Non-Statistical $^{13}$C Fractionation Distinguishes Co-incident and Divergent Steps in the Biosynthesis of the Alkaloids Nicotine and Tropine

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ABSTRACT

During the biosynthesis of natural products, isotopic 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, leading to a non-statistical distribution of isotopes. This can be accessed by isotope ratio monitoring $^{13}$C NMR spectrometry. The solanaceous alkaloids S-(-)-nicotine and hyoscyamine (atropine) are related in having a common intermediate, but downstream enzymatic steps diverge, providing a relevant test case to: (a) elucidate the isotopic affiliation between carbon atoms in the alkaloids and those in the precursors; (b) obtain information about the kinetic isotope effects of as yet undescribed enzymes, thus to make predictions as to their possible mechanism(s). We show that the position-specific $^{13}$C/$^{12}$C ratios in the different moieties of these compounds can satisfactorily be related to their known precursors and to the known kinetic isotope effects of enzymes involved in their biosynthesis, or similar reaction mechanisms. Thus, the pathway to the common intermediate, N-methyl-$\Delta^1$-pyrrolinium, is seen to introduce similar isotope distribution patterns in the two alkaloids independent of plant species, whereas the remaining atoms of each target compound, which are of different origins, reflect their specific metabolic ancestry. We further demonstrate that the measured $^{13}$C distribution pattern can be used to deduce aspects of the reaction mechanism of enzymes still to be identified.

INTRODUCTION

Members of the plant family Solanaceae produce a range of alkaloids derived from L-ornithine or L-lysine, several of which are exploited for their recreational and/or pharmaceutical properties, despite their toxicity. S-(-)-Nicotine, the principle alkaloid of Nicotiana tabacum L. and related species has a long history of use for recreational and ethnopharmaceutical applications. Considered a component of the defensive chemical array of the genus Nicotiana (1,2) nicotine was widely exploited as an insecticide, eventually being phased out as less toxic neonicotinoids became available. Within a closely-related group of plants occurs the tropane alkaloids, of which atropine, hyoscyamine and scopolamine (hyoscine) have a history of use in mystical ceremonies (3). These compounds are now extensively used in modern medicine, atropine and hyoscyamine as mydriatic agents, scopolamine as an anti-emetic for controlling travel sickness.

For both these alkaloid types, the biosynthetic pathways have been extensively investigated at the metabolic, enzymatic and genetic levels (4,5). Despite superficial structural dissimilarities, the pyrrolidine moiety of nicotine and of tropine (of which atropine or hyoscyamine is the tropoyl ester) have a
common precursor in L-ornithine, and share four enzymatic steps to the intermediate \( \text{N-methyl-} \Delta^1 \text{-pyrrolinium} \) salt (Fig. 1). Thereafter, a series of diverse enzymes intervene to condense this intermediate with different groups to render more elaborated structures. A number of the enzymes involved in this pathway are well described: others remain poorly understood.

**FIGURE 1 near here**

In both these alkaloids, the reactions by which \( \text{N-methyl-} \Delta^1 \text{-pyrrolinium} \) salt is further elaborated has resisted a number of attempts at characterization, despite its central role in the pathway. In the case of \( S^-(-)-\text{nicotine} \), a stereospecific decarboxylative condensation with a derivative of nicotinate leads to \( S^-(-)-\text{nicotine} \). An extremely weak ‘nicotine synthase’ has been reported (6), but other authors could not repeat this finding (5). While it has been suggested that this is possibly catalyzed by an enzyme of the berberine-bridge type (7), no details of the nature of this key enzyme nor of its mechanism are available.

In a parallel fashion, condensation with the \( C2 \) position of acetoacetate (probably activated as the \( \text{CoA} \) thioester) followed by decarboxylation (probably spontaneous) leads to tropinone (4). Similarly, despite many efforts, the enzymology of the conversion of \( \text{N-methyl-} \Delta^1 \text{-pyrrolinium} \) to tropinone remains elusive. Tropine is produced therefrom by a stereospecific oxidoreductase (8) and is esterified with phenyllactoyl-\( \text{CoA} \) thioester to form the alkaloid littorine, precursor to hyoscymamine and scopolamine (9-12).

In such cases, where enzymological approaches have proved inadequate, alternative means to probe the biochemical reactions are required. The development of molecular biological approaches has been fruitful (5,13,14) but is challenging where the enzymology is completely unknown. An alternative ‘retro-biosynthetic’ approach involves the measurement and interpretation of the natural fractionation in \( ^2\text{H} \) or \( ^13\text{C} \) isotopes during the course of biosynthetic reactions. During the metabolic processes by which natural products are biosynthesized, isotopic fractionation occurs, due to the sensitivity/insensitivity of the enzymes involved to the presence/absence of one or more heavy atoms in the reaction center. This is the kinetic isotope effect (KIE). As a result, the position-specific isotope ratio in natural products is non-

**RESULTS**

\( ^13\text{C} \) isotopic links in alkaloid biosynthesis

1. Can we predict that similar reaction schemes should lead to similar isotope patterns? Isotopic fractionation should be associated with the KIEs intrinsic to the reaction mechanisms of the implied enzymes, rather than to the plant species involved. This can be tested by comparing the five carbon atoms derived from the common intermediate, \( \text{N-methyl-} \Delta^1 \text{-pyrrolinium salt} \), that these alkaloids have in common in their biosynthetic schemes (Fig. 1).

2. Can we show that the differing origins of the other carbon atoms present that are derived from dissimilar origins—three in tropine and five in \( S^-(-)-\text{nicotine} \)—do not display any particular common features?

3. Can the characteristics of the position-specific \( ^13\text{C}/^12\text{C} \) ratios help indicate mechanistic features of the enzymes that are as yet undescribed for these pathways?
Suitable conditions were established (see EXPERIMENTAL) for the acquisition of $^{13}$C NMR spectra amenable to the calculation of position-specific $^{13}$C/$^{12}$C ratios. Representative spectra are illustrated in Figure 2.

**FIGURE 2 near here**

Subjecting these spectra to total-line-shape curve fitting provided the area under the peak for each carbon atom $i$. From this, the molar fraction was calculated, which gives the extent to which the $^{13}$C/$^{12}$C ratios diverge from a statistical distribution. By combining this with the global value for the whole molecule, $\delta^{13}C_g$ (‰), i.e. the deviation of the carbon isotopic ratio of the whole molecule relative to that of the international standard Vienna Pee Dee Belemnite, (V-PDB), values for the positional isotopic distribution, $\delta^{13}C_i$ (‰), can be calculated (Table 1). The advantage of expressing isotopic deviation as $\delta^{13}C_i$ (‰) is that all molecular targets are related back to the same defined international reference.

**TABLE 1 near here**

For $S$-(-)-nicotine, the range of values obtained for the two samples is -17.3 to -52.3 ‰ and for tropine the range is -9.1 to -52.3 ‰, with standard deviations acceptable for irm-$^{13}$C NMR spectrometry (~1 ‰; range 0.7 to 2.7 ‰). These $\delta^{13}C_i$ (‰) values are within the typical range for natural products from plants using a C$_3$ metabolism (17). Their relative position-specific distributions are illustrated in Figure 4, which shows the extent to which each $\delta^{13}C_i$ (‰) value differs from the $\delta^{13}C_g$ (‰) value.

**DISCUSSION**

Following the successful development of the required protocols, it proved feasible to obtain position-specific $^{13}$C/$^{12}$C ratios for all carbon atoms of the two target alkaloids. Thus, it is possible to exploit these to probe a number of aspects of the biosynthesis of these natural products.

To what extent are isotope ratios determined by the primary precursor molecules giving rise to the N-methyl-$\Delta^1$-pyrrolinium salt?—In $S$-(-)-nicotine the carbon atoms that compose the pyrrolidine ring, C$_2^N$, C$_3^N$, C$_4^N$ and C$_5^N$ are derived from L-ornithine. This involves four known enzymatic steps (Fig. 3) (5). Looking further back into the metabolic precursors, these carbons can be traced to citric acid, a component of the Krebs cycle, thence to oxaloacetate and acetyl-CoA. Therefore, it can be deduced that the C$_{2OX}$ and C$_{3OX}$ of oxaloacetate become the C$_3^N$ and C$_2^N$, respectively, in nicotine. Similarly, the C$_{1A}$ and C$_{2A}$ of acetate are incorporated via citrate into the C$_5^N$ and C$_4^N$, respectively.

**FIGURE 3 near here**

For tropine, by a similar argument, it can be deduced that the C$_{2OX}$ and C$_{3OX}$ of oxaloacetate become the C$_6^T$ and C$_5^T$, respectively, while the C$_{1A}$ and C$_{2A}$ of acetate are incorporated into the C$_{1T}$ and C$_{7T}$, respectively (Fig. 3).

This ancestry will play a significant role in determining the position-specific $^{13}$C/$^{12}$C ratios observed, as they will either essentially be retained, or will be modified due to KIEs in the enzymes involved in the pathway.

Since tropine is spectrally the simpler of the two target molecules, its isotope pattern will be analyzed first. As can be seen from Figure 2A, the pairs of carbons showing the same degree of deshielding, C$_2^T$+C$_4^T$, C$_1^T$+C$_5^T$, and C$_6^T$+C$_7^T$ result in coincident peaks in the $^{13}$C NMR spectrum. The impact of this on interpreting the $^{13}$C/$^{12}$C ratios measured is, however, minimal, as the C$_{1T}$+C$_{5T}$ and C$_{6T}$+C$_{7T}$ pairs of positions are biosynthetically equivalent. Not only is N-methyl-$\Delta^1$-pyrrolinium a symmetrical molecule, but it is derived from the symmetrical molecule, putrescine. As there is no evidence of metabolic tunneling between L-ornithine and N-methylputrescine (21), it can be presumed that methylation of putrescine can occur on either the 1- or the 4-amino group with equal probability. Hence, the (C$_{2O}$;C$_{5O}$) and (C$_{3O}$;C$_{4O}$) positions in L-ornithine become indistinguishable in N-methylputrescine, hence in N-methylpyrrolinium. Furthermore, tautomerism occurs around the quaternary nitrogen in N-methyl-$\Delta^1$-pyrrolinium (22), so no distinction is made as to at which carbon the substitution that leads to the elaborated molecules takes place.

Considering the history of the four carbon atoms derived from N-methyl-$\Delta^1$-pyrrolinium, the C$_6^T$ and C$_7^T$ have undergone no bond-forming reaction since the formation of citrate (Fig. 3). As isotopic fractionation is associated with bond forming and breaking, so the opposite is true: carbon atoms not
undergoing reactions are likely not to significantly fractionated. Hence, at these positions the chances for isotopic fractionation are minimal, and this is reflected in these positions having a relatively high $\delta^{13}C$ (‰) value: -11.9‰ (Fig. 4A, Table 1). The C(1+5)$_T$ is however, relatively depleted, having a $\Delta\delta^{13}C$ (‰) value 10.4‰ lower than the C(6+7)$_T$. This can be explained by two contributing factors. The C(1+5)$_T$ is derived from the carboxyl group (C1$_A$) of acetyl-CoA, which in eukaryotes is enriched relative to the methyl group in conditions in which commitment of acetyl-CoA to synthesis is significant (16,17,23). The degree of this varies. In lipids of eukaryotic origin (Saccharomyces cerevisiae) carbon positions derived from the C1$_A$ are enriched relative to those from the C2$_A$ by ~5‰ (23). Similarly in ethanol from C$_3$ plants, although here the difference is closer to 2‰ (24). In tropine, the impact of this is, however, compensated by the relative depletion at the C2 of citrate (C2c), and, based on estimated values for citrate where it is acting as a metabolic intermediate (16), a $\Delta\delta^{13}C$=[(C(1+5)$_T$−C(6+7)$_T$] value of ~9‰ can be deduced. This is close to the observed differences in tropine.

An addition potential source of fractionation at the C(1+5)$_T$ value (but not the C(6+7)$_T$ value) is the two reactions involved in the final steps of the common pathway forming N-methyl-$\Delta^+$-pyrrolinium, in both of which bond breaking/creation occurs. The ring closure reaction (Fig. 3, step d), in which a C–N bond is formed, is considered spontaneous (13), the two compounds being in equilibrium. This step is unlikely to contribute a significant isotope fractionation as, even if there is an equilibrium isotope effect, the compound in either open or ring-closed structure is fully committed to the pathway. The previous step, in which a C–N bond is broken and a C=O bond is formed is catalyzed by the enzyme N-methylputrescine oxidase (EC 1.4.3.6) (Fig. 3, step c). An intramolecular normal KIE associated with amine oxidase have been reported (25), so a fractionation during the deamination of N-methylputrescine would select in favor of isopomers containing $^{13}C$ at the C1$_T$ position, since it involves an sp$^3$→sp$^3$ conversion (26). This would lead to a $^{13}C$ depletion of the C-atom. However, as the commitment of N-methylputrescine to tropine biosynthesis is very high, this intermediate being a dedicated component of the pathway, isotope fractionation due to this enzyme is unlikely to be significant (see (17) for a detailed discussion of this phenomenon).

These arguments apply equally to the equivalent four carbon atoms of the pyrrolidine ring in $S$-(-)-nicotine biosynthesis (C2$_N$, C3$_N$, C4$_N$, C5$_N$). In this analyte, however, these four carbons are resolved in the $^{13}C$ NMR spectrum (Fig. 2B), which enables a more detailed interpretation (see below). Once again, the C3$_N$ and C4$_N$, derived from citrate essentially without undergoing further reaction, are enriched relative to the C5$_N$ and C2$_N$, which have undergone deaminative oxidation and C–N bond formation, respectively (Fig. 3). Furthermore, the relative depletion at the C(2'+5')$_N$ relative to the C(3'+4')$_N$ of ~7‰ is in the same sense and of a similar size to this comparison in tropine (~10‰).

Hence, it can be concluded (i), that the values obtained in the common compound N-methyl-$\Delta^+$-pyrrolinium can be explained on the basis of the values in their distal precursors and (ii), that this relationship is dictated primarily by the metabolic pathway, and is relatively independent of the plant species involved. To what extent are isotope ratios determined by the primary precursor molecules giving rise to the nicotinic acid-derived moiety of $S$-(-)-nicotine?—Nicotinic acid in plants is considered to be synthesized primarily from aspartate plus dihydroxyacetone phosphate/glyceraldehyde-3-phosphate (DHAP/G3P) (27). The incorporation of [3-14C]aspartate into both the C2$_N$ and C3$_N$ of $S$-(-)-nicotine effectively demonstrated this origin for the pyridine ring (28). Considering that nicotinate is a predominant source of precursor for $S$-(-)-nicotine, which is a major sink for carbon, then some characteristics can potentially be traced back to the precursor pools. C2$_N$ and C3$_N$ are derived from the C1$_{ASP}$ and C2$_{ASP}$, respectively, while the C4$_N$, C5$_N$ and C6$_N$ are from DHAP/G3P (Fig. 5). (Note that the interconversion of DHAP and G3P in primary metabolism means that the C4$_N$ and C6$_N$ are of equivalent origin.) During the synthesis of nicotinic acid, all these positions are involved in reactions, although only the C3$_N$, C4$_N$ and C6$_N$ positions are involved in bond-formation. Again, therefore, it is these three positions that might be expected to show isotopic fractionation.

**FIGURE 5 near here**
First, let us consider the three carbon positions showing the least relative depletion in the pyridine ring, C2N, C4N, and C5N. The C5N ($\delta^{13}C_i = -26.7\%$) is from the keto group of DHAP and undergoes an $sp^3\rightarrow\pi$ transition in the formation of quinolinic acid. The C2N is the most enriched position ($\delta^{13}C_i = -18.9\%$) and, although this carbon is involved in the formation of a C–C bond with the C3N in the formation of quinolinic acid and an $sp^3\rightarrow\pi$ transition, its bonding thereafter is unchanged. In both of these positions, it is apparent that negligible fractionation has occurred, at least relative to the other three positions in the pyridine ring. The C2N is involved in the decarboxylation of quinolinic acid and has a $\delta^{13}C_i \sim 15\%$ and $\sim 7\%$ depleted relative to the C4N and C5N, respectively. Although decarboxylation can take place with negligible $^{13}C$ KIE on the $\alpha$-carbon (29), it would appear that fractionation is occurring here and that decarboxylation is probably a kinetically regulating step.

The C3N at $\delta^{13}C_i \approx -42.8\%$ is $\sim 8\%$ depleted relative to the mean for the pyridine ring. This may be due to two additive effects. The first is the origin of this carbon: the C3OX of oxaloacetate. This carbon is estimated to be markedly depleted (16), by $\sim 11\%$ relative to the other carbons of oxaloacetate (the immediate precursor of L-aspartate). Furthermore, C3N is involved in two bond-forming and one bond-breaking reactions, each of which may lead to selection against $^{13}C$ in this position (see below), hence relative depletion.

However, the most depleted position is the C6N, which at $\delta^{13}C_i = -52\%$ is $\sim 17\%$ depleted relative to the mean for the pyridine ring. This position is involved in the formation of the C=N bond created when it is joined to the imino group of iminospartate by quinolinate synthase (EC 2.5.1.72) (Fig. 5, boxed positions in the first row). Two mechanisms have been proposed for this enzyme, which differ essentially in the order in which the two bonds are formed: there is no experimental evidence to favor one over the other (30). However, in both cases the formation of the new C–C bond involves the nucleophilic attack of either $\text{NH}_2$ or $=\text{NH}_2$ on a $>\text{C}=\text{O}$ group. The degree of electrophilicity of the $>\text{C}=\text{O}$ is considered likely to have a major influence on the $^{13}C/^{12}C$ ratio at this atom (30). Based on our data, a strong normal KIE is indicated.

To what extent do the reactions involving the conversion of N-methyl-$\Delta^1$-pyrrolinium to tropinone influence the observed isotope ratios in tropine?—The elaboration of N-methyl-$\Delta^1$-pyrrolinium to tropinone involves the introduction of the three carbons C2T, C3T and C4T and the creation of two new C–C bonds at the C1T and C5T positions (Fig. 5). These three carbons are derived from acetate, via acetoacetyl-CoA and 4-(1-methyl-2-pyrrolidinyl)-3-oxobutyrte (probably as the CoA thioester) (31). On the basis of the above discussion of the relative enrichments in acetyl-CoA, the C3T, derived from the carboxyl in acetoacetyl-CoA, might be expected to be slightly enriched relative to the C(2+4)T if no other factors come into play. In fact, the $\Delta^{13}C_i = [(C3T)–(C(2+4)T)]$ of $\sim 13\%$ implies a significant KIE associated with the formation of this moiety. This greater relative enrichment is, however, consistent with the characteristic strong $^{13}C$ KIE associated with aldol reactions of acetyl-CoA in the biosynthesis of natural products when there is a high commitment of acetyl-CoA (16). In these cases, a large depletion in the carbons derived from the methylene group is observed. Similarly, in citrus extracted from fruit juice, in which there is again a high commitment of acetyl-CoA to this end-product, the C2CT is relatively depleted, by $\sim 11\%$ (16). Hence, it is probable that these positions in acetoacetyl-CoA are more depleted relative to the carboxyl positions than in acetyl-CoA.

In addition, the condensation of N-methyl-$\Delta^1$-pyrrolinium with acetoacetyl-CoA is likely to have normal $^{13}C$ KIEs for both the formation of the C1T–C2T bond in the synthesis of 4-(1-methyl-2-pyrrolidinyl)-3-oxobutyrte, and for the formation of the C4T–C5T bond created during the synthesis of the 8-azabicyclo[3.2.1]octan-3-ol ring. This should lead to a further relative depletion in the C(2+4)T position, compatible with the observed values. Unfortunately, due to the coincidence of the C2T and C4T in the $^{13}C$ NMR spectrum (Fig. 2A), it is not possible to assess whether the first or second bond formation has a major influence on the $^{13}C/^{12}C$ ratios observed.

To what extent do the reactions involving the conversion of N-methyl-$\Delta^1$-pyrrolinium to S-(-)-nicotine influence the observed isotope ratios?—The elaboration of N-methyl-$\Delta^1$-pyrrolinium to S-(-)-nicotine involves the creation of a new C–C bond linking the C3N and C2N positions, thus joining the nicotine-derived moiety to the N-methyl-$\Delta^1$-pyrrolinium-
derived moiety (Fig. 5). Once again, the symmetry of the N-methyl-Δ1-pyrrolinium salt means that in this precursor the C2\textsuperscript{N} = C5\textsuperscript{N} and C3\textsuperscript{N} = C4\textsuperscript{N}. However, the values observed at the C2\textsuperscript{N} and C5\textsuperscript{N} are not equivalent (Fig. 4B, Table 1): the C2\textsuperscript{N}, the carbon at which the C–C bond with the nicotinate moiety is made, is found to be ~4\% enriched relative to the C5\textsuperscript{N}. The observed difference needs therefore to be accounted for by another cause. It is proposed that this is due to an inverse \textsuperscript{13}C KIE associated with the mechanism of the 'nicotine synthase' reaction condensing the N-methyl-Δ1-pyrrolinium- and nicotinate-derived moieties (Fig. 6). While the mechanism of this reaction is not known, some suggestions can be made on the basis of the isotope fractionation, hence the intrinsic KIE of the reaction. The C2\textsuperscript{N} undergoes a change of state from a semi-aromatic nature with delocalized electrons to a tertiary aliphatic carbon (Fig. 6). Our data indicate that this reaction favors the \textsuperscript{13}C=N+ over the \textsuperscript{12}C=N\textsuperscript{+}, as known for sp\textsuperscript{3} \rightarrow sp\textsuperscript{2} conversions (26). Such a KIE will lead to relative enrichment in the position becoming the C2\textsuperscript{N} and an equivalent relative depletion in the C5\textsuperscript{N} must occur. This is clearly seen in Figure 4B. Furthermore, the C3\textsuperscript{N} position is strongly depleted, which would indicate a selection against \textsuperscript{13}C at this position, compatible with a mechanism in which the C3\textsuperscript{N} undergoes an sp\textsuperscript{3} \rightarrow sp\textsuperscript{2} conversion with kinetic limitation (Fig. 6). Further support for this proposal needs to be sought using specifically-labelled substrates.

What can be deduced about the introduction of the N-methyl group based on the observed isotope ratios?—In addition to having the Δ1-pyrrolinium moiety in common, the N-methyl group in both tropine and S-(−)-nicotine is derived from the common source, S-adenosylmethionine (SAM) and is introduced by the enzyme putrescine N-methyltransferase EC 2.1.1.53) (32). In both compounds, this carbon is found to be depleted relative to the other putrescine-derived positions, with which it should directly be compared (rather than with the whole molecule). In tropine, the extent of depletion is the major difference between the two samples: relative to the putrescine-derive part: ~38\% and ~22\% in TRI-1 and TRI-3, respectively. In nicotine, similarly, when the comparison is made only with the putrescine-derived atoms, then the relative depletion is ~12\%, rather less than in tropine but still well below the average of the other carbon atoms in this moiety.

This relative depletion in the \textsuperscript{13}C/\textsuperscript{12}C isotope ratio of the N-methyl group appears to be characteristic for SAM-derived methylation in natural products, previously recognized for O-methyl groups (33,34). The position-specific analysis of \textsuperscript{13}C/\textsuperscript{12}C isotope ratios of N-methyl group has only recently been investigated by using irm-\textsuperscript{13}C NMR and so far only for a very few alkaloids. Nonetheless, comparison can be made with the purine alkaloids, caffeine and theobromine (35), in which the N-methyl group is SAM-derived (36). In caffeine extracted from coffee the mean of the three N-methyl groups is on average ~36\% depleted relative to the other carbon atoms, while in theobromine extracted from cocoa, the mean of the two N-methyl groups is on average ~23\% below that of the other carbon atoms (35). These values suggest that relative depletion in the N-methyl group is a general phenomenon reflecting the biochemistry of methyl group insertion. The degree of depletion varies, but in all four alkaloids so far analyzed, including tropine and S-(−)-nicotine (Fig. 4), it is marked. This also parallels the relative depletion in a number of O-methyl groups, for example, ferulic acid (33). In tramadol, the N-Me\textsubscript{2} is also depleted relative to the part of the molecule considered to be derived from L-lysine by ~12\% (20), which encouraged us to consider SAM as its probable origin. The O-methyl group of tramadol was also relatively depleted, although only by ~7\%. Overall, then, the depletion found in tropine and S-(−)-nicotine, compares favorably with that in other N- and O-methyl groups, supporting the suggestion that this is a general phenomenon related to SAM metabolism (37).

The application of irm-\textsuperscript{13}C NMR to the study of compounds of known biosynthetic pathways has made possible the interpretation of the origin of the δ\textsuperscript{13}C\textsubscript{i} (\%) values obtained for two alkaloids, S-(−)-nicotine and tropine. Generally, we can conclude that the majority of the carbon positions show an isotope fractionation that is dominated by the position-specific \textsuperscript{13}C/\textsuperscript{12}C isotope ratios in the primary precursors that are exploited by the pathway. Thus, where moieties are incorporated that have other functions in the cell, for example acetate via acetyl-CoA or acetoacetyl-CoA, then isotopic fractionation against the cellular pool can occur, as these precursors fulfill many other metabolic roles. The strength of this link will
reflect the extent to which the primary precursors are committed to the synthesis of the specialized compounds, offering access to improved understanding of the primary/specialized metabolite interface in plant metabolism.

Once a major commitment of the substrate/product cascade to the final product is made, overall fractionation must be expected to have a more limited impact, as there is no branching taking place (17). Nevertheless, some intra-molecular fractionation can be observed when C–C bond formation/breaking takes place. In contrast, isotopic fractionation is minimal for those carbon atoms that undergo no bond-forming reactions. These position-specific observations make possible deductions as to the putative reaction mechanism involved, as in the present case of the ‘nicotine synthase’ condensation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Tropine ((3-endo)-8-methyl-8-azabicyclo[3.2.1]octan-3-ol) (TRI-1, hydrate 98+%, batch N° S21312–474 and S(-)-nicotine ((S)-3-[1-methylpyrrolidin-2-yl]pyridine) (NIC-1, free base >99%, batch N° 1449164V) were obtained from Sigma-Aldrich (www.sigmaaldrich.com); nicotine (((-)-3-[1-methylpyrrolidin-2-yl]pyridine) (NIC-3, free base >99.9%) was obtained from Fluka (www.sigmaaldrich.com); tropane free base (TRI-3, >97%, batch N° 1212025) was obtained from CHEMNOVATIC Ławecki Gęca Sp.j., Lublin, Poland. All these samples are of authenticated natural origin as indicated by their Certificate of Origin (note that the plant species used is not indicated) except for TRI-1, for which no certificate was available. Tris(2,4-pentadionato)chromium-(III) (Cr(Acac)3) was purchased from Merck (www.merck.com). Benzene-d6 and acetonitrile-d3 were obtained from Euriso-top (www.eurisotop.com).

**Isotope ratio monitoring by mass spectrometry**—The global value for the whole molecule, $\delta^{13}C_g$ (‰), is the deviation of the carbon isotopic ratio $R_i$ relative to that of the international standard Vienna Pee Dee Belemnite, (V-PDB), $R_{V-PDB}$. It is determined by isotope ratio monitoring by mass spectrometry and calculated from:

$$\delta^{13}C_g (‰) = \left(\frac{R_s}{R_{V-PDB}} - 1\right) \times 1000$$

where the value of $R_{V-PDB}$ is 0.0111802 (38).

**Isotope ratio monitoring by $^{13}C$ NMR acquisition conditions**—Prior to preparation of samples for irm-$^{13}C$ NMR, purity was confirmed to be superior to 98% by recording a $^1H$ NMR spectrum. Tropine free base from Fluka (TRI-3) was re-crystallized from diethyl ether before use.

For the analysis of nicotine, 250 µL of S-(-)-nicotine was homogenized in 500 µL of acetonitrile-d3. To this was added 100 µL of a solution of relaxation agent Cr(Acac)3 (0.1 M) prepared by dissolving 10.5 mg Cr(Acac)3 in 300 µL of acetonitrile-d3. Under these conditions, $T_i^{\max}=3.74$ s. Spectral acquisition was with $AQ=1.0$ s for 152 scans, giving a signal-to-noise ratio of ~700.

For the analysis of tropine, 150 mg of tropine was dissolved in 600 µL of benzene-d6. As $T_i^{\max}=1.53$ s is relatively short in these conditions, relaxation agent was not required. Spectra acquisition was with $AQ=0.95$ s for 400 scans, giving a signal-to-noise ratio ~550.

Quantitative $^{13}C$ NMR spectra were recorded at 100.6 MHz using a Bruker 400 Avance I NMR spectrometer fitted with a 5 mm 'H/$^{13}C$ dual probe. For S-(-)-nicotine, spectra were also measured on a Bruker 400 Avance III NMR spectrometer fitted with a 5 mm a BBFO probe. The temperature of the probe was set at 303 K for nicotine and 313.2 K for tropine (to decrease the viscosity of the sample). The offsets for both $^{13}C$ and $^1H$ were set at the middle of the frequency range for each analyte. Inverse-gated decoupling was applied and the repetition delay between each 90° pulse was set at 10×$T_i^{\max}$ of the analyte to avoid the nuclear Overhauser effect and to achieve full relaxation of the magnetization. The decoupling sequence used adiabatic full-passage pulses with cosine square amplitude modulation ($v_2^{\max} = 17.6$ kHz) and offset independent adiabaticity with optimized frequency sweep (39). Each measurement consisted of the average of five independently recorded NMR spectra.

**Spectral data processing**—To obtain $S_i$, the area under the $^{13}C$-signal in the irm-$^{13}C$ NMR spectrum for $C$-atom in position $i$, curve fitting based on a total-line-shape analyses (deconvolution) was carried out with a Lorentzian mathematical model using Perch Software (Perch™ NMR Software, http://www.perchsolutions.com). In this procedure, line-shape parameters are optimized in terms of intensities, frequencies, line-width, line-shape (Gaussian/Lorentzian, phase, asymmetry) by iterative fitted to a minimal residue. For each target compound, spectral phasing and line-fitting was performed using the...
same parameters. The positional isotopic distribution, $f/F_i$, was then calculated from the values of $S_i$, hence the $\delta^{13}C_i$ (‰) value, as described previously (19,40,41). For $S$-(−)-nicotine, due to the large range of its spectral shifts, a correction was made to data obtained on the Avance I NMR spectrometer using the data from the Avance III NMR spectrometer (see (42) for a detailed explanation). It should be noted that, since the coupling constants $J(13C,15N)$ are of the order of 10 Hz, no correction for these is required as they are included within the curve fitting applied to the peak.
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Conflict of Interest: The authors declare that they have no conflicts of interest within the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the CNRS, the University of Nantes, or the Łódź University of Technology.

Author contributions: R.J.R. and G.S.R designed the study. K.M.R. and V.S. conducted most of the experiments. R.J.R., G.S.R. and P.P. analyzed and interpreted the data. R.J.R. and K.M.R. wrote the paper.

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**FOOTNOTES**

1 Isotopic fractionation is the selection of one isotopomer versus another during a physical or (bio)chemical process which leads to a non-statistical distribution of isotopes in the population of isotopomers within the final product and the residual substrate.

2 The abbreviations used are: DHAP dihydroxyacetone phosphate, G3P, glyceraldehyde-3-phosphate; irm-$^{13}$C NMR, isotope ratio monitoring by $^{13}$C NMR; KIE, kinetic isotope effect; SAM, S-adenosylmethionine.

3 The notations CXA, CXASP, CXCTR, CXo, CXOX, CXT, CXN indicate the relevant carbon atom in acetate (A), aspartate (ASP), citrate (CIT), L-ornithine (O), oxaloacetate (OX), tropine (T), or nicotine (N), respectively.

4 $\Delta$ is the difference in $^{\delta^{13}}$C ($\‰$) values between two carbon positions. Thus, for example, $\Delta$C4X-C2X means the difference between the $^{\delta^{13}}$C of the C4 position of compound X and the $^{\delta^{13}}$C of the C2 position of compound X. For convenience, $\Delta$ is always attributed a positive sign and the difference described as “relatively depleted” or “relatively enriched”.
FIGURE LEGENDS

Figure 1. Schematic representation of the biosynthetic pathway to nicotine and tropine indicating the common and unique steps. Carbon atoms on the final compounds are numbered according to the IUPAC nomenclature and this numbering is used to indicate the carbon positions from which they are derived in the precursor molecules. Numbers in normal bold font refer to tropine; numbers in italic bold font refer to nicotine. The enzymes indicated are: a, ornithine decarboxylase (EC 4.1.1.17); b, putrescine N-methyltransferase (EC 2.1.1.53); c, N-methylputrescine oxidase (EC 1.4.3.6); d, undescribed activity, probably spontaneous(13); e, nicotine synthase (undescribed activity, but see (6)); f, undescribed activity.

Figure 2. $^{13}$C NMR spectra acquired under quantitative conditions of A) tropine and B) S-(-)-nicotine. Carbon atom displacements are indicated. See EXPERIMENTAL for the acquisition conditions.

Figure 3. Schematic representation of the biosynthetic pathway to N-methyl-$\Delta^1$-pyrrolinium salt from primary precursors. Carbon atoms on the final compounds are numbered according to the IUPAC nomenclature and this numbering is used to indicate the carbon positions from which they are derived in the precursor molecules. Numbers in normal bold font refer to tropine; numbers in italic bold font refer to nicotine. Values for $\delta^{13}C_i$ (‰) of citrate acting as a metabolic intermediate taken from (16) are indicated underlined. For enzymes, see Figure 1.

Figure 4. Position-specific $^{13}$C/$^{12}$C ratios expressed as $\Delta_g \delta^{13}C_i$ (‰) for two samples each of A) tropine and B) S-(-)-nicotine. See EXPERIMENTAL for the acquisition conditions and Table 1 for absolute values. In these graphs the notation $\Delta_g \delta^{13}C_i$ (‰) indicates the difference at the site $i$ of $\delta^{13}C_i$ from the mean overall value of $\delta^{13}C_g$ (‰): i.e. $\Delta_g \delta^{13}C_i$ (‰) = ($\delta^{13}C_i$ - $\delta^{13}C_g$).

Figure 5. Schematic representation of the biosynthetic pathway to S-(-)-nicotine and tropine indicating the origins of the carbon atoms. Those carbon and nitrogen atoms involved in the formation of bonds and therefore the more likely to undergo isotope fractionation are indicated in italics. Carbon atoms on the final compounds are numbered according to the IUPAC nomenclature. For all other compounds, the numbering indicates the position in the analyte in which the given carbon will be found. Numbers in normal bold font refer to tropine; numbers in italic bold font refer to nicotine.

Figure 6. Schematic representation of the proposed mechanism for ‘nicotine synthase’, the last step on the biosynthetic pathway to S-(-)-nicotine, based on the observed site-specific $^{13}$C/$^{12}$C isotopic fractionation. Partial enzymatic steps are given as: e1, the formation of the bond between C2’N and C3N in which the C2’N undergoes an $sp^2 \rightarrow sp^3$ with kinetic limitation leading to selection for $^{13}$C at the C3N position; e2, the decarboxylation at the C3N position, in which the C3N undergoes an $sp^3 \rightarrow sp^2$ with selection for $^{12}$C at the C3N position. Carbon atoms on the final compounds are numbered according to the IUPAC nomenclature. Numbers in normal bold font refer to tropine; numbers in italic bold font refer to nicotine.
Table 1. Data Obtained by irm-\(^{13}\)C NMR for the \(^{13}\)C Distribution in natural tropine or natural S-(−)-nicotine.

| Tropine Sample | TRI-1 | TRI-3 | \(^{15}\)C distribution (‰) | \(^{15}\)C distribution (‰) | \(^{15}\)C distribution (‰) | \(^{15}\)C distribution (‰) |
|----------------|-------|-------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Origin         | Natural | Natural | δ<sub>g</sub> | Mean ± SEM | Mean ± SEM | Average ± SEM |
| N° spectra     | 10     | 10     | -25.56         | -25.11         | -25.77         | -25.54         |
| \(f_{i}/f_{i}'\) C3 | 1.00508879 ± 0.00263500 | 1.00633672 ± 0.00231333 | \(1.00633672 ± 0.00231333\) |
| \(f_{i}/f_{i}'\) C(2+4) | 1.00641651 ± 0.00640000 | 0.99957955 ± 0.00334667 | \(0.99957955 ± 0.00334667\) |
| \(f_{i}/f_{i}'\) C(1+5) | 0.97287264 ± 0.00381500 | 0.98261329 ± 0.00224000 | \(0.98261329 ± 0.00224000\) |
| \(f_{i}/f_{i}'\) C(6+7) | 0.98809680 ± 0.00484000 | 0.99539280 ± 0.00270000 | \(0.99539280 ± 0.00270000\) |
| \(f_{i}/f_{i}'\) CH<sub>3</sub> | 1.01650598 ± 0.00179150 | 1.01055264 ± 0.00139333 | \(1.01055264 ± 0.00139333\) |

| S-(−)-Nicotine Sample | NIC-1 | NIC-3 | \(^{15}\)C distribution (‰) | \(^{15}\)C distribution (‰) | \(^{15}\)C distribution (‰) | \(^{15}\)C distribution (‰) |
|-----------------------|-------|-------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| N° spectra            | 5     | 5     | -29.99                      | -30.93                      | -30.93                      | -30.93                      |
| δ<sub>g</sub>         | Mean ± SEM | Mean ± SEM | Mean ± SEM | SEM |
| \(f_{i}/f_{i}'\) C2  | 1.0000610 ± 0.01095000 | 0.9986821 ± 0.01100000 | \(0.9986821 ± 0.01100000\) |
| \(f_{i}/f_{i}'\) C3  | 0.9897313 ± 0.01261000 | 0.9907477 ± 0.01460000 | \(0.9907477 ± 0.01460000\) |
| \(f_{i}/f_{i}'\) C4  | 1.0136892 ± 0.00450000 | 1.0148068 ± 0.01072000 | \(1.0148068 ± 0.01072000\) |
| \(f_{i}/f_{i}'\) C5  | 1.0074270 ± 0.00690000 | 1.0049678 ± 0.01580000 | \(1.0049678 ± 0.01580000\) |
| \(f_{i}/f_{i}'\) C6  | 0.9806073 ± 0.01357000 | 0.9817312 ± 0.01100000 | \(0.9817312 ± 0.01100000\) |
| \(f_{i}/f_{i}'\) C2’ | 1.0039115 ± 0.00115000 | 1.0065792 ± 0.00720000 | \(1.0065792 ± 0.00720000\) |
| \(f_{i}/f_{i}'\) C3’ | 1.0085256 ± 0.00860000 | 1.0104814 ± 0.01240000 | \(1.0104814 ± 0.01240000\) |
| \(f_{i}/f_{i}'\) C4’ | 1.0034714 ± 0.01106000 | 1.0034401 ± 0.00596000 | \(1.0034401 ± 0.00596000\) |
| \(f_{i}/f_{i}'\) C5’ | 1.0002778 ± 0.00773000 | 0.9977597 ± 0.0000000 | \(0.9977597 ± 0.0000000\) |
| \(f_{i}/f_{i}'\) CH<sub>3</sub> | 0.9922977 ± 0.00562000 | 0.9908040 ± 0.00560000 | \(0.9908040 ± 0.00560000\) |

δ<sub>g</sub> C2 (‰) | -33.09 ± 1.23 | -35.71 ± 0.86 | -34.40 ± 0.86 |
δ<sub>g</sub> C3 (‰) | -42.64 ± 0.70 | -42.87 ± 1.17 | -42.76 ± 1.17 |
δ<sub>g</sub> C4 (‰) | -18.78 ± 0.99 | -19.03 ± 1.71 | -18.90 ± 1.71 |
δ<sub>g</sub> C5 (‰) | -25.76 ± 1.47 | -27.99 ± 1.61 | -26.88 ± 1.61 |
$\delta_i C_6 ($‰$) -51.71 \quad 1.36 \quad -52.26 \quad 0.77 \quad -51.98$

$\delta_i C2' ($‰$) -26.76 \quad 2.09 \quad -23.09 \quad 0.80 \quad -24.93$

$\delta_i C3' ($‰$) -18.01 \quad 0.97 \quad -17.26 \quad 1.79 \quad -17.64$

$\delta_i C4' ($‰$) -22.09 \quad 0.83 \quad -23.39 \quad 1.63 \quad -22.74$

$\delta_i C5' ($‰$) -27.23 \quad 1.82 \quad -31.02 \quad 0.84 \quad -29.12$

$\delta_i CH_3 ($‰$) -33.98 \quad 0.72 \quad -36.81 \quad 0.86 \quad -35.40$

Footnotes:

$^a$ Mean of ten spectral acquisitions obtained as two separate preparations from the same sample (5 spectra per preparation).

$^b$ As these are independent samples, it is not appropriate to use a mean value. Rather, so as to give an indicative value for the text discussion, an average of the two samples each of nicotine and of tropine is given.

$^c$ \(f_i/F_i\) is the positional isotopic distribution, i.e. the variation of the $\delta_i$ from the statistical distribution (see EXPERIMENTAL for details).

$^d$ Mean of five spectral acquisitions. A field-homogenizing correction factor is applied for the data for $S$-(-)-nicotine.(42).
Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.
isomerisation

Figure 6.
Non-Statistical $^{13}\text{C}$ Fractionation Distinguishes Co-incident and Divergent Steps in the Biosynthesis of the Alkaloids Nicotine and Tropine

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