Suicide-Inhibitory Bifunctionally Linked Substrates (SIBLINKS) as Phospholipase A_2 Inhibitors

William N. Washburn*,† and Edward A. Dennis

Department of Chemistry
Univeristy of California, San Diego
La Jolla, California 92093

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We report herein the first example of a specific suicide inhibitor for phospholipase A_2 (PLA_2).[1,2] Prior investigators have reported nonspecific irreversible inhibitors such as *p*-bromophenacyl bromide,[3] manoalide,[4] or manoalogue,[5] competitive inhibitors such as phospholipid or transition-state analogues,[6,7] and a variety of nonspecific inhibitors which prevent enzyme binding to the lipid bilayer.[8]

In the accompanying paper,[9] we introduce the concept of "suicide-inhibitory bifunctionally linked substrates" (SIBLINKS) and report the utilization of 1-decanoyl-2-(*p*-nitrophenyl glutaryl)phosphatidylcholine **1a** to generate a spectroscopic assay for PLA_2. Here, we use this approach to generate specific suicide inhibitors of PLA_2. As outlined in Scheme I, if cyclization of **2** arising from enzymatic hydrolysis of the *sn*-2 glycerol ester of **1** is fast relative to diffusion, a reactive cyclic anhydride would be generated in close proximity to the enzyme active site which could be inactivated upon acylation.

A series of substrates **1b–e** containing succinic and glutaric moieties were prepared by acylation of 1-decanoyl-2-lysophosphatidylcholine with the appropriate anhydride and conversion to the acid chloride and subsequently to the *p*-nitrophenyl ester.[10] The time courses for inactivation of cobra venom (*Naja naja naja*) PLA_2 upon preincubation with 100 μM **1a–e** are shown in Figure 1. In some instances, significant initial activation was observed, possibly due to a detergent effect of the substrate acting to prevent absorption of the hydrophobic PLA_2 enzyme to the surface of the vial.

In the presence of Ca^{2+}, required for PLA_2 activity, irreversible inhibition was observed upon preincubation with all esters except for **1a**.[13] Inhibition was found to parallel release of *p*-nitrophenoxide from each phospholipid. Inhibition was not due to cyclic anhydrides in bulk solution.[14] In the absence of Ca^{2+} (1 mM EGTA), no inhibition or dye release was observed. Also, no PLA_2 inhibition was observed when 20 μM **1b** was added directly to the titrimetric assay containing 5 mM DPPC.

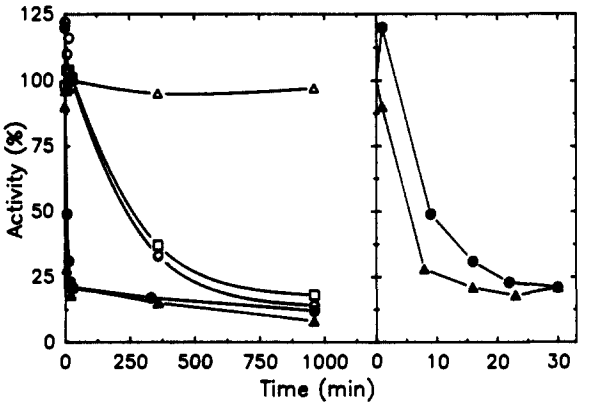

Figure 1. Inhibition of PLA_2 as a function of preincubation time with 100 μM **1a** (△), **1b** (●), **1c** (□), **1d** (▲), and **1e**, (○). Preincubation of a 260:1 (mol/mol) mixture of inhibitor **1** to PLA_2 was at 20 °C in 1 mL of buffer (20 mM Tris·HCl, pH = 8.0, 10 mM $CaCl_2$, and 0.1 M KCl) containing 5 μg mL^{-1} of PLA_2 and 100 μM **1a–e** in vesicles prepared by sonication in 0.1 M KCl followed by centrifugation (9500 g, 25 min, 4 °C). The hydrolysis rate of **1a–e** was followed spectrophotometrically at 400 nm. The extent of PLA_2 inactivation was measured titrimetrically[11,12] by the addition of a 20-μL aliquot of the above solution to 1.7 mL of assay medium containing 5 mM 1,2-dipalmitoylphosphatidylcholine (DPPC), 10 mM $CaCl_2$, and 20 mM Triton X-100 at 40 °C. Right panel shows inhibition by **1b** and **1d** at short times.

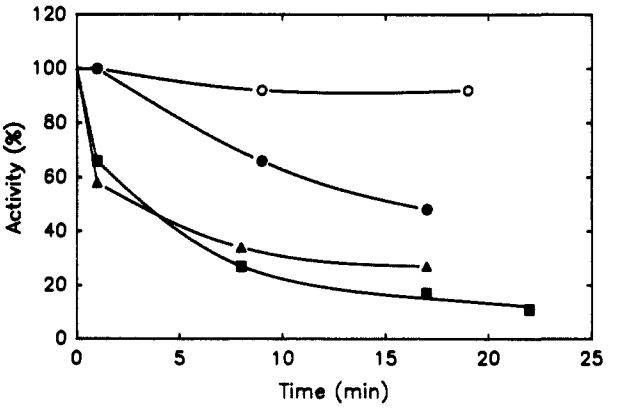

Figure 2. Inactivation of PLA_2 as a function of **1d** concentration. PLA_2 (70 nM), preincubated in the presence of 0.5 μM (○), 1.7 μM (●), 5 μM (▲), and 20 μM (■) **1d**, was assayed as described in Figure 1.

Table I contains the initial velocity for release of *p*-nitrophenoxide from substrates **1a–e** during the first 5% of the hydrolysis. Table I also contains the cyclization $t_{1/2}$ for the five hydrolysis products **2a–e**.

The efficiency of enzymatic inactivation can be expressed as the partition ratio (*P*) as shown in Table I. The extent of inhibition is a function of the preincubation conditions; conditions that favor association of the hydrolysis product with PLA_2 lower the *P*. Raising the temperature from 20 °C to 40 °C during the prein-

* To whom correspondence should be addressed.
† Visiting Scholar UCSD (1988–1989). Permanent address: Life Sciences Research Laboratory, Eastman Kodak Company, Rochester, NY 14650.

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(10) **1b–e** were prepared by using the same procedure described for **1a**.[9] These esters were chromatographed on silica gel by using 2:1 $CHCl_3$/MeOH as the eluant. HPLC using MeOH to elute **1** from a Brownlee Lab C_{18} column removed the remaining trace impurities. The ^1H NMR and high-resolution mass spectra for all four structures were in accord with those reported for **1a**.[9]
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(13) Catalytic activity was not restored upon standing for 24 h at 20 °C after passage of the inactivated PLA_2 through a Pharmacia G-25 PD-10 column to separate enzyme from inhibitor.
(14) PLA_2 activity was measured after preincubation with each of the reaction products to exclude the possibility that **2** or the cyclic anhydride in bulk solution could inhibit PLA_2. 2,2-Dimethylglutaric anhydride, 3,3-dimethylglutaric anhydride, and succinic anhydride at 3 mM gave 50, 50, and 30% inhibition, respectively; no inhibition was observed at 0.3 mM. Inhibition due to **2f** (*p*-nitrophenyl 3,3-dimethylglutarate),[15] a model for the release fragment **2**, was determined to be 20 and 0% at 1.7 mM and 0.15 mM. No inhibition was observed by 300 μM *p*-nitrophenol or 220 μM 2,2-dimethylglutarate.
(15) **2a** and **2f** were synthesized by using the procedure reported by Gaetjens and Morawetz: Gaetjens, E.; Morawetz, H. *J. Am. Chem. Soc.* **1960**, *82*, 3335.
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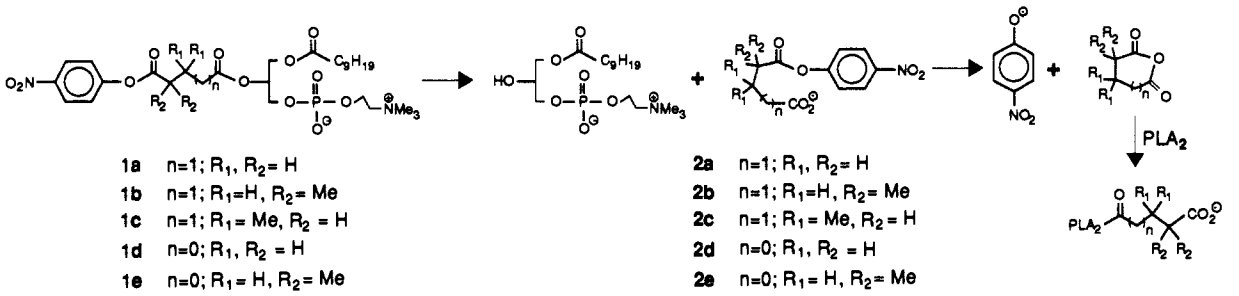
Scheme I



1a n=1; R_1, R_2= H
1b n=1; R_1=H, R_2= Me
1c n=1; R_1= Me, R_2 = H
1d n=0; R_1, R_2 = H
1e n=0; R_1= H, R_2= Me

2a n=1; R_1, R_2= H
2b n=1; R_1=H, R_2= Me
2c n=1; R_1= Me, R_2= H
2d n=0; R_1, R_2 = H
2e n=0; R_1= H, R_2= Me

Table I. Efficiency of Hydrolysis and Inhibition

substrate	V, μmol min^{-1} mg^{-1}	P[a]	release fragment	$t_{1/2}$ cyclization,[b] s
1a	155[c]		**2a**	150
1b	0.11	19	**2b**	40
1c	0.002	9	**2c**	15
1d	0.6	35	**2d**	4
1e	0.005	11	**2e**	1
			2f[d]	30

[a] Partition ratio (P) expressed as moles of dye released per mole of enzyme inactivated. [b] The rate constants for cyclization of *p*-nitrophenyl esters **2a** and **2f**[15] were measured spectrophotometrically at pH 8 at 20 °C and found to be 700 times faster than the literature values[16] for the corresponding phenyl esters. The $t_{1/2}$'s for **2b–e** were calculated from the literature values for the corresponding phenyl esters,[16] assuming that the *p*-nitrophenyl ester cyclized 700 times faster. [c] Taken from ref 9. [d] **2f** is *p*-nitrophenyl 3,3-dimethylglutarate.[15]

cubation of PLA_2 with **1b** or **1d** had little effect on the rate of inactivation, but did increase P. Presumably, higher temperatures favor diffusion of the hydrolysis products **2** away from the enzyme, thereby resulting in the cyclic anhydride being generated in bulk solution. The dimethylglutarates **1b** and **1c** and the dimethylsuccinate **1e** are more efficient suicide inhibitors than **1d**; on average, the enzyme processes 10–20 substrates before being inactivated. Inhibitor **1d** is the best substrate but the least efficient of the inhibitors despite the fact that **2d** cyclizes faster than **2b** or **2c**. Presumably, hydrophobic geminal methyl groups also enhance the association of **2** to the enzyme. The overall rate of inactivation is a reflection of not only susceptibility of **1** to enzymatic hydrolysis and the rate of intramolecular cyclization of **2** but also the rate of diffusion of **2** from the enzyme.

The effect of inhibitor concentration is shown in Figure 2 for succinate **1d**. Inhibition of a 70 nM solution of PLA_2 is quite rapid even at 1.7 μM inhibitor. The 10% inactivation observed for 0.5 μM **1d** was consistent with P = 35 since a 7:1 ratio of **1d**/PLA_2 meant **1d** would be consumed before complete PLA_2 inactivation. Inhibition was observed even at lower concentrations provided the ratio of inhibitor/PLA_2 exceeded 40, indicating that the binding affinity for these inhibitors is high.

Inhibition of other PLA_2 enzymes was also observed. We are further investigating the mechanism by which these exceedingly potent, efficient, and selective inhibitors of PLA_2 function.

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Computer Software Reviews

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Robert D. Walkup, *Texas Tech University*