Phospholipase A_2 Inhibition and Modification by Manoalogue

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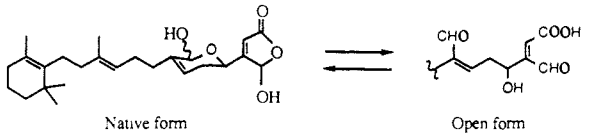
Received December 21, 1987

Abstract: Several analogues of the marine natural product manoalide have been chemically synthesized. One of these compounds, (*E*,*E*)-2-[3-(2,5-dihydro-2-hydroxy-5-oxo-3-furanyl)propylidene]-6,10-dimethyl-5,9-undecadienal, which has been named "manoalogue", causes a partial, irreversible inactivation of cobra venom phospholipase A_2 concomitant with a modification of about three lysine residues. The concentration of manoalogue required for half-maximal inhibition is similar to that observed for manoalide, although it differs in the rate and extent of inactivation. Manoalogue contains the terminal butenolide ring of manoalide as well as the latent α,β-unsaturated aldehyde present in the hemiacetal ring of manoalide. Either reduction of the aldehyde to an alcohol or methylation of the hydroxyl group on the butenolide ring abolished the molecule's ability to irreversibly inhibit phospholipase A_2 and to modify lysine residues. These results demonstrate that the presence of the α,β-unsaturated aldehyde and the opening of the lactone on the butenolide ring are necessary for inactivation to occur. N-terminal sequence analysis of the manoalogue-inhibited protein suggests that Lys-6 is one of the modified residues. A possible mechanism of inactivation of cobra venom phospholipase A_2 by manoalide and manoalogue consistent with these findings is proposed.

Manoalide (**1**) is a marine natural product isolated from the sponge *Luffariella variabilis*,[1] which has been demonstrated to have antiinflammatory activity in vivo. Early experiments by Jacobs and co-workers[2,3] showed that manoalide antagonized



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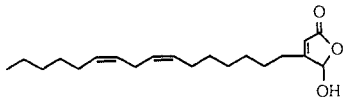
phorbol ester induced local inflammation in murine epidermis, but not that induced by arachidonic acid. The failure to block arachidonic acid induced inflammation suggested that manoalide may act at the level of phospholipase A_2(PLA_2). Manoalide has since been shown to be a potent inhibitor of both cobra[4,5] and bee venom[6,7] PLA_2. It has recently been shown to be less potent toward crude preparations of cytosolic phospholipase A_2's.[8]

Studies on the cobra enzyme in our laboratory showed that manoalide causes a time-dependent, irreversible inactivation of PLA_2, which is accompanied by a modification of about four lysine residues. The inactivation reaches a maximum of 85% with 15% residual activity. This inhibition is also pH-dependent, occurring faster at higher pH. Manoalide contains two cyclic moieties, a γ-lactone ring and a hemiacetal ring, one or both of which may open to generate an α,β-unsaturated aldehyde as shown above.[4,5] It has been suggested that modification of phospholipase A_2 involves a Michael addition of lysine residues to one or both of these α-β unsaturated aldehydes.[4]

Our laboratory is attempting to elucidate the mechanism of inactivation of PLA_2 by manoalide as part of our general aim to understand the mechanism of action of this enzyme.[9] Toward this end, we have previously reported the synthesis of 3-(*cis*,*cis*-7,10-hexadecadienyl)-4-hydroxy-2-butenolide (HDHB, **2**), a



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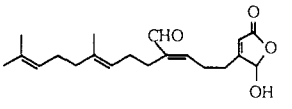
manoalide analogue containing a γ-lactone ring but lacking the hemiacetal ring.[5] The closed and open forms of the γ-lactone ring were shown by NMR experiments to be in rapid equilibrium between pHs 4 and 9, with the open cis form being preferred at pH 9.5. HDHB demonstrated a reversible inhibition of cobra venom PLA_2 that did not change with time, indicating that the γ-lactone ring alone is not sufficient to produce irreversible inactivation. In this paper, we report the synthesis of (*E*,*E*)-2-[3-(2,5-dihydro-2-hydroxy-5-oxo-3-furanyl)propylidene]-6,10-dimethyl-5,9-undecadienal, named "manoalogue" (**3**), a manoalide



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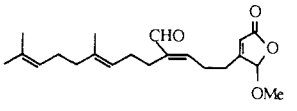
analogue that contains the α,β unsaturated aldehyde portion of the hemiacetal ring in addition to the γ-lactone ring. In contrast to HDHB, this compound causes a partial irreversible inactivation of cobra venom phospholipase A_2. In addition, the activity of this compound is compared to that of two other analogues, methylated manoalogue (**4**), in which the hydroxyl group on the γ-lactone ring is methylated to prevent ring opening, and reduced manoalogue (**5**), in which the free aldehyde is reduced to an alcohol.



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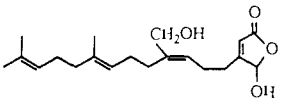

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Experimental Section

Materials. Phospholipase A_2 from cobra venom (*Naja naja naja*) was purchased from the Miami Serpentarium and purified as described elsewhere.[10] 1,2-Bis(decanoylthio)-1,2-dideoxy-*sn*-glycero-3-phosphorylcholine (chiral thio-PC) was prepared as described previous-

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ly.[11,12] 1,2-Bis(decanoylthio)-1,2-dideoxy-*rac*-glycero-3-phosphatidylcholine (racemic thio-PC) was synthesized in the same manner but with racemic tritylglycidol as the starting material. 4,4′-Dithiodipyridine was obtained from Aldrich, Triton X-100 from Sigma, and dipalmitoyl-PC from Calbiochem. All other reagents were analytical reagent grade or better.

Preparation of Keto Acetal 7. Distilled diisopropylamine (1.2 equiv) was placed in 300 mL of dry tetrahydrofuran (THF) and cooled to −10 °C under nitrogen. *n*-Butyllithium (1.1 equiv, 1.6 M solution in hexane) was added dropwise while the temperature was maintained below 0 °C. After addition of 34.53 g (1.1 equiv) of the dimethylhydrazone of pyruvic aldehyde cyclic acetal (prepared by ketalization of methacrolein with 1,3-propanediol, ozonolysis, and hydrazone formation) in 50 mL of THF, the resulting orange solution was stirred for 30 min at 0 °C. Geranyl bromide (39.5 g, 182 mmol) was then added in 50 mL of THF, and the reaction mixture was allowed to warm to room temperature and was stirred overnight. After the mixture was cooled with an ice bath, the reaction was quenched with 20 mL of H_2O and the mixture concentrated. After diethyl ether/water extractions, the organic layers were concentrated. The crude intermediate hydrazone was taken up in 300 mL of cold THF and shaken together with 100 mL of 2 N HCl. This mixture was then diluted with ether, and the layers were separated. Following typical ether/water extractions and drying over $MgSO_4$, the solutions were concentrated. Medium-pressure liquid chromatography[13] (MPLC, 8:1 hexane/ethyl acetate) afforded 33.5 g (69%) of keto acetal **7** (R_f 0.32, 3:1 hexane/ethyl acetate): ^{1}H NMR (300 MHZ, $CDCl_3$) δ 5.08 (m, 2 H), 4.77 (s, 1 H), 4.21 (dd, 2 H), 3.84 (dt, 2 H), 2.64 (t, 2 H), 2.26 (q, 2 H), 2.24–1.97 (m, 5 H), 1.68 (s, 3 H), 1.61 (s, 3 H), 1.59 (s, 3 H), 1.42 (d, 1 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 203.3 (C), 136.1 (C), 131.2 (C), 124.2 (CH), 122.6 (CH), 100.5 (CH), 67.0 (2 CH_2), 39.6 (CH_2), 37.4 (CH_2), 26.6 (CH_2), 25.6 (CH_2, CH_3), 21.4 (CH_2), 17.6 (CH_3), 15.9 (CH_3).

Preparation of Acetal Ester 8. Dry diisopropylamine (1.3 equiv) was placed in 250 mL of dry THF and cooled to −10 °C under nitrogen. *n*-Butyllithium (1.3 equiv, 1.6 M solution in hexane) was added dropwise keeping the temperature below 0 °C. The lithium diisopropylamide (LDA) solution was then cooled to −78 °C, and methyl (trimethylsilyl)acetate (1.3 equiv) was added dropwise. After the mixture was stirred for 1 h, a solution of keto acetal **7** (33.5 g, 126 mmol) in 60 mL of THF was added dropwise over 1 h. The reaction mixture was allowed to warm to room temprature overnight. After being quenched with 20 mL of water, the mixture was concentrated. After ether/water extractions, the organic layers were dried over K_2CO_3 and concentrated. Preparative chromatography (8:1 hexane/ethyl acetate) afforded 30.64 g (76% yield) of a 1:1.2 *E*/*Z* mixture of acetal ester **8**[R_f 0.44 (*E* isomer), 0.40 (*Z* isomer), 3:1 hexane/ethyl acetate]. Anal. Calcd for $C_{19}H_{30}O_4$: C, 70.77; H, 9.38. Found: C, 70.58; H, 9.14.

***E* Isomer**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.08 (s, 1 H), 5.19 (t, 1 H), 5.10 (t, 1 H), 4.92 (s, 1 H), 4.19 (dd, 2 H), 3.84 (dt, 2 H), 3.70 (s, 3 H), 2.65 (t, 2 H), 2.26–1.97 (m, 7 H), 1.65 (s, 3 H), 1.63 (s, 3 H), 1.60 (s, 3 H), 1.39 (d, 1 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 166.6 (C), 156.3 (C), 135.6 (C), 131.1 (C), 124.3 (CH), 123.7 (CH), 117.4 (CH), 101.0 (CH), 67.0 (2 CH_2), 50.9 (CH_3), 39.6 (CH_2), 28.4 (CH_2), 27.4 (CH_2), 26.7 (CH_2), 25.7 (CH_2), 25.6 (CH_3), 17.6 (CH_3), 15.9 (CH_3); IR ($CHCl_3$) 1719 cm^{-1}.

Z Isomer: ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.23 (s, 1 H), 5.75 (s, 1 H), 5.12 (m, 2 H), 4.15 (dd, 2 H), 3.93 (dt, 2 H), 3.71 (s, 3 H), 2.37 (m, 2 H), 2.24–1.98 (m, 7 H), 1.68 (s, 3 H), 1.61 (s, 6 H), 1.40 (d, 1 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.7 (C), 156.8 (C), 135.8 (C), 131.1 (C), 124.3 (CH), 123.3 (CH), 118.0 (CH), 97.6 (CH), 67.0 (2 CH_2), 51.1 (CH_3), 39.6 (CH_2), 31.4 (CH_2), 26.7 (CH_2), 26.3 (CH_2), 26.0 (CH_2), 25.6 (CH_3), 17.6 (CH_3), 15.9 (CH_3); IR ($CHCl_3$) 1717 cm^{-1}.

Preparation of Diacetal Hydrazone 9. Lithium aluminum hydride (0.75 equiv) was suspended in 70 mL of dry ether and cooled to 0 °C under nitrogen. Acetal ester **8** (6 g, 18.6 mmol) was dissolved in 25 mL of dry ether and added dropwise to the suspension. Upon consumption of the starting material, the reaction was quenched by the sequential addition of water (0.51 mL), 15% NaOH solution (0.51 mL), and more water (1.53 mL). After being stirred vigorously, the slurry was filtered and the precipitate washed throughly with dry ether. Concentration of the filtrate and washings provided allylic alcohol (R_f 0.07, 3:1 hexane/ethyl acetate) suitable for the next step.

The alcohol was dissolved in dry CH_2Cl_2 freshly distilled from CaH_2, 0.4 M). (Dimethylamino)pyridine (0.6 equiv), purified *p*-toluenesulfonyl chloride (1.3 equiv), and triethylamine (1.1 equiv) were then added sequentially. After being stirred at room temperature, the mixture was poured into 500 mL of a 1:1 pentane/ether solution. After filtration, the gummy precipitate was rinsed with more of the 1:1 solution. The filtrates were washed with water and brine and dried over K_2CO_3. Concentration afforded the crude allylic chloride (R_f 0.48, 3:1 hexane/ethyl acetate), which is taken on to the next step.

Diisopropylamine (1.2 equiv) was dissolved in dry THF (0.75 M) and cooled to −10 °C under nitrogen. *n*-Butyllithium (1.1 equiv, 1.6 M solution in hexane) was added, keeping the temperature below 0 °C. The dimethyl hydrazone of pyruvic aldehyde dimethyl acetal (1.1 equiv) was added in 10 mL of THF, and the resulting red solution was stirred for 1 h at 0 °C. A solution of the allylic chloride in 20 mL of dry THF was added dropwise, and the reaction mixture was stirred at 0 °C. Upon completion of the reaction, it was quenched with 10 mL of a 1:1 THF/H_2O mixture. After ether/H_2O extractions, the organic layers were dried over K_2CO_3 and concentrated to afford 5.76 g (71%) of diacetal hydrazone **9** (R_f 0.13, 3:1 hexane/ethyl acetate) as a mixture of isomers (2:1 *E*/*Z*): ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.68 (t, 0.67 H, *E*), 5.42 (m, 0.33 H, *Z*), 5.36 (s, 0.33 H, *Z*), 5.21–5.05 (m, 2 H), 4.86 (s, 0.67 H, *E*), 4.60 (s, 0.33 H, *Z*), 4.58 (s, 0.67 H, *E*), 4.17 (dd, 2 H), 3.83 (dt, 2 H), 3.40 (s, 2 H, *Z*), 3.37 (s, 4 H, *E*), 2.57–2.38 (m, 4 H), 2.52 (s, 2 H, *Z*), 2.47 (s, 4 H, *E*), 2.20–1.95 (m, 9 H), 1.70 (s, 3 H), 1.63 (s, 3 H), 1.62 (s, 3 H), 1.36 (d, 1 H).

Preparation of Diacetal *tert*-Butyl Ester 10. Woelm silica gel (2.5 g/mmol, 100–200 μm, activity I) was placed in dry, freshly distilled CH_2Cl_2 (0.1 M). Water (10% based on weight of SiO_2) was added. After adsorption, the diacetal hydrazone **9** (5.76 g, 13.2 mmol) was added and the slurry was stirred at room temperature until completion. The mixture was filtered, and the silica gel was thoroughly washed with acetone. After concentration, ether/water extractions were performed and the ethereal layers were dried over K_2CO_3 and concentrated. Chromatography (MPLC, 2:1 hexane/ethyl acetate) afforded 2.77 g (53%, 3:2 *E*/*Z*) of diacetal ketone (R_f 0.23, 3:1 hexane/ethyl acetate).

***E* Isomer**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.62 (t, 1 H), 5.13 (m, 2 H), 4.83 (s, 1 H), 4.47 (s, 1 H), 4.15 (dd, 2 H), 3.81 (dt, 2 H), 3.39 (s, 6 H), 2.64 (t, 2 H), 2.35 (q, 2 H), 2.17–1.97 (m, 9 H), 1.68 (s, 3 H), 1.61 (s, 6 H), 1.36 (d, 1 H).

***Z* isomer**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.31 (t, 1 H), 5.29 (s, 1 H), 5.11 (m, 2 H), 4.48 (s, 1 H), 4.13 (dd, 2 H), 3.84 (dt, 2 H), 3.40 (s, 6 H), 2.64 (t, 2 H), 2.41 (q, 2 H), 2.18–1.94 (m, 9 H), 1.68 (s, 3 H), 1.60 (s, 6 H), 1.34 (d, 1 H).

Diisopropylamine (1.2 equiv) was dissolved in dry THF (0.5 M) and cooled to −10 °C under nitrogen. *n*-Butyllithium (1.2 equiv, 1.6 M solution in hexane) was added, keeping the temperature below 0 °C. The lithium diisopropylamide (LDA) solution was cooled to −78 °C, and *tert*-butyl (trimethylsilyl)acetate was added dropwise in 5 mL of THF. After the mixture was stirred for 1 h, a solution of diacetal ketone (2.77 g, 7.02 mmol) in 20 mL of dry THF was added dropwise. The reaction was allowed to warm to room temperature overnight. After the mixture was quenched with 20 mL of water, ether/water extractions were performed. The organic layers were dried over K_2CO_3 and concentrated. Chromatography (MPLC, 5:1 hexane/ethyl acetate) afforded 3.15 g (91%) of diacetal *tert*-butyl ester **10** [R_f 0.44 (*Z* isomer), 0.39 (*E* isomer), 3:1 hexane/ethyl acetate] as a mixture of isomers (~2:1 *E*/*Z*) plus minor trans ester isomers.

***Z* Isomer**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.91 (s, 1 H), 5.74 (s, 1 H), 5.34 (t, 1 H), 5.27 (s, 1 H), 5.15 (m, 2 H), 4.15 (dd, 2 H), 3.83 (dt, 2 H), 3.42 (s, 6 H), 2.30 (m, 4 H), 2.15–1.98 (m, 9 H), 1.68 (s, 3 H), 1.60 (s, 6 H), 1.49 (s, 9 H), 1.36 (d, 1 H).

***E* Isomer**: ^{1}H NMR (300 MHz, $CDCl_3$), δ 5.89 (s, 1 H), 5.72 (s, 1 H), 5.65 (t, 1 H), 5.20 (m, 1 H), 5.15 (m, 1 H), 4.84 (s, 1 H), 4.15 (dd, 2 H), 3.86 (dt, 2 H), 3.39 (s, 6 H), 2.28 (m, 4 H), 2.16–1.98 (m, 9 H), 1.68 (s, 3 H), 1.62 (s, 3 H), 1.60 (s, 3 H), 1.49 (s, 9 H), 1.34 (d, 1 H).

Preparation of Methoxybutenolides 11 and 4. Diacetal *tert*-butyl ester **10** (6.69 g, 13.6 mmol) was placed in dry, freshly distilled CH_2Cl_2 (0.1 M) under argon and cooled to 0 °C. Freshly distilled trifluoroacetic acid (1.1 equiv) was added, and the cooling bath was removed. After being stirred for 3 h, the reaction mixture was concentrated. Chromatography (MPLC, 2:1 hexane/ethyl acetate) afforded 3.15 g (57%, 3.8:1 *E*/*Z*) of **11** [R_f 0.45 (*Z*), 0.42 (*E*), 1:1 hexane/ethyl acetate] and 1.0 g (21%) of **4** (R_f 0.38) as well as the unreactive trans ester isomers of the starting material (14%). In later experiments, an alternative workup protocol, which involved pouring the reaction mixture into saturated $NaHCO_3$ before ether extraction, minimized the amount of deprotection and formation of **4** (≤5%).

***E* Isomer of 11**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.89 (s, 1 H), 5.64 (t, 1 H), 5.63 (s, 1 H), 5.13 (m, 2 H), 4.84 (s, 1 H), 4.16 (dd, 2 H), 3.83

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(dt, 2 H), 3.55 (s, 3 H), 2.60–2.34 (m, 4 H), 2.20–1.95 (m, 9 H), 1.68 (s, 3 H), 1.61 (s, 6 H), 1.36 (d, 1 H).

Z Isomer of 11: ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.93 (s, 1 H), 5.66 (s, 1 H), 5.30 (t, 1 H), 5.18 (s, 1 H), 5.11 (m, 2 H), 4.15 (dd, 2 H), 3.80 (dt, 2 H), 3.56 (s, 3 H), 2.58–1.98 (m, 13 H), 1.68 (s, 3 H), 1.60 (s, 6 H), 1.38 (d, 1 H); IR ($CHCl_3$) 1795, 1767, 1651 cm^{-1}.

Lactone Aldehyde 4: ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.39 (s, 1 H), 6.39 (t, 1 H), 5.92 (s, 1 H), 5.65 (s, 1 H), 5.08 (m, 2 H), 3.60 (s, 3 H), 2.71–2.53 (m, 4 H), 2.30 (t, 2 H), 2.11–1.94 (m, 6 H), 1.68 (s, 3 H), 1.60 (s, 3 H), 1.57 (s, 3 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 193.6, 169.0, 164.9, 150.0, 144.0, 135.8, 130.7, 123.6, 122.4, 118.3, 103.7, 56.5, 39.1, 26.2, 26.1, 25.3, 25.0, 23.8, 17.0, 15.4: IR ($CHCl_3$) 1797, 1769, 1682, 1651 cm^{-1}.

Preparation of Hydroxybutenolide 12. Methoxybutenolide **11** (3.15 g, 7.8 mmol) was dissolved in THF (0.1M) and treated with 1 equiv of 1 N NaOH. Heating to 50 °C produced almost complete reaction in 3.5 h. The reaction was cooled and neutralized to pH 7 with 1 N HCl. Ether/H_2O extractions were performed followed by drying over K_2CO_3 and concentration. Chromatography (MPLC, 1:1 hexane/ethyl acetate) afforded 1.74 g (63%) of hydroxybutenolide **12** (R_f 0.22, 1:1 hexane/ethyl acetate) as an ~4:1 E/Z mixture of isomers as well as 10% unreacted starting material: ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.95 (s, 1 H), 5.88 (s, 1 H), 5.68 (t, 0.8 H), 5.35 (t, 0.2 H), 5.13 (s, 0.2 H), 5.12 (m, 2 H), 4.85 (s, 0.8 H), 4.17 (dd, 2 H), 3.83 (dt, 2 H), 2.70–2.39 (m, 4 H), 2.20–1.95 (m, 10 H), 1.68 (s, 3 H), 1.61 (s, 6 H), 1.37 (d, 1 H).

Preparation of (*E*,*E*)-2-[3-(2,5-Dihydro-2-hydroxy-5-oxo-3-furanyl)-propylidene]-6,10-dimethyl-5,9-undecadienal (LY 186559, 3) from 12. Hydroxybutenolide **12** (126 mg, 0.32 mmol) was dissolved in methanol (0.1 M) and treated with 1 equiv of 1 N HCl at room temperature. The reaction was monitored by thin-layer chromatography (TLC). After disappearance of starting material, the reaction was concentrated. Ether/water extractions were performed, and the organic layers were dried over $MgSO_4$ and concentrated to afford 89 mg (83%) of **3** (R_f 0.22, 1:1 hexane/ethyl acetate): ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.38 (s, 1 H, CHO), 6.41 (t, 1 H, H-1′), 6.03 (s, 1 H, H-2″), 5.91 (s, 1 H, H-4″), 5.09 (dt, 2 H, H-5, H-9), 4.02 (br, 1 H, OH), 2.71 (m, 3 H, 2H-2′, H-3′), 2.56 (m, 1 H, H-3′), 2.31 (t, 2 H, H-3), 2.11–1.93 (m, 6 H, H-4, H-7, H-8), 1.68 (s, 3 H), 1.59 (s, 3 H), 1.56 (s, 3 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 194.8 (CH), 170.9 (C), 167.7 (C), 151.4 (CH), 144.5 (C), 136.4 (C), 131.4 (C), 124.1 (CH), 122.9 (CH), 118.1 (CH), 99.0 (CH), 39.7 (CH_2), 26.8 (CH_2), 26.7 (CH_2), 26.6 (CH_2) 25.9 (CH_2), 25.6 (CH_3), 24.37 (CH_2), 17.6 (CH_3), 16.0 (CH_3); IR ($CHCl_3$) 3353, 1767, 1746, 1682, 1651 cm^{-1}. Anal. Calcd for $C_{20}H_{28}O_4$: C, 72.26; H, 8.49. Found: C, 72.14; H, 8.62.

Preparation of Butenolide Alcohol 13. Methoxybutenolide **4** (3.0 g, 8.66 mmol) was dissolved in methanol (0.1 M) and cooled to 0 °C under argon, and sodium borohydride (326 mg, 1 equiv) was added in small portions over 15 min. After 1 h, TLC indicated complete reaction. The mixture was quenched with water, diluted with ether, drained, washed with water and saturated sodium chloride, dried over $MgSO_4$, and concentrated. Chromatography (MPLC, 2:1 hexane/ethyl acetate) afforded 2.6 g (86%) of **13** (R_f 0.23, 1:1 hexane/ethyl acetate): ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.89 (s, 1 H), 5.64 (s, 1 H), 5.42 (t, 1 H), 5.10 (m, 2 H), 4.08 (s, 2 H), 3.57 (s, 3 H), 2.55–2.28 (m, 4 H), 2.15–1.95 (m, 9 H), 1.68 (s, 3 H), 1.60 (s, 6 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.6, 167.1, 140.9, 135.8, 131.4, 124.2, 123.6, 123.5, 118.3, 104.4, 66.4, 56.9, 39.7, 28.3, 27.7, 26.8, 26.6, 25.7, 24.6, 17.7, 16.0; IR ($CHCl_3$) 3708, 3500, 1799, 1764 cm^{-1}. Anal. Calcd for $C_{21}H_{32}O_4$: C, 72.38; H, 9.26. Found: C, 72.11; H, 9.28.

Preparation of Hydroxybutenolide 5. A solution of **13** (2.0 g, 6 mmol) in 60 mL of THF was treated with 1 N NaOH (6 mL, 1 equiv) and heated to 50 °C with stirring for 6 h. TLC showed nearly complete conversion to product. The reaction mixture was cooled in an ice bath and brought to pH 7 by dropwise addition of 1 N HCl. The neutralized mixture was diluted with ether (600 mL), washed with water (3 × 100 mL) and saturated NaCl (100 ml), dried over $MgSO_4$, filtered, and concentrated. Chromatography (MPLC, 1:1 hexane/ethyl acetate) afforded 1.32 g (66%) of **5** (R_f 0.18, 1:1 hexane/ethyl acetate): ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.98 (s, 1 H), 5.88 (s, 1 H), 5.43 (t, 1 H), 5.12 (m, 2 H), 4.07 (s, 2 H), 2.60–2.38 (m, 5 H), 2.20–1.96 (m, 9 H), 1.68 (s, 3 H), 1.60 (s, 6 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.5, 169.2, 140.3, 135.7, 131.1, 124.1, 123.9, 123.4, 117.5, 99.6, 66.3, 39.7, 28.4, 27.6, 26.8, 25.6, 24.6, 17.6, 16.0.

Preparation of 3 from 5. Hydroxybutenolide alcohol **5** (0.32 g, 0.97 mmol) was dissolved in dry CH_2Cl_2 (0.1 M) and cooled to 0 °C. Pyridinium dichromate (1.5 equiv) was added with stirring, and the mixture was allowed to warm to room temperature. Upon consumption of starting material, the reaction mixture was diluted with ether, filtered through Celite, and concentrated. Flash chromatography (2:1 hexane/ethyl acetate) afforded 0.19 g (60%) of **3**.

Preparation of Mixed Micelles. A measured amount of phospholipid or thiophospholipid in chloroform solution was dried under a stream of nitrogen and then in vacuo. Triton X-100 in aqueous solution was added. The mixture was warmed to 40 °C and vortexed several times until a clear solution was obtained.

Enzyme Assays. PLA_2 activity was routinely measured against racemic thio-PC according to the method of Hendrickson and Dennis.[12] Assays were performed on micellar substrate containing 0.5 mM racemic thio-PC and 4 mM Triton X-100 in 25 mM Tris-HCl (pH 8.5), 0.1 M KCl, and 10 mM $CaCl_2$. A 0.3-mL aliquot of this solution was added to a microcuvette (2 × 10 mm) followed by 5 μL of 4,4′-dithiodipyridine (50 mM in ethanol) and equilibriated to 30 °C in the cell compartment of a Perkin-Elmer Model 552 spectrophotometer. The reaction was initiated by addition of 5 μL of PLA_2 (50–125 ng of protein). The liberation of free thiols was monitored by their reaction with 4,4′-dithiodipyridine to form a product that absorbs at 324 nm (ϵ = 19 800 M^{-1} cm^{-1}). Assays with chiral thio-PC were performed in an analogous manner with 0.5 mM chiral thio-PC and 4 mM Triton X-100.

The activity toward dipalmitoyl-PC mixed micelles was determined by using a Radiometer pH-stat with 5 mM dipalmitoyl-PC, 20 mM Triton X-100, and 10 mM $CaCl_2$ at 40 °C and pH 8.0 as described by Deems and Dennis.[14]

Inactivation of Phospholipase A_2. Inactivation of PLA_2 by manoalide analogues was studied by the addition of inhibitor dissolved in methanol (final concentration 40 μM) to a 1 μM solution of phospholipase A_2 in 0.1 M Tris-HCl (pH 8.0). The mixture was immediately vortexed and 5 μL removed and assayed for enzyme activity with thio-PC as a zero-time control. The solutions were incubated at 40 °C and 5-μL aliquots removed at various time points and assayed for remaining phospholipase activity. Incubations were run in duplicate, and their average is reported. Control samples, containing the same amount of methanol but no inhibitors, were also analyzed over the entire time course. The concentration of methanol in the final incubations was always less than 5%. The activity at zero time in the presence of inhibitors in methanol was generally higher than in the absence of inhibitors, presumably due to an effect of the inhibitor/organic solvent on the physical state of the substrate micelles used in the assay. Thus, unless otherwise specified, remaining phospholipase activity and percent inhibition are normalized relative to the activity found at time zero for each reaction.

The dependence of inactivation on the concentration of manoalogue was measured by incubation of 1 μM phospholipase A_2 with varying concentrations of manoalogue at 40 °C. After 150 min, the activity of each sample was measured in the thiol assay. The percent inhibition is expressed relative to a control lacking manoalogue.

The reversibility of the enzyme inhibition was tested by incubation of 20 μM PLA_2 with a 40-fold excess (800 μM) of manoalogue for 22 h at 40 °C. The solution was passed through a Pharmcia PD-10 Sephadex G-25 column and eluted with water to remove excess **3**. The fractions containing enzyme were dialyzed against a 500-fold excess of water at 4 °C for 20 h in Spectrapor 1 dialysis tubing. Protein concentrations were determined by the method of Lowry et al.[15] The remaining enzyme activity was measured in the thio-PC assay and was compared to a control containing methanol that was processed in the same manner. Both control and inhibited enzyme solutions were run in duplicate.

N-Terminal Sequence of Modified PLA_2. Dialyzed, inactivated PLA_2 (5 nmol) was reduced by incubation in a 100-μL volume of 8 M guanidine hydrochloride, 10 mM dithiothreitol, 10 mM EDTA, and 0.1 M Tris-HCl (pH 8.0) at 40 °C for 4 h. The reduced protein was alkylated by addition of 2.1 μmol of [^{14}C]iodoacetamide and incubated in the dark at 40 °C. After 1 h, another 0.7 μmol of iodoacetamide was added, and the solution was incubated for an additional 1 h. The reduced, carboxyamidomethylated protein was purified on a Pharmacia PD-10 G-25 column and eluted with H_2O. The N-terminal sequence of the modified phospholipase A_2 was determined by subjecting the protein to Edman degradation using an Applied Biosystems Model 470A gas-phase sequencer employing the standard 03RPTH program supplied by the manufacturer. PTH derivatives were identified by using an applied Biosystems Model 120 on-line HPLC with a Brownlee analytical C-18 column. Data analysis was accomplished with a Perkin-Elmer 7500 professional computer and CHROM 3 software.

Amino Acid Analysis. PLA_2 was incubated in 0.1 M Tris-HCl buffer (pH 8.0) at a concentration of 20 μM with a 50-fold excess of inhibitor at 40 °C for 20 h. Solutions were applied to a Pharmacia-PD 10 Sephadex G-25 column and eluted with water. PLA_2 fractions were dialyzed exhaustively against water at room temperature in Spectrapor 1 tubing, and the dialysate was lyopholized. Protein was hydrolyzed in

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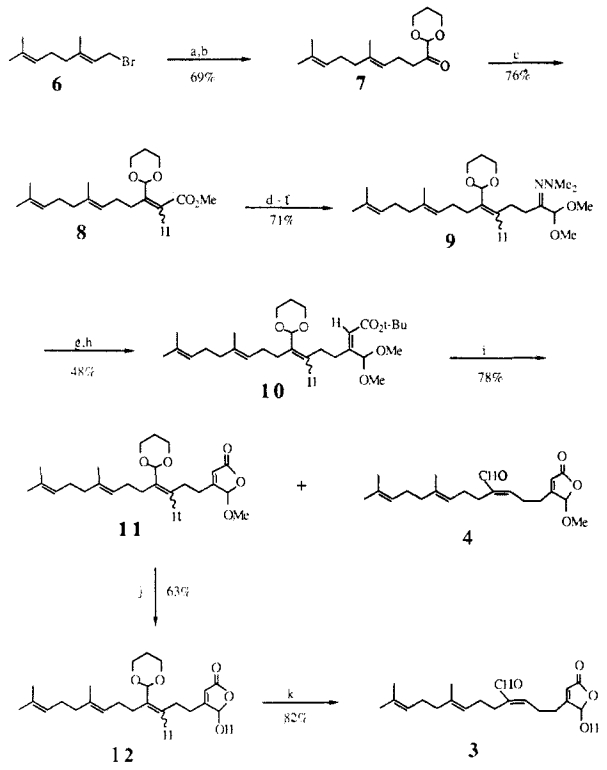

Figure 1. Chemical synthesis and yields of manoalogue (**3**) and the methylated analogue **4**: (a) LDA, $CH_3C{=}NNMe_2CH$ (OCH_2CH_2C-H_2O), THF, 0 → 25 °C; (b) 2 N HCl, THF, 25 °C; (c) LDA, $Me_3SiCH_2CO_2Me$, THF, −78 → +25 °C; (d) $LiAlH_4$, Et_2O, 25 °C. (e) TsCl, Et_3N, (dimethylamino)pyridine, CH_2Cl_2, 25 °C; (f) LDA, $CH_3C{=}NNMe_2CH(OMe)_2$, THF, −15 °C; (g) SiO_2, H_2O, CH_2Cl_2, 25 °C; (h) LDA, $Me_3SiCH_2CO_2t$-Bu, THF, −78 → +25 °C. (i) CF_3CO_2H, CH_2Cl_2, 0 → 25 °C; (j) 1 N NaOH (1 equiv), THF, 50 °C; (k) 1 N HCl, 25 °C.

6 N HCl for 24 h at 120 °C. The amino acid composition was determined on a Beckman 118 amino acid analyzer. The number of residues for each amino acid was calculated by assuming 4.0 valine residues per enzyme molecule. Control enzyme was treated in the same manner as inhibited enzyme. The average of duplicates is reported.

^{1}H NMR. The opening of the lactone ring of manoalogue with increasing pH was determined by ^{1}H NMR at 360 MHz on a modified Varian spectrometer as described previously by Deems et al.[5] Spectra were observed in 20% ^2H_2O/80% DMSO-d_6. The apparent pH values were approximated by pHydrion paper and were adjusted to the desired value with NaO^2H or ^2HCl. Chemical shifts are reported relative to TMS.

Results

Chemical Synthesis. The synthesis of manoalogue (**3**) relied principally on the hydrazone alkylation and butenolide formation methodology of Larcheveque et al.[16] as shown in Figure 1. Hydrazone anions of pyruvic aldehyde acetals were used in the preparation of **7** from **6** and **9** from **8** by the sequence shown. It was necessary to utilize the cyclic acetal in the initial reaction in order for this group to survive the hydrolytic removal of hydrazine (step g) and subsequent butenolide formation (step i). Even so, some deprotection to **4** was generally observed in the latter reaction. As is shown in Figure 2, this material could be efficiently carried on to the desired product. Since **11** is quantitatively converted to **4** by treatment with mild acid or on to **3** uneventfully by way of **12**, the synthesis of manoalogue was achieved.

Inactivation of Phospholipase A_2. Incubation of PLA_2 with manoalogue caused a time-dependent partial inactivation of the enzyme, reaching a maximum of 47% inhibition in 3 h (Figure 3). The extent of this inactivation was not increased by incubation with up to 600 μM manoalogue or by incubation at higher pH (9.0) or temperature (60 °C). The rate of inactivation was not affected by the addition of either 10 mM $CaCl_2$ or 1 mM EGTA. Incubations with HDHB (**2**), the methylated analogue **4**, and the reduced analogue **5** showed a 5–10% loss of phospholipase A_2 activity within the first 5–10 min and no further change in activity thereafter. The control showed a slight increase in activity with time.

With increasing concentrations of manoalogue, inhibition of 1 μM PLA_2 increased and saturation occured at ~40 μM manoalogue. Half-maximal inhibition occured at ~7.5 μM (Figure 4). This inhibition appears to be irreversible since activity was not restored to the inhibited enzyme by passing it through a

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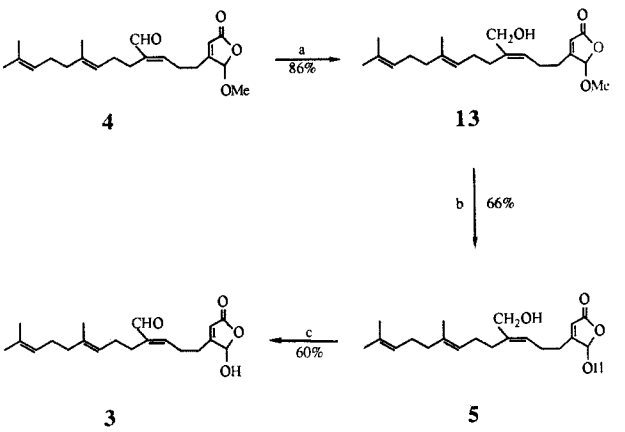

Figure 2. Chemical synthesis and yields of manoalogue (**3**) and the reduced analogue **5** from methylated analogue **4**: (a) $NaBH_4$, MeOH, 0 °C; (b) 1 N NaOH, THF, 50 °C; (c) PDC, CH_2Cl_2, 0 → 25 °C.

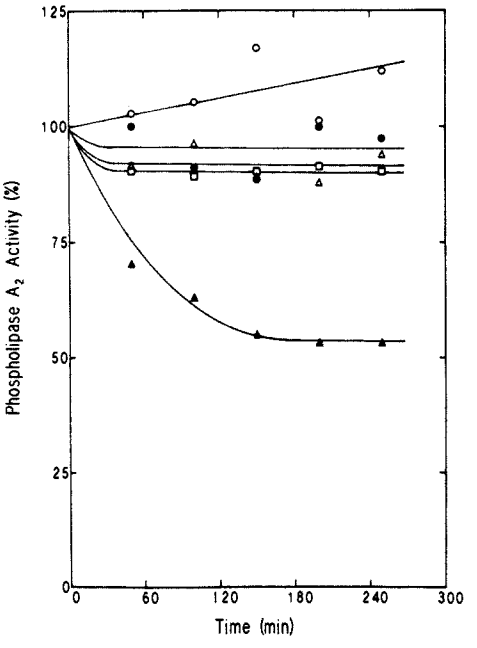

Figure 3. Inhibition of cobra venom phospholipase A_2 by manoalide analogues. Phospholipase A_2 (1 μM) was incubated at 40 °C with 40 μM inhibitor in 0.1 M Tris-HCl (pH 8.0): HDHB (**2**; □), manoalogue (**3**; ▲), methylated manoalogue (**4**; △) or reduced manoalogue (**5**; ●). Aliquots were removed at various times and tested for activity against racemic thio-PC. Phospholipase A_2 activity is expressed relative to the activity of each incubation mixture at time zero. The control sample (○) lacked inhibitor.

Table I. Irreversible Inhibition of Phospholipase A_2 by Manoalogue

condition	sp act., μmol min^{-1} mg^{-1}		% of control
	control	+manoalogue	+ manoalogue
at time zero	29	42	140
after 20 h	32	24	73
after Sephadex G-25	23	17	75
after dialysis	26	17	68

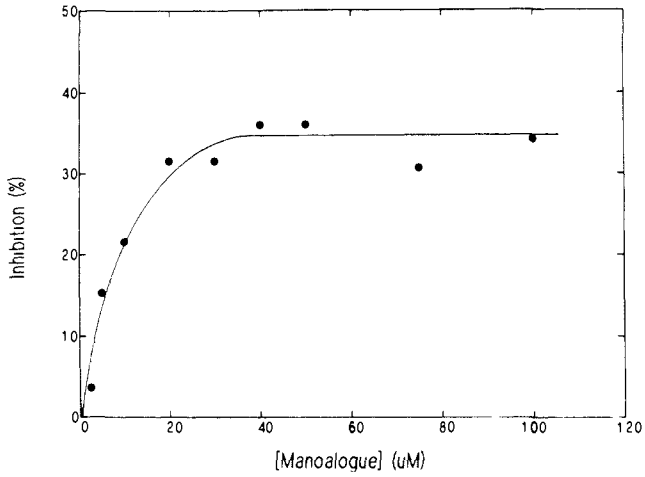

Figure 4. Dependence of phospholipase A_2 inactivation on the concentration of manoalogue. Phospholipase A_2 (1 μM) was incubated with various concentrations of manoalogue at 40 °C in 0.1 M Tris-HCl at pH 8.0. After 150 min, an aliquot was removed and assayed for phospholipase A_2 activity with racemic thio-PC. Inhibition is expressed relative to a control in the absence of manoalogue. The concentrations of manoalogue shown are those present in the preincubation mixture.

Table II. Amino Acid Analysis of Native and Inhibited Phospholipase A_2

amino acid	control	+2	+3	+5
Asp	21.8	22.5	22.6	22.8
Thr	5.2	4.4	4.1	3.3
Ser	7.3	7.8	7.7	8.4
Glu	8.1	8.2	7.5	7.9
Gly	10.0	10.3	10.0	10.1
Ala	11.9	11.9	11.7	11.8
Cys	12.3	12.7	11.5	12.0
Val	4.0	4.0	4.0	4.0
Met	1.4	1.5	1.1	1.3
Ile	4.2	4.5	4.2	4.3
Leu	5.7	6.3	5.6	5.6
Tyr	10.9	10.4	10.3	10.2
Phe	6.2	6.3	5.1	4.6
His	0.9	1.0	0.9	0.9
Lys	6.7	6.6	3.9	5.9
Arg	5.9	6.0	5.6	5.8

Sephadex G-25 column followed by dialysis for 20 h (Table I).

When assaying for enzyme activity, the enzyme solution is diluted 60-fold into the assay mix, bringing the concentration of inhibitor in the cuvette to <1 μM. The observed inhibition of phospholipase A_2 is not due to a competitive inhibition during the assay, since the addition of 1 μM of either **2**, **3**, **4**, or **5** directly to the assay mix did not affect the rate of reaction. Racemic thio-PC was routinely employed in the assay. However, when chiral thio-PC was used to assay enzyme activity in the presence of manoalogue, an inactivation curve identical with that of racemic thio-PC was obtained. In addition, enzyme inhibited with manoalogue demonstrated the same percent inactivation in the pH-stat assay with chiral dipalmitoyl-PC as it did in the thiol assay.

Amino Acid Analysis. Amino acid analysis of PLA_2 inhibited with manoalogue showed a loss of approximately three lysine residues relative to the control, while enzyme incubated with HDHB (**2**) or reduced manoalogue (**5**) showed a loss of less than one lysine (Table II). Phospholipase A_2 incubated with methylated manoalogue (**4**) also showed less than one lysine (0.5) lost relative to the control (data not shown). The number of threonine residues in the inhibited samples also appears slightly low; however, there was a large variability between duplicates (±1 residue), which may be due to the instability of this residue to acid hydrolysis.

N-Terminal Sequence of Modified PLA_2. The amino acid sequence of the first 14 residues on the modified PLA_2 revealed that the N-terminal and Lys-10 were intact (Table III). However, no amino acid was detected at position 6, suggesting that Lys-6 has been modified.

Table III. N-Terminal Sequence of Phospholipase A_2 Modified with Manoalogue

residue	amino acid	pmol	residue	amino acid	pmol
1	Asn	22.6	8	Met	9.3
2	Leu	24.1	9	Ile	8.6
3	Tyr	15.0	10	Lys	5.3
4	Gln	18.4	11	Cys[b]	
5	Phe	14.3	12	Thr	6.6
6	ND[a]	ND[a]	13	Val	2.6
7	Asn	9.3	14	Pro	3.7

[a] ND, not detected. [b] Identified by radioactivity.

Opening of the γ-Lactone Ring. The opening of the lactone ring in HDHB and manoalogue with increasing pH was followed as described by Deems et al.[5] except that spectra were observed in ^2H_2O/DMSO-d_6 instead of ^2H_2O/methanol-d_4. The behavior of HDHB (**2**) in ^2H_2O/DMSO-d_6 was similar to its previously reported behavior in ^2H_2O/methanol-d_4 and was completely in the open form at pH 10.9. For manoalogue, the opening of the lactone ring was observed by following the downfield shift of the vinyl proton from δ 6.05 to 6.9 and of the proton geminal to the hydroxyl (δ 6.1) on conversion to the geminal aldehyde proton (δ 10.2). The lactone ring was completely in the open form at pH 9.2.

Discussion

We have synthesized several manoalide analogues as part of our investigation into the mechanism of inactivation of phospholipase A_2 by manoalide. These analogues have been used to identify which functional groups in the manoalide molecule are essential for inactivation. Preliminary evidence from our laboratory[5] and others[17] has indicated that analogues retaining only the butenolide ring of manoalide, such as HDHB, do not irreversibly inhibit PLA_2 enzymes. These observations are supported by the failure of HDHB to modify lysine residues (Table II).

In our current studies, we have turned our attention to inhibitors that contain the α,β-unsaturated aldehyde portion of the hemiacetal ring in addition to the butenolide ring. Manoalogue contains both of these reactive functional groups. In addition to providing a second reactive functional group, the NMR studies showed that the presence of the α,β-unsaturated carbonyl facilitates the opening of the butenolide ring. Manoalogue demonstrates a saturable, time-dependent, irreversible inactivation of cobra venom PLA_2 concurrent with covalent modification of about three lysine residues. Both the esterification of the butenolide ring to prevent ring opening (**4**) and the reduction of the free aldehyde to an alcohol (**5**) completely abrogated the molecule's ability to irreversibly inhibit the enzyme and to modify lysine residues. These results demonstrate that both reactive groups are necessary for covalent modification of the enzyme.

While we cannot say with certainty that manoalogue exactly mimics manoalide's irreversible inactivation of cobra venom PLA_2, a number of observations support such a hypothesis. Amino acid analysis showed a loss of about three lysines with manoalogue compared to a loss of about four lysines with manoalide.[4] Thus, the number of lysine residues modified by both inhibitors is similar within experimental error. In addition, the concentration of manoalogue required for half-maximal inhibition of the enzyme (7.5 μM) is similar to that of manoalide (2 μM), although the reaction is slower and reaches only 47% inactivation compared to 85% for manoalide.

The structure of manoalogue differs from that of manoalide in two respects. It lacks the hdyroxyl portion of the hemiacetal ring and lacks a portion of the terminal trimethylcyclohexenyl ring. While the presence of the hydroxyl group in manoalide has been implicated in the generation of a chromaphore upon incubation with PLA_2, it has been shown that the time course of this

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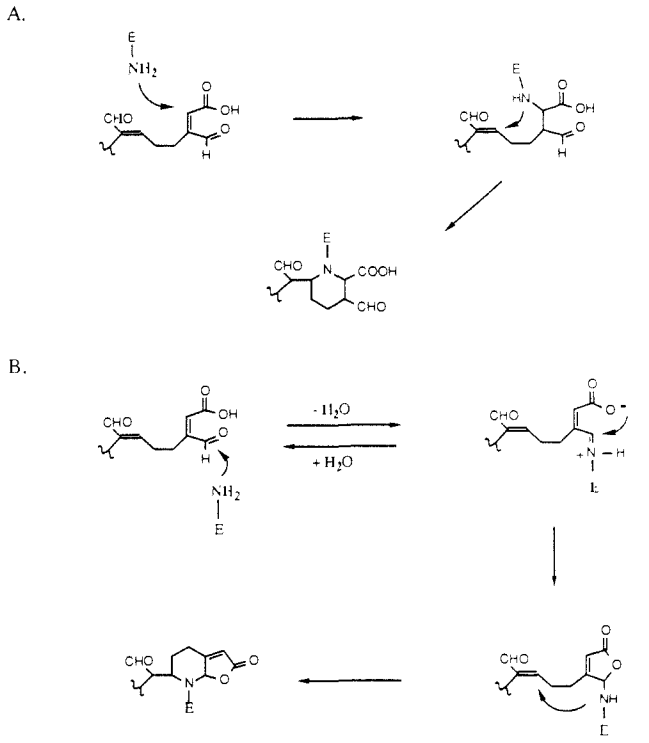

Figure 5. Proposed schemes for the reaction of manoalide and manoalogue with cobra venom phospholipase A_2: (A) involving two conjugate additions or (B) involving conjugate addition following Schiff base formation.

color formation does not correspond to the time course of enzyme inhibition.[7,8] Thus, it is unlikely that the hydroxyl group plays an essential role in the inhibition. Since manoalide-inactivated cobra venom PLA_2 retains 15% of its enzymatic activity, the modified lysine residues must not be essential to catalysis. Instead, it is likely that at least one of the modified lysines is located near the catalytic or activator sites[18,19] and that the manoalide adduct is interfering with phospholipid binding at one of these sites. It is possible that manoalide, with its more bulky and hydrophobic trimethylcyclohexenyl ring, blocks these phospholipid binding sites better than manoalogue and that this effect accounts for the different extent of inactivation seen with the two inhibitors.

Glaser and Jacobs[7] have proposed on the basis of indirect experiments with the bee venom enzyme that inactivation by manoalide requires attack by two lysines on a single inhibitor molecule. The cobra venom enzyme contains six lysine residues, which are observed upon amino acid analysis. N-Terminal sequence analysis of the native enzyme identified two of these as Lys-6 and Lys-10.[20] These are the only two lysines that appear to be close to each other in the primary sequence.[21] The failure to observe an amino acid residue at position 6 in the manoalogue-inhibited PLA_2 suggests that Lys-6 is one of the residues modified. Since Lys-10 was intact in the sequence, we believe it reasonable to postulate that a one-to-one association has occurred between lysine 6 and manoalogue. We cannot yet rule out the possibility that Lys-10 is involved in the modification but forms adducts that are unstable to sequencing. Nor can we exclude the possibility that nucleophiles other than lysine are involved in the mechanism. However, it is reasonable to propose that both manoalide and manoalogue react with single lysine residues.

The studies presented in this article demonstrate that the irreversible inactivation of cobra venom phospholipase A_2 by manoalide and manoalide analogues requires the opening of the lactone ring and the presence of the α,β-unsaturated aldehyde of the hemiacetal ring. In addition, this inactivation is likely to invovle adduct formation between the inhibitor and one lysine residue. Two possible mechanisms of inactivation that take into account these observations are shown in Figure 5. Conjugate addition of the reactive amine of Lys-6 to the ring-opened butenolide would form a β-amino carbonyl compound, which could be in equilibrium with its unsaturated precursor. Ring closure through an additional conjugate addition to the internal olefin would lead to a cyclic structure that would be less prone to reversal and consequently produce a covalently bound enzyme. Alternatively, the amine could form a Schiff base with the aldehyde on the butenolide ring. Conjugate addition to the internal olefin could also occur leading again to a cyclic tertiary amine. Of the inhibitors studied so far, only manoalide and manoalogue have the requisite functionality to undergo these processes. The reversibility of the first addition to the ring-opened butenolide is also consistent with the observation that manoalide analogues containing only the butenolide ring do not cause irreversible inhibition. It is distinctly possible that both manoalide and manoalogue react though such a double conjugate addition or a Schiff base–conjugate addition sequence with a single lysine. Further work is underway in an attempt to evaluate these mechanisms and to isolate and characterize such covalent adducts.

Acknowledgment. Work at the University of California was supported by National Institutes of Health Grant GM-20501 and National Science Foundation Grant DMB 85-18684. L.J.R. is an American Cancer Society postdoctoral fellow. We thank Betty Hendrickson for synthesis of the racemic thio-PC and Lin Yu for synthesis of the chiral thio-PC in our laboratory. We thank Raymond Deems and Richard Engler for NMR measurements on manoalogue, Brian Dwyer and Jack Kyte for conducting the amino acid analysis, and Matt Williamson and Paul Price for obtaining the amino acid sequence data reported here. We also thank Tom Vedvick and Susan Taylor for other sequence data.

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