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Stereoselective Enzyme Cascades: An Efficient Synthesis of Chiral γ-Butyrolactones

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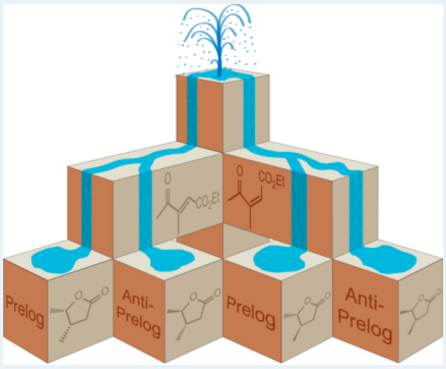
Supporting Information

ABSTRACT: A one-pot consecutive two-enzyme sequential cascade toward chiral γ-butyrolactones using an enoate reductase as well as alcohol dehydrogenases in combination with a glucose dehydrogenase is reported. In this scalable process, the products were obtained in high yield (up to 90%) and with perfect enantioselectivity (98→99% ee). The starting materials, ethyl 4-oxo-pent-2-enoates, are readily accessible via Wittig-type reactions. Furthermore, the stereoselectivity of the enoate reductase catalyzed reaction has been studied in detail, leading to deeper insights into the mechanism of this enzyme.

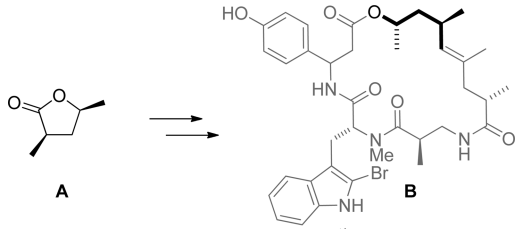


KEYWORDS: *enzymes, enantioselective catalysis, reaction cascades, reductases, lactones*

INTRODUCTION

Substituted γ-butyrolactones are a central motif in many natural compounds and chemical agents such as nephrosteranic acid or arctigenine.[1] In other cases, this moiety is used as a chiral building block. For example, Momose et al. used (2*R*,4*S*)-dimethyl butyrolactone **A** in the syntheses of geodiamolide (**B**) and jasplakinolide starting from (*S*)-propene oxide and propionic acid (Scheme 1). Alternatively, lactic acid from the chiral pool was used to form this particular lactone.[1i,2] The synthesis of (3*R*,4*S*)-dimethyl butyrolactone starting from (*S*)-lactic acid was carried out by Kang et al.[2a] The syntheses are very sophisticated and provided moderate to good yields, although a number of steps are necessary to obtain the butyrolactone. However, there was no concise general route available allowing access to various stereoisomers as well as different substitution patterns.

Scheme 1. Principal Approach towards Geodiamolide (B)[1h]



In general, cascade reactions have the potential to increase the yields of reaction sequences, because the number of tedious workup procedures is reduced. A number of processes have been presented in the recent literature.[3] A chemo-enzymatic approach in which ethyl 4-oxo-pent-2-enoate derivatives were sequentially reduced by an enoate reductase (ER), for example, YqjM, followed by an alcohol dehydrogenase (ADH) would be a viable alternative.[3b,4] This strategy combines the following advantages: Ethyl 4-oxo-pent-2-enoate derivatives are readily available via Wittig or Horner−Wadsworth−Emmons protocols, which provided access to a large number of derivatives. In addition, all stereoisomers of the particular butyrolactone were accessible either by selecting a complementary ADH or by substrate engineering. After our preliminary results, we herein report our new findings in detail.[5]

RESULTS AND DISCUSSION

Synthesis. The general outline of the synthetic approach is depicted in Scheme 2: For the anticipated synthesis of γ-butyrolactones **1**, α,β-unsaturated 4-oxo-ester **2** was required in diastereomerically pure form. They are readily accessible from commercially available starting materials via Wittig-type reactions. The following reduction with an enoate reductase proceeds via a formal antiaddition of hydrogen providing γ-ketoester **3**. Reduction of the ketone **3** with alcohol dehydrogenase provides the γ-hydroxyester **4** that yielded

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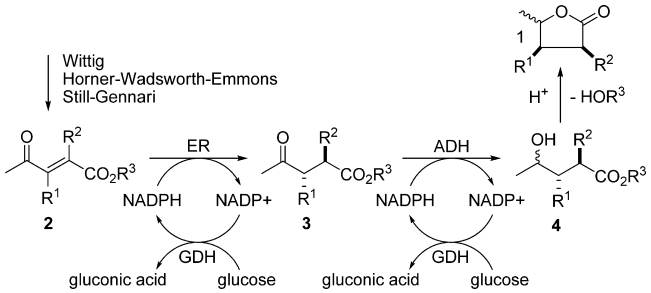
Scheme 2. General Route towards γ-Butyrolactones 1



lactone **1** upon acidification. The cofactor NADPH was recycled using a glucose dehydrogenase (GDH).

The unsaturated dicarbonyl compounds **2** (Scheme 3, Table 1) have been synthesized with good to very good yields

Scheme 3. Overview of the Dicarbonyl Substrate Library

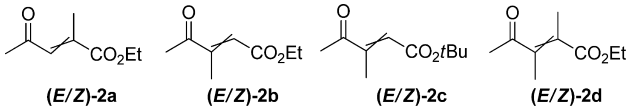


Table 1. Overview on the Syntheses of the Starting Materials

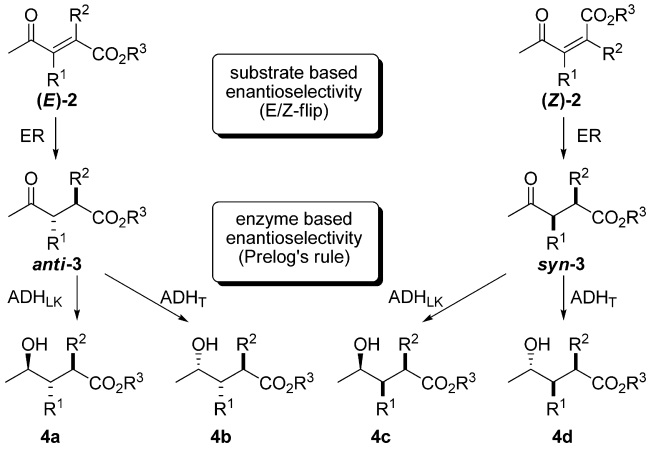
compound	method[a]	yields
2a	Wittig reaction	(*E*)-**2a** 86%; (*Z*)-**2a** 4%
2a	Still−Gennari reaction	(*E*)-**2a** 34%; (*Z*)-**2a** 56%
2a	photoisomerization [(*E*)-**2a**]	(*E*)-**2a** 20%; (*Z*)-**2a** 70%
2b	Wittig reaction	(*E*)-**2b** 86%; (*Z*)-**2b** 4%
2c	Wittig reaction	(*E*)-**2c** 74%; (*Z*)-**2c** 11%
2d	Wittig reaction	(*E*)-**2d** 46%; (*Z*)-**2d** 48%

[a]For reaction conditions, see the Experimental Section.

affording access to both diastereomers. Only isomer (*Z*)-**2a** having the methyl group in 3-position was poorly available via a Wittig reaction. Hence, the (*E*)-isomer (*E*)-**2a**, which can be obtained with very good yield, has been photoisomerized using a UV reactor with its major excitation wavelength of 350 nm. Interestingly, allowing the compound to reach its thermodynamic equilibrium under photoisomerization conditions, the (*Z*)-product (*Z*)-**2a** is the major isomer at ambient temperature (*Z*/*E* = 74:26) and thereby easily accessible as well. The route is especially convenient, because the isomerization was directly performed with the crude product of the Wittig reaction, omitting the purification step of the intermediate.

Enzymatic Conversions. As shown in Scheme 4, unsaturated dicarbonyl compounds can be reduced consecutively by an enoate reductase (ER) followed by an alcohol dehydrogenase (ADH).[6] Because an activation via the ketone moiety takes place in the ER for most of the shown substrates, this reaction must be carried out in the absence of ADH preventing a domino one-pot procedure: In this process, the ADH forms the alcohol first, which is no longer a substrate for the ER.[7] A solution for that problem might be the use of an ADH converting only the saturated ketone rather than the olefinic. In this work, we used commercially available or generic ADHs, making this method accessible for a bigger audience but accepting both the olefinic and the aliphatic compounds, respectively. However, due to this consecutive character, according to Garcia-Junceda,[3e] the presented reaction sequences are classified as one-pot consecutive two-enzyme sequential cascades.

Scheme 4. Concept of the Enantioselective Syntheses of the γ-Hydroxyesters 4



To incorporate flexibility in terms of stereoselectivity in the synthesis of butyrolactones, stereocomplementary ADHs have been used for the reduction of the ketone moiety. Excellent enzyme-based enantioselectivities with enantiomeric excesses greater than 99% were achieved with known ADHs from, for example, *Thermoanaerobacter sp.* (Prelog-ADH) or from *Lactobacillus brevis* (anti-Prelog-ADH).[8] For ERs, neither natural nor artificial general stereocomplementary enzymes are known to date, although several attempts have been carried out to achieve this goal.[3i,9] Nevertheless, stereocomplementarity can be obtained by the choice of the particular (*E*/*Z*)-diastereomer resulting in an inverted stereoselectivity (see Scheme 4).[10] Using this strategy, all stereoisomers of butyrolactones (**1**) resulting from monosubstituted substrates (**2a**−**c**) should be available, and even for the double substituted compounds (**2d**), four of the eight isomers should be accessible.

As depicted in Scheme 5, the olefins **2a**−**d** could be converted in good to very good yields exhibiting excellent enantiomeric excesses. This shows that the one-pot two-step sequential cascade is widely applicable yielding various stereoisomers of the butyrolactones **1a,b,d** as well as the linear *tert*-butyl-ester **4c**, which is not able to cyclize spontaneously. Nevertheless, obviously not all stereoisomers of **1a** and **1d** were accessible by this cascade. As reported previously, both olefins (*E*)-**2a** and (*Z*)-**2a** lead to the aliphatic compound (*R*)-**3a** upon ER reduction.[6,9b] To elucidate the particular binding mode of the substrate in the active site of the ER, reductions in deuterated buffer have been carried out (see Figure 1A−D). From the chemical point of view, the ketone moiety is the most electron-withdrawing group in compound **2a** rather than the ester moiety also juxtaposed to the double bond. Hence, the polarization of the double bond should be dominated by the ketone and enable a hydride transfer toward the 2-position forming the stereogenic center, and the reprotonation should occur in 3-position. In theory, the enzyme will enhance this polarization by forming hydrogen bridges from two histidine (in YqjM: H164 and H167) to the ketone. The deuteration experiments revealed unequivocally that the reduction of olefin (*E*)-**2a** takes place via this route.

Furthermore, in silico docking studies between YqjM and compound (*E*)-**2a** revealed a binding mode that explains both

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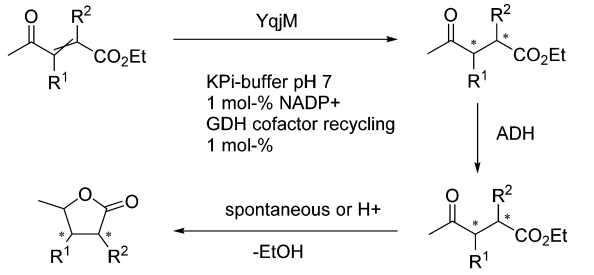
Scheme 5. Results of the Two-Step One-Pot Reductions[a]



starting material	ADH	product	yield ee
(*E*)-**2a**	ADH_T (P)	(2*R*,4*S*)-**1a**	90% >99%
(*E*)-**2a**	ADH_{LK} (AP)	(2*R*,4*R*)-**1a**	80% >99%
(E)-**2b**	evo 1.1.030 (P)	(3*S*,4*S*)-**1b**	82% >99%
(*E*)-**2b**	ADH_{LK} (AP)	(3*S*,4*R*)-**1b**	90% >99%
(*Z*)-**2b**	evo 1.1.030 (P)	(3*R*,4*S*)-**1b**	63% 98%
(*Z*)-**2b**	ADH_{LK} (AP)	(3*R*,4*R*)-**1b**	50% 98%
(*E*)-**2c**	evo 1.1.030 (P)	(3*S*,4*S*)-**4c**	75% >99%
(*E*)-**2c**	ADH_{LK} (AP)	(3*S*,4*R*)-**4c**	85% >99%
(*E*)-**2d**	ADH_T (P)	(2*R*,3*S*,4*S*)-**1d**	70% >99%
(*E*)-**2d**	ADH_{LB} (AP)	(2*R*,3*S*,4*R*)-**1d**	62% >99% de 98% with (2*R*,3*S*,4*R*)-**6**

[a]Conversions of the (*Z*)-compounds are only shown explicitly for compound (*Z*)-**2b**. Other (*Z*)-compounds are either not converted or yielded the same enantiomer as the respective (*E*)-diastereomer (compare Schemes 6 and 7). Prelog-ADHs are indicated by P, whereas AP refers to as anti-Prelog rule ADHs. Enantiomeric excesses have been determined using HPLC, and diastereomers have not been detected unless stated otherwise.

the results of the deuteration experiment and the obtained configuration at the formed stereogenic center (see Figure 1E). In this binding mode, the substrate forms the two proposed hydrogen bridges to H164 and H167. In contrast, the olefin (*Z*)-**2a** is activated by the ester moiety. Here, the stereogenic center is formed by protonation rather than a hydride attack. As shown in Figure 1F, in compound (*Z*)-**2a**, the ester forms the particular hydrogen bridges of the histidines. Upon this binding, the polarization effects toward the double bond are increased, dominating the polarization of the ketone. This effect can be understood as a special form of an umpolung. Because the activated polarization has to oppose the strong ketone activation, this explains why compound (*Z*)-**2a** is converted slower than the diastereomer (*E*)-**2a** (data not shown). The consequences for the stereoselectivity is a double flip: on the one hand, the substrate inherent flip of the double bond using olefin (*Z*)-**2a** rather than (*E*)-**2a**, and on the other hand, a reorientation by 180° of the substrate in the active site. In sum, this leads to the same configuration in the reduction product.

Although the ketone activation is blocked for (*Z*)-**2a** having a methyl group in 2-position, an alternative binding mode via the ester was possible. For the double-methylated substrate **2d**, this is not true. Although compound (*E*)-**2d** is converted entirely by the ER with an ee >99%, the (*Z*)-diastereomer is not accepted as substrate at all. We assume a steric hindrance between the *β*-methyl group (relative to the activated carbonyl moiety) and C26 in the active site, which can be avoided by the above-mentioned carbonyl change in activation. The conversion of olefin (*Z*)-**2d** is not possible, because both carbonyl moieties have a methyl group in *β*-position. Using the mutant YqjM C26G, the enzyme loses its stereocontrol toward this reaction in all described cases but allows a conversion of compound (*Z*)-**2d** (data not shown). In contrast, the ER reduction of compound **2b**, where the methyl group is juxtaposed to the carbonyl moiety, was fully predictable for both diastereomers. This compound showed the *E*/*Z*-flip; although the *E*-diastereomer leads to (*S*)-**3b**, the *Z*-diastereomer has been reduced to (*R*)-**3b**. In both cases, the substrate has been activated via the ketone moiety, like compound (*E*)-**2a** in Scheme 6. Hence, compound **2b** can be converted by the ER yielding both particular enantiomers dependent on the respective diastereomer used (cf. Scheme 7).

In contrast to the ER conversions, the reductions using ADHs was fully predictable for all products in Scheme 7. As a consequence, the one-pot two-step reaction could be carried out with good results in terms of yield as well as enantiomeric excesses.

CONCLUSION

The combination of an enoate reductase and alcohol dehydrogenases was used successfully to produce valuable chiral building blocks in a preparative fashion under strict stereocontrol. Including the preparation of the starting materials, the chiral butyrolactones have been produced in three steps: one chemical and two biocatalytic, which were carried out in a convenient one-pot fashion as the two-step one-pot sequential cascade.

As shown before, the stereoselectivity of the enoate reductase YqjM is excellent in terms of control, but it strongly depends on the substrate and is hardly predictable.[3i,9a,b,10] Our results underline that this enzyme does not follow a rather simple rule of prediction like many ADHs or lipases do.[3d,f] However, the irregularities in stereoselectivity we faced within this work could be studied in detail and finally fully explained.

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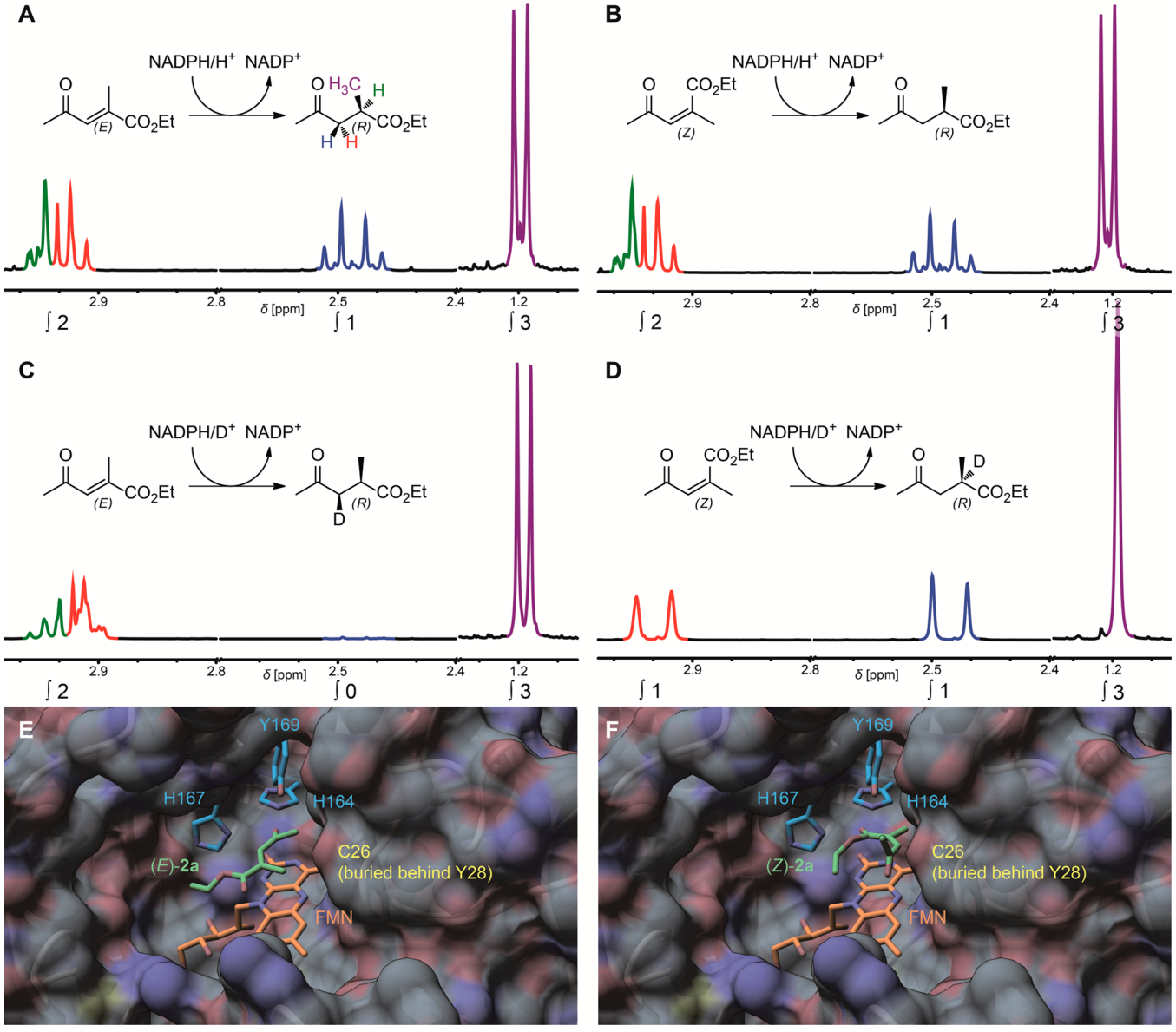

Figure 1. Both, the reduction of compound (*E*)-**2a** and (*Z*)-**2a** using the enoate reductase YqjM resulted in the aliphatic (*R*)-enantiomer. To elucidate the special binding mode of this compound, deuteration experiments have been carried out. Panels A–D show sections of ^{1}H NMR spectra from the conversion of either (*E*)-**2a** (A/C) or (*Z*)-**2a** (B/D) in normal (A/B) as well as deuterated buffer (C/D). The corresponding signals are highlighted according to the color code of panel A. Please note that the signals were marked for illustration rather than representing the correct dissection of the higher order signals. Panels E and D display in silico binding modes of both compounds (green) in YqjM.[11] A solvent-accessible surface is drawn for all residues except for the active site and the FMN, where the structures are shown explicitly.

EXPERIMENTAL SECTION

Materials. Chemicals were purchased from Carl Roth (Karlsruhe, Germany) and Sigma-Aldrich (Steinheim, Germany) in analytical grade. For cloning, enzymes were bought from Fermentas (St. Leon-Roth, Germany) the vector pET22b was purchased from Merck (Darmstadt, Germany), and oligonucleotides were obtained as desalted lyophilizates from Sigma-Aldrich (Steinheim, Germany). Chromatographic columns and matrices were purchased from GE Healthcare (München, Germany) and Qiagen (Hilden, Germany), respectively. Chromatographic steps were carried out on ÄKTA purifier from GE Healthcare (München, Germany). For cell disruption, a FRENCH Press pressure cell from Thermo Fisher Scientific (Schwerte, Germany) was used. $NADP^+$ and NADPH were a generous gift of Codexis (Redwood City, CA). ADH_{LK} crude extracts were obtained from Prof. Dr. W. Hummel (IMET, HHU Düsseldorf). The ADH evo 1.1.030 was a generous gift of Evocatal (Düsseldorf, Germany).

Cloning, Expression, and Purification of YqjM. The *yqjM* gene (identical to gene bank entry CP006881.1 from base 2293711 to 2294727) was amplified from isolated genome of *Bacillus subtilis* DSM168 using the oligonucleotides (Table 2) yqjM_fw and yqjM_rv [5 min, 95 °C; 35 × (1 min 94 °C; 1 min 59 °C; 1 min 72 °C) 10 min 72 °C]. The amplificate was ligated *via NdeI* and *EcoRE* into the likewise restricted vector pET21a. The insert sequence of positive clones pET21::yqjM was verified by sequencing (GATC Biotech AG, Konstanz, Germany).

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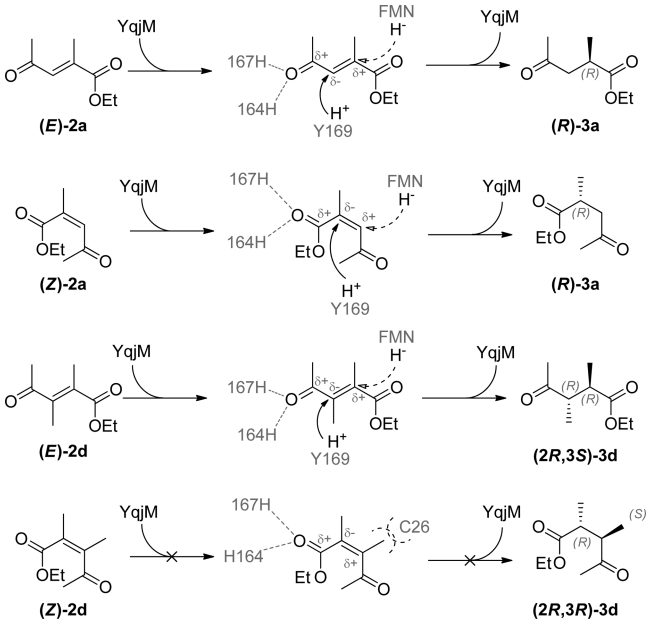
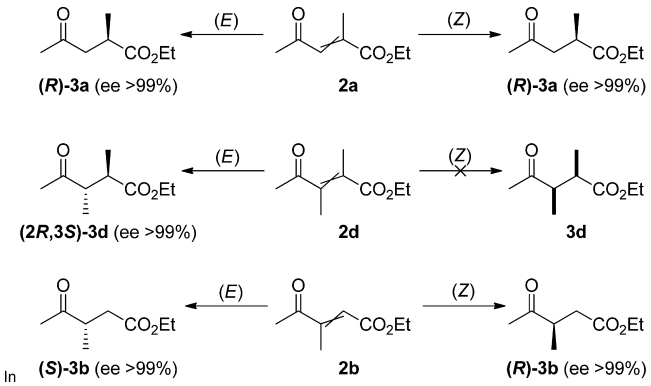
Scheme 6. Schematic Mechanistic Explanations of the Counterintuitive Results



Scheme 7. Results of the Reduction by the Enoate Reductase YqjM Only



A construct with a N-terminal hexa-histidine tag (6xhis) and a tobacco edge virus (TEV) protease recognition site was created as followed: The *yqjM* gene was amplified from pET21a::yqjM using the htyqjM_fw and htyqjM_rv [6 min, 95 °C; 35 × (30 s 95 °C; 30 s 60 °C; 1 min 72 °C) 10 min 72 °C]. The forward primer contained a sequence coding for a hexa-histidine tag (6xHis) followed by a linker sequence for the tripeptide AMT and the tobacco edge virus (TEV) protease recognition site (ENLYFQG). The amplificate was ligated by T4-DNA-ligase via *NdeI* and *XhoI* into the calf intestine alkaline phosphatase dephosphorylated and likewise restricted vector pET22b. The insert sequence of positive clones was verified by sequencing. The obtained vector containing the coding sequence for the YqjM with N-terminally fused 6xHis-tag/TEV-site under lactose inducible T7-promotor for cytosolic expression is referred to as pHT::yqjM in the further text. The protein with tag will be named htYqjM.

Expression was carried out in a 3 L baffled Fernbach flask using 1 L terrific broth (TB) with 100 μg/mL ampicillin inoculated by 1% (v/v) of an overnight culture of *E. coli* BL21(DE3) pHT::yqjM grown at 37 °C. After incubation at 20 °C and 120 rpm for 9 h, the expression was induced with 0.1 mM IPTG followed by further incubation for 15 h. Up to 16 g of wet cell mass was harvested.

One-step purification was carried out using immobilized metal chelate affinity chromatography. Therefore, 10 g of *E. coli* BL21(DE3) pHT::yqjM was suspended 20% (w/v) in purification buffer P_0 (20 mM potassium phosphate, pH 6.5) and disrupted using a FRENCH Press pressure cell on ice. After centrifugation at 18 000 rcf and 4 °C, the cleared supernatant was applied to a 5 mL Ni-NTA cartridge (equilibrated on purification buffer). The column was washed with 6 column volumes (CV) P_0, 6 CV W30 (P_0 with 30 mM imidazole). Finally, the protein was eluted with 6 CV buffer E250 (P_0 with 250 mM imidazole). Flavin mononucleotide (FMN; 5 mM) was applied while the mixture was concentrated to 10 mg/mL using an ultrafiltration unit with a molecular weight cutoff (MWCO) of 10 kDa (Sartorius Stedim Biotech, France). Imidazole and FMN were removed by a PD10 (GE Healthcare, München, Germany) desalting column equilibrated on P_0. A maximum of 250 mg of pure protein (1 U/mg) could be obtained. Long-time storage was carried out as lyophilizate. Therefore, the solution was flash frozen in liquid nitrogen prior to freeze-drying.

Volumetric Activity Assay. Volume activity was measured photometrically in a Shimadzu UV-1800 spectrophotometer with a Peltier heater/cooler (Duisburg, Germany). Therefore, 990 μL of reaction mixture (1 mM cyclohex-2-enone and 0.15 mM NADPH in 20 mM KP_i buffer, pH 6.5) were pretempered to 30 °C. The reaction was started by adding 10 μL of test solution containing the enoate reductase. The decrease of absorbance at 340 nm was recorded and finally the volume activity was calculated by multiplying the slope of the absorbance decrease with the cuvette factor 16.08 U min^{-1} mL^{-1}. Protein concentrations for estimation of specific activity were either determined by the method of Bradford for crude extracts or by measuring the absorbance at 280 and 432 nm or 458 nm.[12] The saturation (S) of YqjM with FMN could be determined from the empirical formula:[13]

$$S = 4.53(A_{432nm}/A_{280nm})$$

$$[\varepsilon]_{280nm} = 1.015S + 0.8[\mathrm{mL\,mg^{-1}\,cm^{-1}}]$$

Dependent on the saturation, S, it was possible to calculate the specific extinction coefficient $[\varepsilon]_{280\ nm}$, which could be used to calculate the mass concentration from the absorption at 280 nm.

Table 2. Oligonucleotides Used in This Work

name	sequence (5′→3′)
yqjM_fw	GGGAATTCCATATGATGGCCAGAAAATTATTTACACCTATTAC
yqjM_rv	CCGGAATTCTTACCAGCCTCTTTCGTATTGAAC
htyqjM_fw	GGGCATATGCACCACCATCATCACCATGCCATGACTGAAAATCTGTATTTTCAGGGCAGCCATGGCCAGAAAATTATTTACACCTATTAC
htyqjM_rv	GGGCTCGAGTTACCAGCCTCTTTCGTATTGAAC

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FMN Complementation. As described above, htYqjM was isolated and desalted. Aliquots of this solution with a final concentration of 2.5 mg/mL, as determined by the Bradford assay, were supplied with varying FMN concentrations (0−1000 μM) and incubated for 1 h on ice.[12] Afterward, the solution was desalted twice with a PD10 desalting column equilibrated with buffer P_0. UV−vis spectra and volume activities were determined for each sample. The data were fitted by the least-squares algorithm using OriginPro 8.5 with the modified hill equation $y = v_{min} + (v_{max} - v_{min})(x/(K + x))$.

Expression and Production of ADH_T and ADH_{LB}. Chemically competent cells of *E. coli* BL21(DE3) were transformed with expression vector containing either the *ADH* gene (synthetically ordered) from either *Lactobacillus brevis* or *Thermoanaerobacter sp.*[8,14] In a 3 L Fernbach flask, 1 L of TB_{Amp} medium was inoculated with 1% (v/v) of an overnight culture. The main culture was incubated at 25 °C, 120 rpm for 9 h, then induced with 0.1 mM IPTG and further incubated for 15 h. The harvested cells were suspended 20% (w/v) in 100 mM KP_i, pH 7 1 mM $MgCl_2$ and disrupted using a FRENCH Press pressure cell. While the crude extract of ADH_T was then heated to 60 °C for 20 min and then centrifuged (20 min, 18000 rcf) to clear the supernatant, the ADH_{LB} was cleared directly. One unit is defined as the amount of enzyme needed for the reduction of 1 μmol/min acetophenone as monitored via the photometric NADPH consumption (340 nm, 30 °C, 100 mM KP_i, pH 7, 1 mM $MgCl_2$, 10.67 mM acetophenone).

Syntheses of the Substrate Library. *(E)-Ethyl 2-methyl-4-oxo-pent-2-enoate [(E)-**2a**], Wittig Reaction.* Under inert conditions 22.06 g (69 mmol, 1.1 equiv) of 1-(triphenylphosphoranylidene)-2-propanone was dissolved in 350 mL of dry toluene, chilled to −78 °C, and 7 mL (63 mmol, 1 equiv) of ethyl pyruvate was added. After the mixture was stirred for 3 h at −78 °C, it was allowed to warm to RT overnight. The reaction was quenched with water and extracted with diethyl ether three times. The combined organic layers were dried with $MgSO_4$, filtered, concentrated under reduced pressure, and finally purified by flash column chromatography (PE/Et_2O = 70:30). A yellow oil (9.2 g, 59 mmol, 86%) was obtained for (*E*)-**2a**, and 0.47 g (3 mmol, 4%) of a clear oil were obtained for (*Z*)-**2a** (*E*/*Z* = 95:5 from crude ^{1}H NMR). The analytical data were in accordance with literature data.[15] GC: t_R[(*E*)-**2a**] = 13.13 min, t_R[(*Z*)-**2a**] = 13.69 min [0.6 bar H_2 Lipodex G; temperature gradient: 60 °C (5 min iso), 5 °C/min to 150 °C (5 min iso)]. (*E*)-**2a**: R_f = 0.6 (PE/Et_2O = 70:30). ^{1}H NMR (600 MHz, $CDCl_3$): δ [ppm] = 1.34 (t, 3 H, $^3J_{2',1'}$ = 7.1 Hz, 2′-H), 2.21 (d, 3 H, $^4J_{Me,3}$ = 1.5 Hz, CH_3), 2.32 (s, 3 H, 5-H), 4.26(q, 2 H, $^3J_{1',2'}$ = 7.1 Hz, 1′-H), 7.08 (q, 1 H, $^4J_{3,Me}$ = 1.5 Hz, 3-H). ^{13}C NMR (151 MHz, $CDCl_3$): δ [ppm] = 14.2 (C-2′), 14.3 (CH_3), 32.1 (C-5), 61.6 (C-1′), 132.4 (C-3), 140.9 (C-2), 167.7 (C-1), 199.5 (C-4). IR (atr film) υ [cm^{-1}] = 2985, 1718, 1693, 1622, 1449, 1425, 1359, 1301, 1250, 1192, 1122, 1028, 966, 888, 863, 739. MS (EI, 70 eV): *m*/*z* (%) = 156 (<1) [$(M)^+$], 141 (9) [$(M - CH_3)^+$], 127 (12) [$(M - C_2H_3)^+$], 113 (24) [$(M - C_2H_3O)^+$], 111 (33) [$(M - C_2H_5O)^+$], 110 (100) [$(C_6H_6O_2)^+$], 85 (30) [$(C_5H_9O)^+$], 82 (44) [$(C_5H_6O)^+$], 67 (10) [$(C_5H_7)^+$]. (*Z*)-**2a**: R_f = 0.4 (PE/Et_2O = 70:30). ^{1}H NMR (600 MHz, $CDCl_3$): δ [ppm] = 1.32 (t, 3 H, $^3J_{2',1'}$ = 7.1 Hz, 2′-H), 2.03 (d, 3 H, $^4J_{Me,3}$ = 1.4 Hz, CH_3), 2.24 (s, 3 H, 5-H), 4.27 (q, 2 H, $^3J_{1',2'}$ = 7.1 Hz, 1′-H), 6.18 (q, 1 H, $^4J_{3,Me}$ = 1.4 Hz, 3-H). ^{13}C NMR (151 MHz, $CDCl_3$): δ [ppm] = 13.9 (C-2′), 20.2 (CH_3), 30.1 (C-5), 61.4 (C-1′), 130.3 (C-3), 141.2 (C-2), 168.9 (C-1), 197.8 (C-4). IR (atr film) υ [cm^{-1}] = 2985, 1728, 1698, 1625, 1445, 1372, 1359, 1252, 1179, 1128, 1018, 959, 862, 762. MS (EI, 70 eV): *m*/*z* (%) = 141 (69) [$(M - CH_3)^+$], 113 (100) [$(M - C_2H_3O)^+$], 111 (56) [$(M - C_2H_5O)^+$], 110 (40), 85 (37) [$(C_5H_9O)^+$], 69 (8) [$(C_4H_5O)^+$].

*(Z)-Ethyl 2-methyl-4-oxo-pent-2-enoate [(Z)-**2a**], Still−Gennari Reaction.* Bis-(2,2,2-trifluorethyl) 2-oxo-propyl-phosphonate (0.5 g, 1.66 mmol, 1 equiv) dissolved in 10 mLof dry THF under inert conditions was chilled to −78 °C, and 0.52 g (1.99 mmol, 1.2 equiv) of 18-crown-6 dissolved in THF was added. A solution of potassium hexamethyldisilazide (0.38 g in 1.8 mL abs THF, 1.82 mmol, 1.1 equiv) was added and stirred for 20 min. Ethyl pyruvate (0.18 mL in 5 mL of THF, 1.66 mmol, 1 equiv) was added dropwise over a period of 10 min, stirred for 3 h at −78 °C, and then warmed to RT. The reaction mixture was quenched with 20 mL of saturated NH_4Cl solution and extracted three times with Et_2O. The combined organic phases were dried with $MgSO_4$, filtered, and the solvent was removed under reduced pressure. The crude product (*E*/*Z* = 58:42, ^{1}H NMR) was purified by flash column chromatography as described above, yielding 134 mg (0.86 mmol, 52%) (*E*)-**2a** and 98 mg (0.63 mmol, 38%) (*Z*)-**2a**. Analytical data vide supra.

*(Z)-Ethyl 2-methyl-4-oxo-pent-2-enoate [(Z)-**2a**], Photoisomerization.* (*E*)-**2a** (1.0 g, 6.4 mmol) dissolved in ethanol (4 mL) was incubated for 5 h in a closed, argon-filled quartz glass reaction in a Rayonet photoreactor RPR-200 (λ = 350 nm, Branford, U.S.A.). The reactant was mixed hourly. The solvent was removed under reduced pressure (*Z*/*E* = 74:26 ^{1}H NMR), and the mixture was purified by flash column chromatography as described above, yielding 700 mg (4.48 mmol, 70%) (*Z*)-**2a** and 200 mg (1.28 mmol, 20%) (*E*)-**2a**.

*Ethyl 3-methyl-4-oxo-pent-2-enoate [(E/Z)-**2b**].* Under inert conditions, 44.6 g (121.6 mmol, 1.1 equiv) of ethyl 2-(triphenylphosphoranylidene)-acetate and 10 mL (110.5 mmol, 1 equiv) of 2,3-butanedione were dissolved in 500 mL of dry CH_2Cl_2 at RT. After the solvent was stirred for 48 h at RT (*E*/*Z* = 70:30 ^{1}H NMR), it was removed under reduced pressure. The precipitate was filtered off prior to the chromatographic purification (PE/Et_2O = 80:20) yielding (*E*)-**2b** as a colorless oil (66%, 73 mmol, 11.35 g) and (*Z*)-**2b** as a colorless oil (26%, 29 mmol, 4.76 g). The analytical data were in accordance with the literature.[16] GC (reduction with ADH): t_R[(*E*)-**2b**] = 15.63 min, t_R[(*E*)-**2b**] = 16.14 min [0.6 bar H_2; Lipodex E; temperature gradient: 60 °C (5 min iso), 5 °C/min to 150 °C (5 min iso)]; GC (reduction with enoate reductase): t_R[(*E*)-**2b**] = 22.88 min, t_R[(*Z*)-**2b**] = 29.03 min [0.6 bar H_2; Hydrodex β-TBDAc; temperature gradient: 85 °C (4 min iso), 1 °C/min to 115 °C (1 min iso)]; GC (reduction with enoate reductase): t_R[(*E*)-**2b**] = 20.66 min, t_R[(*Z*)-**2b**] = 23.27 min [0.6 bar H_2; Lipodex E; temperature gradient: 75 °C (5 min iso), 1 °C/min to 100 °C (1 min iso), 20 °C/min to 120 °C (1 min iso)]; IR (atr film) υ [cm^{-1}] = 2984, 1706, 1644, 1444, 1371, 1357, 1344, 1245, 1159, 1108, 1054, 1023, 984, 858, 729. (*E*)-**2b** R_f = 0.6 (PE/Et_2O = 70:30). ^{1}H NMR (600 MHz, $CDCl_3$): δ [ppm] = 1.33 (t, 3 H, $^3J_{2',1'}$ = 7.1 Hz, 2′-H), 2.21 (d, 3 H, $^4J_{Me,2}$ = 1.5 Hz, CH_3), 2.39 (s, 3 H, 5-H), 4.25 (q, 2 H, $^3J_{1',2'}$ = 7.1 Hz, 1′-H), 6.57 (q, 1 H, $^4J_{2,Me}$ = 1.5 Hz, 2-H). ^{13}C NMR (151 MHz, $CDCl_3$): δ [ppm] = 13.1 (CH_3), 14.2 (C-2′), 26.2 (C-5), 60.8 (C-1′), 126.5 (C-2); 150.4 (C-3), 166.2 (C-1), 199.9 (C-4). MS (EI, 70 eV): *m*/*z* (%) = 156 (<1) [$(M)^+$], 141 (3) [$(M - CH_3)^+$], 111 (41) [$(M - C_2H_5O)^+$], 110 (100) [$(C_6H_6O_2)^+$], 99 (3) [$(C_5H_7O_2)^+$], 85 (15) [$(C_5H_9O)^+$], 67

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(23) [$(C_5H_7)^+$]. (*Z*)-**2b** R_f = 0.4 (PE/Et_2O = 70:30). ^{1}H NMR (600 MHz, $CDCl_3$): δ [ppm] = 1.27 (t, 3 H, $^3J_{2',1'}$ = 7.2 Hz, 2′-H), 1.99 (d, 3 H, $^4J_{Me,2}$ = 1.7 Hz, CH_3), 2.36 (s, 3 H, 5-H), 4.16 (q, 2 H, $^3J_{1',2'}$ = 7.2 Hz, 1′-H), 5.69 (q, 1 H, $^4J_{2,Me}$ = 1.7 Hz, 2-H). ^{13}C NMR (151 MHz, $CDCl_3$): δ [ppm] = 14.1(C-2′), 20.2 (CH_3), 28.6 (C-5), 60.8 (C-1′), 117.1 (C-2), 157.2 (C-3), 165.2 (C-1), 206.2 (C-4). MS (EI, 70 eV): *m*/*z* (%) = 156 (<1) [$(M)^+$], 141 (100) [$(M - CH_3)^+$], 127 (4) [$(M - C_2H_5)^+$], 113 (82) [$(C_5H_5O)^+$], 111 (61) [$(M - C_2H_5O)^+$], 110 (16) [$(C_6H_6O_2)^+$], 99 (15) [$(C_5H_7O_2)^+$], 85 (20) [$(C_5H_9O)^+$], 69 (28) [$(C_4H_5O)^+$], 68 (14) [$(C_4H_4O)^+$].

*tert-Butyl 3-methyl-4-oxo-pent-2-enoate [(E/Z)-**2c**].* Under inert conditions, 1 g (2.6 mmol, 1 equiv) of *tert*-butyl 2-(triphenylphosphoranylidene)-acetate and 0.27 mL (3.12 mmol, 1.2 equiv) 2,3-butanedione were dissolved in 20 mL of dry CH_2Cl_2 at RT. After the solvent was stirred for 48 h at RT (*E*/*Z* = 84:16 ^{1}H NMR), it was removed under reduced pressure. The precipitate was filtered off prior to the chromatographic purification (PE/EE = 90:10) yielding (*E*)-**2c** as a colorless oil (74%, 73 mmol, 11.35 g) and (*Z*)-**2c** as a colorless oil (11%, 28 mmol, 52 mg). The configurations were correlated by chemical shift comparison with (*E*)-**2b** and (*Z*)-**2b**, respectively. GC (for reduction with enoate reductase): t_R[(*E*)-**2c**] = 18.68 min, t_R[(*Z*)-**2c**] = 18.47 [0.6 bar H_2, Hydrodex β-TBDAc, temperature gradient: 80 °C (2 min iso), 2 °C/min to 140 °C (1 min iso)]; t_R[(*E*)-**2c**] = 15.72 min, t_R[(*Z*)-**2c**] = 16.61 [0.6 bar H_2, Lipodex E, temperature gradient: 75 °C (5 min iso), 1 °C/min to 95 °C, 20 °C/min to 150 °C (1 min iso)]; (for reduction with ADH): t_R[(*E*)-**2c**] = 16.66 min, t_R[(*Z*)-**2c**] = 18.38 [0.6 bar H_2, Hydrodex β-TBDAc, temperature gradient: 90 °C (5 min iso), 2 °C/min to 150 °C (1 min iso)]; R_f[(*E*)-**2c**] = 0.46 (PE:EE = 90:10), R_f[(*Z*)-**2c**] = 0.33 (PE:EE = 90:10); IR (atr film) υ[cm^{-1}] = 2980, 1716, 1684, 1637, 1366, 1249, 1213, 1152, 1105, 1027, 877, 858, 703; HRMS calc. 207.224 [$(C_{10}H_{16}O_3Na)^+$] [(M + Na)$^+$] found 207.099 [$(C_{10}H_{16}O_3Na)^+$] [(M + Na)$^+$]; (*E*)-**2c**: MS (EI, 70 eV): *m*/*z* (%) = 128 (68) [$(C_6H_8O_3)^+$], 111 (74) [$(C_6H_7O_2)^+$], 57 (100) [$(C_4H_9)^+$]; ^{1}H NMR (600 MHz, $CDCl_3$): δ [ppm] = 1.52 (s, 9 H, $C(CH_3)_3$), 2.16 (d, 3 H, $^4J_{Me,2}$ = 1.4 Hz, CH_3), 2.37 (s, 3 H, 5-H), 6.51 (q, 1 H, $^4J_{2,Me}$ = 1.4 Hz, 2-H); ^{13}C NMR (151 MHz, $CDCl_3$): δ [ppm] = 12.8 (CH_3), 26.1 (C-5), 28.2 ($C(CH_3)_3$), 81.7 ($C(CH_3)_3$), 128.6 (C-2), 148.9 (C-3), 165.6 (C-1), 200.2 (C-4); (*Z*)-**2c**: MS (EI, 70 eV): *m*/*z* (%) = 169 (15) [$(M - CH_3)^+$], 129 (34) [$(C_6H_9O_3)^+$], 113 (24), [$(C_5H_5O_3)^+$], 111 (100) [$(C_6H_7O_2)^+$], 68 (21), 57 (77) [$(C_4H_9)^+$]. ^{1}H NMR (600 MHz, $CDCl_3$): δ [ppm] = 1.45 (s, 9 H, $C(CH_3)_3$), 1.95 (d, 3 H, $^4J_{Me,2}$ = 1.6 Hz, CH_3), 2.35 (s, 3 H, 5-H), 5.61 (q, 1 H, $^4J_{2,Me}$ = 1.6 Hz, 2-H). ^{13}C NMR (151 MHz, $CDCl_3$): δ [ppm] = 20.1 (CH_3), 28.0 ($C(CH_3)_3$), 28.9 (C-5), 81.4 ($C(CH_3)_3$), 119.1 (C-2), 155.4 (C-3), 164.4 (C-1), 206.3 (C-4).

*Ethyl 2,3-dimethyl-4-oxo-pent-2-enoate [(E/Z)-**2d**].* In 50 mL dry toluene 2.34 g (6.08 mmol, 1.1 equiv) ethyl 2-(triphenyl-phosphoranylidene)-propanoate were dissolved under inert conditions and 0.5 mL (5.53 mmol, 1 equiv) 2,3-butane-dione were added. The mixture was stirred under reflux for 5 h, and then the solvent was removed under reduced pressure. After removal of the precipitate by filtration over Celite, a flash column chromatography was carried out (PE/EE = 95:5).[17] (*E*)-**2d** was obtained as a colorless oil (46%, 2.53 mmol, 431 mg) and (*Z*)-**2d** as a clear oil (48%, 2.67 mmol, 455 mg). GC t_R[(*E*)-**2d**] = 14.56 min, t_R[(*Z*)-**2d**] = 15.74 min; [0.6 bar H_2; Lipodex G; temperature gradient: 60 °C (5 min iso), 5 °C/min to 150 °C (5 min iso)]; t_R[(*E*)-**2d**] = 20.99 min, t_R[(*Z*)-**2d**] = 23.46 min [0.6 bar H_2; Lipodex G; temperature gradient: 60 °C (5 min iso), 5 °C/min to 150 °C (5 min iso)]; t_R[(*E*)-**2d**] = 19.19 min, t_R[(*Z*)-**2d**] = 20.16 min [0.6 bar H_2; Lipodex G; temperature gradient: 60 °C (5 min iso), 2 °C/min to 110 °C, 15 °C/min to 150 °C (1 min iso)]; t_R[(*E*)-**2d**] = 35.88 min, t_R[(*Z*)-**2d**] = 39.58 min [0.6 bar H_2; Hydrodex β-3P; temperature gradient: 70 °C (5 min iso), 1 °C/min to 110 °C, 20 °C/min to 150 °C (1 min iso)]. R_f = 0.31 (PE/EE = 95:5). ^{1}H NMR (600 MHz, $CDCl_3$): δ [ppm] = 1.34 (t, 3 H, $^3J_{2'1'}$ = 7.2 Hz, 2′-H), 1.92 (q, 3 H, $^5J_{2\text{-Me},3\text{-Me}}$ = 1.6 Hz, 2-CH_3), 2.09 (q, 1 H, $^5J_{3\text{-Me},2\text{-Me}}$ = 1.6 Hz, 3-CH_3), 2.32 (s, 3 H, 5-H), 4.26 (qd, 2 H, $^3J_{1'2'}$ = 7.2 Hz, 1′-H); ^{13}C NMR (151 MHz, $CDCl_3$): δ [ppm] = 14.2 (C-2′), 16.6 (2-CH_3), 16.7 (3-CH_3), 29.1 (C-5), 60.8 (C-1′), 126.9 (C-2), 143.9 (C-3), 168.8 (C-1), 205.6 (C-4). (*Z*)-**2d IR** (atr film) υ [cm^{-1}] = 2984, 1700, 1639, 1446, 1366, 1353, 1287, 1243, 1170, 1094, 1024, 950, 857, 771; MS (EI, 70 eV): *m*/*z* (%) = 170 (<1) [$(M)^+$], 155 (85) [$(M - CH_3)^+$], 127 (100) [$(M - C_2H_3O)^+$], 125 (47) [$(M - C_2H_5O)^+$], 113 (6) [$(C_6H_9O_2)^+$], 99 (26) [$(C_5H_7O_2)^+$], 83 (10), [$(C_5H_7O)^+$], 55 (11) [$(C_3H_3O)^+$]. HRMS (positive ion): calcd 193.198 [(M + Na)$^+$], found 193.083 [(M + Na)$^+$]; R_f = 0.14 (PE:EE = 95:5). ^{1}H NMR (600 MHz, $CDCl_3$): δ [ppm] = 1.30 (t, 3 H, $^3J_{2'1'}$ = 7.2 Hz, 2′-H), 1.91 (q, 3 H, $^5J_{2\text{-Me},3\text{-Me}}$ = 1.2 Hz, 2-CH_3), 1.93 (q, 1 H, $^5J_{3\text{-Me},2\text{-Me}}$ = 1.2 Hz, 3-CH_3), 2.32 (s, 3 H, 5-H), 4.21 (q, 2 H, $^3J_{1'2'}$ = 7.2 Hz, 1′-H). ^{13}C NMR (151 MHz, $CDCl_3$): δ [ppm] = 13.9 (C-2′), 14.1 (2-CH_3), 16.5 (3-CH_3), 28.5 (C-5), 61.1 (C 1′), 125.1 (C-2), 148.3 (C-3), 167.5 (C-1), 206.1 (C-4).

Enoate Reductase Catalyzed Reductions. *General Procedure for the Reductions with YqjM (GP 1).* α,β-unsaturated γ-keto ester (100−500 mg) was suspended in 15 mL/mmol KP_i-buffer (100 mM, pH 7) and 250 mM D-glucose, 1 mol % $NADP^+$ 1 U/mmol GDH, 2.5 U/mmol htYqjM. The reaction mixture was shaken at 250 rpm/30 °C while the pH was kept constant by a pH-stat: the solution was continuously titrated with 1 M NaOH, which allows a control of the course of the reaction quantitatively. After complete conversion (GC), the solution was extracted five times with MTBE, the combined organic layers were dried over $MgSO_4$, and the solvent was removed under reduced pressure.

*(2R)-Ethyl 2-methyl-4-oxo-pentanoate [(R)-**3a**].* According to the GP 1, 500 mg (3.2 mmol) of (*E*)-**2a** was reduced. The crude product was purified by flash column chromatography (PE/EE = 80:20) yielding 85−95% (426−474 mg; 2.7−3 mmol) of a colorless oil. $[\alpha]_D^{25}$ = +12.7 (*c* = 1 in $CHCl_3$; ee >99%; GC t_R[(R)-**3a**)] = 11.52 min [0.6 bar H_2; Lipodex G; 60 °C (5 min iso), 5 °C/min to 150 °C (5 min iso)]. Analytical data agreed with those of ***rac*-3a** (vide infra).

*(3S)-Ethyl 3-methyl-4-oxo-pentanoate [(S)-**3b**].* (*E*)-**2b** (1.0 g, 6.4 mmol) was reduced according to the GP 1 yielding 93% (940 mg; 5.9 mmol; 70% for 2.0 g and 78% for 3.0 g) as a colorless oil after ffc (PE/EE = 80:20). The analytical data were in accordance with ***rac*-3b** (vide infra) and literature.[18] $[\alpha]_D^{25}$ = −51.5 (*c* = 1.1 in MeOH; ee >99%); GC t_R[(*S*)-**3b**] = 17.55 min [0.6 bar H_2; Lipodex E; 75 °C (5 min iso), 1 °C/min to 100 °C (1 min iso), 20 °C/min to 120 °C (1 min iso)]. HRMS (positive ion): calcd 181.084 [(M + Na)$^+$], found 181.083 [(M + Na)$^+$].

*(3R)-Ethyl 3-methyl-4-oxo-pentanoate [(R)-**3b**].* (*Z*)-**2b** (1.0 g, 6.4 mmol) was reduced according to the GP 1 yielding 85% (859 mg; 5.44 mmol) after ffc (PE/EE = 80:20). $[\alpha]_D^{25}$ = +42.7 (*c* = 1 in MeOH; ee >99%); GC t_R[(*R*)-**3b**] = 18.13 min

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[0.6 bar H_2; Lipodex E; 75 °C (5 min iso), 1 °C/min to 100 °C (1 min iso), 20 °C/min to 120 °C (1 min iso)]. The analytical data were in accordance with ***rac*-3b** (vide infra).

Sequential One-Pot Syntheses. *Sequential One-Pot Syntheses (GP 2).* α,β-Unsaturated γ-keto ester (3−6.4 mmol) was suspended in 15 mL/mmol KP_i-buffer (100 mM, pH 7), and 250 mM D-glucose, 1 mol % $NADP^+$ 1 U/mmol GDH, 2.5 U/mmol htYqjM were added. The reaction mixture was shaken at 250 rpm/30 °C while the pH was kept constant by a pH-stat; the solution was continuously titrated with 1 M NaOH, which allows a control of the course of the reaction quantitatively. After a complete conversion was observed by chiral GC, 11−25 U/mmol of either ADH_{LK} or ADH_T was added and stirred until complete conversion, which took a maximum of 24 h. The reaction mixture was then extracted five times with MTBE and purified by means of flash column chromatography (PE/Et_2O = 80:20).

(2R,4S)-2,4-Dimethylbutyrolactone [(2R,4S)-1a]. (*E*)-**2a** (1.0, 6.4 mmol) was reduced according to GP 2 with 15 U htYqjM and 70 U ADH_T yielding 90% (656 mg, 5.8 mmol) as a colorless oil after flash column chromatography. The analytical data agreed with those of the literature.[1h,2a,19] $[\alpha]_D^{25}$ = −4.6 (*c* = 1.1 in $CHCl_3$; ee >99%); GC t_R[(**2*R*,4*S*)-3a**] = 10.21 min [0.6 bar H_2; Lipodex G; 60 °C (5 min iso), 5 °C/min to 150 °C (5 min iso)]. HRMS (positive ion): calcd 137.060 [$(M + Na)^+$], found 137.0573; calcd 153.30 [$(M + K)^+$], found 153.0312. ^{1}H NMR (600 MHz, $CDCl_3$): δ [ppm] = 1.28 (d, 3 H, $^3J_{Me,2}$ = 7.1 Hz, 2-CH_3), 1.42 (d, 3 H, $^3J_{Me,4}$ = 6.2 Hz, 4-CH_3), 1.49 (ddd, 1 H, $^2J_{3a,3b}$ = 12.4 Hz, $^3J_{3a,2}$ = 12.1 Hz, $^3J_{3a,4}$ = 10.3 Hz, 3-Ha), 2.50 (ddd, 1 H, $^2J_{3b,3a}$ = 12.4 Hz, $^3J_{3b,2}$ = 8.3 Hz, $^3J_{3b,4}$ = 5.3 Hz, 3-Hb), 2.68 (ddq, 1 H, $^2J_{2,3a}$ = 12.1 Hz, $^3J_{2,3b}$ = 8.3 Hz, $^3J_{2,Me}$ = 7.1 Hz, 2-H), 4.48 (dqd, 1 H, $^3J_{4,3a}$ = 10.3 Hz, $^3J_{4,Me}$ = 6.2 Hz, $^3J_{4,3b}$ = 5.3 Hz, 4-H).

(2R,4R)-2,4-Dimethylbutyrolactone [(2R,4R)-1a]. (*E*)-**2a** (1.0 g, 6.4 mmol) was reduced according to GP 2 with 15 U htYqjM and 160 U ADH_{LK} yielding 80% (583 mg, 5.12 mmol) as a colorless oil after flash column chromatography. The analytical data were in accordance with those of the literature.[1h,2a,19,20] $[\alpha]_D^{25}$ = +36.7 (*c* = 1.0 in $CHCl_3$; ee >99%); GC t_R[(**2*R*,4*R*)-1a**] = 10.1 min [0.6 bar H_2; Lipodex G; 60 °C (5 min iso), 5 °C/min to 150 °C (5 min iso)]. HRMS (positive ion): calcd 137.060 [$(M + Na)^+$], found 137.057. ^{1}H NMR (600 MHz, $CDCl_3$): δ [ppm] = 1.28 (d, 3 H, $^3J_{Me,2}$ = 7.4 Hz, 2-CH_3), 1.38 (d, 3 H, $^3J_{Me,4}$ = 6.4 Hz, 4-CH_3), 2.04 (ddd, 1 H, $^2J_{3a,3b}$ = 12.8 Hz, $^3J_{3a,2}$ = 7.4 Hz, $^3J_{3a,4}$ = 7.4 Hz, 3-H_a), 2.08 (ddd, 1 H, $^2J_{3b,3a}$ = 12.8 Hz, $^3J_{3b,2}$ = 8.8 Hz, $^3J_{3b,4}$ = 4.9 Hz, 3-H_b), 2.73 (dqd, 1 H, $^3J_{2,3b}$ = 8.8 Hz, $^3J_{2,3a}$ = 7.8 Hz, $^3J_{2,6}$ = 7.4 Hz, 2-H), 4.68 (dqd, 1 H, $^3J_{4,3a}$ = 7.4 Hz, $^3J_{4,Me}$ = 6.4 Hz, $^3J_{4,3a}$ = 4.9 Hz, 4-H).

(3S,4R)-3,4-Dimethylbutyrolactone [(3S,4R)-1b]. (*E*)-**2b** (0.5 g, 3.2 mmol) was reduced according to GP 2 with 4.8 U htYqjM and 80 U ADH_{LK} yielding 90% (328 mg, 2.88 mmol) lactone (**3*S*,4*R*)-1b** after flash column chromatography. The analytical data were in accordance with those of the literature.[21] (**3*S*,4*R*)-1b**: $[\alpha]_D^{25}$ = +61.4 (*c* = 1.0 in $CHCl_3$; ee >99%). GC t_R[(**3*S*,4*R*)-1b**] = 10.39 min [0.6 bar H_2; Lipodex G; 60 °C (5 min iso), 5 °C/min to 150 °C (5 min iso)]. HRMS (positive ion): calcd 137.060 [$(M + Na)^+$], found 137.057. ^{1}H NMR (600 MHz, $CDCl_3$): δ [ppm] = 1.14 (d, 3 H, $^3J_{Me,3}$ = 6.5 Hz, 3-CH_3), 1.40 (d, 3 H, $^3J_{Me,4}$ = 6.2 Hz, 4-CH_3), 2.12 − 2.23 (m, 2 H, 2-H_a, 3-CH), 2.68 (dd, 1 H, $^2J_{2b,2a}$ = 16.5 Hz, $^3J_{2b,3}$ = 7.2 Hz, 2-H_b), 4.14 (dq, 1 H, $^3J_{4,3}$ = 7.8 Hz, $^3J_{4,Me}$ = 6.2 Hz, 4-H).

(3S,4S)-3,4-Dimethylbutyrolactone [(3S,4S)-1b]. (*E*)-**2b** (0.5 g, 3.2 mmol) was reduced according to GP 2 with 4.8 U htYqjM and 80 U evo 1.1.030 yielding 82% (299 mg, 2.6 mmol) lactone (**3*S*,4*S*)-1b** after flash column chromatography. The analytical data were in accordance with those of the literature.[21c,22] $[\alpha]_D^{25}$ = +54.5 (*c* = 1.0 in $CHCl_3$; ee >99%); GC t_R[(**3*S*,4*S*)-1b**] = 17.32 min [0.6 bar H_2, Hydrodex β-3P; 60 °C (5 min iso), 5 °C/min to 150 °C (5 min iso)]. ^{1}H NMR (600 MHz, $CDCl_3$): δ [ppm] = 1.03 (d, 3 H, $^3J_{Me,3}$ = 7 Hz, 3-CH_3), 1.29 (d, 3 H, $^3J_{Me,4}$ = 6.5 Hz, 4-CH_3), 2.21 (dd, 1 H, $^2J_{2a,2b}$ = 16.9 Hz, $^3J_{2a,3}$ = 5.6 Hz, 2-H_a), 2.59 (m, 1 H, 3-H), 2.67 (dd, 1 H, $^2J_{2b,2a}$ = 16.9 Hz, $^3J_{2b,3}$ = 8 Hz, 2-H_b), 4.66 (quint., 1 H, $^3J_{4,Me}$ = 6.5 Hz, 4-H).

(3R,4R)-3,4-Dimethylbutyrolactone [(3R,4R)-1b]. (*Z*)-**2b** (0.47 g, 3.0 mmol) was reduced according to GP 2 with 7.5 U htYqjM and 80 U ADH_{LK} yielding 50% (171 mg, 1.5 mmol) lactone (**3*R*,4*R*)-1b** after flash column chromatography (ee >99%). The analytical data were in accordance with those of the literature and (**3*S*,4*S*)-1b**.[21c,22] $[\alpha]_D^{25}$ = +54.2 (*c* = 1.0 in $CHCl_3$; ee >99%); GC t_R[(**3*R*,4*R*)-1b**, ee =98%] = 17.21 min [0.6 bar H_2; Hydrodex β-3P; 60 °C (5 min iso), 5 °C/min to 150 °C (5 min iso)].

(3R,4R)-3,4-Dimethylbutyrolactone [(3R,4S)-1b]. (*Z*)-**2b** (0.47 g, 3.0 mmol) was reduced according to GP 2 with 7.5 U htYqjM and 70 U evo 1.1.030 yielding 63% (272 mg, 1.89 mmol) lactone (**3*R*,4*S*)-1b** after flash column chromatography as yellow oil. The analytical data were in accordance with those in the literature.[21a−c] $[\alpha]_D^{25}$ = +60.1 (*c* = 1.0 in $CHCl_3$; ee >99%); GC t_R[(**3*R*,4*S*-1b**] = 16.18 min [0.6 bar H_2; Hydrodex β-3P; 60 °C (5 min iso), 5 °C/min to 150 °C (5 min iso)]. ^{1}H NMR (600 MHz, $CDCl_3$): δ [ppm] = 1.11 (d, 3 H, $^3J_{Me,3}$ = 6.4 Hz, 3-CH_3), 1.37 (d, 3 H, $^3J_{Me,4}$ = 6.3 Hz, 4-CH_3), 2.10−2.20 (m, 2 H, 2-CH_a, 3-H), 2.65 (dd, 1 H, $^2J_{2b,2a}$ = 16.3 Hz, $^3J_{2b,3}$ = 7.1 Hz, 2-H_b), 4.11 (dq, 1 H, $^3J_{4,3}$ = 7.7 Hz, $^3J_{4,Me}$ = 6.3 Hz, 4-H).

tert-Butyl (3S)-3-methyl-4-oxo-butanoate [(3S)-3c]. This reaction and the following ADH reductions have not been carried out in one step. Five hundred milligrams (2.72 mmol) was reduced according to GP 2 but only with 6 U htYqjM and no ADH. After flash column chromatography (PE/EE = 80:20), 72% (364 mg, 1.96 mmol) of the ester (**3*S*)-3c** was obtained as colorless oil. $[\alpha]_D^{22}$ = −41.6 (*c* = 0.49 in $CHCl_3$) ee =97%; GC: t_R[(**3*S*)-3c**] = 16.29 min [0.6 bar H_2; Hydrodex β-TBDAc; 80 °C (2′ iso), 2 °C/min up to 140 °C (2′ iso)]; R_f = 0.50 (PE:EE = 85:15); IR (atr film): υ [cm^{-1}] = 2972, 2943, 1714, 1458, 1392, 1366, 1355, 1276, 1256, 1216, 1149, 1083, 957, 1083, 957, 846, 759. MS (EI, 70 eV): *m*/*z* (%) = 130 (12) [$(C_6H_{10}O_3)^+$], 113 (100) [$(C_6H_9O_2)^+$], 70 (15) [$(C_4H_6O)^+$], 57 (89) [$(C_4H_9)^+$]. ^{1}H NMR (600 MHz, $CDCl_3$): δ [ppm] = 1.13 (d, 3 H, $^3J_{Me,3}$ = 7.2 Hz, CH_3), 1.42 (s, 9 H, $C(CH_3)_3$), 2.18 (s, 3 H, 5-H), 2.25 (dd, 1 H, $^2J_{2a,2b}$ = 16.5 Hz, $^3J_{2a,3}$ = 5.5 Hz, 2-H_a), 2.68 (dd, 1 H, $^2J_{2b,2a}$ = 16.5 Hz, $^3J_{2b,3}$ = 8.6 Hz, 2-H_b), 2.94 (dqd, 1 H, $^3J_{3,2b}$ = 8.8 Hz, $^3J_{3,Me}$ = 7.2 Hz, $^3J_{3,2a}$ = 5.4 Hz, 3-H); ^{13}C NMR (151 MHz, $CDCl_3$): δ [ppm] = 16.4 (CH_3), 28.1 ($C(CH_3)_3$), 28.4 (C-5), 38.3 (C-2), 43.0 (C-3), 80.7 ($C(CH_3)_3$), 171.5 (C-1), 210.9 (C-4).

tert-Butyl (3R)-3-methyl-4-oxo-butanoate [(3R)-3c]. This reaction and the following ADH reductions have not been carried out in one step. Two hundred milligrams (1.09 mmol) was reduced according to GP 2 but only with 6 U htYqjM and no ADH. After flash column chromatography (PE/EE = 80:20), 76% (150 mg, 0.81 mmol) of the ester (**3*R*)-3c** was obtained as colorless oil. $[\alpha]_D^{22}$ = +31.4 (*c* = 0.48 in $CHCl_3$) ee

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=75%; GC: t_R[(**3*R*)-3c**] = 16.29 min [0.6 bar H_2; Hydrodex *β*-TBDAc; 80 °C (2′ iso), 2 °C/min up to 140 °C (2′ iso)]. Further analytical data were in accordance with (**3*S*)-3c**.

*tert-Butyl (3S,4S)-4-hydroxy-3-methyl-butanoate [(**3S,4R**)-4c]*. (**3*S*)-3c** (186 mg, 1 mmol) was converted according to GP 2 but with only 25 U ADH_{LK} (no htYqjM). After flash column chromatography (PE: ee = 80:20), 85% (159 mg, 0.85 mmol) of the ester (**3*S*,4*R*)-4c** was obtained as colorless oil (crude ^{1}H NMR alcohol/lactone =93:7). IR (atr film) υ [cm^{-1}] = 3443, 2972, 2934, 1727, 1362, 1283, 1255, 1147, 1095, 1014, 997, 958, 928, 843, 761. ^{1}H NMR (600 MHz, $CDCl_3$): δ [ppm] = 0.95 (d, 3 H, $^3J_{Me,3}$ = 9.0 Hz, Me), 1.18 (d, 3 H, $^3J_{5,4}$ = 6.3 Hz, 5-CH_3), 1.45 (s, 9 H, C(CH_3)$_3$), 1.94 (m, 1 H, 3-CH), 2.02 (br, 1 H, OH), 2.13 (dd, 2 H, $^2J_{2a,2b}$ = 15.0 Hz, $^3J_{2a,3}$ = 7.5 Hz, 2-CH_a), 2.41 (dd, 1 H, $^2J_{2b,2a}$ = 15.0 Hz, $^3J_{2b,3}$ = 5.5 Hz, 2-CH_b), 3.59 (m, 1 H, $^3J_{4,5}$ = 6.3 Hz, 4-CH); ^{13}C NMR (151 MHz, $CDCl_3$): δ [ppm] = 16.2 (CH), 20.1 (C-5), 28.1 (C(CH_3)$_3$), 37.8 (C-3), 39.2 (C-2), 71.6 (C 4), 80.4 (C(CH_3)$_3$), 173.2 (C-1); $[\alpha]_D^{20}$ = −9.0 (*c* = 1.0 in $CHCl_3$; 95% pure); GC t_R = 15.70 min (**3*S*,4*R*)-4c** [0.6 bar H_2; Hydrodex ß-3P; 60 °C (5′ iso), 5 °C/min up to 150 °C (5′ iso)]. HRMS (positive ion): calcd 211.123 [($C_{10}H_{20}O_3Na$)$^+$] [(M + Na)$^+$], found: 211.130 [($C_{10}H_{20}O_3Na$)$^+$] [(M + Na)$^+$]. The configuration has been assigned by lactonizing compound (**3*S*,4*R*)-4c** in THF with H_2SO_4 at ambient temperature. The resulting lactone was identical to (**3*S*,4*R*)-1b**.

*tert-Butyl (3S,4R)-4-hydroxy-3-methyl-butanoate [(**3S,4S**)-4c]*. (**3*S*)-3c** (186 mg, 1 mmol) was converted according to GP 2 with 25 U evo 1.1.03 (no htYqjM). After flash column chromatography (PE: ee = 80:20) 75% (139 mg, 0.75 mmol) of the ester (**3*S*,4*S*)-4c** were obtained as colorless oil (crude ^{1}H NMR alcohol/lactone = 93:7). The configuration has been assigned by lactonizing compound (**3*S*,4*S*)-4c** in THF with H_2SO_4 at ambient temperature. The resulting lactone was identical to (**3*S*,4*S*)-1b**. GC t_R[(**3*S*,4*S*)-4c**] = 20.84 min [0.6 bar H_2; Hydrodex ß-3P; 60 °C (5′ iso), 5 °C/min up to 150 °C (5′ iso].

*(2R,3S,4S)-2,3,4-Trimethylbutyrolactone [(**2R,3S,4S**)-1d]*. Olefin (*E*)-**2d** (0.2 g, 0.58 mmol) was reduced according to GP 2 with 15 U htYqjM and 20 U ADH_T yielding 70% (52 mg, 0.41 mmol) lactone (**2*R*,3*S*,4*S*)-1d** after flash column chromatography as a yellow oil. $[\alpha]_D^{20}$ = −20 (*c* = 0.6 in $CHCl_3$; ee >99%); GC t_R[(**3*R*,4*S*)-1d**] = 16.18 min [0.6 bar H_2; Hydrodex *β*-3P; 60 °C (5 min iso), 5 °C/min to 150 °C (5 min iso)]. IR (atr film)]: υ [cm^{-1}] = 2977, 1766, 1456, 1392, 1190, 1172, 1129, 1046, 1005, 933; MS (EI, 70 eV): *m*/*z* (%) = 128 (<1%) [(M)$^+$], 113 (2) [(M − CH_3)$^+$], 84 (21) [(M − CO_2)$^+$], 69 (53), 56 (100) [($C_4H_8^+$)]. ^{1}H NMR (600 MHz, $CDCl_3$): δ [ppm] = 0.86 (d, 3 H, $^3J_{Me,3}$ = 7.3 Hz, 3-CH_3), 1.17 (d, 3 H, $^3J_{Me,2}$ = 7.3 Hz, 2-CH_3), 1.33 (d, 3 H, $^3J_{Me,4}$ = 6.5 Hz, 4-CH_3), 2.47 (qdd, 1 H, $^3J_{3,2}$ = 7.3 Hz, $^3J_{3,3\text{-}Me}$ = 7.3 Hz, $^3J_{3,4}$ = 4.9 Hz, 3-H), 2.82 (dq, 1 H, $^3J_{2,3}$ = 7.3 Hz, $^3J_{2,2\text{-}Me}$ = 7.3 Hz, 2-H), 4.54 (dq, 1 H, $^3J_{4,3}$ = 4.9 Hz, $^3J_{4,4\text{-}Me}$ = 6.5 Hz, 4-H). ^{13}C NMR (151 MHz, $CDCl_3$): δ [ppm] = 8.36 (3-CH_3), 10.10 (2-CH_3), 15.66 (4-CH_3), 38.27 (3-C), 40.81 (2-C), 77.9 (4-C), 179.34 (-CO_2-).

*(2R,3S,4R)-2,3,4-Trimethylbutyrolactone [(**2R,3S,4R**)-1d]*. (*E*)-**2d** (0.2 g, 0.58 mmol) was reduced according to GP 2 with 15 U htYqjM and 20 U ADH_{LB} yielding 62% (46 mg, 0.35 mmol) lactone (**2*R*,3*S*,4*R*)-1d** after flash column chromatography as a yellow oil. $[\alpha]_D^{20}$ = +76 (*c* = 0.27 in $CHCl_3$; ee >99%); GC t_R[(**3*R*,4*S*-1d**] = 16.18 min [0.6 bar H_2; Hydrodex *β*-3P; 60 °C (5 min iso), 5 °C/min to 150 °C (5 min iso)]. IR (atr film): υ [cm^{-1}] = 2976, 1765, 1457, 1386, 1356, 1290, 1247, 1205, 1183, 1156, 1120, 1067, 1021, 997, 939; MS (EI, 70 eV): *m*/*z* (%) = 128 (1%) [(M)$^+$], 113 (9) [(M − CH_3)$^+$], 84 (30) [(M − CO_2)$^+$], 69 (78), 56 (100) [($C_4H_8^+$)]. ^{1}H NMR (600 MHz, $CDCl_3$): δ [ppm] = 1.02 (d, 3 H, $^3J_{Me,3}$ = 7.1 Hz, 3-CH_3), 1.16 (d, 3 H, $^3J_{Me,2}$ = 7.6 Hz, 2-CH_3), 1.38 (d, 3 H, $^3J_{Me,4}$ = 6.3 Hz, 4-CH_3), 2.22 (qdd, 1 H, $^3J_{3,2}$ = na, $^3J_{3,3\text{-}Me}$ = na, $^3J_{3,4}$ = na, 3-H), 2.74 (dq, 1 H, $^3J_{2,3}$ = 7.7 Hz, $^3J_{2,2\text{-}Me}$ = 7.7 Hz, 2-H), 4.21 (dq, 1 H, $^3J_{4,3}$ = 6.3 Hz, $^3J_{4,4\text{-}Me}$ = 6.3 Hz, 4-H). ^{13}C NMR (151 MHz, $CDCl_3$): δ [ppm] = 10.10 (2-CH_3), 13.15 (3-CH_3), 19.23 (4-CH_3), 38.49 (2-C), 40.79 (3-C), 81.5 (4-C), 179.18 (-CO_2-).

Deuteration Experiments. Deuterated KP_i-buffer was produced as follows: A 2 M KP_i buffer in deuterated water (0.0908 mol KH_2PO_4, 0.1091 mol K_2HPO_4, and 100 mL D_2O) was prepared resulting in a pD of 7 (measured from an aliquot to prevent introduction of protons from the pH electrode). This solution was concentrated under reduced pressure to quarter volume and refilled with D_2O again. The resulting solution was freeze-dried over 5 days giving a white salt again, from which the deuterated buffers were prepared by adding D_2O. For the deuteration experiment, 40 μL (~0.25 mmol) of (*E*)-**2a** or (*Z*)-**2b** was emulsified in 4 mL of deuterated and normal KP_i-buffer (20 mM, pD or pH = 7), respectively. The emulsion was supplemented with 200 mg (1 mmol, 4 equiv) of glucose-monohydrate, 2 mg (2.6 μmol, 10 mol ‰), 1 U GDH (10 μL in protonated buffer), and 1 U YqjM (10 μL in protonated buffer). The four reaction tubes (15 mL) were incubated and shaken for 24 h at 30 °C. Each reaction was extracted once with 1.5 mL $CDCl_3$, which was dried consecutively by means of a $MgSO_4$ filter. GC-MS and ^{13}C/^{1}H NMR spectra were taken. MS (***H*-3a**|***D*-3a**, EI, 70 eV): *m*/*z* (%) = 158|159 (6) [(M)$^+$], 143|144 (18) [(M − CH_3)$^+$], 113|114 (100) [(M − C_2H_5O)$^+$], 101 (53) [($C_5H_9O_2$)$^+$], 88|89 (31) [($C_4H_8O_2$)$^+$], 73|74 (46) [($C_3H_5O_2$)$^+$]. ^{1}H NMR (***H*-3a**, 600 MHz, $CDCl_3$): δ [ppm] = 1.18 (d, 3 H, $^3J_{Me,2}$ = 6.8 Hz, CH_3), 1.26 (t, 3 H, $^3J_{2'1'}$ = 7.2 Hz, 2′-H), 2.16 (s, 3 H, 5-H), 2.40 (dd, 1 H, $^2J_{3a,3b}$ = 20.7 Hz, $^3J_{2,3a}$ = 8.3 Hz, 3-H_a), 2.92 (m, 2 H, 2-CH, 3-H_b), 4.13 (q, 2 H, $^3J_{1'2'}$ = 7.2 Hz, 1′-H). ^{13}C NMR (***H*-3a**, 151 MHz, $CDCl_3$): δ [ppm] = 14.0 (C-2′), 16.9 (CH_3), 29.9 (C-5), 34.6 (C-2), 46.5 (C-3), 60.5 (C-1′), 175.6 (C-1), 206.6 (C-4). ^{1}H NMR (***D*-3a** from (*E*)-**2a**, 600 MHz, $CDCl_3$): δ [ppm] = 1.18 (d, 3 H, $^3J_{Me,2}$ = 6.8 Hz, CH_3), 1.26 (t, 3 H, $^3J_{2'1'}$ = 7.2 Hz, 2′-H), 2.16 (s, 3 H, 5-H), 2.92 (m, 2 H, 2-CH, 3-Hb), 4.13 (q, 2 H, $^3J_{1'2'}$ = 7.2 Hz, 1′-H). ^{13}C NMR (***D*-3a** from (*E*)-**2a**, 151 MHz, $CDCl_3$): δ [ppm] = 14.0 (C-2′), 16.9 (CH_3), 29.9 (C-5), 46.5 (C-3), 60.5 (C-1′), 175.6 (C-1), 206.6 (C-4). ^{1}H NMR (***D*-3a** from (*Z*)-**2a**, 600 MHz, $CDCl_3$): δ [ppm] = 1.18 (s, 3 H, CH_3), 1.26 (t, 3 H, $^3J_{2'1'}$ = 7.2 Hz, 2′-H), 2.16 (s, 3 H, 5-H), 2.40 (d, 1 H, $^2J_{3a,3b}$ = 17.8 Hz,, 3-H_a), 2.92 (d, $^2J_{3b,3a}$ = 17.8 Hz, 2 H, 2-CH, 3-H_b), 4.13 (q, 2 H, $^3J_{1'2'}$ = 7.2 Hz, 1′-H). ^{13}C NMR (***D*-3a** from (*Z*)-**2a**, 151 MHz, $CDCl_3$): δ [ppm] = 14.0 (C-2′), 16.9 (CH_3), 29.9 (C-5), 34.6 (small, t, C-2), 60.5 (C-1′), 175.6 (C-1), 206.6 (C-4).

In Silico Docking. Atomic substrate models such as (*E*)-**2a** and (*Z*)-**2a** were prepared using Chem3D Pro 12.0 (CambridgeSoft). The structure of YqjM (pdb:1Z42[11]) has been cleaned in UCSF Chimera (V1.8)[23] in terms of removing crystallographic water, counterions, benzaldehyde as well as substituting seleno−methionine by methionine without changing atomic coordinates. The docking was performed using a

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genetic algorithm implemented in AutoDock4.[24] The cubic search space was oriented having a FMN of one monomer in its center and an edge length of 22.5 Å. Best binding modes in terms of estimated binding energies where selected and compiled to Figure 1 using UCSF Chimera (V1.8)[23] again.

■ ASSOCIATED CONTENT

Supporting Information

Protocols for the expression, purification, and complementation of enoate reductases are provided, as are the experimental procedures and analytical data for racemic and enantiomerically pure reference compounds. This information is available free of charge via the Internet at http://pubs.acs.org/.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

ADH_T, alcohol dehydrogenase from *Thermoanaerobacter wiegelii*; ADH_{LK}, alcohol dehydrogenase from *Lactobacillus kefir*; ee, ethyl acetate; FMN, flavin mononucleotide; GC, gas chromatography; GDH, glucose dehydrogenase; GP, general procedure; IMAC, immobilized metal chelate affinity chromatography; KP_i, potassium phosphate; PE, petroleum ether; MTBE, methyl *tert*-butyl ether; RT, room temperature; TEV, tobacco etch virus; THF, tetrahydrofuran; htYqjM, enoate reductase from *B. subtilis*

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