

University of Kentucky UKnowledge

Pharmaceutical Sciences Faculty Patents

Pharmaceutical Sciences

9-16-2014

High-Activity Mutants of Butyrylcholinesterase for Cocaine Hydrolysis and Method of Generating the Same

Chang-Guo Zhan University of Kentucky, chang-guo.zhan@uky.edu

Hoon Cho University of Kentucky

Hsin-Hsiung Tai University of Kentucky, htai1@uky.edu

Right click to open a feedback form in a new tab to let us know how this document benefits you.

Follow this and additional works at: https://uknowledge.uky.edu/ps patents



Part of the Pharmacy and Pharmaceutical Sciences Commons

Recommended Citation

Zhan, Chang-Guo; Cho, Hoon; and Tai, Hsin-Hsiung, "High-Activity Mutants of Butyrylcholinesterase for Cocaine Hydrolysis and Method of Generating the Same" (2014). Pharmaceutical Sciences Faculty Patents. 21. https://uknowledge.uky.edu/ps_patents/21

This Patent is brought to you for free and open access by the Pharmaceutical Sciences at UKnowledge. It has been accepted for inclusion in Pharmaceutical Sciences Faculty Patents by an authorized administrator of UKnowledge. For more information, please contact UKnowledge@lsv.uky.edu.



(12) United States Patent

Zhan et al.

(54) HIGH-ACTIVITY MUTANTS OF **BUTYRYLCHOLINESTERASE FOR** COCAINE HYDROLYSIS AND METHOD OF GENERATING THE SAME

(71) Applicant: University of Kentucky Research Foundation, Lexington, KY (US)

(72)Inventors: Chang-Guo Zhan, Lexington, KY (US); Hoon Cho, Lexington, KY (US);

Hsin-Hsiung Tai, Lexington, KY (US)

Assignee: University of Kentucky Research Foundation, Lexington, KY (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35

U.S.C. 154(b) by 0 days.

(21) Appl. No.: 13/767,418

(22) Filed: Feb. 14, 2013

Related U.S. Application Data

- (62) Division of application No. 13/449,107, filed on Apr. 17, 2012, now Pat. No. 8,399,644, which is a division of application No. 13/018,641, filed on Feb. 1, 2011, now Pat. No. 8,193,327, which is a division of application No. 12/752,920, filed on Apr. 1, 2010, now Pat. No. 7,919,082, which is a division of application No. 12/192,394, filed on Aug. 15, 2008, now Pat. No. 7,731,957, which is a division of application No. 11/243,111, filed on Oct. 4, 2005, now Pat. No. 7,438,904.
- (51) Int. Cl. C12N 9/16 (2006.01)(2006.01)C07H 21/04
- (52) U.S. Cl.

Field of Classification Search See application file for complete search history.

(56)**References Cited**

U.S. PATENT DOCUMENTS

7,049,121 B2 5/2006 Watkins et al. 2003/0153062 A1 8/2003 Watkins et al. 2004/0120939 A1 6/2004 Watkins et al.

OTHER PUBLICATIONS

Huang, Xiaoqin et al., Reaction Pathway and Free Energy Profile for Prechemical Reaction Step of Human Butyrylcholinesterase-Catalyzed Hydrolysis of (-)-Cocaine by Combined Targeted Molecular Dynamics and Potential of Mean Force Simulations; J. Phys. Chem., 2010, vol. 114, pp. 13545-13554.

Xue, Liu et al., Design, Preparation, and Characterization of High-Activity Mutants of Human Butyrylcholinesterase Specific for Detoxification of Cocaine, Molecular Pharmacology, 2011, vol. 79, pp. 290-297.

Yang, Wenchao et al., Characterization of a high-activity mutant of human butyrylcholinesterase against (-)-cocaine, Chemico-Biological Interactions, 2010, vol. 187, pp. 148-152.

(10) **Patent No.:**

US 8.835,150 B1

(45) **Date of Patent:**

Sep. 16, 2014

Yang, Wenchao et al., Free-Energy Perturbation Simulation on Transition States and Redesign of Butyrylcholinesterase, 2009, vol. 96,

Yang, Wenchao et al., Free Energy Perturbation Simulation on Tran-States and High-Activity Mutants of Human Butyrylcholinesterase for (-)-Cocaine Hydrolysis, J. Phys. Chem., 2010, vol. 114, pp. 10889-10896.

Pan, Yongmei et al., Free Energy Perturbation (FEP) Simulation on the Transition States of Cocaine Hydrolysis Catalyzed by Human Butyrylcholinesterase and its Mutants, J. Am. Chem. Soc., 2007, vol. 129, pp. 13537-13543.

Pan, Yongmei et al., Model of Human Butyrylcholinesterase Tetramer by Homology Modeling and Dynamics Simulation, J. Phys. Chem., 2009, vol. 113, pp. 6543-6552.

Zheng, Fang et al., Rational design of an enzyme mutant for anticocaine therapeutics, J. Comput Aided Mol. Des., 2008, vol. 22, pp. 661-671.

Zheng, Fang et al., Structure-and-mechanism-based design and discovery of therapeutics for cocaine overdose and addiction, Organic & Biomolecular Chemistry, 2008, vol. 6, pp. 836-843.

Zheng, Fang et al., Design of High-Activity Mutants of Human Butyrylcholinesterase against (-)-Cocaine: Structural and Energetic Factors Affecting the Catalytic Efficiency, Biochemistry, 2010, vol. 49, pp. 9113-9119.

Zheng, Fang et al., Recent progress in protein drug design and discovery with a focus on novel approaches to the development of anticocaine medications, Future Med. Chem, 2009), vol. 1, No. 3, pp.

Zheng, Fang et al., Enzyme-therapy approaches for the treatment of drug overdose and addition, Future Med. Chem., 2011, vol. 3, No. 1, pp. 9-13.

Zheng, Fang et al., Most Efficient Cocaine Hydrolase Designed by Virtual Screening of Transition States, J. Am. Chem. Soc., 2008, vol. 130, pp. 12148-12155.

(Continued)

Primary Examiner — Tekchand Saidha (74) Attorney, Agent, or Firm — Stites & Harbison PLLC; Mandy Wilson Decker

(57)ABSTRACT

A novel computational method and generation of mutant butyrylcholinesterase for cocaine hydrolysis is provided. The method includes molecular modeling a possible BChE mutant and conducting molecular dynamics simulations and hybrid quantum mechanical/molecular mechanical calculations thereby providing a screening method of possible BChE mutants by predicting which mutant will lead to a more stable transition state for a rate determining step. Site-directed mutagenesis, protein expression, and protein activity is conducted for mutants determined computationally as being good candidates for possible BChE mutants, i.e., ones predicted to have higher catalytic efficiency as compared with wild-type BChE. In addition, mutants A199S/A328W/ Y332G, A199S/F227A/A328W/Y332G, A199S/S287G/ A328W/Y332G, A199S/F227A/S287G/A328W/Y332G, and A199S/F227A/S287G/A328W/E441D all have enhanced catalytic efficiency for (-)-cocaine compared with wild-type BChE.

(56) References Cited

OTHER PUBLICATIONS

Gao, et al.; Modeling effects of oxyanion hole on the ester hydrolyses catalyzed by human cholinesterases; Phys. Chem. B; 2005; 109; pp. 23070-23076.

Gao, et al; Computational design of a human butyrylcholinesterase mutant for accelerating cocaine hydrolysis based on the transition-state simulation; Angew. Chem. Int. Ed.; 2006; 45; pp. 653-657.

Gao, et al; Modeling evolution of hydrogen bonding and stablization of transition states in the process of cocaine hydrolysis catalyzed by human butyrylcholinesterase; Proteins; 2006; 62; pp. 99-110.

Hamza, et al.; Molecular dynamics simulation of cocaine binding with human butyrylcholinesterase and its mutants; J. Phys. Chem. B.; 2005; 109; pp. 4776-4782.

Pan, et al.; Computational redesign of human butyrylcholinesterase for anti-cocaine medication; G. Proc. Natl. Acad. Sci. USA; 2005; 102; pp. 16656-16661.

Zhan, et al.; Fundamental reaction mechanism for cocaine metabolism in human butyrylcholinesterase; J. Am. Chem. Soc.; 2003; 125; pp. 2462-2474.

Zhan, et al.; Catalytic Mechanism and energy barriers for butyrylcholinesterase-catalyzed hydrolysis of cocaine; Biophysical Journal; 2005; 89; 3863-3872.

Xie, et al.; An improved cocaine hydrolase: the A328Y mutant of human butyrylcholinesterase is 4-fold more efficient; Moleculare Pharm; 1999; 55; pp. 83-91.

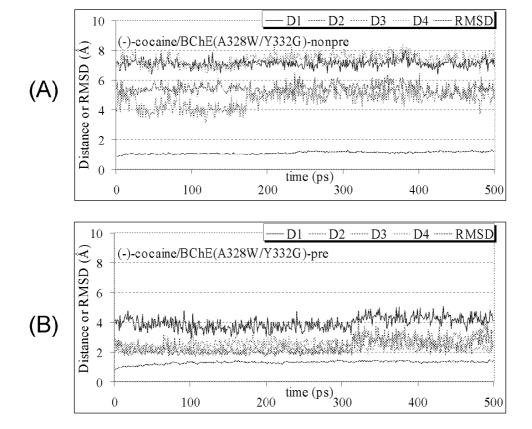
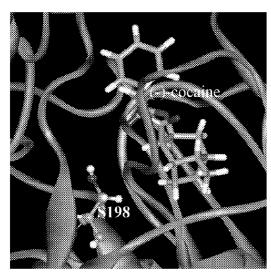
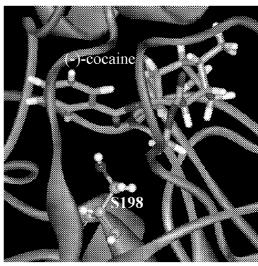


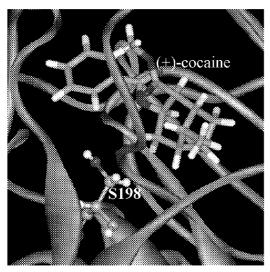
FIGURE 1



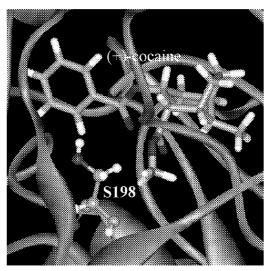
(A) BChE-(-)-cocaine non-prereactive complex



(B) BChE-(-)-cocaine prereactive complex



(C) BChE-(+)-cocaine non-prereactive complex



(D) BChE-(+)-cocaine prereactive complex

FIGURE 2

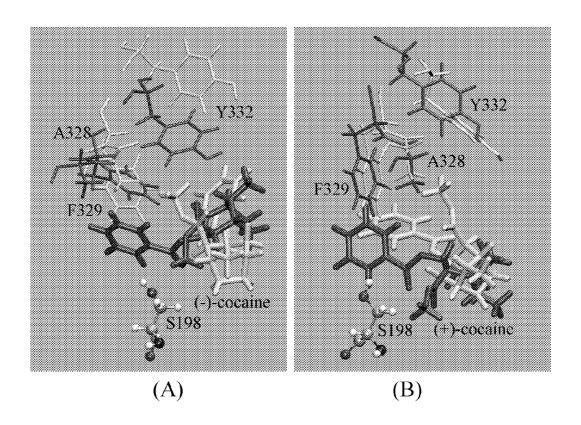


FIGURE 3

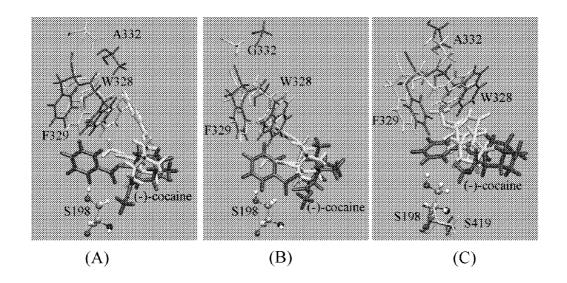


FIGURE 4

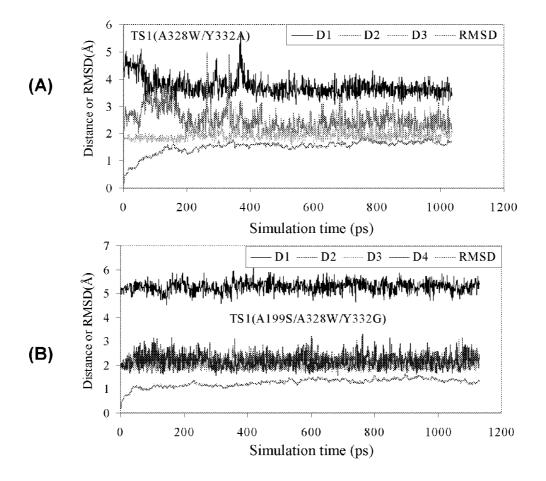


FIGURE 5

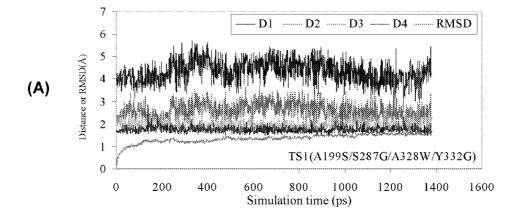


FIGURE 6

35

1

HIGH-ACTIVITY MUTANTS OF **BUTYRYLCHOLINESTERASE FOR** COCAINE HYDROLYSIS AND METHOD OF GENERATING THE SAME

RELATED APPLICATIONS

This application is a division of and claims benefit to U.S. patent application Ser. No. 13/449,107, now allowed, which is a division of and claims benefit to U.S. patent application Ser. No. 13/018,641, now issued as U.S. Pat. No. 8,193,327, filed Feb. 1, 2011, which is a division of and claims benefit to U.S. patent application Ser. No. 12/752,920, now issued as U.S. Pat. No. 7,919,082, filed Apr. 1, 2010, which is a division of and claims benefit to U.S. patent application Ser. No. 15 12/192,394, now issued as U.S. Pat. No. 7,731,957, filed Aug. 15, 2008, which is a division of and claims benefit to U.S. patent application Ser. No. 11/243,111, now issued as U.S. Pat. No. 7,438,904, filed Oct. 4, 2005. The contents of which are incorporated herein by reference in their entirety.

GOVERNMENT INTEREST

Subject matter described herein was made with government support under Grant Number R01DA013930 awarded by the National Institute on Drug Abuse (NIDA) of the National Institutes of Health (NIH). The government has certain rights in the described subject matter.

FIELD OF THE INVENTION

The present invention relates to butyrylcholinesterase variant polypeptides, and in particular, butyrylcholinesterase mutants having amino acid substitutions.

BACKGROUND OF THE INVENTION

Cocaine abuse is a major medical and public health problem that continues to defy treatment. The disastrous medical and social consequences of cocaine addiction, such as violent crime, loss in individual productivity, illness and death, have made the development of an effective pharmacological treatment a high priority. However, cocaine mediates its reinforcing and toxic effects by blocking neurotransmitter reuptake and the classical pharmacodynamic approach has failed to yield small-molecule receptor antagonists due to the difficulties inherent in blocking a blocker. An alternative to receptor- 45 based approaches is to interfere with the delivery of cocaine to its receptors and accelerate its metabolism in the body

The dominant pathway for cocaine metabolism in primates is butyrylcholinesterase (BChE)-catalyzed hydrolysis at the benzoyl ester group (Scheme 1).

2 -continued OCH₃ НÓ BChEΗ2Ο OCH: (+)-cocaine

Scheme 1. Schematic representation of BChE-catalyzed hydrolysis at the benzoyl ester group.

НO

OCH₂

Only 5% of the cocaine is deactivated through oxidation by the liver microsomal cytochrome P450 system. Cocaine hydrolysis at benzoyl ester group yields ecgonine methyl ester, whereas the oxidation produces norcocaine. The metabolite ecgonine methyl ester is a biologically inactive metabolite, whereas the metabolite norcocaine is hepatotoxic and a local anesthetic. BChE is synthesized in the liver and widely distributed in the body, including plasma, brain, and lung. Extensive experimental studies in animals and humans demonstrate that enhancement of BChE activity by administration of exogenous enzyme substantially decreases cocaine half-life.

Enhancement of cocaine metabolism by administration of BChE has been recognized to be a promising pharmacokinetic approach for treatment of cocaine abuse and dependence. However, the catalytic activity of this plasma enzyme is three orders-of-magnitude lower against the naturally 55 occurring (-)-cocaine than that against the biologically inactive (+)-cocaine enantiomer. (+)-cocaine can be cleared from plasma in seconds and prior to partitioning into the central nervous system (CNS), whereas (-)-cocaine has a plasma half-life of approximately 45-90 minutes, long enough for manifestation of the CNS effects which peak in minutes. Hence, BChE mutants with high activity against (-)-cocaine are highly desired for use in humans. Although some BChE mutants with increased catalytic activity over wild-type BChE have previously been generated, there exists a need for 65 mutant BChE with even higher catalytic activity. Thus, prior mutants provide limited enhancement in catalytic activity over wild-type BChE.

Previous studies such as (a) Masson, P.; Legrand, P.; Bartels, C. F.; Froment, M-T.; Schopfer, L. M.; Lockridge, O. Biochemistry 1997, 36, 2266 (b) Masson, P.; Xie, W., Froment, M-T.; Levitsky, V.; Fortier, P.-L.; Albaret, C.; Lockridge, O. Biochim. Biophys. Acta 1999, 1433, 281, (c) Xie, W.; Altamirano, C. V.; Bartels, C. F.; Speirs, R. J.; Cashman, J. R.: Lockridge, O. Mol. Pharmacol. 1999, 55, 83, (d) Duvsen, E. G.; Bartels, C. F.; Lockridge, O. J. Pharmacol. Exp. Ther. 2002, 302, 751, (e) Nachon, F.; Nicolet, Y.; Viguie, N.; Masson, P.; Fontecilla-Camps, J. C.; Lockridge, O. Eur. J. Biochem. 2002, 269, 630, (f) Zhan, C.-G.; Landry, D. W. J. Phys. Chem. A 2001, 105, 1296; Berkman, C. E.; Underiner, G. E.; Cashman, J. R. Biochem. Pharmcol. 1997, 54, 1261; (g) Sun, H.; Yazal, J. E.; Lockridge, O.; Schopfer, L. M.; 15 Brimijoin, S.; Pang, Y.-P. J. Biol. Chem. 2001, 276, 9330, (h) Sun, H.; Shen, M. L.; Pang, Y. P.; Lockridge, O.; Brimijoin, S. J. Pharmacol. Exp. Ther. 2002, 302, 710, (i) Sun, H.; Pang, Y. P.; Lockridge, O.; Brimijoin, S. Mol. Pharmacol. 2002, 62, 220 (hereinafter "Sun et al"); and (j) Zhan, C.-G.; Zheng, F.; 20 Landry, D. W. J. Am. Chem. Soc. 2003, 125, 2462 (hereinafter "Zhan et al"), herein all incorporated by reference, suggested that, for both (-)-cocaine and (+)-cocaine, the BChE-substrate binding involves two different types of complexes: non-prereactive and prereactive BChE-substrate complexes. 25 Whereas the non-prereactive BChE-cocaine complexes were first reported by Sun et al, Zhan et al were the first reporting the prereactive BChE-cocaine complexes and reaction coordinate calculations, disclosed in Zhan et al.

It was demonstrated that (-)/(+)-cocaine first slides down 30 the substrate-binding gorge to bind to W82 and stands vertically in the gorge between D70 and W82 (non-prereactive complex) and then rotates to a position in the catalytic site within a favorable distance for nucleophilic attack and hydrolysis by S198 (prereactive complex). In the prereactive 35 complex, cocaine lies horizontally at the bottom of the gorge. The main structural difference between the BChE-(-)-cocaine complexes and the corresponding BChE-(+)-cocaine complexes exists in the relative position of the cocaine methyl ester group. Reaction coordinate calculations revealed that 40 the rate-determining step of BChE-catalyzed hydrolysis of (+)-cocaine is the chemical reaction process, whereas for (-)-cocaine the change from the non-prereactive complex to the prereactive complex is rate determining. A further analysis of the structural changes from the non-prereactive com- 45 plex to the prereactive complex reveals specific amino acid residues hindering the structural changes, providing initial clues for the rational design of BChE mutants with improved catalytic activity for (-)-cocaine.

Previous molecular dynamics (MD) simulations of prere- 50 active BChE-cocaine binding were limited to wild-type BChE. Even for the non-prereactive BChE-cocaine complex, only one mutant (A328W/Y332A) BChE binding with (-)cocaine was simulated and its catalytic activity for (-)-cocaine was reported by Sun et al. No MD simulation was 55 performed on any prereactive enzyme-substrate complex for (-)- or (+)-cocaine binding with a mutant BChE. In addition, all previous computational studies of Sun et al and Zhan et al of BChE interacting with cocaine were performed based on a homology model of BChE when three-dimensional (3D) X-ray crystal structure was not available for BChE, as taught by Nicolet, Y.; Lockridge, O.; Masson, P.; Fontecilla-Camps, J. C.; Nachon, F. J. Biol. Chem. 2003, 278, 41141 (hereinafter "Nicolet et al"), recently reported 3D X-ray crystal structures of BChE. As expected, the structure of BChE is similar to a 65 previously published theoretical model of this enzyme and to the structure of acetylcholinesterase.

4

The main difference between the experimentally determined BChE structure and its model was found at the acyl binding pocket (acyl loop) that is significantly bigger than expected. It is unclear whether the structural difference at the acyl binding pocket significantly affect BChE binding with (–)-cocaine and (+)-cocaine. Although previous MD simulations of cocaine binding with wild-type BChE and the reaction coordinate calculations point to some amino acid residues that might need to be mutated for the purpose of improving the catalytic activity for (–)-cocaine hydrolysis, it remained unknown which exact amino acid mutations will result in a BChE with a higher catalytic activity for (–)-cocaine.

Computational studies of wild-type BChE and cocaine from Sun, et al, based on a "homology model," suggest that the rate-determining step for BChE-catalyzed hydrolysis of cocaine is the rotation of the cocaine in the active site of BChE. By decreasing the hindrance of the rotation, the rate of the hydrolysis may be enhanced. Sun, et al describes creating an A328W/Y332A BChE mutant by: (1) replacing Tyr332 with Ala, "to reduce the steric hindrance and the π - π interaction that impede rotation," and (2) replacing A1a328 with Trp "to provide a cation- π interaction to restore substrate affinity lost in disabling the π - π interaction."

Sun et al studied the A328W/Y332A BChE mutant using enzyme assays and kinetics. In vitro studies were conducted using human plasma and in vivo studies were conducted using male Sprague-Dawley rats. The mutant was found to have enhanced catalytic properties. The mutant was further studied using molecular modeling. The three dimensional (3D) structure of A328W/Y332A was generated from the computationally generated 3D model of wild-type BChE and changing the relevant residues using commercially available software. Cocaine was docked to the catalytic gorge of the mutant BChE using other commercially available software. The cocaine-enzyme complex was refined by molecular dynamic simulation. The data generated by the molecular modeling studies were consistent with enzyme assays and kinetic data.

It should be noted that all prior computational techniques (molecular docking and molecular dynamics simulation) used by other researchers are based on an empirical force field which cannot be used to perform any necessary reaction coordinate calculation for the detailed understanding of the complicated catalytic reaction process. As it is well-known, it is particularly challenging to model and simulate the detailed reaction pathway and predict the kinetics of such an enzymatic reaction.

U.S. Patent Application Publication Nos. 2004/0121970; 2004/0120939; and 2003/0153062, describe 20+BChE mutants, or "variants," from human and other animals, each having from one to six amino acid alterations and increased cocaine hydrolysis activity. For example, mutants include F227A/A328W; F227A/S287G/A328W; A119S/S287G/A328W; A328W/Y332M/S287G/F227A, A199S/F227A/S/287G/A328W and A119S/F227A/S287G/A328W/Y332M. The mutants have varying increases in catalytic activity, up to 100-fold increase relative to wild-type BChE.

There exists a need in the art for determining which proposed mutant BChEs should have ever increasing catalytic activity and for generating those mutants which should have enhanced catalytic activity.

SUMMARY OF THE INVENTION

The present invention includes five novel human BChE mutants that have unexpected increased catalytic efficiency

for cocaine hydrolysis. The mutants have various unique amino acid residue substitutions which provide the surprising enhanced catalytic activity. These mutants are (1) A199S/ A328W/Y332G mutant (SEQ ID NO: 2), which has a approximately 65-fold improved catalytic efficiency against 5 (-)-cocaine; (2) A199S/F227A/A328W/Y332G mutant (SEQ ID NO: 8), which has an approximately 148-fold improved catalytic efficiency against (-)-cocaine; (3) A199S/ S287G/A328W/Y332G mutant (SEQ ID NO: 14), which has an approximately 456-fold improved catalytic efficiency 10 against (-)-cocaine; (4) A199S/F227A/S287G/A328W/ Y332G mutant (SEQ ID NO: 20), which has an approximately 1.003-fold improved catalytic efficiency against (-)cocaine; and (5) A199S/F227A/S287G/A328W/E441D mutant (SEQ ID NO: 26), which has an approximately 445- 15 fold improved catalytic efficiency against (-)-cocaine.

In addition, the aforementioned mutant amino acid sequences can be truncated without substantially affecting the catalytic activity so that amino acid residues 1-67 and 443-574 can be removed without substantially affecting the 20 catalytic activity of the enzyme. SEQ ID NOS: 4, 10, 16, 22 and 28 are the amino acid sequences for residue 68-142 corresponding to mutants 1-5, respectively. In addition, with regard to mutants 1-4, it was found that amino acid residues before 117 and after 438 could be removed without substantially changing the activity of the mutant enzymes, resulting in truncated amino acid sequences having SEQ ID NOS: 6, 12, 18 and 24, respectively. Finally with regard to mutant 5, amino acid residues before 117 and after 441 could be removed without substantially changing its activity resulting 30 in SEQ ID NO: 30.

These aforementioned truncated sequences all of with similar catalytic activity is based on protein structures.

Further, the present invention is directed to a novel and unique pharmaceutical composition which comprises a 35 butyrylcholinesterase variant, namely mutants 1-5, along with a suitable pharmaceutical carrier. The pharmaceutical composition can be administered to an individual in an effective amount to lower the patient's cocaine blood concentration and in particular (–)-cocaine blood concentration.

In addition, the present invention is directed to a novel and unique method for developing mutants which have enhanced catalytic efficiency. The generation method includes both a computational portion and an experimental portion. With regard to the computational portion, a variety of state of the 45 art computational techniques including molecular modeling, molecular dynamics (MD) simulations and hybrid quantum mechanical/molecular mechanical (QM/MM) calculations, provide a virtual screening of possible BChE mutants. This virtual screening predicts which mutation will lead to a more 50 stable transition state for a rate-determining step compared to the corresponding separated reactants, i.e., free cocaine and free enzyme. The more stable the transition state, the lower the energy barrier, and the higher the catalytic efficiency. Following the computational portion, an experimental test is 55 then conducted on the possible mutants of the computation portion. The experimental test includes site-directed mutagenesis, protein expression, and enzyme activity assay. The experimental tests are conducted on mutants which are predicted to have a high catalytic efficiency against (-)-co- 60 caine than the wild-type BChE and/or other known BChE mutants against (-)-cocaine. Thus, the present method identifies or predicts mutants having high catalytic activity for cocaine hydrolysis by performing molecular modeling and MD simulations on the transition state structures of possible 65 mutants of BChE. This method is an improvement over traditional random-search approaches, which, given the com6

plex catalytic mechanism of cocaine hydrolysis, makes it difficult to improve the catalytic activity of BChE for cocaine hydrolysis

The present invention in one form, concerns a butyrylcholinesterase variant peptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, and 30.

The present invention in another form thereof concerns a nucleic acid molecule comprising a nucleic acid sequence which encodes a butyrylcholinesterase variant peptide, the nucleic acid sequence selected from the group consisting of SEQ ID NOS: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, and 29.

The present invention in another form thereof concerns a pharmaceutical composition comprising a butyrylcholinest-erase variant polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOS. 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, and 30; and a suitable pharmaceutical carrier.

The present invention in another form thereof concerns a method for treating a cocaine-induced condition comprising administering to an individual an effective amount of butyryl-cholinesterase variant peptide having an amino acid sequence selected from the group consisting of SEQ ID NOS. 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, and 30, to lower blood cocaine concentration.

The present invention is another form thereof concerns a method for treating a cocaine induced condition comprising administering to an individual an effective amount of a pharmaceutical composition comprising a butyrylcholinesterase variant having an amino acid sequence selected from the group consisting of SEQ ID NOS. 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, and 30, and suitable pharmaceutical carrier of claim 3 to an individual in an effective amount to lower blood cocaine concentration.

The present invention in yet another form thereof concerns a method for generating butyrylcholinesterase mutants. The method includes generating an initial structure of the transition state structure for the rate-determining step of the cocaine hydrolysis catalyzed by a possible butyrylcholinesterase mutant. A sufficiently long time molecular dynamics simulation is performed on the transition state structure in water to have a stable molecular dynamics trajectory. The molecular dynamics trajectory is analyzed and the hydrogen bonding energies are estimated between the carboxyl oxygen of the (-)-cocaine benzyl ester and the oxyanion hole of the possible butyrylcholinesterase mutant. If the overall hydrogen binding energy between the carboxyl oxygen of the (-)cocaine benzyl ester and the possible butyrylcholinesterase mutant, in the transition state, is stronger than the overall hydrogen binding energy between the carboxyl oxygen of the (-)-cocaine benzyl ester and the wild-type butyrylcholinesterase, optionally, hybrid quantum mechanical/molecular mechanical (QM/MM) geometry optimization is performed to refine the molecular dynamics-simulated structure, the hydrogen binding energies are calculated and the energy barrier is evaluated. Finally, the butyrylcholinesterase mutant is generated.

In various alternative embodiments, the generating an initial structure of the transition state structure is based on reaction coordinate calculations for the wild-type butyrylcholinesterase. The generating butyrylcholinesterase mutant includes performing site-directed mutagenesis on a nucleic acid sequence which includes wild-type butyrylcholinesterase to generate the mutant butyrylcholinesterase nucleic acid sequence. Using the mutant butyrylcholinesterase nucleic acid sequence, the protein encoded by the mutant

nucleic acid sequences is expressed to produce mutant butyrylcholinesterase and catalytic activity assay is performed on the mutant butyrylcholinesterase.

The hybrid quantum mechanical/molecular mechanical geometry optimization may include calculating the hydrogen binding energies and evaluating the energy barriers only if the overall hydrogen binding energy between the carboxyl oxygen of the (-)-cocaine benzyl ester and the possible butyrylcholinesterase mutant, in the transition state, is stronger than known butyrylcholinesterase mutants against (-)-cocaine.

In yet another alternative further embodiment, the method for generating butyrylcholinesterase mutants further includes determining the rate-limiting step in the hydrolysis of (-)cocaine by the possible butyrylcholinesterase mutant by conducting molecular dynamics simulations and quantum mechanical/molecular mechanical calculations relating to the transition states for other reaction steps between (-)-cocaine by the possible butyrylcholinesterase mutant and calculating reaction steps is the rate-determining one.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 are plots of distances in the MD simulation (-)- 25 cocaine binding with A328W/Y332G BChE versus the simulation time, along with root-mean-square deviation (RMSD) in the enzyme-substrate complexes where FIG. 1A represents the non-prereactive enzyme-substrate complexes; and FIG. 1B represents the prereactive enzyme-substrate complexes in 30 accordance with the present invention.

FIG. 2 shows the binding of structures of the simulated non-prereactive and prereactive complexes of wild-type BChE binding with two entomers of cocaine in which FIG. 2B depicts BChE (-)-cocaine prereactive complex, FIG. 2C depictes BChE (+)-cocaine non-prereactive complex; and FIG. 2D depicts BChE (+)-cocaine prereactive complex.

FIG. 3A shows the (-)-cocaine rotation in the BChE active site for the non-prereactive complex to the prereactive complex hindered by some residues at positions Y332, A328, and F329 residues in the non-prereactive complexes which are significantly different from the corresponding positions in the prereactive complex; and FIG. 3B shows the (+)-cocaine rotation in the BChE active site where none of the aforementioned residues hinders the (+)-cocaine rotation in the BChE

8

active site from the non-prereactive complex to the prereactive complex in accordance with the present invention.

FIG. 4A depicts the (-)-cocaine rotation in the active site of A328W/Y332A; FIG. 4B depicts the (-)-cocaine rotation in the active site of A328W/Y332G BChE from the non-preactive complex to the prereactive complex; and FIG. 4C depicts the (–)-cocaine rotation in the active site of wild-type BChE.

FIG. 5A is a plot of the key internuclear distances (in Å) versus the time in the simulated TS1 structure for (-)-cocaine hydrolysis catalyzed by A328W/Y332A; and FIG. 5B for Å199\$/A328W/Y332G BChE.

FIG. 6 is a plot of key internuclear distances (in Å) versus the time in the simulated TS1 structure for (-)-cocaine hydrolysis catalyzed by A199S/S287G/A328W/Y332G BChE.

DETAILED DESCRIPTION OF THE PREFERRED **EMBODIMENTS**

The present invention has two major improvements over respective energy barriers, thereby establishing which of the 20 the prior art. The first is the presently discovered BChE mutants, mutant 1, A199S/A328W/Y332G; mutant 2, A199S/F227A/A328W/Y332G; mutant 3, A199S/S287G/ A328W/Y332G; mutant 4, A199S/F227A/S287G/A328W/ Y332G; and mutant 5, A199S/F227A/S287G/A328W/ E441D each have a significantly higher catalytic efficiency. The second improvement is concerning the mutant designing or discovering process.

> The BChE mutants 1-5 have full length amino acid sequences, SEQ ID NOS: 2, 8, 14, 20, and 26, respectively, which are encoded by nucleic acid sequences having SEQ ID NOS: 1, 7, 13, 19, and 25, respectively. Table 1 summarizes the catalytic efficiency against (-)-cocaine for the five mutants.

In addition to the full length BChE mutants, the respective 2A depicts BChE (-)-cocaine non-prereactive complex; FIG. 35 amino acid sequence can be truncated without substantially affecting the respective catalytic activity. With all mutants, residues 1-67 and 443-574 can be removed without substantially affecting the catalytic activity of the respective mutant BChE. Further, with regard to mutant 1-4, amino acids 1-116 and 439-574 can be omitted without substantially affecting its respective catalytic activity. With regard to mutant 5, amino acid residues 1-116 and 442-574 can be omitted without substantially affecting its catalytic activity. Table 1 also provides a summary of amino acid SEQ ID NOS and corresponding nucleic acid SEQ ID NOS for the aforementioned truncated mutant BChE sequences.

TABLE 1

Mutant Number	Amino Acid Substi- tuting	Nucleic Acid SEQ ID NO.	Amino Acid SEQ ID NO.	Partially Truncated Nucleic Acid Sequence Corresponding To Amino Acid Residues 68-442	SEQ ID NO. for Amino Acid Residues 68-442	SEQ ID NO Corresponding To Amino Acid Residues 117-438/441*	SEQ ID NO. for Amino Acid Residues 117-438/441*	Catalytic Efficiency Against (-)-cocaine
1	A199S/	1	2	3	4	5	6	65-fold
	A328W/							
2	Y332G A199S/F	7	8	9	10	11	12	148-fold
_	227A/A							
	328W/Y							
	332G							
3	A1995/S	13	14	15	16	17	18	456-fold
	287G/A							
	328W/Y							
	332G							

TABLE 1-continued

Mutant Number	Amino Acid Substi- tuting	Nucleic Acid SEQ ID NO.	Amino Acid SEQ ID NO.	Partially Truncated Nucleic Acid Sequence Corresponding To Amino Acid Residues 68-442	SEQ ID NO. for Amino Acid Residues 68-442	SEQ ID NO Corresponding To Amino Acid Residues 117-438/441*	SEQ ID NO. for Amino Acid Residues 117-438/441*	Catalytic Efficiency Against (–)-cocaine
4	A1995/F 227A/S2 87G/A3 28W/Y3 32G	19	20	21	22	22	24	1,003-fold
5	A1995/F 227A/S2 87G/A3 28W/E4 41D	25	26	27	28	29	30	445-fold

(* Amino acid residues 117-438 for mutants 1-4 and residues 117-441 for mutant 5.)

The BChE variant polypeptide, e.g., SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, and 30 can be formulated in a pharmaceutical composition along with a suitable pharmaceutical carrier known to one skilled in the art.

The present BChE variant polypeptides can be used in treating a cocaine-induced condition by administering to an individual, an effective amount of one of the BChE variant polypeptides, i.e., SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, and 30, to lower blood cocaine concentration. The BChE variant polypeptide may be administered in the form of a pharmaceutical composition in which the BChE variant is included with a suitable pharmaceutical carrier. Treatment of a cocaine induced condition using one of the aforementioned BChE variant polypeptides can be done in 35 accordance with Zhan et al., page 2463.

The preferred dose for administration of a butyrylcholinesterase or peptide composition in accordance with the present invention is that amount which will be effective in lowering (-)-cocaine concentration in a patient's blood- 40 stream, and one would readily recognize that this amount will vary greatly depending on the nature of cocaine consumed, e.g., injected or inhaled, and the condition of a patient. An "effective amount" of butyrylcholinesterase mutant or pharmaceutical agent to be used in accordance with the invention 45 is intended to mean a nontoxic but sufficient amount of the agent, such that the desired prophylactic or therapeutic effect is produced. Thus, the exact amount of the enzyme or a particular agent that is required will vary from subject to subject, depending on the species, age, and general condition 50 of the subject, the severity of the condition being treated, the particular carrier or adjuvant being used and its mode of administration, and the like. Similarly, the dosing regimen should also be adjusted to suit the individual to whom the composition is administered and will once again vary with 55 age, weight, metabolism, etc. of the individual. Accordingly, the "effective amount" of any particular butyrylcholinesterase composition will vary based on the particular circumstances, and an appropriate effective amount may be determined in each case of application by one of ordinary skill in 60 the art using only routine experimentation.

A unique method was used to determine potential BChE mutants with projected increased catalytic activity for the hydrolysis of cocaine. The method provides a unique approach which first models the potential BChE mutant interaction with cocaine followed by generating the BChE mutant if the predicted model indicates that the BChE variant should

have enhanced catalytic activity. The method includes gener-20 ating an initial structure of the transition state structure for the rate-determining step for the cocaine hydrolysis catalyzed by a possible BChE mutant. A sufficiently long time molecular dynamics simulation is performed on the transition state structure in water to have a stable molecular dynamics trajectory. The molecular dynamics trajectory is analyzed and the hydrogen binding energies are estimated between the carboxyl oxygen of the (-)-cocaine benzoyl ester and the oxyanion hole of the possible BChE mutant. If the overall hydrogen binding energy between the carboxyl oxygen of the (-)cocaine benzoyl ester and the possible BChE mutant, in the transition state, is stronger than the overall hydrogen binding energy between the carboxyl oxygen of the (-)-cocaine benzoyl ester and the wild-type BChE, hybrid quantum mechanical/molecular mechanical (QM/MM) geometry optimization is performed to refine the molecular dynamics-simulated structure, the hydrogen binding energies are calculated and the energy barrier is evaluated. The QM/MM calculations make the computational predictions more reliable. Finally, the BChE mutant is generated.

With regard to the molecular dynamics (MD) simulations and quantum mechanical/molecular mechanical (QM/MM) calculations, the first chemical reaction step of (–)-cocaine hydrolysis catalyzed by butyrylcholinesterase (BChE) mutants and, when needed, other reaction steps are modeled and calculated using molecular dynamics simulations and QM/MM calculations. Following this modeling, mutant BChE's are created using site-directed mutagenesis followed by protein expression. The aformentioned five mutants were identified by computational analysis and generated by site-directed mutagenesis which have significantly enhanced (–)-cocaine hydrolysis catalytic efficiency compared with wild-type BChE.

In the present method computational analysis in the form of molecular modeling of a potential BChE mutant and MD simulations and QM/MM calculations provide virtual screening of possible BChE mutants which have predicted enhanced catalytic activity for (–)-cocaine. For example, the MD simulations and QM/MM calculations predict which mutation will lead to a more stable transition state for the rate determining step compared to the corresponding separated reagents, i.e., free cocaine and free possible mutant BChE, where the more stable transition state leads to a lower energy barrier and higher predicted catalytic efficiency. Only after the computational analysis predicts enhanced catalytic efficiency, is site-directed mutagenesis conducted on wild-type BChE nucleic acid sequence to generate a mutant nucleic acid sequence which is then used to express a mutant BChE protein. The

10

mutant BChE protein is then used in catalytic assays to determine the catalytic efficiency against (–)-cocaine.

The use of predictive, computational modeling of the present method for identifying mutant BChE candidates and the resulting mutant BChE are novel and unexpected over 5 prior conventional methods which will now be readily apparent to one of ordinary skill in the art.

Using the present method, most discovered new mutants include a specific mutation (Y332G) on residue #332. No prior BChE mutant having the Y332G mutation had ever been 10 reported previously; only mutations Y332A and Y332M on residue #332 had been tested previously by other researchers. Prior to the present invention, there was no reason to expect that a mutant including Y332G mutation should be better than the corresponding mutant including Y332A mutation or 15 Y332M. Thus, the present mutants with a Y332G mutation which have enhanced cataltytic activity represent a surprising and unexpected result over prior BChE mutants.

A Y332G mutant (single mutation) was first tested and found that the Y332G mutant had a slightly low (or approximately equal) catalytic efficiency than the wild-type. So, only an appropriate combination of different mutations on different residues could make the enzyme more active. As seen below, the prior art did not reveal that any of the particular combinations tested was expected to have an improved catalytic efficiency. The present method is based on the present unique, extensive computational modeling and simulations of the detailed catalytic mechanism for both the wild-type BChE and the mutants.

The primary improvement of the present method over the prior art is that high-performance computational modeling and simulations of the detailed catalytic mechanism are performed, which includes modeling how cocaine binds with BChE and the subsequent structural transformation and chemical reaction process. The prior art only considered the cocaine binding with the enzyme (BChE) and was unable to examine the detailed catalytic reaction process after the BChE-cocaine binding. When molecular modeling was limited to studying the BChE-cocaine binding, one could only design a mutation to improve the BChE-cocaine binding without knowing whether the mutation will also speedup the subsequent chemical reaction process or not.

To overcome the obstacles of prior challenges to using computational techniques such as molecular docking and molecular dynamics simulation previously used by others 45 which were based on an empirical force field which cannot be used to perform necessary reaction coordinate calculations for the catalytic reaction process, a variety of state-of-the-art computational techniques of homology modeling, molecular docking, molecular dynamics, quantum mechanics (QM), 50 and hybrid quantum mechanics/molecular mechanics (QM/ MM) were used for the rational design of the BChE mutants. The combined use of these computational techniques, including QM and QM/MM, led to the study of the detailed reaction coordinate of the BChE-catalyzed hydrolysis of cocaine 55 which, for the first time, provided the detailed structures of all transition states and intermediates existing in the reaction process and the corresponding energetics. These extensive computational modeling and simulation studies provided for the rational design of possible BChE mutants that not only 60 can improve the BChE-cocaine binding, but also can speedup the subsequent chemical reaction process. As a result, one can now quickly discover the BChE mutants with the significantly improved catalytic efficiency.

In addition, to the differences mentioned above, the present 65 molecular modeling of the BChE-cocaine binding also differs from the prior modeling. The molecular modeling in the prior

12

art considered only one binding mode for each BChE-cocaine system, without modeling the possible cocaine rotation in the BChE active site after the binding. The present method considers two different binding modes for each BChE-cocaine system and the structural transformation between them: non-prereactive and prereactive BChE-cocaine complexes. The present modeling provides more detailed information about the BChE-cocaine binding and the subsequent structural transformation.

The present method includes molecular dynamics simulations performed on the cocaine binding with both the wild-type BChE and the mutants whereas prior molecular dynamics simulations were only performed on the cocaine binding with the wild-type BChE. As is shown below in the following experiments, the computational prediction could be completely wrong without directly modeling and simulating cocaine binding with the proposed mutants.

The present invention will now be discussed with regard to the following non-limiting examples in the form of experiments which are provided to enhance understanding of the present invention but in no way limit its scope or applicability.

Experiment 1

Computational Study of Cocaine Binding with Wild-Type and Mutant BCHE's for A328W/Y332G, A328W/Y332A, and A328W/Y332A/Y419S

A detailed computational study of cocaine binding with wild-type and mutant BChE's starting from the available X-ray crystal structure of wild-type BChE was performed. The simulated mutants include A328W/Y332G, A328W/Y332A, and A328W/Y332A/Y419S, as simple geometric consideration of the binding site suggests that these mutations could be important for changing the (–)-cocaine rotation from the non-prereactive complex to the prereactive complex. Wet experimental tests were conducted on the catalytic activity of these mutants for (–)-cocaine in order to verify the computational predictions. All of the obtained results clearly demonstrate that molecular modeling and MD simulations of cocaine binding with BChE mutants provide a reliable computational approach to the rational design of high-activity mutants of BChE for the (–)-cocaine hydrolysis.

3D Model of BChE.

The initial coordinates of human BChE used in the computational studies came from the X-ray crystal structure deposited in the Protein Data Bank (pdb code: 1P0P). The missing residues (D2, D3, E255, D378, D379, N455, L530, E531, and M532) in the X-ray crystal structure were built using the automated homology modeling tool Modeler disclosed by Sali, A.; Blundell, T. L. *J. Mol. Biol.* 1990, 212 403, and Sali, A.; Blundell, T. L. *J. Mol. Biol.* 1993, 234, 779, herein incorporated by reference, and InsightII software (Accelrys, Inc.) with the default parameters.

Molecular Docking.

Molecular docking was performed for each non-prereactive protein-ligand binding complex. The binding site was defined as a sphere with an approximately 15 Å radius around the active site residue S198. The amino acid residues included in the binding site model are not contiguous in the protein. Cocaine, considered as a ligand, was initially positioned at 17 Å in front of S198 of the binding site. Each BChE-cocaine binding complex was energy-minimized by using the steepest descent algorithm first until the maximum energy derivative is smaller than 4 kcal/mol/Å and then the conjugated gradient algorithm until the maximum energy derivative is smaller than 0.001 kcal/mol/Å. The energy minimization was fol-

lowed by a 300 ps molecular dynamics (MD) simulation at T=298 K with a time step of 1 fs. During the energy minimization and MD simulation, only cocaine and the residues of BChE included in the binding site were allowed to move, while the remaining part of the protein was fixed. The energyminimization and MD simulation for these processes were performed by using the Amber force field implemented in the Discover_3/InsightII calculation engine, disclosed by Cornell, W. D.; Cieplak, P.; Bayl), C. I.; Gould, I. R.; Merz, Jr., K. M.; Ferguson, D. M.; Spellmeyer, D. C.; Fox, T.; Caldwell, J. W.; Kollman, P. A. J. Am. Chem. Soc. 1995, 117, 5179. The non-bonded cut-off method and the dielectric constant were set up to group based (12 Å cut-off distance) and distance dependent, respectively (∈=4r) in accordance with Harvey, S. C. Proteins 1989, 5, 78-92, herein incorporated by reference. Molecular Dynamic Simulation in Water.

The initial coordinates used in the MD simulation of the non-prereactive complexes were determined by using the molecular docking procedure described above, whereas the initial coordinates used in the MD simulation of the prereactive complexes were obtained from superimposing backbone of the X-ray crystal structure to that of the previously disclosed simulated prereactive complex of Zhan et al between cocaine and a homology model of wild-type BChE. Each BChE-cocaine binding complex was neutralized by adding 25 two chloride counterions and was solvated in a rectangular box of TIP3P water molecules with a minimum solute-wall distance of 10 Å. The general procedure for carrying out the MD simulations in water is similar to that used in our previously reported other computational studies such as those in 30 Zhan et al and (a) Zhan, C.-G.; Norberto de Souza, O.; Rittenhouse, R.; Ornstein, R. L. J. Am. Chem. Soc. 1999, 121, 7279, (b) Koca, J.; Zhan, C.-G.; Rittenhouse, R.; Ornstein, R. L. J. Am. Chem. Soc. 2001, 123, 817, (c) Koca, J.; Zhan, C.-G.; Rittenhouse, R. C.; Ornstein, R. L. J. Comput. Chem. 35 2003, 24, 368, herein all incorporated by reference. These simulations were performed by using the Sander module of Amber7 program as taught by Case, D. A.; Pearlman, D. A.; Caldwell, J. W.; Cheatham III, T. E.; Wang, J.; Ross, W. S.; Simmerling, C. L.; Darden, T. A.; Merz, K. M.; Stanton, R. V.; Cheng, A. L.; Vincent, J. J.; Crowley, M.; Tsui, V.; Gohlke, H.; Radmer, R. J.; Duan, Y.; Pitera, J.; Massova, I.; Seibel, G. L.; Singh, U.C.; Weiner, P.K.; Kollman, P.A. (2002), AMBER 7, University of California, San Francisco, herein incorporated by reference. The solvated system was optimized prior to the MD simulation. First, the protein-ligand was frozen and the 45 solvent molecules with counterions were allowed to move during a 5000-step minimization with the conjugate gradient algorithm and a 5 ps MD run at T=300 K. After full relaxation and the entire solvated system was energy-minimized, the system was slowly heated from T=10 K to T=300 K in 30 ps 50 before the production MD simulation for 500 ps. The full minimization and equilibration procedure was repeated for each mutant. The MD simulations were performed with a periodic boundary condition in the NPT ensemble at T=300 K with Berendsen temperature coupling and constant pressure (P=1 atm) with isotropic molecule-based scaling disclosed in Berendsen, H. C.; Postma, J. P. M.; van Gunsteren, W. F.; DiNola, A.; Haak, J. R. J. Comp. Phys. 1984, 81, 3684, herein incorporated by reference. The SHAKE algorithm of Ryckaert, J. P.; Ciccotti, G.; Berendsen, H. C. J. Comp. Phys. 1977, 23, 327 (herein incorporated by reference) was applied to fix all covalent bonds containing a hydrogen atom, a time step of 2 fs was used, and the non-bond pair list was updated every 10 steps. The pressure was adjusted by isotropic position scaling. The particle mesh Ewald (PME) method of Essmann, U.; Perera, L.; Berkowitz, M. L.; Darden, T. A.; Lee, H., Peder- 65 sen; L. G. J. Chem. Phys. 1995, 98, 10089, herein incorporated by reference, was used to treat long-range electrostatic

14

interactions. A residue-based cutoff of 10 Å was applied to the noncovalent interactions. During the 500 ps production MD simulation, the coordinates of the simulated complex were saved every 1 ps.

Molecular docking and MD simulation procedures described above were performed to study cocaine binding with wild-type BChE and three mutants, i.e., A328W/Y332A, A328W/Y332A/Y419S, and A328W/Y332G. For each protein system (wild-type or mutant BChE), the protein binding with cocaine was considered in both the non-prereactive and prereactive enzyme-substrate complexes.

Most of the MD simulations in water were performed on a supercomputer, Superdome (shared-memory, with 4 nodes and 256 processors), at the Center for Computational Sciences, University of Kentucky. The other computations were carried out on SGI Fuel workstations and a 34-processors IBM x335 Linux cluster.

Experimental Procedure.

Site-directed mutagenesis of human BChE cDNA was per-20 formed by the OuikChange method of Braman, J.: Papworth. C.; Greener, A. Methods Mol. Biol. 1996, 57, 5731, herein incorporated by reference. Mutations were generated from wild-type human BChE in a pRc/CMV expression plasmid in accordance with Xie, W.; Altamirano, C. V.; Bartels, C. F.; Speirs, R. J.; Cashman, J. R.; Lockridge, O. Mol. Pharmacol. 1999, 55, 83, all herein incorporated by reference, kindly provided by Dr. Lockridge at University of Nebraska Medical Center. Using plasmid DNA as template and primers with specific base-pair alterations, mutations were made by polymerase chain reaction with Pfu DNA polymerase, for replication fidelity. The PCR product was treated with Dpn I endonuclease to digest the parental DNA template. Modified plasmid DNA was transformed into Escherichia coli, amplified, and purified. The DNA sequences of the mutants were confirmed by DNA sequencing. BChE mutants were expressed in human embryonic kidney cell line 293T/17. Cells were grown to 80-90% confluence in 6-well dishes and then transfected by Lipofectamine 2000 complexes of 4 μg plasmid DNA per each well. Cells were incubated at 37° C. in a CO₂ incubator for 24 hours and cells were moved to 60-mm culture vessel and cultured for four more days. The culture medium [10% fetal bovine serum in Dulbecco's modified Eagle's medium (DMEM)] was harvested for a BChE activity assay. To measure cocaine and benzoic acid, the product of cocaine hydrolysis by BChE, we used sensitive radiometric assays based on toluene extraction of [3H]cocaine labeled on its benzene ring were used in accordance with Masson, P.; Xie, W., Froment, M-T.; Levitsky, V.; Fortier, P.-L.; Albaret, C.; Lockridge, O. Biochim. Biophys. Acta 1999, 1433, 281, herein incorporated by reference. In brief, to initiate reactions, 100 nCi of [3H]cocaine was mixed with 100 µl of culture medium. Reactions proceeded at 37° C. for varying times. Reactions were stopped by adding 300 µl of 0.02 M HCl, which neutralized the liberated benzoic acid while ensuring a positive charge on the residual cocaine. [3H]benzoic acid was extracted by 1 ml of toluene and measured by scintillation counting. Finally, the measured time-dependent radiometric data were fitted to the kinetic equation so that the catalytic efficiency (k_{cat}/K_M) was determined.

Depicted in FIG. 1 are plots of some important distances in the MD-simulated (–)-cocaine binding with A328W/Y332G BChE versus the simulation time, along with root-mean-square deviation (RMSD) of the coordinates of backbone atoms in the simulated structure from those in the X-ray crystal structure. MD trajectories for other complexes were similar to these two in FIG. 1, although the simulated average distances are different. Summarized in Table 2 are the average values of some important geometric parameters in the simulated complexes.

TABLE 2

	Average values of the geometric parameters ^c										
BChE-cocaine binding ^a	${<\!\!\mathrm{D1}\!\!>_{\!non}}$	<d1></d1>	<d2></d2>	<d3></d3>	<d4></d4>	<Θ>	nonpre	pre			
wild-type wild-type with (+)-cocaine ^b A328W/Y332A A328W/Y332G A328W/Y332A/Y419S	5.60 7.64 7.11 7.06 5.18	3.27 3.69 3.87 3.96 5.84	5.77 2.88 3.30 2.28 5.64	2.71 3.30 2.14 2.52 4.56	3.37 2.83 3.01 2.42 6.97	67 61 51 60 164	1.14 1.15 1.58 1.20 2.66	1.27 1.13 1.65 1.35 2.62			

"Refers to (-)-cocaine binding with wild-type human BChE or (-)-cocaine binding with a mutant BChE, unless indicated otherwise.

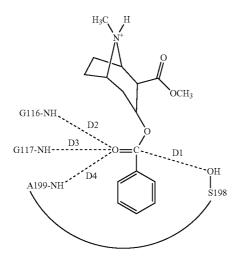
 ${}^b\mathrm{Refers}$ to (+)-cocaine binding with wild-type human BChE.

 c <D1> non and <D1> represent the average distances between the S198 O $^{\gamma}$ atom and the carbonyl carbon of the cocaine benzoyl ester in the simulated non-prereactive and prereactive BChE-cocaine complexes, respectively. <D2>, <D3>, <D4> refer to the average values of the simulated distances from the carbonyl oxygen of the cocaine benzoyl ester to the NH hydrogen atoms of G116, G117, and A199 residues, respectively. <Θ> is the average value of the dihedral angle formed by the S198 O $^{\gamma}$ atom and the plane of the carboxylate group of the cocaine benzoyl ester. See Scheme 2.

^dThe root-mean-square deviation (RMSD) of the coordinates of backbone atoms in the simulated structure from those 30 in the X-ray crystal structure of BChE. "nonpre" and "pre" refer to the non-prereactive and prereactive BChE-cocaine complexes, respectively.

(-)- and (+)-Cocaine Binding with Wild-Type BChE.

FIG. 2 shows the binding structures of the simulated non-prereactive and prereactive complexes of wild-type BChE binding with the two enantiomers of cocaine. In the non-prereactive complexes with (–)- and (+)-cocaine, the methyl ester group of cocaine is positioned at the top of the H438 backbone, while the cocaine benzoyl ester moiety is quasiparallel to the C— O^{γ} side chain of S198 with a dihedral angle Θ of -8° and 140° , respectively.



Scheme 2. Hydrolysis of (–)-cocaine and (+)-cocaine. Here, Θ refers to the dihedral angle formed by S198 Θ^{γ} and 65 the plane of carboxylate group of the cocaine benzoyl ester as shown in the structure diagram below.

The simulated internuclear distances between the carbonyl oxygen of cocaine benzoyl ester group and the NH hydrogen of G116, G117, and A199 are comparable for the two enantiomers. The simulated average distances between the carbonyl carbon of the benzoyl ester and S198 O $^{\gamma}$ are 5.60 Å and 5.18 Å for (–)- and (+)-cocaine, respectively. Comparing the simulated protein backbone structures to the X-ray crystal structure of Nicolet et al, one can see from FIG. 1 that the RMSD values are all smaller than 1.3 Å for the whole protein structures.

The MD simulations of the prereactive complexes reveal that wild-type BChE binding with (–)-cocaine is essentially the same as the binding with (+)-cocaine in the binding site, except for the different positions of methyl ester group of the substrates. The simulated average distances between the carbonyl carbon of the benzoyl ester and S198 O $^{\gamma}$ are 3.27 and 3.69 Å for (–)-cocaine and (+)-cocaine, respectively. Moreover, the (+)-cocaine is stabilized more effectively by the formation of strong hydrogen bonds with the backbone NH of residues G116, G117, and A199 as summarized above in Table 2. The cocaine benzoyl ester moiety is positioned quasiperpendicular to S198 C—O $^{\gamma}$ with a dihedral angle θ of \sim 67° and \sim 61° for (–)- and (+)-cocaine, respectively.

A comparison was made between the currently simulated structures of the BChE-cocaine binding with those simulated previously by using a homology model of BChE and it was noted that two major differences between the two sets of structures. By using the X-ray crystal structure in accordance with Nicolet et al, the acyl loop is positioned on the top of the cocaine benzoyl ester moiety of the cocaine, whereas the acyl loop is far from the cocaine benzoyl ester moiety in the structure simulated starting from the homology model of Zhan et al. The RMSD of the coordinates of backbone atoms in the previously simulated prereactive BChE-(-)-cocaine complex from those in the X-ray crystal structure of BChE is ~2.0 Å for the entire protein and ~3.0 Å for the acyl loop. The RMSD value became ~2.4 Å for the entire protein and ~3.3 Å 50 for the acyl loop, when the X-ray crystal structure was replaced by the MD-simulated prereactive BChE-(-)-cocaine complex starting from the X-ray crystal structure. Despite these structural differences, the benzoyl ester group of the ligand is still close to the key residues (S197, G116, and 55 G117) in the BChE binding site. Some significant differences are associated with the distances between the S198 O^{γ} atom and the carbonyl carbon of the cocaine benzoyl ester in nonprereactive complexes. The average values of this distance in the non-prereactive complexes were ~ 9.5 and ~ 8.5 Å for (–)and (+)-cocaine, respectively, when a homology model was used. Using the X-ray crystal structure to conduct the analysis, corresponding average values became ~5.6 and ~5.2 Å, respectively. Therefore, both (-)- and (+)-cocaine became closer to the binding site when the homology model was replaced by the X-ray crystal structure. However, no significant changes of the binding in the prereactive complexes were observed when the used homology model was replaced by the

X-ray crystal structure. The average values of the simulated distance between the S198 O $^{\gamma}$ atom and the carbonyl carbon of the cocaine benzoyl ester in the prereactive complexes are always close to ~3.5 Å for both (–)- and (+)-cocaine no matter whether the X-ray crystal structure or homology model of 5 BChE was used as the starting structure. The similar computational results obtained from the use of the X-ray crystal structure and homology model of BChE provides evidence that the fundamental structural and mechanistic insights obtained from the previous computational studies of Zhan et al are reliable, despites the previous simulations were performed by using the homology model when the X-ray crystal structure was not available.

17

Further, the simulated structures of the non-prereactive BChE-cocaine complexes were superimposed with the corresponding prereactive complexes. As shown in FIG. 3, the (–)-cocaine rotation in the BChE active site from the non-prereactive complex to the prereactive complex is hindered by some residues as the positions of Y332, A328, and F329 residues in the non-prereactive complex are significantly different from the corresponding positions in the prereactive complex, whereas none of these residues hinders the (+)-cocaine rotation in the BChE active site from the non-prereactive complex to the prereactive complex because these residues stay in nearly the same positions in the two BChE-(+)- 25 cocaine complexes.

(-)-Cocaine Binding with BChE Mutants.

Now that the (-)-cocaine rotation from the non-prereactive complex to the prereactive complex has been known to be the rate-determining step of the BChE-catalyzed hydrolysis of 30 (-)-cocaine as shown by Zhan et al, useful BChE mutants should be designed to specifically accelerate the change from the non-prereactive BChE-(-)-cocaine complex to the prereactive complex. The question is whether MD simulation can be performed to help design BChE mutants that have higher 35 catalytic activity for (-)-cocaine hydrolysis.

In the simulated non-prereactive complex, the average distance between the carbonyl carbon of cocaine benzoyl ester and S198 O^y is 7.6 Å for A328W/Y332A BChE and 7.1 Å for A328W/Y332G BChE, as seen in Table 2 above. In the simulated prereactive complex, the average values of this important internuclear distance become 3.87 and 3.96 Å for A328W/Y332A and A328W/Y332G BChE's, respectively. Compared to the simulated wild-type BChE-(-)-cocaine prereactive complex, the average distances between the carbonyl 45 carbon of the cocaine benzoyl ester and S198 O^γ in the prereactive complex of (-)-cocaine with A328W/Y332A and A328W/Y332G BChE's are all slightly longer, whereas the average distances between the carbonyl oxygen of the cocaine benzoyl ester and the NH of G116, G117, and A199 50 residues are all shorter. This provides evidence that (-)-cocaine more strongly bind with A328W/Y332A and A328W/ Y332G BChE's in the prereactive complexes. More importantly, the (-)-cocaine rotation in the active site of A328W/ Y332A and A328W/Y332G BChE's from the non- 55 prereactive complex to the prereactive complex did not cause considerable changes of the positions of A332 (or G332), W328, and F329 residues as seen in FIG. 4, compared to the (-)-cocaine rotation in the active site of wild-type BChE. These results provide evidence that A328W/Y332A and 60 A328W/Y332G BChE's should be associated with lower energy barriers than the wild-type for the (-)-cocaine rotation from the non-prereactive complex to the prereactive complex. Further, (-)-cocaine binding with A328W/Y332G BChE is very similar to the binding with A328W/Y332A BChE, but 65 the position change of F329 residue caused by the (-)-cocaine rotation was significant only in A328W/Y332A BChE, thus

18

suggesting that the energy barrier for the (–)-cocaine rotation in A328W/Y332G BChE should be slightly lower than that in A328W/Y332A BChE.

Concerning (-)-cocaine binding with A328W/Y332A/ Y419S BChE, Y419 stays deep inside the protein and does not directly contact with the cocaine molecule. The Y419S mutation was tested because it was initially expected that this mutation would further increase the free space of the active site pocket so that the (-)-cocaine rotation could be easier. However, as seen in Table 2 above, the average distance between the carbonyl carbon of cocaine benzoyl ester and S198 O^{γ} atom in the simulated prereactive complex was as long as 5.84 Å. The average distances between the carbonyl oxygen of the cocaine benzoyl ester and the NH hydrolysis atoms of G116, G117, and A199 residues are between 4.56 and 6.97 Å; no any hydrogen bond between them. In addition to the internuclear distances, another interesting geometric parameter is the dihedral angle, Θ , formed by S198 O^{γ} and the plane of the carboxylate group of the cocaine benzoyl ester. As seen in Table 2, the Θ values in the prereactive complexes of cocaine with wild-type BChE and all of the BChE mutants other than A328W/Y332A/Y419S BChE all slightly deviate from the ideal value of 90° for the nucleophilic attack of S198 O^{γ} at the carbonyl carbon of cocaine. The Θ value in the prereactive complex of (-)-cocaine with A328W/Y332A/ Y419S BChE is 164°, which is considerably different from the ideal value of 90°.

Catalytic Activity.

The aforementioned discussion provides evidence that the energy barriers for the (-)-cocaine rotation in A328W/ Y332A and A328W/Y332G BChE's from the non-prereactive complex to the prereactive complex, the rate-determining step for the BChE-catalyzed hydrolysis of (-)-cocaine, should be lower than that in the wild-type BChE. Thus, the MD simulations predict that both A328W/Y332A and A328W/Y332G BChE's should have a higher catalytic activity than the wild-type BChE for (-)-cocaine hydrolysis. Further, the MD simulations also suggest that the energy barrier for the (-)-cocaine rotation in A328W/Y332G BChE should be slightly lower than that in A328W/Y332A BChE and, therefore, the catalytic activity of A328W/Y332G BChE for the (-)-cocaine hydrolysis should be slightly higher than the activity of A328W/Y332A BChE. In addition, the MD simulations predict that A328W/Y332A/Y419S BChE should have no catalytic activity, or have a considerably lower catalytic activity than the wild-type, for (-)-cocaine hydrolysis because (-)-cocaine binds with the mutant BChE in a way that is not suitable for the catalysis.

The catalytic efficiency (k_{cat}/K_M) of A328W/Y332A BChE for (-)-cocaine hydrolysis was reported to be 8.56×10^6 M min⁻¹, which is 9.39 times of the k_{cat}/K_M value (9.11×10⁵ M min⁻¹) of the wild-type BChE. To examine these theoretical predictions of the relative activity for A328W/Y332G and A328W/Y332A/Y419SBChE's, a A328W/Y332A, A328W/ Y332G, and A328W/Y332A/Y419S BChE was produced through site-directed mutagenesis. To minimize the possible systematic experimental errors of the kinetic data, kinetic studies were performed with all of three mutants under the same condition and compared the catalytic efficiency of the A328W/Y332G and A328W/Y332A/Y419S to that of the A328W/Y332A for (-)-cocaine hydrolysis at benzoyl ester group. Based on the kinetic analysis of the measured timedependent radiometric data, the ratio of the k_{cat}/K_M value of A328W/Y332G BChE to the k_{cat}/K_M value of A328W/ Y332A BChE for the (-)-cocaine hydrolysis was determined to be ~2.08, or A328W/Y332G BChE has a k_{cat}/K_M value of $\sim 1.78 \times 10^7$ M min⁻¹ for the (-)-cocaine hydrolysis. The radio-

metric data show no significant catalytic activity for A328W/Y332A/Y419S BChE. These experimental data are consistent with the theoretical predictions based on the MD simulations.

Conclusion.

Molecular modeling, molecular docking, and molecular dynamics (MD) simulations were performed to study cocaine binding with human butyrylcholinesterase (BChE) and its mutants, based on a recently reported X-ray crystal structure of human BChE. The MD simulations of cocaine binding with wild-type BChE led to average BChE-cocaine binding structures similar to those obtained recently from the MD simulations based on a homology model of BChE, despite the significant difference found at the acyl binding pocket. This confirms the fundamental structural and mechanistic insights obtained from the prior computational studies of Zhan et al based on a homology model of BChE, e.g., the rate-determining step for BChE-catalyzed hydrolysis of biologically active (-)-cocaine is the (-)-cocaine rotation in the BChE active site from the non-prereactive BChE-(–)-cocaine complex to the $\,^{20}$ prereactive complex.

The MD simulations further reveal that the (-)-cocaine rotation in the active site of wild-type BChE from the nonprereactive complex to the prereactive complex is hindered by some residues such that the positions of Y332, A328, and F329 residues in the non-prereactive complex are significantly different from those in the prereactive complex. Compared to (-)-cocaine binding with wild-type BChE, (-)-cocaine more strongly bind with A328W/Y332A and A328W/ Y332G BChE's in the prereactive complexes. More importantly, the (-)-cocaine rotation in the active site of A328W/Y332A and A328W/Y332G BChE's from the nonprereactive complex to the prereactive complex did not cause considerable changes of the positions of A332 or G332, W328, and F329 residues. These results provide evidence that A328W/Y332A and A328W/Y332G BChE's are associated with lower energy barriers than wild-type BChE for the (-)cocaine rotation from the non-prereactive complex to the prereactive complex. Further, (-)-cocaine binding with A328W/Y332G BChE is very similar to the binding with

20

A328W/Y332A BChE, but the position change of F329 residue caused by the (–)-cocaine rotation was significant only in A328W/Y332A BChE, thus suggesting that the energy barrier for (–)-cocaine rotation in A328W/Y332G BChE should be slightly lower than that in A328W/Y332A BChE. It has also been demonstrated that (–)-cocaine binds with A328W/Y332A/Y419S BChE in a way that is not suitable for the catalysis.

Based on the computational results, both A328W/Y332A and A328W/Y332G BChE's have catalytic activity for (-)-cocaine hydrolysis higher than that of wild-type BChE and the activity of A328W/Y332G BChE should be slightly higher than that of A328W/Y332A BChE, whereas A328W/Y332A/Y419S BChE is expected to lose the catalytic activity. The computational predictions are completely consistent with the experimental kinetic data, providing evidence that the used computational protocol, including molecular modeling, molecular docking, and MD simulations, is reliable in prediction of the catalytic activity of BChE mutants for (-)-cocaine hydrolysis.

Experiment 2

MD Simulations and Quantum Mechanical/Molecular Mechanical (QM/MM) Calculations Relating to A1995/A328W/Y332G Mutant (Mutant 1) (SEQ ID NO: 1)

Generally speaking, for rational design of a mutant enzyme with a higher catalytic activity for a given substrate, one needs to design a mutation that can accelerate the rate-determining step of the entire catalytic reaction process while the other steps are not slowed down by the mutation. Reported computational modeling and experimental data indicated that the formation of the prereactive BChE-(-)-cocaine complex (ES) is the rate-determining step of (-)-cocaine hydrolysis catalyzed by wild-type BChE as disclosed by Sun et al, Zhan et al and Hamza, A.; Cho, H.; Tai, H.-H.; Zhan, C.-G. *J. Phys. Chem. B* 2005, 109, 4776, herein incorporated by reference, whereas the rate-determining step of the corresponding (+)-cocaine hydrolysis is the chemical reaction process consisting of four individual reaction steps disclosed by Zhan et al and shown in Scheme 3 and Scheme 4 below.

Scheme 3. Schematic representation of BChE-catalyzed hydrolysis of (–)-cocaine. Only QM-treated high-layer part of the reaction system in the QM/MM calculations are drawn. Notation [H] refers to a non-hydrogen atom in the MM-treated low-layer part of the protein and the cut covalent bond 5 with this atom is saturated by a hydrogen atom. The dash lines in the transition state structures represent the transition bonds.

This mechanistic understanding is consistent with the experimental observation of Sun et al, that the catalytic rate constant of wild-type BChE against (+)-cocaine is pH-dependent, whereas that of the same enzyme against (-)-cocaine is independent of the pH. The pH-dependence of the rate constant for (+)-cocaine hydrolysis is clearly associated with the protonation of H438 residue in the catalytic triad (S198, H438, and E325). For the first and third steps of the reaction process, when H438 is protonated, the catalytic triad cannot function and, therefore, the enzyme becomes inactive. The lower the pH of the reaction solution is, the higher the concentration of the protonated H438 is, and the lower the concentration of the active enzyme is. Hence, the rate constant was found to decrease with decreasing the pH of the reaction solution for the enzymatic hydrolysis of (+)-cocaine.

Based on the above mechanistic understanding, the efforts for rational design of the BChE mutants reported in literature have been focused on how to improve the ES formation 25 process. Indeed, several BChE mutants, including A328W, A328W/Y332A, A328W/Y332G, and F227A/S287G/ A328W/Y332M, have been found to have a significantly higher catalytic efficiency (k_{cat}/K_M) against (-)-cocaine; these mutants of BChE have an approximate 9 to 34-fold 30 improved catalytic efficiency against (-)-cocaine. Experimental observation also indicated that the catalytic rate constant of A328W/Y332A BChE is pH-dependent for both (-)and (+)-cocaine. The pH-dependence reveals that for both (-)- and (+)-cocaine, the rate-determining step of the 35 hydrolysis catalyzed by A328W/Y332A BChE should be either the first or the third step of the reaction process. Further, if the third step were rate determining, then the catalytic efficiency of the A328W/Y332A mutant against (-)-cocaine should be as high as that of the same mutant against (+)- 40 cocaine because the (-)- and (+)-cocaine hydrolyses share the same third and fourth steps (see Scheme 3). However, it has also been observed that the A328W/Y332A mutant only has a ~9-fold improved catalytic efficiency against (-)-cocaine, whereas the A328W/Y332A mutation does not change the 45 high catalytic activity against (+)-cocaine. This analysis of the experimental and computational data available in literature clearly shows that the rate-determining step of (-)-cocaine hydrolysis catalyzed by the A328W/Y332A mutant should be the first step of the chemical reaction process. 50 Further, recently reported computational modeling also suggests that the formation of the prereactive BChE-(-)-cocaine complex (ES) is hindered mainly by the bulky side chain of Y332 residue in wild-type BChE, but the hindering can be removed by the Y332A or Y332G mutation. Therefore, start-55 ing from the A328W/Y332A or A328W/Y332G mutant, the truly rational design of further mutation(s) to improve the catalytic efficiency of BChE against (-)-cocaine should aim to decrease the energy barrier for the first reaction step without significantly affecting the ES formation and other chemi- 60 cal reaction steps.

The following rational design of a high-activity mutant of BChE against (-)-cocaine is based on detailed computational modeling of the transition state for the rate-determining step (i.e., the first step of the chemical reaction process). Molecular dynamics (MD) simulations and hybrid quantum mechanical/molecular mechanical (QM/MM) calculations

were performed to model the protein environmental effects on the stabilization of the transition-state structure for BChEcatalyzed hydrolysis of (-)-cocaine. The simulated and calculated results indicate that the transition-state structure can be stabilized better by the protein environment in A199S/ A328W/Y332G mutant of BChE than that in the wild-type. The computational modeling led to a prediction of the higher catalytic efficiency for the A199S/A328W/Y332G mutant against (-)-cocaine. The prediction has been confirmed by wet experimental tests showing that the A199S/A328W/ Y332G mutant has a significantly improved catalytic efficiency against (-)-cocaine. All of the obtained results clearly demonstrate that directly modeling the transition-state structure provides a reliable computational approach to the rational design of a high-activity mutant of BChE against (-)cocaine.

MD Simulations.

It should be stressed that a critical issue exists with regard to any MD simulation on a transition state. In principle, MD simulation using a classical force field (molecular mechanics) can only simulate a stable structure corresponding to a local minimum on the potential energy surface, whereas a transition state during a reaction process is always associated with a first-order saddle point on the potential energy surface. Hence, MD simulation using a classical force field cannot directly simulate a transition state without any restraint on the geometry of the transition state. Nevertheless, if one can technically remove the freedom of imaginary vibration in the transition state structure, then the number of vibrational freedoms (normal vibration modes) for a nonlinear molecule will decrease from 3N-6. The transition state structure is associated with a local minimum on the potential energy surface within a subspace of the reduced vibrational freedoms, although it is associated with a first-order saddle point on the potential energy surface with all of the 3N-6 vibrational freedoms. Theoretically, the vibrational freedom associated with the imaginary vibrational frequency in the transition state structure can be removed by appropriately freezing the reaction coordinate. The reaction coordinate corresponding to the imaginary vibration of the transition state is generally characterized by a combination of some key geometric parameters. These key geometric parameters are bond lengths of the forming and breaking covalent bonds for BChE-catalyzed hydrolysis of cocaine, as seen in Scheme 3. Thus, one just needs to maintain the bond lengths of the forming and breaking covalent bonds during the MD simulation on a transition state. Technically, one can maintain the bond lengths of the forming and breaking covalent bonds by simply fixing all atoms within the reaction center, by using some constraints on the forming and breaking covalent bonds, or by redefining the forming and breaking covalent bonds. It should be pointed out that the only purpose of performing such type of MD simulation on a transition state is to examine the dynamic change of the protein environment surrounding the reaction center and the interaction between the reaction center and the protein environment. For this study, of interest is the simulated structures, as the total energies calculated in this way are meaningless.

The initial BChE structures used in the MD simulations were prepared based on the previous MD simulation in accordance with Hamza et al on the prereactive ES complex for wild-type BChE with (–)-cocaine in water by using Amber7 program package. The previous MD simulations on the prereactive BChE-(–)-cocaine complex (ES) started from the X-ray crystal structure of Nicolet et al deposited in the Protein Data Bank (pdb code: 1P0P). The present MD simulation on the transition state for the first step (TS1) was performed in

such a way that bond lengths of the partially formed and partially broken covalent bonds in the transition state were all constrained to be the same as those obtained from our previous ab initio reaction coordinate calculations on the model reaction system of wild-type BChE in accordance with Zhan et al. The partially formed and partially broken covalent bonds in the transition state will be called "transition" bonds below, for convenience. A sufficiently long MD simulation with the transition bonds constrained should lead to a reasonable protein environment stabilizing the reaction center in the transition-state structure simulated. Further, the simulated TS1 structure for wild-type BChE with (–)-cocaine was used to build the initial structures of TS1 for the examined BChE mutants with (–)-cocaine; only the side chains of mutated residues needed to be changed.

The partial atomic charges for the non-standard residue atoms, including cocaine atoms, in the TS1 structures were calculated by using the RESP protocol implemented in the Antechamber module of the Amber7 package following electrostatic potential (ESP) calculations at ab initio HF/6-31G* level using Gaussian03 program known in the art. The geometries used in the ESP calculations came from those obtained from the previous ab initio reaction coordinate calculations of Zhan et al, but the functional groups representing the oxyan-25 ion hole were removed. Thus, residues G116, G117, and A199 were the standard residues as supplied by Amber7 in the MD simulations. The general procedure for carrying out the MD simulations in water is essentially the same as that used in our previously reported other computational studies including Zhan et al, Hamza et al and (a) Zhan, C.-G.; Norberto de Souza, O.; Rittenhouse, R.; Ornstein, R. L. J. Am. Chem. Soc. 1999, 121, 7279, (b) Koca, J.; Zhan, C.-G.; Rittenhouse, R.; Ornstein, R. L. J. Am. Chem. Soc. 2001, 123, 35 817, (c) Koca, J.; Zhan, C.-G.; Rittenhouse, R. C.; Ornstein, R. L. J. Comput. Chem. 2003, 24, 368, (d) Hamza, A.; Cho, H.; Tai, H.-H.; Zhan, C.-G. Bioorg. Med. Chem. 2005, 13, 4544, herein all incorporated by reference. Each aforementioned starting TS1 structure was neutralized by adding chloride counterions and was solvated in a rectangular box of TIP3P water molecules with a minimum solute-wall distance of 10 Å as described by Jorgensen, W. L.; Chandrasekhar, J.; Madura, J.; Klein, M. L. J. Chem. Phys. 1983, 79, 926, herein 45 incorporated by reference. The total numbers of atoms in the solvated protein structures for the MD simulations are nearly 70,000, although the total number of atoms of BChE and (-)-cocaine is only 8417 (for the wild-type BChE). All of the MD simulations were performed by using the Sander module of Amber7 package. The solvated systems were carefully equilibrated and fully energy minimized. These systems were gradually heated from T=10 K to T=298.15 K in 30 ps before running the MD simulation at T=298.15 K for 1 ns or longer, 55 making sure that a stable MD trajectory was obtained for each of the simulated TS1 structures. The time step used for the MD simulations was 2 fs. Periodic boundary conditions in the NPT ensemble at T=298.15 K with Berendsen temperature coupling and P=1 atm with isotropic molecule-based scaling were applied. The SHAKE algorithm was used to fix all covalent bonds containing hydrogen atoms. The non-bonded pair list was updated every 10 steps. The particle mesh Ewald (PME) method in accordance with Essmann, U.; Perera, L.; 65 Berkowitz, M. L.; Darden, T. A.; Lee, H., Pedersen, L. G. J. Chem. Phys. 1995, 98, 10089, herein incorporated by refer30

ence, was used to treat long-range electrostatic interactions. A residue-based cutoff of 10~Å was utilized to the non-covalent interactions. The coordinates of the simulated systems were collected every 1~ps during the production MD stages.

QM/MM Calculations.

For each TS1 structure examined, after the MD simulation was completed and a stable MD trajectory was obtained, all of the collected snapshots of the simulated structure, excluding those before the trajectory was stabilized, were averaged and further energy-minimized. The energy-minimized average structure (with the transition bonds constrained) was used as an initial geometry to carry out a further geometry optimization by using the ONIOM approach of Dapprich, S.; Komaromi, I.; Byun, K. S.; Morokuma, K.; Frisch, M. J. J. Mol. Struct. (Theochem) 1999, 461, 1-21, herein incorporated by reference, implemented in the Gaussian 03 program of Frisch, M. J. et al Gaussian 03, Revision A.1, Gaussian, Inc., Pittsburgh, Pa., 2003, herein incorporated by reference. Two layers were defined in the present ONIOM calculation: the high layer, as depicted in Scheme 3 above, was calculated quantum mechanically at the ab initio HF/3-21G level, whereas the low layer was calculated molecular mechanically by using the Amber force field as used in our MD simulation and energy minimization with the Amber 7 program. The ONIOM calculations at the HF/3-21G: Amber level in this study are a type of QM/MM calculations of Vreven, T.; Morokuma, K. J. Chem. Phys. 2000, 113, 2969-2975 and Frisch, M.; Vreven, T.; Schlegel, H. B.; Morokuma, K. J. Comput. Chem. 2003, 24, 760-769, herein both incorporated by reference. Previous reaction coordinate calculations with an active site model of wild-type BChE demonstrate that the HF/3-21G level is adequate for the geometry optimization of this enzymatic reaction system shown by Zhan et al, although the final energy calculations for calculating the energy barriers must be carried out at a higher level. As depicted in Scheme 3, for all of these QM/MM calculations, the same part of the enzyme was included in the QM-treated high layer. So, the QM-treated high layer included (-)-cocaine, key functional groups from the catalytic triad (S198, H438, and E325), and the three residues, i.e., G116, G117, and A199 (or S199 for a particular mutant, see Scheme 4), of the possible oxyanion hole, whereas the entire enzyme structure of BChE was included in the MM-treated low layer. A language computer program was developed to automatically generate the input files for the ONIOM calculations following the MD simulations and subsequent energy minimizations in order to make sure that the atom types used for all low-layer atoms are the same as what were used in the Amber7. During the TS1 geometry optimization using the two-layer ONIOM, the length of a key transition C—O bond was fixed which dominates the reaction coordinate; all of the other transition bond lengths were relaxed. The C and O atoms in the key transition O bond are the carbonyl carbon of (-)-cocaine benzoyl ester and the O^y atom of S198, respectively, according to the previous reaction coordinate calculations with an active site model of wild-type BChE of Zhan et al.

Most of the MD simulations and QM/MM calculations were performed in parallel on an HP supercomputer (Superdome, with 256 shared-memory processors) at the Center for Computational Sciences, University of Kentucky. Some of the computations were carried out on a 34-processors IBM x335 Linux cluster and SGI Fuel workstations.

Scheme 4. Schematic representation of the first reaction 65 step for (–)-cocaine hydrolysis catalyzed by a BChE mutant with an A199S mutation.

Experimental Materials.

Cloned pfu DNA polymerase and Dpn I endonuclease were obtained from Stratagene (La Jolla, Calif.). ³H-(–)-cocaine

efficiency $(k_{\it cat}/K_{\it M})$ was determined along with the use of an enzyme-linked immunosorbent assay (ELISA) described

34

(50 Ci/mmol) was purchased from PerkinElmer Life Sciences (Boston, Mass.). The expression plasmid pRc/CMV was a gift from Dr. O. Lockridge, University of Nebraska Medical Center (Omaha, Nebr.). All oligonucleotides were synthesized by the Integrated DNA Technologies, Inc. The OIAprep Spin Plasmid Miniprep Kit and Oiagen plasmid purification kit and OIAquick PCR purification kit were obtained from Qiagen (Santa Clarita, Calif.). Human embryonic kidney 293T/17 cells were from ATCC (Manassas, Va.). Dulbecco's modified Eagle's medium (DMEM) was purchased from Fisher Scientific (Fairlawn, N.J.). Oligonucleotide primers were synthesized by the Integrated DNA Technologies and Analysis Facility of the University of Kentucky. 3,3',5,5'-Tetramethylbenzidine (TMB) was obtained from Sigma (Saint Louis, Mo.). Anti-butyrylcholinesterase (mouse monoclonal antibody, Product #HAH002-01) was purchased from AntibodyShop (Gentofte, Denmark) and Goat antimouse IgG HRP conjugate from Zymed (San Francisco, Calif.).

Enzyme-Linked Immunosorbent Assay (ELISA).

The ELISA buffers used in the present study are the same as those described in the literature such as (a) Brock, A.; Mortensen, V.; Loft, A. G. R.; Nergaard-Pedersen, B. J. Clin. Chem. Clin. Biochem. 1990, 28, 221-224, (b) Khattab, A. D.; Walker, C. H.; Johnston, G.; Siddiqui, M. K. Saphier, P. W. Environmental Toxicology and Chemistry 1994, 13, 1661-1667, herein both incorporated by reference. The coating buffer was 0.1 M sodium carbonate/bicarbonate buffer, pH 9.5. The diluent buffer (EIA buffer) was potassium phosphate monobasic/potassium phosphate monohydrate buffer, pH 7.5, containing 0.9% sodium chloride and 0.1% bovine serum albumin. The washing buffer (PBS-T) was 0.01 M potassium phosphate monobasic/potassium phosphate monohydrate buffer, pH 7.5, containing 0.05% (v/v) Tween-20. All the assays were performed in triplicate. Each well of an ELISA 20 microtiter plate was filled with 100 ul of the mixture buffer consisting of 20 µl culture medium and 80 µl coating buffer. The plate was covered and incubated overnight at 4° C. to allow the antigen to bind to the plate. The solutions were then removed and the wells were washed four times with PBS-T. The washed wells were filled with 200 µl diluent buffer and kept shaking for 1.5 h at room temperature (25° C.). After washing with PBS-T for four times, the wells were filled with 100 µl antibody (1:8000) and were incubated for 1.5 h, followed by washing for four times. Then, the wells were filled with 100 µl goat anti-mouse IgG HRP conjugate complex diluted to a final 1:3000 dilution, and were incubated at room temperature for 1.5 h, followed by washing for four times. The enzyme reactions were started by addition of 100 µl substrate (TMB) solution. The reactions were stopped after 15 min by the addition of 100 μl of 2 M sulfuric acid, and the absorbance was read at 460 nm using a Bio-Rad ELISA plate reader.

Site-Directed Mutagenesis, Protein Expression, and BChE Activity Assay.

Site-directed mutagenesis of human BChE cDNA was performed by using the QuikChange method of Braman et al. Mutations were generated from wild-type human BChE in a 25 pRc/CMV expression plasmid in accordance with (a) Masson, P.; Legrand, P.; Bartels, C. F.; Froment, M.-T.; Schopfer, L. M.; Lockridge, O. Biochemistry 1997, 36, 2266, (b) Masson, P.; Xie, W., Froment, M-T.; Levitsky, V.; Fortier, P.-L.; Albaret, C.; Lockridge, O. Biochim. Biophys. Acta 1999, 1433, 281, (c) Xie, W.; Altamirano, C. V.; Bartels, C. F.; Speirs, R. J.; Cashman, J. R.; Lockridge, O. Mol. Pharmacol. 1999, 55, 83, (d) Duysen, E. G.; Bartels, C. F.; Lockridge, O. J. Pharmacol. Exp. Ther. 2002, 302, 751, (e) Nachon, F.; 35 Nicolet, Y.; Viguie, N.; Masson, P.; Fontecilla-Camps, J. C.; Lockridge, O. Eur. J. Biochem. 2002, 269, 630, herein all incorporated by reference. Using plasmid DNA as template and primers with specific base-pair alterations, mutations merase, for replication fidelity. The PCR product was treated with Dpn I endonuclease to digest the parental DNA template. Modified plasmid DNA was transformed into Escherichia coli, amplified, and purified. The DNA sequences of the mutants were confirmed by DNA sequencing. BChE mutants 45 were expressed in human embryonic kidney cell line 293T/ 17. Cells were grown to 80-90% confluence in 6-well dishes and then transfected by Lipofectamine 2000 complexes of 4 μg plasmid DNA per each well. Cells were incubated at 37° C. in a CO₂ incubator for 24 hours and cells were moved to 50 60-mm culture vessel and cultured for four more days. The culture medium [10% fetal bovine serum in Dulbecco's modified Eagle's medium (DMEM)] was harvested for a BChE activity assay. To measure (-)-cocaine and benzoic acid, the product of (-)-cocaine hydrolysis catalyzed by 55 BChE, sensitive radiometric assays were used based on toluene extraction of [3H]-(-)-cocaine labeled on its benzene ring in accordance with Sun et al. In brief, to initiate the enzymatic reaction, 100 nCi of [³H]-(-)-cocaine was mixed with 100 μl of culture medium. The enzymatic reactions proceeded at 60 room temperature (25° C.) for varying time. The reactions were stopped by adding 300 µl of 0.02 M HCl, which neutralized the liberated benzoic acid while ensuring a positive charge on the residual (-)-cocaine. [3H]benzoic acid was extracted by 1 ml of toluene and measured by scintillation 65 counting. Finally, the measured time-dependent radiometric

data were fitted to the kinetic equation so that the catalytic

Hydrogen Bonding Revealed by the MD Simulations.

In accordance with one aspect of the present invention, were made by polymerase chain reaction with Pfu DNA poly- 40 namely the generation of high-activity mutants of BChE against (-)-cocaine, the present invention includes predicting some possible mutations that can lower the energy of the transition state for the first chemical reaction step (TS1) and, therefore, lower the energy barrier for this critical reaction step. Apparently, a mutant associated with the stronger hydrogen bonding between the carbonyl oxygen of (-)-cocaine benzovl ester and the oxyanion hole of the BChE mutant in the TS1 structure may potentially have a more stable TS1 structure and, therefore, a higher catalytic activity for (-)cocaine hydrolysis. Hence, the hydrogen bonding with the oxyanion hole in the TS1 structure is a crucial factor affecting the transition state stabilization and the catalytic activity. The possible effects of some mutations on the hydrogen bonding were examined by performing MD simulations and QM/MM calculations on the TS1 structures for (–)-cocaine hydrolysis catalyzed by the wild-type and various mutants BChE's.

The MD simulation in water was performed for 1 ns or longer to make sure a stable MD trajectory was obtained for each simulated TS1 structure with the wild-type or mutant BChE. The MD trajectories actually became stable quickly, so were the H . . . O distances involved in the potential hydrogen bonds between the carbonyl oxygen of (-)-cocaine and the oxyanion hole of BChE.

Depicted in FIG. 5 are plots of four important H . . . O distances in the MD-simulated TS1 structure versus the simulation time for (-)-cocaine hydrolysis catalyzed by A328W/ Y332A or A199S/A328W/Y332G BChE, along with the root-mean-square deviation (RMSD) of the simulated positions of backbone atoms from those in the corresponding initial structure. Traces D1, D2, and D3 refer to the distances between the carbonyl oxygen of (-)-cocaine and the NH hydrogen of G116, G117, and 5199, respectively. Trace D4 is the internuclear distance between the carbonyl oxygen of (-)-cocaine and the hydroxyl hydrogen of the 5199 side chain which exists only in A199S/A328W/Y332G BChE. RMSD represents the root-mean-square deviation (in Å) of the simulated positions of the protein backbone atoms from those in the initial structure. The H . . . O distances in the simulated TS1 structures corresponding to the wild-type BChE and the two mutants are summarized in Table 3. The HO distances between the carbonyl oxygen of (-)-cocaine and the peptidic NH hydrogen atoms of G116, G117, and A199 (or S199) of BChE are denoted by D1, D2, and D3, respectively, in Table 2 and FIG. 1. D4 in Table 3 and FIG. 5 refers to the H...O distance between the carbonyl oxygen of (-)-cocaine and the hydroxyl hydrogen of S199 side chain in the simulated TS1 structure corresponding to the A199S/A328W/Y332G 20 mutant, mutant (1) (SEQ ID NO: 2).

Table 3. Summary of the MD-simulated and QM/MM-optimized key distances (in Å) and the calculated total hydrogen-bonding energies (HBE, in kcal/mol) between the oxyanion hole and the carbonyl oxygen of (–)-cocaine benzoyl ester in the first transition state (TS1).

Transition				_Total			
State		Method	D1	D2	D3	D4	${\rm HBE}^b$
TS1 for (-)-	MD	Average	4.59	2.91	1.92		-5.5 (-4.6)
cocaine hydrol-		Maximum	5.73	4.14	2.35		()
ysis catalyzed		Minimum	3.35	1.97	1.61		
by wild-type		Fluctuation	0.35	0.35	0.12		
BChE	QM/N	1M	4.10	2.21	2.05		-4.2
TS1 for (-)-co-	MD	Average	3.62	2.35	1.95		-6.2
							(-4.9)
caine hydroly-		Maximum	4.35	3.37	3.02		
sis catalyzed by		Minimum	2.92	1.78	1.61		
A328W/Y332A		Fluctuation	0.23	0.27	0.17		
mutant	QM/N	1M	3.39	2.05	2.47		-3.3
TS1 for (-)-co-	MD	Average	5.30	2.21	1.94	2.15	-9.7
		_					(-7.4)
caine hydroly-		Maximum	6.08	3.06	2.47	3.27	
sis catalyzed by		Minimum	4.57	1.71	1.66	1.56	
A199S/A328W/		Fluctuation	0.22	0.20	0.13	0.29	
	QM/N	ſМ	4.83	2.09	1.91	2.59	-7.46

^aD1, D2, and D3 represent the internuclear distances between the carbonyl oxygen of cocaine benzoyl ester and the NH hydrogen of residues #116 (i.e., G116), #117 (i.e., G117), 50 and #199 (i.e., A199 or S199) of BChE, respectively. D4 is the internuclear distance between the carbonyl oxygen of cocaine benzoyl ester and the hydroxyl hydrogen of S199 side chain in the A199S/A328W/Y332G mutant.

bThe total HBE value under MD is the average of the HBE values calculated by using the instantaneous distances in all of the snapshots. The value in parenthesis is the total HBE value calculated by using the MD-simulated average distances. The total HBE value under QM/MM was evaluated by using the QM/MM-optimized distances.

As seen in Table 3, the simulated H . . . O distance D1 is always too long for the peptidic NH of G116 to form a N—H . . . O hydrogen bond with the carbonyl oxygen of (-)-cocaine. In the simulated TS1 structure corresponding to wild-type BChE, the carbonyl oxygen of (-)-cocaine formed 65 a firm N—H . . . O hydrogen bond with the peptidic NH hydrogen atom of A199 residue; the simulated H . . . O

distance was 1.61 to 2.35 Å, with an average value of 1.92 Å. Meanwhile, the carbonyl oxygen of (-)-cocaine also had a partial N—H . . . O hydrogen bond with the peptidic NH hydrogen atom of G117 residue; the simulated H . . . O distance was 1.97 to 4.14 Å (the average value: 2.91 Å). In the simulated TS1 structure corresponding to the A328W/Y332A mutant, the simulated average H . . . O distances with the peptidic NH hydrogen of G117 and A199 are 2.35 and 1.95 Å, respectively. These distances suggest a slightly weaker N— H...O hydrogen bond with A199, but a stronger N—H... O hydrogen bond with G117, in the simulated TS1 structure corresponding to the A328W/Y332A mutant. The overall strength of the hydrogen bonding between the carbonyl oxygen of (-)-cocaine and the oxyanion hole of the enzyme is not expected to change considerably when wild-type BChE is replaced by the A328W/Y332A mutant.

However, the story for the simulated TS1 structure associated with the A199S/A328W/Y332G mutant was remarkably different. As one can see from Scheme 4, FIG. 5, and Table 3, when residue #199 becomes a serine (i.e., S199), the hydroxyl group on the side chain of S199 can also hydrogenbond to the carbonyl oxygen of (-)-cocaine to form an O-H . . . O hydrogen bond, in addition to the two N—H . . . O hydrogen bonds with the peptidic NH of G117 and 5199. The simulated average H . . . O distances with the peptidic NH hydrogen of G117, peptidic NH hydrogen of 5199, and hydroxyl hydrogen of 5199 are 2.21, 1.94, and 2.15 Å, respectively. Due to the additional O-H . . . O hydrogen bond, the overall strength of the hydrogen bonding with the modified 30 oxyanion hole of A199S/A328W/Y332G BChE should be significantly stronger than that of the wild-type and A328W/ Y332A BChE's.

To better represent the overall strength of hydrogen bonding between the carbonyl oxygen of (-)-cocaine and the oxya-35 nion hole in a MD-simulated TS1 structure, the hydrogen bonding energy (HBE) associated with each simulated H . . . O distance was estimated by using the empirical HBE equation implemented in AutoDock 3.0 program suite in accordance with (a) Morris, G. M.; Goodsell, D. S.; Halliday, R. S.; 40 Huey, R.; Hart, W. E.; Belew. R. K.; Olson, A. J. J. Comput. Chem. 1998, 19, 1639-1662, and based on the general HBE equation, HBE(r) \approx 5 \in r₀¹²/r¹²-6 \in r₀¹⁰/r¹⁰, in which r is the H...O distance in the considered hydrogen bond and r_0 is the minimum value of the H . . . O distance for which the HBE 45 equation can be used. For the calculation, $r_0=1.52 \text{ Å}$, because it is the shortest H . . . O distance found in all of our MD simulations. The \in value was determined by using the condition that HBE(r)=-5.0 kcal/mol when r=1.90 Å, herein incorporated by reference. Specifically, for each hydrogen bond with the carbonyl oxygen of (-)-cocaine, a HBE value can be evaluated with each snapshot of the MD-simulated structure. The final HBE of the MD-simulated hydrogen bond is considered to be the average HBE value of all snapshots taken from the stable MD trajectory. The estimated total HBE value for the hydrogen bonds between the carbonyl oxygen of (-)-cocaine and the oxyanion hole in each simulated TS1 structure is given in Table 3.

The HBE for each hydrogen bond was estimated by using the MD-simulated average H...O distance. As seen in Table 3, the total hydrogen-bonding energies (i.e., -4.6, -4.9, and -7.4 kcal/mol for the wild-type, A328W/Y332A, and A199S/A328W/Y332G BChE's, respectively) estimated in this way are systematically higher (i.e., less negative) than the corresponding total hydrogen-bonding energies (i.e., -5.5, -6.2, and -9.7 kcal/mol) estimated in the aforementioned way. However, the two sets of total HBE values are qualitatively consistent in terms of the relative hydrogen-bonding

strengths in the three simulated TS1 structures. In particular, the two sets of total HBE values consistently reveal that the overall strength of the hydrogen bonding between the carbonyl oxygen of (–)-cocaine and the oxyanion hole in the simulated TS1 structure for A199S/A328W/Y332G BChE is significantly higher than that for the wild-type and A328W/

Hydrogen Bonding Based on the QM/MM Calculations.

Y332A BChE's.

The above conclusion obtained from the MD simulations was further examined by carrying out QM/MM calculations. Although this enzymatic reaction system is too large to calculate the QM/MM force constant matrix required in the automated search for a first-order saddle point corresponding to TS1, a partial geometry optimization was performed by fixing the length of the transition C—O bond between the carbonyl carbon of (-)-cocaine and the O^{γ} atom of S198 in the QM/MM calculation. This transition C—O bond length dominates the reaction coordinate, according to the previous first-principle reaction coordinate calculations, in accordance 20 with Zhan et al, with an active model of wild-type BChE. In the partial geometry optimization, the transition C—O bond length was fixed at that in the TS1 geometry optimized previously by performing the first-principle reaction coordinate calculation with an active site model of wild-type BChE. This 25 partially optimized geometry should be close to the precisely defined TS1 geometry associated with a first-order saddle point on the potential energy surface, particularly for the hydrogen bonding between the carbonyl oxygen of (-)-cocaine and the oxyanion hole of BChE.

The QM/MM results summarized in Table 3 demonstrate two hydrogen bonds in each of the QM/MM-optimized TS1 structures. Specifically, D2=2.21 Å and D3=2.05 Å in the optimized TS1 structure for wild-type BChE; D2=2.05 Å and D3=2.47 Å in the optimized TS1 structure for the A328W/ Y332A mutant; D2=2.09 Å, D3=1.91 Å, and D4=2.59 Å in the optimized TS1 structure for the A199S/A328W/Y332G mutant. Although the QM/MM-optimized individual H . . . O distances and the estimated HBE values are different from the 40 corresponding values for individual hydrogen bonds, the relative total HBE values (i.e., -4.2, -3.3, and -7.46 kcal/mol for the wild-type, A328W/Y332A, and A199S/A328W/Y332G BChE's, respectively) estimated from these optimized distances are qualitatively consistent with the corresponding 45 total HBE values (i.e., -4.6, -4.9, and -7.4 kcal/mol) estimated from the MD-simulated average H...O distances.

It should be pointed out that the absolute HBE values estimated in this study are not expected to be accurate, as different computational approaches led different HBE values. 50 Nevertheless, for the purpose of our computational design of a high-activity mutant of BChE, one only needs to know the relative strength of the hydrogen bonding based on the estimated relative total HBE values of different mutants. The three sets of total HBE values (Table 3) estimated from the 55 MD-simulated and QM/MM-optimized H . . . O distances all consistently demonstrate: (1) the overall strength of hydrogen bonding between the carbonyl oxygen of (-)-cocaine and the oxyanion hole in the TS1 structure for (-)-cocaine hydrolysis catalyzed by A328W/Y332A BChE should be close to that in 60 the TS1 structure for (-)-cocaine hydrolysis catalyzed by wild-type BChE; (2) the overall hydrogen bonding between the carbonyl oxygen of (-)-cocaine and the oxyanion hole in the TS1 structure for (-)-cocaine hydrolysis catalyzed by A199S/A328W/Y332G BChE should be significantly stron- 65 ger than that in the TS1 structure for (-)-cocaine hydrolysis catalyzed by A328W/Y332A BChE.

38

Catalytic Activity.

The computational results discussed above suggest that the transition state for the first chemical reaction step (TS1) of (-)-cocaine hydrolysis catalyzed by the A199S/A328W/ Y332G mutant should be significantly more stable than that by the A328W/Y332A mutant, due to the significant increase of the overall hydrogen bonding between the carbonyl oxygen of (-)-cocaine and the oxyanion hole of the enzyme in the TS1 structure. The aforementioned analysis of the literature, namely Sun et al and Hamza et al, also indicate that the first chemical reaction step associated with TS1 should be the rate-determining step of (-)-cocaine hydrolysis catalyzed by a BChE mutant including Y332A or Y332G mutation, although the formation of the prereactive enzyme-substrate complex (ES) is the rate-determining step for (-)-cocaine hydrolysis catalyzed by wild-type BChE. This provides evidence of a clear correlation between the TS1 stabilization and the catalytic activity of A328W/Y332A and A199S/A328W/ Y332G BChE's for (-)-cocaine hydrolysis: the more stable the TS1 structure, the lower the energy barrier, and the higher the catalytic activity. Thus, both the MD simulations and QM/MM calculations predict that A199S/A328W/Y332G BChE should have a higher catalytic activity than A328W/ Y332A BChE for (-)-cocaine hydrolysis.

The catalytic efficiency (k_{cat}/K_M) of A328W/Y332A BChE for (-)-cocaine hydrolysis was reported by Sun et al to be $\sim 8.6 \times 10^6$ M min⁻¹, which is ~ 9.4 times of the k_{cat}/K_M value (~9.1×10⁵ M min⁻¹) of wild-type BChE. To examine the theoretical prediction of the higher catalytic activity for A199S/A328W/Y332G BChE, A328W/Y332A and A199S/ A328W/Y332G mutants of BChE were produced through site-directed mutagenesis. To minimize the possible systematic experimental errors of the kinetic data, kinetic studies were performed with the two mutants and wild-type BChE 35 under the same condition and compared the catalytic efficiency of A328W/Y332A and A199S/A328W/Y332G BChE's to that of the wild-type for (-)-cocaine hydrolysis at benzoyl ester group. Based on the kinetic analysis of the measured time-dependent radiometric data and the ELISA data, the ratio of the k_{cat}/K_M value of A328W/Y332A BChE to the k_{cat}/K_M value of wild-type BChE for (-)-cocaine hydrolysis was determined to be ~8.6. The determined catalytic efficiency ratio of ~8.6 is in good agreement with the ratio of ~9.4 determined by Sun et al. Further, by using the same experimental protocol, the ratio of the k_{cat}/K_{M} value of A199S/A328W/Y332G BChE to the k_{cat}/K_{M} value of A328W/Y332A BChE for (-)-cocaine hydrolysis was determined to be ~7.2. These data indicate that the ratio of the k_{cat}/K_M value of A199S/A328W/Y332G BChE to the k_{cat}/K_M K_M value of wild-type BChE for (-)-cocaine hydrolysis should be ~7.2×8.6=~62 or ~7.2×9.4=~68. Thus, A199S/ A328W/Y332G BChE has a ~(65±6)-fold improved catalytic efficiency against (-)-cocaine compared to the wild-type, or A199S/A328W/Y332G BChE has a k_{cat}/K_M value of \sim (5.9±0.5)×10⁷ M min⁻¹ for (–)-cocaine hydrolysis.

Very recently reported F227A/S287G/A328W/Y332M BChE (i.e., AME-359, $k_{car}/K_{M}=3.1\times10^7$ M min⁻¹) has a ~34-fold improved catalytic efficiency for (–)-cocaine hydrolysis. AME-359 has the highest catalytic efficiency against (–)-cocaine within all of the BChE mutants reported in literature prior to the present study. The catalytic efficiency for our A199S/A328W/Y332G BChE is about two times of that for AME-359 against (–)-cocaine.

Conclusion.

Molecular dynamics (MD) simulations and hybrid quantum mechanical/molecular mechanical (QM/MM) calculations on the transition state for the first chemical reaction step

(TS1) of (-)-cocaine hydrolysis catalyzed by butyrylcholinesterase (BChE) mutants lead to a better understanding of the effects of protein environment on the transition state stabilization. All of the computational results consistently demonstrate that the overall hydrogen bonding between the carbonyl oxygen of (-)-cocaine benzovl ester and the oxyanion hole of BChE in the TS1 structure for (-)-cocaine hydrolysis catalyzed by A199S/A328W/Y332G BChE should be significantly stronger than that in the TS1 structure for (-)-cocaine hydrolysis catalyzed by the wild-type and A328W/Y332A BChE's. Thus, both the MD simulations and QM/MM calculations predict that A199S/A328W/Y332G BChE should have a lower energy barrier for the chemical reaction process and, therefore, a higher catalytic efficiency (k_{cat}/K_{M}) for (-)cocaine hydrolysis; A328W/Y332A BChE has been known to have a ~9-fold improved catalytic efficiency for (-)-cocaine hydrolysis. The theoretical prediction has been confirmed by wet experimental tests showing a ~(65±6)-fold improved catalytic efficiency for A199S/A328W/Y332G 20 BChE against (-)-cocaine compared to the wild-type BChE. The k_{cat}/K_M value determined for A199S/A328W/Y332G BChE is about two times of the k_{cat}/K_M value for F227A/ S287G/A328W/Y332M BChE (i.e., AME-359, which has the highest catalytic efficiency within all BChE mutants 25 reported prior to the present study) against (-)-cocaine. The encouraging outcome of this study suggests that the transition-state modeling is a promising approach for rational design of high-activity mutants of BChE as a therapeutic treatment of cocaine abuse.

Experiment 3

MD Simulation and Generation of Mutant A199S/S287G/A328W/Y332G BCHE (Mutant 3) (SEQ ID NO: 14)

The following simulation and mutant generation relate to A199S/S287G/A328W/Y332G (mutant 3) as a rational design of a high-activity mutant of BChE against (–)-cocaine 40 based on detailed computational modeling of the transition state for the rate-determining step (i.e., the first step of the chemical reaction process). Molecular dynamics (MD) simulations were performed to model the protein environmental effects on the stabilization of the transition-state structure for 45 BChE-catalyzed hydrolysis of (-)-cocaine as described above for mutant A199S/A328W/Y332G (mutant 1). The simulated results indicate that the transition-state structure can be stabilized much better by the protein environment in A199S/S287G/A328W/Y332G BChE than that in wild-type 50 BChE and in other BChE mutants examined. The computational modeling led to a prediction of the higher catalytic efficiency for the A199S/S287G/A328W/Y332G mutant against (-)-cocaine. The prediction has been confirmed by wet experimental tests showing that the A199S/S287G/ 55 A328W/Y332G mutant has a remarkably improved catalytic efficiency against (-)-cocaine. All of the obtained results clearly demonstrate that directly modeling the transition-state structure provides a reliable computational approach to the rational design of a high-activity mutant of BChE against 60 (-)-cocaine.

MD Simulations.

As with the A199S/A328W/Y332G mutant, when performing any MD simulation on a transition state, in principle, MD simulation using a classical force field (molecular 65 mechanics) can only simulate a stable structure corresponding to a local minimum on the potential energy surface,

whereas a transition state during a reaction process is always associated with a first-order saddle point on the potential energy surface.

The initial BChE structures used in the MD simulations were prepared based on our previous MD simulation as above with the prior mutant and the prereactive BChE-(-)-cocaine complex (ES) started from the X-ray crystal structure deposited in the Protein Data Bank (pdb code: 1P0P).

The general procedure for carrying out the MD simulations in water is essentially the same as that used in the computational studies for mutant A199S/A328W/Y332G (mutant 1). Site-directed mutagenesis of human BChE cDNA was performed as described before.

The MD simulation in water was performed as described above, on this mutant. Depicted in FIG. 6 are plots of four important H... O distances in the MD-simulated TS1 structure versus the simulation time for (-)-cocaine hydrolysis catalyzed by A199S/S287G/A328W/Y332G BChE, along with the root-mean-square deviation (RMSD) of the simulated positions of backbone atoms from those in the corresponding initial structure. Traces D1, D2, and D3 refer to the distances between the carbonyl oxygen of (-)-cocaine and the NH hydrogen of G116, G117, and 5199, respectively. Trace D4 is the internuclear distance between the carbonyl oxygen of (-)-cocaine and the hydroxyl hydrogen of the S199 side chain in A199S/S287G/A328W/Y332G BChE (mutant 3). RMSD represents the root-mean-square deviation (in Å) of the simulated positions of the protein backbone atoms from those in the initial structure.

The H...O distances in the simulated TS1 structures for wild-type BChE and its three mutants are summarized in Table 4. The H...O distances between the carbonyl oxygen of (–)-cocaine and the peptidic NH hydrogen atoms of G116, G117, and A199 (or S199) of BChE are denoted by D1, D2, and D3, respectively, in Table 4 and FIG. 6. D4 in Table 4 and FIG. 6 refers to the H...O distance between the carbonyl oxygen of (–)-cocaine and the hydroxyl hydrogen of S199 side chain in the simulated TS1 structure corresponding to the A199S/S287G/A328W/Y332G mutant.

Table 4. Summary of the MD-simulated key distances (in Å) and the calculated total hydrogen-bonding energies (HBE, in kcal/mol) between the oxyanion hole and the carbonyl oxygen of (–)-cocaine benzoyl ester in the first transition state (TS1).

		Dis	tance ^a			_
Transition State		D1	D2	D3	D4	Total HBE^b
TS1 structure	Average	4.59	2.91	1.92		-5.5 (-4.6)
for (-)-cocaine	Maximum	5.73	4.14	2.35		
hydrolysis	Minimum	3.35	1.97	1.61		
catalyzed by wild-type	Fluctuation	0.35	0.35	0.12		
TS1 structure	Average	3.62	2.35	1.95		-6.2 (-4.9)
for (-)-cocaine	Maximum	4.35	3.37	3.02		, ,
hydrolysis	Minimum	2.92	1.78	1.61		
catalyzed by	Fluctuation	0.23	0.27	0.17		
TS1 structure for	Average	3.60	2.25	1.97		-6.4 (-5.0)
(-)-cocaine	Maximum	4.24	3.17	2.76		()
hydrolysis	Minimum	2.89	1.77	1.62		
catalyzed by	Fluctuation	0.23	0.24	0.17		
A328W/Y332G mutant of BChE						
TS1 structure for	Average	4.39	2.60	2.01	1.76	-14.0 (-12.0)
(-)-cocaine	Maximum	5.72	4.42	2.68	2.50	, ,
hydrolysis	Minimum	2.87	1.76	1.62	1.48	
catalyzed by	Fluctuation	0.48	0.36	0.17	0.12	
A199S/S287G/ A328W/Y332G						

^aD1, D2, and D3 represent the internuclear distances between the carbonyl oxygen of cocaine benzoyl ester and the NH hydrogen of residues #116 (i.e., G116), #117 (i.e., G117), and #199 (i.e., A199 or S199) of BChE, respectively. D4 is the internuclear distance between the carbonyl oxygen of cocaine benzoyl ester and the hydroxyl hydrogen of S199 side chain in the A199S/S287G/A328W/Y332G mutant.

^bThe total HBE value is the average of the HBE values calculated by using the instantaneous distances in all of the

42

hydrogen bonds with the peptidic NH of G117 and 5199. The simulated average H . . . O distances with the peptidic NH hydrogen of G117, peptidic NH hydrogen of 5199, and hydroxyl hydrogen of 5199 are 2.60, 2.01, and 1.76 Å, respectively. Due to the additional O—H . . . O hydrogen bond, the overall strength of the hydrogen bonding with the modified oxyanion hole of A199S/S287G/A328W/Y332G BChE should be significantly stronger than that of wild-type, A328W/Y332A, and A328W/Y332G BChE's.

snapshots. The value in parenthesis is the total HBE value calculated by using the MD-simulated average distances.

As seen in Table 4, the simulated H . . . O distance D1 is always too long for the peptidic NH of G116 to form a N-H... O hydrogen bond with the carbonyl oxygen of (-)cocaine in all of the simulated TS1 structures. In the simulated TS1 structure for wild-type BChE, the carbonyl oxygen of (-)-cocaine formed a firm N—H . . . O hydrogen bond with the peptidic NH hydrogen atom of A199 residue; the simulated H . . . O distance (D2) was 1.61 to 2.35 Å, with an average D2 value of 1.92 Å. Meanwhile, the carbonyl oxygen of (-)-cocaine also had a partial N—H . . . O hydrogen bond with the peptidic NH hydrogen atom of G117 residue; the simulated H... O distance (D3) was 1.97 to 4.14 Å (the 45 average D3 value: 2.91 Å). The average D2 and D3 values became 2.35 and 1.95 Å, respectively, in the simulated TS1 structure for the A328W/Y332A mutant. These distances suggest a slightly weaker N—H . . . O hydrogen bond with A199, but a stronger N—H...O hydrogen bond with G117, 50 in the simulated TS1 structure for the A328W/Y332A mutant that the corresponding N—H . . . O hydrogen bonds for the wild-type. The average D2 and D3 values (2.25 and 1.97 Å, respectively) in the simulated TS1 structure for the A328W/ Y332G mutant are close to the corresponding distances for 55 the A328W/Y332A mutant. The overall strength of the hydrogen bonding between the carbonyl oxygen of (-)-cocaine and the oxyanion hole of the enzyme is not expected to change considerably when wild-type BChE is replaced by the A328W/Y332A or A328W/Y332G mutant.

However, the story for the simulated TS1 structure for the A199S/S287G/A328W/Y332G mutant was remarkably different. As one can see from Scheme 5, FIG. 6, and Table 4, when residue #199 becomes a serine (i.e., S199), the hydroxyl group on the side chain of S199 can also hydrogenbond to the carbonyl oxygen of (–)-cocaine to form an O—H...O hydrogen bond, in addition to the two N—H...O

Scheme 5. Schematic representation of the transition-state structure for first reaction step for (–)-cocaine hydrolysis catalyzed by a BChE mutant with an A199S mutation.

To better represent the overall strength of hydrogen bonding between the carbonyl oxygen of (-)-cocaine and the oxyanion hole in a MD-simulated TS1 structure, the hydrogen bonding energy (HBE) associated with each simulated H . . . O distance was estimated by using the empirical HBE equation implemented in AutoDock 3.0 program suite of (a) Masson, P.; Legrand, P.; Bartels, C. F.; Froment, M.-T.; Schopfer, L. M.; Lockridge, O. Biochemistry 1997, 36, 2266, (b) Masson, P.; Xie, W., Froment, M-T.; Levitsky, V.; Fortier, P.-L.; Albaret, C.; Lockridge, O. Biochim. Biophys. Acta 1999, 1433, 281, (c) Xie, W.; Altamirano, C. V.; Bartels, C. F.; Speirs, R. J.; Cashman, J. R.; Lockridge, O. Mol. Pharmacol. 1999, 55, 83, (d) Duysen, E. G.; Bartels, C. F.; Lockridge, O. J. Pharmacol. Exp. Ther. 2002, 302, 751, (e) Nachon, F.; Nicolet, Y.; Viguie, N.; Masson, P.; Fontecilla-Camps, J. C.; Lockridge, O. Eur. J. Biochem. 2002, 269, 630, herein all incorporated by reference. Specifically, for each hydrogen bond with the carbonyl oxygen of (-)-cocaine, a HBE value can be evaluated with each snapshot of the MD-simulated structure. The final HBE of the MD-simulated hydrogen bond is considered to be the average HBE value of all snapshots taken from the stable MD trajectory. The estimated total HBE value for the hydrogen bonds between the carbonyl oxygen of (-)-cocaine and the oxyanion hole in each simulated TS1 structure is also listed in Table 4.

The HBE for each hydrogen bond was estimated by using the MD-simulated average H...O distance. As seen in Table 4, the total hydrogen-bonding energies (i.e., -4.6, -4.9, -5.0, and -12.0 kcal/mol for the wild-type, A328W/Y332A, A328W/Y332G, and A199S/S287G/A328W/Y332G BChE's, respectively) estimated in this way are systematically higher (i.e., less negative) than the corresponding total hydrogen-bonding energies (i.e., -5.5, -6.2, -6.4, and -14.0

kcal/mol) estimated in the aforementioned way. However, the two sets of total HBE values are qualitatively consistent with each other in terms of the relative hydrogen-bonding strengths in the three simulated TS1 structures. In particular, the two sets of total HBE values consistently reveal that the overall strength of the hydrogen bonding between the carbonyl oxygen of (-)-cocaine and the oxyanion hole in the simulated TS1 structure for A199S/S287G/A328W/Y332G BChE is significantly higher than that for wild-type, A328W/ Y332A, and A328W/Y332G BChE's.

43

Catalytic Activity.

The computational results discussed above provides evidence that the transition state for the first chemical reaction step (TS1) of (-)-cocaine hydrolysis catalyzed by the A199S/ S287G/A328W/Y332G mutant should be significantly more 15 stable than that by the A328W/Y332A or A328W/Y332G mutant, due to the significant increase of the overall hydrogen bonding between the carbonyl oxygen of (-)-cocaine and the oxyanion hole of the enzyme in the TS1 structure. The aforeal also indicates that the first chemical reaction step associated with TS1 should be the rate-determining step of (-)cocaine hydrolysis catalyzed by a BChE mutant including Y332A or Y332G mutation, although the formation of the prereactive enzyme-substrate complex (ES) is the rate-determining step for (-)-cocaine hydrolysis catalyzed by wildtype BChE. This suggests a clear correlation between the TS1 stabilization and the catalytic activity of A328W/Y332A, A199S/S287G/A328W/Y332G A328W/Y332G, and BChE's for (-)-cocaine hydrolysis: the more stable the TS1 structure, the lower the energy barrier, and the higher the 30 catalytic activity. Thus, the MD simulations predict that A199S/S287G/A328W/Y332G BChE should have a higher catalytic activity than A328W/Y332A or A328W/Y332G BChE for (-)-cocaine hydrolysis.

The catalytic efficiency (k_{cat}/K_M) of A328W/Y332A 35 BChE for (-)-cocaine hydrolysis was reported to be $\sim 8.6 \times 10^6$ M min⁻¹, which is ~9.4 times of the k_{cat}/K_M value (~9.1×10⁵ M min⁻¹) of wild-type BChE for (-)-cocaine hydrolysis. The catalytic efficiency of A328W/Y332G BChE was found to be slightly higher than that of A328W/Y332A BChE for (-)cocaine hydrolysis. To examine the theoretical prediction of the higher catalytic activity for A199S/S287G/A328W/ Y332G BChE, the A328W/Y332A and A199S/S287G/ A328W/Y332G mutants of BChE were produced as previously discussed through site-directed mutagenesis. To minimize the possible systematic experimental errors of the kinetic data, kinetic studies were performed with the two mutants and wild-type BChE under the same condition and compared the catalytic efficiency of A328W/Y332A and A199S/S287G/A328W/Y332G BChE's to that of the wildtype for (–)-cocaine hydrolysis at benzoyl ester group. Based on the kinetic analysis of the measured time-dependent radiometric data and the ELISA data, the ratio of the k_{cat}/K_M value of A328W/Y332A BChE to the k_{cat}/K_M value of wild-type BChE for (-)-cocaine hydrolysis was determined to be ~8.6. The determined catalytic efficiency ratio of ~8.6 is in good agreement with the ratio of ~9.4 determined by Sun et al.

Further, by using the same experimental protocol, the ratio of the k_{cat}/K_M value of A199S/S287G/A328W/Y332G BChE to the k_{cat}/K_M value of A328W/Y332A BChE for (-)-cocaine hydrolysis was determined to be ~50.6. These data indicate that the ratio of the k_{ca}/K_M value of A199S/S287G/A328W/Y332G BChE to the k_{ca}/K_M value of wild-type BChE for (-)-cocaine hydrolysis should be $\sim 50.6 \times 8.6 = \sim 435$ or $\sim 50.6 \times$ 9.4=~476. Thus, A199S/S287G/A328W/Y332G BChE has a ~(456±41)-fold improved catalytic efficiency against (–)-cocaine compared to the wild-type, or A199S/S287G/A328W/ Y332G BChE has a k_{cat}/K_M value of ~(4.15±0.37)×10⁸ M min⁻¹ for (-)-cocaine hydrolysis. The catalytic efficiency of A199S/S287G/A328W/Y332G BChE against (-)-cocaine is much higher than that of AME-359 (i.e., F227A/S287G/ A328W/Y332M BChE, $k_{cat}/K_{M}=3.1\times10^{7} \text{ M min}^{-1}$, whose catalytic efficiency against (-)-cocaine is the highest within all of the previously reported BChE mutants) which has a ~34-fold improved catalytic efficiency against (-)-cocaine compared to wild-type BChE.

By using the designed A199S/S287G/A328W/Y332G mentioned analysis of the literature of Sun et al and Hamza et 20 BChE as an exogenous enzyme in human, when the concentration of this mutant is kept the same as that of the wild-type BChE in plasma, the half-life time of (-)-cocaine in plasma should be reduced from the ~45-90 min to only ~6-12 seconds, considerably shorter than the time required for cocaine crossing the blood-brain barrier to reach CNS. Hence, the outcome of this study could eventually result in a valuable, efficient anti-cocaine medication.

The transition-state simulations demonstrate that the overall hydrogen bonding between the carbonyl oxygen of (-)cocaine benzoyl ester and the oxyanion hole of BChE in the TS1 structure for (-)-cocaine hydrolysis catalyzed by A199S/ S287G/A328W/Y332G BChE should be significantly stronger than that in the TS1 structure for (–)-cocaine hydrolysis catalyzed by the wild-type BChE and other BChE mutants simulated. Thus, the MD simulations predict that A199S/ S287G/A328W/Y332G BChE should have a significantly lower energy barrier for the chemical reaction process and, therefore, a significantly higher catalytic efficiency (k_{cat}/K_{M}) for (-)-cocaine hydrolysis. The theoretical prediction has been confirmed by wet experimental tests showing a ~(456±41)-fold improved catalytic efficiency for A199S/ S287G/A328W/Y332G BChE against (-)-cocaine compared to the wild-type BChE. The k_{cat}/K_M value determined for A199S/S287G/A328W/Y332G BChE is much higher than the k_{cat}/K_M value for AME-359 (i.e., F227A/S287G/A328W/ Y332M BChE, whose catalytic efficiency against (-)-cocaine is the highest within all of the BChE mutants reported previously in literature) which has a ~34-fold improved catalytic efficiency against (-)-cocaine compared to the wild-type BChE. The outcome of this study provides evidence that the transition-state simulation is a novel and unique approach for rational enzyme redesign and drug discovery.

Although the invention has been described in detail with respect to preferred embodiments thereof, it will be apparent that the invention is capable of numerous modifications and variations, apparent to those skilled in the art, without departing from the spirit and scope of the invention.

SEQUENCE LISTING

<210> SEO ID NO 1

<211> LENGTH: 1722

<212> TYPE: DNA

<213> ORGANISM: Artificial

45 -continued

<220> FEATURE:

<223> OTHER INFORMATION: 14-1 mutant A199S/A328W/Y332G of Human butyrylcholinesterase (BChE)

<400> SEOUENCE: 1

gaagatgaca tcataattgc aacaaagaat ggaaaagtca gagggatgaa cttgacagtt 60 tttggtggca cggtaacagc ctttcttgga attccctatg cacagccacc tcttggtaga 120 cttcgattca aaaagccaca gtctctgacc aagtggtctg atatttggaa tgccacaaaa 180 tatgcaaatt cttgctgtca gaacatagat caaagttttc caggcttcca tggatcagag 240 300 atgtggaacc caaacactga cctcagtgaa gactgtttat atctaaatgt atggattcca gcacctaaac caaaaaatgc cactgtattg atatggattt atggtggtgg ttttcaaact 360 ggaacatcat ctttacatgt ttatgatggc aagtttctgg ctcgggttga aagagttatt 420 gtagtgtcaa tgaactatag ggtgggtgcc ctaggattct tagctttgcc aggaaatcct 480 gaggetecag ggaacatggg tttatttgat caacagttgg etetteagtg ggtteaaaaa 540 aatatagcag cetttggtgg aaateetaaa agtgtaacte tetttggaga aagtteegga 600 gcagetteag ttageetgea tttgetttet eetggaagee atteattgtt caecagagee attctgcaaa gtggttcctt taatgctcct tgggcggtaa catctcttta tgaagctagg 720 aacagaacgt tgaacttagc taaattgact ggttgctcta gagagaatga gactgaaata 780 atcaagtgtc ttagaaataa agatccccaa gaaattcttc tgaatgaagc atttgttgtc 840 ccctatggga ctcctttgtc agtaaacttt ggtccgaccg tggatggtga ttttctcact 900 gacatgccag acatattact tgaacttgga caatttaaaa aaacccagat tttggtgggt 960 gttaataaag atgaagggac atggttttta gtcggtggtg ctcctggctt cagcaaagat 1020 aacaatagta tcataactag aaaagaattt caggaaggtt taaaaatatt ttttccagga 1080 qtqaqtqaqt ttqqaaaqqa atccatcctt tttcattaca caqactqqqt aqatqatcaq 1140 1200 agacctgaaa actaccgtga ggccttgggt gatgttgttg gggattataa tttcatatgc cctgccttgg agttcaccaa gaagttctca gaatggggaa ataatgcctt tttctactat 1260 tttgaacacc gatcctccaa acttccgtgg ccagaatgga tgggagtgat gcatggctat 1320 gaaattgaat ttgtctttgg tttacctctg gaaagaagag ataattacac aaaagccgag 1380 gaaattttga gtagatccat agtgaaacgg tgggcaaatt ttgcaaaata tgggaatcca 1440 1500 aatgagactc agaacaatag cacaagctgg cctgtcttca aaagcactga acaaaaatat ctaaccttga atacagagtc aacaagaata atgacgaaac tacgtgctca acaatgtcga 1560 ttctggacat catttttcc aaaagtcttg gaaatgacag gaaatattga tgaagcagaa 1620 tgggagtgga aagcaggatt ccatcgctgg aacaattaca tgatggactg gaaaaatcaa 1680 1722 tttaacgatt acactagcaa gaaagaaagt tgtgtgggtc tc

<210> SEQ ID NO 2

<211> LENGTH: 574

<212> TYPE: PRT

<213 > ORGANISM: Artificial

<220> FEATURE:

<223> OTHER INFORMATION: 14-1 mutant (A199S/A328W/Y332G) aminio acid sequence modified BChE

-continued

_															
< 400)> SI	EQUE	ICE :	2											
Glu 1	Asp	Asp	Ile	Ile 5	Ile	Ala	Thr	ГЛа	Asn 10	Gly	Lys	Val	Arg	Gly 15	Met
Asn	Leu	Thr	Val 20	Phe	Gly	Gly	Thr	Val 25	Thr	Ala	Phe	Leu	Gly 30	Ile	Pro
Tyr	Ala	Gln 35	Pro	Pro	Leu	Gly	Arg 40	Leu	Arg	Phe	Lys	Lys 45	Pro	Gln	Ser
Leu	Thr 50	Lys	Trp	Ser	Asp	Ile 55	Trp	Asn	Ala	Thr	Lys 60	Tyr	Ala	Asn	Ser
Cys	CÀa	Gln	Asn	Ile	Asp 70	Gln	Ser	Phe	Pro	Gly 75	Phe	His	Gly	Ser	Glu 80
Met	Trp	Asn	Pro	Asn 85	Thr	Asp	Leu	Ser	Glu 90	Asp	Cys	Leu	Tyr	Leu 95	Asn
Val	Trp	Ile	Pro 100	Ala	Pro	Lys	Pro	Lys 105	Asn	Ala	Thr	Val	Leu 110	Ile	Trp
Ile	Tyr	Gly 115	Gly	Gly	Phe	Gln	Thr 120	Gly	Thr	Ser	Ser	Leu 125	His	Val	Tyr
Asp	Gly 130	ГÀа	Phe	Leu	Ala	Arg 135	Val	Glu	Arg	Val	Ile 140	Val	Val	Ser	Met
Asn 145	Tyr	Arg	Val	Gly	Ala 150	Leu	Gly	Phe	Leu	Ala 155	Leu	Pro	Gly	Asn	Pro 160
Glu	Ala	Pro	Gly	Asn 165	Met	Gly	Leu	Phe	Asp 170	Gln	Gln	Leu	Ala	Leu 175	Gln
Trp	Val	Gln	Lys 180	Asn	Ile	Ala	Ala	Phe 185	Gly	Gly	Asn	Pro	Lys 190	Ser	Val
Thr	Leu	Phe 195	Gly	Glu	Ser	Ser	Gly 200	Ala	Ala	Ser	Val	Ser 205	Leu	His	Leu
Leu	Ser 210	Pro	Gly	Ser	His	Ser 215	Leu	Phe	Thr	Arg	Ala 220	Ile	Leu	Gln	Ser
Gly 225	Ser	Phe	Asn	Ala	Pro 230	Trp	Ala	Val	Thr	Ser 235	Leu	Tyr	Glu	Ala	Arg 240
Asn	Arg	Thr	Leu	Asn 245	Leu	Ala	Lys	Leu	Thr 250	Gly	CÀa	Ser	Arg	Glu 255	Asn
Glu	Thr	Glu	Ile 260	Ile	Lys	Cys	Leu	Arg 265	Asn	Lys	Asp	Pro	Gln 270	Glu	Ile
Leu	Leu	Asn 275	Glu	Ala	Phe	Val	Val 280	Pro	Tyr	Gly	Thr	Pro 285	Leu	Ser	Val
Asn	Phe 290	Gly	Pro	Thr	Val	Asp 295	Gly	Asp	Phe	Leu	Thr 300	Asp	Met	Pro	Asp
Ile 305	Leu	Leu	Glu	Leu	Gly 310	Gln	Phe	ГЛа	Lys	Thr 315	Gln	Ile	Leu	Val	Gly 320
Val	Asn	Lys	Aap	Glu 325	Gly	Thr	Trp	Phe	Leu 330	Val	Gly	Gly	Ala	Pro 335	Gly
Phe	Ser	Lys	Asp 340	Asn	Asn	Ser	Ile	Ile 345	Thr	Arg	Lys	Glu	Phe 350	Gln	Glu
Gly	Leu	Lys 355	Ile	Phe	Phe	Pro	Gly 360	Val	Ser	Glu	Phe	Gly 365	Lys	Glu	Ser
Ile	Leu 370	Phe	His	Tyr	Thr	Asp 375	Trp	Val	Asp	Asp	Gln 380	Arg	Pro	Glu	Asn
Tyr 385	Arg	Glu	Ala	Leu	Gly 390	Asp	Val	Val	Gly	Asp 395	Tyr	Asn	Phe	Ile	Cys 400
Pro	Ala	Leu	Glu	Phe 405	Thr	Lys	Lys	Phe	Ser 410	Glu	Trp	Gly	Asn	Asn 415	Ala

49 50

-continued

Phe	Phe	Tyr	Tyr 420	Phe	Glu	His	Arg	Ser 425	Ser	ГЛа	Leu	Pro	Trp 430	Pro	Glu	
Trp	Met	Gly 435	Val	Met	His	Gly	Tyr 440	Glu	Ile	Glu	Phe	Val 445	Phe	Gly	Leu	
Pro	Leu 450	Glu	Arg	Arg	Asp	Asn 455	Tyr	Thr	Lys	Ala	Glu 460	Glu	Ile	Leu	Ser	
Arg 465	Ser	Ile	Val	ГÀз	Arg 470	Trp	Ala	Asn	Phe	Ala 475	Lys	Tyr	Gly	Asn	Pro 480	
Asn	Glu	Thr	Gln	Asn 485	Asn	Ser	Thr	Ser	Trp 490	Pro	Val	Phe	Lys	Ser 495	Thr	
Glu	Gln	ГЛа	Tyr 500	Leu	Thr	Leu	Asn	Thr 505	Glu	Ser	Thr	Arg	Ile 510	Met	Thr	
Lys	Leu	Arg 515	Ala	Gln	Gln	Cys	Arg 520	Phe	Trp	Thr	Ser	Phe 525	Phe	Pro	ГЛа	
Val	Leu 530	Glu	Met	Thr	Gly	Asn 535	Ile	Asp	Glu	Ala	Glu 540	Trp	Glu	Trp	Lys	
Ala 545	Gly	Phe	His	Arg	Trp 550	Asn	Asn	Tyr	Met	Met 555	Asp	Trp	Lys	Asn	Gln 560	
Phe	Asn	Asp	Tyr	Thr 565	Ser	Lys	Lys	Glu	Ser 570	Cys	Val	Gly	Leu			
<213 <213 <213 <220 <223	<pre><210> SEQ ID NO 3 <211> LENGTH: 1125 <212> TYPE: DNA <213> ORGANISM: Artificial <220> FEATURE: <223> OTHER INFORMATION: truncated 14-1 (A199S/A328/Y332G) nucleic Acid Sequence for modified BChE corresponding to a.a. residues 68-442 <400> SEQUENCE: 3</pre>															
					cc aç	ggett	ccat	gga	atcag	gaga	tgtg	ggaad	ccc a	aaaca	actgad	c 60
ctca	agtga	aag a	actgi	cttat	a to	ctaaa	atgta	tgg	gatto	ccag	caco	ctaaa	acc a	aaaaa	aatgc	2 120
acto	gtati	ga 1	tatg	gattt	a to	ggtgg	gtggt	: ttt								
tato	gatg	gca a	agtti	ctg	ge to				caac	actg	gaad	catca	atc 1	ttad	catgtt	180
gtg	ggtgo	ccc 1				gggı	tgaa	a aga							catgtt catago	
ttat			tagga	attct	t aç		_	_	agtta	attg	tagt	gtca	aat q	gaact		g 240
	ttga	atc a	-			gettt	gcca	a gga	agtta aaato	attg	t agt	igtea eteca	aat q	gaact gaaca	atago	g 240 : 300
aato	_		aacaq	gttgg	ge to	gettt	gcca gtgg	gga gtt	agtta aaato caaa	attg cctg aaaa	tagt aggo atat	eteca cagea	aat q agg q	gaact gaaca cttto	atagg	g 240 300 a 360
	cctaa	aaa q	aacaq gtgta	gttgg	ge to	gettt ettea	geca agtgg	gga ggtt	agtta aaato ccaaa ctcco	attg cctg aaaa ggag	tagt aggo atat	eteca cagea catea	aat q agg q agc q	gaact gaaca cttto cagco	atagg atgggt	g 240 300 a 360 420
ttg	cctaa cttt	aaa q	aacaq gtgta	gttgg aacto aagco	gc to	gettt ettea ettgg	- gees agtgg gagas	gga ggtt agt	agtta aaato ccaaa ctcco cagao	attg cetg aaaa ggag geca	aggo atat cago ttct	egtea eteea eagea ettea egeaa	agg q agc q agt 1	gaact gaaca ettte cagee cggtt	atagg atgggt ggtgga etgcat	g 240 300 a 360 2 420
ttgo	ectaa ettta	aaa g etc g	aacaq gtgta ctgga gggcq	gttgg aacto aagco ggtaa	ge to	gettt ettea ettge etett	agtgg agtgg gagaa agtto	gga ggtt agt agt	agtta aaato ccaaa ctcco cagao	attg cetg aaaa ggag geea agga	aggo atat cago ttct	etce etce etce ette ecgea gaace	aat q agg q agc q agt 1	gaact gaaca ctttq cagco cggtt gaact	atagg atgggt ggtgga etgcat	240 300 360 420 480 540
ttgo aato aaat	cctaa ctttc gctcc	aaa q ctc q ctt q	aacaq gtgta ctgga gggcq gttga	gttgg aacto aagco ggtaa ctcta	ge to et et ea tt ac at	gettt ettes ettgg etcatt ecetet	agtgg agtgg gagaa gagtto tttat	gga ggtt a agt acc acc	agtta aaato ccaaa ctcco cagao agcta	attg cetg aaaa ggag geea agga ataa	tagt agge atat cage ttet acae	etces etces ettes ettes eggas gaace	agg gage dage dage dage dage dage dage d	gaact gaaca ettte cagee cggtt gaact	catagg atgggt ggtgga ctgcat cccttt	240 300 360 420 480 540 480
ttgo aato aaat gato	cctas ctttc gctcc ctgac	aaa g ete o ett g etg g	aacaq gtgta ctgga gggcq gttga	gttgg aacto aagco aggtaa ctcta	ge to et	gettt ettea ettgg ecatt ectet gagaa aatga	egeca agtgg gagaa egtte ettat atgag	a gga ggtt a agt acc acc gaa gact	agtta aaato ccaaa ctcco cagao agcta cgaaa	attg cctg aaaa ggag gcca agga ataa gtcc	tagt agge atat cage ttet acae tcaae	egtea ettea ettea egeaa egaace gaace agtgt	age of ag	gaact gaaca cttto cagco cggtt gaact cagaa	catagg atgggt ggtgga etgcat ecettt etaget	240 300 360 420 480 540 480 660
aatq aaat gato gtaa	cctaa ctttc gctcc ctgac cccca	aaa g ete o ett g etg g aag a	aacaq gtgtz ctggz gggcq gttgq aaati	gttgg aacto aagco ggtaa ctcta tctto	ge to et et et ea et	getttesettesettesettesettesettesetetetesetetesetesetesetesetesetesetesetesetesetesetesetesetesetesetesetesetes	agtgg gagaa ggtto cttat atgag aagca	a gga g gtt a agt c acc c gaa g act a ttt	agtta aaatc ccaaa ccaaa ccaaa ccaaa ccaaa ccaaa ccaaa ccaaa caaa ccaaa c	anttg cctg aaaaa ggag gcca agga ataa ytcc	tagt aggs atat cags tten acags tten acags ceta	egtes ettes ettes egeas egeas egeas egeas	aaat (gaact gaaca ettte cagett gaact cagaa cacett	catagg atgggt ggtgga ctgcat cccttt ctagct	240 300 360 420 480 540 460 460 720
aate aaat gate gtaa gaae	ectas ettto geteo etgao ecces acett	aaa g ctc o ctt g ctg g aag a	aacaq gtgta ctgga gggcq gttga aaati gtccq	gttgg aacto aagco ggtaa ctcta tctto gacco	ge to et	ettes ettes eccatt ecctet gagas aatgs gatgs	agtgggaaacggtcccttat ttat ttgagaaagca	gga agt agt agt agt agt agt act acc	agtta ccaaa ttccc caaa ccaaaa ccaaaa ccaaaa ccaaaa ccaaaa ccaaaa ccaaaa	actg ggag gcca agga ataa ytcc actg	tagti aggo atati cago ttet acago ttet acago tcas acat ttas	egtes ettes ettes egea egea eges eges	aaat gaagg gaagc caagt 1 aaag 1 gac 1 aaag 1 aaaag 1 aaaa	gaact gaact cggtt gaact cagaa ccctt	catagg atgggt ggtgga ctgcat cccttt ctagct aataaa ctgtca	240 300 360 420 480 540 480 660 720
aato aaat gato gtaa gaao tggt	cetas ettte getee etgae ecces aacti	aaa gete oo ett gete gaag aag aag aag aag aag aag aag aag a	gtgtz gtgtz ctgga gggc gttg aaatt gtcc aattt	gttgg gttgg ggtaa agcc ggtaa tcttc tcttc tcttc tcttc	et ct	getttea ettea ettea eccatt ecctet ecctet gagaa aatga gatga eccatga	cgcca gagagaa gagagaa cgtto tttat atgag aaagca gatto	a gga ggt a agt acc acc gaa ttt ttt ttt;	agtta ccaaa ttccc aggtta cgttc cgttc cctca	anttg ccctg aaaaa ggag ggcca aggca ataa ggtcc actg	tagti aggio atati cago ttoti acago acago ttoti acago a	egtea ettea ettea egea ggaac ggaac ggaac ggaac ggaac gaac	aaat qaagg qaaga 1	gaact gaact ettte cagec ggtt gaact cagaa cataa cagaa	catagg atgggt ggtgga ctgcat cccttt ctagct aataaa ctgtca	240 300 360 420 480 540 480 660 720 480 840
aatq aaat gatq gtaa gaaq tggt	ettte ettte getec ettga ecces aacti ettgg	ctc of the control of	gtgtt ctgggggc gttg aaaatt gtcc aaattt tcggf	gttgg aaactc aaagcc ctctc tcttc tcttc tcgaccc taaaa	ggc to the control of	tttggcttt ccatt cctct ggagaa aatga accca ggatagaa	cgcca agtgg gagaa cgttc tttat ttgag aaagca agatt cattt	gga agga act ttt	agtta ccaaa ttccc caga agcta cgaaa cgttc cctca ggttg ccaaaa cccaa	actg ggag gcca agga ataa gggtg ggtg ggata	tagti aggg atati cagg ttcti acag tcaa acat ttaa acat ttaa acat	cgtca cagca cgcaa agtgt taggca agtgt taggca taga tag	agg sagg sagg sagg sagg sagg sagg sagg	gaact cttti cagcc cggtt cagaact catat cgaac cataa	ataggi ggtgga ccetti tagei aataaa ttgtca tacti	240 300 360 420 480 540 480 660 720 48 780 840
ttgo	cctas etttc ettgac eccccs aacti etttg etttt	aaaa gaaaagaaagaacaaaaaa	gtgtz ctgg: ctgg: gggc; gttg aaatt tcgg: agga: ttca!	gttgg aactc aaagc ggtaa etctc tcttc tcttc aagacc taaaa	ggc to the cut of the	ccatt cccatt cccatt cctct cctct cctct cccctct cccctct aaatga cccctgg accca accctgg	cgcca ggagaa ggagaa cgtto tttat attgag aaagca aaagca aaagca ttgat tcattt	a ggs gtt a agt according to the total accord	agtta aaatc ccaaa acccaaa agcta cgaaa cgaaa ccccaa ccccaa	actg ggag gcca agga agga ataa aggtg ggtg	tagti aggg atati cagg ttct acag ttcaa ccta acat ttaa acaa tgag gacc	cgtca ettca eggaac egga	aaat qaagg saagg saagg saagg saaaag saaga saaga saaga saaga saaga saaaa saaa saa	gaact gaace ettte eggtt gaact eagaa eccett egaac eataa	atagg ggtgga ccctti ttagci ttagci ctacti gggaca actaga	240 300 360 420 480 540 480 660 720 48 340 4900 4960

cttccgtggc cagaatggat gggagtgatg catggctatg aaatt

-continued

<21	0> S1 L> L1 2> T1	ENGTI	H: 3													
<21	3 > OI	RGAN:	ISM:	Art	ific	ial										
		THER	INF							mut	ant	(A19	9S/A	328/	(332G)	amino
	a	cid :	sequ	ence	of t	modi:	fied	BChl	Ε							
< 40	0 > S1	EQUEI	ICE :	4												
Asn 1	Ile	Asp	Gln	Ser 5	Phe	Pro	Gly	Phe	His 10	Gly	Ser	Glu	Met	Trp 15	Asn	
Pro	Asn	Thr	Asp 20	Leu	Ser	Glu	Asp	Сув 25	Leu	Tyr	Leu	Asn	Val 30	Trp	Ile	
Pro	Ala	Pro 35	Lys	Pro	Lys	Asn	Ala 40	Thr	Val	Leu	Ile	Trp 45	Ile	Tyr	Gly	
Gly	Gly 50	Phe	Gln	Thr	Gly	Thr 55	Ser	Ser	Leu	His	Val 60	Tyr	Asp	Gly	ГЛа	
Phe 65	Leu	Ala	Arg	Val	Glu 70	Arg	Val	Ile	Val	Val 75	Ser	Met	Asn	Tyr	Arg 80	
Val	Gly	Ala	Leu	Gly 85	Phe	Leu	Ala	Leu	Pro 90	Gly	Asn	Pro	Glu	Ala 95	Pro	
Gly	Asn	Met	Gly 100	Leu	Phe	Asp	Gln	Gln 105	Leu	Ala	Leu	Gln	Trp 110	Val	Gln	
Lys	Asn	Ile 115	Ala	Ala	Phe	Gly	Gly 120	Asn	Pro	Lys	Ser	Val 125	Thr	Leu	Phe	
Gly	Glu 130	Ser	Ser	Gly	Ala	Ala 135	Ser	Val	Ser	Leu	His 140	Leu	Leu	Ser	Pro	
Gly 145	Ser	His	Ser	Leu	Phe 150	Thr	Arg	Ala	Ile	Leu 155	Gln	Ser	Gly	Ser	Phe 160	
Asn	Ala	Pro	Trp	Ala 165	Val	Thr	Ser	Leu	Tyr 170	Glu	Ala	Arg	Asn	Arg 175	Thr	
Leu	Asn	Leu	Ala 180	Lys	Leu	Thr	Gly	Сув 185	Ser	Arg	Glu	Asn	Glu 190	Thr	Glu	
Ile	Ile	Lys 195	Cys	Leu	Arg	Asn	Lys 200	Asp	Pro	Gln	Glu	Ile 205	Leu	Leu	Asn	
Glu	Ala 210	Phe	Val	Val	Pro	Tyr 215	Gly	Thr	Pro	Leu	Ser 220	Val	Asn	Phe	Gly	
Pro 225	Thr	Val	Asp	Gly	Asp 230	Phe	Leu	Thr	Asp	Met 235	Pro	Asp	Ile	Leu	Leu 240	
Glu	Leu	Gly	Gln	Phe 245	Lys	Lys	Thr	Gln	Ile 250	Leu	Val	Gly	Val	Asn 255	Lys	
Asp	Glu	Gly	Thr 260	Trp	Phe	Leu	Val	Gly 265	Gly	Ala	Pro	Gly	Phe 270	Ser	TÀa	
Asp	Asn	Asn 275	Ser	Ile	Ile	Thr	Arg 280	Lys	Glu	Phe	Gln	Glu 285	Gly	Leu	Lys	
Ile	Phe 290	Phe	Pro	Gly	Val	Ser 295	Glu	Phe	Gly	ГÀв	Glu 300	Ser	Ile	Leu	Phe	
His 305	Tyr	Thr	Asp	Trp	Val 310	Asp	Asp	Gln	Arg	Pro 315	Glu	Asn	Tyr	Arg	Glu 320	
Ala	Leu	Gly	Asp	Val 325	Val	Gly	Asp	Tyr	Asn 330	Phe	Ile	Cys	Pro	Ala 335	Leu	
Glu	Phe	Thr	Lys 340	Lys	Phe	Ser	Glu	Trp 345	Gly	Asn	Asn	Ala	Phe 350	Phe	Tyr	

-continued

```
Tyr Phe Glu His Arg Ser Ser Lys Leu Pro Trp Pro Glu Trp Met Gly
       355
                           360
                                                365
Val Met His Gly Tyr Glu Ile
   370
<210> SEQ ID NO 5
<211> LENGTH: 966
<212> TYPE: DNA
<213 > ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: 14-1 mutant (A199S/A328W/Y332G) BChE nucleic
     acid sequnce corresponding to a.a. residues 117-438
<400> SEQUENCE: 5
ggttttcaaa ctggaacatc atctttacat gtttatgatg gcaagtttct ggctcgggtt
gaaagagtta ttgtagtgtc aatgaactat agggtgggtg ccctaggatt cttagctttg
ccaggaaatc ctgaggctcc agggaacatg ggtttatttg atcaacagtt ggctcttcag
                                                                     180
tgggttcaaa aaaatatagc agcctttggt ggaaatccta aaagtgtaac tctctttgga
                                                                     240
gaaagttccg gagcagcttc agttagcctg catttgcttt ctcctggaag ccattcattg
                                                                     300
ttcaccaqaq ccattctqca aaqtqqttcc tttaatqctc cttqqqcqqt aacatctctt
                                                                     360
tatqaaqcta qqaacaqaac qttqaactta qctaaattqa ctqqttqctc taqaqaqaat
                                                                     420
qaqactqaaa taatcaaqtq tcttaqaaat aaaqatcccc aaqaaattct tctqaatqaa
                                                                     480
gcatttgttg tcccctatgg gactcctttg tcagtaaact ttggtccgac cgtggatggt
                                                                     540
gattttctca ctgacatgcc agacatatta cttgaacttg gacaatttaa aaaaacccag
                                                                     600
attttggtgg gtgttaataa agatgaaggg acatggtttt tagtcggtgg tgctcctggc
                                                                     660
ttcagcaaag ataacaatag tatcataact agaaaagaat ttcaggaagg tttaaaaaata
                                                                     720
ttttttccag gagtgagtga gtttggaaag gaatccatcc tttttcatta cacagactgg
                                                                     780
gtagatgatc agagacctga aaactaccgt gaggccttgg gtgatgttgt tggggattat
                                                                     840
aatttcatat gccctgcctt ggagttcacc aagaagttct cagaatgggg aaataatgcc
                                                                     900
tttttctact attttgaaca ccgatcctcc aaacttccgt ggccagaatg gatgggagtg
                                                                     960
atgcat
                                                                     966
<210> SEQ ID NO 6
<211> LENGTH: 322
<212> TYPE: PRT
<213 > ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: 14-1 mutant (A199S/A328W/Y332G) BChE a.a.
     residues 117-438
<400> SEQUENCE: 6
Gly Phe Gln Thr Gly Thr Ser Ser Leu His Val Tyr Asp Gly Lys Phe
Leu Ala Arg Val Glu Arg Val Ile Val Val Ser Met Asn Tyr Arg Val
                                25
Gly Ala Leu Gly Phe Leu Ala Leu Pro Gly Asn Pro Glu Ala Pro Gly
                            40
Asn Met Gly Leu Phe Asp Gln Gln Leu Ala Leu Gln Trp Val Gln Lys
                        55
Asn Ile Ala Ala Phe Gly Gly Asn Pro Lys Ser Val Thr Leu Phe Gly
                   70
                                        75
Glu Ser Ser Gly Ala Ala Ser Val Ser Leu His Leu Leu Ser Pro Gly
                                    90
```

-	-continued
---	------------

-continued
Ser His Ser Leu Phe Thr Arg Ala Ile Leu Gln Ser Gly Ser Phe Asn 100 105 110
Ala Pro Trp Ala Val Thr Ser Leu Tyr Glu Ala Arg Asn Arg Thr Leu 115 120 125
Asn Leu Ala Lys Leu Thr Gly Cys Ser Arg Glu Asn Glu Thr Glu Ile 130 135 140
Ile Lys Cys Leu Arg Asn Lys Asp Pro Gln Glu Ile Leu Leu Asn Glu 145 150 155 160
Ala Phe Val Val Pro Tyr Gly Thr Pro Leu Ser Val Asn Phe Gly Pro 165 170 175
Thr Val Asp Gly Asp Phe Leu Thr Asp Met Pro Asp Ile Leu Leu Glu 180 185 190
Leu Gly Gln Phe Lys Lys Thr Gln Ile Leu Val Gly Val Asn Lys Asp
Glu Gly Thr Trp Phe Leu Val Gly Gly Ala Pro Gly Phe Ser Lys Asp
Asn Asn Ser Ile Ile Thr Arg Lys Glu Phe Gln Glu Gly Leu Lys Ile
Phe Phe Pro Gly Val Ser Glu Phe Gly Lys Glu Ser Ile Leu Phe His
245 250 255 Tyr Thr Asp Trp Val Asp Asp Gln Arg Pro Glu Asn Tyr Arg Glu Ala
260 265 270 Leu Gly Asp Val Val Gly Asp Tyr Asn Phe Ile Cys Pro Ala Leu Glu
275 280 285 Phe Thr Lys Lys Phe Ser Glu Trp Gly Asn Asn Ala Phe Phe Tyr Tyr
290 295 300
Phe Glu His Arg Ser Ser Lys Leu Pro Trp Pro Glu Trp Met Gly Val 305 310 315 320
Met His
<pre><210> SEQ ID NO 7 <211> LENGTH: 1722 <212> TYPE: DNA <213> ORGANISM: Artificial <220> FEATURE: <223> OTHER INFORMATION: 14-2 mutant (A199S/F227A/A328W/Y332G) BChE, full nucleic acid sequence</pre>
<400> SEQUENCE: 7
gaagatgaca tcataattgc aacaaagaat ggaaaagtca gagggatgaa cttgacagtt 60
tttggtggca cggtaacagc ctttcttgga attccctatg cacagccacc tcttggtaga 120
cttcgattca aaaagccaca gtctctgacc aagtggtctg atatttggaa tgccacaaaa 180
tatgcaaatt cttgctgtca gaacatagat caaagttttc caggcttcca tggatcagag 240
atgtggaacc caaacactga cctcagtgaa gactgtttat atctaaatgt atggattcca 300
gcacctaaac caaaaaatgc cactgtattg atatggattt atggtggtgg ttttcaaact 360
ggaacatcat ctttacatgt ttatgatggc aagtttctgg ctcgggttga aagagttatt 420
gtagtgtcaa tgaactatag ggtgggtgcc ctaggattct tagctttgcc aggaaatcct 480
gaggetecag ggaacatggg tttatttgat caacagttgg etetteagtg ggtteaaaaa 540
aatatagcag cctttggtgg aaatcctaaa agtgtaactc tctttggaga aagttccgga 600
geagetteag ttageetgea tttgetttet eetggaagee atteattgtt eaceagagee 660

attetgeaaa gtggtteege taatgeteet tgggeggtaa catetettta tgaagetagg

-continued

aaca	gaad	gt 1	gaad	cttaç	gc ta	aaati	gact	ggt	tgct	cta	gaga	agaat	ga	gact	gaaat	a 780
atca	agto	gtc 1	taga	aaata	aa a	gatc	cccaa	a gaa	aatto	cttc	tgaa	atgaa	gc	attt	gttgt	c 840
ccct	atg	gga (ctcci	ttgt	cc a	gtaa	acttt	ggt	ccga	accg	tgga	atggt	ga	tttt	ctcac	t 900
gaca	tgc	cag :	acata	atta	ct to	gaact	tgga	a caa	attta	aaaa	aaa	cccag	at	tttg	gtggg	t 960
gtta	ataa	aag	atga	aggga	ac at	tggti	ttta	gto	ggt	ggtg	ctc	ctggc	tt	cagca	aaaga	t 1020
aaca	ataç	gta 1	tcata	aacta	ag a	aaaga	aattt	caç	ggaag	ggtt	taaa	aaata	tt	tttt	ccagg	a 1080
gtga	igtga	agt 1	tgg	aaag	ga at	tccai	cctt	ttt	catt	caca	caga	actgg	gt	agat	gatca	g 1140
agad	ctga	aaa a	acta	ccgt	ga g	gaati	gggt	gat	gtt	gttg	ggg	attat	aa	tttca	atatg	c 1200
ccto	geett	gg	agtto	cacca	aa ga	aagti	ctca	a gaa	atggg	ggaa	ataa	atgcc	tt	tttct	tacta	t 1260
tttç	gaaca	acc q	gatco	ctcca	aa a	cttc	gtgg	g cca	agaat	gga	tgg	gagtg	at	gcat	ggcta	t 1320
gaaa	ittga	aat 1	tgt	cttt	gg ti	ttac	ctctc	g gaa	aagaa	agag	ataa	attac	ac	aaaa	gccga	g 1380
gaaa	attt	ga 🤅	gtaga	atcca	at a	gtga	aacgo	g tg	ggcaa	aatt	ttg	caaaa	ta	tggga	aatcc	a 1440
aato	gagad	etc a	agaa	caata	ag ca	acaa	gctgg	g cct	gtct	tca	aaa	gcact	ga	acaa	aaata	t 1500
ctaa	ecct	ga i	ataca	agagt	c a	acaa	gaata	ato	gacga	aaac	tac	gtgct	ca	acaat	gtcg	a 1560
ttct	ggad	cat (catti	ttt	cc a	aaagi	ctto	g gaa	aatga	acag	gaaa	atatt	ga	tgaaq	gcaga	a 1620
tggg	gagto	gga (aagca	aggat	t c	catc	gctgg	g aad	caatt	caca	tgat	ggad	tg	gaaa	aatca	a 1680
ttta	acga	att a	acact	cagca	aa ga	aaaga	aaagt	t tgt	gtgg	ggtc	tc					1722
<211 <212 <213 <220 <223	L> LI 2> T 3> OI 0> FI 3> O ar	ENGTI YPE: RGAN: EATUI THER mino	ISM: RE:	74 Art: ORMA: d sec	rion	: 14	-2 mu	ıtant	E (A)	199S,	/F22 [.]	7A/A3	28W	/Y332	2G),	full
Glu 1	Asp	Asp	Ile	Ile 5	Ile	Ala	Thr	Lys	Asn 10	Gly	Lys	Val	Arg	Gly 15	Met	
Asn	Leu	Thr	Val 20	Phe	Gly	Gly	Thr	Val 25	Thr	Ala	Phe	Leu	Gly 30	Ile	Pro	
Tyr	Ala	Gln 35	Pro	Pro	Leu	Gly	Arg 40	Leu	Arg	Phe	Lys	Lys 45	Pro	Gln	Ser	
Leu	Thr 50	Lys	Trp	Ser	Asp	Ile 55	Trp	Asn	Ala	Thr	Lys 60	Tyr	Ala	Asn	Ser	
Сув 65	Сув	Gln	Asn	Ile	Asp 70	Gln	Ser	Phe	Pro	Gly 75	Phe	His	Gly	Ser	Glu 80	
Met	Trp	Asn	Pro	Asn 85	Thr	Asp	Leu	Ser	Glu 90	Asp	СЛа	Leu	Tyr	Leu 95	Asn	
Val	Trp	Ile	Pro	Ala	Pro	Lys	Pro	Lys 105	Asn	Ala	Thr	Val	Leu 110		Trp	
Ile	Tyr	Gly 115	Gly	Gly	Phe	Gln	Thr 120	Gly	Thr	Ser	Ser	Leu 125	His	Val	Tyr	
	_	115					120									

Glu Ala Pro Gly As
n Met Gly Leu Phe Asp Gl
n Gl
n Leu Ala Leu Gl
n 165 $$ 170 $$ 175

Trp	Val	Gln	Lys 180	Asn	Ile	Ala	Ala	Phe 185	Gly	Gly	Asn	Pro	Lys 190	Ser	Val
Thr	Leu	Phe 195	Gly	Glu	Ser	Ser	Gly 200	Ala	Ala	Ser	Val	Ser 205	Leu	His	Leu
Leu	Ser 210	Pro	Gly	Ser	His	Ser 215	Leu	Phe	Thr	Arg	Ala 220	Ile	Leu	Gln	Ser
Gly 225	Ser	Ala	Asn	Ala	Pro 230	Trp	Ala	Val	Thr	Ser 235	Leu	Tyr	Glu	Ala	Arg 240
Asn	Arg	Thr	Leu	Asn 245	Leu	Ala	Lys	Leu	Thr 250	Gly	Cys	Ser	Arg	Glu 255	Asn
Glu	Thr	Glu	Ile 260	Ile	Lys	СЛа	Leu	Arg 265	Asn	Lys	Asp	Pro	Gln 270	Glu	Ile
Leu	Leu	Asn 275	Glu	Ala	Phe	Val	Val 280	Pro	Tyr	Gly	Thr	Pro 285	Leu	Ser	Val
Asn	Phe 290	Gly	Pro	Thr	Val	Asp 295	Gly	Asp	Phe	Leu	Thr 300	Asp	Met	Pro	Asp
Ile 305	Leu	Leu	Glu	Leu	Gly 310	Gln	Phe	Lys	Lys	Thr 315	Gln	Ile	Leu	Val	Gly 320
Val	Asn	Lys	Asp	Glu 325	Gly	Thr	Trp	Phe	Leu 330	Val	Gly	Gly	Ala	Pro 335	Gly
Phe	Ser	Lys	Asp 340	Asn	Asn	Ser	Ile	Ile 345	Thr	Arg	Lys	Glu	Phe 350	Gln	Glu
Gly	Leu	Lys 355	Ile	Phe	Phe	Pro	Gly 360	Val	Ser	Glu	Phe	Gly 365	Lys	Glu	Ser
Ile	Leu 370	Phe	His	Tyr	Thr	Asp 375	Trp	Val	Asp	Asp	Gln 380	Arg	Pro	Glu	Asn
Tyr 385	Arg	Glu	Ala	Leu	Gly 390	Asp	Val	Val	Gly	Asp 395	Tyr	Asn	Phe	Ile	Сув 400
Pro	Ala	Leu	Glu	Phe 405	Thr	ГЛа	Lys	Phe	Ser 410	Glu	Trp	Gly	Asn	Asn 415	Ala
Phe	Phe	Tyr	Tyr 420	Phe	Glu	His	Arg	Ser 425	Ser	ГÀв	Leu	Pro	Trp 430	Pro	Glu
Trp	Met	Gly 435	Val	Met	His	Gly	Tyr 440	Glu	Ile	Glu	Phe	Val 445	Phe	Gly	Leu
Pro	Leu 450	Glu	Arg	Arg	Asp	Asn 455	Tyr	Thr	Lys	Ala	Glu 460	Glu	Ile	Leu	Ser
Arg 465	Ser	Ile	Val	Lys	Arg 470	Trp	Ala	Asn	Phe	Ala 475	Lys	Tyr	Gly	Asn	Pro 480
Asn	Glu	Thr	Gln	Asn 485	Asn	Ser	Thr	Ser	Trp 490	Pro	Val	Phe	Lys	Ser 495	Thr
Glu	Gln	Lys	Tyr 500	Leu	Thr	Leu	Asn	Thr 505	Glu	Ser	Thr	Arg	Ile 510	Met	Thr
Lys	Leu	Arg 515	Ala	Gln	Gln	Cys	Arg 520	Phe	Trp	Thr	Ser	Phe 525	Phe	Pro	Lys
Val	Leu 530	Glu	Met	Thr	Gly	Asn 535	Ile	Asp	Glu	Ala	Glu 540	Trp	Glu	Trp	Lys
Ala 545	Gly	Phe	His	Arg	Trp 550	Asn	Asn	Tyr	Met	Met 555	Asp	Trp	Lys	Asn	Gln 560
Phe	Asn	Asp	Tyr	Thr 565	Ser	Lys	Lys	Glu	Ser 570	Сув	Val	Gly	Leu		

-continued

```
<210> SEQ ID NO 9
<211> LENGTH: 1125
<212> TYPE: DNA
<213 > ORGANISM: Artificial
<220> FEATURE:
< 223 > \mathtt{OTHER} \ \mathtt{INFORMATION:} \ 14-2 \ \mathtt{mutant} \ (\mathtt{A199/F227A/A328W/Y322G}) \ \mathtt{BChE}
     nucleic acid sequence for a.a. residues 68-442
<400> SEQUENCE: 9
aacatagatc aaagttttcc aggcttccat ggatcagaga tgtggaaccc aaacactgac
ctcagtgaag actgtttata tctaaatgta tggattccag cacctaaacc aaaaaatgcc
actgtattga tatggattta tggtggtggt tttcaaactg gaacatcatc tttacatgtt
tatgatggca agtttctggc tcgggttgaa agagttattg tagtgtcaat gaactatagg
gtgggtgccc taggattctt agctttgcca ggaaatcctg aggctccagg gaacatgggt
ttatttgatc aacagttggc tcttcagtgg gttcaaaaaa atatagcagc ctttggtgga
                                                                       360
aatcctaaaa qtqtaactct ctttqqaqaa aqttccqqaq caqcttcaqt taqcctqcat
                                                                       420
ttgctttctc ctggaagcca ttcattgttc accagagcca ttctgcaaag tggttccgct
                                                                       480
aatgctcctt gggcggtaac atctctttat gaagctagga acagaacgtt gaacttagct
                                                                       540
aaattgactg gttgctctag agagaatgag actgaaataa tcaagtgtct tagaaataaa
                                                                       600
qatccccaaq aaattcttct qaatqaaqca tttqttqtcc cctatqqqac tcctttqtca
                                                                       660
gtaaactttg gtccgaccgt ggatggtgat tttctcactg acatgccaga catattactt
                                                                       720
gaacttggac aatttaaaaa aacccagatt ttggtgggtg ttaataaaga tgaagggaca
                                                                       780
tggtttttag tcggtggtgc tcctggcttc agcaaagata acaatagtat cataactaga
                                                                       840
aaagaatttc aggaaggttt aaaaatattt tttccaggag tgagtgagtt tggaaaggaa
                                                                       900
tccatccttt ttcattacac agactgggta gatgatcaga gacctgaaaa ctaccgtgag
                                                                       960
gccttgggtg atgttgttgg ggattataat ttcatatgcc ctgccttgga gttcaccaag
                                                                      1020
aagtteteag aatggggaaa taatgeettt ttetaetatt ttgaacaceg atceteeaaa
                                                                      1080
cttccgtggc cagaatggat gggagtgatg catggctatg aaatt
                                                                      1125
<210> SEQ ID NO 10
<211> LENGTH: 375
<212> TYPE: PRT
<213 > ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: 14-2 mutant (A199S/F227A/A328W/Y332G) BChE,
      amino acid sequence for residues 68-442
<400> SEQUENCE: 10
Asn Ile Asp Gln Ser Phe Pro Gly Phe His Gly Ser Glu Met Trp Asn
Pro Asn Thr Asp Leu Ser Glu Asp Cys Leu Tyr Leu Asn Val Trp Ile
Pro Ala Pro Lys Pro Lys Asn Ala Thr Val Leu Ile Trp Ile Tyr Gly
                            40
Gly Gly Phe Gln Thr Gly Thr Ser Ser Leu His Val Tyr Asp Gly Lys
                        55
Phe Leu Ala Arg Val Glu Arg Val Ile Val Val Ser Met Asn Tyr Arg
                    70
Val Gly Ala Leu Gly Phe Leu Ala Leu Pro Gly Asn Pro Glu Ala Pro
                                    90
Gly Asn Met Gly Leu Phe Asp Gln Gln Leu Ala Leu Gln Trp Val Gln
```

105

110

Lys Asn Ile Ala 115	Ala Phe Gly	Gly Asn	Pro Lys	Ser Val	Thr	Leu	Phe	
Gly Glu Ser Ser	Gly Ala Ala	Ser Val	Ser Leu		Leu	Ser	Pro	
Gly Ser His Ser 145	Leu Phe Thr	Arg Ala	Ile Leu 155	Gln Ser	Gly	Ser	Ala 160	
Asn Ala Pro Trp	Ala Val Thr 165	Ser Leu	Tyr Glu 170	Ala Arg	Asn	Arg 175	Thr	
Leu Asn Leu Ala 180	Lys Leu Thr	Gly Cys 185	Ser Arg	Glu Asn	Glu 190	Thr	Glu	
Ile Ile Lys Cys 195	Leu Arg Asr	Lys Asp 200	Pro Gln	Glu Ile 205	Leu	Leu	Asn	
Glu Ala Phe Val 210	Val Pro Tyr 215		Pro Leu	Ser Val 220	Asn	Phe	Gly	
Pro Thr Val Asp 225	Gly Asp Phe	Leu Thr	Asp Met 235	Pro Asp	Ile	Leu	Leu 240	
Glu Leu Gly Gln	Phe Lys Lys 245	Thr Gln	Ile Leu 250	Val Gly	Val	Asn 255	Lys	
Asp Glu Gly Thr 260	Trp Phe Leu	Val Gly 265	Gly Ala	Pro Gly	Phe 270	Ser	Lys	
Asp Asn Asn Ser 275	Ile Ile Thr	Arg Lys 280	Glu Phe	Gln Glu 285	Gly	Leu	Lys	
Ile Phe Phe Pro 290	Gly Val Ser 295		Gly Lys	Glu Ser 300	Ile	Leu	Phe	
His Tyr Thr Asp 305	Trp Val Asp 310	Asp Gln	Arg Pro 315	Glu Asn	Tyr	Arg	Glu 320	
Ala Leu Gly Asp	Val Val Gly 325	Asp Tyr	Asn Phe 330	Ile Cys	Pro	Ala 335	Leu	
Glu Phe Thr Lys 340	Lys Phe Ser	Glu Trp 345	Gly Asn	Asn Ala	Phe 350	Phe	Tyr	
Tyr Phe Glu His 355	Arg Ser Ser	Lys Leu 360	Pro Trp	Pro Glu 365	Trp	Met	Gly	
Val Met His Gly 370	Tyr Glu Ile 375							
<pre><210> SEQ ID NO <211> LENGTH: 96 <212> TYPE: DNA <213> ORGANISM: <220> FEATURE: <223> OTHER INFO</pre>	66 Artificial				328W,	/Y332	:G) BChE	
<400> SEQUENCE:								
ggttttcaaa ctgga gaaagagtta ttgta		_						60 120
ccaggaaatc ctgag								180
tgggttcaaa aaaat								240
gaaagtteeg gagea	agcttc agtta	gcctg ca	tttgcttt	ctcctgg	aag (ccatt	cattg	300
ttcaccagag ccatt	ctgca aagtg	gttcc gc	taatgctc	cttgggc	ggt a	aacat	ctctt	360
tatgaagcta ggaac	cagaac gttga	actta gc	taaattga	ctggttg	ctc 1	agag	gagaat	420
gagactgaaa taatc	aagtg totta	ıgaaat aa	agatecee	aagaaat	tct 1	ctga	atgaa	480
gcatttgttg tcccc	ctatgg gacto	ctttg tc	agtaaact	ttggtcc	gac (egtge	gatggt	540

			US 0,033,130 B1	
	65		-continued	6
gattttctca ctga	catgcc agacat	atta cttgaactt	g gacaatttaa aaaaacccag	600
attttggtgg gtgt	taataa agatga	aggg acatggttt	t tagteggtgg tgeteetgge	660
ttcagcaaag ataa	caatag tatcat	aact agaaaagaa	t ttcaggaagg tttaaaaata	720
ttttttccag gagt	gagtga gtttgg	aaag gaatccato	c tttttcatta cacagactgg	780
gtagatgatc agag	jacctga aaacta	ccgt gaggccttg	g gtgatgttgt tggggattat	840
aatttcatat gccc	tgcctt ggagtt	cacc aagaagtto	t cagaatgggg aaataatgcc	900
tttttctact attt	tgaaca ccgatc	ctcc aaacttcco	t ggccagaatg gatgggagtg	960
atgcat				966
<pre><210> SEQ ID NO <211> LENGTH: 3 <212> TYPE: PRT <213> ORGANISM: <220> FEATURE: <223> OTHER INF</pre>	222 Artificial	2 (A199S/F22 <i>7F</i>	./A328W/Y332G) a.a residue	es
<400> SEQUENCE:	12			
Gly Phe Gln Thr 1	Gly Thr Ser	Ser Leu His Va 10	l Tyr Asp Gly Lys Phe 15	
Leu Ala Arg Val 20	. Glu Arg Val	Ile Val Val Se 25	r Met Asn Tyr Arg Val 30	
Gly Ala Leu Gly 35		Leu Pro Gly As 40	n Pro Glu Ala Pro Gly 45	
Asn Met Gly Leu 50	Phe Asp Gln 55	Gln Leu Ala Le	u Gln Trp Val Gln Lys 60	
Asn Ile Ala Ala 65	Phe Gly Gly .	Asn Pro Lys Se 75	r Val Thr Leu Phe Gly 80	
Glu Ser Ser Gly	Ala Ala Ser 85	Val Ser Leu Hi 90	s Leu Leu Ser Pro Gly 95	
Ser His Ser Leu 100	_	Ala Ile Leu Gl 105	n Ser Gly Ser Ala Asn 110	
Ala Pro Trp Ala 115	Val Thr Ser		a Arg Asn Arg Thr Leu 125	
Asn Leu Ala Lys 130	Leu Thr Gly	Cys Ser Arg Gl	u Asn Glu Thr Glu Ile 140	
Ile Lys Cys Leu 145	ı Arg Asn Lys . 150	Asp Pro Gln Gl 15	u Ile Leu Leu Asn Glu 5 160	
Ala Phe Val Val	Pro Tyr Gly	Thr Pro Leu Se 170	r Val Asn Phe Gly Pro 175	
Thr Val Asp Gly 180	_	Thr Asp Met Pr 185	o Asp Ile Leu Leu Glu 190	
Leu Gly Gln Phe 195		Gln Ile Leu Va 200	l Gly Val Asn Lys Asp 205	
Glu Gly Thr Trp 210	Phe Leu Val	Gly Gly Ala Pr	o Gly Phe Ser Lys Asp 220	
Asn Asn Ser Ile 225	e Ile Thr Arg	Lys Glu Phe Gl 23	n Glu Gly Leu Lys Ile 5 240	

Phe Phe Pro Gly Val Ser Glu Phe Gly Lys Glu Ser Ile Leu Phe His 245 250 255

Tyr Thr Asp Trp Val Asp Asp Gln Arg Pro Glu Asn Tyr Arg Glu Ala 260 $$ 265 $$ 270

-cont	

continued Leu Gly Asp Val Val Gly Asp Tyr Asn Phe Ile Cys Pro Ala Leu Glu 280 Phe Thr Lys Lys Phe Ser Glu Trp Gly Asn Asn Ala Phe Phe Tyr Tyr 295 Phe Glu His Arg Ser Ser Lys Leu Pro Trp Pro Glu Trp Met Gly Val 305 310 315 320 Met His <210> SEQ ID NO 13 <211> LENGTH: 1722 <212> TYPE: DNA <213 > ORGANISM: Artificial <220> FEATURE: <223> OTHER INFORMATION: 14-3 mutant (A199S/S287G/A328W/Y332G) BChE nucleic acid sequence <400> SEQUENCE: 13 qaaqatqaca tcataattqc aacaaaqaat qqaaaaqtca qaqqqatqaa cttqacaqtt 60 tttqqtqqca cqqtaacaqc ctttcttqqa attccctatq cacaqccacc tcttqqtaqa 120 cttcgattca aaaagccaca gtctctgacc aagtggtctg atatttggaa tgccacaaaa 180 tatgcaaatt cttgctgtca gaacatagat caaagttttc caggcttcca tggatcagag 240 atgtggaacc caaacactga cctcagtgaa gactgtttat atctaaatgt atggattcca 300 gcacctaaac caaaaaatgc cactgtattg atatggattt atggtggtgg ttttcaaact 360 ggaacatcat ctttacatgt ttatgatggc aagtttctgg ctcgggttga aagagttatt 420 gtagtgtcaa tgaactatag ggtgggtgcc ctaggattct tagctttgcc aggaaatcct 480 gaggetecag ggaacatggg tttatttgat caacagttgg etetteagtg ggtteaaaaa 540 aatatagcag cetttggtgg aaateetaaa agtgtaacte tetttggaga aagtteegga 600 gcagetteag ttageetgea tttgetttet eetggaagee atteattgtt eaceagagee 660 attotgoaaa gtggttoott taatgotoot tgggeggtaa catototta tgaagotagg 720 aacagaacgt tgaacttagc taaattgact ggttgctcta gagagaatga gactgaaata 780 atcaagtgtc ttagaaataa agatccccaa gaaattcttc tgaatgaagc atttgttgtc 840 ccctatggga ctcctttggg tgtaaacttt ggtccgaccg tggatggtga ttttctcact 900 gacatgccag acatattact tgaacttgga caatttaaaa aaacccagat tttggtgggt 960 gttaataaag atgaagggac atggttttta gtcggtggtg ctcctggctt cagcaaagat 1020 aacaatagta tcataactag aaaagaattt caggaaggtt taaaaatatt ttttccagga 1080 gtgagtgagt ttggaaagga atccatcctt tttcattaca cagactgggt agatgatcag 1140 agacctgaaa actaccgtga ggccttgggt gatgttgttg gggattataa tttcatatgc cctqccttgq agttcaccaa qaaqttctca qaatqqqqaa ataatqcctt tttctactat 1260 1320 tttgaacacc gatcctccaa acttccqtqq ccagaatqqa tqqqaqtqat qcatqqctat gaaattgaat ttgtctttgg tttacctctg gaaagaagag ataattacac aaaagccgag 1380 gaaattttga gtagatccat agtgaaacgg tgggcaaatt ttgcaaaata tgggaatcca aatgagactc agaacaatag cacaagctgg cctgtcttca aaagcactga acaaaaatat 1500 ctaaccttga atacagagtc aacaagaata atgacgaaac tacgtgctca acaatgtcga 1560 ttctggacat catttttcc aaaagtcttg gaaatgacag gaaatattga tgaagcagaa 1620

tgggagtgga aagcaggatt ccatcgctgg aacaattaca tgatggactg gaaaaatcaa

tttaacgatt acactagcaa gaaagaaagt tgtgtgggtc tc

1680

C210 SEQ ID NO 14 C211 LEWSTH: 574 C212 TYPE: PRT TYP	010 00	0 TD 110	7.4										
C213 ORGANISM: Artificial C220 FERTURE:	<211> LE	NGTH: 5											
### ### ### ### ### ### ### ### ### ##	<213> OR	GANISM:	Artific	ial									
Call	<223> OTI	HER INFO			-3 mi	ıtant	(A)	L99S,	/S28′	7G/A:	328W,	/Y332	2G) BChE
1	<400> SE	QUENCE :	14										
20		Asp Ile		Ala	Thr	Lys		Gly	Lys	Val	Arg		Met
Leu Thr Lys Trp Ser Asp Ile Trp Asn Ala Thr Lys Trp Ala Asn Ser 55 Cys Cys Gln Asn Ile Asp Gln Ser Phe Pro Gly Phe His Gly Ser Glu Asp Cys Leu Try Leu Asn 85 Met Trp Asn Pro Asn Thr Asp Leu Ser Glu Asp Cys Leu Try Leu Asn 95 Val Trp Ile Pro Ala Pro Lys Pro Lys Asn Ala Thr Val Leu Ile Trp 100 Ile Tyr Gly Gly Gly Phe Gln Thr Gly Thr Ser Ser Leu His Val Try 115 Asp Gly Lys Phe Leu Ala Arg Val Glu Arg Val Ile Val Val Ser Met 130 Asn Try Arg Val Gly Ala Leu Gly Phe Leu Ala Leu Pro Gly Asn Pro 155 Glu Ala Pro Gly Asn Met Gly Leu Phe Asp Gln Gln Leu Ala Leu Gln 175 Trp Val Gln Lys Asn Ile Ala Ala Phe Gly Gly Asn Pro Lys Ser Val 180 Thr Leu Phe 196 Gly Ser Phe Asn Ala Pro Trp Ala Val Thr Asg Ala Ile Leu Gln Ser 210 Gly Ser Phe Asn Ala Pro Trp Ala Val Thr Ser Leu Tyr Glu Ala Arg 230 Gly Thr Glu Ile Ile Lys Cys Leu Asn Asn Lys Asp Pro Glu Asn 255 Glu Thr Glu Ile Ile Lys Cys Leu Asp Asn Lys Asp Pro Glu Glu Ile 260 Leu Leu Asn Glu Ala Phe Val Val Pro Tyr Gly Thr Pro Leu Gly Val 290 Leu Leu Asn Glu Ala Phe Val Val Pro Tyr Gly Thr Pro Leu Gly Val 290 Leu Leu Leu Glu Leu Gly Gln Phe Val Val Pro Tyr Gly Thr Pro Leu Gly Val 290 Leu Leu Leu Glu Leu Gly Gln Phe Val Val Pro Tyr Gly Thr Pro Leu Gly Val 290 Leu Leu Leu Glu Leu Gly Gln Phe Lys Lys Thr Gln Ile Leu Val Gly 310 310 Gly Leu Lys Asp Asn Asn Ser Ile Ile Thr Arg Lys Glu Phe Gln Glu 300 Gly Leu Lys Asp Asn Asn Ser Ile Ile Thr Arg Lys Glu Phe Gln Glu 340 Gly Leu Lys He Phe Phe Pro Gly Val Ser Glu Phe Gln Glu 345 Gly Leu Lys Ile Phe Phe Pro Gly Val Ser Glu Phe Gln Leu Coll 345 Gly Leu Lys Ile Phe Phe Pro Gly Val Ser Glu Phe Gly Lys Glu Ser Gly Lys Gly Leu Che Gly Val 345 Gly Leu Lys Ile Phe Phe Pro Gly Val Ser Glu Phe Gly Lys Glu Ser Gly Selu Ser Cly Clys Glu Ser Cly Clys Glu Ser Clys Clys Clu Ser Clys Clys Clys Glu Ser Clys Clys Clys Clys Clu Ser Clys Clys Clys Clys Clys Clys Clys Clys	Asn Leu '		Phe Gly	Gly	Thr		Thr	Ala	Phe	Leu	_	Ile	Pro
50 55 60 Cys Cys Gln Asn Ile Asp Gln Ser Phe Pro Gly Phe His Gly Ser Glu 65 70 80 Met Trp Asn Pro Asn Thr Asp Leu Ser Glu Asp Cys Leu Tyr Leu Asn 95 90 80 90 Val Trp Ile Pro Ala Pro Lys Pro Lys Asn Ala Thr Val Leu Ile Trp 1100 11e Tyr Gly Gly Gly Phe Gln Thr Gly Thr Ser Ser Leu His Val Tyr 115 11e Tyr Gly Gly Gly Phe Leu Ala Arg Val Glu Arg Val Ile Val Val Ser Met 130 Asp Gly Lys Phe Leu Ala Arg Val Glu Arg Val Ile Val Val Ser Met 130 140 155 160 Glu Ala Pro Gly Asn Met Gly Leu Phe Asp Gln Gln Leu Ala Leu Gln 165 160 150 160 160 160 Glu Ala Pro Gly Asn Met Gly Leu Phe Asp Gln Gln Leu Ala Leu Gln 175 17e Ser Val 180 180 180 180 180 190 190 160			Pro Leu	Gly		Leu	Arg	Phe	Lys		Pro	Gln	Ser
Met Trp Asn Pro Asn Thr Asp Leu Ser Glu Asp Cys Leu Tyr Leu Asn 90		Lys Trp	Ser Asp		Trp	Asn	Ala	Thr		Tyr	Ala	Asn	Ser
S5 90 95 95 Val Trp Ile Pro Ala Pro Lys Pro Lys Asn Ala Thr Val Leu Ile Trp 110		Gln Asn		Gln	Ser	Phe	Pro	_	Phe	His	Gly	Ser	
100 105 110 110 110 110 110 1110 1115	Met Trp	Asn Pro		Asp	Leu	Ser		Asp	CAa	Leu	Tyr		Asn
Asp Gly Lys Phe Leu Ala Arg Val Glu Arg Val Ile Val Val Ser Met 130	Val Trp		Ala Pro	Lys	Pro		Asn	Ala	Thr	Val		Ile	Trp
Asn Tyr Arg Val Gly Ala Leu Gly Phe Leu Ala Leu Pro Gly Asn Pro 145 Glu Ala Pro Gly Asn Met Gly Leu Phe Asp Gln Gln Leu Ala Leu Gln 175 Trp Val Gln Lys Asn Ile Ala Ala Phe Gly Gly Asn Pro 180 Thr Leu Phe Gly Glu Ser Ser Gly Ala Ala Ser Val Ser Leu His Leu 205 Leu Ser Pro Gly Ser His Ser Leu Phe Thr Arg Ala Ile Leu Gln Ser 210 Gly Ser Phe Asn Ala Pro Trp Ala Val Thr Ser Leu Tyr Glu Ala Arg 245 Glu Thr Glu Ile Ile Lys Cys Leu Arg Asn Lys Asp Pro Gln Glu Ile 275 Asn Pro 190 Leu Leu Asn Glu Ala Phe Val Val Pro Tyr Gly Thr Pro Leu Gly Val 285 Asn Pro 190 Asn Asp Thr Leu Glu Leu Gly Gln Phe Lys Lys Thr Gln Ile Leu Cly Ile Leu Gly 315 Asn Pro 190 Asn Lys Asp Asp Asn Asn Ser Ile Ile Thr Arg Leu Thr Gly Gly Ala Pro Gly 335 Cly Leu Leu Leu Ser Cly Asp Asp Asn Asn Ser Ile Ile Thr Arg Leu Thr Arg Ala Ile Leu Gln Ser 250 Asn Pro Ile Leu Leu Glu Leu Gly Gln Phe Lys Lys Thr Gln Ile Leu Cly Ile Cly 335 Phe Ser Lys Asp Asn Asn Ser Ile Ile Thr Arg Lys Glu Phe Gly Lys Glu Ser			Gly Phe	Gln		Gly	Thr	Ser	Ser		His	Val	Tyr
145		Lys Phe	Leu Ala		Val	Glu	Arg	Val		Val	Val	Ser	Met
Trp Val Gln Lys Asn Ile Ala Ala Phe Gly Gly Asn Pro Lys Ser Val 180 Thr Leu Phe Gly Glu Ser Ser Gly Ala Ala Ser Val Ser Leu His Leu 195 Leu Ser Pro Gly Ser His Ser Leu Phe Thr Arg Ala Ile Leu Gln Ser 210 Gly Ser Phe Asn Ala Pro Trp Ala Val Thr Ser Leu Tyr Glu Ala Arg 225 Glu Thr Glu Ile Ile Lys Cys Leu Arg Asn Lys Asp Pro Gln Glu Ile 260 Leu Leu Asn Glu Ala Phe Val Val Pro Tyr Gly Thr Pro Leu Gly Val 275 Asn Phe Gly Pro Thr Val Asp Gly Asp Phe Leu Thr Asp Met Pro Asp 290 Tle Leu Leu Glu Leu Gly Gln Phe Lys Lys Thr Gln Ile Leu Val Gly 305 Phe Ser Lys Asp Asn Asn Ser Ile Ile Thr Arg Lys Glu Phe Gln Glu Gly Leu Lys Ile Phe Phe Pro Gly Val Ser Glu Phe Gly Lys Glu Ser	_	Arg Val	-		Gly	Phe	Leu		Leu	Pro	Gly	Asn	
Thr Leu Phe Gly Glu Ser Ser Gly Ala Ala Ser Val Ser Leu His Leu 200 Leu Ser Pro Gly Ser His Ser Leu Phe Thr Arg Ala Ile Leu Gln Ser 210 Gly Ser Phe Asn Ala Pro Trp Ala Val Thr Ser Leu Tyr Glu Ala Arg 240 Asn Arg Thr Leu Asn Leu Ala Lys Leu Thr Gly Cys Ser Arg Glu Asn 255 Glu Thr Glu Ile Ile Lys Cys Leu Arg Asn Lys Asp Pro Gln Glu Ile 260 Leu Leu Asn Glu Ala Phe Val Val Pro Tyr Gly Thr Pro Leu Gly Val 275 Asn Phe Gly Pro Thr Val Asp Gly Asp Phe Leu Thr Asp Met Pro Asp 290 Ile Leu Leu Glu Leu Gly Gln Phe Lys Lys Thr Gln Ile Leu Val Gly 305 Val Asn Lys Asp Glu Gly Thr Trp Phe Leu Val Gly Gly Ala Pro Gly 325 Phe Ser Lys Asp Asn Asn Ser Ile Ile Thr Arg Lys Glu Phe Gln Glu Ser Gly Leu Lys Ile Phe Phe Pro Gly Val Ser Glu Phe Gly Lys Glu Ser	Glu Ala 1	Pro Gly		Gly	Leu	Phe	_	Gln	Gln	Leu	Ala		Gln
Leu Ser Pro Gly Ser His Ser Leu Phe Thr Arg Ala Ile Leu Gln Ser 210	Trp Val (Asn Ile	Ala	Ala		Gly	Gly	Asn	Pro	_	Ser	Val
210 215 220 Gly Ser Phe Asn Ala Pro Trp Ala Val Thr Ser Leu Tyr Glu Ala Arg 240 Asn Arg Thr Leu Asn Leu Ala Lys Leu Thr Gly Cys Ser Arg Glu Asn 255 Glu Thr Glu Ile Ile Lys Cys Leu Arg Asn Lys Asp Pro Gln Glu Ile 260 Leu Leu Asn Glu Ala Phe Val Val Pro Tyr Gly Thr Pro Leu Gly Val 275 Asn Phe Gly Pro Thr Val Asp Gly Asp Phe Leu Thr Asp Met Pro Asp 300 Ile Leu Leu Glu Leu Gly Gln Phe Lys Lys Thr Gln Ile Leu Val Gly 305 Val Asn Lys Asp Glu Gly Thr Trp Phe Leu Val Gly Gly Ala Pro Gly 325 Phe Ser Lys Asp Asn Asn Ser Ile Ile Thr Arg Lys Glu Phe Gln Glu Ser Gly Leu Lys Ile Phe Phe Pro Gly Val Ser Glu Phe Gly Lys Glu Ser			Glu Ser	Ser	_	Ala	Ala	Ser	Val		Leu	His	Leu
Asn Arg Thr Leu Asn Leu Ala Lys Leu Thr Gly Cys Ser Arg Glu Asn 255 Glu Thr Glu Ile Ile Lys Cys Leu Arg Asn Lys Asp Pro Gln Glu Ile 260 Leu Leu Asn Glu Ala Phe Val Val Pro Tyr Gly Thr Pro Leu Gly Val 275 Asn Phe Gly Pro Thr Val Asp Gly Asp Phe Leu Thr Asp Met Pro Asp 290 Ile Leu Leu Glu Leu Gly Gln Phe Lys Lys Thr Gln Ile Leu Val Gly 305 Val Asn Lys Asp Glu Gly Thr Trp Phe Leu Val Gly Gly Ala Pro Gly 325 Phe Ser Lys Asp Asn Asn Ser Ile Ile Thr Arg Lys Glu Phe Gln Glu Gly Leu Lys Ile Phe Pro Gly Val Ser Glu Phe Gly Lys Glu Ser		Pro Gly	Ser His		Leu	Phe	Thr	Arg		Ile	Leu	Gln	Ser
Glu Thr Glu Ile Ile Lys Cys Leu Arg Asn Lys Asp Pro Gln Glu Ile 260		Phe Asn			Ala	Val	Thr		Leu	Tyr	Glu	Ala	
Leu Leu Asn Glu Ala Phe Val Val Pro Tyr Gly Thr Pro Leu Gly Val 285 Asn Phe Gly Pro Thr Val Asp Gly Asp Phe Leu Thr Asp Met Pro Asp 290 Ile Leu Leu Glu Leu Gly Gln Phe Lys Lys Thr Gln Ile Leu Val Gly 305 Val Asn Lys Asp Glu Gly Thr Trp Phe Leu Val Gly Gly Ala Pro Gly 325 Phe Ser Lys Asp Asn Asn Ser Ile Ile Thr Arg Lys Glu Phe Gln Glu 340 Gly Leu Lys Ile Phe Phe Pro Gly Val Ser Glu Phe Gly Lys Glu Ser	Asn Arg '	Thr Leu		Ala	Lys	Leu		Gly	Cys	Ser	Arg		Asn
Asn Phe Gly Pro Thr Val Asp Gly Asp Phe Leu Thr Asp Met Pro Asp 290 Ile Leu Leu Glu Leu Gly Gln Phe Lys Lys Thr Gln Ile Leu Val Gly 305 Val Asn Lys Asp Glu Gly Thr Trp Phe Leu Val Gly Gly Ala Pro Gly 325 Phe Ser Lys Asp Asn Asn Ser Ile Ile Thr Arg Lys Glu Phe Gln Glu 340 Gly Leu Lys Ile Phe Phe Pro Gly Val Ser Glu Phe Gly Lys Glu Ser	Glu Thr		Ile Lys	Сла	Leu	_	Asn	Lys	Asp	Pro		Glu	Ile
290 295 300 Ile Leu Leu Glu Leu Gly Gln Phe Lys Lys Thr Gln Ile Leu Val Gly 305 Val Asn Lys Asp Glu Gly Thr Trp Phe Leu Val Gly Gly Ala Pro Gly 325 Phe Ser Lys Asp Asn Asn Ser Ile Ile Thr Arg Lys Glu Phe Gln Glu 340 Gly Leu Lys Ile Phe Phe Pro Gly Val Ser Glu Phe Gly Lys Glu Ser			Ala Phe	Val		Pro	Tyr	Gly	Thr		Leu	Gly	Val
Val Asn Lys Asp Glu Gly Thr Trp Phe Leu Val Gly Gly Ala Pro Gly 325 330 335 Phe Ser Lys Asp Asn Asn Ser Ile Ile Thr Arg Lys Glu Phe Gln Glu 340 345 350 Gly Leu Lys Ile Phe Phe Pro Gly Val Ser Glu Phe Gly Lys Glu Ser		Gly Pro	Thr Val	_	Gly	Asp	Phe	Leu		Asp	Met	Pro	Asp
Phe Ser Lys Asp Asn Asn Ser Ile Ile Thr Arg Lys Glu Phe Gln Glu 340 345 350 Gly Leu Lys Ile Phe Phe Pro Gly Val Ser Glu Phe Gly Lys Glu Ser		Leu Glu	_		Phe	Lys	Lys		Gln	Ile	Leu	Val	_
340 345 350 Gly Leu Lys Ile Phe Phe Pro Gly Val Ser Glu Phe Gly Lys Glu Ser	Val Asn 1	Lya Aap	_	Thr	Trp	Phe		Val	Gly	Gly	Ala		Gly
	Phe Ser		Asn Asn	Ser	Ile		Thr	Arg	Lys	Glu		Gln	Glu
			Phe Phe	Pro		Val	Ser	Glu	Phe		Lys	Glu	Ser

-continued

Ile	Leu 370	Phe	His	Tyr	Thr	Asp 375	Trp	Val	Asp	Asp	Gln 380	Arg	Pro	Glu	Asn	
Tyr 385	Arg	Glu	Ala	Leu	Gly 390	Asp	Val	Val	Gly	Asp 395	Tyr	Asn	Phe	Ile	Сув 400	
Pro	Ala	Leu	Glu	Phe 405	Thr	Lys	Lys	Phe	Ser 410	Glu	Trp	Gly	Asn	Asn 415	Ala	
Phe	Phe	Tyr	Tyr 420	Phe	Glu	His	Arg	Ser 425	Ser	Lys	Leu	Pro	Trp 430	Pro	Glu	
Trp	Met	Gly 435	Val	Met	His	Gly	Tyr 440	Glu	Ile	Glu	Phe	Val 445	Phe	Gly	Leu	
Pro	Leu 450	Glu	Arg	Arg	Asp	Asn 455	Tyr	Thr	Lys	Ala	Glu 460	Glu	Ile	Leu	Ser	
Arg 465	Ser	Ile	Val	Lys	Arg 470	Trp	Ala	Asn	Phe	Ala 475	Lys	Tyr	Gly	Asn	Pro 480	
Asn	Glu	Thr	Gln	Asn 485	Asn	Ser	Thr	Ser	Trp 490	Pro	Val	Phe	Lys	Ser 495	Thr	
Glu	Gln	Lys	Tyr 500	Leu	Thr	Leu	Asn	Thr 505	Glu	Ser	Thr	Arg	Ile 510	Met	Thr	
Lys	Leu	Arg 515	Ala	Gln	Gln	CÀa	Arg 520	Phe	Trp	Thr	Ser	Phe 525	Phe	Pro	Lys	
Val	Leu 530	Glu	Met	Thr	Gly	Asn 535	Ile	Asp	Glu	Ala	Glu 540	Trp	Glu	Trp	Lys	
Ala 545	Gly	Phe	His	Arg	Trp 550	Asn	Asn	Tyr	Met	Met 555	Asp	Trp	Lys	Asn	Gln 560	
Phe	Asn	Asp	Tyr	Thr 565	Ser	Lys	Lys	Glu	Ser 570	Cys	Val	Gly	Leu			
<213 <213 <213 <220 <223	L> LF 2> TY 3> OF 0> FF 3> OT nu	ENGTH YPE: RGAN EATUH THER	ISM: RE: INFO	Art: ORMA:	rion	: 14-			: (Al						2G) BChE	
aaca	ataga	atc a	aaagt	ttt	cc aç	ggctt	ccat	gga	atcaç	gaga	tgtg	ggaa	ccc a	aaaca	actgac	60
ctca	agtga	aag a	actgt	ttat	a to	ctaaa	atgta	a tg	gatto	ccag	caco	ctaaa	acc a	aaaa	aatgcc	120
acto	gtatt	ga t	atg	gattt	ta to	ggtgg	gtggt	ttt	caaa	actg	gaad	catca	atc 1	tttad	catgtt	180
tato	gatgo	gca a	agttt	ctg	gc to	cgggt	tgaa	a aga	agtta	attg	tagt	gtca	aat 🤉	gaact	atagg	240
gtg	ggtgo	ccc t	agga	attct	t aq	gettt	gcca	a gga	aato	cctg	aggo	ctcca	agg (gaaca	atgggt	300
ttat	ttga	atc a	aacaç	gttgg	gc to	ettea	agtgo	ggtt	caaa	aaaa	atat	agca	agc (cttt	ggtgga	360
aato	cctaa	aaa q	gtgta	aacto	ct ct	ttgg	gagaa	a agt	tace	ggag	cago	ette	agt 1	tagco	ctgcat	420
ttg	ettte	ctc o	ctgga	aagco	ca t	catt	gtto	c acc	cagaç	gcca	ttct	gcaa	aag 1	tggtt	ccttt	480
aat	getec	ett ç	gggcg	ggtaa	ac at	ctct	ttat	gaa	agcta	agga	acaç	gaac	gtt 9	gaact	taget	540
aaat	tgad	etg g	gttgo	ctcta	ag ag	gagaa	atgaç	g act	gaaa	ataa	tcaa	agtgt	ct 1	tagaa	aataaa	600
gato	ccca	aag a	aaatt	ctto	et ga	aatga	aagca	a ttt	gttg	gtcc	ccta	atgg	gac 1	tcctt	tgggt	660
gtaa	actt	tg ç	gtaco	gacco	gt g	gatgo	gtgat	: ttt	ctca	actg	acat	gcca	aga (catat	tactt	720
gaad	ettg	gac a	aattt	caaaa	aa aa	accca	agatt	tte	ggtgg	ggtg	ttaa	ataaa	aga 1	tgaaq	gggaca	780
															actaga	840
		-						_				_			-	

aaagaatttc aggaaggttt aaaaatattt tttccaggag tgagtgagtt tggaaaggaa

gocttgggtg atgttgttgg ggattataat ttcatatgc ctgccttgga gtcaccaag aagttctcag aatggggaaa taatgccttt ttctactatt ttgacaccg atccccaaa cttccgtggc cagaatggat gggagtgatg catggctatg aaatt <pre> </pre> <pre> <pre> <pre> </pre> <pre> </pre> <pre> <pre></pre></pre></pre></pre>											-	con	tin	ued	
aagttctcag aatggggaa taatgccttt ttctactatt ttgaacaccg atcctccaaa Cttccctgtggc cagaatggat gggagtgatg catggctatg aaatt <pre> <2100 SEQ ID NO 16 <2111 LENGTH: 375 <2112 TYPE: PRT</pre>	tccatcc	ttt 1	tcat	taca	ac aç	gact	gggta	a gat	gato	caga	gac	ctgaa	aaa (ctaco	cgtgag
CELCCCTGGC Cagaatgat gggagtgatg Catggctatg aaatt	gccttgg	gtg a	atgtt	gtt	gg gg	gatta	ataat	tto	catat	gcc	ctg	cctt	gga (gttca	accaag
Callo SEQ ID NO 16 Callo LENGTH: 375 Calla DENGTH: 375 Calla DENGTH: 375 Calla TYPE: PRT Calla OFF PRATURE: Calla O	aagttct	cag a	aatg	gggaa	aa ta	aatg	cctt	t tto	ctact	att	ttga	aaca	ccg a	atcct	ccaaa
<pre>2212 TYPE PRT 2213 ORGANISM: Artificial 2220 FEATURE: 2230 OTHER INFORMATION: 14-3 mutant (A1995/S287G/A328W/Y332G) BChE amino acid sequence for residues 68-442 </pre> <pre> 4400 SEQUENCE: 16 Asm ITe Asp Gin Ser Phe Pro Gly Phe His Gly Ser Glu Met Trp Asn 15 Pro Asn Thr Asp Leu Ser Glu Asp Cys Leu Tyr Leu Asn Val Trp ITe 25 Pro Ala Pro Lys Pro Lys Asn Ala Thr Val Leu ITe Trp ITe 17 Gly 35 Pro Ala Pro Lys Pro Lys Asn Ala Thr Val Leu ITe Trp ITe Tyr Gly 40 Gly Phe Gln Thr Gly Thr Ser Ser Leu His Val Tyr Asp Gly Lys 50 Phe Leu Ala Arg Val Glu Arg Val ITe Val Val Ser Met Asn Tyr Arg 75 Phe Leu Ala Arg Val Glu Arg Val ITe Val Val Ser Met Asn Tyr Arg 80 Val Gly Ala Leu Gly Phe Leu Ala Leu Pro Gly Asn Pro Glu Ala Pro 95 Gly Asn Met Gly Leu Phe Asp Gln Gln Leu Ala Leu Gln Trp Val Gln 100 Lys Asn ITe Ala Ala Phe Gly Gly Asn Pro Lys Ser Val Thr Leu Phe 115 Gly Glu Ser Ser Gly Ala Ala Ser Val Ser Leu His Leu Leu Ser Pro 140 Gly Ser His Ser Leu Phe Thr Arg Ala ITe Leu Gln Ser Gly Ser Phe 145 Asn Ala Pro Trp Ala Val Thr Ser Leu Tyr Glu Ala Arg Asn Arg Thr 165 Glu Ala Pro Tyr Ala Val Thr Ser Leu Tyr Glu Ala Arg Asn Arg Thr 165 Glu Ala Pro Vys Leu Thr Gly Cys Ser Arg Glu Asn Glu Thr Glu 195 Glu Ala Pro Vys Leu Thr Gly Cys Ser Arg Glu Asn Glu Thr Glu 196 Glu Ala Pro Vys Leu Thr Gly Cys Ser Arg Glu Asn Pro Gly Dan Pro Lys 220 Glu Leu Gly Gln Phe Lys Lys Thr Gln ITe Leu Gly Val Asn Phe Gly 220 Glu Leu Gly Gln Phe Lys Lys Thr Gln ITe Leu Gly Val Asn Phe Gly 225 Asp Asn Asn Ser ITe ITe Thr Arg Lys Glu Phe Glu Glu Gly Leu Lys 275 ITe Phe Phe Pro Gly Val Asp Asp Gln Arg Pro Glu Asn Tyr Arg Glu His Tyr Thr Asp Trp Val Asp Gln Phe Gly Lys Glu Ser ITe Leu Phe 290 His Tyr Thr Asp Trp Val Asp Gln Rap Pro Glu Asn Tyr Arg Glu His Tyr Thr Asp Trp Val Asp Gln Phe Gly Lys Glu Ser ITe Leu Phe 290 His Tyr Thr Asp Trp Val Asp Gln Rap Gln Arg Pro Glu Asn Tyr Arg Glu His Tyr Thr Asp Trp Val Asp Gln Phe Gly Lys Glu Asn Tyr Arg Glu His Tyr Thr Asp Trp Val Asp Asp Gln Arg Pro Glu Asn Tyr Arg Glu His Tyr Thr Asp Trp Val Asp Asp Gln Arg Pro Gl</pre>	cttccgtggc cagaatggat gggagtgatg catggctatg aaatt														
Ash Ile Asp Gln Ser Phe Pro Gly Phe His Gly Ser Glu Met Trp Ash 15 Pro Ash Thr Asp Leu Ser Glu Asp Cys Leu Tyr Leu Ash Val Trp Ile 30 Gly Gly Phe Gln Thr Gly Thr Ser Ser Leu His Val Tyr Asp Gly Lys 60 Gly Phe Gln Thr Gly Thr Ser Ser Leu His Val Tyr Asp Gly Lys 65 Gly Ash Met Gly Phe Leu And Arg Val Glu Asp Cys Pro Gly Ash Pro Gly Cys Pro Gly Ash Pro Gly Ash Pro Gly Cys Pro Gly Ash Pro Gly Cys Cys Leu Ash Pro Gly Cys Cys Leu Arg Ash Lys Ash Pro Gly Cys Cys Leu Ash Pro Gly Cys Cys Cys Leu Ash Pro Gly Cys Cys Leu Ash Pro Gly Cys Cys Cys Cys Cys Cys Cys Cys Cys Cy	<211> L: <212> T <213> O: <220> F: <223> O: <	ENGTI YPE: RGAN: EATUI THER	H: 3' PRT ISM: RE: INFO	75 Art: ORMA	rion	: 14						7G/AC	328W,	/Y332	2G) BChE
1	<400> S	EQUEI	NCE :	16											
Pro Ala Pro Lys Pro Lys Asn Ala Thr Val Leu Ile Trp Ile Tyr Gly Asn Gly Pro Gly Fro So So So So So So So		Asp	Gln		Phe	Pro	Gly	Phe		Gly	Ser	Glu	Met	_	Asn
Signature Sign	Pro Asn	Thr	_	Leu	Ser	Glu	Asp		Leu	Tyr	Leu	Asn		Trp	Ile
So	Pro Ala		ГÀа	Pro	Lys	Asn		Thr	Val	Leu	Ile		Ile	Tyr	Gly
Fig. 10		Phe	Gln	Thr	Gly		Ser	Ser	Leu	His		Tyr	Asp	Gly	Lys
S5		Ala	Arg	Val		Arg	Val	Ile	Val		Ser	Met	Asn	Tyr	-
Lys Asn IIe Ala Ala Phe Gly Gly Asn Pro Lys Ser Val Thr Leu Phe 115	Val Gly	Ala	Leu		Phe	Leu	Ala	Leu		Gly	Asn	Pro	Glu		Pro
115	Gly Asn	Met	_	Leu	Phe	Asp	Gln		Leu	Ala	Leu	Gln	_	Val	Gln
130	Lys Asn		Ala	Ala	Phe	Gly		Asn	Pro	Lys	Ser		Thr	Leu	Phe
145 150 155 160 Asn Ala Pro Trp Ala Val Thr Ser 165 160 Tyr Glu Ala Arg Arg Arg Glu Arg 175 Arg Thr 175 Leu Asn Leu Ala Lys Leu Thr 180 Cys Ser Arg Glu Arg Glu Arg 190 Thr Glu 190 Thr Glu 190 Ile Ile Lys Cys Leu Arg Arg Arg 185 Arg Arg Glu Arg 190 Arg Arg 180 Arg Arg 180 Arg Arg 180 Glu Ala Pro 195 Cys Leu Arg Arg Arg 185 Arg Arg 180 Arg Arg 180 Arg 180 <td></td> <td>Ser</td> <td>Ser</td> <td>Gly</td> <td>Ala</td> <td></td> <td>Ser</td> <td>Val</td> <td>Ser</td> <td>Leu</td> <td></td> <td>Leu</td> <td>Leu</td> <td>Ser</td> <td>Pro</td>		Ser	Ser	Gly	Ala		Ser	Val	Ser	Leu		Leu	Leu	Ser	Pro
170 175 176 177 178 189		His	Ser	Leu		Thr	Arg	Ala	Ile		Gln	Ser	Gly	Ser	
180	Asn Ala	Pro	Trp		Val	Thr	Ser	Leu	_	Glu	Ala	Arg	Asn	_	Thr
Second S	Leu Asn	Leu		Lys	Leu	Thr	Gly		Ser	Arg	Glu	Asn		Thr	Glu
210 215 220 220 220 220 220 220 220 220 220 22	Ile Ile	-	CÀa	Leu	Arg	Asn	-	Asp	Pro	Gln	Glu		Leu	Leu	Asn
230		Phe	Val	Val	Pro	_	Gly	Thr	Pro	Leu	_	Val	Asn	Phe	Gly
245 250 255 Asp Glu Gly Thr Trp Phe Leu Val Gly Gly Ala Pro Gly Phe Ser Lys 265 Asp Asn Asn Ser Ile Ile Thr Arg Lys Glu Phe Gln Glu Gly Leu Lys 285 Ile Phe Phe Pro Gly Val Ser Glu Phe Gly Lys Glu Ser Ile Leu Phe 290 His Tyr Thr Asp Trp Val Asp Asp Gln Arg Pro Glu Asn Tyr Arg Glu		Val	Asp	Gly		Phe	Leu	Thr	Asp		Pro	Asp	Ile	Leu	
Asp Asn Asn Ser Ile Ile Thr Arg Lys Glu Phe Gln Glu Gly Leu Lys 285 Ile Phe Phe Pro Gly Val Ser Glu Phe Gly Lys Glu Ser Ile Leu Phe 290 His Tyr Thr Asp Trp Val Asp Asp Gln Arg Pro Glu Asn Tyr Arg Glu	Glu Leu	Gly	Gln		Lys	Lys	Thr	Gln		Leu	Val	Gly	Val		Lys
275 280 285 Ile Phe Phe Pro Gly Val Ser Glu Phe Gly Lys Glu Ser Ile Leu Phe 290 295 300 His Tyr Thr Asp Trp Val Asp Asp Gln Arg Pro Glu Asn Tyr Arg Glu	Asp Glu	Gly		Trp	Phe	Leu	Val	_	Gly	Ala	Pro	Gly		Ser	Lys
290 295 300 His Tyr Thr Asp Trp Val Asp Asp Gln Arg Pro Glu Asn Tyr Arg Glu	Asp Asn		Ser	Ile	Ile	Thr	_	Lys	Glu	Phe	Gln		Gly	Leu	ГЛа
		Phe	Pro	Gly	Val		Glu	Phe	Gly	Lys		Ser	Ile	Leu	Phe
305 310 315 320	His Tyr 305	Thr	Asp	Trp	Val 310	Asp	Asp	Gln	Arg	Pro 315	Glu	Asn	Tyr	Arg	

-continued

Ala Leu Gly Asp Val Val Gly Asp Tyr Asn Phe Ile Cys Pro Ala Leu 325 330 Glu Phe Thr Lys Lys Phe Ser Glu Trp Gly Asn Asn Ala Phe Phe Tyr Tyr Phe Glu His Arg Ser Ser Lys Leu Pro Trp Pro Glu Trp Met Gly 360 365 Val Met His Gly Tyr Glu Ile 370 <210> SEQ ID NO 17 <211> LENGTH: 966 <212> TYPE: DNA <213> ORGANISM: Artificial <220> FEATURE: <223> OTHER INFORMATION: 14-3 mutant (A199S/S287G/A328W/Y332G) BChE nucleic acid sequence for amino acid residues 117-438 <400> SEQUENCE: 17 ggttttcaaa ctggaacatc atctttacat gtttatgatg gcaagtttct ggctcgggtt 60 gaaagagtta ttgtagtgtc aatgaactat agggtgggtg ccctaggatt cttagctttg 120 ccaqqaaatc ctqaqqctcc aqqqaacatq qqtttatttq atcaacaqtt qqctcttcaq 180 tqqqttcaaa aaaatataqc aqcctttqqt qqaaatccta aaaqtqtaac tctctttqqa 240 gaaagttccg gagcagcttc agttagcctg catttgcttt ctcctggaag ccattcattg 300 ttcaccagag ccattctgca aagtggttcc tttaatgctc cttgggcggt aacatctctt 360 tatgaagcta ggaacagaac gttgaactta gctaaattga ctggttgctc tagagagaat 420 gagactgaaa taatcaagtg tottagaaat aaagatcccc aagaaattot totgaatgaa 480 gcatttgttg tcccctatgg gactcctttg ggtgtaaact ttggtccgac cgtggatggt 540 gattttctca ctgacatgcc agacatatta cttgaacttg gacaatttaa aaaaacccag 600 attttggtgg gtgttaataa agatgaaggg acatggtttt tagtcggtgg tgctcctggc 660 ttcagcaaag ataacaatag tatcataact agaaaagaat ttcaggaagg tttaaaaaata 720 ttttttccag gagtgagtga gtttggaaag gaatccatcc tttttcatta cacagactgg 780 gtagatgatc agagacctga aaactaccgt gaggccttgg gtgatgttgt tggggattat 840 aatttcatat gccctgcctt ggagttcacc aagaagttct cagaatgggg aaataatgcc tttttctact attttgaaca ccgatcctcc aaacttccgt ggccagaatg gatgggagtg atgcat <210> SEQ ID NO 18 <211> LENGTH: 322 <212> TYPE: PRT <213 > ORGANISM: Artificial <220> FEATURE: <223> OTHER INFORMATION: 14-3 mutant (A199S/S287G/A328W/Y332G) BChE amino acid sequence for residues 117-438 <400> SEQUENCE: 18 Gly Phe Gln Thr Gly Thr Ser Ser Leu His Val Tyr Asp Gly Lys Phe 10 Leu Ala Arg Val Glu Arg Val Ile Val Val Ser Met Asn Tyr Arg Val 25 Gly Ala Leu Gly Phe Leu Ala Leu Pro Gly Asn Pro Glu Ala Pro Gly 40 Asn Met Gly Leu Phe Asp Gln Gln Leu Ala Leu Gln Trp Val Gln Lys 55 60

Asn Ile Ala Ala Phe Gly Gly Asn Pro Lys Ser Val Thr Leu Phe Gly 70 75 80	
Glu Ser Ser Gly Ala Ala Ser Val Ser Leu His Leu Leu Ser Pro Gly 85 90 95	
Ser His Ser Leu Phe Thr Arg Ala Ile Leu Gln Ser Gly Ser Phe Asn 100 105 110	
Ala Pro Trp Ala Val Thr Ser Leu Tyr Glu Ala Arg Asn Arg Thr Leu 115 120 125	
Asn Leu Ala Lys Leu Thr Gly Cys Ser Arg Glu Asn Glu Thr Glu Ile 130 135 140	
Ile Lys Cys Leu Arg Asn Lys Asp Pro Gln Glu Ile Leu Leu Asn Glu 145 150 155 160	
Ala Phe Val Val Pro Tyr Gly Thr Pro Leu Gly Val Asn Phe Gly Pro 165 170 175	
Thr Val Asp Gly Asp Phe Leu Thr Asp Met Pro Asp Ile Leu Leu Glu 180 185 190	
Leu Gly Gln Phe Lys Lys Thr Gln Ile Leu Val Gly Val Asn Lys Asp 195 200 205	
Glu Gly Thr Trp Phe Leu Val Gly Gly Ala Pro Gly Phe Ser Lys Asp 210 215 220	
Asn Asn Ser Ile Ile Thr Arg Lys Glu Phe Gln Glu Gly Leu Lys Ile 225 230 235 240	
Phe Phe Pro Gly Val Ser Glu Phe Gly Lys Glu Ser Ile Leu Phe His 245 250 255	
Tyr Thr Asp Trp Val Asp Asp Gln Arg Pro Glu Asn Tyr Arg Glu Ala	
Leu Gly Asp Val Val Gly Asp Tyr Asn Phe Ile Cys Pro Ala Leu Glu 275 280 285	
Phe Thr Lys Lys Phe Ser Glu Trp Gly Asn Asn Ala Phe Phe Tyr Tyr 290 295 300	
Phe Glu His Arg Ser Ser Lys Leu Pro Trp Pro Glu Trp Met Gly Val 305 310 315 320	
Met His	
<210> SEQ ID NO 19 <211> LENGTH: 1722 <212> TYPE: DNA <213> ORGANISM: Artificial <220> FEATURE: <223> OTHER INFORMATION: 12-7 mutant (A199S/F227A/S287G/A328W/Y332G) BChE nucleic acid sequence <400> SEQUENCE: 19	
gaagatgaca tcataattgc aacaaagaat ggaaaagtca gagggatgaa cttgacagtt	60
tttggtggca cggtaacagc ctttcttgga attccctatg cacagccacc tcttggtaga	120
cttcgattca aaaagccaca gtctctgacc aagtggtctg atatttggaa tgccacaaaa	180
	240
	300
	360
	120 180
	540
gaggetecag ggaacatggg tttatttgat caacagttgg etetteagtg ggtteaaaaa 5) -1 ()

-continued

aatatagcag	cctt	tggt	gg a	aatc	ctaaa	agt	gtaa	actc	tct	ttgga	aga	aagti	ccgga	600	
gcagcttcag	ttag	cctg	ca t	ttgci	tttct	cct	ggaa	agcc	att	catt	gtt	cacca	agagcc	660	
attctgcaaa	gtgg	ttcc	gc ta	aatg	ctcct	tgg	ggegg	gtaa	cat	ctctt	ta	tgaaq	gctagg	720	
aacagaacgt	tgaa	cttaç	gc ta	aaati	tgact	ggt	tgct	cta	gag	agaat	ga	gacto	gaaata	780	
atcaagtgtc	ttag	aaata	aa a	gatc	cccaa	gaa	aatto	cttc	tga	atgaa	agc	attt	gttgtc	840	
ccctatggga	ctcc	tttg	gg t	gtaa	acttt	ggt	ccga	accg	tgg	atggt	ga	tttt	ctcact	900	
gacatgccag	acat	atta	ct to	gaact	ttgga	ı caa	attta	aaaa	aaa	cccaç	gat	tttg	gtgggt	960	
gttaataaag	atga	aggga	ac at	tggti	tttta	gto	ggtg	ggtg	ctc	ctgg	ett	cagca	aaagat	1020	
aacaatagta	tcata	aacta	ag a	aaaga	aattt	caç	ggaag	ggtt	taa	aaata	att	tttt	ccagga	1080	
gtgagtgagt	ttgg	aaag	ga at	tccai	tcctt	ttt	catt	caca	cag	actg	ggt	agat	gatcag	1140	
agacctgaaa	acta	ccgt	ga g	gaati	tgggt	gat	gtt	gttg	ggg:	attat	caa	tttca	atatgc	1200	
cctgccttgg	agtt	cacca	aa ga	aagti	tctca	gaa	atggg	ggaa	ata	atgc	ctt	tttct	actat	1260	
tttgaacacc	gatc	ctcca	aa a	cttc	cgtgg	g cca	agaat	gga	tgg	gagt	gat	gcat	ggctat	1320	
gaaattgaat	ttgt	cttt	gg ti	ttac	ctctc	g gaa	agaa	agag	ata	atta	cac	aaaa	gccgag	1380	
gaaattttga	gtag	atcca	at a	gtgaa	aacgg	j tgg	ggcaa	aatt	ttg	caaaa	ata	tggga	aatcca	1440	
aatgagactc	agaa	caata	ag ca	acaa	gctgc	g cct	gtct	tca	aaa	gcact	ga	acaa	aaatat	1500	
ctaaccttga	atac	agagt	c a	acaa	gaata	ato	gacga	aaac	tac	gtgct	cca	acaat	gtcga	1560	
ttctggacat	catt	tttt	cc a	aaagt	tcttg	g gaa	aatga	acag	gaa	atatt	ga	tgaaq	gcagaa	1620	
tgggagtgga	aagc	aggat	it c	catc	gatgo	, aad	caatt	caca	tga	tggad	ctg	gaaaa	aatcaa	1680	
tttaacgatt	acac	tagca	aa g	aaaga	aaagt	tgt:	gtgg	ggtc	tc					1722	
<210> SEQ ID NO 20 <211> LENGTH: 574 <212> TYPE: PRT <213> ORGANISM: Artificial <220> FEATURE: <223> OTHER INFORMATION: 12-7 mutant (A199S/F227A/S287G/A328W/Y332G) BChE amino acid sequence															
<400> SEQU	ENCE:	20													
Glu Asp As	p Ile	Ile 5	Ile	Ala	Thr	Lys	Asn 10	Gly	Lys	Val	Arg	Gly 15	Met		
Asn Leu Th	r Val 20	Phe	Gly	Gly	Thr	Val 25	Thr	Ala	Phe	Leu	Gly 30	Ile	Pro		
Tyr Ala Gl 35		Pro	Leu	Gly	Arg 40	Leu	Arg	Phe	Lys	Lуз 45	Pro	Gln	Ser		
Leu Thr Ly 50	s Trp	Ser	Asp	Ile 55	Trp	Asn	Ala	Thr	Fys	Tyr	Ala	Asn	Ser		
Cys Cys Gl 65	n Asn	Ile	Asp 70	Gln	Ser	Phe	Pro	Gly 75	Phe	His	Gly	Ser	Glu 80		
Met Trp As	n Pro	Asn 85	Thr	Asp	Leu	Ser	Glu 90	Asp	CÀa	Leu	Tyr	Leu 95	Asn		
Val Trp Il	e Pro 100	Ala	Pro	Lys	Pro	Lys 105	Asn	Ala	Thr	Val	Leu 110		Trp		
Ile Tyr Gl		Gly	Phe	Gln	Thr 120	Gly	Thr	Ser	Ser	Leu 125	His	Val	Tyr		

Asp Gly Lys Phe Leu Ala Arg Val Glu Arg Val Ile Val Val Ser Met 130 135 140

-continued

Asn 145	Tyr	Arg	Val	Gly	Ala 150	Leu	Gly	Phe	Leu	Ala 155	Leu	Pro	Gly	Asn	Pro 160
Glu	Ala	Pro	Gly	Asn 165	Met	Gly	Leu	Phe	Asp 170	Gln	Gln	Leu	Ala	Leu 175	Gln
Trp	Val	Gln	Lys 180	Asn	Ile	Ala	Ala	Phe 185	Gly	Gly	Asn	Pro	Lys 190	Ser	Val
Thr	Leu	Phe 195	Gly	Glu	Ser	Ser	Gly 200	Ala	Ala	Ser	Val	Ser 205	Leu	His	Leu
Leu	Ser 210	Pro	Gly	Ser	His	Ser 215	Leu	Phe	Thr	Arg	Ala 220	Ile	Leu	Gln	Ser
Gly 225	Ser	Ala	Asn	Ala	Pro 230	Trp	Ala	Val	Thr	Ser 235	Leu	Tyr	Glu	Ala	Arg 240
Asn	Arg	Thr	Leu	Asn 245	Leu	Ala	Lys	Leu	Thr 250	Gly	Сув	Ser	Arg	Glu 255	Asn
Glu	Thr	Glu	Ile 260	Ile	Lys	CÀa	Leu	Arg 265	Asn	Lys	Asp	Pro	Gln 270	Glu	Ile
Leu	Leu	Asn 275	Glu	Ala	Phe	Val	Val 280	Pro	Tyr	Gly	Thr	Pro 285	Leu	Gly	Val
Asn	Phe 290	Gly	Pro	Thr	Val	Asp 295	Gly	Asp	Phe	Leu	Thr 300	Asp	Met	Pro	Aap
Ile 305	Leu	Leu	Glu	Leu	Gly 310	Gln	Phe	Lys	Lys	Thr 315	Gln	Ile	Leu	Val	Gly 320
Val	Asn	Lys	Asp	Glu 325	Gly	Thr	Trp	Phe	Leu 330	Val	Gly	Gly	Ala	Pro 335	Gly
Phe	Ser	Lys	Asp 340	Asn	Asn	Ser	Ile	Ile 345	Thr	Arg	Lys	Glu	Phe 350	Gln	Glu
Gly	Leu	Lys 355	Ile	Phe	Phe	Pro	Gly 360	Val	Ser	Glu	Phe	Gly 365	Lys	Glu	Ser
Ile	Leu 370	Phe	His	Tyr	Thr	Asp 375	Trp	Val	Asp	Asp	Gln 380	Arg	Pro	Glu	Asn
Tyr 385	Arg	Glu	Ala	Leu	Gly 390	Asp	Val	Val	Gly	Asp 395	Tyr	Asn	Phe	Ile	Сув 400
Pro	Ala	Leu	Glu	Phe 405	Thr	Lys	Lys	Phe	Ser 410	Glu	Trp	Gly	Asn	Asn 415	Ala
Phe	Phe	Tyr	Tyr 420	Phe	Glu	His	Arg	Ser 425	Ser	Lys	Leu	Pro	Trp 430	Pro	Glu
Trp	Met	Gly 435	Val	Met	His	Gly	Tyr 440	Glu	Ile	Glu	Phe	Val 445	Phe	Gly	Leu
Pro	Leu 450	Glu	Arg	Arg	Asp	Asn 455	Tyr	Thr	Lys	Ala	Glu 460	Glu	Ile	Leu	Ser
Arg 465	Ser	Ile	Val	Lys	Arg 470	Trp	Ala	Asn	Phe	Ala 475	Lys	Tyr	Gly	Asn	Pro 480
Asn	Glu	Thr	Gln	Asn 485	Asn	Ser	Thr	Ser	Trp 490	Pro	Val	Phe	Lys	Ser 495	Thr
Glu	Gln	Lys	Tyr 500	Leu	Thr	Leu	Asn	Thr 505	Glu	Ser	Thr	Arg	Ile 510	Met	Thr
Lys	Leu	Arg 515	Ala	Gln	Gln	Cys	Arg 520	Phe	Trp	Thr	Ser	Phe 525	Phe	Pro	Lys
Val	Leu 530	Glu	Met	Thr	Gly	Asn 535	Ile	Asp	Glu	Ala	Glu 540	Trp	Glu	Trp	Lys

-continued

Ala Gly Phe His Arg Trp Asn Asn Tyr Met Met Asp Trp Lys Asn Gln

```
545
                                        555
Phe Asn Asp Tyr Thr Ser Lys Lys Glu Ser Cys Val Gly Leu
                565
<210> SEQ ID NO 21
<211> LENGTH: 1125
<212> TYPE: DNA
<213 > ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: 12-7 mutant (A199S/F227A/S287G/A328W/Y332G)
     BChE nucleic acid sequence for amino acid residues 68-442
<400> SEQUENCE: 21
aacatagatc aaagttttcc aggcttccat ggatcagaga tgtggaaccc aaacactgac
ctcagtgaag actgtttata tctaaatgta tggattccag cacctaaacc aaaaaatgcc
actgtattga tatggattta tggtggtggt tttcaaactg gaacatcatc tttacatgtt
                                                                     180
tatgatggca agtttctggc tcgggttgaa agagttattg tagtgtcaat gaactatagg
                                                                     240
gtgggtgccc taggattctt agctttgcca ggaaatcctg aggctccagg gaacatgggt
                                                                     300
ttatttqatc aacaqttqqc tcttcaqtqq qttcaaaaaa atataqcaqc ctttqqtqqa
                                                                     360
aatcctaaaa qtqtaactct ctttqqaqaa aqttccqqaq caqcttcaqt taqcctqcat
                                                                     420
ttgctttctc ctggaagcca ttcattgttc accagagcca ttctgcaaag tggttccgct
                                                                     480
aatgctcctt gggcggtaac atctctttat gaagctagga acagaacgtt gaacttagct
aaattgactg gttgctctag agagaatgag actgaaataa tcaagtgtct tagaaataaa
                                                                     600
gatccccaag aaattcttct gaatgaagca tttgttgtcc cctatgggac tcctttgggt
qtaaactttq qtccqaccqt qqatqqtqat tttctcactq acatqccaqa catattactt
                                                                     720
gaacttggac aatttaaaaa aacccagatt ttggtgggtg ttaataaaga tgaagggaca
                                                                     780
tggtttttag tcggtggtgc tcctggcttc agcaaagata acaatagtat cataactaga
aaagaatttc aggaaggttt aaaaatattt tttccaggag tgagtgagtt tggaaaggaa
                                                                     900
tccatccttt ttcattacac agactgggta gatgatcaga gacctgaaaa ctaccgtgag
qccttqqqtq atqttqttqq qqattataat ttcatatqcc ctqccttqqa qttcaccaaq
                                                                    1020
aagttctcag aatggggaaa taatgccttt ttctactatt ttgaacaccg atcctccaaa
                                                                    1080
                                                                    1125
cttccgtggc cagaatggat gggagtgatg catggctatg aaatt
<210> SEQ ID NO 22
<211> LENGTH: 375
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: 12-7 mutant (A199S/F227A/S287G/A328W/Y332G)
     BChE amino acid sequence for residues 68-442
<400> SEQUENCE: 22
Asn Ile Asp Gln Ser Phe Pro Gly Phe His Gly Ser Glu Met Trp Asn
                                   10
Pro Asn Thr Asp Leu Ser Glu Asp Cys Leu Tyr Leu Asn Val Trp Ile
Pro Ala Pro Lys Pro Lys Asn Ala Thr Val Leu Ile Trp Ile Tyr Gly
                            40
Gly Gly Phe Gln Thr Gly Thr Ser Ser Leu His Val Tyr Asp Gly Lys
                       55
   50
                                            60
```

Phe 65	Leu	Ala	Arg	Val	Glu 70	Arg	Val	Ile	Val	Val 75	Ser	Met	Asn	Tyr	Arg 80	
Val	Gly	Ala	Leu	Gly 85	Phe	Leu	Ala	Leu	Pro 90	Gly	Asn	Pro	Glu	Ala 95	Pro	
Gly	Asn	Met	Gly 100	Leu	Phe	Asp	Gln	Gln 105	Leu	Ala	Leu	Gln	Trp 110	Val	Gln	
Lys	Asn	Ile 115	Ala	Ala	Phe	Gly	Gly 120	Asn	Pro	Lys	Ser	Val 125	Thr	Leu	Phe	
Gly	Glu 130	Ser	Ser	Gly	Ala	Ala 135	Ser	Val	Ser	Leu	His 140	Leu	Leu	Ser	Pro	
Gly 145	Ser	His	Ser	Leu	Phe 150	Thr	Arg	Ala	Ile	Leu 155	Gln	Ser	Gly	Ser	Ala 160	
Asn	Ala	Pro	Trp	Ala 165	Val	Thr	Ser	Leu	Tyr 170	Glu	Ala	Arg	Asn	Arg 175	Thr	
Leu	Asn	Leu	Ala 180	Lys	Leu	Thr	Gly	Сув 185	Ser	Arg	Glu	Asn	Glu 190	Thr	Glu	
Ile	Ile	Lys 195	Cys	Leu	Arg	Asn	Lys 200	Asp	Pro	Gln	Glu	Ile 205	Leu	Leu	Asn	
Glu	Ala 210	Phe	Val	Val	Pro	Tyr 215	Gly	Thr	Pro	Leu	Gly 220	Val	Asn	Phe	Gly	
Pro 225	Thr	Val	Asp	Gly	Asp 230	Phe	Leu	Thr	Asp	Met 235	Pro	Asp	Ile	Leu	Leu 240	
Glu	Leu	Gly	Gln	Phe 245	Lys	Lys	Thr	Gln	Ile 250	Leu	Val	Gly	Val	Asn 255	Lys	
Asp	Glu	Gly	Thr 260	Trp	Phe	Leu	Val	Gly 265	Gly	Ala	Pro	Gly	Phe 270	Ser	Lys	
Asp	Asn	Asn 275	Ser	Ile	Ile	Thr	Arg 280	Lys	Glu	Phe	Gln	Glu 285	Gly	Leu	Lys	
Ile	Phe 290	Phe	Pro	Gly	Val	Ser 295	Glu	Phe	Gly	Lys	Glu 300	Ser	Ile	Leu	Phe	
His 305	Tyr	Thr	Asp	Trp	Val 310	Asp	Asp	Gln	Arg	Pro 315	Glu	Asn	Tyr	Arg	Glu 320	
Ala	Leu	Gly	Asp	Val 325	Val	Gly	Asp	Tyr	Asn 330	Phe	Ile	Cys	Pro	Ala 335	Leu	
Glu	Phe	Thr	Lys 340	ГЛа	Phe	Ser	Glu	Trp 345	Gly	Asn	Asn	Ala	Phe 350	Phe	Tyr	
Tyr	Phe	Glu 355	His	Arg	Ser	Ser	Lys 360	Leu	Pro	Trp	Pro	Glu 365	Trp	Met	Gly	
Val	Met 370	His	Gly	Tyr	Glu	Ile 375										
<210> SEQ ID NO 23 <211> LENGTH: 966 <212> TYPE: DNA <213> ORGANISM: Artificial <220> FEATURE: <223> OTHER INFORMATION: 12-7 mutant (A199S/F227A/S287G/A328W/Y332G) BChE nucleic acid sequence for amino acid residues 117-438																
		EQUE														
								_		_	_	_			gggtt getttg	60 120
															cttcag	180
	_									_				_	_	
cggg	jctca	aaa a	aaat	acaç	yc ag	jecti	. tggt	- gga	aato	cca	aaag	ytgta	aac 1	ccct	ttgga	240

-continued

			-continued								
gaaagttccg gagca	agcttc agttagcc	ctg catttgcttt	ctcctggaag ccattcattg	300							
ttcaccagag ccati	ctgca aagtggtt	cc gctaatgctc	cttgggcggt aacatctctt	360							
tatgaagcta ggaad	cagaac gttgaact	ta gctaaattga	ctggttgctc tagagagaat	420							
gagactgaaa taato	caagtg tottagaa	aat aaagatcccc	aagaaattct tctgaatgaa	480							
gcatttgttg tccc	ctatgg gactcctt	tg ggtgtaaact	ttggtccgac cgtggatggt	540							
gattttctca ctgad	catgee agaeatat	ta cttgaacttg	gacaatttaa aaaaacccag	600							
attttggtgg gtgt	taataa agatgaag	ggg acatggtttt	tagteggtgg tgeteetgge	660							
ttcagcaaag ataa	caatag tatcataa	act agaaaagaat	ttcaggaagg tttaaaaata	720							
ttttttccag gagtq	gagtga gtttggaa	aag gaatccatcc	tttttcatta cacagactgg	780							
gtagatgatc agaga	acctga aaactacc	gt gaggeettgg	gtgatgttgt tggggattat	840							
aatttcatat gccc	gcett ggagttea	acc aagaagttct	cagaatgggg aaataatgcc	900							
tttttctact attt	gaaca ccgatcct	cc aaacttccgt	ggccagaatg gatgggagtg	960							
atgcat				966							
<210> SEQ ID NO 24 <211> LENGTH: 322 <212> TYPE: PRT <213> ORGANISM: Artificial <220> FEATURE: <223> OTHER INFORMATION: 12-7 mutant (A199S/F227A/S287G/A328W/Y332G) BChE amino acid sequence for residues 117-438											
<400> SEQUENCE:	24										
Gly Phe Gln Thr	Gly Thr Ser Se	er Leu His Val 10	Tyr Asp Gly Lys Phe 15								
Leu Ala Arg Val 20	Glu Arg Val Il	le Val Val Ser 25	Met Asn Tyr Arg Val								
Gly Ala Leu Gly 35	Phe Leu Ala Le	-	Pro Glu Ala Pro Gly 45								
Asn Met Gly Leu 50	Phe Asp Gln Gl 55	ln Leu Ala Leu	Gln Trp Val Gln Lys 60								
Asn Ile Ala Ala	Phe Gly Gly As	sn Pro Lys Ser 75	Val Thr Leu Phe Gly								
Glu Ser Ser Gly	Ala Ala Ser Va	al Ser Leu His 90	Leu Leu Ser Pro Gly 95								
Ser His Ser Leu 100	Phe Thr Arg Al	la Ile Leu Gln 105	Ser Gly Ser Ala Asn								
Ala Pro Trp Ala		eu Tyr Glu Ala	Arg Asn Arg Thr Leu								
_			Asn Glu Thr Glu Ile								
130	135		140								
Ile Lys Cys Leu 145	Arg Asn Lys As	sp Pro Gln Glu 155	Ile Leu Leu Asn Glu 160								
Ala Phe Val Val	Pro Tyr Gly Th	nr Pro Leu Gly 170	Val Asn Phe Gly Pro 175								
Thr Val Asp Gly	Asp Phe Leu Th	nr Asp Met Pro 185	Asp Ile Leu Leu Glu 190								
Leu Gly Gln Phe 195	Lys Lys Thr Gl		Gly Val Asn Lys Asp 205								

-continued

Asn Asn Ser Ile Ile Thr Arg Lys Glu Phe Gln Glu Gly Leu Lys Ile 225 230 235 Phe Phe Pro Gly Val Ser Glu Phe Gly Lys Glu Ser Ile Leu Phe His Tyr Thr Asp Trp Val Asp Asp Gln Arg Pro Glu Asn Tyr Arg Glu Ala Leu Gly Asp Val Val Gly Asp Tyr Asn Phe Ile Cys Pro Ala Leu Glu Phe Thr Lys Lys Phe Ser Glu Trp Gly Asn Asn Ala Phe Phe Tyr Tyr Phe Glu His Arg Ser Ser Lys Leu Pro Trp Pro Glu Trp Met Gly Val Met His <210> SEQ ID NO 25 <211> LENGTH: 1722 <212> TYPE: DNA <213 > ORGANISM: Artificial <220> FEATURE: <223> OTHER INFORMATION: 12-4 mutant (A199S/F227A/S287G/A328W/E441D) BChe nucleic acid sequence <400> SEOUENCE: 25 gaagatgaca tcataattgc aacaaagaat ggaaaagtca gagggatgaa cttgacagtt 60 120 tttqqtqqca cqqtaacaqc ctttcttqqa attccctatq cacaqccacc tcttqqtaqa cttcgattca aaaagccaca gtctctgacc aagtggtctg atatttggaa tgccacaaaa 180 tatgcaaatt cttgctgtca gaacatagat caaagttttc caggcttcca tggatcagag 240 atgtggaacc caaacactga cctcagtgaa gactgtttat atctaaatgt atggattcca 300 gcacctaaac caaaaaatgc cactgtattg atatggattt atggtggtgg ttttcaaact 360 ggaacatcat ctttacatgt ttatgatggc aagtttctgg ctcgggttga aagagttatt 420 gtagtgtcaa tgaactatag ggtgggtgcc ctaggattct tagctttgcc aggaaatcct 480 gaggetecag ggaacatggg tttatttgat caacagttgg etetteagtg ggtteaaaaa 540 aatatagcag cctttggtgg aaatcctaaa agtgtaactc tctttggaga aagttccgga 600 geagetteag ttageetgea tttgetttet eetggaagee atteattgtt eaceagagee 660 attctgcaaa gtggttccgc taatgctcct tgggcggtaa catctcttta tgaagctagg aacagaacgt tgaacttagc taaattgact ggttgctcta gagagaatga gactgaaata 780 atcaagtgtc ttagaaataa agatccccaa gaaattcttc tgaatgaagc atttgttgtc 840 ccctatggga ctcctttggg tgtaaacttt ggtccgaccg tggatggtga ttttctcact 900 gacatgccag acatattact tgaacttgga caatttaaaa aaacccagat tttggtgggt 960 qttaataaaq atqaaqqqac atqqttttta qtctatqqtq ctcctqqctt caqcaaaqat 1020 aacaatagta tcataactag aaaagaattt caggaaggtt taaaaatatt ttttccagga 1080 gtgagtgagt ttggaaagga atccatcctt tttcattaca cagactgggt agatgatcag agacctgaaa actaccgtga ggccttgggt gatgttgttg gggattataa tttcatatgc 1200 cctgccttgg agttcaccaa gaagttctca gaatggggaa ataatgcctt tttctactat 1260 tttqaacacc qatcctccaa acttccqtqq ccaqaatqqa tqqqaqtqat qcatqqctat 1320

gacattgaat ttgtctttgg tttacctctg gaaagaagag ataattacac aaaagccgag

qaaattttqa qtaqatccat aqtqaaacqq tqqqcaaatt ttqcaaaata tqqqaatcca

-continued												
aatgagactc agaacaatag cacaagctgg cetgtettea aaagcactga acaaaaatat 1500)											
ctaaccttga atacagagtc aacaagaata atgacgaaac tacgtgctca acaatgtcga 1560)											
ttctggacat catttttcc aaaagtcttg gaaatgacag gaaatattga tgaagcagaa 1620)											
tgggagtgga aagcaggatt ccatcgctgg aacaattaca tgatggactg gaaaaatcaa 1680)											
tttaacgatt acactagcaa gaaagaaagt tgtgtgggtc tc 1722	2											
<pre><210> SEQ ID NO 26 <211> LENGTH: 574 <212> TYPE: PRT <213> ORGANISM: Artificial <220> FEATURE: <223> OTHER INFORMATION: 12-4 mutant (A199S/F227A/S287G/A328W/E441D)</pre>												
Glu Asp Asp Ile Ile Ile Ala Thr Lys Asn Gly Lys Val Arg Gly Met 1 5 10 15												
Asn Leu Thr Val Phe Gly Gly Thr Val Thr Ala Phe Leu Gly Ile Pro 20 25 30												
Tyr Ala Gln Pro Pro Leu Gly Arg Leu Arg Phe Lys Lys Pro Gln Ser 35 40 45												
Leu Thr Lys Trp Ser Asp Ile Trp Asn Ala Thr Lys Tyr Ala Asn Ser 50 60												
Cys Cys Gln Asn Ile Asp Gln Ser Phe Pro Gly Phe His Gly Ser Glu 65 70 75 80												
Met Trp Asn Pro Asn Thr Asp Leu Ser Glu Asp Cys Leu Tyr Leu Asn 85 90 95												
Val Trp Ile Pro Ala Pro Lys Pro Lys Asn Ala Thr Val Leu Ile Trp 100 105 110												
Ile Tyr Gly Gly Gly Phe Gln Thr Gly Thr Ser Ser Leu His Val Tyr 115 120 125												
Asp Gly Lys Phe Leu Ala Arg Val Glu Arg Val Ile Val Val Ser Met 130 135 140												
Asn Tyr Arg Val Gly Ala Leu Gly Phe Leu Ala Leu Pro Gly Asn Pro 145 150 155 160												
Glu Ala Pro Gly Asn Met Gly Leu Phe Asp Gln Gln Leu Ala Leu Gln 165 170 175												
Trp Val Gln Lys Asn Ile Ala Ala Phe Gly Gly Asn Pro Lys Ser Val 180 185 190												
Thr Leu Phe Gly Glu Ser Ser Gly Ala Ala Ser Val Ser Leu His Leu 195 200 205												
Leu Ser Pro Gly Ser His Ser Leu Phe Thr Arg Ala Ile Leu Gln Ser 210 215 220												
Gly Ser Ala Asn Ala Pro Trp Ala Val Thr Ser Leu Tyr Glu Ala Arg 225 230 235 240												
Asn Arg Thr Leu Asn Leu Ala Lys Leu Thr Gly Cys Ser Arg Glu Asn 245 250 255												
Glu Thr Glu Ile Ile Lys Cys Leu Arg Asn Lys Asp Pro Gln Glu Ile 260 265 270												
Leu Leu Asn Glu Ala Phe Val Val Pro Tyr Gly Thr Pro Leu Gly Val 275 280 285												
Asn Phe Gly Pro Thr Val Asp Gly Asp Phe Leu Thr Asp Met Pro Asp 290 295 300												

Ile Leu Leu Glu Leu Gly Gln Phe Lys Lys Thr Gln Ile Leu Val Gly 305 310 315 320										
Val Asn Lys Asp Glu Gly Thr Trp Phe Leu Val Tyr Gly Ala Pro Gly 325 330 335										
Phe Ser Lys Asp Asn Asn Ser Ile Ile Thr Arg Lys Glu Phe Gln Glu 340 345 350										
Gly Leu Lys Ile Phe Phe Pro Gly Val Ser Glu Phe Gly Lys Glu Ser 355 360 365										
Ile Leu Phe His Tyr Thr Asp Trp Val Asp Asp Gln Arg Pro Glu Asn 370 375 380										
Tyr Arg Glu Ala Leu Gly Asp Val Val Gly Asp Tyr Asn Phe Ile Cys 385 390 395 400										
Pro Ala Leu Glu Phe Thr Lys Lys Phe Ser Glu Trp Gly Asn Asn Ala 405 410 415										
Phe Phe Tyr Tyr Phe Glu His Arg Ser Ser Lys Leu Pro Trp Pro Glu 420 425 430										
Trp Met Gly Val Met His Gly Tyr Asp Ile Glu Phe Val Phe Gly Leu 435 440 445										
Pro Leu Glu Arg Arg Asp Asn Tyr Thr Lys Ala Glu Glu Ile Leu Ser 450 455 460										
Arg Ser Ile Val Lys Arg Trp Ala Asn Phe Ala Lys Tyr Gly Asn Pro 465 470 475 480										
Asn Glu Thr Gln Asn Asn Ser Thr Ser Trp Pro Val Phe Lys Ser Thr 485 490 495										
Glu Gln Lys Tyr Leu Thr Leu Asn Thr Glu Ser Thr Arg Ile Met Thr 500 505 510										
Lys Leu Arg Ala Gln Gln Cys Arg Phe Trp Thr Ser Phe Phe Pro Lys 515 520 525										
Val Leu Glu Met Thr Gly Asn Ile Asp Glu Ala Glu Trp Glu Trp Lys 530 535 540										
Ala Gly Phe His Arg Trp Asn Asn Tyr Met Met Asp Trp Lys Asn Gln 545 550 555 560										
Phe Asn Asp Tyr Thr Ser Lys Lys Glu Ser Cys Val Gly Leu 565 570										
<210> SEQ ID NO 27 <211> LENGTH: 1125 <212> TYPE: DNA <213> ORGANISM: Artificial <220> FEATURE: <223> OTHER INFORMATION: 12-4 mutant (A199S/F227A/S287G/A328W/E441D) BChE nucleic acid sequence for amino acid residues 68-442 <400> SEQUENCE: 27										
aacatagatc aaagttttcc aggcttccat ggatcagaga tgtggaaccc aaacactgac	60									
ctcagtgaag actgtttata tctaaatgta tggattccag cacctaaacc aaaaaatgcc	120									
actgtattga tatggattta tggtggtggt tttcaaactg gaacatcatc tttacatgtt	180									
tatgatggca agtttctggc tcgggttgaa agagttattg tagtgtcaat gaactatagg	240									
gtgggtgccc taggattctt agctttgcca ggaaatcctg aggctccagg gaacatgggt	300									
ttatttgatc aacagttggc tcttcagtgg gttcaaaaaa atatagcagc ctttggtgga	360									
aatootaaaa gtgtaactot otttggagaa agttooggag cagottoagt tagootgoat	420									
ttgctttctc ctggaagcca ttcattgttc accagagcca ttctgcaaag tggttccgct	480									
aatgctcctt gggcggtaac atctctttat gaagctagga acagaacgtt gaacttagct	540									

gatcccaag aaattcttct gaatgaaga ctgaatata toaagtgtot tagaaataaa 600 gatccccaag aaattcttct gaatgaagac tttgttgtoc cctatgggac tcctttgggt 660 gtaaactttg gtccgaccgt ggatggtgat tttctcactg acatgccaga catattactt 720 gaacttggaa aatttaaaa aacccagat ttggtggggt ttaataaaga tgaagggaca 780 tggttttag tctatggtgc tccttggcttc agcaaagata acaatagat cataactaga 780 tggttttag tctatggtgc tcctgggttc agcaagagaa acaataagat tggaagggga 780 caacgattc aggaaggtt tagaaagaaa acaagaattc aggaagggtg 1900 ccctggggg atgttgttg gggattataat ttctacatgc ctgccttgga gttcaccaaa 780 ccctcggggg atgttgtg gggattataat ttcatatgc ctgccttgga gttcaccaaa 780 ccctcgggg cagaatggat gggagtgatg catggctatg acat 780 ccctcgggg cagaatggat gggagtgatg catggctatg acat 780 ccctcgggg cagaatggat gggagtgatg catggctatg acat 780 cccccgtgg agttcaccaaa 780 ccctcgggg cagaatggat gggagtgatg catggctatg acat 780 ccccccg 780 ccccc 780 cccccc 780 ccccccc 780 cccccc 780 cccccc 780 cccccc 780 cccccc 780 cccccc 780 ccccccc 780 ccccccccc 780 cccccccccc												
gtaaactttg gtccgaccgt ggatggtgat tttctcactg acatgccaga catattactt 720 gaacttggac aatttaaaaa aacccagatt ttggtggtg ttaataaaga tgaagggaca 780 tggtttttag tctatggtgc tcctggcttc agcaaagata acaatagtat cataactaga 840 aaagaatttc aggaaggttt aaaaatattt tttccaggag tgagtgagtt tggaaggga 900 tccatccttt ttcattacac agactggtg gatgatcaga gacctgaaaa ctaccgtgag 960 gccttgggtg atgttgttgg ggattataat ttcatatgcc ctgccttgga gttcaccaaa 1020 aagttctcag aatggggaa taatgccttt ttctactatt ttgaacaccg atcctccaaa 1080 cttccgtggc cagaatggat gggggtgatg catggctatg acatt 1125 <210 SEQ ID NO 28 <2111 LENGTH: 375 <1212 TYPE: PRT <1213 ORCANISM: Artificial <220 PENTURE: PRT <2213 ORCANISM: Artificial <220 PENTURE: PRT <2214 ORLE ABORDANISM: TYP ABORD 15 FOR ABORDANISM: Artificial <220 PENTURE: PRT <2213 ORCANISM: Artificial <220 PRO PRT 15 PRO ABORDANISM: Artificial <220 PRO PRT 16 PRO ABORDANISM: Artificial 100 PRO ABORDANISM: Artificial 100 PRO ABORDANISM: Artificial 101 PRO ABORDANISM: Artificial 102 PRO ABORDANISM: Artificial 102 PRO ABORDANISM: Artificial 103 PRO ABORDANISM: Artificial 104 105 PRO ABORDANISM: Artificial 106 PRO ABORDANISM: Artificial 107 PRO ABORDANISM: Artificial 108 PRO ABORDANISM: Artificial 109 PRO ABORDANISM: Artificial 100 PRO ABORDANISM: Artificial 1	aaattgactg gtt	gctctag ag	agaatgag	actgaaataa	tcaagtgtct	tagaaataaa 600						
gaacttggac aatttaaaaa aaccagatt ttggtggtg ttaataaaga tgaagggaca 780 tggtttttag tctatggtgc tcctggcttc agcaaagata acaatagtat cataactaga 840 aaagaatttc aggaaggttt aaaaatattt tttccaggag tgagtgagtt tggaaaggaa 900 tccatccttt ttcattacac agactggta gatgatcaga gacctgaaaa ctaccgtgag 960 gccttgggtg atgttgttgg ggattataat ttcatatgcc ctgccttgga gttcaccaaa 1020 aagttctcag aatggggaa taatgccttt ttctactatt ttgaacaccg atcctccaaa 1080 cttccgtggc cagaatggat gggagtgatg catggctatg acatt 1125 <2210 SEQ ID NO 28 <2111 LENGTH: 375 <2112 TYPE: PRT c2113 ORGANISM: Artificial c2200 PEATURE: PRT c2130 ORGANISM: Artificial c2200 PEATURE: PRT c2130 ORGANISM: Artificial c2200 PEATURE: PRT c110 SEQ UENCE: 28 Absn Ile Asp Gln Ser Phe Pro Gly Phe His Gly Ser Glu Met Trp Asn 1 5 10 15 Pro Asn Thr Asp Leu Ser Glu Asp Cye Leu Tyr Leu Asn Val Trp Ile 2 5 25 OPPO Ala Pro Lys Pro Lys Asn Ala Thr Val Leu Ile Trp Ile Tyr Gly 35 40 Pro Ala Pro Lys Pro Lys Asn Ala Thr Val Leu Ile Trp Ile Tyr Gly 45 Gly Gly Phe Gln Thr Gly Thr Ser Ser Leu His Val Tyr Asp Gly Lys 65 70 75 80 Phe Leu Ala Arg Val Glu Arg Val Ile Val Val Ser Met Asn Tyr Arg 65 70 75 80 Gly Asn Met Gly Leu Phe Asp Gln Gln Leu Ala Leu Gln Trp Val Gln 100 105 110 Lys Asn Met Gly Leu Phe Asp Gln Gln Leu Ala Leu Gln Trp Val Gln 110 105 125 Gly Glu Ser Ser Gly Ala Ala Ser Val Ser Leu His Leu Leu Ser Pro 130 135 Asn Ala Pro Trp Ala Val Thr Ser Leu Tyr Glu Ala Arg Asn Arg Thr 165 150 Asn Ala Pro Trp Ala Val Thr Ser Leu Tyr Glu Ala Arg Asn Arg Thr 165 160 Asn Ala Pro Trp Ala Val Thr Ser Leu Tyr Glu Ala Arg Asn Arg Thr 165 160 Gly Ser His Ser Leu Phe Thr Arg Ala Ile Leu Gln Ser Gly Ser Ala 145 150 125 Glu Ala Leu Ala Lys Leu Thr Gly Cys Ser Arg Glu Asn Glu Thr Glu 180 180 180 180 180 180 180 180 180 180	gatccccaag aaa	ttettet ga	atgaagca	tttgttgtco	cctatgggac	tcctttgggt 660						
tegettttag tetatggtge teetggette ageaaagata aeaatagtat cataactaga 840 aaagaattte aggaaggttt aaaaatattt tttecaggag tgagtgagtt tggaaaggaa 960 tecateettt tteattaeae agaetgggta gatgateaga gacetgaaaa etacegtgag 960 geettgggtg atgttgttgg ggattataaat tteatatgee etgeettgga gtteaceaaa 1080 aagteteag aatggggaaa taatgeettt tetactatt ttgaacaceg atceteeaaa 1080 etteegtgge cagaatggat gggagtgatg catggetatg acatt 1125 <pre> </pre> <pre> <pre> <pre> </pre> <pre> <pre> </pre> <pre> <pre> <pre></pre></pre></pre></pre></pre></pre>	gtaaactttg gtc	cgaccgt gg	atggtgat	tttctcactg	acatgccaga	catattactt 720						
aaagaattto aggaaggtti aaaaatatti titocaggag tigaggagtt tigaaaggaa 960 tocatcetti ticattacac agactgggta gatgatcaga gacctgaaaa ctaccgtgag 960 gcettgggtg atgitigting ggattataat ticatatgge cigcettgga gitcaccaa 1020 aagticicag aatggggaaa taatgcetti tictactatt tigaacaccg atcetccaaa 1080 cttccgtggc cagaatggat gggagtgatg catggctatg acatt 1125 <pre> <pre> <210 > SEQ ID NO 28 <211 > SENTH: 375 <212 > TYPE: PRT <212 > TYPE: PRT <212 > TYPE: PRT <213 > ORGANISM: Artificial <220 > FEATURE: <223 > OTHER INFORMATION: 12 - 4 mutant (Al99S/F227A/S287G/A328W/E441D)</pre></pre>	gaacttggac aat	ttaaaaa aa	cccagatt	ttggtgggtg	ttaataaaga	tgaagggaca 780						
Secretic treathacae agactgggta gatgateaga gactggaaa ctaccgtgag 1020	tggtttttag tct	atggtgc tc	ctggcttc	agcaaagata	acaatagtat	cataactaga 840						
### Secretagging and statisting grants at a treatating of clociting grants grants and process and statistics an	aaagaatttc agg	aaggttt aa	aaatattt	tttccaggag	tgagtgagtt	tggaaaggaa 900						
aagttctcag aatggggaaa taatgccttt ttctactatt ttgaacaccg atcctccaaa 1080 cttccgtggc cagaatggat gggagtgatg catggctatg acatt 1125 <pre> <210 > SEQ ID NO 28</pre>	tocatoottt tto	attacac ag	actgggta	gatgatcaga	gacctgaaaa	ctaccgtgag 960						
### Callone Caga and gaga gaga gaga gaga gaga gaga ga	gccttgggtg atg	ttgttgg gg	attataat	ttcatatgcc	ctgccttgga	gttcaccaag 1020						
<pre> 210 > SEQ ID NO 28 2211> INTPE: PRT 2212> TYPE: PRT 2212> TYPE: PRT 2213 > ORGANISM: Artificial 2220> FEATURE: 2223 > OTHER INPORMATION: 12-4 mutant (A1998/F227A/S287G/A328W/E441D)</pre>	aagttctcag aat	ggggaaa ta	atgccttt	ttctactatt	ttgaacaccg	atcctccaaa 1080						
<pre> 211> LENGTH: 375</pre>	cttccgtggc cagaatggat gggagtgatg catggctatg acatt 1125											
Asn Ile Asp Gln Ser Phe Pro Gly Phe His Gly Ser Glu Met Trp Asn 15 Pro Asn Thr Asp Leu Ser Glu Asp Cys Leu Tyr Leu Asn Val Trp Ile 25 Pro Ala Pro Lys Pro Lys Asn Ala Thr Val Leu Ile Trp Ile Tyr Gly 45 Gly Gly Phe Gln Thr Gly Thr Ser Ser Leu His Val Tyr Asp Gly Lys 60 Phe Leu Ala Arg Val Glu Arg Val Ile Val Val Ser Met Asn Tyr Arg 80 Val Gly Ala Leu Gly Phe Leu Ala Leu Pro Gly Asn Pro Glu Ala Pro 95 Gly Asn Met Gly Leu Phe Asp Gln Gln Leu Ala Leu Gln Trp Val Gln 110 Lys Asn Ile Ala Ala Phe Gly Gly Asn Pro Lys Ser Val Thr Leu Phe 115 Gly Glu Ser Ser Gly Ala Ala Ser Val Ser Leu His Leu Leu Ser Pro 130 Gly Ser His Ser Leu Phe Thr Arg Ala Ile Leu Gln Ser Gly Ser Ala 160 Asn Ala Pro Trp Ala Val Thr Ser Leu Tyr Glu Ala Arg Asn Arg Thr 165 Leu Asn Leu Ala Lys Leu Thr Gly Cys Ser Arg Glu Asn Glu Thr Glu 180 Ile Ile Lys Cys Leu Arg Asn Lys Asp Pro Gln Gln Glu Ile Leu Leu Asn 200 Pro Thr Val Asp Gly Asp Phe Leu Thr Asp Met Pro Asp Ile Leu Leu 240 Glu Leu Gly Gln Phe Lys Lys Thr Gln Ile Leu Val Gly Val Asn Lys	<211> LENGTH: 375 <212> TYPE: PRT <213> ORGANISM: Artificial <220> FEATURE: <223> OTHER INFORMATION: 12-4 mutant (A199S/F227A/S287G/A328W/E441D) BChE amino acid sequence for residues 68-442											
1	~											
20 25 30 30 Pro Ala Pro Lys Pro Lys Asn Ala Thr Val Leu Ile Trp Ile Tyr Gly Asn Ala Thr Val Leu Ile Trp Ile Tyr Gly Asn So So So So So So So S	_		Pro Gly I	-	Ser Glu Met	-						
35		p Leu Ser (Trp Ile						
Phe Leu Ala Arg Val Glu Arg Val Ile Val Val Ser Met Asn Tyr Arg 80	-	s Pro Lys I		Thr Val Leu	-	Tyr Gly						
65		_		Ser Leu His		Gly Lys						
Gly Asn Met Gly Leu Phe Asp Gln Gln Leu Ala Leu Gln Trp Val Gln 110 Lys Asn Ile Ala Ala Phe Gly Gly Asn Pro Lys Ser Val Thr Leu Phe 125 Gly Glu Ser Ser Gly Ala Ala Ser Val Ser Leu His Leu Leu Ser Pro 130 135 The Ser Leu Phe 150 155 The Ala Ala Pro Trp Ala Val Thr Ser Leu Tyr Glu Ala Arg Asn Arg Thr 165 Asn Ala Pro Trp Ala Lys Leu Thr Gly Cys Ser Arg Glu Asn Glu Thr Glu 180 185 Ser Arg Glu Asn Glu Thr Glu 190 The Asn Ala Phe Val Val Pro Tyr Gly Thr Pro Leu Gly Val Asn Phe Gly 210 Thr Val Asp Gly Asp Phe Leu Thr Asp Met Pro Asp Ile Leu Leu Leu 240 Glu Leu Gly Val Asn Lys Lys Thr Glu Ile Leu Val Gly Val Asn Lys		-	Arg Val I		Ser Met Asn							
Lys Asn Ile Ala Ala Phe Gly Gly Asn Pro Lys Ser Val Thr Leu Phe 115	Val Gly Ala Le		Leu Ala I	_	Asn Pro Glu							
Gly Glu Ser Ser Gly Ala Ala Ser Val Ser Leu His Leu Leu Ser Pro 130 135 120 140 Gly Ser His Ser Leu Phe Thr Arg Ala Ile Leu Gln Ser Gly Ser Ala 145 150 160 Asn Ala Pro Trp Ala Val Thr Ser Leu Tyr Glu Ala Arg Asn Arg Thr 165 170 170 175 Leu Asn Leu Ala Lys Leu Thr Gly Cys Ser Arg Glu Asn Glu Thr Glu 180 185 185 Pro Gln Glu Ile Leu Leu Asn 195 200 Pro Gln Glu Ile Leu Leu Asn 205 Glu Ala Phe Val Val Pro Tyr Gly Thr Pro Leu Gly Val Asn Phe Gly 210 Thr Val Asp Gly Asp Phe Leu Thr Asp Met Pro Asp Ile Leu Leu 225 Glu Leu Gly Gln Phe Lys Lys Thr Gln Ile Leu Val Gly Val Asn Lys	-		_		_							
Gly Ser His Ser Leu Phe Thr Arg Ala Ile Leu Gln Ser Gly Ser Ala 145		a Ala Phe (Asn Pro Lys		Leu Phe						
Asn Ala Pro Trp Ala Val Thr Ser Leu Tyr Glu Ala Arg Asn Arg Thr 165 Leu Asn Leu Ala Lys Leu Thr Gly Cys Ser Arg Glu Asn Glu Thr Glu 180 Ile Ile Lys Cys Leu Arg Asn Lys Asp Pro Gln Glu Ile Leu Leu Asn 195 Glu Ala Phe Val Val Pro Tyr Gly Thr Pro Leu Gly Val Asn Phe Gly 210 Pro Thr Val Asp Gly Asp Phe Leu Thr Asp Met Pro Asp Ile Leu Leu 240 Glu Leu Gly Gln Phe Lys Lys Thr Gln Ile Leu Val Gly Val Asn Lys		_		Val Ser Leu		. Ser Pro						
Leu Asn Leu Ala Lys Leu Thr Gly Cys Ser Arg Glu Asn Glu Thr Glu 180	-		Thr Arg A		_							
Ile Ile Lys Cys Leu Arg Asn Lys Asp Pro Gln Glu Ile Leu Leu Asn 195 Glu Ala Phe Val Val Pro Tyr Gly Thr Pro Leu Gly Val Asn Phe Gly 210 Pro Thr Val Asp Gly Asp Phe Leu Thr Asp Met Pro Asp Ile Leu Leu 225 Glu Leu Gly Gln Phe Lys Lys Thr Gln Ile Leu Val Gly Val Asn Lys	Asn Ala Pro Tr		Thr Ser I		. Ala Arg Asn	-						
Glu Ala Phe Val Val Pro Tyr Gly Thr Pro Leu Gly Val Asn Phe Gly 210 215 220 Pro Thr Val Asp Gly Asp Phe Leu Thr Asp Met Pro Asp Ile Leu Leu 225 230 235 240 Glu Leu Gly Gln Phe Lys Lys Thr Gln Ile Leu Val Gly Val Asn Lys		-	_	-								
Pro Thr Val Asp Gly Asp Phe Leu Thr Asp Met Pro Asp Ile Leu Leu 225 230 235 240 Glu Leu Gly Gln Phe Lys Lys Thr Gln Ile Leu Val Gly Val Asn Lys		s Leu Arg 2	-	Asp Pro Glr		. Leu Asn						
225 230 235 240 Glu Leu Gly Gln Phe Lys Lys Thr Gln Ile Leu Val Gly Val Asn Lys			_	Thr Pro Leu	_	Phe Gly						
			Phe Leu :									
	Glu Leu Gly Gl:	_	Lys Thr (. Val Gly Val	-						

```
Asp Glu Gly Thr Trp Phe Leu Val Tyr Gly Ala Pro Gly Phe Ser Lys
           260
Asp Asn Asn Ser Ile Ile Thr Arg Lys Glu Phe Gln Glu Gly Leu Lys
Ile Phe Phe Pro Gly Val Ser Glu Phe Gly Lys Glu Ser Ile Leu Phe
                        295
His Tyr Thr Asp Trp Val Asp Asp Gln Arg Pro Glu Asn Tyr Arg Glu
Ala Leu Gly Asp Val Val Gly Asp Tyr Asn Phe Ile Cys Pro Ala Leu
Glu Phe Thr Lys Lys Phe Ser Glu Trp Gly Asn Asn Ala Phe Phe Tyr
Tyr Phe Glu His Arg Ser Ser Lys Leu Pro Trp Pro Glu Trp Met Gly
Val Met His Gly Tyr Asp Ile
<210> SEQ ID NO 29
<211> LENGTH: 975
<212> TYPE: DNA
<213 > ORGANISM: Artificial
<220> FEATURE:
<223 > OTHER INFORMATION: 12-4 mutant (A199S/F227A/S287G/A328W/E441D)
     BChE nucleic acid sequence for amino acid residues 117-441
<400> SEQUENCE: 29
ggttttcaaa ctggaacatc atctttacat gtttatgatg gcaagtttct ggctcgggtt
                                                                      60
gaaagagtta ttgtagtgtc aatgaactat agggtgggtg ccctaggatt cttagctttg
ccaqqaaatc ctqaqqctcc aqqqaacatq qqtttatttq atcaacaqtt qqctcttcaq
                                                                     180
tgggttcaaa aaaatatagc agcctttggt ggaaatccta aaagtgtaac tctctttgga
                                                                     240
gaaagttccg gagcagcttc agttagcctg catttgcttt ctcctggaag ccattcattg
ttcaccagag ccattctgca aagtggttcc gctaatgctc cttgggcggt aacatctctt
                                                                     360
tatgaagcta ggaacagaac gttgaactta gctaaattga ctggttgctc tagagagaat
qaqactqaaa taatcaaqtq tcttaqaaat aaaqatcccc aaqaaattct tctqaatqaa
                                                                     480
gcatttgttg tcccctatgg gactcctttg ggtgtaaact ttggtccgac cgtggatggt
qattttctca ctqacatqcc aqacatatta cttqaacttq qacaatttaa aaaaacccaq
                                                                     600
attttggtgg gtgttaataa agatgaaggg acatggtttt tagtctatgg tgctcctggc
                                                                     660
ttcagcaaag ataacaatag tatcataact agaaaagaat ttcaggaagg tttaaaaaata
                                                                     720
ttttttccag gagtgagtga gtttggaaag gaatccatcc tttttcatta cacagactgg
                                                                     780
gtagatgatc agagacctga aaactaccgt gaggccttgg gtgatgttgt tggggattat
aatttcatat gccctgcctt ggagttcacc aagaagttct cagaatgggg aaataatgcc
                                                                     900
tttttctact attttgaaca ccgatcctcc aaacttccgt ggccagaatg gatgggagtg
                                                                     960
                                                                     975
atgcatggct atgac
<210> SEQ ID NO 30
<211> LENGTH: 325
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223 > OTHER INFORMATION: 12-4 mutant (A199S/F227A/S287G/A328W/E441D)
```

BChE amino acid sequence for residues 117-441

-continued

_															
< 40	O> SI	EQUEI	ICE :	30											
Gly 1	Phe	Gln	Thr	Gly 5	Thr	Ser	Ser	Leu	His 10	Val	Tyr	Asp	Gly	Lys 15	Phe
Leu	Ala	Arg	Val 20	Glu	Arg	Val	Ile	Val 25	Val	Ser	Met	Asn	Tyr 30	Arg	Val
Gly	Ala	Leu 35	Gly	Phe	Leu	Ala	Leu 40	Pro	Gly	Asn	Pro	Glu 45	Ala	Pro	Gly
Asn	Met 50	Gly	Leu	Phe	Asp	Gln 55	Gln	Leu	Ala	Leu	Gln 60	Trp	Val	Gln	Lys
Asn 65	Ile	Ala	Ala	Phe	Gly 70	Gly	Asn	Pro	Lys	Ser 75	Val	Thr	Leu	Phe	Gly 80
Glu	Ser	Ser	Gly	Ala 85	Ala	Ser	Val	Ser	Leu 90	His	Leu	Leu	Ser	Pro 95	Gly
Ser	His	Ser	Leu 100	Phe	Thr	Arg	Ala	Ile 105	Leu	Gln	Ser	Gly	Ser 110	Ala	Asn
Ala	Pro	Trp 115	Ala	Val	Thr	Ser	Leu 120	Tyr	Glu	Ala	Arg	Asn 125	Arg	Thr	Leu
Asn	Leu 130	Ala	Lys	Leu	Thr	Gly 135	Cys	Ser	Arg	Glu	Asn 140	Glu	Thr	Glu	Ile
Ile 145	ГЛа	CÀa	Leu	Arg	Asn 150	Lys	Asp	Pro	Gln	Glu 155	Ile	Leu	Leu	Asn	Glu 160
Ala	Phe	Val	Val	Pro 165	Tyr	Gly	Thr	Pro	Leu 170	Gly	Val	Asn	Phe	Gly 175	Pro
Thr	Val	Asp	Gly 180	Asp	Phe	Leu	Thr	Asp 185	Met	Pro	Asp	Ile	Leu 190	Leu	Glu
Leu	Gly	Gln 195	Phe	Lys	Lys	Thr	Gln 200	Ile	Leu	Val	Gly	Val 205	Asn	ГÀа	Asp
Glu	Gly 210	Thr	Trp	Phe	Leu	Val 215	Tyr	Gly	Ala	Pro	Gly 220	Phe	Ser	ГЛа	Asp
Asn 225	Asn	Ser	Ile	Ile	Thr 230	Arg	ГÀа	Glu	Phe	Gln 235	Glu	Gly	Leu	ГЛа	Ile 240
Phe	Phe	Pro	Gly	Val 245	Ser	Glu	Phe	Gly	Lys 250	Glu	Ser	Ile	Leu	Phe 255	His
Tyr	Thr	Asp	Trp 260	Val	Asp	Asp	Gln	Arg 265	Pro	Glu	Asn	Tyr	Arg 270	Glu	Ala
Leu	Gly	Asp 275	Val	Val	Gly	Asp	Tyr 280	Asn	Phe	Ile	СЛа	Pro 285	Ala	Leu	Glu
Phe	Thr 290	Lys	Lys	Phe	Ser	Glu 295	Trp	Gly	Asn	Asn	Ala 300	Phe	Phe	Tyr	Tyr
Phe 305	Glu	His	Arg	Ser	Ser 310	ГЛа	Leu	Pro	Trp	Pro 315	Glu	Trp	Met	Gly	Val 320
Met	His	Gly	Tyr	Asp 325											

What is claimed is:

- 1. A nucleic acid molecule comprising a nucleic acid sequence which encodes a butyrylcholinesterase variant peptide, said nucleic acid sequence consisting of SEQ ID NO: 25. $_{60}$
- 2. A nucleic acid molecule comprising a nucleic acid sequence which encodes a butyrylcholinesterase variant peptide comprising the amino acid sequence selected from the group consisting of SEQ ID NOS: 2, 4, 6, 8, 10, 12, 16, 18, 22, 24, 26, 28, and 30.
- 3. A method of treating a cocaine-induced condition comprising administering to an individual an effective amount of the nucleic acid molecule of claim 1 to lower blood cocaine concentration.
- **4**. A method of treating a cocaine-induced condition comprising administering to an individual an effective amount of the nucleic acid molecule of claim **2** to lower blood cocaine concentration.

* * * * *