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Process Patent Protection via Analysis of Stable Isotope Ratios

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ABSTRACT: Process patent protection via the analysis of natural-abundance stable isotopes has been demonstrated as an approach to extend the effective life of bio/pharmaceutical patents. The high specificity of isotope ratio analysis compared to other approaches (for example, concentrations of organic impurities or trace metals) allows the isotopic analysis to differentiate processes that were not previously resolvable by less precise analytical methods. Here we summarize the rationale for, and some selected case studies of, this emerging field. We review: (i) the systematics of stable isotope chemistry, (ii) approaches to instrumental analysis of stable isotopes, (iii) the biogeochemical origin of stable isotopic fingerprints, (iv) equilibrium versus kinetic isotope effects on those fingerprints, (v) categories of application of process patent protection, and (vi) case histories of application. The three reviewed cases include one of nutraceutical false advertising, one of a small molecule antibiotic drug product infringement, and one of wrongful accusation of human drug product infringement, which protected these bio/pharmaceutical products against patent infringement. Finally, we briefly preview some new applications of stable isotopic analysis in the bio/pharmaceutical field including analysis of biologic drugs, continuous monitoring of drug reaction processes, and isotopically directed synthesis.

1. INTRODUCTION

1.1. Stable Isotope Analysis in Process Protection. The analysis of stable isotope ratios is a well-established strategy in forensic biochemistry and geochemistry,[1] with legal criteria and guidelines for its applicability.[2] However, to our knowledge, few reviews address the possibilities afforded by stable isotope analysis for improving process patent protection in relation to pharmaceutical and other value-added products.[3,4]

Recent work in pharmaceutical forensics demonstrates the sensitivity and applicability of stable isotope approaches. Early applications from our own work largely focused on batch manufacturing, in which we established that batches of pharmaceutical materials have distinctive ratios (or "fingerprints") of the stable isotopes of each element present in the final product.[5,6] In 2005, in a blind study commissioned by the US Food and Drug Administration (FDA), Wokovich et al.[7] characterized the ratios of carbon ($^{13}C/^{12}C$) and oxygen ($^{18}O/^{16}O$) isotopes in 26 batches of the pain reliever, naproxen. The FDA wanted to assess the robustness of isotopic fingerprinting to differentiate pharmaceuticals, thereby establishing an important investigative and forensic tool for drug enforcement authorities trying to determine the provenance of a product. The results correctly determined that the batches of naproxen had come from six different manufacturing sites from around the world (Figure 1). Yet there remains significant untapped potential for these approaches: Stable isotopic technologies can identify more than just the uniqueness of product batches[7] or the sources of natural or manufacturing materials[1,2]—the examples we present here show how these technologies can be used to protect the *processes* used during manufacture.

Process protection is distinct from product protection. Extensive capital is expended on developing and improving manufacturing—distinct from the products being produced—

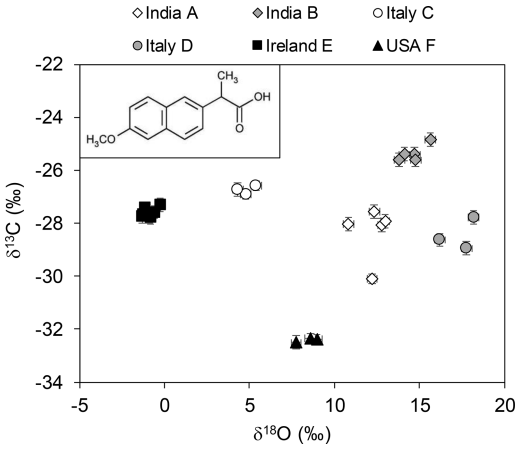

Figure 1. Source identification of naproxen samples: Bivariate plot of the carbon isotopic composition ($\delta^{13}C$) versus the oxygen isotopic composition ($\delta^{18}O$) of 26 batches (A through F) of naproxen from six manufacturers from four countries.[7] This study was performed blind for the United States FDA's Division of Pharmaceutical Analysis (US FDA-DPA).

and these improvements are patentable per statute 35 USC § 101, which states "[w]hoever invents or discovers any new and useful process, machine, manufacture, or composition of matter, or any new and useful improvement thereof, may obtain a patent therefor." However, a simple example illustrates why it can be challenging to prove infringement of a process. Suppose a company synthesizes a drug compound **C**, starting with compound **A**, and going through intermediate **B**, using

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two distinct reagents. A patent claim for this process might read:

A process for manufacturing compound **C** comprising the steps of reaction of:

(a) Compound **A** with reagent 1 to produce compound **B** and,

(b) Compound **B** with reagent 2 to produce compound **C**.

For process infringement to occur, it is necessary to prove that the accused has practiced all steps with the specified materials and in the order recited. If the accused has developed an alternative process, for example without going through Compound **B** or by substituting a specified reagent, they may not be in violation; i.e., neither side is contesting the authenticity or legality of Compound **C**, only how it was produced.

Because of these challenges, process patents have been difficult to defend. Common strategies to prosecute infringement involve evidence of organic impurities and/or trace metals that are argued to be foreign to the established manufacturing process.[8,9] These cases can be challenging due to ambiguities in the interpretation of what constitutes a trace impurity.[10] In contrast, isotopic evidence can be more persuasive than other approaches because it is so quantitatively sensitive, typically requiring samples of less than 0.1 to only a few milligrams of material to be delivered to the mass spectrometer, with high quantitative resolution in the ability to distinguish between materials with different origins and production histories.

The financial benefits to protecting processes can be substantial, especially after the associated product patent has lapsed. It would be valuable to have methodologies that could demonstrate or alternatively, argue against, process infringement. Stable isotopes have permitted a novel and efficient means to protect the period between the expiration dates of composition-of-matter patents and process patents, by identifying *differences in isotopic patterning in addition to bulk isotopic content*.[3,6,11,12] Since litigation is not public, the most relevant cases cannot be disclosed. Due to the importance of the topic, this paper explains the underlying scientific principles and will allow the reader to understand the potential of the technology.

1.2. Background. *1.2.1. Stable Isotope Systematics.* Most synthetic pharmaceuticals, like most biological molecules, are composed predominantly of the elements C, H, N, O, and S. Each of these has multiple, naturally occurring stable isotopes (e.g., ^{12}C, 0.9893; ^{13}C, 0.0107). The distribution of these isotopes in any system of chemical reactions is an inherent property of the bond-level energetics of the specific reactions, and the expression of these effects results in observable fractionation—i.e., sorting—of the isotopes between the various reactants and products.[13]

Historically, the precedent for using stable isotope analysis to record chemical transformations is attributed primarily to Urey,[14−16] who established the "delta notation" approach to isotopic reporting that still is used today. This notation takes an analogous form for all elements, in which δ is expressed as the relative parts-per-thousand (‰, permil) difference between the ratios of the isotopes in a sample vs in a defined standard reference material. To use carbon as an example:

$$\delta^{13}C = 1000 \times \left[\left(\frac{\mathbf{R}_{sample}}{\mathbf{R}_{standard}} \right) - 1 \right] \quad (1)$$

where the ratio **R** is given by

$$\mathbf{R} = \frac{^{13}C}{^{12}C} \quad (2)$$

which is distinct from the fractional abundance **F**, which is given by

$$\mathbf{F} = \frac{^{13}C}{^{12}C + ^{13}C} \quad (3)$$

The standard reference materials and corresponding ratios for C, H, O, N and S are described elsewhere.[17] Again, using the example of carbon, an absolute $\mathbf{R}_{sample}$ = 0.010956 for $^{13}C/^{12}C$ in an organic molecule, relative to $\mathbf{R}_{standard}$ = 0.011237 in the standard reference material Vienna Pee Dee Belemnite (VPDB) (a difference of 0.000281), implies a $\delta^{13}C$ value of −25.0‰ for the sample. One immediately notes, therefore, that to determine values of δ at 0.1‰ precision requires the ability to resolve differences in isotope ratios, **R**, at a precision of 10^{-6}, and this requires dedicated technology.

1.2.2. Analysis of Stable Isotope Ratios. To date, most high-precision stable isotope analyses have been done by multi-collector, magnetic sector mass spectrometry. Known as isotope-ratio mass spectrometry (IRMS), the approach relies on combustion or pyrolysis of the analyte to a low molecular-weight gas, followed by acceleration of that gas to a calibrated set of Faraday cup detectors. Gas molecules containing the higher-mass isotopes (larger m/z) travel through the magnet with a wider radius, separating them from the more abundant lower-mass species (Figure 2). The relative numbers of each mass ion are counted separately, and the ratio is determined

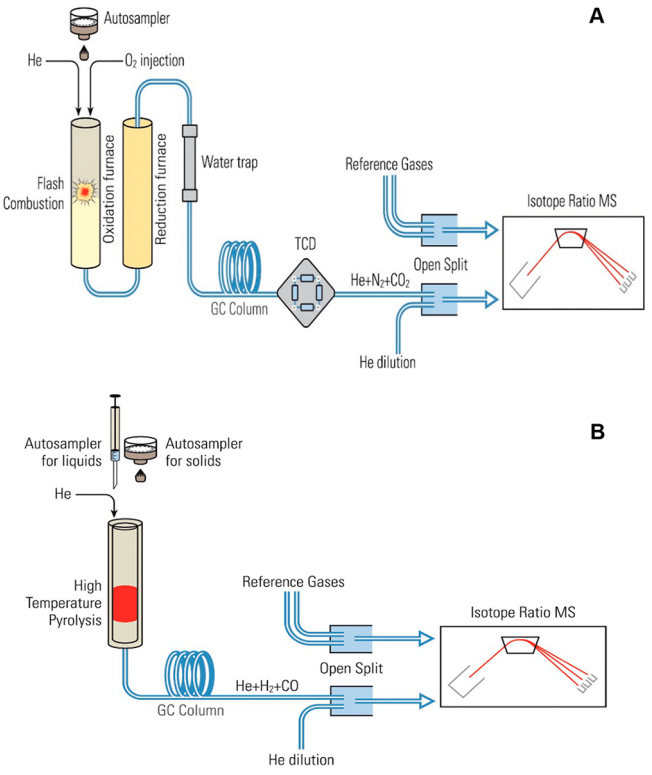

Figure 2. Schematic diagram of typical interfaces for isotope-ratio mass spectrometers: (a) an elemental analyzer/isotope-ratio mass spectrometer (EA-IRMS) and (b) thermal conversion/elemental analyzer/IRMS (TC/EA-IRMS) (figures provided by ThermoFisher).

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relative to the response obtained from the appropriate standard reference material. Extensive reviews are available that detail the physics, electronics, theoretical limits of detection, standardization, and practical application of such instruments (e.g., ref 18).

All IRMS instruments, therefore, require front-end interfaces for converting analytes to purified gases. The three most common interfaces are elemental analyzer (EA; Figure 2a), thermal conversion/elemental analyzer (TC/EA; Figure 2b), and gas chromatograph (GC; not used in the present examples). For EA-IRMS, samples are combusted quantitatively over metal catalysts, usually with excess O_2 added, to produce CO_2, H_2O, and a mixture of NO_x gases.[19] The flow stream is passed through a mild reductant to convert NO_x to N_2 and through a permeable membrane to remove H_2O. Values of $\delta^{13}C$ and $\delta^{15}N$ are obtained from the resulting pure CO_2 and N_2 products, which are separated chromatographically before isotopic analysis. In TC/EA-IRMS, materials are pyrolytically converted without additional oxidant, typically at >1400 °C,[20] yielding CO for analysis of $\delta^{18}O$ values and H_2 for analysis of $\delta^{2}H$ values. Values of $\delta^{34}S$ are obtained by EA-IRMS on SO_2 produced by combustion (not shown in Figure 2). Quantitative yields in these conversions are essential to obtain an accurate determination of the original isotope ratios of the sample.

The resolution of isotope space or uniqueness of an isotope fingerprint depends on the precision and accuracy of δ values obtainable by these techniques (typically 0.1−0.3‰ for C, N, O, S, and 3−5‰ for H) and on the typical range of δ values exhibited by an element (generally <50‰ for C, N, O, S and <500‰ for H). The resolution (precision:range) is therefore $\sim 10^2$ unique values for each element and in a multielement compound the total isotopic resolution is $(\sim 10^2)^n$, where n is the number of elements (e.g., $10^2 \times 10^2 \times 10^2 = 10^6$ for $C_6H_{12}O_6$).[21]

1.2.3. Elemental Source (Biogeochemical) Fingerprints. In addition to providing thousands of unique isotopic combinations to distinguish between organic substances, δ values also have intrinsic meaning that can help illuminate the source history or natural origin of materials used in production.[13,22,23]

Stable carbon isotopes are particularly useful to differentiate organic matter based its original photosynthetic pathway: e.g., C_3-plant versus C_4-plant sources. C_3 plants fix carbon using the Calvin−Benson−Bassham cycle,[24] in which CO_2 and a 5-carbon sugar are converted into two 3-carbon molecules (C_3). C_4 plants fix carbon using the Hatch-Slack pathway,[25] in which the initial product instead is a 4-carbon acid (C_4). C_3 plants strongly favor ^{12}C-containing CO_2 over ^{13}C-containing CO_2, yielding values of $\delta^{13}C$ commonly −25‰ to −30‰; most plants are C_3 and fall in this category. By contrast, the distinctive C_4 physiology is present specifically in heat-tolerant species such as corn and sugar cane; that is, it is prominent in the agricultural sector.[26] This strategy has high biosynthetic efficiency, with little opportunity for isotopic discrimination between ^{12}C and ^{13}C, and the resulting $\delta^{13}C$ range of C_4 plants (−14‰ to −10‰) is nearly equal to the CO_2 that diffuses in from the atmosphere. Thus, the values for C_4 plants have virtually no statistical overlap with C_3 plants.[27]

Two other categories of carbon sources also are noteworthy. The first is a fossil-fuel derived material, which has $\delta^{13}C$ values typically −25‰ to −35‰,[28] as fossil fuels represent the degraded remains of marine and ancient terrestrial biomass. The second category is methane and other natural gas-derived materials, which are < −40‰ if thermogenic and < −60‰ if biogenic;[29] this category may include the methanol used in industrial synthesis processes. Both can be useful signatures of reagents or substrates derived from petrochemicals, thereby distinguishing them from "natural" materials.

Isotopic ratios for oxygen ($\delta^{18}O$) and hydrogen ($\delta^{2}H$) most often are influenced by interaction with water. Values of $\delta^{18}O$ and $\delta^{2}H$ in natural precipitation waters span ranges of approximately 50‰ and 450‰, respectively, from the Earth's equator to poles.[30,31] Values of $\delta^{18}O$ and $\delta^{2}H$ in local fresh water sources resemble VSMOW near the equator and decrease progressively with increasing latitude due to Rayleigh fractionation and distillation during precipitation (described below). This leaves distinct imprints on the biochemicals produced from water at different latitudes or climates or in different industrial settings.

Finally, the stable isotopic composition of nitrogen ($\delta^{15}N$) is highly variable in nature due to the complexity of its inorganic as well as organic reactions.[23] This is due to its many sources both from natural elemental cycling and from commercial fixation using the Haber−Bosch process. In general, however, synthetic nitrogen (Haber-Bosch, e.g., fertilizer) has $\delta^{15}N$ values ≤0‰, while naturally cycling nitrogen has $\delta^{15}N$ values >0‰.[32,33] The stable-isotopic composition of sulfur ($\delta^{34}S$) also demonstrates a very large dynamic range. In pharmaceutical studies, S typically serves as an excellent "fingerprinting" tracer,[34] but the biogeochemical complexity of the global sulfur cycle does not easily yield specific regional or biological interpretations.

1.2.4. Equilibrium vs Kinetic Isotope Effects. Thermodynamic factors govern the sorting of stable isotopes between chemical products that exchange in reversible reaction systems. These exchange reactions result in unequal isotope ratios for the equilibrating molecules, specifically favoring the incorporation of the heavier isotope (e.g., ^{13}C or ^{18}O) into the more stable (lower energy, or stronger) bonding environment. The equilibrium isotope effect, α_{A-B}, describes the distribution of isotopes between the two molecules (**A** ⇔ **B**), given as the isotope ratio of **A** (R_A) divided by the isotope ratio of **B** (R_B):

$$\alpha_{A/B} = R_A/R_B \tag{4}$$

For practical application, values of α are determined from values of δ and are sometimes expressed as ε, the parts-per-thousand equivalent:

$$\alpha_{A/B} = \frac{(1000 + \delta_A)}{(1000 + \delta_B)} \tag{5}$$

$$\varepsilon_{A/B} \equiv 1000(\alpha_{A/B} - 1) \tag{6}$$

$$\varepsilon_{A/B} \approx \delta_A - \delta_B = \Delta\delta_{A/B} \tag{7}$$

In general, when the magnitude of the isotope effect is small (<30‰), the difference between $\Delta\delta$ and ε is negligible (<1‰; eq 7) and may be ignored; however, for precise calculations, the exact form of ε should be used, and we advocate this approach whenever possible. Values of $\alpha_{A/B}$ decrease at higher temperatures, resulting in smaller isotopic differences between **A** and **B** for high-temperature synthesis.

Equilibrium isotope effects can influence exchangeable moieties on organic molecules, particularly in functional positions that are prone to acid- and base-catalyzed substitution reactions, or any other reversible process, e.g., keto−enol tautomerism. Most commonly this affects signatures of oxygen and hydrogen moieties in isotopic process analysis, and it may

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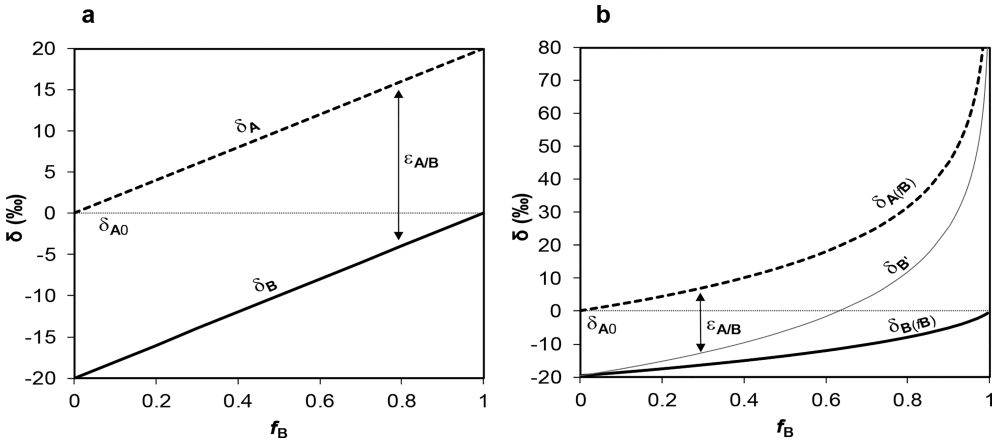

Figure 3. Isotopic distributions between reactant **A** and product **B** in simple closed-batch reactions. (a) In a bidirectional or equilibrium system, δ_A and δ_B are offset by $\varepsilon_{A/B} \approx \delta_A - \delta_B$ (eq 7). When the reaction yield (f_B) is large, δ_B will approach the isotope ratio of the initial starting material (δ_{A0}). (b) In a unidirectional or kinetic system, product is sequestered in **B**, and the isotopic compositions proceed as logarithmic functions of f_B (eqs 16 and 17). The evolving pool of **A** and the instantaneous product **B′** are related by $\varepsilon_{A/B} \approx \delta_{A(fB)} - \delta_{B'}$, but **B′** generally cannot be separated from the accumulated total pool of **B**.

therefore be important to consider that some of these positions may be subject to equilibrium exchange. Inert or integral oxygens (ethers, some ketones), nonacidic alcohols, and alkyl hydrogens do not exchange readily with H_2O at low temperatures,[35] but acidic functional groups may be continuously reactive, especially at elevated temperatures.[36]

In biomolecules and in many cases of synthetic organic molecules, however, the expressed isotopic fractionation primarily is due to kinetic effects. This type of effect results from enzymatically or catalytically mediated unidirectional reactions in which the rate of formation of a product containing a heavier isotope nearly always is slower than that of the same product containing the lighter isotope, because bonds involving heavier isotopes require more energy to dissociate. Thus, kinetic isotope effects (KIEs) are site-specific within molecules, not distributed over the entire molecule. A classic example is the cellular biosynthesis of *n*-acetyl chains. These compounds are formed by polymerizing acetyl groups that originate from the enzymatically mediated decarboxylation of pyruvate. This decarboxylation discriminates against ^{13}C, resulting in position-specific differences in $\delta^{13}C$ values between the acetyl−CoA methyl carbon (not affected by the reaction) and carbonyl carbon (formerly bound to pyruvate), thereby yielding an alternating pattern of isotopic enrichment/depletion in the odd/even positions.[37,38]

KIEs are often described in terms of reactants (**Q**) and products (**P**), using notation analogous to that used to describe equilibrium effects, but transformed into a version that emphasizes the isotopic differences between **Q** and **P**. All equations are analogous to eqs 4−7, above:

$$^{13}\mathbf{Q} \rightarrow {}^{13}\mathbf{P}, \text{ rate } k_1 \tag{8}$$

$$^{12}\mathbf{Q} \rightarrow {}^{12}\mathbf{P}, \text{ rate } k_2 \tag{9}$$

$$\alpha_{\mathbf{Q/P}} = \frac{(1000 + \delta_{\mathbf{Q}})}{(1000 + \delta_{\mathbf{P}})} \tag{10}$$

$$\varepsilon_{\mathbf{Q/P}} \equiv 1000(\alpha_{\mathbf{Q/P}} - 1) \tag{11}$$

$$\varepsilon_{\mathbf{Q/P}} \approx \delta_{\mathbf{Q}} - \delta_{\mathbf{P}} = \Delta\delta_{\mathbf{Q/P}} \tag{12}$$

In unidirectional reactions, the value of $\varepsilon_{Q/P}$ is a positive number because the heavy isotope is enriched in the unused reactants, and products are isotopically "lighter", i.e., $k_2 > k_1$; this is known as a *normal* isotope effect. This normal isotope effect is a manifestation of the fact that light isotopes react more readily than heavy isotopes resulting in an isotopic enrichment in the reactants and by mass balance a relative isotopic depletion in the products.[13] Rare instances of *inverse* KIEs are known but are exceedingly uncommon and result from unusual transition states of reactive intermediates.[39]

1.2.5. Closed vs Open Systems. The preceding types of isotope effects must be interpreted within the context of the type of process being considered. Isotopic distributions between reactants and products can be measured for reactions both in closed (batch) or open (continuous) reaction systems. In addition, kinetic or equilibrium isotope sorting may apply in either system. Although this leads to four combinations of physical scenarios (equilibrium, closed and open; kinetic, closed and open) mathematically the resulting isotope distributions of the reactants and products reduce to only two sets of equations. [The required algebraic simplifications do not always hold true for hydrogen. For further details and more complicated cases, refer to the public document "An Introduction to Isotopic Calculations" http://www.whoi.edu/fileserver.do?id=73289&pt=2&p=74886.] For simplicity below, we will call all reactants **A** and all products **B**.

In closed systems with equilibrium isotope exchange, reactants and products must maintain isotope mass balance at all times. There is no loss of material from the system, and no means to irreversibly sequester isotopes in one of the chemical species. The isotopic distribution is governed by $\alpha_{A/B}$, which when expressed in terms of δ and ε (parts-per-thousand notation), simply describes the isotope mass balance and solves for δ_A and δ_B using the approximation $\varepsilon_{A/B} \approx \delta_A - \delta_B$ (eq 7):

$$\delta_{\text{total}} = f_{\mathbf{A}}\delta_{\mathbf{A}} + f_{\mathbf{B}}\delta_{\mathbf{B}}; \quad \text{fractional abundances } f_{\mathbf{A}} + f_{\mathbf{B}} = 1 \tag{13}$$

$$d_{\mathbf{A}} = \delta_{\mathbf{total}} + f_{\mathbf{B}}\varepsilon_{\mathbf{A/B}}; \quad \delta_{\text{total}} = \delta_{\mathbf{A}} \text{ at } f_{\mathbf{B}} = 0 \tag{14}$$

$$\delta_{\mathbf{B}} = \delta_{\text{total}} - (1 - f_{\mathbf{B}})\varepsilon_{\mathbf{A/B}} \tag{15}$$

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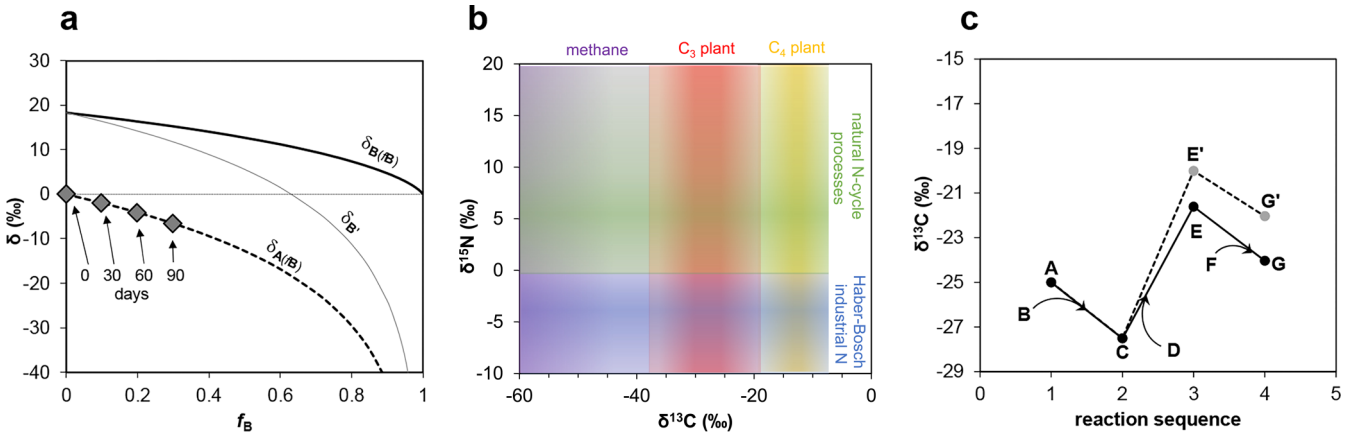

Figure 4. Three general types of process protection study enabled by stable isotopic analyses. (a) During processing, degradation, or distillation, a material's isotopic composition may drift due to loss of original material; isotopically the trajectory follows Rayleigh distillation. (b) In a provenance study, natural isotopic data will reflect the biogeochemical origins of the materials used in manufacture; here the example shows regions of a bivariate carbon and nitrogen isotopic plot—e.g., a product made from corn sugars and synthetically fixed nitrogen would fall in the C_4, Haber–Bosch sector. (c) In a multistep synthesis process study, the production of **G** from **A**, **B**, **D**, and **F** (with measured intermediates **C** and **E**) follows a pathway distinct from simple isotope mass balance (**E**′ and **G**′), due to isotope effects expressed at each step that are quantified by eq 17. Step-wise calculation results are shown in Table 1.

For systems in which equilibrium lies dominantly to the side of the product, **B** (e.g., $f_B = 0.9$), the isotopic composition of **B** will approach that of the initial pool of reactant, **A**, before any reaction had occurred (δ_{A0}). By the isotope mass balance requirement, the leftover **A** will have an isotopic composition that departs strongly from the initial conditions (Figure 3a).

When closed systems undergo reactions that are irreversible (i.e., in kinetic equilibrium), the production of **B** from **A** occurs with the same isotope effect, $\alpha_{A/B}$, but at each stepwise increment of the reaction it is drawing from a successively smaller and more isotopically fractionated pool of **A**. In effect, the reaction is distilling the isotopes at different respective rates as it proceeds. The isotopic trajectories proceed exponentially according to the Rayleigh equations, which in simplified form are as follows (for discussion of the exact forms, see[40]):

$$\delta_{A(fB)} = \delta_{A0} + \varepsilon_{A/B}\ln(1-f) \tag{16}$$

$$\delta_{B(fB)} = \delta_{A0} - \frac{\varepsilon_{A/B}(1-f)\ln(1-f)}{f} \tag{17}$$

Note that now δ_A and δ_B are functions of the fractional extent of irreversible reaction, f_B (Figure 3b). The stable isotopic compositions of the reaction products are sensitive indicators of the progress of the reaction and therefore of the stability of the reaction process if the reaction is performed with consistent yield. Importantly $\delta_{B(fB)}$, above, is the value of the cumulative pool of **B**; the isotopic composition of the instantaneous product ($\delta_{B'}$) at every incremental step is simply $\delta_{A(fB)} - \varepsilon_{A/B}$, but because it is mixed with **B** as it is formed, it cannot be distinguished from the accumulating total.

The scenarios for open systems also can be described using the above equations. In process analysis, they would be applicable—for example—in continuous-process reactors or batch feed designs in which product was being harvested continuously. In the case of a system in which the reaction between **A** and **B** equilibrates, but **B** is removed continuously from the system, the isotopic trajectories evolve as in the Rayleigh case (eqs 16–17; Figure 3b). This is simply another case of distillation, where the system is open on the outlet but not on the inlet. In the case of a continuous process system in which *new A is continuously supplied*, that is, the inlet is also open, then the reaction fractionates the isotopes between **A** and **B** by $\varepsilon_{A/B}$ but always relative to the initial isotopic composition of **A**, which remains stable (i.e., eqs 13–15; Figure 3a).

1.2.6. Isotope Mass Balance and Mixed Processes. The above principles describe the process-level controls on isotope fractionation. In practice, a commercial product is the result of several components and processing steps. But regardless of complexity, the principle of conservation of isotope mass balance is a general requirement. The net isotopic composition is equal to the mass-weighted sum of the parts:

$$\delta_{total} = \sum f_i \delta_I \tag{18}$$

In complex systems of reactions, both kinetic and/or equilibrium isotope fractionation may apply along many steps of the reaction. This may result in isotopic distributions in final reaction products that have been affected by more than one of the processes outlined in sections 1.2.4 and 1.2.5. For example, if a product, **E**, is composed of a mixture of a component **B** that has equilibrated with an excess of **A** (a common example would be equilibration with water), plus two other components **C** and **D** that each have unique biogeochemical sources (section 1.2.3) but experienced no additional isotope fractionation, then the total isotope balance of **E** will be[41]

$$\delta_E = f_B(\alpha_{A/B}(\delta_A + 1000) - 1000) + f_C\delta_C + f_D\delta_D$$
$$\text{where } f_B + f_C + f_D = 1 \tag{19}$$

2. PROCESS PROTECTION APPLICATIONS

2.1. Categories of Application. Examples of process protection can be categorized largely into three groups (Figure 4). In the first, no specific chemical reactions have been performed; rather, the isotopic composition of samples is altered by acts of processing or occurs spontaneously. In the second, the stable isotopic compositions of the final manufactured products are sufficient to establish the necessary conclusions about provenance or process associated with how they were generated ("product authentication"). In the third,

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Table 1. Sequential Isotopic Composition for a Three-Step Synthesis[a]

reagents						conditions				products		
reag. #1	reag. #2	n_1	n_2	δ_1 (‰)	δ_2(‰)	f_1	f_2	ε_1(‰)	ε_2(‰)		δ^*(‰)	δ(‰)
A	**B**	4	4	−25	−30	1	1	10	30	**C**	−27.5	−27.5
C	**D**	8	6	−27.5	−10	0.8	0.5	30	15	**E**	−20.0	−21.6
E	**F**	14	2	−21.6	−25	0.5	0.3	20	35	**G**	−22.0	−24.0

[a]For additional overview, see ref 3.

reaction intermediates as well as final products are studied together to understand, stepwise, the isotopic fractionation associated with both the individual steps and the overall process ("process authentication").

2.1.1. Effects Associated with Processing. As detailed above (section 1.2.5), the differential removal of a distillate—commonly water—will impart an isotopic signature. For example, Bricout[42] used this principle to distinguish natural fruit juices, i.e., those containing only the original plant water, from juices that had been processed to concentrate and then reconstituted using tap water. All juices were authentically juice as claimed by the manufacturers, but the analysis of water isotopes showed that they had been processed differently, thereby distinguishing fresh-squeezed juices from processed products. Other, similar cases are discussed in a recent review on stable isotope forensics.[1]

Operating by similar principles, the degradation of materials involves chemical reactions that have associated isotope effects. During chemical or biological degradation, a fixed pool of material reacts unidirectionally to an end product, again behaving as an open system undergoing distillation. Either the remaining reactant or the accumulating product can be measured as an index of the extent of degradation. This concept has been useful to distinguish natural vs synthetic sources of persistent organic contaminants and the extent and processes by which those sources are degraded.[43] As explained in context in section 1.2.4, these normal isotope effect are a manifestation of the fact that light isotopes react more readily than heavy isotopes resulting in an isotopic enrichment in the reactants and by mass balance a relative isotopic depletion in the products.[13] A further example of this type of application is illustrated in Figure 4a, in which chemical *X*, which is marketed as being stable for a shelf life of 90 days, shows evidence of isotopic distillation indicating breakdown and loss of an active group on a shorter time scale. Here the byproduct (**B**) is isotopically enriched relative to the initial material (**A**) by isotopic fractionation, causing the remaining (degraded) **A** to become more isotopically negative with time ($\alpha_{A/B} < 1$). Note the hypothetical monthly time points that could be measured, which would establish that the claims of product stability are false.

2.1.2. Diagnostic Provenance of Materials. Commonly known as a "product authentication", production and synthesis can leave distinctive isotopic signatures. In this type of study, the primary diagnostic feature is that the stable isotope signature of the final product(s) indicate(s), definitively, something about the materials used in processing. If the retrospective diagnosis of these materials is inconsistent with the patented process, then the litigant is in violation of the process patent.

Examples of this type are common in food science and other forensic applications. For example, in the industrial whole synthesis of artificial vanilla from petrochemicals, or in the production of vanilla by modifying C_3-plant-derived precursors, values of $\delta^{13}C$ and $\delta^{2}H$ are markedly different from the values found in natural vanilla.[44] Similar examples across several different industries have also been detailed.[1] These scenarios are most easily generalized as yielding final products that display isotope ratios that are inconsistent with the basic principles required by their biogeochemical fingerprints (section 1.2.3); i.e., the samples have an incorrect provenance. Regions of carbon and nitrogen isotope space that can help define provenance are shown in Figure 4b. Note that there are six different sectors of predicted material provenance.

2.1.3. Stepwise Analysis of Process Signatures. The most complex application of isotopic analysis to process protection is in the stepwise diagnosis of a multireagent or multistep synthesis (or "process authentication"). This approach also is potentially the most sensitive in that many more variables are measured. As a theoretical example we consider the evolving carbon isotopic composition of a product that is created by a three-step synthesis: **A** + **B** to form **C**; **C** + **D** to form **E**; and **E** + **F** to form **G**. Detailed discussion of the calculations that determine the net isotopic fractionation were described earlier;[3] here we describe the example only for carbon, but additional elements would be treated analogously.

Briefly, in step one of this hypothetical pathway (Figure 4c), two reagents **A** and **B** are combined quantitatively to yield the product, **C**. If the initial isotopic compositions of **A** and **B** are −25‰ and −30‰, respectively, and they contain four carbon atoms each, then by eq 18 the product **C** must be −27.5‰. However, if the next step of the reaction, the addition of **D**, is not quantitative, then the product **E** cannot be assumed to be a simple mass balance addition because the transformation of either reagent (**C** and/or **D**) may have an associated isotope effect. If there is no isotope effect, or if the reaction is quantitative and eq 18 applies, the answer would be **E′** (dotted line, Figure 4c). Yet here the product **E** is different from **E′**. If the reaction yield for each reagent is known, then $\varepsilon_{C/E}$ and $\varepsilon_{D/E}$ can each be determined (eq 17). The isotopic value of **E** depends on the fractional conversion of **C** to **E**, if there is an isotope effect associated with the reaction. Importantly, this difference would remain constant from batch to batch if the reaction has a constant yield efficiency for both reagents, a diagnostic indicator for the patentable process.

This stepwise approach to process analysis is progressively more powerful as the number of diagnosed steps increases: note that the addition of **F** to produce **G** again records an isotope effect for the observed reaction (point **G**) relative to the value predicted solely from isotope mass balance of the relevant reagents (**G′**). Input and yield parameters for all steps of Figure 4c are shown in Table 1.

2.2. Case Studies Litigated by MIT LLC. Stable isotopic approaches represent a powerful litigation tool to the legal community. Our firm, MIT LLC, was retained for its scientific expertise in three major product infringement lawsuits summarized here. Two of the lawsuits were decided in favor of plaintiffs asserting patent infringement against unauthorized

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drug manufacturers. In the third suit, isotopic arguments enabled a defendant to ward off an unfounded claim of patent infringement. We discuss each of these examples in more detail below and how they fit into the general principles of process patent protection.

2.2.1. Case 1, Nutraceutical False Advertising. The first example is one of isotopic provenance, or a *product study* (section 2.1.1). It involved a case of false advertising by a competitor selling a health supplement known as a nutraceutical. The product at the center of this dispute was the nonprotein amino acid, theanine, which is a popular supplement taken to improve memory and mental well-being. The natural source of theanine is almost exclusively from the plant family *Theaceae*, genus *Camellia*, i.e., tea.

The plaintiff had established a successful business manufacturing and selling synthetic theanine, which is produced inexpensively from corn or sugar cane-derived products. A competitor then entered the market and sought to gain market share by claiming to sell natural, superior theanine derived directly from green tea. The plaintiff suspected that the competitor's claims were false, because the very low price of the competitor's product was consistent with a synthetic source.

Carbon and nitrogen isotopic fingerprinting easily resolved the case in favor of the plaintiff. Synthetic theanine derived from corn products and aminated by synthetic (Haber–Bosch) nitrogen would be expected by first-principles to have a value of $\delta^{13}C$ near −12‰ and a value of $\delta^{15}N \leq 0$‰ (section 1.1.3.). The angiosperms (flowering plants) known as *Theaceae*, by contrast, are C_3 plants with expected values of $\delta^{13}C$ and $\delta^{15}N$ near −25‰ and >0‰, respectively.[27] Our analysis of samples obtained from the plaintiff, the competitor, and a verified natural source showed that the theanine of the plaintiff and the competitor were nearly isotopically identical (Figure 5). Importantly, the absolute values also were consistent with the biogeochemical predictions for synthetic (i.e., mineral fertilizer) amination of a C_4 plant source (either corn or sugar cane), versus extraction and purification from a natural C_3 (green tea) plant source. With that, it was impossible that the competitor's product could have resulted from a green tea source; it very likely derived from the aforenoted C_4 sources. Although a plausible hypothesis to validate for a biosynthetic pathway, the observed results were proposed, and the defendant settled the case based solely on the preceding product data. The case was resolved favorably for the plaintiff.

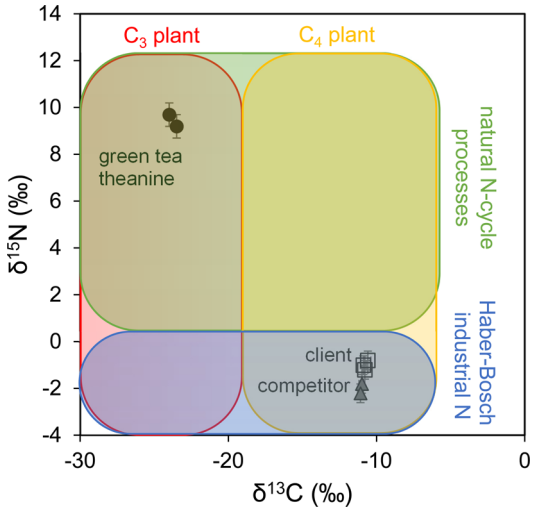

Figure 5. A bivariate plot of nitrogen ($\delta^{15}N$) and carbon ($\delta^{13}C$) isotopic results for an amino acid, theanine, derived from three sources: green tea, the client's product, and the competitor's product. Green tea is a C_3 plant with complex nitrogen sources; in contrast, both the client and competitor are manufacturing theanine from C_4 plant precursors (likely from corn) and Haber–Bosch process nitrogen.

2.2.2. Case 2, Small Molecule Antibiotic Product Infringement. The second example involved determining patterns of isotopic fractionation during stepwise chemical synthesis, i.e., a *process signature* study (section 2.1.2). It involved a case of process patent infringement brought by a plaintiff against a competitor who was selling a generic version of a small molecule antibiotic drug product (allowed, because the product is off composition of matter patent) that was suspected to be manufactured using the plaintiff's patented manufacturing process (not allowed, as the process was still under process patent).

To resolve this case, we established that the patented synthetic pathway would have a predictable isotopic fractionation for both C and N. In a bivariate plot, this translates to a vector of known direction and magnitude between reactants and products (Figure 6). The results of this case are proprietary, so neither the original data nor an accurate magnitude and direction of the vector can be disclosed—Figure 6 is an illustrative diagram. However, the principles shown are accurate: the drug has a specific and predictable isotope fractionation that is induced by the synthetic process. Although the patented manufacturing process comprises multiple steps (illustrated by the dashed vectors), when the drug is synthesized accordingly, it follows the same net fractionation (solid vector) regardless of the isotopic composition of the starting material [e.g., if starting with a C_3 carbon source (scenario 1) or with a petroleum-derived carbon source

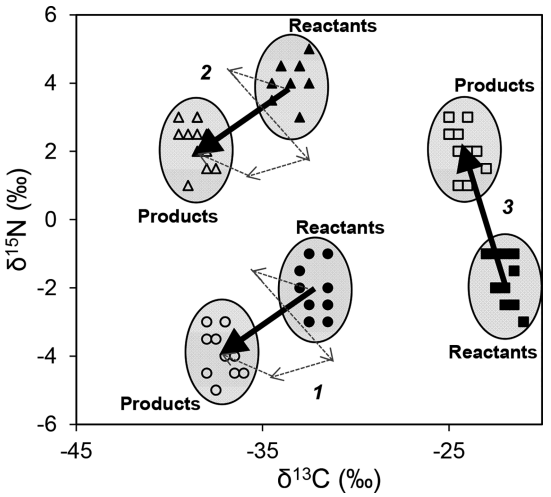

Figure 6. A bivariate plot of nitrogen ($\delta^{15}N$) and carbon ($\delta^{13}C$) isotopic results for a small molecule antibiotic produced by an authentic pathway (scheme 1), by an identical, and therefore infringing, generic pathway (scheme 2), and by a noninfringing generic pathway (scheme 3). The authentic pathway has a net "isotopic vector" (wide black arrows) or a sum of all individual fractionating processes (gray dashed arrows), of −5‰ in $\delta^{13}C$ and −2‰ in $\delta^{15}N$. The infringing generic pathway exhibits the same pattern. By contrast, the legal generic pathway has a distinct vector, −2‰ in $\delta^{13}C$ and +4‰ in $\delta^{15}N$.

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(scenario 2)]. Such scenarios might arise if the drug were produced at two different manufacturing sites with different suppliers of raw materials, but using the same process at both sites. In contrast, when we obtained reactant and product data for a known, noninfringing generic process for synthesizing the drug, we obtained a different overall vector, indicating the net fractionation between reactants and products changes when the steps of synthesis change (scenario 3).

Values of $\delta^{13}C$ and $\delta^{15}N$ were obtained for a key synthetic material and for the final drug for both the plaintiff's product and for the alleged infringer. The data for the defendant's product were consistent with scenario 1, strongly suggesting that the defendant was infringing on the patented process rather than using an alternative synthesis. Based on these data, the defendant settled and agreed to license the manufacturing process from the plaintiff.

2.2.3. Case 3, Human Drug Product Infringement: Wrongful Accusation. The third case also involved determining patterns of isotopic fractionation during stepwise chemical synthesis from an initial precursor, i.e., a *process signature* study (section 2.1.2). In this case, however, our work was for the defendant. MIT LLC was retained by a manufacturer of generic pharmaceuticals who was defending against what they believed to be wrongful accusation of process patent infringement by the major pharmaceutical company selling the branded product.

As in the previous case, we established the trajectory of the isotopic fractionation for synthesis of the generic product, relative to the branded product. However, in this case, the only element of focus was carbon. To establish a robust pattern of fractionation of both carbon and nitrogen isotopes, it was therefore necessary to analyze the steps of product synthesis (dashed vectors) individually, rather than simply the initial precursor → final product relationship (solid vector). We obtained samples of 9 intermediate products in the pathway of synthesis of the final active pharmaceutical ingredient (API), for both the plaintiff and the defendant and performed replicate $\delta^{13}C$ analyses for each (Figure 7). Because the two process profiles were not parallel, the isotopic trajectory showed that the defendant's product had not been manufactured by the patented process as the plaintiff alleged. Based on these results, the defendant was determined to be not guilty of patent infringement, and they were free to bring their product into a major international market.

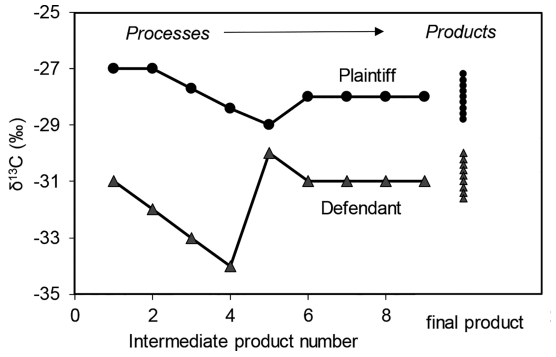

Figure 7. A case of noninfringement of a patented process to produce a pharmaceutical. The carbon ($\delta^{13}C$) isotopic records of two synthetic pathways were diagnosed by measuring reaction intermediates at multiple steps of synthesis. The pathways are not parallel, implying that the isotope effects experienced at each step are not equal. Different isotopic fractionation at *otherwise identical chemical intermediates* implies that the two synthesis *processes* are different.

3. SUMMARY AND OUTLOOK

3.1. Industrial Application and Potential. The pharmaceutical industry is an important segment of the world economy. In 2014, worldwide annual pharmaceutical sales reached $1 trillion and are expected to climb to $1.3 trillion by 2018.[45] These figures do not include over-the-counter medicines, nutritional products, or the raw materials and fine chemical intermediates used in manufacturing. With such huge markets at stake, companies face continuous threat of unauthorized sales of competing counterfeit and patent-infringing products; they have increasingly come to rely on their patent portfolios to protect against the importation of these unauthorized products. Naturally occurring molecular tracers, and process-analytical approaches to trace these fingerprints through the manufacturing chain, provide a powerful strategy to both prosecute and defend against infringement of these rights.

Our isotopic approaches are described in the references, spanning from isotopic analysis to isotopic synthesis.[3,5]

3.2. More Innovation on the Horizon. Looking to the future, we have expanded our technologies to include evaluation of naturally occurring isotopes in high molecular-weight and complex biological molecules. In recent studies presented at international meetings, we demonstrated the successful application of stable-isotopic measurements to biologic compounds in the 6000 to 150 000 Da range.[46] Such high molecular-weight materials can be more than 2 orders of magnitude larger than a typical drug compound and may contain complex mixtures of biologics. In principle, there is no practical upper limit to the size of molecules or complexity of mixtures to which stable isotopic process protection can be applied, but rather ancillary issues such as sample preparation and purification may present some challenges.

Additionally, we have preliminarily tested new technologies for continuously monitoring the progress of chemical and biological reaction processes; these may have a wide range of applications from monitoring the progress of a chemical reaction in a pilot plant to the large-scale production of commodity chemicals. These approaches take advantage of the Rayleigh fractionation principle (sections 1.2.5, 2.2.4) to determine the extent or efficiency of reaction yield (i.e., a direct index of a process). As an example, in the application of fermentation, it would likely be easier to indirectly monitor the yield of ethanol via the carbon-isotopic composition of off-gassed CO_2 as a continuous-process measurement. Such approaches utilize real-time, continuous isotopic monitoring, e.g., the relatively newly developed laser-based spectrophotometric methods (cavity ringdown spectrometer, CRDS[47,48]).

Finally, we also recently have shown the practicality of directed stable-isotopic synthesis of bio/pharmaceutical (or any other) products, known as Molecular Isotopic Engineering. By creating a quantitatively predefined isotopic composition, the approach yields an internal isotopic marker or fingerprint, which can be used, for example, as an in-place security feature, authenticity indicator, or batch-specific bar code for a manufactured compound and may represent a novel, isotopic composition of matter.[41]

3.3. Conclusion. Natural-abundance stable-isotopes ratios provide inherent tracers of product sources and processes. Isotope-ratio mass spectrometry (IRMS) is a proven method

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for the high-precision measurement of such isotope ratios. The unique isotopic fingerprints recorded by the synthetic pathways of all drugs record the histories of the genetic relationships between synthetic precursors and the resulting proprietary products. To demonstrate the utility of these approaches, we have illustrated three case histories of natural-abundance stable isotope ratios in process patent protection. Process patent protection adds value to and may plausibly transform the patent portfolios of the pharmaceutical and consumer products industries.

4. EXPERIMENTAL SECTION

4.1. Sample Composition. Clients provided samples of their proprietary compounds for stable-isotopic analysis. The average elemental composition of these APIs was necessary in advance of isotopic analysis to determine the mass of material needed. In some cases, general or specific structures of the compounds also were required to reveal critical details—e.g., the lability of certain atoms (e.g., H, O, etc.) to isotopic exchange.

4.2. Sampling Frequency. For a typical product study (section 2.1.1), stable-isotope ratios of C, N, H, and O were measured for every sample, with triplicate analysis conducted on approximately every fifth sample to assess analytical precision.[21] A study of 20 samples would thus require 112 measurements (i.e., 20 samples × 4 elements × 1.4 replication). A typical process study (sections 2.1.2 and 2.1.3) required substantially more (~3−10 times as many) analyses, as each element was measured for each individual reaction step, typically also including triplicates for determining precision. Samples typically were measured at commercial fee-for-service laboratories (e.g., www.isotechlabs.com; cost of individual analyses varying by sample type, but typically ~ $100 per measurement) and are always reported relative to accepted international reference materials.

4.3. Carbon and Nitrogen Isotopes. Individual solid samples of ca. 1 mg for $\delta^{13}C$ analysis (0.5 mg C from a 50% C w/w sample) and ca. 4 mg for $\delta^{15}N$ analysis (0.4 mg N from a 10% N w/w sample) were placed into tin capsules (e.g., Costech Analytical, part# 041061) and sealed by crimping. Isotopic analysis was performed on a Carlo Erba 1108 elemental analyzer interfaced to a Thermo Scientific Delta V isotope ratio mass spectrometer (EA-IRMS) (Figure 2a). The EA operated with an oxidation furnace temperature of 1020 °C, reduction furnace temperature of 650 °C, and a packed-column temperature of 70 °C.

4.4. Oxygen and Hydrogen Isotopes. Individual solid samples of ca. 0.3 mg for $\delta^{18}O$ analysis (0.1 mg O from a 30% O w/w sample) and ca. 2 mg for $\delta^{2}H$ analysis (0.2 mg H from a 10% H w/w sample) were weighed and placed into silver boats (e.g., Costech Analytical, part no. 041067) and sealed by crimping. Isotopic analysis was performed on a Finnigan thermal conversion/elemental analyzer interfaced to a Finnigan Delta V Plus isotope-ratio mass spectrometer (TCEA-IRMS) (Figure 2b). Analogous to an EA-IRMS, the TCEA converts samples to the gas phase by pyrolysis (at 1400 °C) instead of oxidative combustion, converting analytes to H_2 and CO, rather than H_2O and CO_2. Analyte gases are chromatographically separated on a packed column at 85 °C.

4.5. Estimates of Uncertainty. Analytical precision was determined from the whole sample suite for each element in each experiment; i.e., a representative standard deviation was generated from the whole raw data set in which small numbers of replicates (typically, $n = 3$) were pooled to generate a representative standard deviation (Jasper, 2001). Typical one sigma (1σ) standard deviations were: $\delta^{13}C$ (±0.08‰), $\delta^{15}N$ (±0.07‰), $\delta^{18}O$ (±0.15‰), and $\delta^{2}H$ (±3‰).

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Notes

The authors declare the following competing financial interest(s): J. P. Jasper holds US patents on product and process authenticity. He has pending US patent applications on biologic authentication, in-process analysis, and molecular isotopic engineering. A. D. Sabatelli and A. Pearson are both board members of MIT LLC and received compensation from MIT LLC for the preparation of this paper. MIT LLC is a client of Dilworth IP, LLC, the firm at which A.D. Sabatelli is a partner.

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This paper is dedicated to the memory of John M. Hayes who was a long-time board member of MIT LLC and who participated in all of the three cases presented here.

REFERENCES

(1) Cerling, T. E.; Barnette, J. E.; Bowen, G. J.; Chesson, L. A.; Ehleringer, J. R.; Remien, C. H.; Shea, P.; Tipple, B. J.; West, J. B. Forensic stable isotope biogeochemistry. *Annu. Rev. Earth Planet. Sci.* **2016**, *44*, 175−206.

(2) Ehlringer, J. R.; Matheson, S. M., Jr. Stable isotopes and the courts. *Utah Law Rev.* **2010**, *2*, 386−442.

(3) Jasper, J. P.; Weaner, L. E.; Hayes, J. M. Process Patent Protection: Characterizing synthetic pathways by stable-isotopic measurements. *Pharm. Technol.* **2007**, *31* (3), 68−73.

(4) Deconinck, E.; van Nederkassel, A. M.; Stanimirova, I.; Daszykowski, M.; Bensaid, F.; Lees, M.; Martin, G. J.; Desmurs, J. R.; Smeyers-Verbeke, J.; Vander Heyden, Y. Isotopic ratios to detect infringements of patents or proprietary processes of pharmaceuticals: two case studies. *J. Pharm. Biomed. Anal.* **2008**, *48* (1), 27−41.

(5) Jasper, J. P.; Westenberger, B. J.; Spencer, J. A.; Buhse, L. F.; Nasr, M. Stable isotopic characterization of active pharmaceutical ingredients. *J. Pharm. Biomed. Anal.* **2004**, *35*, 21−30.

(6) Jasper, J. P.; Lyon, R. C.; Weaner, L. E. Stable isotopes provide a new PAT [Process Analytical Technology] tool. *Pharm. Mfg.* **2005**, *4* (5), 28−33.

(7) Wokovich, A. M.; Spencer, J. A.; Westenberger, B. J.; Buhse, L. F.; Jasper, J. P. Stable isotopic composition of the active pharmaceutical ingredient (API) naproxen. *J. Pharm. Biomed. Anal.* **2005**, *38*, 781−784.

(8) Kirchhoefer, R. D. An FDA laboratory approach to uncovering potential fraud in the generic drug industry. *J. AOAC Int.* **1992**, *75*, 577−580.

(9) Gaudiano, M. C.; Antoniella, E.; Bertocchi, P.; Valvo. Development and validation of a reversed-phase LC method for analysing potentially counterfeit antimalarial medicines. *J. Pharm. Biomed. Anal.* **2006**, *42*, 132−135.

(10) Miller, T. R. Patented compounds inherently coproduced as trace impurities: Issues of inherent anticipations and literal infringement. *Am. Int. Prop. Law Assoc. Quat. Journal* **2004**, *32* (3), 425.

(11) Jasper, J. P.; Pavane, M.; Eyler, D.; Sharma, I.; Lee, A. Process Patent Protection: Protecting intellectual property via natural-abundance stable isotopes. *Am. Intel. Prpty. Law. Assoc.* webinar (November 13, 2012).

DOI: 10.1021/acs.oprd.7b00069
Org. Process Res. Dev. XXXX, XXX, XXX−XXX

(12) Sabatelli, A. D. Nature's indelible marker. *Pharm. Mfg.* **2014**, 15−18.
(13) Hayes, J. M. Factors controlling ^{13}C contents of sedimentary organic compounds: Principles and evidence. *Mar. Geol.* **1993**, *113*, 111−125.
(14) Urey, H. C. Oxygen isotopes in nature and in the laboratory. *Science* **1948**, *108*, 489−496.
(15) McKinney, C. R.; McCrea, J. M.; Epstein, S.; Allen, H. A.; Urey, H. C. Improvements in mass spectrometers for the measurement of small differences in isotope abundance ratios. *Rev. Sci. Instrum.* **1950**, *21*, 724−730.
(16) Urey, H. C.; Lowenstam, H. A.; Epstein, S.; McKinney, C. R. Measurement of paleotemperatures and temperatures of the Upper Cretaceous of England, Denmark, and the Southeastern United States. *Geol. Soc. Am. Bull.* **1951**, *62*, 399−416.
(17) Coplen, T. B.; Kendall, C.; Hopple, J. Comparison of stable isotope reference samples. *Nature* **1983**, *302*, 236−238.
(18) De Groot, P. A. *Handbook of Stable Isotope Analytical Techniques*; Elsevier: Amsterdam, 2004.
(19) Fry, B.; Brand, W.; Mersch, F. J.; Tholke, R. Automated analysis system for coupled $\delta^{13}C$ and $\delta^{15}N$ measurements. *Anal. Chem.* **1992**, *64*, 288−291.
(20) Hilkert, A. W.; Douthitt, C. B.; Schlüter, H. J.; Brand, W. A. Isotope ratio monitoring gas chromatography/mass spectrometry of D/H by high temperature conversion isotope ratio mass spectrometry. *Rapid Commun. Mass Spectrom.* **1999**, *13*, 1226−1230.
(21) Jasper, J. P. Quantitative estimates of precision for molecular isotopic measurements. *Rapid Commun. Mass Spectrom.* **2001**, *15*, 1554−1557.
(22) Smith, B. N.; Epstein, S. Two categories of $^{13}C/^{12}C$ ratios for higher plants. *Plant Physiol.* **1971**, *47*, 380−384.
(23) Fogel, M. L. Variations in abundances of nitrogen isotopes in nature. In *Encyclopedia of Mass Spectrometry*, Vol. *5*; Elsevier: Oxford, UK, 2010.
(24) Calvin, M.; Benson, A. A. The path of carbon in photosynthesis. *Science* **1948**, *107*, 476−480.
(25) Hatch, M. D.; Slack, C. R.; Johnson, H. S. Further studies on a new pathway of photosynthetic CO_2 fixation in sugarcane and its occurrence in other plant species. *Biochem. J.* **1967**, *102*, 417−422.
(26) Tipple, B. J.; Pagani, M. The early origins of terrestrial C_4 photosynthesis. *Annu. Rev. Earth Planet. Sci.* **2007**, *35*, 435−461.
(27) Cerling, T. E.; Harris, J. M. Carbon isotope fractionation between diet and bioapatite in ungulate mammals and implications for ecological and paleoecological studies. *Oecologia* **1999**, *120*, 347−63.
(28) Peters, K. E.; Walters, C. C.; Moldowan, J. M. *The Biomarker Guide*, Vol. *1*: Biomarkers and Isotopes in the Environment and Human History; Cambridge University Press, 2005.
(29) Whiticar, M. J. Carbon and hydrogen isotope systematics of bacterial formation and oxidation of methane. *Chem. Geol.* **1999**, *161*, 291−314.
(30) Craig, H. Isotopic variations in meteoric waters. *Science* **1961**, *133*, 1702−1703.
(31) Criss, R. E. *Principles of Isotope Distributionl* Oxford University Press, 1999.
(32) Sigman, D. M.; Casciotti, K. L. *Nitrogen isotopes in the ocean, in Encyclopedia of Ocean Sciences*; Steele, J. H., Turekian, K. K., Thorpe, S. A., Eds.; Academic Press: London, 2001; p 2449.
(33) Wankel, S. D.; Chen, Y.; Kendall, C.; Post, A. F.; Paytan, A. Sources of aerosol nitrate to the Gulf of Aqaba: Evidence from $\delta^{15}N$ and $\delta^{18}O$ of nitrate and trace metal chemistry. *Mar. Chem.* **2010**, *120*, 90−99.
(34) Jasper, J. P. The increasing use of stable isotopes in the pharmaceutical industry. *Pharm. Technol.* **1999**, *23* (10), 106−114.
(35) Sessions, A. L.; Sylva, S. P.; Summons, R. E.; Hayes, J. M. Isotopic exchange of carbon-bound hydrogen over geologic timescales. *Geochim. Cosmochim. Acta* **2004**, *68*, 1545−1559.
(36) Dias, R. F.; Freeman, K. H.; Lewan, M. D.; Franks, S. G. delta C-13 of low molecular-weight organic acids generated by the hydrous pyrolysis of oil-prone source rocks. *Geochim. Cosmochim. Acta* **2002**, *66*, 2755−2769.
(37) Deniro, M. J.; Epstein, S. Mechanism of carbon isotope fractionation associated with lipid synthesis. *Science* **1977**, *197*, 261−263.
(38) Monson, K. D.; Hayes, J. M. Carbon isotopic fractionation in the biosynthesis of bacterial fatty acids − ozonolysis of unsaturated fatty acids as a means of determining the intramolecular distribution of carbon isotopes. *Geochim. Cosmochim. Acta* **1982**, *46*, 139−149.
(39) Casciotti, K. L. Inverse kinetic isotope fractionation during bacterial nitrite oxidation. *Geochim. Cosmochim. Acta* **2009**, *73*, 2061−2076.
(40) Scott, K. M.; Lu, X.; Cavanaugh, C. M.; Liu, J. Optimal methods for estimating kinetic isotope effects from different forms of the Rayleigh distillation equation. *Geochim. Cosmochim. Acta* **2004**, *68*, 433−442.
(41) Jasper, J. P.; Farina, P.; Pearson, A.; Mezes, P. S.; Sabatelli, A. D. Evaluating practical uses of molecular isotopic engineering. *Pharm. Technol.* **2016**, *40* (7), 34−45.
(42) Bricout, J. Control of authenticity of fruit juices by isotopic analysis. *J. Assoc. Anal. Chem.* **1973**, *56*, 739−42.
(43) Sturchio, N. C.; Böhlke, J. K.; Beloso, A. D.; Streger, S. H.; Heraty, L. J.; Hatzinger, P. B. Oxygen and chlorine isotopic fractionation during perchlorate biodegradation: laboratory results and implications for forensics and natural attenuation studies. *Environ. Sci. Technol.* **2007**, *41*, 2796−2802.
(44) Hansen, A. M. S.; Fromberg, A.; Frandsen, H. L. Authenticity and traceability of vanilla flavors by analysis of stable isotopes of carbon and hydrogen. *J. Agric. Food Chem.* **2014**, *62*, 10326−31.
(45) Miller, P.; Mehta, J. http://thomsonreuters.com/en/articles/2015/global-pharma-sales-reach-above-1-trillion.html. 2015 CMR International Pharmaceutical Summary R&D Executive Summary.
(46) Jasper, J. P.; Eyler, D.; Pavane, M. Protecting bio/pharmaceutical intellectual property via natural-abundance stable isotopes. Abstract O6, *29th North America Chapter Meeting of the International Isotope Society*, Langhorne, PA, USA, Oct 20−21, 2016.
(47) Paldus, B. A.; Kachanov, A. A. Spectroscopic Techniques: Cavity-enhanced methods. *Atomic, Molecular, and Optical Physics Handbook, Part C: Molecules*; Drake, G. W., Ed.; Springer: Berlin, 2004; pp 621−640.
(48) Paldus, B. A.; Kachanov, A. A. An historical overview of cavity enhanced methods (Einstein centennial review article). *Can. J. Phys.* **2005**, *83*, 975−999.

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