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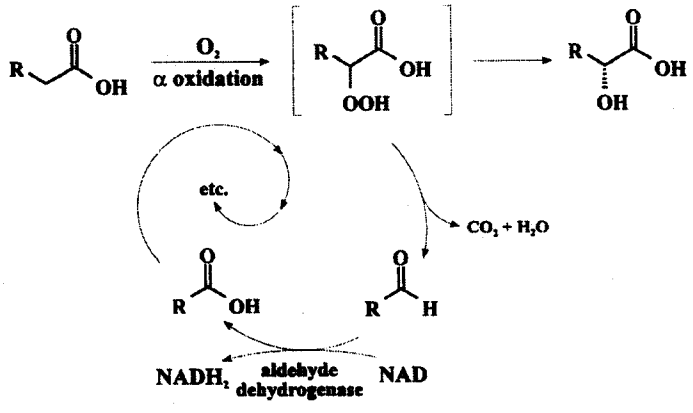
Enantioselective α Hydroxylation of Carboxylic Acids with Molecular Oxygen Catalyzed by the α Oxidation Enzyme System of Young Pea Leaves (*Pisum sativum*): A Substrate Selectivity Study

Waldemar Adam [a]*, Michael Lazarus [b], Chantu R. Saha-Möller [a] and Peter Schreier [b]

[a] Institute of Organic Chemistry and [b] Institute of Pharmacy and Food Chemistry, University of Würzburg, Am Hubland, D-97074 Würzburg, Germany

Abstract: The substrate selectivity of the α oxidation of carboxylic acids **1** by crude homogenate of young pea leaves was investigated. Saturated fatty acids with 7 to 16 carbon atoms and oleic acid were transformed to the enantiomerically pure (*R*)-2-hydroxy acids **2** in the presence of molecular oxygen. Copyright © 1996 Elsevier Science Ltd

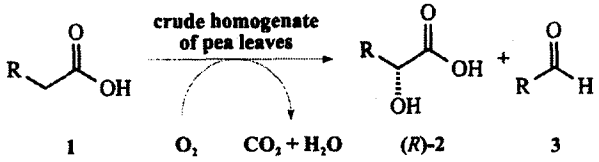
The α oxidation of fatty acids is known for higher plants such as pea leaves (*Pisum sativum*) [1], germinating peanuts (*Arachis hypogaea*) [2], cucumber (*Cucumis sativus*) [3] and potatoes (*Solanum tuberosum*) [4] as well as for simple organisms such as marine green alga (*Ulva pertusa*) [5]. In higher plants, 2-hydroxy fatty acids are formed in the oxidative lipid metabolism by α oxidation of the corresponding acids [2, 6]. The mechanism of this biochemical reaction was worked out by Shine and Stumpf [2] in the seventies (Scheme 1). They have postulated that the flavoprotein-catalyzed oxidation of fatty acid leads to an intermediary



Scheme 1: α Oxidation of fatty acids in higher plants

α-hydroperoxy acid, which preferentially decarboxylates to the corresponding aldehyde in competition with reduction to the 2-hydroxy acid. While the 2-hydroxy acid accumulates, the aldehyde is oxidized by an NAD-dependent aldehyde dehydrogenase to the next lower homologous fatty acid, which in turn functions as a substrate for the α oxidation [2,6]. It was reported that only C_{14} to C_{16} saturated and natural C_{18} unsaturated fatty acids are substrates for the α oxidation enzyme system of higher plants [1-5, 7]. In the case of hexadecanoic (C_{16}) acid (palmitic acid) the enantioselective formation of (*R*)-2-hydroxyhexadecanoic acid was observed [11].

Chiral α-hydroxy acids are important building blocks for the synthesis of optically active glycols [8a], halo esters [8b] and epoxides [8c]. The enzymatic methods employed so far for the synthesis of α-hydroxy-functionalized carboxylic acids are the enantioselective oxidation of 1,2-diols with dehydrogenases [9a], the reduction of α-keto acids with bakers yeast [9b], the oxynitrilases-catalyzed addition of prussic acid to aldehydes [9c] and the kinetic resolution of methyl α-hydroperoxy esters with horseradish peroxidase [9d]. Nevertheless, little is known about the direct enantioselective α hydroxylation of carboxylic acids. To assess the scope of the enzymatic α oxidation for the preparation of enantiomerically pure 2-hydroxy acids, we have studied in detail the substrate selectivity of the α oxidation system of young pea leaves (Scheme 2). The results are given in Table 1.



a-l: R= $CH_3(CH_2)_n$;
a (n=15), b (n=13), c (n=11), d (n=9), e (n=8), f (n=7), g (n=5), h (n=4), i (n=3).
k: R= $CH_3(CH_2)_7CH{=}CH(CH_2)_6$

Scheme 2: α Oxidation of carboxylic acids by a crude homogenate of young pea leaves with molecular oxygen

The α oxidation of saturated carboxylic acids **1b-h** as well as oleic acid **1k** by crude homogenate of pea leaves with molecular oxygen yielded exclusively the corresponding next lower homologous aldehyde **3** and the 2-hydroxy acid **2**, with higher preference for the former (Table 1). To achieve preferential formation of **2**, the enzymatic reaction was optimized by using palmitic acid **1b** as substrate. Under acidic conditions (pH 5-6) the α hydroxylation of palmitic acid is favored over its decarboxylation to the aldehyde **3b** (entries 4 and 5). At pH 7.0 (entry 6), however, the aldehyde **3b** is produced preferentially; therefore, the carboxylic acids **1c-k** were oxidized at pH 6.0. α Oxidation enzymes are regarded to be membrane-bound and, thus, the addition of

the emulsifier Triton X-100 to the phosphate buffer resulted in an increased conversion rate of palmitic

Table 1: α Oxidation of carboxylic acids with crude homogenate of pea leaves [a].

		Substrate		Time	Conversion	Product Ratio (%) [b]	ee (%)
Entry	**1**	C_n	(mmol)	*t* (h)	(%)	**2** : **3**	(*R*)-**2**
1	**1a**	18	0.03	22	0	--	--
2	**1b**	16	0.02 [c]	6	15	23 : 77	> 99
3	**1b**	16	0.02	6	46	40 : 60	> 99
4	**1b**	16	0.02 [c, d]	17	24	56 : 44	> 99
5	**1b**	16	0.02 [c, d]	17	24	55 : 45	> 99
6	**1b**	16	0.02 [c, d]	17	23	29 : 71	> 99
7	**1c**	14	0.02	6	41	31 : 69	> 99
8	**1d**	12	0.02	6	33	38 : 62	> 99
9	**1e**	11	0.02	22	37	38 : 62	> 99
10	**1f**	10	0.02	6	33	48 : 52	> 99
11	**1g**	8	0.03	22	< 10	[e]	> 99
12	**1h**	7	0.03	19	< 10	[e]	> 99
13	**1i**	6	0.03	22	0	--	--
14	**1k**	18	0.03	22	86	24 : 76	> 99 [f]

[a] Crude homogenate of young pea leaves, 0.2 M phosphate buffer (pH 6.0), 0.1% Triton X-100. [b] The product distribution, normalized to 100%, was determined by gas chromatography; column A: J&W DB-5 (30 m x 0.25 mm; df = 0.25 μm), temperature program 60 to 300 °C (5 °C/ min), column B: J&W DB-Wax (30 m x 0.25 mm; df = 0.25 μm), temperature program 50 (3 min isothermally) to 240 °C (4 °C/ min); error limit ± 2%. [c] Phosphate buffer without Triton X-100 was used. [d] The pH value of the phosphate buffer was varied, e.g., for the entries 4-6 pH values of 5.0, 6.0 and 7.0 were used. [e] Only the 2-hydroxy acid was detected as product of the α-oxidation. [f] The absolute configuration is uncertain.

acid **1b**. While the α oxidation of **1b** in the presence of Triton X-100 afforded the 2-hydroxy acid **2b** and the aldehyde **3b** in a ratio of 40:60 at 46% conversion (entry 3), without the emulsifier only 15% conversion and a product ratio of 23:70 (entry 2) were observed. In Table 1 the optimized reaction times are given, beyond which no further conversion of the carboxylic acids **1a-k** was observed.

The results shown in Table 1 reveal that the activity of the crude homogenates of pea leaves is dependent on the chain length of the carboxylic acids. In the homologous series **1b-i** the enzyme activity is significantly diminished with decreasing chain length (entries 11 and 12), in particular for the caprylic **1g** (C_8) and oenanthic acid **1h** (C_7), while the capronic acid **1i** (C_6) is not accepted as substrate by these α oxidation

enzymes. Stearic acid **1a** (C_{18}) marks the upper limit of the saturated carboxylic acids for α oxidation (entry 1). In contrast, the unsaturated oleic acid **1k** represents a good substrate in this enzymatic oxidation to afford the α-hydroxy oleic acid **2k** and the corresponding aldehyde **3k** in a ratio of 24 : 76 at 86% conversion (entry 14).

The enzymatic α hydroxylation of the saturated carboxylic acids **1b-h** and oleic acid **1k** is enantioselective. The enantiomeric excess of the 2-hydroxy acids **2b-h** and **2k** was determined by gas chromatography after their esterification with (*S*)-(+)-α-methoxy-α-trifluoromethylphenylacetyl chloride (Mosher reagent) [10]. A representative chromatogram is depicted in Figure 1.

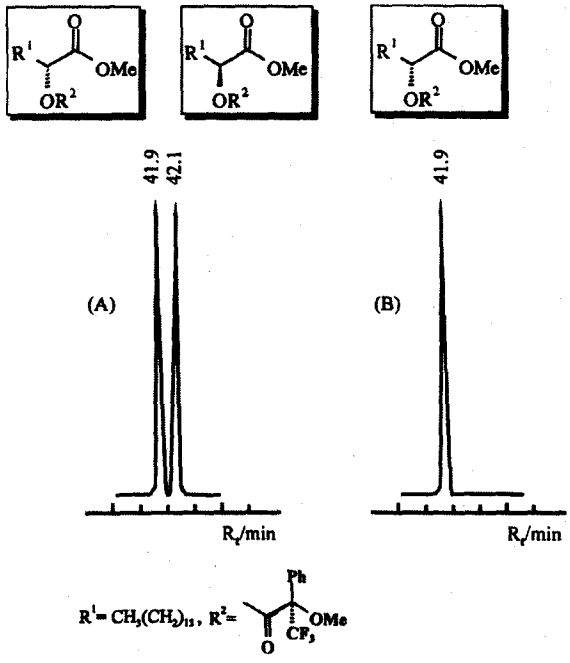


Figure 1: Determination of the enantiomeric excess of the 2-hydroxy acid **2b** after esterification with Mosher reagent; column: J&W DB-5 (30 m x 0.25 mm, df = 0.24 μm); temperature program: 60 - 300 °C, 5 °C/ min; (A) authentic racemic reference compound, (B) Mosher diester of the 2-hydroxy acid **2b** from α oxidation.

The elution order of the diasteromeric Mosher diester of 2-hydroxy acids was ascertained by comparing the gas-chromatographic data with that of the authentic reference compounds methyl (*S*)-2-hydroxybutyrate and (*R*)-2-hydroxyhexadecanoate [9d, 11]. The configurations of the 2-hydroxy acids **2b-h** and **2k** were assigned accordingly.

In summary, our results show that, in contrast to the previous reports [1-5, 7], not only long-chain fatty acids are substrates for α oxidation but a broad variety of saturated acids with 7 to 16 carbon atoms and even the oleic

acid with an unsaturated C_{18} chain are recognized by the α oxidation enzyme system. Thus, readily available carboxylic acids **1** are enantioselectively oxidized by crude homogenates of young pea leaves in the presence of molecular oxygen to the (*R*)-2-hydroxy acids **2**.

Experimental

General procedure. For ease of dissolution of the solid carboxylic acids **1a-f** in the aqueous medium, the particular substrate was first taken up in chloroform (1 mL), the solvent was evaporated under reduced pressure (20 °C/17 Torr), 2 mL of phosphate buffer (pH 6.0), which contained 0.1% Triton X-100, was added to the residue, and the mixture sonicated for 1 min. The liquid carboxylic acids **1g-k** were directly dissolved in the phosphate buffer (pH 6.0) and 0.1% Triton X-100. The crude homogenate was prepared by homogenizing 10-15 g young pea leaves (var. *sativum*, 14 days old) with 150 mL 0.2 M phosphate buffer, which contained 0.1% Triton X-100, in a blender for 45 s. The aqueous carboxylic acid solution and the crude enzyme extract were stirred together for several hours in an ice bath (ca. 4 °C), while a low stream of oxygen gas was passed through the reaction mixture continually. Subsequently, the insoluble materials were removed by filtration, the filtrate was acidified with 6 N hydrochloric acid (pH 3) and extracted with diethyl ether (3 x 75 mL). The combined organic phases were dried over Na_2SO_4 and the solvent was evaporated under reduced presure (20 °C/17 Torr). The free acids **1** and **2** were converted to their methyl esters with diazomethane. After determination of the amount of conversion and product distribution, the crude reaction mixture was submitted to chromatography [ca. 10 g silica gel, 0.032 - 0.062 mesh, petroleum ether/ethyl ether (7:3) as eluent] and the methyl 2-hydroxy ester was isolated in pure form. The enantiomeric excess (ee) of the methyl 2-hydroxy esters was determined after derivatization with (*S*)-(+)-α-methoxy-α-trifluoromethylphenylacetyl chloride (Mosher reagent) [10] by quantitative gas chromatography. The results for the literature known [12] 2-hydroxy acids **2b-h** are given in Table 1.

Acknowledgements

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