4-Pentadecylpyridine: A Competitive Polyphenoloxidase Inhibitor

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4-Pentadecylpyridine was isolated from plums infected by *Taphrina pruni*. This fungal metabolite has been tested for its effect on polyphenoloxidase, using the polyphenoloxidase-catalysed addition of L-proline to 4-methylcatechol in a chromogenic assay. The resulting data prove that 4-pentadecylpyridine is a competitive inhibitor to polyphenoloxidase with an inhibitor constant of $3.70 \pm 0.03 * 10^{-4}$ M. Further comparative measurements have demonstrated that the effect is due to the pyridine moiety. Probably reduced polyphenoloxidase-activity decreases the toxicity of *o*-diphenols to the fungus and thus increases susceptibility of plums to the fungus *Taphrina pruni*.

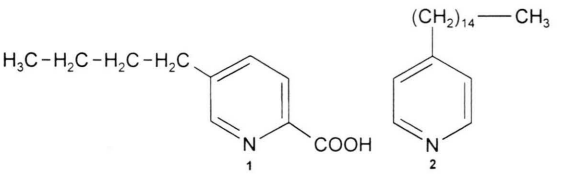
Introduction

Our previous investigations of the constituents of healthy plums and those infected by the fungus *Taphrina pruni* revealed that in affected fruits the content of *o*-diphenols (Fuchs, 1996), especially (+)-catechine, catechine conjugates, proanthocyanidines and caffeoyl-D-quinic acids is increased compared to unaffected fruits for a factor of about 20. Since all of these compounds are phenols they may serve as polyphenoloxidase (PPO)-substrates (Lerch, 1987; Mayer and Harel, 1979; Mayer, 1987).

Polyphenoloxidase (PPO) (tyrosinase, monophenol monooxygenase, catecholoxidase EC 1.14.18.1) is a copper containing enzyme catalysing the *o*-hydroxylation of monophenols to *o*-diphenolic compounds and their further oxidation to the toxic *o*-quinones (Lerch (1987); Scheme 2).

Furthermore, in the course of our investigations, 4-pentadecylpyridine (**2**) has been isolated from the fungus *Taphrina pruni* (Fuchs and Spiteller, 1995). As a weak base bearing long alkyl chains 4-pentadecylpyridine (**2**) is a lysosomotropic detergent (Firestone and Pisano, 1979): It accumulates within lysosomes, ruptures their membranes and releases the contents of the cells into the cytoplasm.

Due to the structural analogy (Scheme 1) of this new natural compound to fusaric acid (**1**), a well-known fungal metabolite from *Fusarium* species, we suspected that **2** may develop similar biological activity as **1**. Since several communications (Becker and Pushkareva, 1972; Kern, 1972) demonstrate that **1** inhibits polyphenoloxidase and thus quinone formation, we investigated the effects of 4-pentadecylpyridine (**2**) on ppo-inhibition, assuming further consequences for the toxicity of the above identified phenols and plum susceptibility (Mason and Wassermann, 1987; Goldstein and Swain, 1963; Hayes, 1989).



Scheme 1. Fusaric acid (**1**) (5-n-butyl-2-picolinic acid) **and 4-pentadecylpyridine** (**2**).

Results

In order to study the effect of 4-pentadecylpyridine (**2**) on ppo-activity a chromogenic assay was

Abbreviations: PPO: Polyphenoloxidase; K_I: inhibitor constant; PMMA: polymethylmethacrylate; LB: Lineweaver-Burk; K_M: Michaelis constant; v_{max}: maximal velocity.

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modified in analogy to Rzepecki *et al.* (Rzepecki and Waite, 1989). This assay is based on the ppo-catalysed addition of L-proline to 4-methylcatechol (simplest monosubstituted *o*-diphenol) (analogous Scheme 2) forming 4-*N*-prolyl-adducts.

The reaction has been used to determine N-terminal proline residues in peptide polymers (Mason and Peterson, 1955) and to detect catecholoxidase activity in nondenaturating thin layer or gel permeation chromatography of polyphenoloxidase enzymes (Jolley and Mason, 1964; Thomas *et al.*, 1978).

Figure 1 shows the Lineweaver-Burk (LB)-plots for the generation of 4-*N*-prolyl adducts after addition of different concentrations of 4-pentadecylpyridine (**2**). Changes of the slope and intercept of the 1/V versus 1/[S] plot indicate competitive respectively non-competitive inhibition. In addition the curve allows conclusions on the binding strength of an inhibitor (Engel, 1977).

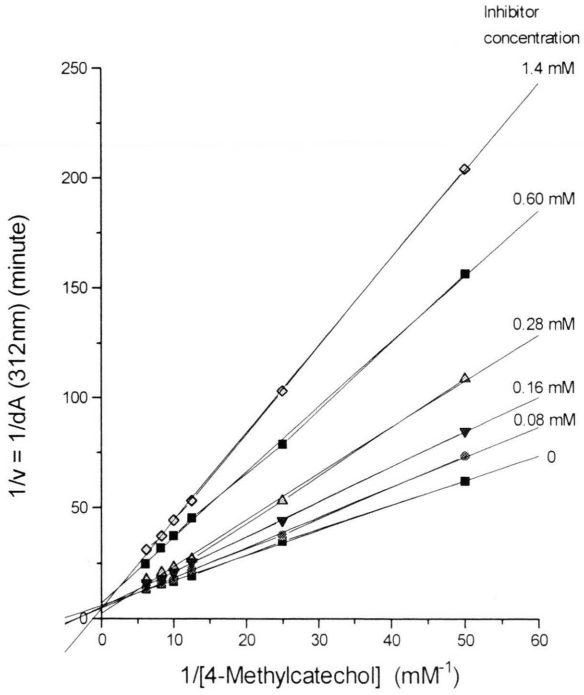


Fig. 1. Lineweaver-Burk-Plot (LB) in presence of different concentrations of 4-pentadecylpyridine (2).

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

According to the formula

$$\mathbf{(1+[\mathit{I}]/\mathit{K}_I)\ \mathit{K}_M/v_{max} = gradient\ of\ the\ LB\text{-}plots}$$

the inhibitor constant was calculated (Henderson, 1992; Lehninger, 1977) as mean value of four measurements with different 4-pentadecylpyridine concentrations:

$$\mathbf{\mathit{K}_I = 0.370*10^{-4} \pm 0.003*10^{-4}\ M\ (Table\ I).}$$

Table I. PPO-inhibition with 4-pentadecylpyridine 2.

Final inhibitor concentration [μM]	Gradient of the LB-plot	$1/K_I$	K_I
0	1.125		
80	1.364	2655	0.000376
160	1.583	2544	0.000393
280	2.079	3029	0.000330
600	1.626	2710	0.000369
1400	3.975	1810	0.000552

We assume that associative effects at higher 4-pentadecylpyridine (**2**) concentrations cause a significant increase of the inhibitor constant (Engel, 1977). For this reason the highest concentration (inhibitor concentration: 1400 μM; K_I: 0.000552 M) was neglected for mean value calculation.

As expected, further comparative measurements with equal concentrations of pyridine (80 μM and 280 μM) proved that the inhibition is exclusively due to the pyridine residue.

Experimental

PPO assay

The ppo-assay was modified in analogy to Rzepecki *et al.* (Rzepecki and Waite, 1989).

Briefly, a PMMA-cuvette was charged with a mixture of 1000 μl phosphate buffer (pH 7,5; 0.1 M), 200 μl L-proline (0.2 M in p-buffer pH 7,5) and 200 μl 4 M NaCl. Then 100 μl of a 1 μM 4-methylcatechol solution was added. Autoxidation of 4-methylcatechol was inhibited by dissolving the substrate in 0.2 M acetic acid. Finally, 700 μl water (reference) respectively X μl 4-pentadecylpyridine (**2**) (X = 50, 100, 175, 300 and 700; 10^{-2} M aqueous solution) and (700-X) μl water were added. The enzymatic reaction was started by the addition of 200 μl ppo-solution (0,614 U; in p-buffer pH 7,5). Kinetics of the 4-*N*-prolyl-adduct formation was determined at 312 nm in a PMMA-cuvette.

The validity of the test system was established with cyanide, a well-known ppo-inhibitor (Wittenberg and Triplett, 1985).

UV spectroscopy

UV measurements were performed with a Shimadzu UV-160 A photometer. The absorbance at 312 nm was measured in intervals of one minute. The molar extinction coefficient (ε) of the 4-*N*-prolyl adduct of 4-methylcatechol is 9630 $M^{-1}cm^{-1}$ (Rzepecki and Waite, 1989).

Chemicals

L-Proline, 4-methylcatechol and tyrosinase (EC 1.14.18.1) were obtained from Fluka Chemie AG, Neu-Ulm.

Synthesis

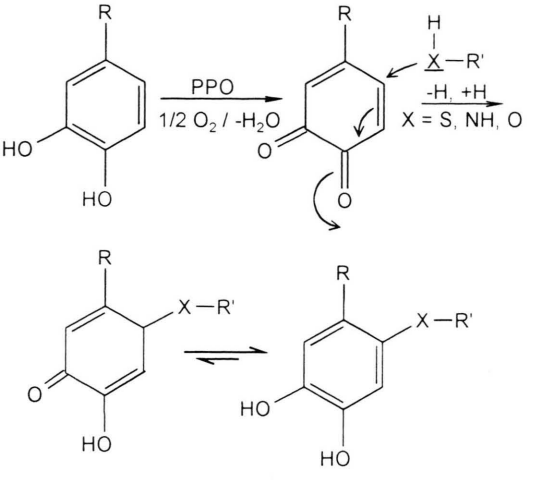
4-Pentadecylpyridine was synthesised as previously described (Fuchs and Spiteller, 1995).

Discussion

PPO-activity increases after infection or mechanical wounding (Matta and Abbattista, 1970; Pitt, 1975; Cheung and Henderson, 1972). A similar increase of activity was observed in galls formed in a number of plants after pathogen infection (Joshi and Tandon, 1984; Tandon and Arya, 1982; Ramawat and Purohit, 1980). Furthermore, an influence on plant hormone regulation, especially indolyl acetic acid, has been implied for PPO by various experiments (Gordon and Paleg, 1961; Tomaszewski and Thimann, 1966; Sondheimer and Griffin, 1960).

Release of free fatty acids generated by membrane wounding (Galliard, 1970) induces the activation of latent PPO (Hutcheson *et al.*, 1980; Golbeck and Cammarata, 1981). Thus enhanced PPO-activity is directly correlated to host resistance respectively pathogen infectibility (Bashan *et al.*, 1987; Leon, 1971).

The assumption that PPO-oxidation plays an important role in plant defence is corroborated by the finding of increased susceptibility of plants to fungi after quinone reduction (Moustafa and Whittenbury, 1970). Furthermore, host resistance in *Ribes* to the pathogen *Spaeotheca* was found to depend on the levels and interaction of polyphenoloxidase, chlorogenic acid and a reducing agent such as ascorbic acid (Trajkowski, 1976). Mukherjee and Gosh demonstrated that ppo-inhibition is directly connected to a decrease in resistance (Mukherjee and Gosh, 1975). This resistance is directly correlated to quinone toxicity and the ability of these quinones to add to substances with active hydrogen atoms, e.g. lysine-, cysteine- or tyrosine residues (Pierpoint *et al.*, 1977; Pierpoint, 1966; Auf' Mkolk, 1985) (Scheme 2).



Scheme 2. Toxicity of *o*-diphenols: Addition of *o*-diquinones to S -, N- and O-containing nucleophils.
PPO: Polyphenoloxidase (EC 1.14.18.1).

Considering that fungal enzymes are S- and N-containing compounds, they are inactivated by the corresponding quinones (Mason and Wassermann, 1987; Goldstein and Swain, 1963; Hayes, 1989). Thus the accumulation of a high concentration of *o*-diphenols in infected plums is a defence response directed to the enzymes of the pathogen. Nevertheless, the effectiveness of this defence response is highly dependent on quinone formation respectively PPO-activity.

While competitive inhibitors influence the binding position of PPO, reagents binding to the copper atom of the enzyme are non-competitive inhibitors (e.g. cyanide and thiourea) (Wittenberg and Triplett, 1985): Our data indicate that 4-pentadecylpyridine (**2**) is a competitive inhibitor, whereas fusaric acid (**1**) (Walter, 1969) shows interaction to the copper atom of the PPO.

In conclusion, 4-pentadecylpyridine (**2**) is a strong competitive inhibitor of PPO. Its binding strength is beyond the magnitude of many natural PPO-substrates, e.g. ferulic acid (inhibitor constant: K_I = 1.3 mM) (Wittenberg and Triplett, 1985; Anosike and Ayaebene, 1982; Walter, 1969; Loeffler and Zenk, 1990).

At least at the site of penetration (and high 4-pentadecylpyridine (**2**) concentration) the fungal

pyridine derivative may significantly reduce the formation of toxic *o*-quinones. Since these o-quinones are directly correlated with defence reactions of affected plants (Mayer, 1987; Bashan *et al.*, 1987; Leon, 1971) they may significantly increase plum susceptibility to the fungus respectively decrease plum resistance to fungal infection.

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