Phosphonate-Containing Phospholipid Analogues as Tight-Binding Inhibitors of Phospholipase-A_2

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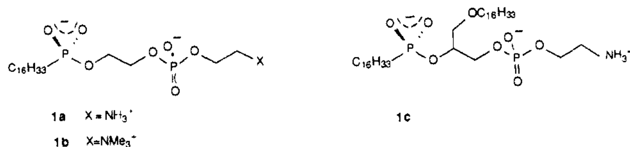
The design and preparation of specific inhibitors of various classes of lipases, especially of phospholipase A_2, is a topic of intense biochemical as well as medicinal interest.[1] For other hydrolytic enzymes such as peptidases and urease, an effective strategy for inhibitor design has been to replace the enzyme-susceptible amide bond with various phosphorus-containing groups such as phosphonates, phosphonamidates, and phosphinates.[2] It has been proposed that the phosphorus-containing species functions to mimic the high-energy tetrahedral intermediates that form in amide bond cleavage.[3] We have recently found that phospholipid analogues that contain a fluorinated ketone as a replacement for the ester are potent phospholipase A_2 inhibitors provided that the fluorinated ketone is hydrated when present in the micelle phase.[4] These results suggested that compounds containing enforced tetrahedral groups would be even better inhibitors of phospholipase A_2. We now describe the preparation of a class of phospholipid analogues **1a–c** which contain a phosphonate group in place of

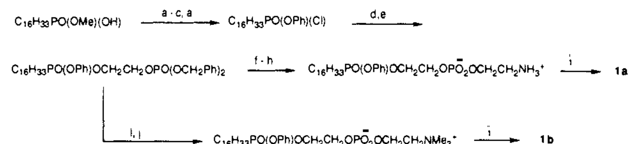


the ester at the 2-position of the glycerol backbone and show that these compounds are very tight binding inhibitors of phospholipase A_2 from cobra venom. We also demonstrate the effectiveness of a mixed micelle assay for the evaluation of inhibitors of lipolytic enzymes.

As an initial evaluation of this approach, the synthetically more accessible single-chain analogues **1a,b** were prepared as described in Scheme I. A more general approach was developed for the preparation of **1c** and is outlined in Scheme II.[5]

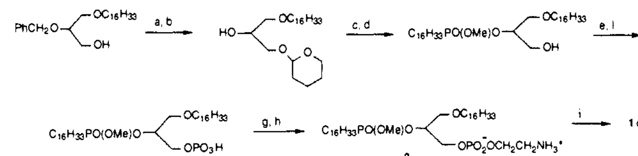
Inhibition studies with lipolytic enzymes are not straightforward to carry out since the assay systems are necessarily heterogeneous due to the water insolubility of the substrates and often the inhibitors. Further complexities arise because the rate of the reaction is a function of not only the chemical structure of the phospholipid substrate but also of the physical structure of the substrate aggregate.[6] Inhibition studies are plagued by the possibility that

Scheme I[a]



[a](a) $SOCl_2$, reflux, 5 h; (b) C_6H_5OH, Et_3N, $CHCl_3$, 45 °C, 12 h; (c) *t*-$BuNH_2$, reflux, 15 h; (d) iodoethanol, Et_3N, $CHCl_3$, 12 h; (e) $AgOPO(OCH_2Ph)_2$, C_6H_6, reflux, 12 h; (f) H_2, Pd–C, CH_3OH, 3 h; (g) $Ph_3CNHCH_2CH_2OH$, 1,4,6-triisopropylbenzenesulfonyl chloride, 40 °C, 5 h; (h) CF_3COOH, 0.3 h; (i) H_2, PtO_2, CH_3OH, 3 h; (j) same as (g) except with choline tetraphenylborate.

Scheme II[a]



[a](a) Dihydropyran, pyridinium tosylate, CH_2Cl_2, 2 h; (b) Na, *t*-BuOH, reflux, 15 h; (c) $C_{16}H_{33}PO(OMe)Cl$, Et_3N, 4-(dimethylamino)pyridine, $CHCl_3$, 50 °C, 12 h; (d) toluenesulfonic acid, CH_3OH, 3 h; (e) $ClPO(OC_6H_5)_2$, pyridine, 12 h; (f) H_2, PtO_2, CH_3OH, 12 h; (g) same as (g) in Scheme I; (h) same as (h) in Scheme II; (i) C_6H_5SH, Et_3N, dioxane, 65 °C, 3 h.

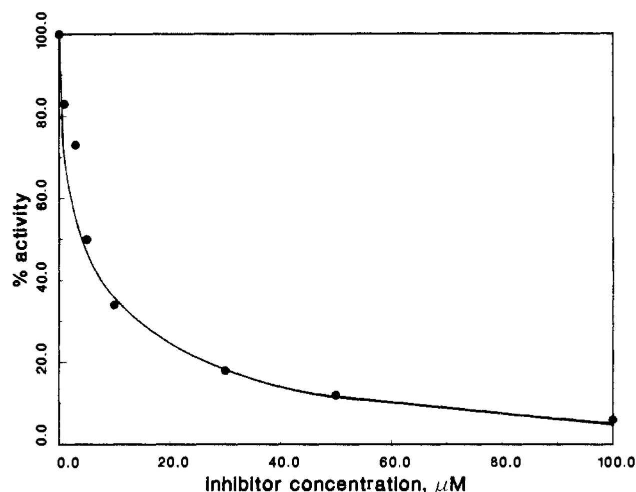


Figure 1. Inhibition of phospholipase A_2 by **1c** (see text for details). The initial reaction velocity is expressed as a percentage of the velocity measured in the absence of inhibitor.

the observed inhibition is not the result of a direct enzyme-inhibitor interaction but rather an effect of the inhibitor on the physical structure of the substrate aggregate.[7] Some known inhibitors of lipolytic enzymes are thought to function by interacting with the substrate rather than with the enzyme.[8] For these reasons, we have carried out our inhibition studies with **1a–c** by using mixed detergent/phospholipid micelles[9] in which small amounts of substrate and inhibitor are "dissolved" in the detergent aggregate. Triton X-100 is the preferred detergent since it forms micelles and mixed micelles of well-defined size.[9] Furthermore, it has been shown that the phospholipid molecules are monodispersed by the detergent in the surface of the mixed micelle provided that a low mole fraction of phospholipid relative to detergent is used.[10] Cobra

(1) For example, see: Lapetina, E. G. *Annu. Rep. Med. Chem.* **1984**, *19*, 213. Blackwell, G. J.; Flower, R. J. *Br. Med. Bull.* **1983**, *39*, 260. Lapetina, E. G. *Trends Pharmaceut. Sci.* **1982**, *3*, 115.0.2.

(2) For example, see: Bartlett, P. A.; Marlowe, C. K. *Science (Washington, D.C.)* **1987**, *235*, 569. Andrews, R. K.; Dexter, A.; Blakeley, R. L.; Zerner, B. *J. Am. Chem. Soc.* **1986**, *108*, 7124. Bartlett, P. A.; Kezer, W. B. *J. Am. Chem. Soc.* **1984**, *106*, 4282. Galardy, R. E.; Kontoyiannidou-Ostrem, V.; Kortylewicz, Z. P. *Biochemistry* **1983**, *22*, 1990. Petrillo, E. W., Jr.; Ondetti, M. A. *Med. Res. Rev.* **1982**, *2*, 1. Thorsett, E. D.; Harris, E. E.; Peterson, E. R.; Greenlee, W. J.; Patchett, A. A.; Ulm, E. H.; Vassil, T. C. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 2176. Holmquist, B.; Vallee, B. L. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 6216. Weaver, L. H.; Kester, W. R.; Matthews, B. W. *J. Mol. Biol.* **1977**, *114*, 119.

(3) Bartlett, P. A.; Marlowe, C. K. *Biochemistry* **1983**, *22*, 4618. Wolfenden, R. *Annu. Rev. Biophys. Bioeng.* **1976**, *5*, 271.

(4) Yuan, W.; Berman, R. J.; Gelb, M. H. *J. Am. Chem. Soc.* **1987**, *109*, 8071. Gelb, M. H. *J. Am. Chem. Soc.* **1986**, *108*, 3146.

(5) Compound **1c** was synthesized in racemic form. The preparation of this inhibitor with the natural *R*-configuration of the glycerol backbone can be accomplished using the readily available chiral glycerol derived from D-mannitol: Chacko, G. K.; Hanahan, D. J. *Biochim. Biophys. Acta* **1968**, *164*, 252.

(6) For example, see: Dennis, E. A. In *The Enzymes*; Academic Press: New York, 1983; Vol. 16, Chapter 9. Volwerk, J. J.; de Haas, G. H. In *Lipid-Protein Interactions*; Just, P. C., Griffith, O. H., Eds.; Wiley: New York, 1982; Vol. 1, pp 69–141. Verger, R. *Methods. Enzymol.* **1980**, *64*, 340.

(7) Inhibition studies using a dispersion of phospholipid substrate in water show that inhibition occurs as the concentration of inhibitor approaches its critical micelle concentration.

(8) For example, see: Davidson, F. F.; Dennis, E. A.; Powell, M.; Glenney, J. R., Jr. *J. Biol. Chem.* **1987**, *262*, 1698. Gräbner, R. *Biochem. Pharmacol.* **1987**, *36*, 1063.

(9) Deems, R. A.; Eaton, B. R.; Dennis, E. A. *J. Biol. Chem.* **1975**, *250*, 9013. Robson, R. J.; Dennis, E. A. *Acc. Chem. Res.* **1983**, *16*, 251.

(10) Roberts, M. F.; Adamich, M.; Robson, R. J.; Dennis, E. A. *Biochemistry* **1979**, *18*, 3301.

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venom phospholipase A_2 binds to the phospholipid substrate in the mixed micelle where the enzymatic reaction occurs.[11] In the present work we show that the mixed micelle system is ideally suited for inhibition studies with phospholipase A_2 since both the inhibitor and substrate can compete for the binding to the active site of the enzyme in the surface of the mixed micelle and is free from the complexities that arise from lipid–lipid interactions. Although the mixed-micelle system is perhaps a poor model of a biological membrane, it provides a reliable method for ranking inhibitors according to their relative free energies of interaction with the enzyme.

Mixed micelles for inhibition studies were prepared by sonicating fixed amounts of Triton X-100 (40 mM) and substrate (dipalmitoyl phosphatidylcholine, 5 mM) with variable amounts of inhibitor in water-containing $CaCl_2$ (10 mM).[12] Enzyme was added, and the reaction velocity was determined in a pH-stat at pH 8.0 and 40 °C.[9] The initial enzymatic velocity as a function of the concentration of inhibitor **1c** is shown in Figure 1. Two-chain analogue **1c** is seen to produce 50% inhibition (IC_{50} value) at a concentration of 5 μM. This compound is the most potent phospholipase A_2 inhibitor reported to date, being significantly more effective than amide analogues[13] and fluorinated ketone analogues.[4] The IC_{50} of methyl phosphonate **2** was found to be 1.25 mM, some 250-fold higher than for **1c**, demonstrating the critical role of the phosphonate anion in the interaction with the enzyme. The IC_{50} values measured for single-chain phosphonates **1a** and **1b** were 0.75 mM and 2.3 mM, respectively, showing that the upper alkyl chain of **1c** plays a significant role in the binding to the enzyme.[14]

Since mixed micelles were used and the fact that the inhibition is seen with levels of **1c** some 1000-fold lower than the amount of substrate make it highly unlikely that the inhibition is due to an inhibitor-induced change in the structure of the substrate aggregate. Rather it appears that these phosphonate-containing phospholipid analogues are interacting tightly with the catalytic site on the enzyme.[15] The phosphonate group may be coordinating to the active-site calcium. Perhaps the most important contribution of this work is the demonstration that the potency of inhibition correlates in a reasonable way with the chemical structure of the inhibitors provided that a mixed micelle assay is used.[7] The possibility of using phosphonates such as **1c** together with sulfur-substituted compounds such as thiophosphonates to probe the role of the calcium ion in the catalysis is particularly intriguing and is under active investigation.

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Supplementary Material Available: Experimental procedures and physical data for the preparation of all new compounds and enzyme inhibition analysis (5 pages). Ordering information is given on any current masthead page.

(11) Roberts, M. F.; Deems, R. A.; Dennis, E. A. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 1950.

(12) Substrate hydrolysis by phospholipase A_2 is totally dependent on enzyme-bound calcium (ref 6).

(13) Davidson, F. F.; Hajdu, J.; Dennis, E. A. *Biochem. Biophys. Res. Commun.* **1986**, *137*, 587.

(14) The higher potency of ethanolamine-containing inhibitor **1a** compared to choline-containing inhibitor **1b** is consistent with our earlier findings (see ref 4 for a complete discussion).

(15) It should be pointed out that the use of bulk concentrations (mol/vol) to express inhibitor potencies is useful in comparing a series of inhibitors according to potency as long as the same mixed micelle assay is used in all cases. However, the kinetics of phospholipase A_2 hydrolysis of mixed micelles is sensitive to both the bulk and surface (mol/surface) substrate concentrations: Hendrickson, H. S.; Dennis, E. A. *J. Biol. Chem.* **1984**, *259*, 5734. In the present study, the bulk concentration of substrate used (5 mM) is sufficient to ensure that all of the enzyme is bound to phospholipid in the micelle. Thus, the observed inhibition is due to a competition between substrate and inhibitor for the binding to the enzyme in the surface of the micelle.

Mechanism of the 1,5-Sigmatropic Hydrogen Shift in 1,3-Pentadiene

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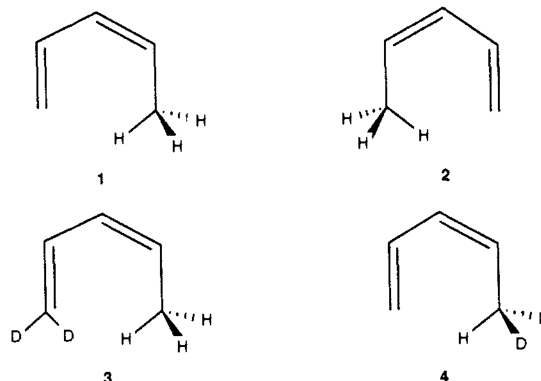
Some years ago Roth and Konig[1] reported a surprisingly large value (5.1 at 473 K) for the primary deuterium kinetic isotope effect (KIE) in the 1,5-sigmatropic rearrangement of 1,3-pentadiene (**1** ⇄ **2**). Extrapolation to room temperature, using the



observed activation parameters, led moreover to a value (12.2) which seemed unreasonably large. MINDO/3[2] calculations predicted a value (2.5) similar to those reported for analogous processes. The reaction therefore seems unlikely to involve a normal thermal rearrangement, and normal ground-state tunnelling seems ruled out by the necessarily large change in geometry. Dewar et al.[2] suggested that the discrepancy might be due to tunnelling from vibrationally excited states (vibrationally assisted tunnelling; VAT), a suggestion supported by an approximate calculation of the VAT rate. The activation energy calculated by MINDO/3 was moreover higher than that observed by 10 kcal/mol, and the KIE corrected for VAT agreed with experiment.

While subsequent ab initio calculations by Dormans and Buck[3] have supported these conclusions, they have recently been challenged by Jensen and Houk[4] on the basis of further ab initio calculations allowing for correlation by Møller–Plesset (MP) perturbation theory.[5] The fact that the calculated activation energy agreed with experiment was taken as evidence that VAT plays a negligible role. The KIE[6] (2.52) calculated by using the 3-21G basis set, but without allowance for correlation, agreed with the MINDO/3 value and was likewise far less than that observed. Jensen and Houk dismissed this discrepancy on the grounds that better agreement with experiment might be expected if correlation were included. They implied that such a calculation would have been impracticable.

The difference between the MINDO/3 and MP activation energies is, however, too small for reliable conclusions to be drawn. It is, for example, the same as the error (10 kcal/mol) in the activation energy calculated by Breulet and Schaefer[7] for a simpler reaction (ring opening of cyclobutene) by using a better ab initio procedure than that of Jensen and Houk. On the other hand,

(1) Roth, W. R.; Konig, J. *Liebigs Ann. Chem.* **1966**, *699*, 24. Roth, W. R. *Chimia* **1966**, *20*, 229.

(2) Dewar, M. J. S.; Merz, K. M., Jr.; Stewart, J. J. P. *J. Chem. Soc., Chem. Commun.* **1985**, 166.

(3) Dormans, G. J. M.; Buck, H. M. *J. Am. Chem. Soc.* **1986**, *108*, 3253. Note that an incorrect geometry was used for the transition state.

(4) Jensen, F.; Houk, K. N. *J. Am. Chem. Soc.* **1987**, *109*, 3139.

(5) Møller, C.; Plesset, M. S. *Phys. Rev.* **1934**, *46*, 618.

(6) Hess, B. A., Jr.; Schaad, L. J.; Pancir, J. *J. Am. Chem. Soc.* **1985**, *107*, 149.

(7) Breulet, J.; Schaefer, H. F., III. *J. Am. Chem. Soc.* **1984**, *106*, 1221.

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