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METABOLITES ASSOCIATED WITH ORGANOPHOSPHONATE C-P BOND CLEAVAGE: CHEMICAL SYNTHESIS AND MICROBIAL DEGRADATION OF [^{32}P]-ETHYLPHOSPHONIC ACID

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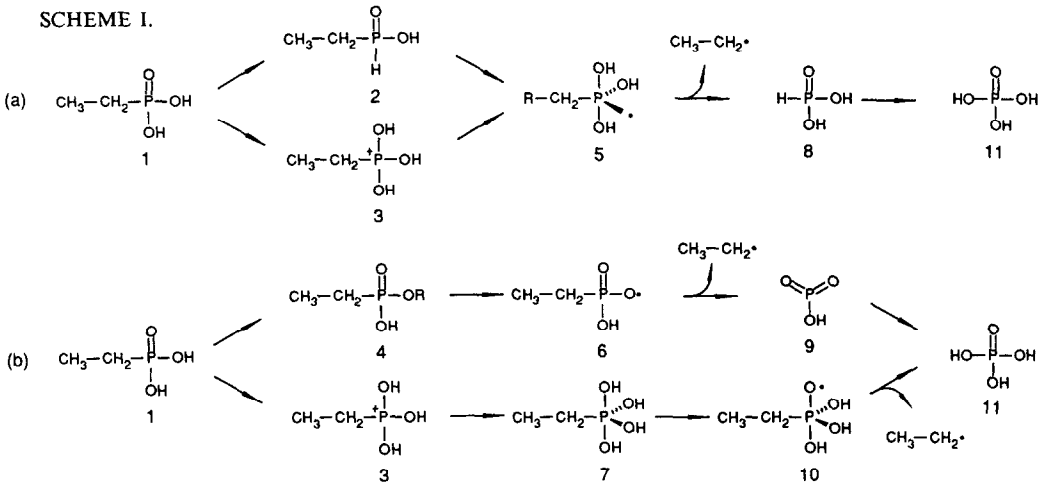
Abstract: Degradation of [^{32}P]-ethylphosphonic acid by *Escherichia coli* has provided the first glimpse of phosphorus-containing metabolites generated during cleavage of organophosphonate carbon to phosphorus (C-P) bonds. Mechanistic ramifications of the observed metabolites are discussed along with the methodology for introduction of ^{32}P into organophosphonates.

The increasing use of organophosphonates as enzyme inhibitors follows from their capacity to function as structural mimics of phosphate esters and transition state analogs of intermediates formed during enzymatic hydrolysis of carboxylic acid derivatives. While the distinguishing carbon to phosphorus (C-P) bond of organophosphonates is chemically stable, microbial cleavage of C-P bonds can be quite facile. Understanding the mechanisms involved in biotic organophosphonate C-P bond cleavage is important for controlling in vivo residence time of these chemicals and avoiding degradation to potentially toxic molecules. Carbon fragments generated during microbial cleavage of the C-P bond of organophosphonates such as ethylphosphonic acid **1** have been postulated to reflect intermediacy of organophosphoranyl radicals[1] (**5**, Scheme Ia) or organophosphonyl radicals[2] (**6** or **10**, Scheme Ib). Unfortunately, the lack of C-P bond cleavage activity in cell lysate has complicated further mechanistic elaboration. A step towards circumventing this impediment has now

SCHEME I.

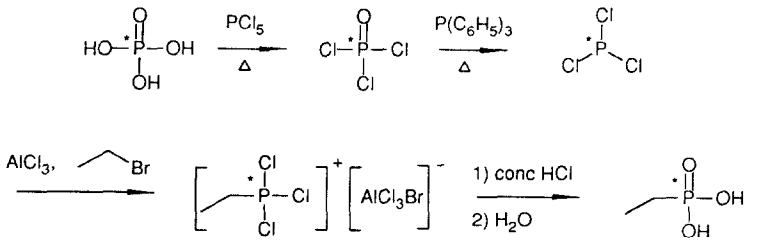


been achieved with the synthesis of [^{32}P]-ethylphosphonic acid. Degradation of [^{32}P]-ethylphosphonate **1** by *Escherichia coli* has facilitated the search for metabolites (such as **2** and **8**,

Scheme I) which could allow differentiation between organophosphoranyl radical and organophosphonyl radical intermediacy. During this search, a unique phosphorus-containing metabolite has been discovered.

Phosphorus trichloride is a useful starting material[3] for organophosphonate synthesis and a logical point for introduction of ^{32}P label. Unfortunately, ^{32}P-labelled phosphorus trichloride is not commercially available or readily prepared by literature[4] methodology. This necessitated development of a procedure (Scheme II) for deriving ^{32}P-labelled phosphorus trichloride from commercially available, ^{32}P-labelled inorganic phosphate. The resulting synthesis of ^{32}P-labelled organophosphonate incorporates 80% of the radiolabel in the starting inorganic phosphate into product ethylphosphonic acid (152 μCi/mmol, 32% overall yield)

SCHEME II.



Degradation of [^{32}P]-ethylphosphonate by *E. coli* RB791 (Figure 1a) and *E. coli* RB791(pSCL90)[5,6,7] (Figure 1b) resulted in the formation of an extracellular, radiolabelled metabolite. Isolation and characterization[8] revealed the metabolite to be α-1-(ethylphosphono)ribose **12** (Figure 1). Although most of this ^{32}P-labelled metabolite was found to accumulate in the extracellular culture supernatant, quantities were also detected in cell lysate. Ribosylated ethylphosphonate was not formed by *E. coli* SL724, a mutant[9] derived from RB791. This indicates that mutation of a genetic locus[10] in *E. coli* RB791 which is essential to C-P bond cleavage also disrupts organophosphonate ribosylation. Neither ^{32}P-labelled inorganic phosphate or ethane are detected when [^{32}P]-α-1-(ethylphosphono)ribose is incubated with *E. coli* cell lysate.

Growth of *E. coli* RB791 in medium lacking inorganic phosphate where unlabelled and ^{32}P-labelled ethylphosphonate were the only sources of phosphorus did not lead to any detectable formation of ^{32}P-labelled ethylphosphonous acid **2** or phosphorous acid **8** in the culture supernatant (Figure 1a) or cell lysate. No extracellular radiolabelled inorganic phosphate was detected (Figure 1a) although substantial quantities were present in cell lysate. In contrast, substantial concentrations of [^{32}P]-ethylphosphonate remained in the culture supernatant (Figure 1a) while none could be detected in cell lysate.

A different radiolabelled solution matrix was observed when *E. coli* RB791(pSCL90) was cultured in LB medium[11] containing inorganic phosphate and [^{32}P]-ethylphosphonate. Extracellular

accumulation (Figure 1b) of ^{32}P-labelled inorganic phosphate in the culture supernatant was observed. This accumulation likely reflects exchange of radiolabelled inorganic phosphate derived from [^{32}P]-ethylphosphonate with the unlabelled excess of inorganic phosphate present in the culture supernatant. Localization of C-P bond cleavage activity on the outer surface of the *E. coli* plasma membrane is also implicated.[12] When *E. coli* RB791(pSCL90) was cultured in LB medium containing phosphorous acid **8** (10 mM) or ethylphosphonous acid **2** (10 mM) in excess to [^{32}P]-labelled ethylphosphonic acid (1.0 mM), no accumulation of ^{32}P-labelled phosphorous or ethylphosphonous acid was detected.

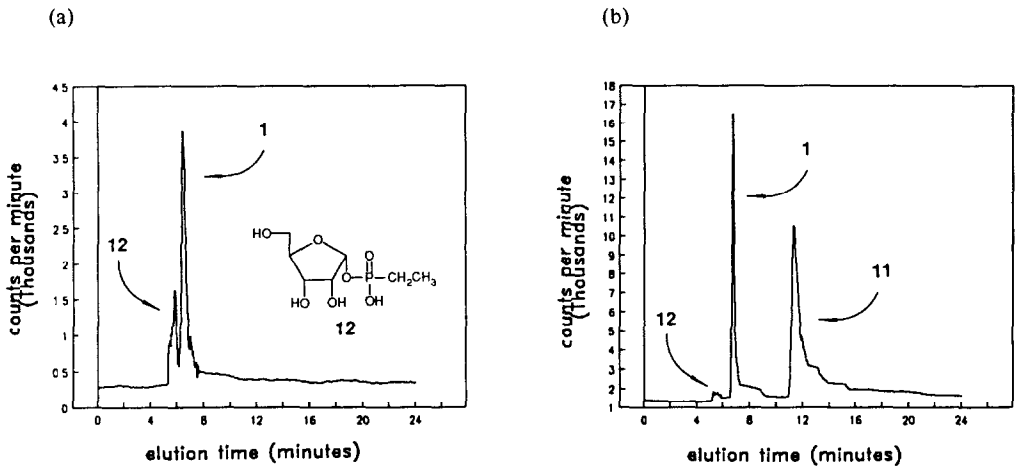


Figure 1. HPLC traces of *E. coli* culture supernatants. Aliquots were injected on a Water Associates HPLC fitted with a Whatman Partisil-10 SAX column and interfaced with a Beckman 171 β-emission detector. Figure 1a: Analysis of the supernatant from *E. coli* RB791 cultured in glucose (26 mM), magnesium sulfate (1 mM), trizma·HCl (64 mM), sodium chloride (8 mM), ammonium chloride (19 mM), and thiamine (0.014 mM). The only source of phosphorus was a 0.4 mM concentration of [^{32}P]-ethylphosphonate (152 μCi/mmol). Figure 1b: Analysis of the supernatant from *E. coli* RB791(pSCL90)[7] cultured in LB medium[11] containing inorganic phosphate (2.2 mM) and [^{32}P]-ethylphosphonate (1.0 mM, 152 μCi/mmol).

Organophosphonyl radical intermediacy (Scheme 1b) is consistent with the absence of phosphorous acid **8** and ethylphosphonous acid **2**. Organophosphoranyl radical intermediacy (Scheme 1a) may still be possible but would likely have to be part of a degradative pathway not involving free phosphorous acid or ethylphosphonous acid. Degradation of [^{32}P]-ethylphosphonic acid by *E. coli* has thus provided a useful refinement of the mechanistic hypotheses relevant to organophosphonate biodegradation. At the same time, identification of α-1-(ethylphosphono)ribose adds a metabolite whose formation must be explained by future mechanistic evaluations of organophosphonate biodegradation.

Acknowledgment. Research was funded by a grant from the Environmental Protection Agency.

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5. Insertion of a genomic fragment essential for C-P bond cleavage into pSU18[6] yields plasmid pSCL90. RB791(pSCL90), as opposed to RB791, retains full C-P bond cleaving activity in medium containing inorganic phosphate [7]

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8. ^{1}H NMR (D_2O, TSP = δ 0.00) δ 1.09 (dt, J = 19, 8 Hz, 3H), 1.66 (dq, J = 19, 8 Hz, 2H), 3.67 (dd, J = 12, 4 Hz, 1H), 3.78 (dd, J = 12, 3 Hz, 1H), 4.10 (m, 2H), 4.22 (m, 1H), 5.64 (dd, J =6, 4 Hz, 1H); ^{13}C NMR (D_2O, CH_3CN = δ 3.69) δ 9.2 (d, J = 6 Hz), 23.3 (d, J = 136 Hz), 64.1, 72.0, 74.2 (d, J = 6 Hz), 87.9, 100.0 (d, J = 6 Hz); ^{31}P NMR (D_2O, 85% H_3PO_4 = δ 0.00) 33.2. Assignment of an α configuration at C-1 is based on comparison with the ^{1}H NMR couplings (dd, J = 6, 4 Hz) of the proton attached to the anomeric carbon of α-1-ribosyl phosphate (Sigma).

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12. (a) Extracellular accumulation of ^{32}P-labelled inorganic phosphate derived from [^{32}P]-ethylphosphonate would be an unlikely result if C-P bond cleavage occurred in the cytosol or on the cytosolic side of the plasma membrane. Control experiments were run where *E. coli* RB791(pSCL90) was grown in LB containing unlabelled ethylphosphonic acid and ^{32}P-labelled inorganic phosphate. These ^{32}P-labelled cells were harvested and then resuspended in LB medium containing unlabelled inorganic phosphate and unlabelled ethylphosphonate. Prolonged incubation of these cells did not lead to detectable levels of ^{32}P-labelled inorganic phosphate in the culture supernatant. (b) Localization of C-P bond cleaving activity in the periplasmic space or on the outer membrane is precluded by the observation of C-P bond cleaving activity in *E. coli* spheroplasts. Avila, L. A.; Frost, J. W. Unpublished results.