Cocaine Benzoyl Thioester: Synthesis, Kinetics of Base Hydrolysis, and Application to the Assay of Cocaine Esterases

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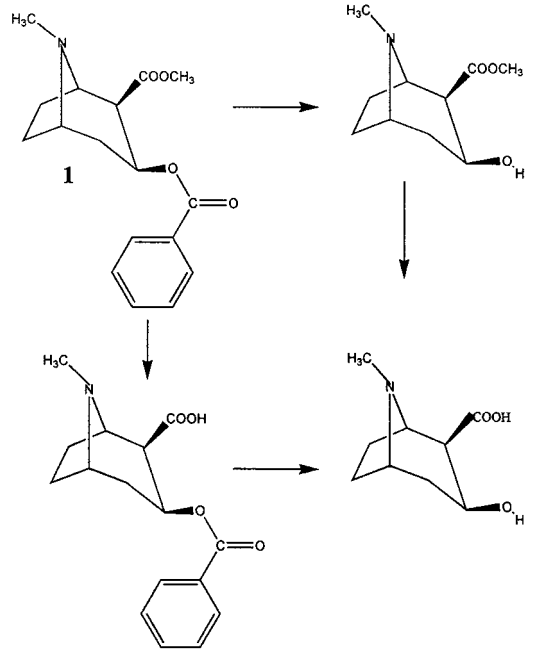
The synthesis and characterization of diastereomers of cocaine benzoyl thioester is described. Allococaine benzoyl thioester and allopseudococaine benzoyl thioester were synthesized by the conjugate addition of *p*-methoxytolyl thiol to ecgonine methyl ester followed by debenzylation and benzoylation. The absolute structure of the hydrochloride salt of the major ecgonine *p*-methoxytolyl sulfide formed was determined by single-crystal diffraction analysis and used to establish the addition geometry. When placed in aqueous solution, the cocaine benzoyl thioester diastereomers hydrolyzed to give thioecgonine methyl ester. The rate of cocaine benzoyl thioester hydrolysis was carefully investigated spectrophotometrically by using the Ellman reagent. At neutral pH, the hydrolysis of the diastereomers was found to proceed at detectable rates. Upon increasing pH, the rate of hydrolysis of cocaine benzoyl thioester diastereomers was increased and the reaction was catalyzed by basic buffer species. In addition to defining the kinetics of hydrolysis in aqueous solution, cocaine benzoyl thioester was utilized as a highly efficient method to monitor the activity of cholinesterases and pig liver esterase. Use of cocaine benzoyl thioester represents a rapid and sensitive way to screen for cocaine esterase activity.

Introduction

The pharmacological action of (−)-cocaine[1] arises from central nervous system stimulation that results in euphoria and activation of the peripheral sympathetic nervous system with tachycardia and blood pressure elevation (*1*). It is possible that repeated use of cocaine to seek the cocaine "high" may lead to progressive cardiovascular toxicity including cocaine-related "sudden death" (*2*−*4*). The pharmacological activity of cocaine is terminated by metabolism to biologically inactive metabolites. Cocaine is hydrolyzed by esterases of the blood, liver, and other organs (*3*, *5*, *6*). After intravenous administration, cocaine has a plasma half-life of about 60 min (*7*, *8*). The relatively short half-life of cocaine in blood is due to its hydrolysis to benzoylecgonine, ecgonine methyl ester, and ecgonine, the major metabolites that appear in the urine (Scheme 1) (*5*−*8*). Although benzoylecgonine is inactive as a psychomotor stimulant, it may contribute to the vasoconstricting effects of cocaine (*9*, *10*). Methylecgonine is largely inactive as a psychomotor and cardiovascular stimulant (*11*). Thus, hydrolysis of the benzoyl ester of cocaine represents a true route of detoxication, in vivo.

In addition to the nonenzymatic (*6*) and enzymatic (*12*, *13*) hydrolysis of the methyl ester, a number of enzymes are capable of hydrolyzing the benzoyl ester of cocaine in humans (*6*, *12*, *14*−*16*). Hydrolysis of the benzoyl ester of cocaine produces ecgonine methyl ester and benzoic acid. This reaction is catalyzed by a serum cholinesterase called butyrylcholinesterase (formerly pseudocholinesterase) (*17*) and a nonspecific carboxylesterase from liver

Scheme 1. Overall Hydrolysis of (−)-Cocaine (1) to Ecgonine Methyl Ester, Benzoylecgonine, and Ecgonine



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[1] Reference to cocaine in this manuscript shall mean the (−) absolute configuration unless otherwise indicated.

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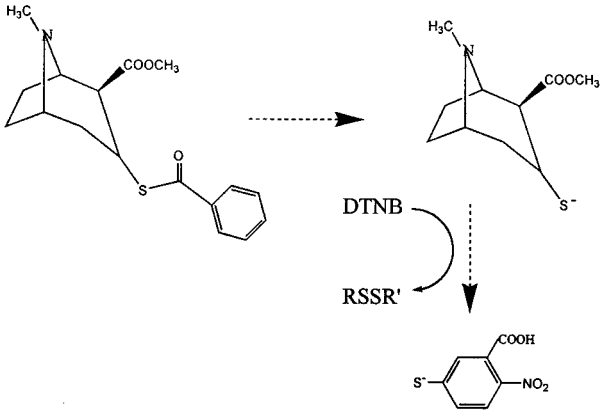
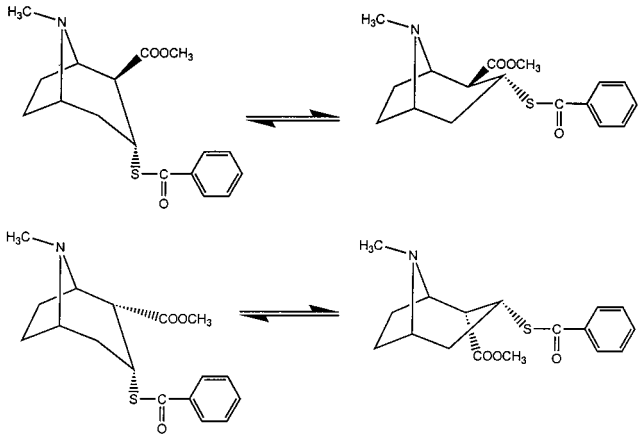


Figure 1. Hydrolysis of cocaine benzoyl thioester to the thiolate anion, which reacts with 5,5′-dithiobis(2-nitrobenzoic acid) (DNTB) to liberate the highly UV-active aryl thiolate anion. The product RSSR′ is composed of a disulfide between 2-nitrobenzoic acid and 3-mercaptoecgonine methyl ester.

Scheme 2. Structural Depiction of Allococaine Benzoyl Thioester (2) and Allopseudococaine Benzoyl Thioester (3) in Normal and Boat-type Conformations



(*6*, *12*). Similar observations have been made for hepatic cocaine hydrolysis in animal preparations including rabbit (*18*), rat (*19*), horse (*20*), and pig.

We have developed an assay for cocaine benzoyl thioester hydrolysis based on the liberation of a chromophoric thiolate anion that follows the general format of the Ellman reagent (*21*) (Figure 1). This method allows for direct continuous analysis of cocaine benzoyl thioester hydrolysis of incubation mixtures without the need for extraction or derivatization. This assay has the further advantage of using a relatively easily synthesized substrate that has the added feature that this substrate can exist as diastereomers (Scheme 2) that can be used to probe the hydrolytic mechanism. Finally, fundamental kinetic studies of cocaine benzoyl thioester hydrolysis have shown that benzoyl thioester cleavage is dependent on pH and buffer strength.

Experimental Procedures

Chemicals. Chemicals were obtained from Aldrich Chemical Co. (Milwaukee, WI) in the highest purity available. Other reagents and chromatography materials were purchased from Fisher Scientific Inc. (Santa Clara, CA). Anhydroecgonine methyl ester was obtained from the Drug Supply Program of the National Institute on Drug Abuse (Rockville, MD). All other chemicals were obtained in the highest purity from commercially available sources.

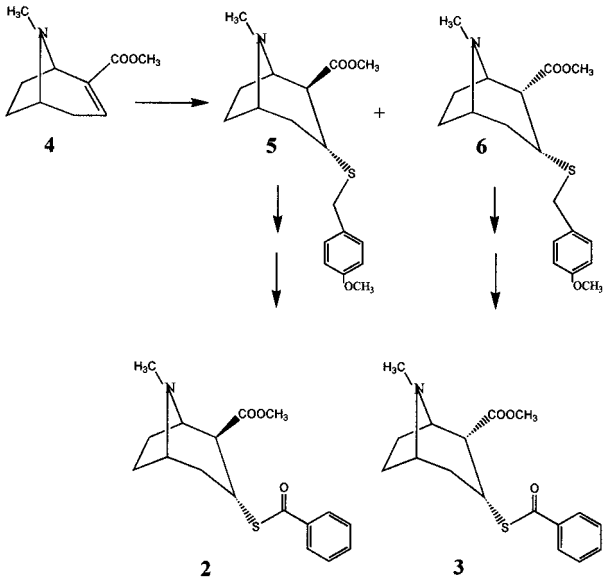


Figure 2. Thiol addition to ecgonine methyl ester (compound **4**) produces a mixture of *p*-methoxytolyl sulfides (compounds **5** and **6**). Debenzylation of the individual isomers, compounds **5** and **6**, and benzoylation with benzoyl chloride gave the cocaine benzoyl thioester isomers, compounds **2** and **3**, respectively.

Instrument Analyses. ^{1}H NMR spectra were recorded with a Varian spectrometer operating at a frequency of 300 MHz. ^{1}H chemical shifts were expressed in parts per million (ppm) downfield from tetramethylsilane. Low-resolution fast atom bombardment (LRFAB) mass spectra were recorded with a Micromass 70 SEQ mass spectrometer. Both instruments were housed at the Department of Medicinal Chemistry, University of Washington (Seattle, WA).

Compounds 5 and 6: 3-(*p*-Methoxytolylsulfide) Ecgonine Methyl Ester. Compounds **5** and **6** have been synthesized previously (*22*). Anhydroecgonine base, compound **4** (1.0 g, 5.52 mmol), was dissolved in MeOH (15 mL) and purged with $Ar_{(g)}$ for 0.5 h. Diisopropylethylamine (DIA)[2] (0.96 mL, 5.52 mmol) and *p*-methoxytoluene thiol (1.54 mL, 11.04 mmol) were each added via syringe and the mixture was allowed to stir under an $Ar_{(g)}$ atmosphere for 48 h. The mixture was concentrated in vacuo to a yellow oil, treated with 10% aqueous HCl (75 mL), and extracted twice with Et_2O (75 mL) to remove unreacted thiol. The aqueous solution was neutralized with NaOH (10 N), basified to pH 10, and extracted twice with CH_2Cl_2 (100 mL each). The organic layers were combined, dried over $MgSO_4$, and concentrated in vacuo to give a pale yellow oil. The products were purified by flash chromatography (silica gel; MeOH/ethyl acetate/NH_4OH (30%) 5:95:0.5 v/v/v; **5**, R_f = 0.4; **6**, R_f = 0.15) to give the products as colorless oils (**5**, 0.715 g, 40% yield; **6**, 0.278 g, 15% yield) (Figure 2). Compound **5**: ^{1}H NMR ($CDCl_3$) δ 7.18 (m, 2 H), 6.8 (m, 2 H), 3.76 (s, 3 H), 3.66 (m, 5 H), 3.52 (m, 1 H), 3.44 (dd, J = 7.8 Hz, 1 H), 3.08 (br s, 1 H), 2.61 (br s, 1 H), 2.3 (m, 1 H), 2.14 (s, 3 H), 1.9−2.1 (m, 3 H), 1.70 (m, 2 H); LRMS(FAB) 336 (M + H, 100%). Compound **6**: ^{1}H NMR ($CDCl_3$) δ 7.16 (m, 2 H), 6.8 (m, 2 H), 3.76 (s, 3 H), 3.63 (m, 5 H), 3.15−3.3 (m, 3 H), 3.12 (t, $J \cong$ 7 Hz, 1 H), 2.6 (m, 1 H), 2.21 (s, 3 H), 1.9−2.07 (m, 3 H), 1.80 (m, 2 H); LRMS-(FAB) 336 (M + H, 100%).

Compound 2: 2*β*,3α-Allococaine Benzoyl Thioester. Compound **5** (0.54 g, 1.6 mmol) was dissolved in trifluoroacetic acid (15 mL) at 0 °C, and $Hg(OAc)_2$ (0.51 g, 1.6 mmol) was added. The reaction mixture became pink in color and was allowed to

[2] Abbreviations: DIA, diisopropylethylamine; EI, electron impact; LRMS(FAB), low-resolution mass spectrometry (fast atom bombardment); TLC, thin-layer chromatography; THF, tetrahydrofuran; TEA, triethylamine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); PLE, pig liver esterase; hBuChE, human butyrylcholinesterase; mAcetylChE, mouse acetylcholinesterase; mBuChE, mouse butyrylcholinesterase.

Table 1. Crystal Data and Structure Refinement for Compound 5

empirical formula	$C_{18}H_{26}ClNO_3S$
formula weight	371.91
temperature	293(2) K
wavelength	0.71073 Å
crystal system	orthorhombic
space group	$P2_12_12_1$
unit cell dimensions	$a = 7.1633(8)$ Å, $\alpha = 90°$ $b = 8.5434(10)$ Å, $\beta = 90°$ $c = 30.933(5)$ Å, $\gamma = 90°$
volume, Z	1893.1(4) Å, 4
density (calculated)	1.305 mg/m^3
absorption coefficient	0.328 mm^{-1}
$F(000)$	792
crystal size	0.06 × 0.32 × 0.46 mm
θ range for data collection	2.47−24.94°
limiting indices	$-7 \le h \le 7$, $-9 \le k \le 10$, $-33 \le l \le 33$
reflections collected	3699
independent reflections	2457 ($R_{int} = 0.0494$)
absorption correction	semiempirical from ψ-scans
max and min transmission	0.8421 and 0.7735
refinement method	full-matrix least-squares on F^2
data/restraints/parameters	2420/0/322
goodness-of-fit on F^2	1.143
final R indices [$I > 2\sigma(I)$]	$R1 = 0.0399$, w$R2 = 0.0927$
R indices (all data)	$R1 = 0.0657$, w$R2 = 0.1923$
absolute structure parameter	−0.08(13)
extinction coefficient	0.006(2)
largest difference peak and hole	0.205 and −0.221 e Å^{-3}

stir at 0 °C for 0.5 h. The mixture was concentrated in vacuo to a semisolid and purified by column chromatography (neutral activity III alumina), first with CH_2Cl_2 until the fast band eluted, followed by MeOH/CH_2Cl_2 (10:90 v/v) to elute the product (0.32 g, 1.49 mmol). This material was dissolved in THF (10 mL) followed by the addition of triethylamine (TEA) (207 μL, 1.49 mmol) and benzoyl chloride (173 μL, 1.49 mmol). The reaction was allowed to stir at room temperature for 2 h. The mixture was concentrated in vacuo to an oil and purified by flash chromatography (silica gel; MeOH/CH_2Cl_2 7.5:92.5 v/v; $R_f = 0.3$). The product was dissolved in CH_2Cl_2 and treated with excess HCl·Et_2O to form the hydrochloride salt as a white precipitate. The solid was filtered and triturated with Et_2O (0.18 g, 32% yield). ^{1}H NMR ($CDCl_3$) δ 7.92 (m, 2 H), 7.48 (m, 3 H), 4.61 (d, $J = 7.4$ Hz, 1 H), 3.77 (s, 3 H), 3.63 (br s, 1 H), 3.17 (m, 1 H), 2.73 (m, 1 H), 2.59 (m, 1 H), 2.19 (s, 3 H), 1.89−2.17 (m, 4 H), 1.72 (m, 1 H); LRMS(FAB) 320 (M + H, 100%).

Compound 3: 2α,3α-Allopseudococaine Benzoyl Thioester Benzyl Thioester. A solution of compound **6** (169 mg, 0.5 mmol) in trifluoroacetic acid (2 mL) at 0 °C was treated with $Hg(OAc)_2$ (160 mg, 0.5 mmol). After 1 h, the solvent was removed under a stream of N_2, and the residue was chromatographed (neutral activity III alumina, CH_2Cl_2/MeOH 9:1). A proton NMR of the residue showed that the product was primarily anhydroecgonine methyl ester. The mixture was taken up in THF and TEA was added (84 μL, 0.6 mmol), followed by benzoyl chloride (70 μL, 0.6 mmol). After stirring overnight, the mixture was evaporated and the residue was purified by preparative TLC (silica, CH_2Cl_2/MeOH 97:3 v/v) to give allopseudococaine benzoyl thioester, compound **3** (8 mg, 4% yield). ^{1}H NMR ($CDCl_3$, δ 7.95 (m, 2 H), 7.45 (m, 3 H), 4.63 (t, $J = 6.4$ Hz, 1 H), 3.65 (m, 1 H), 3.57 (s, 3 H), 3.37 (br s, 1 H), 3.21 (br s, 1 H), 2.75 (m, 1 H), 2.45 (s, 3 H), 2.09−2.32 (m, 4 H), 1.92 (m, 1 H); LRMS(FAB): 320 (M + H, 100%).

X-ray Diffraction Analysis of Compound 5. Crystals of the hydrochloride salt of compound **5** were grown from methanol/diethyl ether. Crystals were examined by optical microscopy and suitable candidates were mounted on a glass fiber with epoxy glue and optically centered in the X-ray beam of an automated Siemens P4 diffractometer. This diffractometer was equipped with a sealed-tube Mo source operated at 60 kV and 40 mA and a graphite monochromator. Crystal quality was evaluated from rotation photographs and scans of representative reflections, and the best crystal was chosen for subsequent diffraction analysis. Data collection and structure solution parameters are given in Table 1. Data were corrected for Lorentz and polarization effects and also for absorption via empirical ψ scans. Structure solution proceeded in a routine fashion using the Siemens SHELXTL structure solution package (*23*). All non-hydrogen atoms were solved with anisotropic displacement parameters. All hydrogen atoms were located on electron density maps and were solved with isotropic displacement parameters. An ORTEP view of the molecule along with selected bond lengths, angles, and torsion angles is given in Figure 3. Complete tables of final atomic coordinates for all non-hydrogen atoms and their equivalent isotropic displacement parameters, anisotropic displacement parameters for the hydrogen atoms, complete listings of bond lengths, angles, torsion angles and structure factors, and additional views of the molecule are available from the authors.

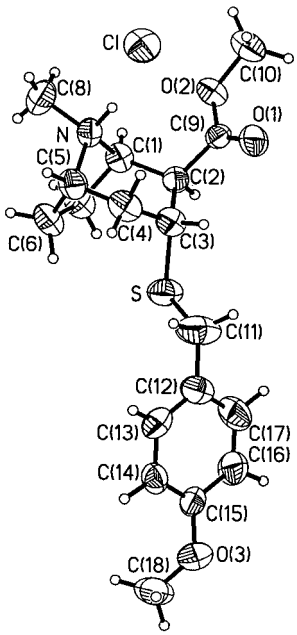


Figure 3. ORTEP plot of compound **5** showing 50% probability ellipsoids. Selected bond lengths (angstroms), angles (degrees), and torsion angles (degrees): C(3)−S, 1.842 (5), C(1)−C(2)−C(9), 115.3 (4), C(1)−C(2)−C(3), 113.3 (4); C(2)−C(3)−C(4), 111.4 (4); C(2)−C(3)−S, 111.1 (3); C(1)−C(2)−C(3)−C(4), 39.0; C(1)−C(2)−C(3)−S, 90.0; and C(4)−C(3)−C(2)−C(9), 91.8.

Measurement of Rate Constants. For the buffer dilution studies, all rate measurements were made at room temperature by using a EL311 BIOTEX Instruments, Inc. (Burlington, VT), microplate reader. The buffer employed was potassium phosphate and cocaine benzoyl thioester and the ionic strength was maintained with 1.0 M KCl. Stock solutions of cocaine benzoyl thioester diastereomers were prepared in 2-propanol immediately before use and aliquots were diluted into the aqueous reaction solutions such that the final concentration of 2-propanol never exceeded 8%. Rate constants (k_{obsd}) were measured under pseudo-first-order conditions by monitoring the formation of thiol formed by a standard Ellman procedure at 405 nm in the presence of 4 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (*21*). A 96-well plate was set up and to each well was added 100 μL of assay solution (4 mM DTNB and 0.25 mM cocaine benzoyl thioester in buffer solution). At specific time points absorbance readings were obtained. Rate constants were measured by monitoring the formation of the nitrobenzoic acid thiolate anion at 405 nm for at least 5 half-lives. The correlation coefficients were in the range 0.76−0.99.

Enzyme Preparations and Kinetic Determinations. Pig liver esterase (PLE) was obtained from Sigma Chemical Co. (St. Louis, MO). Human butyrylcholinesterase (hBuChE) was a generous gift of Professor O. Lockridge (University of Nebraska). Mouse acetylcholinesterase (mAcetylChE) and mouse butyryl-

cholinesterase (mBuChE) were gifts of Professor Palmer Taylor (University of California, San Diego). Enzyme activity was determined at room temperature. K_m determinations were measured spectrophotometrically by a modification of a previously described method (*21*). To each cuvette was added DTNB (880 μL of a 5.55 mM DTNB in 25 mM potassium phosphate buffer, pH 7.4) followed by addition of 100 μL of PLE (7 pmol) or hBuChE (2.3 nmol) or mBuChE (9 pmol) or mAcetylChE (5.0 nmol). The number of active sites per milliliter of esterase was determined by titration with echothiophate. The enzymatic reaction was initiated at room temperature by the addition of 20 μL of various concentrations of allococaine benzoyl thioester (10−200 μM for PLE and 50−1000 μM for the other esterases, in 2-propanol). The initial phase of the linear absorbance increase at 412 nm was monitored for 2 min. The rate of hydrolysis was measured as absorbance increase per minute and was corrected at each substrate concentration for background hydrolysis from a cuvette containing DTNB and allococaine benzoyl thioester without enzyme. To calculate the absolute rate, the following equation was used: absolute rate (moles liter^{-1} minute^{-1}) = (Abs$_{412}$/minute)/(1.36 × 10^4). K_m and V_{max} values were obtained from double-reciprocal plots of 1/rate versus 1/substrate concentration. Nonlinear curve fitting was not used for determination of kinetic constants because, at the concentration of substrate employed, no inhibition or nonlinear kinetics was observed.

Results

The chemical synthesis of cocaine benzoyl thioester diastereomers was done to provide sufficient material for chemical characterization and analysis as a substrate for esterase activity as well as to study the kinetics of hydrolysis. In addition to complete spectral characterization, the single-crystal diffraction analysis of compound **5** showed that the allococaine benzoyl thioester precursor molecule possessed the 2β and 3α absolute stereochemistry. Thus, addition of *p*-methoxytoluene thiol to anhydroecgonine methyl ester gave two diastereomeric conjugate addition products. The hydrochloride salt of the major product, compound **5**, gave crystals of adequate quality to obtain the crystal structure. Efforts to obtain crystals suitable for single-crystal diffraction analysis of the minor product, compound **6**, were not successful.

Compound **5** was well-behaved crystallographically. The final goodness-of-fit parameter was 1.143, the final *R* factor was 0.0657, and the largest peaks on the final electron density difference maps were 0.205 and −0.221 e/Å^3. All atoms were located on electron density maps and, upon refinement, reasonable anisotropic (isotropic for the hydrogen atoms) displacement parameters were observed. In the final structure (see Figure 3), all of the observed bond lengths, angles, and torsion angles fell within the ranges commonly observed for organic compounds and no unusually close intermolecular contacts were observed. The diffraction data were of sufficiently high quality that the absolute structure could be unambiguously determined [giving a final absolute structure parameter of −0.08 (*23*)]. This absolute structure was also consistent with what was expected on the basis of the known absolute configuration of its organic precursor. The geometry of thiol addition in the synthesis of **5** was, however, different from what had been expected (see below) and gave a thioether with the α configuration. While the α substituent is clearly distinct from the naturally occurring β configuration at the C-3 position, the X-ray structure and the NMR provide some evidence for significant contribution from a boat-type conformation (Scheme 2).

Debenzylation of the individually purified compounds **5** and **6** with Hg(OAc)$_2$ gave the thiol, which was treated with benzoyl chloride to form the cocaine benzoyl thioester analogues, compounds **2** and **3**, respectively (Figure 2). In the proton NMR, compound **2** showed a doublet at δ = 4.61 ppm (J = 7.4 Hz) that appeared almost identical to that of allococaine (*24*). In addition, the precursor, thioether **5**, showed a similar doublet at δ = 3.44 ppm (J = 7.8 Hz) that was also consistent with the 2β,3α configuration and this was verified by single-crystal diffraction analysis (Figure 3). Treatment of isomer **6** with Hg(OAc)$_2$ and subsequent benzoylation gave primarily elimination to anhydroecgonine methylester, compound **4**, but also a small amount of the allopseudococaine benzoyl thioester analogue, compound **3**. In the proton NMR an apparent triplet at 4.63 ppm (J = 6.4 Hz) was observed and the spectrum was nearly identical to that observed for allopseudococaine (*24*). Thus, the structure was consistent with a 2α,3α configuration assignment.

That *endo*-face addition predominated in the reaction products observed was somewhat surprising in view of the fact that addition of Grignard reagents has been shown to give mainly addition to the *exo* face, and for tropanes, the *exo* face can be regarded as the less stereochemically hindered site of approach. To test the possiblity that the N-8 tertiary amine moiety was controlling the product stereochemistry, the N-8 carbobenzyloxy-protected anhydronorecgonine methyl ester was prepared by treating norcocaine with concentrated HCl followed by carbobenzyloxy chloride as described previously (*25*). The carbamate-protected N-8 amino group possessed significantly different basicity compared to the amino group of anhydroecgonine methyl ester. However, no addition of *p*-methoxy-α-toluene thiol in methanol was observed with 6 equiv of thiol and either DIA or diazabicycloundecane as a catalyst. Although the presence of the carbobenzyloxy group may alter the conformation of anhydroecgonine methyl ester relative to the *N*-methyl compound, and may cause steric interference at the *exo* face, it was notable that no detectable *endo* addition was observed. This result implies that anomeric assistance from the N-8 amino group may be important for addition of thiol to occur for anhydroecgonine methyl ester or that the thiolate anion avoided the lone pair of the N-8 amino group on the *exo* face.

Reaction of Cocaine Benzoyl Thioester with Base. The reaction of allococaine benzoyl thioester, **2**, and allopseudococaine benzoyl thioester, **3**, with excess hydroxide ion (in the range pH 8−9) was observed to follow pseudo-first-order kinetics for 4−5 half-lives. Specific-base-catalyzed reaction rate constants were determined from the y intercepts of plots of k_{obsd} vs buffer concentration (eq 1) from experiments carried out near pH 7 (Table 2):

$$\text{d[cocaine benzoyl thioester]/d}t = k_{OH}[\text{cocaine benzoyl thioester}]K_w/a\text{H} \quad (1)$$

Second-order rate constants were obtained from the plots of slopes of observed pseudo-first-order rate constants against the concentration of the second reactant or catalyst. Thus, a plot of k_{obsd} versus the activity of hydroxide ion gave linear plots for both cocaine benzoyl thioester diastereomers and the y intercepts of such plots gave the k_0 for the pH-independent or water reaction (Table 2).

Table 2. Rate Constants for Aqueous Hydrolysis of Allococaine Benzoyl Thioester, 2, and Allopseudococaine Benzoyl Thioester, 3[a]

compound	rate constant (M^{-1} min^{-1}) k_{OH^-}	k_{A^-}	K_{AH}	k_0' [b]
allococaine benzoyl thioester, **2**	0.21	62.5	0.87	0.32
allopseudococaine benzoyl thioester, **3**	0.06	44.9	10.1	0.63

[a] Kinetic reactions were done spectrophotometrically at room temperature (μ = 1.0 M) as described in the Materials and Methods section. [b] Dimensional analysis was in units of minutes^{-1}.

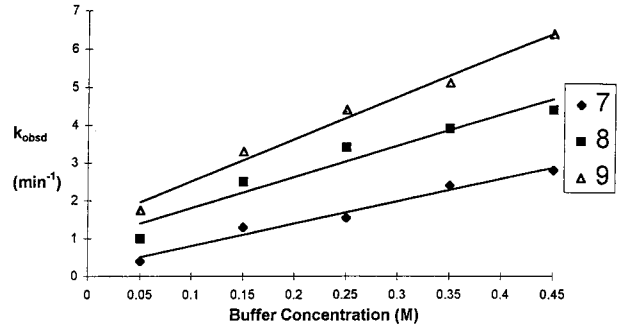


Figure 4. Plot of the observed rate constants (minutes^{-1}) of cocaine benzoyl thioester (compound **2**, 2β,3α-allococaine benzoyl thioester) hydrolysis versus the total phosphate buffer as a function of pH. Ionic strength was kept constant with KCl.

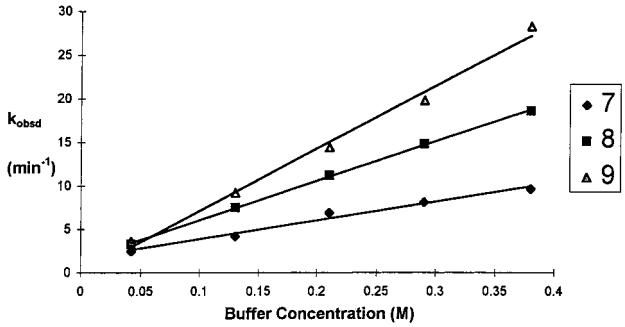


Figure 5. Plot of the observed rate constants (minutes^{-1}) of cocaine benzoyl thioester (compound **3**, 2α,3α-allopseudococaine benzoyl thioester) hydrolysis versus the total phosphate buffer as a function of pH. Ionic strength was kept constant with KCl.

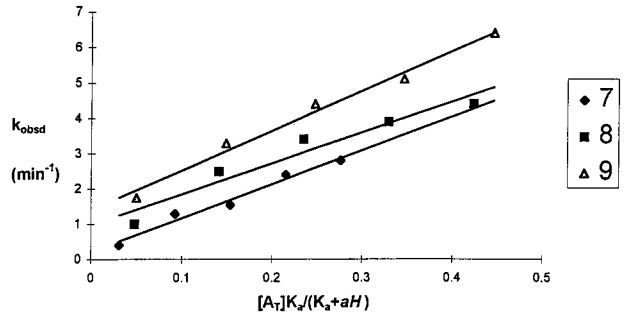


Figure 6. Plots of the observed rate constants (minutes^{-1}) of cocaine benzoyl thioester (compound **2**, 2β,3α-allococaine benzoyl thioester) vs $[A]_T K_a/(K_a + aH)$ as a function of pH and constant ionic strength. $[A]_T$ is the total concentration of buffer.

Buffer Catalysis Studies. Studies with various buffers and buffer concentrations revealed a dependence of allococaine benzoyl thioester and allopseudococaine benzoyl thioester hydrolysis rate on the concentration of buffer at constant pH and ionic strength (Figures 4 and 5). The rate of hydrolysis for either cocaine benzoyl thioester diastereomer in a series of increasing phosphate (pH 6−9) concentration is shown in Figures 4 and 5. The rate increase with increasing buffer concentration, more rapidly at higher pH, indicated that cocaine benzoyl thioester hydrolysis was catalyzed by phosphate ion. Phosphate anion is a more effective catalyst than phosphate, in keeping with the Bronsted catalysis law (*26*). Thus, plots of k_{obsd} versus concentration of buffer (i.e., 0.05−0.45 M) were linear in the pH range 7−9. This result was consistent with the possibility that buffer anion was active as a general base catalyst and that the reaction followed the general rate law of eq 2 where k_0 is the spontaneous rate constant and K_w is the water product. It follows that

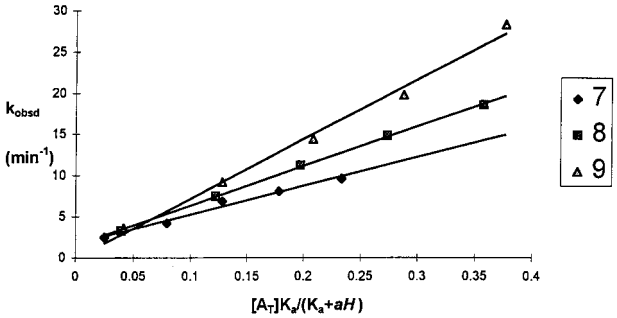


Figure 7. Plots of the observed rate constants (minutes^{-1}) of cocaine benzoyl thioester (compound **3**, 2α,3α-allopseudococaine benzoyl thioester) vs $[A]_T K_a/(K_a + aH)$ as a function of pH and constant ionic strength. $[A]_T$ is the total concentration of buffer.

$$k_{obsd} = k_0 + k_{aH}[H_3O^+] + k_{HA}[HA] + k_{A^-}[A^-] + k_{OH}K_w/aH \quad (2)$$

At regions where very little hydronium or hydroxide ion was present, eq 2 simplifies to

$$k_{obsd} = k_0' + k_{HA}[HA] + k_{A^-}[A^-] \quad (3)$$

From the principle of mass balance (i.e., $[A]_T = [AH] + [A^-]$), where AH is the buffer acid and A^- is the buffer base species, eq 3 can be transformed into eq 4. Equation 4 is a form of eq 3 that is more readily evaluated after simplifying the A^- term by employing the mass balance equation and the equilibrium constant for buffer base and buffer acid species, K_a:

$$k_{obsd} = k_0' + k_{HA}([A^-]aH/K_a) + k_{A^-}[A^-] \quad (4)$$

$$k_{obsd} = k_0' + [k_{HA}(aH/K_a) + k_{A^-}][A^-] \quad (5)$$

$$k_{obsd} = k_0' + [k_{HA}([HA]/[A^-]) + k_{A^-}][A]_T(K_a/(K_a + aH)) \quad (6)$$

Equation 6 predicts that a plot of k_{obsd} versus $[A]_T(K_a/(K_a + aH))$ at fixed pH should be linear, with a slope = $k_{HA}[HA]/[A^-] + k_{A^-}$ and intercept = k_0'. The results for phosphate buffer at room temperature are shown in Figures 6 and 7 for allococaine benzoyl thioester and allopseudococaine benzoyl thioester, respectively. By plotting the slope of these plots versus the buffer ratio $[HA]/[A^-]$, k_{HA} and k_{A^-} can be evaluated as the slope and intercept, respectively:

$$\text{slope} = k_{HA}[HA]/[A^-] + k_{A^-} \quad (7)$$

Table 3. Kinetic Constants for Hydrolysis of Allococaine Benzoyl Thioester in the Presence of Purified Esterases[a]

enzyme	K_{mapp} (μM)	V_{max} (nmol/min)	k_{cat} (min^{-1})
PLE	152	1.68	50.5
mBuChE	182	0.55	59.3
hBuChE	44	0.54	0.24
mAcetylChE	115	0.45	0.09

[a] Kinetic constants were determined by Lineweaver−Burk analysis in 0.025 M potassium phosphate, pH = 7.4 at room temperature. The rate constant k_{cat} was calculated by dividing V_{max} by the concentration of active sites. The correlation coefficients ranged from 0.96 to 0.99. The estimated standard error was approximately 10%.

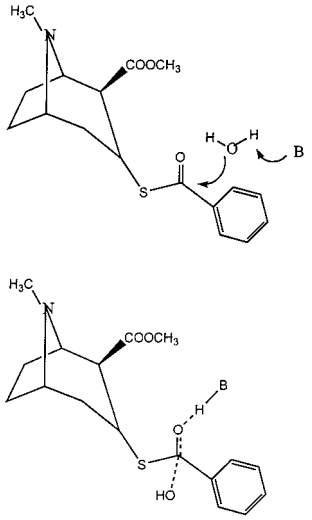
The terms k_{HA} and k_{A^-} are the general acid and general base rate constants, respectively. Such secondary plots are linear (data not shown) and the slopes (k_{HA}) and intercepts (k_{A^-}) are listed in Table 2. Values of k_0' were obtained by subtracting $k_{OH}(K_w/aH)$ from the intercept values of eq 6. These values are also listed in Table 2. From inspection of Table 2, the spontaneous or water-catalyzed rate constants (k_0') were quite small (i.e., 0.06−0.21 M^{-1} min^{-1}) and the general-base-catalyzed rate constants k_{A^-} were much larger (i.e., 44.9−62.5 M^{-1} min^{-1}).

Product Analysis. At a fixed concentration of either cocaine benzoyl thioester diastereomer, TLC analysis of organic extracts of aqueous hydrolysis reaction products showed only one product, 3-mercaptoecgonine methyl ester, was formed. During the relatively short time period of the hydrolysis reaction examined, no apparent hydrolysis of the C-2 methyl ester of either diastereomer was observed. The structure of this isolated material was identical with that of authentic 3-mercaptoecgonine methyl ester as judged by ^1H NMR and mass spectra of authentic 3-mercaptoecgonine methyl ester.

Cocaine Benzoyl Thioester Hydrolysis by Pig Liver Esterase and Choline Esterases. The hydrolysis of allococaine benzoyl thioester, compound **2**, by PLE, hBuChE, mBuChE, and mAcetylChE was examined as a model system to show the utility of the assay for efficiently monitoring hydrolysis. The relatively small amount of allopseudococaine benzoyl thioester, compound **3**, available from chemical synthesis, the relatively rapid rate of spontaneous hydrolysis, and the unfavorable properties as a substrate for hBuChE precluded obtaining kinetic constants of hydrolysis. Hydrolysis of allococaine benzoyl thioester was linear and proportional to purified PLE, hBuChE, mBuChE, and AcetylChE activity. The rate of hydrolysis remained linear for at least 3 min. Under standard incubation conditions, zero time controls showed a minimal amount of hydrolysis of compound **2** at a buffer concentration of 25 mM potassium phosphate buffer, pH = 7.4. The limit of detection of allococaine benzoyl thioester hydrolysis was 442 pmol/assay and this corresponded to a rate of 221 nmol L^{-1} min^{-1}.

When the concentration of allococaine benzoyl thioester was varied, the K_{mapp} and V_{max} values for thioester hydrolysis were obtained from double-reciprocal plots (Table 3). The K_{mapp} and V_{max} values for allococaine benzoyl thioester hydrolysis are comparable with the K_{mapp} and V_{max} values for (−)-cocaine benzoyl hydrolysis. At very long incubation times it is anticipated that some minor amounts of methyl ester hydrolysis could arise from allococaine benzoyl thioester, but we did not examine this point. Allococaine benzoyl thioester was not indefinitely stable at neutral pH and a detectable amount of spontaneous hydrolysis was observed. Nevertheless, normal experimental technique coupled with attentive workup of the incubations can readily provide useful kinetic data employing this substrate.

Chart 1. Two Postulated Transition-State Structures for the Hydrolysis of Cocaine Benzoyl Thioester



Discussion

In good agreement with a study of the synthesis of 3-(phenylthio)tropane-2-carboxylic acid methyl esters (*22*), *endo*-face addition predominated in the conjugate addition of *p*-methoxythiophenol to anhydroecgonine methyl ester. Chemical conversion to allococaine benzoyl thioester afforded a substrate to study the kinetics of nonenzymatic and enzymatic hydrolysis. The hydrolysis of thioesters at neutral pH has been reported to proceed through an anionic as well as a neutral transition state. Thioester hydrolysis increases with an increase in pH and the attack of water on thioesters is subject to general base catalysis (*27*). In accord with the well-accepted mechanism of thioester hydrolysis, the hydrolysis of allococaine benzoyl thioester and allopseudococaine benzoyl thioester was markedly dependent on general base catalysis. In principle, hydrolysis of **2** or **3** could take place by an internal base-promoted mechanism or by an external base-promoted process that may involve stepwise or possibly water-mediated (i.e., Grotthuss-type) mechanisms. Although kinetic data, alone, do not permit an unambiguous choice of mechanism, in the pH range 7−9, a mechanism involving general base catalysis with the involvement of water is favored (Chart 1). An alternative but kinetically equivalent mechanism involving general-acid-catalyzed protonation and hydroxide addition to the thioester (i.e., push−pull catalysis) is not favored because of the anticipated intense steric congestion for the proposed mechanism (Chart 1). While we did not expressly test this mechanism by doing a complete pH−rate profile study, such a mechanism would be expected to give a "bell-shaped" pH−rate profile, and this has not been observed previously in the literature for thioester hydrolysis. In fact, in the acidic portion of pH−rate profiles of thioester hydrolysis, sigmoidal behavior of a decreased hydrolysis below pH 2 has been observed (*28*).

The hydrolysis of allococaine benzoyl thioester has been shown to be catalyzed by PLE, hBuChE, mBuChE, and mAcetylChE, although, in principle, other carboxylesteras-

es could presumably also catalyze the hydrolysis of the cocaine benzoyl thioester diastereomers examined. Allococaine benzoyl thioester was chosen as a prototypical substrate because it is efficiently chemically synthesized in sufficient quantities, it is relatively stable to spontaneous hydrolysis (allopseudococaine benzoyl thioester was hydrolyzed considerably faster than allococaine benzoyl thioester at pH = 7.4), and it gave reliable enzyme kinetic data. The K_{mapp} values for the hydrolysis of allococaine benzoyl thioester by PLE, hBuChE, mBuChE, and mAcetylChE compares with that of the kinetic constants for hydrolysis of (−)-cocaine by the same esterases (*20*) and this suggests that allococaine benzoyl thioester could be a convenient surrogate substrate for studying (−)-cocaine esterases.

Although the configuration at the C-3 position is not the naturally occurring β configuration, it is possible that, due to the achievement of the boat-type conformation, the position of the aromatic group in allococaine benzoyl thioester is in the same general proximity as it exists in (−)-cocaine and, as such, provides a substrate that the enzyme recognizes as not too dissimilar to naturally occurring (−)-cocaine.

In summary, allococaine benzoyl thioester hydrolysis by esterases has several advantages over currently used assays for the study of cocaine esterases. It is sensitive, rapid, and simple. A further advantage to this assay is that enzyme activity can be readily determined even in relatively complex biological matrixes with the proviso that the medium does not contain high concentrations of strongly UV-absorbing materials. The assay described herein does not require any derivatization steps and avoids the use of radioactive substrates. The assay is not as sensitive as a radiometric procedure but is less ambiguous than simply monitoring radioactivity extracted from an enzyme incubation. For the esterases examined, it appears that parallel binding avidity and rate of hydrolysis exists in comparison of allococaine benzoyl thioester and (−)-cocaine and, thus, allococaine benzoyl thioester provides an acceptable surrogate for monitoring cocaine esterases.

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