JOURNAL OF NATURAL PRODUCTS

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J. Nat. Prod., **1992**, 55 (12), 1701-1717• DOI: 10.1021/np50090a001 • Publication Date (Web): 01 July 2004

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Journal of Natural Products
Vol. 55, No. 12, pp. 1701-1717, December 1992

PHOSPHOLIPASE A_2 INHIBITORS FROM MARINE ORGANISMS[1]

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ABSTRACT.—This review of phospholipase A_2 (PLA_2) inhibitors from marine organisms presents a compilation of research in this field over the past decade. As an introduction to the research on marine natural products, there is an overview of the role of PLA_2 in inflammation that provides a rationale for seeking inhibitors of PLA_2 as anti-inflammatory agents. A radiometric assay to measure inhibition of bee venom PLA_2 is described in detail. Examples of marine natural products that inhibit PLA_2 are manoalide and its derivatives, scalaradial and related compounds, the pseudopterosins, the vidalols, and a group of terpenoids that contain masked 1,4-dicarbonyl moieties.

Few human ailments are as painful as those involving inflammation. Arthritis, gout, psoriasis, bee stings, and many chemically induced edemas are all examples of inflammatory conditions that result in considerable pain and swelling of the affected tissues. The inflammatory response is mediated by the biosynthesis of eicosanoids, such as leukotrienes, prostaglandins, and thromboxanes, from arachidonic acid, as well as other autacoids released locally in response to an irritant. Phospholipase A_2 (PLA_2) is an enzyme that specifically catalyzes the hydrolysis of the ester at the *sn*-2 position of a phospholipid to produce a lysophospholipid and a free fatty acid (Figure 1). The release of arachidonic acid from the *sn*-2 position of membrane phospholipids provides the substrate for the biosynthesis of eicosanoids. Thus, compounds that inhibit PLA_2 activity have been targeted as potential therapeutic agents in the treatment of inflammation.

This review presents an account of some research directed toward the discovery of new anti-inflammatory agents from marine organisms. The Marine Chemistry and Pharmacology Program at the University of California has employed a variety of cell and

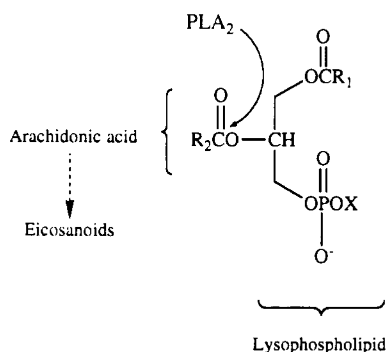


FIGURE 1. Hydrolysis of a phospholipid by PLA_2 occurs at the *sn*-2 position.

[1]An invited review in the series on mechanism-based studies of natural products in drug discovery.

biochemical models to determine the anti-inflammatory properties of several classes of marine natural products. These include an in vitro assay that measures inhibition of bee venom PLA_2 activity and an in vivo assay that measures reduction of chemically induced edema in the mouse ear (1) and reductions in eicosanoid biosynthesis (2). Some of the compounds that were found to be anti-inflammatory were also found to inhibit PLA_2 activity.

THE ROLE OF PLA_2 IN INFLAMMATION.—The inflammatory response is symptomatically characterized by erythema, edema, heat, pain, and tenderness. The symptoms are a manifestation of events occurring at the cellular level, which are in turn chemically modulated (for an overview see Figure 2). A variety of chemicals that modulate inflammation, including eicosanoids, are released by macrophages and neutrophils that migrate to the site of irritation. These autacoids cause a wide variety of physiological responses. The most potent chemotactic factors identified thus far are products of the 5-lipoxygenase pathway, the leukotrienes. The release of leukotrienes and histamine causes

FIGURE 2. An overview of the inflammatory cascade.

the permeability of local venules and capillaries to be increased, and as a result, fluid is leaked into the interstitial spaces, causing edema (3). The vasodilatory effects of certain prostaglandins promote erythema, and the release of thromboxane A_2 triggers platelet aggregation (4). In addition, lytic enzymes may be released into the inflamed site as a result of the disruption of cellular lysosomal membranes. In inflammatory diseases such as rheumatoid arthritis, these lysosomal enzymes cause local tissue damage, increasing the level of inflammation (3).

The prostaglandins, leukotrienes, and thromboxanes are biosynthetic oxidation products of arachidonic acid metabolism. The pathways of eicosanoid biosynthesis are collectively known as the "arachidonic acid cascade" (5). Because eicosanoids are potent mediators of inflammation, selective inhibition of pathways within the arachidonic acid cascade can be expected to modify the inflammatory response (4). For example, indomethacin and the salicylates (aspirin) inhibit the cyclo-oxygenase pathway responsible for converting arachidonic acid into prostaglandins (6).

The increased production of eiconsanoids in response to a variety of stimuli is believed to be controlled by the release of their precursor, arachidonic acid (4, 7–9). Membrane-bound phospholipids are rich in arachidonic acid, but the concentration of free arachidonic acid is low (5,7). In response to a variety of chemical stimuli, arachidonic acid is released from the phospholipids, providing substrate for eicosanoid synthesis (4, 8). Because arachidonic acid is primarily stored in the *sn*-2 position of membrane phospholipids, PLA_2 has been implicated as an important rate-limiting enzyme participating in its release (7–10), although the combined actions of other lipases also have the potential to generate arachidonic acid [for a review, see Irvine (7)]. The lysophospholipid byproduct of phospholipid hydrolysis by PLA_2 is the precursor to yet another inflammatory agent, platelet activating factor (PAF) (8,11). The presence of elevated levels of PLA_2 in inflamed tissues and in a variety of experimental models implies a direct role in inflammation (12–16). For these reasons, PLA_2 has become a target for therapeutic agents in the control of inflammation.

THE PHOSPHOLIPASE A_2 FAMILY OF ENZYMES.—*Extracellular venoms.*—The most well-characterized PLA_2 enzymes are of extracellular origin, reflecting their amenability to purification and mechanistic studies. PLA_2 has been isolated from a variety of sources, including snake venoms, insect venoms, and mammalian exocrine glands such as the pancreas, where it is secreted as a digestive enzyme. These extracellular enzymes share many common characteristics, and their striking homology is revealed by comparisons of amino acid sequences (17,18) and X-ray crystal structures (17–20). The enzymes are small, with an average mol wt of about 14,000, although they differ in their states of aggregation. They are calcium dependent and optimally active in the neutral to alkaline pH range. In terms of sequence homology, residues that are implicated in the active site are conserved. The enzymes are highly stabilized by seven disulfide linkages (17, 18), although bee venom PLA_2 contains only five (21). PLA_2 from bee (*Apis mellifera*) venom is also somewhat larger than other extracellular PLA_2 enymes and contains an oligosaccharide moiety of unknown function (22). For some time, bee venom PLA_2 was considered unrelated to the vertebrate enzymes (18). In light of the revised amino acid sequence (21) and of the X-ray crystal structure of the enzyme in complex with a transition state analogue (20), it is now evident that the essential functional substructures common to other phospholipases are present in bee venom PLA_2 but are contained within a relatively unique framework (20, 21).

The catalytic mechanism by which PLA_2 hydrolyzes its phospholipid substrate (Figure 3) has been described in detail. Elucidation of the currently accepted mechanism has involved a variety of approaches, including studies of inhibition kinetics, chemical

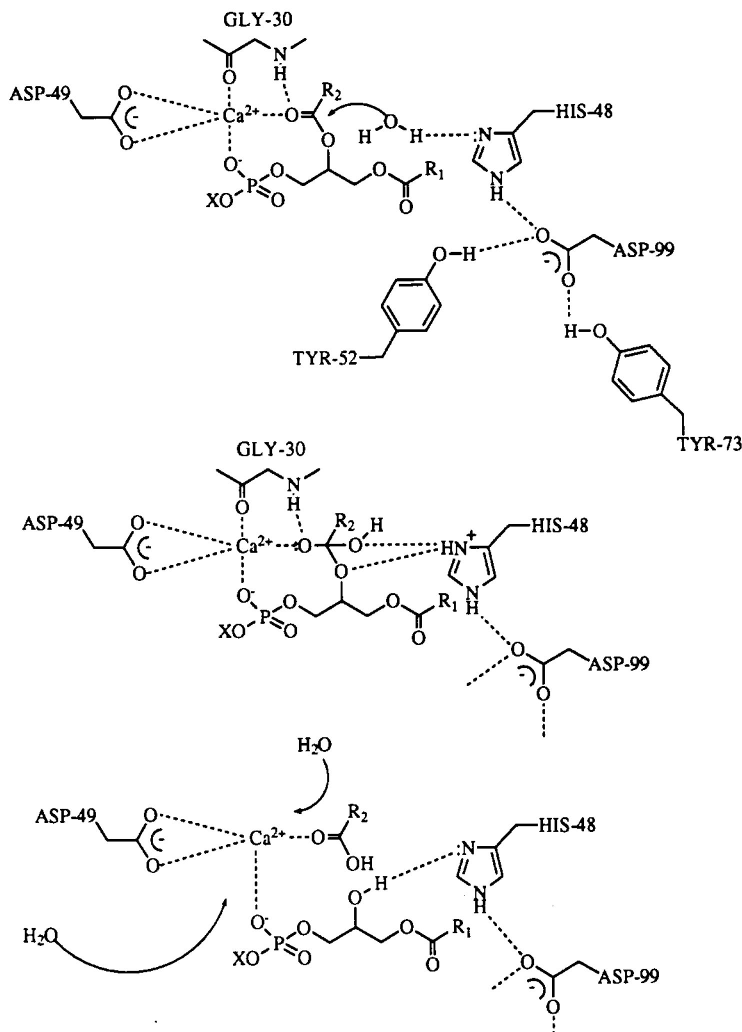


FIGURE 3. The catalytic mechanism of PLA_2 according to Scott *et al.* (24).

modification of the enzyme, evaluation of amino acid sequences, and identification of highly conserved residues, and X-ray crystallography. The most convincing mechanistic evidence has recently been inferred from the X-ray crystal structures of PLA_2 in complex with a transition state analogue.

The catalytic mechanism requires that ASP-99 [the numbering system used is that of Renetseder *et al.* (19)] and HIS-48 be positioned in the enzyme to form a catalytically active "ASP-HIS couple". The HIS-48 imidazole abstracts a proton from the proximal H_2O molecule, promoting nucleophilic attack by H_2O at the substrate *sn*-2 carbonyl to produce a tetrahedral intermediate. The positive charge acquired by the enzyme is delocalized by the ASP-HIS pair. The abstracted proton, ideally situated at the N-1 position of the imidazole, is transferred to the oxygen of the leaving group, promoting the collapse of the tetrahedral intermediate and product formation (see Figure 3) (23–25). Substrate analogue inhibitors of PLA_2 have been developed by replacement of the *sn*-2 ester group with a non-hydrolyzable functionality (26–28).

The essential calcium ion is situated close to HIS-48 and is involved in both catalysis and binding of the substrate. The calcium ion appears to polarize the *sn*-2 substrate

carbonyl group to promote nucleophilic attack by the H_2O molecule and subsequently to stabilize the oxyanion of the tetrahedral intermediate. In terms of substrate binding, it is suggested that calcium interacts with the substrate phosphate group (23–25). The calcium ion is hepta-coordinated, and its ligands form a pentagonal bipyramidal cage around the ion. Together with the ASP-49 carboxylate, three highly conserved backbone carbonyls form the "calcium binding loop." In the absence of substrate, the two remaining sites are occupied by H_2O molecules, but upon substrate binding, the H_2O molecules are replaced by ligands coming from the phospholipid. The *sn*-3 phosphate contributes an axial ligand, and the final donor to calcium is the oxyanion of the tetrahedral intermediate (24). A proton from the backbone NH of GLY-30 is considered to be an additional stabilizing factor for the intermediate oxyanion (24, 25). Two highly conserved tyrosine residues have also been implicated as components of the catalytic network. Although the ASP-HIS couple might be extended by their presence, this function could not be demonstrated experimentally (29). Only one such tyrosine is present in bee venom PLA_2 (20).

A series of hydrophobic residues form a substructure extending from the catalytic site toward the enzyme surface. These hydrophobic residues allow for favorable interactions with the fatty acyl side chains of the substrate, and the phospholipid *sn*-1 and *sn*-2 substituents lie in parallel within this "hydrophobic channel." While the residues are not invariant, their hydrophobic nature is conserved (24). In addition to stabilizing bound substrate during catalysis, the hydrophobic channel allows the substrate access to the catalytic site. The channel contains a flexible residue or "flap" which may facilitate the delivery of substrate to the catalytic site. At the surface of the enzyme surrounding the opening to the channel are the cationic residues of the interfacial recognition site (24) at which the enzyme binds to the substrate aggregate. The interfacial recognition site contains several lysine residues that are thought to bind to PLA_2 inhibitors of the manoalide class (see below).

Intracellular PLA_2 enzymes.—Intracellular PLA_2 enzymes have been found in almost every cell type examined, including bacterial, amoebic, plant, insect, and mammalian tissue cells. The intracellular PLA_2 enzymes have not been as well characterized as their extracellular counterparts, but they have recently received considerable attention owing to their putative role in inflammation (12,30,31). Possible roles have been suggested for intracellular PLA_2 in the absence of an inflammatory response. The substrates for PLA_2, the phospholipids, are the building blocks of subcellular membranes but they are not permanent fixtures; instead, there is a constant turnover whereby existing phospholipids are removed and replaced with newly synthesized molecules. PLA_2 is believed to be active in this turnover process (30). It has been proposed the PLA_2 plays a role in protecting membranes from oxidative damage by preferentially removing peroxidized fatty acids from membrane phospholipids (32). The enzyme is also involved in deacylation/reacylation cycles ("phospholipid remodelling") which actively incorporate alternative side chains into existing phospholipids. Because arachidonic-acid-containing phospholipids are not synthesized de novo, the incorporation of arachidonic acid into the *sn*-2 position of phospholipids represents an important example of this process (7,30).

There has been considerable effort to localize the subcellular origins of the various intracellular PLA_2s. The distribution of PLA_2 in the cell is not restricted to its association with the membranes where substrate phospholipids are stored. Soluble PLA_2 associated with lysosomal compartments and cytosolic fractions has also been identified. Although it has been difficult to provide clear-cut characteristic distinctions among the intracellular enzymes, the membrane-associated enzymes usually require calcium and are optimally active in the neutral-to-alkaline pH range while the soluble enzymes

associated with lysosomes are calcium-independent and optimally active at acidic pH values (12,30,31). It it perhaps too early to categorize the cytosolic enzymes.

Two classes of intracellular PLA_2s have been identified, a low mol wt class (14–16 kDa) and a high mol wt class (60–100 kDa). The low mol wt class has been identified in inflammatory exudates in synovial fluid from patients with rheumatoid arthritis, from platelets, and from the spleen. The large mol wt PLA_2 has been isolated from the cytosolic fraction of a number of cell types including macrophages and kidney cells, and a unique 40 kDa PLA_2 has been isolated from cardiac cells (33–36).

The enzymes secreted from inflammatory cells are a focus of current investigations. Leukocytes, phagocytic cells, platelets, and some other cells secrete PLA_2 in response to a variety of stimuli (12,37–40). Thus, intracellular PLA_2s are somehow mobilized and released extracellularly. Inflamed tissues have been found to contain elevated levels of PLA_2, and secreted PLA_2s have been implicated in the pathogenesis of diseases such as arthritis, psoriasis, pancreatitis, septic shock, and adult respiratory distress syndrome (12–14). Some disease symptoms are expressed upon experimental administration of purified PLA_2(12,13,15,16). The origins, both cellular and subcellular, and characteristics of these PLA_2 enzymes are under active investigation.

Because intracellular PLA_2 enzymes are neither as well characterized nor as readily available as extracellular PLA_2s, in vitro assays of PLA_2 activity generally employ the extracellular enzymes. The majority of research on the inhibition of PLA_2 by marine natural products has involved bee venom PLA_2. Some of the research on the mechanism of action, however, has employed cobra (*Naja naja*) venom PLA_2 (41,42). More recently, attention has been turned toward human synovial fluid PLA_2(HSF-PLA_2), isolated from patients with inflammatory joint disease (rheumatoid arthritis), because this enzyme may be more representative of the PLA_2 enzymes involved in human inflammatory conditions. It is believed that this enzyme may be secreted from synovial cells and, like the extracellular enzymes, HSF-PLA_2 is also calcium-dependent and optimally active at alkaline pH (40). It differs in certain ways from bee venom PLA_2, cobra venon PLA_2, and the high mol wt PLA_2. In most of the research described in this review, bee venom PLA_2 was used as the target for inhibition because it had been extensively studied and the kinetics were well understood. HSF-PLA_2 and crude forms of PLA_2 isolated from murine macrophages have also been employed but to a much lesser extent. The large mol wt form of PLA_2 is not yet available in purified form for general use but it may be produced in the future from a recombinant DNA source.

THE ANTI-INFLAMMATORY BIOASSAYS.—Marine natural products have proved to be a valuable source of PLA_2 inhibitors. Manoalide [**1**] and its structural analogues, both naturally occurring and synthetic, and scalaradial, a recently described PLA_2 inhibitor, are potent anti-inflammatory agents which directly inactivate PLA_2. The selectivity of these compounds, coupled with the large number of marine natural products that show anti-inflammatory activity, suggests that new PLA_2 inhibitors will be discovered from the marine environment in the foreseeable future.

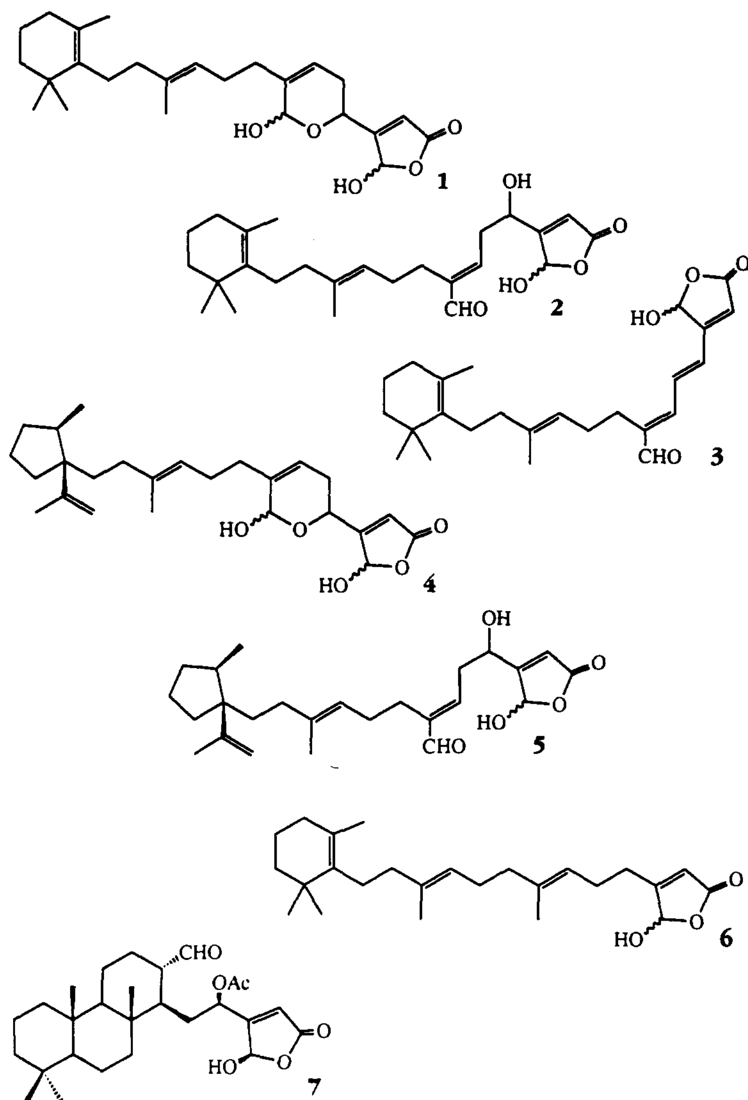
The Marine Chemistry and Pharmacology program at the University of California did not set out to discover new anti-inflammatory agents. Interest in inflammation was stimulated by research to define the mechanism of action of manoalide [**1**], which was initially shown to be an analgesic agent that was highly selective toward chemically induced pain. The analgesic model used involved production of a chemical exudate following injection of phenylquinone and was the only model sensitive to salicylates known at that time. The mouse ear phorbol myristate acetate (PMA) assay is an adaptation of that model. PMA also produces exudate and a pain response similar to phenylquinone when injected; however, unlike phenylquinone, it specifically activates

protein kinase C, which in turn activates cellular PLA_2. This selective agonist was adopted for use in the mouse ear as it provided a powerful model for testing local drug responses on the skin using small amounts of natural product, gave quantitative responses, and spared the animal pain and suffering. This model was sometimes used for screening for cyclooxygenase inhibitors by the pharmaceutical industry. The mouse ear PMA model, however, does require considerable training in its use and it is both expensive and labor intensive, all of which mitigate against its routine use in academic laboratories as a high throughput screen. Furthermore, analysis of the mechanism of action of marine natural products also requires a variety of cell models and sources of PLA_2. In spite of these limitations, the mouse ear model and the bee venom PLA_2 assay continue to be used as primary screens in the authors' laboratories in order to take advantage of the expertise developed over the years and the large data base available for these models.

THE BEE VENOM RADIOMETRIC PLA_2 ASSAY.—Phosphatidylcholine substrate is prepared at 1.36 mM as mixed micelles. Briefly, unlabeled L-α-dipalmitoyl phosphatidylcholine is homogenized in 10 mM HEPES, 1 mM $CaCl_2$ buffer, pH 7.4 at 41°, containing 0.15% (w/v) Triton X-100. ^{3}H-labeled L-α-dipalmitoyl phosphatidylcholine is added to a separate test tube, and organic solvent is removed. Unlabeled substrate solution is added, and the solution is sonicated until clear in an H_2O bath for 45 min. Bee venom PLA_2 is prepared in the above buffer at a bench concentration of 100 units/ml. Bee venom PLA_2 (0.5 ml) is incubated with 8 μl of 10 mg/ml drug stocks for 1 h at 41°. Enzyme/drug mixture (5 μl) is added to 0.5 ml prepared substrate, equilibrated to 41°, to initiate the reaction. The enzyme reactions are allowed to incubate at 41° for 15 sec. The reactions are terminated with the addition of 2 ml of isopropanol-heptane-0.5 M H_2SO_4 (40:10:1, by volume) and placed on ice. Free palmitic acid is extracted according to the method of Dole and Meinertz (43), as modified by Gatt and Barenholz (44). Briefly, phase separation is achieved by the addition of heptane and H_2O, vortexing and then centrifuging to clarify the layers. A portion of the upper heptane layer is removed, added to silica, and vortexed. The silica is pelleted by centrifugation. A portion of the heptane supernatant is removed, added to scintillation fiuid, and counted in a scintillation counter. Extraction efficiency is determined by co-extraction of ^{14}C-labeled palmitic acid in tubes containing no enzyme. Background spontaneous breakdown of substrate is determined in tubes containing no enzyme and subtracted from test sample counts. Total substrate activity is determined by directly counting a small volume of prepared substrate and is used to determine the quantity of palmitic acid released in each reaction. Percent inactivation of enzyme activity by test drugs is determined by comparison to a vehicle control rate of enzyme activity. Each test drug and control is run in triplicate and the results averaged.

MARINE NATURAL PRODUCTS THAT INHIBIT PLA_2.—Manoalide [**1**], a sesterterpenoid isolated from the Indo-Pacific sponge *Laffariella variabilis* (45), is by far the most well-characterized PLA_2 inhibitor from natural sources. Shortly after its discovery, a pharmacological evaluation revealed that manoalide is a potent analgesic and anti-inflammatory agent. Manoalide inhibited chemically induced edema in the mouse ear assay but did not inhibit arachidonic-acid-induced inflammation, indicating that inflammation is inhibited prior to the release of arachidonic acid (46–49), "possibly at the level of PLA_2" (48). The finding led to the discovery that **1** directly and irreversibly inhibits PLA_2 enzymes from bee and cobra venoms (41,48,50).

The significance of inhibiton of extracellular PLA_2 enzymes by manoalide [**1**], in terms of its anti-inflammatory properties, has been addressed in studies aimed at



determining the in vivo pathways that might be affected by manoalide. Manoalide was shown to inhibit the stimulated release of arachidonic acid from activated murine macrophages (51). The drug has also been demonstrated to inhibit phospholipase C (PLC) (52) and to block calcium channels (53,54) in a variety of cell models. Some of the models (52,53), however, did not utilize cells typically involved in the inflammatory process, and some of these non-inflammatory processes affected by **1** may be associated with toxicity. In contrast, membrane-associated PLA_2 isolated from neutrophils was found to be far more sensitive to **1** than was PLC from the same cells (55). Manoalide has also been reported to be a potent inhibitor of a membrane-associated PLA_2 from a macrophage-like cell (56). Another study suggested that intracellular PLA_2 enzymes were relatively insensitive to **1** (57), but these enzymes were obtained from crude cytosolic extracts. Thus, PLA_2 enzymes which are sensitive to **1** appear to be of membrane-associated origin or are isolated from extracellular sources such as venoms, while the relatively insensitive PLA_2 enzymes are from crude cytosolic fractions derived

from cells that are not necessarily inflammatory (58). As noted previously, both groups of manoalide-sensitive enzymes are calcium dependent and optimally active at neutral to alkaline pH. It has recently been reported that **1** is a potent, irreversible inhibitor of low mol wt HSF-PLA_2 isolated from patients with inflammatory joint disease (rheumatoid arthritis) (58). This finding suggests that some of the anti-inflammatory properties of **1** may be at least partly attributed to inhibition of PLA_2. It is not yet known whether this secreted enzyme is of membrane or cytosolic origin, but HSF-PLA_2 is also calcium dependent and optimally active at alkaline pH (40).

In addition to its ability to inhibit phospholipases, other pathways involved in the inflammatory response are affected by manoalide [**1**]. Some enzymes within the arachidonic acid cascade are inhibited by **1** (51,59). Manoalide has also been demonstrated to block calcium channels in neutrophils by a mechanism which is apparently independent of PLC inhibition (54). Thus, the mechanism by which **1** produces an anti-inflammatory response is unclear, since inhibition of PLA_2, PLC, calcium channels, or enzymes involved in the biosynthesis of eicosanoids could each be predicted to directly or indirectly mitigate inflammatory processes. Despite its medicinal potential, it now seems unlikely that **1** itself will be developed as an anti-inflammatory agent. However, it is expected that synthetic compounds based upon the manoalide structure will be developed by the pharmaceutical industry.

Although manoalide [**1**] has received the most attention, *Luffariella variabilis* and related sponges contain other sesterterpenes that inhibit PLA_2. These compounds and some simple derivatives were employed in a structure-activity relationship (SAR) study that defined the molecular architecture required for bee venom PLA_2 inactivation (60). In the SAR study, the potency (IC_{50}; the concentration of drug that causes 50% inhibition of PLA_2 activity) and the efficacy (the maximum % inhibition of PLA_2 activity) of **1** and its analogues as inhibitors of bee venom PLA_2 were compared.

The second major metabolite of *L. variabilis* is secomanoalide [**2**], which is described by de Silva and Scheuer (61). Secomanoalide, which is effectively a geometrical isomer of manoalide [**1**], inhibits bee venom PLA_2 with the same potency (IC_{50}=0.04 μM) and efficacy as **1** (60). Among the minor metabolites of *L. variabilis* there is always a small but variable quantity of (4*E*,6*E*)-dehydromanoalide [**3**] (62) (the geometry at C-6 in [**3**] is incorrect in reference 60), which shows a marked decrease in potency (IC_{50}=0.28 μM). When a large recollection of *L. variabilis* was made in order to provide material for preclinical studies, it was discovered that a small proportion of the sponges contained two new metabolites, luffariellins A [**4**] and B [**5**], in place of or together with **1** and **2** as their major metabolites (63). The luffariellins differ from **1** and **2** only in the "hydrocarbon portion" of the molecules. It is significant that luffariellins A and B have approximately the same potency and efficacy as manoalide and secomanoalide, respectively. This result indicated that the precise structure of the hydrophobic portion of the manoalide analogues was not critical for inhibition of PLA_2 activity. Further results using synthetic compounds have confirmed this observation (64).

Luffariellolide [**6**] is the major metabolite from a closely related species of *Luffariella* that was also collected in Palau (65). The structure of **6** is similar to that of manoalide [**1**] except that the C-24 aldehyde and C-4 alcohol in **1** are absent in **6**. Luffariellolide is a partially reversible inhibitor of bee venom PLA_2 with a potency (IC_{50}=0.23 μM) similar to that of (4*E*,6*E*)-dehydromanoalide [**3**] but, in contrast to **1**, is unable to inactivate that enzyme completely (65). Luffariellolide showed 98% inhibition of HSF-PLA_2 (IC_{50}=5.1 μM) (66) and inhibited PLA_2-induced edema in the mouse paw when co-injected with PLA_2 (16). A minor constituent of the luffariellolide-containing species of *Luffariella* is luffolide [**7**], in which the 6,7-double bond of **1** has been involved in a cyclization to produce a saturated C-24 aldehyde (67). Luffolide causes complete

inactivation of PLA_2 at concentrations of 3–4 μM, which are comparable to the concentrations of **1** required to achieve complete inactivation of PLA_2.

For the SAR study, simple derivatives of manoalide and synthetic γ-hydroxybutenolides derived from diterpene furans were prepared and assayed (60,68). It was concluded that the γ-hydroxybutenolide ring was essential for inactivation of PLA_2 and that a second aldehyde or masked aldehyde was required for complete inactivation of PLA_2 and irreversible binding of the drug to PLA_2. The extract nature of the hydrophobic chain was not important, provided that the chain is sufficiently long to provide the required hydrophobic interactions (64).

Elucidation of the mechanism by which manoalide inhibits PLA_2 may reveal previously undescribed properties of these enzymes. Lombardo and Dennis (41) and Glaser *et al.* (69) independently determined, by different methods, that lysine residues on bee venom and cobra venom PLA_2s are modified by the manoalide-PLA_2 interaction. This has recently been confirmed by other researchers (70). Elucidation of the structure of the manoalide-PLA_2 adduct may explain why modification of these lysine residues causes inhibition of the enzymes, even though there are no lysine residues involved in the active site, and will therefore be instrumental in defining the role of the lysine residues in intact (native) PLA_2. The results of recent experiments by Ghomashchi *et al.* (70) suggest that manoalogue, a synthetic analogue of manoalide (42), reacts with lysine residues at the enzyme's interfacial recognition site and interferes with the desolvation process that occurs when PLA_2 binds to the substrate aggregate. The enzyme can still bind to vesicles but not in a catalytically productive manner (70). The modification of LYS-6 of cobra venom PLA_2 by manoalogue, demonstrated by Reynolds *et al.* (42), is consistent with this hypothesis.

There are several sites on manoalide [**1**] that could potentially react with a lysine residue or residues (Figure 4), and several reaction mechanisms have been proposed. These include Schiff base formation, Michael addition, or amide formation, either singly or in combination (41,42,57,60). Model experiments have demonstrated that the key reaction between a primary amine and **1** is Schiff base formation at C-25 followed by ring closure to the γ-aminobutenolide. Furthermore, in experiments with PLA_2 from bee venom, inhibition by **1**, which cannot be reversed by dilution or dialysis, was substan-

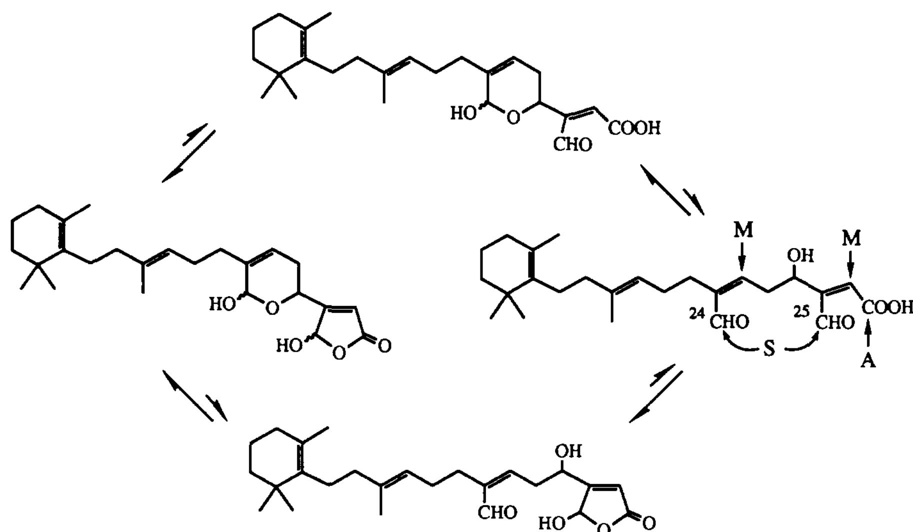
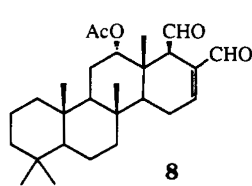


FIGURE 4. Possible sites of reaction of manoalide [**1**] with lysine residues on PLA_2; M=Michael reaction, S=Schiff base formation, A=amide formation (60).

tially reversed by treating the drug-enzyme complex with hydroxylamine (71), a reagent which has historically been used to cleave Schiff bases (72). These data strongly suggest that **1** reacts with PLA_2 to form one or more Schiff bases. Similar experiments were carried out with luffariellolide [**6**] (71). In model studies, amines reacted at C-25 of **6**, and hydroxylamine treatment of the luffariellolide-PLA_2 adduct caused almost complete recovery of the catalytic activity of PLA_2. It was concluded that **6** reacts with PLA_2 in a similar fashion to **1**; however, the data collectively exemplify the importance of the second, potentially reactive aldehyde group of **1** in the inactivation of PLA_2 (71).

The scalaradial group of marine sponge metabolites exhibit potent activity against bee venom PLA_2. Although scalaradial [**8**] was originally isolated from *Cacospongia mollior* and its structure reported in 1974 (73), it has only recently been described as a PLA_2 inhibitor (74). The anti-inflammatory properties of other scalaranes had been known for some time, but their mechanisms of action were not defined (75–78). Scalaradial, however, was found to inhibit PLA_2 from bee venom directly and irreversibly. At low concentrations, **8** completely inactivated the enzyme by a two-step mechanism involving apparent non-covalent binding followed by covalent modification



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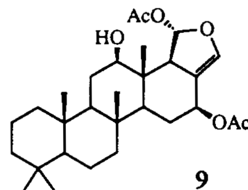

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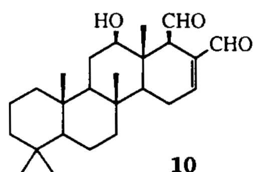

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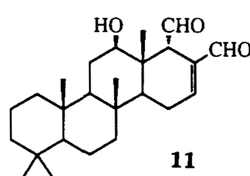

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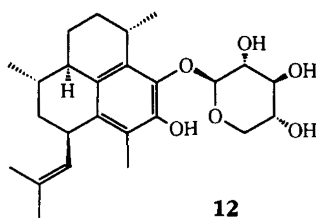

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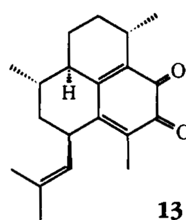

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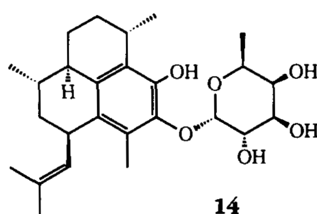

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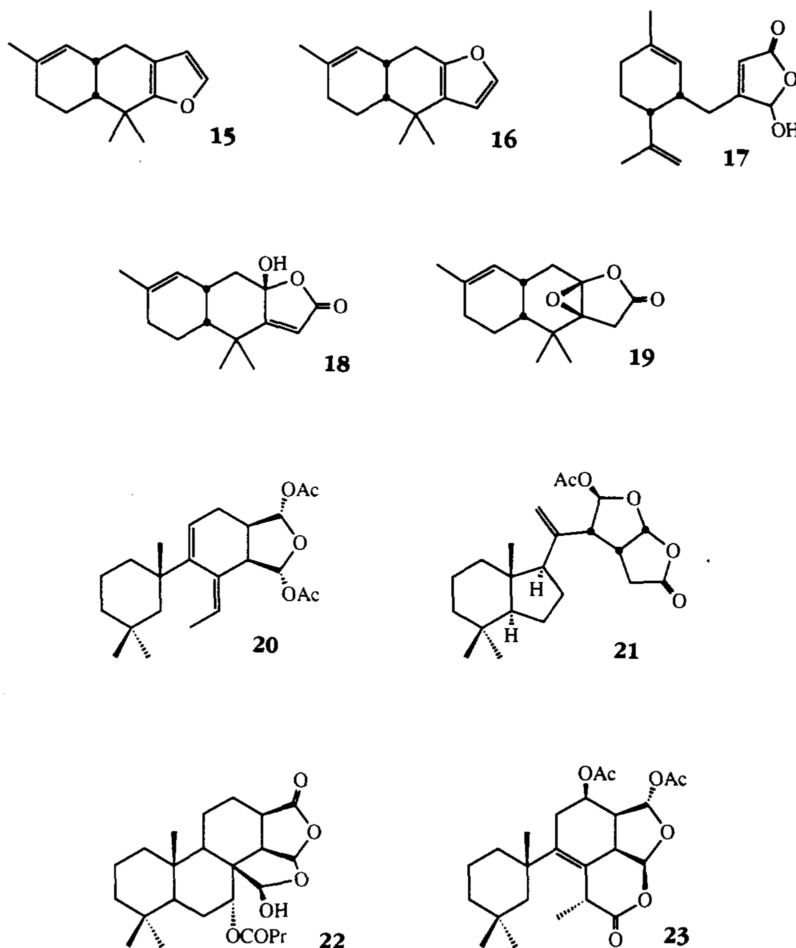
(74). These properties, in addition to the presence of two aldehydes, are reminiscent of manoalide, although the inactivation kinetics suggest that these two natural products inactivate PLA_2 by distinctly different mechanisms. It has been demonstrated that amines react with **8** to form a Schiff base followed by dehydration and aromatization to form a pyrrole adduct (79). Interestingly, inhibition of PLA_2 by **8** can be reversed by treatment with hydroxylamine in early stages of drug-enzyme interaction but not after inactivation is complete, suggesting that **8** reacts with an amine nucleophile on PLA_2 to form a Schiff base (reversible) followed by irreversible pyrrole formation (71). It has recently been demonstrated that **8** directly inactivates macrophage PLA_2 and decreases arachidonic acid release in PMA-stimulated cells (80).

Many other scalaranes have been screened in the bee venom PLA_2 assay. The most frequently encountered scalarane, heteronemin [**9**], which has been found in many Indo-Pacific sponges (81–85), is essentially inactive, demonstrating that a free aldehyde is required for PLA_2 inactivation. Screening of the hydrolysis products of heteronemin, 12-deacetyl-12-*epi*-scalaradial [**10**], which contains a 12β-hydroxyl group, and 12-deacetyl-12,18-*diepi*-scalaradial [**11**], with a 12β-hydroxy group and, more importantly, an axial rather than equatorial C-18 aldehyde group, provided support for the proposed mechanism of inactivation of PLA_2 by scalaradial. 12-Deacetyl-12-*epi*-scalaradial inhibited bee venom PLA_2 in a concentration-dependent manner. It is slightly less potent (IC_{50} 0.2 μM) than scalaradial (IC_{50} 0.07 μM), but it has a similar efficacy and is able to completely inhibit PLA_2. On the other hand, **11** shows much lower potency and efficacy than **10** (<70% inhibition at 4 μM), presumably because the 18-axial geometry of the dialdehyde **11** precludes pyrrole formation (80).

The pseudopterosins are diterpene glycosides isolated from the Caribbean sea whip *Pseudopterogorgia elisabethae* (86,87). The pseudopterosins are potent anti-inflammatory and analgesic agents. They appear to inhibit eicosanoid biosynthesis by inhibition of both PLA_2 (86–88) and 5-lipoxygenase (88). Various purified PLA_2s were originally shown to have a range of sensitivities to pseudopterosin A [**12**] (86); however, an *ortho*-quinone **13** derived from this metabolite was recently demonstrated to be a potent inhibitor of polymorphonuclear leukocyte (PMN) PLA_2, HSF-PLA_2, and bee venom PLA_2 (88). A study comparing pseudopterosins A [**12**] and E [**14**] and the *o*-quinone **13** led to the conclusion that the aglycone is the active form of the drug. The glycosides were active in vivo and in some whole cells, but in crude enzyme preparations, enzyme inhibition was time-dependent and occurred in the presence of fucosidase, suggesting that the formation of the *o*-quinone was required for activation. The *o*-quinone itself was active in crude enzyme preparations and against purified HSF-PLA_2 and bee venom PLA_2, while pseudopterosin E did not inhibit these enzymes. Interestingly, the pseudopterosins inhibit degranulation and leukotriene formation in human neutrophils but do not affect eicosanoid biosynthesis in stimulated murine macrophages in vitro (88). This indicates that this compound inhibits only PMN-PLA_2 and not PLA_2 from other sources. It is thought that the cell type selectivity of the pseudopterosins may well be a function of the glycoside moiety and a novel example of drug targeting.

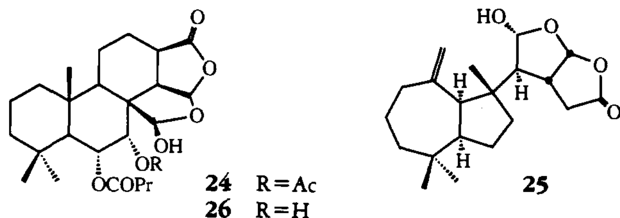
There are additional marine natural products that are known to inhibit PLA_2 activity but most of these have not been studied in detail. Some of the compounds reported below have been shown to exhibit activity in the mouse ear assay as well as inactivating PLA_2. A structural theme in many of the compounds that inactivate PLA_2 is the presence of a γ-hydroxybutenolide ring or masked 1,4-dialdehyde functionality. The nudibranch *Chromodoris funerea* from Palau employs a defensive arsenal of sesquiterpenes that are oxidation products of two sponge metabolites furodysin [**15**] and furodysinin [**16**] (89). While the furans **15** and **16** are inactive, the γ-hydroxybutenolides **17** and **18** both

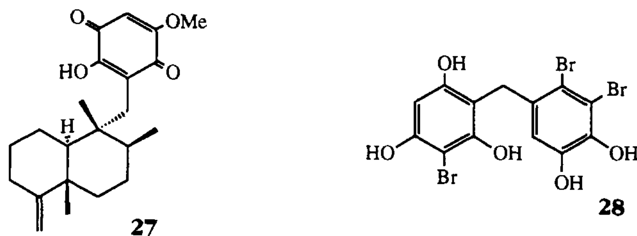


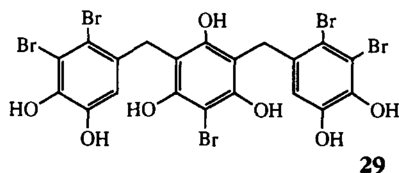
cause about 60% inactivation of PLA_2 at 2 μg/ml. Rather surprisingly, the relatively unstable epoxide **19** was inactive at the same concentration.

A number of diterpenes of the spongian class that were obtained from both Dendroceratid sponges and the nudibranchs that feed upon them (90) have been screened. Many of these compounds inactivate PLA_2 at a concentration of 2 μg/ml. In decreasing order of activity, the compounds are gracilin A [**20**] (69% inactivation) from an *Aplysilla* sp. collected at La Jolla (90,91), norrisolide [**21**] (66%) from the nudibranch *Chromodoris norrisi* (92) and a Palauan sponge of the genus *Dendrilla* (93), aplyroseol 1 [**22**] (65%) from the Australian sponge *Aplysilla rosea* (94), 12-acetoxytetrahydroaplysulphurin 1 [**23**] (60%) from the La Jolla species of *Aplysilla* (90,91), aplyroseol 6 [**24**] (50%) from *Apl. rosea* (94), dendrillolide A [**25**] (48%) from a Palauan species of *Dendrilla* (93), and aplyroseol 5 [**26**] (44%) from, *Apl. rosea* (94). All of these compounds contain the masked 1,4-dialdehyde functionality.

There are some PLA_2 inhibitors that do not contain γ-hydroxybutenolide or masked 1,4-dialdehyde groups. For example, among its many reported pharmacological activities, ilimaquinone [**27**] inactivates PLA_2. Vidalols A [**28**] and B [**29**] are bromophenols that were isolated from the Caribbean red alga *Vidalia obtusaloba* (95). Both **28** and **29** cause 96% inactivation of bee venom PLA_2 at 1.6 μg/ml. These are just a few of the







compounds that exhibited anti-inflammatory activity by a mechanism that probably involves inactivation of PLA_2.

CONCLUSIONS.—The bee venom PLA_2 bioassay is a very convenient bioassay for the screening of marine natural products for anti-inflammatory activity. It can be used to screen crude extracts but is best employed in a routine manner for screening pure compounds and selected column fractions that are approaching purity. Several of the anti-inflammatory agents discovered in this research program are considered to have the potential for commercial development, and in certain cases, development proceeded as far as clinical trials (96). The Sea-Grant-sponsored program continues to yield novel anti-infiammatory agents that will be reported in the future.

ACKNOWLEDGMENTS

The authors owe a great debt of gratitude to all the chemists and pharmacologists who have contributed to this project. In particular, DJF acknowledges the contributions of Dr. Jill E. Hochlowski, who first isolated manoalide for this project, and Joaquin S. Caso and Mary Kay Harper, who together extracted and analyzed over 400 specimens of *L. variabilis.* The Marine Chemistry and Pharmacology Program at the University of California is a collaborative program sponsored by the California Sea Grant College Program involving the research groups of Professors Robert S. Jacobs, Pharmacology, UCSB; Philip Crews, Chemistry, UCSC; D. John Faulkner, Marine Chemistry, UCSD, and William Fenical, Marine Chemistry, UCSD. The collaborative research program has been faithfully funded since 1979 by the California Sea Grant College Program and the authors thank Dr. J.J. Sullivan and his staff for their assistance in coordinating the project. Research in the authors' laboratories was sponsored in part by the National Sea Grant College Program, National Oceanic and Atmospheric Administration, Department of Commerce, under grant number NA89AA-D-SG138, project numbers R/MP-46 and R/MP-47, and by the California State Resources Agency.

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Received 29 June 1992