Two New Sesquiterpene Lactones from the Leaves of *Laurus nobilis*

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As a part of our ongoing interest in new bioactive compounds from natural sources, we studied *Laurus nobilis* (Lauraceae). This plant is widespread in the Mediterranean area and is used for medicinal and economic purposes. Chromatographic separations on active extracts led to the isolation of two new sesquiterpene lactones, 5a,9-dimethyl-3-methylene-3,3a,4,5,5a,6,7,8-octahydro-1-oxacyclopenta[*c*]azulen-2-one (1) and 3β-chlorodehydrocostuslactone (2). The structures of the new compounds were identified by 1D and 2D NMR experiments, as well as high resolution mass spectrometry. The cytotoxic activity was also evaluated against three different tumor cell lines of human origin.

Key words *Laurus nobilis*; Lauraceae; sesquiterpene lactone; cytotoxic activity

Laurus nobilis L. (Lauraceae) is an evergreen tree widespread in the Mediterranean area and Europe, and as a folk medicine, the decoction or tea of bay leaves is often used as a carminative, intestinal and gastric antispasmodic, against diarrhea, for rheumatic pains, in diseases of the respiratory tract, as a cough sedative, to treat asthma and cardiac diseases,[1—3] and in cases of dysmenorrhea (pers. comm.).

Previous phytochemical investigations have led to the isolation of several classes of secondary metabolites of laurel leaves, particularly sesquiterpene lactones,[4,5] alkaloids,[5,6] monoterpene and germacrane alcohols,[5,7,8] catechin and procyanidine derivatives,[5,9] glycosylated flavonoids,[10] and megastigmane glucosides.[11] As well as sesquiterpene lactones, laurenobiolide[12] and costunolide[13] have been identified as the major compounds of *L. nobilis*. Many of these lactone derivatives have been shown to possess various pharmacological effects, with antimicrobial,[14,15] immunomodulating[16] and cytotoxic activities.[17] Three costunolide and germacranolide derivatives, isolated from *L. nobilis*, were recently shown to be able to induce apoptotic chromatin condensation in leukemia cells (HL-60),[18,19] indicating their possibility as leads in the development of new classes of antileukemic drugs. For this reason, we considered this plant in our ongoing studies on antiproliferative agents from vegetal sources. Sequential extracts were prepared using solvents in increasing polarity (petroleum ether, chloroform and methanol), and residues were used to perform preliminary cytotoxic tests against two different cell lines. Activity against Jurkart cell line was established in petroleum ether (PEE) and chloroform extracts, with an IC_{50} of 9.1 and 10.2 μg/ml respectively. Only PEE was active against LoVo with an IC_{50} of 15.1 μg/ml. Fractionation of the extracts led to the isolation of five sesquiterpene lactones, two of which (**1**, **2**) are new natural products.

Compound **1** was obtained as colorless needles. The high resolution (HR)-MS [atmospheric pressure ionization-time of flight (API-TOF)] spectrum showed a protonated molecular ion $[M+H]^+$ at *m/z* 233.1524, corresponding to a molecular formula of $C_{15}H_{20}O_2$. The IR spectrum showed the presence of a γ-lactone ring (1765 cm^{-1}). The ^{1}H-NMR spectrum showed two doublets (each, J=0.8 Hz) at δ 5.33 and 6.10, ascribable to an exocyclic methylene group, and a broad singlet at δ 5.00. Two singlets, each integrating for three protons, were observed at δ 1.10 and 1.47, indicating the presence of two methyl groups (Table 1). The ^{13}C-NMR data revealed the presence of fifteen carbon atoms. Quaternary carbon resonances were obtained by comparison of heteronuclear multiple quantum coherence (HMQC) and ^{13}C data. A carbonyl function at δ_C 171.8 and two quaternary olefin carbons at δ_C 136.8 and 145.9 were observed. Other quaternary resonances were observed at δ_C 74.2 and 46.8. Six methylene groups were observed in HMQC spectrum, one linked to sp^2 carbon resonance at δ_C 124.0 (δ_H 5.33, 6.10) and five to sp^3 carbon resonance at δ_C 43.0 (δ_H 1.43—1.65), 26.8 (δ_H 1.73), 26.5 (δ_H 1.35—1.50), 24.0 (δ_H 1.48), and 30.3 (1.82—1.90). A –CH– group was also observed at δ_C 43.2 (δ_H 2.73). Based on these findings and the index of hydrogen deficiency given by the molecular formula, **1** was suggested to be a tricyclic sesquiterpene lactone. Diagnostic heteronuclear multiple bond connectivity (HMBC) correlations (Fig. 1) were observed between H-13 (δ_H 5.33, 6.10) and C-2 (δ_C 171.8), C-3 (δ_C 145.9) and C-3a (δ_C 43.2). Further correlations were ob-

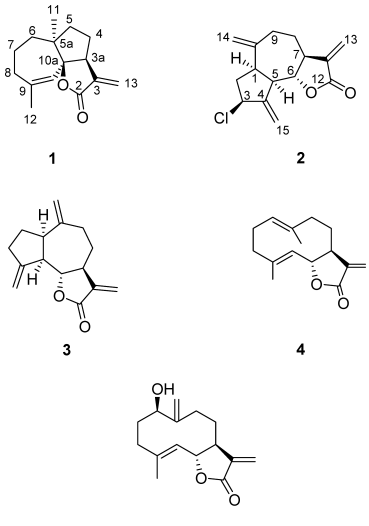

Chart 1. Structures of Isolated Compounds

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Table 1. ^{1}H- and ^{13}C-NMR Data of Compounds **1** and **2** in CD_3OD[a)]

Position	**1**		**2**	
	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
1	—	—	2.92 m	51.9
2	—	171.8	2.15 m 2.30 m	40.9
3a	2.73 dd (10.2, 4.3)	43.2	—	—
3	—	145.9	4.55 dd (2.0, 8.0)	73.8
4	1.35—1.50 m	26.5	—	152.4
5	1.43—1.65 m	43.0	2.83 m	46.4
5a	—	46.8		
6	1.73 m	26.8	4.10 dd (10.0, 10.0)	85.8
7	1.48 m	24.0	2.85 m	45.7
8	1.82—1.90 m	30.3	1.48—2.26 m	32.3
9	—	136.8	2.16—2.51 m	36.4
10	5.00 br s	122.0	—	148.0
10a	—	74.2	—	—
11	1.10 s	25.3	—	139.6
12	1.47 br s	24.0	—	170.6
13	5.33 d (0.8) 6.10 d (0.8)	124.0	5.49 d (2.3) 6.21 d (2.3)	122.3
14			4.94 d (2.0) 5.00 d (2.0)	113.2
15			5.33 d (1.0) 5.46 d (1.0)	116.4

a) Assignments were confirmed by DQF-COSY, HMQC and HMBC experiments.

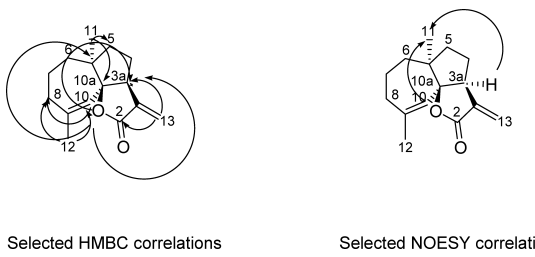

Fig. 1. HMBC and NOESY Correlations for Compound **1**

served between H-3a (δ_H 2.73) and C-2 (δ_C 171.8), C-3 (δ_C 145.9) and C-13 (δ_C 124.0) indicating the presence of the penta-atomic lactone ring. More HMBC correlations were observed between CH_3-11 (δ_H 1.10) and C-5a (δ_C 46.8), C-5 (δ_C 43.0) and C-10a (δ_C 74.2). The latter correlation establishes the link between positions C-5a and C-10a. From a correlation spectroscopy-double quantum filtered (COSY-DQF) spectrum the connectivities between the signals H-3a (δ_H 2.73), H-4 (δ_H 1.35—1.50) and H-5 (δ_H 1.43—1.65) were obtained supporting the presence of a second penta-atomic ring. Other long-range correlations were observed from CH_3-12 (δ_H 1.47) and C-9 (δ_C 136.8), C-10 (δ_C 122.0) and C-8 (δ_C 30.3) and between the H-10 (δ_H 5.00) and C-5a (δ_C 46.8) C-8 (δ_C 30.3) and C-3a (δ_C 43.2). In addition the COSY-DQF data revealed the connectivity between signals H-6 (δ_H 1.73), H-7 (δ_H 1.48) and H-8 (δ_H 1.82—1.90). Those data suggested the presence of the hepta-atomic ring. Data about the relative stereochemistry of the compound were obtained from the nuclear overhauser effect spectroscopy (NOESY) experiment. Correlations were present between H-10 and H-3a, and CH_3-11, indicating the same orientation for these groups. Thus, **1** was identified as 5a,9-dimethyl-3-methylene-3,3a,4,5,5a,6,7,8-octahydro-1-oxacyclopenta[*c*]azulen-2-one, a new sesquiterpene derivative.

Table 2. IC_{50} Values (μM) for Compounds **1**—**5** against the Jurkat (T Lymphoblastoid Leukemia), HL-60 (Promyelocytic Leukemia) and LoVo (Intestinal Adenocarcinoma) Cell Lines

Compound	Cytotoxicity (μM)[a)]		
	Jurkat	HL-60	LoVo
1	5.6±0.5	4.1±0.1	19.5±2.3
2	5.4±0.2	6.9±0.3	12.0±1.3
3	5.0±0.9	7.1±0.6	19.0±1.1
4	>20	>20	>20
5	4.2±0.2	5.5±0.8	>20
Doxorubicin[b)]	N.D.	0.01±0.007	0.2±0.06

a) IC_{50} concentration of compound required to inhibit cell growth by 50% after 72 h of compound exposure, as determined by MTT assay (see Experimental). Data are expressed as means±S.E.M. of three independent experiments; N.D., not determined. *b*) Doxorubicin hydrochloride was used as a reference compound.

Compound **2** was isolated as colorless needles. The HR-MS (APITOF) spectrum displayed a protonated molecular ion at m/z 264.0945, indicating a molecular formula of $C_{15}H_{19}O_2Cl$. The IR spectrum showed the presence of a γ-lactone ring (1767 cm^{-1}). The ^{13}C-NMR spectrum showed fifteen carbon signals consistent with the presence of a sesquiterpene lactone derivative. The ^{1}H-NMR spectrum showed six doublets at δ 6.21, 5.49 (J=2.30 Hz), 5.46, 5.33 (J=1.0 Hz) and 5.00, 4.94 (J=2.0 Hz), indicating three double bonds. The DQF-COSY spectrum revealed the proton connectivity between H-1, H-2 and H-3, and H-9, H-8, H-7 and H-6. Three methylene groups were observed in the HMQC spectrum: C-2 (δ_H 2.15, 2.30; δ_C 40.9), C-9 (δ_H 2.16—2.51; δ_C 36.4) and C-8 (δ_H 1.48—2.26; δ_C 32.3); three –CH– groups were presents: C-1 (δ_H 2.92, δ_C 51.9), C-5 (δ_H 2.83, δ_C 46.4) and C-7 (δ_H 2.85, δ_C 45.7). Two more –CH– groups were observed at δ_H 4.10 (δ_C 85.8) and δ_H 4.55 (δ_C 73.8), and the deshielded values of their carbon resonance supported the presence of linked etheroatoms. Diagnostic long-range correlations in HMBC experiments were observed between H-13 (δ_H 5.49, 6.21) and C-12 (δ_C 170.6), C-11 (δ_C 139.6) and C-7 (δ_C 45.7) indicating a lactone ring. Other long-range correlations were observed between H-15 (δ_H 5.33, 5.46) and C-5 (δ_C 46.4) and C-3 (δ_C 73.8), indicating an electronegative substituent in position 3. The chlorine atom orientation was assumed as β on the basis of NOESY correlations observed from the proton signal in H-3 (δ_H 4.55) and positions H-5 (δ_H 2.83) and H-1 (δ_H 2.92). In addition the chemical shifts of H-3 (δ_H 4.55) and H-6 (δ_H 4.10) appeared upfield and downfield compared to 3α-chlorodehydrocostuslactone values (δ_H 4.89, 3.93, respectively).[20)] The relative stereochemistry of C-1, C-5, C-6 and C-7 was deduced from NOESY data.

According to these spectral data, **2** was established to be 3β-chlorodehydrocostuslactone, a new chlorinated sesquiterpene lactone. Its structure was also confirmed by comparison of its ^{1}H-NMR data in $CDCl_3$ previously published by Wedge *et al.*,[20)] who synthesized this derivative from dehydrocostuslactone.

Compounds (**3**—**5**) are known sesquiterpene lactones and were identified as dehydrocostuslactone (**3**),[21)] artremorin (**4**)[22)] and costunolide (**5**)[13,18)] respectively, by comparison of their spectral data with those in the literature.

The cytotoxic effects of compounds **1**—**5** were tested

in vitro against three human tumor cell lines namely Jurkat (T lymphoblastoid leukaemia), HL-60 (promyelocytic leukaemia) and LoVo (intestinal adenocarcinoma). The cytotoxic data are shown in Table 2. The anticancer agent doxorubicin hydrochloride was used as the positive control. It can be noted that all compounds, except **4** showed a significant cytotoxicity in particular against the leukaemia cell lines. The cytotoxicity activities observed for compounds **1**—**5** were comparable to those reported for other similar compounds,[17] although lower respect to the reference drug these sesquiterpene lactones are potential antitumor candidates.

Experimental

IR spectra were recorded on a Perkin-Elmer 1600 FT-IR spectrometer. NMR spectra in CD_3OD were obtained using a Bruker AMX-300, spectrometer, operating at 300.13 MHz for ^{1}H-NMR and 75.03 MHz for ^{13}C-NMR. 2D experiments, ^{1}H–^{1}H DQF-COSY, NOESY, and inverse-detected ^{1}H–^{13}C HMQC and HMBC spectra were performed using UXNMR software. Exact masses were measured on an API-TOF spectrometer (Mariner Biosystems). Samples were diluted in a mixture of H_2O–AcCN (1 : 1) with 0.1% formic acid and directly injected at a flow rate of 10 μl/min. Sephadex LH 20 and silica gel 60 were used for column chromatography. Silica gel plates were used for analytical and preparative TLC (Merck cat. 5717 and 5715). HPLC analyses were performed on an Agilent 1100 series liquid chromatograph equipped with a series 1100 Diode Array detector using a Merck LiChrospher 100 RP-18 (5 μm) column. Semipreparative HPLC separation was performed on a Gilson series 305 liquid chromatograph using a Merck LiChrospher 100 RP-18 (10 μm, 10×250 mm i.d.) column. The mobile phase was water and acetonitrile in different gradient conditions. The flow rate was 6 ml/min. The separations were monitored at 200 nm.

Plant Material Leaves of *Laurus nobilis* L. (Lauraceae) were collected in November 2003 at S. Basilio (province of Cagliari, Sardinia). The plant was identified by Dr. Maria Cecilia Loi, Department of Botanical Sciences, University of Cagliari, Italy, where a voucher specimen was deposited.

Extraction and Isolation Air-dried powdered leaves of *L. nobilis* (350 g) were exhaustively extracted in a Soxhlet apparatus with petroleum ether, chloroform and methanol, to yield three extracts. The solvents were removed under vacuum. Yields in weight of residue, referring to the weight of dry material extracted, were as follows: petroleum ether 7.70%, chloroform 6.51%, methanol 17.8%.

A portion of the petroleum ether extract (12 g) was chromatographed on a silica gel column (450 g) using as eluent a mixture of ethyl acetate/*n*-hexane (1 : 4). The eluted fractions were pooled and combined in 9 groups according to the TLC pattern. Group 6 (2.3 g) was repeatedly subjected to HPLC separation using water/AcCN (40 : 60) as eluents resulting in the isolation of compounds **3** (22.0 mg) and **4** (24.0 mg).

Group 7 (1.2 g) was chromatographed on a silica gel column (150 g) using mixtures of cyclohexane/EtOAc at increasing polarity from 25% to 50%. The fractions were collected on the basis of their chromatographic behavior in six groups (A—F). Group F was repeatedly subjected to semipreparative HPLC using water/AcCN (gradient elution from 75 to 40% of water in 30 min) as eluents yielding compound **2** (2.1 mg).

From chloroform extract (11 g) by the same analytical procedures were isolated compounds **1** (4.8 mg) and **5** (13.4 mg) together with compounds **2** (2.5 mg) and **3** (2.4 mg).

5a,9-Dimethyl-3-methylene-3,3a,4,5,5a,6,7,8-octahydro-1-oxacyclopenta[*c*]azulen-2-one (**1**): Colorless needles; IR (KBr) ν_{max} 1765 cm^{-1}; ^{1}H- and ^{13}C-NMR data, see Table 1; HR-MS API-TOF *m*/*z* 233.1524 $[M+H]^+$ (Calcd for $C_{15}H_{20}O_2$, 233.1542).

3β-Chlorodehydrocostuslactone (**2**): Colorless needles; IR (KBr) ν_{max} 1767, 814 cm^{-1}; ^{1}H- and ^{13}C-NMR data, see Table 1; HR-MS API-TOF *m*/*z* 264.0945 $[M+H]^+$ (Calcd for $C_{15}H_{19}O_2Cl$, 264.0917).

Antiproliferative Activity Extracts were dissolved in 20 mg/ml DMSO as stock solutions. Compounds were dissolved in 1 mg/ml DMSO as stock solutions. Cell lines were purchased from American Type Culture Collection (ATCC), Human promyelocytic leukemia cells (HL-60) and Jurkat cells were grown in RPMI-1640 medium (Sigma Co. MO, U.S.A.); intestinal adenocarcinoma cells (LoVo) were grown in Ham's F12 medium (Sigma), all supplemented with 115 units/ml of penicillin G (Invitrogen, Milan, Italy), 115 μg/ml streptomycin (Invitrogen) and 10% fetal calf serum (Invitrogen). Doxorubin hydrochloride was used as a reference compound and was purchased from SIGMA. Individual wells of a 96-well tissue culture microtiter plate (Falcon BD) were inoculated with 100 μl of complete medium containing 8×10^3 leukemia and/or LoVo cells. The plates were incubated at 37 °C for 18 h prior to the experiments. After removal of the medium, 100 μl of the tested compound solution at varying concentrations, dissolved in DMSO and diluted with complete medium, was added to each well and incubated at 37 °C for 72 h. Cell viability was assayed by the MTT method.[23] Cell growth at each drug concentration was expressed as a percentage of untreated controls, and the concentration resulting in IC_{50} was determined by linear regression analysis.

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