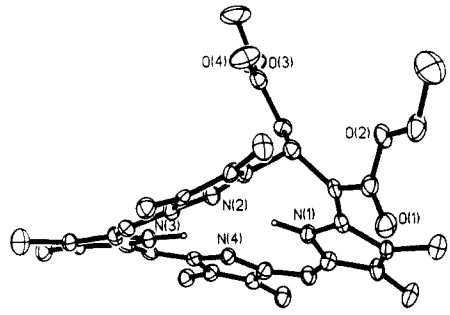
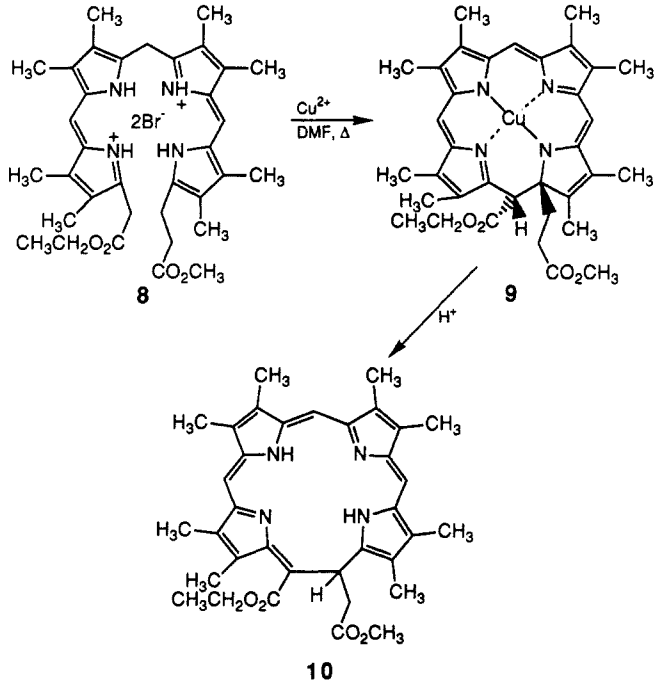


Figure 4. X-ray structure of ring-expanded macrocycle **10**.

of minor products, including a trace of porphyrin. These materials are extremely robust and readily survive even concentrated sulfuric acid without transformation into porphyrin. The optical spectrum of **7** is shown as the broken line in Figure 1, and its X-ray structure is presented in Figure 3.

Use of the unsymmetrically substituted a,c-biladiene salt[12] **8** in the copper(II)-catalyzed cyclization afforded uniquely[13,14] a 36% yield of the copper(II) macrocycle **9**, with an optical spectrum very similar to that of **6**. However, attempts to remove the



chelating copper with sulfuric and trifluoroacetic acids resulted in ring expansion (30%) to give the product **10** with the homoporphyrin carbon skeleton. Figure 4 presents the X-ray structure of this novel material. We presume that the ring expansion proceeds by way of initial ring opening, followed by closure in a different sense, and that the ring opening is favored in the case of **9** (compared with **6**) by the presence of the enolizable proton at the macrocyclic carbon.[14]

(12) Compound **8** was prepared via the tripyrrene route[2,3] from benzyl 5-(benzyloxycarbonyl)-3,3′,4,4′-tetramethyldipyrromethane-5′-carboxylate, 5-[2-(methoxycarbonyl)ethyl]-2-formyl-3,4-dimethylpyrrole, and 5-[2-(ethoxycarbonyl)methyl]-2-formyl-3,4-dimethylpyrrole [NMR ($CDCl_3$) δ 13.43 (br s, 2 H, NH), 13.23, 13.08 (each br s, 1 H, NH), 7.16, 7.12 (each s, 1 H, =CH), 5.19 (s, 2 H, CH_2), 4.23 (s, 2 H, CH_2CO), 4.19 (q, J = 7.2 Hz, 2 H, CH_3CH_2O), 3.66 (s, 3 H, OCH_3), 3.27 (t, J = 7.2 Hz, 2 H, CH_2CH_2CO), 2.99 (t, J = 7.2 Hz, 2 H, CH_2CH_2CO), 2.29, 2.28, 2.23, 2.21, 2.05, 1.98, 1.90, 1.88 (each s, 3 H, CH_3), 1.28 (t, J = 7.2 Hz, 3 H, OCH_2CH_3); vis (CH_2Cl_2) λ_{max} 452 (ϵ 104 200), 528 nm (105 800)].

(13) Though other isomers were possible, only the compound with structure **9** is produced. A crystal structure of this compound, which supports the structure proposed in all respects, has been obtained. See supplementary material.

(14) Complete mechanistic rationalizations will be presented in a full paper.

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Supplementary Material Available: Figures showing structures and tables of crystal data and data collection parameters, atomic coordinates, bond lengths, bond angles, anisotropic thermal parameters, H-atom coordinates, and isotropic displacement coefficients of compounds **6**, **7**, **9**, and **10** (28 pages). Ordering information is given on any current masthead page.

Novel General Approach for the Assay and Inhibition of Hydrolytic Enzymes Utilizing Suicide-Inhibitory Bifunctionally Linked Substrates (SIBLINKS): Exemplified by a Phospholipase A_2 Assay

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Kinetic studies of hydrolytic enzymes such as phospholipase A_2[1] (PLA_2) have been hampered by the lack of a versatile, sensitive, continuous spectroscopic assay. Spectroscopic assays currently employed either have low sensitivity,[2] inhibit some PLA_2's,[3] use exceedingly poor substrates,[4] require extensive synthesis,[4,5] are incompatible with free thiols,[5] or are not continuous.[6] We report herein a convenient spectrophotometric assay for PLA_2 in which the substrate closely resembles a natural phospholipid. In addition, we utilize this approach to design "suicide-inhibitory bifunctionally linked substrates" (SIBLINKS) which are specific irreversible inhibitors for PLA_2[7] as well as discuss extensions to either assay or modulate the activity of other hydrolytic enzymes.

Since direct attachment of a chromophore to the glycerol backbone is precluded by the substrate structural requirements of PLA_2,[1] we employed a dibasic acid to link the lysophospholipid moiety to a dye. Upon PLA_2-catalyzed hydrolysis of the *sn*-2 ester of 1-decanoyl-2-(*p*-nitrophenyl glutaryl)phosphatidylcholine (**1**), nucleophilic catalysis by the nascent carboxylate group of the hydrolysis product **2** releases *p*-nitrophenol via cyclization[8] (Scheme I). The cyclization of **2** should be slow relative to diffusion since $t_{1/2}$ for cyclization of **2** was found to be 140 s at 20 °C. Consequently, the concomitantly formed glutaric anhydride would be formed in bulk solution and would react with H_2O before encountering PLA_2.[9]

The spectrophotometric assay of PLA_2 from cobra venom (*Naja naja naja*[10]) using **1**[11] as substrate is linear for 2 min and is linear

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(9) Inhibition of PLA_2 by exposure to anhydrides in bulk solution is very inefficient. For example, treatment of a 0.35 μM solution of PLA_2 for 5 min with 3, 0.3, and 0.03 mM glutaric anhydride resulted in 80, 14, and 0% inhibition, respectively. These values did not change with time.

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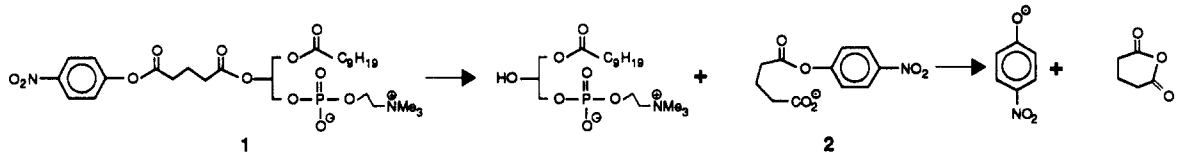
Scheme I



1 2

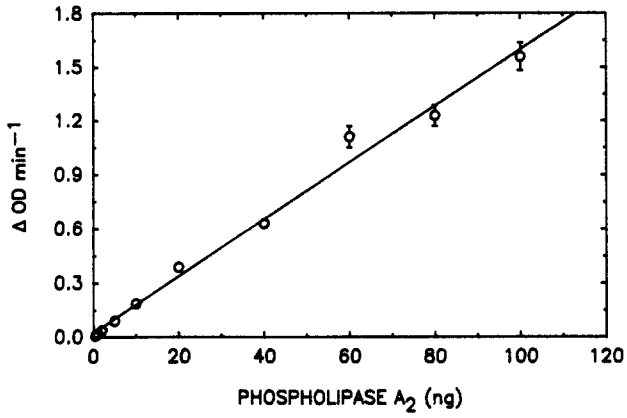


Figure 1. Dependence of velocity on protein concentration. Assay conditions consisted of 400 μM **1** in Triton X-100 mixed micelles (R_e = 1.6) in 0.4 mL of buffer (10 mM Tris·HCl, pH 8.0, containing 10 mM $CaCl_2$ and 100 mM KCl) at 40 °C. Velocity is expressed as ΔOD min^{-1} at 400 nm. Under these conditions, ε for *p*-nitrophenol was 1.5 × 10^4.

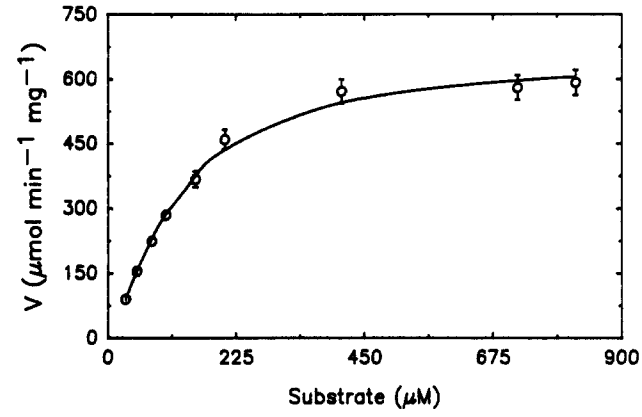


Figure 2. Activity as a function of substrate **1** concentration. Assays were performed under standard conditions defined in Figure 1 with 20 ng of PLA_2 and R_e = 3.

with protein concentration (Figure 1). The observed rate for 0.5 ng of PLA_2 in a 0.4-mL assay was 0.009 ΔOD min^{-1} at 400 nm corresponding to 180 pmol min^{-1} of substrate hydrolyzed. The nonenzymatic hydrolysis rate of 400 μM **1** is 0.001 ΔOD min^{-1} at 40 °C. Thiols are compatible with assays using **1**; for example, a background reaction rate due to 1.1 mM $HSCH_2CH_2OH$ under standard assay conditions (see Figure 1) was 0.06 ΔOD min^{-1}, comparable to that observed for 3 ng of PLA_2.

A plot of velocity (V) versus bulk substrate concentration (Figure 2) was fit to, and the line drawn for, the Hill equation, which gave a V_{max} of about 650 μmol min^{-1} mg^{-1} for **1** in Triton X-100/phospholipid mixed micelles at R_e = 3. R_e is defined as (detergent concentration–detergent cmc)/substrate concentration.[12] Half-maximum velocity was obtained at about 120 μM substrate. The hydrolysis rate is a function of substrate surface concentration[13] in mixed micelles and diminishes by a factor of 2 as R_e increases from 1.6 to 5.2. Small unilamellar vesicles (SUVs) prepared by sonication of **1** in 0.1 M KCl followed by centrifugation (9500*g*, 25 min, 4 °C) were readily hydrolyzed by PLA_2. At 40 °C, a plot of V vs S gave a V_{max} of about 280 μmol min^{-1} mg^{-1} with half-saturation at about 90 μM. V at 40 °C for 400 μM **1** as SUVs was 265 μmol min^{-1} mg^{-1} as compared to 560 μmol min^{-1} mg^{-1} for mixed micelles at R_e = 3.

1 compares favorably with natural substrates. In a titrimetric assay at 40 °C with 5 mM substrate in 4:1 Triton/phospholipid mixed micelles, the relative V's of **1** and 1,2-dipalmitoylphosphatidylcholine (DPPC) were 1:3. Although the apparent V_{max} for **1** was ~$^1/_8$ of that for DPPC,[13] half-saturation occurred with **1** at ~$^1/_5$ the concentration required for DPPC. The advantages to this assay are (a) straightforward three-step substrate synthesis, (b) low amounts of substrate required for an assay, (c) close structural approximation to natural substrate, (d) sensitivity to less than 1 ng of enzyme, and (e) compatibility with thiols.

We anticipate that the SIBLINKS concept can be extended to devise both specific spectrophotometric assays and suicide inhibitors for other hydrolytic enzymes due to the synthetic flexibility inherent in the modular nature of this approach. The critical feature is the use of an appropriate dibasic acid as a spacer to link a molecular ensemble conveying substrate recognition to a leaving group. Upon enzymatic hydrolysis of the "substrate bond", the nascent carboxylate serves as an internal nucleophile to expel the leaving group, thereby forming a five- or six-membered ring. If cyclization is slow relative to product diffusion and the leaving group is a chromophore-linked dye, enzymatic hydrolysis will release a dye, which can be monitored in a spectroscopic assay. Under these conditions, the anhydride, generated in bulk solution, would be trapped by water.

If the cyclization is fast relative to diffusion, the anhydride will be formed at the active site or in the solvation sphere of the enzyme. As illustrated by SIBLINKS-induced PLA_2 inhibition,[7] enzyme acylation would specifically modulate catalytic activity. Nonspecific enzyme acylation is precluded due to facile water trapping of the anhydride. The rate of formation of the anhydride can be modulated by (a) adjustment of the pK_a of the leaving group or (b) modification of the linker chain by introduction of alkyl substituents or incorporation into a cyclic structure.[14]

We are evaluating the use of SIBLINKS for other hydrolytic enzymes as well as exploring alternative dyes to increase the sensitivity of this PLA_2 assay.[15]

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(11) **1** was prepared by acylating 1-decanoyl-2-lysophosphatidylcholine (Avanti) with a 4-fold excess of glutaric anhydride and triethylamine in CH_2Cl_2 at 40 °C. After purification on silica gel using 1:4:30:65 HOAc/H_2O/MeOH/$CHCl_3$, the product half-acid was converted to **1** by sequential treatment in CH_2Cl_2 at 20 °C with (a) an excess of oxalyl chloride and (b) excess *p*-nitrophenol/triethylamine. **1** was purified in 80% overall yield by two selective precipitations induced by dilution of a concentrated CH_2Cl_2 solution 50-fold with Et_2O. Hydrolysis of **1** appears to be catalyzed on silica gel, Florisil, and alumina; efforts to date to separate **1** from *p*-nitrophenol on these supports failed due to the concomitant hydrolysis of **1** during the elution of **1** with 2:1 $CHCl_3$/MeOH. Preparative HPLC using a Brownlee Lab C_{18} column and MeOH as eluant yielded **1** free of trace contaminants: ^1H NMR ($CDCl_3$) δ 0.86 (t, 3 H), 1.29 (s, 12 H), 1.6 (m, 2 H), 2.05 (t, 2 H), 2.28 (m, 2 H), 2.5 (m, 2 H), 2.7 (m, 2 H), 3.35 (s, 9 H), 3.72 (m, 2 H), 3.95 (m, 2 H), 4.15 (m, 1 H), 4.3 (m, 2 H), 4.4 (m, 1 H), 5.2 (m, 1 H), 7.3 (AB q, 2 H), 8.25 (AB q, 2 H); MS (FAB) *m/e* 647; exact mass calcd for $C_{48}N_2O_{12}P$ 647.2945, found 647.2952.

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(15) For example, an assay for chymotrypsin using the bis(*p*-nitrophenyl ester) of glutaric acid is 5 times more sensitive than one with *p*-nitrophenylacetate since twice as much dye is released and the hydrolysis rate of the substrate is approximately 2.5 times higher.