



# Biotransformations

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## Preface

The use of enzymes – employed either as isolated enzymes, crude protein extracts or whole cells – for the transformation of non-natural organic compounds is not an invention of the twentieth century: they have been used for more than one hundred years. However, the object of most of the early research was totally different from that of the present day. Whereas the elucidation of biochemical pathways and enzyme mechanisms was the main driving force for the early studies, in contrast it was mainly during the 1980s that the enormous potential of applying natural catalysts to transform non-natural organic compounds was recognized. This trend was particularly well enhanced by the recommendation of the FDA-guidelines (1992) with respect to the use of chiral bioactive agents in enantiopure form.

During the last two decades, it has been shown that the substrate tolerance of numerous biocatalysts is often much wider than previously believed. Of course, there are many enzymes which are very strictly bound to their natural substrate(s). They play an important role in metabolism and they are generally not applicable for biotransformations. On the other hand, an impressive number of biocatalysts have been shown to possess a wide substrate tolerance by keeping their exquisite catalytic properties with respect to chemo-, regio- and, most important, enantio-selectivity. This made them into the key tools for biotransformations. As a result of this extensive research during the last two decades, biocatalysts have captured an important place in contemporary organic synthesis, which is reflected by the fact that ~8% of all papers on synthetic organic chemistry contained elements of biotransformations as early as in 1991 with an ever-increasing proportion. It is now generally accepted, that biochemical methods represent a powerful synthetic tool to complement other methodologies in modern synthetic organic chemistry.

Whereas several areas of biocatalysis – in particular the use of easy-to-use hydrolases, such as proteases, esterases and lipases – are sufficiently well researched to be applied in every standard laboratory, other types of enzymes are still waiting to be discovered with respect to their applicability in organic-chemistry transformations on a preparative scale. This latter point is stressed in this volume, which concentrates on the “newcomer-enzymes” which show great synthetic potential.

February 1998

Kurt Faber  
Graz, University of Technology

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# Biocatalytic Asymmetric Decarboxylation

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Biocatalytic decarboxylation is a unique reaction, in the sense that it can be considered to be a protonation reaction to a "carbanion equivalent" intermediate in aqueous medium. Thus, if optically active compounds can be prepared via this type of reaction, it would be a very characteristic biotransformation, as compared to ordinary organic reactions. An enzyme isolated from a specific strain of *Alcaligenes bronchisepticus* catalyzes the asymmetric decarboxylation of  $\alpha$ -aryl- $\alpha$ -methylmalonic acid to give optically active  $\alpha$ -arylpropionic acids. The effect of additives revealed that this enzyme requires no biotin, no co-enzyme A, and no ATP, as ordinary decarboxylases and transcarboxylases do. Studies on inhibitors of this enzyme and spectroscopic analysis made it clear that the Cys residue plays an essential role in the present reaction. The unique reaction mechanism based on these results and kinetic data in its support are presented.

**Keywords:** Asymmetric decarboxylation, Enzyme, Reaction mechanism,  $\alpha$ -Arylpropionic acid.

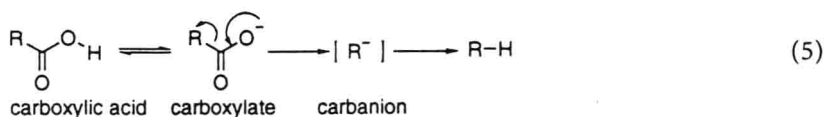
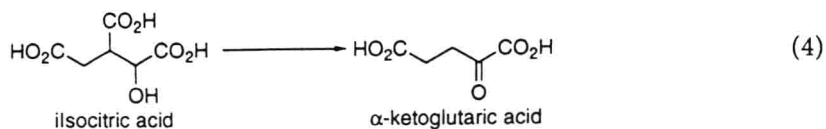
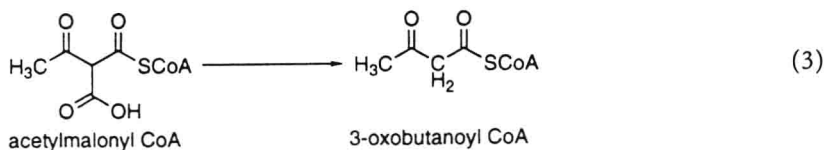
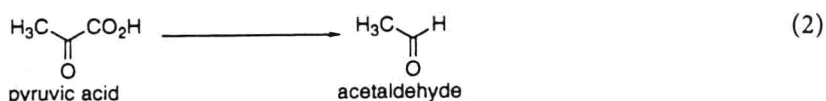
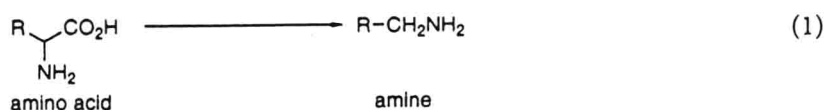
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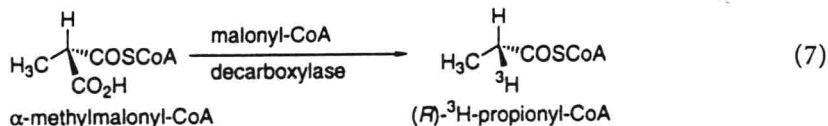
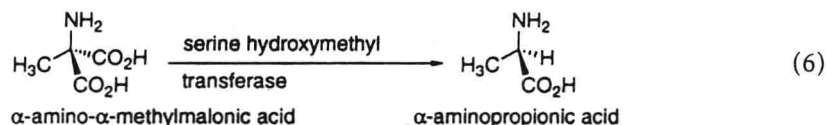
## 1

### Introduction

Biochemical reactions include several types of decarboxylation reactions as shown in Eqs. (1)–(5), because the final product of aerobic metabolism is carbon dioxide. Amino acids result in amines, pyruvic acid and other  $\alpha$ -keto acids form the corresponding aldehydes and carboxylic acids, depending on the co-operating coenzymes. Malonyl-CoA and its derivatives are decarboxylated to acyl-CoA.  $\beta$ -Keto carboxylic acids, and their precursors (for example, the corresponding hydroxy acids) also liberate carbon dioxide under mild reaction conditions.



The most interesting point from the standpoint of organic chemistry is that the intermediate of the decarboxylation reaction should be a carbanion. The first step is the abstraction of the acidic proton of a carboxyl group by some basic amino-acid residue of the enzyme. C–C bond fission will be promoted by the large potential energy gained by formation of carbon dioxide, provided that the other moiety of the intermediate, i.e. the carbanion, is well stabilized by a neighboring carbonyl group, by the inductive effect of the sulfur atom of coenzyme A, or by some functional group of another other coenzyme. Protonation to the carbanion gives the final product. The characteristic feature of this reaction is the fact that a carbanion is formed in aqueous solution. Nonetheless, at least in some cases, the reactions are enantioselective as illustrated in Eqs. (6) and (7).



Serine hydroxymethyl transferase catalyzes the decarboxylation reaction of  $\alpha$ -amino- $\alpha$ -methylmalonic acid to give (*R*)- $\alpha$ -aminopropionic acid with retention of configuration [1]. The reaction of methylmalonyl-CoA catalyzed by malonyl-coenzyme A decarboxylase also proceeds with perfect retention of configuration, but the notation of the absolute configuration is reversed in accordance with the CIP-priority rule [2]. Of course, water is a good proton source and, if it comes in contact with these reactants, the product of decarboxylation should be a one-to-one mixture of the two enantiomers. Thus, the stereoselectivity of the reaction indicates that the reaction environment is highly hydrophobic, so that no free water molecule attacks the intermediate. Even if some water molecules are present in the active site of the enzyme, they are entirely under the control of the enzyme. If this type of reaction can be realized using synthetic substrates, a new method will be developed for the preparation of optically active carboxylic acids that have a chiral center at the  $\alpha$ -position.

## 2

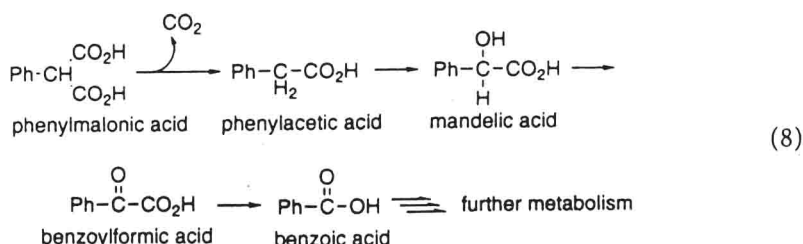
### Screening and Substrate Specificity

At the start of this project, we chose  $\alpha$ -arylpropionic acids as the target molecules, because their *S*-isomers are well established anti-inflammatory agents. When one plans to prepare this class of compounds via an asymmetric decarboxylation reaction, taking advantage of the hydrophobic reaction site of an enzyme, the starting material should be a disubstituted malonic acid having an aryl group on its  $\alpha$ -position.

## 2.1

## Method of Screening

To screen a microorganism which has an ability to decarboxylate  $\alpha$ -aryl- $\alpha$ -methylmalonic acids, a medium was used in which phenylmalonic acid was the sole source of carbon, because we assumed that the first step of the metabolic path would be decarboxylation of the acid to give phenylacetic acid, which would be further metabolized via oxidation at the  $\alpha$ -position. Thus, a



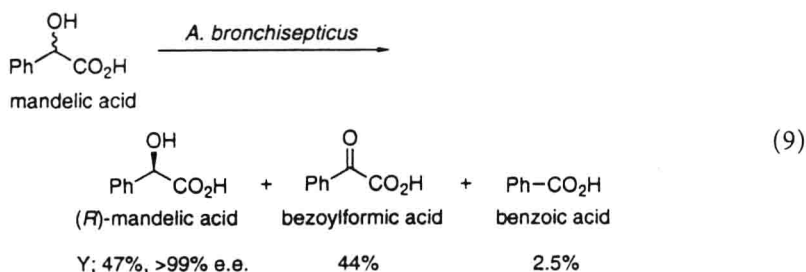
microorganism that has an ability to grow on this medium would be expected to decarboxylate  $\alpha$ -aryl- $\alpha$ -methylmalonic acids, as the only difference in structure between the two molecules is the presence or absence of a methyl group in the  $\alpha$ -position. If the presence of a methyl group inhibits the subsequent oxidation, then the expected monoacid would be obtained. Many soil samples and type cultures were tested and a few strains were found to grow on the medium. We selected a bacterium identified as *Alcaligenes bronchisepticus*, which has the ability to realize the asymmetric decarboxylation of  $\alpha$ -methyl- $\alpha$ -phenylmalonic acid [3]. The decarboxylation activity was observed only when the microorganism was grown in the presence of phenylmalonic acid, indicating that the enzyme is an inducible one.

## 2.2

## Metabolic Path of Phenylmalonic Acid

To elucidate the metabolic pathway of phenylmalonic acid, the incubation broth of *A. bronchisepticus* on phenylmalonic acid was examined at the early stage of cultivation. After a one-day incubation period, phenylmalonic acid was recovered in 80% yield. It is worthy of note that the supposed intermediate, mandelic acid, was obtained in 1.4% yield, as shown in Eq. (8). The absolute configuration of this oxidation product was revealed to be *S*. After 2 days, no metabolite was recovered from the broth. It is highly probable that the intermediary mandelic acid is further oxidized via benzoylformic acid. As the isolated mandelic acid is optically active, the enzyme responsible for the oxidation of the acid is assumed to be *S*-specific. If this assumption is correct, the enzyme should leave the intact *R*-enantiomer behind when a racemic mixture of mandelic acid is subjected to the reaction. This expectation was nicely realized by adding the racemate of mandelic acid to a suspension of *A. bronchisepticus* after a 4-day incubation [4].

As shown in Eq. (9), optically pure (*R*)-mandelic acid was obtained in 47% yield, as well as 44% of benzoylformic acid. Benzoic acid was also isolated, although in very low yield, probably as a result of oxidative decarboxylation of benzoylformic acid.



The present reaction was proven to occur even when the microorganism had been grown on peptone as the sole carbon source. These results lead to the conclusion that this enzyme system is produced constitutively. In the case of mandelate-pathway enzymes in *Pseudomonas putida*, (*S*)-mandelate dehydrogenase was shown to be produced in the presence of an inducer (mandelic acid or benzoylformic acid) [5]. Thus, the expression of the present oxidizing enzyme of *A. bronchisepticus* is different from that of *P. putida*.

When the resulting mixture of benzoylformic acid and (*R*)-mandelic acid was treated with a cell free extract of *Streptomyces faecalis* IFO 12964 in the presence of NADH, the keto acid can be effectively reduced to (*R*)-mandelic acid (Fig. 1). Fortunately the presence of *A. bronchisepticus* and its metabolite had no influence on the reduction of the keto acid. The regeneration of NADH was nicely achieved by coupling the reaction with reduction by formic acid with the aid of formate dehydrogenase. As a whole, the total conversion of racemic mandelic acid to the *R*-enantiomer proceeded with very high chemical and optical yields. The method is very simple and can be performed in a one-pot procedure [6].

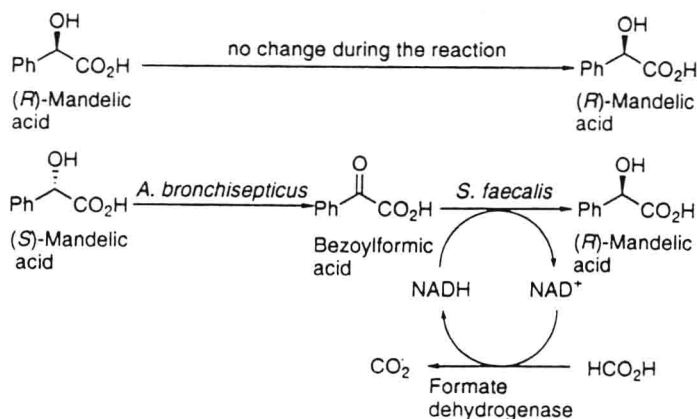


Fig. 1. Conversion of racemic mandelic acid to the (*R*)-enantiomer

## 2.3




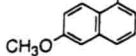
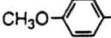



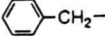





### Substrate Specificity

To a 500-ml. Sakaguchi flask were added 50 ml of the sterilized inorganic medium  $[(\text{NH}_4)_2\text{HPO}_4, 10 \text{ g}; \text{K}_2\text{HPO}_4, 2 \text{ g}; \text{MgSO}_4 \cdot 4\text{H}_2\text{O}, 300 \text{ mg}; \text{FeSO}_4 \cdot 7\text{H}_2\text{O}, 10 \text{ mg}; \text{ZnSO}_4 \cdot 7\text{H}_2\text{O}, 8 \text{ mg}; \text{MnSO}_4 \cdot 4\text{H}_2\text{O}, 8 \text{ mg}; \text{yeast extract}, 200 \text{ mg} \text{ and } \text{D-biotin}, 0.02 \text{ mg in } 1000 \text{ ml } \text{H}_2\text{O}, \text{pH } 7.2]$  containing phenylmalonic acid (250 mg) and peptone (50 mg). The mixture was inoculated with *A. bronchisepticus* and shaken for 4 days at 30 °C. The substrate,  $\alpha$ -methyl- $\alpha$ -phenylmalonic acid was added to the resulting suspension and the incubation was continued for five more days. The broth was acidified, saturated with NaCl, and extracted with ether. After a sequence of washing and drying, the solvent was removed and the residue was treated with an excess of diazomethane. Purification with preparative TLC afforded optically active methyl  $\alpha$ -phenylpropionate. The absolute configuration proved to be *R* by its optical rotation and the enantiomeric excess was determined to be 98% by HPLC, using a column with an optically active immobile phase (Table 1). Since there is no other example of asymmetric decarboxylation, we decided to investigate this new reaction further, although the absolute configuration of the product is opposite to that of anti-inflammatory agents, which is *S*. First, the alkyl group was changed to ethyl instead of methyl, and it was found that the substrate was not affected at all. This difference in reactivity must come from the difference in steric bulk. Thus it is clear that the pocket of the enzyme for binding the alkyl group is very narrow and has little flexibility. Next, variation of the aromatic part was examined. When the aryl group is 4-chlorophenyl or 6-methoxy-2-naphthyl, the substrate was decarboxylated smoothly to afford the optically active monoacid. Also,  $\alpha$ -methyl- $\alpha$ -2-thienylmalonic acid was accepted a good substrate. When the substituent on the phenyl ring was 4-methoxy, the rate of reaction was slower than those of other substrates and the yield was low, although there was no decrease in the enantioselectivity. On the other hand, no decarboxylation was observed when the aryl group was 2-chlorophenyl, 1-naphthyl or benzyl. It is estimated that steric hindrance around the prochiral center is extremely severe and the aryl group must be directly attached to the prochiral center [7].

The  $\alpha$ -fluorinated derivative of  $\alpha$ -phenylmalonic acid also underwent a decarboxylation reaction, resulting in the formation of the corresponding  $\alpha$ -fluoroacetic acid derivative. While the *o*-chloro derivative exhibited no reactivity, the *o*-fluoro compound reacted to give a decarboxylated product, although the reactivity was very low. This fact also supports the thesis that the unreactivity of the *o*-chloro derivative is due to the steric bulk of the chlorine atom at the *o*-position. The *o*-fluoro derivative is estimated to have retained some reactivity because a fluorine atom is smaller than a chlorine atom. The low e.e. of the resulting monocarboxylic acid is probably due to concomitant non-enzymatic decarboxylation, which would produce the racemic product. On the other hand, *meta*- and *para*-fluorophenyl- $\alpha$ -methylmalonic acids smoothly underwent decarboxylation to give the expected products in high optical purity. As is clear from Table 1, the *m*-trifluoromethyl derivative is a good substrate, the chemical and optical yields of the product being practically



**Table 1.** Asymmetric decarboxylation of  $\alpha$ -Alkyl- $\alpha$ -arylmalonic

$  \begin{array}{ccc}  \text{R} & & \text{R} \\    & &   \\  \text{Ar}-\text{C}^* & \xrightarrow{\text{Alcaligenes bronchisepticus}} & \text{Ar}-\text{C}^* \\  / \quad \backslash & & / \quad \backslash \\  \text{CO}_2\text{H} \quad \text{CO}_2\text{H} & & \text{H} \quad \text{CO}_2\text{H} \\  \alpha\text{-alkyl-}\alpha\text{-arylmalonic acid} & & \alpha\text{-ary-}\alpha\text{-alkylacetic acid}  \end{array}  $				
Ar	R	[Sub] (%)	Yield (%)	e. e. (%)
	CH <sub>3</sub>	0.5	80	98
	C <sub>2</sub> H <sub>5</sub>	0.2	0	–
	CH <sub>3</sub>	0.5	95	98
	CH <sub>3</sub>	0.5	96	> 95
	CH <sub>3</sub>	0.1	48	99
	CH <sub>3</sub>	0.3	98	95
	CH <sub>3</sub>	0.2	0	–
	CH <sub>3</sub>	0.2	0	–
	CH <sub>3</sub>	0.2	0	–
	F	0.1	64	95
	CH <sub>3</sub>	0.3	12	54
	CH <sub>3</sub>	0.5	75	97
	CH <sub>3</sub>	0.5	54	98
	CH <sub>3</sub>	0.5	54	98

quantitative. This can be attributed to the strong electron-withdrawing effect of this substituent.

### 3

#### Isolation of the Enzyme and the Gene

The bacterium isolated from a soil sample was shown to catalyze the decarboxylation of disubstituted malonic acid as described above. Although the configuration of the product was opposite to that of physiologically active anti-