Characterization of the Transient Oxaphosphetane BChE Inhibitor Formed from Spontaneously-Activated Ethephon

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**Materials**

Ethephon (97%) was from ChemService. D$_2$O was from Cambridge Isotope Labs. All other compounds were from Sigma Aldrich. Potassium carbonate buffer (2 M) for $^{31}$P/$^1$H NMR time course was prepared in 90% H$_2$O/10% D$_2$O at pH 7.4 and for $^1$H and $^{31}$P NMR in 99% D$_2$O.

**Methods and Results**

*Comparison of Ethephon Preincubated in Potassium Carbonate and Potassium Phosphate Buffers*

Ethephon (10 mM) was dissolved in 100 mM potassium carbonate or potassium phosphate buffers. Aliquots were diluted to 0.11 mM and assayed for BChE inhibitory potency immediately after preparation and after 20.25 h. BChE activity was measured as reported previously (1). Results are given in Figure S1. See main article text for discussion.

![Figure S1](image-url): BChE inhibitory potency of fresh or 20 h incubated eethephon in carbonate (■) or phosphate (♦) buffer.
$^{31}\text{P NMR Kinetic Time Course}$

A 1 ml solution of ethephon (200 mM in 2 M potassium carbonate buffer) was added to an appropriate NMR tube. The sample was kept capped at 25°C and protected from light for the duration of the experiment. Degradation reactions were monitored by 600 MHz $^{31}$P NMR on a Bruker Avance 600 console with Bruker 14 T magnet. Quantitative measurements were made using 32 scans and a 10 s pulse delay. It was determined that these conditions optimized the spectral resolution disrupted by the Nuclear Overhauser effect and the production of gaseous ethylene and CO2 in solution. Spectra were acquired with Topspin 2.1 and processed (automatic Bernstein polynomial baseline correction, followed by manual phase correction, Savitsky-Golay and Whittaker smoothing, manual integration of peak areas, and normalization of total integrated peak area in each spectra to 100 %) using MestreNova 7.1.1. The % peak area was considered a measurement of relative concentration. The magnitudes of spectra in Figure S2 were normalized based on the area of the constant peak at 22.11 ppm. Chemical shifts are reported relative to the designated phosphate peak (Figure S2).

![Figure S2](image)

**Figure S2.** $^{31}$P NMR kinetic time course spectra for 200 mM ethephon degradation in 2 M pH 7.4 potassium carbonate buffer (upper figure) and log concentration-linear time plots of integrations of $^{31}$P NMR experimental data and concentration-time curves from nonlinear least squares fitting of equations 29-33 (see derivation below) to experimental data (lower figures). The average $R^2$ for the fit is 0.887.
Derivation of Concentration-Time Integrated Rate Equations:

The degradation of ethephon in aqueous buffers to produce the species in Scheme S1 could proceed through any of five mechanisms:

![Scheme S1](image)

Mechanism 1 consists of degradation of ethephon A to phosphate and ethylene D. Mechanism 2 consists of degradation of A to D in competition with degradation to the transient inhibitor B which further degrades to the hydrolysis product C. Mechanism 3 incorporates the possibility that B could degrade to D directly by an intramolecular Wittig reaction. Mechanism 4 further incorporates the reaction of B with D to generate a di-phosphate. Mechanism 5 is the special case of Mechanism 4 where no B is converted to D. All of the steps are expected to be irreversible based on the proposed structures.

Mechanism 4 is the most complicated. Mechanisms 1-3 and 5 are special cases of Mechanism 4 where the omitted rate constants are equal to zero. Thus, a derived set of rate equations for Mechanism 4 permits fitting of all of the five mechanisms to experimental data. The differential rate equations for Mechanism 4 are as follows:

\[
\begin{align*}
\frac{dA}{dt} &= -k_1[A] - k_3[A] = -(k_1 + k_3)[A] \\
\frac{dB}{dt} &= k_1[A] - k_2[B] - k_4[B] - k_5[B][D] \\
\frac{dC}{dt} &= k_2[B] \\
\frac{dD}{dt} &= k_3[A] + k_4[B] - k_5[B][D] \\
\frac{dE}{dt} &= k_5[B][D]
\end{align*}
\]

The differential equation \(\frac{dA}{dt}\) has the form of a simple first order differential rate equation with

\[
\frac{dA}{dt} = k_a[A] \quad \text{where} \quad k_a = -(k_1 + k_3):
\]

and therefore the integrated rate equation for the concentration of A from time 0 to t is:

\[
[A] = [A]_0 e^{-(k_a)t}
\]

The \(^{31}\text{P}\) NMR data indicate that at every observed time point in the degradation, the phosphate concentration is at least ten times the concentration of any other species which changes in concentration. Therefore, the second order reaction of B with D can be considered pseudo first order where:

\[
k_5[B][D] \approx k_5'[B]
\]

Substituting equation (7) into equations (2), (4) and (5) and combining the individual rate constants into overall rate constants for each chemical species results in the simplified differential rate equations:
\[ \frac{dB}{dt} = k_1[A] - (k_2 + k_4 + k_5)[B] \]  
(8)

\[ \frac{dB}{dt} = k_5[A] + (k_4 - k_5)[B] \]  
(9)

\[ \frac{dE}{dt} = k_5[B] \]  
(10)

For \( k_c = k_2 + k_4 + k_5 \) and \( k_b = k_1 \), the simplified general form of \( \frac{dB}{dt} \) is:

\[ \frac{dB}{dt} = k_b[A] - k_c[B] \]  
(11)

Substituting equation (6) into equation (11) yields:

\[ \frac{dB}{dt} = k_b[A]_0 e^{-(k_a)t} - k_c[B] \]  
(12)

which rearranges to

\[ \frac{dB}{dt} + k_c[B] = k_b[A]_0 e^{-(k_a)t} \]  
(13)

Assuming that [B] has the form:

\[ [B]_t = y(t) e^{-k_c t} \]  
(14)

differentiating

\[ \frac{d[B]}{dt} = -y(t)(k_c e^{-k_c t}) + e^{-k_c t} \left( \frac{dy}{dt} \right) \]  
(15)

substituting equation (14) into equation (15) and rearranging

\[ \frac{d[B]}{dt} + k_c[B]_t = e^{-k_c t} \left( \frac{dy}{dt} \right) \]  
(16)

equating the right side of equation (16) with the right side of equation (13)

\[ e^{-k_c t} \left( \frac{dy}{dt} \right) = k_b[A]_0 e^{-(k_a)t} \]  
(17)

rearranging

\[ \left( \frac{dy}{dt} \right) = k_b[A]_0 e^{-(k_a)t} (e^{+k_c t}) = k_b[A]_0 e^{(k_c - k_a)t} \]  
(18)

and integrating

\[ y(t) = \frac{k_b[A]_0}{k_c - k_a} e^{(k_c - k_a)t} + C \]  
(19)

At \( t=0, y=0 \) so: \( C = -\frac{k_b[A]_0}{k_c - k_a} \) and therefore:

\[ y(t) = \frac{k_b[A]_0}{k_c - k_a} \left( e^{(k_c - k_a)t} - 1 \right) \]  
(20)

Substituting (14) into (20) and rearranging

\[ [B] = \frac{k_b[A]_0}{k_c - k_a} \left( e^{(k_c - k_a)t} - 1 \right)e^{-k_c t} \]  
(21)

or more simply

\[ [B] = \frac{k_b[A]_0}{k_c - k_a} \left( e^{-k_a t} - e^{-k_c t} \right) \]  
(22)

Substituting (22) into (3) yields:

\[ \frac{dc}{dt} = k_2 \frac{k_b[A]_0}{k_c - k_a} \left( e^{-k_a t} - e^{-k_c t} \right) \]  
(23)

which integrates to:

\[ [C] = \frac{k_2 k_b[A]_0}{k_c - k_a} \left( \frac{e^{-k_c t}}{k_c} - \frac{e^{-k_a t}}{k_a} - \frac{1}{k_c} + \frac{1}{k_a} \right) \]  
(24)
Substituting (22) and (6) into (9) yields:
\[
\frac{dD}{dt} = k_2[A]_0 e^{-k_4t} + (k_4 - k_5') \frac{k_4[A]_0}{k_c-k_a}(e^{-k_4t} - e^{-k_5't})
\]
which integrates to:
\[
[D] = \frac{k_2[A]_0}{k_a} \left( 1 - e^{-k_4t} \right) + \frac{(k_4-k_5')k_4[A]_0}{k_c-k_a} \left( \frac{e^{-k_4t}}{k_c} - \frac{e^{-k_5't}}{k_a} \right) - \frac{1}{k_c} + \frac{1}{k_a}
\]
(26)

Substituting (22) into (10) yields:
\[
\frac{dE}{dt} = k_5' \frac{k_4[A]_0}{k_c-k_a}(e^{-k_4t} - e^{-k_5't})
\]
which integrates to:
\[
[E] = \frac{k_5'k_4[A]_0}{k_c-k_a} \left( \frac{e^{-k_4t}}{k_c} - \frac{e^{-k_5't}}{k_a} \right) - \frac{1}{k_c} + \frac{1}{k_a}
\]
(28)

Therefore the integrated rate equations for Mechanism 4 expressed in terms of the individual rate constants are:
\[
[A] = [A]_0 e^{-(k_1+k_3)t}
\]
(29)
\[
[B] = \frac{k_1[A]_0}{k_2+k_4+k_5'-k_1-k_3} \left( e^{-(k_1+k_3)t} - e^{-(k_2+k_4+k_5')t} \right)
\]
(30)
\[
[C] = \frac{k_2k_1[A]_0}{k_2+k_4+k_5'-k_1-k_3} \left( e^{-(k_2+k_4+k_5')t} - e^{-(k_1+k_3)t} \right) - \frac{1}{k_1+k_3} + \frac{1}{k_2+k_4+k_5'}
\]
(31)
\[
[D] = \frac{k_3[A]_0}{k_1+k_3} \left( 1 - e^{-(k_1+k_3)t} \right) + \frac{(k_4-k_5')k_1[A]_0}{k_2+k_4+k_5'-k_1-k_3} \left( e^{-(k_2+k_4+k_5')t} - e^{-(k_1+k_3)t} \right) - \frac{1}{k_1+k_3} + \frac{1}{k_2+k_4+k_5'}
\]
(32)
\[
[E] = \frac{k_5'k_1[A]_0}{k_2+k_4+k_5'-k_1-k_3} \left( e^{-(k_2+k_4+k_5')t} - e^{-(k_1+k_3)t} \right) - \frac{1}{k_1+k_3} + \frac{1}{k_2+k_4+k_5'}
\]
(33)

**Integrated Rate Equations Fitting and Determination of Parameter Confidence Intervals**

Peak integrations were manually entered into Microsoft Excel and analyzed by nonlinear regression using the Solver feature as reported (2). The concentration-time integrated rate equations were derived. Equations 29-33 were fitted to the assigned peak data sets simultaneously by maximizing the sum of the R^2 values and varying the parameters k_1, k_2, k_3, k_4, and k_5' with initial values of 0.00085, 0.048, 0.029, 0.004 and 0.007 respectively with an initial concentration of ethephon ([A]_0) set to 97.5. The goodness of fit was assessed based on the closeness of the R^2 value to 1 (Table S1). The reported rate constants were determined by Monte Carlo simulation (3). Briefly, the individual root-mean-square error for each chemical species was used to generate 40 virtual data sets per chemical species. Initial parameter values for k_1-k_5' of ± 25% of the fitted value were generated using the RANDBETWEEN function in Excel. The reported average k values (Table S2) are the results obtained after maximizing the sum of R^2 for the virtual data sets to fitted values by modifying k_1-k_5' for each set of virtual data by implementing Solver using 100 iterations, automatic scaling and ‘assume non-negative.’ The corresponding first order or pseudo first order T_{1/2} values were calculated from T_{1/2} = ln(2)/k for each value of k with confidence intervals of 95% (Table S2).
### Table S1: Fitting Results

|        | \( R^2 \) | 95% CI | \( T_{1/2} \), h | type     | max, % |
|--------|-----------|--------|-----------------|----------|-------|
| [A]    | 0.985     | 9.63   | 32.7            | degradation | 97.5  |
| [B]    | 0.917     | 0.19   | 32.9            | formation  | 10.7  |
| [C]    | 0.769     | 1.02   | 45.6            | formation  | 3.19  |
| [D]    | 0.979     | 10.7   | 33.0            | formation  | 93.3  |
| [E]    | 0.784     | 0.80   | 37.1            | formation  | 1.49  |

### Table S2: Rate Constants Derived from Monte Carlo Simulation

| Rate constant | \( k \), h\(^{-1} \) | \( T_{1/2} \), h |
|---------------|------------------------|-----------------|
| \( k_1 \)     | 0.00233, SE 0.000112, LCI 0.00133, UCI 0.00401 | 297, SE 14.2, LCI 173, UCI 520 |
| \( k_2 \)     | 0.0584, SE 0.000565, LCI 0.0522, UCI 0.0646 | 12, SE 0.115, LCI 10.7, UCI 13.3 |
| \( k_3 \)     | 0.0189, SE 0.000207, LCI 0.0167, UCI 0.0210 | 37, SE 0.402, LCI 33.0, UCI 41.5 |
| \( k_4 \)     | 0.139, SE 0.0109, LCI 0.0399, UCI 0.282 | 5, SE 0.390, LCI 2.46, UCI 17.4 |
| \( k_5 \)     | 0.0317, SE 0.000413, LCI 0.0274, UCI 0.0359 | 22, SE 0.285, LCI 19.3, UCI 25.3 |

### Determination of \( T_{1/2} \) Values for all Species

The first order \( T_{1/2} \) value for the degradation of \( A \) was calculated using the equation \( T_{1/2} = \ln(2)/(k_1 + k_3) \). The \( T_{1/2} \) values for formation of \( C \), \( D \) and \( E \) were calculated by solving the corresponding integrated rate equation using the calculated \( k \) values for the time at half maximal concentration. These overall \( T_{1/2} \) values are given in Table S1.

### Determination of Total % Formation for \( B - E \)

The maximum concentrations of \( C \) (\( C_{\text{max}} \)), \( D \) (\( D_{\text{max}} \)), and \( E \) (\( E_{\text{max}} \)) were determined by solving the appropriate rate equation at \( t=10,000 \) h. The total % formation of \( C \) and \( E \) are equivalent to the maximum value. The total formation of \( D \) (\( D_{\text{total}} \)) was calculated as the sum of \( D_{\text{max}} \) and \( E_{\text{max}} \). The maximum concentration of \( D \) formed from \( A \) (\( D_{\text{max}A} \)) was calculated by solving the first term of equation 32 at time \( t=10,000 \) h. The maximum formation of \( D \) from \( B \) (\( D_{\text{max}B} \)) was calculated by subtracting \( D_{\text{max}A} \) from \( D_{\text{total}} \). The total % formation of \( B \) (\( B_{\text{tot}} \)) was calculated as the sum of \( C_{\text{max}} \), \( D_{\text{max}B} \), and \( E_{\text{max}} \). The total % formation for each species is given in Table S1.

### Ethephon Standard Spectrum

2-chloroethylphosphonic acid (ethephon): \(^1\)H NMR (600 MHz, \( D_2\)O) \( \delta \) 3.68 (dt, \( J = 13.5, 7.6 \) Hz, 2H), 1.96 (dt, \( J = 18.0, 7.6 \) Hz, 2H). \(^{31}\)P\{\(^1\)H\} NMR (600 MHz, potassium carbonate buffer in \( H_2O:D_2O \) 90:10, pH=7.4) \( \delta \) 16.65 ppm. \(^{31}\)P NMR (600 MHz, 99% \( D_2\)O, pH = 7.4) \( \delta \) 25.3 ppm.

### Preparation of 2-Hydroxyethylphosphonic acid

Dimethyl 2-hydroxyethylphosphonic acid was refluxed in 6N HCl. Conversion was monitored by \(^{31}\)P NMR and reached 75 % after 120 h. \(^1\)H NMR (400 MHz, \( D_2\)O, pH<4) \( \delta \) 3.48 (dt, \( J = 11.8, 7.5 \) Hz, 2H), 1.71 (dt, \( J = 18.2, 7.6 \) Hz, 3H). \(^{31}\)P\{\(^1\)H\} NMR (400 MHz, \( D_2\)O, pH<4) \( \delta \) 25.3 (s).

### Monomethyl 2-Hydroxyethylphosphonic acid

was visualized by \(^{31}\)P NMR as an intermediate in the hydrolysis of dimethyl 2-hydroxyethylphosphonic acid to 2-hydroxyethylphosphonic acid. \(^{31}\)P\{\(^1\)H\} NMR (400 MHz, \( D_2\)O, pH<4) \( \delta \) 26.9 (s).
2-Oxo-2-hydroxy-1,2-oxaphosphetane was visualized in the degradation time course spectra of ethephon. $^1$H NMR (400 MHz, potassium carbonate buffer in 99% D$_2$O, pH = 7.4) $\delta$ 3.97 (ddt, $J$ = 8.0, 8.0, 7.6 Hz, 2H), 2.85 (dt, $J$ = 18.0, 7.6 Hz, 2H). $^{31}$P{$^1$H} NMR (600 MHz, potassium carbonate buffer in H$_2$O:D$_2$O 90:10, pH = 7.4) $\delta$ 18.1 ppm. $^{31}$P NMR (600 MHz, 99% D$_2$O, pH = 7.4) $\delta$ 18.1 (tt, $J$ = 18.0, 8.0, 8.0 Hz). The J-couplings reported here should be taken with caution as many of the peaks were barely greater than the spectral noise.

$^1$H NMR

The $^1$H NMR spectra of ethephon incubated in pH 7.4 potassium carbonate buffer at 1 - 96 h are shown in Figure S3. The $^1$H NMR spectra of B is expected to be shifted downfield of the $^1$H NMR spectra of C. The transient doublet of triplets at 2.85 ppm corresponds to the alkyl alpha hydrogens of B and is de-shielded compared to the beta hydrogens of C (1.71 ppm) and A (1.96 ppm) consistent with the formation of an oxaphosphetane ring. The H-C-C-H coupling constants are nearly the same for B (7.6 Hz) and C (7.6 Hz) while the H-C-P coupling constant is slightly smaller for B (18.0 Hz) than C (18.2 Hz). The peaks for the alkyl beta hydrogens near 3.5 ppm overlap too much to allow definitive characterization, but it appears that the peak splits into a ddt with two bond splitting to the alpha hydrogens and two sets of three bond splitting to the phosphorus in both directions through the oxaphosphetane ring. The alkyl alpha hydrogens of C and E are also visible in the 21 and 96 h spectra.

Figure S3. $^1$H NMR spectra of ethephon degradation in pH 7.4 potassium carbonate buffer at 1, 21 and 96 h. Insets are expansions of the indicated spectral regions. Letters correspond to the chemical structures in Scheme S1.
**1^H coupled 3^1P NMR**

The 28.11 ppm peaks in the 21 h 1^H coupled 3^1P NMR spectra support the J-coupling calculations above although the magnitude of the peaks is barely above the background requiring caution in interpretation (Figure S4). Also in agreement with the assignments from 1^H uncoupled NMR are the 1^H splitting of the C and E phosphonate peaks but not the E phosphate peak (Figure S4).

![Figure S4](image)

**Figure S4.** 1^H coupled 3^1P NMR spectra of ethephon degradation in pH 7.4 potassium carbonate buffer at 21 h and 96 h. Insets are expansions of the indicated spectral regions. Letters correspond to the chemical structures in Scheme S1.

**References**

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