9-(3,4-Dimethyl-5-pentyl-furan-2-yl) nonanoic Acid and 9-(3,4-Dimethyl-5-propyl-furan-2-yl) nonanoic Acid: New Naturally Occurring Peroxidase Inhibitors

Claus T. Fuchs and Gerhard Spiteller*

Institut für Organische Chemie I, Universität Bayreuth, Universitätsstr. 30, 95440 Bayreuth, Germany. Fax: 0921/552671. E-mail: gerhard.spiteller@uni-bayreuth.de

* Author for correspondence and reprint requests

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Horseradish Peroxidase, 9-(3,4-dimethyl-5-pentyl-furan-2-yl) nonanoic Acid [diMeF(9,5)], 9-(3,4-dimethyl-5-propyl-furan-2-yl) nonanoic Acid [diMeF(9,3)], Competitive Inhibitor, Indole-3-acetic Acid

9-(3,4-Dimethyl-5-pentyl-furan-2-yl) nonanoic acid [diMeF(9,5)] and 9-(3,4-dimethyl-5-propyl-furan-2-yl) nonanoic acid [diMeF(9,3)] and its corresponding methyl esters have been assayed for inhibitory activity on horseradish peroxidase (EC 1.11.1.17) by measuring the peroxidase-catalyse decomposition of indole-3-acetic acid. Both compounds and their methylates are competitive inhibitors to horseradish peroxidase with inhibitor constants (K_I) of $5.0 \pm 0.9 \times 10^{-5}$ M respectively $5.2 \pm 0.8 \times 10^{-5}$ M. Development of inhibitory effect requires not only the presence of the furan heterocycle but also of a polar side chain.

Introduction

Peroxidases (PO) (EC 1.11.1.7) are hemoprotein enzymes catalyzing the oxidation of a number of organic and inorganic compounds, e.g. phenols and indole-3-acetic acid (IAA), with hydrogen peroxide or alkyl peroxides (Dunford and Stillman, 1976).

Considering the structural analogy of furan fatty acids (a five-membered heterocyclic aromate with a carboxylic side chain) to these known PO-substrates we examined whether the furan fatty acids dimethyl-(5-pentyl-furan-2-yl) nonanoic acid [diMeF(9,5)] **1** or 9-(3,4-dimethyl-5-propyl-furan-2-yl) nonanoic acid [diMeF(9,3)]) **2** respectively their corresponding methyl esters dimethyl-(5-pentyl-furan-2-yl) nonanoate **3** and 9-(3,4-dimethyl-5-propyl-furan-2-yl) nonanoate **4**) develop any inhibitory effect on PO-catalyzed IAA-decomposition.

Abbreviations: PO, Peroxidase; K_I, inhibitor constant; LB, Lineweaver-Burk; K_M, Michaelis constant; v_{max}, maximal velocity; HRP, Horseradish peroxidase; nonanoic acid, 9-(3,4-dimethyl-5-pentyl-furan-2-yl) [diMeF(9,5)]; nonanoic acid, 9-(3,4-Dimethyl-5-propyl-furan-2-yl) [diMeF(9, 3)]; IAA, indole-3-acetic acid; TMS, trimethylsilyl.

Furan fatty acids (F-acids) are widely distributed trace components of animal (Glass *et al.,* 1974; Ishii *et al.,* 1988; Ota and Takagi, 1992) and plant (Hasma and Subramaniam, 1978; Hannemann *et al.,* 1989; Guth and Grosch, 1991; Ciminiello *et al.,* 1991; Scheinkönig and Spiteller, 1993) lipids. During recent years both the metabolism (Spiteller *et al.,* 1980; Sand *et al.,* 1983; Lindrup *et al.,* 1991) and the biogenesis (Sand *et al.,* 1984; Batna and Spiteller, 1993; Scheinkönig and Spiteller, 1993; Batna *et al.,* 1993) of these unusual fatty acids were studied, but their biological significance, if any, remained unknown.

Results and Discussion

In order to study the effects of furan fatty acids on PO-activity the chromogenic assay of Krylov *et al.* (1993) was modified. Originally, this assay determined the antioxidative capacity of compounds towards the PO-catalysed decomposition of indole-3-acetic acid (IAA) (Osswald *et al.,* 1988; Waldrum *et al.,* 1981; Gelinas *et al.,* 1973; Wiese, 1986). The IAA-decomposition probably proceeds via an indole-3-methyl-hydroperoxide intermediate (Nakajima and Yamazaki, 1979; Kobayashi *et al.,* 1984) to indole-3-methanol **8** and indole-3-carbaldehyde (Tsurumi and Wada, 1985).

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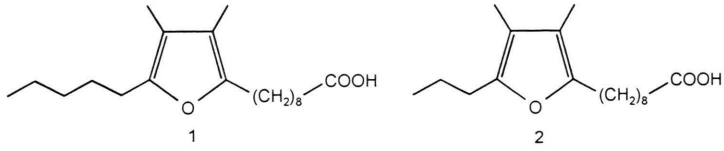


Scheme 1. Dimethyl-(5-pentyl-furan-2-yl) nonanoic acid [(diMeF(9,5)] **1**; 9-(3,4-Dimethyl-5-propyl-furan-2-yl) nonanoic acid [diMeF(9,3)] **2**.

In order to determine IAA decomposition products (mainly indole-3-methanol **8**) (Volpert *et al.*, 1995) we measured the absorbance difference at 242 and 296 nm (wavelengths with the same absorbance for IAA) in comparison to a reference of known IAA-concentration. The resulting absorbance difference corresponds to PO-activity.

Figure 1 shows the Lineweaver-Burk (LB)-plots for the decomposition of IAA after addition of different concentrations of **1**. Changes of the reaction velocity and of the points of intersection allow the determination of the mode of action (competitive or non-competitive) and effectiveness of inhibition.

The LB-plots intersect the ordinate in one point indicating a competitive enzyme inhibition.

According to the formula

$$(1+[I]/K_I)\ K_M / \tilde{\alpha}_{max} = \text{gradient of the LB-plots}$$

the inhibitor constant was calculated (Henderson, 1992) from the mean value of four measurements with different concentrations of **1**, **2**, **3** and **4** (Table I):

Table I. K_I-values for tested compounds.

diMeF(9,5)	**1**	$5.2 \times 10^{-5} \pm 0.8 \times 10^{-5}$ M
diMeF(9,5)-Methylester	**3**	$6.0 \times 10^{-5} \pm 0.6 \times 10^{-5}$ M
diMeF(9,3)	**2**	$5.0 \times 10^{-5} \pm 0.9 \times 10^{-5}$ M
diMeF(9,3)-Methylester	**4**	$5.8 \times 10^{-5} \pm 0.7 \times 10^{-5}$ M

Further investigations with equal concentrations of furan proved that the non-substituted furan heterocycle is about ten times less effective in competitive inhibition (K_I = 3.4 * 10^{-4} ± 0.00009 M). Thus, the PO-inhibitor effectiveness depends on the presence of the heterocycle and a functional group, probably a carboxylic group.

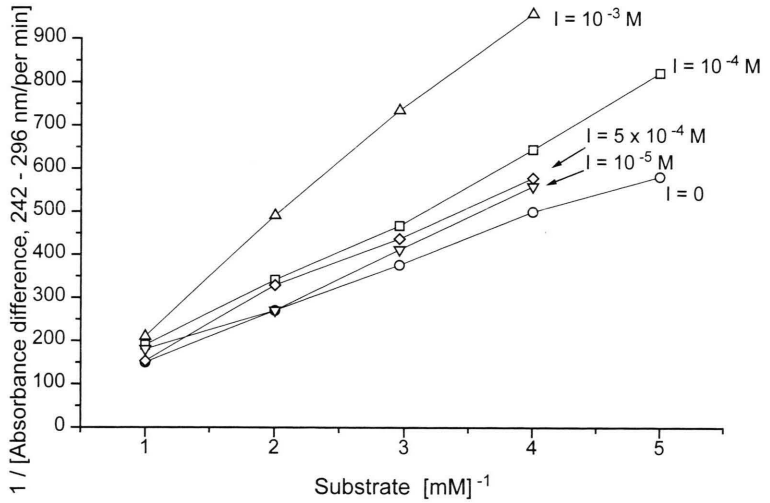


Fig. 1. Lineweaver-Burk-plots (LB) after addition of different concentrations of 9-(3,4-dimethyl-5-pentyl-furan-2-yl) nonanoic acid [diMeF(9,5)] **1**.

In the case of HRP, the binding sites of aromatic substrates were studied by many investigators, revealing that they are located in the vicinity of heme periphery 8-methyl group, the distance from the heme iron being 80–100 nm (Sakurada *et al.*, 1986; Thanabal *et al.*, 1987; Veich and Williams, 1990; Banci *et al.*, 1993; Casella *et al.*, 1991) whereas inorganic substrates bind to the enzyme in the vicinity of the heme at equal distances from 8-methyl and 1-methyl (Sakurada *et al.*, 1987).

Besides a direct competition for the binding at the active site there is another mode of action: The rate of H_2O_2 binding to the heme iron may be reduced by the binding of furan fatty acids to the protein molecule. This has already been reported for EtOH (Dunford and Hewson, 1977).

While partial proteolysis revealed that peroxidase isoenzymes have different peptide sequences (Stephan and van Huystee, 1981) no difference in the substrate specifity were found between isoenzymes. Therefore we speculate that our results may be applicable to other peroxidases and peroxidase isoenzymes too, e.g. human myeloperoxidase.

Experimental

Materials

IAA, horseradish peroxidase (HRP) (EC 1.11.1.17), sodium deoxycholate, maleic acid anhydride and 2-amino-pyridine were obtained from Fluka Chemie AG, Neu-Ulm, Germany.

Sn(IV)-chloride was purchased from Sigma-Aldrich Chemie GmbH, Steinheim, Germany.

PO assay [according to Krylow *et al.*, 1983]

Briefly, a cuvette was charged with a mixture of 1400 µl phosphate buffer (pH 7.4; 67 mM), 200 µl IAA (1 µM in 67 mM phosphate-buffer pH 7.4) and 200 µl of the inhibitor (in 67 mM phosphate-buffer pH 7.4). Furthermore a stock solution of the inhibitor was prepared by addition of desoxy cholic acid sodium salt (1 mM solution in 67 mM phosphate-buffer pH 7.4). The enzymatic reaction was started by the addition of 200 µl PO-solution (0.614 U; in 67 mM phosphate-buffer pH 7.4). The final volume was 2 ml. Kinetics of the IAA decomposition was determined by absorbance difference at 242 nm and 296 nm in a quartz-cuvette. The validity of the test system was established with cyanide, a PO-inhibitor (Tutschek, 1979; Kim *et al.*,1980).

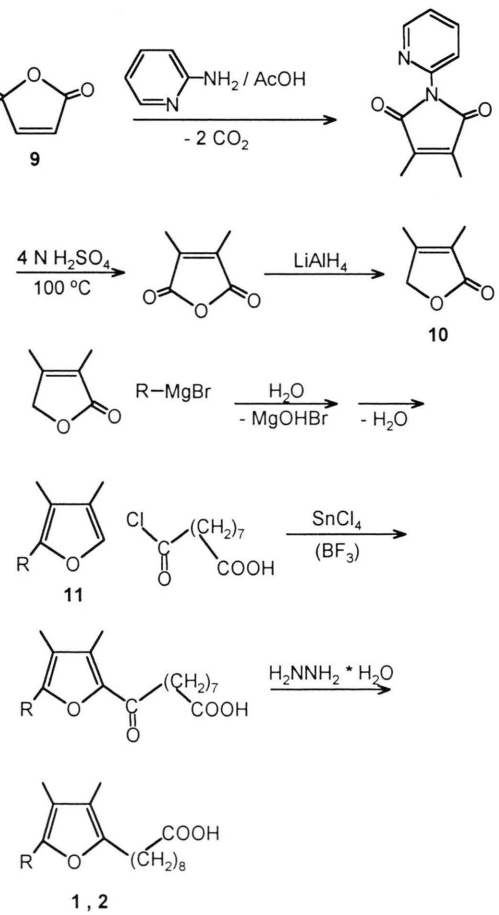
Addition of IAA in excess prevented PO-inhibition.

UV spectroscopy

UV measurements were performed with a Shimadzu UV-160 A photometer. The absorbance at A(242–296) nm was measured in intervals of thirty seconds.

1 and **2** were synthesized (Scheme 2) according to a procedure introduced by Voss, Gerlach and Prasslsberger (Prasslsberger, 1990).

Synthesis of **10** was performed according to Baumann and Bosshard (1978), Baumann *et al.* (1984) and Kayser and Morand (1980).



Scheme 2. Synthesis of 9-(3,4-dimethyl-5-pentyl-furan-2-yl) nonanoic acid [diMeF(9,5)] **1** and 9-(3,4-dimethyl-5-propyl-furan-2-yl) nonanoic acid [diMeF(9,3)] **2**; R = CH_3-$(CH_2)_2$- **2**, CH_3-$(CH_2)_4$- **1**.

Alkylation of **11** and further transformation to **1** and **2** was performed as described by Prasslsberger (1990) and Rahn *et al.* (1979).

The corresponding methyl esters **3**, **4** were generated by addition of an etheral solution of diazomethane to **1** and **2**.

HPLC purification

Purification of **1**, **2**, **3**, **4** was performed by HPLC (detection at 227 nm) on a Bischoff Ultrasep® 100 (6 μm) column. Isocratic separation 45 % A (A: n-hexane/AcOH 100/0.1 v/v) and 55 % B (B: hexane/isopropanol/AcOH 100/5/0.1 v/v/v) at 3 ml/min.

Retention time: **1** 64.218 min.; **2**: 83.515 min.; **3**: 62.357 min; **4**: 80.391 min.

Mass spectra

1: (TMS-derivative): 394 (33), 379(29), 337(46), 193 (12), 179(100), 161(7), 135(14), 123(24), 109(12), 75(23), 73(25), 55(9), 41(16).

2: (TMS-derivative): 376 (33), 361(29), 309(46), 165 (12), 151(100), 135(14), 123(24), 109(12), 75(23), 73(25), 55(9), 41(16).

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