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Enol esters as potential pro-drugs II *. In vitro aqueous stability and enzyme-mediated hydrolysis of several enol esters of acetophenone

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Summary

Several α-acyloxystyrenes were synthesized and evaluated as models of enol esters which might be used in pro-drug design. Their stabilities were evaluated in aqueous buffered solutions at various pH values and temperatures, and in human and rat plasma and tissue homogenates at 37°C. The enol esters studied were relatively stable in neutral aqueous media, but most hydrolyzed rapidly and completely to yield the parent ketone (acetophenone) with the aid of enzymatic catalysis in the biological media used. The aqueous and enzymatic rates of hydrolysis were substantially affected by the nature of the acyl group. The results of this study indicate that enol esters are rather stable yet bioreversible derivatives of ketones and therefore may be useful as pro-drugs of agents containing enolizable carboxyl groups.

Introduction

Bioreversible derivatives of drugs—commonly referred to as pro-drugs—have been used to alter physicochemical properties in order to improve substantially drug stability, solubility, efficacy and safety (Repta, 1975; Stella and Higuchi, 1975; Sinkula and Yalkowsky, 1975; Roche, 1977). Chemical transformation of active drug substances to yield pro-drugs, which revert to the parent compounds within the body, may be relatively simple for drug substances possessing such groups as hydroxyls or carboxyls which may be simply esterified to yield bioreversible derivatives. The identification of a bioreversible derivative of a carbonyl-containing

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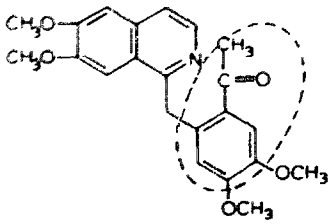
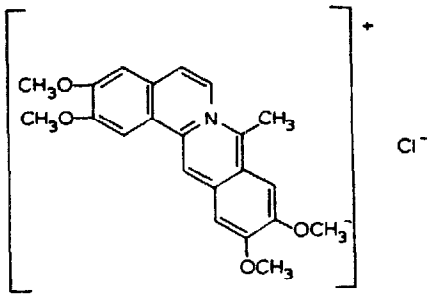
compound is more difficult. The rationale behind the use of enol esters as potential pro-drugs of enolizable ketones has been discussed in a previous communication (Patel and Repta, 1980), in which it was shown that α-acetyloxystyrene exhibited substantial aqueous stability and was a good substrate for the esterases of human and rat plasma and tissue homogenates.

The identification of enol esters of acetophenone as model compounds was based on our interest in prepararing a bio-labile derivative of 6′-acetylpapaverine (I), which is itself an unstable potential pro-drug form of the experimental anticancer agent, coralyne (II). An inspection of the structure of I reveals few possibilities for



6′-Acetylpapaverine, I



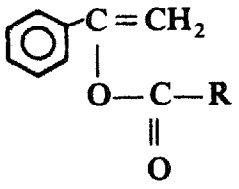
Coralyne chloride, II

formation of a derivative which would be biologically reversible other than chemical modifications of the tertiary amine or carbonyl groups. For a number of reasons, we chose to focus on the carbonyl group and that subfraction of structure I (circled portion) which is chemically very similar to acetophenone.

Since there was little information on the rates of hydrolysis of enol esters at other than highly acidic or alkaline concentrations and nothing on their susceptibility to enzyme-catalyzed hydrolysis, the preliminary study on α-acyloxystyrene was conducted (Patel and Repta, 1980). The encouraging results obtained suggested the need for a more comprehensive study on related model compounds to ascertain the influence of structural and electronic changes in the enol esters on their chemical stability and on their suitability as substrates for enzymatic hydrolysis.

The choice of the enol esters selected (IIIa–h) was predicted on obtaining a breadth of information relating to the general hydrolytic properties of model enol esters in aqueous media and in animal tissues, while maintaining constant some of the essential structural features of the ultimately desired enol esters of 6-acetylpapaverine (I).

Specific choices were made with specific aims in mind. For instance, compounds IIIa–e were chosen to determine the qualitative and quantitative effects of increased steric crowding at the α-carbon of the acyl group. IIIf was selected to obtain some information about the effect of a strong electron withdrawing acyl substituent on the properties of the enol ester. Compound IIIg was included because it might be a likely type of derivative for use in overcoming solubility problems since it is capable of forming a salt. Compound IIIh was chosen since it is probable that use of acyl groups containing an aromatic substituent might be desired to increase lipid solubility and alter partition coefficients in some pro-drug systems.



III

IIIa R = $-CH_3$
IIIb R = $-CH_2-CH_3$
IIIc R = $-CH_2CH_2-CH_3$
IIId R = $-CH(CH_3)_2$
IIIe R = $-C(CH_3)_3$
IIIf R = $-CCl_3$
IIIg R = $-CH_2-N(CH_3)_2$
IIIh R = $-\phi$

The effects of these various acyl substituents on the aqueous stability of the resulting enol esters and their susceptibility to enzymatic hydrolysis were evaluated and are the subject of this report.

Materials and Methods

Materials

Unless otherwise specified all chemicals were analytical grade reagents. All buffer solutions were prepared in deoxygenated de-ionized water obtained by bubbling nitrogen gas through previously boiled and cooled water. Commercial HPLC grade methanol and acetonitrile were used for high-performance liquid chromatography (HPLC) studies.

Silica gel CC-7 (Mallinckrodt Chemical Works, St. Louis, Mo.) was used for purification and separation of model α-acyloxystyrenes by column chromatography. All identification using thin-layer chromatography (TLC) was done on plastic-backed silica gel TLC plates with fluorescent indicator (Polygram Sil G/uv 254, Brinkman Inst., Westbury, N.Y.).

Human serum albumin (HSA), fraction V, was obtained in purified form from United States Biochemicals, Cleveland, Ohio. Male Sprague–Dawley rats (Harlan–Sprague–Dawley, Madison, Wisc.) weighing 225–250 g, were used to obtain plasma, liver and kidney tissue samples. Recovered human plasma was obtained from the Community Blood Center, Kansas City, Mo. The human liver sample used was that of a 66-year-old female, who dies of congestive cardiac failure, and was obtained through the Kansas University Medical Center, Kansas City, Kans. The liver sample, which appeared normal by macroscopic examination, was stored in a freezer (130°C) for less than 10 days prior to use.

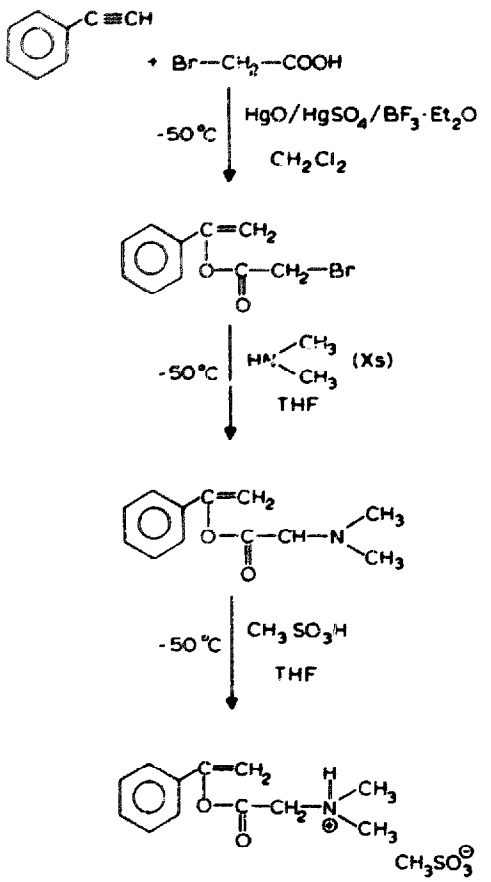
Methods

Synthesis.

α-Acetyloxystyrene (IIIa) was synthesized according to the method of Noyce and

Pollack (1969). α-Propionyloxystyrene (IIIb), α-butyryloxystyrene (IIIc), α-isobutyryloxystyrene (IIId), and α-pivaloyloxystyrene (IIIe) were synthesized according to the methods of House et al. (1969, 1971, 1973) using the desired acid anhydride. α-Trichloroacetyloxystyrene (IIIf) was synthesized by a procedure analogous to that used by Haas et al. (1969) for the synthesis of the corresponding trifluoro derivative. The methods of Lees (1903) and Young et al. (1950) were used to synthesize α-benzoyloxystyrene (IIIh).

The synthesis of α-(N-N-dimethylamino)acetyloxystyrene (IIIg) first required the preparation of α-monobromoacetyloxystyrene, which was subsequently reacted with an excess of dimethylamine to give IIIg. IIIg was subsequently obtained as a salt by neutralization with methane–sulfonic acid. These reactions are shown in Scheme 1.



Scheme 1

Synthetic pathway for IIIg methanesulfonate

The method of Haas et al. (1969) was modified (as described below) to synthesize α-monobromoacetyloxystyrene (α-MBA). Freshly distilled phenylacetylene (25 g, 0.25 mol) was added dropwise to dry methylene chloride (50 ml) containing bromoacetic acid (34.7 g, 0.25 mole), red mercuric oxide (2.7 g), mercuric sulfate (3.71 g) and freshly distilled boron trifluoride etherate (1.5 ml, 0.0125 mol). The temperature during the addition was maintained between −50°C and −60°C using

a dry-ice/acetone bath. The reaction mixture was allowed to warm to room temperature after the addition of phenylacetylene was complete and was subsequently diluted with methanol (50 ml). This mixture was then filtered to remove undesired solids. The filtrate was diluted with hexane (100 ml) and extracted with portions (4 × 100 ml) of a cold saturated aqueous solution of sodium bicarbonate to neutralize unreacted bromoacetic acid. The hexane layer was separated, dried by the addition of anhydrous sodium sulfate (5 g) and filtered after about 15 min. The filtrate was concentrated under reduced pressure and the concentrate applied to a silica gel (30 g) column (60 cm × 1.8 cm i.d.) and eluted with chloroform. The fractions containing the desired product were combined and the chloroform removed under reduced pressure to obtain α-monobromoacetyloxystyrene.

Dimethylamine gas was bubbled into a solution of α-monobromoacetyloxystyrene (2.4 g, 0.01 mol) in dry tetrahydrofuran (THF) (50 ml) maintained at −60°C. As the addition of the dimethylamine gas was continued, the clear solution turned turbid due to precipitation of dimethylamine hydrobromide. An alkaline reaction of the solution was used as an indication of an excess of dimethylamine in solution. The reaction was quite rapid and appeared complete in an hour. The reaction mixture was filtered and the filtrate concentrated under reduced pressure to yield essentially pure α-(N,N-dimethylamino)acetyloxystyrenes (IIIg). To a well stirred solution of IIIg (2.1 g, 0.01 mol) in THF (50 ml) at −60°C, a THF solution (10 ml) of methanesulfonic acid (0.73 ml, 0.01 mol) was added dropwise. The methanesulfonate salt of IIIg, which was insoluble in THF, was removed by filtration and recrystallized from a mixture of THF : methanol (95 : 5).

The elemental analyses of the model α-acyloxystyrenes were in excellent agreement with theory (Table 1). A summary of NMR data, including the peak positions, the type of splitting observed and the number of protons responsible for each peak is shown in Table 2. As evident from this table, all NMR spectra of the model enol

TABLE 1

RESULTS OF ELEMENTAL ANALYSES OF THE α-ACYLOXYSTYRENES

Compound	B.P. (°C) [a] (mm Hg)	M.P. (°C) [a]	Calculated %			Found %		
			C	H	N	C	H	N
IIIa	91–94 (4.5)	–	74.05	6.22	–	74.01	6.26	–
IIIb	105–115 (4)	–	74.98	6.86	–	74.88	6.98	–
IIIc	110 (3.5)	–	75.76	7.42	–	76.10	7.31	–
IIId	82 (0.8)	–	75.76	7.42	–	75.48	7.80	–
IIIe	102 (2.1)	–	76.44	7.89	–	76.31	8.01	–
IIIf	124 (3)	–	–	–	–	– [b]	– [b]	– [b]
IIIg	–	112 (decomp.) [c]	51.81	6.36	4.65	51.68 [c]	6.60 [c]	4.61 [c]
IIIh	190 (9)	37	80.34	5.39	–	80.23	5.45	–

[a] Uncorrected.

[b] Not analyzed.

[c] Results for the methane sulfonate salt.

TABLE 2

MAJOR PEAK POSITIONS FOR THE α-ACYLOXYSTYRENES AS DETERMINED BY NMR ANALYSIS

Key: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet.

Compound	Chemical shift (δ) ppm
IIIa	2.2(s)(3H), 5(d)(1H), 5.4(d)(1H), 7.4(m)(5H)
IIIb	1.2(t)(3H), 2.6(q)(2H), 5(d)(1H), 5.4(d)(1H), 7.4(m)(5H)
IIIc	1.2(t)(3H), 1.8(m)(2H), 2.6(m)(2H), 5(d)(1H), 5.5(d)(1H), 7.4(m)(5H)
IIId	1.3(m)(6H), 2.7(m)(1H), 5(d)(1H), 5.4(d)(1H), 7.4(m)(5H)
IIIe	1.35(s)(9H), 4.95(d)(1H), 5.4(d)(1H), 7.35(m)(5H)
IIIf	5.2(d)(1H), 5.5(d)(1H), 7.4(m)(5H)
IIIg	2.4(s)(6H), 3.4(s)(2H), 5.05(d)(1H), 5.45(d)(1H), 7.35(m)(5H)
IIIh	5.15(d)(1H), 5.6(d)(1H), 7.45(m)(8H), 8.2(m)(2H)

esters showed characteristic vinylic proton absorptions around 5.0 and 5.5 ppm. All of the enol esters exhibited only one spot on silical gel TLC after purification. For all compounds except IIIg, the developing solvent for TLC was chloroform. The compounds and the respective R_f values for the chloroform silica gel system were: IIIa, 0.5; IIIb, 0.53; IIIc, 0.56; IIId, 0.56; IIIe, 0.60; IIIf, 0.57; IIIh, 0.58; and acetophenone, 0.45. In the case of IIIg (methane sulfonate salt) TLC was done using acetone and the R_f value of IIIg and acetophenone was 0.40 and 0.65, respectively.

Kinetic studies

Stock solutions of the α-acyloxystyrenes (IIIa–h) were prepared in dry acetonitrile. An appropriate volume of the stock solution of the enol esters of interest was mixed with aqueous buffers, plasma or tissue supernatant for the kinetic studies. The initial concentration of IIIa–h for all the kinetic studies in various media ranged from 1.8×10^{-5} M to 3.2×10^{-5} M. The final concentration of acetonitrile in all the studies was always $\leq$ 0.5% (v/v). Furthermore, independent studies demonstrated that there was no significant change in the rates of hydrolysis of the α-acyloxystyrenes when the concentration of acetonitrile was varied from 1 to 3% (v/v) in the aqueous buffers, plasma and tissue supernatants.

Unless otherwise noted, all the enzymatic and non-enzymatic rates of hydrolysis were followed spectrophotometrically by monitoring absorbance changes at $\lambda = 280$ nm for IIIa–g and $\lambda = 237$ for IIIh. The pseudo-first-order rate constants were determined from plots of $\log(A_t - A_\infty)$ or $\log(A_\infty - A_t)$ vs. time, where A_t and A_∞ are the absorbance readings at any time t and at infinity (i.e. when the reaction is complete), respectively.

In the rate studies in rat kidney homogenates, rate constants were obtained from initial rates ($\leq$ 5 hydrolysis) based on absorbance changes at 280 nm.

A constant ionic strength was maintained at 0.3 M for each of the buffers used by adding requisite amounts of sodium chloride. Unless otherwise specified, rate constants used in the pH–log rate profiles (Fig. 1) were obtained by linear extrapolation to zero buffer concentration of plots of k_{obs} vs buffer concentration (Table 3).

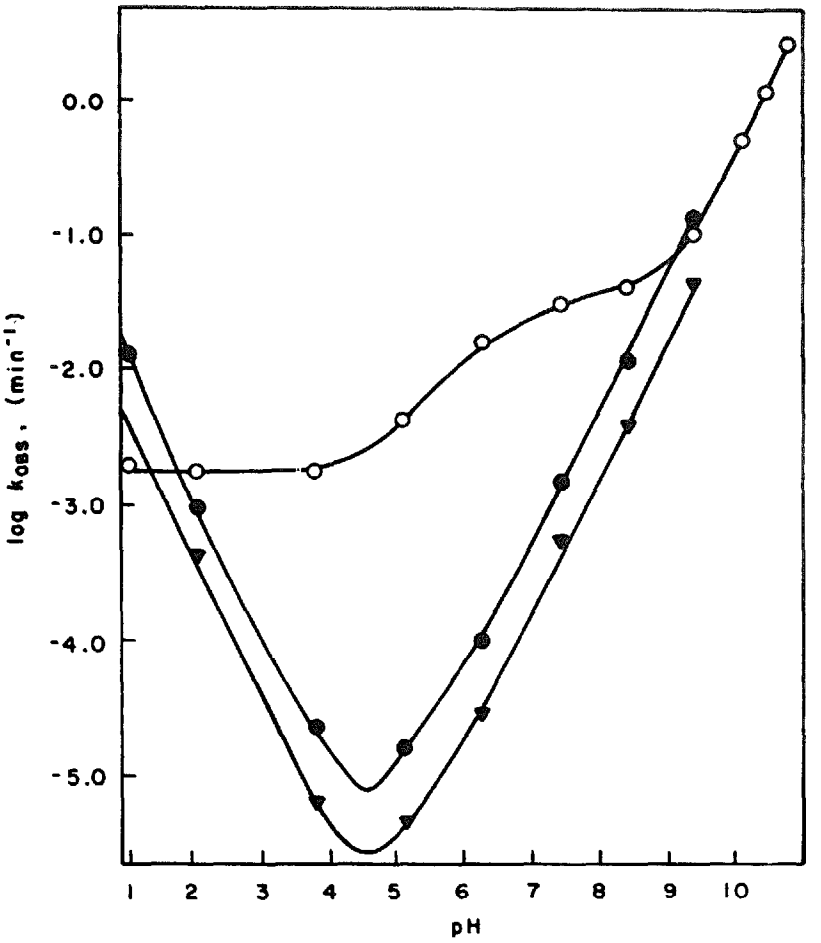


Fig. 1. Log pH–rate profile for the hydrolysis of IIIa (●), IIIg (○) and IIIh (▲) at 60°C, $\mu = 0.3$.

The initial rates of α-acetyloxystyrene (IIIa) and α-benzoyloxystyrene (IIIh) at pH 3.8, 5.05, and 6.25, at 60°C in buffer (0.025 M) were followed by high-performance liquid chromatography (HPLC). Separations were accomplished at ambient temperature (~ 19–22°C) using a Lichrosorb RP18 (25 cm × 4.6 mm i.d.) column (Unimetrics, Anaheim, Calif.) with detection at 254 nm and a sensitivity setting of 0.01 AUFS. Using a mobile phase of methanol : water (1 : 1), at a flow-rate of 1.5 ml/min and an injection volume of 50 μl, acetophenone and α-acetyloxystyrene eluted with retention volumes of 9.8 ml and 17.7 ml,respectively.

In the separation of α-benzoyloxystyrene and acetophenone, the mobile phase was acetonitrile : water (1 : 1) with a flow-rate of 2.5 ml/min. A 25-μl injection volume was used and acetophenone eluted in 6.3 ml while α-benzoyloxystyrene eluted in 35.5 ml. From the HPLC data, rate constants were determined using a method of initial rates in which the area of the hydrolysis product (acetophenone) was monitored as a function of time. The hydrolysis was followed for ≤ 5% of the reaction.

TABLE 3

EFFECT OF BUFFER CONCENTRATION ON THE RATES OF HYDROLYSIS OF MODEL α-ACYLOXYSTYRENES AT VARIOUS pHs ($\mu = 0.3$) AT 60°C

pH (buffer)	Buffer concentration (M)	Pseudo-first-order rate constants [a], K_{obs} (min^{-1})		
		IIIa ($\times 10^4$)	IIIg ($\times 10^3$)	IIIh ($\times 10^5$)
9.35 (Borate)	0.00	1320	114.0	4120
	0.01	– [b]	117.6	4211.6
	0.025	1389	130.8	4232.3
	0.06	1470	147.5	4516.7
	0.10	1582	170.5	4748.3
8.35 (Borate)	0.00	120.0	41.0	410.0
	0.01	126.0	42.6	438.0
	0.025	145.9	48.2	485.6
	0.06	171.2	54.7	578.5
	0.10	215.1	66.6	713.3
7.40 (Phosphate)	0.00	14.0	31.0	57.6
	0.01	14.9	31.5	58.1
	0.025	15.4	32.2	59.6
	0.06	17.1	35.0	62.6
	0.10	19.0	38.1	64.6
6.25 (Phosphate)	0.00	– [b]	16.6	– [b]
	0.01	– [b]	17.7	– [b]
	0.025	1.0 [c]	21.0	2.9 [c]
	0.06	– [b]	25.7	– [b]
	0.10	– [b]	31.7	– [b]
5.05 (Acetate)	0.00	– [b]	4.0	– [b]
	0.01	– [b]	4.3	– [b]
	0.025	0.2 [c]	4.9	0.5 [c]
	0.06	– [b]	5.7	– [b]
	0.10	– [b]	6.6	– [b]
3.8 (Acetate)	0.00	– [b]	1.9	– [b]
	0.01	– [b]	2.0	– [b]
	0.025	0.2 [c]	2.0	0.61 [c]
	0.06	– [b]	2.2	– [b]
	0.10	– [b]	2.6	– [b]

[a] Values at zero concentration obtained by extrapolation of k_{obs} vs concentration.
[b] Not determined.
[c] From initial rate method, see Materials and Methods.

Preparation of tissue homogenates

Blood from male albino Sprague–Dawley rats was withdrawn using a heparinized syringe, transferred to a 10-ml centrifuge tube, and centrifuged at $1000 \times g$ for 15 min after which the plasma was removed. An appropriate volume of rat plasma was diluted with pH 7.4 isotonic Sorenson's phosphate buffer and used in the hydrolytic studies. It was observed that storage of rat plasma at refrigerator temperature for a week did not affect the enzymatic activity toward the enol esters studied.

The human and rat liver, and rat kidney homogenates were prepared in pH 7.4, isotonic Sorenson's phosphate buffer (1 part of tissue to 25 parts buffer), using a mechanically driven glass homogenizer with a teflon pestle having radial serrations. The homogenates were centrifuged for 90 min at 105,000 × *g* at 5°C. The tissue supernatants thus obtained are referred to as 4% (w/v) solutions and were further diluted as needed, using pH 7.4 isotonic Sorenson's phosphate buffer. The temperature was always maintained at 5–10°C during the preparation of the tissue homogenates to avoid denaturation of the enzyme.

Results and Discussion

Kinetic studies in buffered aqueous solution

The rates of loss of the α-acyloxystyrenes (IIIa–h) exhibited pseudo-first-order kinetics in the aqueous media at all of the various temperatures and pH values studied. As expected, the products of the hydrolytic reaction were found to be acetophenone and the respective acid. The rates of hydrolysis of α-acetyloxystyrene (IIIa), α-benzoyloxystyrene (IIIh) and α-(N,N-dimethylamino)acetyloxystyrene (IIIg) were determined at several pH values in various concentrations of acetate, phosphate and borate buffers at 60°C (Table 3). The data demonstrated some buffer dependence in all cases studied. However, in the case of compounds IIIa and IIIh, the effect was rather small and essentially negligible at a buffer concentration of 0.025 M. Consequently, some of the rate data at lower pH values were obtained only at a single buffer concentration. In all cases where several buffer concentrations were used, k_{obs} values at zero buffer concentration were obtained by extrapolation. These k_{obs} values and those obtained directly in hydrochloric acid (pH < 3.8) and sodium hydroxide (pH > 9.35) solution are shown as the data points in the pH–rate profiles in Fig. 1.

The data in Table 3 indicate that catalysis by buffer species occurs in all systems studied with maximum buffer effects being exhibited for the α-(N,N-dimethylamino)acetyloxystyrene (IIIg) system. Substantially lesser effects were observed for the non-ionizable compounds IIIa and IIIh. While the data presented in Table 3 are limited and do not permit extensive quantitative evaluation of the catalytic effects of the various buffer species used, several facts could be abstracted. From graphical and/or mathematical treatment of the data (Jencks, 1969), it was found that for the hydrolysis of all 3 esters the rate constants for the borate ($H_2BO_3^-$) catalyzed reactions were 6–8-fold greater than those for the boric acid-catalyzed reactions. The data for hydrolysis of IIIg in phosphate and acetate buffers showed that while the more acidic species, $H_2PO_4^-$, was a better catalyst than HPO_4^{2-}, the catalytic effect of acetic acid was less than that of acetate ion. Overall, these results suggest that the more catalytic species are those capable of acting as general bases [1].

[1] The data available do not rule out the possibility that the catalysis by the basic species is nucleophilic in nature.

since these α-acyloxystyrenes do not contain any ionizable moieties. The linear segments with unit slopes at low pH (pH < 3) and high pH (pH > 7) indicate specific acid and base catalysis, respectively. While the pH–rate profiles of IIIb–e were not experimentally determined, it is anticipated that on the basis of their structural similarity to IIIa they would be of a shape similar to that observed for IIIa (Fig. 1). However, the values of the apparent rate constants, k_{obs}, might be quite different, due to the differences in the steric nature of the various acyl substituents in IIIa–e. Such differences in values of the rate constants would result n some translation of the V-shaped profiles.

The pH–rate profile of IIIg includes a sigmoidal segment which is attributed to the degree of ionization of the dimethylamine functionality. The ionized and unionized fractions hydrolyze at different rates resulting in the sigmoid portion in the pH–rate profile. The linear portions in the pH–rate profile of IIIg (pH > 10) indicates specific base catalysis. The pH–rate profile for IIIg shows a broad uncatalyzed region (pH 1.5–3.5) and a virtual absence of the specific acid-catalyzed region down to pH = 1.

The pH dependencies of the observed rate constants (k_{obs}) for IIIa and IIIh (Fig. 1), which have no ionizable groups, can be attributed to a pH-independent spontaneous hydrolysis (k_0) (Connors et al., 1979) coupled with specific acid catalysis (k_{H^+}) at low pH values and specific base (k_{OH^-}) catalysis at high pH values. This situation is shown mathematically in Eqn. 1.

$$k_{obs} = k_{H^+}[H^+] + k_{OH^-}[OH^-] + k_0 \tag{1}$$

and would be expected to apply to IIIb–c as well as to IIIa and IIIh.

As mentioned above, the pH–rate profile for IIIg differs from that of IIIa and IIIh (Fig. 1) because of ionization. The data can be qualitatively and quantitatively described by assuming spontaneous or uncatalyzed decomposition of the protonated enol ester, EJ^+, and specific base-catalyzed hydrolysis of both EH^+ and E, the unprotonated enol ester, as shown in Equations 2–4.

$$EH^+ \xrightarrow{k_0} \text{products} \tag{2}$$

$$E + OH^- \xrightarrow{k_{OH^-}} \text{products} \tag{3}$$

$$EH^+ + OH^- \xrightarrow{k'_{OH^-}} \text{products} \tag{4}$$

where $[E]_T$ is the total concentration of the enol ester, IIIg (i.e. $[E]_T = [E] + [EH^+]$). The simplest overall rate equation [2] which accounts for the data is:

$$k_{obs}[E]_T = k_0[EH^+] + k'_{OH^-}[EH^+][OH^-] + k_{OH^-}[E][OH^-] \tag{5}$$

By introducing into Eqn. 5 the identities

$$[E] = \frac{K_a}{a_{H^+} + K_a}[E]_T \tag{6}$$

[2] It is recognized that isokinetic terms may be equally correct and distinguishable.

TABLE 4

VALUES[a] OF THE VARIOUS RATE CONSTANTS FOR THE HYDROLYSIS OF THE α-ACYLOXYSTYRENES, IIIa, IIIg AND IIIh AQUEOUS SOLUTIONS. T=60°C AND μ=0.3

α-Acyloxystyrene	Rate constant			
	K_0 (min^{-1})	k_{H^+} ($M^{-1}\,min^{-1}$)	k_{OH^-} ($M^{-1}\,min^{-1}$)	k_{OH^-} ($M^{-1}\,min^{-1}$)
IIIa	1.0×10^{-5}	0.153	5.45×10^{2}	–[b]
IIIg	1.8×10^{-3}	–[b]	4.28×10^{2}	32.2×10^{5}
IIIh	2.6×10^{-6}	3.98×10^{-2}	1.94×10^{2}	–[b]

[a] These values were used along with either Eqn. 5 or Eqn. 8 to generate the solid lines in Fig. 1.
[b] Not applicable to the particular α-acyloxystyrene.

and

$$[EH^+] = \frac{a_{H^+}}{a_{H^+} + K_a}[E]_T \tag{7}$$

(where K_a is the apparent ionization constant of EH^+ and a_{H^+} is the hydrogen ion activity), the expression for k_{obs} becomes

$$k_{obs} = k_0\frac{a_{H^+}}{K_a + a_{H^+}} + k'_{OH^-}\frac{a_H + [OH]}{K_a + A_{H^+}} + k_{OH^-}\frac{K_a[OH]}{K_a + a_{H^+}} \tag{8}$$

The solid lines in Fig. 1 were obtained using Eqn. 1 (for IIIa and IIIh) and Eqn. 8 (for IIIg) and the rate constants found in Table 4. The apparent ionization constant for the protonated form of IIIg at 60∞C and an ionic strength of 0.3 M was calculated to be 6.15.

Relative rates of hydrolysis of a-acyloxystyrenes

As seen in Fig. 1, k_{obs} differs considerably as a function of pH for IIIa, IIIg and IIIh. The rates of hydrolysis of all of the remaining model α-acyloxystyrenes (except for α-trichloroacetyloxystyrene, IIIf) were studied at 60°C in aqueous borate (0.1 M) buffer (pH 9.35, $\mu = 0.3$). The observed first-order rate constants and corresponding

TABLE 5

APPARENT FIRST-ORDER RATE CONSTANTS AND CORRESPONDING HALF-LIVES FOR THE HYDROLYSIS OF MODEL α-ACYLOXYSTYRENES AT 60°C IN BORATE BUFFER, 0.1 M, pH 9.35 AND μ=0.3

α-Acyloxystyrene	$k_{obs}\times10^3$ (min^{-1})	$t_{1/2}$ (min)
IIIa	154	4.5
IIIb	104	6.7
IIIc	51.7	14
IIId	36.5	19
IIIe	5.0	140
IIIg	151	4.6
IIIh	44.4	16

TABLE 6

RATE CONSTANTS AND HALF-LIVES FOR THE HYDROLYSIS OF α-TRICHLOROACETYLOXYSTYRENE (IIIf) IN WATER, METHANOL–WATER AND BUFFER (0.001 M PHOSPHATE, pH 7.4) AT AMBIENT TEMPERATURE (22°C)

Solvent	$k \times 10^3$ sec^{-1}	$t_{1/2}$ sec
80% (v/v) methanol in water	1.44	480
50% (v/v) methanol in water	4.95	140
20% (v/v) methanol in water	38.5	18
Water	58.0	12
Phosphate buffer (pH 7.4)	58.0	12

half-lives for all of the α-acyloxystyrenes (except IIIf) are presented in Table 5. The importance of steric crowding adjacent to the carbonyl carbon is demonstrated by an ~ 30-fold increase in stability as the bulk of the alkyl substituent increases from methyl (IIIa) to *t*-butyl (IIIe). These effects are similar to those reported by Sun and Connors (1969) for normal saturated esters. α-Benzoyloxystyrene (IIIh) exhibited stability intermediate to that of the *n*-butyric and isobutyric acid esters (IIIc and IIId, respectively). Since all of the simple alkyl and aromatic acyloxystyrenes would be expected to exhibit V-shaped profiles, the rank-order stability observed at pH 9 would pertain at physiological pH and even down to values of pH ~ 5. Maximum stability for IIIa–e and for IIIh would be expected to occur at pH ~ 4.5.

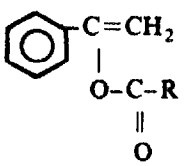
The rate of hydrolysis of α-trichloroacetyloxystyrene (IIIf) was too fast to monitor under the conditions given in Table 5. Even at ~ 22°C, the half-life for hydrolysis in both water and pH 7.4 phosphate buffer was only 12 s. Although these data are limited, the lack of any significant differences in rate at pH 7.4 and in unbuffered water suggest a substantial rate constant for the spontaneous hydrolysis reaction. With the addition of methanol to the aqueous solution, the observed rate constant decreased substantially, probably due in a large part to a decrease in the di-electric constant of the medium. From these data (Table 6) it was obvious that the electron-withdrawing effect of the trichloromethyl group (Hauser, 1962) rendered α-trichloroacetyloxystyrene sufficiently unstable, such that further studies involving enzyme catalysis were neither indicated nor pursued in this work.

The rates of hydrolysis of IIIg and IIIh were studied as a function of temperature in aqueous phosphate buffers (0.025 M, $\mu = 0.3$) at pH 7.4. As was reported previously (Patel and Repta, 1980) for α-acetyloxystyrene, plots of log k_{obs} vs reciprocal temperature (°K) yielded linear relationships. The rate data for all 3 compounds are presented in Table 7 along with the estimated values of k_{obs} and the corresponding half-lives at 25°C. It is clear from Fig. 1, that for all 3 compounds, the hydrolysis reaction at pH 7.4 is due to specific base catalysis. Therefore, the activation parameters (E_a, $\Delta H^{\neq}$, $\Delta S^{\neq}$) obtained from Arrhenius and Eyring plots (Frost and Pearson, 1960) in Table 8 for IIIa [3] and IIIh have been corrected for the

[3] In the earlier publication of Patel and Repta, the values of the activation parameters for α-acetyloxystyrene were reported without correcting for the heat of ionization of water.

TABLE 7

OBSERVED FIRST-ORDER RATE CONSTANTS, ASSOCIATED HALF-LIVES, AND ACTIVATION PARAMETERS [a] FOR THE HYDROXIDE-CATALYZED HYDROLYSIS OF α-ACETYLOXYSTYRENE [b] (IIIa), α-(N,N-DIMETHYLAMINO)ACETYLOXYSTYRENE (IIIg) AND α-BENZOYLOXYSTYRENE (IIIh) IN PHOSPHATE BUFFER (pH 7.4, 0.025 M, μ=0.3) AT VARIOUS TEMPERATURES

α-Acyloxystyrene 	Temperature (°C)	Rate constant [c] k_{obs} (h)	Half-life, $t_{1/2}$ (h)
IIIa	25	3.9×10^{-3} [b]	180
(R=$-CH_3$)	37	1.2×10^{-2}	56.8
	50	4.1×10^{-2}	16.8
	58	8.9×10^{-2}	7.8
	60	9.2×10^{-2}	7.5
	66	16.6×10^{-2}	4.2
	$E_a = 6.1\pm0.4$ kcal/mol		
	$\Delta H^{\neq} = 5.7\pm0.4$ kcal/mol		
	$\Delta S^{\neq} = -23$ e.u.		
IIIg	25	0.14	~5
(R=$-CH_2N(CH_3)_2$)	30	0.20	3.5
	39	0.41	1.7
	50	0.92	0.75
	60	1.81	0.38
	$E_a = 1.6\pm0.7$ [d] kcal/mol		
IIIh	25	3.65×10^{-3}	~1900
(R=)	60	2.80×10^{-2}	24.75
	70	7.91×10^{-2}	8.75
	80	22.50×10^{-2}	3.08
	$E_a = 11.3\pm0.4$ kcal/mol [b]		
$\Delta H^{\neq} = 10.6\pm0.4$ kcal/mol			
$\Delta S^{\neq} = -11$ e.u.			

[a] Activation parameters have been adjusted for heat of ionization of water.
[b] Data taken in part from Patel and Repta (1980).
[c] Values at 25°C were obtained from Arrhenius plots.
[d] This is an apparent value; see text for discussion.

heat of ionization of water, i.e. 13.05 kcal mol^{-1} (Harned and Hamer, 1933). The values of these activation parameters are of a magnitude comparable to those reported by Yrjana (1966) for the enol ester, isopropenylacetate.

In the case of the hydrolysis of IIIg at pH 7.4, it is apparent from Fig. 1 (and Eqn. 8) that two different hydroxide ion-catalyzed reactions are occurring simultaneously. At 60°C, the reaction represented by Eqn. 3 is predominantly responsible for the magnitude of k_{obs}. However, since the observed rate constant is a function of K_a which has an undetermined temperature dependence, the observed linearity of

the Arrhenius plot was unexpected. Although an *apparent* activation energy could be calculated ($E_a = 1.6 \pm 0.07$ kcal/mol) from the slope of the plot, the interpretation of the value obtained is highly questionable and, therefore, no values for $\Delta S^{\neq}$ and $\Delta H^{\neq}$ were calculated.

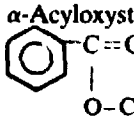
The calculated half-lives at 25°C in pH 7.4 phosphate buffer shown in Table 7 clearly demonstrate that these enol esters are fairly stable in aqueous media at neutral pH values. Furthermore as stated earlier, the data in Table 6 indicate that the various branched and unbranched alkyl enol esters would be expected to be more stable than the corresponding acetate ester. Thus, it may be concluded that all the enol esters studied (except IIIf, Table 5) possess adequate stability in neutral to slightly acidic aqueous media.

In vitro studies in biological media

The relative susceptibility of the various α-acyloxystyrenes to enzymatic hydrolysis was studied in vitro in human and rat plasma and in the supernatant fraction of several tissue homogenates at 37°C. The hydrolysis of all of the enol esters studied was followed directly spectrophotometrically in dilute plasma and liver homogenate supernatants. In these media, the rate of loss appeared to be a first-order process yielding acetophenone. In no instance does any evidence appear of saturation of enzyme activity. The rate data obtained are presented in Tables 8 and 9. In the case of the supernatant of kidney homogenates, the rates were relatively slow, and were determined by initial rate measurements of absorbance changes at $\lambda = 220$ nm. From these initial rate data were calculated the values presented in Table 9.

TABLE 8

RATE CONSTANTS AND HALF-LIVES FOR THE HYDROLYSIS AT 37°C OF THE α-ACYLOXYSTYRENES IN HUMAN AND RAT PLASMA DILUTED WITH pH 7.4 ISOTONIC SORENSON'S PHOSPHATE BUFFER

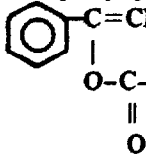
α-Acyloxystyrene 	5% human plasma		1% rat plasma	
	$k \times 10^3$ (min^{-1})	$t_{1/2}$ (min)	$k \times 10^3$ (min^{-1})	$t_{1/2}$ (min)
R = $-CH_3$, IIIa	26.7	26	3.65	1.9
R = $-CH_2-CH_3$, IIIb	53.3	13	13.90	0.5
R = $-CH_2-CH_2-CH_3$, IIIc	57.8	12	9.90	0.7
R = $-CH(CH_3)_2$, IIId	6.30	110	2.24	3.1
R = $-C(CH_3)_3$, IIIe	– [a]	– [a]	3.47	2.0
R = $-CH_2N(CH_3)_2$, IIIg	33.0 [b]	21 [b]	2.67	2.6
R = $-\phi$, IIIh	12.6	55	6.30	1.1

[a] No value determined since hydrolysis was incomplete after 24 h.

[b] In 2% human plasma.

TABLE 9

RATE CONSTANTS AND CORRESPONDING HALF-LIVES FOR THE HYDROLYSIS OF SOME MODEL α-ACYLOXYSTYRENES AT 37°C IN SUPERNATANT OBTAINED AFTER CENTRIFUGATION (AT 105,000×g) OF HOMOGENATES OF HUMAN AND RAT LIVER [a] AND RAT KIDNEY [b]

α-Acyloxystyrene 	Human liver supernatant		Rat liver supernatant		Rat kidney	
	k_{obs} ($\times 10^3$) (sec^{-1})	half-life (min)	k_{obs} ($\times 10^3$) (sec^{-1})	half-life (min)	k_{obs} [c] ($\times 10^3$) (sec^{-1})	half-life [c] (sec)
R=$-CH_3$, IIIa	1.84	6.3	2.02	5.7	0.44	26
R=$-CH_2-CH_3$, IIIb	4.18	2.8	7.97	1.5	0.70	17
R=$-CH_2-CH_2-CH_3$, IIIc	8.35	1.4	6.48	1.8	0.43	27
R=$-CH(CH_3)_2$, IIId	2.42	4.8	4.44	2.6	0.30	39
R=$-C(CH_3)_3$, IIIe	1.04	11.1	1.76	6.6	0.18	64
R=$-CH_2N(CH_3)_2$, IIIg	1.76	6.6	2.86	4.0	– [d]	– [d]
R=$-\phi$, IIIh	5.73	2.0	3.43	3.4	– [d]	– [d]

[a] 197 μg wet tissue/ml of isotonic Sorenson's phosphate buffer, pH 7.4.
[b] 389 μg wet tissue/ml of isotonic Sorenson's phosphate buffer, pH 7.4.
[c] Obtained from calculations based on initial rates determined by spectrophotometry.
[d] Not determined.

Hydrolysis in plasma

The results of preliminary studies (Patel and Repta, 1980) have indicated that the rates of hydrolysis of IIIa in undiluted human plasma were quite rapid. Therefore, diluted plasma was used in these studies. A comparison of the data in Fig. 1 and Tables 5 and 7 with those in Table 8 demonstrates that components of both human and rat plasma greatly catalyze the hydrolysis of the various α-acyloxystyrenes. The observed catalysis of the enol esters is attributed to unspecified esterases present in the plasma.

The data in Table 8 suggests that there is a significant difference in the overall activity and specificity of the esterases of rat and human plasma. All of the model α-acyloxystyrenes studied hydrolyzed significantly faster in rat plasma than in human plasma of comparable concentration. These findings are in general accord with those of Ecobichon (1972) who has shown that the carboxylesterase activity of several mammalian species differed appreciably towards α-naphthylacetate.

α-Pivaloyloxystyrene (IIIe) was a poor substrate for the esterases in dilute human plasma as is evident from the fact that its hydrolysis is not complete in a 24-h period. However, TLC analysis of the chloroform extract from this hydrolysis mixture demonstrated the presence of some acetophenone in addition to the unchanged enol ester. In Sorenson's phosphate buffer alone (no plasma), no detectable loss of the enol ester occurred over a period of 48 h. Thus, the hydrolysis of IIIe was indeed catalyzed by esterases in human plasma, but the magnitude of the catalyzed rate was still extremely small relative to that observed for the other α-acyloxystyrenes studied. This is not the case, however, in rat plasma where IIIe is a good substrate for esterases.

As is evident from the data in Table 8, the half-life for the hydrolysis of α-acyloxystyrenes in both human and rat plasma decreases as the acyl group is changed from acetyl (IIIa) to *n*-propionyl (IIIb), while the latter is similar to the *n*-butyryl ester. Dixon and Webb (1964) found similar results while studying the hydrolysis of acetate, propionate and *n*-butyrate esters of methyl and ethyl alcohol in a purified horse liver carboxylesterase.

The data in Table 9 indicate that an increased branching around the carbonyl carbon decreases the hydrolytic rate. Such decreases may be due to steric factors which result in a poorer fit of the substrate on the active site of the enzyme(s). Similar reductions in rates were obtained for the hydrolysis of the same enol esters in the buffered aqueous media (Table 6). However, the hydrolysis rate of both IIId and IIIe in dilute rat plasma is very similar to that of IIIa, even though the carbonyl group is considerably less sterically hindered in the case of IIIa.

Hydrolysis in tissue supernatant

The hydrolysis of the various α-acyloxystyrenes by soluble components of both human and rat liver and rat kidney homogenates was studied. While enzyme activity would be expected to be associated with both soluble and insoluble (microsomal) fractions, the present studies were confined to the supernatant obtained after centrifugation of the homogenized tissue at $105{,}000 \times g$ for 90 min. Use of the supernatant fraction allowed for convenient monitoring of the hydrolysis rates as

well as allowing for rank-order comparisons of the data with that obtained for the soluble esterases contained in plasma.

Again, comparison of the hydrolysis data obtained in simple aqueous buffer with those in Table 9 demonstrate a large catalytic enhancement by the tissue homogenate supernatant. It is interesting to note that the half-lives for hydrolysis of the various α-acyloxystyrenes are very similar in both the rat and human liver systems. This is in sharp contrast to the results (Table 8) in plasma where (when one considers the different plasma concentrations) the rat plasma was a much more effective hydrolytic medium.

It also appears clear from Table 9 that esterase activity of rat liver supernatant is more than an order of magnitude greater than that of the rat kidney. However, the rank-order of the various α-acyloxystyrenes studied appears to be the same for both rat tissues with maximum rates being observed for IIIb. Among the simple aliphatic compounds (IIIa–e), IIIb was also the compound hydrolyzed most rapidly in rat plasma.

The presence of either the ionizable dimethylamino group of IIIg or the phenyl group of IIIh did not appear to decrease the susceptibility of these compounds to hydrolysis by the various esterases contained in the selected media. In fact, in human plasma the dimethylamino group of IIIg resulted in a sufficient rate enhancement such that a 60% reduction in plasma concentration was used in the kinetic study. These findings are noteworthy since ionizable or aromatic substituents of the types found in IIIg and IIIh may be useful in preparing readily bioreversible enol ester pro-drugs with substantially altered solubility and partitioning properties.

Conclusions

The results of these studies indicate that α-acyloxystyrenes are relatively stable in neutral aqueous solutions. It appears from the limited amount of kinetic data and values of the activation parameters that the mechanisms of hydrolysis of enol esters in mildly acidic and alkaline pH may be similar to those observed for saturated ester (Novak and London, 1977). However, more detailed kinetic studies involving solvent deuterium isotope effects and ^{18}O exchange would have to be done to confirm this hypothesis.

The relative stability of α-acyloxystyrenes is influenced by electronic and steric nature of the acyl group. Increased steric hinderance around the carbonyl carbon leads to a decrease in the rate of hydrolysis. The half-lives of hydrolysis and, therefore, the stability of the enol esters studied can be increased by choosing the more sterically hindered acyl moieties such as the isobutyl and pivaloyl groups. Furthermore, the pH of optimum stability lies between the values of 3 and 5, which is well within a physiologically acceptable range.

In general, the rank-order stabilities of all of the simple aliphatic α-acyloxystyrenes studied were very similar in the biological media used with the single exception of IIIg in rat plasma. Based on the results obtained using human ad rat liver, it appears that there may be some justification for use of rat liver in estimating the hydrolytic

rate to be found in human liver. Additional studies would be required to substantiate this observation and to determine if similar relationships may exist between other tissues.

Overall, the results of this study suggest that the in vivo and in vitro rate of hydrolysis of compounds such as the α-acyloxystyrenes can be substantially altered by careful selection of the acyl group. Consequently, the use of related esters may be of value in achieving enhanced drug delivery of enolizable compounds.

Based on these studies, subsequent efforts in these laboratories have been directed toward the identification and evaluation of some enol esters of 6-acetylpapaverine (I). Encouraging preliminary data have been obtained and will be presented at a later date.

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