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Research Article

Amine Prodrugs Which Utilize Hydroxy Amide Lactonization. II. A Potential Esterase-Sensitive Amide Prodrug

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In an effort to develop esterase-sensitive pro-prodrugs for amines, an amide derivative of 3-(2′-acetoxy-4′,6′-dimethylphenyl)-3,3- dimethylpropionic acid (4-methoxyaniline amide **8**) was synthesized and its stability investigated. This esterified hydroxy amide was found under all conditions to degrade via a two-step process initiated by acetyl ester hydrolysis generating the hydroxy amide intermediate **9a**. The lactonization of this intermediate **9a** in the second step resulted in the formation of 4-methoxyaniline (**10**) and 4,4,5,7-tetramethyl-3,4-dihydrocoumarin (**1a**). The pro-prodrug **8** was observed to possess the following half-lives at 37°C under various conditions: 4030 min in phosphate buffer (50 m*M*, μ = 0.15) fixed to pH 7.4, 11.9 min in the same buffer containing a porcine liver esterase, 53.7 min in plasma, and 475 min in plasma containing diisopropylfluorophosphate. These results suggest that in a biological milieu the ester hydrolysis will occur by the enzymic hydrolysis rather than the chemical hydrolysis and that the enzymic hydrolysis of **8** in plasma is due, in part, to the action of serine-dependent esterases.

KEY WORDS: pro-prodrug; esterified hydroxy amide; esterase; lactonization.

INTRODUCTION

Prodrug formation has been regarded as a useful method of improving the physicochemical properties of a variety of drugs (1,2). While there has been an abundance of work done on ester prodrugs, much less work has been completed on prodrugs of amines (3,4). Esters make excellent prodrugs because at the appropriate pH they are stable *in vitro* while, by virtue of their ability to function as enzyme substrates, they exhibit suitable *in vivo* lability as well. The many attempts to impart these ester characteristics to amines through the use of prodrugs have met with limited success.

This manuscript describes a unique derivatization method for amines which imparts ester qualities (e.g., lability) to amide prodrugs to form pro-prodrugs. A pro-prodrug is a derivative which must undergo two independent reactions in order to regenerate the parent drug (5). In the example shown in Eq. (1), the intermediate prodrug

$$\text{PRO-PRODRUG} \xrightarrow{\text{biological}} \text{PRODRUG} \xrightarrow{\text{chemical}} \text{DRUG} \qquad (1)$$

possesses a high chemical reactivity, allowing it to convert rapidly to the parent drug. However, this chemical reaction cannot occur until the biological conversion of the pro-prodrug (a stable species chemically) to its prodrug form. If the rate of the chemical reaction is sufficiently rapid, the biological reaction will become the rate-determining process in the overall mechanism. A derivative with these qualities has been said to possess an enzymic trigger (5).

We have previously described the synthesis of the highly chemically reactive hydroxy amide **9a** (Fig. 1), which exhibited a half-life of approximately 1 min at near-physiological pH and temperature (6,7). The reactivity of this compound is attributed to the presence of the "trimethyl lock" (methyl groups at positions 3, 3, and 6′). Since hydroxy amide **9a** appeared to possess the requisite chemical reactivity to function as an intermediate prodrug form, it was next of interest to convert **9a** into chemically stable yet enzymatically labile pro-prodrug forms. One potential pro-prodrug form under development in this laboratory is the quinone propionic amide **11**. Other studies from this laboratory (8) have shown that upon reduction of **11** to its hydroquinone form **9b**, the molecule rapidly underwent lactonization, resulting in the release of the amine **10**. Thus, the rate of lactonization was dependent upon the redox potential of the quinone moiety.

2′-Esters of **9a** could constitute another possible pro-prodrug form (e.g., **8**). The ester moiety would serve to prevent lactonization of **9a**; therefore, the rate of lactonization of **9a** would be dependent upon the rate of enzymatic (or chemical) hydrolysis of **8** to **9a** (Fig. 1). Selecting esters that are chemically stable but enzymatically labile would thus impart an "esterase trigger" to this prodrug system for amines. Previous attempts to create derivatives of this type failed because the lactonization reaction proceeded too slowly at physiological pH (9–11). In this article, we report the synthesis and initial hydrolytic stability of compound **8**,

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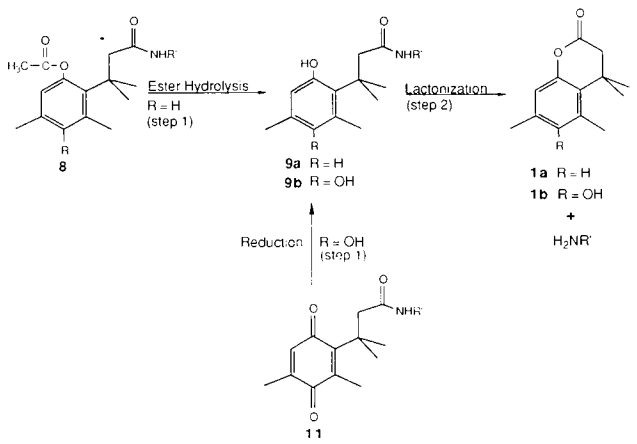


Fig. 1. Proposed conversion mechanisms for esterase and redox-sensitive pro-prodrugs of amines.

the acetyl ester of hydroxy amide **9a**, as a model pro-prodrug form for amines.

MATERIALS AND METHODS

Synthesis

Melting points were determined on a Meltemp apparatus and are reported uncorrected. ^{1}H-NMR spectra were recorded with a Varian FT-80 instrument on $CDCl_3$ solutions with tetramethylsilane employed as an internal standard. UV spectra were obtained on a Shimadzu UV-260 spectrophotometer. Mass spectral analyses were conducted by the University of Kansas Mass Spectral Laboratory and elemental analyses were determined by the University of Kansas Elemental Analysis Laboratory, Lawrence, KS. Column chromatography was performed with silica gel (70–270 mesh) purchased from Aldrich Chemical Co., Milwaukee, WI. Thin-layer chromatography was performed with TLC plates consisting of aluminum sheets precoated with silica gel 60 F_{254} which were purchased from EM Science, Darmstadt, West Germany. Unless otherwise specified all starting materials were obtained commercially from Aldrich Chemical Co.

1-O-t-Butyldimethylsilyl-3-(2'-hydroxy-4',6'-dimethylphenyl)-3,3-dimethylpropanol (**3**). 3-(2'-Hydroxy-4',6'-dimethylphenyl)-3,3-dimethylpropanol (**2**) was prepared by lithium aluminum hydride reduction of 4,4,5,7-tetramethyl-3,4-dihydrocoumarin (**1**) as described previously by our laboratory (7). Compound **2** (5 g, 24 mmol) was dissolved in 50 ml of dimethylformamide along with t-butyldimethylsilyl chloride (4.34 g, 1.2 eq) and imidazole (4.08 g, 2.5 eq). The reaction mixture was stirred at room temperature for 2 hr. The solvent was removed under reduced pressure and the resultant material purified on a silica gel column (30% ethyl acetate:hexane). Recrystallization of the isolated product with ethyl acetate and hexane afforded a white crystalline solid (6.98 g, 90% yield). mp 104–106°C. ^{1}H-NMR ($CDCl_3$) δ 0.03 [6H, s, Si-$(CH_3)_2$], 0.83 (9H, s, t-butyl), 1.50 [6H, s, 3,3-$(CH_3)_2$], 2.04 (2H, t, 1-CH_2) 2.12 (3H, s, 6'-CH_3), 2.39 (3H, s, 4'-CH_3), 3.52 (2H, t, 2-CH_2), 5.40 (1H, s, 2'-OH), 6.35 (2H, dd, ArH); MS (EI), *m/e* 322 (M), 265 (M-t-butyl), 209 (M-TBDMS), 190 (M-O-TBDMS). Anal. Calcd for $C_{19}H_{34}O_2Si$: C, 70.75; H, 10.62. Found: C, 70.44; H, 10.71.

1-O-t-Butyldimethylsilyl-3-(2'-acetoxy-4',6'-dimethylphenyl)-3,3-dimethylpropanol (**4**). A solution of **3** (2.71 g, 8.4 mmol) in dry methylene chloride was mixed with acetic anhydride (1.13 ml, 1.2 eq) and dimethylaminopyridine (0.12 g, 1.2 eq). The reaction mixture was heated to reflux for 4 hr, after which TLC analysis (10% ethyl acetate:hexane) showed the reaction to be complete, with the product appearing at an rf value of 0.57. The reaction mixture was then washed with 100 ml of water, 100 ml of a 5% $NaHCO_3$ solution, and 100 ml of a brine solution. The methylene chloride was removed under vacuum, resulting in a crude liquid product which was distilled to afford a colorless liquid (2.7 g, 88% yield). bp 171–173°C (0.7 mm Hg). ^{1}H-NMR ($CDCl_3$) δ 0.02 (6H, s, Si-$(CH_3)_2$), 0.87 (9H, s, t-butyl), 1.48 [6H, s, 3,3-$(CH_3)_2$], 2.03 (2H, t, 1-CH_2) 2.22 (3H, s, 6'-CH_3), 2.27 (3H, s, acetyl-CH_3), 2.51 (3H, s, 4'-CH_3), 3.48 (2H, t, 2-CH_2), 6.65 (2H, dd, ArH); MS (EI), *m/e* 365 (M + 1), 307 (M-t-butyl), 179 (M-O-TBDMS propanol), 43 (acetyl). Anal. Calcd for $C_{21}H_{36}O_3Si$: C, 69.18; H, 9.95. Found: C, 69.37; H, 9.98.

3-(2'-Acetoxy-4',6'-dimethylphenyl)-3,3-dimethylpropanol (**5**). Compound **4** (2.76 g, 7.6 mmol) was dissolved in 10 ml of tetrahydrofuran along with 30 ml of glacial acetic acid and 10 ml of distilled water. The reaction mixture was stirred for 12 hr at room temperature, after which TLC analysis (30% ethyl acetate:hexane) showed two major products at rf values of 0.37 and 0.67. The compound at rf 0.37 was isolated by separation of the mixture on a silica gel column (20% ethyl acetate:hexane). Evaporation of the solvent resulted in a slightly yellow liquid. This substance was distilled, affording **5** as a colorless liquid (0.67 g, 35% yield). bp 115–117°C (0.4 mm Hg). ^{1}H-NMR ($CDCl_3$) δ 1.50 [6H, s, 3,3-$(CH_3)_2$], 2.21 (3H, s, 6'-CH_3), 2.27 (2H, t, 1-CH_2), 2.30 (3H, s, acetyl-CH_3), 2.47 (3H, s, 4'-CH_3), 3.58 (2H, t, 2-CH_2), 4.24 (1H, t, 1-OH), 6.24, 6.51 (2H, 2s, ArH); MS (EI), *m/e* 207 (M-acetyl), 137 [M-acetyl,$(CH_3)_2$C-CH_2-OH], 43 (acetyl). Anal. Calcd for $C_{15}H_{22}O_3$: C, 71.96; H, 8.86. Found: C, 71.82; H, 8.66.

3-(2'-Acetoxy-4',6'-dimethylphenyl)-3,3-dimethylpropionaldehyde (**6**). Compound **5** (0.49 g, 1.98 mmol) dissolved in 100 ml of methylene chloride was added dropwise to a suspension of pyridinium chlorochromate (0.85 g, 2 eq) also in 100 ml of methylene chloride. This mixture was stirred at room temperature for 4 hr. After this period of time, TLC analysis (30% ethyl acetate:hexane) showed the reaction to be complete, with the product appearing at an rf value of 0.68. The reaction mixture was decanted off a black tar which developed during the course of the reaction. This tar was triturated with 50 ml of methylene chloride (three times) and these portions were combined with the reaction mixture. The combined methylene chloride portions were run through a short silica gel column (100% methylene chloride) in order to remove the most polar impurities. The resulting solution was condensed and further purified on another silica gel column (30% ethyl acetate:hexane). The column solvent was removed from the product under reduced pressure leaving a slightly yellow oil. This substance was distilled, resulting in a colorless liquid (0.43 g, 87% yield). bp 153–156°C (0.3 mm Hg). ^{1}H-NMR ($CDCl_3$) δ 1.57 [6H, s, 3,3-$(CH_3)_2$], 2.18 (3H,

s, 6′-CH_3), 2.25 (3H, s, acetyl-CH_3), 2.49 (3H, s, 4′-CH_3), 2.77 (2H, d, 2-CH_2), 6.53, 6.77 (2H, 2s, ArH), 9.47 (1H, t, ald); MS (EI), *m/e* 248 (M), 205 (M-acetyl), 175 (M-acetyl,ald), 43 (acetyl). Anal. Calcd for $C_{15}H_{20}O_3$: C, 72.55; H, 8.12. Found: C, 72.35; H, 8.33.

3-(2′-Acetoxy-4′,6′-dimethylphenyl)-3,3-dimethylpropionic acid (7). Potassium permanganate (0.58 g, 1 eq) dissolved in a mixture of 15 ml of distilled water and 15 ml of acetone was added dropwise to a solution of compound 6 (0.914 g, 3.68 mmol) dissolved in 60 ml of acetone. The mixture was stirred at room temperature for 13 hr, after which TLC (30% ethyl acetate:hexane) showed the reaction to be complete. The reaction mixture was combined with 100 ml of water and extracted with 75 ml of ether (two times). The ether was then extracted with 75 ml of a 5% $NaHCO_3$ solution (two times). This aqueous solution was washed with 75 ml of ether (two times) and the ether washings were discarded. The aqueous layer was acidified by dropwise addition of a 0.1 *N* HCl solution until the formation of a white precipitate. The cloudy aqueous solution was then extracted with 75 ml of ether (two times) and this ethereal solution was dried with $MgSO_4$. Reduced pressure evaporation of the ether left a solid material which was later recrystallized with acetone and hexane, affording a white crystalline solid (0.96 g, 98% yield). mp 104–107°C. ^{1}H-NMR ($CDCl_3$) δ 1.48 [6H, s, 3.3-(CH_3)$_2$], 2.22 (3H, s, 6′-CH_3), 2.27 (3H, s, acetyl-CH_3), 2.48 (3H, s, 4′-CH_3), 2.95 (2H, s, 2-CH_2), 6.50, 6.73 (2H, 2s, ArH), 7.92 (1H, s, COOH); MS (EI), *m/e* 264 (M), 222 (M-acetyl), 43 (acetyl). Anal. Calcd for $C_{15}H_{20}O_4$: C, 68.16; H, 7.63. Found: C, 67.95; H, 7.69.

4-Methoxyaniline 3-(2′-Acetoxy-4′,6′-dimethylphenyl-3,3-dimethyl-propionic Acid Amide (8). Compound 7 (0.1 g, 0.38 mmol), 4-methoxyaniline (0.07 g, 0.57 mmol), dicyclohexylcarbodiimide (0.117 g, 1.5 eq), and a catalytic amount of dimethylaminopyridine (0.01 g) were dissolved in 40 ml of freshly distilled methylene chloride and the mixture was stirred under N_2 at room temperature for 10 hr. TLC analysis (30% ethyl acetate:hexane) showed that the reaction progressed no further with longer reaction times and the rf value for the product was determined to be 0.44. The methylene chloride was removed by evaporation and the resulting solid was redissolved in ethyl acetate, leaving white needles which were presumably the side product dicyclohexylurea (DCU). This process was repeated two more times until no more DCU could be observed in the reaction mixture. The desired product was isolated from the starting materials with a silica gel column (30% ethyl acetate:hexane). This compound was recrystallized with ethyl acetate and hexane to afford a white crystalline solid (0.02 g, 14% yield). mp 128–130°C. ^{1}H-NMR ($CDCl_3$) δ 1.51 [6 H, s, 3,3-(CH_3)$_2$], 2.25 (3H, s, 6′-CH_3), 2.29 (3H, s, acetyl-CH_3), 2.45 (3H, s, 4′-CH_3), 2.90 (2H, s, 2-CH_2), 3.75 (3H, s, $-OCH_3$), 5.46 (1H, s, NH); 6.75 (6H, m, ArH); MS (EI), *m/e* 369 (M), 326 (M-acetyl), 43 (acetyl). Anal. Calcd for $C_{22}H_{27}NO_4$: C, 71.52; H, 7.37. Found: C, 71.17; H, 7.19.

HPLC Assay

The loss of prodrug 8 as well as the appearance of lactone 1a was monitored with a Shimadzu HPLC system consisting of a SCL-6A system controller, a LC-6A pump, a SPD-6A UV detector, and a C-R6A integrator. The reversed-phase, isocratic assay was done using a C-18 ODS Hypersil column (L = 15 cm, ID = 4.6 mm, particle size = 5 μm) with a mobile phase consisting of 45% acetonitrile in 0.01 *M* phosphate buffer, pH 3. This assay was modified by the addition of a second ODS-Hypersil column (L = 5 cm, ID = 4.6 mm) to act as a guard column during the analysis of plasma samples. The compounds of interest were isolated with retention times ranging from 8 to 20 min. Quantitation of these compounds was achieved using linear calibration curves of peak area versus concentration. The mobile phase consisted of HPLC grade acetonitrile (Fisher Scientific) and water doubly distilled and filtered through a Millipore Milli-Q water purification system. All pH values were determined with a Orion digital Ionalyzer Model 701A and the detection wavelength was 250 nm.

Chemical Kinetics

The rate of chemical conversion of 8 was determined in triplicate (as were the following experiments) in sealed ampoules stored in a Stabil-Therm constant-temperature cabinet set at 37°C. The contents of these ampoules were analyzed by the previously described HPLC assay. The pH was maintained at 7.4 with a 0.05 *M* phosphate buffer and the ionic strength was fixed to 0.15 with NaCl. A 0.01 *M* stock solution of 8 in dimethylsulfoxide was diluted 100 times with the aqueous buffer solution, resulting in a final concentration of 10^{-4} *M* for compound 8 and a final solvent milieu of 1% DMSO in water.

Purified Esterase Kinetics

A purified porcine liver esterase (carboxylic-ester hydrolase; EC 3.1.1.1) was obtained from Sigma as a suspension in a 3.2 *M* $(NH_4)_2SO_4$ solution (pH 8). Five microliters of this suspension (containing 2860 units of enzyme per ml) was diluted to 1 ml with phosphate buffer (0.05 *M*, μ = 0.15, pH 7.4) and then 70 μl of this enzyme solution was added to 920 μl of the same phosphate buffer. Ten microliters of the stock solution of 8 was then combined with this buffer plus enzyme solution and the milieu was maintained at 37 ± 0.5°C by a Precision shaking water bath. Due to the rapid rate of conversion of 8 under these conditions, aliquots were withdrawn from the reaction mixture at various times and immediately frozen in a dry ice/acetone bath, which instantaneously stopped the reaction. These samples were later analyzed by the above-described HPLC assay.

Plasma Kinetics

Plasma was obtained from the Community Blood Center of Greater Kansas City, Kansas City, MO. The stock solution of 8 in dimethylsulfoxide was again diluted 100 times with the plasma and the mixture was maintained at 37 ± 0.5°C by a Precision shaking water bath. No plasma protein precipitation was observed at this concentration of dimethylsulfoxide. The reaction was monitored by withdrawing samples from the reaction mixture at various times and freezing them. These samples were later subjected to the sample preparation method described below and then analyzed with the modified HPLC assay. In a second set of

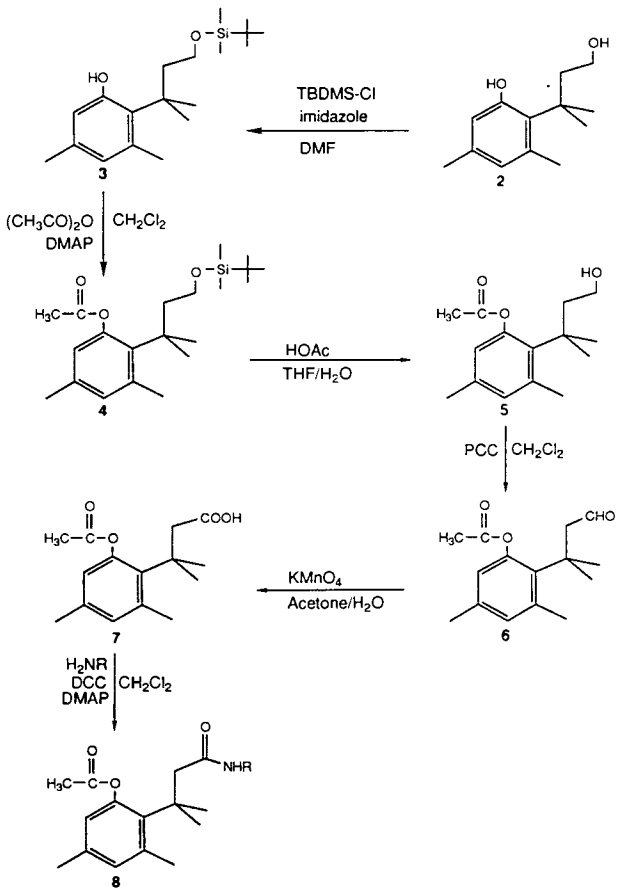
experiments in plasma, the plasma was preincubated with diisopropylfluorophosphate (Aldrich Chemical Co.). Plasma containing a diisopropylfluorophosphate concentration of 2×10^{-4} *M* was allowed to incubate for a period of 10 min before addition of the stock solution of **8**. At this point, the experiment was conducted in the same manner as described for the previous plasma experiment.

Sample Preparation

Plasma samples (0.5 ml) were diluted with an equal volume of ethyl acetate to precipitate the plasma proteins as well as to extract the compounds of interest. After vortex mixing and centrifugation at 800*g* for 1 min, the clear supernatant liquid was directly injected and analyzed. This procedure was found to give consistent and high recovery rates of known amounts of compounds **8** and **1a** spiked into the plasma. The plasma components extracted by this procedure did not interfere with analysis of the compounds of interest.

RESULTS

The pro-prodrug **8** was synthesized according to the reactions described in Scheme I. Because of the tremendous equilibrium favoring the cyclized form, all attempts to hydrolyze 4,4,5,7-tetramethyl-3,4-dihydrocoumarin **1a** and esterify the phenolic hydroxyl group in a single step were unsuccessful. Therefore, it was necessary to open reductively the lactone ring of **1a** to the stable diol **2**. The initial strategy was to acetylate the phenolic hydroxyl of **2** and then reoxidize the side chain alcohol. However, it had previously been demonstrated with a similar compound (compound **2** containing an additional methyl group at position 3′) that acetylation favored the side chain alcohol over the phenolic hydroxyl (12). Under no conditions was selective acetylation of the phenol observed. It was therefore decided to protect the primary alcohol of **2** by selective reaction under mild conditions with t-butyldimethylsilyl chloride, affording **3**, and then in a second step to acetylate the phenolic hydroxyl of **3** with acetic anhydride, yielding **4**. After acetylation, the silyl protecting group of **4** was removed with acetic acid to yield **5**. The side-chain alcohol of **5** was then reoxidized to the aldehyde **6** with pyridinium chlorochromate and **6** was then further oxidized with potassium permanganate to the carboxylic acid **7**. The final step involved coupling of 4-methoxyaniline to **7** with dicyclohexylcarbodiimide and dimethylaminopyridine to yield the target compound **8**. For these preliminary studies, the acetyl ester was chosen because previous work had shown acetyl esters to be reasonable substrates for plasma esterases (10).



Scheme I

The HPLC assay described under Materials and Methods was developed to separate and quantify the pro-prodrug **8**, the intermediate prodrug **9a**, and the products, lactone **1a** and 4-methoxyaniline **10**. Samples of chromatograms generated by this assay are contained in Figs. 2A, 2C, 3A, and 3C.

The kinetics of lactonization of **9a** to **1a** have previously been extensively studied (6,7); therefore, the focus of this work was the evaluation of the stability of the pro-prodrug **8** under various conditions. Our first objective was to determine the rate of chemical conversion of the pro-prodrug **8** to lactone **1a** and 4-methoxyaniline **10** at pH 7.4 and 37°C. The chemical conversion of **8** was measured in a 50 m*M* phosphate buffer with the ionic strength fixed to 0.15. A chromatogram showing the loss of **8** as well as the appearance of **1a** under these conditions is shown in Fig. 2A. Compound **8** was found to disappear in a pseudo first-order fashion with stoichiometric production of **1a** and **10** (Fig. 2B). The intermediate **9a** was not observed under these conditions because the rate of lactonization (determined previously[4] to be 1.06×10^{-2} sec^{-1}) so greatly exceeded the rate of ester hydrolysis. The rate of pro-prodrug disappearance under these conditions is given in Table I, Expt 1.

To determine if the pro-prodrug **8** was susceptible to enzymatic hydrolysis by esterase, the conversion of **8** to **1a** was investigated in the presence of a purified porcine liver esterase. Under these conditions (Table I, Expt 2), the rate of disappearance of **8** was approximately 340 times faster than the rate observed for the chemical conversion. In contrast to the experiments in the previous section, the intermediate **9a** could be observed in the chromatograms (Fig. 2C) for this reaction. The conversion of **8** to **1a** and **10** through the intermediate **9a** was found to obey mass balance, with apparent first-order loss of **8** as shown in Fig. 2D.

It was next necessary to determine if **8** would be con-

[4] This rate constant (see Ref. 7) was determined in a milieu of 20% acetonitrile:water with an ionic strength of 0.3 and a temperature of 30°C, however, we still consider the rate determined under these conditions to be a close approximation of the lactonization rate in this study.

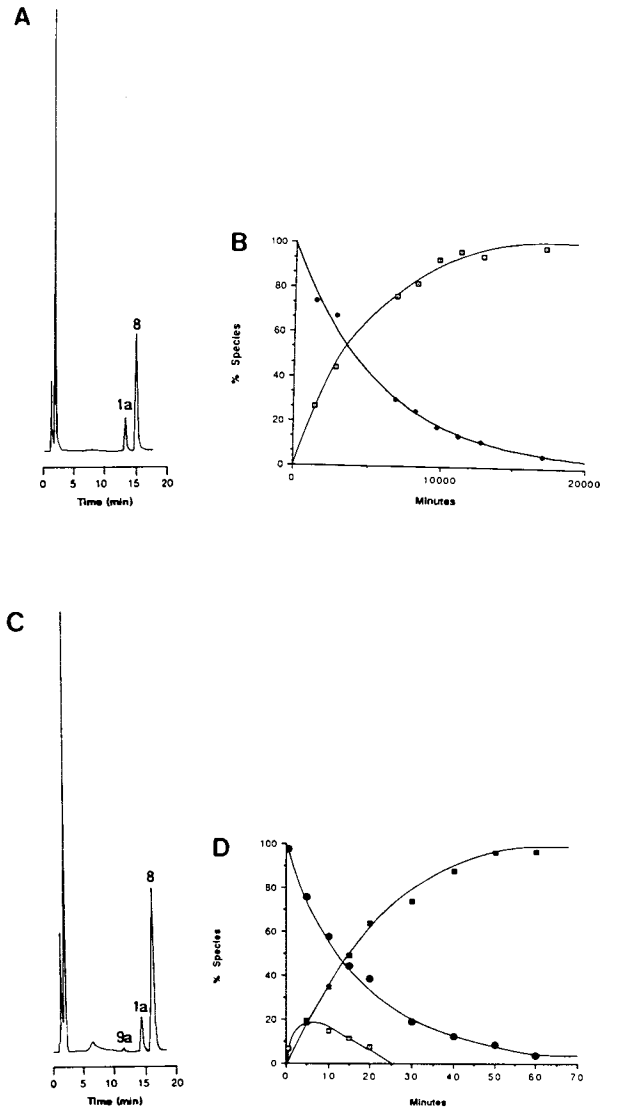


Fig. 2. The stability of 2′-acetyl ester amide 8 in phosphate buffer in the presence and absence of porcine liver esterase. A shows the HPLC chromatogram obtained after incubating 8 in phosphate buffer, pH 7.4 (Expt 1, Table I), for 6840 min and B shows the entire time course for the disappearance of the pro-prodrug 8 (♦) and the appearance of the product **1a** (□). C shows the HPLC chromatogram obtained after incubating 8 in phosphate buffer plus porcine liver esterase, pH 7.4 (Expt 2, Table I), for 20 min, and D shows the entire time course for the disappearance of the pro-prodrug 8 (●), the appearance of the intermediate **9a** (□), and the appearance of the product **1a** (■).

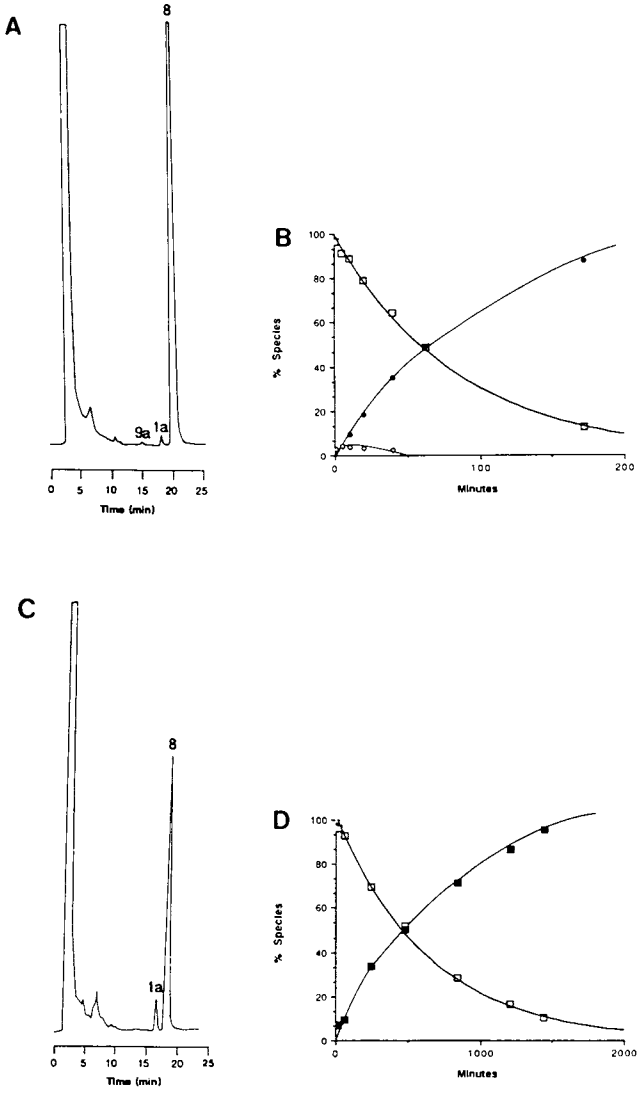


Fig. 3. The stability of the 2′-acetyl ester amide 8 in plasma in the presence and absence of diisopropylfluorophosphate. A shows the HPLC chromatogram obtained after incubating 8 in plasma, pH 7.4 (Expt 3, Table I), for 60 min, and B shows the entire time course for the disappearance of the pro-prodrug 8 (□), the appearance of the intermediate 9a (○), and the appearance of the product **1a** (●). C shows the HPLC chromatogram obtained after incubating 8 in plasma plus DFP, pH 7.4 (Expt 4, Table I), for 240 min, and D shows the entire time course for the disappearance of the pro-prodrug 8 (□) and the appearance of the product **1a** (■).

verted to **1a** and **10** in a biological milieu. The reaction was measured in plasma because it is known to contain an abundance of esterases. Under these conditions, compound 8 disappeared in an apparent first-order fashion (Fig. 3B), resulting in the formation of **1a** and **10** without the appearance of significant amounts of **9a** in the chromatograms (Fig. 3A). The rate of disappearance of 8 under these conditions (Table I, Expt 3) was found to be 75 times faster than the chemical conversion rate but 4.5 times slower than the rate catalyzed by porcine liver esterase.

The final experiment was designed to determine if the conversion of 8 in plasma was due, as expected, to the action of serine-dependent esterases. Serine-dependent esterase activity was inhibited in the plasma by preincubation with diisopropylfluorophosphate—a potent esterase inhibitor (13–15). In this treated plasma, 8 was converted in an apparent first-order fashion to **1a** and **10** without the appearance of **9a** in the chromatograms, a result similar to that observed in the previous plasma experiment. However, this reaction proceeded at a significantly slower rate (Table I, Expt 4) than the rate observed in plasma minus the inhibitor, resulting in an 8.8-fold increase in the half-life of 8. As with all the other

Table I. Conversion Rates for Pro-Prodrug 8 Under Various Conditions

Expt No.	Milieu[a]	k_{obs} (sec^{-1})	$t_{1/2}$ (min)
1	Buffer[b]	$2.87 \pm 0.1 \times 10^{-6}$	4039
2	Buffer[b] + porcine liver esterase	$9.72 \pm 0.83 \times 10^{-6}$	11.9
3	Plasma	$2.15 \pm 0.15 \times 10^{-4}$	53.7
4	Plasma + DFP[c]	$2.43 \pm 0.13 \times 10^{-5}$	475

[a] All experiments were conducted at 37°C.
[b] A 50 m*M* phosphate buffer fixed to pH 7.4, $\mu = 0.15$.
[c] The potent esterase inhibitor diisopropylfluorophosphate.

experiments, there was stoichiometric production of the expected products (Fig. 3D).

DISCUSSION

Acylation has been an attractive potential derivatization reaction for amines because of the ease and versatility of the reaction as well as the tremendous potential for alteration of the physicochemical properties of the amines. In practice, however, amide prodrug forms have not been successful because of their high stability *in vivo*. In fact, only those amide prodrugs which can function as substrates for peptidases are likely to be hydrolyzed to the parent amine in a biological system. Direct chemical hydrolysis of the amide linkage, another possible reaction *in vivo*, requires extreme pH values, high temperatures, and long reaction times and would, therefore, seem to be a physiological impossibility.

One method of increasing the chemical reactivity of an amide has been the incorporation of a neighboring functional group into the molecule to facilitate amide hydrolysis. The most commonly employed functional group has been the hydroxyl group and there are several examples of hydroxy amide lactonization reactions providing much faster rates of amide hydrolysis than the analogous bimolecular reactions (16–18). Further structural modifications of hydroxy amides through the addition of steric bulk (19) or intramolecular general base catalysts (20) have resulted in compounds with even faster rates of lactonization. In our search for novel amine prodrug forms, we were attracted to the potential of esterified hydroxy amides as pro-prodrugs of amines (Fig. 1). A critical requirement for the success of these derivatives is a hydroxy amide of high chemical reactivity. However, we were not aware of any hydroxy amides which would exhibit the necessary reactivity in a biological system. For this reason and others described below, it became apparent that it would be necessary to develop hydroxy amides of greater chemical reactivity.

Previous attempts to develop esterified hydroxy amides as amine pro-prodrugs have failed because the lactonization step was too slow under physiological conditions for the intermediate prodrug to revert to the parent drug within a reasonable period of time. The first attempt at this type of derivative was made by Cain, who synthesized benzoyl esters of several 2-(hydroxymethyl)-benzamides (9). These compounds were found to form the desired products of phthalide lactone and free amines only at the extreme pH values of 2 and 12, making these derivatives unsuitable for a biological system. A later study by Nielsen and Bundgaard employed the same hydroxy amide core but utilized acetyl esters (10). Although the esters were hydrolyzed in plasma, the resulting 2-(hydroxymethyl)-benzamides were found to lactonize too slowly ($t_{1/2}$ of approximately 2700 min) at physiological pH to prove useful as amine prodrug forms. A more recent study in this area investigated the lactonization of a set of structurally diverse hydroxy amides which included some of the most reactive hydroxy amides ever synthesized (11). Exo,exo-2,3-dichloro-endo-3-(hydroxymethyl)bicyclo-[2.2.2]oct-5-ene-endo-2-carboxamide (half-life of 8.62 min at pH 10 and 37°C) and 2-(diphenylhydroxymethyl)-benzamide (half-life of 2.38 min at pH 10 and 37°C), two very reactive compounds, were rejected as prodrugs by the authors because they were considered to be either too toxic or too unreactive to enzymatic hydrolysis. Several 4-hydroxybutenamides containing various alkyl substitutions at the 4 position were ultimately selected as the best prodrug candidates. However, these hydroxy amides exhibited a half-life of approximately 460 min at pH 7.4 and 37°C, which would still seem to be too slow for a pro-prodrug system.

There are many examples of the tremendous rate enhancement provided for intramolecular reactions [i.e., lactonization (21,22), nucleophilic displacement (12), and conjugate addition of carboxylate (23), hydroxyl (24), and aldehyde hemiacetal (25)] of various hydrocinnamic acid analogues by the methyl groups constituting the "trimethyl lock." We speculated that amides of the most reactive hydroxy acids from these studies would also exhibit fast lactonization rates. This was verified in our earlier hydroxy amide studies (6,7), in which **9a** was found to disappear with a half-life of 65 sec at pH 7.5 and 30°C. Possessing a very reactive hydroxy amide, it became our intention to esterify the hydroxyl group, thus producing an amine pro-prodrug.

The experiments in this study have demonstrated that compound **8** is converted to lactone **1a** and amine **10** through the intermediate **9a** as described in Fig. 1. In every instance, the conversion products of **8** were found to be **1a** and **10**. The loss of **8** was also found to exhibit either pseudo or apparent first-order kinetics to completion under all conditions. Inspection of the chromatograms provides evidence that it is hydrolysis of the acetyl ester rather than direct amide hydrolysis which results in amine release. The HPLC assay also possessed the ability to monitor the reaction mixture for the appearance of the synthetic intermediate **7**, which is the direct amide hydrolysis product (chemically or enzymatically) of the model pro-prodrug **8**. Under no conditions was compound **7** observed in the chromatograms, providing strong evidence against a direct hydrolysis mechanism. On the other hand, in experiment 2 where the ester hydrolysis is rapid, the proposed intermediate **9a** was observed. An important observation was the absence of compound **7** in the plasma reactions, indicating that enzymatic cleavage of the amide linkage either does not occur for this pro-prodrug or is insignificant in comparison to that of the esterase reaction. In the absence of direct amide hydrolysis of the pro-prodrug, amine release from the pro-prodrug should occur only as a result of ester hydrolysis followed by lactonization. This type of release selectivity is a necessary characteristic of any successful delivery system.

The results given in Table I elucidate several points concerning this pro-prodrug. First, the results from Experiments 1 and 2 indicate that under identical conditions of pH, temperature, and ionic strength (all simulating the physiological state), the pro-prodrug **8** was over 300 times more stable in the absence of an esterase. This result indicates that in a biological system containing esterase, one could expect amine release to be dictated by the enzymic reaction rather than the chemical reaction.

Having established that **8** is converted to **1a** and **10** by a purified esterase, it was next necessary to determine if the pro-prodrug would be converted in a biological milieu. In experiment 3, **8** was found to be converted in plasma to the expected products of **1a** and **10**. This reaction in plasma, although not as rapid as in experiment 2, was approximately 75 times faster than the *in vitro* chemical reaction, implicating some type of enzymic catalysis. It was next necessary to determine which enzymes were responsible for the conversion. The reaction was found to be approximately 8.8 times slower in the presence of the potent esterase inhibitor diisopropylfluorophosphate, indicating that at least part of the enzymatic conversion of the compound was due to the action of esterases.

An amine pro-prodrug of this type could have several advantages. Because the amine release is triggered by ester hydrolysis, one has essentially created an amide pro-prodrug with the *in vivo* lability of an ester. As stated previously, esters make excellent prodrugs because the compounds generally exhibit good *in vitro* stability along with good *in vivo* reconversion. Pro-prodrugs of this sort could impart these desirable qualities to amine drugs.

There are several synthetic handles in this pro-moiety with which to control amine release rates. The presence or absence of the "trimethyl lock" methyl groups has been found to produce a four order of magnitude range in lactonization rates (7). Structural modification of the ester acyl moiety should also affect amine release rates. It is well established that many esterases possess strict substrate specificities, and in cases where an enzyme is less specific there can be a wide range in velocities for the hydrolytic reaction with different substrates (26).

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