Appendix A: Site-specific Measurement Techniques

Site-specific isotope ratios of alanine derivatives are measured on the Orbitrap Fourier Transform Isotope Ratio Mass Spectrometer ('Orbitrap'). Prior studies have demonstrated that the fragments of alanine N-trifluoroacetyl-O-methyl ester can full constrain the C-1, C-2, and C-3 sites of alanine (Eiler et al., 2017; Chimiak et al., 2020); here we detail the peak analyzed, the conditions in which the Orbitrap measurements in this study were performed, and the rationale for those conditions.

The Orbitrap has two front-end configurations, dual- and single-reservoir. Measurements for fragment verification typically used the dual-reservoir configuration depicted in Figure S1. This configuration has two reservoirs with a pure sample in one and a standard in the other that elutes into the instrument by a helium stream and a front valve (V2) that can change which reservoir elute to the source and which to the vent. Measurements of alanine for this study are made using the single-reservoir system, which operates similarly to an exponential dilution flash and is depicted in Figure S1b. In this study, the reservoir consists of a 30 mL steel reservoir coated with deactivated silica. The single reservoir system has two valves. The first valve (V1) has inputs from the GC column and from a capillary attached to the helium tank by way of a pressure regulator, and outputs are either directed to the second valve (V2) or to the reservoir. V2 has inputs from the V1 and the reservoir and outputs to either the Orbitrap source or to vent.

In single-reservoir analyses, samples are injected into the GC injection port and can be measured as directly as they elute from the GC ('GC direct mode') or they can be selected and broadened in a reservoir ('peak broadening mode'). In this study, the GC is set to start at 50 °C and ramp at 10 °C/minute with a helium flow rate of 1.2 mL/min and have a splitless injection. In both GC direct and peak broadening mode, the initial valve set-up routes pure helium from a tank is directed to the reservoir and the reservoir is directed to the Orbitrap source (the GC eluent is directed to vent).

In GC direct mode, alanine is injected into the Orbitrap and the initial valves are unchanged for 4 minutes while the solvent, hexane, elutes as a failure to vent the solvent creates large backgrounds. After four minutes, GC eluent is routed directly to the Orbitrap source. This mode was used on large mass windows (50-500 Da) to note elution times and find potential fragments using labelled alanine standards and daily on a smaller mass windows (±5 Da) to confirm that the elution time was unchanged and that there was low contamination.

In peak-broadening mode, helium is routed to the reservoir and source for the first 6.0 minutes (Figure S2) as alanine elutes at 7.5 minutes. At this time, the GC eluent is routed to the reservoir, which is routed to the Orbitrap source. At 11 minutes—after which the full alanine peak has eluted—helium is routed to the reservoir for the remainder of the acquisition. All isotope ratio measures for the C-2 and C-3 sites of alanine reported here are measured in peak-broadening mode, in which the alanine peak was captured and measured for 60-minutes per injection.

The path of the sample from the GC front-end to the Orbitrap and its potential impacts on the isotopic measurements is described at length in Eiler et al. (2017). Once gas enters the electron impact (EI) source, electrons from the source impact the analyte and break chemical bonds and/or remove electrons to produce charged fragments. These fragments are then collimated into a beam and filtered by their mass-to-charge ratio via the Advanced Quadrupole Selector (AQS)—a quadrupole mass selector. Typically, fragments have a +1 charge, so the mass-to-charge ratio corresponds to the fragments' mass. We will refer to the mass range that is filtered in the quadrupole as the 'mass window'. Ions in the mass window are collected in an electrostatic trap (C-trap) until a user-defined threshold for the total ion current (TIC) in the C-trap is reached. This threshold measured via the automatic gain control (AGC). Once the TIC in the C-trap reaches the threshold, ions are injected from the C-trap into the Orbitrap where they oscillate and travel back and forth in the Orbitrap at a rate proportional to mass (Makarov, 2000). The time the ions spend in the C-trap prior to injection is referred to as the injection time (IT). If the TIC in the C-trap remains below the threshold for a user-defined amount of time (the max IT), the ions are injected into the Orbitrap and measured. We refer to these scans as ones in

which the IT is 'maxed out.' We refer to each process of injecting an ion packet into the Orbitrap and measuring it in this manner as a scan and the combination of scans that forms a measurement as an acquisition. Using Excalibur, a proprietary software program by Thermo Fisher Scientific, the frequency signal produced by the back and forth travel of ions is deconvolved via a Fourier Transform function and the mass and signal intensity of the ions is reported to a user for each scan in an acquisition. Following acquisitions, all data files are processed through a proprietary software program provided by Thermo Fisher Scientific, 'FT statistic', which extracts information from a RAW file including intensity, peak noise and total ion counts for each scan pertaining to requested masses and converts them into a .csv file. Following extraction in FT statistic, isotopic intensities are converted to counts according to Eiler et al. (2017).

Fragmentation by electron impact is a high energy process, so we anticipate potential recombination reactions occur within the source that could impact the sites that are sampled by each fragment. The carbon sites included in fragments are identified by using a 1:9 mixture of ^{13}C-labelled alanine (at the C-1, C-2, or C-3 site) with unlabeled alanine that is derivatized into a N-trifluoroacetyl-O-methyl ester and diluted to 1:100,000 (parts per volume) in hexane and injected through the GC into the Orbitrap for initial fragment identification. With this analysis, we recognize candidate fragments listed in Table S1 and further explore which alanine sites contribute 102.055, 113.021, 140.032, 184.021, and 200.053 fragments and their ^{13}C-substited versions. For this paper, we will refer to pairs of fragments by the monoisotopic fragment's mass.

Fragments-of-interest were further analyzed using the 1:9 labelled standards in dual-reservoir mode for the 102.055, 140.032, 184.021, and 200.053 fragments and single-reservoir mode for the 113.021 peak. In these analyses, if a fragment contained 100% of the C-1 site, the C-1 labeled compound would have a δ^{13}C that is 11500 ‰ to 2800 ‰ enriched relative to the unlabeled alanine standard for a fragment that contains one to five carbons, respectively. Results from single- and dual-reservoir measurements of the labelled peaks demonstrate that the 102.055 fragment contains the C-3 site and two derivative carbons, the 113.021 fragment contains the C-3

site and two derivative carbons, the 140.032 fragment contains the C-2 and C-3 sites in addition to two derivative carbons, the 184.021 fragment contains the C-1 and C-2 carbons in addition to three derivative carbons, and the 200 and 201 fragments contain all three carbons in alanine and all three derivative carbons (Table S2). In each case, fragments returned either 0% or 100% of a given site's label.

To test Orbitrap acquisition parameters, Alfa Aesar and Strecker alanine standard derivatives were analyzed in dual-reservoir acquisitions. These standards have known $\delta^{13}C_{VPDB}$ values for the C-1 site via ninhydrin decarboxylation, whole molecule $\delta^{13}C_{VPDB}$ from EA measurements, and the $\delta^{13}C_{VPDB}$ averaged across the C-2 and C-3 sites via subtraction. Parameters included AGC, microscans, resolution, and window size (Table S3) were varied. At best, Dual reservoir studies find the following structure of Strecker alanine relative to Alfa Aesar -17.0 ± 8.6 ‰, -9.8 ± 3.9 ‰, and -21.2 ± 1.4 for the C-1, C-2, and C-3 sites, respectively, and the error follows the shot noise limit. For mass windows and fragments considered, only measurements with AGC values below 1 x 10^5 ions accurately replicate the differences between Strecker and Alfa Aesar alanine, so we adopt this AGC value for the measurements presented in this study. Higher resolution and smaller scan windows decreased the ion transmission (Eiler et al., 2017), so 120,000 resolutions are used to ensure the full separation of the singly-substituted ^{13}C and ^{15}N peaks for the masses studied here and scan windows of +/- 5 Da were used to decrease possible contaminant peaks while still keeping transmission high. Differences in microscans do not impact the data, so we use 1 microscan to monitor potential noise.

Measurement of the Alfa-Aesar and Strecker standards with the single-reservoir front-end, demonstrate how IT and timing for peak capture. Data from scans that were conducted at maximum IT were highly fractionated, so in for acquisitions in this study, maximum IT is set to 3000 ms and scans in which the IT maxes out are culled prior to data processing. Peaks are captured in the reservoir via manual valve control. To decrease background, peak should be close to when they elute; however, the strong ^{13}C fractionation along a GC column that might result in the closer valve turns to miss small amounts of the eluted analyte in an inconsistent fashion. Here

we find that acquisitions in which the valve is turned at least before 30s prior to the peak's elution have relative ^{13}R values within 2 standard error, but the acquisitions have a larger as peaks are captured closer to their elution (Table S4). Consequently, measurements here capture alanine peaks from 6.5 minutes to 11 minutes.

Finally, we analyzed the acquisition data for the Alfa Aesar and Strecker alanine standard derivatives by processing the ^{13}R values as an average for the acquisition, an average weighted by counts per scan, an average weighted by TIC of each scan, and the summed singly ^{13}C-substituted peak counts over the monoisotopic peak counts for each acquisition. In each analysis, we further culled each data set according to its peak height (from no culling to culling all scans with the monoisotopic peak at or below 50% of its peak maximum less the background) and according to its monoisotopic peak intensity relative to the TIC (from no culling to culling all scans with the monoisotopic peak intensity at or below 50% of the TIC). For the sample sizes here on the 140.035 Da peak, only analyses in which no cutoffs were used were found to have values that were one standard deviation from the C-2 + C-3 value (Table S5). As the ^{13}R value for the 140.035 Da peak is both most accurate and most precision for a cutoff of the monoisotopic peak's intensity being at least 30% of the TIC for a scan and when calculating ^{13}R as an average weighted by the counts per scan (Table S5), we adopt this as our culling criteria in data processing.

Appendix B: Ninhydrin Decarboxylation

Ninhydrin cleaves amino acids' carbon (the C-1 site), which becomes CO_2 (Donald D. Van Slyke et al., 1941). The ninhydrin reaction followed by capture of the produced CO_2 provides a sample on which to measure an amino acid's $\delta^{13}C$ of the carboxyl site (the C-1 site) (Abelson and Hoering, 1961). We measure the CO_2 produced from the ninhydrin reaction of alanine on an IRMS to constrain $\delta^{13}C$ of the C-1 site for the three alanine standards (See Materials: Alanine Standards).

The ninhydrin reaction (D D Van Slyke et al., 1941) is performed in a custom-made 50 mL borosilicate flask (Figure S3) with one sealable port that attaches to a custom-built gas-line (used similarly to a Schlenk-line in this study) and one port that has a Swagelok fitting and septum. The gas-line has two in-line water traps and two pressure gauges, which enable pressure readings from 1.00 x 10^{-3} mTorr to 760 Torr. The ninhydrin decarboxylation follows methods adapted from Van Slyke et al. (1941). In short, we add 57 mg of ninhydrin, 15 mg alanine, and 92 mg citric acid (exact quantities in Table S6) to a custom-made borosilicate flask. This flask is then attached to a gas-line via a Swagelok with a frit to avoid solid materials from entering the line and is then evacuated. While the flask is being evacuated, 10 mL water is added to a 40 mL vial that is subsequently capped. Helium is bubbled through the water for 10 minutes to remove any carbon dioxide. Once the flask is evacuated (0.005 mTorr or below as read by the pressure gauge), both the port to the reaction flask and to the gas-line are closed. The flask is taken off the line and place in a fume hood where 5 mL of degassed water is injected into the vial through the septum. The flask is then placed in an ethylene glycol bath at 100 °C for 32 minutes after which it is removed from heat and connected to the gas-line. The flask is placed in a -15 °C ethylene glycol-dry ice bath for 20 minutes or until all liquid in the flask is frozen, whichever is longer. During this time, a borosilicate tube is attached to another port on the Gas-line, evacuated, and the port to it is closed. Once the contents of the reaction vessel are frozen, the ports to the pumps are closed and those to the reaction vessel are opened and the pressure is recorded. One of the water traps is then submerged in an ethanol-dry ice bath for 5 minutes or until the pressure reaches 0.005 mTorr. At this point, the port to the reaction vessel is closed, the ethanol-dry ice bath is removed, and the gas equilibrates in the line. We record the pressure, open the port to the borosilicate tube, and immerse the tube in liquid nitrogen. One minute after the pressure reaches 0.005 mTorr, the tube is sealed with a O_2 torch.

Once trapped in a borosilicate tube, CO_2 is measured by IRMS on a Thermo Fisher Delta V with two ethanol-dry ice traps and a GC on the front end for sample purification. To introduce sample into the instrument, borosilicate tubes are scored, and the scored end is sealed into a flexible metal tube via a Swagelok. The other end of the metal tube has a second Swagelok with a frit on

it—this Swagelok is attached the purification line on the front end of the Delta V. After evacuating inlet to the tube, a line is open between it and the first gas trap and the tube is broken. Gas equilibrates for one minute before the inlet port is shut. Measurements of CO_2 for each sample is measured in dual-inlet mode against a CO_2 standard with a known $\delta^{13}C_{VPDB}$ composition.

The C-1 site of three alanine samples are constrained using ninhydrin decarboxylation. The $\delta^{13}C_{VPDB}$ of the C-1 sites for Alfa Aesar and VWR alanine standards are nearly equal at -28.5 ± 0.1 ‰ and 29.6 ± 0.1 ‰, respectively. The Strecker alanine standard's C-1 site has a $\delta^{13}C_{VPDB}$ of -43.5 ± 0.1 ‰, which approximately 15 ‰ below that of the other standards (Eiler et al., 2017). These $\delta^{13}C$ values of the C-1 sites are invariant with respect to yield, reaction time, or the proportion of ninhydrin to alanine. Combining the $\delta^{13}C_{VPDB}$ of the C-1 sites of alanine with the molecular-average $\delta^{13}C_{VPDB}$ measurement, we calculate $\delta^{13}C_{VPDB}$ for the averaged C-2 and C-3 sites for all three standards as well (Table S6).

Appendix C: Equilibrium calculations

The equilibrium constants for the reactions that occur during the equilibration step provide a means to estimate the proportions of species at intermediate steps that we have not directly analyzed. These concentrations include the equilibrium concentration of α-aminopropanenitrile (α-APN), ammonia, acetaldehyde, and cyanide. Equilibrium concentrations of these species are solved for in a series of equilibrium, water activity, and mass balance equations. Equations S1 to S8 are equilibrium equations that relate the relative concentrations of products and reactants to the known values of equilibrium constants (*i.e.,* the ratio of forward and reverse reactions, listed in Table S5) for reactions in which those products and reactants participate (Eqn. S1 to S14). Equation S9 calculates the water activity at the end of the equilibrium step. Equations S10 to S14 are mass balance equations, which assert that the number of moles of an element of interest contained in a specific pool of initial reactants (e.g., the N atoms in ammonium chloride) at the start of the equilibration step (here denoted with a $t = 0$ subscript) must equal the number of moles of that element contained in the residual pool of those reactants plus the number of moles

of that element that were transferred to products at the end of that step (note: no subscript is used for the concentrations at the end of equilibrium).

$$K_1 = \frac{[NH_3][H^+]}{[NH_4^+]} \qquad \textit{(Eqn. S1)}$$

$$K_2 = \frac{[CN^-][H^+]}{[HCN]} \qquad \textit{(Eqn. S2)}$$

$$K_3 = \frac{[CH_3CHO]*a_1}{[CH_3CH(OH)_2]} \qquad \textit{(Eqn. S3)}$$

$$K_4 = \frac{[CH_3CH(NH_2)CN][OH^-]}{[NH_3][CN^-][CH_3CHO]} \qquad \textit{(Eqn. S4)}$$

$$K_5 = \frac{[CH_3CH(NH_2)CN][H^+]}{[CH_3CH(NH_3^+)CN]} \qquad \textit{(Eqn. S5)}$$

$$K_6 = \frac{[CH_3CH(O)CN]}{[CN^-][CH_3CHO]} \qquad \textit{(Eqn. S6)}$$

$$K_7 = \frac{[CH_3CH(O)CN][H^+]}{[CH_3CH(OH^+)CN]} \qquad \textit{(Eqn. S7)}$$

$$K_8 = \frac{[H^+][OH^-]}{a_1} \qquad \textit{(Eqn. S8)}$$

$$a_1 = \frac{n_{H_2O}}{n_{CN} + n_{HCN} + n_{CH_3CHO} + n_{CH_3CH(OH)_2} + n_{NH_3} + n_{NH_4} + n_{CH_3CH(NH_2)CN} + n_{CH_3CH(NH_3)CN} + n_{CH_3CH(O)CN} + n_{CH_3CH(OH)CN}} \qquad \textit{(Eqn. S9)}$$

$$[NH_4Cl]_{t=0} = [NH_4^+] + [NH_3] + [CH_3CH(NH_3^+)CN] + [CH_3CH(NH_2)CN] \qquad \textit{(Eqn. S10)}$$

$NaCN_{t=0} = [HCN] + [CN^-] + [CH_3CH(NH_3^+)CN] + [CH_3CH(NH_2)CN] + [CH_3CH(OH^+)CN] + [CH_3CH(O^-)CN]$ *(Eqn. S11)*

$[CH_3CHO]_{t=0} = [CH_3CH(OH)_2] + [CH_3CHO] + [CH_3CH(NH_3^+)CN] + [CH_3CH(NH_2)CN] + [CH_3CH(OH^+)CN] + [CH_3CH(O^-)CN]$ *(Eqn. S12)*

$[H^+]_{t=0} + [NH_4Cl]_{t=0} + x = [H^+] + [NH_4^+] + [CH_3CH(NH_3^+)CN] + [CH_3CH(OH^+)CN] + [HCN]$ *(Eqn. S13)*

$[OH^-]_{t=0} + x + [CH_3CH(NH_3^+)CN] + [CH_3CH(NH_2)CN] = [OH^-]$ *(Eqn. S14)*

The variable *x* is defined below.

This system of equations accounts for all possible species in solution during the equilibrium step. Initial masses of ammonia and cyanide were weighed and recorded, and volumes of acetaldehyde and water were measured in syringes and recorded from each synthesis (Table 1, main text). For the equation 16, OH^- sources consist of initial OH^-, OH^- produced when α-APN is formed, and OH^- produced from water dissociation. This final OH^- source is denoted as *x* and accounts for the degree of auto-dissociation of water: because the equilibrium constant for water dissociation at a given temperature is always equal to the product of the concentration of H^+ and OH^- divided by the water activity (a_1) (Eqn. S9)—the partial vapor pressure of water in a solution relative to that of pure water, which is roughly equal to 0.98 in our system as defined by equation 12—*x* is the change in $[H^+]$ and $[OH^-]$ from water formation or dissociation that must occur to satisfy equation 11. Gas phase concentrations of reactants were negligible (<0.1%), so we did not include these in our model. Initial water is assumed to have a pH of 7. When calculations were repeated at a pH of 5.6 to account for the uptake of CO_2 from air, the results were within error of those presented here.

Appendix D: Calculation of α-APN formation and degradation during H-1

The α-APN formation and decomposition reactions are the forward and reverse components of reaction 4, respectively (Table S5). We solve for their separate rates using equations 18 and 19 and rate constants from Van Trump (1975) (Table S5):

$$v_{fwd} = k_{f1} * \frac{[\mathrm{RCHO}][\mathrm{CN^-}][\mathrm{NH_3}]}{[\mathrm{OH^-}]} \quad \textit{(Eqn. S15)}$$

$$v_{rev} = k_{r1} * [\alpha\text{-APN}] \quad \textit{(Eqn. S16)}$$

In these equations, v_{fwd} is the rate of α-APN production from reactants (acetaldehyde, cyanide, and ammonia), and v_{rev} is the rate of α-APN decomposition into reactants. Rate constants k_{f1} and k_{r1} (Table S5) are for the reaction rate for the forward and reverse reactions, respectively. The forward and reverse velocities depend on the concentrations α-APN, CN^-, NH_3, and OH^-, all of which are pH dependent, so we solve equations 18 and 19 for two pH conditions, the pH value at the end of equilibrium (pH = 8.2), which we solve for above, and the pH value during hydrolysis (pH < 1).

The quantities [NH_3], [CN^-], and [αAPN] present at the beginning of the hydrolysis step for the two pH conditions listed above are calculated using the relationship:

$$[base] = [acid + base] * \frac{K_a}{K_a + [H^+]} \quad \textit{(Eqn. S17)}$$

where [base] is the concentration of the basic species of a compound of interest, [acid + base] is the total concentration of that compound in solution, and K_a is the acid dissociation constant for the compound (Table S5). In the case of ammonia, [base] is [NH_3], [acid + base] is [NH_4^+ + NH_3], and K_a equals $K_{a,NH4}$, which equals 5.6 x 10^{-10} (Table S5).

Appendix E: Calculation of isotope effects

Isotope effects on nitrogen for the equilibrium step are calculated using equation S18 (subscripts refer to the molecule of interest of f_x and $^{15}F_x$ values and refer to the product/reactant for the α values):

$$^{15}F_{tot} = \frac{f_{NH_3}}{1+\alpha_{\frac{\alpha APN}{NH_3}}\left(\frac{1}{^{15}F_{\alpha APN}}-1\right)} + \frac{f_{NH_4}\,\alpha_{\frac{NH_4}{NH_3}}}{\alpha_{\frac{NH_4}{NH_3}}+\alpha_{\frac{\alpha APN}{NH_3}}\left(\frac{1}{^{15}F_{\alpha APN}}-1\right)} + f_{\alpha APN}\,^{15}F_{\alpha APN}$$

(Eqn. S18)

where $^{15}F_{tot}$ is calculated from the $\delta^{15}N$ value of ammonia added to the reaction vessel and $^{15}F_{\alpha APN}$ is the ^{15}F value of the α-APN pool at equilibrium, which is assumed to be equal to the ^{15}F value of final product alanine, as ^{15}F does not change significantly and consistently with the reaction progress of steps H-1 or H-2 (see Results). The f_x values in equation S18 are the fraction of compound *x* at equilibrium and are equal to the concentration of x calculated at equilibrium divided by the concentration of NH_4Cl added to the reaction vial. The $\alpha_{P/R}$ values for nitrogen, defined as the ratio of $^{15}R_P/^{15}R_R$, are equilibrium isotope fractionations between the product (subscript P) and the reactant (subscript R; ammonia in this case). We use a value of 1.029 for $\alpha_{NH4/NH3}$ (Walters et al., 2019).

Isotopic compositions for the carbon atoms that will ultimately be transferred to the C-2 and C-3 sites of alanine are calculated for the equilibrium step with equation S19:

$$^{13}F_{tot} = \frac{f_{ace}}{1+\alpha_{\frac{\alpha APN}{ace}}\left(\frac{1}{^{13}F_{\alpha APN}}-1\right)} + \frac{f_{ace(OH)_2}\,\alpha_{\frac{ace(OH)_2}{ace}}}{\alpha_{\frac{ace(OH)_2}{ace}}+\alpha_{\frac{\alpha APN}{ace}}\left(\frac{1}{^{13}F_{\alpha APN}}-1\right)} + f_{\alpha APN}\,^{13}F_{\alpha APN}$$

(*Eqn. S19*)

Equation S19 is analogous to equation S18 but considers the carbons transferred from acetaldehyde to αAPN. In equation S19, $^{13}F_{tot}$ is based on the molecular average $\delta^{13}C$ value of acetaldehyde added to the reaction vessel and $^{13}F_{\alpha APN,eq}$ is the ^{13}F value of the carbons in α-APN that will become C-2 and C-3 in alanine. Each f_x value is the concentration of compound x at equilibrium for a specific experiment divided by the concentration of acetaldehyde initially added to the reaction vial for that experiment. Here the compounds are abbreviated as follows: acetaldehyde is 'ace', acetaldehyde hydrate is 'ace$(OH)_2$', and α-APN is αAPN. The $\alpha_{P/R}$ values are as defined in the preceding paragraph but for ^{13}R instead of ^{15}R. As there are no measurements of the equilibrium carbon isotope effect between acetaldehyde and acetaldehyde hydrate, we adopt a value of 1.0034 for $\alpha_{ace(OH)2/ace}$, which is half the value for CO_2 hydration a it would be averaged over two carbons in acetaldehyde (Marlier and O'Leary, 1984).

The equilibrium concentrations calculated in equations 4 to 17 find that effectively all (> 95 %) cyanide added at the start of an experiment is transferred to the C-1 site of α-APN (with the remainder being transferred to the C-1 site of α-hydroxypropionitrile). α-APN and α-hydroxypropionitrile have the same bonding environment at the C-1 site and therefore we adopt the approximation that they have the same equilibrium carbon isotope effects with respect to cyanide. Consequently, we can assert that the C-1 site of α-APN (and α-hydroxypropionitrile) has a $\delta^{13}C$ value at equilibrium closely similar to that of initial HCN reagent. However, we do not have an estimate of the equilibrium fractionation factor between HCN and α-APN (and α-hydroxypropionitrile), and thus we cannot assign a value to the $\delta^{13}C$ of the trace of residual CN at the end of the equilibrium step; however, this uncertainty has no impact on our ability to predict or interpret $\delta^{13}C$ values of other species of interest.

Our experimental results (detailed below) indicate negligible isotopic fractionation of the carbon atoms that will end up in the C-2 and C-3 sites of alanine during the H-2 reaction step because the $\delta^{13}C$ value of these sites are approximately invariant across the progress of this irreversible reaction. This implies that H-2 has negligible secondary and tertiary carbon isotope effects. Consequently, we can use the ^{13}F value for the C-2 and C-3 sites in product alanine and assume

they are the same for initial alaninamide and with these values calculate the ^{13}F and therefore ^{13}R values at the C-1 site in alaninamide and alanine according to equation 3. Combining these C-1 site ^{13}R values and with that of the α-APN's C-1 site provides the ^{13}R values for all of the reactants and products in H-1 and H-2. These values enable us to calculate the isotope effect for H-1 and H-2 by using the Rayleigh equation, written below in equation 23, for each hydrolysis step separately.

$$f^{\frac{\alpha_P - 1}{R}} = \frac{^{13}R_R}{^{13}R_{R,0}} \qquad \textit{(Eqn S20)}$$

where $^{13}R_R$ and $^{13}R_{R,0}$ are the ^{13}R values for the residual and initial reactants (α-APN for H-1 and alaninamide for H-2), f is the fraction of residual to initial reactant, and $\alpha_{P/R}$ is fractionation factor between the product and reactant.

Finally, we calculate molecular-average isotope fractionations for the equilibrium step, H-1, and H-2. As the CN is calculated to have nearly quantitative incorporation into the intermediate α-APN, the equilibrium isotope effect for the full molecular average can be calculated as 2/3 times that of the acetaldehyde's equilibrium isotope effect with α-APN's averaged C-2 and C-3 sites. The molecular-average isotope effects at H-1 are calculated using the Rayleigh equation (Eqn. S20) with α-APN's calculated molecular-average ^{13}R values and the initial alaninamide's calculated ^{13}R values. Finally, the molecular-average isotope effects at H-2 are similarly calculated from the Rayleigh equation (Eqn. S20) and the calculated initial ^{13}R for alaninamide and measured ^{13}R of alanine.

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