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Phospholipase A_2 inhibitors from marine algae

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Abstract

Twelve out of twenty-nine compounds isolated from benthic marine algae from the phyla Chlorophyta, Phaeophyta and Rhodophyta have been found to be potent inhibitors of bee venom derived phospholipase A_2 (PLA_2) (>50%) in the μM range. The compounds investigated were from: *Bryopsis pennata, Rhipocephalus phoenix, Caulerpa prolifera, C. racemosa, C. bikinensis, Cymopolia barbata, Laurencia cf. palisada, Laurencia sp., Ochtodes crockeri, Liagora farinosa, Sphaerococcus coronipifolius, Phacelocarpus labillardieri, Dictyota sp., Bifurcaria galapagensis, Stypopodium zonale, Dictyopteris undulata, Stoechospermum marginatum, Dictyopteris divaricata, Dilophus fasciola* and *Dilophus sp.* This is the first report of bee venom PLA_2 inhibition *in vitro* by pure compounds isolated from marine algae.

Introduction

Phospholipases A_2 [PLA_2] are ubiquitous lipolytic cellular enzymes found in all cell types, as well as bacteria and protozoa, that specifically hydrolyze the 2-acyl ester bond of 1,2-diacyl- sn-3 glycerophosphatides. These enzymes play a major role in both phospholipid turnover and the regulatory mechanisms controlling inflammatory responses, since the release of arachidonic acid by PLA_2 and its subsequent metabolism by the cyclooxygenase or the 5-lipoxygenase enzyme systems leads to the synthesis of the bioactive eicosanoids (prostaglandins, thromboxanes and leukotrienes) (Flower & Blackwell, 1976). The contribution of PLA_2 enzymatic activity to the pathogenesis of human disease has been extensively reviewed (Vadas & Pruzanski, 1986). Enhanced levels of extracellular PLA_2 activity have been observed in septic shock (Vadas *et al.*, 1988), and inflammatory diseases such as pancreatitis, psoriasis and rheumatoid arthritis (Gonzalez-Butirica *et al.*, 1989). After PLA_2 is activated it could mediate pathophysiological processes both by a direct action or indirectly through subsequent transformation of its products, lysophospholipids and arachidonic acid, to eicosanoids (prostaglandins and leukotrienes) and platelet-activating factor (Dennis *et al.*, 1991). These observations provide a strong rationale for the no-

tion that inhibitors of this enzyme could provide novel therapies to alleviate the above mentioned diseases.

At present there is considerable research in developing potent inhibitors of PLA_2 (Mayer *et al.*, 1988) and a number of compounds which represent a diversity of structural types have been reported to inhibit PLA_2 (Chang *et al.*, 1987). These compounds may be broadly described as agents that affect substrate-enzyme interface, agents that modulate cellular calcium levels, non-steroidal anti-inflammatory compounds, natural products, covalent binding agents, substrate or product analogs and compounds derived from screening. Most of these agents are limited in their *in vitro* potency and *in vivo* pharmacological activity (Chang *et al.*, 1987; Glaser & Jacobs, 1986). A few years ago, a survey of the biomedical potential of marine natural products derived from marine invertebrates resulted in the isolation of a novel nonsteroidal sesterterpenoid, manoalide, from the marine sponge *Luffariella variabilis* (deSilva & Scheuer, 1980). Manoalide was subsequently shown to be a potent irreversible inhibitor of bee venom PLA_2 ($IC_{50} = 0.05\ \mu M$) (Glaser & Jacobs, 1986; de Freitas *et al.*, 1984) and human synovial fluid PLA_2 ($IC_{50} = 0.2\ \mu M$) (Jacobson *et al.*, 1990).

Recently, the anti-inflammatory survey was extended to include pure compounds isolated from selected marine algae belonging to the phyla Chlorophyta, Phaeophyta and Rhodophyta as possible sources of extracellular bee-venom PLA_2 inhibitors.

Materials and methods

Isolation of algal metabolites

A variety of compounds (1-29) isolated from 21 species marine algae were used in this study (Figs 1 and 2). All compounds were isolated and purified by standard methods, and their structures verified by NMR and other spectroscopic methods. Each of the compounds, with the exception of compounds 1, 18, 19 and 24, were isolated and described in prior chemical publications. The following references elucidate and describe the structures of metabolites 1-29: 1: Paul & Fenical (1991, personal communication); 2: Sun & Fenical (1979a); 3: Amico *et al.* (1978); 4: Paul & Fenical (1982); 5-6: Hogberg *et al.* (1976); 7: Paul & Fenical (1980a); 8: Shin *et al.* (1986); 9: Fenical *et al.* (1976); 10: Gerwick *et al.* (1979); 11-12: Fenical *et al.* (1973); 13: Sun & Fenical (1979b); 14: Blackman & Wells (1978); 15-17: Paul & Fenical (1980b); 18-19: Capon *et al.* (1981 a,b); 20: Paul & Fenical (1980c); 21: Shin *et al.* (1986); 22: Sun & Fenical (1979b); 23: Gerwick *et al.* (1979); Gerwick & Fenical (1981b); 24: Fenical (personal communication); 25: Suzuki *et al.* (1981) and Suzuki & Kurosawa (1981); 26: Amico *et al.* (1978); 27: Sun *et al.* (1977); 28: Gerwick *et al.* (1981) and Gerwick & Fenical (1981); 29: Sun *et al.* (1980). See Faulkner *et al.* (1984a-b; 1986; 1987; 1988; 1990) for additional references for isolation of compounds.

Phospholipase A_2 assay

The PLA_2 assay currently used in our laboratory has been previously described in detail (de Freitas *et al.*, 1984; Glaser & Jacobs, 1986). Briefly, mixed micelles of 1.35 mM dipalmitoyl phosphatidylcholine, 2.76 mM Triton X-100, and 0.5 μCi of 2-alpha-palmitoyl (2-palmitoyl-9,10-^{3}H) phosphatidylcholine (specific activity 57 mCi $mmol^{-1}$) were prepared in 10 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid, (HEPES) 1 mM $CaCl_2$, pH 7.4 at 41 °C. Bee venom phospholipase A_2 (Boehringer Mannheim, W. Germany) (5 μl of a 50 unit ml^{-1} solution) which had been pre-incubated with a test compound or vehicle (DMSO or MeOH) for 1 hr at 41 °C was added to 0.5 ml of substrate (100-fold dilution of test compound/enzyme complex, final enzyme concentration 1 unit ml^{-1}), incubated for 15 s at 41 °C and extracted as described previously (Glaser & Jacobs, 1986). Released (^{3}H) palmitic acid (heptane phase) was added to 3.0 ml Ecosint (National Diagnostics) and counted in a LKB Rackbeta 1219 liquid scintillation counter. Concentrations of tested compounds are given both as the concentration in the preincubation mixture μM) as well as the concentration in the assay

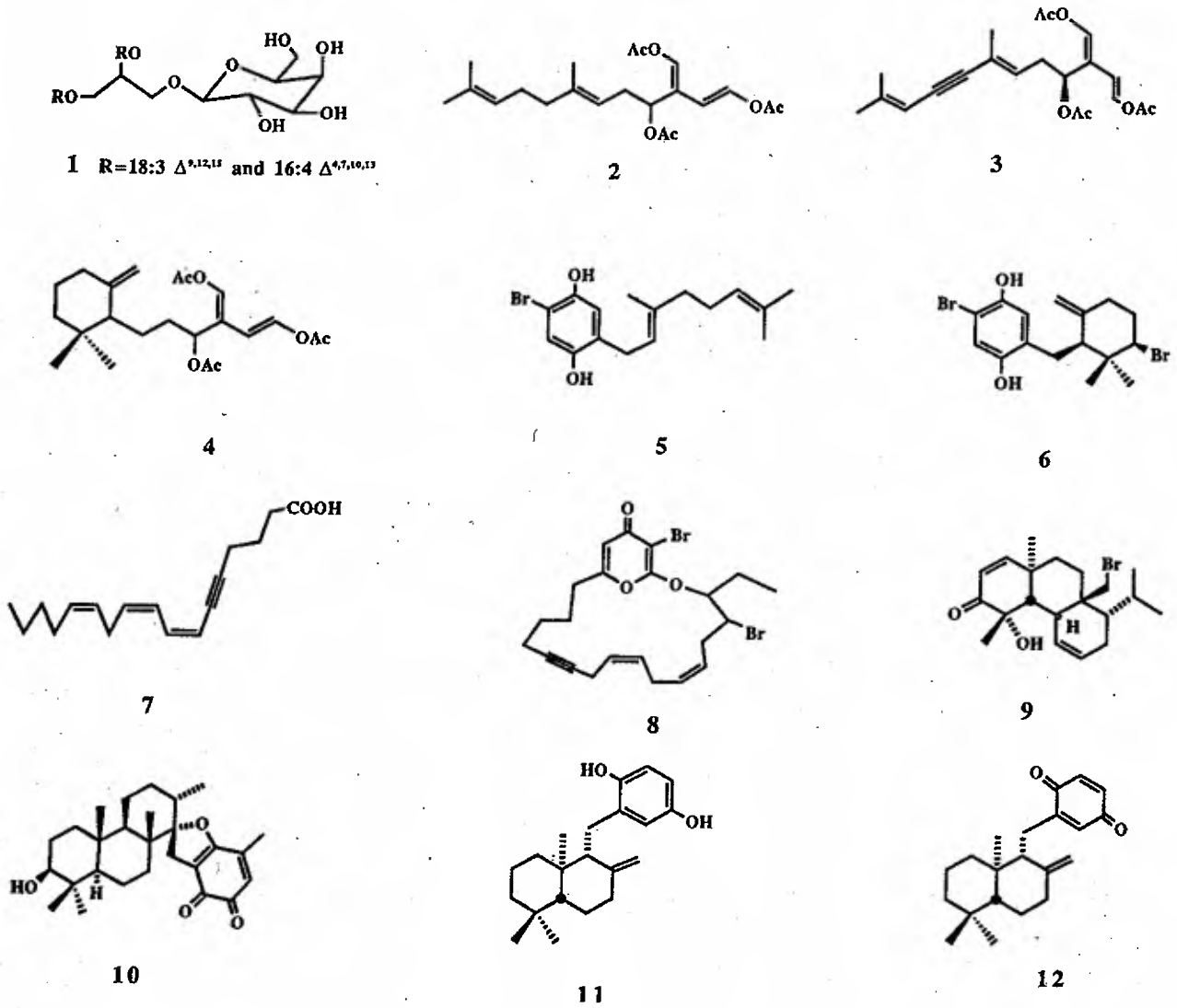
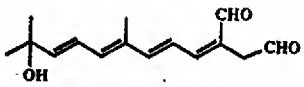


Fig. 1. Algal metabolites that showed greater than 50% inhibition of bee venom phospholipase A^2.

mixture (μM) (Table 1 and 2). The percent inhibition of phosphatidylcholine hydrolysis was determined for each compound, using manoalide (0.25 μm) as a control (100% inhibition of PLA_2) in all the experiments.

Results

Tables 1 and 2 show the degree of inhibition of bee venom PLA_2 observed with the 29 compounds tested. All the compounds reported in Table 1 showed greater than 50% inhibition of bee venom PLA_2. Of these compounds exhibiting active inhibition, 5 were Chlorophyta (*Bryopsis pennata, Rhipocephalus phoenix, Caulerpa prolifera, C. bikinesis* and *Cympolia barbata*), 2 Phaeophyta (*Stypopodium zonale* and *Dictyopteris undulata*), and 3 Rhodophyta (*Liagora farinosa, Phacelocarpus libillardieri,* and *Sphaerococcus coronipifolius*). Seven compounds showed greater than 90% inhibition of PLA_2 within the μM range: Rhiphocephalin (2), a linear sesquiterpenoid from the green alga *Rhipocephalus phoenix* (99% inhi-



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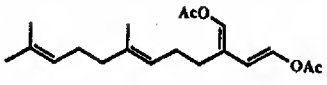

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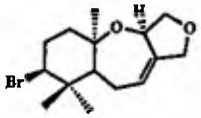

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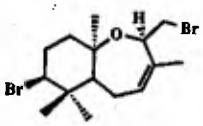

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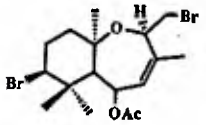

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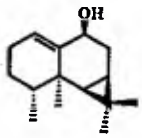

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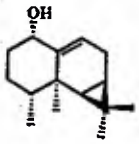

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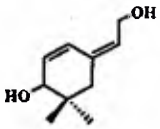

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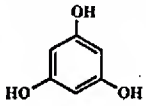

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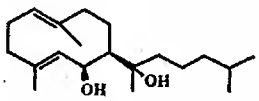

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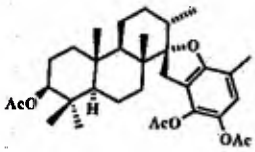

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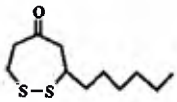

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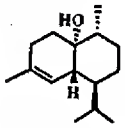

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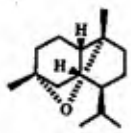

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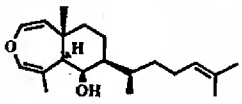

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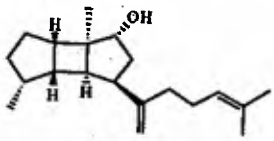

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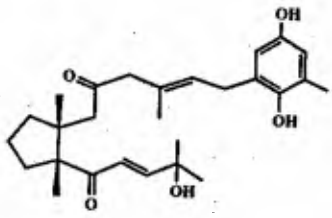

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Fig. 2. Algal metabolites that showed less than 50% inhibition of bee venom phospholipase A_2.

Table 1. Inhibition of bee venom PLA_2 by algal metabolites: active compounds[a]

Species	Compound #	MW[b]	μM[c]	μM[d]	% inhibition[e]
CHLOROPHYTA					
Bryopsidales					
Udotaceae					
Bryopsis pennata Lamouroux	1	494	320	3.2	64
Codiaceae					
Rhipocephalus phoenix (Ellis et Solander) Kuetzing	2	378	410	4.1	99
Caulerpaceae					
Caulerpa prolifera (Forsskål) Lamouroux	3	374	420	4.2	92
Caulerpa bikinensis W. Taylor	4	320	490	4.9	63
Dasycladales					
Dasycladaceae					
Cymopolia barbata (Linnaeus) Lamouroux	5	335	470	4.7	98
Cymopolia barbata (Linnaeus) Lamouroux	6	462	340	3.4	98
RHODOPHYTA					
Nemaliales					
Liagoraceae					
Liagora farinosa Lamouroux	7	274	570	5.7	100
Gigartinales					
Sphaerococcaceae					
Phacelocarpus labillardieri (Mertens) J. Agardh	8	356	440	4.4	93
Sphaerococcus coronopifolius Stackhouse	9	381	410	4.1	69
PHAEOPHYTA					
Dictyotales					
Dictyotaceae					
Stypopodium zonale (Lamouroux) Papenfuss	10	426	370	3.7	100
Dictyopteris undulata Holmes	11	314	500	5	52
Dictyopteris undulata Holmes	12	312	500	5	76

[a]. Greater than 50% inhibition of PLA_2. [b]. Molecular weights of compounds assayed. [c]. Concentration of test compound with PLA_2 in the preincubation mixture. [d]. Concentration of compound with substrate in the assay mixture (100-fold dilution of test compound/enzyme complex). [e]. Percent inhibition of hydrolysis of phosphatidylcholine (manoalide at 0.25 μM used as control for 100% inhibition).

bition; 4.1 μM); caulerpenyne (3), a monocyclic sesquiterpenoid from *Caulerpa prolifera* (92% inhibition; 4.2 μM); two prenylated bromohydroquinones, cymopol (5) and cyclocymopol (6) from *Cymopolia barbata* (98% inhibition; 4.7 and 3.4 μM respectively); an acetylene containing fatty acid derivative (7) from *Liagora farinosa* (100% inhibition; 5.7 μM); a macrocylic enol-ether (8) from *Phacelocarpus labillardieri* (93% inhibition; 4.4 μM); and stypoldione (10), an orthoquinone from *Stypopodium zonale* (100% inhibition; 3.7 μM). Those compounds that showed less than 50% inhibition of bee venom PLA_2 were considered inactive in this investigation (Table 2).

Discussion

Numerous structurally diverse compounds have been shown to inhibit PLA_2 via different mechanisms, but unfortunately most of these are limited in their *in vitro* potency and *in vivo* pharmacological activities (Chang *et al.*, 1987). As a consequence, there is a continued search for new com-

Table 2. Inhibition of bee venom PLA_2 by algal metabolites: inactive compounds[a]

Species	Compound #	MW[b]	μM[c]	μM[d]	% inhibition[e]
CHLOROPHYTA					
Bryopsidales					
Udotaceae					
Rhipocephalus phoenix (Ellis et Solander) Kuetzing	13	248	635	6.3	20
Caulerpaceae					
Caulerpa racemosa (Forsskål) J. Agardh	14	398	390	3.9	26
RHODOPHYTA					
Ceramiales					
Rhodomelaceae					
Laurencia cf. *palisada* Yamada	15	314	501	5.0	1
Laurencia cf. *palisada* Yamada	16	378	416	4.1	0
Laurencia cf. *palisada* Yamada	17	436	361	3.6	6
Laurencia sp.	18	220	716	7.1	27
Laurencia sp.	19	220	716	7.1	10
Gigartinales					
Rhizophyllidaceae					
Ochtodes crockeri Setchell et Gardner	20	168	937	9.4	26
Sphaerococcaceae					
Phacelocarpus labillardieri (Mertens) J. Agardh	21	126	1250	12.50	26
PHAEOPHYTA					
Dictyotales					
Dictyotaceae					
Dictyota sp.	22	306	514	5.1	41
Stypopodium zonale (Lamouroux) Papenfuss	23	552	280	2.8	7
Dictyopteris sp.	24	232	678	6.8	29
Dictyopteris divaricata (Okamura) Nagai	25	222	709	7.1	31
Dilophus fasciola (Roth) Howe	26	222	709	7.1	0
Dilophus sp.	27	304	520	5.2	32
Stoechospermum marginatum (C. Agardh) Kuetzing	28	288	550	5.5	10
Fucales					
Cystoseiraceae					
Bifurcaria galapagensis (Piccone et Grunow) Womersley	29	442	356	3.5	35

[a]. Less than 50% inhibition of PLA_2. [b]. Molecular weights of compounds assayed. [c]. Concentration of test compound with PLA_2 in the preincubation mixture. [d]. Concentration of compound with substrate in the assay mixture (100-fold dilution of test compound/enzyme complex). [e]. Percent inhibition of hydrolysis of phosphatidylcholine (manoalide at 0.25 μM used as control for 100% inhibition).

pounds, both by screening natural sources and by design and synthesis to discover compounds that could alleviate human diseases in which phospholipases have been implicated (Ripka *et al.*, 1989).

As part of an extensive and systematic survey designed to uncover specific and potent PLA_2 inhibitors from tropical and temperate marine organisms, several novel PLA_2 inhibitors have been isolated from marine invertebrates (de Freitas *et al.*, 1984; Kernan *et al.*, 1989) and measurable PLA_2 activity was found in an unusual red alga, *Rhodogorgon* (Burgess & Jacobs, in: Norris & Bucher, 1989). We now extend these findings by reporting that marine algae from three divisions produce structurally diverse compounds which

inhibit bee venom PLA_2 (Tables 1 and 2). In all, compounds from 6 species of Chlorophyta representing 4 families, 9 Phaeophyta of 2 families, and 6 Rhodophyta of 3 families were studied. In particular, we have identified 12 compounds which showed greater than 90% inhibition of bee venom PLA_2 in the μM range. In general, the active compounds are those which have shown bioactivities in several other assays, such as the bisenol ethers 2-4, which are examples of green algal metabolites known to possess antibiotic and cytotoxic effects (Paul & Fenical, 1987; Hay & Fenical, 1988) and the hydroquinones 5, 6 and 11, and the quinones 10 and 12 which are strong fish antifeedants and possess cytotoxic effects (Hay & Fenical, 1988; Hay *et al.*, 1988). Given these observations, it is difficult to understand the weaker activities of metabolites 14, 21 and 29 (see Fig. 2 and Table 2), each of which possess similar functional groups. On the basis of the results obtained, it must be concluded that predicting enzyme inhibition is difficult if not impossible. In *in vitro* testing, enzyme deactivation could occur by numerous mechanisms involving different binding sites and functionalities. Hence, many structural factors could contribute to overall enzyme deactivation. Further research will determine if these compounds inhibit other PLA_2 enzymes, in particular mammalian types, with the same potency, thus providing preliminary evidence of possible *in vivo* pharmacological activity.

In the marine algae, the biosynthesis and distribution of arachidonic acid (Khotimchenko *et al.*, 1991) as well as the production of eicosanoids (Burgess *et al.*, 1991) have been the focus of increased research in recent years. Furthermore, in the Rhodophyta, enzymatic capacity to metabolize polyunsaturated fatty acids in a way analogous to the lipoxygenase pathway in mammalian systems has been recently reported (Bernart & Gerwick, 1988; Burgess & Jacobs, in: Norris & Bucher, 1989). It has also been shown that several of the algal eicosanoids are identical to 12-lipoxygenase products of human metabolism; however, their biological functions in the physiology of the algae are still unknown (Moghaddam *et al.*, 1989). A hypothesis has recently been proposed that eicosanoids may play a role in the osmoregulation, fertilization, growth and tissue development in marine algae (Gerwick *et al.*, 1990). However, in marine invertebrates, the eicosanoids have been shown to mediate a number of biologically important functions such as regulation of ion flux, hatching, reproduction and cell aggregation (Mayer & Jacobs, 1988). Thus, at this, time proposing a physiological role for compounds that inhibit PLA_2 in marine algae must await further research on the biological role of eicosanoids and other bioactive fatty acids in this life form.

Acknowledgements

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