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Use of the Dehydrophos Biosynthetic Enzymes to Prepare Antimicrobial Analogs of Alaphosphin

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Abstract

The C-terminal domain of the dehydrophos biosynthetic enzyme DhpH (DhpH-C) catalyzes the condensation of Leu-tRNA^{Leu} with (*R*)-1-aminoethylphosphonate, the aminophosphonate analog of alanine called Ala(P). The product of this reaction, Leu-Ala(P), is a phosphonodipeptide, a class of compounds that have previously been investigated for use as clinical antibiotics. In this study, we show that DhpH-C is highly substrate tolerant and can condense various aminophosphonates (Gly(P), Ser(P), Val(P), 1-amino-propylphosphonate, and phenylglycine(P)) to Leu. Moreover, the enzyme is also tolerant with respect to the amino acid attached to tRNA^{Leu}. Using a mutant of leucyl tRNA synthetase that is deficient in its proofreading ability allowed the preparation of a series of aminoacyl-tRNA^{Leu} derivatives (Ile, Ala, Val, Met, norvaline, and norleucine). DhpH-C accepted these aminoacyl-tRNA derivatives and condensed the amino acid with L-Ala(P) to form the corresponding phosphonodipeptides. A subset of these peptides displayed antimicrobial activities demonstrating that the enzyme is a versatile biocatalyst for the preparation of antimicrobial peptides. We also investigated another enzyme from the dehydrophos biosynthetic pathway, the 2-oxoglutarate dependent enzyme DhpA. This enzyme oxidizes 2-hydroxyethylphosphonate to 1,2-dihydroxyethylphosphonate en route to L-Ala(P), but longer incubation results in overoxidation to 1-oxo-2-hydroxyethylphosphonate. This α -ketophosphonate was converted by the pyridoxal phosphate dependent enzyme DhpD into L-Ser(P). Thus, the dehydrophos biosynthetic enzymes can generate not only L-Ala(P) but also L-Ser(P).

1-Aminoalkylphosphonates are structural analogues of α -amino acids in which the carboxylic group has been replaced by a phosphonic acid. They often inhibit enzymes involved in primary metabolic processes and thus they have been used successfully as drugs or pesticides in medicine and agriculture.¹ Unmodified aminophosphonates cannot easily

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Conflicts of interest

The authors declare no competing financial interest.

penetrate cell membranes. Therefore, Nature and chemists have used phosphonopeptide derivatives as prodrugs to release the potential biological activity of a simple aminophosphonate inside the targeted cell, an example of a Trojan horse strategy.² Several distinct permeases can transport phosphonopeptides into a cell, where the phosphonate warhead is released upon the action of cytoplasmic peptidases.^{3, 4} The *in vivo* activity of a peptidyl prodrug depends on the chemical structure of the C-P containing warhead, and on the length and nature of the peptide.⁵

Efforts towards understanding the biosynthesis of natural phosphonopeptides^{6, 7} have shown that Nature uses different mechanisms⁸⁻¹⁰ to attach amino acids to an otherwise non-bioavailable aminophosphonate. For example, in the case of phosphinothricin tripeptide (PTT), the warhead is a phosphinic acid mimic of glutamic acid and a potent inhibitor of glutamine synthetase that is ligated to the dipeptide Ala-Ala.¹¹ The assembly of PTT is accomplished by three stand-alone non-ribosomal peptide synthetase modules (Fig. 1a).¹²⁻¹⁵ In the biosynthesis of rhizotocins and plumbemycins the attachment of different proteinogenic amino acids to the threonine synthase antagonist (Z)-L-2-amino-5-phosphono-3-pentenoic acid (APPA) defines the anti-fungal or anti-bacterial activity of these phosphonopeptides.¹⁶⁻¹⁸ In this case, regardless of the exact nature of the side chain, the amino acids are converted to aminoacyl phosphates by ATP-dependent L-amino acid ligases of the ATP-Grasp family¹⁹ before the formation of the desired amide bonds (Fig. 1b).²⁰

Interestingly, in the biosynthesis of dehydrophos in *Streptomyces luridus*, the two enzymes involved in the formation of the two peptide bonds (DhpH and DhpK) do not interact directly with ATP. Instead, aminoacyl-tRNA intermediates are the co-substrates of these biocatalysts (Fig. 1c).^{21, 22} Perhaps this case should still be considered as an ATP-dependent example as the involvement of leucyl- and glycyl-tRNA synthetase requires ATP to charge amino acids to tRNA. A final strategy to generate amide bonds in phosphonopeptides is a combination of different mechanisms such as the biosynthesis of fosfazinomycin in which the Arg-phosphonate linkage appears to be generated by an ATP-GRASP enzyme, but the Val-Arg linkage is formed in a valinyl-tRNA dependent process.^{23, 24}

One of the most extensively studied phosphonopeptides, alaphosphin (Fig. 1d), also known as alafosfalin, is a manmade compound with significant antibacterial properties²⁵ that has been investigated as a prodrug for treatment of urinary tract infections.²⁶ The L-form of alaninephosphonate (L-Ala(P)), the warhead of alafosfalin, is a potent inhibitor of alanine racemases isolated from Gram-positive bacteria.³ However, the compound cannot penetrate the cell membrane. During our investigations of the biosynthesis of the phosphonopeptide dehydrophos we concluded that the generation of L-Ala(P) is necessary for the formation of the final product, because it acts as the acceptor of Leu during DhpH-mediated tRNA-dependent peptide bond formation (Fig. 1c). The generated dipeptide L-Leu-L-Ala(P) is then modified further by other enzymes encoded by genes downstream of *dhpH*. Thus, while L-Leu-L-Ala(P) does not appear to be the final product, this phosphonopeptide is generated as an intermediate and is a natural analogue of synthetic alaphosphin. Interestingly, the extensive structure-activity relationship studies carried out by Roche during the development of alaphosphin^{3, 27} showed that the intracellular amounts of Ala(P) found in six-different microorganisms was much higher when Leu-Ala(P) was administered instead of Ala-Ala(P),

although the former was eliminated from plasma faster than Ala-Ala(P).⁵ The documented bioactivity of phosphonodipeptides as well as the unexplored possibility to generate these molecules enzymatically by using the tRNA-dependent ligase DhpH as catalyst prompted us to investigate its substrate scope.

Results and Discussion

Before investigating the condensation of α -amino acids with aminophosphonates using DhpH, we first reexamined some of the early steps of dehydrophos biosynthesis as we recognized an opportunity to potentially make 1-aminoalkylphosphonates other than Ala(P). The biosynthesis of dehydrophos begins like most phosphonate biosynthetic pathways⁷ with the rearrangement of phosphoenolpyruvate (PEP) to phosphonopyruvate (PnPy) catalyzed by PEP phosphomutase (DhpE) and the subsequent DhpF-mediated decarboxylation of PnPy to phosphonoacetaldehyde (PnAA).²¹ An iron-dependent alcohol dehydrogenase DhpG then reduces PnAA to 2-hydroxyethyl-phosphonate (2-HEP) (Fig. 2a). Subsequently, DhpA, a 2-oxoglutarate/Fe(II)-dependent oxygenase, oxidizes 2-HEP²⁸ to 1,2-dihydroxyethylphosphonate (DHEP). When we examined the reaction mixture upon incubation of 2-HEP with DhpA and α -ketoglutarate (α -KG) for a longer period of time (i.e. 5 h instead of 0.5 h), we observed by ³¹P NMR spectroscopy the formation of a new signal at –1.4 ppm in the ³¹P NMR spectrum (Fig. 3a). Furthermore, when DHEP was incubated directly with DhpA followed by DhpD and alanine, the phosphonic acid analog of serine, Ser(P),²⁹ was formed (Fig. 3b). Reasoning that the signal could be an acyl phosphonate by further oxidation of the secondary alcohol of DHEP to the corresponding carbonyl, we tested if we could produce the same signal by incubating pyruvate and *rac*-Ser(P), with DhpD, a PLP-dependent aminotransferase from the dehydrophos pathway that normally interconverts acetylphosphonate and Ala(P).²¹ Indeed, a ³¹P NMR signal with the same chemical shift was observed (Fig. 3c & d). Thus, we infer from this set of experiments that the ³¹P NMR signal at a –1.4 ppm is due to formation of the unstable acylphosphonate 1-oxo-2-hydroxyethylphosphonate (OH-EP). Therefore, DhpA not only can hydroxylate 2-HEP at the alpha carbon but it can further oxidize the secondary hydroxyl group to the corresponding carbonyl (Fig. 2b). DhpD can then convert this intermediate to the known compound Ser(P).²⁹

We also incubated *rac*-1-HEP instead of DHEP with DhpA and observed formation of acetylphosphonate (AP) as verified with authentic standard. By using an oxygen electrode probe, the steady-state kinetics of hydroxylation of 2-HEP and the overoxidation of *rac*-DHEP and *rac*-1-HEP by DhpA in the presence of 2-oxoglutarate and atmospheric molecular oxygen were determined. DhpA exhibits a $k_{cat}/K_{m,2-HEP}$ ($M^{-1} s^{-1}$) = 8.4×10^4 whereas the catalytic efficiency of DhpA for the oxidation of racemic DHEP and 1-HEP was ca. 10-fold lower (Fig. 4), consistent with the hydroxylation of 2-HEP as the physiological reaction for DhpA.

When using *rac*-1-HEP as substrate, we observed oxidation of roughly half of the substrate to AP by DhpA (Fig. 5b), suggesting that the enzyme accepts only one of the enantiomers. The stereochemical preference of DhpA for the oxidation of 1-HEP was determined as illustrated in Fig. 5a. We incubated *rac*-1-HEP in the presence of atmospheric oxygen with

the *S*-specific non-heme-iron dependent enzyme HEPD.³⁰ As expected based on previous studies, 50% of 1-HEP (i.e. only the *S*-enantiomer) was converted to acetyl phosphate, an unstable intermediate prone to hydrolysis to form phosphate and acetate (Fig. 5a). Thus, the product of this process is mainly (*R*)-1-HEP. Subsequently, after removal of HEPD and lyophilization of the reaction mixture, half of the lyophilized material was re-dissolved in buffer and incubated with excess α -KG and Fe(II)-reconstituted DhpA (Fig. 5c). The other half of the lyophilized material was spiked with *rac*-1-HEP and treated with DhpA in the same manner as the unspiked sample (Fig. 5d). No formation of AP was observed in the non-spiked sample when the DhpA was removed and the ³¹P NMR spectrum was recorded. Thus, we conclude that DhpA preferentially reacts with the *S*-enantiomer of 1-HEP. Indirectly, this also suggests that the DHEP product of DhpA has the *S*-configuration, which was not investigated in previous studies on dehydrophos biosynthesis.

In previous studies, several other phosphonodipeptides with structural changes in the phosphonic acid part have been synthesized chemically and tested for their biological activity. In general replacement of Ala(P) with various phosphono amino acids Xxx(P) lead to loss of antibacterial activity with the exception of L-Ser(P), Gly(P),³¹ L-Phe(P)³² and phosphonate analogues of Glu and α -methylalanine.³³ We tested herein the enzymatic formation by DhpH of L-Leu-L-**Xxx**(P) peptides using commercial available aminophosphonates.³⁴ We first observed that Ser(P) was accepted as substrate by DhpH-C (Fig. 6). We then evaluated whether Gly(P), 1-amino-propylphosphonate, Val(P) and phenylglycine(P) (Phg(P)) could act as Leu acceptors in a leucyl-tRNA dependent process (Fig. 7) in comparison with synthetic standards of the products (see ESI†). In most cases, the reaction went to partial completion when a racemic α -amino phosphonic acid was employed and only one diastereomer of the product was formed presumably due to the stereochemical preference of DhpH-C for L-aminophosphonates as shown by its selectivity for L-Ala(P).²¹ Thus, enzymatic resolution of racemic α -amino phosphonic acids can in principle be accomplished by this method. Another biocatalyzed kinetic resolution of racemic mixtures of chiral α -amino phosphonic acids has been reported recently, but the enzyme responsible for the transformation is unknown and only whole-cells were used.³⁵ In accordance to previous studies,²¹ pSer(P) and 1-hydroxy-2-amino phosphonate were not accepted by DhpH-C. The dianionic form of a phosphonate does not seem to be required, because both the phosphinic form of Ala(P) (Fig. 7e) and the monomethyl ester of Ala(P) were tolerated by DhpH-C (Fig. 7f). However, the tetrahedral geometry of phosphonic acids seems to be important for recognition because Ala(P) could not be replaced by Ala.

We also investigated the tolerance of DhpH-C towards amino acid donors other than Leu. We initially tested if DhpH-C can tolerate a minimal substrate of Leu attached to the 3' hydroxyl group of adenosine as was reported recently for the *Escherichia coli* aminoacyl tRNA transferase.³⁶ Therefore, we synthesized a mixture of 2'- and 3'-leucyl adenosine following the reaction scheme and purification method reported in the literature.³⁶ Unfortunately, neither of the two constitutional isomers was a substrate at 2 mM for DhpH-C in the presence of L-Ala(P). Assuming at this point that the integrity of the tRNA^{Leu} is important for recognition of the donor amino acid covalently attached to it, we employed a different strategy in order to mischarge tRNA^{Leu} with standard and nonstandard amino

acids. Martinis and coworkers reported mutants of *E. coli* leucyl-tRNA synthetase with impaired editing activity which do not hydrolyze misacylated tRNA^{Leu} molecules.³⁷ One of these mutants, LeuRS-Y330A/D342A/D345A from *E. coli*, was used in this study due to its demonstrated high level misacylation activity.³⁷ Indeed, by using the LeuRS triple mutant for charging tRNA^{Leu} instead of wt LeuRS, DhpH-C was able to accept several activated amino acids (Fig. 8). Perhaps the second most interesting dipeptide formed after alaphosphin (Fig. 8e) is norvalyl-1-aminoethylphosphonic acid (Nva-Ala(P), Fig. 8b), which when combined with nocardicin A has been shown to possess potent synergistic antibacterial activity.³⁸ In these assays, the conversion varied from 90% for norvaline and norleucine to less than 10% for alanine. We note that the overall efficiency of these reactions depends on both the efficiency of the triple LeuRS mutant to recognize non-canonical amino acids and the ability of DhpH-C to recognize the mischarged aminoacyl-tRNA^{Leu}. Given the precedent of 1-aminoalkylphosphonic acids as antimicrobial agents, the phosphonodipeptides prepared in this study were tested against *Escherichia coli* ATCC 25922.³⁹ Amino acyl analogs of alaphosphin, such as L-norvaline-L-Ala(P), L-leucine-L-Ala(P), L-norleucine-L-Ala(P) and L-methionine-L-Ala(P), showed antimicrobial activity with clear zones of inhibition by agar disk diffusion assay (see ESI, Figure S2). However, compounds containing other phosphonic acids instead of Ala(P) were inactive against this strain.

Conclusions

We demonstrate in this study that DhpH-C has broad substrate tolerance with respect to its two substrates, leucyl-tRNA and aminophosphonates, and can be used to generate a range of phosphonodipeptides. Other amino acids attached to tRNA^{Leu} are accepted by the enzyme and various L-aminophosphonates could be condensed with Leu. The enzyme only accepts the L-enantiomer of the 1-aminoalkylphosphonate and hence can be used to resolve racemic mixtures. DhpH-C can therefore serve as a biocatalyst for the preparation of phosphonodipeptides that have been shown to be efficient antimicrobial agents in previous studies. This work provides a blueprint for investigating the substrate selectivity of other tRNA-dependent biosynthetic systems.⁴⁰⁻⁴³

Experimental section

General Procedures

DhpH and DhpA were obtained by heterologous expression in *E. coli* of the corresponding genes from *Streptomyces luridus* and the corresponding activity assays were carried out as described previously.^{21, 22} Unless otherwise stated, all reactions to produce authentic standards were performed in oven-dried or flame-dried glassware under an atmosphere of dry nitrogen or argon. Triethylamine and ethyl acetate were distilled from calcium hydride prior to use. Ethyl chloroformate was freshly distilled immediately before use. Reactions were monitored by thin layer chromatography (TLC) on Silicycle Siliaplate™ G TLC plates (250 µm thickness, 60 Å porosity, F-254 indicator) and visualized by UV irradiation and staining with *p*-anisaldehyde, phosphomolybdic acid, or potassium permanganate developing agents. Volatile solvents were removed under reduced pressure using a rotary

evaporator. Flash column chromatography was performed using Silicycle F60 silica gel (60Å, 230-400 mesh, 40-63 µm). Phosphonates were purified using CombiFlash® Rf+ equipped with a 15.5 g RediSep Rf Gold C18Aq column using acetonitrile and water as mobile phases. Proton nuclear magnetic resonance (^1H NMR) and carbon nuclear magnetic resonance (^{13}C NMR) spectra were recorded on a Agilent-600 MHz NMR spectrometer operating respectively at 600 MHz for ^1H , and 150 MHz for ^{13}C . Chemical shifts are reported in parts per million (ppm) with respect to the residual solvent signal CDCl_3 (^1H NMR: $\delta = 7.26$; ^{13}C NMR: $\delta = 77.16$) and D_2O (^1H NMR: $\delta = 4.79$). Peak multiplicities are reported as follows: s = singlet, bs = broad singlet, d = doublet, t = triplet, dd = doublet of doublets, td = triplet of doublets, m = multiplet. app = apparent. High-resolution mass spectra (HRMS) were obtained using a Waters SynApt mass spectrometer.

General procedure for chemical synthesis of phosphonodipeptides.

Phosphonates were prepared using a modified procedure reported by Solodenko and co-workers.⁴⁴ A 10 mL flame-dried reaction tube was charged with aminophosphonate (1.0 equiv). The reaction vessel was evacuated and backfilled with nitrogen (three times in total) followed by the addition of freshly distilled hexamethyldisilazane (5 mL per mmol aminophosphonate). The reaction mixture was heated to 150 °C and kept at this temperature for 5 h or until the aminophosphonate dissolved. The reaction vessel was cooled to room temperature and concentration *in vacuo*. The crude residue was used in the following step without purification. In a separate reaction vessel, an oven-dried round bottom flask was charged with *N*-benzyloxycarbonyl L-amino acid (1 equiv). The reaction vessel was evacuated and backfilled with nitrogen (three times in total), cooled to 0 °C followed by the addition of ethyl acetate (5 mL per mmol amino acid), freshly distilled triethylamine (1 equiv) and then ethyl chloroformate (1 equiv). The reaction mixture was stirred for 30 minutes at 0 °C followed by the dropwise addition of the crude residue containing TMS-protected aminophosphonate in EtOAc. The reaction vessel was maintained at this temperature for 20 minutes, warmed to room temperature for 20 minutes, and finally heated to 80 °C for 1 h. The reaction mixture was quenched with saturated *aq.* NaHCO_3 , and the resulting suspension was acidified with 3 M HCl until pH 1. The crude mixture was extracted with EtOAc (3 × 5 mL) and the combined organic layers were washed with brine, dried over NaSO_4 , and concentrated *in vacuo*. The crude residue was added glacial acetic acid (5 mL per mmol aminophosphonate) followed by 48% hydrobromic acid (5 mL per mmol aminophosphonate) and stirred for 2 h. at room temperature. The reaction mixture was concentrated *in vacuo* and the crude residue was purified by CombiFlash® Rf+ equipped with a 15.5 g RediSep Rf Gold C18Aq column. Acetonitrile and H_2O were used as the mobile phases. The purification method began with 100% H_2O for 5 min and then a gradient of 0-100% *aq.* MeCN was applied over 5 min at 30 mL/min flow rate. Fractions containing the product were concentrated *in vacuo* to afford the pure phosphonodipeptide. For spectroscopic data of synthetic compounds, see the ESI.

Supplementary Material

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Notes and references

1. Mucha A, Kafarski P and Berlicki L, J. Med. Chem, 2011, 54, 5955–5980. [PubMed: 21780776]
2. Klahn P and Bronstrup M, Nat. Prod. Rep, 2017, 34, 832–885. [PubMed: 28530279]
3. Atherton FR, Hall MJ, Hassall CH, Lambert RW, Lloyd WJ and Ringrose PS, Antimicrob. Agents Chemother, 1979, 15, 696–705. [PubMed: 525987]
4. Atherton FR, Hall MJ, Hassall CH, Lambert RW, Lloyd WJ, Lord AV, Ringrose PS and Westmacott D, Antimicrob. Agents Chemother, 1983, 24, 522–528. [PubMed: 6360039]
5. Thomas WA, Biochem. Soc. Trans, 1986, 14, 383–387. [PubMed: 3709951]
6. Metcalf WW and van der Donk WA, Annu. Rev. Biochem, 2009, 78, 65–94. [PubMed: 19489722]
7. Horsman GP and Zechel DL, Chem. Rev, 2017, 117, 5704–5783. [PubMed: 27787975]
8. Giessen TW and Marahiel MA, FEBS Lett, 2012, 586, 2065–2075. [PubMed: 22273582]
9. Hashimoto S, J. Biol. Macromol, 2008, 8, 28–37.
10. Fischbach MA and Walsh CT, Chem. Rev, 2006, 106, 3468–3496. [PubMed: 16895337]
11. Blodgett JA, Thomas PM, Li G, Velasquez JE, van der Donk WA, Kelleher NL and Metcalf WW, Nat. Chem. Biol, 2007, 3, 480–485. [PubMed: 17632514]
12. Schwartz D, Grammel N, Heinzelmann E, Keller U and Wohlleben W, Antimicrob. Agents Chemother, 2005, 49, 4598–4607. [PubMed: 16251301]
13. Grammel N, Schwartz D, Wohlleben W and Keller U, Biochemistry, 1998, 37, 1596–1603. [PubMed: 9484230]
14. Lee JH, Evans BS, Li G, Kelleher NL and van der Donk WA, Biochemistry, 2009, 48, 5054–5056. [PubMed: 19432442]
15. Schinko E, Schad K, Eys S, Keller U and Wohlleben W, Phytochemistry, 2009, 70, 1787–1800. [PubMed: 19878959]
16. Rapp C, Jung G, Kugler M and Loeffler W, Liebigs Ann. Chem, 1988, 655–661.
17. Borisova SA, Circello BT, Zhang JK, van der Donk WA and Metcalf WW, Chem. Biol, 2010, 17, 28–37. [PubMed: 20142038]
18. Gahungu M, Arguelles-Arias A, Fickers P, Zervosen A, Joris B, Damblon C and Luxen A, Bioorg. Med. Chem, 2013, 21, 4958–4967. [PubMed: 23891162]
19. Ogasawara Y and Dairi T, Chemistry, 2017, 23, 10714–10724. [PubMed: 28488371]
20. Kino K, Kotanaka Y, Arai T and Yagasaki M, Biosci. Biotechnol. Biochem, 2009, 73, 901–907. [PubMed: 19352016]
21. Bougioukou DJ, Mukherjee S and van der Donk WA, Proc. Natl. Acad. Sci. U. S. A, 2013, 110, 10952–10957. [PubMed: 23776232]
22. Ulrich EC, Bougioukou DJ and van der Donk WA, ACS Chem. Biol, 2018, 13, 537–541. [PubMed: 29303545]
23. Gao J, Ju KS, Yu X, Velásquez JE, Mukherjee S, Lee J, Zhao C, Evans BS, Doroghazi JR, Metcalf WW and van der Donk WA, Angew. Chem. Int. Ed, 2014, 53, 1334–1337.
24. Huang Z, Wang K-KA and van der Donk WA, Chem. Sci, 2016, 7, 5219–5223. [PubMed: 28070267]
25. Allen JG, Atherton FR, Hall MJ, Hassall CH, Holmes SW, Lambert RW, Nisbet LJ and Ringrose PS, Nature, 1978, 272, 56–58. [PubMed: 628432]
26. Maruyama HB, Arisawa M and Sawada T, Antimicrob. Agents Chemother, 1979, 16, 444–451. [PubMed: 518073]
27. Allen JG, Havas L, Leicht E, Lenox-Smith I and Nisbet LJ, Antimicrob. Agents Chemother, 1979, 16, 306–313. [PubMed: 116591]

28. Circello BT, Eliot AC, Lee J-H, van der Donk WA and Metcalf WW, *Chem. Biol.*, 2010, 17, 402–411. [PubMed: 20416511]
29. Lejczak B, Kafarski P, Soroka M and Mastalerz P, *Synthesis*, 1984, 1984, 577–580.
30. Cicchillo RM, Zhang H, Blodgett JAV, Whitteck JT, Li G, Nair SK, van der Donk WA and Metcalf WW, *Nature*, 2009, 459, 871–874. [PubMed: 19516340]
31. Atherton FR, Hall MJ, Hassall CH, Lambert RW, Lloyd WJ, Ringrose PS and Westmacott D, *Antimicrob. Agents Chemother.*, 1982, 22, 571–578. [PubMed: 6758686]
32. Atherton FR, Hassall CH and Lambert RW, *J. Med. Chem.*, 1986, 29, 29–40. [PubMed: 3510298]
33. Lejczak B, Kafarski P, Sztajer H and Mastalerz P, *J. Med. Chem.*, 1986, 29, 2212–2217. [PubMed: 3783584]
34. Ordonez M, Viveros-Ceballos JL, Cativiela C and Arizpe A, *Curr. Org. Synth.*, 2012, 9, 310–341.
35. Kozyra K, Brzezinska-Rodak M, Klimek-Ochab M and Zymanczyk-Duda E, *J. Mol. Cat. B-Enzym.*, 2013, 91, 32–36.
36. Wagner AM, Fegley MW, Warner JB, Grindley CL, Marotta NP and Petersson EJ, *J. Am. Chem. Soc.*, 2011, 133, 15139–15147. [PubMed: 21894909]
37. Martinis SA, Briggs JM, Mursinna RS, Lee KW, Lincecum TL, Williams AM and Zhai Y. U. S. Patent 7,785,827 B2, 8 31, 2010 *Pat.*, 7,785,827 B2.
38. Angehrn P, Hall MJ, Lloyd WJ and Westmacott D, *Antimicrob. Agents Chemother.*, 1984, 25, 607–611. [PubMed: 6428312]
39. Allen JG, Atherton FR, Hall MJ, Hassall CH, Holmes SW, Lambert RW, Nisbet LJ and Ringrose PS, *Antimicrob. Agents Chemother.*, 1979, 15, 684–695. [PubMed: 43113]
40. Zhang W, Ntai I, Kelleher NL and Walsh CT, *Proc. Natl. Acad. Sci. U. S. A.*, 2011, 108, 12249–12253. [PubMed: 21746899]
41. Maruyama C, Niikura H, Izumikawa M, Hashimoto J, Shin-Ya K, Komatsu M, Ikeda H, Kuroda M, Sekizuka T, Ishikawa J and Hamano Y, *Appl. Environ. Microbiol.*, 2016, 82, 3640–3648. [PubMed: 27084005]
42. Moutiez M, Belin P and Gondry M, *Chem. Rev.*, 2017, 117, 5578–5618. [PubMed: 28060488]
43. Ulrich EC and van der Donk WA, *Curr. Opin. Chem. Biol.*, 2016, 35, 29–36. [PubMed: 27599269]
44. Solodenko V, Kasheva T and Kukhar V, *Syn. Comm.*, 1991, 21, 1631–1641.

Fig. 1.

Different strategies for generating amide bonds in natural phosphonopeptides. **a.** Activation of amino acids *via* formation of an aminoacyl-adenosine monophosphate intermediate during the biosynthesis of PTT. **b.** Activation of amino acids *via* formation of an aminoacyl phosphate intermediate during biosynthesis of rhizoctin B. **c.** Activation of amino acids *via* formation of an aminoacyl-tRNA intermediate in the biosynthesis of dehydrophos. **d.** Other examples of bioactive phosphonopeptides and aminophosphonates.

Fig. 2.

Dehydrophos biosynthetic pathway. **a.** Enzymatic steps involved in biosynthesis of dehydrophos.²¹ **b.** Enzymatic pathway for formation of L-Leu-Ser(P) from DHEP. **c.** Structure of *rac*-1-HEP.

Fig. 3. ^{31}P NMR spectra of DhpA oxidation of *rac*-DHEP and DhpD transamination to produce Ser(P). **a.** ^{31}P NMR spectrum after conversion of *rac*-DHEP (■) into OH-EP (▼) by DhpA in the presence of Fe(II)/ α -KG/ O_2 and L-ascorbic acid. **b.** ^{31}P NMR spectrum of **a** with conversion of OH-EP into Ser(P) (●) by DhpD in the presence of L-alanine. **c.** ^{31}P NMR spectrum after incubation of *rac*-Ser(P) (peak marked with × corresponds to unknown impurity present in Ser(P)) with DhpD in the absence of pyruvate. **d.** ^{31}P NMR spectrum after partial conversion of *rac*-Ser(P) into OH-EP by DhpD in the presence of pyruvate. The unlabeled peak between ~2-3 ppm is orthophosphate.

Fig. 4.

Kinetics of DhpA-mediated oxidation of various substrates at 25 °C. **a.** The assay with 2-HEP contained 0.01 – 0.2 mM 2-HEP, 2 mM α -KG, 0.65 μM DhpA, and 50 mM Na-HEPES, pH 8.0. (slower rates were observed when concentrations higher than 0.25 mM of 2-HEP were used suggesting substrate inhibition). **b.** The assay with *rac*-1-HEP contained: 0.01 – 0.7 mM *rac*-1-HEP, 2 mM α -KG, 3.25 μM DhpA, in 50 mM Na-HEPES, pH 8.0. **c.** The assay with *rac*-DHEP contained: 0.01 – 1.0 mM *rac*-DHEP, 2 mM α -KG, 3.25 μM DhpA, in 50 mM Na-HEPES, pH 8.0. **d.** Summary of kinetic parameters for DhpA (data are not corrected to account for the racemic nature of 1-HEP and DHEP; K_m values for the (*S*) enantiomers would be expected to be two-fold lower).

Fig. 5. Determination of the enantioselectivity of the reaction of DhpA with 1-HEP. **a.** design of experiments to determine the preference of DhpA for the two enantiomers of 1-HEP using the known stereoselectivity upon reaction with HEPD. **b.** ³¹P NMR spectrum of reaction of 1.2 mM *rac*-1-HEP with 75 μM of Fe(II)-reconstituted DhpA in the presence of 5 mM α-KG, 1 mM L-ascorbic acid in 50 mM Na-HEPES, pH 7.5. **c** Assay of 1-HEP remaining after treatment with HEPD and then exposed to 75 μM of Fe(II)-reconstituted DhpA, 5 mM α-KG, 1 mM L-ascorbic acid in 50 mM Na-HEPES, pH 7.5. **d.** Assay of 1-HEP remaining after treatment with HEPD and then spiked with 1.2 mM *rac*-1-HEP (0.6 mM total) treated with 75 μM of Fe(II)-reconstituted DhpA, 5 mM α-KG, 1 mM L-ascorbic acid in 50 mM Na-HEPES, pH 7.5. Signal at ~19.5 ppm corresponds to 1-HEP (●); signal at ~0.5 ppm corresponds to AP (■); the unlabeled signal at ~3 ppm is orthophosphate.

Fig. 6. NMR analysis of L-Leu-Ser(P) (▼). a. ^{31}P NMR spectrum of L-Leu-L-Ser(P) prepared under the following reaction conditions: 10 mM *rac*-Ser(P) (●, peak marked with × corresponds to an impurity of the starting material), 2 mM L-Leu, 4 mM ATP, 2 mg total tRNA (from *E. coli*), 250 μg BSA, 5 μM LeuRS, 20 U TIPP, and 50 μM Dhph-C. b. Enzymatic product in panel a spiked with synthetic L-Leu-*rac*-Ser(P) of panel c. ^{31}P NMR spectrum of L-Leu-*rac*-Ser(P). The products are diastereomeric at the α-carbon of Ser(P). d. ^1H NMR spectrum of synthetic L-Leu-*rac*-Ser(P).

Fig. 7.

^{31}P NMR analysis of DhpH-C activity on other aminophosphonates. For *rac*-1-amino-propylphosphonate and aminomethyl phosphonate each reaction contained: 10 mM aminophosphonate, 4 mM L-Leu, 4 mM ATP, 2 mg total tRNA from *E. coli*, 250 μg BSA, 5 μM LeuRS, 20 U TIPP, and an aliquot of 50 μM DhpH-C in 100 mM Na-HEPES, 10 mM KCl, 20 mM MgCl_2 , pH 7.5. For *rac*-Val(P), *rac*-Phg(P) and *rac*-1-aminoethylphosphinic acid each reaction contained: 5 mM aminophosphonate, 6 mM L-Leu, 6 mM ATP, 1.5 mg total tRNA from *E. coli*, 5 μM LeuRS, 2 U TIPP, and 50 μM DhpH-C in 100 mM Na-HEPES, 10 mM KCl, 20 mM MgCl_2 , pH 7.5. a. Reaction in the presence of aminomethyl phosphonate [Gly(P)]. b. Reaction with *rac*-1-amino-propylphosphonate. c. Reaction with *rac*-Val(P). d. Reaction with *rac*-Phg(P). e. Reaction with *rac*-1-aminoethylphosphinic

acid. f. Reaction with monomethyl Ala(P). Arrow denotes the ^{31}P signal that belongs to phosphonodipeptide product. The chemical shifts of the dipeptides are all downfield of the starting aminophosphonate (for spectral data, see ESI). The peaks at ~ 2 and 3 ppm are phosphate and AMP, respectively.

Fig. 8.

^{31}P NMR analysis of DhpH-C activity on various amino acids with L-Ala(P). Each reaction contained 5 mM Ala(P), 6 mM amino acid, 6 mM ATP, 1.5 mg total tRNA from *E. coli*, 6 μM LeuRS (triple mutant), 10 U TIPP, and DhpH-C (50 μM) in 100 mM Na-HEPES, 10 mM KCl, 20 mM MgCl_2 , pH 7.5 (500 μL total volume). Arrow indicates the expected product. The identity of the products was confirmed by MS. For spectral data, see ESI.