Phosphonopeptide K-26 biosynthetic intermediates in *Astrosporangium hypotensionis*†

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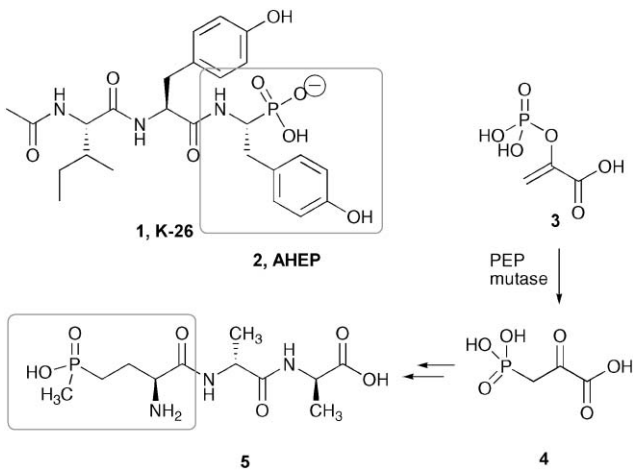
Precursors and advanced intermediates for phosphonopeptide K-26 biosynthesis were synthesized and incorporation studies in *Astrosporangium hypotensionis* suggest a new mechanism of C–P bond formation in aromatic phosphonates.

The angiotensin converting enzyme inhibitor K-26 (**1**) is a naturally occurring *N*-acetylated tripeptide containing isoleucine, tyrosine and the nonproteinogenic amino acid, (*R*)-1-amino-2-(4-hydroxyphenyl)ethylphosphonic acid (AHEP, **2**).[1,2] K-26 is produced by the actinomycete *Astrosporangium hypotensionis* and the terminal phosphonate tyrosine analog is reportedly present in a small group of tripeptide natural products produced by other members of the *Streptosporangiaceae* family.[3,4] This group of natural products possesses potent *in vitro* angiotensin converting enzyme inhibitory activities, which have been demonstrated to translate to effective hypotensive activity *in vivo*.[1,5] To the best of our knowledge, K-26 is the most potent natural product exhibiting this activity, yet the mechanisms by which K-26 and related compounds are biosynthesized remain mainly uncharacterized.

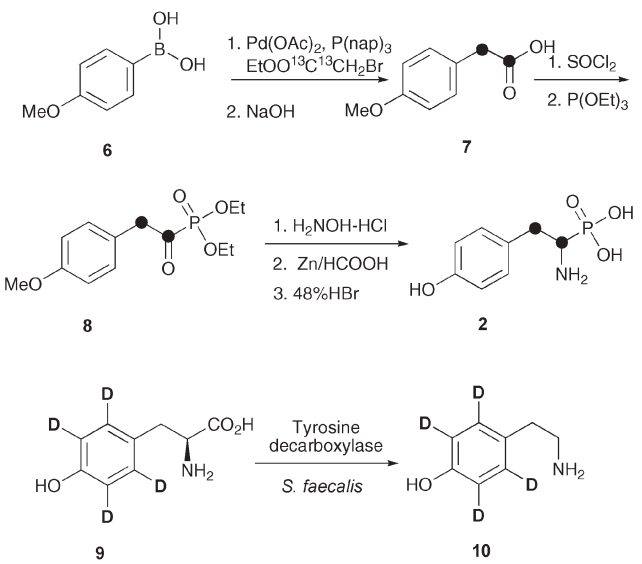
In all C–P bond containing natural product pathways characterized to date, the C–P bond originates *via* phosphoenolpyruvate mutase (PEP mutase) which catalyzes the intramolecular rearrangement of phosphoenolpyruvate to phosphonopyruvate (Scheme 1).[6,7] This endergonic phosphonyl transfer reaction is proposed to proceed through a dissociated metaphosphate intermediate and multiple subsequent elaboration steps are required to produce the final alkylphosphonate natural products. For example, at least 10 steps are required to produce the C–P framework for the γ-phosphonyl amino acid phosphinothricin (**5**) in the herbicidal C–P peptide bialaphos.[8] Due to the positioning of the aromatic ring in AHEP, it is not obvious how the carbon–phosphorus backbone of α-amino phosphonate AHEP could be formed by this mode of phosphonyl migration.



Scheme 1

Recent isotopic incorporation studies have demonstrated incorporation of d_4-tyrosine, ^{15}N-labeled tyrosine and β-d_2-tyrosine into the AHEP moiety of K-26, suggesting that tyrosine is a close primary metabolic precursor of AHEP in the intact peptide.[9] However, these studies were unable to authenticate the intermediacy of free AHEP or determine if C–P bond formation occurs prior or subsequent to peptide bond formation in the biosynthesis of K-26. Also of interest is the timing of acetylation. These data will delineate the interface between primary and secondary metabolism and aid in the elucidation and sequencing of the biosynthetic gene cluster for this potent hypotensive phosphonopeptide.

To determine the substrates and timing of the C–P bond forming step in the biosynthesis of K-26, $^{13}C_2$-labeled AHEP (**2**) was synthesized for isotopic incorporation studies. Based on extant procedures,[10,11] we have developed an efficient synthetic route that facilitates the introduction of heavy atom isotopic labels in *rac*-AHEP (Scheme 2). Suzuki coupling was used to couple



Scheme 2

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4-methoxyphenylboronic acid (**6**) with ethyl $^{13}C_2$-bromoacetate. In a four step one-pot process, the resulting ester was saponified, converted to the corresponding acyl chloride and reacted with triethyl phosphite, resulting in the α-keto phosphonate (**8**). This unstable compound was converted without isolation to the oxime with hydroxylamine, which was immediately reduced *in situ* in the presence of zinc–formic acid, resulting in the respective amine. Global deprotection was achieved with hydrobromic acid and the final product *rac*-AHEP (**2**) was obtained in a 22% overall yield (based on ethyl bromoacetate-$^{13}C_2$) after purification by ion exchange filtration. AHEP was obtained in this two-pot process with only one chromatographic step required and > 95% purity was confirmed by ^{31}P, ^{13}C, ^{1}H NMR and mass spectrometry.

Liquid cultures of *Astrosporangium hypotensionis* were separately supplemented with labeled and unlabeled synthetic racemic AHEP (0.3 mM/day for 4 days). After six days of incubation, K-26 samples were isolated from fermentation supernatant by solid phase extraction with Diaion HP-20 polystyrene resin at pH = 3 and fractionated by 5 K centrifugal molecular weight filtration. K-26 was further separated from co-metabolites by reverse phase HPLC/MS. Cultures that were supplemented with unlabeled AHEP were used as a reference and grew equally well as unsupplemented control cultures. The specific incorporation of $^{13}C_2$-AHEP into K-26 amino acids was determined using the SRM method previously described.[9] The isotopic mass distribution of K-26 from $^{13}C_2$-AHEP fed cultures indicated the major isotopomer at *m/z* = 536, a two Th shift relative to the unlabeled standard (Fig. 1). Deconvolution of mass isotopomer data indicates that $^{13}C_2$-AHEP was incorporated into K-26 at a level of 85% natural abundance.

The high level of incorporation of labeled AHEP in combination with previous data suggests that indeed AHEP is a discrete precursor in the biosynthetic pathway of K-26 and the substrate of the C–P bond forming enzyme is most likely tyrosine or a closely related metabolite. Since decarboxylation of tyrosine is formally required for its conversion to AHEP we also synthesized labeled tyramine *via* decarboxylation of d_4-tyrosine with tyrosine decarboxylase from *Streptococcus faecalis* (Scheme 2).[12] Four separate incorporation studies (at 1.0 mM/day for 4 days) failed to demonstrate any detectable incorporation of d_4-tyramine into K-26. While this result does not absolutely rule out the intermediacy of tyramine in the biosynthesis of K-26, it is most likely that tyrosine is a more direct metabolic precursor than tyramine in the biosynthetic pathway leading to AHEP. If tyrosine is indeed the direct precursor, this also suggests the possibility that decarboxylation is coupled to the C–P bond forming reaction in a single enzymatic reaction. In any event, these data demonstrate that C–P bond forming biochemistry other than PEP mutase is operative in the biosynthetic pathway of K-26.

A priori, it is expected that the K-26 gene cluster will encode an *N*-acetyltransferase enzyme, which may acetylate isoleucine prior or subsequent to peptide coupling. In the biosynthesis of phosphinothricin, the γ-phosphonyl amino acid is acetylated prior to loading onto a nonribosomal peptide synthetase (NRPS) and is subsequently deacetylated at a later stage to produce the bioactive product.[13] To determine if acetylation of isoleucine occurs prior to peptide coupling, as it does in the case of phosphinothricin, feeding studies with $^{13}C_1$-labeled isoleucine and *N*-acetyl isoleucine were designed (Scheme 3). *N*-acetyl isoleucine was synthesized by reacting $^{13}C_2$-labeled acetyl chloride and $^{13}C_1$-isoleucine according to literature precedent.[14] The ^{13}C-labeled isoleucine and *N*-acetyl isoleucine were separately introduced to growing cultures of *Astrosporangium hypotensionis* (0.4 mM/day for 4 days). Samples were prepared as described above and incorporation levels were calculated in the same manner. Incorporation of $^{13}C_1$-isoleucine and $^{13}C_2$-*N*-acetyl $^{13}C_1$-isoleucine into K-26 was calculated to be 24% and 43%, respectively (Fig. 1). Interestingly, $^{13}C_2$-*N*-acetyl $^{13}C_1$-isoleucine was not incorporated intact. That *N*-acetyl isoleucine was incorporated only after being deacetylated was indicated by the enhancement of only the M + 1 peak and not of the M + 3. The higher level of enrichment of the M + 1 peak in the case of $^{13}C_2$-*N*-acetyl $^{13}C_1$-isoleucine relative to isoleucine may be due to more favorable intracellular transport properties of this less charged compound. The requisite acetyltransferase may be a component of an NRPS, in which the acetyltransferase would be the first domain on the megasynthetase. Our results are not inconsistent with this scenario. Alternatively the acetyltransferase enzyme may be discrete from the NRPS, in which case our data suggest that the acetylation would occur subsequent to tripeptide elaboration (in contrast to phosphinothricin).[13] A less typical possibility is that the tripeptide is formed by the consecutive action of two free synthetases, as in the biosynthesis of glutathione.[15] In this instance, our results would suggest that acetylation occurs subsequent to the formation of the first peptide bond.

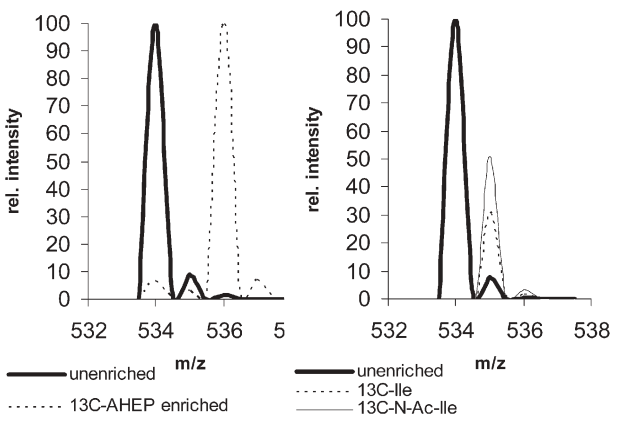
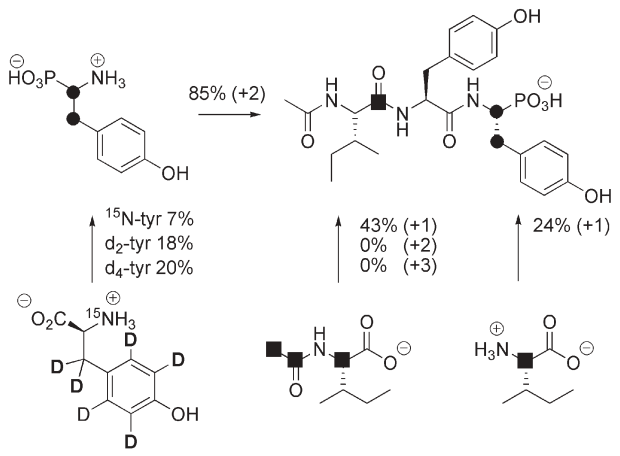


Fig. 1



Scheme 3

These data, in combination with earlier studies delineate a minimal biosynthetic scheme for K-26. AHEP is a discrete intermediate in the biosynthesis of K-26 and tyrosine is a close precursor of AHEP. Tyrosine is not deaminated but it is decarboxylated *en route* to AHEP, but not *via* tyramine. The intermediacy of AHEP suggests that this nonproteinogenic amino acid is appended to a peptide precursor *via* a nonribosomal mechanism. Isoleucine is coupled to tyrosine prior to acetylation unless acetyltransferase activity is a component of an NRPS system. In actinomycetes most small peptide natural products are synthesized by the canonical system of nonribosomal peptide synthetases (NRPS).[16] Whether or not K-26 is synthesized by these systems or by an unrelated synthetase or ligase is unknown at this time. Studies focused on identifying the genes and enzymes involved in the K-26 biosynthesis, including those involved in peptide assembly and C–P bond formation, are in progress.

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