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Novel Irreversible Butyrylcholinesterase Inhibitors: 2-Chloro-1-(substituted-phenyl)ethylphosphonic Acids

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Abstract—2-Chloroethylphosphonic acid (ethephon) as the dianion phosphorylates butyrylcholinesterase (BChE) at its active site. In contrast, the classical organophosphorus esterase inhibitors include substituted-phenyl dialkylphosphates (e.g., paraoxon) with electron-withdrawing aryl substituents. The chloroethyl and substituted-phenyl moieties are combined in this study as 2-chloro-1-(substituted-phenyl)ethylphosphonic acids (**1**) to define the structure–activity relationships and mechanism of BChE inhibition by ethephon and its analogues. Phenyl substituents considered are 3- and 4-nitro, 3- and 4-dimethylamino, and 3- and 4-trimethylammonium. Phosphonic acids **1** were synthesized via the corresponding *O*,*O*-diethyl phosphonate precursors followed by deprotection with trimethylsilyl bromide. They decompose under basic conditions about 100-fold faster than ethephon to yield the corresponding styrene derivatives. Electron-withdrawing substituents on the phenyl ring decrease the hydrolysis rate while electron-donating substituents increase the rate. The 4-trimethylammonium analogue has the highest affinity ($K_i = 180$ μM) and potency ($IC_{50} = 19$ μM) in first binding reversibly at the substrate site (possibly with stabilization in a dianion–monoanion environment) and then progressively and irreversibly inhibiting the enzyme activity. These observations suggest dissociation of chloride as the first and rate-limiting step both in the hydrolysis and by analogy in phosphorylation of BChE by **1** bound at the active site.

Introduction

Butyrylcholinesterase (BChE; EC 3.1.1.8) activity is progressively and irreversibly inhibited on phosphorylation at Ser-198 by the classical triester phosphates such as paraoxon[1–3] and surprisingly also by 2-chloroethylphosphonic acid (ethephon) dianion.[4–6] Ethephon is one of the most important plant growth regulators serving as a source of the phytohormone ethylene on spontaneous decomposition at physiological pH.[7] The irreversible inhibition of BChE activity by ethephon and its close analogues is unique among phosphonic acids since they are usually not reactive species and act instead as reversible enzyme inhibitors.[8] In order to better understand the mechanisms involved, the present investigation considers the design, synthesis and properties of novel irreversible BChE inhibitors based on ethephon as the model.

Many ethephon analogues have been prepared with variations at the 1- or 2 position and enhanced reactivity but not greatly increased BChE inhibitory potency. 2-Substituted ethephon analogues have very short half-life times in neutral and basic aqueous conditions[9] and 2-butylethephon is a poor BChE inhibitor possibly due to rapid hydrolysis ($t_{1/2} < 5$ min) under the enzyme assay conditions (pH 7.4).[6] Thus, only the 1-position was considered as a variable in the present studies. On the other hand, organophosphorus (OP) triester cholinesterase (ChE) inhibitors like paraoxon contain a good leaving group, usually a substituted-phenyl moiety with an electron-withdrawing substituent. This type of structure provides high affinity at the binding site and enhanced reactivity, most probably through interactions with the aromatic residue-rich area in the active site gorge.[10] Accordingly, 2-chloro-1-(substituted-phenyl)-ethylphosphonic acids (**1**) are particularly interesting since they combine the structural features of the paraoxon- and ethephon-type compounds (Fig. 1). Phenyl substituents that proved useful in studying the structure–activity relationships of paraoxon-type inhibitors (specifically nitro, dimethylamino and trimethylammonium

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groups),[1–3] both 3- and 4-positions, were selected for study in the **1** series to identify structural features conferring high potency as BChE inhibitors and to probe the inhibitory mechanism. We show in this investigation that substitution at the 4-position with electron-withdrawing groups provides improved BChE inhibitory potency, not directly related to enhanced hydrolysis rate, and that phosphonic acids **1** first bind competitively then inhibit progressively and irreversibly. On this basis, we propose that BChE phosphorylation by bound **1** involves the reactive species produced during spontaneous chloride dissociation as the first and rate-limiting step.

Results

Synthesis of 2-chloro-1-(substituted-phenyl)ethylphosphonic acids (1a–1g)

The strategy required consideration of the known lability of 2-haloethylphosphonic acids under neutral and basic aqueous conditions[11,12] and difficulties in purifying the acids. Accordingly, in a two-step procedure, diethyl phosphonates (**2**) were prepared as precursors and purified by chromatography, then converted to the target phosphonic acids (**1**) by McKenna reaction (Scheme 1).[13,14] Treatment of **2a**–**2g** with trimethylsilyl bromide (TMSBr) followed by hydrolysis with MeOH/water provided phosphonic acids **1a**–**1g** in essentially pure form and quantitative yields (byproducts were removed completely under reduced pressure). These phosphonic acids were used for chemical reactivity studies and enzyme assay.

Precursor diethyl 2-chloro-1-phenylethylphosphonate (**2a**) can be considered as the parent compound and was prepared as shown in Scheme 2. Diethyl benzylphosphonate was deprotonated by *n*-BuLi and reacted with paraformaldehyde to provide alcohol **3**, which was converted to the chloride **2a** by the general method with triphenylphosphine (Ph_3P) and CCl_4.

This procedure was not suitable to synthesize **2b** and **2c**, possibly due to the lower reactivity of the generated anion to form the corresponding alcohol when treated with paraformaldehyde. An alternative and more general route (Scheme 3) was explored using readily available starting materials. The key step is a coupling reaction of the phosphonate anion and substituted iodobenzene,[15,16] with copper(I) iodide as the catalyst. Thus triethyl phosphonoacetate was deprotonated by NaH and reacted with 3-nitro- or 4-nitroiodobenzene, in the presence of copper(I) iodide,[17] to produce intermediate **4**, which was reduced by borane at room temperature to give the corresponding alcohol (**5**).[18] The alcohol was converted to chloride **2** by reaction with Ph_3P and CCl_4.

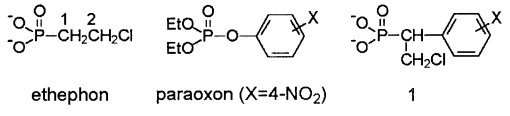
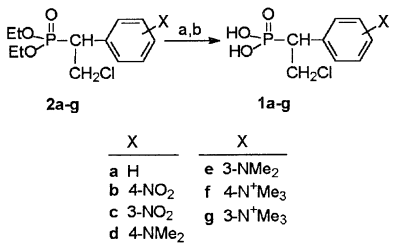


Figure 1. Structures of ethephon (the plant growth regulator) as the dianion, paraoxon (a classical organophosphorus esterase inhibitor), and 2-chloro-1-(substituted-phenyl)ethylphosphonic acids (**1**) which combine the structural features of the first two compounds.



Scheme 1. (a) TMSBr, MeCN, 60 C, overnight; (b) MeOH/H_2O, rt, 1 h.

Syntheses of **2d** and **2e** were achieved from **2b** and **2c** by catalytic hydrogenation to convert the nitro groups to amino groups, which were further transformed by reductive alkylation[19] with formaldehyde and $NaCNBH_3$ to **2d** and **2e**, respectively (Scheme 3). Reaction of **2d** and **2e** with MeI provided the corresponding trimethylammonium-substituted diethylphosphonate precursors **2f** and **2g**.

Relation of structure to hydrolysis rate

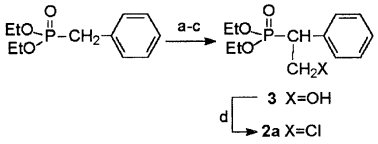
Hydrolysis of 1a–1g at pH 7.4. Phosphonic acids **1a**–**1g** decompose at pH 7.4 where the dianion is the major form observed by ^{31}P NMR. The decomposition products were identified as the corresponding styrene analogues by ^{1}H NMR and as phosphate by ^{31}P NMR in comparison with an authentic standard (Scheme 4). The formation of styrene analogues allowed the reaction to be easily followed by UV absorption change (Fig. 2). Kinetic analysis showed a first-order reaction, that is, the logarithm value of the decreasing concentration of phosphonic acid was linear with reaction time.

Compared with ethephon, analogues **1a**–**1g** decompose 50- to 163-fold faster (Table 1). The substituent on the phenyl ring affects the decomposition to a much less extent than the substituent at the 1-position, following the order of **1d** > **1e** > **1a** > **1b** ≈ **1c** ≈ **1f** ≈ **1g**. Compounds with electron-donating groups decompose fas-

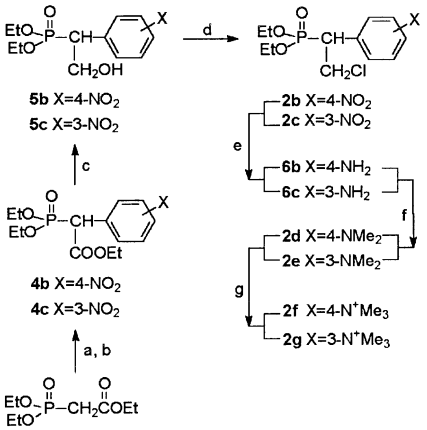
Table 1. Hammett's σ constants and hydrolysis rates at pH 7.4

Compound			Rate[a]	
No.	X	σ[b]	k (min^{-1}) × 10^3	$t_{1/2}$ (min)
1a	H	0	33	21
1b	4-NO_2	0.81	21	32
1c	3-NO_2	0.71	22	32
1d	4-NMe_2	−0.63	65	11
1e	3-NMe_2	−0.10	42	16
1f	4-N^+Me_3	0.82	22	31
1g	3-N^+Me_3	0.88	20	34
Ethephon	—	—	0.4	1735

[a]Determined by rate of formation of styrene derivatives in pH 7.4 phosphate buffer (100 mM) by UV absorption (see Fig. 2) or of phosphate in 2,4,6-collidine (0.7 M)–HCl buffer (pH 7.4) by ^{31}P NMR (for ethephon only), both at 23 °C.
[b]From ref 20.



Scheme 2. (a) *n*-BuLi, THF, −78 °C, 15 min; (b) $(HCHO)_n$, −78 °C to rt; (c) H^+/H_2O; (d) PPh_3, CCl_4, reflux, 1 h.



Scheme 3. (a) NaH, DMF, rt, 10 min; (b) ArI, CuI; (c) BH_3–THF, rt, 2 days; (d) PPh_3, CCl_4, reflux, 1 h; (e) H_2/Pd/C, MeOH, rt, 2 h; (f) HCHO, $NaCNBH_3$, MeCN, HOAc, rt, 2.5 h; (g) MeI, $MeNO_2$, rt, 2 days.

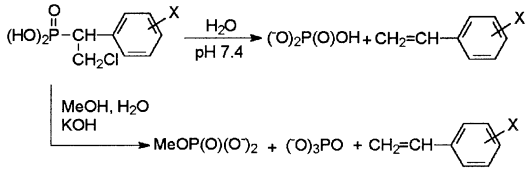
ter. The highest rate is for compound **1d** (X = 4-NMe_2) with a half-life time of 11 min. Compounds with electron withdrawing groups, for example, **1b**, **1c**, **1f**, and **1g**, have slower and almost the same decomposition rates.

Phosphorylation of MeOH. In a solution of MeOH with K_2CO_3, phosphonic acid **1a** decomposes to the styrene derivative with phosphorylation of MeOH to methyl phosphate. Similarly, **1a** in 50% aq MeOH with KOH gives methyl phosphate and phosphate (molar ratio 1:4) (Scheme 4).

Relation of structure to BChE inhibition

IC_{50}. Potencies as IC_{50} values were determined after 90 min incubation of BChE with the inhibitor as a compromise between the relatively short half-lives of the compounds (see above) and slow inhibition rates (considered below). The IC_{50} values ranged from 19 to 468 μM (Table 2). The 4-trimethylammonium compound (**1f**) was most potent, 14-fold better than ethephon, and the 4-nitro compound was second in activity. The least potent were **1e**, with a 3-dimethylamino group, and the parent **1a**. Obviously, an electron-withdrawing group at the 4-position greatly increases the potency (using compound **1a** as the standard for comparison). Introduction of an electron-withdrawing group at the 3- position also increases the potency but to a much less extent based on comparing **1b** with **1c** and **1f** with **1g**.

Progressive and irreversible inhibition. Phosphonic acids **1a**–**1g** (at micromolar concentrations) inhibit BChE (at an extremely low assay level) in a progressive manner;



Scheme 4. Hydrolysis and methanolysis of 2-chloro-1-(substituted-phenyl)ethylphosphonic acids.

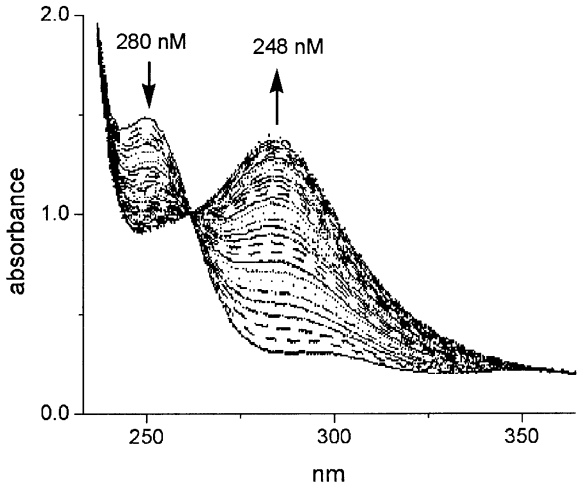


Figure 2. Time course of spectral change for decomposition of phosphonic acid **1d** (λ_{max} 248 nm, 0.1 mM) to 4-dimethylaminostyrene (λ_{max} 280 nm) in pH 7.4 100 mM phosphate buffer. Curves recorded at 1 min intervals.

the enzyme activity decreases as the incubation time increases. As an example, **1a** (0.3 mM) showed an activity loss of 20% after 60 min and 65% after 360 min (Fig. 3). Interestingly, the irreversible inhibition continues to increase even at a time of up to 20-fold the $t_{1/2}$ when almost all of the phosphonate dianion in solution is gone. Although this could conceivably be due to inhibition by the decomposition products rather than the parent compound, this was not the case. When BChE was incubated with the decomposition products, little enzyme inhibition was observed, for example, IC_{50} > 1000 μM with 24 h preincubation of the most potent **1b** or **1f** in buffer prior to addition of BChE. The irreversible manner of the inhibition was also indicated by the lack of recovery of enzyme activity when the inhibitor (**1a**) was removed from the solution.

Table 2. IC_{50} and K_i values

Compound		IC_{50} (μM)	K_i (μM)
No.	X		
1a	H	434±88	1443
1b	4-NO_2	42±2	616
1c	3-NO_2	124±20	1030
1d	4-Nme_2	179±11	593
1e	3-Nme_2	468±6	1076
1f	4-N^+Me_3	19±1	180
1g	3-N^+Me_3	159±25	561
Ethephon	—	267±2[a]	–

[a]From ref 6.

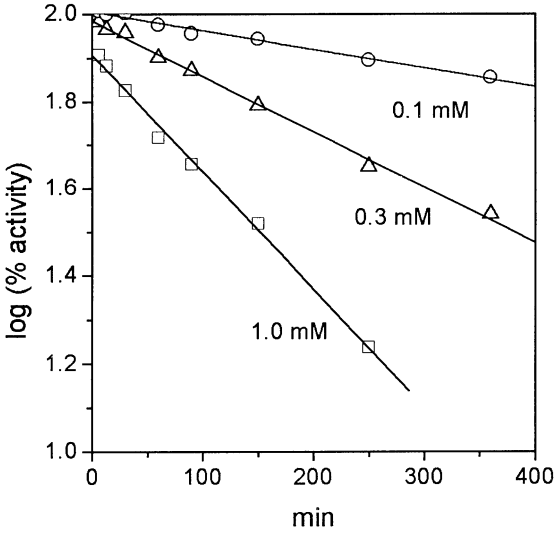


Figure 3. Effect of **1a** concentration on rate of inhibition of BChE activity. Enzyme activity as the logarithm value is plotted as a function of incubation time. In the absence of inhibitor, no deactivation of enzyme was observed during the time scale shown in the plot.

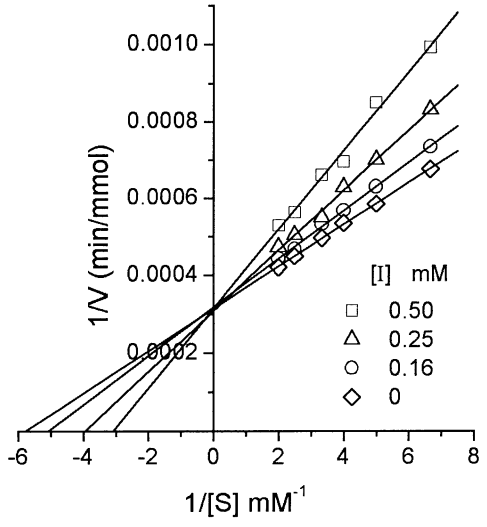


Figure 4. Determination of K_i of phosphonic acid **1g** with BChE at assay times of 0.5–1 min. For each inhibitor concentration, the inverse of the velocity is plotted as a function of the inverse of substrate concentration. $K_i = [I]/[K_m(app)/K_m - 1]$. $K_m(app)$ determined with inhibitor and BTCh and K_m with BTCh alone.

Competitive inhibition. The relatively slow irreversible deactivation by **1a**–**1g** made it possible to determine the binding type and K_i between BChE and inhibitor. On a short time scale, compounds **1a**–**1g** are competitive inhibitors, that is, as inhibitor concentration [I] increases, $K_{m(app)}$ increases but V_{max} remains constant (Fig. 4). K_i varies with the substituents on the phenyl ring. Generally, substitution at the 4-position gives higher affinity than at the 3-position with increasing K_i in the order of **1b** < **1c**, **1d** < **1e** and **1f** < **1g** (Table 2).

Discussion

Mechanism of hydrolysis

Spontaneous decomposition of **1a**–**1g** under physiological conditions involves the unusual property of 2-haloethylphosphonic acids to break the phosphorus–carbon bond. An early suggested mechanism[21] involved intramolecular attack of the phosphonate oxygen anion on the β-carbon to form a 'β-phostone' intermediate but this reaction mechanism was not consistent with later kinetic studies.[22,23] Three other proposed mechanisms are considered here for ethephon and its analogues (Fig. 5). Mechanism (**A**) is similar to the hydrolysis of most OP triesters by attack of a nucleophile (H_2O or serine hydroxyl in BChE) on phosphorus (through a pentacoordinated phosphorus transition state or direct displacement to break the phosphorus–carbon bond) coupled to the release of ethylene and chloride.[11,24] Two observations rule against this mechanism. First, the Hammett correlation[20] for phosphonic acids **1** shows that an electron-withdrawing group on the phenyl ring decreases the hydrolysis rate and an electron-donating group increases the rate with a negative ρ value (−0.32; Fig. 6), supporting an electron-deficient center in the rate-limiting step. This is opposite to the Hammett correlation in the paraoxon series ($\rho > 0$)[3] with an established mechanism by direct attack of a nucleophile on phosphorus, indicating different mechanisms for the hydrolysis of these two types of compounds.[25] Second, considering the steric effect of the substituted-phenyl group, a hydrolysis rate lower than that of ethephon would be expected for phosphonic acids **1** by mechanism (**A**), contrary to the experimental results.

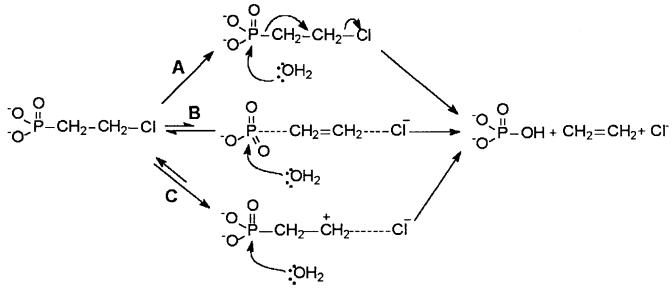


Figure 5. Proposed mechanisms for hydrolysis of ethephon and by analogy for phosphorylation of BChE (serine hydroxyl replaces hydroxyl anion); (A) nucleophilic displacement at phosphorus; (B) concerted dissociation via metaphosphate; (C) dissociation of chloride as first and rate-limiting step.

The other two mechanisms are based on dissociation of the phosphonic acid dianion. Mechanism (**B**) involves a concerted dissociation process to metaphosphate as the highly reactive intermediate,[11,23] which is trapped instantly by solvent (H_2O) or other nucleophile (e.g., serine hydroxyl) to give phosphorylated products. Mechanism (**C**) instead involves stepwise dissociation of chloride as the first and rate-limiting step so that the intermediate is a partially dissociated species rather than free metaphosphate.[9] Both mechanisms (**B**) and (**C**) are consistent with the observed substituent effects due to the developing positive charge from chloride dissociation. The phenyl group can stabilize the positive charge through σ–π conjugation and thus phosphonic acids **1** have much higher hydrolysis rates (about 100-fold) than ethephon. An electron-donating group on the phenyl ring can provide extra stabilization of the positive charge center resulting in a relatively higher reactivity. However, substitution at the 2-position of ethephon has a much greater effect ($\sim 10^4$-fold)[9,23] than substitution at the 1-position (this study), suggesting that the positive charge is located at the 2-carbon not the 1-carbon in the rate-limiting step. In addition, ethephon reacts with water at a very different rate than with isopropanol or *tert*-butanol,[26] implying that the intermediate is still sensitive to steric factors. In this respect, pathway (**C**), in which dissociation of chloride is the first and rate-limiting step and a partially dissociated intermediate serves as the reactive species, appears more consistent with the kinetic behavior.

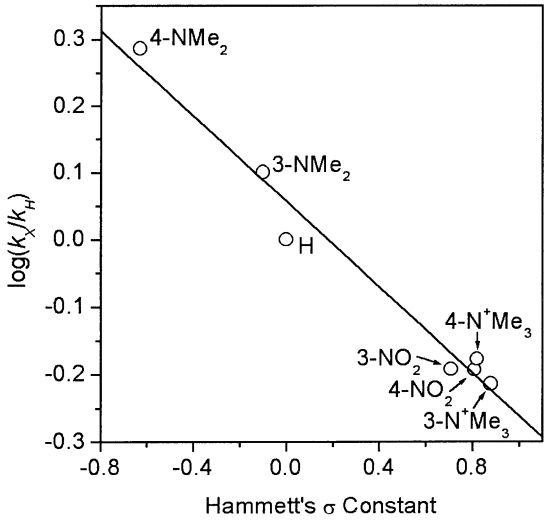


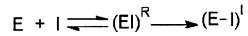
Figure 6. Hammett correlation of the hydrolysis of **1** at pH 7.4. The slope is $\rho = -0.32$, indicating a positive charge developing at the reaction center in the activated complex. See Table 1 for data.

Comparison of BChE inhibition by 1a–1g and triester OPs

Inhibition of ChE by triester OPs involves a two-stage process: reversible binding of inhibitor (I) with enzyme (E) to form the Michaelis complex $(EI)^R$ then phosphorylation of the enzyme to give a poorly reversible or irreversible derivative $(E\text{-}I)^I$ (Scheme 5).[1,27] BChE inhibition by phosphonic acids **1a**–**1g** follows the same course with reversible binding as competitive inhibitors on a short time scale and irreversible inhibition on a long time scale. Compared with the OP triesters, the irreversible inhibition by **1a**–**1g** and ethephon requires hours rather than minutes.

Competitive inhibition of butyrylthiocholine (BTCh) hydrolysis by phosphonic acids **1** shows that they share a common binding site on BChE. No correlation was found between the electronic effects of the phenyl substituents and K_i values. However, higher affinities were observed for 4-substituted than the corresponding 3-substituted compounds.

Inhibitor potency determined as IC_{50} represents the final result of irreversible inhibition as opposed to K_i values for reversible binding. Much enhanced potency



Scheme 5. Two-step process for ChE inhibition by triester OP.

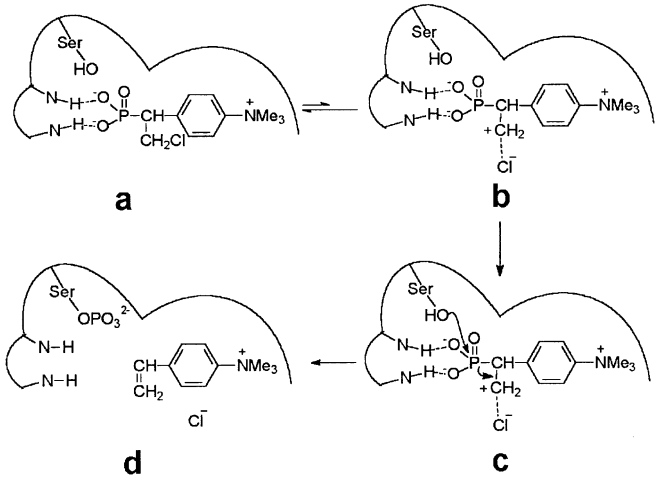


Figure 7. Postulated model for inhibition of BChE by phosphonic acid **1f** showing stabilization of the dianion as a tetrahedral intermediate analogue at the active site (a), undergoing slow dissociation of chloride (b), as the first and rate-limiting step in the phosphorylation (c), and with liberation of the styrene derivative (d).

for **1b** and **1f** fulfills our expectation that electron-withdrawing substituents at the phenyl ring can increase the potency as observed also in the triester OP inhibitor series; however, the higher potency is not related to a higher hydrolysis rate as opposed to the OP triester inhibitors.[3] Compound **1f** with the 4-trimethylammonium group has the highest potency ($IC_{50} = 19$ μM) but the lowest hydrolysis rate. The high potency is presumably due to the high affinity for ammonium cation-active site interactions as observed for many reversible and irreversible inhibitors.[2,3] Compounds **1c** and **1g** also with strong electron-withdrawing groups but at the 3-position have much lower potency, consistent with the profile of the observed K_i values. It appears that the steric effect of the substituent plays an important role in determining potency. Substitution at the 3-position could make the molecule more bulky and then more difficult to access the enzyme active center for irreversible inhibition.

Possible model for interaction between BChE and phosphonic acids 1a–1g

The irreversible inhibition of BChE is presumably through phosphorylation as suggested by postlabeling experiments with a labeled triester OP[6] and established in studies with [^{33}P]ethephon.[28] Similar to hydrolytic decomposition, the phosphonic acid bound at the enzyme active site may produce a highly reactive species serving as the phosphorylating agent for the serine hydroxyl or solvent molecule. The unique inhibition behavior of phosphonic acids **1**, in which inhibition continues to increase even when almost all of the phosphonic acid dianion in solution is gone, suggests some mechanism for localized stabilization of the dianion (Fig. 7). The main chain nitrogens in the oxyanion hole might stabilize the negatively charged phosphonic dianion by hydrogen bonding as they do for a tetrahedral intermediate.[29] The phosphonic acid could also exist in its monoanion form due to its pK_a change in non-aqueous environment[23,30] or the pH of the enzyme microenvironment is different from the solution pH. The monoanion form is much more stable than the dianion form and serves as a potential long-term phosphorylating agent and irreversible inhibitor.

Conclusions

A series of 2-chloro-1-(substituted-phenyl)ethylphosphonic acids **1** has been designed and synthesized as candidate irreversible BChE inhibitors. These phosphonic acids have the features of transition state analogues for reversible binding and triester OPs for irreversible phosphorylation. BChE inhibition by **1** is a two-stage process with reversible binding at the substrate site followed by irreversible phosphorylation, which is presumably through the same mechanism as it hydrolyzes in aqueous media involving dissociation of chloride as the first and rate-limiting step. At physiological pH, phosphonic acids **1** appear to be stabilized by the microenvironment of the enzyme active site. These findings are an example of combining a binding moiety and a reactive center to generate an irreversible inhibitor for BChE, in a manner which may also be applicable to other enzyme systems.

Experimental

Chemicals and general methods

Chemicals were obtained from commercial sources and used without further purification, unless otherwise noted. THF was distilled from sodium-benzophenone ketyl. Column chromatography was performed on silica gel. ^{1}H, ^{13}C and ^{31}P NMR spectra were recorded on a Bruker AMX-300 spectrometer at 300, 75, and 121.5 MHz, respectively. Chemical shifts for ^{1}H and ^{13}C are relative to the solvent chemical shifts and for ^{31}P is relative to 85% H_3PO_4 ($\delta = 0$ ppm). Fast atom bombardment (FAB)-high resolution mass spectrometry (HRMS) was performed by the University of Notre Dame Mass Spectrometry Facility (Notre Dame, IN, USA). Melting points were measured on a Fisher–Johns melting point apparatus without correction. Spectrophotometric determinations of BChE activity and the hydrolysis rate of **1** were made with a Hewlett Packard 8452A diode array spectrometer.

Synthesis

Diethyl 2-hydroxy-1-phenylethylphosphonate (3). A solution of *n*-BuLi (1.6 M, 5 mL) in hexane was added dropwise into diethyl benzylphosphonate (1.27 g, 5.6 mmol) in THF (12.0 mL) at −78 °C under N_2. The solution was stirred at this temperature for 15 min until a bright yellow color appeared. Paraformaldehyde (0.4 g, 13.3 mmol) was then added and the cooling bath was removed. The reaction was continued at room temperature with stirring for 30 min until the yellow color disappeared completely. The solution was acidified by HCl (2 N × 5 mL), extracted with CH_2Cl_2 (3 × 50 mL) and dried with anhydrous Na_2SO_4. Removal of the solvent followed by chromatography with $CHCl_3$ and MeOH (25:1) gave **3** (1.09 g, 76%). ^{31}P NMR ($CDCl_3$): δ +27.2; ^{1}H NMR ($CDCl_3$): δ 7.34–7.30 (m, 5H), 4.22–3.82 (m, 6H), 3.32 (dt, 1H, $J = 6.7$, 21.5 Hz), 1.29 (t, 3H, $J = 7.2$ Hz), 1.13 (t, 3H, $J = 7.2$ Hz); ^{13}C NMR ($CDCl_3$): δ 134.2 (d, $J = 6.7$ Hz), 129.1 (d, $J = 6.7$ Hz), 128.6, 127.5, 62.8 (d, $J = 6.7$ Hz), 62.5, 62.1 (d, $J = 6.7$ Hz), 47.6 (d, $J = 149.8$ Hz), 16.3 (d, $J = 7.8$ Hz), 16.2 (d, $J = 7.8$ Hz); FAB-HRMS calcd for $C_{12}H_{20}O_4P$ (MH^+): 259.1099, found: 259.1107.

Diethyl 2-chloro-1-phenylethylphosphonate (2a). Into a solution of phosphonate **3** (0.9 g, 3.5 mmol) in CCl_4 (10 mL) was added Ph_3P (1.3 g, 5.0 mmol). The solution was refluxed for 1 h and then cooled to room temperature. Hexane (5 mL) was added and the solution was filtered. The residue was washed by a mixture of CCl_4 and hexane (10 mL). Chromatography of the residue from evaporation of the combined filtrate gave **2a** (0.48 g, 50%). ^{31}P NMR ($CDCl_3$) δ +24.4; ^{1}H NMR ($CDCl_3$) δ 7.38–7.37 (m, 5H), 4.23–3.87 (m, 4H), 3.76–3.67 (m, 2H), 3.42 (ddd, 1H, $J = 4.1$, 11.3, 23.1 Hz), 1.31

(t, 3H, = 6.9 Hz), 1.10 (t, 3H, J = 6.9 Hz); ^{13}C NMR ($CDCl_3$) δ 133.6, 129.2 (d, J = 6.7 Hz), 128.6, 127.9, 63.1 (d, J = 6.7 Hz), 62.3 (d, J = 6.7 Hz), 48.2 (d, J = 132.5 Hz), 43.6 (d, J = 6.7 Hz), 16.4, 16.1; FAB-HRMS calcd for $C_{12}H_{19}ClO_3P$ (MH^+): 277.0760, found: 277.0766.

Ethyl α - diethoxyphosphinyl - α - (4 - nitrophenyl)acetate (4b).[17] To a suspension of NaH (80% dispersion in mineral oil, 0.66 g, 22 mmol) in DMF (10 mL) was added a solution of triethyl phosphonoacetate (4.48 g, 20 mmol) at room temperature. After stirring for 10 min, 1-iodo-4-nitrobenzene (1.95 g, 10 mmol) and copper(I) iodide (4.4 g, 23 mmol) were added in turn. The mixture was stirred at 100 °C overnight, after which the reaction was quenched by 10% HCl. The solution was filtered and extracted with EtOAc. The organic layer was combined, dried with Na_2SO_4 and evaporated in vacuum. The residue was subjected to chromatography with $CHCl_3$ and EtOAc (6:1) to give **4b** (0.91 g, 35%). ^{31}P NMR ($CDCl_3$) δ +17.9; ^{1}H NMR ($CDCl_3$) δ 8.20 (d, 2H, J = 8.2 Hz), 7.71 (dd, 2H, J = 2.0, 8.7 Hz), 4.36 (d, 1H, J = 24.1 Hz), 4.27–4.02 (m, 6H), 1.29 (t, 3H, J = 7.2 Hz), 1.28 (t, 3H, J = 6.9 Hz), 1.22 (t, 3H, J = 6.9 Hz); ^{13}C NMR ($CDCl_3$) δ 167.2 (d, J = 4.7 Hz), 148.2, 139.2, 131.3 (d, J = 4.8 Hz), 124.2, 64.3 (d, J = 5.9 Hz), 64.1 (d, J = 6.9 Hz), 63.0, 52.8 (d, J = 131.3 Hz), 16.9, 14.7; FAB-HRMS calcd for $C_{14}H_{21}NO_7P$ (MH^+): 346.1056, found: 346.1066.

Ethyl α - diethoxyphosphinyl - α - (3 - nitrophenyl)acetate (4c).[17] Reaction of triethyl phosphonoacetate with 1-iodo-3-nitrobenzene by the above method gave **5** in 67% yield. ^{31}P NMR ($CDCl_3$) δ +18.0; ^{1}H NMR ($CDCl_3$) δ 8.39 (s, 1H), 8.18 (d, 1H, J = 8.2 Hz), 7.90 (d, 1H, J = 7.7 Hz), 7.54 (dd, 1H, J = 7.7, 8.2 Hz), 4.36 (d, 1H, J = 24.1 Hz), 4.29–4.07 (m, 6H), 1.33–1.22 (m, 9H); ^{13}C NMR ($CDCl_3$) δ 166.7, 148.2, 135.8, 133.4, 129.3, 124.7 (d, J = 6.7 Hz), 122.9, 63.6, 63.4 (d, J = 6.7 Hz), 62.3, 51.8 (d, J = 132.5 Hz), 16.2, 14.0; FAB-HRMS calcd for $C_{14}H_{21}NO_7P$ (MH^+): 346.1056, found: 346.1055.

Diethyl 2 - hydroxy - 1 - (4 - nitrophenyl)ethylphosphonate (5b). Into **4b** (0.91 g, 2.6 mmol) was added a solution of borane in THF (1.0 M × 10 mL). The solution was stirred at room temperature for 2 days, then MeOH (10 mL) was added. The solution was concentrated after the evolution of hydrogen stopped. Chromatography of the residue with $CHCl_3$ and EtOAc (25:1) gave **5b** (0.66 g) in 87% yield. ^{31}P NMR ($CDCl_3$) δ +25.7; ^{1}H NMR ($CDCl_3$) δ 8.21 (d, 2H, J = 8.2 Hz), 7.55 (dd, 2H, J = 2.1, 8.7 Hz), 4.26–3.94 (m, 6H), 3.45 (dt, 1H, J = 6.4, 22.5 Hz), 1.32 (t, 3H, J = 7.2 Hz), 1.19 (t, 3H, J = 7.2 Hz); ^{13}C NMR ($CDCl_3$) δ 147.4, 142.5, 130.1 (d, J = 4.5 Hz), 123.7, 63.1 (d, J = 6.7 Hz), 62.6 (d, J = 6.7 Hz), 62.2, 47.6 (d, J = 134.8 Hz), 16.4 (d, J = 6.7 Hz); FAB-HRMS calcd for $C_{12}H_{19}NO_6P$ (MH^+): 304.0950, found: 304.0960.

Diethyl 2 - hydroxy - 1 - (3 - nitrophenyl)ethylphosphonate (5c). Reduction of **4c** (2.2 g, 6.4 mmol) with borane (1.0 M × 15 mL) at room temperature for 2 days afforded **5c** (1.61 g) in 83% yield. ^{31}P NMR ($CDCl_3$) δ +25.6; ^{1}H NMR ($CDCl_3$) δ 8.23 (d, 1H, J = 2.0 Hz), 8.16 (d, 1H, J = 8.2 Hz), 7.74 (d, 1H, J = 7.7 Hz), 7.53 (dd, 1H, J = 7.7, 8.2 Hz), 4.31–3.96 (m, 6H), 3.45 (dt, 1H, J = 6.7, 22.6 Hz), 1.32 (t, 3H, J = 7.2 Hz), 1.21 (t, 3H, J = 7.2 Hz); ^{13}C NMR ($CDCl_3$) δ 148.3, 136.9, 135.4, 129.5, 124.1 (d, J = 6.7 Hz), 122.5, 62.9 (d, J = 6.7 Hz), 62.6 (d, J = 6.7 Hz), 62.1, 47.2 (d, J = 134.8 Hz), 16.3 (d, J = 6.7 Hz), 16.2 (d, J = 6.7 Hz); FAB-HRMS calcd for $C_{12}H_{19}NO_6P$ (MH^+): 304.0950, found: 304.0955.

Diethyl 2-chloro-1-(4-nitrophenyl)ethylphosphonate (2b). Reaction of **5b** (0.66 g, 2.2 mmol) with Ph_3P (0.86 g, 3.3 mmol) in CCl_4 (10 mL) followed by workup and chromatography with $CHCl_3$ and MeOH (30:1) gave **2b** (0.53 g) in 76% yield. Mp 98–100 °C. ^{31}P NMR ($CDCl_3$) δ +22.4; ^{1}H NMR ($CDCl_3$) δ 8.24 (d, 2H, J = 8.2 Hz), 7.55 (dd, 2H, J = 2.1, 8.7 Hz), 4.24–3.85 (m, 6H), 3.56 (ddd, 1H, J = 4.1, 11.8, 23.6 Hz), 1.32 (t, 3H, J = 7.2 Hz), 1.17 (t, 3H, J = 7.2 Hz); ^{13}C NMR ($CDCl_3$) δ 147.6, 141.3 (d, J = 6.7 Hz), 130.2 (d, J = 4.5 Hz), 123.7, 63.3 (d, J = 6.7 Hz), 62.8 (d, J = 6.7 Hz), 48.2 (d, J = 130.3 Hz), 16.4 (d, J = 6.7 Hz); FAB-HRMS calcd for $C_{12}H_{18}ClNO_5P$ (MH^+): 322.0611, found: 322.0614.

Diethyl 2-chloro-1-(3-nitrophenyl)ethylphosphonate (2c). Reaction of **5c** (1.60 g, 5.3 mmol) with Ph_3P (2.10 g, 8.0 mmol) in CCl_4 (15 mL) followed by workup and chromatography with CH_2Cl_2 and MeOH (30:1) gave **2c** (1.36 g) in 80% yield. ^{31}P NMR ($CDCl_3$) δ +22.6; ^{1}H NMR ($CDCl_3$) δ 8.23 (d, 1H, J = 2.0 Hz), 8.21 (d, 1H, J = 8.7 Hz), 7.73 (d, 1H, J = 6.7 Hz), 7.58 (dd, 1H, J = 6.7, 8.7 Hz), 4.26–3.87 (m, 6H), 3.56 (ddd, 1H, J = 4.1, 11.8, 23.6 Hz), 1.32 (t, 3H, J = 7.2 Hz), 1.20 (t, 3H, J = 7.2 Hz); ^{13}C NMR ($CDCl_3$) δ 148.4, 135.9, 135.3, 129.6, 124.2 (d, J = 6.7 Hz), 122.9, 63.2 (d, J = 6.7 Hz), 62.8 (d, J = 6.7 Hz), 47.9 (d, J = 130.3 Hz), 43.0, 16.4 (d, J = 6.7 Hz), 16.3 (d, J = 6.7 Hz); FAB-HRMS calcd for $C_{12}H_{18}ClNO_5P$ (MH^+): 322.0611, found: 322.0601.

Diethyl 1 - (4 - aminophenyl) - 2 - chloroethylphosphonate (6b). A solution of **2b** (0.52 g, 1.6 mmol) in MeOH was hydrogenated with 10% Pd/C (100 mg) at room temperature for 2 h, after which the solution was filtered and concentrated, providing **6b** as a residue (0.48 g, 100%). Mp 97–98 °C; ^{31}P NMR ($CDCl_3$) δ +25.0; ^{1}H NMR ($CDCl_3$) δ 7.15 (dd, 2H, J = 2.1, 8.7 Hz), 6.69 (d, 2H, J = 8.2 Hz), 4.18–3.36 (m, 6H), 3.29 (ddd, 1H, J = 4.1, 12.3, 23.6 Hz), 1.31 (t, 3H, J = 6.9 Hz), 1.13 (t, 3H, J = 6.9 Hz); ^{13}C NMR ($CDCl_3$) δ 146.0, 130.1 (d, J = 6.7 Hz), 123.0 (d, J = 6.7 Hz), 115.4, 63.1 (d, J = 6.7 Hz), 62.2 (d, J = 9.0 Hz), 47.2 (d, J = 132.5 Hz), 44.0 (d, J = 9.0 Hz), 16.3 (d, J = 9.0 Hz), 16.2 (d, J = 6.7 Hz); FAB-HRMS calcd for $C_{12}H_{20}ClNO_3P$ (MH^+): 292.0869, found: 292.0874.

Diethyl 1 - (3 - aminophenyl) - 2 - chloroethylphosphonate (6c). Hydrogenation of **2c** (1.34 g, 4.2 mmol) in MeOH in the presence of 10% Pd/C (200 mg) gave **6c** (1.17 g) in 97% yield. ^{31}P NMR ($CDCl_3$) δ +24.6; ^{1}H NMR ($CDCl_3$) δ 7.14 (t, 1H, J = 7.7 Hz), 6.74 (d, 1H, J = 7.2 Hz), 6.73 (d, 1H, J = 3.6 Hz), 6.65 (d, 1H, J = 7.2 Hz), 4.17–3.67 (m, 6H), 3.30 (ddd, 1H, J = 4.9, 11.3, 23.6 Hz), 1.31 (t, 3H, J = 7.2 Hz), 1.11 (t, 3H, J = 7.2 Hz); ^{13}C NMR ($CDCl_3$) δ 146.2, 134.4, 129.5, 119.7, 115.9 (d,

$J=6.7$ Hz), 115.0, 63.2 (d, $J=6.7$ Hz), 62.2 (d, $J=6.7$ Hz), 48.0 (d, $J=130.3$ Hz), 43.7, 16.4, 16.2; FAB-HRMS calcd for $C_{12}H_{20}ClNO_3P$ (MH^+): 292.0869, found: 292.0860.

Diethyl 2-chloro-1-(4-dimethylaminophenyl)ethylphosphonate (2d). Into a solution of **6b** (0.29 g, 1.0 mmol), formaldehyde (37% aqueous solution, 1.0 mL) and $NaCNBH_3$ (0.2 g, 3.2 mmol) in MeCN (5.0 mL) was added HOAc (0.1 mL) dropwise. The mixture was stirred at room temperature for 2 h before the addition of another portion of HOAc (0.1 mL) and an additional stirring for 30 min. Ether (15 mL) was then added and the mixture was washed with saturated Na_2CO_3 (5 mL $\times$ 3) and brine (10 mL). The organic layer was dried with anhydrous Na_2SO_4 and solvents were removed by rotary evaporator. The residue was subjected to chromatography with $CHCl_3$ and MeOH (12:1) to give **2d** (0.30 g, 94%). ^{31}P NMR ($CDCl_3$) δ +25.2; ^1H NMR ($CDCl_3$) δ 7.22 (dd, 2H, $J=2.1$, 8.7 Hz), 6.72 (d, 2H, $J=8.7$ Hz), 4.19–3.87 (m, 5H), 3.76–3.67 (m, 1H), 3.31 (ddd, 1H, $J=4.1$, 11.3, 23.6 Hz), 2.95 (s, 6H), 1.31 (t, 3H, $J=7.2$ Hz), 1.13 (t, 3H, $J=6.9$ Hz) ^{13}C NMR ($CDCl_3$) δ 146.0, 130.1 (d, $J=6.7$ Hz), 123.0 (d, $J=6.7$ Hz), 115.4, 63.1 (d, $J=6.7$ Hz), 62.2 (d, $J=9.0$ Hz), 47.2 (d, $J=132.5$ Hz), 44.0 (d, $J=9.0$ Hz), 16.3 (d, $J=9.0$ Hz), 16.2 (d, $J=6.7$ Hz); FAB-HRMS calcd for $C_{14}H_{23}ClNO_3P$ (M^+): 319.1104, found: 319.1110.

Diethyl 2-chloro-1-(3-dimethylaminophenyl)ethylphosphonate (2e). Reductive alkylation of **6c** (1.15 g, 3.9 mmol) with a similar procedure for **2d** gave **2e** in 63% yield. ^{31}P NMR ($CDCl_3$) δ +25.0; ^1H NMR ($CDCl_3$) δ 7.21 (t, 1H, $J=8.2$ Hz), 6.69 (m, 3H), 4.21–3.88 (m, 4H), 3.76–3.68 (m, 2H), 3.35 (ddd, 1H, $J=4.6$, 11.3, 23.6 Hz), 2.96 (s, 6H), 1.32 (t, 3H, $J=6.7$ Hz), 1.12 (t, 3H, $J=6.7$ Hz); ^{13}C NMR ($CDCl_3$) δ 150.7, 134.1 (d, $J=6.7$ Hz), 129.2, 117.2 (d, $J=4.5$ Hz), 113.4 (d, $J=6.7$ Hz), 112.1, 63.1 (d, $J=6.7$ Hz), 62.2 (d, $J=6.7$ Hz), 48.5 (d, $J=130.3$ Hz), 40.5, 16.4 (d, $J=6.7$ Hz), 16.2 (d, $J=6.7$ Hz); FAB-HRMS calcd for $C_{14}H_{23}ClNO_3P$ (M^+): 319.1104, found: 319.1112.

Diethyl 2-chloro-1-(4-trimethylammoniumphenyl)ethylphosphonate iodide (2f). Into a solution of **2d** (0.3 g, 0.94 mmol) in $MeNO_2$ (5.0 mL) was added MeI (0.4 mL). The solution was stirred at room temperature for 60 h and then evaporated. The residue was washed with ether (3 $\times$ 10 mL) and then recrystallized from acetone and ether to give **2f** (0.30 g, 70%) as crystals. ^{31}P NMR (CD_3OD) δ +24.3; ^1H NMR (CD_3OD) δ 7.97 (d, 2H, $J=8.7$ Hz), 7.70 (dd, 2H, $J=2.6$, 9.2 Hz), 4.20–3.97 (m, 6H), 3.72 (s, 9H), 1.32 (t, 3H, $J=7.2$ Hz), 1.22 (t, 3H, $J=7.2$ Hz); ^{13}C NMR (DMSO-d_6) δ 147.1, 137.0, 131.8, 121.0, 64.3 (d, $J=9.0$ Hz), 64.1 (d, $J=9.0$ Hz), 57.6, 46.5 (d, $J=128.0$ Hz), 43.8, 16.9, 16.8. FAB-HRMS calcd for $C_{15}H_{26}ClNO_3P$ $(M-I)^+$: 334.1339, found: 334.1335.

Diethyl 2-chloro-1-(3-trimethylammoniumphenyl)ethylphosphonate iodide (2g). Reaction of **2e** (0.36 g, 1.1 mmol) with MeI (0.4 mL) in $MeNO_2$ (10 mL) at room temperature for 60 h gave **2g** (0.42 g, 80%) by recrystallization from acetone and ether. Mp 122–123 °C. ^{31}P NMR (CD_3OD) δ +24.3; ^1H NMR (CD_3OD) δ 7.99 (br s, 1H), 7.92 (m, 1H), 7.69–7.67 (m, 2H), 4.22–3.98 (m, 7H), 3.72 (s, 9H), 1.32 (t, 3H, $J=7.2$ Hz), 1.22 (t, 3H, $J=7.2$ Hz); ^{13}C NMR (CD_3OD) δ 148.9, 138.6, 132.7 (d, $J=4.5$ Hz), 131.9, 122.8 (d, $J=6.7$ Hz), 120.6, 65.0 (d, $J=9.0$ Hz), 64.7 (d, $J=6.7$ Hz), 58.1, 45.6 (d, $J=128.0$ Hz), 16.8 (d, $J=4.5$ Hz), 16.7 (d, $J=6.7$ Hz); FAB-HRMS calcd for $C_{15}H_{26}ClNO_3P$ $(M-I)^+$: 334.1339, found: 334.1326.

Conversion of phosphonate precursors 2a–2g to phosphonic acids 1a–1g. General procedure. Into a solution of phosphonate **2** ($\sim$ 2 mmol) in MeCN (10 mL) was added TMSBr (2.0 mL). The solution was stirred overnight at 60 °C followed by treatment with MeOH and water (5.0 mL) at room temperature for 1 h. Removal of the solvents gave phosphonic acids **1** as pure products with no pyrophosphonate derivatives as established by ^{31}P NMR.

2-Chloro-1-phenylethylphosphonic acid (1a). ^{31}P NMR (CD_3OD) δ +24.1; ^1H NMR (CD_3OCD_3) δ 7.46–7.29 (m, 5H), 4.32–4.25 (m, 1H), 4.14 (dt, 1H, $J=5.1$, 11.3 Hz), 3.67 (ddd, 1H, $J=3.6$, 11.3, 24.1 Hz); ^{13}C NMR (CD_3OD) δ 134.9, 130.2 (d, $J=6.7$ Hz), 129.2, 128.3, 49.0 (d, $J=128.0$ Hz), 44.3. FAB-HRMS calcd for $C_8H_{11}ClO_3P$ (MH^+) 221.0134, found: 221.0142.

2-Chloro-1-(4-nitrophenyl)ethylphosphonic acid (1b). ^{31}P NMR (CD_3OD) δ +19.8; ^1H NMR (CD_3OD) δ 8.22 (d, 2H, $J=8.2$ Hz), 7.62 (dd, 2H, $J=2.1$, 8.7 Hz), 4.24–3.85 (m, 2H), 3.56 (ddd, 1H, $J=4.1$, 11.8, 23.6 Hz); ^{13}C NMR (DMSO-d_6) δ 146.5, 144.2 (d, $J=6.7$ Hz), 130.4 (d, $J=4.5$ Hz), 123.0, 49.0 (d, $J=123.6$ Hz), 44.3 (d, $J=4.5$ Hz); FAB-HRMS calcd for $C_8H_{10}ClNO_5P$ (MH^+): 265.9985, found: 265.9989.

2-Chloro-1-(3-nitrophenyl)ethylphosphonic acid (1c). ^{31}P NMR ($CDCl_3$) δ +20.3; ^1H NMR ($CDCl_3$) δ 8.29 (d, 1H, $J=2.1$ Hz), 8.19 (br d, 1H, $J=7.7$ Hz), 7.80 (br d, 1H, $J=7.7$ Hz), 7.62 (t, 1H, $J=8.2$ Hz), 4.30–4.08 (m, 2H), 3.63 (ddd, 1H, $J=3.6$, 11.8, 23.6 Hz); ^{13}C NMR (DMSO-d_6) δ 147.7, 138.4 (d, $J=6.7$ Hz), 136.0 (d, $J=4.5$ Hz), 123.6 (d, $J=4.5$ Hz), 121.9, 48.3 (d, $J=123.6$ Hz), 44.3 (d, $J=4.5$ Hz); FAB-HRMS calcd for $C_8H_{10}ClNO_5P$ (MH^+): 265.9985, found: 265.9973.

2-Chloro-1-(4-dimethylaminophenyl)ethylphosphonic acid (1d). ^{31}P NMR (CD_3OD) δ +20.6; ^1H NMR (CD_3OD) δ 7.70 (d, 2H, $J=8.2$ Hz), 7.61 (dd, 2H, $J=2.1$, 8.7 Hz), 4.27–4.05 (m, 2H), 3.55 (ddd, 1H, $J=3.6$, 11.8, 23.6 Hz), 3.33 (s, 6H); ^{13}C NMR (DMSO-d_6) δ 142.2, 136.1, 130.5, 119.7, 48.4 (d, $J=123.6$ Hz), 45.2, 44.6 (d, $J=6.7$ Hz); FAB-HRMS calcd for $C_{10}H_{16}ClNO_3P$ (MH^+): 264.0556; found: 264.0541.

2-Chloro-1-(3-dimethylaminophenyl)ethylphosphonic acid (1e). ^{31}P NMR (CD_3OD) δ +20.6; ^1H NMR (CD_3OD) δ 7.72–7.60 (m, 4H), 4.27–4.09 (m, 2H), 3.59 (ddd, 1H, $J=4.1$, 11.3, 23.6 Hz), 3.33 (s, 6H); ^{13}C NMR (DMSO-d_6) δ 143.4, 138.2, 129.6, 129.1, 120.5, 118.4, 48.8 (d, $J=123.6$ Hz), 45.3, 44.4 (d, $J=6.7$ Hz); FAB-HRMS calcd for $C_{10}H_{16}ClNO_3P$ (MH^+): 264.0556, found: 264.0570.

2-Chloro-1-(4-trimethylammoniumphenyl)ethylphosphonic acid iodide (1f). ^{31}P NMR ($CDCl_3$) δ +20.6; ^{1}H NMR ($CDCl_3$) δ 7.93 (d, 2H, J=8.7 Hz), 7.66 (dd, 2H, J=2.6, 9.2 Hz), 4.27–4.05 (m, 2H), 3.70 (s, 9H), 3.65–3.52 (ddd, 1H, J=4.6, 10.7, 23.6 Hz); ^{13}C NMR (DMSO-d_6) δ 145.8, 138.1, 130.5, 120.0, 56.4, 48.3 (d, J=123.6 Hz), 44.5 (d, J=4.5 Hz); FAB-HRMS calcd for $C_{12}H_{19}ClO_3P$ $(M-I)^+$: 278.0713, found: 278.0715.

2-Chloro-1-(3-trimethylammoniumphenyl)ethylphosphonic acid iodide (1g). ^{31}P NMR ($CDCl_3$) δ +20.6; ^{1}H NMR ($CDCl_3$) δ 7.94 (br s, 1H), 7.89–7.88 (m, 1H), 7.68–7.63 (m, 2H), 4.26–4.15 (m, 2H), 3.71 (s, 9H), 3.65 (ddd, 1H, J=4.6, 10.7, 23.6 Hz); ^{13}C NMR (DMSO-d_6) δ 147.0, 138.4 (d, J=6.7 Hz), 130.6, 129.5, 121.2 (d, J=6.7 Hz), 118.5, 56.4, 48.8 (d, J=123.6 Hz), 44.3 (d, J=4.5 Hz); FAB-HRMS calcd for $C_{12}H_{19}ClO_3P$ $(M-I)^+$: 278.0713, found: 278.0715.

Hydrolysis of 1a–1g at pH 7.4

Identification of decomposition products in pH 7.4 buffer. A solution of **1a** (12 mg) in pH 7.4 phosphate buffer (100 mM, 2.0 mL) was kept at room temperature for 2 h and then extracted with $CDCl_3$. The styrene hydrolysis product in $CDCl_3$ was identified by ^{1}H NMR. The phosphorus-containing product from incubation of **1a** (2 mg) in pH 7.4 2,4,6-collidine–HCl buffer (0.7 M, 1.0 mL) for 2 h was identified as phosphate by ^{31}P NMR comparison with authentic standard.

Determination of hydrolysis rate. The hydrolysis rate of ethephon was determined by ^{31}P NMR in 2,4,6-collidine (0.7 M)–HCl buffer (pH 7.4). Hydrolysis rates of **1** were measured spectrophotometrically. A solution of **1** (10 mM in DMSO, 10 μL) was mixed with pH 7.4 phosphate buffer (100 mM, 1.0 mL). The reaction was followed by the absorbance change at 248 nm (**1a**, **1d**, **1e**, **1f**, **1g**) or 320 nm (**1b**) or 242 nm (**1c**) for 30∼60 min. Kinetic constants for these reactions were calculated according to Kezdy-Swinbourne[25] by plotting A_t versus $A_{t+\tau}$, where A_t is the absorbance at time t, $A_{t+\tau}$ is the absorbance at time $(t+\tau)$ and τ is a constant time interval, set to 1∼1.5 $t_{1/2}$ for greatest accuracy. Kinetic constants were calculated as $k=\ln(\text{slope})/\tau$.

Phosphorylation of MeOH. A solution of **1a** (10 mg) in CD_3OD or MeOH (0.5 mL) was treated with excess solid K_2CO_3 (20 mg) and monitored by ^{1}H or ^{31}P NMR, respectively. This reaction was repeated using MeOH/water (1:1) (1.0 mL) with KOH (20 mg). The molar ratio of methyl phosphate and phosphate formed after 2 h was determined by integration of the appropriate peak areas in the ^{31}P NMR spectra.

Enzyme studies

Materials. BChE from horse serum (highly purified, lyophilized powder, item C 1057), BTCh and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were obtained from Sigma Chemical Co (St. Louis, MO, USA). Stock solutions of phosphonic acids **1** were prepared in DMSO rather than in pH 7.4 phosphate buffer due to their hydrolytic instability.

Determination of IC_{50}. BChE activity was determined spectrophotometrically by the Ellman method.[31] BChE (0.1 units) was incubated with phosphonic acid **1** in pH 7.4 100 mM phosphate buffer (1.0 mL) for 90 min at 23 °C before addition of BTCh (0.3 mM) and DTNB (1.0 mM). Optical density was monitored at 412 nm for 5 min. Inhibitors were tested with a concentration series of 10, 33, 100, 333, 1000 μM in comparison with a control. IC_{50} values are reported as the average and standard error of three experiments. They were determined by plotting inhibitor concentration on a logarithm scale versus percent enzyme activity using iterative nonlinear least-squares regression with the Sigmaplot program (Jandel Scientific Software, San Rafael, CA, USA).

Effect of incubation time. A solution of BChE (2 units) in phosphate buffer (1.0 mL) was incubated with phosphonic acids **1** (100–1000 μM). Enzyme activity was measured by mixing an aliquot (10 μL) of the incubation mixture with DTNB (1.0 mM in 100 mM phosphate buffer, 1.0 mL) and BTCh (0.3 mM in 100 mM phosphate buffer, 1.0 mL). The reaction was followed at 412 nm for 5 min.

Determination of irreversible inhibition. A solution of BChE (2 units) in phosphate buffer (0.5 mL) was incubated with phosphonic acid **1a** (1000 μM) for 90 min and then passed through a desalting column (Bio-Rad, Hercules, CA, USA) to remove inhibitor. Enzyme activity of the eluent (1.0 mL) was measured as above.

Determination of K_i and K_m. K_i was determined by measuring the enzyme activity at various substrate levels in the presence of a known concentration of inhibitor. BChE (0.4 units) in DTNB solution (as above) was mixed with BTCh solution (0.3–1.0 mM in phosphate buffer, 1.0 mL) and inhibitor **1** (10 μL). The reaction was immediately followed for 0.5–1 min. The inhibition type was determined by a Lineweaver–Burk plot and K_m (without inhibitor) and $K_{m(app)}$ (with inhibitor) were obtained in the same way.

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