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A Synthetic p-Nitrophenyl Esterase With Remarkable Substrate Selectivity

by

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Abstract

A new linear polymer, poly(1, 1, 3, 3-tetramethyl-7-(4-pyridinyl)-7-aza-2-oxa-1, 3-disiladecane), **4**, recently prepared and characterized in our laboratory, has been evaluated as a catalyst for hydrolysis of p-nitrophenyl esters. This synthetic material not only exhibits high levels of catalytic efficiency and conforms to Michaelis-Menten model, but it also demonstrates enzyme-like specificity for esters derived from acids of moderate chain length ($C_{12} \rightarrow C_{16}$) with p-nitrophenyl tetradecanoate (C_{14}) the optimum substrate ($V_{max} = 7.5 \times 10^{-7}$ M s^{-1}, $K_M = 2.9 \times 10^{-5}$ M, $k_{cat}/K_M = 1.1 \times 10^4$ M^{-1} s^{-1} at 30 °C in 1 : 1 MeOH-aqueous buffer (pH 8.0) with [Cat] = 2.5×10^{-6} M).

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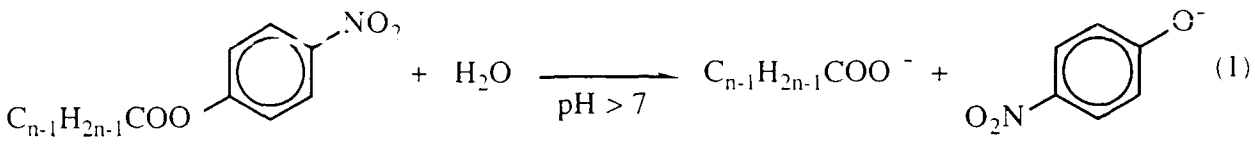
Duplication of the efficiency and selectivity of enzymic catalysis with synthetic materials has been pursued by many investigators for several decades (1-14). Studies directed toward understanding the enhancement of amide and ester hydrolysis have frequently utilized p-nitrophenyl esters, **1**, of aliphatic carboxylic acids as substrates, Eq. 1, to gain insight into these



(1)

1, n = 2, 6, 8, 10, 12, 14, 16, 18

important processes. Most studies have focused on increasing catalyst efficiency to bring activity of synthetic catalyst to levels afforded by relevant enzymes. Our goal has been to examine structural factors in linear, water-soluble, polymeric catalysts that elicit high levels of substrate selectivity as well as notable rate acceleration in the hydrolysis of **1**. For such catalysts to be successful, they must provide selective lipophilic binding sites for **1** and nucleophilic catalytic sites appropriately arranged through structural organization, i.e., self-organization, to function jointly as enzyme-like active sites (15). True enzyme-like behavior in our study requires that these "active sites" discriminate among structurally different p-nitrophenyl esters and effect their hydrolysis at different reaction velocities as well as exhibit the well-known enzymic property of turnover. The structure variable used to differentiate between substrates in this study is the number of carbons in the alkanoate chain. Thus, binding between substrate and catalyst is controlled by hydrophobic-lipophilic interactions (16-20).

The 4-(dialkylamino)pyridines, (DAAP, Fig.1) of which 4-(dimethylamino)pyridine, **2**, is best known,are such highly reactive nucleophilic catalysts in aprotic solvents that they are often referred to as "super-nucleophiles" (13, 14, 21-23). Monomeric and polymeric forms of DAAP are increasingly utilized as catalysts in transacylation reactions. Recently, we have reported on the synthesis of polysiloxanes functionalized with DAAP groups and their catalytic efficiency toward acylation of sterically hindered alcohols in aprotic media (24, 25). Two structurally different backbones have been explored in polymers **3** and **4** which contain pendant and intra-chain DAAP

moieties, respectively. Simple, monomolecular DAAP variants are known to be relatively ineffective nucleophilic catalysts of transacylation reactions in aqueous media. However, Klotz and co-workers (13) discovered that poly(ethylenimine) derivatives functionalized with any one of a variety of DAAP groups exhibit significant catalytic activity in hydrolysis of **1** (n = 6). Rate enhancements of 50-2000-fold over spontaneous hydrolysis were reported for these synthetic catalysts which have different DAAP's covalently linked to poly(ethylenimines) alkylated with dodecyl groups.

More recently, Mathias and Vaidya (14) described a second particularly active polymeric form of DAAP, poly(4-(diallylamino)pyridine, **5**, that exhibited increasing activity toward variants of **1** with increasing length of alkanoate chain ($C_2 \rightarrow C_{12}$). Our two catalysts, **3** and **4**, differ significantly from one another and from the catalysts studied by the Klotz and Mathias groups. Polymer **3** has the well-known methylsiloxane backbone with a pendent DAAP attached to silicon, while **4** has a bis-(disiloxypropyl)amino backbone with DAAP an integral part of the chain. These structural differences offer a potential source of significantly different catalytic behavior within this set of polymeric catalysts. Since all of them utilize DAAP as their catalytic site, they share its propensity for true catalytic behavior, i.e.; it exhibits turnover during reaction with its substrate (21,22).

Hydrolysis of a series of p-nitrophenyl alkanoates, **1**, Eq. 1, catalyzed by **3** and **4** was investigated by methods widely used for evaluation of enzymic catalysis (26). The dependence of hydrolysis rates on catalyst and substrate concentrations, pH, and length of alkanoate chain in substrate were determined. For comparison purposes, similar experiments were run with **2** and **5**. The results from experiments in which hydrolysis of a series of **1**(n = 2 → 18) at 30°C in 1:1 (V/V) MeOH-aqueous buffer (pH 8.0) was catalyzed by **2**, **3**, **4**, and **5** are illustrated in Fig. 2. Polymer **4**, in contrast to **2**, **3**, and **5**, exhibits a clear preference for p-nitrophenyl tetradecanoate, **1** (n = 14), over the other esters studied. *Therefore, this synthetic, linear polymer exhibits enzyme-like substrate selectivity.* Polymer **5**, on the other hand, shows a steady increase in reaction rate with increase in length of alkanoate chain. The dependence of reaction velocity on substrate concentration for the C_6, C_{12}, C_{14}, and C_{16} esters was examined in the presence of **4**, and significantly, the *three longer chain esters exhibited saturation kinetics over the concentration range studied, while the C_6 ester did not.* These results are illustrated in Fig. 3.

As a further test of the ability of **4** to function as an enzyme mimic, the data from hydrolyses of **1**(n = 12, 14, 16) were subjected to Michaelis-Menten analysis (15). The results for C_{14} ester are shown in Fig. 4. The Lineweaver - Burk plot of the reciprocal of the reaction velocity (1/V) versus the reciprocal of the substrate concentration (1/S) is linear as predicted by the

Michaelis-Menten model, Eq. 2, where V_{max} = the maximum reaction velocity and K_M = the Michaelis constant. The values of $V_{max} = 7.5 \times 10^{-7}$ M s^{-1} and $K_M = 2.9 \times 10^{-5}$ M^{-1} are

$$\frac{1}{V} = \frac{K_M}{V_{max}} \cdot \frac{1}{S} + \frac{1}{V_{max}} \qquad (2)$$

consistent with effective binding of substrate to the catalyst and efficient catalysis of the hydrolysis reaction ($t_{1/2} \cong 15$ s at [cat] = 2.5×10^{-6} M). The substrate selectivity of **4** can be assumed to derive from factors believed to control specificity in enzymic catalysis (15). The factor k_{cat}/K_M, where k_{cat} is simply V_{max} / $[cat]_{total}$, has been shown to indicate the specificty of an enzyme for competing substrates. A summary of results from Michaelis-Menten analysis of hydrolysis of **1**(n = 6, 12, 14, 16) catalyzed by **4** is given in Table 1. Substrate optimization at **1**(n = 14) is shown to be due to the enhanced rate of hydrolysis as given by the rate constant, k_{cat}. The values of K_M show a steady decrease with increasing length of alkanoate chain, however, k_{cat}/K_M values maximize at **1**(n = 14).

The pH dependence of reaction velocities, V, for hydrolysis of **1** (n = 12) in 1 : 1 (V/V) methanol - aqueous buffer was measured in the presence of **2**, **3**, and **4** at 30 °C. The reaction velocity increases linearly with pH over the interval pH 7.0 - 9.0 for all three catalysts and it continues to increase at higher pH due to uncatalyzed reaction with hydroxide ion. Significantly, reactions catalyzed by **4** are more than 4-200 times faster than those catalyzed by **2**, **3**, and **5** under similar conditions, Fig. 2. The apparent pKa values for **3** and **4** in the reaction medium [pKa (**3**) = 8.3; pKa (**4**) $\cong 7_{est}$ (see Footnote 28)] are significantly lower than that of DMAP (pKa = 9.5 in water (Ref. 22) and 8.8 in 1 : 1 methanol - water). Therefore, the proportion of free base for **3** and **4** is much higher (approx. 50% and 90%, respectively, at pH 8.0) than that of DMAP (approx. 10% at pH 8.0) over the pH interval used. The kinetic studies routinely used 10-fold excess substrate over catalyst concentrations and reactions were followed to complete reaction (4 - 5 half-lives). The V values are directly proportional to catalyst concentration over the range 0.8×10^{-6} M to 1.0×10^{-5} M. Thus, polymers **3** and **4** like DMAP function as true catalysts.

The performance of catalyst **4** as a p-nitrophenyl esterase was compared with that of two enyzmes, cholesteryl esterase, **6**, (29) and chymotrypsin, **7**, (30), the most active linear, synthetic polymer reported previously poly (DAAP), **5**, (14), and two active micelle/surfactant systems (23, 31), Table 2. Catalyst **4** ranks third below **6** and **7** in catalytic activity and exhibits comparable levels of substrate selectivity, although **6** and **7** show optimum activity for **1**(n = 6) and **1**(n = 7), respectively. An important distinction between synthetic catalyst **4** and enzymes **6** and **7** is that

both enzymes utilize bifunctional catalysis with histidine imidazole and serine hydroxyl groups as key participants in catalysis (15, 29). This mechanism was first mimiced in synthetic polymeric catalysts by Kunitake et al. (8). By contrast, **4** provides a molecular surface that associates strongly with **1**($n > 6$; $K_M < 10^{-4}$ M), and apparently, the relative positioning of **4** and **1** within the association complex is controlled by the length of alkanoate chain. The important consequence of this control may be the optimization of the interaction between the ester carbonyl group of substrate and the strongly nucleophilic pyridine nitrogen of the catalyst to optimize k_{cat}/K_M at **1**($n = 14$). The p-nitrophenyl esters of moderate to long chain carboxylic acids, **1**($n \geq 6$), are known to form aggregates in water (16-20). Work in progress suggests that substrate and catalyst aggregates and partitioning of substrate and/or catalyst among aggregates, co-aggregates, and soluble complexes may play a significant role in the observed substrate selectivity. This feature of catalyst **4** also duplicates reported behavior of enzymes in similar reactions (32). The details of our investigation of effect of medium on solvolysis of **1** in the presence of **4** will be the subject of a future report.

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26. Kinetic Measurements: Reaction mixtures were made up in a 1.00 cm quartz cuvet. The cuvet was filled with 2.97 mL of a 1:1 mixture of methanol and aqueous buffer (0.05 M $H_2PO_4^-$ / HPO_4^{-2}, pH 8.0). A stock solution of catalyst in methanol (usually 5 µL) was added by microsyringe and the solution was equilibrated for 10 min. in the thermostatted cell compartment (30±1°C) of a Hewlett-Packard Model 8450 spectrophotometer. An appropriate aliquot (0.03mL) of a stock soluction of p-nitrophenyl alkanoate in dioxane was added by micro-syringe. The reaction mixture was quickly mixed by shaking,and the absorbance at 400 nm was recorded as a function of time. The pseudo apparent first-order rate constants (k) were obtained as slopes of the plots of $\ln[A_\infty/(A_\infty - A_t)]$ vs. time, where A_∞ and A_t are the absorbances at infinite time and time t, respectively. Duplicate runs generally showed a measurement error of less than 5%. The results of these experiments are displayed in Figs. 2, 3 and 4, and summarized in Table 1.

27. Catalytic activity is taken as equivalent to the reaction velocity, V, i.e., the initial rate of product formation.

28. Solubility limitations and slow equilibration with aqueous buffers make pK determinations for **4** problematic. The results of an investigation of medium effects on proton-transfer between **4** and aqueous buffer are discussed in reference 25 and are the focus of a manuscript in preparation.

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33. We thank the Office of Naval Research for financial support of this work and Prof. L.J. Mathias for the sample of poly(DAAP) used in this study.

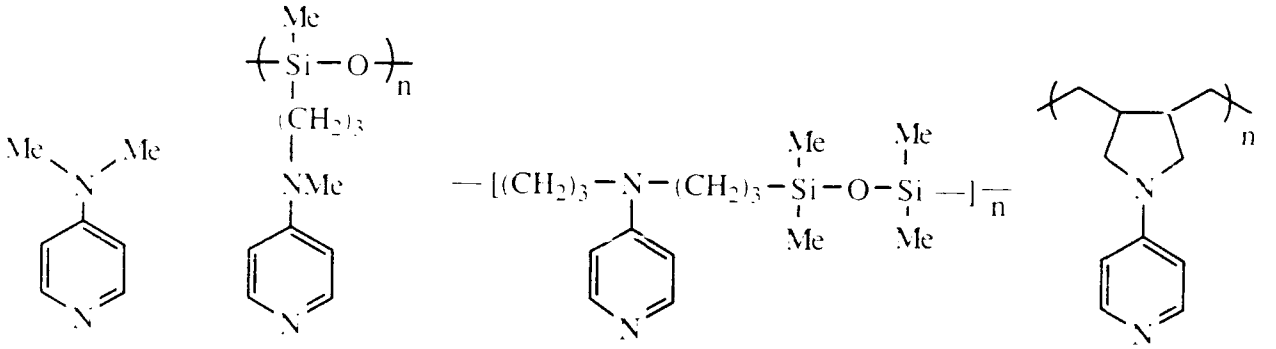
LEGENDS

Fig. 1. Monomeric and polymeric 4-(dialkylamino)pyridines (DAAP): (**2**) 4-(Dimethylamino)pyridine (DMAP); (**3**) Poly(methyl 3-[N-methyl-N-(4-pyridinyl)amino]propylsiloxane); (**4**) Poly(1,1,3,3-tetramethyl-7-(4-pyridinyl)-7-aza-2-oxa-1,3-disiladecane); (**5**) Poly(4-(diallylamino)pyridine) (Poly(DAAP).

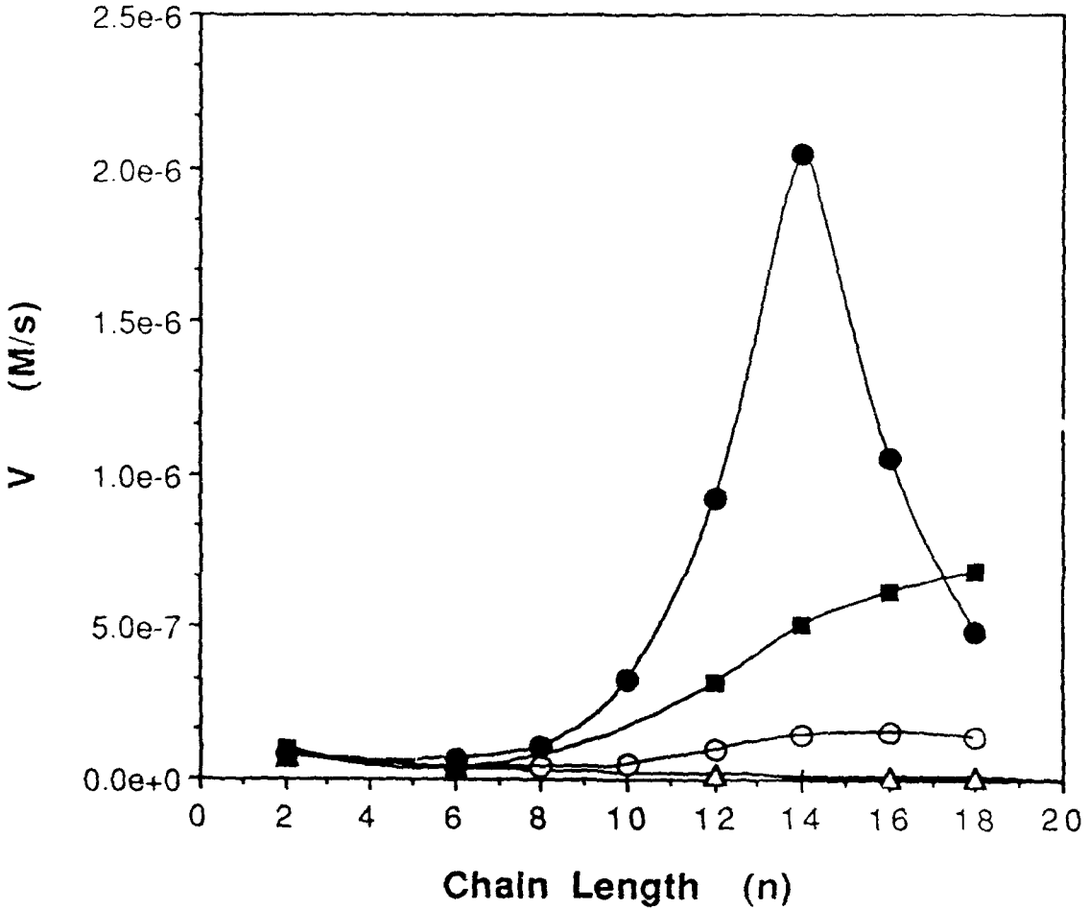
Fig. 2 Dependence of reaction velocity (V) for hydrolysis of **1** on alkanoate chain length (n) at 30 °C in 1:1 (V/V) MeOH-aqueous buffer (0.05 M $H_2PO_4^-$ / HPO_4^{-2}, pH 8.0).
[**1**] = 5 x 10^{-5} M; [cat] = 1.0 x 10^{-5} M. Catalyst: (none) + ; (**2**) Δ; (**3**) ○; (**4**) ●; **5** ■.

Fig. 3 Plots of reaction velocity (V) for hydrolysis of **1** vs. concentration of **1** in the presence of 2.5 x 10^{-6}M **4** 30 °C in 1:1 (V/V) MeOH-aqueous buffer (pH 8.0), **1** (n = 6 ❑; n = 12 ○; n = 14 Δ; n = 16 ●).

Fig. 4. Lineweaver-Burk plot for hydrolysis of **1** (n = 14) in the presence of 2.5 x 10^{-6} M **4** at 30 °C in 1:1 (V/V) MeOH-aqueous buffer (pH 8.0).



2 3 4 5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2.0e-6
1.5e-6
1.0e-6
5.0e-7
0.0e+0
V (M/s)
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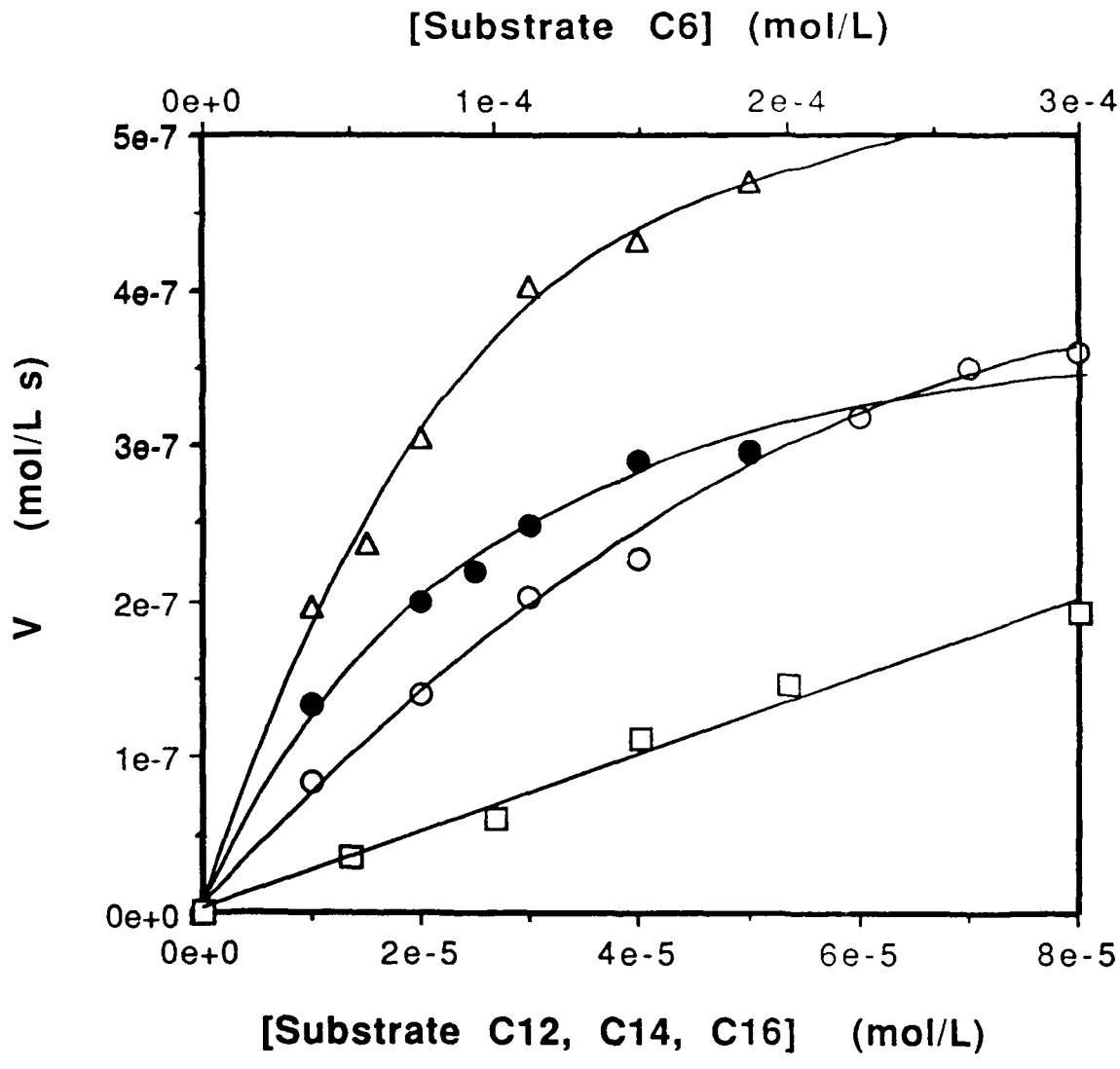
2
4
6
8
10
12
14
16
18
20
Chain Length (n)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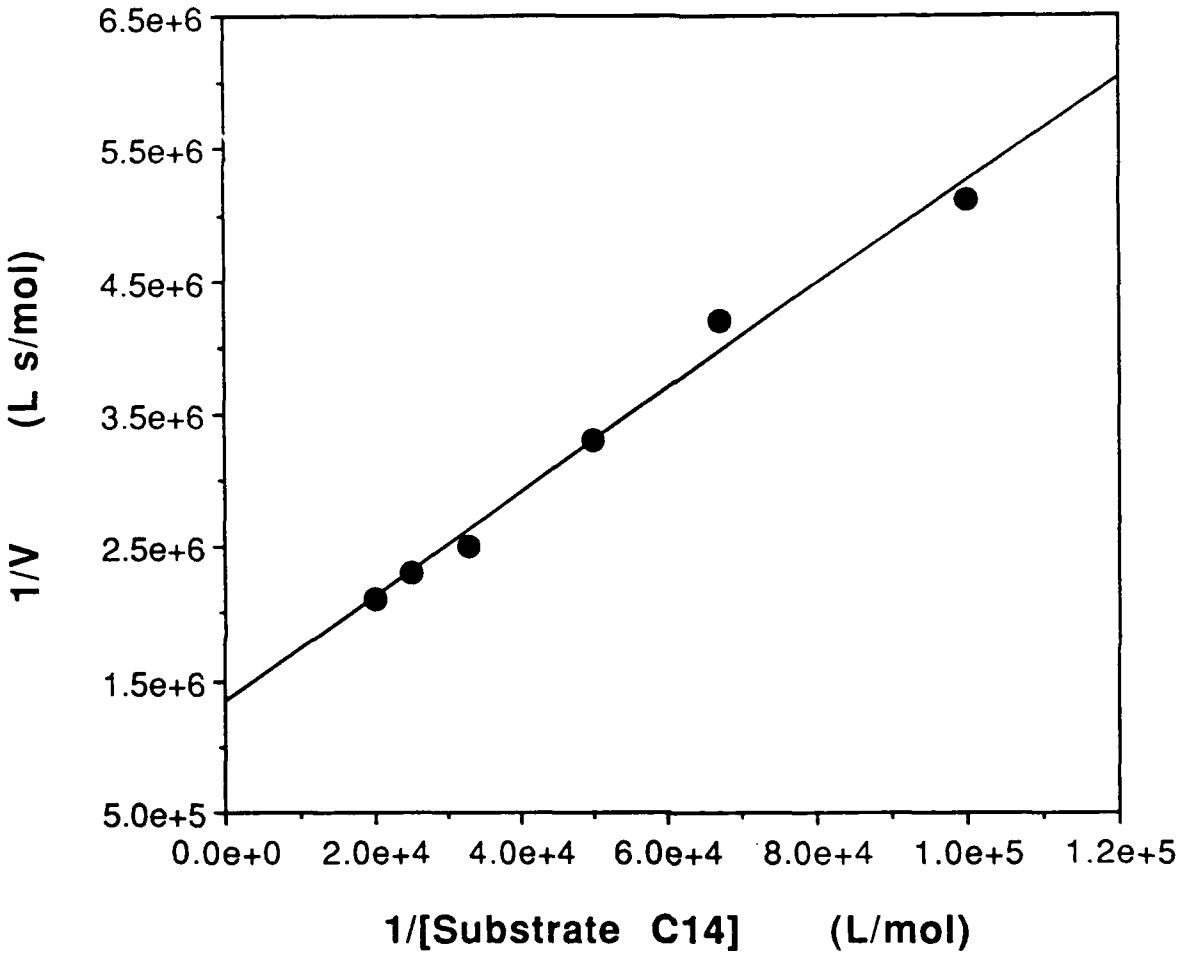
2e-4
3e-4
5e-7
4e-7
3e-7
2e-7
1e-7
0e+0
V (mol/L s)
0e+0
2e-5
4e-5
6e-5
8e-5
[Substrate C12, C14, C16] (mol/L)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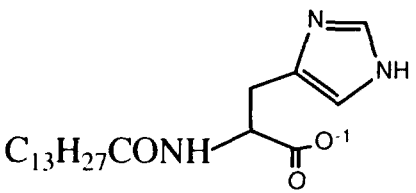
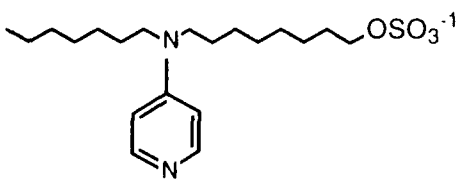
5.5e+6
4.5e+6
3.5e+6
2.5e+6
1.5e+6
5.0e+5
1/V (L s/mol)
0.0e+0
2.0e+4
4.0e+4
6.0e+4
8.0e+4
1.0e+5
1.2e+5
1/[Substrate C14] (L/mol)

Table 1. Summary of Michaelis-Menten parameters for the hydrolysis of 1 (n = 12, 14, 16) catalyzed by 4 (2.5×10^{-6} M) at 30 °C in 1:1 (V/V)MeOH/aqueous buffer (pH 8.0)

Ester (C_n)	V_{max} (M/s)	K_{cat} (s^{-1})	K_M (M)	k_{cat}/K_M ($M^{-1}s^{-1}$)
C_{12}	6.1×10^{-7}	0.27	5.9×10^{-5}	4,600
C_{14}	7.5×10^{-7}	0.33	2.9×10^{-5}	11,400
C_{16}	4.5×10^{-7}	0.20	2.4×10^{-5}	8,300

Table 2. Comparison of enzymes and linear enzyme-mimics as catalysts for hydrolysis of 1.

Catalyst	Alkanoate Chain	Conditions	kcat/KM	Reference
Enzymes	*Ester (C_n)*			
Cholesterol esterase	C_6	pH 7.01, 25 °C, Triton X, H_2O	125×10^4	29
Chymotrypsin	C_7	pH 7.8, 25 °C, H_2O	7.6×10^4	30
Synthetic Polymers				
4	C_{14}	pH 8, 30 °C, 1:1 MeOH/H_2O	1.1×10^4	This work
5	C_6	pH 7.8, 30 °C, 1:1 H_2O	80	14
Micelles				
	C_6	pH 7.2, 25 °C, CTAB-H_2O	122	31
	C_6	pH 8.5, 25 °C, H_2O	38	23