Fragmentation Pathways of Polycyclic Polyisoprenylated Benzophenones and Degradation Profile of Nemorosone by Multiple-Stage Tandem Mass Spectrometry

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Nemorosone is a polycyclic polyisoprenylated benzophenone (PPBs) with strong cytotoxic activity. It is the major constituent of *Clusia rosea* floral resin and brown Cuban propolis. Other PPBs found in Cuban propolis are oxidized and cyclized derivatives of nemorosone. The instability of PPBs carrying an enolizable 1,3-diketone system has been suggested, and the elucidation of this aspect is very fundamental for the evaluation of their biologic activity. Electrospray ionization multistage tandem mass spectrometry (ESI-MSⁿ) was employed to shed light on the origin of these derivatives of nemorosone and to define the stability of this natural product. For this purpose, we initially performed MSⁿ experiments on seven related PPBs to obtain useful information for structural characterization of this class of compounds and to identify the degradation products of nemorosone. The proposed fragmentation pathways, supported by exact mass measurements, allowed the nature of side chains on the bicyclo core and the type and position of their modifications to be established. In a second part, the degradation profile of nemorosone under different conditions was investigated to assess the possible effects of isolation procedures, climatic, and storage conditions on its stability. Our results reveal that nemorosone undergoes rapid degradation in *n*-hexane and chloroform solutions. The degradation products, identified by HPLC-ESI/MSⁿ and NMR, are identical to derivatives of nemorosone previously isolated from propolis and plants. Thus, these PPBs are artefacts formed predominantly during the extraction and purification procedures. (J Am Soc Mass Spectrom 2009, 20, 1688–1698) © 2009 American Society for Mass Spectrometry

Polycyclic polyisoprenylated benzophenones (PPBs) are a class of acylphloroglucinols that is confined to the *Guttiferae*, a plant family almost exclusively tropical, which is a rich source of secondary biologically active metabolites. Many PPBs have an oxygenated and substituted bicyclo-[3.3.1]-nonane-2,4,9-trione core to which a benzoyl group and prenyl or geranyl side chains are attached. Secondary cyclizations involve the β -diketone and pendant olefinic groups affording adamantanes, homoadamantanes and dihydrofuran- or pyran-fused structures [1]. PPBs exhibit a wide variety of biological activities, the most interesting of which are cytoprotection against HIV-1 [2, 3], antibacterial activity against methicillin-resistant *Staphylococcus aureus* [4–6], antioxidant [7–9], and cytotoxic activity [9–14].

Nemorosone (1), a PPB with a bicyclo-[3.3.1]-nonane-2,4,9-trione system of type A (benzoyl group at C1), [1] is found in resins and latex of *Clusia* species [15–17], and it is the major constituent of *C. rosea* floral resin [18] and

brown Cuban propolis [19, 20]. Propolis is a resinous substance produced by honeybees from plant sources and is used to protect the hive against microorganisms and insects. This natural role in defense endows propolis with a broad spectrum of biological activities, such as antibacterial, antiviral, antifungal, anti-inflammatory, antioxidative, and antitumoral properties [21]. Nemorosone is responsible for the antimicrobial activity of *Clusia* spp. resin and propolis [19, 22]. Besides antimicrobial activity, nemorosone exhibits activity as a free radical scavenger of the same order of magnitude as α -tocopherol [19], and has moderate anti-HIV activity [23]. It has also been demonstrated that it exerts a strong cytotoxic activity against a panel of tumor cell lines [19, 24, 25] showing IC₅₀ values comparable to those of doxorubicin [19].

Acylphloroglucinols are labile compounds sensitive to light, oxygen, and heat. Chemical instability of hyperforin and adhyperforin, major phloroglucinol constituents of *Hypericum perforatum* flowers and leaves, leads to changes in the composition of extracts and, until recently, prevented its pharmacological evaluation [26]. A number of oxidized derivatives of hyperforin

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and adhyperforin were identified by HPLC-MS analyses as their degradation products (furohyperforin isomers, furohyperforin hydroperoxide, furoadhyperforin isomers, furoadhyperforin hydroperoxide) [27]. The instability of PPBs that contain the enolizable 1,3-diketone system has also been suggested: organic or inorganic acids rapidly converted these compounds to the corresponding pyran ether derivatives [4, 13, 18]; besides, this reactivity has been hypothesized to be involved in polymerization reactions, which occur in propolis, responsible for the “waterproof” protection [15]. Recently, the chemical lability of nemorosone was observed by Tsukano et al. [28] during its total synthesis. They found that nemorosone was both light- and air-sensitive; it decomposed in chloroform after only a few days at room temperature.

In our previous studies [29, 30], we reported the occurrence, in brown Cuban propolis samples, of oxidized and cyclized derivatives of nemorosone, garcinelliptone I (2), hyperibone B (3), and propolones A–D (4–7), which have a close structural similarity to the degradation products of hyperforin and adhyperforin. This suggests that the PPBs isolated from brown Cuban propolis might be degradation products of nemorosone obtained during the extraction and purification procedures, or the transformation of resins by bees and/or the storage of propolis in the nests. It is essential to determine whether these derivatives of nemorosone are natural products or artefacts, not only for the standardization of brown Cuban propolis extracts, but also for the evaluation of their biological activity, and that of nemorosone. Indeed, the main structural feature of nemorosone is an enolizable β -diketone group, which is absent in the PPBs derivatives (2–7) found in Cuban propolis. This functional group has been often associated with the biological activity of PPBs and nemorosone [1].

To define the origin of Compounds 2–7 and the stability of nemorosone (1), in this study MSⁿ experiments and accurate mass measurements have been used for the elucidation of the fragmentation pathways of these related PPBs and to obtain useful information for the structural characterization of this class of compounds. Subsequently, the stability of nemorosone in different conditions was evaluated by HPLC-ESI/MSⁿ. The stability test conditions were chosen, taking into account the extraction and purification procedures used for the isolation of 2–7 and the climatic conditions at the propolis sampling site. The stability of nemorosone in an ethanol extract of brown Cuban propolis was also examined, to assess its stability in the more widespread commercial form.

Experimental

Standards and Samples

Nemorosone (1) was extracted from the floral resin of *C. rosea* and isolated as previously reported [18]. It was

identified by 1D- and 2D-NMR experiments [18], and its purity was verified by HPLC-DAD and HPLC-MS. Compounds 2–7 were isolated from Cuban propolis samples and identified by spectroscopic methods as previously reported [29, 30]. A 10% ethanol extract of brown Cuban propolis was obtained by maceration of ground propolis samples (10 g) with ethanol (100 mL), in a closed, dark bottle over 2 d. Brown Cuban propolis samples were provided by La Estación Experimental Apícola of Cuba and characterized by spectroscopic techniques [20].

Solvents and Reagents

HPLC-grade acetonitrile, methanol, ethanol, chloroform, and *n*-hexane were purchased from Carlo Erba Reagents (Milan, Italy). HPLC-grade water (18 M Ω) was prepared by a Milli-Q⁵⁰ purification system (Millipore Corp., Bedford, MA, USA).

ESI-MSⁿ Analyses

MS and MSⁿ analyses were performed using a LCQ Advantage ion-trap mass spectrometer (ThermoFinnigan, San Jose, CA, USA) equipped with Xcalibur 3.1 software. Mass spectrometry data were acquired in positive mode, the maximum injection time was 100 ms and the number of microscans was three. Instrumental parameters were tuned for nemorosone (capillary temperature 150 °C, capillary voltage 18 V, spray voltage 5.10 kV, scan range of m/z 200–1000). For MSⁿ analyses, collision energies chosen for each fragmentation step ranged from 15% to 33% of the instrument maximum and the isolation width of parent ion was 2. All compounds were dissolved in methanol and diluted in methanol-water (1:1, vol/vol) to obtain final concentrations of 10 μ g/mL. Samples were infused in the ESI source using a syringe pump (flow rate of 5 μ L/min).

High-Resolution Mass Spectra

High-resolution MS and MS/MS were acquired on a Q-TOF Premier instrument (Waters, Milford, MA, USA), equipped with a nanospray ion source and provided by a lock-mass apparatus to perform a real-time calibration correction mode. To achieve high accuracy mass measurements, both external and internal calibrations of the spectrometer were performed using quercetin (molecular mass 302.0427) or amentoflavone (molecular mass 538.0900) as standards. The Q-TOF instrument operated with a mass resolution of 12,000 (50%) both in MS and MS/MS mode. For high-resolution MS and MS/MS analyses, samples (10 μ g/mL) were dissolved in a water/methanol solution (50/50, vol/vol) and directly infused into the Q-TOF ion source. An ionization energy of 3500, and collision energy of 20 eV were used. Spectra were acquired in profile, positive mode, with a scan time of 1 s. Fragment ion analysis was performed averaging 10 spectra; the

achieved output was elaborated to obtain centroid signals.

Stability Test Conditions

The *n*-hexane, chloroform, methanol, and ethanol solutions of nemorosone (2 mg/mL) were prepared in duplicate. The solutions were vortex-mixed for 1 min and maintained at 25 °C in artificial light. At specific times (every 12 and 24 h for *n*-hexane and chloroform solutions, respectively, and 2 wk for methanol and ethanol solutions), three aliquots from each solution were diluted to 0.02 mg/mL with methanol-water (8:2, vol/vol). For *n*-hexane and chloroform solutions, the solvent was stripped with nitrogen before the dilution. A vial containing about 100 mg of nemorosone was left open to air in a thermostat at 40 °C for 1 wk: three samples of 10 mg were weighed every 24 h and diluted to 0.01 mg/mL with methanol-water (8:2, vol/vol). Two different ethanol extracts of brown Cuban propolis were analyzed in duplicate every 15 d after dilution (methanol-water, 8:2, vol/vol) for a period of 2 mo. The diluted aliquots were analyzed by HPLC-MS using ion trap and triple quadrupole mass spectrometers for qualitative and quantitative analyses, respectively, as reported in the following section. The percentage of residual nemorosone and degradation products in the test solutions at different times were calculated as follows:

$$\text{Residual ratio (\%)} = (A_t/A_0) \times 100$$

where, A_0 is the nemorosone peak area at the initial time and A_t is the area of each peak of the tested sample.

HPLC-ESI-MS Analyses

A HPLC system including a Surveyor Autosampler, Surveyor LC pump LCQ Advantage ion-trap mass spectrometer (ThermoFinnigan, San Jose, CA, USA) was used for characterization of degradation products of nemorosone formed under the stability test conditions. HPLC separation was performed using a Luna C₈ column (150 × 2.0 mm i.d., 5 μm) from Phenomenex (Torrance, CA, USA), protected by a C₈ Guard Cartridge (4 × 2.1 mm i.d., Phenomenex) and isocratic elution (methanol-water 8:2, vol/vol), followed by washing and re-equilibrating the column. The flow rate was 0.2 mL/min and the volume of the injection was 25 μL. Mass conditions were: positive ion mode, capillary temperature 220 °C, capillary voltage 21 V, spray voltage 4.50 kV, sheath and auxiliary gas flow rate 40 and 20 (nitrogen, arbitrary units), respectively, scan range of m/z 200–1000. For ESI-MS and MSⁿ analyses, collision energy of 30% of the instrument maximum was used. For time course studies, nemorosone and its degradation products were quantified using a API 2000 triple

quadrupole spectrometer (Applied Biosystems, Foster City, CA, USA) coupled with an Agilent (Palo Alto, CA, USA) 1100 HPLC system. The HPLC conditions were the same as those used for qualitative analysis. The data were acquired in multiple reaction monitoring (MRM) mode and the selected parent ion → fragment ion transition were 503 → 311 for 1 and 4, 519 → 327 for 2–3, 6–7 and 9, 535 → 343 for 8 and 553 → 535 for 5. The optimized instrumental parameters were: ion spray voltage 5500 V, source declustering potential 28 V, collision energy 30 V for 2–3, 6–7, and 9, and 45 V for 8.

Isolation of Degradation Products

Hexane and chloroform solutions of degraded nemorosone were evaporated to dryness under reduced pressure at 40 °C and dissolved in methanol-water (8:2, vol/vol). HPLC purification of these solutions was performed on an Agilent 1100 series HPLC-PDA system equipped with a Kromasil C₁₈ column (300 × 10 mm i.d., 10 μm, Phenomenex) protected by a C₁₈ guard cartridge (4 mm × 3.0 mm i.d., Phenomenex). Isocratic elution was used (methanol-water 72:28 vol/vol at flow rate of 2.5 mL/min) and PDA data were recorded with a 200–600 nm range with preferential wavelength of 280 nm for the detection.

Results and Discussion

Fragmentation Pathways of PPBs with a Bicyclo-[3.3.1]-Nonane-2,4,9-Trione System

To obtain a useful tool for the structural characterization of PPBs with a bicyclo-[3.3.1]-nonane-2,4,9-trione system (type A) and the identification of degradation products of nemorosone, the fragmentation mechanisms of nemorosone (1) and derivatives (2–7) (Figure 1) were investigated by ESI-MSⁿ experiments. Accurate mass measurements of fragment ions of 1–7, performed by a high-resolution instrument (Q-TOF), were used to support the proposed fragmentation pathways. All the HR-MS spectra are available in the supplementary data, which can be found in the electronic version of this article.

MS analyses were initially performed in both the negative and positive ionization modes, but the latter was preferred as it gave more structural information on the PPBs. In the (+)-ESI-MS spectra, $[M + H]^+$ and $[M + Na]^+$ ions were observed for almost all the analyzed compounds (data not shown) and, without applying CID voltage, no fragments were detected.

The ESI-MS spectrum of nemorosone (1) showed a protonated molecule $[M + H]^+$ at m/z 503. Its fragmentation yielded ions resulting from successive eliminations of the alkyl chains from the bicyclo core. The analysis of MSⁿ spectra enabled the fragmentation pathway of nemorosone (Scheme 1) to be suggested. The MS/MS spectrum of 1 showed fragment ions resulting from losses of side chains at C5 and C3, such as isoprene

Figure 1. The structures of the polycyclic polyisoprenylated benzophenones included in this study.

($[M + H - 68]^+$ at m/z 435) and isobutene ($[M + H - 56]^+$ at m/z 447), respectively (Scheme 1). We proposed that the isoprene molecule originated from a concerted pericyclic mechanism, whereas the loss of isobutene was produced by a six-center concerted mechanism involving the proton of keto-enolic system. In the MS^3 experiment, isolation and fragmentation of the fragment ion at m/z 435 ($[M + H - 68]^+$) resulted in two ions, one from a loss of isobutene at m/z 379 and the other at m/z 311 from a loss of the chain at C7 occurring through

the opening of bicyclo-[3.3.1]-nonane (Scheme 1). The MS^4 spectra of ions at m/z 379 and 311 supported the fragmentation pathway proposed for nemorosone (1) (Scheme 1).

Accurate masses and corresponding assigned elemental compositions of the product ions from MS/MS analysis (Table 1) of nemorosone (1) support this kind of fragmentation. The accuracy for the masses of these ions, determined relative to the values calculated from their assigned structures, was

Scheme 1. Proposed fragmentation pathway for nemorosone (1) and MS^n spectra.

Table 1. Accurate masses for product ions of the PPBs 1–7 and their elemental compositions

	<i>m/z</i> Measured ^a	Elemental composition ^b	<i>m/z</i> Calculated ^c	Δ <i>m/z</i> (ppm)
[M + H] ⁺ ion	503.317	C ₃₃ H ₄₃ O ₄ ⁺	503.316	1.5
Nemorosone (1)	447.246	C ₂₉ H ₃₅ O ₄ ⁺ ^d	447.254	–17.1
	379.191	C ₂₄ H ₂₇ O ₄ ⁺	379.191	–0.4
	311.130	C ₁₉ H ₁₉ O ₄ ⁺	311.128	5.7
	255.067	C ₁₅ H ₁₁ O ₄ ⁺	255.066	4.2
	233.082	C ₁₃ H ₁₃ O ₄ ⁺	233.081	4.8
	177.020	C ₉ H ₅ O ₄ ⁺	177.019	9.1
[M + H] ⁺ ion	519.311	C ₃₃ H ₄₃ O ₅ ⁺	519.311	–0.5
Garcinielliptone I (2)	327.123	C ₁₉ H ₁₉ O ₅ ⁺	327.123	0.5
	309.114	C ₁₉ H ₁₇ O ₄ ⁺	309.113	4.6
	255.066	C ₁₅ H ₁₁ O ₄ ⁺	255.066	6.1
	231.068	C ₁₃ H ₁₁ O ₄ ⁺	231.066	8.1
	177.020	C ₉ H ₅ O ₄ ⁺	177.019	9.7
[M + H] ⁺ ion	519.311	C ₃₃ H ₄₃ O ₅ ⁺	519.311	0.3
Hyperibone B (3)	327.123	C ₁₉ H ₁₉ O ₅ ⁺	327.123	–1.4
	309.11	C ₁₉ H ₁₇ O ₄ ⁺	309.113	4.3
	255.067	C ₁₅ H ₁₁ O ₄ ⁺	255.066	5.4
	231.067	C ₁₃ H ₁₁ O ₄ ⁺	231.066	4.2
	177.021	C ₉ H ₅ O ₄ ⁺	177.019	11.4
[M + H] ⁺ ion	503.315	C ₃₃ H ₄₃ O ₄ ⁺	503.316	–1.7
Propolone A (4)	311.130	C ₁₉ H ₁₉ O ₄ ⁺	311.128	7
	255.067	C ₁₅ H ₁₁ O ₄ ⁺	255.066	4.2
	233.080	C ₁₃ H ₁₃ O ₄ ⁺	233.081	–5.1
	177.020	C ₉ H ₅ O ₄ ⁺	177.019	7.4
[M + H] ⁺ ion	553.317	C ₃₃ H ₄₅ O ₇ ⁺	553.317	0.1
Propolone B (5)	327.130	C ₁₉ H ₁₉ O ₅ ⁺ ^d	327.123	20.3
	309.114	C ₁₉ H ₁₇ O ₄ ⁺	309.113	4.3
	255.069	C ₁₅ H ₁₁ O ₄ ⁺	255.066	5.1
[M + H] ⁺ ion	519.311	C ₃₃ H ₄₃ O ₅ ⁺	519.311	0.1
Propolone C (6)	327.125	C ₁₉ H ₁₉ O ₅ ⁺	327.123	4.7
	309.115	C ₁₉ H ₁₇ O ₄ ⁺	309.113	7.8
	255.067	C ₁₅ H ₁₁ O ₄ ⁺	255.066	6.1
	231.067	C ₁₃ H ₁₁ O ₄ ⁺	231.066	6.8
	177.021	C ₉ H ₅ O ₄ ⁺	177.019	12.5
[M + H] ⁺ ion	519.310	C ₃₃ H ₄₃ O ₅ ⁺	519.311	–1.3
Propolone D (7)	383.184	C ₂₃ H ₂₇ O ₅ ⁺	383.186	–4.6
	327.123	C ₁₉ H ₁₉ O ₅ ⁺	327.123	0.5
	309.114	C ₁₉ H ₁₇ O ₄ ⁺	309.113	4.3
	255.066	C ₁₅ H ₁₁ O ₄ ⁺	255.066	2.2
	231.066	C ₁₃ H ₁₁ O ₄ ⁺	231.066	0.3
	177.020	C ₉ H ₅ O ₄ ⁺	177.019	5.2
[M + H] ⁺ ion	535.308	C ₃₃ H ₄₃ O ₆ ⁺	535.306	3.2
Propolone D hydroperoxide (8)	399.178	C ₂₃ H ₂₇ O ₆ ⁺	399.181	–5.9
	343.119	C ₁₉ H ₁₉ O ₆ ⁺	343.118	3.3
	255.068	C ₁₅ H ₁₁ O ₄ ⁺	255.066	7.3
	177.021	C ₉ H ₅ O ₄ ⁺	177.019	12.5
[M + H] ⁺ ion	519.311	C ₃₃ H ₄₃ O ₅ ⁺	519.311	–0.3
18- <i>epi</i> -propolone C (9)	327.125	C ₁₉ H ₁₉ O ₅ ⁺	327.123	6.6
	309.113	C ₁₉ H ₁₇ O ₄ ⁺	309.113	0.1
	255.067	C ₁₅ H ₁₁ O ₄ ⁺	255.066	4.6
	231.067	C ₁₃ H ₁₁ O ₄ ⁺	231.066	6.8
	177.020	C ₉ H ₅ O ₄ ⁺	177.019	9.1

^aExperimental values obtained using the Q-TOF instrument.^bElemental composition derived from the measured *m/z* with tolerance of 25 ppm. Atom limits were the following: 0 to 60 carbons, 0 to 100 hydrogens, and 0 to 30 oxygens.^cThe *m/z* value calculated for the product ion by the proposed fragmentation pathway or the *m/z* value derived from the elemental composition of PPBs.^dSuggested formula: in these cases, the measured *m/z* values were not accurate enough to allow unambiguous elemental compositions to be defined.

always within 20 ppm error, indicating that the calculated elemental compositions in Table 1 can be considered reliable.

Garcinielliptone I (2) and hyperibone B (3) showed similar ESI-MSⁿ spectra. These nemorosone derivatives, with a five-membered ring in position C3–C4, are

epimers at C18, thus it was not surprising that they displayed the same fragmentation pattern. The fragmentation pathway proposed for **2** and **3** is shown in Scheme 2. The most abundant product ion (m/z 451) detected in MS/MS spectra of $[M + H]^+$ ion (m/z 519) of **2** and **3** was generated by elimination of the prenyl chain at C5 (Scheme 2). As noted for nemorosone, MS³ fragmentation of the ion at m/z 451 produced the ion $[M + H - 68 - 124]^+$ at m/z 327, indicating that the elimination of the chain at C7 occurs only following the loss of isoprene (Scheme 2). This might be explained by the change of hybridization of C5 from sp^3 to sp^2 . These successive fragmentations were observed only for PPBs with the prenyl group at C5 (compounds **1–4**, **6**, and **9**), confirming the mechanisms of C7 chain elimination. The study of MS⁴ fragmentation of the ion at m/z 327 and MS⁵ fragmentations of its product ions (Scheme 2) has enabled us to propose the fragmentation pathway shown in Scheme 2. Accurate mass measurements of compounds **2** and **3** are summarized in Table 1. The elemental composition assigned for ion at m/z 327.123 for **2** and **3** confirmed that this ion was formed by successive losses of C5 and C7 chains.

MS/MS spectra of **2** and **3** also showed a minor fragment ion at m/z 383 ($[M + H - 136]^+$) arising from the loss of a C₁₀ unit (Scheme 2). In this case, a preliminary ring opening between C1 and C8 followed by a concerted pericyclic mechanism was proposed (Scheme 2). This hypothesis was confirmed by the analysis of MS³ spectrum of ion at m/z 383 (Scheme 2). In this second route of fragmentation of the protonated

molecule $[M + H]^+$, the prenyl chain at C5 was eliminated as isobutene (ion at m/z 327 in MS³ 519 → 383).

Propolone C (**6**) and D (**7**) are cyclized derivatives of nemorosone with the tetrahydrofuran ring in position C2–C3 and C4–C5, respectively. ESI-MS^{*n*} data of propolone C (**6**) showed a fragmentation pattern similar to those proposed for garcinielliptone I (**2**) and hyperibone B (**3**), as supported by high-resolution mass data. Thus, it is not possible to distinguish the isomers that have the C₅ unit at C3 involved in a tetrahydrofuran ring. MS^{*n*} experiments performed on propolone D (**7**) indicated a different and simpler fragmentation pathway, compared with **2–3** and **6**, characterized predominantly by the second route of fragmentation observed for cyclized derivatives. The MS/MS spectrum of **7** (Scheme 3) revealed a fragment ion at m/z 383 attributed to the loss of a C₁₀ unit, while the $[M + H - 68]^+$ ion was not observed, in agreement with the proposed mechanism for this elimination of prenyl group at C5 as isoprene. This behavior was explained by the blocking action of the tetrahydrofuran ring on the C5. The elimination of prenyl at C3 by a six-center concerted mechanism occurred only after the loss of a C₁₀ unit ($[M + H - 136 - 56]^+$ ion at m/z 327 in the MS³ 519 → 383 spectrum of **6**, Scheme 3) that restores the enolic group at C2. The fragmentation pathway proposed for propolone D (**7**) is shown in Scheme 3; it is consistent with the accurate mass determinations (Table 1). The product ion at m/z 383.184 (associated with the formula C₂₃H₂₇O₅⁺ with an error of −4.6 ppm) confirms that it is formed after a neutral loss of a C₁₀H₁₆ residue.

Scheme 2. Proposed fragmentation pathway for garcinielliptone I (**2**) and hyperibone B (**3**) and MS^{*n*} spectra of **2**.

Scheme 3. Proposed fragmentation pathway for propolone D (7) and MSⁿ spectra.

As noted in Schemes 2 and 3, the fragmentation pathways of PPBs 2–3 and 6–7 were typically characterized by neutral loss of 72 Da, corresponding to an epoxidic derivative. This neutral loss can be seen as characteristic of PPBs with a 2-(2-hydroxypropyl)-dihydrofuran ring. The accurate mass measurements also validated that the product ions at m/z 255 were produced by loss of a $C_4H_8O_5$ fragment from the ions at m/z 327 (Table 1).

According to the proposed fragmentation mechanisms, the initial elimination of the side-chain at C3 as isobutene ($[M + H - 56]^+$ in the MS/MS spectra) should not be observed for cyclized derivatives of nemorosone, because the hypothetical mechanism involves a free enolic group. However, the MS/MS spectra of some of these compounds showed the fragment ion $[M + H - 56]^+$, although with a very low relative abundance (e.g., MS/MS spectrum of 2, Scheme 2). Probably this fragment ion was derived from a fragmentation mechanism different to the six-center concerted mechanism.

The product ion arising from isobutene loss ($[M + H - 56]^+$) was also detected in the MS/MS spectrum of propolone A (4) (Scheme 4), which differs from other cyclized derivatives of nemorosone by the presence of a dihydropyran ring in place of the tetrahydrofuran ring. In this case, the elimination of isobutene is rationalized by a retro Diels Alder mechanism (rDA) involving the pyran ring. The proposed fragmentation pathway for propolone A (4) (Scheme 4) provides the same fragmentations observed for 2–3 and 6–7 for the substituents of bicyclononane system ($[M + H - 68]^+$, $[M + H - 68 - 124]^+$, $[M + H - 136]^+$ e -78 Da). The MS/MS spectrum

of propolone A (4) (Scheme 4) was similar to those obtained for nemorosone (1), except for the presence of the fragment ion $[M + H - 136]^+$ at m/z 367 arising from elimination of a C_{10} unit. This fragmentation was observed only in MS/MS spectra of cyclized PPBs. The accurate masses measured for fragment ions of 4 (Table 1) agree with the theoretical m/z values expected for structures of proposed fragments (Scheme 4).

The fragmentation pathway of propolone B (5) reported in Scheme 5 further supported the characteristic fragmentations identified for PPBs, since this compound has structural features different from those of the other analyzed compounds, useful to confirm our hypothesis. The substituent at C5 of propolone B (5) is a 2,3-dihydroxy-3-methylbutyl group, and the C_5 unit at C3 forms, with an oxygen atom at C2, a hydroxylated 2,2-dimethyl-dihydropyran ring. The MS/MS spectrum of $[M + H]^+$ ion at m/z 553 showed only a fragment ion at m/z 535 due to loss of a water molecule (Scheme 5). MS³ fragmentation of this ion produced the characteristic fragment ions at m/z 463 $[M + H - 18 - 72]^+$ and 399 $[M + H - 18 - 136]^+$ obtained from a rDA rearrangement of the pyran ring (loss of C_4H_8O fragment) and from elimination of the side chain at C7, respectively (Scheme 5). Instead, the elimination of the isoprenoid group at C5 was not detected: MS³ data of 5 indicated the absence of product ions resulting from loss of this side-chain ($[M + H - 84]^+$, loss of C_5H_8O fragment or $[M + H - 100]^+$, loss of $C_5H_8O_2$ fragment). Again, the MS⁴ spectra of fragment ions at m/z 517 and 399 confirmed the proposed fragmentation pathways (Scheme 5).

Scheme 4. Proposed fragmentation pathway for propolone A (4) and MSⁿ spectra.

Studies of Nemorosone Degradation using HPLC-ESI-MSⁿ

To examine the stability of nemorosone (1) and investigate the nature of its oxidized and cyclized derivatives (2–7), the effects of procedures for the isolation of PPBs, climatic conditions at the sampling site and storage of commercial products of propolis were studied. For this purpose, the degradation of nemorosone was evaluated in different solvents (*n*-hexane, chloroform, methanol and ethanol), in an ethanol extract of brown Cuban propolis and in solid-state at 40 °C by HPLC-ESI-MSⁿ.

The stability studies revealed that nemorosone undergoes degradation in solution, particularly in apolar solvents (hexane and chloroform). The chromatogram reported in Figure 2 (chloroform solution after 168 h storage) clearly shows the degradation of nemorosone

into various products. The same degradation products were observed in *n*-hexane and ethanol solutions.

Degradation products of nemorosone were characterized using ion trap MSⁿ and HR-MS/MS experiments. Identity confirmation was provided by comparison with pure compounds or by their isolation and NMR characterization. The nemorosone derivatives 2–7, previously isolated from propolis and plants, correspond to some degradation products observed in our stability study.

The HPLC elution of the PPBs was as follows: propolone B (5), *m/z* 553, 11.2 min; propolone D (7), *m/z* 519, 21.8 min; hyperibone B (3), *m/z* 519, 28.5 min; garcinielliptone I (2), *m/z* 519, 30.9 min; propolone C (6), *m/z* 519, 35.3 min; nemorosone (1), *m/z* 503, 48.3 min; propolone A (4), *m/z* 503, 72.3 min (Figure 2).

Scheme 5. Proposed fragmentation pathway for propolone B (5) and MSⁿ spectra.

Figure 2. HPLC-MS chromatogram of undegraded nemorosone (grey line) and chloroform solution (2 mg/mL) after 168 h at 25 °C in artificial light (black line). 1 nemorosone, 2 garcinielliptone I, 3 hyperibone B, 4 propolone A, 5 propolone B, 6 propolone C, 7 propolone D, 8 propolone D hydroperoxide, and 9 18-*epi*-propolone C.

Two other degradation products, in addition to 2–7 were also recognized as peaks at 24.0 and 39.0 min. The former showed a protonated molecule $[M + H]^+$ at m/z 535 indicating that it is a dioxidized derivative of nemorosone. The molecular formula $C_{33}H_{43}O_6^+$ was suggested by accurate mass measurement (experimental m/z 535.308, theoretical m/z 535.306). The main product ion of its MS/MS spectrum was $[M + H - 136]^+$ at m/z 399, whereas the fragment ion resulting from initial loss of isoprene was absent. According to the fragmentation pathway for PPBs proposed above, this peak should correspond to a cyclized derivative of nemorosone with the ring in position C4–C5. The MSⁿ spectra of this degradation product (Supplementary Data) exhibited a remarkably similar fragmentation pattern to that of propolone D (7). Thus, we proposed for the peak at 24.0 min, the structure of propolone D hydroperoxide (8) (Figure 1). The high-resolution mass data (Table 1) also supported the proposed structure. Compound 8 was carefully isolated and purified from degraded solutions by HPLC-UV and characterized by nuclear magnetic resonance (NMR) spectroscopy. The NMR data of 8 (Supplementary Data) was very similar to that of propolone D (7) [30], except for the resonances of H-23, C-23, C-24, and C-24 geminal methyls. These observations indicated that 7 and 8 differ only in the replacement of the tertiary hydroxyl at C24 with a hydroperoxyl group [31].

The degradation product eluted at 39.0 min ($[M + H]^+$ at m/z 519) showed MSⁿ spectra almost superimposable with those of garcinielliptone I (2), hyperibone

B (3), and propolone C (6). Considering the possible isomers with the C₅ unit at C3 involved in a tetrahydrofuran ring, for this peak we proposed the structure of 18-*epi*-propolone C (9) (Figure 1). Its identity was supported by accurate mass determinations allowing the assignment of elemental compositions for each fragment ion (Table 1). To confirm the identity of 9, the same purification procedure of degraded nemorosone solutions was carried out, but the recovered amount was not sufficient for an NMR characterization.

The time course studies on nemorosone degradation were performed by a triple quadrupole spectrometer and nemorosone and its derivatives were quantified by LC-MS in MRM mode.

Aliquots of tested solutions (*n*-hexane, chloroform, methanol, ethanol, and ethanol extract propolis) and solid compound were collected at different times and analyzed to establish the degradation profiles of nemorosone.

The profiles of nemorosone degradation under different test conditions are shown in Figure 3. They show that nemorosone proved to be more sensitive to apolar solvents. In hexane solution, its degradation proceeded very rapidly: after 12 h, less than 25% nemorosone remained and after 24 h, complete degradation had occurred. In hexane solution garcinielliptone I (2) and hyperibone B (3) levels increased significantly by 25.0% and 21.8%, respectively, after 24 h. Also propolone D hydroperoxide (8) and 18-*epi*-propolone C (9) showed a progressive increase with time: +12% and +8.1%, respectively, at 12 h, and both 9.1% after 24 h, and these

Figure 3. Stability of nemorosone in test conditions (hexane, chloroform, and ethanol solutions). The data, expressed as residual nemorosone %, are the mean of three replicates for two solutions.

data were in agreement with the rapid degradation of nemorosone under the same conditions.

The degradation process of nemorosone in chloroform was also rapid, although slower than that in hexane solution (15.7% of residual nemorosone after 7 d). In this solvent, the main degradation product is propolone D hydroperoxide (8) (+17.8% after 72 h).

In contrast to apolar solvents, nemorosone was more stable in alcoholic solutions. Degradation in ethanol was very slow: ca. 13% of nemorosone was degraded at 60 d. The decrease of nemorosone produced a small increase of propolone D hydroperoxide (8) which peaked on the 60th day of storage (+5.5%), while, under these conditions, production of compounds 2–7 and 9 was very low (<1% after 60 d). In methanol solution, the nemorosone degradation was negligible (losses of 0.3% and 0.5% after 1 and 2 mo of exposure, respectively), as well as for a ethanol solution of brown Cuban propolis. Also, in the solid-state and under exposure to 40 °C nemorosone was very stable (no degradation was observed after 7 d).

Conclusion

The study showed that electrospray tandem MS is a powerful tool in the analysis of PPBs with a type A bicyclo-[3.3.1]-nonane-2,4,9-trione system. In particular, ESI-MSⁿ techniques provide information about the substitutions in the bicyclo skeleton. The elimination of the

alkylic chains from the bicyclo core is the dominant fragmentation pattern of these compounds and the ESI-MSⁿ spectra give detailed structural information that allow the side chains (C₅H₉ or C₁₀H₁₇) at C3, C5, and C7 and the type and position of modifications or arrangements in the alkylic groups to be assigned. The proposed fragmentation pathways are a useful tool for the development of analytical strategies for the simple and rapid characterization of PPBs in plant extracts.

With regard to the stability of nemorosone, the studies have shown that it is stable in alcohols, both in pure solution or in propolis ethanol extract. However, the compound has proved to be very unstable in apolar solvents such as *n*-hexane and chloroform. The degradation products identified by HPLC-ESI-MSⁿ are oxidized and cyclized derivatives of nemorosone, many of which correspond to PPBs isolated from brown Cuban propolis and plants. The main degradation product is propolone D hydroperoxide, whose structure was deduced by MSⁿ experiments and confirmed by NMR. This evidence demonstrates that these PPB derivatives are not natural products but artefacts produced by degradation of nemorosone. Knowledge of this chemical behavior is very important, because some PPBs described as natural products might well be artefacts formed as a result of the methods used for their isolation or during storage of the natural source or extracts.

The time course studies reveal that nemorosone undergoes a rapid degradation in apolar solutions

(*n*-hexane and chloroform), while it is much more stable in alcoholic solutions (methanol and ethanol). In the solid-state at temperatures similar to the climate of tropical areas, nemorosone seems not to suffer degradation. These results show that degradation products of nemorosone are generated predominantly during the extraction and purification procedures, which usually employ apolar solvents.

Appendix A Supplementary Material

Supplementary material associated with this article may be found in the online version at [doi:10.1016/j.jasms.2009.05.004](https://doi.org/10.1016/j.jasms.2009.05.004).

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