Enzymatic Resolution of Chiral 2-Hydroxy Carboxylic Acids by Enantioselective Oxidation with Molecular Oxygen Catalyzed by the Glycolate Oxidase from Spinach (*Spinacia oleracea*)

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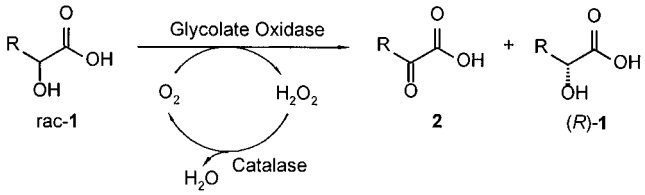
The enzymatic oxidation of a variety of saturated and unsaturated aliphatic derivatives of racemic 2-hydroxy acids **1** to their 2-oxo acids **2** with molecular oxygen catalyzed by the glycolate oxidase from spinach (*Spinacia oleracea*) was shown to proceed highly enantioselectively. Thus, the glycolate oxidase-catalyzed kinetic resolution provides a convenient biocatalytic method for the preparation of enantiomerically pure (*R*)-2-hydroxy acids. The absolute configuration of the (*R*)-2-hydroxy acid **1b** was assigned by comparison of the measured optical rotation value with that of the literature data and by application of the exciton-coupled circular dichroism method (ECCD) on its bichromophoric 2-naphthoate 9-methylanthryl derivative **3b**. These results establish the ECCD method as a convenient microscale chirooptic tool for the configurational assignment of 2-hydroxy acids.

Introduction

The glycolate oxidase (EC 1.1.3.15) is a peroxisomal enzyme, which is found in the leaves of many green plants and in the liver of mammalians. This enzyme has been isolated from a variety of biological sources, including the leaves of spinach,[1a] pea,[1b] pumpkin,[1c] and cucumber cotyledons[1d] and the liver of pigs,[1e] rats,[1f] and humans.[1g,h] The glycolate oxidase was reported to be catalytically active only as tetramers or octamers of identical subunits, which have a molecular mass of 43 000. The extensively studied enzyme has been already expressed in genetically manipulated microorganisms.[2a,b] The enzyme converts glycolic acid to glyoxylic acid (Scheme 1, R = H) and has been employed in organic synthesis as a biocatalyst for the production of glyoxylic acid by using the soluble enzyme,[3a] the enzyme immobilized on a solid support,[3b] or the genetically engineered protein.[3c] Already in the 1960s, it was reported[4a,b] that the glycolate oxidase accepts also 2-hydroxy carboxylic acids as substrates, and the kinetic studies revealed that L-lactic acid is preferentially converted by the catalytically active protein.[4a] However, to date there is no report on the preparation of optically active 2-hydroxy acids by enantioselective oxidation of racemic acids with molecular oxygen catalyzed by the glycolate oxidase.

Scheme 1. Oxidation of 2-Hydroxy Acids with Molecular Oxygen Catalyzed by Glycolate Oxidase from Spinach (*S. oleracea*)



a-d: R= $CH_3(CH_2)_n$;**a** (n=1), **b** (n=2), **c** (n=4), **d** (n=7),
e: R=*i*-Pr, **f**: R= CH_3CHOH(*erythro*)
g: R=$CH_3(CH_2)_4$, **h**: R= $CH_3(CH_2)_4$
i: R= $CH_3(CH_2)_4O(CH_2)_2$

Optically active 2-hydroxy acids are important building blocks for the asymmetric synthesis of glycols,[5a] halo esters,[5b] and epoxides.[5c] Several chemical[6] and enzymatic[7,8] methods have been reported previously on the synthesis of optically active α-hydroxy acids. The enzy-

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matic methods employed so far for the synthesis of α-hydroxy-functionalized carboxylic acids are the enantioselective oxidation of 1,2-diols with dehydrogenases,[7a] the reduction of α-keto acids with baker's yeast,[7b] the oxynitrilase-catalyzed addition of prussic acid to aldehydes,[7c] the stereoselective esterification of 2-hydroxy acids with lipase,[7d] the reduction of α-keto acids with lactate dehydrogenases,[7e,f] and the kinetic resolution of methyl α-hydroperoxy esters with horseradish peroxidase.[7g] Recently, we have reported the enzymatic α-oxidation system of young pea leaves as a suitable biocatalyst for the preparation of enantiomerically pure 2-hydroxy acids.[8]

The disadvantages of the methods reported so far entail substrate selectivity,[7a−c,e−g,8] requirement of cosubstrates[7c,g] or coenzymes,[7a,e−f] the limitation to organic solvents,[7d] or the accessibility of enzyme on a large scale.[8] These facts demand the development of alternative methods for the preparation of optically active 2-hydroxy acids. Here we describe for the first time the efficient enzymatic resolution of 2-hydroxy acids (Scheme 1) by the enantioselective oxidation with molecular oxygen catalyzed by the glycolate oxidase from spinach (*Spinacia oleracea*). This novel enzymatic transformation affords optically active 2-hydroxy acids in excellent ee values and high chemical yields.

Results and Discussion

The biocatalytic oxidation of the racemic 2-hydroxy acids **1a**−**i** with glycolate oxidase by molecular oxygen was carried out in 0.1 M ethylenediamine (EDA) buffer in the presence of 0.2 mM flavin mononucleotide (FMN) and catalase (Table 1). The oxidation of 2-hydroxy to 2-oxo acids proceeds by a mechanism similar to that observed for other flavoprotein oxidases.[9,10] After the binding of 2-hydroxy acid to the active site of the enzyme, the oxidation is effected by means of an overall two-electron transfer from the substrate to FMN. The reoxidation of the reduced FMN by reaction with molecular oxygen produces the byproduct hydrogen peroxide. To supress the destruction of the 2-oxo acid with hydrogen peroxide, catalase is added to reduce the latter to water (Scheme 1). Since the glycolate oxidase does not persist in solution, the coenzyme FMN is added to stabilize the enzyme activity in solution.[3]

The results in Table 1 reveal that the biocatalytic production of a broad variety of (*R*)-2-hydroxy acids proceeds in a high degree of enantioselectivity by preferential oxidation of the *S* enantiomers in racemic mixtures with glycolate oxidase. To assess the substrate selectivity of the glycolate oxidase, the enzymatic oxidation of saturated 2-hydroxy acids **1a**−**e** with different chain length, as well as the unsaturated and oxo-functionalized derivatives **1f**−**i**, has been studied in detail. The derivatives **1a**−**d**,**g**−**i** of the 2-hydroxy acids are accepted well as substrates by the glycolate oxidase, and the *S* enantiomer is converted to the corresponding 2-oxo acids **2a**−**d**,**g**−**i**, to leave behind the respective (*R*)-2-hydroxy acids in high enantiomeric purity (ee values 91−99%).

The oxidation of the 2-hydroxy carboxylic acids **1a**−**i** were performed either with the pure glycolate oxidase (GOX) or with spinach acetone powder (SAP), which contains glycolate oxidase besides other enzymes. Since the enzymatic resolution of the 2-hydroxyvaleric acid (**1b**) with both enzyme preparations afforded enantiomerically pure products (cf. Table 1, entries 2 and 3), we have for economical reasons applied the inexpensive spinach acetone powder in our further studies to assess the substrate selectivity of the 2-hydroxy acids on the analytical scale.

Table 1. Oxidation of Racemic 2-Hydroxy Acids with Glycolate Oxidase from Spinach[a]

entry	substrate (mmol)	GOX[b] (IU)	SAP[c] (mg)	time (h)	conversion[d] (%)	ee (%)[e] (*R*)-**1**
1	**1a** (0.05)		1000	22	50	>99[f,g]
2	**1b** (0.01)	2		22	49	98[h]
3	**1b** (0.01)		500	22	50	>99
4	**1b** (0.7)	5		48	50	>99[h]
5	**1c** (0.02)		500	22	48	91[f]
6	**1d** (0.03)		500	22	50	>99[h]
7	**1e** (0.02)	2		22	0	−[h]
8	**1f** (0.03)		500	22	0	−[h]
9	**1g** (0.02)	2		22	50	>99[g]
10	**1h** (0.03)		500	22	50	>99[f−h]
11	**1i** (0.05)	3		60	47	86[h]

[a] Reactions were carried out in 0.1 M EDA buffer (pH 7.8) in the presence of 0.2 mM FMN and 1600 IU of catalase. [b] Pure glycolate oxidase from Sigma. [c] Spinach acetone powder from Sigma (3.5×10^{-3} IU/mg). [d] Conversion was determined by GC analysis (DB-Wax column; error limit ±2%). [e] Enantiomeric excess (ee values) was determined by GC analysis. [f] After esterification with Mosher reagent (DB-5 column). [g] After esterification with (−)-menthyl chloroformate (DB-Wax column; error limit ±2%). [h] Determined by multidimensional GC analysis (cf. Table 2 in Supporting Information); error limit ±2%.

In the homologous series **1a**−**d** this enzyme oxidizes enantioselectively not only the short-chain 2-hydroxy acids **1a**,**b** (entries 1−3) but also the long-chain derivatives **1c**,**d** (entries 5 and 6). In contrast, the 2-hydroxyisobutyric acid (**1f**) and the *erythro*-2,3-dihydroxybutyric (**1e**) acids, with substantial steric demand in close proximity to the α-hydroxy functionality, are not accepted by the oxidase (entries 7 and 8). Nevertheless, the enzymatic resolution of the unsaturated *cis*- and *trans*-2-hydroxydec-4-enoic acids (**1g**,**h**) (entries 9 and 10) takes place with total enantioselectivity. Furthermore, the enzymatic resolution of the racemic 2-hydroxy-4-pentoxybutyric acid (**1i**) was achieved in high enantioselectivity with the glycolate oxidase (entry 11); however, compared to 2-hydroxycaprylic (**1d**) and the *cis*- and *trans*-2-hydroxydec-4-enoic (**1g**,**h**) acids, the enzyme activity toward the ether derivative **1i** is diminished. Thus, the latter requires a 3 times longer reaction time for ca. 50% conversion, and the enantioselectivity is with 86% ee significantly lower (entry 11).

To illustrate the synthetic value of this novel enzymatic preparation of optically active (*R*)-2-hydroxy acids, we have, as a representative case, conducted the enzymatic resolution of the 2-hydroxyvaleric acid (**1b**) on a preparative scale (entry 4). After two days of reaction time in the presence of the pure glycolate oxidase (5 IU), the resulting 2-hydroxyvaleric acid (**1b**) was obtained enantiomerically pure at 50% conversion of the racemic mixture and isolated in 41% yield.

According to the literature,[4] the oxidation of 2-hydroxy acids with glycolate oxidase takes place with a high degree of preference for the *S* enantiomer. In analogy, we have assigned the configuration of the 2-hydroxyvaleric acid (**1b**) in a chirooptical manner by measuring its optical rotation. The comparison of the measured optical

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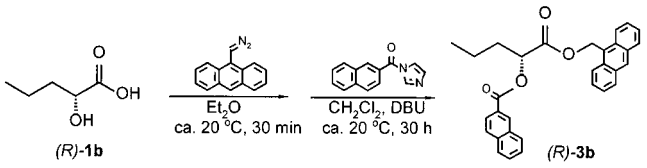
Scheme 2. Functionalization of the 2-Hydroxy Acid (*R*)-1b to the Corresponding Bichromophoric Derivative (*R*)-3b



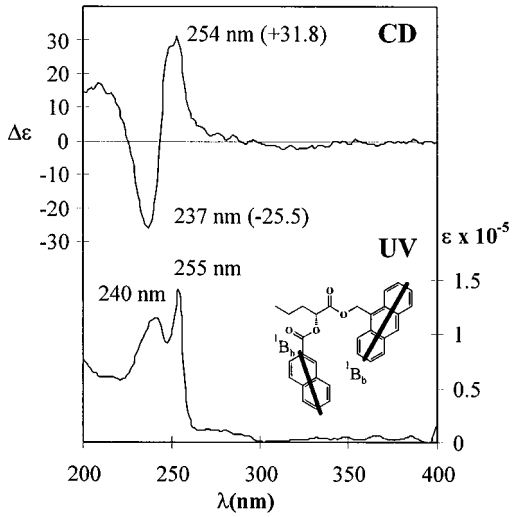


Figure 1. CD and UV spectra of the bichromophoric derivative (*R*)-**3b** in MeCN (1-cm cell). The bold lines represent the direction of the transition dipoles.

rotation value, $[\alpha]^{25}_D = -4.4$ ($c = 1.0$, $CHCl_3$), with the literature data[11] confirms the *R* configuration for the 2-hydroxyvaleric acid (**1b**).

We have also employed the exciton-coupled circular dichroism (ECCD) method for this purpose, which has been recently developed for the assignment of the absolute configuration of acyclic α- and *β*-hydroxy acids.[12] For the determination of the configuration by the ECCD method, two chromophores suitable for exciton coupling are required in the substrate. After derivatization (Scheme 2) of the enantiomerically pure 2-hydroxyvaleric acid (**1b**) to the corresponding 9′-methylanthryl (*R*)-2-(2″-naphthoyloxy)pentanoate (**3b**), the CD and UV[13] spectra of (*R*)-**3b** were recorded (Figure 1). In the bichromophoric derivative **3b**, the quite intense long axis ^1B_b transition of the 9-anthryl chromophore couples with the ^1B_b band of the 2-naphthoate chromophore to give a positive split CD curve with extrema at 254 nm ($\Delta\epsilon = +31.8$) and 237 nm ($\Delta\epsilon = -25.5$) and an amplitude *A* of +57.3. This positive CD behavior shows that the electric transition dipoles (^1B_b) of the 9-anthryl and the 2-naphthoate chromophore possess a "positive chirality",[14] which suggests the *R* configuration.[12] Thus, the ECCD method provides a convenient microscale method for the assignment of the absolute configuration of 2-hydroxy acids.

The enantiomeric excesses of the 2-hydroxy acids **1a**,**c**,**g**,**h** were determined by gas chromatography after the esterification of their methyl esters with either (*S*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (Mosher reagent)[15] or (−)-menthyl chloroformate.[16] The ee values of the 2-hydroxy acids **1b**,**d**−**f**,**h**,**i** were determined by multidimensional gas chromatography (MDGC) on a polar, achiral column in series with a chiral main column (the details of the MDGC analysis are given as Supporting Information). The elution order of the diastereomeric derivatives of 2-hydroxy acids **1a**,**c**,**g**,**h** on an achiral stationary phase and the optically active 2-hydroxy acids **1b**,**d**−**f**,**h**,**i** on a chiral stationary phase was ascertained by comparing the gas chromatographic data with that of the authentic reference compounds methyl (*S*)-2-hydroxybutyrate, (*R*)-2-hydroxyheptanoate, and (*R*)-2-hydroxydecanoate.[6e,8] The configurations of the 2-hydroxy acids **1g**−**i** were assigned accordingly.

In summary, our results show that the saturated (*R*)-2-hydroxy acids **1a**−**d**, the unsaturated derivatives **1g**,**h**, and the 2-hydroxy-4-pentoxybutyric acid (**1i**) may be obtained in very high enantiomeric purity from the corresponding racemic mixtures by enantioselective oxidation with molecular oxygen catalyzed by glycolate oxidase from spinach (*S. oleracea*). The assignment of the absolute configuration of the 2-hydroxy acid **1b** by the ECCD method confirms the exclusive preference of the glycolate oxidase for the *S* enantiomer and demonstrates the applicability of this microscale method for the configurational assignment of 2-hydroxy acids.

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Supporting Information Available: Experimental Section (8 pages). This material is contained in libraries on microfiche, immmediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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