrazine (IS) | Standard          | 3.8                  |
| Tetramethylpyrazine (IS) | Highboilers       | 1.5                  |

Retention times of the compounds analysed after cascade reactions can be found in Table S5. For the HPLC parameters, see the General section.

| Compound                      | Structure                        | Retention time (min) |
|-------------------------------|----------------------------------|----------------------|
| Oxime 1a (substrate)          | ![Structure](image)               | 8.03                 |
| Pyrazine 2a (product from non-cascade pathway) | ![Structure](image)               | 8.89                 |
| Acetanilide (internal standard) | ![Structure](image)               | 13.9                 |
| N-benzoyl-L-threonine ethyl ester (L-5) | ![Structure](image)               | 15.1                 |
| N-benzoyl-D-threonine ethyl ester (D-5) | ![Structure](image)               | 19.0                 |
| N-benzoyl-L-allothreonine ethyl ester (L-allo-5) | ![Structure](image)               | 22.2                 |
A chromatogram showing the pyrazine, internal standard and all four enantiomers of the threonine product is shown in Figure S3. NB: an peak due to ethyl acetate is present at 4.25 min.

Figure S3: HPLC-UV chromatogram of pyrazine 2a, acetanilide (IS) and the four stereoisomers of derivatized threonine 5.

Figure S4: HPLC-UV chromatogram of cascade biotransformation (using OYE3 and ADH-A), showing the internal standard (13.6 min), D-5 (18.9 min, confirmed also by spiking). Unidentified side product at 23.4 min.
NMR and MS spectra

Figure S5: $^1$H-NMR of oxime 1a.

Figure S6: $^{13}$C-NMR of oxime 1a.
Figure S7: $^1$H-NMR of oxime 1b.

Figure S8: $^{13}$C-NMR of oxime 1b.
Figure S9: $^1$H-NMR of oxime 1c.

Figure S10: $^{13}$C-NMR of oxime 1c.
Figure S11: $^1$H-NMR of oxime 1d.

Figure S12: $^{13}$C-NMR of oxime 1d
Figure S13: $^1$H-NMR of oxime 1e.

Figure S14: $^{13}$C-NMR of oxime 1e.
Figure S15: $^1$H-NMR of oxime 1f.

Figure S16: $^{13}$C-NMR of oxime 1f.
Figure S17: $^1$H-NMR of oxime 1g.

Figure S18: $^{13}$C-NMR of oxime 1g.
Figure S19: $^1$H-NMR of oxime 1h.

Figure S20: $^{13}$C-NMR of oxime 1h.
Figure S21: $^1$H-NMR of oxime 1i.

Figure S22: $^{13}$C-NMR of oxime 1i.
Figure S23: $^1$H-NMR of oxime 1j.

Figure S24: $^{13}$C-NMR of oxime 1j.
Figure S25: $^1$H-NMR of oxime 1k.

Figure S26: $^{13}$C-NMR of oxime 1k.
Figure S27: $^1$H-NMR of oxime 1I.

Figure S28: $^{13}$C-NMR of oxime 1I.
Figure S29: $^1$H-NMR of oxime 1m.

Figure S30: $^{13}$C-NMR of oxime 1m.
Figure S31: $^1$H-NMR of oxime 1n.

Figure S32: $^{13}$C-NMR of oxime 1n.
Figure S33: $^1$H-NMR of pyrazine 2a.

Figure S34: $^{13}$C-NMR of pyrazine 2a.
Figure S35: $^1$H-NMR of pyrazine 2b.

Figure S36: $^{13}$C-NMR of pyrazine 2b.
Figure S37: $^1$H-NMR of pyrazine 2c.

Figure S38: $^{13}$C-NMR of pyrazine 2c.
Figure S39: $^1$H-NMR of pyrazine 2d.

Figure S40: $^{13}$C-NMR of pyrazine 2d.
Figure S41: $^1$H-NMR of pyrazine 2e.

Figure S42: $^{13}$C-NMR of pyrazine 2e.
Figure S43: $^1$H-NMR of pyrazine 2f.

Figure S44: $^{13}$C-NMR of pyrazine 2f.
Figure S45: $^1$H-NMR of pyrazine 2g.

Figure S46: $^{13}$C-NMR of pyrazine 2g.
Figure S47: $^1$H-NMR of pyrazine 2h.

Figure S48: $^{13}$C-NMR of pyrazine 2h.
Figure S49: $^1$H-NMR of liguzinediol 5.

Figure S50: $^{13}$C-NMR of liguzinediol 5.
Figure S51: NMR of the biotransformation of oxime 1a. The red spectrum represents the start of the biotransformation, the turquoise spectrum the end of the measurement at 60 min (solvent: D$_2$O, conditions: 100 µg/mL ene reductase, 0.5 mM NADPH, 50 mM glucose, 1 mg GDH, 10 mM oxime).
Figure S52: $^1$H and $^{13}$C-NMR of N-benzoyl-L-threonine.
Figure S53: $^1$H and $^{13}$C-NMR of $N$-benzoyl-D-threonine.
Figure S54: $^1$H and $^{13}$C-NMR of $N$-benzoyl-L-threonine ethyl ester.
Figure S55: $^1$H and $^{13}$C-NMR of N-benzoyl-D-threoneine ethyl ester.
Figure S56: $^1$H and $^{13}$C-NMR of N-benzoyl-L-allo-threonine ethyl ester.
Figure S57: $^1$H and $^{13}$C-NMR of N-benzoyl-D-allo-threonine ethyl ester.
Figure S58: $^1$H and $^{13}$C-NMR of ethyl-3-hydroxy-2-(hydroxylimino)butanoate.
Figure S59: MS scan of the ADH-ER cascade (A) and the ER-mediated biotransformation (B).

Figure S60: Fragmentation spectrum of 4 of the ADH-ER cascade with assigned fragments.
Figure S61: GC-MS chromatogram of the ER-ADH cascade and mass spectrum of compound 4 (peak at 7.439 min) found in the reaction. In this case the intermediate was analyzed as N-acetyl derivative, therefore the masses of the fragments differ from the HR-MS measurement depicted in Figure S59.

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