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

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Supplementary Material

Fermentation. The K-26 producing strain (*Actinomycetes sp K26*) was obtained from the Agricultural Research Service (NRRL 12379). The production protocol suggested by Yamato *et al*[1] was followed with minor modifications. The seed medium consisted of dextrose, 0.1g/L; Difco soluble starch, 0.1g/L; Bacto beef extract, 0.05g/L; Bacto yeast extract, 0.05g/L; Bacto tryptone, 0.05g/L; and $CaCO_3$, 0.02g/L dissolved in distilled water and was adjusted to pH 7.2 before autoclaving. Fermentation was initiated by aseptically inoculating a loopful of K-26 grown on an agar plate into a sterile 50-mL Falcon tube containing 10 mL seed medium (Phase I). The Falcon tube was incubated for 10-12 days at 28^{o}C in a shaker incubator. In the second phase, 3 mL of the phase I seed culture were transferred into a 300-mL flask containing 30 mL of seed medium. The flask was incubated for 3-4 days at 28^{o}C in a shaker incubator. In phase III, 30 mL of phase II seed culture were transferred into a 3000-mL Fernbach flask containing 300 mL production medium. The flask was incubated for 5-6 days at 28^{o}C in a shaker incubator. The production medium contained Difco soluble starch, 0.4g/L; soy bean meal (Wild Oat), 0.3g/L; corn steep liquor (Sigma), 0.05g/L; K_2HPO_4, 5mg/L; $MgSO_4$, 2.4mg/mL; KCl, 3mg/L; and $CaCO_3$, 0.03g/L dissolved in distilled water and was adjusted to pH 7.8 before autoclaving.

Chemicals. All stable isotopically labeled precursors were purchased from Cambridge Isotope Laboratories.

Pulse Feeding Experiments. One millimolar amounts (based on the culture volume) of each precursor were dissolved in 5 mL water and administered separately to phase III culture through a sterile syringe filter in every 24 hours for 5 days.

K-26 purification. The production culture was centrifuged and the supernatant was acidified to pH 3.00. To one liter of supernatant were added thirty grams of the hydrophobic

resin Diaion HP-20 (activated by stirring with methanol and rinsing with water) and the suspension was stirred for 30 minutes. The resin was filtered and washed with water (2x30mL). The washed resin beads were placed in a 50% methanol-50% water solution and stirred for 10 min. The suspension was then filtered, washed with 50% methanol (2x30 mL), and discarded. The filtrate was concentrated to 40 mL and neutralized. K-26 was further purified using Millipore Ultra 5000 MWCO centrifugal filter devices and centrifuging at 3450*g* for 30 minutes. The filtrate was concentrated to ca. one mL by rotary evaporation.

ACE assay. To each well of a 96-well plate were added 100 µL FAPGG reagent, 60 µL ACE extract and 60 µL sample. The FAPGG reagent consisted of 3.4 mg furanacryloyl Phe-Gly-Gly, 0.5 mL Tris buffer (1M, pH 8.0), 6 mL NaCl (1M) solution, and 3.5 mL H_2O. ACE was extracted from rabbit lung acetone powder by suspending 0.2 g in 6 mL potassium phosphate (50 mM, pH 8.3) buffer followed by centrifuging at 1400 rpm for 30 minutes. The supernatant is the ACE extract mentioned above. Absorbance readings were made with a Spectramax UV/Vis spectrometer at 340 nm every 10 seconds over a period of 5 minutes.

Mass spectrometry

General. Mass spectrometry was performed using ThermoFinnigan (San Jose, CA) TSQ® Quantum triple quadrupole mass spectrometer equipped with a standard electrospray ionization source outfitted with a 100-µm I.D. deactivated fused Si capillary. Data acquisition and spectral analysis were conducted with Xcalibur™ Software, version 1.3, from ThermoFinnigan (San Jose, CA), on a Dell Optiplex GX270 computer running the Microsoft® Windows 2000 operating system. The source spray head was oriented at an angle of 90^o to the ion-transfer-tube. Nitrogen was used for both the sheath and auxiliary gas. The sheath and auxiliary gases were set to 33 and 14 (arbitrary units) respectively. Samples were introduced by

HPLC. A Surveyor® Autosampler and a Surveyor® MS Pump from ThermoFinnigan (San Jose, CA) were used. The injection volume was 10µL. K-26 was separated from co-metabolites using a Jupiter™ minibore 5µm C18 column (2.0mm × 15cm) with a linear water-acetonitrile gradient (ranging from 95:5 to 5:95 H_2O:CH_3CN) containing 10mM ammonium acetate. The flow rate was 0.2mL/min.

Comparison of biogenic K-26 to synthetically prepared sample. Synthetic K-26 was prepared, with minor modification, according to the procedure of: Kasai, M.; Yoshida, N.; Hirayama, N.; Shirahata, K. *Symposium Papers, The 27th Symposium on the Chemistry of Natural Products* **1985**, 577. (Data to be published elsewhere). Biogenic K-26 was identical to synthetic sample as shown in figure 1.

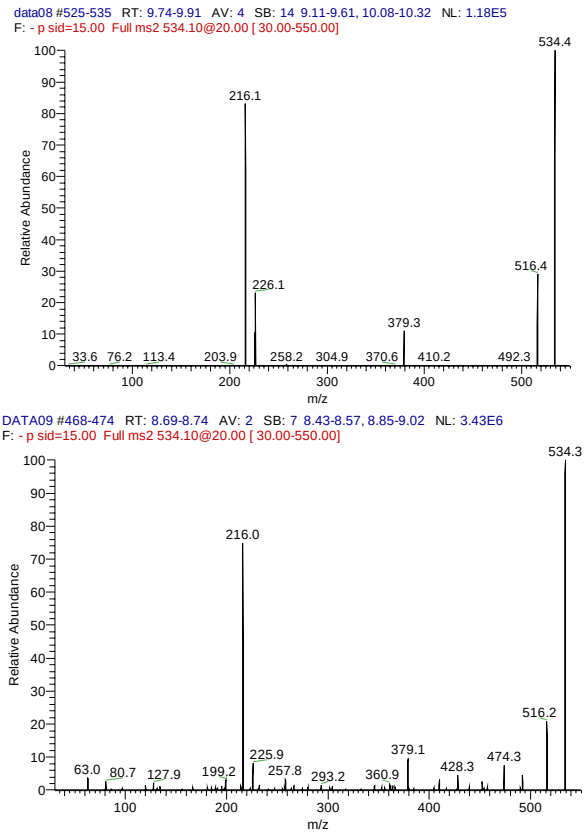

Figure 1. MS^2 of 534 ion of synthetic (above) versus biogenic (below) K-26.

Ring-d_4-tyrosine. The mass spectrometer was operated in the positive ion mode and the electrospray needle was maintained at 4200V. The ion transfer tube was operated at 35V and 300^{o}C. The tube lens voltage was set to 131V. Source CID (offset voltage between skimmer and the first ion quide, Q00) was used at 15V. The selected reaction monitoring (SRM) mode was used. Ions were collisionally activated with argon at an indicated pressure of 1.5mT. The mass-spectral resolution was set to a peak width (full width at half maximum, FWHM) of 0.70u and 0.70u for precursor and product ions respectively. Mass transitions at the specified collision energy (m/z 536→218; 20eV), (m/z 540→218; 20eV), and (m/z 540→222; 20eV) were monitored for unenriched K-26, enrichment in the central tyrosine and enrichment in AHEP, respectively. The scan width for product ions was 1.000u and the cycle time for each ion was 0.25 seconds. The electron multiplier gain was set to 2 × 10^6. Data were acquired in profile mode. The resulting chromatogram is illustrated in figure 2.

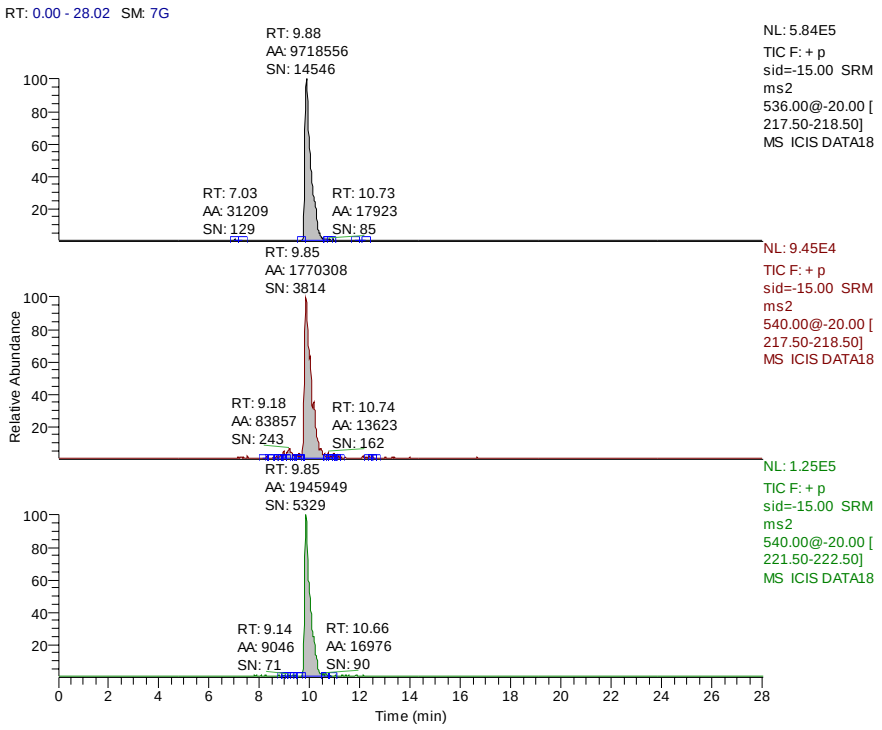

Figure 2. Chromatograms used for the calculation of ring-d_4-tyrosine incorporation

Table 1. Calculation of ring-d_4-tyrosine incorporation

Transition	Area			Isotope Distribution				
	1	2	3	1	2	3	average	SD
536 --> 218	9701469	9674024	9736478	100	100	100	100	0
540 --> 218	1719261	1658059	1755359	17.72	17.13	18.03	17.63	0.45
540 --> 222	1983006	1954870	1962925	20.44	20.21	20.16	20.27	0.15

The isotope distribution was calculated by assigning the 536→218 peak an area of 100 and normalizing the other peaks accordingly.

^{15}N-tyrosine. The mass spectrometer was operated in the negative ion mode and the electrospray needle was maintained at 4200V. The ion transfer tube was operated at -35V and 350^{o}C. The tube lens voltage was set to -150V. Source CID (offset voltage between skimmer and the first ion quide, Q00) was used at 15V. The selected reaction monitoring (SRM) mode was used. Ions were collisionally activated with argon at an indicated pressure of 1.4mT. The mass-spectral resolution was set to a peak width (full width at half maximum, FWHM) of 0.50u and 0.50u for precursor and product ions respectively. Mass transitions at the specified collision energy (m/z 534→216; 35eV), and (m/z 535→217; 35eV) were monitored for AHEP and AHEP+1, respectively. Transitions (m/z 534→216; 35eV), (m/z 535→216; 35eV), and (m/z 536→216; 35eV) were monitored for *N*-Ac-Ile-Tyr, *N*-Ac-Ile-Tyr+1, and *N*-Ac-Ile-Tyr+2, respectively. Transitions (m/z 534→379; 35eV), (m/z 535→380; 35eV), and (m/z 536→381; 35eV) were monitored for Tyr-AHEP, Tyr-AHEP+1, and Tyr-AHEP+2, respectively. Finally, transitions (m/z 534→379; 35eV), (m/z 535→379; 35eV), and (m/z 536→379; 35eV) were monitored for *N*-Ac-Ile, *N*-Ac-Ile+1, and *N*-Ac-Ile+2, respectively. The scan width for product ions was 1.000u and the cycle time for each ion was 0.15 seconds. The electron multiplier gain was set to 2×10^6. Data were acquired in profile mode. The resulting

chromatograms are illustrated in figures 3,4 and 5. Both a ^{15}N-tyrosine enriched sample and an unenriched sample were scanned. The unenriched sample was used to create theoretical curves of ratios of mass isotopomer abundance in K-26 to correct the isotopomer distribution of the enriched sample.

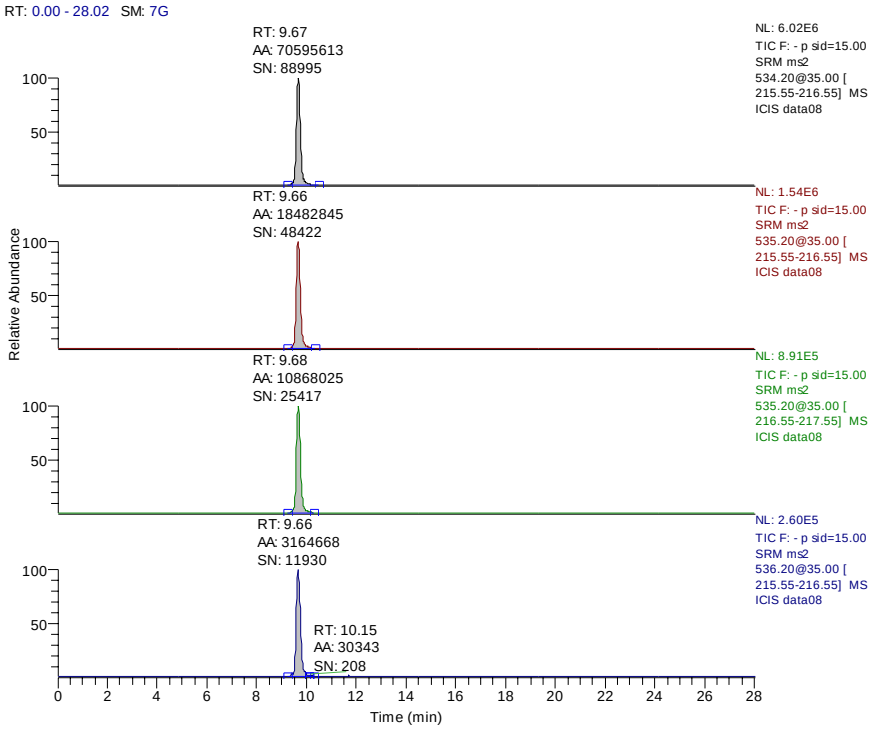

Figure 3. Chromatograms used in the calculation of ^{15}N-tyrosine incorporation in AHEP

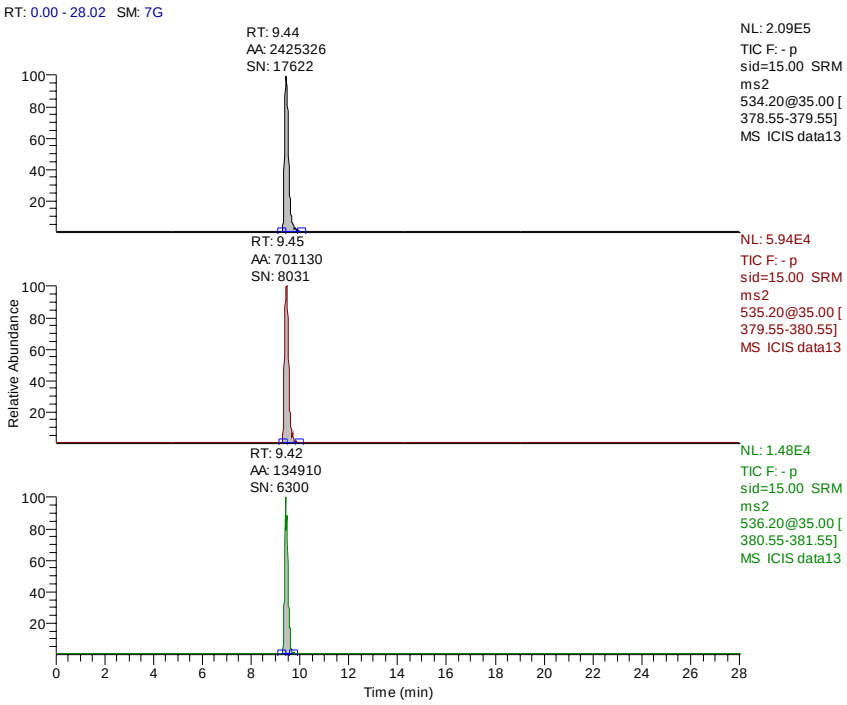

Figure 4. Chromatograms used in the calculation of ^{15}N-tyrosine incorporation in Tyr-AHEP

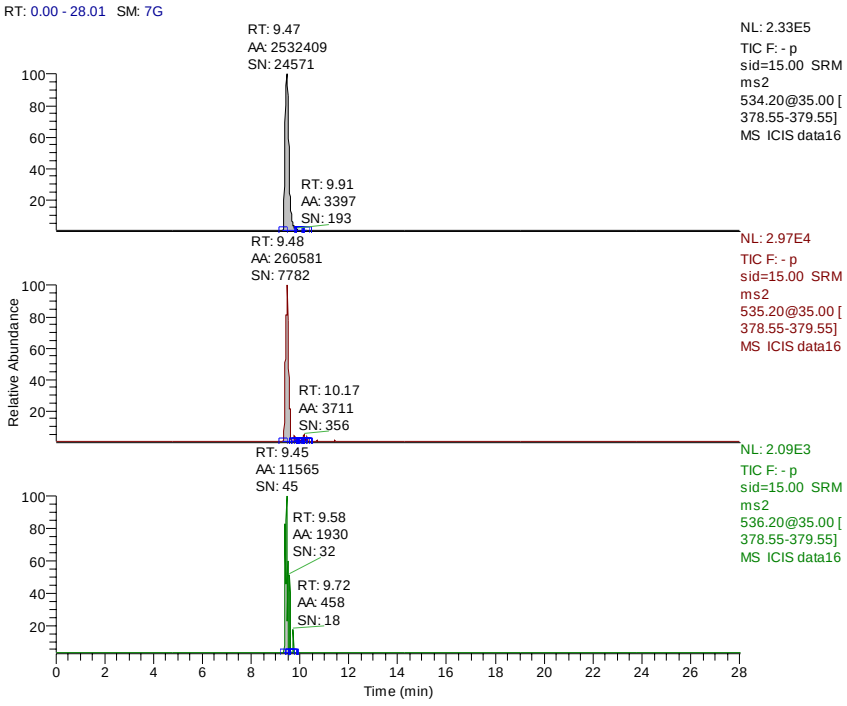

Figure 5. Chromatograms used in the calculation of ^{15}N-tyrosine incorporation in N-Ac-Ile-Tyr

Calculation of ^{15}N enrichment example. The tabulated numbers below (Calculation 1-4) resulted from the following calculations. Since all the calculations (1-4) are similar, the deconvolution of tandem MS data in order to determine ^{15}N enrichment in Tyr-AHEP (Calculation 4) exemplifies the method. In this example, the **theoretical isotope matrix** was constructed using the relative mass spectral ion intensities of transitions 534→379, 535→380, and 536→381 measured for an unenriched sample. In the absence of a pure standard for $^{15}N_1$-K-26 and $^{15}N_2$-K-26, we assumed that the theoretical distribution of unenriched K-26 would be as follows.

	Theoretical Isotope Ratio (x100)		
m/z	**$^{15}N_0$-PA**	**$^{15}N_1$-PA**	**$^{15}N_2$-PA**
534 -> 379	100.00	0.00	0.00
535 -> 380	16.89	100.00	0.00
536 -> 381	1.86	16.53	100.00
		1.86	16.17
			1.86

The **mole fraction matrix** was obtained by normalizing the relative ion intensities to the sum of the ion intensities for each species ($^{15}N_0$, $^{15}N_1$, and $^{15}N_2$, the tracer substances). The mole fraction matrix was transposed resulting in the **isotope design matrix**, in order that the final result would be along the same row instead of down the same column. This set of normalized mass spectral ion intensities can be used as a set of fixed coefficients in a system of linear equations that describe the relative ion abundances of the different tracers. The general form of the equation is

$$I_j = A_{i,j} \times B_i \qquad \text{equation 1}$$

Where I_j is the intensity of transition j, $A_{i,j}$ is the normalized ion abundance of tracer substance i during transition j (isotope design matrix), and B_i is the unknown mole fraction of tracer

substance i. The system has to include one equation for each tracer and each equation must contain one term for each tracer. This results in the following set of linear equations:

$$I_{534\rightarrow 379} = 84.213B_{15N0} + 14.219B_{15N1} + 1.568B_{15N2}$$

$$I_{535\rightarrow 380} = 0.000B_{15N0} + 84.467B_{15N1} + 13.962B_{15N2}$$

$$I_{536\rightarrow 381} = 0.000B_{15N0} + 0.000B_{15N1} + 84.724B_{15N2}$$

In order to solve this set of linear equations, the **pseudoinverse matrix** was calculated according to the following equation.

$$P = (A^T \times A)^{-1} \times A^T \qquad \text{equation 2}$$

Where P is the pseudoinverse matrix, A is the isotope design matrix and A^T is the transpose of A. Multiplying P with the **measured isotope ratio matrix** (normalized measured ion intensities) gives the **corrected isotopic abundances and ratio**. This calculation was performed for both unenriched and enriched samples to check for errors in the method. The corrected isotopic abundances were normalized to 100. The unenriched samples had corrected tracer: tracee ratio of 100:0:0 for $^{15}N_0$:$^{15}N_1$:$^{15}N_2$ while the enriched sample had a ratio of 100:12.49:1.43, clearly indicating ^{15}N enrichment. Simulated isotopic distributions based on least squares fitting of isotopomer data in Calculations 1-4 are shown in figure 6.

Calculation 1: Presence of 15N in the N-terminal Isoleucine or the central Tyrosine
Note: Neutral loss isotopic enrichment model

Theoretical Isotope Ratio (x100)

m/z	$^{15}N_0$-PA	$^{15}N_1$-PA	$^{15}N_2$-PA
534 -> 216	100.00	0.00	0.00
535 -> 216	17.88	100.00	0.00
536 -> 216	2.40	17.52	100.00
		2.40	17.16
			2.40

Mole Fraction (x100)

m/z	$^{15}N_0$-PA	$^{15}N_1$-PA	$^{15}N_2$-PA
534 -> 216	83.13	0.00	0.00
535 -> 216	14.87	83.39	0.00
536 -> 216	2.00	14.61	83.64

Isotope Design Matrix

	$^{15}N_0$-PA	$^{15}N_1$-PA	$^{15}N_2$-PA
534 -> 216	83.135	14.866	1.999
535 -> 216	0.000	83.389	14.610
536 -> 216	0.000	0.000	83.640

Pseudoinverse Matrix

	$^{15}N_0$-PA	$^{15}N_1$-PA	$^{15}N_2$-PA
534 -> 216	0.0120	-0.0021	0.0001
535 -> 216	0.0000	0.0120	-0.0021
536 -> 216	0.0000	0.0000	0.0120

Sample	Measured Isotope Ratio (x100) $^{15}N_0$-PA	$^{15}N_1$-PA	$^{15}N_2$-PA
1	100.000	17.831	2.370
2	100.000	17.823	2.427
3	100.000	17.992	2.416
Mean	100.000	17.882	2.405
S.D.	0.000	0.095	0.030
1	100.000	26.970	4.595
2	100.000	26.181	4.526
3	100.000	26.848	4.421
Mean	100.000	26.666	4.514
S.D.	0.000	0.424	0.088

Corrected, Tracer:Tracee Ratio (x100)

	$^{15}N_0$-PA	$^{15}N_1$-PA	$^{15}N_2$-PA
1	100.00	-0.05	-0.03
2	100.00	-0.06	0.03
3	100.00	0.11	-0.01
Mean	100.00	0.00	0.00
S.D.	0.00	0.09	0.03
1	100.00	9.06	0.60
2	100.00	8.27	0.66
3	100.00	8.94	0.44
Mean	100.00	8.76	0.57
S.D.	0.00	0.42	0.11

Corrected Isotopic Abundances & Ratio

[M]	[M + 1]	[M + 2]
1.203	-0.001	0.000
1.203	-0.001	0.000
1.203	0.001	0.000
1.203	0.109	0.007
1.203	0.100	0.008
1.203	0.108	0.005

Calculation 2: Presence of 15N in the AHEP moiety
Note: Charged residue isotopic enrichment model

Theoretical Isotope Ratio (x100)

m/z	$^{15}N_0$-PA	$^{15}N_1$-PA	$^{15}N_2$-PA
534 -> 216	100.00	0.00	0.00
535 -> 217	8.56	100.00	0.00
536 -> 218	0.00	8.20	100.00
		0.00	8.20
			0.00

Mole Fraction (x100)

m/z	$^{15}N_0$-PA	$^{15}N_1$-PA	$^{15}N_2$-PA
534 -> 216	92.12	0.00	0.00
535 -> 217	7.88	92.42	0.00
536 -> 218	0.00	7.58	92.42

Isotope Design Matrix

	$^{15}N_0$-PA	$^{15}N_1$-PA	$^{15}N_2$-PA
534 -> 216	92.115	7.885	0.000
535 -> 217	0.000	92.421	7.579
536 -> 218	0.000	0.000	92.421

Pseudoinverse Matrix

	$^{15}N_0$-PA	$^{15}N_1$-PA	$^{15}N_2$-PA
534 -> 216	0.0109	-0.0009	0.0001
535 -> 217	0.0000	0.0108	-0.0009
536 -> 218	0.0000	0.0000	0.0108

Sample	**Measured Isotope Ratio (x100)** $^{15}N_0$-PA	$^{15}N_1$-PA	$^{15}N_2$-PA	**Corrected, Tracer:Tracee Ratio (x100)** $^{15}N_0$-PA	$^{15}N_1$-PA	$^{15}N_2$-PA
1	100.000	8.509	0.000	100.00	-0.05	0.00
2	100.000	8.656	0.000	100.00	0.10	-0.01
3	100.000	8.515	0.000	100.00	-0.04	0.00
Mean	100.000	8.560	0.000	100.00	0.00	0.00
S.D.	0.000	0.083	0.000	0.00	0.08	0.01
1	100.000	16.107	0.000	100.00	7.52	-0.62
2	100.000	15.395	0.000	100.00	6.81	-0.56
3	100.000	15.439	0.000	100.00	6.86	-0.56
Mean	100.000	15.647	0.000	100.00	7.06	-0.58
S.D.	0.000	0.399	0.000	0.00	0.40	0.03

Corrected Isotopic Abundances & Ratio

[M]	[M + 1]	[M + 2]
1.086	-0.001	0.000
1.086	0.001	0.000
1.086	0.000	0.000
1.086	0.082	-0.007
1.086	0.074	-0.006
1.086	0.074	-0.006

Calculation 3: Presence of 15N in the N-terminal Isoleucine
Note: Neutral loss isotopic enrichment model

Theoretical Isotope Ratio (x100)

m/z	$^{15}N_0$-PA	$^{15}N_1$-PA	$^{15}N_2$-PA
534 -> 379	100.00	0.00	0.00
535 -> 379	8.01	100.00	0.00
536 -> 379	0.26	7.65	100.00
		0.26	7.29
			0.26

Mole Fraction (x100)

m/z	$^{15}N_0$-PA	$^{15}N_1$-PA	$^{15}N_2$-PA
534 -> 379	92.36	0.00	0.00
535 -> 379	7.40	92.67	0.00
536 -> 379	0.24	7.09	92.98

Isotope Design Matrix

	$^{15}N_0$-PA	$^{15}N_1$-PA	$^{15}N_2$-PA
534 -> 379	92.361	7.395	0.244
535 -> 379	0.000	92.670	7.089
536 -> 379	0.000	0.000	92.980

Pseudoinverse Matrix

	$^{15}N_0$-PA	$^{15}N_1$-PA	$^{15}N_2$-PA
534 -> 379	0.0108	-0.0009	0.0000
535 -> 379	0.0000	0.0108	-0.0008
536 -> 379	0.0000	0.0000	0.0108

Sample	Measured Isotope Ratio (x100) $^{15}N_0$-PA	$^{15}N_1$-PA	$^{15}N_2$-PA
1	100.000	7.880	0.180
2	100.000	7.621	0.283
3	100.000	8.520	0.331
Mean	100.000	8.007	0.265
S.D.	0.000	0.463	0.077
1	100.000	10.484	0.355
2	100.000	11.149	0.373
3	100.000	10.290	0.457
Mean	100.000	10.641	0.395
S.D.	0.000	0.451	0.054

Corrected, Tracer:Tracee Ratio (x100)

	$^{15}N_0$-PA	$^{15}N_1$-PA	$^{15}N_2$-PA
1	100.00	-0.13	-0.07
2	100.00	-0.38	0.05
3	100.00	0.51	0.03
Mean	100.000	0.000	0.000
S.D.	0.000	0.461	0.065
1	100.00	2.47	-0.10
2	100.00	3.13	-0.13
3	100.00	2.28	0.02
Mean	100.000	2.625	-0.070
S.D.	0.000	0.449	0.078

Corrected Isotopic Abundances & Ratio

[M]	[M + 1]	[M + 2]
1.083	-0.001	-0.001
1.083	-0.004	0.001
1.083	0.006	0.000
1.083	0.027	-0.001
1.083	0.034	-0.001
1.083	0.025	0.000

Calculation 4: Presence of 15N in the central Tyrosine or the AHEP moiety
Note: Charged residue isotopic enrichment model

Theoretical Isotope Ratio (x100)

m/z	$^{15}N_0$-PA	$^{15}N_1$-PA	$^{15}N_2$-PA
534 -> 379	100.00	0.00	0.00
535 -> 380	16.89	100.00	0.00
536 -> 381	1.86	16.53	100.00
		1.86	16.17
			1.86

Mole Fraction (x100)

m/z	$^{15}N_0$-PA	$^{15}N_1$-PA	$^{15}N_2$-PA
534 -> 379	84.21	0.00	0.00
535 -> 380	14.22	84.47	0.00
536 -> 381	1.57	13.96	84.72

Isotope Design Matrix

	$^{15}N_0$-PA	$^{15}N_1$-PA	$^{15}N_2$-PA
534 -> 379	84.213	14.219	1.568
535 -> 380	0.000	84.467	13.962
536 -> 381	0.000	0.000	84.724

Pseudoinverse Matrix

	$^{15}N_0$-PA	$^{15}N_1$-PA	$^{15}N_2$-PA
534 -> 379	0.0119	-0.0020	0.0001
535 -> 380	0.0000	0.0118	-0.0020
536 -> 381	0.0000	0.0000	0.0118

Sample	Measured Isotope Ratio (x100) $^{15}N_0$-PA	$^{15}N_1$-PA	$^{15}N_2$-PA
1	100.000	16.567	1.841
2	100.000	16.904	1.821
3	100.000	17.185	1.923
Mean	100.000	16.885	1.862
S.D.	0.000	0.310	0.054
1	100.000	31.980	5.447
2	100.000	27.237	5.079
3	100.000	29.020	5.584
Mean	100.000	29.412	5.370
S.D.	0.000	2.396	0.261

Corrected, Tracer:Tracee Ratio (x100)

	$^{15}N_0$-PA	$^{15}N_1$-PA	$^{15}N_2$-PA
1	100.00	-0.32	0.03
2	100.00	0.02	-0.04
3	100.00	0.30	0.01
Mean	100.000	0.000	0.000
S.D.	0.000	0.309	0.039
1	100.00	15.05	1.08
2	100.00	10.32	1.50
3	100.00	12.10	1.71
Mean	100.000	12.489	1.429
S.D.	0.000	2.388	0.317

Corrected Isotopic Abundances & Ratio

[M]	[M + 1]	[M + 2]
1.187	-0.004	0.000
1.187	0.000	-0.001
1.187	0.004	0.000
1.187	0.179	0.013
1.187	0.123	0.018
1.187	0.144	0.020

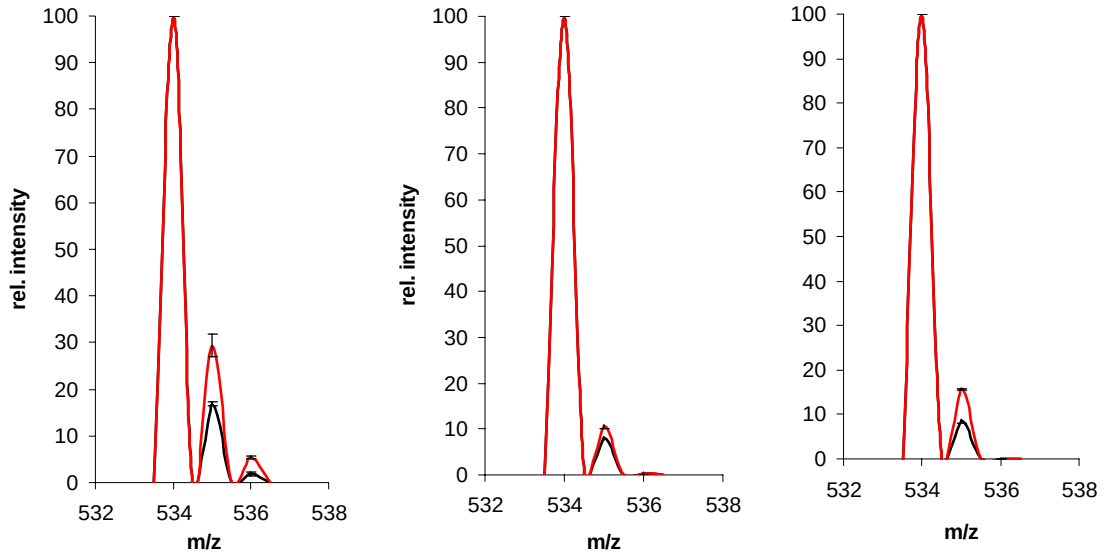

Figure 6. Simulated isotopic distributions based on least squares fitting of isotopomer data in Calculations 1-4. Red is labeled black is unlabeled. *Left:* $\%^{15}N$ enrichment in Tyr-AHEP fragment. *Middle:* $\%^{15}N$ enrichment in N-Ac-Ile fragment. *Right:* $\%^{15}N$ in AHEP alone.

***d_2*-tyrosine.** The mass spectrometer was operated in the positive ion mode and the electrospray needle was maintained at 4200V. The ion transfer tube was operated at 35V and 300^{o}C. The tube lens voltage was set to 131V. Source CID (offset voltage between skimmer and the first ion quide, Q00) was used at 15V. The selected reaction monitoring (SRM) mode was used. Ions were collisionally activated with argon at an indicated pressure of 1.5mT. The mass-spectral resolution was set to a peak width (full width at half maximum, FWHM) of 0.50u and 0.50u for precursor and product ions respectively. Mass transitions at the specified collision energy (m/z 536→218; 20eV), (m/z 537→219; 20eV), (m/z 538→220; 20eV), and (m/z 539→221; 20eV) were monitored for AHEP, AHEP+1, AHEP+2, and AHEP+3, respectively. Additionally, transitions at the specified collision energy (m/z 536→381; 20eV), (m/z 537→382; 20eV), (m/z 538→383; 20eV), (m/z 539→384; 20eV), and (m/z 540→385;

20eV) were monitored for Tyr+AHEP, Tyr+AHEP+1, Tyr+AHEP+2, Tyr+AHEP+3, and Tyr+AHEP+4, respectively. The scan width for product ions was 1.000u and the cycle time for each ion was 0.15 seconds. The electron multiplier gain was set to 2×10^6. Data were acquired in profile mode. The resulting chromatograms are illustrated in figures 7 and 8. Both a d_2-tyrosine enriched sample and an unenriched sample were scanned. The unenriched sample was used to create a least squares curve for the data. The same method of calculation as in the ^{15}N-tyrosine experiment was used for determining the amount and position of incorporation. The enrichment level in the central tyrosine was calculated by subtracting the AHEP enrichment from the Tyr-AHEP enrichment. Simulated isotopic distributions based on least squares fitting of isotopomer data are shown in figure 9.

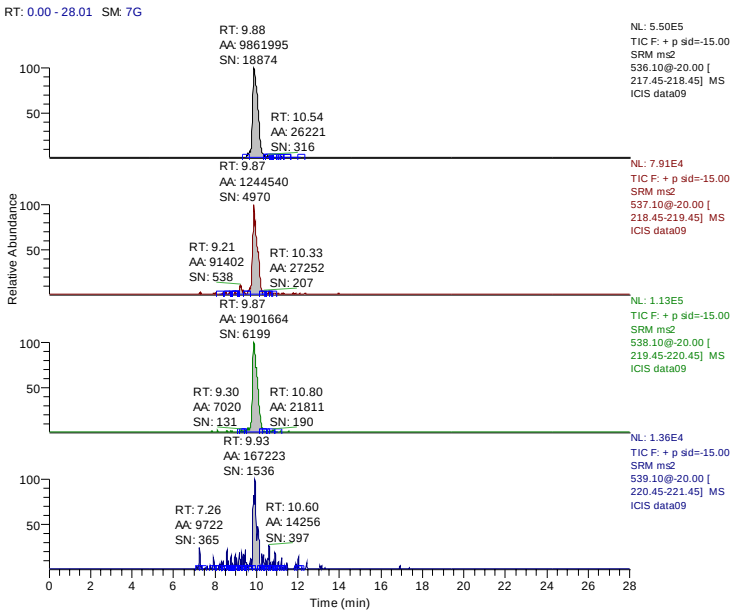

Figure 7. Chromatograms used in the calculation of 3,3-d_2-tyrosine incorporation in AHEP fragment.

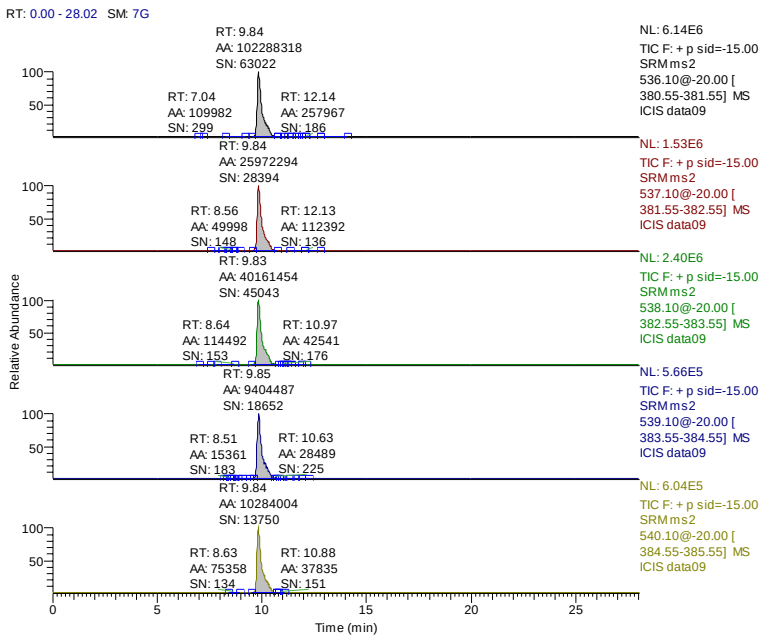

Figure 8. Chromatograms used in the calculation of 3,3-d_2-tyrosine incorporation in Tyr-AHEP dipeptide fragment.

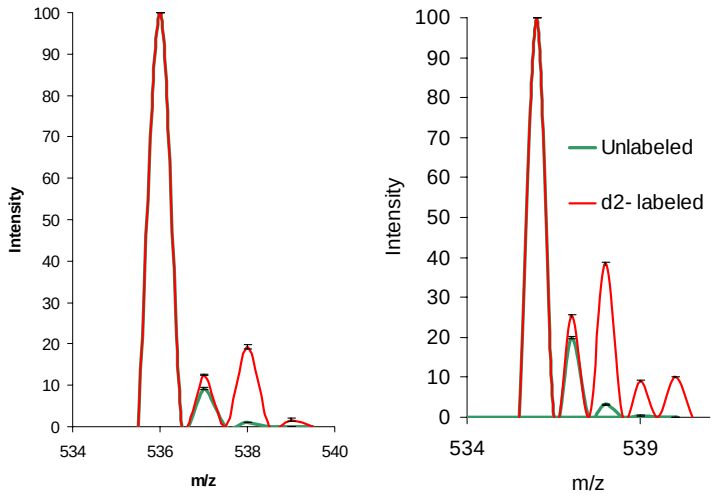

Figure 9. Simulated isotopic distributions based on least squares fitting of isotopomer data from chromatograms in figure 7 and 8. Red is labeled sample, green is unlabeled. *Left:* calculated % β–d_2-Tyrosine in AHEP fragment. These data clearly show conservation of both deuteriums into AHEP and very little evidence of loss of single deuterium. *Right:* % β–d_2-Tyrosine in Tyr-AHEP fragment confirming double incorporation.