LC-MS-SPE-NMR for the Isolation and Characterization of *neo*-Clerodane Diterpenoids from *Teucrium luteum* subsp. *flavovirens*[⊥]

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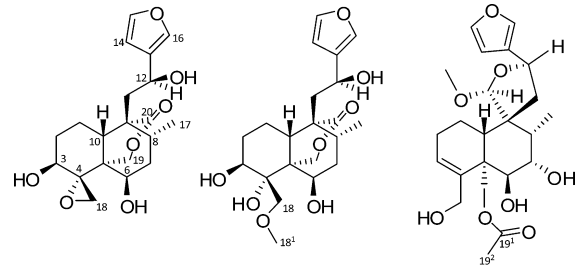
neo-Clerodane diterpenes of plant origin are molecules difficult to monitor due to their nonspecific UV/vis absorption. The present work describes for the first time the application of the LC-MS-SPE-NMR technique for the isolation and characterization of three new *neo*-clerodane diterpenes, 3β-hydroxyteucroxylepin and teuluteumin A and teuluteumin B, from *Teucrium luteum* subsp. *flavovirens*, harvested from two different locations.

neo-Clerodane diterpenes are present in different plant families,[1−3] and a wide range of bioactivities have been reported for these molecules. Insect antifeedant properties are their most extensively studied biological effects.[4] The genera *Scutellaria* and *Ajuga* (Lamiaceae) produce potent *neo*-clerodane antifeedants. In *Scutellaria*, jodrellin B (occurring in *S. albida*, *S. galericulata*, *S. grossa*, *S. polyodon*, and *S. woronowii*) and scutecyprol B (found in *S. columnae*, *S. cypria*, *S. grossa*, and *S. rubicunda*) show the highest antifeedant indices against *Spodoptera littoralis*.[5] From *Ajuga pseudoiva* leaves, 14,15-dihydroajugapitin displayed the most potent activity (AI > 90 even at a 1 ppm dose) among several highly active compounds isolated.[6]

A number of *neo*-clerodane diterpenes have also been found to be cytotoxic for cancer cell lines.[7−15] Recent studies have led to reports of new structures with such activity from *Croton* spp., *Casearia* spp., and *Scutellaria barbata*. One single report deals with cytotoxic activity among *Teucrium* clerodanes, namely, teumarin B.[15]

The genus *Teucrium* (Lamiaceae) is one of the richest sources of *neo*-clerodane diterpenes, and new natural products are found on a regular basis.[16] Most of the reported structures display a furan ring system in the side chain, with an oxygen-bearing C-12. Those isolated from *T. fruticans* with a fruticolone-like structure are unusual,[17−20] since C-11 and C-12 appear unsubstituted. Altogether, more than 200 diterpenoids have been isolated and characterized from the genus *Teucrium*.[16]

The section *Polium* (Mill.) Schreb. is morphologically the most diversified of the seven sections of the genus *Teucrium*. This includes over half of the species of the genus and is concentrated in the Mediterranean region. Only one phytochemical study has been reported on *Teucrium luteum,* showing the presence of caffeic acid esters and flavonoids.[21] In the present work, an LC-MS-SPE-NMR system has been applied to prepurified extracts from *T. luteum* subsp. *flavovirens,* collected from two different locations, for both the isolation and characterization of *neo*-clerodane diterpenes. The nonspecific UV/vis absorption of these molecules prompted the use of mass spectrometry as a monitoring system for the chromatographic separation.



1 **2** **3**

Compound **1**, which gave an elemental formula of $C_{20}H_{26}O_7$, showed the presence of typical signals for a side-chain furanyl *neo*-clerodane diterpene in the ^{1}H and ^{13}C NMR spectra (δ 6.48, 7.44, and 7.47 for protons and δ 109.7, 144.6, and 140.0 for carbons at C-14, C-15, and C-16, respectively; in addition, a quaternary sp^2 carbon atom resonance occurred at δ 132.1 for C-13). The presence of only one methyl signal (a characteristic doublet at δ 0.75 assigned to C-17) pointed to oxygenated substituents at C-18, C-19, and C-20. Furthermore, ^{1}H−^{13}C HMBC cross-peaks from the furan protons at δ 6.48 and 7.47 to the carbon resonance at δ 63.2 were consistent with an oxygenated substituent at C-12 (the δ 4.86 overlapped signal in the ^{1}H NMR spectrum was apparent in the ^{1}H−^{13}C HSQC spectrum). Starting at H-12, the ^{1}H−^{13}C HMBC spectrum showed a methylene group signal (C-11: δ 37.4, 2.06, 2.39) and a quaternary carbon (C-9: δ 50.9). The C-17 methyl doublet (δ 0.75) was correlated to a CH (C-8: δ 2.53) in the ^{1}H−^{1}H COSY spectrum and to the above-mentioned quaternary carbon (C-9: δ 50.9) in the ^{1}H−^{13}C HMBC spectrum. ^{1}H−^{1}H ROESY signals between δ 2.53 (H-8) and 2.94 (H-10) indicated the axial β-position of both. ^{1}H−^{1}H COSY signals from H-8 to δ 1.72/1.51 (H_2-7) and from these signals to δ 3.54 (triplet-like, $J = 2.7$ Hz) unequivocally showed H-6 as an equatorial substituent at an oxygenated C-6 (δ 67.5) as the rationale for the H-7α signal multiplicity [triplet-like doublets, $J = 13.7$, 2.1 Hz; only two large J values: one axial−axial (H-8) and one geminal (H-7β) coupling constants]. The ^{1}H−^{13}C HSQC correlation for the δ 4.67/3.96 A/B system and an oxygenated carbon signal at δ 73.1 and the ^{1}H−^{13}C HMBC cross-peak between this carbon signal and δ 3.54 (H-6) were used to establish the chemical shifts for H_2C-19. The formation of a lactone ring between C-19 and C-20 was postulated from the ^{1}H−^{13}C HMBC correlation between the H_2-19 signals and that at δ 175.2. As expected, the previously assigned H-8, H-10, and H-11 signals all showed a long-distance correlation with the δ 175.2 (C-20) resonance.

The H_2-1 and H_2-2 ^{1}H NMR assignments of **1** were derived from a spin system, H_1−H_2−H_2−H_1, in the ^{1}H−^{1}H COSY spectrum,

[⊥] Dedicated to Professor Josep Font Cierco of the Universitat Autònoma de Barcelona on the occasion of his 70th birthday.

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starting at both ends: δ 4.00 and 2.94 (H-3 and H-10, respectively, previously assigned from the HMBC spectrum). The observed multiplicity (doublet of doublets) and one large coupling constant of the δ 4.00 absorption required an axial H−C(3) and, thus, an equatorial hydroxy substituent. The ^{1}H−^{13}C HMBC correlation from δ 4.00 to δ 50.3 with attached protons at δ 3.33/3.20 (from the ^{1}H−^{13}C HSQC spectrum) was supportive of an oxirane ring across C-4 and C-18. In the ^{1}H−^{1}H ROESY spectrum, cross-peaks of the H-19b proton demonstrated the α and axial nature of H-1ax and H-3, while H-12 interacted with H-8, H-10, and H-17. The NMR data were consistent with those reported for teucroxylepin[22] and related 12*S*-hydroxylated *neo*-clerodane diterpenoids[23] rather than those for the 12*R* isomers, which led to the assignment of compound **1** as (12*S*)-4,18;15,16-diepoxy-3β,6β,12-trihydroxy-*neo*-cleroda-13(16),14-dien-20,19-olide (3β-hydroxyteucroxylepin).

Compound **2** gave a molecular formula of $C_{21}H_{30}O_8$ and shared many features in common with compound **1** in both the ^{1}H and ^{13}C NMR spectra. Thus, the proposed structure of **2** could be assigned with the same substitution pattern and configuration for the side chain and B ring of the decalin system, by direct comparison of most of the signals with data obtained for **1**. The NMR differences between compounds **1** and **2** involved mainly the A ring around C-4, as the result of a formal addition of methanol to the oxirane ring. Thus, the signals assigned from the ^{1}H−^{13}C HSQC and ^{1}H−^{1}H COSY spectra for positions 2 (δ 1.83, 1.41, and 30.2), 3 (δ 4.03 and 73.2), and 6 (δ 4.11 and 69.5) were shifted slightly. Furthermore, in the ^{1}H−^{13}C HMBC spectrum, H-3 and H-6 showed correlations with quaternary carbon signals at δ 77.6 (C-4) and 46.4 (C-5), with the former supporting an oxymethylene as the substituent (H_2C-18: δ 3.83, 4.08, and 71.6), as indicated by the reciprocal correlation of H-3 to C-18 and H_2-18 to C-3. A cross-peak of the δ 3.49 methoxy singlet with the C-18 signal (δ 71.6) indicated the presence of a methoxy-methylene substituent. The possibility of an oxirane ring between C-3 and C-4, based on the mass spectroscopic data (*m*/*z* 393.1927 $[M + H - H_2O]^+$), was excluded by the ^{13}C NMR spectrum of **2**. Analysis of the HRESIMS of the pure compound showed a $[M + HCOO]^-$ peak in the negative mode, supporting the molecular formula. Thus, the structure proposed for **2** is (12*S*)-15,16-epoxy-3β,4α,6β,12-tetrahydroxy-18-methoxy-*neo*-cleroda-13(16),14-dien-20,19-olide.

Naturally occurring compounds resulting from a formal intramolecular attack of a hydroxy group to the 4α,18-oxirane ring (10 to 4 or 18; 19 to 4) or addition of H_2O, HAcO, or HCl has been reported,[16] but that of methanol as in the isolated compound **2** is unprecedented. A methoxy anion attack to the less hindered C-18 position of the oxirane ring of teucroxylepin was the rationale for the partial formation of an addition compound when teucjaponin A was treated with potassium carbonate in an aqueous methanol solution.[22] Related oxirane-opening reactions of teucroxylepin and teucjaponin B have been studied to assess the effects of neighboring groups on the stereochemical outcome of the addition/rearrangement processes to the resulting compounds.[24]

Compound **3** gave a molecular formula of $C_{23}H_{32}O_8$ and displayed a different structure from compounds **1** and **2**, although the furan ring on the side chain and one methyl doublet (C-17; pointing once more to oxygenated functions for C-18, C-19, and C-20) were again observed. Two methyl singlets (δ 2.02 and 3.25) showed the presence of an acetoxy group and a methoxy substituent. The correlations between a carbon signal at δ 108.9 and protons at δ 5.36 (in the HSQC spectrum) and δ 3.25 (in the HMBC spectrum) supported the presence of an acetal function, with the methoxy group being part of this. The HMBC spectrum correlations from H-8 (δ 1.82), H-10 (δ 2.23), and H_2-11 (δ 1.81/2.35) indicated C-20 as being the carbonyl part of the acetal function. From H-8 (δ 1.82 ppm), the ^{1}H−^{1}H COSY spectrum showed HC(O)-7 at δ 3.72 (proton in equatorial configuration from two small coupling constants) and HC(O)-6 at δ 4.14 from H-7. The ^{1}H−^{1}H ROESY H-6/H-19b correlation (δ 4.14/4.54) indicated H-6 as an α-substituent. The presence of a double bond across C-3 and C-4 (δ 132.8 and 141.5) was based on the HMBC cross-peaks to these sp^2 carbons of the A/B proton system at δ 3.99/4.37 (assigned as H_2-18). The *C*(=O) of the acetoxy substituent (δ 173.0) correlated with the H_2C(O)-19 A/B system (δ 4.33/4.54). The H/C-12 chemical shift changes supported a ring formation across C-20 and C-12, and the ^{1}H−^{1}H ROESY cross-peaks from the H-20 acetal proton (δ 5.36) with H-12, H-17, and H_2-19 established the absolute configuration as 12*R*,20*S*. The ROESY spectrum displayed a cross-peak, H-6/H-18b, and the possibility of a 6:18 oxolane ring was considered, but no evidence of this structure was found from the HMBC spectrum. Moreover, the MS results fit the proposed empirical formula, rather than the [M − 18] ion. Thus, compound **3** was assigned as (12*R*,20*S*)-19-acetoxy-12,20;15,16-diepoxy-20-methoxy-*neo*-cleroda-3,13(16),14-trien-6β,7α,18-triol. Whereas a 20,12-hemiacetal or a (20-*O*-acetyl)-20,12-hemiacetal is a rather common structural feature among *Teucrium* clerodane diterpenoids, the occurrence of methoxy derivatives was reported only recently, during an attempt to separate the corresponding epimeric mixture of hemiacetals by the $CHCl_3$/MeOH solvent system.[25]

The present report of three new structures (**1**−**3**) from a single species collected in two different locations demonstrates once more that environment may influence plant secondary metabolite production within the same species to afford different resultant compounds. The most common substituent in *Teucrium* species, a furan ring, appeared in all compounds **1**−**3**, but the 20,19-olide functionality found in **1** and **2** is rare.[16] A few reported 20,19-olide clerodanes display a 3-en-18-hydroxy unit but not a 4-hydroxy-18-OR system, although some examples contain a 4-hydroxy-6,18-epoxy moiety.[16] Conversely, there are examples of clerodane diterpenoids with a 4α,18-epoxy-3-hydroxy moiety in 20,12-hemiacetal units.[16]

The LC-MS-SPE-NMR reported here for the first time for *neo*-clerodanes has been proved to be an optimal system for the rapid and reliable isolation and characterization of these secondary metabolites from small amounts of plant material. The use of MS (more sensitive than ELS) overcomes the lack of UV−vis monitoring of compound separation, allowing a lower detection limit and also the discrimination between possible co-eluting signals.

Experimental Section

General Experimental Procedures. LC-MS-SPE-NMR measurements were carried out on an LC separation system (Agilent, Waldbronn, Germany), consisting of an HPLC quaternary pump, an autosampler, a column oven, and a diode array detector, and a MicrOTOF mass spectrometer (Bruker) coupled to a Bruker Avance III 600 (ultrashielded) NMR spectrometer equipped with a dual inverse ^{13}C−^{1}H cryoflow probe with an active volume of 30 μL. A more detailed description of this instrumental setup has been published elsewhere.[26,27] 1D and 2D NMR spectra were recorded under standard conditions and with gradient-enhanced pulse sequences. Chemical shifts are given in ppm referenced to solvent signal (CD_3OD at 3.31 and 49.1 ppm for ^{1}H and ^{13}C, respectively). ^{1}H NMR spectroscopic analysis and parameters (^{1}H chemical shifts and coupling constants) for compounds **1** and **2** were based on software calculations with PERCH (PERCH Solutions Ltd., Kuopio, Finland), while for compound **3** they were derived from the experimental spectrum. MS measurements were performed on a MicrOTOF mass spectrometer, equipped with an electrospray ion source, from Bruker Daltonics (Bremen, Germany). Five percent of the eluent was split to the MS, 95% to the SPE unit. MS data were acquired in a scan range between 50 and 1000 Da with an electrospray interface under positive-ionization conditions. Isolated compounds **1**−**3** were further injected in an ACQUITY UPLC (Waters Corporation, Milford, MA) with a Q-Tof Premier mass spectrometer detector (Waters Corporation) in a range between 50 and 1500 Da under positive- and negative-ionization conditions.

Chemicals. Solvents used for extraction and column chromatography (HPLC grade) were from Merck (Darmstadt, Germany). Acetonitrile (Chromasolv quality) for HPLC was obtained from Riedel-de-Haen (Seelze, Germany). Formic acid was purchased from Merck (Haarlem,

Table 1. ^{1}H and ^{13}C NMR Spectroscopic Data for Compounds **1**−**3** in CD_3OD [δ in ppm, mult. (J in Hz)]

	1		**2**		**3**	
position	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1_{ax}	24.3	1.21 q^{a}d (13.1, 3.1)	25.3	1.12 q^{a}d (13.1, 3.2)	22.0	2.36[b]
1_{eq}		2.47 dq^a (13.1, 3.7)		2.44 dq^a (13.1, 3.6)		1.86[b]
2_{ax}	33.1	1.46 q^{a}d (12.6, 3.4)	30.2	1.41 q^{a}d (12.9, 3.4)	28.9	2.03[b]
2_{eq}		2.07[b]		1.83[b]		2.17 dt (10.1, 5.3)
3	67.9	4.00 dd (12.3, 4.6)	73.2	4.03 dd (12.6, 4.6)	132.8	5.95 d[c] (3.4)
4	63.6		77.6		141.5	
5	42.8		46.4		47.2	
6	67.5	3.54 t[c] (2.7)	69.5	4.11 t[c] (2.6)	72.0	4.14 d (3.2)
7_{ax}	37.7	1.51 t^{a}d (13.7, 2.1)	37.5	1.59 t^{a}d (13.5, 2.8)	76.1	
7_{eq}		1.72 dt^a (14.7, 3.9)		1.79 dt^a (14.3, 3.4)		3.72 t^a (3.0)
8	30.5	2.53 dquint (12.7, 6.8)	30.3	2.60 dquint^a (12.8, 6.9)	41.5	1.82 qd (7.2, 3.0)
9	50.9		51.4		53.8	
10	37.9	2.94 dd (13.1, 4.1)	35.8	2.91 dd (13.2, 3.6)	45.5	2.23 d (11.9)
11a	37.4	2.06[b]	37.3	2.04[b]	46.5	1.81 dd (12.9, 10.3)
11b		2.39 dd (15.8, 3.8)		2.36 dd (15.8, 3.3)		2.35[b]
12	63.2	4.86[d]	63.4	4.91[d]	70.3	4.91 dd (10.2, 7.2)
13	132.1		132.2		126.7	
14	109.7	6.48 t[c] (1.5)	109.7	6.50 t[c] (1.5)	110.0	6.50 m
15	144.6	7.44 t[c] (1.7)	144.6	7.47 t[c] (1.7)	144.4	7.48 t^a (1.6)
16	140.0	7.47 t[c] (1.3)	139.9	7.49 t[c] (1.3)	140.6	7.52 d (0.7)
17	16.6	0.75 d (6.8)	16.8	0.79 d (6.9)	14.5	1.11 d (7.3)
18a	50.3	3.20 d (5.2)	71.6	3.83 d (10.2)	64.35	3.99 d (12.0)
18b		3.33 d (5.2)		4.08 d (10.2)		4.37 d (12.0)
18^1			59.9	3.49 s		
19a	73.1	3.96 d (13.4)	74. 5	3.96 d (13.9)	70.0	4.33 d (11.7)
19b		4.67 d (13.4)		4.73 d (13.9)		4.54 d (11.8)
19^1					173.0	
19^2					21.0	2.02 s
20	175.2		175.8		108.9	5.36 s
20^1					54.4	3.25

[a] Apparent multiplicity (t^a = dd with $J_1 \approx J_2$; q^a = ddd with $J_1 \approx J_2 \approx J_3$; quint^a = qd with $J_1 \approx J_2$). [b] Overlapped signal. [c] Broad signal. [d] Chemical shift deduced from HSQC.

The Netherlands), and water was produced in-house (Milli-Q Water Purification System, Millipore). CD_3OD (99.8% D) was purchased from CortecNet (Paris, France).

Plant Material. The aerial parts of *Teucrium luteum* subsp. *flavovirens* (Batt.) Greuter & Burdet were collected in the Meknès-Tafilalt region in Morocco in July 2007. A sample labeled TPL was located at 1880 masl, latitude 32°35′04.9″ N and longitude 4°44′26.5″ W. A second sample labeled TPO was harvested at 1750 masl, latitude 32°37′04.3″ N and longitude 4°48′12.4″ W. Collection and identification were carried out by Dr. J. M. Montserrat (Institut Botànic de Barcelona, IBB) as part of a cooperative project IBB (Barcelona)−Institut Scientifique Université Mohamed V, Rabat (Semclimed: Interreg IIIB Medocc-2005-05-4.1-E-110), and voucher specimens have been deposited at the IBB (JMM-12111 and JMM-12121, respectively).

Extraction and Isolation. Ground leaves and flowers (24.1 g for TPL and 30.0 g for TPO) were extracted with dichloromethane (×2; 250 and 300 mL, respectively) for three days at room temperature in the dark. Extracts were combined and dried under vacuum at 40 °C, yielding residues of 0.985 and 1.167 g, respectively. Crude extracts were defatted by hexane and then centrifuged (×10 v/w; 10 min sonication/15 min 3000 rpm). The residues were then treated with MeOH and again centrifuged (30 mL; 10 min sonication/15 min 3000 rpm). The supernatants were dried under vacuum at 40 °C, yielding residues of 471 and 506 mg, respectively. The residues were then digested/centrifuged sequentially (15 min sonication/15 min 3000 rpm) in 30, 60, and 90% methanol/water mixtures (10 mL each). Each solution was filtered (fraction F) through a 10 g Strata C_{18} column (Phenomenex, Torrance, CA), followed by a further elution with 10 mL of the corresponding fresh methanol/water mixture (fraction E), affording fractions labeled as 30F, 30E, 60F, 60E, 90F, and 90E, respectively. Previous experience has shown that *neo*-clerodane diterpenes are contained mainly in fractions 60E and 90F.[19,20]

Compound isolation was performed using LC-MS-SPE-NMR equipment, with acetonitrile/water mixtures with 0.1% formic acid as mobile phase, a 1 mL/min flow rate, and 1 mg of mixture per injection. Collected peaks were selected by intensity of the MS detector, based on previous experience. The LC was equipped with a Grace Alltima HP C_{18} 3 μm, 4.6 × 150 mm column. The following gradient was used for the TPL 60E fraction (51.1 mg): 12% acetonitrile for 20 min, gradient to 20% for 5 min, held for 5 min, gradient to 95% acetonitrile in 1 min, held 7 min, and back to the initial conditions (1 min) and column re-equilibration (5 min). Peaks with retention times of 18.24 (**1**) and 24.87 (**2**) min were collected six times using the SPE manual mode. A gradient from 28 to 30% acetonitrile for 30 min, followed by a gradient to 95% in 1 min, held for 5 min, then back to initial conditions and column re-equilibration as above, was used for the TPO 90F fraction (50.4 mg). Similar to the above, a peak with retention time 22.80 min (**3**) was collected manually (six times) on the SPE system. After a drying step the analytes were transferred with CD_3OD to the NMR probe and the spectra were acquired.

Pure compounds **1**−**3** were dissolved in 100 μL of MeOH, and 3 μL of each was injected in a UPLC-MS with a UPLC BEH C_{18} 1.7 μm, 2.1 × 100 mm column. Chromatographic conditions used at 30 °C were 0.3 mL/min flow methanol/0.1% v/v formic acid. Positive- and negative-electrospray ionization modes were used.

3β-Hydroxyteucroxylepin (1): ^{1}H and ^{13}C NMR data, see Table 1; HRESIMS m/z 361.1508 $[M + H - H_2O]^+$, 401.1437 $[M + Na]^+$, 779.2993 $[2\ M + Na]^+$, 1157.4514 $[3\ M + Na]^+$; 377.1989 $[M - H]^-$, 423.1985 $[M + HCOO]^-$, 755.3896 $[2\ M - H]^-$ (calcd for $C_{20}H_{26}O_7Na$, 401.1576, and $C_{20}H_{25}O_7$, 377.1600).

Teuluteumin A (2): ^{1}H and ^{13}C NMR data, see Table 1; HRESIMS m/z 455.2117 $[M + HCOO]^-$ (calcd for $C_{22}H_{31}O_{10}$, 455.1917).

Teuluteumin B (3): ^{1}H and ^{13}C NMR data, see Table 1; HRESIMS m/z 459.1860 $[M + Na]^+$, 895.3855 $[2\ M + Na]^+$, 1331.5840 $[3\ M + Na]^+$, 435.2635 $[M - H]^-$, 481.2747 $[M + HCOO]^-$, 917.5475 $[2\ M + HCOO]^-$, 1353.8149 $[3\ M + HCOO]^-$ (calcd for $C_{23}H_{32}O_8Na$, 459.1995, and $C_{23}H_{31}O_8$, 435.2019).

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Supporting Information Available: Tables of ^{1}H and ^{13}C NMR data, including 2D NMR spectroscopic correlations (Tables S1−S3), and NMR 1D and 2SD spectra of the isolated compounds **1**−**3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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