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Preparation of Both Enantiomers of Malic and Citramalic Acid and Other Hydroxysuccinic Acid Derivatives by Stereospecific Hydrations of *cis* or *trans* 2-Butene-1,4-dioic Acids with Resting Cells of *Clostridium formicoaceticum*

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Abstract: (*R*)-Malic , (*S*)-malic, (*R*)-citramalic, (*S*)-citramalic, (2*R*,3*S*)-2-hydroxy-3-methyl-succinic and (2*R*,3*S*)-2,3-dimethyl-2-hydroxysuccinic acid were prepared on scales up to 25 mmol by stereospecific addition of water to different 2-butene-1,4-dioic acid derivatives catalyzed by resting cells of *Clostridium formicoaceticum* (Scheme 1). The (3*R*)-monodeuterio (*R*)- and (*S*)-malic acid as well as (*R*)- and (*S*)-citramalic acid were prepared using freeze-dried cells in ^2H_2O-buffer. The stereochemical purity of the products was in most cases ≥ 99 %.

Introduction

(*R*)-and (*S*)-malic acid (**2a** and **1a**) are important compounds of the ' chiral pool '[1a]. However, **2a** is about thirty times more expensive than the (*S*)-enantiomer. Only a few recent examples of their application in the synthesis of chiral products such as carnitine[1b], butenolides[1c], 3,5-dihydroxy-6-oxohexanoates[1d], 1,2- or 1,3-diols[1e] and oxiracetam[1f] shall be mentioned. For other uses of these synthons in organic synthesis see l.c.[1a,g,h].

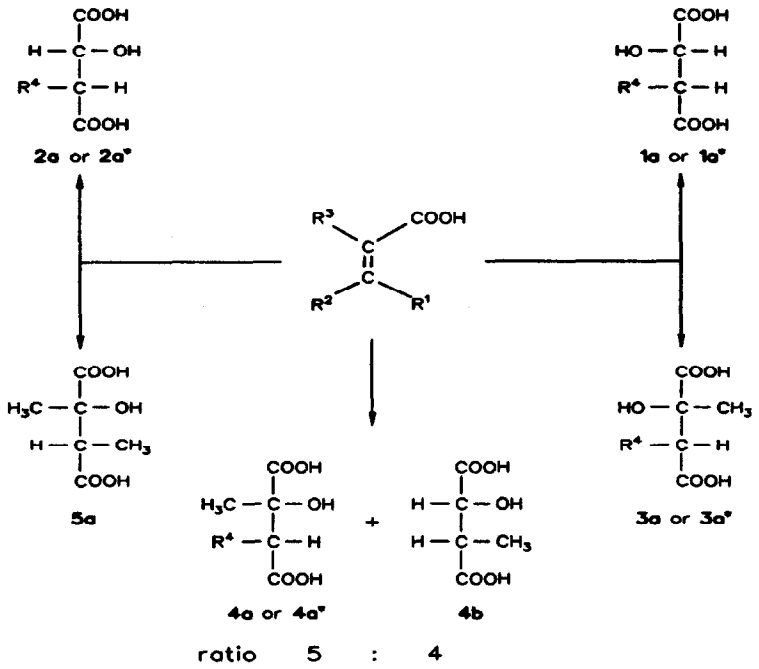
Further chiral synthons from 2-butene-1,4-dioic acids useful as synthons are the enantiomers of citramalic acid (2-hydroxy-2-methylsuccinic acid), 2,3-dimethyl-2-hydroxysuccinic acid and 2-hydroxy-3-methylsuccinic acid. (*R*)-Citramalic acid (**4a**) and the (*S*)-enantiomer (**3a**) are synthons for pheromones[2a,b]. (2*R*,3*S*)-2,3-Dimethyl-2-hydroxysuccinic acid (**5a**) has been used for the synthesis of the alkaloid jacobine[2c], whereas (2*R*,3*S*)-2-hydroxy-3-methylsuccinic acid (**4b**) is a synthon for 2-methyl-3-hydroxy-butyrolactones[2d]. For additional examples, in which **3a**, **4a** or **4b** were used as synthons see l.c.[2d,e]. Many chemical, microbiological or enzymatic methods are known for the preparation of the aforementioned products[1a,b,g,h,2d,e,3a-i].

We describe the simple preparation of both enantiomers of malic and citramalic acid as well as the (2*R*,3*S*)-enantiomers of 2-hydroxy-3-methyl- and 2,3-dimethyl-2-hydroxysuccinic acid with resting cells of *Clostridium formicoaceticum* (Scheme 1). *Clostridia* are used for the industrial synthesis of butanol, acetone, acetic acid and butyric acid[4a,b]. On a laboratory scale they are useful for many chiral carboxylates and chiral secondary alcohols[4c-f]. In the preceding paper *C. formicoaceticum* grown on fructose and fumarate was used for the stereospecific reduction of 2-substituted fumarates and dimethyl 2-methyl-

fumarate with formate as electron donor in the presence of catalytic concentrations of artificial electron mediators[4g].

Unless stated otherwise, the cells applied here were grown on fructose as the only carbon source. Therefore reductions play hardly any role.

Scheme 1: Hydrations of five substrates with *Clostridium formicoaceticum*. Product numbers marked with an asterisk were prepared in ^2H_2O, leading to a stereospecifically ^2H-labelled product.



Substrate	R^1	R^2	R^3	R^4	Product
1	H	COOH	H	H	**1a**
1	H	COOH	H	^2H	**1a***
2	COOH	H	H	H	**2a**
2	COOH	H	H	^2H	**2a***
3	H	COOH	CH_3	H	**3a**
3	H	COOH	CH_3	^2H	**3a***
4	COOH	H	CH_3	H	**4a**
4	COOH	H	CH_3	^2H	**4a***
4	COOH	H	CH_3	H	**4b**
5	COOH	CH_3	CH_3	H	**5a**

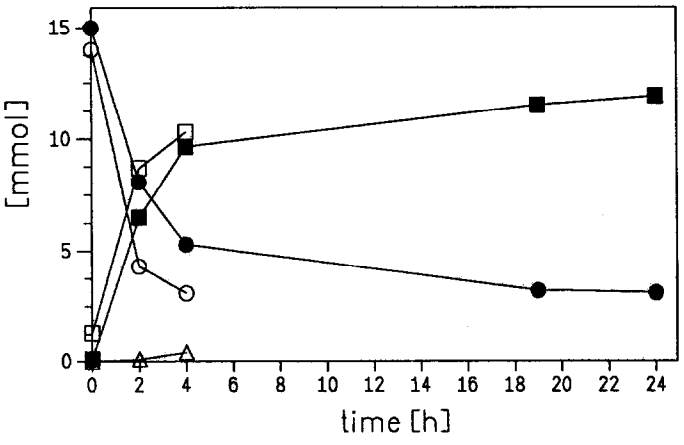
(*S*)-Malate hydro-lyase (fumarase)[5a] and (*S*)-2-methylmalate hydro-lyase (mesaconase)[6a-d] catalyze the reversible hydration of fumarate **1** to **1a**. (*R*)-malate hydro-lyase (malease)[7a-c] and (*R*)-2-methylmalate hydro-lyase (citraconase)[8a,b] catalyze irreversibly the hydration of maleate **2**[9] or 2-methylmaleate **4**[8a], respectively. The existence of a (2*R*,3*S*)-2,3-dimethylmalate hydro-lyase is assumed to be present in a *Clostridium* species by Tsai and Stadtman[10a] as well as Pirzer *et al.*[3i] in *C. barkeri*. At least three different hydro-lyases for 2-butene-1,4-dioic acids seem to be present in *C. formicoaceticum*.

Results and discussion

The water addition to various 2-butene-1,4-dioic acids catalyzed by resting cells of *C. formicoaceticum* are shown in Scheme 1.

Formation of (*S*)-malate and (*S*)-citramalate. Figure 1 shows the time course of the formation of **1a** and **3a** and the disappearance of **1** and **3**. In both cases 15 mmol of substrate were applied. For both water addition reactions two equilibrium constants were reported: for the system **1-1a** 6.2 (25°C and pH 8.4)[6a] and 3.7 (30°C and pH 8.0)[6d], and for the system **3-3a** 5.8 (25°C and pH 8.4)[6a] and 5.0 (30°C and pH 8.0)[6d]. Therefore, a complete conversion could not be achieved. Productivity numbers (PN)[11] after 4 h and after completion of the reaction as well as yield and ee-value of the products are shown in Table 1. The stereochemically pure compounds **1a** and **3a** could be produced by *C. formicoaceticum* with a PN of 2130 and 420, respectively. The reaction yields are 70 % and 83% with regard to the substrates **1** and **3**.

Figure 1: Time course of the transformation of 15 mmol **1** (–○–) to **1a** (–□–) and succinate (–△–) as well as of **3** (–●–) to **3a** (–■–) with 6.0 g wet packed resting cells of *C. formicoaceticum* in 100 ml 50 mM potassium phosphate buffer pH 8.0 at 37°C.



No water was added by *C. formicoaceticum* to 2-ethyl-, 2-propyl-, 2-isopropyl-, 2-phenyl-, 2-chloro-, 2-bromo-, 2-iodo- and 2,3-dimethylfumarate. Teipel *et al.*[4a] as well as Findeis and Whitesides[5b]

describe the substrate specificity of (*S*)-malate hydro-lyase from pig heart muscle. This enzyme transforms 2-methyl-, 2-chloro- and 2-bromofumarate to (2*S*,3*R*)-2-hydroxy-3-methyl-, (2*R*,3*S*)-2-chloro-3-hydroxy- and (2*R*,3*S*)-2-bromo-3-hydroxysuccinate. Since the product of water addition to **3** by *C. formicoaceticum* is **3a** and not (2*S*,3*R*)-2-hydroxy-3-methylsuccinate, an (*S*)-2-methylmalate hydro-lyase and not an (*S*)-malate hydro-lyase is present in *C. formicoaceticum*. An (*S*)-2-methylmalate hydro-lyase was also isolated from *C. tetanomorphum*[6a]

Table 1: Formation of 2-hydroxysuccinates and (*S*)-2-methylsuccinate with *C. formicoaceticum*. For details see also Table 4.

Substrate [mM]	Product	Reaction yield substrate/product [mM]	ee-Value	PN after 4 h	Final PN[1)]
1 (150)	**1a**	36/105	99.9 %	≥ 2130	≥ 2130 (4 h)
2 (150)	**2a**	2/147	99.9 %	2770	≥ 660 (18 h)
3 (150)	**3a**	23/124	99.9 %	1740	420 (24 h)
4 (300)	**4a** and **4b**	89/90 and 71	both 99.9 %	400 and 340	90 and 60 (142 h)
5 (100)	**5a**	34/65	99.9 %	250	≥ 150 (21 h)
(*RS*)-citramalic acid (133)	**4a** and (*S*)-2-methylsuccinic acid	67/65	96.6 and 99.9 %	320	≥ 180 (69 h)

1)The final PN could not be determined exactly since the reaction came to an end during night.

The PN after 4 h (PN_{4h}) for **3a** is 1740 and after 24 h (the equilibrium is almost reached) 420. Formate and 1 mM concentrations of methylviologen (MV) or anthraquinone-2,6-disulphonate (AQ-2,6-DS) acting as electron mediators were added to the reaction solutions for the optimization of hydration of **1** and **3**, because the hydration of **1** and **3** without the addition of formate led, in addition to the desired products **1a** and **3a**, to succinate or (*S*)-2-methylsuccinate and acetate. The formation of acetate during the hydration of **1** and **3** in the absence of formate is about 70 % and only 9 % in the presence of formate. Comparing the by-products succinate or (*S*)-2-methylsuccinate for the two conditions, they are 60 % or 80 % with formate. Presumably, in the absence of a reducing agent **1a** is dehydrogenated to oxaloacetate by malate dehydrogenase[12], whereas **3a** is probably lyased to pyruvate and glyoxylate by the (*S*)-citramalate lyase[13]. The electrons available from the dehydrogenation of **1a** or **3a** could be utilized by fumarate reductase to reduce **1** to succinate or **3** to (*S*)-2-methylsuccinate. To prevent the catabolism of **1a** and **3a** dehydrogenations must be avoided. The addition of formate decreases the formation of acetate in both cases, especially the (*S*)-citramalate lyase is inhibited. The production of succinate could be prevented by using *C. formicoaceticum* cells with a low fumarate reductase activity[4g], by treating the cells with bile extract[3b] or malonate[3c] or by terminating the reaction after 4 h before dehydrogenation of **1a** is observed. Of course the last method would decrease the yield.

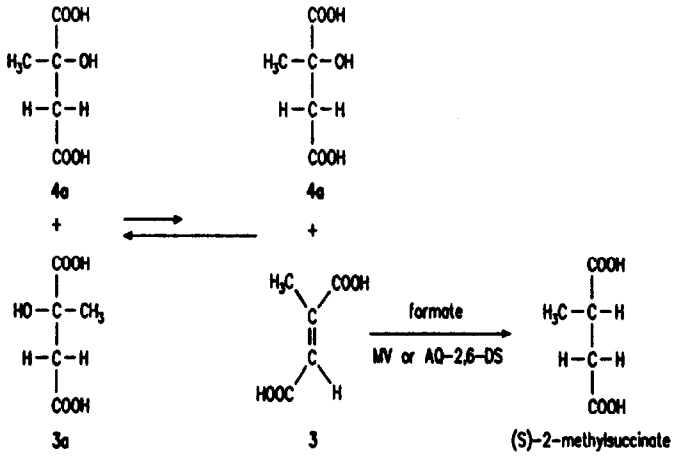
The PN_{1h}, which is not given in Table 1, was used for comparison of the different reaction conditions. At that time the thermodynamic equilibrium between **1** and **1a** or **3** and **3a** had not been reached.

The dependence on temperature and pH of the hydration was optimized for the hydrations of **1**, **2**, **3** and **4**. The temperature optimum for substrate **3** was about 35°C. The PNs_{1h} at 30°C and 40°C were about 15 % or 10 % lower. The pH optimum was about 8.0. The PNs at pH 7.5 and 8.5 were 38 % and 14 % lower.

Barker and Blair[14] used a crude extract of *Clostridium tetanomorphum*, while Subramanian and Raghavendra Rao[3g] used a crude extract of a *Pseudomonas* species for preparing **3a**. They had to add Fe^{2+}-salts and a compound with a sulphuryl group to reactivate and stabilize the enzyme. The addition of both compounds had no influence on the reaction rate of *C. formicoaceticum*.

Preparation of (*R*)-citramalate and (*S*)-2-methylsuccinate. The (*S*)-enantiomer of racemic citramalate was stereoselectively converted to **3** leaving **4a** unreacted. The enantiomeric excess of **4a**, that could be reached by this water elimination, was about 71 %, because the equilibrium constant for the addition of water to **3** leading to **3a** is 5.8[6a]. The equilibrium could be shifted by irreversible reduction of **3** to (*S*)-2-methylsuccinate with fumarate reductase, which is present in fructose/fumarate grown *C. formicoaceticum* cells[4g] (Scheme 2). The PN (180) of (*S*)-2-methylsuccinate, which is also valid for **4b**, is limited by the rate of water elimination from **3a**. The obtained ee-value of **4b** (96.6 %) could be further improved by longer reaction times. The fumarate reductase[4g] and the (*S*)-2-methylmalate hydro-lyase (Table 1) are both strictly enantioselective. The separation of **4b** and (*S*)-2-methylsuccinate is possible by anion exchange chromatography[15] or preparative HPLC and the yields after isolation were 82 % and 85 %.

Scheme 2: Formation of **4a** and (*S*)-2-methylsuccinate from (*RS*)-citramalate by *C. formicoaceticum* in the presence of formate and catalytic concentrations (1 mM) of MV or AQ-2,6-DS (see also l.c.[4g])



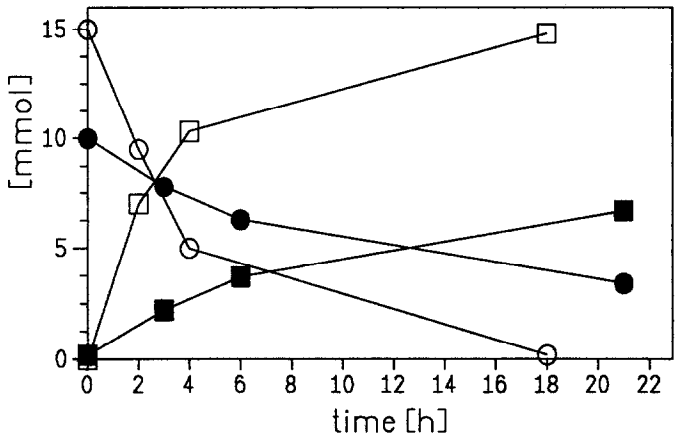
Preparative hydration of maleate and maleate derivatives. The maleates **2**, **4** and **5** are substrates for the hydration by *C. formicoaceticum* (Scheme 1). 2-Ethyl-, 2-propyl-, 2-isopropyl-, 2-phenyl-, 2-chloro- and 2,3-dichloromaleate did not react. The hydration of **2** is probably catalyzed by a (*R*)-malate hydrolyase[7a-c]. The enzyme present in microorganisms such as *Pseudomonas species* catalyzes the hydration but not the dehydration of **2a**[9]. Table 1 shows that **2a** can be stereoselectively prepared from **2** with a final PN of 660. The time course of the reaction is depicted in Figure 2. The reaction yield is ≥ 98 %. The yield of 75 % for the isolated product could be increased by a more efficient ether extraction from the water phase. The pH-optimum of the hydration of **2** by *C. formicoaceticum* is about pH 7.5. The PNs observed at pH 7.0 and 8.0 are respectively 50 % and 13 % lower than those at pH 7.5 (1400).

The final PN for the preparation of **5a** is about 150 (Table 1). The pH and the temperature optimum of the hydration of **5** were not optimized. The reaction yield of 67 % was determined by the thermodynamic equilibrium constant, which is about 2.1 (Table 1).

The hydration of **4** by *C. formicoaceticum* leads enantioselectively to **4a** and **4b** in a ratio of 5 : 4 (Scheme 1 and Table 1) with PNs of 90 and 60. The preparative use of this transformation is limited by the low PNs and the difficult separation of both acids. After reaction of the dimethyl ester of **4b**[16] with *tert*-butyldimethylsilyl chloride[17] the dimethyl ester of **4a** can be isolated with a yield of 47 %, whereas after the desilylation with tetrabutyl ammonium fluoride[17] the methyl ester of **4b** is still contaminated by about 10 % of the methyl ester of **4a**.

It is not known whether **4a** and **4b** are formed from **4** by one or two different enzymes.

Figure 2: Preparative synthesis of **2a** (–□–) from **2** (–○–) and **5a** (–■–) from **5** (–●–) by *C. formicoaceticum*.

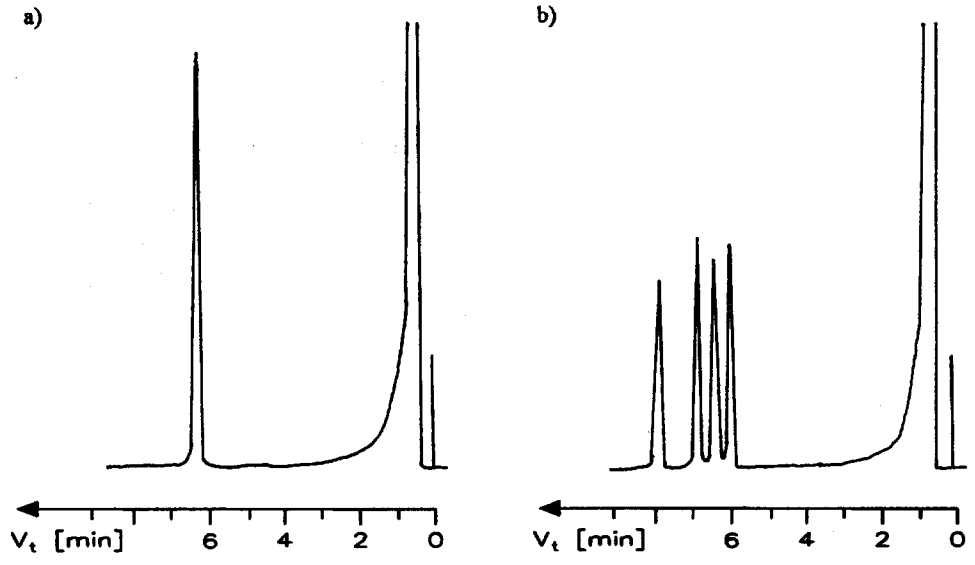


Enantiomeric excess of the products. The optical rotation and the glc-analysis on a chiral glass capillary column were used to determine the stereochemistry and the enantiomeric excess of all products. It was essential for glc-analysis to produce the dimethyl esters of the hydroxy acids[16]. The enantiomeric excess of

the products was ≥ 99 %. The only exception is **4a**, which was formed from (*RS*)-citramalate with an enantiomeric excess of 96.6 % (Table 1).

Figure 3 shows the glc-determination of the enantiomeric excess of the dimethyl ester of **5a**, racemic *erythro* and racemic *threo* 2,3-dimethyl-2-hydroxysuccinate[18]. The coinjection of the dimethyl ester of (2*R*,3*S*)-2,3-dimethyl-2-hydroxysuccinate[18] and of **5a** results in only one peak. Therefore, **5a** is (2*R*,3*S*)-dimethyl-2-hydroxysuccinic acid and not the enantiomer or one of the diastereomers.

Figure 3: Glc-analysis of the dimethyl esters of **5a**, racemic *threo* and racemic *erythro* 2,3-dimethyl-2-hydroxysuccinate.



Glc-analysis on a 8 m glass capillary column filled with heptakis(2,6-di-O-methyl-3-O-pentyl)-β-cyclodextrin as chiral stationary phase. Carrier gas: hydrogen gas (0.6 bar), column temperature: 100°C. a) Coinjection of the dimethylesters of **5a** and (2*R*,3*S*)-2,3-dimethyl-2-hydroxysuccinate[18]. b) Racemic dimethyl *erythro* and *threo* 2,3-dimethyl-2-hydroxysuccinate.

The (3*R*)-[^{2}H]-isomers of (*R*)-malate, (*S*)-malate, (*R*)-citramalate and (*S*)-citramalate. The hydrations described can also be carried out in ^2H_2O-buffer leading to the (3*R*)-monodeuterio hydroxysuccinates (Scheme 1). In order to eliminate ordinary water the cells must be freeze-dried before suspending them in 99.9 % ^2H_2O-buffer. The hydration rates observed with freeze-dried (60 mg) and wet packed (300 mg) cells in 3 ml of ^2H_2O-buffer are nearly equal. The hydrations in ^2H_2O-buffers were not optimized and were carried out on a scale of 0.6 mmol. The products were not isolated, but the ^{1}H-NMR spectra were recorded after centrifugation of the suspension. Only one proton could be detected in the 3-position of the labelled acids by ^{1}H-NMR spectroscopy (Table 2). Comparing the ^{1}H-NMR spectra with those given in the literature, the hydro-lyases present in *C. formicoaceticum* add ^2H_2O also *via* a *trans* addition[7c,8a,19]. It is astonishing that the hydration of **4** in ^2H_2O-buffer produces only one product instead of two, which

could be observed in H_2O-buffer. The ^{1}H-NMR-spectra of all four products show a deuteration grade of ≥ 95 %.

Table 2: ^{1}H-NMR spectra of (*3R*)-monodeuterio and non labelled malates and citramalates.

Products	^{1}H-NMR Signals (ppm, in $C^2H_3O^2H$)		
malate	4.30 (dd, 1H)	2.65 (dd, 1H)	2.37 (dd, 1H)
(2*S*,3*R*)-[3-^{2}H]-malate	4.32 (d, 1H)	——	2.36 (d, 1H)
(2*R*,3*R*)-[3-^{2}H]-malate	4.29 (s, 1H)	2.65 (s, 1H)	——
citramalate	2.72 (d, 1H)	2.42 (d, 1H)	1.31 (s, 3H)
(2*S*,3*R*)-[3-^{2}H]-citramalate	2.75 (s, 1H)	——	1.37 (s, 3H)
(2*R*,3*R*)-[3-^{2}H]-citramalate	——	2.46 (s, 1H)	1.41 (s, 3H)

Concluding remarks and comparison of the results with other malic or citramalic acid syntheses

With resting cells of *C. formicoaceticum* it is possible to prepare enantioselectively (*R*)- and (*S*)-malic acid as well as (*R*)- and (*S*)-citramalic acid and (2*R*,3*S*)-2,3-dimethyl-2-hydroxysuccinic acid. (*R*)-Citramalate and (*S*)-2-methylsuccinate can be prepared from (*RS*)-citramalate because *Clostridium formicoaceticum* posseses a reversible (*S*)-2-methylmalate hydro-lyase and an, under practical conditions, irreversible fumarate reductase, which converts 2-methylfumarate to (*S*)-2-methylsuccinate[4g]. This method for the preparation of (*R*)-citramalic acid may be of commercial interest because a (*RS*)-citramalic acid synthesis with a high yield is known[20]. (*R*)-citramalic acid and (2*R*,3*S*)-2-hydroxy-3-methylsuccinic acid are formed simultaneously from 2-methylmaleic acid. The complete separation of both hydroxy acids is not simple even after silylation of the more reactive (2*R*,3*S*)-3-methyl-2-hydroxysuccinic acid with *tert*-butyldimethylsilyl chloride. By using freeze-dried *C. formicoaceticum* cells in ^2H_2O-buffer the (*3R*)-monodeuterium labelled forms of (*R*)- and (*S*)-malic as well as of (*R*)- and (*S*)-citramalic acid can be prepared.

Other chemical, enzymatic or microbiological methods for these hydroxysuccinic acid syntheses are listed in Table 3. The chemical malic acid synthesis is almost enantioselective but (*R*)- and (*S*)-quinidine is expensive[3a]. Chemical citramalic acid syntheses show a low to moderate enantiomeric excess and low yields[21]. The chemical synthesis of (2*R*,3*S*)-2,3-dimethyl-2-hydroxysuccinic acid gives moderate yield and uses an expensive chiral compound as synthon[3h]. The industrial microbiological synthesis of (*S*)-malate proceeds with a productivity number five times as high as our preparation[3b]. The preparation of (*R*)-malate described here has a higher reaction yield and productivity number as those reported[1a,g]. The productivity number could not always be calculated from published work[1a,b,g] (Table 3). With *C. formicoaceticum* both enantiomers of citramalate could be prepared stereoselectively. This could also be done with a *Pseudomonas* species[3e], even with higher productivity numbers. The preparation of **3a** from itaconate by *Alcaligenes denitrificans* reaches the same enantiomeric excess and yield at the same substrate concentration (180 mM)[3f]. The chemoenzymatic synthesis of (*R*)- and (*S*)-citramalic acid has a low yield

and the ee-value of the (*S*)-enantiomer is only 77 %[2e]. The microbiological synthesis of (2*R*,3*S*)-2-hydroxy-3-methylsuccinic acid with *Candida albicans* proceeds with a moderate stereochemical purity of 77 % and the desired product has to be separated from the (2*S*,3*S*)- and (2*R*,3*R*)-diastereomers by chromatography after lactonization[3g]. The enzymatic preparation of (2*R*,3*S*)-2,3-dimethyl-2-hydroxy-succinic acid from propionate and pyruvate by 2,3-dimethylmalate-lyase has two disadvantages. For thermodynamic reasons the yield is only 40 % and it can be conducted only with the isolated enzyme from *C. barkeri*[3i].

With *C. formicoaceticum* six stereoselective hydroxysuccinic acids could be prepared without isolation of the enzymes. Using the freeze-dried cells in ^2H_2O-buffer both (3*R*)-monodeuterated enantiomers of malic acid and citramalic acid could be synthesized. Whereas the formation of (2*S*,3*R*)-[3-^{2}H]-citramalate[19], (2*S*,3*R*)-[3-^{2}H]-malate[22a,b] and (2*R*,3*R*)-[3-^{2}H]-malate[22c] are described in the literature, the formation of (2*R*,3*R*)-[3-^{2}H]-citramalate does not seem to have been reported.

Table 3: Chemical, enzymatic or microbiological syntheses of various 2-hydroxysuccinic acids

Enantiomer	Method	ee-Value	Yield	Catalyst, enzyme or microorganism[a)]
1a or **2a**	chemical	98 %	79 %	(*S*)- or (*R*)-quinidine reacting with keten and chloral[3a]
1a	microb.	≥ 99.9 %	n.g.	*Brevibacterium flavum* (10500)[3b]
1a	microb.	n.g.	n.g.	*Saccharomyces cerevisiae*[3c]
2a	enzymatic	≥ 99 %	20 %	*Rhizopus* lipase[1g]
2a	microb.	100 %	50 %	*Acinetobacter lwofii*[1a]
2a	microb.	100 %	n.g.	*Brevibacterium helvolum*[1b]
3a or **4a**	chemical	≥ 95 %	79 %	(*S*)- or (*R*)-malic acid and pivalaldehyde[3d]
3a or **4a**	chemo-enzymatic	77 or > 97 %	34 %	ethylmethacrylate, ethylchloro(hydroxy-imino)acetate and *Aspergillus oryzae*[2e]
3a or **4a**	microb.	≥ 99 %	62 %	*Pseudomonas* species (1100)[3e]
3a	microb.	99.9 %	69 %	*Alcaligenes denitrificans* MCI2775[3f]
4b	microb.	77 %	84 %	*Candida albicans*[3g]
5a	chemical	≥ 99 %	81 %	BMD[b)], (E)-1-nitropropene and (*R*)-lactic acid[3h]
5a	enzymatic	≥ 99 %	40 %	2,3-dimethylmalate-lyase from *C. barkeri*[3i]

a) The PN is noted in parentheses if it could be calculated from the details in the literatures. b) BMD: (2*S*,5*S*)-2-(*tert*-butyl)-5-methyl-1,3-dioxolan-4-one. microb.: microbiological. n.g.: not given.

Experimental

General procedures. Fumarate, maleate, 2-methylfumarate, 2-methylmaleate and 2,3-dimethylmaleate are commercially available from Aldrich (D-Steinheim). The 2-ethyl-, 2-propyl-, 2-isopropyl- and 2-phenyl homologs of fumarate and maleate were synthesized as described[4g]. 2-Chloro-, 2-bromo- and 2-iodofumarate[23] as well as 2,3-dimethylfumarate could be obtained by the method of Ott[24]. A mixture of *erythro* and *threo* 2-hydroxy-3-methylsuccinic acid (ratio 6:1) was obtained by the reduction of diethyl

(*RS*)-3-methyl-2-oxosuccinate with potassium borohydride in absolute ethanol and hydrolysis of the ester with barium hydroxide. The configuration of the four isomers could be assigned after the synthesis of the dimethyl (2*S*,3*R*)- and (2*S*,3*S*)-2-hydroxy-3-methylsuccinate[25]. Racemic *threo*, racemic *erythro* and (2*R*,3*S*)-2,3-dimethyl-2-hydroxysuccinic acids were a gift from Prof. Eggerer (TU Munich), who prepared these acids as described[3i,18].

^{1}H-NMR spectra were recorded on a WP 200 or AM 360 spectrometer from Bruker and the ^{13}C-NMR spectra on the AM 360 spectrometer at 90.556 MHz. Unless mentioned otherwise, the solvent was $C^2H_3O^2H$.

The optical rotation measurements were carried out with the ORD spectral photometer J5 of Jasco (J-Tokyo) or a polarimeter 241 MC of Perkin Elmer.

The time course of the reactions and the homogeneity of the isolated products were controlled with HPLC-columns (4.6x250 mm) filled with 5 µm or 10 µm Nucleosil RP-18® (Macherey & Nagel, D-Düren). The products **4a** and **4b** could not be separated completely with the 5 µm RP-18 column, so that their exact concentration could not be determined. Depending on the compounds 0.1 % aqueous phosphoric acid containing 0-30 % methanol was used as eluent. The refractive index and UV absorption (214 or 254 nm) were simultaneously recorded. Samples (100 µl) containing cells were acidified with 4 µl of 6 N sulphuric acid, centrifuged and injected into the HPLC-system.

The enantiomeric excess of the products was measured by glc (Carlo Erba, D-Lorsbach) equipped with an 8 m glass capillary column filled with heptakis(2,6-di-O-methyl-3-O-pentyl)-β-cyclodextrin as chiral stationary phase[26].

All reaction flasks were shaken in a shaker model SW 1 combined with a thermostat model Julabo EM (Julabo, D-Sellbach) with about 70 strokes per minute.

Preparative separations of (*S*)-2-methylsuccinate and **4a** as well as **5** and **5a** were performed with HPLC apparatus DuPont model 830 (USA-Wilmington), combined with an UV-detector and differential refractometer. The column (16x250 mm) was filled with Nucleosil RP-18® (Macherey & Nagel, D-Düren).

Microorganism. Cells of *C. formicoaceticum* (DSM 92, Deutsche Sammlung von Mikroorganismen, D-Braunschweig) were grown on fructose as carbon source[27] (fructose grown cells). A modified medium containing 100 mM **1** instead of $NaHCO_3$ at a pH-value between 8.0 - 8.5 was used for growing fructose/fumarate cells. After reaching the stationary phase about 6.4 g wet packed fructose/fumarate grown cells and 5.5 g wet packed fructose grown cells per ml medium could be isolated after centrifugation from the continuous flow system. The cells were stored in wet packed or in freeze-dried form under exclusion of oxygen at −18°C.

Optimization of the water additions. Oxygen was excluded from the *C. formicoaceticum* cells. Buffers were boiled and cooled down under an atmosphere of nitrogen. Bulbs were flushed with nitrogen and sealed with rubber septa (Verneret, F-Ivry). Reactions were carried out in the presence of 10 µM of tetracycline.

Screening experiments were conducted in a total volume of 3 ml of 0.3 M phosphate buffer pH 7.5 containing 0.3 mmol of substrate, 0.9 mmol of formate, 3 µmol of MV or AQ-2,6-DS and 300 mg of wet packed cells of *C. formicoaceticum*. The suspensions were shaken at 30°C in 10 ml bulbs sealed with rubber septa. At different times small aliquots were taken *via* a syringe. The reactions were optimized by varying the temperature and the pH value. Ferrous ammonium sulfate (3 µmol) and/or glutathione (3 µmol) were added to two transformations of **3**. The water addition leading to **2a**, **4a** and **5a** was carried out without formate and an electron mediator in contrast to the water addition to **1** and **3**.

Preparative hydrations. The preparative transformations of all substrates were carried out by shaking 200 ml volumes under a nitrogen atmosphere at 37°C in 0.1 M of phosphate buffer pH 8.0. The substrates and the reaction conditions are summarized in Table 4. For the transformation of (*RS*)-citramalate to **4b** and (*S*)-2-methylsuccinate a thermostated tube was used with a pH control[4g].

Table 4: Substrates and reaction conditions of the water additions by *C. formicoaceticum* in a preparative scale.

Substrate (mmol)	Formate (mmol)	Total volume	Wet packed cells of *C. formicoaceticum*	Reaction time
1 (15.0)	(15.0)	100 ml	6.0 g	4 h
2 (15.0)	——	100 ml	6.1 g	18 h
3 (15.0)	(15.0)	100 ml	6.0 g	24 h
4 (25.0)	——	85 ml	4.3 g	142 h
5 (10.0)	——	100 ml	12.0 g	21 h
(*RS*)-citramalate (10.0)	(20.0)	70 ml	2.1 g*)	69 h

*) Fructose/fumarate grown cells were used. The reaction solution contained also 70 µmol of MV.

Product isolation. All preparative transformations were quenched by acidification with conc. hydrochloric acid to pH 1.5 and centrifugation at 38000 g for 20 min. The sediment was washed with 20 ml water and centrifuged again. If formic acid was present the supernatants were lyophilized. The residue was dissolved in 30 ml of water and extracted once or twice with 30 ml of diethyl ether, which contained the unsaturated dicarboxylic acids and about 3 % of the hydroxydicarboxylic acids. After continuous extraction of the aqueous solutions with diethyl ether (48-72 h) the ether solution was dried with magnesium sulphate and evaporated. The enantiomeric excess was determined from the residue and afterwards the product was recrystallized.

Isolation of **4a** and **4b**: the residue of the diethyl ether layers contained a mixture of **4a** : **4b** : **4** in a ratio of 4 : 5 : 5. The separation of **4a** and **4b** from **4** was carried out by chromatograpy on a Dowex 1x8 column (200-400 mesh, 3.5 cm x 26 cm, formate form)[15]. The 2.33 g oil of **4a** and **4b** was transformed with diazomethane[16] to the methyl esters. The secondary hydroxyl group of **4b** was silylated with *tert*-butyldimethylsilyl chloride in dimethylformamide (DMF), while the tertiary hydroxyl group of **4a** did not react[17]. The mixture was dissolved in 100 ml of diethyl ether and the dimethyl ester of **4a** was extracted

five times with 20 ml of water. The silylated methyl ester of **4b**, DMF and about 10 % of **4a** were present in the diethyl ether. The aqueous layer containing **4a** and DMF was evaporated in vacuo.

Isolation of **4a** and (*S*)-2-methylsuccinate: After diethyl ether extraction the mixture of (*S*)-2-methylsuccinic acid, **4a** and some acetic acid (48:45:7) was separated by preparative RP-18 HPLC (eluent: 0.1 % phosphoric acid; flow: 20 ml/min). The aqueous fractions were concentrated to 30 ml and continuously extracted with diethyl ether. After drying with magnesium sulphate and evaporation of the solvent (*S*)-2-methylsuccinate was recrystallized from a ligroin-diethyl ether mixture (2:1) and the non-crystalline **4a** was evaporated at 50°C (0.1 mm Hg) and crystallized after adding three drops of ethyl acetate.

Isolation of **5a**: After centrifugation the supernatant was concentrated to about 50 ml. By continuous extraction with diethyl ether **5a** and **5** were separated *via* preparative RP-18 HPLC (eluent: 0.1 % phosphoric acid with 15 % methanol; flow: 20 ml/min). The fraction 1700-2300 ml of the elution volume was concentrated to about 80 ml and then continuously extracted with diethyl ether.

Addition of ^2H_2O using lyophilized, fructose/fumarate grown cells. The reactions were carried out in 25 ml flasks at 35°C. About 0.6 mmol of **1**, **2**, **3** or **4** and 160 mg of dry weight cells were suspended in 6 ml of 50 mM phosphate buffer p^{2}H 8.0. The solutions of **1** and **3** contained 1.8 mmol of formate. After 69 h the reactions were quenched by centrifugation at 38000 g for 30 min without acidification. The ^{1}H-NMR-spectra were recorded from each supernatant.

Characterisation of the products. **1a**: yield 0.9 g (52 %); Mp. 100-101°C; $[\alpha]_D$ = –25.8° (c = 5.5, pyridine); ($[\alpha]_D$ = –27°)[28]; ^{1}H-NMR: δ 4.49 (dd, J_1 = 7.64 Hz, J_2 = 4.36 Hz, 1H), 2.80 (dd, J_1 = 16.22 Hz, J_2 = 4.38 Hz, 1H) and 2.65 (dd, J_1 = 16.17 Hz, J_2 = 7.69 Hz, 1H); ^{13}C-NMR: δ 176.6, 174.3, 68.5 and 40.0; Anal. calcd. for $C_4H_6O_5$: C, 35.82; H, 4.51; Found: C, 35.92; H, 4.55.

2a: yield 1.45 g (75 %); Mp. 99-100°C; $[\alpha]_D$ = +25.5° (c = 5.5, pyridine); ^{1}H-NMR and ^{13}C-NMR see **1a**; Anal. calcd for $C_4H_6O_5$: C, 35.82; H, 4.51; Found: C, 35.95; H, 4.35.

3a: yield 1.24 g (69.8 %; oil); The chiral glc-analysis showed an enantiomeric excess of ≥ 99.9 %; ^{1}H-NMR: δ 2.92 (d, J = 16.18 Hz, 1H), 2.62 (d, J = 16.13 Hz, 1H) and 1.43 (s, 3H); ^{13}C-NMR: δ 178.7, 174.2, 73.4. 45.1 and 26.7.

Dimethyl ester of **4a**: yield 0.31 g (7.2 %); $[\alpha]_D$ = –24.3° (c = 2.25, $CHCl_3$); ($[\alpha]_D$ = –25.4°)[21]; ^{1}H-NMR: δ 3.80 (s, 3H), 3.69 (s, 3H), 2.98 (d, J = 16.37 Hz, 1H), 2.69 (d, J = 16.40 Hz) and 1.45 (s, 3H); ^{13}C-NMR: δ 176.1, 171.5, 72.6, 53.0, 51.9, 44.0 and 26.3; Anal. calcd. for $C_7H_{12}O_5$: C, 47.72; H, 6.87; Found: C, 47.33; H, 6.99.

Dimethyl ester of **4b**: The chiral glc-analysis showed a contamination of about 10 % by the dimethyl ester of **4a** and an enantiomeric excess for both acids of ≥ 99 %.

4a (from (*RS*)-citramalate): yield 0.61 g (81.8 %); Mp. 107-109°C; $[\alpha]_D$ = –22.1° (c = 3.0, water); ($[\alpha]_D$ = –24° (c = 4.4 , water)[3g]); ^{1}H-NMR and ^{13}C-NMR see **3a**; Anal. calcd. for $C_5H_8O_5$: C, 40.54; H, 5.45; Found: C, 40.64; H, 5.64.

(*S*)-2-methylsuccinic acid (from (*RS*)-citramalate): yield 0.56 g (84.8 %); Mp. 116°C; $[\alpha]_D$ = –16.0° (c = 4.4, ethanol); ((*R*)-2-methylsuccinic acid: $[\alpha]_D$ = +16.5°)[29]; ^{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, 45.62; H, 6.19.

5a: yield 0.75 g (67.6 %); Mp. 95-97°C; $[\alpha]_D$ = −15.8° (c = 2.34, methanol); ($[\alpha]_D$ = −16.4° (c = 5, water)[18]); ^{1}H-NMR (^2H_2O with 5 % $C^2H_3O^2H$): δ 3.07 (q, J = 7.32 Hz, 1H), 1.42 (s, 3H) and 1.25 (d, J = 7.33 Hz, 3H); ^{13}C-NMR (^2H_2O with 5 % $C^2H_3O^2H$): δ 182.1, 180.5, 77.9, 48.9, 25.8 and 12.7; Anal. calcd. for $C_6H_{10}O_5$: C, 44.44; H, 6.22; Found: C, 44.48; H, 6.27.

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