MARINE ORGANISMS AS SOURCES OF C_4-WEED-SPECIFIC HERBICIDES

Lyndon E. Llewellyn from the Australian Institute of Marine Sciences and James N. Burnell from the Department of Biochemistry and Molecular Biology, James Cook University (both in Townsville, Queensland, Australia) suggest that compounds from Great Barrier Reef coral reefs could have herbicidal application

Imagine that you are a plant propagule looking for a home. You find a nice warm sunny place where the nutrients wash over you and think that you have arrived in paradise. And, what is more, there are few other plants with which to compete. However there are many animals close by in the form of corals and sponges and a variety of animals which make up the Great Barrier Reef. If you were on land, where plants occupy much of the available surface area over which animals wander, you would seem less out of place. Yet on healthy and pristine coral reefs found on the outer shelf of the Great Barrier Reef off the north east coast of Australia, only 20–28% of the available surface area comprises plants, *i.e.* algae (Sweatman *et al.*, 1998) (Figure 1). Sea grass and kelp beds are more reminiscent of the terrestrial situation. Why is this so? Maybe coral reefs are, in fact, not a good place for plants to grow. But if this were the case, why then would the space be dominated by animals reliant for much of their nutrition upon symbiotic relationships with plants such as are found in corals and their symbiotic unicellular plants, zooxanthellae. What then, keeps the number of free-growing plants low on coral reefs relative to the land? Does chemical warfare by the animals play some role? And if so, can these chemicals be developed for use as herbicide in the terrestrial environment?

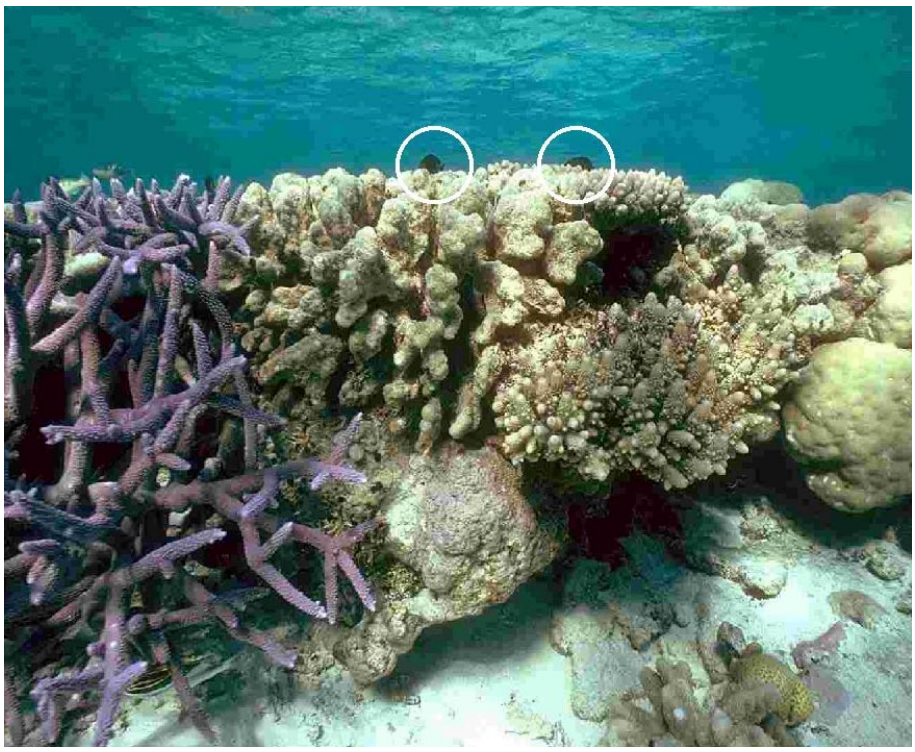

Figure 1. **Typical areas on a healthy and pristine coral reef. Note the dearth of plant life with the only alga evident being the small green tufts of *Chlorodesmis* highlighted with the white circles.**

Marine organisms as sources of potential herbicides

Compared to the search for new pharmaceutical compounds, very little effort has been devoted to the exploration of agrochemical compounds from marine natural products (Fenical, 1997). Nereistoxin, is probably the ocean's only major claim to fame for an agrochemical with it and its analogues (bensultap, cartap, thiocyclam) being used as insecticides in some parts of the world.

One of the major impediments to successful herbicide development is obtaining compounds capable of penetrating the cell membranes of target plants. Coral reef organisms are a likely source of compounds "predesigned", for want of a better word, to pass easily through cell barriers. The limited amount of space available on coral reefs has placed selection pressures on the development of strategies whereby sessile reef organisms produce chemicals detrimental to organisms that may compete for essential resources. If an animal secretes a chemical targeted against a plant it must be able to penetrate the cell membrane of the target plant to be effective. These compounds must also be effective at low concentration because of their immediate dilution in the water column between the sessile marine animal and target organism.

Once inside the plant cell, what metabolic pathways are the best target for these compounds?

Carbon fixation in plants

Rubisco (ribulose bisphosphate carboxylase/oxygenase) is one of the world's most abundant enzymes, being present in all plants and responsible for the incorporation of inorganic carbon into carbohydrates. Rubisco, however, is an inefficient enzyme in that it not only fixes CO_2 but also oxygen. When oxygen is incorporated into organic compounds in place of CO_2 plants expend energy to

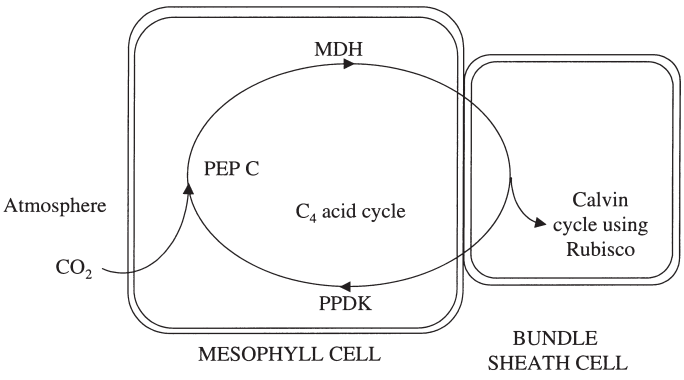

Figure 2. **The C4 acid cycle showing the physical separation of Rubisco and the Calvin cycle from the atmosphere and the cellular location of the enzymes involved in C4 photosynthesis, MDH - malate dehydrogenase; PEP C, PEP carboxylase; PPDK, pyruvate P_i dikinase.**

recover the carbon from the compounds formed in a process known as photorespiration. This process is energetically wasteful. The predominance of oxygen over CO_2 in the atmosphere and the fact that oxygen is released during the light reactions of photosynthesis ensures that photorespiration takes place in many plants. However, not all plants are the same. Plants can be divided into three main groups depending on how they fix inorganic carbon into sugars. These three groups are the C_3 plants, the C_4 plants and the crassulacean acid metabolism (CAM) plants; CAM plants will not be discussed further. C_3 plants fix CO_2 into sugars using Rubisco and are photosynthetically inefficient due to photorespiration. C_4 plants have evolved two mechanisms that serve to eliminate photorespiration (Ashton *et al.*, 1990). These plants have evolved a distinct leaf anatomy and developed an additional biochemical pathway (the C_4 acid pathway) (Figure 2) that acts as a biochemical appendage to the C_3 pathway. The unique leaf anatomy and the additional biochemical pathway has allowed C_4 plants to eliminate the deleterious effects of photorespiration. They have achieved this by using a different enzyme initially to fix inorganic carbon into organic molecules. This enzyme, PEP carboxylase, uses bicarbonate as its inorganic carbon substrate rather than CO_2 thus eliminating competition between the inorganic carbon substrate and oxygen. Also, they have essentially physically separated the location of the oxygen-producing light reaction and Rubisco. So by initially fixing inorganic carbon in the form of bicarbonate and then releasing the CO_2 in a low oxygen environment, the C_4 acid cycle serves to pump CO_2 into an inner ring of cells where Rubisco operates to fix the CO_2 efficiently without the wasteful reactions of photorespiration. Because C_4 plants fix carbon so efficiently, they require less rubisco and, therefore, have to invest less nitrogen in this essential protein. Compared with C_3 plants, not only are C_4 plants more efficient at fixing CO_2 but they are also more efficient in their use of both water and nitrogen. However, this efficiency comes at a cost in that C_4 plants require more light energy per molecule of CO_2 fixed and so C_4 plants require high light conditions to grow successfully. One of the enzymes critical to the C_4 photosynthetic pathway, pyruvate orthophosphate dikinase (PPDK) is generally recognised as being the rate-limiting enzyme in C_4 photosynthesis. This is supported by experiments that showed that C_4 plants in which the level of PPDK produced was decreased by antisense technology could only survive if the transformed plants were grown in high CO_2 conditions to compensate for the inactivity of PPDK (Maroco *et al.*, 1998). PPDK, however, is a temperature sensitive enzyme, with the subunits dissociating at low temperatures. The dual requirements of high light and warm temperatures restricts C_4 plants to the tropics or to growing in the warmer months in temperate regions.

Agriculturally speaking, some of the world's most important crops are C_3 plants, namely wheat, rice, oats and barley. Only maize, sugar cane, sorghum and millet are economically important C_4 crops. In warm regions where C_4 plants can flourish, they outcompete C_3 plants, becoming weeds within the C_3 crops. Some examples of economically important C_4 weeds are nutgrass or purple nut sedge (*Cyperus rotundus*), couch or Bermuda grass (*Cynodon dactylon*), barnyard grass (*Echinochloa spp.*), Johnson grass (*Sorghum halepense*) and Goose grass (*Eleusine indica*). In fact, Cyperus rotundus is one of the world's worst weeds, being recognised as a pest in more than 100 countries, affecting more than 50 crop species. To further highlight the problem, it should be noted that of the world's top 18 weeds, 14 of them are C_4 plants (Table 1).

Focusing the herbicide search

So, combining the above information and rationales, coral reef organisms were targeted as a potential source of chemicals to provide lead compounds for the development of herbicides. Specifically, activity against C_4 plants was targeted in the identification of potential herbicidal compounds since a compound which selectively blocks the

Table 1. The world's worst weeds and whether they are C_3 or C_4 plants (Holm *et al.*, 1977).

Species	C_4 or C_3
Cyperus rotundus	C_4
Cynodon dactylon	C_4
Echinocloa crusgalli	C_4
Echinochloa colonum	C_4
Eleusine indica	C_4
Sorghum halepense	C_4
Imperata cylindrica	C_4
Eichornia crassipes	C_3
Portulaca oleracea	C_4
Chenopodium album	C_3
Digitaria sanguinalis	C_4
Convolvulus arvensis	C_3
Avena fatua	C_3
Amaranthus hybridus	C_4
Amaranthus spinosus	C_4
Cyperus esculentus	C_4
Paspalum conjugatum	C_4
Rottboellia exaltata	C_4

Table 2. Taxonomic composition of marine macro-organism samples screened which caused >80% inhibition in screening assay and which were then found to be PPDK selective

Taxonomic group	no. screened	no. causing >80% inhibition	no. selective for PPDK
Green Algae	113	3	0
Red Algae	116	1	0
Brown Algae	66	0	0
Cyanophytes	10	0	0
Mangrove trees & sea grasses	21	5	0
Hard & soft corals, anemones	885	17	2
Sea squirts	654	34	3
Sponges	1472	212	19
Starfish, brittle stars, sea cucumbers, sea urchins	668	33	4
Molluscs	421	13	0
Diatoms	10	1	1
Minor phyla such as echiurans, sipunculids & brachiopods	18	0	0
Annelid worms and flatworms	104	0	0
Bryozoans (lace coral)	181	0	0
Crustaceans	284	0	0
Total	5023	319	29

C_4 acid cycle could be used amongst C_3 crops without the need to engineer genetically resistant crop plants. Aquatic and marine organisms possessing enzymes of the C_4 acid pathway have been reported while, to date, only the C_3 photosynthetic pathway has been described in organisms involved in a symbiotic relationship. Therefore, a marine animal in a symbiotic relationship with a C_3 microorganism would benefit if it were able to produce a compound which inhibited the growth of C_4 plants with the potential to overgrow and, therefore, shade the animal.

The validation of PPDK as a critical enzyme for the growth of C_4 plants and its almost exclusive production in C_4 plants make it a highly attractive target for development of a C_4 plant specific herbicide with little impact not only on other plants, but also animals. The absence of PPDK from both vertebrates and invertebrates reduces the likelihood of PPDK active compounds having adverse toxicological or environmental impacts.

Our research is not the first such attempt to develop a C_4 specific herbicide. In the 1980s, the Division of Plant Industry at the Australian CSIRO attempted to develop a C_4 specific herbicide by synthesising analogues of substrates, products and effectors (Jenkins *et al*., 1987; McFadden *et al*., 1987) of enzymes of the C_4 acid cycle. A single compound which exhibited a high degree of specificity towards C_4 PEP carboxylase in an *in vitro* assay was developed but this compound was ineffective when applied to the leaves of C_4 plants.

How the search is conducted

A simple spectrophotometric assay which allows the rapid screening of three C_4 acid cycle enzymes simultaneously, including PPDK, with a large number of compounds has been developed and a patent application to protect the assay has been filed. It is not uncommon when screening natural product extracts, however, to detect compounds with general degradative effects on proteins, being biologically reactive rather than just active. By innovative manipulation of this biomolecular assay, we can identify extracts and compounds that selectively inhibit the individual enzymes in the assay and eliminate these poor quality hits. We then test extracts and compounds in a seedling assay conducted in 96 well plates using both C_4 and C_3 plants. By this means, we can measure whether lead extracts and compounds can be taken up via the roots and translocated to the foliage or whether they can act as pre-emergent herbicides. Coupling this with our ability to measure the effect of extracts and compounds selectively upon C_4 photosynthesis in leaf slices directly, we are able to demonstrate the link between herbicidal activity and its biomolecular activity (Table 2).

Subsequent testing of extracts which were selective inhibitors of PPDK *in vitro* revealed that application to the roots of plants also exhibited selective herbicidal activity towards C_4 plants. C_4 plants such as Bermuda grass, *Urochloa mozambicensis* and *Digitaria ciliaris* were killed with little or no effect on C_3 plants such as rye grass or barley even when tested on C_3 plants at 10 times the concentration used on C_4 plants. This supported our contention that bioactive compounds from marine organisms may be predesigned to enable them to pass through cell membranes and cell walls. From these extracts several pure compounds have now been isolated and their structures elucidated. In addition we have also identified compounds that exhibit C_4 plant selective phytotoxic activity at concentrations far below that normally used for compounds such as glyphosate.

To test the likelihood of the C_4 selective herbicidal compounds producing side effects, extracts were tested against a variety of enzymes that use either substrates or products used by PPDK. These included pyruvate carboxylase, pyruvate kinase, lactate dehydrogenase and

PEP carboxykinase. None of these enzymes was inhibited by compounds shown to be active against PPDK even at ten-fold higher concentrations.

Interestingly, enzyme kinetic studies with compounds and extracts shown to inhibit PPDK selectively demonstrated that PPDK was inhibited by a variety of mechanisms, namely uncompetitive, non-competitive and competitive inhibition.

The future

Natural products have been the basis for over 30 agrochemicals (Copping, 1998) ranging from abamectin which is used as an insecticide, the fungicide blasticidin and the herbicide bilanafos. There is a perception however that the often complex structure of natural products make them difficult to develop as agrochemicals as they are less amenable to large scale process chemistry and, therefore, bulk manufacture. Constantly improving synthetic methods necessitate regular reappraisal of this attitude. It may be possible that substructures of lead compounds may be all that is necessary for agrochemical activity. This may lead to a simpler manufacturing process thus making a previously non-viable product commercially viable. Compounds already found in this study represent a structurally diverse battery of lead compounds that may result in the successful development of a herbicide selective for C_4 weeds in C_3 crops. And what makes this research even more exciting, is that we have only screened 5000 extracts and have another 5000 waiting to be tested. In addition, there are many more species yet to be collected from both the Great Barrier Reef and other coral reef systems to be tested in this and other herbicide discovery projects. We also possess a collection of over 8000 microbial isolates that we are presently extracting for testing for C_4 plant selective phytotoxins.

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