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Supporting Information

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The complete stereochemistry of the enzymatic dehydration of 4-hydroxybutyryl-CoA to crotonyl-CoA

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General

All NMR-spectra were recorded at 300 Mhz for ^{1}H and 75 Mhz for ^{13}C.

1. Elucidation of the Stereochemistry at C-2

Organic syntheses

Experimental for scheme 2:

3-Benzyloxypropionic acid, reaction **I**

Preparation of Jones' reagent: powdered chromium(VI) oxide (13.4 g, 134 mmol) was dissolved in conc. sulfuric acid (11.5 ml). Water was added carefully over 30 min whilst stirring to give a volume of 50 ml.

To 3-benzyloxy-1-propanol (Sigma, 10.0 g, 60 mmol) in 150 ml acetone was added Jones' reagent slowly over 80 min until an orange colour persisted (ca. 37 ml). The reaction was stirred for 15 min and the green-blue precipitate was filtered. The orange filtrate was concentrated under vacuum. To the biphasic residual liquid was added 50 ml ethyl acetate, and the resulting mixture was washed with water (2 × 20 ml) and 20 ml brine, and dried over $MgSO_4$. The solvent was removed to give light yellow/colourless oil (10.7 g, 59.4 mmol, 94% yield). ^{1}H NMR ($CDCl_3$) δ 2.67 (2H, t, J = 6.33, CH_2), 3.76 (2H, t, J = 6.33, OCH_2), 5.56 (2H, s, CH_2O), 7.34 (5H, m, C_6H_5).

3-Benzyloxy-1-[1-$^{2}H_2$]propanol (**3**), reaction **II**

To 3-benzyloxypropionic acid (5.0 g, 27.6 mmol) in 100 ml diethyl ether cooled to 0 °C was added dropwise $LiAlD_4$ in diethyl ether (1 M, 27.3 mmol). The mixture was stirred for 1 h at 0 °C, allowed to warm up to room temperature (10 min), cooled to 0 °C and quenched with 100 ml brine. For easier workup, the mixture was split into two. Each part was extracted with diethyl ether (3 × 60 ml). The combined organic phases were washed in portions of 150 ml with water (2 × 50 ml). The organic phase was dried with $MgSO_4$, filtered and the solvent was removed to give 3.24 g product (22.3 mmol, 81% yield.) ^{1}H NMR ($CDCl_3$) δ 1.90 (2H, t, J = 5.67, CH_2), 3.67 (2H, t, J = 5.76, OCH_2), 4.53 (2H, s, CH_2O), 7.34 (5H, m, C_6H_5).

3-Benzyloxy[1-^{2}H]propanal (**4**), reaction **III**

To oxalyl chloride (1.84 ml, 21.3 mmol) in dry 60 ml dichloromethane stirred at –80 °C under N_2 was added dropwise 3.03 ml DMSO (42.7 mmol). After 15 min 1.72 ml 3-benzyloxy-1-[1-$^{2}H_2$]propanol (10.8 mmol) in 15 ml dry DCM was added over 5 min. Stirring was continued at –80 °C for 15 min. More 3-benzyloxy-1-[1-$^{2}H_2$]-propanol (7.41 ml, 53.6

mmol) was added dropwise. After 10 min the cooling was removed and stirring was continued at room temperature for 2 h. Water (10 ml) was added and the mixture was stirred for 10 min. The two layers were separated and the aqueous phase was extracted with DCM (2 × 10 ml). The combined organic layers were washed with brine (2 × 10 ml), dried with $MgSO_4$, filtered and the solvent was removed to give 2.7 g crude product (16.4 mmol), which was used directly in the next step assuming 100% yield.

(*S*)-3-Benzyloxy[1-$^2H_1$]propan-1-ol (*S*-**3**)[1], reaction **IV**

R-ALPINE BORANE™ solution was cooled to –78 °C (1.1 equivalent, 23.8 ml, 11.9 mmol) under N_2 while stirring for 15 min. 3-Benzyloxy[1-$^2H_1$]propanal (10.8 mmol, assuming 100% yield of **4** from **III**) in 10 ml THF was added drop-wise. The reaction was stirred for 3 h at –78 °C. The resulting slightly yellow solution was brought to 0 °C and 977 µl ethanolamine (1.5 equiv., 16.3 mmol) was added dropwise. Stirring was continued at 0 °C for 30 min before quenching with 10 ml water. The phases were separated and the aqueous phase was extracted with diethyl ether (3 × 20 ml). The combined organic layers were washed with 50 ml 1 M H_2SO_4, dried with $MgSO_4$, filtered and the solvent was removed to give the 6.15 g crude product. Two consecutive columns with silica gel [elution with diethyl ether/petrol (1:2)] gave 1.0 g colourless oil (6.0 mmol, 55% yield). ^{1}H NMR ($CDCl_3$) δ 1.87 (2H, dt, J = 5.67, 5.71, CH_2), 3.67 (2H, t, J = 5.85, OCH_2), 3.78 (1H, m, CD*H*OH), 4.53 (2H, s, CH_2O), 7.34 (5H, m, C_6H_5); ^{13}C ($CDCl_3$) NMR δ 32.04 (CH_2), 61.60 (t, J = 21.96, CDHOH), 69.39 (OCH_2), 73.30 ($C_6H_5CH_2O$), 127.64, 127.71, 128.45, 138.11 (C_6H_5).

(*R*)-3-Benzyloxy[1-$^2H_1$]propan-1-ol (*R*-**3**)

The preparation was done in a similar manner to that of (*S*-**3**) using *S*-ALPINE BORANE™ to yield 0.98 g colourless oil (5.87 mmol, 54% yield). ^{1}H NMR was identical to that of the (*S*)-isomer.

(*S*)-3-Benzyloxy[1-$^2H_1$]propanoyl tosylate, reaction **V**

To (*S*)-3-benzyloxy[1-$^2H_1$]propan-1-ol (1.0 g, 5.99 mmol, 957 µl) in dry pyridine (6 ml), cooled to 0 °C, was added 4.13 g *p*-toluenesulfonyl chloride (3.63 equivalents, 21.7 mmol) and the reaction was stirred for 1 h. The mixture was quenched in 20 ml ice water and extracted with diethyl ether (4 × 10 ml). The combined ether phases were washed with 3 M HCl (2 × 5 ml), dried with $MgSO_4$, filtered and the solvent was removed to give 1.61 g tosylate (5.01 mmol, 84% yield). ^{1}H NMR ($CDCl_3$) δ 1.94 (2H, dt, J = 5.98, 6.00, CH_2), 2.43 (3H, s, CH_3), 3.50 (2H, t, J = 5.95, OCH_2), 4.15 (1H, m, CD*H*OS), 4.40 (2H, s, CH_2O), 7.59 (9H, m, C_6H_4, C_6H_5).

(*R*)-3-Benzyloxy[1-$^2H_1$]propanoyl tosylate

(*R*)-3-Benzyloxy[1-$^2H_1$]propan-1-ol (*R*-**3**) (1.0 g, 5.99 mmol, 957 µl) was reacted in a similar manner to that described above to give 1.34 g tosylate (4.17 mmol, 71% yield). ^{1}H NMR ($CDCl_3$) δ 1.94 (2H, dt, J = 5.98, 5.95, CH_2), 2.42 (3H, s, CH_3), 3.50 (2H, t, J = 5.95, OCH_2), 4.16 (1H, m, CDHOS), 4.40 (2H, s, CH_2O), 7.52 (9H, m, C_6H_4, C_6H_5); ^{13}C ($CDCl_3$) NMR δ 21.60 (CH_2), 29.28 (CH_3), 65.67 (OCH_2), 67.39 (t, J = 22.54, CDHOS), 73.06 ($C_6H_5CH_2O$), 127.54, 127.64, 127.90, 128.37, 129.81, 129.87, 133.17, 138.09, 144.66 (C_6H_5, C_6H_4).

(*R*)-4-Benzyloxy[2-$^2H_1$]butane nitrile (*R*-**5**), reaction **VI**

Ice-cold saturated NaCN in 40 ml dimethylformamide (DMF) was added to 1.61 g *S*-tosylate (5.0 mmol). Solid NaCN (ca. 2 g) was added and the mixture was stirred at room temperature for 24 h. The resulting slurry was diluted with 100 ml ice water and extracted with diethyl ether (5 × 30ml). The combined ether phases were washed with water (2 × 25

ml), saturated $KHCO_3$ (25 ml) and brine (25 ml). After drying with $MgSO_4$ and filtration the solvent was removed to give 0.90 g nitrile (5.1 mmol, 100%). ^{1}H NMR ($CDCl_3$) δ 1.94 (2H, dt, J = 6.36, 6.09, CH_2), 2.49 (1H, m, CDHCN), 3.59 (2H, t, J = 5.76, OCH_2), 4.52 (2H, s, CH_2O), 7.33 (5H, m, C_6H_5); ^{13}C ($CDCl_3$) NMR δ 25.41 (CH_2), 32.43 (t, J = 19.36, CDH), 69.34 (OCH_2), 73.01 ($C_6H_5CH_2O$), 127.72, 128.49, 138.31 (C_6H_5) 175.07 (CN).

(*S*)-4-Benzyloxy[2-$^2H_1$]butane nitrile (*S*-**5**)

The *R*-tosylate was reacted in the manner described above to give 0.81 g nitrile (4.6 mmol, 100%). The ^{1}H and ^{13}C NMR spectra were identical to those of the (*R*)-isomer.

(*R*)-4-Benzyloxy[2-$^2H_1$]butyryl amide (*R*-**6**), reaction VII[2]

Acetamide (1.169 g, 19.79 mmol) and 102 mg Pd(II) acetate (0.45 mmol) were dissolved in a mixture of 15 ml tetrahydrofuran (THF) and 5 ml water. Nitrile (*R*-**5**) (900 mg, 818 µl, 5.11 mmol) was added and the reaction was stirred for 2 days. The mixture was diluted with 10 ml diethyl ether, washed with saturated $KHCO_3$ (2 × 5 ml), dried with $MgSO_4$, filtered and the solvent was removed to give 1.1 g crude yellow semi-crystalline amide. Recrystallisation from diethyl ether/petrol gave 360 mg white crystals (1.85 mmol, 36% yield) and mother liquor containing unreacted starting material. The mother liquor was reacted as described above (623 mg acetamide, 55 mg Pd acetate, in 7.5 ml THF and 2.5 ml water for 2 days). Workup gave crude 0.4 g product (2.05 mmol, 75% yield). Recrystallisation from diethyl ether/petrol gave 170 mg white crystals (0.87 mmol). The combined crystalline products (500 mg, 2.56 mmol, 50% yield) were used in the next step. ^{1}H NMR ($CDCl_3$) δ 1.95 (2H, dt, J = 6.42, 6.18, CH_2), 2.35 (1H, m, CD*H*$CONH_2$), 3.55 (2H, t, J = 5.95, OCH_2), 4.51 (2H, s, CH_2O), 5.45 (2H, m, NH_2), 7.34 (5H, m, C_6H_5); ^{13}C ($CDCl_3$) NMR δ 25.41 (CH_2), 32.43 (t, J = 19.36, CDH), 69.34 (OCH_2), 73.0075 ($C_6H_5CH_2O$), 127.72, 128.45, 138.31 (C_6H_5) 175.07 ($CONH_2$).

(*S*)-4-Benzyloxy[2-$^2H_1$]butyryl amide (*S*-**6**)[2]

Using similar conditions as given above but with nitrile (*S*-**5**) gave 510 mg combined crystalline fractions (2.62 mmol, 57% yield), which were used in the next step. The ^{1}H and ^{13}C NMR spectra were identical to those of the (*R*)-isomer.

(*R*)-4-Hydroxy[2-$^2H_1$]butyryl amide, reaction **VIII**

To (*R*)-4-benzyloxy[2-$^2H_1$]butyryl amide (*R*-**6**) (500 mg, 2.56 mmol) in 7.5 ml methanol was added 621 mg palladium catalyst (10% Pd/C) and the headspace was exchanged four times with hydrogen while stirring. The reaction was left overnight at room temperature stirring under hydrogen. The suspension was filtered through silica and the solvent was removed to yield 0.26 g colourless oil (2.5 mmol, 98% yield), which was directly used for next step. NMR-characterisation was done with the *S*-isomer (see below).

(*S*)-4-Hydroxy[2-$^2H_1$]butyryl amide

Recrystallised (*S*)-4-benzyloxy[2-$^2H_1$]butyryl amide (*S*-**6**) (510 mg, 2.62 mmol) was reacted in a similar manner to that described above to yield 0.25 g colourless oil (2.4 mmol, 92% yield). ^{1}H NMR (D_2O) δ 1.70 (2H, dt, J = 6.74, 6.75, CH_2), 2.21 (1H, m, CD*H*$CONH_2$), 3.49 (2H, t, J = 6.52, HOCH_2); ^{13}C ($CDCl_3$) NMR δ 27.58 (CH_2), 31.33 (t, J = 19.94, CDH), 60.86 (OCH_2), 179.53 ($CONH_2$).

(*R*)-γ-[2-$^2H_1$]butyrolactone (2*R*-**7**), reaction **IX**

(*R*)-4-Hydroxy[2-$^2H_1$]butyryl amide (0.26 g, 2.5 mmol) was dissolved in 6 ml 1 M HCl and stirred vigorously with 15 ml DCM overnight. The DCM was removed and another 15 ml DCM was added and the mixture was stirred vigorously.

This action was repeated once more. The three organic layers were combined, dried with $MgSO_4$, filtered and the solvent was removed to give 250 mg (*R*)-lactone (2.87 mmol, 100% yield). ^{1}H NMR ($CDCl_3$) δ 2.26 (2H, dt, *J* = 7.99, 7.27, CH_2), 2.49 (1H, m, CD*H*CO_2), 4.3567 (2H, t, *J* = 6.99, OCH_2); ^{13}C ($CDCl_3$) NMR δ 22.0779 (CH_2), 27.48 (t, *J* = 20.52, CDH), 68.44 (*C*H_2CO), 177.62 (COO).

(*S*)-γ-[2-$^2H_1$]butyrolactone (2*S*-**7**)

(*S*)-4-Hydroxy[2-$^2H_1$]butyryl amide (0.25 g, 2.4 mmol) was lactonised as described above to give 250 mg (*S*)-lactone (2.87 mmol, 100% yield). The ^{1}H and ^{13}C NMR spectra were identical to those of the (*R*)-isomer.

(*R*)-[$^2H_1$]Succinic acid, reaction **X**

(*R*)-γ-[2-$^2H_1$]butyrolactone (2*R*-**7**) (22.6 mg, 0.26 mmol) was stirred for 5 min with 0.1 ml 5 M NaOH. Sodium phosphate buffer (1 ml, 0.67 M) was added and the pH was adjusted to 6.8 (5 M HCl). Acetonitrile (1.33 ml) and TEMPO (3.3 mg, 0.182 mmol) were added and the mixture was heated to 35 °C. Over a period of 2.5 h sodium hypochlorite solution (0.33 ml in 1.83 ml H_2O, 0.24 mM) and $NaClO_2$ (60 mg, 0.53 mmol in 260 µl H_2O) were added dropwise from separate syringes at 35°C while stirring (Caution: do not mix sodium hypochlorite solution and $NaClO_2$ before adding to the reaction!). At room temperature, the pH was adjusted to 8.5 with 5 M NaOH. The mixture was quenched with Na_2SO_3 (1.33 ml, 0.24 g in 4 ml water) at 0 °C and the resulting solution was stirred at room temperature for 0.5 h (pH 8.5- 9.0). After acidification to pH 1 and extraction with methyl tert-butyl ether (3 × 5 ml), the combined extracts were dried over $MgSO_4$, filtered and the solvent was removed to give 16 mg white crystalline solid (0.13 mmol, 52% yield) ^{1}H NMR (D_2O) δ 2.53 (2H, s, CH_2), 2.54 (1H, m, CD*H*); ^{13}C (D_2O) NMR δ 28.50 (t, *J* = 19.65, CDH), 28.70 (CH_2), 177.01 (COO). CD: Θ = – 4.0 ± 0.4 mDeg / 0.1 cm.

(*S*)-[$^2H_1$]Succinic acid

(*S*)-γ-[2-$^2H_1$]Butyrolactone (2*S*-**7**) was oxidized as described above to give a white 18 mg crystalline solid (0.15 mmol, 58% yield). The ^{1}H NMR spectrum was identical to that of the (*R*)-isomer. CD: Θ = + 4.5 ± 0.4 mDeg / 0.1 cm.

Both succinic acids were analysed by CD-spectroscopy (JASCO J-810 Spectropolarimeter, Jasco Labor und Datentechnik GMBH, Germany, d = 0.1 cm, 24 °C, 4.0 mg /ml, pH = 2.5, λ = 206 nm).

Enzymatic methods

The purifications of 4-hydroxybutyrate CoA-transferase[3] and 4-hydroxybutyryl-CoA dehydratase[4] from *Clostridium aminobutyricum* were performed as described; 1 unit (U) is defined as conversion of 1 µmol substrate min^{-1}.

Enzymatic synthesis of 4-hydroxybutyryl-CoA (**1**): The lactone (**7**, 0.1 mmol) hydrolysed with 1 ml 0.1 M NaOH for 5 min at r. t. was incubated with 100 mM potassium phosphate pH 7.4, 2.5 mM acetyl-CoA, 5 U 4-hydroxybutyrate CoA-transferase in a total volume of 1 ml for 20 min at 37 °C. The reaction was acidified with 0.2 ml 5 M HCl to pH 2 and passed through a C-18 column (50 mg, SepPak™, Waters), which was conditioned with 500 µl methanol and washed with 1 ml 0.1% trifluoroacetic acid (TFA). After loading the sample, the column was washed with 1 ml 0.1% TFA. The CoA thioesters were eluted with 50% acetonitrile in 0.1% TFA and analysed by MALDI-TOF MS.

Equilibration with 4-hydroxybutyryl-CoA dehydratase: before passing the acidified reaction mixture (see above) through the C-18 column, 0.5 ml of the solution were neutralised with 0.1 ml 5 M NaOH and, after addition of 6 U 4-hydroxybutyryl-CoA dehydratase, incubated for 20 min at 37 °C. The CoA thioesters were purified as described above.

2. Chiral Methyl Experiments

Organic synthesis

Ethyl [^{3}H]formate:

To unlabeled sodium formate (1.7 g, 25 mmol) and sodium [^{3}H]formate (500 μCi, 2 Ci/mmol) in 75 ml ethanol was added 2 ml conc. sulfuric acid and the solution was stirred for 2 days at room temperature in a sealed flask. The reaction mixture was distilled at atmospheric pressure with a −80 °C cooling bath applied to the collecting flask. The product distilled off (52 -75 °C): 5.4 g (contains ethanol, 58.7 μCi, 12% radiochemical yield). No NMR spectra were taken because of the presence of tritium.

Diethyl [^{2}H]formylsuccinate[5-7]

To sodium ethoxide (2.28 g, 33.5 mmol) in 6 ml dry toluene cooled to 0°C were added a mixture of 4.73 g diethyl succinate (27.5 mmol) and 2.05 g commercially available methyl [^{2}H]formate (Sigma-Aldrich, 33.5 mmol) portion-wise while stirring. After 72 h at room temperature 5 g crushed ice was added and acidified with H_2SO_4 (pH 1). The aqueous layer was extracted with diethyl ether (2 × 30 ml). The ether extracts were combined with the toluene solution and washed to neutrality with 1 M $KHCO_3$ (2 × 25 ml) followed by water (2 × 25 ml). The solution was dried ($MgSO_4$), filtered and the solvent was removed to yield 7.77 g crude yellow oil. Purification on a silica column with petrol (40-60)/ethyl acetate (3:2) as eluent (R_f = 0.71) gave 4.58 g colorless oil (22.6 mmol, 83% yield). The NMR data showed a keto-enol tautomeric distribution of 3:2. ^{1}H NMR ($CDCl_3$): keto tautomer, δ 4.33 – 4.07 (4H, m, 2 × C*H*$_2$CH$_3$), 3.70 (1H, m, C*H*CDO), 2.92 + 2.90 (2H, d, *J* = 6.04 – 6.23, CH_2), 1.2 (6H, m, 2 × CH$_2$C*H*$_3$); enol tautomer, 4.33 – 4.07 (4H, m, 2 × C*H*$_2$CH$_3$), 3.05 (2H, s, CH_2), 1.26 (6H, m, 2 × CH$_2$C*H*$_3$). The unlabeled ester (<10%) gave additional signals (see below).

Diethyl formylsuccinate [5, 6, 7]

In a similar manner 4.17 g unlabeled diethyl formylsuccinate was obtained (20.64 mmol, 75% yield). The NMR data showed a mixture of keto and enol forms in a 3:2 ratio. ^{1}H NMR ($CDCl_3$): keto tautomer, δ 9.92 (1H, s, CHO), 4.33 – 4.08 (4H, m, 2 × C*H*$_2$CH$_3$), 3.79 (1H, t, *J* = 6,14 C*H*CHO), 2.92 + 2.90 (2H, d, *J* = 6.04 – 6.23, CH_2), 1.28 (6H, m, 2 × CH$_2$C*H*$_3$); enol tautomer, δ 11.53 (H$_Z$, d, *J* = 12.65, C*H*COH), 7.09 (H$_E$, d, *J* = 12.65, C*H*COH), 4.33 – 4.08 (4H, m, 2 × C*H*$_2$CH$_3$), 3.05 (2H, s, CH_2), 1.28 (6H, m, 2 × CH$_2$C*H*$_3$). ^{13}C NMR ($CDCl_3$): both tautomers, δ 195.74 (CHO), 167.97 (CH*C*OOCH$_2$CH$_3$), 162.63 (CH$_2$*C*OOCH$_2$CH$_3$), 61.10 (CH), 54.03 (CH_2), 33.01 (CHCOO*C*H$_2$CH$_3$), 29.95 (CH$_2$COO*C*H$_2$CH$_3$), 14.14 (CHCOOCH$_2$*C*H$_3$), 14.08 (CH$_2$COOCH$_2$*C*H$_3$).

Diethyl [^{3}H]formylsuccinate was similarly synthesized from 3.92 g ethyl [^{3}H]formate (19.4 mmol, 97% yield). The specific activity of the product was determined to be 1914 cpm/μmol (0.9 μCi/mmol, 1.5% radiochemical yield). No NMR spectra were taken because of the presence of tritium.

γ-Ethoxy[4-^{2}H]butyrolactone[5, 6, 8]

In a 50 ml Claisen flask were placed 4.58 g diethyl [^{2}H]formylsuccinate (22.6 mmol), 4.6 g ethyl orthoformate (31.0 mmol), 55 mg *p*-toluenesulfonic acid (0.51 mmol) and 3 ml ethanol. The mixture was heated at 60 °C for 2 h whereby some of the ethyl formate was distilled off. A solution of 3.83 g KOH pellets (72 mmol) in 25 ml ethanol was added to the cooled reaction mixture and heated to reflux for 2 h. The ethanol was removed under reduced pressure and the resulting potassium salt was dissolved in 10 ml D_2O. The aqueous phase was washed with diethyl ether (2 × 10 ml) to elimi-

nate neutral by-products, acidified with conc. H_2SO_4 (to minimize H_2O content) to pH = 1 and extracted with diethyl ether (3 × 20 ml). The combined organic phases were dried with $MgSO_4$ and the solvent was removed to give 2.8 g crude oil (21.5 mmol, 95% yield). This was transferred to a 25 ml Claisen flask fitted with a short Vigreux column and distilled at water pump pressure. Two fractions were collected after removal of ethanol and decarboxylation (occurring at 95 °C): 1st at 120-130 °C as 0.26 g slightly yellow liquid (1.98 mmol, 9% yield); 2nd residues in the Vigreux column after cooling down (not in the reservoir flask) as 0.23 g colourless liquid (1.76 mmol, 8% yield); ^{1}H NMR ($CDCl_3$) δ from fraction 1: 3.87 + 3.61 (2H, m, C*H*$_2$CH$_3$), 2.67 (1H, m, H-3), 2.41 (2H, m, H-3 und H-4), 2.14 (1H, m, H-4), 1.24 (3H, t, *J* = 6.99, CH$_2$C*H*$_3$); ^{13}C ($CDCl_3$) NMR δ 176.70 (CO), 103.97 (t, *J* = 26, C-4), 65.10 (d, *J* = 4.05, *C*H$_2$CH$_3$), 28.75 (d, *J* = 10.4, C-3), 26.80 (t, *J* = 6.36, C-2), 14.90 (CH$_2$*C*H$_3$). The ^{1}H-NMR spectrum shows that the acylal hydrogen (H-5) is missing, because it is almost completely substituted by deuterium. Hydrogens 1-4 are partially deuterated, also indicated by the couplings observed in the ^{13}C-NMR spectrum. The other fraction was characterized by formation of succinic semialdehyde after hydrolysis. The aldehyde was determined enzymatically with NADH and 4-hydroxybutyrate dehydrogenase.

Unlabeled γ-ethoxybutyrolactone was synthesized in the same manner (240 mg, 1.83 mmol, 8.9% yield) ^{1}H NMR ($CDCl_3$) δ 5.47 (1H, m, H-5), 3.80 + 3.54 (2H, m, C*H*$_2$CH$_3$), 2,59 (1H, m, H-3), 2.37 (2H, m, H-3 und H-4), 2.07 (1H, m, H-4), 1.17 (3H, t, *J* = 7.18, CH$_2$C*H*$_3$); ^{13}C NMR ($CDCl_3$) δ 175.58 (CO), 103.07 (C-4), 64.20 (*C*H$_2$CH$_3$), 27.82 (C-3), 25.83 (t, *J* = 6.36, C-2), 13.91 (CH$_2$*C*H$_3$).

For the synthesis of 108 mg tritium labeled γ-ethoxybutyrolactone, diethyl [^{3}H]formylsuccinate was used (831 µmol, specific activity 2719 cpm/µmol, 1.2 µCi/mmol, 100% yield). 4-Oxo[4-^{3}H]butyric acid was prepared by hydrolysis of this lactone with water (see below).

4-Oxo[4-^{2}H]butyric acid, [4-^{2}H]succinic semialdehyde[5]

γ-Ethoxy[4-^{2}H]butyrolactone (1.33 µl, 10 µmol) was hydrolysed in 100 µl H_2O for 40 min at room temperature to 4-oxo[4-^{2}H$_1$]butyric acid and ethanol. The ethanol content was lowered under reduced pressure. Caution: The 4-oxobutyric acid must not be concentrated too much for it can polymerize very easily. The residual aqueous solution was directly used for the next step. ^{1}H NMR (D_2O) δ 3.49 (2H, q, *J* = 7.11, HOC*H*$_2$CH$_3$), 2.30 (2H, m, CH$_2$C*H*$_2$COOH), 1.73 (2H, m, C*H*$_2$CH$_2$COOH), 1.02 (3H, t, *J* = 7.08, HOCH$_2$C*H*$_3$).

4-Oxobutyric acid

Unlabeled 4-oxobutyric acid was prepared similarly from γ-ethoxybutyrolactone. ^{1}H NMR (D_2O) δ 4.89 (1H, m), 3.49 (2H, q, *J* = 7.11, HOC*H*$_2$CH$_3$), 2.31 (2H, m, CH$_2$C*H*$_2$COOH), 1.74 (2H, m, C*H*$_2$CH$_2$COOH), 1.02 (3H, t, *J* = 7.18, HOCH$_2$C*H*$_3$); ^{13}C NMR (D_2O) δ 177.01 (CO), 90.06 (hydrate CH(OH)$_2$), 57.45 (HO*C*H$_2$CH$_3$), 32.60 (*C*H$_2$CH$_2$COOH), 29.58 (CH$_2$*C*H$_2$COOH), 16.80 (HOCH$_2$*C*H$_3$).

Enzymatic methods

Acetyl-CoA synthase (Sigma-Aldrich), alcohol dehydrogenase from horse liver (ADH, Sigma-Aldrich) and fumarase (Roche/Boehringer Mannheim) were commercially available. 4-Hydroxybutyrate dehydrogenase (4-HBDH) was purified from *C. aminobutyricum*.[9, 10] A C-terminal His-tagged version of malate synthase G from *Escherichia coli* was obtained by overexpression of p-MSG-B in *E. coli* Bl 21 and purification by affinity chromatography on a Ni-NTA Aga-

rose column.[11] The concentration of 4-hydroxybutyrate was determined enzymatically using 4-HBDH, NAD^+, iodonitrosotetrazolium chloride, and Meldola Blau, cf.[12]

(*R*)-γ-[4-^{3}H,4-$^2H_1$]Butyrolactone

A cuvette (d = 1 cm) contained in total volume of 1.0 ml 100 mM potassium phosphate pH 7.4, 10 mM 4-oxo[4-^{2}H]butyric acid, 0.25 mM NAD^+, 0.11 µmol [^{3}H]formate (20 µCi), and 7 U formate dehydrogenase. When the absorbance at 340 nm reached 0.8, addition of 4-HBDH (0.8 U raised to 4 U continually over time) caused a decrease in the absorbance to 0.1. Further addition of unlabeled formate (19 µmol) and formate dehydrogenase (3 U) stabilized the absorbance at 0.6. The reaction mixture was incubated at ambient temperature for 1 h. When the aldehyde was completely reduced, the absorbance rose again. The reaction mixture was acidified and the product was extracted into DCM (5 × 3 ml), yield 9.72 µmol, 420 µCi/mmol. In a similar reaction 4-HBDH replaced alcohol dehydrogenase from horse liver, yield 3.75 µmol, 189 µCi/mmol.

(*S*)-γ-[4-^{3}H,4-$^2H_1$]Butyrolactone

The reaction was done as described above but using 26 mg 4-oxo[4-^{3}H]butyric acid (200 µmol) and 1 mmol [^{2}H]formate in the reaction. The yield of the reaction with 4-hydroxybutrate dehydrogenase was 60.6 µmol (specific activity 2.0 µCi/mmol). When alcohol dehydrogenase from horse liver replaced 4-hydroxybutrate dehydrogenase, 39 µmol (specific activity, 1.1 µCi/mmol) were obtained.

(*R*)-γ-[4-$^2H_1$]Butyrolactone

The reaction was done as described above but 147 µmol unlabeled 4-oxobutyric acid (100 µl of an 15% aqueous solution) and 1.0 mmol [^{2}H]formate (75 µl) were used, yield 18 mg (contains denatured proteins, maximum theoretical yield 13 mg). ^{1}H NMR ($CDCl_3$) δ 4.34 (1H, tt, *J* = 7.05, 1.35, CD*H*O), 2.50 (2H, td, *J* = 8.12, 0.94, CH_2COO), 2.26 (2H, qd, *J* = 7.81, 0.94, CH_2); ^{13}C NMR ($CDCl_3$) δ 176.66 (COO), 67.16 (t, *J* = 23.12, CDHO), 26.77 (CH_2COO), 21.05 (CH_2).

[^{3}H,$^2H_1$]Acetic acid from (*R*)-γ-[4-^{3}H,4-$^2H_1$]butyrolactone:

(*R*)-γ-[4-^{3}H,4-$^2H_1$]Butyrolactone (9.72 µmol, 116 µCi/mmol, generated with 4-hydroxybutyrate dehydrogenase) was converted to the 4-hydroxybutyric acid sodium salt with equimolar sodium hydroxide and dissolved in 1.0 ml 100 mM potassium phosphate pH 7.4, 2 mM EDTA, 2 mM dithiothreitol, 0.1 mM coenzyme A, 0.1 mM acetyl phosphate, 11.1 mM NAD^+ and 0.5 mM acetyl coenzyme A. The reaction was catalyzed by an enzyme mixture from *Acidaminococcus fermentas* [crotonase (**III**, Scheme 4), (*S*)-3-hydroxybutyryl-CoA dehydrogenase (**IV**), thiolase (**V**), phosphotransacetylase (**VI**); 0.2 mg protein],[13] 0.6 U 4-hydroxybutyrate CoA-transferase and 0.5 U 4-hydroxybutyryl-CoA dehydratase, both from *C. aminobutyricum*. The acetic acid produced was isolated by steam distillation at pH 1. The collecting flask contained 1 ml, 0.1 M $NaHCO_3$. The condensate was brought to pH 10 with NaOH and dried under reduced pressure to give 4.9 µmol sodium acetate (specific activity 42 µCi/mmol). A similar reaction was done with γ-butyrolactone (53 µCi/mmol) generated with horse liver alcohol dehydrogenase yielding 4.5 µmol sodium acetate (specific activity 11 µCi/mmol). Acetate was determined enzymatically using ATP, phosphoenolpyruvate, NADH, acetyl-CoA synthase, myokinase, pyruvate kinase and lactate dehydrogenase.[14] An error occurs through the addition of 0.6 µmol of unlabeled acetate via acetyl-CoA and acetylphosphate. The specific activities of the acetates deriving from the lactones are therefore corrected to be 47 µCi/mmol (4-HBDH) and 13 µCi/mmol. The unexpected loss of specific activ-

ity can be explained by the presence of [^{3}H]formate used to introduce the label into 4*R*-7. This exess tritum is eliminated at the stage of malate by purification over a Dowex 1 x 8 column.[15-17]

From (*S*)-γ-[4-^{3}H,4-^{2}H$_1$]butyrolactone:

A similar set of reactions was repeated with (*S*)-γ-[4-^{3}H,4-^{2}H$_1$]butyrolactone generated with 4-hydroxybutyrate dehydrogenase (60.6 µmol, specific activity 2.0 µCi/mmol) and alcohol dehydrogenase (39 µmol, specific activity 1.1 µCi/mmol), respectively. The yields achieved with 4-hydroxybutyrate dehydrogenase were 7.3 µmol (specific activity, 1.1 µCi/mmol) and with alcohol dehydrogenase 12.1 µmol (specific activity 0.50 µCi/mmol).

Butyrolactones	Enzyme used for lactone production	specific Activities [µCi / mmol]		
		lactones	acetates	malates (before fumarase)
(*R*)-γ-[4-^{3}H,4-^{2}H$_1$]-**7**	4-HBDH	116	42	17.8
(*R*)-γ-[4-^{3}H,4-^{2}H$_1$]-**7**	ADH	53	11.1	not determined
(*S*)-γ-[4-^{3}H,4-^{2}H$_1$]-**7**	4-HBDH	2.0	1.1	0.9
(*S*)-γ-[4-^{3}H,4-^{2}H$_1$]-**7**	ADH	1.1	0.5	not determined

Table 1. Specific activities during the course of the chiral-methyl experiment.

The stereochemical analyses of the chiral acetates were done enzymatically via acetyl-CoA (acetyl-CoA synthetase) condensation with glyoxylate to (*S*)-malate (malate synthase) and further equilibration with fumarase using protocols given in previous publications.[15-17]

References

[1] A. M. Valentine, B. Wilkinson, K. E. Liu, S. Kornar-Panicucci, N. D. Priestley, P. G. Williams, H. Morimoto, H. G. Floss, S. J. Lippard, *J. Am. Chem. Soc.* **1997**, *119*, 1818.
[2] S. I. Maffioli, E. Marzorati, A. Marazzi, *Org Lett* **2005**, *7*, 5237.
[3] U. Scherf, W. Buckel, *Appl Environ Microbiol* **1991**, *57*, 2699.
[4] U. Scherf, W. Buckel, *Eur J Biochem* **1993**, *215*, 421.
[5] U. Näser, PhD thesis, University of Newcastle upon Tyne (Newcastle upon Tyne, UK), **2004**.
[6] C. G. Wermuth, *J. Org. Chem.* **1979**, *44*, 2406.
[7] Carriere, *Ann.Chim.(Paris)* **1922**, *17*, 47.
[8] L. Claisen, *Ber. Dtsch. Chem. Res.* **1907**, *40*, 3903.
[9] J. K. Hardman, *Methods Enzymol* **1962**, *5*, 778–783.
[10] A. Gerhardt, I. Cinkaya, D. Linder, G. Huisman, W. Buckel, *Arch Microbiol* **2000**, *174*, 189.
[11] B. R. Howard, J. A. Endrizzi, S. J. Remington, *Biochemistry* **2000**, *39*, 3156.
[12] H.-O. Beutler, G. Michal, in *Methods of Enzymatic Analysis, Vol. II*, 3rd ed. (Ed.: H. U. Bergmeyer), VCH, Weinheim, **1974**, pp. 1753.
[13] W. Buckel, *Methods Enzymol.* **1986**, *125*, 547.
[14] H.-O. Beutler, in *Methods of Enzymatic Analysis, Vol. VI*, 3rd ed. (Ed.: H. U. Bergmeyer), VCH, Weinheim, **1974**, p. 639.
[15] J. W. Cornforth, J. W. Redmond, H. Eggerer, W. Buckel, C. Gutschow, *Nature* **1969**, *221*, 1212.
[16] J. Lüthy, J. Retey, D. Arigoni, *Nature* **1969**, *221*, 1213.
[17] A. J. Pierik, D. Ciceri, G. Bröker, C. H. Edwards, W. McFarlane, J. Winter, W. Buckel, B. T. Golding, *J Am Chem Soc* **2002**, *124*, 14039.