ACS Medicinal Chemistry Letters

Letter

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ACS Med. Chem. Lett., **Just Accepted Manuscript** • Publication Date (Web): 08 May 2017

ACS Publications

ACS Medicinal Chemistry Letters is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Study of *para*-Quinone Methide Precursors towards the Realkylation of Aged Acetylcholinesterase

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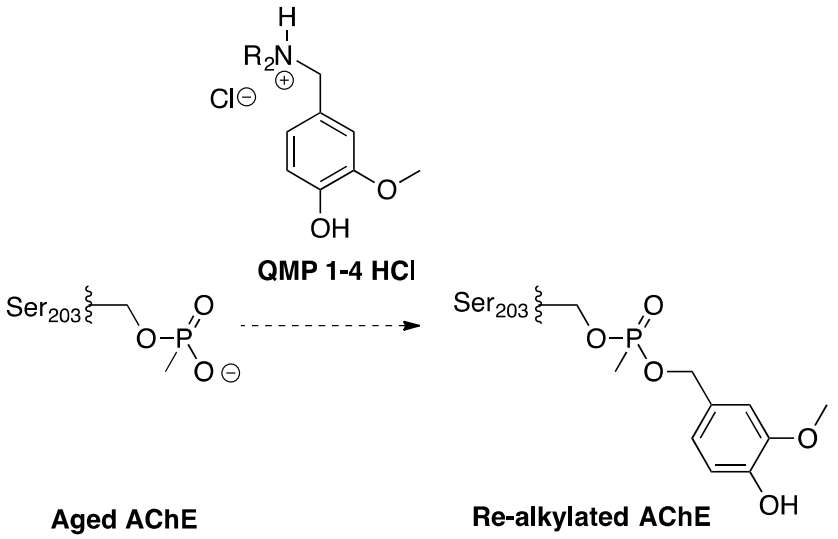
Department of Chemistry and Biochemistry, The Ohio State University, 100 West 18th Avenue, Columbus, OH 43210, USA

[#]Department of Chemistry and Biochemistry, The Ohio State University, Marion Campus, 1465 Mt. Vernon Avenue, Marion, OH 43302, USA

[†]College of Pharmacy, The Ohio State University, 500 West 12th Avenue, Columbus, OH 43210, USA

[‡]Department of Chemistry and Biochemistry, The Ohio State University, Newark Campus, 1179 University Drive, Newark, OH 43055, USA

KEYWORDS Acetylcholinesterase, Organophosphorus chemical nerve agents, Quinone methide.

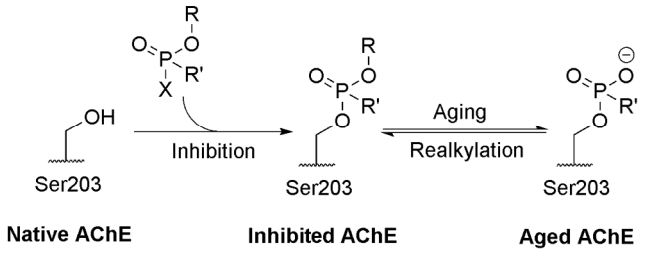


ABSTRACT: Acetylcholinesterase (AChE) is an essential enzyme that can be targeted by organophosphorus (OP) compounds, including nerve agents. Following exposure to OPs, AChE becomes phosphylated (inhibited) and undergoes a subsequent aging process where the OP-AChE adduct is dealkylated. The aged AChE is unable to hydrolyze acetylcholine, resulting in accumulation of the neurotransmitter in the central nervous system (CNS) and elsewhere. Current therapeutics are only capable of reactivating inhibited AChE. There are no known therapeutic agents to reverse the aging process or treat aged AChE. Quinone methides (QMs) have been shown to alkylate phosphates under physiological conditions. In this study, a small library of novel quinone methide precursors (QMPs) has been synthesized and examined as potential alkylating agents against model nucleophiles, including a model phosphonate. Computational studies have been performed to evaluate the affinity of QMPs for the aged AChE active site, and preliminary testing with electric eel AChE has been performed.

Organophosphorus (OP) compounds containing a phosphoryl group have been used as pesticides and chemical warfare agents.[1-4] These compounds are toxic due to their inhibition of the enzyme acetylcholinesterase (AChE), a serine hydrolase typically found in the central and peripheral nervous system that regulates concentrations of the neurotransmitter acetylcholine.[5,6] As illustrated in Scheme 1, when the central phosphorus atom is attacked by the catalytic serine, a leaving group is substituted, leaving a phosphyl group covalently attached to the serine, which blocks the active site. Inhibition of AChE can lead to death by respiratory failure due to overstimulation of muscarinic acetylcholine receptors at the neuromus-

cular junctions.[7,8] There are known therapeutics, specifically oxime-containing compounds, to reverse OP inhibition of AChE.[9-11]

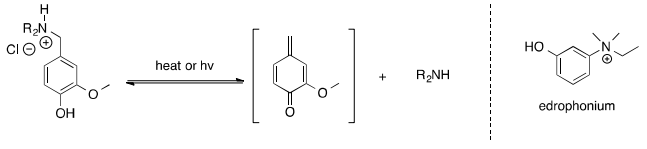
If left untreated after OP inhibition, the *O*-alkyl group of the AChE-OP adduct can undergo a dealkylation reaction, leaving a phosphonate (or phosphate) anion in the AChE active site in a process known as "aging" (Scheme 1).[11-14] The rate of aging varies from minutes to hours between different OPs.[15-17] Currently there is no known therapeutic to reactivate aged AChE.[17] Previous investigations utilized alkyl sulfonates[19,20] and phenacyl bromides[21] to alkylate a model phosphonate, but attempts to reactivate the aged enzyme were unsuccessful.



Scheme 1. Inhibition, aging and realkylation of AChE. The leaving group X is substituted during inhibition. The alkyl group R is lost during aging.

Thus, the need for an effective therapeutic to reverse the effects of aging in OP-inhibited AChE has not been met. Importantly, a single alkylating agent will treat the identical aged structures of each of the common OPs, except for tabun, which result in the same methylphosphonate structure after aging. Consequently, the same oxime should be effective for many realkylated OPs.

The preferred alkylating agent must be reactive enough to alkylate the phosphonate anion in the aged AChE enzyme, but selective enough to minimize alkylation of other biomolecules *in vivo*. Quinone methides (QMs) have been used as alkylating agents for other biological applications *in vitro*, including the alkylation of phosphodiesters at physiological conditions.[22] QMs have also been shown to reversibly alkylate DNA and block transcription.[23] Additionally, QMs have been shown to possess highly tunable reactivity through modification of substituents on the aromatic ring,[24] or the amine leaving group.[25] The structure of potential quinone methide precursors (QMPs) also strongly mimics edrophonium, an oxyanilinium-based inhibitor of AChE that is known to bind in the (native) AChE active site.[26] Another potentially useful aspect of QMs is their ability to be delivered in an unreactive prodrug form where a reactive QM could be generated selectively within the AChE active site (Scheme 2).



Scheme 2. Potential Quinone Methide Precursor (QMP) Prodrug and Quinone Methide Intermediate

Recent studies have shown the generation of QMs from benzyl and naphthyl derivatives via silyl cleavage.[27] QMs have predominantly been generated by oxidative[28] or photochemical[29] means due to higher yield of the reactive intermediate, but for *in vivo* applications, the QM intermediate must be generated thermally. Therefore, we decided to focus on a subset of *para*-QMPs as candidates for the realkylation of aged AChE (Figure 1). Initial computational modeling (see below) suggested that these QMPs would have some affinity for the active site of aged AChE. As a proof-of-concept, we will focus on finding the optimum QMP through quantitative alkylation experiments with a variety of nucleophiles, including a model phosphonate, in addition to molecular modeling and kinetic experiments with native and aged AChE.

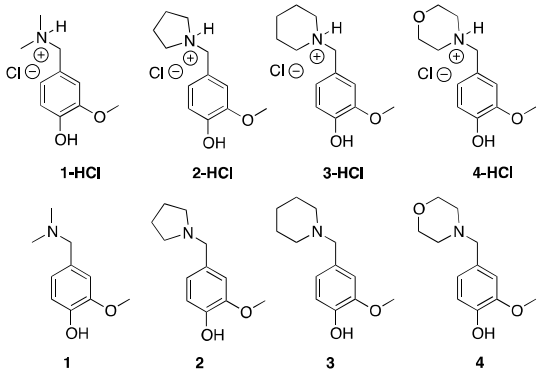
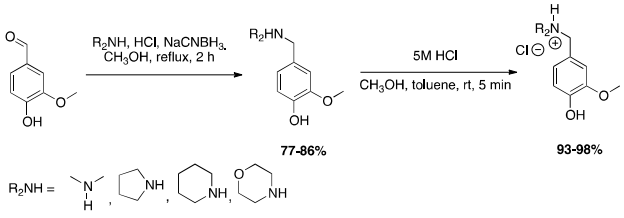


Figure 1. Target Quinone Methide Precursors

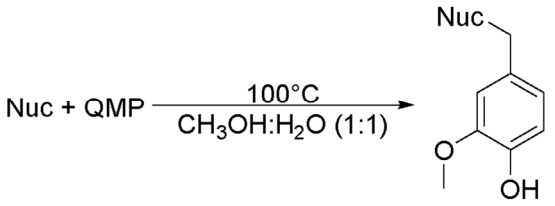
A reliable method for producing amines via reductive amination has been developed (Scheme 3). In the process, secondary amines were reacted with the aldehyde substrate to produce a conjugated iminium ion which was reduced in situ by sodium cyanoborohydride (77–86%). The resulting amines (**1**–**4**) were isolated via column chromatography and then dissolved in methanol and toluene followed by slow addition of methanolic hydrochloric acid to yield the corresponding hydrochloride ammonium salts in 93–98% yield (**1**–**4 HCl**).



Scheme 3. Reductive Amination for the Preparation of QMPs

Studies to evaluate the general effectiveness of the amines and ammonium salts towards nucleophilic substitution were undertaken. Each of the compounds shown in Figure 1 were separately subjected to reaction with 4-methylbenzenethiol, piperidine/pyrrolidine and benzyl alcohol at 100 °C for 3h. These nucleophiles were chosen as representative thiol, amines and alcohol. We chose this high temperature because in the absence of assistance of desolvation and proximity, the energy barrier in bulk solvent could be higher than that at the active site of the enzyme. Both the neutral and the protonated forms of QMPs were tested because the active sites of enzymes are normally highly desolvated, and can exhibit a dif-

ferent pH environment due to the surrounding dissociable residues compared to that in the bulk solvent. All compounds alkylated more than 65% of the thiol and the amine, as shown in Table 1. None alkylated more than 20% of the alcohol. This test implies that the candidate QMPs can react as alkylators of nucleophiles with moderate reactivity.



Scheme 4. Alkylation of model nucleophiles by QMPs

Table 1. Percentage yields of alkylation of model nucleophiles by QMPs

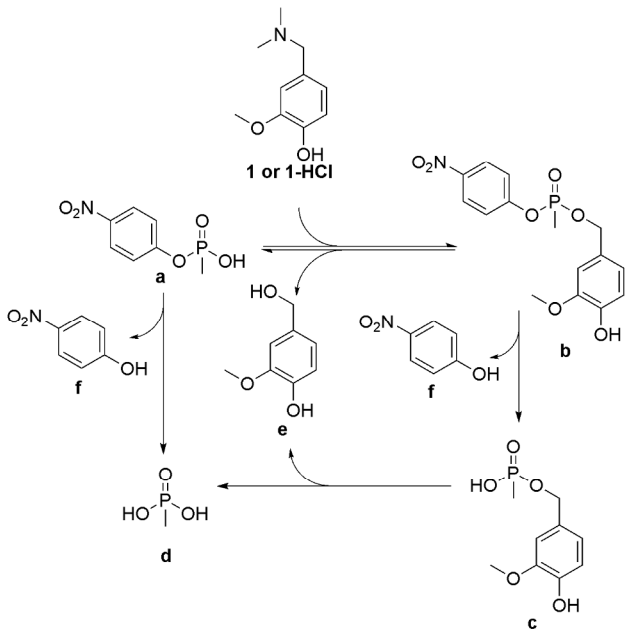
QMP	Nucleophile		
	4-methylbenzenethiol	piperidine*	benzyl alcohol
1	84%	80%	0%
2	87%	77%	8%
3	83%	81%	11%
4	83%	79%	13%
1-HCl	83%	78%	8%
2-HCl	83%	78%	12%
3-HCl	85%	81%	10%
4-HCl	84%	69%	16%

*__3__ and __3-HCl__ were reacted with pyrrolidine because of their piperidinyl groups in the existing structure.

In order to test the efficacy of our precursors against a model phosphonate as a simple model for the aged AChE active site, we sought to replicate a study from Steinberg[21] to indirectly monitor alkylation by UV-Vis spectroscopy. By appending a model phosphonate with a *p*-nitrophenol leaving group, alkylation could be indirectly monitored after addition of base to the synthesized alkylated product.

However, after following Steinberg's protocol, our results proved unreliable over the course of replicate trials with large levels of background signal (Supporting Information). Thus, our efforts moved away from the indirect UV-vis screen and were refocused on confirming the alkylation of a model phosphonate by our QMPs.

In order to confirm alkylation of the model phosphonate by QMPs, the reaction was monitored by tandem HPLC/ESI-MS-TOF. These techniques would allow for a better opportunity to monitor the potential products generated, and perhaps other previously unconsidered pathways that may be responsible for the degradation of the alkylated product. A representative QMP of the neutral (**1**) and protonated (**1-HCl**) forms were each reacted with the model phosphonate in water at 100 °C for 3 h and analyzed using the HPLC/ESI-MS-TOF to identify each of the species in solution after heating. The reaction pathways are illustrated in Scheme 5. For both **1** and **1-HCl**, only trace amounts of the intact alkylated product (**b**) were observed. The major peaks observed in each reaction were **a**, **c**, **e** and **f**. Species **c** indirectly implied the formation of **b** via alkylation.



Scheme 5. Reactions between 1 or 1-HCl and the model phosphonate (a). Protonation states are not specified.

To further examine the alkylation of the model phosphonate by our QMPs, ^{31}P NMR spectroscopy was used to follow the different phosphorus species generated from the same reactions (Scheme 5) in D_2O. The results indicated the presence of all four different phosphorus species (**a-d**) after heating. The major species identified in the product were **a** (25.1 ppm) and **d** (20.6 ppm). **b** and **c** were also observed, indicating the occurrence of alkylation.

In parallel, we used computational approaches to evaluate this QMP approach. Past efforts towards understanding the effect of OP nerve agents by molecular modeling studies have been performed by our group.[30] In order to better understand the potential interaction between our QMPs and the target aged AChE active site, molecular docking simulations were performed using AutoDock 4.0. The ligands were initially optimized using Gaussian 09[31] at the B3LYP[32,33]/6-311+G** level of theory, and then submitted to a Merz-Kollman charge calculation to prepare the substrates for docking. In docking simulations with aged AChE, the receptor was kept mostly rigid and the ligand flexible, and each of the poses was analyzed to determine if it would present the potential for a favorable interaction between the aged serine residue and the benzylic carbon of our QMPs. Specifically, we evaluated docked poses in which the reactive benzylic carbon was within 5.0 Å of the anionic O-(P=O) of the aged serine residue. The aged AChE was prepared by *in silico* methods from the human iso-

form (PDB: 1B41[a]) and details are provided in the supporting information.

Examination of molecular docking snapshots revealed some variability in the specific orientation of the amine leaving group (Supporting Information). It was found that the pyrrolidine (**2**/**2-HCl**) and piperidine (**3**/**3-HCl**) amine leaving groups were the most promising of this library of ligands with the highest percentage of docking poses in which the benzylic carbon is within 5.0 Å of the aged serine residue. While the majority of poses are outside of this distance cutoff, a significant portion do exist within this distance criterion, suggesting that the ligands can access the aged active site. Thus, these QMPs may allow for a therapeutic reaction to occur whether alkylation were to take place through *in situ* generation of a quinone methide or by a direct displacement at the benzylic position. Also, among the poses not within the distance criterion, the vast majority were still within the enzyme itself, but further away from the aged serine towards the gorge bottleneck. Interestingly, the smaller dimethylamine group (**1**/**1-HCl**) and the similarly sized morpholine group (**4**/**4-HCl**) did not have as strong a recognition for the enzyme's active site.

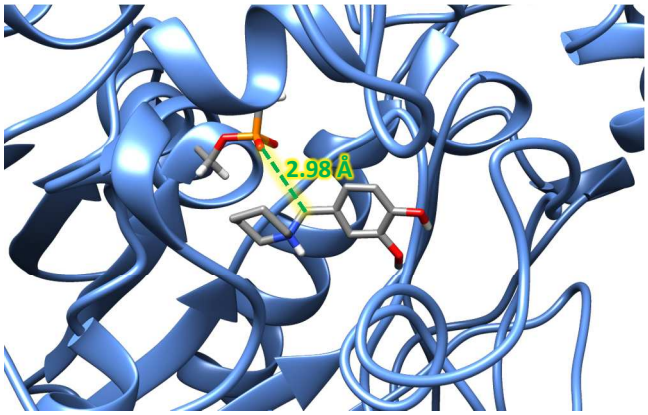


Figure 2. Docked pose of **3-HCl** in the aged AChE active site, displaying the putative reactive orientation of the benzylic carbon being less than 5 Å from aged serine residue.

With molecular docking results in hand, molecular dynamics (MD) simulations were performed subsequently to examine how the ligands noncovalently interact with the enzyme over the course of time. For each ligand, the three lowest energy snapshots from each of the thirteen aged AChE frames were chosen as the starting points for our MD simulations. Once again, of interest was the interaction between the reactive benzylic carbon of our QMPs and the target, phosphylated serine residue of aged AChE. Over the course of a brief 1 ns simulation for each starting orientation from docking, the QMP ligands could be further examined for their preference towards the aged active site versus other areas of the enzyme by establishing zoning criteria. Interestingly, once again the pyrrolidine (**2-HCl**) amine leaving group was the most promising ligand. Also, there was a strong preference for the protonated leaving groups to spend more time in the active site while the neutral leaving groups rarely stayed in the enzyme's active site (see Supporting Information).

We sought to evaluate whether our QMP ligands could bind into the active site of aged AChE; however, we were unable to develop a protocol to evaluate that binding as the aged AChE is not "active" to any known substrate. Instead, the binding affinity and selectivity of QMPs to the active site of AChE can be revealed by kinetics of reversible inhibition of native AChE, and as measured by competition kinetics for AChE's substrate. Indeed, the activity of electric eel AChE was monitored at room temperature using Ellman's assay in the presence of QMPs at varied concentrations ranging from 0 to 1.5 mM.[34] Electric eel AChE is a commercially available AChE homolog. Despite the difference between the gorge shapes of human and non-human AChE homologs, in this proof-of-concept study it is acceptable to qualitatively estimate the binding affinity using electric eel AChE. The concentrations of acetylthiocholine (ATC, used as the AChE substrate) were varied from 0 to 1.1 mM independently. Acquired data were processed with GraphPad Prism 6 for nonlinear regression. Details of the experimental procedure and calculations are included in the Supporting Information.

Table 2. Molecular Dynamics Results and Inhibition kinetics of electric eel AChE by QMPs

QMP	1-HCl	2-HCl	3-HCl	4-HCl
MD AS%*	44.8 (11.8)	50.5 (24.1)	44.6 (6.0)	37.4 (8.9)
V_{max} **(nM/s)**	61.2 ± 2.2	104.9 ± 4.2	80.3 ± 3.9	141.0 ± 8.9
K_m **(mM)**	0.032 ± 0.014	0.079 ± 0.014	0.062 ± 0.015	0.189 ± 0.037
K_i **(mM)**	0.080 ± 0.040	0.095 ± 0.024	0.106 ± 0.042	1.57 ± 0.83
α	34.9 ± 22.9	8.7 ± 4.0	3.1 ± 1.7	1.24 ± 0.94
IC_{50} **(mM)****	0.919	0.403	0.227	1.83

*Over a 1 ns simulation, the percentage of time ligand spent in the aged AChE active site (defined as the benzylic carbon of the QMP being 0-5 Å from the phosphylated serine residue (Supporting Information). Values of the corresponding neutral compounds are displayed in parentheses. **[acetylthiocholine] = 0.5 mM

As illustrated in Table 2, the K_i values ranged from 0.08 to 1.57 mM indicate overall weak inhibition of native AChE. The constant α is the ratio between the dissociation equilibrium constants of the enzyme-inhibitor-substrate complex and the enzyme-inhibitor complex. The greater α is than 1, the closer the inhibition mechanism is to competitive inhibition.[35] All QMPs showed α values greater than 1, especially **1-HCl** and **2-HCl**; thus, they are competitive inhibitors or near-competitive mix-type inhibitors[35] that selectively bind to the active site of native AChE.

The four hydrochloride compounds were subject to an *in vitro* test against methylphosphonate-aged AChE, which is the common aged product of many authentic V-agents and G-

[a] A newer structure (PDB ID: 4EY4), which is of higher resolution, was published after we initiated this study. However, alignment shows only trivial difference in coordinates (backbone RMSD = 0.708 Å).

agents. Electric eel AChE (Sigma Aldrich) was used in these studies. Each tested QMP (4 mM) was added to AChE that was aged with a soman analogue, originally reported by Amitai *et al*.[36] The negative and the positive controls prepared in parallel were aged and native AChE without QMPs. To determine the amount of residual inhibited AChE after the aging process, a 2-pralidoxime chloride (2-PAM) control was also prepared by mixing aged AChE with 4 mM 2-PAM.

In a similar manner to 2-PAM, fluoride ion at high concentration can function as a reactivator of AChE.[37,38] As an anion with simple structure, it is speculated to reactivate all inhibited/realkylated AChE without bias and not to compete with QMPs for AChE's active site. Therefore, NH_4F (4 mM) was also added to the reaction mixture to reactivate the realkylated AChE, in order to avoid re-aging after alkylation. After reaction for 24 h at 37 °C and pH 8.0, all samples were treated with 4 mM of 2-PAM to ensure the reactivation of any aged AChE that was (hopefully) realkylated. The resulting AChE activity was determined with Ellman's assay with ATC as a substrate. The relative activities of QMP-treated samples, the negative control and the 2-PAM control compared to the positive control are displayed in Figure 3 (see the Supporting Information for specific procedures and additional details). Unfortunately, none of our QMP substrates appear to realkylate the aged form of AChE to any significant extent; indeed, the QMP-treated samples have similar activities in the Ellman's assay and are less than 2-PAM by itself (Figure 3). (2-PAM is known not to reactivate the aged form of AChE.)[11-14]

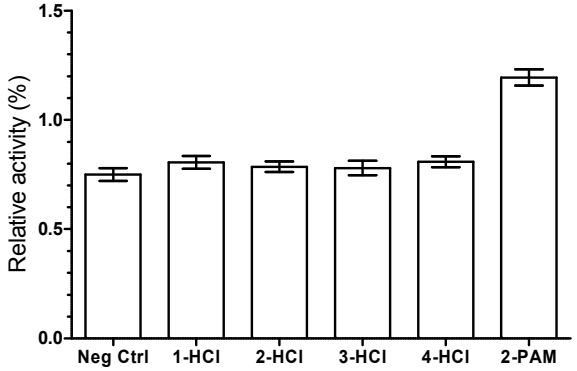


Figure 3. Result of AChE realkylation test. The relative activity of 2-PAM control was 1.2%, implying the near completeness of aging. Aged AChE samples treated with tested QMPs were not obviously more active than the negative control (Neg Ctrl).

In conclusion, we have shown the ability of a novel class of Quinone Methide Precursors (QMPs) to act as alkylating agents with a variety of model nucleophiles, including a model phosphonate to mimic the aged AChE active site. Molecular modeling studies confirm that this moiety has some affinity for the aged AChE active site, with a significant contribution of poses oriented in a potentially reactive conformation. The varied results of our experiments also suggest the tunability within our initial scaffold to modulate reactivity. With confirmation of the alkylated model phosphonate and preliminary computational and kinetic data completed, the hydrochloride QMPs described herein were further investigated to determine their efficacy towards reactivating aged AChE. Though not effective at present, this proof-of-concept model demonstrates the feasibility to develop potentially effective countermeasures against OP exposure using QMP-based AChE realkylators. Further studies are in progress in order to optimize this potential strategy and to reverse the effects of aging of AChE by OP agents.

Supporting Information. Please find further experimental details and computational protocols in the supporting information. This material is available free of charge via the Internet at http://pubs.acs.org. The content includes:

Synthesis and Characterization of QMPs
Alkylation of Model Nucleophiles
UV-Vis Studies
HPLC Data
Computational Methods
Determination of Inhibition Characteristics of QMPs against Native AChE
Procedures for Aging of AChE
Procedures for Realkylation and Reactivation of Aged AChE
NMR Spectra
Atom Coordinates and Parameters

AUTHOR INFORMATION

Corresponding Author

hadad.1@osu.edu

Funding Sources

National Institutes of Health (U01–NS087983) and the US Army (W81XWH-10-2-0044).

Notes

Any additional relevant notes should be placed here.

ACKNOWLEDGMENT

The authors would like to acknowledge financial support from the National Institutes of Health (U01–NS087983) and computing resources from the Ohio Supercomputer Center. Initial financial support of this project was obtained with resources from the US Army (W81XWH-10-2-0044).

ABBREVIATIONS

AChE: acetylcholinesterase. ATC: acetylthiocholine. MD: molecular dynamics. OP: organophosphorus. QM: quinone methide. QMP: quinone methide precursor. 2-PAM: 2-pyridine aldoxime methyl chloride.

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