of these systems, like the $Mn^{2+}Cu^{2+}$ bimetallic chains, could represent quite an important step in the perspective of molecular ferromagnets. The quantitative interpretation of the magnetic properties as well as the investigation of the other physical properties will be developed in a subsequent paper.

Registry No. 1·2Na, 61344-73-8; 2, 101935-07-3; 3·2Na, 101954-74-9; 4, 101935-08-4; NiCu(pba)·$3H_2O$, 101935-09-5; ethyl oxamate, 617-36-7; *N,N'*-bis(aminopropylene)oxamate, 19980-60-0; *N,N'*-bis(oxamato-1,3-propylene)oxamide, 101954-75-0.

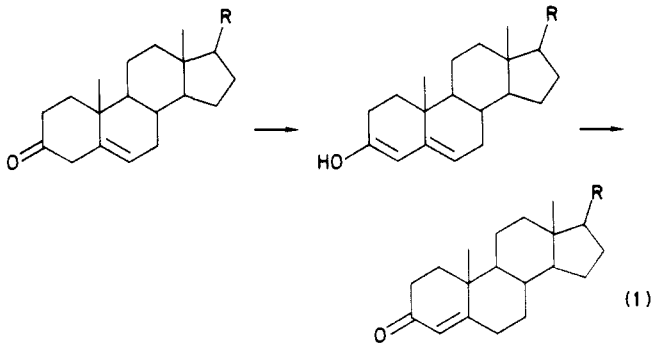
Supplementary Material Available: Atomic parameters and listing of structure factor amplitudes (9 pages). Ordering information is given on any current masthead page.

Evidence for an Enol Intermediate in the 3-Oxo-Δ^5-steroid Isomerase Catalyzed Isomerization of Δ^5 Ketones

Shanta Bantia and Ralph M. Pollack*

Laboratory for Chemical Dynamics
University of Maryland Baltimore County
Baltimore, Maryland 21228
Received January 21, 1986

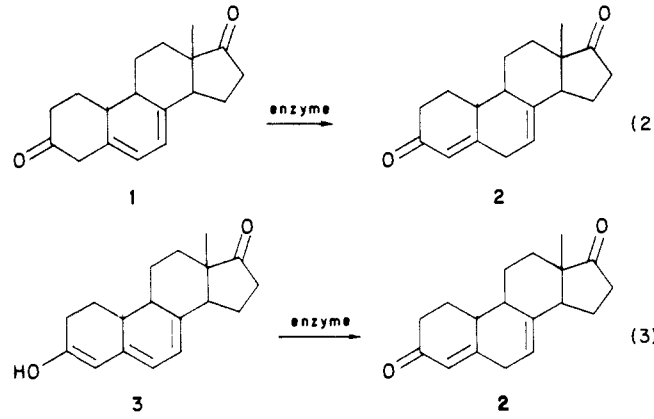
The 3-oxo-Δ^5-steroid isomerase (EC 5.3.3.1) from *Pseudomonas testosteroni* catalyzes the isomerization of a variety of $\Delta^{5,6}$ and $\Delta^{5,10}$ steroids to their Δ^4 isomers.[1,2] This enzyme is of particular interest due to its extremely high activity ($k_{cat} = 4.4 \times 10^6$ min^{-1} at pH 7 and 25 °C with 5-androstene-3,17-dione as the substrate).[1] The mechanism has been extensively investigated and both Asp-38 and Asn-57 have been identified at the active site.[3-7] The proposed catalytic mechanism involves protonation of the carbonyl followed by proton transfer of the 4β-hydrogen to the 6β-position through the intermediate formation of a dienol (eq 1).[9] Although model



(1)

reactions have demonstrated that a mechanism involving a Schiff base intermediate can account for a substantial fraction of the catalytic activity,[10-12] no evidence has been found which confirms an intermediate of this type.[1]

We now wish to report that the isomerase catalyzes the interconversion of 5,7-estradiene-3,17-dione (**1**) to 4,7-estradiene-3,17-dione (**2**). Furthermore, the putative intermediate trienol **3** also serves as a substrate for the isomerase, implicating an enol intermediate in the overall catalytic process.



Trienol **3** was synthesized according to a published procedure[13] by quenching the enolate of 4,6-estradiene-3,17-dione (**4**) in acetic acid–water (1:1). Although **3** is unstable in solution, it can be stored for several days as a solid under vacuum. The ultraviolet spectrum of our sample showed $\lambda_{max}^{MeOH} = 320$ nm, ϵ 13 000 (lit.[13] 320 nm, ϵ 15 000) with no evidence of substantial absorbance due to contamination by **1** (λ_{max}^{MeOH} 281 nm, ϵ 8600),[13] **2** (λ_{max}^{MeOH} 238 nm, ϵ 14 950),[13] or **4** (λ_{max}^{MeOH} 280 nm, ϵ 26 300).[14] Quenching the enolate of **4** in acetic acid/benzene (1:2) gave a mixture of **1**, **2**, and **4**, with **1** as the major product on the basis of the ultraviolet spectrum.[13] Alternatively, **1** could be prepared in situ from trienol **3**. When **3** is added to acetate buffer at 25 °C and pH 4.5, the predominant product formed after about 5 min is **1** (ca. 90%) with some contamination by **4** (ca. 10%). This latter procedure proved to be the most convenient, as **1** decomposes to **4** when stored in solution. Reproducible results were most easily obtained by generating **1** in situ immediately before the kinetic measurements.

The 5,7-dienone **1** was examined as a substrate for the isomerase (pH 4.49, 0.034 M acetate, 1.7% methanol) at 25.0 and 10.0 C.[15] Initial rates were determined at substrate concentrations ranging from 20 to 150 μM and an enzyme concentration of 4.3×10^{-11} M (10 °C) or 2.9×10^{-11} M (25 °C) by monitoring the increase in absorbance due to appearance of the 4,7-dienone **2** (λ_{max}^{MeOH} = 246 nm). Values of k_{cat}/K_m were determined from weighted least-squares analysis of plots of $1/V$ vs. $1/[S]$ and found to be $(6.6 \pm 0.5) \times 10^9$ M^{-1} min^{-1} (25 °C) and $(2.5 \pm 0.2) \times 10^9$ M^{-1} min^{-1} (10 °C). Although the unavoidable presence of small amounts of the competitive inhibitor **4** ($K_I = 18$ μM) complicates the interpretation of the kinetics, the ratio k_{cat}/K_m may be obtained from the slope of a plot of $1/V$ vs. $1/[S]$.[16] Lower limits for both k_{cat} and K_m are given by k_{cat}^{app} (6.2×10^5 min^{-1} at 25 °C and 1.90×10^5 min^{-1} at 10 °C) and K_m^{app} (95 μM at 25 °C and 75 μM at 10 °C). A comparison with k_{cat}/K_m at pH 4.5 and 25 °C

(1) Batzold, F. H.; Benson, A. M.; Covey, D. F.; Robinson, C. H.; Talalay, P. *Adv. Enzyme Regul.* **1976**, *14*, 243–267.
(2) Talalay, P.; Benson, A. M. In *The Enzymes*, 3rd ed.; Boyer, P. D., Ed.; Academic Press: New York, 1972; Vol. 6, pp 591–618.
(3) Kayser, R. H.; Bounds, P. L.; Bevins, C. L.; Pollack, R. M. *J. Biol. Chem.* **1983**, *258*, 909–915.
(4) Martyr, R. J.; Benisek, W. F. *J. Biol. Chem.* **1975**, *250*, 1218–1222.
(5) Ogez, J. R.; Tivol. W. F.; Benisek, W. F. *J. Biol. Chem.* **1977**, *252*, 6151–6155.
(6) Bantia, S.; Bevins, C. L.; Pollack, R. M. *Biochemistry* **1985**, *24*, 2606–2609.
(7) There is some disagreement whether the residue at position 38 is Asp or Asn.[3-6,8]
(8) Benson, A. M.; Jarabak, R.; Talalay, P. *J. Biol. Chem.* **1971**, *246*, 7514–7525.
(9) Malhotra, S. K.; Ringold, H. J. *J. Am. Chem. Soc.* **1965**, *87*, 3228–3236.
(10) Kayser, R. H.; Pollack, R. M. *J. Am. Chem. Soc.* **1975**, *97*, 952–953.
(11) Pollack, R. M.; Kayser, R. H. *J. Am. Chem. Soc.* **1976**, *98*, 4174–4181.
(12) Benisek, W. F; Jacobson, A. *Bioorg. Chem.* **1975**, *4*, 41–57.
(13) Kruger, G. *J. Org. Chem.* **1968**, *33*, 1750–1753.
(14) Zderic, J. A.; Carpio, H.; Bowers, A.; Djerassi, C. *Steroids* **1963**, *1*, 233–249.
(15) Both **1** and **3** are quite labile to isomerization, particularly in neutral and basic solutions. In order to minimize the background rate it was necessary to examine the enzyme-catalyzed reaction of **2** and **3** at pH 4.5 and, in the case of **3**, 10.0 °C. The isomerase activity at pH 4.5 is approximately half of that at pH 7 toward a variety of other substrates.
(16) If the ratio [I]/[S] is constant then

$$V = \frac{V_{max}[S]}{[S] + K_m(1 + [I]/K_i)} = \frac{V_{max}[S]}{[S] + C[S]K_m/K_i + K_m}$$

where $C = [I]/[S]$. Inverting gives

$$\frac{1}{V} = \frac{K_m}{V_{max}}\frac{1}{[S]} + \frac{1}{V_{max}}\left(C\frac{K_m}{K_i} + 1\right)$$

A plot of $1/V$ vs. $1/[S]$ gives K_m/V_{max} as its slope. The intercept divided by the slope is equal to $1/K_m^{app} = 1/K_m + C/K_i$. Accurate determination of k_{cat} and K_m requires a knowledge of the exact value of C. Our value is not sufficiently precise to allow a meaningful calculation.

0002-7863/86/1508-3145$01.50/0 © 1986 American Chemical Society

for the specific substrate 5-androstene-3,17-dione (7.5×10^9 M^{-1} min^{-1}) shows that **1** is an excellent substrate.[17]

When the trienol **3** was incubated with the isomerase at pH 4.52 (0.0025 M acetate, 1.7% methanol) and 10.0 °C, a first-order decay was observed at $\lambda = 320$ nm with substrate concentrations of 7–15 μM and an enzyme concentration of 4.4×10^{-10} M. The observed rate was approximately 6-fold greater than the buffer-catalyzed rate. Calculation of k_{cat}/K_m gave $(7.2 \pm 0.4) \times 10^9$ M^{-1} min^{-1}. A spectral scan of the product showed that it consisted almost exclusively of 4,7-estradiene-3,17-dione (**2**). Addition of 60 μM of the competitive inhibitor 19-nortestosterone in the presence of 7.2% methanol caused a marked decrease (ca. 90%) in the enzyme-catalyzed rate, as expected for a reaction occurring at the active site.

The ability of the isomerase to catalyze the conversion of trienol **3** to the α,β-unsaturated ketone **2** is strong evidence for the existence of an enol intermediate in the normal catalytic reaction. Furthermore **3** is trapped by the isomerase ca. 3 times faster than **1** is converted to **2**. The observation of a first-order decay with **3**, coupled with the magnitude of the rate constant, suggests that the reaction is diffusion-controlled with the rate-limiting step being association of **3** and the enzyme.

Acknowledgment. This work was supported by a grant from the National Institutes of Health (GM 33059). We thank Professors Donald J. Creighton and Dale L. Whalen for helpful discussions.

(17) The rapid enzyme-catalyzed isomerization of **1** to **2** is somewhat surprising in view of the report that the isomerase is incapable of utilizing 5(10),9(11)-dien-3ones as substrates.[18]

(18) Weintraub, H.; Baulieu, E.-E.; Alfsen, A. *Biochem. J.* **1980**, *185*, 723–732.

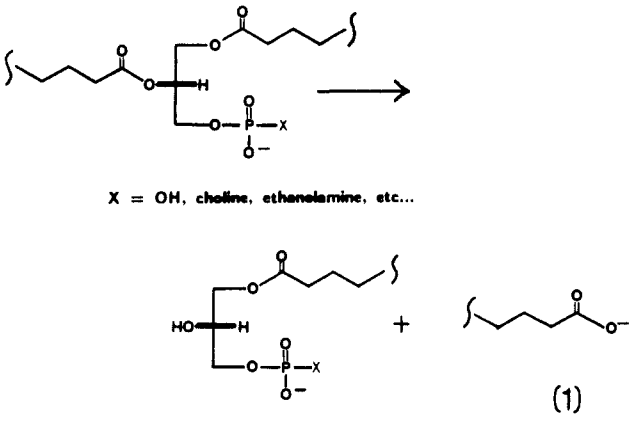
Fluoro Ketone Phospholipid Analogues: New Inhibitors of Phospholipase A_2

Michael H. Gelb

Daniel Bagley Laboratories, Department of Chemistry University of Washington, Seattle, Washington 98195

Received January 31, 1986

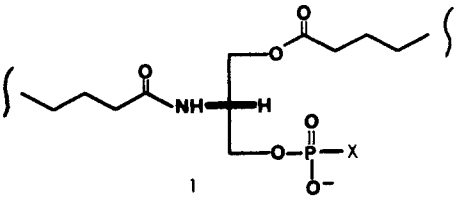
The design of inhibitors of phospholipase A_2 is of recent medicinal interest since this enzyme catalyzes the liberation of arachidonic acid from the phospholipid membrane pool.[1] This represents the rate-determining step in the biosynthesis of prostaglandins, leukotrienes, thromboxanes, and prostacyclin.[2] This enzyme has been isolated from a number of sources and cleaves *sn*-glycero phospholipids specifically at the 2-position as shown in eq 1.[3] Very few rationally designed phospholipase A_2 inhibitors



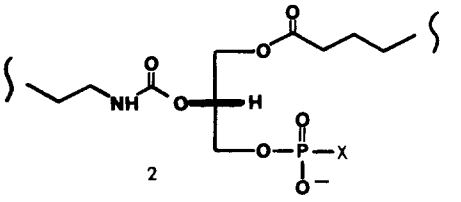
(1) Lapetina, E. G. *Annu. Rep. Med. Chem.* **1984**, *19*, 213–221. Blackwell, G. J.; Flower, R. J. *Br. Med. Bull.* **1983**, *39*, 260–264. Wallach, D. P.; Brown, V. J. R. *Biochem. Pharmacol.* **1981**, *30*, 1315–1324.

(2) Lapetina, E. G. *Trends Pharmaceut. Sci.* **1982**, *3*, 115–118. Cross, P. E. *Annu. Rep. Med. Chem.* **1982**, *17*, 79–88.

have been reported. The most extensively studied have been the phospholipid analogues **1** and **2** in which the enzyme-susceptible



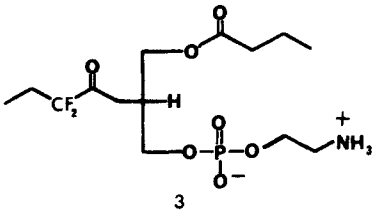
1



2

ester linkage has been replaced with an amide[4] or carbamate.[5] The K_i values for these inhibitors are similar in magnitude to the K_m values for the analogous substrates.

We now report a difluoromethylene ketone phospholipid analogue **3** and show that this compound is a tight-binding inhibitor



3

of phospholipase A_2. Substrate analogues containing polarized ketones including fluoro ketones and 1,2-diketones have been shown to inhibit hydrolytic enzymes.[6] Since difluoromethylene ketones are predominantly hydrated in aqueous solution, **3** might mimic the tetrahedral intermediate that forms during phospholipase-catalyzed lipolysis and might therefore be considered a transition-state analogue inhibitor.[7] The use of **3** is particularly appealing in the present case since it can bind to the enzyme as either the hydrate **4** or as a hemiketal **5** involving a nucleophile

4 5

present at the active site of the enzyme. This point is important since the structure of the tetrahedral intermediate that forms during phospholipase-catalyzed substrate hydrolysis has not been established, although current evidence favors a mechanism involving the enzyme-assisted attack of a water molecule onto the ester carbonyl group (mimicked by **4**) as opposed to the formation of a covalent acyl-enzyme intermediate[3] (mimicked by **5**).

Compound **3** is an analogue of a short-chain phosphatidylethanolamine and was chosen for this initial study since, unlike long-chain phospholipids, it will have monomeric solution prop-

(3) Review: Dennis, E. A. *The Enzymes*; Academic Press: New York; 1983; Vol. 16, Chapter 9.

(4) Bonsen, P. P. M.; de Haas, G. H.; Pieterson, W. A.; Van Deenen, L. L. M. *Biochim. Biophys. Acta* **1972**, *270*, 364–382. Chandrakumar, N. S.; Hajdn, J. *Tetrahedron Lett.* **1981**, *22*, 2949–2952; *J. Org. Chem.* **1982**, *47*, 2144–2147.

(5) Plückthun, A.; Rohlfs, R.; Davidson, F. F.; Dennis, E. A. *Biochemistry* **1985**, *24*, 4201–4208.

(6) Gelb, M. H.; Svaren, J. P.; Abeles, R. H. *Biochemistry* **1985**, *24*, 1813–1817. Hammock, B. D.; Wing, K. D.; McLaughlin, J.; Lovell, V. M.; Sparks, T. C. *Pestic. Biochem. Physiol.* **1982**, *17*, 76–88. Brodbeck, V.; Schweikert, K.; Gentinetta, R.; Rothenberg, M. *Biochim. Biophys. Acta* **1979**, *567*, 357–369. Berndt, M. C.; de Jersey, J.; Zerner, B. *J. Am. Chem. Soc.* **1977**, *99*, 8334–8335. Prestwich, G. D., Eng, W.-S.; Roe, R. M.; Hammock, B. D. *Arch. Biochem. Biophys.* **1984**, *230*, 639.

(7) Wolfenden, R. *Annu. Rev. Biophys. Bioeng.* **1976**, *5*, 271–306. Bartlett, P. B.; Marlowe, C. K. *Biochemistry* **1983**, *22*, 4618–4624.

0002-7863/86/1508-3146$01.50/0 © 1986 American Chemical Society