Journal of Medicinal Chemistry

Article

pubs.acs.org/jmc

Metaproterenol, Isoproterenol, and Their Bisdimethylcarbamate Derivatives as Human Cholinesterase Inhibitors

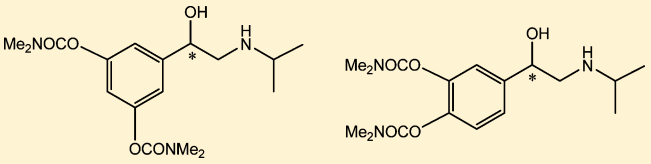
Anita Bosak,[†] Ivana Gazić Smilović,[‡] Goran Šinko,[†] Vladimir Vinković,[‡] and Zrinka Kovarik*[,†]

[†]Institute for Medical Research and Occupational Health, Ksaverska cesta 2, HR-10000 Zagreb, Croatia

[‡]Ruđer Bošković Institute, Bijenička 54, HR-10000 Zagreb, Croatia

Supporting Information

ABSTRACT: Metaproterenol and isoproterenol are bronchodilators that provide a structural basis for many other bronchodilators currently in use. One of these structurally related bronchodilators is terbutaline; it is administered as a prodrug, bambuterol, and is metabolized (bioconverted) into terbutaline by butyrylcholinesterase (BChE). The metabolism rate can be affected by BChE gene polymorphism in the human population and BChE stereoselectivity. The aim of our study was to investigate inhibition of human BChE and acetylcholinesterase (AChE) with metaproterenol, isoproterenol, and newly synthesized racemic bisdimethylcarbamate derivatives of metaproterenol (metacarb) and isoproterenol (isocarb) and their (*R*)-enantiomers to see if their bioconversion is affected by BChE inhibition in the same way as that for bambuterol. Metacarb and isocarb proved to be selective BChE inhibitors, as they progressively inhibited AChE 960 to 80 times more slowly than $BChE_{UU}$. All studied cholinesterases displayed poor affinity for metaproterenol and isoproterenol, yet $BChE_{UU}$ had an affinity about five times higher than that of AChE.



1. INTRODUCTION

Metaproterenol (also known as orciprenaline) is a bronchodilator currently used to treat asthma, bronchitis, and emphysema. Isoproterenol (also known as isoprenaline) is mostly used to treat bradycardia and heart block, while its use as a bronchodilator is rare. Metaproterenol and isoproterenol are structurally related to terbutaline, a fast-acting bronchodilator and tocolytic for delaying premature labor. All three compounds are β-amino alcohol benzenediols that constitute the elementary pattern for most bronchodilators in use or with a potential for use.[1] The side effects of the marketed bronchodilators include tachycardia, hypertension, and tremor, and their therapeutic effect is often short.[1] To prolong the therapeutic effect, some formulations use a prodrug such as bambuterol, a bisdimethylcarbamate ester of terbutaline.[1,2] In vivo conversion of bambuterol involves hydrolysis by butyrylcholinesterase (BChE EC 3.1.1.8), which is present in the organism in high concentrations and is capable of hydrolyzing a variety of esters.[3] BChE and the related enzyme acetylcholinesterase (AChE; EC 3.1.1.7) hydrolyze carbamates by forming a covalent bond between the carbamic group of the carbamate ester and the hydroxy group of the catalytic serine.[4] However, because of a slow decarbamylation rate, carbamates represent progressive inhibitors of cholinesterases.[4]

The high therapeutic index of bambuterol is the result of its inhibitory preference for BChE over AChE.[5−7] It inhibits usual human BChE about 20000 times faster than human AChE and mouse BChE about 8600 times faster than mouse AChE.[6,8,9] BChE is also involved in the bioconversion of other ester-based prodrugs.[10] The efficacy of a drug or a prodrug that requires BChE action is affected by mutations of the human *BCHE* gene. More than 56 mutations of the *BCHE* gene have been identified to date, and several BChE variants possess different catalytic properties or are expressed at lower levels than usual BChE ($BChE_{UU}$).[3,11] For example, people with atypical BChE ($BChE_{AA}$) are not able to hydrolyze succinylcholine and can experience prolonged apnea if this muscle relaxant is administered.[12]

Among the many chiral drugs, one enantiomer is often more active than the other in treating a medical condition. The (*R*)-enantiomer of the short-acting bronchodilator albuterol is approximately 80 times more potent as a β-adrenoceptor agonist than the (*S*)-enantiomer, and the administration of pure (*R*)-albuterol improves therapeutic effects and reduce side effects.[13] Similar in vivo results were obtained with the enantiomers of terbutaline.[14] Both AChE and BChE are stereoselective in the interaction with various esters such as phosphonates, acetate derivatives of quinuclidin-3-ols, and carbamates such as physostigmine and bambuterol.[7−9,15,16]

In this paper, we describe the synthesis of racemic bisdimethylcarbamate derivatives of metaproterenol (metacarb) and isoproterenol (isocarb) and their (*R*)-enatiomers (Figure 1). The aim was to determine the inhibition rates of five BChE variants, expected by analogy to bambuterol to critically affect the rates of metacarb and isocarb bioconversion. We also determined inhibition rates of AChE to establish metacarb and isocarb inhibition selectivity between AChE and BChE, expecting it to be similar to that of bambuterol. The stereoselectivity of AChE and four BChE variants was

Received: November 11, 2011
Published: July 20, 2012

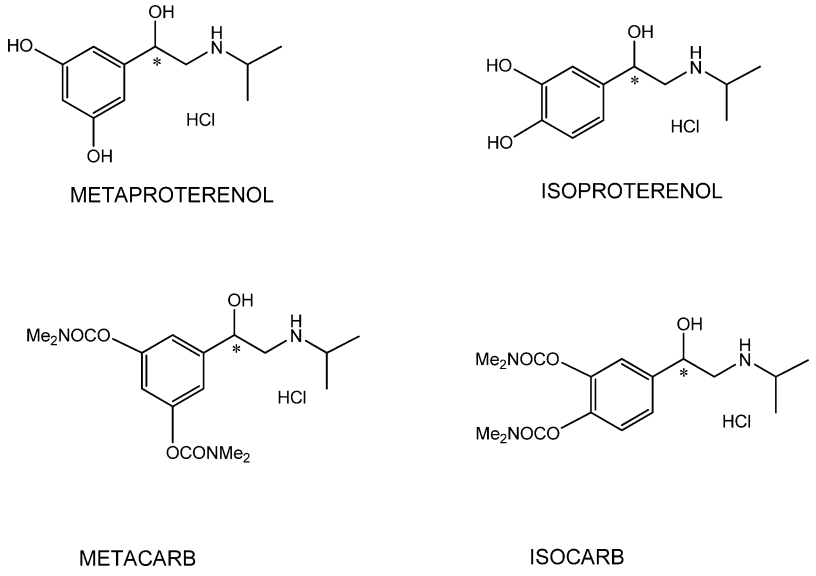


Figure 1. Chemical structures of tested inhibitors. Asterisk denotes a chiral center.

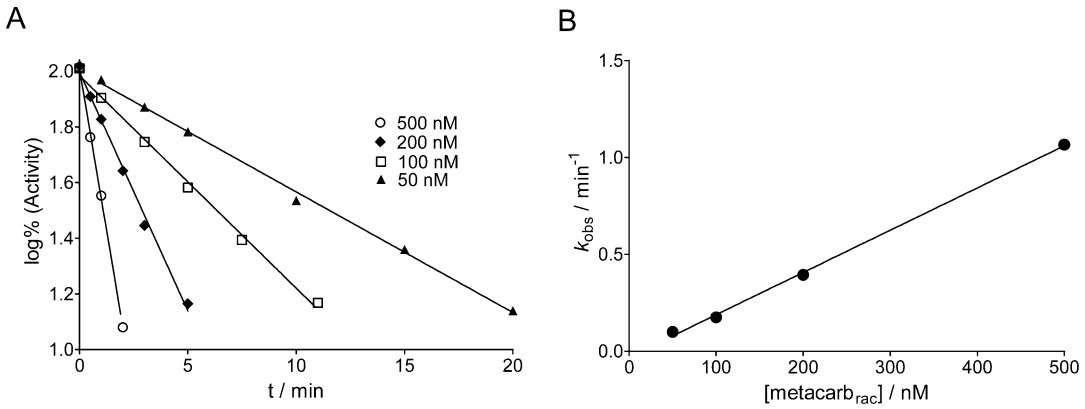


Figure 2. Representative progressive inhibition of $BChE_{UU}$ by racemic metacarb. (A) Points indicate the logarithm of residual activity, while slope of the lines determines the first-order rate constant k_{obs}. (B) k_{obs} was plotted as a function of $metacarb_{rac}$ concentration wherefrom the second-order rate constant, k_i, was calculated.

estimated based on comparison of inhibition rate constants determined for racemic and (*R*)-enantiomers of bisdimethylcarbamates. Inhibition selectivity of metacarb and isocarb and stereoselectivity of $BChE_{UU}$ were analyzed by molecular modeling of the transition state of carbamylation by (*R*)-enantiomers.

2. RESULTS AND DISCUSSION

2.1. Inhibition by Metacarb and Isocarb. Bisdimethylcarbamates, metacarb and isocarb, displayed time-dependent inhibition of all the tested BChE variants. Inhibition followed first-order kinetics at any given inhibitor concentration (Figure 2A), and the first-order rate constant ($k_{[AB]}$) was a linear function of metacarb and isocarb concentration (Figure 2B). The overall inhibition rate constant (k_i) expresses the first step in metacarb and isocarb hydrolysis (Table 1) and represents a measure of metacarb and isocarb inhibition potency. The order of magnitude of k_i constants for the tested bisdimethylcarbamates and BChE variants was 10^6 M^{-1} min^{-1}, which makes both compounds very fast BChE inhibitors. The fastest inhibition by both metacarb and isocarb was obtained for $BChE_{UU}$, and the slowest for $BChE_{AA}$. The difference in inhibition rates of $BChE_{UU}$ and $BChE_{AA}$ is primarily the result

Table 1. Progressive Inhibition of Human BChE and AChE by Racemic Metacarb and Isocarb

	k_i (10^3 M^{-1} min^{-1})	
enzyme	$metacarb_{rac}$	$isocarb_{rac}$
$BChE_{UU}$	2200 ± 40	200 ± 7.9
$BChE_{AA}$	40 ± 8.8	21 ± 0.48
$BChE_{UA}$	1400 ± 46	160 ± 8.5
$BChE_{FF}$	600 ± 16	140 ± 2.6
$BChE_{AF}$	440 ± 16	87 ± 0.60
AChE		
k_i (10^3 M^{-1} min^{-1})	2.3 ± 0.51	2.5 ± 0.91
k_{max} (min^{-1})	1.6 ± 0.14	3.5 ± 0.57
K_i (mM)	0.71 ± 0.14	1.4 ± 0.46

of amino acid substitution at position 70, where acidic amino acid aspartate is replaced with aliphatic glycine (D70G). This is in agreement with previous studies which showed that charged inhibitors such as bambuterol, dibucaine, and Ro 02-0683 reduced their inhibition potency toward the atypical BChE variant.[6,8,17] Inhibition of fluoride-resistant BChE ($BChE_{FF}$) by metacarb or isocarb was about three times or about 30% lower than the inhibition of $BChE_{UU}$. This drop could be attributed to

dx.doi.org/10.1021/jm300289k | J. Med. Chem. 2012, 55, 6716−6723

fluoride-resistant variant mutations T243M or G390V that, even when far from the enzyme active center, could cause conformational changes resulting in lower inhibition potency of positively charged ligands.[6,18]

The rate of cholinesterase carbamylation is determined by the entrance of carbamate and its orientation into the active site of the enzyme.[4] Metacarb and isocarb differ in disposition of carbamate groups on the benzene ring: meta-position in metacarb vs ortho-position in isocarb (Figure 3). Because

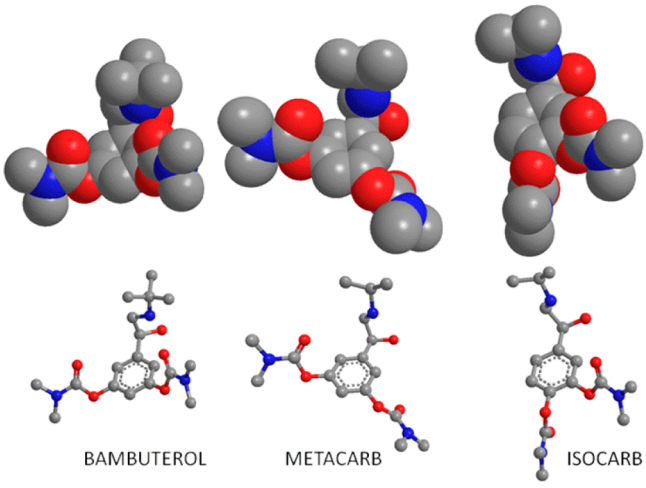


Figure 3. Minimized structures of bambuterol, metacarb, and isocarb. For better visibility, hydrogen atoms are not displayed.

metacarb was up to 11 times more potent toward BChE inhibition than isocarb (Table 1), it seems that the meta-position of carbamate groups on the benzene ring is preferred. This is supported by the fact that the overall inhibition rate of metacarb and bambuterol, where carbamate groups are also meta-positioned (Figure 3), is within the same order of magnitude.[8,17] Two times slower inhibition of usual BChE by metacarb compared to inhibition by bambuterol could be attributed to the different size of the alcohol moiety of these two biscarbamates: isopropyl group of metacarb vs *tert*-butyl group of bambuterol.[8] To elucidate the inhibition potency of the studied biscarbamates, we used molecular modeling to study the transition state of the carbamylation reaction. Figure 4 presents a simulation of the transition state between the hydroxyl group of the active site serine (Ser198) of human $BChE_{UU}$ (PDB code 2PM8) and the carbamic group on the C2 atom of the benzene ring of (*R*)-metacarb (Figure 4A), (*R*)-isocarb (Figure 4B), and (*R*)-bambuterol (Figure 4C). The transition states of carbamylation by three carbamates were stabilized by the cation−π interaction between the protonated nitrogen of carbamates and the indole ring of Trp82, and by the hydrogen bond between the oxygen of the carbamic group of carbamates and the amino group of Gly117. Differences in carbamylation rate by metacarb, isocarb, and bambuterol can be attributed to additional stabilization typical for each carbamate. The transition state of (*R*)-metacarb was additionally stabilized by two hydrogen bonds between the hydroxyl group on the chiral center and residues His438 and Glu197 (Figure 4A), and the transition state of (*R*)-isocarb was stabilized by the hydrogen bond between protonated nitrogen and Glu197. The transition state of (*R*)-bambuterol was stabilized by the cation−π interaction between protonated nitrogen and Tyr440

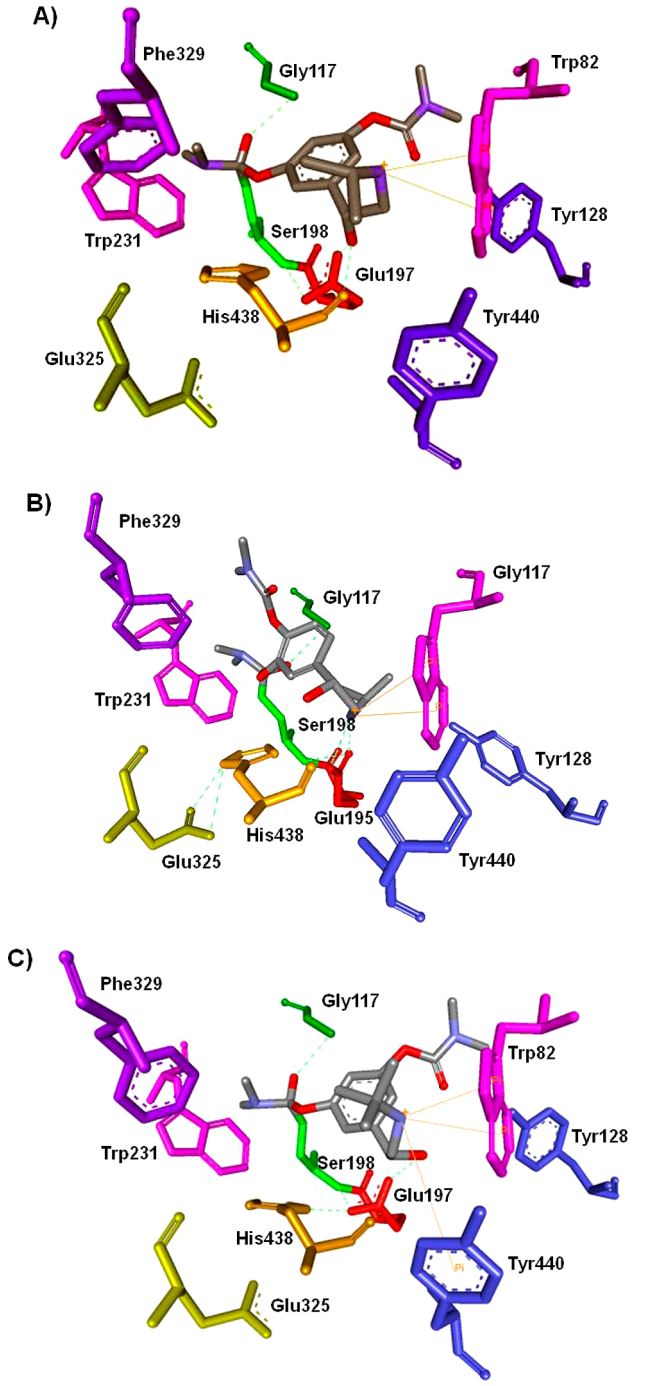


Figure 4. Computational molecular modeling of the transition states of human BChE and (*R*)-metacarb (A), (*R*)-isocarb (B), and (*R*)-bambuterol (C). Amino acid residues that stabilize the transition state are displayed. Ser198, His438, and Glu325 represent the catalytic triad. The structure was generated using the Accelrys Discovery Studio program[26] and the BChE structure (PDB ID code 2PM8).[27].

and by the hydrogen bond between Glu197 and the hydroxyl group on the chiral center (Figure 4C).

Dimethylcarbamates, metacarb, and isocarb, also inhibited AChE time-dependently but relatively slowly. The order of magnitude of overall inhibition rate constants was 10^3 M^{-1} min^{-1}, which was about ten times higher than previously determined for bambuterol, classifying metacarb and isocarb as inhibitors with low AChE inhibition potency.[7,8] The relation between the first-order rate constant ($k_{[AB]}$) and inhibitor

dx.doi.org/10.1021/jm300289k | J. Med. Chem. 2012, 55, 6716−6723

Table 2. Progressive Inhibition of Human BChE and AChE by *R*-Metacarb and *R*-Isocarb

enzyme	(*R*)-metacarb		(*R*)-isocarb		bambuterol
	k_i (10^3 $M^{-1}min^{-1}$)	$k_{i(R)}/k_{i(rac)}$	k_i (10^3 $M^{-1}min^{-1}$)	$k_{i(R)}/k_{i(rac)}$	[a] $k_{i(R)}/k_{i(rac)}$
$BChE_{UU}$	3200 ± 300	1.5	340 ± 8.7	1.7	1.5
$BChE_{AA}$	76 ± 2.3	1.9	31 ± 0.53	1.5	1.3
$BChE_{UA}$	2200 ± 77	1.6	210 ± 8.6	1.3	1.4
$BChE_{FF}$	1100 ± 42	1.8	150 ± 6.3	1.1	1.6
$BChE_{AF}$	550 ± 19	1.3	100 ± 3.0	1.1	1.5
AChE					
k_i (10^3 $M^{-1}min^{-1}$)	4.7 ± 1.2	2.0	2.7 ± 0.78	1.1	2.0
k_{max} (min^{-1})	1.9 ± 0.18		3.1 ± 0.41		
K_i (mM)	0.39 ± 0.096		1.2 ± 0.31		

[a]Reference 8.

Table 3. Reversible Inhibition of Human AChE, BChE, and Four BChE Variants by Metaproterenol and Isoproterenol

enzyme	metaproterenol			isoproterenol		
	[s] (mM)	$K_{(I)}$ (mM)	$K_{(S)}$ (mM)	[s] (mM)	$K_{(I)}$ (mM)	$K_{(S)}$ (mM)
$BChE_{UU}$	0.05–0.30	0.55 ± 0.11	0.098 ± 0.022	0.05–0.35	0.47 ± 0.21	0.095 ± 0.047
$BChE_{AA}$	0.05–1.0	6.1 ± 0.71	0.26 ± 0.035	0.05–1.0	1.6 ± 0.25	0.21 ± 0.039
$BChE_{FF}$	0.05–0.50	0.87 ± 0.18	0.14 ± 0.031	0.05–1.0	0.99 ± 0.12	0.42 ± 0.064
$BChE_{UA}$	0.05–0.50	0.65 ± 0.11	0.12 ± 0.021	0.05–0.5	0.72 ± 0.082	0.42 ± 0.057
AChE	0.03–0.20	3.1 ± 1.2	0.035 ± 0.014	0.05–1.0	2.5 ± 1.0	0.54 ± 0.31

concentration deviated from linearity, allowing determination of the maximal first-order rate constant of carbamylation, k_{max}, and dissociation constant, K_i (Table 1). Although overall metacarb and isocarb inhibition rate constants of AChE were very similar, intrinsic carbamylation constants differed. AChE had about two times higher k_{max} for isocarb than metacarb, while due to high standard deviations of the K_i constants, higher affinity ($1/K_i$) of AChE for metacarb than isocarb must be considered with a caution. Poor inhibition of AChE may have to do with $\pi-\pi$ interactions between the aromatic ring of bisdimethylcarbamates and aromatic residues of the active site that, due to steric constraints, disabled proper orientation of the carbamate group to catalytic serine, stabilizing the Michaelis type of bisdimethylcarbamate–AChE complex. The final result is the slow carbamylation rate.

The ratio of overall inhibition rate constants for BChE and AChE describes the inhibition selectivity of metacarb and isocarb (Table 1). Both bisdimethylcarbamates showed distinct BChE selectivity, as they inhibited AChE 80 to 960 times more slowly than $BChE_{UU}$. The highest BChE inhibition selectivity was determined for $BChE_{UU}$ while the lowest was for $BChE_{AA}$ by both bisdimethylcarbamates. In general, metacarb was more selective than isocarb. However, both compounds were less selective inhibitors compared to bambuterol, which could affect their possible future use as bronchodilators.

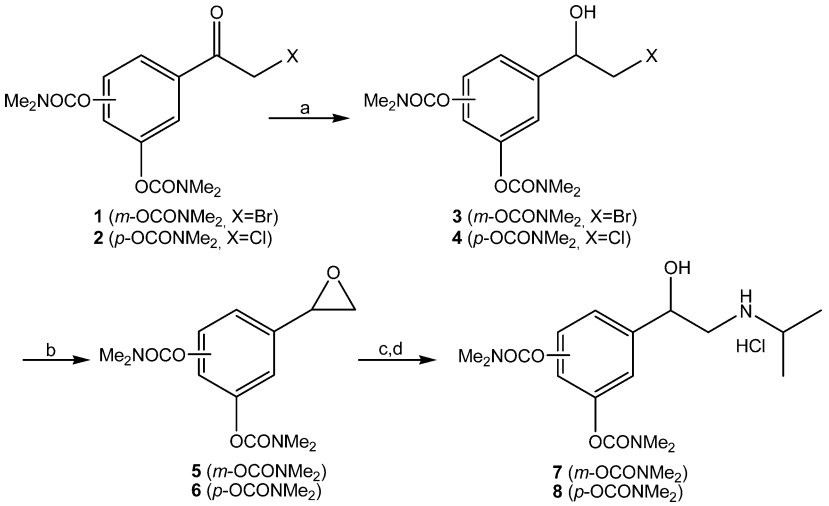
2.2. Inhibition by (*R*)-Enantiomers of Metacarb and Isocarb. As metacarb and isocarb are chiral molecules with the chiral center on the alcohol part of the molecule, stereoselectivity of cholinesterases could be expected as result of different inhibition rates by enantiomers (Figure 1). Due to difficulties in the synthesis of (*S*)-enantiomers, the stereoselectivity of cholinesterases was estimated from the ratio of the inhibition rate constant for racemate and (*R*)-enantiomer of metacarb and isocarb (Table 2). (*R*)-Metacarb was 1.3–2.0 times more potent than the racemate toward inhibition. Inhibition by (*R*)-isocarb was 1.3–1.7 times faster than the racemate for $BChE_{UU}$, $BChE_{AA}$, and $BChE_{UA}$ (heterozygote of usual and atypical BChE), while no difference was shown for $BChE_{FF}$, $BChE_{UF}$ (heterozygote of usual and fluoride resistant BChE), and AChE (Table 2). Stereoselectivity of human cholinesterases for bambuterol was determined previously when all the studied BChE variants as well as AChE revealed a four times higher preference for (*R*)- over (*S*)-bambuterol.[8] A comparison of the k_i ratio obtained for (*R*)-bambuterol and the racemate showed that cholinesterase stereoselectivity for metacarb and isocarb could be similar to those for bambuterol (Table 2).[8]

A molecular modeling study of the transition state of the carbamylation reaction was done for (*R*)-metacarb, (*R*)-isocarb, and (*R*)-bambuterol (Figure 4). Stabilization of the transition state of the (*S*)-enantiomer of bisdimethylcarbamates involves spatial reorientation of the hydroxyl group, which could lead to disappearance of the hydroxyl group interaction that stabilized the transition state of (*R*)-enantiomers.

2.3. Inhibition by Metaproterenol and Isoproterenol. Metaproterenol and isoproterenol are the final products of metacarb and isocarb hydrolysis, respectively. Both reversibly inhibited BChE and AChE, forming noncovalent interactions within the active site of the enzyme. To measure metaproterenol and isoproterenol inhibition potency, we determined the dissociation constants of the enzyme–inhibitor complex ($K_{(I)}$) and dissociation constants of the enzyme–substrate complex ($K_{(S)}$) (Table 3). $K_{(I)}$ constants ranged from 0.5 mM to 6.0 mM, typical for low-potency inhibitors. Usual BChE showed the highest affinity ($1/K_{(I)}$) for both inhibitors, and atypical BChE the lowest affinity. Furthermore, the affinity of $BChE_{UU}$, $BChE_{UA}$, and $BChE_{FF}$ was similar for metaproterenol and isoproterenol. The only exception was $BChE_{AA}$ with four times higher affinity for isoproterenol. The affinity of AChE to metaproterenol or isoproterenol was about four times lower than the affinity of the tested BChE variants. A comparison with the affinity to terbutaline, the final product of bambuterol hydrolysis, showed that usual BChE had about three times higher affinity to terbutaline than to metaproterenol or isoproterenol, while $BChE_{AA}$ had the highest affinity to isoproterenol, about two times higher than to terbutaline.[17]

dx.doi.org/10.1021/jm300289k | J. Med. Chem. 2012, 55, 6716–6723

Scheme 1. Synthesis of Racemic Metacarb (7) and Isocarb (8)[a]



[a](a) $NaBH_4$, MeOH; (b) NaOH aq, EtOH; (c) isopropylamine; (d) HCl in *i*-PrOH.

The determined $K_{(S)}$ constants were in good agreement with the constants corresponding to the Michaelis type complex or to the enzyme–substrate dissociation constants for binding at the catalytic site of the enzyme as reported previously.[17,19] This implies that at given substrate concentrations, both inhibitors bind to the catalytic site of the enzymes.

3. CONCLUSION

A very desirable characteristic for an asthma drug is prolonged action, so that a patient can have a whole night's sleep. Bambuterol, a bisdimethylcarbamate of terbutaline, ensures release of terbutaline over 24 h. The high therapeutic index of bambuterol is associated with its extremely selective inhibition of BChE compared to AChE. Dimethylcarbamates studied in this paper, metacarb and isocarb, are structurally similar to bambuterol, and we expected that they should display a similar selective inhibition of BChE. However, both proved far less selective for BChE than bambuterol and therefore less likely to be used as prodrugs of bronchodilating agents. Both bisdimethylcarbamates have about 50 times higher affinity for the $BChE_{UU}$ than for $BChE_{AA}$, as was earlier demonstrated for bambuterol.[8,17] This study has also shown that metaproterenol and isoproterenol are reversible cholinesterase inhibitors. The affinity of both for any of the BChE variants was too low to interfere with metacarb and isocarb inhibition. Our findings provide new information on the kinetics of bisdimethylcarbamates by BChE. It seems that the meta-position of the two carbamate groups on the benzene ring allows easier entrance of bisdimethylcarbamate compounds and its orientation into the active site of the BChE. In addition, the *tert*-butyl group on the alcohol moiety of the ester, as it is in bambuterol, ensures efficient carbamylation of BChE by bisdimethycarbamates. BChE is stereoselective, preferring (*R*)-bisdimethylcarbamates over the (*S*)-enantiomer, probably because the stabilization of the transition state of the (*S*)-enantiomer involves spatial reorientation of the hydroxyl group.

4. EXPERIMENTAL SECTION

4.1. Chemicals. Racemic isoproterenol bitartarate and metaproterenol hemisulfate were purchased from Sigma-Aldridge (Sigma Chimica, Italy) and converted to hydrochlorides by treating them with an aqueous solution of Na_2CO_3 and by adding a solution of HCl in *i*-PrOH to form salts. All chemicals, reagents and solvents for the preparation of metaproterenol and isoproterenol bisdimethylcarbamate derivatives were purchased from commercial sources. Acetylthiocholine iodide (ATCh), propionylthiocholine iodide (PTCh), and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Sigma Chemical Co., St. Louis, MO. All inhibitors and substrates were dissolved in water, while DTNB was dissolved in 0.1 M sodium phosphate buffer (pH 7.4).

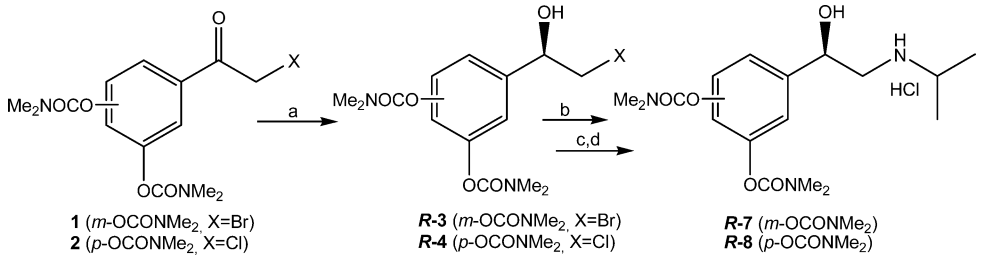
4.2. Synthesis. The racemic bisdimethylcarbamate derivate of metaproterenol (metacarb) was prepared following slightly modified protocols as described in patent US7495028B2 (Scheme 1).[20] 2′-Bromo-3,5-di(*N*,*N*-dimethylcarbamyloxy)acetophenone (**1**) was prepared (80% yield) by the reaction of copper(II) bromide with 3,5-di(*N*,*N*-dimethylcarbamyloxy)acetophenone, which was prepared from commercially available 3,5-dihydroxyacetophenone. The racemic bisdimethylcarbamate derivate of isoproterenol (isocarb) was prepared in a way (Scheme 1) similar to that for 2′-chloro-3,4-di(*N*,*N*-dimethylcarbamyloxy)acetophenone (**2**), which was prepared from commercially available 2′-chloro-3,4-dihydroxyacetophenone. The substituted acetophenones **1** and **2** were reduced with $NaBH_4$ to respective alcohols **3** and **4**, which were further reacted with NaOH to form their epoxides **5** and **6**, respectively (details in Supporting Information).

*3,5-Bis(N,N-dimethylcarbamyloxy)metaproterenol Hydrochloride (**7**).* 2-[3,5-Di(*N*,*N*-dimethylcarbamyloxy)phenyl]oxirane (5) (1.0 mmol) was treated with isopropylamine under reflux overnight. The mixture was evaporated to dryness, diluted with water, and extracted with ethyl acetate (2 × 10 mL). The combined organic layers were washed with water, dried over $MgSO_4$, filtered off, and evaporated to dryness. The residue was purified by column chromatography (dichloromethane/methanol/triethylamine 20:1:0.04) to provide a free base in 75% yield as an oil. Purity of the compound was 98.79% (determined by RP HPLC). ^1H NMR ($CDCl_3$, 600 MHz) δ = 1.12 (d, J = 6.3 Hz, 6H), 2.70 (dd, J = 12.3 and 9.0 Hz, 1H), 2.85–2.94 (m, 2H), 2.99 (s, 6H), 3.07 (s, 6H), 4.73 (dd, J = 8.9 and 5.6 Hz, 1H), 6.68 (t, 2.6 Hz, 1H), 7.01 (d, J = 2.3 Hz, 2H) ppm. ^{13}C NMR ($CDCl_3$, 100 MHz) δ = 25.1, 36.1, 36.5, 47.6, 56.4, 78.2, 113.9, 116.9, 141.3, 153.2, 157.4 ppm. A solution of the obtained base (1.0 mmol) in *i*-PrOH (3 mL) was treated with a solution of HCl in *i*-PrOH (pH = 3) to give the hydrochloride salt of the metaproterenol bisdimethylcarbamate derivate.

*3,4-Bis(N,N-dimethylcarbamyloxy)isoproterenol Hydrochloride (**8**).* 2-[3,4-di(*N*,*N*-Dimethylcarbamyloxy)phenyl]oxirane (6) (1.0 mmol) was treated with isopropylamine under reflux overnight. The mixture was evaporated to dryness, diluted with water, and extracted with ethyl acetate (2 × 10 mL). The combined organic layers were

dx.doi.org/10.1021/jm300289k | J. Med. Chem. 2012, 55, 6716–6723

Scheme 2. Synthesis of (*R*)-Metacarb (*R*-7) and (*R*)-Isocarb (*R*-8)[a]



[a](a) (-)-DIP-chloride, THF, diethanolamine; (b) NaOH (aq), EtOH; (c) isopropylamine; (d) HCl in *i*-PrOH.

washed with water, dried over $MgSO_4$, filtered off, and evaporated to dryness. The residue was purified by column chromatography (dichloromethane/methanol/triethylamine 20:1:0.04) to provide a free base in 71% yield as an oil. Purity of compound was 98.65% (determined by RP HPLC). ^{1}H NMR ($CDCl_3$, 600 MHz) δ = 1.08 (d, J = 6.3 Hz, 6H), 2.66−2.91 (m, 3H), 3.01 (s, 6H), 3.07 (s, 6H), 4.69 (dd, J = 8.7 and 5.1 Hz, 1H), 7.20 (s, 2H), 7.25 (s, 1H) ppm. ^{13}C NMR ($CDCl_3$, 100 MHz) δ = 25.0, 36.4, 36.4, 48.1, 55.9, 78.1, 121.0, 121.4, 123.1, 136.2, 145.1, 154.6 ppm. A solution of the obtained base (1.0 mmol) in *i*-PrOH (3 mL) was treated with a solution of HCl in *i*-PrOH (pH = 3) to give hydrochloride salt of the isoproterenol bisdimethylcarbamate derivate.

(*R*)-Enantiomers of metacarb and isocarb were prepared following the above procedure. The chiral center was introduced in the reduction step (Scheme 2) using the enantiomerically pure reagent, (−)-chlorodiisopinocamphenylborane ((−)-DIP-chloride). This reagent provided the (*R*)-absolute configuration of metacarb and isocarb.

(R)-3,5-Bis(N,N-dimethylcarbamyloxy)metaproterenol hydrochloride (R-7). *R*-7 was prepared from (*R*)-2-[3,5-di(*N*,*N*-dimethylcarbamyloxy)phenyl]oxirane (*R*-**5**) following the same procedure as for the preparation of the racemic metaproterenol derivate, in 80% yield, 98.65% pure (determined by RP HPLC), and 99.54% ee (determined by HPLC, column Chiralpak-AD). NMR spectra were the same as for the racemic product.

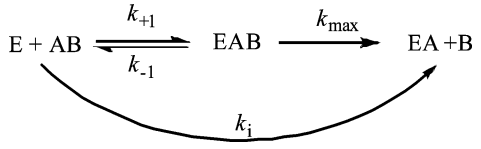
(R)-3,4-Bis(N,N-dimethylcarbamyloxy)isoproterenol hydrochloride (R-8). *R*-**8** was prepared from (*R*)-2-[3,4-di(*N*,*N*-dimethylcarbamyloxy)phenyl]oxirane (*R*-**6**) following the same procedure as for the preparation of the racemic isoproterenol derivate in 70% yield, 98.48% (determined by RP HPLC) and 83.51% ee (determined by HPLC, column Chiralcel-OD). NMR spectra were the same as for the racemic product.

4.3. Enzymes. The source of BChE was human native plasma and the source of AChE was human hemolyzed erythrocytes. Blood from donors with the usual BChE variant were collected at the Institute for Medical Research and Occupational Health, Zagreb, Croatia. BChE was obtained from heparinized blood after centrifugation (20 min, 2500 rpm, 25 °C) as supernatant and was phenotyped as described previously.[21] Sedimented erythrocytes were made up to the volume of whole blood by phosphate buffer. Atypical BChE ($BChE_{AA}$) was a gift from Dr. O. Lockridge (University of Nebraska Medical Center, Eppley Institute, Omaha, NE). Specimens of fluoride-resistant BChE ($BChE_{FF}$) and heterozygotes $BChE_{UA}$ and $BChE_{AF}$ were donated by Dr. R. T. Evans (Cholinesterase Investigation Unit, St. James's University Hospital, Leeds, Great Britain). All enzymes were used without further purification, and all enzyme dilutions were performed in phosphate buffer.

4.4. Spectrophotometric Measurements. Enzyme activities were measured spectrophotometrically by the Ellman method at 412 nm using DTNB (final concentration 0.3 mM) as thiol reagent and ATCh (AChE activity) or PTCh (BChE activity) as substrate.[22,23] Erythrocytes were diluted 600 times, while plasma was diluted 150−300 times for the enzyme activity measurements. All experiments were performed in 0.1 M phosphate buffer, pH 7.4, at 25 °C on a CARY 300 spectrophotometer equipped with a temperature controller (Varian Inc., Australia).

Progressive Inhibition by Carbamates. Progressive inhibition by carbamates proceeds according to Scheme 3 where E, AB, EAB, EA, and B stand for the free enzyme, carbamate, Michaelis-type complex between enzyme and carbamate, carbamylated enzyme, and leaving group, respectively. k_{+1}, k_{-1}, and k_{max} are rate constants of the respective reactions, while k_i is the overall inhibition rate constant.

Scheme 3. Progressive Inhibition of Cholinesterases by Carbamates



Enzyme samples were incubated for ≤30 min with the carbamates. The inhibition reaction was stopped by the addition of ATCh (1.0 mM final concentration) or PTCh (4.0 mM final concentration), and the extent of inhibition was determined by measuring the residual activity. At least three different concentrations of carbamates were used in at least two experiments. The first-order rate constants ($k_{[AB]}$) were calculated by linear regression analysis at any given carbamate concentration [AB] (Figure 2A):[24]

$$\ln \frac{v_0}{v} = k_{[AB]} t_i$$

where v_0 and v_i stand for the enzyme activity in the absence and in the presence of carbamate at the time of inhibition t_i. When $k_{[AB]}$ was a linear function of [AB] (Figure 2B), the overall inhibition rate constant k_i was calculated by linear regression analysis from:

$$k_i = \frac{k_{[AB]}}{[AB]}$$

The nonlinear relationship between $k_{[AB]}$ and [AB] indicates the presence of a reversible Michaelis-type complex between enzyme and carbamate. Then, the maximal first-order rate constant of carbamylation (k_{max}) and dissociation constant of the Michaelis-type complex (K_i) were calculated by:

$$\ln \frac{v_0}{v} = \frac{k_{max}[AB]}{K_i + [AB]} t_i = k_{[AB]} t_i$$

The overall inhibition rate constant (k_i) corresponds to the ratio k_{max}/K_i.

Reversible Inhibition by Metaproterenol and Isoproterenol. Reversible inhibition by metaproterenol and isoproterenol were measured at different substrate concentrations ([S]; Table 3) in the absence (v_0) and in the presence of the inhibitor (v_i) of a given inhibitor concentrations ([I]; 0.5−10 mM.). For each substrate concentration, the apparent dissociation constant ($K_{i,app}$) was calculated by the Hunter−Downs equation using linear regression analysis:[25]

dx.doi.org/10.1021/jm300289k | J. Med. Chem. 2012, 55, 6716−6723

$$K_{i,app} = \frac{v_i}{v_0 - v_i}[I] = K_{(I)} + \frac{K_{(I)}}{K_{(S)}}[S]$$

The y-intercept determines the enzyme−inhibitor dissociation constants (K_i), while the x-intercept determines the enzyme−substrate dissociation constant, $K_{(S)}$ (Figure 5).

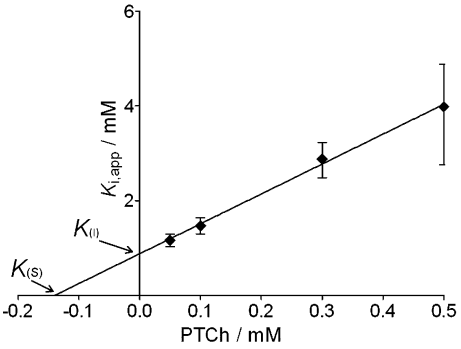


Figure 5. Reversible inhibition of human $BChE_{FF}$ by metaproterenol. Points indicate the average apparent enzyme−inhibitor constant at a given substrate (PTCh) concentration, $K_{i,app}$ (±standard deviation).

4.5. Molecular Modeling. The studied carbamates were docked using the Accelrys Discovery Studio 2.1 CDOCKER docking protocol and CHARMm force field.[26] The crystal structure deposited in the Protein Data Bank (PDB ID 2PM8) was used for the structural model of BChE.[27] Water molecules were removed from this structure. Minimized structures of three studied carbamates were docked into the active site of BChE. The protocol resulted in clusters of carbamate poses. Poses were selected based on the orientation of a single carbamate group and its distance from the catalytic serine hydroxyl group. The criterion was that after the nucleophilic attack of the catalytic serine on the carbon atom from the carbonyl group, the newly formed asymmetrical carbon had to form the (*S*) configuration because of stabilization of carbamyl oxygen in the oxyanion hole.

The transition state of BChE carbamylation was simulated using the crystal structure of tabun-phosphorylated BChE (PDB ID 3DJY) as a template.[28] The phosphorus atom was replaced with a carbon atom, the phosphorus−oxygen double bond was then converted into a carbon−oxygen single bond, and the charge on the oxygen was set to −1. The ethoxy group of the tabun·BChE conjugate was also modified by deleting the ethyl part of the ethoxy group. Then, two crystal structures of BChE (2PM8 and 3DJY) were superimposed with the purpose of translating the carbamate molecule from the non-phosphorylated 2PM8 structure into a phosphorylated 3DJY structure. The resulting complex of 3DJY structure and carbamate was additionally modified by the formation of a new bond between the oxygen atom from the ethoxy group and carbon from the carbamate benzene ring. Prior to minimization, the position of the following atoms was fixed to remain as they were in phosphorylated BChE: asymmetrical carbon with the surrounding catalytic serine oxygen, negatively charged oxygen, and nitrogen and oxygen connected to carbon from the benzene ring. The structure of this conjugate, a transition state analogue, was minimized except for the fixed atoms. Upon minimization, the resulting structure was subjected to a 10 ps NVT dynamic simulation including 13 000 production steps with heating, equilibration, and dynamics simulation.[26] This simulation was repeated, and interactions between BChE residues and the carbamyl part of the molecule were evaluated from the final structure. During simulations, the CHARMM force field with full potential minimization was used, heating target temperature was 700 K, equilibration target temperature was 310 K, and the target temperature for dynamics simulation was 310 K.

■ ASSOCIATED CONTENT

Supporting Information

Detailed description of purity analysis of compounds, synthesis of compounds **1**−**6**, RP HPLC chromatograms of metacarb (**7**), isocarb (**8**), (*R*)-metacarb (*R*-**7**), and (*R*)-isocarb. This material is available free of charge via the Internet at http://pubs.acs.org.

Accession Codes

PDB ID codes: 2PM8 and 3DJY.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +385 1 4682500. Fax: +385 1 4673303. E-mail: zkovarik@imi.hr.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This study was supported by the Ministry of Science, Education and Sports, Republic of Croatia (grants 022-0222148-2889 and 098-0982904-2910)

■ ABBREVIATIONS USED

AChE, acetylcholinesterase; BChE, butyrylcholinesterase; metacarb, bisdimethylcarbamate ester of metaproterenol; isocarb, bisdimethylcarbamate ester of isoproterenol; ATCh, acetylthiocholine iodide; PTCh, propionylthiocholine iodide; DTNB, 5,5́-dithiobis(2-nitrobenzoic acid); $BChE_{UU}$, usual BChE variant; $BChE_{AA}$, atypical BChE variant; $BChE_{FF}$, fluoride resistant BChE variant; $BChE_{UA}$, heterozygote of usual and atypical BChE variants; $BChE_{AF}$, heterozygote of atypical and fluoride resistant BChE variants; NMR, nuclear magnetic resonance; HPLC, high performance liquid chromatography; TMS, tetramethylsilane

■ REFERENCES

(1) Waldeck, B. *β*-Adrenoceptor agonists and asthma-100 years of development. *Eur. J. Pharmacol.* **2002**, *445*, 1−12.
(2) Testa, B. Prodrug research: futile or fertile? *Biochem. Pharmacol.* **2004**, *68*, 2097−2106.
(3) Lockridge, O.; Masson, P. Pesticides and susceptible populations: people with butyrylcholinesterase genetic variants may be at risk. *Neurotoxicology* **2000**, *21*, 113−126.
(4) Reiner, E.; Radić, Z. Mechanism of action of cholinesterase inhibitor. In *Cholinesterases and Cholinesterase Inhibitors*, 1st ed.; Giacobini, E., Ed.; Martin Dunitz Ltd.: London, 2000; pp 103−121.
(5) Tunek, A.; Svensson, L.-Å. Bambuterol, a carbamate ester prodrug of terbutaline, as inhibitor of cholinesterases in human blood. *Drug Metab. Dispos.* **1988**, *16*, 759−764.
(6) Kovarik, Z.; Radić, Z.; Grgas, B.; Škrinjarić-Špoljarić, M.; Reiner, E.; Simeon-Rudolf, V. Amino acids residues involved in the interaction of acetylcholinesterase with carbamates Ro 02-0683 and bambuterol, and with terbutaline. *Biochim. Biophys. Acta* **1999**, *1433*, 261−271.
(7) Bosak, A.; Gazić, I.; Vinković, V.; Kovarik, Z. Amino acids involved in stereoselective inhibition of cholinesterases with bambuterol. *Arch. Biochem. Biophys.* **2008**, *471*, 72−76.
(8) Gazić, I.; Bosak, A.; Šinko, G.; Vinković, V.; Kovarik, Z. Preparative HPLC separation and stereoselective inhibition of human cholinesterases. *Anal. Bioanal. Chem.* **2006**, *385*, 1513−1519.
(9) Bosak, A.; Gazić, I.; Vinković, V.; Kovarik, Z. Stereoselective inhibition of human, mouse, and horse cholinesterases by bambuterol enantiomers. *Chem.-Biol. Interact.* **2008**, *175*, 192−195.

(10) Liederer, B. M.; Borchardt, R. T. Enzymes involved in the bioconversion of ester-based prodrugs. *J. Pharm. Sci.* **2006**, *95*, 1177−1195.
(11) Souza, R. L. R.; Mikami, L. R.; Maegawa, R. O. B.; Chautard-Freire-Maia, E. A. Four new mutations in the BCHE gene of human butyrylcholinesterase in a Brazilian blood donor sample. *Mol. Genet. Metab.* **2005**, *84*, 349−353.
(12) Jensen, F. S.; Viby-Mogensen, J. Plasma cholinesterase and abnormal reaction to succinylcholine: twenty years' experience with the Danish Cholinesterase Research Unit. Acta. *Anaesthesiol. Scand.* **1995**, *39*, 150−156.
(13) Hartley, D.; Middlemis, D. Absolute configuration of the optical isomers of salbutamol. *J. Med. Chem.* **1971**, *14*, 895−896.
(14) Källström, B.-L.; Sjöberg, J.; Waldeck, B. Steric aspects of formoterol and terbutalin: Is there an adverse effect of the distomer on airway smooth muscle function? *Chirality* **1996**, *8*, 567−573.
(15) Kovarik, Z.; Radić, Z.; Berman, H. A.; Simeon-Rudolf, V.; Reiner, E.; Taylor, P. Acetylcholinesterase active centre and gorge conformation analysed by combinatorial mutations and enantiomeric phosphonates. *Biochem. J.* **2003**, *373*, 33−40.
(16) Bosak, A.; Primožič, I.; Oršulić, M.; Tomić, S.; Simeon-Rudolf, V. Enantiomers of quinuclidin-3-ol derivatives: Resolution and interactions with human cholinesterases. *Croat. Chem. Acta* **2005**, *78*, 121−128.
(17) Kovarik, Z.; Simeon-Rudolf, V. Interaction of human butyrylcholinesterase variants with bambuterol and terbutaline. *J. Enzyme Inhib. Med. Chem.* **2004**, *19*, 113−117.
(18) Masson, P.; Adkins, S.; Gouet, P.; Lockridge, O. Recombinant human butyrylcholinesterase G390V, the fluoride-2 variant, expressed in Chinese hamster ovary cells, is a low affinity variant. *J. Biol. Chem.* **1993**, *268*, 14329−14341.
(19) Simeon-Rudolf, V.; Juršić, B. Heterogeneity of human serum cholinesterase revealed by thiocholine substrates. *Period. Biol.* **1996**, *98*, 331−335.
(20) Tan, W.; Cheng, J. L. R-bambuterol, its preparation and therapeutic uses. U.S. Patent US7495028B2, 2009.
(21) Kovarik, Z.; Simeon-Rudolf, V. An improvement in segregation of human butyrylcholinesterase phenotypes having the fluoride-resistant variants. *Arh. Hig. Rada Toksikol.* **2003**, *54*, 239−244.
(22) Ellman, G. L.; Courtney, K. D.; Andres, V.; Featherstone, R. M. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* **1961**, *7*, 88−95.
(23) Eyer, P.; Worek, F.; Kiderlen, D.; Sinko, G.; Stuglin, A.; Simeon-Rudolf, V.; Reiner, E. Molar absorption coefficients for the reduced Ellman reagent: reassessment. *Anal. Biochem.* **2003**, *312*, 224−227.
(24) Main, R. Affinity and phosphorylation constants for the inhibition of esterases by organophosphates. *Science* **1964**, *14*, 992−993.
(25) Aldridge, W. N.; Reiner, E. *Enzyme Inhibitors as Substrates. Interaction of Esterases with Esters of Organophosphorus and Carbamic Acids*; North-Holland: New York, 1972.
(26) Accelrys Software Inc., San Diego, CA, 2005−2009.
(27) Ngamelue, M. N.; Homma, K.; Lockridge, O.; Asojoa, O. A. Crystallization and X-ray structure of full-length recombinant human butyrylcholinesterase. *Acta Crystallogr., Sect. F: Struct. Biol. Cryst. Commun.* **2007**, *F63*, 723−727.
(28) Carletti, E.; Li, H.; Li, B.; Ekström, F.; Nicolet, Y.; Loiodice, M.; Gillon, E.; Froment, M. T.; Lockridge, O.; Schopfer, L. M.; Masson, P.; Nachon, F. Aging of cholinesterases phosphylated by tabun proceeds through O-dealkylation. *J. Am. Chem. Soc.* **2008**, *130*, 16011−16020.