Chemical Mechanism of Inactivation of Bee Venom Phospholipase A_2 by the Marine Natural Products Manoalide, Luffariellolide, and Scalaradial

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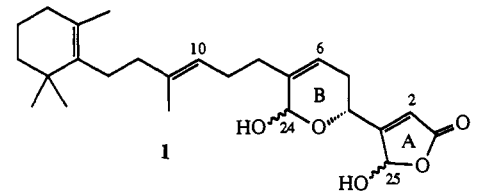
Abstract: Inhibition of bee venom phospholipase A_2 (PLA_2) by manoalide (**1**) and luffariellolide (**2**) involves the initial formation of a Schiff base (imine) between a lysine residue on PLA_2 and an aldehyde group on each of the drugs. Model reactions employing a primary amine in place of the lysine residue were studied by ^1H NMR spectroscopy. Amines reacted at the γ-hydroxybutenolide ring of **2** to produce γ-(alkylamino)butenolides **6a,b**, which are cyclized forms of the corresponding Schiff bases. Manoalide methyl analogue (MMA, **12**), which is a simple analogue of the reactive portion of **1**, reacted similarly. The γ-(*n*-butylamino)butenolide **6b** reacted with hydroxylamine to form the oxime **8** with concomitant release of *n*-butylamine. When the luffariellolide–PLA_2 and manoalide–PLA_2 adducts were treated with hydroxylamine, the PLA_2 activity was substantially recovered, but the activity was not recovered if the luffariellolide–PLA_2 adduct was reduced with sodium borohydride prior to hydroxylamine treatment. PLA_2 activity could be significantly recovered by treatment of the initial scalaradial **3a**–PLA_2 adduct with hydroxylamine but the final adduct, which is proposed to be a pyrrole (**20**), could not be cleaved.

The marine natural products manoalide (**1**), isolated from the sponge *Luffariella variabilis*,[1] luffariellolide (**2**), from the sponge *Luffariella* sp.,[2] and scalaradial (**3a**), from the sponge *Cacospongia*



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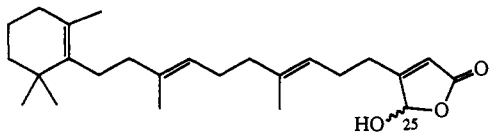

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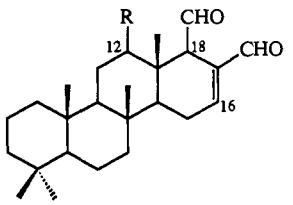

3a 18β-CHO; R=12α-OAc
3b 18β-CHO; R=12β-OH
3c 18α-CHO; R=12β-OH

mollior,[3] are potent anti-inflammatory agents[2,4,5] and inhibitors of various secreted forms of phospholipase A_2 (PLA_2).[2,5-12] Of the three inhibitors, manoalide has been most extensively characterized.[1,4,7-19] Of particular interest to this study are the reports of Dennis[9] and Jacobs,[15] who independently demonstrated by different methods that a fraction of the lysine residues in PLA_2 are modified by the manoalide–PLA_2 interaction. These data, coupled with the time-dependent, irreversible nature of the inhibition,[9,10] demonstrate that manoalide forms a covalent adduct with PLA_2. On the basis of these observations and on structure–activity relationship studies employing analogues of manoalide,[13,20-22] several mechanisms of chemical inactivation of PLA_2 by manoalide have been suggested.[9,11,13,21] There have, however, been no experiments reported to date to test any of these proposals. In this paper, we demonstrate that the primary reaction involved in the inactivation of bee venom PLA_2 by manoalide, luffariellolide, and scalaradial is Schiff base (imine) formation and that enzyme activity can be significantly recovered if the

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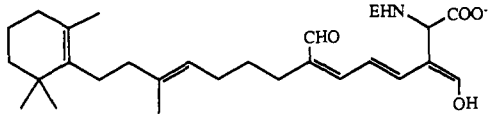
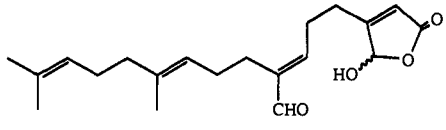
drug–enzyme adducts are treated with hydroxylamine.

Manoalide is a sesterterpene containing a hydrophobic chain joined to two potentially reactive rings, a γ-hydroxybutenolide (A) ring and a δ-lactol (B) ring.[1] The importance of the hydrophobic chain in the inhibition of bee venom PLA_2 has been demonstrated,[13,22] suggesting that it may be involved in noncovalent, hydrophobic binding to PLA_2,[13] while rings A and B are thought to react with lysine residues.[9,13,14] Since the rings can open to generate two α,β-unsaturated aldehydes and an α,β-unsaturated carboxylic acid, there are several possible mechanisms by which lysine could react with manoalide. These mechanisms involve imine formation at the aldehyde groups, Michael addition at the β position of the α,β-unsaturated aldehydes or carboxylic acid, and amide formation, either singly or in combination. Since **2**, which contains only the γ-hydroxybutenolide ring, is a partially reversible inhibitor of bee venom PLA_2 and produces up to 70% inhibition[2,7] while **1** is an irreversible inhibitor[9,10] which can completely inactivate bee venom PLA_2,[10] it was proposed that the second functional group in ring B is necessary to effect irreversible inactivation.[2,14,20,21] Chemical models have been proposed to explain these observations. Faulkner and Jacobs have suggested that the C-25 aldehyde reacts with a lysine residue to form an imine, thereby reversibly binding manoalide to PLA_2. A second lysine can then add at C-24 to produce an adduct in which manoalide is effectively irreversibly bound to PLA_2. The imines or iminium salts can be further stabilized by reclosure of rings A and B (Scheme I).[13] Bennett et al. proposed Michael addition of a lysine residue at C-2 of ring A to initially form an enolate. After elimination of the C-4 hydroxyl group, a highly conjugated enol adduct (**4**) is produced which could account for the chromophore observed when manoalide is incubated with PLA_2.[11] Dennis et al. provided an alternate proposal for manoalide and manoalogue (**5a**), a structural analogue of manoalide, suggesting that either Michael addition or imine formation at ring A is followed by Michael addition of the same lysine to the second α,β-unsaturated aldehyde (**5b,c**).[21]



4 E = Enzyme



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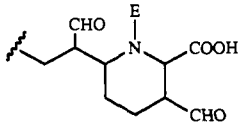
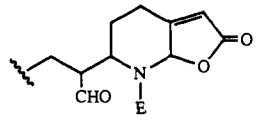


5b E = Enzyme



5c E = Enzyme

We initially approached the problem of testing these hypotheses by studying the model reactions of drugs and drug analogues with primary amines. Manoalide methyl analogue (MMA, **12**) and **2** were each reacted with primary amines in order to assess their reactivity. The products of these reactions were analyzed by ^1H NMR and other spectroscopic methods. Cimino et al. had already studied the reactions of primary amines with **3a**.[23] These model studies strongly influenced the course of our research on the reaction of PLA_2 with manoalide, luffariellolide, and scalaradial. In this paper, we report that treatment of the drug–PLA_2 complexes with hydroxylamine reactivates the enzyme, thereby demonstrating that manoalide, luffariellolide, and scalaradial initially react with bee venom PLA_2 to form imines, as expected from the model studies.

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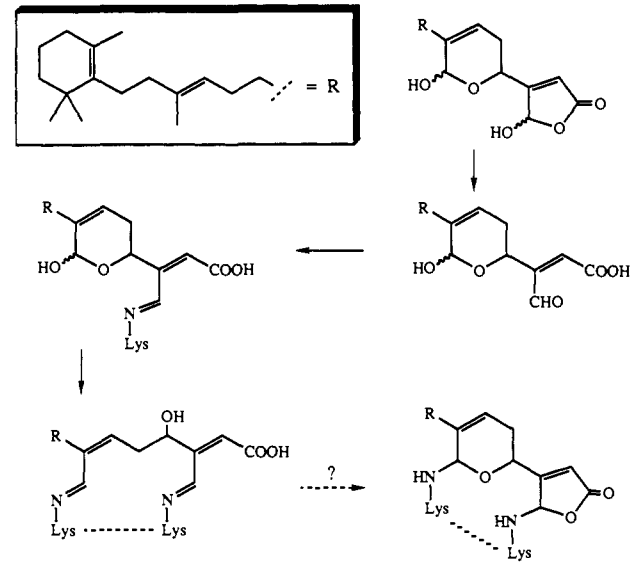
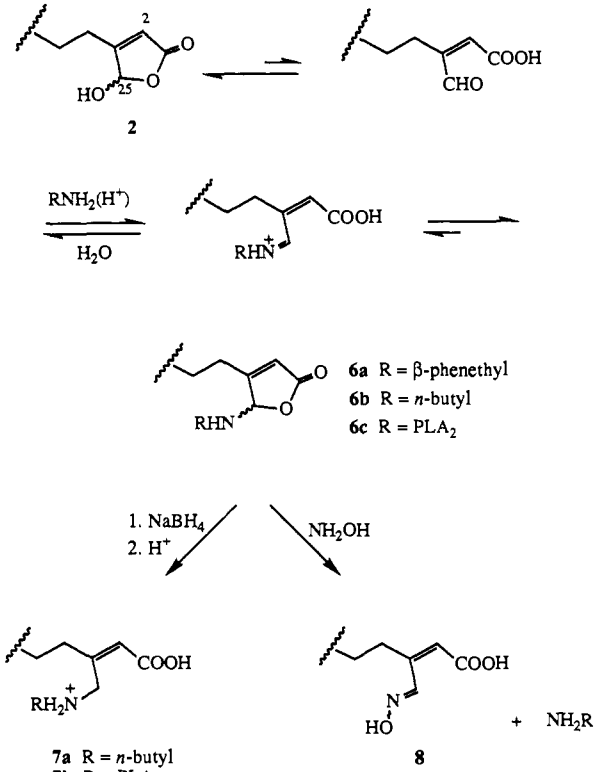
Scheme I. Proposed Reaction between Manoalide (**1**) and Lysine Residues on PLA_2, Adapted from Glaser et al.[13]



Scheme II. Sequence of the Reaction of Luffariellolide (**2**) with Amines or with a Lysine Residue on PLA_2, and Reduction Products and Hydroxylamine Cleavage Products



7a R = *n*-butyl
7b R = PLA_2

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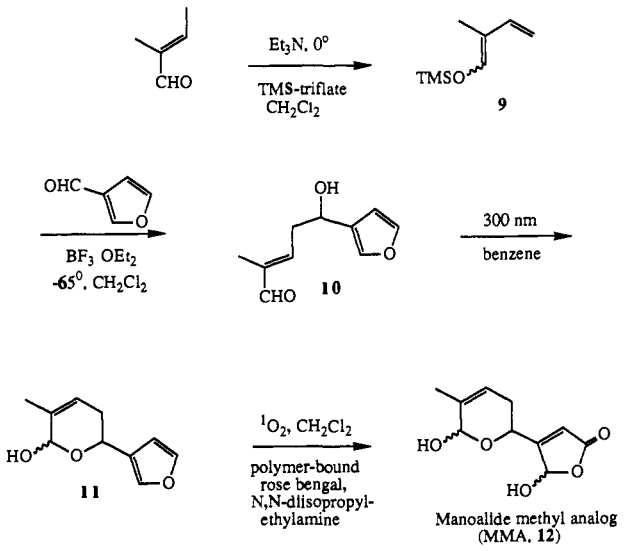
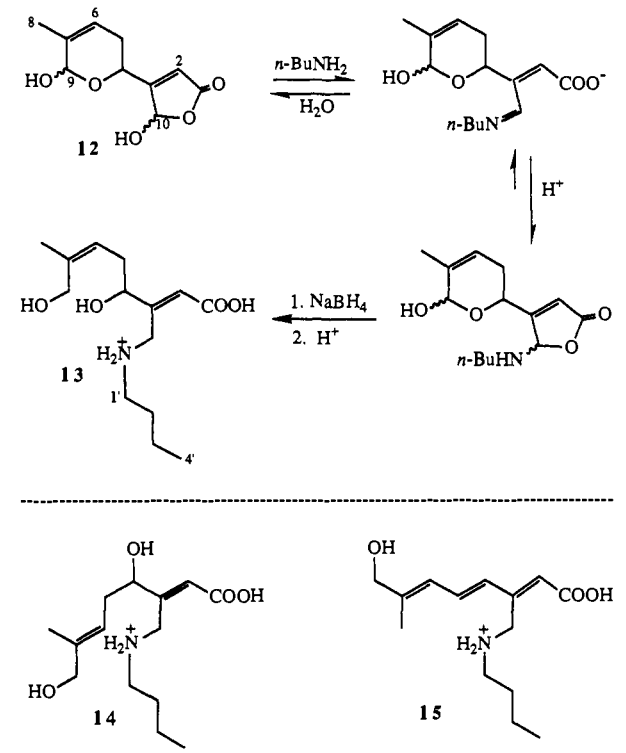
Results and Discussion

Luffariellolide Model Studies. Luffariellolide was reacted with 1 equiv of β-phenylethylamine or *n*-butylamine in the presence or absence of 1 equiv of weak acid (acetic acid) to yield γ-(alkylamino)butenolide derivatives **6a** and **6b**, respectively, which form via imine formation at C-25 followed by ring closure (Scheme II). This reaction has been monitored by ^1H NMR spectroscopy. During the course of the reaction, the C-25 proton signal of **2** at δ 6.04 (CD_3OD) diminished while a new signal appeared at 5.74, due to the C-25 proton of the newly formed γ-(alkylamino)butenolide. Furthermore, the presence of the imine intermediate

Scheme III. Synthesis of MMA (**12**), Based on Work by Garst et al.[24] and by Kernan and Faulkner[25]



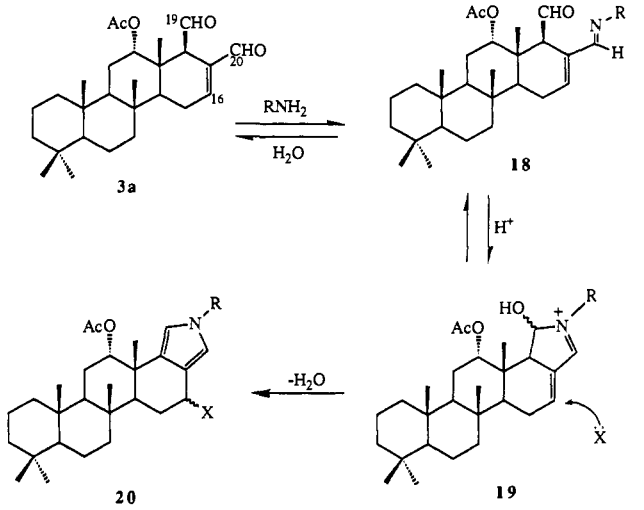
Scheme IV. Sequence of Reaction of MMA (**12**) with *n*-Butylamine followed by Reductive Workup To Give **13** and the Minor Products **14** and **15**



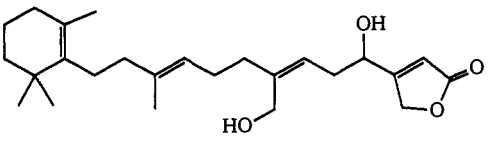
(open-ring form) was detected by observation of the C-25 imine proton signal at δ 8.17. There is no evidence for Michael addition at C-2 or for any other reaction. Adduct **6b** was reduced with $NaBH_4$ to produce the γ-(alkylamino) acid **7a** (Scheme II).

Manoalide Model Studies. Our initial attempts to react manoalide with primary amines in protic solvents resulted in very complex mixtures of products. We therefore chose to use **12** as a substitute for **1** because the reactions of **12** could be more easily analyzed by ^1H NMR spectroscopy. **12** had been synthesized previously[22] and was prepared for this study by employing the methods of Garst et al.[24] together with those of Kernan and Faulkner[25] (Scheme III). The reaction of **12** with *n*-butylamine gave a complex mixture in protic solvents but proceeded cleanly

Scheme V. Sequence of the Reaction of Scalaradial (**3a**) with Amine, as Described by Cimino et al. (R = Amine, X = H_2O)[23] and Proposed Here for the Reaction of Scalaradial with PLA_2 (R = PLA_2, X = H_2O or Nucleophile on PLA_2)

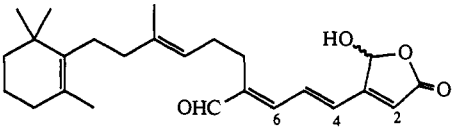


in dichloromethane or in acetonitrile solution. After quenching the reaction with $NaBH_4$, the γ-(alkylamino) acids **13** and **14** were isolated, indicating that stable products are formed by reaction of amines at the C-10 hemiacetal group on the γ-hydroxybutenolide ring (Scheme IV). The ^1H NMR chemical shifts of these products were compared to model compounds **7a** and manoalide diol **16**.[13] Compounds **13** and **14** were distinguished by



16

nuclear Overhauser effect difference spectroscopy (NOEDS). Irradiation of the C-8 methyl group resulted in a 3% enhancement of the H-6 signal of compound **13** at 5.32 ppm. The minor product **15**, which was produced in small and variable quantities, was identified by comparison of its ^1H NMR spectrum to that of the model compound (4*E*,6*E*)-dehydromanoalide (**17**).[26] Addition



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of *n*-butylamine at C-10 on ring A of **12** was not followed by Michael addition to the α,β-unsaturated aldehyde of ring B (**5c**), suggesting that the irreversible nature of inactivation of PLA_2 by manoalide cannot be explained by this proposed mechanism.[21] The reaction of **12** with 2 equiv of *n*-butylamine in dichloromethane or acetonitrile solution gave complex mixtures of products which have yet to be fully analyzed. The formation of the (6*E*)-isomer **14** and the dehydration product **15** in addition to the expected (6*Z*)-isomer **13** suggested that their formation may be catalyzed by reversible imine formation at C-9.[27]

Scalaradial Model Studies. The reaction of scalaradial with methylamine has been reported by Cimino et al.[23] The reaction sequence consists of condensation at C-20 to produce an intermediate imine **18** followed by intramolecular addition to the C-19 aldehyde to give a transient iminium ion **19** which rapidly aro-

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(26) Potts, B. C. M.; Capon, R. J.; Faulkner, D. J. *J. Org. Chem.*, in press.
(27) This isomerization has been observed for the related compound **11** that contains only the δ-lactol ring.

Table I. Inhibition of PLA_2 by Drug and Reversal of Inhibition with Hydroxylamine

drug	preincubation time, min	% inhibition[a] for postincubation treatment: buffer	% inhibition[a] for postincubation treatment: hydroxylamine	% reversal of inhibition
1	60	93 ± 0.2	42 ± 9	55
1	64	94 ± 1	26 ± 3	72
2	60	67 ± 2	12 ± 13	82
2[b]	60	54 ± 1	2 ± 6	96
3a	10	25 ± 1	8 ± 2	68
3a	60	75 ± 1	75 ± 3	0

[a] Data represent mean ± standard error (N = 4). [b] PLA_2 was preincubated with the drug in borate buffer (pH 9) and subsequently dialyzed against HEPES buffer, and the PLA_2 activity was measured (see text); thereafter, the sample was diluted 2-fold with HEPES buffer or hydroxylamine, as indicated in the table, and the PLA_2 activity was measured (N = 3).

matizes to form the pyrrole **20** (Scheme V). Although it has not been demonstrated that lysine is involved in the inactivation of PLA_2 by scalaradial, we have included it in our studies because it possesses two aldehyde groups, is an irreversible inhibitor of bee venom PLA_2, and has similar potency and a similar inactivation profile to manoalide, i.e., apparent noncovalent binding followed by covalent modification, with the ability to completely inactivate bee venom PLA_2.[6]

Enzyme Studies: Rationale. The model studies indicate that imine formation is the preferred reaction of primary amines with manoalide, luffariellolide, and scalaradial. In order to demonstrate that these compounds react with bee venom PLA_2 in the same or similar manner, we employed the methodology used previously to cleave Schiff bases in studies of the chemistry of rhodopsin. Retinal is covalently bound to a lysine residue of opsin via a protonated Schiff base (iminium ion).[28-31] The retinals are dissociated from the protein by exposing the complex to light ("bleaching") and allowing it to react with hydroxylamine.[32] Hydroxylamine reacts with the Schiff base to form retinal oxime, thereby releasing retinal from the lysine residue of opsin. If manoalide is bound to PLA_2 by one or more Schiff bases (imines) or their stabilized ring-closed forms (γ-aminobutenolides), the manoalide–PLA_2 adduct should be cleaved by treating it with hydroxylamine and the PLA_2 activity should be recovered. Similarly, treating the inactivated luffariellolide–PLA_2 adduct with hydroxylamine would reactivate the PLA_2. In order to demonstrate that the γ-(alkylamino)butenolide behaves like an imine and could by cleaved by hydroxylamine under the reaction conditions, the γ-(*n*-butylamino)butenolide **6b** was reacted with hydroxylamine (Scheme II). The product proved to be identical to luffariellolide oxime (**8**), which was readily prepared by treating luffariellolide with hydroxylamine. In the case of scalaradial, intermediate imines **18** and **19** should be cleaved by reaction with hydroxylamine, but the pyrrole **20** should be stable to hydroxylamine treatment at physiological temperatures. Thus, the scalaradial–PLA_2 complex should be dissociated by hydroxylamine only in early stages of the reaction but not after the pyrrole has formed.

Luffariellolide–PLA_2 Inhibition/Reactivation. Inhibition of PLA_2 by **2** was almost completely reversed with hydroxylamine (Table I). At 67% (approximately maximal) inhibition, 82% of the activity was recovered. PLA_2 preincubated with luffariellolide, dialyzed, and then treated with hydroxylamine was essentially completely reactivated (2% inhibition). The luffariellolide–PLA_2 adduct was readily cleaved by hydroxylamine under these reaction conditions, as would be expected for the γ-aminobutenolide structure (**6c**) proposed. Oxime **8** did not inhibit PLA_2 activity.

Table II. Inhibition of PLA_2 by Manoalide over a Range of Preincubation Times and Reversal of Inhibition with Hydroxylamine

preincubation time, min	% inhibition[a] for postincubation treatment: buffer	% inhibition[a] for postincubation treatment: hydroxylamine	% reversal of inhibition
4	60 ± 5	5 ± 8	92
8	77 ± 3	22 ± 2	71
16	86 ± 2	44 ± 1	49
64	87 ± 2	56 ± 1	36

[a] Data represent mean ± standard error (N = 4).

Manoalide–PLA_2 Inhibition/Reactivation. The inhibition of PLA_2 by **1** was markedly reversed by hydroxylamine treatment (Table I). Up to 72% reversal of inhibition was achieved for PLA_2 preincubated with manoalide for 1 h. PLA_2 pretreated with hydroxylamine and then incubated with manoalide was not inhibited, demonstrating that manoalide oxime is not a PLA_2 inhibitor. (Any manoalide in solution, either approaching or reaching the site of inhibition, would be immediately exposed to hydroxylamine, forming the oxime of manoalide.) We made an interesting observation regarding the chromophore which develops when manoalide is incubated with PLA_2:[7,11,14] hydroxylamine treatment reverses chromophore formation as well as inhibition.[33]

In an attempt to resolve the stepwise formation of the manoalide–PLA_2 adduct, the reversal of PLA_2 inhibition by manoalide was measured over a range of drug–enzyme preincubation times (Table II). Clearly, inhibition can be most substantially reversed in the early stages of the manoalide–PLA_2 reaction. At the 4-min preincubation point, the data are similar to those obtained for the luffariellolide–PLA_2 complex (Table I), suggesting that the majority of the manoalide–PLA_2 adduct is linked by an imine or stabilized imine at ring A at this time. The percent reversal achieved decreased linearly up to 16 min of preincubation and leveled off (data not shown) once inhibition was complete. The formation of the final product probably involves three rapid, consecutive steps occurring during the first 15–20 min of preincubation: immediate noncovalent binding of drug to enzyme through hydrophobic interactions,[9,13] γ-(alkylamino)butenolide formation between a lysine residue and the C-25 hemiacetal of manoalide during the first minutes of preincubation, and a subsequent interaction between ring B and PLA_2 to form the final product which can be only partially reactivated by hydroxylamine.

Although the presence of a second functional group is required to completely inactivate bee venom PLA_2,[2,14] the nature of the interaction of ring B with PLA_2 remains undefined. Inability to completely reactivate manoalide-treated PLA_2 may reflect incomplete reaction with hydroxylamine and/or an adduct in which hydroxylamine cleaves the linkage at ring A while the linkage (or interaction) with ring B remains intact, yielding a drug–PLA_2 adduct which is only partially inhibited. Several possibilities, either singly or in combination, could account for these results. (i) After forming a γ-(alkylamino)butenolide with a lysine residue at C-25, a second lysine residue may react at C-24 to form an adduct with two imine linkages (Scheme I). There is evidence which suggests the necessity for two nucleophiles in the reaction of manoalide with PLA_2.[14] (ii) A second nucleophile on PLA_2 other than a lysine may form a linkage with ring B which cannot be cleaved by hydroxylamine but requires a different mechanism of cleavage such as hydrolysis. (iii) As suggested by the formation of model compounds **14** and **15**, the manoalide–PLA_2 adduct may isomerize to give the (6*E*)-geometry or may undergo dehydration to yield a 4,5-double bond, either reaction rendering an adduct which cannot be readily cleaved under the reaction conditions. This is analogous to rhodopsin (11-*cis*-retinal linked to opsin), which cannot be cleaved with hydroxylamine unless it is simultaneously bleached.[34] (iv) Ring B may be involved in hydrogen bonding

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(33) The results of these and related observations will be reported elsewhere.
(34) The bleaching induces double-bond isomerization(s) accompanied by conformational changes in the retinal–opsin complex, thereby exposing the imine linkage to hydroxylamine.[28,32]

with other amino acids at the adduct site.[35] (v) After reacting with manoalide, the enzyme may undergo a conformational change which renders it completely inactive and in which the linkage to manoalide is not readily approached by hydroxylamine. This is, however, unlikely since conformational changes should be restricted due to the presence of five disulfide bridges.[36]

Approximately 3 of 12 lysine residues on bee venom PLA_2 are modified by manoalide.[15] It is therefore possible that a fraction of manoalide molecules bound to specific lysine residues of PLA_2 are being cleaved by hydroxylamine while another fraction bound to a different residue are not. The results of recent experiments by Gelb et al.[37] suggest that **5a** reacts with lysines at the enzyme's interfacial recognition site and interferes with the desolvation process which occurs when PLA_2 binds to the substrate aggregate. The enzyme can still bind tightly to vescicles but not in a catalytically productive manner.[37] The modification of lysine-6 of cobra venom PLA_2 by manoalogue, demonstrated by Dennis et al.,[21] is consistent with this hypothesis.

Our data strongly suggest the presence of imine linkages between manoalide and bee venom PLA_2. This conclusion is in contrast to the early results of Lombardo and Dennis,[9] who found that acid hydrolysis of the manoalide–cobra venom PLA_2 adduct renders a fraction of lysine residues permanently modified, data which do not support the presence of imine linkages between manoalide and cobra venom PLA_2. This result could, however, be explained if a secondary reaction or rearrangement occurs at >100 °C to form a product that is resistant to hydrolysis, as has previously been suggested.[9] It was also recently reported that hydroxylamine treatment of manoalogue or manoalide-inactivated PLA_2's resulted in 0–20% recoveries of enzymatic activities with poor reproducibility.[38] In our experiments, we have exercised caution in using concentrations of manoalide which produce submaximal (~95%) inhibition and have observed marked recoveries of activity under these conditions (Table I).

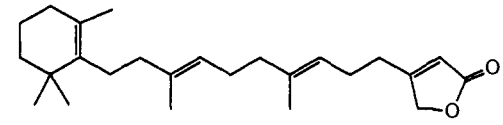
Scalaradial–PLA_2 Inhibition/Reactivation. Our data support a two-step mechanism for the reaction of **3a** with a lysine residue of PLA_2, which is analogous to that described by Cimino et al.[23] for the reaction of methylamine with scalaradial (Scheme V). Because we have shown that the inhibition of PLA_2 by scalaradial can be reversed with hydroxylamine in the early stages of inhibition (Table I), the initial reaction of scalaradial with PLA_2 probably involves the formation of an imine between the C-20 aldehyde and a lysine residue, followed by an intramolecular reaction involving the second aldehyde at C-19, a reaction which can also be reversed by treatment with hydroxylamine. Once pyrrole formation occurs with addition of a nucleophile at C-16, the scalaradial–PLA_2 adduct can no longer be cleaved with hydroxylamine at 41 °C (Table I). In the model reaction, the nucleophile which adds at C-16 is water (or hydroxide ion).[23] When scalaradial reacts with PLA_2, it is possible that the nucleophile is either water or a second nucleophile on PLA_2, perhaps another lysine residue. Structure–activity relationship studies on analogues of scalaradial further support the hypothesis that pyrrole formation plays an important role in inactivating PLA_2. 12-*epi*-12-Desacetylscalaradial (**3b**), in which the 12-α-acetate of scalaradial is replaced by a 12-β-hydroxy group, can completely inactivate the enzyme at similar doses to **3a**, although it is less potent.[5] 12,18-*diepi*-12-Desacetylscalaradial (**3c**) differs from **3a** in two ways: in addition to the structural difference at C-12, the aldehyde at C-18 is α (axial) rather than β (equitorial). Cimino et al.[23] reported that no reaction occurred between methylamine and 1,4-dialdehydes with the 18-*epi* geometry, explaining that the difference in reactivity toward epimeric 1,4-dialdehydes was dependent on the distance between the aldehyde groups. Interestingly, epimerization at C-18 results in significant loss in potency as well as loss of the ability to completely inactivate bee venom PLA_2.[5]

Table III. Inhibition of PLA_2 by Luffariellolide and Sodium Borohydride Treatment, Dialysis, and Reversal of Inhibition with Hydroxylamine

enzyme treatment	% inhibition predialysis[a]	% inhibition postdialysis[b]	% inhibition post-hydroxylamine treatment[b]
2	60 ± 1	54 ± 1	2 ± 6
2/$NaBH_4$	62 ± 2	56 ± 4	44 ± 8

[a] Data represent mean ± standard error (N = 4). [b] Data represent mean ± standard error (N = 3).

Sodium Borohydride Reduction of the Luffariellolide–PLA_2 Complex: Formation of an Irreversibly Bound Inhibitor. Manoalide contains two reactive rings, and the inhibition of PLA_2 by manoalide cannot be reversed by dilution or dialysis.[10] Luffariellolide contains only the γ-hydroxybutenolide ring (ring A), which has been demonstrated to be critical to the potency of manoalide analogues.[13,22] Furthermore, we have demonstrated using **12** that when both rings are present, amines preferentially react at ring A to form a stable adduct, indicating that luffariellolide possesses the key pharmacophore. Glaser observed that inhibition of PLA_2 with luffariellolide can be partially reversed by dialysis.[2,7] This is not surprising because Schiff base formation is a reversible process, and dialysis would be expected to remove any free luffariellolide, thereby driving the reaction toward hydrolysis. We had observed that reduction of the model γ-aminobutenolide **6b** with $NaBH_4$ produced the γ-amino acid **7a** (Scheme II). We therefore reacted the luffariellolide–PLA_2 complex **6c** with sodium borohydride to form an irreversibly bound adduct **7b**. PLA_2 was preincubated with luffariellolide, the solution was diluted either with borate buffer or with buffered $NaBH_4$, and samples were dialyzed to remove the reagent. The percent inhibition was measured against control samples, which were preincubated with methanol instead of luffariellolide but were otherwise treated identically. The samples were then treated with hydroxylamine and PLA_2 activity was again measured (Table III). The PLA_2 which was treated with luffariellolide alone was essentially completely reactivated (2% inhibition). Conversely, when the $NaBH_4$-reduced complex was treated with hydroxylamine, the enzyme was 44% inhibited. This indicates that reduction with $NaBH_4$ produced a substantial quantity of the secondary amine **7b**, which cannot be cleaved with hydroxylamine. Preincubation of PLA_2 with luffariellolide γ-lactone (**21**), which is the



21

$NaBH_4$-reduction product of luffariellolide, did not inhibit the enzyme. The data strongly suggest that when PLA_2 is treated with luffariellolide, it forms a complex in which the majority of luffariellolide is covalently bound to lysine via an imine or a γ-aminobutenolide (**6c**) linkage and that the adduct can be reduced with $NaBH_4$ to permanently attach the drug to the enzyme.

Conclusions

The chemical mechanisms by which manoalide and luffariellolide inactivate PLA_2 have eluded researchers for some time. Scalaradial, which has only recently been described as a PLA_2 inhibitor,[5,6] will undoubtedly become the focus of future investigations. Model reactions suggested that the primary reaction involved in inhibition of PLA_2 by these compounds is imine formation between the hemiacetal or aldehyde functionalities in the inhibitors and lysine residues in PLA_2. We therefore tested the hypothesis that imine formation was involved in PLA_2 inhibition by applying a methodology historically used to cleave

(35) We have observed strong hydrogen-bonding interactions between MMA (**12**) and *N*,*N*-diisopropylformamide but not between luffariellolide (**2**) and the amide.

(36) Kuchler, K.; Gmachl, M.; Sippl, M. J.; Kreil, G. *Eur. J. Biochem.* **1989**, *184*, 249–254.
(37) Ghomashchi, F.; Yu, B.; Mihelich, E. D.; Jain, M. K.; Gelb, M. H. *Biochemistry* **1991**, *30*, 9559–9569.
(38) Reynolds, L. J.; Mihelich, E. D.; Dennis, E. A. *J. Biol. Chem.* **1991**, *266*, 16512–16517.

Schiff bases. Since we have successfully reversed the inhibition with hydroxylamine, the role of imine formation in the inhibition of bee venom PLA_2 by these drugs has been demonstrated.

The experimental results are in accord with our proposed mechanism for the drug–enzyme reaction involved in the inhibition of bee venom PLA_2. We have shown that the initial reaction which forms a covalent linkage between bee venom PLA_2 and manoalide, luffariellolide, or scalaradial is imine formation. The data also support the hypotheses that scalaradial reacts further to form a pyrrole adduct, luffariellolide reacts with lysine to form a γ-(alkylamino)butenolide **6c**, and that manoalide reacts in a stepwise fashion to form a γ-(alkylamino)butenolide at ring A followed by a second interaction involving ring B to form a drug–enzyme adduct which can be substantially reactivated by hydroxylamine. These data, together with knowledge of the location of the manoalide–PLA_2 binding site,[15] will be instrumental in elucidating the mechanism by which manoalide inactivates bee venom PLA_2. The ability to reverse the binding of luffariellolide to PLA_2 allows us for the first time to design materials that can be used for purification of PLA_2 enzymes by affinity chromatography.

The chemical reactivity of the marine natural products studied herein can be correlated with their enzyme inhibition profiles. Both manoalide and scalaradial form irreversible adducts with bee venom PLA_2 and can completely inactivate the enzyme (100% inhibition).[6,7,10] On the other hand, both luffariellolide and 12-desacetyl-12,18-*diepi*-scalaradial, which can form only a single Schiff base with a lysine residue on PLA_2, cause only 70% inhibition of PLA_2 from bee venom.[2,5,7] The inability to completely inactivate bee venom PLA_2 may therefore reflect an equilibrium mixture of bound and unbound drug. Alternatively, it may reflect greater flexibility of the drug bound to the enzyme at only a single point of attachment. The flexibility could prevent the drugs from acting as effectively as manoalide and scalaradial, which are more rigidly bound to the enzyme as a result of multiple drug–enzyme reactions and which may interfere with desolvation at the microinterface between the enzyme and the substrate aggregate, as proposed in the Gelb model.[37]

Experimental Section

Materials. Bee venom PLA_2 (1160 units/mg of protein) and D,L-α-phosphatidylcholine dipalmitoyl were obtained from the Sigma Chemical Co. L-α-Dipalmitoyl[2-palmitoyl-9,10-$^3H(N)$]phosphatidylcholine (specific activity 50 Ci/mmol) and 1-[^{14}C]palmitic acid (specific activity 8.9 mCi/mmol) were obtained from New England Nuclear. Spectra/Por dialysis tubing (MWCO 10000) was purchased from Fisher Scientific. Manoalide (**1**) was a generous gift from Allergan Pharmaceuticals, Inc. Luffariellolide (**2**) was isolated and purified as described previously.[2] Scalaradial (**3a**) was a generous gift from Dr. Valerie Paul (University of Guam).

Luffariellolide–β-Phenylethylamine Adduct (6a). Luffariellolide (**2**, 26 mg, 48 mM) was dissolved in ethanol (1.5 mL). β-Phenethylammonium acetate (1 equiv, 69 μL of a 1 M solution in ethanol) was added, and the solution was stirred at room temperature for 2 h when the solvent was evaporated. The mixture was chromatographed by HPLC on Partisil, eluting with 3:1 hexane:ethyl acetate to obtain **6a** (21 mg, 64% yield). UV ($CHCl_3$) 240 nm (ε 2400); IR ($CHCl_3$) 2930, 2910, 1740 (br) cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.20 (m, 5 H), 5.83 (s, 1 H, H-2), 5.57 (s, 1 H, H-25), 5.10 (br m, 2 H, H-6, H-10), 3.00 (m, 2 H, H-1′), 2.80 (t, 2 H, J = 7 Hz, H-2′), 1.65 (s, 3 H, H-22), 1.61 (br s, 6 H, H-23, H-24), 1.00 (s, 6 H, H-20, H-21). The ^{1}H NMR spectrum was subsequently recorded in $CDCl_3$ with a drop of d_1-TFA. Initially, an NH signal appeared as a broad triplet at 5.1 ppm. The broad multiplet assigned to H-1′ shifted downfield (3.65 ppm) upon addition of the acid. When the NH signal was irradiated, the H-1′ signal collapsed to a triplet. With time, the NH proton exchanged with deuterium. As the NH signal exchanged, the H-1′ signal sharpened to a triplet. ^{13}C NMR (50 MHz, $CDCl_3$) δ 170.8 (s, C-1), 168.5 (s, C-3), 139.1 (s, C-3′), 137.1 (2 s, C-7, C-11), 136.2 (s, C-14), 128.7 (2 d, C-5′, C-7′), 128.5 (2 d, C-4′, C-8′), 126.9 (s, C-15), 126.3 (d, C-6′), 123.3 (d, C-10/6), 122.1 (d, C-6/10), 118.5 (d, C-2), 95.1 (d, C-25), 45.4 (t, C-1′), 40.3 (t), 39.8 (t), 39.6 (t, C-18), 36.7 (t, C-2′), 34.8 (s, C-19), 32.7 (t, C-16), 28.6 (2 q, C-20, C-21), 28.4 (t), 27.9 (t), 26.5 (t), 25.3 (t), 19.8 (q), 19.5 (t, C-17), 16.2 (q), 16.0 (q); HREIMS obsd m/z 489.3608, $C_{33}H_{47}NO_2$ requires 489.3607.

Luffariellolide–n-Butylamine Adduct (6b). Luffariellolide (**2**, 90.6 mg) was dissolved in ethanol (5 mL). *n*-Butylammonium acetate (1 equiv of a 1 M solution in ethanol) was added and the solution stirred at room temperature. After 14 h, the solvent was evaporated. The almost pure product was purified by chromatography on silica gel, eluting with 3:1 hexane:ethyl acetate to obtain **6b** (80 mg, 77% yield). UV (MeOH) 218 nm (ε 8600); IR ($CHCl_3$) 3000, 2930, 2915, 2830, 1740 (strong) cm^{-1}; ^{1}H NMR δ 5.84 (s, 1 H, H-2), 5.59 (s, 1 H, H-25), 5.08 (t, 2 H, J = 6 Hz, H-6, H-10), 2.71 (t, 2 H, J = 7 Hz, H-1′), 2.38 (br m, 2 H), 2.28 (br t, 2 H, J = 7 Hz), 1.94 (m, 8 H), 1.87 (t, 2 H, J = 6 Hz), 1.60 (br s, 6 H), 1.57 (s, 3 H), 1.44 (m, 8 H), 0.96 (s, 6 H), 0.88 (t, 3 H, J = 7 Hz, H-4′); ^{13}C NMR (50 MHz, $CDCl_3$) δ 171.8 (s, C-1), 168.5 (s, C-3), 2 × 137.0 (s, C-7, C-11), 136.1 (s, C-14), 126.8 (s, C-15), 123.1 (d, C-10/6), 122.1 (d, C-6/10), 118.3 (d, C-2), 95.2 (d, C-25), 43.8 (t, C-1′), 40.2 (t), 39.7 (t), 39.5 (t, C-18), 34.8 (s, C-19), 32.6 (t, C-16), 32.2 (t, C-2′), 2 × 28.5 (q, C-20, C-21), 28.4 (t), 27.8 (t), 26.4 (t), 25.2 (t), 20.1 (t, C-3′), 19.7 (q), 19.4 (t, C-17), 16.1 (q), 15.9 (q), 13.7 (q, C-4′); HRFABMS obsd m/z 574.2655, $C_{29}H_{47}NO_2Cs$ (M + Cs)$^+$ requires 574.2661.

Reduction of Luffariellolide–n-Butylamine Adduct. The adduct **6b** (80 mg) was dissolved in dry MeOH (5 mL). The solution was stirred under argon at 0 °C, and $NaBH_4$ (7 mg, 1 equiv) was added. After 1 h, the reaction mixture was quenched with dilute aqueous HCl solution. The solvent was evaporated, and the mixture was redissolved in ethyl acetate and extracted with a dilute aqueous HCl solution. The organic extract was dried over anhydrous Na_2SO_4, and the solvent was evaporated. Hexane:ethyl acetate (2:1) was added to precipitate the γ-amino acid **7a** (34.7 mg). The remaining material (a yellow oil) was purified by HPLC on Partisil, eluting with 2:1 hexane:ethyl acetate to obtain an additional 22.6 mg of **7a** (total of 57.2 mg of **7a**, 71% yield). UV ($CHCl_3$) 241 nm (ε 4500); IR ($CHCl_3$) 1675, 1650 cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$) 9.52 (br s, 1 H), 6.10 (s, 1 H, H-2), 5.06 (br t, 2 H, H-6, H-10), 3.77 (m, 2 H, H-25), 2.90 (m, 2 H, H-1′), 2.37 (m, 2 H), 2.20 (m, 2 H), 2.00 (m, 8 H), 1.88 (t, 2 H, J = 6 Hz), 1.76 (m, 2 H), 1.61 (s, 3 H), 1.59 (br s, 6 H), 1.40 (m, 6 H), 0.97 (s, 6 H), 0.92 (t, 3 H, J = 7 Hz, H-4′); ^{13}C NMR (50 MHz, $CDCl_3$) δ 170.0 (s, C-1), 152.3 (s, C-3), 137.3 (s, C-14), 137.0 (s, C-7/11), 136.2 (s, C-11/7), 126.8 (s, C-15), 123.3 (d, C-6/10), 123.1 (d, C-10/6), 121.0 (d, C-2), 47.5 (t, C-25/1′), 46.0 (t, C-1′/25), 40.2 (t, C-8/12/4), 40.1 (t, C-12/4/8), 39.7 (t, C-4/8/12), 39.6 (t, C-18), 34.9 (s, C-19), 32.7 (t, C-16), 2 × 28.5 (q, C-20, C-21), 28.3 (t, C-9/5/13/2′), 27.8 (t, C-9/5/13/2′), 26.5 (t, C-9/5/13/2′), 25.5 (t, C-9/5/13/2′), 19.81 (t, C-3′), 19.77 (q, C-22), 19.5 (t, C-17), 16.2 (q, C-23/24), 16.0 (q, C-24/23), 13.4 (q, C-4′); HRFABMS obsd m/z 444.3818, $C_{29}H_{50}O_2N$ (MH)$^+$ requires 444.3842.

Luffariellolide Oxime (8). Luffariellolide (**2**, 29.6 mg) was dissolved in ethanol (0.8 mL). Na_2CO_3 (0.56 equiv, 43 μL of a 1 M aqueous solution) was added while stirring at room temperature. Hydroxylamine hydrochloride (1 equiv, 79 μL of a 1 M aqueous solution) was added dropwise. The reaction was allowed to proceed for 1 h, and a few drops of dilute aqueous HCl were added. The solvent was evaporated, the material was dissolved in chloroform, and the inorganic salts were removed by filtration. This procedure resulted in quantitative conversion of luffariellolide (**2**) to luffariellolide oxime (**8**, 31.7 mg, >99% yield). Oxime **8** was also obtained by similar treatment of luffariellolide–*n*-butylamine adduct **6b** (20 mg) in ethanol (0.6 mL). The hydroxylamine treatment resulted in quantitative conversion of **6b** to **8** with concomitant release of *n*-butylamine: the ^{1}H NMR spectrum of the products (19.2 mg) contained signals due to **8** and to *n*-butylammonium chloride. The ammonium salt was removed by chromatography on silica gel with significant loss of the product **8** (10.6 mg, 55% recovery). UV ($CHCl_3$) 255 nm (ε 1270); IR ($CHCl_3$) 3260, 2930, 1690, 1625 cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$) δ 9.03 (s, 1 H), 5.97 (s, 1 H), 5.09 (br t, 2 H), 1.61 (s, 3 H), 1.58 (s, 6 H), 0.97 (s, 6 H); HRCIMS obsd m/z 401.2914, $C_{25}H_{39}NO_3$ requires 401.2930. Several signals in the ^{13}C NMR were too broad to be observed, and **8** was therefore converted by treatment with diazomethane to its methyl ester: UV ($CHCl_3$) 255 nm (ε 1360); IR ($CHCl_3$) 3320, 3020, 2930, 2870, 1710, 1630 cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$) δ 9.00 (s, 1 H), 5.91 (s, 1 H), 5.10 (t, 2 H, J = 6 Hz), 3.71 (s, 3 H), 1.61 (s, 3 H), 1.58 (br s, 6 H), 0.97 (s, 6 H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 165.9 (s), 150.0 (s), 148.9 (d), 137.2 (s), 136.5 (s), 136.1 (s), 126.9 (s), 123.5 (d), 122.8 (d), 121.3 (d), 51.5 (q), 40.3 (t), 39.8 (t), 39.7 (t), 35.0 (s), 33.1 (t), 32.7 (t), 28.6 (2 q), 27.9 (t), 27.2 (t), 26.6 (t), 19.8 (q), 19.5 (t), 16.1 (q), 16.0 (q); HRCIMS obsd m/z 416.3153 (M + H)$^+$, $C_{26}H_{42}NO_3$ requires 416.3165.

Synthesis of Manoalide Methyl Analogue (12). MMA (**12**) was prepared by methods described by Garst[24] and by Kernan and Faulkner.[25] Tiglic aldehyde silyl enol ether (**9**) was prepared as follows: triethylamine (8.56 mL, 1.5 equiv) was added to dichloromethane (300 mL freshly distilled over CaH_2, under argon), and the solution was stirred under argon at 0 °C. Tiglic aldehyde (3.95 mL, 1 equiv) was added followed by trimethylsilyl-triflate (10 g). After 70 min, the reaction mixture was partitioned with the following chilled (0 °C) aqueous solutions: 5%

$NaHCO_3$ (250 mL), 1 N HCl (250 mL), 2 × 5% $NaHCO_3$ (200 mL each). The organic phase was dried over anhydrous Na_2SO_4 and the solvent evaporated under reduced pressure at room temperature to obtain tiglic aldehyde silyl enol ether (**9**, 5.94 g, 91% yield). ^1H NMR (200 MHz, $CDCl_3$) δ 6.38 (s, 1 H), 6.30 (dd, 1 H, J = 11, 17 Hz), 4.97 (d, 1 H, J = 17 Hz), 4.82 (d, 1 H, J = 11 Hz), 1.69 (s, 3 H), 0.19 (s, 9 H).

9 (2.89 g, 1.1 equiv) was added to dichloromethane (500 mL freshly distilled over CaH_2) under an atmosphere of argon. The solution was cooled to −65 °C, and 3-furaldehyde (1.45 mL, 1 equiv) was added, followed by $BF_3{\cdot}OEt_2$ (2.1 mL, 1 equiv). After 21 h, the reaction was quenched with 5% aqueous $NaHCO_3$ and allowed to warm to room temperature. The solution was partitioned with 5% aqueous $NaHCO_3$ (2 × 200 mL). The dichloromethane extract was dried over anhydrous Na_2SO_4, and the solvent was evaporated. The extract was purified by flash chromatography on silica gel, eluting with 1:1 hexane:ethyl acetate to obtain the *trans*-furan **10** (1.84 g, 55% yield based on **9**): ^1H NMR (200 MHz, $CDCl_3$) δ 9.34 (d, 1 H, J = 2 Hz, H-9), 7.39 (s, 1 H, H-1 or H-10), 7.38 (s, 1 H, H-10 or H-1), 6.55 (br t, 1 H, J = 7 Hz, H-6), 6.40 (s, 1 H, H-2), 4.85 (t, 1 H, J = 7 Hz, H-4), 2.78 (br t, 2 H, J = 7 Hz, H-5), 2.48 (br s, OH), 1.71 (s, 3 H, H-8); ^{13}C NMR ($CDCl_3$, 50 MHz) δ 195.2 (d, C-9), 149.7 (d, C-6), 143.6 (d, C-1), 140.9 (s, C-7), 139.0 (d, C-10), 128.2 (s, C-3), 108.2 (d, C-2), 65.6 (d, C-4), 37.2 (t, C-5), 9.3 (q, C-8); HREIMS obsd m/z 180.0780, $C_{10}H_{12}O_3$ requires 180.0787.

The *trans*-furan **10** (620 mg) was dissolved in benzene (600 mL). Argon gas was bubbled through the solution for 30 min to remove oxygen. The solution was irradiated at 300 nm (by using a 254-nm lamp filtered through a Pyrex flask) for 22.5 h. The solvent was evaporated, and the mixture was chromatographed on a silica gel flash column, eluting with 3:2 hexane:ethyl acetate to obtain furan **10** (111 mg, 18% recovery) and the hemiacetal **11** (455 mg, 73% yield): ^1H NMR ($CDCl_3$, 200 MHz) δ 7.43 (s, 1 H, H-1 or H-10), 7.40 (s, 1 H, H-10 or H-1), 6.43 (s, 1 H, H-2), 5.69 (br t, 1 H, H-6), 5.25 (s, 1 H, H-9), 4.96 (dd, 1 H, J = 4, 11 Hz, H-4), 3.20 (br s, 1 H, OH), 2.19 (m, 2 H, H-5), 1.77 (s, 3 H, H-8); ^{13}C NMR ($CDCl_3$, 50 MHz) δ 143.2 (d, C-1), 139.4 (d, C-10), 133.0 (s, C-7), 126.3 (s, C-3), 122.6 (d, C-6), 108.9 (d, C-2), 92.6 (d, C-9), 61.7 (d, C-4), 31.5 (t, C-5), 19.0 (q, C-8); HRFABMS obsd m/z 179.0701, $C_{10}H_{11}O_3$ $(M - H)^+$ requires 179.0708.

N,N-Diisopropylethylamine (78 μL, 2 equiv) was added to a solution of **11** (55 mg, 0.02 M) in dichloromethane (15 mL). Oxygen gas was bubbled through the solution for 10 min; thereafter, the solution was kept under an atmosphere of oxygen. Polymer-bound rose bengal catalyst was added, and the solution was cooled to −78 °C and irradiated with a 500-W tungsten incandescent lamp for 4 h. After this time, the solution was allowed to warm to room temperature. The photosensitizer was removed by filtration, and the dichloromethane solution was washed with aqueous NaH_2PO_4 buffer (2 × 10 mL, 1 M, pH 4.3). The combined aqueous extracts were extracted with ethyl acetate (4 × 15 mL). The organic extracts were dried over anhydrous Na_2SO_4, and the solvents were evaporated under reduced pressure. The ethyl acetate extract contained MMA (**12**, 48.2 mg, 74% yield based on **11**): UV (MeOH) 211 nm (ϵ 7500); IR ($CHCl_3$) 3360, 3020, 2920, 1760 cm^{-1}; ^1H NMR (200 MHz, CD_3OD) δ 6.26 (s, 0.5 H, H-10), 6.12 (s, 0.5 H, H-2 or H-10), 6.07 (s, 0.5 H, H-2 or H-10), 6.03 (s, 0.5 H, H-2), 5.71 (br t, 1 H, H-6), 5.27 (s, 0.5 H, H-9), 5.24 (s, 0.5 H, H-9), 4.87 (br m, 1 H, H-4), 2.23 (br m, 2 H, H-5), 1.78 (s, 3 H, H-8); ^{13}C NMR (50 MHz, CD_3OD) δ 172.9 (C-1), 170.8 (C-3), 135.3 (C-7), 122.1 (C-6), 117.8 (C-2), 100.1 (C-10), 93.1 (C-9), 64.0 (C-4), 30.2 (C-5), 19.2 (C-8); HRCIMS obsd m/z 212.0933, $C_{10}H_{14}NO_4$ $(M + NH_4 - H_2O)^+$ requires 212.0923.

Preparation of 13–15. *n*-Butylamine (13 μL, 1 equiv) was added to a solution of MMA (**12**, 28 mg) in CH_3CN (6.7 mL). The mixture was stirred at room temperature for 15 h. The solvent was evaporated and the mixture partitioned between dichloromethane (15 mL) and 0.1 M NaH_2PO_4 buffer (4 × 12 mL). The dichloromethane extract was dried over anhydrous Na_2SO_4, and the solvent was evaporated. The ^1H NMR spectrum indicated that the mixture contained the precursors of **13–15** (unreduced forms). The mixture was dissolved in methanol (5 mL) and cooled to 0 °C under an atmosphere of argon. $NaBH_4$ (9 mg, 2 equiv) was added. After 1 h, the reaction was quenched by addition of a few drops each of 0.1 M NaH_2PO_4 and dilute HCl solution. The solvent was removed, and the reduction product was partitioned between 0.1 M phosphate buffer (15 mL) and dichloromethane (3 × 15 mL). The combined dichloromethane extracts were back-extracted with H_2O (15 mL). The dichloromethane extract amounted to only 2.6 mg of material. The combined aqueous extracts were evaporated to dryness and triturated with MeOH. The methanol-soluble material (29.5 mg) contained some salts. This mixture was chromatographed by RP HPLC, eluting with 3:2 H_2O:MeOH at 2.3 mL/min to obtain MMA–γ-(*n*-butylamino) acid (**13**, 2.7 mg), seco-MMA–γ-(*n*-butylamino) acid (**14**, 3.1 mg), and dehydro-seco-MMA-γ-(*n*-butylamino) acid (**15**, 0.9 mg).

MMA–γ-(*n*-Butylamino) Acid (13). UV (MeOH) 207 nm (ϵ 11 000); IR (solid) 1648 cm^{-1}; ^1H NMR (500 MHz, CD_3OD) δ 6.13 (s, 1 H, H-2), 5.32 (t, 1 H, J = 7 Hz, H-6), 4.13 (t, 1 H, J = 7 Hz, H-4), 4.07 (d, 1 H, J = 13 Hz, H-9), 4.03 (d, 1 H, J = 13 Hz, H-9), 3.73 (d, 1 H, J = 14 Hz, H-10), 3.68 (d, 1 H, J = 14 Hz, H-10), 2.98 (m, 2 H, H-1′), 2.36 (t, 1 H, J = 7 Hz, H-5), 1.79 (s, 3 H, H-8), 1.67 (m, 2 H, H-2′), 1.44 (m, 2 H, H-3′), 0.98 (t, 3 H, J = 8 Hz, H-4′); ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.7, 145.2, 138.7, 133.1 (br), 124.0, 76.2, 61.5, 47.7, 45.2, 34.7, 29.5, 22.0, 20.8, 13.9; HRFABMS obsd m/z 272.1844, $C_{14}H_{26}O_4N^+$ $(MH)^+$ requires 272.1862.

Seco-MMA–γ-(*n*-Butylamino) Acid (14). UV (MeOH) 205 nm (ϵ 7300); IR (solid) 1648 cm^{-1}; ^1H NMR (500 MHz, CD_3OD) δ 6.14 (s, 1 H, H-2), 5.42 (t, 1 H, J = 7 Hz, H-6), 4.16 (t, 1 H, J = 7 Hz, H-4), 3.93 (s, 2 H, H-9), 3.76 (d, 1 H, J = 14 Hz, H-10), 3.73 (d, 1 H, J = 14 Hz, H-10), 3.00 (m, 2 H, H-1′), 2.32 (dt, 1 H, J = 7, 13 Hz, H-5), 1.68 (m, 2 H, H-2′), 1.66 (s, 3 H, H-8), 1.44 (m, 2 H, H-3′), 0.98 (t, 3 H, J = 8 Hz, H-4′); HRFABMS obsd m/z 272.1850, $C_{14}H_{26}O_4N$ $(M + H)^+$ requires 272.1862.

Dehydroseco-MMA-γ-(*n*-Butylamino) Acid (15). ^1H NMR (500 MHz, CD_3OD) δ 6.96 (dd, 1 H, J = 11, 15 Hz, H-5), 6.31 (d, 1 H, J = 15 Hz, H-4), 6.23 (d, 1 H, J = 11 Hz, H-6), 6.14 (s, 1 H, H-2), 4.04 (s, 2 H, H-9), 3.97 (s, 2 H, H-10), 3.02 (t, 2 H, H-1′), 1.87 (s, 3 H, H-8), 1.69 (m, 2 H, H-2′), 1.44 (m, 2 H, H-3′), 0.98 (t, 3 H, J = 8, H-4′).

Luffariellolide γ-Lactone (21). γ-Lactone **20** was prepared by treating luffariellolide (**2**, 23.1 mg) in methanol (3 mL) with excess $NaBH_4$ (3.4 mg, 1.5 equiv) at 0 °C for 1 h. The reaction was quenched with dilute HCl solution, and the solvent was evaporated. The product was purified by partitioning between water (10 mL) and ethyl acetate (3 × 10 mL). The organic extract was dried over anhydrous Na_2SO_4, and the solvent was evaporated to obtain luffariellolide γ-lactone (**21**, 17.7 mg, 80% yield). UV (MeOH) 218 nm (ϵ 9700); IR ($CHCl_3$) 2930, 2870, 1785, 1750 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 5.82 (s, 1 H), 5.08 (t, 2 H, J = 6 Hz), 4.71 (s, 2 H), 2.41 (m, 2 H), 2.29 (m, 2 H), 1.99 (m, 6 H), 1.88 (t, 2 H, J = 6 Hz), 1.61 (br s, 6 H), 1.57 (s, 3 H), 1.52 (m, 4 H), 1.37 (m, 2 H), 0.96 (s, 6 H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 174.1 (s), 170.3 (s), 137.4 (s), 137.0 (s), 136.2 (s), 126.8 (s), 123.1 (d), 121.8 (d), 115.4 (d), 73.1 (t), 40.2 (t), 39.7 (t), 39.5 (t), 34.9 (s), 32.6 (t), 28.7 (t), 2 × 28.5 (q), 27.8 (t), 26.3 (t), 25.6 (t), 19.7 (q), 19.4 (t), 16.1 (q), 16.0 (q); HRDCIMS obsd m/z 371.2964, $C_{25}H_{38}O_2$ $(M + H)^+$ requires 371.2950.

PLA_2 Radiometric Assay. PLA_2 activity was measured using a radioassay as described previously.[10] Protein concentration was determined by the Bradford method.[39]

Inhibition/Reactivation Studies. The inhibition/reactivation experiments were carried out with PLA_2 from bee venom (*Apis mellifera*). All experiments including controls were carried out in duplicate, and each sample was assayed for PLA_2 activity in duplicate, thereby generating four data points for each experimental condition. PLA_2 (16 μM) was preincubated at 41 °C with manoalide (**1**, 25 μM), luffariellolide (**2**, 50 μM), or scalaradial (**3a**, 25 μM) or with methanol (control) in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (10 mM HEPES, 1 mM $CaCl_2$, pH 7.4 at 41 °C) for 1 h unless otherwise indicated. The control and drug-treated enzyme solutions were then diluted 2-fold with HEPES buffer or with hydroxylamine hydrochloride in HEPES buffer (final concentration of hydroxylamine 50 mM, pH 7.4) and incubated for 2 h at 41 °C, after which time 5-μL aliquots were taken to measure the PLA_2 activity (final concentration of drug under assay conditions: 0.125 μM manoalide, 0.25 μM luffariellolide, 0.125 μM scalaradial).

Hydroxylamine-Pretreated PLA_2. Hydroxylamine hydrochloride was added to HEPES buffer (10 mM HEPES, 1 mM $CaCl_2$, pH 7.4 at 41 °C) to a final concentration of 50 mM hydroxylamine. The pH was adjusted to 7.4 with sodium hydroxide. PLA_2 (16 μM) was dissolved in this solution. The hydroxylamine-pretreated PLA_2 was incubated with manoalide (25 μM) or with methanol (control) at 41 °C for 1 h. Samples were then diluted 2-fold with 50 mM hydroxylamine solution and incubated for an additional 2 h at 41 °C. Aliquots (5 μL) were then taken to measure PLA_2 activity.

Luffariellolide–PLA_2 Sodium Borohydride Reduction. PLA_2 (21 μM) was preincubated with **2** (50 μM), the γ-lactone **14** (50 μM), or methanol (control) at 41 °C in borate buffer (pH 9) for 1 h. The control and drug-treated enzyme solutions were then diluted 2-fold with borate buffer and incubated at 0 °C for 2 h, after which time 5-μL aliquots were taken to measure the PLA_2 activity (final concentration of drug under assay conditions: 0.25 μM). Alternatively, the control and drug-treated enzyme solutions were diluted 2-fold with borate buffer containing $NaBH_4$ (final concentration of $NaBH_4$: 4 mM) and incubated at 0 °C for 2 h,

(39) Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248–254.

after which time 5-μL aliquots were taken to measure the PLA_2 activity (final concentration of drug under assay conditions: 0.25 μM). Controls and samples which had been preincubated with luffariellolide, both treated with or without $NaBH_4$, were dialyzed against HEPES buffer (pH 7.4, 0 °C, 17 h with two buffer changes), and the postdialysis activity was measured. The samples were then diluted 2-fold with HEPES or with hydroxylamine in HEPES (final concentration of hydroxylamine: 50 mM) and incubated at 41 °C for 2 h, after which time 10-μL aliquots were taken to measure the PLA_2 activity.

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Structure and Reactivity of Lithium Diisopropylamide in the Presence of *N,N,N',N'*-Tetramethylethylenediamine

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Abstract: Lithium diisopropylamide (LDA) crystallizes from *N,N,N',N'*-tetramethylethylenediamine (TMEDA)/hexane mixtures as an infinite array of dimers linked by bridging (nonchelating) TMEDA ligands. ^6Li and ^{15}N NMR spectroscopic studies reveal that LDA in neat TMEDA exists as a cyclic dimer bearing a single η^1-coordinated TMEDA ligand on each lithium. The equilibrium of solvent-free LDA and the TMEDA-solvated dimer shows a very strong temperature dependence. TMEDA coordinates readily only at low temperature. High TMEDA concentrations are required to saturate the lithium coordination spheres at ambient temperatures. One equivalent of THF readily displaces TMEDA from the coordination sphere to produce the dimeric LDA/THF solvate characterized previously. Kinetics of metalation of 2-methylcyclohexanone *N,N*-dimethylhydrazone monitored by Fourier-transform infrared (FT-IR) spectroscopy are consistent with a rapid, spectroscopically undetectable dimer–monomer preequilibrium followed by rate-limiting proton transfer. The reaction rate is independent of the TMEDA concentration. Related spectroscopic and rate studies using dimethylethylamine lacking the capacity to chelate show strong parallels with the TMEDA data. We conclude that TMEDA chelates of LDA are of no measurable consequence throughout the reaction coordinate. MNDO calculations of THF and TMEDA solvates of Me_2NLi and LDA monomers and dimers qualitatively support the conclusions derived from the experimental data.

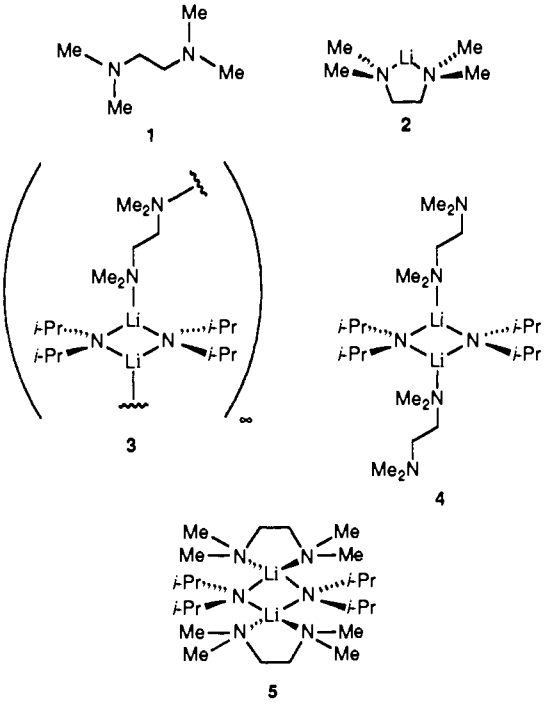
Introduction

Upon taking a casual survey of the literature of organolithium chemistry, one is struck by the central role played by *N,N,N',N'*-tetramethylethylenediamine (TMEDA; **1**). The crystallographic literature is replete with the Li–TMEDA chelate substructure **2**.[1] The kinetic consequences of TMEDA chelates are even more prominent. TMEDA dramatically accelerates organolithium reaction rates, improves product yields, and alters product distributions.[2] The consensus is that the two Lewis basic dimethylamino moieties function cooperatively to make TMEDA a truly outstanding ligand for the Li^+ ion.

We describe below structure and reactivity studies of lithium diisopropylamide (LDA) in the presence of TMEDA. An X-ray crystal structure reveals that LDA crystallizes from TMEDA/hexane as an infinite array of dimer units connected by η^1-linkages (e.g., **3**). We further demonstrate that TMEDA-solvated LDA is dimeric and desolvates at ambient temperatures *even in the absence of other donor ligands*. Less direct evidence implicates dimer **4** rather than the anticipated doubly chelated dimer **5** as the stable solution structure in the limit of high TMEDA concentration. Rate studies of the metalation of *N,N*-dimethylhydrazone **6** by LDA in neat TMEDA are interpreted in the context of the mechanism illustrated in Scheme I without invoking TMEDA chelates at any point along the reaction coordinate.[3]

Results

We will begin the Results section with a discussion of NMR spectroscopic studies of LDA in the presence of TMEDA. It is instructive to note, however, that the metalation rate data described subsequently signaled several structural subtleties that may have otherwise gone undetected.



Solution Structure of LDA in TMEDA. ^6Li and ^{15}N NMR spectra of analytically pure [^6Li,^{15}N]LDA[4] dissolved in neat

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