p-Hydroxyphenylpyruvate dioxygenase is a herbicidal target site for β-triketones from *Leptospermum scoparium*

Franck E. Dayan [a,*], Stephen O. Duke [a], Audrey Sauldubois [b], Nidhi Singh [c], Christopher McCurdy [c], Charles Cantrell [a]

[a] *United States Department of Agriculture, Agricultural Research Service, Natural Products Utilization Research Unit, P.O. Box 8048, University, MS 38677, USA*
[b] *UFR des Sciences, Université d'Angers, 2, Boulevard Lavoisier, 49045 Angers Cedex 01, France*
[c] *Department of Medicinal Chemistry, University of Mississippi, University, MS 38677, USA*

Abstract

p-Hydroxyphenylpyruvate dioxygenase (HPPD) is a key enzyme in tyrosine catabolism and is the molecular target site of β-triketone pharmacophores used to treat hypertyrosinemia in humans. In plants, HPPD is involved in the biosynthesis of prenyl quinones and tocopherols, and is the target site of β-triketone herbicides. The β-triketone-rich essential oil of manuka (*Leptospermum scoparium*), and its components leptospermone, grandiflorone and flavesone were tested for their activity in whole-plant bioassays and for their potency against HPPD. The achlorophyllous phenotype of developing plants exposed to manuka oil or its purified β-triketone components was similar to that of plants exposed to the synthetic HPPD inhibitor sulcotrione. The triketone-rich fraction and leptospermone were approximatively 10 times more active than that of the crude manuka oil, with I_{50} values of 1.45, 0.96 and 11.5 μg mL^{-1}, respectively. The effect of these samples on carotenoid levels was similar. Unlike their synthetic counterpart, steady-state O_2 consumption experiments revealed that the natural triketones were competitive reversible inhibitors of HPPD. Dose–response curves against the enzyme activity of HPPD provided apparent I_{50} values 15.0, 4.02, 3.14, 0.22 μg mL^{-1} for manuka oil, triketone-rich fraction, leptospermone and grandiflorone, respectively. Flavesone was not active. Structure–activity relationships indicate that the size and lipophilicity of the side-chain affected the potency of the compounds. Computational analysis of the catalytic domain of HPPD indicates that a lipophilic domain proximate from the Fe^{2+} favors the binding of ligands with lipophilic moieties.
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Keywords: *Leptospermum scoparium*; Leptospermone; Manuka oil; Myrtaceae; Essential oil; Grandiflorone; Herbicide; *p*-Hydroxyphenylpyruvate dioxygenase; HPPD; Mode of action; Phytotoxins; Triketones

1. Introduction

p-Hydroxyphenylpyruvate dioxygenase (HPPD, EC 1.13.11.27, EC 1.14.2.2) is involved in pigment synthesis and tyrosine catabolism in most organisms. This non-heme, iron II containing, α-keto acid-dependent enzyme catalyzes a complex reaction involving the oxidative decarboxylation of the 2-oxoacid side-chain of 4-hydroxyphenylpyruvate (4-HPP), the subsequent hydroxylation of the aromatic ring, and a 1,2 (ortho) rearrangement of the

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carboxymethyl group, to yield homogentisic acid (HGA) (Que and Ho, 1996; Crouch et al., 1997; Pascal et al., 1985).

HPPD is a molecular target site for pharmaceutical products in the treatment of the hypertyrosinemia, a hereditary disease that results in the unregulated degradation of tyrosine. More specifically, a deficiency in the enzyme fumarylacetoacetase causes abnormal accumulation of succinylacetoacetate, succinylacetone and 5-aminolevulinic acid, which are subsequently degraded by the liver HPPD into toxic metabolites (Lindblad et al., 1977). Inhibiting HPPD activity by β-triketone pharmacophores, such as 2-(2-nitro-4-trifluoromethylbenzoyl)cyclohexane-1,3-dione (NTBC) (1) (Fig. 1), reduces or prevents the formation of these harmful by-products (Lindblad et al., 1977).

HPPD is also the target site in plants of β-triketone herbicides [e.g. sulcotrione (2-[2-chloro-4-methanesulfonyl benzoyl]-cyclohexane-1,3-dione) (2) and mesotrione (2-[4-methylsulfonyl-2-nitrobenzoyl]-cyclohexane-1,3-dione) (3)] (Schulz et al., 1993; Lee et al., 1997, 1998; Pallett et al.,

Fig. 1. Structures of the reference compounds NTBC (1), sulcotrione (2) and mesotrione (3), as well as the β-triketones isolated from manuka oil that were tested in this study, leptospermone (4), isoleptospermone (5), flavesone (6), and grandiflorone (7).

1998; Viviani et al., 1998). Inhibition of this enzyme disrupts the biosynthesis of carotenoids and results in bleaching (loss of chlorophyll) of the foliage of treated plants. Such phenotypic response is similar to that observed on plants treated with inhibitors of phytoene desaturase (Lee et al., 1997). However, inhibition of HPPD has a different mechanism of action. In plants, HPPD catalyzes the formation of HGA, which is a key precursor of the eight different tocochromanols (tocopherols and tocotrienols) and prenyl quinones. The latter prenylquinone is a required cofactor for phytoene desaturase (Norris et al., 1995). Therefore, inhibition of HPPD indirectly reduces phytoene desaturase activity by reducing the pool of available plastoquinone (Pallett et al., 1998). The subsequent decrease in carotenoid levels results in the destabilization of the photosynthetic apparatus. Therefore, under high light intensity, excess energy is no longer quenched, chlorophyll molecules are destroyed, and the foliage appears bleached.

The triketone herbicides were apparently derived from the structural backbone of natural β-triketones following the discovery of the herbicidal properties of leptospermone (4) (Fig. 1) from the bottlebrush plant (*Callistemon* spp.) (Lee et al., 1997, 1998; Gray et al., 1980). The private sector has invested millions of dollars in developing and commercializing structural analogues of leptospermone (4) as herbicides. The mode of action of these synthetic compounds has been well studied, yet, there are no published reports on the mode of action of this natural β-triketone. The only published reports of the effect of natural products on HPPD are concerned with usnic acid (a lichen secondary metabolite), sorgoleone (a plant lipid benzoquinone) and juglone (a plant naphthoquinone) (Meazza et al., 2002; Romagni et al., 2000), which provide indirect information regarding the structural requirement for inhibition of plant HPPD by natural products.

The essential oils of several woody plants originating from New Zealand and Australia (e.g., *Leptospermum*, *Eucalyptus*, and *Callistemon* spp.) contain relatively large amounts of natural β-triketones (e.g., leptospermone (4), isoleptospermone (5), flavesone (6), and grandiflorone (7)) (Douglas et al., 2004; Hellyer, 1968). These oils and their components have antifungal, antimicrobial (Christoph et al., 2000; Spooner-Hart and Basta, 2002; van Klink et al., 2005), antiviral (Reichling et al., 2005; Spooner-Hart and Basta, 2002), and insecticidal and molluscicidal activities (Spooner-Hart and Basta, 2002). Leptospermone (4), one of the primary components of these oils, is also phytotoxic and causes bleaching of grass and broadleaf weeds, while maize is inherently more resistant (Gray et al., 1980; Knudsen et al., 2000).

We report here that essential oil distilled from the leaves of manuka (*Leptospermum scoparium* J.R. and G. Forst) and the β-triketone leptospermone (4) isolated from this oil cause bleaching symptoms very similar to those caused by synthetic triketone herbicides. The molecular target site of the natural β-triketones isolated from
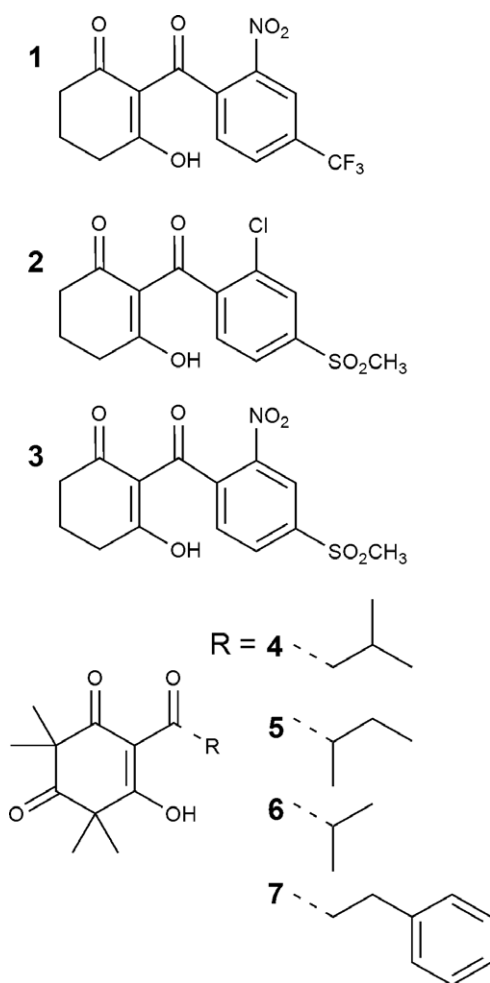
manuka oil (e.g., leptospermone (**4**), flavesone (**6**) and grandiflorone (**7**)) was determined to be the enzyme HPPD. Molecular modeling techniques were applied to study the interaction of these compounds to the catalytic site of HPPD.

2. Results and discussion

2.1. Isolation of β-triketones from L. scoparium

The essential oil distilled from the leaves and twigs of one regional chemotype of manuka (*L. scoparium*), a shrub from the Myrtaceae family, is rich in β-triketone (Douglas et al., 2004; Hellyer, 1968; Christoph et al., 1999). This oil contained approximately 18.4% triketones as determined by GC-MS TIC analysis (Fig. 2A) using percentage of total peak area. The initial liquid/liquid purification steps reported by Hellyer and Pinhey (1966) worked well for the purification of a β-triketone-rich fraction, which was composed of 72.9% leptospermone (**4**), 18.4% isoleptospermone (**5**), 7.1% flavesone (**6**), and <1% grandiflorone (**7**) (Figs. 2B and 1).

A preparative HPLC method was used to isolate individual components. Multiple injections of the β-triketone-rich fraction (total amount was 4.4 g) yielded fractions containing pure leptospermone (**4**) (394 mg), flavesone (**6**) (3.9 mg), and grandiflorone (**7**) (33 mg). The isoleptospermone (**5**) isolated (26 mg) was contaminated with approximately 37% leptospermone (**4**).

2.2. Phytotoxic effects of β-triketones on lettuce

Manuka oil, the triketone-rich fraction, and its main component (**1**) were tested on lettuce seedlings for their effect on chlorophyll and carotenoid content. The synthetic triketone herbicide sulcotrione (**2**) was used as a positive control (Garcia et al., 1997). All of the fractions caused a similar bleaching phenotype in the treated seedlings (Fig. 3A), which is consistent with data reported on essential oil of bottlebrush plant (Gray et al., 1980). Carotenoid and chlorophyll levels decreased in a dose-dependent manner (Fig. 3B and C). Manuka oil was the least active on chlorophyll levels, with an I_{50} of 11.5 μg mL^{-1}. The triketone-rich fraction and leptospermone (**4**) were approximatively 10 times more active,
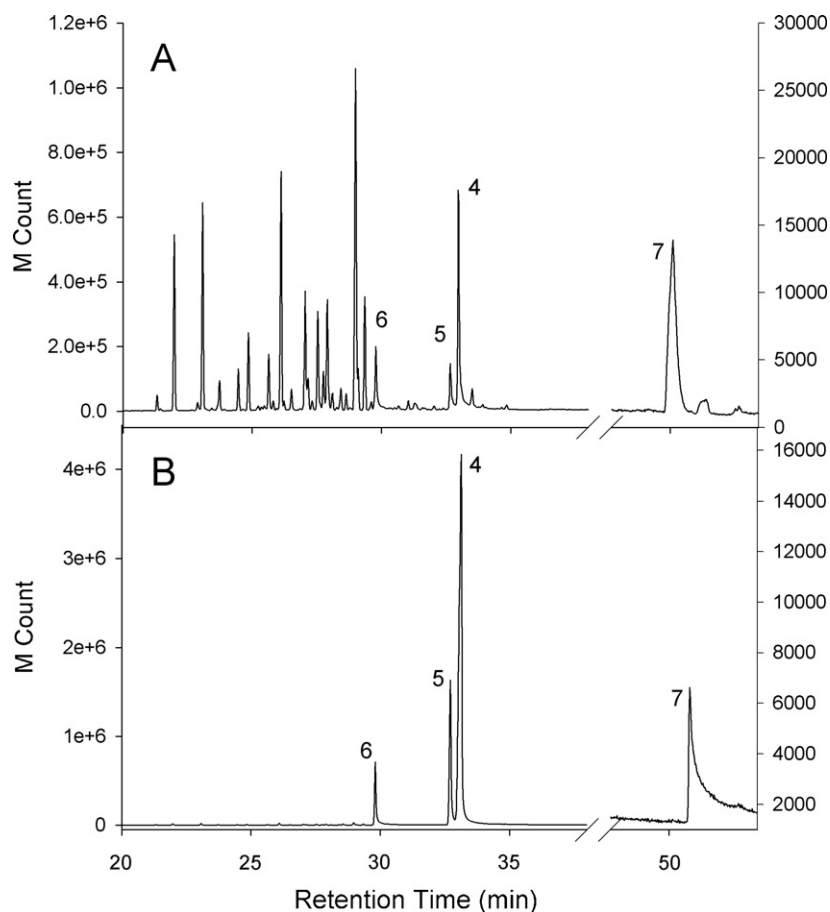

Fig. 2. GC-MS total ion chromatograms of (A) crude manuka oil containing 12.1% leptospermone (**4**), 2.6% isoleptospermone (**5**), 3.4% flavesone (**6**) and 0.1% grandiflorone (**7**), and (B) the triketone-rich fraction containing 72.9% leptospermone (**4**), 18.4% isoleptospermone (**5**), 7.1% flavesone (**6**) and <1% grandiflorone (**7**).
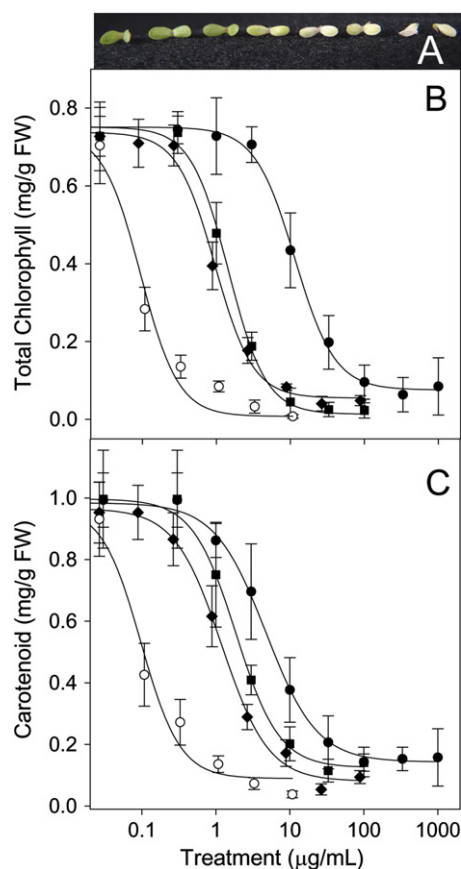
Fig. 3. (A) Bleaching symptoms caused by manuka oil on lettuce at increasing concentrations (see X axis). Effect of manuka oil (●), its triketone-rich fraction (■) and purified leptospermone (4) (◆) on (B) chlorophyll and (C) carotenoid accumulation in lettuce. Sulcotrione (2) (○), a synthetic herbicide known to affect chlorophyll and carotenoid levels, was used as a positive control. The pigments were extracted from 10 mg of tissues. Each data point represents the mean of two independent experiment ± 1 SD ($N = 6$).

Table 1
Inhibitory activity of manuka oil and its components as measured *in vivo* on chlorophyll and carotenoid accumulation and *in vitro* on *p*-hydroxyphenylpyruvate dioxygenase activity

Compound	clogP	Chlorophyll I_{50}[a] ($\mu g\ mL^{-1}$)	Carotenoid I_{50}[a] ($\mu g\ mL^{-1}$)	HPPD I_{50}[a] ($\mu g\ mL^{-1}$ (μM))
Manuka oil	–[b]	11.5 ± 1.25	4.75 ± 0.68	15.0 ± 1.27
Triketone fraction	–	1.45 ± 0.10	1.87 ± 0.26	4.02 ± 0.57
Leptospermone (4)	2.56	0.96 ± 0.07	1.20 ± 0.12	3.14 ± 0.58 (12.1 ± 2.24)
Isoleptospermone (5)	2.34	–	–	–
Flavesone (6)	1.88	–	–	>100
Grandiflorone (7)	2.91	–	–	0.22 ± 0.02 (0.68 ± 0.05)
Sulcotrione (2)	1.73	0.09 ± 0.01	0.10 ± 0.01	0.07 ± 0.01 (0.22 ± 0.02)

[a] I_{50} = concentration required for 50% inhibition followed by standard deviation.
[b] Not applicable or not determined. The synthetic herbicide sulcotrione (2), a known inhibitor of *p*-hydroxyphenylpyruvate dioxygenase, was used as a positive control.

2.3. Inhibitory activity of β-triketones against HPPD

The inhibitory activity of manuka oil and its components provided some insight into the structure–activity relationships of the β-triketone inhibitors with HPPD (Fig. 4). Manuka oil, which possessed ca. 18% β-triketone, had an apparent I_{50} (I_{50} app) value of nearly 15.0 μg mL^{-1} against HPPD (Table 1). As expected, the triketone-rich fraction was much more active, with an I_{50} app of 4 μg mL^{-1} (Table 1). The three purified natural β-triketones differed dramatically in their ability to inhibit HPPD. The I_{50} app of leptospermone (4) was similar to that of the

with I_{50} values of 1.45 and 0.96 μg mL^{-1}, respectively (Table 1), suggesting that the biological activity of manuka oil is associated with the presence of bioactive triketones. Since the potency of the triketone-rich fraction was similar to that of the purified leptospermone (4), the enriched fraction may potentially be used as an effective natural herbicide without requiring further purification. The synthetic triketone herbicide was more than 125 times more potent than manuka oil, with an I_{50} of 0.09 μg mL^{-1}.

Overall, these compounds had similar effect on carotenoid and chlorophyll levels, whereas manuka oil was approximately twice as potent on carotenoids than chlorophyll levels (Table 1). The physiological and biochemical factors responsible for this are not clear since leptospermone (4) is the primary constituent of the triketone fraction. Isoleptospermone (5), flavesone (6), and grandiflorone (7) were not tested in these assays due to the limited amount of each of these compounds that were obtained through purification.
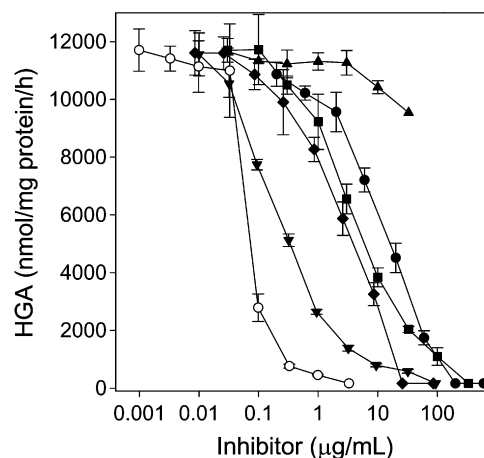

Fig. 4. Inhibition of HPPD by manuka oil (●), the β-triketone-rich fraction (■), and its individual β-triketone components: leptospermone (4) (◆), flavesone (6) (▲), and grandiflorone (7) (▼). The synthetic herbicide sulcotrione (2) (○) was added as positive control. Each data point represents the mean of two independent experiment ± 1 SD ($N = 6$). HGA = homogentisic acid.

triketone-rich fraction, at 3.14 µg mL^{-1} (12.1 µM). However, grandiflorone (**7**) was much more active, with an I_{50} app value of 0.22 µg mL^{-1} (0.68 µM). On the other hand, flavesone (**6**) did not effectively inhibit HPPD with an $I_{50} > 100$ µg mL^{-1} (Fig. 4). It should be noted that none of the natural β-triketones were as potent as the synthetic herbicide sulcotrione (**2**), which had an I_{50} app value of 0.07 µg mL^{-1} (0.22 µM) against HPPD (Table 1).

Values of clogP were calculated for the triketones using PreADMET (http://preadmet.bmdrc.org/preadmet/index. php). While the activity of the natural β-triketones appeared to be related to the lipophilicity of their side-chain, this parameter alone did not provide a general correlation when sulcotrione (**2**) is added (Table 1). This may be due to the fact that the presence of a strong electron-withdrawing group ($-SO_2CH_3$) in the *para-* position of the phenyl ring of sulcotrione (**2**) increased its activity by 40 fold (Lee et al., 1998; Ellis et al., 1996).

Previous work reported that the phytotoxicity of the synthetic β-triketones was greatly affected by the nature of the side chain attached to the triketone backbone. Compounds with shorter, less lipophilic tail than that of leptospermone (**4**) were significantly less active (Knudsen et al., 2000). Our data support this observation, with flavesone (**6**), the β-triketone with the shortest side-chain, having no activity against HPPD (Fig. 4), whereas grandiflorone (**7**), with its long hydrophobic side chain, was the most active natural β-triketone against HPPD (Fig. 4). A similar observation was reported recently on the antimicrobial activity a set of synthetic derivative of triketones (van Klink et al., 2005).

Steady-state observation of O$_2$ consumption during the formation of HGA by HPPD suggested that unlike their synthetic counterparts (Ellis et al., 1996), the natural β-triketones leptospermone (**4**) and grandiflorone (**7**) did not bind tightly to the enzyme (Fig. 5A and B). The inhibition of O$_2$ consumption did not appear to be time-dependent,

suggesting that the natural β-triketones acted as competitive reversible inhibitors. While the mechanism responsible for this is not clearly understood, structure–activity studies on synthetic inhibitors of HPPD have shown that the presence of an electron-withdrawing group, such as $-SO_2CH_3$ in sulcotrione (**2**), are required to impart tight-binding properties to β-triketones, and that compounds lacking such groups behave as competitive inhibitors (Ellis et al., 1996; Lee et al., 1998). Therefore, the fact that β-triketones isolated from manuka oil behave as competitive reversible inhibitors may be due to the lack of electron-withdrawing groups in their side chains. While several other natural products apparently act as reversible inhibitors of HPPD (Meazza et al., 2002), some natural triketones have tight-binding properties on HPPD (Romagni et al., 2000).

2.4. Modeling of the interaction between the β-triketones and the catalytic site of HPPD

The X-ray structure of the bacterial enzyme (*Sa*-HPPD) is in complex with the triketone inhibitor NTBC (**1**) and the required metal ion, Fe^{2+}, at the catalytic center of the active enzyme (Brownlee et al., 2004). Since the chemical structure of NTBC (**1**) is similar to that of the natural triketones isolated from manuka oil (Fig. 1), it was assumed that the protein–ligand interactions between the keto groups of the ligands and the Fe^{2+} ion in the catalytic domain were similar.

Despite relatively low sequence identity (∼35%) between *Sa*-HPPD and the plant *Arabidopsis thaliana* (*At*-HPPD) (Yang et al., 2004), their secondary structure and topology are nonetheless very similar. The overall sequence similarity between the plant and bacterial enzyme is approximately 63%. Furthermore, superimposition of the two models shows that the active site architecture is conserved, and that the residues contacting NTBC (**1**) in *Sa*-HPPD are identical in the plant enzyme. The *At*-HPPD-metal-inhibitor complex was generated by superimposing different ligands onto NTBC (**1**) within the binding pocket of *At*-HPPD one at a time while taking care that the coordination environment around the catalytic Fe^{2+} ion is maintained. The coordinate of NTBC (**1**) complexed with Fe^{2+} ion of *Sa*-HPPD (Brownlee et al., 2004) were used instead of those of the pyrazole present in the plant *At*-HPPD crystal structure (Yang et al., 2004) because of the greater similarity (both in structure and in size) between the natural triketones used in this study and NTBC (**1**), than with the pyrazoles.

Docking of all the structures into the active site of plant HPPD was investigated. Flexidock docked NTBC (**1**) within the active site in the same horizontal plane as that observed in the crystal structure. However, in order to keep the coordination geometry around the Fe^{2+} ion intact, some distance restraints between the diketone moiety and the metal ion were established.

An analysis of the protein–ligand complexes revealed that the natural β-triketones had similar binding orientation
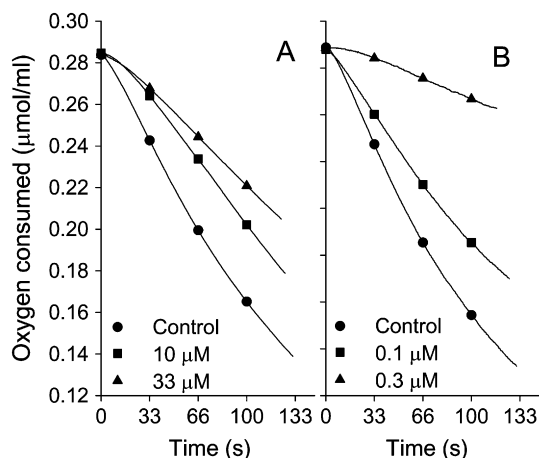

Fig. 5. Steady-state observation of O$_2$ consumption by HPPD in the presence of either (A) leptospermone (**4**) or (B) grandiflorone (**7**). Each reaction was initiated by adding 250 µM HPP and the test compound simultaneously.

around the Fe^{2+} ion, which is probably due to the structural similarity existing among these compounds. The 1,3-diketone moiety of all the docked inhibitors coordinated Fe^{2+} ion still formed an octahedral complex with three strictly conserved active site residues (Glu373, His287 and His205) and a critical binding site H_2O molecule, providing a strong ligand orientation and binding force (Figs. 6A, 7A and 8A). These interactions are consistent with those established with several classes of potent 1,3-diketone-type HPPD inhibitors.

The triketone functionality of the inhibitors resembles the α-keto acid moiety of 4-HPP and competes for the binding site of the natural substrate (Pascal et al., 1985). Inhibition kinetic analysis indicates that most synthetic β-triketones bind slowly but very tightly (nearly irreversibly) to the catalytic site of HPPD (Ellis et al., 1995).

The benzoyl moiety of sulcotrione (2) was localized between the aromatic rings of two phenylalanine residues (Phe360 and Phe403) which are located in a pronounced hydrophobic pocket, as visualized as Molecular Lipophilic Potential generated by MOLCAD (Fig. 6) (Brickmann, 1997; Heiden et al., 1993). Due to the presence of additional carbon atoms between the two rings, the aromatic ring of grandiflorone (7) enters even further into the hydrophobic region (Fig. 8). However, this π-stacking interaction is missing in leptospermone (4) (Fig. 7) and flavesone (6) (data not shown), which may explain the lower affinity of such compounds towards HPPD. This observation is in
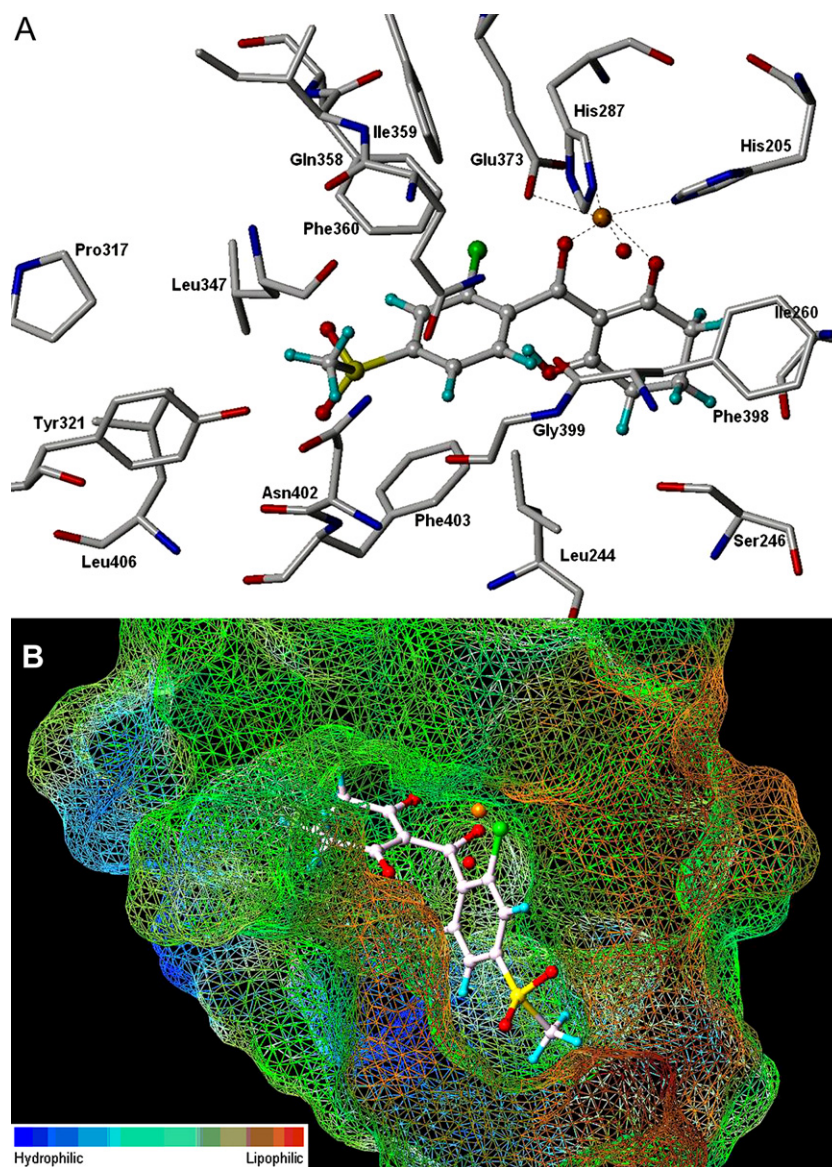

Fig. 6. (A) Binding mode of sulcotrione (2) docked into the putative active site of At-HPPD. Sulcotrione (2) (colored by atom type) shown as ball and stick model; Fe^{2+} ion in orange; binding site H_2O in red. The metal ion coordination is depicted in black dotted lines. (B) A MOLCAD generated MLP surface of At-HPPD showing binding mode of sulcotrione (2) within the putative active pocket. The metal ion lies at the base of the active pocket. Sulcotrione (2) is represented as capped stick model; metal ion and H_2O molecule in orange and red, respectively.
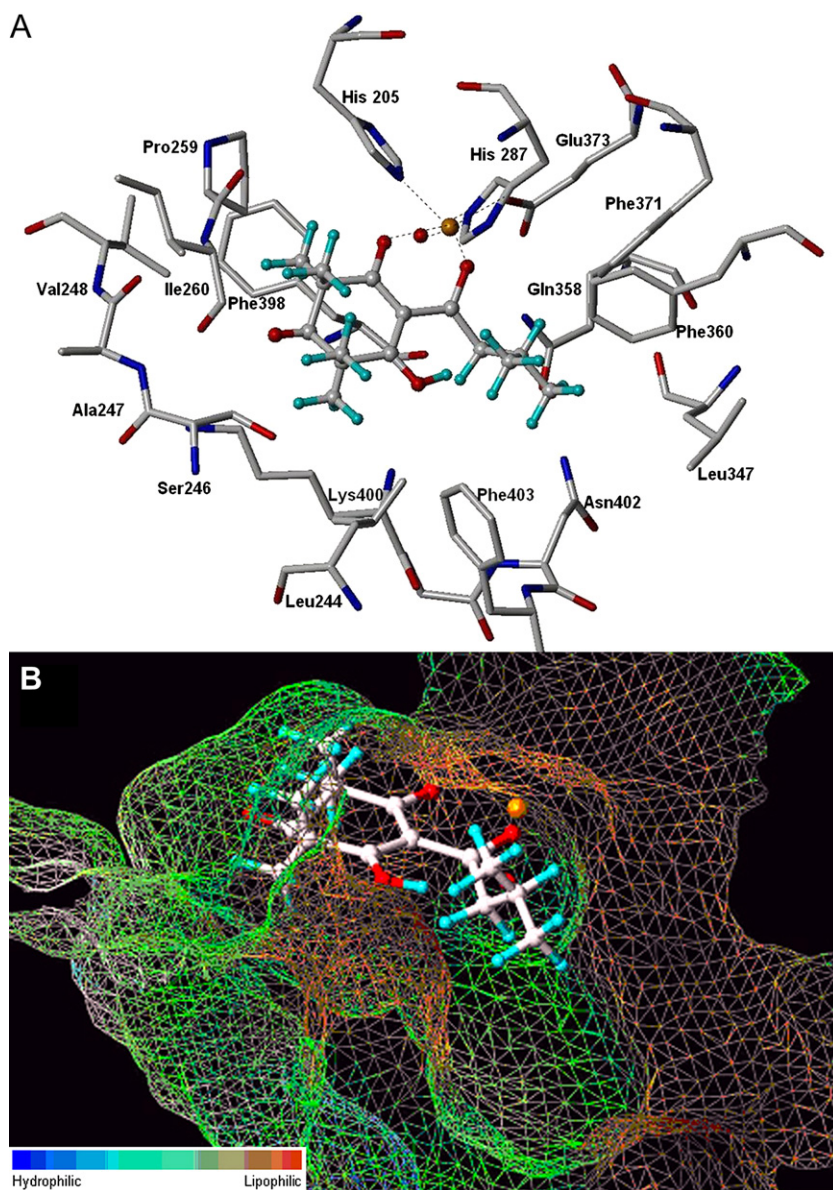
Fig. 7. (A) Binding mode of leptospermone (**4**) docked into the putative active site of *At*-HPPD. For the purpose of clarity, amino acid residue Asn 216 has been omitted. (B) A MOLCAD generated MLP surface of protein showing binding mode of leptospermone (**4**) within the active pocket of *At*-HPPD.

agreement with the results from a recent study by Neidig et al. (2005) that held the combination of two distinct binding interactions, bidentate coordination and π-stacking, responsible for high affinity of NTBC (**1**) for HPPD. None of the inhibitors showed hydrogen bonding interactions with amino acid residues within the active site.

The nature of the substituent at the 2-position of the phloroglucinol ring is known to determine whether the compounds behave as time-dependent or time-independent inhibitors (Ellis et al., 1996). For example, a phenyl substitution at this position results in a competitive reversible inhibitor, whereas having a 2-nitro, 4-trifluoromethyl-phenyl ring resulted in a tight-binding inhibitor. Consequently, the natural β-triketone leptospermone (**4**) and grandiflorone (**7**) should behave as time-independent reversible

inhibitors. Our steady-state O_2 consumption experiment confirmed that hypothesis.

2.5. Concluding remarks

Synthetic triketone-based HPPD inhibitors were discovered following a study on the phytotoxic properties of leptospermone (**4**) (Knudsen et al., 2000). These herbicides were later found to target HPPD, but the inhibitory activity of leptospermone (**4**) and other natural triketones had not been reported. This study confirms that the phytotoxic activity of leptospermone (**4**) is due to its ability to inhibit HPPD. Grandiflorone (**7**) was significantly more active than leptospermone (**4**), but it accounts for <1% so its contribution to the overall activity of the triketone-rich
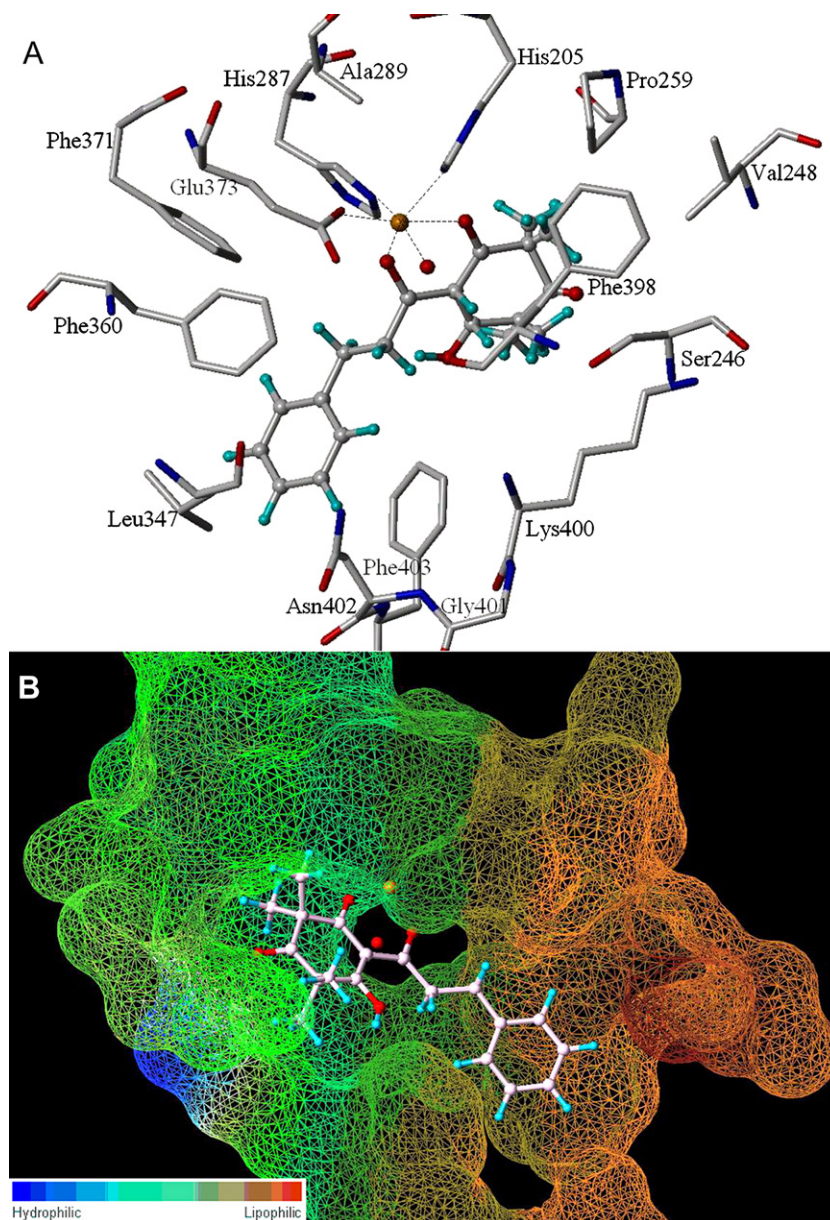
Fig. 8. (A) Binding mode of grandiflorone (**7**) docked into the putative active site of *At*-HPPD. (B) A MOLCAD generated MLP surface of protein showing binding mode of leptospermone (**4**) within the active pocket of *At*-HPPD.

fraction is probably masked by the abundance of leptospermone (**4**). Our data suggest that, unlike their commercial synthetic derivatives that are slow tight-binding inhibitors, these natural β-triketones are competitive, reversible inhibitors.

3. Experimental

3.1. Sources of natural products and chemicals

Manuka oil (1 L) was obtained from Tairawhiti Pharmaceuticals Ltd. (Felton, CA) on January 4th, 2005. Information from the manufacturer indicated that the oil was steam distilled from *L. scoparium*. All other chemicals were purchased from Sigma–Aldrich (Milwaukee, WI) or Fisher Scientific (Pittsburgh, PA).

3.2. Instrumentation

^{1}H- and ^{13}C-NMR spectra were recorded in $CDCl_3$ on a Bruker Avance 400 MHz spectrometer (Billerica, MA). HPLC method development work was performed using an Agilent 1100 system (Palo Alto, CA) equipped with a quaternary pump, autosampler, diode-array detector, and vacuum degasser. Semi-preparative HPLC purifications were performed using a Waters Corporation Delta-Prep system (Milford, MA) equipped with a diode-array detector and a binary pump.

Manuka oil and fractions were analyzed by GC-MS on a Varian CP-3800 GC coupled to a Varian Saturn 2000 MS/MS (Palo Alto, CA). GC was equipped with a DB-5 column (30 m × 0.25 mm fused silica capillary column, film thickness of 0.25 μm) operated using the following conditions: injector temperature, 240 °C; column temperature, 60–240 °C at 3 °C/min then held at 240 °C for 5 min; carrier gas, He; injection volume, 1 μL (splitless).

3.3. Purification of β-triketone components

Crude manuka oil (50.3 g) was dissolved in Et_2O (100 mL) and extracted once with satd. $NaHCO_3$ (100 mL) followed by extraction with 5% Na_2CO_3 solution (3 × 100 mL). The combined Na_2CO_3 extracts were neutralized with 30% aqueous HCl and subsequently partitioned with Et_2O (2 × 200 mL). The Et_2O fractions were combined, dried ($MgSO_4$) and concentrated in vacuo (Hellyer and Pinhey, 1966).

3.4. Purification of β-triketones

A reversed phase HPLC method was developed for the purification of the compounds present in the β-triketone fraction. A Zorbax SB-C18, 5 μm, 4.6 × 250 mm (Palo Alto, CA) column was used with a flow rate of 1 mL min^{-1} and the mobile phase consisted of a 6:4 (v/v) ratio of MeCN:(deionized) H_2O containing 0.1% CF_3CO_2H. Runs were conducted over 30 min. Preparative purifications were performed in a similar manner using a Zorbax SB-C18, 7 μm, 21.2 × 250 mm column with a flow rate of 20 mL min^{-1}. Multiple 500 μL injections of the β-triketone fraction (838 mg) in MeOH (10 mL) were made until all material had been injected while separately collecting four fractions at 254 nm. MeCN was removed from fractions leaving an acidic aqueous solution which was subsequently extracted thrice using Et_2O followed by drying with $MgSO_4$ and removal of the Et_2O by rotary evaporation. This procedure was repeated for each fraction to obtain pure leptospermone (4) (394 mg), flavesone (6) (3.9 mg), and grandiflorone (7) (33 mg). Isoleptospermone (5) (26 mg) was also obtained but was contaminated with 37% leptospermone (4). All compounds were identified by comparison of mass spectrometry and ^{13}C NMR spectroscopic data with that reported in the literature (van Klink et al., 1999).

3.5. Dose–response curves in lettuce (Lactuca cv. Iceberg) bioassays

The test solutions (1 mL) were added to 5 cm diameter Petri dishes lined with a filter paper. Twenty lettuce seeds were placed on top of the filter paper. The plates were sealed with parafilm and placed in an incubator (Percival Scientific, Boone, IA) set up at 25 °C with a 16 h/8 h day night light cycle. The light intensity was 100 ± 5 μmol m^{-2} s^{-1}. Dose–response curves for concentrations ranging from 0 to

1 mg mL^{-1} were obtained for manuka oil, triketone-rich fraction. Leptospermone (4) and sulcotrione (2) were tested at concentrations ranging from 0 to 333 μM. Plant responses to the test compounds included measurement of chlorophyll and carotenoid contents in cotyledons 7 d after treatment. Chlorophyll was extracted from cotyledons (10 mg) per treatment in dimethyl sulfoxide (3 mL), and chlorophyll concentrations were determined spectrophotometrically (Hiscox and Israelstam, 1979). Carotenoids were extracted from cotyledons (10 mg) and total carotenoid concentrations were determined spectrophotometrically according to Sandmann and Böger (1983) with an extinction coefficient of E445 2,500 (% w/v).

3.6. Expression and extraction of HPPD

Recombinant HPPD from A. thaliana was overexpressed 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^{-1} of carbenicillin and 100 μg mL^{-1} streptomycin. 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 (6000g). 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, Branson Ultrasonics Corporation, Danbury, CT, USA). A cell-free supernatant was obtained by centrifugation at 35,000g for 30 min.

3.7. Assay of HPPD activity

HPPD activity was measured at a protein concentration of 5 mg mL^{-1} in the cell-free extract. The assay mixture (contained 0.25 mg mL^{-1} in 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 half-log increments. The reaction was initiated by adding 4-HPP (5 μL, 10 mM in MeOH) to afford 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 assay was incubated at 30 °C for 15 min and the reaction was stopped by addition of perchloric acid (70 μL, 20% (v/v)). The precipitated protein was removed by centrifugation at 20,000g for 5 min. The supernatant was subjected to HPLC analysis for the determination of HGA produced.

3.8. HPLC protocol

The HPLC system used to measure enzyme activity was composed of a Waters Corporation system (Milford, MA) 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 Nova-Pak C18 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 7 min, 70% to 100% B from 7 to 9 min, 100% B from 9 to 10 min, 100% to 0% B from 10 to 12 min and 0% B from 12 to 22 min. The flow rate was 1 mL min^{-1} and the injection volume was 100 µL.

Solvent A was ddH$_2$O containing 0.1% (v/v) CF$_3$CO$_2$H and solvent B was a 4:1 ratio of HPLC-grade MeCH:ddH$_2$O containing 0.07% (v/v) CF$_3$CO$_2$H. HGA was detected by the UV absorbance at 288 nm (Garcia et al., 1999). A calibration curve was established by injecting various concentrations of HGA.

3.9. Steady-state observation

For the steady-state observation, assays were performed using a model DW1 Hansatech Oxygraph oxygen electrode (Hansatech, Norfolk, UK). The reaction mixture (3 mL) contained 10 µM ferrous sulfate and 1 mM dithiothreitol in 20 mM HEPES buffer at pH 7.0. A 75 µl aliquot of crude HPPD preparation (25 µg protein) was added. After initial observation of the non-enzymatic rate of oxygen consumption, the reaction was initiated by adding 250 µM HPP and the test compound simultaneously. The reaction was monitored for 130 s. Leptospermone (**4**) was tested at 10 and 33 µM, whereas grandiflorone (**7**) was tested at 0.1 and 0.3 µM, respectively.

3.10. Statistical analysis

Data from dose–response experiments were analyzed with the add-on package for dose–response curves, drc (Ritz and Streibig, 2005), for R version 2.2.1 (R Development Core Team, 2005) using a four-parameter logistic function. Means and standard deviations were obtained using the raw data, and I_{50} values were one of the parameters in the regression curves. The regression curves were imported into SigmaPlot version 10 (Systat Software Inc., San Jose, CA).

3.11. Modeling of the binding of leptospermone (4) and other β-triketones to HPPD

Molecular modeling was performed using Sybyl v.7.1 software from Tripos Inc. (St. Louis, MO) on a Silicon Graphics Octane 2 workstation, equipped with two parallel R12000 processors. The crystal structures of bacterial HPPD from *Streptomyces avermitilis* (Sa-HPPD; pdb: 1T47) (Brownlee et al., 2004) and plant HPPD from *A. thaliana* (At-HPPD; pdb: 1TFZ) (Yang et al., 2004) at 2.5 and 1.8 Å resolution, respectively, were obtained from the Protein Data Bank (Bernstein et al., 1977). Sequence alignment was derived using CLUSTALW v.1.8 package (Thompson et al., 1994) using the GONNET scoring matrices. The parameter for GAP OPEN, GAP EXTENSION and

GAP DISTANCE was set equal to 10, 0.2 and 8, respectively.

Hydrogens were added and all H$_2$O molecules were deleted from the two crystal structures. The two enzymes were then superimposed and the coordinates of NTBC (**1**) along with the complexed metal ion from Sa-HPPD were extracted into the active site of the At-HDDP. Similarly, the critical H$_2$O molecule required to complete the octahedral geometry of the coordinated metal ion was extracted from the other protomers of Sa-HPPD into the active site of plant enzyme. A subsequent minimization was performed on the transferred ligands with the Tripos force field, as implemented in Biopolymer module of *Sybyl*.

The initial structures of β-triketone analogues were derived from the coordinates of NTBC (**1**), minimized for 1000 steps each of steepest descent, followed by conjugate gradient, and finally by the BFGS method to a gradient of 0.001 kcal/mol/Å or less. The charges were added to the molecules using the *Sybyl* Gasteiger–Hückel algorithm. These analogues were docked to the active site of At-HPPD using the Flexidock (Genetic algorithm-based flexible docking) routine. FlexiDock explores the conformational and orientational space that defines possible interactions between the ligand and its binding site. The active site for docking was defined as a spherical region of 6.5 Å radius centered from the β-triketone ligand, NTBC (**1**). This sphere encompasses all residues known to be involved in inhibitor binding (Brownlee et al., 2004). The default *Sybyl*/Flexidock parameters were used. Of the several possible conformations obtained for each ligand, the conformation with the lowest binding energy to At-HPPD was selected for further analyses.

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