Biosynthesis of salvinorin A

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Abstract. *Salvia divinorum* plants were exposed to a synthetic atmosphere containing 600 ppm
$^{13}$CO$_2$ for 4 hours with white light illumination and were subsequently cultured for a chase period
of 10 days under standard greenhouse conditions. Salvinorin A was obtained by solvent
extraction of the leaves and was purified chromatographically. The isotopologue composition of
salvinorin A was determined by NMR spectroscopy. The presence of multiply $^{13}$C-labeled
isotopologues in considerable excess over their natural occurrence showed that the $^{13}$C labeling
pulse had afforded multiply $^{13}$C-labeled biosynthetic. Consecutive catabolic and anabolic
reactions during the chase period afforded salvinorin A that was a mosaic revealing its origin
from a mixture of labeled and unlabeled precursors. The presence of triple labeled isotopologues
demonstrated that the IPP and DMAPP precursors of the diterpene, salvinorin A, were
predominantly biosynthesized via the recently discovered non-mevalonate pathway.

1. Introduction

Green technology is moving from an option to a must in modern industrial processing. Solvents are the
core of the food, pharmaceutical, cosmetic, agrochemical, chemical, and biotechnological process
technologies [1, 2, 3].

Metabolic pathways can be studied in plants using stable-isotope-labelled precursors followed by a
NMR-based analysis of labelling patterns. Very general carbon-13 labelled precursors, such as acetate,
glucose or carbon dioxide, are applied to growing plant cells or even whole plants to study incorporation
into the compounds of interest.

The label of the general precursor is diverted through the metabolic network by the reaction of the
central metabolism, such as glycolysis, the pentose phosphate cycle or citrate cycle, and finally results
in specific labelling patterns of metabolic products such as amino acids or secondary metabolites.

Starting from general $^{13}$C-labeled feed supplements (e.g. [U-$^{13}$C$_6$]glucose), the distribution of label
throughout the metabolic network of an organism is monitored by quantitative NMR spectroscopy.
NMR spectroscopy is characterized by high positional resolution but low sensitivity. On this basis, NMR
signals of amino acids from a labelling experiment can be assigned and quantified. Based on the
isotopologue abundances of target compounds (for example, secondary metabolites) and certain hubs from
the metabolic network (reconstructed by retrobiosynthetic analysis of amino acids) the biosynthetic
history of each compound under study can be assessed. This methodological concept is illustrated with
the determination of carbohydrate flux during starch biosynthesis in developing maize kernels.
Surprisingly, high rates of metabolic cycling of glucose were observed prior to its conversion into starch.

Isotopolog profiling was also used to quantitatively delineate the biosynthetic origin of the basic
building blocks of terpenoids, isopentenyl diphosphate and dimethylallyl diphosphate. Using plant cell
cultures, plant organs or whole plants it was found that the vast majority of plant terpenoids are derived via 1-deoxyxylulose 5-phosphate. Isotopolog editing was crucial to determine the functions of the enzymes involved in this basic metabolic pathway. Therefore, the enzymes of the pathway are attractive targets for the development of novel antibiotics, antimalarials and herbicides.

Stable isotopes play a central role in the elucidation of metabolic pathways. Historically, isotope incorporation studies were interpreted as if metabolism proceeds in a linear and unidirectional fashion. However, metabolism is a complex network, and metabolic flux can occur from any node in the network to virtually any other node. This fact is taken into account by an experimental approach which is based on perturbation/relaxation of isotopolog compositions in living organisms [4, 5].

With the goal of phytochemistry at hand, namely that it shall afford a functional description of the chemistry and biochemistry in plants, NMR contributes to a concise analysis and structure determination of metabolites. On the other hand, mass spectrometry is very sensitive, but is hampered by low positional resolution of isotopologue determination. Since NMR and MS provide complementary information, both methods are used for isotopologue profiling.

In addition, metabolite flux analysis based on NMR spectroscopy and mass spectrometry becomes more and more important. With this in mind, it is immediately obvious that NMR is a key technology that enables a better understanding of plant chemistry and biology.

Very general carbon-13 labelled precursors, such as acetate, glucose or carbon dioxide, are applied to growing plant cells or even whole plants to study incorporation into the compounds of interest.

The label of the general precursor is diverted through the metabolic network by the reaction of the central metabolism, such as glycolysis, the pentose phosphate cycle or citrate cycle, and finally results in specific labelling patterns of metabolic products such as amino acids or secondary metabolites [1, 8].

One of the key questions in metabolic phytochemistry is what happens under physiological conditions. In order to address this question we have performed labelling experiments with whole plants using carbon-13 labelled CO₂ as carbon source in a closed chamber as shown on the left of the slide. In this approach, the plants are incubated in an atmosphere containing C-13 CO₂.

During this pulse period of feeding, totally carbon-13 labelled intermediates are formed. After the pulse feeding, the plants are taken out off the chamber and cultivated under standard greenhouse conditions with unlabelled CO₂. During this chase period unlabelled intermediates are formed.

The result of pulse and chase period is a mixture of totally labelled carbohydrates and unlabelled carbohydrates.

2. Conclusion

As an example of understanding of biochemical fluxes in living organisms is a Mexican plant *Salvia divinorum* L. The plant has not been well-studied in high-quality clinical data, little is known about its toxicology and pharmacology activity or safety over long-term consumption.

*Salvia divinorum* (Labiatae) is a psychoactive plant that has been used in traditional spiritual practices by the Mazatec Indians of Oaxaca, Mexico for many centuries. The main active ingredient of *S. divinorum* is a neoclerodane diterpene salvinorin A (figure1).

![Figure 1. Structure of salvinorin A.](image-url)
This compound has been identified as an extremely potent and highly selective kappa-opioid receptor agonist [3].

The biosynthesis of salvinorin A previously was studied by labelling experiments with [1-\textsuperscript{13}C]glucose, [Me\textsuperscript{13}C]methionine, and [1,4,13C; 3,4,2-H\textsubscript{2}]-1-deoxy-D-xylulose. NMR spectroscopic analysis indicated that salvinorin A is biosynthesized via 1-deoxy-D-xylulose-5-phosphate pathway [6].

In order to address the question: what happens under physiological conditions, we have performed labelling experiments with whole plants using carbon-13 labelled CO\textsubscript{2} as carbon source in a closed chamber. In this approach, the plants are incubated in an atmosphere containing C\textsubscript{13}O\textsubscript{2}. During this pulse period of feeding, totally carbon-13 labelled intermediates such as sugars are formed.

After the pulse feeding, the plants are taken out of the chamber and cultivated under standard greenhouse conditions with unlabelled CO\textsubscript{2}. During this chase period unlabelled intermediates are formed.

The result of pulse and chase period is then a mixture of totally labelled carbohydrates and unlabelled carbohydrates, which give rise of specific labelling profiles in all downstream products, such as secondary metabolites [7].

The relative intensities of the satellite signals were well above those characteristic of the natural abundance (1.1% for each adjacent \textsuperscript{13}C atom in natural abundance material). The relative integrals of the satellite signals afforded relative abundances of the multiple \textsuperscript{13}C-labeled isotopologs.

Some of the coupling satellites showed a fine structure caused by \textsuperscript{13}C\textsuperscript{13}C coupling via two or three carbon bonds. From the relative intensities, the abundances of these specimens can also be calculated. Triple-labelled isotopologs are shown as filled squares and arrows connecting \textsuperscript{13}C atoms within the same molecule.

In our experiments, \textit{Salvia divinorum} plants were exposed to a synthetic atmosphere containing 600 ppm \textsuperscript{13}CO\textsubscript{2} for 4 hours with white light illumination and were subsequently cultured for a chase phase of 10 days under standard greenhouse conditions. A growing plant of \textit{Salvia divinorum} with a height of about 20 cm was placed in a closed plant growth chamber (BIOBOX, GWS, Berlin. During an adaption light period of 6 hours at 26 °C and a dark period of 12 hours at 22 °C and 66 % humidity, the plant was exposed to synthetic air containing 20.5 % oxygen and 700 ppm CO\textsubscript{2}. Prior to the labeling period, the chamber was flushed with synthetic air containing only oxygen and nitrogen. Then, the plants were exposed to synthetic air containing 600 ppm \textsuperscript{13}CO\textsubscript{2} (Campro Scientific, Berlin, Germany, > 99 % \textsuperscript{13}C abundance) as the only carbon source and were illuminated with white light (about 20,000 lux) at 26 °C for 4 hours. Subsequently, the plants were kept for 18 hours in the dark and were then allowed to grow under standard greenhouse conditions at ambient temperature for 10 days. Biomass was then harvested and lyophilized [8].
Figure 2. Several labeled $^{13}$C NMR signals of salvinorin A from the experiment with $^{13}$CO$_2$ [8].

![Figure 2](image)

Figure 3. Biosynthesis of salvinorin A from DXP.

Table 1. NMR signal assignment of salvinorin A [8].

| Position | Chemical shift $^{13}$C $\delta$, ppm | $^1$H $\delta$, ppm | Coupling constant COSY J$_{CC}$, Hz | HMBC |
|----------|---------------------------------------|---------------------|-----------------------------------|------|
| 1        | 202.0                                 | 42.0(6)             | 2,10,3                            |
| 2        | 75.0                                  | 36.5(3)             | 3                                 |
| 3        | 30.7                                  | 36.0(2)             | 2,4,22                            |
|          |                                       | 2.9(18)             | 2,4,22                            |
| 4        | 53.5                                  | 19                  | 3,5                               |
| 5        | 42.0                                  | 36.0(6)             | 3,5,18                            |
| 6,6'(α)  | 38.1                                  | 34.7(5)             | 10,8,19                           |
|          |                                       | 3.1(1)              |                                   |
| 7(β)     | 18.1                                  | 33.4(11)            | 6,8                               |
| 8        | 51.3                                  | 52.6(17)            | 7                                 |
|          |                                       | 2.0(20?)            | 7,9,17                            |
| 9        | 35.4                                  | 32.3(11)            | 8,10,20                           |
| 10       | 64.0                                  | 2.19(br s)          | 4,9,11,19,20                      |
| 11(α)    | 43.3                                  | 33.4(9)             | 8,9,10,12,20                      |
| 12       | 72.0                                  | 5.54(dd)            | n.d.                              |
|          |                                       | 72.6(16)            | 11,12,14,15,16                    |
| 13       | 125.2                                 | 70.3(15)            | 13,15                             |
| 14       | 108.3                                 | 3.0(12)             | 12,15,16                          |
| 15       | 143.7                                 | 70.3(14)            | 14                                |
| 16       | 139.4                                 | 73.8(13)            | 13,15                             |
| 17       | 171.1                                 | 51.1(8)             | 8                                 |
| 18       | 171.5                                 | 3.7(12)             | 3,4,23                            |
| 19       | 16.4                                  | 7.1(3)              |                                   |
| 20       | 15.2                                  | 35.1(4or5)          | 4,10                              |
| 21       | 169.9                                 | 35.1(9)             | 9                                 |
| 22       | 20.5                                  | 59.7(22)            | 2,22                              |
|          |                                       | 59.9(21)            |                                   |
Salvinorin A was obtained by solvent extraction of the leaves and was purified chromatographically. The isotopologue composition of salvinorin A was determined by NMR spectroscopy (figure 2). The presence of multiply $^{13}$C-labeled isotopologues in considerable excess over their natural occurrence showed that the $^{13}$C labelling pulse had afforded multiply $^{13}$C-labeled biosynthate. Consecutive catabolic and anabolic reactions during the chase phase period afforded salvinorin A that was a mosaic revealing its origin from a mixture of labelled and unlabelled precursors. The presence of triple labelled isotopologues demonstrated that the IPP and DMAPP precursors of the diterpene, salvinorin A, were predominantly biosynthesized via the recently discovered non-mevalonate pathway under the in vivo conditions.

The interpretation of the satellite signatures depends crucially on unequivocal signal assignment for all 20 carbon atoms of the salvinorin A skeleton. The published signal assignments were confirmed by two-dimensional COSY, HMQC and HMBC experiments (table 1).

On basis of the $^{13}$C assignments, the coupling patterns in Figure 2 and Table 1 indicate the presence of 6 pairs of directly adjacent $^{13}$C atoms. For example, the satellite lines of the signal of C-16 were separated by $^{13}$C-coupling with a coupling constant of 73.8 Hz. This indicates the presence, at relatively high abundance, of an isotopologue carrying $^{13}$C in position 16 and in the directly adjacent position 13. This interpretation is confirmed by the signature of C-13 which is also characterized by a doublet with a $^{13}$C-coupling of 73.8 Hz confirming the presence of the [13,16-$^{13}$C$_2$]-isotopologue.

The presence of multiply $^{13}$C-labeled isotopologues in considerable excess over their natural occurrence showed that the $^{13}$C labelling pulse had afforded multiply $^{13}$C-labeled biosynthate [7, 8]. Consecutive catabolic and anabolic reactions during the chase phase period afforded salvinorin A that was a mosaic revealing its origin from a mixture of labelled and unlabelled precursors. The presence of triple labelled isotopologues demonstrated that the IPP and DMAPP precursors of the diterpene, salvinorin A, were predominantly biosynthesized via the recently discovered non-mevalonate pathway under the in vivo conditions (figure 3).

The example illustrates that NMR in combination with labelling techniques is a very powerful tool to delineate biosynthetic pathways and, without any doubt, the technique will play a dominant role in phytochemistry [9, 10].

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