ORGANIC LETTERS

2005
Vol. 7, No. 13
2763−2765

Biosynthetic Origins of C–P Bond Containing Tripeptide K-26

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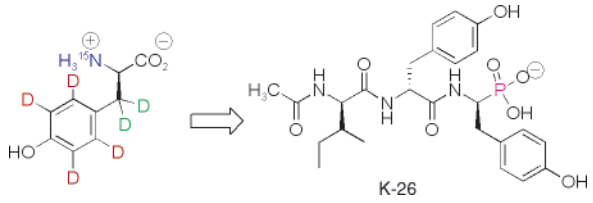
Received May 11, 2005

ABSTRACT



Primary metabolic precursors for K-26, a naturally occurring tripeptide phosphonic acid from *Actinomyces* sp. K-26, are investigated by heavy-atom isotope labeled substrate incorporation experiments. A highly sensitive selected reaction monitoring (SRM)-based method for isotopic incorporation estimation in natural products is reported. The incorporation of heavy-atom isotope labeled tyrosine compounds into the (*R*)-1-amino-2-(4-hydroxyphenyl)-ethylphosphonic acid moiety of compound K-26 suggests a new mechanism of biosynthesis of phosphonate functionality in natural products.

Carbon−phosphorus (C−P) bond containing natural products are remarkable for their diverse and potent biological activities and unique biological roles in primary and secondary metabolism. Examples of a few targets of natural phosphonates and related compounds include glutamine synthetase, β-lactamase, angiotensin converting enzyme (ACE), and mevalonic acid biosynthesis, which result in herbicidal, antibacterial, antihypertensive, and antiparasitic activity, respectively.[1] The biosynthetic origins of several C−P bond containing natural products, including phosphinothricin (**1**) and fosfomycin (**2**), have been investigated, providing a number of biosynthetic enzymes with surprising mechanisms. The central C−P bond forming enzyme in the biosynthesis of these and all C−P compounds investigated to date is phosphoenolpyruvate (PEP) mutase.[2]

K-26 (**3**) from *Actinomycete* sp. K-26 (NRRL 12379) is representative of an uninvestigated class of natural phosphonates that incorporate a phosphonic acid analogue of tyrosine. K-26 is reported to possess angiotensin converting enzyme inhibitory activity with an IC_{50} value of 12.5 nM, comparable to the widely prescribed antihypertensive drug Captopril.[3] K-26 was initially discovered[4] via ACE bioassay guided fractionation of extracts of *Actinomycete* sp. K-26. NMR, mass spectrometry, and degradation and synthetic studies have demonstrated that K-26 is composed of *N*-acetylated L-isoleucine, tyrosine, and the nonproteinogenic amino acid (*R*)-1-amino-2-(4-hydroxyphenyl)ethylphosphonic acid (AHEP). This tyrosine-phosphonate moeity is shared among several ACE active K-26 analogues produced by *Streptosporangium*[5] and *Actinomadura*[6] species.

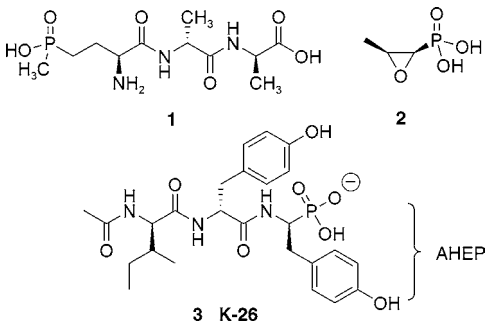


Figure 1. C−P bond containing natural products.

(1) Seto, H.; Kuzuyama, T. *Nat. Prod. Rep.* **1999**, *16*, 589−96.
(2) (a) Liu, S.; Lu, Z.; Jia, Y.; Dunaway-Mariano, D.; Herzberg, O. *Biochemistry* **2002**, *41*, 10270−6. (b) Lee, S. H.; Hidaka, T.; Nakashita, H.; Seto, H. *Gene* **1995**, *153* (1), 143−144.

10.1021/ol051091d CCC: $30.25
Published on Web 05/27/2005

Despite the potent hypotensive activity of K-26 and related compounds, the biosynthetic pathways by which K-26 and more specifically AHEP are biosynthesized still remain uncharacterized. The aromatic amino acid functionality of AHEP suggests that its origin lies in the shikimic acid pathway. However, it is difficult to rationalize how a PEP mutase generated precursor or analogous mutase reaction can be integrated into classical amino acid metabolism to generate AHEP. To investigate the biosynthetic origin of the atoms in the aromatic ring and side chain of AHEP, a series of incorporation studies with isotopically labeled tyrosines was undertaken. Our results demonstrate that the K-26 biosynthetic system is distinct from previously investigated C−P-containing natural products.

Extremely low production levels of K-26 precluded classical NMR-based isotope estimation and required the development of a method with high sensitivity to accurately measure the incorporation ofproposed primary metabolites into K-26. Less than 10 μg/L of K-26 was detected in the fermentation broth, based on ACE inhibitory activity based estimations. We developed tandem mass spectrometric methods using selected reaction monitoring (SRM)[7] in which the [M−H] precursor ions of K-26 were fragmented by collision-induced dissociation. By judicious selection of precursor and product isotopomer masses in an experiment, quantitation of the isotopic enrichment in the charge-containing product ions *and* in the neutral loss fragments was possible. In effect, the easily selected precursor ion was used to indirectly observe the relative isotopic abundances in fragments. This application of SRM is widely applicable to natural product biosynthetic studies, providing a highly sensitive and specific means of determining incorporation of monoisotopic or polyisotopic substitution in fragments by mass spectrometry.

Samples were prepared by pulse feeding growing 0.6−1.2 L cultures of *Actinomycete* sp. *K-26* (1 mM/day for 5 days) with ring-d_4-tyrosine (**4**), 3,3-d_2-tyrosine (**5**), and ^{15}N-tyrosine (**6**). After 6 days of incubation, K-26 samples were isolated by solid-phase extraction on HP-20 resin and enriched by centrifugal molecular weight filtration. K-26 was further separated from co-metabolites by reversed phase HPLC. Initial studies employing ACE bioassay guided fractionation by reported methodologies[8] unambiguously identified K-26 by ESI-MS/MS and biological activity. Characteristic MS/MS fragmentation reactions, in comparison to an authentic synthetic sample,[4b] verified the sequence of the unusual tripeptide and position of phosphonate moiety by loss of PO_3 from the C-terminal amino acid. These data enabled subsequent isotopic enrichment measurements that were performed on a triple quadrupole mass spectrometer using electrospray ionization (ESI), collision-induced dissociation (CID), and selected reaction monitoring (SRM).

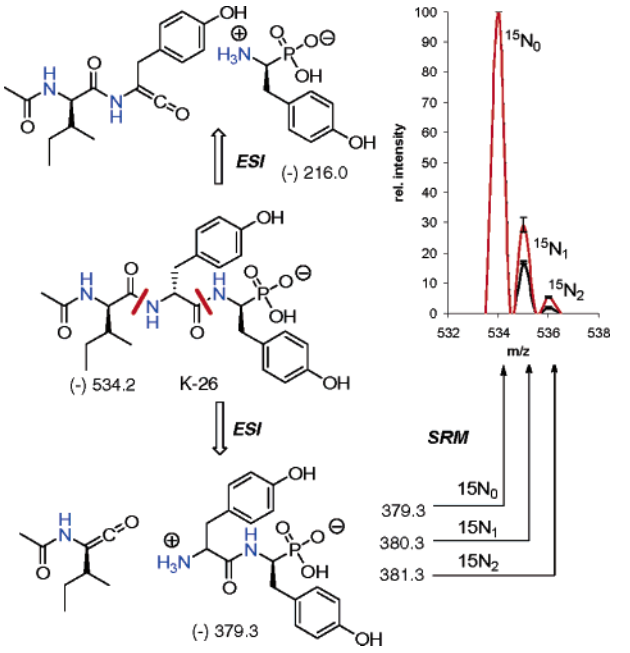


Figure 2. Selected ESI fragmentation reactions used for ^{15}N SRM isotopic abundance calculations. Inset shows isotopomer deconvolution for ^{15}N-tyrosine incorporation (red) vs control unenriched sample (black) into the charged Tyr-AHEP fragment.

The incorporation of ^{15}N-tyrosine into K-26 amino acids was determined by numerical deconvolution using the theoretical mass isotopomer abundance of K-26 to compute the enrichment profiles in the tracer studies.[9] To accurately measure the isotopomer distribution in the individual amino acids, two CID reactions were monitored. First, we selected a specific precursor mass for K-26 (*m*/*z* 534, 535, 536) and monitored two characteristic product ions *m*/*z* 216 and 217, which correspond to AHEP and AHEP + 1, respectively. To determine if the ^{15}N was incorporated into the Ile or the central Tyr, the same precursor ions were chosen and we monitored product ions *m*/*z* 379, 380, and 381. SRM experiments of these selected peptide fragmentations were used to accurately measure the isotope distribution ratios of enriched and unenriched samples using either neutral loss or charged residue fragments. Least-squares fitting of labeled isotopomer data permitted the extraction of relative isotopic enrichments of each amino acid in the tripeptide K-26. The internal control of ^{15}N incorporation into isoleucine demonstrates the robustness of the applied isotopomer abundance back calculation technique and is presumably the result of transamination via α-keto-3-methyl valerate (Scheme 1).

(3) Hirayama, N.; Kasai, M.; Shirahata, K. *Int. J. Pept. Protein Res.* **1991**, *38*, 20−24.

(4) (a) Yamato, M.; Koguchi, T.; Okachi, R.; Yamada, K.; Nakayama, K.; Kase, H.; Karasawa, A.; Shuto, K. *J. Antibiot.* **1986**, *39*, 44−52. (b) Kasai, M.; Yoshida, N.; Hirayama, N.; Shirahata, K. *Symposium Papers*; The 27th Symposium on the Chemistry of Natural Products, 1985, 577.

(5) Ohuchio, S.; Kurihara, K.; Shinohara, A.; Takei, T.; Yoshida, J.; Amano, S.; Miyadoh, S.; Matsushida, Y.; Somura, T.; Sezaki, M. *Sci. Rep. Meiji Seika Kaisha* **1988**, *27*, 46−54.

(6) Koguchi, T.; Yamada, K.; Yamato, M.; Okachi, R.; Nakayama, K.; Kase, H. *J. Antibiot.* **1986**, *39*, 364−371.

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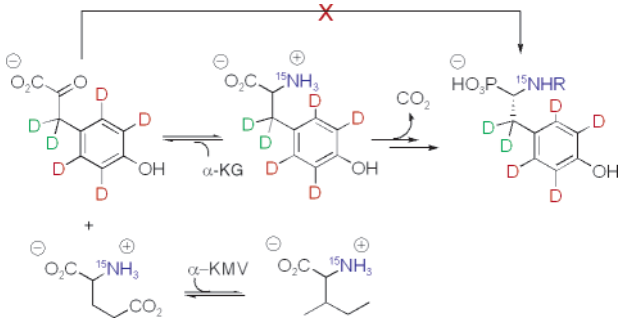
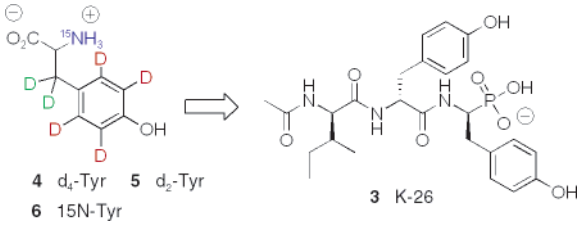
Scheme 1. Summary of Isotopic Enrichment Experiments



When ring-d_4-tyrosine-enriched samples were analyzed by MS, new pseudomolecular ion peaks appeared at the +4 and +8 position, indicating double incorporation of labeled tyrosine into K-26. SRM experiments were designed in order to decouple these data and determine the relative extent of incorporation into AHEP and tyrosine moieties (Scheme 1). Transitions 536 → 218, 540 → 218, 540 → 222 were monitored in order to determine the relative amounts of unenriched K-26, incorporation in the central tyrosine, and incorporation in AHEP, respectively. Similarly, 3,3-d_2-tyrosine incorporation was measured in order to determine whether the biosynthesis of K-26 proceeds through an elimination intermediate. These data are summarized in Table 1.

The trio of tyrosine incorporation studies clearly demonstrate that the nonproteinogenic amino acid AHEP is derived from tyrosine. Since dearomatization of chorismate is highly unlikely, intact ring d_4-tyrosine incorporation suggests that AHEP biosynthesis branches from primary metabolism after chorismic acid. ^{15}N-Tyrosine incorporation data suggest that hydroxyphenylpyruvate is not an immediate progenitor for AHEP, as this would likely be evidenced by a washing out of ^{15}N abundance in AHEP relative to tyrosine, which is not observed. The d_2-tyrosine incorporation data (Supporting Information) indicate that, in addition to intact incorporation, the AHEP + 1 peak was only weakly enhanced (3.26 ± 0.1%), leading us to conclude that β-deuteriums are retained in the transformation of tyrosine to AHEP. In summary, these data unambiguously establish that enzymatic

Table 1. Percent Intact Incorporation of Isotope-Labeled Tyrosines into K-26 Amino Acids Measured by SRM



precursor	Ile (%)	Tyr (%)	AHEP (%)
d_4-Tyr	nd	17.6 ± 0.5	20.2 ± 0.2
d_2-Tyr	nd	16.1 ± 0.6[b]	18.7 ± 0.6
^{15}N-Tyr	2.6 ± 0.1	6.2 ± 0.6[a] (5.4 ± 1.4)[b]	7.1 ± 0.4

[a] Determined by subtraction of %Ile from %Ile-Tyr. [b] Determined by subtraction of %AHEP from % Tyr-AHEP.

phosphonylation occurs after tyrosine biosynthesis and does not proceed via an eliminated intermediate. The substrate for phosphonylation may be tyrosine, a tyrosine metabolite or the tripeptide *N*-Ac-Ile-Tyr-Tyr. Any of these alternatives would indicate that the K-26 biosynthetic system is distinct from previously studied C−P-containing natural products.

These results will inform ongoing studies concerning the genetics and biochemistry of the biosynthesis of K-26 and other carbon−phosphorus bond containing natural peptides. The ability to incorporate stable reduced phosphate functionality into non-ribosomally encoded peptides and amino acids has the potential to expand the biosynthetic repertoire and utility of these systems, which are already yielding new compounds via recombinant biocatalysis.

Acknowledgment. The Vanderbilt Institute of Chemical Biology provided financial support.

Supporting Information Available: Additional information and data concerning fermentation, isolation, and MS methodology. This material is available free of charge via the Internet at http://pubs.acs.org.

OL051091D