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NEW PHENOLIC INHIBITORS OF THE PEROXIDASE-CATALYSED OXIDATION OF INDOLE-3-ACETIC ACID

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Abstract—Previously we reported two metabolites of the insecticide carbofuran as persistent inhibitors of the peroxidase-catalysed oxidation of indole-3-acetic acid. In searching for more active inhibitors of this type, we have found that 5-hydroxy-2,2-dimethylchromene (β-tubanol), 2′,6′-dihydroxyacetophenone oxime, 5-hydroxy-2,2-dimethylchroman, 2′,6′-dihydroxyacetophenone and 2,6-dihydroxybenzoic acid methyl ester were more active than the carbofuran metabolite 7-hydroxy-2,2-dimethyl-3-oxo-2,3-dihydrobenzofuran. Resorcinol, 5-hydroxy-2,2-dimethylchroman-4-one, 3-hydroxy-5-methoxy-2,2-dimethylchroman-4-one and 5-hydroxy-2-methylchrom-4-one were also inhibitory but with less activity. The new inhibitors differed from the well-known phenolic inhibitors such as caffeic acid in inhibition kinetics as demonstrated by the rate of disappearance of indole-3-acetic acid, the rate of formation of the oxidation products, and the transient spectral change in the enzyme.

INTRODUCTION

It is well known that 3,4-dihydroxyphenols such as caffeic acid are strong inhibitors of the *in vivo* and *in vitro* oxidation of indole-3-acetic acid (IAA) by enzymes. However, these diphenols cause only a temporary inhibition after which IAA oxidation resumes at the same rate as the control [1]. Recently we found that two of four metabolites of the insecticide carbofuran, which inhibit IAA oxidation, showed kinetics distinctly different from these common phenolic inhibitors in that they cause a sustained rather than a temporary inhibition of IAA oxidation [2]. This appeared to be the first reported instance of sustained inhibition of IAA oxidation by a phenol. Since the metabolites of carbofuran such as 7-hydroxy-2,2-dimethyl-3-oxo-2,3-dihydrobenzofuran (**1**) showed some growth-regulating activity in the presence of IAA [3], the question arose as to whether plant growth can be effectively regulated by application of more active compounds of this type. As a result of our search, we report in this paper nine compounds which show sustained inhibition to IAA oxidation; five were more active than the carbofuran metabolite (**1**). Some of the compounds tested were selected on the basis of preliminary indications of possible structural requirements. However, the data obtained do not reveal any clear structure–activity relationships.

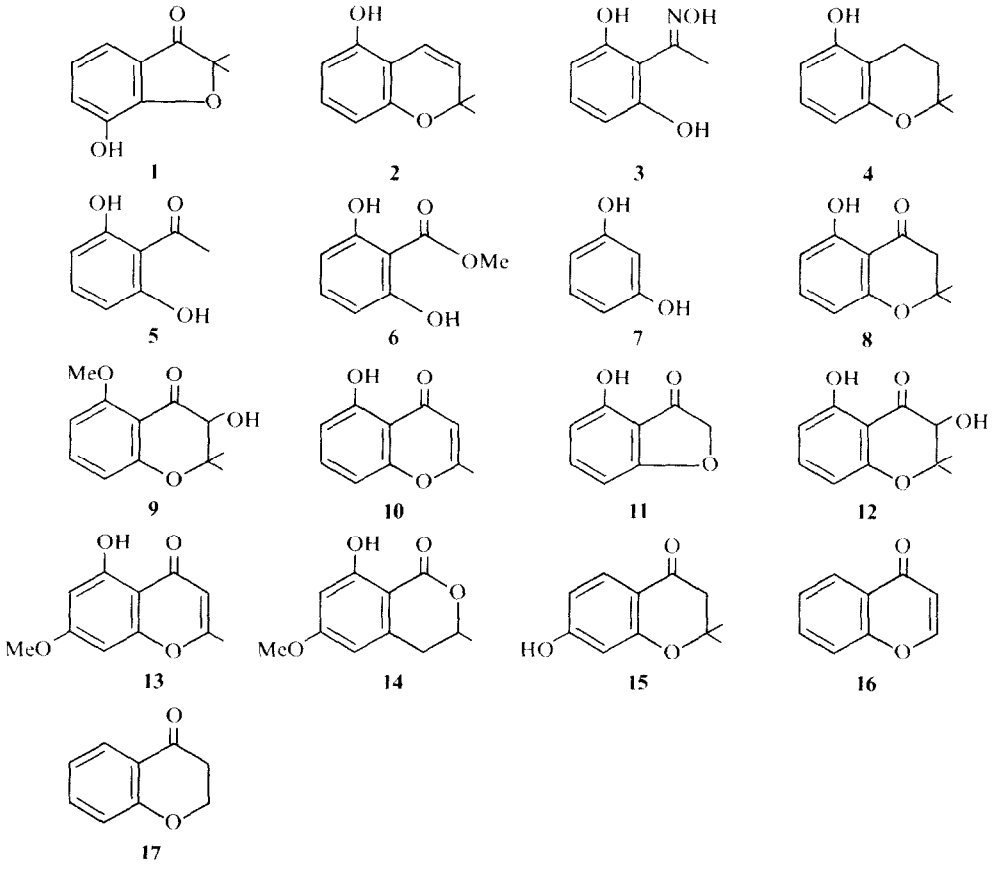
RESULTS AND DISCUSSION

Among the compounds tested, 5-hydroxy-2,2-dimethylchromene (β-tubanol) (**2**) was the most active inhibitor of IAA oxidation and its activity was 100% higher than that of **1** as measured by decarboxylation of IAA (Table 1).

Table 1. Relative activity of the persistent-type inhibitors of the peroxidase-catalysed decarboxylation of IAA

No.	Compound	% Inhibition
1	7-Hydroxy-2,2-dimethyl-3-oxo-2,3-dihydrobenzofuran	42.8
2	5-Hydroxy-2,2-dimethylchromene	80.2
3	2′,6′-Dihydroxyacetophenone oxime	68.9
4	5-Hydroxy-2,2-dimethylchroman	68.8
5	2′,6′-Dihydroxyacetophenone	61.9
6	2,6-Dihydroxybenzoic acid methyl ester	55.0
7	Resorcinol	33.3
8	5-Hydroxy-2,2-dimethylchroman-4-one	24.7
9	3-Hydroxy-5-methoxy-2,2-dimethylchroman-4-one	13.1
10	5-Hydroxy-2-methylchrom-4-one	7.5

The reaction soln (2.05 ml) had the molar composition: IAA (0.2 mM containing 0.33 μM [1-^{14}C]-IAA), 2,4-dichlorophenol (0.1 mM), $MnCl_2$ (0.1 mM), K-Pi buffer (50 mM, pH 6), and the inhibitors (0.1 mM), and contained 0.25 μg peroxidase. The reaction was run at 25° for 7 min. $^{14}CO_2$ collected from the control without inhibitor had 14 000–16 000 dpm.



2′,6′-Dihydroxyacetophenone oxime (**3**), 5-hydroxy-2,2-dimethylchroman (**4**), 2′,6′-dihydroxyacetophenone (**5**) and 2,6-dihydroxybenzoic acid methyl ester (**6**) were also more active than **1**. Resorcinol (**7**), 5-hydroxy-2,2-dimethylchroman-4-one (**8**), 3-hydroxy-5-methoxy-2,2-dimethylchroman-4-one (**9**) and 5-hydroxy-2-methylchrom-4-one (**10**) were less active inhibitors. Five other compounds which showed little or no inhibitor activity when tested at the same concentration were: 4-hydroxycoumaran-3-one (**11**), eugenin (**13**), 7-hydroxy-2,2-dimethylchroman-4-one (**15**), chromone (**16**) and 4-chromanone (**17**). In contrast, 3,5-dihydroxy-2,2-dimethylchroman-4-one (**12**) and 6-methoxymellein (**14**) promoted IAA oxidation.

The new inhibitors differed from the caffeic acid type in inhibition kinetics as shown by data presented in Figs. 1–3 which compare compound **2** with caffeic acid. Figure 1 shows the rate of formation of the oxidation products of IAA as affected by the two types of inhibitors added at zero time. Compound **2** caused a sustained inhibition even at 10 μM. In contrast, caffeic acid caused a complete inhibition but only for a limited period of time, after which IAA oxidation resumed at the same rate as the control.

Figure 2 shows the difference between compound **2** and caffeic acid in the inhibition of IAA oxidation when the compounds were added 30 or 60 sec after the start. When addition was delayed, caffeic acid at 2.8 μM was no longer inhibitory. However, compound **2** inhibited the reaction as soon as it was added and the inhibition continued thereafter.

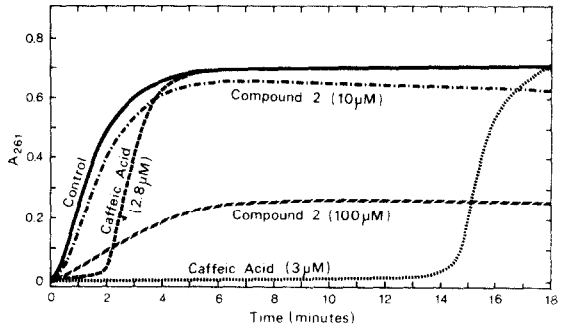


Fig. 1. Time course of IAA oxidation as affected by the two types of inhibitors. The composition of the reaction solution (3 ml) was 0.2 mM IAA, 50 μM each of 2,4-dichlorophenol and $MnCl_2$, 50 mM K-Pi buffer (pH 6), inhibitor and 2 μg peroxidase. The inhibitors were added before peroxidase.

Figure 3 illustrates differences in the spectral change of the prosthetic moiety of the enzyme when reacting with IAA in the presence of the two types of inhibitors. The IAA-induced spectral change in the enzyme molecule has been attributed to formation of enzyme intermediates and ΔA_{418} has been used as an indicator for the transient formation of HRP-II [4, 5]. When caffeic acid was present at zero time, the increase in A_{418} was delayed by 4 min, after which the rate of increase was similar to that of the control

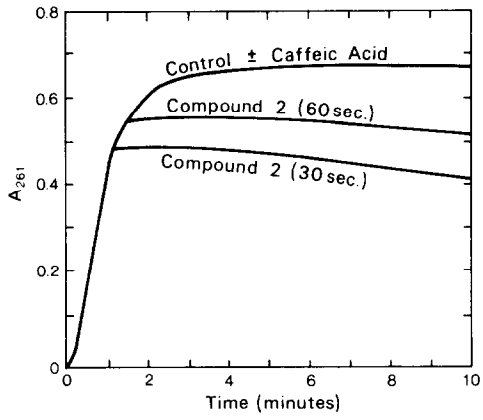


Fig. 2. Time course of IAA oxidation as affected by the two types of inhibitors added 30 or 60 sec after zero time. The composition of the reaction solution was the same as that shown in Fig. 1, except that the concentration of 5-hydroxy-2,2-dimethylchrom-3-ene was 0.1 mM and that of the caffeic acid was 2.8 μM.

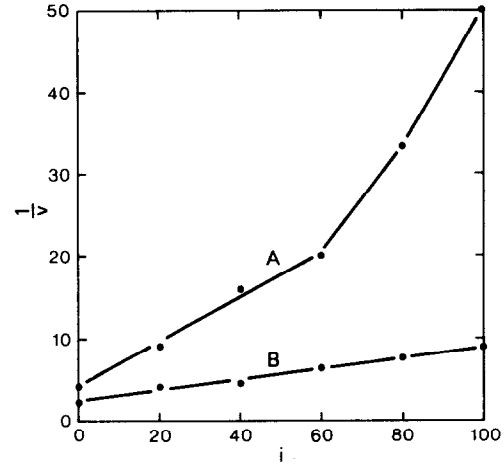


Fig. 4. Effect of inhibitor concentration on the rate of IAA oxidation. v represents $A_{261} \cdot \min^{-1}$ and i the concentration of 5-hydroxy-2,2-dimethylchrom-3-ene in μM. The basic composition of the reaction solution (3 ml) was 0.1 mM (line A) or 0.2 mM (line B) IAA, 50 μM each 2,4-dichlorophenol and $MnCl_2$, 50 mM K-Pi buffer (pH 6), and 0.5 μg peroxidase.

without caffeic acid. The shape and the height of the curves, with and without caffeic acid, were identical, confirming that the inhibition by caffeic acid was only temporary. The effect of **2**, however, was different. Absorbance at 418 nm increased slowly, reached a peak much later and decreased thereafter more quickly than that of the control. The changes, as illustrated in Fig. 3, suggest an interaction of the phenolic inhibitors with the heme moiety of peroxidase and a difference in reactivity and stability between the two types of inhibitors. The caffeic acid type inhibitors are known to be unstable in active IAA-oxidizing enzyme systems [1, 2, 6–9]. The stability of compound **2** and others listed in Table 1 has not been investigated.

The effect of inhibitor concentration on the rate of IAA oxidation at two levels of IAA was determined. The $1/v$ vs i plot (Fig. 4) shows basically a competitive inhibition although the nonlinearity suggests that the reaction is complex. The plot is similar to that for **1** [1]. As previously noted these inhibitors probably compete with IAA at various steps in the reaction, at which different enzyme intermediates may vary in affinity for the inhibitor and IAA.

In summary, all compounds in Table 1 in addition to 2,2-dimethyl-3-oxo-2,3-dihydrobenzofuranyl-7-*N*-methylcarbamate, another metabolite of carbofuran [2], are persistent-type inhibitors of IAA oxidation. The temporary type includes the common phenolic inhibitors, such as caffeic acid, catechol, protocatechuic acid and scopoletin, the phytoalexin orchinol [6] and two metabolites of carbofuran, 7-hydroxy-2,2-dimethyl-2,3-dihydrobenzofuran and 3,7-dihydroxy-2,2-dimethyl-2,3-dihydrobenzofuran [2]. In the present work we found that 2′,5′-dihydroxyacetophenone, 2′,3′,4′-trihydroxyacetophenone and 3,4-dihydroxyphenylacetic acid also belong to this group.

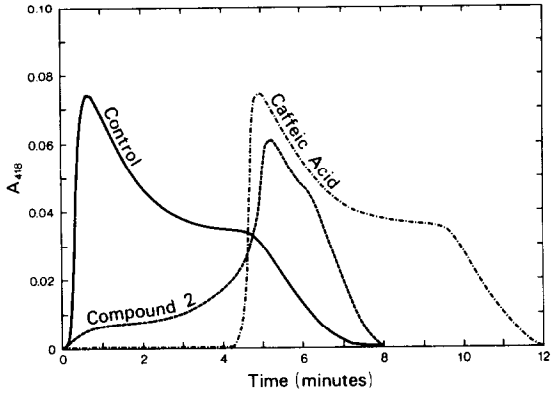


Fig. 3. Transformation of horseradish peroxidase during reaction with IAA as affected by the two types of inhibitors. The composition of the reaction solution (2.5 ml) was 5 μM peroxidase, 16 μM IAA, 50 mM K-Pi buffer (pH 6) and 4.8 μM 5-hydroxy-2,2-dimethylchrom-3-ene or 24 nM caffeic acid.

EXPERIMENTAL

Assay for oxidation of IAA. Oxidation of IAA was assayed by three methods as previously described [3, 4]. The first measured decarboxylation of [1-^{14}C]-IAA (60 μCi/μmol, New England Nuclear) by liquid scintillation counting of $^{14}CO_2$; the second recorded automatically the rate of product formation as indicated by an increase in absorbance at 261 nm; and the third determined the disappearance of IAA by color reaction with the modified Salkowski reagent. The composition and concn of the reaction soln are given in the table and figures. Reactions were run at 25° and horseradish peroxidase (EC 1.11.1.7, Sigma Type VI) was used throughout. Spectral change at 418 nm, used by Yamazaki and Yamazaki [4] and by Ricard and Job [5] to demonstrate the transient formation of the enzyme intermediate II, was employed as an additional parameter to monitor the difference between the two types of inhibitors.

Chemicals. **1** was prepared by Dr. R. A. Chapman [2]. 5-Hydroxy-2,2-dimethylchromene (**2**) was obtained from its acetate as described in the literature but, contrary to the earlier report [10], crystallized, mp 130–133° (from C_6H_6–petrol). The ^{1}H NMR spectrum was in accord with the assigned structure.

Compound **6** was obtained by methylation ($MeOH–H_2SO_4$) of 2,6-dihydroxybenzoic acid (Pfaltz & Bauer). The prepns of **3**, **4**, **8**–**12**, and **15** have been described [11–15]. **13** and **14** were available from an unrelated study [16]. 2′,6′-Dihydroxyacetophenone (**5**) (Aldrich) was purified by chromatography over a column of silicic acid–celite (4:1) using CH_2Cl_2 as solvent. Resorcinol (**7**) (B.D.H.) was recrystallized before testing. **16**, **17**, 2′,5′-dihydroxyacetophenone and 2′,3′,4′-trihydroxyacetophenone (Aldrich), 3,4-dihydroxyphenylacetic acid and horseradish peroxidase (Sigma), and IAA (Calbiochem) were used as received.

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