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The inhibitory activity of natural products on plant *p*-hydroxyphenylpyruvate dioxygenase

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Abstract

The inhibitory activity of 34 natural products of various structural classes on hydroxyphenylpyruvate dioxygenase (HPPD), the target site for triketone herbicides, and the mode of interaction of selected natural products were investigated. Recombinant HPPD from arabidopsis is sensitive to several classes of natural compounds including, in decreasing order of sensitivity, triketones, benzoquinones, naphthoquinones and anthraquinones. The triketone natural products acted as competitive tight-binding inhibitors, whereas the benzoquinones and naphthoquinones did not appear to bind tightly to HPPD. While these natural products may not have optimal structural features required for in vivo herbicidal activity, the differences in their kinetic behavior suggest that novel classes of HPPD inhibitors may be developed based on their structural backbones. © 2002 Published by Elsevier Science Ltd.

Keywords: Herbicide; HPPD; Mode of action; Natural products; Phytotoxins; Structure–activity relationships; Benzoquinone; Naphthoquinone; Triketone

1. Introduction

p-Hydroxyphenylpyruvate dioxygenase (HPPD, EC 1.13.11.27, EC 1.14.2.2) is a relatively recent target site for herbicides (Schulz et al., 1993; Lee et al., 1997, 1998; Pallett et al., 1998; Viviani et al., 1998). Inhibition of this enzyme disrupts the biosynthesis of carotenoids and results in foliage bleaching (loss of chlorophyll). While these symptoms are similar to those observed in plants treated with inhibitors of phytoene desaturase (Lee et al., 1997), HPPD inhibition is a distinct mechanism of action.

HPPD catalyzes the conversion of *p*-hydroxyphenyl pyruvate (4-HPP) to homogentisate (HGA) (Crouch et al., 1997; Pascal et al., 1985; Que and Ho, 1996), which is a key precursor of α-tocopherol and plastoquinone. Inhibition of HPPD indirectly affects phytoene desaturase activity by reducing the pool of available plastoquinone, a required cofactor (Pallett et al., 1998). The subsequent decrease in carotenoid levels causes foliage bleaching because the photosynthetic apparatus is no longer stabilized by these pigments. Under high light intensity, excess energy is not quenched and chlorophyll molecules are destroyed.

HPPD inhibitors have introduced new classes of herbicides based on the triketone backbone which apparently mimics a reaction intermediate. These compounds are time-dependent (tight-binding) inhibitors of this enzyme. Sulcotrione (2-[2-chloro-4-methanesulfonyl benzoyl]-cyclohexane-1,3-dione) and isoxaflutole (5-cyclopropyl-4-(4-trifluoromethyl-2-methanesulfonyl-benzoyl)isoxazole) are commercial products belonging to two of such classes. Sulcotrione possesses the classic triketone structure required for inhibition of HPPD, while the isoxaflutole must be activated to the conjugated diketonitrile moiety after opening of the isoxazole ring

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(Viviani et al., 1998). These are potent bleaching herbicides used in pre- and post-emergence control of broadleaf weeds in corn. Examples include sulcotrione (Mikado®), used in post-emergence broadleaf weed control in corn, and mesotrione (Callisto®), a newer selective herbicide developed for pre- and post-emergence management of annual grasses and broadleaf, including sulfonylurea resistant, weeds (Mitchell et al., 1999). Because of their favorable environmental and toxicological profiles, HPPD inhibitors are considered 'reduced risk pesticides' by the U.S. Environmental Protection Agency.

Other synthetic HPPD inhibitors, such as 2-(2-nitro-4-trifluoromethylbenzoyl)cyclohexane-1,3-dione (NTBC), may have useful pharmaceutical applications in the treatment of the tyrosinemia type I disease, by preventing the degradation of tyrosine (Ellis et al., 1995). In tyrosinemia type I disease, a deficiency in the enzyme fumarylacetoacetase leads to an accumulation of succinylacetoacetate, succinylacetone and 5-aminolevulinic acid, which are degraded into toxic metabolites by HPPD in the liver. Inhibition of HPPD reduces or prevents the formation of these toxic by-products.

Leptospermone, a natural triketone isolated from bottlebrush plant (*Calispermon* spp.) (Hellyer, 1968), is herbicidal and causes bleaching of the foliage (Knudsen et al., 2000). The herbicides sulcotrione and mesotrione (2-[4-methylsulfonyl-2-nitrobenzoyl]-cyclohexane-1,3-dione) are synthetic triketones structurally similar to leptospermone and are potent inhibitors of HPPD (Prysbilla et al., 1993; Beraud et al., 1993; Wichert et al., 1999). Therefore, it has been postulated that HPPD can be inhibited by natural triketones, but no data were available in the literature until the activity of usnic acid, a natural triketone isolated from the lichen *Alectoria sarmentosa* (Ach.) Ach., was reported by our laboratory (Romagni et al., 2000).

This paper reports a structure-activity study of the inhibitory activity of several classes of natural products including benzoquinone, naphthoquinone, anthraquinones, triketones and other related structures on HPPD from *Arabidopsis thaliana*, expressed in recombinant form. The putative mode of interaction between selected inhibitors and the target site was also investigated.

2. Results and discussion

Since most synthetic triketone herbicides targeting HPPD are competitive tight-binding inhibitors, their binding to the catalytic site is for all practical purposes irreversible (Ellis et al., 1995; Garcia et al., 2000). That is, equilibrium can be established so inhibition of the enzyme increases over time, as the inhibitors occupy more sites. Hence, for tight-binding inhibitors, traditional dose–response curves can only yield apparent I_{50} since this value will be time-dependent. For this reason, the dose–response curves were performed carefully to ensure that the incubation time with the inhibitor and the subsequent enzymatic assays lasted precisely the same duration for each experiment. Under these conditions, the I_{50} values obtained were highly reproducible and can be used as a relative value to compare the potency of the compounds tested.

2.1. Structure–activity relationships of HPPD inhibitors based on apparent I_{50}

2.1.1. Natural benzoquinones

All of the natural plant benzoquinones (**1**–**34**) tested were inhibitory toward HPPD, with I_{50} vaules ranging from 0.3 to 20 μM (Table 1). The difference in activity between sorgoleone (**1**) and maesanin (**5**), which differ only in the number and positions of the double bonds in the 3-alkyl chain (Fig. 1), is very slight. The degree of unsaturation in the C15 aliphatic "tail" does not have a great effect on the affinity of the benzoquinone "head" for HPPD. However, when the side-chain is fully saturated (**4**), the activity was reduced, indicating that the positions of the unsaturation in the side-chain of **5** may facilitate binding of the quinone to the catalytic site of HPPD compared to the fully saturated side chain of **4**.

Substitution of the methyl group with an ethyl group in the 5-position of the benzoquinone ring of **2**, relative to **1**, also lowered the activity. The presence of two methyl groups in the 2 and 5-positions (**3**) decreased the activity, relative to **1**, and an even more pronounced decrease was observed with **6**, the dimethoxy derivative of **5**. The quinone moiety of **3** and **6** retained the structural requirements to inhibit HPPD, and their lower activities may be due to greater lipophilicity. Substitution of the methoxyl group in the 5 position with the 2-hydroxyethylamino group of **8** caused a 10-fold decrease in inhibitory activity, relative to methoxyl analog **5**. The activity of maesanol (**7**), which lacks the 5-methoxyl group and has a 6-methyl group (Fig. 1), was between that of **5** and **6**.

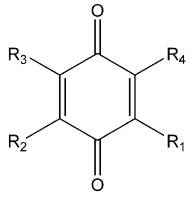
Since none of the benzoquinones tested lacked an oxygen at the 2 position (Fig. 1), it is difficult to evaluate the importance of this atom for the activity. Nonetheless, it is clear that the two least active benzoquinones (**3** and **6**) were those with more bulky, less reactive methoxyl groups. The potential role of the hydroxyl group for the binding to HPPD catalytic site is discussed in later sections.

2.1.2. Natural naphthoquinones

The overall activity of the naphthoquinones was lower than that of the benzoquinones tested. Their apparent I_{50} ranged from 1.2 to >100 μM. All of the most active naphthoquinones tested, juglone (**10**), **12**, and shikonin (**17**) possessed a hydroxy group in the

Table 1
Common name, class and inhibitory activity of natural products

No.	Common name	Class	I_{50}
1	Sorgoleone	Benzoquinone	0.4
2	Ethoxysorgoleone	Benzoquinone	3
3	Dimethoxysorgoleone	Benzoquinone	9
4	Dihydromaesanin	Benzoquinone	2
5	Maesanin	Benzoquinone	0.3
6	Dimethoxymaesanin	Benzoquinone	20
7	Maesanol	Benzoquinone	1.5
8	MSNM-4	Benzoquinone	2
9	Lapachol	Naphthoquinone	>100
10	Juglone	Naphthoquinone	1.3
11	Plumbagine	Naphthoquinone	2
12	5,8-Dihydroxy-*p*-naphthoquinone	Naphthoquinone	1.3
13	2-Hydroxy-*p*-naphthoquinone	Naphthoquinone	>100
14	*p*-Naphthoquinone	Naphthoquinone	4
15	2,5-Dihydroxy-*p*-naphthoquinone	Naphthoquinone	>100
16	Menadione	Naphthoquinone	15
17	Shikonin	Naphthoquinone	1.2
18	1,2-Naphthoquinone	Naphthoquinone	10
19	1,8-Dihydroxyanthraquinone	Anthraquinone	>100
20	Emodin	Anthraquinone	>100
21	1,4-Dihydroxyanthraquinone	Anthraquinone	>100
22	Alizarin	Anthraquinone	>100
23	(-)-Usnic acid	β-Triketone	0.08
24	(+)-Usnic acid	β-Triketone	0.3
25	2-Palmitoyl-1,3-cyclohexanedione	β-Triketone	8
26	3-Acetyl-3,4-dihydro-2,4-dioxo-2*H*-pyran-5-carboxylic acid	β-Triketone	>100
27	Tenuazonic acid	β-Triketone	18
28	Leucoquinizarin	α,β-unsaturated β-Hydroxyketone	>100
29	Atranorin	β′β-Dihydroxyaldehyde	60
30	Maclurin	β′β-Dihydroxybenzophenone	>100
31	β′-Alectoronic acid	Diphenyl ether	80
32	Eriodictyol	Flavanone	>100
33	Isosalipurpol	Chalcone	30
34	Lupulone	β-Triketone	>100



N	R_1	R_2	R_3	R_4
1	8(*Z*),11(*Z*),14-pentadecatrienyl	OCH_3	H	OH
2	8(*Z*),11(*Z*),14-pentadecatrienyl	OCH_2CH_3	H	OH
3	8(*Z*),11(*Z*),14-pentadecatrienyl	OCH_3	H	OCH_3
4	pentadecanyl	OCH_3	H	OH
5	10(*Z*)-pentadecenyl	OCH_3	H	OH
6	10(*Z*)-pentadecenyl	OCH_3	H	OCH_3
7	10(*Z*)-pentadecenyl	H	CH_3	OH
8	10(*Z*)-pentadecenyl	$NHCH_2CH_2OH$	H	OH

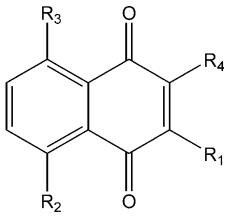
Fig. 1. Structures of the benzoquinones used in this study.

5-position (Fig. 2). Compounds **12** and **17** also have an additional hydroxyl group in the 8-position. The presence of a hydroxyl group in the 2-position of the naphthoquinone ring yielded totally inactive compounds (**9**, **13**, and **15**). This was unexpected since all of the active benzoquinones had such a hydroxyl group in a similar position. This data suggests that the binding mechanisms of benzoquinones and naphthoquinones on HPPD may be different.

1,4-Naphthoquinone (**14**) has a relatively good activity, which decreased upon introduction of a 2-methyl group in **16**. With the 5-hydroxy and 5,8-dihydroxy substituted naphthoquinones **11** and **17**, respectively, the presence of an alkyl chain in the 3-position is well tolerated (Table 1). The assymetric 1,2-naphthoquinone (**18**) also retained some inhibitory activity on HPPD.

2.1.3. Anthraquinones

None of the anthraquinones **19**–**22** inhibited HPPD, giving good indication that the presence of a quinone moiety is not sufficient for binding to this target site. In fact, all of the anthraquinones tested had hydroxyl groups in position 1, which is structurally similar to that observed in the most active naphthoquinones (**10**–**12**) (Figs. 2 and 3). Similarly, 1,4-dihydroxyanthraquinone (**21**) and the quinone-like leucoquinizarin (**28**) (Fig. 5) have part of the same structural characteristics of **12** but are completely inactive. The reason for the lack of



N	R_1	R_2	R_3	R_4
9	3-methyl-but-2-enyl	H	H	OH
10	H	OH	H	H
11	H	OH	H	CH_3
12	H	OH	OH	H
13	H	H	H	OH
14	H	H	H	H
15	H	OH	H	OH
16	H	H	H	CH_3
17	H	OH	OH	1-hydroxy-4-methyl-pent-3-enyl

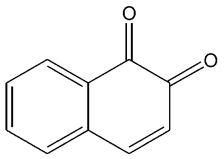


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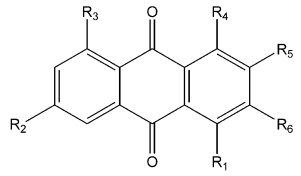
Fig. 2. Structures of the naphthoquinones used in this study.



N	R_1	R_2	R_3	R_4	R_5	R_6
19	H	H	OH	OH	H	H
20	H	CH_3	OH	OH	H	OH
21	OH	H	H	OH	H	H
22	H	H	H	OH	OH	H

Fig. 3. Structures of the anthraquinones used in this study.

activity of the anthraquinones is not clear based on available data.

2.1.4. β-Triketones

The activity of the triketones tested varied greatly, with I_{50} ranging from 0.08 to >100 μM (Table 1). (−)-Usnic acid (**23**) (Fig. 4) was the most active compound tested in this study, with an I_{50} of 0.08 μM. Its enantiomer (+)-usnic acid (**24**) was much less active. 2-Palmitoyl-1,3-cyclohexanedione (**25**) with a long lipophilic alkyl chain and tenuazonic acid (**27**) were not very active. Compound **26**, which possesses a highly polar carboxylic acid group, is completely inactive.

2.1.5. Others

The other miscellaneous compounds tested (**28**–**34**) have the recognized minimum structural requirements for the inhibition of HPPD, namely the 2-ketoethen-1-ol framework (Fig. 5) (Lee et al., 1997). However, most of the compounds in this group were either poorly active or completely inactive. It is worthy of note that whilst maclurin (**30**) has no activity, the relatively similar chalcone analog isosalipurpol (**33**) had some inhibitory activity on HPPD. The lack of activity of lupulone (**34**) is probably due to steric hindrance associated with the numerous sidechains surrounding the β-triketone framework.

2.2. Mechanism of interaction between the natural products and HPPD

As mentioned above, most known inhibitors of HPPD bind to the enzyme very tightly with $T_{\frac{1}{2}}$ of dissociation ranging from a few hours to several days, as opposed to milliseconds for traditional reversible inhibitors. The compounds reported in the literature almost exclusively belong to the triketone class (Lee et al., 1998; Ellis et al., 1995, 1996; Garcia et al., 2000; Pallett et al., 1998). This study used a structurally more diverse group of compounds including numerous natural products. We previously reported that the triketones **23** and **24** behaved as expected for compounds of this class by binding tightly to HPPD (Romagni et al., 2000) and similar results were obtained in this study (Fig. 6 Fig. 6A).

The binding kinetics observed with sorgoleone (**1**) and juglone (**10**) were different from that observed with the triketone class of natural products, with the lines intersecting at their point of origin (Fig. 6B and C). This suggests that the benzoquinones and naphthoquinones do not interact with HPPD in a time-dependent tight-binding manner. The anthraquinones were not tested since none of the compounds in our data set were very active on HPPD.

HPPD is a non-heme, iron II containing, α-keto acid-dependent enzyme (Lindblad et al., 1970; Que and Ho, 1996). The reaction catalyzed by HPPD is complex, involving oxidative decarboxylation of the 2-oxoacid side chain of 4-HPP, accompanied by hydroxylation of the aromatic ring, and a 1,2 (*ortho*) migration of the carboxymethyl group (Crouch et al., 1997; Pascal et al., 1985; Que and Ho, 1996). Inhibition kinetic analysis of triketone herbicides indicate that these compounds bind slowly but very tightly to the catalytic site of HPPD (nearly irreversibly) and competitively with respect to 4-HPP (Romagni et al., 2000; Viviani et al., 1998). Complicating the analysis of kinetic data is that some HPPD inhibitors, such as the diketonitrile isoxaflutole, exhibit half-site reactivity where binding to one catalytic site of the dimer completely inhibits the enzyme (Garcia et al., 2000). Other triketone herbicides have a stoichiometric relationship where binding to one of the two sites provides only 50% inhibition.

The fact that the triketone-type natural products, such as **23**, bind tightly to HPPD can readily be explained by previous research on synthetic triketones.

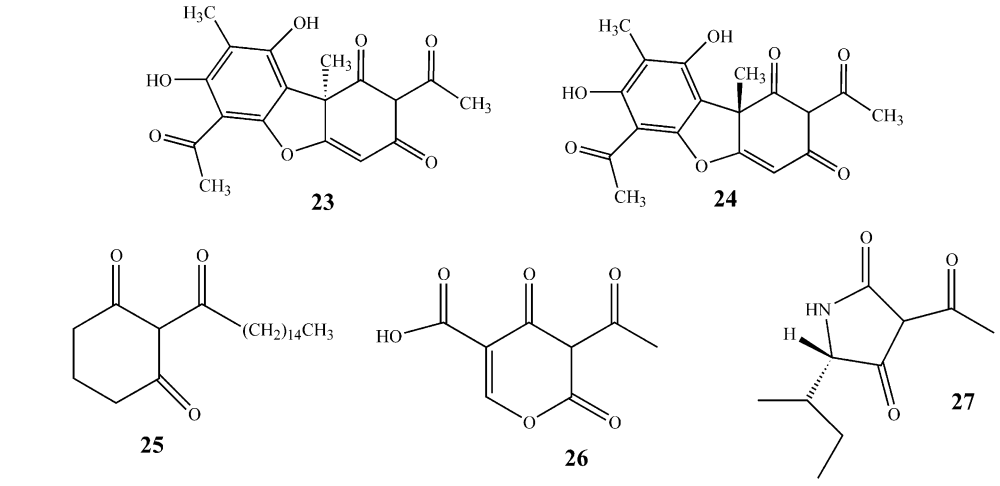


Fig. 4. Structures of triketones used in this study.

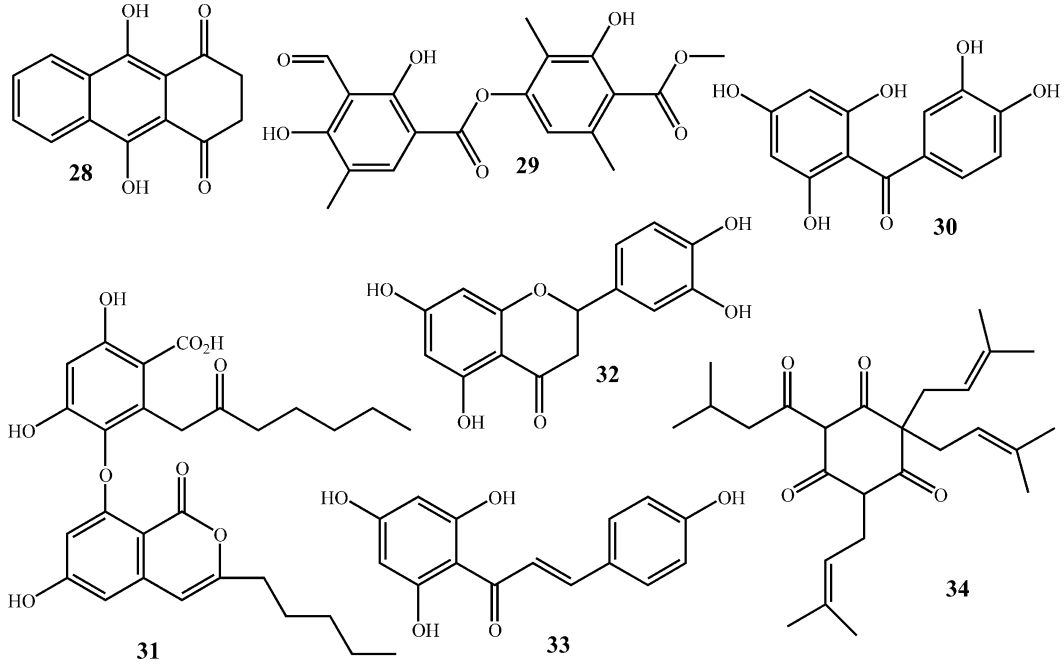


Fig. 5. Structures of miscellaneous natural products tested.

The triketone functionality of the inhibitors mimic the α-keto acid moiety of 4-HPP and compete for the binding site of the natural substrate (Pascal et al., 1985; Garcia et al., 2000). These intermediates form stable enzyme-complex intermediates in association with iron II in the catalytic site. On the other hand, benzoquinones and naphthoquinones do not behave as tight-binding inhibitors (Fig. 6B and C). This may be due to the fact that these inhibitors are structurally more rigid (planar) than the triketones and may not mimic the α-keto acid moiety of 4-HPP as well. Therefore, these compounds may not form a stable reaction intermediate. Instead, their backbones may resemble the conformation of one of the later intermediates in the reaction mechanism of HPPD. Furthermore, the C-2 hydroxyl group has dramatically opposite effects on the potency of the *p*-benzoquinones and *p*-naphthoquinones. The activity of the *p*-benzoquinones was positively affected by the presence of a 2-hydroxyl group, whereas *p*-naphthoquinones with a C-2 hydroxyl group were completely inactive. Finally, the activity was reduced in the methoxy derivatives, suggesting that oxygen atoms may still interact with the metal iron of HPPD.

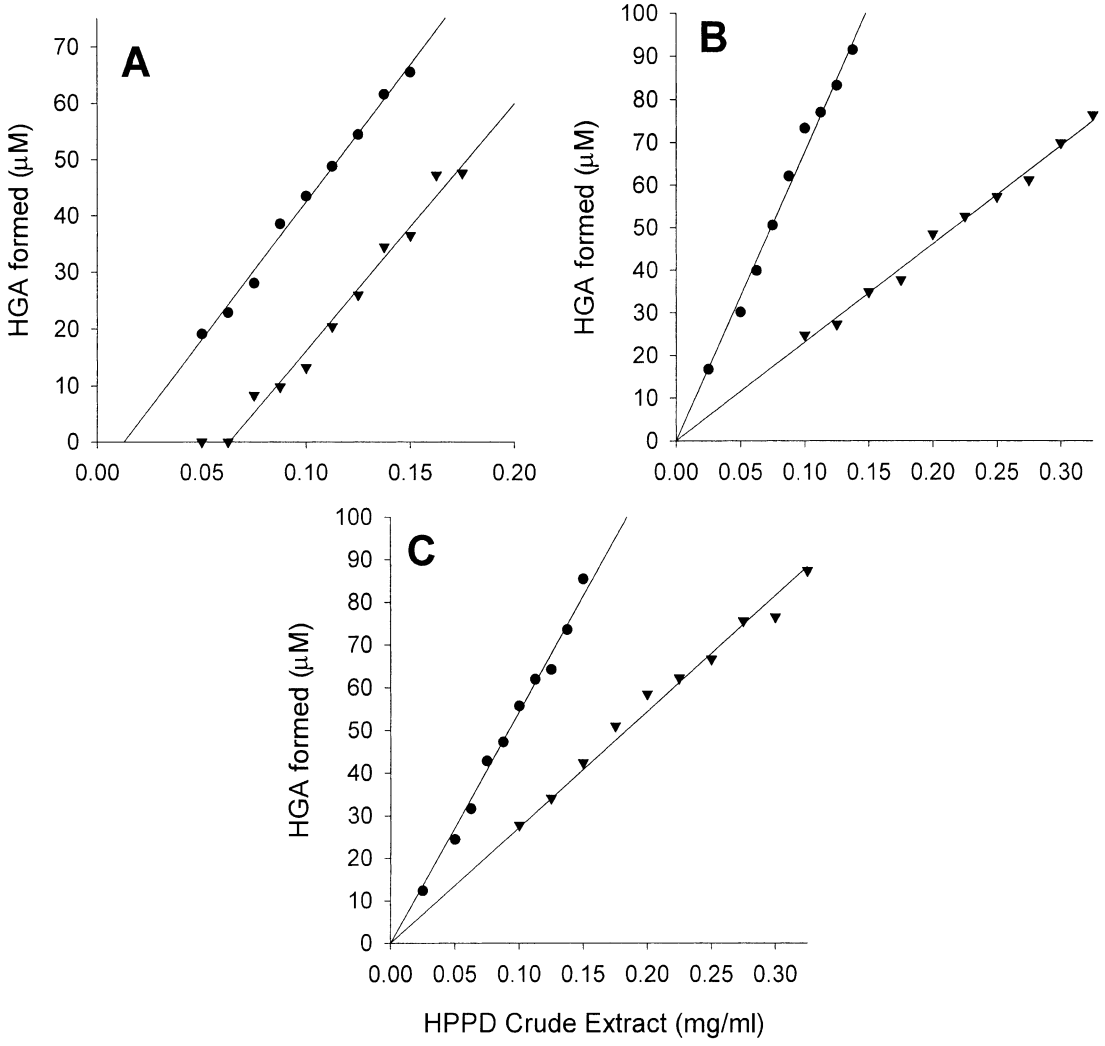


Fig. 6. HPPD inhibition kinetics of (A) the β-triketone usnic acid **23**, (B) the *p*-benzoquinone sorgoleone **1**, and (C) the *p*-naphthoquinone juglone **10**. ●, no inhibitor; ▲, 0.03 μm(–)-usnic acid, 1 μM sorgoleone, or 10 μM juglone, respectively.

3. Conclusion

Recombinant HPPD from *A. thaliana* is sensitive to several classes of natural compounds, including benzoquinones, naphthoquinones and triketones, but not anthraquinones. While these natural products may not have optimal structural features required for in vivo herbicidal activity, differences in their kinetic behavior suggest that novel classes of HPPD inhibitors may be developed based on their structural backbones. Ongoing research will provide more insight into the mechanism of interaction between quinones and HPPD.

4. Experimental

4.1. Sources of natural products and chemicals

(+)-Usnic acid **24** was purchased from Aldrich (Sigma-Aldrich, Milwaukee, WI). (−)-Usnic acid **23** and β-alectoronic acid **31** were isolated from *Alectoria sarmentosa* (Ach.) Ach. collected in Coeur d'Aléne, ID in August, 1999. The sorgoleone analogues **1**–**3** were isolated from root extracts of *Sorghum bicolor* according to Rimando et al. (1998). The maesanin analogues **4**–**8** were isolated from *Maesa lanceolata*. Menadione **16**, emodin **20**, leucoquinizarin **28**, 5-carboxydehydroacetic acid, copper salt of tenuazonic acid **27**, atranorin **29**, maclurin **30** were from Sigma/Aldrich. 1,8-dihydroxyanthraquinone **19** and 1,4-dihydroxyanthraquinone **21**, 1,2-naphthoquinone **18** were from Fluka, and shikonin **17**, 5,8-dihydroxy-*p*-naphthoquinone **12** and alizarin **22** were from TCI. Eriodictyol **32**, isosalipurpol **33**, and lupulone **34** were from APIN chemical (UK) and 2,5-dihydroxy-*p*-naphthoquinone **15** and 2-palmitoyl-1,3-cyclohexanedione **25** were purchased from Maybridge Chemical Company (UK). All other quinones (**9**–**11**, **13**–**19**, **26**) were purchased from the specialty library of Aldrich Chemical (Milwaukee, WI).

4.2. Expression and extraction of HPPD

Recombinant HPPD from *A. thaliana* was over-expressed in *E. coli* JM105 cells with pTrc 99A-AT4-HPPD plasmid. The cells were grown at 37 °C in Luria Bertani broth supplemented with 100 μg/ml of carbenicillin and 100 μg/ml streptomycin according to Maniatis et al. (1982). Expression of the vector was induced by IPTG (1 mM) when bacterial growth was equivalent to an A_{600} of 0.6. The cells were grown for another 17 h at 30 °C and harvested by centrifugation (6000 *g*). The pellet was resuspended in buffer (20 mM potassium phosphate, pH 6.8, 1 mM EDTA, 1 mM DTT, 1 mM 6-aminohexanoic acid, 1 mM benzamidine) and sonicated using a Branson sonicator (Sonifier 450, Danbury, CT, USA). A crude cell-free supernatant was obtained by centrifugation at 35,000×*g* for 30 min.

4.3. Assay of HPPD activity

HPPD activity was measured at a protein concentration of 5 mg/ml in the cell-free extract. The assay mixture (contained 0.25 mg/ml in a 200 μl of assay volume) was incubated for 15 min on ice with various concentrations of inhibitors applied in a 4 μl volume. The final concentration of the test compounds ranged from 0.01 to 100 μM in a half-log increment. The reaction was initiated by adding 5 μl of 4-HPP (10 mM in MeOH) for a total volume of 200 μl. The assay buffer contained 50 mM sodium ascorbate in 100 mM Tris–HCl, pH 7.5. Controls contained the same volume of solvent used to deliver the inhibitor (acetone or MeOH).

The samples were incubated at 30 °C for 15 min and the reaction was stopped by addition of 70 μl of perchloric acid 20% (v/v). The protein precipitated was removed by centrifugation at 20,000×*g* for 5 min. The supernatant was subjected to HPLC analysis for the determination of homogentisic acid produced.

4.4. HPLC protocol

The HPLC system was composed of a Waters Associates system (Milford, MA 01757, USA) which included a Model 600E pump, a Model 717 autosampler, a Millenium 2010 controller and a Model 996 photodiode detector equipped with a 3.9 mm×15 cm Pico Tag 4 μm reversed phase column preceded by a Bio-Rad ODS-5S guard column. The solvent system consisted of a linear gradient beginning at 0% (100% A) to 70% B from 0 to 17 min, 70% to 100% B from 17 to 20 min 100% B from 20 to 24 min, 100% to 0% B from 24 to 28 min and 0% B from 28 to 35 min. The flow rate was 1 ml/min and the injection volume was 100 μl.

Solvent A was 0.1% (v/v) trifluoroacetic acid in ddH_2O and solvent B was 0.07% (v/v) trifluoroacetic acid in 80% (v/v) HPLC-grade acetonitrile/ddH_2O. Homogentisic acid was detected by UV absorbance at 288 nm. A calibration curve was established by injecting various concentrations of homogentisic acid.

4.5. Time course experiments

4-HPP and the selected inhibitors were co-administered to the assay solution containing 0.25 mg/ml protein from the crude cell-free extract in a final assay volume of 1.5 ml. Aliquots of the assay reaction (200 μl) were collected after 0, 1, 5, 10, 15, 20, and 25 min incubation at 30 °C and placed in vials containing 70 μl of $HClO_4$ acid to quench the reaction. HGA formed in the samples was analyzed as described above.

4.6. Kinetic study of binding mechanisms

The nature of the interaction between the natural products and their binding site on HPPD was determined according to Ellis et al. (1995) by incubating various concentrations of HPPD 15 min with either 0.03 μM (−)-usnic acid **23**, 1 μM of sorgoleone **1** or 10 μM juglone **10**. The concentrations of HPPD used were 0.25 mg/ml protein in the crude cell-free extract. HPPD activity was then measured as described above.

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