A retro-biosynthetic approach to the prediction of biosynthetic pathways from position-specific isotope analysis as shown for tramadol

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Tramadol, previously only known as a synthetic analgesic, has now been found in the bark and wood of roots of the African medicinal tree *Nauclea latifolia*. At present, no direct evidence is available as to the biosynthetic pathway of its unusual skeleton. To provide guidance as to possible biosynthetic precursors, we have adopted a novel approach of retro-biosynthesis based on the position-specific distribution of isotopes in the extracted compound. Relatively recent developments in isotope ratio monitoring by ^{13}C NMR spectrometry make possible the measurement of the nonstatistical position-specific natural abundance distribution of ^{13}C ($\delta^{13}C_i$) within the molecule with better than 1‰ precision. Very substantial variation in the ^{13}C positional distribution is found: between $\delta^{13}C_i = -11$ and −53‰. Distribution is not random and it is argued that the pattern observed can substantially be interpreted in relation to known causes of isotope fractionation in natural products. Thus, a plausible biosynthetic scheme based on sound biosynthetic principals of precursor–substrate relationships can be proposed. In addition, data obtained from the ^{18}O/^{16}O ratios in the oxygen atoms of the compound add support to the deductions made from the carbon isotope analysis. This paper shows how the use of ^{13}C NMR at natural abundance can help with proposing a biosynthetic route to compounds newly found in nature or those difficult to tackle by conventional means.

NMR spectrometry | position-specific isotope analysis | retro-biosynthesis | tramadol | *Nauclea latifolia*

The bark and wood from roots of the West African medicinal tree *Nauclea latifolia* Sm. is widely exploited in ethnomedicine for various treatments, including for pain relief. A blind bioassay-guided search for the active analgesic ingredient led to the isolation of a single compound, present at low concentration in some samples but at 0.4% d/w in a tree recognized as particularly effective by the local ethnopharmacists (1). Systematic fractional purification led to the isolation of racemic (1*R*,2*R*)-2-[(dimethylamino)methyl]-1-(3-methoxyphenyl)cyclohexanol as the active principle (1). This finding caused considerable interest in both the scientific (2) and public domains (3), principally because this compound is the well-known synthetic worldwide-prescribed analgesic drug, tramadol. The level of accumulation shows great variability with geographical location (4) as well as with the age of trees. The absence or only trace amounts of tramadol in some samples [<0.00002% (wt/wt)] and its detection in soils near to trees [<0.00015% (wt/wt)] has led to the suggestion that its presence might be due to anthropogenic contamination, such as from cattle grazing (4). Equally plausibly, this later could be due to dispersal by baboons and monkeys known to feed on *N. latifolia* fruits and leaves, or by its known use in ethnoveterinary practices (5). This could also be the cause for the occurrence of the traces of tramadol metabolites found by Kusari et al. (4). However, it cannot explain the high level at which it was found to occur in the samples obtained from bark and wood of roots of a plant growing in a section of the Benoué biosphere reserve in which human activity and livestock grazing are prohibited (1), a level independently confirmed (4).

As a recently identified natural product, tramadol shows some interesting features, but to elucidate a biosynthetic pathway by which it is produced is problematical. The classic approach of feeding experiments with labeled putative precursors requires using in vitro material or, rather impractically, trees in the field. In addition, the structural features could arise from a number of putative pathways. To obtain guidance as to the possible primary precursors and intermediates involved, we have taken a novel approach based on studying the nonstatistical distribution of isotopes within the molecule. For ^{13}C, the powerful technique of isotope ratio monitoring by ^{13}C NMR (irm-^{13}C NMR) spectrometry at natural abundance (6) makes possible the determination of the nonstatistical distribution of ^{13}C and the observation of individual ^{13}C isotopomers, thus the intramolecular distributions of ^{13}C [$\delta^{13}C_i$ (‰)]. To this can be added the values for the δ^{18}O, indicating the probable origins of the oxygen atoms present (7). The patterns observed can then be related to those already established in a number of plant compounds and to the isotopic fractionation introduced due to known kinetic and equilibrium isotope effects for the postulated enzymes (8), thus allowing a plausible hypothesis to be made as to the origins of the carbon and oxygen atoms. Hence, this approach generates data that will greatly facilitate a targeted approach to precursor feeding experiments.

Significance

The extraction of tramadol from the roots of *Nauclea latifolia* excited great interest worldwide. This was the first time that a widely marketed synthetic drug was found as an apparently natural product at high abundance. In this article, we describe the position-specific distribution of ^{13}C in this tramadol. As a conventional approach to study its probable biosynthetic precursors, enzymatic steps, and intermediate metabolites is not currently feasible, we herein propose the concept of a retro-biosynthesis by examining the position-specific isotope distribution within the molecule and rationally interpreting the data in terms of known plant biochemical processes that may be involved in a biosynthesis of tramadol. Thus, clear guidance is given for future labeling studies.

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Likewise, it can provide evidence to distinguish between different pathways to obtain the same product (9, 10).

The phenomenon of isotopic fractionation during reactions is well established as a method to characterize reaction mechanisms and help distinguish different (bio)synthetic origins (11–13). The information that can be obtained from examining the global isotope deviations ($\delta^{13}C_g$) in whole molecules (14, 15) by isotope ratio monitoring by mass spectrometry (irm-MS) is inadequate because this technique, in which the target is combusted to CO_2 in an O_2-rich atmosphere, has the disadvantage of only determining the average distribution of ^{13}C among the isotopomers ($\delta^{13}C_g$‰). This leads to considerable loss of information about isotope fractionation, because not only are heavy isotopes unevenly distributed within a compound, but position-specific isotopic fractionation at natural abundance is defined by the specific reactions taking place during biosynthesis (12, 16, 17).

It has been more than 30 y since it was recognized that "isotopic contrasts between compound classes. . . must be the attenuated and superficial manifestations of isotopic differences within molecules" (18). Only recently, however, has it become possible to access these intramolecular distributions of ^{13}C in whole molecules through the power of irm-^{13}C NMR at natural abundance to give direct measurement of position-specific $^{13}C/^{12}C$ ratios and to obtain quantification of all individual isotopomers (19). This technique has been exploited in authenticity and in examining differences in fermentation pathways.

The definite origin of the naturally occurring tramadol is still under investigation, and unequivocal evidence of its in planta biosynthesis requires rigorous tracer experiments following feeding with suitable labeled precursors. To provide logical guidance as to what tracer experiments might be fruitful, we have used the isotope pattern in the extracted tramadol to deduce a potential biosynthetic pathway. This is the first time (to our knowledge) that position-specific $^{13}C/^{12}C$ ratios have been used to make metabolism-guided deductions in relation to deducing a probable biosynthetic route.

Results and Discussion

The objective of this work was first to obtain by irm-^{13}C NMR the position-specific$^{13}C/^{12}C$ ratios in isotopomers of tramadol extracted from the root bark of *N. latifolia* and second to probe the potential biosynthetic causes of the extensive nonstatistical distribution observed.

Establishing the Methodology for Tramadol Analysis. The methodology of irm-^{13}C NMR spectrometry needs fine-tuning for each target compound in relation to the relaxation properties, solubility, stability, and purification of the target molecule. Suitable conditions for irm-^{13}C NMR spectrometry on tramadol were established and validated on free tramadol base obtained by basification of a synthetic commercial sample of tramadol·HCl. Tramadol of purity >99.5% (wt/wt) was obtained by crystallization from aqueous acetone, a technique that has the advantage that it does not introduce isotopic fractionation (20, 21). Irm-^{13}C NMR spectra were acquired using 200 mg of purified synthetic tramadol in slightly aqueous acetone. Spectra were obtained with a signal-to-noise ratio of ∼650, from which the distribution of ^{13}C at each carbon position, the reduced molar fraction f_i/F_i, was calculated from the areas under the peaks. This was then converted to $\delta^{13}C_i$ (‰) values using the $\delta^{13}C_g$ (‰) obtained by irm-MS (Table 1), as previously described (10, 19).

Five separate acquisitions, each of five spectra, showed good repeatability with the worst SD of 2.4‰ for the C4′ position. Hence, it was verified that the protocol gives position-specific values of the distribution of ^{13}C with sufficient accuracy.

Analysis of the Position-Specific $^{13}C/^{12}C$ Isotope Ratio in Tramadol Isolated from the Root Bark of *Nauclea latifolia*. Attention was then turned to the tramadol extracted from root bark of *N. latifolia*. Tramadol was obtained in sufficient quantities for irm-^{13}C NMR spectrometry by extraction (1) from a source of root bark from a tree identified by local ethnic doctors as providing a particularly potent source for their decoctions. This bark contained 0.4% d/w tramadol (1). Purification including a final crystallization from aqueous acetone yielded pure [>99.5% (wt/wt)] tramadol. Irm-^{13}C NMR spectra were acquired using 200 mg of pure tramadol from the field collection in slightly aqueous acetone and the $\delta^{13}C_i$ (‰) values computed as described above (10, 19).

Two independent isolations and purifications from the same biological source of *N. latifolia* root bark were carried out. The profiles shown are reproducible and show that tramadol from *N. latifolia* has a wide range of $\delta^{13}C_i$ (‰) values, varying from −11‰ to −54‰ (Fig. 1*A*). The majority of the positions showed no significant difference in the $\delta^{13}C_i$ (‰) values for the two extractions, with the worst SD of 2.9‰ for the C6′.

The range of values determined fall within that to be anticipated for a compound obtained from a natural source (22). Before developing how these can be related on a position-specific basis to a biosynthetic origin, a plausible scheme is presented.

Proposed Biosynthesis of Tramadol. First, it should be noted that all of the structural features seen in tramadol (Fig. 1*B*) have been characterized in various natural products. The *N*,*N*-dimethyl group occurs in many alkaloids, such as hordenine [4-(2-dimethylaminoethyl)phenol] (23), and products possessing a *N*,*N*-dimethyl-2-aminomethyl-cyclohexane moiety are known (24). Many phenolic natural products derived from a number of biosynthetic routes contain a *meta*-methoxyl group, such as gallic acid, ferulic acid, vanillin, stilbenes, and aurones (25). 3-Methoxyacetophenone (**1**) and its precursor 3′-hydroxyacetophenone have been reported in various plant species (26–28). Additionally, an alkaloid with a 3-methoxyphenyl moiety, probably derived from 3-methoxyacetophenone, has been isolated from *Lobelia siphilitica* (29).

Second, drawing on these known natural structures and on a sound knowledge of parallel biochemistry, several hypothetical pathways can be proposed: the more plausible are given in Fig. 2 *A* and *B*. Two potential biosynthetic origins can be put forward as the origin of the aromatic ring: an acetyl-CoA–derived polyketide or a shikimate-derived amino acid. Feeding studies in *Camellia sinensis* flowers (30) and in the fungus *Bjerkandera adusta* (31) have shown

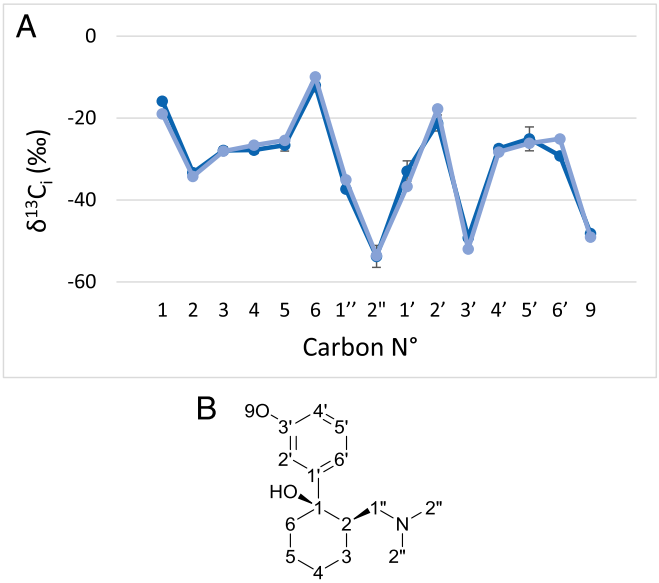

Fig. 1. The $\delta^{13}C_i$ (‰) values for tramadol obtained by irm-^{13}C NMR from two independent extractions of the root bark of *N. latifolia*. (*A*) The $\delta^{13}C_i$ (‰) values of the 15 carbon positions. (*B*) The structure of tramadol.

that the acetophenone skeleton can be biosynthesized from L-Phe, probably via a β-oxidation pathway from cinnamic acid. Plant enzymes that lead to acetophenone derivatives from feruloyl-CoA (32) are known, and the production of L-3′-hydroxyphenylalanine (3′-OH-Phe), an important nonprotein amino acid described in the grass *Festuca rubra* (33) implicates a role for phenylalanine 3-hydroxylase, an enzyme that has so far only been characterized in a streptomycete (34). Thus, 3′-oxidation followed by methylation through S-adenosylmethionine (SAM) methyltransferase would introduce the 3′-methoxyl group and β-oxidation (31) would lead to **1**. However, an alternative pathway from acetyl-CoA was demonstrated in *Kniphofia pumila* (35). Discriminating between these alternative pathways is discussed in relation to the experimental results.

The biosynthesis of **2** could follow one of two pathways from L-Lys. The first is the route common to the biosynthesis of a number of alkaloids in which decarboxylation of L-Lys leads to the symmetrical diamine, cadaverine (1,5-diaminopentane), which undergoes oxidative deamination to 5-aminopentanal (Fig. 2*A*) (36, 37). However, the 5-carbon unit could equally well be incorporated from L-Lys, via another degradation product, L-2-amino-adipic-6-semialdehyde, a pathway in which oxidative deamination precedes decarboxylation. In either case, *N*,*N*-dimethylation of the amine group leads to **2** (38). Again, a distinction between these alternative pathways can be deduced from the experimental results.

Subsequently, two key steps are invoked. The first is the condensation of **1** with **2** involving an aldolization to form **3**: the second is a cyclization converting **4**/**4a** to tramadol.

Does the Position-Specific $^{13}C/^{12}C$ Isotope Ratio in Tramadol Obtained from the Root Bark of *N. latifolia* Support a Proposed Biosynthetic Pathway? Considering those atoms proposed to be derived from L-Lys, these give a mean $\delta^{13}C_g$ value of −30.2‰ (Table 1), compatible with literature values for amino acids from C_3 plants (39). The key difference between the two postulated alternative routes–via cadaverine or via L-2-amino-adipic-6-semialdehyde–is that the former involves a symmetrical intermediate, whereas the latter does not. Biosynthesis via a symmetrical compound would lead to scrambling of the original $C2_L$ and $C6_L$ of L-Lys (yielding $C1''_T$ and $C5_T$ in Fig. 2*A*) and of the $C3_L$ and $C5_L$ positions (yielding atoms $C2_T$ and $C4_T$ in Fig. 2*B*). [The notation CX_L, CX_P, CX_F, and CX_T indicate the relevant carbon atom in L-lysine (L), L-phenylalanine (P), ferulic acid (F), or tramadol (T), respectively.] Therefore, the $\delta^{13}C$ values of the $C2_T$ and $C4_T$ should be identical, which is clearly not the case ($\Delta C4_T - C2_T$ = 6.6‰). [Δ is the difference in $\delta^{13}C_i$ (‰) values between two carbon positions. Thus $\Delta C4_T - C2_T$ means the difference between the $\delta^{13}C_i$ of the C4 position of tramadol and the $\delta^{13}C_i$ of the C2 position of tramadol. For convenience, Δ is always attributed a positive sign.] In addition, the oxidative deamination of cadaverine should imply an intramolecular kinetic carbon isotope effect (40). As a result, competition between equivalent positions of the symmetrical precursors should select in favor of isotopomers containing ^{12}C at this position, leading to a ^{13}C depletion of the C atom bearing the oxygen function ($C5_T$) relative to that bound to N ($C1''_T$). Both these predictions are not met: on the contrary, $C1''_T$ is depleted relative to $C5_T$ ($\Delta C5_T - C1''_T$ = 10.2‰). Hence, the ^{13}C isotope data argue strongly in favor of the precursor being a nonsymmetrical molecule. Incorporation of L-Lys via L-2-amino-adipic-6-semialdehyde, in contrast, would not invoke scrambling of the original $C2_L$ and $C6_L$ positions of L-Lys and is fully compatible with the observed isotope distribution pattern. Furthermore, the relative depletion of the $C1''_T$ is in accord with a general ^{13}C depletion at the α-atom of amino acids from auxotrophs (41).

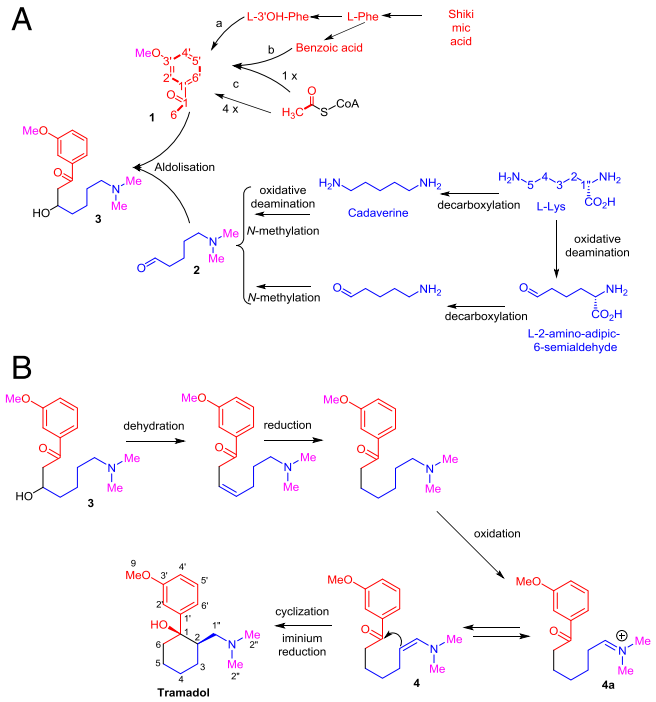

Fig. 2. Proposed origins of the carbon atoms in biosynthetic tramadol. (*A*) Condensation of the carbon skeleton. (*B*) Reactions leading to ring closure and tramadol. Racemic (1*R*,2*R*)-tramadol is likely to be due to the final cyclization/reduction step. Origins from L-Phe in red, L-Lys in blue, and SAM in violet. All carbons are numbered by their respective positions in the tramadol molecule. The bold bonds in **1** show those that would be retained intact in a polyketide-derived structure.

Turning to the origin of moiety **1**, the acetophenone skeleton, two alternatives have been indicated: an acetyl-CoA–derived polyketide or a phenylpropanoid from the shikimic acid pathway. In general, a *meta*-substituent oxygen function in an aromatic ring of a natural product can suggest its origin from a polyketide (14, 35). However, the *meta* positioning of this oxygen function to that in the side chain is not in agreement with the expected alternating sequence for a polyketide, which would be predicted to carry oxygen substitutions on even positions, those derived from the carbonyl of acetyl-CoA (35). Hence, this origin can be argued as incompatible with the presence of a $C3'_T$ methoxyl substituent. The analysis of the ^{13}C isotope distribution data supports this conclusion in several ways (Fig. 3). First, the mean $\delta^{13}C_g$ values for the aromatic ring is −26.7‰ (Table 1), compatible with literature values for aromatic amino acids from C_3 plants (39) but richer than those for acetyl-CoA–derived compounds (14). Second, the condensation of four units of acetate via acetyl-CoA generates a sequential pattern of two-carbon units (a pattern indicated as bold bonds in Fig. 2*A*) with the CH_3-derived $\delta^{13}C_i$ value higher than the (impoverished) C=O-derived value (43), due to the kinetic isotope effects associated with the pyruvate decarboxylase reaction (44). The resulting pattern in tramadol should be C1′>C2′, C3′>C4′, and C5′>C6′. This is clearly not the case (Fig. 3).

What evidence then can be put forward to support an origin from the shikimic acid pathway? Focusing first on the $\delta^{13}C_i$ pattern of the aromatic ring of tramadol, it is apparent that the $C4'_T$, $C5'_T$, and $C6'_T$ have values closely related to those obtained from vanillin and ferulic acid (16, 45, 46), derived from L-Phe, despite the different compounds having been obtained from

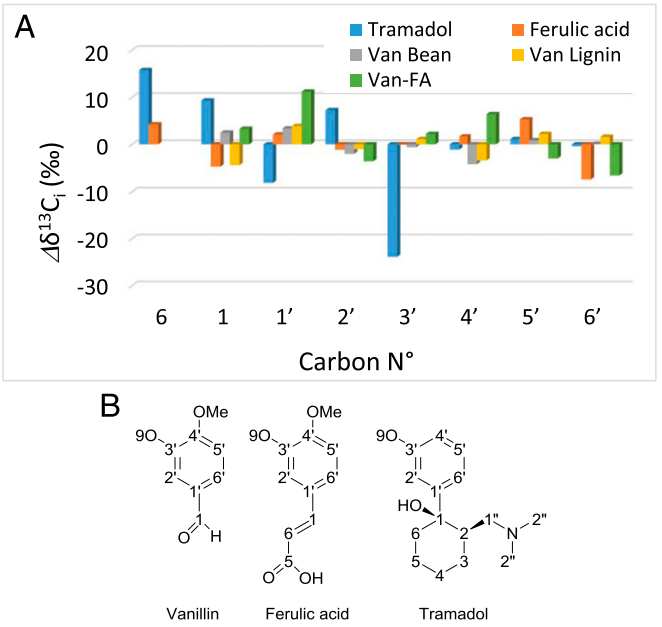

Fig. 3. Variation in $\Delta\delta^{13}C_i$ (‰) of the aromatic ring of tramadol from *N. latifolia*, ferulic acid from *Oryza sativa*, vanillin from *Vanilla planifolia* (Van bean), and vanillin synthesized from natural lignin from *Picea* sp. (Van lignin). (*A*) Values illustrated as the $\Delta\delta^{13}C_i$ relative to the mean for the positions being considered, because $\Delta\delta^{13}C_i$ expresses the deviation for a given position *i* independently of the value $\delta^{13}C_g$ (‰) for each molecule. Numbering as for tramadol. (*B*) Structures of tramadol, ferulic acid, and vanillin with carbon positions numbered as for tramadol. Additional data are from refs. 15, 44, and 45.

different plants and from different geographical areas. However, at the $C1'_T$, and $C2'_T$ positions, the match is less evident, whereas the $C3'_T$ is distinguished by being very impoverished relative to the equivalent position of the other 3-hydroxylated compounds. As, currently, neither isotopic nor enzyme mechanistic evidence is available for the *m*-hydroxylation, interpreting this observation requires further investigation. In particular, data from other *m*-hydroxylated compounds, notably the 3′-OH-Phe found in *F. rubra* (33), would be valuable. It might indicate a mechanism for 3-hydroxylation in tramadol biosynthesis different from the well-described 3-coumaroyl-CoA hydroxylase of phenylpropanoid metabolism (25), which require 4-hydroxylation before 3-hydroxylation. It should be noted that the 3-positioned O atom in vanillin and ferulic acid is in effect introduced by an *ortho*-hydroxylation relative to the first oxygen function in the 4-position. It has been shown from ^2H analysis that this can have fundamental effects on isotope patterns (11).

For the positions that are assumed to be derived from the nonaromatic part of the C_6–C_3 molecule, the enrichment of the $C6_T$ position relative to the $C1_T$ ($\Delta C6_T - C1_T = 6.5‰$) is consistent with the known enrichment in the carbon derived from the α-C (C2) of aromatic amino acids (47). An enrichment relative to the β-C (C3) of the same order is observed (Fig. 3) in natural ferulic acid ($\Delta C6_F - C1_F = 9.0‰$) (16) derived from L-Phe. Similarly, the $C1_T$ of both tramadol and natural vanillin show enrichment, whereas that in ferulic acid and lignin-derived vanillin do not. This is compatible with the involvement of a >C=O at the equivalent position to the $C1_T$ in the two former but not in the two latter compounds. However, an alternative option that must be considered is that the aromatic ring and $C1_T$ are derived from L-Phe via benzoic acid (Fig. 2*A*, option b) or even from an earlier intermediate of the shikimic acid pathway, as shown for gallic acid (48). These options will be further considered in the following paragraph.

Does the $^{18}O/^{16}O$ Isotope Ratio in Tramadol Isolated from the Root Bark of *N. latifolia* Support the Proposed Biosynthetic Pathway? The values of $\delta^{18}O$ have been obtained for three samples of pure tramadol, for the whole-root tissue from which the compound was obtained, and from other tissues of the same trees (Table 2). The values, in the range of 23.5–28.3‰, are typical for above-ground dry matter of C3 plants from a moderate to semiarid climate and meet the generally used isotopic discrimination Δ for cellulose of 27 ± 4‰ above leaf water (49). The slightly lower value for root and fruit may be due to either higher concentrations of other material [e.g., lignin that has $\delta^{18}O \sim 12‰$ (50)] or to more impoverished source water, respectively. Although the isotopic discrimination Δ of 27 ± 4‰ is also generally taken for carbonyl groups fully equilibrated with source water, for many carboxyl groups isotopic discrimination Δ values of 19 ± 2‰ are found (7).

In contrast, the oxygen atom of hydroxyl groups of aromatic compounds originate from atmospheric O_2 ($\delta^{18}O = 23.8‰$) and is introduced by monooxygenase reactions, accompanied by a KIE of ∼1.018, leading to $\delta^{18}O$ values of 6 ± 1‰. Water addition to >C=C< is catalyzed by lyases, implying large KIEs (>1.01), leading to $\delta^{18}O$ values of hydroxyl groups of more than −10‰ below water (51). Turning to the tramadol extracted from the root tissue, it is found that the values for the three samples are closely similar, giving a mean of 11.0 ± 0.3‰. According to the proposed biosynthetic biosynthesis of tramadol (Fig. 2), both oxygen atoms originate from precursor **1**: the methoxyl group on position $C3'_T$ by aromatic hydroxylation ($\delta^{18}O = 6‰$) and that in position $C1_T$ from water, either via the carboxyl group of a benzoic acid intermediate (Δ = 19‰) (7) or from the carbonyl group of precursor **1** itself (Δ = 27‰).

Data from northern Cameroon give shallow groundwater values of −2.42 ± 1.56‰ ($n = 19$) and deep groundwater values of −4.86 ± 0.55‰ ($n = 24$) (52), giving $\delta^{18}O$ values for dry matter closely compatible with those determined (Table 2). The $\delta^{18}O$ value of the source water in the roots of *N. latifolia* should be near to those of the groundwater, and from here, the $\delta^{18}O$ of the oxygen atom on position $C1_T$ of tramadol as a product of the plant roots should be correlated to that of the groundwater, by one or the other correlations given above. A calculation of the bulk $\delta^{18}O$ value of tramadol has to take into account the range limits of the source water and the alternative equilibrations with either a carboxyl or a carbonyl group, respectively. This yields in ‰, with the source of the oxygen atom on C-1 being as follows:

$$\text{A carboxyl group}: 0.5X(-2.4+19+6) = 11.3 \text{ or}$$
$$0.5X(-4.9+19+6) = 10.1;$$

$$\text{A carbonyl group}: 0.5X(-2.4+27+6) = 15.3 \text{ or}$$
$$0.5X(-4.9+27+6) = 14.1.$$

The mean bulk experimental value for tramadol of 11.0 + 0.3‰ (Table 2) agrees excellently with the reaction sequence b in Fig. 2*A*, the synthesis of **1** via degradation of the side chain of L-Phe to benzoic acid and its methoxylation. However, the first alternative reaction sequence (a in Fig. 2*A*) cannot be excluded, as the cyclization reaction of intermediate **4** (Fig. 2*B*) could imply an ^{18}O KIE contributing to an additional ^{18}O depletion in the case of incomplete conversion.

In contrast, an origin via malonyl-CoA would lead to a value closer to that of both oxygens being from water, giving $\delta^{18}O \sim 18‰$. Hence, from the bulk $\delta^{18}O$ analysis it can be clearly deduced that the oxygen atoms in tramadol have as origin one from water and one from O_2, in line with the proposed biosynthetic pathway. Positional $\delta^{18}O$ analysis should provide further evidence to support this deduction.

Conclusion

Evidence is presented that, for a natural product of unknown biosynthetic pathway, an analysis of the pattern of $\delta^{13}C_i$ values obtained by irm-^{13}C NMR can be used to differentiate between alternative putative biosynthetic schemes. Working from a plausible biosynthetic scheme (Fig. 2), the position-specific isotopic distribution provides crucial evidence that supports the proposed biochemistry, while inevitably leaving some questions unanswered. A close examination of the $\delta^{13}C_i$ values highlights significant positional differences that can largely be rationally interpreted in terms of biochemical reactions. Of particular interest is the convincing evidence presented by the methyl groups, which show relatively depleted values of $\delta^{13}C_i$, as to be expected for methyl groups introduced from SAM (53). In addition, the isotope data strongly indicate that a pathway from L-Lys and the aromatic amino acid pathway is credible, and that an origin for the aromatic ring from acetyl-CoA is not. Furthermore, the $\delta^{18}O$ values provide support to the pathway in terms of the putative origin of the two oxygen atoms.

The elaborated ^{13}C pattern of plant-extracted tramadol is fully compatible with a proposed biosynthetic origin of the compound. To provide direct evidence for tramadol biosynthesis in *N. latifolia* requires that the biosynthesis is probed in vivo, notably by showing the incorporation of ^{13}C-enriched precursors. The present study of the position-specific ^{13}C isotope distribution pattern and of the bulk ^{18}O values in the tramadol extracted from *N. latifolia* provides clear pointers as to how such feeding experiments should be approached and which precursors should be tested. Further analysis of field and synthetic samples is now needed to probe the biosynthetic route to this previously unknown natural product.

Methods

Materials. Commercial tramadol·HCl (99%, Product 42965) was purchased from Sigma-Aldrich (www.sigmaaldrich.com/). Acetone-d_6 was purchased from EurisoTop (www.eurisotop.com). Silica gel was obtained from Merck. All other reagents were of Analar quality or equivalent grade.

The samples of *Nauclea latifolia* Sm. wood and bark from roots from which the tramadol was extracted were collected in the Benoué National Park, Cameroon, a United Nations Educational, Scientific, and Cultural Organization-recognized biosphere reserve, in that part of the park in which livestock grazing and human habitation is prohibited. It should be noted that the park contains wild animals, including baboons and other monkeys.

Extraction and Purification of Tramadol. Tramadol was extracted from freshly collected root bark and wood as described previously (1). This extract was further purified by column chromatography on Si gel (63 mesh) eluted with AcOEt/MeOH (9:1) and crystallization of the pooled fractions from aqueous acetone.

Reference tramadol was obtained as a free base from commercial samples by extraction of a basic solution (5 g of tramadol·HCl in 50 mL of water with 1 g of $NaHCO_3$) with $CHCl_3$ (3× 1 vol). After drying the combined organic phases over anhydrous $MgSO_4$, the solvent was removed by exhaustive drying in vacuum.

Purified tramadol was rinsed in a small volume of water, and then the damp powder was taken into a minimal volume of ice-cold acetone and left for 30 min on ice until the appearance of white crystals, after which it was left for 48–96 h in the refrigerator (4 °C). The white crystals were collected and dried in vacuum. Purity of the final compound used for ^{13}C NMR was >99.5% as assessed by ^{1}H NMR.

Table 1. Values for $\delta^{13}C_g$ (‰) for different parts of the tramadol molecule obtained by irm-^{13}C NMR spectrometry

	$\delta^{13}C$, ‰	
Sample	Sigma	*N. latifolia*
N	4	2
$^{13}C\ \delta_{g(all)}$	−29.08	−31.15
$^{13}C\ \delta_{g(phe)}$	−25.38	−26.72
$^{13}C\ \delta_{g(lys)}$	−31.26	−30.23
$^{13}C\ \delta_{g(Me)}$	−38.42	−51.12

Table 2. Values for $\delta^{18}O$ (‰) measured by irm-EA/pyrolysis/MS for tramadol purified from three different root samples (L, 6, B) and for the dry matter obtained from various tissues of *N. latifolia*

	$\delta^{18}O$ (‰)	
Sample	Mean	SD
Fruit pericarp	23.47	0.31
Root total dry matter	26.51	0.07
Leaf dry matter	28.33	0.05
Leaf dry matter (deproteinated)	25.03	0.39
Root-derived tramadol (L)	10.68	0.46
Root-derived tramadol (6)	11.10	0.49
Root-derived tramadol (B)	11.21	0.30
Commercial tramadol (Sigma)	7.01	0.62

Isotope Ratio Monitoring by ^{13}C NMR Spectral Conditions. Samples were prepared by dissolving 200 mg of purified tramadol in 600 μL of acetone-d_6 and adding 10 mg of water. The resulting solution was thoroughly mixed and transferred to a 5-mm NMR tube via a filter (Pasteur pipette with cotton wool).

Quantitative ^{13}C NMR spectra were recorded on a Bruker 500 Avance III spectrometer fitted with a 5-mm-i.d. $^{13}C/^{1}H$ dual cryoprobe carefully tuned to the recording frequency of 125.76 MHz. The temperature of the probe was set at 303 K. The experimental parameters for spectral acquisition were the following (19): pulse width, 11.3 μs (90°); sampling period, 1 s. The offsets for both ^{13}C and ^{1}H were set at the middle of the frequency range for tramadol. Twenty-four scans were acquired using a repetition delay of 120 s leading to a signal-to-noise ratio of ∼650. Inverse-gated decoupling was applied to avoid nuclear Overhauser effect. The decoupling sequence used a cosine adiabatic pulse with appropriate phase cycles, as described previously (54). Each measurement consisted of the average of five independently recorded NMR spectra. In addition, a quantitative ^{13}C NMR reference spectrum was recorded on a Bruker 400 Avance III spectrometer fitted with a BBFO+ probe carefully tuned to the recording frequency of 100.62 MHz, which was used to calibrate the 500-MHz spectrometer (46, 55).

Chemical shifts were reported in parts per million (δ), with the residual acetone signal (δ_C 29.84) used as internal reference. Acetone-d_6 was used as deuterium lock. Free induction decay was submitted to an exponential multiplication inducing a line broadening of 1.4 Hz. The curve fitting was carried out with a Lorentzian mathematical model using Perch Software (Perch NMR Software; University of Kuopio, Kuopio, Finland).

Data were processed as described previously, and the $\delta^{13}C_i$ (‰) values were calculated from the reduced molar fraction f_i/F_i and the $\delta^{13}C_g$ (‰) obtained by isotope ratio measurement by mass spectrometry (10, 19).

Isotope Ratio Monitoring by Mass Spectrometry. The $\delta^{13}C_g$ (‰) values were measured using irm–elemental analyzer (EA)/MS. Approximately 1.0 mg of tramadol was weighed into each of two tin capsules (solids "light" 5 × 9 mm; Thermo Fisher Scientific) and the $^{13}C/^{12}C$ ratios measured using a Sigma2 mass spectrometer (Sercon Instruments, www.sercongroup.com) linked to a Sercon elemental analyzer. Isotope ratios [$\delta^{13}C_g$ (‰)] were expressed relative to $\delta^{13}C_{VPDB}$ (‰). The instrument was calibrated for $\delta^{13}C$ using the international reference materials NBS-22 ($\delta^{13}C_{PDB}$ = −30.03‰), SUCROSE-C6 ($\delta^{13}C_{PDB}$ = −10.80‰), and IAEA-CH-7 PEF-1 ($\delta^{13}C_{PDB}$ = −32.15‰) (International Atomic Energy Agency, Vienna, Austria), and instrumental deviation followed via a laboratory standard of glutamic acid.

The $\delta\ ^{18}O$ (‰) values were obtained by irm-EA/pyrolysis/MS in continuous flow as described in ref. 56. Approximately 1.0 mg of tramadol was weighed into each of three silver capsules, and the $^{18}O/^{16}O$ ratios were determined using a EuroVector EuroEA3028-HT system (EuroVector SpA) interfaced to an IsoPrime mass spectrometer working in continuous-flow mode. For each sample, the first value was discarded to avoid memory effects. Calibration was by the working standard NBS120c, calibrated against the international standard $\delta\ ^{18}O_{V\text{-}SMOW}$ (21.7‰).

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