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Research Article

Amine Prodrugs Which Utilize Hydroxy Amide Lactonization. I. A Potential Redox-Sensitive Amide Prodrug

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Several amides of 3-(3′,6′-dioxo-2′,4′-dimethylcyclohexa-1′,4′-diene)-3,3-dimethylpropionic acid (**2**) have been synthesized and tested as model redox-sensitive pro-prodrugs of amines. The reduction of these model pro-prodrugs generated hydroxy amide intermediates **4a–4h**, the lactonization of which resulted in amine release. The rates of lactonization of **4a–4h** were investigated at pH 7.4 and 37°C. The half-lives for appearance of the product lactone **1a** from these intermediates were found to range from 1.4 to 3.4 min. With such rapid lactonization rates, it is believed that reduction will be the rate-limiting step in the two-step conversion of the pro-prodrug to the amine.

KEY WORDS: amine pro-prodrugs; redox potential; quinone; hydroxy amide; lactonization.

INTRODUCTION

Prodrug formation has been regarded as a useful means 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 have dominated prodrug research because they possess ideal characteristics for bioderivatives. They exhibit reasonable chemical stability *in vitro,* which allows them to be prepared as formulations with adequate shelf lives; yet by virtue of their ability to function as enzyme substrates, they exhibit a suitable *in vivo* lability as well. Many attempts to impart these characteristics to amines through the use of prodrugs have met with limited success.

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 amine drugs. However, simple amides are generally too stable *in vivo* to be useful prodrug forms for amines. Therefore, many investigators have attempted to prepare amides which utilize an intramolecular reaction (e.g., lactonization) to increase the lability of the amide bond, thus facilitating release of the amine (5–7). These approaches, however, have resulted in amine release rates which are still considered to be too slow at physiological pH.

Recently, our laboratory described the synthesis of the highly chemically reactive hydroxy amide **7** (Fig. 1), which lactonized with a half-life of approximately 1 min at near-physiological pH and temperature (8,9). The reactivity of this compound is attributed to the presence of the "trimethyl lock" (methyl groups at positions 3, 3, and 6′). In order to transform **7** into a useful prodrug system for amines, methods had to be developed for converting this hydroxy amide into chemically stable yet enzymatically labile pro-prodrug forms. A pro-prodrug is a derivative which must undergo two independent reactions in order to regenerate the parent drug (10). In the example shown in Eq. (1), the intermediate prodrug possesses a high chemical reactivity, allowing it to rapidly convert to the parent drug (the characteristics exhibited in **7**). However, this chemical reaction cannot occur until the pro-prodrug (a chemically stable species) is converted to its prodrug form via a biological reaction (e.g., enzymatic). If the rate of the chemical reaction is sufficiently rapid, the biological reaction will become the rate-determining process in the overall drug regeneration mechanism. A derivative with these qualities has been said to possess an enzymic trigger (10).

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

One potential pro-prodrug form of **7** under development in our laboratory involves esterification of the 2′-hydroxyl group (e.g., **6**, Fig. 1). We have recently shown that **6** undergoes rate-limiting hydrolysis of the 2′-acetyl ester, generating the hydroxy amide intermediate **7**, which then rapidly lactonizes to yield **1b** and amine **5** (11). Therefore, the rate of lactonization of **7** is dependent upon the rate of hydrolysis of the 2′-acetyl ester. Thus, we have developed an amide derivative that has the enzymatic (and chemical) lability of an ester.

In this report, we describe another potential method for controlling the rate of hydroxy amide lactonization which involves a "redox trigger." This pro-prodrug system consists of amides **3a–3h** (Fig. 1) of 3-(3′,6′-dioxo-2′,4′-

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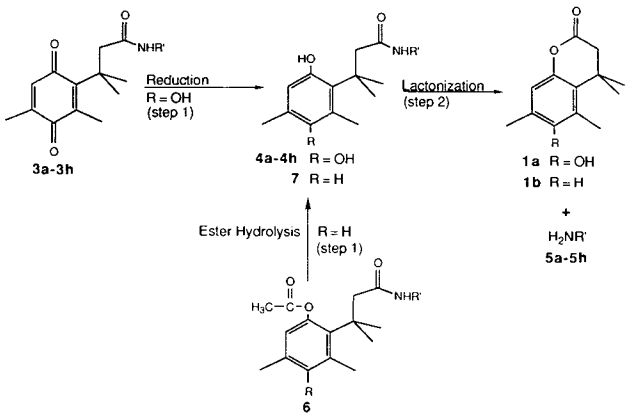


Fig. 1. Proposed conversion mechanisms for redox-sensitive **3a–3h** and esterase-sensitive **6** model pro-prodrugs of amines.

dimethyl-1',4'-cyclohexadiene)-3,3-dimethylpropionic acid (**2**). The reduction of the quinone portion of **3a–3h** generates the intermediate hydroquinones **4a–4h** (essentially hydroxy amide analogues of 7), which rapidly lactonize, yielding **1a** and the amines **5a–5h** (Fig. 1). The result is a derivative form in which quinone reduction controls the rate of hydrolysis of an amide prodrug.

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. All starting materials, unless otherwise specified, were obtained commercially from Aldrich Chemical Co.

*6-Hydroxy-4,4,5,7-tetramethyl-3,4-dihydrocoumarin (**1a**).* 2,6-Dimethyl-1,4-benzoquinone (5.66 g, 42 mmol) was dissolved in 200 ml of ether and washed (two times) with 200 ml of aqueous sodium hydrosulfite (14.5 g, 2 eq). The ethereal layer, initially a bright yellow solution, changed first to a dark brown solution upon shaking with the sodium hydrosulfite and eventually to a light yellow solution signaling the end of the reaction. The washings were conducted over a period of 15 min. The ether layer was then washed two times with 200 ml of brine solution and dried with $MgSO_4$. Removal of the desiccant by filtration and evaporation of the ether under reduced pressure afforded the hydroquinone—1,4-dihydroxy-2,6-dimethylbenzene as a white solid (5.15 g, 89% yield). mp 152.5–154°C [lit. (12) mp].

The synthesis of methyl 3,3-dimethylpropionate has previously been reported from this laboratory (9). 1,4-Dihydroxy-2,6-dimethylbenzene (6.23 g, 45 mmol) and methyl 3,3-dimethylpropionate (10.29 g, 2 eq) were dissolved in 250 ml of toluene along with 2.5 ml of concentrated sulfuric acid, and the mixture was heated at reflux for 2 hr. The reaction milieu was then washed with 200 ml of water, 200 ml of 5% $NaHCO_3$ solution (two times), and finally, 200 ml of brine solution. The toluene layer was then dried with $MgSO_4$. The desiccant was removed by filtration and the toluene was removed under reduced pressure. Recrystallization of the resulting solid with toluene afforded a white crystalline solid (5.9 g, 60% yield). mp 140–142°C. ^{1}H-NMR ($CDCl_3$) δ1.45 (6H, s, 4,4-$(CH_3)_2$), 2.20 (3H, s, 5-(CH_3)), 2.30 (3H, s, 7-(CH_3)), 2.50 (2H, s, CH_2), 4.55 (1H, s, 6-OH), 6.65 (1H, s, Ar-H); MS (EI), *m/e* 220 (M), 205 (M-CH_3). *Anal.* Calcd for $C_{13}H_{16}O_3$: C, 70.88; H, 7.32. Found: C, 70.60; H, 7.39.

3-(3',6'-Dioxo-2',4'-dimethylcyclohexa-1',4'-diene)-3,3-dimethylpropionic Acid (2). A solution of *N*-bromosuccinimide (0.166 g, 0.94 mmol) dissolved in 30 ml of water was added dropwise to a solution of compound **1a** (0.21g, 0.94 mmol) dissolved in 30 ml of acetonitrile. The mixture was stirred for 45 min during which time the solution changed from colorless to yellow, indicating that the quinone was being produced. The reaction milieu was then extracted with 50 ml of ether and the ethereal solution was subsequently extracted with 50 ml of a 5% $NaHCO_3$ solution (two times). The $NaHCO_3$ solution was then washed with 50 ml of ether (two times) to remove any nonionized compounds. This aqueous layer was acidified by dropwise addition of 0.1 *N* HCl until the appearance of a precipitate. The product was extracted from the aqueous solution with 100 ml of ether (two times). The ether was then dried with $MgSO_4$. After filtration of the desiccant, the ether was removed by reduced pressure evaporation, leaving a yellow oil. Recrystallization of this compound with chloroform and hexane afforded a yellow crystalline solid (0.216 g, 98% yield). mp 100–102°C. ^{1}H-NMR ($CDCl_3$) δ1.40 (6H, s, 3,3-$(CH_3)_2$), 1.90 (3H, s, 4'-(CH_3)), 2.10 (3H, s, 2'-(CH_3)), 2.95 (2H, s, CH_2), 6.25 (1H, s, Ar-H); MS (EI), *m/e* 237 (M + 1), 219 (M-OH), 203 (M-OOH), 191 (M-COOH), 176 (M-CH_2COOH). *Anal.* Calcd for $C_{13}H_{16}O_4$: C, 66.08; H, 6.83. Found: C, 66.42; H, 6.99.

*4-Methoxyaniline 3-(3',6'-dioxo-2',4'-dimethyl cyclohexa-1',4'-diene)-3,3-dimethylpropionic Acid Amide (**3a**).* Compound **2** (0.2 g, 0.85 mmol) was dissolved in 30 ml of freshly distilled methylene chloride along with 4-methoxyaniline (0.16 g, 1.5 eq), dicyclohexylcarbodiimide (0.28 g, 1.5 eq) and 4-dimethylaminopyridine (0.01 g). The reaction was stirred for 4 hr under N_2. TLC (30% ethyl acetate:hexane) of the reaction mixture showed it to be unchanged with longer reaction times. The methylene chloride was removed by reduced pressure evaporation, resulting in a yellow solid. The yellow compound—the presumed product—was then dissolved in 10 ml of ethyl acetate, leaving some insoluble white needles—presumably the undesired side product dicyclohexylurea (DCU) which were then removed by filtration. Evaporation of the ethyl acetate again left the product along with a smaller amount of DCU, which was filtered off. In this way, the slightly soluble DCU was

removed from the product. Repetition of this procedure three times removed all visible amounts of the DCU from the product mixture. This mixture was then purified on a silica gel column (30% ethyl acetate:hexane) with the separation resulting in three major portions of material [rf values 0.9, 0.35, and 0.25 on TLC analysis (30% ethyl acetate:hexane)]. The isolation of the middle portion (rf = 0.35) and evaporation of the mobile phase resulted in a yellow solid, compound **3a**. Recrystallization of this material with ethyl acetate and hexane afforded a yellow crystalline solid (0.075 g, 26% yield). mp 147–148°C. ^{1}-H-NMR ($CDCl_3$) δ1.50 (6H, s, 3,3-$(CH_3)_2$), 1.93 (3H, s, 4'-(CH_3)), 2.15 (3H, s, 2'-(CH_3)), 2.95 (2H, s, CH_2), 3.70 (3H, s, OCH_3), 6.43 (1H, s, Ar-H), 6.67–7.23 (4H, dd, Ar-H); MS (EI), *m/e* 341 (M), 219, 191, 177, 122 (amine). *Anal.* Calcd for $C_{20}H_{23}NO_4$: C, 70.36; H, 6.79; N, 4.10. Found: C, 70.53; H, 6.86; N, 4.23.

Aniline 3-(3',6'-dioxo-2',4'-dimethylcyclohexa-1',4'-diene)-3,3-dimethylpropionic Acid Amide (3b). Compound **2** (0.2 g, 0.85 mmol), aniline (0.12 g, 1.5 eq), dicyclohexylcarbodiimide (0.28 g, 1.5 eq), and 4-dimethylaminopyridine (0.01 g) were treated according to the procedure for the formation of compound **3a**. The reaction afforded a yellow crystalline solid (0.042 g, 16% yield). mp 136–137°C. ^{1}H-NMR ($CDCl_3$) δ1.47 (6H, s, 3,3-$(CH_3)_2$), 1.91 (3H, s, 4'-(CH_3)), 2.13 (3H, s, 2'-(CH_3)), 2.96 (2H, s, CH_2), 6.39 (1H, s, Ar-H), 7.25 (5H, s, Ar-H); MS (EI), *m/e* 311 (M), 219, 191, 177, 93 (amine). *Anal.* Calcd for $C_{19}H_{21}NO_3$: C, 73.28; H, 6.80; N, 4.50. Found: C, 73.16; H, 6.88; N, 4.72.

4-Nitroaniline 3-(3',6'-dioxo-2',4'-dimethyl cyclohexa-1',4'-diene)-3,3-dimethylpropionic Acid Amide (3c). Compound **2** (0.2 g, 0.85 mmol), 4-nitroaniline (0.18 g, 1.5 eq), dicyclohexylcarbodiimide (0.28 g, 1.5 eq), and 4-dimethylaminopyridine (0.01 g) were treated according to the procedure for the formation of compound **3a**. The reaction afforded a yellow crystalline solid (0.027 g, 9% yield). mp 124–126°C. ^{1}H-NMR ($CDCl_3$) δ1.50 (6H, s, 3,3-$(CH_3)_2$), 1.92 (3H, s, 4'-(CH_3)), 2.14 (3H, s, 2'-(CH_3)), 2.93 (2H, s, CH_2), 5.47 (1H, s, NH), 6.43 (1H, s, Ar-H), 6.54–7.08 (4H, dd, Ar-H); MS (EI), *m/e* 356 (M), 219, 191, 176, 137 (amine). *Anal.* Calcd for $C_{19}H_{20}N_2O_5$: C, 64.03; H, 5.66; N, 7.86. Found: C, 64.31; H, 5.81; N, 7.98.

Benzylamine 3-(3',6'-dioxo-2',4'-dimethyl cyclohexa-1',4'-diene)-3,3-dimethylpropionic Acid Amide (3d). Compound **2** (0.2 g, 0.85 mmol), benzylamine (0.14 g, 1.5 eq), dicyclohexylcarbodiimide (0.28 g, 1.5 eq), and 4-dimethylaminopyridine (0.01 g) were treated according to the procedure for the formation of compound **3a**. The reaction afforded a yellow crystalline solid (0.047 g, 17% yield). mp 149–150°C. ^{1}H-NMR ($CDCl_3$) δ1.43 (6H, s, 3,3-$(CH_3)_2$), 1.88 (3H, s, 4'-(CH_3)), 2.08 (3H, s, 2'-(CH_3)), 2.77 (2H, s, CH_2), 4.19 (1H, s, benzylic), 4.26 (1H, s, benzylic), 5.50 (1H, s, NH), 6.33 (1H, s, Ar-H), 7.14 (5H, s, Ar-H); MS (EI), *m/e* 325 (M), 219, 191, 176, 105 (amine). *Anal.* Calcd for $C_{20}H_{23}NO_3$: C, 73.82; H, 7.12; N, 4.30. Found: C, 73.66; H, 7.21; N, 4.08.

2-Phenylethylamine 3-(3',6'-dioxo-2',4'-dimethyl cyclohexa-1',4'-diene)-3,3-dimethylpropionic Acid Amide (3e). Compound **2** (0.2 g, 0.85 mmol), 2-phenylethylamine (0.155 g, 1.5 eq), dicyclohexylcarbodiimide (0.28 g, 1.5 eq), and 4-dimethylaminopyridine (0.01 g) were treated according to the procedure for the formation of compound **3a**. The reaction afforded a yellow crystalline solid (0.06 g, 21% yield). mp 101–103°C. ^{1}H-NMR ($CDCl_3$) δ1.43 (6H, s, 3,3-$(CH_3)_2$), 1.98 (3H, s, 4'-(CH_3)), 2.12 (3H, s, 2'-(CH_3)), 2.70 (2H, t, benzylic), 2.75 (2H, s, CH_2), 3.40 (2H, q, $NHCH_2$), 5.33 (1H, s, NH), 6.45 (1H, s, Ar-H), 7.21 (5H, s, Ar-H); MS (EI), *m/e* 339 (M), 324 (M-CH_3), 219, 191, 177, 120 (amine). *Anal.* Calcd for $C_{21}H_{25}NO_3$:C, 74.31; H, 7.42; N, 4.13. Found: C, 74.56; H, 7.59; N, 4.35.

N-Methylaniline 3-(3',6'-dioxo-2',4'-dimethyl cyclohexa-1',4'-diene)-3,3-dimethylpropionic Acid Amide (3f). Compound **2** (0.2 g, 0.85 mmol), *N*-methylaniline (0.14 g, 1.5 eq), dicyclohexylcarbodiimide (0.28 g, 1.5 eq), and 4-dimethylaminopyridine (0.01 g) were treated according to the procedure for the formation of compound **3a**. The reaction afforded a yellow crystalline solid (0.041 g, 15% yield). mp 135–137°C. ^{1}H-NMR ($CDCl_3$) δ1.48 (6H, s, 3,3-$(CH_3)_2$), 1.95 (3H, s, 4'-(CH_3)), 2.21 (3H, s, 2'-(CH_3)), 2.95 (2H, s, CH_2), 3.70 (3H, s, NCH_3), 6.40 (1H, s, Ar-H), 7.13 (5H, s, Ar-H); MS (EI), *m/e* 325 (M), 219, 191, 176, 106 (amine). *Anal.* Calcd for $C_{20}H_{23}NO_3$: C, 73.82; H, 7.12; N, 4.30. Found: C, 74.09; H, 7.15; N, 4.47.

n-Butylamine 3-(3'6'-dioxo-2',4'-dimethyl cyclohexa-1',4'-diene)-3,3-dimethylpropionic Acid Amide (3g). Compound **2** (0.2 g, 0.85 mmol), *n*-butylamine (0.094 g, 1.5 eq), dicyclohexylcarbodiimide (0.28 g, 1.5 eq), and 4-dimethylaminopyridine (0.01 g) were treated according to the procedure for the formation of compound **3a**. The reaction afforded a yellow crystalline solid (0.044 g, 18% yield). mp 122–123°C. ^{1}H-NMR ($CDCl_3$) δ0.84 (3H, m, CH_3), 1.30 (4H, m, CH_2CH_2), 1.45 (6H, s, 3,3-$(CH)_3)_2$), 1.93 (3H, s, 4'-(CH_3)), 2.12 (3H, s, 2'-(CH_3)), 2.77 (2H, s, CH_2), 3.05 (2H, m, $NHCH_2$), 5.20 (1H, s, NH), 6.44 (1H, s, Ar-H); MS (EI), *m/e* 291 (M), 219, 191, 176, 71 (amine). *Anal.* Calcd for $C_{17}H_{25}NO_3$: C, 70.07; H, 8.65; N, 4.81. Found: C, 70.34; H, 8.80; N, 5.03.

Leucine β-Naphthylamide 3-(3'6'-dioxo-2',4'-dimethyl cyclohexa-1',4'-diene)-3,3-dimethylpropionic Acid Amide (3h). Compound **2** (0.2 g, 0.85 mmol), leucine β-naphthylamide (0.33 g, 1.5 eq), dicyclohexylcarbodiimide (0.28 g, 1.5 eq), and 4-dimethylaminopyridine (0.01 g) were treated according to the procedure for the formation of compound **3a**. The reaction afforded a yellow crystalline solid (0.052 g, 13% yield). mp 187–188°C. ^{1}H-NMR ($CDCl_3$) δ0.93 (8H, m, isoprop.), 1.40 (6H, d, 3,3-$(CH_3)_2$), 1.75 (3H, d, 4'-(CH_3)), 2.07 (3H, s, 2'-(CH_3)), 2.85 (2H, s, 2-CH_2), 4.60 (1H, q, α-c), 5.83 (1H, d, NH), 7.25–7.75 (4H, dq, Ar-6,9-H), 7.58 (1H, s, Ar-4-H), 8.02 (1H, d, Ar-3-H), 8.47 (1H, s, Ar-1-H); MS (EI), *m/e* 474 (M), 332 (M-$NHC_{10}H_7$), 304 (M-$CONHC_{10}H_7$), 220, 143 ($NHC_{10}H_7$). *Anal.* Calcd for $C_{29}H_{34}N_2O_4$: C, 73.39; H, 7.22; N, 5.90. Found: C, 73.34; H, 7.40; N, 5.73.

Formation and Reduction of Prodrug Stock Solutions

The quinone amides **3a–3h** were kept in the solid state in amber bottles until needed for kinetics. The compounds were then dissolved in an appropriate volume of acetonitrile to result in a 10^{-3} *M* stock solution. These solutions were kept in amber bottles in the dark and at a temperature of approximately 4°C to increase their stability. Prior to a kinetic experiment, 200 μl of the stock solution was combined with 20 μl of a $NaBH_4$ solution to effect the reduction. The

$NaBH_4$ was dissolved in an appropriate volume of the phosphate buffer described below to result in a 0.1 *M* solution. The combination of these two solutions resulted in complete and rapid reduction of the quinone to its hydroquinone form as demonstrated by HPLC with the assay described below. The combination resulted in a 10-fold excess of the reducing agent, which ensured that the relatively facile reoxidation of the hydroquinone would not occur during the course of the cyclization kinetic experiments.

Kinetic Measurements

The previously reduced stock solutions were diluted 10 times with aqueous buffer, resulting in a final prodrug concentration of 1×10^{-4} *M* and a final solvent milieu of 10% acetonitrile in water. All cyclization kinetic experiments were conducted in 0.05 *M* phosphate buffer, pH 7.4 and μ = 0.15. The phosphate buffer solutions were prepared with commercial reagent-grade materials and water doubly distilled and filtered by a Milli Q Water System. Measurements of pH were made with an Orion Model 701 A digital Ionalyzer. The reactions were conducted at a temperature of 37 ± 0.5°C maintained by a Precision shaking water bath. The ionic strength was fixed to 0.15 with NaCl. Aliquots were removed from the reaction mixture at various times and frozen in a dry ice/acetone bath, stopping the reaction instantaneously. These samples were later analyzed by the HPLC assay described below.

HPLC Assay Conditions

The appearance of lactone **1a** in the samples was monitored through the use of a Shimadzu HPLC system consisting of a SCL-6A system controller, LC-6A pump, SPD-6A UV detector, and C-R6A integrator. The lactone **1a** was quantified by measuring peak areas of unknown quantities in relation to those of standards chromatographed under the same conditions. A detection wavelength of 250 nm was used. The assay was also capable of identifying the model pro-prodrugs and their respective hydroquinone forms. The isocratic assay was conducted on an ODS Hypersil C-18 column with a mobile phase consisting of 50% acetonitrile (HPLC grade obtained from Fisher Scientific) in pH 3, 0.01 *M* phosphate buffer, which resulted in retention times of less than 12 min for all compounds.

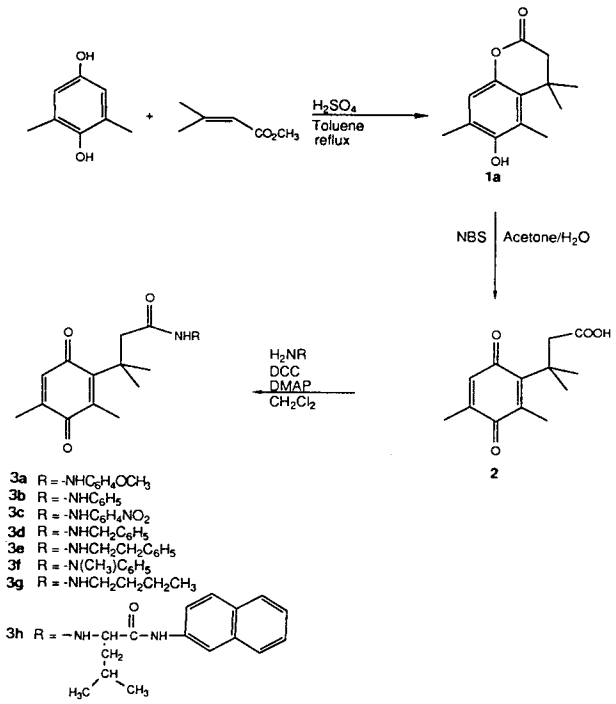
Cyclic Voltammetry

Stock solutions of **3g**, **2**, and **1a** were prepared at a concentration of 1×10^{-3} *M* in acetonitrile. These stock solutions were combined either 1:10 or 1:1 with a pH 7.4 sodium phosphate buffer [0.05 *M*. μ = 0.15 (fixed with NaCl)]. The experiments were run at room temperature. All equipment was obtained from Bioanalytical Systems, West Lafayette, IN. The potentiostat was a three-electrode CV-24 voltammograph (Model NY-009) which included a glassy carbon working electrode (MF-2012), a Ag/AgCl reference electrode (MF-2020), and a Pt auxiliary electrode (MF-1052). An *X–Y* recorder (model MF-8050) was used to record the CV scans.

RESULTS

The model redox-sensitive prodrugs were synthesized by the reactions outlined in Scheme I. 2,6-Dimethylbenzoquinone, which was obtained commercially, was reduced with sodium hydrosulfite to its hydroquinone form—1,4-dihydroxy-2,6-dimethylbenzene. This hydroquinone was then condensed under acidic conditions at reflux with methyl 3,3-dimethylpropionate to form 6-hydroxy-4,4,5,7-tetramethyl-3,4-dihydrocoumarin (**1a**). The lactone **1a** was then oxidized with *N*-bromosuccinimide in aqueous acetonitrile to yield the benzoquinone propionic acid **2**. In the final step, the appropriate amines were reacted with **2** in the presence of dicyclohexylcarbodiimide and dimethylaminopyridine to yield the amides **3a–3h**. This reaction was employed because of our previous lack of success in forming the acid chloride of similar side-chain propionic acids containing the 3,3-geminal dimethyl groups (9). All quinonoid compounds were expected to be light sensitive and were, therefore, kept in amber bottles and in the dark.

Having generated the model pro-prodrugs **3a–3h**, it was necessary to determine whether or not the compounds, upon reduction, would rapidly cyclize to lactone **1a** and amines **5a–5h** (Fig. 1). To accomplish this objective, an HPLC assay was developed which allowed for the separation and quantification of the pro-prodrugs **3a–3h**, their hydroquinone intermediates **4a–4h**, and the product lactone **1a**. A representative chromatogram showing the separation of **3g**, **4g**, and **1a** is shown in Fig. 2. This HPLC method was primarily used to determine the rates of lactonization of the hydroquinone intermediates **4a–4h**. It was also used to verify that the $NaBH_4$ reduction of **3a–3h** was complete. In all cases, injection of the pro-prodrug stock solutions onto the HPLC system immediately after addition of the $NaBH_4$ showed that the pro-prodrugs were completely converted to compounds having shorter retention times. It was believed that these



Scheme I

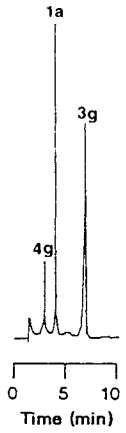


Fig. 2. Representative chromatogram showing the separation of the model pro-prodrug **3g** from its reduced form **4g** and from its product lactone **1a**.

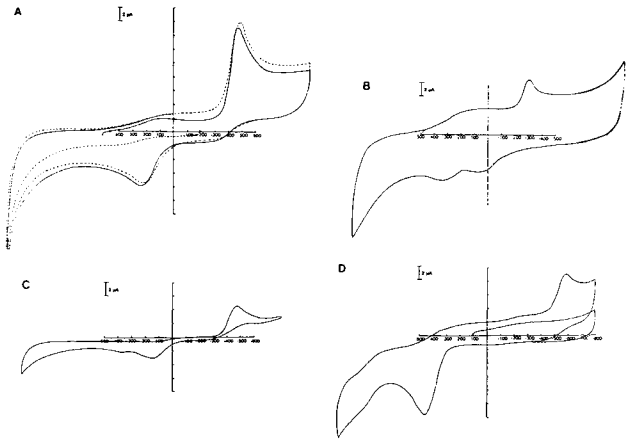


Fig. 3. Cyclic voltammograms (CV) of **3g**, **2**, and **1a** at room temperature in acetonitrile and phosphate buffer (0.05 *M*, μ = 0.15, pH 7.4). A contains the CV scan of **3g** in 50% acetonitrile/buffer scanning at a rate of 0.2 V/sec. The solid trace corresponds to scanning in the reductive direction beginning at +0.5V and the dashed trace corresponds to scanning in the oxidative direction beginning at −0.2V. B contains the CV scan of **3g** in 10% acetonitrile/buffer beginning at 0.5 V and scanning in the reductive direction at a rate of 0.2 V/sec. C contains the CV scan of **2** in 50% acetonitrile/buffer beginning at 0.1 V and scanning in the reductive direction at a rate of 0.01 V/sec. D contains the CV scan of **1a** in 50% acetonitrile/buffer beginning at 0.1 V and scanning in the reductive direction at a rate of 0.2 V/sec.

compounds were the more hydrophilic hydroquinone forms **4a–4h** of the pro-prodrugs **3a–3h**.

The unstable nature of the hydroquinones **4a–4h** did not permit the characterization of these intermediates by standard methods. However, UV spectroscopy studies verified that the pro-prodrugs **3a–3h** exhibited λ_{max} values between 320 and 340 nm which are characteristic of benzoquinones. For example, 2,6-dimethylbenzoquinone has a λ_{max} at 317 nm which is absent from the spectrum of its hydroquinone form—1,4-dihydroxy-2,6-dimethylbenzene. The absorbances at 320–340 nm for the pro-prodrugs **3a–3h** were lost upon reduction with $NaBH_4$, confirming the formation of the corresponding hydroquinones **4a–4h**.

Cyclic voltammetry (CV) studies were conducted on solutions of **3g**, **2**, and **1a** in acetonitrile and phosphate buffer (0.05 *M*, pH 7.4, μ = 0.15) in order to determine the oxidation and reduction potentials of the reversible quinone/hydroquinone redox couple as well as to provide evidence that reduction of the pro-prodrugs follows the mechanism described in Fig. 1. The CV scans are shown in Figs. 3A–D. Figure 3A shows a CV scan of **3g** at a concentration of 5×10^{-4} *M* in 50% acetonitrile in buffer and a scan rate of 0.2 V/sec. The solid line shows a tracing in the reductive direction and exhibits a reduction potential of −0.47 V for the quinone and an oxidation potential of +0.24 V for the hydroquinone. When the scan is conducted in the oxidative direction, no wave is observed at +0.24 V; however, a similar reduction potential is observed near −0.47 V, and following this reduction the oxidation potential at +0.24 V reappears. Figure 3B shows a CV scan of **3g** at a concentration of 1×10^{-4} *M* in 10% acetonitrile in buffer and determined at a scanning rate of 0.2 V/sec. When scanning in the reductive direction, a reduction potential corresponding to the quinone reduction is observed at −0.29 V, while there are now two waves observed in the oxidative direction, one at +0.05V and the second at +0.32 V. It is believed that the first oxidation potential corresponds to oxidation of the hydroquinone and that the second corresponds to oxidation of the lactone **1a** which had time to form from the hydroquinone **4g** during the scan. Figure 3C shows a CV scan of **2** at a concentration of 5×10^{-4} *M* in 50% acetonitrile in buffer and a scan rate of 0.01 V/sec. When scanning in the reductive direction, a reduction potential corresponding to the quinone reduction is observed at −0.47 V, and there are two oxidation waves observed during the return, one at +0.14V and the other at +0.37 V. Again, it is reasonable to assume that the first wave corresponds to hydroquinone oxidation and the second to oxidation of the lactone **1a**. When this sample was scanned at faster rates, the oxidation wave at +0.37 V was not observed (data not shown). Figure 3D contains a CV scan of **1a** at a concentration of 5×10^{-4} *M* in 50% acetonitrile in buffer and a scan rate of 0.2 V/sec. When scanning in the reductive direction no waves are observed, while on the return there is an oxidation wave at +0.46 V, which corresponds to oxidation of the lactone **1a**. Following this oxidation, there is a reduction wave occurring at −0.58 V, which corresponds to reduction of some oxidized form of **1a**. It was observed that the aromatic amines themselves underwent a reductive conversion which complicated the CV scans and prevented the examination of most of the model pro-prodrugs in this study. Therefore, the CV data on compound **3g** is presented as being representative of the set of pro-prodrugs.

In order to evaluate whether or not these pro-prodrugs would experience rate-limiting reduction in their conversion reactions, it was necessary first to determine the rates of lactonization of the reduced forms **4a–4h** under near-physiological conditions. Using the HPLC assay, it was possible to determine the amount of **1a** present at any time during the reaction. Figure 4 is a representative plot showing that the formation of lactone **1a** from **4g** follows first-order kinetics for product formation. Similar results were obtained for the other hydroquinones (**4a–4f** and **4h**) studied (data not shown). With all of the hydroquinones, the only products

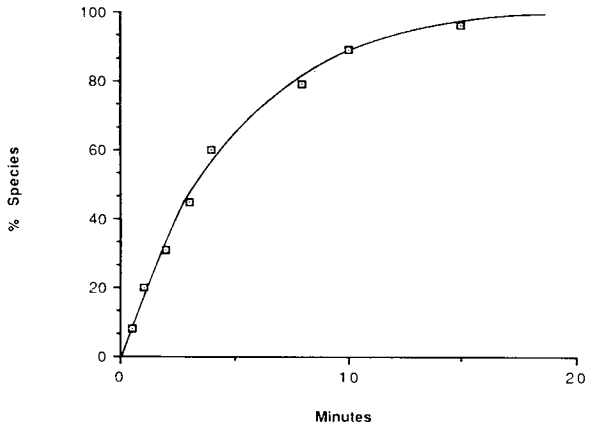


Fig. 4. Representative time course for the appearance of the product lactone **1a** from the lactonization of the reduced form of the pro-prodrug **3g** at pH 7.4 and 37°C.

observed were the lactone **1a** and amines **5a–5h**. The observed rate constants (Table I) for the appearance of lactone **1a** from the reduced prodrugs **4a–4h** were determined by fitting the data to Eq. (2)—an equation for first-order product formation.

$$\ln (A_\infty - A_t) = k_{obs}t \qquad (2)$$

In Eq. (2), A_∞ refers to the peak area for **1a** after the reaction is complete and A_t refers to the peak area for **1a** at any time during the course of the reaction.

DISCUSSION

The majority of prodrugs relies on some type of hydrolase for bioconversion to the parent drug. There are few prodrugs which utilize a bioreductive conversion to regenerate the drug. One example of a prodrug which requires reduction to its active metabolite is the antiinflammatory drug Sulindac. Sulindac is a sulfoxide prodrug which has been shown to possess negligible intrinsic activity. Its pharmacological effect is expressed upon NADPH-dependent reduction of the sulfoxide to the sulfide (12). Another example of a redox prodrug is pro-2-PAM, a dihydropyridine derivative of *N*-methylpyridinium-2-carbaldoxime salt (2-PAM). 2-PAM, an antidote for organophosphate poisoning, is a quaternary amine and, therefore, possesses a positive charge at physiological pH. As a result of this charge, 2-PAM suffers from a short biological half-life and low permeability through biological membranes. Since the toxic effects of organophosphates are felt most acutely in the CNS, it was of interest to improve the delivery of 2-PAM to the brain. The lipophilicity of 2-PAM was increased dramatically by reducing its pyridine ring to the dihydropyridine form (pro-2-PAM) and thereby converting the molecule into a tertiary amine at physiological pH (13). It was proposed that the pro-2-PAM would be susceptible to oxidation *in vivo* which would regenerate the 2-PAM. It was found that the iv administration of pro-2-PAM resulted in a 50% increase in the biological half-life of 2-PAM (14) and a 13-fold increase in the amount of 2-PAM delivered to the brain (15). Later the concept was expanded by simply using the pyridine/dihydropyridine system for the carrier molecule (trigonelline was the actual carrier molecule employed) in a redox-sensitive pro-prodrug fashion to improve the delivery of a variety of drugs to the brain (16).

Table I. Rates of Appearance of Lactone **1a** from the Intermediate Hydroquinones **4a–4b**

Model pro-prodrug[a]	k_{obs} ($\times 10^3$) (sec^{-1})[b]	$t_{1/2}$ (sec)
3a	6.54 ± 0.31	106
3b	7.86 ± 0.42	88
3c	6.73 ± 0.34	103
3d	7.21 ± 0.45	96
3e	8.10 ± 0.56	85
3f	7.39 ± 0.53	94
3g	3.45 ± 0.28	201
3h	6.22 ± 0.40	111

[a] The quinone amide pro-prodrugs **3a–3h** were instantaneously and completely converted into their hydroquinone forms **4a–4b** according to the procedure described under Materials and Methods.

[b] The observed rate constants for the appearance of lactone **1a** were determined at pH 7.4, 37°C by plotting the amount of **1a** versus time according to Eq. (2).

The most abundant source of redox prodrugs may come from the literature of anticancer drugs. There are several examples of antineoplastic agents which are reductively activated to cytotoxic species—usually alkylating agents. The best example is the naturally occurring agent mitomycin C, which has been shown to form a powerful alkylating agent upon reduction of its quinone moiety. Another class of compounds, of which Misonidazole is a member, contains a nitro group *ortho* to a good leaving group. The reduction of this nitro group transforms the molecule into an alkylating agent. These compounds have generally been designed for selective toxicity to the hypoxic regions of tumors. Hypoxic tumor regions are of interest because they have been shown to resist conventional cancer treatment (17–19) and because hypoxia is an unusual cellular state; therefore, the targeting of agents to these tissues could provide a novel basis for tumor selectivity (20,21). Evidence has been presented which indicates that there is a positive correlation between low redox potential and selective toxicity in hypoxic tumor cells with agents which generate a cytotoxic species via a reductive mechanism (21).

Having observed the reactivity of hydroxy amide 7 toward lactonization to amine **5** and lactone **1b**, it was of interest to explore the potential of controlling this reaction using a redox reaction. A closely related quinone propionic acid (**2** with an additional methyl group at the 5′ position) was originally synthesized by Borchardt and Cohen (22) in order to provide another example of the tremendous rate enhancement for intramolecular reactions generated by the "trimethyl lock." The authors expected the trimethyl lock to force the compound into a hemiketal ring tautomer; however, the molecule was found to undergo an unexpected ring tautomerism. The carboxyl group underwent intramolecular conjugate addition to the quinone—a reaction which is rarely observed—forming the spirolactone 4,4,7,8,10-pentamethyl-1-oxaspiro-[4.5]dec-7-ene-2,6,9-trione. Kinetic experiments revealed that this addition reaction was reversible over a wide pH range as nearly complete interconversion from the quinone acid anion at pH 8.7 (0.5% spirolactone

present) to the spirolactone at pH 3.8 (88% spirolactone present) was observed.

The potential of this quinone propionic acid system as a possible redox-sensitive pro-prodrug form was attractive. However, when the quinone acids were initially investigated, no carboxylic acid derivatives were studied. It seemed logical to expect that quinone propionic amides containing the trimethyl lock would rapidly undergo lactonization following reduction of the quinone portion of the pro-prodrug (Fig. 1). If the lactonization rates of these reduced forms approached the rates previously observed with the corresponding hydroxy amides (8,9), then it was reasonable to expect this pro-moiety to result in a derivative form in which drug regeneration is triggered by the reduction of the quinone.

Table I contains the half-lives for the appearance of lactone **1a** from the hydroquinone prodrug intermediates **4a–4h**. The versatility of **2** as a pro-moiety is discussed first. Quinone propionic acid **2** was successfully coupled to a wide variety of amines ranging from aromatic to aliphatic amines and including a secondary amine and a model dipeptide. The observed rate constants for lactonization of **4a–4h** showed little dependence on the nature of the amine, indicating that the leaving group exerts little effect on the rate of lactone formation. These two facts indicate not only that this derivative form could be applied to a wide range of compounds, but that one could expect the pro-prodrug conversion rates to be highly predictable and independent of the attached amine drug. These cyclization rates for **4a–4h** are very fast and are in close agreement with the values determined for the model hydroxy amides (8,9). During the preparation of our manuscript, another laboratory (23) demonstrated similar release rates for amines and alcohols following reductive lactonization of an analogous pro-moiety (compound **2** with an additional methyl group in the 5′ position).

The CV data reveal several important facts about these molecules. From Fig. 3A, it is evident that the potential of +0.24 V must correspond to the oxidation of the hydroquinone, as this value was observed only following reduction of the original compound **3g**. In Figs. 3B and C, the CV scans of **3g** and **2** show a second oxidation wave of higher potential which we attribute to the lactone **1a**. The fact that this second oxidation wave is observed only following passage through the reductive wave is good evidence that quinone reduction is a requirement for lactone formation. A CV scan of **1a** alone in Fig. 3D demonstrates that its oxidation potential is considerably higher than that of the hydroquinone.

It is clear that reduction of the benzoquinone is required for release of the amine. The cyclic voltammetry results indicate that the pro-prodrug does pass through the hydroquinone intermediate, resulting in the formation of the lactone **1a**. In these studies the prodrug has been reduced either by a chemical reducing agent (sodium borohydride or sodium hydrosulfite) or electrochemically. A question of critical interest to the success of this compound as a pro-prodrug of amines is whether or not the molecule will be reduced *in vivo*. Without direct biological evidence, we believe that the molecule will be reduced in a biological system, as there are many examples of biological reduction of benzoquinones in the literature (24–27).

The possibility exists that the redox potential of the benzoquinone system is too positive for this system to be successful for the treatment of tumors. The determined redox potential of −0.47 V indicates that the molecule would be susceptible to reduction by such ubiquitous systems as NADH or NADPH, lessening the possibility that the pro-prodrug would reach the hypoxic tumor site intact. A more appropriate system may be the analogous naphthoquinone system, which would certainly exhibit a lower redox potential. In a study employing reductively activated cytotoxic agents, the lower redox potential naphthoquinones were found to be more selectively cytotoxic against hypoxic tumor cells (28).

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