The antibiotic dehydrophos is converted to a toxic pyruvate analog by peptide bond cleavage in *Salmonella enterica*

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Running title: Activation of dehydrophos

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The metabolic processing of dehydrophos, a broad-spectrum peptide antibiotic containing an unusual vinyl-phosphonate moiety, was examined using a panel of Salmonella enterica mutants deficient in peptide uptake and catabolism. Dehydrophos bioactivity is lost in opp, tpp double mutants, demonstrating a requirement for uptake via non-specific oligopeptide permeases. Dehydrophos bioactivity is also abolished in a quadruple Salmonella mutant lacking the genes encoding peptidases A, B, D, and N, showing that hydrolysis of the peptide bond is required for activity. $^{31}$P NMR spectroscopy was used to assess the fate of dehydrophos following in vitro digestion of the antibiotic with purified PepA. The results suggest that the initial product of peptidase processing is 1-aminoethylphosphonate O-methyl ester. This phosphonate analogue of dehydroalanine undergoes rearrangement to the more stable imine, followed by spontaneous hydrolysis to yield O-methyl-acetylphosphonate, a structural analogue of pyruvate. This compound is a known inhibitor of pyruvate dehydrogenase and pyruvate oxidase and is probably the active species responsible for dehydrophos bioactivity.
Dehydrophos (formerly A53868) is an unusual antibiotic produced by *Streptomyces luridus*. This compound has broad spectrum activity *in vitro*, and is effective *in vivo* in *Salmonella* challenged chickens (15). Dehydrophos consists of a glycine-leucine dipeptide linked to an O-methylated aminovinylphosphonate (28) (Figure 1). The cellular target of the compound has yet to be established and it is unknown whether it acts as a tripeptide, or whether it is metabolized by the target cell to release a bioactive product. Structure-activity relationships of several dehydrophos derivatives clearly revealed the importance of the O-methyl and vinyl moieties, but did not allow conclusions to be made regarding whether peptide bond cleavage is required for bioactivity (16).

The coupling of phosphonic acids to amino acids has been observed in numerous bioactive natural products. In the case of rhizotinic, plumbemycin, bialaphos and phosalacine, these amino acids convert a toxic phosphonate molecule into an inactive “trojan horse” that is readily taken up by target organisms. Once inside the cell, peptidase activity serves to release the bioactive phosphonate moiety (6, 17). In other cases the entire peptide is required for bioactivity. For example, the angiotensin converting enzyme inhibitor K-26 binds to its target as a tripeptide consisting of Ile-Tyr coupled to (R)-1-amino-2-(4-hydroxyphenyl)-ethylphosphonic acid (AHEP) (2, 23).

Two mutually exclusive mechanisms for dehydrophos bioactivity can be envisioned based on whether the peptide bonds arecleaved or remain intact. In reporting the revised structure of antibiotic, Whitteck *et al* noted that cleavage of the peptide bond would produce an unstable enamine phosphonate, expected to undergo tautomeric rearrangement followed by spontaneous hydrolysis (Figure 1) (28). The
expected product of this transformation would be methyl acetylphosphonate (MAP), a structural analog of pyruvate and a known inhibitor of both pyruvate oxidase and pyruvate dehydrogenase (24). Alternatively, the vinylphosphonate moiety of dehydrophos could undergo a Michael-type addition to sulfhydryl-containing target enzymes in a reaction analogous to that which occurs during the inactivation of cysteine dependent proteases by vinyl sulfones (25). This mechanism would require that the peptide bond remain intact to prevent the rearrangement and hydrolysis reactions described above.

Both peptide transport and catabolism have been extensively characterized in *Salmonella enterica*, providing a facile experimental system to distinguish between the two models for dehydrophos action. *S. enterica* contains three distinct systems for peptide uptake encoded the *opp*, *tpp*, and *dpp* operons, which encode oligopeptide, tripeptide, and dipeptide permeases, respectively (10). The Opp permease is the primary peptide uptake system, transporting molecules containing up to 5 amino acids (11-13). The Tpp permease is relatively specific, preferring hydrophobic residues, and requiring anaerobic conditions for expression (7, 16), whereas the Dpp permease transports dipeptide substrates and acts as the signal receptor for peptide chemotaxis (1). Mutant strains lacking all three permeases are unable to assimilate most peptides (10). Peptides are converted to free amino acids in *S. enterica* via the action of eight major peptidases, designated A, B, D, E, N, P, Q, and T, with overlapping specificities. Peptidases A, B and D possess broad substrate specificities, with A and B belonging to the leucyl aminopeptidase family (19, 21). Peptidase N also has broad substrate specificity, but belongs to the lysyl aminopeptidase family and appears subject to a
more complex regulation scheme. The remaining peptidases display more restricted
substrate ranges and/or are only expressed under certain conditions (20, 21).

Here we report the permease- and peptidase-dependent nature of dehydrophos
activity in *S. enterica*. Consistent with the ‘trojan horse’ model of activity we also
demonstrate in vitro peptidase-dependent generation of the toxic pyruvate mimic methyl
acetylphosphonate and a derived metabolite, 1-hydroxyethylphosphonate monomethyl
ester (1-HEP O-Me), following hydrolysis of dehydrophos. These data suggest that
dehydrophos functions by generation of a pyruvate analogue capable of inhibiting
multiple targets, a useful feature in minimizing bacterial antibiotic resistance.

**Materials and Methods**

**Bacterial strains, plasmids and culture conditions.** The strains used in this
study are listed in Table 1. *Streptomyces* strains were propagated on ISP4 medium
(Difco, Becton Dickinson Microbiology Systems, Sparks, Maryland) at 30 °C unless
otherwise noted. Antibiotic production cultures were inoculated with 50 µL of a spore
suspension (~10⁹ colony forming units) and incubated in 500 mL baffled flasks
containing 100 mL of nutrient broth (Difco) agitated at 250 rpm for 48 hours prior to
analysis of the supernatant. *S. enterica* was routinely grown in Luria-Bertani broth (LB)
or on 1.5% agar LB plates supplemented with appropriate antibiotics. Antibiotics were
used at the following concentrations: ampicillin 100 µg/mL, chloramphenicol 10 µg/mL,
apramycin 37.5 µg/mL, kanamycin 50 µg/mL.

**Bioassay of dehydrophos growth inhibition.** Lawns of the appropriate
*Salmonella* strain were made by plating 0.1 mL of an exponential phase culture (OD₆₀₀
85 ~0.1) suspended in molten (~40 °C) ‘E’ medium (25) top agar (0.5 %) onto ‘E’ medium 86 agar (1.5%) plates containing succinate (40 mM) and leucine (50 µM). Agar cores were 87 then taken from cultures of Streptomyces luridus NRRL 15101 grown for 48 hrs at 30 °C 88 on ISP4 plates. Alternatively, filter disks were spotted with 10 µL of an authentic 89 standard dehydrophos (1 mg/mL). These cores and/or disks were then placed onto the 90 freshly inoculated bioassay plates, incubated at 37 °C for 12 hours, and then scored for 91 bioactivity.

**Overexpression, purification, and activity assay of PepA.** PepA was purified 92 using a modified version of the Vogt purification of *E. coli* aminopeptidase I (27). A 500 93 mL culture of the peptidase A overproducer *S. enterica* TN2216 was grown for 16 hours 94 at 37 °C and the cells were then harvested by centrifugation at 5000 X g for ten 95 minutes. The cell pellet was resuspended in 20 mL of ice cold buffer 1 (50 mM Tris-HCl 96 pH 7.8, 200 mM KCl, 10 mM MgCl₂, 100 mM EDTA, 100 mM DTT and 5% glycerol) and 97 disrupted by a single passage through a chilled French press cell at 20,000 psi. Solid 98 debris was removed by centrifugation at 13,000 × g for 30 minutes at 4 °C. The 99 resuspended cells were then placed into a 70 °C water bath and continuously swirled 100 until the temperature reached 65 °C. Precipitated proteins were removed by 101 centrifugation at 13,000 X g for 15 minutes at 4 °C. The supernatant was dialyzed 102 (Pierce 10K MWCO) against 2 L of 0.1M phosphate buffer pH 7.6 for 18 hours at 4 °C 103 with one change of buffer. Following dialysis the extract was centrifuged again at 13,000 104 × g for 15 minutes at 4 °C to remove insoluble material. The supernatant was then 105 dialyzed against buffer 2 (20 mM Tris-HCl pH 8.4, 10 mM KCl, 1 mM Mg(OAc)₂, 0.1 mM 106 EDTA) for 18 hours. The precipitate that formed was collected and redissolved in Buffer
3 (20 mM KHCO\textsubscript{3} pH 10.3, 50 mM KCl, 1 mM Mg(OAc)\textsubscript{2}, 0.1 mM EDTA). This precipitate failed to completely solubilize in Buffer 3, leaving the solution turbid. PepA purity was evaluated by visual inspection of an SDS-PAGE gel stained with Coomassie Brilliant Blue (estimated to be at least 95% pure). PepA action on dehydrophos was assayed at 37 °C over 18 hours by direct addition of dehydrophos (7 mM) to dissolved PepA. The reaction was monitored by \textsuperscript{31}P NMR spectroscopy.

\textbf{\textsuperscript{31}P NMR spectroscopy based identification of dehydrophos metabolites, and monitoring of the hydrolysis reaction.} Culture supernatant was passed through a 0.2 micron filter, concentrated twenty fold by evaporation, and D\textsubscript{2}O was added to 25% as a lock solvent. \textsuperscript{31}P NMR spectra were acquired on a Varian Unity Inova 600 spectrophotometer equipped with a 5 mm AutoTuneX probe at the Varian Oxford Instrument Center for Excellence at the University of Illinois Urbana-Champaign. An external standard of 85% phosphoric acid was defined as 0 ppm. Experiments to confirm the identity of proposed metabolites were routinely performed by adding 1-5 mM of standard to the sample and then reacquiring the spectra.

\textbf{Results}

\textbf{Dehydrophos is transported into \textit{S. enterica} via the Opp and Tpp transporters.} To determine which peptide transport systems are involved in the uptake of dehydrophos, we tested the ability of the antibiotic to inhibit the growth of \textit{S. enterica} permease mutants. The mutants used are well-characterized strains that have been previously used to characterize peptide uptake in \textit{S. enterica} (7). TN1379 encodes each of the known peptide permease systems, TN1890 contains both the Dpp and Tpp
permease systems (\textit{opp}) and TN2271 contains only the Dpp system (\textit{opp}, \textit{tpp}). As expected, dehydrophos inhibits TN1379; however, it fails to inhibit TN2271 and shows only a very small zone of inhibition with TN1890 (Figure 2). These results indicate that the Opp peptide transporter is the major transporter of dehydrophos, with Tpp transporter playing a relatively minor role. Dpp is unable to transport dehydrophos. This result also shows that a cytoplasmic target or processing step is required for bioactivity.

**Dehydrophos action requires peptidase activity.** To test whether peptide bond cleavage was required for dehydrophos activity, we examined bioactivity against a series of characterized \textit{S. enterica} peptidase mutants (22). A quadruple mutant lacking PepA, PepD, PepB and PepN was resistant to dehydrophos, whereas the isogenic strain containing all of the missing peptidases was sensitive to the antibiotic, indicating that dehydrophos action is dependent on peptidase cleavage. Strains carrying any one of the deleted peptidases were also sensitive to dehydrophos; however, the severity of the growth inhibition varied depending on the peptidase present. Strains carrying peptidase A or B displayed growth inhibition similar to wild-type, while a strain carrying peptidase D had a slightly smaller zone of inhibition, and a strain carrying only peptidase N had a significantly smaller zone of inhibition (Figure 4).

**In vitro peptidase cleavage of dehydrophos by \textit{Salmonella} peptidase A results in the generation of methyl acetylphosphonate.** As described above, hydrolysis of the peptide bonds in dehydrophos is expected to yield methyl acetylphosphonate via a series of uncatalyzed chemical reactions. To test this prediction, we incubated synthetically prepared dehydrophos with purified PepA and monitored the products of the cleavage reaction with phosphorus NMR spectroscopy.
Under the conditions used, the reaction proceeded slowly, which allowed for the detection of discrete intermediates. After one hour of incubation, four separate peaks were observed. Based on a spiking experiment with a synthetic standard, the peak with a chemical shift of 10.2 ppm corresponds to unaltered dehydrophos. The peak at 10 ppm is presumed to be dehydrophos lacking the N-terminal glycine (desglycyldehydrophos); however, this could not be rigorously established due to the lack of an appropriate standard. After three hours of incubation, the dehydrophos peak decreased in intensity, while the putative desglycyldehydrophos peak increased. Following overnight incubation, the desglycyldehydrophos peak disappeared and the dehydrophos peak was greatly diminished. Two other peaks were seen in the spectra; one located at 6.2 ppm, and the other at 1 ppm. The peak at 6.2 ppm was transiently observed during the first two time points, but was not observed after the overnight incubation, whereas the peak at 1 ppm increased in intensity over time, and following overnight incubation represented the most abundant phosphorus containing species in the reaction mixture. Spiking of the assay with a synthetic standard identified the 1 ppm peak as MAP. The 6.2 ppm peak was not identified.

**Dehydrophos bioactivity is not relieved by acetate.** Because MAP is a known inhibitor of pyruvate dehydrogenase and pyruvate oxidase, we examined whether the bioactivity of dehydrophos could be relieved by supplementation with acetate, which is required for growth of *pdh, pox* double mutants (4). To this end, we performed a bioassay in the presence of dehydrophos and acetate. The zone of inhibition remained unaltered, consistent with the idea that dehydrophos has multiple, essential cellular targets (Figure 6).
1-HEP O-monomethyl ester is a product of dehydrophos breakdown. Early investigations into dehydrophos biosynthesis were confounded by the presence of an unidentified phosphonate observed by $^{31}$P NMR spectroscopy in the culture supernatants of native and heterologous dehydrophos producers (5). Our observation of MAP as the product of in vivo peptidase processing of dehydrophos suggested a potential source and identity of this unknown phosphonate. Spiking of samples containing the unidentified phosphonate with authentic standards showed that the unknown compound was not MAP (data not shown), leading to the hypothesis that cells might detoxify MAP via reduction to form 1-HEP O-Me, analogous to the thermodynamically favorable reduction of pyruvate to lactate by lactate dehydrogenase.

To test this idea, we fed dehydrophos to *S. lividans* 66 and monitored the culture supernatant with phosphorus NMR spectroscopy. Over time the phosphorus peak associated with dehydrophos diminishes in intensity corresponding with the appearance and growth of a second peak, which was confirmed to be 1-HEP O-Me by addition of a synthetic standard (Figure 7).

**Discussion**

Many known phosphonate antibiotics utilize short peptides as a ‘trojan horse’ to obtain access into the cellular environment. The data presented here clearly indicate that dehydrophos can be added to this list. The in vivo requirement for both peptide transport and hydrolysis, coupled with the results of in vitro peptidase digestion, strongly suggest that the bioactive agent derived from dehydrophos is MAP, a structural analog of pyruvate. MAP is a known inhibitor of both pyruvate dehydrogenase and pyruvate
oxidase. With a $K_i$ of $5 \times 10^{-8}$ M, MAP binds to pyruvate dehydrogenase 10,000 times better than the natural substrate, pyruvate (24). MAP has relatively modest $K_i$ for pyruvate oxidase (1 mM, pH 7.0), which is ten-fold lower affinity than the enzyme has for its natural substrate pyruvate (8, 24). Mutants lacking both pyruvate dehydrogenase and pyruvate oxidase are incapable of growth unless the media are supplemented with acetate, potentially explaining the mechanisms of growth inhibition by dehydrophos. Nevertheless, these two enzymes cannot be the sole targets of dehydrophos, because supplementation of the media with acetate fails to relieve growth inhibition. This finding is not altogether surprising given the number of essential cellular processes that involve pyruvate. Indeed, while MAP is best known as a potent pyruvate dehydrogenase inhibitor, it has also been shown to inhibit other pyruvate utilizing enzymes (24).

Our model for in vivo production of MAP involves enzymatic cleavage to produce an unstable compound that undergoes spontaneous chemical conversion to the pyruvate analog. Interestingly, our $^{31}$P NMR data show transient accumulation of a phosphonate intermediate at 6.2 ppm that could correspond to the unstable intermediate, which could be either 1-aminovinylphosphonate $O$-methyl ester or 1-iminovinylphosphonate $O$-methyl ester (Figure 1). Although we could not rigorously establish the identity of this compound, we believe the intermediate is probably the latter compound, because known examples of enamine/imine tautomerization occur rapidly and favor the imine form (17). Regardless of which intermediate accumulates, the observation of this transient peak in the $^{31}$P NMR spectrum raises interesting questions regarding the mode of action of dehydrophos. It is possible that one of the transient reactive intermediates produced during dehydrophos catabolism might contribute to
toxicity of dehydrophos. Importantly, these data suggest that the peptide linkages have
a dual role in the antibiotic activity of dehydrophos. Not only do they promote uptake of
the inactive tripeptide, they also stabilize an unstable phosphonate moiety by preventing
the enamine/imine tautomerization of the vinyl amine functional group.

Finally, it should be noted that our results suggest that there is significant
substrate specificity in both uptake and processing of dehydrophos in S. enterica. These
findings are reminiscent of specificity seen in other phosphonate tripeptide antibiotics.
For example, rhizocticin and plumbemycin contain the same bioactive component, but
differ in the amino acid side chains. Interestingly, rhizocticin is an antifungal compound
with little activity towards bacteria, whereas plumbemycin is antibacterial, with little
activity towards fungi. It has been proposed that the differences in bioactivity are a
reflection of substrate specificity in either uptake or processing (3, 6). This observation
has significant implications in the development and design of new, more specific
antibiotics. Given the varied nature of oligopeptide uptake and cleavage, an intriguing
idea would be the attachment of small molecule inhibitors to multiple peptide scaffolds.
Applied appropriately, this could open the door for treatment of specific infections with
specific antibiotics, while avoiding the deleterious effects on the natural host-associated
microbial community.

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References


Figure 1. A Trojan horse model for bioactivity of dehydrophos. Cleavage of the peptide bonds in dehydrophos releases 1-aminovinylphosphonate O-monomethyl ester, which rearranges to the preferred imine. This imine is then subject to attack by a water molecule to yield methyl acetylphosphonate (MAP), a toxic pyruvate mimic.

Figure 2. The oligopeptide permease is required for dehydrophos activity. Bioassays were performed as described by spotting synthetic dehydrophos onto a filter disc and assaying growth inhibition against various Salmonella strains: (center) strain TN1379, contains the Opp, Dpp and Tpp permease systems; (left) strain TN2271 carries only the Dpp permeases (i.e. opp-, tpp-); and (right) strain TN1890 carries both the Dpp and Tpp oligopeptide permeases (i.e. opp).

Figure 3. The antibiotic activity of dehydrophos is peptidase dependent. Dehydrophos fails to inhibit a S. enterica TN215 lacking Peptidases A, B, D, and N when applied as either an agar core from the native producer, or as pure synthetic compound (a). In contrast, S. enterica TN1379 containing the full complement of peptidases exhibits the typically seen growth inhibition (b).

Figure 4. Reintroduction of any single peptidase allele is sufficient to restore dehydrophos bioactivity. Bioassay plates examining the effects of reintroduction of either PepA (Strain TN272, b), PepB (Strain TN273, c), PepN (Strain TN271, d), PepD (Strain TN274, e), or to a quadruple mutant of the aforementioned peptidases (Strain TN271, a).
Figure 5. PepA catalyzed degradation of dehydrophos. A time course following the fate of dehydrophos, monitored by $^{31}$P NMR, incubated with purified *S. enterica* PepA is shown. After one hour, a large peak representing unreacted dehydrophos (10.2 ppm) is present along with a substantial amount of the putative dipeptide (10 ppm) (a). After three hours, the putative dipeptide peak dominates the spectrum and the peak at 1 ppm has increased in intensity (b). Following an overnight incubation only two peaks remain, a large peak at 1 ppm and peak representing unreacted dehydrophos at 10.2 ppm (c). Addition of a synthetic standard identifies the peak at 1 ppm as methylacetylphosphonate (MAP) (d).

Figure 6. Acetate fails to relieve antibiotic inhibition generated by dehydrophos. The central filter disc was spotted with synthetic dehydrophos, while the outer disc contained 10 µl of a 40 mM acetate solution.

Figure 7. *S. lividans* 66 converts exogenous dehydrophos to 1-HEP 0-Me. $^{31}$P NMR spectra depicting the fate of dehydrophos fed to *S. lividans* 66 over time. Prior to inoculation dehydrophos can be observed at a chemical shift of 10.2 ppm (a). Following inoculation of *S. lividans* 66 and 48 hours of growth the peak corresponding to dehydrophos has decreased in intensity, and a second peak has appeared at 24.5 ppm (b). Addition of a synthetic standard increased the intensity of the 24.5 ppm peak and confirmed its identity as 1-hydroxyethylphosphonate 0-methyl ester (1-HEP 0-Me) (c).
Table 1: Strains used in this study

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<th>Strain</th>
<th>Genotype/ relevant features</th>
<th>Source</th>
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<td>S. enterica TN271</td>
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<td>(22)</td>
</tr>
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<td>(9)</td>
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<td>S. lividans WM4467</td>
<td>S. lividans 66 with dehydrophos gene cluster fosmid integrated into ϕC31 attB site</td>
<td>(5)</td>
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<tr>
<td>S. luridus NRRL 15101</td>
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All S. enterica strains are derivatives of Salmonella enterica serovar typhimurium LT2, except TN2271, which is a derivative of Salmonella enterica serovar typhimurium CH379.