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Translation of in Vitro Inhibition by Marine Natural Products of the C_4 Acid Cycle Enzyme Pyruvate P_i Dikinase to in Vivo C_4 Plant Tissue Death

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Marine organism derived extracts, previously identified as containing compounds that inhibited the C_4 acid cycle enzyme pyruvate P_i dikinase (PPDK), were assessed for their ability to exhibit an effect on the C_4 plants *Digitaria ciliaris* and *Echinochloa crus-galli*. Oxygen electrode studies revealed that over half of these extracts inhibited C_4 acid driven photosynthesis in leaf slices. Seventeen extracts had a deleterious effect on C_4 plants in vivo within 24 h, whereas 36 caused an observable phytotoxic response in one or both of the C_4 plants used for in vivo testing. None of the extracts inhibited PPDK metabolism of pyruvate via a directly competitive mechanism, instead hindering the enzyme by either mixed or uncompetitive means. This screening strategy, using a suite of assays, led to the isolation and identification of the herbicidal marine natural product ilimaquinone.

KEYWORDS: Herbicide; PPDK; C_4 plant; oxygen evolution

INTRODUCTION

In recent times, global use of biologically active compounds as herbicides or plant growth regulators approached 1,000,000 tonnes per annum (*1*). Most act by targeting the plant enzymes acetolactate synthase (e.g., the sulfonylureas) or acetyl CoA carboxylase (e.g., aryloxyphenoxypropionates, cyclohexanediones) as well as various sites within photosystem II (e.g., atrazine) (*2*). Add to this the large and increasing usage of the amino acid synthesis inhibitor, glyphosate (*3*), and the herbicide market is fast becoming homogeneous with regard to chemical diversity and biological action. This imposes an enormous selective pressure, resulting in herbicide resistance. Chemical diversification of herbicides is necessary to reduce the likelihood of the continued emergence of herbicide resistance. Although several strategies have been employed whereby herbicides that act via different mechanisms are applied on a rotational basis (*4*), these can be greatly improved by pursuing new chemical classes that act via novel mechanisms.

Despite the plethora of metabolic processes within plants, the number of physiological mechanisms targeted by today's herbicides are few (*4*). Historically, herbicide discovery has relied on direct application of compounds to whole plants. This has the advantage of demonstrating early in the discovery process that the compound is able to penetrate the plant's defenses and elicit a deleterious effect (*5*). This approach is hampered, however, by the need to source large enough quantities of the compound to conduct these tests. Furthermore, if the compounds being tested are derived from a synthetic or combinatorial library, diversity is limited by the variety of chemical templates amenable to such syntheses. In recent times, in vitro target-based screening has been growing in use (*2*, *5*). This approach to herbicide discovery requires much less test material, enabling a larger variety of materials to be tested. This has increased the screening of natural product extracts for herbicidal potential (*6*, *7*). Once extracts of interest have been identified, bioassay-guided isolation of bioactive compounds is undertaken. This process, although hampered by the slow turnaround of results from whole plant testing, is significantly enhanced by the use of an in vitro assay. It should be noted, however, that in vitro screening is unable to determine whether those compounds would also act against the whole organism (*1*, *2*, *8*, *9*). Although both approaches have their advantages, the best strategy would be to integrate the in vivo and in vitro screenings as early as is practicable in the discovery process.

A recent in vitro screening program conducted in our laboratories led to the discovery of a number of marine organism derived extracts containing compounds that selectively inhibited the rate-limiting enzyme of the C_4 acid cycle, pyruvate P_i dikinase (PPDK, EC 2.7.9.1) (*10*). It is possible that this targeted molecular action will translate to the death of C_4 plants (*10*, *11*), which happen to comprise the world's most prolific weeds (*12*). Several physical and biochemical barriers add further complexity and thwart the translation of in vitro activity to an in vivo effect. These include the ability to penetrate the leaf cuticle, cell walls, and cell membranes and to survive the intracellular defense mechanisms against exogenous compounds.

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Given that the C_4 acid pathway generally becomes functional only following differentiation of Krantz anatomy, death of C_4 plants by inhibition of PPDK would require postemergent application of a herbicide (*13*). Successful development of a C_4 plant specific herbicide would depend on its ability to penetrate the leaf cuticle, translocate throughout the plant, and elicit an effect over a period of time while being exposed to a number of fluctuating environmental conditions, such as light, temperature, and moisture. To identify putative C_4 plant specific herbicides with a greater likelihood of development success, we employed an integrated approach of measuring their ability to inhibit C_4 acid driven photosynthesis (i.e., pyruvate-dependent) in leaf slices. This maintains the integrity of the photosynthetic apparatus while eliminating the need to penetrate the cuticle. Direct application of the extracts on the leaves of whole plants was also undertaken to measure their prospective ability as an effective herbicide.

MATERIALS AND METHODS

Reagents. All buffers, general chemicals, and deuterated chloroform ($CDCl_3$) were sourced from Sigma-Aldrich (St. Louis, MO). NAD−malate dehydrogenase (NAD-MDH; EC 1.1.1.37) was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Microtiter plates were flat-based polystyrene plates from Falcon (catalog no. 353072) (BD Biosciences, Sydney, Australia). Purified water was obtained from a Milli-Q water purification system (Millipore, Billerica, MA). PEPC (EC 4.1.1.31) was isolated and purified from maize leaves, and recombinant maize pyruvate P_i dikinase (PPDK, EC 2.7.9.1; Genbank accession no. J03901) was expressed as described previously (*10*). The herbicide formulation Uptake was supplied by Nufarm (Melbourne, Australia). Compressed gases were purchased from BOC Gases (Townsville, Australia) and were at least 99.99% pure. Analytical grade ethanol, acetone, DMSO, and methanol (MeOH) were purchased from Biolab (Melbourne, Victoria, Australia). HPLC grade MeOH, dichloromethane (DCM), hexane, and ethyl acetate (EtOAc) were from Mallinckrodt (Hazelwood, MO). Kieselgel 60G silica gel for open column chromatography was purchased from Merck (Melbourne, Australia).

Marine Organism Derived Extracts. Approximately 1 g (wet weight) of biomass of each of 56 marine organisms, determined to selectively inhibit PPDK (*10*), was lyophilized, covered with 5 mL of ethanol, sonicated for 80 min, and left to steep for 2 days. Each extract was filtered and solvent removed by gentle heating at 30 °C under a stream of nitrogen gas, followed by lyophilization. All extracts were prepared at 10 mg mL^{-1} in specified solvents.

Inhibition of Oxygen Evolution during C_4 Acid Cycle Driven Photosynthesis. C_4 acid cycle driven oxygen (O_2) evolution was measured using a method derived from ref *14*. Buffer (1 mL) containing 0.33 M sorbitol, 2.5 mM $MgCl_2$, 2.5 mM NaH_2PO_4, 25 mM Hepes−KOH (pH 7.5), 50 μM $MnCl_2$, and 2.5 mM DTT was added to the reaction chambers of a Hansatech O_2 electrode, and O_2 was removed from the solution by bubbling with nitrogen. Leaf slices of ~1 mm thickness of the C_4 plant *Digitaria ciliaris* were added to the buffer, and the recording commenced. When the system had stabilized (as indicated by a steady minimal rate of O_2 evolution), the reaction chambers were illuminated, using Osram Halogen Photo Optic lamps (1200 μmol m^{-2} s^{-1}), and the system was again allowed to stabilize. C_4 acid cycle driven O_2 evolution was initiated by the addition of 4 mM $NaHCO_3$ followed by 4 mM pyruvate and the C_4 acid cycle driven O_2 evolution calculated from the pyruvate-dependent increase in O_2 production. $NaHCO_3$-dependent O_2 evolution was negligible in leaf slices of C_4 plants.

To standardize experiments, chlorophyll (chl) content of leaf slices were determined, and rates of O_2 evolution were expressed as micromoles of O_2 per minute per milligram of chlorophyll. Following determination of rates of O_2 evolution, leaf slices were removed from the O_2 electrode chamber, placed in 1 mL of 80% acetone, and stored in the dark at 4 °C for a minimum of 48 h prior to spectrophotometric determination of chl concentration in a Beckman DU650 spectrophotometer. Chl levels were calculated using the following formula (*15*):

$$\text{total chl (mg L}^{-1}) = (20.2 \times \text{OD}_{645}) + (8.02 \times \text{OD}_{663})$$

O_2 evolution, generally at a rate of 1−3 μmol of O_2 min^{-1} mg of chl^{-1}, was recorded for 5−7 min prior to the addition of test material to determine the control rate. Test material (10 mg mL^{-1} in MeOH) was added to the reaction mixture to give a final concentration of 50 μg mL^{-1}. Photosynthetic rates were measured for a further 5−7 min to record the changes in the rates of O_2 evolution caused by the presence of test material. The rate of O_2 evolution in the presence of marine organism derived extracts or compounds was expressed as percent inhibition calculated as follows:

$$\% \text{ inhibition} = 100 - [(O_2 \text{ evolution in the presence of test material} \times 100/O_2 \text{ evolution in the absence of test material})]$$

C_4 Plant Phytotoxicity. Seedlings of the C_4 plants *D. ciliaris* and *Echinochloa crus-galli* were grown in a glasshouse, in seed trays divided into individual circular pots 5 cm in diameter, containing potting mix (1:1 v/v vermiculite/peat moss). Seedlings were watered once during their growth period with a complete liquid fertilizer, and the potting mix was kept damp at all times. When the flag leaf was at least the same size as the seed leaf (length of time depends on season and weather conditions), seedlings were deemed to be no longer reliant on the seed for nutrients and ready for herbicidal testing. The length of the leaves ranged from 2.0 to 2.5 cm and the width usually approximated 0.5 cm. The day before treatment, plant numbers in each plot were reduced to three and watered immediately before testing, ensuring both leaves and potting mix were wet.

Test material, prepared in either (a) 0.05% Tween 20 in MeOH or (b) 0.5% DMSO and 0.08% Uptake in MeOH to give a final concentration of 8 mg mL^{-1}, was applied (5 μL) to individual leaves on duplicate plants. Control plants were treated with formulation only. Test materials were applied to the tip of the leaf using a micropipet, and the solution was spread over the leaf, ensuring that the leaf base remained untreated.

Plants were observed within 6 h of application of test material and then at 24 h intervals for 4 days. All physical changes of the leaves resulting from photosynthesis interruption were recorded and ranked according to potency of effect. Descriptions of these effects and their potency score are listed in the legend of **Table 1**.

Mechanism of PPDK Inhibition. PPDK activity was measured in reaction mixtures (0.5 mL) containing 10 mM DTT, 8 mM $MgSO_4$, 10 mM $NaHCO_3$, 1 mM glucose-6-phosphate, 5 mM $(NH_4)_2SO_4$, 0.2 mM $NADH_2$, 25 mM Hepes−KOH (pH 8), 5 mM pyruvate, 2.5 mM NaH_2PO_4, 1 mM ATP, at least 2 units of PEPC, 2 units of NAD-MDH, and sufficient PPDK to give a reaction rate corresponding to a decrease in A_{340} of between 0.075 and 0.085 AU min^{-1}. All experiments were conducted at 30 °C in a temperature-regulated Beckman DU650 spectrophotometer. Adenylate kinase (1 unit) and pyrophosphatase (1 unit) were added to reaction mixtures to eliminate end-product inhibition of PPDK. MeOH was added to all nil inhibitor reference reactions in volumes equivalent to those used when test materials were added.

Nil inhibitor reference kinetic experiments containing MeOH were conducted in the presence of six substrate concentrations. For pyruvate, these concentrations were 40, 80, 160, 320, 480, and 720 μM. For ATP, the concentrations used were 20, 40, 60, 120, 240, and 480 μM. For phosphate, the concentrations used were 0.3, 0.6, 1.2, 1.8, 2.7, and 4.32 mM.

Due to the constraint of available test material, enzyme inhibitor experiments were conducted using only three of the above-mentioned substrate concentrations: 80, 360, and 720 μM pyruvate; 40, 120, and 480 μM ATP; and 0.6, 1.8, and 4.32 mM phosphate.

Maximum rates of reaction were calculated, and the mechanism of inhibition of PPDK by the test material with respect to pyruvate, phosphate, and ATP was graphically determined (*16*).

Compound Purification and Identification. The lead extract for isolation of a bioactive compound was selected on the basis of potent

Table 1. Summary of Bioassay Data

sample	source	PPDK inhibition[a,b] Pyr	ATP	Pi	O_2 electrode[c]	*D. ciliaris*[d]	*E. crus-galli*[d]
1	soft coral	U/M	nd	nd	■	■■■■ (3)	
2	sponge	nd	nd	nd	■■■■		
3	sponge	U/M	nd	nd	■■■■	■■■■ (3)	
4	ascidian	U/M	U/M	U/M		■■■■ (1)	■ (1)
5	ascidian	U/M	nd	nd	■■■	■■■■ (2)	
6	ascidian	U/M	nd	nd	■■■■	■■■■ (2)	
7	sponge	U/M	nd	nd	■■■		
8	sponge	U/M	U/M	U/M	■■■■	■■■■ (6)	■■■■ (6)
9	sponge	U/M	nd	nd		■■ (1)	■■■ (1)
10	sponge	nd	nd	nd	■■		
11	sponge	U/M	U/M	U/M	■■■■	■■■ (1)	
12	sponge	nd	nd	nd		■■■ (1)	
13	sponge	U/M	nd	nd	■■■■	■■■■ (2)	
14	ascidian	U/M	nd	nd	■■		
15	sponge	U/M	nd	nd	■■■■	■■■■ (3)	
16	sponge	U/M	nd	nd	■■■■	■■ (1)	
17	sponge	U/M	nd	nd	■■■■	■■ (5)	■■ (1)
18	sponge	U/M	nd	nd	■■■■	■■ (1)	■■ (6)
19	sponge	nd	nd	nd	■■	■■ (1)	■■■ (1)
20	sponge	U/M	nd	nd	■■■		■■■ (1)
21	sponge	nd	nd	nd	■	■■■ (1)	
22	sponge	U/M	U/M	U/M	■■		
23	sponge	U/M	U/M	U/M	■■		
24	sponge	U/M	nd	nd	■■■		
25	sponge	nd	nd	nd	■		
26	sponge	nd	nd	nd	■		
27	feather star	U/M	nd	nd	■■■■	■■■■ (6)	■■■■ (6)
28	sponge	U/M	U/M	U/M	■■■■		
29	sponge	U/M	C	U/M	■■■■		
30	starfish	U/M	nd	nd	■■■■	■■■■ (1)	■■■■ (3)
31	sponge	U/M	C	U/M	■■■■	■■■■ (6)	■■■■ (6)
32	ascidian	U/M	U/M	U/M	■■■■	■■■■ (6)	■■ (5)
33	sponge	U/M	U/M	U/M	■■■■		
34	sponge	U/M	U/M	U/M	■■■		■■■ (3)
35	ascidian	nd	nd	nd	■■	■■■■ (3)	■ (1)
36	starfish	U/M	nd	nd	■	■■■■ (3)	■■■ (1)
37	sponge	nd	nd	nd	■■		
38	sponge	U/M	nd	nd	■	■■ (1)	
39	sponge	U/M	nd	nd	■■■■	■■■■ (2)	
40	sponge	U/M	C	U/M	■■■■		
41	sponge	U/M	U/M	U/M	■■■	■■ (1)	
42	hard coral	U/M	U/M	U/M	■■■■		■ (6)
43	mollusc	U/M			■■■■		
44	ascidian	U/M	U/M	U/M	■■■■		
45	starfish	U/M	U/M	U/M	■■■■		■■■■ (3)
46	sponge	U/M			■■■	■■ (1)	
47	sponge	U/M	U/M	U/M	■■■■		
48	sponge	U/M	U/M	U/M	■■■■	■■ (1)	■■ (1)
49	sponge	U/M	U/M	U/M	■■		
50	sponge	nd	nd	nd		■■ (1)	
51	ascidian	U/M	nd	nd	■■■		
52	sponge	nd	nd	nd	■	■■■■ (1)	■■■■ (3)
53	sponge	U/M	U/M	U/M	■■	■■ (1)	
54	sponge	nd	nd	nd	■		
55	ascidian	U/M	U/M	U/M	■■■■	■■ (1)	■ (1)
56	ascidian	U/M	U/M	U/M	■■	■■■ (1)	

[a] All extracts are potent inhibitors of the enzyme PPDK (*10*). [b] Type of inhibition of PPDK with respect to Pyr (pyruvate), ATP, and P_i: C, competitive; U/M, uncompetitive or mixed; nd, not determined. [c] O_2 electrode activity categories from percent inhibition values: 0–19, blank; 20–39, ■; 40–59, ■■; 60–79, ■■■; >80, ■■■■. [d] Effect on plant observed within 24 h, ■■■■; 48 h, ■■■; 72 h, ■■; 96 h, ■; no effect left blank. Number in parentheses is phytotoxicity score, where 1 denotes fading of green leaf color; 2, leaf tip shriveled; 3, leaf shriveled over area of application; 4, dead patches on leaf where sample was applied; 5, leaf tip dead; 6, leaf dead over area to which sample was applied.

phytotoxicity to both C_4 test plant species, exhibiting potent activity against C_4 acid cycle driven photosynthesis in leaf slices, and not being a competitive inhibitor of PPDK with respect to any of the three substrates.

The sponge, *Rhopaloeides* sp. (family Spongiidae, order Dictyoceratida, class Demospongiae), was collected by dredging at a depth of 30−42 m from Shelburne Bay off the northeastern coast of Australia.

Bulk frozen material (73.9 g) was lyophilized (dry weight = 13.4 g) and exhaustively extracted with DCM followed by MeOH. The DCM extract (187.4 mg) exhibited PPDK inhibitory activity. Isolation of the active component from this extract was undertaken.

The DCM extract (187.4 mg) was chromatographed on Kieselgel 60G silica gel (8 × 5 cm) and eluted in a step gradient: hexane → DCM → EtOAc → MeOH. Twenty fractions were collected (150 mL each) and assayed. The three active fractions were eluted with a gradient of hexane/DCM (from 9:1 to 8:2) and pooled. Recrystallization from cold MeOH yielded 21.6 mg of a bright orange compound. Structural determination was performed by analysis of the low-resolution positive mode ESI spectrum, ^1H and ^{13}C NMR spectra, and their comparison with literature data.

Mass spectra of the active compound were recorded on a Bruker BioApex 47e FT-ICR mass spectrometer with an electrospray Analytica of Branford source. Ions were detected in positive mode within a mass range of *m*/*z* 200−1000. Direct infusion of the sample (0.2 mg mL^{-1}) was carried out using a Cole Palmer 74900 syringe pump at a flow rate of 80 $\mu L\ h^{-1}$. The low-resolution positive mode ESI mass spectrum contained two major signals at *m*/*z* 739.40 and 381.20. The *m*/*z* 739.40 signal was determined to be the sodiated dimer, $[2M + Na]^+$, of the parent ion M^+, *m*/*z* 358.20. Although the parent ion was not detected, a signal corresponding to the sodiated species, *m*/*z* 381.2 $[M + Na]^+$, was observed.

The NMR spectra were acquired on a Bruker AC 300 spectrometer operating at 300 MHz for ^1H and at 75 MHz for ^{13}C and using $CDCl_3$ (δ 7.27 and 77.0, respectively).

Theoretical Chemical Property Calculations. The chemical structure of the isolated natural product was analyzed using chemometric approaches. The theoretical logarithm of the *n*-octanol/water partition coefficient (log *P*) was calculated using the online ClogP program provided by Daylight Chemical Information Systems, which implements the method of Hansch and Leo (*17*) and the LOGKOW program (*18*) of the Environmental Science Centre of the Syracuse Research Corp. (Syracuse, NY). The numbers of hydrogen bond donors and hydrogen bond acceptors were calculated as described by Lipinski et al. (*19*). The number of rotatable bonds was calculated using the interactive software provided by Molinspiration Cheminformatics (Bratislava, Slovak Republic). This same software was also used to calculate the molecule's polar surface area (PSA) on the basis of method of Ertl et al. (*20*). This calculation was independently conducted using the Marvin software (ver 3.4.2) produced by Chemaxon (Budapest, Hungary).

RESULTS

Effects of Marine Organism Derived Extracts on C_4 Acid Driven Photosynthesis in Leaf Slices. Marine organism derived extracts were redissolved in MeOH and tested for their ability to inhibit C_4 acid driven photosynthesis in leaf slices. MeOH had a minimal effect, with O_2 evolution reducing to 93 ± 18% of control rate (n = 10 experiments).

Twenty-six of the 56 extracts inhibited C_4 acid driven photosynthesis by ⩾80%, whereas only three extracts inhibited C_4 acid driven photosynthesis by <20% (**Table 1**).

Effects of Marine Organism Derived Extracts on *D. ciliaris* and *E. crus-galli*. Sixteen of the 56 extracts exhibited photosynthetic effects on *D. ciliaris* leaves within the first 24 h after sample application (**Figure 1**; **Table 1**). Three of these 16 extracts caused plant tissue death within 24 h of application. Another 16 extracts caused deleterious effects within the 4 day observation period after extract application. The remaining 24 extracts, however, had no measurable effect on the whole plant during the course of the experiments.

Fewer extracts had an effect on *E. crus-galli* leaves, with only five extracts producing an observable effect on the plants within the first 24 h after sample application (**Figure 1**; **Table**

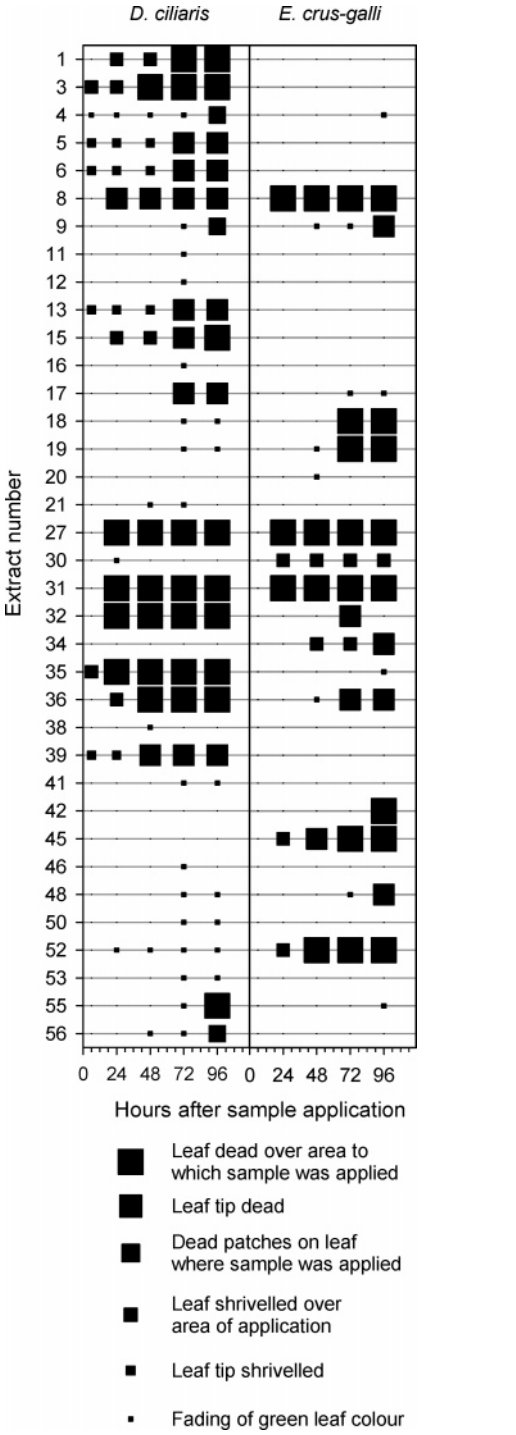
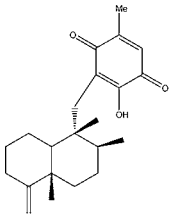


Figure 1. Effects of those extracts that adversely affected the C_4 plants, *D. ciliaris* and *E. crus-galli* (**Table 2**). Observations were made 6, 24, 48, 72, and 96 h after sample application. Increasing symbol size reflects increasing phytotoxic effect on treated plants.

1), and only two of these extracts caused death. Only 14 other extracts had an observable effect on treated plants, leaving 37 extracts not having any observed effect on *E. crus-galli* leaves. Four extracts, however, adversely affected *E. crus-galli* leaves with no observable action on *D. ciliaris* leaves.

In most cases, when a photosynthetic effect was observed on a treated plant, this effect continued for the duration of the experiment with increasing severity (**Figure 1**). Within the first 6 h after application, seven extracts caused an observable effect on *D. ciliaris* leaves, whereas no effect was observed on *E. crus-galli* leaves. Treated plants were able to recover from a



Parameter	Desired Range	Ilimaquinone
Molecular mass	≥150 and ≤500	342.5
ClogP	≤3.5	6.7
No. of hydrogen bond donors	≤3	1
No. of hydrogen bond acceptors	≥2 and ≤12	3
No. of rotatable bonds	≤12	2
PSA (Å^2)	50-60	54.4

Figure 2. Structure of ilimaquinone isolated from extract 8 and calculated theoretical properties

Table 2. Bioactivity Profile for Ilimaquinone and the Extract from Which It Was Purified

bioactivity property	extract 8	ilimaquinone
IC_{50} versus PPDK	not done	292 ± 23 μM
PPDK inhibition mechanism relative to pyruvate	uncompetitive/mixed	uncompetitive/mixed
PPDK inhibition mechanism relative to phosphate	uncompetitive/mixed	uncompetitive/mixed
PPDK inhibition mechanism relative to ATP	uncompetitive/mixed	uncompetitive/mixed
% inhibition of C_4 acid cycle O_2 evolution	87	48
effect on *D. ciliaris* leaves	potency = 6 within 24 h	potency = 6 within 24 h
effect on *E. crus-galli* leaves	potency = 6 within 24 h	potency = 6 within 48 h

weak effect produced by extracts 11, 12, 21, 38, and 46 with respect to *D. ciliaris* and by extract 20 with respect to *E. crus-galli* (**Figure 1**).

Mechanism of PPDK Inhibition. Precipitation occurred for 12 of the extracts when added to the reaction mixture, preventing accurate absorbance measurements. Of the remaining extracts, none exhibited competitive inhibition with respect to pyruvate (**Table 1**). The majority of extracts exhibited mixed/uncompetitive inhibition with respect to pyruvate. A select number of extracts were tested to ascertain the mechanism by which PPDK inhibition occurred with respect to ATP and phosphate. None of these extracts inhibited PPDK by the same mechanism for each of pyruvate, ATP, or phosphate (**Table 1**).

Identification of the PPDK Inhibitory Compound from Extract 8 and Its in Vivo Activity. Bioassay-guided fractionation of extract 8, identified using the integrated screening strategy (PPDK, leaf slice and whole plant assays) as fulfilling all of the necessary criteria (**Table 2**), resulted in the isolation of a PPDK inhibitory compound. Analysis of the ^1H and ^{13}C NMR spectra indicated the compound was a sesquiterpene with a rearranged drimane skeleton. This compound was identified as ilimaquinone (**Figure 2**) by comparison of the NMR and mass spectral data with literature values (*21*).

The IC_{50} of ilimaquinone against PPDK was 292 ± 23 μM. Ilimaquinone was an uncompetitive inhibitor of PPDK with respect to pyruvate and ATP, and mixed competition was observed with regard to phosphate (**Table 2**). O_2 evolution was inhibited by slightly >50% at a final concentration of 146 μM ilimaquinone. Treatment of flag leaves of both C_4 plants with 8 mg mL^{-1} ilimaquinone resulted in leaf death within 24 h of application (**Figure 3**).

Chemical Property Calculations. Ilimaquinone has a ClogP of 6.7 and log K_{OW} of 6.0 (*18*). It possesses a single hydrogen bond donor, three hydrogen bond acceptors, and two rotatable bonds. Both software platforms used resulted in a calculated PSA of 54.4 Å^2 (**Figure 2**).

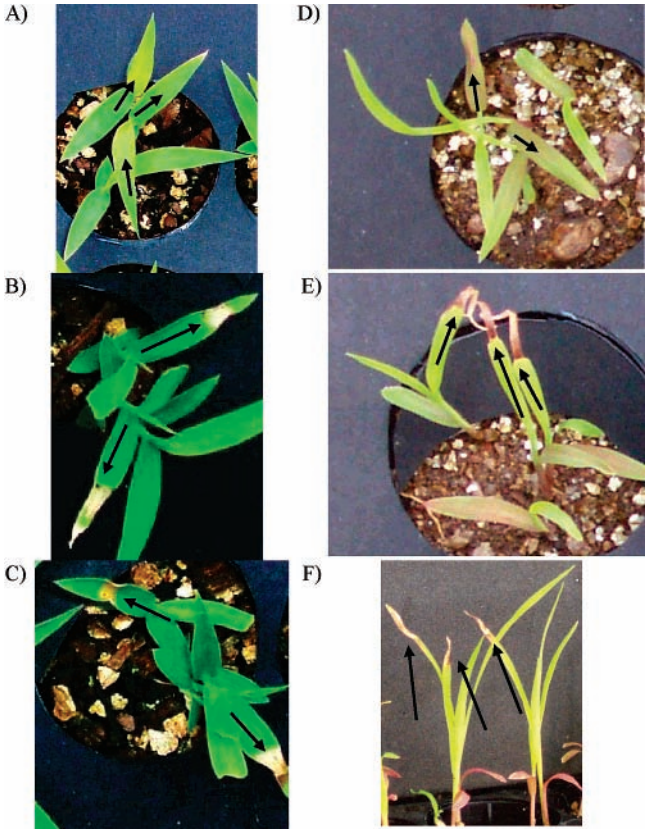


Figure 3. Effect of the crude extract 8 and ilimaquinone on *D. ciliaris* (**A**–**C**) and *E. crus-galli* (**D**–**F**). The formulation in all cases was 0.5% DMSO and 0.08% Uptake in MeOH. Samples applied were formulation only control (**A**, **D**), 8 mg mL^{-1} crude extract 8 (**B**, **E**), and 8 mg mL^{-1} ilimaquinone (**C**, **F**). Sites of sample application are indicated by arrows.

DISCUSSION

In vitro assays identify bioactive compounds that may not necessarily penetrate or translocate within the intact plant. The O_2 electrode assay, where leaf slices are used, removes a major barrier to herbicide penetration while maintaining the integrity of the inter- and intracellular distribution of the C_4 photosynthetic apparatus. This enables inhibitors of a variety of metabolic pathways to elicit their effect while surrounded by the cellular milieu, adding yet another layer of complexity by virtue of additional targets being available for herbicides that may not be absolutely selective for a single site of action. Furthermore, conditions used in enzyme assays are usually designed to ensure experimental requirements imposed by the law of mass action (*16*) for valid mathematical analysis and interpretation. This includes the requirement for the concentration of enzyme to be significantly lower than that of the substrate and/or inhibitor, and this may not reflect the natural situation. In vivo inhibition must occur when the enzyme and substrate concentrations are at naturally occurring levels and with enough potency so that the effect is observable in the whole plant.

This research was designed to identify natural products that killed plants by inhibiting the C_4 acid cycle, specifically the PPDK enzyme, which catalyzes the rate-limiting step. There are no commercially available herbicides that target this pathway. We previously utilized a rapid-throughput enzyme assay to identify marine organism derived extracts that selectively inhibited PPDK (*10*); however, this bioactivity may not necessarily translate to whole plant activity due to a plant's physical and/or biochemical barriers to xenobiotics (*1*, *2*, *8*, *9*). Prior to embarking on the laborious and costly process of isolating a natural product from a crude mixture, we developed an integrated strategy to identify extracts that not only inhibited PPDK but that also exhibited several important properties: inhibition of the C_4 acid cycle photosynthetic process to which PPDK is critical, the propensity to enter intact plant leaves and cause death, and an uncompetitive mode of inhibition with respect to PPDK substrates. With regard to the last point, the mechanism by which a compound inhibits an enzyme, that is, un- or noncompetitive versus competitive inhibition, may be related to the in vivo expression of herbicidal activity (*22*). In this particular case, during periods of shade and darkness, pyruvate accumulates in C_4 leaves. This increased concentration of PPDK substrate may outcompete an inhibitor that acts directly on the pyruvate-binding site; hence, any extract that competitively inhibited PPDK would be of lesser interest. No extract in this study was found to directly compete with either pyruvate or phosphate. Several extracts did, however, competitively inhibit PPDK with respect to ATP.

Penetration of the leaf cuticle is the preferred mechanism of entry for postemergent herbicides (*23*). Once inside the leaf, the plasmodesmata of bundle sheath cells of C_4 plants provide an avenue for chemical passage through plant cell walls with a size exclusion limit of ~900 Da (*24*). The limiting steps in the leaf slice assay are the rate of inhibitor diffusion through the reaction mixture, diffusion rate through the plasmodesmata, the distribution time of the compound throughout the cell, and, finally, the rate of binding to the molecular site of action. By removal of the cuticular barrier, the compounds were seen to elicit their effect within minutes of administration of the extract to the O_2 electrode reaction mixture. If compounds with in vitro activity are unable to penetrate the leaf, the cuticular resistance can be decreased by the use of adjuvants such as surfactants, oils, fertilizers, and pH modifiers (*25*).

Of the 56 extracts tested in the whole plant assays, extracts 19 and 32 caused phytotoxic effects on *E. crus-galli* and *D. ciliaris*, respectively. This may reflect chemical and/or physical differences between these two plant species and hence their sensitivity to potentially phytotoxic compounds. Interestingly, two extracts (4 and 9) that caused leaf death on the whole plant did not inhibit C_4 acid driven photosynthesis in the leaf slices. This indicates they contain compounds that act as broad-spectrum herbicides rather than C_4-selective herbicides. Despite their ability to inhibit PPDK, they did not inhibit C_4 acid driven O_2 evolution, and the link between enzymic inhibition and plant tissue toxicity does not exist. In this instance, it is possible that the PPDK inhibitory compound present in the extract was unable to diffuse through the chloroplast membrane and into the stroma, where the C_4 acid cycle operates.

Of the marine organisms investigated, extracts of three (no. 8, 27, and 31) caused a rapid and potent effect on both C_4 plants used in this study as well as C_4 acid driven O_2 evolution. To further prioritize these extracts, we investigated the pattern of PPDK inhibition with respect to all three of its substrates. None of the three extracts competitively inhibited PPDK with respect to pyruvate. Extract 27 caused precipitation during determination of the kinetic parameters for phosphate and ATP but not for pyruvate. Extract 31 competitively inhibited PPDK with respect to ATP. Because ATP is ubiquitous in biological systems, this eliminated extract 31 from further investigation because of the increased potential of widespread toxicity. Extract 8 did not compete directly with either phosphate or ATP. With due consideration to the view of Gerwick et al. (*22*), extract 8 was identified as the primary candidate for further investigation.

Ilimaquinone, isolated from the sponge *Rhopaloeides* sp., exhibited a bioactivity profile similar to that of its crude extract (extract 8, **Table 2**). It is useful to consider the potential of ilimaquinone as a postemergent, PPDK-selective herbicide in light of Lipinski's "rule of five" for drug candidates (*19*) as applied to postemergent herbicides (*23*). Ilimaquinone satisfies all but one of these criteria (**Figure 2**) as well as another important descriptor used to predict herbicide potential, namely, having a PSA between 50 and 60 $Å^2$ (*26*). To further develop this compound as a postemergent herbicide, structure-based design may be a useful approach in improving its partition coefficient without compromising other parameters. Ilimaquinone belongs to a class of compounds that exhibit a wide range of bioactivity including cytotoxicity to a range of animal cells and antimicrobial and antiviral actions (*27*−*34*). Although it is possible to synthesize ilimaquinone and derivatives, the likelihood of producing a PPDK-selective inhibitor with no cytotoxic activity is remote. Although ilimaquinone's broad-spectrum bioactivity might preclude its further development as a herbicide, inhibition of similar molecular targets in animals may not have the same deleterious effect due to different modes of metabolism and/or excretion (*35*). In fact, herbicidal compounds may be beneficial to mammals such as the triketone inhibitors of 4-hydroxyphenylpyruvate dioxygenase, which exert phytotoxicity by inhibiting carotenoid biosynthesis in plants and tyrosinemia in mammals (*35*).

There have been previous attempts to develop a C_4 plant-selective herbicide by designing structural analogues of C_4 acid cycle enzyme substrates (*36*−*38*). The synthetic compound 3,3-dichloro-2-(dihydroxyphosphinoylmethyl)propenoate was found to specifically inhibit PEPC, but the compound had no effect on intact C_4 plant tissue. During the present study, we found a significant number of marine organism derived extracts containing compounds of unknown concentration and potency that were able to penetrate intact leaves of C_4 plants and cause localized phytotoxicity. This integrated in vitro/in vivo screening strategy led to the purification of ilimaquinone from one of the active extracts. This approach underlies the continued isolation of natural product inhibitors of PPDK in the remaining marine organisms described in this study, which, like ilimaquinone, are potential leads for C_4 weed selective herbicides.

ABBREVIATIONS USED

chl, chlorophyll; ClogP, calculated log *P*; $CDCl_3$, deuterated chloroform; DCM, dichloromethane; EtOAc, ethyl acetate; IC_{50}, concentration that causes 50% inhibition; MeOH, methanol; NAD-MDH, NAD−malate dehydrogenase (EC 1.1.1.37); OD, optical density; PEPC, phosphoenolpyruvate carboxylase (EC 4.1.1.31); PPDK, pyruvate P_i dikinase (EC 2.7.9.1); PSA, polar surface area.

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