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Preparation of (*S*)-2-Substituted Succinates by Stereospecific Reductions of Fumarate and Derivatives with Resting Cells of *Clostridium formicoaceticum*

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Abstract: Fumarate derivatives have been reduced to (*S*)-2-methylsuccinate **2a**, (*S*)-2-ethylsuccinate **3a** and (*S*)-2-chlorosuccinate **4a** in up to 1 M concentrations with *Clostridium formicoaceticum*. Formate was the electron donor and viologens or anthraquinone-2,6-disulphonate acted as artificial electron mediators. Reductions with freeze-dried cells in ^2H_2O-buffers led to the (2*R*,3*S*)-[2,3-^{2}H]-dideuterated succinate derivatives. The productivity numbers[1] ranged from 450 to 5000 and the enantiomeric excess of all (*S*)-2-substituted succinates was ≥ 99 %.

Introduction

2-Alkylsuccinates are synthons for natural and other chiral products[2,3]. The synthesis of (*R*)-, (*S*)-2-methylsuccinate **2a** and (*S*)-2-ethylsuccinate **3a** by enantioselective homogeneous catalysis with transition metal compounds[4a,b] is well known. The ee values range from 3 to 99 %. **2a** was also prepared by enzymatic regio- and stereoselective ester hydrolysis[5a,b] or microbiological reduction[6]. Both enantiomers of 2-chlorosuccinate can be obtained by substitution of the hydroxyl groups of (*R*)- or (*S*)-malate with chlorine[7].

We describe the preparative reduction of fumarate and of 2-substituted fumarates in the ordinary way or in ^2H_2O-buffer with *Clostridium formicoaceticum* at the expense of formate. In addition the substrate specificity of fumarate reductase was examined, because only fumarate **1** and 2-methylfumarate **2** have been described as substrates[6], whereas (*S*)-2-chlorosuccinate or 2-alkylsuccinates (R = methyl, ethyl) are substrates of succinate dehydrogenase[8a,b]. However, these studies were not conducted under preparative conditions. *C. formicoaceticum* was also used for the reduction of dimethyl 2-methylfumarate **5**, dimethyl fumarate and dimethyl maleate. The reduction of the last two dimethyl esters was not interesting for preparative purposes. *C. formicoaceticum* cells have to be grown on a fructose/fumarate medium to increase fumarate reductase activity[9].

Results and discussion

Synthesis of fumaric and maleic acid derivatives. 2-Alkylfumaric acids can be obtained in good yields by isomerization of 2-alkylmaleic acids[10a,c,d]. Diethyl 2-alkylmaleates (R = ethyl, propyl, *iso*-propyl) and diethyl 2-phenylmaleate were prepared from ethyl 2-oxocarboxylates and the ylide of triethyl

phosphonoacetate by Horner-Emmons reaction[11a,b]. The ratio of the formed diethyl esters of the maleate, fumarate and itaconate derivatives, the yield of the Horner-Emmons reactions and the yield of the isomerization of 2-alkyl- and 2-phenylmaleic acids are shown in Table 1.

Table 1: Ratio of the formed diethyl esters of 2-alkyl- and 2-phenylmaleic, -fumaric and 2-alkylidene-succinic acids by Horner-Emmons reaction.

R[a)]	Method[b)]	2-Alkylmaleate	2-Alkylfumarate	2-Alkylidenesuccinate	Yield[c)]	Yield[d)]
C_2H_5	A	90 %	≤ 1 %	10 %	95.8 %	90.6 %
C_2H_5	B	24 %	≤ 1 %	76 %	75.9 %	
n-C_3H_7	A	98 %	≤ 1 %	2 %	91.3 %	84.4 %
n-C_3H_7	B	44 %	≤ 1 %	56 %	74.6 %	
iso-C_3H_7	A	97 %	≤ 1 %	3 %	85.9 %	< 10 %
iso-C_3H_7	B	2 %	≤ 1 %	98 %	72.2 %	
C_6H_5	A	100 %	——	——	97.8 %	< 1 %
C_6H_5	B	100 %	——	——	97.0 %	

a) R is the substituent on C-2 position of 2-butene-1,4-dioic acid. b) Method A: reaction in diethyl ether at –78°C; method B: reaction at reflux in benzene. c) Yield of the isolated diethyl ester mixture of the Horner-Emmons reaction. d) Yield of the isomerization of 2-alkyl- and 2-phenylmaleic acid by the method of Fittig[10a].

Only maleate or alkylidenesuccinate and practically no fumarate derivatives were formed. In the literature the formation of diethyl 2-anisylfumarate[11c] and diethyl 2-alkoxy-fumarates[11c] has been reported.

The 2-alkylidenesuccinates may be formed from the 2-alkylmaleates *via* a sigmatropic [1,3]-hydrogen shift. Jugded by ^{1}H-NMR under conditions A and B 100 % of 2-phenylmaleate resulted.

The isomerizations of the 2-ethyl- and 2-propylmaleates to the 2-alkylfumarates succeeded in excellent yields[10a] (91 % and 85 %), whereas the isomerization of 2-*iso*-propylmaleic acid and 2-phenylmaleic acid with ultraviolet light (254 nm) occurred with less than 10 % or not at all, respectively. Therefore, the synthesis of 2-*iso*-propyl-[10b], 2,3-dimethyl-[10c] and 2-phenylfumarate[10d] was conducted according to the indicated literature.

Reduction of fumarate derivatives. The reductions of the substrates **1** - **5** to the products **1a** - **5a** (Scheme 1) at the expense of formate catalyzed by resting cells of *C. formicoaceticum* grown on fructose/fumarate (*vide infra*) proceed in a consecutive way:

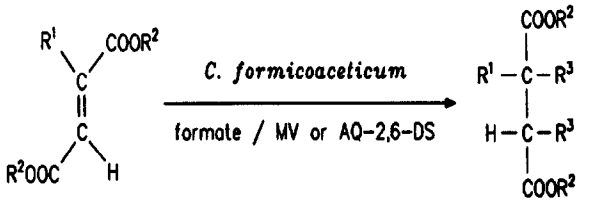
$$HCOO^- + Med_{ox} \longrightarrow CO_2 + H^+ + Med_{red} \quad (1)$$

$$substrate + Med_{red} \longrightarrow product + Med_{ox} \quad (2)$$

Present in 1 mM concentration, viologens or anthraquinone-2,6-disulphonate (AQ-2,6-DS) were used as electron mediators (Med).

The non-pyridine nucleotide dependent formate dehydrogenase present in *C. formicoaceticum* delivers electrons to the artificial mediator, which may be methylviologen (MV) or AQ-2,6-DS (reaction 1). The reduced mediators pass the electrons to fumarate, maleate diester or fumarate diester reducing enzymes which reduce the substrates (reaction 2). If viologens are used as mediators the relative rate of reactions (1) and (2) can be differentiated. As long as reaction (1) is rate limiting the solutions are not coloured. However, if reaction (2) is rate limiting the solutions are intensely violet due to the reduced viologens. Besides the reduction (reaction 2), the substrates **1** and **2** are reversibly hydrated. But this does not prevent the quantitative reduction.

Scheme 1: Preparation of 2-substituted succinates and their [2,3-^{2}H]-derivatives.



Substrate	R^1	R^2	R^3	Product
1	H	H	H	**1a**
1	H	H	^{2}H	**1a***
2	CH_3	H	H	**2a**
2	CH_3	H	^{2}H	**2a***
3	C_2H_5	H	H	**3a**
3	C_2H_5	H	^{2}H	**3a***
4	Cl	H	H	**4a**
5	CH_3	CH_3	H	**5a**
5	CH_3	CH_3	^{2}H	**5a***

Instead of AQ-2,6-DS or MV benzylviologen as well as carbamoylviologen[12] can be used (Table 2). Comparing experiment 2 and 3 (Table 2), the cheap and stable AQ-2,6-DS is equal or superior to MV. In experiments not described here it was shown that MV and AQ-2,6-DS lead to the same PNs if cells from the same growth experiment were used[13]. If instead of 1 mM (PN after 1 h: PN_{1h} = 2600) 0.3, 0.6 or 3 mM AQ-2,6-DS was used, PNs_{1h} of 1780, 2070 and 2620 were observed; these experiments are not described here. This shows that 1 mM AQ-2,6-DS is sufficient.

Compound **2** was reduced in a concentration of 1 M without decrease of the PN_{3h}. This means, that under these conditions neither product nor substrate inhibition takes place. The temperature increase from 40°C to 45°C leads to an increase of PN_{3h} from 1770 to 2180. At 50°C the reduction rate decreased

probably due to denaturation of the enzymes of this mesophilic microorganism. The rate of the reduction of **2** is limited by the simultaneous addition of water to **2** forming (*S*)-citramalate. So after 3 h the main product is (*S*)-citramalate[14]. Since under the applied conditions the water addition is reversible and the reduction is irreversible, a complete formation of **2a** from **2** can be reached. Probably also **3a** and **4a** can be formed in concentrations up to 1 M or more. An intermediate water addition does not seem to take place with the substrates **3** - **5**.

Table 2: Substrates, concentrations, temperature, mediators and yields of the reduction of **1** - **5** to **1a** - **5a** with fructose/fumarate grown *C. formicoaceticum* cells. Productivity numbers PN are given for 3 h and after complete reduction.

Exp. no.	Product [mM]	Temperature	Mediator[a)]	PN_{3h}	Final PN[b)]	Yield[c)]
1	**1a** (300)	40°C	MV	3000	≥ 1800	—[d)]
2	**2a** (300)	40°C	MV	1130	≥ 660	92.1 %
3	**2a** (300)	40°C	AQ-2,6-DS	1800	≥ 830	89.0 %
4	**2a** (1000)	40°C	AQ-2,6-DS	1770	≥ 1020	89.4 %
5	**2a** (1000)	45°C	AQ-2,6-DS	2180	≥ 1030	85.8 %
6	**3a** (200)	40°C	MV	1000	≥ 450	88.7 %
7	**4a** (150)	30°C	MV	≥ 5000	≥ 5000	90.0 %
8	**5a** (150)	40°C	MV	—[e)]	≥ 130	56.5 %

a) The concentration of the mediator was always 1 mM. b) The reductions were usually carried out overnight, in the morning the reactions were finished. c) The yields for isolated products are given. The reductions proceed with ≥ 99 %. d) The product was not isolated. e) The low solubility of the dimethyl esters prevents exact determination of their concentrations.

Reaction (2) is rate limiting for the reduction of **2**, **3** and **5**. In contrast reaction 1 is rate limiting for the reduction of **4**. A rise in temperature from 30°C to 45°C decreases the PN_{3h} for **4a** formation to about 85 %. The higher temperature may inactivate formate dehydrogenase, which is in this case the rate limiting enzyme activity.

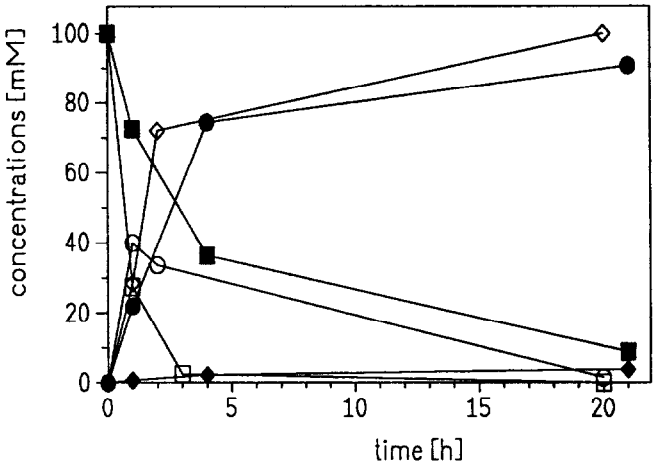
2-Propyl-, 2-*iso*-propyl-, 2-phenyl-, 2,3-dimethylfumarate, 2-methyl-, 2-ethyl-, 2-propyl-, 2-*iso*-propyl-, 2-phenyl-, 2,3-dimethyl-, 2-chloromaleate, maleate and glutaconate could not be reduced with *C. formicoaceticum* cells. 2-Bromo- and 2-iodofumarate could be reduced, but their products decomposed under the reduction conditions. As tested, the spontaneous degradation of 2-bromosuccinate is only about 20 % of the degradation observed in the presence of cells.

Comparison of fructose and fructose/fumarate grown *C. formicoaceticum* cells. Figure 1 shows that for the reduction of **2** only cells grown on fructose and fumarate are suitable. Cells grown on fructose only form (*S*)-citramalate. Fructose grown cells form **2a** only with 3 % of the rate of fructose/fumarate grown cells. The inducement of fumarate reductase by fumarate was already shown by Dorn et al.[9]. The cells grown on fructose were only used for water additions[14], because with these cells the reduction product was formed in amounts less than 5 % (experiments not shown).

Reduction of the dimethyl esters of fumarate, 2-methylfumarate and maleate. Dimethyl fumarate, dimethyl 2-methylfumarate and dimethyl maleate were reduced by *C. formicoaceticum*. The ethyl esters were not substrates. Dimethyl (*S*)-2-methylsuccinate **5a** was formed with an enantiomeric excess of ≥ 99.9 % and a PN of ≥ 130. The water solubility of only 30 mM of **5** may be one reason for the low PN. We have isolated the dimethyl maleate reductase, which reduced only dimethyl maleate (K_M = 3.8 mM; determined by the method of Wilkinson[15]), but not the dimethyl esters of **1** or **2**. So in *C. formicoaceticum* an additional dimethyl reductase may be present, which has a narrow substrate specificity. Both dimethyl ester reductases are contained in the fructose and fructose/fumarate grown cells with nearly the same activities.

During the reduction of **5**, dimethyl fumarate and dimethyl maleate one (15-20 %) or two (~ 5 %) ester groups were hydrolysed. The ester hydrolysis by *C. formicoaceticum* could be repressed by 50 % by addition of 5 vol.-% of ethanol, but this addition decreases the fumarate reductase activity by 20 %. Only MV ($E^{o'}$ = −440 mV) is a suitable mediator for this reduction. AQ-2,6-DS ($E^{o'}$ = −184 mV) or carbamoylviologen[12] ($E^{o'}$ = −296 mV) showed no activity with **5**, whereas both compounds are effective electron mediators for the reduction of **2** by *C formicoaceticum* (experiments not described here).

Figure 1: Transformation of **2** with fructose (filled signs) and fructose/fumarate (open signs) grown cells: The substrate is **2** (■ and □) and the products **2a** (◆ and ◇) and (*S*)-2-citramalate (● and ○).



(2*R*,3*S*)-[2,3-^{2}H]-succinate derivatives. If the reduction is carried out in ^2H_2O-buffer, made from 99.9 % ^2H_2O, (2*R*,3*S*)-[2,3-^{2}H]-succinate **1a***, (2*S*,3*S*)-[2,3-^{2}H]-2-methylsuccinate **2a***, (2*S*,3*S*)-[2,3-^{2}H]-ethylsuccinate **3a*** and dimethyl (2*S*,3*S*)-[2,3-^{2}H]-2-methylsuccinate **5a*** could be prepared by *trans* addition of deuterium (Scheme 1)[6]. The PNs of freeze-dried and normal cells are approximately the same. The deuteration of all products is measured *via* ^{1}H-NMR-spectroscopy and is ≥ 95 % for the indicated positions. We assume that the synthesis of (2*S*,3*S*)-[2,3-^{2}H]-2-chlorosuccinate is also possible. The pre-

paration of **1a*** and **2a*** by chemical[16a-e] and microbiological[6] synthesis has been described. Both methods are also characterized by excellent yields and a deuteration of ≥ 95 %. The chemical methods use deuterium gas[16a-d] or dideuterated diimide[16e] as deuterium source, whereas the microbiological method uses ^2H_2O[6].

Conclusion

(*S*)-2-Methyl-, (*S*)-2-ethyl- and (*S*)-2-chlorosuccinate can be prepared in concentrations of 1 M or higher with *Clostridium formicoaceticum* grown on fructose and fumarate. The enantiomeric excess of the products was higher than for most of the homogeneous chemical catalyses[3a,b]. The reduction with formate is effective and very simple from a technical point of view. The product of formate dehydrogenation is carbon dioxide, which makes product isolation easier since no by-product from the regeneration of the reduced artifical mediator has to be separated. Some time ago we observed with *Proteus vulgaris* a higher productivity number for the reduction of 2-methylfumarate (1500 instead of 1080), but we tested no additional substrates[6].

The stereoselective dideuteration of the substrates is possible by using freeze-dried *C. formicoaceticum* cells, which show nearly the same rate in ^2H_2O and H_2O.

Fructose and fructose/fumarate grown *C. formicoaceticum* cells contain a dimethyl maleate and a dimethyl fumarate reductase. To our knowledge both enzymes have not been described in the literature so far.

The examples described here show clearly that non pyridine nucleotide dependent reductases can be very effective for the stereospecific reduction of unsaturated compounds. In contrast to pyridine nucleotide dependent oxidoreductases they are often able to accept single electrons from various artificial redox active mediators. Nevertheless, their stereoselectivity is extremely high[17a,b].

It is not necessary to isolate the enzymes from the microorganisms and the productivity numbers can be increased by cheap artifical mediators applied in 1 mM concentrations by 1 - 2 orders of magnitude. Besides the artifical mediators mentioned here safranine T in 0.75 mM concentration shows about 80 % of the activity observed with 1 mM MV or 1 mM AQ-2,6-DS for the reduction of **2**. The absence of a mediator decreases the reduction rate to 3 - 4 %. If reductions can also be conducted with yeasts the observed productivity numbers are usualy 1 - 3 orders of magnitude lower[17a].

Experimental

General procedures. Fumarate, maleate, 2-methylfumarate, 2-methylmaleate, 2,3-dimethylmaleate, 2-chloromaleate, glutaconate, methylviologen, benzylviologen, AQ-2,6-DS and safranine T are available from Aldrich (D-Steinheim). 2-Chloro-, 2-bromo- and 2-iodofumarate as well as 2,3-dimethylfumarate could be synthesised as described in the following paper[14]. The isomerizations of the 2-alkyl- and 2-phenylmaleate to the corresponding fumarates are described in the literature[10a-d].

Synthesis of 2-alkyl- and 2-phenylmaleic acids. The general reaction conditions are described for the synthesis of 2-ethylmaleic acid.

2-Ethylmaleic acid: To a solution of sodium hydride (3.0 g, 121 mmol) in 70 ml of absolute diethyl ether triethyl phosphonoacetate (27.1 g, 121 mmol) was added during 30 min at 0°C. After cooling the solution to -78°C ethyl 2-oxobutyrate was added slowly. After 3 h the solution was allowed to warm up to room temperature overnight. The diethyl ether insoluble phosphonate was dissolved in 100 ml water. After phase separation the aqueous layer was extracted three times with 40 ml of diethyl ether, the combined organic layers were dried with magnesium sulphate and the solvent was distilled off. At reflux the diethyl ester was hydrolyzed in 60 ml of an ethanol-water mixture (1:1) with a threefold excess of potassium hydroxide. Ethanol was evaporated, the residue acidified with conc. hydrochloric acid to pH 1.5 and extracted with diethyl ether. The mixture of ethylmaleic acid (90 %), 2-ethylidenesuccinic acid (10 %) and 2-ethylfumaric acid (< 1%) was separated by adding cold chloroform and filtering off the insoluble 2-ethylidenesuccinic acid. 2-Ethylmaleic acid was isolated after concentrating the solution.

Yield of the mixture of diethyl 2-ethylmaleate, diethyl 2-ethylfumarate and diethyl 2-ethylidenesuccinate was 95.8 %. 2-Ethylmaleic acid: M.p.: 96-98°C, ^{1}H-NMR: δ 5.80 (tr, J = 1.5 Hz, 1H), 2.39 (dq, J_1 = 1.5 Hz, J_2 = 7.4 Hz, 2H) and 1.12 (tr, J = 7.4 Hz, 3H). ^{13}C-NMR: δ 172.6, 168.3, 153.7, 119.4, 28.5 and 12.0. Anal. calcd. for $C_6H_8O_4$: C, 50.00; H, 5.60; Found: C, 49.81; H, 5.45.

2-Propylmaleic acid was prepared on a 10 mmol scale using ethyl 2-oxovalerate. Yield of the mixture of diethyl 2-propylmaleate, diethyl 2-propylfumarate and diethyl 2-propylidenesuccinate was 91.3 %. 2-Propylmaleic acid: M.p.: 91-92°C, ^{1}H-NMR: δ 5.81 (tr, J = 1.0 Hz, 1H), 2.33 (dtr, J_1 = 1.0 Hz, J_2 = 7.5 Hz, 2H), 1.54 (sextett, J = 7.5 Hz, 2H) and, 0.97 (tr, J = 7.4 Hz, 3H). ^{13}C-NMR: δ 172.6, 168.1, 152.1, 120.4, 37.5, 21.5 and 13.7. Anal. calcd. for $C_7H_{10}O_4$: C, 53.16; H, 6.37; Found: C, 52.93; H, 6.37.

2-*iso*-Propylmaleic acid was prepared on a 10 mmol scale using ethyl 3-methyl-2-oxobutyrate. Yield of the mixture of diethyl 2-*iso*-propylmaleatediethyl, diethyl 2-*iso*-propylfumarate and diethyl 2-*iso*-propylidenesuccinate was 95.8 %. 2-*iso*-Propylmaleic acid: M.p.: 92°C, ^{1}H-NMR: δ 5.77 (d, J = 1.3 Hz, 1H), 2.65 (dseptett, J_1 = 1.2 Hz, J_2 = 7.0 Hz, 1H) and 1.15 (tr, J = 7.0 Hz, 6H). ^{13}C-NMR: δ 172.6, 168.5, 158.6, 118.0, 34.1, 21.2 and 21.2. Anal. calcd. for $C_7H_{10}O_4$: C, 53.16; H, 6.37; Found: C, 53.28; H, 6.49.

2-Phenylmaleic acid was prepared on a 125 mmol scale in a yield of 95.8 % using ethyl phenylglyoxalate. M.p.: 126°C, ^{1}H-NMR: δ 7.57-7.41 (m, 5H) and 6.34 (s, 1H). ^{13}C-NMR: δ 171.8, 168.0, 151.1, 135.0, 131.6, 130.1 and 117.8. Anal. calcd. for $C_{10}H_8O_4$: C, 62.50; H, 4.20; Found: C, 62.64; H, 4.32.

Media for the fructose and fructose/fumarate grown *C. formicoaceticum* cells and further general procedures can be taken from the following paper[14].

The time course of the reaction and the homogeneity of the isolated products were controlled with HPLC on columns (4.6x250 mm) filled with 10 µm Nucleosil RP18 (Macherey & Nagel, D-Düren). Depending on the compounds 0.1 % aqueous phosphoric acid containing 0-70 % methanol was used as eluent. The refractive index and UV absorption (214 or 254 nm) were recorded simultaneously. Samples (100 µl) were acidified with 4 µl of 6 N sulphuric acid, centrifuged and injected into the HPLC-system.

The solvent for the NMR-measurements was $C^2H_3O^2H$.

The enantiomeric excess of the products was determined with a gas chromatograph (Carlo Erba, D-Lorsbach) with a 8 m glass capillary column filled with heptakis(2,6-di-O-methyl-3-O-pentyl)-β-cyclodextrin or a 25 m glass capillary column filled with octakis(3-O-butyryl-2,6-di-O-pentyl)-γ-cyclodextrin as chiral stationary phases[18].

Preparative reductions. All reductions were carried out in a 100 ml thermostated tube under an atmosphere of nitrogen. The pH was continuously controlled and regulated with 2 N formic acid. Reductions which lasted over 24 h contained also 10 µM tetracycline. Further conditions of the reductions are listed in Table 3.

Product isolation. All reductions were stopped by acidification with conc. sulphuric acid to pH 1.5 and the suspension was centrifuged at 38000 g for 20 min. The sediment was washed with 20 ml water and centrifuged again. The supernatants were stirred with charcoal to absorb the mediator and afterwards lyophilized to remove the formic acid. The residue was dissolved in 30 ml of water, the solution extracted continuously with diethyl ether (12 h) and the ether, after drying with magnesium sulphate, evaporated. The enantiomeric excess of the products was determined after transformation of a sample of the residue to the dimethyl ester with diazomethane[19]. The rest was recrystallised from a mixture of ligroin and diethyl ether (2:1).

Table 3: Substrates and reaction conditions of preparative reductions.

Substrate (mmol)	Product	Formate (mmol)	Temperature	Total volume	Wet packed cells of *C. formicoaceticum*	Reaction time and reaction yield
1 (0.9)	**1a**	1.4	40°C	3 ml	0.4 g	7 h (≥ 99 %)
2 (30.0)	**2a**	45.0	45°C	30 ml	5.6 g	30 h (≥ 99 %)
3 (14.0)	**3a**	24.2	40°C	70 ml	3.9 g	45 h (≥ 99 %)
4 (10.5)	**4a**	15.0	30°C	70 ml	3.9 g	2 h (≥ 99 %)
5 (10.5)	**5a**	16.5	40°C	70 ml	4.0 g	100 h (≥ 99 %)

Characterisation of the products. 2a: M.p. 116-117°C, $[\alpha]_D$ = -16.0° (c = 4.4, ethanol); ((*R*)-2-methylsuccinic acid: $[\alpha]_D$ = +16.5°)[20], ^{1}H-NMR: δ 2.83 (m, 1H), 2.66 (dd, J_1 = 16.65 Hz, J_2 = 8.24 Hz, 1H), 2.46 (dd, J_1 = 16.67 Hz, J_2 = 5.88 Hz, 1H) and 1.21 (d, J = 7.34 Hz, 3H). ^{13}C-NMR: δ 179.3, 175.6, 38.4, 37.0 and 17.5. Anal. calcd. for $C_5H_8O_4$: C, 45.45 %; H, 6.10 %; found: C, 45.52 %; H, 6.15 %.
3a: M.p. 94-95°C, $[\alpha]_D$ = -22.3° (c = 4.6, acetone); ($[\alpha]_D$ = -20.8°)[21], ^{1}H-NMR: δ 2.75-2.65 (m, 1H), 2.65 (dd, J_1 = 16.14 Hz, J_2 = 9.22 Hz, 1H), 2.42 (dd, J_1 = 16.14 Hz, J_2 = 4.67 Hz, 1H), 1.72-1.57 (m, 2H) and 0.95 (tr, J = 7.48 Hz, 3H). ^{13}C-NMR: δ 178.7, 175.8, 43.9, 36.4, 26.0 and 11.6. Anal. calcd. for $C_6H_{10}O_4$: C, 49.31 %; H, 6.90 %; found: C, 49.45 %; H, 7.00 %.

4a: M.p. 173-175°C (decomposition), $[\alpha]_D$ = -18.9° (c = 9.3, water); ($[\alpha]_D$ = -22.1° (c = 4.2, water))[8b], ^{1}H-NMR: δ 4.63 (tr, J = 7.06, 1H), 3.09 (dd, J_1 = 16.96 Hz, J_2 = 7.32 Hz, 1H) and 2.89 (dd, J_1 = 16.95 Hz, J_2 = 6.74 Hz, 1H),. ^{13}C-NMR: δ 172.9, 171.9, 53.2 and 40.5. Anal. calcd. for $C_4H_5ClO_4$: C, 31.50 %; H, 3.30 %; found: C, 31.80 %; H, 3.50 %.

5a: B.p. 80°C/ 13-14 mm Hg, $[\alpha]_D$ = -6.4° (neat); (dimethyl (*R*)-2-methylsuccinate: ($[\alpha]_D$ = +6.44°)[22], ^{1}H-NMR: δ 3.70 (s, 3H), 3.68 (s,3H), 2.93 (sextett, J = 7.04, 1H), 2.71 (dd, J_1 = 16.52 Hz, J_2 = 8.10 Hz, 1H), 2.49 (dd, J_1 = 16.52 Hz, J_2 = 6.05 Hz, 1H) and 1.22 (d, J = 7.17 Hz, 3H). ^{13}C-NMR: δ 175.7, 172.3, 51.9, 51.7, 37.4, 35.8 and 17.0. Anal. calcd. for $C_7H_{12}O_4$: C, 52.49 %; H, 7.55 %; found: C, 52.24 %; H, 7.24 %.

Synthesis of 2,3-dideuterated succinate derivatives. At 35°C the following reactions were carried out in 24 ml of 0.1 M potassium phosphate buffer of p^{2}H 7.0, which was made of 99.9 % ^2H_2O: 100 mM substrate, 300 mM formate, 1 mM MV, 10 μM tetracycline and 640 mg of fructose/fumarate grown *C. formicoaceticum* cells (dry weight). Substrates were fumarate, 2-methyl-, 2-ethylfumarate and diethyl 2-methylfumarate. As determined by HPLC the educt disappeared over 69 h. The reaction was stopped by acidification with 6 N sulphuric acid to pH 1.5, the suspension was centrifuged at 38000 g for 30 min, the supernatant stirred with charcoal and then extracted continuously with diethyl ether for 72 h. The solution was dried with sodium sulphate and the solvent was evaporated.

Characterisation of the deuterated products. The ^{13}C-NMR spectra were naturally identical with the nondeuterated products except the C-atoms on which the deuteriums are bound. Only the signals of these C-atoms are listed.

1a*: $[\alpha]_D$ no rotation. $[\alpha]_{260}$ = +2.0° (c = 0.43, water). ^{1}H-NMR: δ 2.54 (s, broad signal). ^{13}C-NMR: δ 29.5 (tr, J = 19.80 Hz).

2a*: $[\alpha]_D$ = −9.8° (c = 2.97, methanol). $[\alpha]_{260}$ = −146.0° (c = 2.97, methanol)). ^{1}H-NMR: δ 2.63 (s, broad signal, 1H) and 1.20 (s, broad signal, 3H). ^{13}C-NMR: δ 38.0 (tr, J = 19.69 Hz) and 36.6 (tr, J = 20.05 Hz).

3a*: $[\alpha]_D$ = −15.9° (c = 2.58, methanol). $[\alpha]_{260}$ = −220.0° (c = 2.58, methanol). ^{1}H-NMR: δ 2.61 (s, broad signal, 1H), 1.68-1.57 (m, 2H) and 0.95 (tr, J = 7.49 Hz, 3H). ^{13}C-NMR: δ 43.5 (tr, J = 19.99) and 36.1 (tr, J = 19.61 Hz).

5a*: $[\alpha]_{290}$ = −163.0° (c = 1.15, methanol). ^{1}H-NMR: δ 3.69 (s, 3H), 3.37 (s, 3H), 2.71 (s, broad signal, 1H) and 1.21 (s, 3H). ^{13}C-NMR: δ 37.1 (tr, J = 19.85 Hz) and 35.4 (tr, J = 20.30 Hz).

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References and Notes

1. Productivity number PN = mmol product formed per kg dry weight biocatalyst and hour.
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