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Mini review

Non-statistical isotope fractionation as a novel "retro-biosynthetic" approach to understanding alkaloid metabolic pathways

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ABSTRACT

During the biosynthesis of natural products, isotope fractionation occurs due to the selectivity of enzymes for the heavier or lighter isotopomers. As only some of the positions in the molecule are implicated in a given reaction mechanism, position-specific fractionation occurs. Thus, the position-specific $^{13}C/^{12}C$ ratios in these compounds can be related to their known precursors and to the known isotope effects of enzymes involved in their biosynthesis, or similar reaction mechanisms. This can be accessed by isotope ratio monitoring NMR spectrometry. In this short review, how isotope fractionation occurs and when it is manifest is described. Then, the way that ^{13}C NMR spectrometry has been applied to study certain aspects of the biosynthesis of the solanaceous alkaloids *S*-(−)-nicotine and tropine is outlined. Notably, it is shown how similar isotope fractionation is found in the steps of the pathway to the common intermediate, *N*-methyl-Δ^1-pyrrolinium, but that in the moieties derived from different origins no such similarity is found, the isotopic composition of these atoms reflecting their specific metabolic ancestry. In a second example, tramadol, it is shown how this technique can be used in retro-biosynthesis to give direction as to what precursors and pathway intermediates are probable. It is shown how the observed fractionation in the site-specific $^{13}C/^{12}C$ ratios can be effectively explained by known metabolism and the properties of enzymes proposed for the pathway. Furthermore, it can give indications of possible mechanisms of those enzymes that are as yet to be described for a number of key steps.

Contents

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1. Introduction

1.1. Causes of isotope fractionation in natural products

Isotope fractionation[1] is the degree of selection of one isotope over another and, in the example of carbon, causes variation in the $^{13}C/^{12}C$ ratios. It occurs most prevalently in open systems under natural conditions. As biological systems can be described as open, ultimately unidirectional, and irreversible, isotope fractionation can substantially contribute to the formation of isotopic patterns and the degree of variation will reflect the processes involved (Schmidt et al., 2015). It takes an incomplete or branched substrate conversion to cause an isotope discrimination[1] *in vivo*. To maintain an isotopic balance, the isotope shifts in the alternative products need to be mutually counteractive, i.e. to be opposed and to be in relation to their chemical yields (Schmidt, 2003). However, isotope discrimination *in vivo* within a metabolic network will not be manifest by a non-branched and quantitative process, despite the fact that the process by which it is carried out (e.g. catalysed by an enzyme), may indicate *in vitro* kinetic and/or equilibrium isotope effects.[1]

The most common classification of isotope effects (IEs) is division into kinetic or thermodynamic but, in relation to studying biological processes, the real distinction is between non-equilibrium (KIE) and equilibrium (EIE) situations. The balance of two KIEs at chemical equilibrium is represented by thermodynamic effects, which are generally smaller than individual kinetic effects. ^{13}C KIEs (i.e. a discrimination that alters the $^{13}C/^{12}C$ ratio) are expressed during the formation and fission of bonds that involve a carbon atom. However, KIEs and EIEs can also be manifest in other processes, such as diffusion or volatilisation, which are also affected by mass.

As a relatively simple example of a non-reactional isotope effect, the difference between the binary diffusivity of $^{13}CO_2$ and that of $^{12}CO_2$ in air can be given. The overall abundance of ^{13}C in atmospheric CO_2 is greater than in plant tissues, which gives an indication that carbon isotope discrimination occurs in the incorporation of CO_2 into plant biomass. This has a major impact on the $^{13}C/^{12}C$ ratio of natural metabolites from different plant species, since the diffusion pathway for CO_2 from the ambient to the site of fixation can differ in different types of assimilatory tissue and is influenced by the degree of opening of the stomata (Farquhar et al., 1989a,b). In addition, CO_2 assimilation is influenced by reaction-related IEs: the difference between the kinetic constants for the reaction of $^{12}CO_2$ and $^{13}CO_2$ with the assimilatory enzymes, ribulose bisphosphate carboxylase-oxygenase (RuBisCO, EC 4.1.1.39) and phosphenolpyruvate carboxylase (EC 4.1.1.31) (Tcherkez and Farquhar, 2005; Tcherkez et al., 2006). The former of these enzymes discriminates against the heavier isotope by about 25–30‰, the latter only by about 2‰. However phosphenolpyruvate carboxylase uses HCO_3^- as substrate, which is enriched in ^{13}C due to the EIE of the interconversion of CO_2 and HCO_3^-. The degree of enrichment in ^{13}C relative to atmospheric CO_2 depends on the extent to which diffusion through the stomata limits the reaction rate, but is typically of the order of 8‰. The impact of these processes–diffusion, equilibration and assimilation–on the $^{13}C/^{12}C$ ratios of plant metabolites is fundamental and is a means of distinguishing between those of C_3 metabolism, C_4 metabolism or Crassulacean acid metabolism (CAM).

1.2. Isotope fractionation in natural products

Post-photosynthetic metabolism leads to further modifications in the $^{13}C/^{12}C$ ratios and these are necessarily consistent with a thermodynamic order. Furthermore, they vary depending on the organism. For example, in specialised metabolites such as terpenes (Martin et al., 2004) and phenylpropanoids (Schmidt et al., 2006), pathway-dependent ^{2}H and ^{13}C patterns are observed, yet thermodynamic considerations predict for these pathways an absolute independence of isotopic patterns. Reaction mechanisms and KIEs are thus combining to create a reproducible and potentially predictable 'order' in metabolism. Hence, metabolite isotopic patterns of 'transient' molecules (intermediates) are often preserved in 'permanent' (sink) products. If a given product can be synthesized by several pathways, differing isotopic patterns are likely to result. A parallel relative depletion of the heavy isotopes for carbon and hydrogen is not necessarily observed in metabolic steps or chains. This effect occurs only occasionally, because the involved reactions and KIEs strongly differ from each other.

Extrinsic (pools of primary precursors, temperature, and pressure) and intrinsic factors (metabolite pools, alternative pathways, turnover rates, formation and fission of bonds) are modulating the *in vivo* effectiveness of thermodynamic and kinetic IEs on enzyme-catalysed reactions (expressed as isotope discrimination). The assignment of alternative/competing biochemical pathways can be achieved through a proper understanding of the *in vivo* isotope discrimination and application of this knowledge to the intrinsic and extrinsic conditions of the biosynthetic origin of natural products (Schmidt, 2003). In the enzyme-catalysed reactions involved in metabolism, the KIEs can only manifest themselves as an isotope fractionation (*in vivo*) in cases of incomplete substrate conversion. Thus, when the substrate is converted into two or more products by metabolic branching (Hayes, 2004), then the substrate can become enriched or depleted in the heavier isotopomer[2] by a dominant branching route: as a result, the isotope shift values are inverted in the other branch, as required to maintain an isotopic balance. Hence, isotope discriminations coupled with irreversible or unidirectional processes permit KIEs to come into play and this leads to isotope fractionation. When the isotope shifts in the products are following an isotopic balance (e.g. incomplete turnover or metabolic branching events, rate-limiting reactions, or an isotope-sensitive step in a reaction mechanism), KIEs linked with them can become effective *in vivo*. If, from a common precursor pool, several reactions are competing with each other, one or more of them may achieve *in vivo* isotope discrimination, irrespective of the *in vitro* KIEs of each pathway.

1.3. Overall effect of position-specific fractionation in carbon on isotope patterns

In natural organic compounds the backbone is a chain of carbon atoms. The formation and fission of carbon-carbon bonds and carbon-hydrogen bonds are the major processes that are occurring during anabolic and catabolic reactions in plants. The last product of a (reductive) synthesis chain is most commonly the one which is the most depleted in heavy atoms of carbon and hydrogen, taking into consideration the assumption that KIEs are implicated in the steps in the biosynthesis, and that this is associated with metabolic branching. In general, due to isotope fractionation in plant

[1] **Isotope discrimination** is a general observation that a process selects for one isotope in preference to the other. **Isotope fractionation** is the observed difference between a given compound and another compound (usually derived from it) and may refer to a process or to a whole series of processes (e.g. a metabolic pathway). **Isotope effect** is the intrinsic selectivity for/against heavy isotopes of a catalyst, in this context, an enzyme.

[2] Isotopomers are molecules that have the same number of each isotope of a given element but with the heavy isotope in different positions.

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