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# Stereoselective Inhibition of Human Butyrylcholinesterase by Phosphonothiolate Analogs of (+)- and (−)-Cocaine

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**ABSTRACT.** The hydrolysis of cocaine (benzoylecgonine methyl ester) to ecgonine methyl ester by human butyrylcholinesterase (BuChE; EC 3.1.1.8) has been shown previously to constitute an important means to detoxicate this material to pharmacologically inactive metabolites. The naturally occurring (−)-cocaine is hydrolyzed to ecgonine methyl ester approximately 2000 times slower than the unnatural (+)-cocaine isomer. In good agreement with previous studies, (−)-cocaine bound to human BuChE with relatively good affinity and competitively inhibited the hydrolysis of the spectrophotometric substrate butyrylthiocholine with a $K_i$ value of 8.0 μM. Similarly, (+)-cocaine also showed relatively high affinity for the human BuChE and competitively inhibited butyrylthiocholine hydrolysis with a $K_i$ value of 5.4 μM. The phosphonothiolates corresponding to the transition state analogs for both (−)- and (+)-cocaine hydrolysis were synthesized and tested as inhibitors of human BuChE-catalyzed hydrolysis of butyrylthiocholine. The phosphonothiolate corresponding to the transition state for (−)-cocaine hydrolysis was a competitive inhibitor with a $K_i$ value of 55.8 μM. The phosphonothiolate corresponding to the transition state for (+)-cocaine hydrolysis gave a $K_i$ value of 25.9 μM, but, in addition, it also showed irreversible inhibition with a $k_i$ of inactivation of 68.8 $min^{-1}$ $M^{-1}$. It is likely that the mechanism-based inhibitor described herein may find use as a mechanistic probe of butyrylcholinesterase action and also possibly aid in the purification of this class of esterases. BIOCHEM PHARMACOL **54**;11: 1261–1266, 1997. © 1997 Elsevier Science Inc.

**KEY WORDS.** human butyrylcholinesterase mechanism; (+)- and (−)-phosphonothiolate inhibitors; stereoselective enzyme inhibition; transition-state mechanism-based inhibitors; cocaine detoxication

Overuse of and addiction to the psychomotor stimulant (−)-cocaine are significant public health problems in the United States [1]. In a frightening trend for the economic and social consequences of future generations, after remaining constant between 1992 and 1993, a small but significant increase in cocaine use has been observed for eighth and tenth graders [2]. The use of illicit drugs by high school seniors in 1994 (i.e. 45.6%) is up from 1993 (i.e. 42.9%), although it is still less than the peak year of 1981 (i.e. 65.6%). The complex picture of the pharmacology of (−)-cocaine action is thought to involve binding to the dopamine transporter and inhibition of dopamine re-uptake, as well as interaction with other receptors and transporters [3, 4]. Naturally occurring (−)-cocaine is the levo enantiomer. The C-2 and C-3 centers of chirality of cocaine make a significant contribution to the pharmacological properties of cocaine. For example, synthetic (+)-cocaine is a much weaker inhibitor of the dopamine transporter [5] and lacks the behavioral properties of (−)-cocaine [3, 6]. The duration of action of cocaine is primarily controlled by hydrolysis to benzoylecgonine and ecgonine methyl ester by serum and hepatic esterases [7–9]. Both benzoylecgonine and ecgonine methyl ester are inactive as stimulants when administered to humans [10]. The prominent enzyme responsible for the debenzoylation of cocaine in human serum is BuChE† (EC 3.1.1.8). The craving for and pharmacological response to cocaine are probably related to the short duration of action of (−)-cocaine. In contrast, (+)-cocaine, the unnatural isomer, is hydrolyzed over 2000-fold faster than (−)-cocaine [11] by horse BuChE. Rapid hydrolysis of (+)-cocaine and the weak inhibitory properties against dopamine re-uptake may account for the lack of pharmacological activity of (+)-cocaine.

The objectives of the present study were to determine: (a) the kinetic properties of (+)- and (−)-cocaine in the presence of human BuChE, and (b) the mechanism and potency of phosphonothiolate analogs of the transition state of (+)- and (−)-cocaine hydrolysis as selective inhibitors of human BuChE (Fig. 1). The conclusion from this study was that (+)- and (−)-phosphonothiolate ana-

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† *Abbreviations:* BuChE, butyrylcholinesterase; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); MS(FAB), fast atom bombardment mass spectrometry; BuTCh, butyrylthiocholine; and hCE-1, human carboxylesterase (form 1).

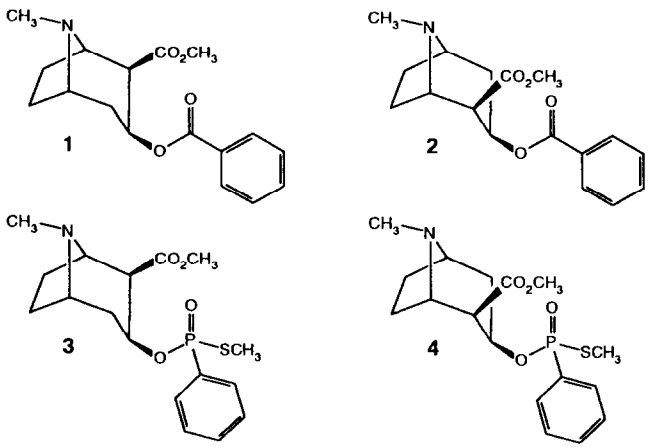


**FIG. 1. Structures of cocaine and phosphonothiolate isomers used in this study. Compound 1 is (−)-cocaine, compound 2 is (+)-cocaine, compound 3 is (−)-3β-(methyl phenylphosphonothioyloxy)-8-azabicycl[3.2.1]octane-2β-carboxylic methyl ester, and compound 4 is (+)-3β-(methyl phenylphosphonothioyloxy)-8-azabicycl[3.2.1]octane-2β-carboxylic methyl ester.**

logs of cocaine hydrolysis were selective inhibitors of human BuChE acting by, respectively, irreversible and competitive mechanisms. Such reagents could find use as mechanistic probes and as selective binding agents in the study of cholinesterases.

## MATERIALS AND METHODS

### *Chemicals*

(+)- and (−)-Cocaine and (+)- and (−)-ecgonine methyl ester were provided by the NIDA Drug Supply Program, National Institute of Health, Rockville, MD. BuTCh, DTNB, potassium ethylxanthate, dichlorophenylphosphine sulfide, tetrazole, and (S)-(−)-carbomethoxytropinone were obtained from the Aldrich Chemical Co. (Milwaukee, WI). All chemicals, buffers, and reagents were obtained from Fisher Scientific (Richmond, CA). All other chemicals were of the highest purity available and were purchased from commercial sources.

### *Chemical Synthesis*

COMPOUND 6. *R*-(−)-Ecgonine methyl ester (**5**) (0.50 g, 2.51 mmol) and tetrazole (0.018 g, 0.25 mmol) were dissolved in $CH_2Cl_2$ (10 mL) under an atmosphere of Ar (g). Diisopropylethylamine (685 μL, 5.52 mmol) was added via syringe followed by the addition of dichlorophenylphosphine sulfide (429 μL, 2.76 mmol), and the reaction mixture was stirred for 3 hr. Methanol (167 μL, 3.76 mmol) was added, and the reaction was stirred for an additional 1 hr. The solvent was removed *in vacuo*, and the product was purified by flash chromatography (silica gel; MeOH: $CH_2Cl_2$: $NH_4OH$ (30%) 10:90:0.04, by vol., $R_f$ = 0.55) to give a colorless oil (0.352 g, 38% yield). $^1$H NMR: δ 1.48–1.70 m, 3H; 1.82–2.12 m, 2H; 2.16 s, 3H; 2.35 and 2.50 dt, 1H, $J$ = 3.2, 11.7; 2.72 and 3.02 s, 1H; 3.16 and 3.26 s, 1H; 3.40 and 3.49 s, 1H; 3.55 and 3.73 s, 3H; 3.59 and 3.65 d, 3H, $J$ = 13.8; 4.72–4.94 m, 1H; 7.37–7.54 m, 3H; 7.77–7.95 m, 2H. $^{31}$P NMR: δ 88.21, 88.95.

COMPOUND 3. Potassium ethylxanthate (0.056 g, 345 μmol) and **6** (0.116 g, 314 μmol) were dissolved in dry acetone and refluxed for 10 hr. The procedure was a modification of a previous report for a related compound [12]. Thereafter, dimethylsulfate (33 μL, 345 μmol) was added, and the reaction mixture was refluxed for an additional 0.5 hr. The solvent was removed *in vacuo*, and the product was purified by flash chromatography (silica gel; MeOH:$CH_2Cl_2$, 10:90, v/v, $R_f$ = 0.25) to give a pale yellow oil (0.070 g, 61% yield). $^1$H NMR: δ 1.54–1.70 m, 2H; 1.82 s (br), 1H; 1.92–2.16 m, 2H; 2.04 and 2.08 d, 3H, $J$ = 13.7; 2.18 s, 3H; 2.30–2.60 m, 1H; 2.96 and 3.08 s, 1H; 3.20 and 3.26 s, 1H; 3.49 s, 1H; 3.73 and 3.65 s, 3H; 4.78–4.92 m, 1H; 7.38–7.56 m, 3H; 7.76–7.90 m, 2H. $^{31}$P NMR: δ 44.62, 44.95. MS(FAB) $m/z$ 370 (M + H).

COMPOUND 9. *S*-(+)-Ecgonine methyl ester (**8**) was prepared from *S*-(−)-carbomethoxytropinone by sodium-mercury amalgam reduction in 26% yield as described previously [13]. (*S*)-Phosphonothionate (**9**) was prepared as described above for compound **6** starting with *S*-(+)-ecgonine methyl ester, compound **8**. $^1$H NMR and $^{31}$P NMR for compound **9** were identical to those of compound **6**.

COMPOUND 4. (*S*)-Phosphonothiolate (**4**) was prepared as described above for compound **3** starting with the (*S*)-phosphonothionate, compound **9**. $^1$H NMR and $^{31}$P NMR for compound **9** were identical to those of compound **3**.

### *Enzyme Preparations*

Human BuChE was isolated and purified as described previously [14]. The wild-type human BuChE had an activity of 20.8 μmol of benzoylcholine hydrolyzed/min/mL. Enzyme activities were determined at room temperature. $K_m$ determinations for each BuChE incubation were measured spectrophotometrically by a modification of a previously described method [15]. To each cuvette was added DTNB (900 μL of 5.55 mM DTNB in 50 mM potassium phosphate buffer, pH 7.4) followed by the addition of BuTCh (25 μL of a methanolic solution of BuTCh of varying concentration). The enzymatic reaction was initiated at 25° by the addition of enzyme (75 μL of human BuChE, appropriately diluted in 50 mM, pH 7.4, potassium phosphate buffer to give 0.005 units based on benzoylcholine hydrolysis), and absorbance increases were monitored at 412 nm for 2 min as described previously [16]. The rate of hydrolysis for each substrate concentration was measured as absorbance increase per minute and was corrected for background hydrolysis from a cuvette containing DTNB and BuTCh without enzyme. $K_m$ and $V_{max}$ values were

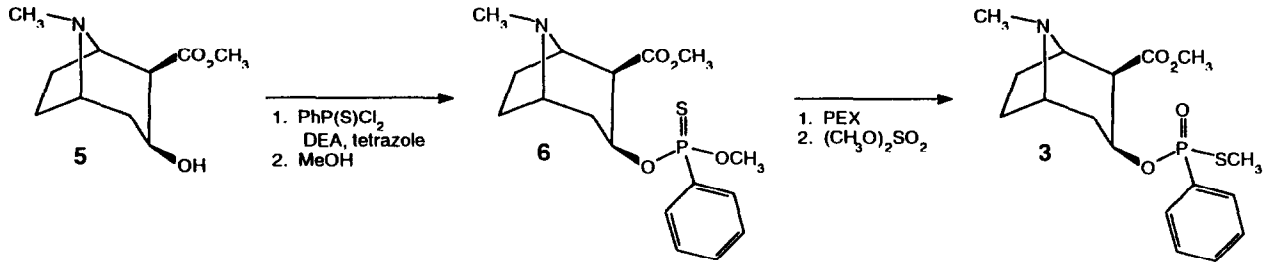


**FIG. 2. Chemical synthesis of the phosphonothiolate (compound 3) of a proposed transition state analog of (−)-cocaine hydrolysis.**

obtained from double-reciprocal plots of 1/rate versus 1/substrate concentration.

To determine the effects of the compounds as reversible inhibitors of human BuChE, the above-described procedure was followed except that a methanolic solution containing both substrate and inhibitor at varying concentrations was used: final substrate concentration varied from 25 to 100 μM; final cocaine-inhibitor concentration varied from 0 to 16 μM, and final phosphonothiolate-inhibitor varied from 0 to 25 μM. The absorbance increase was monitored at 412 nm for 2 min, and the rate of hydrolysis for each incubation condition was measured as absorbance increase per minute and was corrected for background hydrolysis from a cuvette containing DTNB and BuTCh without enzyme. Values for $K_i$ were obtained from the average of three Dixon plots of 1/rate versus inhibitor concentration.

To determine the extent of irreversible inactivation of human BuChE, the following procedure was used. A 375-μL solution of human BuChE (0.0667 units/mL benzoylcholine activity) was incubated with a solution of inhibitor (concentration of inhibitor chosen to give approximately 90% inactivation after a 12-min incubation). At selected time intervals from 0 to 12 min, 100 μL of the enzyme–inhibitor mixture was withdrawn and placed in a cuvette containing 900 μL of a solution containing DTNB and BuTCh (5.55 mM DTNB and 0.278 mM BuTCh in 50 mM, pH 7.4, potassium phosphate buffer). The remaining enzyme activity for each incubation time was determined by measuring the absorbance increase monitored at 412 nm for 2 min. The rate of hydrolysis for each incubation time was determined as absorbance increase per minute and was corrected for background hydrolysis from a cuvette containing DTNB and BuTCh without enzyme. Values for $k_i$ were obtained from the average of three plots of $\ln(A_0/A_t)$ versus time, where $A_0$ was the rate of hydrolysis at time = 0 and $A_t$ was the rate of hydrolysis at time = $t$.

Although (+)-phosphonothiolate (4) showed irreversible inactivation of human BuChE, it is important to note that 4 exists as a mixture of diastereomers (racemic at the asymmetric phosphorus center) and that stereoselective inactivation of human BuChE for these diastereomers is a likely possibility. Resolution of these diastereomers will permit studies for stereoselective inactivation and may provide insight into the structure of the human BuChE active site.

## RESULTS

To examine the stereoselectivity requirements for inhibition of human BuChE by cocaine, several phosphorous-containing analogs of (+)- and (−)-cocaine were chemically synthesized. The synthesis of the phosphonothiolate analogs of (−)- and (+)-cocaine is described in Figs. 2 and 3, respectively. Briefly, (+)- or, separately, (−)-ecgonine methylester (i.e. 8 and 5, respectively) was treated with dichlorophenylphosphine sulfide in the presence of diisopropylethylamine and tetrazole followed by methanol to produce the (+)- or (−)-phosphonothionates, compounds 9 and 6, respectively. As observed previously [17], the dimeric phosphonothionate was also formed. Treatment of compound 6 or compound 9 with potassium ethylxanthate followed by re-alkylation with dimethylsulfate gave the desired (−)- and (+)-phosphonothiolate, compounds 3 and 4, respectively (Figs. 2 and 3, respectively). It should be noted that the phosphonothiolates 3 and 4 were formed as diastereomeric mixtures, racemic at the asymmetric phosphorus center, and this fact was confirmed by $^{31}P$ NMR and $^{1}H$ NMR. Each synthetic compound was completely characterized spectroscopically.

Preliminary studies showed that CHO cell-expressed human BuChE catalyzed the hydrolysis of a number of esters. As a standard for comparison, the hydrolysis of

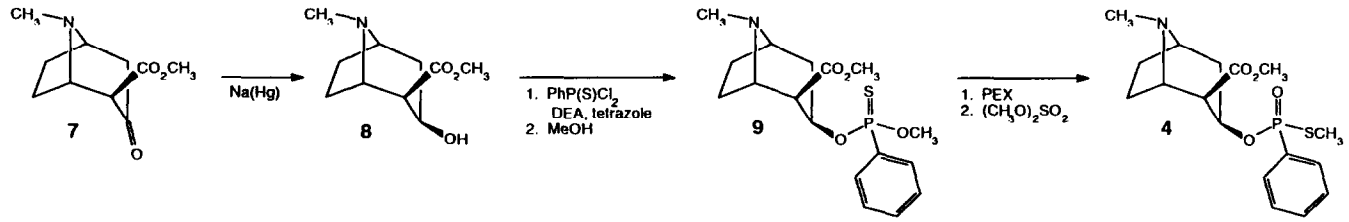


**FIG. 3. Chemical synthesis of the phosphonothiolate (compound 4) of a proposed transition state analog of (+)-cocaine hydrolysis.**

**TABLE 1. Kinetic values for the stereoselective inhibition of human BuChE by cocaine and phosphonothiolate isomers of cocaine***

| Compound | $K_i$ (μM) |
|---|---|
| (−)-Cocaine, **1** | 8.0 |
| (+)-Cocaine, **2** | 5.4 |
| (−)-Thiolate, **3** | 55.8 |
| (+)-Thiolate, **4** | 25.9 |

* Incubations were done as described in "Materials and Methods."

benzoylcholine was examined. Under standard conditions, human BuChE hydrolyzed benzoylcholine at a rate of 20.5 μmol/min/mL of enzyme. For BuTCh, hydrolysis was a linear function of human BuChE protein concentration (0 to 7.5 mU of protein) and of time (0–3 min) (data not shown).

Enzyme kinetic studies showed that BuTCh was a good substrate for human BuChE expressed in CHO cells. The substrate concentration required for half-maximal activity (i.e. $K_m$) was 21.4 μM and the $V_{max}$ at infinite BuTCh substrate concentration was 7.65 μmol/mL/min as determined by double-reciprocal plots. These values were similar to values reported before [18].

In good agreement with a previous report [11], the results herein showed that (+)-cocaine was a much better substrate than (−)-cocaine for human BuChE-catalyzed benzoate hydrolysis. However, (+)-cocaine inhibited BuTCh hydrolysis approximately as efficiently as (−)-cocaine. Thus, in the presence of various concentrations of (−)- or (+)-cocaine, a series of reciprocal plots of 1/velocity of BuTCh hydrolysis versus 1/substrate concentration gave a series of intersecting Lineweaver–Burk plots. Secondary plots of 1/slope versus inhibitor concentration provided $K_i$ values of 8.0 and 5.4 μM for (−)- and (+)-cocaine, respectively (Table 1).

Preliminary studies examining the inhibitory properties of (−)- and (+)-phosphonothiolates (**3** and **4**, respectively) showed that (−)-phosphonothiolate (**3**) followed competitive inhibition kinetics and (+)-phosphonothiolate (**4**) showed irreversible inhibition kinetics. Thus, reciprocal plots of 1/velocity versus 1/substrate at various concentrations of **3** showed a common intercept in 1/velocity and no hint that inhibition was time dependent. Additional studies incubating **3** for protracted time periods also indicated no observable irreversible inhibition of human BuChE. The $K_i$ value determined for **3** was 55.8 μM. In contrast, the (+)-phosphonothiolate, compound **4**, showed time-dependent inactivation of human BuChE-catalyzed hydrolysis of BuTCh. From a series of $\ln(A_0/A_t)$ versus time plots, the inactivation rate constant, $k_i$ was determined to be 68.8 $min^{-1}$ $M^{-1}$. Replots of the primary kinetic data as reciprocal plots of 1/velocity versus of 1/substrate at different concentrations of inhibitor **4** also provided kinetic constants for the competitive inhibition component of compound **4**. The $K_i$ value was determined to be 25.9 μM, less than half the value for the (−)-phosphonothiolate isomer, compound **3** (Table 1).

## DISCUSSION

In good agreement with previous observations, both (−)- and (+)-cocaine bound relatively efficiently to human BuChE as shown by their similar $K_i$ values (i.e. 8.0 and 5.4 μM, respectively) [7, 18–20]. However, (+)-cocaine binding leads to much more efficient hydrolysis because the hydrolysis of (+)-cocaine is over 1000-fold faster than (−)-cocaine for human BuChE [11]. We extended these observations concerning the influence of stereochemistry on the selectivity of cocaine hydrolysis by designing and testing the phosphonothiolate analog inhibitors of (+)- and (−)-cocaine (i.e. compounds **4** and **3**, respectively) as mechanistic probes of human BuChE.

During the course of the human BuChE-catalyzed hydrolysis of the benzoyl ester of cocaine, the planar carbonyl is changed into a center with tetrahedral geometry. One way to mimic the proposed tetrahedral nature of the hydrolytic transition state with a chemically stable analog is with phosphonate structures. Based on the fact that binding of (−)-cocaine does not efficiently lead to cocaine debenzoylation but binding of (+)-cocaine does, we tested the hypothesis that the (+)-phosphonothiolate (**4**) would function as a mechanism-based irreversible inactivator and (−)-phosphonothiolate (**3**) would serve only as a competitive inhibitor. In practice, this was what was observed: **3** was shown to be a competitive inhibitor and **4** was shown to possess irreversible inhibitor properties. The conclusion is that changing the geometry of the benzoyl group of the cocaine isomers to a tetrahedral arrangement by utilizing a phosphonothiolate moiety does not abrogate the inhibitory activity of the isomers and, in the case of the (+)-phosphonothiolate analog of (+)-cocaine, actually markedly enhances the irreversible nature of the interaction. This result supports the notion that the transition state for the hydrolysis of the benzoyl ester of (−)- and (+)-cocaine is closely related to the tetrahedral-like structures of **3** and **4**, respectively. Because **3** and **4** possess a thiomethoxy substituent attached to phosphorous, they are not true tetrahedral intermediate analogs. However, what is revealed is that proper orientation of the C-2 methyl ester group relative to the benzoyl moiety in the tetrahedral intermediate of the hydrolysis of cocaine must be an important prerequisite for productive hydrolysis of cocaine by human BuChE. Although the $K_i$ values for **3** and **4** are larger relative to (−)- and (+)-cocaine, respectively, the C-3 group must be important for binding of the cocaine isomers to the human BuChE for inhibitory and catalytic activity. Had the inhibition constants not been different for **3** and **4**, the result would have suggested that the benzoyl group was less important for enzyme binding than the configuration of the C-2 methyl ester. Thus, the benzoyl group may serve to anchor the cocaine molecule at the active site, and the relative orientation of the C-2 methyl

ester in the active site may determine whether the hydrolytic mechanism proceeds highly efficiently or rather sluggishly. The prediction is that the relative position of the C-2 methyl ester is the structural determinant for the *hydrolysis* of cocaine but not necessarily the determinant for *binding* efficiency. Stated another way, the C-2 methyl ester of (−)-cocaine may interfere with some important catalytic residue, but the C-2 methyl ester of (+)-cocaine is rotated away from the critical residue and hydrolysis of (+)-cocaine is observed. The binding region may not be in the same position needed for catalysis, and both isomers of cocaine may be accepted for binding with the same approximate affinity.

Recently, Masson *et al.* [21] proposed a mechanism for human BuChE with three discrete enzyme–substrate complexes. A separate study described a model depicting three distinct domains for mouse acetylcholinesterase and BuChE [22]. For the human BuChE model, the first enzyme–substrate complex, ES1, was proposed to be formed when substrate binds to Asp70 of human BuChE, which is located on the rim of the substrate binding gorge. The second complex, ES2, is proposed to arise when the substrate slides down the substrate-binding gorge and binds to Trp 82. Trp 82 is the key factor in the stabilization of positively charged substrates in the ES2 and ES3 complexes, although this interaction should be more properly named a cation-Π interaction [23]. The third complex, ES3, forms when the substrate rotates to a position within a favorable distance for nucleophilic attack and hydrolysis by Ser 198. The most stable complex, ES2, is probably the one that is measured and provides the values that are reported in Table 1. From the data, both (−)- and (+)-cocaine bind with similar affinity to ES2. On the basis of the model, the hypothesis is that (+)-cocaine readily fits near Ser 198 to form ES3 and is efficiently hydrolyzed at the benzoyl ester. On the other hand, (−)-cocaine apparently has difficulty rotating into position proximal to Ser 198, and only very few molecules assume the correct orientation that allows for hydrolysis of the benzoyl ester. The apparent limitation on the rotation of (−)-cocaine for correct fit near Ser 198 is also manifested in the observation that (−)-phosphonothiolate (**3**) shows only competitive inhibition and does not get close enough to Ser 198 to work as a mechanism-based inactivator. The (+)-phosphonothiolate (**4**) apparently does correctly fit near Ser 198 because **4** irreversibly inactivates human BuChE by efficient participation of Ser 198 and covalent modification.

It is notable that the human liver cocaine esterase (hCE-1) [9] is also inhibited by compound **3** (i.e. $K_i$ value = 170 μM) [17]. The hCE-1 hydrolyzes the C-2 methyl ester of cocaine, but it possesses the opposite stereoselectivity for inhibition [i.e. (−)-cocaine inhibits hCE-1 with a $K_i$ value of 10 μM and (+)-cocaine inhibits hCE-1 with a $K_i$ value of >10 mM]. Increased lipophilicity of the substituent at the C-3 position also tends to enhance the inhibitory activity (i.e. the corresponding thione and dithiophosphonate of **3** inhibited hCE-1 with $K_i$ values of 30 and 10 μM, respectively). Thus, an increase in lipophilicity at the C-3 position increases the inhibitory potency toward hCE-1. Irreversible inhibition was not observed for hCE-1 in the presence of **3** or its phosphonothio-containing analogs [17].

The fact that the (+)-phosphonothiolate, compound **4**, is a mechanism-based inactivator of human BuChE and a tight-binding competitive inhibitor of hCE-1 suggests that this compound could serve as a mechanistic probe of cocaine hydrolysis. The (−)-phosphonothiolate, compound **3**, could serve as an efficient affinity ligand in the purification of cocaine esterases from human serum. Future studies will be directed at examining the selectivity of enzyme inhibition and the stereoselectivity of the mechanism. For example, it is possible that one of the phosphonothiolate diastereomers is considerably more potent at inactivating human BuChE and, as has been shown before for related systems [16], purification of phosphorothiolate diastereomers could provide novel stereochemical probes of cholinesterase action.

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