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On the active site for hydrolysis of aryl amides and choline esters by human cholinesterases

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Abstract—Cholinesterases, in addition to their well-known esterase action, also show an aryl acylamidase (AAA) activity whereby they catalyze the hydrolysis of amides of certain aromatic amines. The biological function of this catalysis is not known. Furthermore, it is not known whether the esterase catalytic site is involved in the AAA activity of cholinesterases. It has been speculated that the AAA activity, especially that of butyrylcholinesterase (BuChE), may be important in the development of the nervous system and in pathological processes such as formation of neuritic plaques in Alzheimer's disease (AD). The substrate generally used to study the AAA activity of cholinesterases is *N*-(2-nitrophenyl)acetamide. However, use of this substrate requires high concentrations of enzyme and substrate, and prolonged periods of incubation at elevated temperature. As a consequence, difficulties in performing kinetic analysis of AAA activity associated with cholinesterases have hampered understanding this activity. Because of its potential biological importance, we sought to develop a more efficient and specific substrate for use in studying the AAA activity associated with BuChE, and for exploring the catalytic site for this hydrolysis. Here, we describe the structure–activity relationships for hydrolysis of anilides by cholinesterases. These studies led to a substrate, *N*-(2-nitrophenyl)trifluoroacetamide, that was hydrolyzed several orders of magnitude faster than *N*-(2-nitrophenyl)acetamide by cholinesterases. Also, larger *N*-(2-nitrophenyl)alkylamides were found to be more rapidly hydrolyzed by BuChE than *N*-(2-nitrophenyl)acetamide and, in addition, were more specific for hydrolysis by BuChE. Thus, *N*-(2-nitrophenyl)alkylamides with six to eight carbon atoms in the acyl group represent suitable specific substrates to investigate further the function of the AAA activity of BuChE. Based on the substrate structure–activity relationships and kinetic studies, the hydrolysis of anilides and esters of choline appears to utilize the same catalytic site in BuChE.

1. Introduction

Acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (BuChE, EC 3.1.1.8), in addition to their well-known esterase action,[1,2] also have an aryl acylamidase (AAA) activity whereby they catalyze the hydrolysis of amides of certain aromatic amines.[3] The biological function of this catalysis is not known. However, fluctuations of this enzyme activity over the course of the development of the nervous system have led to speculation that the AAA activity of cholinesterases, especially that of BuChE, is involved in this process.[4,5] The AAA activity of BuChE has also been implicated in the pathology of Alzheimer's disease (AD). In AD, there is a significant increase in the levels of BuChE in the brain and this enzyme activity has been detected in all the neuropathological hallmarks of this disorder, including neuritic plaques and neurofibrillary tangles.[6–13]

Enzymes from a variety of animal sources have been found to exhibit the ability to hydrolyze acid derivatives of aromatic amines (anilides) such as acetanilide, first reported by Mirkowski in 1909 (cited in Ref. 14) and sulfa drugs.[15] Detection of this aryl acylamidase activity (AAA, EC 3.1.5.13) was facilitated by the use of the chromogenic substrate, *N*-(2-nitrophenyl)acetamide.[16] Using this substrate, it was later shown that AAA activity could be detected in mammalian brain and that this

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hydrolytic activity was associated with AChE,[3,17] the enzyme known to catalyze the breakdown of the neurotransmitter acetylcholine.[1,2] Subsequently, BuChE was also shown to have this ability to hydrolyze anilides.[18]

Within the context of multiple catalytic activities of cholinesterases, it is unclear whether the esterase and AAA activities utilize the same active site. However, it has been suggested that the AAA catalytic site overlaps with the esteratic site in cholinesterases.[19,20] Given the possible biological significance of the AAA activity of BuChE, further study of this hydrolytic activity was undertaken.

The most commonly used substrate for examining the AAA activity is *N*-(2-nitrophenyl)acetamide (**2**) (Table 1).[16] The major limitation in the use of this compound as a cholinesterase substrate is that the reaction is slow, especially for BuChE, and requires high concentrations of enzyme and substrate, relative to what is required for monitoring esterase activity.[21] Furthermore, measurement of enzymatic activity is difficult with this compound because it absorbs strongly in the same region of the visible spectrum as the product, 2-nitroaniline, the formation of which is used to monitor the catalytic hydrolysis of the substrate by cholinesterases.

The present study was undertaken to assess a series of analogues of *N*-(2-nitrophenyl)acetamide with a view to exploring the requirements for hydrolysis and for obtaining more efficient and enzyme-specific substrates for studying aryl acylamidase activity of BuChE. Through structure–activity explorations involving kinetic studies, calculations of molecular parameters, and NMR analysis, a number of substrate features are identified that are essential for, or facilitate hydrolysis by, aryl acylamidase-type hydrolysis by cholinesterases.

Here we show that longer acyl chain anilides are more efficient substrates for the AAA activity of BuChE than is *N*-(2-nitrophenyl)acetamide (**2**). Furthermore, based on kinetic studies and the comparison of anilide hydrolysis with that of analogous long chain thioesters of choline, we suggest that the hydrolysis of acyl anilides and esters of choline occurs at the same catalytic site.

2. Results and discussion

A total of 22 compounds (Table 1) were examined as potential substrates for hydrolysis by aryl acylamidase activity of AChE, BuChE, and the enzyme aryl acylamidase from *Pseudomonas fluorescens* (AAA_{Pf}, EC 3.1.1.13) (Table 2).

2.1. Synthetic chemistry

2.1.1. Anilide derivatives. Compounds **1**, **2**, and **4** were purchased from commercial sources and the rest were synthesized by standard methods (see Section 4). Physical data for the synthetic compounds corresponded well with the published data (where available), and the spectral data were consistent with the structures of the analogues.

2.1.2. Thiocholine derivatives. All thiocholine derivatives used in this study have been described before.[2,31] Acetylthiocholine (**I**), propionylthiocholine (**II**), and butyrylthiocholine (**III**) were purchased commercially. Octanoylthiocholine (**IV**) was synthesized according to a published procedure,[31] and the spectral data were consistent with the structure.

2.2. General evaluation of anilide substrates

All compounds were first monitored for susceptibility to hydrolysis by doing repetitive UV–vis scans in the presence of either BuChE, AChE or AAA_{Pf} for up to 7 h. Since one of the main objectives of this study was to obtain a more efficient anilide substrate for BuChE, such repetitive scans were able to show which substrates were more rapidly hydrolyzed than the substrate commonly used, namely, *N*-(2-nitrophenyl)acetamide (**2**). For example, Figure 1 shows two repetitive scans, in the presence of BuChE, of *N*-(2-nitrophenyl)acetamide (**2**) (Fig. 1a) and *N*-(2-nitrophenyl)hexanamide (**12**) (Fig. 1b). These scans indicate that compound (**12**) is hydrolyzed 50 times more rapidly by BuChE than (**2**).

Of the 22 compounds tested (Table 2), twelve compounds were hydrolyzed by BuChE (**2**, **7**–**14**, **16**, **18**, and **22**), eight were hydrolyzed by AChE (**2**, **8**, **9**, **11**, **13**, **14**, **16**, and **22**) and twelve by AAA_{Pf} (**1**–**5**, **7**–**12**, and **22**). Five compounds (**6**, **15**, **17**, **19**, and **21**) were not hydrolyzed by any of these enzymes.

2.2.1. The effect of the 2-nitro group of the aniline ring. The role of the nitro group in providing a chromophore for the spectrophotometric detection of the AAA activity of cholinesterases is well established.[3,18] However, its function in affecting the hydrolysis of the aryl amide bond is unknown. As summarized in Table 2, no hydrolysis by cholinesterases was observed, even after 7 h of exposure to AChE or BuChE, for compounds such as acetanilide (**1**), benzanilide (**17**), and *N*-phenyltrifluoroacetamide (**21**), compounds that do not have a nitro group on the aniline ring.

When the nitro group is present in position 2 on the aniline ring, as in compounds (**2**, **18**, and **22**), hydrolysis of these compounds by at least one of the cholinesterases occurred. This implies a more important function for the nitro group in influencing the hydrolysis of the amide bond, beyond simply increasing the sensitivity for detection of anilide hydrolysis.

To determine whether the position of the nitro group in the aniline ring is important in promoting the hydrolysis of the amide bond, compounds (**3**) and (**4**) with the nitro group in 3- or 4-position, were compared with the isomeric lead compound **2**. Changing the position of the nitro group from the 2-position led to loss of hydrolysis by BuChE and AChE, although AAA_{Pf} hydrolysis was enhanced (Table 2). These results indicate that a nitro group in the 2-position of the aniline ring is important

Table 1. Structures of anilides tested

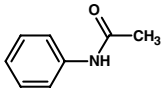
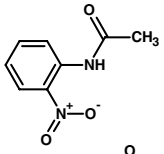
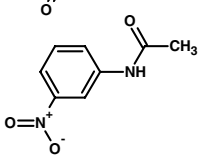
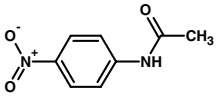
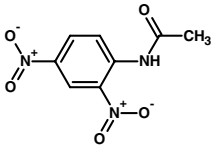
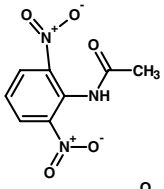
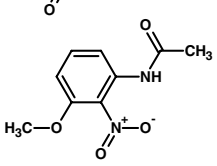
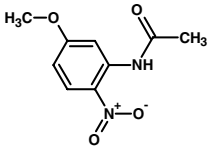
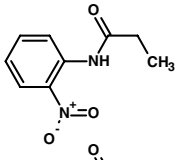
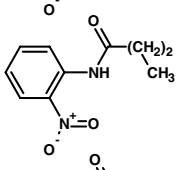
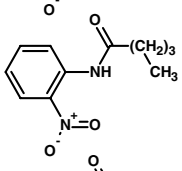
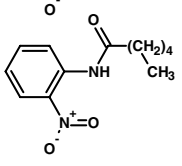
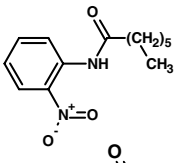
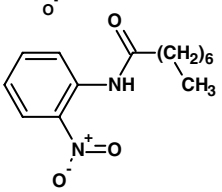
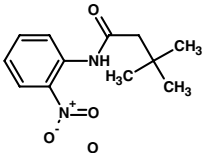
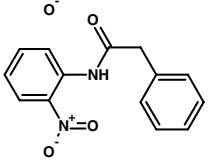
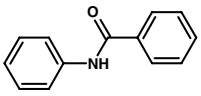
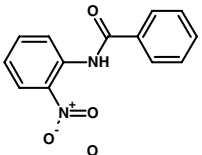
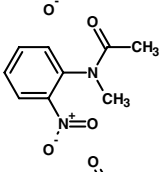
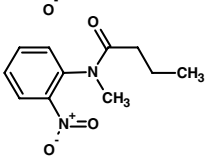
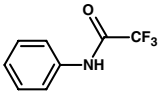
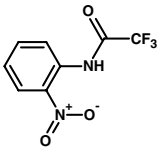
Compound	Name	Structure
1	*N*-Phenylacetamide	
2	*N*-(2-Nitrophenyl)acetamide	
3	*N*-(3-Nitrophenyl)acetamide	
4	*N*-(4-Nitrophenyl)acetamide	
5	*N*-(2,4-Dinitrophenyl)acetamide	
6	*N*-(2,6-Dinitrophenyl)acetamide	
7	*N*-(3-Methoxy-2-nitrophenyl)acetamide	
8	*N*-(5-Methoxy-2-nitrophenyl)acetamide	
9	*N*-(2-Nitrophenyl)propanamide	
10	*N*-(2-Nitrophenyl)butanamide	
11	*N*-(2-Nitrophenyl)pentanamide	
12	*N*-(2-Nitrophenyl)hexanamide	

Table 1 (*continued*)

Compound	Name	Structure
13	*N*-(2-Nitrophenyl)heptanamide	
14	*N*-(2-Nitrophenyl)octanamide	
15	*N*-(2-Nitrophenyl)-3,3-dimethylbutanamide	
16	*N*-(2-Nitrophenyl)phenylacetamide	
17	*N*-Phenylbenzamide	
18	*N*-(2-Nitrophenyl)benzamide	
19	*N*-Methyl-*N*-(2-nitrophenyl)acetamide	
20	*N*-Methyl-*N*-(2-nitrophenyl)butanamide	
21	*N*-Phenyltrifluoroacetamide	
22	*N*-(2-Nitrophenyl)trifluoroacetamide	

for the hydrolysis of anilides by cholinesterases. A nitro group in either the 2- or 4-position has an electron-withdrawing effect on the benzene ring.[32] Therefore, the action of the 2-nitro group in promoting anilide hydrolysis by cholinesterases is thus probably not an electron-withdrawing one since the 4-nitro analogue (**4**), which is electronically similar to compound **2**, is not hydrolyzed (Table 2). Instead, these results suggest a more direct involvement of the 2-nitro group in promoting cholinesterase-catalyzed anilide hydrolysis. This direct effect may be through the formation of a six-membered cyclic species, involving intramolecular hydrogen bonding, between the oxygen of the nitro group and the hydrogen of the amide moiety (Fig. 2). Hydrogen bonding in 2-nitroanilides was suggested earlier, based on spectrophotometric studies.[33–35] We postulated that

Table 2. Initial rates (ΔA/h) of hydrolysis of selected acyl anilides by human butyrylcholinesterase (BuChE), acetylcholinesterase (AChE), and aryl acylamidase from *Pseudomonas fluorescens* (AAA_{Pf})

Compound	BuChE	AChE	AAA
1	X	X	1.0341
2	0.1101	0.2540	1.3835
3	X	X	0.3150
4	X	X	6.2998
5	X	X	9.7167
6	X	X	X
7	0.0133	X	0.2310
8	0.0746	0.3819	0.0480
9	0.0995	0.0692	0.8070
10	0.9124	X	0.3749
11	2.9244	0.0226	0.3666
12	4.8789	X	0.1491
13	0.5499[a]	0.0074[a]	X
14	1.4312[a]	0.0631[a]	X
15	X	X	X
16	0.1136	0.0184	X
17	X	X	X
18	0.0241	X	X
19	X	X	X
20	X	X	X
21	X	X	X
22	14.3079	0.9445	0.2823

All assays carried out at 430 nm except for **1** (280 nm), **3** (360 nm), and **5** (340 nm).

X: No observed hydrolysis of substrate. Assays contained 10 units (0.16 nm) of BuChE, or 10 units (0.07 nm) AChE or 0.125 units AAA_{Pf}.

[a] Initial rates of hydrolysis determined at 3.33×10^{-5} M due to solubility. All other substrates were 3.33×10^{-4} M.

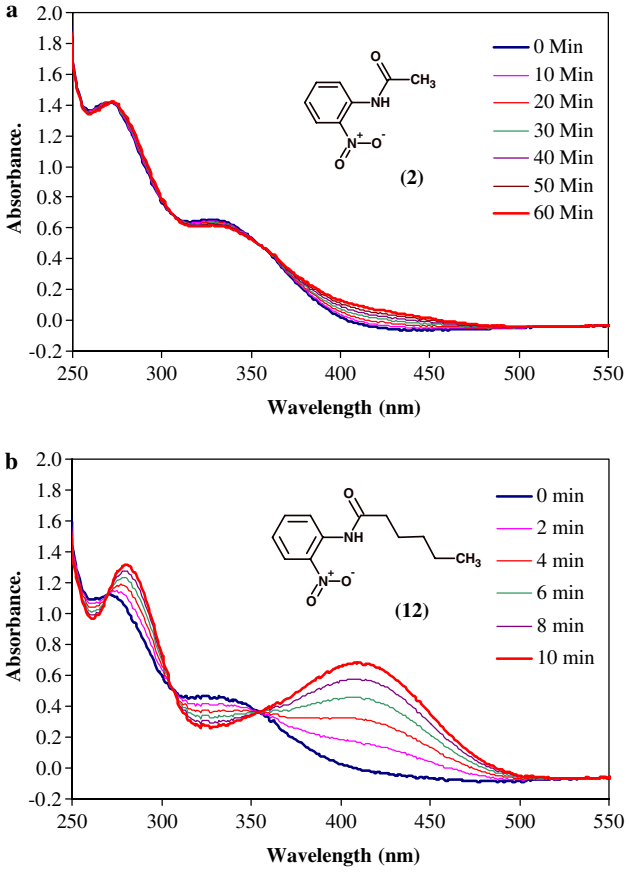


Figure 1. Repetitive scans using 3.33×10^{-4} M of: (a) *N*-(2-nitrophenyl)acetamide (**2**) (total time 60 min) and (b) *N*-(2-nitrophenyl)hexanamide (**12**) (total time 10 min), as substrate for BuChE.

this intramolecular hydrogen bond could facilitate hydrolysis of the amide bond.

To test further the contribution of hydrogen bonding in facilitating hydrolysis, two analogues without the amide hydrogen were prepared and examined. Replacement of the hydrogen on the nitrogen of the amide with a methyl group, as in *N*-methyl-*N*-(2-nitrophenyl)acetamide (**19**) and *N*-methyl-*N*-(2-nitrophenyl)butanamide (**20**), compounds that are unable to form the intramolecular hydrogen bond, led to complete loss of hydrolysis (Table 2).

In an attempt to strengthen the hydrogen bond and facilitate hydrolysis, compounds with an additional nitro group in the 4-position, as in *N*-(2,4-dinitrophenyl)acetamide (**5**), or 6-position, as in *N*-(2,6-dinitrophenyl)acetamide (**6**), were synthesized and examined. Neither of these compounds was hydrolyzed by cholinesterases (Table 2). Another approach was to add an electron-donating group at the 3-position as in *N*-(3-methoxy-2-nitrophenyl)acetamide (**7**), or 5-position, as in *N*-(5-methoxy-2-nitrophenyl)acetamide (**8**). Once again, neither of these compounds showed significant improvement in hydrolysis by cholinesterases over the lead compound, **2**. These observations suggest that the presence of additional functional groups on the aniline ring may disrupt the formation of the hydrogen bond or alter other molecular parameters that may be important in the hydrolytic process. In an effort to clarify these

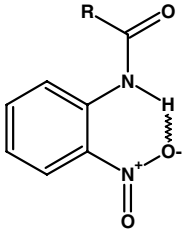


Figure 2. Hydrogen bonding in *N*-2-nitroanilides.

questions, ^{1}H NMR analyses and molecular calculation studies were undertaken.

2.2.2. NMR analysis. Comparison of the ^{1}H NMR spectra of the three isomeric substrates, *N*-(2-nitrophenyl)acetamide (**2**), *N*-(3-nitrophenyl)acetamide (**3**), and *N*-(4-nitrophenyl)acetamide (**4**), in dilute $CDCl_3$ solution revealed a unique feature of the presence of a nitro group in the 2-position. Each spectrum contained a broad singlet that integrated for one proton and was assigned to the N–H proton of the amide moiety. The position of this signal was first established for the parent compound *N*-phenylacetamide (**1**). The signal for this proton appeared at δ 7.47 for this compound (Table 3). The signal for the amide proton appeared in a comparable position for the *N*-(3-nitrophenyl)acetamide (**3**)

Table 3. Physical parameters of compounds examined as potential substrates for aryl acylamidase activity of butyrylcholinesterase

Compound	Name	Chem shift N–H δ (ppm)	H-bond distance (Å)	Atomic charges	
				Amide-N	Amide-H
1	*N*-Phenylacetamide	7.47	NA	−0.709	+0.328
2	*N*-(2-Nitrophenyl)acetamide	10.32	2.63	−0.736	+0.385
3	*N*-(3-Nitrophenyl)acetamide	7.44	NA	−0.710	+0.337
4	*N*-(4-Nitrophenyl)acetamide	7.42	NA	−0.718	+0.345
5	*N*-(2,4-Dinitrophenyl)acetamide	10.64	2.63	−0.741	+0.390
6	*N*-(2,6-Dinitrophenyl)acetamide	9.48	2.65	−0.714	+0.380
7	*N*-(3-Methoxy-2-nitrophenyl)acetamide	8.27	2.67	−0.740	+0.378
8	*N*-(5-Methoxy-2-nitrophenyl)acetamide	10.76	2.63	−0.738	+0.385
9	*N*-(2-Nitrophenyl)propanamide	10.37	2.63	−0.755	+0.386
10	*N*-(2-Nitrophenyl)butanamide	10.36	2.63	−0.758	+0.386
11	*N*-(2-Nitrophenyl)pentanamide	10.36	2.63	−0.758	+0.386
12	*N*-(2-Nitrophenyl)hexanamide	10.36	2.63	−0.759	+0.386
13	*N*-(2-Nitrophenyl)heptanamide	10.36	2.63	−0.759	+0.386
14	*N*-(2-Nitrophenyl)octanamide	10.36	2.62	−0.759	+0.386
15	*N*-(2-Nitrophenyl)-3,3-dimethylbutanamide	10.31	2.63	−0.752	+0.390
16	*N*-(2-Nitrophenyl)phenylacetamide	10.25	2.63	−0.743	+0.388
17	*N*-Phenylbenzamide	7.85	NA	−0.737	+0.333
18	*N*-(2-Nitrophenyl)benzamide	11.34	2.63	−0.784	+0.403
19	*N*-Methyl-*N*-(2-nitrophenyl)acetamide	NA	NA	−0.505	NA
20	*N*-Methyl-*N*-(2-nitrophenyl)butanamide	NA	NA	−0.519	NA
21	*N*-Phenyltrifluoroacetamide	8.21	NA	−0.723	+0.353
22	*N*-(2-Nitrophenyl)trifluoroacetamide	11.38	2.62	−0.762	+0.406

and *N*-(4-nitrophenyl)acetamide (**4**) (δ7.44 and 7.42 ppm, respectively). However, in *N*-2-nitrophenylacetamide (**2**), this signal appeared much further downfield at δ 10.32 ppm (Table 3). This downfield shift of δ 2.9 ppm for the N–H proton in compound **2** is interpreted as indicating the presence of a deshielding intramolecular hydrogen bond in this isomer, which cannot be present in either 3- or 4-nitro isomers or in *N*-phenylacetamide (**1**). These ^{1}H NMR analyses support the notion that intramolecular hydrogen bonding (Fig. 2) is an important feature of 2-nitroanilides that facilitates their hydrolysis by cholinesterases (Table 2). However, the observation that *N*-(2,4-dinitrophenyl)acetamide (**5**), *N*-(2,6-dinitrophenyl)acetamide (**6**), and *N*-(2-nitrophenyl)-3,3-dimethylbutanamide (**15**), all show the downfield N–H shift indicative of hydrogen bonding (Table 3), but are not readily hydrolyzed by cholinesterases (Table 2), suggests that other factors, in addition to hydrogen bonding, must also be important in determining whether substrate hydrolysis will occur.

2.2.3. Computational analysis. To explore the possible contribution of molecular parameters, such as preferred conformation, atomic distances, and atomic charges, to the facilitation of anilide hydrolysis, computational analyses were carried out.

Geometry optimization of the molecules involved obtaining the lowest energy conformer, first using force-field methods (PC Spartan Pro, Wavefunction, Inc., 18401 Von Karman, Suite 370, Irvine, CA 92612), ultimately led to an optimized geometry at the higher B3LYP/6-31G(d) level of theory.[36–38]

Figure 3a shows optimized geometry of one of the substrates, *N*-(2-nitrophenyl)acetamide (**2**), that is hydrolyzed by cholinesterases. As is shown in Figure 3a, the 2-nitro group oxygen atom and the amide N–H of compound **2** are directed toward each other in the proper orientation to form an intramolecular hydrogen bond.

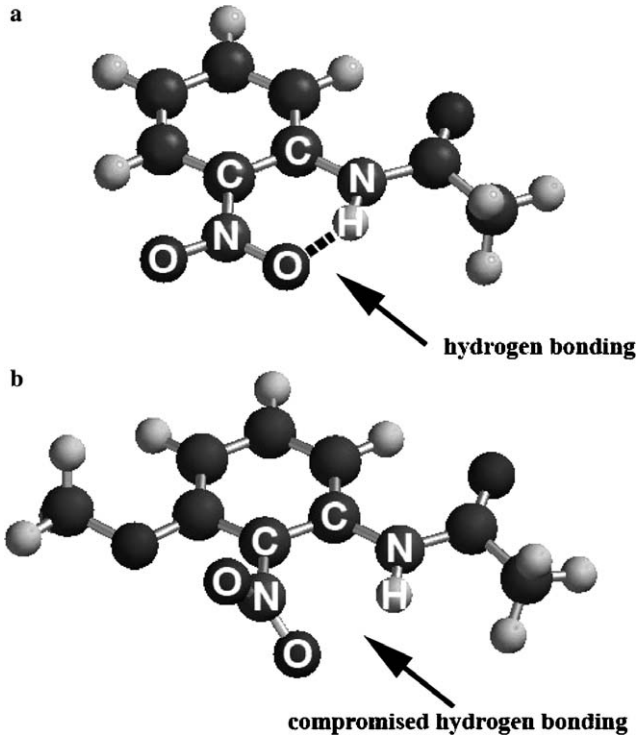


Figure 3. Calculated optimal geometries of anilide derivatives: (a) *N*-(2-nitrophenyl)acetamide (**2**) showing hydrogen bonding with all relevant atoms in plane and (b) *N*-(3-methoxy-2-nitrophenyl)acetamide (**7**) with the nitro oxygen out of plane (52.51°) and concomitant weakening of hydrogen bonding.

It is significant to note that the most common occurrence of intramolecular hydrogen bonding arises when the H-bonded species can form a six-membered ring, in which the hydrogen atom is one of the six atoms.[32] As can be seen for the representative compound in Figure 3a, the 2-nitroanilides that are hydrolyzed by cholinesterases adopt a conformation such that, with the hydrogen bond, a planar, six-membered ring is produced. The preferred conformation of *N*-(3-methoxy-2-nitrophenyl)acetamide (**7**) indicates displacement of the nitro group oxygen atom out of the plane of the amide hydrogen (Fig. 3b). This compound is not readily hydrolyzed by cholinesterases. In fact, the rate of hydrolysis of compound (**7**) by BuChE is much slower than that of the lead compound (**2**), and it is not hydrolyzed by AChE. The resulting weaker hydrogen bonding can be considered a factor that adversely affects hydrolysis of **7** by AChE and BuChE (Tables 2 and 3).

For optimum hydrogen bond interactions between an amide nitrogen and an oxygen atom, the donor (N) and acceptor (O) atoms should be at a distance from each other not greater than 2.9Å.[39] As the results in Table 3 indicate, the hydrogen bond distances for the compounds that are hydrolyzed by AChE and BuChE range between 2.61 and 2.63 Å, which are well within the range for hydrogen bonding to take place. However, there are exceptions. For example, compounds **5**, **6**, **15**, and **18** are not hydrolyzed despite optimal hydrogen bond distances. This suggests that intramolecular hydrogen bonding alone is not sufficient to effect hydrolysis by cholinesterases.

Other parameters such as atomic charges on the atoms in the vicinity of the amide bond were also considered as potential indicators of the ability of particular substrates to undergo hydrolysis. Calculated atomic charges for the amide nitrogen and amide hydrogen are summarized in Table 3. These calculated atomic charges, like the hydrogen bond distance determinations, did not provide a clear explanation of the patterns for substrate hydrolysis noted in Table 2. However, if one assumes that increasing the electron density on the amide nitrogen should facilitate proton transfer to this nitrogen, to create the amino leaving group, then some observations can be made with respect to this parameter in the hydrolytic process. For example, *N*-phenylacetamide (**1**), not hydrolyzed by cholinesterases, has an atomic charge on the amide nitrogen of −0.709, while that for the lead compound, *N*-(2-nitrophenyl)acetamide (**2**), is more electron-rich at −0.736 (Table 3). The 3- and 4-nitro isomers, that is, compounds **3** and **4**, which cannot form an intramolecular hydrogen bond and do not undergo cholinesterase hydrolysis, also show diminished amide nitrogen electron density, at −0.710 and −0.718, respectively. On the other hand, compounds that show improved cholinesterase hydrolysis over that of compound **2**, such as compounds **9**–**14** and **22**, all show a greater negative charge on the amide nitrogen (Table 3). That having an amide nitrogen charge of −0.740 to −0.760 is not sufficient to guarantee efficient hydrolysis by cholinesterases is evident from the very negative amide nitrogen (−0.784) calculated for *N*-(2-nitrophenyl)benzamide (**18**). This compound was only hydrolyzed by BuChE and at a very slow rate (Table 2). Thus, like the NMR and bond distance results described above, an electron-rich amide nitrogen appears to be an important contributing factor for the efficient hydrolysis of anilides by cholinesterases.

2.2.4. The effect of the acyl group on anilide hydrolysis. The hydrolysis of esters of choline by cholinesterases has long been known to be affected by the size of the acyl function of the molecule.[2] For example, AChE and BuChE readily hydrolyze acetylcholine. However, as the number of carbon atoms is increased through butyrylcholine, hydrolysis by AChE is diminished considerably, while the rate of hydrolysis by BuChE is enhanced. In this context, the effect of the size of the acyl group was examined in a series of 2-nitroanilide derivatives. Increasing the number of carbons from the lead compound, *N*-(2-nitrophenyl)acetamide (**2**), was found to diminish the hydrolysis of the anilide by AChE, while the hydrolysis by BuChE was considerably enhanced (Table 2). For example, compound **2** was hydrolyzed by both AChE and BuChE, while *N*-(2-nitrophenyl)butanamide (**10**) was hydrolyzed only by BuChE. Furthermore, comparison of compounds **9** and **12**, in which the number of acyl carbons is doubled, indicates a 50-fold increase in the rate of anilide hydrolysis by BuChE (Table 2). Figure 4 shows a bar diagram summarizing the rates of hydrolysis of straight chain 2-nitroanilides as the number of carbons in the acyl group is increased from two to eight. As has been observed for the esters of choline, increasing the number of carbons in the acyl group leads to diminished hydrolysis by AChE. In the present work, it was observed that with the *N*-2-nitroanilides there was indeed loss of hydrolysis by AChE as the number of acyl carbons was increased from 3 to 6 (compounds **9**–**12**). However, for the acyl anilides with seven and eight carbon atoms in the acyl side chain (compounds **13** and **14**), some hydrolysis by AChE was observed (Fig. 4). Based on the crystal structure of AChE, [40] it is recognized that the active site is at the bottom of a 20 Å deep gorge. Hydrolysis involves a

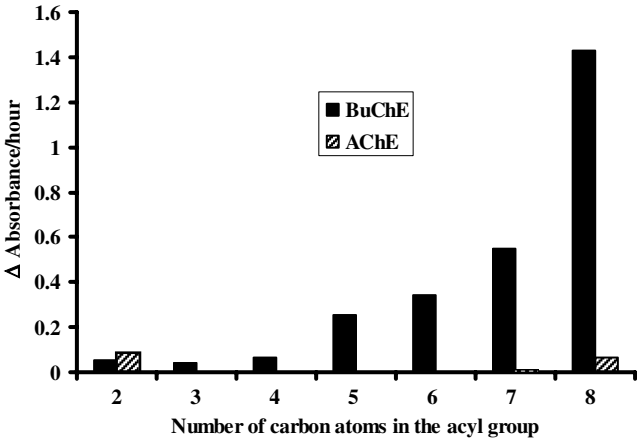


Figure 4. Initial rates of hydrolysis of alkyl acyl anilides with 2–8 carbon atoms in the acyl group (compounds **2** and **9**–**14**, 3.33×10^{-5} M) by human cholinesterases (10 units).

catalytic triad of serine, histidine, and glutamate residues, as well as an acyl pocket that accommodates the acyl group during catalysis. Although the catalytic triad is common in AChE and BuChE, the acyl pocket in AChE is small, relative to that in BuChE,[41] which explains, in part, why substrates with larger acyl groups are not hydrolyzed by AChE, but are hydrolyzed by BuChE. If the catalytic site for the hydrolysis of esters of choline and anilides is the same, then the observation that compounds **13** and **14** are hydrolyzed by AChE, albeit slightly, suggests that the larger substrates may be hydrolyzed by an alternate mechanism.[31]

The esteratic substrate benzoylcholine has long been known to be specifically hydrolyzed by BuChE, but at a slower rate than the analogous alkyl esters.[2] To test whether an anilide derivative behaved similarly, *N*-(2-nitrophenyl)benzamide (**18**) was prepared and tested. Once again, this was hydrolyzed only by BuChE, and at a very low rate compared to the alkyl derivatives, **2** and **9**–**14** (Table 2). In order to examine whether this was partly a consequence of the highly conjugated rigid system in **18**, compound **16**, with a more flexible methylene bridge between the carbonyl and the benzene ring in the acyl moiety, was examined. This compound showed enhanced (5-fold) hydrolysis by BuChE and even permitted hydrolysis by AChE (Table 2). To test the effect of branching in the acyl group, hydrolysis of *N*-(2-nitrophenyl)hexanamide (**12**) was compared to that of the highly branched 3,3-dimethylbutanamide derivative (**15**), which also has a six carbon acyl group. This compound (**15**) was not hydrolyzed by BuChE, suggesting that a highly branched moiety cannot be accommodated by the binding mechanism of this enzyme.

The effect on hydrolysis of electron-withdrawing groups in the vicinity of the carbonyl in the acyl moiety was examined by using *N*-(2-nitrophenyl)trifluoroacetamide (**22**). This compound was very rapidly hydrolyzed by cholinesterases, especially BuChE (Table 2). The rate of hydrolysis of compound **22** by AChE was about 4 times faster than that of the analogous lead compound, **2**, by the same enzyme. On the other hand, the hydrolysis of **22** by BuChE was roughly 100 times faster than the hydrolysis of **2** by this enzyme. A comparison of the rates of hydrolyses of *N*-(2-nitrophenyl)trifluoroacetamide (**22**) by the two cholinesterases (Table 2) showed that this compound was hydrolyzed about 15 times faster by BuChE than AChE. These results indicate that the electron-withdrawing fluorine atoms on the carbon adjacent to the carbonyl facilitate the hydrolysis of the amide bond by cholinesterases. NMR analysis, calculated charge on the amide nitrogen and atomic distances for hydrogen bonding (Table 3), all point toward favorable hydrolysis of this compound (**22**) by cholinesterases. Furthermore, replacement of the hydrogen atoms by larger fluorine atoms may account for the more rapid hydrolysis of **22** by BuChE than by AChE.

2.2.5. *N*-2-Nitroanilide kinetic constants. The straight chain 2-nitroanilide substrates that were effectively hydrolyzed by cholinesterases (**2**, **9**–**14**, and **22**) were examined for their BuChE affinities and relative maximum velocities of hydrolysis under identical conditions. The K_m and V_{max} values obtained through Lineweaver–Burk plots are summarized in Table 4. Although there were no large differences in the BuChE affinity constants for most of the anilides, that of *N*-(2-nitrophenyl)trifluoroacetamide (**22**) was distinctly different, this substrate showing some 1000-fold lower affinity for BuChE than the straight-chain alkyl 2-nitroanilides (**2** and **9**–**14**). On the other hand, the rate of hydrolysis of this substrate was found to be much higher. For example, although the K_m values reveal that **22** has a 200-fold lower affinity for BuChE than **2**, it is hydrolyzed about 40,000 times faster than the reference compound **2**. These differences in the kinetic behavior of various 2-nitroanilides are depicted in Figure 5. These Michaelis–Menten plots are based on the observed K_m and V_{max} values for BuChE (Table 4) with each substrate. Using the Michaelis–Menten equation, and the observed K_m and V_{max} values, the rate of product formation at each substrate concentration was calculated. This simulation shows that, as the number of carbon atoms is increased in these unsubstituted straight chain acyl anilides (**2** and **9**–**14**), the rate of hydrolysis increases proportionately. The exception is compound **22** in which the presence of the electron-withdrawing fluorine atoms results in a dramatic increase in the rate of hydrolysis by BuChE.

2.2.6. Comparison of anilide and thioester hydrolysis. As with anilide hydrolysis (Fig. 4), cleavage of thioesters of choline by AChE drops off rapidly from the acetyl (**I**) to the propanoyl (**II**) derivative and is virtually lost for the butanoyl (**III**) substrate (Fig. 6). On the other hand, the hydrolysis of esters of choline by BuChE is found to increase from the acetyl to the butanoyl ester (Table 5), as has been observed previously.[2] Because *N*-(2-nitrophenyl)octanamide (**14**) was hydrolyzed by BuChE, even more rapidly than *N*-(2-nitrophenyl)butanamide (**10**)

Table 4. Kinetic parameters of substrates for aryl acylamidase activity of butyrylcholinesterase

Compound	Name	K_m ($\times 10^{-4}$ M)	V_{max} ($\times 10^{-6}$ M/min)	k_{cat} (min^{-1})
2	*N*-(2-Nitrophenyl)acetamide	6.03 ± 0.34	0.29 ± 0.03	3
9	*N*-(2-Nitrophenyl)propanamide	17.8 ± 1.9	4.88 ± 0.62	46
10	*N*-(2-Nitrophenyl)butanamide	3.55 ± 0.74	3.77 ± 0.54	35
11	*N*-(2-Nitrophenyl)pentanamide	5.62 ± 1.58	18.3 ± 5.0	172
12	*N*-(2-Nitrophenyl)hexanamide	4.85 ± 0.95	24.5 ± 3.6	230
13	*N*-(2-Nitrophenyl)heptanamide	3.77 ± 0.70	31.9 ± 6.3	299
14	*N*-(2-Nitrophenyl)octanamide	1.43 ± 0.03	34.8 ± 3.8	326
22	*N*-(2-Nitrophenyl)trifluoroacetamide	1410 ± 110	10,700 ± 3000	100,312

The k_{cat} values were calculated by dividing V_{max} by the moles of BuChE (0.16 nm) in the assay and multiplying by the reaction volume of 1.5 mL.

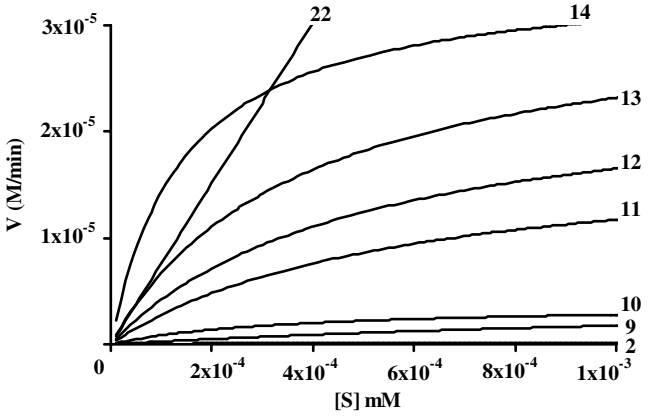


Figure 5. Simulated plots showing calculated velocities of hydrolysis of 2-nitroanilide substrates based on observed affinity constants (K_m) and maximum velocity (V_{max}) values.

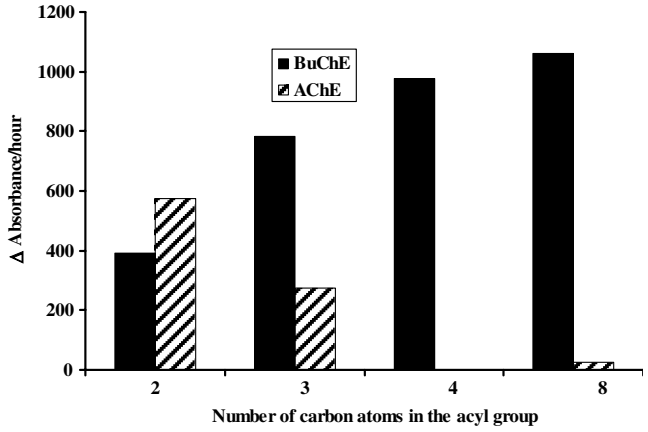


Figure 6. Initial rates of hydrolysis of thiocholine esters with 2–4 and 8 carbon atoms in the acyl group (compounds **I**–**IV**) (3.33×10^{-5} M) by human cholinesterases (0.04 units).

(Figs. 4 and 5), the octanoylthiocholine derivative (**IV**) was synthesized and its initial rate of hydrolysis was compared with those of the acetyl (**I**), propanoyl (**II**), and butanoyl (**III**) choline esters. As observed earlier[2] and, as with the 2-nitroanilide derivatives (Table 4), the rate of hydrolysis by AChE decreased rapidly from the acetyl- (**I**) through the butanoyl- (**III**) derivatives, whereas the reverse was true for BuChE (Fig. 6). The octanoyl ester (**IV**), like its anilide counterpart (**14**), was also found to be efficiently hydrolyzed by BuChE. Furthermore, like the *N*-(2-nitrophenyl)octanamide (**14**), the analogous octanoylthiocholine (**IV**) was also slightly hydrolyzed by AChE (Fig. 6). This parallel between the two classes of substrates suggests that the anilides and choline esters may undergo hydrolysis at the same catalytic site via a similar mechanism. However, the mechanism of hydrolysis for longer acyl derivatives of both classes of substrates by AChE may differ from that utilized in the hydrolysis of derivatives with shorter chain acyl groups.

The broad spectrum of compounds that BuChE is capable of hydrolyzing may indicate the involvement of this enzyme in other important biological functions. For example, BuChE has been suggested to be involved in the deactivation of the acylated form of the growth hormone secretagogue ghrelin.[42] The hormone is a 28 amino acid residue protein which is active when the third serine residue from the amino terminal is an octanoyl ester. This active form of the hormone is reported to be deactivated via deoctanoylation by BuChE.[42]

2.2.7. Probing the active site of butyrylcholinesterase with anilides and esters of choline. When hydrolysis of butyrylthiocholine by BuChE is examined in the presence and absence of the comparable, non-chromogenic substrate, butyrylcholine a competition between the substrates is observed.[2] Lineweaver–Burk plots of the hydrolysis of butyrylthiocholine in the presence of butyrylcholine exhibit an apparent competitive 'inhibition' ('K_i' = 1.42×10^{-6} M). It was reasoned that hydrolysis of an anilide substrate in the presence of butyrylcholine, may give an indication of whether these two substrates may be competing for the same catalytic site in BuChE. When the rapidly hydrolyzed *N*-2-nitrophenyltrifluoroacetamide (**22**) was incubated with BuChE in the presence of varying concentrations of butyrylcholine, the time of the appearance of the hydrolysis product of **22**, namely, 2-nitroaniline, increased with increasing amounts of butyrylcholine present (Fig. 7). This suggests that the ester and anilide substrates compete for the some catalytic site and that no significant hydrolysis of the anilide (**22**) takes place until the preferred choline ester hydrolysis has been completed.

3. Conclusions

N-2-Nitroanilides with longer straight chain acyl groups represent efficient and highly specific substrates for examining the aryl acylamide activity of BuChE. The hydrolysis of anilides by cholinesterases has a requirement for intramolecular hydrogen bonding between the amide hydrogen and an adjacent group (e.g., nitro) on the aniline ring. Hydrolysis is further enhanced by electron-withdrawing groups (e.g., fluoro) adjacent to

Table 5. Hydrolysis of thiocholine esters by human butyrylcholinesterase

Compound	Name	K_m ($\times 10^{-5}$ M)	V_{max} ($\times 10^{-5}$ M/min^{-1})	k_{cat} (min^{-1})
I	Acetylthiocholine	5.07 ± 0.58	1.92 ± 0.11	45,000
II	Propionylthiocholine	4.02 ± 0.55	3.06 ± 0.85	72,000
III	Butyrylthiocholine	3.47 ± 1.38	3.77 ± 0.19	88,000
IV	Octanoylthiocholine	1.99 ± 0.80	3.00 ± 0.21	70,000

The k_{cat} values were calculated by dividing V_{max} by the moles of BuChE (0.64 pm) in the assay and multiplying by the reaction volume of 1.5 mL.

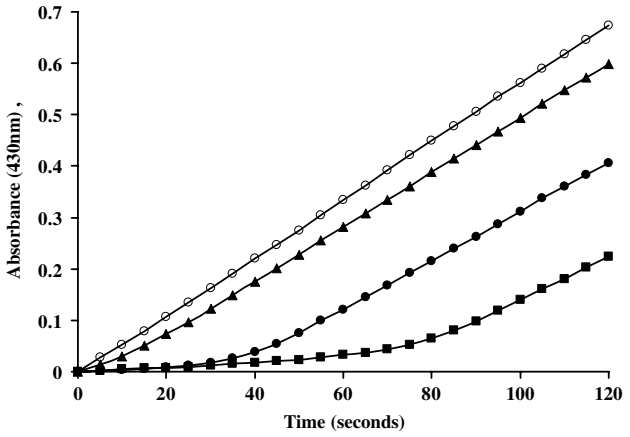


Figure 7. Delay in hydrolysis of *N*-(2-nitrophenyl)trifluoroacetamide (**22**) (3.3×10^{-4} M) caused by increasing concentrations of butyrylcholine. No butyrylcholine (○), 0.3 mM butyrylcholine (▲), 1.7 mM butyrylcholine (●), and 3.3 mM butyrylcholine (■).

the carbonyl in the acyl moiety. Comparison of the cholinesterase hydrolysis of esters of choline and 2-nitroanilides suggests that both classes of substrates generally utilize the same acyl binding pocket and the same catalytic triad as part of the hydrolytic mechanism.

The data presented here, with more efficient and enzyme-specific 2-nitroanilides, provide a basis to aid in further elucidation of the biochemical nature and possible biological function of the aryl acylamidase activity of BuChE.

4. Experimental

4.1. Materials

Recombinant human acetylcholinesterase (AChE, EC 3.1.1.7), aryl acylamidase from *P. fluorescens* (EC 3.1.1.13), *N*-phenylacetamide (acetanilide) (**1**), *N*-(4-nitrophenyl)acetamide (4-nitroacetanilide) (**4**), and all the reactants used to synthesize the anilide substrates, except those indicated below, were purchased from Sigma–Aldrich (St. Louis, MO). *N*-(2-Nitrophenyl)acetamide (**2**) was purchased from TCI America (Portland, OR), 3-chloro-2-nitroaniline from Frinton Laboratories (Vineland, NJ), butyryl chloride from Acros Laboratories (NJ), phenylacetyl chloride from Lancaster Synthesis (Pelham, NH), and benzoyl chloride from Eastman Kodak (Rochester, NY). Purified human wild-type butyrylcholinesterase (BuChE, EC 3.1.1.8) was a gift from Dr. Oksana Lockridge (University of Nebraska Medical Center).

4.2. Synthesis of substrates

4.2.1. Anilides. All of the anilides were synthesized by one of the standard procedures.[22–30] Briefly, to a well-stirred solution containing equivalent quantities of the substituted aniline and triethylamine in dichloromethane was added slowly 2–4 equiv of the acid chloride or trifluoroacetic anhydride also in dichloromethane solution. The solution was stirred at room temperature (or at reflux for the slower reactions) until thin-layer chromatography revealed that all aniline had reacted. The solution was poured slowly into sufficient 5% aqueous sodium bicarbonate to neutralize all the acid produced. After vigorous stirring for 1–2 h, the layers were separated and the organic layer washed with a second portion of bicarbonate solution, followed by water. The organic layer was dried ($MgSO_4$) and the solvent removed on a rotary evaporator. The crude anilide product was purified by column chromatography and/or recrystallization from a dichloromethane/petroleum ether mixture.

An alternate procedure was found to be superior for the highly deactivated anilines (2,4- and 2,6-dinitroaniline). These compounds were acetylated by stirring the aniline in a 10- to 15-fold excess of pure acetyl chloride at room temperature. Workup was achieved by pouring the reaction mixture into excess ice-cold 5% aqueous sodium bicarbonate and stirring the mixture until all gas evolution had ceased. The precipitated crude anilides were purified by recrystallization from ethanol–water.

In each case, the purified anilides were found to be homogeneous by thin-layer chromatography, had melting points which agreed well with literature values (where available) and had spectral data (IR, ^{1}H NMR, ^{13}C NMR, and mass spectra) that were consistent with the structure.

4.2.2. Thiocholines. Of the four thiocholine derivatives used in this study, three (**I**–**III**) were commercially available. The fourth, octanoylthiocholine (**IV**), was synthesized by a previously reported procedure.[31]

4.3. Analysis of synthesized compounds

Melting points were determined on a Mel-Temp II apparatus and are uncorrected. Thin-layer chromatography was carried out using silica gel sheets with fluorescent indicator (0.20 mm thickness; Macherey-Nagel) and dichloromethane or a dichloromethane/ethyl acetate mixture as developing solvent. Plates were visualized using a short wavelength UV lamp. Infrared spectra were recorded as Nujol mulls or thin films between sodium chloride plates on a Nicolet Avatar 330 FT-IR spectrometer. Peak positions were obtained in the 'find peaks' mode and were reproducible within 1–2 cm^{-1}. Nuclear magnetic resonance spectra were recorded at the Atlantic Region Magnetic Resonance Centre at Dalhousie University on a Bruker Avance spectrometer, operating at 500 MHz for proton and 125 MHz for carbon. Chemical shifts are reported in parts per million relative to TMS, usually in $CDCl_3$ solution. Mass spectra were recorded at Dalhousie University on a CEC 21-110B spectrometer using electron ionization at 70 V and an appropriate source temperature with samples being introduced by means of a heatable port probe. Accurate mass measurements were also made on this machine operated at a mass resolution of 8000 by computer controlled peak-matching to appropriate PFK reference ions. Mass measurements were routinely within 3 ppm of the calculated value.

4.4. Analytical data

4.4.1. *N*-(3-Nitrophenyl)acetamide (3). Pale yellow crystals, mp 150–151 °C (lit. mp 151–153 °C).[23] IR (Nujol): 3262, 3192, 3130, 3097, 1674, 1600, 1551, 1530, 1351, 1326, 1295, 887, 824, 806, 742 cm^{-1}. ^1H NMR ($CDCl_3$): 2.24 (s, 3H), 7.44 (br s, 1H), 7.49 (t, $J = 8.2$ Hz, 1H), 7.94–7.98 (m, 2H), 8.34 (m, 1H). ^{13}C NMR (acetone-d_6): 24.3, 114.3, 118.4, 125.5, 130.7, 141.6, 149.5, 169.6. EI-MS (*m*/*z*): 180 (M^+), 139, 138 (base), 108, 92, 80, 65. HR-MS: M^+ found, 180.0543; calcd for $C_8H_8N_2O_3$, 180.0535.

4.4.2. *N*-(2,4-Dinitrophenyl)acetamide (5). Beige needles, mp 119–121 °C (lit. mp 119–120 °C).[22] IR (Nujol): 3339, 1711, 1600, 1347, 1306, 1264, 1222, 1136, 924, 857, 841, 768, 744 cm^{-1}. ^1H NMR ($CDCl_3$): 2.38 (s, 3H), 8.48 (dd, $J = 9.5$ and 2.6 Hz, 1H), 9.10 (d, $J = 9.5$ Hz, 1H), 9.14 (d, $J = 2.6$ Hz, 1H), 10.64 (br s, 1H). ^{13}C NMR (acetone-d_6): 25.2, 122.4, 123.6, 130.2, 137.6, 140.1, 142.7, 170.1. EI-MS (*m*/*z*): 225 (M^+), 184, 183 (base), 167, 153, 137, 107, 91, 69, 63. HR-MS (EI): M^+ found, 225.0386; calcd for $C_8H_7N_3O_5$, 225.0385.

4.4.3. *N*-(2,6-Dinitrophenyl)acetamide (6). Greenish-yellow needles, mp 196–197 °C (lit. mp 193–195 °C).[23] IR (Nujol): 3283, 1677, 1609, 1583, 1535, 1347, 1299, 1262, 907, 819, 739, 716 cm^{-1}. ^1H NMR ($CDCl_3$): 2.27 (s, 3H), 7.46 (t, $J = 8.2$ Hz, 1H), 8.29 (d, $J = 8.2$ Hz, 2H), 9.48 (br s, 1H). ^{13}C NMR (acetone-d_6): 24.0, 127.0, 127.5, 130.8, 147.0, 170.0. EI-MS (*m*/*z*): 225 (M^+), 184, 183 (base), 179, 166, 153, 121, 107, 91, 75, 63. HR-MS (EI): M^+ found, 225.0385; calcd for $C_8H_7N_3O_5$, 225.0385.

4.4.4. *N*-(3-Methoxy-2-nitrophenyl)acetamide (7). This compound was prepared from 3-chloro-2-nitroaniline by the procedure of Nozary et al.[28] Reddish-brown crystals, mp 129–131.5 °C. IR (Nujol): 3255, 1667, 1590, 1521, 1278, 1098, 972, 855 cm^{-1}. ^1H NMR ($CDCl_3$): 2.19 (s, 3H), 3.91 (s, 3H), 6.81 (d, $J = 8.4$ Hz, 1H), 7.43 (t, $J = 8.4$ Hz, 1H), 7.86 (br d, $J = 8$ Hz, 1H), 8.27 (br s, 1H). ^{13}C NMR ($CDCl_3$): 24.8, 56.7, 108.1, 115.1, 131.9, 132.7 (2 peaks), 152.7, 168.5. EI-MS (*m*/*z*): 210 (M^+), 174, 172, 168, 164 (base), 121, 110, 108, 107, 95, 80, 77, 67, 65, 64, 63, 53. HR-MS (EI): M^+ found, 210.0639; calcd for $C_9H_{10}N_2O_4$, 210.0640.

4.4.5. *N*-(5-Methoxy-2-nitrophenyl)acetamide (8). This compound was prepared from 5-chloro-2-nitroaniline by the procedure of Nozary et al.[28] Yellow crystals. mp 122–125 °C (lit. mp 123–125°).[28] IR (Nujol): 3313, 1696, 1607, 1587, 1326, 1274, 1240, 1218, 1090, 1014, 865, 760 cm^{-1}. ^1H NMR ($CDCl_3$): 2.30 (s, 3H), 3.91 (s, 3H), 6.55 (dd, $J = 9.4$ and 2.8 Hz, 1H), 8.19 (d, $J = 9.4$ Hz, 1H), 8.42 (d, $J = 2.8$ Hz, 1H), 10.76 (br s, 1H). ^{13}C NMR ($CDCl_3$): 25.8, 56.1, 104.2, 110.7, 128.0, 129.4, 137.8, 165.7, 169.4 ppm. EI-MS (*m*/*z*): 210 (M^+), 169, 168 (base), 165, 164, 138, 122, 120, 79, 63. HR-MS (EI): M^+ found, 210.0639; calcd for $C_9H_{10}N_2O_4$, 210.0640.

4.4.6. *N*-(2-Nitrophenyl)propanamide (9). Bright yellow needles, mp 62.7–63.6 °C (lit. mp 64 °C).[27] IR (Nujol): 3356, 1721, 1606, 1587, 1499, 1315, 1270, 1147, 1070, 741 cm^{-1}. ^1H NMR ($CDCl_3$): 1.29 (t, $J = 7.6$ Hz, 3H), 2.54 (q, $J = 7.6$ Hz, 2H), 7.17 (m, 1H), 7.64 (m, 1H), 8.20 (dd, $J = 8.6$ and 1.6 Hz, 1H), 8.80 (dd, $J = 8.6$ and 1.6 Hz, 1H), 10.37 (br s, 1H). ^{13}C NMR ($CDCl_3$): 9.3, 31.7, 122.1, 123.0, 125.7, 135.0, 136.0, 136.2, 172.8. EI-MS (*m*/*z*): 194 (M^+), 148, 138 (base), 108, 92, 90, 80, 65, 63, 57. HR-MS (EI): M^+ found, 194.0695; calcd for $C_9H_{10}N_2O_3$, 194.0691.

4.4.7. *N*-(2-Nitrophenyl)butanamide (10). Nearly colorless needles, mp 52–54 °C (lit. mp 49–49.5 °C).[29] IR (Nujol): 3282, 1665, 1590, 1517, 1500, 1273, 1200, 777, 740 cm^{-1}. ^1H NMR ($CDCl_3$): 1.04 (t, $J = 7.2$ Hz, 3H), 1.81 (sextet, $J = 7.4$ Hz, 2H), 2.48 (t, $J = 7.5$ Hz, 2H), 7.17 (dt, $J = 8.6$ and 1.4 Hz, 1H), 7.65 (dt, $J = 8.6$ and 1.4 Hz, 1H), 8.21 (dd, $J = 8.6$ and 1.4 Hz, 1H), 8.81 (dd, $J = 8.6$ and 1.4 Hz, 1H), 10.36 (br s, 1H). ^{13}C NMR ($CDCl_3$): 13.7, 18.8, 40.6, 122.2, 123.1, 125.7, 135.0, 136.0, 136.2, 172.1. EI-MS (*m*/*z*): 208 (M^+), 162, 138 (base), 121, 108, 91, 90, 79, 71, 62. HR-MS (EI): M^+ found, 208.0835; calcd for $C_{10}H_{12}N_2O_3$, 208.0848.

4.4.8. *N*-(2-Nitrophenyl)pentanamide (11). Pale yellow crystals, mp 42.4–43.7 °C. IR (Nujol): 3363, 1717, 1610, 1586, 1500, 1338, 1273, 742 cm^{-1}. ^1H NMR ($CDCl_3$): 0.97 (t, $J = 7.4$ Hz, 3Hz), 1.40–1.48 (m, 2H), 1.72–1.78 (m, 2H), 2.50 (t, $J = 7.6$ Hz, 2H), 7.15–7.19 (m, 1H), 7.62–7.66 (m, 1H), 8.21 (dd, $J = 8.5$ and 1.5H), 8.80 (dd, $J = 8.5$ and 1.2 Hz, 1H), 10.36 (br s, 1H). ^{13}C NMR ($CDCl_3$): 13.8, 22.3, 27.4, 38.4, 122.2, 123.0, 125.7, 135.1, 136.0, 136.3, 172.3. EI-MS (*m*/*z*): 222 (M^+), 180, 139, 138 (base), 92, 85, 57, 56. HR-MS (EI): M^+ found, 222.1003; calcd for $C_{11}H_{14}N_2O_3$, 222.1004.

4.4.9. *N*-(2-Nitrophenyl)hexanamide (12). Pale yellow crystals, mp 46.4–48.5 °C. IR (Nujol): 3250, 1665, 1591, 1499, 1359, 1270, 1252, 1190, 784 cm^{-1}. ^1H NMR ($CDCl_3$): 0.91–0.95 (m, 3H), 1.36–1.42 (m, 4H), 1.74–1.80 (m, 2H), 2.49 (t, $J = 7.5$Hz, 2H), 7.15–7.18 (m, 1H), 7.62–7.66 (m, 1H), 8.21 (dd, $J = 8.4$ and 1.5 Hz, 1H), 8.81 (dd, $J = 8.6$ and 1.2 Hz, 1H), 10.36 (br s, 1H). ^{13}C NMR ($CDCl_3$): 13.9, 22.4, 25.1, 31.3 38.7, 122.2, 123.1, 125.7, 135.1, 136.0, 136.3, 172.3. EI-MS (*m*/*z*): 236 (M^+), 152, 151, 138, 134, 133, 122 (base), 108, 100, 99, 93, 92. HR-MS (EI): M^+ found, 236.1159; calcd for $C_{12}H_{16}N_2O_3$, 236.1161.

4.4.10. *N*-(2-Nitrophenyl)heptanamide (13). Pale yellow crystals, mp 32.5–33.5 °C. IR (thin film): 3363, 2924, 2854, 1716, 1610, 1586, 1499, 1458, 1377, 1338, 1273, 1147, 742 cm^{-1}. ^1H NMR ($CDCl_3$): 0.88–0.91 (m, 3H), 1.30–1.43 (m, 6H), 1.73–1.79 (m, 2H), 2.49 (t, $J = 7.5$Hz, 2H), 7.15–7.19 (m, 1H), 7.63–7.66 (m, 1H), 8.21 (dd, $J = 8.4$ and 1.5 Hz, 1H), 8.81 (dd, $J = 8.5$ and 1.2 Hz, 1H), 10.36 (br s, 1H). ^{13}C NMR ($CDCl_3$): 14.0, 22.4, 25.3, 28.8, 31.5, 38.7, 122.2, 123.0, 125.7, 135.0, 135.9, 136.2, 172.3. EI-MS (*m*/*z*): 250 (M^+), 180, 151, 139, 138 (base), 122, 113, 90, 85, 65, 55. HR-MS

(EI): M found, 250.1315; calcd for $C_{13}H_{18}N_2O_3$, 250.1317.

4.4.11. *N*-(2-Nitrophenyl)octanamide (14). Pale yellow crystals, mp 40.0–41.8 °C. IR (Nujol): 3287, 1662, 1591, 1536, 1518, 1495, 1362, 778 cm^{-1}. ^1H NMR ($CDCl_3$): 0.87–0.90 (m, 3H), 1.29–1.43 (overlapping m, 8H), 1.73–1.79 (m, 2H), 2.49 (t, $J = 7.6$ Hz, 2H), 7.15–7.18 (m, 1H), 7.62–7.66 (m, 1H), 8.21 (dd, $J = 8.4$ and 1.5 Hz, 1H), 8.81 (dd, $J = 8.6$ and 1.2 Hz, 1H), 10.36 (br s, 1H). ^{13}C NMR ($CDCl_3$): 14.0, 22.5, 25.3, 28.9, 29.0, 31.6, 38.7, 122.1, 123.0, 125.7, 135.0, 135.9, 136.2, 172.2. EI-MS (*m*/*z*): 264 (M^+), 218, 180, 151, 139, 138 (base), 127, 122, 92, 90, 57, 55. HR-MS (EI): M^+ found, 264.1479; calcd for $C_{14}H_{20}N_2O_3$, 264.1474.

4.4.12. *N*-(2-Nitrophenyl)-3,3-dimethylbutanamide (15). Bright yellow crystals, mp 64.0–66.2 °C. IR (Nujol): 3360, 1692, 1609, 1586, 1500, 1341, 1272, 1147, 1119, 748 cm^{-1}. ^1H NMR ($CDCl_3$): 1.12 (s, 9H), 2.35 (s, 2H), 7.15–7.19 (m, 1H), 7.62–7.66 (m, 1H), 8.21 (dd, $J = 8.6$ and 1.5 Hz, 1H), 8.82 (dd, $J = 8.5$ and 1.1 Hz, 1H), 10.30 (br s, 1H). ^{13}C NMR ($CDCl_3$): 29.8, 31.4, 52.7, 122.1, 123.1, 125.7, 135.0, 136.0, 136.3, 170.9. EI-MS (*m*/*z*): 236 (M^+), 221, 180, 139, 138 (base), 99, 92, 83, 71, 57. HR-MS (EI): M^+ found, 236.1157; calcd For $C_{12}H_{16}N_2O_3$, 236.1161.

4.4.13. *N*-(2-Nitrophenyl)phenylacetamide (16). Bright yellow crystals, mp 81.2–82.6 °C. IR (Nujol): 3360, 1705, 1608, 1584, 1494, 1339, 1272, 1240, 1162, 1131, 1073, 1033, 853, 786, 743 cm^{-1}. ^1H NMR ($CDCl_3$): 3.81 (s, 2H), 7.11–7.14 (m, 1H), 7.35–7.44 (overlapping multiplets, 5H), 7.58–7.62 (m, 1H), 8.13 (dd, $J = 8.4$ and 1.5 Hz, 1H), 8.78 (dd, $J = 8.6$ and 1.2 Hz, 1H), 10.25 (br s, 1H). ^{13}C NMR ($CDCl_3$): 45.8, 122.0, 123.3, 125.7, 128.0, 129.3, 129.7, 133.3, 134.7, 135.9, 136.3, 170.3. EI-MS (*m*/*z*): 256 (M^+, base), 210, 119, 118, 92, 91, 90, 89, 65. HR-MS (EI): M^+ found, 256.0839; calcd for $C_{14}H_{12}N_2O_3$, 256.0848.

4.4.14. *N*-Phenylbenzamide (17). Colorless crystals, mp 162–163 °C (lit. mp 163°).[26] IR (Nujol): 3342, 1655, 1599, 1578, 1529, 1321, 1260, 749, 716, 690 cm^{-1}. ^1H NMR ($CDCl_3$): 7.15 (t, $J = 7.4$ Hz, 1H), 7.37 (t, $J = 8.0$ Hz, 2H), 7.48 (t, $J = 7.4$ Hz, 2H), 7.55 (m, 1H), 7.86 (br s, 1H), 7.87 (d, $J = 8$ Hz, 2H). ^{13}C NMR ($CDCl_3$): 120.4, 124.8, 127.2, 129.0, 129.3, 132.0, 135.2, 138.1, 165.9. EI-MS (*m*/*z*): 197 (M^+), 106, 105 (base), 77, 76, 65. HR-MS (EI): M^+ found, 197.0845; calcd for $C_{13}H_{11}NO$, 197.0841.

4.4.15. *N*-(2-Nitrophenyl)benzamide (18). Bright yellow needles, mp 89.5–91.5 °C (lit. mp 90–92 °C).[22,24] IR (Nujol): 3361, 1683, 1606, 1589, 1502, 1341, 1278, 742, 706 cm^{-1}. ^1H NMR ($CDCl_3$): 7.22 (d, $J = 7.6$ Hz and 1.3 Hz, 1H), 7.54 (t, $J = 7.5$ Hz, 2H), 7.61 (m, 1H), 7.71 (dt, $J = 7.8$ Hz and 1.3 Hz, 1H), 8.00 (dd, $J = 7.1$ Hz and 1.3 Hz, 1H), 8.27 (dd, $J = 8.4$ Hz and 1.5 Hz, 1H), 9.01 (dd, $J = 8.5$ Hz and 1.3 Hz, 1H), 11.34 (br s, 1H). ^{13}C NMR ($CDCl_3$): 122.1, 123.3, 125.9, 127.4, 129.1, 132.6, 134.1, 135.4, 136.2, 136.5, 165.7. EI-MS (*m*/*z*): 242 (M^+), 196, 106, 105 (base), 77. HR-MS (EI): M^+ found, 242.0694; calcd for $C_{13}H_{10}N_2O_3$: 242.0691.

4.4.16. *N*-Methyl-*N*-(2-nitrophenyl)acetamide (19). Yellow crystals, mp 69.1–70.2 °C (lit. mp 70–71.5 °C).[34] IR (Nujol): 1664, 1602, 1524, 1346, 1309, 1142, 1070, 973, 794, 708 cm^{-1}. ^1H NMR ($CDCl_3$): product exists as a 3.3:1 mixture of two amide conformers. Major component (76%): 1.82 (s, 3H), 3.22 (s, 3H), 7.42 (dd, $J = 7.9$ and 1.3 Hz, 1H), 7.57–7.61 (m, 1H), 7.71–7.75 (m, 1H), 8.02 (dd, $J = 8.1$ and 1.4 Hz, 1H). Minor component (24%): 2.25 (s, 3H), 3.45 (s, 3H), 7.34 (dd, $J = 8.0$ and 1.3 Hz, 1H), 7.43–7.47 (overlapping m, 1H), 7.65–7.69 (m, 1H), 7.97 (dd, $J = 8.2$ and 1.3 Hz, 1H). ^{13}C NMR: (a) major conformer: 22.0, 36.7, 125.6, 129.5, 131.0, 134.5, 137.6, 146.8, 169.8 ppm; (b) minor conformer: 22.0, 39.3, 125.1, 128.0, 129.3, 134.2, 137.4, 146.5, 171.2 ppm. EI-MS (*m*/*z*): 194 (M^+), 153, 152, 148 (base), 135, 106, 105, 104, 79, 78, 77. HR-MS: M^+ found, 194.0692; calcd for $C_9H_{10}N_2O_3$, 194.0691.

4.4.17. *N*-Methyl-*N*-(2-nitrophenyl)butanamide (20). Yellow liquid. IR (thin film): 2967, 1710, 1670, 1602, 1530, 1488, 1352, 1293, 1192, 1136, 1081, 1043, 851, 785, 760, 707 cm^{-1}. ^1H NMR ($CDCl_3$): product exists as 3.5:1 mixture of amide conformers. Major component (78%): 0.97 (t, $J = 7.5$ Hz, 3H), 1.65–1.73 (m, 2H), 2.33 (t, $J = 7.4$ Hz, 2H), 3.23 (s, 3H), 7.39 (dd, $J = 8.7$ and 1.2 Hz, 1H), 7.57–7.60 (m, 1H), 7.70–7.74 (m, 1H), 8.02 (dd, $J = 8.3$ and 1.3 Hz, 1H). Minor component (22%): 1.00 (t, $J = 7.5$ Hz, 3H), 1.56–1.62 (m, 2H), 2.47 (t, $J = 7.4$ Hz, 2H), 3.43 (s, 3H), 7.32–7.34 (m, 1H), 7.43–7.46 (m, 1H), 7.64–7.67 (m, 1H), 7.96–7.98 (m, 1H). ^{13}C NMR: (a) major conformer: 13.6. 18.2, 18.5, 35.9, 125.7, 129.5, 131.2, 134.1, 134.5, 137.6, 172.74; (b) minor conformer: 13.9, 17.9, 36.9, 125.1, 128.0, 129.4, 137.3, 173.8. EI-MS (*m*/*z*): 222 (M^+), 176, 153, 152 (base), 106, 105, 104, 79, 77, 71. HR-MS (EI): M^+ found, 222.1004; calcd for $C_{11}H_{14}N_2O_3$, 222.1004.

4.4.18. *N*-Phenyltrifluoroacetamide (21). Colorless needles, mp 86.5–88.9 °C (lit. mp 85–86 °C).[30] IR (Nujol): 3318, 1704, 1603, 1551, 1308, 1287, 1244, 1152, 1078, 921, 896, 755, 731, 690 cm^{-1}. ^1H NMR ($CDCl_3$): 7.23 (t, $J = 7.7$ Hz, 1H), 7.36 (m, 2H), 7.55 (d, $J = 7.8$ Hz, 2H), 8.21 (br s, 1H). ^{13}C NMR (proton-decoupled; $CDCl_3$): 115.7 (q, $J^1_{CF} = 288$ Hz), 120.7, 126.4, 129.3, 135.0, 155.0 (q, $J^2_{CF} = 37$ Hz). EI-MS (*m*/*z*): 189 (M^+) (base), 121, 120, 119, 94, 92, 91, 77, 69, 65, 52. HR-MS (EI): M^+ found, 189.0405; calcd for $C_8H_6NOF_3$, 189.0401.

4.4.19. *N*-(2-Nitrophenyl)trifluoroacetamide (22). Pale yellow needles, mp 88–89 °C (lit. mp 89–90 °C).[25] IR (Nujol): 3298, 1728, 1596, 1519, 1275, 1171, 1158, 1139, 740 cm^{-1}. ^1H NMR ($CDCl_3$): 7.38 (dt, $J = 8.5$ and 1.4 Hz, 1H), 7.77 (dt, $J = 8.5$ and 1.4 Hz, 1H), 8.32 (dd, $J = 8.5$ and 1.4 Hz, 1H), 8.74 (dd, $J = 8.5$ and 1.4 Hz, 1H), 11.38 (br s, 1H). ^{13}C NMR (proton-decoupled; $CDCl_3$): 115.4 (q, $J^1_{CF} = 288$ Hz), 122.2, 125.6, 126.2, 132.1, 136.4, 137.0, 155.4 (q, $J^2_{CF} = 38$ Hz). EI-MS (*m*/*z*): 234 (M^+), 188 (base peak), 168, 165, 148,

121, 120, 102, 91, 90, 89, 77, 69, 64, 62. HR-MS (EI): M^+ found, 234.0268; calcd for $C_8H_5N_2O_3F_3$, 234.0252.

4.4.20. Octanoylthiocholine (IV). Colorless powder, mp 129.6–133.8 °C. IR (Nujol): 1694, 1417, 1124, 1048, 1017, 968, 932, 909 cm^{-1}. ^1H NMR (D_2O): 0.84–0.87 (m, 3H), 1.2–1.3 (overlapping m, 8H), 1.55–1.61 (m, 2H), 2.64 (t, $J = 7.3$ Hz, 2H), 3.14 (s, 9H), 3.24–3.27 (m, 2H), 3.40–3.44 (m, 2H). ^{13}C NMR (D_2O): 13.8, 21.2, 21.9, 24.8, 28.0, 28.2, 30.9, 43.1, 52.1, 63.6, 197.8. ESI-MS (*m*/*z*): 246.1 (M^+), 188, 187, 127, 109.

4.5. Enzyme assays

4.5.1. Aryl acylamidase activity. The aryl acylamidase activity of purified human recombinant AChE and purified human plasma BuChE, as well as aryl acylamidase from *P. fluorescens* (AAA_{Pf}) was studied using a modified Fujimoto spectrophotometric method.[3] Briefly, in a quartz cuvette, with a 1 cm path-length, were placed 1.35 mL of 0.06 M Tris–HCl buffer (pH 8.0), 0.05 mL of 50% aqueous acetonitrile or 0.05 mL of inhibitor in this solvent or H_2O, and 0.05 mL of up to 10 mM substrate in 50% acetonitrile. The reagents were mixed and zeroed at the appropriate wavelength, 0.05 mL of enzyme (10 units, defined below), of AChE or BuChE or 0.125 unit of AAA_{Pf} were added (final volume 1.50 mL), mixed by inversion, and the absorbance measured every minute for an appropriate time period, most often 10 min, using a Milton Roy 1200 UV–vis spectrophotometer at 23 °C.

Under the conditions employed, 0.1 unit of BuChE was defined as that amount of enzyme that led to a change of absorbance of 1.00 in one minute in the presence of 1.60×10^{-4} M butyrylthiocholine. This represents a rate of butyrylthiocholine hydrolysis of 2.1×10^{-7} M/min. Under the conditions employed, 0.1 unit of AChE was defined as above, but using 1.60×10^{-4} M acetylthiocholine as the substrate. For AAA_{Pf}, 1 unit is defined as that amount of enzyme that will convert 1.0×10^{-6} M/min of *N*-acetyl-*p*-aminophenol to *p*-aminophenol at pH 9.0 and 37 °C, as defined by the supplier.

Repetitive scans for detecting AAA activity of cholinesterases and AAA_{Pf} were performed using a reaction mixture containing 1.4 mL of 0.06 M Tris buffer, pH 8, and 0.05 mL of enzyme as above. The reactions were commenced with the addition of 0.05 mL of anilide compound (10 mM or 1 mM, depending on solubility) dissolved in 50% aqueous acetonitrile. Changes in absorbance were monitored over a spectral range of 200–600 nm using an Ultrospec 2100 UV–vis spectrophotometer with Swift II applications software. The time interval for these scans varied depending on the rate of hydrolysis.

Determination of kinetic constants for hydrolysis of anilide substrates by BuChE was done using reaction mixture containing 1.4 mL of 0.06 M Tris buffer, pH 8, and 0.05 mL of enzyme. The reactions were commenced with the addition of 0.05 mL of substrate dissolved in 50% aqueous acetonitrile. Final substrate concentrations ranged from 8×10^{-6} to 3.5×10^{-4} M in the reaction mixture. The rate of formation of the aniline product was determined using a Milton Roy 1200 UV–vis spectrophotometer or an Ultrospec 2100 pro UV–vis spectrophotometer with Swift II applications software. The molar extinction coefficient (ε) for 2-nitro aniline used to convert the change in absorbance at $\lambda = 430$ nm to moles of product was 3954 M^{-1} cm^{-1}. Kinetic constants (K_m and V_{max}) were determined using the modified-direct linear plot method.[43] The k_{cat} values were calculated by dividing V_{max} by the moles of BuChE (0.16 nm) in the assay and multiplying by the reaction volume of 1.5 mL.

4.5.2. Esterase activity. The esterase activity of AChE and BuChE was determined by a modification[44] of the method described by Ellman et al.[45] Briefly, 1.35 mL of buffered 5,5′-dithio-bis(2-nitrobenzoic acid) solution (pH 8.0), 0.05 mL of AChE (0.03 units) or BuChE (0.04 units) in 0.1% aqueous gelatin, and 0.05 mL of 50% aqueous acetonitrile were mixed in a quartz cuvette of 1 cm path-length. The mixture was zeroed at 412 nm, and the reaction was initiated by the addition of a thiocholine in an aqueous solution to give a final substrate concentration of 2.0×10^{-4} M or less. The reactions were performed at 23 °C. The rate of change of absorbance (ΔA/min), reflecting the rate of hydrolysis of the thiocholine, was recorded every 5 s for 1 min, using a Milton-Roy 1201 UV–vis spectrophotometer. The molar extinction coefficient (ε) for the Ellman product used to convert the change in absorbance at $\lambda = 412$ nm to moles of product was 14,150 M^{-1} cm^{-1}. These experiments were generally done at least in triplicate and the values averaged. Modified-direct linear plots[43] were generated by using a fixed amount of cholinesterase and varying amounts of substrate (2.0×10^{-5} to 2.0×10^{-4} M).

4.6. Calculations of molecular parameters

Geometry optimization of the anilide substrates was carried out in a three-step process. First, the lowest energy conformer was obtained by the molecular mechanics (MM) method using the MMFF94 force-field (PC Spartan Pro, Wavefunction, Inc., 18401 Von Karman, Suite 370, Irvine, CA, 92612). Second, the geometry was optimized at the Hartree–Fock (HF) level using the STO-3G basis set starting from the preferred MM conformer. In step three, the HF/STO-3G optimized geometry became the starting point for an optimization at a higher level of theory using the B3LYP functional with 6-31G(d) basis set.[36–38] The latter two steps were carried out using the Gaussian 98 suite of programs.[46] HF and B3LYP geometry optimizations were followed by a frequency analysis to ascertain that the optimized species were the true minima on their potential surfaces. In some cases, the optimization was on the basis of negligible forces. The rationale for carrying out higher-level quantum chemical calculations was 2-fold. First, it was noted that B3LYP/6-31G(d) geometries more closely resembled X-ray crystallographic data than did their counterparts obtained at MM level of theory.[47] Second, any study of the relationship between electronic properties and

activity would be impossible at the force-field level since electrons are not explicitly included in force-field calculations. Atomic charges were calculated via Mulliken population analysis. Calculated properties included lowest energy conformations, extent of linearity between donor and acceptor atoms, extent of planarity of the anilide derivatives, and atomic charges.

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