Structural Determinants of Haloenol Lactone-Mediated Suicide Inhibition of Canine Myocardial Calcium-Independent Phospholipase A_2[†]

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Haloenol lactones are potent mechanism-based inhibitors of a novel class of calcium-independent phospholipases A_2 which have been implicated as the enzymic mediators of membrane dysfunction during myocardial ischemia (Hazen, S. L.; et al. *J. Biol. Chem.* **1991**, *266*, 7227-7232). Herein we demonstrate that the ring size, hydrophobic group, and cryptic electrophile in the haloenol lactone moiety are important and modifiable determinants of the inhibitory potency of haloenol lactone-mediated inhibition of calcium-independent phospholipase A_2. Direct comparisons between haloenol lactone-mediated inhibition of calcium-independent phospholipase A_2 and the absence of inhibition with calcium-dependent phospholipase A_2 further underscore the marked differences in the catalytic strategy employed by these two classes of intracellular phospholipases A_2.

Introduction

Intracellular phospholipases A_2 represent attractive targets for pharmacologic manipulation since they catalyze the rate-determining step in the formation of biologically active eicosanoids and modulate membrane physical properties. Recently, intracellular calcium-independent phospholipases A_2 have been identified[1,2] and purified[3,4] which possess physical and kinetic characteristics that differ dramatically from their calcium-dependent counterparts. Activation of at least one member of this family of calcium-independent phospholipases A_2 has been implicated in the pathogenesis of membrane dysfunction during myocardial ischemia.[5-8] Accordingly, identification of an inhibitor which selectively targets myocardial calcium-independent phospholipases A_2 is of interest since it could define the scope of calcium-independent phospholipases in cellular biology and potentially attenuate some of the adverse sequelae of myocardial ischemia (e.g., lethal ventricular arrhythmias and/or myocytic cellular necrosis).

Suicide inhibitors possess distinct advantages as specific pharmacologic agents since they exploit the specificity inherent in enzyme-substrate recognition.[9] Recently, we demonstrated that one member of a class of compounds (haloenol lactones) initially described by Katzenellenbogen et al.[10,11] is a potent suicide inhibitor of canine myocardial calcium-independent phospholipase A_2 and is over 1000-fold selective for inhibition of calcium-independent phospholipase A_2 in comparison to calcium-dependent phospholipases A_2.[12] The potential utility of these compounds as pharmaceutical agents which specifically inhibit calcium-independent phospholipase A_2 is dependent upon the identification of critical structure-activity relationships which facilitate selective interactions with this novel class of phospholipases A_2. We now report that the inhibitory potency of haloenol lactones toward calcium-independent phospholipase A_2 is critically dependent upon the ring size of the lactone, the nature of the hydrophobic group, and the conformation and nature of the cryptic reactive electrophilic site.

Chemistry

The series of haloenol lactones (**1a-g**) were prepared as shown in Scheme I. The methodology, as described by Katzenellenbogen et al.,[10] employed alkylation of the carbanion of a 2-arylacetic acid with 1-bromo-3-butyne, followed by base-catalyzed halolactonization. Both the cis (**1g**) and trans (**1f**) iodoenol lactones were isolated in the lactonization of α-3-butynyl-1-naphthaleneacetic acid with iodine and potassium carbonate in acetonitrile.[10] In the preparation of compound **1d**, ethyl 3-pyridylacetate was alkylated in high yield with 3-butynyl triflate, as the reaction would not occur using 3-pyridylacetic acid or 1-bromo-3-butyne. Base hydrolysis of the alkylated ester, followed by lactonization with *N*-bromosuccinimide and tetrabutylammonium hydroxide in methylene chloride and water, gave compound **1d**. The five-membered ring bromoenol lactone (compound **2**) was prepared as described by Daniels et al.[11] in a procedure similar to that shown in Scheme I except that propargyl chloride was utilized as the alkylating agent. The ynenol lactone (compound **3**) was prepared using methodology described by Spencer et al.[13] as outlined in Scheme II. Coupling of the iodoenol lactone (**1f**) with (trimethylsilyl)acetylene was achieved in good yield using bis(triphenylphosphine)-palladium(II) chloride and cuprous chloride. Desilylation was accomplished with aqueous silver nitrate and potassium cyanide.

Biology

Plasmalogens represent the major phospholipid constituents of the electrically responsive membranes in

[†] Abbreviations: CI = chemical ionization, DCC = 1,3-dicyclohexylcarbodiimide, DMAP = 4-(dimethylamino)pyridine, DTT = dithiothreitol, EGTA = ethylene glycol bis(2-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, EI = electron impact, GPC = *sn*-glycero-3-phosphocholine, HPLC = high-performance liquid chromatography, Np = 1-naphthyl, Ph = phenyl, OPh = phenoxy, THF = tetrahydrofuran, TLC = thin-layer chromatography, 16:0,18:1 plasmenylcholine = 1-*O*-(*Z*)-hexadec-1'-enyl-2-*O*-octadec-9'-enoyl-GPC, 16:0,[^{3}H]18:1 plasmenylcholine = 1-*O*-(*Z*)-hexadec-1'-enyl-2-*O*-[9,10-^{3}H]octadec-9'-enoyl-GPC.

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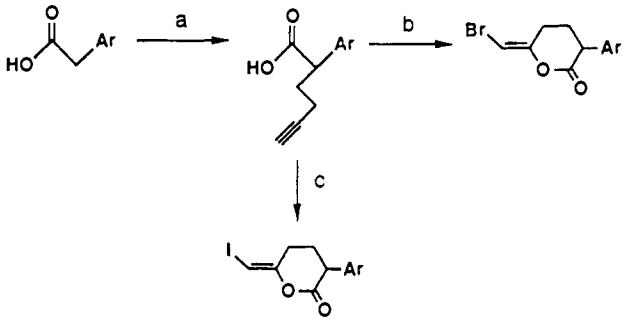
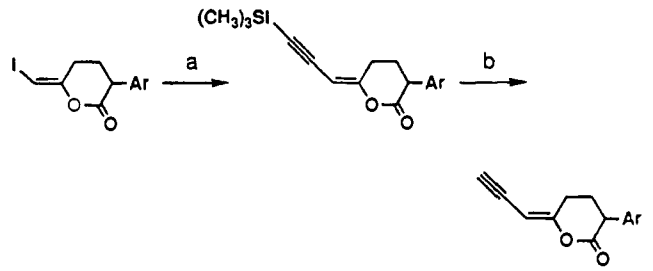
Scheme I [a]



[a] (a) Lithium diisopropylamide, 3-butynyl bromide or triflate, tetrahydrofuran; (b) *N*-bromosuccinimide, tetrabutylammonium hydroxide, methylene chloride, water; (c) iodine, potassium bicarbonate, acetonitrile.

Scheme II [a]



[a] (a) Bis(triphenylphosphine)palladium(II) chloride, cuprous chloride, (trimethylsilyl)acetylene, triethylamine; (b) silver nitrate, potassium cyanide, ethanol, water.

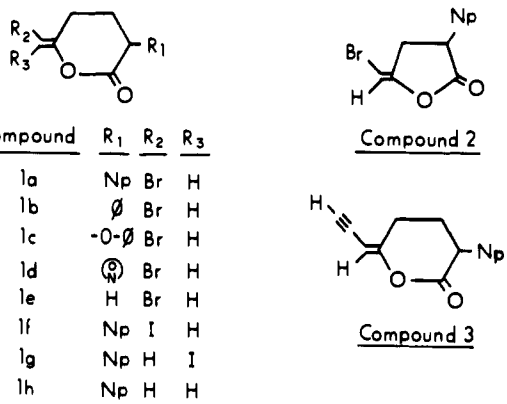
myocardium.[14,15] Since the majority of measurable phospholipase A_2 activity present in myocardium is highly selective for plasmalogen substrate,[1] all assays employed synthetically-prepared, HPLC-purified, homogeneous plasmalogen molecular species. Furthermore, since most tissues (including myocardium and platelets) typically contain several different phospholipase activities,[16-18] the enzymes utilized in this study were extensively purified by sequential column chromatography employing, in the final purification step, either an affinity matrix or fast protein liquid chromatography.[19] All assays quantifying phospholipase A_2 activity were performed utilizing physiologically relevant macromolecular aggregates of plasmenylcholine substrate (liquid–crystalline bilayers) at physiologic temperature (37 °C) and pH (7.2). Assays performed in this manner are essentially free of artifacts arising from lipid mixing and isotope dilution which are typically encountered when utilizing crude preparations or membrane-associated phospholipase A_2.

Results and Discussion

Initial experiments assessed the importance of the hydrophobic constituent at the 3-position of the lactone to characterize the stereoelectronic relationships that facilitate the efficient interaction of inhibitor with the active site of canine myocardial cytosolic calcium-independent phospholipase A_2. Comparisons of the inhibitory potency of compounds containing 1-naphthyl, phenyl, phenoxy, 3-pyridyl, or hydrogen side groups (Chart I; compounds **1a–e**) demonstrated that inhibition was critically dependent upon the nature of the substituent at the 3-position of the lactone (Figure 1). Specifically, the bromoenol lactone containing the naphthyl moiety was the most potent inhibitor in this group, possessing an IC_{50} of 140 nM under the conditions employed. Substitution

Figure 1. Comparison of the inhibitory potency of haloenol lactones differing in the nature of the hydrophobic substituent or ring size for myocardial calcium-independent phospholipase A_2. Canine myocardial phospholipase A_2 (10 ng from the affinity column eluent) was incubated with the indicated concentration of inhibitor for 5 min at 25 °C in 210 mM Tris·Cl buffer containing 1 mM EGTA (pH 7.4). Phospholipase A_2 was subsequently assessed after injection of 1 μM 1-*O*-(*Z*)-hexadec-1′-enyl-2-*O*-[9,10-^{3}H]octadec-9-enoyl-*sn*-glycero-3-phosphocholine (16:0,[^{3}H]18:1 plasmenylcholine) and incubation for 5 min at 37 °C. Released [^{3}H]oleic acid was extracted into butanol, separated by TLC, and quantified by scintillation spectrometry. Data represent the mean of duplicate determinations which each agreed within 10% of the indicated values.

Chart I



Compound	R_1	R_2	R_3
1a	Np	Br	H
1b	Ø	Br	H
1c	-O-Ø	Br	H
1d	(9-N)	Br	H
1e	H	Br	H
1f	Np	I	H
1g	Np	H	I
1h	Np	H	H

Compound 2

Compound 3

of a phenyl group at the 3-position resulted in an increase in the IC_{50} to 250 nM. Remarkably, introduction of the phenoxy substituent at the 3-position reduced the inhibitory potency of the haloenol lactone by nearly 2 orders of magnitude. Finally, bromoenol lactones containing either pyridine or hydrogen at the 3-position did not substantially inhibit canine myocardial calcium-independent phospholipase A_2. Compounds **1a** and **1f**, the two most potent analogs evaluated in the 3-substituted lactone series, did not inhibit intracellular calcium-dependent phopholipase A_2 purified from sheep platelet cytosol (i.e., $IC_{50} > 100$ μM). Collectively, these results demonstrate that the inhibitory potencies of these haloenol lactones for myocardial calcium-independent phospholipase A_2 are highly influenced by the size, hydrophobicity, and electron-withdrawing properties of the constituent at the 3-position.

Since previous studies demonstrated that the ring size of the lactone moiety is an important determinant of inhibitory potency for this family of suicide inhibitors against chymotrypsin,[11] experiments were performed to compare the inhibitory potencies of compounds **1a** and **2** utilizing purified myocardial cytosolic phospholipase A_2. Reduction of ring size from a 6- to a 5-membered lactone resulted in a 1 order of magnitude decrease in the inhibitory potency of the haloenol lactone against myocardial phos-

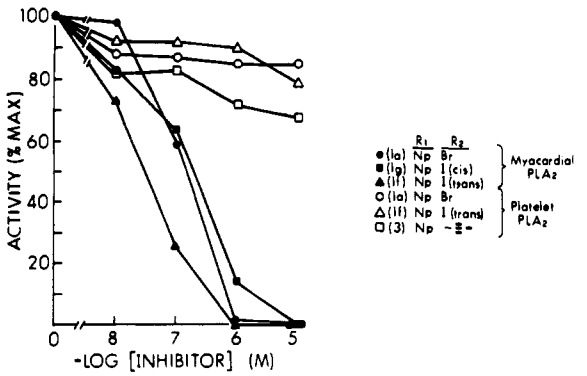


Figure 2. Comparison between haloenol lactone-mediated inhibition of myocardial calcium-independent phospholipase A_2 and platelet calcium-dependent phospholipase A_2 and the effects of alterations in the cryptic electrophilic site. Either canine myocardial phospholipase A_2 (10 ng) or platelet phospholipase A_2 (10 μg) was preincubated with the indicated haloenol lactone for 5 min at 25 °C in 210 mM Tris·Cl buffer containing 1 mM EGTA (in the case of the myocardial enzyme) or 1 μM $CaCl_2$ (in the case of the platelet enzyme). After preincubation, phospholipase A_2 activity was assessed after injection of 16:0,[^{3}H]-18:1 plasmenylcholine (1 μM) and incubation for an additional 5 min at 37 °C. Released radiolabeled fatty acids were extracted into butanol, separated by TLC, and quantified by scintillation spectrometry. Results represent the mean of duplicate determinations which each agree within 10% of the indicated values.

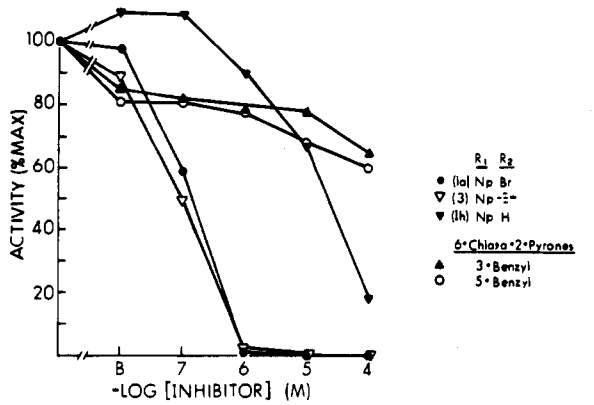


Figure 3. Comparison of haloenol lactone-, ynenol lactone- and 6-chloro-2-pyrone-mediated inhibition of calcium-independent phospholipase A_2. Canine myocardial phospholipase A_2 (10 ng) was incubated at 25 °C for 5 min with the indicated concentrations of haloenol lactone (●), ynenol lactone (▽), diproteoenol lactone (▼), 6-chloro-3-benzo-2-pyrone (▲), or 6-chloro-5-benzo-2-pyrone (○) at the indicated concentrations in 210 mM Tris·Cl buffer containing 1 mM EGTA (pH 7.4). Phospholipase A_2 activity was assessed after preincubation for 5 min at 25 °C by injection of 16:0,[^{3}H]18:1 plasmenylcholine (1 μM) and subsequent incubation for 5 min at 37 °C. Released [^{3}H]oleic acid was subsequently quantified after extraction into butanol, separation by TLC and scintillation spectrometry. Results represent the mean of duplicate determinations which each agree within 10% of the indicated value.

pholipase A_2 (Figure 1). Such alterations in ring size result in a different geometric relationship between the hydrolyzable lactone and the latent electrophilic site destined to covalently bind to the phospholipase A_2. The differences in inhibitory potency between 5- and 6-membered ring lactones suggest that such geometric considerations may modulate the intrinsic hydrolytic rate of the inhibitor, the rate of productive interactions of the ring-opened inhibitor with the active site, or the subsequent rate of nucleophilic attack of the tethered α-bromomethyl ketone by an active site nucleophile.

It has been previously demonstrated that alterations in the electrophilicity of the functionality revealed at the active site in this class of inhibitors result in substantial changes in inhibitory potency.[10,11] Since iodine is inherently more polarizable than bromine, it was reasoned that the iodoenol lactone would be more potent than its corresponding bromine analog. Accordingly, we compared the inhibitory potency of compounds **1a** and **1f** utilizing purified canine myocardial cytosolic phospholipase A_2 (Figure 2). As anticipated, the iodoenol lactone (compound **1f**) was over 5-fold more potent than the bromoenol lactone (compound **1a**), suggesting that the greater reactivity of the cryptic iodomethyl ketone facilitates rapid covalent binding to nucleophiles at or near the active site.

When the iodoenol lactone is hydrolyzed within the active site of the enzyme, it is subject to steric constraints imposed by the catalytic cleft. Accordingly, the stereoselectivity of inhibition of the cis/trans isomers of the iodoenol lactone were compared. The relative potency of the 6(*E*)-iodoenol lactone was 6-fold greater than that of the 6(*Z*)-iodoenol lactone (i.e., 30 vs 190 nM for compound **1f** vs compound **1g**). To further assess the importance of the reactivity of the electrophilic moiety generated after lactone hydrolysis, the ynenol lactone (compound **3**) was synthesized since hydrolysis of this class of compounds by elastase is known to generate an electrophilic allenone during mechanism-based inhibition.[13] Incubation of compound **3** with purified canine myocardial phospholipase A_2 demonstrated that the ynenol lactone was a potent inhibitor of this enzyme (i.e., IC_{50} = 95 nM under the conditions employed) (Figure 3). Sheep platelet cytosolic phospholipase A_2, a calcium-dependent enzyme, was not inhibited at comparable concentrations by either the iodoenol lactone (compound **1f**) or the ynenol lactone (compound **3**) (i.e., $IC_{50} > 40\ \mu M$). Finally, the diproteoenol lactone (compound **1h**) was only weakly inhibitory against canine phospholipase A_2 and did not inhibit sheep platelet phospholipase A_2, demonstrating the obligatory requirement for a latent reactive functionality in the inhibitor to allow its covalent binding to the active site after enzyme-mediated cleavage of the lactone.

Previous studies have demonstrated that 6-chloropyrones and 3-alkoxy-7-amino-4-chloroisocoumarins inhibit serine esterases. Although serine esterases (e.g., chymotrypsin) are efficiently inhibited by both the haloenol lactone and the 6-chloro-2-pyrone families of suicide inhibitors, myocardial phospholipase A_2 was not inhibited by 6-chloropyrones [6-chloro-3-(phenylmethyl)-2*H*-pyran-2-one or 6-chloro-5-(phenylmethyl)-2*H*-pyran-2-one] even at concentrations of 100 μM (Figure 3). The mechanism-based trypsin inhibitor 3-[(7-amino-4-chloro-1-oxo-1*H*-2-benzopyran-3-yl)oxy]propyl carbamimidothioate monohydrobromide failed to inhibit myocardial phospholipase A_2 even at a concentration of 100 μM. Thus, although the active site of the phospholipase A_2 can recognize some suicide substrates with a specificity similar to chymotrypsin (i.e., haloenol lactones), myocardial calcium-independent phospholipase A_2 possesses separate and distinct stereochemical requirements for mechanism-based inhibition. Similarly, ring-opened bromomethyl ketone inhibits chymotrypsin through pseudo-mechanism-based inhibition but fails to inhibit myocardial calcium-independent phospholipase A_2, thereby further underscoring the structural and kinetic differences inherent in the active site of phospholipases and proteases.

Conclusion

The results of the present study demonstrate that the precise stereoelectronic configurations of constituents in substituted haloenol lactone suicide inhibitors are important and modifiable determinants of their interaction with myocardial cytosolic calcium-independent phospholipase A_2. By merely substituting the more polarizable iodine constituent for the bromine atom, we have identified one of the most potent inhibitors of phospholipase A_2 activity known to date (IC_{50} = 30 nM). Accordingly, compound **1f** is a potentially useful pharmacologic tool which will likely facilitate identification of the roles of calcium-independent phospholipases A_2 in myocardium and other tissues during both physiologic and pathophysiologic perturbations. Since conservative modifications of the constituents in the haloenol lactone family of suicide inhibitors have dramatic effects on their inhibitory potencies, it seems likely that therapeutically useful pharmacologic agents can be designed which selectively (or specifically) interact with this novel class of calcium-independent phospholipases A_2.

Experimental Section

Methods. Purification of Canine Myocardial Calcium-Independent Phospholipase A_2 and Sheep Platelet Calcium-Dependent Phospholipase A_2. Canine myocardial cytosolic calcium-independent phospholipase A_2 was purified to near homogeneity by sequential DEAE Sephacel, chromatofocusing, and ATP-affinity chromatographies as previously described.[19] Canine myocardial cytosolic phospholipase A_2 activity obtained from affinity chromatography was 52 000-fold purified and possessed a specific activity of 67 μmol/mg·min utilizing 16:0,-[^{3}H]18:1 plasmenylcholine substrate.[3] Purified myocardial phospholipase A_2 was stored in liquid nitrogen in individual aliquots and was rapidly thawed immediately prior to use. No differences between fresh and previously frozen phospholipase A_2 preparations were observed when the enzyme was stored for up to 6 months.

Sheep platelet cytosolic calcium-dependent phospholipase A_2 was purified through tandem Superose 12 gel filtration chromatography as previously described.[16] Sheep platelet cytosolic calcium-dependent phospholipase A_2 eluted from Superose 12 resin with an apparent molecular weight of 60 kDa and possessed a specific activity of 7 nmol/mg·min utilizing 16:0,[^{3}H]18:1 plasmenylcholine substrate.

Preparation of Synthetic Phospholipids. Synthesis of homogeneous 16:0,[^{3}H]18:1 plasmenylcholine was performed by dicyclohexylcarbodiimide-mediated synthesis of radiolabeled fatty acid anhydride followed by its condensation with 1-*O*-(*Z*)-hexadec-1′-enyl-GPC utilizing *N*,*N*′-dimethyl-4-aminopyridine as catalyst as previously described.[23] Phospholipids were initially purified by preparative TLC and subsequently by strong-cation-exchange HPLC utilizing a Whatman Partisil SCX column.[24] The structure and purity of the radiolabeled synthetic product was confirmed by TLC in two solvent systems, by straight-phase HPLC and by comigration with authentic standards on reverse-phase HPLC.[15,23]

Phospholipase A_2 Assays. Myocardial phospholipase A_2 was assayed in 210 mM Tris.Cl (pH 7.0), 1 mM EGTA while platelet cytosolic phospholipase A_2 was assayed in the presence of 210 mM Tris.Cl (pH 7.2) and 1 μM $CaCl_2$. Selected concentrations of inhibitor were preincubated with enzyme for 5 min at 25 °C in the appropriate assay buffer. Remaining phospholipase activity was subsequently quantified following addition of 2 μM 16:0,-[^{3}H]18:1 plasmenylcholine (600 Ci/mol) and incubation at 37 °C for either 2 min (platelet phospholipase A_2) or 5 min (myocardial phospholipase A_2). Reaction products were extracted into butanol and subsequently isolated and quantified by TLC and scintillation spectrometry, respectively.[1]

Miscellaneous Procedures and Sources of Materials. Protein content was assessed utilizing a Bio-Rad protein assay kit with bovine serum albumin as standard. All radiolabeled starting materials were purchased from Du Pont-New England Nuclear. Bovine heart lecithin was obained from Avanti Polar Lipids and oleic acid was obtained from Nu Chek Prep, Inc. DEAE-cellulose was obtained from Whatman LabSales while DEAE-Sephacel, PBE-94, PB74, PB96, and Superose 12 were obtained from Pharmacia-LKB Biotechnology, Inc. *N*,*N*′-Dicyclohexylcarbodiimide and *N*,*N*-dimethyl-4-aminopyridine were obtained from Aldrich Chemical Co. All other reagents were obtained from Sigma Chemical Co.

Synthesis and Characterization of Inhibitors. Compounds **1a**,[10] **1b**,[10] **1e**,[25] **1f**,[10] **1h**,[11] **2**,[11] 6-chloro-3-(phenylmethyl)-2*H*-pyran-2-one,[20] 6-chloro-5-(phenylmethyl)-2*H*-pyran-2-one,[20] 3-[(7-amino-4-chloro-1-oxo-1*H*-2-benzopyran-3-yl)oxy]propyl carbamimidothioate, monohydrobromide,[21] 1-bromo-3-butyne,[10] 3-butynyl triflate,[26] and α-3-butynyl-1-naphthaleneacetic acid[10] were prepared according to previously described procedures. Chemicals were reagent grade and obtained from Aldrich Chemical Co. Preparative reverse-phase HPLC was performed employing a cis stationary phase (Waters 5.7 × 30 cm PrepPak Vydac C_{18} column (15–20 um)), a mobile phase comprised of acetonitrile/water (60/40), and a flow rate of 50 mL/min. ^{1}H NMR spectra (300 MHz) were obtained utilizing a Varian VXR300 FT spectrometer and are relative to TMS (δ = 0.0). The geometries of the vinyl substituents of the enol lactones were determined by proton nuclear Overhauser effect studies utilizing a Varian VXR400 FT spectrometer. All melting points are uncorrected.

(*E*)-6-(Bromomethylene)tetrahydro-3-phenoxy-2*H*-pyran-2-one (1c). In a flame-dried apparatus, a solution of 2.5 M *n*-butyllithium in hexanes (23.5 mL, 58.8 mmol) was added to a stirred solution of anhydrous diisopropylamine (8.24 mL, 5.95 g, 58.8 mmol) in anhydrous THF (20 mL) at 0 °C over argon. The mixture was cooled to −78 °C. A solution of phenoxyacetic acid (2.24 g, 14.7 mmol) in THF (8 mL) was added to the stirred mixture. A solution of 1-bromo-3-butyne (2.8 mL, 4.0 g, 30 mmol) in THF (8 mL) was then added and, after stirring for 1 h at −78 °C, the mixture was poured into ice (300 g). The mixture was then acidified to pH 2 with 3 N HCl and extracted with ethyl ether. The extract was dried (magnesium sulfate) and the solvent was removed in vacuo. The residue was purified by preparative reverse-phase HPLC to give 129 mg (9% yield) of α-3-butynylphenoxyacetic acid as a white solid. Recrystallization from methylene chloride/hexane gave white needles: mp 92–93 °C; ^{1}H NMR ($CDCl_3$) δ 1.99 (t, *J* = 2.6 Hz, 1 H), 2.20 (m, 2 H), 2.48 (m, 2 H), 4.84 (t, *J* = 6.4 Hz, 1 H), 6.92 (d, *J* = 7.8 Hz, 2 H), 7.00 (t, *J* = 6.9 Hz, 1 H), 7.28 (m, 2 H).

A mixture of α-3-butynylphenoxyacetic acid (74 mg, 0.36 mmol), *N*-bromosuccinimide (132 mg, 0.720 mmol), potassium bicarbonate (98 mg, 0.90 mmol), and 0.4 M aqueous tetrabutylammonium hydroxide (0.11 mL, 0.044 mmol) in methylene chloride (8 mL) was stirred at room temperature for 7 h in the dark. The mixture was washed with 5% sodium thiosulfate and saturated NaCl. The organic layer was dried (magnesium sulfate) and the solvent was removed in vacuo. The oil was purified by preparative reverse-phase HPLC to give 6 mg (6% yield) of **1c** as a colorless oil: ^{1}H NMR ($CDCl_3$) δ 2.23 (m, 1 H), 2.48 (m, 1 H), 2.98 (m, 2 H), 5.41 (dd, *J* = 10.9, 5.3 Hz, 1 H), 6.21 (t, *J* = 1.9 Hz, 1 H), 7.02 (m, 3 H), 7.30 (m, 2 H). HRMS calcd 283.1287, found 283.1299.

(*E*)-6-(Bromomethylene)tetrahydro-3-(3-pyridyl)-2*H*-pyran-2-one (1d). To a stirred solution of anhydrous diisopropylamine (8.91 mL, 6.43 g, 63.5 mmol) in anhydrous THF (100 mL) was added 2.5 M *n*-butyllithium in hexanes (25.4 mL, 63.5 mmol) at −20 °C under a dry argon atmosphere in an oven-dried apparatus. The resultant solution was stirred for 30 min at −20 °C. A solution of ethyl 3-pyridylacetate (9.21 mL, 10.0 g, 60.5 mmol) in anhydrous THF (70 mL) was added to the solution at −20 °C. Hexamethylphosphoramide (20 mL) was added to dissolve the dark yellow precipitate. The solution was stirred for 30 min at −20 °C and then for 30 min at −78 °C. A solution of 3-butynyl triflate (12.9 g, 63.5 mmol) in anhydrous THF (30 mL) was added dropwise at −78 °C. The resultant dark red solution was allowed to warm to room temperature and was subsequently stirred for 18 h. The reaction mixture was quenched by pouring it onto saturated ammonium chloride (500 mL). The mixture was then extracted with ethyl ether. The combined

extracts were washed with H_2O and then saturated NaCl and were subsequently dried (magnesium sulfate). The solvent was removed in vacuo to give an oil. Purification by silica gel chromatography using chloroform/methanol (99/1, v/v) afforded 10.6 g (81% yield) of ethyl α-3-butynyl-3-pyridylacetate as a yellow oil: ^{1}H NMR ($CDCl_3$) δ 1.22 (t, J = 7.1 Hz, 3 H), 2.15 (m, 4 H), 3.82 (t, J = 7.6 Hz, 1 H), 4.15 (m, 2 H), 7.27 (dd, J = 8.2, 5.1 Hz, 1 H), 7.67 (dt, J = 8.0, 2.0 Hz, 1 H), 8.54 (dd, J = 4.8, 1.6 Hz, 1 H), 8.57 (d, J = 1.7 Hz, 1 H).

Sodium hydroxide (150 mL, 1 N) was added dropwise to a stirred solution of ethyl α-3-butynyl-3-pyridylacetate (10.6 g, 48.8 mmol) in methanol (150 mL) at room temperature. The resultant yellow solution was stirred for 30 min. The methanol was removed in vacuo and the pH was adjusted to 5 with 1 N HCl. The solution was concentrated in vacuo and then extracted into chloroform. The extract was dried (disodium sulfate) and the solvent was removed under reduced pressure to give an oil. Purification by recrystallization from methylene chloride/hexane afforded 7.93 g (86% yield) of α-3-butynyl-3-pyridylacetic acid as a white crystalline solid: mp 109–111 °C; ^{1}H NMR ($CDCl_3$) δ 2.19 (m, 5 H), 3.88 (t, J = 7.5 Hz, 1 H), 7.38 (dd, J = 7.7, 5.1 Hz, 1 H), 7.86 (dd, J = 8.1, 1.8 Hz, 1 H), 8.52 (dd, J = 5.0, 1.4 Hz, 1 H), 8.61 (s, 1 H).

A mixture of *N*-bromosuccinimide (3.76 g, 21.1 mmol), potassium bicarbonate (2.12 g, 21.1 mmol), and α-3-butynyl-3-pyridylacetic acid (4.00 g, 21.1 mmol) in methylene chloride (350 mL) was stirred for 4 h at room temperature in the dark. The mixture was added to 5% sodium thiosulfate (300 mL) to quench the reaction. The organic layer was washed with H_2O and saturated NaCl and subsequently dried (magnesium sulfate). The solvent was removed in vacuo. Purification by silica gel chromatography using methylene chloride/ethyl acetate (2:1, v/v) gave 0.60 g (11% yield) of **1d** as an oil. Compound **1d** was characterized as its hydrochloride salt which was recrystallized twice from methanol/ethyl ether to give colorless needles: mp 170–171 °C; ^{1}H NMR ($CDCl_3$) δ 2.22 (m, 1 H), 2.34 (m, 1 H), 2.76 (m, 1 H), 2.97 (m, 1 H), 4.43 (dd, J = 12.8, 5.5 Hz, 1 H), 5.24 (br s, 1 H), 6.47 (t, J = 2.0 Hz, 1 H), 8.00 (dd, J = 8.1, 5.5 Hz, 1 H), 8.48 (dt, J = 8.1, 1.7 Hz, 1 H), 8.85 (dd, J = 4.4, 1.1 Hz, 1 H), 8.90 (d, J = 1.8 Hz, 1 H).

Tetrahydro-(*Z*)-6-(iodomethylene)-3-(1-naphthalenyl)-2*H*-pyran-2-one (1g). To a stirred solution of α-3-butynyl-1-naphthaleneacetic acid (9.4 g, 39 mmol) in acetonitrile (100 mL) were added iodine (10.2 g, 40.0 mmol) and potassium bicarbonate (40 g, 40 mmol). The mixture was stirred for 50 h at room temperature. A solution of 5% sodium thiosulfate (300 mL) was added to remove excess iodine. The mixture was extracted into methylene chloride. The combined extracts were dried (magnesium sulfate), and the solvent was removed in vacuo. The residue was applied to a silica gel column and eluted with methylene chloride/hexane (50:50, v/v) to remove the (*E*)-iodoenol lactone. Elution of the column with methylene chloride/hexane (60:40, v/v) afforded 0.65 g of (*Z*)-iodoenol lactone as a colorless oil (5% yield). An analytical sample was obtained by preparative reverse-phase HPLC: ^{1}H NMR ($CDCl_3$) δ 2.32 (m, 2 H), 2.95 (m, 2 H), 4.86 (dd, J = 9.9, 6.3 Hz, 1 H), 5.60 (t, J = 0.9 Hz, 1 H), 7.52 (m, 4 H), 7.88 (d, J = 7.7 Hz, 1 H), 7.98 (m, 2 H). HRMS calcd 363.9960, found 363.9975.

Tetrahydro-3-(1-naphthalenyl)-(*E*)-6-(prop-2-ynylidene)-2*H*-pyran-2-one (3). To a stirred mixture of compound **1f** (330 mg, 0.906 mmol) in anhydrous triethylamine (10 mL) were added bis(triphenylphosphine)palladium(II) chloride (12.7 mg, 0.016 mmol) and cuprous chloride (7.1 mg, 0.071 mmol) at room temperature in a flame-dried apparatus over a dry argon atmosphere. To the cloudy mixture was added (trimethylsilyl)-acetylene (0.132 mL, 0.937 mmol) at room temperature. The mixture was stirred vigorously for 4 h. The solvent and volatile materials were removed in vacuo. The oil was purified by flash chromatography (silica gel) using methylene chloride/hexane (50:50, v/v) as eluant to give 182 mg (67% yield) of tetrahydro-3-(1-naphthalenyl)-(*E*)-6-[3-(trimethylsilyl)prop-2-ynylidene]-2*H*-pyran-2-one as an orange oil: ^{1}H NMR ($CDCl_3$) δ 0.20 (s, 9 H), 2.34 (m, 2 H), 2.83 (m, 1 H), 3.00 (m, 1 H), 4.58 (t, J = 7.2 Hz, 1 H), 5.46 (s, 1 H), 7.31 (d, J = 6.6 Hz, 1 H), 7.50 (m, 3 H), 7.85 (m, 3 H).

To a stirred solution of tetrahydro-3-(1-naphthalenyl)-(*E*)-6-[3-(trimethylsilyl)prop-2-ynylidene]-2*H*-pyran-2-one (1.29 g, 3.86 mmol) in ethyl alcohol (20 mL) was added a solution of silver nitrate (2.63 g, 15.5 mmol) in H_2O (21 mL) at room temperature over argon. The mixture was stirred for 1 h at room temperature. Methylene chloride (20 mL) was added and an orange precipitate formed. The mixture was poured into a stirred solution of potassium cyanide (6.0 g, 92 mmol) in H_2O (60 mL). The mixture was stirred for 1 h at room temperature and then extracted with methylene chloride. The combined extracts were dried (magnesium sulfate), and the solvent was removed in vacuo. The oil was purified by preparative reverse-phase HPLC to give 464 mg (51% yield) of compound **3** as a waxy orange solid: ^{1}H NMR ($CDCl_3$) δ 2.34 (dd, J = 13.5, 7.5 Hz, 2 H), 2.84 (m, 1 H), 3.03 (m, 1 H), 3.13 (d, J = 2.1 Hz, 1 H), 4.59 (t, J = 7.5 Hz, 1 H), 5.42 (s, 1 H), 7.32 (d, J = 6.6 Hz, 1 H), 7.50 (m, 3 H), 7.86 (m, 3 H). HRMS calcd 262.0993, found 262.0987.

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References

(1) Wolf, R. A.; Gross, R. W. Identification of Neutral Active Phospholipase C which Hydrolyzes Choline Glycerophospholipids and Plasmalogen Selective Phospholipase A_2 in Canine Myocardium. *J. Biol. Chem.* **1985**, *260*, 7295–7303.

(2) Angle, M. J.; Paltauf, F.; Johnston, J. M. Selective Hydrolysis of Ether-containing Glycerophospholipids by Phospholipase A_2 in Rabbit Lung. *Biochim. Biophys. Acta* **1988**, *962*, 234–240.

(3) Hazen, S. L.; Stuppy, R. J.; Gross, R. W. Purification and Characterization of Canine Myocardial Cytosolic Phospholipase A_2: A Calcium Independent Phospholipase with Absolute *sn*-2 Regiospecificity for Diradyl Glycerophospholipids. *J. Biol. Chem.* **1990**, *265*, 10622–10630.

(4) Gassama-Diagne, A.; Fauvel, J.; Chap, H. Purification of a New, Calcium-independent, High Molecular Weight Phospholipase A_2/Lysophospholipase (Phospholipase B) from Guinea Pig Intestinal Brush-border Membrane. *J. Biol. Chem.* **1989**, *264*, 9470–9475.

(5) Katz, A. M.; Messineo, F. C. Lipid-membrane Interactions and the Pathogenesis of Ischemic Damage in the Myocardium. *Circ. Res.* **1981**, *48*, 1–16.

(6) Corr, P. B.; Gross, R. W.; Sobel, B. E. Amphipathic Metabolites and Membrane Dysfunction in Ischemic Myocardium. *Circ. Res.* **1984**, *55*, 135–154.

(7) Hazen, S. L.; Gross, R. W. Principles of Membrane Biochemistry and Their Application to the Pathophysiology of Cardiovascular Disease. In *The Heart and Cardiovascular System*, 2nd ed.; Fozzard, H. A., Haber, E., Jennings, R. B., Katz, A. M., Morgan, H. E., Eds.; Raven Press, Ltd: New York, 1991; pp 839–860.

(8) Gross, R. W. Myocardial Phospholipases A_2 and Their Membrane Substrates. *Trends Cardiovasc. Med.* **1992**, *2*, 115–121.

(9) Bloch, K. Enzymatic Synthesis of Monounsaturated Fatty Acids. *Acc. Chem. Res.* **1969**, *2*, 193–202.

(10) Daniels, S. B.; Cooney, E.; Sofia, M. J.; Chakravarty, P. K.; Katzenellenbogen, J. A. Haloenol Lactones, Potent Enzyme-Activated Irreversible Inhibitors for α-Chymotrypsin. *J. Biol. Chem.* **1983**, *258*, 15046–15053.

(11) Daniels, S. B.; Katzenellenbogen, J. A. Halo Enol Lactones: Studies on the Mechanism of Inactivation of α-Chymotrypsin. *Biochemistry* **1986**, *25*, 1436–1444.

(12) Hazen, S. L.; Zupan, L. A.; Weiss, R. H.; Getman, D. P.; Gross, R. W. Suicide Inhibition of Canine Myocardial Cytosolic Calcium-Independent Phospholipase A_2. *J. Biol. Chem.* **1991**, *266*, 7227–7232.

(13) Spencer, K. W.; Tam, T. F.; Thomas, E.; Robinson, V. J.; Krantz, A. Ynenol Lactones: Synthesis and Investigation of Reactions Relevant to Their Inactivation of Serine Proteases. *J. Am. Chem. Soc.* **1986**, *108*, 5589–5597.

(14) Gross, R. W. High Plasmalogen and Arachidonic Acid Content of Canine Myocardial Sarcolemma: A Fast Atom Bombardment Mass Spectroscopic and Gas Chromatography-Mass Spectroscopic Characterization. *Biochemistry* **1984**, *23*, 158–165.

(15) Gross, R. W. Identification of Plasmalogen as the Major Phospholipid Constituent of Cardiac Sarcoplasmic Reticulum. *Biochemistry* **1985**, *24*, 1662–1668.

(16) Loeb, L. A.; Gross, R. W. Identification and Purification of Sheep Platelet Phospholipase A_2 Isoforms: Activation by Physiologic

Concentrations of Calcium Ion. *J. Biol. Chem.* **1986**, *261*, 10467–10470.
(17) Kramer, R. M.; Jakubowski, J. A.; Deykin, D. Hydrolysis of 1-alkyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine, A Common Precursor of Platelet-activating Factor and Eicosanoids, by Human Platelet Phospholipase A_2. *Biochim. Biophys. Acta* **1988**, *959*, 269–279.
(18) Clark, J. D.; Milona, N.; Knopf, J. L. Purification of a 110-kilodalton Cytosolic Phospholipase A_2 from the Human Monocytic Cell Line U937. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 7708–7712.
(19) Hazen, S. L.; Loeb, L. A.; Gross, R. W. Purification and Characterization of Novel Cytosolic Phospholipase A_2 Activities from Canine Myocardium and Sheep Platelets. In *Methods in Enzymology*; Dennis, E. A., Eds.; Academic Press, Inc.: San Diego, 1991; pp 400–411.
(20) Westkaemper, R. B.; Abeles, R. H. Novel Inactivators of Serine Proteases Based on 6-Chloro-2-pyrone. *Biochemistry* **1983**, *22*, 3256–3264.
(21) Boulanger, W. A.; Katzenellenbogen, J. A. Structure-activity Study of 6-substituted 2-pyranones as Inactivators of α-Chymotrypsin. *J. Med. Chem.* **1986**, *29*, 1159–1163.
(22) Kam, C.-M.; Fujikawa, K.; Powers, J. C. Mechanism-based Isocoumarin Inhibitors for Trypsin and Blood Coagulation Serine Proteases: New Anticoagulants. *Biochemistry* **1988**, *27*, 2547–2557.
(23) Han, X.; Zupan, L. A.; Hazen, S. L.; Gross, R. W. Semi-synthesis and Purification of Homogeneous Plasmenylcholine Molecular Species. *Anal. Biochem.* **1992**, *200*, 119–124.
(24) Gross, R. W.; Sobel, B. E. Isocratic High-performance Liquid Chromatography Separation of Phosphoglycerides and Lysophosphoglycerides. *J. Chromatogr.* **1980**, *197*, 79–85.
(25) Krafft, G. A.; Katzenellenbogen, J. A. Synthesis of Halo Enol Lactones: Mechanism-based Inactivators of Serine Proteases. *J. Am. Chem. Soc.* **1981**, *103*, 5459–5466.
(26) Hummel, K.; Hanack, M. *Liebigs Ann. Chem.* **1971**, *746*, 211–213.