Natural Products

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Occurrence of the Synthetic Analgesic Tramadol in an African Medicinal Plant**

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Initiating a research program aimed at discovering natural products as pain relievers, we focused our attention on the sub-Saharan *Nauclea latifolia* Sm. (Rubiaceae) plant, commonly known as African peach or pincushion tree. This plant has a long tradition of use by local populations for the treatment of a wide diversity of illnesses, including severe digestive problems, neurological disorders and infectious diseases.[1–3] In Cameroon, the plant is used to treat pain, malaria, fever, epilepsy, and infantile convulsions. We were particularly interested in the traditional use of the root bark of *N. latifolia* in pain relief. Previous phytochemical investigation of *N. latifolia* led to the identification of alkaloids, mostly naucleamides, as the main constituents.[4] Extracts from this plant have shown antipyretic,[5] antinociceptive,[5–7] anti-inflammatory[6] and antimalarial activities.[6] In this study, a fractionation of the crude extract, guided by the antinociceptive bioactivity, led to the isolation of a potent analgesic compound, which was identified as (±)-*cis*-2-[(dimethylamino)methyl]-1-(3-methoxyphenyl)cyclohexanol, commonly known by its international non-proprietary name (INN), tramadol. Tramadol was first manufactured by Grünenthal GmbH (Germany) and was brought into clinical usage in the late 1970s. It is now used worldwide for the treatment of moderate to severe pain without any known side effects.[8,9] It was designed by a simplification of the structure of morphine that kept the pharmacophoric elements responsible for the analgesic effect. Herein, we describe the isolation of tramadol from the root bark of *N. latifolia*, and the different methods used to prove the authenticity of its natural origin. The methanolic extract of the root bark of *N. latifolia* showed potent analgesic activity in an antinociceptive in vivo assay. To identify the active compounds, the extract was investigated through a bio-guided isolation procedure. The methanolic extract was submitted to HPLC fractionation using a reverse-phase column (Supporting Information, Figure 1 a). The resulting fractions were submitted to the anti-nociceptive assay, which revealed that the bioactivity resided within fractions F25 to F29, with a peak of activity in fraction F27 (Figure S1 b). F27 produced a dose-dependent (8, 16, or 32 mg kg^{-1}, oral administration) inhibition of acetic-acid-induced abdominal constrictions on mice (Table S1). The mean ID_{50} value for oral administration of F27 was 14.1 mg kg^{-1} (5.5–41.9, at a 95% confidence limit) and the maximal inhibition was 56.8% [$F(6, 78) = 101.42$; $p < 0.001$]. Naloxone partially antagonized the antinociceptive effect of this fraction (Figure S1 c). We further confirmed the analgesic

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activity of F27 using additional nociceptive tests on mice. Indeed, with this fraction, we observed significant effects in the formalin test (Figure S2a), the hot-plate test (Figure S2b and Table S1), the tail-flick test (Figure S2c and Table S2), and the glutamate-induced nociception test (Figure S3).

On further examination, the most active fraction was found to contain predominantly an oily yellow transparent compound (>95%). Its high-resolution ESI-TOF-MS (electrospray ionization time-of-flight mass spectrometry) exhibited a $[M+H]^+$ ion at m/z 264.1972. From these data the elemental composition was deduced to be $C_{16}H_{26}NO_2$, which corresponds to five double-bond equivalents. The ^{1}H NMR spectrum displayed four aromatic protons, with a pattern corresponding to a 1,3-disubstituted benzene ring. The presence of a methoxy group linked to the benzene ring was deduced from a signal at $\delta_H = 3.77$, with an integrated area corresponding to three protons. The complex spin system at high field ($\delta_H = 1.35$-2.65) suggested the presence of a cyclic hexyl chain in the molecule, which is in agreement with the degree of unsaturation attributed to the isolated compound. The ^{13}C NMR and DEPT spectra revealed the presence of three quaternary carbons ($\delta_C = 158.49$, 150.99, 75.68). The quaternary carbon at 75.68 ppm was assigned to an oxygenated sp^3 carbon atom on the basis of HRMS and both 1D and 2D NMR spectroscopy (Figure S4). The structure was therefore assigned as 2-(dimethylaminomethyl)-1-(3-methoxyphenyl)cyclohexanol (Figure 1a). A crystal of the

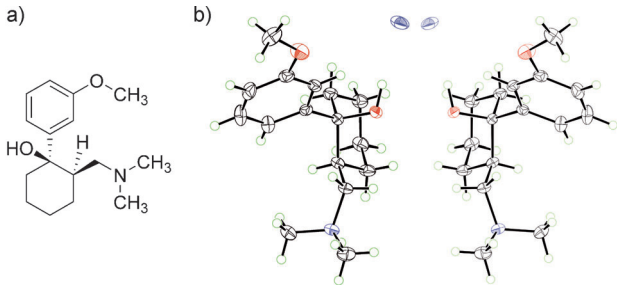

Figure 1. Chemical structure of the isolated anti-pain compound. a) Chemical structure of the natural compound. b) Crystal structure of the natural compound. ORTEP drawing of the two isomers in the cell packing. Thermal ellipsoids are drawn at the 20% probability level. Cl purple, N blue, O red.

hydrochloride salt of this compound was obtained in acetonitrile. Single-crystal X-ray crystallography confirmed the proposed structure and allowed the assignment of the relative stereochemistry of the two chiral carbon centers (Figure 1b). The isolated compound had no optical activity, as shown by measurements of the optical rotation ($[\alpha]_D^{25} = 0$ ($c = 1$, CH_2Cl_2)), thus indicating that it occurs as a racemic mixture. Based on X-ray diffraction analysis, the stereochemistry was confirmed to correspond to (±)-*cis*-2-[(dimethylamino)methyl]-1-(3-methoxyphenyl)cyclohexanol (Figure 1b). Surprisingly, this structure proved to be identical with that of a synthetic analgesic, tramadol. To unambiguously assess the enantiomeric composition of the natural compound, it was submitted to HPLC analysis on a chiral stationary phase. Each enantiomer of a sample of commercial tramadol, which is sold as the (±)-*cis* isomer, was obtained by chemical separation from a racemate, according to a well-established method.[10] The high enantiomeric excess of each enantiomer was demonstrated by HPLC on a chiral stationary phase (Daicel AD-H column; Figure 2a). When the extracted material was analyzed accordingly, it showed an elution profile (Figure 2b) identical with that observed for synthetic

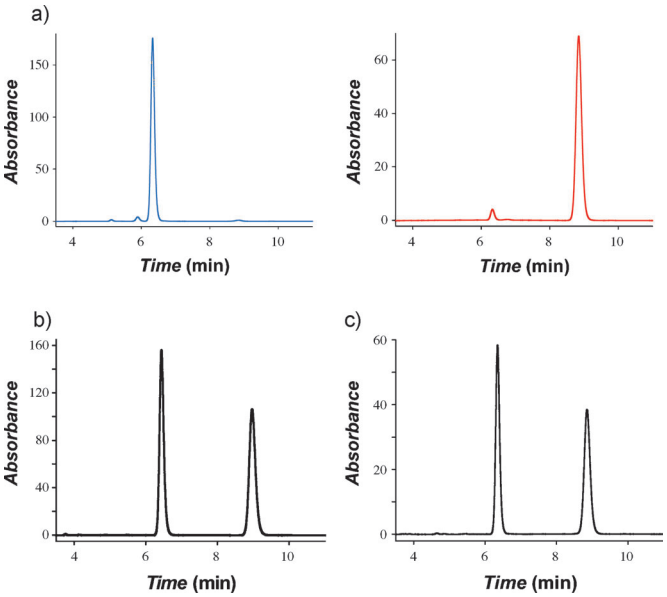

Figure 2. HPLC analysis of natural tramadol on a chiral stationary phase. a) HPLC profile of each enantiomer purified from racemic tramadol. b) HPLC profiles of commercial tramadol. c) HPLC profile of the isolated compound.

tramadol (Figure 2c). This indicates that the enantiomers are present in the same relative amounts in natural and synthetic tramadol. The racemic character was preserved regardless of the extraction procedure or solvent used. In addition, we submitted the (+)-*cis* isomer to incubation in ethanol for 24 h, both with and without heating, and observed no racemization, which indicates that the extraction procedure had no influence on the racemic character of the natural compound. Relatively few natural products occur as racemic mixtures (less than 1% of the metabolome of the biosphere), but nevertheless they do occur (for example, anabasine) and their occurrence is supported by appropriate biogenetic mechanisms.[11] We are currently working on validating a plausible biosynthetic pathway (Figure S6) that involves the formation of two key intermediates, an acetophenone (part A) and an enaminopentanal (part B). Moiety **A** could originate from either acetyl CoA or phenylalanine, whereas moiety **B** could be derived from lysine. A cascade of reactions, involving aldolization/crotonization, addition, and reduction, would provide tramadol with the racemates being produced during the cyclization step.

This unexpected discovery prompted us to validate our findings by three independent laboratories, working on three different samples collected at three different periods of the

year. All results converge and confirm the occurrence of tramadol in the root bark of *N. latifolia*. Tramadol could not, in contrast, be detected in any of the aerial parts of the plant (leaves, trunk, and branches). The presence of basic nitrogen within the structure of the isolated compound confers its alkaloidal characteristics. Hence, our extraction was simplified by macerating the root bark of *N. latifolia* in ethanol followed by an alkaloid-specific extraction procedure (see the Supporting Information). The sample obtained was profiled by high resolution ultrahigh-performance liquid chromatography (UHPLC)-TOF-MS. An intense LC peak at R_t = 5.91 min exhibited an $[M+H]^+$ signal of 264.1971, which corresponds to the molecular formula of tramadol (Figure 3a). Injecting the isolated compound and synthetic tramadol under the same LC conditions resulting in them eluting with the same R_t, thus confirming the presence of tramadol in the crude extract (data not shown). In addition, a series of alkaloids already described in *N. latifolia*[4,6] were also detected in this extract: nauclechine demethoxycarbonyl ($[M+H]^+ = 306.1609$; R_t = 7.35 min), nauclefine ($[M+H]^+ = 288.1113$; $R_t = 8.30$ min), vinconsamide ($[M+H]^+ = 499.2101$; $R_t = 9.41$ min), and naucleamide E ($[M-H]^- = 337.1554$; $R_t = 11.73$ min). The molecular formulae of all these alkaloids were ascertained based on the high mass accuracy of the TOF-MS detection (< 5 ppm), a comparison of the isotope peak ratios, and heuristic filtering according to the method of Funari and collaborators[12] (Figure S5).

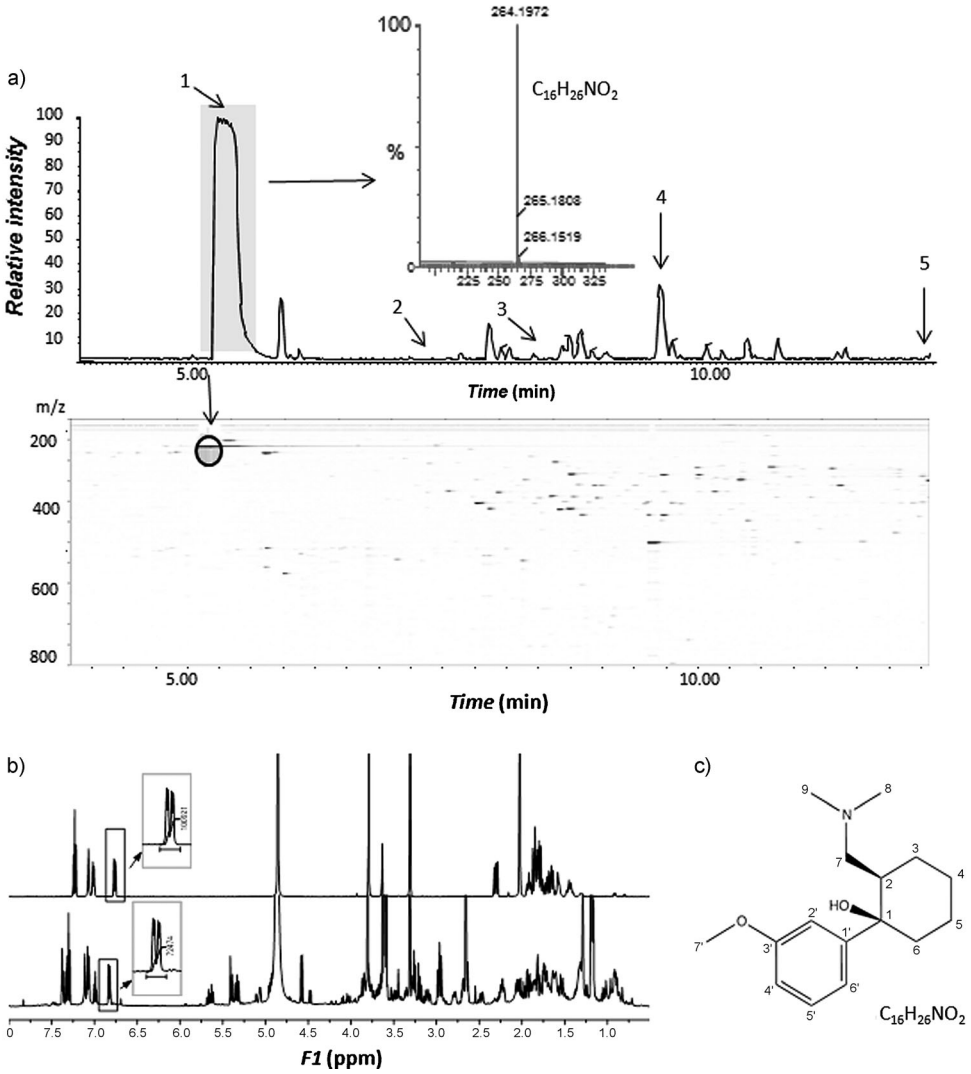

Figure 3. NMR analysis and UHPLC-TOF-MS profiling of the crude extract from *N. latifolia* for identification and quantification of tramadol. a) UHPLC-TOF-MS profiling of the crude ethanolic extract of *N. latifolia* with a label for compounds dereplicated (zoomed into the 0–12 min retention domain). Top panel: TOF-MS spectra of tramadol in the crude extract. Bottom panel: 2D ion map of the crude extract of *N. latifolia* displaying all recorded ions. b) The absolute integration of the ^{1}H NMR signal at $\delta_H = 6.77$ (ddd, 8.0, 2.6, 0.9 Hz, H-4′) of commercial tramadol in a CD_3OD solution at 263.4 mM was used as an external reference (top panel) to quantify the amount of natural tramadol in an ethanolic extract of *N. latifolia* (bottom panel) using the PULCON method. c) Structure of tramadol.

To further exclude the possibility of an unintentional cross-contamination of the root-bark samples of *N. latifolia* with synthetic tramadol, we purposely isolated a root bark sample from the inner core of the bark and confirmed the presence of the natural compound with similar yields.

Finally, a last attempt was made to dismiss the very unlikely possibility that the tramadol obtained from the root bark of *N. latifolia* was due to contamination with synthetic tramadol. We compared the $^{15}N/^{14}N$ and $^{13}C/^{12}C$ isotope ratios in two independent extracts of natural tramadol and in four samples of tramadol obtained from two different commercial sources. The results are presented in Table 1.

Negligible differences can be seen between the natural and commercial samples on the basis of the $\delta^{13}C$ values, which indicates that the majority of the carbon atoms in the commercial samples probably derive from a C_3 plant biological origin. In contrast, a range of values are found for the $\delta^{15}N$ values, depending on the source of the sample. The wide range of $^{15}N/^{14}N$ ratios in the commercial tramadol could

Table 1: Isotope ratios $^{15}N/^{14}N$ and $^{13}C/^{12}C$ in extracts of natural tramadol and in samples of commercial tramadol.

Sample	$\delta^{15}N$ [ppt]	Range	$\delta^{13}C$ [ppt]	Range
commercial 1-1	−2.61	0.05	−29.97	0.15
commercial 1-2	−9.24	0.84	−29.73	0.20
commercial 1-3	5.68	0.30	−29.61	0.12
commercial 2-1	−1.79	0.10	−31.97	0.10
natural 1	−3.22	0.45	−32.68	0.10
natural 2	−3.13	0.23	−31.98	0.04

result from the use of different batches of methylamine for its synthesis, as found for the synthesis of *N*-methyl-3,4-methyldioxyphenylisopropylamine (MDMA, ecstasy).[13] Although the natural samples of tramadol do not fall within a distinctly different range of $\delta^{15}N$ values, they both have the same isotope ratio, which is different from all the values obtained for the commercial samples. As these two samples 1) were sourced from two different plant collections, 2) had a one-year interval between extractions, 3) were collected by different people, and 4) were extracted from the inner core of the bark, the likelihood that a source of synthetic tramadol contaminated all these *N. latifolia* samples can be considered negligible.

To estimate the quantities of tramadol in the root bark of *N. latifolia* and further confirm its presence by an orthogonal analytical method, the ethanolic extract was submitted to metabolite fingerprinting by NMR spectroscopy. The extract was directly and completely solubilized in CD_3OD and a synthetic tramadol sample was analyzed under the same conditions. In the NMR spectrum of the crude ethanolic extract recorded in a phosphate buffer (pH 6, to avoid NMR shifts), the characteristic signals of the aromatic proton resonances $\delta_H = 6.9$ (ddd, $J = 8.0$, 2.5, 0.7 Hz, 1H, H6′), 7.10 (m, 2H, H2′, H4′), 7.37 (t, $J = 8.0$ Hz, 1H, H5′) were clearly seen to be aligned with the signals of the synthetic tramadol (Figure 3*b*). As NMR spectroscopy provides an absolute quantitative method, it was used with optimized parameters according to the pulse-length-based concentration (PULCON) method, to provide a good estimate of the amount of compound in the root bark. The quantitative measurement was performed by comparing the integration of the isolated H-6′ signal in the extract with the corresponding signal of tramadol used as an external standard. This indicated a tramadol concentration of 3.9% (w/w) in the ethanolic extract, which gives a content of 0.4% (w/w) in the dried root bark (Figure 3b, c).

In conclusion, based on an isolation guided by antinociceptive bioactivity, we have made the surprising discovery that *N. latifolia* root bark contains a high level of tramadol, a synthetic drug, largely used as an analgesic. This finding justifies the traditional use of this medicinal plant for pain relief. Complete structural elucidation of tramadol was performed by a combination of HRMS, 1D and 2D NMR experiments, and X-ray crystallography. Its natural origin was confirmed by LC-MS and NMR spectroscopic profiling of the crude extract, and by isotope ratio analysis. Although the data obtained by this arsenal of analyses can be confidently used to show the natural presence of tramadol in the root bark of *N. latifolia*, further evidence to complement and strengthen the present findings will be sought. This may well include 1) probing the biosynthetic pathway in plants by feeding isotopically-labelled putative precursors, 2) isolation of precursors proposed to be involved in the biosynthetic pathway, and 3) measuring the site-specific $^{13}C/^{12}C$ and $^{2}H/^{1}H$ isotope ratios to look for the isotopic distribution patterns characteristic of natural products.[14,15]

Our results validate the traditional usage of decoctions of root bark of *N. latifolia*, but not of other parts of this plant for the treatment of pain. Furthermore, within an ethnopharmaceutical context, they should provide consumers with a better understanding of how to use these extracts, and indicate that the plant can be considered as an inexpensive and readily available source of tramadol. This is the third reported case of the occurrence of a synthetic and clinically used drug that has been identified in natural sources. Former cases include clinically used benzodiazepines, which were isolated from plants, albeit at trace concentrations,[16,17] and fluorouracil, an anticancer drug obtained from the marine sponge *Phakellia fus*ca.[18] In addition, a number of other cases have been reported, in which synthetic flavonoids and alkaloids (obtained during total synthesis) were subsequently discovered in natural sources, but these compounds were not used as drugs or end-products of synthesis. To the best of our knowledge, however, this is the first reported case of the occurrence of a synthetic drug present at clinically relevant concentrations in a plant source. Finally, it should be highlighted that up to ten species of *Nauclea* are known in Africa, which suggests that a screening of all species to ascertain the presence or absence of tramadol would be valuable.

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