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Manoalide: Structure-Activity Studies and Definition of the Pharmacophore for Phospholipase A_2 Inactivation

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SUMMARY

Manoalide is a potent antiinflammatory marine natural product and a direct inactivator of venom phospholipase A_2 (PLA_2; EC 3.1.1.4). Manoalide has been shown to irreversibly inhibit PLA_2, with the corresponding modification of a selective number of lysine residues. The mechanism of inactivation has not yet been elucidated and structure-activity relationship studies were, therefore, performed in order to determine the contributions of the various functional groups incorporated in the γ-hydroxybutenolide, α-hydroxydihydropyran, and trimethylcyclohexenyl ring systems to the efficacy (irreversibility) and potency of this series of inhibitors. These studies indicate that 1) the presence of the hemiacetal in the α-hydroxydihydropyran ring is required for irreversible binding of manoalide, 2) the γ-hydroxybutenolide ring is involved in the initial interaction of manoalide with PLA_2, and 3) the hydrophobic nature of the trimethylcyclohexenyl ring system allows nonbonded interactions between manoalide and PLA_2 that enhance the potency of these analogs. These structure-activity relationship studies suggest that the closed ring form of manoalide is the predominant molecular species that accounts for the selective and potent inhibition of PLA_2 by manoalide. Elucidation of the mechanism awaits further detailed physicochemical studies on the structure of the manoalide (analog)-protein adducts in model systems and using PLA_2.

Manoalide (1) is the parent compound of a series of marine sponge metabolites that possess potent antiinflammatory properties (2–4) and inhibit the enzyme PLA_2 (3, 5–7). The important role of PLA_2 in the release of arachidonic acid, which is the biosynthetic precursor of proinflammatory eicosanoids (8, 9), suggests that understanding the structure-activity relationships of inactivation of PLA_2 by manoalide will ultimately lead to a better understanding of PLA_2 function in the whole cell and possibly the mechanism of hydrolysis of phospholipid substrates.

Manoalide is a sesterterpenoid from the marine sponge *Luffariella variabilis* (1). The antiinflammatory activity of manoalide was demonstrated against phorbol ester-induced but not arachidonic acid-induced local inflammation (2), which suggested that the mechanism of action of manoalide was proximal to the metabolism of arachidonic acid, i.e., the release mechanism. Subsequently, manoalide was shown to inhibit the hydrolysis of phosphatidylcholine by bee venom PLA_2 (5) by an irreversible mechanism (6, 10). Manoalide has also been shown to inhibit PLA_2 from several different sources (7, 11). Inhibition of cobra venom PLA_2 is accompanied by the modification of four of the six lysine residues present (7). Inhibition of bee venom PLA_2 also results in the loss of only lysine residues but, in contrast to cobra venom PLA_2, only three of 11 possible lysine residues are modified (12). The reactivity of manoalide with the enzyme may require the presence or specific orientation of at least two lysine (nucleophilic) residues that react in an ordered mechanism to produce irreversible binding (13). Although manoalide modifies lysine residues and inhibits several different sources of phospholipase (6, 7, 11), its mechanism of action does not involve nonselective modification of the lysine residues (11, 12), i.e., manoalide appears to have a specific binding site on PLA_2 and reacts with a selective number of lysine residues on PLA_2.

Although some of the conditions necessary for manoalide reactivity have been determined, the mechanism by which manoalide inactivates PLA_2 has not been elucidated. Several models for the mechanism of manoalide have been proposed. One model suggested that manoalide can exist in a ring-opened form (Fig. 1, **1D**), which would undergo Schiff base formation and/or Michael addition with a lysine residue on PLA_2 to

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ABBREVIATIONS: PLA_2, phospholipase A_2; HPLC, high performance liquid chromatography; HRMS, high resolution mass spectrometry; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

produce the adducts shown in Fig. 2 (**a** and **b**), which would cause the observed irreversible inactivation (7, 14). The presence of a Michael adduct, however, has never been detected in model experiments.[1] Another model proposed a Michael adduct formation at C-2 with concomitant elimination of the C-4 hydroxyl to produce an adduct (Fig. 2, **c**) that possesses a chromophore (λ_{max} = 435 nm) (11, 13). However, the relationship of the chromophore production to the inactivation of PLA_2 is a causal not temporal one (13) and this proposed model does not consider the necessity of the C-24 hemiacetal for irreversible inhibition (3).

Our hypothesis, illustrated in Fig. 3, is one whereby the selectivity and reactivity of manoalide with PLA_2 results from the reaction of the closed ring form of manoalide, the predominant species at neutral pH as suggested by the chemical equilibrium (Fig. 1), with the PLA_2 active pocket to generate the C-25 aldehyde group, which may undergo Schiff base formation with an adjacent lysine residue. The anchoring of the γ-hydroxybutenolide ring allows the proper orientation of the pyran moiety, which then undergoes a second addition to produce irreversible binding.

The present structure-activity study was designed, using marine natural product analogs of manoalide or chemical derivatives of manoalide, to further characterize the structural features of the manoalide molecule that are necessary for both potency and efficacy (irreversibility) of this novel phospholipase inhibitor. The structural modifications of the manoalide molecule being investigated may be grouped according to alterations of 1) the γ-hydroxybutenolide (γ-lactone) ring system (Fig. 1, **1A**, ring A), 2) the α-hydroxydihydropyran ring (Fig. 1, **1A**, ring B), 3) the hydrophobic trimethylcyclohexenyl ring system (Fig. 1, **1A**, ring C), and 4) the overall length of the carbon backbone of the molecule (e.g., C-20 versus C-25). Our results demonstrate the overall importance of both the γ-hydroxybutenolide and the C-24 aldehyde (or hemiacetal) in the mechanism of inactivation. Loss of the C-25 aldehyde group (the ring A aldehyde) has a greater effect on the potency (relative IC_{50}) of these analogs, whereas loss of the C-24 aldehyde (the ring B aldehyde) has a greater effect on the efficacy (irreversibility).

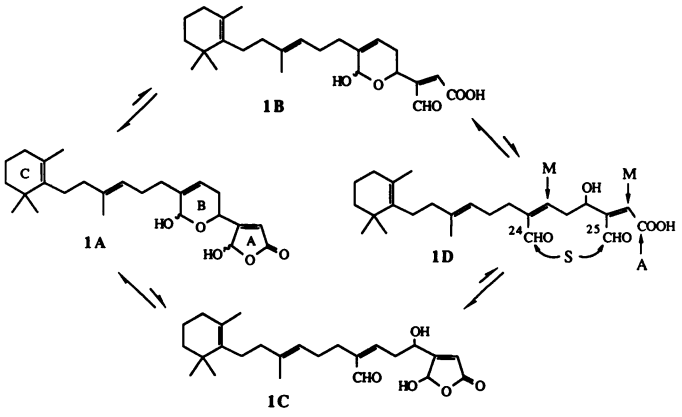


Fig. 1. Equilibration of manoalide (**1A**) with ring-opened form, showing the generation of aldehyde groups at C-25 (**1B**), at C-24 (**1C**), and at both C-24 and C-25 (**1D**). The dialdehyde form (**1D**) may react with primary amines to form an amide (*A*) or Schiff bases (*S*) or by a Michael addition (*M*).

[1] D. J. Faulkner, unpublished observations.

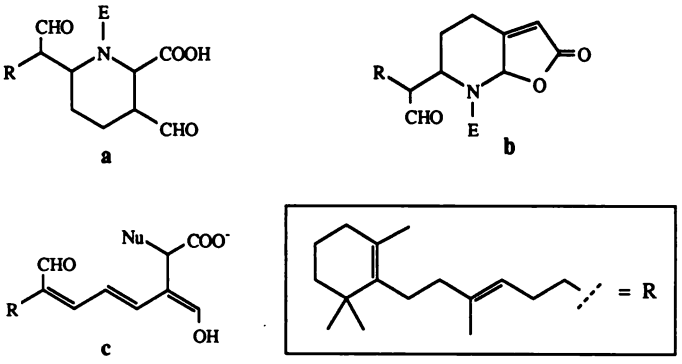


Fig. 2. Reaction products between manoalide (or analogues) and PLA_2 proposed by Reynolds *et al.* (14) (**a** and **b**) and Bennett *et al.* (11) (**c**)

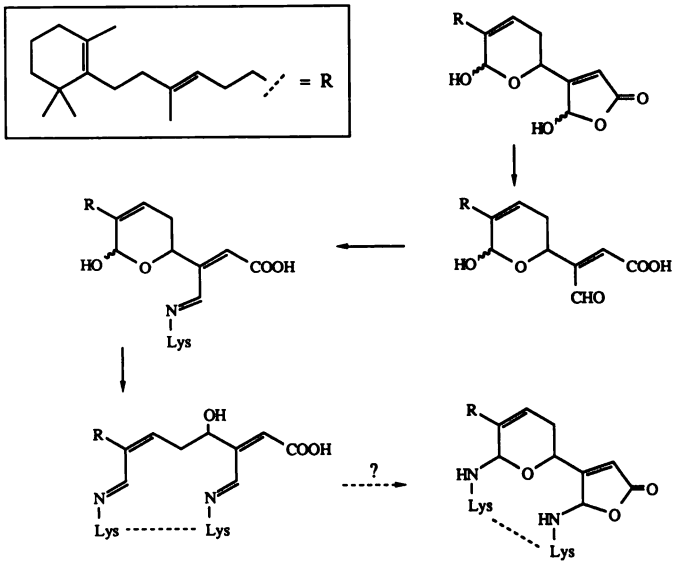


Fig. 3. Proposed mechanism for the reaction of lysine residues on PLA_2 with manoalide.

Experimental Procedures

Isolation and Synthesis of Compounds Used in this Study

Manoalide (Fig. 4, **1**)[2] and secomanoalide (**4**) were isolated from specimens of the sponge *L. variabilis* according to the procedures outlined by de Silva and Scheuer (1, 15). During the final chromatographic separation on silica gel, variable amounts of dehydromanoalide (**5**) were obtained from a yellow band that eluted just before manoalide (**1**). The procedures for the isolation of luffariellins A (**9**) and B (**10**) from selected specimens of *L. variabilis* (4) and luffariellolide (**6**) (3) and luffolide (**14**) (16) from a different species of *Luffariella* have all been reported elsewhere. The syntheses of the γ-hydroxybutenolide (**12**) from ambliol A (**13**), which was isolated from *Dysidea amblia* (17), and of the γ-hydroxybutenolide (**11**) and γ-aminobutenolide (**15**) were reported by Kernan and Faulkner (18).

Preparation of lactone (2). A solution of Jones's reagent (prepared from 6.7 g of chromium trioxide and 6 ml of sulfuric acid) was added dropwise to a stirred solution of manoalide (30 mg, 0.07 mM) in distilled acetone (20 ml) at 25° until the solution remained brown. After 5 min, the reaction mixture was filtered through a short column of silica gel and the solvent was evaporated to obtain an oil. The product was chromatographed by HPLC to obtain the manoalide δ-lactone as a mixture of two diastereoisomers. Yield, 15 mg (50% theoretical); oil;

[2] Boldface numbers after each compound refer to the corresponding compound numbers in Fig. 4.

IR (film): 3300, 1770, 1750 cm^{-1}, UV (methanol): 208.5 nm (ϵ 10,350); ^1H NMR ($CDCl_3$): δ 0.99 (s, 6 H), 1.60 (s, 3 H), 1.65 (s, 3 H), 5.10 (m, 1 H), 5.26 (dd, 0.5 H, J = 12, 5 Hz), 5.37 (dd, 0.5 H, J = 12, 5 Hz) 6.15 (s, 0.5 H), 6.20 (d, 0.5 H, J = 7 Hz), 6.23 (s, 0.5 H), 6.35 (d, 0.5 H, J = 7 Hz), 6.62 (m, 0.5 H), 6.65 (m, 0.5 H); HRMS: m/z 414.2384, $C_{25}H_{34}O_5$ requires 414.2406.

Preparation of acetate (3). Lactone (**2**) (15 mg, 0.04 mM) was dissolved in acetic anhydride (0.5 ml) and pyridine (1.0 ml) and the mixture was stirred at 25° for 4 hr. The solvents were removed under high vacuum and the residue was dissolved in ether and filtered through a silica gel plug to obtain a clear oil. The oil was chromatographed by HPLC to obtain a mixture of diastereoisomeric acetates. Yield, 16 mg (quantitative); oil; IR (film): 1880, 1770, 1725 cm^{-1}; UV (methanol): 208 nm (ϵ 10,600); ^1H NMR ($CDCl_3$): δ 0.99 (s, 6 H), 1.59 (s, 3 H), 1.65 (s, 3 H), 2.18 (s, 3 H), 5.10 (t, 1 H, J = 7 Hz), 5.21 (m, 1 H), 6.26 (s, 0.4 H), 6.34 (s, 0.6 H), 6.61 (m, 1 H), 6.98 (s, 1 H); HRMS: m/z 456.2514, $C_{27}C_{36}O_6$ requires 456.2512.

The acetate is a 6:4 mixture of two diastereoisomers. The diastereoisomers can be separated, but the material assayed was the mixture of isomers.

Preparation of the diol (8). Excess sodium borohydride (300 mg, 7.9 mmol) was added in small portions to a stirred solution of manoalide (**1**) (136 mg, 0.33 mmol) in isopropanol (20 ml) at 0°. The mixture was stirred at 0° for 1 hr. Excess reagent was destroyed by dropwise addition of 2% hydrochloric acid until hydrogen evolution ceased. The product was partitioned between water (100 ml) and ether (2 × 100 ml), the ether extract was dried over sodium sulfate, and the solvent was removed to obtain an oil. The product was purified by HPLC to obtain the diol (**8**) (75 mg, 55% yield); oil; ^1H NMR ($CDCl_3$): δ 0.99 (s, 6 H), 1.60 (s, 3 H), 1.65 (s, 3 H), 4.11 (d, 1 H, J = 14 Hz), 4.17 (d, 1 H, J = 14 Hz), 4.64 (m, 1 H), 4.88 (br s, 2 H), 5.13 (t, 1 H, J = 7 Hz), 5.39 (t, 1 H, J = 7 Hz), 5.98 (br s, 1 H).

Preparation of 25-deoxymanoalide (7). A solution of the diol (**8**) (29.2 mg) in dry dichloromethane (150 μl) was added to a stirred suspension of pyridinium chlorochromate (33 mg) in dry dichloromethane (250 μl) at room temperature. A black precipitate formed after 25 min and dry ether (2 ml) was added to the reaction mixture. The solvent was decanted and the precipitate was washed with ether (4 × 2 ml). The ether solution was filtered through a Sep-Pak cartridge and the solvent was removed to yield an oil (9.5 mg). The oil was purified by liquid chromatography on silica gel (7:4 hexane/ethyl acetate) to obtain 25-deoxymanoalide (**7**) (7 mg, 24% yield); oil; ^1H NMR ($CDCl_3$): δ 0.99 (s, 6 H), 1.60 (s, 3 H), 1.65 (s, 3 H), 3.02 (br s, OH), 4.88 (d, 1 H, J = 16 Hz), 4.92 (d, 1 H, J = 16 Hz), 4.93 (m, 1 H), 5.14 (t, 1 H, J = 7 Hz), 5.32 (s, 1 H), 5.70 (br s, 1 H), 6.01 (s, 1 H).

PLA_2 Radiometric Assay

Bee venom PLA_2 (Sigma; St. Louis, MO) activity was determined using mixed micelles of 1.35 mM dipalmitoyl phosphatidylcholine, 2.76 mM Triton X-100, and 0.5 μCi of 2-α-palmitoyl[2-*palmitoyl*-9,10-^{3}H] phosphatidylcholine (specific activity, 57 mCi/mmol) prepared in 10 mM HEPES, 1 mM $CaCl_2$, pH 7.4, at 41°. Bee venom PLA_2 (5 μl; final assay concentration, 25 nM) that had been preincubated with inhibitor or vehicle for 1 hr at 41° (6) was added to 500 μl of substrate (a 100-fold dilution of inhibitor/enzyme complex), incubated for 15 sec at 41°, and extracted as described previously (6). Released [^{3}H]palmitic acid (heptane phase) was added to 3.0 ml of Ecosint (National Diagnostics) and counted in a LKB Rackbeta 1219 liquid scintillation counter. Under these conditions the initial rate of hydrolysis was linear and ≤7% of the substrate was hydrolyzed, ensuring initial rate kinetics. All inhibitor concentrations and IC_{50} values are given as the final concentration of inhibitor in the assay mixture, because the mechanism of most of these analogs is unknown relative to manoalide and range from irreversible to partially reversible inhibition of PLA_2. Due to the complex inhibition kinetics of manoalide, the inability to produce pseudo-first-order kinetics under various experimental conditions (6, 7), the structure-activity relationship studies are reported herein as relative IC_{50} values to allow comparison of the structurally diverse compounds.

Reversibility Studies

Bee venom PLA_2 (25 μM) was preincubated with various concentrations of manoalide analogs for 1 hr at 41° to achieve maximal inactivation (6). An aliquot was removed and assayed for predialysis activity as described above. Preincubation mixtures were placed in Spectra/Por dialysis tubing (MWCO 11-12000) and dialyzed for 16–24 hr at 4°. After dialysis, the samples were assayed for postdialysis activity as described above. Control samples, containing solvent, were treated identically to analog-treated samples.

Statistical analyses of the differences between pre- and postdialysis samples were performed using Student's t test for paired observations ($p < 0.05$ and 0.01).

Results

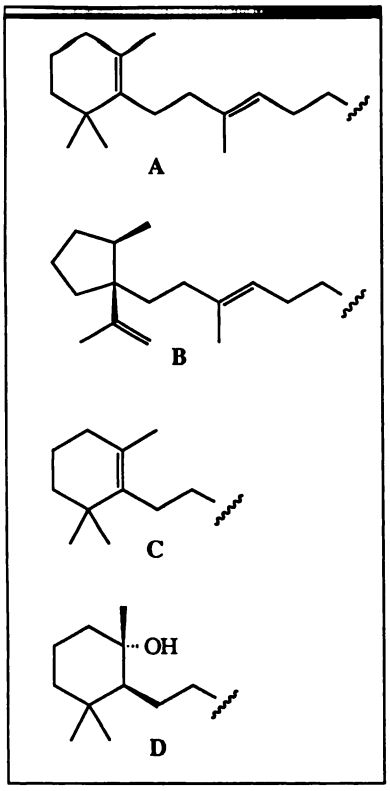
Structural effects on efficacy: comparison of manoalide, secomanoalide, dehydromanoalide, and luffariellolide. The parent compound of this series, manoalide (**1**), acts via an irreversible mechanism that completely inactivates bee venom PLA_2 (IC_{50} of 30 nM at 25 nM enzyme) (6). Of the series of manoalide analogs thus far examined (Fig. 4), manoalide appears to be the most potent and reactive (13). With bee venom PLA_2, the efficacy of manoalide is the complete inactivation of enzymatic activity (Fig. 5).

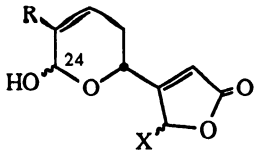
Secomanoalide (**4**), which is a geometrical isomer of manoalide that has a C-4 hydroxyl and an α,β-unsaturated aldehyde at C-24, has the same potency (IC_{50}) and efficacy as manoalide (**1**) (data not shown). The geometry of the 6*Z* double bond does not allow pyran ring formation under the assay conditions and equilibration between secomanoalide (**4**) and manoalide (**1**) has only been observed under photolysis conditions (15). These data suggest that the efficacy of the inhibition reaction is not dependent on a rigid geometrical relationship between the C-24 aldehyde and the second lysine residue (13).

Dehydration of manoalide at C-4 to form the (4*Z*)-dehydromanoalide (**5**) results in a marked decrease in potency (IC_{50} = 0.28 μM) without affecting the efficacy of this compound (Fig. 5). Therefore, it is possible that the formation of the 4*Z* double bond, which results in a structure that is more sterically rigid, may hinder the interaction of (**5**) with the active pocket of PLA_2.

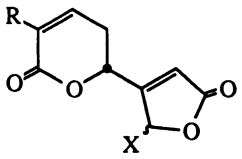
In compounds such as luffariellolide (**6**) that lack the C-24 aldehyde or its equivalent, there is a complete alteration of the kinetic mechanism of the reaction with PLA_2. Luffariellolide (**6**) is a partially reversible inhibitor of PLA_2 (3, 13) with a potency similar to that of dehydromanoalide (**5**) [IC_{50} of luffariellolide (**6**) = 0.25 μM]. The presence of the aldehyde at C-24 appears to be essential for irreversible inhibition in the manoalide series (3, 13). Reversible inhibition of PLA_2 has also been observed for a synthetic analog of manoalide (19) that lacks the C-24 aldehyde.

Modification of functional moieties of manoalide. The greatest effects on the potency of analogs arise from modifications at C-24 and C-25. However, changing the C-24 hemiacetal to a δ-lactone (**2**) has no observable effect on the potency of this compound (IC_{50} = 0.04 μM). Further modification by acetylation of the C-25 hydroxyl (**3**) causes a 2-fold decrease in potency. These observations do not contradict the proposed mechanisms by which manoalide may react with lysine resi-

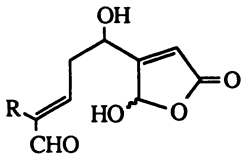




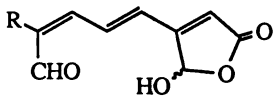
1 R = A X = OH IC_{50} = 0.03
***7** R = A X = H IC_{50} = 1.0
9 R = B X = OH IC_{50} = 0.06



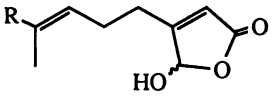
***2** R = A X = OH IC_{50} = 0.04
***3** R = A X = OAc IC_{50} = 0.1



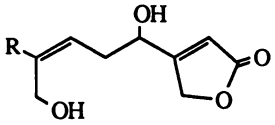
4 R = A IC_{50} = 0.04
10 R = B IC_{50} = 0.06



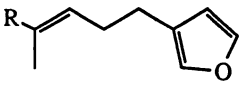
5 R = A IC_{50} = 0.28



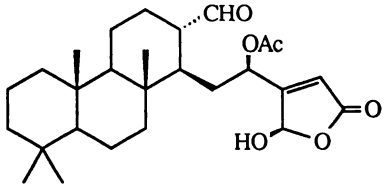
6 R = A IC_{50} = 0.23
***11** R = C IC_{50} = 0.13
***12** R = D IC_{50} = 2.0



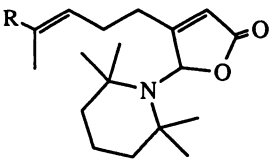
***8** R = A IC_{50} = 1.4



13 R = D IC_{50} > 4.0



14 IC_{50} < 3.5



***15** R = D inactive

Fig. 4. PLA_2 inhibition by manoalide and analogs. IC_{50}, final concentration of inhibitor in the assay that reduces PLA_2 activity by 50%, as determined from the linear portion of the dose-response curve. *Compounds that are chemical derivatives of manoalide.

dues, because amide formation may replace Schiff base formation in these derivatives and the presence of the acetate may hinder a possible PLA_2-catalyzed step in the inhibition (19).

Removal of the C-25 hydroxyl, forming the γ-lactone (**7**), results in a considerable decrease in potency, approximately 20-fold (IC_{50} = 1.0 μM), without apparently changing the efficacy (Fig. 6). Reductive opening of the pyran ring of manoalide to produce the diol (**8**) slightly changes the potency (IC_{50} = 1.41 μM) and reduces the efficacy, a similar effect to that observed for the reversible inhibitor luffariellolide (**6**). These observations further support the involvement of the C-24 aldehyde in the irreversible mechanism of manoalide. Reversibility studies (Table 1) demonstrate that, at concentrations of the γ-lactone below 1 μM (<50% inhibition), the inhibition appears to be partially reversible (31% inhibition predialysis and 19% postdialysis), as seen with luffariellolide (3). At concentrations >1 μM (>50% inhibition), the inhibition appears to be essentially irreversible, as seen with manoalide (6). The 20-fold decrease in potency associated with loss of the C-25 hydroxyl demonstrates the importance of the intact γ-hydroxybutenolide ring system.

Effects of carbon chain length and changes in hydrophobicity. As seen in Fig. 7, analogs of manoalide in which the trimethylcyclohexenyl ring is replaced by the isomeric 1-isopropenyl-2-methylcyclopentane ring possess similar potency and efficacy [luffariellin A (**9**) IC_{50} = 0.056 μM and luffariellin B (**10**) IC_{50} = 0.06 μM]. This type of structural alteration would not appreciably affect the hydrophobicity of this region, which

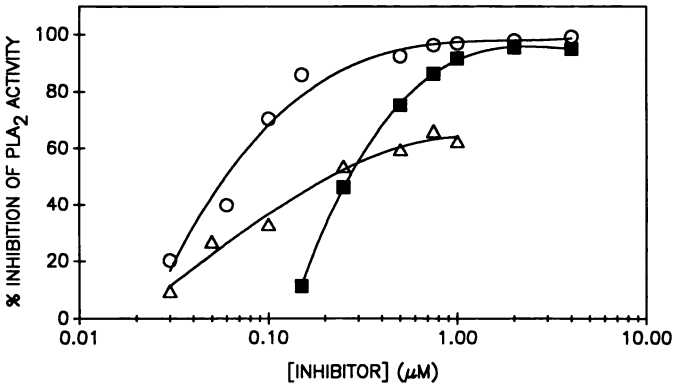


Fig. 5. Inhibition of PLA_2 by manoalide (**1**) (○), dehydromanoalide (**5**) (■), and luffariellolide (**6**) (△).

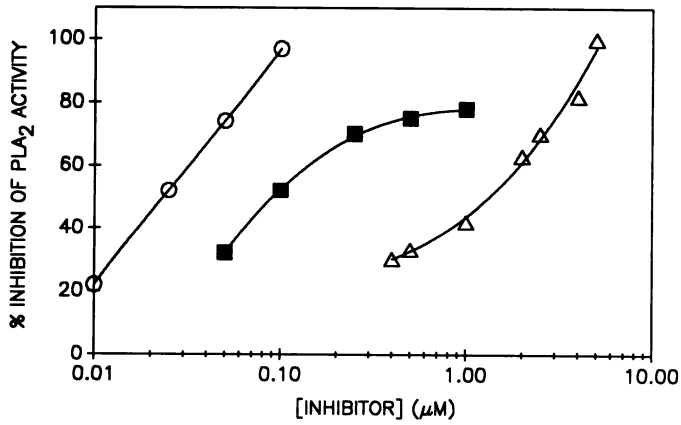


Fig. 6. Inhibition of PLA_2 by the γ-lactone (**7**) (△), manoalide (**1**) (○), and luffariellolide (**6**) (■).

TABLE 1

Reversibility studies for manoalide, luffariellolide, and γ-lactone

Compounds were preincubated for 1 hr at 41° before predialysis activity was determined. Dialysis was performed in Spectra/Por MWCO/12-1400 cellulose tubing at 4° in 10 mM HEPES (pH 7.4) buffer for 16–24 hr, with two buffer changes before postdialysis activity was determined.

Compound	Concentration	Inhibition		Change
		Predialysis	Postdialysis	
	μM	%		%
Manoalide (**1**)	0.05	68	65	−4
Luffariellolide (**6**)	1.0	74	64	−14
γ-Lactone (**7**)	1.0	31	19	−39
	2.0	63	69	+10

may be involved in hydrophobic interactions (binding) and/or the directed orientation due to hydrophobic forces and no effect on the potency or efficacy of these compounds would therefore be expected.

Decreasing the overall carbon chain length from the sesterterpene luffariellolide (**6**) to the corresponding diterpene (**11**) has little effect on the potency (IC_{50} value for (**6**) = 0.23 μM and (**11**) = 0.13 μM). However, when the hydrophobicity of the trimethylcyclohexenyl moiety is altered by addition of a hydroxyl group, there is a 15-fold decrease in potency (IC_{50} = 0.13 μM for (**11**) to 2.0 μM for (**12**)). This demonstrates the importance of this hydrophobic region in the interaction of manoalide and manoalide analogs with PLA_2.

The furan analog (**13**), which lacks any of the reactive groups, is effectively inactive. Formation of a bulky Schiff base

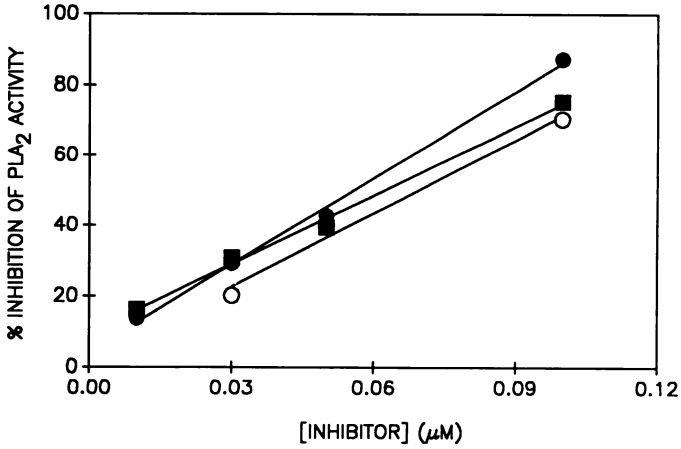


Fig. 7. Inhibition of PLA_2 by luffariellin A (**9**) (●), luffariellin B (**10**) (■), and manoalide (**1**) (○).

derivative at the C-25 aldehyde produces a compound, (**15**), that no longer inhibits PLA_2. These data support the role of the γ-hydroxybutenolide in the recognition and initial binding of manoalide (and analogs) to PLA_2, which leads to inhibition of PLA_2 activity.

Analogs lacking α,β-unsaturated aldehydes. The tricyclic analog of manoalide, luffolide (16) (**14**) lacks an α,β-unsaturation at the aldehyde position corresponding to C-24 of manoalide, therefore eliminating the possibility of Michael addition. The aldehydes still present in this analog are able to participate in Schiff base formation and complete inactivation of PLA_2 occurs at concentrations of 3–4 μM, which are comparable to the concentrations of manoalide (**1**) necessary to achieve complete inactivation of PLA_2 (6).

Discussion

It has been suggested that the interaction of manoalide with PLA_2 initially takes place at the γ-hydroxybutenolide ring, followed by a second reaction at the α-hydroxydihydropyran ring that causes irreversible inhibition (13). Recently, this type of mechanism has also been proposed for the manoalide-like analog manoalogue (14). Our hypothesis, based upon the reactivity of manoalide (13) and these structure-activity relationship studies, implicates the closed ring form of manoalide (Fig. 1, **1A**) as the molecular species that would most selectively interact with PLA_2. With the array of manoalide-like natural products and chemical derivatives of manoalide (analogs) presented herein, we can now attempt to define the contribution of each functional group to the inhibitory activity of manoalide. These data will aid in the elucidation of the mechanism of inactivation of PLA_2 by manoalide.

First, it is important to note that, from the physicochemical data obtained on manoalide (7), this molecule may exist in an equilibrium between closed and open ring forms (Fig. 1). In all inhibition studies reporting reactions carried out at neutral pH (physiological pH), the closed ring form of manoalide (Fig. 1, **1A**) is the predominant molecular species. The partially and fully open ring forms exist in nominal concentrations under neutral conditions and, therefore, would not be expected to contribute significantly to the irreversible inhibition of PLA_2. From the data with bee venom PLA_2 (12), it is apparent that the manoalide binding site is selective and it is possible that increased selectivity would result from the interaction of the

dominant form of manoalide with PLA_2. The partially or fully open ring forms might be expected to undergo rapid nucleophilic attack at any exposed lysine residue or similar nucleophile, resulting in nonselective interactions, which are not observed for manoalide (12) or related analogs (14).

The butenolide ring of the γ-lactone (**7**) cannot exist in equilibrium with the corresponding 4-hydroxy acid (open ring form) under normal assay conditions; however, at sufficiently high concentrations the analog irreversibly inactivates the enzyme. The partial reversibility of inhibition by the γ-lactone at lower concentrations is compatible with a mechanism involving the formation of a single Schiff base at C-24 following orientation of the molecule by interaction with the active pocket of PLA_2. The decreased potency of this analog may be due to the reduced ability of the butenolide ring to bind to PLA_2 and possibly to a slower rate of "hydrolysis" of the butenolide, as compared with the γ-hydroxybutenolide. This "PLA_2-catalyzed"-type inhibition has been suggested (19); however, the analog of manoalide that was studied lacked both the α-hydroxydihydropyran and trimethylcyclohexenyl rings and did not provide conclusive evidence for this mechanism.

The importance of the butenolide structure in the reaction mechanism is also supported by the absence of inhibitory activity of the analog (**15**), where the C-25 hydroxyl is replaced by the bulky tetramethylpiperidine group, which may prevent access to the active pocket of PLA_2. A similar but much smaller effect is observed by comparing data for the δ-lactone (**2**) with those of the corresponding acetate (**3**). Hydrolysis of the butenolide ring of (**3**) by PLA_2 with concomitant loss of the acetate group leads to the same intermediate that would be formed from (**2**). It would be of interest to determine the kinetics of inhibition of PLA_2 by these analogs in order to elucidate the possible mechanisms for the inactivation of PLA_2. Kinetic studies are presently underway in several laboratories.

The suggestion of a possible PLA_2-catalyzed step in the inhibition in part comes from the demonstration that the rate and extent of inhibition by manoalide are highly dependent on pH. Increasing the pH from 6.0 to 8.0 causes an increased rate and extent of inhibition of both bee venom and cobra venom PLA_2 by manoalide. This pH range also corresponds to the pH optimum for bee venom (pH optimum, 7.1) and cobra venom (pH optimum, 8.0) PLA_2. Lombardo and Dennis (7) suggested that this also corresponds to formation of carbonyl functionalities on the manoalide molecule. However, at pH 7.0–7.5, conditions under which the maximum extent of inactivation of PLA_2 is observed, NMR studies demonstrated that the predominant species was the closed ring form of manoalide (Fig. 1, **1A**) (7). Using cobra venom PLA_2, the rate of inhibition is increased slightly with added Ca^{2+} during the preincubation, possibly suggesting that enzyme activity enhances the rate of inactivation by manoalide.

Irreversibility is an important feature of the mechanism of manoalide inhibition of bee (6) and cobra (7) venom PLA_2. The efficacy or irreversibility of the manoalide series has been associated with the C-24 aldehyde or the corresponding hemiacetal present in the α-hydroxydihydropyran ring system (3, 6, 7, 13, 19). The importance of the C-24 aldehyde is most emphatically demonstrated by the data for luffariellolide (**6**), which totally lacks this reactive group and, therefore, lacks the irreversible character of the manoalide series (3, 13). The data for secomanoalide (**4**) and dehydromanoalide (**5**) demonstrate that the C-24 aldehyde need not be protected as a hemiacetal to achieve efficacy. However, protection of the C-24 aldehyde as a hemiacetal leads to an increase in the potency of inhibition, presumably because the aldehyde is not available to react in a random manner with other available lysine residues.

The tricyclic analog luffolide (**14**) completely inhibits bee venom PLA_2 at 3.5 μM (16), a concentration equivalent to that required for complete inactivation of PLA_2 by manoalide (**1**). This analog contains a saturated C-24 aldehyde in place of the α,β-unsaturated aldehyde (C-6, C-7, C-24) in manoalide (**1**) that would be required for a Michael addition at C-6 according to the previously proposed mechanisms for irreversibility (7, 14). Having eliminated the possibility of Michael addition in luffolide (**14**) only Schiff base formation at the C-24 aldehyde of manoalide and analogs (or amide formation in the case of δ-lactones (**2**) and (**3**)) remains as a reasonable explanation for irreversible inhibition.

From the studies with the luffariellins (**9**) and (**10**), it is apparent that a minor change in the structure of the hydrophobic region of the molecule does not affect potency. However, introduction of a hydrophilic group, such as a hydroxyl, on the trimethylcyclohexenyl ring drastically decreased the potency of the analog (**12**). This demonstrates the importance of nonbonded interactions between PLA_2 and the hydrophobic region of manoalide (**1**) to the potency of inhibition. This aspect has not previously been considered to be as important as demonstrated herein and may account for some of the differences observed in results obtained in similar systems by replacing manoalide with manoalide-like analogs that lack a bulky hydrophobic region (14, 19). The hydrophobic region of manoalide (**1**) may mimic the hydrophobic acyl chains on the phospholipid substrates of PLA_2 and may partially account for the selectivity of manoalide and its analogs.

In conclusion, several dominant molecular features that contribute to the potency and efficacy of manoalide (**1**) have been described. These include the γ-hydroxybutenolide ring system, the C-24 aldehyde (hemiacetal), and the hydrophobic alkyl region. From these structure-activity relationship studies it has been demonstrated that 1) the ring-closed form of manoalide (**1**) acts as the selective inhibitor of PLA_2, i.e., selectivity has been demonstrated for both bee (12) and cobra venom (14) PLA_2 in which only 2–3 of the possible lysine residues are modified by manoalide; 2) the initial reaction of manoalide (**1**) with PLA_2 is dependent on a concerted interaction between both the γ-hydroxybutenolide ring and the hydrophobic alkyl region and the enzyme; 3) there may be a PLA_2-catalyzed step in the inactivation mechanism; 4) the C-24 aldehyde is essential for irreversible inhibition of PLA_2 by manoalide; and 5) Schiff base formation at C-24 is more likely than Michael addition at C-6 to account for the irreversible inhibition. It is apparent that manoalide (**1**) is the most potent and efficacious of the analogs thus far examined. The complexity of the interaction of manoalide (**1**) with PLA_2 is apparent from both the inactivation kinetics (6, 7) and these studies. The actual mechanism by which manoalide reacts with PLA_2 will not be determined until suitable labeled species are synthesized and detailed physicochemical studies of the manoalide-PLA_2 interaction are performed.

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