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Rapid Report

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

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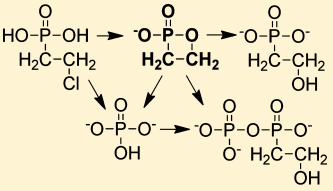
Supporting Information

ABSTRACT: The major plant growth regulator ethephon degrades to ethylene and phosphate in aqueous solutions and plants and is spontaneously activated to a butyrylcholinesterase (BChE) inhibitor in alkaline solutions and animal tissues. In the present ^{31}P NMR kinetic study of the reactions of ethephon in pH 7.4 carbonate buffer, we observed a transient peak at 28.11 ppm. The time course for the appearance and disappearance of this peak matches the activation/degradation kinetic profile of the BChE inhibitor, and the chemical shift supports the proposed 2-oxo-2-hydroxy-1,2-oxaphosphetane structure.



Ethephon (2-chloroethylphosphonic acid, **A** in Figure 1) is widely used as an ethylene-generating plant growth

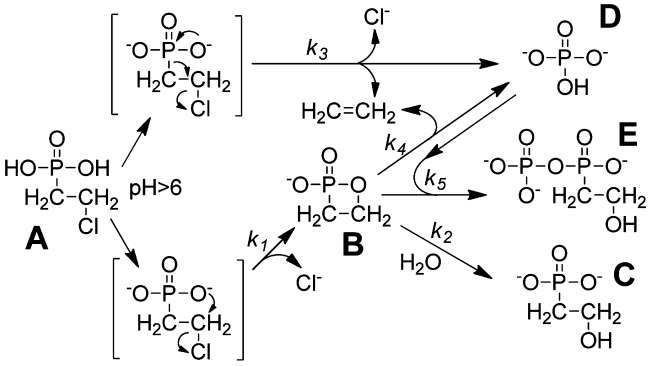


Figure 1. Ethephon degradation pathways.

regulator with an intriguing toxicological observation that it inhibits butyrylcholinesterase (BChE) *in vitro* and *in vivo* in rats and humans.[1,2] Even more surprising, ethephon is not direct-acting but undergoes spontaneous activation in aqueous alkaline solutions to the activated BChE inhibitor.[1] The ethephon-inhibited BChE is covalently derivatized with a transfer of the phosphorus from ethephon.[2] The active site Ser198 contains a mass adduct of 108 Da thought to be the 2-hydroxyethylphosphonate (**C**) derivative which forms from the ring-opening of 2-oxo-2-hydroxy-1,2-oxaphosphetane (the proposed activated inhibitor, **B**).[1] Degradation of **B** in aqueous media in the absence of BChE by electrophilic attack on the phosphorus by water or phosphate would produce the hydrolysis product **C** or the phospholysis product 2-hydroxyethylphosphonyl phosphate (**E**), respectively. The reported 3−5 mol % trapping of **B** by BChE[1] suggests that the formation of **B** likely competes with the formation of phosphate (**D**) and ethylene from **A**. All reactions are expected to be irreversible.

The possibilities for the degradation of **A** in aqueous solution with pH > 6 are depicted in Figure 1. The presence of phosphorus in each chemical species permits ^{31}P NMR monitoring of the concentrations of each product from ethephon degradation. Fitting of concentration−time rate equations to kinetic data can be used to model proposed reaction mechanisms and facilitates the calculation of individual reaction rates from experimental data for complex mechanisms.[3]

To establish an appropriate buffer for ^{31}P NMR monitoring of degradation, ethephon (10 mM) was dissolved in 100 mM potassium carbonate or potassium phosphate buffer. Aliquots were diluted to 0.11 mM and assayed as reported[1] for BChE inhibitory potency immediately after preparation and after 20 h. Preincubation of ethephon at high concentration in phosphate or carbonate buffer gave nearly the same activation to a BChE inhibitor (Supporting Information).

^{31}P NMR monitoring of degradation utilized 200 mM ethephon in 2 M potassium carbonate buffer (1 mL volume). Spectra were recorded from 0.1 to 166 h, and peaks were integrated and related to % total area. The time course spectra (Supporting Information) show degradation of ethephon (16.65 ppm, s) and formation of phosphate (2.49 ppm, s). Minor impurities not changing in concentration are evident at 10.94 ppm (s), 22.11 ppm (s), and 24.18 ppm (s). One peak (28.11 ppm (s)) increases and then decreases in concentration, and three peaks (−6.29 (d), 13.51 (d), and 18.76 (s) ppm) increase in concentration over the time course (Figure 2). ^{31}P NMR chemical shifts of phosphorus acids are sensitive to pH, and standards are often reported in highly acidic solutions complicating peak identification in our spectra. However, a change from pH < 4 to pH > 7 induces an ∼8 ppm upfield shift of phosphonate peaks in ^{31}P NMR as illustrated by ethephon in acidic D_2O at 25.19 ppm[4] and in pH 7.4 carbonate buffer at 16.65 ppm. **C** has a ^{31}P NMR chemical shift of 25.30 ppm in

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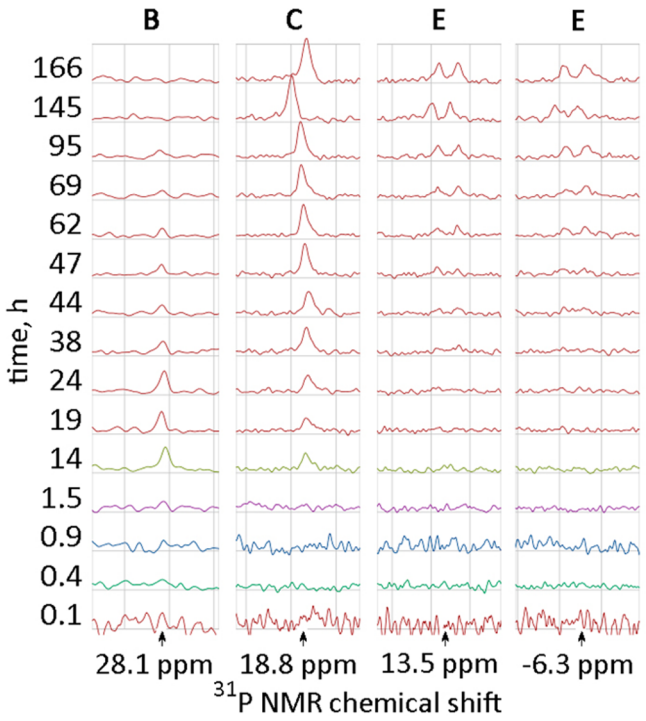

Figure 2. Time course spectra for low concentration products **B**, **C**, and **E** formed from the degradation of **A** at pH 7.4.

acidic D_2O. The singlet peak at 18.76 ppm is thus in the expected region for **C** at pH 7.4 and is assigned as such. The doublets at −6.29 and 13.51 ppm have *J* couplings of 24 Hz and integrate nearly equally at all time points. The spectra are ^{1}H decoupled, and peak splitting results from P−P spin spin coupling only. *J* = 24 Hz matches two bond P−P coupling of a diphosphate.[5] Two doublets indicate unequal phosphorus atoms corresponding to a phosphate (−6.29 ppm) and a phosphonate (13.51 ppm). We therefore assign these peaks as the phosphorus atoms in **E**. All peak assignments are corroborated by ^{1}H NMR and ^{1}H coupled ^{31}P NMR spectra (Supporting Information).

The transient ^{31}P NMR peak at 28.11 ppm (designated **B**) is of particular interest. The atom connectivity to phosphorus in **B** is similar to that in monoalkyl-**C**. We have observed monomethyl 2-hydroxyethylphosphonic acid (26.9 ppm) as an intermediate in the hydrolysis of dimethyl 2-hydroxyethylphosphonic acid (35.87 ppm) to **C** (25.3 ppm) in refluxing HCl. Thus, oxaphosphetane **B** is expected to have a chemical shift downfield of the 18.76 ppm **C** signal. Although numerous mechanistic ^{31}P NMR studies have proposed an oxaphosphetane intermediate in the Wittig reaction, to our knowledge, only one of these studies[6] examined the phosphonate modification of the Wittig reaction where the oxaphosphetane intermediates would be most similar to **B**. ^{31}P NMR chemical shifts of 35 and 32 ppm were reported for these dialkyl oxaphosphetanes.[6] Thus, a chemical shift at 28.11 ppm, slightly upfield of similar dialkyl oxaphosphetanes, is as expected for 2-oxo-2-hydroxy-1,2-oxaphosphetane (**B**).

The reactions in Figure 1 were mathematically modeled using irreversible first order and pseudo-first-order kinetics for $k_1 \neq k_2 \neq k_3 \neq k_4 \neq k_5'$ and $B_0 = C_0 = D_0 = E_0 = 0$ to derive integrated concentration−time rate equations (see Supporting Information). These equations were fit simultaneously to the assigned peak data sets by maximizing the sum of the r^2 values using nonlinear regression as reported.[7] Rate constants were determined by the Monte Carlo simulation.[8] 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%. The $T_{1/2}$ values for individual species were calculated using the % total conversion (see Supporting Information).

The overall fit of the ^{31}P NMR data to the integrated rate equations is good (average r^2 = 0.887). The r^2 values for **C** (0.771) and **E** (0.783) are lower than those for **A** (0.985), **B** (0.917), and **D** (0.979). This likely results in part from error due to the pseudo-first-order approximation ($k_5[D] \approx k_5'$) used in deriving the integrated rate equations. While the concentration of **D** is always at least 10 times that of **B**, it varies from 0 to 93 mol %, and thus, k_5' is an approximation of the average value over time. At 0 h, the relative conversion of **B** to **C** or **E** is expected to shift toward the formation of **C** and as the concentration of **D** increases over time, the conversion of **B** to **E** would increase. Furthermore, in phosphate buffer, a higher percentage conversion to **E** is expected.

The $T_{1/2}$ degradation rate of **A** (32.7 h) and formation rate of **D** (33.0 h) (Supporting Information) are in agreement with reported rates (11−58 h) summarized previously.[1] Rates derived from measurements of ethephon degradation,[4] phosphate formation, or ethylene generation[9,10] are all reported as a measure of the rate of ethephon hydrolysis. This study supports that approximation as the measured overall hydrolysis rate of **A** does not differ from the formation rate of **D**. The measured rate for the formation of **D** from **A** ($T_{1/2}$ = 37 h) is 10 times faster than the rate for the formation of **B** from **A** ($T_{1/2}$ = 297 h) indicating that the overall rate of ethephon hydrolysis is governed by the rate of phosphate formation (k_3). The rate of ethylene generation would also approximately equal the measured rate of phosphate generation.

2-Hydroxyethylphosphonate (**C**) has been reported previously as a degradation product of **A** in buffered solutions, formed in 1% and 8% yield at pH 7.4 and 13.8, respectively,[4] suggesting either differential formation or degradation of **B** at varying pH. Up to 10 mol % **C** forms from photolysis or soil degradation of **A** and up to 3.7 mol % is formed from anaerobic degradation,[11] likely by direct SN_2 reaction at the β-carbon rather than via the intermediacy of **B**. To the best of our knowledge, **E** is previously unreported as a degradation product of **A**.

The BChE inhibitor forms spontaneously at neutral to alkaline pH and is short-lived. The concentration−time curves based on enzyme inhibition[1] or ^{31}P NMR analysis (this study) are almost identical (Figure 3) indicating that the two methods measure the same compound (the transient BChE inhibitor, **B**). The results of our kinetic modeling suggest that in pH 7.4 carbonate buffer in the absence of BChE, the total conversion to **B** is 10.7 mol % and that **B** degrades to **D** (6 mol %, k_4 = 0.139 h^{-1}), **C** (3.2 mol %, k_2 = 0.0584 h^{-1}), and **E** (1.5 mol %, k_5' = 0.0317 h^{-1}). In the presence of BChE, 3−5 mol % of **A** was trapped as a BChE adduct over 168 h.[1] The difference in values for % conversion to **B** likely results from a 30−50% efficiency for the BChE trap with the remainder of **B** degraded to **C**, **D**, and **E** under the conditions of the trapping experiments. Although the formation and degradation of **B** depends on conditions, the high agreement (Figure 3) between the concentration−time curves generated from BChE trapping[1] and direct ^{31}P NMR monitoring of ethephon degradation (this

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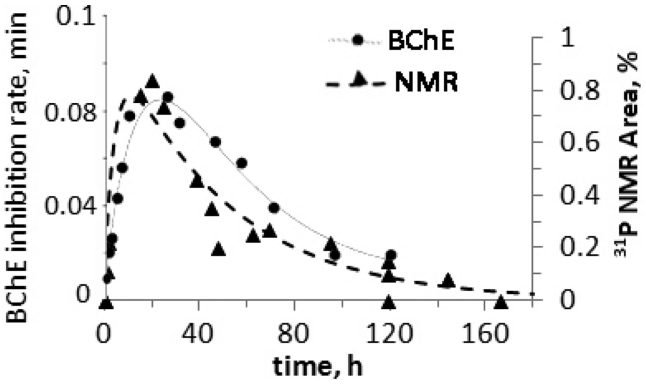

Figure 3. Comparison of BChE inhibition rate[1] and ^{31}P NMR area (this study) for oxaphosphetane **B** over time.

study) suggests that the efficiency of the BChE trap is likely limited by competitive degradation of **B**.

There are two separate mechanisms for ethephon degradation: direct production of ethylene (and phosphate) and formation of the BChE inhibitor. In both forks of the degradation pathway, the first step is halide dissociation (Figure 1) which is in agreement with the observed trend of higher BChE inhibitory potency for bromo and iodo analogues of **A**[2] as well as analogues with electron withdrawing β-substituents.[12] Degradation of **A** under different conditions might alter the percent formation of the BChE inhibitor **B** and the degradation products **C**, **D**, and **E**. In biological systems, additional derivatives of many nucleophilic species could form, but **B** reacts fairly specifically with BChE compared to numerous other esterases.[13] Thus, inhibited BChE is a useful monitor of ethephon exposure.

■ ASSOCIATED CONTENT

Supporting Information

Detailed methods, buffer comparison, full spectral data, fitting results, and derivation of integrated rate equations. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

This rapid report has not been formally reviewed by EPA, and the views expressed are solely those of the authors. EPA does not endorse any products or commercial services mentioned in this rapid report.

The authors declare no competing financial interest.

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■ ABBREVIATIONS

BChE, butyrylcholinesterase; EPA, Environmental Protection Agency; NMR, nuclear magnetic resonance

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