Lab. d'Ecologie, Systématique et Evolution
Université Paris-Sud, Paris-Saclay, CNRS, UMR 8079,
Bâtiment 362 - 91400 Orsay, France
http://www.ese.u-psud.fr

Orsay, November 16th, 2016

Report on the PhD Thesis of Katarzyna M. Romek

The PhD Thesis of Katarzyna Romek deals with "Studies of isotopic fractionation of ^{13}C during biosynthesis and enzymatic reactions". This is a very interesting research topic and the NMR methodology used to determine the position-specific ^{13}C distribution within different metabolites is relevant for biosynthetic pathways for a range of natural and synthetic compounds. The methodology used opens new insights in plant (eco)physiological studies for understanding the physiological functioning of different plants/organs under varying environmental conditions. I was impressed by the scientific quality and the huge amount of theoretical and experimental work done by Katarzyna Romek as well as the analysis of the literature and of her results.

This PhD Thesis document is composed of six Chapters. **Chapter I** is a one-page relevant summary of the objectives, methods as well as the results. **Chapter II** is a valuable introduction to the basics on stable isotopes and isotopic fractionation in natural products, including definitions, descriptions and calculations of related parameters, and mechanisms of both kinetic and thermodynamic isotope effects. Carbon isotope fractionation during primary carbon metabolism as well as fractionations during post-photosynthetic metabolism, mainly during biosynthetic pathways are also described. This Chapter also contains detailed methodologies/protocols used, i.e. IRMS (for measuring the $\delta^{13}C$ of the whole molecules) and NMR (for determination of intra-molecular ^{13}C patterns).

As underlined by Katarzyna, the initial work of Rossmann et al (1991) on the intra-molecular ^{13}C distribution in glucose molecules using the chemical degradation of the molecule opened the way to new concepts of observing isotope fractionation in metabolic pathways. This indeed is demonstrated by the recent progress in the use of NMR technology to analyse ^{13}C patterns in different natural compounds by the group of Richard Robins in Nantes University, including the PhD work of Katarzyna, with even more novelty in methodological approaches.

Chapter III deals with the ^{13}C distribution in amino acids starting with an overview of different metabolic pathways leading to the synthesis of different categories of amino acids. The amino acids selected for intra-molecular ^{13}C distribution analyses (i.e. alanine, valine, serine, isoleucine, methionine, glutamic acid, and tyrosine) are representatives of different biosynthetic pathways, clearly presented by metabolic schemes. They are chosen as examples for a hydroxyl, an amine, a carboxylic acid, a phenolic or an S-methyl function, thus presenting a wide range of structural properties as suitable test group for tracking isotopic variation in relation to biosynthesis. Indeed, the understanding of the causes of intra-molecular isotopic fractionation during the processes of amino acids metabolism is useful for elucidation of biosynthetic pathways in plants. Differential partitioning of metabolic fluxes at branching points also influences the isotopic composition of metabolites as well as the intra-molecular isotopic patterns. This is thus a relevant work undertaken by Katarzyna.

Since amino acids are structurally not amenable to ^{13}C-NMR (because poorly soluble in polar solvents) she developed a strategy for derivatization of the carboxyl function rending the amino acid less polar and more soluble in organic solvents with good properties for NMR spectroscopy. The methylation, solvent choice and NMR methodology and spectral acquisition are described and the isotopic composition of the whole molecule as well as intra-molecular ^{13}C pattern for each of them are presented and enzymatic reactions with corresponding (potential) isotope fractionations leading to such patterns are discussed.

The amino acids used have animal protein origin (indicated by the suppliers). However, their ^{13}C isotope compositions measured by IRMS indicate if animals were fed by C3 or C4 plants. Interestingly, huge position-specific ^{13}C difference within each amino acid molecule is observed, e.g. for valine (with C3 plant imprint), the intra-molecular variation is up to about 30‰ between C=O and CH3 functions. As expected, ^{13}C enriched values for C=O group compared to CH2 is observed for isoleucine, glutamic acid and tyrosine. But surprisingly, serine and valine do not correspond to this pattern, with C=O highly impoverished being around -54‰ in valine (the whole molecule being around -32‰). The origins (precursors) and the biosynthesis pathways and corresponding isotope effects, which could explain these intra-molecular ^{13}C distribution in different amino acids studied are discussed. A good example is the similar ^{13}C pattern observed for tyrosine and phenylpropanoid ferulic acid. These two compounds have the same origin (the shikimate pathway) and clearly the pattern of the precursor is transferred to these two different compounds.

Chapter IV deals with the isotopic fractionation in methionine biosynthesis and is well documented on this sulphur containing amino acid, which has a central position in cell metabolism. Its role in plant metabolism, and the enzymes involved in its biosynthesis pathway are described, as well as the origin of the highly heterogeneous intra-molecular ^{13}C distribution observed (strong ^{13}C depletion in methyl group, due to isotope fractionation during methyl transfer) are discussed.

One of the enzymes involved in methionine biosynthesis was selected, and Katarzyna used the 3-dimentional structure of this enzymes available in the Protein Data Bank to build up different models of the possible reaction mechanism of the enzyme using theoretical calculations. She used the cobalamin-independent methionine synthase expressed in *E. coli* for starting the structure for simulation. She describes the methodology and analyses the results, which show a good agreement between the experimental data (she obtained by NMR and IRMS) for kinetic isotope effect for methyl group transfer in the formation of methionine and the values predicted by her theoretical model. Her experimental data together with calculations strongly support the hypothesis that the observed ^{13}C depletion in methyl groups in natural products can be tracked back to the formation of methionine by this enzyme.

Chapter V deals with the isotopic fractionation in natural alkaloids, which are of great importance as specialized metabolites, many of them are pharmacologically active and some have been used for medicinal purposes since several thousands of years. In most cases, their heterocyclic nitrogen originates from amino acids and they are classified on the basis of their ring system. She gives an interesting introduction on some important alkaloids, and then presents the nice work she conducted on nicotine, tropine and tramadol.

Since the enzymology approach of conversion of the precursors (with N-methyl group) to tropine and nicotine remains unknown despite many efforts, she adopted an alternative “retro-biosynthesis” approach involving the measurements and interpretation of the natural fractionation in ^{13}C (and ^{2}H) isotopes during the course of biosynthesis. Indeed, during the metabolic processes by which the natural products are biosynthesised, kinetic isotopic fractionations occur. As a result, the position specific isotope ratio in natural products is non-

statistically distributed. Therefore, the position-specific $^{13}C/^{12}C$ ratios are of special interest because they reflect the carbon skeleton(s) already present in the precursor(s) and the biochemical processing that each position has undergone at different steps in the pathway. The study of the position-specific $^{13}C/^{12}C$ ratios should help thus to interpret the observed values of the known biochemistry. This approach has been proved to be remarkably accurate for some primary metabolites.

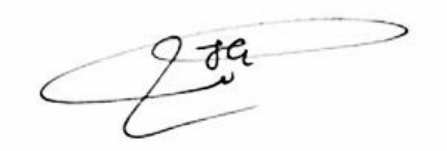
She thus realised the "retro-biosynthesis" of nicotine and tropine using the precursors and compared the position-specific $^{13}C/^{12}C$ ratios obtained to those measured on the products from natural origin. Interestingly, the ^{13}C-NMR spectral analysis showed quite similar results for both natural products and those obtained by "retro-biosynthesis" in both alkaloids. Then she discusses in detail the potential isotope effects during the pathway leading to such position-specific ^{13}C- distribution (for each position) and to which extent they are determined by the precursor molecules. Similar work is also presented for tramadol. This novel approach (retro-biosynthesis) combined with NMR analysis is of great relevance for understanding the biosynthetic pathways of different metabolites.

Chapter VI synthesises the main findings with conclusions on the power and relevance of the methodologies/approaches used (^{13}C-NMR, retro-biosynthesis, theoretical calculations) used with an example for each of them and proposes some perspectives for future work.

Although Katarzyna has already published two papers on her PhD results in highly ranking international journals (PNAS and the Journal of Biological Chemistry, both as first author), her Thesis has a great merit to be a complete PhD document including different chapters on the whole work she has conducted (rather than a compilation of papers). The Thesis is well documented and well written and it will be very useful for students and researchers who will continue the work on intra-molecular isotope distribution understanding and methods. Although the cited references are listed at the end of the document, I much appreciated the corresponding references of each page listed as footnotes. This is a beautiful contribution to both scientific and technical progress in this domain. I suggest to add the papers as annexes to this document. She is also the co-author of 2 other papers in similar subject (published in RCMS and Amino Acids).

On the basis of the above mentioned highlights of the tremendous work (both scientifically and methodologically) she has conducted and presented in her Thesis document as well as in her papers, which are of great importance for understanding the isotope fractionation mechanisms of enzymes involved in intra-molecular ^{13}C distribution and the associated metabolic pathways, and which open new avenues for plant physiological studies too, I wholly agree that Katarzyna Romek can defend her PhD Thesis on the 9th December 2016, as foreseen, and that it's an honour for me to take part of her PhD committee and Thesis Jury.

Sincerely,



Jaleh Ghashghaie (Professor)
Phone : +33(0)1-69-15-63-59
E-mail : jaleh.ghashghaie@u-psud.fr