

Direct Identification and Quantitative Determination of Costunolide and Dehydrocostuslactone in the Fixed Oil of *Laurus novocanariensis* by ^{13}C -NMR Spectroscopy

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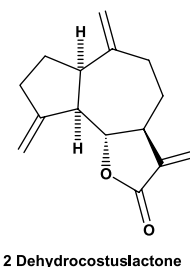
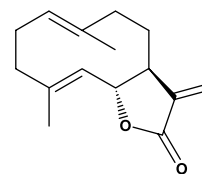
The fixed oil of *Laurus novocanariensis* (previously *L. azorica*) contains mostly glycerides together with minor non-saponifiable compounds. The direct identification and quantitative determination of costunolide and dehydrocostuslactone, two sesquiterpene lactones components of the oil that exhibit biological activities, is described. The analysis was carried out using ^{13}C -NMR spectroscopy (signal acquisition with inverse gated decoupling of protons; diglyme as internal standard) without separation, derivatisation or any sample preparation. Copyright © 2005 John Wiley & Sons, Ltd.

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INTRODUCTION

The leaves of *Laurus azorica*, harvested from laurels endemic to the macaronesian archipelagos of Madeira, Azores and Canaries, are much used in traditional medicine owing to attributed anti-ulcer and blood depurative properties. Recent publications have shown that these laurels exhibit anti-thrombin activity (Medeiros *et al.*, 2000). The hexane extract of the leaves contains two lactones, costunolide (**1**) and dehydrocostuslactone (**2**) (Tinoco, 2000) that are known to possess plant growth regulatory and cytotoxic properties (Sun *et al.*, 2003). These sesquiterpene lactones, which are mainly found in the Asteraceae but also occur infrequently in other families of higher and lower plants, have recently received renewed attention since both **1** and **2**, isolated from bay leaves (*L. nobilis*), were found to inhibit inducible nitric oxide synthase (iNOS; Matsuda *et al.*, 2000) and, furthermore, **1** was shown to have inhibitory effects on blood-ethanol elevation (Matsuda *et al.*, 2002), to inhibit the RAS-farnesyl-protein-transferase (Park *et al.*, 2001), to induce differentiation in human leukaemia HL-60 cells (Choi *et al.*, 2002a), and to trigger apoptosis in human leukaemia U937 cells (Choi *et al.*, 2002b).

Until 2002, it was considered that the laurels endemic to the archipelagos of Madeira, Azores and Canaries constituted a single species referred to as *Laurus azorica* (Seub.) Franco. Recently it was determined that there



were sufficient distinctions to consider the laurels from Madeira and Canaries as a separated taxon, now classified as *Laurus novocanariensis* Rivas Mart., Lousã, Fern. Prieto, E. Días, J. C. Costa and C. Aguiar (Rivas-Martínez *et al.*, 2002).

On the island of Madeira, the fixed oil, traditionally obtained by boiling and crushing the fruits (drupes) of *L. novocanariensis*, is used in external application for its cicatrising and anti-rheumatic properties and it is taken internally for the treatment of apoplexy (Rivera and Obón, 1995). The main fatty acid chains present in the oil have been analysed by GC after *trans*-esterification, and were found to be oleic, palmitic, linoleic and lauric in descending order of abundance (P. Castilho, unpublished results). The oil also contains the sesquiterpene lactones costunolide (**1**) and dehydrocostuslactone (**2**)

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and these have been isolated by a combination of CC and TLC (see Experimental section).

The aim of the present work was to establish a method, based on the analysis of the ^{13}C -NMR spectrum, that would allow the direct identification and quantitative determination of the lactones **1** and **2** without previous separation from the principal constituents (glycerides) of the fixed oil of *L. novocanariensis*.

EXPERIMENTAL

Fractionation of the oil and isolation of the lactones.

Commercial samples of the fixed oil from *L. novocanariensis* were prepared and supplied by local producers on the island of Madeira. A sample was submitted to column chromatography over silica gel (200–500 μm) eluted with pentane and increasing amounts of diethyl ether (100:0 to 0:100) to yield six fractions, *F1*–*F6*. Fraction *F1*, which eluted with pentane, contained terpene hydrocarbons; *F2* and *F3* were composed exclusively of triacylglycerides (TG); and fraction *F4* contained, apart from some TG, mainly 1,2- and 1,3-diacylglycerides (DG). Although still containing some glycerides (TG, 1,2- and 1,3-DG), the ^{13}C -NMR spectrum of *F5* exhibited the characteristic signals of costunolide (**1**) and dehydrocostuslactone (**2**) (Jacobsson *et al.*, 1995; Yuuya *et al.*, 1999). Fraction *F6* appeared to be a complex mixture of TGs and DGs, whilst monoacylglycerides (MGs) were detected in small amounts together with other minor components. Further purification of *F5* by preparative TLC yielded the isolated lactones **1** and **2** in sufficient quantities to record ^{13}C -NMR spectra. Identifications were established by comparison of the chemical shifts with those reported in the literature (Jacobsson *et al.*, 1995; Yuuya *et al.*, 1999).

Liquid–liquid partition. A sample of the fixed oil (50.19 g) was stripped of its volatile components by hydrodistillation in a Clevenger-type apparatus to yield 5.0 g of an odourless residue containing the lipids and other non-volatile compounds. The residue was dissolved in *n*-hexane and filtered through activated charcoal to remove the chlorophylls. The filtrate was extracted with methanol (4 \times 20 mL). The portion that was insoluble in methanol contained lipid components. The combined methanolic extracts were dried under vacuum at room temperature in a rotary evaporator, the residue taken up in 10 mL of acetonitrile and the solution washed with *n*-hexane (3 \times 10 mL) to remove fats and waxes. The acetonitrile solution was dried under vacuum at room temperature in a rotary evaporator and the residue (2.62 g; 5.2% w/w of the fixed oil) taken up in 1 mL methanol and analysed by TLC. The methanolic fraction contained mainly the lactones **1** and **2**.

^{13}C -NMR analysis. All ^{13}C -NMR spectra were recorded on a Bruker (Wissembourg, France) AC 200 Fourier transform spectrometer, operating at 50.323 MHz for ^{13}C , equipped with an Aspect 3000 computer and a 10 (or 5) mm probe. The spectra were recorded in deuteriochloroform (CDCl_3) and all shift values (δ) were referred to the internal standard (TMS). The spectra of the six chromatographic fractions were recorded with the following parameters: pulse width (PW), 5 (or 3) μs (flip

angle 45°); acquisition time, 1.3 s; relaxation delay (D_1), 2 s (total recycling time 3.3 s) for 32 K data table with a spectral width (SW) of 12,500 Hz (250 ppm); composite phase decoupling (CPD) of the proton band; and digital resolution, 0.763 Hz/pt. Exponential line broadening multiplication (LB = 1 Hz) of the free induction decay (FID) was applied before Fourier transform. The number of accumulated acquisitions was 3000–5000 depending on the quantity of product: 200 (or 70) mg of the mixture in 2 (or 0.5) mL CDCl_3 .

The quantitative analysis of two samples of fixed oil of *L. novocanariensis* (188–296 mg of oil; 2 mL CDCl_3 ; 10 mm probe) was carried out using the Bruker micro-program (signal acquisition with inverse gated decoupling of protons, pulse width = 90°; pulse delay $5 \times T_1 = 25$ s).

T_1 measurements. The longitudinal relaxation delays of the ^{13}C nuclei (T_1 values) of **1** were determined by the inversion-recovery method using the standard sequence: $180^\circ - \tau - 90^\circ - D_1$, with a relaxation delay D_1 of 20 s.

RESULTS AND DISCUSSION

Identification of sesquiterpene lactones in the fixed oil from drupes of *L. novocanariensis*

The most important signals in the ^{13}C -NMR spectrum of a sample of fixed oil of *L. novocanariensis* (Fig. 1) corresponded to fatty acid chains. A comparison of the signal intensities of the olefinic carbons of the unsaturated fatty acids confirmed that the oleic chain (C18:1 $\Delta 9\text{Z}$) was predominant over the linoleic (C18:2 $\Delta 9,12 \text{ZZ}$) chain, and that the linolenic chain (C18:3 $\Delta 9,12,15 \text{ZZZ}$) was present in only very small amounts. These results are in good agreement with those obtained by GC analysis of the *trans*-esterified oil (P. Castilho, unpublished results). Characteristic signals of TGs and 1,3-DGs (in a ratio of approximately 96:4) were observed together with signals associated with trace amounts of 1,2-DG. Signals corresponding to minor components, assignable to the lactones costunolide (**1**) and dehydrocostuslactone (**2**) were also detected (Fig. 1). Using the chemical shift values determined from the analysis of the purified sesquiterpene lactones (see the Experimental section), it was possible to detect all of the carbon signals associated with the two lactones in the ^{13}C -NMR spectrum of the total fixed oil, thus permitting their identification without the need for purification.

Quantitative analysis of **1** and **2**

A further aim of the study was to develop a method for the quantification of the lactones **1** and **2** by direct ^{13}C -NMR analysis of the vegetable oil without performing a previous separation. Although several techniques can be employed for the ^{13}C -NMR quantification of mixed components, the most common pulse sequence was chosen for the quantitative determination. This consists of a 90° pulse angle, and a delay between pulses that was five times longer than the largest longitudinal relaxation time (T_1) associated with the inverse gated decoupling of the protons (Mooney, 1989). This method allows the total

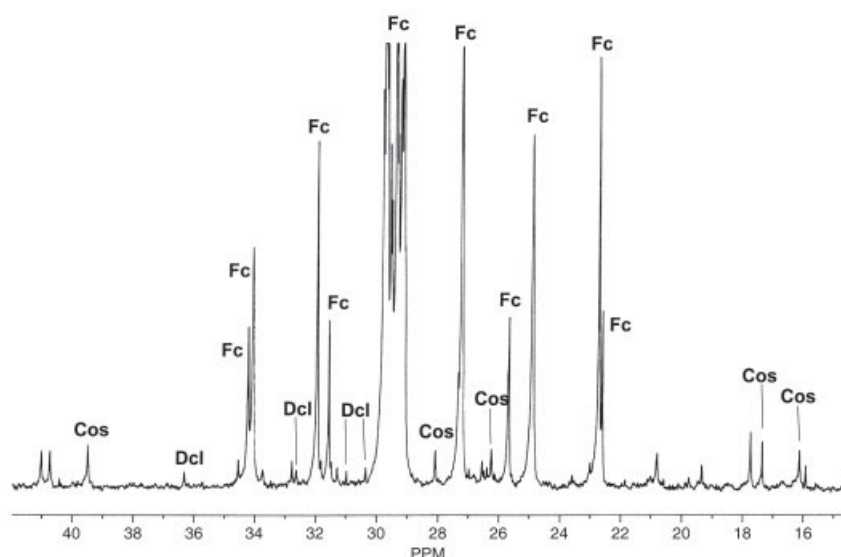


Figure 1. Portion of the ^{13}C -NMR spectrum of the fixed oil of *Laurus novocanariensis*. Peaks labelled 'Cos' are associated with costunolide (**1**), those labelled 'Dcl' with dehydrocostuslactone (**2**), and those labelled 'Fc' with fatty acid chains.

Table 1. ^{13}C -NMR chemical shifts (δ , ppm) of costunolide (**1**) and dehydrocostuslactone (**2**) and relaxation times (T_1) of the protonated carbons of **1**

Carbon	1		2
	(δ , ppm)	T_1 (s)	(δ , ppm)
1	127.26	2.2	47.61
2	26.20	1.2	30.31
3	39.46	2.2	32.61
4	141.51	—	151.24
5	127.05	2.2	52.02
6	81.95	3.5	85.29
7	50.41	4.0	45.12
8	28.05	2.2	30.94
9	40.99	1.4	36.28
10	136.96	—	149.23
11	140.07	—	139.74
12	170.55	—	170.33
13	119.71	1.2	120.23
14	16.12	4.5	109.61
15	17.36	5.0	112.62

relaxation of all of the carbon atoms as well as the suppression of the nuclear Overhauser effect (NOE effect). Only the signals of the protonated carbons were taken into account. Diglyme was used as internal standard since (i) the NMR signals of this molecule did not overlap with those of the lactones to be quantified, and (ii) the relaxation times of the protonated carbons in **1**, chosen as a model, were between 1.2 and 5.0 s (Table 1) and compatible with those of diglyme (the methylene carbons exhibit $T_1 = 3.8$ s).

The determination of the mass of each lactone (m_L) in the sample was calculated from equation (1):

$$m_L = \frac{2I_L \times M_L \times m_D}{I_D \times M_D} \quad (1)$$

where I_L is the mean value of the intensities of the signals belonging to the protonated carbons C1–C3, C5–

C9 and C13–C15 of the considered lactone, M_L is the molar mass of the considered lactone (232 g/mol for **1** and 230 g/mol for **2**), m_D is the mass (mg) of diglyme, I_D is the mean value of the intensities of the signals of the two methylene carbons of diglyme, and M_D is the molar mass of diglyme (134 g/mol). The factor 2 has to be introduced because of the symmetry of the molecule of diglyme.

Two samples of the fixed oil of *L. novocanariensis* obtained from different local producers, were submitted to NMR analysis. The masses of costunolide (**1**) m_{COS} and dehydrocostuslactone (**2**) m_{DCL} were determined for the first oil sample using data obtained from three independent ^{13}C -NMR experiments in order to ensure the precision of the analysis. The mean values ($n = 3$) for the contents of the lactones in the oil (expressed as percentages) were 3.8% for **1** (standard deviation 0.1) and 1.5% for **2** (standard deviation = 0.2). As expected, the precision of the measurement was better for **1**, the more abundant lactone, than for **2** (Table 2). The contents of the two lactones were slightly different for the second sample of oil, being 3.4 and 1.4%, respectively.

These findings are in very good agreement with results derived from the much less accurate method for determining total sesquiterpene lactones involving liquid–liquid partition of the oil between *n*-hexane and methanol. This partition gave $5 \pm 0.5\%$ of a methanolic extract that consisted mainly of lactones (see Experimental section): the ^{13}C -NMR signals of the lactones were totally absent from the spectrum of the *n*-hexane fraction.

The contents of **1** and **2** in the fixed oil of *L. novocanariensis* are quite significant compared with the amounts present in the methanolic extract from bay leaves (*L. nobilis*) that are, respectively, 0.9 and 0.04% (Matsuda *et al.*, 2000). However, the levels of **1** and **2** determined in *L. novocanariensis* are considerably lower than those in *Costus* resinoid, the commercially available extract from *Costus* roots (*Saussurea lappa*), which is the usual source of these lactones, 9 and 18.4%, respectively (Kobayashi *et al.*, 2001).

Table 2. Quantitative determination of costunolide (1) and dehydrocostuslactone (2) in the fixed oil of *L. novocanariensis* using ¹³C-NMR spectroscopy and equation (1)

Parameter ^a	Experiment 1	Sample 1 Experiment 2	Experiment 3	Sample 2 Experiment 1
Mass of oil (mg)	188.1	221.0	194.6	296.5
m_D (mg)	14.0	22.1	18.1	6.2
I_D	1.48	3.96	2.63	0.76
I_{COS}	0.22	0.45	0.30	0.36
m_{COS} (mg)	7.2	8.6	7.1	10.2
% _{COS}	3.8	3.9	3.7	3.4
I_{DCL}	0.10	0.16	0.11	0.15
m_{DCL} (mg)	3.2	3.0	2.6	4.2
% _{DCL}	1.7	1.4	1.3	1.4

^a m_D , mass (mg) of diglyme; I_D , mean value of the intensities of the signals of the two methylene carbons of diglyme; I_{COS} and I_{DCL} , mean values of the intensities of the signals belonging to the protonated carbons (C1–C3, C5–C9, C13–C15) of costunolide (1) and dehydrocostuslactone (2), respectively. m_{COS} and m_{DCL} , mass of 1 and 2 in the sample; %_{COS} and %_{DCL}, percentages of 1 and 2 in the sample.

The present study shows that ¹³C-NMR spectroscopy can be used for the identification and quantification of costunolide and dehydrocostuslactone in the fixed oil of *L. novocanariensis* without the need for the separation of the saponifiable and non-saponifiable components, or derivatisation, or indeed any sample preparation.

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